CELL CYCLE REGULATION VIA GROWTH FACTOR- AND STRESS-INDUCED PATHWAYS

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ACADEMIC DISSERTATION

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To family and friends
Summary

Cell division is the basis of all life forms and reproduction, and it is regulated by the activity of the CDKs, the cyclin dependent kinases. p27^Kip1 is a CDK inhibitor, which can promote cell cycle arrest by binding and inhibiting CDKs. A specific form of p27^Kip1 increases after TGF-β treatment or during serum starvation, but is not bound to CDKs. We studied the regulation of this form of p27^Kip1 (p27^{NCDK} for non-CDK-bound) by TGF-β. In addition to being increased by the growth inhibitory TGF-β, inhibition of growth promoting pathways such as MEK or PI3K caused an increase in p27^{NCDK} levels, while activation of the Akt pathway decreased p27^{NCDK} levels, indicating its involvement in the growth arrested status of the cell. We further discovered that cellular stress increased p27^{NCDK} levels, and pinpointed the involvement of AMPK pathway in this process.

The crosstalk of Akt and TGF-β pathways was further investigated at the level of TGF-β signal transducers. We discovered that Akt pathway can oppose the TGF-β by phosphorylating Ski. Phosphorylation led to destabilisation of Ski, which relieved its inhibitory effect on the Smad7 promoter. Induction of Smad7 levels led to repression of the TGF-β receptors and inhibition of the TGF-β signalling, providing another example of the cross talk between the Akt and TGF-β pathways.

A biological context where these two pathways interact is the prostate, where TGF-β affects cancer cell migration and invasion. We discovered that unlike most cell types, normal prostate epithelial cells do not undergo cell cycle arrest in response to irradiation despite having functional ATM-Chk2 mediated DNA damage signaling. However, these cells did not display inhibitory Tyr15 phosphorylation of CDK2 after irradiation, allowing cell cycle to proceed. We discovered that the levels of the Wee1 kinase were low compared to many other cell types, including tumor cells. This could potentially be the underlying cause for hyperproliferative disorders of the prostate and multifocality in prostate cancer.

Altogether, the studies presented here identify new mechanisms how cells integrate environmental inputs such as stress and growth conditions via signaling pathways to cell cycle machinery.
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This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.


## Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACC</td>
<td>Acetyl CoA-Carboxylase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AKT</td>
<td>Oncogene isolated from AKT8 retrovirus</td>
</tr>
<tr>
<td>AMP</td>
<td>5' Adenosine Monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated Protein Kinase</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia Mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia-Telangiectasia and Rad3-Related</td>
</tr>
<tr>
<td>CAK</td>
<td>CDK-activating Kinase</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin Dependent Kinase</td>
</tr>
<tr>
<td>CDKN1B</td>
<td>p27Kip1-gene</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>CRM1</td>
<td>Chromosome Region Maintenance 1</td>
</tr>
<tr>
<td>DSB</td>
<td>Double Strand Break</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-Mesenchymal Transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead Box O proteins</td>
</tr>
<tr>
<td>gamma-H2AX</td>
<td>gamma-histone2AX</td>
</tr>
<tr>
<td>HEJ</td>
<td>Homologous End Joining</td>
</tr>
<tr>
<td>HPEC</td>
<td>Human Prostatic Epithelial Cells</td>
</tr>
<tr>
<td>HRR</td>
<td>Homologous Recombination Repair</td>
</tr>
<tr>
<td>INK4</td>
<td>Inhibitor of CDK4</td>
</tr>
<tr>
<td>IR</td>
<td>Ionising radiation</td>
</tr>
<tr>
<td>KPC</td>
<td>Kip1-ubiquitination-promoting complex</td>
</tr>
<tr>
<td>LKB1</td>
<td>Liver Kinase B1</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
</tr>
<tr>
<td>MEN</td>
<td>Multiple Endocrine Hyperplasia</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic Target of Rapamycin</td>
</tr>
<tr>
<td>Mv1Lu</td>
<td>Mink Lung Epithelial Eells</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide Excision Repair</td>
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<tr>
<td>NEJ</td>
<td>Non-homologous End Joining</td>
</tr>
<tr>
<td>p27NCDK</td>
<td>Non-CDK-bound-p27Kip1</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PH</td>
<td>Plextrin Homology Domain</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidyl Inositol 3-Kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>inositol-3-phosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homologue</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>-------------</td>
<td>----------------------------------</td>
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<tr>
<td>R-Smad</td>
<td>Receptor-Smad</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>Ski</td>
<td>Sloan-Kettering Institute Protein</td>
</tr>
<tr>
<td>SMAD</td>
<td>Suppressor of Mothers Against Decapentaplegic</td>
</tr>
<tr>
<td>SnoN</td>
<td>Ski-related Nuclear Oncoprotein</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>Tricarbocyclic Acid Cycle</td>
</tr>
<tr>
<td>TGF-beta</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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All animals, including humans, are multicellular organisms, resulting from the well-controlled and programmed cell divisions of the fertilized oocyte. To produce more cells of any type, the cells go through a process called the cell cycle. It starts with a single cell, and through intricately controlled and sequential steps leads to the formation of two daughter cells. As a result, there are more than 37 trillion cells in human body (Bianconi et al., 2013). Despite the single origin and genetic identity, our cells perform hugely different functions depending on where in the body they are located. For example, the morphology and function of the cells in the lung epithelium are very different from the ones in our liver or prostate. The number of cells and the decision to divide or not needs to be maintained under tight control. This is accomplished by cell cycle control, which operates on demand in all cell types.

Cells and organisms must also respond to the changing environment, levels of nutrients and stresses. This often involves increasing or decreasing the number of new cells produced and is achieved through various signaling pathways able to respond to these changes and communicate this information to the key controllers of the cell cycle.

At any given time, most cells in an organism are in a quiescent, resting state. They need extracellular stimuli to enter the cell cycle and start dividing. Such stimuli can be provided in the form of growth factors or hormones. Typically, binding of these molecules to a receptor on the cell surface triggers a signalling cascade, which eventually leads to expression of early G1-cyclins and other proteins necessary for cell division. There are several signalling pathways involved in cell cycle regulation, including but not restricted to mitogen activated kinase (MAPK), phosphatidylinositol-3-phosphate kinase (PI3K) and transforming growth factor beta (TGF-β) pathways. First two are considered growth stimulating, while TGF-β is mostly anti-proliferative, although the precise effects of TGF-β depend on the cell type and context.

There are also situations where the surroundings of the cell or the organism are unfavourable for growth or division, e.g. under low nutrient or high energy-expenditure conditions. The main signalling pathways activated under these conditions will try to halt or slow cell
division. The 5’-adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway and the DNA damage-induced responses are perhaps the most well-known of such stress-induced examples.

Maintaining appropriate regulation of cell cycle and signaling pathways is not only important for the normal development and maintenance of the organisms, but is also relevant to diseases as uncontrolled cell division is the first hallmark of cancer. Many of these signalling pathways directly regulate the cell cycle machinery to prevent uncontrolled division, and also impinge on other cancer cornerstones such as cell migration, which may lead to cell invasion and metastasis. Loss of appropriate responses to extracellular cues - positive and negative - is also a characteristic feature of cancer and can be achieved by e.g. mutations in the pathway components.

In recent years we have come to appreciate the importance of these pathways and started to unravel the complexity of signalling crosstalk. As cells are constantly under the influence of both positive and negative growth signals, predicting the end result of targeting or modulating a single player is often challenging as proven by the numerous failed clinical trials with new drug targets. Attempts for finding a modality to target uncontrolled cell division in cancer are still ongoing. Decades of work on the fundamental cell cycle regulation and results from several intriguing mouse models have recently been translated into clinical success. Small molecule inhibitors against proteins driving the cell cycle, Cdk4/6, are showing high efficacy and extended survival time in treatment of breast cancer in particular. As breast cancer is the most common type of cancer in women, this treatment modality may benefit a large number of patients. Also, use of the compounds in other types of cancers is being investigated, potentially widening the scope for their utilisation in treatment.

Therefore, understanding more about cell cycle control and cell-cycle-related signalling pathways is proving more important than ever. Since many of the pathways implicated in cancer and investigated in this research converge at the level of cell cycle, it is reasonable to assume targeting the regulators of the cycle could provide a unified strategy to fight cancer.
Review of the literature

Part 1 The Cell Cycle

Phases of the Cell cycle

Many of the initial cell cycle studies were performed using yeast as model system (Lee and Nurse, 1988). However, yeast are single cell eukaryotic organisms whose main activity is to proliferate as opposed to animals, which are multicellular and composed of a number of different cell types, many of which are non-dividing. Therefore, the events leading to and regulating the initial decision to divide are more complex in animals, as the outcome must benefit the whole organism rather than an individual cell. Nevertheless, the fact that many of the core factors controlling cell cycle are amazingly similar in multicellular and unicellular organisms has been widely utilised in cell cycle research. Consequently, yeast experiments laid solid foundations for the field (Lee and Nurse, 1988), as studying gene function in unicellular organisms was, and still is, more straightforward both for technical and genetic reasons. For example, while yeast often has one gene for a specific function, higher organisms may possess several variants of a particular gene, which also possibly display tissue specific expression. This complexity of the cell cycle has been addressed with mouse knock-out models, which have provided valuable and sometimes unexpected information regarding the roles of the cell cycle regulators (Satyanarayana and Kaldis, 2009).

The cell cycle consists of four key stages - Synthesis (S), Mitosis (M) and two gap phases (G1 and G2), which separate the DNA synthesis and mitosis. The G1-, S- and G2-phases combined are often referred to as interphase, the time spent between two mitoses. The total length of the cell cycle is highly variable and depends on the cell type and the cellular environment. For example, the time required to complete the G1- and G2-phases may vary from non-existent to years (Morgan, 2007). In addition cells, which are not dividing, enter a quiescent state called G0, from which they can return to the cycle again by entering G1.

The cell cycle is initiated in G1-phase with a decision to divide, after which production of the building blocks required for cell duplication begins and cell growth commences. The cell then enters S-phase, where DNA is synthesised and a full copy of the genome is created. Between S- and M-phases lies the G2-phase, which provides time for further growth and also
serves as a control point for the quality of the duplicated DNA before mitosis starts. Mitosis is further divided into distinct sub-phases; chromosomes begin to condense in prophase; then align along the central plane and attach their kinetochores to the spindle poles, which form in the cytoplasm, in metaphase; in anaphase the sister chromosomes get pulled to the opposite ends of the cell, and mitosis is completed in telophase, where the chromosomes begin to decondense and a nuclear membrane forms around the two daughter cells. The chromosomes must be carefully managed during mitosis. During interphase, chromosomes are being held together by a protein called cohesin. At the onset of anaphase, cohesin is cleaved by a protease called separase, which has been kept inactive via its association to yet another protein dubbed securin. Dephosphorylation and phosphorylation events lead to the degradation of securin and activation of separase, which by cleaving cohesin releases the tension between the two chromosomes (Holt et al., 2008). Two copies of the chromosomes can then be pulled to the opposite poles of the cell by spindles attached to centrosomes. After this stage, the cell cycle is completed in cytokinesis, where the cytosolic material is split and encapsulated within the separate cell membranes of the two daughter cells (Hartwell and Weinert, 1989; Nurse, 1987) (Figure 1).

The key regulatory steps in the cell cycle are the transitions to the S and M phases, which are governed by cell cycle checkpoints. The first checkpoint controls entry into the S phase to ensure that the cell has adequate resources to complete the cell cycle (Hartwell and Weinert, 1989). Another checkpoint before entering mitosis is required as unevenly distributed genetic material creates chromosomally abnormal cells, which may eventually become cancerous (Paulovich et al., 1997). Furthermore, there are various molecular mechanisms during mitosis to ensure the correct sequence of events, for example the spindle checkpoint, where wrongly attached chromosome will create a signal to halt the cell division until all chromosomes are correctly aligned and can be pulled towards the opposite poles (Musacchio and Hardwick, 2002). For the scope of this thesis I will focus on the early stages of cell cycle, primarily the G1 phase regulation.
Figure 1. Cell division cycle. The cell cycle consists of two Gap (G) phases, which separate the DNA Synthesis (S) and Mitosis (M) phases. Cells in G0 phase are not actively dividing and are quiescent. These cells may re-enter the cycle at G1 phase after receiving appropriate stimulus. Sequential activation of the different CDK/Cyclin complexes during the cycle ensures the cell cycle events are executed in the correct order. CDK inhibitors can bind different CDKs at various stages of the cell cycle and thereby inhibit cell cycle progression. For simplicity, only p27 of these inhibitors is shown.

Molecular machinery of the cell cycle

CDK/Cyclin complexes

The main proteins regulating the ordered progression through the cell cycle are CDKs, cyclin dependent kinases, and their heterodimeric partners, cyclins (Hunt, 1991; Morgan, 1997). Several CDKs are activated sequentially during the cell cycle. Their activation requires the binding of a specific cyclin, which are usually expressed and available only during the corresponding cell cycle phase (Jeffrey et al., 1995). There are many different subgroups of cyclins, and in some species several isoforms of a single cyclin type. The CDK/cyclin complexes are named and classified according to the cell cycle stage they are active in, for example G1, G1/S, S and M. In mammalian cells these are CDK4/6-cyclinD (G1), CDK2-CyclinE (G1/S), CDK2-CyclinA (S), CDK2 and CDK1-cyclin B (M). The same CDK can be involved in several phases by changing the cyclin partner, thereby changing the substrate
specificity and/or localisation of the kinase complex, the latter of which can also be the determining factor in substrate phosphorylation. For example, CDK2 can bind either cyclin E or cyclin A, being a G1/S-phase kinase complex with cyclin E, but an S-phase complex with cyclin A. (Figure 1)

For full activity, the CDK also needs to be activated by phosphorylation by CDK-activating kinase (CAK) in the C-terminus of the CDK protein (Desai et al., 1992; Gould et al., 1991; Solomon et al., 1992). The identity and function of the CAK in different species is one of the main points of evolutionary divergence in cell cycle control. In budding yeast CAK is called Cak1 and is only distantly related to Cdk5. In mammalian cells cyclin binding precedes phosphorylation by CAK, but in yeast cells the phosphorylation can occur prior to cyclin binding, cyclin binding being the rate limiting step. Surprisingly, in mammalian cells no Cak1 homolog was found but instead the CDK-activating kinase activity was found to reside within the Cdk7-CyclinH-Mat1 complex (Fesquet et al., 1993; Fisher and Morgan, 1994; Poon et al., 1993; Solomon et al., 1993). As this complex is also responsible for the phosphorylation of TFIH, a factor of the basal transcription machinery that phosphorylates the C-terminal domain of RNA polymerase II, (Feaver et al., 1993; Roy et al., 1994), these two rather separate functions and non-conserved target sequence initially cast doubt as to whether Cdk7-CyclinH-Mat1 really is the metazoan CAK. However, mounting evidence and eventually structural studies which also demonstrated 300-fold activation of the CDK2-CyclinA complex by Cdk7-CyclinH-Mat1(Russo et al., 1996) solidified the case.

Another puzzling discovery was that in most mammalian cells CAK activity does not seem to be regulated, making it somewhat of a conundrum as to why it exists in the first place (Harper and Elledge, 1998). Instead, a commonly observed way to negatively affect the CDK activity is by phosphorylation. For example, when the integrity of the DNA has been compromised, Wee1/Myt1 kinases can be activated leading to phosphorylation of tyrosine 15 residue and subsequent inhibition of the mitotic kinase CDK1 (McGowan and Russell, 1995; O'Connell et al., 1997; Parker and Piwnica-Worms, 1992). This Wee1/Myt1 mediated phosphorylation prevents the CDK1 mediated phosphorylation of targets crucial for the completion of mitosis, thereby allowing time to correct errors before completing the cell cycle (Jin et al., 1996).
In addition to inhibitory phosphorylation, CDK activity is also commonly regulated through binding of specific CDK-inhibitor proteins, CDKIs. These can be divided into two groups, the INK4 and the Cip/Kip proteins (Sherr and Roberts, 1999). The INK4 proteins - p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p18\textsuperscript{INK4c} and p19\textsuperscript{INK4d} - bind only the kinase part of the complex, thereby preventing cyclin binding. Another feature of the Ink4 proteins is that they bind and inhibit only the early G1 CDKs, i.e. CDK4/6 in mammalian cells. The biological relevance of the inhibition of CDKs by INK4 proteins has become apparent by the discovery that germline mutations in the p16 \textit{CDKN2A} gene lead to increased risk of skin, pancreatic and lung cancer (Kamb et al., 1994; Nobori et al., 1994; Zuo et al., 1996).

The Cip/Kip inhibitors consist of p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2} proteins. They bind both components of the CDK/cyclin complexes, affecting the activity of D-, A, E- and B-type cyclin complexes. Their role is also not as strictly inhibitory as that of INK4-proteins. They also seem to function as essential assembly factors for the Cyclin D-type complexes such that CDK4-Cyclin D-p27 complexes maintain kinase activity towards RB (Blain et al., 1997; LaBaer et al., 1997). Additionally, the Cip/Kip proteins seem to have roles beyond cell cycle regulation. There are multiple reports describing their role in cell migration, apoptosis, transcriptional regulation and DNA damage response (Sherr, 1993; Sherr and Roberts, 2004).

The activity of the CDK inhibitors is commonly regulated through signalling pathways. For example, TGF-\(\beta\) can induce the expression of p15, resulting in binding of p15 to CDK4/6-cyclin D complexes and release of p27/p21 from these complexes thereby allowing more of these proteins to bind by Cdk2 complexes, which leads to inhibition of Cdk2 activity (Reynisdottir and Massague, 1997; Reynisdottir et al., 1995; Sandhu et al., 1997).

\textit{p27Kip1}

P27 is a small protein with 198 amino acid residues and a relatively simple domain structure (Figure 2) with an apparent molecular weight of 27 kDa on SDS-PAGE gels. It was first discovered as a Cdk-inhibitory activity in TGF-\(\beta\) treated cells and in growth arrested cells (Hengst et al., 1994; Polyak et al., 1994a; Slingerland et al., 1994). Overexpression of p27
was found to cause G1 arrest in both Mv1Lu mink lung epithelial and human osteosarcoma cells (Saos-2) (Polyak et al., 1994b; Toyoshima and Hunter, 1994). This inhibition was overcome by excess addition of Cyclin E, while addition of excess CDK4-CyclinD into these cells was able to activate CDK2-Cyclin E complexes, providing the first clue to the dual role of p27Kip1 (Polyak et al., 1994a). Levels of p27^Kip^ do not show high degree of variation throughout the cell cycle apart from accumulation in G0, and p27^Kip^ is found in complexes with CDK4-CyclinD also in proliferating cells (Blain et al., 1997; Soos et al., 1996). As mouse fibroblasts lacking both p21 and p27 were found to have reduced CDK4 activity and defects in assembling CDK4-CyclinD as well as in CyclinD nuclear transport (Cheng et al., 1999), an understanding of p27^Kip^ and p21^Cip1^ as dual-function proteins emerged. Their role in inhibiting CDK2 was not disputed, but the mounting evidence suggested that they were, at least in some conditions, not inhibiting CDK4 activity. Instead, they could even facilitate the complex formation between Cyclin D and its associating kinase (CDK4 or CDK6) as cells lacking p21^Cip1^ and p27^Kip1^ were not able to assemble functional complexes with Cyclin D-associated kinase activity (Cheng et al., 1999; LaBaer et al., 1997). The assembly factor function was disputed as cells lacking p27^Kip1^ and p21^Cip1^ were shown to have Cyclin D3-associated kinase activity, which was inhibited by addition of p27^Kip1^ (Bagui et al., 2000; Bagui et al., 2003). CDK4-CyclinD complexes were also more stable in the presence of the disputed inhibitor. In addition, recombinant p27^Kip1^, when added to cell extracts, was able to inhibit Cdk4-Cyclin D complexes, as was p27^Kip1^ from contact inhibited cells but not from proliferating cells (Blain et al., 1997; James et al., 2008). The conflicting data seemed to promote both scenarios, or as later emerged, support the bifunctional role of p27^Kip1^ as both a facilitator and inhibitor of CDK4/6 complexes. The mechanisms enabling this are due to post-translational modifications on p27^Kip1^, which will be discussed later.
Figure 2. Regulation of p27Kip protein by phosphorylation. p27 is an intrinsically unstructured protein, its two main domains are the kinase inhibitory domain (KID) and the C-terminal QT-domain, which is an evolutionarily conserved stretch of glutamine and threonine residues. The QT-domain contains three threonines that serve as phospho-acceptors; Thr157, Thr187 and Thr198. Known kinases and their phosphorylation sites in p27 are shown. Phosphorylation of Thr157 leads to nuclear export, while Thr187 phosphorylation by Cdk2 targets p27 for degradation. Thr198 has been reported to be phosphorylated by multiple kinases and this regulates autophagy, cell motility and p27 stability. Ser10 phosphorylation regulates subcellular localisation and stability of the protein. Tyrosine phosphorylation of p27 within the kinase inhibitory domain modulates CDK-inhibitory activity, enables kinase activation and promotes assembly of CDK-cyclin complexes.

p27Kip1 is deregulated in multiple human tumors and cancer cell lines suggesting a role as a tumor suppressor. Mutations in the gene, however, were initially rarely detected (Blain et al., 2003; Fero et al., 1998; Ponce-Castaneda et al., 1995; Slingerland and Pagano, 2000). Yet decrease in p27Kip1 levels occurs in roughly half of carcinomas and often correlates with poor prognosis (see (Slingerland and Pagano, 2000) and references therein). In general it seems that low p27Kip1 levels correlate with poor survival, increased tumor progression, increased malignancy of the tumor and poor prognosis (Lloyd et al., 1999) (Chu et al., 2008).

Although germline mutations in p27Kip1 gene (CDKN1B) are rare and misregulation more commonly arises at the level of translation or degradation, mutations were first identified in a syndrome called multiple endocrine hyperplasia (MEN). There are four subtypes of MEN and p27Kip1 mutations have been found to be the underlying cause of MEN, type 4. This
syndrome affects different endocrine, i.e. hormone-producing organs, of the body. MEN4 is characterised by hyperparathyroidism and pancreatic and pituitary tumors (Thakker, 2014). Rats also have a recessive multiple endocrine neoplasia-like syndrome called MENX and the disease locus was mapped to a region encompassing CDKN1B (Pellegata et al., 2006).

The advances in whole genome sequencing and screening of sporadic tumors challenged the view of p27Kip1 deregulation occurring only at the protein level. Instead it has now been found that mutations in the p27Kip1 gene, CDKN1B, do contribute significantly to development of some cancer types. Mutations in CDKN1B were detected in prostate cancer (Barbieri et al., 2012), in small intestine neuroendocrine tumors (SI-NETs) (Francis et al., 2013), and in particular sporadic luminal breast cancer (Ellis et al., 2012). Another analysis confirmed p27Kip1 gene to be frequently mutated in breast cancer, and placed CDKN1B mutations as an early, driver event. Mutations were found in both ER-positive and ER-negative tumors, but less so in the metastatic growths (Yates et al., 2017). A meta-analysis of related research confirmed the findings and further pointed out that several of the mutations in CDKN1B found in breast cancer are located in the C-terminal portion of the protein, potentially resulting in altered subcellular localisation (Cusan et al., 2018). It is also interesting to notice that these cancer types are all hormone-driven, suggesting p27Kip1 might be involved in the paracrine cell-cell communication as well as the intracellular signalling pathways.

Mouse knockout models have been extensively used to study the biological relevance of p27Kip1. Engineered mice nullizygous for p27Kip1 are larger in size than their wild type littermates and show pituitary hyperplasia and adenomas (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). The p27Kip1-deficient animals display increased cell numbers across a spectrum of organs highlighting the role of p27Kip1 as a negative regulation of proliferation in vivo. The mice also display thymic hyperplasia with increased proliferation of T-cells and enhanced proliferation of hematopoietic stem cells. Pituitary tumors are also frequently observed (Fero et al., 1996; Nakayama et al., 1996).

An interesting phenomenon was discovered when p27Kip1 +/- mice were compared with the p27Kip1 -/- mice. The heterozygous mice were found to be more prone to tumorigenesis than the mice lacking p27Kip1 both in prostate and breast cancer models, suggesting that the remaining p27Kip1 allele plays an active role in tumorigenesis (Gao et al., 2004; Muraoka et
al., 2002). This was further highlighted by the knock-in mouse model, where the wild type p27Kip1 was replaced with a p27CK- mutant unable to bind to CDKs. Instead of the rather restricted pituitary adenomas seen in p27Kip1 -/- mice, these mice exhibited hyperplastic lesions in multiple organs. This was attributed to the amplification of various stem and progenitor cell populations (Besson et al., 2007).

Deletion of p27Kip1 in combination with Cdks uncovered many compensatory mechanisms and unpredicted complexes within the cells. When Cdk4(R24C), which abolishes the ability of CDK4 to bind to p16INK4a, was combined with p27Kip1 loss, this resulted in more complete penetrance of pituitary tumors, indicating co-operation of the two proteins in tumor promotion (Sotillo et al., 2005). Unexpectedly, ablation of Cdk2 in p27Kip1 -/- cells did not suppress the phenotypic defects of p27Kip1, suggesting Cdk2 is not an essential target of p27Kip1 in proliferation (Martin et al., 2005). Furthermore, p27Kip1 was found in complexes with Cdk1, cyclin A2 and cyclin B1 in cells lacking Cdk2 and in wild type cells (Aleem et al., 2005).

Studies on p27Kip1 protein with mutations in specific amino acids of critical phosphorylated residues have further highlighted important regulatory aspects of p27Kip1 function. These will be discussed in detail below.

**Regulation of p27Kip1 abundance, phosphorylation and localisation**

Regulation of p27Kip1 occurs at several levels. Transcription of p27Kip1 is regulated by the oncoprotein Myc, which binds p27Kip1 promoter and represses its expression (Yang et al., 2001). Forkhead box O proteins (FOXO) transcription factors induce p27Kip1 expression leading to G0/G1 arrest (Chandramohan et al., 2004; Stahl et al., 2002).

However, p27Kip1 levels are predominantly regulated at a post-transcriptional level, both through phosphorylation and ubiquitin-dependent degradation. P27Kip1 protein is subject to phosphorylation by Cdk2-Cyclin E, and this phosphorylation primes and targets it for degradation (Sheaff et al., 1997; Vlach et al., 1997). Phosphorylation of T187 creates a binding site for the F-box protein SKP2, and will lead to the ubiquitin-dependent degradation of p27Kip1 primarily through SCFSkp2 E3 ubiquitin ligase and occurs in the nucleus (Carrano
et al., 1999). A knock-in mouse model where the p27<sub>Kip1</sub> was replaced with unphosphorylatable p27<sub>Kip1</sub> (T187A) revealed that this pathway regulated p27<sub>Kip1</sub> during S and G2 phases but degradation of p27<sub>Kip1</sub> during G1 was unaffected (Malek et al., 2001). This suggested the existence of a second pathway regulating p27<sub>Kip1</sub> during entry into and in G1 phase. This second activity leading to p27<sub>Kip1</sub> destruction was found to reside in the cytoplasm and was named KPC (Kip1 ubiquitination-promoting complex), containing KPC1 and KPC2 (Kamura et al., 2004; Kotoshiba et al., 2005). The ubiquitination occurs in a phosphorylation dependent manner involving Ser 10 of p27<sub>Kip1</sub>. This phosphorylation has also been shown to mediate the translocation of p27<sub>Kip1</sub> protein from nucleus to cytoplasm through binding to CRM1 (Chromosome region maintenance 1) (Ishida et al., 2002).

Subcellular localisation of p27<sub>Kip1</sub> is regulated by several kinases phosphorylating different residues on p27<sub>Kip1</sub>. In addition to Ser10 mentioned above, Thr157 within the nuclear localisation sequence of p27<sub>Kip1</sub> was identified as a target site for Akt/PKB kinase. Phosphorylation of the site blocks the nuclear import of p27<sub>Kip1</sub> (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002). The resulting cytoplasmic localisation prevents p27<sub>Kip1</sub> from inhibiting its target Cdk5 in the nucleus. High levels of p27<sub>Kip1</sub> in the cytoplasm have been shown to correlate with poor prognosis in breast cancer patients (Liang et al., 2002). Thus, cancer cells could utilise these mechanisms and become resistant to inhibitory signals. Liang and co-workers indeed showed that cells that had become resistant to TGF-β could be rendered TGF-β sensitive by inhibition of Akt/PKB (Liang et al., 2002). The biological relevance of the Thr157 phosphorylation remains unclear as mice lack the corresponding threonine residue complicating the analysis of this residue using mouse models.

The most abundantly phosphorylated residue in p27<sub>Kip1</sub> is Ser10, and phosphorylation of the site leads to nuclear export in CRM1 dependent manner as explained above. Several kinases have been assigned as p27<sub>Kip1</sub> Ser10 kinases, including Akt, KIS and Mirk1/Dyrk (Boehm et al., 2002; Deng et al., 2004; Fujita et al., 2002). In contrast to others Fujita <i>et al.</i> suggested that phosphorylation does not change the nuclear localisation of p27<sub>Kip1</sub>, while two groups showed that Ser10 phosphorylation is a prerequisite to CRM1 mediated export. Mice harbouring the unphosphorylatable knock-in mutation S10A are normal in body size but have decreased levels of p27<sub>Kip1</sub> protein in several organs (Kotake et al., 2005). The stability of the p27<sub>Kip1</sub> (S10A) protein was particularly affected in G0 cells but not in S phase. Furthermore,
the nuclear export of p27^Kip1 at the G0-G1 transition occurred normally in the p27^(S10A/S10A) mouse embryonic fibroblasts (MEFs), suggesting it is not a prerequisite for export (Kotake et al., 2005).

While p27^Kip1 is a CDK-inhibitor, it was also shown that p27^Kip1 is able to bind Cdk4/6-Cyclin D complexes without inhibition of the kinase activity, as discussed above (Cheng et al., 1999; Sherr and Roberts, 1999). The discrepancy between the two seemingly opposite modes was rather controversial. Insight into this unexpected binding mode was provided by the findings that Tyr74, Tyr88 and Tyr89, which are located within the CDK-binding region of p27, can be phosphorylated by many growth-promoting kinases such as ABL, SRC, LYN and YES (Chu et al., 2007; Grimmler et al., 2007; James et al., 2008; Kardinal et al., 2006). Being an intrinsically unstructured protein, p27^Kip1 can acquire several different conformations, often only upon binding to another protein.

It has been shown that phosphorylation of the residues within CDK-binding region (Figure 2) push the p27^Kip1 tail away from the catalytic cleft of CDK4/6 allowing entry of substrate and ATP and reducing the inhibitory qualities of p27^Kip1 towards CDKs. On the other hand, p27^Kip1 isolated from arrested cells is not inhibitory towards CDK4 complexes, while p27^Kip1 from proliferating cells is (James et al., 2008). P27^Kip1 inhibitory activity is dependent on its tyrosine phosphorylation on residues 88 and 89 by kinases described above. These phosphorylations deactivate the inhibitory property of p27^Kip1.

One of the mechanisms that may contribute to the inhibitory activity of p27^Kip1 (in addition to active site inhibition), has been proposed to be the prevention of activating phosphorylation of the CDK by CAK via a yet unidentified mechanism (Bockstaele et al., 2006a; Bockstaele et al., 2006b; Kato et al., 1994). Combined, these observations suggested that tyrosine phosphorylation of p27^Kip1 within the CDK-binding region alters the conformation of the protein and promotes phosphorylation by the CDK-activating kinase CAK on Thr172 of CDK4 (Blain, 2008; James et al., 2008). This may in part explain how p27^Kip1 can execute its dual role both as an activator and inhibitor of CDK activity depending on the growth status of the cell and the extracellular signals.

The C-terminus of p27^Kip1 can also be phosphorylated. The last amino acid, Thr198, was reported to be the target of AMPK pathway (Liang et al., 2007) regulating decision between
apoptosis and autophagy. Autophagy can be induced by energy deprivation and AMPK activation. Previously, the same residue had been reported to be phosphorylated by AKT (Fujita et al., 2002), generating a binding site for 14-3-3 proteins. The 14-3-3 proteins generally sequester other proteins in the cytoplasm, and this was found to be the case with p27Kip1 as well (Fujita et al., 2002; Fujita et al., 2003), presenting another mechanism for AKT to exert its pro-proliferative functions on the cell cycle by removing an inhibitor of CDK-Cyclin complexes from the nucleus.

The C-terminal Thr198 residue can also be phosphorylated by RSK1 (Fujita et al., 2003; Larrea et al., 2009), which is an effector of both Ras/MEK/MAPK and PI3K/PDK1 pathways. The RSK1 mediated phosphorylation is an example of regulation of p27Kip1 functions beyond the cell cycle. Larrea et al. showed that RSK1 phosphorylation of p27Kip1 leads to increased RhoA binding, preventing RhoA activation of a signalling cascade involving ROCK and LIM kinase. The inhibition leads to activation of cofilin, which is an actin filament depolymerising factor. Thereby increased p27Kip1 phosphorylation by RSK1 leads to cytoplasmic accumulation of p27Kip1, reduction in stress fiber stabilisation and reduced focal adhesion formation resulting in increased cell motility. As increased motility is a feature of metastatic potential of the cells, and as an increase in MAPK and PI3K signalling is often observed in human tumors, this suggests a potential role for p27Kip1 in motility and metastasis via activation of these signalling pathways, possibly independently of its role in cell cycle progression.

**Mammalian cell cycle entry**

Almost all mammalian cells within an organism are not normally dividing but have exited the cell cycle and are resting in a non-dividing state called G0. To re-enter the cycle a stimulus such as growth factor signalling or change of environment e.g. an increase in nutrients or space, needs to occur. This will lead to expression of D-type cyclins and activation of CDK4/CyclinD complexes. They will in turn phosphorylate the retinoblastoma (RB) protein. In resting cells, phosphorylated retinoblastoma protein binds E2F, a key transcription factor driving S phase specific gene expression, and actively represses the transcription of genes required for cell cycle progression. Like CDKs, E2F is a family of transcription factors, typically in a heteromeric complex with a member from another transcription factor family,
DP. E2Fs 1-3a are activators, while E2Fs 3b and 4-8 are repressors (Attwooll et al., 2004; Trimarchi and Lees, 2002). In quiescent cells the promoters are bound by the repressor E2Fs and RB further recruits chromatin remodelling proteins to ensure repression of the gene transcription (Shao and Robbins, 1995), thus maintaining the G0 state.

G0 is revoked by activation of growth factor pathways which results in phosphorylation of RB as above. This in turn causes replacement of the repressor E2F/DP1 transcription factors with activating E2F/DP1 in the RB-complex, binding of the complexes to DNA and expression of their target genes such as the activator E2Fs themselves (causing a positive feedback loop that ensures irreversibility in the cell cycle transition), cyclin E and A and proteins required for e.g. DNA synthesis such as TK (thymidine kinase) and PCNA (proliferating cell nuclear antigen) (Morgan, 2007).

Retinoblastoma protein was the first tumor suppressor protein identified, first discovered in a childhood cancer syndrome affecting the retina, and later identified as the causative mutation in a variety of cancers including small cell lung carcinoma and osteosarcoma (Dunn et al., 1988; Friend et al., 1986). It has been estimated that loss of RB or deregulation of the RB pathway by mutations in other pathway components happens in most cancers.

Mice lacking both copies of the RB gene die in utero, while mice lacking one allele develop pituitary tumors (Jacks et al., 1992; Lee et al., 1992). Loss of RB in mouse embryonic fibroblasts leads to shortened G1, and cells lacking RB show defects in cell cycle exit under stress or damage conditions (Herrera et al., 1996). A study using E2F activation at a single cell level as a marker, demonstrated that the length of the G1 is directly controlled by the activity of CDK4/6-CyclinD (Dong et al., 2018), further enhancing the link between CDK4/6, RB and cell cycle progression.

Loss of repressor function and hyperphosphorylation of RB in cancer cells can also result from loss of Cdk4 inhibitors such as p16 or p15, overexpression of cyclin D or specific mutations such as R24C in Cdk4 that renders it resistant to inhibition by Ink4 inhibitors. This highlights the importance of loss of G1 control in the development of cancer (Classon and Harlow, 2002).
Two proteins related to RB: p107 and p130, can compensate for RB in case of its loss-of-function mutations. The three are often referred to as the pocket proteins, due to a pocket structure that can bind E2Fs. Despite the structural and functional similarities, they also have distinct roles in cell cycle regulation. Like RB, they repress transcription of E2F target genes but do so mainly by acting as co-repressors for the repressor E2Fs. In addition, p107 and p130 bind cyclin E-CDK2 and cyclin A-CDK2 through a domain not present in RB but similar to one found in p21\(^{Cip1}\) and p27\(^{Kip1}\). By doing so, they also exert inhibitory effects on the cell cycle kinases (Ewen et al., 1992; Faha et al., 1992; Smith and Nevins, 1995; Zhu et al., 1993).

Until recently it was thought that RB is found either in non-phosphorylated state or in variable multiphosphorylated forms with all of its 14-residues hyperphosphorylated. This dogma was challenged by a study which showed that mono-phosphorylated RB, phosphorylated by CDK4/6-CyclinD, is the only RB isoform detected in the early G1 phase. (Narasimha et al., 2014).

**Essential (or not?) regulators of the cell cycle**

A breadth of experiments has helped to construct the current model of the cell cycle and also to prove the vital role of the Cdks in cell cycle regulation. The signal transduction pathways inducing cyclin D expression in G1, and hence Cdk4/6 activity have been well documented (Sherr, 1993). Mutations in CDK-inhibitors have been found in human cancers (Hirama and Koeffler, 1995; Wolfel et al., 1995; Zuo et al., 1996) and injection of antibodies directed to cyclins D, E or A have been shown to block DNA synthesis (Pagano et al., 1992). Therefore, it was surprising when the first study showed that cancer cell growth was refractory to inhibition of Cdk2 by antisense oligonucleotides or expression of a dominant negative form of Cdk2 (Tetsu and McCormick, 2003). This was followed by even more striking observations that ablation of individual CDKs or cyclins seemed to have very little effect in mouse development. Not only were embryonic cell cycles of the knock out animals still occurring, but live mice with only minor observable phenotypes were born (Malumbres and Barbacid, 2009; Satyanarayana and Kaldis, 2009).
In fact, to observe the expected embryonic lethality, several cell cycle regulators had to be knocked out simultaneously. For example, mice lacking any one of the type D cyclins (D1, D2, D3) were viable demonstrating defects only in certain tissues, often corresponding with the expression pattern of the cyclin. For example, cyclin D1 knock-out mice had neurological abnormalities and hypoplastic retinas. Mammary glands of the female Cyclin D1 deficient mice did not undergo normal developmental proliferative changes during pregnancy despite normal hormone levels, but were otherwise normal (Fantl et al., 1995; Sicinski et al., 1995). Cyclin D2-null females were sterile and males had hypoplastic testes (Sicinski et al., 1996), but showed defects only in specific tissues such as cerebellar development (Huard et al., 1999) and pancreatic β-cell proliferation (Georgia and Bhushan, 2004; Kushner et al., 2005). Only when all three were deleted was the animal viability compromised (Kozar et al., 2004).

Similar results were obtained with knocking out CDKs 4 and 6. Deletion of Cdk4 resulted in small mice with proliferation defects in postnatal pancreatic β-cells and pituitary lactotrophs (Mettus and Rane, 2003; Rane et al., 1999; Tsutsui et al., 1999), while Cdk6 null mice display defective erythroid lineage development (Malumbres et al., 2004). Deletion of both results in embryonic lethality but only around day 18.5, suggesting that cell proliferation and organogenesis in the early embryonic development can occur largely unaffected without key G1 CDKs.

The fact that also Cdk2, which was thought to be a master regulator of the mammalian cell cycle, proved to be non-essential for life was another unexpected discovery (Berthet et al., 2003; Ortega et al., 2003). Mice lacking Cdk2 were infertile due to problems in meiosis, which uncovered a previously unknown function for Cdk2 protein. More than a decade later it was uncovered that Cdk2 also controls the structure of the nuclear envelope and the telomere-led chromosome movements essential for homolog pairing in mouse spermatocytes (Viera et al., 2015). This observation further emphasized the vast scope of targets of the central cell cycle kinases.

Indeed, the only essential Cdk based on the knock-out studies turned out to be Cdk1 (Malumbres, 2005). Various reasons for CDK redundancy have been speculated. The highly conserved nature of the proteins probably enables them to substitute for one another in the absence of one of the family members. This has indeed been found to be true as in mouse
embryonic fibroblasts lacking Cdk4 and Cdk6, where Cdk2 was found in complexes with cyclin D (Malumbres et al., 2004). These cells showed delayed entry into cell cycle after G0 arrest, which could be due to the fact that Cdk2 is expressed later in the cycle than Cdk4/6.

**Cell cycle and Cancer**

The results of these various CDK knock-out models took the cell cycle field by surprise. But it soon became apparent that we do not need to fully rewrite the text books and abandon the old models as these mouse models merely suggest that the system has built-in redundancy to ensure a functional cell cycle even when one component should fail. In addition, these mouse models demonstrate that even though e.g. Cdk2 can be substituted in the somatic (but not meiotic) (Berthet et al., 2003; Ortega et al., 2003) cell cycles, regulation of its activity is still a major point of control under normal conditions - and loss of that control can lead to hyperproliferation, one of the hallmarks of cancer.

As cancer is characterized by uncontrolled division of the cells, it is essentially a disease of the cell cycle. Indeed, many of the familial cancer syndromes and somatic mutations in the cell cycle genes demonstrate the role and importance of this control mechanism. Mutations can occur either in the core cell cycle proteins, or the upstream effectors and signalling components regulating the activity of the cell cycle. For example, to acquire uncontrolled growth properties, cancer has to inactivate the RB protein. This can be achieved via several mechanisms, one being mutations in the \(RB\) gene itself. Other methods include hyperactivation of Cdk4 or Cdk6 kinases, as seen in glioblastomas, where Cdk4 is amplified in approximately half of the tumors (Schmidt et al., 1994). This tumor type also demonstrates the key role of this pathway as tumors with no alteration in Cdk4 activity displayed mutations in the \(RB\) gene itself (Ichimura et al., 1996). In addition to amplification, the kinases undergo mutations influencing their activity. In cases of familial melanoma a mutation in Cdk4 (R24C at the p15/p16 binding site) renders it insensitive to inhibitors (Zuo et al., 1996), thereby maintaining RB in phosphorylated state even under growth-restricting conditions.

Deletion or inactivation of CDK-inhibitors also activates the CDKs and thereby inactivates RB. Deletion of the CDKN2A gene, encoding both the p16 CDK-inhibitor and p14ARF tumor suppressor, is the most frequently deleted locus in human cancer, often also silenced
by promoter methylation (Beroukhim et al., 2010). On the other hand, mutations in the CDK-inhibitor p27Kip1 are not frequently found in all tumor types. Yet in the absence of mutations the levels of the protein are often downregulated via enhanced degradation, which has been found to correlate with poor prognosis (Catzavelos et al., 1997; Loda et al., 1997; Porter et al., 1997).

Targeting CDK-activity was proposed as a cancer therapy early on, and many studies have explored this possibility. The first CDK-inhibitors developed were broad spectrum inhibitors with little specificity to individual CDKs. Among these are flavopiridol and R-roscovitine. Although flavopiridol showed antitumour activity in preclinical studies (Arguello et al., 1998), in clinical studies it proved to have insufficient efficacy and low therapeutic index. Likewise, R-roscovitine had a low therapeutic index and did not show significant therapeutic advantage in clinical studies. This is likely caused by the off-target effects of the drugs, causing toxicity due to general inhibition of all CDKs at concentrations required for clinical benefits. Whether these broad-spectrum CDK-inhibitors will show effects at lower concentrations when combined with other conventional therapies such as radiation therapy has not been demonstrated.

The first compounds selective to CDKs are CDK4/6 inhibitors palbociclib, ribociclib, and abemaciclib (Sherr et al., 2016). Palbociclib has been shown to prevent RB phosphorylation by inhibiting CDK4/6 while having little effect on the activity of other CDKs. It showed antitumor activity against several types of human cancers in xenograft models and also crossed the blood/brain barrier and prevented tumor progression in a glioblastoma model (Michaud et al., 2010). Unlike flavopiridol and roscovitine, palbociclib has shown great promise in clinical trials. A study in mantle cell lymphoma patients demonstrated partial response or stable disease in 59% of patients (Leonard et al., 2012). Further studies showed positive responses also in germ cell tumours, non-small-cell lung carcinoma and CDK4-amplified liposarcoma. Screening of different cancer types revealed luminal-type breast cancer cells expressing oestrogen receptor (ER+) to be particularly sensitive to palbociclib (Finn et al., 2009), suggesting that this patient group especially might benefit from palbociclib treatment.

The clinical utility with Palbociclib was unequivocally demonstrated in a trial involving postmenopausal women with ER+ advanced breast cancer. This study compared treatment
with palbociclib combined with the standard-of-care letrozole, an aromatase inhibitor, with
treatment with letrozole alone. Palbociclib nearly doubled the median progression free
survival (from 10.2 months to 20.2 months) (Finn et al., 2015). This prompted an accelerated
approval from the US Food and Drug Administration (FDA), and palbociclib is now used in
the clinic while undergoing several additional clinical trials for other cancer types.

Along with palbociclib several other compounds targeting cell cycle proteins are undergoing
clinical or pre-clinical trials. These include the other CDK4/6 inhibitors ribociclib and
abemaciclib and inhibitors targeting CHK1, WEE1, Aurora A and B or PLKs, kinases that
control the activity of CDKs via various mechanisms. What is clear from these trials is that
none of the inhibitors are suitable for all cancers. Instead, they may prove beneficial for
specific types of cancers and in specific genetic backgrounds, especially when combined with
other or more conventional cancer therapies. Personalised medicine, the development of
genetic diagnostics for individuals, will likely be helpful in tailoring the best treatment
modality and combination of cell cycle inhibitors and other cancer drugs for each cancer and
each patient. Therefore, targeting the cell cycle proteins by small molecule inhibitors, a
strategy considered for cancer treatment initially and then strongly disputed, remains a valid
approach as demonstrated by the recent success (Shapiro, 2006).
TGF-β pathway

TGF-β belongs to the cytokine group of growth factors. It is a secreted molecule of about 25 kDa that elicits responses highly versatile and central in processes such as cell cycle, differentiation, morphogenesis, tissue homeostasis and regeneration (Massague et al., 1992). Deregulation of TGF-β signalling may give rise to severe diseases including fibrosis, hypertension, problems of the reproductive tract (Persistent Müllerian duct syndrome) and cancer (Massague et al., 2000).

The cellular response to TGF-β varies depending on the cell type, state of differentiation and presence of other growth factors (Ravitz and Wenner, 1997). It is a growth promoter in mesenchymal cells, particularly fibroblasts, but inhibits the growth of epithelial, endothelial and lymphoid cells (Massague, 2000). Even in similar cell types it can cause different effects depending on growth conditions or cellular context. An example is provided by the inhibitor of differentiation 1 (ID) gene, which is repressed by TGF-β in mammary epithelial cells (Kang et al., 2003), but induced in metastatic breast cancer cells (Padua et al., 2008).

TGF-β receptor is a heterodimer consisting of type I and type II proteins. The ligand binds the type II dimers, which upon ligand binding recruit the type I receptors forming an heterotetramer. The type II receptors then phosphorylate the type I, leading to receptor activation (Wrana et al., 1992). The phosphorylated receptor binds the cytosolic signal transducers, Smad-proteins. The canonical pathway involves Smads 2 and 3 (S2/3), phosphorylation of which by the activated type I receptors opens the structure to allow binding of the Co-Smad, Smad 4. The trimeric complex is then translocated to the nucleus, where it binds the promoters of the target genes (Massague, 1998) (Figure 3).

The variety of the effects induced by TGF-β signaling is perplexing considering the relatively straightforward pathway induced by the binding of TGF-β ligand to its receptors on the cell surface. Nevertheless, the target genes of this pathway vary depending on the cellular context and also the particular TGF-β ligand/receptor type in question. In the case of TGF-β1 the
target genes are generally involved in apoptosis, extracellular matrix regeneration, immunosuppression and G1 cell cycle arrest (Massague and Wotton, 2000).

**Figure 3. TGF-β pathway.** TGF-β receptor consists of two type I and two type II receptors. Binding of the extracellular ligand activates the heterotetrameric complex and leads to phosphorylation of the R (Receptor)-Smads, most commonly Smad 2/3. The phosphorylated R-Smad then binds the Co-Smad (Smad4) and the complex translocates to the nucleus. In the nucleus the R-Smad/Co-Smad complex binds the TREs (TGF-β-responsive elements) on the target gene promoters and promotes their transcription. One of the best characterised target genes is the CKD inhibitor p15Ink4a.

**PI3-Kinase-PKB/Akt pathway**

The inositol phosphate pathway is a central regulator of cell metabolism and proliferation. It is activated at the cell membrane when binding of a ligand, such as a growth factor, to receptor tyrosine kinase causes the formation of inositol-3-phosphate via PI3 kinase activity. Proteins with plextrin homology (PH) domain bind PIP3 (Phosphatidylinositol (3,4,5)-trisphosphate) and become activated - these include PDK1 (Phosphoinositide-dependent-kinase) and the serine/threonine kinase PKB/Akt (Protein kinase B/Akt). When recruited to
the plasma membrane, PDK1 phosphorylates Akt on Thr308, priming it for second activating phosphorylation of Ser473. The identity of the Ser473-kinase was elusive for a long time, until mTOR (mechanistic Target of Rapamycin) was identified (Jacinto et al., 2006; Sarbassov et al., 2005). It has also been postulated that Ser473 can be phosphorylated by DNA-PK in response to DNA damage (Bozulic et al., 2008). Phosphorylated and activated Akt then translocates from the membrane to the cytosol and nucleus resulting in phosphorylation of its target proteins in these cellular locations.

PKB/Akt is a family of three proteins in humans; PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3. Akt1 is the most ubiquitously expressed and has the broadest range of substrates. Most tissue types express at least one Akt but show some specific patterns - Akt2 being most abundant in insulin responsive tissues and Akt3 in brain. Mouse knock out models recapitulate the pattern. Akt1−/− mice display a general growth retardation and perinatal lethality (Chen et al., 2001), (Cho et al., 2001b), while Akt2−/− mice develop a diabetes-like syndrome (Cho et al., 2001a). Consistent with the expression of Akt3 in brain, the brains of the Akt3−/− mice are smaller than those of the wild type mice (Easton et al., 2005; Tschopp et al., 2005).

Akt is implicated in several essential cellular processes, most of them considered pro-survival/anti-apoptotic. A well-documented example is the FOXO transcription factors, which are direct targets of Akt. Phosphorylation by Akt leads to cytoplasmic translocation and degradation, i.e. deactivation of the gene expression. FOXO-target genes regulate processes such as cell survival (e.g. TRAIL), proliferation (p27, p21), growth (Sestrin3), metabolism (G6PC) and feedback signalling by expression of insulin receptor or Insulin Receptor Substrate 2 expression (Manning and Toker, 2017). One of the first Akt targets discovered was Glycogen synthase 3β (GSK-3β). In addition to Akt signaling, it is involved in glucose metabolism, Wnt/ B-catenin pathway as well as cell cycle regulation. GSK-3β affects the cell cycle directly by phosphorylating cyclin D1 and enhancing its degradation. Phosphorylation of GSK-3β by Akt will inhibit its activity and promote cell cycle progression (Diehl et al., 1998). Akt also regulates cell migration e.g. by direct phosphorylation of actin (Xue et al., 2018). Thus, in the context of cell growth and cell cycle regulation, AKT has a large number of direct and indirect (via FOXO) targets (Figure 4).
The PI3K-Akt axis is negatively regulated by PTEN (phosphatase and tensin homologue), a tumor suppressor frequently mutated in cancer. Upregulation of the PI3K-Akt pathway is commonly observed in cancers. PTEN is one of the most commonly lost tumor suppressor genes, estimated to be deleted or mutated in half of all cancers and being especially prevalent in prostate tumors, while Akt/mTOR mutations are rare (Cairns et al., 1997; Suzuki et al., 1998; Wang et al., 1998).

PTEN dephosphorylates the phosphoinositides turning off the PI3K signals and inhibiting Akt activation. Early studies showed that overexpression of PTEN inhibits the tumorigenicity of human glioblastoma cells and causes G1 cell cycle arrest (Li and Sun, 1998). This effect was found to be exerted by increased levels of p27 and concomitant inhibition of CDK-cyclin complexes.

**Figure 4. PI3-Kinase pathway.** Binding of growth factors to their extracellular receptors leads to production of phosphatidylinositol phosphates, which activate the PI3-kinase. This in turn promotes translocation of Akt to the plasma membrane and causes conformational changes, which enable activating phosphorylations of Akt to occur. Akt is phosphorylated on Thr308 by PDK1 and Ser473 by mTORC2. One of the first Akt substrates identified were the
FOXO proteins. Phosphorylation by Akt leads to the translocation of FOXO from cytoplasm to the nucleus, where it can exert its effect as a transcription factor. Among other Akt substrates known to date are GSK-3β and the CDK-inhibitor p27Kip1.

AMPK pathway

The 5'-adenosine monophosphate (AMP)-activated protein kinase is, as its name indicates, a kinase that monitors the cellular energy status through the low energy adenine nucleotide AMP and gets activated when the AMP/ATP or ADP/ATP ratio in the cells increases, usually as a result of energy expenditure or nutrient deprivation (Hardie et al., 2000). It is a trimeric kinase consisting of a catalytic α and regulatory β and γ subunits (Hardie, 2014; Hardie et al., 2012). There are two isoforms of the α and β subunit and three of the γ subunit in humans, providing several heterotrimeric combinations and divergence of regulation.

In addition to AMP binding to the γ subunit the enzyme activity is increased by phosphorylation of T172 in the regulatory loop of the catalytic subunit by LKB1 (Liver Kinase B1) or CaMKKβ kinases. As mentioned, the activation occurs during e.g. energy stress and leads to phosphorylation of target proteins on both catabolic and anabolic pathways, stimulating the catabolic i.e. energy consuming and inhibiting the anabolic i.e. growth stimulatory processes (Hardie et al., 2012).

AMPK has been implicated in many cellular processes, including but not limited to glucose homeostasis, lipid metabolism, cell growth, autophagy and cell polarity (Mihaylova and Shaw, 2011) (Figure 5). In addition to increased AMP levels or energy deprivation resulting from prolonged exercise, AMPK can be activated by the commonly used painkiller aspirin, or the drug metformin, which is the most widely used drug for type 2 diabetes. Metformin activates AMPK by inhibiting mitochondrial respiration (Hawley et al., 2010). More recently other types of cellular stresses have also been shown to activate AMPK. These include challenges in growth factors, nutrients, oxygen, adhesion or pH that are unfavourable for cell proliferation. Also, more recently added to these challenges of AMPK activating conditions are oncogenic stressors such as Ras and Myc deregulation (Liang and Mills, 2013).
**Figure 5. AMPK pathway.** The AMP-activated protein kinase is activated under energy deprivation. Phosphorylation by LKB1 (or CaMKK2β, not shown) activates AMPK, which in turn phosphorylates and regulates proteins involved in energy metabolism (including fatty acid (FA) metabolism), protein synthesis and cell growth. Other more recently identified substrates for AMPK are cell cycle regulatory proteins such as p27Kip1 and Cdc25.

AMPK is activated by LKB1, which is mutated in the familial Peutz-Jehgers syndrome (Hemminki et al., 1998) (Figure 5) and characterised by the occurrence of multiple intestinal polyps. This was one of the first implications that AMPK pathway could also be misregulated in tumors (Hawley et al., 2003). Many tumor cells switch their metabolism to aerobic glycolysis, also known as Warburg-effect (Liberti and Locasale, 2016; Warburg, 1927), where lactate is produced in the presence of oxygen. This metabolic shift attributed to the high metabolic rate and nutrient demand of the rapidly proliferating tumor cells, further points to a role of AMPK in regulating tumor growth. However, mutations in AMPK are rarely found in tumors. This has been suggested to be due to the requirement for AMPK activity during metabolic stress often found in cancerous tissue (Hardie, 2015). Instead, downregulation of AMPK activity to a level that would still promote tumor growth has been
suggested to occur through different mechanisms, one being the LKB1 mutation, which still allows for some AMPK activity through the activation by an alternative kinase, CaMKK2β. Decreased staining for Thr172 phosphorylation, the key residue required for AMPK activity, in immunohistochemistry analysis of human breast cancer tissue compared to surrounding normal tissue was found in 90% cases investigated (Hadad et al., 2009). Reduced expression of AMPK -alpha2 subunit is also found in hepatocellular carcinoma (Lee et al., 2012).

Consistent with the diverse roles of AMPK in different cellular conditions, the AMPK target proteins are just as diverse (Figure 5). Many of them lie on the core metabolic pathways. Some of the first ones to be identified were acetyl CoA-carboxylase (ACC) (Carlson and Kim, 1973) and 3-hydroxy-3methylglutaryl-CoA reductase (HMGR) (Beg et al., 1973), which regulate fatty acid and cholesterol biosynthesis, respectively. These enzymes, particularly ACC, are still commonly used as an indicator of AMPK activity. However, it must be acknowledged that the AMPK activity towards its various substrates varies depending on the cellular context and the type, duration and magnitude of the stress (Hardie et al., 2016).

In addition to several metabolic enzymes controlling processes such as glucose uptake, glycolysis, β-oxidation and thereby general energy homeostasis (Hardie et al., 2016), a classical target of AMPK lies in another important stress regulatory pathway, the mTOR pathway. The name stands for mechanistic (formerly mammalian) target of rapamycin. Rapamycin is a compound discovered on the Easter Islands (locally known as Rapa Nui), that was found to have anti-fungal, immunosuppressive, and anti-tumor properties. Studies later showed that rapamycin binds FKBP12 to inhibit mTOR, which is required for cell growth and proliferation and regulates several metabolic and protein synthesis and turnover processes (Saxton and Sabatini, 2017).

AMPK was found to phosphorylate the TSC2 protein, also known as tuberin, which is mutated in familial tumor predisposing syndrome called tuberous sclerosis and lies upstream of mTOR. TSC2 and TSC1 proteins form a complex, which acts as a GTPase activating protein (GAP) for Rheb, promoting the exchange of GTP (active) to GDP (inactive) form. This will keep the mTORC1 activity in check, but this function is lost in TSC2 mutants,
causing elevated mTOR activity (Parmar and Tamanoi, 2010). Consistently, AMPK phosphorylation of TSC2 causes inhibition of mTOR activity.

mTORC1 is a complex, the core of which is formed by three components; the catalytic mTOR, regulatory-associated protein of target of rapamycin (RAPTOR) and mammalian lethal with SEC13 protein 8 (mLST8), also known as GβL. The classical mTOR targets are proteins involved in mRNA translation, metabolism and protein turnover. Often used as a marker for mTOR activity are the phosphorylation statuses of p70S6K1 and eIF4E Binding Protein (4EBP). S6K influences the phosphorylation and activation of several substrates involved in mRNA translation initiation (Holz et al., 2005). 4EBP on the other hand inhibits translation by sequestering the eIF4E, and mTOR relieves this inhibition allowing 5’cap-dependent mRNA translation to occur (Brunn et al., 1997; Gingras et al., 1999).

There are two distinct complexes of mTOR; TORC1, which is the rapamycin-sensitive TOR complex, and the rapamycin-insensitive TOR complex 2 (TORC2). The mTORC2 complex consists of RICTOR (rapamycin-insensitive companion of target of rapamycin) in place of RAPTOR and in addition also contains an additional protein called mammalian stress-activated protein kinase interacting protein (mSIN1). While mTORC1 is viewed as the main regulator of growth and metabolism, mTORC2 tends to regulate processes involved in proliferation and survival (Oh and Jacinto, 2011). It does this via two main routes discovered to date – phosphorylation of the AGC family of protein kinases, as exemplified by PKCα, which regulates the actin cytoskeleton (Jacinto et al., 2004; Sarbassov et al., 2004). The discovery of mTORC2 as the Ser473 kinase of Akt/PKB (Jacinto et al., 2006; Sarbassov et al., 2005) provided an important missing link in the regulation of the PI3K-pathway, and proved another example of the crosstalk between different growth promoting and inhibiting pathways, which will be discussed in more detail later.
DNA damage pathways in cell cycle regulation

While cancer is basically the result of uncontrolled cell proliferation, another hallmark of cancer cells is their genomic instability (Jackson and Bartek, 2009). DNA synthesis has also been one of the main targets of cancer therapy for decades, utilising radiation or DNA-damaging agents to target the rapidly dividing cancer cells (Lord and Ashworth, 2012). This has, however, had the obvious drawback of damaging the healthy cells alike, causing chemotherapy side-effects resulting from the damage to rapidly cycling cells in the body like hair follicles or intestinal epithelia.

All cells encounter various DNA insults daily. These include e.g. UV from sunlight, genotoxic compounds in the environment (e.g. tobacco smoke), reactive oxygen species produced by cellular metabolic processes, and in particular, DNA metabolism. If the damage in the DNA is left unrepaired, the cells accumulate a growing number of lesions, which could eventually confer growth advantage and cell pre-malignant states. To maintain genomic integrity, several partly overlapping DNA lesion surveillance and repair systems exist (Jackson and Bartek, 2009). Upon recognition of DNA damage, the surveillance pathways are activated and cause the cell cycle to halt until the damage has been repaired by the dedicated repair pathways. Alternatively, if the damage is too severe, a cellular suicide program will be initiated leading to apoptosis, i.e. programmed cell death, to prevent the propagation of the damaged genome (Morgan, 2007). While the core of the model still holds true, a far more complex and context-dependent picture has emerged in recent years.

A key concept, intricately connected to the cell cycle, is the activation of DNA damage checkpoints. These are monitoring and decision points that enforce arrest of the cell cycle only at specific stages of the cycle. By doing so they ensure both time for the repair as well as prohibit inheritance of genetically altered genomes.

It has been long recognised that the signalling pathways that are activated as a result of genomic insult vary depending on the insult. UV irradiation from sunlight and cisplatin (used in cancer therapy), for example, cause bulky DNA adducts, 6-4 photoproducts and pyrimidine dimers (Ravanat et al., 2001). These are usually recognised by the nucleotide excision repair machinery (NER). The damage caused by ionising radiation (IR), on the other
hand, results in double strand breaks, which, when left unattended, can cause severe problems during chromosome segregation in mitosis. In the presence of undamaged sister chromatid the homologous recombination repair (HRR) pathway, which is considered rather accurate and efficient, is activated (Moynahan and Jasin, 2010). In the absence of such template non-homologous end-joining (NHEJ) pathway can be utilised, although it is considered more error-prone (Ceccaldi et al., 2016). HRR pathway is active in S/G2 when cells have two copies of each chromosome, while the cells in G1 without the homologous sister chromatid will have to rely on the NHEJ pathway.

Minor DNA damage may not cause cell cycle arrest or apoptosis if the damage can be enzymatically reversed or repaired quickly, like in case of base modifications, single nucleotide changes or deletions. However, when the damage is more extensive, two specific kinases, ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia and Rad3-related), are recruited to the site of damage. They activate a series of events, the outcome of which depends on the extent and type of damage as well as on the stage of the cell cycle.

In the event of ionising radiation, which generates double strand breaks, accessory protein ATRIP recruits ATM to the site of damage, that is recognised by MRN-complex composed of three proteins: Mre11-Rad50-Nbs1. ATM phosphorylates the S139 on histone variant H2AX on the DNA adjacent to a DSB. The appearance of the γH2AX is often used as a marker for double strand breaks. In G1 activated ATM phosphorylates Chk1 kinase, which in turn results in the stabilisation of the tumor suppressor p53 and expression of its target genes, among which is the CDK inhibitor p21. Induction of p21 leads to cell cycle arrest until the damage has been repaired. If the damage is too severe for repair, apoptotic p53 target genes such as Puma and Bax get activated.

Different mechanisms exist during S phase, when cells also shift from the NHEJ to the HEJ if new template is available. During S phase single stranded DNA is also present around the replication fork, causing activation of ATR in addition to ATM. ATR phosphorylates Chk1 and initiates another signalling cascade leading to phosphorylation and ubiquitinylation of Cdc25A phosphatase. Cdc25A would normally remove the inhibitory phosphorylation at Thr14/Tyr15 in Cdk2 and Cdk1, so activation of Chk1 will lead to inactivation of the Cdks and cell cycle arrest.
Interestingly, Cdk activity is not inhibited by p21Cip1 during S phase, as p21Cip1 is constantly degraded by a ubiquitin ligase present on the replication fork (Abbas et al., 2008; Havens and Walter, 2011; You et al., 2002). Interfering with p21Cip1 degradation during S phase leads to re-replication, a phenotype also resulting from CDK1 inactivation (Diril et al., 2012). Instead, during S phase the cell cycle checkpoint relies on Wee1 kinase, which is expressed in S phase and will keep the inhibitory Thr14/Tyr15 phosphorylation on Cdk1 and Cdk2 kinases until DNA synthesis is complete (Chow et al., 2003). Wee1 may also have a major role in checkpoint control in G2, given that Wee1 inhibitors override the G2 checkpoint in p53 mutant cells (Hirai et al., 2009). In addition, degradation of p21Cip1 is no longer inhibited after S phase, which ensures the prolonged cell cycle arrest in p53 positive cells after damage (Bunz et al., 1998).

DDR is actively inhibited during mitosis. Instead, the cell operates a spindle checkpoint, which assures the correct attachment of sister chromatids to the mitotic spindle. A single unattached kinetochore is sufficient to generate a “wait anaphase” signal until all chromosomes are attached and correctly aligned. This is achieved by inhibiting APC^Cdc20 and thereby securing destruction. Upon securing degradation separase is released and will degrade cohesin, leading to release of sister chromatid cohesion (Pinsky and Biggins, 2005).

**Crosstalk of growth, survival and nutrient sensing signalling pathways**

It has become evident in recent years that the linear and unidirectional models of signalling cascades initially depicted are rather simplified. The pathways diverge into different directions and outputs depending on the combination of signals and stimulus in the cellular surroundings. In addition, there are multiple points of crosstalk between the pathways, whereby one signalling pathway can affect another. This can be achieved via a shared component, most often a protein, that exists on both pathways and can influence either or both, depending on the signal(s) received. I will next review the pairwise crosstalk between the key cell cycle regulating signaling pathways.
**TGF-β and AKT**

TGF-β and PI3K/Akt-pathways both control an overlapping array of cellular processes from survival to apoptosis and migration to proliferation. Both pathways are heavily involved at various stages of carcinogenesis. Initially it seemed that the linear models of the pathways antagonised each other in many aspects, TGF-β being growth inhibitory and PI3K growth promoting. The crosstalk of the two pathways which has emerged has changed this notion and provides an excellent example of the multiple levels and feedbacks different pathways can exert on each other and also the effects it may have on cellular and organismal level.

Firstly, TGF-β signalling can integrate with the AKT pathway at transcriptional level. The Smads partner with the Akt-substrate FOXO-proteins (Seoane et al., 2004) and induce a specific set of target genes. In human keratinocytes 11 genes out of 115 induced by TGF-β were FOXO-dependent (Gomis et al., 2006), demonstrating signal diversion and specificity in this cell type. On the other hand, the FOXO gene is also a target the TGF-β-SMAD signalling pathway (Naka et al., 2010) providing potentially multiple layers of regulation and crosstalk between the signals.

TGF-β has a dual role in cancer, being growth-restrictive in normal cells but becoming tumour-promoting during later stages of carcinogenesis. One process involved in the tumour-promoting aspects of TGF-β is epithelial-to-mesenchymal transition (EMT), which also includes many points of crosstalk between the pathways. In EMT epithelial cells acquire mesenchymal properties, such as decreased adhesion and increased migration – phenotypes often linked to their increased metastatic potential. EMT requires the activation of cytokines, and among these, TGF-β is known to be the most potent EMT-inducing agent. The PI3K-pathway, on the other hand, had been established as cancer promoting via its pro-proliferative and anti-apoptotic effects. However, it was shown that the AKT pathway is required for TGF-β induced EMT (Bakin et al., 2000). Induction of EMT by bone morphogenic protein 2 (BMP-2), another TGF-β family member, is also dependent on Akt activation (Chen et al., 2011). One mechanism by which the induction of Akt-pathway by TGF-β can occur is by direct association of the PI3K-subunit p85 with the TGF-β receptor type II (Yi et al., 2005). This binding occurs during TGF-β stimulation and leads to activation of the mTOR pathway (Lamouille and Derynck, 2011).
Recently the AMPK activator metformin was shown to prevent fibrosis by downregulating TGF-β signalling (Zheng et al., 2017). Also, TGF-β1 pathway may intersect in hepatic gluconeogenesis via the AMPK-FoxO1 signalling (Yadav et al., 2017).

**PI3K and AMPK**

The effects of Akt and AMPK on cellular levels are intertwined and often opposing. Even though both kinases can stimulate glucose uptake in metabolic tissues, they do so in response to different stimuli; Akt in response to insulin and AMPK in response to ATP depletion during energy stress, although both lead to the increased translocation of the Glut4 transporter to the plasma membrane (Cong et al., 1997; Lund et al., 1998; Summers et al., 1998). The signalling converges at the point of Rab-GAPs TBC1D1 and TBC1D4 (AS160), which are differentially expressed in adipose tissue and skeletal muscle, TBC1D1 being predominantly expressed in skeletal muscle while AS160 is more abundant in adipose tissue. Phosphorylation by the kinases leads to stimulation of RAB-mediated GLUT4 translocation (Chavez et al., 2008; Eguez et al., 2005; Sano et al., 2003), apparently mediated by AMPK-TBC1D1 in muscle in response to energy stress during exercise and via AS160 in adipose tissue in response to insulin signalling and Akt activation.

Regarding energy consumption the two pathways seem to be the polar opposites – AKT promoting the energy-consuming anabolic processes while activated AMPK blocks anabolic metabolism and favours ATP-producing catabolic processes (Mihaylova and Shaw, 2011). They also have opposing effects on processes such as protein and lipid synthesis, autophagy and glycogen synthesis. The AMPK energy-sensing pathway and the growth-promoting phosphoinositide 3-kinase (PI3K)–AKT cascade converge on mTOR with opposing regulatory effects, with the effect of AMPK activation being dominant over AKT-dependent pathways (Hahn-Windgassen et al., 2005).

The central convergence point is the TSC complex that negatively regulates mTOR, and TSC2 was identified as a direct Akt substrate based on consensus sequence prediction and *in vivo* data, also demonstrating that in cells lacking PTEN the Akt-sites were constitutively phosphorylated leading to loss of mTOR inhibition (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002), suggesting that AKT activation functions to stimulate mTOR under
appropriate growth conditions. Furthermore, TSC2 was found to be directly phosphorylated by AMPK, and these modifications were found to be critical in mediating the AMPK-dependent inhibition of mTOR signalling in inhibiting cell growth and in protection against apoptosis during glucose starvation (Inoki et al., 2003; Manning et al., 2002). Yet these results also pointed out that there might be other regulatory inputs from AMPK to mTOR as AMPK was able to inhibit mTOR also in cells lacking TSC2, even though not to the same extent. This was resolved when it was shown that AMPK can also target the Raptor subunit of the mTORC1 complex and this phosphorylation causes inhibition of mTOR activity (Gwinn et al., 2008). Interestingly, this phosphorylation was found to be required for cell cycle arrest in response to energy stress, suggesting that AMPK could function to prevent entry into S phase if the energy supplies are insufficient. This suggests the existence of a metabolic checkpoint in G1. Further enhancing the model of AMPK as a metabolic checkpoint sensor are the findings that AMPK can also delay mitotic entry by phosphorylating Cdc25C phosphatase. This prevents the dephosphorylation of the inhibitory phosphorylation Tyr14/Thr15 on Cdk1 (by Wee1 kinase) preventing Cdk1-targeted phosphorylation of critical mitotic substrates (Shen et al., 2018).

Akt has been reported to phosphorylate AMPK directly, even though the site is a slightly modified version of the AKT consensus motif. It was found that phosphorylation at Ser485 (in rats, Ser 487 in humans), antagonises or prevents the T172 phosphorylation and activation by LKB1 (Horman et al., 2006). This phosphorylation appears to occur mainly in the α1 subunit and to downregulate AMPK activity in tumor cells (Hawley et al., 2014).

Akt and AMPK also converge at transcriptional level, both regulating the FOXO transcription factor. As mentioned above, FOXO was one of the first AKT substrates to be identified and Akt phosphorylation creates a recognition site for 14-3-3 proteins and results in cytoplasmic translocation of FOXO. This downregulates the activity of processes regulated by FOXO proteins such as cell cycle (via induction of p27Kip1/p21Cip1 cell cycle inhibitors), apoptosis (BIM/PUMA) or catabolism and growth inhibition (Sestrin 3, MAP1LC3B) (Webb and Brunet, 2014). In contrast to AKT, AMPK phosphorylation of FOXO does not influence its subcellular localisation but instead increases its activity (Greer et al., 2007), having an antagonistic effect on the processes mentioned above in comparison with AKT.
Another AKT substrate that features on the AMPK-mTOR pathway is GSK3β, the first AKT substrate to be described (Cross et al., 1995). GSK3β phosphorylates a variety of substrates involved in survival (PTEN), proliferation (c-Jun, c-Myc) and metabolism (TSC2, HIF1α). GSK3β generally recognises proteins that have been phosphorylated by a priming kinase, and the additional phosphorylation by GSK3β leads to their degradation. Phosphorylation of GSK3β by AKT creates a pseudosubstrate within the enzyme, blocking access of the substrate and preventing their degradation (Dajani et al., 2001; Frame et al., 2001; ter Haar et al., 2001). Thereby AKT promotes the processes that GSK3β inhibits. In this manner GSK3β phosphorylates glycogen synthase to inhibit glycogen synthesis under energy stress, while AKT phosphorylation prevents the degradation of glycogen synthase. AMPK functions in a manner analogous to GSK3β and antagonistic to AKT in relation to glycogen synthase, similar to the many metabolic processes they regulate. Therefore, although an interesting suggestion, the report that GSK3β can directly phosphorylate AMPK to inhibit it’s activity (Suzuki et al., 2013), has been questioned as the physiological context and relevance seems to be obscure (Hardie, 2014).

**DNA damage and AMPK**

The canonical AMPK pathway places the kinase upstream of TSC and mTOR and points to its role in control of cellular energy metabolism. However, recent work suggests that roles of AMPK may expand beyond this and it could play a role in cellular responses to DNA damage. One of the main cellular effectors induced after genotoxic insult is the p53 protein that regulates both cell cycle arrest or apoptosis. AMPK activation was found to induce the phosphorylation of p53 leading to cell cycle arrest (Jones et al., 2005), and this enhanced cell survival after glucose deprivation.

Another link between metabolism and p53-mediated genotoxic stress was discovered by investigating Sestrins, poorly characterised proteins that had previously been linked to oxidative stress (Budanov et al., 2004). Sestrins were found to be p53 target genes whose expression activated AMPK and AMPK phosphorylation of TSC2, thereby inhibiting mTOR (Budanov and Karin, 2008). Sestrin-deficient cells were shown to have decreased AMPK and heightened mTORC1 activity both under normal growth conditions and after stress (Budanov and Karin, 2008; Lee et al., 2010; Wempe et al., 2010). In mammalian cells Sestrin2 was
found in a complex with TSC1, TSC2, and AMPK (Budanov and Karin, 2008), although the exact mechanism by which it activates AMPK is not clear. However, these discoveries would indicate that after genotoxic stress AMPK could play a role in the adaptation of cellular processes that mediate cell survival and/or metabolic responses such as shutting down mTOR and anabolic processes.

Interestingly, ionising radiation (IR) induces phosphorylation of LKB1, the AMPK-activating kinase (Sapkota et al., 2002), but the effects on AMPK were not investigated at the time. Another group showed activation of AMPK by etoposide, a DNA damaging agent (Fu et al., 2008). As this response was absent in ATM deficient cell lines it led to the assumption of existence of a kinase cascade from ATM to LKB1 to AMPK. Sanli and colleagues also demonstrated activation of AMPK by IR (Sanli et al., 2014). However, their data showed that this activation occurred also in a LKB1 deficient cell line A549. This led other investigators to address the issue and determine that AMPK was activated in several cell types that lacked LKB1, as well as in some expressing LKB1, while they initially detected no activation in LKB1-negative HEK293 cells (Vara-Ciruelos et al., 2018). Further analysis, however, showed that AMPK phosphorylation was increased also in these cells by etoposide, but only within the nuclear fraction. The phosphorylation was dependent on the increase in nuclear Ca²⁺, which causes activation of CaMKK2. This restricts the activation of AMPK to the nucleus and is also specific for the alpha1 subunit, making an interesting point as alpha1, but not alpha2, is amplified in human cancer (Monteverde et al., 2015). Others also demonstrated the increase in the activating phosphorylation of AMPK-alpha1 (T172) by a variety of stresses including UV- or gamma-irradiation and camptothecin (Bungard et al., 2010). The authors further demonstrated that under genotoxic stress AMPK colocalised with chromatin and phosphorylated histone H2B (Ser36). Both AMPK and histone H2B phosphorylation colocalised at the promoters and transcribed regions of the genes known to be activated by AMPK pathway. This provides yet another level at which AMPK can regulate cellular responses to metabolic stress.

Other points of crosstalk relevant to cell cycle

Sestrins have also been found to be linked with the TGF-β pathway, as cells lacking Sesn2 show not only elevated mTOR activity but also increased phosphorylation of TGF-β target
genes (Wempe et al., 2010) Combined deletion of Sesn2 and a TGF-β regulatory protein LTB4S (Latent TGF-β binding protein 4, small isoform) rescued the COPD-like phenotype of the LTB4S-knockout mice (Wempe et al., 2010), suggesting cross-regulation of these two pathways at least in the context of COPD (Chronic Obstructive Pulmonary Disease). This would place Sestrins both on the DNA damage and TGF-β pathways.

It is logical that metabolic stress should impact the cell cycle progression, as production of two cells requires vast amounts of amino acids, nucleotides and other building blocks. AMPK was shown to regulate many of these processes leading to G1 arrest, often by induction of p53 and its target gene p21 (Jones et al., 2005). Prolonged activation of AMPK and persistent p21 expression on the other hand lead to cellular senescence.

The regulation of G2/M transition by AMPK was less well established until recently, when it was shown that AMPK can exert an inhibitory phosphorylation on the Cdc25C phosphatase. This promotes the activity of Wee1 and inhibits G2/M transition, as mentioned in previous chapter (Shen et al., 2018).

Another interesting aspect of the growth signals influencing AMPK activity is the finding that the cell cycle kinase CDK4 phosphorylates the AMPKα2 subunit thereby promoting anaerobic glycolysis and repressing fatty acid oxidation (Lopez-Mejia et al., 2017). Considering the frequent perturbations in cancer that interfere with CDK4 activity, such as its overexpression or inhibitor inactivation, and the fact that since the times of Otto Warburg aerobic glycolysis has been considered a central feature of cancer cells (Warburg, 1927), this provides a molecular link between the control of the cell cycle control and metabolism. Signals can also travel from the cell cycle machinery to AMPK. Cdk4 knockout mice show altered glucose homeostasis, mitochondrial defects and a diabetic phenotype due to proliferation defects of pancreatic β-cells (Rane et al., 1999). Interestingly in normal pancreatic β-cells Cdk4 is induced by insulin, suggesting that Cdk4 may play a role in certain metabolic processes. Furthermore, it has been shown that Cdk4 promotes glycolysis and inhibits fatty acid oxidation, demonstrating the influence of Cdk4 on cellular energy homeostasis (Lopez-Mejia et al., 2017). This study also found that this was achieved by direct phosphorylation of AMPK and in particular the alpha2 subunit, by CDK4-CyclinD3.
kinase complex. This provides an example on the specificity of the Cdk-cyclin complexes, but also identifies the substrate-specificity of the AMPK subunits. Similar to the selective activation of AMPKalpha1 in the nuclear compartment after genotoxic stress, the activation of AMPKalpha2 by CDK4 to regulate fatty acid metabolism, reveals the diversity and complexity of the regulatory pathways involved both in cell cycle control and cellular metabolism.
Conclusions

The purpose of the molecular machinery described above is to respond to different external and internal cues and to maintain the homeostasis of cell size and numbers within a tissue. When this control fails, it may lead to hyperproliferation under conditions where it is not desired. In the absence of further control mechanisms that can eliminate these cells by apoptosis or other forms of cell death, hyperproliferation and accrual of other genetic changes will eventually lead to cancer.

Although the mouse knock-out models have demonstrated that individual Cdk, cyclins or even their combinations, are redundant for cell cycle progression *per se*, it has been disputed that such redundancy does not undermine the potential importance of the individual genes and proteins in normal cell cycle or cancer. Indeed, while another Cdk could easily substitute for a family member that had been artificially removed from the genome, under normal conditions gain-of-function mutations of positive regulators and loss-of-function mutations of inhibitors are important events in progression of individual cancers.

Appropriate control of the cell cycle is required for the maintenance of tissue homeostasis within an organism and preventing cancer growth. Forty years after the first discovery of the molecular regulators of the cell division machinery, we now understand more than ever about the complexity of the regulatory processes. In the recent years, this has led to new therapeutics and clinical successes in cancer treatment. Yet the complexity of cancers, the cell division cycle and the related signalling pathways means that more potential therapeutic targets and modalities await their discovery.
Aims of the present study

This study aims to investigate the effects of external growth and stress signals on the cell cycle and its core regulators, and to elaborate mechanisms of crosstalk between the pathways. The specific aims are:

1) To characterise the function of the non-cdk-bound form of p27 (p27\textsuperscript{NCDK}) and examine its role in cell cycle regulation after different growth- or stress stimuli.

2) To investigate the crosstalk between TGF-β and PI3-kinase in regulating p27\textsuperscript{NCDK}, and uncover the mechanisms by which insulin may influence TGF-β signalling.

3) To analyse changes in the cell cycle regulators that occur after genotoxic stress in a human prostate model and explore the underlying features contributing to the multifocality of this cancer type.
Materials and Methods

Cell Culture

The cell lines used in this study are listed in the table below:

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description and reference</th>
<th>Used in</th>
<th>Culture medium</th>
</tr>
</thead>
</table>
| Mv1Lu      | Mink lung epithelial cell line  
(CCL-64, ATCC) | I       | DMEM + 10% FCS                                       |
| HeLa       | Human ovarian cancer cell line  
(ATCC) | I       | DMEM + 10% FCS                                       |
| AMPK α1−/α2lox/lox | Mouse embryonic fibroblasts  
(Study I) | I       | DMEM (high glucose) + 10% FCS  
glutamine, puromycin, hygromycin |
| U-2 OS     | Human osteosarcoma  
(ATCC) | II      | DMEM + 15% FCS                                       |
| 293T       | Human kidney epithelia  
(ATCC) | II      | DMEM + 10% FCS                                       |
| Cos7       | African green monkey kidney fibroblast-like  
(ATCC) | II      | DMEM + 10% FCS                                       |
| MDA-MB 435 | Human melanoma  
(ATCC) | II      | RPMI + 10% FCS                                       |
| HPEC       | Human Prostate Epithelial Cells  
(ATCC) | III     | MCDB 105 with epithelial cell-specific supplements  |

All cells were cultured in humidified atmosphere containing 5% CO2 at +37°C.
Methods

Methods and reagents used are described within the original publications and summarised in the table below:

<table>
<thead>
<tr>
<th>Method</th>
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<tr>
<td>Transfections</td>
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<td>Immunoblotting and preparation of cell lysates</td>
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<td>RT-PCR</td>
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<tr>
<td>Gamma irradiation</td>
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<tr>
<td><em>Ex vivo</em> Prostate Tissue Cultures</td>
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</table>
Results and Discussion

Multiple pathways regulate the CDK-inhibitor p27Kip1 (I)

*p27Kip1* CDK-inhibitor binds to all CDK-cyclin complexes. However, the outcome of these interactions depends on the CDK and its cyclin partner. While being a strong inhibitor of CDK2-cyclin E complexes, *p27Kip1* can act as an assembly factor for the CDK4 - cyclin D complexes. Inhibitor-of-CDK4 (Ink4) proteins, as their name suggests, only bind to CDK4/6. *p15Ink4* belongs to the Ink4-family of proteins, and is induced by TGF-β. TGF-β was also found to induce a form of *p27Kip1* detected by a specific antibody. This form was not found in complexes with CDKs as immunoprecipitation and kinase assay experiments did not detect any associated CDKs or kinase activity that would have precipitated with the *p27Kip1* recognised by the antibody (*p27NCDK* for non-CDK-bound *p27*) (Taipale et al., 2000). We therefore hypothesised that this form could represent the *p27Kip1* that is replaced from CDK4/6 complexes by *p15Ink4* and could be the result of a post translational modification or complex reorganisation, as induction of the form was only evident under non-denaturing conditions. To test this, we used a cell culture model, where different kinase combinations were transfected into mink lung epithelial (Mv1Lu) cells (I, Fig 1). Overexpression of CDK-cyclin complexes diminished the amount and induction of *p27NCDK*, while *p15Ink4* increased the basal levels of *p27NCDK* and abolished the induction by TGF-β. This finding provided support to the notion that the CDK-cyclin bind several inhibitors possibly in a competitive manner.

We wanted to extend the initial observation of TGF-β regulation of *p27NCDK* further by investigating the potential role of other signalling pathways. We discovered that *p27NCDK* was also induced by compounds inhibiting the PI3-kinase and MEK growth-promoting pathways (I, Fig. 2). The induction was both time- and concentration-dependent.

As *p27Kip1* was reported to be the target of Akt/PKB kinase downstream of PI3K (Fujita et al., 2002; Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002), we wanted to analyse the role of Akt/PKB in more detail. We found that LY-294002, a small molecule inhibitor against Akt/PKB, induced *p27NCDK* alone and also potentiated the TGF-β effect. We also discovered that the basal levels of *p27NCDK* varied between cell lines. While most cell lines
had low basal levels of p27\textsuperscript{NCDK} (below 10% positive in exponentially growing cells), some cell lines like the ovarian-cancer-derived HeLa cells and melanoma cell line G361 had substantially higher percentage of p27\textsuperscript{NCDK} positive cells under normal growth conditions, as assessed by immunofluorescence (unpublished data, Björklund et al.). Interestingly, we found that overexpression of a constitutively active Akt/PKB construct in the HeLa cell line with high basal p27\textsuperscript{NCDK} levels downregulated p27\textsuperscript{NCDK} levels (I, Fig. 3c and d), suggesting that differences in the basal signal regulation play an important role.

The fast proliferation of cancer cells means higher requirements for many building blocks and therefore a high metabolic rate. The altered metabolism and aerobic glycolysis of cancer cells is a well-known feature of the disease, and the roles of many metabolic regulators such as AMPK has become increasingly recognised in cancer. As AMPK was suggested to phosphorylate p27\textsuperscript{Kip1} (Liang et al., 2007), we wanted to investigate the effect of the conditions inducing this kinase on p27\textsuperscript{NCDK}. We discovered that many types of cellular stresses known to activate AMPK also caused an induction of p27\textsuperscript{NCDK}, including osmotic and respiratory stresses (I, Fig. 4a). Further analysis using small molecule activators for AMPK also demonstrated p27\textsuperscript{NCDK} induction, suggesting that AMPK may regulate p27 either directly or indirectly (I, Fig. 4b). We then used AMPK knock-out cell lines and observed that p27\textsuperscript{NCDK} induction by cellular stress was attenuated in the absence of AMPK activity (I, Fig. 4a). On the other hand, ablation of growth factors induced p27\textsuperscript{NCDK} both in the presence and absence of AMPK activity. However, some responses with cellular stress remained and the effects on p27\textsuperscript{NCDK} seemed to be independent of the effects on the cell cycle phase distribution. Strongest connection between p27\textsuperscript{NCDK} induction and AMPK activity was found in relation to metabolic stress and PI3K-inhibition (I, Fig. 5f).

To be able to further dissect the role and identity of p27\textsuperscript{NCDK}, we wanted to confirm that it represents the non-cdk-bound pool of p27\textsuperscript{Kip1} in the cell. As it was detected by a monoclonal antibody, we used phage display to map the epitope of the antibody. This approach confirmed that the epitope of the antibody resides at the CDK-binding region of p27\textsuperscript{Kip1}, so can therefore detect p27\textsuperscript{Kip1} only when the epitope is not masked by CDK-proteins (unpublished data and I, Fig. 1a).

Next, we wanted to produce polyclonal antibodies against this region as the affinity of the monoclonal antibody was frequently insufficient for some applications such as
immunoprecipitation. We used two different peptides for immunisation of rabbits; the sequence of the peptides was based on the results of the phage display and in silico analysis of the immunogenicity and represented the CDK-binding region and epitope of the monoclonal antibody. Unfortunately, we were unable to obtain high affinity antibodies that would have been specific enough for $p27^{NCDK}$ (unpublished data).

To characterise whether $p27^{NCDK}$ was bound to other known or unknown interaction partners, we used gel filtration chromatography followed by immunoprecipitations with the $27^{NCDK}$ antibody from the fractions. Cells were either exponentially growing or treated with TGF-$\beta$. However, the levels of $p27^{NCDK}$ after TGF-$\beta$ treatment, filtration and immunoprecipitation followed by western blotting, proved to be too low for detection. As the induction of $p27^{NCDK}$ after TGF-$\beta$ is more modest than after serum starvation, we next decided to use serum starved cells. However, also this approach did not yield sufficiently consistent results; although we were able to identify $p27^{NCDK}$ from the low molecular weight fractions after starvation, which would represent the unbound form. A higher molecular weight complex was detected in one experiment, possibly representing $p27^{Kip1}$ bound to heat shock proteins or other chaperones, but the identity of this complex remained elusive (unpublished results).

It was plausible to speculate that post-translational modifications in response to different growth- and stress-stimulus would play a role in $p27^{NCDK}$ induction. We therefore first investigated if phosphorylation status of $p27^{Kip1}$ would play a role in the recognition by the antibody. For this aim we constructed several different $p27^{Kip1}$ mutants with either unphosphorylatable alanine or phospho-mimicing aspartic acid in place of the amino acid targeted for phosphorylation. These constructs were made into GFP-, HA- and HIS-tagged vectors and included mutants for Ser10, Thr157 and Thr187 and combinations of them. It turned out that the high expression levels of all constructs lead to high abundance of "free" $p27^{Kip1}$ available for antibody recognition. Under overexpression conditions no differences between the constructs were detected in regard to $p27^{NCDK}$ status (unpublished data). We also addressed the question of sumoylation in $p27^{NCDK}$ regulation, and although we could detect sumoylated $p27^{Kip1}$ when Sumo-1 was overexpressed, we were not able to pinpoint any correlation with $p27^{NCDK}$ and sumoylation (unpublished data).

As several growth promoting and inhibiting pathways had been identified as regulators of $p27^{NCDK}$, we wanted to expand the potential network of regulators and used a human kinome
Open Reading Frame (ORF) expression (Varjosalo et al., 2008) library to find potential new pathways affecting p27\textsuperscript{NCDK} abundance. High-content automated microscopy was used to analyse the effect of individual kinases. Mv1Lu cells were co-transfected with the kinome library and GFP in duplicate and followed by staining for p27\textsuperscript{NCDK} by immunofluorescence. The amount of double positive cells in the sample was used as an endpoint. The approach proved to have some caveats as the general transfection toxicity alone caused an increase in p27\textsuperscript{NCDK}. High expression of GFP also increased p27\textsuperscript{NCDK}. The levels of the kinases could also vary, although being expressed from the same plasmid backbone, potential differences in translation and degradation rate could influence the amount of active kinase in the cells. To be able to better assess this, the kinases having showed the highest induction of p27\textsuperscript{NCDK} in duplicate samples were cloned into HA-vector. These were then transfected and co-staining for p27\textsuperscript{NCDK} and HA was performed followed by analysis with high content microscope (unpublished data). Difficulties in data interpretation were caused by high variability in the p27\textsuperscript{NCDK} background level. Various reasons for this were speculated; potentially the transfection toxicity was highly dependent on the cell confluency at the time of transfection. Although the same number of cells was plated in all experiments and transfections were performed the day after plating, cell doubling time may have differed depending on the passage number of the cells. The toxicity experienced by the cells could account for the increase in the basal p27\textsuperscript{NCDK}. This caused a background variation between 5-40 \% of positives in cells transfected with GFP alone. This may in part have contributed to the fact that the "hits" identified in the screen varied between experiments. A pathway analysis to highlight any potential new or known pathways as regulators of p27\textsuperscript{NCDK} proved inconclusive. Some of the more consistently identified hits from the screen are shown in the Table I below:
TABLE 1
Putative p27\textsuperscript{NCDK} -regulating kinases from a human kinome screen.

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Description</th>
<th>Group</th>
</tr>
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<tbody>
<tr>
<td>ADRBK1</td>
<td>adrenergic, beta, receptor kinase 1</td>
<td>AGC</td>
</tr>
<tr>
<td>ACVR2B</td>
<td>activin A receptor, type IIb</td>
<td>TKL</td>
</tr>
<tr>
<td>GSK3A</td>
<td>glycogen synthase kinase 3 alpha</td>
<td>CMGC</td>
</tr>
<tr>
<td>PRKX</td>
<td>protein kinase, X-linked</td>
<td>AGC</td>
</tr>
<tr>
<td>PAK6</td>
<td>p21(CDKN1A)-activated kinase 6</td>
<td>STE</td>
</tr>
<tr>
<td>PRKY</td>
<td>protein kinase, Y-linked</td>
<td>AGC</td>
</tr>
<tr>
<td>GPRK5</td>
<td>G protein-coupled receptor kinase 5</td>
<td>AGC</td>
</tr>
<tr>
<td>CAMKK2</td>
<td>calcium/calmodulin-dependent protein kinase kinase 2, beta</td>
<td>Other</td>
</tr>
<tr>
<td>PIM1</td>
<td>pim-1 oncogene</td>
<td>CAMK</td>
</tr>
<tr>
<td>EPHB1</td>
<td>EphB1</td>
<td>TK</td>
</tr>
<tr>
<td>SNARK</td>
<td>likely ortholog of rat SNF1/AMP-activated protein kinase</td>
<td>CAMK</td>
</tr>
<tr>
<td>RPS6KA1</td>
<td>ribosomal protein S6 kinase, 90kDa, polypeptide 1</td>
<td>AGC</td>
</tr>
<tr>
<td>BRD3</td>
<td>bromodomain containing 3</td>
<td>Atypical</td>
</tr>
<tr>
<td>BMPR2</td>
<td>bone morphogenetic protein receptor, type II (serine/threonine kinase)</td>
<td>TKL</td>
</tr>
<tr>
<td>PHKG2</td>
<td>phosphorylase kinase gamma 2 (testis)</td>
<td>CAMK</td>
</tr>
<tr>
<td>MAPK14</td>
<td>mitogen-activated protein kinase 14</td>
<td>CMGC</td>
</tr>
<tr>
<td>CCRK</td>
<td>cell cycle related kinase</td>
<td>CMGC</td>
</tr>
<tr>
<td>FLJ20574</td>
<td>hypothetical protein FLJ20574</td>
<td>Other</td>
</tr>
<tr>
<td>MAP3K7</td>
<td>mitogen-activated protein kinase kinase 7</td>
<td>TKL</td>
</tr>
<tr>
<td>TRIM28</td>
<td>tripartite motif-containing 28</td>
<td>Atypical</td>
</tr>
<tr>
<td>DYRK2</td>
<td>dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2</td>
<td>CMGC</td>
</tr>
</tbody>
</table>

Despite the extensive studies we were unfortunately unable to molecularly characterise the identity of p27\textsuperscript{NCDK}. However, these results suggested that there were multiple, possibly overlapping connections between cell cycle promoting and inhibiting pathways, resulting in regulation of p27\textsuperscript{Kip1} and p27\textsuperscript{NCDK} distribution as demonstrated by TGF-\(\beta\), HGF, and stress-induced AMPK pathway as well as growth promoting PI3K-pathway.

**PI3-kinase/ AKT pathway regulates TGF-\(\beta\) pathway (II)**

In study I, we showed that AMPK and TGF-\(\beta\) pathways promote p27\textsuperscript{NCDK} levels, while PI3K-Akt/PKB activity appears to negatively regulate p27\textsuperscript{NCDK}, inhibition of PI3K -pathway causing an increase in p27\textsuperscript{NCDK} as seen with TGF-\(\beta\). PI3K-pathway is growth promoting and had been reported to suppress some of the antiproliferative responses of TGF-\(\beta\), including apoptosis and phosphorylation of the Smad transcription factors required e.g. for induction of the CDK-inhibitor p15\textsuperscript{Ink4} in response to TGF-\(\beta\) (Chen et al., 1998; Song et al., 2006). Smad7
is a negative regulator of TGF-β pathway. Smad7 binds and inactivates type I TGF-β receptor and blocks transcription of TGF-β target genes by preventing formation of Smad-DNA complex at the promoter sites (Massague and Wotton, 2000). Other negative regulators of the pathway are Ski and SnoN, which inhibit TGF-β responses via multiple mechanisms. They prevent the association of R-Smads with Smad4 and the complex formation between Smads and co-activators. Ski was shown to bind Smad3, interfere with its association with the transcriptional coactivator p300 and recruit histone deacetylases to the promoters of target genes (Akiyoshi et al., 1999; Luo et al., 1999). Multiple interacting partners have been described for Ski, yet most of these associations have not been verified physiologically. Based on these studies Ski appears to mediate its effects via protein-protein interactions as it seems to lack an intrinsic ability to bind DNA (summarised in (Tecalco-Cruz et al., 2018)).

Looking for possible points of crosstalk between the TGF-β and Akt/PKB pathways we noted that c-Ski contains a consensus sequence for Akt phosphorylation. This suggested that potential phosphorylation of Ski by Akt could present a novel regulation mechanism between the TGF-β and Akt/PKB pathways. To investigate this possibility, we conducted co-immunoprecipitation studies between Ski and Akt isoforms Akt1, Akt2 and Akt3. Ski was found to interact with Akt1 and Akt2 and to a lesser extent with Akt3 (II, Fig. 1a, b). Given that Akt3 is mainly expressed in the brain these results suggested that the interaction may have evolved in specialized tissues. We also tested if the interaction of Ski with Akt required Akt-kinase activity by co-expression of Ski with Akt wt, Myr-Akt (constitutively active) and kinase dead (KD)-Akt, and found that the interaction was not dependent on the kinase activity of Akt (II, Fig. 1c).

The putative Akt phosphorylation site (Thr458) on Ski was mutated to a non-phosphorylatable alanine (A) or a phosphomimicing aspartic acid (D). Using an antibody that recognises the Akt-consensus site when phosphorylated at serine or threonine residue, we found that the antibody recognised the wild type Ski and its D-mutant but not the non-phosphorylatable T458A -mutant (II, Fig. 2b). These studies demonstrated that this site is a putative Akt phosphorylation site. Further, the phosphorylated form of c-Ski was found both in the nucleus and cytoplasm (II, Fig. 4).

In order to downregulate Akt activity we also used chemical inhibitors of Akt such as LY294002 and Tricibine. These decreased the phosphorylation of Ski as detected by the Akt-
substrate antibody. Furthermore, activation of Akt by insulin or growth factors induced phosphorylation of the residue (II, Fig. 3). TGF-β stimulation causes degradation of c-Ski thereby relieving the repression on the Smad-regulated target genes (Le Scolan et al., 2008; Nagano et al., 2007). Hence it became relevant to address whether phosphorylation of c-Ski by Akt could influence this process. We investigated this in cells stably expressing the wild type and the non-phosphorylatable Ski proteins. Insulin treatment was found to decrease the levels of Ski (wt) but not Ski (A), suggesting that phosphorylation of Thr458 by Akt destabilises Ski (II, Fig.6). To support this, treatment with PI3K/Akt inhibitor increased the level of Ski (wt) but not of the Ski (A) mutant, suggesting phosphorylation by Akt destabilises Ski. However, pretreatment with insulin did not augment the degradation induced by TGF-β (II, Fig. 7)).

Among the transcriptional targets inhibited by Ski is the inhibitory Smad7. The potential effects of Akt induced phosphorylation of Ski on the transcription of Smad7 were investigated. Insulin-induced decrease in the levels of Ski protein preceded the induction of Smad7 mRNA and protein levels, suggesting that Akt can affect Smad7 levels via regulation of Ski protein stability (II, Fig. 8). As Smad7 can influence the activity of TGF-β receptors (Ebisawa et al., 2001; Kavsak et al., 2000; Shi et al., 2004), we evaluated the effect of insulin on Smad2 and Smad3 phosphorylation. Pretreatment of cells with insulin before TGF-β stimulation caused an attenuation of Smad2 and Smad3 phosphorylation without affecting the total protein levels, demonstrating decreased TGF-β activity in the presence of insulin and Akt-signalling pathway. One of the most characterised downstream targets of the TGF-β pathway is the CDK-inhibitor p15 that binds CDK4/6 thereby inhibiting cell cycle progression. It was therefore of interest to determine whether the observed effects of insulin on Ski stability, Smad7 induction and Smad2/3 phosphorylation were transferrable to the transcriptional targets of TGF-β and thereby to cell cycle regulation. We found that insulin pretreatment of cells inhibited the induction of p15 cell cycle inhibitor by TGF-β. This was a novel finding linking the pro-survival insulin-Akt and growth-inhibitory TGF-β pathways at the level of Ski protein stability (II). This parallels the findings in (I) where TGF-β-p15 axis and the PI3K -signalling pathway were shown to regulate p27NCDK abundance.

Mice overexpressing c-Ski demonstrate increased muscle mass (Sutrave et al., 1990), making Ski a key factor in the induction of myogenesis. Elevated levels of Ski in the muscle resulted
in downregulation of Smad1, 4 and 7 as well as myostatin expression (Leong et al., 2010). These mice also have decreased body fat and increased lean body mass. The skeletal muscle of the mice was shown to have an enhanced fatty acid oxidative capacity while Ski repressed Srebp1 and Pparγ, genes involved in metabolism.

Another study utilising chicken embryonic fibroblasts transformed with c-Ski illustrated that unlike most transformed cells, cells overexpressing c-Ski did not demonstrate the Warburg effect. Instead of switching their metabolism to glycolysis they displayed reduced lactate production and enhanced TCA cycle activity. Many of the effects appear to be regulated via the transcription factor PPARγ, whose levels were elevated in the chicken embryonic fibroblasts transformed by Ski (Ye et al., 2011).

When the c-Ski overexpressing mice were challenged with a high fat (HF) diet, they showed partial resistance to the weight gain induced by the HF-diet in wt mice. Interestingly, c-Ski mice showed impaired Akt phosphorylation in response to insulin and concomitantly dampened gene responses (Diaz et al., 2012). Furthermore, while insulin caused a significant increase in glucose uptake in the muscle of Ski (wt) mice, this increase was not observed in the mice overexpressing Ski. This suggest that while insulin via Akt activation can destabilise Ski and thereby control the expression of Ski target genes (II), Ski also regulates Akt activation and physiological insulin responses. Similar to mice overexpressing c-Ski, mice lacking Smad3 were protected from HF-diet induced obesity and diabetes (Yadav et al., 2011), further strengthening the role of TGF-β pathway in the control of insulin signalling and metabolic pathways.

This also places c-Ski both on the TGF-β and Akt-signalling pathways and links it with metabolism and cell cycle in a way similar to p27NCDK as was presented in (I).

**Regulation of cell cycle and signalling pathways in prostate (III)**

Loss of PTEN is known to be a frequent event in tumor initiation and progression especially in the prostate, occurring in about 30% of PIN (prostate intraepithelial neoplasia) and 60% of metastatic prostate cancer (Cairns et al., 1997; Suzuki et al., 1998). Loss of PTEN in mice results in metastatic prostate cancer (Ma et al., 2005; Trotman et al., 2003; Wang et al.,
Lack of PTEN in the mouse prostate was shown to lead to increased Smad4 levels and Smad2 phosphorylation in the nucleus (Bjerke et al., 2014). This suggested that TGF-β might play a role in restraining metastasis in the prostate during oncogenic signalling. This was further assessed by deleting the TGF-β receptor II in the presence of constitutively activated Akt signalling in the prostate. Deletion of TGF-β RII allowed progression of the non-metastatic PIN lesions of Akt-overexpressing prostate to invasive cancer. The metastatic state was also refractory to castration, i.e. hormone independent. This suggests that TGF-β may provide a metastatic barrier in cancer progression (Bjerke et al., 2014).

Interestingly, invasion in the above model was slower than in the Pten-null models, suggesting additional proteins or pathways regulated by PTEN loss. One such player known to play a role in prostate cancer is p27^Kip1. In a mouse model, additional loss of Cdkn1b in PTEN +/- background enhances the tumorigenesis induced by loss of one PTEN allele, resulting in complete penetrance of cancer in the prostate by the age of three months (Di Cristofano et al., 2001). PTEN may thereby regulate tumor progression at multiple levels from cell proliferation to metastasis.

Prostate cancer is the second most common type of cancer in men in the western world and also the second leading cause of death from cancer (Siegel et al., 2019). The mortality rate for prostate cancer saw a steep decline over two decades which has been attributed to PSA (prostate specific antigen) testing, early diagnosis and advances in treatment. Yet the benefits of screening for prostate cancer are being disputed, as although some types can be aggressive, it is often known to be non-metastatic and slow-advancing (Ilic et al., 2013).

Multifocality is a common feature of prostate cancer, yet reasons behind this have remained obscure. The frequent occurrence of neoplastic foci is indicative of a relaxed cell cycle control mechanism. As DNA damage usually enforces a cell cycle arrest for the duration of DNA repair, it was of interest to investigate how cells of the prostate would behave when challenged with genotoxic treatment. Commonly used prostate cancer cell lines are not an informative option for this end point as they already harbour several mutations contributing
to the transformation of the cells. Given this we chose to use primary cells and human prostate tissues to outline and map cell cycle and signalling responses to DNA damage caused by ionising radiation (IR) to study the activation of DNA damage checkpoints. For this purpose, tissues were obtained from radical prostatectomies and primary cultures were established. The human prostatic epithelial cells (HPECs) represent the prostate progenitor or transit-amplifying cells (Peehl, 1992). They show characteristics of both basal and luminal epithelial cells and are thought to be the cells of origin in prostate cancer (Hallstrom and Laiho, 2008).

When encountered with genotoxic insult most cells will activate their DNA damage control mechanisms. This will lead to cell cycle arrest to allow for repair of the damage, or - when damage is assessed as too severe for repair - instigation of the cell suicide program, apoptosis. In study (III) this was indeed observed for most cell lines, yet HPECs continued cycling even after high doses of ionising radiation, and showed no signs of commencing the apoptotic program (III, Fig. 1). This could be due to defects in mechanisms detecting the damaged DNA. To assess this, we investigated activation of early DNA damage signalling pathways. We observed that the initial detection of DNA damage was intact as determined by activation and recruitment of DNA damage sensors γH2AX, Nbs1, and Rad50 (III, Fig. 2a). However, clearance of the damage foci in HPECs was significantly slower than in the control cell lines (III, Fig. 2b, c). This is suggestive of altered checkpoint function and persistence of DNA damage after genotoxic stress. If this is the case, it could predispose to accrual of genetic lesions and partially explain the mechanism behind the multifocal nature of prostatic cancer.

To investigate the functionality of the DNA damage response in HPECs we analysed the pathway components in more detail. ATM target proteins Chk2 and p53 were phosphorylated on their target residues and phosphatase Cdc25A was degraded as predicted by the existing model (III, Fig. 3b). However, despite phosphorylation, p53 stabilisation or transcriptional activation as assessed by the expression of its target genes, could not be detected (III, Fig. 3a and Supporting information Fig. 6). The absence of p53 stabilisation in response to IR in the primary prostate cells was surprising, yet in accordance with other studies in the prostate, breast and thyroid epithelial cells (Flatt et al., 1998; Gadbois and Lehnert, 1997; Hong and Stambrook, 2004; Meyer et al., 1999). This suggested that these cell types that have low
proliferation indexes in normal tissues do not rely on p53 pathway for their checkpoint control.

In cells exposed to IR, decreased Cdk2-Cyclin E activity is detected resulting in cell cycle arrest. This is mainly due to degradation of Cdc25A, which under normal growth conditions maintains CDK activity by removing the inhibitory phosphorylation on CDK2 (Tyr15). We therefore analysed Tyr15 phosphorylation of Cdk2 in HPECs. We observed that IR did not induce Tyr15 phosphorylation of Cdk2, which translated to continued Cdk2 activation in HPECs after genotoxic stress as assessed by in vitro kinase assay (III, Fig. 3e, f). We also tested turnover of Cdc25A phosphatase and observed its degradation in HPECs after IR in a manner similar to other cell types (III, Fig. 3b). This led us to investigate the kinase phosphorylating Tyr15 of Cdk2, Wee1A. Interestingly, the levels of Wee1A turned out to be extremely low in HPECs compared to other cell lines (III, Fig. 4a). To determine expression of Wee1A in the human prostate tissue we performed immunohistochemistry for Wee1A. Wee1A levels in the prostate were similarly low or absent in the luminal (i.e. secretory cells) compartment, but detectable in basal cells. In basal cells it was interestingly localised both in the nucleus and cytoplasm despite being considered a nuclear protein (III, Fig. 4b).

It therefore appears that low Wee1A levels and thereby lack of inhibitory Cdk2 phosphorylation may cause or contribute to the lack of cell cycle arrest after genotoxic stress. To test if Wee1A is indeed rate limiting for this, we induced the levels of Wee1A and Tyr15-phosphorylated Cdk2 in HPECs by transfecting HPEC cells with expression plasmids for Wee1A (wt or non-degradable mutant) and Cdk2. Ectopic expression of Wee1A alone or in combination with Cdk2 restored Cdk2-Tyr15 phosphorylation in response to IR, which in turn resulted in cell cycle arrest, suggesting that the low levels of Wee1 critically contribute to the lack of cell cycle response to ionising radiation (III, Fig. 4G).

Lack of Cdk2-Tyr15 phosphorylation in response to IR was also evident in fresh prostate tissues. P53 response to IR was likewise missing in the prostate tissue obtained from radical prostatectomies. Detectable γH2AX foci was evident after IR in the basal cells, while it and other stress responses to IR were missing in the luminal cells (III, Fig. 5). This reveals a fascinating cell type specificity of normal tissue selectivity in response to DNA insults and
suggests one possible mechanism that contributes to prostate cancer and explain its high incidence and multifocality.

Prostate therefore provides an example about the importance of the regulation of signalling pathways by cellular stress responses, and translation of it to cell cycle control mechanism. The dampened response to extracellular damage seen in the prostate may predispose it to the hyperproliferative changes underlying tumor development.

**Concluding remarks**

Cell cycle research has come a long way from the discovery of the genes regulating duplication of the genome and the cell to dissecting the intricate mechanisms and events of the process. Forty years after discovering the cyclins and CDKs in sea urchin and yeast cells, we now understand a lot more about their role in mammalian development and human disease. It was apparent early on that these mechanisms and genes are key factors in development of cancer, which ultimately results from uncontrolled cell division. Mutations in the core cell cycle proteins were subsequently discovered in several cancer types, and also in the signalling pathways regulating cell cycle progression.

Cancer is not a single entity but rather an umbrella term for a multitude of hyperproliferation disorders. Investigations of the signalling pathways and affected cell cycle components have revealed that their function may vary between different organs and even within different cell types within the organ. These pathways are not simply one-directional, but they also intersect and different signals may influence the same pathway positively or negatively. This was demonstrated in study I, where effects of external stress or growth stimulus on cell cycle and in particular cell cycle inhibitor p27\textsuperscript{Kipl} were investigated. These studies showed how different pathways can converge at the level of a single protein, or even a particular subset of the protein, such as p27\textsuperscript{NCDK}, which represents a form that is unbound to CDKs, and therefore demonstrates a function beyond the previously known functions of this protein. Crosstalk of the pathways and convergence on single protein level was also the focus of study II. This study looked at TGF-β and insulin pathways and identified the TGF-β pathway inhibitor Ski as a target for the Akt/PKB kinase.
Cancer therapy by targeting the cell cycle pharmaceutically has proved challenging. The first compounds developed to inhibit CDKs had little specificity towards any particular CDK, which led to side-effects and toxicity. In recent years more specific CDK inhibitors have been developed, and these have shown great benefits in certain cancer types. Inhibition of CDK2 activity in a manner similar to CDK4/6 inhibition in breast cancer could be beneficial for treatment of prostate cancer, as prostate has low levels of Wee1 kinase which normally keeps CDK2 activity in check (III). This specific feature of the prostate, having downregulated Wee1 kinase activity and thereby diminished cell cycle arrest responses to external stresses, could provide a treatment modality for this cancer type, which is the most prevalent cancer in males in the western world. As hormonal therapy is widely used in prostate cancer treatment, combination therapy with CDK inhibitors could further provide clinical benefits similar to breast cancer.

The major metabolic sensor, AMPK, is activated when cells encounter energy stress during energy-consuming processes, including high proliferation of cancer cells. A role for AMPK in cell cycle control has also emerged, and we demonstrated that extracellular stresses may mediate their functions on the CDK inhibitor \( p27^{Kip1} \) via AMPK, ultimately leading to cell cycle inhibition (I). An interesting observation from a clinical point of view is, that patients receiving metformin treatment, which is used in diabetes and known to activate AMPK, have lower rates of cancer. Some of the antiproliferative effects of metformin on cancer cells have been demonstrated to involve \( p27^{Kip1} \) (Cai et al., 2015; Liu et al., 2016). This points to the role of metabolism and \( p27^{Kip1} \) in cancer growth and raises the question if, at least in certain cancer types, metformin could also be used in combination therapy with specific CDK-inhibitors that might mimic the function of \( p27^{Kip1} \).

It has become evident that there will not be one drug or one target that will be able to cure all cancers. The encouraging results from combination of CDK inhibitors and hormone therapy as well as metformin already demonstrate that we need to understand the basic processes and mechanisms related to cell cycle and the associated signaling pathways regulating it before we can tackle these diseases.
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