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INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR, NOVEL FUNCTIONS AND FUTURE POSSIBILITIES

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To all my friends who were in Tahrir and Rabia squares….

ABSTRACT

Insulin like growth factor-1 receptor (IGF-1R) has been shown to be important for cancer cell growth and survival, and is often overexpressed in malignant and premalignant tissues. Ligand binding to IGF-1R induces transphosphorylation and activation of the receptor, leading to subsequent activation of the phosphatidyl inositol-3 kinase (PI3K), the mitogen-activated protein kinase (MAPK) and the 14-3-3 pathways. Most of these pathways are shared by other receptor tyrosine kinases. Inhibition of these pathways using specific IGF-1R antibodies has, however, failed in large trials on cancer patients. This opens the possibilities for alternative signaling pathways. Recently, IGF-1R was shown to be SUMOylated and translocated to the cell nucleus. In the nucleus it binds to enhancer-like regions, and regulates expression of genes including *CCND1 and AXIN2*, and phosphorylates Histone3. The expression of nuclear IGF-1R has also been linked to increased cell growth and aggressive phenotype in cancer.

In paper I the effects of picropodophyllin (PPP), an inhibitor of IGF-1R, on cell cycle progression were studied. Previous studies have indicated that PPP treated cells arrest in G2/M. We found that PPP induced G2/M arrest through interfering with microtubule dynamics causing prolonged mitotic arrest and mitotic catastrophe in an IGF-1R independent manner. This mechanism of PPP may contribute to its efficacy in treatment of cancer patients. In paper II the roles of SUMOylated IGF-1R in regulating cell proliferation and cell cycle progression were investigated. We provided evidence that SUMOylation of IGF-1R increases G1/S phase transition through inducing expression of cyclins (D, A and B) and upregulating CDK2. Cells expressing SUMOylated IGF-1R also proliferated faster and formed more colonies in soft agar compared to cells expressing *IGF1R* with mutated SUMO-binding sites. In paper III we investigated potential binding partners to nuclear IGF-1R in human embryonic stem cells (hESC). We found that nuclear IGF-1R associates with PCNA and phosphorylates it, not only in hESCs but also in other cell types. The nuclear IGF-1R-induced PCNA phosphorylation was followed by ubiquitination of PCNA, probably through DNA damage tolerance (DDT)-dependent E2/E3 ligases (e.g. Rad18 and UBC13). Our data suggest that IGF-1R may contribute to activation of DDT, as externally induced DNA damage in IGF-1R negative cells led to G1 cell cycle arrest and larger S-phase fork stalling compared to cells expressing IGF-1R. In summary, the achieved results may contribute in understanding the complexity of IGF-1R's roles in cell growth and maintenance of genome stability, as well as the shown mitotic block induced by PPP may be a mechanism that favors anti-IGF-1R treatment in cancer.

LIST OF SCIENTIFIC PAPERS

I. **Waraky, A**., Akopyan, K., Parrow, V., Stromberg, T., Axelson, M., Abrahmsen, L., Lindqvist, A., Larsson, O., and Aleem, E. (2014) Picropodophyllin causes mitotic arrest and catastrophe by depolymerizing microtubules via insulin-like growth factor-1 receptor-independent mechanism. Oncotarget 5, 8379-8392.

II. Lin, Y., Liu, H*., **Waraky, A.*,** Haglund, F., Agarwal, P., Jernberg-Wiklund, H., Warsito, D., and Larsson, O. (2017) SUMO-modified insulin-like growth factor 1 receptor (IGF-1R) increases cell cycle progression and cell proliferation. Journal of Cellular Physiology 232, 2722-2730.

III. **Waraky, A.,** Lin, Y., Warsito, D., Haglund, F., Aleem, E., Larsson, O. (2017) Nuclear insulin-like growth factor 1 receptor phosphorylates proliferating cell nuclear antigen and rescues stalled replication forks after DNA damage. Submitted.

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RELATED PUBLICATIONS NOT INCLUDED IN THESIS

Waraky, A., Aleem, E., and Larsson, O. (2016) Downregulation of IGF-1 receptor occurs after hepatic linage commitment during hepatocyte differentiation from human embryonic stem cells. Biochemical and Biophysical Research Communications 478, 1575-1581.

TABLE OF CONTENTS

1. List of abbreviations

53BP1	:	Tumor suppressor p53-binding protein 1
ALS	:	Acid labile subunit
APC	:	Anaphase promoting complex
APIM	:	AlkB homolog 2 PCNA-interacting motif
ATM	:	Ataxia telangiectasia mutated
ATP	:	Adenosine tri-phosphate
ATR	:	*ATM*- and Rad3-Related
ATRIP	:	ATR-interacting protein
Bad	:	Bcl associated death promoter
Bak	:	Bcl-2 Homologous Antagonist-Killer Protein
Bax	:	Bcl-2 associated X Protein
Bcl	:	B-cell leukemia protein
BRCA	:	Breast cancer susceptibility gene
CAK	:	Cdk activating kinase
Caspase	:	Cystine aspartic acid specific protease
CDC	:	Cell division cycle protein 2 homolog
CDK	:	Cyclin-dependent protein kinase
cDNA	:	Complementary DNA (DNA copy of mRNA)
CDT1	:	CDC10-dependent transcript 1
Chk	:	Checkpoint kinase
Chk2	:	Checkpoint kinase 2
CKI	:	Cyclin dependent kinase inhibitor
Co-IP	:	Co-immunoprecipitation
DDR	:	DNA damage response

DDS	:	DNA damage silencing
DNA	:	Deoxyribonucleic acid
DNA-PK	:	DNA-dependent protein kinase, catalytic subunit
DSB	:	Double strand break
E2F	:	Transcription factor activating adenovirus E2 gene
EGF	:	Epidermal growth factor
EGFR	:	Epidermal growth factor receptor
ERK1/2	:	Extracellular signal-regulated kinase 1/2
FGF	:	Fibroblast growth factor
FGFR	:	Fibroblast growth factor receptor
GDP	:	Guanosine diphosphate
GH	:	Growth hormone
Grb2	:	Growth factor receptor-bound protein 2
HCC	:	Hepatocellular carcinoma
HLTF	:	Helicase-like transcription factor
ICD	:	Intracellular domain
ICF	:	Intermediate cell foci
IGF-1	:	Insulin-like growth factor 1
IGF-1R	:	Insulin-like growth factor 1 receptor
IGF-2	:	Insulin-like growth factor 2
IGF-2R	:	Insulin-like growth factor 2 receptor
IGFBP	:	Insulin-like growth factor binding proteins
INFS	:	Integrative nuclear FGFR1 signaling
INK4	:	Inhibitor of cdk4
INTERNET	:	Integral trafficking from the ER to the nuclear envelope transport
IR	:	Insulin receptor
IR-A/B	:	Insulin receptor isoform A/B

IRS1-4	:	Insulin receptor substrate 1-4
kDa	:	Kilo Dalton
KIP	:	Cdk inhibitory protein
M6P	:	Mannos-6-phosphate
mAb	:	Monoclonal antibodies
MAPK	:	Mitogen activated protein kinase
MCL-1	:	Myeloid leukemia cell differentiation protein
MCM	:	Minichromosome maintenance
MDC1	:	Mediator of DNA damage checkpoint protein 1
MDM2	:	Murine double minute 2
MEK	:	Mitogen-activated/Extracellular Regulated kinase
MMS	:	Methyl methanesulfonate
mRNA	:	Messenger ribonucleic acid
mTOR	:	Mammalian target of rapamycin
Nedd4	:	Neural precursor cells-expressed developmentally down-regulated 4
NHEJ	:	Non-homologous end joining repair
NLS	:	Nuclear localization sequence
NSCLC	:	Non-small cell lung cancer
ORC	:	Origin recognition protein complex
PCNA	:	Proliferation cell nuclear antigen
PDGF	:	Platelet-derived growth factor
PDGFR	:	Platelet-derived growth factor receptor
PDK	:	Phosphoinositide-dependent protein kinase
PI3K	:	Phosphatidylinositol-3’-kinase
PI3P	:	Phosphatidylinositol 3,4,5-triphosphate
PKB	:	Protein kinase B
PLA	:	Proximity ligation assay

PLK1	:	Polo-like kinase 1
Post-RC	:	Post-replication complex
PPP	:	Picropodophyllin
Pre-IC	:	Pre-initiation complex
PTB	:	Phospho-tyrosine binding domain
Raf	:	V-raf-1 murine leukemia viral oncogene homolog 1
Ras	:	Human homologue of Rat sarcoma
RB	:	Retinoblastoma
RFC	:	Replication factor C
RING	:	Really interesting new gene
RNA	:	Ribonucleic acid
RNF	:	Ring finger protein
RPA	:	Replication protein A
rRNA	:	Ribosomal RNA
RSK1	:	Ribosome S6 kinase 1
RT	:	Room temperature
RTK	:	Receptor tyrosine kinase
SCF	:	Skp, Cullin, Fbox
SH	:	Src homology
Shc	:	Src homology and collagen
SHPRH	:	SNF2 histone linker PHD RING helicase
Sos	:	Son of sevenless
Src	:	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
SSB	:	Single strand break
ssDNA	:	Single strand DNA
SUMO	:	Small ubiquitin-related modifier

TK	:	Tyrosine kinase
TLS	:	Translesion synthesis
TOPBP1	:	Topoisomerase binding protein 1
TOPO	:	Topoisomerase
Ub	:	Ubiquitin
UBC	:	E2 ubiqution-conjugating protein
UV	:	Ultra-violet
VEGFR	:	Vascular endothelial growth factor receptor

Introduction

2. Chapter 1

Cancer

2.1 How cancer arises

Normally human adult cells are specialized, i.e. each cell type has a specific function to preform within the tissue. As cells grow old or become damaged, they will die and new cells will divide and grow to replace them. This process is usually controlled by signals that regulate the division process and later on the specialization of the newly formed cells. As cancer develops, this control process breaks down and damaged cells continue to grow and divide in un-controlled manner, giving rise to a benign or a malignant tumor.

A tumor is defined malignant if the cancerous cells start to invade neighboring tissues or use the blood or the lymph system to travel and spread throughout the body. A benign tumor does not spread or invade other tissues, and when removed usually does not recur.

2.2 Hallmarks of cancer

The traits normal cells acquire during transformation process to cancerous cells are known as the hallmarks of cancer as defined by Hananhan & Weinberg (1,2), which include six main hallmarks, discussed below, and four emerging hallmarks including genome instability, deregulated metabolism, evading immune system and inflammation.

2.2.1 Sustained proliferation signaling

Normally the division of cells is controlled by signals that maintain the hemostasis of the cell number and function within a tissue. These signals are mediated in large, by growth factors and hormones that bind to cell surface receptors and transmit the signals intracellularly through cascades of protein interactions known as signaling pathways for the proper respond.

Cancer cells on the other hand, are the masters of their own destiny. They evade these controlling signals through a variety of mechanisms which include: 1) Producing the growth factor ligands themselves; 2) Stimulating the normal cells within the tumor stroma, known as tumor-associated fibroblasts, to produce growth factors; 3) Increasing number of growth factor receptors, or acquiring structural alteration in the receptor molecules that facilitate ligand-independent activation; and 4) Constant activation through somatic mutations of components of signaling pathway components

downstream of these receptors. These components are known as cancer drivers and are either proto-oncogenes, DNA repair genes or tumor suppressor genes.

Proto-oncogenes are genes regulating normal cell proliferation. When altered in certain ways or become more active than normal, they may become cancer-causing genes (or oncogenes). DNA repair genes are involved in repairing damaged or mutated DNA. Tumor suppressor genes are discussed below.

2.2.2 Evading growth suppressors

In addition to mutations in proto-oncogenes which induce and increase cellular proliferation, cancer cells must acquire mutations in another group of cancer drivers known as tumor suppressor genes. Tumor suppressor genes are involved in negatively regulating cell proliferation in a manner opposite to oncogenes, serving to protect cells from uncontrolled division. Cells with certain alterations in tumor suppressor genes, resulting from loss or reduction of functions, may divide in an uncontrolled manner.

2.2.3 Resisting cell death

Apoptosis is a process of controlled and regulated cell death when cells become old, malfunctional, no longer needed or pose a threat to the organism. The apoptotic machinery is composed of upstream regulators and downstream effectors. The regulators are either receiving extracellular death-inducing signals (extrinsic apoptotic program; Fas ligand/Fas receptor), or sensing a variety of intracellular signals (intrinsic apoptotic program) including DNA damage, insufficient survival factors or hyperactivation of certain oncoproteins. Both regulators induce apoptosis through activation of downstream latent protease effectors known as caspases that leads to dissembling and consumption of the cell by both its neighbor cells and phagocytic cells. Cancer cells have evolved a variety of mechanism to evade apoptosis including: 1) Loss of DNA damage sensors; 2) Increased expression of antiapoptic regulators; 3) Downregulation pro-apoptotic factors.

2.2.4 Enabling replicative immortality

Normal cells are able to proliferate through a limited number of cell divisions before entering a non-proliferative state known as senescence, or entering into a state called crisis which involves cell death. This limited number of cell division is linked to telomeres, multiple hexanucleuotide tandem repeats that protect the end of chromosomes from chromosomal DNA end-to-end fusion. The length of telomeric DNA is shortened through every round of cell division, driving the cells into a state of senescence or crisis after a certain number of cell divisions. Cancer cells, and other immortalized cells

nay process an enzyme called telomerase, a specialized DNA polymerase that add more segments to the end of telomeres, resulting in evading senescence and acquiring unlimited proliferation capacity.

2.2.5 Inducing angiogenesis

Like normal cells, cancer cells need the continuous supply of oxygen and nutrients and the ability to get rid of metabolic wastes. This is performed by the ability of cancer cells to induce angiogenesis or the formation of new blood vessels. The formation of new blood vessels is essential during embryological development, and postnatal in certain physiological processes, like wound healing and female reproductive cycle. However, in cancer cells the angiogenic switch is always turned on.

2.2.6 Activating invasion and metastasis

Metastasis is the ability of cancer cells to invade and spread throughout the body. It starts with local invasion, then intravasation through a nearby blood or lymphatic vessel, and use them to travel and spread to distant tissues. Then the escape of cancer cells from lumen of the vessels to the parenchyma of the distant tissues starts, leading to formation of nodules of cancer cells.

3. Chapter 2
Growth factors and cell signaling

3.1 Ligands

In multi-cellular organisms, cells communicate with each other though physical interactions or via a group of communication molecules known as ligands. These two types of interactions allow cells to receive signals from outside and deliver them intracellularly for the proper respond and co-ordination with other cells.

These ligands are divided into two groups; hydrophobic molecules that can pass directly through the phopholipid layer of the plasma membrane, and interact with intracellular proteins, and hydrophilic molecules which cannot pass through the phopholipid layer and must interacts instead with plasma membrane-associated proteins.

3.2 Receptor tyrosine kinase family

One of the largest classes of membrane receptors are a group of trans-membrane proteins known as enzyme-linked receptors. Ligand binding to the extracellular side of the receptor activates the receptor enzymatic activity on the intracellular side, and allows the transmission of the signal through a cascade of interactions between intracellular proteins. The majority of receptors in this class belong to receptor tyrosine kinase (RTKs) family, a group of membrane associated receptors with protein tyrosine kinase enzymatic activity, which catalyze the transfer of a phosphate group from ATP to certain tyrosine residues on the target protein.

The general structure of RTKs is very similar, with an intracellular domain that possess the tyrosine kinase activity, an extracellular domain to which ligands bind to and a hydrophobic transmembrane domain connecting them together. In the non-activated form, majority of RTKs exist as monomers in the cell membrane, with the exception of insulin-like growth factor 1 receptor (IGF-1R)/insulin receptor (IR) family that exist as disulfide linked dimers of two different polypeptide chains (α and β) forming a heterodimer. Ligand binding induces receptor dimerization and autophosphorylation by bringing two monomers together, allowing the kinase domain of one RTK monomer to phosphorylate the other and *vice versa .(3)*

RTKs are further subdivided into 20 different families based on the similarity in the primary structure and the functional domain composition in the extracellular and intracellular domains of the receptor.

3.2.1 The IGF Family

The IGF system consists of three ligands (insulin, IGF-1 and IGF-2), at least four cell membrane receptors (IR, with two isoforms [IRA, IRB], IGF-1R and IGF-2R) and six high-affinity binding proteins (IGFBP 1-6) in addition to the IGF-1R/IR hybrid receptor (4,5).

3.2.1.1 Insulin

The human insulin gene, *INS*, is located on the short arm of chromosome 11. Insulin is produced in the pancreas by beta cells of the islets of Langerhans as proinsulin (6). However, the biologically active form of insulin is a monomer consisting of two chains, an A chain composed of 21 amino acids and a B chain composed of 30 amino acids, linked by two disulfide bridges.

The binding of the hormone to its receptor results in the increased uptake of glucose into the cells, where it is converted into metabolic energy or stored as glycogen or fat (7). Most studies suggest that the metabolic functions of insulin are mainly mediated through insulin receptor isoform B (IR-B), and the mitogenic functions are mediated through the insulin receptor isoform A (IR-A) (8-10). They both exhibit the same affinity for insulin ligand; however, IRA exhibits a higher affinity for IGFs, especially for IGF-2 (9).

3.2.1.2 Insulin-like growth factor 1 (IGF-1)

The human IGF-1 gene, *IGF1*, is located on the long arm of chromosome 12 (11). *IGF1* has two promoter sites, and initiation of transcription from different promoters, in addition to alternative splicing creates multiple transcripts of *IGF1*(12). IGF-1 is a 70 amino acid polypeptide with a molecular mass of about 7.5 kDa, sharing 49% homology with insulin and 62% homology with IGF-2 (13,14). IGF-1 production is regulated mainly by growth hormone (GH), in addition to other hormones and growth factors including follicle-stimulating hormone (FSH) and epidermal growth factor (EGF) (15,16). Levels of IGF-1 in the blood varies between 100-200 ng/ml in adults, but also varies substantially with age, increasing slowly from birth to puberty, surging during puberty and declining thereafter (17). IGF-1 mediates its effect through binding to IGF-1R, where it can regulate proliferation, differentiation, apoptosis and anabolic functions by increasing amino acids uptake (15,16).

3.2.1.3 Insulin-like growth factor 2 (IGF-2)

The human *IGF-2* gene is located on the distal end of the short arm of chromosome 11, 1.4 kb downstream of the insulin gene (18). IGF-2, like IGF-1, is a single polypeptide, with a molecular mass of about 74 kDa (19). *IGF-2* displays parental imprinting. Thus, *IGF-2* is expressed only from the paternal allele, while the maternal allele being transcriptionally silent (20). *IGF-2* has four promoter

sites, with the first promoter expressed mainly in adults and promoters 3 and 4 in fetal development (12). Levels of IGF-2 in the blood varies between 400-600 ng/ml in adults, and mostly stable after puberty (21). However, GH has no regulatory role on *IGF-2* (12).

Like IGF-1, IGF-2 mediate its action through binding to IGF-1R, however IGF-1 binds to IGF-1R with 2-15 folds higher affinity than IGF-2 (12). IGF-2 plays an important role in embryonic and fetal development, as mitogenic and anti-apoptotic ligand. Whereas its role in the postnatal appears to be less important (22).

3.2.1.4 IGF binding proteins and IGFBP proteases

IGFs are usually found associated with one of the six known high-affinity binding proteins (IGFBP-1 to 6) in the bloodstream and other biological fluids and tissues. IGFBPs function in regulating the interaction between IGFs and IGF-1R, in protecting IGFs from degradation and in transportation of IGFs throughout the blood (23-25). IGFBPs expression are regulated by a number of hormones including GH, FSH, estrogen, insulin, vitamin D, in addition to growth factors like FGF, EGF, PDGF and IGFs as well (12).

IGFBP-3 is the predominant IGFBP in serum. Most circulating IGF-1 and IGF-2 form a ternary complex with IGFBP-3 and a third component, the acid-labile subunit (ALS), in a 1:1:1 molar ratio (26-28). In addition to regulating IGF bioavailability, IGFBPs have also IGF independent functions. IGFPB-3 and IGFBP-5, in particular, have been shown to regulate proliferation and apoptosis independent of their effects on IGF-1R signaling (27).

IGFBP proteases are a group of proteases which can cleave intact IGFBPs into small fragments, thereby altering their IGF-binding capacities. This family of molecules is heterogeneous, including serine proteases, cathepsins, and matrix metalloproteinases (16,26). For example, prostate-specific antigen (PSA), a serine protease, is able to cleave IGFBP-3 and 5, reducing their affinities towards IGF-1 and restoring the mitogenic activity of IGF-1(12). The regulation of IGFBP proteolysis remains poorly understood, some of these proteases act preferentially within specific tissues, whereas others function in the bloodstream and extracellular space (12).

3.2.1.5 IR/IGF receptors

IGF-1R and IR belong to receptor tyrosine kinases (RTK) super family. IGF-1R/IR mediates the biological effects of the IGF ligands; IGF-1 functions primarily by activating the IGF-1R, insulin by activating IR, whereas IGF-2 can act through either the IGF-1R or through the IR-A isoform (9). IGF-R and IR share a high homology of (70%) especially within the tyrosine kinase domain (84%) (29,30), whereas IGF-1R and IGF-2R differ completely in structure. The structure of IGF-1R is show in (Fig.1)

The IGF-1R is synthesized as a single pre-protein with a 30-amino acid signal peptide that is cleaved after translation. The protein is then glycosylated, dimerized and transported to the Golgi apparatus, where it is processed as α- and β-subunits. These subunits, form a tetramer (β-α-α-β) through disulfide bonds, and then transported to the plasma membrane (31-33). The mature cell membrane-bound IGF-1R consists of two 130- to 135-kDa extracellular α-chains, to which ligands bind to, and two 90- to 95-kDa β-chains, which contain the tyrosine kinase domain, with several α–α and α–β disulfide bridges (34).

The α-subunit is entirely extracellular and forms a dimer with the other α-subunit. The α-subunit contains two homologous domains, L1 and L2, separated by a cysteine-rich domain. (Fig.1). The cysteine-rich domain is also conserved in the IR and the ligand binding pockets of IGF-1R and IR are formed by the extracellular α-subunits and possibly some extracellular portions of the β-subunits (30,35,36). Differences in receptor-ligand specificities are likely to be dictated by sequence differences within these regions. Indeed, lower homology between IGF-1R and IR is found in the amino-acid sequences of the C-terminal of the α-subunits (47%), extracellular cysteine-rich domains (48%), and N-terminal portion of the β-subunits (41%).

The β-subunit spans the plasma membrane and has three domains; the extracellular, transmembrane and intracellular domains (Fig.1). The intracellular part of β-subunit can be divided into a juxtamembrane, a tyrosine kinase (TK) and a C-terminal domain (Fig.1). The homology between IGF-1R and IR at these domains are different. The TK domain exhibits the highest homology between the two receptors (84%) and the juxtamembrane domain shares 61% of homology, whereas the C-terminal domain shares only 44% (30). Despite this high degree of homology, experimental evidences suggest that the two receptors have distinct biological roles.

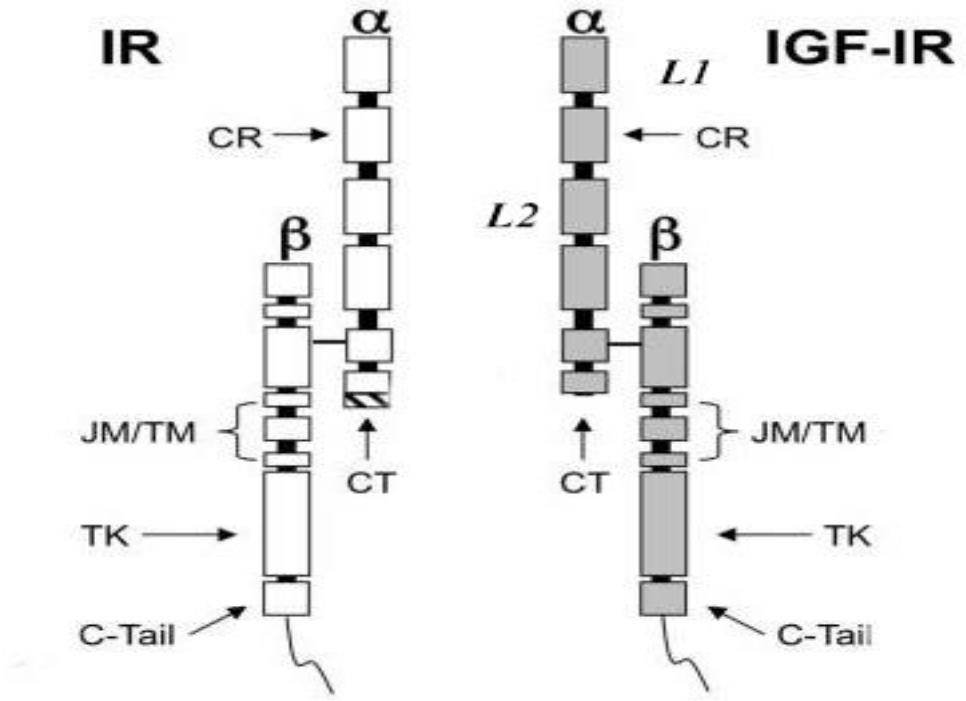

Figure 1. Schematic representation of IGF-1R/IR structure. IGF-1R/IR are composed of two α-chains and two β-chains connected by disulphide bridges. The α-subunit is composed of L1, L2 and cysteine rich domains (CR), while the β-subunit is composed of juxtamembrane domain (JM), tyrosine kinase domain (TK) and C-terminal domain (C-Tail) (37).

The IGF-2R, also called the cation-independent mannose-6-phosphate receptor (CI-MPR), is a multiple ligand-binding cell surface receptor. IGF-2R ligands are divided into those containing mannose-6-phosphate (M-6-P) and those that are mannose-6-phosphate free. Some M-6-P-bearing ligands include leukemia inhibitory factor, proliferin and cathepsin D(38). Among IGF-2R ligands without M-6-P, including insulin-like growth factor 2, and retinoic acid (38). IGF-2R functions to clear IGF-2 from the cell surface to attenuate signaling. After binding IGF-2 at the cell surface, IGF-2R accumulates in forming clathrin-coated vesicles and is internalized (39,40).

3.2.2 IGF signaling

The multiple cellular responses that IGF-1 elicits could be explained by the fact that IGF-1R is able to activate at least three distinct downstream signaling pathways (Fig. 2). The first step in the IGF-1 signaling pathway is the activation of the receptor. Mutations of the three tyrosines of the TK domain (Y1131, Y1135/1136) result in an almost but not completely, inactive receptor (41), while mutations at the K1003, in the ATP binding site, result in a dead receptor (42-44).

As a consequence of ligand-induced autophosphorylation, the receptor will acquire and display a characteristic array of phosphotyrosine residues on its cytoplasmic tail. These phosphotyrosines

become recruiting sites for various cytoplasmic proteins, which will then transmit the signal through signaling pathways to mediate the effects of the ligand.

3.2.2.1 The phosphoinositide 3-kinases, PI3-kinase (PI3K)/AKT pathway

One of the first molecules that reach full activation after phosphorylation of tyrosine residues of intracellular subunit of IGF-1R is the insulin receptor substrate -1 (IRS-1). IRS-1 has three domains: an N-terminal pleckstrin homology (PH) domain, a phosphotyrosine-binding domain (PTB) and a C-terminal domain with more than 20 potential phosphorylation sites interacting with SH2 domain-containing proteins, including Grb2 and regulatory subunit of PI3K (p85) (45).

IRS-1 interaction with p85, leads to activation of catalytic subunit p110 of PI3K and inducing the production of phosphoinsitol 3,4,5 triphosphate (PI3P) (46). In addition, p85 can also bind directly to the phosphorylated intra-cytoplasmic region of IGF-1R. These PI3P will serve as docking sites for pleckstrin-homology (PH) domain-containing proteins including AKT (Protein Kinase B, PKB), and its upstream activator 3-phosphoinositide-dependent protein-kinase 1 (PDK1). Akt/PKB is a serine/threonine kinase, which interacts with PI3P causing its translocation to the inner membrane and then phosphorylated by PDK1 on T308. For full activation AKT needs to be activated on S473 by target of rapamycin complex 2 (TORC2) (47), and DNA-activated protein kinase (DNA-PK) (48).

The activated AKT/PKB will then activate several targets, including phosphorylation of and sequestration of the pro-apoptotic factor Forkhead-1 in the cytoplasm (49,50), inhibition of other pro-apoptotic factors including BAD (51), activation of MDM2 leading to its translocation inside the nucleus, where it binds P53 and targets it for ubiquitination and degradation (52). Akt activation can also induce activation of TORC1, that will lead to phosphorylation of the 40S ribosomal S6 protein by the p70S6 kinase (53), affecting protein-synthesis machinery and cell cycle machinery (54-56).

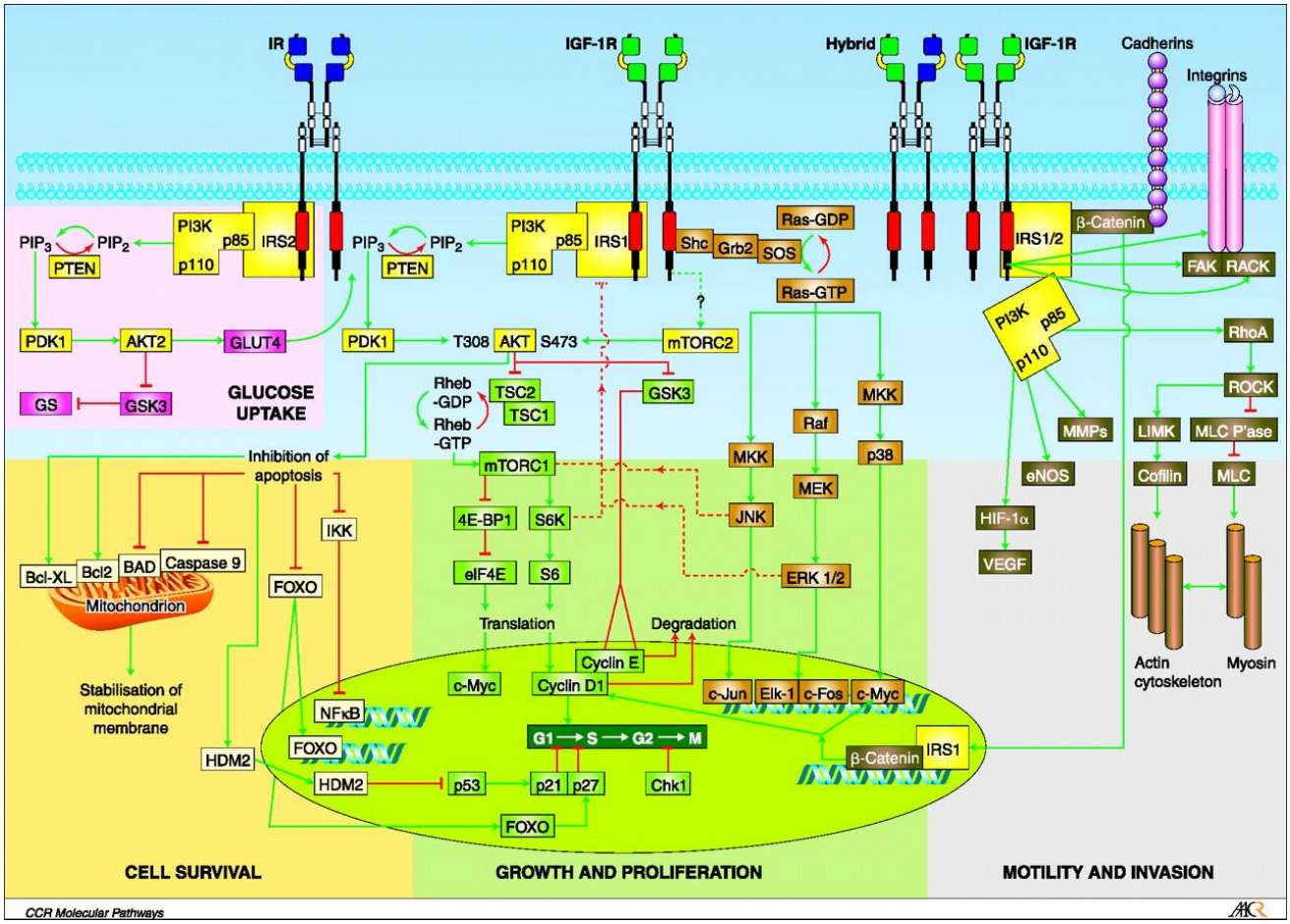

Figure 2. Signaling pathway stream of the IGF-1R. IGF-1R/IR autophosphorylation induced by ligand binding leads to activation of two main pathways the MAPK pathway and the PI3K/AKT pathway. These two pathways then mediate the cellular functions of the IGF-1R, for example induction of cell growth and proliferation, cell survival and motility (57).

3.2.2.2 The Mitogen activated protein kinase (MAPK) pathway

Following activation of the IGF-1R kinase, SH2-containing protein Src homology and collagen (Shc) becomes activated by phosphorylation and forms a complex with the second adaptor protein, the growth factor receptor-binding protein 2 (Grb2). Grb2 can be activated also by binding directly to IRS-1 via its SH2 domain. Grb2 binds to a guanine nucleotide exchange protein called the son of sevenless (SOS), binding of Grb2 to SOS leads to the activation of SOS activity and stimulate the exchange of GDP into GTP from a member of the Ras subfamily (58). Ras protein is able to interact physically with several alternative downstream signaling partners. The first of Ras effectors is the Raf serine/threonine kinase. The activation of Raf by Ras depends upon the relocalization of Raf within the cytoplasm (Ras proteins are always anchored to the inner surface of the plasma membrane through their C-terminal

hydrophobic tails) (59). Once activated, Raf proceeds to phosphorylate and activate a second kinase known as MAP kinase kinase (MEK). MEK, a serine/threonine and tyrosine kinase will phosphorylate two other kinases, the extracellular signal-regulated kinases 1 and 2, commonly referred to as ERK1 and ERK2 (60,61). ERK kinases then phosphorylate several cytoplasmic substrates including Mnk1 kinase as well as translocate to the nucleus, where they can phosphorylate several transcription factors including Ets and Jun (62,63).

3.2.2.3 The 14-3-3 pathway

The 14-3-3 is a family of regulatory proteins that bind to serine/threonine-phosphorylated residues. By interacting with various regulatory proteins, 14-3-3 participates in diverse signal transduction pathways, although the role of 14-3-3 in many cases remains elusive. 14-3-3 can interact with phosphorylated IRS-1 or with the phosphoserine residues within the C-terminus of the IGF-1R. Some insights have been gained from investigations involving 14-3-3 functions on Raf-1 and BAD (64-66).

14-3-3 maintains Raf-1 in an inactive state in the absence of activation signals, by binding to the N-terminal domains, and promotes Raf-1 activation , by binding to the C-terminal domain, when such signals are received (64,65). 14-3-3 is also involved in preventing apoptosis through inhibiting the interaction of BAD with Bcl-X_L and Bcl-2. When Bad is phosphorylated, by survival signals-stimulated kinases, such as PKB, Raf-1, it becomes complexed with 14-3-3 in the cytosol, sequestered from mitochondrially localized Bcl-X_L/Bcl-2 and unable to induce apoptosis (66).

4. Chapter 3

Nuclear translocation of RTKs

4.1 Endocytosis and Ubiquitination of RTKs

Following ligand binding, virtually all RTKs are rapidly endocytosed. Most of the RTKs are endocytosed through clathrin-coated pits pathway, which then shed the clathrin and fuse with an endosome to form an early endosome. From the early endosome, the internalized receptor can then be recycled back to the membrane if the ligand can be dissociated from the receptor in the low pH of the early endosome, or sorted into late endosome and vesicles within the multi-vascular bodies (MVBs) for lysosomal degradation (67)

In addition to clathrin mediated endocytosis, RTKs can be internalized through several clathrin-independent endocytic mechanisms, including macropinocytosis, phagocytosis, and lipid raft-mediated (e.g., caveolin-mediated) endocytosis, and among them, macropinocytosis and lipid raft-mediated endocytosis have been reported for RTK internalization (68)

The leading model for RTK clathrin mediated endocytosis suggests ubiquitination as the mechanism to recruit RTKs to clathrin-coated pit through interaction with proteins containing ubiquitin-binding domains (UBDs) in the clathrin-coated pit (67). Ligand binding to RTKs induces autophosphorylation of tyrosine residues in the cytoplasmic domain, which serves as binding site for RTK ubiquitination enzymes, a posttranslational modification by the covalent binding of the ubiquitin polypeptide to lysine residues of the target protein. The model assumes that ubiquitin modification recruits RTKs to the clathrin-coated pit through interaction with three UBD-containing proteins residing in clathrin-coated pits (epsin-1/2, Eps15, and Eps15R) (67). However, there are contradicting data regarding the importance of these proteins in EGFR and other RTKs' internalization (69,70).

One large family of E3 ligases that ubiquitinate RTKs is the RING finger containing ubiquitin ligases, such as Cbl. Cbl was shown to bind to and ubiquitinate EGFR, PDGFR, VEGFR, FGFR, IGF-1R, hepatocyte growth factor (c-Met), as well as other RTKs (71-74). c-Cbl knockout, siRNA depletion, or overexpression of inactive Cbl mutants inhibit internalization of EGFR and several other RTKs (67,75,76).

Two other E3 ligases, Nedd4 and Mdm2, have been shown to mediate IGF-1R ubiquitination (77-80). Although exactly how c-Cbl interacts with IGF-1R remains largely unknown, Mdm2 and Nedd4 have been shown to bind to adaptor molecules β-arrestin and Grb 10, respectively (77,79,80). However, other studies showed that ubiquitin-conjugation sites of EGFR, FGFR-1 were not essential for ubiquitination, but only essential for IGF-1R internalization and endocytosis (76,81,82).

4.2 Nuclear and sub-cellular trafficking of RTKs

In addition to recycling of the receptor and lysosomal degradation, internalized receptor can undergo retrograde trafficking, the transport of protein and lipid from the cell surface to Golgi and from the Golgi to Endoplasmic reticulum (ER) in coat protein complex I (COPI) coated vesicles, or even to the nucleus (68,83,84).

The first example of retrograde trafficking was shown for EGFR. In response to EGF stimulation, a portion of EGFR would redistribute to the Golgi and the ER, with its C-terminus exposed to the cytoplasm and the N-terminus resides within the lumen of Golgi/ER. Blockage of the Golgi-to-ER retrograde trafficking through dissembling COPI complex inhibited EGFR transport to both the ER and the nucleus (84). In addition, inhibition of receptor endocytosis blocked the nuclear transport of EGFR, ErbB-2 and FGFR. Furthermore, ErbB-2 and EGFR have been shown to be associated with endosomal marker in the nucleus, suggesting the nuclear transport to be mediated by early endosomal sorting (85).After reaching the ER, RTKs can be transported to the nucleus via two importin-β-dependent pathways, integrative nuclear FGFR-1 signaling (INFS) and integral trafficking from the ER to the nuclear envelope transport (INTERNET). For the INFS pathway, characteristic of FGFR (86), the RTK is pumped through the Sec61 complex, a protein complex that transports proteins across ER, into the cytosol where it binds to cytosolic importin and transported into nucleus through the importin-nuclear pore complex interaction. For the INTERNET pathway, characteristic of EGFR and ERbB-2 (87), the receptor is transported along ER, translocated from the outer nuclear membrane to the inner nuclear membrane by binding to ER-associated importin, all members of EGFR family contain nuclear localization signal (NLS) required for complex formation with importin-β. The RTK is then released into the nucleoplasm by the Sec61 complex (68).

To date, 18 different RTKs have been reported to translocate to cell nucleus in respond to ligand stimulation. In addition to the nuclear transport of the intact receptor, several RTKs translocate in the form of receptor fragment called intracellular domain (ICD), generated by proteolysis, internal translation initiation or messenger RNA (mRNA) splicing (88).

4.3 Nuclear trafficking of IGF-1R

The nuclear localization of IGF-1R has been shown in a number of previous studies (89-93) and nuclear IGF-1R has been associated with many types of cancers including lung, breast, and prostate cancers, and hepatocyte carcinoma (89,93). However, until recently the exact mechanism of nuclear translocation of IGF-1R remained largely unknown. Earlier studies showed that IGF-1R and IR could be cleaved by γ-secretase complex to produce intra-cellular domain (ICD) fragments (94,95), with the

IR-ICD fragment localized to the cell nucleus (95). However, more recent studies have showed the translocation of the intact IGF-1R to the cell nucleus after SUMOylation on three lysine residues (Lys1025, Lys1100 and Lys1120) (90). Nuclear translocation of IGF-1R was shown to be mediated through nuclear pore complex after interacting with β-importin and SUMOylation of the receptor was shown to be required for the interaction with RanBP2 nuclear pore complex protein (91). However, further investigation is needed to identify mechanism of transportation of IGF-1R from endocytic vesicle to nuclear pore complex.

4.4 Nuclear functions of RTKs

Nuclear RTKs, either as intact receptors or as truncated fragments of the receptors, have been shown to mediate several nuclear functions, including modification and remodeling of histones (96-98), regulating DNA repair and DNA replication (99-101), transcription co-activation of several genes including cyclin D (92,102,103) and cyclooxygenase-2 (Cox-2) (104,105) through binding to transcriptions factors as STAT3&5 (106,107), LEF (92).

Nuclear functions of IGF-1R are associated mainly with the translocation of the intact receptor, with the exception of the single study showing the accumulation of α-subunit of IGF-1R after IGF-1 treatment in Graves' disease fibroblasts. Nuclear IGF-1R was shown to associate with LEF-1 transcription factor to regulate cyclin D1 expression (92), and regulate histone remodeling through phosphorylating histone 3 at tyrosine 41(96). Furthermore, in the current study we showed nuclear IGF-1R to associate with and phosphorylate PCNA, regulating PCNA ubiquitination and DNA damage response and to regulate cell cycle progression through increasing expression of cyclins D, A,B and CDK2 (108).

5. Chapter 4

Targeting RTKs as therapeutic approach

5.1 Targeted therapy

Surgery, chemotherapy and radiotherapy alone or in combination have been the conventional methods for cancer treatment. However due to the toxic effect of conventional treatments on normal cells, more interest is drawn towards a new treatment method called targeted therapy (109). Targeted therapy aim to block specific proteins or pathways related to carcinogenesis and tumor growth that is highly upregulated in that type of tumor, instead of targeting both normal and tumor cells (110). Various targeted therapies have been approved for cancer treatment including, apoptosis inducers, angiogenesis inhibitors and signal transduction inhibitors (109,110). Two main approaches for targeted therapy are used; small molecule inhibitors and monoclonal antibodies (mAbs).

5.1.1 Small molecule inhibitors

Small molecule inhibitors are usually organic compounds with low molecular weight that are able to penetrate the cell membrane. They are specifically designed to act on specific protein targets or to interfere with the signaling pathways. Small molecule inhibitors could be specific for a protein target, or have a broad spectrum of targets. The generic names for the most of small molecules are suffixed with “ib” (Imatinib, Sorafenib, etc.), whereas mAbs drugs are suffixed with “mab” (Cetuximab, Trastuzmab, etc.) (109,110).

5.1.2 Monoclonal antibodies

Initially the generation of mAbs was done in mouse, producing mAbs that were entirely comprised of murine protein and were highly antigenic causing hypersensitivity in humans. To overcome these problems, recent mAbs are modified to have a minimum murine proportion and a maximum human proportion. For example, human antibodies contain 100%, humanized antibodies contain 95% and chimeric antibodies contain 65% human components. The suffix of the antibody indicates the percentage of human component, e.g., -umab (human), -zumab (humanized), -ximab (chimeric) and -omab (murine) (109,110). The mAbs mediate their actions either through 1) Inhibiting ligand-receptor interaction, 2) Activating immune system, 3) Delivering an immunotoxin (111). mAbs are more specific than small molecule inhibitors, however the obstacles in using monoclonal antibodies include requirement for systemic administration, which can be painful to patient, they can only target extracellular antigens as they cannot penetrate the plasma membrane, relatively expensive to

manufacture, and may trigger a host immune response (111). Approved small molecule inhibitors and mAbs targeting RTKs are summarized in Table 1.

Drug type	**Name**	**Targets**	**Disease**
Antibody	Trastuzmab (Herceptin)	HER2	Breast cancer
	Bevacizumab (Avastin)	VEGFR	Colorectal cancer
	Cetuximab (Erbitux)	EGFR	Colorectal cancer
Small molecule	Imatinib	BCR-ABL, c-KIT, PDGF	CML, GIST
	Gefitinib	EGFR	Non small-cell lung carcinoma
	Sorafenib	VEGFR, PDGFR	Renal cell cancer
	Laptinib	EGFR, HER2	HER2-positive breast cancer

Table 1. Approved small molecule inhibitors and monoclonal antibodies targeting members of RTKs in cancer (112,113). CML: Chronic myeloid leukemia, GIST: Gastrointestinal stromal cell tumor.

5.2 RTKs in Cancer

Targeted therapies for RTKs have gained much interest due to their oncogenic properties. In addition to regulating major signaling pathways that control cellular proliferation, apoptosis, and migration, mutations or overexpression of RTKs have been reported in several types of tumors (112,114-116). For example overexpression of HER2 and EGFR have been reported in 25% of breast cancer cases, 30–50% of glioblastoma and 25–82% of colorectal cancer (115). These mutations generally result in a constitutive active receptor that can result from: 1) Mutations in the active loop of the receptor as in EGFR kinase domain mutation in lung cancer stabilizing the activity of the kinase domain (117), 2) Mutation in the extracellular domain as in HER2 mutation in pulmonary neoplasm resulting in a constitutive dimerization of the receptor (118), 3) Mutation in the juxta-membrane domain as in c-Kit and PDGFR in gastrointestinal stromal cell tumors (GIST) (119). In addition to these mutations overexpression of RTKs can also result from DNA translocation, as in the BCR-ABL translocation in chronic myeloid leukemia (CML), or from genomic amplification of EGFR in breast cancer and lung adenocarcinoma (116,120).

Although activation mutations of IGF1R have not been reported and gene amplification of IGF1R family is extremely rare (121), elevated IGF-1 levels have been shown in prostate and breast cancer, loss of genomic imprinting of IGF-2 have been reported in colorectal cancer and childhood acute lymphoblastic leukemia (122), and elevated levels of IGF-2 and IGF-1R in synovial sarcoma as a product of SS18-SSX fusion (123). In addition, extensive crosstalk between IGF-1R and other members of RTKs including EGFR and HER2 have been shown (122,124,125), and significant role for IGF-1R in HER2/EGFR resistance to targeted therapy have been reported (124-126). Furthermore, the recent role of nuclear translocation of IGF-1R and the association of nuclear IGF-1R with many types of cancers including lung, breast, and prostate cancers, and hepatocyte carcinoma (89,93) provide further significance for targeting IGF-1R in cancer.

5.2.1 Targeting IGF-1R

Three main approaches are used for IGF-1R targeted therapy: 1) Antibodies targeting IGF-1R, IR/IGF-1R hybrid, 2) Neutralizing antibodies against IGF-1 and IGF-2 ligands, 3) small molecule tyrosine kinase inhibitors (122,127). Antibodies targeting IGF-1R mediate their actions either through blocking IGF-1R from ligand binding, or mediate IGF-1R, IR/IGF-1R hybrid receptor degradation without affecting IR-A or IR-B. Neutralizing antibodies against IGF ligands inhibit the binding of ligands to both IGF-1R and IR-A without affecting IR-B, and small molecule inhibitors inhibit the kinase activity of the receptor (122).

5.2.1.1 Monoclonal Antibodies for IGF-1R targeted therapy

The monoclonal antibodies against IGF-1R are well tolerated as a montotherapy, with no maximum dose achieved at the end of Phase I, and only recorded side effect was hyperglycemia in 20% of the cases (122,127). Table.2 summarizes the most prominent monoclonal antibodies that target IGF-1R pathway. Most notable effect of mAbs in phase I and II were shown with sarcomas specially Ewing sarcoma and non-small cell lung cancer with reports of complete response (CR), partial response (PR) and prolonged stable disease (SD) with R1507 (128), IMC-A12 (129,130) and CP-751(131,132).

mAb	Status	Trail
CP-751 (Figitumumab)	Discontinued after phase III no benefit from combination with carboplatin/paclitaxel and increased toxicity	Phase I/II: Soft tissue sarcoma, Ewing Sarcoma Phase II: Prostate cancer
R1507	Phase II	Phase I: Ewing Sarcoma Phase II: Rhabdomyosarcoma, osteosarcoma, Ewing Sarcoma
IMC-A12 (Cixutumumab)	Phase II	Phase II: Advanced non-small cell lung cancer Prostate cancer
MK0646 (Dalotuzumab)	Phase III	Phase I: Solid tumors Phase II: Neuroendocrine tumor

Table 2. Most prominent monoclonal antibodies that target IGF-1R pathway (122,127-132).

5.2.1.2 Small molecule inhibitors for IGF-1R targeted therapy

Due to the high homology between IR and IGF-1R tyrosine kinase domain, most small molecule inhibitors inhibit the activity of IR as well. However, co-inhibition of IR and IGF-1R might provide a better antitumor effect, as the IR signaling have been implicated in a number of tumor models (122). Currently the most promising small molecular inhibitors are OSI-906 (Linsitinib) in phase III, BMS-754807 in phase II and AXL1717 (picropodophyllin) phase II.

5.2.1.2.1 Picropodophyllin

Picropodophyllin (PPP) belongs to cyclolignans, which also include podophyllotoxin (PPT) that earlier has been described as an antineoplastic compound (133). This kind of chemical compounds are also known as phytoestrogens (plant-derived compounds that have estrogenic properties when consumed) (134). PPP has been demonstrated to potently inhibit IGF-1R autophosphorylation (IC50 of 0.4 μM) in

intact cells and was selective against a panel of other receptor tyrosine kinases including IR (135). The PPP-induced inhibition of IGF-1R phosphorylation did not interfere with the IGF-1R tyrosine kinase at the ATP binding site (135), instead it is suggested to interfere with phosphorylation at substrate level (136). Furthermore, PPP was shown to induce IGF-1R downregulation through MDM2 and β-arrestin, without affecting IR or other RTKs (79). Inhibition of the kinase activity of the IGF-1R caused reduction in phosphorylation of Akt and ERKs in IGF-I stimulated cells (135). Furthermore, PPP rapidly caused complete regression of autografts derived from IGF-1R positive cells and did not affect the tumors derived from IGF-1R negative cells (135). PPP in its oral formulation is named AXL1717. AXL1717 has showed promising results in a phase I/II study involving a number of patients with advanced non-small cell lung cancer of adenocarcinoma subtype, in which a progression-free survival of 31 weeks and an overall survival 60 weeks were reported (137). PPP has also been shown to induce G2/M arrest in treated cells (138).

Chapter 5

Molecular regulation of the mammalian cell cycle

6.1 Cyclin-dependent kinases

The process of cell proliferation is especially important for cancer cells. A hallmark of cancer is the abnormal regulation of cell cycle. In order for a cell to complete one round of cell division and reproduce an exact duplicate of itself, it has to perform five tasks in a highly ordered fashion: first to grow in size (G1 phase), replicate its DNA (S phase), undergo a second phase of growth (G2 phase), equally segregating the duplicated DNA (M phase) and finally divide into two equal daughter cells (139). All these crucial decisions are made by a set of information processors (cell cycle machinery) that integrate extracellular and intracellular signals to coordinate cell cycle events.

The cell cycle molecular core machinery consists of a family of serine/threonine protein kinases known as cyclin-dependent kinases (CDKs). These are catalytic subunits, which are activated by associating with the regulatory subunits called cyclins. For example, CDK4/CDK6 binds to cyclin D, CDK2 binds to Cyclin E, A and CDK1 binds to Cyclin A, B. The activity of CDK/cyclin complexes is further regulated on several levels as discussed below (140).

I) Cyclin-dependent kinase inhibitors: CDK activity is regulated by two families of inhibitors; the Cip/Kip family, composed of $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ and the INK4 proteins, composed of $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$. INK4 proteins inhibit cyclin-CDK interaction, while Cip/ Kip proteins binds to CDK–cyclin complexes and disrupt ATP binding and substrate access (141).

II) Phosphorylation of the CDK subunit can have both positive and negatives effects on its activity. For example, phosphorylation of threonine 161 (T161) in Cdc2 is required in order for the full activity of the CDK/cyclin complex (142). However, phosphorylation of threonine 14 (T14) and tyrosine 15 (Y15) of Cdc2, inhibits CDK activity even when it is phosphorylated at T161.

III) Proteasome protein degradation: Two types of E3 ubiquitin ligases have important roles in regulating cell cycle the Skp1–Cul1–F-box protein (SCF) complex family and the anaphase promoting complex/cyclosome (APC/C). The SCF and APC/C complexes belong to a broader family of cullin–RING ubiquitin ligases (CRLs), and have important functions in regulating cell cycle through targeting cell cycle proteins for ubiquitination and subsequent proteasome degradation. The SCF complex is composed of four components; Skp1, cullin protein Cdc3/Cul1, an F-box containing protein, and the RING protein Rbx/Roc1/Hrt1. The F-box protein determines the specificity of the complex, and access to F-box proteins is

usually regulated to control ubiquitination of the target protein. For example, in many substrates pre-phosphorylation is required for the interaction of SCF complex with its cognate F-box protein, and/or the F-box protein are usually targeted for degradation through autoubiquitination. The APC/C is active throughout mitosis and G1 phase, it is a large, multicomponent protein complex composed of more than a dozen individual proteins. The activity of the APC/C is regulated by CDK phosphorylation of core APC/C components and pseudo-substrate inhibitors.

6.2 Regulation of cell cycle during G1 phase:

In early G1 phase, extracellular mitogenic signals stimulate the release of INK4 inhibitors from the CDK4/6, allowing the association of CDK4/6 with cyclin D. CDK4/6 being active induces the phosphorylation of a group of proteins called pocket proteins, including the retinoblastoma protein (Rb), the retinoblastoma-like protein 1 (p107) and the retinoblastoma-like protein 2 (p130). In the non-dividing state the Rb protein binds to the activator E2F family (E2F 1,2,3), a family of transcription factors that regulate expression of G1/S phase proteins through binding to E2F-responsive promoters, and inhibit them from binding to the cognate E2F-responsive promoters. At the same time p107 and p130 bind to repressor E2F family of proteins (E2F 4,5), allowing them to bind to E2F-responsive promoters and inhibit cell cycle protein expression. Phosphorylation by CDK 4/6 allow the release of E2F proteins from Rb and pocket proteins, allowing the binding of activator E2F proteins and inducing expression of G1/S cell cycle proteins including cyclin D1, cyclin A, cyclin E (143,144), $p21^{CIP}$, CDK2, Cdc2, and Cdc25c (145). Activator E2F proteins induce the expression of $SCF^{Fbx4/alphaB\text{-}crystallin}$ protein complex which targets ubiquitination and degradation of cyclin D (146), as well as the expression of repressor E2F proteins (6,7) acting as a negative feedback loop. In addition to inducing the expression of SCF^{SKP2} complex required for p21 and p27 degradation to allow the accumulation of cyclin E which marks the ends of early G1 phase and the start of transition towards G1/S phase (reviewed in(147)).

6.3 Regulation of cell cycle during G1/S phase:

During G1/S-phase a group of protein called pre-replication complex (pre-RC) has to be bound to the origin of replication to initiate replication during S-phase. The pre-RC is composed of origin recognition protein complex (ORC), minichromosome maintenance protein complex (MCM 2-7), CDC6 and CDC10-dependent transcript 1(CDT1). The ORC is bound to the origin of replication throughout most of the cell cycle to form the post-replication complex (post-RC). By the end of mitosis, and through anaphase the degradation of CDK1 and another protein called Geminin by the

APC/C, allow the binding of CDT1 and CDC6 to the ORC. Geminin inhibits CDT1 activity and prevent from binding to the ORC, and the high CDK activity phosphorylates CDT1 and marks it for degradation by SCF^{SKP2}. Binding of CDC6 and CDT1 to the ORC create a footprint for MCM loading for the formation of pre-RC. For replication firing during S-phase, the MCM 2-7 helicase needs to be activated and the pre-initiation complex (pre-IC) is required to be formed. The high CDK2, and the DBF4-dependent kinase activity (DDK; also known as the CDC7–DBF4 complex) during S-phase is required for phosphorylation and activation of the MCM 2-7 complex, in addition to phosphorylation of several other factors including (CDC45, GINS, treslin, DNA topoisomerase 2-binding protein 1 (TOPBP1) and DNA polymerase ε (Pol ε)) which are required for formation of pre-IC. CDC45 and GINS are required for the activation of the MCM helicase, and the formation of CMG complex at the replication fork (CDC45, MCM, GINS). The phosphorylation of TOPBP1 and treslin is required for loading of CDC45 and GINS to the pre-IC. Replication firing then recruits additional factors including proliferating cell nuclear-antigen (PCNA), replication factor C (RFC), replication protein A (RPA) and DNA polymerases. To prevent origin re-replication, binding of Cdt1 to the PIP domain of the PCNA marks the Cdt1 for proteasome degradation, and the high level of CDK during S-phase induce activation of Geminin and inhibition of CDT1, in addition to nuclear export of CDC6 (reviewed in (148,149)).

6.4 Regulation of cell cycle during mitosis

The M phase is regulated by CDK1 kinase and the associated cyclin B or M-phase promoting factor. Cyclin B expression start to increase by late S phase through activation of NF-Y, B-Myb, FoxM1 transcription factors induced by CDK2/Cyclin A expression, peak by the beginning of mitosis and start to decline by the end of mitosis through proteosomal degradation by APC/C. CDK1/Cyclin B activation is mainly regulated by phosphorylation, through inhibitory phosphorylation by Wee1/Myt1 kinases on T14/Y15 and by activating phosphorylation on T161 by CDK-activating kinase (CAK). CAK is fairly active throughout mitosis, suggesting that regulation is mainly mediated by Wee1/Myt1 kinase. At the beginning of mitosis Wee1/Myt1 inhibition of CDK1/Cyclin B is dominant, the activation of CDC25 phosphatase allow the removal of the inhibitory phosphorylation from CDK1, which in turn phosphorylate CDC25 for further activation and phosphorylate Wee1 for proteasomal degradation. The Wee1/CDC phosphatase are regulated by another protein called Polo-like kinase 1 (PLK1). The PLK1 composed of two domains (Polo-box domain) and kinase domain, separated by linker domain. Phosphorylation of kinase domain by Aurora A kinase activates PLK1 kinase, which then bind to phospho-site on the target protein through its Polo-box domain, inducing conformational changes in the PLK1 kinase allowing the additional phosphorylation of the target

protein by the kinase domain. The PLK1 add additional phosphorylation, after the initial phosphorylation by CDK1/cyclin B, to CDC25 for full activation and to Wee1 kinase for ubiquitination by F-box E3 ligases and proteasomal degradation. In addition, PLK1 have other cell cycle functions including centrosome maturation, chromosome attachment and cytokinesis.

7. Chapter 6
Maintaining genome stability

7.1 Genomic instability

Genomic instability is the inability of cancer cells to maintain proper number and structure of chromosomes. Genome instability arises if cells initiate mitosis with partially replicated chromosomes, miss-segregated chromosomes or unrepaired DNA-damage such as double strand breaks (DSB). Some DNA aberrations arise *via* physiological processes, such as DNA mismatches occasionally occurring during DNA replication, and DNA strand breaks caused by non-enzymatic methylations, or by reactive-oxygen compounds. DNA damage can be also caused by environmental factors such as ultraviolet light (UV), ionizing radiation (IR), or through DNA-damaging chemicals such as aflatoxins or tobacco products (150,151).

To avoid transmission of genome alterations to daughter cells, cells have evolved checkpoint pathways during cell cycle divisions to arrest cell cycle progression and promote DNA repair or, in case of unrepairable damage, stimulate cell death and/or senescence. In response to DNA damage, cell cycle checkpoints can be activated in G1 phase, in S phase and at the G2/M transition in addition to the spindle assembly check point for chromosome segregation during mitosis. The overall cellular response to DNA damage is known as DNA damage response (DDR), and involves sensor proteins that detect DNA damage and transmit the signal through mediator proteins to downstream effectors that, in turn, arrest cell cycle progression and promote repair on induce apoptosis (reviewed in (152-155) .

7.1.1 ATR DNA damage response signaling pathway

The cellular responses to DNA damage during cell cycle checkpoints are controlled by the sensor proteins ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related). These proteins belong to a family of Ser/Thr protein kinases called phosphoinositide-3 –kinase related kinase (PIKKs), which also include DNA-PK (DNA-dependent protein kinase) that plays an important role during DNA repair (156). The ATR generally recognizes single strand breaks (SSB) and replication stalling during S-phase and G2/M phase of cell cycle. Replication stalling, or single strand breaks result in free single stranded DNA (ssDNA), which is soon coated by RPA. The RPA-ssDNA recruits ATR, where ATR associates with RPA through its targeting subunit ATR interacting protein (ATRIP). DNA damage is recognized independently by a heterotrimeric ring-shaped protein complex related in structure and sequence to PCNA known as the 9-1-1 (Rad9–Hus1–Rad1) checkpoint clamp. As with PCNA, the 9-

1-1 complex is loaded through a damage-specific clamp loader Rad17 and RFC. The 9-1-1 complex associates with TopBP1, which is required for ATR activation (152,157). Activated ATR then phosphorylates an adaptor protein called Claspin, phosphorylated Claspin act to recruit and bring CHK1 kinase to ATR for phosphorylation and activation on $S^{345/317}$. CHK1 acts as the mediator protein in the DNA damage response of ATR signaling, transmitting the signal from ATR to effector proteins. Phosphorylated Claspin also binds to phosphorylated Rad17, which is required for sustained CHK1 phosphorylation. CHK1 being activated leaves the chromatin to the nucleoplasm where it phosphorylates CDC25A and target it for proteasome degradation, or CDC25C and target it for association with 14-3-3 and cytoplasm export. In addition, CHK1 phosphorylates and activates Wee1 kinase. This functions in preventing cell cycle progression by inhibiting CDK2 and CDK1, allowing the cell to repair DNA damage (158).

In addition to inhibiting cell cycle progression, CHK1 participate in homologous recombination by recruiting Rad51 (159), in regulating PCNA monoubiquitination and Translesion synthesis (TLS) pathway (160), discussed further below, and in stabilizing replication fork and/or the restart of replication fork (158).

7.1.2 ATM DNA damage response signaling pathway

ATM exists as inactive homo-dimer, and generally recognizes DSBs through association with Mre11/Rad50/Nbs1 (MRN complex). The association of ATM to MRN complex induces autophosphorylation of ATM and dissociation into active monomers (156). The activated ATM then phosphorylate the mediator protein CHK2 and histone 2X variant known as γH2AX, which act as recruitment platform for mediator of DNA damage checkpoint protein 1 (MDC1). MDC1 acts to amplify the signal by recruiting additional MRN complex which further recruits additional ATM (156).Activated CHK2 then, phosphorylates several nuclear proteins including p53, DNA repair proteins (BRCA1, BRCA2) and cell cycle proteins (CDC25A, CDC25C, RB). This leads to cell cycle arrest by the degradation of CDC25A and CDC25C, or through upregulation of p21 through activation of p53 which then binds to and inhibits CDK/Cyclin complexes (161).

MDC1 is also phosphorylated by ATM which results in recruitment of E3 ligases RNF8 and RNF168 that leads to ubiquitination of histone 2. Histone 2 ubiquitination and histone 4 lysine 20 (H4K20) methylation recruit 53BP1 to the damage site which is further phosphorylated by ATM and act to activate the non-homologous end joining repair pathway (NHEJ) (155). During S phase the elevated CDK activity result in phosphorylation of NBS1 subunit of MRN complex, in addition to the DNA-end processing factor CtIP and BRCA1. Phosphorylated NBS1 binds to CtIP which then recruits and binds

to BRCA1. BRCA1 counteracts and inhibits the action of 53BP1 and activates repair through homologous recombination (155).

Although ATR and ATM are generally known to respond to single and double-strand DNA damage respectively, their signaling pathways are interconnected. For example, double-strand breaks activate ATM, which along with CtIP 5-3' resection activity, generates ssDNA leading to consequent ATR activation (162). On the other hand, ATR activated in response to UV-induced DNA damage activate ATM by phosphorylating ATM at its autophosphorylation site S1981 (163).

7.1.3 PCNA and DNA damage tolerance pathway

The conformation of DNA polymerases does not allow for stable interaction with the template DNA. To strengthen the interaction between DNA template and polymerases, DNA sliding clamp is required (149). In eukaryotes, this sliding clamp is the PCNA, which forms a homotrimer ring-like structure, each monomer contains 2 domains I, comprised of residues 1 to 117, domain II, comprised of residues 135 to 258, connected by the interdomain-connecting loop (IDCL) , comprised of residues 118 to 134 (Fig. 3) (164).

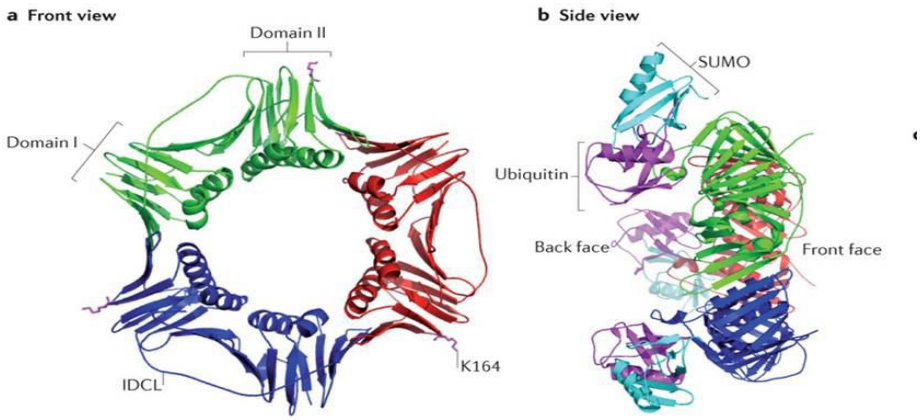

Figure 3. Structure of PCNA. (A) Front view showing Domain I, II and IDCL. (B) Side view showing ubiquitin site (165).

The PCNA homotrimer ring has a front face with C-terminus, where most interactions with DNA polymerases and other proteins occur (166-168) and back face with N-terminus, where K-164 site for ubiquitination and SUMOylation is found (169). Most proteins bind to PCNA through a conserved sequence called PIP (PCNA interacting peptide) box, at the hydrophobic pocket beneath the IDCL on the front face. PIP motifs are sequences of eight amino acids, Q-x-x-J-x-x-w-w, in which Q is a conserved glutamine, J is a moderately aliphatic residue (leucine, isoleucine, or methionine) and w is an

aromatic residue (phenylalanine or tyrosine) (170,171). In addition a second interacting motif termed AlkB homolog 2 PCNA-interacting motif (APIM) is also common among DNA repair proteins (170,171).

7.1.3.1 PCNA and DNA replication

After the formation of the pre-initiation complex during S-phase, see chapter 5, the CMG complex starts unwinding the DNA and the ssDNA soon will be coated with RPA. DNA polymerase α/primase start forming a short DNA/RNA hybrid primer, which is recognized by the RFC that loads the PCNA in the proper orientation. The PCNA acts as the processivity factor (the average number of nucleotides added before dissociation from DNA) for pol ε in the leading strand and for pol δ in the lagging strand (148,149,164,171). In yeast PCNA is SUMOylated on K-164 by the E2/E3 ligases Ubc9/Siz1 (169,172), this prevents recombination during S-phase (173,174).

7.1.3.2 DNA damage tolerance pathway

In addition to DNA replication, PCNA has additional roles in DNA repair and the recently identified DNA damage tolerance pathway. During S-phase, replication-blocking lesions must be repaired or bypassed in order to complete DNA replication. Prolonged stalling of replication forks can lead to fork collapse, DSBs, chromosomal rearrangements and genomic instability (175-177). DNA damage tolerance mechanisms, or post-replication repair processes, allow the cell to replicate over polymerase-blocking lesions. There are three types of DNA-damage tolerance: 1) An error-prone translesion DNA synthesis (TLS), 2) An error-free TLS and 3) An error-free DNA damage silencing (DDS), or template switching. All three pathways require PCNA and depend on the ubiquitination status of PCNA (178,179).

TLS is a conserved process that allows the replication machinery to bypass DNA lesions through the exchange of the high-fidelity DNA polymerases to low-fidelity DNA polymerases (178,179). Unlike the high-fidelity DNA polymerases such as Polα, Polδ and Polε, low-fidelity or Y-family of TLS polymerases including Polη (eta), Polκ (kappa), Polι (iota) and Rev1 contain larger active sites capable of accommodating distorted bases and mismatched base pair (180,181). Thus, the general conception that TLS polymerases are often considered error-prone. However several TLS polymerases have been shown to display proper base pairing opposite to specific lesions (180,181), for example, the Polη preferentially inserts two "A"'s opposite a thymine dimer, a common UV photoproduct (182,183) and Polκ has been shown to accurately bypass benzopyrene-induced guanine adducts (184,185). Thus, TLS can be either mutagenic or accurate, depending on the type of lesion and which TLS polymerase is used (178).

Much less is known about DNA damage silencing or template switching, an error-free form of DNA damage tolerance. As the name implies, template switching is thought to utilize the undamaged sister chromatid as the template to carry out limited DNA replication. Two models for template switching have been proposed, one involving fork regression using the nascent sister chromatid, leading to the formation of what is called chicken foot structure, and the other involving invasion of the sister chromatid in a manner similar to homologous recombination (178,179,186,187).

Interestingly, all DNA damage tolerance pathways require PCNA, and different covalent modifications of PCNA by ubiquitin (Ub) determine which tolerance pathway will be utilized. Mono- and polyubiquitination of PCNA are mediated by two distinct sets of E2–E3 enzymes that operate in a linear fashion (188). PCNA is monoubiquitinated by the E2/E3 RING ligases Rad6/Rad18 on K-164 on the back face of the homotrimer PCNA ring (169,189,190). PCNA monoubiquitination is required for the subsequent polyubiquitination mediated by the E2 Mms2-Ubc13 and the E3 SNF2 histone linker PHD RING helicase (SHPRH), helicase-like transcription factor (HLTF) (equivalent to *S. cerevisiae*. Rad5) ligases (175,178,191-193). Polyubiquitination of PCNA is mediated by the attachment of ubiquitin through the K63-linkages, which is different from the conventional K48-linked ubiquitin chains that target proteins for degradation by the 26S proteasome (194,195). Recent data have showed that polyubiquitination of PCNA functions to prevent the binding of pol δ to PCNA, and thus prevents normal replication (196).

7.1.4 Spindle assembly checkpoint

The spindle assembly checkpoint (SAC) is a mitotic checkpoint that monitors the attachment of chromosomes to the kinetochore spindles and prevents the progression of anaphase until all chromosomes are correctly attached to the bipolar spindle. Upon spindle attachment, Cdc20 and APC ubiquitin ligase are activated, leading to ubiquitination and proteolytic degradation of cyclin B, and securin, which inhibits separase. Following degradation of securin, the activated separase targets cohesin, a protein complex that holds sister chromatids together, causing sister chromatid separation, and, anaphase onset.

7.1.5 Anti-mitotic drugs and mitotic catastrophe

Mitotic catastrophe, as defined in 2012 by the International Nomenclature Committee on Cell Death, is a bona fide intrinsic oncosuppressive mechanism that senses mitotic failure and responds by driving the cell to an irreversible anti-proliferative fate of death or senescence, thus preventing aneuploidy and genome instability. This form of cell death does not require caspase 9 or 3 and can still occur in the presence of caspase inhibitors. Instead it requires CDK1 and BCL-2 family activity (197-201). BCL-2

is a large family of proteins that include prosurvival proteins (Bcl-2, Bcl-$_{XL}$, BCL-$_{W}$, Mcl1, A1, and Bcl-B), proapoptotic effector proteins (Bax, Bak, and Bok) as well as BH3-only proteins (Bim, PUMA, Bad, NOXA, Bik, Hrk, Bmf, and tBid) (202). Active CDK1 phosphorylates Bcl-2, Bcl-$_{XL}$ blocking heterodimer formation with proapoptotic members, Bax and Bak, resulting in oligomerization of the proapoptotic factors at the outer mitochondrial membrane, release of cytochrome C, and thereby apoptosis (203). In addition, CDK1 also phosphorylates Mcl-1 and targets it for ubiquitination and proteasome degradation, leading to proapoptotic factors oligomerization at the outer mitochondrial membrane and apoptosis (198,199).

It is then proposed that during a typical mitotic division, the levels of CDK1 activity are not sufficient enough to promote cell death through phosphorylation and degradation of Mcl-1, due to the degradation of cyclin B by the APC. However, during a mitotic catastrophe, the prolonged mitotic arrest results in prolonged CDK1 activation which is sufficient enough to promote cell death (199). Thus the proposed model that the fate of cells during mitotic catastrophe will be determined through a balance of CDK1 activity and apoptotic signals, leading to three possible anti-proliferative fates: 1) If levels of cyclin B1 are high, resulting in high CDK1 activity, the cell will undergo death in mitosis (204), 2) If the levels of cyclin B start to decline mitotic slippage will occur and the cell will undergo death in G1 phase (205), 3) Cells can undergo senescence after mitotic slippage (199). Although it was shown that caspase activity was not required for mitotic catastrophe or cell death after mitotic slippage (201), cell death can also occur in a caspase-independent manner (206). However, the molecular signals that link these events during mitotic catastrophe remain poorly understood.

Mitotic catastrophe is usually accompanied by prolonged mitotic arrest, and drugs mostly induce mitotic catastrophe through interfering with microtubules. However, due to the side effect of microtubule-targeting agents, including neutropenia, neuropathy, and reversible myelosuppression (207,208), recent research efforts have focused on the development of non-microtubule antimitotic agents, such as those targeting mitotic motor proteins and mitotic kinases (199,200). These can cause mitotic catastrophe through : 1) Abrogating G2 checkpoint or inducing persistent DNA damage during chromosomal segregation, 2) Interfering with spindle formation or mitotic machinery that activate the SAC, 3) Inhibiting cytokinesis that will lead to mitotic catastrophe in the next round of mitosis (199).

7.1.5.1 Microtubule- targeting agents

Microtubules are dynamic structures composed of α and β–tubulin molecules that are constantly integrated by polymerization, named the plus end, or shed off by depolymerization, named the minus end, at both ends of microtubule (209). Drugs that act on microtubules can be divided into two groups: microtubule polymerizing agents that bind to taxoid binding site on beta tubulin, including taxanes (paclitaxel and docetaxel), and the microtubule depolymerizing agents, which bind to one of two domains on tubulin, the "vinca" domain and the "colchicine" domain. The Vinca-domain agents include the vinca alkaloids (vinblastine and vincristine) and the colchicine-domain agents include colchicine and its analogs, podophyllotoxin (207). Both classes of drugs alter microtubule dynamics, thus preventing the formation of normal bipolar spindle and chromosome biorientation, leading to mitotic catastrophe (199,200).

7.1.5.2 Mitotic kinesins

Mitotic kinesins are a group of motor proteins that function exclusively in mitosis and are involved in bi-polar spindle assembly. This includes Eg5, KIFC1 and KIF2A. Eg5 is a plus end motor protein, moves towards the plus end of the microtubule. Active Eg5 is a homotetramer that cross-link and translocate along two adjacent microtubules with each dimer interacting with a single microtubule fiber (210,211). Eg5 creates necessary forces for spindle formation, thus Eg5 inhibition results in the formation of monoaster spindles leading to mitotic arrest and apoptosis or mitotic catastrophe (199,200).

7.1.5.3 Aurora and polo-like kinases

The aurora kinase is a family of serine/threonine protein kinases including Aurora A, Aurora B and Aurora C kinases. Aurora A is localized to centrosomes during interphase and to spindles poles and spindle microtubules during mitosis, where it participates in several processes required for bipolar spindle formation including PLK1 activation, centrosome separation and microtubule dynamics. Aurora B localizes to kinetochores from prophase to metaphase and to the spindle midzone in cytokinesis where it forms a part of the Chromosome Passenger Complex (CPX) that plays critical roles during chromosome segregation and microtubule-to-kinetochore attachments (212,213). Little is known about Aurora C, but it is mainly expressed in the testis. Inhibition of the Aurora B kinase causes mitotic slippage without cytokinesis, causing aneuploidy, and continued inhibition results in very large multiploid cells that eventually undergo apoptosis or senescence (200,214-216). Inhibition of Aurora A kinase causes defects in mitotic spindle assembly, mitotic arrest and apoptosis (212).

Polo-like kinases are yet another family of Serine/Threonine (Ser/Thr) kinases with key roles in mitosis. Mechanistically, PLK1 is activated by Aurora-A, as explained in chapter 5, and functions to promote CDK1 activation, checkpoint recovery and centrosome separation. The inhibition of PLK1 kinase does not block mitotic entry; instead induces a metaphase arrest due to monopolar spindle formation (217).

Results and Discussion

8. Chapter 7

Results and Discussion

8.1 Paper I

PICROPODOPHYLLIN CAUSES MITOTIC ARREST AND CATASTROPHE BY DEPOLYMERIZING MICROTUBULES VIA INSULIN-LIKE GROWTH FACTOR-1 RECEPTOR-INDEPENDENT MECHANISM.

PPP has been demonstrated to inhibit IGF-1R and/or its signaling in numerous cell systems (218,219), and in tumor xenografts (220,221), as well as in granulocytes in treated patients (137). A unique feature for PPP is that it also induces G2/M arrest in treated cells (138). We sought to investigate the mechanism behind PPP-induced G2/M arrest in detail. Using fluorescence microscopy, flow cytometry and time-lapsed video microscopy, we demonstrated the mitotic block of PPP in various cell lines and xenograft, even in *igf1r*$^{-/-}$ cells (R-) cells, but not in normal hepatocytes or normal cells from A549 xenografts, indicating that the mitotic arrest was IGF-1R independent. Trying to reveal the mechanism through which PPP induces the mitotic arrest, we found a high activation of CDK1, increased CDK1 phosphorylation of T161, along with increased expression and association with cyclin B. A prolonged mitotic arrest with increased cyclin B expression represents a new mechanism of cell death called mitotic catastrophe, a caspase independent cell death pathway (199). With this hypothesis in mind, we investigated the apoptotic effect of PPP and found it to be independent of caspases, but occurred through inhibition of MCL-1, as has been reported for mitotic catastrophe (198). We also found PPP to interfere with centrosome and mitotic spindle formation during mitosis. This effect was shown to be independent from PLK1 kinase or Eg5 kinesin but through interference with microtubule dynamics. However, PPP was shown to not bind to microtubules. This is compatible with results from clinical studies, in which PPP has not shown any neurotoxicity. Neurotoxicity is a quite common and serious adverse effect of microtubule inhibitors used in cancer therapy.

8.2 Paper II

SUMO-MODIFIED INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR (IGF-1R) INCREASES CELL CYCLE PROGRESSION AND CELL PROLIFERATION.

IGF-1R has been shown to be SUMOylated on three lysine residues (Lys1025, Lys1100 and Lys1120), and this SUMOylation was shown to be important for the nuclear translocation of the receptor (90,92), where it can bind to several enhancer regions and regulate gene expression (96,108). Nuclear IGF-1R has been associated with many types of cancers including lung, breast, and prostate cancers, and hepatocyte carcinoma (89,93), suggesting a possible role for nuclear IGF-1R in regulating tumorigenicity. To verify such hypothesis, we investigated the effect of IGF-1R SUMOylation and nuclear translocation on cellular proliferation.

We transfected *igf1r*$^{-/-}$ knockout cell line with either wild type (WT), triple SUMOylated mutant *IGF1R* (TSM) or empty plasmid (R-), and used these cell lines as a model in our study. We showed that both the WT and TSM cell line could maintain same level of AKT and ERK phosphorylation, indicating that the canonical pathway of IGF-1R was not affected. However, we found an unexpected but low level of TSM translocation to the nucleus, which could be mediated by dimerization to insulin receptor (IR), as shown by co-immunoprecipitation (co-IP). We found that WT transfected cell line had higher proliferation rate than the TSM or the R- cells, and that this effect was not mediated through difference in cell death. To investigate the mechanisms behind the higher proliferation rate in WT cells, cell cycle progression was followed after cellular synchronization using flowcytometry. We found that WT cells have a much higher G1/S progression. This was shown to be due to increased G1 phase cyclin D expression, followed by increased expression of G1/S CDK2 and cyclins A and B, along with increased inhibition of p27, suggesting a potential role for SUMO/nuclear IGF-1R in regulating cell cycle.

8.3 Paper III

INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR PHOSPHORYLATES PROLIFERATING NUCLEAR ANTIGEN AND RESCUES STALLED REPLICATION FORKS AFTER DNA DAMAGE.

In this study, we tried to identify novel binding partners of nuclear IGF-1R. Initially we made co-IP of IGF-1R from the hESC line WA01, and the associated proteins were identified by mass spectrometry. PCNA was identified as one of main proteins binding to IGF-1R, which was validated in several cell types by co-IP after cell fractionation. Using *igf1r*$^{-/-}$ knockout mouse cells (R-) and an R- stable transfectant with *IGF1R* (R+), we found that PCNA from R+ was tyrosine phosphorylated and ubiquitinated but not PCNA from R- cells. IGF-1R mediated PCNA ubiquitination was correlated to recruitment of E2/E3 ligases mediating DDT (Rad18, UBC13/SHPRH, HLTF) (178,188,222,223). We investigated the effect of DNA damage, using ultraviolet radiation (UV) and methyl methanesulfonate (MMS) on PCNA ubiquitination. While it was found to be absent in R- , PCNA ubiquitination was strongly increased in R+ cells upon DNA damage. However, this response disappeared/decreased in R+ cells after treatment with an IGF-1R inhibitor. A potential role of IGF-1R in the DDT pathway was further supported by results from replication fork restart analysis using DNA fiber assay and S-phase progression using cell cycle analysis of DNA damage-exposed R+ and R- cells.

9. Chapter 8
Concluding Remarks

Since the 90s IGF-1R has been increasingly reported to be important for cancer cell growth and survival, as well as the receptor exhibits substantial expression in malignant tissues. This has raised expectations of its use as a target for cancer therapy. Accordingly, several pharmaceutical companies started producing specific antibodies against the receptor. Several phase 3 clinical trials were performed, but all of them reported disappointed results (224). One explanation could be that the antibodies can only target IGF-1R localized on the cell membrane. Cell membrane IGF-1R activates the so-called canonical pathways, which other receptor tyrosine kinases also do. However, recent discovery of and studies on nuclear IGF-1R have provided new insights into actions of IGF-1R.

The mechanisms by which PPP causes G2/M arrest were here characterized in detail. PPP induced mitotic arrest causing mitotic catastrophe. These events were confirmed to be independent to IGF-1R. The mitotic arrest and catastrophe induced by PPP may potentiate its anti-cancer efficacy, without leading to irreversible adverse effects.

SUMOylation of IGF-1R has earlier been shown to be required for nuclear IGF-1R's transactivating effect. Here it is shown that SUMOylation of IGF-1R increases cell proliferation through directing expression of cyclins and related cell cycle proteins. Evidence that nuclear IGF-1R regulates DNA damage tolerance pathway through posttranslational modifications of PCNA is also provided.

These identified novel properties of nuclear IGF-1R support involvement of new mechanisms regulating cell proliferation and genetic stabilization, independent to the canonical IGF-1R pathways. Such mechanisms may contribute to increased understanding of IGF-1R actions in cancer and possibly in development of improved therapy. The mitotic block and catastrophe induced by PPP may explain a part of its antitumor efficacy.

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References

1. Hanahan, D., and Weinberg, R. A. (2000) The hallmarks of cancer. *Cell* **100**, 57-70
2. Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of cancer: the next generation. *Cell* **144**, 646-674
3. Lemmon, M. A., and Schlessinger, J. (2010) Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117-1134
4. Treadway, J. L., Morrison, B. D., Goldfine, I. D., and Pessin, J. E. (1989) Assembly of insulin/insulin-like growth factor-1 hybrid receptors in vitro. *J Biol Chem* **264**, 21450-21453
5. Frattali, A. L., Treadway, J. L., and Pessin, J. E. (1992) Insulin/IGF-1 hybrid receptors: implications for the dominant-negative phenotype in syndromes of insulin resistance. *Journal of cellular biochemistry* **48**, 43-50
6. Steiner, D. F., and Oyer, P. E. (1967) The biosynthesis of insulin and a probable precursor of insulin by a human islet cell adenoma. *Proc Natl Acad Sci U S A* **57**, 473-480
7. Wing, S. S. (2008) The UPS in diabetes and obesity. *BMC Biochem* **9 Suppl 1**, S6
8. Giorgino, F., Belfiore, A., Milazzo, G., Costantino, A., Maddux, B., Whittaker, J., Goldfine, I. D., and Vigneri, R. (1991) Overexpression of insulin receptors in fibroblast and ovary cells induces a ligand-mediated transformed phenotype. *Mol Endocrinol* **5**, 452-459
9. Frasca, F., Pandini, G., Scalia, P., Sciacca, L., Mineo, R., Costantino, A., Goldfine, I. D., Belfiore, A., and Vigneri, R. (1999) Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. *Molecular and cellular biology* **19**, 3278-3288
10. Seino, S., and Bell, G. I. (1989) Alternative splicing of human insulin receptor messenger RNA. *Biochemical and biophysical research communications* **159**, 312-316
11. Sara, V. R., and Hall, K. (1990) Insulin-like growth factors and their binding proteins. *Physiol Rev* **70**, 591-614
12. Yu, H., and Rohan, T. (2000) Role of the insulin-like growth factor family in cancer development and progression. *Journal of the National Cancer Institute* **92**, 1472-1489
13. Daughaday, W. H., and Rotwein, P. (1989) Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr Rev* **10**, 68-91
14. Furstenberger, G., and Senn, H. J. (2002) Insulin-like growth factors and cancer. *Lancet Oncol* **3**, 298-302
15. Bach, L. A. (1999) The insulin-like growth factor system: basic and clinical aspects. *Aust N Z J Med* **29**, 355-361
16. Le Roith, D. (2003) The insulin-like growth factor system. *Exp Diabesity Res* **4**, 205-212
17. Juul, A. (2003) Serum levels of insulin-like growth factor I and its binding proteins in health and disease. *Growth Horm IGF Res* **13**, 113-170
18. Paulsen, I. T., Sliwinski, M. K., and Saier, M. H., Jr. (1998) Microbial genome analyses: global comparisons of transport capabilities based on phylogenies, bioenergetics and substrate specificities. *J Mol Biol* **277**, 573-592
19. Rosenfeld, R. G., Kofoed, E., Little, B., Woods, K., Buckway, C., Pratt, K., and Hwa, V. (2004) Growth hormone insensitivity resulting from post-GH receptor defects. *Growth Horm IGF Res* **14 Suppl A**, S35-38
20. DeChiara, T. M., Robertson, E. J., and Efstratiadis, A. (1991) Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* **64**, 849-859
21. Bennett, A., Wilson, D. M., Liu, F., Nagashima, R., Rosenfeld, R. G., and Hintz, R. L. (1983) Levels of insulin-like growth factors I and II in human cord blood. *J Clin Endocrinol Metab* **57**, 609-612
22. Baker, J., Liu, J. P., Robertson, E. J., and Efstratiadis, A. (1993) Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* **75**, 73-82
23. Baxter, R. C. (1994) Insulin-like growth factor binding proteins in the human circulation: a review. *Horm Res* **42**, 140-144
24. Rajah, R., Khare, A., Lee, P. D., and Cohen, P. (1999) Insulin-like growth factor-binding protein-3 is partially responsible for high-serum-induced apoptosis in PC-3 prostate cancer cells. *J Endocrinol* **163**, 487-494

25. Zapf, J. (1995) Physiological role of the insulin-like growth factor binding proteins. *Eur J Endocrinol* **132**, 645-654
26. LeRoith, D., and Roberts, C. T., Jr. (2003) The insulin-like growth factor system and cancer. *Cancer letters* **195**, 127-137
27. Baxter, R. C. (2000) Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *American journal of physiology. Endocrinology and metabolism* **278**, E967-976
28. Boisclair, Y. R., Hurst, K. R., Ueki, I., Tremblay, M. L., and Ooi, G. T. (2000) Regulation and role of the acid-labile subunit of the 150-kilodalton insulin-like growth factor complex in the mouse. *Pediatr Nephrol* **14**, 562-566
29. Sepp-Lorenzino, L. (1998) Structure and function of the insulin-like growth factor I receptor. *Breast Cancer Res Treat* **47**, 235-253
30. Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., and et al. (1986) Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J* **5**, 2503-2512
31. Carlberg, M., Dricu, A., Blegen, H., Wang, M., Hjertman, M., Zickert, P., Hoog, A., and Larsson, O. (1996) Mevalonic acid is limiting for N-linked glycosylation and translocation of the insulin-like growth factor-1 receptor to the cell surface. Evidence for a new link between 3-hydroxy-3-methylglutaryl-coenzyme a reductase and cell growth. *The Journal of biological chemistry* **271**, 17453-17462
32. Jansson, M., Hallen, D., Koho, H., Andersson, G., Berghard, L., Heidrich, J., Nyberg, E., Uhlen, M., Kordel, J., and Nilsson, B. (1997) Characterization of ligand binding of a soluble human insulin-like growth factor I receptor variant suggests a ligand-induced conformational change. *J Biol Chem* **272**, 8189-8197
33. Wang, M., Xie, Y., Girnita, L., Nilsson, G., Dricu, A., Wejde, J., and Larsson, O. (1999) Regulatory role of mevalonate and N-linked glycosylation in proliferation and expression of the EWS/FLI-1 fusion protein in Ewing's sarcoma cells. *Experimental cell research* **246**, 38-46
34. Massague, J., and Czech, M. P. (1982) The subunit structures of two distinct receptors for insulin-like growth factors I and II and their relationship to the insulin receptor. *J Biol Chem* **257**, 5038-5045
35. Ward, C. W., Garrett, T. P., McKern, N. M., Lou, M., Cosgrove, L. J., Sparrow, L. G., Frenkel, M. J., Hoyne, P. A., Elleman, T. C., Adams, T. E., Lovrecz, G. O., Lawrence, L. J., and Tulloch, P. A. (2001) The three dimensional structure of the type I insulin-like growth factor receptor. *Mol Pathol* **54**, 125-132
36. Lawrence, M. C., McKern, N. M., and Ward, C. W. (2007) Insulin receptor structure and its implications for the IGF-1 receptor. *Current opinion in structural biology* **17**, 699-705
37. Malaguarnera, R., and Belfiore, A. (2011) The insulin receptor: a new target for cancer therapy. *Frontiers in endocrinology* **2**, 93
38. Martin-Kleiner, I., and Gall Troselj, K. (2010) Mannose-6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) in carcinogenesis. *Cancer letters* **289**, 11-22
39. Brown, J., Delaine, C., Zaccheo, O. J., Siebold, C., Gilbert, R. J., van Boxel, G., Denley, A., Wallace, J. C., Hassan, A. B., Forbes, B. E., and Jones, E. Y. (2008) Structure and functional analysis of the IGF-II/IGF2R interaction. *The EMBO journal* **27**, 265-276
40. Brown, J., Jones, E. Y., and Forbes, B. E. (2009) Keeping IGF-II under control: lessons from the IGF-II-IGF2R crystal structure. *Trends in biochemical sciences* **34**, 612-619
41. Baserga, R. (2000) The contradictions of the insulin-like growth factor 1 receptor. *Oncogene* **19**, 5574-5581
42. Gronborg, M., Wulff, B. S., Rasmussen, J. S., Kjeldsen, T., and Gammeltoft, S. (1993) Structure-function relationship of the insulin-like growth factor-I receptor tyrosine kinase. *J Biol Chem* **268**, 23435-23440
43. Coppola, D., Ferber, A., Miura, M., Sell, C., D'Ambrosio, C., Rubin, R., and Baserga, R. (1994) A functional insulin-like growth factor I receptor is required for the mitogenic and transforming activities of the epidermal growth factor receptor. *Mol Cell Biol* **14**, 4588-4595
44. Li, S., Ferber, A., Miura, M., and Baserga, R. (1994) Mitogenicity and transforming activity of the insulin-like growth factor-I receptor with mutations in the tyrosine kinase domain. *J Biol Chem* **269**, 32558-32564

45. Wang, L. M., Myers, M. G., Jr., Sun, X. J., Aaronson, S. A., White, M., and Pierce, J. H. (1993) IRS-1: essential for insulin- and IL-4-stimulated mitogenesis in hematopoietic cells. *Science* **261**, 1591-1594
46. Vanhaesebroeck, B., Leevers, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J., and Waterfield, M. D. (2001) Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem* **70**, 535-602
47. Sarbassov, D. D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**, 1098-1101
48. Feng, J., Park, J., Cron, P., Hess, D., and Hemmings, B. A. (2004) Identification of a PKB/Akt hydrophobic motif Ser-473 kinase as DNA-dependent protein kinase. *The Journal of biological chemistry* **279**, 41189-41196
49. Zheng, W. H., Kar, S., and Quirion, R. (2002) Insulin-like growth factor-1-induced phosphorylation of transcription factor FKHRL1 is mediated by phosphatidylinositol 3-kinase/Akt kinase and role of this pathway in insulin-like growth factor-1-induced survival of cultured hippocampal neurons. *Mol Pharmacol* **62**, 225-233
50. Leinninger, G. M., Backus, C., Uhler, M. D., Lentz, S. I., and Feldman, E. L. (2004) Phosphatidylinositol 3-kinase and Akt effectors mediate insulin-like growth factor-I neuroprotection in dorsal root ganglia neurons. *FASEB J* **18**, 1544-1546
51. Bouchard, V. J., Rouleau, M., and Poirier, G. G. (2003) PARP-1, a determinant of cell survival in response to DNA damage. *Exp Hematol* **31**, 446-454
52. Mayo, L. D., and Donner, D. B. (2001) A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci U S A* **98**, 11598-11603
53. Dupont, J., Pierre, A., Froment, P., and Moreau, C. (2003) The insulin-like growth factor axis in cell cycle progression. *Horm Metab Res* **35**, 740-750
54. Blume-Jensen, P., and Hunter, T. (2001) Oncogenic kinase signalling. *Nature* **411**, 355-365
55. Viglietto, G., Motti, M. L., Bruni, P., Melillo, R. M., D'Alessio, A., Califano, D., Vinci, F., Chiappetta, G., Tsichlis, P., Bellacosa, A., Fusco, A., and Santoro, M. (2002) Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. *Nat Med* **8**, 1136-1144
56. Zhou, B. P., Liao, Y., Xia, W., Spohn, B., Lee, M. H., and Hung, M. C. (2001) Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat Cell Biol* **3**, 245-252
57. Chitnis, M. M., Yuen, J. S., Protheroe, A. S., Pollak, M., and Macaulay, V. M. (2008) The type 1 insulin-like growth factor receptor pathway. *Clinical cancer research : an official journal of the American Association for Cancer Research* **14**, 6364-6370
58. Bar-Sagi, D. (1994) The Sos (Son of sevenless) protein. *Trends Endocrinol Metab* **5**, 165-169
59. Molina, J. R., and Adjei, A. A. (2006) The Ras/Raf/MAPK pathway. *J Thorac Oncol* **1**, 7-9
60. Robbins, D. J., Cheng, M., Zhen, E., Vanderbilt, C. A., Feig, L. A., and Cobb, M. H. (1992) Evidence for a Ras-dependent extracellular signal-regulated protein kinase (ERK) cascade. *Proc Natl Acad Sci U S A* **89**, 6924-6928
61. Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H., and Yancopoulos, G. D. (1991) ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* **65**, 663-675
62. Seger, R., and Krebs, E. G. (1995) The MAPK signaling cascade. *FASEB J* **9**, 726-735
63. Lenormand, P., Brondello, J. M., Brunet, A., and Pouyssegur, J. (1998) Growth factor-induced p42/p44 MAPK nuclear translocation and retention requires both MAPK activation and neosynthesis of nuclear anchoring proteins. *J Cell Biol* **142**, 625-633
64. Michaud, N. R., Fabian, J. R., Mathes, K. D., and Morrison, D. K. (1995) 14-3-3 is not essential for Raf-1 function: identification of Raf-1 proteins that are biologically activated in a 14-3-3- and Ras-independent manner. *Mol Cell Biol* **15**, 3390-3397
65. Morrison, D. K., and Cutler, R. E. (1997) The complexity of Raf-1 regulation. *Curr Opin Cell Biol* **9**, 174-179

66. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* **87**, 619-628
67. Goh, L. K., and Sorkin, A. (2013) Endocytosis of receptor tyrosine kinases. *Cold Spring Harbor perspectives in biology* **5**, a017459
68. Chen, M. K., and Hung, M. C. (2015) Proteolytic cleavage, trafficking, and functions of nuclear receptor tyrosine kinases. *The FEBS journal* **282**, 3693-3721
69. Kazazic, M., Bertelsen, V., Pedersen, K. W., Vuong, T. T., Grandal, M. V., Rodland, M. S., Traub, L. M., Stang, E., and Madshus, I. H. (2009) Epsin 1 is involved in recruitment of ubiquitinated EGF receptors into clathrin-coated pits. *Traffic* **10**, 235-245
70. Sigismund, S., Woelk, T., Puri, C., Maspero, E., Tacchetti, C., Transidico, P., Di Fiore, P. P., and Polo, S. (2005) Clathrin-independent endocytosis of ubiquitinated cargos. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 2760-2765
71. Miyake, S., Mullane-Robinson, K. P., Lill, N. L., Douillard, P., and Band, H. (1999) Cbl-mediated negative regulation of platelet-derived growth factor receptor-dependent cell proliferation. A critical role for Cbl tyrosine kinase-binding domain. *The Journal of biological chemistry* **274**, 16619-16628
72. Duval, M., Bedard-Goulet, S., Delisle, C., and Gratton, J. P. (2003) Vascular endothelial growth factor-dependent down-regulation of Flk-1/KDR involves Cbl-mediated ubiquitination. Consequences on nitric oxide production from endothelial cells. *The Journal of biological chemistry* **278**, 20091-20097
73. Penengo, L., Rubin, C., Yarden, Y., and Gaudino, G. (2003) c-Cbl is a critical modulator of the Ron tyrosine kinase receptor. *Oncogene* **22**, 3669-3679
74. Marmor, M. D., and Yarden, Y. (2004) Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. *Oncogene* **23**, 2057-2070
75. Zeng, S., Xu, Z., Lipkowitz, S., and Longley, J. B. (2005) Regulation of stem cell factor receptor signaling by Cbl family proteins (Cbl-b/c-Cbl). *Blood* **105**, 226-232
76. Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S., and Sorkin, A. (2006) Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. *Molecular cell* **21**, 737-748
77. Girnita, L., Girnita, A., and Larsson, O. (2003) Mdm2-dependent ubiquitination and degradation of the insulin-like growth factor 1 receptor. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 8247-8252
78. Sehat, B., Andersson, S., Girnita, L., and Larsson, O. (2008) Identification of c-Cbl as a new ligase for insulin-like growth factor-I receptor with distinct roles from Mdm2 in receptor ubiquitination and endocytosis. *Cancer research* **68**, 5669-5677
79. Vasilcanu, R., Vasilcanu, D., Rosengren, L., Natalishvili, N., Sehat, B., Yin, S., Girnita, A., Axelson, M., Girnita, L., and Larsson, O. (2008) Picropodophyllin induces downregulation of the insulin-like growth factor 1 receptor: potential mechanistic involvement of Mdm2 and beta-arrestin1. *Oncogene* **27**, 1629-1638
80. Vecchione, A., Marchese, A., Henry, P., Rotin, D., and Morrione, A. (2003) The Grb10/Nedd4 complex regulates ligand-induced ubiquitination and stability of the insulin-like growth factor I receptor. *Molecular and cellular biology* **23**, 3363-3372
81. Haugsten, E. M., Malecki, J., Bjorklund, S. M., Olsnes, S., and Wesche, J. (2008) Ubiquitination of fibroblast growth factor receptor 1 is required for its intracellular sorting but not for its endocytosis. *Molecular biology of the cell* **19**, 3390-3403
82. Mao, Y., Shang, Y., Pham, V. C., Ernst, J. A., Lill, J. R., Scales, S. J., and Zha, J. (2011) Polyubiquitination of insulin-like growth factor I receptor (IGF-IR) activation loop promotes antibody-induced receptor internalization and down-regulation. *The Journal of biological chemistry* **286**, 41852-41861
83. Massie, C., and Mills, I. G. (2006) The developing role of receptors and adaptors. *Nature reviews. Cancer* **6**, 403-409
84. Wang, Y. N., Yamaguchi, H., Hsu, J. M., and Hung, M. C. (2010) Nuclear trafficking of the epidermal growth factor receptor family membrane proteins. *Oncogene* **29**, 3997-4006
85. Wang, Y. N., and Hung, M. C. (2012) Nuclear functions and subcellular trafficking mechanisms of the epidermal growth factor receptor family. *Cell & bioscience* **2**, 13

86. Stachowiak, M. K., Maher, P. A., and Stachowiak, E. K. (2007) Integrative nuclear signaling in cell development--a role for FGF receptor-1. *DNA and cell biology* **26**, 811-826
87. Wang, Y. N., Lee, H. H., Lee, H. J., Du, Y., Yamaguchi, H., and Hung, M. C. (2012) Membrane-bound trafficking regulates nuclear transport of integral epidermal growth factor receptor (EGFR) and ErbB-2. *The Journal of biological chemistry* **287**, 16869-16879
88. Carpenter, G., and Liao, H. J. (2013) Receptor tyrosine kinases in the nucleus. *Cold Spring Harbor perspectives in biology* **5**, a008979
89. Deng, H., Lin, Y., Badin, M., Vasilcanu, D., Stromberg, T., Jernberg-Wiklund, H., Sehat, B., and Larsson, O. (2011) Over-accumulation of nuclear IGF-1 receptor in tumor cells requires elevated expression of the receptor and the SUMO-conjugating enzyme Ubc9. *Biochemical and biophysical research communications* **404**, 667-671
90. Sehat, B., Tofigh, A., Lin, Y., Trocme, E., Liljedahl, U., Lagergren, J., and Larsson, O. (2010) SUMOylation mediates the nuclear translocation and signaling of the IGF-1 receptor. *Science signaling* **3**, ra10
91. Packham, S., Warsito, D., Lin, Y., Sadi, S., Karlsson, R., Sehat, B., and Larsson, O. (2015) Nuclear translocation of IGF-1R via p150(Glued) and an importin-beta/RanBP2-dependent pathway in cancer cells. *Oncogene* **34**, 2227-2238
92. Warsito, D., Sjostrom, S., Andersson, S., Larsson, O., and Sehat, B. (2012) Nuclear IGF1R is a transcriptional co-activator of LEF1/TCF. *EMBO reports* **13**, 244-250
93. Aleksic, T., Chitnis, M. M., Perestenko, O. V., Gao, S., Thomas, P. H., Turner, G. D., Protheroe, A. S., Howarth, M., and Macaulay, V. M. (2010) Type 1 insulin-like growth factor receptor translocates to the nucleus of human tumor cells. *Cancer research* **70**, 6412-6419
94. McElroy, B., Powell, J. C., and McCarthy, J. V. (2007) The insulin-like growth factor 1 (IGF-1) receptor is a substrate for gamma-secretase-mediated intramembrane proteolysis. *Biochemical and biophysical research communications* **358**, 1136-1141
95. Kasuga, K., Kaneko, H., Nishizawa, M., Onodera, O., and Ikeuchi, T. (2007) Generation of intracellular domain of insulin receptor tyrosine kinase by gamma-secretase. *Biochemical and biophysical research communications* **360**, 90-96
96. Warsito, D., Lin, Y., Gnirck, A. C., Sehat, B., and Larsson, O. (2016) Nuclearly translocated insulin-like growth factor 1 receptor phosphorylates histone H3 at tyrosine 41 and induces SNAI2 expression via Brg1 chromatin remodeling protein. *Oncotarget* **7**, 42288-42302
97. Chou, R. H., Wang, Y. N., Hsieh, Y. H., Li, L. Y., Xia, W., Chang, W. C., Chang, L. C., Cheng, C. C., Lai, C. C., Hsu, J. L., Chang, W. J., Chiang, S. Y., Lee, H. J., Liao, H. W., Chuang, P. H., Chen, H. Y., Wang, H. L., Kuo, S. C., Chen, C. H., Yu, Y. L., and Hung, M. C. (2014) EGFR modulates DNA synthesis and repair through Tyr phosphorylation of histone H4. *Developmental cell* **30**, 224-237
98. Hu, Y., Fang, X., Dunham, S. M., Prada, C., Stachowiak, E. K., and Stachowiak, M. K. (2004) 90-kDa ribosomal S6 kinase is a direct target for the nuclear fibroblast growth factor receptor 1 (FGFR1): role in FGFR1 signaling. *The Journal of biological chemistry* **279**, 29325-29335
99. Lee, H. J., Lan, L., Peng, G., Chang, W. C., Hsu, M. C., Wang, Y. N., Cheng, C. C., Wei, L., Nakajima, S., Chang, S. S., Liao, H. W., Chen, C. H., Lavin, M., Ang, K. K., Lin, S. Y., and Hung, M. C. (2015) Tyrosine 370 phosphorylation of ATM positively regulates DNA damage response. *Cell research* **25**, 225-236
100. Ortega, J., Li, J. Y., Lee, S., Tong, D., Gu, L., and Li, G. M. (2015) Phosphorylation of PCNA by EGFR inhibits mismatch repair and promotes misincorporation during DNA synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 5667-5672
101. Wang, S. C., Nakajima, Y., Yu, Y. L., Xia, W., Chen, C. T., Yang, C. C., McIntush, E. W., Li, L. Y., Hawke, D. H., Kobayashi, R., and Hung, M. C. (2006) Tyrosine phosphorylation controls PCNA function through protein stability. *Nature cell biology* **8**, 1359-1368
102. Beguelin, W., Diaz Flaque, M. C., Proietti, C. J., Cayrol, F., Rivas, M. A., Tkach, M., Rosemblit, C., Tocci, J. M., Charreau, E. H., Schillaci, R., and Elizalde, P. V. (2010) Progesterone receptor induces ErbB-2 nuclear translocation to promote breast cancer growth via a novel transcriptional effect: ErbB-2 function as a coactivator of Stat3. *Molecular and cellular biology* **30**, 5456-5472
103. Andrique, L., Fauvin, D., El Maassarani, M., Colasson, H., Vannier, B., and Seite, P. (2012) ErbB3(80 kDa), a nuclear variant of the ErbB3 receptor, binds to the Cyclin D1 promoter to activate cell proliferation but is negatively controlled by p14ARF. *Cellular signalling* **24**, 1074-1085

104. Lo, H. W., Cao, X., Zhu, H., and Ali-Osman, F. (2010) Cyclooxygenase-2 is a novel transcriptional target of the nuclear EGFR-STAT3 and EGFRvIII-STAT3 signaling axes. *Molecular cancer research : MCR* **8**, 232-245
105. Wang, S. C., Lien, H. C., Xia, W., Chen, I. F., Lo, H. W., Wang, Z., Ali-Seyed, M., Lee, D. F., Bartholomeusz, G., Ou-Yang, F., Giri, D. K., and Hung, M. C. (2004) Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. *Cancer cell* **6**, 251-261
106. Hung, L. Y., Tseng, J. T., Lee, Y. C., Xia, W., Wang, Y. N., Wu, M. L., Chuang, Y. H., Lai, C. H., and Chang, W. C. (2008) Nuclear epidermal growth factor receptor (EGFR) interacts with signal transducer and activator of transcription 5 (STAT5) in activating Aurora-A gene expression. *Nucleic acids research* **36**, 4337-4351
107. Lo, H. W., Hsu, S. C., Ali-Seyed, M., Gunduz, M., Xia, W., Wei, Y., Bartholomeusz, G., Shih, J. Y., and Hung, M. C. (2005) Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. *Cancer cell* **7**, 575-589
108. Lin, Y., Liu, H., Waraky, A., Haglund, F., Agarwal, P., Jernberg-Wiklund, H., Warsito, D., and Larsson, O. (2017) SUMO-modified insulin-like growth factor 1 receptor (IGF-1R) increases cell cycle progression and cell proliferation. *Journal of cellular physiology* **232**, 2722-2730
109. Gerber, D. E. (2008) Targeted therapies: a new generation of cancer treatments. *American family physician* **77**, 311-319
110. Joo, W. D., Visintin, I., and Mor, G. (2013) Targeted cancer therapy--are the days of systemic chemotherapy numbered? *Maturitas* **76**, 308-314
111. Baudino, T. A. (2015) Targeted Cancer Therapy: The Next Generation of Cancer Treatment. *Current drug discovery technologies* **12**, 3-20
112. Takeuchi, K., and Ito, F. (2011) Receptor tyrosine kinases and targeted cancer therapeutics. *Biological & pharmaceutical bulletin* **34**, 1774-1780
113. Gschwind, A., Fischer, O. M., and Ullrich, A. (2004) The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nature reviews. Cancer* **4**, 361-370
114. Zwick, E., Bange, J., and Ullrich, A. (2001) Receptor tyrosine kinase signalling as a target for cancer intervention strategies. *Endocrine-related cancer* **8**, 161-173
115. Regad, T. (2015) Targeting RTK Signaling Pathways in Cancer. *Cancers* **7**, 1758-1784
116. Raval, S. H., Singh, R. D., Joshi, D. V., Patel, H. B., and Mody, S. K. (2016) Recent developments in receptor tyrosine kinases targeted anticancer therapy. *Veterinary world* **9**, 80-90
117. Pao, W., and Chmielecki, J. (2010) Rational, biologically based treatment of EGFR-mutant non-small-cell lung cancer. *Nature reviews. Cancer* **10**, 760-774
118. Greulich, H., Kaplan, B., Mertins, P., Chen, T. H., Tanaka, K. E., Yun, C. H., Zhang, X., Lee, S. H., Cho, J., Ambrogio, L., Liao, R., Imielinski, M., Banerji, S., Berger, A. H., Lawrence, M. S., Zhang, J., Pho, N. H., Walker, S. R., Winckler, W., Getz, G., Frank, D., Hahn, W. C., Eck, M. J., Mani, D. R., Jaffe, J. D., Carr, S. A., Wong, K. K., and Meyerson, M. (2012) Functional analysis of receptor tyrosine kinase mutations in lung cancer identifies oncogenic extracellular domain mutations of ERBB2. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 14476-14481
119. Hirota, S., Isozaki, K., Moriyama, Y., Hashimoto, K., Nishida, T., Ishiguro, S., Kawano, K., Hanada, M., Kurata, A., Takeda, M., Muhammad Tunio, G., Matsuzawa, Y., Kanakura, Y., Shinomura, Y., and Kitamura, Y. (1998) Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. *Science* **279**, 577-580
120. Drake, J. M., Lee, J. K., and Witte, O. N. (2014) Clinical targeting of mutated and wild-type protein tyrosine kinases in cancer. *Molecular and cellular biology* **34**, 1722-1732
121. Berns, E. M., Klijn, J. G., van Staveren, I. L., Portengen, H., and Foekens, J. A. (1992) Sporadic amplification of the insulin-like growth factor 1 receptor gene in human breast tumors. *Cancer research* **52**, 1036-1039
122. Chen, H. X., and Sharon, E. (2013) IGF-1R as an anti-cancer target--trials and tribulations. *Chinese journal of cancer* **32**, 242-252
123. Przybyl, J., Jurkowska, M., Rutkowski, P., Debiec-Rychter, M., and Siedlecki, J. A. (2012) Downstream and intermediate interactions of synovial sarcoma-associated fusion oncoproteins and their implication for targeted therapy. *Sarcoma* **2012**, 249219

124. Gee, J. M., Robertson, J. F., Gutteridge, E., Ellis, I. O., Pinder, S. E., Rubini, M., and Nicholson, R. I. (2005) Epidermal growth factor receptor/HER2/insulin-like growth factor receptor signalling and oestrogen receptor activity in clinical breast cancer. *Endocrine-related cancer* **12 Suppl 1**, S99-S111
125. Shin, D. H., Min, H. Y., El-Naggar, A. K., Lippman, S. M., Glisson, B., and Lee, H. Y. (2011) Akt/mTOR counteract the antitumor activities of cixutumumab, an anti-insulin-like growth factor I receptor monoclonal antibody. *Molecular cancer therapeutics* **10**, 2437-2448
126. Bodzin, A. S., Wei, Z., Hurtt, R., Gu, T., and Doria, C. (2012) Gefitinib resistance in HCC mahlavu cells: upregulation of CD133 expression, activation of IGF-1R signaling pathway, and enhancement of IGF-1R nuclear translocation. *Journal of cellular physiology* **227**, 2947-2952
127. You, L., Liu, C., Tang, H., Liao, Y., and Fu, S. (2014) Advances in targeting insulin-like growth factor signaling pathway in cancer treatment. *Current pharmaceutical design* **20**, 2899-2911
128. Pappo, A. S., Patel, S. R., Crowley, J., Reinke, D. K., Kuenkele, K. P., Chawla, S. P., Toner, G. C., Maki, R. G., Meyers, P. A., Chugh, R., Ganjoo, K. N., Schuetze, S. M., Juergens, H., Leahy, M. G., Geoerger, B., Benjamin, R. S., Helman, L. J., and Baker, L. H. (2011) R1507, a monoclonal antibody to the insulin-like growth factor 1 receptor, in patients with recurrent or refractory Ewing sarcoma family of tumors: results of a phase II Sarcoma Alliance for Research through Collaboration study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **29**, 4541-4547
129. Naing, A., LoRusso, P., Fu, S., Hong, D. S., Anderson, P., Benjamin, R. S., Ludwig, J., Chen, H. X., Doyle, L. A., and Kurzrock, R. (2012) Insulin growth factor-receptor (IGF-1R) antibody cixutumumab combined with the mTOR inhibitor temsirolimus in patients with refractory Ewing's sarcoma family tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research* **18**, 2625-2631
130. Weickhardt, A., Doebele, R., Oton, A., Lettieri, J., Maxson, D., Reynolds, M., Brown, A., Jackson, M. K., Dy, G., Adjei, A., Fetterly, G., Lu, X., Franklin, W., Varella-Garcia, M., Hirsch, F. R., Wynes, M. W., Youssoufian, H., Adjei, A., and Camidge, D. R. (2012) A phase I/II study of erlotinib in combination with the anti-insulin-like growth factor-1 receptor monoclonal antibody IMC-A12 (cixutumumab) in patients with advanced non-small cell lung cancer. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer* **7**, 419-426
131. Schmitz, S., Kaminsky-Forrett, M. C., Henry, S., Zanetta, S., Geoffrois, L., Bompas, E., Moxhon, A., Mignion, L., Guigay, J., Knoops, L., Hamoir, M., and Machiels, J. P. (2012) Phase II study of figitumumab in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck: clinical activity and molecular response (GORTEC 2008-02). *Annals of oncology : official journal of the European Society for Medical Oncology* **23**, 2153-2161
132. Juergens, H., Daw, N. C., Geoerger, B., Ferrari, S., Villarroel, M., Aerts, I., Whelan, J., Dirksen, U., Hixon, M. L., Yin, D., Wang, T., Green, S., Paccagnella, L., and Gualberto, A. (2011) Preliminary efficacy of the anti-insulin-like growth factor type 1 receptor antibody figitumumab in patients with refractory Ewing sarcoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **29**, 4534-4540
133. Kelly, M., and Hartwell, J. L. (1954) The biological effects and the chemical composition of podophyllin: a review. *J Natl Cancer Inst* **14**, 967-1010
134. Ward, R. S. (1995) Lignans, neolignans, and related compounds. *Nat Prod Rep* **12**, 183-205
135. Girnita, A., Girnita, L., del Prete, F., Bartolazzi, A., Larsson, O., and Axelson, M. (2004) Cyclolignans as inhibitors of the insulin-like growth factor-1 receptor and malignant cell growth. *Cancer research* **64**, 236-242
136. Vasilcanu, D., Girnita, A., Girnita, L., Vasilcanu, R., Axelson, M., and Larsson, O. (2004) The cyclolignan PPP induces activation loop-specific inhibition of tyrosine phosphorylation of the insulin-like growth factor-1 receptor. Link to the phosphatidyl inositol-3 kinase/Akt apoptotic pathway. *Oncogene* **23**, 7854-7862
137. Ekman, S., Harmenberg, J., Frodin, J. E., Bergstrom, S., Wassberg, C., Eksborg, S., Larsson, O., Axelson, M., Jerling, M., Abrahmsen, L., Hedlund, A., Alvfors, C., Stahl, B., and Bergqvist, M. (2016) A novel oral insulin-like growth factor-1 receptor pathway modulator and its implications for patients with non-small cell lung carcinoma: A phase I clinical trial. *Acta oncologica* **55**, 140-148
138. Stromberg, T., Ekman, S., Girnita, L., Dimberg, L. Y., Larsson, O., Axelson, M., Lennartsson, J., Hellman, U., Carlson, K., Osterborg, A., Vanderkerken, K., Nilsson, K., and Jernberg-Wiklund, H.

(2006) IGF-1 receptor tyrosine kinase inhibition by the cyclolignan PPP induces G2/M-phase accumulation and apoptosis in multiple myeloma cells. *Blood* **107**, 669-678
139. Mitchison, J. M., and Creanor, J. (1971) Further measurements of DNA synthesis and enzyme potential during cell cycle of fission yeast Schizosaccharomyces pombe. *Exp Cell Res* **69**, 244-247
140. Aleem, E., and Kaldis, P. (2006) Mouse models of cell cycle regulators: new paradigms. *Results and problems in cell differentiation* **42**, 271-328
141. Malumbres, M., and Barbacid, M. (2005) Mammalian cyclin-dependent kinases. *Trends Biochem Sci* **30**, 630-641
142. Harper, J. W., and Elledge, S. J. (1998) The role of Cdk7 in CAK function, a retro-retrospective. *Genes Dev* **12**, 285-289
143. Soucek, T., Pusch, O., Hengstschlager-Ottnad, E., Adams, P. D., and Hengstschlager, M. (1997) Deregulated expression of E2F-1 induces cyclin A- and E-associated kinase activities independently from cell cycle position. *Oncogene* **14**, 2251-2257
144. Geng, Y., Eaton, E. N., Picon, M., Roberts, J. M., Lundberg, A. S., Gifford, A., Sardet, C., and Weinberg, R. A. (1996) Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. *Oncogene* **12**, 1173-1180
145. Cobrinik, D. (2005) Pocket proteins and cell cycle control. *Oncogene* **24**, 2796-2809
146. Barbash, O., Lin, D. I., and Diehl, J. A. (2007) SCF Fbx4/alphaB-crystallin cyclin D1 ubiquitin ligase: a license to destroy. *Cell division* **2**, 2
147. Bertoli, C., Skotheim, J. M., and de Bruin, R. A. (2013) Control of cell cycle transcription during G1 and S phases. *Nature reviews. Molecular cell biology* **14**, 518-528
148. Fragkos, M., Ganier, O., Coulombe, P., and Mechali, M. (2015) DNA replication origin activation in space and time. *Nature reviews. Molecular cell biology* **16**, 360-374
149. Leman, A. R., and Noguchi, E. (2013) The replication fork: understanding the eukaryotic replication machinery and the challenges to genome duplication. *Genes* **4**, 1-32
150. Abbas, T., Keaton, M. A., and Dutta, A. (2013) Genomic instability in cancer. *Cold Spring Harbor perspectives in biology* **5**, a012914
151. Aguilera, A., and Garcia-Muse, T. (2013) Causes of genome instability. *Annual review of genetics* **47**, 1-32
152. Rhind, N., and Russell, P. (2012) Signaling pathways that regulate cell division. *Cold Spring Harbor perspectives in biology* **4**
153. Visconti, R., Della Monica, R., and Grieco, D. (2016) Cell cycle checkpoint in cancer: a therapeutically targetable double-edged sword. *Journal of experimental & clinical cancer research : CR* **35**, 153
154. Jackson, S. P., and Bartek, J. (2009) The DNA-damage response in human biology and disease. *Nature* **461**, 1071-1078
155. Hustedt, N., and Durocher, D. (2016) The control of DNA repair by the cell cycle. *Nature cell biology* **19**, 1-9
156. Marechal, A., and Zou, L. (2013) DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harbor perspectives in biology* **5**
157. Cimprich, K. A., and Cortez, D. (2008) ATR: an essential regulator of genome integrity. *Nature reviews. Molecular cell biology* **9**, 616-627
158. Patil, M., Pabla, N., and Dong, Z. (2013) Checkpoint kinase 1 in DNA damage response and cell cycle regulation. *Cellular and molecular life sciences : CMLS* **70**, 4009-4021
159. Sorensen, C. S., Hansen, L. T., Dziegielewski, J., Syljuasen, R. G., Lundin, C., Bartek, J., and Helleday, T. (2005) The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. *Nature cell biology* **7**, 195-201
160. Yang, X. H., Shiotani, B., Classon, M., and Zou, L. (2008) Chk1 and Claspin potentiate PCNA ubiquitination. *Genes & development* **22**, 1147-1152
161. Zannini, L., Delia, D., and Buscemi, G. (2014) CHK2 kinase in the DNA damage response and beyond. *Journal of molecular cell biology* **6**, 442-457
162. Cuadrado, M., Martinez-Pastor, B., Murga, M., Toledo, L. I., Gutierrez-Martinez, P., Lopez, E., and Fernandez-Capetillo, O. (2006) ATM regulates ATR chromatin loading in response to DNA double-strand breaks. *The Journal of experimental medicine* **203**, 297-303

163. Stiff, T., Walker, S. A., Cerosaletti, K., Goodarzi, A. A., Petermann, E., Concannon, P., O'Driscoll, M., and Jeggo, P. A. (2006) ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. *The EMBO journal* **25**, 5775-5782
164. Boehm, E. M., Gildenberg, M. S., and Washington, M. T. (2016) The Many Roles of PCNA in Eukaryotic DNA Replication. *The Enzymes* **39**, 231-254
165. Mailand, N., Gibbs-Seymour, I., and Bekker-Jensen, S. (2013) Regulation of PCNA-protein interactions for genome stability. *Nature reviews. Molecular cell biology* **14**, 269-282
166. Hishiki, A., Hashimoto, H., Hanafusa, T., Kamei, K., Ohashi, E., Shimizu, T., Ohmori, H., and Sato, M. (2009) Structural basis for novel interactions between human translesion synthesis polymerases and proliferating cell nuclear antigen. *The Journal of biological chemistry* **284**, 10552-10560
167. Hishiki, A., Shimizu, T., Hanafusa, T., Ohmori, H., Sato, M., and Hashimoto, H. (2008) Initial crystallographic study of human PCNA in complex with a peptide containing the noncanonical PIP-box sequence of human DNA polymerase iota. *Acta crystallographica. Section F, Structural biology and crystallization communications* **64**, 954-956
168. Gulbis, J. M., Kelman, Z., Hurwitz, J., O'Donnell, M., and Kuriyan, J. (1996) Structure of the C-terminal region of p21(WAF1/CIP1) complexed with human PCNA. *Cell* **87**, 297-306
169. Hoege, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G., and Jentsch, S. (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419**, 135-141
170. Pedley, A. M., Lill, M. A., and Davisson, V. J. (2014) Flexibility of PCNA-protein interface accommodates differential binding partners. *PloS one* **9**, e102481
171. Choe, K. N., and Moldovan, G. L. (2017) Forging Ahead through Darkness: PCNA, Still the Principal Conductor at the Replication Fork. *Molecular cell* **65**, 380-392
172. Watts, F. Z. (2006) Sumoylation of PCNA: Wrestling with recombination at stalled replication forks. *DNA repair* **5**, 399-403
173. Pfander, B., Moldovan, G. L., Sacher, M., Hoege, C., and Jentsch, S. (2005) SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase. *Nature* **436**, 428-433
174. Papouli, E., Chen, S., Davies, A. A., Huttner, D., Krejci, L., Sung, P., and Ulrich, H. D. (2005) Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p. *Molecular cell* **19**, 123-133
175. Motegi, A., Liaw, H. J., Lee, K. Y., Roest, H. P., Maas, A., Wu, X., Moinova, H., Markowitz, S. D., Ding, H., Hoeijmakers, J. H., and Myung, K. (2008) Polyubiquitination of proliferating cell nuclear antigen by HLTF and SHPRH prevents genomic instability from stalled replication forks. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 12411-12416
176. Carr, A. M., Paek, A. L., and Weinert, T. (2011) DNA replication: failures and inverted fusions. *Seminars in cell & developmental biology* **22**, 866-874
177. Liefshitz, B., Steinlauf, R., Friedl, A., Eckardt-Schupp, F., and Kupiec, M. (1998) Genetic interactions between mutants of the 'error-prone' repair group of Saccharomyces cerevisiae and their effect on recombination and mutagenesis. *Mutation research* **407**, 135-145
178. Chang, D. J., and Cimprich, K. A. (2009) DNA damage tolerance: when it's OK to make mistakes. *Nature chemical biology* **5**, 82-90
179. Friedberg, E. C. (2005) Suffering in silence: the tolerance of DNA damage. *Nature reviews. Molecular cell biology* **6**, 943-953
180. Yang, W., and Woodgate, R. (2007) What a difference a decade makes: insights into translesion DNA synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 15591-15598
181. McCulloch, S. D., and Kunkel, T. A. (2008) The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases. *Cell research* **18**, 148-161
182. Johnson, R. E., Prakash, S., and Prakash, L. (1999) Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Poleta. *Science* **283**, 1001-1004
183. McCulloch, S. D., Kokoska, R. J., Masutani, C., Iwai, S., Hanaoka, F., and Kunkel, T. A. (2004) Preferential cis-syn thymine dimer bypass by DNA polymerase eta occurs with biased fidelity. *Nature* **428**, 97-100
184. Zhang, Y., Yuan, F., Wu, X., Rechkoblit, O., Taylor, J. S., Geacintov, N. E., and Wang, Z. (2000) Error-prone lesion bypass by human DNA polymerase eta. *Nucleic acids research* **28**, 4717-4724

185. Ogi, T., Shinkai, Y., Tanaka, K., and Ohmori, H. (2002) Polkappa protects mammalian cells against the lethal and mutagenic effects of benzo[a]pyrene. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 15548-15553
186. Unk, I., Hajdu, I., Blastyak, A., and Haracska, L. (2010) Role of yeast Rad5 and its human orthologs, HLTF and SHPRH in DNA damage tolerance. *DNA repair* **9**, 257-267
187. Vanoli, F., Fumasoni, M., Szakal, B., Maloisel, L., and Branzei, D. (2010) Replication and recombination factors contributing to recombination-dependent bypass of DNA lesions by template switch. *PLoS genetics* **6**, e1001205
188. Andersen, P. L., Xu, F., and Xiao, W. (2008) Eukaryotic DNA damage tolerance and translesion synthesis through covalent modifications of PCNA. *Cell research* **18**, 162-173
189. Stelter, P., and Ulrich, H. D. (2003) Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature* **425**, 188-191
190. Watanabe, K., Tateishi, S., Kawasuji, M., Tsurimoto, T., Inoue, H., and Yamaizumi, M. (2004) Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination. *The EMBO journal* **23**, 3886-3896
191. Unk, I., Hajdu, I., Fatyol, K., Hurwitz, J., Yoon, J. H., Prakash, L., Prakash, S., and Haracska, L. (2008) Human HLTF functions as a ubiquitin ligase for proliferating cell nuclear antigen polyubiquitination. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 3768-3773
192. Unk, I., Hajdu, I., Fatyol, K., Szakal, B., Blastyak, A., Bermudez, V., Hurwitz, J., Prakash, L., Prakash, S., and Haracska, L. (2006) Human SHPRH is a ubiquitin ligase for Mms2-Ubc13-dependent polyubiquitylation of proliferating cell nuclear antigen. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 18107-18112
193. Motegi, A., Sood, R., Moinova, H., Markowitz, S. D., Liu, P. P., and Myung, K. (2006) Human SHPRH suppresses genomic instability through proliferating cell nuclear antigen polyubiquitination. *The Journal of cell biology* **175**, 703-708
194. Pickart, C. M., and Fushman, D. (2004) Polyubiquitin chains: polymeric protein signals. *Current opinion in chemical biology* **8**, 610-616
195. Hochstrasser, M. (1996) Ubiquitin-dependent protein degradation. *Annual review of genetics* **30**, 405-439
196. Yang, K., Gong, P., Gokhale, P., and Zhuang, Z. (2014) Chemical protein polyubiquitination reveals the role of a noncanonical polyubiquitin chain in DNA damage tolerance. *ACS chemical biology* **9**, 1685-1691
197. Terrano, D. T., Upreti, M., and Chambers, T. C. (2010) Cyclin-dependent kinase 1-mediated Bcl-xL/Bcl-2 phosphorylation acts as a functional link coupling mitotic arrest and apoptosis. *Molecular and cellular biology* **30**, 640-656
198. Harley, M. E., Allan, L. A., Sanderson, H. S., and Clarke, P. R. (2010) Phosphorylation of Mcl-1 by CDK1-cyclin B1 initiates its Cdc20-dependent destruction during mitotic arrest. *The EMBO journal* **29**, 2407-2420
199. Mc Gee, M. M. (2015) Targeting the Mitotic Catastrophe Signaling Pathway in Cancer. *Mediators of inflammation* **2015**, 146282
200. Jackson, J. R., Patrick, D. R., Dar, M. M., and Huang, P. S. (2007) Targeted anti-mitotic therapies: can we improve on tubulin agents? *Nature reviews. Cancer* **7**, 107-117
201. Lee, K., Kenny, A. E., and Rieder, C. L. (2011) Caspase activity is not required for the mitotic checkpoint or mitotic slippage in human cells. *Molecular biology of the cell* **22**, 2470-2479
202. Czabotar, P. E., Lessene, G., Strasser, A., and Adams, J. M. (2014) Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nature reviews. Molecular cell biology* **15**, 49-63
203. Han, C. R., Jun do, Y., Lee, J. Y., and Kim, Y. H. (2014) Prometaphase arrest-dependent phosphorylation of Bcl-2 and Bim reduces the association of Bcl-2 with Bak or Bim, provoking Bak activation and mitochondrial apoptosis in nocodazole-treated Jurkat T cells. *Apoptosis : an international journal on programmed cell death* **19**, 224-240
204. Gascoigne, K. E., and Taylor, S. S. (2008) Cancer cells display profound intra- and interline variation following prolonged exposure to antimitotic drugs. *Cancer cell* **14**, 111-122

205. Brito, D. A., and Rieder, C. L. (2006) Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint. *Current biology : CB* **16**, 1194-1200
206. Tait, S. W., and Green, D. R. (2008) Caspase-independent cell death: leaving the set without the final cut. *Oncogene* **27**, 6452-6461
207. Dumontet, C., and Jordan, M. A. (2010) Microtubule-binding agents: a dynamic field of cancer therapeutics. *Nature reviews. Drug discovery* **9**, 790-803
208. Mukhtar, E., Adhami, V. M., and Mukhtar, H. (2014) Targeting microtubules by natural agents for cancer therapy. *Molecular cancer therapeutics* **13**, 275-284
209. Jordan, M. A., and Wilson, L. (2004) Microtubules as a target for anticancer drugs. *Nature reviews. Cancer* **4**, 253-265
210. Tao, W. (2011) KSP Mitotic Spindle Motor Protein. in *Encyclopedia of Cancer* (Schwab, M. ed.), Springer Berlin Heidelberg, Berlin, Heidelberg. pp 1960-1963
211. Huszar, D., Theoclitou, M. E., Skolnik, J., and Herbst, R. (2009) Kinesin motor proteins as targets for cancer therapy. *Cancer metastasis reviews* **28**, 197-208
212. Bavetsias, V., and Linardopoulos, S. (2015) Aurora Kinase Inhibitors: Current Status and Outlook. *Frontiers in oncology* **5**, 278
213. Giet, R., and Prigent, C. (1999) Aurora/Ipl1p-related kinases, a new oncogenic family of mitotic serine-threonine kinases. *Journal of cell science* **112 (Pt 21)**, 3591-3601
214. Adams, R. R., Maiato, H., Earnshaw, W. C., and Carmena, M. (2001) Essential roles of Drosophila inner centromere protein (INCENP) and aurora B in histone H3 phosphorylation, metaphase chromosome alignment, kinetochore disjunction, and chromosome segregation. *The Journal of cell biology* **153**, 865-880
215. Hauf, S., Cole, R. W., LaTerra, S., Zimmer, C., Schnapp, G., Walter, R., Heckel, A., van Meel, J., Rieder, C. L., and Peters, J. M. (2003) The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *The Journal of cell biology* **161**, 281-294
216. Gizatullin, F., Yao, Y., Kung, V., Harding, M. W., Loda, M., and Shapiro, G. I. (2006) The Aurora kinase inhibitor VX-680 induces endoreduplication and apoptosis preferentially in cells with compromised p53-dependent postmitotic checkpoint function. *Cancer research* **66**, 7668-7677
217. Lenart, P., Petronczki, M., Steegmaier, M., Di Fiore, B., Lipp, J. J., Hoffmann, M., Rettig, W. J., Kraut, N., and Peters, J. M. (2007) The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. *Current biology : CB* **17**, 304-315
218. Fulzele, K., DiGirolamo, D. J., Liu, Z., Xu, J., Messina, J. L., and Clemens, T. L. (2007) Disruption of the insulin-like growth factor type 1 receptor in osteoblasts enhances insulin signaling and action. *The Journal of biological chemistry* **282**, 25649-25658
219. Lu, X., Wang, L., Mei, J., Wang, X., Zhu, X., Zhang, Q., and Lv, J. (2013) Picropodophyllin inhibits epithelial ovarian cancer cells in vitro and in vivo. *Biochemical and biophysical research communications* **435**, 385-390
220. Yin, S., Girnita, A., Stromberg, T., Khan, Z., Andersson, S., Zheng, H., Ericsson, C., Axelson, M., Nister, M., Larsson, O., Ekstrom, T. J., and Girnita, L. (2010) Targeting the insulin-like growth factor-1 receptor by picropodophyllin as a treatment option for glioblastoma. *Neuro-oncology* **12**, 19-27
221. Osuka, S., Sampetrean, O., Shimizu, T., Saga, I., Onishi, N., Sugihara, E., Okubo, J., Fujita, S., Takano, S., Matsumura, A., and Saya, H. (2013) IGF1 receptor signaling regulates adaptive radioprotection in glioma stem cells. *Stem cells* **31**, 627-640
222. Blastyak, A. (2014) DNA replication: damage tolerance at the assembly line. *Trends in biochemical sciences* **39**, 301-304
223. Chen, J., Bozza, W., and Zhuang, Z. (2011) Ubiquitination of PCNA and its essential role in eukaryotic translesion synthesis. *Cell biochemistry and biophysics* **60**, 47-60
224. Baserga, R. (2013) The decline and fall of the IGF-I receptor. *Journal of cellular physiology* **228**, 675-679