Transcriptional regulation of the cell cycle.

Transcriptionele regulatie van de celcyclus.

(met een samenvatting in het Nederlands)

Proefschrift

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For all those who do not have access to (higher) education)...
A ceux qui n'ont pas accès à l'enseignement (supérieur)...

" Apprendre est difficile. Apprendre sans surestimer les vertus du savoir l'est encore plus."

Didier Nordon (A bas le savoir!)

Contents.

General introduction:

Cell cycle, cancer and transcription.

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The English naturalist Robert Hooke coined the term “cell” after viewing slices of cork through a microscope. The term came from the Latin word *cella* which means “storeroom” or “small container”. He documented his work in the Micrographia, written in 1665. Two hundred years later, this concept was included in a theory, the cellular theory (1839), which proposed that the cell is the fundamental element of all organisms, both animal and vegetal. The origin of the cell, however, was misinterpreted until Rudolf Virshow established that all cells come from one cell (“Omnis cellula e cellula”) in 1858. However, many more years of research, supported by great technological improvements [1], have been necessary to reach a better understanding of the transmission of the genetic information from one cell’s generation to the next one.

What is the cell cycle?

In order to propagate, cells need to execute a cell division cycle, or cell cycle. The notion of the cell cycle refers to the ordered sequence of events that controls and coordinates the accurate duplication and segregation of the genetic information of a cell that guarantees the genetic stability between one cell and its progeny.

The cell cycle is composed of four phases (see Figure **1)**: the G1 and G2 gap-phases, the Synthetic S-phase, and the Mitotic M-phase. However, it can be summarized by the oscillation of the cell between two states: S-phase, during which the cell duplicates its DNA, and M-phase during which it proceeds to the segregation of theses two copies of DNA in the two daughter cells. The mechanism by which this oscillation is induced and regulated forms the basis for the research done in the cell cycle field.

The cell cycle autonomous oscillator.

The cell cycle machinery is driven by an autonomous oscillator commanded by events in the cell cycle [2, 3]. As the cell progresses through the cell cycle, the levels of cyclin-dependent kinase (Cdk) activity rise and fall. This oscillation leads to cyclical changes in the phosphorylation of key-proteins that regulate or execute major cell cycle events, such as DNA duplication, DNA segregation and cell division (see Figure 2).

Although the levels of Cdk activity vary during the cell cycle, their protein levels remain quite constant. However, they associate with an activating partner, the cyclin, whose protein levels vary during the cell cycle, thereby explaining their cyclic activation. During each cell cycle, cyclins are synthesised and subsequently degraded [4]. Furthermore, in addition to their role in Cdk activation, cyclins also provide a Cdk with substrate specificity. By binding to a specific subset of cell cycle proteins, cyclins bring these proteins into the vicinity of the active Cdk, which catalyses their phosphorylation [5].

In mammalian cells, the autonomous cell cycle machinery is primarily composed of the oscillatory activity of Cdk2 and Cdk1 (also known as Cdc2). These can associate to multiple cyclin moieties (mostly Cyclin A, B and E), each of which in turn associates to a distinct subset of cell cycle proteins. As synthesis and degradation of the different cyclins is separately regulated, this creates a complex pacemaker that allows timed execution of the different cell cycle events.

What are the checkpoints?

The faithful duplication of the human genome takes about 8 to 12 hours, a very short period of time, considering that its size is of about

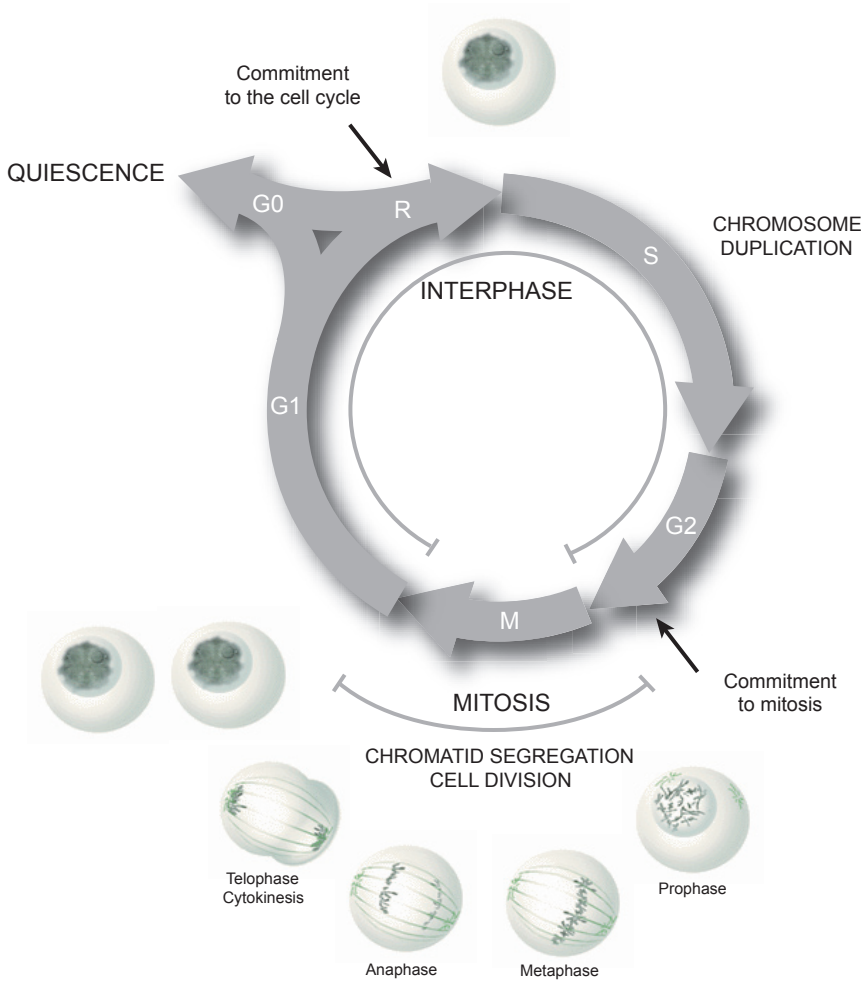

Figure 1. The cell cycle. The cell cycle is composed of four phases: G1 (Gap 1), S (DNA Synthesis), G2 (Gap 2) and M (Mitosis). The S-phase and its two flanking gap-phases form the interphase. During the cell cycle, the cell needs to coordinate two major events: the faithful duplication of its chromosomes during S-phase and the equal segregation of these two copies of DNA during mitosis. Completion of mitosis, which is subdivided into the intermediary stages prophase, metaphase, anaphase and telophase / cytokinesis, results in the formation of two daughter-cells with the exact same genetic information as the initial cell. The gap phases play an important role in the coordination of these two events. In G2-phase, the presence of errors in the DNA, or the presence of unreplicated or re-replicated DNA sequences are monitored and control the onset of mitosis. Only after errors and misreplicated sequences have been cleared, can the cell commit to mitosis. This guarantees the genetic stability from one cell's generation to another. In G1, and before the restriction point (R), a cell can either decide to proliferate or to exit the cell cycle. In the presence of mitogenic cues, the cell can commit to cell cycle by moving across the restriction point after which cell cycle progression is no longer dependent on the presence of mitogenic cues. However, in the absence of mitogenic cues, for example when subjected to low levels of nutrients or growth factors, the cell enters a

3.2 billion base pairs. However, the normal rate of acquisition of mutations is quite low, only 10^{-7} to 10^{-8} per nucleotide per cell division [6]. Likewise, it takes only about an hour to equally segregate the two copies of 23 pairs of chromosomes during a typical mitosis, with an error rate of less than one in 10^5 events [7]. Therefore, there must be safeguard mechanisms that protect against DNA duplication/segregation errors and prevent their transmission. These mechanisms are provided by the existence of points of control, also called checkpoints, spanning all phases of the cell cycle, that ensure that each cell cycle event has occurred correctly and completely before proceeding to the next one (see figure 2) [8]. Checkpoints actively stall cell cycle progression when "errors" have been detected, and are relieved only when the prior stage is completed correctly. As such, they provide time for error correction and prevent the accumulation of genetic defects.

Checkpoints rely on the activity of so-called checkpoint proteins, which form complex cascades that sense "errors" and transduce a signal to downstream effector checkpoint proteins. These cascades integrate incoming signals and need to instantly adjust their activation status in response to any defect or error. When irreversible damage has occurred, some of these checkpoint proteins can induce cell death.

The G1 gap-phase

The designation "cell cycle" refers in general to the "chromosome cycle", as opposed to the "cell growth cycle". However, in order to maintain the cell's size constant from one cell generation to another, the length of the chromosome division cycle must match the time necessary to double the mass of the cell. The G1 and G2 gap-phases are transitional stages of the cell cycle of variable duration which provide for a time delay that allows cell growth, i.e. growth of the cytoplasmic mass and of the cellular components [9]. In addition, they also prepare the cell for the successful completion of the S- and M-phases respectively, and monitor and integrate cues from both the internal and external environment.

The most important role of the gap phase G1 is to check whether the trophic conditions are favourable to complete one round of cell division. In this respect, Cyclin D plays a crucial role during the G1-phase. Expression of cyclin D does not have the typical cell-cycle-dependent pattern of expression that the other cyclins generally have. Rather than following the oscillations of the cell cycle, Cyclin D levels depend on oscillations of mitogenic stimuli. Cyclin D is expressed as cells re-enter the cell cycle in response to a mitogenic stimulus, persists as long as the mitogenic stimulation is present, and is rapidly degraded following withdrawal of this stimulation [10]. Cyclin D turnover depends on separate signalling pathways that are regulated by mitogenic cues [11, 12]. The presence of mitogenic signalling increases Cyclin D protein levels by concomitantly increasing gene transcription, protein translation and protein stability [13]. On the contrary, absence of a mitogenic stimulus, by preventing Cyclin D gene transcription, protein translation and by increasing its nuclear export and subsequent degradation, leads to the very rapid disappearance of Cyclin D, regardless of the cell cycle stage. Thus, Cyclin D represents a sensor for mitogenic cues.

In early G1, Cyclin D associates with either Cdk4 or Cdk6 and initiates cell cycle progression by partially phosphorylating the pocket proteins, i.e. the retinoblastoma protein (pRb), p107 and p130 [14]. These G1 checkpoint proteins associate with various E2F transcription factors and recruit co-repressors, such as histone deacetylases and DNA remodelling enzymes [15]. Activity of the

pocket proteins results in the transcriptional repression of genes which are essential for DNA synthesis, among which the Cyclin E gene [16, 17]. However, in G1, these interactions are disrupted by phosphorylation, initiated by Cyclin D/Cdk complexes. After the partial inactivation of pocket proteins by Cyclin D/Cdk complexes, Cyclin E/Cdk2 complexes become active. Unlike Cyclin D, Cyclin E is expressed periodically, peaking at the G1/S transition, while it is degraded after S-phase onset. Cyclin E synthesis is directly regulated by the E2F transcription factors and partial inactivation of the pocket proteins leads to increased synthesis of Cyclin E, among other gene products. In turn, Cyclin E/Cdk2 collaborates with Cyclin D/Cdk to complete phosphorylation and subsequent inactivation of the pocket proteins. This creates a positive feedback loop where Cyclin D/Cdk activation by mitogenic stimulation leads to the activation of Cyclin E/Cdk2, thereby promoting progression through the G1/S boundary [18, 19].

In late G1, the activation of Cyclin E/Cdk2 drives the cell through the restriction point which corresponds to a cell cycle commitment checkpoint after which cell cycle progression is no longer affected by the presence or the absence of mitogenic signalling until the following G1-phase. Therefore, the shift between the Cyclin D-mediated hypophosphorylation to the Cyclin E-mediated hyperphosphorylation of the pocket proteins corresponds to the transition between a mitogen-sensitive to a mitogen-insensitive cell cycle state. Thus, although not exactly taking part in the autonomous cell cycle oscillator, Cyclin D/Cdk complexes link mitogenic signalling to cell cycle entry.

Before the G1 restriction point, and in the absence of proper mitogenic signalling, the cell can exit the cell cycle by entering the G0-phase. This process offers the cell the possibility to survive a long period of time in an almost inactive state that is also called “quiescence”. The quiescent state can be induced upon poor environmental conditions or in the absence of appropriate mitogenic signals. Only when more favourable conditions arise, can the quiescent cell re-enter the cell cycle at G1 and commit to divide. Induction of quiescence not only requires a block of E2F-mediated transcriptional activation by the pocket proteins, but also active repression by pocket protein-E2F complexes. In this respect, the p130 pocket protein plays a critical role in inducing and maintaining the G0-state. pRb and p107 are more important during G1 [20]. Therefore, the composition of the pocket proteins-E2F complexes and their phosphorylation by the Cdk’s are determinant to regulate cell cycle exit and entry.

Interestingly Cyclin D/Cdk complexes have another, non-catalytic role in G1. They can sequester the cyclin-dependent kinase inhibitors (CKI) $p27^{Kip1}$ and $p21^{Cip1}$, two potent inhibitors of Cdk2. This binding stabilizes Cyclin D/Cdk complexes without interfering with their kinase activity. After mitogenic stimulation, Cyclin D levels are induced, and although CKI protein levels may represent an inhibitory threshold for Cyclin E/Cdk2 activation, their sequestration by Cyclin D lowers their effective levels for Cdk2 inhibition, thereby facilitating Cyclin E/Cdk2 activation. This creates a feedback loop where $p27^{Kip1}$ stabilizes Cyclin D/Cdk complexes and therefore indirectly promotes Cyclin E-Cdk2 activation, which in turn facilitates its own activation by phosphorylating $p27^{Kip1}$ and targeting it for degradation [21].

The p16INK4 protein is another important CKI that regulates progression through the G1-phase. It binds to Cdk4 and competes for its binding to Cyclin D, thereby altering Cdk4 distribution. p16INK4A can prevent cell cycle progression by several means. First, it prevents the assembly of Cyclin D/Cdk complexes and therefore lowers the effective concentration

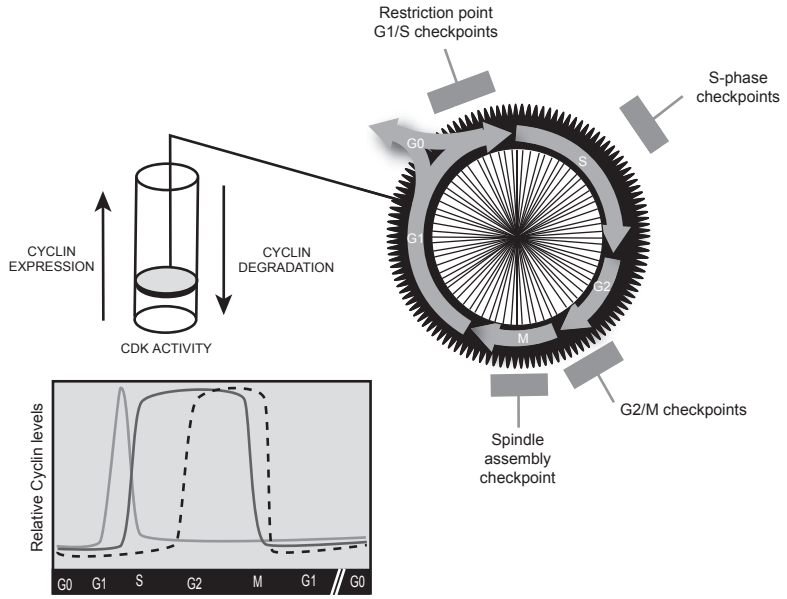

Figure 2. The cell cycle autonomous oscillator. In an engine, the oscillating movement of the piston in the cylinder operates the movement of the wheel. The frequency of this oscillation defines the speed at which the wheel rotates, and can be adjusted by the accelerator and brake pedals. Like the engine, the cell cycle machinery is an autonomous oscillator and progression of the cell cycle (wheel rotation) is driven by the oscillatory activity of Cyclin-dependent kinases Cdk1 and Cdk2 (piston), which regulate the regulatory and mechanistic aspects of DNA replication and of mitosis. The cell cycle checkpoints are acting as brakes on cell cycle progression. Similarly to the oscillatory movement of the piston, which induces rotation of the wheel, the regular fluctuations in the levels of cyclins and in the activity of their associated Cdk's (cyclin-dependent kinases) induce cell cycle progression. As shown in the graph. Cdk2 associates with Cyclin E (light grey line) and Cyclin A (dark grey line). Cyclin E is synthesised in G1, peaks at the G1/S transition and is degraded early after S-phase entry. Cyclin A is synthesised somewhat later at the G1/S transition, peaks in S-phase and is degraded just after mitotic entry, in prophase. Cdk1 (also known as Cdc2) associates with Cyclin A, and more importantly with Cyclin B (dashed line). Cyclin B is synthesised in late S-phase, peaks at the G2/M transition and is degraded in mitosis at the metaphase-to-anaphase transition.

of active Cyclin D/Cdk complexes. Second, because Cyclin D stability is lower, when it is not in complex with a Cdk, p16INK4A can affect Cyclin D turnover. Thirdly, $p27^{Kip1}$ and $p21^{Cip1}$can only bind to Cyclin D in complex with a Cdk, and therefore interaction of p16 with Cdk4, leads to the release of $p27^{Kip1}$ and $p21^{Cip1}$ from the disrupted cyclin D/Cdk complexes, which are then available for Cyclin E/Cdk2 inhibition. Taken together, the global consequence of p16INK4A action is a very efficient inhibition of all G1 Cyclin-dependent kinase activities and the maintenance of the pocket proteins in their non-phosphorylated growth-suppressive form [21].

Surprisingly, Cyclin D, Cdk4, Cdk6, Cyclin E or Cdk2 are individually not essential for cell proliferation and mitogenic responses [22-24]. A certain degree of functional overlap can be observed in cells lacking one or several of these components. Similarly, functional compensation can be observed between

the different pocket proteins [25]. Functional overlap, redundancy and compensation are likely to provide a safeguard mechanism against acquired genetic defects that affect the function of these proteins which are crucial for the decision-making process governing proliferation [26].

The S-phase - Regulation of DNA duplication.

After entry into S-phase, Cyclin E is rapidly degraded, and Cyclin A replaces it in complex with Cdk2. Like Cyclin E, Cyclin A displays a periodical expression pattern. It is expressed early in S-phase and is degraded after the onset of mitosis. Throughout S-phase, Cdk2 promotes cell cycle progression by phosphorylating key proteins of the replication machinery [27].

S-phase is not discernible microscopically. Its beginning and end are defined by the initiation and completion of DNA duplication. During S-phase, checkpoints ensure that: 1) the whole genomic DNA is been fully copied (replication checkpoint), 2) only a single copy of the genome is made during each cell cycle (replication licensing), and 3) no damage to the DNA has occurred during replication (intra-S-phase DNA damage checkpoint).

Due to the size of their genome, eukaryotic cells initiate replication from multiple locations throughout the chromosomes, called replication origins. In order to ensure duplication of the entire genome, there is an excess of replication origins, and neighbouring replication origins may be activated if one fails to fire. Replication origins are bound by the Origin Recognition Complex (ORC), a multiprotein complex that serves as a platform for the recruitment of the pre-replicative complexes (pre-RCs). These pre-RCs are assembled prior to S-phase in a highly ordered and regulated manner, and are converted into replication forks during the transition from G1-to S-phase. This step is triggered by the action of kinases, the two most important ones being Cdk2 and Cdc7/Dbf4. In S-phase, replication is initiated by promoting unwinding of the origin, stabilization of the single-stranded DNA threads formed, and by recruitment of the DNA polymerases. Replication origins are then fired and replication takes place at the speed of 500 nucleotides per minute with an error rate of 1 nucleotide per billion [6].

However, events such as depletion of the deoxyribonucleotide (dNTP) pool or presence of diverse DNA lesions can stall the elongation of the replication forks. Stalled replication forks, as well as DNA breaks, generate stretches of single-stranded DNA, which are coated with Replication Protein A (RPA). RPA accumulation recruits a variety of checkpoint signalling molecules and is therefore the initial event causing activation of the replication checkpoint [6]. This checkpoint has three main functions. First, it prevents thus far unfired origins to initiate replication. Second, it stabilizes the stalled replication forks in order to improve the recovery of cell cycle progression once the dNTP pool is restored or the DNA damage is repaired. Finally, it prevents entry into mitosis of each cell which would have incompletely replicated DNA [28] [29].

Replication origins are fired only once per cell cycle in order to prevent re-replication. This is due to replication licensing. In G1 the DNA is licensed for replication, while passage through S-phase removes the license, rendering the DNA refractory to any additional round of replication. Only completion of cell division can restore the license, thereby ensuring the association between one round of replication with one round of cell division [30]. The nature of this license is Cdk activity. Indeed, Cdk's can phosphorylate components of the pre-RC's and thereby prevent their assembly. Therefore, during the S, G2 and M-phases, the high Cdk activity prevents re-initiation of

DNA replication by blocking reassembly of pre-RC's, and pre-RC formation is restricted to the G1-phase during which Cdk activity is low. This is a conserved mechanism to suppress re-replication in eukaryotes. Furthermore, higher eukaryotes have acquired an additional, Cdk-independent, mechanism to prevent re-replication which utilizes Geminin, an inhibitor of pre-RC formation. Geminin protein levels are high in S, G2 and M, during which pre-RC formation is restricted. At the end of the M-phase, geminin is degraded allowing new pre-RC assembly for the next S-phase [31].

The G2 gap phase.

The gap phase G2 coordinates the S- and M-phases of the cell cycle, and thus DNA duplication and DNA segregation. This phase is very important to delay the onset of M-phase when errors have been detected in the genome before the latter is divided between the two daughter-cells. The errors that are detected can be replication errors, presence of unreplicated or re-replicated DNA, or damage inflicted to the DNA. Indeed, the constant metabolic activity of the cell, as well as normal and abnormal environmental aggressions, can generate diverse forms of DNA damage, which are monitored throughout the cell cycle. The DNA damage response coordinates DNA damage sensors with, on the one hand, the cell cycle machinery and, on the other hand, the DNA repair machinery [32]. The gain of time induced by activation of the cell cycle checkpoints then allows the DNA repair machinery to correct these errors. This is particularly important before mitosis in order to prevent the transmission of these errors to the progeny [29].

Several types of DNA damage response can be distinguished, all involving the ATM/ATR kinases, which are activated directly or indirectly by DNA damage or stalled replication forks [33]. For one, in S-phase, the ATM/ATR cascade can activate a replication-independent checkpoint, which is especially important for lesions outside of the replicating areas which are not able to induce the replication checkpoint [28]. This intra S-phase DNA damage checkpoint induces a delay in S-phase progression, by inhibiting Cdc7/Dbf4; one of the kinases required for replication initiation in the unfired replication origins [34]. However, this checkpoint does not alter the extension of the actively progressing replication forks. In G2, ATM/ATR can also delay cell cycle progression by interfering with the activation of the mitotic cyclin-dependent kinase Cdk1. ATM/ATR downstream signalling regulates multiple pathways responsible for Cdk1 activation. As such, regulation of several kinases such as Chk1, Chk2 and Plk1 is required for ATM/ATR downstream signalling. Activation of Chk1/Chk2 and inhibition of Plk1 by DNA damage leads to the inhibition of Cdc25 protein phosphatases and activation of Wee-1 kinase. Cdc25 removes inhibitory phosphate groups on Cdk1, whereas Wee1 adds these inhibitory phosphate groups. Thus, DNA damage through inhibition of Cdc25 and activation of Wee1 lead to the inhibition of Cdk1, thereby preventing mitotic entry. These type of responses are very rapid and are responsible for the immediate cellular response to DNA damage. Subsequent to this rapid response, a more delayed response ensues that involves the transcription factor p53. p53 is quickly stabilized following DNA damage, but the execution of its effect involves transcriptional upregulation of broad variety of target genes. Induction of these latter genes takes several hours, and therefore the p53-dependent response is particularly important to maintain an initial cell cycle arrest, or to induce cell death if excessive damage has been inflicted. One important p53 gene target with respect to cell cycle progression is the CKI $p21^{Cip1}$, which can mediate a cell cycle arrest in G1 or G2 through inhibition of Cdk2

or Cdc2 [35, 36] respectively.

The M-phase (Mitosis) - Regulation of DNA segregation and cell division.

The other highly controlled phase of the cell cycle is mitosis, during which Cdk1 activity plays a central role. Cdk1 promotes progression through mitosis, by binding with Cyclin A and, most importantly, Cyclin B. Like Cyclin E and Cyclin A, Cyclin B is expressed periodically. It is synthesised late in S-phase, peaks at the G2/M transition and is eventually degraded in late mitosis. Cdk1 kinase activity is critical for the proper timing of mitosis. Indeed, it phosphorylates substrates that are critical for mitotic entry, and its inactivation in late mitosis, due to mitotic cyclin destruction, allows DNA segregation and cell division [37-39].

Mitosis itself is a sequence of highly regulated events (see Figure 1). First, during prophase, the DNA starts to condense to form visible chromosomes. Concomitantly, the centrosomes, which were also duplicated during S-phase, start to separate and migrate towards the opposite poles of the cell. Each centrosome is important for the nucleation and organisation of an aster of microtubules. Together, the two asters form the spindle, which is required for the capture and the correct movements of the chromosomes. Finally, the nuclear envelope is broken down. Cyclin A/Cdk2 has been shown to be required for mitotic entry and progression until late prophase [40] and Cyclin A is degraded after nuclear envelope breakdown [41].

Following prophase is prometaphase, during which the chromosomes get attached to the spindle. This attachment involves the kinetochores, a proteinaceous structure placed at the centromere of the chromosome, which function is to capture microtubules. Eventually, each kinetochore is attached to microtubules. If attachment is bipolar, i.e. if opposite kinetochores of paired sister-chromatids have attached to microtubules originating from the opposite poles of the cell, then forces are generated that bring kinetochores under tension. The equilibration of these forces aligns all the chromosomes at the centre of the spindle. Other non-bipolar kinetochore-microtubule attachments are possible (see Figure 4), but need to be resolved to allow the eventual establishment bipolar attachment in order to generate tension on the kinetochore [42, 43].

Metaphase is a very discrete stage, when all chromosomes are perfectly aligned at the centre of the cell, on the so-called metaphase plate. During prometaphase, the spindle assembly checkpoint prevents mitotic progression as long as the metaphase plate is not fully formed (see Figure 4). The molecular mechanism underlying the spindle assembly checkpoint will be addressed later in this chapter [44]. Furthermore, Cyclin A degradation, that is initiated in prophase, is important because presence of Cyclin A in prometaphase delays metaphase onset and anaphase [45]. During prometaphase, Cyclin A destruction is completed, an event that is not affected by the spindle assembly checkpoint [41].

Once all kinetochores are under tension, the spindle assembly checkpoint is relieved and mitosis can progress to the next stage: anaphase. The sister-chromatids of each chromosome are separated in anaphase to opposite poles of the dividing cell. Microtubule-binding proteins are then recruited in the zone between the separated chromosomes called mid-zone, and guide the ingression furrow for the execution of the last mitotic steps: the splitting of the cytoplasm content in two, following the axis of the ingression furrow (telophase) and the pinching-off of the two daughter cells (cytokinesis) [46].

Cancer cells: a product of deregulated cell cycles.

During cell cycle regulation, lower and higher organisms are confronted to different realities. Unicellular organisms, such as yeast, need to take decisions that will affect their individual survival. In this case, the appearance of mutations may allow acquisition of new characteristics and adaptation to a changing environment. As such, these mutations may give a substantial growth advantage. On the contrary, in multicellular organisms (metazoans), the decisions that are taken affect the survival of one single cell, in the context of billions of other cells. Here, the appearance of mutations may lead to a life-threatening risk for the organism. Indeed, a subset of cells may diverge genetically and lose its capacity to be controlled, thereby leading to unscheduled proliferation, and possibly to cancer [47].

Cancers are typically initiated by acquired alterations to the genome of the cancer cell such as chromosomal translocations, mutations, and deletions. These acquired genetic changes in turn alter and override the normal mechanisms controlling cellular proliferation. Tumorigenesis in humans is a multistep process, each step reflecting a genetic alteration and corresponding to progression from normality to malignancy. Malignant growth is usually characterised by [48, 49:

1) self-sufficiency in growth factors,
2) resistance to growth-inhibitory factors,
3) resistance to programmed cell death (apoptosis),
4) unlimited replicative potential,
5) sustained angiogenesis
6) capacity to invade tissues and relocate (metastasis)].

In order to obtain these characteristics, the cancer cell acquires sequential mutations affecting key signalling pathways. The consequent impairment of these pathways participates to uncontrolled and unscheduled cell proliferation. These pathways are recurrently affected during carcinogenesis, underscoring their importance for cellular homeostasis. However, in normal cells, the occurrence of expressed mutations is low. This is due to the action of the cell cycle checkpoints and the DNA repair machinery which prevent their formation. However, should there be any non-repaired mutation, the chance for it to be of serious consequence is low due to the abundance of intergenic DNA, to redundancy in the genetic code, and to the chance of forming silent mutations. To become cancerous, a cell therefore needs to target the integrity of these safeguards in order to acquire new mutations (genetic instability). For this reason, cancer is more and more viewed as a cell cycle disease [50]. For example, it is known since a long time that tumours contain a lot of abnormal mitotic figures. These mitotic defects are due to loss of the mitotic checkpoint function and lead to alterations of the number of chromosomes, also known as aneuploidy [51]. However, the question whether genetic instability is the cause or an indirect byproduct of neoplastic transformation remains very controversial. Most likely, aneuploidy is caused by mutations acquired during malignant transformation, and is a hallmark of neoplasia [52]. Nevertheless, not only are aneuploidy and genetic instability produced by the loss of checkpoint function, they also participate to the acquisition of new genetic alterations, by accelerating the rate of acquisition of new genetic defects. However, to which extent aneuploidy contributes to carcinogenesis is still an open-question.

Mechanisms underlying cell cycle oscillations

Cell cycle integrity relies mostly on oscillations of Cdk1 and Cdk2 activities, secondary to

oscillation in Cyclin protein levels. These are mainly due to regulation of gene expression and of protein stability, although other mechanisms such as regulation of transcript stability and posttranslational modifications exist.

Protein degradation in mitosis.

In eukaryotes, cell cycle progression is partly controlled at the level of protein stability. In combination with timed gene expression, timed protein degradation participates to the oscillation of different cell cycle regulator protein levels.

Active protein degradation involves modification of lysine residues by attachment of ubiquitin moieties, or ubiquitination [53]. However, ubiquitination can also participate to other cellular processes such as signalling, and transcriptional regulation, but it involves a different type of ubiquitin modification (i.e. monoubiquitination). Polyubiquitination is the marker for protein degradation. Polyubiquitinated proteins are recognized by the 26s proteasome, a cylinder-shaped multiprotein complex. At the entrance of the cylinder, polyubiquitinated proteins are stripped of their ubiquitin moieties, which can be recycled. Subsequently, they enter the cylinder where they are destroyed by the proteases present inside of the proteasome (see Figure 3).

Ubiquitination is an active ATP-dependent process, which requires a sequence of highly regulated events. The ubiquitin moiety is first activated by an ubiquitin-activating enzyme (E1) in an ATP-dependent manner, and then transferred to an ubiquitin-conjugating enzyme (UBC or E2). Finally, an ubiquitin-protein ligase (or E3) catalyses the transfer of the ubiquityl group from the E2 to the target protein. This process can be repeated until the target protein is tagged with a polyubiquitin chain [54].

Ubiquitination-mediated protein degradation is involved in many aspects of cell biology. The E3-ligases are largely responsible for the target specificity of protein ubiquitination. Several E3-ligases have been implicated in regulation of the cell cycle. Most notably, the Anaphase-Promoting Complex or Cyclosome (APC/C) and the [Skp1/Cullin/F-box protein]-related complexes (SCF) [55] have been shown to perform crucial functions at various stages in the cell cycle. Whereas the APC/C is active in mitosis and in G1, the SCF has a less well defined function, although it also regulates cell cycle progression. The first evidence for the involvement of the SCF in cell cycle regulation came from the identification of SCF-related complexes in yeast, which mediate the degradation of the G1 cyclins and the $p27^{Kip1}$/$p21^{Cip1}$ yeast homolog Sic1 [56, 57]. The APC/C on the other hand, was originally identified as a large multiprotein complex mediating the degradation of the mitotic cyclins [58, 59] (see Figure 4).

As mentioned above, Cdk1 activation promotes mitotic entry. In prophase, high Cdk1 activity in turn leads to APC/C activation by promoting the binding of a co-activator, Cdc20. APC^{Cdc20} mediates degradation of a subset of early target proteins, among which Cyclin A [41]. Then, at the metaphase-to-anaphase transition, a second subset of proteins, comprising Cyclin B, is degraded. After anaphase onset, all mitotic cyclins have been degraded and thus Cdk1 activity is low again. This allows a second co-activator of the APC/C, namely Cdh1, to take over the function of Cdc20. Prior to anaphase Cdk1 phosphorylates and represses Cdh1, but the drop in Cdk1 activity that takes place in metaphase leads to a switch from APC^{Cdc20} to APC^{Cdh1}. This confers specificity for the third subset of “late” protein targets, among which Cdc20 itself. Thus, mitotic cyclins, by activating the APC/C, initiate their own degradation. Similarly, Cdc20, by promoting Cyclin B degradation, initiates its own degradation

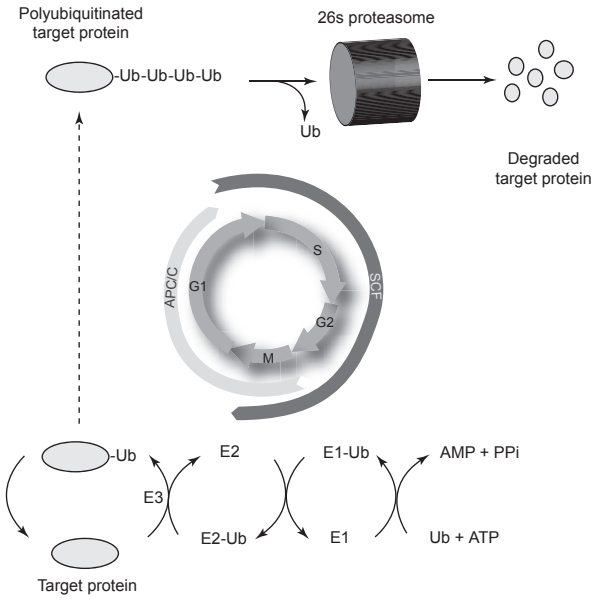

Figure 3: Protein degradation by the ubiquitin-proteasome system. A target protein becomes polyubiquitinated after undergoing several rounds of ubiquitination, which is an active process that requires the hydrolysis of ATP. Ubiquitination is a biochemical reaction leading to the ligation of an ubiquitin moiety. This reaction is catalysed by an E1 ubiquitin-activating enzyme. The activated ubiquitin can be transferred from the E1 to an E2 ubiquitin-conjugating enzyme, and then to the target protein owing to the action of an E3 ubiquitin-ligase, which determines the specificity of this process by recognizing specific protein targets. Polyubiquitinated proteins are targeted for proteolytic degradation by the 26s proteasome. The 26s proteasome is a barrel-shaped multi-protein complex. Polyubiquitinated target proteins enter the proteasome structure. Ubiquitin is released, and possibly re-used in further ubiquination reactions, whereas target proteins are degraded by proteases hidden in the proteasome's lumen.

Two classes of ubiquitin-ligating complexes regulate cell cycle progression: the SCF and the APC/C. The SCF becomes active in late G1-phase (upon Cdk2 activation) and peaks in S-G2. By using multiple E3-ligases, the SCF targets numerous proteins, among which the cyclin-dependent kinase inhibitor $p27^{Kip1}$. Unlike the SCF, whose role is not restricted to cell cycle regulation, the APC/C plays a major role in the regulation of mitosis and mitotic exit.

[60]. This type of negative feedback loop is very frequent during the cell cycle. Indeed, this regulatory process allows the coordinated sequence of key-events and resets the cell cycle "clock" after completion of a specific event.

The name given to the APC/C (Anaphase promoting complex/ cyclosome) stems from the fact that its activity promotes the metaphase-to-anaphase transition (DNA segregation). This transition can only occur when two requirements are fulfilled: 1) all the chromosomes have captured microtubules, and 2) the attachment created is bipolar in order to generate tension (see Figure 4). These events are closely monitored by the spindle assembly checkpoint, which can delay anaphase onset as long as these two requirements have not been met [44]. The activity of the spindle assembly checkpoint controls the degradation of Securin by APC^{Cdc20} [60]. Securin is an inhibitor of anaphase that acts by protecting the "glue" along the chromosome from proteolytic cleavage. This glue, which contributes to the cohesion of the sister-chromatids, is composed of Cohesin sub-units. Thus, the presence of Cohesin renders disjunction of the sister-chromatids and thus their segregation impossible. Securin prevents anaphase by sequestering the protease Separase, which is responsible for the proteolytic cleavage of Cohesin. However, as soon as the spindle assembly checkpoint is silenced, the APC/C is allowed to degrade Securin. This leads to the release of Separase and the subsequent cleavage of Cohesin sub-units, thereby allowing sister-

chromatid separation.

How are attachment and tension monitored, and how does the spindle assembly checkpoint control APC/C activity? In order to be fully functional, the APC/C needs to be activated by an activating subunit, either Cdc20 or Cdh1. Early in mitosis, the APC/C binds to Cdc20 and forms the APC^{Cdc20}. In the presence of any unattached kinetochore, or in the absence of tension, the spindle assembly checkpoint inhibits the activity of APC^{Cdc20} [61]. For that, it relies on the activity of the checkpoint proteins Mad1, Mad2, Bub3 and BubR1. These proteins are all four essential for the activity of the spindle assembly checkpoint and they form multiple complexes, most of them being constitutively present, regardless of kinetochore attachment or tension. Therefore, formation of the checkpoint protein complexes cannot be sufficient for the arrest. Of these checkpoint proteins, Mad2 seems to play a key role in the inhibition of the APC^{Cdc20}. Mad2 can interact with Cdc20, and both of them can associate with the unattached kinetochore with swift exchange rates [62]. The Mad1 checkpoint protein stably interacts with the unattached kinetochores where it recruits Mad2. Mad2 subsequently undergoes a conformational change that appears to facilitate the interaction between Mad2 and Cdc20 [63]. On the other hand, Bub3 and BubR1 also form a complex with Cdc20 upon checkpoint activation [64]. Therefore, the spindle assembly checkpoint appears to catalyse the formation of an inhibitory MCC (Mitotic checkpoint Complex) between Mad2, Bub3, BubR1 and Cdc20 [7].

However, some questions remain to be answered. For example, what is the initial event sensed by the spindle assembly checkpoint? Is it only the lack of tension or is it also the lack of microtubule attachment? How is this initial event leading to the recruitment of the MCC to unattached kinetochores? How can APC^{Cdc20} inhibition be a spatially limited event? What distinguishes the APC^{Cdc20} that mediates Cyclin A degradation, from the APC^{Cdc20} that mediates Cyclin B degradation? Which mechanism prevents the APC^{Cdc20} active in prophase from targeting Cyclin B as well?

It has been suggested that both attachment and tension are monitored by the spindle assembly checkpoint, but their relative contribution may be different from one cell-type to another [65]. However, tension cannot build in the absence of stable bi-orientated microtubule-kinetochore attachments. In fact, we now know that Aurora-B kinase functions by monitoring microtubule-kinetochore interactions and by correcting inadequate attachments, causing the depolymerisation of attached microtubules in the absence of tension. Recent findings also point to a role for Aurora-B in the establishment of a stable association between spindle checkpoint proteins with the kinetochore in the absence of tension, thereby linking spindle assembly checkpoint signalling, microtubule attachment, and generation of tension [66-68].

A single unattached kinetochore has been suggested to be sufficient to delay the anaphase onset for all chromosomes [69]. This implies that there must be a catalytic amplification of the signal from the single unattached kinetochore to the whole cell. The MCC is not likely to provide for this catalytic amplification as it is a stoichiometric inhibitor of Cdc20. Therefore, the kinetochore is suggested to have a catalytic role in Cdc20 inhibition. However, the question whether a single unattached kinetochore is indeed sufficient to activate the spindle assembly checkpoint, i.e. to generate enough MCC to inhibit APC activity, cannot be answered assertively and quantitative studies are required to address this issue [61]. Interestingly, in an artificial cell containing two independent spindles, a single unattached kinetochore in the first spindle fails to inhibit cell cycle

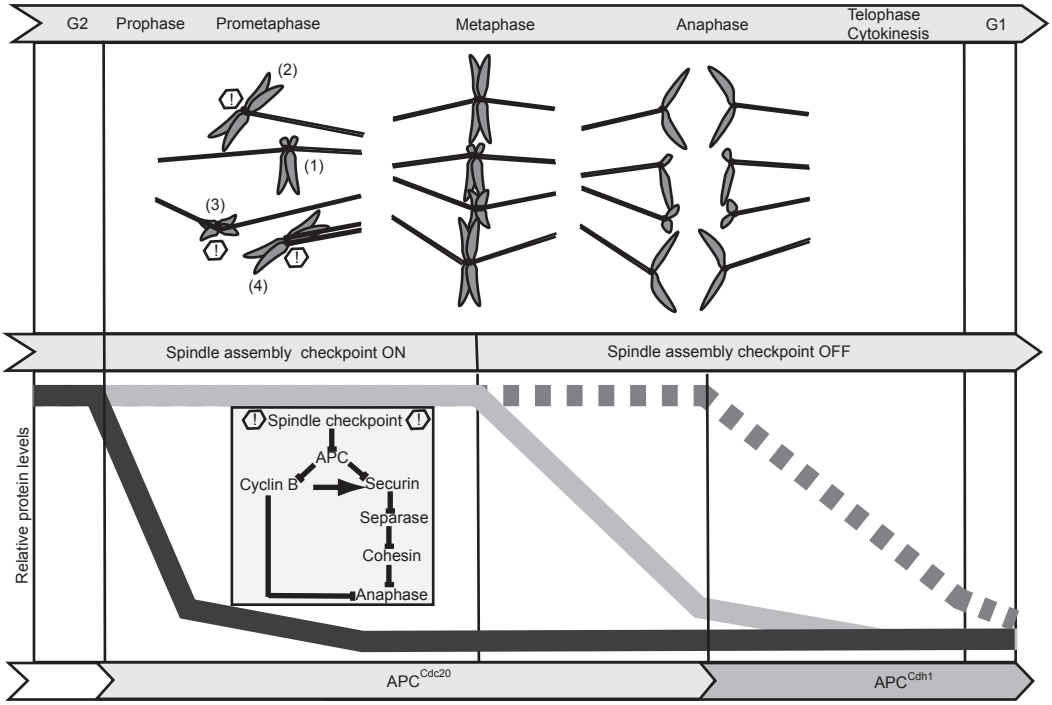

Figure 4: Progression through mitosis is dependent on the activity of the APC/C. One remarkable feature of mitosis is that its progression is delayed until all sister-kinetochores are attached to spindle microtubules (upper panel) in a bi-orientated manner, or amphitelic attachment (1). Defective attachments may occur: only one kinetochore of the pair may be attached (monotelic attachment) (2), one kinetochore may be attached to microtubules coming from both poles (merotelic attachment) (3), or both kinetochores may be attached to microtubules originated from the same pole (syntelic attachment) (4). However, these have to be corrected to bi-orientated attachments (1) in order to resume mitotic progress through anaphase. In the absence of correct attachments, the spindle assembly checkpoint is responsible for the delay of anaphase onset. This checkpoint functions by preventing APC^{Cdc20}-mediated protein degradation. As shown in the inserted frame, activation of the spindle assembly checkpoint leads to APC^{Cdc20} inhibition. When all chromosomes are properly attached, the spindle assembly checkpoint is relieved. Then, APC^{Cdc20} induces degradation of Cyclin B and Securin, which eventually leads to the proteolytic cleavage of Cohesin, a protein involved in sister-chromatid cohesion. During mitosis, the APC/C mediates sequential waves of protein degradation (lower panel). As cells enter prophase, a first subset of proteins, among which Cyclin A and Nek-2 (black line), are degraded in an APC^{Cdc20}-dependent manner. The degradation of the first subset is not affected by the activity of the spindle assembly checkpoint. The second subset of proteins contains Cyclin B and Securin (grey line). The APCCdc20 mediates their degradation only after the spindle assembly checkpoint is turned off as described above. After anaphase onset, Cyclin B degradation leads to a switch in the activation subunit of the APC/C ($APC^{Cdc20} \rightarrow APC^{Cdh1}$). Cdh1 provides specificity for the third subset of APC/C protein targets, among which Cdc20 itself, but also Plk and Aurora-A (dashed line). The degradation of these proteins in late mitosis and in early G1 is important for proper mitotic exit and entry into S-phase.

progression in the other spindle even if all of its kinetochores are attached and under tension [70]. Therefore, regulation of Cdc20 during mitosis is not only temporally but also spatially limited. Consistent with this, Cyclin A degradation in prophase by APC^{Cdc20} is not affected by the spindle assembly checkpoint [41]. This suggests that there are several pools of Cdc20 in the mitotic cell, one in the vicinity of the spindle that would be subjected to the spindle assembly checkpoint, and one in the cytoplasm that is not subjected to the spindle assembly checkpoint.

Transcriptional programs regulating cell cycle progression.

Cell cycle progression is also partly controlled at the transcriptional level. Cell cycle gene expression programs have been well studied in yeasts, in which microarray technology has allowed the identification of a surprisingly large number of cell cycle-regulated genes that display a periodical expression pattern [71]. These genes can be grouped in clusters of co-regulated genes, which, in many cases, are controlled by a single cell cycle transcription factor [72] [73]. It is hypothesized that the serial regulation of a limited number of transcription factors governs a continuous cycle of transcriptional waves. In this model, transcription factors expressed during a certain phase of the cell cycle and belonging to one cell cycle-regulated gene-cluster govern the expression of other transcription factors in the next phase and gene-cluster, thereby building sequential and inter-dependent cell-cycle transcriptional programs.

The genome sequence, gene expression data and genome-wide location analysis data can be combined in order to define models of cell cycle transcriptional regulatory networks [74]. These analyses have led to a better understanding of the interconnections between the cell cycle and the transcriptional machinery in yeast, and in the case of conserved mechanisms, this corpus of information can be extrapolated, at least to a certain extent, to higher eukaryotes. Interestingly, there is a rather limited number of conserved cell cycle-regulated genes [75]. This suggests that this subset of cell cycle genes is crucially important for driving progression through the cell cycle, and that in general, transcriptional control in evolution is a plastic mechanism (see Figure 5).

As highlighted before, replication and mitosis are two crucial phases of the cell cycle, and they are both partly regulated at the transcriptional level. In *Saccharomyces cerevisiae*, they are respectively controlled by the transcriptional complexes SBF/MBF at the G1/S transition [76] and FKH/Mcm1/Ndd1 at the G2/M transition [77, 78]. The expression pattern of these transcription factors defines two transcriptional programs. The G1/S transcriptional program controls the CLN2/FKS1 gene cluster, which comprises genes involved in S-phase entry and in DNA replication. Similarly, the G2/M transcriptional program controls the CLB2 gene cluster, involved in the entry and execution of mitosis. The CLB2 cluster contains 33 genes, among which the mitotic cyclins Clb1 and Clb2 [77]. Comparable to the SBF/MBF in yeast, the E2F transcription factors control the G1/S transcriptional program in mammalian cells [24] involving the expression of genes encoding DNA replication proteins, enzymes of the deoxyribonucleotide (dNTP) biosynthesis, replication origin proteins, and kinases that activate replication initiation. However, the G2/M transcriptional program is much less well characterized in mammalian cells than it is in yeast. Nevertheless, a recent study done in our lab has uncovered the role of the transcription factor FoxM1 in the regulation of numerous mitotic genes [79]. As Fkh proteins in yeast, FoxM1 is a member of the Forkhead family of transcription factors. Interestingly,

the group of FoxM1-regulated genes overlaps with the CLB2 cluster.

Entry into and withdrawal from the cell cycle are also important events that are at least partially determined at the transcriptional level. Quiescence (G0-phase), as mentioned before, is a developmental program that allows survival under poor nutrient conditions. It is a non-responsive state characterized by longevity and stress resistance. However, it is reversible, meaning that quiescent cells can re-enter the cell cycle upon appropriate stimulation. In eukaryotes, quiescence is regulated, at least partly, by the highly conserved insulin/IGF1 signalling pathway. The insulin-like growth factor receptor signalling pathway has been shown to regulate longevity in the worm *Caenorhabditis*

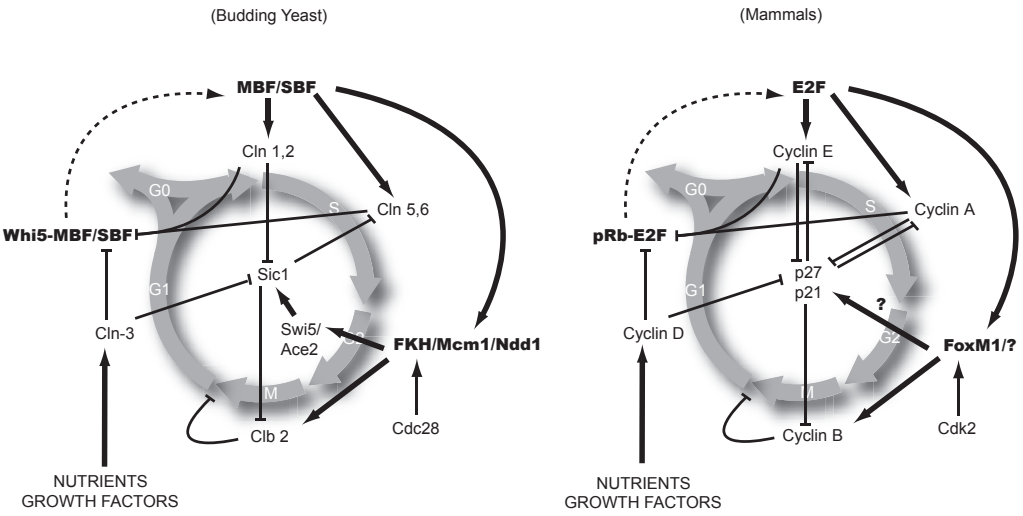

Figure 5: Transcriptional programs regulating the cell cycle. Cell cycle progression is regulated by interdependent waves of transcription. In this diagram, cell cycle transcriptional programs from yeast and higher eukaryotes are compared to highlight the degree of conservation of the most essential mechanisms that coordinate cell cycle progression. In bold are represented transcription factors responsible for the G1/S and G2/M transcriptional programs, respectively MBF/SBF//E2F and FKH/Mcm1/Ndd1//FoxM1/?. Transcriptional activation is depicted by the thick arrows, whereas thin arrows correspond to post-transcriptional activation and thin T-bars post-transcriptional inhibition. Dashed arrows designate modification of the transcriptional complex. Favourable conditions, i.e. nutrients and/or growth factors, stimulate cell cycle entry through signalling cascades that lead to synthesis and stabilization of the early G1 cyclin Cln3/Cyclin D. These can inhibit the repressors Whi5/pRb and thus activate the G1 transcriptional program. Activated MBF/SBF//E2F induce the expression of the late G1 cyclins Cln 1,2/Cyclin E and the S-phase cyclins Cln 5,6/Cyclin A and thus promote cell cycle progression. It also regulates the expression of transcription factors of the G2/M transcriptional programs, which in turn induce expression of the mitotic cyclin Clb2/Cyclin B. Cyclin/Cdk inhibitors Sic1//p27/p21 are synthesised/activated and then degraded/inactivated at each phase of the cell cycle, insuring proper coordination of the different phases. Their endogenous inhibitory role also makes of them powerful emergency brakes that can stall cell cycle progression. This diagram emphasizes the fact that these regulatory mechanisms have been conserved throughout evolution. Even though some of their molecular components may have diverged, the functional conservation underscores their indispensability and their efficacy.

elegans. Under normal conditions, the insulin-like receptor DAF-2 signals through the AGE-1/AKT pathway (PI3K/PKB) and inhibits the transcription factor DAF-16 [80] [81]. However, in adverse environmental conditions, the absence of DAF-2 signalling leads to the inhibition of the AGE-1/AKT pathway, and the subsequent activation of DAF-16, which in turn induces dauer formation. The dauer state is an "arrested" larval developmental stage in which the worm can survive starvation. When environmental conditions become more favourable again, the worm re-enters its normal life cycle to form a normal adult worm. The DAF-2/AGE-1/AKT/DAF-16 pathway has been shown to be well conserved in higher organisms, where a variety of growth factor receptors can activate the PI3K/PKB pathway, which in turn inhibits the activity of three of the members of the FoxO subfamily, FoxO1, FoxO3a, FoxO4 [82]. PKB-dependent phosphorylation of FoxO leads to its nuclear exclusion and its sequestration in the cytoplasm by interaction with 14-3-3 proteins. In the absence of PI3K/PKB signalling, FoxO can relocate to the nucleus and transactivate its target gene promoters [83].

FoxO transcription factors regulate the expression of genes that are involved in the induction of a quiescent arrest. In mammalian cells, FoxO induces the CKI $p27^{Kip1}$ and the pocket protein p130 [82, 84], and represses the expression of D-type cyclins [85]. Furthermore, enforced expression of FoxO causes growth arrest in a variety of cell-lines, including tumour cell-lines [84, 86]. Finally, FoxO can induce the expression of enzymatic activities that play a role in protection against oxidative stress [84]. In actively cycling cells, PKB is active and provides an important stimulus for cell survival. However, in quiescent cells, PI3K/PKB signalling is down and these cells may suffer of extensive oxidative damage when arrested for long periods of time. Therefore, FoxO activity may extend the life-span of quiescent cells by participating in a compensatory mechanism by enhancing protection against oxidative stress.

Outline of this thesis.

Transcriptional regulators play an important role during cell cycle progression. A subset of these even seems to have a critical function in regulating cell cycle transitions. In this thesis, I have addressed the importance of transcriptional control in the regulation of cell cycle progression, in particular at two critical transitions: the G0/G1 transition (cell cycle entry/exit) and the G2/M transition (coordination between DNA replication and segregation). In particular, my attention has focussed on the role of transcription factors of the Forkhead family in these transitions.

In the first part of the thesis (chapters 1 to 3), the role of transcriptional regulation in cell cycle entry/exit was investigated. The background information on cell cycle entry/exit in the context of the immune compartment will be presented in Chapter 1, as T cells were used as experimental system in order to investigate this question. In particular, human primary T cells were used as they represent a particularly nice model for cell cycle entry of quiescent cells upon TCR stimulation. In addition, the mechanisms by which proliferation can be suppressed in inadequately proliferating T cells such as leukaemic T cells have been addressed in more detail. This can be achieved at different levels: by preventing T cell activation, by inducing a cell cycle arrest, or by inducing apoptosis. The cAMP-dependent pathway is known to suppress T cell proliferation and to induce tolerance in normal T cells. This is targeted by numerous drugs used in the clinic for immunosuppression or anti-cancer therapy. In Chapter 2, induction of cAMP-dependent signalling was examined as a way to suppress proliferation in leukaemic T

cells. In Chapter 3, the role of FoxO Forkhead transcription factors in the regulation of cell cycle progression and apoptosis was studied in normal T cells. In particular, attention was focussed on their role during/after T cell activation.

In the second part of this thesis (chapters 4 and 5), the role of another Forkhead transcription factor, FoxM1, in G2/M was investigated, and in particular, the regulation of FoxM1 transcriptional activity during the cell cycle. After reviewing the role of FoxM1 in cell cycle regulation and carcinogenesis in Chapter 4, the mechanisms of activation and inactivation of FoxM1 during cell cycle progression are discussed in Chapter 5. More specifically, the focus of the studies has been on the role of Cyclin A in FoxM1 activation during S/G2-phases and the mechanisms of inactivation of FoxM1 during mitotic exit. The observations made in these studies can explain the low levels of FoxM1 protein and activity during the G1-phase of the cell cycle. These mechanisms may involve FoxM1 dephosphorylation and degradation during mitotic exit. Finally, the main findings of these studies and their implications for further research will be summarized and discussed in Chapter 6.

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Chapter 1:
Transcriptional regulation of cell cycle and cell death in lymphocytes.

Transcriptional regulation of cell cycle and cell death in lymphocytes.

T lymphocytes are essential actors of the adaptive immune system. Engagement of their T Cell Receptor (TCR) has a variety of outcomes, ranging from activation and subsequent proliferation, cytokine production or cell cytotoxicity, to anergy and apoptosis.

T cell activation and subsequent proliferation.

Most circulating T lymphocytes are naive T cells, for they have not yet been in contact with any activating antigen. These cells are "long-lived" cells that are arrested in the G0-phase of the cell cycle. The event that induces re-entry of the lymphocytes into the cell cycle is called T cell activation. A full immune response builds up only after stimulation by a specific antigen presented by Major Histocompatibility Complex (MHC) molecules expressed on the surface of an antigen-presenting cell (APC), and in the context of activated co-receptors (such as CD28 bound to its ligand B7, also expressed by the APC).
First, engagement of the TCR leads to the transition of the T cell from the G0 to the G1-phase of the cell cycle. This has as main consequence the early induction of effector functions that characterize the activated state, namely the expression of both the cytokine Interleukine-2 (IL-2) and the high-affinity IL-2 receptor. Then, activation of IL-2 signalling pushes the activated cell to commit to proliferation, i.e. to progression through the G1 into the S-phases of the cell cycle.

Molecular events following T cell receptor engagement.
TCR engagement results in a cascade of signalling events including protein tyrosine kinase (PTK) recruitment and activation, substrate phosphorylation, subsequent mobilisation of adapter proteins and activation of second-messenger cascades (see Figure1).
Within seconds after TCR engagement, Lck, a PTK of the Src family, is recruited to and phosphorylates the TCR immune receptor-based activation motifs (ITAMs). These phosphorylated motifs in turn recruit the PTK Zap-70, which phosphorylates multiple substrates including the protein Linker for Activation of T cells (LAT). LAT plays a critical role in coupling the proximal events to a variety of downstream signalling pathways [1]. This transmembrane protein has a long cytoplasmic tail containing tyrosine residues, which, upon phosphorylation by Zap-70, become docking sites for adapter proteins. The most important adapter proteins with respect to TCR signalling are Grb2, Gads, p85 regulatory subunit of Phosphatidylinositol-3OH kinase (PI3K), and Phospholipase Cγ (PLC$\tilde{\gamma}$) [2][3].
Grb2 associates with Sos (Son of sevenless), a Ras guanine nucleotide exchange factor (GEF), which activates the small G-protein Ras. Consequently, Ras triggers activation of the Raf/ERK Mitogen-Activated Protein Kinase (MAPK) pathway [4]. This pathway is crucial for the integration of mitogenic signals, mediated at least partly by TCR signalling.
Gads recruits the adapter SLP-76 which binds both Grb2 and Vav, leading to the activation of Rho family of guanine-triphosphate phosphatases (GTPases). These proteins, in particular Cdc42 and Rac, regulate cellular shape by controlling the actin cytoskeleton. Their activation following TCR engagement leads to a large-scale rearrangement of the actin cytoskeleton and to the activation of integrin signalling. This participates to the acquisition of a closer and tighter contact between the activated T cell and the APC. This reorganisation is accompanied by the congression of surface receptors, adapters and signalling molecules at the T cell/APC interface, known as immunological synapse [5].
PI3K binds to phosphatidylinositides and phosphorylates their position-3 hydroxyl group [6]. Activation of PI3K leads to formation of the signalling membrane phospholipids

phosphatidylinositol-(3,4,5)triphosphate (PIP3), and phosphatidylinositol-(3,4)biphosphate (PIP2), which bind and activate plekstrin homology (PH) domain-containing proteins such as Plekstrin Domain Kinase 1 (PDK1) or Protein Kinase B (PKB)/Akt [7]. However, it seems that TCR signalling alone is not sufficient to activate PKB and the role of co-activators such as CD28 or the IL-2 receptor appears to be fundamental here [8][9]. Furthermore, PI3K is also known to positively regulate Vav, and helps to fully activate PLCγ.

PLCγ activation is central in TCR signalling. It hydrolyses the membrane phospholipids PIP2 to produce inositol 3-phosphate (IP3) and diacylglycerol (DAG) second messengers. On the one hand, IP3 leads to mobilisation of the intracellular stores of calcium and to increased free intracellular Ca^{2+} concentration [10]. On the other hand, DAG activates the Protein Kinase Cθ (PKCθ), and the Ras GEF RasGRP, which might represent an alternative pathway for Ras activation in T cells [11][12].

Several central players of T cell activation, such as LAT, TCRζ chain and PKCθ, are sequestered within specialised lipid microdomains called “lipid rafts”. These lipid rafts have been observed to cluster at the immunological synapse. Thus, it seems evident that not only does TCR signalling orchestrate protein-protein interactions, but it also leads to a major re-organisation in the membrane, in order to allow sustained TCR engagement and therefore execution of complex functions such as T cell proliferation, which requires signalling for longer periods of time.

Transcriptional regulation following TCR engagement.

The consequence of TCR engagement and concomitant activation of these signalling molecules is the dynamic regulation of more than 100 activation-specific genes that are required for cell cycle progression and proliferation. These activation-specific genes are regulated by transcription factors activated downstream to the previously described signalling cascades [13]. Not only do these transcription factors need to be activated, but they also need to bind simultaneously in order to coordinate expression of the activation-specific genes. One of the most studied activation-specific genes is the IL-2 gene [14]. IL-2 expression following TCR engagement is mediated by the inducible transcription factors Activation Factor 1 (AP1), Nuclear Factor of Activated T cells (NFAT) and Nuclear Factor κB (NFκB) (see Figure 1). Their individual binding onto IL-2 promoter is important, and each of them is regulated separately. However, all three must be coordinately activated in order to stimulate IL-2 production.

AP1 is the main transcription factor activated by MAPK signalling and it is involved in many cellular processes, including cell cycle progression [15]. AP1 is composed of two subunits, Jun and Fos. In resting T cells, Jun is cytoplasmic. Upon activation, it translocates to the nucleus where it associates with Fos to form a transcriptionally active AP1 complex. Again, the transcription factor NFAT is cytoplasmic in resting cells, and translocates to the nucleus upon activation. However, NFAT is mainly regulated by calcium signalling [16]. After activation, its nuclear import is induced by dephosphorylation by Calcineurin, a Ca^{2+}-dependent phosphatase. Once in the nucleus, NFAT cooperates with transcription factors of the AP1 family to induce the expression of T cell activation-associated genes such as the IL-2 gene. Yet, the activation of AP1 transcription factors only occurs during a productive T cell activation, when both TCR and co-stimulatory signals (such as CD28 triggering) are integrated and potentiate activation of the MAPK pathways. The transcription factor NFκB is essential for T cell activation [17]. Similarly to AP1 and NFAT, although using a different mechanism, NFκB is cytoplasmic in resting T cells and translocates to the nucleus following TCR engagement. NFκB is sequestered in the cytoplasm by physical interaction with the protein Inhibitor of κB (IκB). Upon phosphorylation by IκB kinase β (IKKβ), IκB is degraded, releasing NFκB. IKKβ is itself regulated by several kinases, but PKCθ is required for activation of NFκB downstream to TCR signalling [18]. However, AP1, NFAT and NFκB transcription factors also regulate other genes, in a coordinated

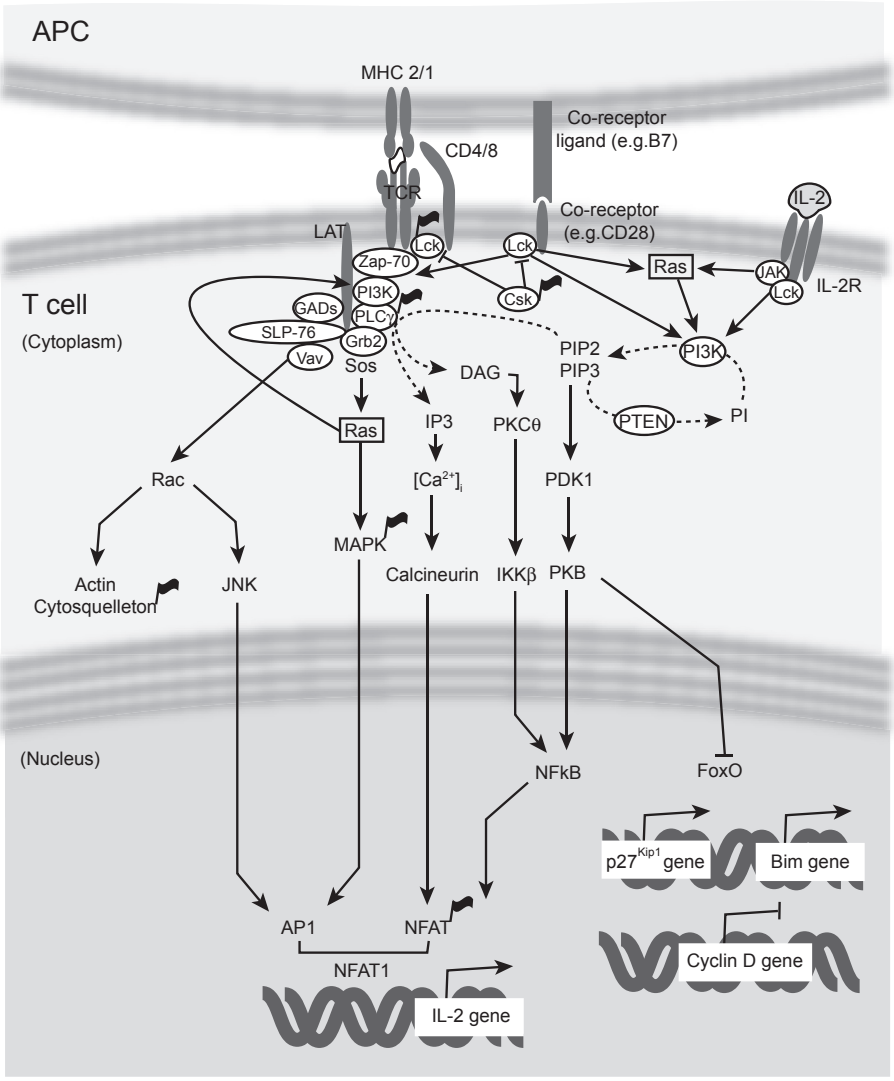

Figure 1: Molecular cascade following T cell activation. Full T cell activation requires TCR signalling accompanied by either co-stimulatory receptor signalling (e.g. CD28) or cytokine signalling (e.g. IL-2). They synergize and converge to critical signal transduction cascades, downstream to proximal protein tyrosine kinases (PTK's) such as Lck. The main mitogenic effect of T cell activation is due to activation of the Ras/Raf/ERK/MAPK pathway. PI3K/PKB pathway is activated mostly downstream to CD28 or IL-2 receptors. It is particularly important with respect to T cell survival. This pathway acts through the inhibition of FoxO transcription factors that are known to induce cell cycle arrest and apoptosis. Finally, TCR signalling also leads to the activation of PLCγ, which in turn activates PKCθ and calcium signalling, both of which are critical for T cell activation. The first is important for the activation of the NFAT transcription factor, and the second for activation of the NFκB transcription factor. NFAT and NFκB collaborate together with AP1 transcription factors to induce the expression of IL-2, a critical step for T cell activation. In the clinic, drugs that increase cAMP levels or activate cAMP signalling are known to modulate immunity. PKA is the most important cellular receptor for cAMP, and it indeed mediates modulation of TCR signalling. The protein targets of PKA immunomodulation are labelled with the black flag.

fashion or independent of each other. AP1, for instance, is important for the proliferative response of the activated T cell, not only because of its effect on IL-2 expression, but also because of its effect on the expression of components of the cell cycle machinery such as Cyclin D or Myc (see following paragraph). Moreover, NFAT/AP1 complexes and NFκB regulate the expression of many other activation-associated genes, in particular of cytokines such as IL-3, IL-4, IL-13, GM-CSF and TNFα.

Finally, the cyclic-AMP Response Element Binding protein CREB is another important transcription factor activated following TCR engagement. In resting T cells, CREB is transcriptionnally inactive. During T cell activation, CREB rapidly gets phosphorylated, mostly downstream to Ras/MAPK and calcium/Calmodulin kinase signalling cascades. Activated CREB binds to the co-activator CREB-binding protein (CBP) and transactivates T cell-specific genes such as TCR components. In addition, CREB induces AP1 components Jun and Fos. Moreover, in the absence of functional CREB, T cells are arrested at the G1/S checkpoint and undergo programmed cell death in response to several activation signals. Therefore, CREB plays a critical role during T cell activation, mostly due to its role in the expression of AP1 and consequently of IL-2 [19].

Modulation of TCR signalling by PKA.

Cell cycle progression and proliferation must be tightly regulated in order to prevent exaggerated immune response. Therefore, dynamic inhibitory mechanisms are required in order to control, adjust the adaptive immune reaction. One of them is the cyclic AMP-dependent serine-threonine protein kinase A (PKA), a major modulator of immunity [20]. PKA is the principal cyclic 3'-5'-adenosine monophosphate (cAMP) receptor in cells. In mature lymphocytes, increased intracellular levels of cAMP mediate a general inhibitory signal. cAMP is synthesised by adenylate cyclases (ACs), and degraded by phosphodiesterases (PDEs). In T cells, TCR/CD28-stimulated expression of PDE7 is necessary for the increase in IL-2 production [21], suggesting that cAMP levels, and therefore PKA activity, are potent modulators of immunity.

PKA acts by regulating TCR signalling at different levels (see Figure 1). First, PKA can modulate TCR proximal signalling. Csk is a key-player in the control and fine-tuning of the proximal TCR. In resting peripheral T cells, this PTK is constitutively associated to the lipid rafts, where it inhibits Lck by phosphorylation. Upon TCR engagement, Csk dissociates from the lipid rafts, and loses its activity, thereby allowing activation of Lck and TCR downstream signalling. After activation, Lck phosphorylates the protein anchor of Csk in the lipid raft, allowing its re-association of Csk to the lipid raft which turns TCR signalling off [22]. PKA is also present in the lipid rafts, where it can activate Csk by phosphorylation [23]. This provides a powerful mechanism for terminating activation.

In addition, PKA can also act on a variety of signal transduction cascades. PKA can phosphorylate PLCγ and suppress phosphatidylinositol hydrolysis and calcium mobilization upon T cell activation [24][25]. The inhibitory regulation of TCR signalling by PKA through Csk and PLCγ seems to the most potent, and certainly are responsible for the general inhibitory effect of PKA. Furthermore, PKA can interfere with MAPK cascade. PKA has been shown to inhibit the activity of Raf-1, an essential kinase for mitogenic signalling [26][27], and to activate Rap1, a known Raf-1 inhibitor. PKA also prevent integrin activation in leukocytes through inhibition of RhoA GTPase [28][29]. This has as main consequence. Because stable interactions are required in order to build up the immune reaction, PKA and cAMP, by causing less effective cell adhesion, prevent T cell activation. On the other hand, PKA can activate MAPK signalling. First PKA can activate B-Raf in a Rap1-dependent manner [30]. Then, PKA can also increase activation and nuclear translocation of Erk1/2 and p38 MAPK through the inhibition of tyrosine-protein phosphatases, notably in haematopoietic cells [31][32]. Thus, PKA has various effects on the cellular components activated by TCR signalling. That is the reason why PKA is

considered as a modulator and not only an inhibitor of immunity. However, keeping MAPK signalling is important to induce T cell death during antigen-activated T cell death [33] (this will be address later in this chapter), and thus, PKA positive regulation of MAPK may fully take part in modulation of immunity

Finally, PKA can also interfere with the activity of various TCR-regulated transcription factors. PKA exerts its activity by phosphorylating NFAT on a residue adjacent to the nuclear localization signal (NLS) that regulates association with 14.3.3 proteins, leading to its inhibition through sequestration in the cytoplasm [34]. In that sense, PKA and Calcineurin exert opposing effects by regulating NFAT cytoplasmic retention by association with 14.3.3.

IL-2-dependent signalling.

Once the T-cell has re-entered from G0 into G1, the activated state is further regulated by cytokines, and mostly by IL-2. IL-2 is a bona fide lymphocyte growth factor which downstream signalling controls T cell survival, proliferation and functional differentiation [14]. Therefore, concomitant expression of IL-2 and of its high-affinity receptor is a key event after which the T cell becomes sensitive to the trophic action of IL-2 following TCR engagement. This mechanism generates a positive feedback loop, which explains the extremely intense clonal proliferation of the effector T cell pools after antigen recognition.

The IL-2 receptor is composed of α, β, and γ subunits [35], and heterodimerisation of the β and γ subunits [36] and activation upon binding with IL-2 induces PTK-dependent pathways involving Janus kinases and Src family, which in turn leads to the activation of the mitogenic protein Ras and PI3K (see **Figure** 1) after recruitment of Grb2 adapter protein, similarly as in TCR signalling. The PI3K/PKB pathway is thought to be critical for regulating IL-2-dependent proliferation of T cells, since PI3K inhibitors such as LY294002 can block proliferation of primary T cells [37][38][39]. But, not only is the PI3K/PKB pathway important for the IL-2-dependent proliferation, it is also known to regulate survival of numerous cell types, including lymphoid cells (see next).

Apoptosis and anergy: safeguards of immunological homeostasis.

Because of the potentially deleterious effects of uncontrolled immune reactions, some mechanisms prevail in order to guarantee protection against overactive T lymphocytes.

T lymphocytes are subjected to life-and-death decisions throughout their differentiation, during their maturation in the thymus as well as in the periphery. These decisions aim at the deletion of every self-toxic T lymphocyte. This process is referred to as self-tolerance [40]. It occurs first during T cell development through deletion by apoptosis of auto-reactive T cells that are specific for ubiquitous self-antigens. Furthermore, in the periphery, deletion of lymphocytes by apoptosis occurs after a prolonged antigenic stimulation or when cytokine levels are becoming limiting, at the end of an immune reaction. Self-tolerance is also acquired in the periphery using another mechanism than apoptosis. Indeed, auto-reactive T cells can also be inactivated by anergy following exposure to tissue-specific antigens in the absence of co-stimulatory signals.

Apoptosis in T lymphocytes.

Apoptosis is a conserved and indispensable mechanism by which cell death is actively induced, notably during developmental programs. It is also known as programmed cell death (PCD) or cell suicide. It has several phenotypic features among which blebbing of the plasmatic membrane, exposure of specific phospholipids on the cell surface, cell shrinkage, nuclear DNA fragmentation. Interestingly, unlike necrotic cells, apoptotic cells do not burst and their content remains enclosed in the plasmatic membrane. Therefore, apoptotic bodies do not cause local inflammation and are silently resorbed through phagocytosis [41][42].

In T cells, apoptosis preserves peripheral homeostasis by controlling both number and specificity of the lymphocytes activated by diverse antigens [43]. T cell apoptosis occurs mainly by two processes: either by lymphokine withdrawal or by an antigen-

driven mechanism. Furthermore, two cellular pathways, the mitochondrial pathway, and the death receptor pathway can induce apoptosis [44]. Both pathways lead to the activation of effector cystein proteases, named caspases, which are, at least partly, responsible for the phenotypic features of apoptosis [45] (see Figure 2).

During T cell death induced by cytokine withdrawal, members of the Bcl-2 family play an essential role in the initiation of the apoptotic response. Bcl-2 family contains pro- apoptotic (Bax, Bad, Bim, etc) and anti-apoptotic (Bcl-2, Bcl-XL, etc) members that can dimerise [46]. The balance between these two classes governs the release of cytochrome C from the mitochondria and the subsequent recruitment of the adapter Apaf-1 required for the activation of the initiator caspase-9. In turn, caspase-9 activates the downstream caspase cascade.

Although IL-2 signalling is essential for both survival and proliferation of activated T cells, it also renders them exceptionally susceptible to apoptosis once they have entered the cell cycle in response to antigen stimulation. This concept is called propriocidal regulation of the immune response and is based on the idea that, distinctly from its effect on proliferation, IL-2 exerts a negative feedback control through apoptosis in mature lymphocytes receiving TCR stimulation [47]. Indeed, after clearance of the infection, antigen stimulation ceases, leading to lower IL-2 production and lower IL-2 receptor expression. This curbs the most important survival signal of the activated T cell and inexorably leads to “passive” apoptosis due to cytokine withdrawal. This is thought to be the major mechanism of deletion of the activated T cells after clearance of the antigen.

However, although most of the activated T cells die rapidly after clearance of the infection, it is not well understood yet which mechanism protects a minor population (5-10%) of activated T cells from death. They form the pool of long-lived memory T cells [48]. After clearance of the infection, these cells enter a low metabolic state, comparable to quiescence. In contrast to that of naïve T cells, memory T cell survival and longevity does not require “tickling” by MHC molecules [49]. There is evidence to suggest that memory CD8+ T cell survival is dependent on IL-15 stimulation and on enhanced expression of the IL-15 receptor, which may ultimately act via maintaining of the expression of various anti-apoptotic proteins [50]. On the other hand, memory CD4+ T cell survival is dependent, similarly as naïve T cells, on IL-7 stimulation [51].

Antigen-induced cell death (AICD) is very distinct from cytokine withdrawal-induced apoptosis, both from the molecular point of view as well as from the physiological point of view. Whereas cytokine withdrawal-induced apoptosis is a pathway by which lymphocytes are deleted when the infection source has been eliminated, AICD occurs during prolonged exposure of the activated T cells to their activating antigen. This may represent the default pathway for every activated T cell that fails to be rescued by proper survival signals, such as co-stimulation by CD28 or IL-2 [52].

From the molecular point of view, AICD is induced using the death receptor pathway [53]. Death receptors such as Fas or TNF receptor, upon activation by their respective ligand Fas ligand (FasL) and TNFα, oligomerise and bind to the adapter FADD through their so-called death domains (DD). This leads to the activation of Caspase-8. On the one hand, caspase-8 activates the downstream caspase cascade, and on the other hand, caspase-8 activates by cleavage the pro-apoptotic Bcl-2 member Bid [54]. Cleaved Bid interacts with Bcl-2 and therefore modifies the Bcl-2 family member balance leading to activation of the mitochondrial pathway of apoptosis [55].

Anergy.

Anergy is an alternative mechanism to apoptosis by which T cells can be rendered inactive and non-responsive. T cell anergy corresponds to failure for an antigen to activate its specific T cell due to the absence of co-stimulatory signals [56]. Therefore, anergic cells fail to activate the MAPK/AP1 pathway and to IL-2 production following TCR engagement. In the absence of binding of AP1 dimers, the IL-2 gene promoter is

repressed by CREB complexes. Furthermore, NFAT transcription factor, which are activated downstream to calcium signalling, cannot transactivate the IL-2 gene in absence of cooperation with AP1 transcription factors. Instead, it transactivates a distinct set of genes. These calcium-induced genes associated with reduced responsiveness are negative regulators of the TCR signalling that act by maintaining the anergic arrest [57]. Therefore, calcium signalling is clearly critical for induction of anergy. Interestingly, some of these genes encode for E3 ubiquitin ligases which have been shown to interfere with TCR proximal signalling, in particular with PLCγ, PKCθ and Ras activation [58].

The anergic T cell cycle arrest also correlates with increased levels of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor, which promotes a cell cycle arrest in the G1-phase. $p27^{Kip1}$ protein levels are controlled by the PI3K/PKB pathway. The absence of co-stimulatory signals, and thereby the lack of PI3K/PKB signalling might therefore lead to increased levels of $p27^{Kip1}$ and maintain the anergic state. Not only do $p27^{Kip1}$ protein levels inhibit cell cycle progression by inhibiting the G1 cyclin-cdk kinase activity, but it has also been shown to interfere with AP1 activation in anergic cells, and therefore with IL-2 transcription [59].

Clinical implications.

Lymphocyte activity should be limited in volume and time, otherwise the immune response, which purpose is to maintain the organism healthy, lapses into disease. On the one hand, deregulated lymphocyte proliferation gives rise to a number of biologically and clinically different haematological malignancies. Indeed, defects in the regulation of proliferation and/or apoptosis at various stages of normal lymphocyte differentiation can lead to distinct cancers. On the other hand, some antigens may elicit inappropriate immune responses, hence leading to various disorders such as allergies and autoimmune diseases [60]. Autoimmune pathologies are characterized by an adaptive (auto-) immune response that is mounted against the host. The concept of "horror autotoxicus" was stated as immunologists realized early that the immune response could have dreadful consequences if turned against the "self" since it involves potent and potentially toxic effector functions. As a matter of fact, autoimmune reactions are rather common, but only when persistent and leading to severe tissue/organ damage do they become of medical resort.

As described above, apoptosis and anergy are two natural processes that can prevent exaggerate expansion of T cell pools or inappropriate T cell activation. Pharmacologically-induced apoptosis or anergy represent an attractive therapeutic tool in the clinic for the treatment of immunological disorders [61]. Inflammatory mediators (prostaglandin E) and pharmacological agents (corticosteroids) that were first observed to alter hypersensitive immune reactions are now known to modify cAMP levels, and therefore PKA signalling [62][63]. At present, drugs that induce anergy are used in the clinic to induce immunosuppression in the treatment of patients with severe inflammatory or autoimmune diseases (lupus, arthritis, etc) and of patients receiving organ transplant. The most commonly used are Cyclosporine A and Tacrolimus (FK506), both shown to inhibit calcineurin, and thereby interfere with Calcium signalling and IL-2 expression and thus phenocopy the action of PKA [64]. Finally, in the human body, about 80% of the immune cells are present in the mucosa-associated lymphoid tissue. Already since a long time, a number of vaccines are delivered orally, basing their action on mucosal immunity and its capacity to induce systemic tolerance to oral antigens. Currently, there is a growing interest to use mucosal-induced tolerance as immunomodulatory treatment for certain autoimmune diseases and allergies [65].

Regulation of cell cycle progression and apoptosis at the G0-G1 transition in lymphocytes.

As mentioned before, most circulating T lymphocytes are long-lived cells that are arrested in the G0-phase of the cell cycle. This state is called quiescence. Quiescence is a non-proliferative state that is actively maintained, rather than a default pathway in

the absence of any stimulation [66][67][68].

Cell cycle arrest and resistance to apoptosis in quiescent cells.

In peripheral blood T lymphocytes, key cell cycle regulators maintain the quiescent state, in particular the E2F4-p130 repressor complex [69][70]. Indeed, induction of the retinoblastoma member p130 has been shown to be important for cell cycle exit and maintenance of a quiescent G0 state [71]. It binds E2F4, a repressor E2F family member [72]. However, p130 and the other retinoblastoma members such as p107 show functional compensation, and thus p130 is not strictly required for the establishment and maintenance of the quiescent state [73].

Quiescence is also characterized by a resistance to apoptosis, and for that reason can be distinguished from anergy, which is highly sensitive to death stimuli. Of course, the pool of naïve T cells represents the whole potential of the organism's resistance to pathogens. Therefore, there should be a mechanism that protects them from dying. It has been shown that survival of resting naïve T cells requires continuous contact or "tickling" by MHC combined with exposure to IL-7 [74]. It seems likely that this mechanism provides a low-level signalling sufficient to keep the cells alive although insufficient to induce their re-entry into the cell cycle. Furthermore, Bcl-2 is expressed at relatively high levels in naïve and CD8+ memory T cells, and is required for quiescent naïve T cells survival [75][76]. Conversely, Bcl2 levels drop dramatically upon TCR stimulation, and this is necessary to render activated T cells to AICD [77]. In activated T cells, however, co-stimulation by CD28 or IL-2 through the PI3K/PKB pathway increases Bcl-XL levels, protecting them from propriocidal apoptosis [78][79].

Unlike anergy, quiescence is a reversible cell fate, and understanding the molecular switches that regulate it will lead to a better comprehension of both cell cycle control in normal lymphocyte and haematological tumour development. Indeed, factors important for the regulation of quiescence represent potential tumour suppressor gene products, whose alteration can lead to malignant development, and one could think that these factors could be exploited in the future in order to enforce quiescence in leukaemia's or lymphomas.

The quiescence transcriptional program.

A growing body of evidence points in the direction of a transcriptional program regulating quiescence in lymphocytes. These transcriptional regulators control the expression of intermediate transcription factors and thereby influence the expression of crucial cell cycle regulators [80]. The "quiescence factors" have three characteristics: 1) they are expressed at high levels in various quiescent T cells; 2) their expression is abrogated by appropriate T cell activation; and 3) their overexpression can lead to enforced quiescence in normal or malignant cell-lines. As such, these factors are both required for maintenance of T cell homeostasis and sufficient to induce quiescence in leukaemic T cells. Mutations in these quiescence factors or in their target genes could lead to the development of haematological malignancies and/or autoimmune pathologies. In lymphocytes, there are two major "quiescence (transcription) factors", the Lung Kruppel-like factor (LKLF) and proteins of the FoxO subfamily of Forkhead transcription factors.

LKLF is a member of the family of Kruppel-like factors (KLFs), and has been identified as a quiescence factor. Its expression pattern, for instance, is consistent with a role in quiescence: it is highly expressed in naïve T cells, it is rapidly lost after T cell activation, but is recovered in memory T cells, another important quiescent T cell population (Schober et al, 1999). Furthermore, overexpression of LKLF is sufficient to enforce a quiescent arrest in tumour T cell-lines [81]. In a mouse model, LKLF-deficient T cells have a spontaneously activated phenotype and are rapidly eliminated by Fas-mediated apoptosis in the lymphoid organs, resulting in a reduced number of circulating T cells [82]. However, these circulating LKLF-deficient T cells show increased proliferation, increased cell size and increased expression of surface activation markers [81]. Thus, LKLF appears to be required for execution of the quiescent

program in mature T cells.

Forkhead box proteins, and particularly those belonging to the FoxO subfamily, are transcription factors that have a broad influence on cell cycle regulation and cell survival. Forkhead transcription factors belong to a large family of transcription factors with a highly conserved DNA binding domain, also called the Forkhead box, or winged-helix domain. Forkhead genes are found in species ranging from yeast to humans, and over 100 members (Fox) have been identified, segregated in 18 subfamilies (FoxA to R) [83]. The first member of the FoxO subfamily to be identified in *C. elegans* was DAF-16. DAF-16 acts downstream of the insulin-like growth factor receptor signalling to to regulate dauer formation and longevity [84] [85]. The DAF-2/AGE-1/AKT/DAF-16 pathway has been shown to be well conserved in higher organisms, where a variety of growth factor receptors can activate the PI3K/PKB pathway, which in turn inhibits the activity of three of the members of the FoxO subfamily, FoxO1, FoxO3a, FoxO4 [86].

The literature is consistent with a role for FoxOs in the regulation of quiescence in lymphocytes. Indeed, in mammalian cells, FoxO activity is low in normally growing cells due to inhibition by the PI3K/PKB pathway. FoxO activation regulates the expression of genes that are involved in the establishment of a quiescent arrest, by inducing the CKI $p27^{Kip1}$ and the pocket protein p130 [86][87], and by repressing the expression of D-type cyclins [88].

Furthermore, enforced expression of FoxO causes growth arrest in a variety of cell-lines, including tumour cell-lines [87][89]. Finally, FoxO can induce the expression of enzymatic activities that play a role in protection against oxidative stress [90]. This also may justify the designation of FoxO as “quiescence factor” as FoxO activity may extend the life-span of quiescent cells by participating to a compensatory mechanism that enhances protection against oxidative stress in the absence of growth factor signalling.

Several lines of evidence indicate that inactivation of FoxO plays a role during tumour development. First, FoxO factors are implicated in chromosomal translocations in alveolar rhabdomyosarcoma [91][92] and in acute leukaemia [93]. In these tumours, the FoxO transactivation domain is fused with the DNA-binding domain of PAX3/PAX7 transcription factors or to MLL, and is required for the transforming activity of the fusion proteins. Although this transforming activity may be mediated through transcriptional regulation, it may also result from the functional knock-out resulting from the chromosomal translocation. Indeed, the translocation itself leads to the inactivation of one allele of one FoxO gene. However, FoxO4 (AFX) gene is located on the X chromosome. Therefore, in male individuals, it leads to a full knock-out for this gene, which may participate in carcinogenesis. Furthermore, PAX3-FoxO fusion has been shown to upregulate a growth factor receptor, which is a very potent activator of PI3K pathway, which in turn inactivates FoxO transcription factors. Therefore, not only does the chromosomal translocation leading to PAX3-FoxO1 fusion protein inactivate one FoxO1 allele, but it also leads to the secondary inactivation of all FoxO factors through up-regulation of PI3K pathway. Therefore, FoxO loss of function may participate to carcinogenesis, also suggesting that FoxO may be a protein with tumour suppressor activity.

In addition, FoxO activity is positively regulated by the tumour suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) [94]. PTEN is a phosphoinositide-3OH phosphatase, which antagonizes PI3K activity [95]. It is found mutated or deleted in many tumours and tumour-derived cell-lines. In PTEN-negative cancer cell-lines, the increased PIP2 and PIP3 levels correlate with activation of PKB, increased proliferation and decreased sensitivity to cell death. Furthermore, FoxO is found in its inactive cytoplasmic form. Similarly, PTEN heterozygous mice display increased proliferation and impaired Fas-mediated apoptosis that leads to neoplasm formation and to the development of a lethal polyclonal autoimmune disorder. Re-introduction of PTEN in PTEN-negative tumour cells causes inhibition of PKB, growth suppression due to increased $p27^{Kip1}$ levels,

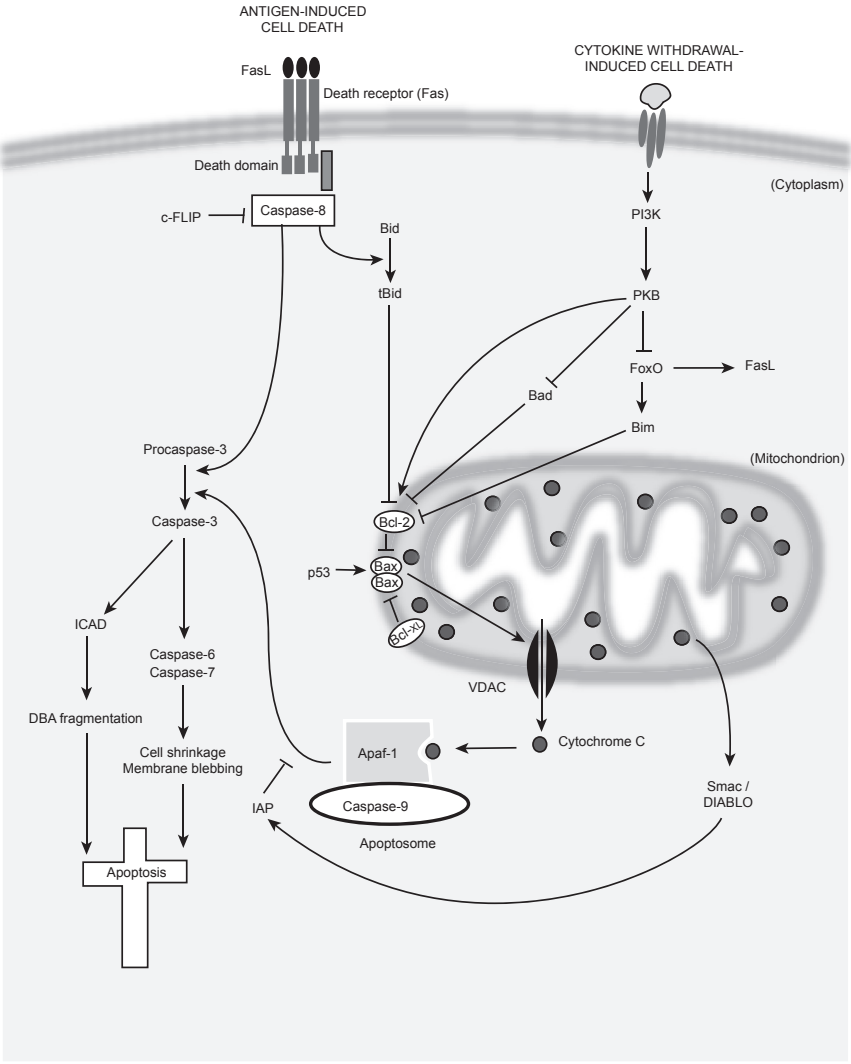

Figure 2: Overview of the apoptotic molecular cascades. In T cells, apoptosis can be induced in two manners. First, antigen-induced cell death (AICD) utilizes the death receptor pathway. Upon ligand binding, these receptors oligomerize and recruit Caspase-8 through the adapter FADD. Caspase-8 is a protease that induces proteolysis of another caspase, Caspase-3. On the other hand, cytokine withdrawal can induce cell death, through loss of PI3K/PKB pathway activation, and consequent activation of FoxO transcription factors. FoxO, when activated, can mediate expression of potent apoptotic proteins such as Bim and Fas ligand, in parallel to their effect on cell cycle progression. The lack of PKB-mediated phosphorylation also leads to activation of pro-apoptotic proteins such as Bad and Caspase-9, as well as inhibition of the anti-apoptotic protein Bcl-2. Pro- (Bax, Bad, Bim, Bid) and anti-apoptotic proteins of the Bcl-2 (Bcl-2, Bcl-XL) can dimerize and their balance regulate the activity of the voltage-dependent anion channel (VDAC). After cytokine withdrawal, strong activation of pro-apoptotic members and/or inhibition of anti-apoptotic members command the release of Cytochrome C from the mitochondrion through the VDAC channel. Cytochrome C associates with Apaf-1 and Caspase-9 to form the apoptosome, which also leads to activation of Caspase-3 by proteolysis. Active Caspase-3 then mediates proteolysis-mediated activation of several effectors that are responsible for the phenotypic features of apoptosis: DNA fragmentation, cell shrinkage and membrane blebbing.

and increased sensitivity to apoptotic stimuli. Overexpression of FoxO, like re-introduction of PTEN can induce a cell cycle arrest in G1 and/or apoptosis. Therefore, FoxO "constitutive" inactivation due to loss of function of PTEN may account for, at least partially, for tumour development, owing to its inability to induce expression of $p27^{Kip1}$ [96].

Summary and perspectives.

Several pathways are naturally able to control T cell proliferation and survival, either by inducing apoptosis, or by inducing an unresponsive state (anergy), or finally by promoting cell cycle exit (quiescence). Therefore, by high-jacking these pathways, it is theoretically possible to control T cell proliferation and survival. Some seem particularly interesting on that point of view: for example PKA, as it is a potent immunomodulator. Quiescent factors are also interesting to induce long-term cell cycle arrest of cancer cells. In that respect, FoxO are attractive candidate as they belong to a very well-known pathway, which is potentially easy to target in the clinic (specific drugs, monoclonal antibodies or blocking peptides for growth factor receptors, etc).

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Chapter 2:

Protein Kinase A regulates expression of p27/Kip1 and Cyclin D3 to suppress proliferation of leukemic T cell lines .

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Protein Kinase A Regulates Expression of p27^{kip1} and Cyclin D3 to Suppress Proliferation of Leukemic T Cell Lines*

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Peripheral homeostasis and tolerance requires the suppression or removal of excessive or harmful T lymphocytes. This can occur either by apoptosis through active antigen-induced death or cytokine withdrawal. Alternatively, T cell activation can be suppressed by agents that activate the cAMP-dependent protein kinase (PKA) signaling pathway, such as prostaglandin E2. Stimulation of PKA inhibits lymphocyte proliferation and immune effector functions. Here we have investigated the mechanism by which activation of PKA induces inhibition of proliferation in human leukemic T cell lines. Using a variety of agents that stimulate PKA, we can arrest Jurkat and H9 leukemic T cells in the G_1 phase of the cell cycle, whereas cell viability is hardly affected. This G_1 arrest is associated with an inhibition of cyclin D/Cdk and cyclin E/Cdk kinase activity. Interestingly, expression of cyclin D3 is rapidly reduced by PKA activation, whereas expression of the Cdk inhibitor p27^{kip1} is induced. Ectopic expression of cyclin D3 can override the growth suppression induced by PKA activation to some extent, indicating that growth inhibition of leukemic T cells by PKA activation is partially dependent on down-regulation of cyclin D3 expression. Taken together our data suggest that immunosuppression by protein kinase A involves regulation of both cyclin D3 and p27^{kip1} expression.

Exposure to an antigen elicits a dramatic expansion of resting peripheral T lymphocytes that specifically recognize and target this antigen. However, the proliferative capacity and effector functions of the evolving T cell population need to be kept in check in order to ensure a restricted reactivity and protect the host from an uncontrolled immune response. To this end, several mechanisms have evolved that can cause deletion or suppression of reactive mature T cells. One mechanism involves apoptosis of the activated T cell population that can occur either via restimulation of the T cell receptor (TCR)[1] with the appropriate antigen or through cytokine deprivation (1). Another mechanism involves suppression of T cell proliferation and effector functions by glucocorticoid hormones and prostaglandins of the E series (PGE) (2). These agents are thought to regulate the intensity of the immune response at the site of inflammation. For example, PGE2 is secreted by many cell types, such as IL-1-stimulated macrophages, and is a potent activator of the cAMP-dependent protein kinase (PKA) pathway in lymphocytes (3). Stimulation of PKA results in the inhibition of T cell proliferation and dampens T cell effector functions (2). As such, PGE2 conveys an inhibitory signal to produce a properly tuned immunoreactivity.

At the onset of the immune response, resting peripheral T lymphocytes enter the cell cycle from quiescence (G_o). At this time D-type cyclins and cyclin E are sequentially synthesized during the G_1 interval (4–6), both being rate limiting for S phase entry (7). Three D-type cyclins have been identified, of which cyclins D2 and D3 are predominantly expressed in T cells (5, 8). Cyclins D2 and D3 can form an active kinase complex with the cyclin-dependent kinase (Cdk)4 or Cdk6, and the resulting kinase complexes are involved in phosphorylation of the retinoblastoma protein (pRb) (8, 9). Phosphorylation of pRb results in its functional inactivation and allows progression of the cell through the late G_1 restriction point and subsequent entry into S phase (10). However, induction of cyclin D expression alone is not sufficient to drive resting T cells into S phase. This is due to the fact that resting T cells express abundant amounts of the Cdk inhibitor p27^{kip1} that associates to the assembled cyclin D/Cdk complexes and inhibits their kinase activity (11, 12). Expression of p27^{kip1} is down-regulated as cells progress through G_1, but this process requires the presence of a co-stimulatory signal supplied by IL-2 or B7–1 and B7–2, the ligands for CD28 (6, 11, 12). Thus, only the combination of T cell receptor (TCR) complex activation and co-stimulation with IL-2 or B7 molecules allows for the formation of active cyclin D/Cdk complexes and initiation of T cell proliferation.

Leukemic T cell lines, such as Jurkat or H9 cells, are not dependent on TCR activation or co-stimulatory signals for their proliferation, and these cells express low levels of p27^{kip1} (6). Nevertheless, cell proliferation can be blocked in these cell lines upon restimulation of the TCR complex, and this is associated with activation-induced cell death (13–15). As such, these cells have been used as a model system to study the signaling events triggered by TCR activation that induce apoptosis in cycling T cells. Using these cell lines, we have previously shown that down-regulation of cyclin D3 is crucial to TCR-induced apoptosis (13). Here we have used these cell lines as a model to study PKA-mediated immunosuppression. We show that these cells are inhibited in their growth by agents that activate PKA and show that expression of cyclin D3 is down-regulated in leukemic T cell lines in response to activation of PKA. This down-regulation of cyclin D3 plays an important role in the PKA-mediated growth inhibition, since ectopic expression of cyclin D3 could overcome the suppressive effects

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[1] The abbreviations used are: TCR, T cell receptor; PGE, prostaglandins of the E series; PKA, cAMP-dependent protein kinase; Cdk, cyclin-dependent kinase; pRb, retinoblastoma protein; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

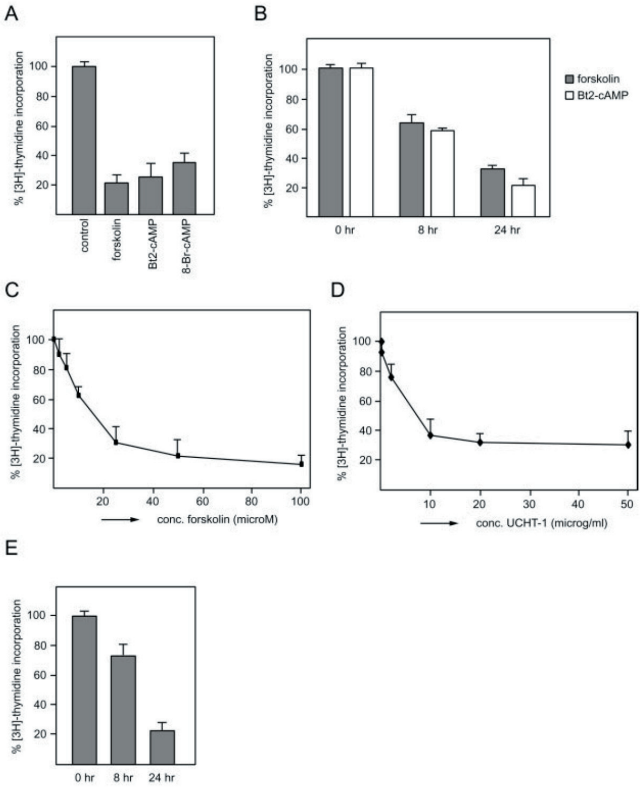

FIG. 1. **Activation of PKA causes growth inhibition in Jurkat leukemic T cells.** *A*, Jurkat T cells were stimulated with forskolin (50 μM), dibutyryl cAMP (1 mM), or 8-bromo-cAMP (1 mM) for 24 h or left untreated. *B*, Jurkat cells were treated for 8 or 24 h with forskolin (50 μM) (*gray bars*) or dibutyryl cAMP (1 mM) (*white bars*). *C*, Jurkat cells were treated with increasing amounts of forskolin for 24 h. *D*, Jurkat T cells were incubated for 24 h on tissue culture plates coated with an α-CD3 monoclonal antibody (UCHT-1) at the indicated concentrations. *E*, Jurkat cells were incubated on tissue culture plates coated with α-CD3 monoclonal antibody (UCHT-1) (10 μg/ml) for 8 or 24 h. In all cases cell proliferation was determined by [^{3}H]thymidine incorporation and expressed as a percentage of the untreated control population. *Error bars* represent S.E. and are derived from at least three independent experiments.

of PKA to some extent. However, ectopic expression of cyclin D3 was unable to fully prevent the growth suppression that occurred in response to activation of PKA. Indeed, we could show that PKA activation also results in the induction of p27^{kip1} expression, independent of its effect on cyclin D3, indicating that PKA can cause growth suppression in T cells via multiple independent pathways.

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Reagents—Human Jurkat and H9 leukemic T cell lines were routinely cultured as described (13). Jurkat T cells stably expressing cyclin D3 (clones JD-I and JD-II) have been described previously (13). Anti-pRb (G3-245) was obtained from PharMingen (Hamburg, Germany), anti-p27^{kip1} (clone 57) from Transduction Laboratories (Lexington, KY), anti-cyclin D3 (Ab-2) from Calbiochem (San Diego, CA), anti-Cdk2 (M2), anti-cyclin E (HE-12 for immunoblotting and HE-111 for immunoprecipitation and *in vitro* kinase activity) were all from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cyclin D2 (DCS-5) was a kind gift from Dr. Jiri Lukas (Danish Cancer Society, Copenhagen, Denmark). Propidium iodide, *N*-6,2′-*O*-dibutyryl-cAMP (dibutyryl cAMP) and 8-bromo-cAMP were purchased from Sigma, and protein A/G-Sepharose beads from Santa Cruz Biotechnology.

Cell Stimulation—All cells were plated in fresh medium at a density of 2×10^5 cells/ml at 24 h prior to cell stimulation to ensure that they were growing exponentially at the time of stimulation. Cells were counted the next day and replated in the presence or absence of forskolin, dibutyryl cAMP, and 8-bromo-cAMP at the indicated concentrations or on plates coated with anti-CD3 monoclonal antibodies (mAb) (UCHT-1) as previously described (13).

Cell Cycle Analysis and Viability—Cells were plated at a density of 2×10^5 cells/ml in 96-well plates and grown with or without forskolin, dibutyryl cAMP, or 8-bromo-cAMP for the indicated times. Alternatively, cells were plated on anti-CD3-coated 96-well plates and grown for the indicated times. During the last 2 h, 0.5 μCi [^{3}H]thymidine (Amersham Pharmacia Biotech) was added to each well, after which cells were harvested and [^{3}H]thymidine incorporation was determined in a scintillation counter. To determine which phase of the cell cycle was affected by activation of protein kinase A or TCR activation, cells were grown at a density of 2×10^5 cells/ml on 12-well tissue culture plates (Costar, Cambridge, MA) and treated with forskolin or anti-CD3 monoclonal antibodies or left untreated. At different time points after the stimulation, cells were collected and washed with ice-cold phosphate-buffered saline, after which they were fixed overnight in 70% ethanol at 4 °C. Cells were then pelleted by centrifugation, and cell pellets were washed once with ice-cold phosphate-buffered saline. Cells were stained with propidium iodide as described (13). Cell viability after stimulation for different time periods with forskolin or anti-CD3 monoclonal antibodies was determined by staining whole cells with propidium iodide as described (13). Samples were analyzed on a FACScalibur and cell viability was subsequently determined using Cell Quest software (BD PharMingen).

Immunoprecipitations and in Vitro Kinase Assays—Cells were stimulated with forskolin or anti-CD3 monoclonal antibodies or left unstimulated and harvested at the indicated time points. Cells were washed once with ice-cold phosphate-buffered saline, and cell pellets were resuspended and lysed in E1A lysis buffer as described previously (6). Protein concentrations were determined, and equal amounts of protein were used for immunoprecipitation, using 100 μg of whole cell lysate, 20 μl of protein A/G-Sepharose beads with 4 μl of anti-cyclin E antibody, or 2 μl of anti-Cdk2 antibody. The resulting immunocomplexes were washed three times with ELB lysis buffer and used in *in vitro* kinase assays using histone H1 as substrate as described previously (16). Samples were analyzed on SDS-PAGE gels and exposed to PhosphorImager plates (Molecular Dynamics).

Immunoblotting—After proper stimulation, cells were harvested and washed once with ice-cold phosphate-buffered saline. Cell pellets were then lysed in 2× Laemmli buffer without β-mercaptoethanol and bromphenol blue. Samples were then incubated at 95 °C for 5 min. Protein concentrations were determined using a standard Lowry protein deter-

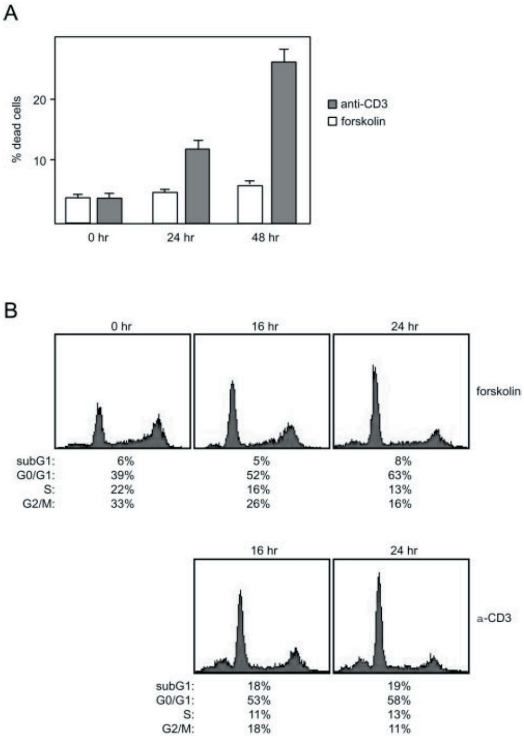

FIG. 2. **PKA activation leads to a cell cycle arrest without apparent apoptosis.** *A*, Jurkat T cells were cultured (2×10^5 cells/well) in the presence of 50 μM forskolin (*white bars*) or in tissue culture plates coated with 10 μg/ml anti-CD3 antibody UCHT-1 (*gray bars*) for the indicated times. The percentage of dead cells was determined by propidium iodide staining of whole cells followed by flow cytometric analysis. *B*, Jurkat T cells were cultured in the presence of 50 μM forskolin (*top panels*) or in tissue culture plates coated with 10 μg/ml anti-CD3 antibody UCHT-1 (*bottom panels*) for the indicated times. Cells were harvested, fixed in ethanol, and stained with propidium iodide. Cell cycle profiles were analyzed by flow cytometry and the percentages of cells in the sub-G_1 (apoptotic cells), G_1, S, and G_2/M phase of the cell cycle are indicated below each histogram.

mination, after which β-mercaptoethanol and bromphenol blue were added to final concentrations of 10 and 0.01%, respectively. Samples were then separated on appropriate SDS-PAGE gels and blotted to a nitrocellulose membrane. Proteins were then detected by ECL using standard protocols.

RESULTS

Inhibition of Cell Proliferation of Human Leukemic T Cell Lines by Activation of PKA—Activation of PKA can suppress T cell activation and causes growth inhibition in a large variety of lymphocytes, including leukemic T cell lines. We set out to study the mechanism of PKA-mediated growth inhibition in more detail. To this end, we stimulated Jurkat leukemic T cells with a variety of agents known to activate PKA. Treatment of Jurkat T cells with forskolin, which directly activates the catalytic subunit of adenylate cyclase, resulted in marked inhibition of proliferation, as measured by [^{3}H]thymidine incorporation (Fig. 1*A*). A similar inhibition was seen after addition of dibutyryl cAMP or 8-bromo-cAMP (Fig. 1*A*), both membrane-permeable cAMP analogs known to efficiently stimulate PKA activity when added to cells. Inhibition of cell proliferation in response to forskolin or dibutyryl cAMP was apparent as early as 8 h after stimulation and reached >75% inhibition after 24 h (Fig. 1*B*). Inhibition of [^{3}H]thymidine incorporation was dependent on the concentration of forskolin added to the cells (Fig. 1*C*). Because 50 μM forskolin caused an efficient growth inhibition, we chose to use this concentration in our further experiments.

In addition to immunosuppression by agents that modulate the activity of PKA, tolerance of mature T lymphocytes can be induced through antigen-driven cell death (1). This latter process has been studied extensively in leukemic T cell lines that undergo apoptosis upon direct activation of the TCR complex (13–15). Therefore, we compared growth inhibition mediated by PKA with that observed in response to TCR activation. To this end we incubated cells with a cross-linked anti-CD3 mAb in increasing concentrations (13) and could observe an efficient inhibition of [^{3}H]thymidine incorporation after a 24 h stimulation, similar to the effects of forskolin (Fig. 1*D*). Similar to PKA-induced inhibition, the suppression of thymidine incorporation was visible as early as 8 h after stimulation with UCHT-1 and reached ~75% after 24 h (Fig. 1*E*).

PKA Stimulation Causes a G_1 Arrest but Does Not Affect Cell Viability—Cross-linking of the CD3 complex on leukemic T cell lines can result in programmed cell death (13–15), and we previously showed that the TCR-induced growth inhibition of Jurkat cells is in part due to a reduction in cell viability (13). Therefore, we wanted to determine the contribution of cell death to the growth inhibition seen upon activation of PKA. To this end, we cultured the cells in the presence of forskolin for 24 or 48 h and measured cell viability by propidium iodide uptake. Under these experimental conditions, forskolin induced only minor increases in the percentage of dead cells (from 4 to 6% after 48 h) (Fig. 2*A*), indicating that the effect of PKA cannot be explained by programmed cell death in these cells. In the same experiments, we observed a more significant increase in the number of dead cells in response to TCR activation, from 4% in the control population to 27% after 48 h (Fig. 2*A*).

Because activation of PKA induced a significant growth inhibition in the absence of apoptosis, we decided to study the effects of PKA activation on the cell cycle progression in more detail. We, therefore, stimulated Jurkat cells for 24 or 48 h with forskolin and analyzed the DNA profiles using propidium iodide staining and flow cytometry. As shown in Fig. 2*B*, the percentage of cells in the G_0/G_1 phase rises from 39 to 52% within 16 h of forskolin treatment and reaches ~63% at 24 h after treatment. As can been seen in Fig. 2*B*, the fraction of cells with a sub-G_1 DNA content (apoptotic cells) remains unchanged after treatment with forskolin (Fig. 2*B*), consistent with our data on cell viability (Fig. 2*A*). Stimulation with anti-CD3 mAbs resulted in a similar increase in the percentage of cells in G_1 (Fig. 2*B*). In contrast with forskolin, anti-CD3 mAbs induced a clear increase in the fraction of apoptotic cells ($<2n$), from 6% in the unstimulated cells to 19% in the cells stimulated for 24 h, similar to the percentages obtained by measuring cell viability.

PKA-mediated Inhibition of Cyclin/Cdk Activity—To investigate at which point in G_1 cells arrest in response to PKA activation, we examined the phosphorylation state of pRb after treatment of cells with forskolin. In untreated, asynchronous cultures of Jurkat cells, pRb is mostly present in its slower migrating hyperphosphorylated form (Fig. 3*A*, *lane 1*). After 24 h of stimulation with forskolin we observed a clear mobility shift in the pRb protein from the hyperphosphorylated to the hypophosphorylated form (Fig. 3*A*, *lane 2*). In order to rule out that these effects of PKA activation were restricted to Jurkat leukemic cells, we analyzed the effects of PKA activation on pRb phosphorylation in another human leukemic cell line, H9. Indeed, proliferation of H9 leukemic cells was also efficiently

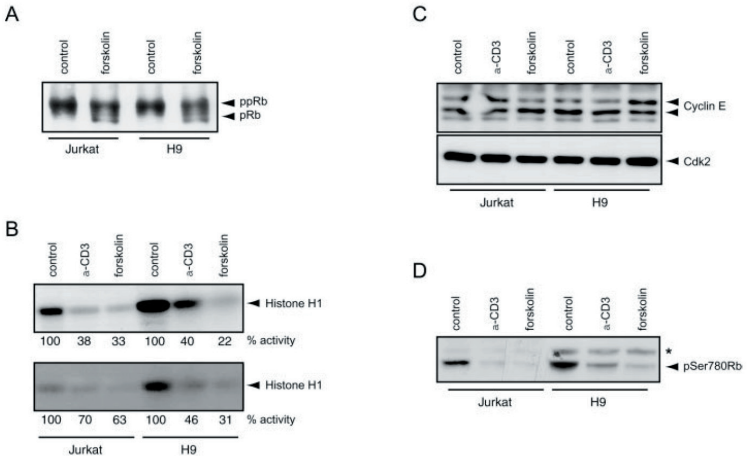

FIG. 3. **Effect of PKA activation on G_1 cyclin/Cdk activity.** *A*, phosphorylation state of pRb in Jurkat (*lanes 1* and *2*) or H9 cells (*lanes 3* and *4*) after stimulation with forskolin for 24 h. Total lysates were separated on SDS-PAGE gels for the analysis of Rb phosphorylation on Western blots. *Arrows* indicate the hyper- and hypophosphorylated forms of pRb. *B*, kinase activity of Cdk2 (*top panel*) or cyclin E immunoprecipitates (*bottom panel*) prepared from Jurkat or H9 cells after stimulation with forskolin or α-CD3 antibody for 24 h. To determine Cdk2 kinase activity, cyclin E or Cdk2 were immunoprecipitated from total cell lysates and immune complexes were then used for *in vitro* kinase assays using histone H1 as substrate. The *arrow* indicates ^{32}P-phosphorylated histone H1. The percentage of remaining kinase activity (relative to the untreated control) is indicated below each *lane*. *C*, after stimulation with the indicated agents, cells were lysed, and total lysates were separated on SDS-PAGE gels to determine the expression levels of cyclin E (*top panel*) and Cdk2 (*bottom panel*). Equal amounts of protein were loaded in each lane. *D*, Ser^{780} phosphorylation of pRb was analyzed in Jurkat and H9 cells after stimulation with forskolin or α-CD3 for 24 h. Ser^{780} phosphorylation was determined by Western blotting of total lysates with an antibody specific for Ser^{780}-phosphorylated pRb[22]. *Asterisk* indicates a nonspecific background band present in lysates prepared from H9 cells. Equal amounts of protein were loaded in each lane.

inhibited by forskolin (data not shown), and a similar inhibition of pRb phosphorylation was observed in response to forskolin (Fig. 3*A*, *lanes 3* and *4*).

Phosphorylation of pRb is mediated by cyclin/Cdk complexes (10), and therefore, we measured the kinase activity of cyclin E-Cdk2 complexes, normally induced late in G_1. For this purpose, cyclin E or Cdk2 were immunoprecipitated from whole cell lysates prepared from control and forskolin- or anti-CD3-stimulated Jurkat or H9 cells. The obtained immunocomplexes were then subjected to *in vitro* kinase reactions using histone H1 as a substrate. After a 24 h stimulation with forskolin or anti-CD3 mAbs, both cyclin E as well as Cdk2-associated kinase activity were inhibited (Fig. 3*B*). Expression of cyclin E and Cdk2 were unaltered upon stimulation with forskolin or anti-CD3 mAbs (Fig. 3*C*), demonstrating that regulation of cyclin E expression is not involved in the inhibition of cyclin E-Cdk2 kinase activity.

In addition to phosphorylation by cyclin E-Cdk2, Rb phosphorylation also requires the activity of cyclin D-Cdk4 or -Cdk6 complexes (17). Cyclin D/Cdk complexes phosphorylate residues on pRb that are distinct from the residues phosphorylated by cyclin E- or cyclin A-Cdk2 complexes (18). For example, Ser^{780} is uniquely phosphorylated by cyclin D/Cdk complexes, and its phosphorylation state is, therefore, a good measure of cyclin D/Cdk kinase activity. Using an antibody recognizing Ser^{780}-phosphorylated pRb (anti-phospho-Ser^{780}), we noted that treatment of Jurkat cells with forskolin for 24 h resulted in the loss of phosphorylated pRbSer^{780}, similar to what was observed after stimulation with anti-CD3 mAbs (Fig. 3*D*). These experiments clearly demonstrate that PKA stimulation results in the loss of cyclin D- and cyclin E-associated kinase activities from the cell.

PKA Activation Induces Down-regulation of Cyclin D3 and Up-regulation of p27^{kip1}—We have previously shown that Jurkat and H9 cells express abundant amounts of cyclin D3 but no cyclin D2 and that its expression is down-regulated upon activation of the TCR (13). Therefore, we next decided to analyze the effect of PKA activation on expression of the cyclin D3. Expression of cyclin D3 was dramatically reduced after 24 h in the presence of forskolin or anti-CD3 mAbs in Jurkat and H9 cells (Fig. 4*A*). The timing of cyclin D3 down-regulation appears to be similar after stimulation with anti-CD3 mAbs or forskolin. In both cases, a reduction in cyclin D3 expression is first visible after 8 h of stimulation and expression continues to decline up to 24 h (Fig. 4*B*). This timing slightly precedes the decrease in Ser^{780} dephosphorylation on pRb (Fig. 4*C*), suggesting that the reduction in cyclin D3 expression is responsible for the observed pRb dephosphorylation.

To examine if changes in the levels of cyclin-dependent kinase inhibitors (Cdki) contribute to the observed inhibition of cyclin/Cdk activity, we examined if PKA activation could induce expression of p27^{kip1} or p21^{cip1} in leukemic T cells. Whole cell lysates were prepared from control cells and from cells stimulated for 24 h with anti-CD3 mAbs or forskolin. As shown in Fig. 4*D*, expression of p27^{kip1} did not change significantly upon activation of the T cells with anti-CD3 mAbs. In contrast, stimulation of PKA did result in an increase in p27^{kip1} protein levels. Induction of p27^{kip1} in response to activation of PKA was visible after 2 h of stimulation with forskolin, indicating that this is a rather rapid response (Fig. 4*E*). We next checked expression of p21^{cip1} but could not detect p21^{cip1} in control or forskolin-stimulated Jurkat cells (data not shown), nor in cells treated with anti-CD3 mAbs (13). Thus, it seems unlikely that induced expression of p21^{cip1} causes the observed inhibition of cyclin/Cdk complexes in response to TCR or PKA activation, but it appears that PKA-mediated growth inhibition is due to combined regulation of both cyclin D3 and p27^{kip1} expression.

Ectopic Expression of Cyclin D3 Partially Overrides Growth Suppression by cAMP—We have previously shown that the TCR-induced growth arrest in Jurkat cells is critically dependent on down-regulation of cyclin D3. In order to investigate if the PKA-induced growth suppression shows a similar depend-

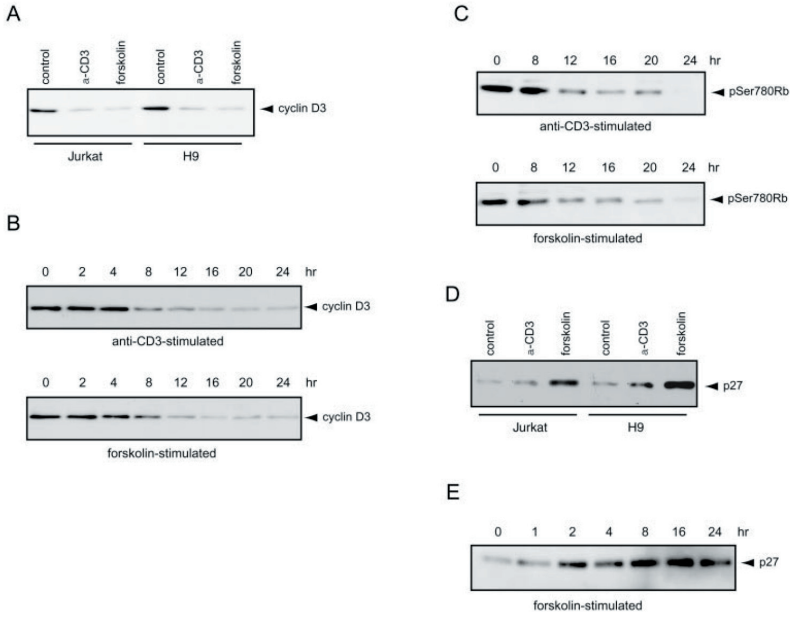

FIG. 4. **PKA activation results in the induction of p27^{kip1} and a reduction in cyclin D3 expression.** Total lysates prepared from Jurkat or H9 cells were separated on SDS-PAGE gels for the analysis of cyclin D3 expression (*A*, *B*), pSer^{780} phosphorylation of pRb (*C*), or p27^{kip1} expression levels (***D***, *E*) on Western blots. Jurkat (*A* – *E*) or H9 cells (***A***, *D*) were left untreated or stimulated with forskolin, α-CD3 for 24 h (*A*, *D*) or a time course of induction (*B*, *C*, *E*) with forskolin or α-CD3 was performed as indicated. Equal amounts of protein were loaded in each lane. Kinetics of the loss of Ser^{780} phosphorylation of pRb was analyzed in Jurkat cells after stimulation with forskolin or α-CD3 for the indicated time (*C*). Ser^{780} phosphorylation was determined by Western blotting of total lysates as described in the legend to Fig. 3.

ence, we studied PKA-mediated growth inhibition in Jurkat-derived cell lines stably overexpressing cyclin D3. To this end, we transfected a plasmid encoding human cyclin D3 into Jurkat cells and isolated single cell colonies by limiting dilution. Of several lines isolated, two lines were selected for further studies, JD3.I and JD3.II, which express moderate levels of exogenous cyclin D3 (13). To confirm that cyclin D3 was indeed constitutively expressed in these cell lines, also in the presence of forskolin, we analyzed cyclin D3 expression by Western blot analysis. Whereas cyclin D3 is efficiently down-regulated in the parental Jurkat cell line, no effect on cyclin D3 expression by forskolin or anti-CD3 mAbs is seen in JD3.I or JD3.II (Fig. 5*A*). As can be seen in Fig. 5*C*, the growth inhibitory response to anti-CD3 mAbs was almost completely abolished when we measured [^{3}H]thymidine incorporation in the JD3.I and JD3.II clones, consistent with our previous report (13). In contrast, although a significant reduction in the forskolin-induced inhibition of thymidine incorporation was observed in JD3.I and JD3.II, ectopic expression of cyclin D3 was unable to fully revert growth inhibition by forskolin. These data show that down-regulation of cyclin D3 plays an important role in both TCR- and PKA-mediated growth suppression, but also indicate that PKA must affect other cell cycle regulatory proteins besides cyclin D3. Our data suggest that this other pathway involves regulation of p27^{kip1} (see Fig. 4*D*). p27^{kip1} binds to multiple G_1 cyclin/Cdk complexes and is a potent Cdk inhibitor for complexes containing Cdk2, although it does not seem to inhibit cyclin D/Cdk complexes (19). As such, p27^{kip1} can inhibit cell cycle progression in G_1 by inhibition of cyclin/Cdk complexes other than cyclin D-Cdk4 or -Cdk6, such as cyclin E-Cdk2. Thus, regulation of p27^{kip1} expression, independent from cyclin D, could very well constitute a separate pathway of growth inhibition in these PKA-stimulated T cell lines. Indeed, when analyzing p27^{kip1} expression, we found that the ability of forskolin to induce p27^{kip1} expression was retained in the JD3.I and JD3.II clones (Fig. 5*B*), demonstrating that regulation of p27^{kip1} by PKA occurs independently of its effects on cyclin D3 expression.

DISCUSSION

Agents that activate the PKA signaling cascade act *in vivo* to down-regulate the immune response in order to prevent excessive reactivity. In this report we have addressed the mechanism of suppression of T cell proliferation in response to activation of PKA. To this end, we have used leukemic T cell lines that are no longer dependent on activating stimuli for their proliferation. Rather, these T cell lines resemble activated mature T cells, in that apoptosis can be induced upon activation of the TCR complex, and their proliferation is dramatically inhibited by agents that cause a rise in the intracellular cAMP levels. These properties allowed us to use these cell lines to study PKA-mediated immunosuppression in more detail and to compare this to immunosuppression by stimulation of the TCR complex on the same T cell lines. We could show that the PKA-mediated block in cell proliferation was due to an arrest of cell cycle progression at some point in the G_1 phase, but no dramatic reduction in cell viability was observed. The G_1 arrest induced by forskolin was associated with the appearance of dephosphorylated pRb, indicating an arrest at, or prior to, the G_1 restriction point. In agreement with this notion was the finding that the kinase activity of cyclin E and Cdk2 complexes was reduced. In order to address the mechanism by which the reduction in kinase activity of these complexes occurs, we analyzed expression of the various G_1 cyclins. Expression of cyclin E is usually induced late in G_1 as a function of cell cycle progression. No reduction in the expression level of cyclin E

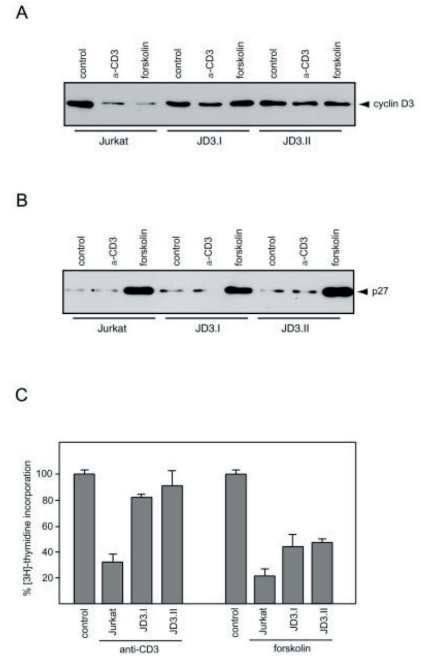

FIG. 5. **Ectopic expression of cyclin D3 is unable to fully override growth inhibition in response to PKA activation.** *A* and *B*, effect of PKA activation on cyclin D3 expression (*A*) and $p27^{kip1}$ expression (*B*) was compared in parental Jurkat cells *versus* two cell lines stably overexpressing cyclin D3, JD3.I and JD3.II. Cells were stimulated with forskolin or α-CD3 mAb for 24 h, and total lysates were prepared to determine cyclin D3 (*A*) and $p27^{kip1}$ (*B*) protein levels by Western blotting. *C*, rescue from PKA- or TCR-mediated growth suppression by ectopic expression of cyclin D3 was determined by analysis of [^{3}H]thymidine incorporation in parental Jurkat *versus* the JD3.I and JD3.II cell lines. Cells were stimulated for 24 h with forskolin or α-CD3 before the addition of [^{3}H]thymidine. *Bars* indicate the percentage of inhibition of thymidine incorporation relative to the untreated control. *Error bars* represent the S.E. determined from three independent experiments.

could be detected within 24 h after treatment with forskolin, indicating that the initial inhibition of cyclin E-associated kinase occurs through active repression. Therefore, these data indicate that the reduction in cyclin E-associated kinase activity is not a mere consequence of the arrest in G_1 but is affected more directly by PKA activation. Further analysis of expression of G_1 cyclins showed that expression of cyclin D3 was reduced significantly after treatment with forskolin.

The reduction in cyclin D3 expression was correlated with a loss of cyclin D-specific pRb phosphorylation, as measured by Ser^{780} phosphorylation. Since expression of the D-type cyclins is not a function of cell cycle progression but depends on the presence of sufficient mitogens in the cellular environment (20), it is very unlikely that the reduction in cyclin D3 expression is a secondary effect of the induced arrest in G_1. Rather, these data indicate that the arrest in G_1 is caused by the reduction in expression of cyclin D3, which will result in inhibition of pRb phosphorylation and a block in cell cycle progression. Indeed, ectopic expression of cyclin D3 could prevent the forskolin-induced growth suppression of Jurkat cells to some extent. The effect of cyclin D3 on repression of PKA-mediated inhibition of proliferation was not as extensive as seen in response to TCR activation, indicating that PKA affects other cell cycle regulatory proteins in addition to cyclin D3.

Analysis of the expression of other cell cycle regulatory proteins that play a role in the progression from G_1 to S phase showed that expression of the Cdk inhibitor $p27^{kip1}$ is induced after stimulation with forskolin. This rise in $p27^{kip1}$ occurs independently of the reduction of cyclin D3, since it is not seen in response to TCR activation and, more importantly, is not affected in the JD3.I and JD3.II cell lines that fail to down-regulate cyclin D3 in response to forskolin. At this point, we cannot completely rule out the possibility that the effects of PKA on $p27^{kip1}$ are an indirect consequence of cell cycle arrest. However, expression of $p27^{kip1}$ is not induced by TCR activation, although this leads to a similar block in proliferation. Also, the rapid kinetics of $p27^{kip1}$ induction would argue against this possibility. Thus, regulation of $p27^{kip1}$ expression appears to distinguish the PKA-mediated growth suppression from TCR-mediated inhibition of leukemic T cell growth. Taken together, our data indicate that PKA activation in leukemic T cell lines affects multiple pathways to elicit an efficient inhibition of proliferation. Interference with one of the PKA targets, namely cyclin D3, is not sufficient to fully override growth suppression, indicating that other targets play an equally important role in the PKA response.

It was recently reported that PKA activation can also affect cyclin D3 expression in primary T lymphocytes (21). However, $p27^{kip1}$ levels were not rapidly induced after PKA activation in primary T lymphocytes, although we find induction of $p27^{kip1}$ expression as early as 2 h after stimulation with forskolin in leukemic T cells. This discrepancy could be due to a fundamental difference in PKA-mediated growth suppression in primary lymphocytes *versus* the leukemic T cell lines studied here. Unfortunately, the importance of down-regulation of cyclin D3 to PKA-mediated growth suppression of primary T lymphocytes has not been addressed, making it impossible to draw conclusions as to the existence of other potential PKA targets in primary T cells. Nevertheless, our experiments have clearly shown that down-regulation of cyclin D3 is not solely responsible for PKA-mediated growth suppression of leukemic T cells, because a failure to down-regulate cyclin D3 does not result in a complete loss of responsiveness to forskolin. The data presented here suggest that one other crucial target may be $p27^{kip1}$, which is induced upon activation of PKA in the leukemic cell lines studied here.

Regulation of $p27^{kip1}$ expression in a fashion independent from cyclin D expression, as seen here for PKA activation in Jurkat T cells, truly constitutes a separate pathway of growth suppression. Although $p27^{kip1}$ binds with high affinity to cyclin D/Cdk complexes, it is clear that these complexes remain catalytically active, even in the presence of high levels of $p27^{kip1}$ (22, 23). In contrast, cyclin E-Cdk2 complexes are very efficiently inhibited by $p27^{kip1}$ (19). Based on these observations, it has been proposed that cyclin D/Cdk complexes function to sequester $p27^{kip1}$ away from cyclin E-Cdk2 complexes (19). Therefore, whereas down-regulation of cyclin D3 expression by PKA will inhibit G_1 progression at the level of cyclin D activity, up-regulation of $p27^{kip1}$ will exert its inhibition at the level of cyclin E-associated kinase activity. This way, activation of PKA can lead to a more tight suppression of T cell proliferation, responsible for the efficient immunosuppressive activities of agents known to activate PKA in T lymphocytes. It will be interesting to determine at which point after PKA activation this bifurcation toward cyclin D3 and $p27^{kip1}$ occurs, and future experiments will be directed toward a better understanding of the mechanism by which PKA can exert its effects on $p27^{kip1}$ expression.

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Chapter 3:

The Forkhead transcription factor FoxO regulates transcriotion of p27/Kip1 and Bim in response to IL-2.

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The Forkhead Transcription Factor FoxO Regulates Transcription of p27^{Kip1} and Bim in Response to IL-2[1]

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The cytokine IL-2 plays a very important role in the proliferation and survival of activated T cells. These effects of IL-2 are dependent on signaling through the phosphatidylinositol 3-kinase (PI3K) pathway. We and others have shown that PI3K, through activation of protein kinase B/Akt, inhibits transcriptional activation by a number of forkhead transcription factors (FoxO1, FoxO3, and FoxO4). In this study we have investigated the role of these forkhead transcription factors in the IL-2-induced T cell proliferation and survival. We show that IL-2 regulates phosphorylation of FoxO3 in a PI3K-dependent fashion. Phosphorylation and inactivation of FoxO3 appears to play an important role in IL-2-mediated T cell survival, because mere activation of FoxO3 is sufficient to trigger apoptosis in T cells. Indeed, active FoxO3 can induce expression of IL-2-regulated genes, such as the *cdk* inhibitor p27^{Kip1} and the proapoptotic Bcl-2 family member Bim. Furthermore, we show that IL-2 triggers a rapid, PI3K-dependent, phosphorylation of FoxO1a in primary T cells. Thus, we propose that inactivation of FoxO transcription factors by IL-2 plays a critical role in T cell proliferation and survival. ***The Journal of Immunology,*** **2002, 168: 5024–5031.**

Optimal activation of mature resting T lymphocytes requires engagement of the TCR complex, accompanied by a costimulatory signal that can be provided by either CD28 or IL-2 (1, 2). TCR engagement causes the activation of a number of genes, including the high-affinity IL-2Rα chain (or CD25) gene. The expression of CD25 on the cell surface of activated T cells leads to IL-2 responsiveness (3). This is a critical event for the onset of the immune response, because IL-2 is a potent mitogen for the T cells and promotes rapid proliferation of the activated T cells (2–4). Importantly, TCR stimulation also triggers IL-2 gene activation, leading to an autocrine loop of activation (5).

In addition to stimulating T cell proliferation, IL-2 also functions as an important survival factor for T cells. This function of IL-2 has been shown to depend on signaling through the phosphatidylinositol 3-kinase (PI3K)[3] pathway. One important target of PI3K signaling in this respect is protein kinase B (PKB)/Akt (6, 7). PKB activation has been implicated in many metabolic processes and, importantly, in a strong promotion of cell survival (8, 9). Such PKB-dependent cell survival has been proposed to occur through a variety of mechanisms, and most notably through activation of antiapoptotic proteins, such as Bcl-2 (9), and inhibition of proapoptotic proteins, such as Bad (10, 11). Although it is well established that IL-2 promotes T cell survival and proliferation through the activation of PKB, the molecular events downstream of PKB that are involved in these responses remain unclear.

Several lines of evidence have demonstrated that PKB inhibits transcriptional activation of a number of related forkhead transcription factors (FKHR/FKHR-L1/AFX) (12, 13), now referred to as FoxO1, FoxO3, and FoxO4 (14). These forkhead transcription factors recognize a common DNA-binding element (15) that is highly related to insulin response elements (16). Each of these forkhead factors contains conserved phosphorylation sites for PKB, and PKB-mediated phosphorylation was shown to result in translocation of these factors to the cytoplasm (17–19). A conserved pathway is present in the nematode *Caenorhabditis elegans*, where the PI3K and PKB homologs (AGE-1 and AKT, respectively) regulate the activity of a forkhead transcription factor, DAF-16, in a pathway involved in the regulation of survival in response to nutrient starvation (20).

We have recently shown that PKB-regulated forkhead transcription factors are involved in regulation of the *cdk* inhibitor p27^{Kip1} and Bim, a proapoptotic member of the Bcl-2 family (21–23). This indicates that these forkhead factors control expression of genes involved in the regulation of cell cycle progression as well as apoptosis. Therefore, we investigated the role of FoxO proteins in IL-2-dependent T cell proliferation and survival. In this report, we present evidence that points to a role for FoxO3 in IL-2-mediated survival. We found that IL-2 withdrawal leads to activation of FoxO3 and up-regulation of p27^{Kip1} and Bim levels, and that activation of FoxO3 alone is sufficient to mimic the effects of IL-2 withdrawal. Furthermore, in primary T cells, we observed an IL-2-dependent inhibition of the forkhead transcription factors that may potentiate the required down-regulation of p27^{Kip1} for cell cycle reentry.

Materials and Methods

Plasmids and reagents

pCMV-p27^{Kip1} was a kind gift of Dr. R. Bernards (Netherlands Cancer Institute, Amsterdam, The Netherlands), and pCMV-EGFP-spectrin was a kind gift of Dr. A. Beavis (Princeton University, Princeton, NJ). pCMV-FoxO3(A3)ER (or pCMV-FKHR-L1(A3)ER) has been described (23). LY294002 was from Biomol (Plymouth Meeting, PA). Actinomycin D and 4-hydroxy-tamoxifen (4OH-T) were from Sigma-Aldrich (Steinhem, Germany) and Ficoll-Paque was purchased from Amersham Pharmacia Biotech (Little Chalfont, U.K.).

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[3] Abbreviations used in this paper: PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; 4OH-T, 4-hydroxy-tamoxifen; ER, estrogen receptor.

Cell culture

Murine CTLL-2 T lymphocytes were cultured in RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with 10% FCS, 1% penicillin-streptomycin-2-ME, and recombinant human IL-2 (100 IU/ml). CTLL-2-FoxO3(A3)ER stable cell lines were obtained by electroporation of a pCMV-FoxO3(A3)ER construct at 320 V and 960 μF. Stable transfectants were selected in medium containing 500 μg/ml G418/neomycin (Calbiochem, La Jolla, CA), and single-cell clones were obtained by limiting dilution. Primary human lymphocytes were isolated from healthy donor blood on a Ficoll-Paque gradient and cultured in complemented RPMI medium in absence of IL-2. After purification of the T cells, the population was analyzed for CD3 expression by flow cytometry. Cells were only used for further experimentation if the percentage of CD3 positivity was >95%. Cells were activated by addition of recombinant human IL-2 (final concentration, 5 IU/ml) in combination with anti-CD3 mAb (OKT-3 or UHCT-1) as described (24). 4OH-T was diluted in RPMI 1640 and added to the cells at a final concentration of 500 nM. LY294002 was dissolved in DMSO and used at a final concentration of 40 μM.

RNA preparation and Northern blotting

CTLL-2 cells were lysed in a guanidine-isothiocyanate lysis buffer and total RNAs were isolated. Equal amounts were loaded on an agarose-formaldehyde gel, and the blot was probed with $p27^{Kip1}$ or GAPDH cDNA probes.

Abs and Western blotting

$p27^{Kip1}$ and Fas ligand mAb were purchased from Transduction Laboratories (Lexington, KY) and Bim polyclonal Ab were purchased from Affinity BioReagents (Golden, CO). Anti-phospho-Thr^{24}-FoxO1/phospho-Thr^{32}-FoxO3 rabbit Ab (cross-reactive with phospho-Thr^{28}-FoxO4), anti-FoxO3, anti-PKB, and anti-phospho-Ser^{473}-PKB were purchased from Cell Signaling Technology (Beverly, MA). Anti-FoxO4 (N-19) goat Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-hemagglutinin Ab was 12CA5 hybridoma supernatant. Cells were lysed in Laemmli buffer (120 mM Tris (pH 6.8), 4% SDS, 20% glycerol) at room temperature, and DNA was sheared by passing through a syringe. The protein content was determined using a Lowry assay, equal amounts of proteins were analyzed by SDS-PAGE, and blots were probed with the appropriate Abs. Proteins were visualized by standard ECL (Amersham Pharmacia Biotech).

FACS analysis

For cell cycle analysis, the harvested cells were washed with PBS and fixed in 70% ethanol for at least 2 h on ice. Cells were spun down for 5 min at 480 × *g*, labeling buffer was added (0.25 mg/ml RNase and 10 μg/ml propidium iodide in PBS), and cells were incubated for 10 min in the dark. The cell population was viewed using a FACSCalibur (BD Biosciences, Mountain View, CA) and analyzed using CellQuest software (BD Biosciences). For determination of $CD3^+$ or $CD25^+$ cells, cells were harvested and incubated for 20 min on ice in the corresponding Ab solution (anti-CD3-FITC or anti-CD25-PE; BD Immunocytometry Systems, San Jose, CA). Cells were washed, resuspended in PBS, and analyzed by flow cytometry.

Results

PI3K/PKB signaling promotes survival of IL-2-dependent T cells

IL-2 induces T cell progression through G_1 into S phase of the cell cycle and controls T cell survival and clonal expansion (2). In addition, many hematopoietic cells have a default program for cell death and require a constant supply of cytokines to promote their survival. The CTLL-2 cell line is dependent on IL-2 for its survival and proliferation and is thus a good model to study IL-2-regulated genes implicated in regulation of survival and proliferation.

When CTLL-2 are cultured in the absence of IL-2, a cell cycle arrest in G_0/G_1 is observed that is most evident after 24 h of IL-2 deprivation (Fig. 1*A*). This initial growth arrest is then followed by apoptosis, so that the majority of the cells in the population have undergone programmed cell death after 48 h of IL-2 deprivation, as indicated by the rise in cells with a sub-G_1 DNA content (Fig. 1*A*).

As mentioned above, cytokine-mediated cell survival was shown to be dependent on PI3K/PKB signaling (8). Activation of PKB correlates with its phosphorylation on certain residues, notably on Ser^{473}. Using a phosphospecific Ab that specifically recognizes Ser^{473}-phosphorylated PKB, we observed that PKB was gradually losing its phosphorylation on Ser^{473} in response to IL-2 deprivation of CTLL-2 cells (Fig. 1*B*). Clearly, it takes ~2 h of IL-2 deprivation to see a significant drop in PKB phosphorylation. These kinetics are most likely determined in large part by the time required to turn over the IL-2 bound to the cells, and the subsequent down-modulation of IL-2R signaling. The decrease in Ser^{473} phosphorylation was not due to a reduction in PKB expression levels, as this was constant throughout the course of this experiment (Fig. 1*B*).

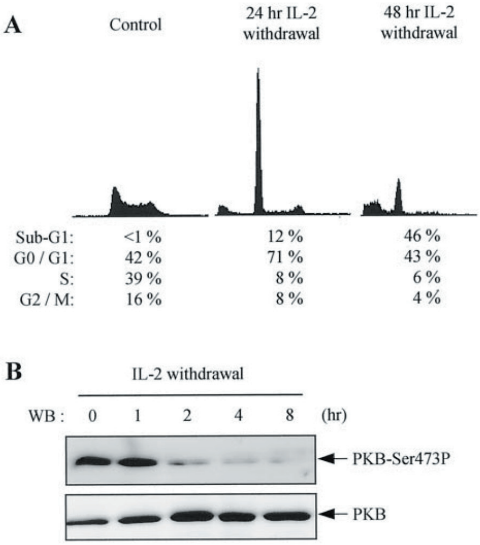

FIGURE 1. IL-2 promotes survival of IL-2-dependent T cells via the PI3K/PKB pathway. *A*, IL-2-dependent CTLL-2 mouse T cells were grown in the absence of IL-2. After 24 and 48 h they were harvested, fixed in ethanol, and labeled with propidium iodide to determine their DNA profiles by flow cytometry. *B*, CTLL-2 were deprived of IL-2 for different periods of time and harvested. They were monitored for PKB expression by Western blotting using a rabbit anti-phospho-Ser^{473}-PKB Ab, recognizing active PKB (*upper panel*), or an Ab directed against total PKB (*lower panel*).

To further investigate the requirement for PI3K/PKB signaling in IL-2-mediated survival of CTLL-2 cells, we treated CTLL-2 with LY294002, a potent inhibitor of PI3K. After a 24-h treatment with LY294002, we observed a prominent arrest in G_0/G_1 (Fig. 2*A*). At 48 h after addition of LY294002, a large fraction of the cells had fragmented DNA, as demonstrated by the rise in cells with a sub-G_1 DNA content (Fig. 2*A*), comparable to what was seen after IL-2 withdrawal (Fig. 1*A*). As expected, addition of LY294002 resulted in efficient inhibition of PI3K-mediated signaling, as demonstrated by the rapid reduction in the levels of activated PKB (Fig. 2*B*). Taken together, these data are consistent with the established role for PI3K/PKB signaling in cytokine-dependent cell survival, and show that these CTLL-2 cells critically depend on PI3K activity for their maintenance in culture.

IL-2-mediated cell survival is linked to inactivation of FoxO3

PKB is known to directly phosphorylate a number of forkhead transcription factors belonging to the FoxO subfamily (12, 13, 17). Phosphorylation of these forkhead factors results in their exclusion from the nucleus and a subsequent inhibition in transcriptional

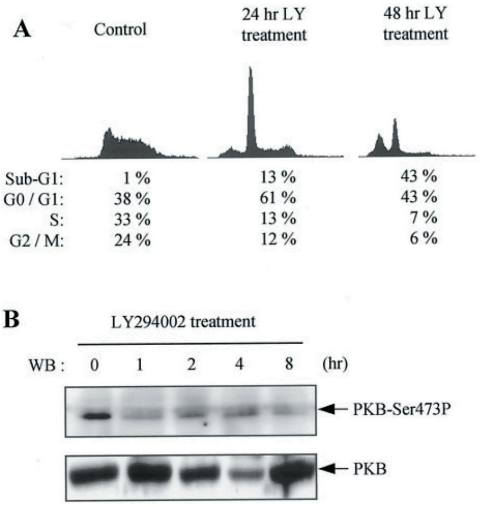

FIGURE 2. Specific interference with the PI3K/PKB pathway mimics IL-2 deprivation. *A*, CTLL-2 mouse T cells were treated with LY294002 for 24 and 48 h in the presence of IL-2. They were harvested, fixed, and labeled as described in Fig. 1*A* for FACS analysis. *B*, CTLL-2 were treated with LY294002 for the corresponding periods of time in presence of IL-2, harvested, and lysed. PKB activity and total PKB were determined using the same Abs as in Fig. 1*B*.

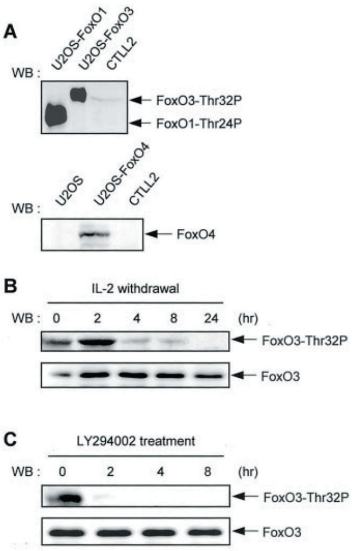

FIGURE 3. IL-2 signaling regulates forkhead transcription factor activity via the PI3K/PKB pathway. *A*, The expression pattern of different FoxO forkhead transcription factors was determined in CTLL-2 mouse T cells. For this purpose, CTLL-2 cells were lysed and analyzed by Western blotting using an anti-phospho-Thr^{24}-FoxO1a/phospho-Thr^{32}-FoxO3 (/phospho-Thr^{28}-FoxO4) Ab (*upper panel*) or an anti-total FoxO4 Ab (*lower panel*). Controls consisting of FoxO4-, FoxO1-, and FoxO3-transfected U2OS cells were loaded in parallel. *B*, CTLL-2 were deprived of IL-2, lysed after different periods of time, and analyzed by Western blotting using a rabbit anti-Thr^{32}-phospho-FoxO3 Ab (*upper panel*) or an Ab recognizing total FoxO3 (*lower panel*). *C*, CTLL-2 were treated for different periods of time with LY294002, lysed, and analyzed by Western blotting using the same Abs as in *B*.

activation of forkhead target genes (18, 19). Moreover, we and others (22, 23, 25) have shown that these forkhead factors can cause apoptosis when expressed at high levels in T and B cell lines. Therefore, we wanted to determine whether the IL-2-mediated survival of CTLL-2 cells was coupled to regulation of FoxO activity. To this end, we analyzed expression of the different PKB-regulated forkhead factors in CTLL-2 cells. Apparently, CTLL-2 cells express relative high levels of FoxO3, while we were unable to detect expression of the related forkhead members FoxO1 and FoxO4 (Fig. 3*A*). We next analyzed PKB-mediated phosphorylation of FoxO3, reflected by its phosphorylation on residue Thr^{32}. As expected, FoxO3 was rapidly dephosphorylated upon IL-2 withdrawal, within 4 h (Fig. 3*B*). Similarly, upon treatment with LY294002, we also observed decreased phosphorylation of FoxO3 (Fig. 3*C*). These results indicate that IL-2 regulates FoxO3 activity through the PI3K/PKB pathway in CTLL-2 cells.

IL-2 mediates p27^{Kip1} and Bim regulation

We have recently shown that FoxO3 controls p27^{Kip1} and Bim levels in a PKB-regulated pathway (22, 23). p27^{Kip1} is a well-known regulator of the G_1/S transition through its cyclin-dependent kinase inhibitory activity, which blocks the cell in G_1 phase by preventing *cdk*-dependent phosphorylation of pRb (26, 27). Bim is a proapoptotic member of the Bcl-2 family (28, 29), and ectopic expression of Bim is sufficient to trigger apoptosis in a variety of cell types, including CTLL-2 cells (data not shown). To study whether p27^{Kip1} and Bim expression is regulated in an IL-2-dependent fashion in CTLL-2 cells, we analyzed their respective protein levels after IL-2 deprivation or addition of LY294002. We observed an up-regulation of both p27^{Kip1} and Bim protein levels in response to IL-2 starvation (Fig. 4*A*, *upper panels*) as well as after treatment with LY294002 (Fig. 4*A*, *lower panels*). It should be noted that another reported proapoptotic target of FoxO3, Fas ligand (25), does not seem to be an important mediator of cell death after cytokine withdrawal, because we could not observe significant changes in the expression of Fas ligand upon IL-2 withdrawal (Fig. 4*B*) or treatment with LY294002 (data not shown).

Because p27^{Kip1} was reported to be a direct target of FoxO3 transactivation (23), we speculated that increased expression of p27^{Kip1} following IL-2 withdrawal is due to transcriptional activation. Indeed, p27^{Kip1} RNA was up-regulated upon IL-2 deprivation in CTLL-2 (Fig. 4*C*). This induction was blocked by addition of actinomycin D, an inhibitor of transcription, indicating that the rise in p27^{Kip1} RNA levels was not due to stabilization of the messenger.

Activation of FoxO3 is sufficient to trigger apoptosis in CTLL-2 cells

The data described above show that IL-2-mediated survival is coupled to FoxO3 inactivation in CTLL-2 cells and that IL-2 withdrawal results in a rapid activation of FoxO3a as determined by its phosphorylation status. However, this does not allow any conclusion as to the importance of FoxO3a activation in the apoptotic program of CTLL-2 cells. To determine whether mere activation of FoxO3 alone is sufficient to mimic the effects of IL-2 deprivation in these cells, we established CTLL-2-derived cell lines expressing a FoxO3-estrogen receptor (ER) fusion protein. In this chimeric protein, the ER module binds heat shock proteins, which preclude translocation to the nucleus. Upon interaction between the ER module and 4OH-T, the heat shock protein mantle is disrupted and the fusion protein is shuttled to the nucleus, where it

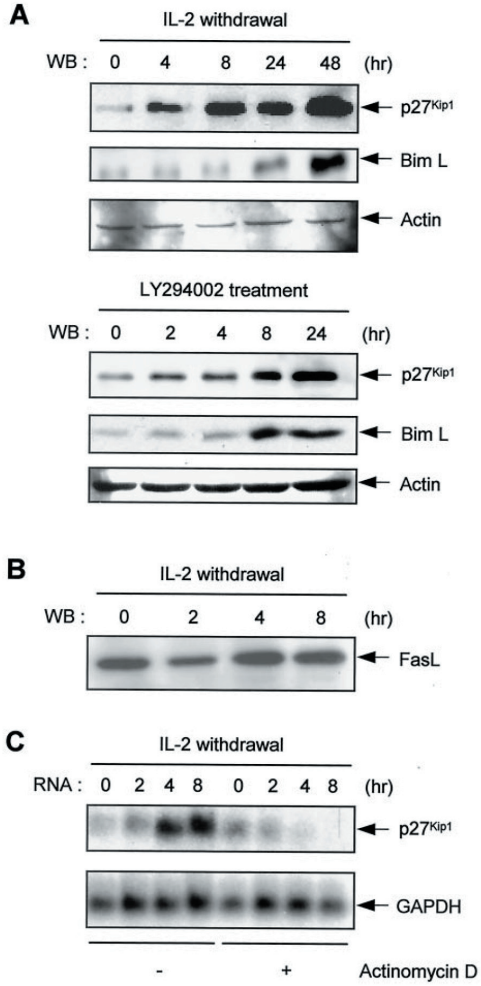

FIGURE 4. FoxO3 directly regulates $p27^{Kip1}$ and Bim expression. *A*, CTLL-2 were either deprived of IL-2 (*upper panels*) or treated with LY294002 (*lower panels*) for different periods of time, harvested, and lysed. $p27^{Kip1}$, Bim, and actin protein levels were monitored by Western blotting using the appropriate Abs. *B*, CTLL-2 cells were deprived of IL-2 for different periods of time and lysed. Using an anti-Fas ligand Ab, the expression levels of this protein were analyzed by Western blotting to determine whether forkhead transcription factors induce other important proapoptotic target genes in these cells. *C*, CTLL-2 were deprived of IL-2 for the time indicated and treated or not with actinomycin D, a transcription inhibitor. RNAs were extracted at the indicated time points after IL-2 withdrawal and analyzed by Northern blotting using a $p27^{Kip1}$ cDNA probe and a GAPDH cDNA probe as loading control.

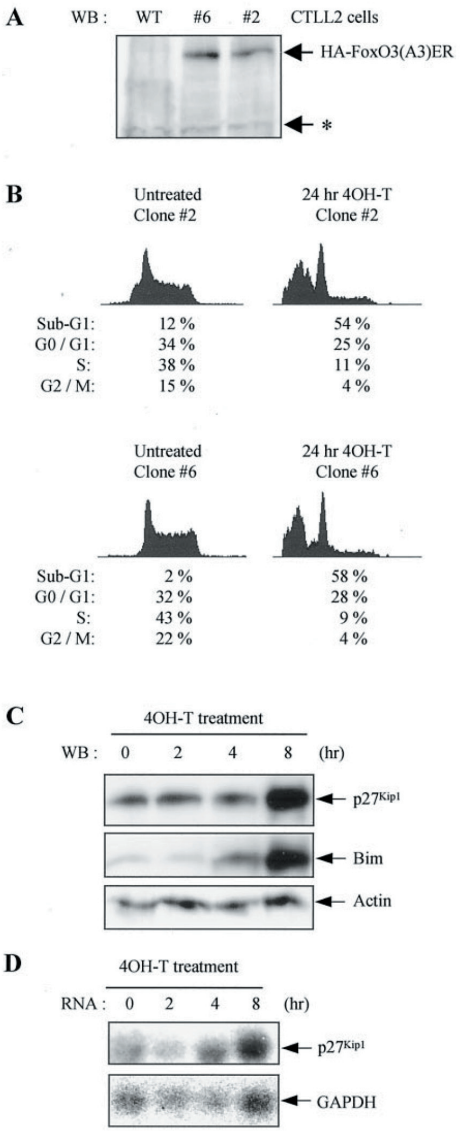

FIGURE 5. PKB-independent activation of FoxO3 mimics IL-2 withdrawal. *A*, CTLL-2 cells were electroporated with FoxO3(A3)ER construct. Clonal cell lines expressing this construct in a stable manner were selected on G418. The clones were harvested, lysed, and analyzed by Western blotting using an anti-hemagglutinin mAb. Clones 2 and 6 appeared to express similar levels of FoxO3(A3)ER. *B*, Clones 2 and 6 were treated with 4OH-T for 24 h in presence of IL-2 and analyzed by FACS as described in Fig. 1*A*. *C*, Clone 6 was treated with 4OH-T in the presence of IL-2 for different time lapses and analyzed by Western blotting using the appropriate Abs. *D*, Clone 6 was treated with 4OH-T in presence of IL-2 for the reported time lapses, harvested, and lysed. RNAs were isolated and equal amounts were analyzed by Northern blotting as described in Fig. 4*C*.

can now exert FoxO3 transcriptional activity. To ensure that the fusion protein could not be inactivated by PKB-mediated phosphorylation, the three previously identified PKB phosphorylation sites (Thr^{32}, Ser^{253}, and Ser^{315}) were mutated into alanine residues. This enabled us to trigger FoxO3 activation in the presence of IL-2. Two independent clones (clones 2 and 6) that expressed similar levels of the FoxO3(A3)-ER fusion protein were selected (Fig. 5*A*).

Addition of 4OH-T induced a block in cell cycle progression at the G_1/S transition, as evidenced by a reduction of cells in S and G_2/M in both of these clones (Fig. 5*B*). In addition, a clear increase

in apoptosis was observed at 24 h after addition of 4-OHT (Fig. 5*B*). A certain basal level of apoptosis was observed in clone 2, even in the absence of 4OH-T, suggesting that this clone was somewhat leaky. Indeed, expression of FoxO3(A3)ER was lost over time in clone 2; therefore, clone 6 was used for further experimentation.

We next wanted to study whether activation of FoxO3 alone would result in the induction of the same genes that are up-regulated after withdrawal of IL-2. Indeed, activation of FoxO3 by 4OH-T treatment was able to induce both $p27^{Kip1}$ and Bim protein expression in presence of IL-2 (Fig. 5*C*). Moreover, we were able to confirm that up-regulation of $p27^{Kip1}$ occurred at the transcriptional level, because we could observe a rise in $p27^{Kip1}$ mRNA levels (Fig. 5*D*). These data demonstrate that the sole activation of FoxO3 is sufficient to mimic the effects on $p27^{Kip1}$ and Bim that are normally seen after IL-2 withdrawal, and is sufficient for initiation of programmed cell death.

IL-2 signals rapid inactivation of FoxO in activated T cells

IL-2 signaling is critical throughout T cell development and maturation. The majority of peripheral T cells are quiescent cells but can be stimulated to reenter the cell cycle upon activation of the TCR. However, efficient cell cycle reentry of such resting T cells requires the presence of a costimulatory signal that can be provided by IL-2. Because FoxO3 appears to be such an important target of the IL-2 signaling with respect to T cell survival, we hypothesized that FoxO phosphorylation could be involved during activation of peripheral quiescent T cells upon combined TCR/IL-2 stimulation as well. To address this question, we isolated primary T cells from peripheral blood and activated them with immobilized anti-CD3 mAbs together with IL-2. At 24–48 h after stimulation we could observe that a large proportion of the activated cells had reentered the cell cycle (Fig. 6*A*), but that this cell cycle reentry was efficiently prevented by treatment with

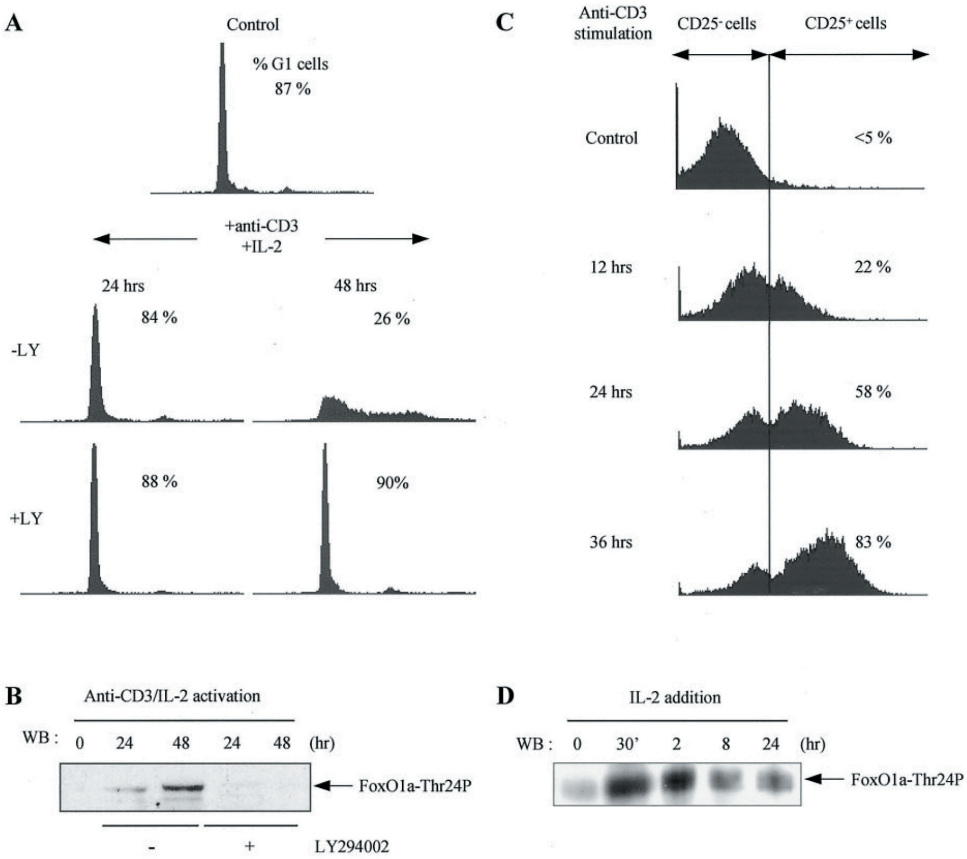

FIGURE 6. Activation of peripheral T lymphocytes is linked with PI3K/PKB-dependent phosphorylation of FoxO1. *A*, Primary T cells were isolated from fresh blood. The cells were stimulated with anti-CD3 mAbs and IL-2 in the presence or absence of LY294002. The cells were harvested after 24 and 48 h, fixed, and stained for FACS analysis as described in Fig. 1*A*. *B*, Protein samples prepared from the cultures shown in *A* were separated on polyacrylamide gels, and FoxO1a-Thr^{24}P levels were detected on Western blot. *C*, Primary T cells were isolated from fresh human blood and stimulated on UCHT-1 anti-CD3-coated dishes for different periods of time. The $CD25^+$ cell population was determined by FACS analysis by incubation with anti-CD25-PE Abs. *D*, Primary T cells were stimulated with anti-CD3 Abs for 24 h, and then with IL-2 for the indicated times. Cells were harvested, lysed, and analyzed by Western blotting with an anti-phospho-Thr^{24}-FoxO1a Ab.

LY294002 (Fig. 6*A*), confirming that activation of resting T cells is dependent on PI3K signaling.

Analysis of the expression of the different FoxO family members showed that these peripheral T cells express relatively high levels of FoxO1a and very low levels of FoxO3a and FoxO4 (data not shown). Phosphorylation of FoxO1a was induced in cells treated with anti-CD3/IL-2 (Fig. 6*B*), indicating that T cell activation correlates with PKB-mediated phosphorylation of FoxO factors. This phosphorylation event was fully dependent on PI3K signaling, because it was prevented when cells were treated with LY294002 in combination with anti-CD3/IL-2. This indicates that PI3K signaling is essential for FoxO phosphorylation in primary T cells. However, because anti-CD3 and IL-2 were added simultaneously in this experiment, and because it takes several hours to induce expression of the IL-2R, we were unable to properly study the kinetics of these events. Therefore, we stimulated the primary T cells with anti-CD3 alone to allow up-regulation of the IL-2R before adding IL-2. As shown in Fig. 6*C*, the high-affinity α-chain of IL-2R (CD25) is not present on resting peripheral T cells but was expressed on 58% of the T cells after 24 h and on ~83% of the cells after 36 h of activation with anti-CD3. To minimize effects of autocrine IL-2 produced by the activated T cells themselves, we used peripheral T cells that had been activated for 24 h only and analyzed the effects of IL-2 addition on FoxO1a phosphorylation. Under these circumstances, FoxO1a was phosphorylated within 30 min, and the extent of phosphorylation peaked at 2 h after stimulation (Fig. 6*D*). At later time points, FoxO1a phosphorylation dropped but was still elevated after 24 h of stimulation with IL-2 compared with the non-IL-2-stimulated population (Fig. 6*D*).

Discussion

IL-2 is a major regulator of T cells throughout their development and differentiation. In this report, we have addressed the molecular mechanism of the IL-2-mediated regulation of T cell proliferation and survival. For this purpose, we have studied IL-2 signaling in the murine T cell line CTLL-2, which is dependent on IL-2 for its proliferation and survival and is therefore a useful model to study IL-2-regulated events. As mentioned before, CTLL-2 cells arrest in the G_1 phase of the cell cycle and undergo apoptosis upon withdrawal of IL-2 from the culture medium. Interestingly, the execution of apoptosis was apparent only at ~24 h subsequent to the onset of a G_1 arrest, suggesting that the onset of apoptosis may require a prolonged arrest in the G_1 phase of the cell cycle to be irreversibly established. In addition, we observed up-regulation of $p27^{Kip1}$ levels upon IL-2 deprivation. $p27^{Kip1}$ is a *cdk* inhibitor that associates with G_1 cyclin/*cdk* complexes and inhibits their enzymatic activity. When $p27^{Kip1}$ protein levels increase, the cyclinE/*cdk2*-associated form of $p27^{Kip1}$ increases and *cdk2* is no longer able to phosphorylate pRb, leading to an arrest in the G_1 phase of the cell cycle (27).

Furthermore, we observed an up-regulation of Bim protein levels upon IL-2 withdrawal from CTLL-2. Bim is a proapoptotic BH3 domain-only member of the Bcl-2 family (28). Interestingly, Bim up-regulation upon IL-2 withdrawal occurred much later than $p27^{Kip1}$ up-regulation, which could explain our findings that the arrest in G_0/G_1 precedes the onset of apoptosis.

The Bcl-2 family consists of pro- and antiapoptotic members that can either homodimerize to promote a common function or heterodimerize to titrate their opposite functions (30, 31). In this model, either up-regulation of proapoptotic member or down-regulation of antiapoptotic member protein levels may lead to the induction of mitochondrial apoptosis. In this respect, it is worth noting that PKB has been described to induce the expression of the antiapoptotic Bcl-2 (9) and to inactivate the proapoptotic Bad by means of phosphorylation (32). Thus, PKB-mediated cell survival and protection from apoptosis occurs notably through interfering with the balance of pro- and antiapoptotic Bcl-2 family members. In this work, we show that proapoptotic Bim is down-regulated in a PI3K-dependent fashion in response to IL-2. This down-regulation appears to require inactivation of FoxO3, because expression of a mutant form of FoxO3 that can no longer be inactivated by the PI3K/PKB signaling pathway is sufficient to induce Bim expression. Bim appears to be an important mediator of the apoptosis seen in response to FoxO3 activation. For one, small changes in the expression of another proapoptotic FoxO3 target gene, namely Fas ligand, are observed upon IL-2 withdrawal or activation of FoxO3 in these cells, consistent with the general idea that apoptosis induced by cytokine withdrawal does not involve Fas signaling (33). Moreover, ectopic expression of Bim very efficiently kills these CTLL-2 cells (data not shown), indicating that the sole up-regulation of Bim is sufficient to drive these cells into apoptosis. Nevertheless, our data do not provide rigorous proof for an involvement of Bim in apoptosis caused by cytokine deprivation, and at present we cannot rule out that other targets of FoxO factors may exist that play a role in this.

Previously, we have reported a role for FoxO3 in IL-3-dependent survival signals in the Ba/F3 pre-B cell line (22). We show in this report that the effect of IL-2 on T cell survival is mediated, at least partly, by FoxO3, indicating that cytokine-mediated cell survival critically depends on the inactivation of FoxO3 or related forkhead factors. Taken together, these observations suggest that there is a conserved pathway from *C. elegans* to mammalians by which PI3K/PKB signaling regulates forkhead transcription factor activity to adapt cell survival and proliferation to the environmental conditions (nutrient, growth factors, and cytokine availability).

When an immune response is initiated, a resting T cell encounters Ag in the context of an APC and subsequent ligation of the TCR together with costimulatory molecules activates the T cell. This leads to expression of a functional IL-2R (through up-regulation of CD25) and secretion of IL-2. Autocrine stimulation by IL-2 now triggers the T cells to proliferate, thereby giving rise to an increased size of the Ag-specific T cell pool. Our results show that in Ag-triggered T cells IL-2 stimulation rapidly activates the PI3K/PKB pathway, which inactivates the forkhead transcription factors, leading to $p27^{Kip1}$ down-regulation (Fig. 7). This down-regulation results in the release of active cyclin-dependent kinase activity, allowing T cells to pass through the G_1 restriction point and to complete a full cell division.

Although a large pool of Ag-primed T cells is necessary to eliminate an invading pathogen, after its eradication the Ag-challenged T cell pool needs to reduce to its normal size to avoid excessive T cell accumulation. Next to a cell death pathway involving the death receptor Fas, the gradual loss of cytokines such as IL-2 has been suggested to be responsible for shutting off an immune response (33). Interestingly, T cell deletion by IL-2 depletion was found to require new gene expression because it can be blocked by actinomycin D and cycloheximide (33). Our data in both primary T cells and the IL-2-dependent CTLL-2 T cell line imply that, in this end stage of an immune response, IL-2 deprivation of the activated T cells leads to inhibition of PKB, activation and nuclear translocation of the forkhead transcription factors, and, finally, expression of $p27^{Kip1}$ and Bim (Fig. 7). The importance of (at least) Bim in immune homeostasis is underscored by the fact that Bim knockout mice accumulate T and B cells and develop signs of autoimmunity later in life (34).

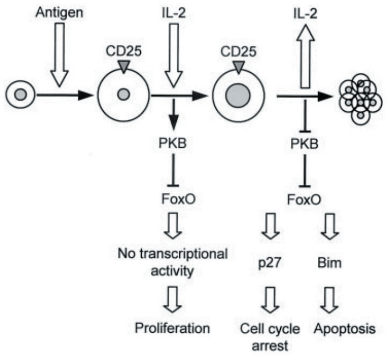

FIGURE 7. The IL-2 downstream pathway bifurcates at the level of the forkhead transcription factors to modulate both cell cycle and apoptosis. Early in an immune response, IL-2 stimulation promotes proliferation of newly Ag-primed $CD25^+$ T lymphocytes through activation of the PI3K/PKB pathway. Activated PKB phosphorylates FoxO members of the forkhead transcription factor family, thereby preventing their translocation to the nucleus and thus transcription of the cell cycle inhibitor $p27^{Kip1}$, allowing the T cells to proliferate. Later in the immune response, when Ag and IL-2 become limiting, withdrawal of IL-2 shuts down the PI3K/PKB pathway, releasing active FoxO forkhead transcription factors which can in turn activate transcription of target genes, such as $p27^{Kip1}$ and Bim. The Cdk inhibitor $p27^{Kip1}$, via its brake activity on the cell cycle progression, induces an arrest in G_1 phase. Bim, a proapoptotic member of the Bcl-2 family, can induce apoptosis in the activated T cell pool. In conclusion, the IL-2 signaling pathway regulates the FoxO members of the forkhead transcription factor family and bifurcates at that level to exert a dual effect on both cell cycle and cell death via $p27^{Kip1}$ and Bim, respectively.

Interestingly, in contrast to activated T cells, Bim cannot be detected in quiescent primary T cells, although our data suggest that these cells do contain active FoxO1a. Thus, in comparison to cytokine-depleted CTLL-2, transactivation of the Bim promoter by FoxO factors appears to be repressed in resting mature T cells. From a physiological point of view, the reason for such repression is evident, because elevated Bim levels could result in the death of peripheral quiescent T cells, as well as newly Ag-activated T cells. This would severely reduce their potential to generate an adapted immune response to a specific Ag. Nevertheless, the underlying mechanism for the lack of Bim induction is unclear at present. Obviously, the difference could be due to the distinct FoxO factors expressed in these two cell types (FoxO3 in CTLL-2 and FoxO1a in primary T cells), or one could speculate that distinct cofactors must exist that are involved in the activation or repression of the various FoxO target genes.

Taken together, our study indicates that the FoxO transcription factors may play an important role in IL-2-mediated effects on cell cycle progression as well as cell survival. This bifurcation of the IL-2 signaling pathway at the forkhead level enables coupling of G_1 progression to apoptosis in T cells. Regulation of the proliferation/death balance in T lymphocytes is critical to insure a fine-tuned immune reaction. Therefore, forkhead transcription factors, being at the crossroad of cell cycle regulation and apoptosis, might be critical modulators of the proliferation/death balance, and interference with their activity might destabilize the proliferation/apoptosis balance and lead to severe pathological disorders.

Acknowledgments

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Chapter 4:

Review

FoxM1: on a tight rope between ageing and cancer.

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# The authors contributed equally to this work.

To be submitted

FoxM1: on a tight rope between ageing and cancer

Summary

Forkhead transcription factors are intimately involved in the regulation of organismal development, cell differentiation and proliferation. Here we review the current knowledge of the role played by FoxM1 in these various processes. This particular member of the Forkhead family is crucial for cell cycle-dependent gene expression in the G2 phase of the cell cycle. Moreover, FoxM1 appears to play a crucial role in the demise of proliferative capacity that comes with age. In addition, FoxM1 has been shown to be essential for the fidelity of the cell division process, and inhibition of FoxM1 activity results in overt aneuploidy, a condition typical for human cancer. These aspects of FoxM1 function will be discussed, as well as their implication for our understanding of ageing and age-associated diseases, such as cancer

Cell cycle dependent transcription.

Cell cycle progression is, at least partly, controlled by transcriptional programs. These have been well-studied in yeast, in which many cell cycle-regulated genes displaying a periodical expression pattern have been identified [1]. These genes can be grouped in clusters of co-regulated genes, which are usually controlled by a single transcription factor complex [2] [3].

The cell cycle can be envisioned as the oscillation between two phases, DNA replication and mitosis. In the yeast *Saccharomyces cerevisiae*, two major cell cycle transcriptional programs have been identified: the G1/S transcriptional program that depends on the activity of the SBF/MBF transcriptional complex, and the G2/M transcriptional program that depends on the activity of FKH/Mcm1/Ndd1 transcriptional complex [4-6]. In yeast as well as in higher eukaryotes, the G1/S transcriptional program regulates the expression of genes involved in entry and execution of the S-phase, encoding DNA replication proteins, enzymes of the deoxyribonucleotide (dNTP) biosynthesis, replication origin proteins, and kinases that activate replication initiation.

The yeast G2/M transcriptional program is referred to as the CLB2 gene cluster, involved in entry and execution of mitosis. This cluster contains 33 genes, among which the Clb1 and Clb2 mitotic cyclins and the Cdc5 Polo kinase [5]. Evidence that a similar G2/M transcriptional program is also present in higher eukaryotes is scarce. A recent study done in our lab has uncovered a role for the FoxM1 transcription factor in the regulation of numerous mitotic genes that overlap with the CLB2 cluster [7], and thus in a potential mammalian G2/M transcriptional program. In light of this, we will discuss in this review the function of FoxM1 in regulation of cell cycle progression and its involvement in carcinogenesis.

FoxM1 is a transcription factor of the Forkhead family.

FoxM1 is a transcription factor of the Forkhead family. It is also known in the literature as Trident (in mouse), HFH-11 (in human), WIN or INS-1 (in rat), MPP-2 (partial human cDNA) or FKHL-16. In order to keep some clarity and homogeneity, and according to the proposed nomenclature of the Forkhead family members [8], we will refer to it as FoxM1 in this review.

The Forkhead family comprises a large number of transcription factors defined by a conserved DNA binding domain called Forkhead or winged-helix domain. The FoxM1 gene was cloned in an attempt to identify novel Forkhead genes involved in T cell differentiation. For that, Korver et al. screened a mouse thymus cDNA library with degenerate primers for homologues with a conserved DNA-binding domain [9]. Simultaneously, Ye et al. and Yao et al. identified FoxM1 using a similar strategy to study the involvement of Forkhead factors in intestinal differentiation [10] and to determine their expression in the endocrine pancreas [11]. The FoxM1 gene was revealed to encode a more distant Forkhead transcription factor family member that exhibits 45% identity in the DNA-binding domain with five of its closest related Forkhead

members, namely FoxA3 (HNF-3γ, FoxC1 (fkh-1), FoxF2 (FREAC-2), FoxK1 (ILF) and FoxN2 (HTLF) [9].

Originally, the rat FoxM1 C-terminal region was found to have homology (76% identity) with a human partial cDNA encoding an open reading-frame of 221 amino acids, termed MPP-2 [11]. MPP-2 stands for MPM-2-reactive phosphoprotein-2 and was identified after screening a lymphoblast cell line cDNA library with the MPM-2 monoclonal antibody, which binds specifically to epitopes on mitotic proteins that are phosphorylated in a phosphoserine-proline dependent manner [12]. MPP-2 reacted to the MPM-2 antibody only after phosphorylation by M-phase Cyclin B-Cdk1 kinase. However, it is not excluded that other serine/threonine kinases active in mitosis, such as Cyclin A/Cdk complexes, mitogen-activated (MAP) kinase or Plk-1, may also be involved in MPP2 phosphorylation (Figure 1). *In vivo* phosphorylation of FoxM1 during M-phase of the cell cycle was further confirmed by Korver et al. [9].

FoxM1 is alternatively spliced.

The human FoxM1 gene is a 10-exon structure spanning approximately 25kb on the 12p13-3 chromosomal band (telomeric position) [13]. Two exons, named exons Va and VIIa, also referred to as exon A1 (or rat exon 6) and A2 respectively, are alternatively spliced [10, 11, 13]. Exon Va encodes a 15 amino-acid insertion within the C-terminal part of the DNA binding-domain, and is not seen in any of the other Forkhead transcription factor family members. Exon VIIa represents a 38 amino-acid insertion within the C-terminus of the protein. Strikingly, one rat form of FoxM1 also expresses a small exon, rat exon 4, which is exclusive to the rat and is not present in other species. The presence of this exon leads to lower expression of the transcript [11].

Differential splicing of these two exons gives rise to three classes of transcripts, class A containing both alternative exons, class B containing none of the alternative exons, and class C in which exon Va only is retained. Both FoxM1B and C are transcriptionally active, whereas FoxM1A is transcriptionally inactive. This can be explained by the fact that the C-terminal region is required for transcriptional activation (trans-activation domain), and that insertion of exon VIIa in the C-terminus of the protein suppresses transcriptional activity as shown by transcriptional activity using deletion mutants of the different splice variants [10]. FoxM1A variant seems to show a dominant-negative effect [10], since it has retained the DNA binding domain in the absence of functional transactivation domain.

The presence of this natural splice variant may play a role by modulating the activity of functional FoxM1 variants under certain conditions. Therefore, it would be interesting to address in more detail the expression pattern of FoxM1A and its biological effect. Furthermore, knowing the stimulating effect of FoM1B and FoxM1C on cellular

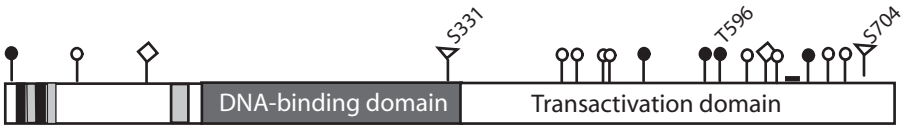

Figure 1. Schematic representation of FoxM1 protein. FoxM1 is a member of the Forkhead family of transcription factors, and thus comprises a conserved "Forkhead box" DNA binding domain in its N-terminal region, and a transactivation domain in its C-terminal region. Based on the described consensus sequences, we could identify two RXL cyclin binding motifs (white boxes) in the N-terminal region of FoxM1. Similarly, we could identify conserved putative phosphorylation sites for cyclin/cdc2 complexes (S/T-P-X-K/R consensus, black-rounded pins), for other cyclin/cdk complexes (S/T-P sites, white-rounded pins), for Plk1 mitotic kinase (D/E-X-S/T-Φ consensus, squared pins) and for mitogen-activated protein kinases (MAPK) (P-X-S/T-P consensus, triangular pins). The residues detailed above the pins have been previously described for their importance in FoxM1 activation, namely by Cyclin B (T596, Major et al) and by MAPK cascade (S331 and S704, Ma et al) Finally, we could also identify putative proteasome-dependent protein degradation boxes (light grey boxes), namely two N-terminal d-boxes (consensus RXXL) and a more C-terminal KEN box.

proliferation, induction of FoxM1A variant may be an interesting mean to curb increased proliferation in cells overexpressing FoxM1, such as cancer cells.
On the other hand, insertion of exon Va although disrupting part of the winged-helix structure [11] does not seem to affect binding of the protein to DNA. Thus, one could think that FoxM1C, which we have been studying in Chapter 5, and FoxM1B may have differential target genes due to a difference in DNA binding specificity resulting from the presence or the absence of Exon Va respectively. Interestingly, splicing of both exons Va and VIIa leads to the formation of the natural FoxM1B splice variant, which is present mostly in the testis [11, 14]. The fact that both exons are spliced out in a tissue-specific manner suggests that splicing of FoxM1 transcripts is differentially regulated. It would be interesting to address this for example by comparing microarray data obtained from cells expressing ectopic FoxM1B versus cells expressing ectopic FoxM1C. This would possibly reveal the differentially-expressed genes and also give more information of the respective role of these two transcription factors.

FoxM1 is expressed in actively proliferating cells.

FoxM1 is expressed in all embryonic tissues [9], and in particular in proliferating cells of epithelial and mesenchymal origin [10]. In adult tissues, FoxM1 is only observed in the thymus and testis at high levels, and in the lung and intestine at moderate levels [9], all of which represent proliferating adult tissues. In addition, FoxM1 is expressed in a wide variety of cell lines and cancer-derived cell lines [15]. Furthermore, during T cell development, FoxM1 is expressed in the thymus in immature single positive (SP) and double positive (DP) T lymphocytes, which are actively proliferating cell populations, but not in mature SP lymphocytes, which form the resting peripheral T cell population [15]. Taken together, these observations suggest that FoxM1 expression levels correlate with the proliferation rate of a cell.
Furthermore, FoxM1 has been shown to be present in neural stem cell germinal centres in mammalian cells and to be subsequently downregulated as cells differentiate [16, 17]. This suggests that FoxM1 may play a role during brain development, but most importantly that FoxM1 may generally be required during cell proliferation and that FoxM1 downregulation may conversely be necessary to allow differentiation.
Interestingly, FoxM1 expression is induced when serum-starved cells are allowed to re-enter the cell cycle [9]. A 2.4kB fragment of FoxM1 gene promoter, mapping the region upstream to the identified start site, was cloned in a luciferase reporter. It showed a significant activity, and more importantly appeared to be serum-inducible [13]. Further analysis of the promoter showed that only the 300 bp fragment upstream to the start site was required for the serum response of the promoter. An E2F binding site and Myc E-box were found about –550 bp and -40 bp respectively in FoxM1 promoter, and could at least partly be responsible for the serum-responsive nature of the FoxM1 promoter. We identified another potential E2F binding site which is conserved in mouse and human at about -20 bp. However, it remains to be tested if G1/S transcription factors such as E2F and Myc can regulate FoxM1 expression. Interestingly, in yeast, the cell cycle transcriptional programs are interdependent entities, and for instance, the G1/S SBF/MBF transcriptional complex has been shown to promote activation of the G2/M FKH/Mcm1/Ndd1 transcriptional complexes [5, 6].
In conclusion, FoxM1 is a true proliferation-specific protein: its expression is induced when cells are incited to enter the cell cycle; its levels are high in proliferating cells; and FoxM1 is specifically phosphorylated during mitosis. On the contrary, FoxM1 is negatively regulated in quiescent or terminally-differentiated cells [9].

FoxM1 and organogenesis.

Recently, the cloning of *Xenopus laevis* FoxM1 cDNA has allowed the determination of FoxM1 expression patterns in space and time during early development [18]. Maternally-expressed FoxM1 transcripts are present at the animal pole during early cleavage stages. However, until the blastula stage,

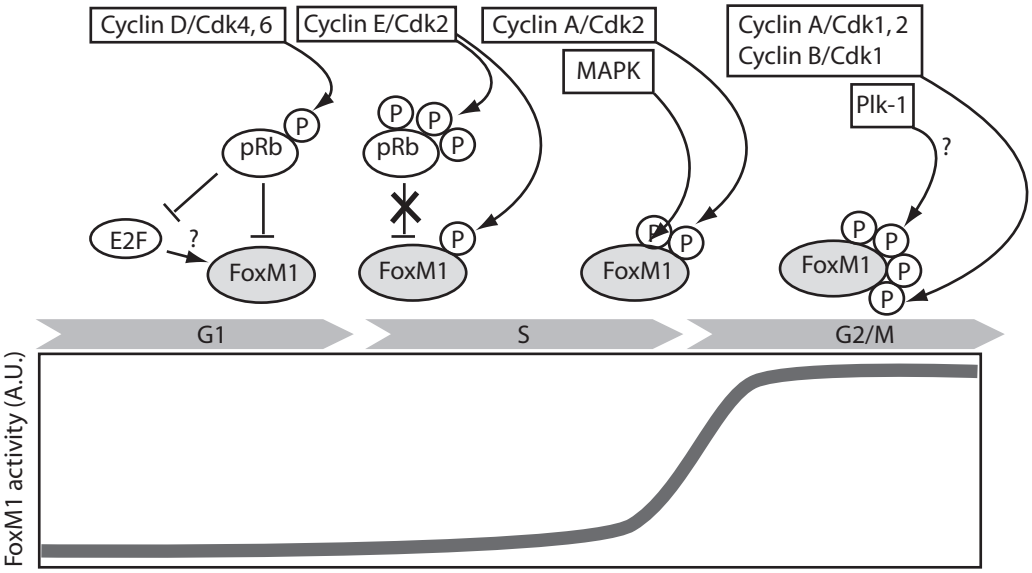

Figure 2. Cell cycle-dependent regulation of FoxM1 transcriptional activity. FoxM1 protein starts to be expressed in late-G1, possibly in an E2F-dependent manner. However, FoxM1 activity does not rise significantly before G2/M (lower panel). This may be due to pRb-dependent inhibition of FoxM1. Upon mitogenic stimulation, Cyclin D/Cdk4, 6 and Cyclin E/Cdk2 inactivate pRb, allowing the cells to progress to S-phase, but also relieving inhibition of FoxM1 by pRb. Enhanced FoxM1 transcriptional activity correlates with its hyperphosphorylation, suggesting that phosphorylation plays an important role in FoxM1 activation. FoxM1 is subjected to waves of phosphorylation by the subsequent cyclin/cdk complexes that are activated during cell cycle progression from the G1/S transition to the entry in mitosis (as indicated in the white boxes in the upper part of the figure). Phosphorylation during the S-phase does not seem to be critical for FoxM1 activation since FoxM1 activity in S-phase remains low. However, it may be a priming event that is required for the critical phosphorylation steps that occur during G2/M. These lead to the full activation of FoxM1, and are possibly mediated by the mitotic kinases Cyclin A/Cdk2 or Cdk1, Cyclin B/Cdk1, Plk-1 and by the mitogen-activated protein kinase (MAPK) cascade.

these mRNAs are unstable and degraded, suggesting that FoxM1 may not play a crucial role before gastrulation. However, during organogenesis, zygotic xlFoxM1 transcripts start to be expressed during neurulation at the dorsal pole of the animal in the neural folds. Later, FoxM1 mRNAs are found in structures of the developing neuro-sensory system [18]. As mentioned before, FoxM1 has also been suggested to play a role in the development of the central nervous system in mammalian cells [16, 17].

FoxM1 knock-out (KO) mouse models further confirm the role of FoxM1 during organogenesis. Mice containing FoxM1 targeted alleles that have inserted the targeting PGK-Neomycin gene cassette in exon 3 were generated [19]. Homozygous disruption of FoxM1 gene is lethal, but only around birth time, while heterozygous mutants appear to be normal. This confirms that FoxM1 does not play a role in the early stages of embryogenesis, but rather later during organogenesis.

Histological analyses of homozygous mutants have shown abnormalities in the heart and liver. The heart is dilated, with a significantly thinner myocardium. Microscopic analyses reveal irregularities in cardiomyocyte orientation as well as enlarged nuclei, suggestive of polyploidy. Liver cells also have enlarged nuclei. The DNA content of both cardiomyocytes and hepatocytes of FoxM1$^{-/-}$ fetuses has been assessed and shows respectively a 50-fold and 6-fold increase in DNA content (polyploidy).

Although heart and liver are known to be naturally polyploid tissues in the adult, polyploidization only occurs after birth and is therefore normally not observed in

embryos or fetuses. Thus, this suggests that, in the mouse, FoxM1 activity is required during development to prevent premature endoreplication *in vivo*. Accordingly, primary Mouse Embryonic Fibroblasts (MEFs) derived from FoxM1$^{-/-}$ embryos also have abnormal chromosome number [7], revealing a more profound effect of FoxM1 loss on the cell's DNA content. This suggests that FoxM1 plays a role in coordinating replication and mitosis and may be implicated in genomic stability.

Recently, a FoxM1 knock-out mouse was generated using another approach (deletion by gene targeting of the exons coding for both DNA-binding and transcriptional activation domains) [20]. Similarly as in the previously described FoxM1 KO model, these mice die in-utero shortly before birth (18.5 days after fertilization) and show abnormal liver development. Pathological analysis of these new FoxM1-/- embryos has revealed a significantly reduced hepatoblast proliferation, which contributes to hepatic failure, reduced liver perfusion and absence of intrahepatic bile ducts, suggesting that FoxM1 is critical for hepatoblast proliferation and differentiation toward biliary epithelial cell lineages. However, the FoxM1 expression pattern during organogenesis suggests that FoxM1 may play a role in proliferation and differentiation of progenitor cells in other tissues too. Indeed, a later study reports pulmonary defects in these FoxM1-/- mice, namely hypertrophic arteriolar smooth muscle cells and defective peripheral pulmonary capillaries [21]. These defects are due to the lack of proliferation of the embryonic pulmonary mesenchyme but not of the cells of epithelial origin. These defects were found to correlate with diminished TGF-β signalling and severe reduction in pulmonary levels of Laminin-α2 and -α4, both of which are essential for epithelial-mesenchymal signalling and found to be direct transcriptional targets of FoxM1 [21]. FoxM1-/- lungs also displayed reduced expression of additional target genes essential for lung vascular development including Platelet endothelial cell adhesion molecule-1 (Pecam-1), VEGF receptor type 1(Flt1), and the Forkhead transcription factor FoxF1. Thus, this report points at a role for FoxM1 notably in mesenchymal proliferation and vasculogenesis.

FoxM1 promotes cell proliferation.

As mentioned above, studies in FoxM1-/- mice have revealed defects in various organs due to the lack of progenitor cell proliferation, suggesting that FoxM1 may not only be expressed in proliferating cells, but may also be required for proper cell proliferation. Consistent with the observed decreased cell proliferation, FoxM1 deficiency has been associated to reduced expression of Cyclin A2, Cdc25B phosphatase, Cyclin B1, Polo-like kinase 1 (Plk-1) and Aurora-B [20-23]. These gene products are known to potently promote progression through the S-, G2- and M-phases of the cell cycle.

In the liver, FoxM1 is induced in proliferating hepatocytes during liver regeneration following partial hepatectomy [10]. This assay consists in the ablation of a portion of liver, which results in hepatocyte proliferation and regeneration of the liver to its initial size. This procedure is a commonly used protocol to study cell cycle re-entry of quiescent differentiated cells *in vivo*. This suggests that not only does FoxM1 stimulate progenitor cell proliferation, but under certain conditions, it may also induce proliferation of differentiated cells.

FoxM1 transgenic (Tg) mice were created by expressing a FoxM1 transgene solely in the adult liver under the control of transthyretin (TTR) promoter [24]. Premature expression of FoxM1 in regenerating hepatocytes of TTR-FoxM1 transgenic mice leads to premature onset of DNA replication and mitosis. However, in non-regenerating hepatocytes, expression of the TTR-FoxM1 transgene does not elicit abnormal proliferation. This is due to the inactivation of FoxM1 by sequestration in the cytoplasm, far from the DNA where it normally plays its role of transcriptional regulator. Similar results have been obtained in regenerating livers following carbon tetrachloride injury [25]. This reveals two important points: that FoxM1 promotes cell proliferation, and that FoxM1 needs to be properly activated by mitogenic cues. From the molecular point of view, TTR-FoxM1 transgene expression correlates with increased expression of numerous cell cycle regulatory genes, such as Cyclin B, Cyclin A and Cdc25B, as well as with downregulation of the Cdk inhibitor p21^{Cip1} [24, 25].

Expression and disruption of FoxM1 in the liver have unveiled an essential role in liver cell proliferation. However, the importance of FoxM1 for cell proliferation has been shown to apply to other tissues as well. For example, FoxM1 is also induced in lung cells of both mesenchymal and epithelial origin following chemical insult [26], where it correlates with cell proliferation and tissue repair. The fact that FoxM1 is induced during cell proliferation following tissue injury may also suggest that FoxM1 expression is important for tissue regeneration and repair.

Ubiquitous FoxM1 transgenic mice were produced by expressing FoxM1 under the control of the ubiquitous promoter Rosa 26. In this model, premature FoxM1 expression correlated with accelerated lung cell proliferation following tissue injury, here, with butylated hydroxytoluene, similarly as seen in FoxM1 liver-specific transgenic mice after chemical or surgical injury. Interestingly, this acceleration of cell proliferation involved different lung cell lineages such as alveolar and bronchial epithelial cells, smooth muscle cells and endothelial cells. Furthermore, it also correlated with earlier expression of cell cycle genes required for DNA replication and mitosis [27]. This suggests that FoxM1 expression is capable of stimulating different differentiated cell lineages to proliferate following certain mitogenic stimuli. Although the authors of this study emphasize mostly the fact that maintaining FoxM1 expression may be used in the prevention of ageing-related defects due to decreased cell proliferation, one could think that deregulation of FoxM1 expression may be a means by which a malignant cell can attain an increased proliferation rate. This specific issue will be addressed later in this review.

FoxM1 and ageing.

Ageing is characterized by a decreased cell proliferation rate, due to the accumulation of mitotic defects and chromosomal instability [28]. The mitotic defects observed in cells from elderly patients as well as from patients with progeria (syndrome of premature ageing) have been associated to decreased expression of cell cycle regulatory genes, one of the strongest outliers being FoxM1 [28]. Increased levels of FoxM1 in elder TTR-FoxM1 transgenic mice correlated with increased hepatocyte proliferation rate after partial hepatectomy, to similar levels as in young mice. This was associated with increased levels of cell cycle regulatory proteins such as Cyclin D1, Cyclin A2, Cyclin B1 and B2, Cyclin F and Cdc25B [29]. Similarly, acute delivery of FoxM1 in old mice also increased hepatocyte proliferation in response to partial hepatectomy and correlated with increased expression of Cyclin A, Cyclin B, Cdc25B and decreased levels of $p27^{Kip1}$ [23]. This study suggested that reduced FoxM1 levels contribute to the mitotic defects seen in ageing cells. If so, then loss of FoxM1 is expected to result in decreased hepatocyte proliferation after partial hepatectomy. Because a full FoxM1 knock-out is lethal, conditional knock-outs with liver-specific disruption of FoxM1 genes were produced in order to address this question. For this purpose, Cre recombinase was expressed under the control of the albumin promoter, mediating liver-specific deletion of FoxM1 gene by promoting recombination with the targeting construct. Liver-specific deletion of FoxM1 in the adult mouse led to a significant reduction in hepatocyte proliferation after partial hepatectomy. This was correlated with high levels of $p21^{Cip1}$, lower expression of Cdc25A and Cdc25B and delayed accumulation of Cyclin B, and subsequent decreased Cdk2 and Cdk1 activity [23]. This suggests that FoxM1 is essential for hepatocyte cell cycle progression.

Reduced cell proliferation in elderly patients has been associated with reduced levels of Growth Hormone (GH). In fact, GH treatment in elderly patients has been shown to restore some cell proliferation. Also, it has been shown that GH plays a role in hepatocyte proliferation during liver regeneration, notably by promoting nuclear translocation of FoxM1. GH treatment could restore hepatocyte proliferation after partial hepatectomy in old-aged mice. This observation correlated with restored induction of FoxM1 [30]. After disruption of FoxM1 in the liver, GH failed to restore proliferation, providing a mechanism by which GH can stimulate proliferation in old-aged organisms [20]. However, the response to mitogens

such as the hepatic mitogen TCPOBOP was comparable in aged versus young mice, in which induction of FoxM1 as well as other cell cycle-associated proteins was similar [31]. These observations suggest that differentiated hepatocytes retain their capacity to proliferate throughout life, possibly through upregulation of FoxM1. Thus, not only is FoxM1 specifically expressed in proliferating cells, but it also promotes cell cycle progression, most likely by direct transcriptional regulation of key cell cycle components.

FoxM1 regulates cell cycle progression.

Although many reports suggested that FoxM1 acts through transcriptional regulation of important cell cycle regulators such as Cyclin B, no evidence was actually supporting a direct effect of FoxM1 on the expression of these genes. Recently, expression profiling led to the identification of a FoxM1-regulated cluster of genes among which many were known G2/M-specific genes (Cyclin B, Nek-2, CENP-F, etc). For the most significant of these genes, direct binding of FoxM1 to the promoter was confirmed by chromatin-IP, demonstrating that FoxM1 indeed regulates these genes directly [7]. These results confirmed above-mentioned studies that reported FoxM1 expression to correlate with expression of many G2/M genes [20-23]. More importantly, this analysis revealed a new set of genes that appear to be controlled by FoxM1, implying that FoxM1 exerts a multiplicity of functions in regulating the cell cycle machinery.

In FoxM1-deficient MEFs as well as in FoxM1-depleted human cancer cells (FoxM1 RNAi), expression of FoxM1 target genes is impaired. Moreover, these cells display pleotropic cell cycle defects including severe delay in mitotic entry, chromosome missegregation, defective mitotic spindle assembly checkpoint, polyploidization and/or mitotic catastrophy catastrophy [7, 32, 33]. In FoxM1-deficient MEFs, this was associated with dramatic changes in chromosome numbers and premature senescence, suggesting that, in addition to promote cell cycle progression, FoxM1 is also required to maintain chromosomal stability.

Several target genes can account for these defects. Indeed, FoxM1-deficient cells fail to accumulate G2/M phase-promoting genes such as Cyclin B, Plk-1 and Aurora-A and -B kinases [7, 20-23], all known for their importance in mitotic progression. Furthermore, FoxM1 activates expression of the CENP-F gene, by direct binding to its promoter. CENP-F is a kinetochore binding protein and therefore appears to be an important component of the mitotic checkpoint machinery [34-37]. Expression of CENP-F is seen to be decreased in cells lacking FoxM1 [7]. More importantly, CENP-F-depleted cells display chromosome segregation defects, very similar to those found in FoxM1-deficient cells. Because levels of BubR1 and Mad2 proteins, two important component of the mitotic spindle assembly checkpoint were reduced in CENP-F-depleted cells, it is most likely that CENP-F is required to maintain recruitment of mitotic checkpoint components to kinetochores in order to allow proper chromosome segregation.

The role of FoxM1 versus FoxO in cell cycle regulation.

All Forkhead transcription factors share a common highly conserved DNA binding domain and can potentially bind similar consensus sites in the promoter of genes. In addition to FoxM1, other members of the Forkhead family have been shown to be involved in cell cycle regulation, most notably the FoxO subfamily members FoxO1 (also known as FKHR), FoxO3a (FKHR-L1) and FoxO4 (AFX). Their function has mainly been attributed to regulation of the G1-phase [38, 39], but some reports have implicated them in the regulation of later stages of the cell cycle. For example, the G2/M checkpoint protein Gadd45 was reported to be regulated in a FoxO-dependent manner in response to oxidative stress and/or DNA damage [40, 41]. This suggests that FoxO may have a role in the regulation of G2/M under conditions of cellular stress. In addition, another study has reported that FoxO plays an important role in the transcription of mitotic regulators, such as Plk1 and Cyclin B1 [42]. These two views on the potential role of FoxO factors seem rather contradictory, as one proposes

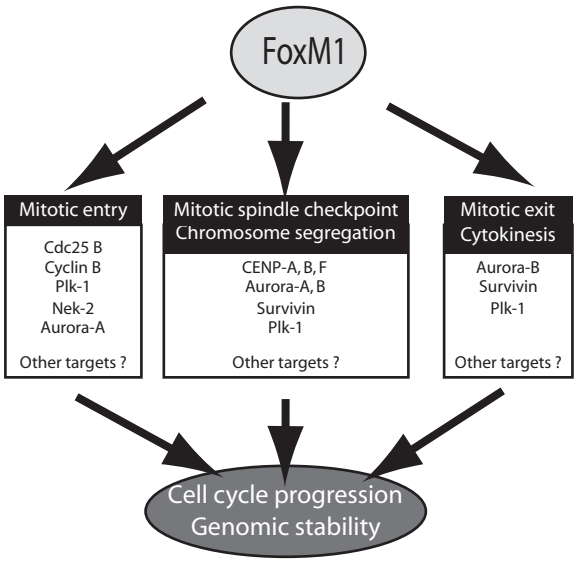

Figure 3. Involvement of FoxM1 target genes in the regulation of cell cycle progression and maintenance of the genomic stability. Several microarray analyses studies have revealed numerous FoxM1 target genes. The most significant of these genes can be clustered in function of their role in the regulation of the cell cycle, more specifically of the G2/M-phase of the cell cycle. We have set three clusters: the genes that are mainly involved in mitotic entry, those that are regulating the mitotic spindle checkpoint and/or chromosome segregation regulation, and finally, those that play a role in cytokinesis and mitotic exit. The proper coordination of the expression of these genes in space and time participate to proper cell cycle progression and maintenance of the genomic stability.

FoxO factors to inhibit cell cycle progression in response to stress [40, 41], while the other describes a major cell cycle promoting role to FoxOs [42]. The mitotic phenotypes of FoxM1-deficient cells render it unlikely that FoxM1 and FoxO act in a redundant fashion during G2. However, it should be noted that related Forkhead factors can bind to similar consensus sites, and therefore it is not unlikely that FoxO can transactivate FoxM1 target genes when expressed at high levels. Nonetheless, in the absence of functional FoxM1, expression of genes such as Cyclin B1, Plk-1 or CENP-F is dramatically reduced and these levels do not recover upon ectopic expression of active FoxO [7]. This indicates that FoxM1 is required for the expression of these genes and that activation of FoxO cannot compensate for FoxM1. Moreover, FoxO and FoxM1 transcriptional activities were compared in G1/S versus G2/M using a reporter containing a general Forkhead-responsive element (6xDB), or a reporter containing the Plk-1 promoter. This comparison clearly showed that although a constitutively active mutant of FoxO displays some activity in G2 on the general Forkhead-responsive element, it fails to transactivate the Plk-1 promoter [7]. Furthermore, activation of endogenous FoxO in G2 by treatment with a PI3-kinase inhibitor did not result in induction of the Plk-1 or CENP-F promoters in a FoxM1-deficient background, although it did result in induction of 6xDB promoter activity. Taken together, these data demonstrate that FoxM1 is the predominant Forkhead transcription factor that regulates G2/M genes, and if FoxO does contribute to transcriptional activation of the same target genes, its overall contribution is expected to be very low.

Regulation of FoxM1 activity.

The FoxM1 gene exhibits a specific expression pattern during the cell cycle. Both FoxM1 mRNA and protein levels are barely detectable in quiescent cells, whereas they increase in cells stimulated to re-enter the cell cycle [9]. This increase in FoxM1 expression is initiated at the onset of S-phase and continues throughout G2-phase and mitosis [9]. Further studies have shown that transcriptional activity of FoxM1 is also cell cycle-specific, and correlates with increased phosphorylation of the protein, reaching maximum levels in the G2-phase of the cell cycle. While the earliest findings have reported that phosphorylation of FoxM1 occurs mainly during mitosis, others have shown that phosphorylation of FoxM1 is initiated by Cyclin-Cdk complexes in early G1 and continued during G2- and M-phases of the cell cycle [43]. Based on the putative consensus phosphorylation sites, kinases such as Cdk2, Cdk1, mitogen-activated protein

kinase (MAPK) and Plk-1 may be involved in FoxM1 phosphorylation (Figure 1).

FoxM1 transcriptional activity was shown to require binding to Cyclins through an LXL Cdk docking sequence in the C-terminal region (639 to 641 in the human FoxM1) in order to allow efficient phosphorylation of FoxM1 on residue T596 by Cyclin-dependent kinases [43] (Figure 1). FoxM1 displays increased association with Cyclin E-Cdk2 complexes in G1- and S-phases of the cell cycle, whereas FoxM1 preferentially binds the Cyclin B-Cdk1 complex in G2 phase [43] (Figure 2). The FoxM1 T596 Cdk site was shown to be particularly important with respect to transcriptional activation as its phosphorylated form serves to recruit a transcriptional co-activator, the histone deacetylase p300/CREB binding protein (p300/CBP). Indeed, mutation of threonine 596 to an unphosphorylatable alanine residue (FoxM1 T596A) caused a strong decrease in transcriptional activity of FoxM1. Furthermore, FoxM1 has been shown to interact with Cyclin-Cdk in complex with the Cdk inhibitor $p27^{Kip1}$. Because of p27-dependent inhibition of Cyclin-Cdk kinase acitivity, such an interaction might not lead to phosphorylation of T596 and thus prevents activation of FoxM1. We may even extrapolate and foresee this as a repression mechanism for FoxM1 activity during the G1-phase of the cell cycle. Moreover, our recent data indicate that Cyclin A/cdk complexes play an important role in the regulation of FoxM1 transcriptional activity through direct binding and phosphorylation of both N-terminal and C-terminal regions of FoxM1 protein (Stahl et al., manuscript in preparation) (Figure 2).

We mentioned before that FoxM1 also requires activation by mitogenic signals, and the Ras-MAPK pathway seems to play an important role in this respect. MAPK signalling-mediated FoxM1 phosphorylation was shown to regulate FoxM1 cellular localization and transcriptional activation [44] (Figure 1 and 2). Indeed, inhibition of Ras-MAPK signalling pathway resulted in strong reduction of FoxM1-dependent transcription. Moreover, Raf/MEK/MAPK signalling was shown to stimulate FoxM1 nuclear translocation through phosphorylation in late S-phase [44]. This suggests that multiple FoxM1 phosphorylations may be catalyzed by multiple kinases at different stages of the cell cycle.

In addition to association with Cyclin-Cdk complexes, FoxM1 has also been shown to bind other cell cycle-regulatory proteins at specific stages of the cell cycle. FoxM1 was shown to associate to the cell cycle-inhibitory pocket protein pRb and to the Cdk-activating phosphatase Cdc25B in G1 and in G1/S respectively [43]. These proteins are known to be important cell cycle regulators and regulate FoxM1 transcriptional activity via their effect on Cdk kinase activity. Interestingly, activation of FoxM1 by Cdc25B creates a positive feedback-loop, since FoxM1 in turn regulates transcriptional activation of Cdc25B [43]. Finally, it was shown that p19ARF, through its residues 24 to 37, interacts with the FoxM1 C-terminal transactivation domain and inhibits its transcriptional activity. Similarly, the peptide (D-Arg)9-p19ARF 26–44 was shown to inhibit FoxM1 transcriptional activity. One mechanism that could account for p19ARF-dependent FoxM1 inhibition is direct interference with FoxM1 transactivation domain. Alternatively, p19 ARF could act through interference with the localization of FoxM1 within the nucleus. Indeed, p19ARF was shown to direct FoxM1 to the nucleolus, possibly precluding interaction of FoxM1 with its target genes.

In conclusion, both expression and transcriptional activity of the FoxM1 transcription factor are dependent on cell cycle progression. Initiating and maintaining FoxM1 transcriptional activity clearly requires binding of activated Cdk-Cyclin complexes, subsequent phosphorylation by mitogenic kinases and Cdk-Cyclin complexes, as well as recruitment of regulatory proteins such as p300/CBP.

FoxM1 contribution to cancer.

As mentioned before, FoxM1 is considered as a proliferation marker. Thus, it is not surprising that FoxM1 is expressed in numerous tumour-derived cell-lines [9]. However, there is accumulating evidence that not only is FoxM1 highly expressed in tumour cells because they display high proliferation rates, but that FoxM1 also actively participates in

tumour development through its proliferation-stimulating activity.

FoxM1 was shown to contribute to cellular transformation by the high risk human papillomavirus-16 (HPV-16) E7 protein [45]. HPV-16 is commonly associated with pre-malignant and malignant lesions, most notably in cervical squamous carcinomas [46]. FoxM1 was identified in a two-hybrid screen as an interactor of HPV16 E7. This interaction was shown to increase E7 transforming activity, as well as FoxM1 transcriptional activity. FoxM1 may therefore be an important target for E7-mediated transformation. Interestingly, FoxM1 was shown to interact with the cell cycle inhibitory pocket protein pRb in its hypophosphorylated form in G1 [43]. E7 is known to abrogate pRb function, and this targeting is required for both HPV-26 viral replication and virus-induced transformation in mammalian cells [47]. E7 binds to pRb via an LXCXE motif, interferes with E2F binding to pRb via its C-terminal domain, and may recruit factors that target pRb for proteolysis. It is very well possible that E7 uses a similar strategy with FoxM1-pRb complexes, and E7-mediated pRb inactivation may account for FoxM1 transcriptional activation. Finally, the human FoxM1 gene is located on the chromosomal band 12p13 [13]. This band is commonly amplified in advanced-stage cervical squamous carcinomas, in 50% of which high-risk HPV-16 was detected [48]. In this particular context, amplification of FoxM1 gene in the presence of high-risk HPV-16 may increase the proliferation rate while potentiating HPV transforming activity to extremely high levels, and thus lead to cancer development.

Several studies report FoxM1 as a differentially-expressed gene in solid tumours [49-51]. For example, in basal cell carcinoma (BCC) skin tumours, FoxM1 expression and activity were shown to be significantly elevated [49]. Interestingly, the increase in FoxM1 mRNA levels was specific for BCCs and not a reflection of increased cell proliferation, in that no such up-regulation was seen in skin squamous cell carcinomas or proliferating primary human keratinocyte cultures. Activation of Sonic Hedgehog (Shh) signalling is known to play a key-role in the development of skin BCCs in humans [52];[53]. Interestingly, the Shh downstream target Glioma transcription factor-1 (Gli1) was shown to upregulate FoxM1 at the transcriptional level [49]. This infers that upregulation of FoxM1 in BCC may be one mechanism by which Shh signalling exerts a mitogenic effect towards tumour development.

Increased expression of FoxM1 has also been implicated in brain tumours (anaplastic astrocytomas and glioblastomas), not for tumour formation per se but rather for malignant progression to more agressive forms of the disease [50]. Interestingly, Shh has also been implicated in glioma tumorigenesis [53], again pointing at the potential importance of FoxM1 in Shh signalling with respect to cell proliferation.

In addition to skin BCCs and brain tumours, Shh signalling is pivotal in many other human cancers, such as gastric, pancreatic, colorectal, breast, and prostate cancers. Interestingly, a recent study has identified FoxM1 as differentially expressed in most carcinomas [51]. In this study, the authors have analyzed complete microarray datasets for the differentially expressed genes in carcinomas originating from different tissues (prostate, breast, lung, ovary, colon, pancreas, stomach, bladder, liver and kidney) and grouped these that are differentially expressed in the majority of carcinomas. In this analysis, FoxM1 ranks among the top 100 genes that have been identified as commonly upregulated in carcinomas.

Comparative genomic hybridization (CGH) analyses have shown amplifications of the 12p13 chromosomal band, comprising the FoxM1 gene, in numerous tumours mostly solid tumours such as cervical squamous carcinomas [48], breast adenocarcinomas [54], nasopharyngeal carcinomas [55], and head and neck squamous cell carcinomas [56], but also in peripheral cytotoxic T cell lymphomas not otherwise specified (PTCL-NOS) [57].

Furthermore, FoxM1 is also overexpressed in hepatocellular carcinoma (HCC) [58]. Although FoxM1 overexpression in the liver (TTR-FoxM1 transgenic mouse) was shown not to influence the incidence of HCC after treatment with the carcinogenic cocktail

Diethylnitrosamine/Phenobarbital (DEN/PB), it did lead to an increase in size of the pre-neoplastic and early neoplastic lesions [59]. Conversely, FoxM1-deficient hepatocytes displayed resistance to tumour development in a similar experimental set-up [22]. This was due to the failure of FoxM1-deficient hepatocytes to undergo extensive proliferation and was associated with increased nuclear levels of $p27^{Kip1}$ and decreased protein levels of Cdc25B. Interestingly, targeted mutations to other transcription factors such as c-Jun or E2F1 did not have such a profound effect in blocking cell proliferation as compared to FoxM1 deficiency [22, 60, 61]. This suggests that FoxM1 is required for the proliferative expansion necessary for tumour progression, particularly in HCCs.

Moreover, this study has also revealed the role of p19ARF in the negative regulation of FoxM1. Interestingly, DEN/PN exposure was shown to cause a transient induction of p19ARF, which was found to bind and inhibit FoxM1 [22], suggesting that p19ARF inhibits FoxM1 in response to oncogenic stimulation. However, at later times after DEN/PB exposure, p19ARF was extinguished, suggesting that FoxM1 may participate to tumour development by promoting cell proliferation in cells where p19ARF has been inactivated [22]. Furthermore, FoxM1 was shown to stimulate colony-formation of U2OS human osteosarcoma cells on soft-agar, suggesting that FoxM1 is able to stimulate cellular proliferation [22]. Unfortunately, U2OS being cancer cells and thus already transformed, this assay could not address the question whether FoxM1 may be required for cellular transformation. Interestingly, the (D-Arg)9-p19ARF 26–44 peptide was also shown to prevent FoxM1-mediated stimulation of cellular proliferation of U2OS cells in soft agar.

In summary, it appears that FoxM1 is often overexpressed in tumours, in particular in different types of carcinomas. FoxM1 may be up-regulated, for example by Shh signalling, but its increased expression may also be the reflection of amplification of the chromosomal band 12p13. Interestingly, this genomic region has in fact been described as often subjected to rearrangements, such as amplifications, breakpoints, deletions and loss of heterozygocity (LOH) [62-65], suggesting that FoxM1 activity may not only be altered by gene amplification but also by other genomic modifications. However, the FoxM1 gene has never been directly implicated in these rearrangements. Despite the presence of genes potentially implicated in cancer development in this chromosomal band, it is very well possible that FoxM1 function is altered as a consequence of these rearrangements. Indeed, a few genes in this region can be linked to cancer development, such as TEL/ETV6 oncogene. Cyclin D2 gene and p27Kip1 and ING4 tumour suppressor genes. However, it has been suggested that modification of other yet unidentified genes lying in this region may also occur [66-68].

Finally, FoxM1 has been shown to be downregulated during the process of transdifferentiation of prostate fibroblasts into myofibroblasts in response to TGF-β1. This process can be observed during remodeling of the stromal tissue surrounding benign prostatic hyperplasias and in prostate cancers. We now know the importance of stromal tissue in cancer development [69], and FoxM1 downregulation may therefore indirectly participate to tumour development through stromal modification.

FoxM1 lies at the bottom of several cascades that can be drastically modified in cancer cells. In particular, FoxM1 is activated by potential oncogenes, and is inhibited by tumour suppressor genes which function is often lost during carcinogenesis. This position makes of FoxM1 an interesting protein with regards to its role in cellular proliferation and regulation of genomic stability, and thus to its role in tumour development.

Future directions.

Our current understanding of FoxM1 has given it a central role in the regulation of the cell division process. FoxM1 executes this function primarily through regulation of essential mitotic regulatory proteins that are part of the G2/M transcriptional cluster of mammalian cells. Nonetheless, many of the targets of FoxM1 have currently no mitotic function ascribed to them and it will

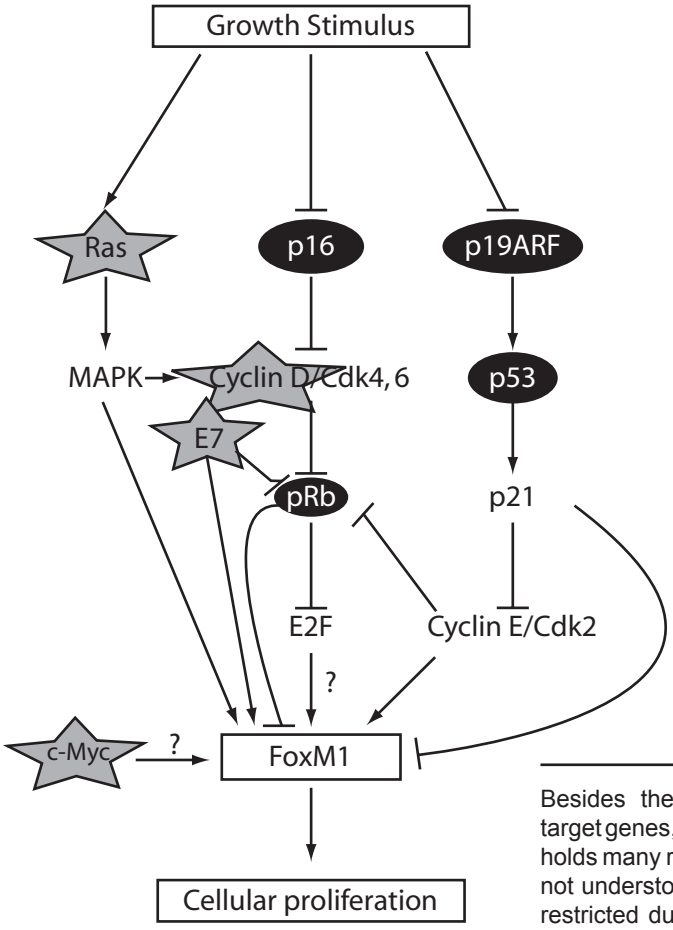

Figure 4. Schematic representation of tunour suppressor gene and oncogene pathwatys that regulate FoxM1. FoxM1 expression has been correlated with cancer development. Analysis of FoxM1 regulatory pathways revealed that FoxM1 lies within key-pathways that are affected during carcinogenesis (grey stars for proto-oncogenes and dark ovals for tumour suppressor genes). It remains to be answered whether overexpression of FoxM1 in cancer cells is only a consequence of cell transformation because of its proliferation-stimulating effect, or whether overexpression of FoxM1 also collaborates with oncogenes in order to induce cell transformation.

be interesting to find out if any of these do regulate cell division. Alternatively, FoxM1 may have additional roles besides regulation of cell division. In this respect, it is interesting to note that its main target gene, Gas1, has been associated with cellular quiescence and is implicated in Sonic Hedgehog (Shh) signalling. As FoxM1 itself was shown to be regulated by Gli-transcription factors, which in turn are responsive to Shh, this opens the door to a feedback system that could play an important role during the developmental processes driven by Shh, such as brain development. However, it should be noted, that Gas1 has been annotated as part of the G2/M cluster in human cells, suggesting Gas1 may also be a mitotic target of FoxM1 with a role in mitosis that awaits discovery.

Besides the undiscovered roles of FoxM1 target genes, the regulation of FoxM1 itself also holds many remaining mysteries. It is currently not understood how exactly FoxM1 activity is restricted during an ongoing cell cycle. Also, it remains to be addressed whether FoxM1 alone, or, like its ancestor Fhk1/2 in yeast, in complex with other transcription factors acts to transactivate its target genes.

Another aspect of FoxM1 that will have to be addressed in the future is its role during ageing. Clearly, FoxM1 activity is reduced as cells age, but how this comes about is completely unclear. On the other hand, ectopic expression of FoxM1, can revert some of the proliferation defects that come with age, but it remains to be seen if overall organism age can be affected by altering FoxM1 expression. In parallel, if and how the function of FoxM1 is affected during tumorigenesis is not well understood. As interference with FoxM1 function leads to aneuploidy, a condition almost invariably seen in human tumors, this seems an interesting avenue to explore.

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Chapter 5:

Cell cycle-dependent regulation of the Forkhead transcription factor FoxM1.

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To be submitted

Cell cycle-dependent regulation of FoxM1 activity.

Abstract.

The Forkhead transcription factor FoxM1 is an important regulator of gene expression during the G2-phase of the cell cycle. Expression of FoxM1 itself is low in quiescent cells and is induced upon stimulation with mitogens. FoxM1 expression reaches maximum levels as cells enter S-phase, but most known FoxM1 target genes are not induced until a cell reaches G2. Here, we have investigated the regulation of FoxM1 activity during an ongoing cell cycle. We show that transactivation by FoxM1 is indeed largely restricted to G2. Specific activation of FoxM1 during the G2-phase appears to involve Cdk-dependent relief of a transcriptional repressor function, exerted by the N-terminus of FoxM1. Moreover, we find that FoxM1 binds directly to Cyclins A and B, and complexes containing these cyclins can phosphorylate N- and C-terminal domains of FoxM1 with different specificities. Based on these observations, we propose a model in which Cyclin A/Cdk1 is required to activate FoxM1 by inactivating the N-terminal repressor domain. Finally, we demonstrate that FoxM1 stability is differentially regulated during the cell cycle and that FoxM1 is actively degraded during exit from mitosis. Taken together, these data show that multiple levels of control are enforced upon FoxM1 to limit is transcriptional activity to the G2 phase of the cell cycle.

Introduction.

Cell cycle progression is powered by the sequential oscillations in cyclin-dependent kinase (Cdk) activity, themselves being dependent on the sequential oscillations in the cyclin protein levels [1]. Cyclins have a typical cell cycle expression pattern: they are expressed in order to regulate the execution of specific cell cycle events, and they are degraded after the completion of these events. Therefore, timing of both cyclin expression and subsequent cyclin degradation sets the cell cycle clock to ensure the proper sequential timing of the cell cycle events.

Cdk activation through interaction with a specific cyclin partner allows regulation of specific subsets of cell cycle-regulated proteins through phosphorylation. Currently, five main cyclin/Cdk complexes are recognized to be activated and inactivated during the cell cycle: Cyclin D in complex with Cdk4 or 6 is active from early to mid-G1, Cyclin E/Cdk2 is active from mid-G1 to early S-phase, Cyclin A/Cdk2 is active in late-G1 and throughout S-phase, Cyclin A/Cdk1 is active from G2 until early mitosis and finally Cyclin B1/Cdk1 is activated upon entry in mitosis and inactivated at the metaphase/anaphase transition. Each of these cyclin/Cdk complexes phosphorylate different subsets of cell cycle-regulated proteins.

The cell cycle-dependent degradation of cyclins is the result of their ubiquitination and their subsequent proteolysis by the 26S proteasome complex [2] {Ciechanover, 2000 #14}. Ubiquitination of target proteins such as the cyclins occurs as the consequence of an enzymatic cascade leading to the formation of a covalent bond of one or several Ubiquitin moieties either with the target protein or with previously attached Ubiquitins. This reaction involves three different types of enzymes. First, Ubiquitin is activated in an ATP-dependent fashion by the E1 Ubiquitin-activating enzyme, which transfers it to the E2 Ubiquitin-binding protein (UBP). Finally, the Ubiquitin is transferred from the E2 to the target protein as a result of the intervention of an E3 Ubiquitin-ligase. Although, ubiquitination can occur either as mono- or poly-ubiquitination, only polyubiquitination is recognized by the proteasome and leads to degradation of the target protein. During the cell cycle, many proteins are degraded by the proteasome in a cell cycle-dependent manner. The E3-ligase is the factor that provides for the specificity as well as the timing of degradation of the ubiquitinated target protein. These proteins may exert both temporal and spatial action in order to regulate properly cell cycle progression.

FoxM1 is a transcription factor of the Forkhead

family. FoxM1 protein levels are known to vary during cell cycle progression [3]. In Rat-1 cells that have been synchronized in the G0-phase of the cell cycle by serum starvation, FoxM1 mRNA and protein are not detectable. However, if serum is re-added to the culture medium, cells re-enter the cell cycle. FoxM1 mRNA is detectable as early as 2 hours after serum stimulation and FoxM1 protein after 6 to 9 hours [3]. FoxM1 expression reaches maximum levels sometime in late G1 or early S-phase, and is sustained at these levels throughout G2 and mitosis. At later time-points (21 to 24 hours) after serum stimulation, slower-migrating bands of FoxM1 can be observed, suggesting that FoxM1 is phosphorylated in mitosis [3]. Thus, the literature is consistent with FoxM1 being expressed in late G1, with levels attaining their maximum in S-phase, and the appearance of a phosphorylated band in mitosis. Similar to Rat-1 cells, MEFs display comparable expression patterns for FoxM1 [4]. Thus, although FoxM1 is only present in cycling cells, it is expressed as early as late G1, while many of its target genes are induced in G2. Therefore, FoxM1 activity must somehow be inhibited during early S-phase and mitotic exit in order to prevent the unscheduled expression of mitotic proteins in S and G1. In this study, we set out to study this aspect of FoxM1 function through analysis of the regulation of FoxM1 at the post-transcriptional level during an ongoing cell cycle.

Results.

FoxM1 protein levels vary during the cell cycle.

In order to examine regulation of FoxM1 activity throughout the cell cycle, we set out to examine FoxM1 expression levels at different phases of the cell cycle in human cells. U2OS osteosarcoma cells that have been synchronized for 24 hrs at the G1/S transition by thymidine treatment do express FoxM1 to significant levels (Figure 1A). However, FoxM1 protein levels increase as the cells are released from the thymidine block, suggesting that FoxM1 transcription is further activated as cells progress through S-phase. Furthermore, FoxM1 is hyperphosphorylated during mitosis, as evidenced by western-blot by the appearance of a slower migrating band 10 hrs after release (Figure 1A). This corresponds to a time when most cells are in G2 (data not shown). This shifted band is also present in extracts prepared from cells trapped in mitosis by nocodazole treatment (Figure 1B) and is indeed due to phosphorylation since phosphatase treatment prevents this shift (Figure 1). This confirms earlier studies showing that FoxM1 is phosphorylated during mitosis [3]. Interestingly, a minor shifted form of FoxM1 is also seen in thymidine-blocked cells (Figure 1), suggesting that phosphorylation of FoxM1 is a multistep process that starts as early as late G1, as already proposed in [7].

FoxM1 transcriptional activity varies during cell cycle progression.

Parallel to variations and modifications of the FoxM1 protein during cell cycle progression, one might expect FoxM1 transcriptional activity to be modified accordingly. In order to address this issue, we have used the 6xDB Forkhead consensus reporter construct [4] and analyzed transactivation by FoxM1 in different phases of the cell cycle. For this purpose, U2OS cells were synchronized with thymidine, and subsequently allowed to resume cell cycle progression after thymidine block release. There was a clear increase in FoxM1 transactivation between 10 and 14 hrs after release (Figure 2A), which corresponds to a time where the majority of cells are in G2 (data not shown). This suggests that FoxM1 is specifically activated in G2, even when it is constitutively expressed throughout the cell cycle. Interestingly, we could also observe a mild induction in 6xDB promoter activity between 10 and 14 hrs after release in the absence of overexpressed FoxM1 (Figure 2A), possibly due to endogenous FoxM1 activity present in these cells. Similar induction of promoter activity in G2 was observed using the CENP-F promoter (Figure 2A), an established in vivo target of FoxM1 [4]. Likewise, blocking cells at different stages of the cell cycle using different synchronizing agents confirmed that FoxM1 activity is highest in G2 (Figure 2B). In the absence of serum, causing cells to arrest

in G0, FoxM1 transcriptional activity was very low (Figure 2B). In contrast, treatment of cells with nocodazole, which blocks cell cycle progression in G2 and mitosis, had a slightly stimulating effect on FoxM1 activity (Figure 2B), confirming that FoxM1 is indeed active in G2. In contrast, thymidine (Figure 2B) and aphidicolin (data not shown), two drugs that block DNA cells in S-phase, caused an inhibition in FoxM1 activity. Taken together, our data suggest that although FoxM1 is expressed in late G1- and S-phases, its activity is somehow restricted to G2. This finding is further strengthened by our finding that forced overexpression of FoxM1 in resting cells, or cells arrested at the G1/S transition does not result in full transcriptional activation.

Regulation of FoxM1 activity by Cyclin A.

The mechanism regulating cell cycle progression is based on the interaction of the Cdk kinases with their cyclin partner. FoxM1 has been shown to interact with cyclin/Cdk complexes through an LXL motif present in its C-terminal region [7]. Such an interaction was shown to be important for proper Cdk-dependent phosphorylation of FoxM1 on residue T596 and subsequent recruitment of the transcriptional co-activator p300, and thus required for FoxM1 transcriptional activity [7]. Therefore, we wanted to address the effect of blocking cyclin-dependent kinase activity on the function of FoxM1. Expression of the Cdk-inhibitors p16 and p21 efficiently blocked transactivation by FoxM1 (Figure 3A), similarly as in cells chemically-blocked in G1 (Figure 2B). To extend these observations, we tested the effect of dominant-negative Cdk-mutants on FoxM1 transactivation. Expression of a dominant negative form of Cdk2 caused a dramatic reduction in FoxM1 activity (Figure 3B), consistent with the effect of the Cdk-inhibitors p16 and p21. Similarly, expression of a dominant-negative version of Cdk1 (or Cdc2) also caused a similar reduction in FoxM1 activity (Figure 3B). Interestingly, the effect of dominant-negative Cdk1 was reverted when Cyclin A was co-expressed, while Cyclin A overexpression was unable to revert the effect of dominant-negative Cdk2 (Figure 3B). The mechanism underlying this difference is difficult to understand, as these dominant negative mutants act via competition with the endogenous proteins and therefore might disrupt multiple cyclin/Cdk complexes. Nonetheless, these data suggest that the effect of Cdk1 on FoxM1 transactivation is mediated via Cyclin A. Therefore, we tested the requirement for Cyclin A in transactivation by FoxM1 by knocking-down endogenous Cyclin A by means of RNAi. Depletion of Cyclin A was able to cause a very significant inhibition of FoxM1 transcriptional activity, although slightly less than dominant-negative Cdk2 (Figure 3C). This confirms that Cyclin A plays a major role in FoxM1 activation, although the involvement of additional cyclin/Cdk complexes cannot be excluded.

Cyclin/Cdk complexes bind directly and phosphorylate FoxM1.

As mentioned before, FoxM1 has been shown to interact physically with cyclin/Cdk complexes through an LXL domain in the C-terminal region of FoxM1 [7]. Interestingly, cyclin binding consensuses are also present in the very N-terminal region of FoxM1 (see Chapter 4, Figure 1). Therefore, we tested if cyclins could directly bind to the N-terminus of FoxM1. Indeed, we find that the N-terminal domain of FoxM1 binds to Cyclin A and Cyclin B1, but has no appreciable affinity for Cyclin E (Figure 4A). In order to examine if this interaction could result in direct phosphorylation of FoxM1, we performed kinase assays using GST–FoxM1 variants as bait. We find that Cyclin A can phosphorylate both N-terminal and C-terminal regions of FoxM1 (Figure 4B), while Cdk2, one of the interaction partners of Cyclin A, only phosphorylates the C-terminal fragment (Figure 4C). This suggests that the N-terminus of FoxM1 is phosphorylated by Cyclin A in complex with Cdk1. Moreover, Cyclin B1, which forms complexes with Cdk1 only, could bind and phosphorylate the N-terminal region of FoxM1, albeit weakly (Figure 4D). Thus, it seems likely that Cyclin A/Cdk2 complexes phosphorylate FoxM1 in its C-terminal region whereas Cyclin A/Cdk1 complexes bind and phosphorylate the N-terminal part of FoxM1.

The N-terminal domain of FoxM1 acts as

transcriptional repressor.

As the N-terminus of FoxM1 appears to be phosphorylated by Cyclin A-containing complexes, and Cyclin A is required to activate FoxM1, we wondered what the contribution of the N-terminus was to transcriptional activation by FoxM1. Interestingly, deletion of the N-terminal domain, right up to the start of the DNA binding domain (ΔN-FoxM1) resulted in a protein with increased activity (Figure 5A). In addition, transactivation by this mutant was not greatly affected by the introduction of dominant negative Cdk1 (Figure 5A) Moreover, ΔN-FoxM1 activity was not greatly affected upon synchronization of cells by serum-starvation or by the addition of thymidine (Figure 5B). These data indicate that the N-terminus of FoxM1 carries repressor activity, which can be relieved through the action of Cdk1, likely in complex with. Cyclin A. This would cause FoxM1 activity to be high under conditions of high Cyclin A/Cdk1 activity, timing of which coincides with G2. Obviously, it is hard to completely rule out Cyclin A/Cdk2 complexes in this respect, but it should be noted that Cyclin A/Cdk2 activity is extremely high in thymidine-blocked cultures (data not shown), while FoxM1 activity is inhibited during a thymidine block. Thus, although Cyclin A/Cdk2 can contribute to activation of FoxM1 [7], this points to the existence of additional mechanisms that control transactivation by FoxM1, allowing a tight restriction of FoxM1 transactivation to the G2 phase of the cell cycle.

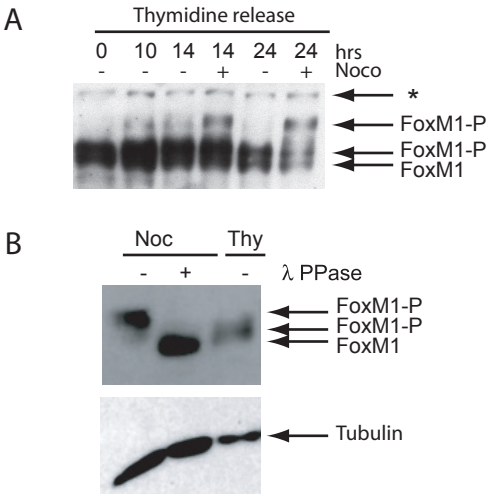

Figure 1. FoxM1 is expressed in G1, accumulates as cells progress through the cell cycle and is phosphorylated in G2/M. (A) U2OS osteosarcoma cells were synchronized at the G1/S transition by thymidine treatment and allowed to progress. Endogenous FoxM1 protein levels in these cells are shown by western-blot. (B) U2OS cells were blocked at the G1/S transition by thymidine treatment, or in G2/M by nocodazole treatment. The nocodazole-blocked cell lysates were then treated or not by the λ phosphatase. Endogenous FoxM1 protein levels were viewed by western-blot.

FoxM1 is degraded during mitotic exit.

Our data show that FoxM1 protein levels and activity are low in early G1. This strongly suggests that there may be mechanism in place that actively act to shut down FoxM1 activity, and thus the expression of its target genes, during the G1-phase of the cell cycle. One likely option is active degradation of FoxM1 during the G1-phase of the cell cycle. In order to investigate FoxM1 protein stability, we treated U2OS cells with the protein synthesis inhibitor Cycloheximide (CHX) at different stages in the cell cycle. In cells blocked in mitosis by nocodazole treatment, or in cells blocked at the G1/S transition by thymidine treatment, FoxM1 appears to be stable in contrast to asynchronously growing cells (Figure 6A). Thus, the FoxM1 protein is stabilized at the G1/S transition, and during mitosis, but must be significantly less stable in one or more other phases during an ongoing cell cycle.

A well-known mechanism that leads to the downregulation of mitotic proteins is the proteasome-mediated protein degradation during mitotic exit [8]. In order to determine whether FoxM1 is degraded during mitotic exit, we analyzed FoxM1 protein levels during mitotic exit. For this purpose, nocodazole-arrested mitotic cells were released from the block and allowed to enter the next G1. As shown in Figure 6B (upper left panel), we could observe two processes. First, the endogenous phosphorylated band of FoxM1 starts disappearing during exit from mitosis as early as 1 hour after nocodazole

release. Second, overall endogenous FoxM1 protein levels decreased after 3 hours of release. These time-points correspond well with mitotic exit as demonstrated by the decrease in Cyclin B1 protein levels (Figure 6B, middle left panel) and the mitotic marker phospho-Histone H3 (Figure 6B, lower left panel). Importantly, FoxM1 degradation as well as Cyclin B1 degradation and H3 phosphorylation were rescued by treatment with MG132, suggesting that the decrease in FoxM1 protein levels is mediated by the proteasome. In order to confirm this, HA-tagged Ubiquitin was also transfected in these cells. We observed that FoxM1 is increasingly ubiquitinated during mitotic exit (Figure 6B, right panel), supporting the hypothesis that FoxM1 is degraded during mitotic exit in a proteasome-dependent manner.

FoxM1-GFP is degraded after the onset of anaphase.

We next constructed a FoxM1-GFP fusion, to allow us to analyze FoxM1 degradation in living cells. The resulting FoxM1-GFP fusion protein appeared to be functional, as demonstrated by its transactivation properties, when compared to a HA-tagged form of FoxM1 that we routinely use in our laboratory (Figure 7A). In a luciferase assay using the 6xDB reporter for Forkhead transcription factor activity, transactivation by FoxM1-GFP was about 2-fold, and about 6-fold upon Cyclin A co-expression. Furthermore, it was completely repressed when co-expression of a dominant-negative form of Cdk2, in the same manner as HA-FoxM1 (Figure 7A).

The FoxM1-GFP construct was next transfected in U2OS cells, cells were synchronized with thymidine, and analyzed using time-lapse microscopy after release from the block. FoxM1-GFP levels rise steadily as cells progress to mitosis (data not shown), possibly as a consequence of continued *de novo* synthesis of FoxM1 driven by the ectopic CMV-promoter. However, FoxM1-GFP fluorescence clearly starts to decrease at the onset of anaphase and this process continues as cells complete cell division. About 105 min after anaphase onset, the cells have entered the next G1, and the initial fluorescence decreased by about 60%, indicating that the majority of the protein is degraded upon mitotic exit (Figure 7B).

During mitosis, the major E3-ligase activity is the APC/C. This big protein complex can be activated by either activating subunits Cdc20 or Cdh1, which associate successively with

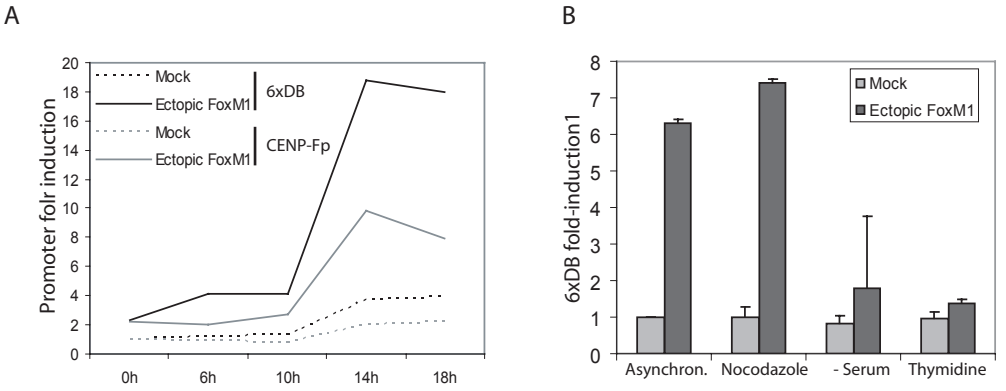

Figure 2. FoxM1 transcriptional activity during the cell cycle was analysed by expressing the Forkhead 6xDB luciferase reporter construct together with the renilla reference construct in U2OS cells. (A) U2OS cells were synchronized in G1/S as described in Figure 1A, and allowed to resume cell cycle progression. Transactivation of the 6xDB construct (black lines) and of the CENP-F promoter (CENP-Fp) (grey lines) by endogenous FoxM1 (Mock, dashed lines) or by ectopic FoxM1 (full lines) were measured after release at the times indicated on the X axis. (B) Transactivation of 6xDB by FoxM1 was measured in U2OS cells that were either asynchronously growing (Asynchron.), serum-deprived (- Serum) or synchronized by Nocodazole or Thymidine treatment.

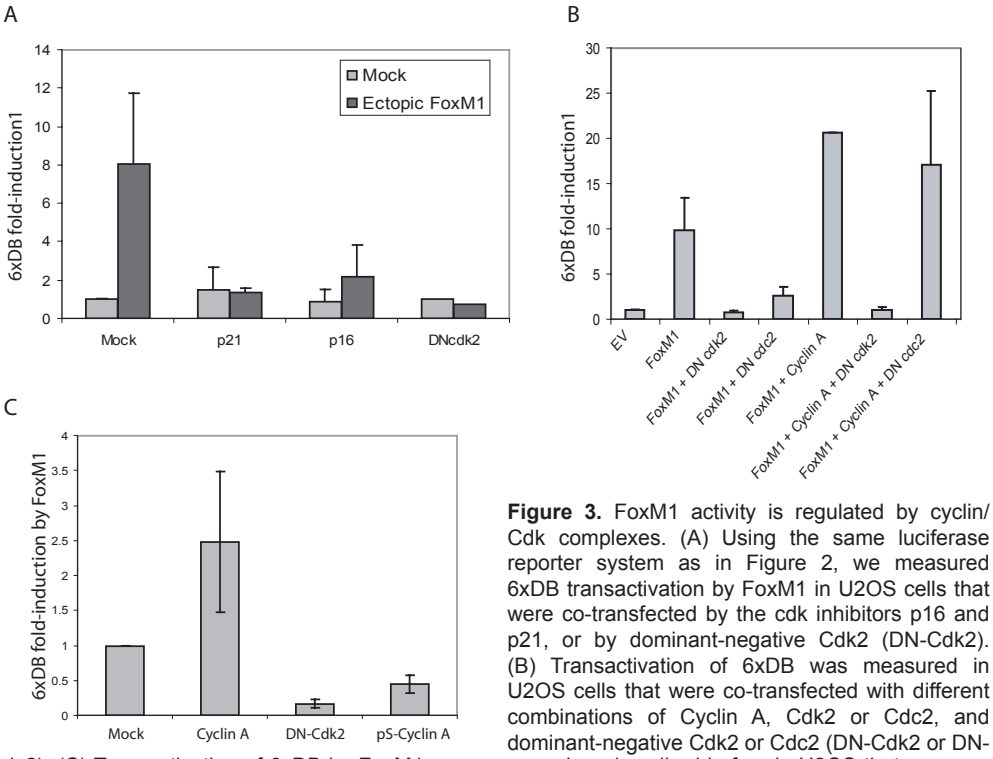

Figure 3. FoxM1 activity is regulated by cyclin/Cdk complexes. (A) Using the same luciferase reporter system as in Figure 2, we measured 6xDB transactivation by FoxM1 in U2OS cells that were co-transfected by the cdk inhibitors p16 and p21, or by dominant-negative Cdk2 (DN-Cdk2). (B) Transactivation of 6xDB was measured in U2OS cells that were co-transfected with different combinations of Cyclin A, Cdk2 or Cdc2, and dominant-negative Cdk2 or Cdc2 (DN-Cdk2 or DN-Cdc2). (C) Transactivation of 6xDB by FoxM1 was measured as described before in U2OS that were co-transfected with Cyclin A, dominant-negative Cdk2 (DN-Cdk2) or an RNAi-expressing construct targeting Cyclin A mRNA (pS-Cyclin A).

the APC/C in a cell cycle-dependent manner [8]. These two subunits are thought to direct the APC/C to distinct subsets of protein targets that need to be degraded. Cdc20-regulated events take place before anaphase onset, and particularly at metaphase (inactivation of the mitotic spindle checkpoint), whereas Cdh1-regulated events take place from anaphase on, until G1-phase. The fact that FoxM1 degradation starts at the onset of anaphase is suggestive of degradation mediated by APC^{Cdh1}. Therefore, we quantified FoxM1-GFP degradation in cells depleted of Cdh1, using the knock-down construct pS-Cdh1. In Cdh1-depleted cells, FoxM1 protein degradation appeared to be blocked (Figure 7C), strongly suggesting that FoxM1 degradation is indeed mediated by the APC/C^{Cdh1}.

To extend these observations to endogenous FoxM1, we either depleted Cdh1 or overexpressed Cdh1 in asynchronously growing cells (Figure 7D). Clearly, our pS-Cdh1 construct was able to knock-down endogenous as well as exogenous Cdh1 protein levels (Figure 7D, lower panel), and to induce a delay in mitosis (Figure 7D, table: second and fifth columns). However, Cdh1 knock-down did not seem to cause an increase in endogenous FoxM1 protein levels as we would have expected (Figure 7D, upper panel, third, fourth and fifth lanes). Also, analysis of endogenous FoxM1 degradation during mitotic exit showed no significant change in cells depleted of Cdh1 (Figure 7E, upper panel), although Cdh1 was clearly knocked down (Figure 7E, middle panel), and one of its known targets, Aurora-A, was stabilized (Figure 7E, lower panel). These data suggest that Cdh1-independent mechanisms to degrade FoxM1 must exist. However, overexpression of Cdh1, did lead to a clear reduction in FoxM1 protein levels

(Figure 7D, upper panel), indicating that Cdh1 does play a role in the stability of FoxM1. This was not due to an indirect effect of Cdh1 overexpression on cell cycle progression, since overexpression of Cdh1 did not affect the cell cycle distribution of asynchronously-growing U2OS cells (Figure 7D, table: third and fourth columns).

It is now well-established that Cdh1 recruits its target proteins to the APC/C through the recognition of a specific signal called the KEN box, accompanied or not by a D-box [9]. Sequence analysis of different FoxM1 homologues showed that FoxM1 contains a conserved KEN box, as well as several putative D-boxes, in the N-terminal part of the protein (see Chapter 4, Figure 1). This finding underscores FoxM1 protein as a putative APC^{Cdh1} target. Therefore, we decided to mutate the KEN box **to** KAA (as described in [10]) in the FoxM1-GFP construct. FoxM1-(KAA)-GFP transactivation of 6xDB was slightly higher but showed a similar behaviour as wild-type FoxM1-GFP with respect to regulation by Cyclin A and DN-Cdk2/Cdk2 regulation (Figure 7F). Interestingly, mutation of the KEN-box was able to stabilize FoxM1 protein during mitotic exit, as determined by time-lapse analysis (preliminary results, data not shown). Furthermore, using the same experimental set-up as in Figure 6B, we compared the degradation of the wild-type and KEN-box mutant of FoxM1-GFP during mitotic exit (Figure 7G). The FoxM1 KEN-box mutant seems to be stabilized during mitotic exit, as compared to FoxM1-GFP, since expression is elevated at 2 hours release from the nocodazole block. This suggests that inactivation of the KEN-box prevented FoxM1 degradation during mitotic exit and that FoxM1 degradation may indeed be Cdh1-dependent. Consistent with this notion is our observation

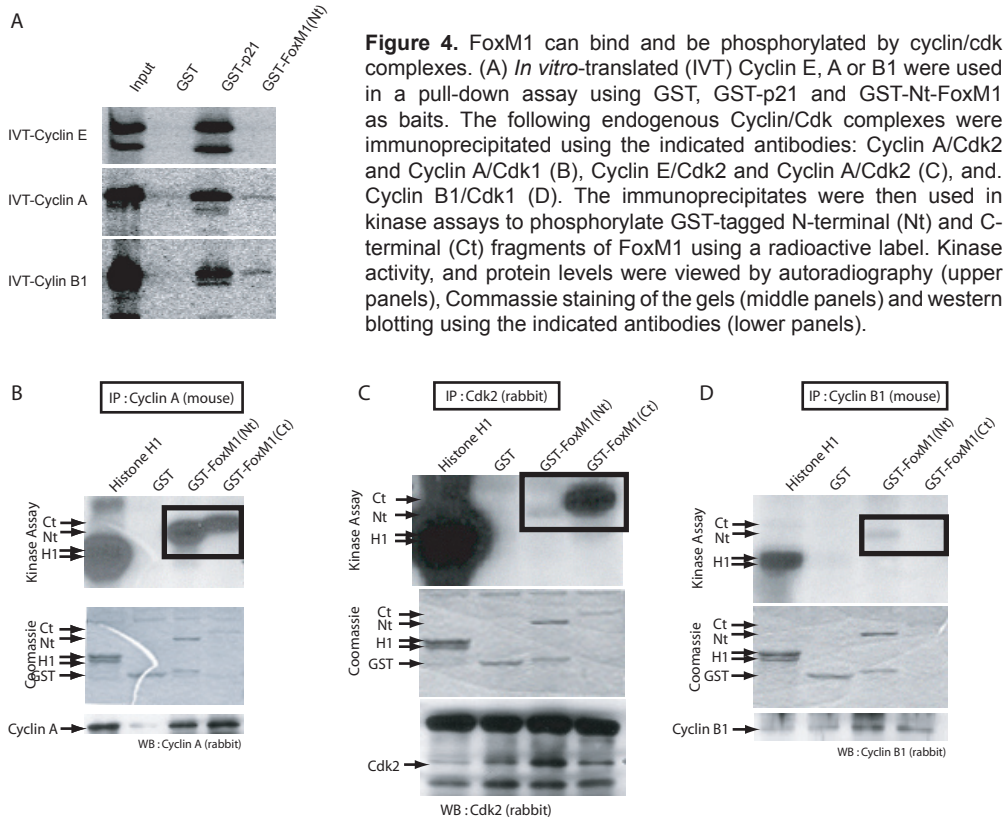

Figure 4. FoxM1 can bind and be phosphorylated by cyclin/cdk complexes. (A) *In vitro*-translated (IVT) Cyclin E, A or B1 were used in a pull-down assay using GST, GST-p21 and GST-Nt-FoxM1 as baits. The following endogenous Cyclin/Cdk complexes were immunoprecipitated using the indicated antibodies: Cyclin A/Cdk2 and Cyclin A/Cdk1 (B), Cyclin E/Cdk2 and Cyclin A/Cdk2 (C), and. Cyclin B1/Cdk1 (D). The immunoprecipitates were then used in kinase assays to phosphorylate GST-tagged N-terminal (Nt) and C-terminal (Ct) fragments of FoxM1 using a radioactive label. Kinase activity, and protein levels were viewed by autoradiography (upper panels), Commassie staining of the gels (middle panels) and western blotting using the indicated antibodies (lower panels).

that expression of the KEN-box mutant is not elevated by treatment with MG132, indicating this mutant is indeed not sensitive to proteasome-dependent degradation (Figure 7G).

Discussion.

In this study, we aimed to gain more insight on how FoxM1 activity is regulated during an ongoing cell cycle. We show that FoxM1 is already expressed in late G1- and in S-phase, but its transcriptional activity is kept low until entry in G2. Based on these observations, we wanted to address the following two questions: 1) what triggers FoxM1 activation in G2/M, and 2) what happens with FoxM1 during mitotic exit and early G1 in cycling cells.

First, we have studied the regulation of FoxM1 transcriptional activity during cell cycle progression. We found that FoxM1 is active in cells blocked in G2/M, corresponding to a time in the cell cycle during which FoxM1 is hyperphosphorylated. Indeed, several conserved putative phosphorylation sites for kinases that are active in G2/M such as cyclin/cdk complexes and Plk-1 can be identified in FoxM1. In fact, Major and coworkers have previously shown that cyclin/cdk complexes can bind FoxM1 in order to phosphorylate its residue T596, which is critical for recruitment of the transcriptional co-activator p300 and thus for FoxM1 transcriptional activity [7]. Our data indicate that Cyclin A plays an important role in the regulation of FoxM1 transcriptional activity, as Cyclin A can bind directly to FoxM1, and promote its phosphorylation. Moreover, removal of Cyclin A through RNAi-mediated depletion leads to a strong reduction in FoxM1 activity, similar to what is observed after expression of dominant negative versions of Cdk1 and Cdk2. Obviously, it is difficult to discriminate which Cyclin/Cdk complex is most crucial for FoxM1 activation on the basis of these observations. However, the timing of FoxM1 activation corresponds to a time in the cell cycle where Cdk1 is active. Because Cyclin E/Cdk2 and Cyclin A/Cdk2 activities are limited to the S-phase of the cell cycle, they may not be sufficient for full activation of FoxM1, but could prime FoxM1 for eventual activation by Cyclin A/Cdk1 complexes in

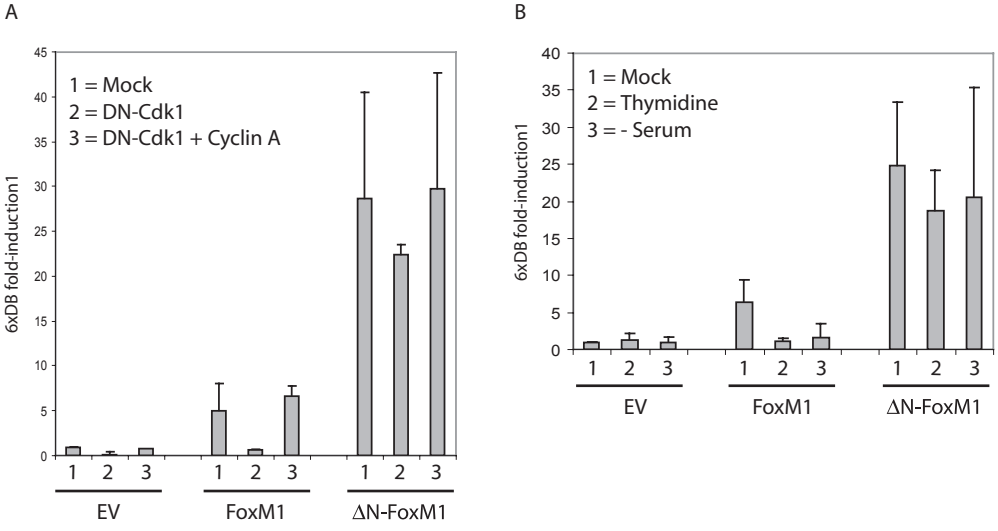

Figure 5. FoxM1 N-terminal region contains a repressor function. (A) Using luciferase reporter assay as in Figure 2, transactivation of 6xDB by FoxM1 or ΔN-FoxM1 were measured in U2OS cells co-expressing dominant-negative Cdk1 (DN-Cdk1) alone or together with Cyclin A. (B) Similarly, transactivation of 6xDB by FoxM1 or ΔN-FoxM1 were measured in U2OS cells blocked in G1/S by thymidine treatment or by serum starvation (- Serum).

G2. Interestingly, we find that the N-terminal domain of FoxM1 can be phosphorylated by Cyclin A-containing complexes, yet this domain is not readily phosphorylated by Cdk2. Thus, it seems most likely that Cyclin A/Cdk1 complexes are responsible for phosphorylation of the N-terminus. This could directly impact on FoxM1 transactivation, as we could show removal of the N-terminus leads to a hyperactive form of FoxM1 that can no longer be efficiently inhibited by dominant-negative Cdk1 or G1/S synchronizing agents. Therefore, our data are most consistent with a model in which FoxM1 activity is kept low due to the negative action of its own N-terminal domain, while this repressive function can be relieved through direct phosphorylation by Cyclin A/Cdk1. This hypothetical model of FoxM1 regulation displays striking similarities to what has been proposed previously for B-Myb, a completely unrelated transcription factor that is involved in the regulation of genes that are specifically expressed in the G2-phase [11, 12]. B-Myb is dependent on activation by Cyclin A/Cdk complexes, and this activation relieves an autorepressive function present within the C-terminus of B-Myb. This suggests a common regulatory system is responsible for the activation of diverse transcription factors that regulate the transcription of genes during the G2 phase of the cell cycle. However, the repressive function present in FoxM1 N-terminal region may also be due to interaction with inhitory partners such as the pocket protein pRb, as suggested previously [7]

In addition to this, we have addressed the fate of FoxM1 during mitotic exit. Because FoxM1 stimulates the expression of genes that are important for G2/M progression, it seems critical that FoxM1 is inactivated during mitotic exit in order to prevent the presence of active FoxM1 in G1. We have observed that, as cells progress from mitosis to the next G1-phase, the hyperphosphorylated form of FoxM1 is rapidly removed. At this stage, we do not know the impact of this event on FoxM1 function, but it seems likely that dephosphorylation

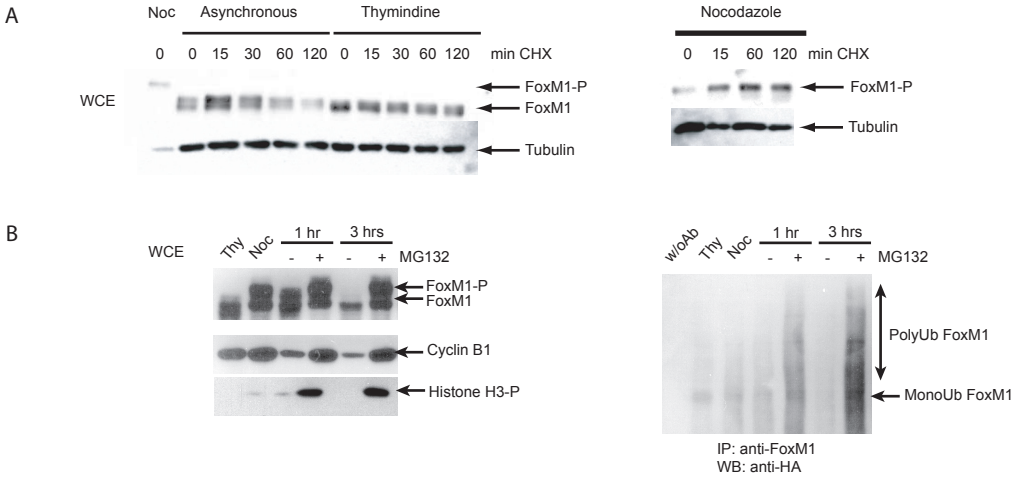

Figure 6. FoxM1 protein stability during the cell cycle. (A) U2OS cells that are asynchronously growing, or blocked in G1/S or G2/M by thymidine or nocodazole treatments respectively were treated for the indicated periods of time by the translation inhibitor Cycloheximide (CHX), and endogenous FoxM1 (upper panels) and tubulin (lower panels) protein levels were viewed by western-blot. (B) HA-Ubiquitin was transfected in U2OS cells, which were subsequently synchronized by nocodazole treatment and allowed to resume cell cycle progression in the presence or absence of the proteasome inhibitor MG132 for the indicated periods of time. Endogenous FoxM1 protein levels were then analysed by western-blot (upper left panel). Similarly, endogenous Cyclin B1 (middle left panel) and phosphorylated histone H3 (lower left panel) levels were assessed. In parallel, immunoprecipitation using an anti-FoxM1 antibody were performed in these cell extracts, and HA-ubiquitin levels were determined by western-blotting with anti-HA antibody (right panel).

of the residues phosphorylated by Cyclin/Cdk complexes leads to inactivation of FoxM1. Interestingly, in yeast, the mitotic exit network (MEN) promotes mitotic exit through activation of the phosphatase Cdc14 [13]. Ccd14 homologues have been identified in mammalian cells, but their exact role is still elusive. Thus, it would be interesting to address whether dephosphorylation of FoxM1 could be an active Cdc14-dependent process, or whether it occurs as a consequence of the default phosphatase activity after silencing of Cdk kinase activity in late mitosis.

Besides the rapid reduction in the phosphorylated forms of FoxM1, we also found the protein to be actively degraded by the proteasome at anaphase onset. This active degradation removed up to 60% of the protein during progression from anaphase to G1. Our data suggest that this occurs, at least partly, in a Cdh1-dependent manner, as knocking-down Cdh1 by means of a RNAi construct could prevent this degradation. Moreover, overexpression of Cdh1 resulted in a dramatic reduction of FoxM1 protein levels. In further support of Cdh1-dependent FoxM1 degradation is our observation that a KEN-box mutant of FoxM1 is stabilized. Thus, it seems that inactivation of FoxM1 before entry in G1 may require several complementary mechanisms: protein degradation through APC/Cdh1-dependent ubiquitination, and active inactivation of the remaining protein by dephosphorylation.

Unfortunately, although our data on FoxM1 degradation seem consistent with a main role for Cdh1 in this process, we were never able to observe stabilization of endogenous FoxM1 upon Cdh1 knock-down. This strongly suggests that redundant (Cdh1-independent) pathways may exist that can promote degradation of FoxM1 in early G1. Thus, while reducing Cdh1 in cells overexpressing FoxM1-GFP clearly does lead to a limitation in total FoxM1 turnover, this elicits no difference in situations where only endogenous FoxM1 protein is present. This suggests that the redundant pathway(s) that promote FoxM1 degradation during mitotic exit are not able to compensate for Cdh1 loss in cells expressing high levels of FoxM1. One way to strengthen our hypothesis that FoxM1 degradation is Cdh1-dependent would be to show that FoxM1 can bind the APC, possibly in complex with Cdh1. If not, then it would be interesting to address the other potential mediators of FoxM1 protein degradation, such as the SCF.

Materials and methods.

Cell culture.

U2OS cells were maintained in DMEM medium supplemented with 10% FCS and antibiotics. Thymidine, nocodazole and MG132 were added at a final concentration of 2.5 mM, 250 ng/ml and 5 μM respectively, and were all purchased from Sigma.

Plasmids and oligonucleotides.

HA-FoxM1, pS-FoxM1, 6xDB and pCENP-Fp have been described previously [4]. GST, GST-Nt-FoxM1 and GST-Ct-FoxM1 constructs were from W. Korver, and corresponding proteins were purified from bacterial cultures. GFP-FoxM1 was obtained by sub-cloning the complete FoxM1 cDNA insert (BamH1 restriction fragment) from pcDNA3-FoxM1-ER (described in [4]) into a peGFP-N1 vector (Clontech). FoxM1(KAA)-GFP was obtained by site-directed mutagenesis using the following

Figure 7. FoxM1 is degraded during cell cycle exit. (A) We constructed a GFP-tagged wild-type FoxM1 expression construct and analysed its transcriptional activity using the same setting as in Figure 2E. (B) U2OS cells expressing FoxM1-GFP were analysed by time-lapse microscopy. Total green fluorescence of the cells was measured every 3 minutes, corrected for background fluorescence value, and all the results were plotted on a graph. (C) A similar experiment was performed as in Figure 5B, in U2OS cells co-expressing the RNAi-expressing construct targeting Cdh1 mRNA (pS-Cdh1). (D) Asynchronously growing U2OS cells were transfected with empty vector pSuper, RNAi-expressing construct targeting Cdh1 (pS-Cdh1) or FoxM1 (pS-FoxM1) mRNAs, or with the HA-tagged Cdh1 expression construct (HA-Cdh1). Endogenous protein levels of FoxM1 and endogenous and exogenous protein levels of Cdh1 were assessed by western-blotting. In parallel, the cells of each sample were analysed by FACS and the percentage of cells in each phase of the cell cycle was determined as indicated below the western-blot. (E) U2OS cells expressing either the empty vector pSuper (left panels) or the Cdh1-targeting construct pS-Cdh1 (right panels) were synchronized by nocodazole treatment and released using a similar experiment set-up as in Figure 4B. Endogenous protein

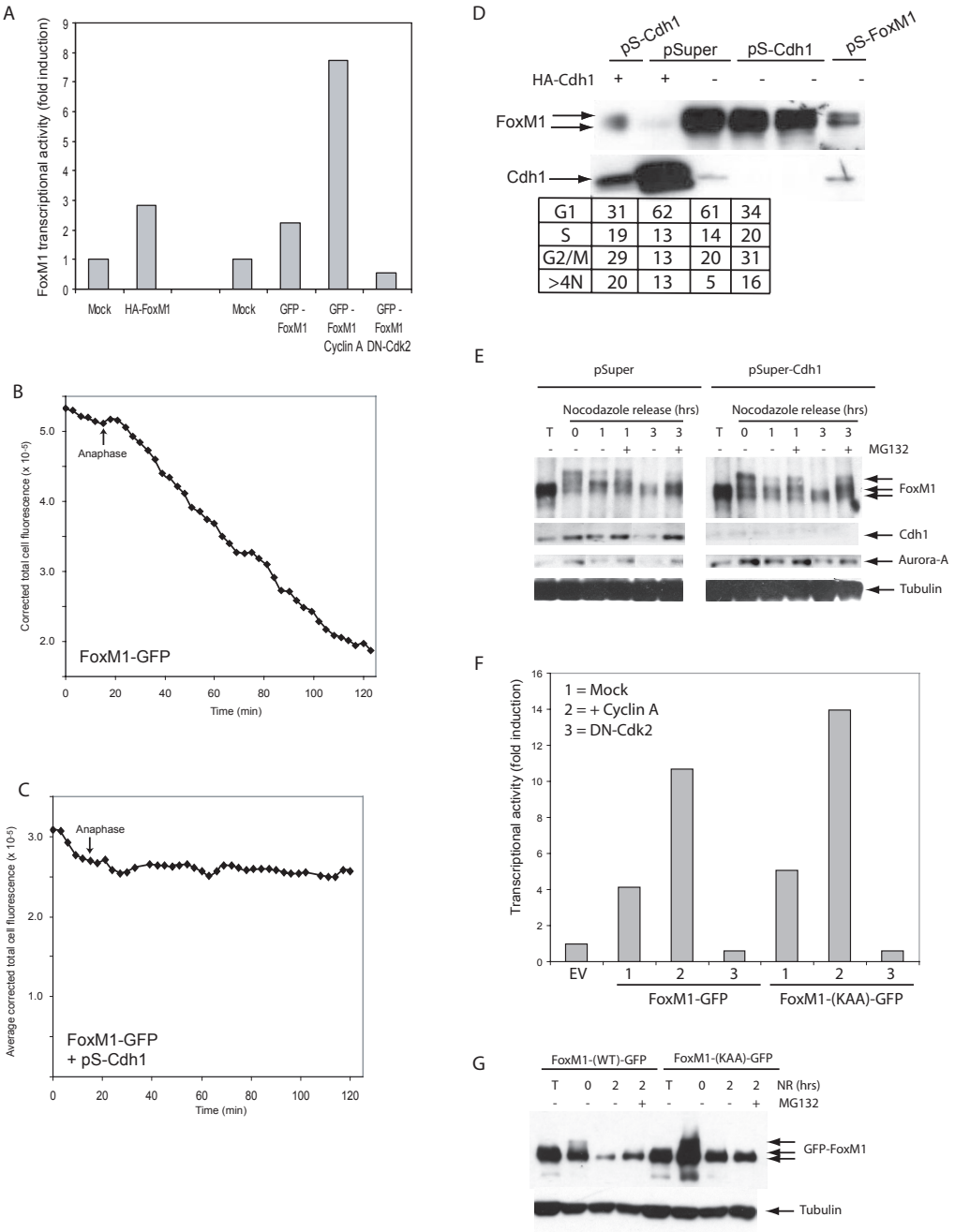

Figure 7 (suite)- levels of FoxM1, Cdh1, Aurora-A and tubulin were determined by western-blotting. (F) We constructed a KEN-box mutant (KAA) GFP-FoxM1 expression construct and analysed its transcriptional activity using the same setting as in Figure 2E and compared it to wild-type GFP-FoxM1. (G) U2OS cells expressing either the wild-type (WT) (left panel) or the KEN-box mutant (KAA) (right panel) forms of GFP-tagged FoxM1 were synchronized and released as in Figure 4B. Exogenous levels of FoxM1 were determined by western-blotting, using an anti-GFP antibody.

primer set:
5'-GAACTGGAAGAGCGGGCGGCTTGTCACCTG GAG-3'
5'-CTCCAGGTGACAAGCCGCCCGCTCTTCCAG TTC-3'.
Correctly mutated plasmids were identified through direct sequence analysis.
Expression constructs for Cyclin A, Cyclin B1, Cyclin D1, Cyclin E, Cdk1/Cdc2, Cdk2 were all described previously [4]. pSuper and pS-Cdh1, pS-Cyclin A, HA-Cdh1, Histone H2B-dsRed and HA-Ubiquitin, and DN-Cdk2 and DN-Cdk1 were kind gifts from R. Agami, M. van Vugt, R. Wolthuis and S. van de Heuvel respectively.

Reporter assays.
Cells were transfected using the standard calcium phosphate transfection protocol. Luciferase activity was determined 48 hours after transfection, using the Dual luciferase kit (Promega) according to the manufacturer's instructions. Relative luciferase activity was expressed as a ratio of firefly luciferase activity to control Renilla luciferase activity.

Antibodies.
The following primary antibodies were used: anti-Aurora-A and mouse anti-Cdh1 from Neomarker; rabbit anti-Cdk2, anti-Cyclin A, anti-Cyclin B1, anti-Cyclin E, rabbit anti-FoxM1 and rabbit anti-HA from Santa Cruz; biotinylated rat anti-HA from Roche; rabbit anti-phospho-Histone H3 from Upstate Biotech, and rabbit anti-tubulin from Sigma. The following secondary antibodies were used: goat anti-rabbit/Alexa 488 was from Molecular Probes and peroxidase-conjugated goat anti-rabbit and goat anti-mouse were from DAKO.

Western blot, FACS and immunofluorescence analyses.
Western blot analysis was performed as described [5]. For IP/Kinase assays, cells were lysed in ELB and 100 µg of protein was used for immunoprecipitation with the appropriate antibody coupled to protein A/G agarose beads. Immunoprecipitates were extensively washed and incubated in kinase buffer (Hepes pH7.5 50 mM, $MgCl_2$ 5 mM, $MnCl_2$ 2.5 mM, DTT 1 mM): with 1 µl of substrate (Histone H1, GST, GST-Nt-FoxM1 or GST-Ct-FoxM1), 50 mM cold ATP, and 2.5 µCi ^{32}P-µATP for 30 min at 30°C. Samples were then denatured in sample buffer and loaded on SDS-PAGE. Results were visualized by Coomassie blue staining of the gel followed by autoradiography. FACS analysis was performed as described [4].

Time-lapse microscopy.
Time-lapse analysis was performed essentially as described [6]. Briefly, cells plated on 35 mm glass-bottom culture dishes (Willco-Wells, Amsterdam, the Netherlands) were transfected with FoxM1-GFP or FoxM1(KAA)-GFP together with pSuper or pS-Cdh1 in combination with Histone H2B-dsRed. 12 hours after thymidine release, dishes were transferred to a heated culture chamber (37°C, 5% CO_2) on a Zeiss Axiovert 200M microscope equipped with a 40x Plan-Neo DIC 1.3 N.A. objective. Images were taken every 3 minutes and further processed using MetaMorph software (Universal Imaging).

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Discussion

Discussion.

In yeast, it is long known that timed waves of transcription regulate cell cycle progression. The object of this thesis was to study the similar regulatory mechanisms that exist in mammalian cells. We have mainly focused our attention on transcriptional regulation of cell cycle entry/exit (G0-G1 transition) (Chapters 1 to 3) as well as on transcriptional regulation of the coordination between DNA replication (S-phase) and cell division (M-phase) (Chapters 4 and 5). More specifically, in the first part of this thesis, we have addressed the mechanisms by which cell cycle exit can be induced in inadequately proliferating T cells such as leukaemic T cells, by means of cAMP signalling and/or activation of FoxO Forkhead transcription factors. In the second part, we have studied the mechanisms of regulation of FoxM1, another Forkhead transcription factor, which is known to be important for coordinating the S- (replication) and M-phases (cell division) of the cell cycle. Here, we discuss the implications of our findings.

It is known since a long time that natural molecules such as prostaglandin E2, or pharmacological compounds such as forskolin, increase cAMP levels in T cells and suppress T cell proliferation [1, 2]. Therefore, strategies to increase cAMP levels or activate the pathway downstream to cAMP have been developed in order to curb proliferation of inadequately activated T cells [3]. Our study (Chapter 2) describes the molecular mechanisms by which cAMP, through activation of its principal cellular receptor, protein kinase A (PKA), inhibits proliferation of leukaemic T cells. We have shown that PKA activates distinct pathways that lead to inhibition of Cyclin D3 and induction of $p27^{Kip1}$ expression and thereby, induce a cell cycle arrest in leukaemic T cells [4].

cAMP is known to interfere with Cyclin D and $p27^{Kip1}$ expression [5-8], thereby leading to general inhibition of G1 cyclin-cdk complexes and subsequent Rb activation and E2F sequestration. It was first proposed that this effect was mediated through PKA-mediated interference with MAPK signalling [9-13], which is known to induce Cyclin D and downregulate $p27^{Kip1}$ [14, 15]. However, the Ras/Raf/MAPK cascade alone cannot account for cAMP-mediated cell cycle arrest in G1, since long-term exposure of cells to elevated cAMP levels does not lead to inhibition of p44 MAPK [16]. Furthermore, expression of cAMP-insensitive versions of Raf do not prevent the cAMP-induced cell cycle arrest [17, 18]. Thus, cAMP may affect other pathways, such as PI3K, which is also activated downstream to growth factor receptors and known to regulate cell cycle progression. Indded, it was shown that cAMP has a direct effect on the membrane localization of p85 PI3K regulatory sub-unit, which is also required for proper phosphorylation of the membrane phospholipids and production of PIP2 and PIP3 [19]. Furthermore, one report suggested that cAMP inhibits PI3K lipid kinase activity, thereby decreasing the levels of the phospholipids PIP2 and PIP3 *in vivo*. PIP2 and PIP3 are critical for the membrane localization and subsequent activation of the serine/threonine kinases PDK1 and PKB, and as a consequence, PKB signalling is attenuated [20]. As PKB, through regulation of FoxO activity, is an important regulator of Cyclin D and $p27^{Kip1}$ (see below), this pathway could play an important role in the inhibitory response to cAMP.

In Chapter 3, we have studied the role of FoxO Forkhead transcription factors in the regulation of T cell cycle exit and apoptosis. We have shown that inhibition of FoxO transcription factors is important for T cell cycle progression and survival, and that this inhibition is mediated by the PI3K/PKB pathway downstream of the IL-2 receptor. Our data point at a role for FoxO transcription factors in the regulation of T cell cycle exit and propriocidal death upon IL-2 withdrawal, through the upregulation of $p27^{Kip1}$ and Bim genes. Furthermore, we have evidence to prove that FoxO inactivation is critical for cell cycle re-entry of quiescent T cells upon TCR ligation in the presence of proper co-stimulation [21].

In T cells, sustained PI3K signalling after antigen stimulation is important to initiate cell cycle progression. This can be achieved through recruitment of PI3K to the membrane,

in particular at the sitel of the immunological synapse [22]. Therefore, cAMP, which interferes with membrane localization of PI3K, PDK1 and PKB, may act by preventing stable PI3K signalling for long periods of time, which is necessary for sustained inhibition of FoxO transcription factors, during T cell activation. Thus, it would be interesting to verify if cAMP in T cells regulates Cyclin D and $p27^{Kip1}$ levels through FoxO activation as a consequence of interfering with the PI3K/PKB pathway. Interestingly, in MEFs, the PKB/FoxO pathway has been shown to contribute greatly to cAMP-induced cell cycle arrest [23]. Not only cAMP mediates Cyclin D1 downregulation in MAPK-dependent and –independent fashions, but it also potently increases $p27^{Kip1}$ levels through PKB inhibition and subsequent FoxO activation. Thus it would be interesting to address whether FoxO also regulates p27Kip1 and Cyclin D expression downstream to cAMP in lymphocytes. In lymphocytes, we have shown that cAMP represses Cyclin D3, while the cell types used to demonstrate regulation of Cyclin D by FoxO factors only express Cyclin D1 and/or D2 [23, 24]. Thus, the regulation of Cyclin D3 by FoxO factors was not resolved in these studies.

FoxO is considered as a potent protein for the induction of quiescence, notably in naïve T cells. Activation of FoxO is known to be critical for cell cycle exit to the G0-phase of the cell cycle, through repression of Cyclin D [24] and induction of p27Kip1 [25]. Furthermore, FoxO has been shown to regulate the expression of the pocket protein p130 [26]. Thus, FoxO is likely to play a role in both establishment and maintenance of a quiescent state in memory T cells, although this still needs to be addressed experimentally. Consistent with this hypothesis, FoxO has among its numerous target genes the transcriptional repressor Bcl-6 [27]. Bcl-6 is known to regulate differentiation of B- and T-cells. In particular, its expression is required for generation and maintenance of memory T cells [28]. Therefore, if FoxO should be proven to have a role in memory T cells, this may involve upregulation of Bcl-6.

We and others have shown that activated FoxO induces apoptosis through increasing Bim protein levels in various cell types, among which haematopoietic cells (Capter 3) [21, 29-34]. Other mechanisms account for FoxO-induced cell death, involving Fas ligand (FasL), and Bcl-6, which mediates repression of Bcl-XL, an anti-apoptotic member of the Bcl-2 family. The fact that FoxO induces cell death is quite puzzling as FoxO also emerges as an important protein with respect to induction and maintenance of quiescence as well as life-span/survival of quiescent cells. Although this may seem paradoxical, parallel mechanisms exist that prevent cell death in quiescent cells such as naïve and probably memory T cells. These mechanisms involve compensatory regulation of the expression of anti-apoptotic proteins to counteract the effect of FoxO-induced pro-apoptotic proteins. For example, it has been shown that Bcl-2 is expressed to relatively high levels in naïve and CD8+ memory T cells, and is required for quiescent naïve T cell survival [35, 36]. Conversely, Bcl2 levels drop dramatically upon TCR stimulation, and this is necessary to render activated T cells sensitive to activation-induced cell death [37]. In activated T cells, however, co-stimulation by CD28 or IL-2 through the PI3K/PKB pathway increases Bcl-XL levels, protecting them from propriocidal apoptosis [38, 39]. Furthermore, it has been proposed that certain cytokines are necessary for the survival and the homeostasis of these cells, for example, IL-7 and IL-15 [40]. It seems likely that this mechanism provides a low-level signalling sufficient to keep the cells alive although insufficient to induce their re-entry into the cell cycle.

In the second part of this thesis (Chapters 4 and 5), we have focussed on the Forkhead transcription factor FoxM1, which is known to play an important role in the regulation and coordination of replication and mitosis [41]. In particular, we have studied the regulation of FoxM1 transcriptional activity during cell cycle progression. First, we have addressed FoxM1 regulation through phosphorylation by cell cycle-regulated kinases. Previous reports show that FoxM1 C-terminal region can be bound by cyclin/cdk complexes through an LXL motif and consequently phosphorylated on residue T596 [42]. However, our data suggest that additional levels of regulation exist. Indeed, we have shown that FoxM1

transcriptional activity is increased upon stimulation by Cyclin E and Cyclin (see Chapter 5, Figure 2C). However, FoxM1 activity is low in thymidine-blocked cells although Cdk2 actvity is high (see Chapter 5, Figure 2A and 2B), suggesting that FoxM1 is rather activated by Cdk1 than by Cdk2. Furthermore, we have observed that the N-terminus deletion mutant of FoxM1 (ΔN-FoxM1) displays a hyper-active phenotype and shows a reduced sensitivity to Cyclin A stimulation or to synchronization in G1 (see Chapter 5, Figure 3x). This indicates that the N-terminus of FoxM1 is important for regulation by Cyclin A/Cdk1, and carries a potent repressor activity. This repression appears to be relieved by the action of Cyclin A/Cdk1, indicating that FoxM1 activity is high under conditions of high Cyclin A/Cdk1 activity. This strongly suggests that FoxM1 transactivation is somehow restricted to G2/M. Interestingly, the N-terminal region of FoxM1 comprises two specific RXL cyclin-binding consensuses as well as only two conserved putative phosphorylation sites for cyclin/cdk complexes (one of which being a putative Cdk1 phosphorylation site).

The FoxM1 protein sequence is punctuated by conserved putative cyclin/cdk phosphorylation sites. Thus, it would be interesting to address which of these sites are, in addition to residue T596, the critical cyclin/cdk phosphorylation sites. Furthermore, these may be involved in either positive or negative regulation of FoxM1 transcriptional activity. In addition to regulation by cyclin/cdk complexes, FoxM1 had been shown to be regulated by the Raf/MAPK cascade on the conserved residues S331 and S704 [43]. This phosphorylation results in the activation of FoxM1 transcriptional activation through its translocation from the cytoplasm to the nucleus. Also, we have identified two conserved putative Plk-1 phosphorylation sites, one in the N-terminal part of the protein, the other one in the C-terminal part. However, phosphorylation of FoxM1 by Plk-1 has never been addressed. Interestingly, Plk-1 is one FoxM1 transcriptional targets. Plk-1 is inhibited following DNA damage and plays an important role in cell recovery following DNA damage [44]. Interestingly, preliminary observations that FoxM1 activity is decreased following DNA damage inflicted 6 hrs after thymidine release (late S-phase, early G2-phase) suggest that FoxM1 may be inhibited following DNA damage. Thus, it would be interesting to address putative phosphorylation of FoxM1 by Plk1, possibly during recovery from DNA damage, or more generally in G2/M. Furthermore, if Plk-1 should be shown to regulate the activity of FoxM1, it would unveil a positive feedback-loop, a mechanism often seen in cell cycle regulation.

On the other hand, we have addressed regulation of FoxM1 by proteasome-dependent protein degradation. Analysis of FoxM1 protein sequence reveals the presence of three degradation motifs in its N-terminal portion. These are two D-boxes (RXXL) and a KEN-box, which are recognition motifs for the APC^{Cdc20} and the APC^{Cdh1} and for the APC^{Cdh1} respectively. Our data show that FoxM1 is degraded during mitotic exit in a proteasome-dependent manner and suggest that the mechanism underlying this degradation may be Cdh1-dependent, although this should need further confirmation. However, not all FoxM1 (up to 70%) is inactivated by protein degradation during mitotic exit. Therefore, there should be an additional mechanism in order to prevent active FoxM1 to be present in G1. We have suggested in Chapter 5 that FoxM1 may also be actively dephosphorylated during mitotic exit, so that a small pool of FoxM1 is already present in early G1-phase in cycling cells but in an unactive form. We have hypothesized that FoxM1 dephosphorylation during mitotic exit could be due to a Cdc14-like activity, although it also could result from the activity of default phosphatases after that Cdk1 has been inactivated after anaphase.

From the published literature, FoxM1 is emerging as an important protein for cancer cell proliferation (see Chapter 4). More specifically, FoxM1 transcriptional activity has been shown to be stimulated by Ras/Raf/MAPK pathway, implying that expression of oncogenic Ras may lead to constitutive activation of FoxM1 transcriptional activity. On the other hand, FoxM1 is inhibited by the tumour suppressor pRb in G1. One could think that loss of pRb, which is observed in many cancers, may lead to untimely activation of FoxM1. Furthermore, the oncogenic viral protein E7, which is known to inhibit pRb, has been shown to increase

FoxM1 transcriptional activity. Conversely, FoxM1 has been shown to be required for E7 transforming activity. Finally, the p19ARF tumour suppressor, which often inactivated in cancer through methylation of the INK4 locus, has been shown to inhibit FoxM1 activity by sequestration in the nucleolus. Therefore, it would be interesting to address whether FoxM1 is only regulated downstream to oncogenic and tumour suppressor pathways and participates in carcinogenesis through its proliferation-stimulating function, or whether FoxM1 truly cooperates with oncogenic pathways for cellular transformation.

An E-box c-Myc binding domain has been identified on FoxM1 promoter [45]. We would expect that c-Myc would stimulate expression of FoxM1, since c-Mys is a proliferation-stimulating protein. However, c-Myc is a puzzling protein that does many things in the cell, and it seems that c-Myc represses FoxM1 promoter transactivation (personal communication of J. Laoukili). Interestingly, in the liver of transgenic mice that express an inducible form of c-Myc (c-Myc-ER) under the control of the hepatic-specific Albumin promoter, activation of c-Myc causes a transient increase in hepatocyte proliferation with increased BrdU incorporation and phosphor-histone H3 staining. However, at later time-points, a decrease in proliferation is observed, correlating with an increased G2/M cell population, reduced phospho-histone H3 staining and increased polyploidization. Some of the remaining p-H3-positive hepatocytes show defective mitosis with chromosome missegregation, and tripolar spindles as compared to control animals, in which c-Myc-ER was not activated (personal communication T. Dansen). This phenotype is very similar to the one observed in cells in which expression of FoxM1 is impaired. Thus, it would be interesting to address whether, in hepatocytes where c-Myc is active, overexpression of FoxM1 could prevent these mitotic defects, restore normal cell cycle progression, and possibly restore the increased proliferation seen in early time-points. Furthermore, it is known that c-Myc and oncogenic Ras cooperate to transform mouse primary cells. Thus, it would be interesting to address whether overexpression of FoxM1 could substitute for oncogenic Ras and cooperate with c-Myc in cell transformation.

FoxM1 emerges as an important regulator of the mitotic progression through its crucial role in the regulation of the G2/M transcriptional cluster. Thus, FoxM1 (over)expression is likely to be important for proliferation-associated diseases such as cancer.

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Samenvatting (ook voor niet-specialisten)

Inleiding

Het menselijk lichaam is opgebouwd uit vele biljoenen cellen, waarvan de meeste in een rustende staat verkeren. Hoewel deze rustende cellen niet delen, zijn ze wel in staat hun eigen, specifieke taak uit te voeren. Bijvoorbeeld de cellen in het netvlies die licht kunnen detecteren, cellen in de alvleesklier die insuline produceren, of de cellen die verantwoordelijk zijn voor het samentrekken van onze spieren. Maar onder bepaalde (extreme) condities kan het belangrijk zijn dat deze rustende cellen weer aangezet kunnen worden tot actieve celdeling. Zo is het essentieel dat rustende cellen van het immuunsysteem actief gaan delen wanneer er een infectie optreedt, of cellen moeten gaan delen om een botbreuk te herstellen, of het bloed aan te vullen. Om te kunnen delen, moet een rustende cel de celcyclus opstarten, een proces dat volledig geblokkeerd is in de rustende staat.

De celcyclus representeert de levenscyclus van een cel, van geboorte tot aan de deling in twee nieuwe dochtercellen. Eenmaal terug in de celcyclus, duurt het ongeveer 10 tot 24 uur om een celdeling te completeren. Dit is behoorlijk snel, als men in overweging neemt dat er een volledige kopie van het gehele genoom gemaakt moet worden. Het menselijk genoom is namelijk opgebouwd uit lange ketens van totaal zo'n 3.2 biljoen nucleotide paren. Die bevatten samen alle genetische informatie, ofwel ongeveer 30.000 genen, verdeeld over 23 chromosoomparen. Als de cel eenmaal een exacte kopie van het genoom heeft weten te produceren, moeten origineel en kopie precies gescheiden worden, zodat elke dochtercel een complete en volledig identieke set chromosomen ontvangt. Dit proces noemen we mitose.

Om een stabiel genoom te behouden, waarin de genetische opmaak niet veranderd, is het essentieel dat de duplicatie van het genoom, alsook de scheiding ervan, zonder fouten en in de juiste volgorde uitgevoerd worden. Om dit te bewerkstelligen, heeft de cel de beschikking over verschillende "checkpoints". Zodra er fouten in het duplicatieproces, of tijdens de scheiding van de chromosomen worden gesignaleerd, zorgen deze checkpoints ervoor dat het proces gestopt wordt. Zo is er vlodoende tijd om de fouten te herstellen voordat de deling plaatsvindt. Dit mechanisme is zeer efficiënt, en voorkomt foutieve celdeling die zou kunnen leiden tot het ontstaan van gemuteerde cellen die mogelijk uit kunnen groeien tot een tumor.

Kanker ontstaat als gevolg van een ophoping van niet gerepareerde fouten (mutaties) in het DNA van één of meerdere cellen. Deze mutaties stellen de cel in staat om nieuwe eigenschappen te ontwikkelen die bijdragen aan de kwaadaardigheid van de tumor. Voorbeelden van dergelijke eigenschappen zijn; de capaciteit om oneindig door te delen, ongevoeligheid voor groeiremmende factoren, de capaciteit om te ontsnappen aan het afweersysteem, of de capaciteit om los te breken van het oorspronkelijke orgaan om op een andere plek in het lichaam door te groeien (metastasering). Om een dergelijk gemuteerde staat te bereiken moet een cel de checkpoints omzeilen die het ontstaan van mutaties trachten te voorkomen.

De processen die door de cel worden uitgevoerd worden in grote mate bepaald door de genen die op dat moment actief zijn. Activiteit van de verschillende genen wordt gereguleerd door een grote verscheidenheid aan transcriptiefactoren. Transcriptiefactoren zijn eiwitten die zorgen voor de translatie van de genetische informatie naar een observeerbare functie. Zo bevat het insuline gen de informatie die de cel in staat stelt dit hormoon aan te maken, essentieel voor de suikerhuishouding. Maar het insuline gen wordt alleen vertaald wanneer de juiste transcriptiefactoren geactiveerd zijn. Zodoende spelen transcriptiefactoren en kritieke rol in het functioneren van de cel. Transcriptiefactoren kunnen gereguleerd worden door signalen vanuit de omgeving van de cel, hun activiteit kan bijvoorbeeld veranderen onder invloed van bepaalde hormonen of medicijnen. Zo vormen ze een link tussen aangeboren eigenschappen (de genen, die nauwelijks veranderen) en verworven eigenschappen (interacties met

een omgeving die constant veranderd).
Het werk beschreven in dit proefschrift heeft betrekking op de rol van Forkhead transcriptiefactoren in de regulatie van het proces van celdeling. Het was reeds bekend dat voor de uitvoering van een volledige celdeling, activering van verschillende “golven” van genexpressie nodig is, maar er was nog weinig bekend over de transcriptiefactoren die verantwoordelijk zijn voor de geprogrammeerde celcyclus-afhankelijke volgorde in genexpressie. Een golf correspondeert met een groep genen (cluster), wiens activering leidt tot de activering van de volgende golf van genexpressie. Zo draagt de productie van specifieke transcriptiefactoren in de eerste golf van genexpressie bij aan de expressie van het opeenvolgende cluster. Elk cluster reguleert mechanistische (DNA replicatie, DNA reparatie) en regulatoire (checkpoints, transcriptiefactoren) aspecten van de specifieke fase van de celcyclus waarin zij actief is. Daarnaast zijn veel van de genproducten in een cluster onmisbaar voor het completeren van het specifieke stadium in de celcyclus waarin zij actief zijn. Deze opeenvolgende activering van verschillende clusters van genen zorgt ervoor dat de verschillende stadia in de celcyclus in de juiste volgorde worden doorlopen.

Hoe wordt de celcyclus gestart en gestopt?

In het **eerste gedeelte** van mijn proefschrift beschrijf ik de rol van transcriptiefactoren in de overgang van de rustende fase naar een actieve celdeling. Oftewel, hoe kunnen transcriptiefactoren de celcyclus starten, en hoe kan dit proces omgekeerd worden zodat delende cellen in een rustende staat kunnen worden gebracht. Een beter begrip van deze overgang kan leiden tot een beter inzicht in het proces van ongecontroleerde celdeling (kanker) en hoe dit voorkomen kan worden. In het onderzoek naar de regulatie van de transitie van rust naar deling, hebben we vooral gebruik gemaakt van cellen van het afweersysteem. Deze cellen maken van nature de transitie van een rustende staat naar een actieve celdeling wanneer er sprake is van een infectie of na vaccinatie. We hebben bij ons onderzoek gebruik gemaakt van gezonde T cellen, die normaal reageren op signalen vanuit de omgeving, en we hebben gebruik gemaakt van leukemische T cellen, waarin een zekere mate van groeicontrole verloren is gegaan. Gebruikmakend van deze cellen hebben we twee strategieën gevolgd om te trachten de ongecontroleerde celgroei te remmen.
In de eerste strategie hebben we gebruik gemaakt van een artificiële manier om de celcyclus van leukemische cellen een halt toe te roepen. In **hoofdstuk 2** beschrijven we hoe een aantal natuurlijke en farmacologische substanties de productie van cAMP (cyclisch adenosine monofosfaat) stimuleren, en zo de groei van leukemische cellijnen kunnen remmen. We hebben het verantwoordelijke mechanisme van deze groeiremming verder onderzocht, en hebben kunnen aantonen dat cAMP werkt via negatieve regulatie van de expressie van Cycline D3 en stimulatie van p27kip1. Cycline D3 induceert de start van de celcyclus, terwijl p27kip1 de start juist blokkeert. Door de toename in het niveau van Cycline D3, en een afname in de hoeveelheid p27kip1 zorgt cAMP voor een zeer efficiënt blok in de celcyclus van deze leukemische cellijnen.
De **tweede strategie** om de ongecontroleerde celdeling te voorkomen, was via verwijdering van essentiële groei- of overlevingsfactoren. IL-2 (interleukine-2) is een belangrijk hormoon voor cellen van het afweersysteem dat het celdelingsproces stimuleert, en de levensvatbaarheid van actief delende cellen bevordert. IL-2 wordt geproduceerd wanneer cellen van het afweersysteem worden geactiveerd, bijvoorbeeld gedurende een infectie van het organisme met een microbe. Wij hebben de rol van transcriptiefactoren van de FoxO familie gedurende de IL-2-gestimuleerde celgroei onderzocht. In **hoofdstuk 3** laten we zien dat toevoeging van IL-2 leidt tot de afschakeling van FoxO factoren. Daartegenover leidt artificiële activering van FoxO in afwezigheid van IL-2 tot een snelle stop in de celdeling, waarna de cel sterft. Wij tonen aan dat dit het gevolg is van stimulering van de expressie van p27kip1 (de “rem” op de celdeling die hierboven al genoemd is), en Bim (een “killer” eiwit) door

FoxO. Op grond van deze bevindingen, hypothetiseren wij dat FoxO in cellen van het afweersysteem een belangrijke rol speelt in de beslissing om te delen of het delingsproces af te breken.

Implicaties van deze resultaten

We hebben aangetoond dat cAMP, via proteïne kinase A, de expressie van Cycline D3 en p27kip1 reguleert. Omdat het inmiddels bekend is dat FoxO deze beide genen direct reguleert, zou het interessant zijn om te onderzoeken of FoxO factoren deze effecten van cAMP mediëren. We hebben kunnen laten zien dat FoxO onder controle staat van IL-2, en deze controle uit zich inderdaad in een verandering van de expressie van p27kip1. Aan de hand van deze resultaten komen we tot de conclusie dat FoxO belangrijk zou kunnen zijn om cellen in een rustende staat te houden. Daarnaast vinden we dat FoxO factoren, naast hun effect op de celcyclus, ook celdood induceren. Daarom zullen er voor een langdurig verblijf in een rustende staat ook overlevingsmechanismen geactiveerd moeten worden die de bevordering van de celdood door FoxO kunnen tegengaan. Het is wel interessant om op te merken dat een snelle inductie van celdood, ofwel lange overleving in een staat van rust, zeer belangrijk is in het afweersysteem. Snelle celdood is belangrijk om reactieve T cellen uit het lichaam te verwijderen, wanneer een infectie is genezen. Lange overleving in een staat van rust is belangrijk voor het “geheugen” van het afweersysteem. Memory T cellen vormen een kleine populatie in het afweersysteem van een individu. Zij bevatten de informatie die nodig is voor herkenning van pathogenen waaraan het individu in het verleden al eens is blootgesteld. Zij zijn betrokken bij de ontwikkeling van immuniteit voor bepaalde ziektes. Zonder deze memory T cellen T zouden vaccins niet werkzaam zijn. Deze memory T cellen kunnen voor zeer lange tijd in een rustende staat overleven in het lichaam, om slechts te gaan delen wanneer het individu opnieuw in aanraking komt met hetzelfde pathogeen. Onze bevindingen met FoxO factoren suggereren dat zij een heel belangrijke rol zouden kunnen spelen in de verwijdering van reactieve T cellen, evenals de langdurige overleving van memory T cellen. Het is natuurlijk van groot belang om in de toekomst de achterliggende mechanismen, die verantwoordelijk zijn voor het onderscheid tussen celdood of overleving in een staat van rust, te ontrafelen.

Regulatie van het mitotische gencluster

In het **tweede gedeelte** van dit proefschrift (**hoofdstuk 5**), beschrijven we ons onderzoek naar de verschillende mechanismen die de activiteit van de Forkhead transcriptiefactor FoxM1 gedurende verschillende stadia van de celcyclus. Het was reeds bekend dat FoxM1 betrokken is bij de regulatie van de celcyclus, via de regulatie van een cluster van genen die essentieel zijn voor de uitvoering van de mitose. FoxM1 is uitsluitend actief in bepaalde stadia van de celcyclus, en deze beperking in FoxM1 activiteit is belangrijk voor een juiste celdeling. Maar hoe de beperking van FoxM1 activiteit tot stand komt is niet helemaal duidelijk. Wij hebben aangetoond dat Cycline A, een belangrijk celcyclus regulerend eiwit, FoxM1 activiteit stimuleert. Onze bevindingen suggereren dat Cycline A nodig is voor de activering van FoxM1, door opheffing van een remmende functie aanwezig in het N-terminale gedeelte van FoxM1. Dat wijst erop dat FoxM1 activiteit niet uitsluitend gereguleerd wordt via expressie van FoxM1. Inductie van FoxM1 expressie in het cluster van genen dat tot expressie komt tijdens de duplicatie van het DNA, is dus niet voldoende om het mitotische cluster van genen te activeren. Voor dit laatste is verdere activering van FoxM1 nodig, meest waarschijnlijk via fosforylering door Cycline A/Cdk1 complexen, die niet gevormd kunnen worden zolang een cel zijn DNA replicatie nog niet voltooid heeft. Zodanig zal het mitotische cluster niet geactiveerd kunnen worden zolang de DNA replicatie nog niet compleet is, en is mitose dus niet mogelijk voordat het volledige genoom gekopieerd is.
Naast fosforylering door Cycline A/Cdk1, bevat FoxM1 ook mogelijke sites voor fosforylering door Polo-like kinase-1 (Plk-1), een ander belangrijk mitotisch kinase. Hierdoor zou het mogelijk zijn dat FoxM1 activering ook afhankelijk is van andere celcyclus

regulerende kinasen, die uitsluitend actief worden wanneer DNA replicatie is voltooid. Daarnaast speelt Plk-1 ook een cruciale rol tijdens herstel van cellen die DNA schade hebben opgelopen. Het is te verwachten dat FoxM1 activiteit in dergelijke cellen laag zal zijn, als gevolg van de remming van Cycline/Cdk activiteit door DNA schade. Het is daarom interessant om verder te bestuderen of FoxM1 daadwerkelijk door Plk-1 gereguleerd wordt, vooral in cellen die herstellen van DNA schade. Wij hebben aangetoond dat Plk-1 zelf door FoxM1 gereguleerd wordt, en dit zou een aantrekkelijke feedback creëren om de herstart na schade te bevorderen na herstel van de DNA schade. Ten slotte hebben we ook aangetoond dat de stabiliteit van het FoxM1 eiwit varieert gedurende de celcyclus, en dat ongeveer 70% van het totale FoxM1 eiwit wordt afgebroken in de late stadia van mitose. Dit, in combinatie met het verlies van Cycline A gedurende mitose, zorgt voor een omzet in het transcriptionele apparaat die voorkomt dat FoxM1 het mitotische cluster al kan activeren in de vroege stadia van de volgende celdelingscyclus.

De rol van FoxM1 in kanker

FoxM1 komt alsmaar sterker naar voren in de wetenschappelijke literatuur als een eiwit dat een belangrijke rol speelt in de celdeling, met name de deling van kankercellen. Vele eiwitten die gemuteerd zijn in kanker zijn bekende regulatoren van FoxM1. Daarom is het voor de toekomst van belang om te onderzoeken of FoxM1 zelf participeert in kanker, en hoe. Is dit simpelweg het gevolg van de stimulering van de celdeling door FoxM1, of kan FoxM1 ook samenwerken met andere bekende transformerende eiwitten om de checkpoints van de cel dusdanig te ontwrichten dat

Summary (also for non-specialists)

Introduction.

The human body is composed of billions of cells, most of which are in a resting state (quiescence). Although these quiescent cells do not divide, they are capable of performing their own specific task. For example, some detect light in the retina, some produce insulin in the pancreas, and some other generate muscular contractions. However, it is important that they are allowed to divide under specific conditions. For example, some cells will be asked to divide in order to prevent an infection, repair a broken bone or reconstitute the blood. In order to divide, a cell needs to re-start the cell division cycle (or cell cycle) that is blocked in the resting state.

The cell cycle corresponds to the cycle of life of a cell, from its “birth” until its division into two new daughter cells. Once back in the cell cycle, it takes between 10 to 24 hours for a cell to divide and form two new daughter cells. This is extremely swift if one considers that a cell has to copy (duplicate) its entire DNA (deoxyribonucleic acid), a double chain (the double helix) formed by about 3.2 billions of nucleotide pairs. The DNA contains the genetic information, i.e. about 30 000 genes, spread out over 23 pairs of chromosomes. Once a cell has duplicated its DNA, it then has to precisely segregate the two copies of the genome, such that each daughter cell receives a complete and identical set of chromosomes. This process is known as mitosis.

To maintain a stable genome, DNA duplication and mitosis need to be error-proof processes that occur in a defined sequence. To make sure this happens correctly, several cell cycle checkpoints have evolved. If errors in DNA duplication, chromosome segregation or the order of events are detected, these checkpoints stall cell cycle progression in order to provide time to correct the errors. This is a very efficient mechanism to prevent erroneous cell division that might lead to the generation of mutant, potentially cancerous cells.

Cancer is the consequence of the accumulation of non-repaired errors (or mutations) in the DNA of one or more cells. These mutations allow the cell to acquire new characteristics that contribute to its tumorigenic potential. Examples of such characteristics are: immortality, loss of proper growth control, the

capacity of the cell to escape the immune system, or its capacity to move in the body and invade organs where it is normally not present (metastasis). In order to do accomplish this mutant state, a cell needs to overcome the cell cycle checkpoints that act to prevent mutations.

The actions undertaken by a cell are in large part determined by the genes that are active at a given time. Gene activity is determined by a large variety of transcription factors. Transcription factors are proteins which function is to regulate gene expression, i.e. the translation of the genetic information (the recipe) into an "observable" character (the cake). For example, the insulin gene contains the information that allows the cell to make this hormone, essential for sugar metabolism. However, the insulin gene is only actively transcribed when the proper transcription factors are activated. As such, transcription factors play a critical role in cellular function. Transcription factors can be under the influence of signals from the "environment", for example, their activity can be modified by certain drugs. They form a link between the innate (the genes, which barely change) and the acquired (interactions with an environment constantly changing).

The work described in my thesis addresses the role of members of the Forkhead family of transcription factors in the regulation of cell division. It was well known that completion of a cell division cycle requires the activation of subsequent "waves" of gene transcription, but little was known about the transcription factors that are responsible for this programmed cell cycle-dependent gene activity. A wave corresponds to a group of genes (cluster), whose activation leads to the production of the following wave/group of proteins. For instance, transcription factors produced during one wave of the cell cycle will lead to the production of (among other proteins) the transcription factors of the following wave. Each cluster controls mechanistic (e.g. DNA duplication, DNA repair machineries) and regulatory (checkpoints, transcription factors) aspects of the particular cell cycle stage in which it is activated, and many genes in the cluster will be essential for the completion of this stage. This sequential activation of different gene clusters ensures that subsequent events of the cell cycle are properly timed and orderly executed.

How to start and stop the cell cycle?

In the **first part** of this thesis, I describe the role of transcription factors in the transition of cells from a resting state into the cell division cycle. In other words, how transcription factors regulate the start of an active cell division, and how this process can be turned around to drive cells into a resting state. A better comprehension of this transition can lead to a better understanding of the process by which a cell can attain a state of uncontrolled cell division (i.e. cancer), and how this can be prevented. This part of my thesis work was performed with cells of the immune system, because they naturally go back and forth between a resting and an actively cycling state, whenever there is an infection or a vaccination. The immune cells that we used for these studies were either healthy T lymphocytes that respond normally to cues from the environment, or leukaemic cancer cells that have lost the proper control mechanisms. We have studied two strategies by which we can prevent uncontrolled cell division.

The **first strategy** was to artificially promote a cell cycle exit in leukaemic cancer cells. In **Chapter 2**, I describe how certain natural or pharmacological compounds can stimulate the production of a molecule called cAMP (cyclic adenosine monophosphate) and prevent cell division in leukaemic cell lines. My colleagues and I then went on studying the mechanism by which activation of protein PKA (Protein Kinase A) by cAMP induces suppression of cell division in T lymphocytes (immunosuppression). We show that PKA is capable of stopping cell cycle progression in leukaemic cell by down-regulating the expression of Cyclin D3 and up-regulating p27/Kip1. Cyclin D3 acts to promote the start of the cell cycle, while p27kip1 acts to stop the cell cycle and promotes entry into a resting state. By co-regulating these two important cell cycle regulatory proteins, cAMP can potently block cell division of these leukaemic cells.

The **second strategy** to prevent uncontrolled cell division that we have studied was by removal of essential growth and/or survival factors. IL-2 (Interleukine-2) is an important hormone for cells of the immune system (T-lymphocytes) that induces entry into the cell cycle and maintains cell viability in actively dividing cells. IL-2 is produced when cells of the immune system are activated, for example upon infection of an organism with a microbe. In **Chapter 3**, we have studied the role of the transcription factors of the FoxO family of transcription factors in IL-2-mediated stimulation of cell division. We show that IL-2 leads to the inhibition of FoxO factors. Conversely, artifical activation of FoxO, or activation of FoxO in the absence of IL-2 leads to a rapid exit from the cell cycle, followed by cell death. We demonstrate that this is due to stimulation of the expression of p27/Kip1 (the cell cycle "brake" mentioned above) and of Bim (a "killer" protein) by FoxO. Based on these observations, we propose that in cells of the immune system, FoxO plays a crucial role in the decision to start or stop the cell division process.

Implications of this line of research

We have demonstrated that cAMP, via PKA, regulates the expression of Cyclin D3 and p27/Kip1. As FoxO transcription factors are known to regulate the expression of these same genes, it would be interesting to address whether FoxO factors regulate their expression in response to cAMP in cells of the immune system. FoxO is regulated by IL-2, and this regulation does result in altered expression of p27kip1. Based on these data, we hypothesize that activation of FoxO transcription factors is important to maintain cells in a resting state. However, because we find that FoxO factors, in addition to promoting cell cycle exit, also induce proteins that promote cell death, a long-term arrest in a resting state will require the induction of a survival mechanism to counteract the cell death promoting activities of FoxO. Interestingly, it is equally important for cells of the immune system to be able to induce rapid cell death or to promote a long-term arrest in a resting state. The first is important to clear the highly reactive toxic T lymphocytes that are produced during an infection. The second is important for immunological memory. Memory cells form in a tiny cell population of the immune system of an individual. They represent the memory of the immune history of this individual, i.e. they store the memory of how to fight a specific pathogen that has already affected this individual in the past. Thus, without memory cells, vaccines would not have any efficacy. These memory cells can survive in a resting state throughout the entire lifespan and can be reactivated whenever the pathogen for which they are specialized re-challenges the individual. Our findings on FoxO factors suggest that they may play a very important role in both the removal of cytotoxic T lymphocytes, as well as the long-term survival of memory cells. Of course it will be very interesting to dissect how the discrimination between these two very different outcomes is regulated.

Regulation of the mitotic gene cluster.

In the **second part** of this thesis (**Chapter 5**), I describe my studies on the multiple regulatory mechanisms affecting the activity of the transcription factor FoxM1 during cell cycle progression. FoxM1 is known to regulate the cell division process. In particular, FoxM1 was shown to be important for the regulation and completion of mitosis. It is active only during a limited period of time in the cell cycle (stages preceding mitosis and mitosis), and temporal limitation of its activity is important for proper cell cycle progression. We show that Cyclin A, a cell cycle regulatory protein, stimulates FoxM1 activity. Based on our observations, it seems that Cyclin A is required for FoxM1 activation by inactivating a repressive function present at one extremity (N-terminus) of FoxM1 protein. Our data show that FoxM1 activity is not solely determined by expression of FoxM1 alone. That is, induction of FoxM1 expression within the cluster of genes that is activated during DNA replication is not sufficient to activate the cluster of genes that regulates mitosis. Activation of this latter cluster requires the further activation of FoxM1, most likely through phosphorylation by Cyclin A/Cdk1 complexes that do not form

until a cell has completed S-phase. This way, the mitotic cluster is only properly induced once cells have finished replicating their DNA, and in doing this a cell makes sure that mitosis is not possible until DNA replication is completed.
Interestingly, FoxM1 also contains potential regulatory sites for Plk-1, another cell cycle regulatory protein. This raises the possibility that FoxM1 activation also depends on additional cell cycle regulated kinases that are only activated following completion of DNA replication. Also, Plk-1 is important for the recovery of cells that have experienced DNA damage. One would expect FoxM1 activity to decrease in damaged cells, as a result of the inhibition of cyclin/cdk complexes. Thus, it would be interesting to address whether Plk-1 is indeed a regulator of FoxM1 activity, particularly in cells recovering from DNA damage. We have shown that Plk-1 expression is itself regulated by FoxM1, and therefore this would create an interesting feedback loop to regulate cell cycle re-initiation following DNA damage repair. Finally, we show that FoxM1 stability varies during the cell cycle, and that about 70% of the total FoxM1 protein is degraded during exit from mitosis. This, combined with loss of Cyclin A during mitosis, resets the transcriptional machinery of the cell cycle to prevent the inadequate activation of FoxM1 during the early stages of the following cell division cycle.

Role of FoxM1 transcription factor in cancer

FoxM1 is emerging in the scientific literature as a protein that plays an important role in cell division, in the division of cancer cells. Many proteins that are mutated in cancer cells are known regulators of FoxM1 activity. Thus, it would be interesting in the future to study whether FoxM1 participates in cancer development only as a consequence of its stimulating function on cell division, or whether FoxM1 can also cooperate with other known mutant proteins to promote cancer development.

Résumé (aussi pour les non-spécialistes)

Introduction.

Notre organisme est constitué de milliards de cellules. La plupart d'entre elles est en dormance (ou quiescence). En effet, ces cellules ne prolifèrent pas, mais elles continuent malgré tout de remplir leur rôle au sein de l'organisme, par exemple détecter la lumière dans la rétine de l'oeil, fabriquer de l'insuline dans le pancréas ou encore permettre la contraction musculaire. Parfois pourtant, il est important que certaines cellules puissent proliférer de manière contrôlée, par exemple pour pouvoir lutter contre une infection, pour réparer un os brisé ou pour reconstituer le sang après un don. Pour pouvoir se diviser, ces cellules doivent redémarrer le cycle cellulaire qui est bloqué dans la phase de dormance.
Le cycle cellulaire correspond au cycle de vie d'une cellule, de sa formation à sa division en deux cellules appelées cellules-fille. Cela prend environ 10 à 24 heures avant qu'une cellule qui est retournée dans le cycle cellulaire se soit divisée. C'est en effet très rapide si l'on considère que pour se diviser, une cellule doit d'abord copier (dupliquer) son ADN (acide désoxyribonucléique), une sorte de chaîne double (la double hélice), constituée de 3,2 milliards de paires de maillons. L'ADN contient l'information génétique, sous la forme de 30 000 gènes environ, répartis sur 23 paires de chromosomes. Après avoir dupliqué cet ADN, la cellule va devoir séparer les deux copies de manière très précise, pour que chacune des cellules-fille ait une copie complète et parfaitement identique. Ce processus, pendant lequel les deux copies d'ADN sont séparées et les deux cellules formées, se nomme mitose.
Il va sans dire que la duplication de l'ADN, ainsi que la mitose, ne doivent comporter aucune erreur. Pour cela, l'organisme s'est équipé de points de contrôle (ou checkpoints), dont le role est de détecter les erreurs qui pourraient se produire. Ces points de contrôle agissent

en stoppant la progression du cycle cellulaire d'une cellule présentant une/des erreur/s, soit dans la duplication ou la ségrégation de l'ADN, soit dans l'ordre des évènements. D'une part, cela donne du temps à la cellule pour lui permettre de se réparer. D'autre part, c'est un excellent moyen pour prévenir la prolifération de cellules contenant des mutations, qui présentent un risque accru de devenir cancéreuses.

L'apparition d'un cancer est donc la conséquence de l'accumulation d'erreurs non-réparées (ou mutations) dans le matériel génétique d'une ou de plusieurs cellules. En effet, les mutations permettent à la cellule d'acquérir des caractéristiques qui contribuent à l'évolution tumorale, par exemple, acquérir l'immortalité, échapper au contrôle de la croissance exercé par l'organisme ou à la toxicité du système immunitaire, ou encore se déplacer dans l'organisme et coloniser des organes où elle n'est normalement pas présente (métastase). Pour devenir mutante et acquérir ces caractéristiques, une cellule doit donc à un moment ou à un autre inactiver les points de contrôle du cycle cellulaire qui luttent contre l'acquisition des mutations.

Les actions entreprises par la cellule sont en large partie déterminées par les gènes qui sont actifs à un moment donné. A leur tour, l'activité de ces gènes est elle-même determinée par une grande variété de facteurs de transcription. Les facteurs de transcription sont une catégorie de protéines dont la fonction est de réguler l'expression des gènes, c'est-à-dire le processus de traduction de l'information contenue dans ces gènes (la recette) en des caractères « observables » (le gateau). Par exemple, le gène de l'insuline contient l'information nécessaire pour fabriquer cette hormone essentielle pour le métabolisme du glucose. Mais le gène de l'insuline n'est exprimé que lorsque les facteurs de transcription appropriés sont activés, par exemple quand le taux de sucre dans le sang est trop élevé. Dans cette mesure, les facteurs de transcription jouent donc un rôle capital dans la cellule. De plus, ces facteurs subissent des influences de l'« environnement », par exemple leur activité peut être modifiée par certains médicaments. Ils représentent donc une charnière entre l'inné (l'information génétique qui ne change pas, ou peu), et l'acquis (l'intéraction avec un environnement constamment changeant).

Pendant ma thèse, j'ai étudié le rôle de facteurs de transcription de la famille Forkhead dans la régulation du cycle cellulaire. La progression du cycle cellulaire est alimentée par la succession de « vagues »successives dans l'expression des gènes. Peu est su, par contre, sur l'identité des facteurs de transcription responsables des différentes vagues d'activité des gènes pendant le cycle cellulaire. Une vague correspond à un groupe de protéines, qui, à son tour, produit le groupe de protéines suivant. Chaque groupe contrôle les aspects mécaniques (machinerie de duplication ou de réparation de l'ADN) et régulateurs (points de contrôle, facteurs de transcription) d'une étape du cycle celluaire pendant laquelle il est activé, et est essentiel pour le bon déroulement de cette étape. L'activation successive de ces différents groupes garantit que chaque étape du cycle cellulaire est effectuée au moment approprié et dans l'ordre.

Comment arrêter et démarrer le cycle cellulaire ?

Dans la **première partie** de cette thèse, je décris mes recherches sur le rôle de facteurs de transcription dans la transition des cellules entre un état de prolifération et un état de dormance, c'est-à-dire comment les facteurs de transcription régulent le démarrage d'un cycle actif de division cellulaire, et comment ce processus peut être inversé pour ramener les cellules dans un état de dormance. Une meilleure compréhension de cette transition pourrait permettre de mieux comprendre comment une cellule peut se mettre à proliférer de manière incontrolable (par exemple, cellule cancéreuse), et comment l'en empêcher. Ces recherches ont été effectuées dans des cellules du système immunitaire qui transitent naturellement entre cet état de dormance et cet état de prolifération, lors d'une infection ou après une vaccination. Les cellules immunitaires que nous avons utilisées sont soit des lymphocytes T sains, purifiés à partir du sang d'un donneur sain, qui répondent normalement aux stimulations

de l'environnement, soit des cellules cancéreuses leucémiques, qui ont perdu leurs mécanismes de contrôle. Nous avons étudié deux stratégies pour empêcher la prolifération anormale de cellules.

La **première stratégie** est de plonger artificiellement dans un état de dormance ces cellules leucémiques cancéreuses. Ainsi, dans le **Chapitre 2**, je décris comment certains procédés naturels ou pharmacologiques qui activent la production par la cellule d'un composé appelé AMPc (Adénosine Monophosphate cyclique) préviennent la prolifération de cellules immunitaires leucémiques. Mes collègues et moi-même avons étudié le mécanisme par lequel l'activation de la protéine PKA (Protein Kinase A) par l'AMPc induit une suppression de la prolifération des lymphocytes T (immunosuppression). Nous avons montré que cette protéine est capable de stopper la progression dans le cycle cellulaire des cellules leucémiques en diminuant l'expression de la Cycline D3 et en stimulant celle de p27/Kip1. La Cycline D3 est un stimulateur du redémarrage du cycle cellulaire, alors que p27/Kip1 est un frein au cycle cellulaire et stimule l'entrée dans l'état de dormance. En co-régulant ces deux importantes protéines du cycle cellulaire, l'AMPc peut ainsi bloquer efficacement la division cellulaire de ces cellules leucémiques.

Nous avons étudié une **seconde stratégie** pour bloquer la prolifération anormale des cellules, qui consiste à retirer des facteurs essentiels à la croissance et à la survie des cellules. L'IL-2 (interleukine-2) est une sorte d'hormone du système immunitaire qui induit la prolifération active et la survie des lymphocytes T en réponse à une agression microbienne. Dans le **Chapitre 3**, nous avons étudié le rôle des facteurs de transcription de la famille FoxO dans la régulation par l'IL-2 de la prolifération et la survie de cellules immunitaires. Nous avons montré que l'IL-2 inhibe les facteurs FoxO. Inversement, l'activation artificielle des facteurs FoxO ou l'activation des facteurs FoxO en l'absence d'IL-2, conduit rapidement à l'arrêt de la prolifération des cellules, puis à leur mort. Nous avons démontré que ceci est dû à la stimulation de l'expression par les facteurs FoxO de p27/Kip1 (le « frein » du cycle cellulaire décrit plus haut) et de Bim (une protéine « tueuse »). Sur la base de nos observations, nous avons proposé que, dans les cellules du système immunitaire, les facteurs FoxO jouent un rôle capital dans la prise de décision concernant l'entrée dansou la sortie du cycle cellulaire.

Mise en perspective de ces recherches.

Nous avons montré que l''AMPc, via PKA, régule indirectement l'expression des gènes de la Cycline D3 et de p27/Kip1. Comme les facteurs de transcription FoxO régulent l'expression de ces mêmes gènes, il serait intéressant à l'avenir d'étudier si les facteurs FoxO régulent leur expression en réponse à des stimulations par l'AMPc dans les cellules du système immunitaire. FoxO est régulé par l'IL-2, et cela résulte dans l'altération de l'expression de p27/Kip1. De là, nous avons émis l'hypothèse que l'activation des facteurs FoxO est importante pour maintenir les cellules dans un état de dormance. Mais, comme nous avons trouvé que les facteurs FoxO, en plus de stimuler la sortie du cycle cellulaire, induisent aussi la mort cellulaire, un arrêt prolongé dans cet état de dormance nécessite aussi la présence d'un mécanisme de protection contre la mort cellulaire. Il est important de noter qu'il est aussi critique pour les cellules du système immunitaire de pouvoir mourir rapidement (on parle de suicide cellulaire) comme de pouvoir rentrer de manière prolongée en dormance. Le premier mécanisme permet de détruire rapidement les lymphocytes T, des cellules hautement réactives, qui sont produites pendant une infection. Le second mécanisme est important pour l'établissement de la mémoire immunologique. Les cellules-mémoire forment une infime proportion des cellules immunitaires d'un individu. Elles représentent la mémoire immunologique de cet individu, c'est-à-dire qu'elles conservent une trace du comment-lutter contre un pathogène qui a déjà agressé cet individu auparavant. Sans elles, les vaccinations n'auraient donc aucun effet. De telles cellules survivent tout au long de la vie de cet individu et se réactivent au moindre contact avec le pathogène pour lequel elles sont spécialisées. Nos résultats suggèrent

que les facteurs FoxO pourraient jouer un rôle dans la destruction des lymphocytes T, ainsi que dans la survie prolongée des cellules mémoire. Il serait intéressant dans le futur de comprendre comment s'effectue la discrimination entre ces deux rôles très distincts.

Régulation de l'expression des gènes impliqués dans la mitose.

Dans la **seconde partie** de cette thèse (**Chapitre 5**), je décris l'étude que j'ai effectué sur les multiples mécanismes de régulation de l'activité du facteur de transcription FoxM1 au cours du cycle cellulaire. Ce facteur de transcription est une protéine qui stimule la prolifération cellulaire. En particulier, FoxM1 est très important pour la régulation et le bon déroulement de la mitose. Il est actif seulement pendant une fenêtre de temps très limitée du cycle cellulaire (phase qui précède la mitose et mitose). La limitation temporelle de l'activité de FoxM1 est très importante pour que la progression du cycle cellulaire se fasse sans heurt. Nous avons montré que la Cycline A, un régulateur du cycle cellulaire, stimule l'activité du facteur FoxM1. Sur la base de nos observations, il semble que la Cycline A soit nécessaire à l'activation de FoxM1, en inactivant une fonction répressive située à l'extrémité (N-terminale) de FoxM1. Nos données montrent que l'activité de FoxM1 n'est pas seulement déterminée par son niveau d'expression. Cela signifie que l'expression de FoxM1, qui est induite pendant la période où l'ADN est dupliqué, n'est pas suffisante pour activer l'expression des gènes qui régulent la mitose. Cela n'est possible qu'après l'activation de FoxM1, vraisemblablement par la Cycline A, qui n'est elle-même active qu'une fois la duplication de l'ADN terminée. De cette manière, les gènes nécessaires pour la mitose ne sont exprimés que lorsque la cellules a terminé de copier son ADN et, de cette manière, la cellule garantit que la mitose ne soitt possible qu'une fois la duplication de l'ADN terminée.
Nous avons trouvé sur FoxM1 des sites potentiels de régulation par une autre protéine régulatrice du cycle cellulaire, Plk-1. Cela suggère que l'activité de FoxM1 peut dépendre d'autres régulateurs du cycle cellulaire qui sont présents une fois l'ADN dupliqué. Plk-1 est une protéine importante pour la convalescence des cellules après que leur ADN ait été endommagé. Dans ces cellules convalescentes, il semblerait que l'activité de FoxM1 soit diminuée, participant au ralentissement du cycle cellulaire nécessaire à ces cellules pour récupérer. Il serait donc intéressant de savoir si Plk-1 est en effet un régulateur de l'activité de FoxM1, en particulier dans ces cellules convalescentes. De plus, parce que l'expression de Plk-1 elle-même dépend en partie de l'activité du facteur FoxM1, cela formerait une intéressante boucle de régulation rétro-active qui régulerait le redémarrage du cycle cellulaire après la réparation de l'ADN endommagé. Enfin, nous avons montré que la stabilité de FoxM1 varie pendant le cycle cellulaire et que 70% de FoxM1 est en fait détruit dès la fin de la mitose. Il semble donc que l'inactivation de FoxM1 en l'absence de Cyclin A ainsi que sa dégradation en fin de mitose soient des mécanismes qui agissent ensemble pour prévenir l'activation inopportune de FoxM1 dans certaines phases du cycle cellulaire.

Rôle du facteur de transcription FoxM1 dans le cancer.

FoxM1 émerge dans la littérature scientifique comme une protéine qui joue un rôle important dans la prolifération des cellules, et en particulier des cellules cancéreuses. De nombreux facteurs mutés dans les cellules cancéreuses sont des régulateurs connus de FoxM1. Dans le futur, il serait donc intéressant d'étudier si FoxM1 joue un rôle dans l'apparition d'un cancer par sa fonction stimulatrice de la prolifération cellulaire, ou alors si FoxM1 peut coopérer avec ces protéines souvent mutées dans les cellules cancéreuses pour stimuler le processus de formation du cancer.

Curriculum Vitae

Marie Stahl was born on October 24th 1977 in Montpellier (France). After finishing her secondary education in 1994 at Foch high school in Rodez (France), she started her academic studies at the Faculty of Medicine, University of Montpellier. Then, she went to the Faculty of Science, University of Montpellier to study Biochemistry, Molecular and Cellular Biology. During her Master, she took part to the Erasmus/Socrated European exchange program in oder to be trained in the lab of René Medema as undergraduate student. After obtaining her Master degree, she decided to stay in the Netherlands to start working as a PhD student in the lab of René Medema. Her research was started in November 2000 at the University Medical Centre of Utrecht (UMC-U), department of Haematology, then at the Dutch Cancer Institute (NKI) in Amsterdam, department of Molecular Biology, from January 2001 till January 2005, and finally finished at the UMC-U, department of Experimental Oncology.

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# These authors contributed equally.

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