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Escola de Ciências

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Dissertação de Mestrado
Mestrado em Genética Molecular

Trabalho realizado sob orientação da
Professora Doutora Cândida Lucas
e da
Doutora Célia Ferreira

Janeiro de 2013

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Título da Tese: Identification of the *Saccharomyces cerevisiae* Target of Cetuximab/Erbitux®, the Anti-EGFR Antibody Used in the Treatment of Colorectal Cancer

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Designação do Mestrado: Genética Molecular

Universidade do Minho, ___/___/___

Assinatura: _____

ACKNOWLEDGMENTS

I would like to thank to everyone who has contributed in some way to this project.

To my supervisors, Prof Cândida Lucas and Célia Ferreira, for the scientific guidelines and counseling, thesis proof reading and for all the opportunities.

To Fábio for the all the scientific discussions, endless support and friendship.

To the lab colleagues, especially Paulo for friendship and good times spent together.

To the Crime Partners. You know who you are ;-)

À família: Obrigada pelo apoio, por todos os sacrifícios, pelos conselhos sempre sábios, pela compreensão, por tudo aquilo que me ensinaram e me ajudaram a alcançar.

We thank Merck Serono S.A. (Merck KGaA, Darmstad, Germany) for agreeing in establishing the MTA that provided the generous amounts of cetuximab/Erbitux® used in this study.

Identification of the *Saccharomyces cerevisiae* Target of Cetuximab/Erbitux®, the Anti-EGFR Antibody Used in the Treatment of Colorectal Cancer

ABSTRACT

Colorectal cancer (CRC) is one of the most common malignancies affecting mankind. CRC cells over-express epidermal growth factor receptor (EGFR), which usually correlates with disease poor prognosis and reduced response to therapy. Hence, several therapeutic agents against EGFR were developed, *viz.* the monoclonal antibody cetuximab/Erbitux®. Such drug competes with EGFR ligands for binding to L2/III domain, which results in EGFR internalization and subsequent degradation, leading to inhibition of cell growth and angiogenesis, and induction of apoptosis. Yet, cancer patients may display or acquire resistance-inducing mutations in EGFR, as well as in its downstream effectors. These contribute to a significant degree of ineffectiveness of treatment, being one of the most prominent problems in CRC clinical assessment.

Given the high degree of conservation of eukaryotic cellular processes, yeast has been a model of choice for research in many human pathologies. In this line, this work aimed at the identification of *S. cerevisiae* surface target of cetuximab, ultimately seeking for the possible EGFR yeast counterpart. Two different strategies were used: (1) *in silico* sequence and structure homology search, and (2) immune recognition in a cetuximab-based Western blot.

The first approach pointed to proteins from the yeast Sporulation specific family, especially Sps2p and Sps22p. These have some structural resemblance with EGFR leucin-rich L-domains, along with cell-surface localization. Conversely, the Western blot clearly identified the Pdc1p (pyruvate decarboxylase isoform 1) as cetuximab antigen. The subsequent detailed analysis of protein features revealed that Pdc1p, as well as its close homologue Pdc5p, present some similarity with EGFR epitope sequence. Moreover, Pdc and EGFR also present some functional pathway overlapping, more evident in malignantly transformed cells. The recognition of Pdc1/5p as cetuximab antigen, combined with its extracellular localization described before, suggests that Pdc1p may have distinct functions beyond glycolytic catalysis/regulation. The double deletion of Sps and Pdc, and the use of diploid genetic background, will be needed to devise the true existence of growth phenotypes induced by cetuximab. However, this work opens a large window as to future research in novel pathways in yeast, beyond the continued exploration of yeast for the aim of generating a tool for CRC patients' theranostics.

Identificação em *Saccharomyces cerevisiae* do Alvo do Cetuximab/Erbitux®, o Anticorpo Anti-EGFR Utilizado no Tratamento do Cancro Colo-rectal

RESUMO

O cancro colo-rectal (CRC) é uma das enfermidades mais comuns no mundo. Células de CRC apresentam sobre-expressão do recetor do fator de crescimento epidérmico (EGFR), normalmente associada a um pior prognóstico e uma resposta reduzida à terapia. Desta forma, vários agentes contra EGFR foram desenvolvidos, tal como o anticorpo monoclonal cetuximab/Erbitux®. Este compete com os ligandos do EGFR para o domínio L2/III, resultando na internalização e degradação do EGFR. Isto leva à inibição da proliferação celular e angiogénese e, à indução de apoptose. No entanto, os pacientes com CRC podem ter ou adquirir mutações no EGFR, ou nos efetores da sinalização a jusante, que induzem resistência às opções terapêuticas. Estas situações contribuem para uma significativa ineficácia no tratamento do CRC, tornando-se um dos principais problemas da assistência médica a estes doentes.

A elevada conservação de processos celulares eucarióticos tornou a levedura um modelo privilegiado no estudo de muitas patologias humanas. Nesse sentido, este trabalho visou a identificação do alvo do cetuximab em *S. cerevisiae*, em última instância, contribuindo para uma possível identificação da proteína de levedura correspondente ao EGFR. Foram usadas duas estratégias: (1) procura *in silico* de homologia de sequência e estrutura e, (2) imuno-reconhecimento pelo cetuximab (Western blot).

A primeira abordagem apontou para a família de proteínas específicas de esporulação (Sporulation specific), nomeadamente as proteínas Sps2p e Sps22p. Estas apresentam alguma semelhança estrutural com os domínios do EGFR ricos em leucinas (domínios L), além de também serem proteínas da superfície celular. Por outro lado, por Western blot identificou-se a Pdc1p (piruvato descarboxilase isoforma 1) como antigénio do cetuximab. Uma análise detalhada subsequente da composição aminoacídica revelou que a Pdc1p, bem como a sua homóloga Pdc5p, apresentam alguma similaridade com a sequência do epítopo do EGFR. Além disso, Pdc e EGFR também apresentam alguma sobreposição funcional, em particular no que diz respeito ao metabolismo das células malignas. O reconhecimento da Pdc1/5p como antigénio do cetuximab, em combinação com a descrição anterior desta proteína na superfície da célula, sugere que a Pdc1p pode ter outras funções para além do seu papel catalítico e regulador no âmbito da glicólise. Para aceder ao fenótipo que o cetuximab possa induzir no crescimento e viabilidade da levedura serão necessárias deleções duplas dos membros das famílias Sps e Pdc, bem como a utilização de estirpes diplóides de levedura. Apesar disso, e para além de permitir a prossecução do objetivo de transformar a levedura numa ferramenta para teranóstico, o presente trabalho abre uma grande janela na investigação em novas vias de sinalização em levedura.

TABLE OF CONTENTS

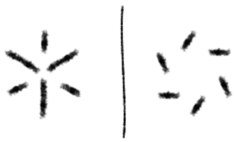
ABBREVIATIONS

Akt	Also known as Protein Kinase B
APC	Adenomatous polyposis coli
AR	Amphiregulin
ATP	Adenosine triphosphate
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-x_L	B-cell lymphoma-extra large
BCR	Breakpoint cluster region
BLAST	Basic local alignment search tool
BLOSUM	Blocks of amino acid substitution matrix
BRAF	v-raf murine sarcoma viral oncogene homologue B1
BTC	Betacellulin
c-ABL	c-abl oncogene 1, non-receptor tyrosine kinase
CAM	Cell adhesion molecule
CAMIIK	Calmodulin II kinase
c-Kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue
CRC	Colorectal cancer
CREB	cAMP response element-binding protein
DAG	Diacyl glycerol
DER	*Drosophila EGFR*
E Value	Expect Value
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	EGFR variant III
eIF2B	eukaryotic initiation factor 2
EMT	Epithelial mesenchymal transition
EPR	Epiregulin
ERBB1	See EGFR
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homologue 2
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homologue 3
ERBB4	v-erb-a erythroblastic leukemia viral oncogene homologue 4
ERK	Extracellular regulated kinase
FAb	Fragment antigen binding
Fc	Fragment constant
FGF	Fibroblast growth factor
Fv	Fragment variable (see FAb)
GDP	Guanosine diphosphate

GO	Gene ontology
GPI	Glycosylphosphatidylinositol
GRB2	Factor receptor-bound protein 2
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HACL1	2-Hydroxyacyl-CoA lyase 1
HB-EGF	Heparin-binding EGF
HER1	See EGFR
HER2	See ERBB2
HER3	See ERBB3
HER4	See ERBB4
HMM	Hidden Markov models
HOG	High osmolarity glycerol
HRG	Heregulins
HRP	Horseradish peroxidase
IGF	Insulin growth factor
IGF1R	Insulin-like growth factor-1 receptor
IgG1	Immunoglobulin G subclass 1
IP3	Inositol 1,4,5-triphosphate
IR	Insulin receptor
JAK	Janus kinase
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue
mAb	Monoclonal antibody
MALDI	Matrix Assisted Laser Desorption Ionization
MAPK	Mitogen-activated protein kinases
miRNA	microRNA
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
NADH	Nicotinamide adenine dinucleotide
NCBI	National center for biotechnology information
NDF	Neu differentiation factors
NEU	See ERBB2 or HER2
NF-kB	Nuclear factor kappa B
NGF	Nerve growth factor
NRDB	Non-redundant database
OD	Optical density
ORF	Open reading frame
p53	Protein 53
Pdc	Pyruvate decarboxylase
PDGFR	Platelet-derived growth factor receptor
PDK1	3-phosphoinositide-dependent protein kinase-1

PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PIR	Proteins with internal repeats
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein kinase C
PLC	Phosphoinositide phospholipase C
PTB	Phosphotyrosine binding
PtdIns(3,4,5)P3	See PIP3
PtdIns(4,5)P2	See PIP2
Puma	p53 upregulated modulator of apoptosis
Rb	Retinoblastoma
RET	ret proto-oncogene
ROS	Reactive oxygen species
Rpl	Ribosomal protein of the large subunit
RTK	Receptor tyrosine kinase
SCOP	Structural classification of proteins
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGD	*Saccharomyces* genome database
SH1	Src homology domain 1
SH2	Src homology 2
Sps	Sporulation specific
STAT	Signal transducer and activator of transcription
TBS	Tris buffer saline
TBST	Tris buffer saline tween
TCA	Tricarboxylic acid cycle
TGF-α	Transforming growth factor-α
TOF	Time of flight
TOR	Target of rapamycin
TP53	Tumour protein 53
VEGFR	Vascular epidermal growth factor receptor

Introduction



1. Biological Information Processing

All forms of communication between humans have long been recognized as a condition for mutual understanding, transfer of knowledge, and productive development of societies. Similarly to humans, cell communication is crucial to coordinate the myriad of activities needed for any organism (unicellular or multicellular, prokaryote or eukaryote) to grow, develop and function [1, 2]. Throughout a set of cell signalling mechanisms, cells communicate by sending and receiving signals that may arrive from the environment or from other cells. These signal information is then processed in order to appropriate responses within the cellular environment can be made [3, 4]. The mechanisms that enable one cell to influence the behaviour of another almost certainly existed in the world of unicellular organisms long before multicellular organisms appeared on Earth. For instance, bacteria sense and respond to a wide range of physicochemical signals such as temperature, light, and oxygen tension from the ever-changing environment [5]. These prokaryotes also communicate between them using chemical signal molecules. This involves producing, releasing, detecting, and responding to small hormone-like molecules termed autoinducers. This process, termed *quorum sensing*, allows bacteria to coordinate their behaviour on a population-wide scale in what concerns mobility, biofilm formation, antibiotic production, spore formation and sexual conjugation [6-9]. Yeasts, another unicellular organism, evolved autonomous mechanisms for adapting to drastic environmental changes such as fluctuations in the types and quantities of available nutrients, temperature, osmolarity and acidity of their environment, and the variable presence of noxious agents, such as radiation and toxic chemicals [10]. Furthermore, yeasts influence each other's behaviour in preparation for mating. In the budding yeast *Saccharomyces cerevisiae*, for example, when a haploid individual is ready to mate, it secretes a peptide mating factor that signals cells of the opposite mating type to stop proliferating and prepare to mate [11]. Yeast colonies, organised multicellular structures, can as well communicate at long distance by means of volatile ammonia [12]. Plant cells, as in any other multicellular organism, communicate to coordinate their activities in response to changing conditions either in the external environment or in the microenvironment surrounding the organ, the tissue or the cell [13]. For example, in the human body, pancreatic cells release insulin to inform muscle cells to take up sugar from the blood for energy [14]. Identically, cells of the immune system instruct other immune cells to attack invaders [15], and cells of the nervous

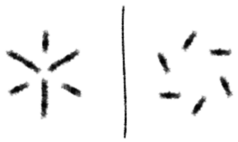
system rapidly fire messages to and from the brain [16]. Cells also exchange information in what regards patterning/morphogenesis of a wide variety of tissues in an embryo [17]. Those messages elicit the right responses only because they are transmitted accurately far into a recipient cell and to the exact molecules able to carry out the directives commanding a pattern of differentiation. Globally, exchanges of information between neighbouring cells occurs either directly, when cells are compactly packed in a tissue, or through the surrounding extracellular matrix that provides support and anchorage and regulates the type and range of diffusion of signalling molecules [2, 18].

The ability of cells to perceive and correctly respond to their microenvironment is the basis of development, tissue repair, and immunity as well as normal tissue homeostasis. Errors in cellular information processing are responsible for diseases such as autoimmunity, diabetes and the leading cause of death worldwide – cancer.

1.1. Cancer, When Cellular Communication Goes Wrong

1.1.1. The Origin and Burden

Human beings have had cancer throughout recorded history, although the word cancer was not used. Some of the earliest evidence of cancer was found among fossilized bone tumours, human mummies in ancient Egypt, and ancient manuscripts. The oldest description of cancer was discovered in Egypt and dates back to about 3000 BC. The origin of the word cancer is credited to the Greek physician Hippocrates (460-370 BC), who used the terms *carcinos* and *carcinoma* to describe non-ulcer forming and ulcer-forming tumours. The Roman physician, Celsus (28-50 BC), later translated the Greek term into *cancer*, the Latin word for crab (the finger-like spreading projections reminded a crab). Galen (130-200 AD), another Roman physician, used the word *oncos* (Greek for swelling) to describe tumours. Although the crab analogy of Hippocrates and Celsus is still used to describe malignant tumours, Galen's term is now used as a part of the name for cancer specialists - the oncologists. While there were some additions to the medicinal understanding of cancer in the Middle Ages, it was not until the nineteenth century that microscopic work by German pathologists including Müller and Virchow, identified the cellular origins of cancer [19, 20].



Cancer is a leading cause of death worldwide [21]. Based on the GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 [22]. Colorectal cancer (CRC), with over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred worldwide in 2008, is the third most frequent cancer in men, after prostate and lung, and the second most common in women, after breast [21, 23]. The burden of cancer is increasing in economically developing countries as a result of population growth, and in economically developed countries as a consequence of an increase in population life span/age, adding to, increasingly, an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and ''westernized'' diets [24-28]. It should also be noted that cancer tends to be diagnosed at late stages in many developing countries compared with developed countries. This, combined with reduced access to appropriate therapeutic facilities and drugs, has an adverse effect on survival [23]. Current data on Portuguese cancer patients is limited, and there are divergences in methods of data collection and treatment amongst regional cancer registries. However, the available data from 2009 help to understand the dimension of CRC, with an incidence of 37 per 100,000 and mortality of 31 per 100,000 annually [29].

1.1.2. Definition and Genetics

It is difficult to define cancer with precision. Still, cancer can be described as an abnormal growth of cells caused by multiples changes in gene expression leading to an unregulated balance of cell proliferation and cell death. Eventually, this evolves into a population of cells able to invade and metastasize to adjacent tissues, resulting in morbidity and even death of the host if not treated. Cancer is not one disease but many diseases. There are more than 100 different types of cancer, and subtypes of tumours can be found within specific organs [30]. Boveri, in the early 1900s, was the first one to propose that malignant tumours would probably arise from a defect in the nucleus, at the chromosome level. Therefore cancer would be a genetic disease of somatic cells. He also predicted what would be later called oncogenes and tumour-suppressor genes [31].

Normal cells can derive into cancer cells by several steps, a process known as carcinogenesis, which comprises a progression of changes on cellular and genetic level that ultimately reprograms the cells. To cause the development of a cancer, usually, a single genetic

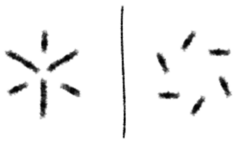
alteration/mutation is not enough. Cancer is a multi-step and multi-gene pathology, involving sequential alterations in several oncogenes, tumour-suppressor genes, and microRNA (miRNA) genes [32, 33]. Oncogenes result from gain-of-function mutations in proto-oncogenes, the normal function of which is to drive cell proliferation and/or apoptosis in the appropriate contexts [34]. There are several mechanisms by which proto-oncogenes can be altered, for example: amplification, point mutation and chromosomal translocation. Several oncogenes are found in a diversity of human solid malignancies, with variations on the mechanism of activation and biochemical functions [32]. Tumour-suppressor genes are called like this once their protein products normally inhibit cell proliferation and are inactivated through loss-of-function mutations: point mutations, microdeletions or insertions, large deletions or even translocations [32, 35]. Implication of miRNA in cancer development has been shown to occur through processes of gain and loss of function [36, 37]. In what concerns to modifications of miRNA, these can be a consequence of amplifications, deletions, or mutations involving miRNA *loci*, epigenetic silencing or deregulation of transcription factors that target specific miRNA [37].

Cancer origin can be associated with environmental factors, such as diet or life style as mentioned above, but as a genetic disease, cancer can also be hereditary. In 1971, Knudson studied cases of retinoblastoma (cancer that develops in the cells of retina), hypothesizing that this form of cancer was caused by two mutational events: the two hits model, as it is known, postulates that in the hereditary form the first hit (mutation) is inherited from germ-line cells and the second hit occur in somatic cells. In non-inherited retinoblastoma both hits take place in somatic cells [38, 39].

1.1.3. Cancer Hallmarks

The vast catalogue of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth and these are: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Fig. 1) [40, 41].

Normal tissues carefully control the production and release of growth-promoting signals that are carried through the cell membrane, binding cell-surface receptors (normally having



intracellular tyrosine kinase domains) [42]. These signalling molecules instruct entry into and progression through the cell growth and division cycle, thereby ensuring a homeostasis of cell number and thus maintenance of normal tissue architecture and function. Malignant cells have acquired the capability of maintain a proliferative state. This occurs (i) through the production of autocrine growth factor ligands; (ii) by sending signals to make normal cells, within the tumour-associated environment, produce various growth factors [43]; (iii) by deregulating receptor signalling, *i.e.*, elevating levels of receptors proteins displayed at the cancer cell surface, turning cells highly responsive to growth factor ligands; and (iv) by changing the structure of receptor molecules or constitutively active components of signalling pathways (downstream of receptors) [41]. This last case applies to the pathway responding to the Ras signal transducer (for details, see section 2.1.1 - *Signalling Pathways Downstream of EGFR* ahead in this Introduction).

Another common capacity in tumours cells is the insensitivity to growth-inhibitory signals, *i.e.* evading growth suppressors. Many tumour-suppressor genes that limit cell growth and proliferation are inactivated. For example, the p53 and Rb proteins are encoded by tumour-suppressor genes, and are responsible for cell-cycle progression. Changes in Rb, APC (adenomatous polyposis coli) and p53 function allow persistent cell proliferation [41, 44].

Cancer cells have found a variety of strategies to diminish apoptosis and as a result resist cell death. The loss of *TP53* tumour-suppressor function, which in normal cells induces apoptosis in response to DNA damage, is one of the alterations that allow these cells to avoid death [45]. Plus, tumour cells can get the same results by (i) increasing the expression of antiapoptotic regulators (Bcl-2, Bcl-x_L), (ii) by downregulating proapoptotic factors (Bax, Bim, Puma) or (iii) by interrupting the extrinsic ligand-induced death pathway [41]. Apoptosis, autophagy and cellular homeostasis have been shown to share some regulatory pathways. Thus, the PI3K (phosphatidylinositol 3-kinase), Akt (also known as Protein Kinase B - PKB) and mTOR (mammalian Target of Rapamycin) kinases, implicated in a signalling pathway stimulated by survival signals to block apoptosis, also inhibit the autophagy process. As a result, in a situation where survival signals are not sufficient, the PI3K signalling pathway is downregulated and therefore autophagy and/or apoptosis may be induced [46, 47]. In the context of neoplasia, cell death by necrosis can also occur and can lead to the release of bioactive regulatory factors, which can directly stimulate neighbouring viable cells to proliferate, with the potential, once again, to facilitate neoplastic progression. Additionally, necrotic cells can recruit immune

inflammatory cells that can be actively tumour promoting, given that such cells are capable of fostering angiogenesis, cancer cell proliferation, and invasiveness [48].

Malignant cells are also known for their immortality. The immortalization potential has been attributed to cells ability to maintain adequate telomeric DNA lengths to avoid senescence or apoptosis. This hallmark is a result of an upregulation of the telomerase enzyme expression using a recombination-based telomere maintenance mechanism [41, 49].

Tumours, just like normal tissues, need to obtain nutrients and oxygen, and get rid of carbon dioxide and waste compounds produced by the metabolic activity. The tumour-associated neovasculature, generated by the process of angiogenesis, addresses these needs. During tumour progression, angiogenesis is almost always activated and remains on, which make the normally quiescent vasculature to continually sprout new vessels that help sustain expanding neoplastic growths [50].

Another cancer hallmark is the ability to invade other tissues and metastize which are important mechanisms for disease outcome. New blood vessels created by angiogenesis or even lymphatic system provide an escape for tumours cells, allowing them to enter into the blood stream (intravasion). The following step is extravasation, in which cells quit the blood stream to the surrounding tissues, forming small nodules (micrometastases) and growing until macroscopic tumours (colonization) [51]. A fundamental process to cells acquire this capacity is the loss of E-cadherin (cell-to-cell adhesion molecule) that helps to assemble epithelial cell sheets and maintain the quiescent state of cells. Furthermore, in aggressive carcinomas, expression genes encoding cell-to-cell and cell-to-extracellular matrix molecules is perceptibly altered. For instance, E-cadherin, the prototypical adhesion molecule of epithelia, is frequently lost in epithelial malignancies, whereas the related N-cadherin, absent in normal epithelia, is upregulated in many invasive tumours [52, 53]. The epithelial-mesenchymal transition has also been highly implicated as a means by which transformed epithelial cells can acquire the ability to invade, to resist apoptosis, and to disseminate [54].

These hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. Underlying these hallmarks are genome instability [41, 55, 56], which generates the genetic diversity that expedites their acquisition, and inflammation, which fosters multiple hallmark functions supplying the tumour microenvironment with bioactive molecules,

such as growth factors, which will maintain proliferation, survival factors (limiting cell death) among others [57-60].

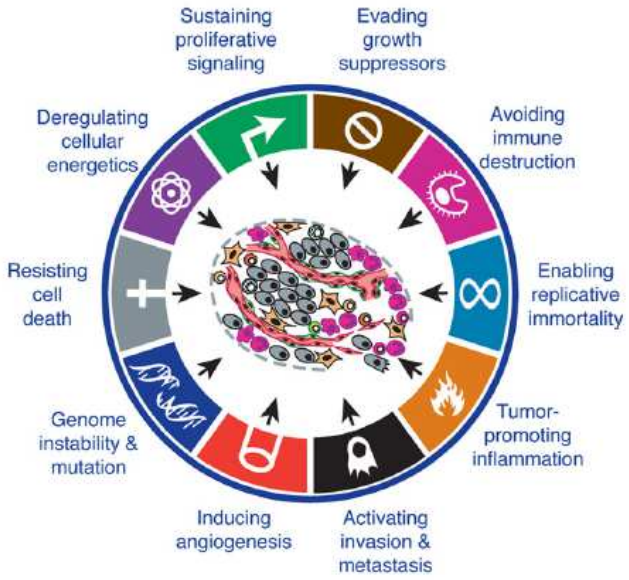


Figure 1 - The hallmarks of cancer. Adapted from [41].

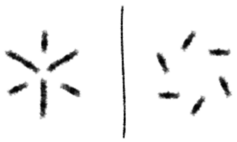
Conceptual progress in the last decade has added two emerging hallmarks: reprogramming of energy metabolism and evading immune destruction (Fig. 1) [41]. The first involves major reprogramming of cellular energy metabolism in order to support continuous cell growth and proliferation, replacing the metabolic program that operates in most normal tissues and fuels the physiological operations of the associated cells. Some tumours have been found to contain two subpopulations of cancer cells that differ in their energy-generating pathways. One subpopulation consists of glucose-dependent (Warburg-effect) cells that secrete lactate, whereas cells of the second subpopulation preferentially import and utilize the lactate produced by their neighbours as their main energy source, establishing what can be a relation of intratumoural symbiosis [61, 62]. The second emerging hallmark involves active evasion of the cancer cells from the attack and elimination by immune cells, mostly by T and B lymphocytes, macrophages and natural killer cells [63, 64]. Both of these capabilities may well prove to facilitate the development and progression of many forms of human cancer and therefore can be considered to be emerging hallmarks of cancer [41]. In addition to cancer cells, tumours exhibit another dimension of complexity: they contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the “tumour microenvironment”.

Recognition of the widespread applicability of these concepts will increasingly affect the development of new means to treat human cancer [41].

1.1.4. Tumour Therapy

Successful accomplishments have been made in tumour therapy strategy by inhibiting some oncogenes achieving tumour cell death, differentiation or senescence. Drugs targeting protein kinase oncogenes such as BCR-ABL (imatinib/Gleevec®), EGFR (cetuximab/Erbitux®) and HER2 (trastuzumab/Herceptin®) have been used in some types of cancer, including CRC [65-67]. A multi-kinase inhibitor sorafenib/Nexavar® (BAY 43-9006) prevents tumour growth by inhibiting tumour cell proliferation and angiogenesis, and/or pro-apoptotic effects [68]. This agent blocks tyrosine kinase receptors signalling (VEGFR, PDGFR, c-Kit and RET) and, consequently, inhibits downstream Ras serine/threonine kinase activity [69, 70]. Imatinib, another c-Kit tyrosine kinase inhibitor, has a therapeutic effect in CRC cells expressing the *c-Kit* proto-oncogene by inhibiting cell proliferation and inducing apoptosis *in vitro* [71]. miRNAs appear to have a role in regulation of oncogenes and tumour-suppressor genes involved in several pathways in CRC [72, 73], thus being a further option for CRC therapy.

New biological agents acting on receptor kinases, counteracting epigenetic abnormalities, tumour vasculature and microenvironment particularities, are also being developed, and could help to improve the existent chemotherapy [74]. An up-to-date example relies on exploring metabolic specificities of malignant cells, namely in glycolytic flux regulation. Cancer cells activate glycolysis to meet their energy demands and use oxygen (O_2) to generate excessive levels of the reactive oxygen species (ROS) namely hydrogen peroxide (H_2O_2) [75]. Therefore tumour cells can be killed in a selective way by increasing cellular levels of H_2O_2 and/or diminishing glycolysis, using pro-oxidant agents and glycolysis inhibitors [76]. Additionally, solid tumours contain regions at very low oxygen concentrations (hypoxia). The cells in these hypoxic regions are resistant to both radiotherapy and chemotherapy. The bacterium *Clostridium* has been considered as an alternative strategy to selectively target and destroy cancer cells especially for the treatment of solid tumours. Scientific research has shown that various non-pathogenic strains of *Clostridium* are able to infiltrate and selectively replicate within solid tumours, therefore, recombinant



anaerobic clostridial spores could in the future be used as highly selective vectors for delivery of therapeutic proteins directly and specifically to the solid tumour [77-79].

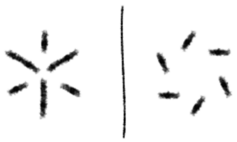
Despite the advances in medical practices and the progress obtained with the introduction of new cytotoxic agents, there are still a high number of cancer situations in which treatment is not effective. Failure of therapy can be due to the development of resistance to anticancer drugs as a result of host factors or a result of genetic and/or epigenetic changes in cancer cells. Thus, resistance can be intrinsic to the cancer or it can be acquired. The major mechanism of multidrug resistance seems to be associated to an energy-dependent drug efflux pump called P-glycoprotein, product of the *MDR1* gene, belonging to the ABC family of transporters. Proteins from the same family like MRP1 (multidrug resistance associated protein 1) are also shown to be overexpressed in anticancer resistance drugs situations [80]. Additionally, some points of evidence suggested that therapy resistance can also be a consequence of survival pathways activation during carcinogenesis by oncogenic transformation. Some examples of oncogenes that can activate survival pathways are Ras, Raf, c-Kit, HER2 and EGFR [81].

2. Mechanisms of Cell Communication and the Role of Cell Surface Receptors

Even the simplest organisms can detect and respond to events in their ever-changing environment [2]. Similarly, within a multicellular organism, cells are surrounded by an extracellular environment from which signals are received and responded to. Cell-cell and cell-matrix interaction are crucial for the development and proper functioning not only for complex multicellular organisms as also for of single celled ones [82]. Unlike free-living cells, which supposedly compete to survive, the cells of a multicellular organism are committed to collaboration. Any mutation that gives rise to selfish behaviour by individual members of the community will compromise the future of the whole enterprise. Mutation, competition, and natural selection operating within the population of somatic cells are the basic ingredients of cancer: it is a disease in which individual mutant cells begin by prospering at the expense of their neighbours but in the end destroy the whole cellular society and die [41].

Within multicellular organisms cells may interact with each other directly, requiring cell-cell contact, or indirectly, via molecules secreted by one cell, which are then carried away to target cells [11]. The extracellular signal molecules include proteins, small peptides, amino acids, nucleotides, steroids, retinoids, fatty acid derivatives, and even dissolved gases such as nitric oxide and carbon monoxide. Most of these signal molecules are secreted from the signalling cell into the extracellular space by exocytosis or by diffusion through the plasma membrane. Signalling via secreted signalling molecules can be paracrine (acting on neighbouring cells) and/or autocrine (acting on the cell that secretes the signalling molecule) [11, 83]. Cancer cells, for example, often use this strategy to stimulate their own survival and proliferation. Intercellular communication over large distances is achieved through endocrine signalling (hormones) or electrical signalling (between neurons or between a neuron and a target cell). Some signalling molecules, however, are exposed to the extracellular space while remaining tightly bound to the signalling cell's surface providing a signal to other cells only when they make contact. This type of signalling is particularly important between immune cells, where it forms the basis of antigen presentation and the initiation of the immune response. Cells may also communicate directly with their immediate neighbour through gap junctions that connect the cytoplasm of neighbouring cells via protein channels allowing the passage of ions and small molecules between them (*e.g.*, gap junctions allow the coordinated contraction of cardiac muscle cells) [11, 83].

Cell signalling requires not only extracellular signal molecules, but also a complementary set of receptor proteins in each cell that enable it to bind and respond to the signal molecules in a characteristic way. These cell-surface receptor proteins act as signal transducers. They convert an extracellular ligand-binding event into intracellular signals that alter the behaviour of the target cell [84, 85]. The extracellular signal molecules often act at very low concentrations and the receptors that recognize them usually bind them with high affinity. In most cases, the receptors are transmembrane proteins on the target cell surface. When these proteins bind an extracellular signal molecule (a ligand), they become activated and generate various intracellular signals that alter the behaviour of the cell. In other cases, the receptor proteins are inside the target cell, and the signal molecule has to enter the cell to bind to them: this requires that the signal molecule be sufficiently small and hydrophobic to diffuse across the target cell's plasma membrane [11, 83].



2.1. The Classes of Cell Surface Receptors

The concept of receptors was first introduced in the context of the mechanisms of action of drugs, and the term was used long before the molecular nature of the various receptors was known [86]. Most cell-surface receptor proteins are defined by their transduction mechanism [83, 87]. In ion-channel-coupled receptors, ligand binding changes the conformation of the receptor so that specific ions flow through it; the resultant ion movements alter the electric potential across the cell membrane. The acetylcholine receptor at the nerve-muscle junction is an example [11, 83, 87].

G-protein-coupled receptors act by indirectly regulating the activity of a separate plasma-membrane-bound target protein, which is generally either an enzyme or an ion channel. A trimeric GTP-binding protein (G protein) mediates the interaction between the activated receptor and this target protein. The receptors for epinephrine, serotonin, and glucagon are examples [11, 83, 87].

Enzyme-coupled receptors either function directly as enzymes or associate directly with enzymes that they activate. They are usually single pass transmembrane proteins that have their ligand-binding site outside the cell and their catalytic or enzyme-binding site inside. The great majority, however, are either protein kinases or associate with protein kinases, which phosphorylate specific sets of proteins in the target cell when activated leading to the activation of signal transduction pathways that often terminate in the regulation of transcription and gene expression [11, 87]. The receptor tyrosine kinases (RTKs) are a large superfamily of receptors that function as the receptors for a wide array of growth factors, including epidermal growth factor (EGF), nerve growth factor (NGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin and the insulin-like growth factors (IGF), and the ephrins and angiopoietins [87]. RTKs are essential components of cellular signalling pathways that are active during embryonic development and adult homeostasis. Because of their roles as growth factor receptors, many RTKs have been implicated in the onset or progression of various cancers, either through receptor gain-of-function mutations or through receptor/ligand overexpression [88].

Furthermore, there are also several types of adhesion receptors such as the integrin family of adhesion molecules, the selectins, cadherins, and the Ig cell adhesion molecules (CAMs).

These receptor molecules play important roles in a number of basic processes including cell proliferation, migration, development, and tissue remodelling in adults [87].

The varied receptors allow cells to respond to a wide range of stimuli ranging from ions to large ECM proteins that lead to activation of specific signalling processes and changes in cellular behaviour. Any mutation that leads to unregulated or inactive signalling can lead to pathologies. Thus, cell surface receptors are key to the mechanism of many chemical toxicants and serve as targets for the development of drugs [87].

2.1.1. EGFR and ERBB/HER Family

The flow of information from the extracellular environment into the cell is at the core of a functional biological system. Receptor tyrosine kinases (RTKs) are primary mediators of many of these signals and thus determine whether the cell grows, differentiates, migrates, or dies. Moreover, carcinogenesis is a multi-step process that requires the accumulation of several genetic and functional alterations in a single cell. Among numerous factors, carcinogenesis involves the activation of oncogenes such as the epidermal growth factor receptor (EGFR), also known as ERBB1/HER1 [89, 90]. This receptor belongs to the ERBB/HER family of ligand-activated RTKs, which also comprises ERBB2/NEU/HER2, ERBB3/HER3 and ERBB4/HER4 (Fig. 2). All of them can be alternatively spliced to give rise to various partial protein products [91, 92]. These receptors are anchored in the cytoplasmic membrane and share a similar structure, composed by an extracellular ligand-binding domain, a short transmembrane domain, and an intra-cytoplasmic tyrosine kinase domain [89, 93, 94]. The overall amino acid identity between these proteins is about 50%, and mammals contain the four members of the family, which transduce extracellular signals by EGF family of peptide growth factors [95].

EGFR has an almost ubiquitous expression in normal epithelial tissues along with an important role in directing and coordinating many normal processes, including growth and development, normal tissue turnover and wound healing. As a result, null mutations lead to embryo lethality [90, 96]. The degree of conservation observed in many other organisms is in accordance with the important biologic functions of EGFR. For instance, signalling from the EGFR also plays a critical role in the development of the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*, each of which contain only one gene encoding EGFR orthologues,

called LET-23 and DER respectively (Fig. 2) [97, 98]. LET-23 is necessary for the induction of vulva [99] and DER fulfils many roles during development, including oogenesis, proliferation and differentiation [100].The overall amino acid identity between the human EGFR and LET-23 proteins is 29% and that between DER and EGFR is 38% [95].

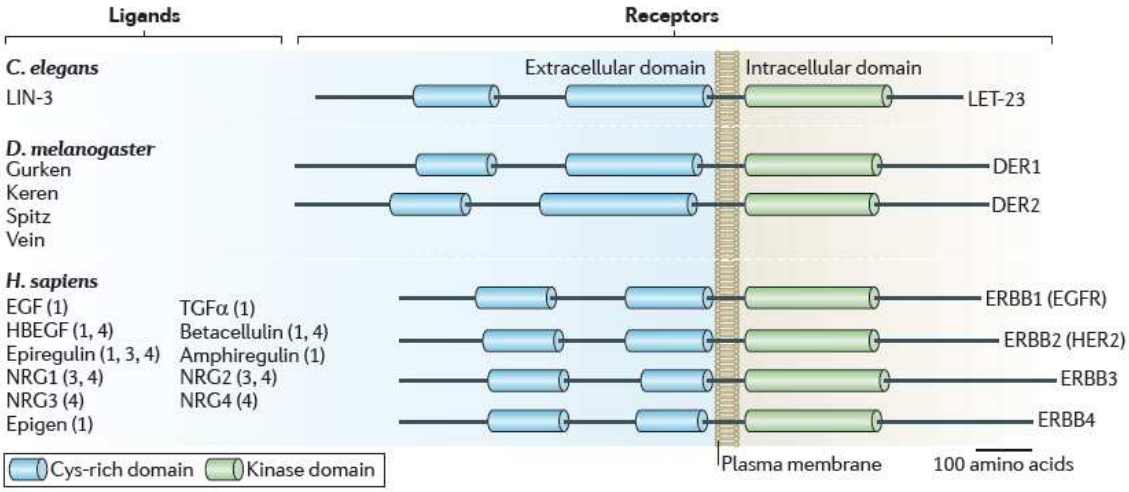


Figure 2 - ERBB family of receptors. In the course of evolution, the ERBB family expanded from the nematode's single ligand (LIN-3) and receptor (LET-23) to a group of four receptors and 11 ligands in vertebrates. Similarly, the domain structure of all four receptors is well conserved and includes a ligand-binding extracellular domain that is linked through a single transmembrane region to the cytoplasmic tyrosine kinase domain. The two insect receptors represent splicing isoforms of the *D. melanogaster* EGF receptor (DER1 and DER2). Unlike LIN-3, the vertebrate ligands specifically bind to more than one receptor, but ERBB2 binds no known ligand and ERBB3 cannot signal when present alone, because the respective kinase domain is practically inactive. Withdrawn from [101].

Deregulation of ERBB/HER family members activity stimulates key processes associated with tumourigenesis such as tumour growth and progression, including proliferation, angiogenesis, invasion, and metastasis [93, 102, 103]. For these reasons, the EGFR are amongst the cell-surface markers most frequently implicated in the development of cancer. This is the case of many epithelial cancers, including CRC, breast, ovarian, prostate, lung, gastric, head and neck in which cases EGFR is over-expressed. Moreover, patients with altered EGFR activity tend to have a more aggressive disease, associated with a poor clinical outcome [90, 91, 104, 105]. Therefore, EGFR has become an attractive target for therapy development with two classes of biologic agents, the anti-EGFR monoclonal antibodies and the tyrosine kinase inhibitors [106].

The ERBB/HER Growth Factor Ligands

Interactions between receptors, and the existence of a wide group of ligands, underlie the enormous potential for diversification of the biological messages mediated by the ERBB family (Fig. 3). These peptide ligands are produced as transmembrane precursors, and the ectodomains are processed by proteolysis, which leads to the release of soluble growth factors [107, 108]. There are several ERBB-specific ligands (Fig. 3), all sharing an EGF-like motif of 45-55 amino acids and including six cysteine residues that interact covalently. This region of HER ligand proteins is probably the most important, conferring the binding specificity that underlies their classification in three groups. The first group includes EGF, amphiregulin (AR), and transforming growth factor-α (TGF-α), which bind specifically to EGFR/ERBB1/HER1. The second group includes betacellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin (EPR) [109], which exhibit dual specificity for EGFR and ERBB4/HER4. The third group is composed of the neuregulins (NRG, also called NEU differentiation factors, NDFs, or heregulins, HRG) and includes two subgroups based on their capacity to bind ERBB3/HER3 and ERBB4/HER4 (NRG-1 and NRG-2) or only ERBB4/HER4 (NRG-3 and NRG-4) [110, 111].

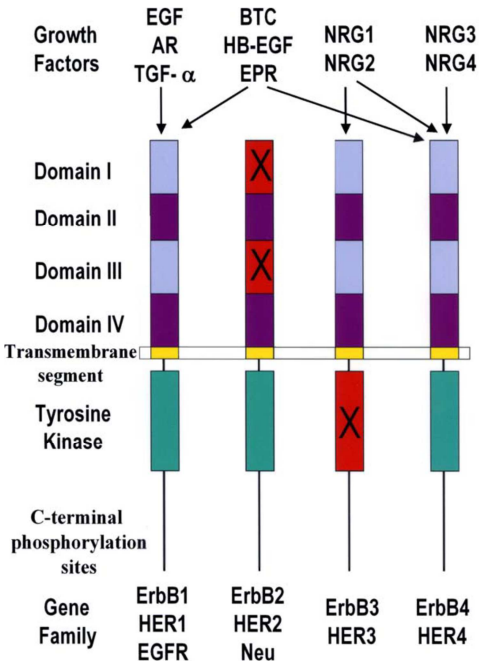
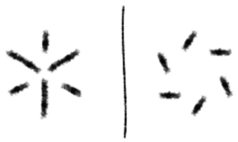


Figure 3 - Epidermal growth factor family of ligands and the ERBB/HER family. The topology of the receptor proteins is indicated. The inactive ligand-binding domains of ERBB2 and the inactive kinase domain of ERBB3 are denoted with an X. Withdrawn from [92].



Each of these many ligands has a different preference for stabilizing distinct receptor dimers. The members of the ERBB/HER family can form four homodimers and six heterodimers for a total of 10 distinct states [92]. Each receptor dimer has a different set of tyrosine autophosphorylation sites, which serve as docking sites for specific intracellular proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains, thus recruiting different combinations of signalling molecules and initiating a wide variety of signalling cascades [109, 112-114].

EGFR–ERBB2 heterodimers are associated with a more robust signal then EGFR–EGFR homodimers [115]. *EGFR* is overexpressed in bladder, breast, head and neck, kidney, non-small cell lung, and prostate cancers [93]. Similar to EGFR, the EGFRvIII (EGFR variant III; a truncated form of EGFR) is primarily localized on the cell-surface where it activates several signalling modules. However, unlike EGFR, EGFRvIII is constitutively active, independently of ligand stimulation, in part due to the loss of a portion of the ligand-binding domain. EGFRvIII is predominantly detected in malignant gliomas [116-119].

Hitherto, there is no evidence that any growth factor binds to ERBB2 homodimers. Rather, ERBB2 forms heterodimers with each of the other family members, and such heterodimers can bind growth factors [115, 120]. *ERBB2* is overexpressed in breast, cervix, colon, endometrial, esophageal, lung, and pancreatic cancers [88, 93].

ERBB3 instead, and although it has a tyrosine kinase domain that is highly homologous to those of the other family members, has no kinase activity [120, 121]. ERBB3 can form heterodimers with the other three family members. Owing to the lack of protein kinase activity of ERBB3, for cell signalling is required the trans-phosphorylation by other members of the EGF receptor family. *ERBB3* is overexpressed in breast, colon, prostate, and stomach malignancies [88, 93]. The last member of the family is ERBB4 [122].

Regarding the other family members, the identity of ERBB4 transmembrane residues is approximately 75%, 70% for the intracellular catalytic domain (276 residues), and 20% for the carboxyterminus. *ERBB4* is overexpressed in breast cancer and granulosa cell tumours of the ovary [88].

EGFR Structure and Regulation of Activity

Human *EGFR* gene locates at chromosome 7p11-13 and the mature protein is synthesized from a 1210-residue polypeptide precursor. This, after cleavage of the N-terminal sequence and other post-translational modifications originates a highly glycosylated 170kDa membrane spanning protein of a single 1186 amino acids polypeptide chain (Fig. 4) [90, 94, 123, 124].

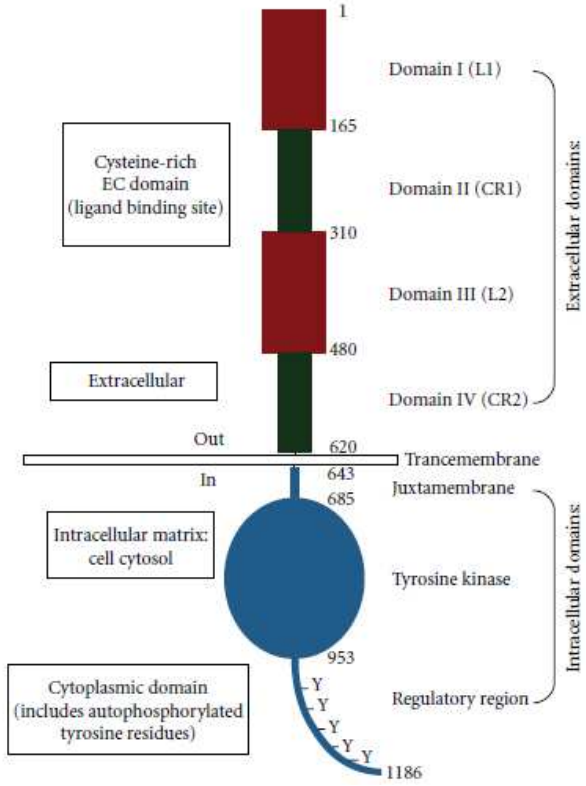
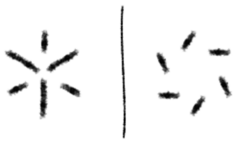


Figure 4 - Basic Structure of EGFR displaying the relevant domains. (I) The extracellular domains: (1) domain I/L1; (2) domain II/CR1; domain III/L2; domain IV/CR2. (II) Transmembrane domains. (III) The intracellular domains (1) juxtamembrane domain; (2) tyrosine kinase domain; (3) regulatory region domain. The phosphorylation of several substrates by the tyrosine kinase domain of the EGFR receptor is responsible for activating the various signalling cascades. Withdrawn from [124].

Like all RTKs, as referenced above, EGFR is characterized by three main domains [94]. The extracellular domain of the mature receptor contains 621 amino acids, followed by a single transmembrane domain (amino acids 622–644), and a juxtamembrane domain (amino acids 645–682). The tyrosine kinase domain extends from amino acids 683 to 958, whereas all the autophosphorylation sites are located between amino acids 992–1186 [123]. The extracellular domain of the EGFR can be further divided into four subdomains, I or L1 (L stands for leucine-



rich) from residues 1–165, II or CR1 (CR stands for cysteine-rich) from residues 166–310, III or L2 from residues 311–480, and IV or CR2 from residues 481–621 (Fig. 4). Domain I/L1 shares sequence and structural homology with the domain III/L2, both of which are involved in ligand binding, based on site directed mutagenesis and deletion mutation studies [125]. Domains II/CR1 and IV/CR2 have predisposition to form disulphide bridges, and are important in facilitating the overall conformational change induced by the EGFR [124]. ERBB proteins contain two cysteine-rich domains, except for their invertebrate counterparts, LET-23 and DER, that have an additional CR motif [95].

Crystallographic studies of the EGFR extracellular domain complexed to its ligands have shown that the domains I, II and III form a ligand-binding pocket [126, 127]. In the absence of ligand, EGFR exist as monomers on the cell surface. Binding of ligand to EGFR leads to the formation of receptor homo- and heterodimers, depending on whether EGFR dimerizes with another EGFR or with other ERBB family members, respectively [42, 128]. EGFR dimerization is entirely receptor-mediated, with no contacts between the two growth factor molecules in the dimeric complex [126]. By binding simultaneously to two sites (within domains I and III) in the extracellular region of the receptor, the growth factor alters the special arrangement of the domains (as shown schematically in Fig. 5). This domain rearrangement exposes a critical region of domain II that is otherwise hidden by an intramolecular interaction (or tether) within domain IV (Fig. 5A). The region thus exposed is known as the dimerization arm, and forms the core of the dimer interface in Fig. 5D. Growth factors bind preferentially to the extended or untethered forms of EGFR (Fig. 5B) and “trap” the receptor in the conformation that can dimerize through the exposed dimerization arm (Fig. 5C), thus driving the equilibrium towards the activated complex (Fig. 5D) [127].

The transmembrane domain plays an important role in anchoring the receptor to the cell membrane, with high affinity for caveolae or lipid rafts [125], resulting in the enrichment of defined patches of the membrane in EGFR, and hence allowing a faster receptor dimerization following binding of the ligand [129]. Adjacent to the transmembrane domain, and facing the interior of the cell, there is the juxtamembrane domain, which is believed to regulate various functional aspects of EGFR including control of the tyrosine kinase activity, downregulation of the EGFR, ligand internalization, and receptor sorting. Of note, this domain also has binding motifs that allow it to interact with second messengers like calmodulin [125].

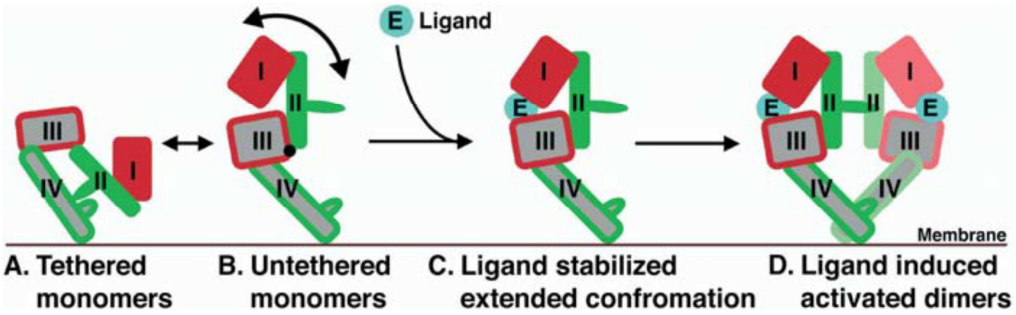
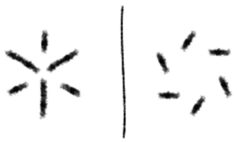


Figure 5 - Mechanism of ligand-induced EGFR dimerization. About 95% of the unliganded EGFR exists in a compact autoinhibited or tethered conformation, in which domains II and IV form an intramolecular interaction or tether (A). In the remaining 5% of the unliganded molecules, this tether is broken, and the soluble extracellular region of EGFR (sEGFR) can adopt a range of untethered conformations (B), some of which will be more extended. Ligand binds preferentially to untethered molecules, and interacts simultaneously with domains I and III, stabilizing the particular extended form in which domain II is exposed and the receptor can dimerize (C). Dimerization is entirely receptor-mediated and dominated by domain II interactions (D). Withdrawn from [127].

Adjacent to the juxtamembrane domain there is the tyrosine kinase domain, a Src homology domain 1, SH1. This is the most conserved region among the EGFR protein family, except in ERBB3 [95]. Activation of the EGFR tyrosine kinase phosphorylates numerous targets, including itself (autophosphorylation), a different EGFR (homodimerization), another member of the family (heterodimerization), and non-receptor substrates, which in turn initiate the signalling cascades [95, 124]. Tyrosine kinase activity of EGFR is tightly regulated via its own internal regulatory region located at the C-terminal tail of the structure, which involves the tyrosine residue cluster with the potential of being transphosphorylated during EGFR dimerization [124]. The degree of evolutionary conservation is yet significantly reduced in the carboxy-terminal tail, in spite of (or because of) their important regulatory functions [95]. EGFR dimerization induces phosphorylation of six tyrosine residues creating docking sites for the recruitment of other adaptor molecules and signalling proteins with SH2 or PTB domains to the membrane. These attributes suggest that the tyrosine-rich C-terminal tail is a phosphorilable, mobile structure connected to a relatively stationary TKD [124]. Finally, the carboxy-terminal tail contains motifs for internalization and degradation of the receptor [95].

The binding of the ligands to the ectodomain results in allosteric transitions leading to receptor dimerization, protein kinase activation, trans-autophosphorylation, and activation of several intracellular signalling pathways downstream of the receptor. Some of the pathways downstream of EGFR activation include those mediated by Ras/Raf/MEK, PI3K/Akt/mTOR, PLC-



γ/PKC and JAK2/STAT3 which upon activation lead to cell proliferation, motility and survival [102]. All four members of the ERBB family have the potential to stimulate the Raf/MEK/ERK protein kinase cascade [120, 122]. However, phospholipase Cγ binds to specific phosphotyrosines of EGFR/ERBB1, but not the other ERBB family members. Furthermore, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) binds to specific phosphotyrosines on ERBB3 and ERBB4, which leads to enzyme activation [92]. Although members of the same family, each receptor and each kinase has its own distinguishing features [92].

Inactivation of the EGFR can be mediated either by receptor dephosphorylation by phosphotyrosine phosphatases or receptor downregulation. Receptor downregulation is the most prominent regulator of EGFR signal attenuation and involves the internalization by endocytosis and subsequent degradation of the ligand-receptor complex in the lysosomes [90, 130, 131]. It was determinate that the half-life of the EGFR in the absence of EGF in cell culture is 6.5h, and it is 1.5h in the presence of EGF [132]. These findings led to the study of the downregulation of a variety of receptors. In cells expressing ERBB2/ERBB3 or ERBB4, in contrast to EGFR, these are not downregulated by stimulatory ligands [132].

Signalling Pathways Downstream of EGFR

Depending upon the type of ligand and the EGFR dimerization partner, several different signal transduction pathways can be engaged. The best-studied pathways include the Ras/Raf/MEK/ERK and PI3K/Akt, but the PLC-γ and JAK/STAT pathways can also be engaged by activated EGFR (Fig. 6).

The Ras/extracellular signal regulated kinase (ERK) pathway is a critically important route that regulates cell proliferation and survival [133]. Activation of the EGFR receptor leads to the phosphorylation of key tyrosine residues within its COOH-terminal portion and, as a result, provides specific docking sites for cytoplasmic proteins containing SH2 or PTB binding domains [124]. Growth factor receptor-bound protein 2 (GRB2) is an SH2/SH3 domain-containing protein that binds EGFR either directly or through the association with the adaptor molecule Shc, and acts as a common adapter protein in a majority of growth factor related signalling events. GRB2 binding to phosphotyrosine residues changes its conformation and allows it to bind to proline-rich sequences in the carboxy terminal tail of Sos, a GDP-GTP exchange protein (Fig. 6) [134, 135].

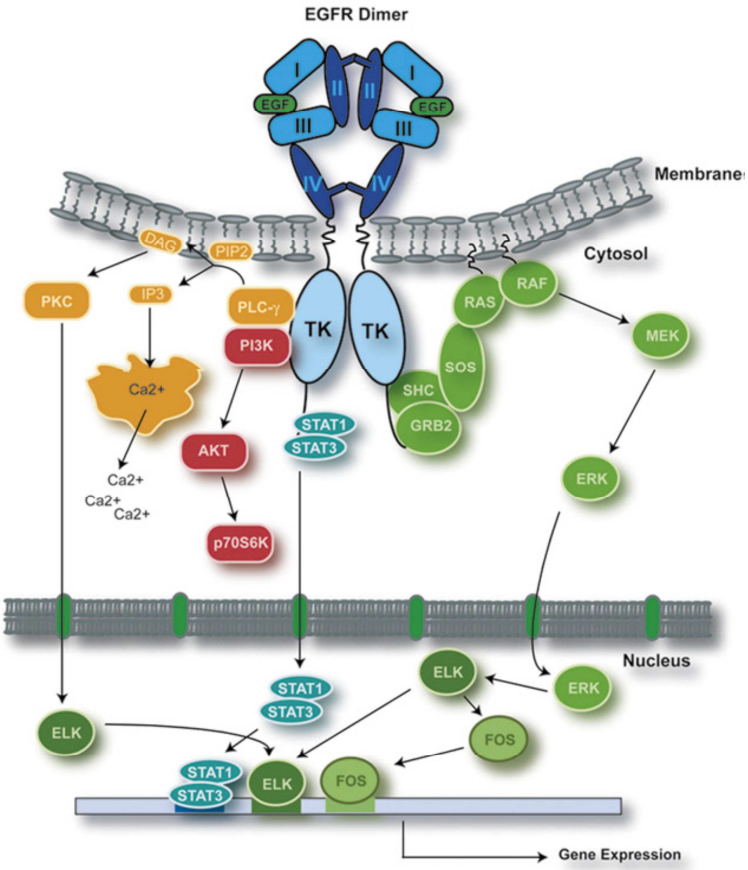
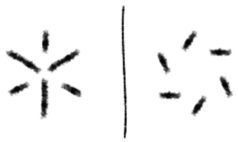


Figure 6 - EGFR Signalling. Binding of ligand to EGFR leads to receptor dimerization, autophosphorylation and activation of several downstream signalling pathways. Withdrawn from [90].

This binding displaces an inhibitory domain in Sos and allows its activation. It then translocates to the plasma membrane where it activates Ras (a family of oncogenes that include *KRAS*, *HRAS*, and *NRAS)* through an exchange of GDP for GTP [136]. Ras binds to the N-terminus domain of the Raf serine/threonine kinases (RAF1, ARAF and BRAF) [137], and recruits this protein complex to the plasma membrane inner surface, where Raf subsequently phosphorylates and activates MEK1 and MEK2 dual-specificity protein kinases, which in turn activate a third protein kinase called ERK1/2. These are then imported into the nucleus where they phosphorylate specific transcription factors promoting the increased transcription of Bcl-2 family members and proteins that inhibit apoptosis, thereby increasing cell survival [138-140].

The PI3K/Akt signalling pathway affects many cellular processes including cell proliferation, apoptosis and invasion [141, 142]. Phosphatidylinositol 3 kinases (PI3Ks) are lipidic kinases that usually exist as heterodimers of a p110 catalytic subunit and a p85 regulatory



subunit that can be activated by recruitment to the cell membrane. This occurs via the anchorage to ERBB receptor specific docking sites or Ras protein [141, 142]. Active PI3Ks phosphorylate phosphatidylinositol 4,5-bisphosphate (also known as PtdIns(4,5)P_2, or more simply PIP2) to phosphatidylinositol triphosphate (PtdIns(3,4,5)P_3 or PIP3). PIP3 acts as an anchor for the Akt serine/threonine kinase and for 3-phosphoinositide-dependent protein kinase-1 (PDK1). Once at the membrane, PDK1 phosphorylates and activates Akt, which then regulates a range of target proteins involved in a variety of intracellular processes [141-144]. Akt promotes cell survival through the transcription of anti-apoptotic proteins. Intermediate transcription factors involved in this process are NF-kB and CREB. Another downstream target of Akt is glycogen synthase kinase 3 (GSK3). Under basal conditions the constitutive activity of GSK3 leads to the phosphorylation and inhibition of a guanine nucleotide exchange factor eIF2B, which regulates the initiation of protein translation. Therefore, upon inactivation of GSK3 by Akt, eIF2B is dephosphorylated resulting in the promotion of protein synthesis and the storage of amino acids [145]. Akt also activates mammalian target of rapamycin (mTOR), which promotes protein synthesis through p70 ribosomal S6 kinase (p70s6k) and inhibition of eIF-4E binding protein (4E-BP1) [146]. Collectively, these processes all promote cell growth and survival in response to EGF.

Phospholipase Cγ interacts directly with activated EGFR and cleaves PIP2 at the plasma membrane, resulting in the production of the second messengers diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP3). DAG activates members of the protein kinase C (PKC) family at the membrane [147]. These PKC isoforms variously enhance cell polarization, migration and invasion by enhancing the activity of MET and integrins, and promoting cell survival throughout MAPK and c-Jun NH2-terminal kinase activation [148, 149]. IP3 diffuses through the cytoplasm, where one of its more important activities is the binding of an IP3 receptor to trigger Ca^{2+} ion release from intracellular stores in the endoplasmic reticulum. Increased intracellular Ca^{2+} activates calmodulin II kinase (CAMIIK) and calcineurin, and directly binds and induces conformational changes in other proteins to regulate their activity. Cumulatively, the perturbed Ca^{2+} signalling that is common in cancer cells also supports the cell cycle progression and survival of these cells [150].

Another signalling cascade initiated by EGF is the JAK/STAT (Janus kinase/Signal transducer and activator of transcription) pathway, which is also implicated in cell survival responses [151, 152]. JAK phosphorylates STAT proteins localized at the plasma membrane.

STAT proteins also interact with phosphotyrosine residues via their SH2 domains and, on dimerization, translocate to the nucleus where they activate the transcription of genes associated with cell survival [153]. EGFR-activated STAT3 has been shown to activate the expression of an E-cadherin transcriptional repressor, TWIST, and thereby, promote epithelial-mesenchymal transition [154].

The interlinked Ras/MAPK and PI3K/Akt signalling pathways play an important role in tumourigenesis via phosphorylation of various proteins and transcription factors that directly control cell growth, differentiation, and apoptosis [93, 143, 155]. Furthermore, mutation in *KRAS*, *BRAF*, or *PIK3CA* results in continuous activation of the downstream Ras/MAPK or PI3K pathways, regardless of whether the EGFR is activated or pharmacologically blocked. Such activation in turn enhances transcription of various oncogenes, including *MYC*, *CREB*, and the gene for NF-κB [93, 143, 155].

EGFR Functionality Can Be Dependent of Its Sub-Cellular Location

Recent evidences indicate that EGFR functionality can be dependent on its sub-cellular location [102]. In this regard, EGFR undergoes translocation into different organelles, where it elicits functions besides its best known activity as a plasma membrane-bound receptor tyrosine kinase. At plasma membrane level, it mediates cellular processes dependently and independently of its kinase activity. EGFR can also be shuttled into the cell nucleus and mitochondrion upon ligand binding, radiation, EGFR-targeted therapy and other stimuli. Nuclear EGFR behaves as transcriptional regulator, tyrosine kinase, and mediator of other physiological processes. The role of mitochondrial EGFR remains poorly understood but it appears to regulate apoptosis and autophagy [156]. Studies using patient tumours have shown nuclear EGFR to be an indicator for poor clinical outcomes in cancer patients, however, the impact of mitochondrial EGFR on tumour behaviour and patient prognosis remains to be defined.

The Cell Surface and Cytoplasmic EGFR Signalling

Upon ligand binding, activated EGFR recruits, phosphorylates and activates a number of important signalling molecules such as PLC-γ, Ras, PI3K and JAK2, as well as the signal

transducer and activator of transcription STAT3, promoting its dimerization, nuclear transport, and subsequent gene regulation [157-162]. However, EGFR can also mediate cellular processes independent of its kinase activity or ligand activation, mostly through its ability to physically interact with other proteins (Fig. 7).

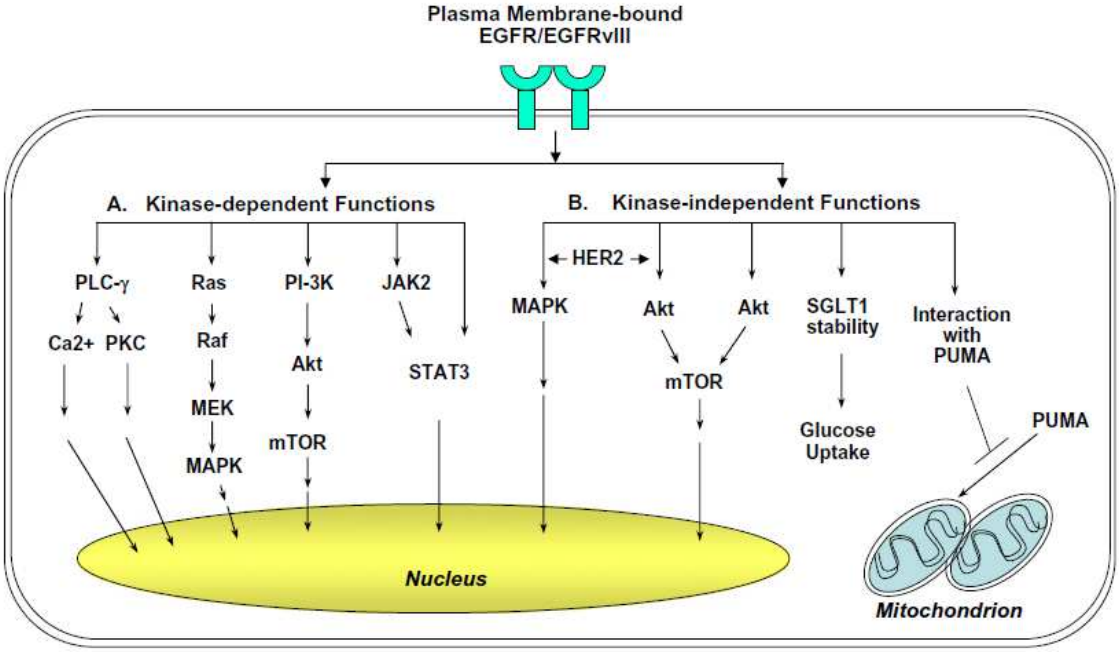


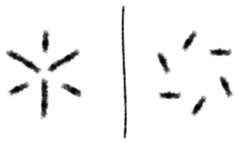
Figure 7 - The plasma membrane-bound EGFR/EGFRvIII signalling consists on the kinase-dependent and -independent modes of actions. **A: Kinase-dependent functions.** Upon ligand binding, EGFR becomes activated and phosphorylated at multiple tyrosine residues including those within its kinase domain. Phosphorylated EGFR then recruits and phosphorylates downstream signalling molecules. The major pathways downstream of EGFR include those mediated by PLC-γ/PKC, Ras/Raf/MEK, PI3K/Akt/mTOR and JAK2/STAT3. In addition, EGFR can directly interact with and phosphorylate STAT3 transcription factor. EGFRvIII is constitutively active independently of ligand stimulation. **B: Kinase-independent functions.** Co-expression of the kinase-dead EGFR mutant with HER2 rescued the inability of the mutant EGFR to activate Akt and MAPK. Kinase dead EGFR mutant may activate Akt via undefined mechanisms. Independent of its kinase activity, EGFR also interacts with and stabilizes plasma membrane-bound SGLT1, leading to glucose uptake and increased intracellular glucose levels. EGFR and EGFRvIII associates and sequesters the pro-apoptotic protein PUMA in the cytoplasm, independently of EGF stimulation or its kinase activity. The EGFR–PUMA and EGFRvIII–PUMA interactions contribute to reduced apoptosis and survival. Withdrawn from [102].

Studies showed that a kinase-dead EGFR mutant retained the ability to stimulate DNA synthesis. In addition, co-expression of the kinase-dead EGFR mutant with HER2 rescued the inability of the mutant EGFR to activate Akt and MAPK, suggesting that hetero-dimerization with other members of the ERBB family of receptors may help support the kinase-independent

function of EGFR [163]. Cell-surface EGFR was also found to physically interact with and stabilize sodium/glucose co-transporter 1, SGLT1, a plasma membrane-bound protein that mediates glucose uptake, contributing to the maintenance of high glucose levels inside the cells. Conversely, EGFR expression knockdown, led to SGLT1 degradation, reduction of intracellular glucose and subsequent autophagic cell death [164]. In support of these observations, co-expression of EGFR and SGLT1 was found to be frequent in cell lines and specimens of oral squamous cell carcinoma [165].

Through physical associations, regardless of kinase activity, EGFR can modulate protein trafficking. Studies reported that both EGFR and EGFRvIII associate with p53-upregulated modulator of apoptosis (PUMA), a proapoptotic member of the Bcl-2 family of proteins primarily located on the mitochondria [166]. PUMA is a potent apoptosis inducer that binds to and inhibits the anti-apoptotic members of the Bcl-2 family of proteins [167, 168] and also directly binds to the apoptotic executor Bax [169, 170] to induce mitochondrial outer membrane permeabilization. It was further demonstrated that the EGFR–PUMA and EGFRvIII–PUMA interactions are independent of EGF stimulation or kinase activity and that these interactions are constitutive and only modestly reduced following apoptotic stress [166]. As a consequence of the EGFR–PUMA and EGFRvIII–PUMA interactions, PUMA is sequestered in the cytoplasm and unable to translocate onto the mitochondria to initiate apoptosis. This is in agreement with the evidence showing that PUMA is highly co-expressed with EGFR/EGFRvIII in cell lines and primary specimens of malignant gliomas, but also with the fact that this particular tumour type is highly resistant to apoptosis inducing treatments [166].

Studies showed that EGFR localized within the lipid raft microdomain of the plasma membrane could activate Akt without the need for kinase activity [171, 172]. It has also been shown that PI3K and c-Src co-localized and associated with EGFR in the lipid rafts [171]. These findings suggest that the lipid raft microdomain may serve as a platform for EGFR and other signalling molecules to interact with each other to transmit survival signals, independent of EGFR kinase function, and that pharmacological inhibitors for cholesterol biosynthesis may be useful in targeting some of the kinase-independent activities of cell-surface localized EGFR [102].



The Nuclear EGFR signalling

Nuclear existence of EGFR as well as EGFR ligands, namely EGF and pro-TGF-α was first observed in hepatocytes [173-175]. Nuclear expression of EGFR was further detected in other types of normal cells and tissues, such as placenta, thyroid, immortalized epithelial cells of ovary and kidney origins, and keratinocytes [176-179]. More recently, nuclear EGFR has been shown to be detected in many different types of cancer cells and specimens, including those of breast, epidermoid, bladder, ovary, oral cavity, lungs, and pancreas, and also in malignant gliomas [102].

Evidence to date indicates nuclear EGFR to be the full-length receptor that originates from the cell-surface [176, 177, 180]. The mechanisms underlying nuclear transport of EGFR begin with endocytosis, which occurs following ligand-induced activation, as the ligand-bound receptors are internalized through clathrin-coated pits that pinch off from the plasma membrane in a dynamin-dependent manner [181]. After the endocytic vesicle fuses with the early endosome, the internalized EGFR can be recycled back to the plasma membrane, or sorted to late endosomes and, eventually, to lysosomes for degradation, or further transported into the nucleus. Nuclear EGFR can be localized within the nucleoplasm [176, 177] and on the inner nuclear membrane [179].

Nuclear EGFR was defined as a transcriptional co-factor that contains a transactivation domain in its C-terminus, similarly to rat Neu [176]. As summarized in Fig. 8, the transcriptional targets of nuclear EGFR that have been identified to date include: i) cyclin D1 (important regulatory protein responsible for transition through the G1 chekpoint in the cell cycle) [176], ii) inducible nitric oxide synthase (iNOS) (key enzyme responsible for cellular production of nitric oxide, a potent signalling molecule known to influence metastasis and angiogenisis) [177], iii) B-Myb (proto-oncogene that plays a role in progression through the G1/S phase of the cell cycle) [182], iv) cyclooxygenase-2 (COX-2) (enzyme responsible for formation of important biological mediators called prostanoids involved in the process of inflammation and pain) [183], v) Aurora A (a serine/threonine kinase that associates with the centrossome during mitotic spindle formation to ensure proper spindle formation, chromatid separation, and fidelity of the spindle checkpoint) [184], vi) c-Myc (regulator gene that codes for a transcription factor, which activates expression of a great number of genes involved in cell proliferation) [185], and vii) breast cancer resistance

protein, BCRP (an ATP binding cassette half transporter that forms homo- or heterodimers in the plasma membrane of various cell types to actively pump macromolecules out of cells) [186]. Through increasing the expression of these target genes, nuclear EGFR has been linked to several malignant phenotypes of human cancers, including proliferation, inflammation and tumour drug resistance [187, 188].

Extensive efforts have been focused on finding EGFR transcriptional co-regulators with DNA-binding capability since EGFR lacks a DNA-binding domain [177]. It was found that nuclear EGFR is able to associate with STAT3 oncogenic transcription factor to enhance expression of iNOS, a protein involved in inflammation, tumour progression and metastasis. It was further reported that nuclear EGFR interacted with E2F1 to activate human B-Myb gene expression, leading to uncontrolled proliferation [182]. Nuclear EGFR has also been shown to also interact with STAT5 in order to enhance human Aurora A gene expression, leading to chromosome instability [184]. Another mechanism for nuclear EGFR-associated transcriptional regulation was also suggested [189] - RNA helicase A serves as a DNA-binding partner for nuclear EGFR. Interestingly, a recent study showed that EGFR, Src and STAT3 form a heteromeric complex in the nucleus [185]. This nuclear complex is bound to the *c-Myc* gene, which may contribute to *c-Myc* gene overexpression in pancreatic cancer cells. Also of note and indicative of a possible mechanism underlying the ability of nuclear EGFR to regulate gene transcription, is the ability of nuclear EGFR to interact with MUC1. This interaction may promote both the accumulation of chromatin bound EGFR and the significant co-localization of EGFR with phosphorylated RNA polymerase II [190].

HER2 can also be detected in the cell nucleus and activates *COX-2* gene expression, as well as upregulates cyclin D1 gene expression [191, 192]. Nuclear HER2 enhanced translation by activating transcription of ribosomal RNA genes [193]. Taken together, these findings indicate that nuclear EGFR and EGFRvIII function as transcriptional regulators, which cooperate with their transcriptional co-factors to mediate the expression of a number of important cancer-related genes and thereby, regulate many physiological and pathological processes [102].

Nuclear EGFR phosphorylates proliferating cell nuclear antigen (PCNA) to promote cell proliferation and DNA repair and that indicates nuclear EGFR retains its tyrosine kinase activity [194]. Furthermore, nuclear EGFR also plays an essential role in DNA repair following radiation therapy [195, 196], which was the inducer for EGFR nuclear entry. Then, nuclear EGFR interacts

with DNA-dependent protein kinase (DNA-PK), leading to repair of radiation-induced DNA double-strand breaks in bronchial carcinoma cells. Similar to EGFR, HER2 nuclear transport can be induced by radiation [197].

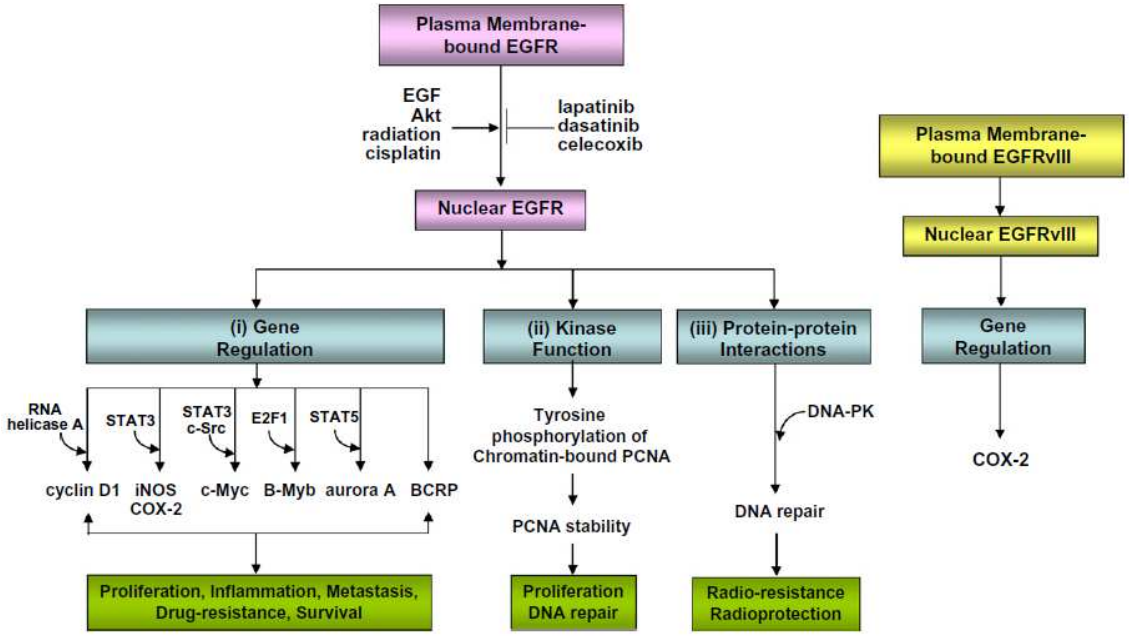


Figure 8 - The nuclear mode of EGFR/EGFRvIII signalling network. EGFR nuclear transport can be induced by EGF, Akt phosphorylation, radiation and cisplatin, and conversely, inhibited by lapatinib, dasatinib and celecoxib. Nuclear EGFR has three major functions: (i) gene regulation, (ii) kinase function, and (iii) protein–protein interactions. Via these actions, nuclear EGFR is implicated in a number of physiological and pathological processes, such as proliferation, inflammation, metastasis, DNA repair, and resistance to DNA-damaging radiation and alkylating anti-cancer agents. Nuclear EGFRvIII activates COX-2 gene expression. Withdrawn from [102].

The Mitochondrial EGFR Signalling

In 2004 it was demonstrated that EGFR translocated to the mitochondria after EGF stimulation (Fig. 9) [198]. While localized in the mitochondria, EGFR interacts with cytochrome c oxidase subunit II (CoxII), a mitochondrion-encoded protein and a critical component of the oxidative phosphorylation pathway [199]. EGFR translocation to the mitochondria has been reported to occur through clathrin-mediated endocytosis, suggesting that the origin of mitochondrial EGFR may be plasma membrane-bound EGFR [199]. However, another study reported that EGFR mitochondrial transport is independent of endocytosis [200]. In addition, a potential mitochondrial localization signal contained in the juxtamembrane region of EGFR was

identified (residues 645–666) [199]. However, the nuclear localization signal of EGFR is also found in the same juxtamembrane region (residues 645–657), overlapping with the potential mitochondrial localization signal (residues 645–666) [177, 183, 201]. Another work reported that EGFR mitochondrial translocation could be increased by a mTOR inhibitor, rapamycin [202].

Both EGFR and EGFRvIII are constitutively present in the mitochondria. Importantly, the degrees of EGFR and EGFRvIII mitochondrial import were greatly enhanced following treatments with the apoptosis inducers, staurosporine and anisomycin, and with an EGFR kinase inhibitor, Iressa (Fig. 7) [102]. Additionally, the mitochondrial transport of EGFR and/or EGFRvIII can be constitutive and further enhanced by EGF, rapamycin, apoptosis inducers, c-Src and EGFR inhibition (Fig. 7). Conversely, the receptor mitochondrial import can be suppressed by 3-methyladenine and by etoposide. The origin of mitochondrial EGFR and EGFRvIII remains to be defined given the mixed results. While localized to the mitochondria, EGFR interacts with and phosphorylates CoxII, but its impact on CoxII and CoxII-mediated ATP biosynthesis is still not known. Accumulation of EGFR and EGFRvIII in the tumour mitochondria could contribute to tumour resistance to apoptosis although the underlying mechanisms have yet to be defined. Overall, the nature and consequences of the mitochondrial mode of EGFR signalling are still elusive.

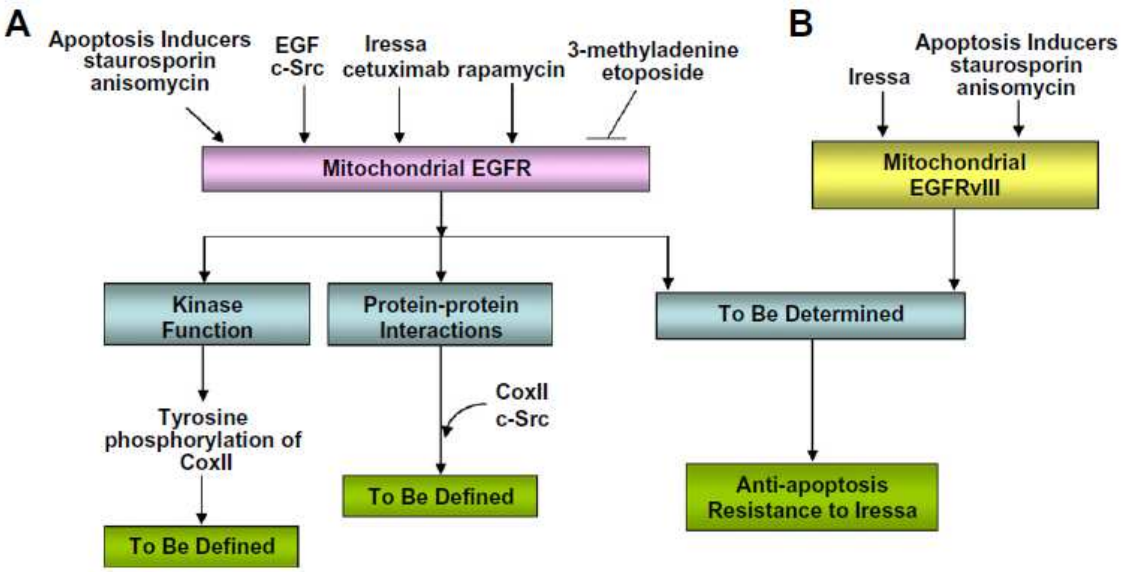
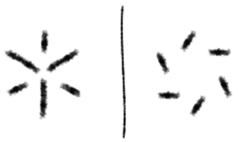


Figure 9 - The mitochondrial mode of EGFR/EGFRvIII signalling pathway. (A) EGFR mitochondrial import can be constitutive and the extent can be enhanced by apoptosis inducers (staurosporine and anisomycin), EGF, c-Src, Iressa, cetuximab and rapamycin. Conversely, EGFR mitochondrial transport can be blocked by 3-methyadenine (inhibitor of autophagy and PI3K) and etoposide. Mitochondrial EGFR retains its tyrosine kinase activity and



phosphorylates CoxII; however, the consequence of the phosphorylation is not yet defined. The mitochondrial EGFR–CoxII complex can include c-Src; however, the effects of this interaction are still unknown. Furthermore, mitochondrial accumulation of EGFR led to compromised apoptotic response and resistance to Iressa treatments, while the underlying mechanisms are still undetermined. (B) EGFRvIII mitochondrial import is constitutive and can be further enhanced by apoptosis inducers (staurosporine and anisomycin) and by Iressa. Mitochondrial accumulation of EGFRvIII rendered tumour cells highly resistant to apoptotic death and to Iressa treatments, though the process remains to be elucidated. Withdrawn from [102].

EGFR Signalling System Is Unbalanced in Human Malignancies

Being EGFR involved in the regulation of cell proliferation, motility and survival; an imbalance in the EGFR-ligand system giving rise to increased EGFR signalling can lead to neoplastic transformation [203, 204]. There are quite a few mechanisms by which the tight regulation of the EGFR-ligand system can be abrogated (Fig. 10). These include: 1) increased production of ligands, 2) increased levels of EGFR protein, 3) EGFR mutations giving rise to constitutively active variants, 4) defective downregulation of EGFR and 5) cross-talk with heterologous receptor systems [90].

TGF-α and EGF are frequently found co-expressed with EGFR in various types of cancer, and they are considered to act in an autocrine/paracrine manner, leading to deregulated EGFR activation and uncontrolled tumour growth (Fig. 10) [205, 206]. Deregulated EGFR activation is often associated with overexpression of EGFR (Fig. 10). This transforming ability of overexpressed EGFR is likely due to constitutive receptor activation caused by spontaneous dimerization, which in turn is a result of high EGFR levels on the cell surface. This leads to a continuous activation of downstream signalling pathways, conducing to a more malignant phenotype [207, 208]. Amplification of the EGFR gene is a way to increase EGFR levels. This has been observed in several cancer types [209, 210]. EGFR might also be overexpressed in the absence of gene amplification owing to a variety of mechanisms, which include increased activity of the EGFR promoter or deregulation at the translational and post-translational levels. For instance, wild type and mutant p53 proteins have been shown to directly activate EGFR transcription by binding to specific response sites in the promoter [211, 212]. As the level of mutant p53 proteins are usually high in tumour cells, it may lead to strong and continuous activation of the EGFR promoter and thus receptor expression [213].

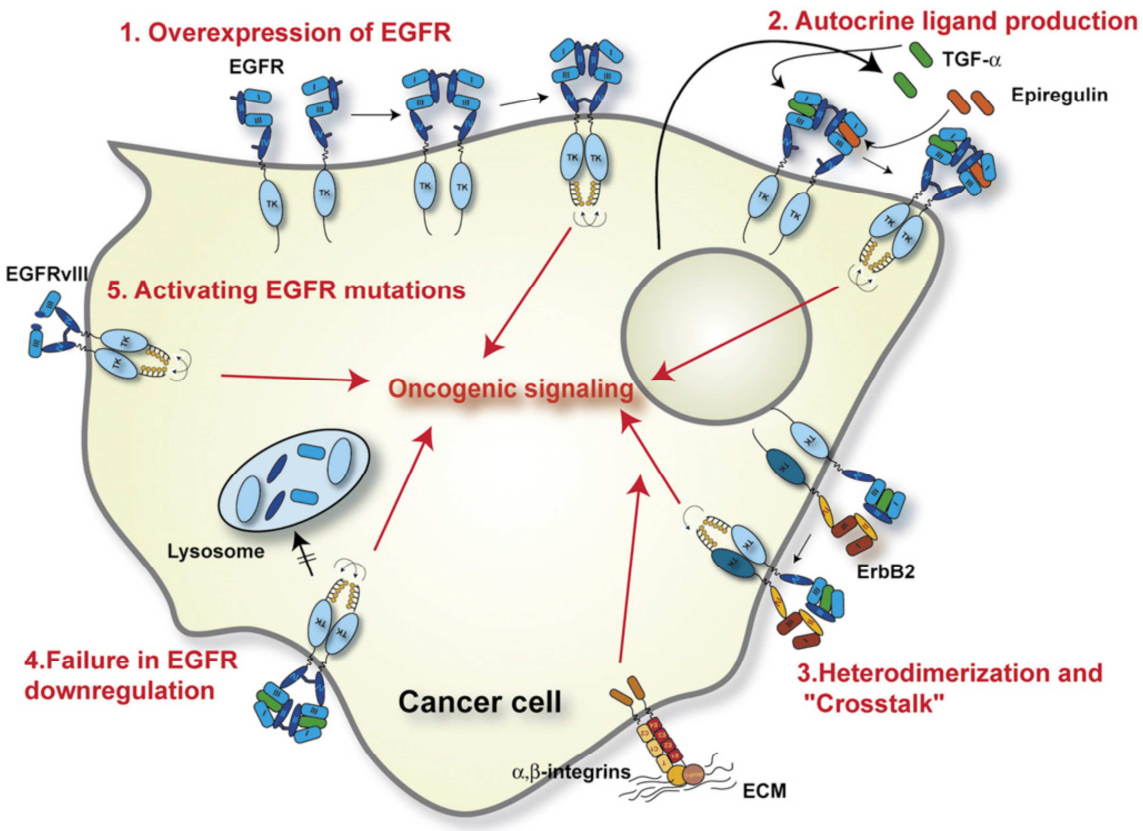
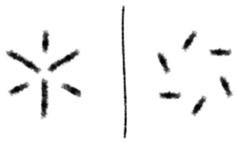


Figure 10 - Mechanisms leading to EGFR oncogenic signalling. There are several mechanisms by which EGFR becomes oncogenic including: 1) increased EGFR levels, 2) autocrine and/or paracrine growth factor loops 3) heterodimerization with other EGFR family members and cross-talk with heterologous receptor systems 4) defective receptor downregulation and 5) activating mutations. Withdrawn from [90].

Mutations in the EGFR gene are frequent in human cancers. These mutations can lead to changes in the extracellular domain, in the intracellular domain and in the intracellular tyrosine kinase domain [90]. Several mutations have been reported in the extracellular domain, usually due to large deletions or duplications of specific exons, encoding all or parts of the extracellular domain [118, 214]. These mutated genes give rise to truncated receptors (EGFRvI, EGFRvII, EGFRvIII, EGFRvIII/D12-13, EGFR.TDM/2-7), which are constitutively activated and escape regulation [90]. However, the molecular mechanism(s), which lead to these changes, remain elusive. The mutations in the intracellular portion of the receptor consist of either large deletions and/or duplications of exons. Both EGFRvIV and EGFRvV carry deletions in the C-terminal part of the receptor [214]. EGFR.TDM/18-25 and EGFR.TDM/18-26 contain duplications of exons which code for most of the C-terminal part of the receptor including the tyrosine kinase [90]. The last



group of the EGFR mutations lead to changes in the intracellular tyrosine kinase domain, these include deletions, missense mutations and small duplications or insertions [215, 216].

Aberrant EGFR signalling due to defective receptor down-regulation has also been linked to neoplastic cell transformation. As mentioned above, EGFR downregulation is a mechanism by which EGFR signalling is attenuated and it involves the internalization and subsequent lysosomal degradation, mediated by tagging with ubiquitin, of the activated receptor [130, 131]. Proper downregulation of EGFR does not only seem to be dependent on a functional intracellular EGFR domain, but also on a functional c-Cbl protein that binds to the EGFR but fails to ubiquitinate the receptor [90]. Unlike ligand-activated EGFR, activated ERBB2 is not downregulated, and when expressed together with EGFR at high levels, ERBB2 also inhibits the downregulation of EGFR [217]. The ligand bound to EGFR can also have a strong influence on receptor degradation. EGFR is efficiently degraded upon EGF stimulation but not upon incubation with TGF-α. When TGF-α bound EGFR reaches the endosomes, the ligand is largely dissociated from the receptor and the receptor is recycled back to the cell surface [218-220]. Another mechanism by which EGFR signalling is prolonged without subsequent receptor downregulation occurs when EGFR is exposed to oxidative stress in the form of hydrogen peroxide (H_2O_2) [221, 222]. H_2O_2 is a ubiquitous molecule, present in several air pollutants, including the cigarette smoke, and can cross cell membranes freely. Like EGF, H_2O_2 induces EGFR phosphorylation but to a lower extent than EGF [222]. Thus, H_2O_2 seems to induce tumour formation by activating EGFR and uncoupling the activated receptor from normal downregulation, thereby leading to continuous downstream signalling and hence cell proliferation [90].

The functional role of EGFR in cancer cells is widely affected by other cell-surface receptors. A cross-talk has been described between EGFR and other members of the ERBB family, as well as other receptor tyrosine kinases, cell adhesion molecules, cytokine receptors, ion channels, and G-protein coupled receptors (GPCR). Activation of certain integrin molecules by extracellular matrix proteins has been demonstrated to induce EGFR tyrosine phosphorylation independent of EGFR ligands [223, 224]. The mechanism by which integrins activate EGFR is uncertain, but they seem to associate with EGFR on the cell membrane in macromolecular complexes involving adaptor and signalling molecules (Fig. 10) [90]. Integrin induced EGFR activation could be involved in cell survival through pathways that engage PI3K activation [224, 225] and in cell proliferation through activation of MAPK [225, 226]. In cancer cells, EGFR

transactivation has been shown to induce DNA synthesis, cell cycle progression and cell migration, suggesting that EGFR transactivation by GPCR ligands may play an important role in tumour onset [227]. A proposed ligand-independent mechanism involves GPCR-mediated recruitment of EGFR to a complex with intracellular tyrosine kinases leading to EGFR phosphorylation (Fig. 10) [228]. Moreover, a suggested ligand-dependent mechanism of EGFR transactivation involves GPCR-dependent stimulation of membrane bound matrix metalloproteinases (MMPs), which subsequently cleave membrane-tethered EGFR precursor ligands. This include the precursors to heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), or the transforming growth factor-α (TGF-α), thereby releasing the ligand to bind EGFR (Fig. 5) [90, 229].

EGFR as a Therapeutic Target: Cetuximab

To date, five EGFR-targeted agents have been approved by the FDA for treating cancer patients. Among them, three are small molecule inhibitors of the EGFR tyrosine kinase enzymatic activity, directed against the intracellular kinase domain. The other two of these are monoclonal antibodies directed against the extracellular domain of the EGFR, to block ligand binding and receptor activation [102]. Gefitinib (ZD1839, Iressa®) [230] and Erlotinib (OSI-774, Tarceva®) [231], are small molecular weight EGFR kinase inhibitors and are being used for locally advanced and metastatic non-small cell lung cancer (NSCLC). Lapatinib (GW572016, Tykerb/Tyverb®) [232] is an EGFR/HER2-dual targeting small molecule inhibitor and has been used in the treatment of breast cancer. Cetuximab (C225, Erbitux®) and panitumumab (ABX-EGF, Vectibix®), are humanized monoclonal antibodies used to treat metastatic colorectal cancer upon chemotherapy [233] or other therapies [234] failure.

As mentioned above, cetuximab was approved by the FDA in 2004 for squamous cell carcinoma of the head and neck, and advanced-stage EGFR-expressing colorectal cancer [235]. It is a chimeric mouse/human monoclonal antibody of the immunoglobulin G1 (IgG1) class that recognizes the extracellular domain of both EGFR [127] and EGFRvIII [236]. It is composed of four polypeptide chains: two identical heavy (lambda) chains, each consisting of 449 amino acids and two identical light (kappa) chains, each consisting of 214 amino acids. The four chains are held together by a combination of covalent and non-covalent bonds. The Fv/Fab (fragment

variable, antigen-binding), of cetuximab is composed of regions of the 225 murine EGFR monoclonal antibody, specific for the N-terminal portion of human EGFR, combined with human IgG1 heavy and light chain constant regions (Fig. 11). Furthermore, cetuximab has two N-linked carbohydrates on both heavy chains and its molecular weight is approximately 152 kDa including carbohydrates [237-240]. Glycosylation, generally talking, plays a critical role in the biological and physiochemical properties of an antibody, influencing resistance to proteases, binding to monocyte Fc receptors, complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and circulatory half-life *in vivo* [241-243].

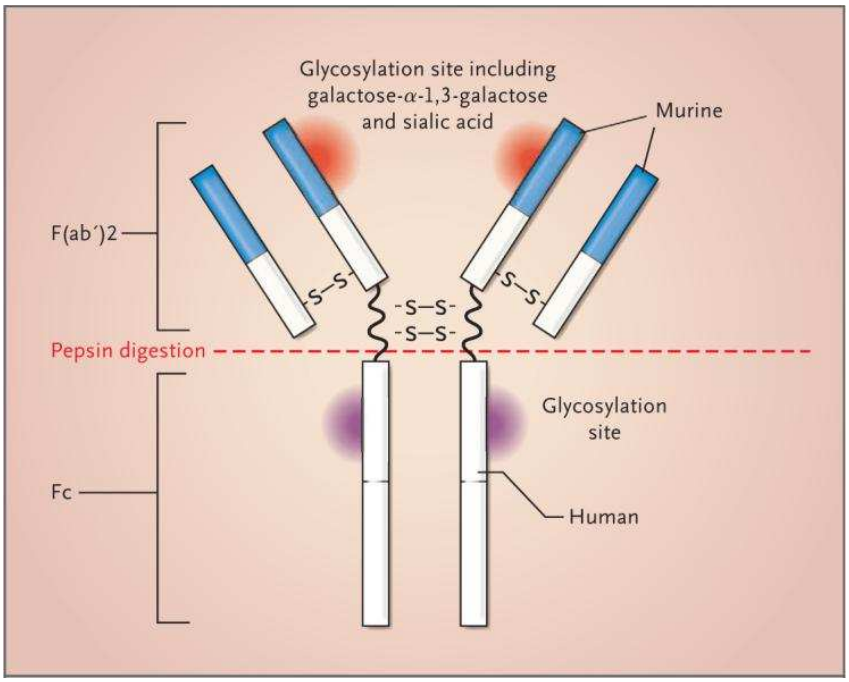


Figure 11 - Structure of cetuximab. Cetuximab is a chimeric IgG1 monoclonal antibody that targets the ligand-binding domain of the epidermal growth factor receptor (EGFR). Cetuximab is composed of the Fv regions of a murine anti-EGFR antibody with human IgG1 heavy and kappa light chain constant regions and has an approximate molecular weight of 152kDa. The sugars on the Fab portion include galactose-α-1,3-galactose and the sialic acid *N*-glycolylneuraminic acid and glycosylation of the Fc portion of the heavy chain includes only oligosaccharides that are commonly present on human proteins. S–S denotes a disulphide bond. Withdrawn from [244].

Cetuximab binds specifically to EGFR on both normal and tumour cells with an affinity that is approximately 5- to 10-fold higher than that of endogenous ligands, therefore competitively inhibiting binding of endogenous ligands, such as EGF and TGF-α Ultimately, cetuximab inhibits EGFR activation and the associated downstream intracellular signalling [245, 246]. The crystal structure of the cetuximab Fab fragment bound to the EGFR extracellular region shows that the

antibody binds to a site on domain III of the receptor that overlaps the EGF binding site (Fig. 12). Both the heavy and the light chains of cetuximab participate in the interaction with domain III. In addition, cetuximab steric binding prevents the EGFR extracellular region from adopting the dimerization-competent extended configuration [127].

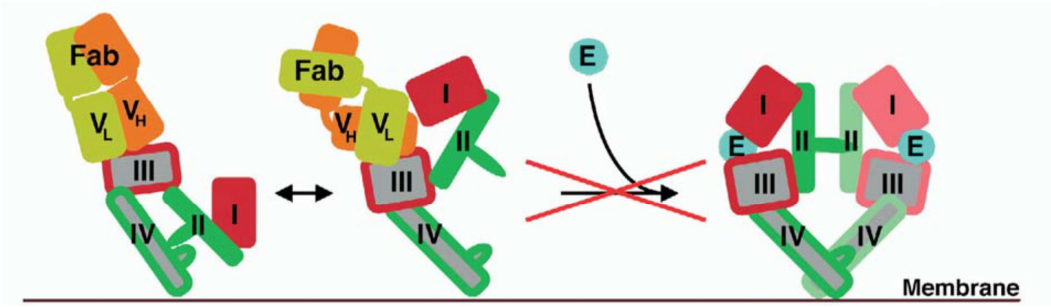
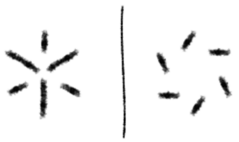


Figure 12 - Model for inhibition of ligand-induced dimerization by cetuximab. In addition to blocking the domain III ligand binding site, FabC225 prevents the receptor from adopting the extended conformation required for high-affinity ligand binding and dimerization. Withdrawn from [127].

The direct mechanism of action of cetuximab is therefore the blockade of ligand-receptor binding and consequent inhibition of ligand-mediated activation of the EGFR tyrosine kinase [127]. As result, a variety of processes regulated by the EGFR-signalling pathways relevant for tumour development or onset are disrupted [247]. These include EGFR downregulation [248, 249], inhibition of cell cycle progression [250-254], induction of apoptosis [254-256], inhibition of DNA repair [254, 257], inhibition of angiogenesis [258-260], and inhibition of tumour cell motility, invasion and metastasis [260-262]. Stimulation of antibody-dependent cellular cytotoxicity (ADCC) has also been described [263].

Cetuximab blocks cell-cycle progression by inducing G1 arrest. This occurs through an increase in the levels of the p27kip1 inhibitor of cyclin-dependent kinases [251, 264, 265]. Cetuximab's potentiation of apoptosis is correlated with the induction of Bax and the increase in expression of caspases [254-256]. The inhibition of tumour induced angiogenesis is probably due to reduced tumour production of angiogenic factors, including TGF-α, vascular endothelial growth factor (VEGF), interleukin-8 and basic fibroblast growth factor, leading to reduced tumour microvessel density, and inhibition of invasion and metastases by inhibiting matrix metalloproteinases [260, 261, 266, 267].



As an IgG1 monoclonal antibody, cetuximab also has the potential to kill tumour cells through antibody-dependent cell-mediated cytotoxicity (ADCC). As mentioned above, the specificity of cetuximab for the EGFR is determined by its antigen-binding region, whereas its Fc region is characteristic of other IgG1 immunoglobulins. After binding to EGFR, the Fc region of cetuximab remains exposed, and may be recognized by specific Fc receptors for IgG (FcγR) located on the surface of monocytes, natural killer cells, and other immune effectors leading to activation of effector cell functions. For antibody-opsonized tumour cells, activation of ADCC provides a mechanism for tumouricidal action [268, 269]. A synergistic enhancement of anti-tumour effects has been observed in cell lines, and patients treated with cetuximab in combination with different cytotoxic drugs [102, 233, 237, 260, 270, 271].

Treatment of CRC patients with cetuximab, improves overall survival and progression-free survival and preserves the quality of life when the disease did not respond to chemotherapy [272]. Although, patients may acquire resistance-mediating mutations within the extracellular EGFR domain, consequently the exact binding sites of EGFR targeting antibodies may help predict treatment responses [273]. Additionally, mutations in downstream effectors of the EGFR signalling pathways also affect negatively the response to anti-EGFR antibodies [274-276]. A population-based study of 586 patients with colon adenocarcinomas found mutations in *KRAS*, *BRAF*, and/or *PIK3CA* in 316 (56%) of the 586 tumours studied [277]. These mutations result in continuous activation of the downstream Ras/MAPK or PI3K pathways, regardless of whether the EGFR is activated or pharmacologically blocked. Such activation in turn enhances transcription of various oncogenes, including *MYC*, *CREB*, and NF-κB [93, 143, 155].

KRAS mutation is thought to be an early event in tumourigenesis [278-280], plus it is the most commonly mutated gene, with mutations in 35%-45% of colorectal adenocarcinomas; mutations in *PIK3CA* (≤ 20%) and *BRAF* (<15%) are less common [277, 281-285].

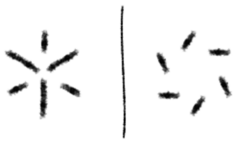
The Ras oncoproteins, namely H-, N- and KRAS are very important in carcinogenesis controlling cellular proliferation and differentiation [286, 287]. Mutations in codon 12 (82%), 13 (17%) and 61 (4%) of KRAS protein have been implicated in resistance to treatment in CRC patients [288]. Thus, the mutation status of the *KRAS* gene in the tumour may affect the response to cetuximab and have treatment-independent prognostic value. Patients with a colorectal tumour bearing mutated *KRAS* did not benefit from cetuximab, contrarily to patients

with a tumour bearing wild-type *KRAS* [289]. In contrast to the widely accepted predictive role of *KRAS* mutation in identifying resistance to anti-EGFR therapy, the prognostic role of KRAS mutation in CRC remains uncertain. Several studies have compared the prognostic roles of *KRAS* codon 12 mutations with those of codon 13. Nonetheless, there is a lack of agreement as to the prognostic difference between *KRAS* codon 12 and codon 13 mutations in colorectal cancer [276, 290-294].

Although the *KRAS* wild-type state seems to be a condition for response, most patients with *KRAS* codon 12 and 13 wild type tumours do not respond to anti-EGFR monoclonal antibodies [289, 295, 296]. Consequently mutations in other downstream effectors of the EGFR signalling pathway, such as *BRAF*, *NRAS*, and *PI3KCA*, might also have a negative effect on response to anti-EGFR antibodies [274-276]. Raf proteins are components of a conserved signalling pathway that regulates cellular responses to extracellular signals. Over 40 different missense mutations in *BRAF*, involving 24 different codons, have been identified. Most mutations are extremely rare, accounting for 0.1%-2% of all cases. However, the mutation V600E within the kinase activation domain of *BRAF* predominates [297]. This mutation impairs the therapeutic potential of cetuximab and panitumumab in CRC cells. Nevertheless, *BRAF*-mutated CRC cells can potentially respond to EGFR-targeted monoclonal antibodies if the BRAF inhibitor sorafenib is administered concomitantly with cetuximab or panitumumab [298]. The histology and clinical characteristics of *BRAF*-mutant tumours are different from *KRAS*-mutant tumours [299], which also suggests specificity of the mutation for tumour subtypes.

In CRC, *PIK3CA* activating mutations have been described at frequencies of 10%-20% [285, 300, 301], and prevalence of *PTEN* mutations has been reported to vary between 1% and 29% [302-307]. The loss of the tumour suppressor *PTEN*, which dephosphorylates the lipid product of PI3Ks results in high levels of PIP3 and constitutive activation of the PI3K pathway [141-144]. *PIK3CA* mutations and PTEN protein deregulation are mutually exclusive [283]. Additionally, loss of expression of the PTEN protein has been reported to confer tumour resistance to EGFR tyrosine kinase inhibitors *in vitro* [308].

Mutations in *PIK3CA* and *KRAS* or *BRAF* may coexist within the same tumour [277, 284, 285, 309], but *KRAS* and *BRAF* mutations appear to be mutually exclusive [281, 282, 298, 310], as were *KRAS* and *NRAS* mutations, and *BRAF* and *NRAS* mutations [292].



The identification of each patient *KRAS* mutation status allows to make a prognostic as to the patients who are likely to benefit from cetuximab and avoid a costly and potentially toxic administration of this treatment in non-responder patients [282]. The toxicity profile of EGFR-targeted mAbs excludes many of the severe side effects commonly observed with cytotoxic chemotherapy. Still, as a class of EGFR inhibitors are characterized by cutaneous adverse effects such as skin rash [311]. The condition can negatively affect treatment compliance and quality of life. In addition to leaving skin vulnerable to bacterial overgrowth and serious infection, skin rash can lead to dose modification or treatment discontinuation, thus potentially affecting the overall clinical benefits of this form of therapy [311]. Additionally, also *BRAF* mutation analysis could be used as an additional tool for the selection of mCRC patients who might benefit from EGFR targeted mAb therapies [298]. All taken, the genetic profiling of individual tumours can lead to personalized medicine with improved therapeutical results.

3. Yeast as a Model Organism for Cancer Study

3.1. Yeast as a Model Organism

The budding yeast *Saccharomyces cerevisiae* is being widely used as a model for investigating fundamental processes affected by genetic and epigenetic alterations in cancer, such as cell cycle progression, DNA replication and segregation, maintenance of genomic integrity and stress responses. *S. cerevisiae* is one of the simplest eukaryotic organisms. It has a life cycle of ±90 minutes, it is quite inexpensive to maintain and grow, and it is stable in both haploid and diploid forms [312]. Its haploid genome fully sequenced and annotated since more than 10 years, has a small size and relatively low complexity as compared to higher Eukaryotes, and it is packaged into 16 well characterised chromosomes [313].

Yeasts reproduce young and die old, or by apoptosis caused by several possible stimulus [314]. They live as individual cells or in colonies, in which case they behave heterogeneously, forming a tissue-like structure [315]. Cells can differentiate in response to environmental cues into pseudo or true hyphae, which formation is related to the invasion and adhesion of natural or artificial surfaces [316]. Yeast has become over the years a prominent model for human

diseases and pathways. There are several reasons for this success, one of which is that at least 31% of the proteins encoded in the yeast genome have a human orthologue, and nearly 50% of human disease genes exhibit yeast orthologues [317]. Therefore, yeasts have also been widely used to unveil the basic molecular mechanisms derived from these proteins and their mutations. This organism has been used to study human pathologies, like neurological diseases [318, 319], mitochondrial diseases [320], as well as insulin signalling [321], cell cycle regulation and ageing [321, 322]. More recently it is being tentatively used to approach obesity and dyslipidemia [323, 324]. Yeast has been used to express successfully human proteins like β-catenin [325], dimerized EGFR [326], as well as other RTKs [327], and the PKC isoforms [314]. Furthermore, yeast has been the testing ground of new genomic technologies: gene expression profiling of drug action [328], synthetic lethal screens [329], drug-induced haploinsufficiency [330], and drug-induced phenotypic responses [331].

Moreover, yeasts harbour well conserved pathways, like TOR, PKC and, Calcineurin, stress responsive, secretory and protein sorting pathways, as well as the Ras/cAMP/PKA [332]. Prominent among yeast signalling pathways are the mitogen-activated protein kinase cascades, allowing a quickly responding/adaptation to a changing environment [333, 334]. The MAPK cascades, found in animals [335, 336], plants [337], and fungi [333, 338], regulate transcription factors by MAPK-mediated phosphorylation. Presently, the budding yeast *S. cerevisiae* has five recognized MAPK pathways [339, 340] (Fig. 13): the mating-pheromone response [341], the filamentation-invasion pathway [342], the high osmolarity glycerol (HOG) stress response [343], and the cell integrity pathway [344]. All of them are active in vegetative cells. The spore wall assembly pathway [345] operates during sporulation and regulates the correspondent developmental process [340].

When establishing disease (cancer or other) related protein models, different approaches are adopted depending on the degree of conservation of the protein under study. If the gene codifying for the protein is conserved in yeast, it is possible to directly study its function [346]. If the gene has no orthologue in yeast, the heterologous expression of the human gene in this organism (the so called “humanized yeast”) can still be highly informative because yeast may conserve protein interactions that can indicate its function and pathobiology [346]. An example of this strategy is the expression in yeast of the tumour suppressor p53 [346, 347]. Moreover the introduction of the human gene into yeast cells often leads to disease-relevant phenotypes

because yeast and mammalian cells may respond similarly to the appearance of such mutant genes [319]. In addition, the lack of endogenous proteins or an entire pathway, *S. cerevisiae* can provide an ideal heterologous system to study the function of these proteins in a null background environment. The protein can be studied in a simpler eukaryotic environment, without the interference of other proteins with similar or overlapping functions, as well as its endogenous regulators. For example, yeast has been used for the independent analysis of each isoform of the protein kinase C (PKC) family [314, 346, 347].

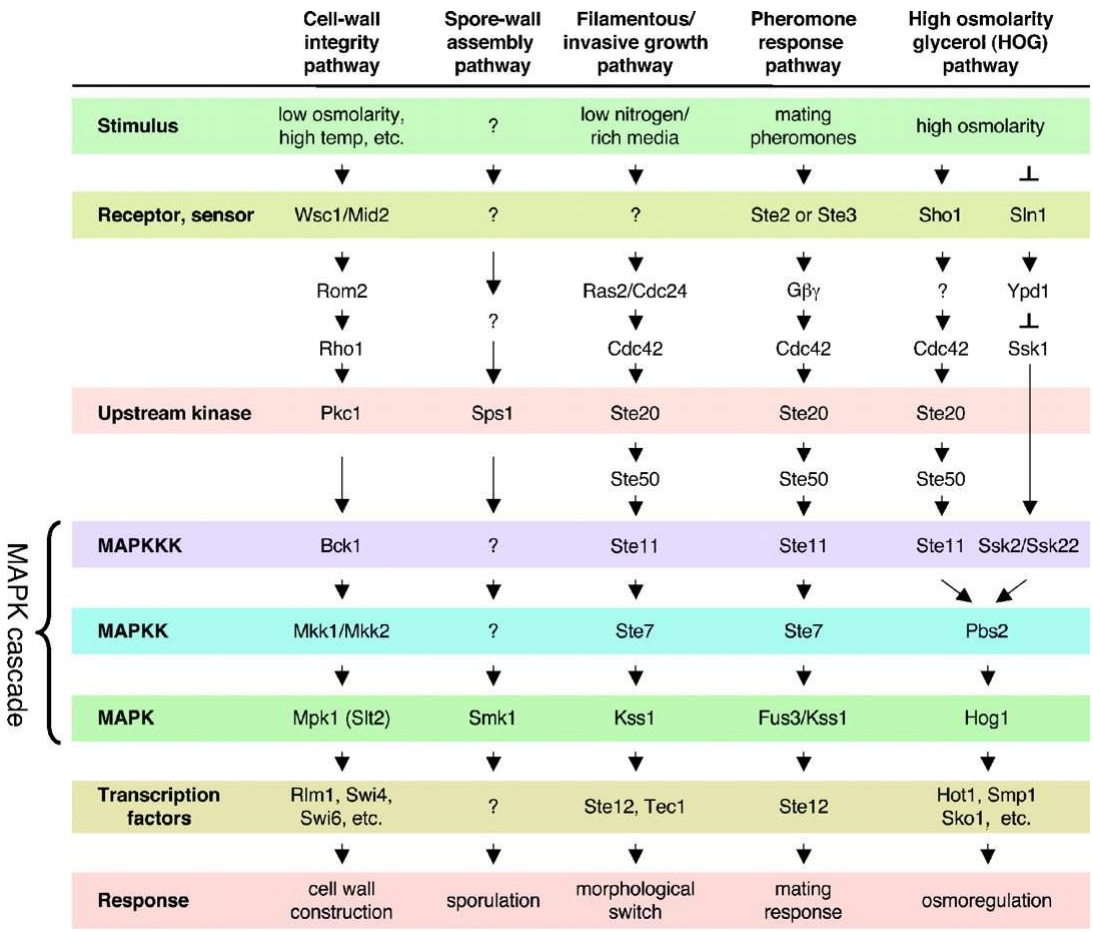


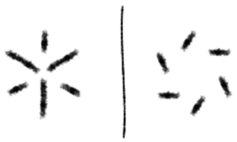
Figure 13 - MAPK pathways in yeast *S. cerevisiae*. The four MAPK pathways present in vegetative cells are the mating-pheromone response pathway, filamentation-invasion pathway, high osmolarity growth, and cell integrity pathway. The fifth one plays a role in spore wall assembly. Withdrawn from [348].

3.2. Yeast and Cancer-Related Signalling

As a cell model for human diseases, yeast has provided insights into the basic processes underlying pathogenesis. However, as a unicellular organism, the analysis of the disease aspects that rely on multicellularity and cell-cell interactions has been seen as a limitation of this cell system, in particular because yeasts' multicellularity is mostly unstudied [349]. Also, as a less complex system, some relevant genes involved in the pathology might not be present in the yeast genome. Even so, yeast cell system has already proved its value as a first-line tool in the discovery of mechanistic processes involved in the disease due to the molecular interaction networks that are largely conserved from yeast to humans [350].

Dozens of different cancer-causing mutations have been identified up to date. Most of these mutations occur in genes that have some role in regulating the cell cycle, or in the series of events that undergo as DNA is replicated and cell divides. The majority of these mutations were discovered in other species, like yeast, before their role in human cancer was realized. Leland H. Hartwell won 2001 Nobel Prize in Physiology or Medicine for his contributions to the understanding of the cell cycle regulation in particular the identification of cyclins and cyclin-dependent kinases that act as cell cycle checkpoints, using *S. cerevisiae* as biological model [351]. Hartwell L.H. identified in yeast more than 100 genes involved in cell cycle control, generally known as the cell division cycle genes (CDC). As it turns out, the same genes that control the cell cycle in baker's yeast, exist and identically control cell cycle progression in human cells, and malfunction in tumour cells [351-353]. Hartwell L.H. shared his Nobel Prize with Paul Nurse, who is credited for discovering the first human equivalent of yeast's CDC genes: cyclin-dependent kinase 1, or the *CDK1* gene [354]. CDC genes and the molecular pathways they control are actually highly conserved through evolution [264, 355-358].

CDCs are shown to regulate the cell cycle by either stimulating or inhibiting cell division in response to the signals that cells constantly receive from their environment. In cancer cells, these are designated as proto-oncogenes and tumour suppressor genes, respectively. Importantly, their malfunction implicates the malfunction of *e.g.* cell cycle checkpoints. The checkpoint molecular machinery detects serious anomalies, like damage in DNA or chromosomes, and promotes the arrest of the cell cycle until the damage is repaired, opening the way for cell self-destruction through programmed cell death events, like anoikis and apoptosis [359, 360], if repair is not



possible. Therefore, mutation on a checkpoint protein (*e.g.* p53) is often associated with cancer ontology and progression [351-353].

Many of the genes that are frequently altered in tumours have structural or functional orthologues in model genetic systems, including the yeast *S. cerevisiae* [361]. As mentioned above, yeast presents a considerable degree of homology to the human proteome, including two onco-Ras genes *RAS*1 and *RAS*2 [362, 363]. Yeast Ras1p and Ras2p proteins (small GTPases) are highly homologous and phenotyipically partially redundant. The N-terminal portions of these proteins have significant homology to the mammalian Ras, including some short sequences of amino acids that are involved in the recognition of guanine nucleotide and phosphate [364]. On the other hand, yeast Ras proteins C-terminal diverges significantly with the mammalian Ras, especially in the 4 terminal amino acids that are important for post-translational modifications that facilitate their membrane association [365]. Mammalian *HRAS* can suppress the loss of either yeast counterparts supporting the functional similarity observed between the yeast and mammalian genes [366, 367]. On the other hand, a modified yeast *RAS*1 gene can be biologically active in mammalian cells since it induces morphologic transformation of mouse NIH3T3 cells [366].

Ras1p and Ras2p have similar functions, but their expressions differ. *RAS*1*,* but not *RAS*2*,* is repressed when cells are grown on non-fermentable carbon sources such as glycerol or pyruvate [368]. Therefore, a strain deleted in *RAS2* will behave in this situation as a double deleted mutant, since both Ras1p and Ras2p proteins are absent. Otherwise, the actual *ras*1Δ*ras*2Δ double mutant is nonviable [369, 370]. Cells with a temperature-sensitive *RAS*2 mutation or *ras*1Δ deletion are blocked in the G1 phase of the cell cycle and do not bud at non-permissive temperatures [365]. Mutations of the *RAS*2 gene cause accumulation of storage carbohydrates and concomitant sporulation increase, even on rich media [365]. Conversely, yeast cells expressing an activating mutant of Ras2p, $Ras2^{val19}$, exhibit (i) reduced sporulation and decreased glycogen storage level, (ii) sensitivity to heat shock, and (iii) sensitivity to nutrient starvation [369]. Also, the amount of cAMP inside the cell is decreased in the *ras* mutants, and increased in the mutant expressing $Ras2^{val19}$ [369].

The Ras/cAMP pathway in *S. cerevisiae* (Fig. 14) plays a major role in the control of growth, proliferation, metabolism, stress resistance, aging, morphogenesis and development according to nutrients availability, specially the glucose that can act as a signalling molecule to

regulate multiple aspects of yeast physiology [371]. In response to nutrients, the guanine nucleotide exchange factors (GEF) Cdc25p and Sdc25p activate the redundant Ras1p and Ras2p GTPases. The GTPase activating proteins (GAPs), encoded by the *IRA*1 and *IRA*2 genes, downregulate Ras proteins by promoting GTP hydrolysis, resulting in the accumulation of Ras 1/2-GDP, the inactive form of Ras 1/2p. On the other hand, GEFs encoded by *CDC25* and *SDC25* promote Ras 1/2p activation by facilitating GTP charging of Ras [372]. When in an active state both Ras1p and Ras2p function to activate adenylate cyclase (Cyr1p) which is associated with a protein called CAP [370], resulting in the production of cAMP from ATP and activation of protein kinase A (PKA). Binding of the secondary messenger cAMP to the regulatory PKA subunit Bcy1p induces its dissociation from the PKA catalytic subunits (redundantly encoded by the *TPK1*, *TPK2*, and *TPK3* genes) resulting in their activation [371, 372]. Subsequently, the phosphorylation of several substrates leads to the regulation of a variety of functions including cell cycle progression [371, 372]. Recently, the complexity of Ras/cAMP signalling has increased as a result of the discovery of a G-protein-coupled receptor system, consisting of Gpr1p-Gpa2p complex, that appears to act upstream of adenylate cyclase to stimulate cAMP production, probably in response to glucose [371, 372].

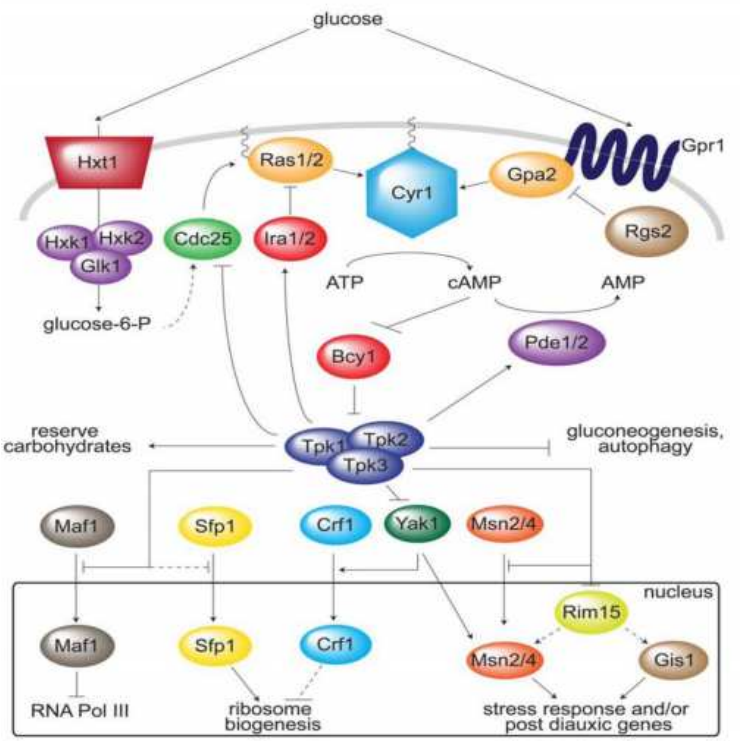
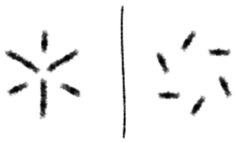


Figure 14 - The cAMP-PKA pathway in *S. cerevisiae*. Addition of glucose to glucose-starved, respiring cells triggers the rapid synthesis of cAMP and, subsequently, the activation of PKA. Glucose-induced cAMP synthesis



requires two sensing systems: (i) extracellular detection of glucose via the Gpr1p–Gpa2p system and (ii) intracellular detection of glucose, which requires uptake and phosphorylation of the sugar. The intracellular sensing system most probably transduces signals via the guanine nucleotide exchange factor (GEF) protein Cdc25p and the Ras proteins. Activated PKA (Tpk1p, Tpk2p, Tpk3p) mediates the fast transition from respiratory to fermentative growth via the modulation of numerous downstream targets, thereby allowing cells to make the necessary adaptations for fermentative growth. These include the upregulation of glycolysis, the stimulation of cell growth and cell cycle progression, the downregulation of stress resistance and gluconeogenesis, and the mobilization of the reserve carbohydrate glycogen and the stress protectant trehalose. Arrows and bars represent positive and negative interactions, respectively. Dashed lines represent putative or indirect interactions. Withdrawn from [372].

The Ras/cAMP pathway regulates a variety of processes including cell cycle progression and life span [369, 370, 373]. Recent studies identified a number of other cellular processes regulated by the Ras/cAMP pathway. For example, this signalling pathway is reported to regulate the polarity of actin cytoskeleton through the stress response pathway [374]. It also controls spore morphogenesis [375], and the activity of the amino acid transporter Gap1p permease [376] which has a crucial role in nitrogen regulation in yeast (for a review see [377]). Ras/cAMP signalling has also been implicated in the nutrient-mediated control of ribosome biogenesis [378]. Conversely, when the reported phosphorylation of Ras proteins is repressed [379] that results in enhanced sensitivity to heat shock, reduced glycogen, and increased cAMP. This may suggest that phosphorylation is involved in the feedback regulation of the Ras/cAMP pathway [365]. Importantly, Ras signalling deregulation compromises DNA damage checkpoint recovery [380].

In addition to the Ras/cAMP/PKA signalling cascade, the other major nutrient-responsive, growth-controlling pathway in yeast is the TOR network (Fig. 15) [371, 372]. Tor (Target of Rapamycin - a macrolide drug that binds to TOR suppressing its interaction with target substrates) serine/threonine kinases, Tor1p and Tor2p, belong to the phosphatidylinositol-3 kinase (PI3K) family and exert their functions in two distinct multiproteic complexes: TOR Complex 1 (TORC1) which control various aspects of yeast growth and cell proliferation, and TORC2 which regulates cell polarity and organization of the actin cytoskeleton [371, 372]. The two complexes are structurally and functionally conserved in all the eukaryotes [381]. TORC1 activity responds to the nutritional status, primarily the quality of the nitrogen source, and to a wide variety of stress conditions, apparently relaying amino acid concentrations, glucose, and perhaps other nutrient signals to the cellular machinery, including growth factors (insulin/IGF) in mammals [371, 381]. Its major function appears to be the regulation of translation capacity in

response to environmental signals by promoting ribosome biogenesis, amino acid availability, and translation efficiency [371, 372]. The specific inhibition of TORC1 by rapamycin mimics nutrient starvation and causes G1 arrest, inhibition of protein synthesis, glycogen accumulation, induction of autophagy and entry into quiescence [371, 372].

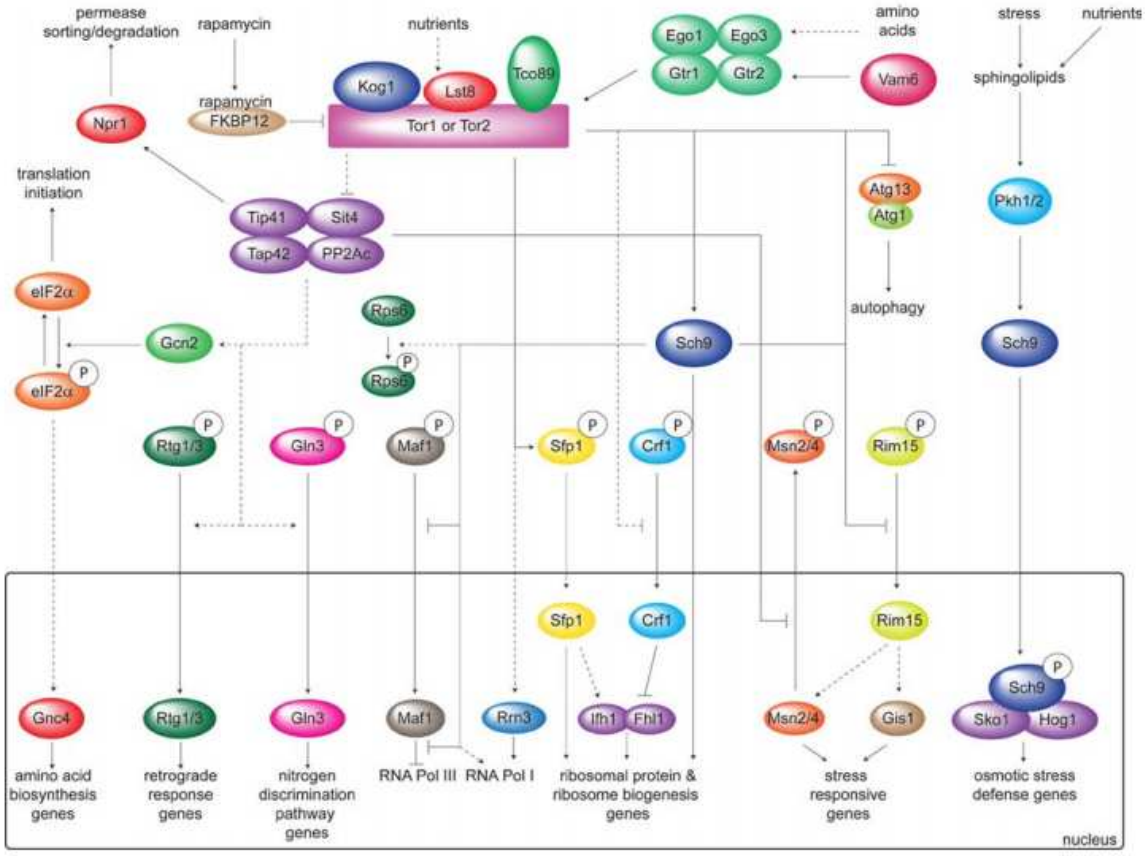
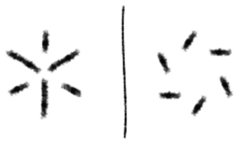


Figure 15 - The TORC1 pathway in *S. cerevisiae*. Nutrients activate TORC1, resulting in the stimulation of protein synthesis and the inhibition of stress response genes, autophagy and several pathways that allow growth on poor nitrogen sources. A major part of these processes is regulated by the rapamycin-sensitive TORC1 complex either via the Tap42p-Sit4p/PPA2c or by the recently identified Sch9p branches. The activity of Sch9p is additionally regulated by Pkh1p and Pkh2p. Note that Sch9p functions both in the cytoplasm and the nucleus. Withdrawn from [372].

In the literature, several functional interactions between TOR and the Ras/cAMP pathway have been described [371]. For example, the activation of the Ras/cAMP signalling pathway confers pronounced resistance to rapamycin. Additionally, the constitutive activation of the Ras/cAMP pathway prevents several rapamycin-induced responses, such as the induction of



stress genes, the accumulation of glycogen, the induction of autophagy, the downregulation of ribosome biogenesis, and the downregulation of some glucose transporters. Finally, and importantly, TOR controls the subcellular localization of both the PKA catalytic subunit Tpk1p and the Ras/cAMP signalling-related kinase Yak1p [371, 372, 382]. Overall, the exact relationship between the TOR and Ras/cAMP/PKA networks is still not fully understood. It has been suggested that the TOR and PKA signalling cascades independently coordinate the expression of several genes. As an alternative model, it has been proposed that TOR may act upstream of Ras to regulate PKA activity, thus the Ras/cAMP pathway may actually be a novel TOR effector branch [371, 372, 382].

The mammalian TOR (mTOR) is a critical target of EGFR signalling, linking growth factor abundance to cell growth and proliferation. Generally, it is the PI3K/Akt pathway [383] (see Fig. 7), but also the PLC-γ/PKC pathway [384], that connects EGFR and mTOR. In addition, signalling through mTOR is stimulated by defects in the pathway components upstream of mTOR, such as growth factor receptors, PI3K, Akt, PTEN, or by stimulation of PI3K by effectors of the mutant Ras/Raf/MAPK pathway [385]. Overall, mTOR is a key intracellular kinase integrating proliferation, survival and angiogenic pathways and has been implicated in the resistance to EGFR inhibitors. Thus, mTOR blockade is pursued to interfere at multiple levels with tumour growth [383].

The true dimension and conservation degree of the PI3K signalling is still not well perceived in yeasts [386]. The only detectable PI3K from *S. cerevisiae* is Vps34p (Vacuolar Protein-Sorting-34) that is present in two different complexes to carry out autophagy - Complex I - and Carboxypeptidase-Y sorting - Complex II. Vps34p is involved with trafficking of proteins from Golgi to vacuole in yeast and also catalyses the conversion of PtdIns to PtdIns(3)P [387, 388]. The Vps34p-mediated PtdIns(3)P production recruits effector proteins that function in budding [389, 390]. Nonetheless, in higher eukaryotes, the PI3K and PTEN are major positive and negative regulators, respectively, of the PI3K/Akt pathway, controlling growth, survival, and proliferation [391, 392]. In *S. cerevisiae*, Tep1p [393, 394] (orthologue of human tumour suppressor gene *PTEN*) inhibits the downstream functions mediated by the Vps34p pathway, such as cell survival, cell proliferation and activation of Sch9p (serine/threonine protein kinase and an Akt/PKB orthologue), which is activated by cAMP, and regulates longevity and stress resistance in yeast [388, 389].

There are several lines of evidence that Sch9p plays a central role in nutrient-mediated signaling in the budding yeast [372, 395]. It acts in parallel with the PKA pathway and is directly phosphorylated by TORC1, mediating many of the TORC1-regulated processes. Nevertheless, Sch9p also acts independently of TORC1, and can even exert opposite effects to TORC1 in the adaptation to stressful conditions (Fig. 15 and 16) [372]. Sch9p integrates nutrient signals with cell size regulation [396], along with being an activator of ribosomal proteins and ribosomal biogenesis [396]. Sch9p is also a negative regulator of both chronological and replicative aging [397, 398] and has recently been shown to similarly regulate mitochondrial respiration [399]. It was demonstrated that the *sch9*Δ mutation upregulates electron transport chain gene expression and that this is associated with an increase in mitochondrial respiration [399], further strengthening the fact that yeast orthologues of oncogenes play a role in the regulation of cell metabolism [400]. It has recently been shown that yeast Sch9p is a central component of a network that controls a common set of genes implicated in a metabolic switch from the TCA cycle and respiration to glycolysis and glycerol biosynthesis. During chronological survival, mutants lacking *SCH9* depleted extracellular ethanol and reduced stored lipids, but synthesized and released glycerol, suggesting that glycerol production, in addition to the regulation of stress resistance systems, optimizes life span extension [401].

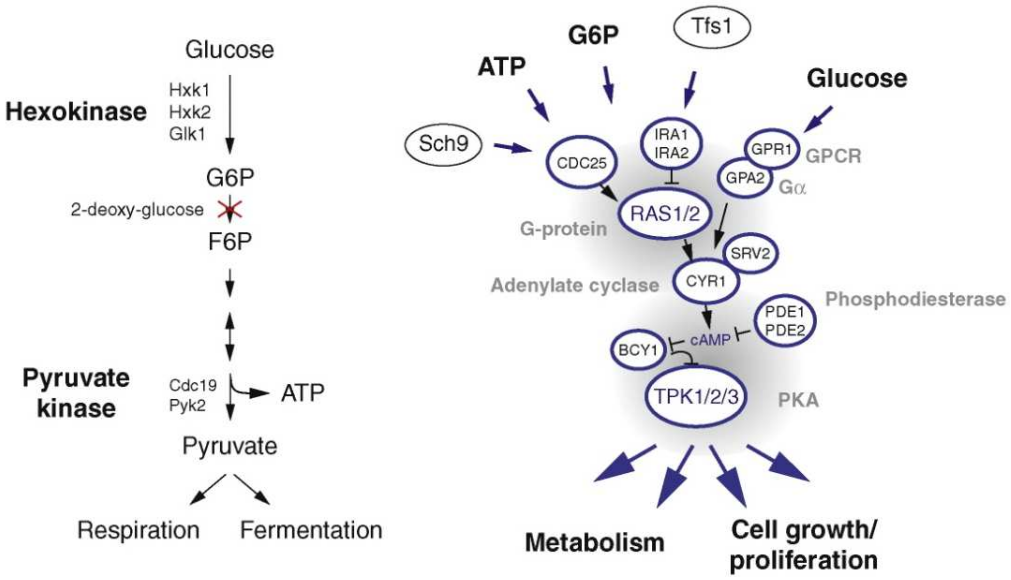
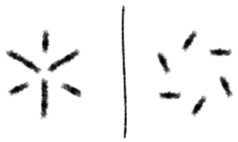


Figure 16 - PKA responds to extracellular and intracellular glucose signals. Putative upstream regulators of the Ras/PKA pathway deriving from glycolysis. Withdrawn from [402].



3.3. Metabolic Similarities Between Cancer Cells and Yeast

In order to proliferate, cells must comply with the energy demand imposed by vital processes such as macromolecule biosynthesis, DNA replication, ion gradients generation and cell structure maintenance. Hence, mitochondria play an important role in energy metabolism as they synthesize most of the cellular ATP through oxidative phosphorylation. However, it was suggested that cancer cells suppress mitochondrial metabolism [400, 403, 404]. Otto Warburg back in 1920s registered that the metabolism of cancer cells is shifted from the oxidation of glucose to carbon dioxide and respiration-driven ATP production to fermentative reduction of pyruvate to lactate. Under these conditions, ATP is mainly derived from cytosolic glycolysis, which, however, is a much less efficient pathway to generate energy compared to mitochondrial respiration [400, 403, 404]. In spite of the decrease in energy yield, as a consequence of the "glycolytic phenotype", this seems to allow an increase in cell proliferation rate and be applicable to other fast growing cells [405]. Because the repression of oxidative metabolism occurs even in the presence of oxygen, this metabolic phenomenon is known as "aerobic glycolysis" or the "Warburg effect" [400]. The specific advantages that cancer cells acquire by undergoing this metabolic switch are unknown. Moreover, it is unclear whether "aerobic glycolysis" is a prerequisite for a cell to become neoplastic or if this dramatic switch in metabolism occurs concomitantly to, or after malignant transformation, although a correlation between the glycolytic phenotype and tumour invasiveness has also been suggested [406]. It has also been suggested that "aerobic glycolysis", which is accompanied by high glucose uptake rates and acidification of the extracellular environment, may actually be a response to hypoxic conditions, providing growth advantage. Similarly, aerobically fermenting yeasts, also display Warburg effect, converting glucose to ethanol and acetic acid at high rates in the presence of oxygen [407]. The concomitant reduced mitochondrial activity might contribute to the ability of cancer cells to evade apoptosis [408-410].

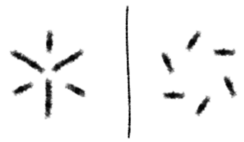
The exact molecular mechanisms underlying the "Warburg effect" are unknown. Nonetheless, it is possible that the enhanced expression of glycolysis enzymes and glucose transporters, along with a downregulation of mitochondrial metabolism could be at the basis of the glycolytic phenotype of tumour cells [403]. In these cells, the HIF-1 transcription factor regulates hypoxia-induced metabolic reprogramming through the expression of glycolysis

enzymes, glucose transporters and several other tumour-related genes [411]. Although aerobic glycolysis generates only 2 molecules of ATP per molecule of glucose, the overall rate of ATP production might indeed be higher in aerobic glycolysis than in mitochondrial respiration due to lower costs for enzyme production or higher activities of the fermenting enzymes compared to TCA cycle and respiratory chain enzymes and cofactors [412, 413]. Nonetheless, some glioma, hepatoma and breast cancer cell lines possess functional mitochondria and obtain their ATP mainly from oxidative phosphorylation [414, 415], posing questions as to the actual biological role of this metabolic behaviour. Moreover, some cancer cells can reversibly switch between fermentation and oxidative metabolism, depending on the absence or the presence of glucose and the environmental conditions [416, 417].

Herbert G. Crabtree back in 1929 showed that mitochondrial respiration in neoplastic tissue is repressed at physiological glucose concentrations [418]. Interestingly, a recent model proposed that "glycolytic" cells could establish a metabolic symbiosis with the "oxidative" ones through lactate shuttling [419]. Regarding this, a well-defined feature of some cancer cells is the glucose-induced suppression of respiration and oxidative phosphorylation [404]. This is a short-term and reversible event and is referred to as the "Crabtree effect". Crabtree effect is long and well known to yeast biotechnologists who deal with industrial fermentations since *S. cerevisiae*, as Crabtree positive yeast, ferments glucose aerobically in the presence of extremely high extracellular glucose concentrations instead of producing biomass via the TCA cycle [420, 421]. Increasing concentrations of glucose accelerate glycolysis. This provokes an increase in the ATP yield through substrate phosphorylation, decreasing the need for oxidative phosphorylation through the TCA cycle, and therefore oxygen consumption, and ultimately leading to the repression of respiratory enzymes [422, 423]

The reversible switch between fermentation and oxidative metabolism might represent an advantage of cancer cells *in vivo*, as it would allow them to adapt their metabolism to the rather heterogeneous microenvironments in malignant solid overgrowths. It is therefore crucial to clearly understand the long-term metabolic reprogramming of cancer cells (the Warburg effect) and the short-term adaptation mechanisms (the Crabtree effect) as the targeting of both would lead to much more effective therapeutic strategies [400].

Yeast cells when grown in the presence of oxidative carbon sources (*e.g.* lactate, acetate or glycerol) contain competent and well differentiated mitochondria [424]. In contrast, when glucose



is present, as mentioned above, mitochondrial morphology changes and oxidative, catabolite repression or glucose repression [425]. From the metabolic point of view it is very similar to the Warburg effect. Glucose repression acts on an enormous variety of genes, which expression only occurs when the amounts of extracellular glucose reduce to levels below ±0.3% (w/v) [426]. Therefore, when yeasts are proliferating in a glucose rich medium, a considerable part of the genome is shut down, especially genes related to metabolism of alternative substrates, respiration, mitochondrial activities, and gluconeogenesis [427]. When the glucose concentration on the growth medium decreases below the referred threshold, repression on transcription alleviates and many genes become induced, including transporters for respirable carbon sources, and enzymes from catabolism that allow the consumption of other carbon sources [428]. The transition period between a high glucose, fermentative metabolism to a low or no glucose respiratory metabolism is called "diauxic shift" [429]. This nomenclature derives from the intermediate growth arrest of a microbial culture that is observed after glucose is exhausted and while cells re-programme their proteome for the consumption of a respirable carbon source, allowing a second fast growth period, thus producing a microbial culture diauxic growth curve [371, 372]. The same mechanisms that could explain the Crabtree effect induction in tumour cells may equally apply to the yeast model: limitations in ADP and Pi levels [106], Ca^{2+}-induced decrease in respiration [107], reduced permeability of the mitochondrial outer membrane [108] and fructose 1,6-biphosphate mediated inhibition of the respiratory chain [404].

For a summary of the parallels/similarities between the glucose-induced repression of oxidative metabolism of yeast and the "aerobic glycolysis" of tumour cells see Fig. 17:

- In both cell types, the downregulation of oxidative metabolism is observed along with an enhanced fermentation despite the presence of oxygen [404].
- Yeast shares with cancer cells the metabolic features that are identified as the underlying causes of the Warburg effect [404].
- As cancer cells, fermenting yeast over-express all glycolytic enzymes in response to high glucose, and regardless to the presence of oxygen - Crabtree effect [430, 431].

Therefore, *S. cerevisiae* presently emerges as a suitable model for the screening of metabolism-targeted drugs employed for anti-tumour therapy [400, 404], in spite of the debate originated from yeast not possessing the genetic defects identified in cancer cells that ultimately

implicate changes in metabolism regulation [361]. Yet, the genome and proteome similarities between *S. cerevisiae* and human cells, in particular at the level of fundamental/conserved cellular and molecular processes allied to the huge knowledge on *S. cerevisiae* metabolism and regulation, derived from biotechnology, will most certainly contribute to faster understanding of similar intricate circumstances in human cells.

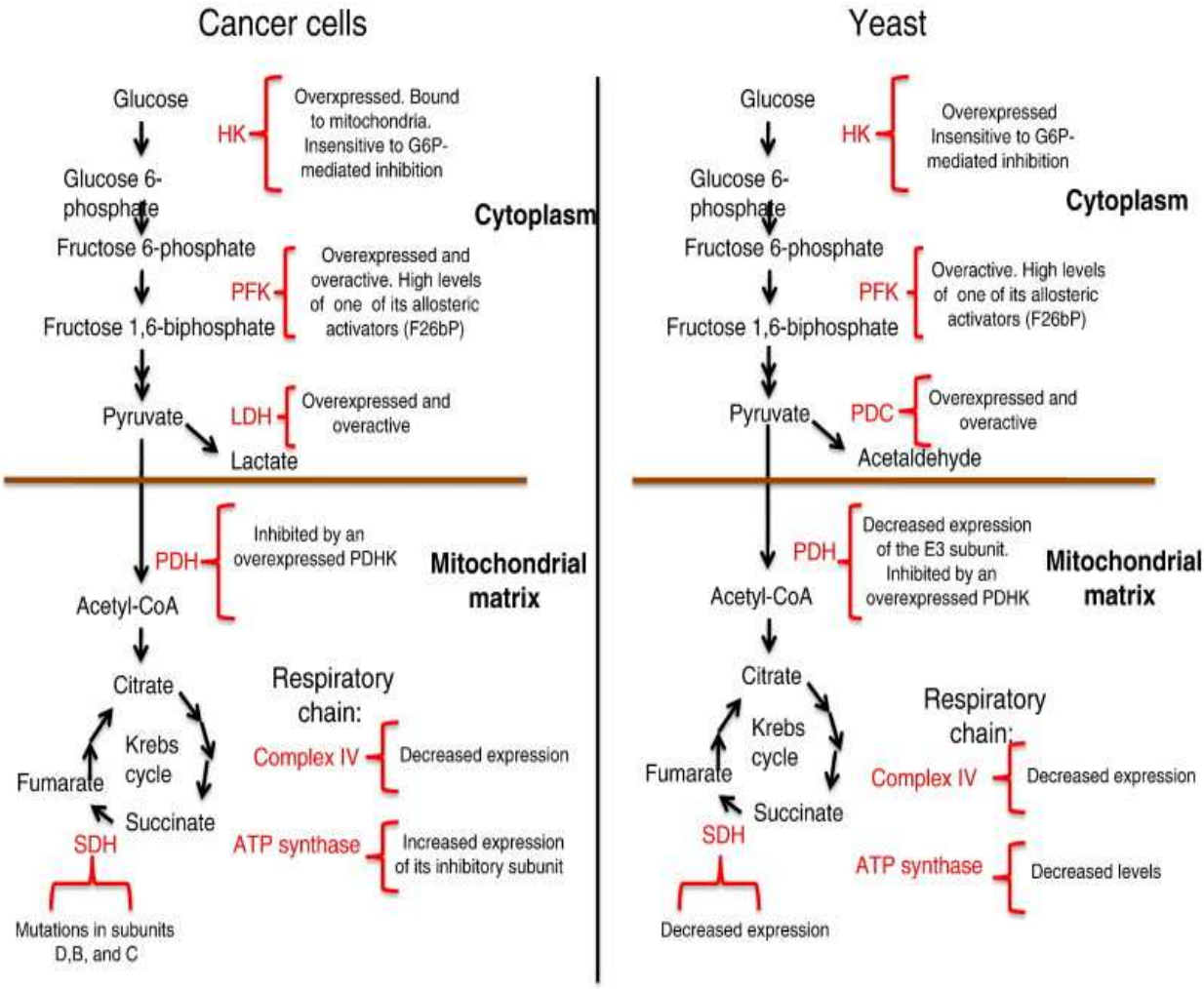
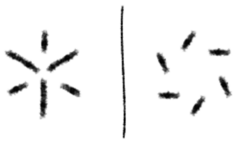


Figure 17 - Metabolic similarities between cancer cells and yeast. Hexokinase (HK) isoforms insensitive to glucose 6-phosphate-mediated inhibition are overexpressed. Phosphofructokinase (PFK) is activated by higher levels of one of its allosteric activators: fructose 2,6-biphosphate (F26bP). The enzymes that metabolize pyruvate in the cell cytoplasm are overexpressed: Lactate dehydrogenase (LDH) in the case of cancer cells and pyruvate decarboxylase in yeast (PDC). Pyruvate dehydrogenase complex (PDH) is inhibited through phosphorylation by an overexpressed pyruvate dehydrogenase kinase (PDHK). Succinate dehydrogenase (SDH) has mutations in different subunits in cancer cells and its expression is downregulated in yeast. In both cases, a decreased expression of mitochondrial complex IV is observed as well as a downregulation of the ATP synthase. Withdrawn from [400].



3.4. Using Yeast to Study Cell Communication Through RTKs

Yeasts, as all cells, display plenty of surface proteins involved in sensing and signalling. These include GTP-binding proteins and protein kinases with close relatives among the receptors and intracellular signalling proteins in animal cells [11]. The human genome contains more than 1,500 genes that encode receptor proteins, and the number of different receptor proteins is further increased by alternative RNA splicing and posttranslational modifications. The large numbers of signal proteins, receptors, and intracellular signalling proteins used by animals can be grouped into a much smaller number of protein families, most of which have been highly conserved in evolution. Flies, worms, fungi and mammals all use essentially similar machinery for cell communication [11, 83].

Deregulated RTK signalling is critically involved in the development and progression of human cancer. Also in this case, *S. cerevisiae* represents an inexpensive and rapid alternative for measuring the activity of RTKs in a heterologous, yet eukaryotic environment [326, 346, 432]. The fact that yeast genome apparently does not display any RTK-like protein, offers the advantage of a null background for the expression of mammalian RTKs and for the measurement of the effects of compounds on the specific target [326].

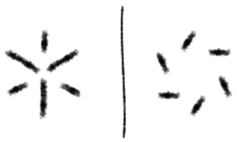
S. cerevisiae has been used to study several features of EGFR and its inhibitors after EGFR expression and incorporation into the cell surface [433-436]. In one of these studies, large fragments, some encompassing multiple domains of EGFR, were expressed and properly folded on the surface of yeast due to the protein folding and quality control machinery in the endoplasmic reticulum. These fragments were used to localize antibody binding to particular domains of EGFR [433]. In another study, a yeast-displayed library of single point mutants of an EGFR ectodomain fragment (residues 273-621) was constructed by random mutagenesis and was screened for reduced binding to EGFR mAbs. If an EGFR mutant showed loss of binding to a mAb, this suggested that the mutated residue was potentially a contact residue [434]. A subsequent study used directed evolution by random mutagenesis and recombination followed by yeast surface display to isolate mutants that exhibit proper protein folding for the soluble expression of EGFR's ectodomain in *S. cerevisiae.* As screening probes, several conformationally-specific mAbs against EGFR's ectodomain were employed [435]. Monoclonal antibody 225 (the predecessor of the chimeric human/murine antibody IMC-C225 or cetuximab/Erbitux®) binds to

domain III of the receptor, between 294 and 475, and it is reactive towards conformational or discontinuous epitopes of the receptor [433, 435]. Moreover, the amino acids Lys465 and Ile467 were identified as important residues for mAb 225 binding to EGFR. These residues are adjacent to the EGFR ligand-binding site, which is consistent with the ability of mAb 225 to block binding of EGF and TGF-α ligands [434].

Alternatively, Gunde and co-workers have reconstituted aspects of the EGFR signalling pathway in yeast *S. cerevisiae* [326]. The temperature sensitive *cdc25-2* yeast strain does not grow at 37°C, because the mutant Cdc25p protein (the yeast orthologue of mammalian son of sevenless (Sos) and a Ras guanyl nucleotide exchange factor (Ras-GEF) functioning upstream of the Ras proteins in *S. cerevisiae*) is unable to activate the endogenous Ras protein at the restrictive temperature. The co-expression of dimerizing mammalian EGFR and EGFR derivatives, together with a cytoplasmic GRB2 adaptor fused to the membrane-localized and constitutively active human Ras rescues growth of the *cdc25-2* mutant yeast strain at the nonpermissive temperature. Using kinase-defective RTK mutants and selective EGFR kinase inhibitors, it is demonstrated that growth rate of this yeast strain correlates with kinase activity of the EGFR derivatives [326].

Busti and co-workers [432] showed that the EGFR module (constitutively activated EGFR which activation is independent of ligand stimulation, GRB2 and hSos1) can be functionally linked to the Ras/cAMP/PKA pathway (see above) in a *S. cerevisiae cdc25*^{ts} (temperature sensitive) strain. Several independent biological readouts showed a significant delay in inactivation of the Ras/cAMP/PKA pathway, including drop of budding index, decrease of cAMP level, acquisition of thermotolerance and arrest of cell division [432]. Auto-phosphorylation of the EGFR is a necessary step in coupling RTK expression to yeast Ras activation. In fact, no significant effect was observed in cells transformed with a kinase defective version of the receptor or treated with a drug specifically inhibiting the kinase activity of the receptor. Although significant, the physiological coupling of the EGFR module to the Ras/cAMP pathway is short-lived. This could be due to EGFR being expressed in yeast in a partially misfolded state [435] that in turn could limit receptor autophosphorylation [432]. Therefore, appropriately engineered yeast can serve as screening tool for identifying RTK inhibitors by combining advantages of *in vitro* (target-specific read-out) with those of cell-based assays (physiological environment).

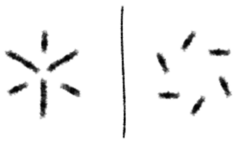
OBJECTIVES



Drugs and molecular effectors used against the most frequent cancers have a statistically significant degree of ineffectiveness in patient's population, being this one of the most prominent problems identified in clinical practice. Thus, it is imperative the adjustment of therapeutic strategies to the individual cancer patient in a clinically manageable period of time. Yeasts are being extensively used worldwide as tools for drug discovery, drug target identification, and drug mode of action study. This is due to the high degree of conservation between yeast cellular processes and those of human cells, as well as their versatile genetic malleability, becoming the model of choice for research in molecular and cell biology. In this context, the present work is included in a broader aim of establishing *Saccharomyces cerevisiae* as a tool for theranostics.

In view of the high percentage of colorectal cancer (CRC) patients insensitive to routine treatment with cetuximab/Erbitux®, a monoclonal antibody against epidermal growth factor receptor (EGFR) from Merck Serono, this EGFR antagonist was chosen as a first case study. Previous preliminary results from our team showed that yeast is sensitive to the presence of cetuximab/Erbitux®. Therefore, the work primarily dedicated to the identification of this antibody *S. cerevisiae* surface target, ultimately seeking the possible EGFR counterpart in yeast, the downstream effectors and therein regulated genes.

Material & Methods



1. *In Silico* Survey to Discover the EGFR Counterpart in Yeast

1.1. Yeast Cell Surface Proteins Identification

The *Saccharomyces* Genome Database SGD (www.yeastgenome.org) was used to collect the sub set of yeast genes corresponding to annotated proteins known to localize at the cell wall and at the plasma membrane. For this purpose, the search was performed using several key words, such as “cell wall”, “plasma membrane”, “permease”, “pump”, “channel”, “transporter”, “receptor”, “cell periphery”, “extracellular region”, “periplasm”, “transmembrane”, “integral to membrane”, “extrinsic to membrane”, “plasma membrane enriched fraction”, “internal side of plasma membrane”, “extrinsic to internal site of plasma membrane”, “kinase”, “ras”, “raft”, “antigen”, “actin cortical patch”, “GPI”, “manoprotein”, “glycoprotein” and “PIR” (CD-ROM at the back cover of the final printed version - Supplementary Material - Table I). To check the hydrophobicity of putative plasma membrane integral proteins, it was used the net-available servers, based on Kite and Doolittle method for the prediction of transmembrane helices in proteins: TMHMM Server v.2.0 (www.cbs.dtu.dk/services/ TMHMM/), PredictProtein server (www.predictprotein.org). Additionally, also the Transporter Classification Database (www.tcdb.org) was used.

Protein BLAST was performed on SGD database using as bait (i) the EGFR total protein sequence (epidermal growth factor receptor isoform a precursor; accession: NP_005219.2 GI: 29725609), as well as the (ii) extracellular domain (residues 25-645), (iii) domain I/L1 (residues 25-189), (iv) domain II/CR1 (residues 190-334), (v) domain III/L2 (residues 335-504), (vi) domain IV/CR2 (residues 505-645), (vii) cytoplasmic domain (residues 669-1210), (viii) domain tyrosine kinase (residues 707-982), all sequences from EGFR protein. The SGD protein sequence dataset was the yeast “gene: protein encoding (strains)”, among these are BY4741, W303, CEN.PK and other well-known laboratory strains. Occasionally, the Génolévures dataset, which includes other *S. cerevisiae* genetic backgrounds, was also used (cbi.labri.fr/Genolevures/). All searches were performed with the BLOSUM 62 scoring matrix, coupled with default parameters (Oct, 2011; for more information see also Table I from supplementary materials).

1.2. Search for Similar EGFR Domain Architectures in Yeast Proteins

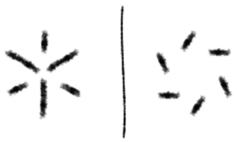
EGFR conserved domain architecture was explored in the whole yeast proteome using all the available data on *S. cerevisiae* strains at NCBI Conserved Domain Architecture Retrieval Tool (www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi) and InterPro database (www.ebi.ac.uk/interpro/). To investigate yeast proteins that have L domains, a database of structural and functional annotation for all proteins and genomes - SUPERFAMILY database v.1.75 (supfam.org/SUPERFAMILY/ index.html) was used. Multiple sequence alignments were performed using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2/). Proteins' tri-dimensional structure was obtained using Swiss-PdbViewer v.4.01.

2. Yeast Strains and Culture Media

S. cerevisiae strains used for the experiments were wild type (wt) BY4741 (*MAT*a *his*3Δ1, *leu2*Δ0, *met*15Δ0, *ura*3Δ0) obtained from EUROSCARF (Frankfurt, Germany) and W303-1A (*MAT*a *leu2*-3/112, *ura*3-1, *trp*1-1, *his*3-11/15, *ade2*-1, *can*1-100) [437]. The mutants used throughout the work were obtained from the *S. cerevisiae* single deletion strains collection (EUROSCARF), in the BY4741 background [438]. Strains were routinely kept at 4ºC in YPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar).

3. Cetuximab

The monoclonal antibody cetuximab/Erbitux® was supplied by Merk (Germany) in its clinical presentation of 5mg/mL (32.89μM), and stored at 4ºC. Besides the cetuximab (active ingredient) the other components of the Erbitux® solution are sodium chloride (isotonicity agent), sodium dihydrogen phosphate dehydrate (buffer), disodium phosphate dehydrate (buffer) and water. This solution was diluted in medium to the desired concentrations for the drug assays performed.



4. Cetuximab Susceptibility Assays

4.1. Yeast Growth Curves

The susceptibility of the parental strains, BY4741 and W303-1A, to cetuximab was assessed by comparing the growth in liquid cultures supplemented with a range of concentrations of cetuximab. Yeasts were grown at 30°C in YNB medium (0.5% ammonium sulphate, 0.17% YNB, 2% glucose) supplemented with the appropriate auxotrophic requirements (10g/L leucine, 10g/L methionine, 10g/L histidine, 2g/L uracil for BY4741 or 10g/L leucine, 10g/L histidine, 2g/L uracil, 2g/L adenine, 5g/L tryptophan for W303-1A). Cells were allowed to grow until exponential phase of growth (OD_{600nm} 0.4-0.8), and then diluted to an OD of ±0.1 for further incubation in the same media containing several concentrations of cetuximab, 30nM, 150nM, 300nM, 900nM and a blank culture without cetuximab. Incubation was performed in an orbital shaker (200rpm), with an air/liquid ratio of 2:1. Growth was monitored spectrophotometrically (OD_{600nm}) during 24h.

4.2. Measurement of Intracellular Concentration of Ethanol by HPLC

Cell cultures were grown at 30°C in YNB medium without cetuximab and in the same medium supplemented with 30nM, 150nM and 900nM of the monoclonal antibody. Ethanol production was assessed by HPLC (High Performance Liquid Chromatography) at 0, 6, 12 and 24h of culture growth. At the times indicated, the samples from the growth assays (see 4.1 from Material & Methods) were used for biomass quantification, by spectrophotometry (OD_{600nm}), and for the ethanol quantification. This last was obtained centrifuging the samples for 2min at 7,000rpm for separation of the suspended cells, and incubating the supernatant at 4°C for 30min with perchloric acid 2% (v/v) for removal of proteins and/or cellular contaminants. The mixture was then centrifuged for 10min at 12,000rpm at 4°C and the supernatant was filtrated with sterile filters of 0.22µm. The samples were stored at -20°C until use. Quantification was performed in a Gilson HPLC with a column Merck Polyspher OA KC Cat. 51270, maintained at 50°C, and using 2.5mM sulphuric acid in ultra-pure water as a mobile phase at a flow rate of 0.5mL/min. As internal standard, to allow integration of the peaks, a solution of 5g/L of

arabinose was used. As initial pattern solutions, for recognition of the retention times of the compounds, 3g/L of arabinose and 3g/L of ethanol were also used. Prior to a 25µL injection, both the initial pattern and the experimental samples were diluted in a 1:1 ratio with the internal standard solution. The data acquisition program used was Gilson System Controller 712.

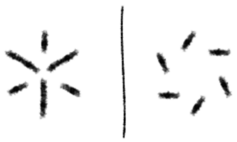
5. Western Blot Analysis

5.1. Whole Yeast Protein Extraction

For immunoblot analysis, total proteins from *S. cerevisiae* BY4741 were extracted using the trichloroacetic acid (TCA) method. Briefly, cells were grown in YNB media, at 30°C and 200rpm until exponential phase. Cell culture at an OD of ±1 (OD_{600}), which corresponds to approximately 0.4mg cell dry mass and 1.4 x 10^7 cells/ml, was collected, and cells were harvested by centrifugation at 4,000g for 5min. The pellet was resuspended in 200µL of NaOH 0.2M and 2% of β-mercaptoethanol, incubated on ice for 10min and centrifuged at 13,000rpm for 5 min. The pellet obtained was washed with 500µL of acetone and centrifuged at 13,000rpm for 5min. The new precipitate was allowed to dry for 10min at 4°C, and the proteins were resuspended in Laemmli sample buffer [439] with modifications according to the group's routine protocols: 4% SDS, 2% 2-mercaptoehtanol, 20% glycerol, 0.01% bromophenol blue, 0.125 M Tris HCl, pH 6.8. The samples were stored at -20°C until use.

5.2. Cellular Fractioning: Cell Wall, Plasma Membrane and Cytosol Extraction

Cell wall and plasma membrane were obtained as described in the literature [440-442], with modifications. *S. cerevisiae* was grown in YNB at 30°C, 200 rpm and collected, at exponential phase, by centrifugation at 4,000rpm for 5min at 4°C. The supernatant was discarded and the cell pellet was washed three times with cold deionized water. The supernatant was discarded and the cells were resuspended in 50mM Tris-HCl buffer (pH 8.5) containing 150mM NaCl and the appropriate inhibitors, added just before use (5mM EDTA, 1mM PMSF,



50µg/mL aprotinin, 10µg/mL leupeptin, 6µg/mL pepstatin A). A volume of 200µL of the cell suspension was transferred to screw-capped 2mL eppendorf tubes compatible with a mini-bead beater. Equal volume of 0.425mm diameter acid-washed glass beads was added to the cells that were disrupted mechanically in a Kaiser homogenizer (B. Braun) set at 6,800rpm for 30sec, six times alternating these with 30sec periods on ice. The beads were spun down by a short centrifugation at 500g for 5min. The supernatant was saved and the glass beads were washed at least 5 times with the same buffer by vortexing the cell suspension briefly and then spinning down the beads (500g for 1min) after each wash. Occasionally, it was performed one or two additional washings of the beads until the washing solution became limpid, meaning that there was no longer cell debris bound to the beads. The collected supernatants were centrifuged at 500g for 5min to precipitate unwanted and unbroken cells. After that the cell walls were harvested by centrifugation for 20min at 9,600g, and resuspended in the modified Laemmli sample buffer (above), while the supernatant was centrifuged for 45min at 18,000g to obtain the membranes that were resuspended in the same buffer.

The supernatant proteins of the remaining cell debris were precipitated according to the method described in [443]. Briefly, the supernatant was mixed with equal volume of a TCA (20% w/v), acetone (90% v/v) and DTT (20mM) mixture, vortexed thoroughly and allowed to precipitate overnight at -20°C. This was followed by a centrifugation at 15,000rpm for 4°C during 30min. The supernatant was decanted and the pellet was washed twice, first with cold acetone (90% v/v) containing 20mM DTT and the second wash with cold acetone (80% v/v) containing 10mM DTT. For each wash, the pellet was sonicated until the whole pellet was fully broken. The pellet suspension was then placed at -20°C for 20min and subsequently centrifuged at 4°C for 5min at 15,000rpm. The pellet was allowed to dry and it was resuspended in modified Laemmli sample buffer (above), and stored at -20°C until Western blot analysis.

5.3. Western Blot Assay

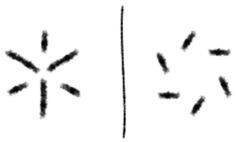
The protein extracts were denatured for 5min at 95°C before application on 10.5% SDS-PAGE gels. The gels were made in duplicate. To each, it was loaded 3-10µL of total protein extract per well. The gels were submerged in migration buffer (25mM Tris; 192mM glycine; 0,1% SDS, pH 8.3) and the electrophoresis allowed to run for over 2h at 100 volts or until the

"migration front" reaches the bottom of the gel, in a Bio-Rad system (MiniProtean). After electrophoresis, one of the SDS-PAGE gels was stained with Coomassie Brilliant Blue (50% ethanol, 10% acetic acid and 0.25% Coomassie R258), while the other one was electro blotted onto PVDF membrane (Roche), in a protein transfer system (BIORAD). The transfer was performed in wet conditions, where the gel and membrane are sandwiched between sponge and paper. The sandwich was submerged in transfer buffer (192mM glycine, 25mM Tris, pH 8.3, 20% ethanol), for 2h at 4°C and 10V. The nonspecific sites of the membrane were saturated for 2h at room temperature in blocking solution (5% non-fat milk in Tris Buffer Saline Tween20 (TBST)) to prevent non-specific background binding of the primary and/or secondary antibodies to the membrane. Subsequently, the membrane was washed two times with TBST for 10min and then incubated with primary antibody (cetuximab at 1:1000) overnight, at 4°C, with agitation. The first antibody was removed by briefly washing the membrane twice with TBST and incubated at room temperature in TBST for 15min. Two more successive washes with TBS (Tris Buffer Saline) were performed for 15min each at room temperature before incubating the membrane with the secondary antibody coupled with horseradish peroxidase (rabbit anti-mouse antibody at 1:5000). This was done for 1-2h at room temperature with agitation. Expired that period, the membrane was briefly washed two times with TBST, and incubated at room temperature in TBST for 15min. Then, the membrane was washed twice with TBS for 15min at room temperature, to remove residual antibodies. The immunoreactive bands were detected using ECL Plus Western blotting Detection System (Amersham Biosciences) in an Image Analysis System ChemiDoc XRS (Bio-Rad, Laboratories Inc.) bearing Quantity-One 4.5.0 Software (Bio-Rad, Laboratories Inc.). For the identification and sequencing of the immunoreactive proteins, the interest bands from the SDS-PAGE gel were excised and shipped to Alphalyse, Denmark (www.alphalyse.com).

5.4. Dot Blot Assay

A volume of 5µL of the cetuximab stock solution of 5mg/mL and the same solution diluted 5, 20, 50 and 100 times in ultra-pure water were placed on PVDF membrane (Roche). This membrane was transferred to a block solution and further incubated with HRP-conjugated anti-mouse antibody, following the same procedure described above.

Results & Discussion



1. An *In Silico* Approach to Discover the EGFR Counterpart in Yeast

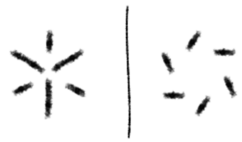
1.1. The Search and Collection of Yeast Cell's Surface Proteins

The *S. cerevisiae* genome is the most well-characterized eukaryotic genome and one of the simplest for identifying open reading frames (ORFs), being its annotation continually updated since its initial release in 1996 [444]. The *Saccharomyces* Genome Database (www.yeastgenome.org), integrates functional information about budding yeast genes and their products with a set of analysis tools that facilitate exploring their biological details [445]. According to the degree of certainty that each ORF encodes a functional protein, SGD classifies all *S. cerevisiae* ORFs into one of three groups: i) "dubious" (785 ORFs, 11.88%), referring to those ORFs that are unlikely to encode a protein; ii) "uncharacterized" (845 ORFs, 12.79%), those that are likely, but not yet fully established, to encode a protein; and iii) "verified" (4977 ORFs, 75.33%), those for which there is clear experimental evidence for the presence of a protein-encoding gene (Oct 18, 2012) [445, 446]. It should be noted that these ORF classifications are a working hypothesis, and the designations change based on published experimental results [445, 447]. Overall, the SGD's goal is to provide its users with detailed information about the roles of gene products in the cell (including protein localization, phenotypes, and structural specificities), and their relationship to other gene products in yeast and other organisms, namely through physical and non-physical interactions. To this end, SGD is annotating genes to the Gene Ontology (GO), a structured representation of biological knowledge that can be shared across species [448, 449]. The GO consists of three separate ontologies describing molecular function, biological process and cellular component. Thus, the purpose is to use published information to associate each characterized *S. cerevisiae* gene product with one or more GO terms from each of the three ontologies [445, 447, 450].

The mammalian EGFR, as well the *C. elegans* and *D. melanogaster* orthologues localise at the cell plasma membrane [101]. In analogy, it was thought that the potential counterpart in yeast could be located somewhere on the yeast cell surface. This comprises the plasma membrane, but also and importantly, the cell wall. The SGD was used to list the yeast gene products annotated to the cell wall and plasma membrane (Table I - Supplementary Material -

CD-ROM at the back cover of the final printed version). As mentioned in the Materials & Methods, membrane-associated proteins were identified, and once one was not present on the Transporters Database (www.tcdb.org), the protein amino acid sequence was evaluated as to the putative existence of putative transmembrane domains (hydrophobic portions of the polypeptide sequence), as well as to the protein predicted localization.

There are a total of 6607 ORFs in SGD, 5584 of which are currently annotated to the GO term cellular component (Oct 19, 2012) [445, 446]. We identified around 1,000 gene products associated with yeast cell's surface (Oct and Nov, 2011; Table I - Supplementary Material). This number reflects the importance of cell wall and plasma membrane in yeast physiology [451]. The cell wall is needed for maintenance of shape, protection against osmotic and physical stress, therefore, for the interaction with the environment and other cells, as well as reproductive processes. Many cell wall proteins are known. Some of them are soluble cell wall proteins, however, most of them are covalently attached to the wall glucan layer [440]. These include the two major classes of cell surface glycoproteins that comprise the outer cell wall layer: GPI (glycosylphosphatidylinositoll) proteins [452] and the PIR proteins (proteins with internal repeats) [453]. GPI anchored proteins allow physical interaction between the plasma membrane and the wall, possibly contributing to an open highway of signalling throughout the cell surface into the intracellular space cytoskeleton. This is suggested by the fact that many GPI anchored proteins localize in specific areas of the membrane rich in ceramides, phosphoinositol-based sphingolipids and ergosterol, known as rafts [454, 455]. Rafts in yeast were quite controversial for some time, in particular due to their small size and difficult assessment [456]. Presently, indirect evidence suggests they have a role in signalling mediation in yeast, as well in protein sorting, secretion, endocytosis, and cell polarity [455, 457, 458]. On the other hand, in association with plasma membrane, there is a set of membrane proteins that enables the membrane to carry out its distinctive activities. Some proteins are bound only to the membrane surface, whereas others are partly buried within the membrane [459]. Protein domains on the extracellular membrane surface are generally involved in cell-cell signalling or interactions with other proteins or substrates of many different chemical families [460]. Domains within the membrane are hydrophobic, since the plasma membrane forms a lipid bilayer which is a relatively impermeable barrier for hydrophilic molecules, and mediate the selective uptake and/or secretion of solutes across the membrane [459]. Examples of these specialized proteins are the transporter



molecules, such as (1) carriers mediating uniport - facilitated diffusion, symport – co-transport and antiport - exchange diffusion, (2) channels, mostly mediating water and ions translocation, and (3) pumps, like the ATP-dependent ion pumps and the ABC (ATP binding cassette) transporters. In general transporters are the main constituents of the yeast plasma membrane. In particular the plasma membrane ATPase accounts for almost 50% of the total plasma membrane associated protein [459]. Finally, the domains on the cytosolic face of the membrane have a wide range of functions, from anchoring cytoskeletal proteins to the membrane to trigger intracellular signalling pathways [459]. Other plasma membrane proteins are involved in cell wall synthesis [459]. In many cases, the function of a membrane protein and the topology of its polypeptide chain in the membrane can be predicted based on its homology with other such well-characterized protein [460]. Besides, the prediction of the hydrophobicity of large parts of the protein can suggest imbibing in the lipid bilayer. Both cell wall and plasma membrane undergo profound changes according to growth phase, nutrient availability, temperature, pH and oxygen levels, which implies a temporal and spatial control of its composition, especially for the expression and incorporation of proteins [451, 459].

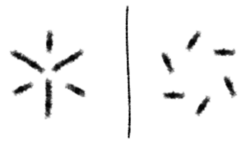
1.2. Sequence Similarity Search

In order to narrow the number of the candidates for EGFR counterparts in relation to the proteins identified in the previous approach (Table I from Supplementary Material (1.1.)) a protein sequence similarity search was performed using (i) the full amino acid sequence of EGFR protein, (ii) the extracellular domain, (iii) the sub-domains responsible for binding of the ligand (I/L1 and III/L2) [125] and cetuximab (III/L2) [127], and (iv) the EGFR's intracellular domain (Table I). The protein BLASTs were performed using the BLOSUM 62 matrix that is calculated from comparisons of sequences with no less than 62% divergence. Though it also performs well in detecting closer relationships, it is tailored for comparisons of moderately distant proteins. BLOSUM matrices with low numbers are designated for comparisons of distantly related proteins [461]. Additionally, the E Value (Expect Value) describes the likelihood that a sequence with a similar score will occur in the database by chance - the smaller the E Value, the more significant the alignment. For example, an alignment with a very low E value means that a sequence with a similar score is very unlikely to occur simply by chance [462].

Table I - Sequence similarity searching between EGFR and *S. cerevisiae*. Protein BLASTs were performed in SGD using as query sequences the amino acid sequence of EGFR, the EGFR's extracellular domain, the ligand binding domains (I/L1 and III/L2) and the cetuximab binding domain (III/L2), as well as the EGFR's intracellular domain against the *S. cerevisiae* BY4741 protein database. All searches were performed with the BLOSUM 62 scoring matrix, coupled with default parameters (October 2011). Some of the best alignments are shown (top 10 for each BLASTP performed), and the genes listed in bold refer to proteins that are located on the yeast's cell surface as were found in the keyword search previously performed (see Table I from Supplementary Material).

EGFR

E value	Standard Name	Systematic Name	Description
$4.8e^{-17}$	***SKM1***	**YOL113W**	**Member of the PAK family of serine/threonine protein kinases with similarity to Ste20p and Cla4p; involved in downregulation of sterol uptake; proposed to be a downstream effector of Cdc42p during polarized growth**
$5.0e^{-17}$	***KIN1***	**YDR122W**	**Serine/threonine protein kinase involved in regulation of exocytosis; localizes to the cytoplasmic face of the plasma membrane; closely related to Kin2p**
$3.3e^{-16}$	*CLA4*	YNL298W	Cdc42p-activated signal transducing kinase of the PAK (p21-activated kinase) family, along with Ste20p and Skm1p; involved in septin ring assembly, vacuole inheritance, cytokinesis, sterol uptake regulation; phosphorylates Cdc3p and Cdc10p
$4.6e^{-16}$	***KIN2***	**YLR096W**	**Serine/threonine protein kinase involved in regulation of exocytosis; localizes to the cytoplasmic face of the plasma membrane; closely related to Kin1p**
$7.9e^{-15}$	*KIC1*	YHR102W	Protein kinase of the PAK/Ste20 family, required for cell integrity; physically interacts with Cdc31p (centrin), which is a component of the spindle pole body; part of the RAM network that regulates cellular polarity and morphogenesis
$2.5e^{-14}$	*CDC28*	YBR160W	Catalytic subunit of the main cell cycle cyclin-dependent kinase (CDK); alternately associates with G1 cyclins (CLNs) and G2/M cyclins (CLBs) which direct the CDK to specific substrates

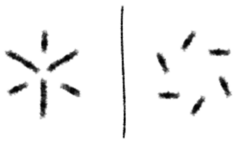


$3.3e^{-14}$	***STE20***	**YHL007C**	**Cdc42p-activated signal transducing kinase of the PAK (p21-activated kinase) family; involved in pheromone response, pseudohyphal/invasive growth, vacuole inheritance, downregulation of sterol uptake; GBB motif binds Ste4p**
$3.5e^{-14}$	*CDC15*	YAR019C	Protein kinase of the Mitotic Exit Network that is localized to the spindle pole bodies at late anaphase; promotes mitotic exit by directly switching on the kinase activity of Dbf2p; required for spindle disassembly after meiosis II
$6.7e^{-14}$	*TPK2*	YPL203W	cAMP-dependent protein kinase catalytic subunit; promotes vegetative growth in response to nutrients via the Ras-cAMP signalling pathway; partially redundant with Tpk1p and Tpk3p; localizes to P-bodies during stationary phase
$2.2e^{-13}$	*IPL1*	YPL209C	Aurora kinase subunit of the conserved chromosomal passenger complex (CPC; Ipl1p-Sli15p-Bir1p-Nbl1p), involved in regulating kinetochore-microtubule attachments; helps maintain condensed chromosomes during anaphase and early telophase; required for SPB cohesion and prevention of multipolar spindle formation

EGFR Extracellular Domain

0.23	*CRS5*	YOR031W	Copper-binding metallothionein, required for wild-type copper resistance
0.23	*CUP1-1*	YHR053C	Metallothionein, binds copper and mediates resistance to high concentrations of copper and cadmium; locus is variably amplified in different strains, with two copies, CUP1-1 and CUP1-2, in the genomic sequence reference strain S288C
0.23	*CUP1-2*	YHR055C	Metallothionein, binds copper and mediates resistance to high concentrations of copper and cadmium; locus is variably amplified in different strains, with two copies, CUP1-1 and CUP1-2, in the genomic sequence reference strain S288C
0.24	*NCS2*	YNL119W	Protein required for thiolation of the uridine at the wobble position of Lys(UUU) and Glu(UUC) tRNAs; has a role in urmylation and in invasive and pseudohyphal growth; inhibits replication of Brome mosaic virus in *S. cerevisiae*
0.93	*LIP5*	YOR196C	Protein involved in biosynthesis of the coenzyme lipoic acid, has similarity to *E. coli* lipoic acid synthase

0.98	*HRT1*	YOL133W	RING finger containing subunit of Skp1-Cullin-F-box ubiquitin protein ligases (SCF); required for Gic2p, Far1p, Sic1p and Cln2p degradation; may tether Cdc34p (a ubiquitin conjugating enzyme or E2) and Cdc53p (a cullin) subunits of SCF
0.98		YLR286W-A (Dubious)	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; overlaps the verified gene CTS1
0.99	*PGM2*	YMR105C	Phosphoglucomutase, catalyzes the conversion from glucose-1-phosphate to glucose-6-phosphate, which is a key step in hexose metabolism; functions as the acceptor for a Glc-phosphotransferase
1	*COA3*	YJL062W-A	Mitochondrial inner membrane protein that participates in regulation of COX1 translation, Cox1p stabilization, and cytochrome oxidase assembly
1	*CDD1*	YLR245C	Cytidine deaminase; catalyzes the modification of cytidine to uridine in vitro but native RNA substrates have not been identified, localizes to both the nucleus and cytoplasm
Domain I (L1 domain)			
0.028	*NCS2*	YNL119W	Protein required for thiolation of the uridine at the wobble position of Lys(UUU) and Glu(UUC) tRNAs; has a role in urmylation and in invasive and pseudohyphal growth; inhibits replication of Brome mosaic virus in *S. cerevisiae*
0.58	*DYN1*	YKR054C	Cytoplasmic heavy chain dynein, microtubule motor protein, required for anaphase spindle elongation; involved in spindle assembly, chromosome movement, and spindle orientation during cell division, targeted to microtubule tips by Pac1p
0.77	***SPS22***	**YCL048W**	**Protein of unknown function, redundant with Sps2p for the organization of the beta-glucan layer of the spore wall**
0.82		YKL133C (Uncharacterized)	Putative protein of unknown function; has similarity to Mgr3p, but unlike MGR3, is not required for growth of cells lacking the mitochondrial genome (null mutation does not confer a petite-negative phenotype)

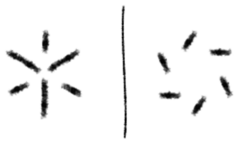


0.82	*FRT2*	YAL028W	Tail-anchored ER membrane protein, interacts with homologue Frt1p; promotes growth in conditions of high Na^+, alkaline pH, or cell wall stress, possibly via a role in posttranslational translocation; potential Cdc28p substrate
0.96	*SPE1*	YKL184W	Ornithine decarboxylase, catalyzes the first step in polyamine biosynthesis; degraded in a proteasome-dependent manner in the presence of excess polyamines; deletion decreases lifespan, and increases necrotic cell death and ROS generation
0.96	*GAD1*	YMR250W	Glutamate decarboxylase, converts glutamate into gamma-aminobutyric acid (GABA) during glutamate catabolism; involved in response to oxidative stress
0.97	***SPS2***	**YDR522C**	**Protein expressed during sporulation, redundant with Sps22p for organization of the beta-glucan layer of the spore wall; *Schizosaccharomyces pombe* orthologue is a spore wall component**
1	*NIP100*	YPL174C	Large subunit of the dynactin complex, which is involved in partitioning the mitotic spindle between mother and daughter cells; putative orthologue of mammalian p150(glued)
1	*MAD1*	YGL086W	Coiled-coil protein involved in the spindle-assembly checkpoint; phosphorylated by Mps1p upon checkpoint activation which leads to inhibition of the activity of the anaphase promoting complex; forms a complex with Mad2p
Domain III (L2 domain)			
0.42	*PGM2*	YMR105C	Phosphoglucomutase, catalyzes the conversion from glucose-1-phosphate to glucose-6-phosphate, which is a key step in hexose metabolism; functions as the acceptor for a Glc-phosphotransferase
0.62	*POL4*	YCR014C	DNA polymerase IV, undergoes pair-wise interactions with Dnl4p-Lif1p and Rad27p to mediate repair of DNA double-strand breaks by non-homologous end joining (NHEJ); homologous to mammalian DNA polymerase beta
0.74	*COA3*	YJL062W-A	Mitochondrial inner membrane protein that participates in regulation of COX1 translation, Cox1p stabilization, and cytochrome oxidase assembly

0.97		YLR286W-A (Dubious)	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; overlaps the verified gene CTS1
1		YLR434C (Dubious)	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data; partially overlaps the verified ORF TSR2/YLR435W
1	*QNS1*	YHR074W	Glutamine-dependent NAD(+) synthetase, essential for the formation of NAD(+) from nicotinic acid adenine dinucleotide
1	*HBT1*	YDL223C	Substrate of the Hub1p ubiquitin-like protein that localizes to the shmoo tip (mating projection); mutants are defective for mating projection formation, thereby implicating Hbt1p in polarized cell morphogenesis
1	*CTR9*	YOL145C	Component of the Paf1p complex involved in transcription elongation; binds to and modulates the activity of RNA polymerases I and II; required for expression of a sub-set of genes, including cyclin genes; involved in SER3 repression by helping to maintain SRG1 transcription-dependent nucleosome occupancy; contains TPR repeats

EGFR Intracellular Domain

1.0e^{-17}	***SKM1***	**YOL113W**	**Member of the PAK family of serine/threonine protein kinases with similarity to Ste20p and Cla4p; involved in downregulation of sterol uptake; proposed to be a downstream effector of Cdc42p during polarized growth**
1.0e^{-17}	***KIN1***	**YDR122W**	**Serine/threonine protein kinase involved in regulation of exocytosis; localizes to the cytoplasmic face of the plasma membrane; closely related to Kin2p**
2.1e^{-17}	***KIN2***	**YLR096W**	**Serine/threonine protein kinase involved in regulation of exocytosis; localizes to the cytoplasmic face of the plasma membrane; closely related to Kin1p**
7.1e^{-17}	*CLA4*	YNL298W	Cdc42p-activated signal transducing kinase of the PAK (p21-activated kinase) family, along with Ste20p and Skm1p; involved in septin ring assembly, vacuole inheritance, cytokinesis, sterol uptake regulation; phosphorylates Cdc3p and Cdc10p



$4.6e^{-16}$	*KIC1*	YHR102W	Protein kinase of the PAK/Ste20 family, required for cell integrity; physically interacts with Cdc31p (centrin), which is a component of the spindle pole body; part of the RAM network that regulates cellular polarity and morphogenesis
$6.5e^{-15}$	*CDC28*	YBR160W	Catalytic subunit of the main cell cycle cyclin-dependent kinase (CDK); alternately associates with G1 cyclins (CLNs) and G2/M cyclins (CLBs) which direct the CDK to specific substrates
$8.0e^{-15}$	***STE20***	**YHL007C**	**Cdc42p-activated signal transducing kinase of the PAK (p21-activated kinase) family; involved in pheromone response, pseudohyphal/invasive growth, vacuole inheritance, downregulation of sterol uptake; GBB motif binds Ste4p**
$8.4e^{-15}$	*CDC15*	YAR019C	Protein kinase of the Mitotic Exit Network that is localized to the spindle pole bodies at late anaphase; promotes mitotic exit by directly switching on the kinase activity of Dbf2p; required for spindle disassembly after meiosis II
$1.7e^{-14}$	*TPK2*	YPL203W	cAMP-dependent protein kinase catalytic subunit; promotes vegetative growth in response to nutrients via the Ras-cAMP signalling pathway; partially redundant with Tpk1p and Tpk3p; localizes to P-bodies during stationary phase
$5.7e^{-14}$	*IPL1*	YPL209C	Aurora kinase subunit of the conserved chromosomal passenger complex (CPC; Ipl1p-Sli15p-Bir1p-Nbl1p), involved in regulating kinetochore-microtubule attachments; helps maintain condensed chromosomes during anaphase and early telophase; required for SPB cohesion and prevention of multipolar spindle formation

The result of the protein BLAST using as query sequence the EGFR membrane spanning protein of 1210 amino acids, revealed that among the top ten best alignments were four yeast proteins located at the cell's surface (Skm1p, Kin1p, Kin2p, Ste20p - showed in bold in the Table I). All the four proteins are kinases, as well as most of the remaining peptides in the result. As protein kinases, these peptides have a certain degree of similarity to the tyrosine kinase domain of the EGFR, which is confirmed by the very similar alignment results obtained when using as query sequence the intracellular domain of EGFR (see Table I), as well as the residues of the

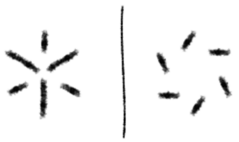
tyrosine kinase domain of EGFR (data not shown). These two last alignments showed a smaller E Value, then a more significant alignment, than the ones using the full protein.

In the results obtained with the extracellular domain of EGFR, the E Value is quite high (Table I), suggesting the alignment is not very significant and constituting low evidence for homology. Plus, none of the top ten results is a protein putatively localized at the cell's surface. Confining the query sequence used for the BLASTP to the domain I/L1 of EGFR, between the top of the best alignments came up proteins Sps22p and Sps2p (Table I), which have a putative location at the cell's surface, in spite of the high E value obtained in the BLASTP. The protein BLAST using domain III/L2 sequence did not show any significant result.

1.3. Motifs Are Conserved Regions of a Protein That Usually Share Similar Structure and Function

The protein domains, usually segments of continuous amino acids within a protein, are the structural, functional and evolutionary units of the protein [463, 464]. They can occur on their own in single-domain proteins or in combination with different partner domains making multidomain proteins. In this case, they can fold independently into a stable core structure. The domains within a protein are often also structurally and functionally independent. The ones that are related to each other by descent from a common ancestor are members of the same superfamily [463, 464]. Alternatively, the protein domain architecture is the pattern of linear and sequential domains in a given protein. Usually, proteins with the same or similar architectures are close homologues, while different proteins possess distinct domain architectures [465]. Yet, domain recurrences among tri-dimensional structures consistently reveal that protein structure is more conserved than sequence. There are many examples of domains adopting highly similar tri-dimensional structures despite no apparent similarity in sequence. For many of these examples, proteins have diverged beyond the limits of sequence similarity detection methods but have nevertheless retained a common structure and similar function [464].

EGFR architecture (Fig. 4), contemplates the EGFR extracellular portion (or ectodomain), which consists of four domains that are known as the I/L1, II/CR1, III/L2, and IV/CR2 domains [124-127, 466, 467]. The structure determinations of ectodomain fragments of the EGFR show the L1 and L2 domains (rich in leucine, therefore designated L domains) to consist of a single-



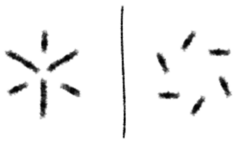
stranded right hand β-helix, which resembles the corresponding domains of the insulin-like growth factor-1 (IGF-1) receptor [126, 466]. The CR1 and CR2 domains (rich in cysteine, hence the designation of CR) consist of a number of small modules, each appearing to be held together by disulphide bonds [126]. In spite of the low sequence identity between the ectodomains of IGF1R and the EGFR, the tri-dimensional structure of the first three domains of the IGF1R was used to build a comparative (or homology) model of the four domains of the EGFR ectodomain [466]. Additionally, the EGFR transmembrane domain and the beginning of the cytoplasmic domain (known as juxtamembrane domain) are α-helical [468], and the tri-dimensional structure of the EGFR kinase domain is similar to other tyrosine kinases [469]. By analogy with protein kinase structures, the ATP *sits* between the N-terminal lobe (dominated by a β-sheet) and the larger C-terminal lobe (mainly α-helical) [470].

Hence, we searched for similar domain architectures between EGFR and all the *S. cerevisiae* strains available at NCBI *Conserved Domain Architecture Retrieval Tool*. Our search showed that none of the proteins belonging to the available yeast strains are predicted to present domain architecture similar to EGFR's. As happened in the protein BLAST above mentioned, most of the results of the domain architecture recognised were of proteins with kinase domains, due to the percentage of identity of these proteins to the tyrosine kinase domain of EGFR. However, we were particularly interested in the extracellular portion of EGFR, due to binding of ligands, including cetuximab to this region, and because we were looking for a molecule in yeast able to be recognized by cetuximab that can be functionally orthologous to EGFR. No yeast proteins having similar domain architecture to ectodomain of EGFR were found. The same happened with the CR domains (II and IV). Nevertheless, regarding the L domains (I and III), nine yeast strains presented one protein with an L domain - Sps2p already identified on Table I. Additionally, it was also used InterPro (www.ebi.ac.uk/interpro/) which provides functional analysis of proteins by classifying them into families and predicting domains and important sites, to access whether the CR and L domains were present in any other *S. cerevisiae* protein besides the Sps2p identified using NCBI tool. The protein Sps2p was again identified, and so was its paralogue Sps22p (Table I). No further proteins with L domains were found. Similarly to the search operated at NCBI, no proteins harbouring CR domains were found. On the contrary, both NCBI and InterPro databases show CR regions and L domains in a variety of proteins from more complex eukaryotes, such as humans (*Homo sapiens*), mouse (*Mus musculus*), zebrafish (*Danio*

rerio), fruit fly (*Drosophila melanogaster*) and worm (*Caenorhabditis elegans*). Noticeable, both engines identify as well these domains in the Cnidarian *Hydra vulgaris.* In this organism a gene encoding a receptor protein-tyrosine kinase closely related to the vertebrate insulin receptor has been identified [471]. This finding supports the idea that an intercellular signalling pathway involving an insulin-like molecule was a component of the earliest multi-cellular animals. Although no insulin-signalling-like pathway has been described in yeast, this appear to have precursors of such a metabolic control pathway that function in the glucose/nutrient-signalling cascade and are orthologous to the serine/threonine kinase Akt/PKB of insulin-signalling pathways in *C. elegans* and mammals [321].

Given the Sps2p and Sps22p results concerning domain analysis, we confirmed the existence of yeast proteins that contain L domains using a database of structural and functional annotation for all proteins and genomes – SUPERFAMILY database (Table II). The SUPERFAMILY database contains a library of hidden Markov models (HMMs) based on the domains of known tri-dimensional structure, and the assignments made by these HMMs to the predicted proteins of all completely sequenced genomes. The SUPERFAMILY database takes the definition of domains from the structural classification of proteins (SCOP) database, using the superfamily level of classification, which groups together domains sharing a common evolutionary ancestor [472, 473].

The yeast proteins in Table II display L domains, characteristic of some mammalian receptors, such as the IGF1R and the IR. Two L domains from these receptors make up the bilobal ligand binding site [466]. The proteins presented in Table II show the highest similarity in pairs: Pst1p is most similar to Ecm33p, and Sps2p to Sps22p [474]. Ecm33p has the same features as Pst1p, and both display typical features of GPI-anchored proteins [452, 474]. GPI proteins are widely found in lower and higher eukaryotic organisms [475]. In mammals, although functionally diverse, many of these proteins have, or are predicted to have, hydrolytic activity, or serve as receptors or adhesive proteins [476]. They are also important for the cell surface display of a variety of proteins and glycol-conjugates in parasitic protozoa [477]. In yeast, GPI-proteins are found at the cell surface, either attached to the plasma membrane or as an intrinsic part of the cell wall [452, 474], where they may be involved in cell wall biosynthesis and cell wall remodelling. They may also determine surface hydrophobicity and antigenicity, and are thought to have a role in adhesion and virulence [452, 476, 478]. Interestingly, all the four proteins,



Ecm33p, Pst1p, Sps2p and Sps22p, have been grouped in the so-called SPS2 family [452], named after the first described member. According to our survey and also to the literature [474], the SPS2 family contains the only *S. cerevisiae* proteins where L domains has been detected. Some proteins from SPS2 family are conserved amongst fungi [474].

Table II - *S. cerevisiae* proteins harbouring L domains. Domain assignments to the following gene products were taken from the SUPERFAMILY database version 1.75. All the below proteins are located on the yeast's cell surface as were found in the keyword search and harbour L domains.

Standard name	Systematic name	E Value	Region	Domain Family	Description
ECM33	YBR078W	$2.24e^{-14}$	35-136	L domain	GPI-anchored protein of unknown function, has a possible role in apical bud growth; GPI-anchoring on the plasma membrane crucial to function; phosphorylated in mitochondria; similar to Sps2p and Pst1p
		$8.6e^{-11}$	161-291	L domain	
SPS22	YCL048W	$9.27e^{-14}$	286-398	L domain	Protein of unknown function, redundant with Sps2p for the organization of the beta-glucan layer of the spore wall
		$1.36e^{-12}$	72-172	L domain	
PST1	YDR055W	$1.5e^{-12}$	32-129	L domain	Cell wall protein that contains a putative GPI-attachment site; secreted by regenerating protoplasts; up-regulated by activation of the cell integrity pathway, as mediated by Rlm1p; upregulated by cell wall damage via disruption of FKS1
		$1.75e^{-10}$	199-284	L domain	
		$2.91e^{-08}$	257-355	L domain	
		0.0808	144-202	Internalin LRR domain[1]	
SPS2	YDR522C	$6.28e^{-14}$	280-425	L domain	Protein expressed during sporulation, redundant with Sps22p for organization of the beta-glucan layer of the spore wall; *S. pombe* orthologue is a spore wall component
		$3.8e^{-12}$	101-214	L domain	

Note: 1. Both L domain and Internalin LRR (leucine-rich repeat) domain families belong to the L domain-like superfamily.

Overall, yeast proteins Sps2p and Sps22p were a common result. They are present in the cell's surface (Table I from Supplementary Material), they emerged in the protein BLAST when the domain I/L1 was used as a query sequence (Table I), although, the alignment had a high E value representing low evidence for homology. But, most importantly, they have two L domains resembling domains I/L1 and III/L2 of EGFR (table II). Thus, accordingly to this bioinformatics approach, these proteins appear as suitable candidates for EGFR yeast counterparts.

2. Identification of the Yeast Target of Cetuximab by Western Blot

Cetuximab, as a monoclonal antibody (mAb), recognizes a single epitope on an antigen, differently from a polyclonal antibody that recognizes several different epitopes on each antigen. Because of their specificity, mAbs are excellent as primary antibodies in an assay, since cross-reactions with other proteins are less likely to occur [479]. The recombinant human/mouse chimeric mAb cetuximab has a high affinity for EGFR located on the cells surface [239].

Based on the high specificity assumption, we performed a Western blot using cetuximab as primary antibody and the whole yeast proteome of *S. cerevisiae* BY4741 strain, as well as proteic fractions of plasma membrane, cell wall and cytosol, obtained as described in the Material & Methods (representative results in Fig. 18A). Three immunoreactive bands appeared (black arrows), one of molecular weight around 63kDa and two around 20kDa. They were present in all of the cellular fractions (lanes 1-3) as well as on the whole cell extract (lane 4), although considerably more evident on the cytosol fraction (lane 3). Moreover, a similar experiment containing the same samples was performed using only the HRP-conjugated anti-mouse antibody (Fig. 18B) to discriminate if any of the bands that appeared in Fig. 18A was a result of the secondary antibody hybridization and not directly due to cetuximab recognition. As observed in Fig. 18B lane 3 (grey arrows), the two bands around 20kDa also appeared in the cytosol fraction of the membrane incubated only with HRP-conjugated anti-mouse antibody.

In a similar way to our experimental approach, a Western blot assay using cetuximab as a primary mAb and a secondary HRP-conjugated anti-human antibody allowed to successfully measure EGFR expression level in seven tumour types [480]. The anti-human secondary antibody

recognized the human and free constant regions (Fc) of the heavy and light chains of cetuximab that match to 2/3 of the antibody. The region that is recognized by the anti-mouse antibody is the cetuximab's variable region (Fv) of the heavy and light chains, both from a mouse anti-EGFR monoclonal antibody. The variable region of cetuximab is responsible for binding the antigen and only corresponds to 1/3 of the antibody. Still, the HRP-conjugated anti-mouse antibody we used was also able to detect several concentrations of cetuximab as can be seen in the dot blot in Fig. 18C.

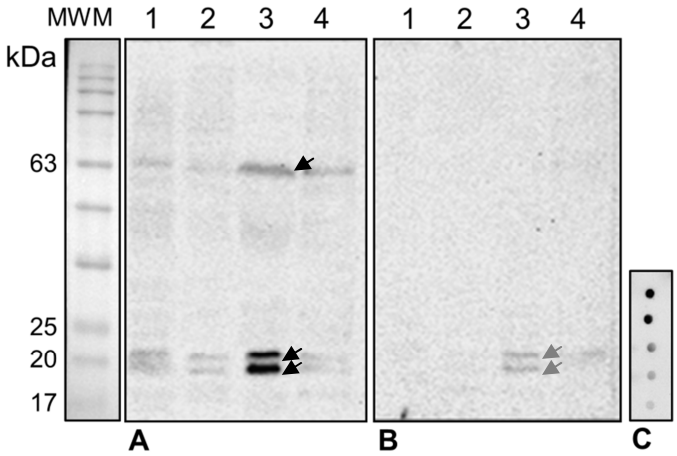


Figure 18 - Identification of yeast target of monoclonal antibody cetuximab by Western blot. SDS-PAGE followed by immunoblotting of *S. cerevisiae* BY4741 plasma membrane fraction (lane 1), cell wall fraction (lane 2), cytosol fraction (lane 3) and whole cell extract (lane 4), obtained as described in material and methods section. (**A**) Membrane incubated with the mAb cetuximab as primary antibody and with a HRP-conjugated anti-mouse antibody as secondary antibody. The black arrows indicate the bands that were excised from the SDS-PAGE and sent to protein identification. (**B**) Membrane incubated only with HRP-conjugated anti-mouse antibody. The immunoreactive bands obtained are indicated by the gray arrows. (**C**) Dot blot: 5µL of the Erbitux® stock solution of 5mg/mL and the same solution diluted 5, 20, 50 and 100 times (from top to bottom) were placed on PVDF membrane further incubated with HRP-conjugated anti-mouse antibody.

The 3 immunoreactive bands (indicated by the black arrows and further named, from top to bottom, as "prot 1", "prot 2" and "prot 3") were excised from the SDS-PAGE and sent to Alphalyse for protein analysis. According to the protein identification report supplied by the company, the protein samples were reduced and alkylated with iodoacetamide, *i.e.* carbamidomethylated, and subsequently digested with trypsin that cleaves after lysine and arginine residues. The resulting peptides were concentrated on a ZipTip micropurification column and eluted onto an anchorchip target for analysis on a Bruker Autoflex Speed MALDI TOF/TOF

instrument. The peptide mixture was analysed in positive reflector mode for accurate peptide mass determination. MALDI MS/MS was performed on 15 peptides for peptide fragmentation analysis, *i.e* partial sequencing. The MS and MS/MS spectra were combined and used for database searching using the Mascot software. The data were then BLASTED against in-house protein databases downloaded from NCBI, including the NRDB database containing more than 17 million known non-redundant protein sequences. The results from protein identification are shown in Table III.

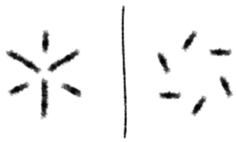
Table III - Protein identification by MS peptide mapping and sequencing analysis.

Sample name	Protein found in database	GI-number	MW	Score	Seq. cov.
Prot 1	Pdc1p [*S. cerevisiae* VL3]	gi|323353922	69666	218	18%
Prot 2	Rpl16bp [*S. cerevisiae* S288c]	gi|6324260	22235	85	36%
Prot 2[1]	Putative D-Ala-D-Ala carboxypeptidas 3 (S13) [*Synechococcus sp.* RS9916] [1]	gi|116074849 [1]	47198 [1]	66 [1]	19% [1]
Prot 3	Rpl19ap [*S. cerevisiae* FostersB]	gi|323306117	18878	86	6%
Prot 3	Rpl20bp [*S. cerevisiae* FostersB]	gi|323303008	17314	76	43%

Note: 1. The identification of this protein is uncertain because the score is outside the 95% confidence level.

A) Putative Ribosomal Peptides (Prot. 2 and 3)

Rpl16bp (YNL069C) [481-486], Rpl19ap (YBR084C-A) [482-484, 486, 487] and Rpl20bp (YOR312C) [482-484, 486] code for the ribosomal 60S subunit proteins L16B, L19A and L20B, and each of the proteins is orthololegous to mammalian ribosomal protein L13A, L19 and L18A, respectively. The sub-cellular localization of the three ribosomal proteins according to the SGD database is at the cytosolic large ribosomal subunit where they participate in translation. Yet, several ribosomal proteins were detected in the *surfome* (cell surface proteome) of a wild type *S.*



cerevisiae laboratory yeast strain [488] and in a wild type wine strain [489]. Nevertheless, and probably due to differences in strain and growth conditions, only a few ribosomal proteins appeared in both works. This suggests that there might be a sort of strain specific signature for the cell surface proteins, depending also of factors such as growth conditions and/or stress. Although the exact role for the incorporation of this group of proteins in yeast cell wall at the moment can only be speculated, several lines of evidence indicate that mutations in ribosomal genes may result in altered organization of cells or their buds, abnormal cell morphology and altered cell shape [490-492], which is not consistent with the exclusive participation in an indiscriminate molecular process of translation.

B) Pyruvate decarboxylase (Prot. 1)

In *S. cerevisiae,* Pdc1p (YLR044C) is the major of three pyruvate decarboxylase isoenzymes. It is a key enzyme in alcoholic fermentation, decarboxylating pyruvate to acetaldehyde, and it is also involved in amino acid catabolism [493-496]. Moreover, Pdc1p sub-cellular localization, according to databases, is known to be at cytoplasm and nucleus. Yet, studies concerning the proteomic analysis of yeast *surfome*, revealed the presence of Pdc1p on the cell wall of *S. cerevisiae* [488, 489, 497, 498] and also *C. albicans* [499-501]. More specifically, Pdc1p has been found among the cell surface-exposed peptides of log phase cells of glucose fermenting *S. cerevisiae* BY4741 [488]. However, in *S. cerevisiae* K310, a wild type wine strain, Pdc1p was only detected at the beginning of fermentation [489]. Given this dual location, it remains to be elucidated if Pdc1p actually is a "moonlighting protein", *i.e.* a protein that display different functions depending on sub-cellular localization, expression, state, concentration of ligand, cofactor or substrate [502]. As components of the cell surface, some glycolytic enzymes can act as binding receptors to extracellular matrix proteins [503], immunodominant antigens [504, 505], or constitutive proteins of the cell wall [505], as well as working as enzymatically active proteins [506, 507]. Hence, the dual location of proteins "classically" considered to be confined to the cytosol, might imply the presence of alternative secretory pathway(s) besides the classical endoplasmic reticulum - Golgi pathway which is driven by a canonical N-terminal signal peptide [508].

In vivo, the yeast cell wall and the plasma membrane determine yeast permeability to several molecules. Many reports on secretion describe that the rigid walls of growing cells of *S.*

cerevisiae allow the exit of molecules with relative masses as great as 400kDa, that often diffuse quite freely through intact yeast cell walls [509], while the molecular weight limit for easy entry through the intact yeast cell wall seems to be about 700Da [510]. This value depends markedly on the yeast strain and physiological conditions [509]. Since cetuximab has an approximated molecular weight of 152kDa, the yeast envelope would probably prevent the direct interaction of cetuximab with proteins in the membrane or even farther, in the intracellular space. Considering that Pcd1p may localize to the cell wall it is probable that the putative recognition is done at the cell surface and not otherwise.

Imatinib mesylate is an effective inhibitor of tyrosine kinases, such as c-KIT, c-ABL, and platelet-derived growth factor receptor (PDGFR), which are important regulators of cell growth [511-514]. These proteins have important roles in certain leukemia and sarcomas development [515, 516]. *S. cerevisiae* is naturally sensitive to imatinib that inhibits its growth [517]. The survey of the whole single deletion mutant collection EUROSCARF identified an array of mutants loosing this sensitivity. Among the genes required for imatinib resistance is one component of the large (60S) ribosomal subunit, Rpl27Ap, but Pdc1p is not included [517]. However, a subsequent study of the yeast phospho-proteome in response to imatinib showed that several glycolytic proteins, including Pdc1p, presented a reduced level of phosphorylation in response to imatinib [518]. Nonetheless, the treatment of head and neck squamous carcinoma cells with clinically relevant concentrations of imatinib, induced changes in cell morphology and growth similar to changes associated with EGFR activation. Those changes were blocked with the EGFR antagonist cetuximab, which suggested direct involvement of EGFR in this process [519]. An *in vitro* kinase assay showed that imatinib did not directly affect EGFR kinase activity, suggesting involvement of EGFR-activating molecules. In other words, imatinib affects EGFR activation and signalling pathways through rapid release and increased expression of endogenous EGFR-activating ligands. Although, imatinib primarily inhibits tyrosine kinases, it also stimulates the activity of EGFR tyrosine kinase in head and neck squamous tumours [519].

Pdc1p has a human orthologue named HACL1 (2-Hydroxyacyl-CoA lyase 1) (E-value $2.0e^{-20}$) [518]. Yeast Pdc1p participates in glucose fermentation to ethanol as mentioned above, acetoin biosynthesis II pathway, degradation of isoleucine, tryptophan, valine and phenylalanine, as well as in the superpathway of acetoin and butanediol biosynthesis (Fig 18) [495, 520]. On

the other hand, human HACL1 has a role in peroxisome, alpha-oxidation of phytanate and peroxisomal lipid metabolism [521, 522].

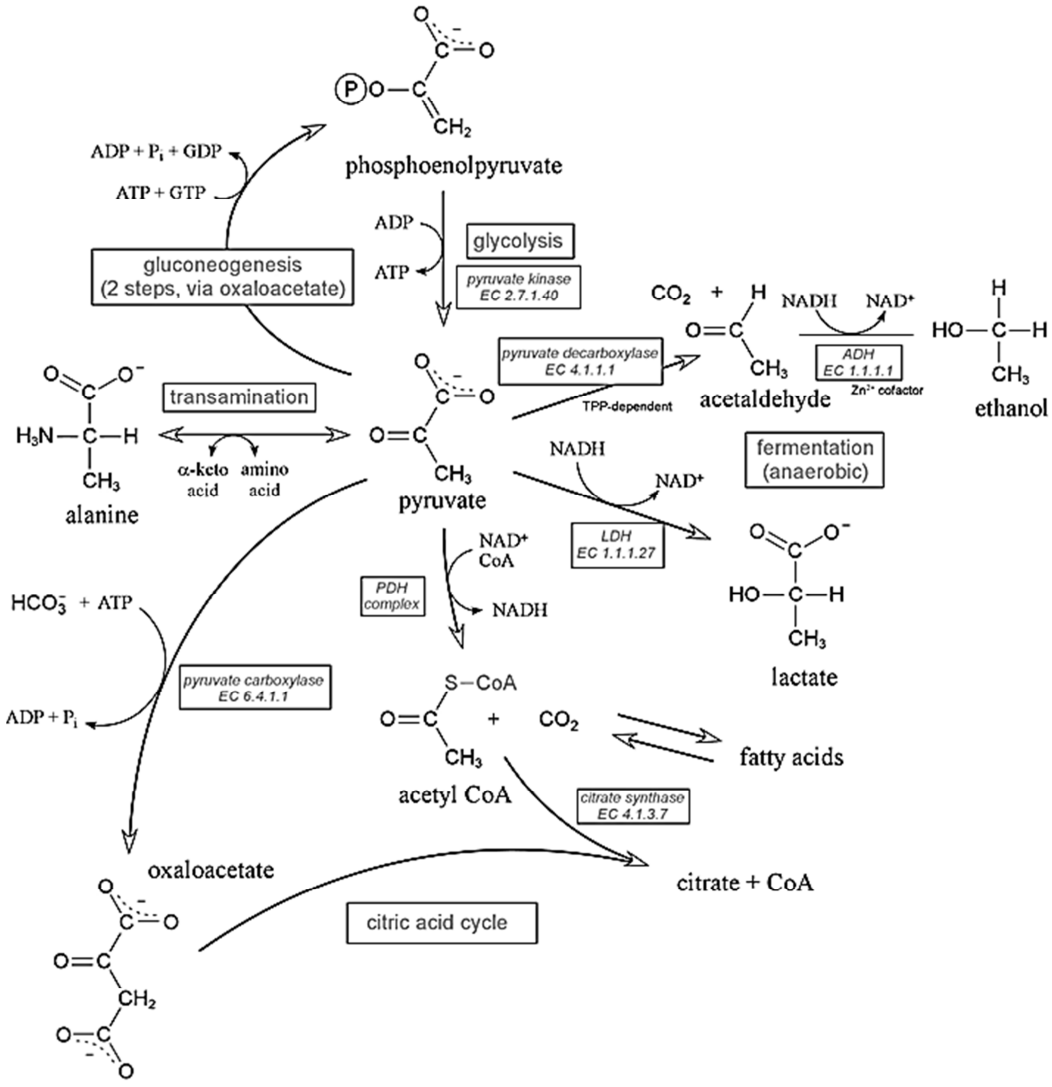


Figure 19 - The possible fates of pyruvate. The fate of pyruvate, a highly-oxidized product of glycolysis, in intermediary metabolism varies depending on the organism or tissue and on whether conditions are anaerobic or aerobic. In anaerobic conditions, pyruvate can be reduced by NADH, as catalyzed by lactate dehydrogenase yielding lactate and regenerating $NAD^{\cdot}$. Alternatively, pyruvate is first decarboxylated to acetaldehyde by pyruvate decarboxylase, which then is reduced to ethanol by alcohol dehydrogenase. The processes occurring anaerobically that lead from pyruvate to lactate or ethanol are characterisic of fermentation, *i.e.* glycolysis under anerobic conditions. Under aerobic conditions, pyruvate undergoes oxidative decarboxylation by pyruvate dehydrogenase (PDH), ultimately yielding acetyl CoA, which feeds directly into the citric acid cycle. Both pyruvate and phosphoenolpyruvate can undergo carboxylation reactions yielding oxaloacetate. These three compounds are intermediates of the gluconeogenic pathway. Adapted from [523].

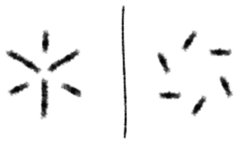
Yeast and cancer cells rely on increased glucose uptake and high glycolytic activity to fuel the biosynthesis of biomass as already discussed previously in introduction. For lipid synthesis, the major carbon precursor is acetyl-CoA, derived from the ATP:citrate lyase reaction in humans and from the Pdc activity in *S. cerevisiae* [524]. Both cancer cells and yeast maintain high glycolytic rates for biomass formation and for ATP production by substrate-level phosphorylation.

Most of the NADH derived from glycolysis is reoxidized through cytosolic reactions: in cancer cells, pyruvate is reduced to lactate by the lactate dehydrogenase. In yeast, reduction is preceded by decarboxylation of pyruvate to acetaldehyde by Pdc, and yields ethanol, which is excreted from the cell, as happens with lactate in cancer cells [400, 404]. Lactate dehydrogenase is also present in yeast: two isoforms are located in the mitochondrial inner membrane [525, 526]. The mitochondrial forms irreversibly oxidize lactate to pyruvate and participate in the respiratory chain [525, 527], being the role of the cytoplasmic isoform still unknown. The reduction of pyruvate metabolism in cytosol depends mainly on Pdc [494]. Even though the cytoplasmic pyruvate metabolism is not strictly the same as that of tumour cells, an increased flow through this pathway inhibits the substrate supply to oxidative metabolism, just as seen with the increased activity of lactate dehydrogenase in cancer cells [400].

3. Testing Yeast Susceptibility to Cetuximab

3.1. Effect of Monoclonal Antibody Cetuximab on Yeast Growth

Yeast has been broadly used to test toxicity of certain drugs and compounds, as well as to identify new drugs [320, 517, 528, 529]. In what concerns to anticancer agents, such as Cisplatin (a DNA-damaging agent) [530, 531] and imatinib (a tyrosine kinase inhibitor of the oncogenic kinases) [517] among several others [532-535], *S. cerevisiae* has been used as eukaryotic model system to better understand the mode of action, as well as potential mechanisms of resistance to these drugs. Regarding the antibodies used in the treatment of neoplastic diseases [536], such as the mouse-human chimeric IgG1 cetuximab or the fully human IgG2 panitumumab, both targeting domain III of EGFR, to our knowledge, yeast has never been used to study these compounds.



Cetuximab inhibits cell proliferation, in a dose-dependent fashion, of various tumour cell lines in culture or xenografts, including cell lines derived from cancers of the vulva [248, 537, 538], breast [539, 540], prostate [251], ovarian [541], bladder [261], kidney [542], lung [543, 544], colon [264, 545], head and neck [254, 255], gastric [546], among other EGFR-overexpressing cancers. In several studies, and also depending of the cell line, concentrations up to 100μg/mL of cetuximab have been used showing anti-tumour effects mediated either by inhibition of cellular proliferation and angiogenesis, and by enhancement of apoptosis [540, 544, 546-548]. According to the manufacturer and following the FDA-recommended dose regimen (400mg/m^2 initial dose; 250mg/m^2 weekly dose) [549], cetuximab concentrations reached steady-state levels, in the human body, by the third weekly infusion with mean peak and trough concentrations across studies ranging from 168 to 235 and 41 to 85μg/mL, respectively. Hence, a cetuximab's concentration of 100μg/mL is likely to have been utilized in many studies to reflect an intermediate point of these values.

Preliminary results from our laboratory suggested that *S. cerevisiae* might be naturally sensitive to cetuximab, much like what has been described to happen with imatinib. These previous trials pointed to an optimal concentration of antibody causing a moderate effect in a very narrow range of concentrations, around 30nM. This was tested under "standard" growth conditions, *i.e.,* 30°C, 50% aeration and liquid minimal medium [550].

The effect of this drug was herein examined in *S. cerevisiae* parental strain from EUROSCARF collection BY4741 and the mutants defective in the genes identified as the best candidates for cetuximab targets by the *in silico* survey - Sps2p and Sps22p - as well as through Western blot - Pdc1, Rpl16p and Rpl20p. A substantial difference between the susceptibility of wt and any of the mutants should indicate that the deleted gene has a role in the recognition of cetuximab. Yeast cells express *SPS2* only during the middle/late stage of sporulation [551], when it plays a role in spore wall formation [552]. As to Sps22p there is some evidence indicating that it functions redundantly with Sps2p in the organization of the β-glucan layer of the spore wall [552-554]. Only diploid a/α cells of the budding yeast *S. cerevisiae* undergo a specialized developmental program termed sporulation when transferred to a nitrogen-free medium containing potassium acetate as non-fermentable carbon source. The final product of sporulation is an *ascus* that consists of four haploid spores surrounded by the *ascus* wall, the former vegetative cell wall [555]. Even though BY4741 strain is haploid, and so it cannot

sporulate, we decided to test *sps2Δ* and *sps22Δ* susceptibility to cetuximab since, as mentioned, their roles have been scarcely studied. It remains to be elucidated whether it would be more appropriate to test a diploid strain and the correspondent null mutant on *SPS2, SPS22* or both.

BY4741wt and the mutants *sps*2Δ, *sps*22Δ, *pdc*1Δ, *rpl*16Δ and *rpl*20Δ were cultivated at 30°C in minimal medium supplemented with cetuximab 30nM, 150nM, 300nM and 900nM (Fig. 20). Under these conditions, incubation of cetuximab with yeast cells for approximately 24h did not achieve a significant effect in any of the strains. In fact, we observed that, under these experimental conditions, the culture growth is not influenced by any of the drug concentrations tested, being growth pattern almost indistinguishable from the one obtained in the absence of drug (Fig. 20). Another wt strain of *S. cerevisiae,* W303-1A, was included in this susceptibility test to evaluate if the genetic background was a factor to consider. Yet, this wt strain behaved identically to BY4741 (data not shown).

To better understand the results obtained in what concerns yeast growth in the presence of cetuximab, we looked into the literature on how antibodies, particularly cetuximab, could lose activity due to, for example, fragmentation, and how yeast could interfere in this process. Antibodies are usually recognized by several receptors that have binding affinities to either the protein and carbohydrate moieties on the Fc region. The binding of the antibodies to these receptors is usually followed by its internalization and further catabolism. All of these pathways involve the biodegradation of the antibody to smaller peptides or even amino acids [556, 557]. Several pathways have been described that may contribute to the metabolism of antibodies in human cells. Targeted antibodies such as cetuximab are metabolized via a specific, saturable target (antigen) through an elimination process based on receptor/ligand internalization [372-374]. At a certain serum concentration, the EGFRs will become saturated. At this point, a second, non-saturable, unspecific elimination occurs, and this is common to all antibodies [558].
A key component for either endogenous antibodies or therapeutic mAbs to link antigen to immune effector cells is the structural integrity of the hinge region. Although antibodies are generally regarded as highly resistant to proteolytic-mediated breakdown, diverse studies have shown that antibodies are in fact susceptible to proteolytic breakdown by multiple physiologically-relevant bacterial or mammalian proteases [559-561]. Candidate extracellular proteases included

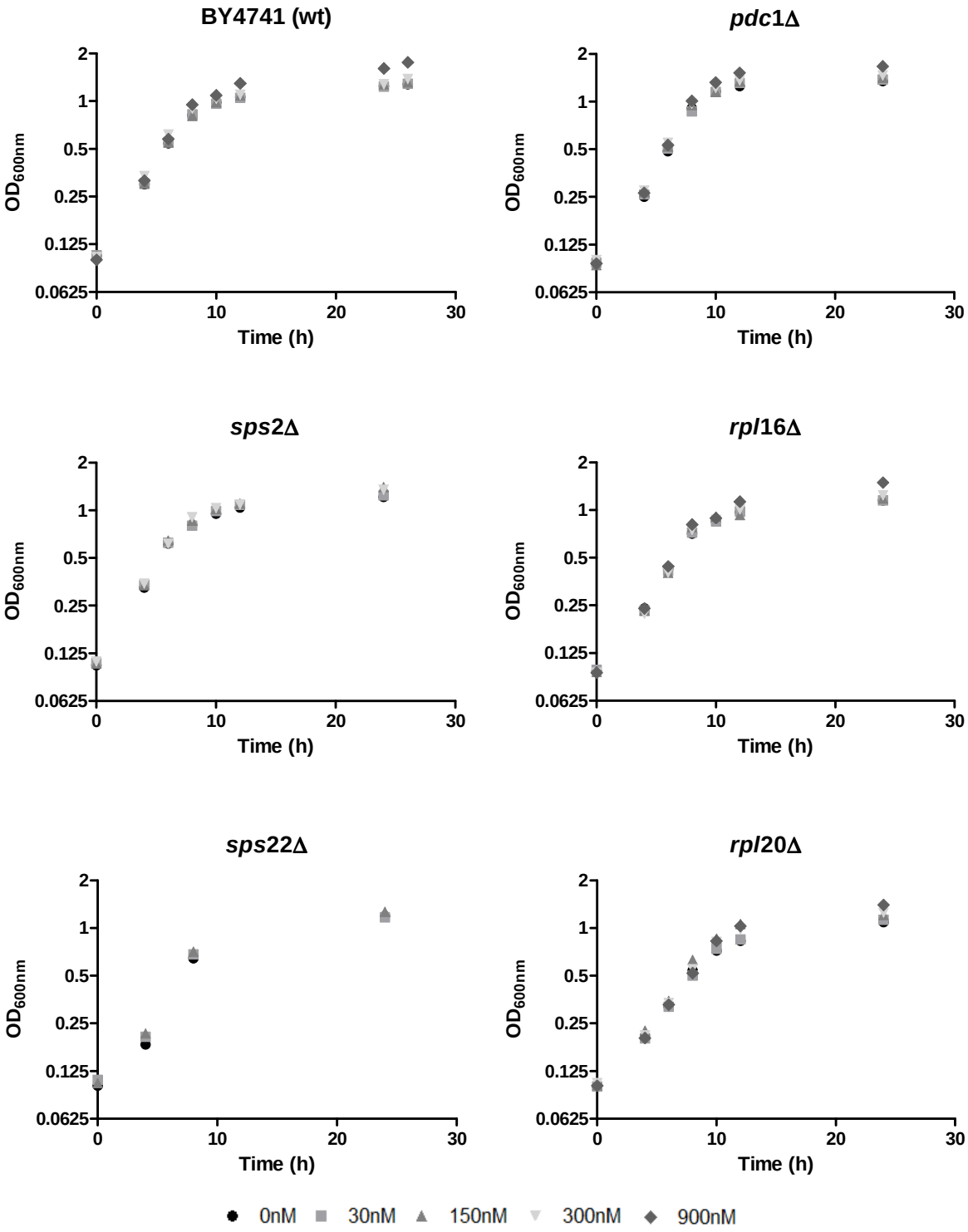


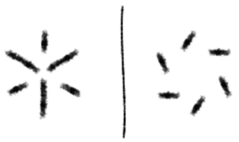
Figure 20 - Effect of monoclonal antibody cetuximab on yeast growth. Growth curves of parental strain BY4741 and mutants *sps*2Δ, *sps*22Δ, *pdc*1Δ, *rpl*16Δ and *rpl*20Δ in YNB medium and in the same basal medium supplemented with 30nM, 150nM, 300nM and 900nM of cetuximab. Growth at 30°C was followed by measuring OD_{600nm}. The results are representative of at least three independent growth experiments.

several matrix metalloproteinases that are elaborated in tumour and wound healing environments [562, 563].Some proteases associated with cancer, in particular the matrix metalloproteinases, have the ability to cleave IgGs, [559, 560, 564, 565] therefore, it has been speculated that antibody cleavage could be another method of tumour immune evasion [566]. In addition, a number of microorganisms express immunoglobulin-degrading proteases that have been implicated as virulence factors in aiding bacterial colonization [561, 567, 568]. Also, yeasts are able to secrete extracellular proteases [569, 570], which may play important roles in the virulence of pathogenic yeast strains, namely from *Candida sp* [571-573]. Proteases, secreted by different fungal genera, have multiple biological functions, ranging from the regulation of cellular processes to the degradation of exogenous proteins for obtaining nutrients, and/or the adaptation to various external habitats [574-576]. A study shows that protease secretion by *S. cerevisiae* was induced in the presence of yeast extract, or of purified proteins, such as bovine serum albumin, casein, or ovalbumin, and some proteolytic activity was present also without protein inducer. This same study also found that properties of proteinases induced under cultivation conditions were different in various aspects (temperature- and pH-dependencies, substrate specificities, sensitivities to proteinase inhibitors). This variability in the secretion of proteolytic enzymes is not purely concordant with their nutritive function and may reflect some other, *e.g.*, recognition aspects [570]. Further studies are needed to clarify whether the proteases secreted by yeast are capable of degrading cetuximab to smaller molecules, such as small peptides or amino acids. If the IgG1 mAb cetuximab does not maintain integrity during the growth assay due to the action of proteases there cannot be any phenotype associated.

3.2. Effect of Monoclonal Antibody Cetuximab on Ethanol Production

Yeasts are historically recognized for their fermentative capabilities and have been used in the elaboration of food and beverages for human consumption in practically all civilizations [577, 578]. With the advent of industrial fermentation technology, these microorganisms have been used by the food industry mostly for their production of ethanol and carbon dioxide, which are important to the brewing, winemaking and baking processes .{Faria-Oliveira, 2013 #7538}

Most organisms use glycolysis as a major means of metabolizing hexoses. This pathway produces pyruvate, which, in the yeast *S. cerevisiae,* either enters fermentation to yield carbon



dioxide and ethanol, or is channelled into different fates as above mentioned, from which the TCA cycle/aerobic respiration (pyruvate converted to acetyl-CoA by the pyruvate dehydrogenase complex) [581] is the most prominent. However, most of the glucose entering the cells is metabolized through fermentation, even under aerobic growth conditions [582] - the so-called Crabtree effect mentioned in the Introduction. Pyruvate decarboxylase (Pdc), which catalyses the decarboxylation of pyruvate to acetaldehyde, further reduced to ethanol can be attributed to is the axis role that this enzyme plays, committing the glycolytic flux towards either ethanol or other fates including its reversion through gluconeogenesis (pyruvate converted to oxaloacetate, by pyruvate carboxylase) (Fig. 19) [520]. Pyruvate decarboxylase can also decarboxylate other 2-oxo acids such as indolepyruvate and 2-keto-3-methyl-valerate, and this activity contributes to the catabolism of the amino acids isoleucine, phenylalanine, tryptophan, and valine [495].

In *S. cerevisiae*, six *PDC* genes have been identified, of which three are structural genes (*PDC*1, *PDC*5 and *PDC*6) and encode for active Pdc enzymes independently. The regulatory genes *PDC*2, *PDC*3 and *PDC*4 encode proteins that are probably involved in the regulation of *PDC*1 and *PDC*5 expression [494]. Structurally, the catalytically active Pdc enzyme is a tetramer composed of two dimers, each consisting of two subunits that are identical and tightly bound. Each subunit has a molecular mass of approximately 60kDa making up a tetramer of 250kDa [583]. The catalytic activity of the Pdc1 enzyme requires the presence of thiamine diphosphate (ThDP) and the metal ion Mg^{2+} as cofactors [584] with the optimum activity of the tetramer at pH 6.2. Increasing pH towards alkalinity results in the dissociation of the Pdc tetramer into inactive dimers, and this dissociation is pH-dependent and reversible [585, 586]. Yeast Pdc enzyme is activated by its substrate, *i.e.* pyruvate, but P_i is a competitive inhibitor [587, 588].

The Pdc1p was recognized by cetuximab in the Western blot assay (Fig. 18A). Bearing in mind that Pdc is a key enzyme in alcoholic fermentation, prompted us to evaluate the effect of cetuximab in ethanol production. This was done using in the wt and *pdc*1Δ mutant from BY4741 background. Cells were grown at 30°C in minimal medium without cetuximab, and in the same medium supplemented with 30nM, 150nM e 900nM of the monoclonal antibody (Fig. 21). Ethanol production was assessed at T0h (latency phase), T6h (exponential growth phase), T12h (stationary phase) and T24h (late stationary phase) of culture growth. This preliminary experiment displayed, as expected, that ethanol production increases over time in the wt culture without cetuximab in the medium. In the presence of 30nM, 150nm and 900nM of cetuximab, the ethanol production increased identically, the presence of the drug not causing significant

differences (Fig. 21). The *pdc*1Δ mutant behaved identically to wt with one exception. The *pdc*1Δ growth in the presence of 900nM of cetuximab at T6h, and at T12h, presented a lower production of ethanol than the wt strain in the same circumstances, respectively 30,4% and 27,8% less, which appears to be recovered after 24h growth. The statistical significance and meaning of these differences remain to be confirmed and explored in the future.

Pdc1p is the major Pdc isoenzyme and is strongly expressed in actively fermenting yeast cells. However, under the same conditions the nearly identical Pdc5p is also expressed, even if its expression is hardly detected, or not detectable at all according to some studies [494, 589-592]. Still, the expression of *PDC*1 and *PDC*5 genes is enhanced during growth on glucose with *PDC*1 mRNA levels being five-fold higher than that of the *PDC5* mRNA levels [589]. This should be the case of wt BY4741 strain, in which case Pdc1p should the main responsible for the production of ethanol while the Pdc5p should be poorly expressed.

Conversely, in the absence of *PDC*1, actively fermenting yeast cells strongly express Pdc5p [593], which redundantly substitutes the missing Pdc1 in alcoholic fermentation, justifying the identical ethanol production observed in both wt and *pdc1Δ* strains (Fig. 21). The deletion in *PDC*5 does not cause any decrease in the specific pyruvate decarboxylase activity, while deletion of *PDC*1 gene results in a five-fold increase in the transcription of *PDC*5 mRNA, yielding a decrease of only approximately 20% of the wild type Pdc activity [494, 590, 591, 593]. Since the promoter activity of *PDC*1 is stimulated in a mutant lacking the coding region of *PDC1*, this phenomenon has been termed Pdc auto-regulation [494, 496, 591]. It was also demonstrated that Pdc1p (but not its catalytic activity) is required to mediate repression of *PDC*5. Thus, a property of Pdc1p that is independent of catalysis appears to be to mediate auto-regulation [591]. This may be even more complex considering that both *PDC*1 and *PDC*5 are expressed under thiamine limitation [594]. All taken, in the *pdc*1Δ mutant grown on glucose, *PDC*5 becomes strongly expressed [593], becoming responsible for the ethanol production observed in the *pdc*1Δ mutant strain (Fig. 21).

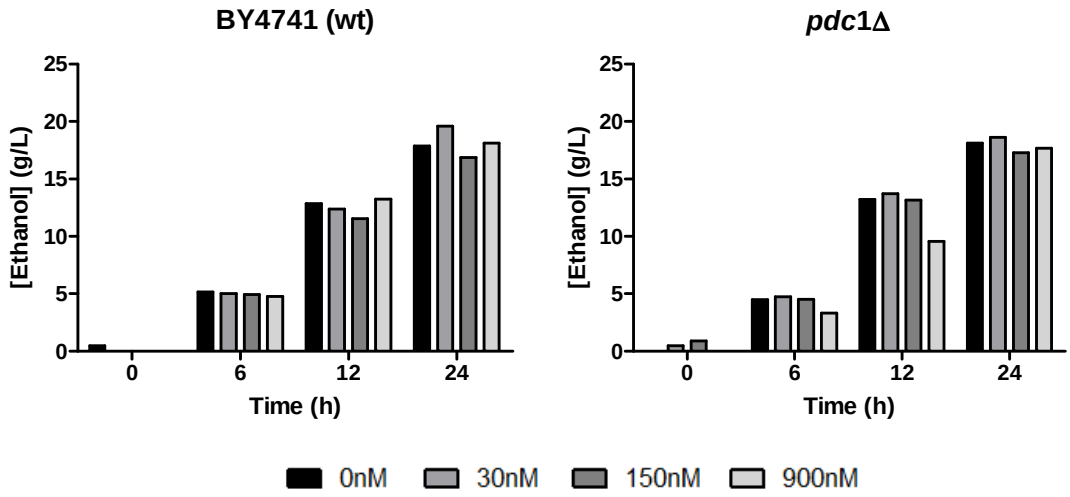


Figure 21 - Effect of monoclonal antibody cetuximab on ethanol production. BY4741 wt strain and the *pdc*1Δ mutant were grown at 30°C in YNB medium without cetuximab and in the same medium supplemented with 30nM, 150nM e 900nM of the monoclonal antibody. Ethanol production was assessed by HPLC at 0, 6, 12 and 24h of culture growth. The ethanol production by the wt culture and the *pdc*1Δ mutant culture increase over time, either in the absence or presence of the 30nM, 150nM e 900nM of cetuximab. The amount of ethanol produced by the two isogenic cell cultures is also similar, except in the presence of 900nM at 6h and at 12h where the mutant produces less ethanol than the wt strain (approximately 30%) in the same condition, as well as the *pdc*1Δ cultures with lower drug concentration. The results displayed refer only to an independent experiment. The experiment must be repeated to reach a suitable number of replicates in order to achieve statistical significance.

Subsequently, we performed a Western blot assay using the whole protein extract of the *pdc*1Δ mutant and cetuximab as primary antibody (Fig. 22), employing an identical procedure to the one in Fig. 18. An immunoreactive band of similar localization to the band identified as Pdc1p in the wt strain (Fig. 22A) was observed in the *pdc*1Δ strain (Fig. 22 B). As *pdc*1Δ deletion strain does not express Pdc1p, we can guess that the band corresponds to Pdc5p, which is consistent with the above-mentioned overexpression of *PDC*5 in *pdc*1Δ mutant [593]. The very similar molecular weight between Pdc1p and Pdc5p, respectively 61,495 Da and 61,912 Da, can sustain undistinguishable SDS-PAGE results. More importantly, the amino acid sequences of Pdc1p and Pdc5p are 88% identical as well as the tri-dimensional structure which is highly similar [494]. This, and in spite of the very affirmative result of peptide band identification reported by Alphalyze, we can postulate that cetuximab can recognize either or both Pdc1 and Pdc5p. The further construction of a double mutant *pdc*1Δ*pdc*5Δ would be ideal to confirm this possibility.

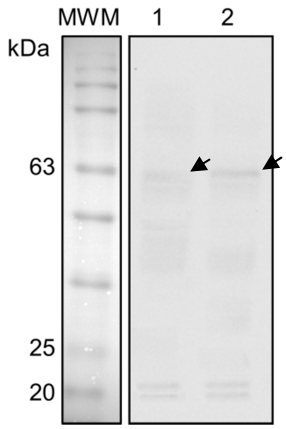
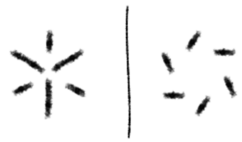


Figure 22 - Both yeast Pdc1p and Pdc5p seem to be recognized by the monoclonal antibody cetuximab. Western blot assay was performed using the whole yeast proteome of wt *S. cerevisiae* BY4741 (lane 1) and the isogenic Pdc-negative strain - *pdc*1Δ (lane 2), and cetuximab as primary antibody. In both conditions it was obtained the same pattern of immunoreactive bands observed in Fig. 18 (see for more details). As *pdc*1Δ strain is deleted in Pdc1p, which is highly similar to Pdc5p that is expressed in the absence of the first, it is probably Pdc5p protein that is being recognized by cetuximab (lane 2).

4. Pdc1p and Pdc5p Structural Characteristics

In the yeast *S. cerevisiae* the major structural gene *PDC1* codes for a 563 amino acids protein [493]. Still, as said above, the catalytically active Pdc enzyme is a tetramer composed of two dimers [583]. Each monomer is built up of three independent domains, which were denoted as pyrimidine (PYR) binding domain, regulatory (R) domain and diphosphate (PP) binding domain [595]. Two subunits form a dimer in the asymmetric unit and the R domains participate in the dimer-dimer interactions [584]. Furthermore, the loops comprising residues 104-113 and 290-304 take part in the closing of the substrate-binding sites and are crucial components in the new dimer-dimer interface formed in the tetramer assembly of form B Pdcp (according to the crystallization of yeast Pdcp in the presence of the activator pyruvamide [596]). As a result, these chain segments undergo a disorder-order transition resulting in the closure of two active sites in the dimer [584].

Optimal sequence alignments between EGFR and isoenzymes Pdc1p and Pdc5p were generated using ClustalW2 (Fig. 23). This program produces biologically meaningful multiple



sequence alignments of divergent sequences and it calculates the best match for the selected sequences. In a simplistic way, the alignment of the EGFR protein and Pdc isoenzymes (Fig. 23) shows that Pdc proteins begin to align in the region of EGFR domain II (CR1), followed by domain III (L2), domain IV (CR2), the transmembrane and juxtamembrane domains and the tyrosine kinase domain as well a portion of the autoregulatory region of EGFR. Pdc1p residues 104-113 (red), that are important in dimer-dimer interaction, align with EGFR domain that binds cetuximab domain III/L2 (yellow), while residues 290-304 (red), also important in dimer-dimer interaction, align in the region of the transmembrane (light gray) and juxtamembrane (dark gray) EGFR's domains. Furthermore, in Fig. 23 also the amino acid residues of EGFR important for the binding of cetuximab were highlighted The crystal structure of the EGFR-cetuximab complex revealed that the epitope covers a large surface on domain III (yellow) of the EGFR [127], in which some functionally critical residues have been identified by diverse groups: Q384, Q408, H409, K443, K465, I467, and S468 (dark blue, black residues) [127, 434, 597, 598]. More recently, some more domain III residues were identified (F352, D355, P387) (dark blue, white residues), which according to the literature should be involved in cetuximab binding as well, since their mutation resulted in a significant decrease (>50%) of antibody binding ability. Positions P349, P362, W386, E388, R390, F412, and I438 (light blue) seem to be of some minor importance for the EGFR-cetuximab interaction as their mutation results in a binding reduction of 30% to 50% [273].

```
Pdc1p       ------------------------------------------------------------
Pdc5p       ------------------------------------------------------------
EGFR        LEEKKVCQGTSNKLTQLGTFEDHFLSLQRMFNNCEVVLGNLEITYVQRNYDLSFLKTIQE 60

Pdc1p       ------------------------------------------------------------
Pdc5p       ------------------------------------------------------------
EGFR        VAGYVLIALNTVERIPLENLQIIRGNMYYENSYALAVLSNYDANKTGLKELPMRNLQEIL 120

Pdc1p       ------------------------------------------------------------
Pdc5p       ------------------------------------------------------------
EGFR        HGAVRFSNNPALCNVESIQWRDIVSSDFLSNMSMDFQNHLGSCQKCDPSCPNGSCWGAGE 180

Pdc1p       ------------------------------------------------------------
Pdc5p       ------------------------------------------------------------
EGFR        ENCQKLTKIICAQQCSGRCRGKSPSDCCHNQCAAGCTGPRESDCLVCRKFRDEATCKDTC 240

Pdc1p       ------------MSEITLGKYLFERLKQVNVNTVFGLP---------------------- 26
Pdc5p       ------------MSEITLGKYLFERLSQVNCNTVFGLP---------------------- 26
EGFR        PPLMLYNPTTYQMDVNPEGKYSFGATCVKKCPRNYVVTDHGSCVRACGADSYEMEEDGVR 300
                         *.  . *** *      :    : :.
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```
Pdc1p        ------------------GDFNLSLLDKIYEVEGMR----WAGNANELNAAYAADGYARI 64
Pdc5p        ------------------GDFNLSLLDKLYEVKGMR----WAGNANELNAAYAADGYARI 64
EGFR         KCKKCEGPCRKVCNGIGIGEFKDSLSINATNIKHFKNCTSISGDLHILPVAFRGDSFTHT 360
                             *:*: **  :  ::: ::     :*: : * .*: .*.:::

Pdc1p        KGMS----------------CIITTFG-----VGELSALNGIAGSYAEH----------- 92
Pdc5p        KGMS----------------CIITTFG-----VGELSALNGIAGSYAEH----------- 92
EGFR         PPLDPQELDILKTVKEITGFLLIQAWPENRTDLHAFENLEIIRGRTKQHGQFSLAVVSLN 420
               :.                 :* ::      :  :. *: * *   :*

Pdc1p        VGVLHVVGVPSISAQAKQLLLHHTLGNGDFTVFHRMSANISETTAMITDIATAPAEIDRC 152
Pdc5p        VGVLHVVGVPSISSQAKQLLLHHTLGNGDFTVFHRMSANISETTAMITDIANAPAEIDRC 152
EGFR         ITSLGLRSLKEISDGDVIISGNKNLCYANTINWKKLFGTSGQKTKIISNRGENSCKATGQ 480
             :  * : .: .**     :  ::.*  .:   :::: .. .:.* :*:: .  ..:

Pdc1p        IRTTYVTQRPVYLGLPANLVDLNVPAK----------LLQTPIDMSLKPNDAESEKEVID 202
Pdc5p        IRTTYTTQRPVYLGLPANLVDLNVPAK----------LLETPIDLSLKPNDAEAEAEVVR 202
EGFR         VCHALCSPEGCWGPEPRDCVSCRNVSRGRECVDKCNLLEGEPREFVENSECIQCHPECLP 540
             :  :  : .  :   * : *. .  ::          *   * ::  :.:  :.. * :

Pdc1p        TILALVKDAKNP---VILADACCSRHDVKAETKKLIDLTQFPAFVTPMGKGSIDEQHP-- 257
Pdc5p        TVVELIKDAKNP---VILADACASRHDVKAETKKLMDLTQFPVYVTPMGKGAIDEQHP-- 257
EGFR         QAMNITCTGRGPDNCIQCAHYIDGPHCVKTCPAGVMGENNTLVWKYADAGHVCHLCHPNC 600
               : :   .:.*   :  *.   . * **: .  ::. .:  .:  . .    .  **

Pdc1p        -------RYGGVYVGTLSKPEVKEAVESADLILSVGALLSDFNTG--------SFSYSYK 302
Pdc5p        -------RYGGVYVGTLSRPEVKKAVESADLILSIGALLSDFNTG--------SFSYSYK 302
EGFR         TYGCTGPGLEGCPTNGPKIPSIATGMVGALLLLLVVALGIGLFMRRRHIVRKRTLRRLLQ 660
                       *  ..  . *.:  .: .* *:* : **  .:           ::    :

Pdc1p        TKNIVEFHSDHMKIRNATFPGVQMKFVLQKLLTT-------------IADAAKGYKPVAV 349
Pdc5p        TKNIVEFHSDHIKIRNATFPGVQMKFALQKLLDA-------------IPEVVKDYKPVAV 349
EGFR         ERELVEPLTPSGEAPNQALLRILKETEFKKIKVLGSGAFGTVYKGLWIPEGEKVKIPVAI 720
              :::**  :   :  * ::  :  :  ::*:               *.:  *   ***:

Pdc1p        PARTPANAAVPASTPLKQEWMWNQLGN---------FLQEGDVVIAETGTSAFGINQTTF 400
Pdc5p        PARVPITKSTPANTPMKQEWMWNHLGN---------FLREGDIVIAETGTSAFGINQTTF 400
EGFR         KELREATSPKANKEILDEAYVMASVDNPHVCRLLGICLTSTVQLITQLMPFGCLLDYVRE 780
                   . . . .  :.: ::   :.*          * .   :*::  . .  :: .

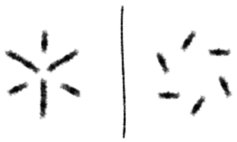
Pdc1p        PNNTYGISQVLWGSIGFTTGATLGAAFAAEEIDPKKRVILFIGDGSLQLTVQEISTMIRW 460
Pdc5p        PTDVYAIVQVLWGSIGFTVGALLGATMAAEELDPKKRVILFIGDGSLQLTVQEISTMIRW 460
EGFR         HKDNIGSQYLLNWCVQIAKGMNYLEDRRLVHRDLAARNVLVKTPQHVKITDFGLAKLLGA 840
              .:  .   :*  .: :: *          . *   * :*.     :::*   ::.::

Pdc1p        GLKPYLFVLN-----------------------NDGYTIEKLIHGPKAQYNEIQGWDHL 496
Pdc5p        GLKPYIFVLN-----------------------NNGYTIEKLIHGPHAEYNEIQGWDHL 496
EGFR         EEKEYHAEGGKVPIKWMALESILHRIYTHQSDVWSYGVTVWELMTFGSKPYDGIPASEIS 900
               * *    .                         . * *: :*:      *: * . :

Pdc1p        SLLPTFG---------------------------------------AKDYETHRVATTG 516
Pdc5p        ALLPTFG---------------------------------------ARNYETHRVATTG 516
EGFR         SILEKGERLPQPPICTIDVYMIMVKCWMIDADSRPKFRELIIEFSKMARDPQRYLVIQGD 960
             ::* .                                         *:: : : *   .

Pdc1p        EWDKLTQDKSFNDNSKIRMIEIMLPVFDAPQNLVEQAKLTAATNAKQ------------ 563
Pdc5p        EWEKLTQDKDFQDNSKIRMIEVMLPVFDAPQNLVKQAQLTAATNAKQ------------ 563
EGFR         ERMHLPSPTDSNFYRALMDEEDMDDVVDADEYLIPQQGFFSSPSTSRTPLLSSLSATSNN 1020
             *  :*.. .. :    :   * *  *.** : *: *  : ::.:.:

Pdc1p        ------------------------------------------------------------
Pdc5p        ------------------------------------------------------------
EGFR         STVACIDRNGLQSCPIKEDSFLQRYSSDPTGALTEDSIDDTFLPVPEYINQSVPKRPAGS 1080
```



```
Pdc1p       ------------------------------------------------------------
Pdc5p       ------------------------------------------------------------
EGFR        VQNPVYHNQPLNPAPSRDPHYQDPHSTAVGNPEYLNTVQPTCVNSTFDSPAHWAQKGSHQ 1140

Pdc1p       -------------------------------------------
Pdc5p       -------------------------------------------
EGFR        ISLDNPDYQQDFFPKEAKPNGIFKGSTAENAEYLRVAPQSSEFIGA 1186
```

Figure 23 - Alignment between human EGFR and *S. cerevisiae* protein Pdc1p and its close homologue Pdc5p. EGFR protein sequence of 1186 amino acids was obtained from NCBI (accession: NP_005219.2 GI: 29725609), that corresponds to the EGFR isoform a precursor, and from which it was removed the signal sequence (24 residues) to simplify further analysis. Pdc isoenzymes sequences, both of 563 amino acids, were obtained from SGD. **Red** – important domains to dimer-dimer interaction in Pdc1p; **Orange** – EGFR L1/I domain; **Yellow** - EGFR L2/III domain; **Light grey** – EGFR transmembrane domain; **Dark grey** – EGFR juxtamembrane domain; **Dark blue** i) black residues – critical residues involved in cetuximab binding; ii) white residues – mutation in this residues results in an abrogation (>50%) of antibody binding to EGFR; **Light blue** – residues of minor importance for the EGFR-cetuximab interaction. The sequences were aligned using clustalW2 coupled with the default parameters. An alignment will display by default the following symbols denoting the degree of conservation observed in each column: ***** - the residues in that column are identical in all sequences in the alignment; **: -** conserved substitutions have been observed; **. -** semi-conserved substitutions are observed.

Using the same strategy outlined above, it was performed an alignment between the EGFR and yeast proteins Sps2p and Sps22p (Fig. 24), which resulted from the bioinformatics approach as the best candidates to be the yeast counterpart of EGFR. We started by using the 1186 amino acid sequence of EGFR, but the alignment had many gaps and did not seem the most appropriate situation (data not shown). Instead, it was decided to use only the extracellular domain of EGFR, since the results from the *in silico* survey indicated that these proteins were possibly more similar to the EGFR ectodomain than any other region of the receptor. Furthermore, it is actually this region that contains the important residues for binding of ligands and cetuximab. In general, the alignment of the proteins is fairly good, including the L domains of the different proteins partially overlap between them. But, regarding to the region that would be important since it is the epitope of cetuximab (blue residues in the Fig. 24), there are several (big) gaps, and only 4 amino acids (of a total of 17) are conserved in Sps proteins.

```
Sps2p       MPIWKTQTFFTSISVIQIVNKETKVSTKKEKDSMLNQLNTILRFLFLFLQLIKSSAAVEP 60
Sps22p      ------------------MNRITRKS---------CLFAIIFASLFVTHALG---AAIDP 30
EGFR        --------LEEKKVCQGTSNKLTQLG-------TFEDHFLSLQRMFNNCEVVLGNLEITY 45
                             *: *: .               :  :*    :      :

Sps2p       NGGPNILDHNIMLVNTN----ATIPKKEQT---DFEVIS--PTKQTQVDEDCKKGLYHIE 111
Sps22p      PRRPHNVKP---FHNGN----LELQRRANEPFFEIDVKS--LNTNSPISELCKKDLHVIE 81
EGFR        VQRNYDLSFLKTIQEVAGYVLIALNTVERIPLENLQIIRGNMYYENSYALAVLSNYDANK 105
                  :.    : :        :    .    ::::       :.       ..    :
```

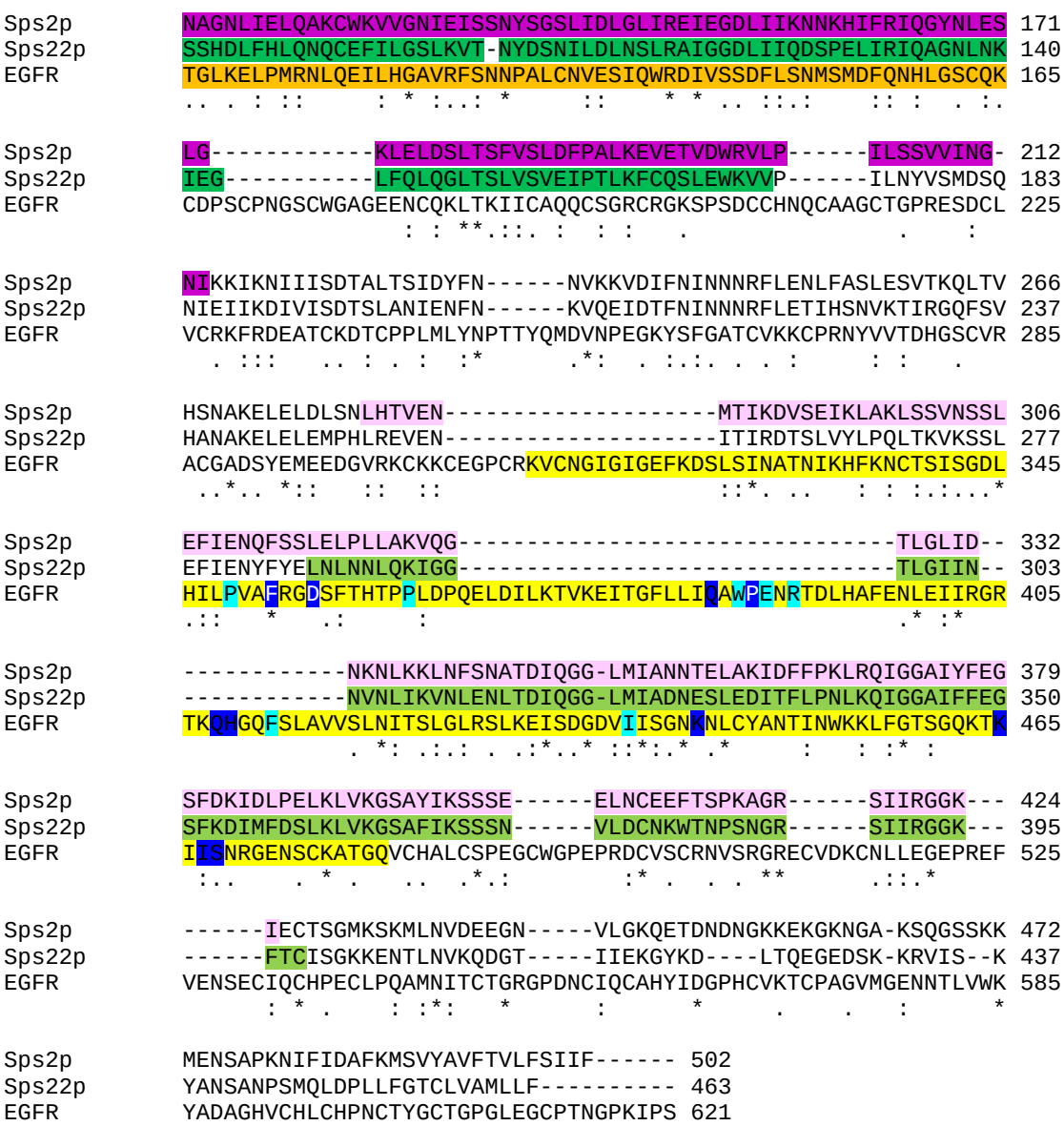


Figure 24 – Alignment between EGFR ectodomain and *S. cerevisiae* proteins Sps2p and Sps22p. EGFR extracellular protein sequence of 621 amino acids was obtained from NCBI (accession: NP_005219.2 GI: 29725609 - EGFR isoform A precursor residues 25-645). Sps2p and Sps22p sequences were obtained from SGD. **Orange** – EGFR L1/I domain; **Yellow** - EGFR L2/III domain; **Violet** – Sps2p L domain domain (see Table II for more information concerning Sps proteins domains); **Pink** – Sps2p L domain; **Drak green** - Sps22p L domain; **Light green** - Sps22p L domain. **Dark blue** i) black residues – critical residues involved in cetuximab binding; ii) white residues – mutation in this residues results in an abrogation (>50%) of antibody binding to EGFR; **Light blue** – residues of minor importance for the EGFR-cetuximab interaction. The sequences were aligned using clustalW2 coupled with the default parameters. ***** - the residues in that column are identical in all sequences in the alignment; **:** - conserved substitutions have been observed; **.** - semi-conserved substitutions are observed.

Conversely, five of the seven amino acid residues critical for the binding of cetuximab to EGFR are present in Pdc1p and Pdc5p or a semi-conserved substitution is observed. Concerning

the amino acid residues whose mutation results in an abrogation (> 50%) of antibody binding to EGFR, two of the three amino acids are present in Pdc isoforms or semi-conserved substitutions are observed. Regarding the seven EGFR residues of minor importance for the EGFR-cetuximab interaction, only one is semi-conserved in Pdc. Moreover, in the absence of substrate the enzyme is in the open, nonactivated state, with four equivalent active sites. In this state, the loop regions 104-113 and 290-304 are disordered. In the activated state, the tetramer assembly has rearranged, with substrate bound at the active site and with two of the four active sites in the closed conformation. The loops 104-113 and 290-304 are now ordered in the active subunits and take part in the closing of the substrate-binding sites and in the new dimer-dimer interactions. After turnover at the two active sites, the tetramer might form an analogous assembly in which the other two substrate-binding sites are activated [584]. Thus, if cetuximab is interacting with Pdc1p (and/or Pdc5p), *in vivo*, near one of the sites responsible for the dimer-dimer interaction namely the 104-113 residues region as suggested in the alignment performed (see Fig. 23) and observing the superficial location of this region in the tri-dimensional structure of Pdc dimer (Fig. 25A) and consequently of easy access by cetuximab, it is most probably interfering with the formation of the tetramer (Fig. 25B), which is the catalytically active form of the enzyme.

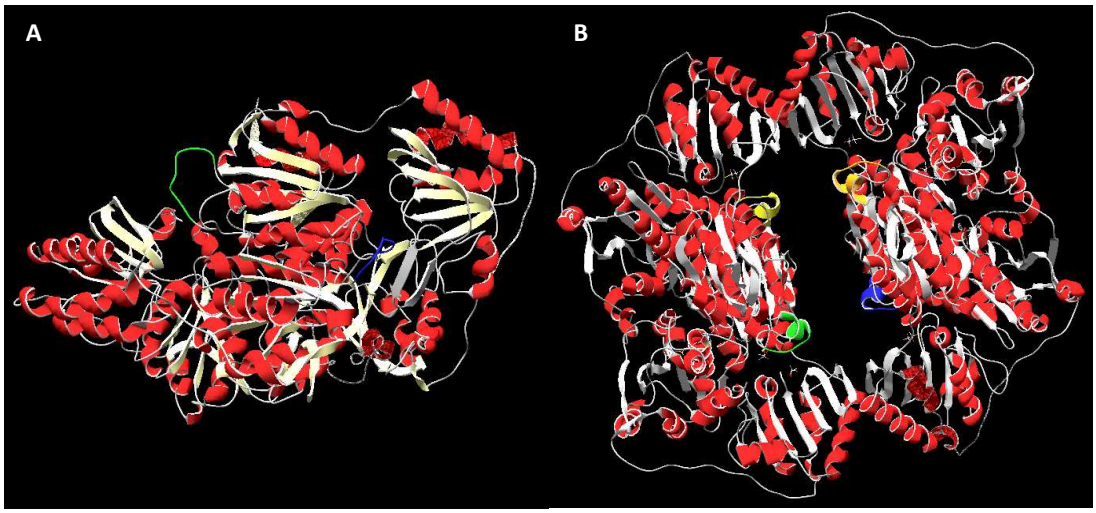
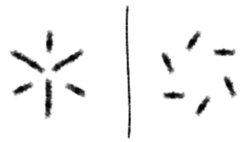


Figure 25 - Tri-dimensional structure of *S. cerevisiae* Pdc1p/Pdc5p. (**A**) Pdc dimer and (**B**) Pdc tetramer tri-dimensional structure were obtained using Swiss-PdbViewer version 4.01 [599]. The loop comprising residues 104-113 (green, blue, orange, dark yellow – each monomer has its colour) take part in the closing of the substrate-binding sites and is a crucial component in the new dimer-dimer interface formed in the tetramer assembly, as well the residues 290-304 (not shown).

Another possibility can be considered, that cetuximab is actually interfering with the catalytic domain substrate recognition/binding site of the enzyme, as the sequences displayed in red (Fig. 23) are involved in the closure of two active sites in the dimer. Besides, one of these loops (104-113 and 290-304) in each monomer is part of the active site in the activated molecule [584].

Final Remarks



Considering the results from the *in silico* survey of *S. cerevisiae* proteome, proteins from the yeast SPorulation Specific family (SPS2 family), in particular proteins Sps2p and Sps22p appeared as the most suitable candidates for EGFR yeast counterparts. These yeast proteins localize to the cell-surface, as EGFR in mammalian cells [102], and the *C. elegans* and *D. melanogaster* orthologues [101]. Moreover, similarly to EGFR's ligand-binding domains I and III [125], and the cetuximab-binding domain III [127], Sps2p and Sps22p belong to the sole *S. cerevisiae* family of proteins (SPS2) displaying L domains*, i.e.,* leucine-rich aminoacid repeats (10-16%) [600]. Furthermore, the tri-dimensional structure of EGFR's ectodomain shows the L1 and L2 domains to consist of single-stranded right-handed β-helices [126, 466]. In the SUPERFAMILY database the L domains of Sps2 and Sps22 are annotated as right-handed β-α superhelix. Databases dedicated to protein structural information (www.yeastgenome.org/cgi-bin/protein/get3) use the information on proteins of known tri-dimensional structure with sequence similarity to a query protein to predict its structure throughout model building by homology. There is no structural information for Sps22p, yet the structure of *S. cerevisiae* protein Sps2p can be theoretically inferred from similarity with internalin (InlA) of *Listeria monocytogenes* [601]. Finally, the alignment between these proteins and EGFR's ectodomain showed that a small number of residues important for binding of cetuximab are conserved in these proteins. Considering that the expression pattern of these proteins is related to sporulation, which operates through meiosis therefore requiring diploidy, it remains to be elucidated whether cetuximab has any effect over phenotypes on a *S. cerevisiae* diploid strain, namely on sporulation and mating processes.

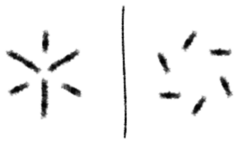
Probing *S. cerevisiae* total proteins with the monoclonal antibody cetuximab identified Pdc1p as the recognized antigen. Ribossomal 60S subunit proteins Rpl16bp, Rpl19ap and Rpl20bp were also identified. However, due to the fact that they were also identified by the secondary antibody used in the Western blot and produced a dubious identification, the results were inconclusive. Therefore, at this stage, Pdc1p was chosen to further analysis and discussion. This is the most expressed isoform - 1 - of the glycolytic enzyme Pyruvate Decarboxylase. This enzyme operates at the level of cytosol as a tetramer, catalysing the conversion of pyruvate to acetaldehyde, ultimately yielding ethanol. It is the most important enzyme in channelling glycolytic flux towards alcoholic fermentation [520]. Besides whole protein and cytosol extracts,

Pdc1p was recognized by cetuximab in extracts from cell wall and plasma membrane. This protein has been reported to appear in yeasts cell surface, where its function and structure remain unknown [488, 489]. As mentioned above, Pdc1p is strongly expressed in fermenting yeast cells, but the isoenzyme Pdc5p is also expressed, even if at low levels, taking over the fermentation needs when *PDC1* is deleted [494, 589-593]. In addition to their functional redundancy, these two proteins are very similar. From the six Pdc proteins, Pdc5p is the closest to Pdc1p, with 86% identity. For this reason in the future it will be necessary to double delete *PDC1* and *PDC5* genes in order to actually evaluate whether cetuximab is able to equally recognize either protein monomer.

Additionally, intracellular Pdc activity has been also used as an indicator of viability in fermenting cells [602]. Therefore, in this study the susceptibility of the wt strain to the presence of cetuximab was compared to the one of the mutant defective in *PDC1*. Preliminary results showed that *pdc*1Δ strain was sensitive to cetuximab at high concentrations, 900nM, and only at the level of ethanol production. Cells cultivated this way presented a 30% lower production of ethanol than the wt strain in the same circumstances. Otherwise, growth seemed not to be influenced by any of the drug concentrations tested (30nM to 900nM). This raised the question whether the proteases secreted by yeasts might cause degradation of cetuximab [569, 570], or if any other cause might be affecting the response of the cells to cetuximab, including the fact that it may affect other phenotypes not assayed within the scope of this thesis. Either way the recognition of Pdc monomer by cetuximab raises even more and wider questions.

At the moment we can only hypothesize that cetuximab can recognize either or both Pdc1p and Pdc5p, and the consequences this might bring to the catalytically active form of the enzyme. The further use of the *pdc*5Δ and the construction of a double mutant *pdc*1Δ*pdc*5Δ would be ideal to clarify by Western blot which Pdc, or if both, are recognized by cetuximab. Nevertheless, due to the presence of the yeast cell envelope that prevents cetuximab diffusion to the cell interior, it should be only the Pdc secreted to the cell exterior that is recognized (and hypothetically inhibited) by cetuximab and that should not impair the intracellular function of the protein. There will be always a good fraction of Pdc inside the cell to perform in fermentation and in ethanol production as well as other cellular processes, in spite of cetuximab presence in the exterior.

The protein alignments between EGFR, Pdc1p and Pdc5p showed that the several residues that constitute cetuximab epitope [127, 273] are present in Pdc proteins, next to the residues



104-113 that (i) are part of the active site, (ii) have a role in the closing of the substrate-binding site, and (iii) are also essential components in the dimer-dimer interface formed in the tetramer assembly [584]. Consequently, if cetuximab is interacting with Pdc1p (and/or Pdc5p) near the site responsible for the dimer-dimer interaction, it is most probably interfering with the formation of the tetramer, assuming its occurrence in the cell exterior, which is the catalytically active form of the enzyme and/or interfering with the catalytic domain substrate recognition/binding site of the enzyme. Unlike the Sps2p and Sps22p proteins, databases do not recognize the putative existence of L domains in Pdc1p. On the other hand, cetuximab is specific for EGFR, not recognizing other human proteins that have L domains such as the IR. Furthermore, similarly to our approach, a Western blot assay using cetuximab as a primary antibody allowed to successfully measure EGFR expression level in seven tumour types [480]. In a Western blot, the proteins are essentially denatured and therefore cannot assume their natural tri-dimensional conformations. Hence, antibodies that recognize linear epitopes instead of conformational epitopes are chosen for immunodetection. Even so, contrarily to monoclonal antibody 225 (the predecessor of the cetuximab) that also binds to domain III of the EGFR but has conformational specificity, since heat denaturation of the receptor abolished the antibody binding to EGFR [433], cetuximab is able to recognize its epitope independently of tri-dimensional structure (at least *in vitro*).

Throughout this work cetuximab was used as antibody, being therefore supposed to attach only to an extracellular protein which might inhibit its activity. With the recognition of a major glycolytic enzyme monomer, this work opens the way to a whole new range of possible functions for this and other glycolytic enzymes that were recently described at the cell surface and whose function remains unknown. This comes at hand with the emerging cancer hallmark, the reprogramming of energy metabolism, namely at the level of glucose respiration [41]. It is widely accepted that tumours display enhanced glycolytic activity and impaired oxidative phosphorylation (Warburg effect) [400], reason why the disruption of glycolysis might be a promising candidate for specific anti-cancer therapy [400, 404, 603]. Tumour cells and fermenting yeast *S. cerevisiae* share several features, including metabolism regulation by genes that are orthologues to known oncogenes [400]. Additionally, imatinib, when administered in yeast *S. cerevisiae*, affects the expression and phosphorilation of many proteins, including glycolytic enzymes [518]. Concerning our study, there is no knowledge of any positive or negative

regulatory function that may be attributed to Pdc at cell surface, either as monomer, dimer or tetramer. The Ras/cAMP/PKA pathway is known to mediate the response of yeast to glucose through activation of glycolytic enzymes, which in turn contributes with intracellular glucose signals and other molecules that might act as putative upstream regulators of the Ras/cAMP/PKA pathway [402] [604]. Therefore, similarly to what happens with EGFR and the downstream regulation of Ras/Raf/MAPK pathway in mammalian cells, other molecules, equally susceptible to cetuximab, may act in yeast as upstream regulators of the Ras/cAMP/PKA pathway, this way controlling metabolism, stress resistance, cell cycle, growth, and transcription. This remains to be assessed in the future.

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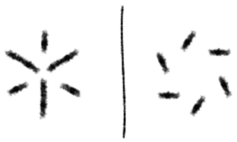

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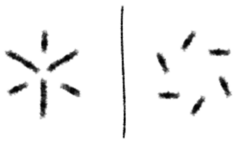

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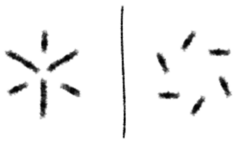

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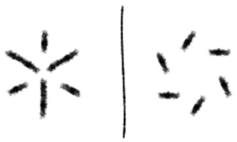

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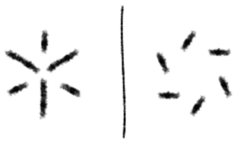

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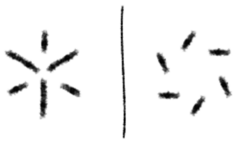

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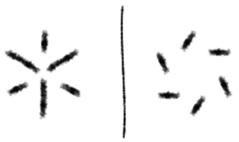

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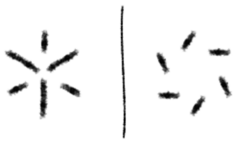

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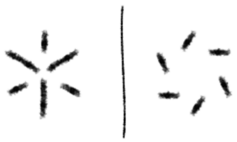

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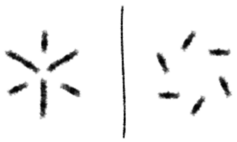

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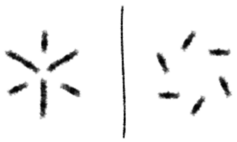

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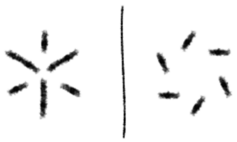

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