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First molecular and biochemical characterization of the extracellular matrix of *Saccharomyces cerevisiae*

Programa Doutoral em Biologia Molecular e Ambiental
Especialidade de Biotecnologia Molecular

Trabalho realizado sob orientação da
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- Faria-Oliveira, F., Carvalho, J., Belmiro, J., Ramalho, G., Ferreira, C., Pavão, M. and Lucas, C. First approach to the chemical nature of the polysaccharides in the extracellular matrix (ECM) of the yeast *Saccharomyces cerevisiae*. *(Submited)*

The further publication of the contents of Chapter 4 is under preparation. The detailed material from the Introduction will be used to build a review that includes for the first time results from *S. cerevisiae* ECM.

Resumo

A levedura *Saccharomyces cerevisiae*, tal como todos os microrganismos, é usualmente considerada como um organismo unicelular. Contudo, os microrganismos formam mais frequentemente comunidades multicelulares macroscópicas que apresentam diferenciação celular, e são coordenadas por um complexo sistema de comunicação, e suportadas por uma matriz extracelular (MEC). A presença deste tipo de suporte das comunidades multicelulares de *S. cerevisiae* foi descrita no início deste século. Apesar disso, a informação relacionada com a sua composição e organização tridimensional é escassa. Assim, o principal objetivo deste trabalho foi realizar a primeira abordagem sistemática aos principais componentes da MEC de levedura. Para o efeito, foram desenvolvidas metodologias para (1) obter de forma reprodutível uma considerável e homogénea biomassa de leveduras produtora de MEC, e (2) extrair e fracionar a MEC produzida de forma a obter frações analiticamente puras de proteínas e polissacáridos, compatíveis com a aplicação de metodologias analíticas de alto-débito como o GC-MS e o DIGE.

A análise detalhada da fração proteica permitiu a identificação de mais de 600 proteínas. A maioria destas tem função e localização intracelulares, e é aqui identificada extracelularmente pela primeira vez, o que pode indicar um *moonlighting* surpreendentemente elevado. A presença de todas as enzimas associadas à glicólise e à fermentação, assim como ao ciclo do glioxilato, levanta suspeitas sobre a possibilidade de haver metabolismo extracelular. Além disso, um grande número de proteínas associadas à síntese, remodelação e degradação de outras proteínas foi identificado, incluindo elementos da família HSP70 e várias proteases. De realçar a presença das exopeptidases Lap4, Dug1 e Ecm14, e das metaloproteinases Prd1, Ape2 e Zps1, que partilham um domínio funcional *zincin* com as metaloproteinases da MEC de Eucariotas superiores. A presença adicional de proteínas intervenientes em várias vias de sinalização, como as Bmh1 e Bmh2, e da *homing* endonuclease Vde, que partilha o domínio Hedgehog/inteína com os morfogenos de Eucariotas superiores, sugere que a MEC de levedura poderá, tal como nesses organismos, mediar sinalização intercelular.

As análises cromatográfica e eletroforética da fração glicosídica revelaram claramente a presença de dois polissacáridos. A análise por espectrometria de massa identificou glucose, manose e galactose na composição destes polissacáridos. Foram ainda observados indícios da presença de ácido urónico. A indução de metacromasia sugeriu que os polissacáridos detetados apresentam substituição química. A

possibilidade desta corresponder a sulfatação foi testada através de um teste de atividade anticoagulante. Das diversas amostras de MEC de diferentes estirpes de levedura usadas, o duplo mutante *gup1Δgup2Δ* apresentou, ao contrário da estirpe Wt, razoável atividade anticoagulante indicadora da presença de grupos sulfato.

Os efeitos da deleção do gene *GUP1* na composição da MEC de levedura proporcionaram uma perspectiva mais detalhada da composição molecular e mecanismos a ela associados. Observaram-se alterações nas frações protéica e glicosídica. A deleção resultou na ausência de várias proteínas, associadas principalmente com o metabolismo de fontes de carbono, defesa e resgate da célula, bem como síntese, modificação e degradação de proteínas, e organização celular. Adicionalmente, a deleção deste gene também teve um grande impacto na composição glicosídica da matriz, levando ao desaparecimento do polissacárido de maior peso molecular detetado na estirpe Wt. Globalmente, os efeitos da deleção do *GUP1* na MEC mostram que a estrutura desta é muito dinâmica e que se encontra sob controlo apertado das células que compõem o agregado multicelular.

As funções sugeridas para as proteínas ortólogas das Gup1 e Gup2 de levedura, respetivamente Hhatl e Hhat, nas vias de sinalização de Eucariotas superiores esteve na origem da construção de uma bateria de estirpes de levedura recombinantes transformadas com os ortólogos da via Hedgehog de ratinho, mosca e homem, para futura avaliação. Da mesma forma, foram clonados em *S. cerevisiae* os recetores de mamífero para o ácido hialurónico (AH), CD44 e HMMR. Estes transformantes foram submetidos ao crescimento na presença de AH de diferentes tamanhos moleculares. As estirpes exprimindo ambos os recetores foram igualmente sensíveis à presença de AH de elevado peso molecular, mas foram diferentemente sensíveis à presença de AH de tamanho molecular intermédio. As células expressando o recetor CD44 mostraram-se, tal como em Eucariotas superiores, sensíveis à presença de AH 50 kDa, apresentando uma forte redução da taxa específica de crescimento. Isto indica a expressão funcional dos recetores de AH em levedura e a provável conservação da maquinaria celular de resposta a este componente da MEC dos Eucariotas superiores.

Este trabalho é o primeiro a apresentar um estudo detalhado sobre as frações protéica e glicosídica secretadas para a matriz extracelular de *S. cerevisiae* durante o seu crescimento em comunidades multicelulares, oferecendo a primeira abordagem proteómica e glicómica da sua composição e organização. Globalmente, este trabalho permite prever que a MEC de levedura exerça funções equivalentes às conhecidas da MEC de Eucariotas superiores.

Abstract

The yeast *Saccharomyces cerevisiae*, as all microbes, is generally regarded as a unicellular organism. However, microorganisms live more frequently in macroscopic multicellular aggregates, presenting cellular differentiation, coordinated by complex communication, and supported by an extracellular matrix (ECM). The presence of this type of structure supporting multicellular life-style of *S. cerevisiae* was first described early this century. However, the information available on the yeast ECM components and three-dimensional spatial organization is scarce. Hence, this work aimed to provide a first methodical insight into the molecular composition of the yeast ECM major components. A methodology was developed capable of reproducibly obtaining ECM-producing homogenous yeast mats, and extracting and fractionating the yeast ECM into analytical-grade fractions. This was developed in order to be fully compatible with the application of high-throughput analytical techniques, like GC-MS and DIGE.

The in-depth analysis of the proteins in the yeast ECM identified more than 600 proteins, most of which being ascribed to intracellular functions and localization, and therefore found extracellularly for the first time. This might indicate unexpectedly extensive *moonlighting*. The entire sets of enzymes from glycolysis and fermentation, as well as gluconeogenesis through glyoxylate cycle were highly represented, raising considerable reason for doubt as whether extracellular metabolism might exist. Moreover, a large number of proteins associated with protein fate and remodelling were found. These included several proteins from the HSP70 family, and proteases, importantly, the exopeptidases Lap4, Dug1 and Ecm14, and the metalloproteinases Prd1, Ape2 and Zps1, sharing a functional *zincin* domain with higher Eukaryotes ECM metalloproteinases. The further presence of the broad signalling cross-talkers Bmh1 and Bmh2, as well as the homing endonuclease Vde that shares a Hedgehog/intein domain with the Hh morphogens from higher Eukaryotes, suggest that analogously to the tissues in these organisms, yeast ECM is mediating signalling events.

The chromatographic and electrophoretic analysis of the sugar fraction revealed the clear presence of two distinct polysaccharides. Mass spectrometry identified glucose, mannose and galactose in their composition. Evidence was also obtained of the presence of uronic acids. Both polysaccharides showed chemical substitution, as

indicated by metachromasia, and the existence of sulphate groups was assessed through an anticoagulant activity test. From several ECM samples from different yeasts strains surveyed, the double mutant *gup1Δgup2Δ* displayed a relatively high anticoagulant activity, which was not observed in Wt, likely related to the presence of sulphate groups.

The effects of the deletion of *GUP1* gene in the composition of yeast ECM were also assessed, providing a more in-depth perspective of the ECM components and molecular mechanisms associated. Alterations in both protein and sugar fractions were observed. The deletion of *GUP1* led to the absence of several ECM proteins, mainly associated with the carbon metabolism, cell rescue and defence, protein fate and cellular organization. Additionally, the disruption of this gene impacted in the composition of the ECM sugar fraction, through the disappearance of the higher molecular weight polysaccharide that had been detected in the Wt sample. The effects of *GUP1* deletion on the ECM show that its structure is very dynamic, and that it is under the tight control of the cells composing the aggregate.

S. cerevisiae Gup1 and Gup2 orthologues have suggested regulatory roles in the Hedgehog signalling pathway from higher Eukaryotes, in which organisms these proteins are known as Hhatl and Hhat, respectively. This led to the engineering the yeast mutants defective on either or both *GUP1* and *GUP2* by expressing these genes orthologues from fly, human and mouse, yielding a collection of transformants for future assessment. Similarly, the mammalian receptors of hyaluronic acid (HA), CD44 and HMMR, were cloned into the yeast *S. cerevisiae*. The engineered strains were subjected to growth in the presence of different molecular sizes of HA, and were identically and differentially sensitive to, respectively, high and intermediate molecular weight HA. The strain expressing CD44 presented a high growth sensitivity to the presence of 50 kDa HA as in high Eukaryotes. The HA receptors are therefore functional in the yeast cell, and the cellular machinery to respond to HA *stimuli* appears to be fairly conserved.

The present work is the first to present a comprehensive detailed study on the protein and polysaccharide fractions secreted during growth in *S. cerevisiae* multicellular aggregates. Overall, this work gives a first insight of the multicellular communities of *S. cerevisiae* proteomics and glycomics, ascertaining yeast ECM with putative roles derived from its components that resemble ECM from higher Eukaryotes.

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"In life, love and business, we need to do three things:
communicate, communicate and communicate"
Anonymous

1. GENERAL INTRODUCTION

Life in community

Communication is probably one of the most fundamental processes of any living organism. From the complex behaviours of higher eukaryotes populations to the response of a single cell microorganism to an extracellular stimulus, almost everything is about understanding what is around and how to behave towards that. How this information exchange is performed and processed greatly influences the organism survival.

Regarding the several factors influencing the signalling process, namely the signal nature and target, one of the most important factors is the medium through which the communication is performed, the environment surrounding the cell. However, almost every cell has its own particular environment, with wide variations in osmolarity, pH, oxygen as well as nutrients. Therefore, several strategies have been adopted by the cells to cope with these changes, from spore formation to increase the genetic pool of the population and survive extreme conditions in the yeast *Saccharomyces cerevisiae* [1] to social cooperation to form a multicellular structure in response to starvation in the amoeba *Dictyostelium discoideum* [2]. In multicellular organisms tissues [3], as well as in microbial biofilms [4], extracellular environment actually consists in an extracellular matrix (ECM) in which the cells are imbedded, that provides support and connects the cells. This scaffolding structure, besides storing water and offering physical protection, promotes cell communication, provides substrates to cell migration and can act as signals source, either by releasing stored molecules or by interacting with cells and generating new signals [5, 6]. Present across all levels of multicellular organization complexity, ECM has been receiving increasing attention.

Mammalian ECM – The space-filler that stole the spotlight

Mammals are amongst the most complex life-forms on Earth. Yet, their survival as organisms keeps closely linked to the needs of individual cells, regardless of their localization/specialization in tissues, organs and systems. In order to meet those needs, cells have to be fed nutrients and oxygen and get rid of wastes. This operates through a tight regulated process across the whole organism. The coordination of the resulting

homeostasis ultimately relies in the communication of stimuli between distant cells. A good example is the production of insulin in the pancreas, which stimulates the liver and muscle cells to take up the glucose in the blood, which otherwise becomes toxic [7]. Besides this *long distance talk*, the *local communication* between neighbouring cells is no less vital. In fact, the coordinated response to a remote stimulus is dependent of an efficient synchronization of the target cells, through signals exchanged between nearby cells. In this regard, the ECM is an especially important mediator, as it can block, delay or promote the signal [3].

Chemically, the ECM is mainly composed of water, proteins and polysaccharides, produced by the resident cells of a particular tissue and further modified by cells as needed to respond to particular stimuli. As such, its composition is actually very dynamic and tissue-specific [8], at the same time being modulated by and modulating almost all cell processes, through integration of mechanical stimulus and activation of specific receptors, as well as the regulation of growth factors storage and presentation [5, 6]. Thus, any major response or cell rearrangement depends on an efficient communication between these two partners, as the matrix can modulate the communication between cells and the cells can alter and reorganize the matrix. The ECM main players of this biochemical and biomechanical dialogue are always the same: the fibrous proteins, as collagens or fibronectin [5, 9], and the branched proteoglycans, as perlecan [10]. Interactions between the different ECM building blocks form a three-dimensional structure that provides support and shape to tissues and organs, and substrate to cell migration [11, 12]. Actually, there are two major types of structurally different mammalian ECM: the interstitial stroma, a fibrous and porous matrix supporting the cells through thread-like fibrils, and the basement membrane, a sheet-like structure supporting epithelia that divides tissue compartments. The misregulation of the cell-cell and cell-matrix interactions have a major impact in the organism survival, with a wide range of pathologies described [12-16].

The Collagen family

Collagens are the main family of fibrous proteins in vertebrates, being the main components of constitutive tissue and amounting to 30% of whole-body protein [8]. The 28 collagenous proteins described so far, numbered in Roman numerals (I-XXVIII) [17],

are trimeric molecules organized in a right-handed triple helix (Fig. 1) [18]. Such quaternary structure is formed by either identical (homotrimer) or two/three different monomers (heterotrimer) twisted around the same central axis (Table 1). Each collagen monomer, named α-chain, is composed of collagenous domains (Col domains) and flanking non collagenous regions (NC domains). Col domains consist of repeating tripeptide that naturally self-organizes in a left-handed helix due to the high content in glycine and proline [19, 20]. The most common motifs in Col domains are Glycine-Proline-X and Glycine-X-Hydroxyproline, where X can be any amino acid except glycine, proline or hydroxyproline. The presence of glycine every three residues, with its small side chain –H, allows a tightly packed triple helix. The presence of proline stabilizes the collagen at higher temperatures, coupled with the hydrogen bridges between chains and strong electrostatic interactions [19]. The NC domains are capable of interfering with several cellular processes, as angiogenesis [21] and tumour growth [22], and interacting with other ECM molecules, such as fibronectin and a laminin 5/6 complex [23].

The fibril forming collagens are the types I, II, III, V, XI, XXIV and XXVII, and usually present a helical domain with a perfect Gly-X-Y repetition over 1,000 amino acids long, the major helix. These collagens usually present one small triple helical domain in the amino end, the minor helix. These molecules undergo processing, once the major helix is formed, and associate and align in a quarter stagger alignment forming banded fibrils (Fig. 1). Such process is aided and regulated by other fibrillar collagens, type V and XI nucleate fibrils of Col I and II regulating fibril diameter [17, 18, 20]. Collagens XXIV and XXVII are rather unique members of the fibrillar collagens sub-family, presenting interruptions in their major helix [17]. The exact number of interruptions is not unanimously accepted, with some controversy regarding the presence of a small helix [24-26]. These molecules have been discovery recently [25, 26], but present structural resemblances to invertebrates' collagens, implying a probable ancient origin [24, 27]. However, not all collagen molecules form fibrils. Actually, most collagenous proteins feature several interruptions in the helical domain and are unable to form a fibril, being subdivided in several subgroups according to their nature and structural function [17, 18].

The Fibril Associated Collagens with Interrupted Triple helices, FACITs, are the larger group of non-fibrillar collagens, particularly abundant in the basement membrane.

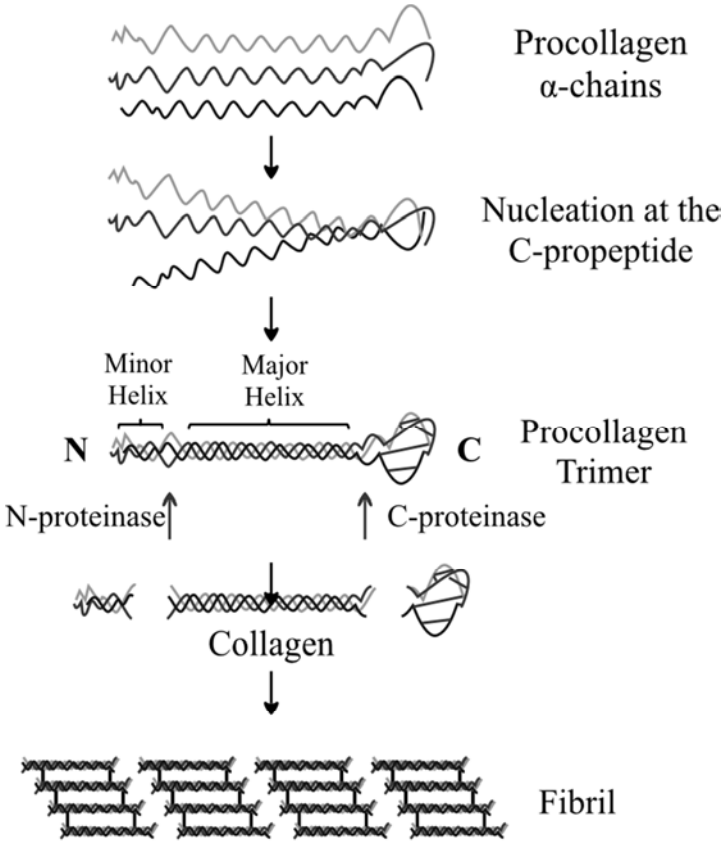


Figure 1. Overview of the steps involved in the production of collagen fibrils. Procollagen α-chains are synthesized in the ER, where a large number of post-translational modifications occur (not depicted). These monomers fold to form a rod-like triple-helical domain through interactions between the C-propeptides, usually presenting major and minor helices. After the full formation of the major helix, the procollagen undergoes the removal of the N- and C-propeptides, accomplished in the Golgi. The collagens are then able to interact and form ultrastructures, namely fibrils. Adapted from [28]

Comprising types IX, XII, XIV, XVI, XIX, XX, XXI and XXII, the FACITs are able to cross-link the surface of fibrillar collagens, producing distinct fibril surface properties and contributing to the biomechanical diversity of banded fibrils [29]. These collagens present several triple helical domains linked by short NC domains, as well as a large amino end domain featuring a thrombospondin sub domain [29, 30]. But their most distinctive features are the two G-X-Y imperfections in the Col2 domain and the two highly conserved cysteine residues separated by four amino acids in the NC1-Col1 domain boundary. Some of these molecules are able to interact with other matrix components, *e.g.*, ColXII can covalently bound glycosaminoglycans and form proteoglycans.

Non-fibrillar collagens are able to form several ultrastructures depending on the tissue properties, namely networks, beaded filaments and anchoring fibrils [31], the distinction between these structural groups is rather difficult as some collagens can assume more than one tri-dimensional ultrastructure, *e.g.*, collagen VI [18].

Network forming collagens, particularly type IV, are especially abundant in the basement membranes. This particular ECM type occurs in several body locations, with significant differences in the biomechanical properties of the tissues, contributing to the diversity of network-like structures. [31]. These collagens, IV, VIII and X, usually present an N-terminal 7S domain, responsible for inter-collagen interactions, a Col domain featuring around 20 interruptions and a C-terminal NC domain. These collagens network are formed by the combination of 4 molecules 7S domains into an antiparallel tetramer. The NC domains of each collagen molecule interact with the NC domain of other tetramer molecules to form a dimer [17, 31]. These inter-collagen interactions form a bi-dimensional grid-like structure. These collagen monomers can interact in different manners, yielding different supramolecular assemblies, namely hexagonal networks [31].

The single member of the anchoring fibril forming group, collagen VII, is responsible for connecting epidermis and dermis, tethering the basement membrane to the dermis [32]. This large collagen presents two NC domains flanking a large Col domain, at the N- and C-terminal ends, which assemble into anchoring fibrils. These fibrils, structurally different from the banded fibrils, are formed by antiparallel dimers connected by overlapping C- terminal ends. Several dimers assemble into a non-staggered fibril through lateral association, after proteolytic processing of the NC2 domain [17, 18]. These collagen ultrastructures are stabilized by transglutaminase cross-links. Collagen VII usually forms homotrimers, and present a very low affinity for other molecular collagens; nonetheless, the anchoring role results from tight interactions with dermal fibrils, showing that some interactions are only possible in the supramolecular level [20, 31].

Types VI, XXVI and XXVIII homotrimers assemble into beaded filaments, a thread-like ultrastructure [20, 31]. These proteins present a relatively short triple helical domain, featuring two interruptions, flanked by two globular domains [33]. These globular domains are especially important in the lateral association that leads to tetramerization, which for collagen VI occurs still inside the cell [34]. Two monomers

dimerize through the central overlapping of the C- terminal globular with the helical domain. This staggered dimer is stabilized by the formation of a supercoil between the helical domains of the monomers, reinforced by disulphide bonds near the ends of the overlapped region [35]. The dimmers associate into tetramers through lateral association, which in turn can form end-to-end linear aggregates, beaded filaments, or networks, hexagonal lattices. Such collagens, especially type VI, interact with a wide range of ECM molecules, including other collagens, non-collagenous proteins and glycosaminoglycans [31], which greatly influence the formation of these supramolecular structures, *e.g.*, the hexagonal lattice is favoured by the presence of byglycan. These interactions are particularly important for the formation of different architectures in adjacent regions or tissues [31], allowing a tight control on different mechanisms and functions occurring in such close locations.

While most collagens are secreted, a particular group consists of membrane spanning proteins [17]. Collagens XIII, XVII, XXIII, and XXV all form homotrimers, with a single hydrophobic transmembrane domain and a cytoplasmic N- terminal end domain [17, 31]. The extracellular C- terminus domain contains several COL domains with NC interruptions, increasing flexibility. The extracellular helical domain assembly is performed from N- to C- terminus, unlike the fibrillar collagens [31]. This ectodomain is involved in the epithelial cells anchoring to the basement membrane, extending from the cell to bind laminin [17], complementing the anchor-forming type VII action, that also interacts with laminin to anchor the dermis. All these type II transmembrane proteins are subject of proteolytic shedding between the membrane and the first COL domain, originating soluble forms [36].

Table 1. The Collagen family. Genes, trimers composition and associated pathologies.

Type of Collagen	Genes	Molecular Structure	Diseases and Disorders
I	*COL1A1*, *COL1A2*	$\alpha1(I)_2\alpha2(I)$ $\alpha1(I)3$	Osteogenesis imperfecta; Ehlers – Danlos syndrome; Infantile cortical hyperostosis [37-40].
II	*COL2A1* [a]	$\alpha1(II)3$	Spondyloepiphyseal dysplasia; Spondyloepimetaphyseal dysplasia; Stickler syndrome [40-42].
III	*COL3A1*	$\alpha1(III)3$	Ehlers–Danlos syndrome; Dupuytren's contracture [37, 38, 43].
IV	*COL4A1*, *COL4A2*, *COL4A3*, *COL4A4*, *COL4A5*, *COL4A6*	$\alpha1(IV)_2\alpha2(IV)$ $\alpha3(IV)\alpha4(IV)\alpha5(IV)$ $\alpha5(IV)_2\alpha6(IV)$	Alport syndrome; Goodpasture's syndrome [44, 45].

V	*COL*5*A*1, *COL*5*A*2, *COL*5*A*3 [a]	$\alpha1(V)_2\alpha2(V)$ $\alpha1(V)_3$ $\alpha1(V)\alpha2(V)\alpha3(V)$	Ehlers–Danlos syndrome (Classical) [46].
VI	*COL*6*A*1, *COL*6*A*2, *COL*6*A*3, *COL*6*A*5 [a]*	$\alpha1(VI)\alpha2(VI)\alpha3(VI)$ $\alpha1(VI)\alpha2(VI)\alpha4(VI)$ $\alpha1(VI)\alpha2(VI)\alpha5(VI)$ $\alpha1(VI)\alpha2(VI)\alpha6(VI)$	Ulrich myopathy; Bethlem myopathy; Atopic dermatitis [47, 48].
VII	*COL*7*A*1 [a]	$\alpha1(VII)_3$	Epidermolysis bullosa dystrophica [49, 50].
VIII	*COL*8*A*1, *COL*8*A*2	$\alpha1(VIII)_2\ \alpha2(VIII)$ $\alpha1(VIII)_3$ $\alpha2(VIII)_3$	Corneal endothelial dystrophies [51].
IX	*COL*9*A*1, *COL*9*A*2, *COL*9*A*3 [a]	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	Multiple epiphyseal dysplasia; Autosomal recessive Stickler syndrome [40, 52].
X	*COL*10*A*1	$\alpha1(X)_3$	Schmid metaphyseal dysplasia [53, 54]
XI	*COL*11*A*1, *COL*11*A*2 [a] *COL*2*A*1 [b]	$\alpha1(XI)\ \alpha2(XI)\ \alpha3(XI)$	Collagenopathy, types II and XI; Stickler syndrome [52, 55, 56].
XII	*COL*12*A*1 [a]	$\alpha1(XII)_3$	
XIII	*COL*13*A*1 [a]	$\alpha1(XIII)_3$	
XIV	*COL*14*A*1 [a]	$\alpha1(XIV)_3$	
XV	*COL*15*A*1	$\alpha1(XV)_3$	
XVI	*COL*16*A*1	$\alpha1(XVI)_3$	
XVII	*COL*17*A*1	$\alpha1(XVII)_3$	Junctional epidermolysis bullosa [57].
XVIII	*COL*18*A*1 [a]	$\alpha1(XVIII)_3$	Knobloch syndrome [58, 59].
XIX	*COL*19*A*1	$\alpha1(XIX)_3$	
XX	*COL*20*A*1	$\alpha1(XX)_3$	
XXI	*COL*21*A*1	$\alpha1(XXI)_3$	
XXII	*COL*22*A*1	$\alpha1(XXII)_3$	
XXIII	*COL*23*A*1	$\alpha1(XXIII)_3$	
XXIV	*COL*24*A*1	$\alpha1(XXIV)_3$	
XXV	*COL*25*A*1 [a]	$\alpha1(XXV)_3$	
XXVI	*EMID*2	$\alpha1(XXVI)_3$	
XXVII	*COL*27*A*1	$\alpha1(XXVII)_3$	
XXVIII	*COL*28*A*1	$\alpha1(XXVIII)_3$	
XXIX	*COL*29*A*1 (*COL*6*A*5)	*	

a. Several gene products by alternative splicing.
b. *COL*2*IA*1 product is known as α3(XI) when assemble in a type XI collagen heterotrimer.
* Collagen XXIX was described by [60] and later proved to be Collagen VI α-chain 5 by [61].

Multiplexin collagens, including type XV and XVIII, are basement membrane molecules that undergo proteolytic cleavage to yield antiangiogenic endostatins. As full-length molecules, these collagens present a central COL domain flanked by NC domains, at both N- and C- terminus [62]. These collagens present high homology between their COL and NC domains; however, present different tissues distribution, type XV is mainly expressed in the heart and skeletal muscle, whereas type XVIII is the main multiplexin in the smooth muscle [17]. The carboxyl terminal domain can be cleaved to generate endostatin and restin, which present distinct antiangiogenic properties [63]. Furthermore, both collagens XV and XVIII molecules are proteoglycan core proteins with an attached chondroitin sulphate and heparan sulphate glycosaminoglycan, respectively [64, 65]. The structural role of these multiplexins, as well as the diverse functions of the soluble shed forms help these collagenous proteins to regulate a wide range of cell-cell and cell-matrix processes [31].

In all, collagens play important roles in the ECM structural and signalling functions. Accordingly, a high number of syndromes result from mutations in these molecules (Table 1).

Non-Collagenous proteins

Much of the ECM regulatory functions are accomplished by non-collagenous glycoproteins [8]. Most of them present several functional domains, which allows a tight interaction with specific receptors and other ECM molecules, this way mediating important cell-cell and cell-matrix communication [66-68]. These domains are frequently coded by a single exonic unit, allowing a genomic shuffle throughout evolution [69]. Multidomain glycoproteins, like laminins, thrombospondins, fibronectin, tenascins, elastins or fibrilins (Fig. 2, Fig. 3 and Fig. 4), regulate cell adhesion, structural elements rearrangement. They also intervene in cell-matrix adhesion or cytoskeleton mechanical coupling [8, 9, 70]. These proteins are frequently subject of alternative splicing, which increases the number of forms [69], *e.g.*, the fibronectin gene originates around 20 different proteins through alternative splicing (Fig. 2) [71]. Such diversity allows the cells to exert complex fine-tuning of the ECM processes in a tissue specific manner.

Fibronectins (FNs) are dimeric glycoproteins composed by highly similar 250 kDa monomers [72]. These monomers are composed of three repeating domains, functionally

and structurally different: type I (FN-I) is a 40 amino acids domain repeated 12 times; type II (FN-II) presents two repeats of 60 amino acids long chain; and type III (FN-III) are 90 amino acids long sequences that, due to alternative splicing, may be present 15-17 times (Fig. 2 A). All domains present two antiparallel β-strands. However, types I and II are stabilized by intra-chain disulphide bonds, while FN-III forms a flexible seven stranded barrel that undergoes conformational changes [71]. A single gene, *FN1*, encodes for all the described FN proteins [73], these proteins result from the alternative splicing of the single pre-mRNA [71, 73]. In humans, 20 different FN forms arise from three major splicing sites in the FN transcript: two extra type III domains between FN-III 7 and 8, domain EIII-B, and between FN-III 11 and 12, domain EIII-A; and a variable domain between FN-III 14 and 15, V domain (Fig. 2 A) [69, 74]. These extra type III domains, EIII-A and EIII-B, may be both included or just one in the FN sequence, as in cellular FNs, or both absent, as occurs in the plasma FN [72]. This happens because each extra domain is coded by a single exonic unit that may be included or skipped during alternative splicing. However, the V domain is coded by a large exon that undergoes subdivision, yielding several different size domains [9]. The

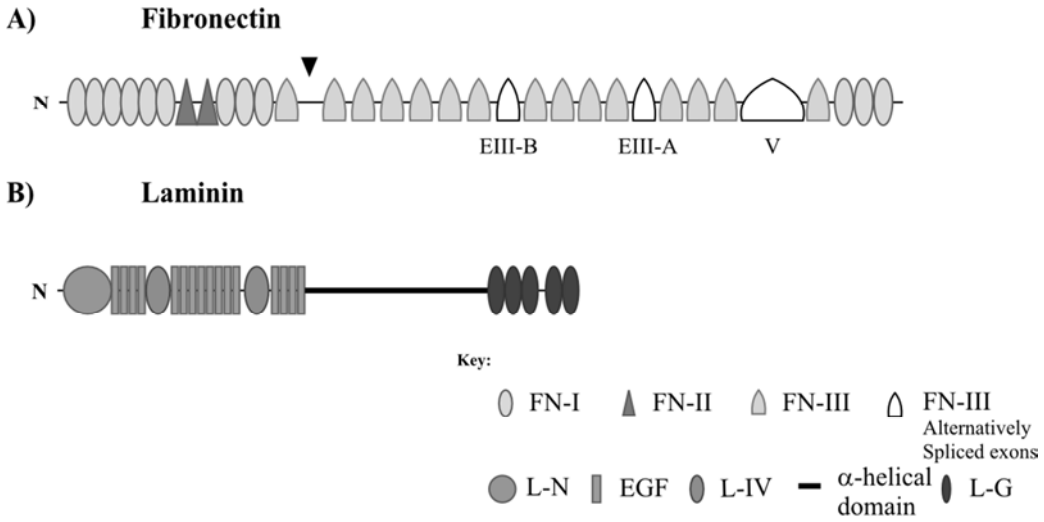


Figure 2. Domain architecture for Fibronectin (A), and Laminin (B)**.** These proteins present several domains, typically encoded by different exonic units that are subject to alternative splicing and generate different protein forms (as the EIII-A, EIII-B and V domains indicated for fibronectin). Extra-large linker indicated by arrow head. Adapted from [69]and [75].

different FN monomers, resulting from the alternative splicing of these three regions, present differences regarding solubility and cell adhesive or ligand binding ability. Monomers assemble into antiparallel dimers through the interaction formation of disulphide bonds between two cysteine residues, present near the C- terminal end of each chain [74]. The disulphide isomerase activity described for the last FN-I modules mays facilitate this reaction [76]. The covalently linked FN dimer is secreted as a soluble and compact protein [72, 74]. Such compact configuration depends on intramolecular ionic interactions between FN-III domains 2-3 and FN-III 12-14 [77], and prevents FN fibril formation in solution [78]. The fibrillogenesis occurs after FN interaction with integrin cell surface receptors, αβ heterodimers with two transmembrane domains [79, 80]. Several integrins interact with FN and aid the cell adhesion process, but only integrin α5β1 is able recruit and bind soluble FN [81]. The FN binding to α5β1 activates the receptor intracellular domain, which interacts with the actin cytoskeleton and promotes receptor clustering. The receptor-dimer clusters promote FN-FN interactions, and the initially compact dimers undergo a conformational extension [82]. The FN-I 1-5 modules are especially important to inter-chain interactions [78]. After dimer extension, these five modules recognize and bind several regions within FN. Fibrillogenesis depends on the interaction between these amino end domain, FN-I 1-5, and the first two FN-III. These two FN-III modules are connected by an extra-large linker that allows conformational changes within the FN molecule and regulates FN interactions (Fig. 2 A, arrow head) [72]. FN fibrils can further associate and form thicker bundles, stabilizing the ECM [9]. Besides FN-FN binding regions, FN presents motifs that interact with several other ECM molecules, namely heparin and fibrin [71]. FN different domains allow simultaneous binding to cell receptors and ECM constituents, integrating cell-matrix communication. The three dimensional organization of the multiple domains and their flanking residues contribute to regulate this information exchange [9].

Laminins are main constituents of the basal lamina, one of the stratified layers composing the basement membrane, promoting cell differentiation and regulating cell migration and adhesion [83]. These glycoproteins are composed of three structurally and functionally distinct polypeptide chains, referred as α-, β- and a γ-, which assemble into a parallel coiled-coil trimeric structure [75, 83, 84]. Similarly to most ECM proteins, laminin monomers are composed by tandem repeats of several functional domains (Fig. 2 B) [84]. The interaction of the different variants of each subunit, five α-, three β- and

three γ-, produces the great biological diversity of laminins [85, 86]. The laminin family presents a characteristic domain architecture: a large amino end globular domain (L-N), several EGF-like domains interspersed by globular domains (L-IV), an α -helical coiled-coil domain, the family defining domain, and a large carboxyl end globular L-G domain (Fig. 2 B) [75, 84]. Each monomer subtype presents small differences regarding this typical architecture: α- chains present 2 globular L-IV domains, some truncated forms lack L-IV domains, and exhibit five subdivisions of the L-G domain (Fig. 2 B); β-chains present small interruptions in the coiled-coil domain, termed β-knob, and lack L-G domain. Subunit β3 misses L-IV domain, while β1 and β2 present a different globular domain (L-F); and γ-chains present a singular L-IV domain and do not possess the carboxyl end L-G domains, while subunit γ2 also lacks N- terminus L-N globular domain [75, 87]. The C- terminus L-G domain, present in α-chains, is extremely important in interactions with cell receptors. It presents two functionally different subdivisions (L-G 1-3 and L-G 4-5) that can assume different configurations and interact with several ECM molecules. Intracellularly, the trimer assembly initiates with the β- and γ-chains coiled-coil domains interaction and dimerization. A particular γ-chain C- terminus 10 amino acids motif is critical for this step. This dimer intermediate is retained in the cytoplasm until α-chain recruitment and binding, after which it undergoes secretion [75, 86]. Secreted laminins may go through modifications, namely proteolytic cleavage and glycosylation, which modulate signals and change interaction with cell receptors. The L-G 4-5 domain cleavage, by the action of proteases, can modulate the signal from promoting cell migration to supporting cell adhesion [88, 89]. The secreted and processed laminins can form different supramolecular structures, like fibrils and meshes. The kind of laminin structure depends on a biophysical and biochemical dialogue between cells and ECM. Non-migrating keratinocytes assemble rosette-like structures, while migrating keratinocytes form laminin trails that guide and promote cell movement [90]. In turn, fibrillar laminin integrates the cell cytoskeleton with the ECM structure and help transmit biomechanical signals that regulate tissue properties [91]. Laminin ultrastructure assembly occurs when its L-G domains interacts with cell receptors and receptor-like molecules, namely integrins and dystroglycan. The cell-bound heterotrimers start interacting with each other, through small arms L-N domains, and reorganize to form a three dimensional network [92], which promotes tissues structural coordination. Laminin supramolecular assemblies can present a single

laminin type, or several laminin family members. Temperature and Ca^{2+} greatly influence these assemblies by promoting conformational changes in the L-N domains [86], therefore, environmental changes cause cellular responses that implicate in the ECM structure and composition.

Tenascins are a small family of ECM glycoproteins, comprising four members, which present adhesion-modulating properties [70, 93]. Tenascin shares some structural similarities with FN, *i.e.*, several tandem repeats of FN-III modules (Fig. 3 A) that interact frequently. However, tenascin and FN present antagonizing functions, influencing each other actions [94]. These molecules mediate the cell-matrix interactions and promote cell motility, influencing cell proliferation and differentiation and some types of programmed cell death, namely anoikis [94], which is also a cell motility-related process. Present mostly in the central nervous system, these proteins exhibit several functional domains organized in a conserved manner: three to four small amino end α-helical repeats (TA), extremely important to oligomerization, several EGF-like repeats followed by FN-III modules, and a carboxyl end fibrinogen-like globular domain (FBG) (Fig. 3 A). These monomeric chains can assemble into trimers, which can further associate into a hexabrachion ultrastructure, whereas some splice variants form dimers [70, 93]. The formation of this hexameric structure depends on two steps: firstly, three monomers associate through the TA helical repeats and form a short triple stranded coiled coil, the trimer is stabilized by intrachain disulphide bonds formed between cysteine residues; secondly, two different trimers TA domains interact and associate homophilically [70]. The resulting hexamer presents six arms with a terminal globular domain (FBG) originating from a central core; the proximal portions of the arms, composed EGF-like repeats, are thin and rigid, whereas the distal portions, composed of FN-III modules, are thick and flexible. These molecules, present exclusively in vertebrates, are able to interact with several cell surface proteins, integrins and cell adhesion molecules, as well as with ECM molecules, aggrecan, versican or neuron. Tenascins are also able to modulate the action of several serine proteases and matrix metalloproteinases, leading to ECM reorganization [70]. Such interactions occur mainly through FN-III and FBG domains; nevertheless, EGF-like domains were shown to act as low affinity ligands for EGF receptors [93]. As such, tenascins are capable of complex interactions with both the cells and other molecules, regulating focal adhesion kinase- and Rho-mediated signalling pathways [95], and

influencing morphogenetic pathways [96]. Given the broad field of action of these proteins, namely promoting cell migration, the deregulation of the associated metabolism and functions relates with several pathologies, namely cancer and Ehlers–Danlos syndrome [97, 98].

Thrombospondin family comprises multidomain calcium-binding glycoproteins [99], interacting with a wide range of cell surface components, ECM molecules or growth factors, and modulating cell-cell and cell-matrix communications. These ECM proteins present several tissue specific functions, from wound healing and angiogenesis to inflammatory response [100]. Thrombospondin family members are divided in two subgroups, according to their domain architecture and oligomerization [101]. All thrombospondins present a highly conserved domain organization in the carboxyl portion of each polypeptide [100], comprising three EGF-like domains followed by several calcium binding thrombospondin type III domains (TSP-3), and a C- terminal end globular domain (TSP-C; Fig. 3 B). However, the N-terminal portion of these molecules presents several differences. The subgroup A members present an N-terminal globular domain (TSP-N), important for oligomerization, a von Willebrand factor module, also present in some collagens, and three thrombospondin type I repeats (TSP-1). The subgroup B presents a distinct amino end domain and an extra EGF-like repeat, lacking both the von Willebrand and TSP-1 domains [102-105]. The differences in the amino end domains influence these molecules oligomerization. The subgroup A monomers TSP-N interact and assemble into a triple left handed coiled-coil, whereas subgroup B TSP-N domains form a pentamer [99, 101]. Both structures present high flexibility, which changes according to the amount of calcium ions bound to TSP-3. After assembly, these molecules undergo secretion and are either incorporated in the matrix or suffer proteolytic cleavage to yield anti-inflammatory and antiangiogenic fragments [106]. ECM-integrated thrombospondin may interact with integrins to modulate cell attachment and migration, or with some growth factors, TGF-β interacts specifically with subgroup A thrombospondin TSP-1 domains [107, 108]. The thrombospondin role in angiogenesis and cell migration is of special importance in pathological conditions as cancer or vascular diseases, reason why it has been considered as a potential therapeutic target [109].

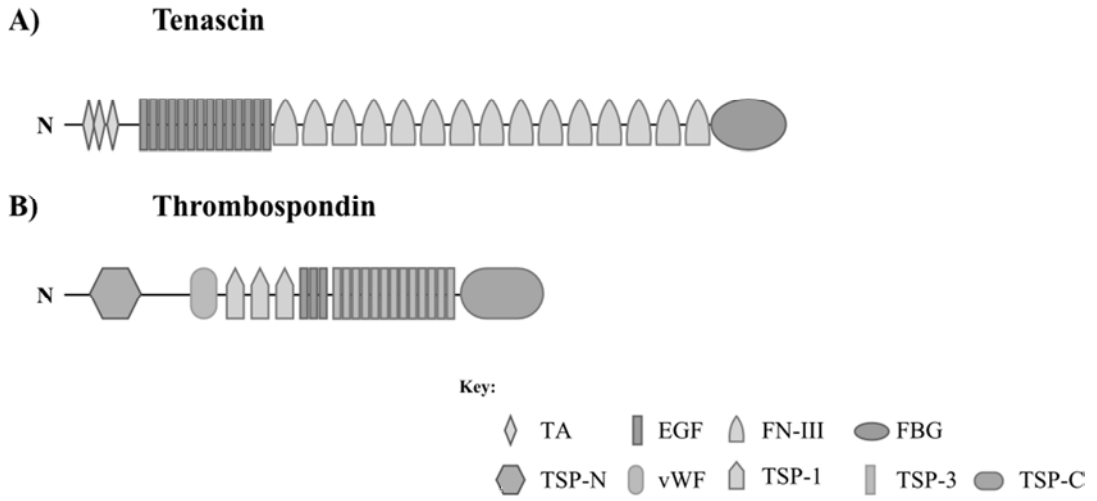


Figure 3. Domain architecture for Tenascin (A), and Thrombospondin (B). These glycoproteins present tandem repeats of several functional domains that allow interactions with other ECM molecules, *e.g.*, von Willebrand factor (vWF) interacts with several growth factors and influences cell response to several stimuli. Adapted from [69].

Fibrillin family comprises large and highly homologous secreted glycoproteins that are unusually rich in cysteine (12-13%) [110, 111]. These glycoproteins are secreted as a large 350 kDa proprotein. Convertases from the Furin family cleave the small N-terminal peptide (14-48 amino acids) and the larger C- terminal sequence (120-140 amino acids). The small size of the amino end peptide hinders its study, and almost all information available on the profibrillin processing is about the carboxyl end domain excision [112, 113]. These domains present several conserved Cys residues that may help stabilize the profibrillin structure in the first stages after secretion [114]. Currently, there are three known fibrillin isoforms, fibrillin -1, -2 and -3 [115-117]. Similarly to other ECM proteins, these isoforms present several functional domains organized in a highly conserved fashion, ≈100% homology [110]. Fibrillins present a large number of EGF-like functional domains (Fig. 4 A), most of which (42-43) contain specific amino acid residues that mediate calcium binding (cb-EGF) [118-120]. The amino acid motif responsible for calcium binding is conserved, but the affinity changes significantly between individual domains [121, 122]. Calcium plays a vital function in the structural stabilization of fibrillin [119, 123, 124], as well as in the protection of proteolytic degradation [125]; or the regulation of interactions with ligands [126-128]. The EGF-like

domains present six conserved Cys residues that form intradomain disulphide bonds, stabilizing the structure. Such three-dimensional organization, helped by interdomain interactions and short linker sequences, leads to a rod-like structure formed by tandem repeats of calcium loaded EGF domains [110].

The fibrillin protein presents other domains interspersing the EGF-like domain repetitions, namely the TGF-β-binding protein-like domains (TB) (Fig. 4 A), which interact with growth factors, mainly TGF-β and BMP [129]. The TB domains present four disulphide bonds between conserved Cys residues. Some EGF-like domains present some homology to TB domains, especially in the amino half of the protein, and are sometimes named *hybrid* domains [130, 131]. The *hybrid* domains are particularly important to the establishment of intermolecular connections. These domains present nine Cys residues, and the ninth residue is free and solvent accessible, enabling the formation of higher-order assemblies [132].

The main distinguishing characteristic among the different isoforms is a domain which structure is unknown, the Proline Rich Region (PRR) (Fig. 4 A). These domains present low homology between the different isoforms; the fibrillin -1 isoform is especially enriched in Pro residues, whereas the fibrillin -2 protein present a Gly rich domain, and the fibrillin -3 PRR's domain is enriched in both Pro and Gly residues [110]. Other structural differences include the number and position of the integrin binding site motif Arg-Gly-Asp, the predicted tyrosine sulphation sites and the predicted *N*- and *O*-glycosylation sites. Early metabolic labelling experiments dismissed the presence of sulphation [133], whereas the role of glycosylation remains unknown. The interaction of fibrillin with several integrins, namely αvβ3, α5β1 and αvβ6, through the Arg-Gly-Asp was reported [134-137].

Despite many efforts and a wide range of techniques used, the molecular organization of individual fibrillin monomers in microfibrils is not completely resolved. Hence, most of the information available is focused in the multicomponent microfibrils, whose main constituent is fibrillin. The assembly of fibrillin in such structures may depend on self-interactions. Fibrillin self assembles into multimers in solution, forming heterodimers between fibrillin -1 and -2 [138], and truncated forms lacking either the N- or C- terminal halves show high affinity for each other [139]. Interactions of fibrillin with other ECM components, namely fibronectin and heparan sulphate proteoglycans (HSPG), also influences the assembly of microfibrils. Perlecan, an HSPG, interacts with

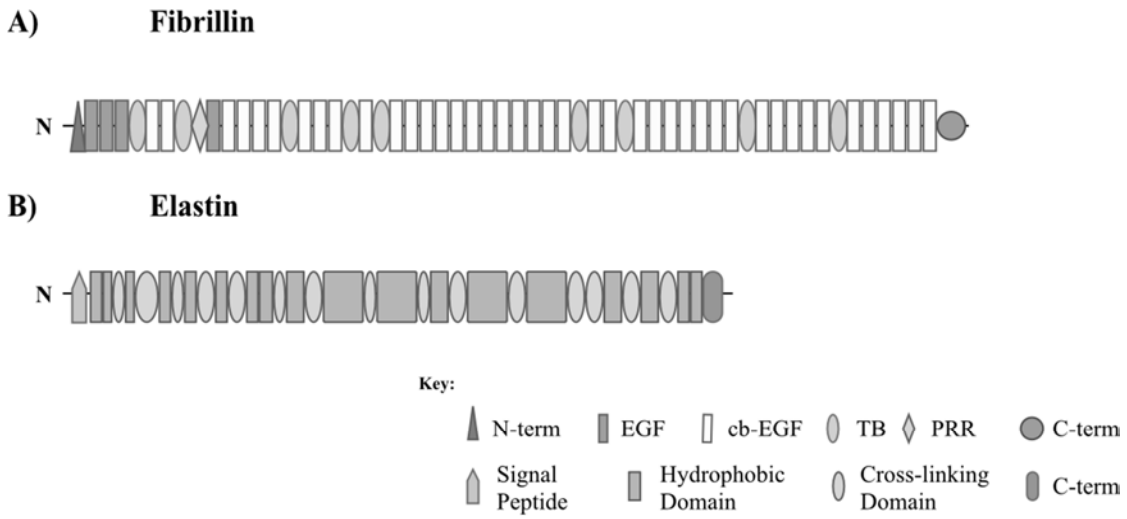


Figure 4. Domain architecture for fibrillin (A) and elastin (B)**.** These proteins present several domains, which allow the interaction between microfibrils and elastin monomers to form elastic fibres. Adapted from [140] and [141].

fibrillin through several identified heparan sulphate binding sites and promotes microfibril formation [142], whereas free heparan sulphate or heparin compete for the binding sites and strongly inhibits the microfibril formation [127, 143, 144]. The assembly of a fibronectin network is crucial for the deposition of microfibrils.

Interactions between fibronectin, integrins and fibrillin regulate the microfibril assembly into bundles [145, 146]. The three-dimensional structure of mature microfibrils is maintained by a high degree of cross-linking. Disulphide bonds between Cys resides, and ε (γ-glutamyl)-lysine cross-links catalysed by transglutaminases, are the main intermolecular cross-links [147, 148]. Fibrillins present 361 different Cys residues, and the ones responsible for the intermolecular disulphide bonds are still unknown, although the ninth residue from the *hybrid* domains is a likely candidate. The disulphide bonds are formed after a few hours of secretion and help the stabilization of the molecule in the early phases [110]. The ε (γ-glutamyl)-lysine cross-links are formed in later stages of the maturation process in an irreversible way [148, 149], and as much as 15% of all lysine residues may be cross-linked [148].

Fibrillin interacts directly with the ECM proteoglycan perlecan, allowing the tethering of microfibrils to the basement membrane components, and acting as stress-bearing entities to ensure tissue integrity [142]. Some evidence shows that the basement membrane may provide nucleation sites for microfibrils formation [150]. In tissues, microfibrils present a uniform appearance, forming thread-like structures of 10-12 nm

of diameter organized in bundles. However, the microfibrils extracted from tissues present a different structural organization, displaying a beads-on-a-string ultrastructure on rotary shadowing electron microscopy [151-153]. The experimental procedures may remove or lead to the partial loss of protein components of the microfibrils from the interbead regions [154]. Nevertheless, this beads-on-a-string structure displays remarkable elastic properties; it can be stretched up to 100 nm in a reversible manner, and only higher periodicities lead to permanent deformation [155-157].

Microfibrils are ubiquitously distributed in the ECM of most tissues and contribute to their physical properties. Microfibrils are particularly important in the connective tissue present in several types of fibrous tissue, underlying differences regarding density and cellularity. It can also be found in more specialized and recognizable variants of connective tissue, like bone, tendons, cartilage and adipose tissue [158]. In blood vessels, lungs and skin, microfibrils act as scaffolds for the deposition of elastin in early stages of elastic fibres formation, and decorating the surface of mature fibres [159]. As mentioned above, microfibrils are able to interact with several ECM proteins, regulating elastic fibre synthesis and cross-linking formation [160, 161]. These supramolecular aggregates are also present in tissues lacking elastin [162]. In kidneys or the ciliary zonules of eyes, microfibrils assemble into bundles and provide tensile strength and shear stress resistance to tissues [111]. The vital role of fibrillin in the cardiovascular, skeletal and ocular systems is intimately connected with the severity of the syndromes arising from mutations on this ECM protein. The most common fibrillin-associated pathology is the connective tissue disorder known as the Marfan syndrome. It corresponds to several symptoms, including mitral valve disease, progressive dilatation of the aortic root, dolichostenomelia, arachnodactyly, scoliosis and *ectopia lentis*. More than 1.000 distinct mutations were identified in the *FBN1* gene, coding for fibrillin-1 [163]. The Marfan syndrome may derive from insufficient expression of the protein or its exaggerated degradation, or from the incorporation of mutated or truncated forms of the protein in the microfibrils compromising its function [110].

Elastin is synthesized almost exclusively during specific developmental stages – from mid-gestation to postnatal [164-166]. In aorta, elastin expression decreases when blood pressure stabilizes after birth, and almost no synthesis of elastin is found in adult tissue [167-169].

The hydrophobicity and insolubility of elastin is a major obstacle to its structural characterization. Most of the available information derives from the soluble tropoelastin monomers, elastin proteins before the extensive cross-linking. Tropoelastin is trypsin-sensitive, allowing the study of the structural organization of the molecules. The tryptic digestion of this monomer revealed the presence of two main classes of peptides composing the protein: (1) small peptides rich in alanine, deriving from the regions protected by cross-links in elastin; and (2) large peptides rich in hydrophobic amino acids, responsible from the biologically relevant elastic properties [170-173]. Accordingly, the later analysis of the tropoelastin cDNA showed that this protein presents alternating hydrophobic and lysine-rich domains [174-176]. The lysine rich domains are important for the cross-linking of tropoelastin, and proper functioning of the elastic fibres. The secretion and deposition of elastin in the ECM and formation of highly cross-linked multimeric proteins is not fully understood. The synthesis of tropoelastin happens in membrane-bound polysomes, and the protein is transported along the Golgi apparatus to the secretory vesicles [177, 178]. The tropoelastin secretion occurs through a distinct mechanism from other ECM proteins. The protein reaches the extracellular space through an acidic endosomes in the presence of a 67 kDa chaperone [179-181]. Once in the extracellular space, tropoelastin forms small aggregates on the cell surface, initiating cross-linking [182-184]. This reaction is mediated by the enzyme protein lysine-6-oxidase, which oxidizes selective lysine residues in peptide linkage to α-aminoadipic δ-semialdehyde, also known as allysine [185].

Two main cross-links can be found in elastin: (1) the condensation of an allysine residue with and lysine residue, dehydrolysinonorleucine, or (2) the condensation of two allysine residues, allysine aldol [186-188]. These two cross-links can further condense with each other to form more complex cross-links, desmosine or isodesmosine [189]. There is also evidence that desmosine/isodesmosine cross-links can be oxidized by reactive oxygen species resulting in dihydrooxopyridine forms [190]. The interaction with other ECM molecules, namely proteoglycans, facilitates the self-association of tropoelastin monomers [191]. Interactions with proteoglycans mediate the releasing of the chaperone protein and initiation of cross-linking [181, 192].

Elastin is a protein with high longevity, and resistant to several proteases, but not to elastases. These proteases are responsible for the turnover of elastin, targeting amino acids with small hydrophobic chains [193]. Elastases are produced by pro-inflammatory

cells, but some bacteria can produce some potent elastases that mediate the infectious process [194, 195]. The degradation of elastin releases peptides capable of signalling to several cell types [196-199]. From these peptides, the most active biologically, Val-Gly-Val-Ala-Pro-Gly, regulates several pathways and processes, namely protein kinase C [200] and Ras-independent ERK1/2 pathways [201], as well as the G-protein associated opening of l-type calcium channels [202]. Abnormal elastin degradation can trigger responses and lead to pathologies. However, the major pathologies are related to elastin loss-of-function mutations, either leading to proteins that lack the capacity to assemble into fibres [203-205], or severely mutated proteins that are marked for degradation by regulatory mechanisms of the cells [206]. The Supravalvular Aortic Stenosis, the major elastin-related pathology, is autosomal dominant disease, and mutations leading to this condition include small deletions, removing multiple exons from the elastin gene, and nonsense or frameshift mutations [185].

Matrix Metalloproteinases (MMPs)

ECM dynamic environment results from the constant remodelling of its components, with a tightly regulated synthesis and degradation. While synthesis is under the responsibility of cells, like fibroblast, the degradation is performed by matrix metalloproteinases (MMPs) [207]. These tissue specific metallopeptidases, also known as matrixins, are capable of degrading virtually all ECM components, influencing tissue structure, growth factor release and cell migration [208]. Under normal conditions, MMPs present very low activity, being activated by the presence of cytokines, growth factors or hormones. MMPs untimely activation is associated with several pathological conditions [209], which is in accordance with these extracellular multidomain enzymes being primarily translated into inactive pre-proenzymes (Fig. 5).

These proteins typically present a signal peptide directing towards secretion, a 80 amino acids long propeptide, followed by a catalytic domain with Zn^{2+} affinity connected to a hemopexin-like domain through a linker, or hinge domain (Fig. 5 A). These proteins undergo ER-Golgi secretion, during which the signal peptide is excised, and are released in an inactive proenzyme state, proMMP. The propeptide presents a “cysteine switch” motif that binds to the Zn-binding domain and keeps the MMP inactive [208, 210]. The proMMPs activation is a tightly regulated step-wise process. The

propeptide presents a proteinase susceptible “bait sequence” that can be excised by several proteinases, both endogenous and exogenous. The initial propeptide degradation allows its complete removal by self-catalysis or by the action of other active MMP, and inherent enzyme activation, [210].

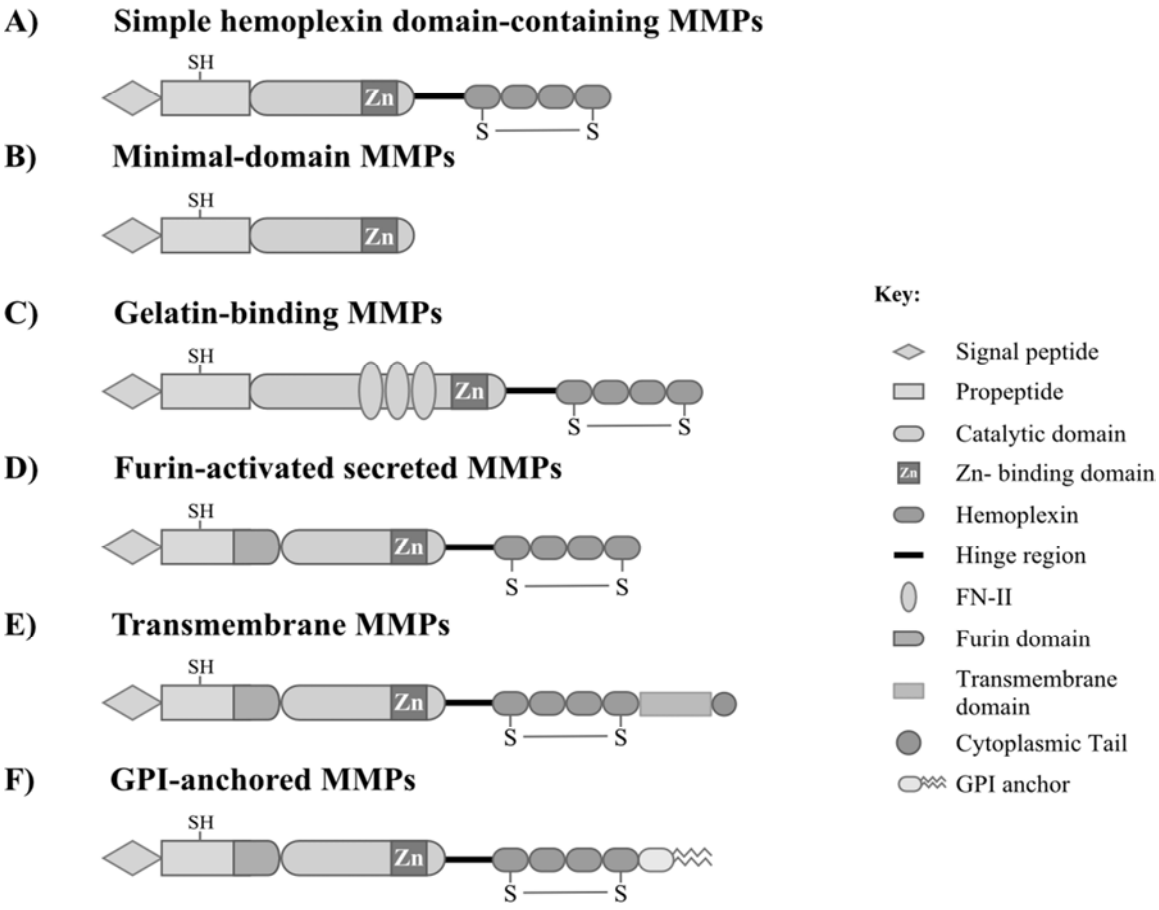


Figure 5. Matrix metalloproteinases (MMPs) structural organization. MMPs are present in the ECM either as secreted forms (A-D) or membrane tethered forms (E-F). Adapted from [211].

MMPs present structural diversity depending on their substrate. MMP-7 and -26, also known as matrilysins, do not present the linker and the hemopexin-like domain and are unable to degrade interstitial collagen (Fig. 5 B) [212]. Gelatinases, MMP-2 and -9, present three fibronectin type II domains within the catalytic domain (Fig. 5 C). These domains allow the cleavage of type IV collagen, elastin, and gelatins [210]. The

hemopexin-like domain is vital for the triple helical collagen unfolding. Collagenases mutated in this domain are able to degrade non-collagenous proteins but are unable to unfold and degrade collagen [213]. Some matrixins present a furin-recognition domain within the propeptide (Fig. 5 D), which allows the intracellular activation of the MMP by furin proconvertase, and subsequent secretion in an active form [118]. Some stromelysins, namely MMP-11, present the furin recognition site, are active both intracellularly and extracellularly. Membrane-tethered MMPs, both membrane spanning proteins (Fig. 5 E) and GPI anchored (Fig. 5 F), also present furin recognition site and are inserted in the cell surface in an active form. They present collagenolytic activity and are responsible for the processing of several proMMPs [210]. Given the MMPs profound impact in tissue organization, cell motility and overall ECM metabolism, cells present several mechanisms to regulate these proteinases activity, from timely regulation of cellular location to endogenous inhibitors, and ultimately proteolysis [210].

Endogenous inhibitors, like α-macroglobulin and tissue inhibitors of MMPs (TIMPs), allow a higher control and are the main regulatory system of MMP activity. α-macroglobulin is a high molecular weight tetramer. It inhibits most proteinases by entrapping the enzyme inside its four subunits and directing the complex for receptor-mediated endocytosis [210]. TIMPs are small proteins, 180-190 amino acids long, presenting a wedge-like structure divided in two subdomains, an amino- end domain and a carboxyl end domain. The N- terminal end domain slots into the MMPs active site and chelate the ionic zinc. TIMPs are able to inhibit MMPs to different extents. For example, TIMP-1 is a poor inhibitor of membrane tethered MMPs [207, 210]. As for other ECM proteins, engineered forms of MMPs inhibitors were generated to serve as pharmacological treatments for numerous diseases [209, 214]. Ultimately tissues homeostasis depends on MMPs and MMPs inhibitors constant interplay.

Proteoglycans and Glycosaminoglycans

Proteoglycans (PGs) and glycosaminoglycans (GAGs) are another important group of functional molecules of the mammalian ECM vital for structural and signalling purposes. PGs are composed of a core protein, substituted with one or more covalently attached GAGs. GAGs/mucopolysaccharides are long linear heteropolysaccharides composed of a hexosamine (glucosamine or galactosamine, frequently *N*-substituted)

and a hexuronic acid (glucuronic acid or iduronic acid), attached to the protein core through a conserved oligosaccharide [215]. The newly synthesized GAG chains may undergo several modifications: *O*-sulphation of hydroxyl groups, deacetylation and subsequent *N*- sulphation, and epimerization of glucuronic acid to iduronic acid. These linear mucopolysaccharides contribute the most for the PGs high size and weight, and as such its properties tend to dominate the chemical properties of the PGs [10].

Hyaluronan

Hyaluronan is in several ways exceptional in regard to the other GAGs. It is the only non-sulphated GAG, composed by a repetition of a dimer of glucuronic acid (GlcA) and *N*-acetyl-glucosamine (GlcNAc) (Table 2). HA is not attached to a peptide, although it interacts with several proteins presenting HA binding motifs, namely the PG hyalectans (Fig. 6) and the membrane receptors hyaladherins.

HA is synthesized in the plasma membrane, by opposition to the Golgi apparatus as happens with the remaining glycosaminoglycans [216]. The enzymes responsible for its synthesis, Hyaluronan synthases (HAS), are capable of establish both β-1,3 and β-1,4 linkages, and simultaneously export the newly synthesized HA chain to the extracellular space, through a pore constituted by the enzyme itself. Again in opposition to the other GAGs, the increase in size of the HA chain is obtained by addition of new residues to the reducing end of the chain [217]. There are several HAS with different properties and expression patterns, in particular their specificity as to the different size of the synthesized products [216, 218-220]. The HA turnover and production of HA fragments biologically relevant is the role of a different set of enzymes, the hyaluronidase (HYAL) family [219]. It is the concerted action of these different HAS and HYAL enzymes that maintain the amount and size of HA within physiological boundaries for ECM structural and functional homeostasis, *i.e.*, in the correct size to fulfil scaffolding or signalling tasks. The maintenance of HA homeostasis can occur through three different pathways:

(1) *local cellular turnover* - the specific HA membrane receptors being the main responsible molecules for the binding of HA that precedes internalization and degradation;

(2) *tissue level turnover* - where HA is drained into the vascular and lymphatic systems and guided to liver and kidneys for degradation;

(3) *free radicals-dependent turnover* - under oxidative conditions the scission of HA can be promoted by divalent cations [219, 221].

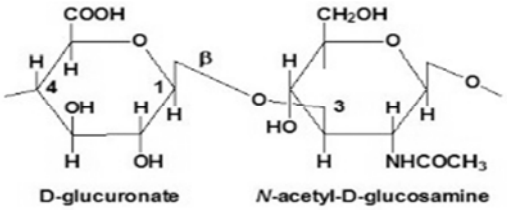
Alterations in this tightly regulated equilibrium between synthesis and degradation can lead to developmental defects or tumourigenesis [221-223]. HA is required for proper craniofacial development, as deregulation of its biosynthetic process leads to severe defects during *Xenopus laevis* embryogenesis [224]. On the other hand, increased HA overproduction promotes cell invasion and metastasis formation [225]. Besides its role in the disease progression, HA also presents several biomedical applications given its biophysical and biochemical properties, particularly in joint injury recovery [226], tissue engineering [223] or cartilage regeneration [227]. Additionally, it has been recognizably important in skin regeneration after burning or other serious injuries, as well as anti-ageing treatment, facial aesthetics, and other cosmetic applications. This non sulphated GAG is a vital ECM molecule, interacting with several cell surface receptors as well as other ECM molecules and regulating several cellular processes [219].

Sulphated Glycosaminoglycans

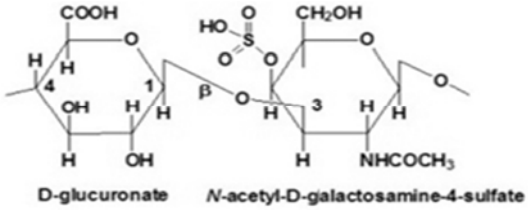
Chondroitin Sulphate (CS) is a sulphated GAG composed of GlcA and *N*- Acetyl-Galactosamine (GalNAc; Table 2). This GAG is found connected to a protein core through a conserved oligosaccharide linker – Xyl-Gal-Gal-GlcA [228] forming a CS-PG. Firstly, a xylopyranoside is added to a serine residue in the core protein, through the action of a xylosyl transferase in the Endoplasmic Reticulum (ER) [229]. Secondly, two galactose residues are sequentially added to the nascent chain [228]. Such process occurs in the early Golgi, and it is the result of the action of two different galactosyl transferases, Gal I and Gal II transferases [230]. Finally, the addition of GlcA occurs in the late Golgi by the action of GlcA I transferase [231]. When this process is completed, the CS chain grows by the alternate addition of GalNAc and GlcA. However, in opposition to HA synthesis, where a single enzyme performs all steps, the CS chain elongation requires the coordination of several enzymes that cooperate to catalyse each step [228, 232]. These newly formed chains can present several modifications; phosphorylation of the xylose residue in the linker oligosaccharide and sulphation of the

Table 2. Chemical composition of the glycosaminoglycans. Adapted from [233].

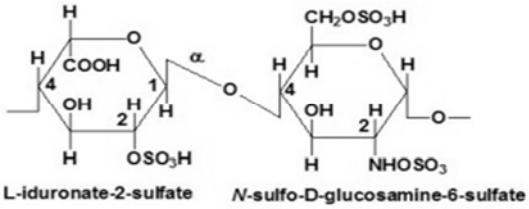
Hyaluronan



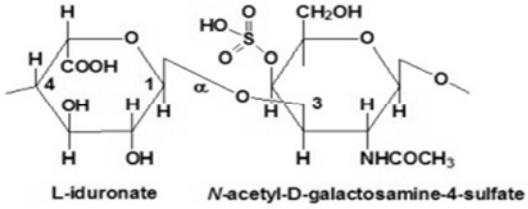
Chondroitin Sulphate



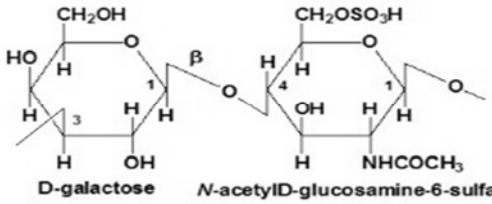
Heparan Sulphate



Dermatan Sulphate



Keratan Sulphate



GalNAc and GlcA residues are the most commons [232]. CS frequently presents 4- and /or 6-*O*-sulphation of the GalNAc, and 2- or more rarely 3-*O*-sulphation of the GlcA [228]. Sulphation introduces another level of complexity to the CS-PGs molecules. Fibroblast growth factors bind to highly sulphated CS, while proteins like netrin and semaphorins interact with CS in a sulphation pattern dependent manner [234]. Such selective binding process allows the control of several molecules availability, regulating several cellular processes. CS-PGs regulate processes so diverse as skeletal morphogenesis and cartilage organization or cell differentiation and motility [235].

The most common CS-PGs are members of the hyalectan subfamily, comprising aggrecan, neurocan, brevican and versican. These CS-PGs present HA binding motifs

and are involved in brain development and regulate tissue growth and plasticity [236, 237]. Brevican is associated with the myelination process [238], and it regulates the nodal matrix assembly [239]. Alteration of CS-PGs synthesis/degradation is associated with the disease process in cancer, with increased cell motility, angiogenesis and metastasis, and atherosclerosis, favouring lipoprotein oxidation and accumulation [240].

Dermatan sulphate (DS) is the main GAG in the skin and results from the epimerization of some GlcA residues in CS to Iduronic acid (IdoA; Table 2). Similarly to CS, DS is found connected to a protein core through a conserved oligosaccharide linker – Xyl-Gal-Gal-GlcA [228], forming DS-PG. Besides the IdoA epimerization, DS is also modified by 4- and/or 6-*O*-sulphation of the GalNAc, and 2-*O*-sulphation of IdoA [241]. These alterations, through a complex enzymatic system, result in three distinct hexuronic forms, and four possible hexosamine residues present in the chain, increasing the amount of information present within the sugar moiety. Such complexity is associated with the role of DS in the anti-coagulation process, as well as cell proliferation and migration, infection and wound repair [242]. DS is capable of forming a stable complex between serine protease inhibitor Heparin Cofactor II (HCII) and thrombin. Thrombin is a procoagulant protease that starts the blood-clotting cascade. The DS-mediated interaction of this protease with HCII inhibits this process [243]. DS also inhibits the clotting process by enhancing the activity of an endogenous inhibitor, the Activated Protein C (APC). APC interacts with different GAGs, but DS has the most potent effect on its activity [241]. Some studies describe a potent antithrombotic effect of DS [244-246], significantly higher than heparin. The correspondent exact mechanism still remains unknown [242].

DS-PGs are particularly expressed in skin, cardiovascular system and central nervous system [241, 242]. In skin, the normal tensile strength is regulated by the interaction between collagen fibrils and tenascin-X. This interaction is mediated by the DS-PG decorin. Decorin core protein binds to fibrillar collagen and tenascin-X binds to the sugar moiety. The disruption of this interaction results in increased skin fragility [247, 248]. Decorin role in atherosclerosis plaque formation was also described [249]. This proteoglycan interacts with low-density lipoproteins and helps its docking process to collagen. DS-PGs are also involved in arterial mechanical strain and inflammation-mediated angiogenesis [241]. In the central nervous system of patients with several

diseases, the levels of CS/DS-PGs are elevated, *e.g.*, in Alzheimer these PGs localize to the lesions [250, 251], being powerful enhancers of amyloid fibrillogenesis [252].

Heparan sulphate (HS) is the main GAG of the cellular surface, substituted in transmembrane and GPI anchored PGs, and heparin (highly sulphated HS) is the main GAG present in intracellular storage granules [253]. This sulphated GAG is constituted by a repeating disaccharide subunit comprising a Glucosamine residue and a GlcA/ IdoA residue, where some GlcA residues are epimerized during chain elongation to IdoA (Table 2). However, the glucosamine residue presents more possible chemical substitutions than other GAGs groups. It can be *N*- acetylated, *N*- sulphated or unmodified. These disaccharide units present variable *O*- sulphation. The glucosamine presents 3- and/or 6-*O* sulphation, whereas the IdoA residues may present 2-*O*-sulphation [254]. Similarly to CS and DS, the HS chain elongation occurs after the assembly of a Xyl-Gal-Gal-GlcA oligosaccharide linker. Firstly, a GlcNAc is transferred by the action of the EXTL glycosyl transferase family [255]. The three known isoforms are able to attach the first residue to the non-reducing end of the chain, but EXTL3 is the main isoform *in vivo* [256]. The chain elongation happens by the alternate addition of GlcA and GlcNAc residues, by the action of the HS polymerases Ext1 and Ext2 [254]. During chain polymerization several alterations occur. The *N*-deacetylation/ *N*-sulphation reaction appears to be the first, generating several *N*-Acetylated portions and *N*- Sulphated portions [257]. The epimerization of the C5 hydroxyl group of some GlcA residues, and 2-O sulphation of most of the resulting IdoA residues also appear to occur in an early step of the chain elongation, through a coordinated interaction of the responsible enzymes [258]. The 3- and 6-O sulphation of the glucosamine occurs after these initial steps. However, evidence suggest that sulphation of the growing HS chain stimulates the elongation process and results in increased chain length [259].

HS is present in the surface of every mammalian cell. It forms a polysaccharide coating that mediates many of the cell interactions with other cells, as well as with growth factors, chemokines, morphogens and enzymes. The dynamic modification of this envelope allows the cell to change its sensibility to some signals and modulate its responses. HS and heparin regulatory role is especially important in cellular processes, like membrane trafficking and signalling, or whole-organism processes, like embryogenesis, as well as in pathophysiological conditions, metastasis and angiogenesis

[253]. In fact, evidence shows that HS can behave as pro or anti-tumorigenic based solely in its presence as a membrane tethered PG or a free soluble GAG chain in the ECM [260]. Heparin presents high anti-inflammatory and antimetastatic activities [246, 261]. However, the main utilization of this GAG is as anticoagulant and antithrombotic, being commonly used to treat thromboembolic disorders [253]. This highly sulphated GAG interacts and mediates the inhibition of thrombin by the HCII [241] and the cell surface P-selectin recognition by platelets during metastasis formation [262]. Besides, HS also interacts with several ECM proteins through conserved sugar motifs, namely TGF-β or FGF family [263-265], promoting and mediating several protein-protein interactions, *e.g.* between the FGF 2 and its receptor [266]. HS-PGs also influence the Wnt morphogenic pathway and have a role in the embryo patterning [267], since deregulations of these PGs sulphation patterns are associated with developmental defects [268] and tumourigenesis [269].

Finally, keratan sulphate (KS) is a very unusual GAG since it does not present the uronic acid subunit, therefore consisting of a poly-N-acetyllactosamine, a linear chain of GlcNAc repetitions (Table 2). It is synthesized by the alternate and sequential steps of galactosylation and *N*-acetyl–glucosamylation, and followed by an extensive sulphation [270]. KS presents a wide array of chain sizes and sulphation pattern, leading to a great variability of the global charges of KS substituted molecules. It is attached to the core protein through several oligosaccharides, distinct from CS, DS and HS linker [271, 272].

KS is the main GAG in the cornea, where it regulates the collagen matrix assembly and the cornea water content, but it is also present in the brain and cartilage. The KS location is closely related with its organization, especially with its oligosaccharide linker structure [273]. KSI, the main glycoform in the cornea, is *N*- attached to an Asn residue in the core protein through a complex branched linker; the KSI chain elongates from the C6 branch of a mannose residue, whereas the C3 branch is capped with a lactosamine disaccharide and a syalic acid. This GAG presents two non-sulphated disaccharides in the reducing end, followed by 10-12 disaccharide units sulphated in the GlcNAc residue. Its non-reducing end consists of a domain of variable length (8-34 residues) composed of disulphated disaccharides. Nonetheless, the structure of this KS subtype and its modifications may be more tissue dependent than type specific. KSI from different origins, namely cartilage, can present elongation of the mannose C3 chain, fucosylation of some residues, as well as different sulphation pattern [274].

Another KS subtype, KSII, is attached to the core protein through a linker very similar to the mucin core-2, a GalNAc residue *O*-kinked to a Ser/Thr residue [275]. This KS subtype also presents a branched structure; the C3 of the GlcNAc is attached to a Gal residue and capped with a syalic acid, while the C6 elongates with sulphated lactosamine units. Typically, KSII is composed by 5-11 highly sulphated disaccharides; it consists almost entirely of disulphated subunits and some interspersed monosulphated disaccharides [276]. The KSII chain is terminated by a neuraminic acid residue, after a terminal GlcNAc; several of GlcNAc residues are fucosylated. KSII is found substituted only in aggrecan core proteins; this core protein present amino acid motifs in its sequence that correlates with KS substitution [277]. KS subtype III, KSIII, is present mostly in the brain, where proteins are frequently substituted with *O*- linked short lactosamines; KSII chain are attached to Ser residues through a *O*- linked mannose linker, and are considered an extended and sulphated versions of such glycoforms. Some points of evidence show a possible role of KS-PGs in Alzheimer's disease [278, 279], arthritis [280] and ocular defects [281].

Proteoglycans

GAGs are highly functional molecules, and tend to dominate the biochemical properties of glycoconjugates. Nevertheless, the protein core of each GAG will determine its distribution, function and molecular interactions, as well as performing regulatory functions PGs can be substituted by a single GAG, as decorin [282], or by several units of different GAGs , as versican and aggrecan [283, 284]. A few of these GAGs are excreted to the extracellular space, as the small leucine-rich PGs (SLRPs) or the large molecular weight aggrecan (Fig. 6). Some are membrane tethered, through a GPI anchor, like the heparan sulphate PG glypican, or by a membrane spanning protein core, as syndecan [10, 285]. The molecular diversity arising from the different combinations of PGs protein cores with one or more types of GAGs leads to a wide variety of biological roles.

a) Intracellular PGs

Serglycin, named after the serine-glycine rich motif along its sequence, is an intracellular PG stored in secretory granules, stored in secretory granules (Fig. 6) [286,

287]. This intracellular PG is mostly frequent in hematopoietic cells, mast cells and T lymphocytes [288], but can also be found in endothelial or smooth muscle cells.

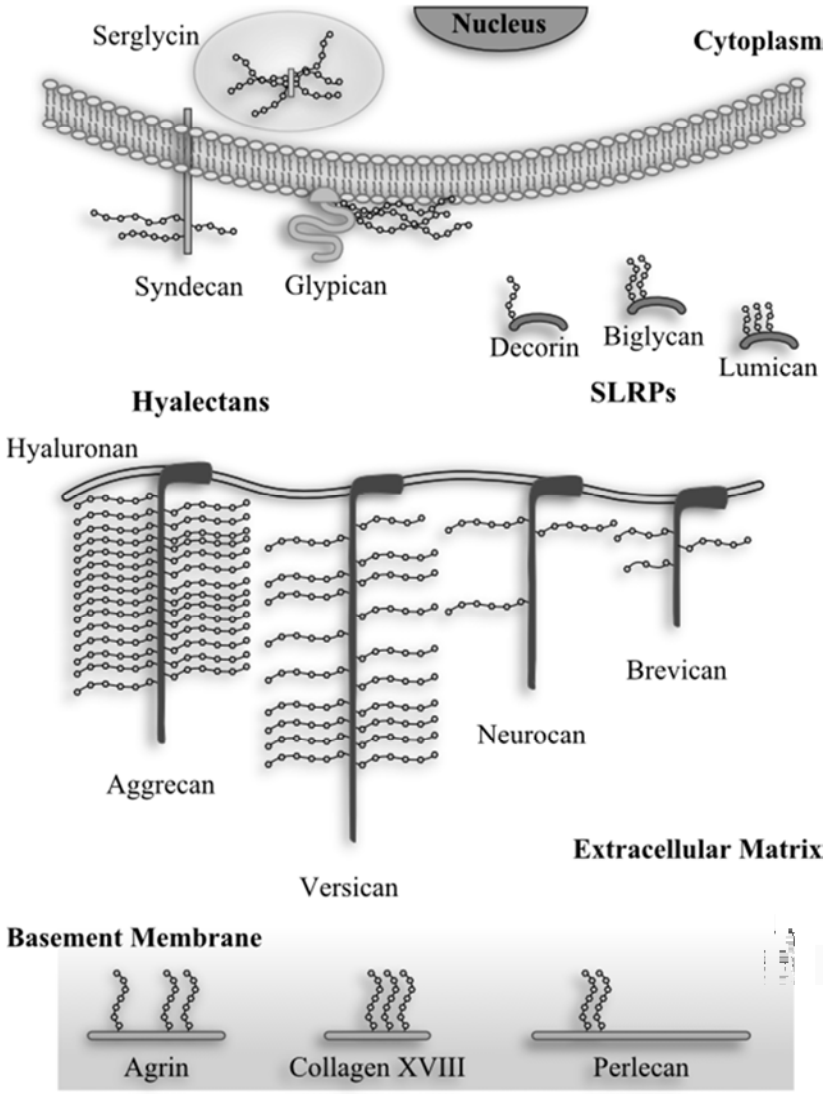


Figure 6. Proteoglycans families and their associated locations. All mammalian cells produce proteoglycans, which are then secreted, inserted into the membrane or stored in secretory granules. The two major families of the surface-associated proteoglycans are syndecans, transmembrane heparan sulphate PGs, and glypicans, GPI-anchored PGs. Serglycin is an intracellular PG, substituted with heparin chains, found in secretory granules of mast and endothelial cells. The secreted extracellular PGs include the hyalectans – aggrecan, versican, neurocan and brevican – which present hyaluronan binding domains, the SLRPs – decorin, biglycan and lumican – which interact with several ECM proteins, namely collagen, and basement membrane PGs, agrin and collagen XVIII and perlecan. Adapted from [289].

Serglycin was initially labelled as a macromolecular form of heparin [290], and later described as a protease resistant core protein for CS and heparin substituted intracellular PGs [291]. All serglycin family members present a highly conserved amino end sequence and a characteristic long extension of Ser-Gly repeats [286]. These Ser residues act as GAGs substitution sites and their close location originates a densely substituted PG, the presence of a tightly packed sugar coat is involved in the protease resistance [291].

Serglycin PGs present a wide variety of GAGs substitution and sulphation degree; mast cells present serglycin substituted with highly sulphated heparin, whereas circulating cells, like T lymphocytes, present less sulphated GAGs [287]. This intracellular PG is very important for granulopoiesis, the formation of intracellular secretory granules [286]. These granules are important for the storage of preformed molecules, such as histamine, serotonin and several proteases, enabling the quick release of immune active molecules in response to certain stimuli, namely inflammation. The high anionic charge of this heparin substituted PG seems vital for the formation and organization of these granules; the storage of several molecules is dependent of interactions with the GAG chains [287]. The activation of serglycin storing cells, namely mast cells, leads to the secretion of this PG and its associated molecules. Several molecules remain associated with serglycin GAG chains after secretion; this provides protection from proteolytic cleavage or enables selective substrate presentation. Alterations on serglycin synthesis or sulphation result in alterations of secretory granules morphology, changes in the typical electron dense regions and metachromatic properties [288, 292], affecting the storage of several molecules [293].

Accordingly, the lack of *N*-deacetylase/ N-sulphotransferase, the enzyme responsible for IdoA 2-*N*-sulphation, leads a phenotype similar to serglycin deficiency [294]. Animals lacking serglycin synthesis present several immune defects, whereas several myeloma cell lines present increased serglycin expression. Myeloma derived serglycin seems to interfere in bone mineralization, with frequent reports of myeloma associated osteoporosis. Altered expression of this PG is also associated with nasopharyngeal carcinoma, associated with poor prognosis, or acute myeloid leukaemia.

b) Membrane tethered PGs

Membrane tethered PGs comprise single spanning transmembrane syndecans and GPI-anchored glypicans (Fig. 6). These PGs interact with several cytokines, chemokines, growth factors and morphogens, through HS and CS chains, and act as reservoirs of such molecules [295]. The selective degradation of these chains allows the formation of morphogens functional gradients, with a significant role in embryogenesis and morphogenesis [296, 297]. Syndecan and glypican cooperate with several cell surface receptors, namely integrins, acting as co-receptors and modulating several cell-cell and cell-matrix interactions [298]. These PGs play an important role in the internalization of several bound ligands, relevant for lipoprotein metabolism in liver [299]. While these PGs share some functional roles, mostly due to similar HS substitution, they present inherent structural and functional differences.

Syndecans are type I transmembrane core proteins that present HS or CS substitutions (Fig. 6). This PG family is composed of four members, syndecans 1-4, that share a conserved structure. These PGs present a short cytoplasmic tail, followed by a single spanning transmembrane domain, and an extracellular domain, ectodomain, presenting three to five GAG attachment sites [300]. The cytoplasmic tail presents two conserved regions (C1 and C2) interrupted by a variable region (V). The region C1, immediately after the membrane, is highly conserved in the four members of this family[301]; it is vital for dimerization of syndecans [120] and interactions with several proteins, namely ezrin and Src kinase [302, 303]. The other conserved region, C2, comprises the distal portion of the cytoplasmic tail; it presents two conserved tyrosine residues and a post synaptic density-95/ disc large protein / zonula occludens-1 (PDZ)-binding site at the carboxyl end. Such PDZ-binding site is extremely important for interactions with several proteins presenting PDZ domains, as syntenin, synectin, synbindin and calcium/ calmodulin dependent serine protein kinase (CASK/LIN-2) [304-307]. The variable region is highly heterogeneous. Syndecan-4, the best studied case, presents a phosphatidylinositol-4,5-biphosphate (PIP_2) binding site involved in dimerization and syndecan recycling [308]. The transmembrane domain is composed of a single transmembrane span with a SDS resistant motif, GXXXG. This motif is highly conserved and enhances the dimerization of syndecans [309]. The ectodomain presents a small motif responsible, contiguous the membrane surface, for enhancing self-

association and interaction with several proteins, promoting protein kinase C activation [310, 311].

Syndecans interact with a wide range of functional molecules, growth factors and morphogens, regulating and modulating several vital processes, namely wound healing, inflammation, angiogenesis or neural patterning [312]. Most of the functions associated with syndecan are performed by its CS or HS chains. However, the ectodomain is capable of interactions with several ECM molecules, as well as with cell surface receptors [313, 314]. MMPs promote the proteolytic release of some of the membrane bound syndecan [315], reducing transmembrane signal transduction [316]. Ectodomains proteolytic shedding produces soluble molecules that compete with bound syndecan for the same ligands [317]. Shed syndecan is present in high amounts in fluids surrounding lesions, regulating inflammation during wound healing [318]. Syndecans modulate chemokine gradients an regulate leukocyte recruitment during inflammatory response [319]. In colitis animal model, animals deficient for syndecan-1 show prolonged and excessive leukocyte recruitment [320]. These PGs modulate the action of several growth factors, as well as morphogens, and disruptions of such processes are associated with tumour progression. Syndecan expression is altered in prostate [321], breast [322] or colon [323] cancer. Low syndecan-1 expression is associated with worst prognosis in lung [324] and colorectal cancer [325], while myeloma presents high amounts of these proteins [326, 327]. Being important cell surface components, syndecans are also target by virus, bacteria and parasites during infection [319].

Glypicans are HS-PGs tethered to the outer membrane leaflet through a GPI anchor (Fig. 6) [328]. Glypican proteins are encoded by 6 known genes in humans and several homologues across metazoa. Glypican proteins usually present 555-580 amino acids and are divided in two subgroups, due to sequence similarities: glypicans -1, -2, -4 and -6 compose subgroup I; whereas glypicans -3 and -5 belong to subgroup II. These PGs present 14 conserved cysteine residues that stabilize the chain through disulphide bonds [329]. However, there is no crystallographic data available and the three-dimensional structure is unknown [328]. Glypicans present a cysteine rich domain (CRD), which presents a weak homology to the cysteine-rich domain of Frizzled proteins [330]. This domain is frequently subject of proteolytic cleavage by a furin-like convertase, by which way a core protein may be produced, composed of two subunits bound together by disulphide bonds [331]. Glypicans are HS substituted near the membrane, maybe

mediating interactions with other cell surface molecules. These GPI anchored proteins are distributed by the lipid ordered domains, lipid rafts, across the membrane, but, unlike other GPI proteins, a significant amount of glypican can be found outside rafts, which may be due to GAG chains interactions [332]. The GPI anchor may be cleaved by the action of a lipase [333], producing soluble forms that regulate the formation of several signalling molecules gradients.

Glypicans interact with several growth factors, namely Fibroblast Growth Factor and Bone Morphogenetic Proteins (BMPs), but are particularly important in the regulation of Wnt and Hedgehog (Hh) morphogenic pathways [296, 334]. The shedded forms of glypican are vital for Wnt and Hh transport and gradient formation, promoting or inhibiting these signalling pathways. Glypican promotes Wnt signalling, by stabilizing the interaction between Wnt and its receptor Frizzled [334, 335], but it inhibits Hh signalling by competing with Patched, the Hh receptor, for Hh binding [296]. Given its important role in several signalling pathways, it is not surprising the role of altered expression of glypicans in pathophysiological processes [336-339]. Glypicans seem involved in prion protein conversion in lipid rafts, with evidence of interactions of prions with Glypican GAG chains [338, 340]. Mutations in Glypican -3 and -4 are associated with a overgrowth syndrome, Simpson-Golabi-Behme [336], which includes both prenatal and postnatal overgrowth due to increased cell proliferation and alteration in apoptosis, a process regulated by these glypican PGs [337, 341]. Similarly, the increased cell proliferation and apoptosis repression in the absence of glypican has been described in several cancers, as mesothelioma, ovary, and breast cancers cancer [337]. Nevertheless, overexpression of glypican is associated with hepatocellular carcinomas [342].

c) Secreted/ECM PGs

Several PGs are secreted to the ECM (Fig. 6). These include the SLRPs that associate with collagen fibrils, the hyalectans that bind to HA, and the basement membrane PGs that are responsible for the basement membrane homeostasis. SLRPs are ubiquitous PGs, presenting relatively small core proteins (36-42 kDa). These PGs are subdivided in five different groups, based on evolutionary conservation, structural homology and chromosome organization [343]. Typically, these PG present a N-terminus CRD, four cysteine residues separated by a variable number of amino acids,

several leucine rich repeats (LRRs) and a C- terminus capping motif encompassing two LRR and the canonical “ear repeat” [344, 345]. LRRs are subunits of 24 amino acids with a characteristic pattern of hydrophobic residues, forming a short β-sheet. These repeats assemble into a curved, solenoid structure composed of several short β-sheets, connected by a turn and a more variable region[343]. SLRPs are able to interact with several ECM proteins by a LRR mediated process, where the inner side chains of the residues composing the β-sheets interact with specific proteins. According to the last review on these proteins classification [343, 344], SLRPs are organized into five distinct groups.

SLRPs Group I includes decorin, biglycan, asporin and ECM-2. These present the typical CRD forming two intrachain disulphide bonds, the “ear repeat” between the last two LRRs, and can be substituted by CS or DS. Asporin lacks the typical peptide motif required for glycanation [346, 347]. GAG substitution seems to be tissue specific. Decorin and biglycan mostly present CS chains in bone, while in skin are substituted by DS [343]. Decorin is the model SLRP. its protein core is a Zn^{2+} metalloproteinase that binds to collagen α1(I) and helps maintain both intrafibrillar space in corneal collagen, essential for transparency, and mechanical coupling in tendon and skin [348]. Decorin interacts with fibrillar collagen in a periodic manner, forming a surface coat in fibril, which coat regulates proper fibril assembly and protects collagen from degradation, limiting MMPs access to cleavage sites. Asporin also binds to collagen I, and it competes with decorin for the same binding sites. However, decorin interacts with collagen through its LLR 7; whereas in asporin, these interactions are mediated by LRR 10-12 [349].

SLRPs Group II members - lumican, fibromodulin, PRELP, Keratocan and osteoadherin - are typically substituted by KS or polylactosamine, an unsulphated form of KS, and present a tyrosine clusters at the amino end. Fibromodulin and lumican also associate with collagen, regulating its fibril assembly. These SLRPs interact with collagen fibrils through the same LRR 5-7, but only fibromodulin intervenes in the process of fibril maturation.

SLRPs Group III comprises epiphycan, opticin and osteoglycin. These SLRPs present a relatively low number of LRRs and may exist as glycoproteins. Osteoglycin is typically substituted by KS, while epiphycan may present CS and DS substitutions. As for opticin, it does not present GAG chain.

SLRPs Groups IV and V, considered non canonical SLRPs, main distinctive features are the lack of “ear repeat” and the absence of GAG substitutions [344]. Group IV features chondroadherin, nyctolopin and the recently described tsukushi [350]. Chondroadherin presents a KS substitution, being the only member presenting a GAG chain. Nyctolopin, implicated in stationary night blindness, is the first GPI-anchored SLRP described [351]. Group V is composed by podocan and the highly homologous podocan-like protein 1. These SLRPs present a distinct amino end cysteine rich motif and 20 LRRs very similar to those of groups I and II [345].

As mentioned, several SLRPs are associated with collagen assembly, and as such, mutations in these PGs are associated with skin and cartilage diseases and ocular defects. A particular asporin mutation, D14, is associated with high risk of osteoarthritis in Asian individuals [347], whereas mutations of lumican are associated with myopia [352], and alterations in keratocan cause *cornea plana,* a condition ultimately resulting in hypermetropia and astigmatism [353]. Decorin deficiency, for instance, is connected with enhanced skin fragility, and decorin truncated forms are associated to congenital dystrophy of the cornea [354].

Hyalectans are a small family of PGs family known for interacting with HA (Fig. 6) [236]. These high molecular weight PGs comprise aggrecan, neurocan, brevican, versican and neurocan. These CS substituted molecules are particularly abundant in cartilage and neural tissues [236]. These PGs, also known as lecticans, feature two globular regions, at C- and N- termini, interconnected by a structurally diverse central domain, presenting attachment sites for CS [355]. Such globular regions present several functional domains that promote and regulate several interactions. The N- terminal region presents the HA binding motif [356]. The amino end globular region, also known as G1 region, features an immunoglobulin-like loop and two link protein-like tandem repeats, also named proteoglycans tandem repeats (PTRs). The immunoglobulin-like loop presents two conserved Cys residues, while the PTRs present 4 conserved Cys. The PTR domain form a double loop stabilized by disulphide bonds between these residues [236]. The HA binding activity of these domains is dependent of a proper protein folding, and the presence of both PTRs, as a single PTR does not present significant HA binding [357]. In cartilage, the G1 region also presents the binding site for the cartilage link protein; this 50 kDa glycoprotein stabilizes the complex of aggrecan and HA [236].

Aggrecan presents an extra globular region (G2), containing two additional PTRs but without the immunoglobulin-like loop. The carboxyl end globular region (G3) comprises a C-type lectin domain flanked by two EGF-like repeats and a complement regulatory protein-like motif (CRP) [356]. These domains are also found in the some adhesion molecules, namely the selectin family, although organized in a distinct manner [358]. The C- terminus globular region interacts with simple sugars, as fucose, galactose or GlcNAc [359] [360, 361], and other GAGs [362] through the C-type lectin-like domain. The C-type lectin like domain also binds with tenascin-R, through interactions with tenascin-R FN repeats [361]. The lecticans central domain presents high variability in size and sequence. Brevican presents a 300 amino acids long sequence, versican splice variants can lack the entire central domain or present a 1700 amino acid long sequence. This domain presents all the potential CS-GAG chains attachment sites; aggrecan presents 120 sites, versican can be substituted at 20 sites, neurocan features seven attachment sites and brevican only presents three potential substitution sites [236].

Lecticans are widely present in metazoa organisms. Aggrecan is the main PG of the cartilage, neurocan and brevican are the most abundant proteins of the central nervous system, and versican is present in most connective tissues [236, 355, 356]. Lecticans, as other ECM molecules, are subject of MMP cleavage, yielding new functional molecules. For example, the glial HA-binding protein is a 60 kDa fragment of versican [363]. Alterations in these proteoglycans turnover are associated with pathophysiological conditions. The abnormal degradation of aggrecan by a non-MMP enzyme is associated with the pathogenesis of osteoarthritis [364, 365]. Lecticans, mostly neurocan and brevican, are expressed in the central nervous system and regulate cell adhesion and migration, controlling and guiding axon growth [239, 366]. These PGs form a barrier to guide axon development and neural crest cell migration [367]. Deregulations in hyalectans synthesis and turnover have been associated with Alzheimer's disease [368], prostate cancer [369] and vascular disease [370].

Basement membrane PGs interact and modulate the action of several growth factors, influencing processes like angiogenesis and keeping basement membrane homeostasis [371]. These HS substituted PGs influence cell adhesion, interact with cell surface receptors and regulate angiogenesis [372]. The best characterized basement membrane PGs are: the hybrid collagen PG from the multiplexin family, Collagen XVIII [373, 374]; the high molecular weight modular PG, perlecan [375, 376]; and the

neuromuscular junction PG, agrin [377]. These PGs have an especially active role in the modulation of angiogenesis; their GAG chains can either promote angiogenesis - binding growth factors and presenting them to cell surface receptors – or inhibit this process - restricting growth factor diffusion and inhibiting signalling [372]. Proteolytic cleavage of carboxyl end domains of these PGs can release new functional molecules with potent anti-angiogenic activity, as endostatin and endorepellin [63, 378, 379]. Collagen XVIII is a member of the multiplexin subfamily of collagens; the first evidence of its role as PG came from the presence of several Ser-Gly residues in NC domains [380]. These PGs plays a crucial role in eye development and ocular functions; animals deficient for collagen XVIII expression present iris disruption and degeneration of retinal epithelial cells [381, 382]. Alterations in the synthesis of collagen XVIII result in structural defects in the basement membrane; collagen null animals present basement membrane thickening [383], and collagen XVIII alterations in the choroid plexus basement membrane leads to abnormal accumulation of cerebrospinal fluid in the ventricles of the brain, hydrocephalus pathology [384]. Collagen XVIII is widely distributed through vasculature basement membranes, but the absences or mutation of collagen XVIII is only associated with defects in the eye vasculature [385]. The lack of collagen XVIII, and consequent lack of endostatin, is associated with increasing of angiogenesis [386]; however, some reports also showed that the absence of this PG is connected with neovascularization and maintenance of vascular permeability [387]. Perlecan is one of the largest natural proteins, 470 kDa, and can reach 800 kDa when substituted with GAG chains [372]. This PG presents 46 functional domains organized in five modules; these domains, as well as GAG chains, interact with several basement membrane growth factors as FGF, vascular endothelial growth factor (VEGF) or platelet-derived growth factor (PDGF) [371]. Perlecan is present in the basement membrane of most epithelia and endothelia [388], especially vascular endothelia; but it is also present in avascular tissues like cartilage [389] or connective tissue stromas [390]. The presence of perlecan in the cell surface enables the interaction with cell receptors, as integrins, and the modulation of their ligands [388]; perlecan regulates the action of Hh and FGF during the synthesis of cartilage [376]. Perlecan is vital for the integrity of basement membranes; almost half of the animals deficient for perlecan die from haemorrhage in the pericardial cavity, this happens due to the deterioration of the basement membrane in regions of increased mechanical stress [391]. In humans,

mutations in perlecan lead to cartilage and brain defects and are associated with severe dwarfism [392]. In cancer, perlecan supports tumour blood vessels development [393]; deletion of perlecan or perlecan GAG chains leads to decreased tumour growth and angiogenesis [394]. Agrin is a modular PG that, similarly to perlecan and collagen XVIII, presents an anti-angiogenic carboxyl terminal module, endorepellin [379]. This PG is involved in the acetylcholine receptor clustering [395], being special relevant in neuromuscular junction [377]. Agrin is highly expressed in the brain, lungs and kidneys [396], especially in the blood vessels around these organs [397, 398]. Agrin I extremely important for the proper establishment of neuromuscular and immunological synapses [377, 398]; animals lacking agrin die neonatally due to respiratory failure from impaired diaphragm excitation [399]. This modular PG presents four splicing sites; a splice variant present a transmembrane domain at the amino end [371]. Altered distribution of agrin has been associated with Alzheimer's disease [400, 401] and its presence in liver has been used as an angiogenesis marker [402].

Secreted Morphogens

In higher eukaryotes, each cell must acquire positional information that specifies its relative position in tissues, organ and body. In embryonic development, or any other major tissue rearrangement, such information is delivered to the cell through proteins secreted to the ECM – morphogens [403]. These proteins are capable of both short-range contact-dependent signalling and long distance communication [404]. Morphogens control critical processes during embryogenesis, as patterning, differentiation, proliferation, and cell fate, and their malfunction underlies severe developmental defects and many types of cancer [405, 406].

Several morphogen families are currently known, including Hedgehog (Hh), Wingless-Int (Wnt)[1], and Bone Morphogenetic Protein (BMP). Each of these morphogens corresponds to signalling cascades, known to control specific processes, but sharing some degree of cross-talking, this way fine-tuning the processes together.

[1] The name Wnt was coined as a combination of Wg (wingless) and Int. The Wg gene had originally been identified as a segment polarity gene in *D. melanogaster* which functions during embryogenesis and adult limb formation. The INT genes were originally identified as vertebrate genes near several integration sites of mouse mammary tumor virus (MMTV). The Int-1 gene and the Wg gene were found to be homologous, with a common evolutionary origin evidenced by similar amino acid sequences of their encoded proteins.

Hedgehog family of morphogens comprises the original Hedgehog protein from *Drosophila melanogaster* (Rasp), and three members from vertebrates, the Sonic (Shh), the Indian (Ihh) and the Desert (Dhh) Hedgehog proteins. Shh is especially important in limb bud development, and notochord patterning. The bone and cartilage development are under the control of Ihh, which is partially redundant with Shh. Dhh mediates the germ cell development in the testis, as well as the peripheral nerve sheath formation [407]. Most information regarding these proteins processing and secretion is yet derived from the Hh from *D. melanogaster* Rasp and the vertebrate homologue Shh protein.

The Hh morphogen is a highly post-translationally modified protein. It is translated as a 45 KDa precursor (Fig. 7), which undergoes intramolecular processing to yield a secreted 25 kDa C-terminal fragment (Hh-C), and a 20 kDa N-terminal fragment (Hh-N) attached covalently to a cholesterol moiety in its C-terminal [408-412]. The cholesterol modification allows association of Hh-N with the cell membrane and is essential for proper Hh function and secretion [411, 412]. Actually, the cholesterol modification is indispensable to operate the nucleophilic attack that further allows transesterification-mediated cleavage of the peptide bond between two highly conserved amino acid residues, Cys-Phe. The Hh signal secreted to the ECM is further modified through palmitoylation of its N-terminal (Fig. 7) [411, 413]. *In vitro* studies show that this modification can be performed before or after the cholesterol attack [414]. The Hh palmitoylation is operated by the Hedgehog Acyltransferase (Hhat) from the MBOAT family of membrane bound *O*-acyl transferases [415]. On the other hand, the highly similar protein Hedgehog Acyltransferase-Like (Hhatl) regulates negatively Hh signal by competing with Hhat to bind the Hh ligand, rendering the palmitoylation impossible [416].

The secretion of processed Hh-N (Fig. 7), hereafter named simply as Hh, is mediated by the membrane-spanning Dispatched (Disp), a conserved protein with similarities to transmembrane transporters, that presents a SRR that interacts with the cholesterol moiety [417, 418]. In organisms lacking Disp, the secreting cells retain Hh and all its elicited responses are lost, except for the cell-cell communication mediated by the membrane-tethered Hh [417].

The above mentioned lipid moieties are critical for Hh-N association with the membrane and secretion. In the absence of cholesterol modification, Hh does not

associate with cell membrane, which leads to an enhanced spreading of the Hh-N signal, and abnormal long-range signalling, originating severe development defects [419-421].

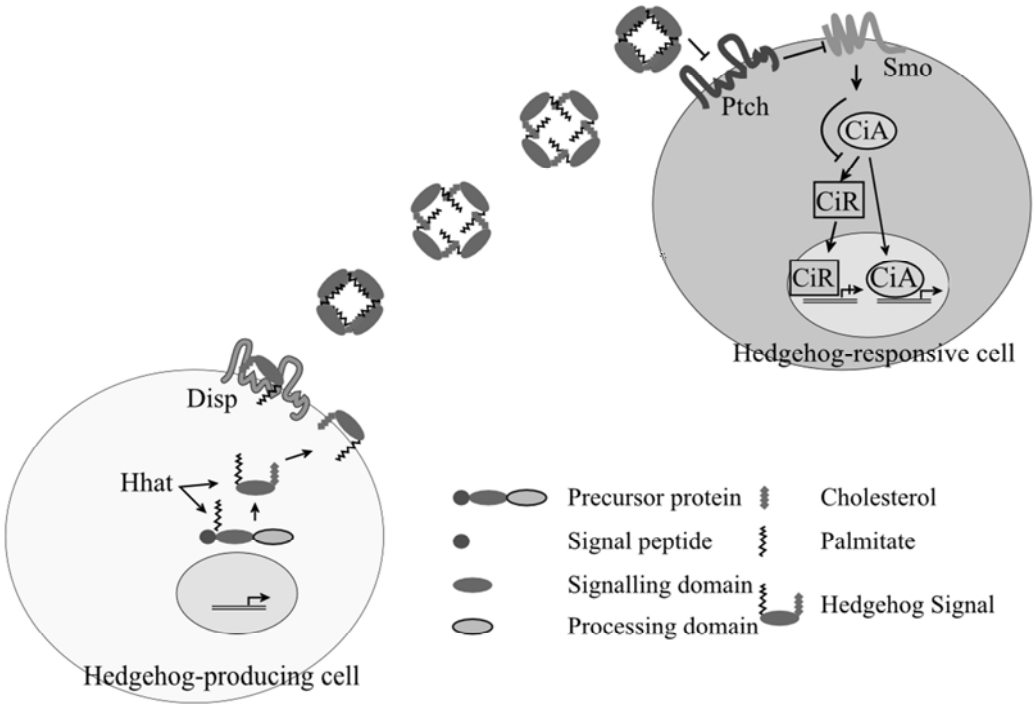


Figure 7. Hedgehog protein lipid modification and signalling. In Hh-producing cells, the Hh precursor protein undergoes processing, yielding a cholesterol-modified signalling domain and a processing domain that is ubiquitinated and directed for degradation. The molecule is further processed by the action of Hhat that transfers a palmitate to the amino end of the molecule; this step can be performed before or after the autoprocessing step. The mature hedgehog signal is directed for the plasma membrane and it is inserted in the lipid rafts. The transmembrane protein Dispatched helps the secretion of the signals as multimeric complexes, which are soluble and can be detected in the extracellular environment. Hh multimeric complex is likely the vehicle to be transported to Hh-responsive cells to achieve long-range signalling, although the mechanism by which transport is achieved is unknown. The Hedgehog signal binds to the Patched transmembrane protein of the-responsive cell and relieves the Smo inhibition. This promotes the transcription of the Hedgehog response genes. Adapted from [422].

Nevertheless, Hh peptides associated with the membrane are only capable of juxtacrine signalling. The release of Hh-N to the ECM is indispensable for long-range signalling which is diffusional gradient-dependent (Fig. 7). After secretion, the Hh-N concentration gradient is mediated by several ECM macromolecules, namely HSPGs

and Hh-interacting protein (Hip). Embryos lacking the HS synthesizing enzymes from the EXT family do not present Hh migration across the tissues [423, 424]. Whereas Hip binds to Hh with high affinity and restricts its diffusion, regulating its action range [425]. The Hh peptide elicits a response when encounters a cell expressing the membrane receptor Patched (Ptch), a 12 span transmembrane protein with some homology to the bacterial RND transmembrane transporter family [403]. However, the transcriptional response to Hh depends on the activity of the seven-span membrane protein Smoothened (Smo) [426, 427]. The Ptch protein inhibits activation of Smo and subsequent downstream signalling (Fig .7). This process is not fully understood in mammals, but it has been characterized in *D. melanogaster*. In the absence of Hh ligand, the protein cubitus interruptus (Ci) is retained in the cytoplasm by the complex formed by Costal-2 (Cos2), Fused (Fu) and "Suppressor of Fused" protein (SuFu). The Ci protein is phosphorylated by protein kinase A (PKA), Glycogen synthase kinase 3 beta (GSK3β) and casein kinase I (CKI), a process mediated by Cos2, and associates with the Slimb/βTrCP E3 ubiquitin ligase. The complex is directed to the proteasome, where Ci is processed to a repressor form (CiR), and represses the expression of Hh-target genes [428]. Ultimately, the activation of the Hh elicited response depends on the inhibition of Ptch (Fig. 7). When Hh is present, the ligand binds to the two large extracellular domains of Ptch, and blocks the action of Ptch on Smo [429]. The Hh association with Ptch is enhanced and stabilized by the action of membrane tethered glypican [430], and by the "Cell adhesion molecule, down-regulated by oncogenes" (CDO) and "brother of CDO" (BOC) membrane proteins [431]. In *D. melanogaster*, the activation of Smo occurs by phosphorylation of 26 serine/threonine residues of its carboxyl end cytoplasmic tail, by PKA and CKI [432, 433]; however, none of these residues are conserved in mammals [434]. The activated Smo accumulates in the membrane, leading to an enhanced association with Cos2/Fu. The association with Smo inhibits the Cos2/Fu/Sufu mediated processing of Ci; the full-length protein enters the nucleus as a transcriptional activator (CiA) and promotes the expression of Hh-target genes [403, 428], including the genes coding for Ptch, Patched 2 (Ptch2), HIP1 and GLI1(vertebrate homologue of Ci) that participate directly in the pathway [435-437].

In each tissue a specific sub-set of genes are activated. In the *D. melanogaster* wing development, Hh gradient promotes the differential activation of several genes, namely *decapentaplegic* (dpp), *collier* and *engrailed* [438]. Some studies show that the

activation of some genes require distinct ratios of CiA/CiR [439, 440]. Different concentration of Hh ligand might lead to distinct intracellular levels of Ci processing [441, 442], creating a Ci activity gradient that further increases the signalling gradient and enhances tissue patterning. Some studies show that the time of exposure to Hh ligand may be just as important [443-445]. All this information leads to the proposal of a model in which the Ci/GLI activity behaves as a component in the regulatory networks mediating patterning. Both the level and timing of Ci/GLI activity influence when and where genes are activated [446].

Hh signalling also induces the response from other signalling pathways, namely Wnt and BMP as referred above. The abnormal appearance of *D. melanogaster* larvae lacking Hh is the result of the loss of reciprocal feedback between cells expressing the Wnt family member Wingless (Wg) and Hh [447]. The reciprocal feedback helps to maintain the expression of these proteins along the anterior-posterior axis, guiding the embryo development. Similarly, in vertebrates limb development, a set of feedback signalling loops of Shh, BMP and Gremlin 1 (Grem1) regulate limb outgrowth and patterning, while the expression of Grem1 is stimulated by Shh, limiting the BMP signalling [448].

Besides its role as a morphogen, Hh is also a very important player in the modulation of numerous tissue progenitor and stem cell populations [449]. In the central nervous system (CNS), Hh signalling is important in the regulation of adult neural stem cells, providing continuous supply of new neurons [450, 451]. However, in the developing cerebellum the Hh signalling elicit a different response. The Shh ligands secreted by Purkinje cells will promote the proliferation of granule cell precursors, and activate the expression of several stem cell and proliferative genes, including genes encoding MYC, cyclin D1, insulin-like growth factor 2 and BMI1 [452, 453]. Hh has also been associated with the maintenance of adult stem cells in several tissues, from the hair follicle to the haematopoietic system, but more importantly, it is involved in injury healing, modulating the injury-dependent regeneration of numerous organs, namely exocrine pancreas, prostate and bladder [449].

The critical role of Hh signalling in the numerous developing tissues and organs is intimately connected with several severe congenital abnormalities that result from genetic defects in the pathway components [407]. Some defects are ligand-independent, including constitutive activation or repression of some pathway component; whereas

others are ligand-dependent, namely abnormal ligand secretion or diffusion. The heritable Gorlin's syndrome belongs to the former; this pathology, also known as nevoid basal cell carcinoma syndrome, presents a mutation in the *PTCH* gene that blocks the Ptch-mediated Smo inhibition [454, 455]. Patients with this syndrome presence a high incidence of the skin cancer basal cell carcinoma and the cerebellum cancer medulloblastoma [407]. The abnormal production of Hh ligand by tumour cells themselves,[456] promoting proliferation and tumour growth [457]. Hh signalling may work in two different ways; the autocrine and juxtacrine Hh signalling may mediate communication and promote cell growth inside the tumour [458], but the tumour cells may also signal to the surrounding environment, which will then signal back and also promote cancer progression [459]. Some drugs that target the Hh production have been used to treat some ligand-dependent cancers [460]; however, the simple ligand inhibition does not completely blocks cell proliferation, suggesting a more complex signalling network promoting the tumour growth.

Morphogens were subject of intense study in the last decade, however, there is still much to be understood. A recent review [407], summarized the current 10 great questions about Hh signalling and its physiological role. From the mechanism that allows Hh diffusion in tissues to the mechanism of Smo inhibition by Ptch or the tissue specific gene regulation after Hh elicitation, much needs to be unveiled until we have a clear picture of all the mechanisms and roles of Hh signalling and the putative influence of the other ECM components in this.

Microbial ECM – *the slime of the slimy microbes*

Life forms come in all shapes and sizes, and microbes are surely counted among the smallest and simplest. Nevertheless, these "simple" organisms present a wider ecological distribution than other more complex life forms, being capable of surviving and growing in the presence of high concentrations of certain chemical compounds or extreme temperatures and pressures (reviewed in [461-463]).

The term "microorganism" is commonly associated with simple unicellular entities swimming freely in watery environments, contaminating food, or causing disease. However, these solitary planktonic cells rarely exist in nature. Over 90% of all microorganisms on Earth live as multicellular communities [464-466], organized distinctly as biofilms [465], colonies [467, 468] and UV-induced stalks [469, 470]. These types of multicellular organization are common to bacteria, microalgae, protozoa and fungi (including yeasts), occurring more or less frequently according to environmental constraints. Such organized communities can be found in environments so diverse as mine sediments [471] and wastewater treatment plants [472, 473] or man-made equipment and facilities [474, 475]. While the occurrence of microbial colonization of medical devices or marine facilities equipment carries severe health and economic losses [476-478], the development of these communities is actually helpful in several processes, like for example the microbial-enhanced recovery of minerals and oil, the bioremediation of soils, rivers and groundwater, or wastewater treatment [479, 480].

Bacterial biofilms

Bacteria, distributed across a wide range of ecological niches, form highly dense multicellular communities - biofilms [481]. Bacterial biofilms are heterogeneous communities embedded in a polysaccharide-rich ECM that mediates attachment to biotic and abiotic substrates [482]. But the multicellular aggregation of bacterial cells brings several advantages over the planktonic cell lifestyle; biofilm ECM provides surface adhesion, mechanical support, physical and chemical protection against environmental variations, while promoting cell-cell communication and enabling community-based gene regulation and metabolic cooperation [482]. Being a highly complex structure, the ECM provides physical protection for the cells against shear

forces or UV damage. Although this is still not known how, bacterial ECM also mediates cell-cell communication, since it enables biofilm gene expression synchronization [483]. As in mammals, it presents a high water retention capacity, keeping a stable hydration level against environmental variations, and retains several organic and inorganic compounds, providing a nutrient source and protecting against xenobiotics [484-486] [487, 488]. Furthermore, it apparently also is able to modulate extracellular enzymatic activity, and mediate horizontal transfer of genetic material [481, 489, 490].

Bacterial biofilms development occurs in sequential steps of colonization, proliferation, and differentiation. Cellular adhesion to a biotic or abiotic substrate is the first step of bacterial colonization (Fig. 8 A). Planktonic cells interact with surfaces through the action of fibrillar adhesins or polysaccharides. Some of these molecules, like the fibrillar *pilli* or *flagella*, interact directly with the *substratum*, while others modulate the cell surface hydrophobic properties to enhance the interaction between the

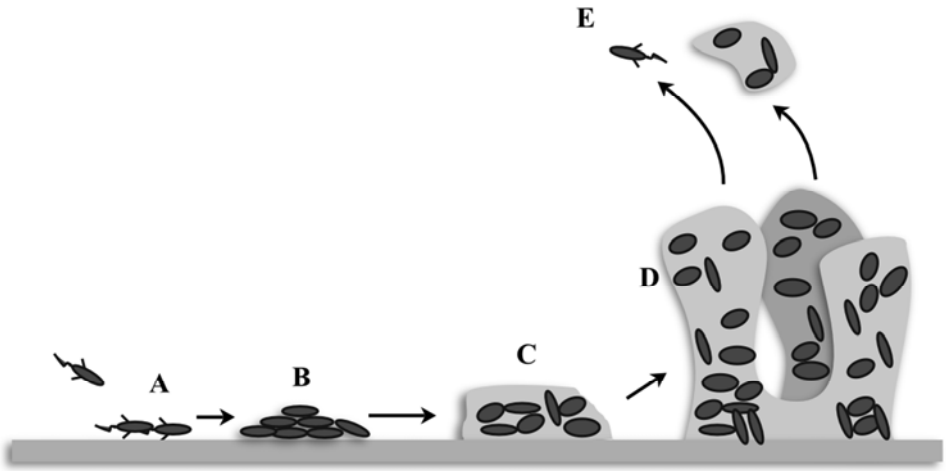


Figure 8. Bacterial biofilm formation and development. Planktonic cells attach to biotic and abiotic substrates through cell surface adhesins, after environmental variations (A). Cell motility and division lead to the formation of microcolonies, enabling cell-cell communication and metabolic synchronization (B). The differentiated community produces an ECM that supports and protects the developing biofilm (C). Under continuously changing environmental conditions, the biofilm further develops into a mature and highly differentiated community completely included in a protective ECM (D). Extracellular enzymes mediate the degradation of ECM biopolymers, leading to the release of planktonic cells that might colonize new substrates (E). Adapted from [491] and [492].

cell and the biological or artificial surface [493]. These initial interactions are frequently non-specific and reversible. The permanent attachment depends of physical constraints, like surface roughness and shear stress of water or air against the surface, and non-covalent binding forces, like electrostatic interactions and hydrogen bonds [465]. After a stable surface attachment, cells start proliferating and form microcolonies (Fig. 8 B). Cells presenting motile appendages may form microcolonies through agglomeration of several roaming cells [491]. The cells within the microcolony undergo differentiation and simultaneously initiate production and secretion of extracellular polymeric substances (EPS) that accumulate and form the ECM (Fig. 8 C). This secreted ECM enhances cell surface adhesion and intercellular cohesion, resulting in an irreversible attachment to the substrate. The onset of bacterial ECM production may occur upon diverse environmental insults, namely biotic stresses like competition or predation, nutrient availability or shear forces intensity [465, 481, 490]. In face of these environmental challenges, constant biopolymers production and secretion is maintained, until all the cells are embedded in ECM (Fig. 8 D). In bacterial biofilms, the ECM may account for over 90% of the total dry weight [494, 495].

The bacterial ECM is composed of several biopolymers, *viz.* polysaccharides, proteins, lipids, extracellular DNA (eDNA) and humic substances [481]. The ECM composition is highly dependent on the microorganism and on the environmental causes that guided its development [465]. A mature biofilm presents an uneven distribution of cells, these being organized in layers and clusters supported and connected by the ECM. The three dimensional structure of a mature biofilm contains voids depleted of cells, cavities, channels, pores, filaments and other structures that mediate a two phase system: 1) a solid network of polymers enclosing bacterial cells, and 2) free interstitial water that conducts the nutrients flow and exerts physical pressure [488]. As the biofilm grows and becomes more complex, there is remodelling and recycling of several components, through the action of some secreted enzymes. These ECM degrading enzymes are therefore responsible for the remodelling of the biofilm and in ultimately the reutilization of polysaccharides and proteins as nutrient sources [481]. The action of these secreted proteins also enables the release of planktonic cells (Fig. 8 E), which can migrate and colonize new and sometimes completely different surfaces.

The bacterial biofilms, as mentioned above, are composed of a wide range of biopolymers, each microorganism species, or even strain, producing and secreting a

different type of polysaccharides, proteins or eDNA [481]. The role of each component also seems to be species-dependent. Otherwise, the polysaccharide poly-*N*-acetyl-glucosamine in *Staphylococcus epidermidis*, and some types of eDNA in its close relative *S. aureus*, play the same structural function in biofilms [496]. Extracellular polysaccharides, or exopolysaccharides, are the main class of biopolymers in bacterial biofilms [497, 498], comprising long molecules, linear or branched, with high molecular weight [481]. Some of these are composed by repetitions of a single monosaccharide, like the fructans and glucans from *Streptococcus spp.*, or the cellulose from *Escherichia coli* [2] [499], while others result from the combinations of several different sugar units, *e.g.*, the heteropolysaccharides from *Proteobacteria* [500]. The biochemical properties of these molecules depend on their overall charge and hydrophobicity. Several Gram-negative bacteria present neutral or polyanionic exopolysaccharides, rich in uronic acids or ketal-linked pyruvates or more rarely sulphate [481], while in some Gram-positive strains is possible to detect exopolysaccharides primarily cationic, as the poly-*N*-acetyl glucosamine with partly deacetylated residues from *S. aureus* and *S. epidermidis* [464, 495, 501]. The presence of several organic and inorganic substituents can change the physical and biochemical properties of polysaccharides. These molecules are frequently *N*- and *O*-acylated, presenting substitution with *N*-acetyl or *O*-succinyl groups [502, 503].

The production of polysaccharide is vital for proper biofilm development. In fact, strains that do not produce exopolysaccharides are unable to form a biofilm, even if they adhere to a substrate [504, 505]. However, some polysaccharides types are not fundamental structural elements of a biofilm although their presence changes the biofilm structure radically. *Pseudomonas aeruginosa* strains that colonize and produce biofilms in the lungs of patients with cystic fibrosis present a typical mucoid phenotype [506, 507]. These strains produce an increased amount of alginate, a polysaccharide composed by mannuronic acid and guluronic acid, that helps to sustain a more complex biofilm and plays an important role in the surface adhesion [508, 509].

Proteins are another very important component of bacterial biofilms. The molecules can even exceed the polysaccharide in a mass basis [497]. Biofilms have several extracellular enzymes, many of which are associated with the modification or

[2] Cellulose can be classified into plant cellulose and bacterial cellulose, both of which are naturally occurring. They have basically the same chemical nature, but they differ significantly as to the macromolecular properties and characteristics. In general, microbial cellulose is chemically more homogenous, containing no hemicellulose or lignin. It has higher water holding capacity, and greater tensile strength, resulting from a larger amount of polymerization and ultrafine network architecture.

degradation of their polymeric constituents, both water-soluble – proteins and polysaccharides - and water-insoluble - cellulose, chitin and lipids [481]. These enzymes are particularly important during starvation periods or for planktonic cells release, for which the remodelling and recycling of the ECM components are vital [510]. Actually, the release of planktonic cells is dependent on environmental changes, either the depletion of nutrients in the substrate where the biofilm is set, or the elsewhere-nutrient availability that will facilitate the colonization of new surfaces [510, 511]. In *Actinobacillus actinomycetemcomitans* biofilms, the cells secrete an *N*-acetyl-β-hexosaminidase that mediates ECM/biofilm exopolysaccharides degradation and cell release [512]. These proteins are kept inside the biofilm by interactions with polysaccharide and other proteins, maintaining a dynamic turnover of ECM components and structure. In *P. aeruginosa* biofilms, alginate molecules interact with several enzymes. Such interactions keep the enzymes inside the biofilm and promote the biochemical activation of the biofilm constituents by the attached enzymes [513].

Bacterial ECM enzymes target exopolysaccharides belonging to the biofilm of the producing bacteria as mentioned above, or otherwise degrade the polysaccharides produced by another bacteria [514]. Some of these proteins have industrial applications, namely in food and pharmaceutical industries [515, 516]. The growth inhibition effect of some these enzymes against other bacteria is frequently used to reduce food spoilage and improve shelf-life [517, 518]. One such example is the bacterial cell wall hydrolase family (BCWHs), enzymes that degrade peptidoglycan. Hydrolysis of peptidoglycan by BCHWs results in cell lysis, since this cross-linked cell wall component confers mechanical strength and resistance against external turgor pressure [519]. ECM secreted enzymes are also object of active studies in bacterial infection. They act as virulence factors in mammals and plant hosts, for the advantages they provide the infectious microorganism against the host defence system [520, 521].

Biofilms also present several non-enzymatic proteins that play a structural role, usually interacting with cell membranes or presenting carbohydrate-binding domains. Such proteins include lectin-like and biofilm-associated surface proteins (Bap), which help to establish a connection between the cell surface and the biofilm structure. In oral biofilms, the pathogen *Streptococcus mutans* secretes several glucan-binding proteins [522, 523]. *P. aeruginosa* also secretes some lectin-like proteins during biofilm formation, the galactose-specific lectin lecA and the fucose-specific lectin lecB [524,

525]. These lectin-like proteins mediate biofilm formation and stabilization. The selective inhibition of LecB hinders biofilm formation and promotes complete dispersion of established biofilms [526]. The protein CdrA, present in *P. aeruginosa* biofilms, is attached to the bacterial cell surface but interacts with several exopolysaccharides, namely Psl, anchoring the cells to the matrix. The shedded forms of these proteins interact directly with exopolysaccharides, cross-linking and reinforcing the biofilm network [527]. Other common biofilm constituents are amyloid proteins; these fibrous adhesins comprise several repeats of protein molecules forming a cross-β structure, in which the β-strands are perpendicular to the fibre axis. These proteins are involved in abiotic surfaces adhesion, and can also function as cytotoxins for host cells or other bacterial cells [528, 529]. In *Bacillus subtilis* biofilms, amyloid fibres provide structural integrity and bind the cells in the biofilm [530]. These proteins present a very ubiquitous distribution, being found in freshwater lakes, brackish water, drinking-water reservoirs or wastewater treatment plants [528, 529]. The presence of motility appendages, *pilli* or flagella, stabilizes the biofilm through interactions with other ECM components. In several bacterial biofilms, namely *E. coli* and *Salmonella typhimurium*, the presence of interactions between fimbriae and cellulose results in a rigid and hydrophobic ECM, whereas that the absence of these appendages results in a cellulose-based fragile network [499].

The presence of eDNA is very frequent in bacterial biofilms; however, for some time the structural role of eDNA in biofilm ECM was not recognized, being accepted as residual material from lysed cells [481]. Evidence of its importance for biofilm integrity and structure has lately piled up [531-533]. The importance of eDNA in microbial aggregation was initially surveyed in bacteria from the genus *Rhodovulum*. These bacteria aggregate in *flocs* and produce an ECM composed of polysaccharides, proteins and nucleic acids. These aggregates were treated with degrading enzymes, selectively targeting polysaccharides, proteins or DNA. Surprisingly, only the action of nucleolytic enzymes resulted in deflocculation, the other treatments had no effect [534]. eDNA is also particularly important in the development of *P. aeruginosa* biofilms, where it acts as a structural connector [535], evidenced by the treatment with DNase inhibiting new biofilm formation and destabilizing mature biofilms [531, 536]. The amount, localization and role of eDNA in biofilms greatly differ between bacterial species. In *S. aureus* biofilms, eDNA is an important structural constituent present in high amounts and

forming a grid-like structure across the biofilm [537], whereas in the closely related *S. epidermidis* biofilms, eDNA is only a residual component [496]. In *Haemophilus influenza* biofilms, eDNA is organized in a dense network composed of fine strands that provides structural support to the microcolonies. Occasionally, thick rope-like strands of dsDNA, crossing over and through the channels that conduct water, bridge different parts of the densely populated bacterial biofilm [538].

Gammaproteobacterium strain F8 presents a filamentous network of eDNA supporting its biofilms [489]. Some bacteria present eDNA identical to the genomic DNA, namely *P. aeruginosa* and *P. putida*, which can be used as a pool of genetic information and enable the horizontal transfer of genes [489], whereas others species, namely *Gammaproteobacterium* strain F8, present eDNA with particular properties, indicating that its biofilm eDNA is not simply released by lysed cells but undergo some specific modifications [537]. In *S. epidermidis* biofilms, eDNA is released by the action of an autolysin that promotes the controlled lysis of a particular subpopulation of cells, promoting biofilm formation of the remaining cells [539].

Most polymeric substances present in bacterial biofilms ECM are hydrophilic, as polysaccharides and DNA, but some species present highly hydrophobic components in their ECM. The production of hydrophobic biopolymers, both lipids and substituted polysaccharide, is frequently associated with adhesion, nutrient utilization or resistance to antibiotics. Several bacteria, including *Thiobacillus ferrooxidans*, produce energy from the oxidation of reduced sulphur compounds, colonizing and forming biofilms in mine sediments. These minerals, mainly pyrite, present high surface hydrophobicity and so, this bacteria biofilm is rich in lipopolysaccharides, presenting hydrophobic features and mediating the attachment to the surface of pyrite [540]. *Serratia marcescens* is a bacterium that colonizes lipid rich environments, namely gasoline or diesel fuel contaminations these bacteria secrete several lipids with surface-active properties, named “serrawettins”, that disperse hydrophobic substances and render them bioavailable [541]. These bacteria have biotechnological potential in bioremediation in oil spills [542].

K. C. Marshall, one of the pioneers in biofilms research, defined biofilms as “stiff water” [481, 543]. Such statement reveals the importance of water for biofilms physiology. Biofilms ECM is a highly hydrated environment that provides the proper pH, temperature and molecules diffusion conditions for cells to survive. The ECM is

highly hygroscopic and retains water entropically, drying more slowly than its surroundings therefore protecting against desiccation. Desiccation reduces biofilm volume, concentrating the matrix and exposing new binding sites, promoting changes in molecules interactions and responses. As such, desiccation is one of the stimuli for bacteria to produce ECM [544, 545].

The combination of different biopolymers, and their distinct interactions with water, makes the biofilm a highly complex structure. All these components contribute for the mechanical properties of biofilms. Cohesive and adhesive properties, mediated by polysaccharides, proteins and eDNA, are particularly important in ECM stability and biofilm resistance to physical and chemical removal [546]. The combined environmental conditions of shear stress, temperature and water availability, and interactions with multivalent inorganic ions, may reinforce the ECM network. Also the presence of more than one species of microorganism, a single bacterial species or a mixture of different bacteria and yeast/fungi, greatly influences the properties of biofilms. Biofilms from stable environments, like stagnant waters, are easily disrupted by low shear force as were not formed to resist such conditions. Otherwise, biofilms from the family *Podostemaceae* present high stability, rubber-like appearance, and colonize waterfall rocks [547]. A biofilm can reinforce the structure of its ECM to respond to mechanical insults by increasing biopolymers synthesis and secretion [548]. The biofilm ECM can act as a molecular sieve, sequestering cations, anions, and apolar particles. The biopolymers secreted during biofilm development and maturation contain a diversity of structural elements, uronic acids or amino sugars, that allow tight interactions with water phase compounds restricting access to the cells [549]. In activated sludge, hydrophobic compounds, as benzene or toluene, are retained by the biofilm ECM, whereas heavy metals, as Zn^{2+}, Cd^{2+} or Ni^{2+}, bind to cell walls of bacteria [550]. The molecular sieving provided by the biofilm ECM plays an important role in restricting the access of foreign substances to the cells, namely compounds that modulate biofilm structure and composition [551]. The role of biofilm biopolymers in antibiotics resistance is of particular interest. Bacteria producing biofilms produce chronic infections with high human and economic implications [552]. Biofilm response to xenobiotics is distinct of planktonic cells'. A bacterial biofilm comprises different metabolically and physiologically subpopulations, expressing high amounts of efflux drug pumps and presenting distinct susceptibility to drugs [484-486], and its ECM

interferes with diffusion rates of antibiotics [487, 488] and presents extracellular enzymes that may degrade them [553, 554].

Within bacterial communities the response to environmental stimuli, the metabolic synchronization, the aggregation in multicellular biofilms, the production of the extracellular biopolymers, and the release of DNA, are mediated by cell-cell communication [483]. The most studied bacterial communication mechanism is the quorum sensing (QS) [483, 555, 556], which regulates the expression of specific genes in correlation to population density [465]. Bacterial cells produce and secrete signalling molecules, named autoinducers, which diffuse and accumulate in the surrounding environment. The autoinducers accumulate until a specific critical concentration is reached. The larger the population producing the autoinducers is, the faster this concentration is reached. After reaching this threshold, the autoinducers interact with receptors on the cell surface or are imported into the cell and interact with receptors in the cytoplasm, which act as transcription factors and change the expression of genes [556, 557]. Several systems of bacterial QS are known, like the signalling through *N*-acyl-homoserine lactone (AHL) system of Gram negative bacteria [558, 559], the modified oligopeptides from Gram positive bacteria [560, 561], and the autoinducer type II interspecific system [562-564].

The first and best-characterized QS system is the AHL-mediated communication in *Vibrio fischeri* [565-567], being the QS paradigm for communication in Gram negative bacteria [465, 483]. This QS system comprises two proteins, LuxI and LuxR, which regulate the expression of the Luciferase operon, responsible for the light production characteristic of this species. The protein LuxI is an autoinducer synthase that produces *N*-(3-oxohexanoyl)-*L*-homoserine lactone, requiring S-adenosylmethionine, whereas the LuxR is a cytoplasmic receptor that once activated, binds to DNA and initiates the transcription of the Luciferase genes [565, 568]. *V. fischeri* also recognizes and responds to *N*-octanoyl-*L*-homoserine lactone [569]. Similarly, *P. aeruginosa* also presents two AHL-based communication systems that regulate virulence and biofilm development [570, 571]. Two LuxI type synthases, LasI and RhlI, produce *N*-(3-oxododecanoyl)-*L*-homoserine lactone and *N*-butyryl-*L*-homoserine lactone, respectively. These AHL inducers cross the membrane and interact with LasR and RhlR receptors, LuxR type transcription activators that activate specific genes and promote the phenotypic change [572-575]. Mutants on QS systems produce thinner and more densely populated biofilms,

while mutation of LasI produces abnormal and undifferentiated biofilms [576]. The lipopolysaccharides synthesis in *A. ferrooxidans* is regulated by the AfeI/AfeR system [577, 578]. Other Gram negative bacteria present AHL-mediated communication. In *Pectobacterium carotovorum*, QS communication mediates carbapenem antibiotic production [579], while in *Agrobacterium tumefaciens* regulates Ti plasmid conjugal transfer [580]. The multicellular aggregation of *Serratia liquefaciens* [581], as well as the virulence and production of exopolysaccharides in *Pantoea stewartii* [582] are also regulated by AHL-based systems. While the AHL system was a broad distribution in bacterial species, the sequence homology between LuxI type and LuxR type proteins is fairly low. Some species produce AHL degrading enzymes, while others produce small molecules that block the AHL. The former include oxireductases, AHL aminoacylases that cleave the amine bond and AHL lactonases that open the lactone ring [583, 584]. The latter include brominated furanones that bind to the active centre of LuxR type receptors and block the action of the AHL inducer [585, 586]. The production of quorum quenching molecules gives the producing bacteria competitive advantage, as it can inhibit biofilm development or genetic information exchange from other species.

In Gram positive bacteria, communication is carried out by modified oligopeptides that interact with membrane bound sensor histidine kinases. Typically, a newly synthesized pre-protein undergoes proteolytic cleavage and exported from the cell. In the extracellular milieu, these oligopeptides interact with the cell surface receptor, generating a signal that is communicated by several phosphorylated intermediates [560]. Such signals are highly specific; the chemical structure of the signal is defined by the amino acid sequence, which can be further modified to increase specificity, *e.g.*, the formation of a thiolactone ring in the *S. aureus* oligopeptides [587]. Such high specificity allows distinct signalling between different strains of the same bacterial species. In *S. aureus*, several strains produce signalling peptides that able to cross-communicate with other strains. In several strains, the signal produced by a group of cells, stimulates the production of more of the same signal, while it inhibits the production of different signals. Arguably, these types of cross-strain communication could demonstrate cooperative behaviour and intraspecific competition [588].

The interspecific QS system is present in Gram positive and negative bacteria [562, 589, 590]. This very simple system is based on the signal synthase LuxS, that produces 4,5-dihydroxy-2,3- petanedione in chemical equilibrium with several furanones [591,

592], and in the LuxPC sensor that recognizes the signal and promotes the gene expression change [589]. Different species and strains are able to recognize different stereoisomers of the molecule and respond in distinct ways, while others respond in the same way to both chemical species. This lack of directionality in the promoted responses leads to an inefficient information transfer.

Multicellular bacterial communities are remarkable structures in which unicellular microorganims organise a multicellular way of life. This complex life style provides improved nutrient supply and protection against environmental stresses, such as antibiotics. The cell-cell communication that undergoes within these communities and that allows metabolic synchronization and concerted responses is still little understood.

Fungal biofilms

Similarly to bacteria, yeast and other fungi organize themselves in multicellular aggregates embedded in a polymeric ECM [466]. In response to environmental insults fungal cells assemble into several structurally distinct communities – biofilms, colonies and stalks [467]. While most yeasts and fungi species are able to form some kind of multicellular community, biofilms are the most studied [495] mostly for their role in infection and pathogenesis. Adherence and colonization of inert surfaces from medical devices are important for clinically relevant fungal pathogens [593] and constitute a critical source of hospital-acquired infections. On the other hand, yeast and fungi infections usually proceed through adherence and colonization of mucosa. In immunosuppressed patients, serious infection progresses by overcoming the hematopoietic barrier and colonizing the blood stream causing systemic infection and eventual death [594, 595]. This is particularly so in patients receiving transplants, who are immunodepressed on purpose in order to lower the chances of organ rejection, as well as patients under medical-assisted life extension procedures, adding the immunodepression caused by HIV, cancer or other diseases [596]. Altogether, the numbers have reached a world-wide significant high impact with large associated costs on lives, health care systems and ultimately economy. The high tolerance of biofilms to antifungal drugs is yet another important issue to public health [597, 598].

Some of these major offenders are fungal human commensals of mucosal surfaces and intestinal tract, like the fungus *Aspergillus fumigatus* or the yeasts *Candida albicans*

and *Cryptococcus neoformans*. The shift from commensality to pathogenicity is a very complex process that has been mostly addressed in the yeast *C. albicans.*

a) The fungus Aspergillus fumigatus

A. fumigatus is an opportunistic filamentous mould, being the second most common fungal infection found in hospitalized patients, after *C. albicans* [599]. This mould forms biofilms in both biotic and abiotic surfaces, through the germination of small spores called conidia. The conidia are easily dispersed through the air, remaining active in the atmosphere for long periods, and being frequently inhaled by humans [600]. In a healthy host, conidia are easily and quickly eliminated by the immune system. In immunodepressed patients, especially those with cystic fibrosis, these cells can cause a wide range of systemic problems, including severe pulmonary infections [601]. After the initial colonization, the conidia start differentiating and producing hyphae. During maturation, the mycelium continues to develop and the ECM accumulates until it envelops the entire biofilm [478, 602]. *A. fumigatus* infections can be subdivided according to their distinct hyphal organization [478] into (1) the aspergilloma, a tightly packed spheroid mass of hyphae that promotes localized infection of pre-existing parenchymal cavities or in chronically obstructed paranasal sinuses in the upper-airways, and (2) the invasive aspergillosis, a widespread and multifocal invasion of host tissues leading to systemic infection. Such infections result in elevated mortality rates, over 50% [603]. The ECM has a particularly important role in these infectious processes. The presence of polysaccharides (galactomannan, α-1,3 glucan), melanin, proteins (major antigens and hydrophobins) and monosaccharides was described for agar-grown biofilm's ECM of *A. fumigatus* [604]. Later studies in vivo showed differences between the ECM of lung aspergilloma and aspergillosis. Both hyphal aggregates presented galactomannan and galactosaminogalactan, but α-1,3 glucan was detected only in aspergilloma [478]. Additionally, components of the ECM interact with and sequester antifungal drugs, mainly azoles [605].

b) The yeast Cryptococcus neoformans

The yeast *C. neoformans* is an encapsulated opportunistic yeast pathogen that colonizes the central nervous system of immunocompromised individuals, causing life-

endangering meningoencephalitis [606]. Similarly to *A. fumigatus*, host colonization may happen by inhalation of air disperse small spores or planktonic cells, as it presents a very ubiquitous distribution [607, 608]. This pathogenic yeast is capable of colonizing several hosts, including plants [609, 610], protozoans [611], nematodes [612], insects [613, 614], birds [615], and mammals, including house pets [616, 617] or humans [606]. Clinically, *C. neoformans* forms biofilms in ventricular shunts, peritoneal dialysis fistulas or cardiac valves [618, 619]. Similarly to other fungal and bacterial pathogens, cryptococcal biofilms are less susceptible to anti-fungal agents, as well as to host immune system [618, 619]. *Cryptococcus spp*. enhanced resistance to antifungals and virulence derives from the presence of an unusual polysaccharide capsule. This provides protection against phagocytosis and diminishes the effect of antimicrobial agents through these molecules sequestering and reduced diffusion [619, 620]. The capsule forms a dense, highly hydrated, gelatinous layer that prevents contact between cellular components and the host defences, avoiding antibody binding and subsequent activation of complement system [621]. Natural occurring *Cryptococcus* strains that present defective capsule production or no capsule at all are little virulent or avirulent [622, 623].

Studies on *Cryptococcus* biofilms are mostly performed *in vitro*, including adherence onto glass surfaces or wells of polystyrene plates [608, 618, 619, 624], and information on the capsule composition has been inferred based largely on analysis of shed exopolysaccharides that accumulate in culture supernatants [625]. The capsule is mainly formed by glucuronoxylomannan, galactoxylomannan and mannoproteins [626]. However, the role of these polysaccharides in the capsule structure is unknown. *C. neoformans* is also capable of synthesizing hyaluronan. The gene *CPS*1 encodes a hyaluronan synthase. The presence of this GAG in the capsule is extremely important for the yeast infection *in vitro* and *in vivo,* as strains lacking HA production are less pathogenic [627]. The capsule size depends on the environmental conditions, including host immune response; incubation with serum, increasing in CO_2 concentration, iron deprivation and pH variations stimulate capsular components production and the enlargement of this structure [621, 628-631].

c) The Candida *yeast species*

The pathogenic species of the genus *Candida* are the major agents causing hospital-acquired infections [632]. The most common species include *C. glabrata* and *C.*

parapsilosis but mostly *C. albicans*. *C. albicans* usually acts as a commensal of human skin, gastrointestinal tract, and vaginal and urinary mucosa. However, under favourable environmental conditions, this yeast behaviour shifts towards pathogenicity, causing several pathologies, namely stomatitis, thrush, nosocomial pneumonias and urinary tract-infections. Moreover, as the other fungi and yeasts, in immunocompromised patients, it provokes life-threatening systemic infections [633]. To study *C. albicans* biofilm formation several *in vivo* and *in vitro* models were developed, including several animal models, namely for venous catheters, oral and denture colonization; abiotic surfaces like polystyrene; or special apparatus that allow the control of all the different biofilm developmental phases like the Calgary biofilm device [477, 634-637].

C. albicans biofilm development occurs in sequential steps of adherence, proliferation and ECM secretion (Fig. 9). In a natural occurring biofilm, these steps may happen simultaneously rather than sequentially, in different regions of the same cellular aggregate. This complex process is controlled by genes regulating (1) the morphological transition from yeast to hyphal form, (2) the production and response to QS molecules, and (3) the cell wall composition [638-643], while initiation is regulated by nutrient availability and cell-cell communication (Fig. 9 A). Most proteins involved in this step are involved in the regulation of the adherence to plastic or protein-coated surfaces. A group of cell wall proteins - adhesins - is responsible for the cell-cell and cell-substrate interactions. Adhesins include the Eap1, Hwp1 and the two closely Als1 and Als3. Eap1 is the main adhesin involved in adherence to inert substrate It is a GPI anchored protein rich in serine and threonine that presents internal repeats of Trp-Pro-Cys-Leu, common in fungal surface proteins [644, 645]. The Ser and Thr residues are potential glycosylation sites [645]. The deletion of the *EAP*1 gene results in reduced adherence to polystyrene and defective biofilm formation, both *in vivo* and *in vitro*. Analogously, the heterologous expression of this protein in non-adherent *S. cerevisiae* strains promoted the adhesion to polystyrene [644, 646]. The role of the closely related adhesins Als1 and Als3 was unveiled through the phenotypical analysis of mutant strains for both adhesins, and non-adherent *S. cerevisiae* strains heterologously expressing these proteins. *C. albicans* mutant strains for these proteins were unable to produce biofilm in the surface of a catheter inoculated with the double mutant [647]. Expression of Als1 and Als3 in *S. cerevisiae* strains promoted the adherence of cells to several protein-coated substrates [648]. Eap1 and Als1 are expressed in both yeast-form and hyphal cells [649,

650]. Als3 is present exclusively in hyphae [649]. The expression of *C. albicans* adhesins is under the control of several transcription factors, which respond to QS signals and nutrient availability. These include Efg1, a transcription factor with a basic helix–loop–helix motif and a homologue of *S. cerevisiae* Sok2 [651], and Yac1, a DYRK transcription factor family member [652, 653]. Efg1 is the major regulator of cell wall proteins expression [641, 654, 655], and Yac1 regulates the expression of several adhesins and initiation and maintenance of hyphal growth.

Recently, a further signalling pathway regulating adherence was described, the Mating Factor Response pathway [656]. *C. albicans* a/a cells respond to α-factor by

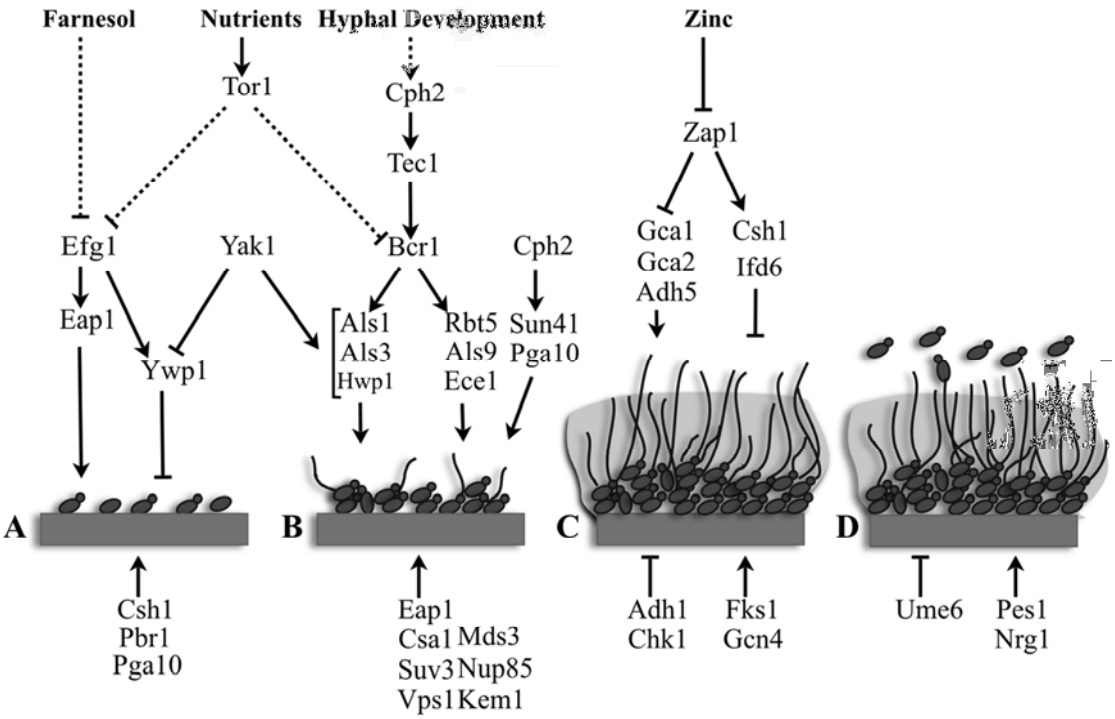


Figure 9. *C. albicans* biofilm development phases and regulatory proteins. Similarly to bacterial biofilms, adherence to the substrate is the first step in surface colonization and biofilm development (A). Cells propagate to form microcolonies, and start differentiation into pseudo- and true hyphae (B). As the biofilm matures, the extracellular matrix accumulates and the overall drug resistance increases (C). In the dispersal step, yeast-form cells are released to colonize the surrounding environment (D). Proteins and pathways involved in biofilm development are depicted; known pathway relationships (upper half) and proteins with known function in a specific step but not be connected to a known pathway (lower half) are represented. Dashed T-shaped bars indicate repression by an indirect mechanism. Adapted from [657].

forming a biofilm. These responsive cells are unable to mate with α/α cells because they have not underwent the epigenetic transition from white, mating-incompetent, to opaque, mating-competent cells [658]. During biofilm formation, white cells present several upregulated genes, including the adhesin coding *EAP1* and the *PGA10*, *PBR1* and *CSH1* that are fundamental for the proper adherence of pheromone-induced biofilms [659].

While some external and internal factors may stimulate adherence and modulate gene expression, the process of adherence itself promotes changes in gene expression. Microarray comparative analysis between planktonic and adherent cells shows changes in gene expression around 30 minutes after the beginning of the process [660]. Even faster responses were reported, the adherence to glass modulates the expression of efflux pumps Cdr1 and Mdr1 just a few minutes after adhesion, promoting antifungal resistance in an early phase of biofilm development [661].

The adherent cells start proliferating and clustering, beginning the yeast-to-hyphae transition (Fig. 9 B). These processes are mediated by cell-substrate and cell-cell adhesins [662]. The proteins Als3 and Hwp1 are the main adhesins in cell-cell adhesion. These proteins are complementary and interact to mediate intercellular adhesion [650, 663]. Mutants for these adhesins are unable to form biofilms, but the mixture of *C. albicans* mutant strains, each lacking one of these adhesins, results in strong and dense biofilms [647]. This mutation complementation derives from the overexpression of Als3 in an *eap*1*Δ* mutant strain, indicating these proteins probably have overlapping functions [664]. The expression of adhesins is regulated by several transcription factors, including Bcr1, a C_2H_2 zinc finger protein, and Tec1, a TEA/ATTS transcription factor family member. Bcr1 mediates cell-substrate adhesion, being fundamental for biofilm formation [642, 665-667], while Tec1 regulates the expression of several hyphal cell wall proteins, and is involved in the yeast-to-hyphae transition [668-670] and the white cells response to the pheromone signalling [671]. The *bcr1Δ* mutant strain is unable to form biofilms because of the lack of expression of the adhesins Als3 and Hwp1. The over-expression of these proteins restores the capacity to develop biofilms, both *in vivo* and *in vitro* [642, 663].

There are several genes know to be involved in the yeast-to hyphae transition and biofilm development. These include the genes *GUP1*, *RBT2*, *HWP2*, *SUN41* and *PGA10* all of which encoding proteins without fully characterized function. In

particular, the pleotropic gene *GUP1* codes for an *O*-acyltransferase [415], which deletion results in defective hyphal and biofilm development and affects the strain virulence and antifungal resistance [633]. The simultaneous deletion of either *RBT2* or *HWP2* and *HWP1* results in an increasingly defective adhesion phenotype, while the deletion of *SUN41* and *PGA10* genes increases the sensibility to cell wall inhibitors, indicating a potential role in cell wall architecture [672, 673].

The development of microcolonies interconnected by hyphae leads to the production of a supportive and protective ECM (Fig. 9 C). The study of this ECM revealed the presence of carbohydrates, proteins, hexosamines, uronic acids and DNA [4, 674, 675]. A major component is the carbohydrate β-1,3 glucan. This molecule synthesis is significantly increased in biofilm cells and might play a role in antifungal resistance [676, 677]. A more detailed analysis of some the *C. albicans* biofilm components revealed an ECM exopolysaccharide composed of α-*D*-glucose and β-*D*-glucose, α-*D*-mannose, α-*L*-rhamnose and *N*-acetyl glucosamine [678]. Yet, the molecular identification of most of the biofilm components has focused on proteins and was unveiled in proteomic surveys [679-681]. *C. albicans* biofilms ECM displays a large number of very diverse proteins included in the most diverse cellular processes: metabolic process, protein synthesis, folding and degradation, cell rescue, defence and virulence, and biogenesis of cellular components [679]. A sticking revelation was the presence of so many of supposedly intracellular-only proteins, such as the glycolytic enzymes Pgk1, Pfk2, Eno1, Fba1, Tdh3, Tpi1, Gpm1 and Hxk2. The abundant presence of these enzymes in the biofilm ECM overcame their hypothetic origin from lysis of dead cells in the biofilm, and opened the way to consider protein moonlighting [682, 683].

Finally, and similarly to bacterial biofilms, *C. albicans* biofilms present eDNA, which appears to play a structural role in the fungal multicellular aggregates, since exogenously added DNA promotes biofilm growth and the addition of DNase resulted in the dissociation of the biofilm [675].

Some environmental factors, namely water availability or the presence of certain chemicals, regulate the production of structural components of the biofilm ECM. The presence of ionic zinc is one of the best studied environmental inputs, seeming to negatively regulate ECM production [684]. The zinc responsive transcription factor Zap1 activates the expression of Csh1 and Ifd6, and represses the expression of Gca1 and

Gca2 and Adh5. The mutant *zap*Δ forms biofilms with elevated production of β-1,3 glucans in the ECM. The glucoamylases Gca1 and Gca2 might play a role in the β-1,3 glucans hydrolysis, and the proteins Adh5, Csh1 and Ifd6 are involved in the synthesis of acyl- and aryl- alcohols, putatively acting as QS signals [684, 685]. The biofilm maturation, and the production of ECM components, both contribute to high-level resistance to antifungals. Several mechanisms underlie this resistance, namely (1) the upregulation of the drug efflux pumps genes *CDR1*, *CDR2* and *MDR1* early in biofilm development [686], (2) the reduced amount of membrane sterols and elevated expression of sterol synthesis proteins, comparatively to planktonic cells [660, 686-688], (3) the binding and sequestering of antifungal agents by the ECM β-1,3 glucans [676], and (4) the existence of subpopulations of persister cells, metabolically quiescent cells that resist to a range of drug concentrations otherwise lethal to planktonic cells [689, 690].

The release of cells from biofilms occurs to allow colonization of new substrates or dissemination into the host tissues (Fig. 9 D). These cells are mainly yeast-form cells that present increased adherence and filamentous capacity, becoming highly virulent [691]. The regulation of this process is not yet fully understood, but the role of the transcription factors Ume6, Pes1 and Nrg1 was described. The overexpression of Ume6 yielded lower levels of released cells, whereas overexpression of either Pes1 or Nrg1 resulted in higher release of yeast-form cells [691, 692].

The environmental inputs that regulate most of these processes are not yet fully characterized, but most of these surface colonization and biofilm formation mechanisms depend on cell-cell communication and behaviour synchronization. Similarly to bacteria, fungal pathogens present an auto-regulatory communication system [693, 694]. In *C. neoformans*, QS signalling is performed through a peptide-mediated mechanism. The signalling peptide is coded by the gene *QSP*1, under direct control of Gat201 DNA-binding regulator, being translated as a larger precursor that undergoes proteolytic processing before being secreted. Both production and secretion-mediating are currently unknown [695, 696]. The Qsp1 peptide is important in overcoming the inhibition of growth at low culture density lag phase. However, only mutants for the repressor Tup1 present an effect dependent on Qsp1 concentration [695]. In *C. albicans* biofilms, QS signalling is mediated by farnesol and tyrosol [639, 697]; farnesol is an intermediate in sterol biosynthesis, whereas tyrosol derives from aromatic amino acids biosynthetic pathway. The biosynthesis, as well as the secretion machinery remain uncharacterized

[693]. Farnesol regulates cell adherence (Fig. 9 A), increasing amounts of farnesol inhibited the cell adherence to substrates, yet it had no effect in biofilm development if presented after adhesion was in an advanced stage. An inhibitory effect on cell germination was also reported [698], farnesol might mediate yeast-form cells detachment and dissemination. Tyrosol-mediated signalling, in turn, influences the lag phase of diluted cultures of *C. albicans*, a microarray analysis showed that tyrosol induces the expression of genes involved in DNA replication [697]. However, tyrosol promoting effect cannot overcome farnesol inhibitory activity, being a secondary signalling system [699, 700]. Unlike in bacteria, where some systems have all intermediates characterized and the environmental and internal inputs identified, the QS communication in fungal species is still in its first steps. Much more research is needed to understand the full effects of QS in multicellular communities' metabolism and behaviour, and to identify the mechanisms that activate or repress such communication.

Saccharomyces cerevisiae

S. cerevisiae is one of mankind oldest domestications, providing food and beverages since ancient times [701, 702]. One can think of *S. cerevisiae* as one of those friendly neighbours that always has the right tool for the job, or in this particular case, it is the right tool for the job. This yeast, useful in so many industrial and biotechnological applications, has also lent its many "talents" to biological and medical research, contributing with knowledge to understand several fundamental cellular processes, as cell cycle [703], providing tools for biomolecules production and manipulation [704, 705], and shedding light in some intricate disease processes as a model eukaryote [706-708].

Similarly to other microorganisms, *S. cerevisiae* is capable of forming multicellular aggregates. Structured communities include flocs, the peculiar stalks, mats or biofilms, and, of course, colonies [467, 469, 709-711]. Flocs are industrially relevant aggregates of yeast cells, produced in response to environmental variations [712, 713]. However, arguably, this transitory behaviour in liquid growth might not be a true multicellular community. Most of the times, flocculation can be easily reverted by the action of EDTA or mannose [711, 714]. As such, the study of this microorganism growth on solid media provided the most important evidences on its ability to be more than an undifferentiated cluster of cells, including the production and secretion of a yet

uncharacterized ECM. As in the other organisms, this polymeric structure provides protection against external insults, interconnects yeast cells with each other mediating cell-cell communication, and offers an suitable environment for cellular survival [467]. Nevertheless *S. cerevisiae* multicellular aggregates received little attention for a long time. The widely spread biotechnological utilization of this yeast in liquid cultures, tightly connected to its fermentative capacity in batch, fed-batch or continuous culture, has diverted the attention from *S. cerevisiae* multicellularity-associated abilities. Quite surprisingly, in support of this omission, there are reports that claim this yeast being unable to excrete an extracellular matrix [713, 715].

a) Yeast Stalks

Yeast stalks were the first multicellular community that received detailed attention in the works of Engelberg and collaborators [469, 470]. These authors described the morphological structure, the three-dimensional organization, and assessed the physiological state of the composing cells. They reported the presence of upright, long and thin multicellular aggregates achieving a centimetre size scale, when yeast cells were inoculated in high percentage agar media and irradiated with UV light (Fig. 10 A). Highly dense lawns of cells ($2x10^6$ cells/plate) were exposed to UV radiation until 99,95% of the cells were dead. The cells that accumulated in tiny pits formed by air bubbles in the agar surface were protected by the layers of cells above and survived. Under such conditions, the vast majority of the surviving colonies formed stalks. Several other species were able to produce stalks in these same conditions, including *C. albicans* and *E. coli*, which led the authors to suggest that a merely mechanical model was responsible for stalk formation. The surviving cells in contact with nutrients, in the bottom and the walls of the air bubble pit, started proliferating and the top layers of the colony were extruded the cellular mass through the pit hole, forming the stalk. However, this environmental and mechanical model was proved to be, at least, incomplete [470]. The same group reported the structural organization and cellular differentiation across the stalk, an inner core formed by vital yeast cells and spores, was enveloped by a physically separated outer shell composed mainly by dying or dead cells. The protective shell cells presented very thick cell walls, a large number of vesicles. The inner core was described as being composed of cells similar to colony cells but presenting several vesicles or fat bodies, and cells presenting 1-2 spores. The

spores-containing cells were present only in the top half of the inner core. The cell differentiation and structural organization presented by these aggregates goes against the initially described model of mere mechanical extrusion. The authors suggested the possibility of the cells differentiation occurring in a latter phase of development, after the initial stalk formation. The study of this multicellular aggregated halted after these first reports, and information regarding these structures supporting ECM, and its composition, is not currently available. The study of these peculiar structures may provide insight on early mechanisms of multicellular communities' formation, and in the environmental and genetic driving vectors behind them.

b) Yeast Mats/Biofilms

Yeast mats, more recently referred as biofilms, are other structural organizations that have been receiving great attention [709, 710, 716-721]. Such aggregates were first described as multicellular communities grown on low-agar plates, presenting an "elaborated pattern" [709]. Yeast cells in a low-agar media (0.3%) for several days form

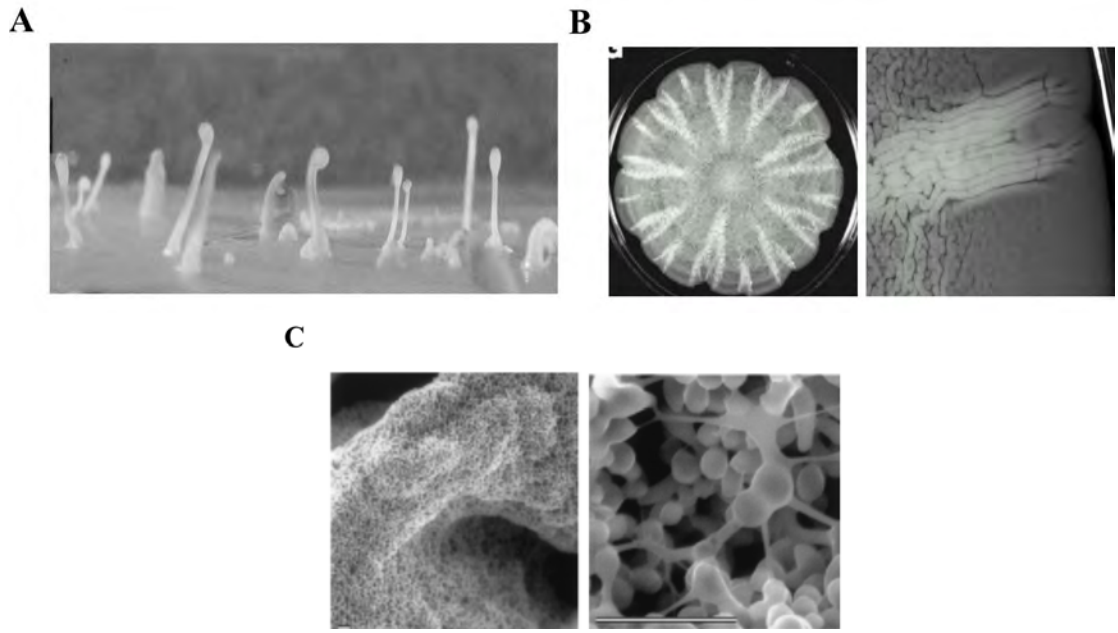


Figure 10. *S. cerevisiae* multicellular communities. Microphotography of stalks [469](A); mat or biofilm(left panel) and detail of mat spokes (right panel) [709](B); and colony structure (left panel) and ECM (right panel) [722](C).

wide confluent mats, spreading radially until most of the area was covered. These communities are organized in two visually distinct populations: a central hub composed of a network of "cables", emanating a bundle of spokes, and a rim of less organized cells (Fig. 10 B) [709]. The radial symmetry and the number of spokes were conserved under several conditions, indicating a putative programmed developmental event.

The role of the mucin Flo11 was assessed in *S. cerevisiae* adhesion to plastic surfaces. Cells lacking *FLO11* and its regulatory protein *FLO8* showed poor adherence to plastic substrates. Flo11 is similar to the glycopeptidolipids (GPL) of *Mycobacterium smegmatis*, which depend on these proteins to display sliding motility[3] [723, 724]. The similarity between Flo11 and the GPLs raised the question as to whether Flo11 might mediate biofilm formation and adhesion. The mutant *flo11*Δ lacked adherence to agar, and decreased Flo11 expression was detected in mutants for glucose responsive pathways, presenting blocked mat formation and alterations in adherence ability. Although Flo11 expression was found in both the rim and the hub cells, the mutant *flo11*Δ loses the ability to form confluent mats above described [710]. Additionally, high pH in the rim area decreased the Flo11 adhesion capacity and allows the detachment of these cells. The low adherence capacity of the rim cells might be fundamental for the colonization of new substrates, in response to changes in glucose levels across the mat.

Moreover, under glucose limiting conditions, yeast cells were able to adhere and form a thin-layered biofilm in several plastic surfaces. Similarly to mat formation, the adhesion to plastic is dependent on glucose levels and the presence of Flo11.

Another study analysed the molecular pathways that may regulate mat formation, performing whole-genome transcriptional profiling to compare cells growing as a mat and planktonic cells [716]. Gene disruptions of *INO2*, *INO4*, and *OPI1* revealed that Opi1 has a significant effect on mat formation, not presenting spokes and showing a poorly developed hub, and *INO2* and *INO4* have minor effects. Given the important role of Flo11 and nutrient limitation conditions in mat formation, the Flo11 expression levels and invasive growth in an *opi*1Δ mutant strain were assessed. The mutant presented low levels of Flo11 expression and defective invasive capacity. Both phenotypes were dependent on the transcriptional activator Ino2p. These results indicate that Opi1 affects mat formation and invasive growth by participating in the regulation of *FLO11*. Later,

[3] Sliding motility is a form of surface motility of entire colonies obtained by expansive forces produced by the growing bacterial population in combination with cell surface physical properties of reduced friction between the cells and the substrate [723].

the presence of Flo11-independent regulatory mechanisms of mat formation was reported [719, 725]. Martineau and collaborators showed that the disruption of genes of the Hsp70 family molecular chaperones and nucleotide exchange factors resulted in defective mat formation, but do not interfered with Flo11 expression, localization or invasive growth. The effect of the chaperone system disruption in Flo11 folding or post-translational modifications, such as *O*-glycosylation, cannot be discarded. Nonetheless, this was the first report of a Flo11 independent regulatory mechanism in mat development, as the previous reports always referred to mutations that decreased or abolished *FLO11* expression, affecting invasive growth [709, 716].

Until recently, mat formation in low-agar media was studied separately from invasive growth since the softness of low-agar surface could not withstand the wash under running water. The latest work on mat formation reported conditions to enable the evaluation of both the development of the multicellular aggregate in the plate surface, and the invasive growth on agar [721]. This work showed that the same pathways regulate both processes, and that such mechanisms are two faces of the same response to environmental stimuli, regulating *S. cerevisiae* social behaviour.

c) Yeast Colonies

Colonies of *S. cerevisiae* or any other microorganism have been known and used since the dawn of Microbiology. They are recognized as multicellular communities, but were mostly considered just unorganized, unstructured lumps of cells. The study of the structural organization of these communities started barely a decade ago, when the first glimpse of its scaffolding infrastructure was first reported (Fig. 10 C) [722]. Interestingly, the first approaches to study colonies as a whole unveiled a cell-cell communication system that promoted a synchronized response to environmental signals [726-728]. Palková and her group showed that *S. cerevisiae* colonies exhibit a periodic behaviour, transitioning from an active growth “acidic” phase, to an “alkali” phase presenting transiently inhibited growth, and back to an “acidic” phase where growth is resumed. The transition from “acidic” phase to “alkali” is mediated by amino acid depletion, as assessed by the study of mutants in amino acid uptake that were unable to make the acidic-to-alkali transition and the transient changes in intracellular amino acid concentrations during this transition [728, 729]. During the “alkali” phase, ammonia is released and this volatile compound acts as a long-range signal between neighbouring

colonies. The ammonia produced by colonies in “alkali” phase induces the production of this volatile compound in the neighbouring colonies. The concentration of ammonia is especially higher in adjacent regions, where neighbouring colonies growth became concurrently inhibited. As the production of ammonia declines, cells resume growth and enter the next “acidic” phase. The authors suggested that the ammonia-mediated signalling helps synchronize the acid/alkali pulses of neighbour colonies and directs their growth to free space. The same group reported that, during colony development, some programmed cell death features were observed in an ammonia-dependent manner [730]. A volatile signal produced by aging colonies promotes differential cell death distribution across the colony, only in the colony centre. The remaining cells might utilize the released resources and survive for longer periods. The role of ammonia was further highlighted by the survival defect and cell fragility of cells mutant in the gene *SOK2* [731]. Cells lacking this transcription factor are unable to produce ammonia. Genome-wide analysis on gene expression differences between *sok2*Δ and Wt colonies revealed that mutant colonies are not able to switch on the genes of adaptive metabolisms effectively, and displayed impaired amino acid metabolism and insufficient activation of genes for the putative ammonium exporter Ato. The differential expression of several genes during colony morphogenesis, including *CCR4*, *PAM1*, *MEP3*, *ADE5,7* and *CAT2*, was also reported [732]. Concurrently, the absence of Mca1p metacaspase or Aif1p was shown not to prevent programmed cell death in yeast colonies [730], which occurs differentially according to cells position in the colony.

The first exploration of *S. cerevisiae* colonies structure and composition was reported by Kuthan and his research group [722]. The authors analysed the colonies complex structure presented by wild strains and the progressive loss of this complexity during the process of laboratory “domestication”. The authors showed that the continuous growth under laboratory nutrient-rich conditions of nature isolated *S. cerevisiae* strains leads to the progressive decrease in secreted polymers that interconnect and support the colony. The wild strains presented a complex structure (Fig. 10 C), named *fluffy* by the authors, presenting intercellular fibrils and very rich in proteins and glycoproteins, whereas, the colonies grown under laboratory conditions were lean and structurally poor, presenting significantly less proteins and glycoproteins. The presence of long, pseudo-hyphae-like cells in the fluffy colonies was later reported by the same group [733].

The great influence of ECM production and the high expression of *FLO11* and *AQY*1 in three-dimensional colony architecture were also emphasised. Similarly to mats, the adhesin Flo11, when present in high levels, could mediate cell–cell adhesion. Otherwise, the channel aquaporin Aqy1p can influence water permeability and the surface properties. The lack of *FLO11* expression in the vast majority of laboratory strains due to a mutation in the Flo8 transcriptional activator [734], may be associated with the incapacity to produce a complex colony. All *fluffy* colonies presented high amounts of ECM (Fig. 10 C; right panel). The authors mention a method to extract the yeast from these colonies [722], but no detailed compositional study was ever reported. More recent works showed the importance of Flo11 to intercellular connections inside de colony, especially in the aerial parts and cavities architecture, and reinforced the predicted role of ECM in the protection of the colony, presenting low-permeability even to small molecules [735]. The presence of drug efflux pumps in the top layers of the mature colonies and elongated cells anchoring the community were also described in the same report. The latest study on *S. cerevisiae* colony structure and ECM composition focused on the metabolically distinct subpopulations of these communities [736]. The authors were able to identify two major subpopulations in a growing colony, U and L cells occupying the upper and lower colony regions, respectively. These subpopulations were distinct in cellular ultrastructure, physiology, gene expression, and metabolism and the authors suggested that metabolite exchange occurs between these different yeast cell types. The population closer to the substrate, L cells, displayed features of stressed and nutrient starved cells, presenting active degradative mechanisms to recycle cellular components. The upper subpopulation, U cells, presented an active metabolism controlled by TOR pathway, amino acid sensing systems and mitochondrial-mediated signalling; promoting adaptation to nutrient limitations and scavenging of L cells metabolites.

The study of *S. cerevisiae* multicellular aggregates is still in its very early stages as compared to bacteria or fungal pathogens. The future developments will no doubt contribute to unveil the nature and roles of ECM in the radical change between cellular behaviour when alone or in a community, and understand the very roots of multicellularity in Eukaryotes, further allowing the use of yeast as a model also for higher eukaryotes ECM-related pathologies.

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2. OBJECTIVES

Objectives

The yeast *Saccharomyces cerevisiae* is a well-known eukaryote that has provided crucial information in most cellular processes, contributing to understand other more complex organisms and their malfunctions. Also important is the role that this microbe plays in the food industry, as well as in so many biotechnological applications, contributing daily to the fulfilment of human needs. However, the multicellular communities formed by this microorganism are barely known, on one hand because traditionally a microorganism is by definition regarded a unicellular being, and in another hand because it actually presents some technical difficulties to assess the multicellular properties and behaviour.

In this context, the primary objective of this thesis was to develop, and credit experimentally, the essential protocols that allow the assessment of the extracellular matrix produced by the yeast *S. cerevisiae*. The focus on the extracellular matrix derived from the recognition that in other more complex eukaryotes, ECM is in the centre of most of the fundamental biological processes controlling existence. Hence, the work dedicated to pioneer:

I. the establishment of a reproducible methodology for the generation of a young yeast cells matt that allowed the retrieval of analysable amounts of ECM;
II. the characterization of the proteins secreted by yeast to the ECM; and
III. the characterization of the glycosidic constituents of the yeast ECM.

For this purpose, we chose to use *S. cerevisiae* W303-1A strain, as well as the derived mutant strains deficient in genes encoding for the putative morphogen-modifying enzymes, the Gup1p and Gup2p. These proteins are orthologous to high eukaryotes acyltransferases that modulate morphogens from the Hedgehog pathway, main players in ECM biological roles. Finally, and as additional preparation for future yeast ECM-related work, also the mammalian receptors of hyaluronic acid, a major regulator of ECM biophysical properties, were cloned into the same strains.

3. THE PROTEOME OF *SACCHAROMYCES CEREVISIAE* EXTRACELLULAR MATRIX

Abstract

Multicellularity depends on a complex balance between the needs of individual cells and those of the organism. It is maintained by a tightly controlled communication network between cells mediated by the extracellular matrix (ECM) in which the cells are embedded. ECM provides tissues with a cell-supporting scaffold, mediating molecules diffusion and cell migration, and its components have an active role in each cell fate. Microorganisms like *Saccharomyces cerevisiae* are mostly regarded as unicellular organisms. However, they can form large communities – colonies, biofilms and stalks - in which cells display complex multicellular-type behavior. These communities function as proto-tissues: yeast cells organize hierarchically to form basic supra-cellular structures that maintain the group shape and actively promote its survival, while individual cells roles and fates become differentiated. We extracted *S. cerevisiae* ECM and characterized its proteome. More than 600 proteins were identified; most being ascribed to intracellular functions and localization and found extracellularly for the first time. This might indicate unexpected extensive moonlighting. The entire sets of enzymes from glycolysis and fermentation, as well as gluconeogenesis through glyoxylate cycle were highly represented, raising considerable reason for doubt as whether there is extracellular metabolism. Moreover, a large number of proteins associated with protein fate and remodeling were found. These included several proteins from the HSP70 family, and proteases, importantly, the exopeptidases Lap4, Dug1 and Ecm14, and the metalloproteinases Prd1, Ape2 and Zps1, sharing a functional zincin domain with higher Eukaryotes ECM metalloproteinases. The further presence of the broad signaling cross-talkers Bmh1 and Bmh2, as well as the homing endonuclease Vde that shares a Hedgehog/intein domain with the Hh morphogens from higher Eukaryotes, suggest that analogously to the tissues in these organisms, yeast ECM is mediating signaling events.

Introduction

The yeast *Saccharomyces cerevisiae* is the most studied and best-known lower Eukaryote. As all microorganisms, it is mostly regarded as a unicellular organism. Yet, *S. cerevisiae*, as other yeasts, can form large multicellular communities: colonies, biofilms, and stalks [1]. All these are formed by extremely large numbers of cells, sustained by a scaffolding extracellular polymeric substance (EPS), forming a network of channels conducting water and nutrients to the cells farther from the surrounding medium [2-5]. This is accompanied by large differences in gene expression, metabolic performance between cells in different layers [4, 6-8], namely, the different biological behaviour of cells in what regards apoptosis and therefore cell fate [4, 6-8]. These observations suggest that yeast multicellular aggregates display multicellular-type behaviour and may therefore be regarded as *proto-tissues*. In tissues from higher Eukaryotes, cells are embedded in the extracellular matrix (ECM) that, besides providing these with a supporting scaffold, also mediates molecules diffusion and cell migration, and which components have an active role in each cell fate [9]. Identically, the existence of a microbial ECM in colonies and biofilms, frequently referred as extracellular polymeric substance (EPS), with active roles in the protection against xenobiotics and dissecation has been described in *S. cerevisiae* [4, 7], and *C. albicans* [10]. This encompasses a new conceptualization of microbial life, taking colonies and other multicellular structures as the simplest forms of multicellular organization, with tissue-like behaviour, ensuring spatial organization and group survival.

The molecular characterization of the yeast ECM is still incipient. The ECM of *S. cerevisiae* colonies display large amounts of glycoproteins [2], namely the flocculin Flo11 [8], while *C. albicans* biofilms have been reported to contain proteins, sugars and DNA [11, 12]. Several proteins from carbon metabolism were identified, namely several glycolytic and fermentative enzymes, as well as members of the HSP70 family [13, 14].

Our group developed a methodology to retrieve amounts of *S. cerevisiae* ECM large enough to be assessed in detail. In this work we report, for the first time to the best of our knowledge, the molecular characterization of yeast *S. cerevisiae* ECM proteome. We identified the proteins secreted in yeast ECM grown to homogenous overlay/mat, and compared these with the ones secreted in identical liquid media samples.

Importantly, most of the proteins identified are annotated to cellular compartments, and are now reported to appear extracellularly for the first time. Moreover, entire sets of main metabolic pathways were found, and identically to higher Eukaryotes' ECM, a large number of chaperones and metalloproteinases were identified. This work contributes to the assertion of yeast ECM importance in the development of multicellular aggregates through a first comprehensive image of its molecular composition.

Materials and Methods

Strains and Media

S. cerevisiae strain W303-1A (MATa; leu2-3; leu2-112; ura3-1; trp1-1; his3-11; his3-15; ade2-1; can1-100) was used in this work [15]. Cells were grown on rich medium, YPD (yeast extract 1%; peptone 2%; glucose 2%; adenine hemisulphate 0.005%) on an orbital shaker, 200 rpm, at 30°C. Growth was monitored by optical density (OD) at 600 nm. Solid growth was performed in agarose (2%, w/v) supplemented YPD*a* plates. All ingredients percentages were calculated as weight per volume units.

Yeast Overlay Development and Matrix Extraction

The development of a uniform, ±3 mm thick homogenous yeast culture mat was obtained spreading evenly 1.5 ml of YPD batch cultures at OD_{600} 1 in Ø 90 mm YPD*a* plates and incubating for 7 days at 30 °C [2]. The cellular biomass was gently swapped into a 50 ml Falcon tube. The suspension was washed with PBS buffer (NaCl 100 mM; KCl 2.7 mM; $Na_2HPO_4.2H_2O$ 10 mM; KH_2PO_4 2.0 mM; pH 7.4), supplemented with a protease inhibitor cocktail (PMSF 0.2 µg/ml; Aprotinin 0.32 µg/ml; Pepstatin 1 µg/ml; Leupeptin 1 µg/ml), and incubated for 10 min with constant rotation in a tube roller (SRT1; Stuart, Staffordshire, UK). The suspension, containing cells and ECM, was spun down for 10 min at 15,000 rpm and 4 °C in a Sigma 4-16K centrifuge (Sigma, Osterode, Germany). The supernatant was collected and freeze-dried. The proteins were precipitated using the chloroform/methanol protocol as before [16]. Overnight batch

cultures on liquid YPD with an air:liquid ratio of 2:1 were used as control, to assess the proteins from the extracellular growth medium. Cultures were centrifuged for 10 min at 5000 rpm, and the supernatants collected and processed in the same way as the ECM samples.

Cellular viability assessment

Membrane integrity was assessed by cytometry as described before [17]. Briefly, cells were harvested and added 4 µg/ml PI (Sigma). After a 10 min incubation in the dark at room temperature, the samples were analysed in an Epics® XL™ (Beckman Coulter) flow cytometer.

Proteomic analysis

The SDS-PAGE was carried out in a 10% homogeneous gel [18]. Protein expression was analysed through Western Blot as described before [19]. Total protein extract (50 µg) was brought up to 40 µl with MilliQ water and mixed with loading buffer 5X [18]. The samples were loaded and run at low voltage (25 V) until the migration front reached 2-3 mm above the resolving gel. Two dimensions electrophoresis (2DE) were performed as described before [20], with minor modifications. The samples were cup loaded in isoelectric focusing (IEF) dry strips (24 cm, pH 3-11 NL), and the IEF performed at 20 °C, according to the following program: 120 V for 1 h; 500 V for 2 h; 500-1,000 V in gradient for 2 h; 1,000-5,000 V in gradient for 6 h; and 5,000 V for 10 h. Second dimension SDS-PAGE were run on homogeneous polyacrylamide gel (12% T, 2.6% C). Gels were stained with Colloidal Coomassie Blue as before [21].

The bands containing protein total extract, as well as the chosen spots from the 2DE, were excised and in-gel digested as described in the literature [22]. Samples were digested overnight at 37 °C with 12.5 ng/µl and 1 µg/20µg protein of sequencing grade trypsin (Roche Biochemicals), and in 25 mM ammonium bicarbonate (pH 8.5), for spots and bands, respectively. After digestion, the supernatant from the excised protein bands were analysed by LC-MS/MS and the spots assessed by MALDI-TOF.

Protein Identification

LC-MS/MS

The total extract samples (5 µl), in 0.1% formic acid for a final concentration of 1 µg/µl, were loaded onto a C18-A1 ASY-Column 2 cm pre-column (Thermo Scientific) and then eluted onto a Biosphere C18 column (inner diameter 75 µm, 15 cm long, 3 µm particle size) (NanoSeparations). The proteins were separated using a gradient on a nanoEasy HPLC (Proxeon) coupled to a nanoelectrospray ion source (Proxeon), at a flow-rate of 250 nl/min. The mobile phase A consisted of 0.1% formic acid in 2% CAN and mobile phase B was 0.1% formic acid in 100% CAN. A solvent gradient was applied for 140 min, from 0% to 35% phase B. Mass spectra were acquired on the LTQ-Orbitrap Velos (ThermoScientific) in the positive ion mode. Full-scan MS spectra (m/z 400-1800) were acquired with a target value of 1,000,000 at a resolution of 30,000 at m/z 400 and the 15 most intense ions were selected for collision induced dissociation (CID) fragmentation in the LTQ with a target value of 10,000 and normalized collision energy of 38%. Precursor ion charge state screening, and monoisotopic precursor ion selection, were enabled. Singly charged ions and unassigned charge states were rejected. Dynamic exclusion was enabled with a repeat count of 1 and exclusion duration of 30 ms.

Proteome discoverer 1.2 with MASCOT 2.3 was used as search engine to search in the Uniprot/Swissprot (taxonomy *Saccharomyces cerevisiae*) database (7,798 sequences). The search parameters used were the following: peptide tolerance - 10 ppm; fragment ion tolerance - 0.8 Da; missed cleavage sites - 2; fixed modification, carbamidomethyl cysteine and variable modifications, and methionine oxidation. Mascot ion score 20 and a 99% peptide confidence were set as filters.

MALDI-TOF/TOF

The supernatants from spots excised from 2DE gels were collected and 1 µl was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Subsequently, 0.5 µl of α-cyano-4-hydroxytranscinnamic acid matrix (3 mg/ml in 50% (v/v) acetonitrile (Sigma Aldrich)) was added to the dried peptide digest spots, and allowed to air-dry again at room temperature. Analyses were performed in a 4800 Plus

MALDI TOF/TOF™ and Proteomics Analyzer (Applied Biosystems. MDS Sciex, Toronto, Canada), using 4000 Series Explorer™v 3.5 software (ABSciex). The instrument was operated in reflector mode, with an accelerating voltage of 20,000 V. All mass spectra were internally calibrated using peptides from the auto-digestion of the trypsin. The MS spectra of all the spotted fractions were acquired in positive reflector mode for peak selection (S/N>12). The suitable precursors for MS/MS sequencing analysis were selected, and fragmentation was carried out using the CID (atmospheric gas) on 1 Kv ion reflector mode, and precursor mass Windows +/− 4 Da. The plate model and default calibration were optimized for the MS-MS spectra processing.

The search of peptides was performed in batch mode, using GPS Explorer v3.5 software (ABSciex), 2.3 of MASCOT version (www.matrixscience.com), using the NCBInr database (date: 08052012; 17919084 sequences; 6150218869 residues). The MASCOT search parameters were: (1) species - *S. cerevisiae*, (2) allowed number of missed cleavages – 1, (3) fixed modification - carbamidomethyl cysteine, (4) variable modifications - methionine oxidation, (5) peptide tolerance - ±50 ppm for PMF and 80 ppm for MSMS searches, (6) MS/MS tolerance - ±0.3 Da, and (7) peptide charge - +1. In all identified proteins, the probability score was $p<0.05$, *i.e.*, greater than the one fixed by Mascot as significant.

Western blot

The western blot was performed as described before [19]. The blot was revealed using the peroxidase subtract 3,3'-diaminobenzidine (Sigma, UK). The rabbit anti-VDE antibody was kindly provided by Professor Yoshi Ohya (University of Tokyo, Japan).

Results and Discussion

Experimental Strategy

Our approach to study the yeast ECM proteome started with the standardization of a methodology to multiply the cells into an homogenous overlay/mat of solid growth that can be reproducibly obtained, and that is manageable for the extraction of usable

amounts of ECM. Colonies are too small to handle in that sense. The cells in the mat were primarily separated from the surrounding ECM, and fractioned into analytical-grade protein and sugar fractions. As control for the detection of proteins that correspond exclusively to the yeast ECM, we examined the secreted proteins during batch growth in identical liquid medium (Table 1).

Cells were inoculated so as to fill a whole Petri dish, thus providing a cellular environment similar to a colony [23, 24] yet greatly increasing the yield in biomass. The development of a thick homogenous mat was achieved after 7 days, similarly to other reports [2, 23]. Outer layers of cells from yeast colonies are usually composed of dying cells [25]. A similar observation was reported in *S. cerevisiae* stalks [26]. The viability of the cells during this growth process was followed by cytometry (not shown). The results indicated that in the 7 days-mat, ±15% of the cells were dead or presenting compromised membrane integrity. As much as the existence of dead cells may be natural *in vivo*, eventually contributing to colony or biofilm regular life, the presence of dead cells debris in the ECM samples affects the variety and number of intracellular molecules identified. Therefore, to avoid that intracellular, plasma membrane and cell wall proteins are unduly released into the ECM, a mild non-enzymatic method was chosen to separate and extract the ECM components. To achieve this, cells were gently emulsionized in PBS buffer and centrifuged. The protein fraction was obtained with a protein precipitation protocol known to remove most salts and detergents [16], The use of this protocol was particularly important to avoid interference of buffer salts in the downstream analysis by polyacrylamide gel-based electrophoresis (2DE and SDS-PAGE) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

Proteins Identification

The ECM protein extracts were analysed and compared with batch cultures extracellular medium. The range of protein separation obtained by a large size 2DE was complemented by further using LC-MS/MS. The first encompassed 17 - 120 KDa and 4 - 10 pI proteins. The second allowed the separation of proteins from 3 - 430 KDa and 3.88 - 11.36 pI [20]. The extracts were also preliminarily assessed through SDS-PAGE, comparing the total protein extract against the proteins secreted during solid and liquid

growth (Fig. 1 A). The ECM extract band pattern was clearly different from both total protein and liquid grown samples. The 2DE were identical in 4 independent assays (Fig. 1 B), reinforcing the reproducibility of the ECM extraction methods. With both approaches 693 proteins were unequivocally identified (Table 2). These proteins belong to very diverse pathways, mechanisms and structures. The ECM thus emerges as a dynamic and rich environment, with high molecular diversity. The majority of the proteins identified are ascribed to intracellular compartments in databases. In fact, only 106 were already annotated to the cell surface, cell wall and/or plasma membrane (Table 2), whereas 27 have no ascribed

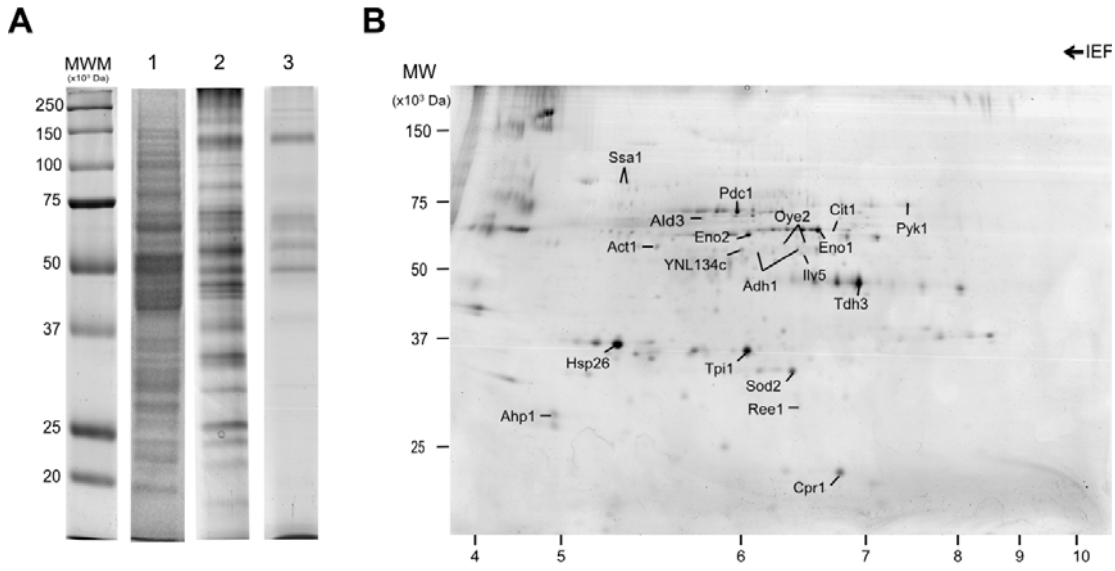

Figure 1. SDS-PAGE and Two-dimensions electrophoretic (2DE) separation of the Yeast ECM proteome. The SDS-PAGE analysis (A) of total protein extract (1), ECM proteome (2), and liquid growth secreted proteins (3) reveal three distinct profiles. The ECM proteome presents a different band pattern from both (1) and (2). The intensity of some bands indicates that the ECM is enriched in some protein species. The 2DE (B) revealed the presence of several glycolytic enzymes, as well as stress response. Some of these proteins should present post-translational modifications, as the predicted pI is different from the experimental determined pI.

location. Nevertheless, some of the supposedly intracellular proteins were already reported to appear in the cell surface, namely several of the glycolytic/ fermentation enzymes [20, 27]. Actually, in yeasts, proteins unconventional localization appears to be more common than thought [28], and might correspond to *moonlighting* proteins for

their unexpected roles, although this designation has been mainly applied to proteins of higher Eukaryotes [29]. A good example of such a protein is *S. cerevisiae* enolase [30] that was also identified in yeast ECM in the present work.

The subcellular localization-related information is often generated using fluorescence-tagged proteins in liquid grown cells [31, 32], although non-tagged proteins may localize differently [33, 34]. Additionally, also different cultivation conditions interfere with protein localization [35]. Computer assisted analysis of protein sequences to predict subcellular location and secretion based on the recognition of signal peptides can be surpassed by the biological mechanism. Most proteins in eukaryotic cells are targeted to the membrane and extracellular space through a tightly regulated process, the canonical Endoplasmic Reticulum (ER)/ Golgi-dependent secretory pathway [36]. However, some proteins are secreted through unconventional mechanisms [28, 37, 38], that mediate the transfer for the extracellular space of two kinds of proteins, (i) those that do not have a recognized signal sequence, and (ii) those presenting the signal peptide, but suffering modifications during the trafficking being diverted from the classical secretory mechanism [37]. Therefore the realization that many proteins actual path during their lifetime is mostly unknown [28].

Functional Classification and ECM Relevant Families

From the yeast ECM 693 proteins (Table 2), 630 have known or predicted roles spread by a wide range of functional groups, while 63 are presently either uncharacterized ORFs or have no attributed function (Fig. 2). From these functional groups, metabolism and protein fate account for 46% of the ECM proteins. Moreover, almost 19% accounts for processes of cellular response, including stress response (Fig. 2).

Metabolism

The carbon metabolism group includes all the enzymes necessary to accomplish glycolysis and alcoholic fermentation, as well as the gluconeogenic phosphoenolpyruvate carboxykinase Pck1 (Table 2). Furthermore, the enzymes Aco1,

Cit1, Mls1, Mdh1, Idh1, Icl2, Idh2 and Idp2[4] from either or both the glyoxylate and TCA cycles were also found (Table 2). Besides carbon, the metabolism of amino acids, nucleotides and lipids are also represented (Table 2).

The presence of a whole metabolic pathway in biofilms had already been suggested to occur in *C. albicans* [13]. Besides, several glycolytic enzymes (Tdh3, Eno1/2, Pgk1, Pyk1 and Fba1[5]) were localized to the cell surface [22, 39]. These proteins may be moonlighters. Tdh3 was detected in the outermost layer of the *C. albicans* cell wall and suggested to act as fibronectin and laminin-binding protein [40], mediating this yeast adhesion to tissues during pathogenic invasion. Accordingly, Tdh3 is found overexpressed *C. albicans* biofilms, being suggested to be implicated in their development [13]. On the other hand, based on results from the *ICL1* deletion mutant, the glyoxylate cycle has been suggested as mandatory for *C. albicans* virulence [41], although its enzymes were never reported to appear extracellularly. The glyoxylate cycle is used in yeasts for gluconeogenesis from fatty acids. Glyoxylate and acetyl-CoA are ultimately converted into phosphoenolpyruvate, which is the product of Pck1, the first enzyme in gluconeogenesis, which is also present in yeast ECM.

S. cerevisiae is Crabtree positive. It preferably utilizes high amounts of glucose to form ethanol, even in the presence of oxygen [42]. Glucose exerts repression on the genes that enable the consumption of alternative carbon sources, such as ethanol [43]. These include a number of genes involved in mitochondrial and peroxisomal functions. The presence in the yeast ECM of proteins which genes are known to be *in vivo* under glucose repression, like Pck1, Mls1 and Icl2, could suggest that the yeast cells mat generated two types of cellular population, one using glucose in the growth medium, and the other putatively feeding on the ethanol generated by the first. In colonies of *S. cerevisiae*, there has been reference to the existence of two sub populations of cells metabolically diverse, promoting nutrients flow inside the colony [44]. Identically, *C. albicans* biofilms display sub-populations of morphologically and metabolically distinct cells [45]. In liquid grown yeasts, where there is unrestricted access to nutrients, some of these glycolytic enzymes were also reported at the cell surface [20, 21, 27]. At this stage we can only speculate what the true function of these proteins in the ECM might be,

4 Aco1 – aconitase; Cit1 - citrate synthase; Mls1 – malate synthase; Mdh1 – malate dehydrogenase; Icl2 – isocitrate liase; Idh1/ Idh2/ Idp2– isocitrate dehydrogenases.

5 Tdh3 - glyceraldehyde-3-phosphate dehydrogenase; Eno1/2 – enolase; Pgk1 - 3-phosphoglycerate kinase; Pyk1 - Pyruvate kinase; Fba1 - Fructose 1,6-bisphosphate aldolase.

namely, whether there is extracellular metabolism as the finding of complete pathways might suggest.

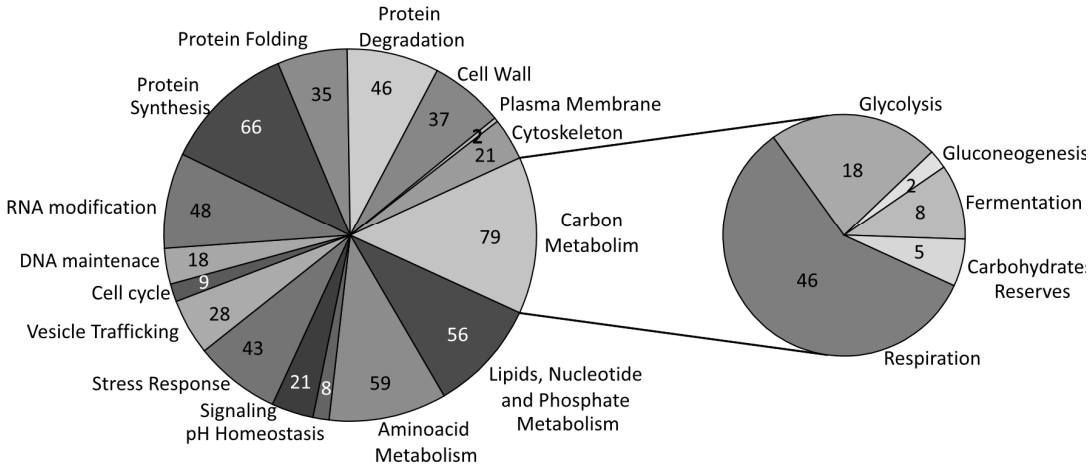

Figure 2. Functional distribution of the proteins from *S. cerevisiae* extracellular matrix.

Protein fate

Of all *S. cerevisiae* ECM proteins, 147 are involved in the synthesis, folding and degradation of other proteins (Fig. 2; Table 2). These include the proteins from the HSP70 family, Ssa1/2/3/4, Ssb1, Ssc1, Sse1/2 and Kar2, and the proteases, Lap4, Dug1, Ecm14, Ape2, Prd1 and Zps1. The HSP70 family includes chaperones that are responsible for the folding and membrane translocation of other proteins [46]. In particular, Ssa1 and Ssa2 are implicated in the biosynthesis and assembly of the cell wall [47]. These two proteins, as well as Ssb2 and Sse1 were previously reported to be present in the cell surface in both *S. cerevisiae* and *C. albicans* [20, 21, 27].

In mammalian ECM, metalloproteinases ensure constant remodelling of the glycoproteins [48]. Yeasts do not have a group of recognized metalloproteinases. Nevertheless, from the proteases found in *S. cerevisiae* ECM, Lap4 is a Zn-metalloproteinase, Dug1 is a metallo-di-peptidase, Ecm14 is a Zn-carboxipeptidase, Prd1 is a metalloendopeptidase, and Ape2 is an aminopeptidase. Lap4, Dug1 and

Ecm14 share the functional domain Zn–dependent exopeptidase, therefore belonging to the same Zn-dependent exopeptidases superfamily (53187), which includes another 11 yeast members not found in the ECM survey. On the other hand, Prd1 and Ape2 both have a functional zincin domain. This domain is also present in another protein identified in yeast ECM with unknown function, Zps1. Prd1, Ape2 and Zps1, together with 6 other yeast proteins not found in the ECM survey, belong to the Metalloproteases ("zincins") superfamily (55486) that includes, importantly, mammalian ECM metalloproteinases [49]. In higher Eukaryotes, ECM demands for constant remodelling. Analogously, we suggest that such a function in *S. cerevisiae* ECM is performed by the hereby-identified metalloproteinases Lap4, Dug1 and Ecm14 from the Zn-dependent exopeptidases superfamily, and Prd1, Ape2 and Zps1 from the metalloproteases ("zincins") superfamily, as well as the HSP70 chaperones Ssa1/2/3/4, Ssb1, Ssc1, Sse1/2 and Kar2.

Once considering the parallelism between yeast and higher Eukaryotes ECM, we should expect to find a protein able to perform the structural roles of collagen and proteoglycans. Springer and co-workers [50] showed that the pathogenic yeast *Cryptococcus gattii* formed extensive extracellular fibrils sensitive to cytoskeleton protein inhibitors [50]. Also in *S. cerevisiae*, inter-cellular filaments were reported in 3 weeks starved colonies [51], although there was no reference as to their molecular nature. In the present survey, actin (Act1) was well represented (Table 2), and tubulin (Tub2) was also found although in trace amounts (Table 2).

Cellular response

From the group of proteins that were ascertained to the broad designation of Cellular Response, the presence of ROS-related proteins, the signalling Bmh1 and Bmh2, and the Subunit A of the V1 domain of the vacuolar ATPase Tfp1 stand out. Response to oxidative stress is another function well represented in the yeast ECM through the presence of Sod1 and Sod2, Ctt1, Trx1 and Trx2 and Trr1[6]. These enzymes are involved in the response to several reactive oxygen species (ROS), namely superoxide and both hydrogen and organic peroxides. Cells deficient in *SOD1* grow poorly in liquid media [52], yet, both these and the Δ*sod2* mutants keep producing

[6] Sod1 / Sod2 – superoxide dismutase; Ctt1 – catalase; Trx1 / Trx2 – thioredoxin; Trr1 - thioredoxin reductase.

colonies identically to Wt. This could indicate that the response to oxidative stress is not working as such in a multicellular environment. In accordance, Cap and co-workers [53] showed that colonies lifespan depends more on the maintenance of cellular metabolic differentiation, and Sod2 is necessary for this differentiation, since the deletion of this gene affects cell ageing and viability across the whole colony [53].

The Bmh1 and Bmh2 found in yeast ECM are also known as the 14-3-3 proteins. They are transversal to a large number of signalling pathways. These include the TOR pathway and retrograde response, HOG, PKC and Ras, pathways as well as the chitin synthesis control [54, 55]. In *S. cerevisiae* the double deletion of these two genes is lethal [56]. Bmh1 and Bmh2 have been observed in the cell surface of both *S. cerevisiae* and *C. albicans* [20, 22, 27]. The presence of such broad cross talking controllers of signalling in the yeast ECM suggests that, identically to higher Eukaryotes, signalling events occur through the matrix, therefore implying cell-to-cell communication.

Tfp1 is the designation of the ORF that actually encodes for two proteins, the vacuolar membrane ATPase Vma1, and the intein homing endonuclease Vde, also known as PI-SceI. In the present survey, the peptides that originated the identification of Tfp1 correspond to three different parts of this ORF, two inside the Vma1 sequence (in the beginning and in the end of the protein), and the other inside the Vde sequence. This means that the yeast ECM may actually harbour either or all Tfp1, Vma1 and Vde.

The presence of the Vde protein in the ECM was further surveyed by Western Blot with a rabbit anti-Vde antibody. This antibody is capable of recognizing Vde before and after the auto-excision process, yielding several molecular size bands. Tfp1 has 120 kDa, Vma1 70 kDa and Vde 50 kDa [57]. In the total cell protein extract (including all the intracellular proteins), a thick band of 50 kDa (Fig. 3, lane 1, arrow head) indicated the presence of large amounts of Vde. Additionally, this sample also presents the predicted higher and lower molecular weight intermediates of 90 kDa, 80 kDa, 40 kDa and 30 kDa corresponding to Vde+Vma1-C', Vde+Vma1-N', Vma1-C'and Vma1-N' respectively (Fig. 3, lane 1, arrows). This is in agreement with the intracellular processing mechanism of Tfp1 to yield Vma1 and Vde [57]. However, the lower molecular weight intermediates visible in lane 1 are absent from ECM protein sample (Fig. 3, lane 2), as well as from lane 3 which shows residual amounts of Vde secreted during liquid growth (Fig. 3, lane 3). In view of these results, the presence of this protein in the ECM seems to occur through a specific mechanism, as the lower

molecular weight bands should result from misprocessed proteins during intein excision. As such, the presence of just the intein (50 kDa band) and some higher molecular weight intermediates suggests that only the correctly processed forms are secreted, indicating the presence of some kind of regulatory mechanism.

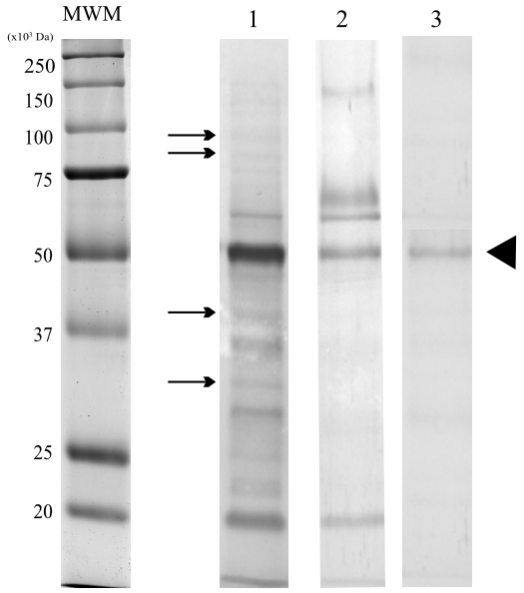

Figure 3. Yeast intein Vde presence assessed through Western Blot. The intein Vde was present in all tested conditions, total cell protein extract (1), ECM proteome (2) and liquid growth secreted proteins (3). The antibody detected and bound the main 50 kDa form (arrow head), as well as all the higher molecular weight intermediates forms and the miscleaved smaller peptides (arrows). The ECM extract presented both the mains 50 kDa protein and the higher intermediates, but completely lacked the smaller size peptides. Only the 50 kDa protein was above detection level in (3).

The Vde is the only recognized intein from *S. cerevisiae* proteome, and belongs to the Hedgehog/intein (Hint) domain superfamily 51294. This superfamily includes the self-splicing Hedgehog (Hh) proteins from higher Eukaryotes. The Hh proteins command crucial events during embryogenesis and wound healing, namely cellular differentiation, patterning and migration [58]. The pathway operates on a cell-to-cell signalling basis. The signalling Hh protein diffuses through the ECM generating a molecular gradient according to proximity [59]. Considering that *S. cerevisiae* colonies and *C. albicans* biofilms display sub-populations of metabolically, molecularly and

morphologically differentiated cells [10, 44], it would not be surprising if a Hedgehog-like pathway could operate through yeast ECM. The proteins that modify the Hh secreted signal in numerous high Eukaryotes, Hhat and Hhatl, are orthologous to the *S. cerevisiae* MBOAT *O*-acyltransferases Gup1 [60] and Gup2 [61-63] and *C. albicans* Gup1 [64]. The putative presence of Tfp1, Vma1 and/or Vde in the yeast ECM, along with the Bmh1 and Bmh2, is a strong indicator that broad signalling events implicating cell-to-cell communication might operate in a Hedgehog pathway-like manner in yeast ECM.

Conclusions

The acknowledgement of the existence of structural organization, and differential expression of genes with concomitant metabolic and morphological specialization across a yeast colonies, biofilms or stalks, suggests that yeasts have a complex multicellular behaviour. The existence of an extracellular matrix that, in analogy to the higher Eukaryotes, may operate as scaffold but also actively contribute to this behaviour agrees with the suggested complexity. The present work presents the first identification of the proteome of *S. cerevisiae* ECM.

It stands out the presence of sets of intracellular enzymes covering whole metabolic pathways, glycolysis and gluconeogenesis through the glyoxylate cycle. Although some of these enzymes have been previously described as *moonlight* proteins, the presence of entire pathway enzyme sets generates doubts as whether these might actually be metabolically active in the yeast ECM. Additionally, the simultaneous presence of glycolytic and glucose repressible enzymes could derive from two metabolically distinct sub-populations of cells coexisting in the multicellular aggregates. This would also be compatible with the extracellular presence of proteins from oxidative stress response that have been associated with cellular differentiation and ageing in yeast colonies. Furthermore, from the proteins identified, it also stands out the presence in *S. cerevisiae* ECM of chaperones and metalloproteinases that could be active on protein remodelling, identically to the metalloproteinases from higher Eukaryotes ECM. Further extending this parallel, broad signalling events could originate from the diffusion of proteins found in yeast ECM, able to control many pathways and to possibly also command differentiation and spatial distribution events. By this work, a door is opened to deepen

the understanding of yeast extracellular matrix as a model for multicellular life, taking yeast colonies, biofilms, or other types of large aggregates like the overlays used in this work as tissue-like communities.

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4. YEAST ECM POLYSACCHARIDES

Abstract

In a multicellular organism, the extracellular matrix (ECM) provides a cell-supporting scaffold and helps maintaining the biophysical integrity of tissues and organs, at the same time playing crucial roles in cellular communication and signalling, implicating in spatial organization, motility and differentiation. Similarly, the presence of an ECM-like extracellular polymeric substance is known to support and protect bacterial and fungal multicellular aggregates such as biofilms or colonies. However, the roles and composition of this microbial ECM are still poorly understood. In the present work, we report a protocol for obtaining a homogenous mat of young cells of *Saccharomyces cerevisiae* and from this the extraction and separation into analytical purity of the main molecular fractions of yeast ECM. The analysis of the glycosidic fraction by anion exchange chromatography, diaminopropane agarose and polyacrylamide electrophoresis, suggests the presence of two well-defined low molecular weight polysaccharides. The mass spectrometry data indicates that these are composed of glucose, galactose, mannose and uronic acids. Our results also suggest the presence of possible sulphate substitution, given the metachromatic shift of DMMB and toluidine blue, as well as the anticoagulant effect variably displayed by the ECM extracts of some yeast mutant strains. These results pioneer the study of yeast surface glycomics beyond the cell wall, opening a whole range of unsolved questions for the future, and positioning *S. cerevisiae* as a possible model for the study of eukaryotic ECM.

Introduction

In a multicellular organism, the maintenance of the homeostatic balance required for regular function ultimately depends on the extracellular matrix (ECM) where cells are embedded. The ECM provides a cell-supporting scaffold and helps maintaining the biophysical integrity of tissues and organs. ECM is under constant remodelling, changing rigidity, porosity and spatial disposition, therefore orienting cell movement and tissue growing [1, 2]. Moreover, the diffusion of molecules from cell to cell depends on ECM, that in this way influences directly the availability of signalling effectors of all kinds, including growth factors and hormones [3, 4], playing crucial roles in all tissue rearrangements [5].

Mammalian ECM presents a great number of functional molecules, biochemically and biophysically diverse, including proteins, glycoproteins, glycosaminoglycans (GAGs), and proteoglycans (PGs). PGs are vital structural and signalling molecules composed of a core protein with one or more covalently attached GAGs [6, 7], which can be a single GAG, as decorin [8], or several units of different GAGs, as versican and aggrecan [9, 10]. GAGs are linear heteropolysaccharides consisting of repeating disaccharide units, composed of a hexosamine (glucosamine or galactosamine, frequently *N*-substituted) and a hexuronic acid (glucuronic acid or iduronic acid). Exceptionally, in keratan sulphate, *N*-acetyl-glucosamine associates with galactose instead. GAGs are generally multi-sulphated, with the exception of hyaluronan [6, 7, 11-13]. In mammals, a few PGs are secreted to the extracellular space, like the large molecular weight aggrecan, or the small leucine-rich PGs [14-16]. Some are membrane tethered, through a glycosylphosphatidylinositol anchor, like glypican, or by a membrane spanning protein core, as syndecan [7, 17]. A high molecular diversity arises from the different combinations of PGs protein cores with one or more types of GAGs, eventually leading to a wide variety of biological roles. As such, disturbances in PGs synthesis and turnover lead to severe pathologies [5]. Moreover, important medical properties were also reported for some ECM components. Heparin for example, presents anti-thrombotic, anti-coagulant, anti-inflammatory and anti-metastatic properties [18-20].

The presence of ECM-like extracellular polymeric substances, is known to support and protect multicellular bacterial and fungal aggregates [21-27]. *Saccharomyces*

cerevisiae yeast cells are able to form complex multicellular aggregates: stalk-like structures [28, 29], mats/biofilms [30, 31] and colonies [27, 32]. These last display a high level of cellular organization [27, 33-35], and an ECM-like glycosidic substance [27, 35] involved in the conduction of nutrients to the top layers of cells [27], while providing protection against xenobiotics [35], and dissecation [32]. Within a colony, physiologically different subpopulations are present [36], a substantial part of which is in a non-dividing and quiescent state [37, 38]. Like in mammals, these cells present condensed chromosomes [39] and unreplicated genomes [40], with low rates of translation [41, 42], getting most of their energy from respiration [43]. A genomic analysis showed that different genes are expressed accordingly with the area that the cell occupies in the colony. In fact, even apoptosis is coordinated differently within the same colony [34, 44, 45].

The complex chemical nature of the microbial ECM has been poorly assessed [46, 47]. In *Candida albicans* biofilms, ECM was shown to contain proteins, polysaccharides, and DNA [48-50]. In *S. cerevisiae*, some preliminary data showed the existence of glycoproteins [27] [35]. A more recent work showed ECM to be highly glycosylated, and highlighted the presence of high amounts of secreted Flo11p [35].

In the present work, a protocol was devised to obtain a young homogenous mat-type growth of *S. cerevisiae*, and separate the ECM main glycosidic fraction to analytical purity allowing chemical analysis of its components. This revealed the presence of two low molecular weight polysaccharides, basically composed of glucose, mannose, galactose and uronic acid. The metachromatic shift induced by the ECM extracts, as well as the anticoagulant activity showed by some yeast strains ECM, suggests the further presence of sulphate groups in *S. cerevisiae* ECM polysaccharides. Although the chemical nature of the glycosidic bonds, and the macromolecular structure of the polysaccharides are still under study, this work pioneers the acknowledgement of *S. cerevisiae* ECM polysaccharides main components and their complexity.

Materials and Methods

Strains and Media

S. cerevisiae strains used in this work are listed in Table 1. Liquid batch cultures were done on YPD (yeast extract 1%; peptone 2%; glucose 2%; adenine hemisulphate 0.005%) on an orbital shaker (200 rpm), at 30 ºC and an air: liquid ratio of 2:1. Growth was monitored by measuring OD at 600 nm. Yeast mat development was obtained on YPD supplemented with 2% electrophoretic grade agarose instead of agar (YPDa). All media constituents are expressed in weight /volume percentages.

Table 1. *S. cerevisiae* strains used in the present study.

Strain	Genotypes	Origin
W303-1A	*MAT***a***leu2-3 leu2-112 ura3-1 trp1-1 his3-11 his3-15 ade2-1 can1-100*	[51]
BHY54	Isogenic to W303-1A but *gup1::His5*$^{+}$	[52]
Cly5	Isogenic to W303-1A but *gup2::KanMX*	[52]
Cly3	Isogenic to W303-1A but *gup1::His5*$^{+}$*gup2::KanMX*	[52]

Yeast mat development and ECM extraction

The development of a uniform yeast culture overlay/mat was obtained spreading evenly 1.5 ml of YPD batch cultures at OD600=1 in Ø 90 mm YPDa plates and incubating for 7 days at 30 °C. The cellular overlay was carefully removed from the plates into a 50 ml Falcon tube, suspended in PBS buffer and homogenised for 10 min with constant gentle rotation in a tube roller (SRT1, Stuart, UK). The suspension, containing cells and ECM, was then spun down for 10 min at 10,000 rpm and 4 °C (Sigma4-16K, Germany). The supernatant, containing the water-soluble ECM, was collected and freeze-dried (Christ Alpha 2-4 Christ LDC-1m, B.Braun, Germany).

Protein fraction collection and Western Blot

The ECM protein sample was collected and treated as described before (Ch. 3). The proteins were separated in a 10% PAGE and blotted in a PVDF membrane and probed with Concanavalin-A – Horseradish Peroxidase conjugate as previously described [27].

Polysaccharide Precipitation and Fractionation

The lyophilized supernatant was resuspended in digesting buffer (0.1 M sodium acetate; 5 mM EDTA; 5 mM cysteine; pH 5.5) in a proportion of 20 ml for each 1 g of lyophilized supernatant. Double-crystalized papain was added to the mixture (10 mg/ml) and incubated at 60 °C overnight. The mixture was centrifuged (3,000 rpm for 10 min at room temperature), and the clear supernatant was collected. The recovery of yeast ECM polysaccharides was achieved through ethanol precipitation. Three volumes of ethanol (95-99% v/v) were added to the supernatant and incubated overnight at 4 ºC. The precipitate was collected by centrifugation, 10 min at 3,000 rpm, and left to evaporate the residual ethanol. An aliquot of the resulting pellet was resuspended in deionised water and stored at 4 ºC for (1) electrophoretic analysis and (2) evaluation of total sugar content, by the reaction of phenol-sulphuric acid with hexoses [53], (3) presence of hexuronic acids, through carbazole method [54], and (4) metachromatic shift of 1,9-dimethylmethylene blue (DMMB), indicative of chemically substituted polysaccharides [55]. Another aliquot of the ethanol-precipitated pellet (~20 mg) was resuspended in 1 ml MilliQ water, filtered through a syringe filter (0.22 µm) for analysis in a FPLC system (Pharmacia Biotech, Sweden). The filtered sample was applied to a Hitrap Q-XL-FPLC column, equilibrated with elution buffer (20 mM Tris-HCl; pH 8.6). The glycosaminoglycans were eluted by a linear gradient of 0–3.0M NaCl (10 ml) at a flow rate of 0.50 ml/min. Fractions of 0.5 ml were collected and hexuronic acid [54], and total sugars [53] were quantified. Additionally, sulphated polysaccharides were also assessed [55]. The fractions comprising the peaks detected were collected and dialysed against MilliQ water for 48 hours and subsequently freeze dried.

Sugar Fraction Chemical Analysis

a) Mass Spectrometry

The ratio of monosaccharides in the extract of yeast ECM was determined by GC/MS, analysing the corresponding alditol acetate derivatives, according to the previously described method [56]. Briefly, the samples were briefly hydrolysed with trifluoroacetic acid at 100° C for 4 h. The monosaccharides released were converted into alditol acetate by successive reduction with NaBH4 and acetylation with Ac2O pyridine, allowing identification by GC/MS. The samples were analysed by GC-MS system (Shimadzu QP2010 Plus, Japan) equipped with a Restek RTX-5MS column. Ultrapure helium (99.999%) was used as the carrier gas at a constant flow of 1.0 ml/min. The oven temperature was programmed to increase from 110 °C to 260 °C in a 70 min period. The ion-source temperature was 200 °C and the interface temperature was 230 ºC.

b) GAG Agarose Gel Electrophoresis

ECM total polysaccharide, as well as the respective fractions, were submitted to agarose gel electrophoresis as described previously [13]. Briefly, about 1.5 µg of the sample was applied to a 0.5% agarose gel in a buffer composed of 50 mM 1,3-diaminopropane acetate (pH 9.0), and run for 1 h at 100 V. As standard, a mixture of GAGs, containing chondroitin sulphate, dermatan sulphate, and heparan sulphate (1.5 µg of each) was used. The GAGs were fixed with aqueous 0.1% cetyl-trimethylammonium bromide solution (Cetavlon®), allowed to dry, and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v/v).

c) GAG PAGE

PAGE was used to estimate the MW of polysaccharide molecules. Samples (10 µg) were applied to a 1-mm-thick 6% polyacrylamide gel in 3X PAGE running buffer (60 mM Tris-HCl; pH 8.6), and run at 100 V. The gel was stained with 0.1% toluidine blue

in 1% acetic acid. After staining, the gel was washed overnight in 1% acetic acid. The molecular mass markers were dextran sulphate 8 (average MW 8,000 Da), chondroitin-4-sulphate (average MW 36,000 Da), and dextran sulphate 100 (average MW 100,000 Da).

Anticoagulant Action Measured by Activated Partial Thromboplastin Time (aPTT)

Activated partial thromboplastin clotting time assays were carried out incubating at 37°C for 1 min, 100 µl of human plasma with 10 µl of the yeast ECM polysaccharide sample. As a control, the incubation was performed with a solution standard of heparin (50µg/ml). Subsequently, 100 µl of cephalin were added, and the mixtures re-incubated for 2 min, after which 100 µl of CaCl2 (0.25 M) were added and the clotting time recorded in a coagulometer (KC4A, Amelung) [57].

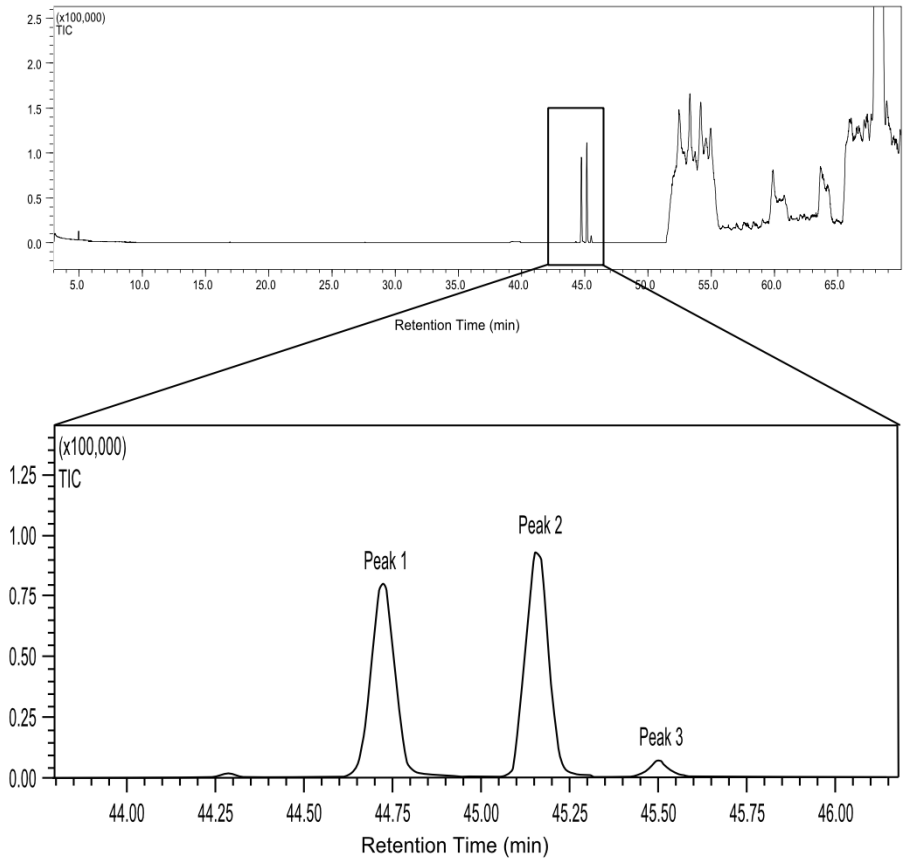
Results and Discussion

S. cerevisiae polysaccharides in the ECM

The extraction and characterization of yeast ECM protein fraction was recently described by our group (Ch. 3). In this work we applied a complementary methodology to extract considerable amounts of polysaccharides from yeast ECM. The production of a *S. cerevisiae* young cells mat and extraction of the correspondent ECM (Faria-Oliveira *et al.*, submitted), was combined with a widely used protocol to recover and identify the polysaccharides of mammalian ECM [57]. The ECM total extracts, containing both proteins and sugars, were treated with a broad range proteinase, double crystalized papain, to eliminate all protein residues, as described before for higher eukaryotes tissues [57]. Then the polysaccharides were recovered by ethanol precipitation [57]. This approach yields a pure sugar fraction that allows chemical downstream analyses aimed at the characterization of the ECM polysaccharide components.

The yeast ECM total polysaccharides were initially characterized by mass spectrometry (Fig. 1). The results indicate that the polysaccharides present are mainly

composed by glucose (52%), mannose (44%), and galactose (4%). The presence of such monomers suggests that these ECM polysaccharides might be similar to the glucans and/or mannans present in yeast and fungi cell walls. In part, this is in concert with the studies on *C. albicans* biofilms, which revealed the presence of several polysaccharides: β-1,3 glucan, a major biofilm component presenting a putative role in antifungal resistance [58], and a polysaccharide composed of α-D-glucose and β-D-glucose, α-D-



Peek number	Retention time (tR - min)	Proportion (% of total)	Sugar	*m/z* (GC-MS)
1	44,7	44	Mannose	43, 85, 115, 127, 145, 187, 217
2	45,1	52	Glucose	43, 85, 115, 127, 145, 187, 217
3	45,5	4	Galactose	43, 85, 115, 127, 145, 187, 217

Figure 1. Yeast polysaccharides composition. Mass spectrometry of the ECM glycosidic constituents: the presence of glucose, mannose and galactose was revealed. The retention times and main mass-to-charge ratios for each monomer are included in the table.

mannose, α-L-rhamnose and *N*-acetyl glucosamine [25]. Nevertheless, the presence of galactose on *S. cerevisiae* ECM hints for the presence of more unconventional carbohydrates, as some galactoglycans described for algae and ascidians [59, 60].

ECM polysaccharides present chemical substitution

Samples of the ECM papain-treated total polysaccharide extract induced metachromasia in both DMMB and toluidine blue dyes, and tested positive for the presence of uronic acids through the carbazole method (not shown). The induction of metachromasia is frequent in chemically substituted polysaccharides, namely the highly sulphated glycosaminoglycans from higher eukaryotes ECM [61], whereas, polysaccharides rich in uronic acids are common in both high eukaryotes and bacteria ECM [6, 24].

These same samples were submitted to PAGE, and their components separated according to the molecular weight. ECM papain-treated total polysaccharide extract presented two distinct polydisperse bands (Fig. 2A, black arrows), the more abundant band showing low molecular weight (<8 kDa), and a lighter band showing an average size of 35-40 kDa. The abundant low molecular weight band might be composed by oligosaccharides, with just some disaccharide units, putatively associated with protein cores, similarly to what happens with the higher eukaryotes PGs. To test this hypothesis, we analysed the glycosylation level of the ECM proteins with Concanavalin-A (Fig. 2B), which recognizes glycosyl and mannosyl groups. The results (Fig. 2B) showed that the ECM protein extract presented a highly glycosylated pattern. Several intense bands were detected and could be easily attributed to protein bands on the SDS- PAGE. The indication of several polysaccharides in the yeast ECM composition led to the samples fractionation in an anionic exchange column (Fig. 3A) and analysis by diaminopropane agarose (Fig. 3B).

The ECM papain-treated total polysaccharides were loaded in the column and a NaCl gradient (0-3M) was used to separate the differently charged molecules. Fractions (0.5 ml) were collected and each tested once more for metachromasia, uronic acids, as well as total sugars. The fractionation of the yeast ECM extract yielded two major sugar peaks that tested positive for uronic acid, presenting as well metachromasia in DMMB

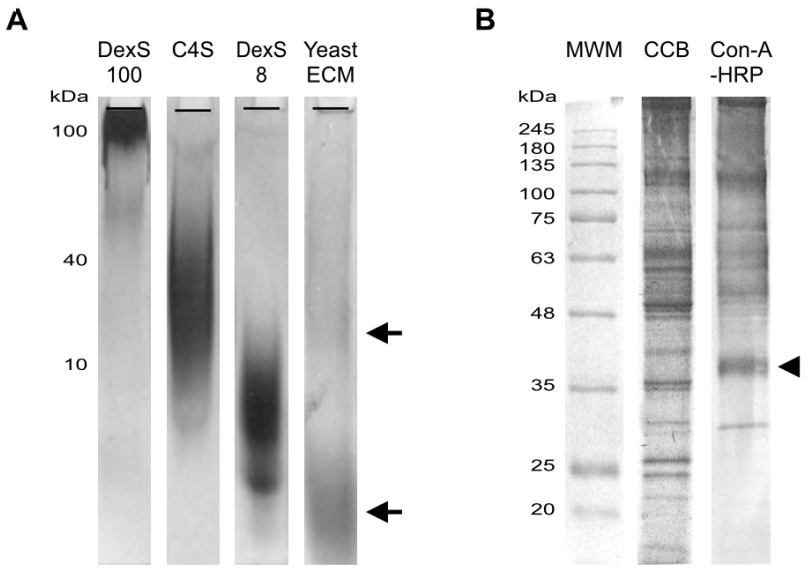


Figure 2. Yeast ECM molecular size and association to proteins. (A) The molecular weight assessment revealed two components presenting distinct sizes (arrows): one fraction weighing less than 8 kDa, and another component in small amounts with an average weight of 35-40 kDa. (B) The yeast ECM revealed a wide range of glycosylated proteins, particularly a highly glycosylated band (arrow). STD – GAG standards: DexS100 - Dextran Sulphate MW 100,000; CS4 – Chondroitin-4-Sulphate MW 36,000; DexS8 – Dextran Sulphate MW 8,000. CCB – Colloidal Coomassie Blue staining; Con-A-HRP – Concanavalin-A - Horseradish Peroxidase conjugate.

(Fig. 3A). This indicates that two compounds were differentially eluted by the NaCl gradient. The presence of metachromasia in ECM polysaccharides is mostly associated with the presence of sulphate groups [6], however, metachromasia may also be induced by a wide range of polyanions [62-64]. These later compounds, also known as chromotropes, include polyphosphates, polyacrylates, polysulphates, carboxylated polysaccharides, and include some proteins and nucleic acids as well [62]. While some of these compounds may be present in the yeast ECM and therefore induce the metachromatic shift, the chromatographic fractionation revealed a “clean” profile (Fig. 3A), which suggests the presence of just two major compounds. The compounds constituting these two peaks also tested positive for the presence of some type of uronic acid (Fig. 3A). Accordingly, glucuronic and iduronic acids are common on in higher eukaryotes ECM [6], and the presence of mannuronic and guluronic acid was already reported in *Pseudomonas aeruginosa* biofilms [24]. The fractions composing each of the two major peaks were collected (P1 and P2) and submitted to electrophoretic analysis

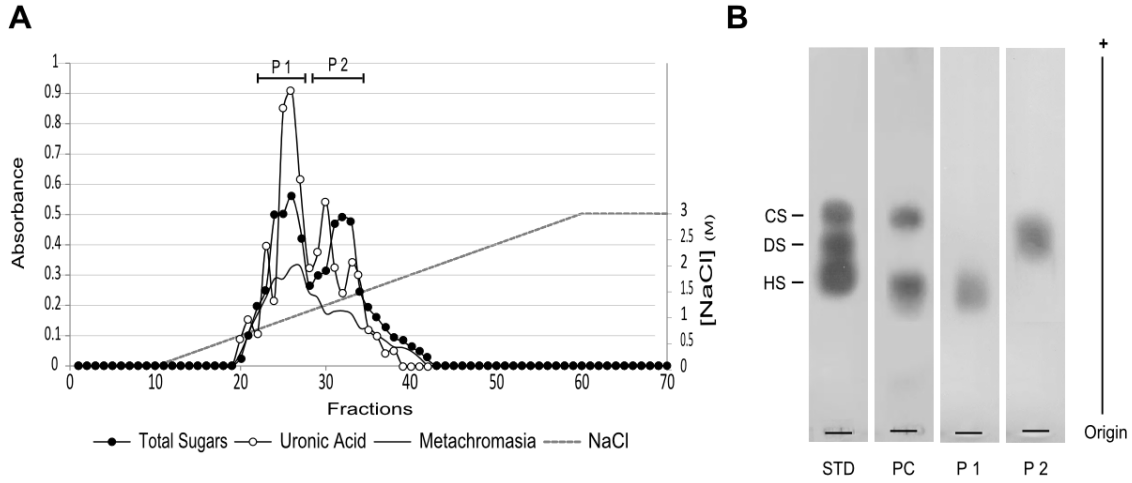


Figure 3. Chromatographic fractionation and electrophoretic profiles of yeast polysaccharides. The total ECM sample was submitted to anionic exchange fractionation and yielded two main compounds fraction P1 and P2 (A). The same sample was analysed through diaminopropane agarose electrophoresis before the FPLC fractionation (PC), together with fractions P1 and P2 (B). STD – GAG standard (see Materials and Methods)..

(Fig. 3B). The diaminopropane agarose electrophoresis is a powerful tool to separate compounds with different degrees of chemical substitution, usually sulphated polysaccharides [65]. The charged molecules have high affinity for the diaminopropane matrix in the agarose gel. The higher is the affinity/charge of the migrating compound, the shorter is the migration path. The pre-chromatography sample (PC), as well as the fractions composing the two peaks (P1 and P2), were submitted to this electrophoretic separation (Fig. 3B). The PC sample presented two clearly distinct bands; two metachromatic compounds migrated similarly to chondroitin sulphate (CS) and heparan sulphate (HS), putatively presenting a chemical substitution degree similar to these GAGs. These two compounds were efficiently separated through FPLC (Fig. 3B), even though a little more dispersion is visible. Similarly to other reports using both FPLC fractionation and 1,3- diaminopropane agarose electrophoresis for GAG analysis [61], the first eluted ECM component (P1) matched the shortest migration in the agarose electrophoresis. These two fractions might be the same molecule with different degrees of chemical substitution, or more likely two different molecules. The latter is supported by the molecular weight assessment in PAGE (Fig. 2A).

Anticoagulant activity displayed by yeast substituted-polysaccharides

Several studies showed the importance of sulphate substitution in the anticoagulant effect of heparin [66], as well as of DS [67], or tunicates analogues [13, 18, 57]. Therefore, assessing the yeast ECM components for anti-coagulant properties may be an indirect way of confirming this type of residue substitution in ECM polysaccharides. This was done using the same ECM total extracts above tested. The yeast putative morphogens Gup1p and Gup2p were previously described to interfere with cell wall and plasma membrane composition and function [68-70], including wall polysaccharide assembly, lipid metabolism, and rafts and GPI anchors integrity. The role of Gup1 in the colony morphology, as well as in invasive and filamentous growth, was also reported in *C. albicans* [70]. Therefore, the glycosidic fraction of the ECM extracted form Wt was compared to the one from these genes single and double mutant strains (Fig. 4). As a control, bovine heparin anticoagulant action was used.

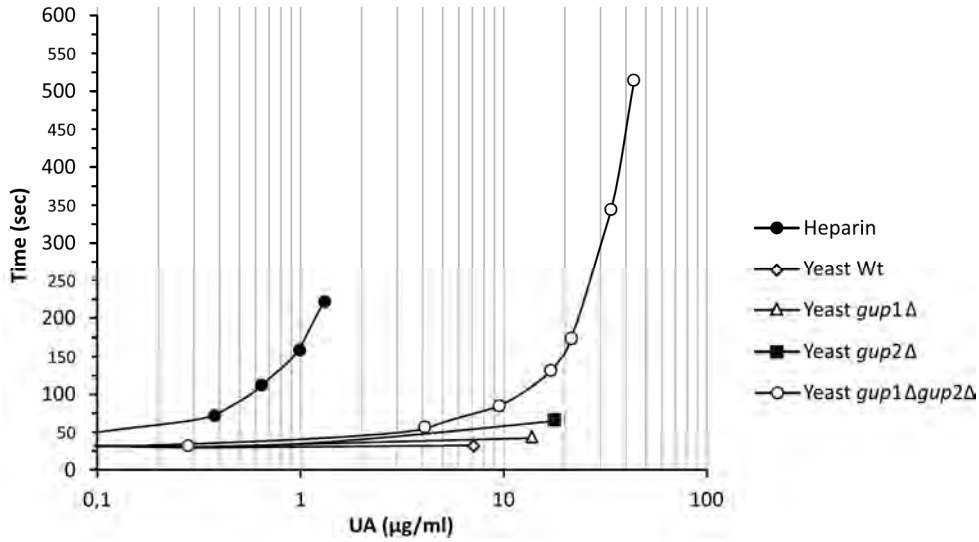


Figure 4. Anticoagulant effect of yeast ECM extracts. Anticoagulant effect of ECM extracts from several yeast strains. Yeast Wt was compared to the mutant strains defective in the putative morphogens Gup1p and Gup2p, known to interfere with cell wall and plasma membrane composition and functions [68-70], including rafts and GPI anchors integrity, as well as in colony morphology, and invasive and filamentous growth [70]. These strains were assessed for their ECM polysaccharides chemical substitution through the aPTT test. The differences in anticoagulant effect represent an indirect measurement of the presence of sulphate groups. Heparin (50µg/ml) was used as control. UA- Uronic acid concentration.

ECM papain-treated total polysaccharide extracts from several yeast strains (Table 1) were tested for anticoagulant properties. The double mutant *gup1Δgup2Δ* extract presented the highest anticoagulant effect (Fig. 4). Given the role of sulphation pattern and degree in anticoagulant effect of mammalian heparin and ascidian analogues [18], the effect displayed by this mutant suggests that the disruption of these genes influences the chemical substitution present on the ECM polysaccharides, supposedly increasing sulphation. These results suggest that some sulphate substitution is present in the polysaccharides, but also that a dynamic substitution pattern regulated by genetic elements is observed. The nature of the chemical substitution in the yeast ECM is currently being examined using tools with higher analytic power, including the methylation analysis of such compounds and NMR.

Conclusions

This work presents the first report on the nature of *S. cerevisiae* ECM polysaccharides. Hints on the presence of uronic acids, besides the common sugars of glucose, mannose and galactose, indicate that yeast ECM polysaccharides are complex molecules. Our results also suggest the presence of sulphate substitution in the yeast ECM, given the metachromatic shift of DMMB and toluidine blue, as well as the anticoagulant effect displayed by the mutants ECM extract. However, as mentioned before, several chemical groups may induce the metachromasia of DMMB and toluidine blue, and the presence of classic sulphotransferases, as well as the chemical intermediates, has not been so far reported in *S. cerevisiae*. These results pioneer the study of yeast surface glycomics beyond the cell wall, opening a whole range of unsolved questions for the future, and positioning *S. cerevisiae* as a possible model for the study of eukaryotic ECM.

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5. THE EFFECT OF THE DELETION OF *GUP1* GENE ON YEAST ECM COMPOSITION

Abstract

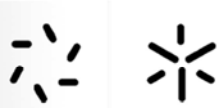
The study of yeast colonies extracellular matrix (ECM) structure, composition and properties is still in an early stage. In order to better understand the mechanisms underlying the ECM assembly and maintenance, we assessed the multicellular aggregates formed by cells lacking the pleiotropic gene *GUP1,* known to affect cellular processes so diverse as cell wall and plasma membrane composition and organization, cytoskeleton assembly, or apoptosis. The mass spectrometry analysis of the ECM proteome revealed that the mutant's presented less 106 proteins than the Wt. The common proteins were assessed for differences between strains through the highly sensitive DIGE methodology. A total of 56 proteins were present in statistically different amounts. Seven spots presented a 2-fold increase in abundance in the mutant, whereas eight were more abundant in the Wt. Both the absent and differentially present proteins belong mainly to the functional classes: carbon metabolism, cellular rescue and defence, protein fate, and cellular organization. The absence of such proteins concurs with several of the known phenotypes of *gup1Δ,* namely the impaired response to stress and diminished ability to utilize non-fermentable carbon sources. Nevertheless, we cannot overlook the possibility that all or most of these protein are moonlighting, *i.e.*, performing completely new functions in the ECM, its maintenance and biological activity. Similarly, the absence of Gup1 also has a high impact in the polysaccharide components of the ECM. The mutant presents a low molecular weight oligosaccharide, but lacks the 35-40 kDa polysaccharide also observed in the Wt. Additionally, the deletion of *GUP1* severely compromised the synthesis and/or chemical substitution of the ECM polysaccharides. The unveiling of Gup1 and Gup2 roles in the ECM composition, structure and chemical properties will certainly contribute for the understanding of the *S. cerevisiae* multicellular lifestyle.

GUP1 – *A Jack of all trades, a master of none*

Saccharomyces cerevisiae was the first eukaryote with the genome fully sequenced [1]. The next step was the annotation of all 6000 potential protein-encoding genes. Presently, 85% of these have their proteins and biological function described [2]. However, the complete annotation of the remaining 15% coding ORFs is probably the most difficult part. Some of the remaining unannotated genes are pleiotropic, with effects in apparently unrelated phenotypical traits, which turn very difficult the unveiling of their biological role. *GUP*1, named after glycerol uptake protein 1, gene is one such case.

*GUP*1, and its close homologue *GUP*2, according to sequence similarity and conserved domains, encode transmembrane-spanning proteins that belong to the membrane-bound *O*-acyltransferase (MBOAT) superfamily [3] [4, 5]. However, Gup1 was initially reported to have a role in *S. cerevisiae* glycerol active uptake [6]. The defective growth of cells lacking *GUP*1 gene in both glycerol based media and glucose-based media supplemented with high amounts of salt, suggested a putative function relating to the glycerol transport across the membrane. Moreover, the deletion of *GUP*1 in a mutant deficient in glycerol consumption and dissimilation, through the mutation of the *GUT1* (glycerol kinase) and *GPD1* (glycerol 3P dehydrogenase), impaired glycerol uptake. However, other mutant combinations involving the deletion of *GUP1* were still able to actively take up glycerol [7]. Additionally, the expression of both *GUP*1 and *GUP*2 did not match glycerol transport regulation [8].

The Gup1p localization was predicted to be in the plasma membrane due to the putative existence of 10-12 transmembrane domains [6]. In the study by Blève and his collaborators [9], Gup1 protein was tagged with the green fluorescent protein (GFP) and its subcellular localization was found to be the plasma membrane, but also the endoplasmic reticulum (ER). Experiments of cellular fractionation by Hölst and collaborators [6] identically showed possible multiple localizations of Gup1p, in the plasma membrane, in the ER and in the mitochondrion [6]. The immuno-electron microscopy analysis [9] suggested that Gup1 presents a carboxyl end cytoplasmic tail, and an amino end extended to the periplasmic space. The mechanism of Gup1-GFP proper localization and its removal from the membrane was analysed in a collection of yeast lacking genes in specific steps of the secretory and endocytic pathways,



highlighting the role of Sec6 and End3 in, respectively, the plasma membrane localization and turnover of this protein [9]. Shortly after, the transmembrane spanning Stl1 was beyond doubt recognized as the active glycerol/H^+ symporter [10]. The role of Gup1p in the regulation of the Stl1 glycerol symporter, that justifies the glycerol transport-related phenotypes [6], is not at expression level [10]. Rather, the absence of Gup1 affects the proper localization and activity of ATPase, and therefore the resulting proton motive force [11]. Considering that the glycerol transporter is a symport with protons, this might be the cause affecting Stl1 activity. Nevertheless, the hypothesis was never tested in other H^+ symporters.

A considerable amount of the information available for Gup1 derives from the survey of *gup*1Δ phenotypes. In view of their large amount and diversity, Gup1 function remains elusive. From genome-wide surveys it was possible to identify that Gup1 influences anaerobic sterol uptake [12], cytoskeleton polarization and bud side selection pattern [13, 14], telomeres length [15], secretory/ endocytic pathway performance [16], as well as arsenic [17] and imatinib resistance [18]. The role of Gup1 in the integrity and composition of the plasma membrane and cell wall was also reported [4]. In *gup*1Δ an increase in total triglycerides and diacylglycerol and concomitant decrease in phospholipids percentage was reported, implying Gup1 in the maintenance of the membrane lipids. Moreover, Ferreira and collaborators (2006) [19] reported a wide array of altered cell wall phenotypes in *gup*1Δ cells. Cells lacking *GUP*1 presented increased amounts of chitin and β1,3-glucans and decreased amounts of mannoproteins. The mutant cell wall was also loose and more easily extractable. Cells presented altered morphology and exhibited increased sensitivity to weak acids, detergents, caffeine, cell wall degrading enzymes, and heat. The mutant cells also displayed and increased sedimentation/aggregation phenotype.

According to Bosson and collaborators [20, 21] the role of Gup1 relies in the remodelling of glycosylphosphatidylinositol (GPI) anchors, both in *S. cerevisiae* and in the fungus *Trypanosoma brucei*. *S. cerevisiae* GPI anchors have two different lipid moieties, ceramides and diacylglycerol. These lipids are introduced by remodelling of the primary GPI lipid, during which C16- and C18- diacylglycerol are modified or replaced. The authors analysed the lipid moieties of GPI anchored proteins in thin layer chromatography (TLC) and reported that *gup*1Δ cells presented a high accumulation of primary anchors. The mutant cells seemed to perform a different remodelling on the

GPI anchors. The analysis of Gas1, a very well-known diacylglycerol GPI-anchored protein, revealed that in Wt cells most of the protein was detergent resistant, whereas the Gas1 from *gup*1Δ cells were detergent soluble. In view of these results, the authors suggested that Gup1 perform the acylation on sn-2 position of GPI anchors. Other reports supporting the role of *GUP*1 on the remodelling of GPI anchored are available [22-25]. Nevertheless, the existence of a GPI remodelling branch independent of Gup1 was suggested by Ferreira and co-workers [11] and recently confirmed [26], indicates that the role of Gup1 in that regard might not actually be the suggested acylation on sn-2 position of GPI anchors [20, 21].

The *gup*1Δ elicits changes in plasma membrane lipid composition [4], and cell wall components amounts [19], as well as GPI anchors [20, 21], and cytoskeleton polarization [13]. This prompted the study of the role of Gup1 in the integrity of lipid order microdomains, lipid rafts. The sphingolipid-sterol-ordered domains integrity/assembly is disrupted by the deletion of *GUP1* [11]. The cells lacking *GUP*1 yield 40% less Detergent Resistant Membrane domains (DRMs) than the Wt cells. Moreover, the mutant DRMs presented fewer proteins, namely the plasma membrane H^+-ATPase Pma1 and the GPI-anchored Gas1 [11].

The *gup*1Δ mutant showed altered lipid metabolism [11], as cells lacking Gup1 presented increased resistance to several ergosterol synthesis inhibitors targeting different biosynthetic steps, and increased sensitivity to some sphingolipid biosynthesis inhibitors. But perhaps more important is the role of Gup1 in the maintenance of the structural integrity and maintenance of lipid rafts. Their analysis through staining with the fluorescent dye filipin, showed in Wt cells a typical patched distribution, as well as the presence of some of these microdomains in some intracellular membranes, whereas in the strain lacking Gup1, the cells presented an unusual uniform distribution with occasional patches in some cells. The deregulation of lipid rafts integrity may cause the otherwise reported phenotypes on GPI-anchoring [20] and cytoskeleton polarization [13].

More recent works also implied the role of Gup1 in mechanisms of programed cell death [27, 28], however, each suggests opposite roles for this *O*-acyltransferase. Li and collaborators [27] analysed the effect of calorie restriction in the lifespan of *S. cerevisiae* and tried to identify related mutants with similar effects on life span. Typically, life span in yeast is studied in two distinct manners: replicative lifespan (RLS), measuring the number of yeast cell divisions before senescence; and chronological lifespan (CLS), that

measures the length of time cells remain viable in the post-diauxic and stationary phases [29, 30]. Calorie restriction has a positive effect in both CLS and RLS. The *gup*1Δ cells presented especially extended CLS and RLS. Actually, *GUP*1 disruption increased RLS, in particular under calorie restriction. On the other hand, the analysis of cell viability in stationary phase showed that the disruption of *GUP*1 extends CLS beyond Wt cells, but not compared to Wt cells under calorie restriction. This way, the Gup1p appears to have an effect over replicative life span rather than over chronological life span. The increase in extension of RLS in cells lacking Gup1 is prevented by the disruption of cytochrome c1 gene, *CYT*1.

On the other hand, Δ*gup1* cells, when challenged with conditions of acetic acid-inducing programmed cell death (PCD) [31], were unable to undergo proper apoptosis and died by necrosis, presenting shorter CLS [28]. The authors analysed several typical apoptosis markers, namely plasma membrane integrity and mitochondrial membrane potential, phosphatidylserine externalization and chromatin condensation. The Wt cells progressively followed the typical hallmarks of PCD, while the *gup*1Δ mutant presented faster loss of membrane integrity, the presence of abnormal chromatin condensation and multiple nuclei, and a high percentage of necrotic cells. However, mitochondrial membrane potential decreased similarly in both the mutant and the Wt. Additionally, the authors also showed that Δ*gup1* is highly sensitive to acetic acid [28], in agreement with the previous report of sensitivity against weak acids [19].

The differences between the reports of Li and collaborators [27] and Tulha and collaborators [28], may be due to different genetic backgrounds of yeast strains, BY4742 *vs.* W303-1A, or to distinct experimental procedures. Both performed the measurement of CLS in minimal media supplemented with auxotrophic elements, however, Li and collaborators [27] supplemented the media with 4 times more auxotrophic requirements than Tulha and collaborators [28], which may alter the mutant nitrogen metabolism and cause different responses.

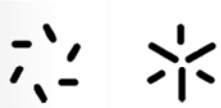
The role of Gup1 was also studied in the pathogenic yeast *C. albicans*. Similarly to what happens in *S. cerevisiae*, Ferreira and collaborators [32] reported that the *C. albicans gup1Δ* null mutant displayed altered sensitivity to specific ergosterol biosynthesis inhibitors [11], and disruption of lipid rafts [11]. More importantly, they also reported the loss of virulence and yeast-to-hyphal transition in cells lacking CaGup1. The disruption reduces the virulence factors of *C. albicans*: the *gup1Δ* strain is unable to

form true hyphae, to adhere, to invade different substrates and to form biofilms. Concurrently, this strain presents aberrant colony morphology and absence of filamentous growth in the colony periphery [32]. The mutant colonies presented a more complex architecture than Wt, with a radial pattern and no filamentous growth around the colony.

Gup1 and Gup2 are highly conserved genes, with orthologues in many organisms [5, 33]. Presently, a search in databases can easily yield more than 150 putative orthologues in yeasts, fungi, mammals, insects, fish, nematodes, and even plants. Namely, Gup1 and Gup2 orthologues are recognized in mouse (*Mus musculus*) [33], rat (*Rattus novergicus)* [34] and man [35]; the bony fish *Danio rerio* [36], the frogs *Xenopus laevis* and *X. tropicalis* [37], the higher plant *Zea mays* [38], the alga *Dunaliella tertiolecta* [39], and the parasite *Trypanosoma cruzi* [40]. Otherwise, in the fly *D. melanogaster* [41] and the nematode *Caenorhabditis elegans* [42], only a orthologue for Gup1 is present, whereas in several fungi, namely the basidiomycete *Puccinia graminis* [43], and the yeast *C. albicans* [44], only the Gup1 orthologue is described.

All these proteins putatively belong to the MBOAT family for possessing the correspondent conserved domain. All the Gup2-similar proteins present the conserved His412 residue in the active centre of the acyltransferases from this family, whereas only *S. cerevisiae* Gup1 maintains this residue [3]. All the remaining Gup1-similar proteins do not possess this residue.

In line with this high similarity, the Gup1 orthologue from mouse (*Mus musculus*), MmGup1, was shown to be responsible for the negative regulation of the palmitoylation of the secreted Sonic Hedgehog (Shh) protein from the morphogenic Hedgehog (Hh) pathway [33]. In high eukaryotes, several morphogenic processes are regulated through secreted proteins that function as long distance signals, namely Hh and Wnt (see Chapter 1). For the proper secretion and extracellular diffusion of the Hh signalling proteins, these undergo several post-translational modifications, including the attachment of a cholesterol molecule to the carboxyl end, and the palmitoylation of the amino end. This is mediated by a specific acyl transferase, Hhat (Hedgehog Acyl Transferase). Actually, mouse Hhat is 27% similar to the yeast Gup2p. MmGup1 instead is 26% similar to yeast Gup1p, and is presently known by the designation of Hhatl (Hedgehog Acyl Transferase Like protein). Hhatl (MmGup1) and Hhat (MmGup2) differ in which Hhatl does not present the conserved His residue in the



active centre typical of the MBOATs *O*-acyl transferases, and conserved in yeast Gup1 and Gup2, and is therefore unable to execute the transfer of a palmitate group. Hhatl and Hhat compete for binding Shh signal, and the inhibition of the palmitoylation can preclude secretion of the Shh signal, and block Hh signalling altogether.

Several attempts are under way to complement the yeast Δ*gup1,* Δ*gup2* and double mutants with the orthologous genes from mouse, man and fly (Ch. 6). A successful complementation, single trial, was obtained using MmGup1 in *C. albicans gup1Δ*- cells (personal communication from Ferreira, C.). This yielded partial complementation of the above mentioned morphogenic defects of hyphae formation and invasive growth, suggesting that a putative morphogenic pathway, similar to the mammalian Hedgehog, could be present in yeast.

In view of the putative involvement of the Gup proteins in morphogenic processes, it became likely that their absence could affect the yeast extracellular matrix formation in some way. The study of the effects of deletion of *GUP1* on the structure and composition of multicellular aggregates might contribute for unveiling its roles on cellular metabolism, and at the same time provide important hints on the ECM structural organization and metabolic mechanisms.

Materials and Methods

Strains and Media

S. cerevisiae strains W303-1A (MATa; leu2-3; leu2-112; ura3-1; trp1-1; his3-11; his3-15; ade2-1; can1-100) and BHY54 (isogenic to W303-1A but *gup1::HIS5*$^{+}$) were used in this work [6, 45]. Cells were grown on rich medium, YPDA (1% yeast extract; 2% peptone; 2% glucose; 0.005% adenine hemisulphate) on an orbital shaker, 200 rpm, at 30 °C. Growth was monitored by optical density (OD) at 600 nm. Cells were maintained in solid YPDA medium supplemented with agarose (2% (w/v)), grown at 30 °C for 48 h and kept at 4 °C up to 5 days. All ingredients percentages were calculated as weight per volume units.

Yeast ECM production, extraction and fractionation

a) Yeast ECM production and extraction

The development of the yeast cell mat and the ECM extraction were performed as described previously (Ch. 3 and 4). For differential gel electrophoresis, four separate batch cultures per strain were treated independently up to the ECM extraction step and freeze drying.

b) Sugar precipitation and Gel electrophoresis

The polysaccharides precipitation and recovery, the as well as the 1,3-diaminopropane acetate and polyacrylamide gel electrophoresis were performed as described previously (Ch. 3).

c) Protein precipitation and recovery

The freeze dried extracts were resuspended in MilliQ water (just enough to completely solubilize the overlay components). The proteins were precipitated using the chloroform/methanol protocol [46]. The protein pellets were left at room temperature to evaporate the remaining methanol, and resuspended in DIGE compatible buffer (30 mM Tris at pH 8.9; 7 M urea; 2 M thiourea; 2% (w/v) CHAPS). Protein was quantified with Bio-Rad Protein Assay (Bio-Rad, Richmond, CA, USA) as recommended by the manufacturer.

Protein Labelling

Four biological replicates were used for the Wt and mutant strain, thus generating eight individual samples Proteins in each sample were fluorescently labelled with Cy dye derivatives according to the scheme in Table 1 as described before [47]. Labelling was done according to manufacturer instructions (GE Healthcare Life Sciences, USA). Briefly, the protein sample (50 μg) was labelled with 400 pmol of Cy dye in 1 μl of anhydrous N,N-dimethylformamide (DMF, Sigma). The mixture as was incubated on

ice for 30 min of incubation, in the dark. The reaction was stopped with the addition of lysine (10 mM), and a second incubation of 10 min on ice in the dark.

Table 1. Experimental design indicating the CyDye labelling in each of the four replicates for each condition (indicated as "1-4") and the samples that are mixed in each of the gels 1 to 4.

	Cy5	**Cy3**	**Cy2**
Gel 1	Wt (1)	*gup1Δ* (4)	Internal standard
Gel 2	Wt (2)	*gup1Δ* (1)	Internal standard
Gel 3	*gup1Δ* (2)	Wt (3)	Internal standard
Gel 4	*gup1Δ* (3)	Wt (4)	Internal standard

Two-Dimensional Differential Gel Electrophoresis (DIGE)

The DIGE was performed using GE Healthcare reagents and equipment. Labelled samples were combined as in Table 1. Dye swaps were performed to avoid dye-dependent differences. Each gel contained a pair of Cy3 and Cy5 labelled samples, corresponding to the Wt and *Δgup*1 mutant, and a Cy2 labelled pooled standard, a mixture of equal amounts of all the samples used for each DIGE experiment. To each mixture, an equal volume of 2x hydratation buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (w/v) dithiothreitol (DTT), 4% (v/v) pharmalytes at pH 3-11) was added for the cup loading process.

For first dimension, 24 cm IPG strips in the pH range of 3-11 were used. The strips were previously hydrated overnight with 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 100 mM DeStreak, and 2% (v/v) pharmalytes at pH 4-7. Isoelectric focusing was performed at 20º C using the following program: 120 V for 1 h, 500 V for 2 h, 500-1000 V for 2 h, 1000-5000 V for 6 h, and 5000 V for 10 h. Subsequently, strips were equilibrated for 12 min in reducing solution (6 M urea, 50 mM Tris-HCl at pH 6.8, 30% (v/v) glycerol, 2% (w/v) SDS, and 2% (w/v) DTT) and then for 5 min in alkylating solution (6 M urea, 50 mM Tris-HCl at pH 6.8, 30% (v/v) glycerol, 2% (w/v) SDS, and 2.5% (w/v) iodoacetamide). The second-dimension SDS-PAGE was run on homogeneous 10% T and 2.6% C polyacrylamide gels casted in low-fluorescent glass

plates. Electrophoresis was carried out in the dark at 20 ºC, with a potency of 2 W/gel for 18 h, using an Ettan-Dalt six unit.

Image acquisition and DIGE analysis

Proteins were visualized using a Typhoon 9400™ scanner (GE Healthcare) with CyDye filters. For the Cy3, Cy5 and Cy2 image acquisition, the 532 nm/580 nm, 633 nm/670 nm and 488 nm/520 nm excitation/emission wavelengths were used, respectively, and 100 µm as pixel size. Image analysis was carried out with DeCyder™ differential analysis software v6.5 (GE Healthcare). The Differential In-gel Analysis (DIA) module was used to assign spot boundaries and to calculate parameters such as normalized spot volumes. Inter-gel variability was corrected by matching and normalization of the internal standard spot maps in the biological variance analysis (BVA) module. The internal standard image gel with the greatest number of spots was used as a master gel. Comparisons between Wt and *Δgup*1 mutant were carried out using average ratio and unpaired Student's t-test. In order to reduce the false positive, False Discovery Rate was applied [48]. Protein spots were considered as differentially present with statistical significance between the extracts under comparison, if presenting: (1) a 1,5-fold difference in the average ratio; and (2) a p value less than 0.05. Principal Component Analyses (PCA), Hierarchical Cluster Analysis (HCA) were performed using the DeCyder Extended Data Analysis (EDA) module on the group of spots identified as significantly changed. Based on collective comparison of expression patterns from the set of proteins, these multivariate analyses clustered the individual Cy3- and Cy5-labeled samples.

Colloidal Coomassie Blue Staining and Protein Digestion and Identification

The procedures for gel staining and protein digestion and identification were performed as described previously (Ch. 2).

Results and Discussion

Deletion of GUP1 affects ECM composition and complexity

Compared analysis of Δ*gup1* and Wt ECM proteome by LC-MS/MS

The analysis of the *gup1Δ* total ECM proteins, as well as the proteins secreted during liquid growth, was performed through liquid chromatography coupled with mass spectrometry as described for the Wt samples (Ch. 2). This methodology allowed an in-depth analysis of the effects on *GUP1* deletion on the ECM proteome.

a) Comparative identification of the proteins secreted during liquid batch culturing

The analysis of the proteins secreted by mutant cells *gup1Δ* grown in liquid batch cultures had previously been briefly assessed [11]. The results now obtained confirmed the mutant secretes substantially more proteins than the Wt in identical conditions (Fig. 1 A), respectively 311 against 80 (Table S3). From these, 68 were common to both strains (Table S3). The *gup1Δ* samples presented a great number of surface proteins, including cell wall and plasma membrane proteins. These include plasma membrane integral proteins as Pma1, and glycosylphosphatidylinositol-anchored proteins, namely several members of the Gas family that are crucial for the cell wall remodelling. Gas1 had already been identified as one of the major proteins secreted by the mutant [11]. Both Gas1 and Pma1 are usual markers of lipid rafts integrity. The abnormally high release to the extracellular medium of these proteins, as well as Pir1, Pir2 and Pir3 required for cell wall organization and maintenance, is in agreement with the compromised membrane and cell wall composition and structure reported for the *gup1Δ* mutant cells [4, 11, 19, 20].

The *gup1Δ* samples also presented a high number of proteins intervening in the carbon metabolism (Table S3), namely Eno1 (enolase), Fba1 (fructose-1,6-bisphosphatase) or Tpi1 (triosephosphate isomerase). While these proteins were already reported in the cell surface of other yeast strains during liquid growth [49, 50], they were never reported to actually appear in the extracellular medium, and were absent from the Wt sample in identical conditions. Additionally, a great number of proteins associated

with lipid synthesis, ergosterol and fatty acids metabolism, was also present in the *gup1Δ* mutant ECM sample (Table S3). Whether the unintended release of proteins belonging to the Erg and Fas families might contribute to the altered composition of the mutant plasma membrane, or might be the cause of such defective condition, is still not fully understood.

b) Comparative identification of the proteins secreted to the yeast ECM

In opposition to liquid batch cultures, mutant cells mats secreted 15% less proteins to their ECM than Wt (Fig. 1 B), respectively 587 against 693 (Table S4). From these, 406 were common to both strains (Table S4). As occurs in the liquid growth, the mutant ECM comprises a great number of proteins involved in the cell wall remodelling that were not found in the Wt ECM. It includes the homologous GPI-anchored proteins Dcw1 and Dfg1, putative mannosidases required for cell wall biosynthesis, the Utr2, Kre6 and Krt2 proteins, involved in the biosynthesis of β-glucans, the Pir1 and Pir2 proteins, involved in the stabilization of the cell wall, or the GPI-anchored protease Yps1, required for the cell wall growth and maintenance (Table S4).

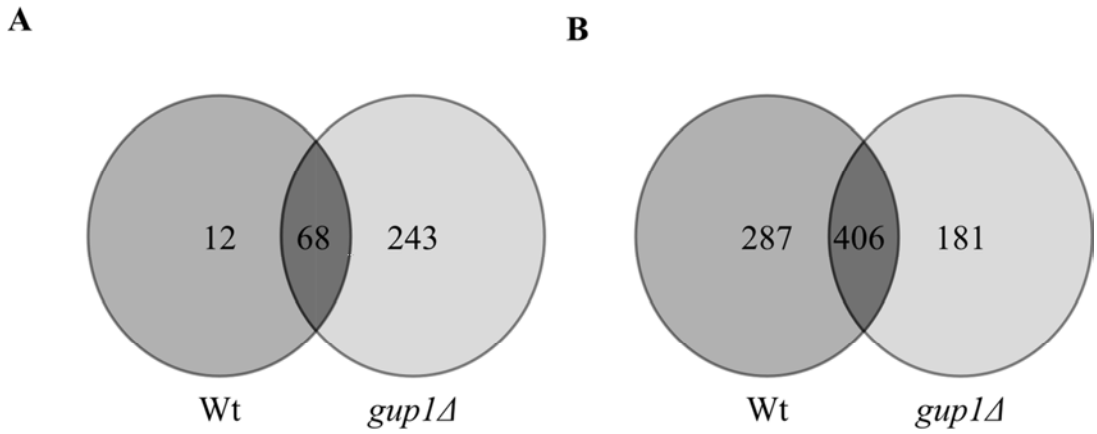


Figure 1. Venn charts of the distribution of identified proteins. Proteins released during growth on liquid media (A), and during the ECM development (B). Overlapping area represent proteins common to both samples.

The proteins absent in the mutant ECM that were identified in Wt (Ch. 2) comprise several functional classes: carbon metabolism, cell rescue and defence, protein fate and cellular organization. In fact, the Wt ECM presented all the proteins composing the glycolysis, gluconeogenesis and glyoxylate cycle (Ch. 2). However, the mutant is lacking some pivotal proteins that play crucial roles in the regulation of these pathways, namely the Fbp1 (fructose-1,6-bisphosphatase) and Pyc2 (pyruvate carboxylase).

Fbp1 protein is the key regulatory protein of gluconeogenesis [51], and it is under tight glucose regulation [52]. A recent report [53] shows that the addition of glucose to starved cells performing gluconeogenesis leads to the degradation of both intracellular and extracellular Fbp1, suggesting that the protein might be performing the same role in the cell surface. The absence of this protein in the ECM might suggest that a glucose repression could still be in place, which is not in accordance with the outside presence of other glucose repressed proteins like Pck1 (phosphoenolpyruvate carboxykinase) and Mls1 (malate synthase). More plausibly, the apparent absence of relation between the physiology of the cell and the extracellular appearance of these proteins, suggests they might have distinct roles once outside the cell.

Pyc2 protein is responsible for the conversion of pyruvate to oxaloacetate that initiates the gluconeogenesis, but also plays a role in the tricarboxylic acid (TCA) and glyoxylate cycles [54, 55]. Evidence of this protein presence in the cell surface is available in the pathogenic yeast *C. albicans* [56], but this is the first report in *S. cerevisiae*. These results show a class difference between the ECM protein lot of mutant and Wt that corresponds to the absence of the gluconeogenic enzymes, the physiological significance of which remains for the moment obscure.

The *gup1Δ* ECM is also lacking the presence of pyruvate decarboxylase isoforms Pdc5 and Pdc6. These proteins are involved in the glucose fermentation into ethanol, and their expression is usually repressed by the presence of another isoform, Pdc1 [57, 58]. However, the presence of the three isoforms was detected in the Wt sample (Ch. 2), suggesting the presence of subpopulations with distinct expression patterns. However, that expression heterogeneity is absent in the mutant ECM, which might be indicative of a loss of differentiation/specialization with the yeast colony.

Another large set of proteins are absent from the *gup1Δ* ECM sample, the ones involved on the metabolism of intermediates of the TCA cycle. This includes the Acs1, Pda1 and Pdx1 from the Acetyl-CoA biosynthesis, and the Gor1, Kgd1, Lsc3 and Sdh2

enzymes responsible for the glyoxylate, α-ketoglutarate, succinyl-CoA and succinate metabolism, respectively. The effect of the absence of these proteins on the yeast ECM function and structure is unknown.

A great reduction in the proteins performing roles of cellular organization was also observed. The *gup1Δ* ECM samples do not present several proteins involved in the cytoskeleton organization, namely the tubulin encoding Tub2, the Arc19, Arc35, Arp2, Ent2 and Ent3 proteins involved in the assembly of actin cortical patches, or Rvs161 or VPS that modulate the cytoskeleton assembly. All these were present in Wt ECM (Ch. 2). Considering that Δ*gup1* has altered cytoskeleton and bud-site selection [13], one cannot avoid considering the possibility that there might be some relation. Actually, the actin (Act1) protein present in both Wt and mutant ECM samples, might play some structural role in ECM edification, similarly to what happens in *Staphylococcus gatii*, which extracellular filaments that are sensitive to cytoskeleton protein inhibitors [59]. Nevertheless, no other known cytoskeleton proteins able to interact with and reinforce actin were found.

The *gup1Δ* ECM also presents considerable less number of proteins from stress response and secretory pathway. Several proteins associated with the vacuole acidification, namely the ATPase isoforms Vma7 and Vma6, as well as its V_0 subunit Vph1, are absent from the mutant ECM. Following an identical rationale as above for cytoskeleton phenotypes, this might relate to the abnormal vacuolar morphology described for *gup1Δ* cells [60]. Similarly, proteins associated with oxidative stress response and glutathione metabolism, namely Ctt1, Ecm4, Ecm38 and Gto1, could not be detected in the mutant ECM, again ultimately implicating in *gup1Δ* mutant altered oxidative stress response that results in cell death [28].

Another functional class that is substantially diminished in the *gup1Δ* ECM is the one of protein fate, especially protein degradation. Several proteins involved in ubiquitination and sumoylation, namely Fub1, Aos1, Pib1 and several members of the Rpn family, and that direct proteins from degradation are absent from the mutant sample. Similarly, proteins involved in the folding of other proteins are also missing from the mutant ECM, especially the chaperone Cct3 that is involved in the assembly of actin and tubulin filaments.

Compared analysis of Δ*gup1* and Wt ECM protein abundance by DIGE

In order to confirm and better characterize the differences in ECM protein secretion caused by *GUP1* deletion on the secretion of proteins to the ECM, we further submitted the same samples to DIGE. This highly sensitive technique allowed us the direct comparison of both mutant and Wt samples in the same gel. At the same time it demonstrated the robustness of the ECM extraction and fractionation methodologies.

The preparatory SDS-PAGE (Fig. 2 A) was followed by 2D DIGE technique, which enables the use of a unique experimental design that eliminates the inter-gel comparison bias. A crucial characteristic of this technique is the presence of the internal standard labelled with Cy2 dye, which is formed by equal amounts of all samples, and is included in all gels, allowing the normalization of measured fluorescence. In order to avoid dye-dependent variations, namely labelling efficiency and fluorescence emission, half of the samples from each strain ECM protein fraction were labelled with Cy3 dye, and the other half was labelled with Cy5 dye (Table 1). This allowed to eliminate most of the inherent variation between the gels, and allowed a statistically validated analysis, the four gels acting as independent replicates.

The proteomic profile, analysed in the DeCyder Software, revealed an average of 1400 spots per sample, which include all the protein isoforms present. The biological variation analysis matched a total of 1200 spots that were present in every sample. This spot set was used to study the proteins that are differentially abundant in the ECM of the Wt and *gup1Δ* ECM samples. A total of 56 protein spots presented significant different ($p<0.05$) abundance variation 1.5-fold or greater between the two strains. The mutant strain presented 28 spots with increased abundance, and 28 spots that were significantly less abundant than in the Wt. Among these 56 protein spots, the 15 protein spots presenting an abundance variation of 2-fold or greater between the two strains were excised from 2D GE gels staining with CCB, subjected to tryptic digestion, and the resulting peptides analysed by mass spectrometry (Table 1). Seven spots presented a 2-fold increased abundance in the mutant sample, and 8 were more abundant in the Wt sample (Table 2).

A pattern analysis was performed to assess the differential abundance of proteins in the several replicates and validate the reproducibility of the extraction methodology (Fig. 3). The Principal Component Analysis displays the two principal components that

distinguish the two largest sources of variation within the data set. The data sets from the four replicates were grouped together with the first component (PC1) in the loading plot (Fig. 3 A; top panel), showing that the samples present a low variance within the same strain. The score plot show that eight protein are more present in the Wt, while seven are more abundant in the mutant (Fig. 3 A, lower panel). Next, the similarities of protein abundance between samples was analysed through the Hierarchical Clustering Analysis (Fig. 3 B). As expected, the samples formed two main clusters, one including all the Wt replicates and other including all the *gup1Δ* replicates. The variations between each replicate are displayed as and abundance matrix, represented in a standardized logarithmic scale of abundance ranging from -0.5 (green) to +0.5 (red).

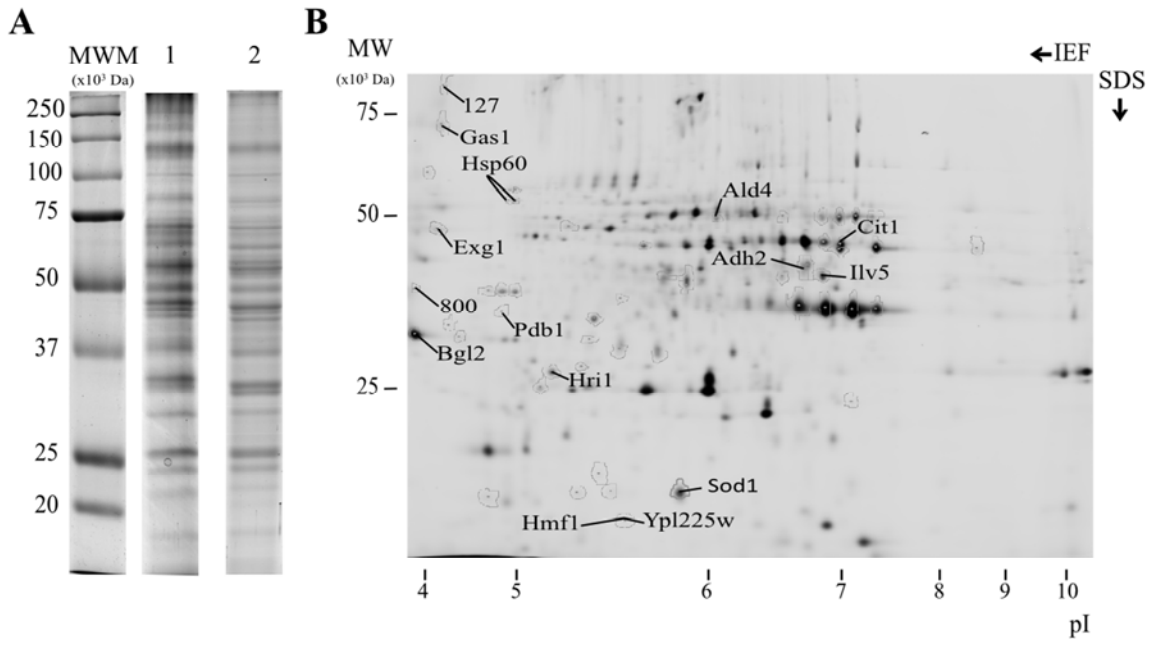


Figure 2. Electrophoretic separation of the ECM proteins. Preparatory one-dimensional analysis (A) of the Wt (lane 1) and *gup1Δ* (lane 2) samples. Two-dimensional DIGE analysis (B) and identification of the protein spots whose abundance varied 2-fold or more and present $p < 0.05$.

The proteins present in higher abundance in the *gup1Δ* mutant were mainly associated with cell wall assembly and maintenance: Gas1 β-1,3 (glucanosyltransferase) [61], Exg1 (exo-1,3-beta-glucanase) [62], Bgl2 (endo-beta-1,3-glucanase) [63], and Sod1 (superoxide dismutase) [64]. As predicted [11], the most secreted protein by the mutant cells to the ECM was Gas1, presenting an increase of abundance more than 8-fold

greater than the Wt ECM. Both Exg1 and Bgl2 proteins were previously described in the cell surface [49]. From these, only Exg1 was reported in the extracellular region [65], but the presence of Bgl2 in the extracellular space is predicted through bioinformatics approaches [66]. Sod1 was also found in increased amounts in the mutant ECM. The presence of this protein in the cell surface of *S. cerevisiae*, where it plays a role in the cell wall maintenance, was already described [49, 50, 64]. As mentioned above, the altered membrane and cell wall composition and structure is most certainly associated with an unintended release of surface proteins. The function performed by these proteins in the ECM is still unknown. Nevertheless, increased amounts of cell wall remodelling enzymes in the extracellular space might affect the structure of the wall periphery but also of ECM, through the modification of the structural components or interference with their normal turnover.

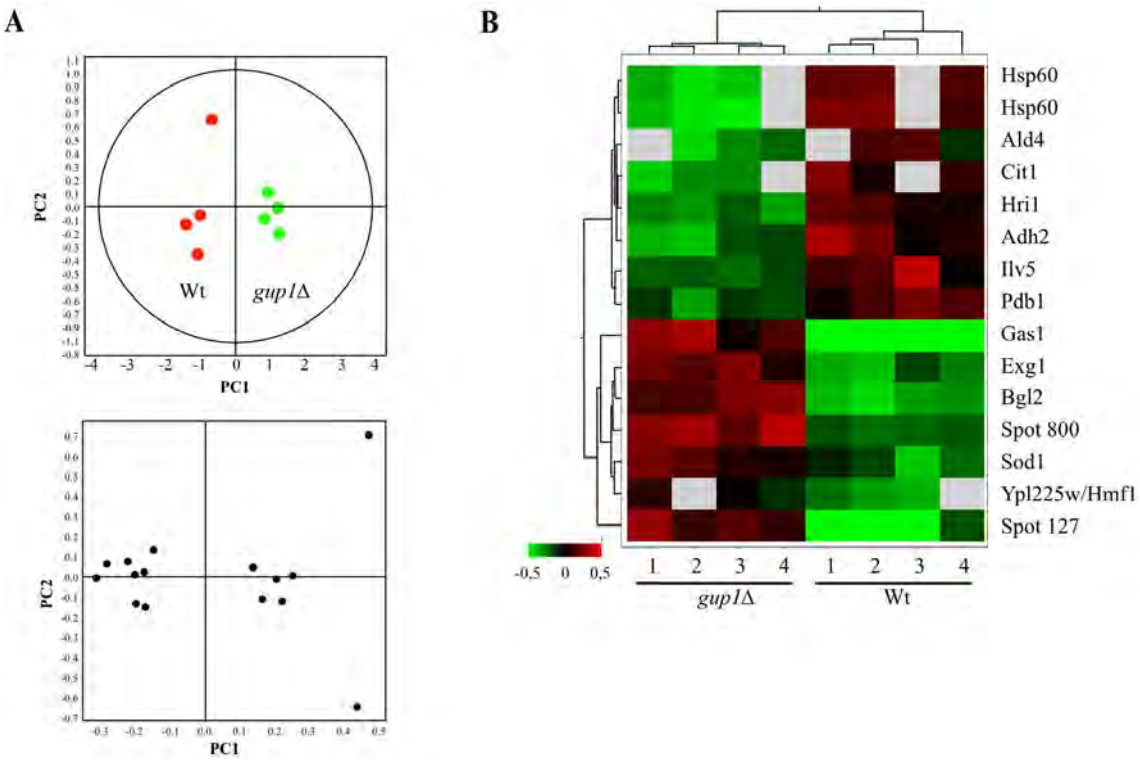


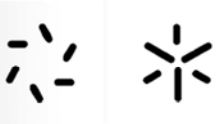
Figure 3. Unsupervised multivariate analysis of data from the 2D DIGE experiment. (A) PCA loading and score (bottom) plots show the clustering of the eight individual Cy3- and Cy5-labeled DIGE spot maps and the subset of proteins whose ratios varied 2-fold or more and in which $p < 0.05$ respectively, in the two principle components. (B) Hierarchical clustering settings are Pearson distance measurements and average linkage. The dendrogram of eight individual spot maps clustering is shown at the top, and that of individual proteins is shown on the left, with relative expression values being displayed in a heat map.

Two uncharacterized proteins, Hmf1 and Ypl225w, were also identified. These two proteins were not sufficiently resolved in the 2D electrophoresis and were identified from the same spot (Fig. 1 B), being difficult to assess if both or only one are affected by the *GUP1* deletion. Finally, the protein spots nº 127 and nº 800 were not identified, as it was not possible to extract enough protein to perform an unequivocal identity assignment.

On the other hand, the proteins that were significantly less present in the mutant ECM can be ascribed to several functional processes: metabolism of ethanol (Ald4 and Adh2) and Acetyl-CoA (Cit1 and Pdb1), protein folding and targeting (Hsp60 and Hri1), and maintenance of mitochondrial DNA (Ilv5). A partial overlap of the functional classes of proteins absent, and the proteins significantly reduced, can be observed in the mutant ECM. The most striking difference between the Wt and mutant ECM proteins relies in the many enzymes from carbon metabolism absent of significantly less represented, namely from ethanol metabolism.

In addition to the alcohol dehydrogenases Adh4 and Adh6 that are absent, which are responsible for the formation of several alcohols [67, 68], the *gup1*Δ sample presents only residual amounts of another isoform, Adh2 (Table 2). This enzyme is glucose repressible and catalyses the conversion of ethanol to aldehyde, *i.e.* the initial step in the utilization of ethanol as a carbon source [69]. Accordingly, the *gup1*Δ ECM lacks the aldehyde dehydrogenase Ald4 and presents reduced amounts of the isoform Ald2, which catalyse the next step in ethanol consumption [70]. A role in the detoxification of harmful aldehydes was also reported for these enzymes [71]. This suggests that, inside Wt multicellular aggregates, two metabolically distinct subpopulations might coexist, one fermenting the glucose present in the media and the other respiring the ethanol and glycerol produced by the first. The data now obtained for the *gup1Δ* multicellular aggregates, suggests this metabolic differentiation is partially lost or significantly reduced by *GUP1* deletion.

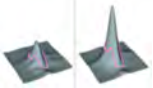
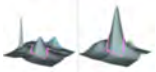
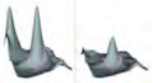
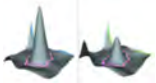
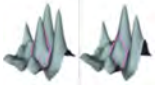
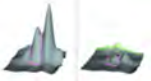
Another group of proteins that are significantly altered in the mutant ECM are the proteins responsible for the Acetyl-Co-A metabolism. The *gup1Δ* sample presents low amounts of Pdb1 (Table 1), and completely lacks Pda1. These two proteins comprise the subunit E1 of the pyruvate dehydrogenase, responsible for the conversion of pyruvate to acetyl-CoA [72]. The abundance of the citrate synthase Cit1 in the mutant sample is also significantly decreased (Table 2). Being the rate-limiting enzyme of the TCA cycle, this



enzyme is responsible for the condensation of acetyl-CoA and oxaloacetate into citrate [73]. The function of such proteins in the ECM is still unknown. The presence of complete pathways may suggest that some part of the metabolic steps could be performed outside the cell. Proteins involved in the maturation and targeting of other proteins, Hsp60 and Hri1 (Table 2), are also significantly decreased in the ECM of *gup1Δ* colonies. The protein Hri1 is a protein of unknown function that interacts with the broad protein kinase Hrr25 [74], and that is associated with the protein targeting to the membrane. On the other hand, the chaperone Hsp60 is vital for the cell survival, cells lacking the gene encoding for this protein are unviable [75]. Hsp60 is a mitochondrial chaperone involved in the *de novo* folding of mitochondrial proteins and respiration complexes assembly [76]. The two isoforms Hsp60 detected were the proteins whose abundance in the ECM most decreased with the deletion of *GUP1* (-3.73 and -4.38 fold). The presence of this Hsp60 in the ECM raises high expectations as to a possible role in the maintenance of protein function and extracellular metabolism.

Finally, the acetohydroxy acid reductoisomerase Ilv5 is also significantly less abundant in the mutant sample (Table 2). Intracellularly, this protein is responsible for the mitochondrial DNA maintenance [77], and is involved in the biosynthesis of branched-chain amino acids, like leucine, isoleucine, and valine [78]. In spite that in the present work the presence of DNA in the yeast ECM was not assessed, it most probably exists (see Ch. 1). Given Ilv5 DNA-binding properties, the presence of proteins involved in DNA modification are not unexpected.

Table 2. Summary of the differentially present proteins. Statistical analysis, Identification data and 3D representation of the spots.

Master number	Protein Name	Mr	pI	*gup1*Δ/Wt		Wt	*gup1*Δ	Score	N Mass Matched	MS/MS mass ion	MS/MS ion score	Sequence coverage
				t-test	Avg ratio							
26	Gas1	60343	4,46	0,0077	8,33			59	47	-	-	21
127	127	-	-	0,023	5,73			-	-	-	-	-
954	Bgl2	34325	4,16	0,0065	3,51			62	9	-	-	40
800	800	-	-	0,0065	3			-	-	-	-	-
518	Exg1	51735	4,57	0,011	2,79			66	13	-	-	24
1239	Sod1	15959	5,62	0,025	2,11			83	8	-	-	77
1274	YPL225w	17491	5,24	0,029	2,01			190	-	1257.673; 1.951.892	59; 75	45
1274	Hmf1	14011	5,28	0,029	2,01			119	-	1133.593; 1.304.658	40; 34	62
880	Pdb1	40086	5,23	0,023	-2,01			78	10	-	-	37
744	Ilv5	44512	9,83	0,023	-2,33			163	19	-	-	45
1048	Hri1	27541	5,1	0,0077	-2,37			71	8	-	-	51
455	Ald4	56973	6,31	0,044	-2,38			102	15	-	-	30
687	Adh2	37165	6,26	0,023	-2,62			66	10	-	-	35
599	Cit1	53384	8,23	0,023	-2,85			53	9	-	-	24
345	Hsp60	60999	5,23	0,0068	-3,73			91	14	-	-	31
346	Hsp60	60999	5,23	0,0077	-4,38			113	16	-	-	34

Deletion of *GUP1* induces differences on polysaccharides of the ECM

The ECM polysaccharides from cells lacking Gup1 were analysed as described for the Wt strain (Ch. 3). The polysaccharides were recovered through ethanol precipitation and analysed in both agarose and acrylamide electrophoresis (Fig. 3). The polysaccharides extracted from *gup1Δ* ECM were remarkably different from the Wt (Ch. 3). Furthermore, the 1,3-diaminopropane agarose electrophoresis showed that the deletion of *GUP1* possibly induces a loss of complexity in the polysaccharides from the ECM (Fig. 3 A). The sample from Wt ECM polysaccharides clearly presents two metachromatic compounds, one of which close to the control chondroitin sulphate, while in the *gup1Δ* sample, only one band close to this last was observed. Different migration patterns in 1,3-diaminopropane agarose electrophoresis occur due to differences in the degree of sulphation, which increases towards the start point of the run. Therefore, the lack of Gup1 either affects the synthesis of a core compound, or the chemical substitution, originating a compound that does not induce metachromasia. The further molecular weight separation of compounds by PAGE (Fig. 3 B) yielded a similar result. The samples from *gup1Δ* mutants presented a single polydisperse band in the 5-8 kDa range (arrow), compared to wt in which samples a further 35-40 kDa was present (arrows). This suggests that the Gup1 protein might play a role in the production, secretion and/or processing of such compounds.

In the literature, several reports show that *gup1Δ* mutant cells presented altered plasma membrane and cell wall composition and integrity [4, 19], compromised lipid metabolism and lipid rafts integrity [11]. These phenotypes might influence the efficient production and secretion of ECM components. Nevertheless, the putative role of Gup1 in chemical substitution of ECM polysaccharides cannot be discarded. The protein analysis revealed that several polysaccharide modifying or degrading enzymes are released to the yeast ECM that may alter the ECM polysaccharides, namely the exo-1,3-beta-glucanase Exg2, a chitin trans-glycosylase involved in the β-glucan processing, as well as the putative mannosidases Dcw1 and Dfg5 (Table S4). Moreover, the double mutant disrupted in both *GUP1* and its close homologue

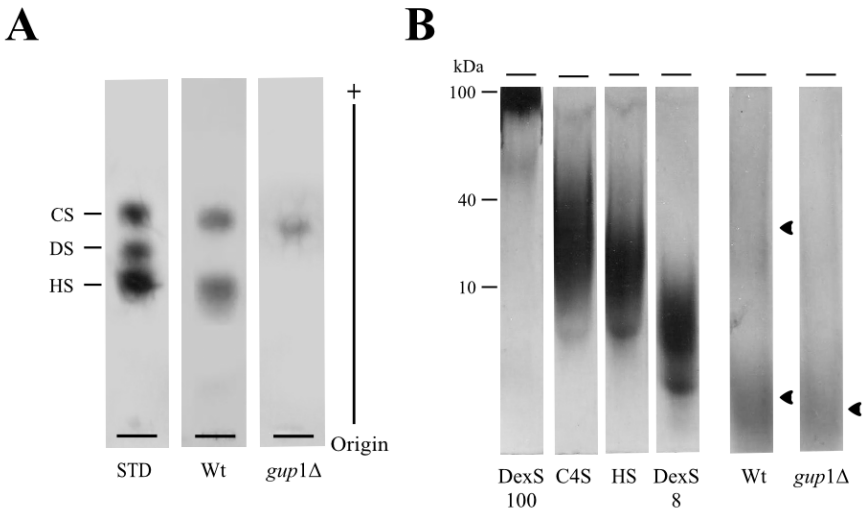


Figure 4. Electrophoretic profiles of Wt and *gup1Δ* ECM polysaccharides. The 1,3-diaminopropane acetate agarose (A) and polyacrylamide (B) electrophoresis shows that the deletion of *GUP1* induces the loss of the 35-40 kDa chemically substituted polysaccharide present in the Wt ECM.

GUP2 produced ECM polysaccharides that showed anticoagulant properties, inducing an extended activated partial thromboplastin time (shown in Ch. 3). Bearing in mind that Wt sample induced metachromasia, and is chemically substituted, while not presenting anticoagulant properties, it is possible to consider that the two Gup proteins are most likely intervening together with the regulation of the polysaccharide modification.

In the future, the utilization of other staining systems may also prove helpful. The Stain All dye detects acidic compounds, being able to stain both hyaluronan and sulphated glycosaminoglycans [79, 80].

Conclusions

The yeast ECM is a dynamic environment that is greatly influenced by the cells physiological state. The deletion of the pleiotropic *GUP1* gene introduced profound changes in the cell physiology and structure. Therefore, it is not unexpected that the ECM components are altered by *GUP1* and *GUP2* deletions. Moreover, the putative morphogenic aptitude of the Gup proteins, stressed by the similarity with the Hedgehog acyltransferases, suggests as much.

Alterations in both protein identities and abundances were observed, a great number of proteins were absent, and the remaining proteins were in significantly different amounts. The careful analysis of the functions performed by these proteins inside the cell might give some indications as to their functions on the ECM. Nevertheless, we cannot overlook the possibility that all or most of these proteins are actually moonlighting, *i.e.*, performing completely new functions in the ECM. Furthermore, the deletion of *GUP1* severely compromised the synthesis and/or chemical substitution of the ECM polysaccharides, disappearance the polysaccharide of higher molecular weight.

A whole new range of unanswered questions arose from this work. Further unveiling the Gup1 and Gup2 roles in the ECM composition, structure and chemical properties, will certainly contribute for the understanding of the *S. cerevisiae* multicellular lifestyle.

Acknowledgments

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6. HETEROLOGOUS EXPRESSION OF HIGHER EUKARYOTES YEAST GUP GENES ORTHOLOGUES

Abstract

In higher Eukaryotes, at tissue level, major events like patterning during embryogenesis or remodelling during wound healing, are regulated by molecules named morphogens. These molecules comprise several families, including the Hedgehog (Hh) signalling pathway. The Hh signal is a long-range signal transmitted from one cell to the other through the ECM. It is translated into a precursor protein that undergoes extensive post-translational modifications, including palmitoylation of the amino end. The protein that palmytoylates the Hh signal (Hhat Hedgehog acyltransferase) is similar to yeast Gup2, while the negative inhibitor of this modification (Hhatl Hedgehog acyltransferase-like) is recognizably similar to yeast Gup1. In view of the high conservation of these proteins in the most diverse organisms, from man to plants, insects and nematodes, the complementation of the GUP mutants with the Gup1 and Gup2 orthologues from mouse, man and fly was attempted. The constructions involved transformation of the Δgup1 and Δgup2 and Δgup1Δgup2 mutants with the mouse mmGUP1/HHATL, the human HHAT and HHATL and the fly RASP. The preliminary complementation assays under stress that highly impairs the growth of these mutants did not yield phenotype reversion. Further assays will show whether the proteins are expressing correctly, and whether a change of expression system will be needed.

Higher eukaryotes Gup- like acyltranferases

Saccharomyces cerevisiae presents several orthologues of human genes, a great number of which are associated with diseases [1-3]. Yeast models of protein-misfolding disorders are allowing a better understanding of disease underlying mechanisms [4], and identification of compounds with potential therapeutic effect. Namely, studies in spongiform encephalopathies are being conducted in *S. cerevisiae* due to the presence of proteins that behave like mammalian prions [5]. These proteins (PrPC), consisting mainly by α-helices in their native form, can be founded in a misfolded prion-like form (PrPSc), which develops pathological aggregates, due to their capacity to catalyse the transformation of all other endogenous proteins into an identical, pathological, form[6]. The identification of specific drugs that solubilize the protein aggregates and reduce its toxic effect is the main objective [5]. Similarly, alterations in the mechanisms of protein folding, accumulation and sorting underlying diseases like Huntington, Parkinson or Alzheimer's [7]. *S. cerevisiae* helped shed some light on the basis of malfunction of misfolded proteins removal processes, or protein sorting, as well as on the role of oxidative stress, thus contributing for the understanding of numerous neurodegenerative diseases [7-10]. Moreover, yeast has also provided information regarding human mitochondrial disorders like Leigh syndrome. The respiratory chain defects, metabolism regulation or abnormal modifications of mitochondrial DNA are some of the subjects studied in *S. cerevisiae* [11]. Additionally, yeast has been giving a great deal of help to uncover the function of many mammalian proteins through their successful heterologous expression [12]. When these proteins are human this is frequently designated as yeast *humanization*. The other way around is less frequent, but may also happen. For example, several oncogenes were firstly known in mammalian models, and subsequently identified in yeast, where their functions have been shown to be rather similar to the ones in the original organism. This is the case of the *RAS1* and *RAS2* oncogenes [1].

GUP1 and its close homologue *GUP2* were initially identified as participants of the glycerol active transport mechanism [13]. Later, *GUP*1 has been implicated in several critical cellular mechanisms: plasma membrane and cell wall composition and integrity

[14, 15]; cytoskeleton polarization and bud side selection pattern [16, 17]; telomeres length [18]; lipid metabolism and lipid rafts integrity [19]; anaerobic sterol uptake [20]; secretory/endocytic pathway [21], GPI-anchor remodelling [22, 23]; and programmed cell death [24, 25]. Studies on the pathogenic yeast *Candida albicans* additionally showed that the disruption of *CaGUP*1 reduces the virulence, since the *gup1Δ* strain is unable to form true hyphae, to adhere and to invade to different substrates and to form biofilms. Similarly to what happens in *S. cerevisiae*, the cells lacking *CaGUP*1 displayed altered sensitivity to specific ergosterol biosynthesis inhibitors [19], and distribution of lipid rafts [19].

Both Gup1 and Gup2 are members of the MBOAT family of membrane bound *O*-acyltransferases [14]. These two proteins share a high degree of similarity with each other (53% of identity between sequences) and with the other members of the same family from yeast [14, 26]. Moreover, they also share a high degree of similarity with proteins from other eukaryotic organisms, namely other yeasts and fungi, but importantly, from high eukaryotes, like the fly, the mouse and man [14, 27] (Fig. 1).In these last cases, the fact that the Gup-similar proteins were identified as regulators of the Hedgehog pathway [28], yielded the nomenclature by which Gup1 orthologues from high eukaryotes are known, Hhat (Hedgehog Acyl Transferase) for Gup2, and Hhatl (Hedgehog Acyl Transferase-Like) for Gup1. Hhat is responsible for the palmitoylation of the Sonic Hedgehog, while Hhatl competes with Hhat to inhibits its function, this way greatly diminishing Sonic hedgehog extracellular mobility and compromising signalling proper course [28]. Unpublished results from our group (kindly supplied by C. Ferreira) show that the mouse Hhatl can partially complement the phenotypes generated by *gup*1Δ deletion in *C. albicans*, namely hyphae production and invasive growth. The complementation of the morphology phenotypes raises the question of whether yeast might possess a morphogenic signalling pathway equivalent to Hedgehog from high eukaryotes. Therefore, the functional complementation of *gup*1Δ and *gup*2Δ mutations in *S. cerevisiae* by the high eukaryotic orthologues was attempted.

A

```
hsHHATL         -------------MGIKTALP-----------------AAELGLYSLVLSGALAYAG-RGLLEASQDGAHRKAFRESVRPGWEYIGRKMD
mmHHATL         -------------MGIKTALP-----------------AAELGLYSLVLSGALAYAG-RGLLEASQDGAHRKAFRESVRPGWEYLGRKMD
yeastGUP1       MSLISILSPLITSEGLDSRIKPSPKKDASTTTKPSLWKTTEFKFYYIAFLVVVPLMFYAGLQASSPENPNYARYERLLSQGWLFG-RKVD
                             *:.: :                ::*: :* :.:  .:.     **  :* :..:   :.. :  ** :  **:*

hsHHATL         VADFEWVMWFTSFRNVIIFALSGHVLFAKLCTMVAPKLRSWMYAVYGALAVMGTMGPWYLLLLLGHCVGLYVASLLG--QPWLCLGLGLA
mmHHATL         VADFEWVMWFTNFRNVIVFALSGHVLFAKLCTMVAPQLRSWMYAVYGVLAVVGTMGPWYLLLLLGHCMVLYVASLLG--QRWLCLALGLA
yeastGUP1       NSDSQYRFFRDNFALLSVLMLV-HTSIKRIVLYSTNITKLRFDLIFGLIFLVAAHGVNSIRILAHMLILYAIAHVLKNFRRIATISIWIY
                 :* :: ::  .*  : :: *  *. : ::    :   :  :  ::* : ::.: *   : :*    :   :* :*   :    :.: :

hsHHATL         SLASFKMDP-LISWQSGFVTGTFDLQEVLFHGGS-------SFTVLRCTSFALES-------------------------------CA
mmHHATL         SLASFKVDP-GISWQSGFVTGTFDLQDVLFHGGS-------SFTVLRCTSFALES-------------------------------CA
yeastGUP1       GISTLFINDNFRAYPFGNICSFLSPLDHWYRGIIPRWDVFFNFTLLRVLSYNLDFLERWENLQKKKSPSYESKEAKSAILLNERARLTAA
                .:::: ::    ::  * : . :.  :  ::*         .**:**  *: *:                                  .*

hsHHATL         HPDRHYSLADLLKYNFYLPFFFFGPIMTFDRFHAQVSQVEPVRREGELWHIRAQAGLSVVAIMAVDIFFHHFFYILTIPSDLKFANRLPDS
mmHHATL         HPDRRYSLADLLKYNFYLPFFFFGPIMTFDRFHAQVSQ-EPVRPEGELWHIQAQAGLSAAAIVAVDVFFHHFFYILTIPSDLKFASRLPDS
yeastGUP1       HPIQDYSLMNYIAYVTYTPLFIAGPIITFNDYVYQSKHTLPSIN---FKFIFYYAVRFVIALLSMEFILHFLHVVAISKTKAWENDTP-F
                ** : *** : : *  * *:*: ***:**: :  * .:  *      : .*   *   . *:::::.::**:::::*..   : .  *

hsHHATL         ALAGLAYSNLVYDWVKAAVLFGVVNTVACLDHLDPPQ-PPKCITALYVFAETH--FDRGINDWLCKYVYNHIGGEHSAVIPELAATVATF
mmHHATL         ALAGLAYSNLVYDWVKAAVLFGVVNTVARLDHLDPPQ-PPKCITALYVFGETH--FDRGINDWLCKYVYDHIGGDHSTVIPELAASVATF
yeastGUP1       QISMIGLFNLNIIWLKLLIPWRLFRLWALLDGIDTPENMIRCVDNNYSSLAFWRAWHRSYNKWVVRYIYIPLGGSKNRVL----TSLAVF
                 :: :.  **   *:*  : : :..  * ** :*.*:   :*:   *        :.*. *.*: :*:*  :**.:. *:    :::*.*

hsHHATL         AITTLWLGPCDIVYLWSFLNCFGLNFELWMQKLAEWGPLARIEASLSVQMSRRVRALFGAMNFWAIIMYNLVSLNSLKFTELVARRLLLT
mmHHATL         VVTTLWLGPCDIVYLWSVLNCFGLNFELWVQKLAERGPLAQIEARLSEQMSRRVRALCGAVNFWAIIMYNLVSLNSLEFTELVARRLILT
yeastGUP1       SFVAIWHDIELKLLLWGWLIVLFLLPEIFATQIFS--------HYTDAVWYRHVCAVGAVFNIWVMMIANLFGF----CLGSDGTKKLLS
                 ..::* .    : **. *  : *  *:: :: .           .    *:* *: ...*:*.::: **..:         . : :*:
hsHHATL         GFPQTTLSILFVTYCGVQLVKERERTLALEEEQKQDKEKPE- 504
mmHHATL         GFPQTTLAVLFVTYCGVQLVKERERSLALEEEQRQDREKLE- 503
yeastGUP1       DMFCTVSGFKFVILASVSLFIAVQIMFEIREEEKRHGIYLKC 560
                .:  *. .. **  ..*.*.   :  : :.**:::.    :
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B

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hsHHAT          ------------------------------------------------------------MLPRWELALYLLASLGFHFYSFYEVY
mmHHAT          ------------------------------------------------------------MLPGWELTLCLLVSLGFHFRSFYEVY
dmRASP          ----------------------------------------------------MSRLPDRSLLTRCEIFVYFGVYIAYIVVGLYKIY
yeastGUP2       MSMLRIWSCIVHFFSVQALDSRIKPDIEFKRRQRIFINSSKEENGSSSSAVTVTRNPVLSSNSPSPPLWNTWEFRLYYLAFTVVVPFMIK
                                                                          .  :       :.: .   : :

hsHHAT          KVSREHEEELDQEFELETDTLFGGLKKDATDFEWSFWMEWGK--QWLVWLLLGHMVVSQMATLLARKHRPWILMLYGMWACWCVLGTP--
mmHHAT          KVSREHEEELDQEFELEMDTLFGGLKKDPTDFEWNFWMEWGK--RRLVWLFIGHMAVSQLATLLTKKHRPWIVMVYGMWACWCVLGAP--
dmRASP          GLR--DHIVKEAKFQFPEGWSLYPFSQRRRDDSNDELENFGD--FIVSFWPFYLLHVAVQGFIRWKRPRLQCLGFIGVCALALSVNLD--
yeastGUP2       AALATSSESNPNYYKFSGLLAHGWILGRKVDNSDPQYRFFRSNFFLLAILLLQIILKKVFVKFSKIPKTKFDFACGLVFVCFMYGINSV
                              :::       :     * .     : .    :    :  : :       :  :       *:       .

hsHHAT          -GVAMVLLHTTIS--FCVAQFRSQLLTWLCSLLLLSTLRLQG---------VEEVKRRWYKTENEYYLLQFTLTVRCLYYTSFSLELCWQ
mmHHAT          -GVVMVLLHSTIA--FCVAQFRSVLLSWLCSLLLLSTLRLQS---------VEEVKRRWYKTENEYYLLQFTLTVRCLYYTSFSLELCRQ
dmRASP          -WSSMVLLVTLIASYYIVSLLSLKFLVWLLSAGWILCINVMQ---------KNVWWTDRVG-YTEYVLVIVTMSWSVLRGCSYSLSKIGA
yeastGUP2       KLFTHAFIFFTLAHSLKRKRLIAAFAIWSYGIFTLFINQKMKNLPFNNIAIILSPMDQWYKGIVPRWDFFFNFTLLRLLSYSMDFLERWH
                     .::   ::       :   :  *  .   :   .                              . ..::   *   * .:

hsHHAT          QLPAAST-----------------------------------------SYSFPWMLAYVFYYPVLHNGPILSFSEFIKQMQQQEHDS
mmHHAT          PPSAQPTPSAQG-------------------------------------ASHSYPWLLTYVFYYPVFHNGPILNFPEFFRQMQQPELNS
dmRASP          KQEDLTR-------------------------------------------YSLVQYLGYAMYFPCLTYGPIISYQRFAARREDEVQNW
yeastGUP2       EQLSRQPSIDYDDRRPEFRKSLSGSTLQTIYESGKNVLEEKERLVAEHHIQDYNFINFIAYITYAPLFLVGPIITFNDYLYQSENKLPSL
                                                          :.    : *  * * :  ***:.:  :  : ::   .

hsHHAT          LKASLCVLALGLGRLLCWWWLAELMAHLMYMHAIYSSIPLLETVS-CWTLGGLALAQVLFFYVKYLVLFGVPALLMRLDGLTPPA-LPRC
mmHHAT          LQHSLCIVAKGLGRLLCWWWLAELMVHLMYMHALYSSAPLLESVS-CWTLGGLALAQVLFFYVKYLVLFGVPALLMRLDGLTPPP-LPRC
dmRASP          LG-----FVGGVLRSAIWWLVMQCALHYFYIHYMSRDVRMVEMMDSVFWQHSAGYFMGQFFFLYYVVTYGLGIAFAVQDGIPAPN-RPRC
yeastGUP2       TKKN---IGFYALKVFSSLLLMEIILHYIYVGAIARTKAWNNDTP--LQQAMIALFNLNIMYLKLLIPWRLFRLWAMVDGIDAPENMLRC
                       .     :      : :   * :*:  :       :           .     :  :::  :: : :     **: .*    **

hsHHAT          VSTMFSFTGMWRYFDVGLHNFLIRYVYIPVGGSQHGLLGTLFSTAMTFAFVSYWHGGYDYLWCWAALNWLGVTVENGVRRLVETPCIQDS
mmHHAT          VSTMFSFTGMWRYFDVGLHNFLIRYVYIPL-------------------------------------------------------------
dmRASP          IGRIHFYSDMWKYFDEGLYEFLFQNIYAELCGKRSSAAAKFGATALTFAFVFVWHGCYTYVLIWSILNFLCLAAEKVFKTFTAMPEYQRW
yeastGUP2       VDNNYSTVGFWRAWHTSFNKWVIRYIYVPFGGSNN----KILTSFAVFSFVAIWHDIQLRVLFWGWLTVLLLLGETYITN---------C
                :.  .   .:*: :. .: ::::: :*  .

hsHHAT          LARYFSPQARRRFHAALASCSTSMLILSNLV-FLGGNEVGKTYWNRIFIQGWPWVTLSVLGFLYCYSHVGIAWAQTYATD-------
mmHHAT          LARHLSPQAHHRLHALLAACSTSMLILFNLV-FLGGIQVGKTYWNRIFLQAAFR-----------------------------------
dmRASP          TQRHLGAVGAQRLYAMLATQLFIPAAFSNVY-FIGGQEIGDFLMRGAYLSGVGNYVALCFCSYCFFQCSELLLTKSDGRSKTKTF--
yeastGUP2       FSRYRFRSWYRFVCGIGAAINICMMMIINVYGFCLGAEGTKLLLKGIFNNSHSPEFLTAVMVSLFIAVQVMFEIREEEKRHGINLKC
                  *:      : . .  *:       : *:  *  * :  .   .  : ..
```

Figure 1. Alignments of the *S. cerevisiae* Gup1 protein with its orthologues from *H. sapiens* and *M. musculus* (mouse)(A); and Gup2 protein with its orthologues from *D. melanogaster* (fly) and *H. sapiens* (B).

Materials and Methods

Strains and growth conditions

S. cerevisiae strains used in the present work are listed in Table 1. Plasmid cloning and propagation was performed in the *Escherichia coli* strain XL1Blue (*endA1 gyrA96 (nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10proAB$^+$lacI^{q}Δ(lacZ)M15] hsdR17(r_K^- m_K^+)).*Yeast batch cultures were grown aerobically in minimal medium YNBD (0.67 % yeast nitrogen base and 2 % glucose). Incubation was performed at 30 °C, 200 rpm, orbital shaking and air/liquid ratio 2:1. Wt and mutant strains maintenance was done on solid rich medium YPD (1% yeast extract; 2% peptone; 2% glucose and 2 %agar), and the transformed strains were kept in solid minimal medium (with 2% agar), grown at 30 ºC for 48 h and kept at 4 ºC up to five days. Bacterial strains were grown in LB (2 % tryptone; 0.5 % yeast extract; 0.5 % NaCl; pH 7.2), at 37 °C, 200 rpm, orbital shaking, The maintenance of positive transformants was done in solid LB medium (with 2% agar), grown at 37 ºC overnight and kept at 4 ºC up to five days.

Dropout tests were performed from mid-exponential YNBD cultures containing approximately 1×10^6 cells/ml. Ten-fold serial dilutions were then made, and 5 µl of each suspension was applied on YNB medium supplemented with 2% glucose or 2% glycerol, and 2% glucose supplemented with 50 mM $CaCl_2$, 200 µg/ml rapamycin, 50 mM Congo Red, 0.01% SDS, 1 M sorbitol and 0.01% SDS + 1 M sorbitol. Results were scored after 48h of incubation at 30 °C, unless mentioned otherwise.

All ingredients percentages were calculated as weight per volume units. Amino acids and antibiotics supplementation were done according to strains auxotrophic markers and plasmid maintenance needs.

Plasmid constructions

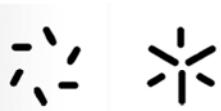
The cloning and expression of the higher eukaryotes cDNAs was carried out using the plasmid p426GPD. This multi-copy plasmid presents the strong GPD promoter and the *URA*3 gene for auxotrophic selection [29]. The *Drosophila melanogaster RASP*

cDNA, also known as *dmGUP2*, was provided already cloned in this vector with the *Hind*III/*Xho*I restriction sites (kindly supplied by F. Grieco from the Instituto di Scienze Delle Produzioni Alimentari, Lecce, Italy). The *Mus musculus HHATL* cDNA, referred as mmgup1, presented an eGFP tag and it was cloned in another plasmid [28]. This was kindly supplied by Y. Abe from the Keio University, Tokyo, Japan. The gene from pGup1-eGFP was digested with *BamH*I and *Bgl*II and the fragment was recovered with a GenElute Gel Extraction Kit (Sigma). The fragment was inserted in a *BamH*I digested p426GPD plasmid; proper orientation was evaluated through *EcoR*I digestion. The digestion of a correct oriented gene originated to 2 fragments: 7800 bp (plasmid + 1430 bp *mmGUP1*) and 250 bp (250 bp *mmGUP1*). The human cDNAs from *HHAT* and

Table 1. *S. cerevisiae* strains used in the present study.

Strain	Genotypes	Origin
W303-1A	*MAT***a***leu2-3 leu2-112 ura3-1 trp1-1 his3-11 his3-15 ade2-1 can1-100*	[30]
BHY54	Isogenic to W303-1A but *gup1::His5*$^{+}$	[13]
Cly5	Isogenic to W303-1A but *gup2::KanMX*	[13]
Cly3	Isogenic to W303-1A but *gup1::His5*$^{+}$*gup2::KanMX*	[13]
FFOY011	Isogenic to BHY54 but transformed with p426GPD+*mmGUP1*	This study.
FFOY012	Isogenic to BHY54 but transformed with p426GPD+*hsGUP1*	This study.
FFOY021	Isogenic to Cly5 but transformed with p426GPD+*dmGUP2*	This study.
FFOY022	Isogenic to Cly5 but transformed with p426GPD+*hsGUP2*	This study.
FFOY121	Isogenic to Cly3 but transformed with p426GPD+*mmGUP1*	This study.
FFOY122	Isogenic to Cly3 but transformed with p426GPD+*hsGUP1*	This study.
FFOY123	Isogenic to Cly3 but transformed with p426GPD+*dmGUP2*	This study.
FFOY124	Isogenic to Cly3 but transformed with p426GPD+*hsGUP2*	This study.

HHATL, respectively *hsGUP2* and *hsGUP1*, were inserted in a pDonor Gateway system® plasmid [31] and supplied by the Human ORFeome Collection of the Dana



Faber Cancer Institute, University of Harvard, USA. These genes were PCR isolated with specific primers (Table 2). The primers were designed to include the *Hind*III and *Xho*I restriction sites to enable the cloning in the plasmid p426GPD.The genes were inserted in the p426GPD plasmid through the action of a T4 ligase (Roche).

Table 2. Primers used in PCR reactions.

Primer	Sequence
hs*GUP1*_fw	5'-GCG AAG CTT ATG GGC ATC AAG ACA GCA TTG CC -3'
hs*GUP1*_rv	5'- GCG CTC GAG CTC CGG CTT CTC TTT GTC CTG C-3'
mm*GUP1*_fw	5'- ATA AAG CTT GCC ATG GGC ATC AAG ACA GC-3'
mm*GUP1*_rv	5'-ATA GGA TCC CTC CAG CTT CTC TCT GTC CTG-3'
hs*GUP2*_fw	5'- GCG AAG CTT ATG CTG CCC CGA TGG GAA CT-3'
hs*GUP2*_rv	5'- GCG CTC GAG GTC CGT GGC GTA GGT CTG GG-3'
dm*GUP2*_fw	5'-ATA AAG CTT CCA AAT CGG TGG TGT AGT G-3'
dm*GUP2*_rv	5'-CGC CGC GGA TCC ATA TAC AAT TAT ATA TTT-3'

Transformation and DNA manipulation

Escherichia coli cells were transformed with a $CaCl_2$/heat shock-based protocol [32]. Cells were incubated in LB medium supplemented with ampicillin (100 µg/ml) for selection. Positive transformants were grown overnight and the presence of the desired plasmid was assessed through miniprep and digestion with restriction endonucleases. Positive transformants were used to plasmid storage and propagation. *S. cerevisiae* cells were transformed using the lithium acetate methodology [32]. Transformants were selected through incubation in minimal medium without uracil.

Results and Discussion

Stress response and functional complementation

The *S. cerevisiae GUP1*and *2* mutants were complemented according to the following scheme:

	Ø plasmid	dmRASP	mmHHATL	hsHHATL	hsHHAT
*gup1*Δ	X		X	X	
*gup2*Δ	X	X			X
*gup1*Δ*gup2*Δ	X	X	X	X	X

The transformants were submitted to phenotypic tests according to the results previously obtained in *S. cerevisiae* [15].The transformants were incubated in glucose and glycerol, and submitted to several types of stress, including high temperature (37 ºC), cell wall perturbing agents (SDS and Congo Red), signalling pathways disturbing compounds (50 mM $CaCl_2$ and 200 µg/ml rapamycin), osmotic pressure (1 M sorbitol), as well as remediation of 1 M of Sorbitol through the combination of 0,01% SDS[15]. The Wt strain, as well as the *gup1*Δ, *gup2*Δ and *gup1*Δ*gup2*Δ mutants, responded to these tests similarly to previously [15]. The transformants on the other hand, did not complement the mutants phenotypes under the conditions tested (Fig. 1, 2 and 3).

In fact, it is possible to observe a discrete loss of fitness in all the strains harbouring plasmids. These were assessed for the plasmid presence through growth in solid minimal media supplemented with the auxotrophic needs except uracil (not shown). All strains were able to grow as expected in supplemented YNB, but, surprisingly, lost the ability to grow on this selective medium after one passage. Therefore, the presence of the plasmid was thereafter checked by PCR, using the primers in Table 2. Nonetheless, the expression of the heterologous proteins was not assessed through Western Blot, as there were no available antibodies. Although these have been recently commercialized (see https://www.scbt.com/pt/), raising a whole new range of possibilities, they have not yet been tested in yeast.

The successful complementation of *Δgup1* mutant achieved in *C. albicans* by *mmHHATL* cDNA above referenced was obtained using the p414GPD. This expression plasmid belongs to the same vector family as p426, but it is a low-copy plasmid, and presents the tryptophan auxotrophic marker instead of uracil. In what regards these markers, both have recorded low level of reversal, and should present similar selective strength [33]. Identically, the presence of the strong constitutive GPD promoter on both cloning vectors should result in similar levels of expression for each plasmid copy.

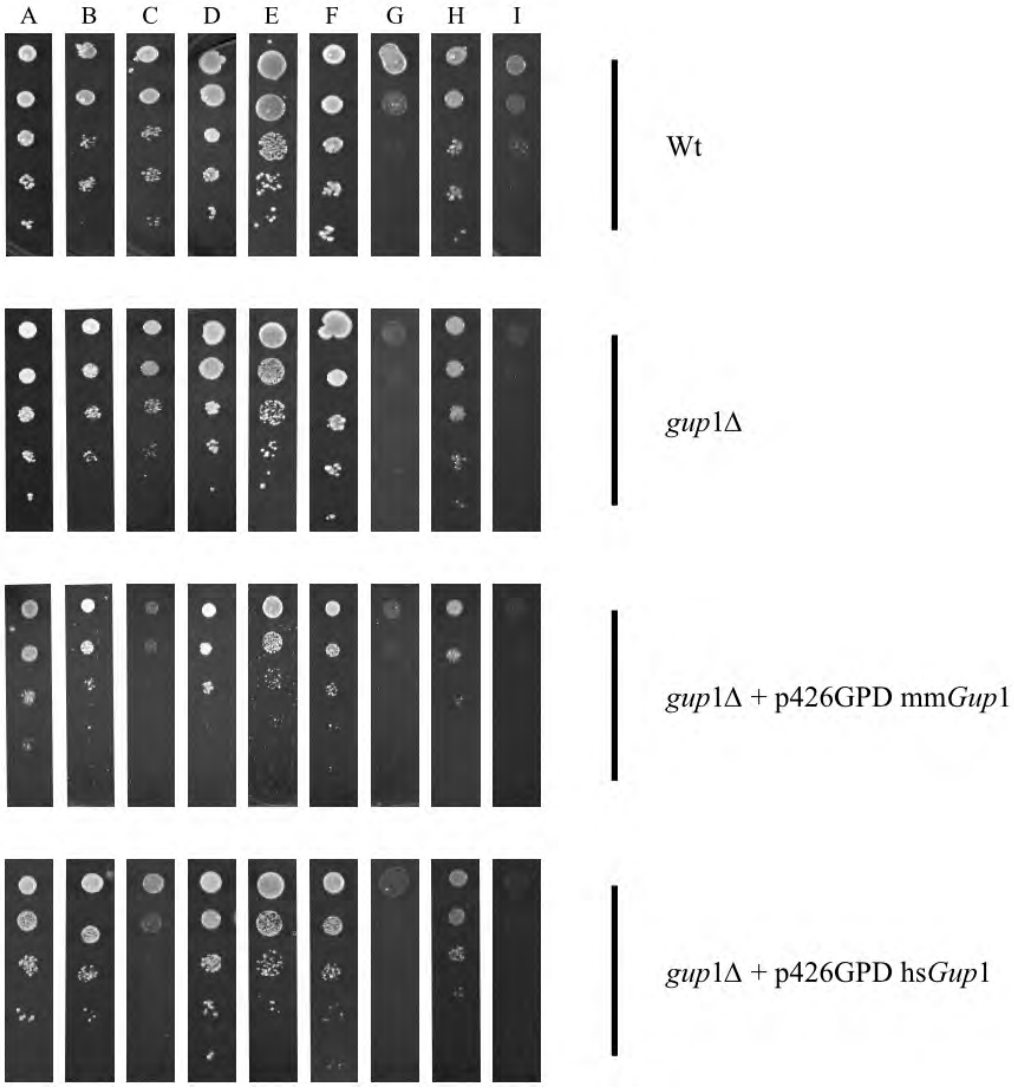


Figure 1. Functional complementation of *gup*1Δ mutation by mouse and human acyltransferase-like genes. Ten-fold serial dilutions of cells were spotted in minimal media with 2% glucose at 30 ºC (Column A) or at 37 ºC (Column B) and 2% glycerol at 30 ºC (Column C), or 2% glucose supplemented with 50 mM CaCl2 (Column D), 200µg/mL Rapamycin (Column E), 50 mM Congo Red (Column F), 0.01% SDS (Column G), 1 M Sorbitol (Column H) and 0.01% SDS + 1 M Sorbitol (Column I).

It is known that a high number of plasmids might lead to the expression of saturating levels of heterologous protein inside the cells, triggering the proteolytic degradation of the protein of interest [34], which might be responsible for the lack of complementation and putatively of expression. However, the differences in growth conditions may also play a role. The minimal media and auxotrophic requirements limit the growth of the

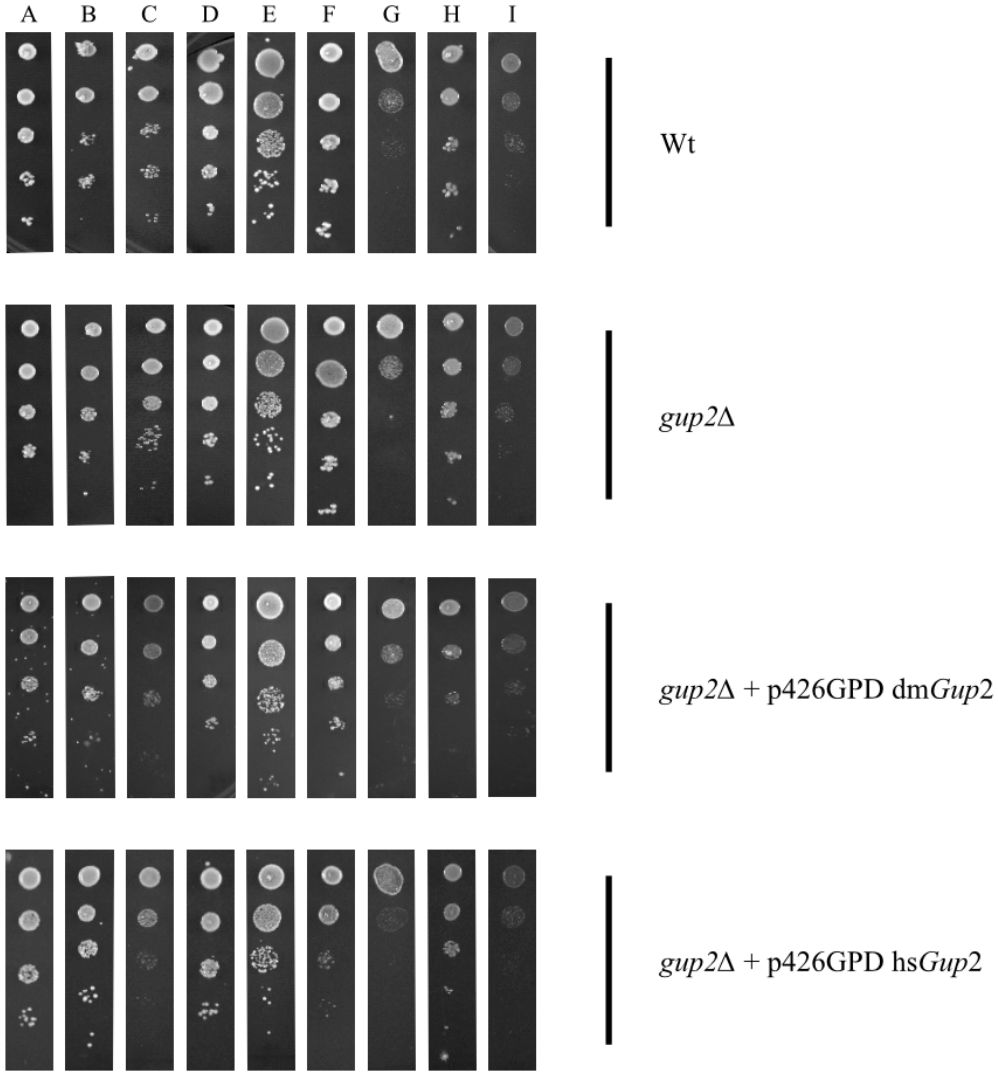


Figure 2. Functional complementation of *gup*2Δ mutation by fly and human acyltransferase genes. Ten-fold serial dilutions of cells were spotted in minimal media with 2% glucose at 30 ºC (Column A) or at 37 ºC (Column B) and 2% glycerol at 30 ºC (Column C), or 2% glucose supplemented with 50 mM CaCl2 (Column D), 200µg/mL Rapamycin (Column E), 50 mM Congo Red (Column F), 0.01% SDS (Column G), 1 M Sorbitol (Column H) and 0.01% SDS + 1 M Sorbitol (Column I).

colonies, yielding less biomass than rich media in the same conditions. Hence, if only a mild or partial functional complementation is occurring, it could be masked by fewer colonies observed in the minimal media.

Still, the strains made available in this pioneering work will be further studied. They will be used to confirm the expression of the heterologous proteins using the antibodies presently available commercially against Rasp, mmHhatl, hsHhatl and hsHhat. If necessary the constructions will be shifted to low copy number plasmids for a new phenotypic check. Additionally, several drugs have been described as targeting components of the Hh pathway, namely exo-cyclopamine that selectively inhibits the Smoothened protein and interferes with the patterning during development [35]. The effects of such compounds that target proteins downstream of the Hh signalling pathway should overlap, at least partially, with the effects of mutations in the yeast acyltransferases, giving important hints about the cell-cell communication mediated by the putative yeast pathway.

All the available information in the components of Hh pathway in mammals, and the available tools for the treatment of Hh-related disorders may become powerful tools to uncover the function of yeast Gup1 and Gup2 proteins, and the putative extracellular signalling pathway.

Acknowledgments

We thank Francesco Grieco from the Instituto di Scienze Delle Produzioni Alimentari, Lecce, Italy, for providing the fly *Gup2* gene already cloned into p426GPD, Yoichiro Abe from the University of Keio, Tokyo, Japan for sending us the mouse *HHATL* cDNA, and Kourosh Salehi-Ashtiani, presently at the University of New York in Abu Dhabi, Emirates, for granting us access to the Dana Farber Institute - Human Orfeome collection and allow the work with the human *HHAT* and *HHATL* genes.

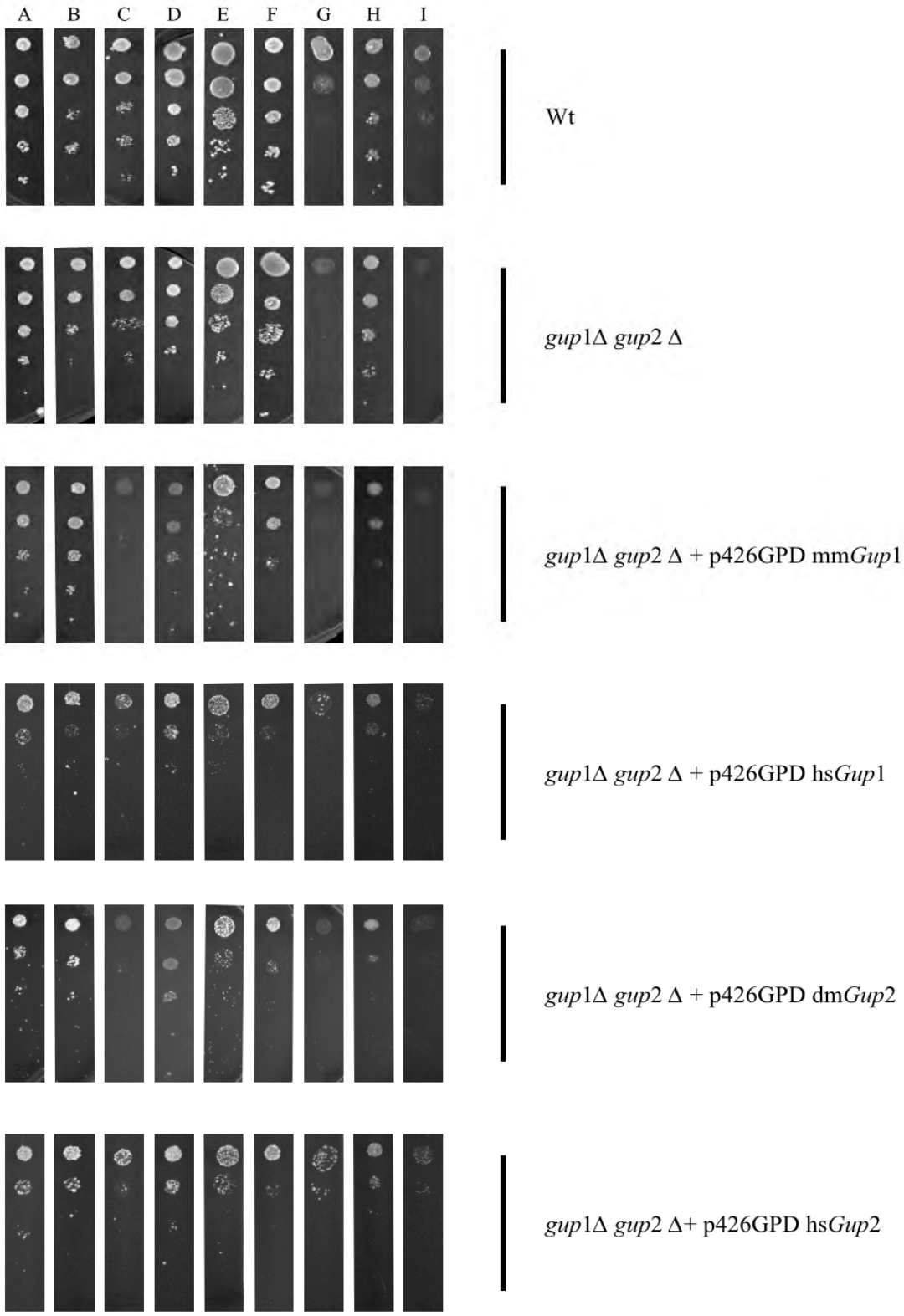


Figure 3. Functional complementation of *gup*1Δ*gup*2Δ mutation by fly, mouse and human acyltransferase genes. Ten-fold serial dilutions of cells were spotted in minimal media with 2% glucose at 30 ºC (Column A) or at 37 ºC (Column B) and 2% glycerol at 30 ºC (Column C), or 2% glucose supplemented with 50 mM CaCl2 (Column D), 200µg/mL Rapamycin (Column E), 50 mM Congo Red (Column F), 0.01% SDS (Column G), 1 M Sorbitol (Column H) and 0.01% SDS + 1 M Sorbitol (Column I).

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7. EFFECT OF MAMMALIAN ECM COMPONENT HYALURONAN ON YEAST

Abstract

Hyaluronan (HA) is a non-sulphated glycosaminoglycan (GAG) present in the extracellular matrix (ECM) of the higher Eukaryotes, regulating major cellular processes as apoptosis, angiogenesis or cell migration and proliferation. HA is present in the ECM in a wide range of biologically active sizes, which modulate the biophysical and biochemical properties of tissues. This GAG intervenes in several signalling pathways through its receptors CD44 and RHAM. Several microorganisms produce proteins involved in the metabolism of HA, namely *Candida albicans* and *Streptococcus spp.*, which are associated with infection/colonization processes. The effects of different size HA in *S. cerevisiae* were assessed in W303-1A strains heterologously expressing the HA receptors, CD44 and HMMR. The engineered strains were subjected during cultivation to the presence of HA with 2 to 1000 kDa. The HA of higher molecular weights produced a mild effect on growth parameters of CD44 and HMMR expressing strains. However, in accordance with the literature the strain expressing CD44 presented a high growth reduction in the presence of 50 kDa HA. These results suggest that the receptors are functional in the cell, and the cellular machinery to respond to HA *stimuli* is fairly conserved. The further testing of *S. cerevisiae* strains expressing the HA receptors will provide crucial information on the role of yeast interactions with high molecular weight polysaccharides, and the role of those interactions on several cellular processes, namely differentiation and invasive growth.

Hyaluronan and hyaladherins

Hyaluronan (HA) is one of the most important components of the extracellular matrix (ECM) of high eukaryotes, and it plays several structural and metabolic roles in the ECM [1]. Its average molecular weight ranges from 1 to 5 million Daltons, and it is composed of a homogenous linear repetition of the dimmer [glucuronic acid (β1,3) /N-acetyl- glucosamine (β1,4)][2]. This glycosaminoglycan is not attached to a peptide, and its synthesis is performed in the plasma membrane by opposition to the Golgi apparatus as the remaining glycosaminoglycans [3]. Also, the new residues are added to the reducing end of the chain, whereas in the case of the other glycosaminoglycans the addition occurs at the non-reducing end [4]. Moreover, the hyaluronan synthase, aside from being capable of establish both β-1,3 and β-1,4 linkages, is also responsible for the exportation of the newly synthesized chain to the extracellular space through a pore constituted by the enzyme itself [5].

HA regulates and intervenes in several important intracellular pathways [6] through the numerous and disparate biological-relevant molecular sizes, resulting from very dynamic and controlled processes of synthesis and degradation/ turnover [1, 3, 6].These different size molecules interact with specific receptors, hyaladherins, promoting signal transduction and HA internalization [7]. The hyaladherins, namely HMMR (Hyaluronan-Mediated Motility Receptor, also known in the literature as RHAMM, Receptor for Hyaluronan-Mediated Motility) and CD44, present several different isoforms, resulting from alternative splicing, that interact with different HA molecular sizes and control cell migration, aggregation and proliferation [6]. Being present in the cell surface and in intracellular compartments, these receptors may perform a role in the information flow between the cell genome and the extracellular environment [3].

The lack of information on yeast ECM and the presence of enzymes related to HA turnover in yeast and bacteria, namely *Candida albicans* and *Streptococcus spp*. [8-11], prompted the study of HA and its metabolism in *S. cerevisiae*. The growth of yeast strains expressing cDNAs from the human hyaladherins HMMR and CD44 was differently affected by the presence of several HA molecular sizes. This study allows us to infer the possibility of potential conserved pathways to integrate HA receptors signals, a probable result of glycosaminoglycans presence in yeast ECM.

Materials and Methods

Strains and growth conditions

S. cerevisiae strains used in the present work are listed in Table 1. Plasmid cloning and propagation was performed in the *Escherichia coli* strain XL1Blue (*endA1 gyrA96 (nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10proAB$^+$lacI^{q}Δ(lacZ)M15] hsdR17($r_K m_K^+$)).*Yeast batch cultures were grown aerobically in minimal medium YNBD (0.67 % yeast nitrogen base; 2 % glucose). Incubation was performed at 30 °C, 200 rpm, orbital shaking and air/liquid ratio 2:1. Wt and mutant strains maintenance was done on solid rich medium YPD (1% yeast extract; 2% peptone; 2% glucose; 2 %agar) and transformants strains were kept in solid minimal medium (with 2% agar), grown at 30 ºC for 48h and kept at 4 ºC up to five days. Bacterial strains were grown in LB (2 % tryptone; 0.5 % yeast extract; 0.5 % NaCl; pH 7.2), at 37 °C, 200 rpm, orbital shaking. The maintenance of positive transformants was done in solid LB medium (with 2% agar), grown at 37ºC overnight and kept at 4ºC up to five days.

The growth assays in the presence of 0.1% HA were performed in 96 well plates. Cells from mid-exponential YNBD cultures were collected through centrifugation and resuspended in fresh YNBD (OD_{600nm}=0.5). The cells were diluted to an initial OD of 0.05 (200µl of suspension per well), and incubated in the presence of HA (2, 10, 200, 1000 kDa), at 30ºC with orbital shaking for 10h. Growth was monitored spectrophotometrically every hour at 600 nm in a SpectraMax microplate reader (Molecular Devices). Data represents the average of three independent experiments.

Table 1. *S. cerevisiae* strains used in the present study.

Strain	Genotypes	Origin
W303-1A	*MAT***a***leu2-3 leu2-112 ura3-1 trp1-1 his3-11 his3-15 ade2-1 can1-100*	[12]
FFOY000	Isogenic to W303-1A but transformed with empty p426GPD	This study.
FFOY001	Isogenic to W303-1A but transformed with p426GPD+*CD44*	This study.
FFOY002	Isogenic to W303-1A but transformed with p426GPD+*HMMR*	This study.

All ingredients were calculated as weight per volume units. Amino acids and antibiotics supplementation were done according to strains auxotrophic markers and plasmid maintenance needs.

Plasmid constructions

The cloning and expression of the HA receptors was carried out using the plasmid p426GPD. This multi-copy plasmid presents the strong GPD promoter and the URA3 gene for auxotrophic selection [13].

The cDNAs from human CD44 and HMMR were received in a pDonor Gateway system® plasmid [14]. These genes were PCR isolated with specific primers (Table 2). The primers were designed to include the *Hind*III and *Xho*I restriction sites for the CD44 gene, and the *Hind*III and *Spe*I for the HMMR gene, to enable the cloning in the plasmid p426GPD. The cDNAs were inserted in the p426GPD plasmid through the action of a T4 ligase (Roche).

Table 2. Primers used in PCR reactions.

Primer	Sequence
hs*CD44*_fw	5'-GCGAAGCTTATGGACAAGTTTTGGTGGCA-3'
hs*CD44*_rv	5'- GCGCTCGAGCACCCCAATCTTCATGTCCACATTGTGC-3'
hs*HMMR*_fw	5'- GCGACTAGTATGTCCTTTCCTAAGGCGCCC-3'
hs*HMMR*_rv	5'- GCGCTCGAGCTTCCATGATTCTTGACACTCCATA-3'

Transformation and DNA manipulation

E. coli cells were transformed with a $CaCl_2$/heat shock-based protocol [15]. Cells were incubated in LB medium supplemented with ampicillin (100 µg/ml) for selection. Positive transformants were grown overnight and the presence of the desired plasmid was assessed through miniprep and digestion with restriction endonucleases. Positive transformants were used to plasmid storage and propagation. *S. cerevisiae* cells were

transformed using the lithium acetate methodology [15]. Transformants were selected through incubation in minimal medium without uracil.

Results and Discussion

Hyaladherins expression and growth defects

In order to assess the effect of HA in *S. cerevisiae* expressing HA specific receptors, the cloning of the genes coding for these proteins was performed in the multi-copy plasmid p426GPD. As before (Chapter 6), these cDNAs originated from the Dana Farber Human ORFeome Collection, and were retrieved from the Gateway system-based plasmids through PCR with specific primers (Table 2). The p426GPD plasmid containing the cDNAs encoding for the HA receptors were transformed into the W303-1A yeast strain. The strains were assessed for the plasmid presence through growth in uracil-less selective medium (not shown). The strains were able to grow in supplemented YNB after the transformation, and identically to what happened with the construction from Chapter 6, after a passage in YPD, the strains were no longer able to grow on YNB lacking uracil (not shown). As mentioned before (Chapter 6), the plasmid stability in the transformants was an issue, impossible to assess by Western Blotting. Some transformants did not respond to HA, but presented enhanced adherence to agar (not shown). Only transformants that responded to HA were selected to proceed with the tests.

The several strains were grown in selective medium in the presence of 0.1% HA from a wide range of molecular sizes: ≈2, 10, 50, 200 and 1000 kDa (Fig. 4). Each culture growth was followed throughout exponential and post-diauxic phases. As control, the strains were incubated without HA (Fig. 4 A). Results showed that the CD44 and the HMMR expressing yeast strains presented identical performance. Moreover, in the presence of the HA lower molecular weights (≈2 kDa and 10 kDa) (Fig. 4 B, C), growth was basically indistinguishable from the control cultures in the absence of HA (Fig. 4 A). The low HA MW, in the present conditions, namely HA concentration, did not produce a growth phenotype.

Otherwise, the HA higher molecular weights (200 and 1000 kDa) (Fig. 4 E, F), produced a mild effect on *lag* phase which increased to 2 h time, as well as on μ_g that

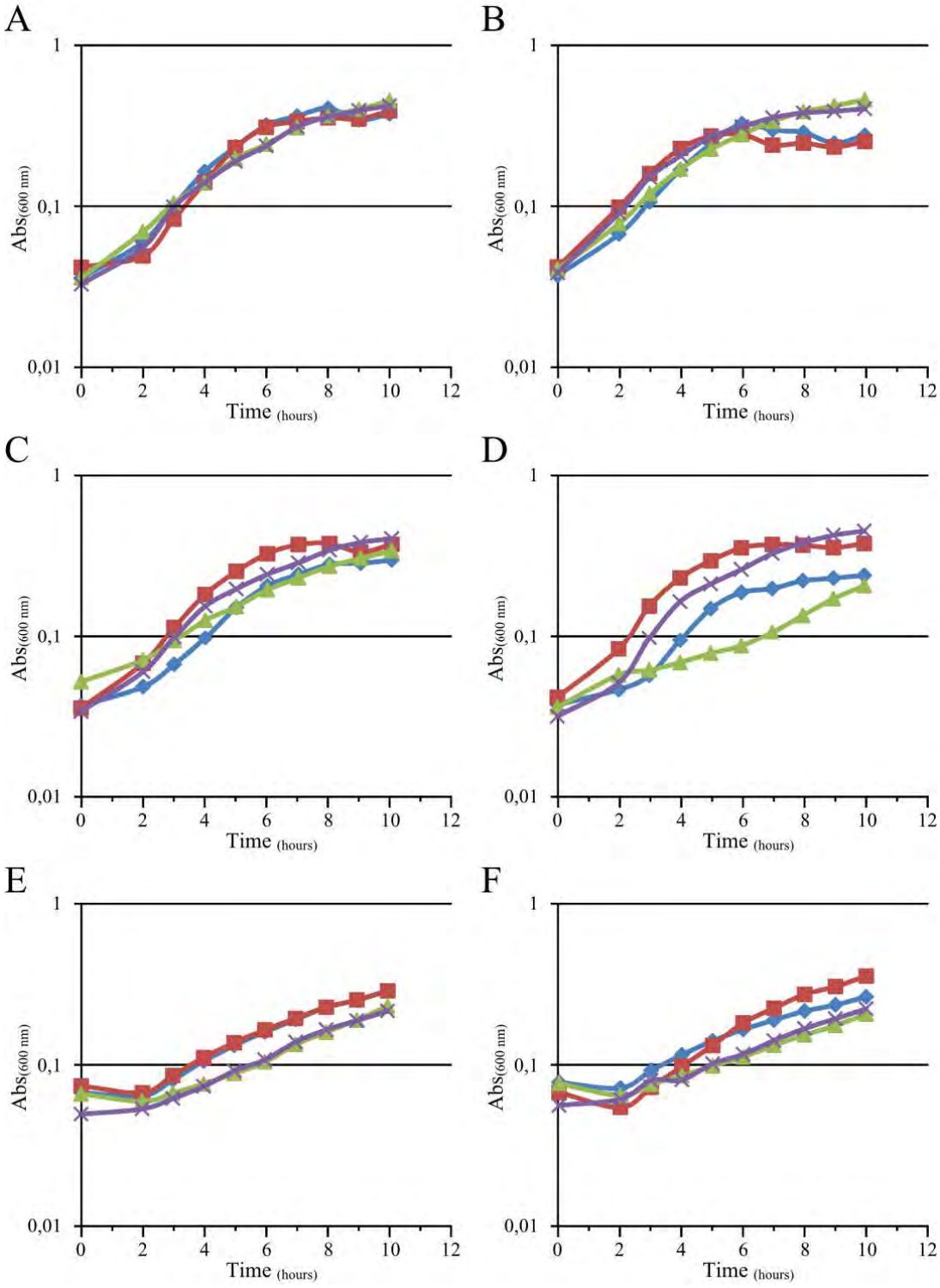
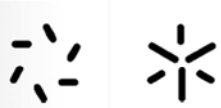


Figure 1. Hyaluronan influence on cell growth in the yeast strains expressing CD44 and HMMR. Growth curves in YNB alone (A), or supplemented with 0.1% HA ≈2 kDa (B), 10 kDa (C), 50 kDa (D), 200 kDa (E) and 1000 kDa (F). Strains: W303-1A (—◆—), W303-1A p426GPD (—■—), W303-1Ap426GPD-CD44 (—▲—) and W303-1A p426GPD-HMMR (—✕—).



was 0.14 h^{-1} and 0.18 h^{-1} in CD44 and HMMR expressing strains respectively, that is, 40% and 20% slower as compared to 0.23 $h^{-1}$from both control strains in the same growth conditions. An identically mild effect was observed in biomass achieved entering stationary phase. The fact that this effect was not too afar in both the CD44 and the HMMR expressing strains could arguably be due to a non-specific inhibition caused by the increase in osmotic stress derived from the presence of the very high MW HA, which has high water retention ability and/or the high viscosity of these media. Finally, 0.1% 50 kDa HA had the most effect (Fig. 4 D), affecting especially the CD44 expressing strain. These cells presented a decreased in growth, being exponential phase μ_g 0.11 h^{-1}, ±70% lower than all the other strains (HMMR - 0.40 h^{-1}, empty plasmid - 0.35h^{-1}, and Wt- 0.37 h^{-1}) (Fig. 4 D).

These results are quite preliminary. No WB was possible to confirm the expression of the two proteins in yeast cells. Nevertheless, the different response of each strain to the several HA concentrations suggests that the proteins are functionally expressed. Actually, the growth sensitivity of the CD44 expressing strain to 50 kDa HA is somehow expected since this receptor in mammalian cells presents high affinity for HA in the range of 30-40 kDa MW [16]. Moreover, other HA concentrations were not tested. Although that would not be possible to perform for the higher MW because of the limits of solubility and/or exceptional viscosity of the media, it cannot be disregarded that for the smaller MW effects could be observed in other range of concentrations. Additionally, it is known that the physiological state of the cell, namely glucose metabolism, or other environmental factors, may affect HA turnover if not the response to HA [3, 17].

The *S. cerevisiae* strains humanized by expressing the hyaluronan receptors will be further assessed, hopefully becoming a tool for the study of the roles and effects of hyaluronan in yeast cell response and yeast ECM production and characteristics.

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8. GENERAL DISCUSSION

General discussion

The yeast *Saccharomyces cerevisiae*, as all microbes, is generally regarded as a unicellular organism. The biotechnological applications of this yeast mostly relying on liquid batch, fed-batch or continuous cultures contributed to strengthen that concept. Essentially, microbes are regarded as unicellular because they do not form enduring and self-complying multicellular aggregates like higher organisms. However, in nature, the existence of planktonic cells is transitory. Actually, microorganisms live more frequently in spontaneously forming macroscopic aggregates of many cells that easily could be considered as proto-tissues. They present differentiated and specialized cells [1, 2], spatially organized into functional structures [3, 4], coordinated by complex communications systems [5, 6], and supported by a complex extracellular matrix (ECM) [7-10]. The ECM is a crucial structure for the development of multicellularity. It provides protection against environmental variations and stresses, and acts as a scaffold for the three-dimensional organization of cells [11, 12]. Evidence of the presence of this type of structure supporting the life of *S. cerevisiae* within colonies emerged barely a decade ago [4]. Since then, new information from *S. cerevisiae*, as well as knowledge gathered from other microorganisms, including bacteria, has been accumulating [2-4, 13-22]. However, the identity of most protein and sugars basic components supporting this life-style, as well as their biological roles, are still unknown, although complexity is expected in view of the 3D spatial organization of wild type yeasts colonies [4, 19]. Hence, the work developed under the scope of this thesis aimed to provide a first systematic insight in the composition and molecular characterization of yeast ECM components.

The inexistence of a robust methodology for the extraction and fractionation into chemical purity of yeast ECM to enable the identification of the major components from different chemical families was not available. The extraction of the protein fraction of the colonies ECM had already been described, but only a very preliminary analysis by SDS PAGE was presented [4, 19], therefore raising doubts as to the feasibility of the correspondent protocol for proteomic approaches. A more in-depth analysis, based in powerful methodologies, was a requirement to initiate the systematic study of the yeast mat ECM proteins and their biological roles. In this work a methodology was devised to

(1) reproducibly obtain young yeast cells grown into homogenous mats; (2) extract the ECM and (3) fractionate the ECM into analytical-grade protein and sugar fractions.

The high throughput mass spectrometry analysis of the proteins in the yeast ECM identified an unsuspected large number and diversity of proteins, most of which usually assigned to the cytoplasm or intracellular organelles. These proteins were empirically organized into the following classes: control of cellular organization, cell rescue and defence, DNA/RNA maintenance, protein fate and components metabolism. The cell integrity assessment showed that in a 7 days grown-mat, 10-15% of cells had compromised plasma membrane. The lysis of some cells during the development of the mat is inevitable, and certainly contributes to increase the number and/or amount of intracellular proteins that were found. As much as it may theoretically be desirable to avoid, one cannot discard the notion that the actual existence of intracellular contents from lysed cells might be important for the mat or biofilm development. The role of such proteins is for the moment unknown. They might contribute to the scaffolding of the ECM, to the formation of signalling gradients, or simply to act as a nutrient source. However, some of these proteins have already been reported in the cell surface, namely glycolytic enzymes [23-25]. In another yeast, *Candida albicans*, the same proteins were shown to present a completely different function [26, 27], and therefore nicknamed *moonlighting proteins* [28, 29]. Moreover, a large number of proteins associated with protein fate and remodeling were found. These included several proteins from the HSP70 family, and proteases, importantly, the exopeptidases Lap4, Dug1 and Ecm14, and the metalloproteinases Prd1, Ape2 and Zps1, sharing a functional zincin domain with higher Eukaryotes ECM metalloproteinases.

The information regarding the ECM sugar fraction available in the literature [4, 19] was considerably smaller than the protein-related data. The methodologies underlying polysaccharides characterization are not very common and present a considerable degree of difficulty and uncertainty. The information available on yeast ECM polysaccharides was reduced to the acknowledgement of the present of glycoproteins [4, 19], and the sugar monomers composing a exopolysaccharide in *C. albicans* biofilms[30]. Our methodology allowed obtaining a sugar rich fraction that once treated with a broad range proteinase, yielded a sugar fraction with high purity. The mass spectrometry analysis revealed the presence of glucose, mannose and galactose, suggesting the presence of polysaccharides similar to the glucans and/or mannans

present in yeast and fungi cell walls [31], or to the galactoglycans described for algae and ascidians [32, 33]. The presence of uronic acids was also observed in the yeast ECM sample, whose occurrence is frequent in the ECM of both high Eukaryotes [34], and microbial biofilms [35]. The electrophoretic and chromatographic separation revealed the existence of two distinct polysaccharides, a low molecular weight oligosaccharide, and a 35-40 kDa polysaccharide. Both these compounds presented chemical substitution, as indicated by the metachromatic shift induced in dimethymethylene blue and toluidine blue dyes. The putative existence of sulphate groups in the yeast ECM led to the analysis of the anticoagulant activity of several ECM samples from different yeasts strains. A particular strain, mutant for both *GUP1* and *GUP1* genes, presented a relatively high anticoagulant activity, which was not observed in Wt cells. Given the role of sulphation pattern in anticoagulant effect [36], the chemical substitution detected in the yeast ECM is mostly likely sulphate groups. However, several other chemical groups may induce the metachromatic shift of the analysed dyes, and the presence of classic sulphotransferases, as well as the chemical intermediates, has not been so far reported in *S. cerevisiae*. The nature of the chemical substitution in the yeast ECM polysaccharides, as well as its macromolecular structure, is currently being examined using tools with higher analytic power, including the methylation analysis of such compounds and NMR.

The *GUP1* gene is a very pleiotropic gene that influences a great deal of cellular processes, from plasma membrane and cell wall composition and organization to cytoskeleton assembly [37-42]. In *C. albicans*, the disruption of this gene resulted in changes in colony morphology, and loss of invasive growth capacity and hyphae formation [43]. The effects of the deletion of *GUP1* gene in the composition of yeast ECM were assessed, providing a more in-depth perspective of the ECM structural organization and metabolic mechanisms. Alterations in both protein identities and abundances were observed, a great number of proteins were absent and the remaining proteins amounts were significantly different. The deletion of *GUP1* made disappear from the yeast ECM mainly proteins associated with the carbon metabolism, cell rescue and defence, protein fate and cellular organization. The functions of these proteins in the ECM are still unknown. The deletion of *GUP1* additionally had a profound impact in the composition of the ECM sugar fraction, through the disappearance of the higher molecular weight polysaccharide that was detected in the Wt sample, or alternative

elimination of the sulphation and subsequent loss of metachromasia. The *GUP1* effects on the ECM shows that its structure is very dynamic, and that it is under the tight control of the cells composing the aggregate. The possible relation between the ECM molecular composition and the phenotypes observed previously in this mutant (lipids, rafts [40], wall[39], cytoskeleton [38], life span and death [41, 42], invasiveness and differentiation [43]) point to the biological roles known to higher Eukaryotes ECM.

In higher Eukaryotes, tissue patterning and remodelling during embryogenesis or wound healing are regulated by molecules named Morphogens [44]. These molecules comprise several families, including the Hedgehog (Hh), Wnt or the Bone Morphogenetic Protein (BMP) [45-47]. The Hh signal is translated into a precursor protein that undergoes auto-excision and extensive post-translational modifications, including the addition of cholesterol and palmitate to the protein extremities [47]. The palmitoylation of Hh is the role of a membrane bound *O*-acyltransferase known as the Hedgehog acyltransferase (Hhat) [48]. Another protein, Hedgehog acyltransferases-like (Hhatl) competes for the Hh molecule and negatively regulates the process [49]. These proteins are similar to, respectively, Gup2 and Gup1. The high homology between yeast Gup1 and Hhatl, and Gup2 and Hhat, suggests the presence of a signalling pathway equivalent to the morphogenic Hedgehog pathway, mediated by a modified protein extracellular bound signal. Unpublished results from our group, showed that the Hhatl from mouse is capable of functionally complement the *GUP1* deletion in *C. albicans*. This led to the engineering the yeast mutants defective on either or both *GUP1* and *GUP2* by expressing the fly, human and mouse orthologues. This strain set was preliminarily assessed in stress conditions known to produce severe phenotypic changes in cells lacking Gup1, and the functional complementation evaluated. The different strains did not present reversion of the mutant phenotype. The assessment of protein expression through WB, as well as the trial of alternative expression systems, will be continued in the future.

Finally, as an additional preparation for future yeast ECM-related work, also the mammalian receptors of hyaluronic acid (HA), CD44 and HMMR, were cloned into the yeast *S. cerevisiae*. Several microorganisms produce proteins involved in the metabolism of HA, mostly human pathogens [50-52]. The presence of such proteins in yeast and bacteria, as well as the lack of information regarding yeast ECM prompted the study of HA and its metabolism in *S. cerevisiae*. The engineered strains were subjected

to the presence during cultivation of HA from 2 to 1000 kDa. Nevertheless, the presence of these HA higher molecular weights (produced a mild effect on lag phase, as well as on μg that was 40% and 20% slower in CD44 and HMMR expressing strains, respectively. However, in accordance with the literature [53], the strain expressing CD44 presented a high growth reduction in the presence of 50 kDa HA. These results suggest that the receptors are functional in the cell, and the cellular machinery to respond to HA *stimuli* is fairly conserved. The further testing of *S. cerevisiae* strains expressing the hyaluronan receptors will provide crucial information on the role of yeast interactions with high molecular weight polysaccharides, and the role of those interactions on several cellular processes, namely differentiation and invasive growth.

The study of the multicellular life-style of the yeast *S. cerevisiae* is still very recent. The knowledge on the ECM composition, organization and metabolism is still reduced. The methodology described in this work is able to provide large amounts of yeast ECM, in a simple and reproducible manner, allowing the analysis of both the protein and sugar components. So far, the study of the yeast ECM was limited due to the low amount of starting materials. Our methodology will help to overcome this limitation. The establishment of standardized inoculation, growing conditions, and ECM extraction procedures, ensures reproducibility, which became patent in the results obtained using highly sensitive techniques, namely DIGE. Several independent replicates were analysed and shown to behave similarly.

The present work is innovative and ground breaking since (1) this is the first time that a detailed survey on the identity of the proteins secreted during growth in a yeast multicellular aggregate is presented, and (2) this is the first time a more in-depth approach into the polysaccharide composition of the correspondent glycosidic fraction is obtained. Moreover, this work contributed with the first description of the effect of the deletion of genes involved in several cellular processes in the development of the yeast ECM, identifying the proteins affected positively and negatively by the mutation. Finally, this work is also a pioneer for suggesting the possibility of polysaccharide chemical substitution, this way adding to the complexity of yeast ECM. Overall, this work contributes with a large step to give the future insight of the multicellular life-style of *S. cerevisiae.*

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SUPPLEMENTARY DATA

Supplementary Data

Table S1. Proteins secreted during liquid growth by *S. cerevisiae* W303-1A cells.

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
Q04951	SCW10	32,65	35	10	389	40,4	4,65	1398,20
P53334	SCW4	31,61	31	10	386	40,1	4,83	1072,14
P00445	SOD1	55,84	35	8	154	15,8	6,00	1021,79
P38288	TOS1	17,80	23	5	455	48,0	4,67	972,76
P23776	EXG1	40,40	25	12	448	51,3	4,75	799,49
P17967	PDI1	30,08	22	11	522	58,2	4,53	770,07
P42835	EGT2	8,55	15	5	1041	108,5	4,82	755,08
P00925	ENO2	40,05	18	11	437	46,9	6,00	694,00
Q12408	NPC2	22,54	24	3	173	19,1	4,44	591,44
P00044	CYC1	32,11	17	3	109	12,2	9,42	583,90
P15703	BGL2	23,32	15	6	313	34,1	4,51	571,11
A7A003	UTH1	16,30	15	5	362	36,7	4,79	415,10
P0CG63	UBI4	40,68	14	4	381	42,8	7,58	378,33
P35842	PHO11	13,28	9	5	467	52,7	5,17	367,21
P40472	RPL10	16,81	11	4	476	48,2	4,60	359,16
P06169	PDC1	14,03	8	6	563	61,5	6,19	312,63
P00359	TDH3	22,89	9	6	332	35,7	6,96	306,08
P53616	SUN4	11,90	8	2	420	43,4	4,36	296,34
P02994	TEF1	8,73	10	4	458	50,0	9,04	271,49
B5VL27	CIS3	11,56	10	3	225	23,0	4,68	249,94
P00560	PGK1	21,15	10	8	416	44,7	7,61	246,64
P36110	PRY2	5,17	8	1	329	33,8	4,60	218,62
P00729	PRC1	12,97	8	6	532	59,8	4,73	185,14
P00549	PYK1	11,20	6	5	500	54,5	7,68	181,40
P26263	PDC6	5,33	4	2	563	61,5	6,19	170,63
P12709	PGI1	4,69	7	2	554	61,3	6,46	167,72
P10592	SSA2	8,29	4	3	639	69,4	5,06	136,17
Q05902	ECM38	7,58	5	3	660	73,1	5,88	129,25
P60010	ACT1	9,87	5	3	375	41,7	5,68	125,61
P16474	KAR2	4,69	5	3	682	74,4	4,93	124,36
P25296	CNB1	10,86	4	2	175	19,6	4,55	122,03
P22146	GAS1	9,12	4	3	559	59,5	4,67	108,20
P06367	RPS14A	24,82	4	3	137	14,5	10,73	99,61
P02365	RPS6A	13,56	2	2	236	27,0	10,45	96,75
P00330	ADH1	2,30	3	1	348	36,8	6,68	95,82
P17076	RPL8A	7,42	3	2	256	28,1	10,04	95,27
P11484	SSB1	5,22	3	3	613	66,6	5,44	93,39
P04456	RPL25	9,15	1	1	142	15,7	10,11	90,63
P38013	AHP1	11,36	1	1	176	19,1	5,16	89,71

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
A6ZL22	ECM33	3,50	3	1	429	43,7	4,91	87,41
P35271	RPS18A	7,53	4	1	146	17,0	10,27	86,76
P17079	RPL12A	14,55	3	2	165	17,8	9,41	79,28
P38836	ECM14	3,49	2	1	430	49,8	5,30	77,69
P32603	SPR1	2,25	3	1	445	51,8	5,81	75,35
Q12335	PST2	7,58	2	1	198	21,0	5,73	72,17
P05317	RPP0	3,53	2	1	312	33,7	4,83	69,26
P26783	RPS5	6,67	1	1	225	25,0	8,59	68,78
P29029	CTS1	1,78	2	1	562	59,0	4,55	67,79
Q08108	PLB3	1,75	2	1	686	75,0	5,03	65,86
P00950	GPM1	8,91	2	2	247	27,6	8,84	64,82
P07267	PEP4	2,22	2	1	405	44,5	4,84	61,50
P00635	PHO5	3,43	2	2	467	52,8	4,83	60,66
P38011	ASC1	3,76	2	1	319	34,8	6,24	60,28
P41805	RPL10	4,98	2	1	221	25,3	10,02	59,72
P04807	HXK2	3,50	1	1	486	53,9	5,30	57,22
P40213	RPS16A	8,39	1	1	143	15,8	10,26	56,08
P15108	HSC82	1,28	2	1	705	80,8	4,83	55,06
P25349	YCP4	3,24	2	1	247	26,3	8,19	55,05
P00175	CYB2	2,20	2	1	591	65,5	8,41	52,48
P19882	HSP60	2,10	1	1	572	60,7	5,31	51,84
P14904	LAP4	1,95	1	1	514	57,1	5,83	51,49
P25443	RPS2	4,33	2	1	254	27,4	10,43	48,96
P05750	RPS3	5,42	1	1	240	26,5	9,41	46,27
P47143	ADO1	3,53	1	1	340	36,3	5,16	45,08
P05739	RPL6B	8,52	1	1	176	20,0	10,08	42,34
P39931	SSP120	5,13	1	1	234	27,3	5,11	41,16
P22803	TRX2	11,54	1	1	104	11,2	4,93	38,63
P26786	RPS7A	7,37	1	1	190	21,6	9,83	37,46
P54115	ALD6	2,20	1	1	500	54,4	5,44	37,04
P26782	RPS24A	6,67	1	1	135	15,3	10,51	35,89
P05737	RPL7A	3,69	1	1	244	27,6	10,15	35,24
P0C2H6	RPL27A	10,29	1	1	136	15,5	10,36	33,09
P05753	RPS4A	3,83	1	1	261	29,4	10,08	32,65
P05743	RPL26A	6,30	1	1	127	14,2	10,58	31,51
P06168	ILV5	2,53	1	1	395	44,3	9,04	27,85
Q12074	SPE3	4,44	1	1	293	33,3	5,53	27,81
Q03558	OYE2	2,75	1	1	400	45,0	6,57	26,80
P28319	CWP1	4,60	1	1	239	24,3	4,67	25,30
P32589	SSE1	1,44	1	1	693	77,3	5,22	24,96
P48589	RPS12	13,29	1	1	143	15,5	4,73	20,36

Table S2. Proteins from *S. cerevisiae* W303-A extracellular matrix.

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P00924	ENO1	62,70	305	20	437	46,8	6,62	10412,91
P00925	ENO2	67,28	281	21	437	46,9	6,00	10025,98
P53334	SCW4	46,63	278	12	386	40,1	4,83	9452,71
P00359	TDH3	74,10	221	20	332	35,7	6,96	7415,04
P14540	FBA1	54,32	169	12	359	39,6	5,78	5530,25
P10591	SSA1	54,98	162	24	642	69,6	5,11	4923,62
P38013	AHP1	76,14	125	8	176	19,1	5,16	4717,40
P15703	BGL2	59,74	152	11	313	34,1	4,51	4558,72
P10592	SSA2	57,12	148	23	639	69,4	5,06	4239,94
P00560	PGK1	68,27	138	23	416	44,7	7,61	3638,17
P00360	TDH1	71,08	125	21	332	35,7	8,28	3610,70
P00549	PYK1	62,80	122	23	500	54,5	7,68	3226,01
P06169	PDC1	54,71	109	22	563	61,5	6,19	3021,53
P00358	TDH2	54,52	97	17	332	35,8	6,96	2885,88
P38288	TOS1	23,52	107	6	455	48,0	4,67	2811,27
P12709	PGI1	51,44	81	19	554	61,3	6,46	2746,63
P23776	EXG1	59,38	85	16	448	51,3	4,75	2615,98
P00942	TPI1	81,45	83	13	248	26,8	6,01	2371,82
P02829	HSP82	42,45	77	26	709	81,4	4,91	2186,28
P15992	HSP26	66,82	66	10	214	23,9	5,53	2181,80
P15108	HSC82	43,97	77	27	705	80,8	4,83	2175,96
Q04951	SCW10	43,44	67	12	389	40,4	4,65	2111,81
P31539	HSP104	41,63	74	30	908	102,0	5,45	1961,32
P09435	SSA3	50,39	72	23	649	70,5	5,17	1909,38
P53252	PIL1	51,03	41	12	339	38,3	4,63	1721,15
Q12230	LSP1	43,99	38	12	341	38,0	4,70	1511,81
P00830	ATP2	46,97	51	16	511	54,8	5,71	1502,95
P46367	ALD4	60,50	58	23	519	56,7	6,74	1494,66
P11484	SSB1	44,05	48	17	613	66,6	5,44	1447,13
A7A003	UTH1	26,24	37	6	362	36,7	4,79	1447,06
P00890	CIT1	41,96	51	15	479	53,3	8,29	1425,57
P42835	EGT2	13,74	39	8	1041	108,5	4,82	1359,54
P02994	TEF1	43,89	48	13	458	50,0	9,04	1358,73
P0CX10	ERR1	48,28	39	13	437	47,3	5,29	1346,42
P22202	SSA4	38,47	53	17	642	69,6	5,14	1344,40
P60010	ACT1	49,33	51	13	375	41,7	5,68	1328,07
P32324	EFT1	38,24	45	22	842	93,2	6,32	1313,92
P17967	PDI1	33,33	36	13	522	58,2	4,53	1311,36
P09624	LPD1	50,10	45	18	499	54,0	8,03	1308,86
P04806	HXK1	50,93	46	16	485	53,7	5,45	1307,31
P53912	YNL134C	51,86	47	11	376	41,1	6,21	1275,46
P40472	SIM1	23,11	35	7	476	48,2	4,60	1250,50

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P07257	QCR2	63,04	39	17	368	40,5	7,96	1172,81
P17505	MDH1	65,57	42	14	334	35,6	8,47	1155,69
Q04432	HSP31	62,03	39	10	237	25,7	5,50	1138,91
P00950	GPM1	65,99	45	12	247	27,6	8,84	1125,13
P34760	TSA1	72,45	44	10	196	21,6	5,14	1108,43
P26263	PDC6	34,64	35	15	563	61,5	6,19	1057,44
Q12363	WTM1	49,43	38	14	437	48,4	5,36	1045,15
P22803	TRX2	73,08	31	6	104	11,2	4,93	1037,86
P19882	HSP60	37,59	38	17	572	60,7	5,31	1036,47
P05694	MET6	33,77	39	18	767	85,8	6,47	1030,13
P39708	GDH3	41,36	33	14	457	49,6	5,47	969,03
P37012	PGM2	49,91	43	20	569	63,0	6,62	931,42
P29311	BMH1	50,94	36	11	267	30,1	4,88	928,41
Q12512	ZPS1	48,19	32	8	249	27,5	5,02	928,35
P48589	RPS12	28,67	23	3	143	15,5	4,73	841,07
P17709	GLK1	28,80	28	10	500	55,3	6,19	838,52
P34730	BMH2	49,82	35	11	273	31,0	4,88	824,90
P15019	TAL1	46,27	29	13	335	37,0	6,43	813,60
P07262	GDH1	33,70	25	12	454	49,5	5,69	803,97
P41921	GLR1	38,72	28	11	483	53,4	7,83	781,98
P09938	RNR2	34,34	24	11	399	46,1	5,25	779,80
P00330	ADH1	52,30	33	14	348	36,8	6,68	772,50
P15705	STI1	34,47	27	17	589	66,2	5,59	766,54
P38715	GRE3	44,04	25	11	327	37,1	7,08	755,48
P54114	ALD3	38,34	29	15	506	55,4	5,76	752,51
Q06494	YPR127W	53,33	24	13	345	38,6	5,99	742,34
Q07653	HBT1	19,22	21	12	1046	113,5	6,38	735,79
P17255	TFP1	18,39	26	15	1071	118,6	6,16	731,32
P07267	PEP4	28,64	23	8	405	44,5	4,84	719,05
A6ZRW6	MLS1	35,56	31	13	554	62,8	7,03	711,76
P04840	POR1	68,55	29	15	283	30,4	7,93	710,71
A6ZTT5	ADH4	36,39	25	9	382	41,1	6,28	705,25
P38720	GND1	31,08	26	11	489	53,5	6,64	705,12
P16474	KAR2	29,47	35	17	682	74,4	4,93	700,23
P35842	PHO11	24,41	21	9	467	52,7	5,17	697,79
P14904	LAP4	38,33	28	13	514	57,1	5,83	683,95
P16861	PFK1	22,19	29	17	987	107,9	6,39	652,65
P12398	SSC1	26,61	28	14	654	70,6	5,59	635,89
P53184	PNC1	38,43	19	5	216	25,0	6,27	634,16
P37291	SHM2	25,80	24	11	469	52,2	7,43	627,93
Q03558	OYE2	27,75	23	9	400	45,0	6,57	626,75
P22146	GAS1	15,74	20	7	559	59,5	4,67	626,29
Q01574	ACS1	22,30	22	12	713	79,1	6,62	619,30

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P38804	RTC3	52,25	18	5	111	12,0	5,07	618,00
P53228	NQM1	52,25	29	13	333	37,2	6,38	617,89
P00331	ADH2	48,56	25	12	348	36,7	6,74	607,44
Q12408	NPC2	25,43	35	4	173	19,1	4,44	604,85
P49723	RNR4	46,96	26	13	345	40,0	5,21	600,62
P54070	KTR6	33,63	23	11	446	52,1	5,50	597,27
Q08108	PLB3	18,51	25	10	686	75,0	5,03	595,45
P53319	GND2	28,05	20	10	492	53,9	7,30	586,24
P10963	PCK1	21,49	20	8	549	60,9	6,34	577,12
P07251	ATP1	29,36	24	12	545	58,6	9,04	574,18
P46955	NCA3	16,32	15	5	337	35,4	4,46	572,71
P53753	DSE4	8,77	19	7	1117	121,0	4,53	553,08
A6ZZG1	PIR3	49,16	15	5	415	41,5	5,74	549,97
P47771	ALD2	32,81	23	13	506	55,2	5,60	547,47
P26783	RPS5	28,89	13	4	225	25,0	8,59	541,24
P00817	IPP1	43,90	18	10	287	32,3	5,58	534,30
P32590	SSE2	25,25	22	15	693	77,6	5,63	530,84
P36139	PET10	18,02	14	4	283	31,2	8,16	526,91
P32589	SSE1	21,50	25	13	693	77,3	5,22	520,73
P16120	THR4	32,49	21	12	514	57,4	5,64	513,22
P46655	GUS1	22,03	21	12	708	80,8	7,53	511,48
P16140	VMA2	27,85	19	11	517	57,7	5,07	509,36
P19414	ACO1	14,52	22	9	778	85,3	8,07	503,98
P28240	ICL2	29,80	20	11	557	62,4	6,42	503,26
Q07551	YDL124W	37,18	18	9	312	35,5	6,19	497,66
P40185	MMF1	74,48	18	7	145	15,9	9,28	496,98
P09620	KEX1	16,87	17	9	729	82,2	4,56	496,62
P32316	ACH1	30,80	21	11	526	58,7	6,79	495,58
P23301	HYP2	30,57	15	4	157	17,1	4,96	489,82
A6ZQJ1	TIF1	31,65	14	9	395	44,7	5,12	489,55
P23285	CPR2	47,32	21	7	205	22,8	6,13	488,74
P16467	PDC5	11,72	15	5	563	61,9	6,43	486,58
P22217	TRX1	58,25	13	5	103	11,2	4,93	479,78
Q03048	COF1	38,46	15	4	143	15,9	5,20	479,09
P00445	SOD1	59,09	17	6	154	15,8	6,00	463,38
Q08911	FDH1	30,59	16	9	376	41,7	6,47	461,21
P04807	HXK2	23,05	16	7	486	53,9	5,30	456,13
P33315	TKL2	19,97	17	9	681	75,0	6,14	453,75
P04802	DPS1	24,78	17	11	557	63,5	6,58	453,19
Q08969	GRE1	29,17	11	3	168	19,0	4,77	452,89
P16547	OM45	30,03	17	10	393	44,6	8,59	450,11
P25694	CDC48	10,30	17	6	835	91,9	4,94	448,03
Q00055	GPD1	26,09	15	7	391	42,8	5,47	441,80

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P14065	GCY1	45,19	21	10	312	35,1	7,99	440,96
P00729	PRC1	27,63	23	12	532	59,8	4,73	438,89
P32454	APE2	12,61	18	10	952	107,7	8,03	430,14
P27809	KRE1	31,00	21	9	442	51,4	5,40	428,03
P14832	CPR1	38,89	19	5	162	17,4	7,44	426,51
Q03104	MSC1	22,22	13	9	513	59,6	7,58	424,54
P06168	ILV5	32,66	18	10	395	44,3	9,04	412,07
P28834	IDH1	41,94	18	11	360	39,3	9,00	409,55
Q12068	GRE2	17,25	15	5	342	38,1	6,15	408,21
P33416	HSP78	10,85	14	7	811	91,3	8,05	407,45
P05750	RPS3	37,50	16	7	240	26,5	9,41	400,69
P41939	IDP2	30,34	15	11	412	46,5	6,19	396,76
P29509	TRR1	43,57	12	7	319	34,2	5,94	393,07
P0CG63	UBI4	56,43	15	4	381	42,8	7,58	391,84
P24031	PHO3	16,70	15	6	467	52,7	4,63	390,77
P40531	GVP36	42,33	13	9	326	36,6	4,97	376,66
P07991	CAR2	34,91	13	10	424	46,1	6,95	374,58
P32861	UGP1	12,42	12	5	499	56,0	7,44	371,58
P36110	PRY2	9,73	12	2	329	33,8	4,60	370,91
P41338	ERG10	21,36	13	7	398	41,7	7,39	368,55
P38616	YGP1	16,67	18	5	354	37,3	5,44	364,86
P36008	TEF4	12,62	10	4	412	46,5	7,87	360,06
P41816	OYE3	27,00	19	10	400	44,9	5,60	358,74
P11076	ARF1	40,33	13	6	181	20,5	7,34	347,77
P49090	ASN2	17,66	13	8	572	64,6	5,87	342,19
P49089	ASN1	19,76	16	9	572	64,4	6,11	336,09
Q04792	GAD1	18,80	12	8	585	65,9	6,62	335,22
Q00764	TPS1	14,14	11	5	495	56,1	6,09	334,82
P00447	SOD2	57,94	14	7	233	25,8	8,48	331,07
P39931	SSP120	32,05	12	6	234	27,3	5,11	327,46
P28241	IDH2	36,59	17	9	369	39,7	8,69	326,93
P06115	CTT1	22,95	18	10	562	64,5	6,54	326,62
Q04409	EMI2	16,80	13	6	500	55,9	6,27	326,55
Q12303	YPS3	21,06	12	6	508	54,5	8,69	325,29
A6ZV70	CTT1	23,84	19	10	562	64,5	6,54	322,71
P29547	CAM1	23,61	15	7	415	47,1	8,38	321,18
A6ZZH2	MCR1	24,83	16	6	302	34,1	8,65	320,54
P08524	ERG20	23,01	10	6	352	40,5	5,47	318,52
P29029	CTS1	5,87	11	3	562	59,0	4,55	309,27
P34227	PRX1	31,03	12	7	261	29,5	8,87	307,56
P40165	YNL200C	31,30	10	5	246	27,5	8,29	303,65
P38115	ARA1	38,08	11	9	344	38,9	5,96	302,18
P04076	ARG4	19,22	13	8	463	52,0	5,73	301,47

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P00724	SUC2	12,78	12	6	532	60,6	4,75	300,83
B5VL27	CIS3	11,56	13	3	225	23,0	4,68	299,74
P11412	ZWF1	25,35	16	10	505	57,5	6,30	299,07
O74302	YDR261C-C	27,50	13	7	440	49,0	7,62	298,33
Q12335	PST2	39,90	12	4	198	21,0	5,73	292,72
P07256	COR1	18,82	9	6	457	50,2	7,30	291,42
P06106	MET17	17,57	13	7	444	48,6	6,43	289,93
P25294	SIS1	30,40	12	8	352	37,6	9,03	286,13
Q05911	ADE13	19,29	14	7	482	54,5	6,43	284,67
P53978	HEF3	2,59	8	2	1044	115,8	6,23	283,79
P32582	CYS4	25,05	12	9	507	56,0	6,70	277,14
P38011	ASC1	20,06	10	5	319	34,8	6,24	275,17
P26321	RPL5	30,98	8	5	297	33,7	6,83	274,62
P25375	PRD1	7,58	7	4	712	81,9	5,80	271,68
P38069	MNN2	11,56	7	5	597	67,7	6,27	268,61
P31116	HOM6	32,87	10	7	359	38,5	7,44	267,14
P07274	PFY1	34,13	11	3	126	13,7	5,80	264,90
P05317	RPP0	19,55	8	4	312	33,7	4,83	264,38
P22515	UBA1	15,82	14	11	1024	114,2	5,11	262,06
P35691	TMA19	20,96	7	3	167	18,7	4,56	261,67
P28319	CWP1	29,29	11	5	239	24,3	4,67	260,42
Q04304	YMR090W	44,93	11	7	227	24,9	5,80	258,69
P06738	GPH1	10,53	13	7	902	103,2	5,62	256,97
P10659	SAM1	25,39	9	7	382	41,8	5,22	255,29
P32327	PYC2	6,44	9	5	1180	130,1	6,51	254,64
P41940	MPG1	21,88	12	5	361	39,5	6,34	253,44
Q12428	PDH1	20,35	9	7	516	57,6	9,07	252,83
P41277	GPP1	34,40	9	6	250	27,9	5,55	252,39
P39954	SAH1	17,37	9	6	449	49,1	6,24	250,75
P13663	HOM2	21,92	12	6	365	39,5	6,73	248,81
P00635	PHO5	20,56	16	7	467	52,8	4,83	244,82
Q05016	YMR226C	16,48	8	3	267	29,1	6,81	243,31
P22943	HSP12	34,86	7	3	109	11,7	5,38	242,78
B3LT19	RPS0B	39,68	12	7	252	27,9	4,75	242,17
P18239	AAC2	21,38	7	5	318	34,4	9,79	240,22
A6ZLF4	SDS24	11,95	8	4	527	57,2	8,91	239,92
P00044	CYC1	32,11	10	3	109	12,2	9,42	239,70
P54838	DAK1	20,38	12	8	584	62,2	5,41	239,42
P53616	SUN4	19,05	7	3	420	43,4	4,36	234,73
P53301	CRH1	10,65	9	5	507	52,7	4,65	226,54
P25349	YCP4	23,89	7	3	247	26,3	8,19	225,21
P26637	CDC60	8,07	10	7	1090	124,1	5,85	224,95
P23254	TKL1	16,32	10	7	680	73,8	7,01	222,95

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P48016	ATH1	5,95	11	6	1211	136,8	5,43	222,28
P38230	ZTA1	18,26	7	5	334	37,0	8,73	221,44
P43616	DUG1	4,16	3	1	481	52,8	5,67	220,53
P22203	VMA4	27,47	8	5	233	26,5	5,36	220,24
Q08193	GAS5	13,22	10	5	484	51,8	4,64	220,01
P07284	SES1	13,85	7	4	462	53,3	6,09	219,72
P02365	RPS6A	13,56	6	2	236	27,0	10,45	217,08
P54115	ALD6	20,60	10	8	500	54,4	5,44	216,77
P21954	IDP1	16,12	7	6	428	48,2	8,76	214,82
P38009	ADE17	21,11	10	9	592	65,2	6,55	210,95
P36010	YNK1	47,71	12	6	153	17,2	8,60	209,47
Q12331	YDR262W	23,90	8	5	272	30,6	5,41	206,92
P40510	SER33	13,22	8	5	469	51,2	6,42	206,92
A6ZRK4	LAP3	21,12	9	7	483	55,5	8,75	206,62
P40893	REE1	24,75	6	4	198	22,0	6,39	206,56
P38067	UGA2	9,26	5	2	497	54,2	6,65	206,32
P47143	ADO1	26,18	10	5	340	36,3	5,16	205,11
P35271	RPS18A	26,71	7	4	146	17,0	10,27	203,76
P38836	ECM14	16,98	10	5	430	49,8	5,30	203,46
P54113	ADE16	15,74	8	7	591	65,2	6,55	203,34
Q3E841	YNR034W-A	40,82	4	3	98	10,8	8,97	198,78
P36046	MIA40	3,97	3	1	403	44,5	4,56	196,37
P19358	SAM2	18,49	8	6	384	42,2	5,38	194,45
P00431	CCP1	15,51	5	4	361	40,3	6,38	192,06
A6ZRW8	SCY_4679	5,59	4	3	644	74,1	6,71	191,60
P43635	CIT3	10,70	9	4	486	53,8	8,59	189,36
P12695	PDA2	13,90	8	5	482	51,8	7,80	186,95
P25296	CNB1	6,86	5	1	175	19,6	4,55	186,03
P23638	PRE9	13,57	7	3	258	28,7	5,22	184,82
P05756	RPS13	15,89	5	2	151	17,0	10,43	184,31
P26786	RPS7A	26,32	5	3	190	21,6	9,83	181,99
Q05902	ECM38	10,15	7	5	660	73,1	5,88	179,66
P17695	GRX2	25,17	7	3	143	15,9	7,28	179,47
P04147	PAB1	9,19	5	4	577	64,3	5,97	178,46
P38764	RPN1	7,05	7	6	993	109,4	4,63	178,15
P39721	AIM29	12,60	8	2	246	27,1	6,28	175,91
P23542	AAT2	18,42	7	6	418	46,0	8,32	174,73
P23641	MIR1	12,22	4	3	311	32,8	9,31	167,64
P0C0W1	RPS22A	12,31	3	1	130	14,6	9,94	166,74
A6ZYI0	ADK1	28,38	8	5	222	24,2	6,70	166,38
P39676	YHB1	7,27	4	2	399	44,6	6,28	164,30
P07244	ADE5,7	5,74	5	3	802	86,0	5,27	162,19
P06208	LEU4	9,53	6	5	619	68,4	6,01	161,78

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P38131	KTR4	12,93	9	5	464	54,5	4,84	156,58
P38891	BAT1	17,30	8	5	393	43,6	8,91	156,12
P19262	KGD2	12,10	7	5	463	50,4	8,85	156,01
P31373	CYS3	17,26	6	4	394	42,5	6,54	155,77
P25373	GRX1	39,09	7	3	110	12,4	5,06	153,93
P39101	CAJ1	6,91	4	2	391	44,8	5,80	153,05
P40213	RPS16A	18,88	4	2	143	15,8	10,26	153,01
P35719	MRP8	14,61	4	3	219	25,1	4,79	152,27
P13130	SPS100	13,50	7	2	326	34,2	5,41	151,82
P32471	EFB1	39,81	8	5	206	22,6	4,45	151,68
P53598	LSC1	20,06	7	4	329	35,0	8,46	150,91
P47176	BAT2	13,56	8	4	376	41,6	7,30	150,06
P40582	GTT1	18,80	5	4	234	26,8	6,65	149,00
P54839	ERG13	7,33	4	3	491	55,0	8,16	148,87
P32445	RIM1	34,81	5	4	135	15,4	8,34	148,63
Q04902	SNO4	17,30	6	3	237	26,0	8,07	148,10
P32614	FRD1	7,87	5	3	470	50,8	6,33	141,91
P07143	CYT1	9,39	5	2	309	34,0	8,12	141,30
P17079	RPL12A	21,82	5	3	165	17,8	9,41	141,16
P38707	DED81	4,69	3	2	554	62,2	5,85	141,13
P38841	YHR138C	45,61	7	4	114	12,7	5,06	140,63
Q06151	DCS1	7,71	4	2	350	40,7	6,25	140,57
P16862	PFK2	7,61	7	6	959	104,6	6,67	140,36
P32775	GLC3	9,38	8	7	704	81,1	6,16	138,63
P14306	TFS1	21,00	5	3	219	24,3	6,54	138,59
P04801	THS1	4,09	6	3	734	84,5	7,03	138,39
P38788	SSZ1	16,91	7	6	538	58,2	5,05	137,87
P07245	ADE3	8,14	7	6	946	102,1	6,84	137,68
P41805	RPL10	18,10	3	2	221	25,3	10,02	137,31
P17555	SRV2	7,98	5	3	526	57,5	5,64	136,50
P43590	YFR006W	9,35	4	3	535	61,7	6,16	135,24
Q12449	AHA1	19,43	5	5	350	39,4	7,47	132,92
P08417	FUM1	7,79	5	3	488	53,1	8,25	132,62
Q06703	CDA2	9,29	4	2	312	35,7	5,38	132,37
P17649	UGA1	16,14	6	5	471	52,9	6,80	132,13
P40054	SER3	10,87	5	4	469	51,2	5,57	131,38
P32835	GSP1	21,92	6	4	219	24,8	6,55	130,52
P02557	TUB2	10,94	6	3	457	50,9	4,75	130,06
P33297	RPT5	9,68	4	3	434	48,2	5,06	129,91
P25293	NAP1	19,42	9	5	417	47,9	4,34	129,00
P43593	UBP6	6,81	4	3	499	57,1	7,14	128,03
P09232	PRB1	8,50	7	5	635	69,6	6,39	127,62
Q08971	YPL225W	15,07	3	2	146	17,4	5,30	127,29

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P22133	MDH2	19,89	6	4	377	40,7	6,90	127,18
A6ZRM0	ADE12	17,55	8	7	433	48,2	8,35	127,03
P40513	MAM33	13,91	3	2	266	30,1	4,58	125,85
P0C0W9	RPL11A	12,64	3	2	174	19,7	9,92	125,79
P07283	SEC53	36,22	10	9	254	29,0	5,24	124,79
P19097	FAS2	2,81	5	3	1887	206,8	5,44	124,45
P25659	FUB1	9,60	3	2	250	26,7	5,02	123,24
Q04947	RTN1	5,76	2	1	295	32,9	8,98	122,55
A6ZY20	PST1	10,59	4	3	444	45,8	9,19	121,74
P07263	HTS1	4,40	5	2	546	59,9	7,71	121,47
Q03161	YMR099C	20,20	6	5	297	33,9	6,13	120,57
P38701	RPS20	31,40	4	4	121	13,9	9,52	120,28
P00498	HIS1	13,47	5	3	297	32,2	6,15	119,54
P32191	GUT2	5,70	8	4	649	72,3	7,90	119,07
P39005	KRE9	13,77	5	2	276	30,0	9,20	118,80
Q12458	YPR1	12,50	6	3	312	34,7	7,12	116,37
P53090	ARO8	7,00	5	3	500	56,1	6,01	115,47
Q03655	GAS3	6,49	4	3	524	56,8	4,79	114,93
P07278	BCY1	12,02	5	4	416	47,2	7,94	114,28
P07806	VAS1	3,89	5	4	1104	125,7	6,96	114,03
P22768	ARG1	10,95	7	4	420	46,9	5,62	113,93
A6ZS33	SCY_4744	3,87	2	2	671	77,3	6,58	113,38
P38137	PCS60	9,76	6	4	543	60,5	9,20	112,50
Q12118	SGT2	8,96	4	2	346	37,2	4,79	110,74
P36105	RPL14A	13,77	4	2	138	15,2	10,93	110,58
P13517	CAP2	12,54	4	3	287	32,6	4,72	109,79
P25491	YDJ1	14,43	5	4	409	44,6	6,30	109,27
P21242	PRE10	8,68	4	2	288	31,5	5,19	108,95
P14120	RPL30	29,52	6	2	105	11,4	9,80	108,50
P10664	RPL4A	12,43	4	3	362	39,1	10,64	108,48
Q04336	YMR196W	5,06	4	4	1088	126,5	5,45	107,46
P40302	PRE5	23,50	5	4	234	25,6	7,39	106,88
Q05506	YDR341C	7,74	6	5	607	69,5	6,79	106,55
P20967	KGD1	5,92	5	4	1014	114,3	7,21	105,73
Q12447	PAA1	11,52	5	2	191	21,9	5,82	105,71
P48015	GCV1	5,25	4	2	400	44,4	8,84	105,43
P40495	LYS12	14,56	4	4	371	40,0	8,02	105,38
P01120	RAS2	13,04	3	3	322	34,7	7,27	104,64
P10869	HOM3	5,69	5	3	527	58,1	6,67	104,10
P07560	SEC4	27,44	6	4	215	23,5	7,09	103,48
P33330	SER1	5,82	3	2	395	43,4	6,54	102,13
P38624	PRE3	18,60	5	4	215	23,5	5,90	101,68
P21243	SCL1	13,49	3	3	252	28,0	6,24	100,67

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P41911	GPD2	5,23	3	2	440	49,4	7,09	100,35
P05737	RPL7A	15,98	4	3	244	27,6	10,15	99,90
P38077	ATP3	19,29	6	3	311	34,3	9,31	99,81
P06780	RHO1	16,27	5	3	209	23,1	6,23	99,26
P07275	PUT2	5,22	4	2	575	64,4	7,01	99,10
P32603	SPR1	2,25	3	1	445	51,8	5,81	98,93
P13298	URA5	16,81	5	3	226	24,6	6,05	98,33
P17076	RPL8A	8,20	3	2	256	28,1	10,04	97,98
P29952	PMI40	4,90	2	1	429	48,2	5,99	97,91
P32366	VMA6	8,41	3	2	345	39,8	4,60	97,53
A6ZL22	ECM33	5,83	4	2	429	43,7	4,91	94,70
Q03940	RVB1	4,75	4	2	463	50,4	5,87	93,97
P16550	APA1	8,72	3	2	321	36,5	5,02	92,60
P05744	RPL33A	31,78	5	3	107	12,1	11,08	92,17
B3LRI4	RGI1	19,25	3	2	161	19,0	6,18	92,14
P06367	RPS14A	17,52	4	2	137	14,5	10,73	92,14
Q12123	DCS2	12,46	5	4	353	40,9	6,64	92,00
P07280	RPS19A	6,25	2	1	144	15,9	9,61	91,82
P04456	RPL25	9,15	1	1	142	15,7	10,11	91,59
P53312	LSC2	10,54	5	4	427	46,9	7,47	91,44
P05626	ATP4	12,30	3	2	244	26,9	9,13	91,03
P39990	SNU13	19,05	2	1	126	13,6	7,85	90,27
Q12122	LYS21	10,00	4	4	440	48,6	6,30	89,71
Q01939	RPT6	6,17	3	2	405	45,2	9,01	89,64
P08067	RIP1	9,77	3	2	215	23,3	8,07	89,19
P40581	HYR1	7,98	2	1	163	18,6	8,19	89,19
P22855	AMS1	2,12	2	2	1083	124,4	7,25	88,98
P21576	VPS1	1,70	2	1	704	78,7	7,91	88,82
P52290	DIA3	7,05	4	3	468	53,0	5,53	88,23
P24280	SEC14	10,86	4	3	304	34,9	5,49	87,86
P32419	MDH3	11,66	3	2	343	37,2	9,20	87,57
P35189	TAF12	6,56	2	1	244	27,4	5,29	87,54
Q99258	RIB3	11,06	4	2	208	22,6	5,78	86,38
P0C2I0	RPL20A	18,02	5	3	172	20,4	10,30	86,15
P53189	SCW11	8,30	3	3	542	56,4	4,48	85,21
P36059	YKL151C	15,13	6	4	337	37,3	7,55	84,99
P53691	CPR6	7,82	4	2	371	42,0	6,16	84,63
P46672	ARC1	22,07	6	5	376	42,1	7,88	84,63
P40037	HMF1	30,23	4	3	129	13,9	5,41	84,61
P54837	ERV25	7,58	1	1	211	24,1	5,52	84,39
P33327	GDH2	2,01	3	2	1092	124,3	5,73	84,31
P53130	GPG1	21,43	3	2	126	14,9	4,75	82,93
P32381	ARP2	4,09	5	2	391	44,0	5,78	82,55

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P30656	PRE2	9,41	3	2	287	31,6	6,23	82,21
P05755	RPS9B	15,90	4	4	195	22,3	10,10	82,20
P00931	TRP5	3,68	3	2	707	76,6	6,51	82,08
P38910	HSP10	19,81	3	2	106	11,4	9,00	81,48
P00045	CYC7	19,47	5	2	113	12,5	9,58	81,13
P23724	PRE7	13,28	3	3	241	26,9	6,07	79,59
P00175	CYB2	7,78	4	2	591	65,5	8,41	77,88
P25372	TRX3	14,96	3	2	127	14,4	8,90	77,58
P40168	YNL195C	12,64	3	3	261	28,3	6,46	77,30
A6ZWD3	DBP1	4,21	3	2	617	67,9	8,62	77,08
P25443	RPS2	10,24	6	2	254	27,4	10,43	76,60
P53622	COP1	1,67	3	1	1201	135,5	5,99	76,50
P05739	RPL6B	8,52	4	1	176	20,0	10,08	75,60
Q12377	RPN6	5,30	2	2	434	49,7	6,28	75,44
P20081	FPR1	30,70	2	2	114	12,2	6,04	75,21
P20459	SUI2	8,88	3	2	304	34,7	5,02	75,03
P40029	MXR1	10,87	3	2	184	21,1	6,99	74,43
P15625	FRS2	12,92	3	3	503	57,5	5,78	74,06
P47096	BNA1	5,65	3	1	177	20,2	5,78	73,69
P25572	YCL042W	7,56	3	1	119	13,4	10,56	73,06
P32598	GLC7	6,73	4	2	312	35,9	5,49	72,56
P26782	RPS24A	14,81	3	2	135	15,3	10,51	72,40
P40471	AYR1	5,39	1	1	297	32,8	9,16	71,56
Q03102	YML131W	4,66	1	1	365	40,0	8,13	71,37
P47137	YJR096W	17,38	5	3	282	32,3	6,93	71,01
P32599	SAC6	10,44	5	5	642	71,7	5,48	70,97
P33299	RPT1	2,78	1	1	467	52,0	5,47	70,55
P14843	ARO3	3,51	2	1	370	41,0	7,39	70,54
P15873	POL30	7,75	3	2	258	28,9	4,59	70,05
P32449	ARO4	10,27	3	3	370	39,7	6,95	69,42
Q12513	TMA17	7,33	2	1	150	16,8	4,73	68,46
P40509	SEC28	7,43	1	1	296	33,8	4,55	68,06
Q06263	VTA1	9,70	3	2	330	37,3	4,63	67,73
Q96VH4	HBN1	13,99	2	1	193	21,0	6,95	67,69
P15624	FRS1	3,19	4	2	595	67,3	5,78	67,48
P01095	PBI2	16,00	2	1	75	8,6	6,80	66,91
P36421	TYS1	3,81	1	1	394	44,0	8,44	66,70
A6ZXP4	SUB2	3,36	2	1	446	50,2	5,52	66,29
P25087	ERG6	4,44	1	1	383	43,4	5,77	65,89
P14747	CMP2	4,64	3	2	604	68,5	6,35	65,52
A6ZPE5	NOP58	3,33	2	1	511	56,9	8,94	65,01
Q12177	YLL056C	4,36	1	1	298	32,1	7,15	63,35
P46784	RPS10B	13,33	3	1	105	12,7	9,07	63,30

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P27472	GSY2	1,13	2	1	705	80,0	6,35	63,00
Q03148	SNZ1	9,76	3	3	297	31,8	5,58	62,97
Q02046	MTD1	5,94	3	2	320	36,2	6,99	62,34
P32642	GRX4	3,28	2	1	244	27,5	4,65	62,31
P05736	RPL2A	19,29	3	3	254	27,4	11,11	62,22
P06105	SCP160	1,39	3	1	1222	134,7	5,85	62,07
P38934	BFR1	5,74	2	2	470	54,6	9,19	62,00
A6ZRR2	MDG1	7,38	2	2	366	40,3	5,80	61,77
Q12434	RDI1	8,91	1	1	202	23,1	6,04	61,67
P39958	GDI1	10,20	4	4	451	51,2	5,99	61,65
P32499	NUP2	2,36	1	1	720	77,8	7,20	61,20
P38219	OLA1	8,63	4	3	394	44,1	7,43	61,04
P05753	RPS4A	10,34	4	3	261	29,4	10,08	60,80
A6ZPQ6	MPM1	17,06	3	3	252	28,5	5,92	60,60
P42936	YGR201C	5,78	1	1	225	26,3	5,55	60,58
P32473	PDB1	12,84	3	3	366	40,0	5,30	60,18
P11491	PHO8	2,65	2	1	566	63,0	5,59	60,08
P08019	SGA1	2,00	1	1	549	61,4	5,07	59,83
P38886	RPN10	7,84	2	1	268	29,7	4,82	59,64
Q07505	YDL086W	4,76	1	1	273	30,8	6,34	59,36
P32379	PUP2	9,23	2	2	260	28,6	4,73	59,27
P33298	RPT3	7,24	2	2	428	47,9	5,53	58,41
Q06142	KAP95	1,74	1	1	861	94,7	4,64	58,32
P18961	YPK2	1,33	3	1	677	76,6	7,65	58,31
P38427	TSL1	2,82	2	2	1098	122,9	6,64	57,78
P47117	ARP3	4,90	2	2	449	49,5	5,80	57,57
Q12464	RVB2	4,46	4	2	471	51,6	5,31	57,46
P30624	FAA1	3,29	4	2	700	77,8	7,62	57,45
P05030	PMA1	2,29	2	2	918	99,6	5,11	56,92
P38081	YBR056W	2,40	1	1	501	57,8	6,46	56,49
P38972	ADE6	1,18	2	1	1358	148,8	5,27	56,43
P49435	APT1	12,83	2	1	187	20,6	5,10	56,41
Q12460	NOP56	3,97	2	2	504	56,8	8,90	56,31
P38129	TAF5	3,26	2	1	798	88,9	7,40	56,18
P13134	KEX2	1,23	3	1	814	89,9	4,98	55,87
P47068	BBC1	0,95	2	1	1157	128,2	5,26	55,03
P38911	FPR3	2,68	2	1	411	46,5	4,46	54,59
P01123	YPT1	10,68	2	2	206	23,2	5,33	54,41
P16521	YEF3	4,31	5	3	1044	115,9	6,05	54,27
Q04458	HFD1	4,32	2	2	532	59,9	6,76	53,17
P10080	SBP1	3,74	2	1	294	33,0	5,66	53,16
P31383	TPD3	3,78	1	1	635	70,9	4,72	52,66
P50107	GLO1	3,07	1	1	326	37,2	6,84	52,51

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P0C0V8	RPS21A	26,44	3	2	87	9,7	6,06	52,38
P30402	URA10	7,93	3	2	227	24,8	8,51	52,28
P19454	CKA2	4,42	2	1	339	39,4	8,53	51,94
B3LLZ8	SPG4	20,87	2	2	115	13,2	6,80	51,89
P43639	CKB1	3,60	1	1	278	32,2	4,61	51,65
P16387	PDA1	2,62	2	1	420	46,3	8,10	51,63
Q06651	PIB1	6,64	2	1	286	32,7	5,88	51,59
P23639	PRE8	11,20	3	2	250	27,1	5,72	51,27
P34251	YKL107W	9,39	2	2	309	34,5	6,58	51,17
Q12277	RRP42	5,66	1	1	265	29,0	5,45	49,59
Q04491	SEC13	14,81	3	3	297	33,0	5,73	49,51
P54885	PRO2	3,51	2	1	456	49,7	5,59	49,32
A6ZX97	AIM6	3,59	1	1	390	44,4	5,59	49,29
Q08723	RPN8	3,55	1	1	338	38,3	5,64	49,10
P36047	SDS22	15,98	3	3	338	38,9	5,52	49,07
P10622	RPP1B	15,09	1	1	106	10,7	4,01	49,04
Q02753	RPL21A	11,25	2	2	160	18,2	10,39	48,91
P46992	YJL171C	4,29	2	1	396	42,9	5,06	48,64
P22141	PRE1	12,63	2	2	198	22,5	6,23	48,48
P38235	YBR053C	3,35	2	1	358	40,3	5,06	48,37
P22137	CHC1	2,18	2	2	1653	187,1	5,24	48,05
P15303	SEC23	1,30	1	1	768	85,3	5,66	47,87
P50095	IMD3	5,93	2	2	523	56,5	7,40	47,83
P09201	FBP1	9,48	2	2	348	38,2	6,01	47,82
P43588	RPN11	7,52	3	2	306	34,4	6,19	47,64
Q01560	NPL3	5,31	1	1	414	45,4	5,54	47,63
P37302	APE3	7,45	3	3	537	60,1	5,31	46,96
P04449	RPL24A	5,16	2	1	155	17,6	11,28	46,33
A6ZT99	EGD2	15,52	2	2	174	18,7	4,94	46,31
Q06624	AOS1	7,20	1	1	347	39,2	5,12	46,17
P38755	OSH7	1,83	2	1	437	49,8	7,23	45,84
Q06336	GGA1	1,97	1	1	557	62,3	5,62	45,57
P02407	RPS17A	8,82	1	1	136	15,8	10,51	45,56
P47037	SMC3	0,89	2	1	1230	141,2	5,81	45,06
P47160	ENT3	2,45	2	1	408	45,1	4,84	45,05
P38088	GRS1	3,77	2	2	690	78,1	6,52	44,67
P31412	VMA5	8,67	3	3	392	44,2	6,67	44,48
P09457	ATP5	5,19	2	1	212	22,8	9,57	44,37
P02293	HBT1	11,45	1	1	131	14,2	10,10	44,27
P11745	RNA1	1,97	2	1	407	45,8	4,61	43,84
P32340	NDI1	2,14	1	1	513	57,2	9,44	43,63
P48240	MTR3	5,60	2	1	250	27,6	4,98	43,52
P16451	PDX1	2,68	2	1	410	45,3	5,73	43,46

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P40075	SCS2	5,74	1	1	244	26,9	4,89	42,99
P38892	CRG1	3,44	2	1	291	33,8	6,00	42,98
P02309	HHF1	17,48	2	2	103	11,4	11,36	42,91
P20606	SAR1	5,79	2	1	190	21,4	5,38	42,45
P40009	YND1	3,02	1	1	630	71,8	6,24	42,06
P0C0X0	RPS28B	17,91	2	1	67	7,6	10,78	42,04
P53860	PDR1	7,69	2	2	351	40,7	7,96	41,83
P25619	HSP30	3,01	1	1	332	37,0	5,21	41,71
P32074	SEC21	2,35	1	1	935	104,8	5,12	41,33
P27616	ADE1	3,92	1	1	306	34,6	5,95	41,13
P48837	NUP57	2,22	1	1	541	57,5	9,58	40,93
Q04062	RPN9	3,05	2	1	393	45,8	5,78	40,74
P17423	THR1	5,04	2	2	357	38,7	5,41	40,65
B3RHI0	HRI1	4,92	1	1	244	27,5	5,21	40,07
P40215	NDE1	1,61	1	1	560	62,7	9,28	39,72
P36156	ECM4	6,49	1	1	370	43,2	6,89	39,22
P32527	ZUO1	5,77	2	2	433	49,0	8,25	39,07
P38071	ETR1	2,63	3	1	380	42,0	9,00	38,91
P36017	VPS21	10,48	2	2	210	23,1	5,33	38,90
P43555	EMP47	2,70	1	1	445	50,3	5,97	38,69
P53303	ZPR1	2,67	1	1	486	55,0	4,86	38,48
Q12207	NCE102	5,78	1	1	173	19,0	9,39	38,37
P42884	AAD14	2,39	2	1	376	42,0	6,74	38,35
A6ZQF6	ATG27	4,43	1	1	271	30,2	5,77	38,32
Q08977	YPL260W	2,54	1	1	551	62,7	5,12	38,19
P03962	URA3	4,49	1	1	267	29,2	7,36	37,62
A6ZWL1	EGD1	19,11	1	1	157	17,0	6,55	37,41
P28777	ARO2	8,24	2	2	376	40,8	7,80	37,30
P27466	CMK1	2,24	1	1	446	50,3	6,11	37,29
P0C2H6	RPL27A	10,29	1	1	136	15,5	10,36	37,05
P32911	SUI1	13,89	1	1	108	12,3	7,97	36,71
P08536	MET3	4,70	1	1	511	57,7	5,82	36,67
Q06505	SPN1	2,93	2	1	410	46,1	7,52	36,14
P53731	ARC35	5,26	2	2	342	39,5	6,81	35,89
A6ZLG8	OM14	19,40	1	1	134	14,6	8,75	35,62
P02406	RPL28	16,11	3	2	149	16,7	10,62	35,38
Q02933	RNY1	6,45	3	2	434	50,1	8,05	35,22
P36521	MRPL11	4,82	1	1	249	28,5	9,70	35,21
P38114	TBS1	0,64	2	1	1094	126,8	5,43	35,18
P07342	ILV2	1,02	2	1	687	74,9	8,51	35,14
Q12250	RPN5	2,47	1	1	445	51,7	6,13	34,52
P25605	ILV6	6,80	1	1	309	34,0	6,52	34,41
P35997	RPS27A	19,51	1	1	82	8,9	9,14	34,24

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P34167	TIF3	1,83	2	1	436	48,5	5,29	34,17
P32377	MVD1	3,28	2	1	396	44,1	5,76	34,12
P05743	RPL26A	12,60	2	2	127	14,2	10,58	33,68
P38109	YBR139W	3,35	1	1	508	57,6	5,31	33,67
Q06205	FPR4	2,04	1	1	392	43,9	4,69	33,61
P30822	CRM1	0,92	1	1	1084	124,0	5,48	33,53
P32337	PSE1	2,02	1	1	1089	121,0	4,74	33,43
Q02821	SRP1	2,03	1	1	542	60,4	4,91	33,37
P40303	PRE6	4,33	2	1	254	28,4	7,36	33,36
P27796	POT1	3,12	1	1	417	44,7	7,61	33,29
Q01976	YSA1	6,49	1	1	231	26,1	6,38	33,05
A6ZN26	NIP1	2,71	1	1	812	93,2	5,00	33,04
P38264	PHO88	7,45	1	1	188	21,1	9,19	32,99
P04046	ADE4	2,16	1	1	510	56,7	6,28	32,48
Q12402	YOP1	4,44	1	1	180	20,3	9,03	32,23
P38840	ARO9	1,95	1	1	513	58,5	5,52	32,10
A6ZN14	LIP1	4,00	1	1	150	17,2	6,67	32,06
B3LI04	SPG1	11,58	1	1	95	10,5	8,46	32,04
P30657	PRE4	3,38	1	1	266	29,4	5,99	32,00
Q03690	CLU1	0,94	1	1	1277	145,1	6,39	31,88
P40069	KAP123	1,53	1	1	1113	122,5	4,63	31,79
P48239	GTO1	3,09	1	1	356	41,3	8,84	31,61
P47018	MTC1	3,35	1	1	478	53,4	4,59	31,17
P53315	SOL4	5,88	2	1	255	28,4	5,35	31,09
P00128	QCR7	17,32	2	2	127	14,6	5,88	31,05
P38708	YHR020W	3,05	2	2	688	77,3	6,40	31,01
Q12305	YOR285W	21,58	2	2	139	15,4	6,38	30,92
P15496	IDI1	4,51	1	1	288	33,3	4,98	30,61
Q03761	TAF12	4,64	1	1	539	61,0	9,55	30,61
B3LSS7	SOL3	13,65	2	2	249	27,8	5,50	30,54
P32288	GLN1	4,86	1	1	370	41,7	6,34	30,51
Q05785	ENT2	1,63	1	1	613	71,8	5,47	30,43
P38273	CCZ1	1,56	2	1	704	80,7	5,26	30,43
O14467	MBF1	7,95	1	1	151	16,4	10,61	30,28
P35176	CPR5	4,44	1	1	225	25,3	5,60	30,21
P39077	CCT3	2,25	1	1	534	58,8	6,11	30,07
P07149	FAS1	1,02	1	1	2051	228,5	5,92	30,02
P53008	CWH41	1,44	1	1	833	96,4	5,07	29,96
P32329	YPS1	1,58	2	1	569	60,0	4,89	29,93
Q07454	YDL073W	0,81	1	1	984	113,8	7,94	29,91
Q12074	SPE3	4,44	1	1	293	33,3	5,53	29,87
Q12125	GET4	3,85	2	1	312	36,3	5,20	29,86
P05318	RPP1A	20,75	1	1	106	10,9	3,88	29,79

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P52488	UBA2	1,42	2	1	636	71,2	5,14	29,43
B3LLS9	EIS1	1,66	2	1	843	93,3	6,34	29,24
P38882	UTP9	1,22	2	1	575	65,2	4,86	29,14
B3LGZ3	GET3	4,52	1	1	354	39,3	5,00	29,13
P20485	CKI1	1,20	1	1	582	66,3	5,67	29,01
P43123	UAP1	2,94	1	1	477	53,4	7,42	28,88
Q03435	NHP10	5,42	1	1	203	23,8	7,87	28,85
P38795	QNS1	2,52	1	1	714	80,6	6,54	28,75
P33204	ARC19	4,68	1	1	171	19,9	5,40	28,30
P49954	NIT3	4,47	1	1	291	32,5	7,55	28,15
P32891	DLD1	1,87	1	1	587	65,3	6,83	28,12
Q07648	DTD1	7,33	1	1	150	16,7	7,42	28,12
P36015	YKT6	5,00	1	1	200	22,7	5,69	27,97
A6ZP88	CLP1	2,47	1	1	445	50,2	5,87	27,68
Q00362	CDC55	4,75	1	1	526	59,6	6,39	27,64
Q07629	YDL218W	3,15	1	1	317	34,4	8,00	27,37
P40363	YJL068C	6,69	2	2	299	33,9	6,71	27,12
P41811	SEC27	1,12	1	1	889	99,4	4,74	27,00
P14126	RPL3	3,88	1	1	387	43,7	10,29	26,74
P00937	TRP3	2,48	1	1	484	53,5	6,92	26,69
P47035	NET1	0,93	2	1	1189	128,5	7,80	26,62
Q12329	HSP42	4,27	1	1	375	42,8	5,08	26,44
P33734	HIS7	1,99	1	1	552	61,0	5,49	26,40
A6ZRI9	RTC4	2,74	1	1	401	46,1	7,14	26,33
Q02895	YPL088W	3,51	1	1	342	39,7	7,31	26,24
O43137	YBR085C-A	14,12	1	1	85	9,4	5,35	26,23
P05738	RPL9A	10,47	2	2	191	21,6	9,73	26,18
P38913	FAD1	2,94	1	1	306	35,5	5,30	26,13
A6ZP58	AIM41	3,78	1	1	185	21,2	9,60	25,81
Q02785	PDR12	0,46	1	1	1511	171,0	6,84	25,81
P07260	TIF45	12,21	2	2	213	24,2	5,49	25,75
P50086	NAS6	4,39	1	1	228	25,6	6,44	25,53
P32588	PUB1	1,99	1	1	453	50,7	5,11	25,48
P41920	YRB1	3,48	1	1	201	22,9	6,10	25,45
Q04178	HPT1	4,98	1	1	221	25,2	5,69	25,42
P43619	BNA6	2,71	1	1	295	32,3	5,85	25,19
P21801	SDH2	5,26	1	1	266	30,2	8,82	25,16
P15454	GUK1	10,70	2	2	187	20,6	7,18	25,11
P32356	NTH1	1,07	1	1	751	85,8	7,77	25,06
P53905	LSM7	6,96	1	1	115	13,0	9,31	24,78
P25567	SRO9	4,61	1	1	434	48,0	8,90	24,70
P40825	ALA1	0,92	1	1	983	110,0	5,71	24,67
O13546	YLR232W	7,83	1	1	115	12,2	6,51	24,61

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
Q02948	VPS30	1,26	1	1	557	63,2	5,07	24,56
P15274	AMD1	1,23	1	1	810	93,2	6,61	24,46
P04911	HTA1	6,82	1	1	132	14,0	10,67	24,46
P47130	CSN12	3,55	1	1	423	49,5	9,17	24,27
Q06103	RPN7	2,33	1	1	429	48,9	5,25	23,99
P53049	YOR1	0,74	1	1	1477	166,6	7,68	23,96
P14742	GFA1	3,07	1	1	717	80,0	6,43	23,93
P38821	APE4	2,86	1	1	490	54,1	7,02	23,73
Q08446	SGT1	2,53	1	1	395	44,8	6,49	23,59
Q08245	ZEO1	11,50	1	1	113	12,6	5,43	23,47
P25343	RVS161	4,91	1	1	265	30,2	6,62	23,44
P38912	TIF11	7,19	1	1	153	17,4	4,83	23,31
P39984	HAT2	2,24	1	1	401	45,0	4,84	23,21
Q03976	RSM24	3,76	1	1	319	37,4	9,23	23,18
P53981	YNL010W	4,15	1	1	241	27,5	5,45	23,14
P47032	PRY1	3,34	1	1	299	30,6	4,63	23,13
P15646	NOP1	4,28	1	1	327	34,4	10,24	23,11
P38234	RFS1	4,29	1	1	210	22,9	5,29	23,09
P07264	LEU1	2,18	1	1	779	85,7	5,90	23,07
P53289	RTS3	2,66	1	1	263	29,2	9,98	22,98
Q04894	ADH6	2,22	1	1	360	39,6	6,74	22,94
P32563	VPH1	1,19	1	1	840	95,5	5,48	22,91
P39726	GCV3	8,24	1	1	170	18,8	4,73	22,87
P53839	GOR1	3,43	1	1	350	38,8	6,34	22,82
P16649	TUP1	0,98	1	1	713	78,3	5,68	22,81
P11978	SIR4	0,81	1	1	1358	152,0	9,00	22,71
P53155	YGL082W	1,84	1	1	381	43,3	6,27	22,70
P32565	RPN2	1,16	1	1	945	104,2	6,18	22,49
P12904	SNF4	3,73	1	1	322	36,4	5,78	22,43
Q06554	IRC20	0,45	1	1	1556	180,2	6,54	22,15
P32804	ZRT1	6,12	1	1	376	41,6	5,81	22,11
Q06096	COG4	1,05	1	1	861	98,6	6,10	22,11
P53969	SAM50	1,45	1	1	484	54,4	8,51	22,08
Q05080	HOF1	1,05	1	1	669	76,2	9,07	21,93
P38249	TIF32	0,93	1	1	964	110,3	6,30	21,83
P40482	SEC24	0,86	1	1	926	103,6	6,23	21,68
P09436	ILS1	1,31	1	1	1072	122,9	6,06	21,57
Q08968	FMP40	2,62	1	1	688	78,3	5,44	21,29
P53141	MLC1	5,37	1	1	149	16,4	4,73	21,13
P27476	NSR1	2,66	1	1	414	44,5	4,93	21,10
P12612	TCP1	3,04	1	1	559	60,4	6,49	21,08
P0C5L5	YBR191W-A	25,00	1	1	24	3,1	10,55	21,07
P40580	IRC24	3,04	1	1	263	28,8	6,37	21,02

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P40477	NUP159	0,82	1	1	1460	158,8	4,83	20,92
P38811	TRA1	0,37	1	1	3744	432,9	6,55	20,84
P38887	YHR202W	1,99	1	1	602	69,0	6,23	20,72
P39743	RVS167	1,87	1	1	482	52,7	6,01	20,69
P50942	INP52	1,69	1	1	1183	133,2	8,63	20,69
P32457	CDC39	1,92	1	1	520	60,0	5,48	20,68

Table S3. Proteins secreted during liquid growth by *S. cerevisiae* W303-1A *gup1Δ* cells.

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P40472	SIM1	37,61	265	9	476	48,2	4,60	12468,33
P53334	SCW4	36,01	130	10	386	40,1	4,83	4968,56
P15703	BGL2	57,51	133	10	313	34,1	4,51	4239,87
P00925	ENO2	71,40	128	20	437	46,9	6,00	4131,25
P00924	ENO1	59,95	113	16	437	46,8	6,62	3676,89
P38288	TOS1	35,38	120	8	455	48,0	4,67	3606,71
P28319	CWP1	42,26	66	9	239	24,3	4,67	2699,88
Q12408	NPC2	44,51	122	6	173	19,1	4,44	2311,12
Q04951	SCW10	45,50	70	13	389	40,4	4,65	1974,18
P42835	EGT2	12,39	38	6	1041	108,5	4,82	1783,29
P23776	EXG1	57,37	58	16	448	51,3	4,75	1730,79
P22146	GAS1	21,47	58	9	559	59,5	4,67	1728,31
P06169	PDC1	35,88	56	13	563	61,5	6,19	1631,39
P53753	DSE4	14,32	40	8	1117	121,0	4,53	1488,32
P00560	PGK1	49,76	43	15	416	44,7	7,61	1257,74
P00549	PYK1	46,40	48	17	500	54,5	7,68	1208,25
P00359	TDH3	39,46	39	8	332	35,7	6,96	1196,61
Q12303	YPS3	25,00	36	8	508	54,5	8,69	989,45
P11484	SSB1	29,69	25	11	613	66,6	5,44	935,75
P14540	FBA1	39,00	26	7	359	39,6	5,78	887,01
A6ZY20	PST1	14,41	26	4	444	45,8	9,19	843,74
P15108	HSC82	29,65	30	15	705	80,8	4,83	830,30
P53616	SUN4	34,76	28	6	420	43,4	4,36	823,87
P32324	EFT1	25,77	25	13	842	93,2	6,32	799,33
P00942	TPI1	56,45	34	9	248	26,8	6,01	792,74
P10596	SUC4	11,28	26	6	532	60,5	4,88	790,58
P12709	PGI1	5,42	16	2	554	61,3	6,46	744,88
P05694	MET6	28,55	34	17	767	85,8	6,47	743,04
A6ZL22	ECM33	9,79	28	5	429	43,7	4,91	724,36
B3LQU1	PIR3	8,79	21	2	307	31,2	5,66	632,44
P02829	HSP82	23,55	24	13	709	81,4	4,91	600,58
P02994	TEF1	31,44	27	9	458	50,0	9,04	586,99
P24031	PHO3	24,84	19	6	467	52,7	4,63	573,87
P39105	PLB1	15,21	24	8	664	71,6	4,73	561,01
P00358	TDH2	39,16	23	8	332	35,8	6,96	557,81
A7A003	UTH1	26,24	22	6	362	36,7	4,79	549,11
P16521	YEF3	13,98	20	9	1044	115,9	6,05	549,00
P36110	PRY2	12,77	19	3	329	33,8	4,60	548,68
P06787	CMD1	29,93	10	3	147	16,1	4,30	539,76
P07267	PEP4	27,16	18	8	405	44,5	4,84	517,72
P00445	SOD1	35,06	15	4	154	15,8	6,00	516,38

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P00830	ATP2	21,72	14	8	511	54,8	5,71	495,17
P39954	SAH1	19,60	17	6	449	49,1	6,24	465,61
P46992	YJL171C	14,65	12	3	396	42,9	5,06	460,30
P48589	RPS12	13,29	11	1	143	15,5	4,73	458,88
P26321	RPL5	33,33	13	5	297	33,7	6,83	445,85
P32334	MSB2	2,30	12	2	1306	133,0	4,22	432,42
Q08193	GAS5	10,54	11	3	484	51,8	4,64	421,26
P38616	YGP1	22,88	15	5	354	37,3	5,44	411,60
P00330	ADH1	20,98	21	7	348	36,8	6,68	400,20
Q08108	PLB3	12,39	12	6	686	75,0	5,03	394,10
P05750	RPS3	24,58	11	4	240	26,5	9,41	380,33
P10664	RPL4A	26,24	13	5	362	39,1	10,64	358,84
P15019	TAL1	14,63	6	3	335	37,0	6,43	358,80
P34760	TSA1	28,06	10	4	196	21,6	5,14	347,88
P26783	RPS5	19,11	8	2	225	25,0	8,59	333,82
P12630	BAR1	8,52	13	3	587	63,7	4,78	330,79
P00360	TDH1	27,11	10	6	332	35,7	8,28	329,70
B5VL27	CIS3	11,56	15	3	225	23,0	4,68	321,49
P38013	AHP1	28,98	12	2	176	19,1	5,16	320,79
P04807	HXK2	19,75	11	5	486	53,9	5,30	319,20
P08067	RIP1	18,60	9	4	215	23,3	8,07	315,94
P00950	GPM1	51,82	19	8	247	27,6	8,84	313,80
P26786	RPS7A	36,32	11	4	190	21,6	9,83	304,23
P10592	SSA2	21,60	15	8	639	69,4	5,06	296,04
P07262	GDH1	14,10	12	5	454	49,5	5,69	295,79
Q03655	GAS3	18,13	11	6	524	56,8	4,79	289,95
P36105	RPL14A	21,74	7	2	138	15,2	10,93	285,96
P10591	SSA1	17,91	11	7	642	69,6	5,11	273,08
P07251	ATP1	12,66	12	6	545	58,6	9,04	271,37
P41805	RPL10	18,10	7	2	221	25,3	10,02	269,56
P29453	RPL8B	31,64	9	6	256	28,1	10,02	259,23
P02365	RPS6A	25,42	9	4	236	27,0	10,45	257,59
P39676	YHB1	26,82	10	5	399	44,6	6,28	255,36
P05754	RPS8A	13,50	7	2	200	22,5	10,67	247,92
P23301	HYP2	30,57	9	3	157	17,1	4,96	238,53
P05737	RPL7A	21,72	9	4	244	27,6	10,15	236,80
P00890	CIT1	18,37	9	5	479	53,3	8,29	236,65
P19358	SAM2	20,31	7	5	384	42,2	5,38	236,32
P25694	CDC48	7,19	9	4	835	91,9	4,94	234,17
P38720	GND1	11,04	10	4	489	53,5	6,64	230,94
P17079	RPL12A	16,97	7	2	165	17,8	9,41	226,60
P05030	PMA1	6,97	6	3	918	99,6	5,11	225,56
P05759	RPS31	36,18	12	6	152	17,2	9,86	220,93
P25443	RPS2	5,91	6	1	254	27,4	10,43	215,94
Q03048	COF1	28,67	6	3	143	15,9	5,20	210,42

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
A6ZQJ1	TIF1	16,46	5	4	395	44,7	5,12	209,70
P17967	PDI1	5,17	5	2	522	58,2	4,53	206,77
P14120	RPL30	45,71	7	3	105	11,4	9,80	203,29
P05739	RPL6B	19,32	9	3	176	20,0	10,08	202,88
P53301	CRH1	6,31	9	3	507	52,7	4,65	202,41
P36008	TEF4	9,71	5	3	412	46,5	7,87	201,47
P53379	MKC7	8,39	8	3	596	64,2	4,75	200,08
O13547	CCW14	17,65	3	2	238	23,3	5,94	198,51
P07149	FAS1	4,49	11	7	2051	228,5	5,92	192,98
B3LI22	RPS0A	21,43	8	3	252	28,0	4,72	192,58
P52911	EXG2	7,65	7	4	562	63,5	5,38	189,80
P41940	MPG1	7,20	6	2	361	39,5	6,34	186,80
P00044	CYC1	17,43	6	2	109	12,2	9,42	185,54
P35691	TMA19	20,96	5	3	167	18,7	4,56	184,49
P40213	RPS16A	28,67	6	3	143	15,8	10,26	184,43
P29311	BMH1	15,73	6	2	267	30,1	4,88	175,58
P11986	INO1	14,82	7	5	533	59,6	5,92	173,54
P06106	MET17	20,72	8	6	444	48,6	6,43	171,29
P41338	ERG10	12,81	7	4	398	41,7	7,39	170,91
P02293	HBT1	11,45	3	1	131	14,2	10,10	168,82
P07279	RPL18A	11,29	4	2	186	20,6	11,71	165,64
B5VL26	HSP150	5,94	9	2	303	30,5	5,59	162,65
P18239	AAC2	13,84	7	4	318	34,4	9,79	159,52
P32827	RPS23A	7,59	3	1	145	16,0	10,73	157,51
P05753	RPS4A	15,33	8	3	261	29,4	10,08	156,33
P26782	RPS24A	22,96	7	5	135	15,3	10,51	155,28
P31373	CYS3	16,50	6	3	394	42,5	6,54	155,24
P05317	RPP0	19,55	7	4	312	33,7	4,83	155,06
P0C0W1	RPS22A	30,00	6	3	130	14,6	9,94	151,84
P14904	LAP4	5,25	4	2	514	57,1	5,83	149,81
P54115	ALD6	5,40	4	2	500	54,4	5,44	149,57
P61830	HHT1	23,53	2	1	136	15,3	11,43	148,70
P22217	TRX1	35,92	4	3	103	11,2	4,93	147,30
P00729	PRC1	13,72	8	5	532	59,8	4,73	140,95
P07280	RPS19A	25,69	7	3	144	15,9	9,61	140,19
Q00955	FAS3	4,39	10	8	2233	250,2	6,28	139,08
A6ZZG0	PIR1	5,28	7	2	341	34,6	6,55	138,78
P23641	MIR1	9,97	6	3	311	32,8	9,31	129,53
P32329	YPS1	7,38	6	2	569	60,0	4,89	128,45
P05756	RPS13	30,46	8	4	151	17,0	10,43	128,40
P40185	MMF1	17,93	3	2	145	15,9	9,28	128,25
Q12118	SGT2	4,91	5	1	346	37,2	4,79	127,30
P14832	CPR1	13,58	6	2	162	17,4	7,44	126,27
P04801	THS1	2,45	4	2	734	84,5	7,03	124,71
Q06549	CDD1	39,44	5	4	142	15,5	7,17	116,46

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P0C2H8	RPL31A	23,89	4	2	113	12,9	9,99	114,69
P22203	VMA4	12,02	3	2	233	26,5	5,36	112,67
A6ZTT5	ADH4	4,45	2	1	382	41,1	6,28	112,53
Q12335	PST2	7,58	4	1	198	21,0	5,73	111,74
P25371	ADP1	2,48	4	2	1049	117,2	5,16	110,28
P17255	TFP1	5,60	5	4	1071	118,6	6,16	109,49
P16120	THR4	10,89	4	4	514	57,4	5,64	106,34
P00447	SOD2	18,88	4	3	233	25,8	8,48	105,18
P05736	RPL2A	16,14	3	2	254	27,4	11,11	105,04
P49167	RPL38	8,97	3	1	78	8,8	10,93	104,24
P05748	RPL15A	20,10	6	4	204	24,4	11,39	103,98
P09624	LPD1	8,82	4	3	499	54,0	8,03	102,94
P04456	RPL25	9,15	2	1	142	15,7	10,11	101,08
A6ZPE5	NOP58	3,33	3	1	511	56,9	8,94	100,94
P07259	URA2	1,85	6	4	2214	244,9	5,83	99,51
P17505	MDH1	13,47	4	3	334	35,6	8,47	99,46
P10622	RPP1B	15,09	3	1	106	10,7	4,01	99,09
P0C0V8	RPS21A	11,49	3	1	87	9,7	6,06	98,50
P02400	RPP2B	42,73	3	3	110	11,0	4,15	98,40
P39976	DLD3	12,10	5	3	496	55,2	6,89	97,69
P05735	RPL18A	4,76	2	1	189	21,7	11,36	97,03
P32589	SSE1	5,63	5	3	693	77,3	5,22	96,22
P35271	RPS18A	9,59	4	2	146	17,0	10,27	96,10
P38841	YHR138C	24,56	5	2	114	12,7	5,06	95,73
P41277	GPP1	11,20	3	2	250	27,9	5,55	95,53
Q05016	YMR226C	6,37	2	1	267	29,1	6,81	93,02
P11076	ARF1	10,50	4	2	181	20,5	7,34	92,94
P19097	FAS2	2,23	3	2	1887	206,8	5,44	92,89
P40106	GPP2	20,00	3	2	250	27,8	6,16	92,71
P04449	RPL24A	14,19	4	2	155	17,6	11,28	91,70
P02309	HHF1	17,48	3	2	103	11,4	11,36	91,00
P28240	ICL2	5,03	3	2	557	62,4	6,42	90,47
P53189	SCW11	5,72	3	2	542	56,4	4,48	89,52
P05744	RPL33A	7,48	4	1	107	12,1	11,08	89,11
P32582	CYS4	8,48	3	3	507	56,0	6,70	87,77
P02406	RPL28	12,08	5	2	149	16,7	10,62	87,74
Q07551	YDL124W	8,33	3	2	312	35,5	6,19	82,63
Q03558	OYE2	11,50	5	3	400	45,0	6,57	82,20
P32642	GRX4	3,28	3	1	244	27,5	4,65	81,89
P23254	TKL1	9,56	4	4	680	73,8	7,01	80,73
P16140	VMA2	2,71	2	1	517	57,7	5,07	79,27
P05318	RPP1A	20,75	3	1	106	10,9	3,88	79,13
P04911	HTA1	24,24	3	2	132	14,0	10,67	78,36
P25087	ERG6	3,39	1	1	383	43,4	5,77	76,65

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P08524	ERG20	4,83	4	2	352	40,5	5,47	76,31
P49090	ASN2	10,84	3	3	572	64,6	5,87	76,27
Q96VH4	HBN1	13,99	2	1	193	21,0	6,95	75,78
P29029	CTS1	4,45	3	2	562	59,0	4,55	75,61
P31787	ACB1	39,08	5	2	87	10,1	4,92	75,29
P16861	PFK1	7,19	4	4	987	107,9	6,39	74,41
P17709	GLK1	5,20	3	2	500	55,3	6,19	74,16
P02407	RPS17A	10,29	3	1	136	15,8	10,51	74,07
P38804	RTC3	15,32	3	1	111	12,0	5,07	71,38
P14126	RPL3	9,04	5	3	387	43,7	10,29	70,86
P11412	ZWF1	1,98	2	1	505	57,5	6,30	70,47
P26781	RPS11A	6,41	2	1	156	17,7	10,78	69,47
P29509	TRR1	3,76	2	1	319	34,2	5,94	69,38
B3LLJ2	RPS1B	6,27	4	2	255	28,8	10,02	67,95
P38836	ECM14	6,51	2	2	430	49,8	5,30	66,59
P00817	IPP1	13,94	2	2	287	32,3	5,58	66,25
P35997	RPS27A	19,51	2	1	82	8,9	9,14	66,17
P19882	HSP60	5,07	3	2	572	60,7	5,31	65,75
P39741	RPL35A	9,17	2	1	120	13,9	10,58	65,61
P0C2H6	RPL27A	28,68	3	3	136	15,5	10,36	65,23
P07246	ADH3	2,13	2	1	375	40,3	8,43	64,87
P16474	KAR2	6,01	3	3	682	74,4	4,93	64,70
P53090	ARO8	2,00	2	1	500	56,1	6,01	64,21
P05755	RPS9B	8,72	3	2	195	22,3	10,10	63,92
P36139	PET10	3,89	1	1	283	31,2	8,16	59,67
P0C2I0	RPL20A	8,14	1	1	172	20,4	10,30	59,07
P15873	POL30	4,26	2	1	258	28,9	4,59	58,70
P22943	HSP12	10,09	2	1	109	11,7	5,38	58,25
P25349	YCP4	8,91	2	2	247	26,3	8,19	57,99
P06168	ILV5	5,57	3	2	395	44,3	9,04	57,81
P32471	EFB1	22,33	3	2	206	22,6	4,45	57,80
P05319	RPP2A	10,38	2	1	106	10,7	4,04	57,27
P38011	ASC1	5,64	2	1	319	34,8	6,24	56,82
P25296	CNB1	4,00	3	1	175	19,6	4,55	56,46
P32449	ARO4	3,51	2	1	370	39,7	6,95	55,97
P05740	RPL17A	14,13	2	2	184	20,5	10,92	54,43
P49089	ASN1	5,07	3	2	572	64,4	6,11	54,21
P46367	ALD4	5,97	2	2	519	56,7	6,74	53,25
P07265	MAL62	4,28	4	2	584	68,1	5,81	50,55
P52910	ACS2	4,25	2	2	683	75,4	6,68	48,74
Q12447	PAA1	6,81	1	1	191	21,9	5,82	48,10
P22803	TRX2	35,58	3	3	104	11,2	4,93	47,82
Q99258	RIB3	11,06	2	2	208	22,6	5,78	47,81
P31116	HOM6	3,34	2	1	359	38,5	7,44	46,46
Q08822	CIR2	2,22	1	1	631	69,6	7,83	46,23

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P40054	SER3	3,20	1	1	469	51,2	5,57	45,98
P32891	DLD1	2,56	2	1	587	65,3	6,83	45,96
P53252	PIL1	5,90	3	2	339	38,3	4,63	43,91
P02557	TUB2	2,19	2	1	457	50,9	4,75	43,90
Q12127	CCW12	12,78	2	1	133	13,0	4,64	43,80
P04840	POR1	4,59	1	1	283	30,4	7,93	43,63
P40069	KAP123	1,53	1	1	1113	122,5	4,63	43,56
P46784	RPS10B	13,33	2	1	105	12,7	9,07	43,49
P40212	RPL13B	13,07	2	1	199	22,5	11,08	43,25
P54070	KTR6	4,04	3	2	446	52,1	5,50	42,80
Q12460	NOP56	2,18	1	1	504	56,8	8,90	42,23
P39938	RPS26A	7,56	1	1	119	13,5	10,76	42,15
Q06325	YPS7	2,85	1	1	596	64,5	4,96	41,92
P47037	SMC3	0,89	2	1	1230	141,2	5,81	40,97
P00498	HIS1	2,36	2	1	297	32,2	6,15	40,33
P54839	ERG13	4,07	2	2	491	55,0	8,16	40,33
P32590	SSE2	3,61	3	2	693	77,6	5,63	40,13
P53030	RPL1A	5,07	2	1	217	24,5	9,72	39,98
P03962	URA3	4,49	1	1	267	29,2	7,36	39,63
P53289	RTS3	2,66	2	1	263	29,2	9,98	39,59
P07257	QCR2	4,35	1	1	368	40,5	7,96	39,04
P07342	ILV2	1,02	1	1	687	74,9	8,51	38,85
P26637	CDC60	1,65	2	2	1090	124,1	5,85	38,68
P38701	RPS20	9,92	1	1	121	13,9	9,52	38,55
Q02979	GDE1	0,57	3	1	1223	137,9	6,79	38,54
Q3E7Z5	YIL002W-A	14,49	1	1	69	7,7	4,67	38,03
P22137	CHC1	1,51	2	2	1653	187,1	5,24	36,44
P41057	RPS29A	19,64	1	1	56	6,7	10,27	36,30
P32835	GSP1	11,42	3	2	219	24,8	6,55	36,11
Q01560	NPL3	5,31	1	1	414	45,4	5,54	35,86
P05738	RPL9A	7,85	2	1	191	21,6	9,73	35,63
A6ZX97	AIM6	3,59	1	1	390	44,4	5,59	35,03
P60010	ACT1	5,87	2	2	375	41,7	5,68	34,93
P48570	LYS20	5,37	2	2	428	47,1	7,27	34,63
Q03674	PLB2	3,26	1	1	706	75,4	4,70	34,13
Q12680	GLT1	0,33	2	1	2145	238,0	6,58	33,76
P07260	TIF45	2,82	3	1	213	24,2	5,49	33,51
P0C0X0	RPS28B	17,91	1	1	67	7,6	10,78	33,26
P49723	RNR4	10,43	2	2	345	40,0	5,21	33,08
P07283	SEC53	5,51	2	2	254	29,0	5,24	33,01
P36010	YNK1	5,88	1	1	153	17,2	8,60	32,74
P15992	HSP26	7,48	1	1	214	23,9	5,53	32,07
P09938	RNR2	3,01	1	1	399	46,1	5,25	31,65
P0C5L5	YBR191W-A	25,00	2	1	24	3,1	10,55	31,58

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P40513	MAM33	10,15	2	2	266	30,1	4,58	31,33
P04802	DPS1	1,62	2	1	557	63,5	6,58	30,92
P04451	RPL24A	5,84	1	1	137	14,5	10,33	30,66
P33775	PMT1	3,30	1	1	817	92,6	6,68	30,25
P34167	TIF3	2,52	1	1	436	48,5	5,29	30,24
P33331	NTF2	17,60	1	1	125	14,4	4,70	29,98
P41921	GLR1	2,90	1	1	483	53,4	7,83	29,88
A6ZZH2	MCR1	5,30	2	2	302	34,1	8,65	29,63
A7A024	OAF3	0,81	1	1	863	101,8	6,96	29,36
Q12306	SMT3	6,93	1	1	101	11,6	5,02	28,68
P25846	MSH1	0,83	1	1	959	109,3	8,73	28,41
P11745	RNA1	1,97	1	1	407	45,8	4,61	27,94
P38882	UTP9	1,22	2	1	575	65,2	4,86	27,60
P25572	YCL042W	7,56	1	1	119	13,4	10,56	27,56
P47035	NET1	0,93	1	1	1189	128,5	7,80	27,33
P39077	CCT3	1,50	1	1	534	58,8	6,11	27,13
P39968	VAC8	1,38	2	1	578	63,2	5,14	27,07
A6ZZ25	TIF35	4,38	1	1	274	30,5	6,81	26,86
P27614	CPS1	2,26	1	1	576	64,6	5,53	26,82
Q08300	YOL159C	7,02	1	1	171	19,6	4,45	26,00
P38788	SSZ1	1,67	1	1	538	58,2	5,05	25,99
P13298	URA5	6,64	1	1	226	24,6	6,05	25,77
P27476	NSR1	2,17	1	1	414	44,5	4,93	25,71
P53278	YGR130C	0,98	1	1	816	92,6	5,03	25,70
Q02794	STD1	2,70	1	1	444	50,2	8,98	25,51
P32466	HXT3	4,06	1	1	567	62,5	7,08	25,42
P07274	PFY1	11,11	1	1	126	13,7	5,80	25,23
P38273	CCZ1	1,56	1	1	704	80,7	5,26	25,19
Q05902	ECM38	1,52	1	1	660	73,1	5,88	25,17
P32074	SEC21	1,93	1	1	935	104,8	5,12	25,00
P26785	RPL16B	7,07	2	2	198	22,2	10,55	24,75
P10869	HOM3	2,09	1	1	527	58,1	6,67	24,58
P38114	TBS1	0,64	1	1	1094	126,8	5,43	24,26
P37012	PGM2	1,58	1	1	569	63,0	6,62	24,25
Q02753	RPL21A	6,88	1	1	160	18,2	10,39	24,07
P46655	GUS1	1,13	1	1	708	80,8	7,53	24,04
P00410	COX2	7,97	1	1	251	28,5	4,58	23,86
P20051	URA4	2,20	1	1	364	40,3	6,54	23,60
P17695	GRX2	9,79	1	1	143	15,9	7,28	22,88
P13663	HOM2	3,84	1	1	365	39,5	6,73	22,65
P37291	SHM2	2,99	1	1	469	52,2	7,43	22,53
P22768	ARG1	4,52	1	1	420	46,9	5,62	22,27
P08964	MYO1	0,36	1	1	1928	223,5	6,40	22,13
P28707	SBA1	5,09	1	1	216	24,1	4,59	21,47
P38625	GUA1	2,86	1	1	525	58,4	6,49	20,33

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P22515	UBA1	1,95	1	1	1024	114,2	5,11	20,18
Q03361	JIP4	0,80	1	1	876	98,6	9,57	20,03

Table S4. Proteins from *S. cerevisiae* W303-1A *gup1Δ* extracellular matrix.

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P00942	TPI1	81,85	331	17	248	26,8	6,01	10008,87
P0CG63	UBI4	61,68	261	5	381	42,8	7,58	9226,87
P15703	BGL2	58,15	305	11	313	34,1	4,51	8874,51
P00925	ENO2	74,60	228	26	437	46,9	6,00	7250,28
P00359	TDH3	73,19	213	15	332	35,7	6,96	7205,30
P00924	ENO1	69,79	212	23	437	46,8	6,62	6606,57
P28240	ICL2	60,50	185	24	557	62,4	6,42	5548,13
P22146	GAS1	21,65	192	11	559	59,5	4,67	5324,49
P38013	AHP1	85,80	140	10	176	19,1	5,16	5012,38
P34760	TSA1	82,65	177	12	196	21,6	5,14	4971,22
P09624	LPD1	73,35	170	28	499	54,0	8,03	4736,74
P49089	ASN1	70,63	167	31	572	64,4	6,11	4696,46
P00560	PGK1	76,20	189	27	416	44,7	7,61	4550,29
P00549	PYK1	73,80	166	31	500	54,5	7,68	4496,47
P00830	ATP2	72,21	149	26	511	54,8	5,71	4331,32
P00358	TDH2	48,80	122	12	332	35,8	6,96	4173,84
P00360	TDH1	49,10	89	13	332	35,7	8,28	3455,51
P49090	ASN2	70,28	117	28	572	64,6	5,87	3376,05
P17255	TFP1	22,60	132	19	1071	118,6	6,16	3107,60
P10591	SSA1	62,62	108	30	642	69,6	5,11	3047,31
P23776	EXG1	66,96	93	19	448	51,3	4,75	2972,04
P47143	ADO1	72,94	98	15	340	36,3	5,16	2900,13
P37302	APE3	41,15	114	14	537	60,1	5,31	2555,84
P06169	PDC1	65,90	94	25	563	61,5	6,19	2531,04
P38288	TOS1	40,00	91	9	455	48,0	4,67	2516,57
P07262	GDH1	67,18	69	21	454	49,5	5,69	2449,33
P40472	SIM1	30,04	64	8	476	48,2	4,60	2240,70
P10592	SSA2	55,87	88	26	639	69,4	5,06	2221,73
P29509	TRR1	65,20	61	13	319	34,2	5,94	2221,50
P15019	TAL1	57,61	67	18	335	37,0	6,43	2193,12
P00890	CIT1	47,60	81	19	479	53,3	8,29	2126,16
P38115	ARA1	66,57	73	18	344	38,9	5,96	2020,09
P07267	PEP4	48,64	65	13	405	44,5	4,84	2014,64
P17505	MDH1	72,46	69	16	334	35,6	8,47	1975,78
P53912	YNL134C	63,03	65	14	376	41,1	6,21	1872,57
P22803	TRX2	80,77	64	7	104	11,2	4,93	1859,39
P07257	QCR2	69,84	56	20	368	40,5	7,96	1848,45
P07274	PFY1	68,25	87	4	126	13,7	5,80	1800,03
P41338	ERG10	52,76	60	16	398	41,7	7,39	1748,53
P07251	ATP1	39,82	53	15	545	58,6	9,04	1699,49
P39708	GDH3	57,99	53	18	457	49,6	5,47	1682,95

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P40185	MMF1	82,76	63	9	145	15,9	9,28	1655,37
P00447	SOD2	65,24	46	7	233	25,8	8,48	1643,40
P00950	GPM1	69,64	63	14	247	27,6	8,84	1568,68
P12709	PGI1	44,22	45	16	554	61,3	6,46	1560,46
P43590	YFR006W	41,87	46	16	535	61,7	6,16	1550,96
Q03655	GAS3	41,03	48	15	524	56,8	4,79	1505,32
P00445	SOD1	81,82	47	10	154	15,8	6,00	1470,61
P28319	CWP1	38,08	40	8	239	24,3	4,67	1456,95
P41921	GLR1	59,01	60	20	483	53,4	7,83	1443,61
P14540	FBA1	50,14	43	10	359	39,6	5,78	1437,95
Q12363	WTM1	55,84	53	19	437	48,4	5,36	1424,40
P38616	YGP1	30,23	58	7	354	37,3	5,44	1383,15
P46367	ALD4	57,80	50	21	519	56,7	6,74	1379,41
Q07505	YDL086W	64,47	41	12	273	30,8	6,34	1368,06
Q04432	HSP31	83,54	40	13	237	25,7	5,50	1349,53
P38804	RTC3	67,57	43	7	111	12,0	5,07	1294,25
P15108	HSC82	28,37	42	15	705	80,8	4,83	1263,06
P14904	LAP4	48,64	45	17	514	57,1	5,83	1253,69
P00498	HIS1	55,89	44	12	297	32,2	6,15	1250,24
P46992	YJL171C	23,99	31	6	396	42,9	5,06	1242,38
P16120	THR4	40,47	33	13	514	57,4	5,64	1179,87
P38720	GND1	49,69	43	18	489	53,5	6,64	1131,59
P32445	RIM1	56,30	40	7	135	15,4	8,34	1127,45
P38715	GRE3	62,69	41	19	327	37,1	7,08	1118,46
P05694	MET6	44,85	51	24	767	85,8	6,47	1108,27
P21954	IDP1	53,27	42	20	428	48,2	8,76	1097,24
P02829	HSP82	28,21	38	15	709	81,4	4,91	1081,49
P00729	PRC1	41,54	45	14	532	59,8	4,73	1066,27
P38836	ECM14	41,86	39	13	430	49,8	5,30	1064,22
P53753	DSE4	14,32	33	8	1117	121,0	4,53	1058,03
Q03048	COF1	72,03	31	6	143	15,9	5,20	1049,08
P39721	AIM29	70,73	34	12	246	27,1	6,28	1043,96
Q08193	GAS5	23,97	31	8	484	51,8	4,64	1025,53
P00431	CCP1	54,29	40	16	361	40,3	6,38	1023,27
P04807	HXK2	40,95	42	14	486	53,9	5,30	1015,51
P24031	PHO3	37,04	36	11	467	52,7	4,63	992,46
P39105	PLB1	17,02	42	10	664	71,6	4,73	977,67
P40513	MAM33	37,22	35	8	266	30,1	4,58	958,45
P39954	SAH1	40,76	36	15	449	49,1	6,24	955,50
P27614	CPS1	42,36	44	20	576	64,6	5,53	949,60
P33331	NTF2	88,00	33	6	125	14,4	4,70	935,38
P53301	CRH1	19,72	25	11	507	52,7	4,65	867,25
P11412	ZWF1	42,97	30	15	505	57,5	6,30	867,07
P00817	IPP1	63,76	28	12	287	32,3	5,58	865,40
P09232	PRB1	25,20	31	10	635	69,6	6,39	857,29

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P28834	IDH1	50,56	39	13	360	39,3	9,00	855,06
Q02933	RNY1	50,69	30	15	434	50,1	8,05	842,48
P22217	TRX1	80,58	27	8	103	11,2	4,93	841,76
P38891	BAT1	54,20	35	13	393	43,6	8,91	823,06
P25605	ILV6	35,60	29	9	309	34,0	6,52	818,69
P02309	HHF1	41,75	33	5	103	11,4	11,36	805,74
P29311	BMH1	48,31	30	10	267	30,1	4,88	794,46
P09938	RNR2	36,59	28	10	399	46,1	5,25	782,49
P14832	CPR1	46,30	29	7	162	17,4	7,44	777,50
P31539	HSP104	23,79	34	15	908	102,0	5,45	736,74
B5VL27	CIS3	8,00	54	2	225	23,0	4,68	720,99
P02293	HBT1	34,35	23	5	131	14,2	10,10	712,95
P32324	EFT1	16,51	22	12	842	93,2	6,32	702,28
P07275	PUT2	26,78	22	11	575	64,4	7,01	701,95
Q03558	OYE2	46,25	29	12	400	45,0	6,57	698,09
P49723	RNR4	36,52	24	9	345	40,0	5,21	682,85
P23301	HYP2	48,41	20	5	157	17,1	4,96	675,80
P54115	ALD6	47,00	30	17	500	54,4	5,44	671,37
P14065	GCY1	48,40	26	10	312	35,1	7,99	665,95
P53334	SCW4	24,35	20	7	386	40,1	4,83	660,49
Q08971	YPL225W	57,53	23	8	146	17,4	5,30	652,25
B3LLV5	ARG7	30,16	22	11	441	47,8	7,14	637,81
P13130	SPS100	14,11	23	3	326	34,2	5,41	637,17
P19414	ACO1	28,53	28	15	778	85,3	8,07	634,52
P25373	GRX1	74,55	18	6	110	12,4	5,06	631,77
P38816	TRR2	34,21	15	6	342	37,1	6,87	627,55
P13663	HOM2	39,45	26	11	365	39,5	6,73	624,30
P53228	NQM1	42,04	21	10	333	37,2	6,38	618,70
P15873	POL30	58,14	21	11	258	28,9	4,59	617,12
Q05016	YMR226C	69,29	27	13	267	29,1	6,81	609,48
Q99258	RIB3	74,04	29	12	208	22,6	5,78	606,16
A6ZL22	ECM33	9,79	24	5	429	43,7	4,91	602,03
P15992	HSP26	50,93	21	7	214	23,9	5,53	588,09
P39726	GCV3	34,71	14	5	170	18,8	4,73	583,99
P36015	YKT6	60,00	25	10	200	22,7	5,69	582,41
P40303	PRE6	62,60	25	9	254	28,4	7,36	578,44
Q08245	ZEO1	50,44	27	9	113	12,6	5,43	578,27
P00044	CYC1	34,86	17	5	109	12,2	9,42	574,47
P22943	HSP12	36,70	16	4	109	11,7	5,38	571,59
P28241	IDH2	29,54	21	7	369	39,7	8,69	571,18
P34730	BMH2	47,25	26	10	273	31,0	4,88	562,43
A6ZY20	PST1	14,86	17	6	444	45,8	9,19	562,04
P50095	IMD3	18,55	14	6	523	56,5	7,40	557,44
P41939	IDP2	26,70	22	9	412	46,5	6,19	555,57
P43616	DUG1	15,80	13	4	481	52,8	5,67	551,11

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P00635	PHO5	30,41	22	9	467	52,8	4,83	550,50
P36110	PRY2	12,77	18	3	329	33,8	4,60	549,32
P47176	BAT2	32,71	23	8	376	41,6	7,30	547,59
P22202	SSA4	18,54	29	10	642	69,6	5,14	520,63
P36010	YNK1	61,44	21	8	153	17,2	8,60	519,96
P04806	HXK1	29,69	15	9	485	53,7	5,45	519,21
A7A003	UTH1	26,24	21	6	362	36,7	4,79	504,18
P13298	URA5	42,04	16	6	226	24,6	6,05	503,30
P38145	RIB5	42,44	16	8	238	26,2	5,17	494,04
P09435	SSA3	12,17	25	7	649	70,5	5,17	493,72
P40302	PRE5	35,47	16	6	234	25,6	7,39	485,18
P17709	GLK1	17,40	17	6	500	55,3	6,19	484,68
Q02895	YPL088W	27,19	21	9	342	39,7	7,31	480,60
P21243	SCL1	46,03	18	10	252	28,0	6,24	480,20
P06168	ILV5	32,15	17	9	395	44,3	9,04	474,41
P32454	APE2	12,50	13	9	952	107,7	8,03	472,47
P53184	PNC1	62,50	19	7	216	25,0	6,27	465,20
O13547	CCW14	18,07	14	4	238	23,3	5,94	464,54
P32891	DLD1	23,17	17	10	587	65,3	6,83	462,29
B3RHI0	HRI1	41,80	15	8	244	27,5	5,21	454,30
E7KS00	HRI1	41,80	15	8	244	27,5	5,21	454,30
A6ZYI0	ADK1	41,44	16	7	222	24,2	6,70	450,56
P23638	PRE9	34,88	11	8	258	28,7	5,22	447,29
P31787	ACB1	67,82	19	6	87	10,1	4,92	428,33
Q06252	YLR179C	47,26	17	6	201	22,1	4,94	425,07
Q06494	YPR127W	29,57	16	8	345	38,6	5,99	420,39
Q04951	SCW10	30,85	14	9	389	40,4	4,65	419,17
P40029	MXR1	35,33	12	5	184	21,1	6,99	418,61
P40893	REE1	43,94	15	6	198	22,0	6,39	418,12
P06208	LEU4	31,66	16	12	619	68,4	6,01	409,76
B3LSS7	SOL3	44,58	17	7	249	27,8	5,50	393,72
P32377	MVD1	35,10	13	8	396	44,1	5,76	389,91
P53982	IDP3	12,38	12	5	420	47,8	9,22	385,30
P12398	SSC1	11,62	14	6	654	70,6	5,59	384,13
P32471	EFB1	50,49	17	5	206	22,6	4,45	383,56
P48016	ATH1	10,98	17	10	1211	136,8	5,43	379,80
P40165	YNL200C	27,24	12	4	246	27,5	8,29	376,50
Q12458	YPR1	51,60	18	11	312	34,7	7,12	376,07
P40037	HMF1	59,69	16	7	129	13,9	5,41	373,86
P17555	SRV2	7,03	8	3	526	57,5	5,64	368,97
B3LQU1	PIR3	8,79	12	2	307	31,2	5,66	366,55
P17695	GRX2	44,06	12	5	143	15,9	7,28	366,05
P17967	PDI1	21,46	13	7	522	58,2	4,53	364,41
P32582	CYS4	16,37	10	5	507	56,0	6,70	363,35

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
B3LRI4	RGI1	36,02	12	3	161	19,0	6,18	361,19
P25372	TRX3	59,06	17	7	127	14,4	8,90	357,80
P02994	TEF1	24,45	21	8	458	50,0	9,04	357,37
Q06151	DCS1	19,14	11	5	350	40,7	6,25	354,79
Q12434	RDI1	40,59	15	5	202	23,1	6,04	353,40
P04911	HTA1	29,55	13	3	132	14,0	10,67	351,75
P25451	PUP3	39,51	10	6	205	22,6	5,15	351,72
P53252	PIL1	30,68	9	7	339	38,3	4,63	349,95
P08524	ERG20	42,61	12	9	352	40,5	5,47	346,73
P11484	SSB1	17,29	12	8	613	66,6	5,44	339,70
P08679	CIT2	6,96	9	2	460	51,4	6,38	334,74
P38841	YHR138C	54,39	14	7	114	12,7	5,06	332,28
Q12123	DCS2	22,10	13	6	353	40,9	6,64	323,38
P0CX10	ERR1	14,19	10	4	437	47,3	5,29	321,33
P19882	HSP60	26,22	17	11	572	60,7	5,31	319,38
Q04902	SNO4	19,83	9	3	237	26,0	8,07	316,71
Q07551	YDL124W	49,68	18	10	312	35,5	6,19	313,91
P23254	TKL1	24,26	19	11	680	73,8	7,01	311,82
Q03161	YMR099C	36,70	16	9	297	33,9	6,13	310,91
P35691	TMA19	27,54	9	4	167	18,7	4,56	307,96
P31116	HOM6	37,33	13	7	359	38,5	7,44	305,63
P32379	PUP2	36,54	11	7	260	28,6	4,73	302,57
P38081	YBR056W	24,95	11	9	501	57,8	6,46	300,51
Q12305	YOR285W	42,45	13	4	139	15,4	6,38	297,71
Q04341	MIC14	11,57	8	1	121	13,9	5,63	295,04
Q04304	YMR090W	45,81	15	7	227	24,9	5,80	293,44
A6ZQJ1	TIF1	20,51	10	6	395	44,7	5,12	292,55
P43577	GNA1	50,94	12	5	159	18,1	5,24	292,25
Q12306	SMT3	46,53	11	4	101	11,6	5,02	291,79
Q12230	LSP1	25,51	14	8	341	38,0	4,70	287,92
P61830	HHT1	28,68	6	2	136	15,3	11,43	281,68
Q99312	ISN1	20,00	13	7	450	51,3	5,43	275,65
Q12513	TMA17	23,33	6	3	150	16,8	4,73	274,49
P53616	SUN4	13,57	8	3	420	43,4	4,36	273,59
P04840	POR1	36,04	9	7	283	30,4	7,93	271,57
P27882	ERV1	26,98	12	4	189	21,6	8,21	266,68
P29952	PMI40	20,75	9	6	429	48,2	5,99	266,53
P23542	AAT2	43,06	14	10	418	46,0	8,32	262,83
P54839	ERG13	22,81	12	7	491	55,0	8,16	262,39
O43137	YBR085C-A	41,18	9	3	85	9,4	5,35	262,23
P38707	DED81	9,57	8	4	554	62,2	5,85	262,19
P36008	TEF4	10,68	6	3	412	46,5	7,87	258,61
P32329	YPS1	7,38	13	2	569	60,0	4,89	258,23
P41277	GPP1	30,40	9	5	250	27,9	5,55	256,07
P38817	GGA2	9,74	9	4	585	64,3	6,49	251,92

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
A6ZRW6	MLS1	25,99	15	10	554	62,8	7,03	251,72
P38624	PRE3	36,28	9	5	215	23,5	5,90	250,68
P10963	PCK1	18,76	8	6	549	60,9	6,34	250,40
Q07648	DTD1	20,00	10	3	150	16,7	7,42	249,48
B3LHB3	AIM6	27,95	10	6	390	44,3	5,71	243,71
P21242	PRE10	11,46	7	3	288	31,5	5,19	242,22
P00724	SUC2	12,22	8	6	532	60,6	4,75	241,32
P15705	STI1	12,56	8	5	589	66,2	5,59	240,76
Q05911	ADE13	25,52	14	9	482	54,5	6,43	236,60
P32334	MSB2	2,30	7	2	1306	133,0	4,22	229,79
P39926	SSO2	16,61	9	4	295	33,7	4,91	229,43
Q03629	YML079W	46,27	9	5	201	22,4	5,40	228,63
P07256	COR1	21,44	11	7	457	50,2	7,30	225,59
Q06178	NMA1	12,97	9	4	401	45,8	6,90	222,12
P15891	ABP1	6,76	8	3	592	65,5	4,68	221,93
P00330	ADH1	22,13	12	7	348	36,8	6,68	221,60
Q12118	SGT2	25,43	10	6	346	37,2	4,79	221,30
P30657	PRE4	16,54	5	3	266	29,4	5,99	220,23
P53141	MLC1	37,58	8	4	149	16,4	4,73	217,86
P48606	RBL2	44,34	8	4	106	12,4	5,10	217,01
Q05031	DFG5	13,10	5	4	458	50,5	4,75	209,35
P35842	PHO11	13,28	7	4	467	52,7	5,17	208,50
P54838	DAK1	13,87	7	5	584	62,2	5,41	206,31
P22133	MDH2	26,79	8	6	377	40,7	6,90	204,52
P60010	ACT1	17,87	8	4	375	41,7	5,68	202,35
P00175	CYB2	14,21	7	5	591	65,5	8,41	201,53
P00331	ADH2	22,70	10	6	348	36,7	6,74	201,32
P17536	TPM1	25,63	7	4	199	23,5	4,61	200,16
P02400	RPP2B	33,64	7	3	110	11,0	4,15	197,93
P07283	SEC53	16,14	10	5	254	29,0	5,24	196,69
P53622	COP1	1,67	4	1	1201	135,5	5,99	193,91
P53204	NMA2	21,01	10	5	395	44,9	5,90	193,62
P52911	EXG2	14,06	9	7	562	63,5	5,38	189,45
P23639	PRE8	34,00	10	6	250	27,1	5,72	189,32
P00045	CYC7	27,43	6	3	113	12,5	9,58	186,95
P36059	YKL151C	28,78	11	7	337	37,3	7,55	186,94
P14843	ARO3	20,81	8	5	370	41,0	7,39	186,13
Q04178	HPT1	31,22	11	5	221	25,2	5,69	183,05
P22768	ARG1	16,43	9	5	420	46,9	5,62	181,30
P47123	MOG1	23,39	5	3	218	24,3	4,68	180,99
P16474	KAR2	11,14	13	6	682	74,4	4,93	180,69
P54114	ALD3	18,38	6	6	506	55,4	5,76	180,60
Q12449	AHA1	15,71	8	5	350	39,4	7,47	179,22
P32449	ARO4	15,95	6	4	370	39,7	6,95	178,72

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
A6ZPE5	NOP58	8,41	6	3	511	56,9	8,94	175,84
P20081	FPR1	41,23	6	3	114	12,2	6,04	175,78
P29453	RPL8B	17,97	6	3	256	28,1	10,02	175,09
P01095	PBI2	72,00	10	6	75	8,6	6,80	174,49
P26783	RPS5	19,11	3	2	225	25,0	8,59	172,30
P53090	ARO8	11,60	7	4	500	56,1	6,01	172,20
P05739	RPL6B	14,20	7	2	176	20,0	10,08	171,85
P53889	FMP41	18,53	6	3	259	28,8	8,62	171,74
P46655	GUS1	11,30	9	6	708	80,8	7,53	170,49
P37012	PGM2	8,44	7	4	569	63,0	6,62	169,29
P17649	UGA1	17,41	7	5	471	52,9	6,80	168,90
P14306	TFS1	26,48	7	4	219	24,3	6,54	168,72
Q12447	PAA1	11,52	4	2	191	21,9	5,82	168,17
P52893	ALT1	14,86	8	4	592	66,4	7,02	166,60
P16547	OM45	18,83	8	6	393	44,6	8,59	165,84
P28707	SBA1	26,39	9	4	216	24,1	4,59	165,72
A6ZT99	EGD2	16,67	6	2	174	18,7	4,94	165,42
Q12303	YPS3	12,99	6	4	508	54,5	8,69	164,80
P36139	PET10	9,54	3	2	283	31,2	8,16	164,64
P05319	RPP2A	39,62	5	3	106	10,7	4,04	162,21
P40531	GVP36	32,52	8	6	326	36,6	4,97	161,98
P39979	HPA3	16,20	10	3	179	20,7	7,59	158,81
P48015	GCV1	23,75	6	5	400	44,4	8,84	158,63
P38281	APD1	25,95	11	5	316	35,7	7,18	158,05
Q08911	FDH1	18,62	6	4	376	41,7	6,47	155,85
P22203	VMA4	18,45	5	3	233	26,5	5,36	155,76
P28272	URA1	22,61	6	3	314	34,8	6,05	153,87
P06106	MET17	9,91	6	4	444	48,6	6,43	152,16
Q12074	SPE3	17,75	6	4	293	33,3	5,53	149,98
P49435	APT1	12,83	3	2	187	20,6	5,10	149,14
P23724	PRE7	19,50	7	3	241	26,9	6,07	147,46
Q03104	MSC1	8,58	5	3	513	59,6	7,58	146,53
Q3E841	YNR034W-A	52,04	5	4	98	10,8	8,97	146,41
Q04344	HNT1	46,20	9	5	158	17,7	6,95	144,78
P40581	HYR1	19,63	5	2	163	18,6	8,19	141,60
P25694	CDC48	6,23	6	3	835	91,9	4,94	140,06
P48589	RPS12	13,29	3	1	143	15,5	4,73	136,70
P38235	YBR053C	20,39	6	5	358	40,3	5,06	134,53
P32614	FRD1	6,38	8	2	470	50,8	6,33	133,94
P17423	THR1	19,05	7	5	357	38,7	5,41	132,85
P34216	EDE1	3,19	4	3	1381	150,7	4,72	131,87
Q00055	GPD1	15,60	6	4	391	42,8	5,47	130,23
P32469	DPH5	12,33	5	2	300	33,8	4,92	129,96
Q12314	YOR021C	26,76	5	4	213	24,7	5,07	129,72

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P40075	SCS2	18,85	4	3	244	26,9	4,89	125,72
P05749	RPL22A	32,23	3	2	121	13,7	6,14	125,47
P19097	FAS2	1,64	2	1	1887	206,8	5,44	122,66
P38821	APE4	15,92	7	6	490	54,1	7,02	122,00
B5VL26	HSP150	5,94	6	2	303	30,5	5,59	121,68
P07244	ADE5,7	8,48	4	3	802	86,0	5,27	121,57
P53095	DSD1	7,48	4	3	428	47,8	7,59	120,59
P31373	CYS3	15,23	6	3	394	42,5	6,54	119,31
P29029	CTS1	4,45	3	2	562	59,0	4,55	118,15
P50107	GLO1	3,07	2	1	326	37,2	6,84	117,36
P36091	DCW1	5,35	4	2	449	49,5	4,67	116,16
P06738	GPH1	7,43	8	5	902	103,2	5,62	115,90
P42938	TDA10	15,52	3	3	290	33,3	5,41	114,78
Q06892	POS5	6,28	3	2	414	46,2	8,97	114,56
P32861	UGP1	9,62	5	4	499	56,0	7,44	110,64
P25043	PUP1	12,26	2	1	261	28,3	6,61	110,49
A6ZRW1	NMA111	2,21	4	2	997	110,8	5,96	109,02
P47117	ARP3	2,45	2	1	449	49,5	5,80	108,86
P08466	NUC1	21,28	7	5	329	37,2	8,78	108,31
P39676	YHB1	18,55	3	3	399	44,6	6,28	108,16
P38067	UGA2	4,23	2	1	497	54,2	6,65	107,09
P36154	AIM29	24,52	4	3	155	18,4	6,40	106,86
P53314	CPD1	5,02	2	1	239	26,7	7,47	106,09
P25036	YSP3	3,14	2	1	478	52,1	5,43	105,76
P32486	KRE6	3,89	3	2	720	80,1	4,69	103,89
P12630	BAR1	10,56	7	4	587	63,7	4,78	102,82
Q01976	YSA1	11,69	3	2	231	26,1	6,38	101,46
P12695	PDA2	6,64	5	3	482	51,8	7,80	100,94
P39990	SNU13	25,40	3	2	126	13,6	7,85	100,88
P43635	CIT3	3,91	3	1	486	53,8	8,59	100,49
Q05926	GRX8	10,09	2	1	109	12,5	7,99	99,29
A6ZZG0	PIR1	5,28	6	2	341	34,6	6,55	98,64
Q03103	ERO1	2,31	2	1	563	65,0	5,68	98,40
O13297	CET1	10,38	6	3	549	61,8	5,60	98,09
Q12068	GRE2	16,37	4	4	342	38,1	6,15	96,70
A6ZRM0	ADE12	8,08	4	3	433	48,2	8,35	96,50
P15454	GUK1	22,99	4	3	187	20,6	7,18	94,82
P47018	MTC1	7,53	4	3	478	53,4	4,59	93,92
P48510	DSK2	11,53	4	2	373	39,3	4,96	93,74
P35195	ECM15	56,73	8	2	104	11,5	6,68	93,67
P07245	ADE3	5,81	4	3	946	102,1	6,84	92,27
P50861	RIB4	9,47	4	1	169	18,5	6,54	92,17
P36105	RPL14A	7,97	2	1	138	15,2	10,93	90,43
P38886	RPN10	7,09	1	1	268	29,7	4,82	90,24

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P32642	GRX4	3,28	3	1	244	27,5	4,65	89,86
P27810	KTR1	17,30	7	5	393	46,0	6,68	88,93
P47039	BNA3	11,26	6	3	444	50,1	6,57	87,93
P32589	SSE1	7,22	4	4	693	77,3	5,22	87,16
Q06991	PUN1	6,84	3	1	263	29,2	8,51	85,97
P40509	SEC28	8,78	3	2	296	33,8	4,55	84,93
P05318	RPP1A	20,75	2	1	106	10,9	3,88	84,51
Q08220	GSH2	5,09	3	2	491	55,8	5,66	84,16
Q05979	BNA5	11,48	3	3	453	51,0	5,29	83,96
P18562	FUR1	25,00	4	4	216	24,6	5,74	82,39
P41805	RPL10	4,98	2	1	221	25,3	10,02	81,71
P38197	YBL036C	29,57	7	6	257	29,1	6,43	81,66
A6ZQF6	ATG27	6,64	1	1	271	30,2	5,77	80,02
P22141	PRE1	19,70	4	3	198	22,5	6,23	79,95
P25719	CPR3	18,68	3	3	182	19,9	8,70	79,49
P07991	CAR2	5,66	2	2	424	46,1	6,95	79,28
P43567	AGX1	16,88	5	4	385	41,9	8,00	77,92
P02365	RPS6A	6,36	2	1	236	27,0	10,45	77,91
P24280	SEC14	6,91	4	2	304	34,9	5,49	77,46
P38137	PCS60	5,34	4	2	543	60,5	9,20	75,75
P48439	OST3	6,86	4	2	350	39,5	8,48	75,48
P37291	SHM2	2,99	2	1	469	52,2	7,43	75,42
P07215	CUP1-1	31,15	2	1	61	6,6	5,91	74,51
Q12692	HTZ1	12,69	3	2	134	14,3	10,65	74,40
Q04409	EMI2	5,20	4	2	500	55,9	6,27	74,23
P07284	SES1	12,99	5	4	462	53,3	6,09	74,15
P04449	RPL24A	10,97	3	2	155	17,6	11,28	73,93
P32603	SPR1	2,25	2	1	445	51,8	5,81	72,55
P04456	RPL25	9,15	1	1	142	15,7	10,11	72,24
P40495	LYS12	4,58	1	1	371	40,0	8,02	71,75
P30656	PRE2	8,01	2	2	287	31,6	6,23	69,01
P22434	PDE1	5,69	2	2	369	42,0	6,13	68,72
Q96VH4	HBN1	13,99	2	1	193	21,0	6,95	68,58
P46681	DLD2	12,64	5	4	530	59,2	6,13	68,38
P05740	RPL17A	8,70	2	1	184	20,5	10,92	68,19
P05373	HEM2	10,53	3	3	342	37,7	6,14	66,81
P32590	SSE2	5,19	3	3	693	77,6	5,63	65,86
P16862	PFK2	1,88	1	1	959	104,6	6,67	65,70
P47120	LIA1	10,46	3	2	325	36,1	4,87	65,67
P46672	ARC1	9,84	2	2	376	42,1	7,88	65,56
P47085	YJR008W	5,62	1	1	338	38,5	6,84	65,53
P39976	DLD3	5,65	2	1	496	55,2	6,89	65,47
Q12428	PDH1	4,84	2	2	516	57,6	9,07	65,21
P32473	PDB1	7,38	4	2	366	40,0	5,30	64,69
P06780	RHO1	9,09	3	2	209	23,1	6,23	64,55

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P07280	RPS19A	6,25	2	1	144	15,9	9,61	64,19
P54113	ADE16	3,05	2	1	591	65,2	6,55	64,00
P39101	CAJ1	4,35	1	1	391	44,8	5,80	63,76
P06787	CMD1	25,17	2	2	147	16,1	4,30	63,13
P53598	LSC1	4,26	1	1	329	35,0	8,46	60,76
P53853	VPS75	7,20	2	2	264	30,6	4,75	59,94
P22855	AMS1	3,05	4	3	1083	124,4	7,25	59,44
P32316	ACH1	5,51	1	1	526	58,7	6,79	58,91
P53860	PDR1	11,68	5	4	351	40,7	7,96	58,20
P0C0W1	RPS22A	12,31	1	1	130	14,6	9,94	58,02
P32463	ACP1	12,80	2	1	125	13,9	4,97	57,17
P25375	PRD1	1,54	1	1	712	81,9	5,80	57,02
P32074	SEC21	2,35	2	1	935	104,8	5,12	56,74
P38075	PDX3	15,35	2	2	228	26,9	7,49	56,06
P33317	DUT1	8,16	1	1	147	15,3	7,25	56,06
P53332	CAB4	8,20	3	2	305	34,3	6,32	55,98
P26782	RPS24A	13,33	3	2	135	15,3	10,51	55,35
P32775	GLC1	2,56	2	2	704	81,1	6,16	54,69
P32835	GSP1	16,44	5	3	219	24,8	6,55	53,92
Q08108	PLB3	3,35	2	2	686	75,0	5,03	53,72
Q08977	YPL260W	1,81	1	1	551	62,7	5,12	53,41
P40202	CCS1	6,02	1	1	249	27,3	6,67	53,24
P38636	ATX1	23,29	2	1	73	8,2	8,44	53,01
P01094	PAI3	39,71	3	2	68	7,7	7,40	52,82
P10622	RPP1B	15,09	1	1	106	10,7	4,01	52,59
P38911	FPR3	2,68	1	1	411	46,5	4,46	52,47
Q04371	YMR027W	3,62	3	2	470	54,1	5,64	52,30
P38109	YBR139W	8,86	2	2	508	57,6	5,31	51,81
P53044	UFD1	6,37	4	2	361	39,8	6,21	51,34
P38972	ADE6	1,18	2	1	1358	148,8	5,27	51,22
P37292	SHM1	2,86	2	1	490	53,7	8,72	50,13
P38131	KTR4	2,16	2	1	464	54,5	4,84	50,03
P47068	BBC1	1,04	2	1	1157	128,2	5,26	49,94
P39003	HXT6	4,04	1	1	570	62,7	7,75	49,28
P50086	NAS6	4,39	2	1	228	25,6	6,44	48,92
P40363	YJL068C	7,36	3	2	299	33,9	6,71	48,84
P40414	TPM2	8,70	1	1	161	19,1	4,51	48,75
P11632	NHP6A	13,98	1	1	93	10,8	9,76	48,55
P05750	RPS3	7,08	2	1	240	26,5	9,41	48,09
Q12408	NPC2	6,36	3	1	173	19,1	4,44	47,69
P40213	RPS16A	8,39	1	1	143	15,8	10,26	47,61
P53315	SOL4	5,88	2	1	255	28,4	5,35	47,58
P39743	RVS167	2,28	1	1	482	52,7	6,01	47,56
Q04013	YHM2	2,55	2	1	314	34,2	9,88	47,56

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
Q12329	HSP42	2,93	1	1	375	42,8	5,08	47,52
P05735	RPL18A	4,76	1	1	189	21,7	11,36	47,28
P38773	DOG2	8,54	4	2	246	27,1	5,74	47,21
P53600	RET3	14,29	2	2	189	21,7	5,16	46,81
P35127	YUH1	6,36	1	1	236	26,4	4,56	46,69
P40212	RPL13B	5,53	2	1	199	22,5	11,08	46,66
P0C0W9	RPL11A	8,05	1	1	174	19,7	9,92	46,50
P32527	ZUO1	8,31	2	2	433	49,0	8,25	46,35
Q03674	PLB2	4,82	3	2	706	75,4	4,70	46,05
A6ZRK4	LAP3	6,42	3	2	483	55,5	8,75	45,93
P00427	COX6	12,84	1	1	148	17,3	6,05	45,48
P35176	CPR5	27,11	5	4	225	25,3	5,60	45,22
P43321	SMD3	8,91	2	1	101	11,2	9,99	44,46
Q08647	PUS7	2,07	1	1	676	77,0	7,80	44,45
P13517	CAP2	8,01	2	2	287	32,6	4,72	44,39
P27616	ADE1	9,48	3	3	306	34,6	5,95	43,89
P38922	HRB1	2,64	1	1	454	52,1	7,59	43,89
P04802	DPS1	3,59	2	2	557	63,5	6,58	43,46
Q06325	YPS7	4,36	3	2	596	64,5	4,96	43,41
P39522	ILV3	2,05	1	1	585	62,8	7,83	43,41
P43619	BNA6	13,56	2	2	295	32,3	5,85	43,28
Q12012	YOR289W	3,98	1	1	251	29,1	8,34	43,03
Q99321	DDP1	18,09	2	2	188	21,6	8,15	42,90
P35271	RPS18A	11,64	3	2	146	17,0	10,27	42,40
Q12031	ICL2	1,57	2	1	575	64,9	7,56	42,25
P40518	ARC15	7,14	2	1	154	17,1	5,31	41,94
P32599	SAC6	2,49	2	1	642	71,7	5,48	41,82
P32191	GUT2	1,23	1	1	649	72,3	7,90	41,73
P06367	RPS14A	9,49	1	1	137	14,5	10,73	41,48
P33330	SER1	2,53	1	1	395	43,4	6,54	41,48
Q08412	CUE5	9,49	2	2	411	46,8	4,81	41,01
Q12335	PST2	15,66	1	1	198	21,0	5,73	40,70
P40093	YER156C	2,96	1	1	338	38,2	7,49	40,60
P06786	TOP2	0,98	1	1	1428	164,1	7,03	40,40
Q12165	ATP16	12,50	1	1	160	17,0	6,55	39,80
Q04792	GAD1	1,88	1	1	585	65,9	6,62	39,75
P39940	RSP5	3,09	2	2	809	91,8	6,70	39,53
Q06217	SMD2	7,27	2	1	110	12,8	9,16	39,29
Q06625	GDB1	1,76	2	2	1536	174,9	5,77	39,10
P32623	UTR2	5,57	2	2	467	49,9	4,78	38,65
P53691	CPR6	3,50	1	1	371	42,0	6,16	38,50
P08417	FUM1	2,66	1	1	488	53,1	8,25	38,49
P27809	KRE1	3,85	2	1	442	51,4	5,40	37,88
Q04638	ITT1	1,51	2	1	464	54,1	5,55	37,80
P32787	MGM101	10,41	2	2	269	30,1	9,32	37,38

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P40582	GTT1	5,13	1	1	234	26,8	6,65	37,16
P38910	HSP10	10,38	1	1	106	11,4	9,00	37,05
A6ZZH2	MCR1	5,30	2	2	302	34,1	8,65	37,00
P06101	CDC37	8,70	3	3	506	58,3	5,06	36,63
P47037	SMC3	0,89	2	1	1230	141,2	5,81	36,04
P05030	PMA1	2,18	1	1	918	99,6	5,11	35,97
P16140	VMA2	2,71	1	1	517	57,7	5,07	35,67
P32368	SAC1	2,89	1	1	623	71,1	7,71	35,35
P54070	KTR6	1,79	2	1	446	52,1	5,50	34,59
P53189	SCW11	2,40	2	1	542	56,4	4,48	34,22
P39015	STM1	4,76	2	1	273	30,0	9,66	33,91
P02406	RPL28	6,71	2	2	149	16,7	10,62	33,72
P14120	RPL30	18,10	2	1	105	11,4	9,80	33,69
P32472	FPR2	7,41	1	1	135	14,5	5,50	33,22
P46680	AIP1	3,09	2	2	615	67,3	5,59	32,99
P16861	PFK1	0,91	1	1	987	107,9	6,39	32,93
A6ZV70	CTT1	2,67	1	1	562	64,5	6,54	32,72
P38011	ASC1	2,82	1	1	319	34,8	6,24	32,58
P20051	URA4	5,77	3	2	364	40,3	6,54	32,11
P38777	FSH1	7,82	1	1	243	27,3	6,35	32,01
P28777	ARO2	5,59	1	1	376	40,8	7,80	31,90
P0CW40	IMA3	1,70	1	1	589	68,7	5,59	31,74
P29547	CAM1	2,65	1	1	415	47,1	8,38	31,60
P53299	TIM13	10,48	2	1	105	11,3	8,16	31,58
A6ZUT6	PXR1	2,58	2	1	271	31,4	9,86	31,54
A6ZS33	SCY_4744	1,19	1	1	671	77,3	6,58	31,47
P19262	KGD2	1,94	2	1	463	50,4	8,85	30,83
A6ZT54	CRP1	2,58	1	1	465	51,1	7,34	30,78
P25654	YCR090C	3,85	1	1	182	20,7	4,88	30,73
P19358	SAM2	2,08	1	1	384	42,2	5,38	30,66
P53163	MNP1	5,67	1	1	194	20,6	9,19	30,16
P33416	HSP78	1,23	1	1	811	91,3	8,05	30,07
P11745	RNA1	1,97	1	1	407	45,8	4,61	30,06
P04076	ARG4	6,70	2	2	463	52,0	5,73	29,98
P25491	YDJ1	3,42	1	1	409	44,6	6,30	29,89
P32867	SSO1	8,62	2	2	290	33,1	5,30	29,59
P32558	SPT16	1,35	1	1	1035	118,6	5,10	29,28
Q99288	DET1	4,49	1	1	334	39,1	7,34	29,10
Q12287	COX17	18,84	2	1	69	8,1	4,92	28,24
P00812	CAR1	2,40	1	1	333	35,6	5,64	28,01
P32914	SPT4	14,71	1	1	102	11,2	5,12	28,00
P42884	AAD14	2,39	1	1	376	42,0	6,74	27,78
P49954	NIT3	4,47	1	1	291	32,5	7,55	27,71
P0C2H6	RPL27A	5,15	1	1	136	15,5	10,36	27,54

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P53303	ZPR1	2,06	1	1	486	55,0	4,86	27,54
P05626	ATP4	6,15	1	1	244	26,9	9,13	27,50
P32917	STE5	1,09	1	1	917	102,7	5,45	27,31
P25332	RBK1	3,00	1	1	333	36,9	5,29	27,30
Q04212	ARA2	2,69	1	1	335	38,2	5,57	27,16
P25294	SIS1	3,98	1	1	352	37,6	9,03	27,10
Q6Q546	HUB1	16,44	1	1	73	8,3	6,54	27,08
Q06549	CDD1	6,34	1	1	142	15,5	7,17	26,92
P43569	CAF16	3,11	1	1	289	33,1	6,65	26,92
P32447	ASF1	7,89	1	1	279	31,6	3,99	26,82
A6ZVM6	CBR1	7,39	2	1	284	31,4	8,78	26,72
P25443	RPS2	5,91	1	1	254	27,4	10,43	26,51
A6ZNU5	RKI1	5,43	1	1	258	28,2	5,92	26,17
Q05778	PBA1	7,61	1	1	276	30,7	4,81	26,13
P28273	OXP1	1,09	1	1	1286	140,3	6,77	26,02
P46969	RPE1	2,94	1	1	238	26,0	6,28	25,98
P38071	ETR1	2,37	1	1	380	42,0	9,00	25,96
P34227	PRX1	3,83	1	1	261	29,5	8,87	25,92
P05737	RPL7A	8,61	1	1	244	27,6	10,15	25,83
A5Z2X5	YPR010C-A	22,22	1	1	72	7,9	8,73	25,55
P05748	RPL15A	6,86	1	1	204	24,4	11,39	25,45
P41920	YRB1	3,48	1	1	201	22,9	6,10	25,31
P05736	RPL2A	4,72	1	1	254	27,4	11,11	25,25
P11978	SIR4	0,81	1	1	1358	152,0	9,00	25,03
P41697	BUD6	1,40	1	1	788	88,8	7,78	24,76
Q12247	FYV7	5,96	1	1	151	18,2	10,26	24,40
P25293	NAP1	5,76	1	1	417	47,9	4,34	24,37
P11914	MAS2	1,45	1	1	482	53,3	6,34	24,35
P10869	HOM3	2,09	1	1	527	58,1	6,67	24,19
Q05788	PNP1	4,50	1	1	311	33,7	7,27	24,06
O14455	RPL36B	7,00	1	1	100	11,1	11,59	23,80
P53981	YNL010W	4,15	1	1	241	27,5	5,45	23,78
P33315	TKL2	1,76	1	1	681	75,0	6,14	23,21
P14747	CMP2	1,82	1	1	604	68,5	6,35	23,21
P38041	BOI1	0,92	1	1	980	109,2	9,10	23,05
P22082	SNF2	0,65	1	1	1703	193,9	7,01	22,95
P47075	VTC4	1,25	1	1	721	83,1	6,83	22,92
Q04869	YMR315W	5,44	1	1	349	38,2	6,62	22,79
P33734	HIS7	1,99	1	1	552	61,0	5,49	22,78
Q04947	RTN1	4,07	1	1	295	32,9	8,98	22,77
P38283	SLI15	1,29	1	1	698	79,1	9,92	22,69
P36126	SPO14	0,36	1	1	1683	195,1	7,59	22,49
P07260	TIF45	2,82	1	1	213	24,2	5,49	22,49
Q04458	HFD1	1,69	1	1	532	59,9	6,76	22,31
P15496	IDI1	4,51	1	1	288	33,3	4,98	22,29

Accession	Protein	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score
P14284	REV3	1,60	1	1	1504	172,8	8,65	21,87
P15202	CTA1	4,47	1	1	515	58,5	7,44	21,85
Q03868	SPO7	0,48	1	1	1245	145,1	9,13	21,66
Q07451	YET3	5,91	1	1	203	22,9	7,14	21,56
P38262	SIF2	2,24	1	1	535	59,1	4,96	21,50
Q02794	STD1	2,70	1	1	444	50,2	8,98	21,26
P38077	ATP3	3,54	1	1	311	34,3	9,31	21,17
A6ZPQ6	MPM1	7,14	1	1	252	28,5	5,92	21,14
P87284	PMP3	34,55	1	1	55	6,1	4,46	21,08
P00815	HIS4	2,13	1	1	799	87,7	5,29	20,92
Q02753	RPL21A	5,63	1	1	160	18,2	10,39	20,71
P26781	RPS11A	6,41	1	1	156	17,7	10,78	20,05