Molecular mechanisms of anti-proliferative effects of the Traditional Chinese Medicinal compound Rocaglamide A on cancers

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And how do you benefit if you gain the whole world
but lose your own soul in the process?
Is anything more worth than your soul?

Matthew 16:26

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Abstract

Cell cycle deregulation has been considered as one of the ten described hallmarks of cancer. Targeting cell cycle deregulation is therefore thought to be one of the promising strategies for cancer treatment. In Traditional Chinese Medicine herbal extracts are by far the most common elements used. Several active compounds, the rocaglamide derivatives, have been isolated from the genus *Aglaia* of the plant family Meliaceae. These compounds have been shown to exhibit anti-cancer activities *via* inhibition of tumour proliferation *in vitro* and *in vivo* and, as such, they have the potential to be developed into new anti-cancer drugs.

The rocaglamide derivative Roc A exerts anti-proliferative effects on tumour cells. However, the direct mechanism remained elusive. Therefore, this study aimed at elucidating the mechanism of Roc A-mediated inhibition of tumour cell proliferation. It could be shown that Roc A, the first identified Roc compound, induced G0/G1 cell cycle arrest in haematological cancer cells through two independent pathways. Rapid cell cycle arrest is achieved *via* Cdc25A downregulation. Cdc25A is an essential protein for regulation of G1-S cell cycle transition. Downregulation of this protein will lead to immediate halt in cell cycle progression. Further investigation of the molecular mechanisms by which Roc A downregulates Cdc25A revealed a new signalling pathway triggered by Roc A that resembles the DNA damage response pathway. It could be shown that the cell cycle checkpoint kinases Chk1 and Chk2 are involved in the Roc A-mediated downregulation of Cdc25A. Investigation how the molecular target of Roc A, PHB1, may be involved in the observed cell cycle arrest showed that in PHB1-deficient cells the Erk-pathway is downregulated and through this protein *de novo* synthesis is diminished. This subsequently leads to G0/G1 arrest in PHB1-deficient cells and to decreased expression of cell cycle proteins.

In summary, this study revealed a new Roc A-induced signalling pathway that leads to inhibition of cell proliferation *via* rapid downregulation of Cdc25A. In addition, prolonged cell cycle arrest is achieved through decreased protein *de novo* synthesis of necessary cell cycle proteins. Thus, this study further supports Roc A as a potential new anti-cancer drug.

Zusammenfassung

Die Fehlregulation des Zellzyklus ist eine von zehn beschriebenen Merkmalen von Krebs. Daher ist eine vielversprechende Strategie, in der Krebsbehandlung diese Fehlregulation anzugreifen. Die Traditionelle Chinesische Medizin setzt hauptsächlich auf den Einsatz pflanzlicher Extrakte. Aus der Pflanzenfamilie Meliaceae, insbesondere dem Genus *Aglaia*, wurden verschiedene aktive Wirkstoffe isoliert, die sogenannten Rocaglamide Derivate. Durch ihre proliferationsinhibierende Wirkweise auf Tumorzellen, die in *in vitro* und *in vivo* Studien gezeigt wurde, hemmen sie das Wachstum von Krebs und sind so potentielle, neue Medikamente in der Krebstherapie.

Insbesondere das Rocaglamide Derivat Roc A verfügt über proliferationsinhibierende Effekte auf Tumorzellen, doch bisher war der molekulare Mechanismus unbekannt. Um den Mechanismus der Roc A vermittelten Hemmung der Proliferation von Krebszellen zu untersuchen, wurden verschiedene Krebszelllinien des blutbildenden Gewebes nach Behandlung mit Roc A untersucht. Es wurde gezeigt, dass Roc A durch die Herunterregulation von Cdc25A einen schnellen G0/G1 Zellzyklusarrest induziert. Cdc25A ist ein essentielles Protein, welches den Übergang der G1 in die S Phase im Zellzyklus reguliert. Die Herunterregulation dieses Proteins führt daher zu einem sofortigen Stopp des Zellzyklus. Weitere Untersuchungen am molekularen Wirkmechanismus von Roc A konnten zeigen, dass Roc A einen ähnlichen Signalweg induziert, wie er nach Schädigung von DNA auftritt. Es konnte dargestellt werden, dass die beiden Zellzyklus Checkpoint Kinasen, Chk1 und Chk2, an der Herunterregulation von Cdc25A beteiligt sind. Nachforschungen, auf welche Weise der molekulare Interaktionspartner von Roc A, PHB1, an dem beobachteten Zellzyklusarrest beteiligt ist, haben ergeben, dass in PHB1-defizienten Zellen der Erk-Signalweg herunter reguliert ist. Dies führt zu einer verminderten Proteinneusynthese, welches eine verringerte Expression von wichtigen Proteinen des Zellzyklus zur Folge hat. Hierdurch wird ein langsamerer aber beständiger Zellzyklusarrest ausgelöst.

Zusammenfassend lässt sich sagen, dass in dieser Studie ein neuer von Roc A vermittelter molekularer Mechanismus identifiziert wurde, der durch die Herunterregulation von Cdc25A zu einer schnellen Hemmung der Zellproliferation führt. Ein beständiger Zellzyklusarrest wird durch die Interaktion von Roc A mit seinem molekularen Interaktionspartner PHB1 ausgelöst. Die vorliegenden Untersuchungen fördern den Ansatz, Roc A zu einem neuen, potentiellen Medikament in der Krebstherapie zu entwickeln.

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1 Introduction

1.1 Apoptosis

This chapter is adapted from (Bleumink, 2007), (Sass, 2010), and (Mendelsohn *et al.*, 2008).

One of the fundamental characteristics of multicellular organisms is that some cells must die for proper development and to maintain homeostasis and health (Danial & Korsmeyer, 2004; Krammer, 2000; Thompson, 1995). This propensity to die for the good of the organism has evolved so that cells are systematically dismantled through a complex response termed *programmed cell death* (PCD). In mammals, billions of cells die every day, sustaining an exquisite balance between proper cell proliferation, differentiation, and cell death. A prominent example how PCD is involved in preserving homeostasis of individual tissue can be found in vertebrate development. During the sculpting of fingers the cells between the digits must be cleared out to maintain the correct size of the tissue and its proper function (Glücksmann, 1951). Similarly, cell death plays an important role in the selective removal of autoreactive lymphocytes (Surh & Sprent, 1994; Thompson, 1995) and in regulating blood cell numbers (Krammer, 2000).

As early as 1842, Carl Vogt described that cells can die in a regulated process (Vogt, 1842). Subsequently, the term *apoptosis* (from Greek: apo = off; ptosis = falling; depicting the way leaves fall off a tree) was proposed by Kerr, Wyllie and Currie to describe the process of PCD in 1972 (Kerr *et al.*, 1972). Cells undergoing apoptosis can be distinguished by a set of unique morphologic and biochemical changes from other cell death processes, such as necrosis, necroptosis, paraptosis, autophagy, and others. The classical morphological features of apoptotic cells are nuclear shrinkage (pyknosis), chromatin condensation and DNA fragmentation (karyorrhexis) resolved on gels as characteristic DNA ladders (Robertson *et al.*, 2000; Steller, 1995; Wyllie, 1980). An important feature of apoptotic cells is that their membranes remain intact but they are portioned into many small membrane vesicles, called apoptotic bodies, that contain the cytosol, the condensed chromatin, and organelles. Therefore, apoptotic cell death is considered as non-immunogenic cell death (Savill *et al.*, 1993). In contrast, during necrosis membrane swelling, rupture, and release of intracellular content can be observed, activating the immune system and inducing inflammation (Majno & Joris, 1995). This mainly happens in an uncontrolled fashion resulting from severe and

acute injury (Kroemer *et al.*, 1998); however, controlled necrosis has also been described leading to a similar phenotype as apoptosis (Krysko *et al.*, 2008).

Apoptosis is initiated and executed by the action of certain cysteine proteases called caspases (Thornberry & Lazebnik, 1998). Caspases are synthesised as inactive zymogens, called pro-caspases, and upon activation they cleave substrates on the carboxyl-side of an aspartate residue (Cohen, 1997; Stennicke & Salvesen, 1998; Thornberry & Lazebnik, 1998). Until now, 14 mammalian caspases have been identified, and these can be subdivided into the two classes of initiator and effector caspases based on their function in apoptosis. Upon death signals, the initiator caspases, such as caspase-8, -9, and -10, are processed and cleaved. In turn, they activate the effector caspases, including caspase-3, -6, and -7, which ultimately leads to the cleavage of specific substrates such as nuclear LAMINS or CAD (caspase-activated deoxyribonuclease, DFF40). Nuclear LAMINS are involved in chromatin condensation and nuclear shrinkage, and CAD causes the release of the endonuclease, which travels to the nucleus to fragment DNA (Igney & Krammer, 2002). Induction of apoptosis and activation of caspases can be achieved by external or internal stimuli *via* the induction of two major pathways, namely the death receptor (extrinsic) cell death pathway which is initiated by engagement of extracellular death receptors (DRs), or the mitochondrial (intrinsic) cell death pathway which is mainly dependent on mitochondrial changes. An overview is shown in Figure 1.1.

Apoptosis must be tightly regulated, as deregulation leads to a variety of disorders. Several diseases are associated with the induction of too much apoptosis including the acquired immunodeficiency syndrome (AIDS), neurodegenerative disorders such as Parkinson's, Alzheimer's, and Huntington's disease, and ischemic injury. Failure in the apoptotic process may result in autoimmune diseases like Lupus erythematosus or Atopic dermatitis, and the spreading of viral infections and cancer (Thompson, 1995). In cancer, the resistance of tumour cells to death is not complete, but rather confers an enhanced ability to survive under conditions of cellular stress. In particular, late-stage, metastatic malignancies correlate with acquired resistance to apoptosis (Mendelsohn *et al.*, 2008). The major difficulty in cancer treatment is to induce selective apoptosis in cancer cells over normal, healthy tissue.

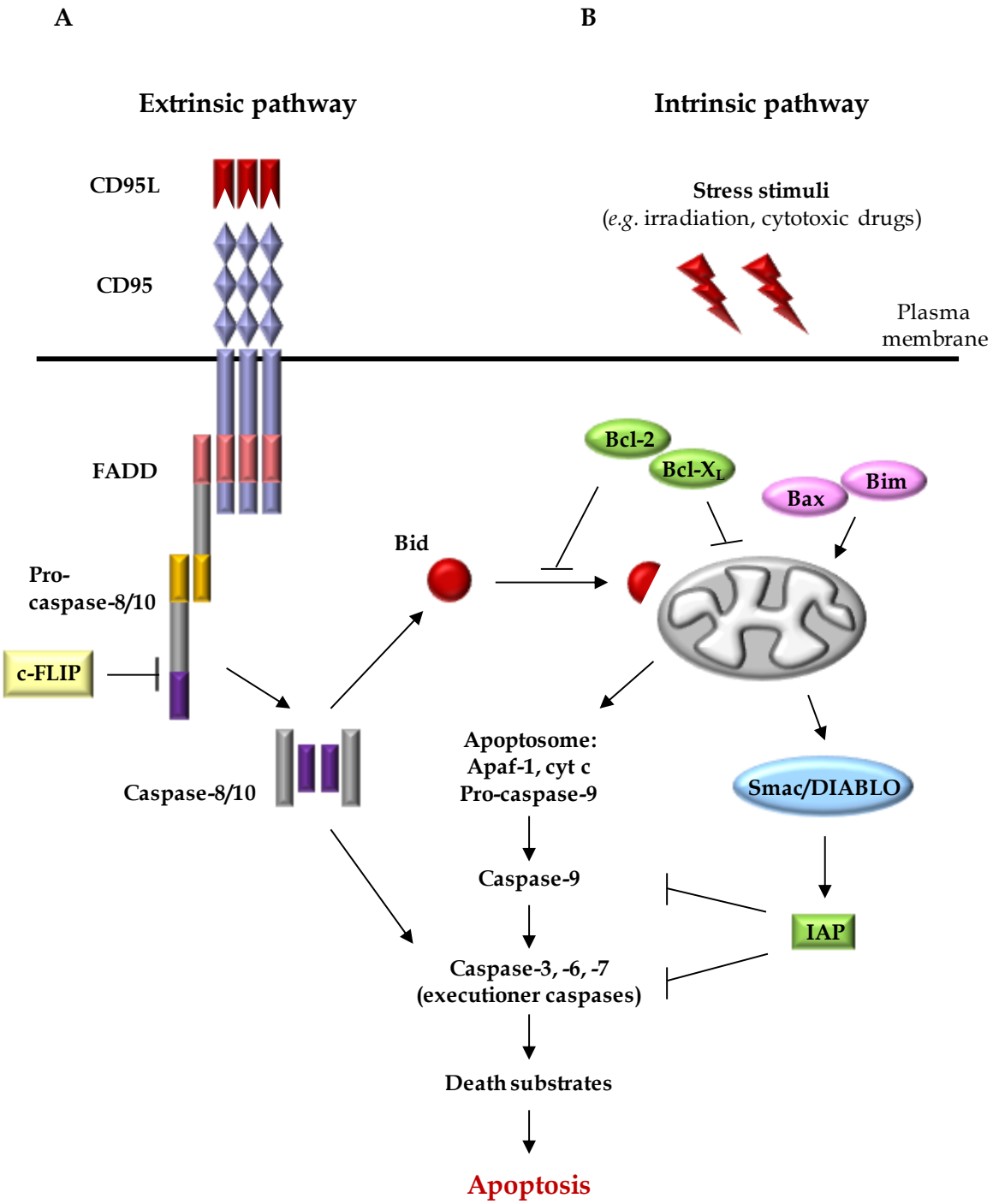


Figure 1.1 | The two main apoptotic signalling pathways.

(A) The extrinsic apoptotic pathway: apoptosis signalling upon engagement of death receptors. **(B) The intrinsic apoptotic pathway**: apoptosis signalling through mitochondria. See text for details. Adapted from (Igney & Krammer, 2002).

1.1.1 The extrinsic apoptotic pathway

Initiation of the apoptotic program *via* the extrinsic apoptotic pathway relies on the interactions of extracellular ligands with specific transmembrane DRs, which are characterised by a cysteine-rich extracellular domain and a short cytoplasmic (~80 kDa residue) domain that contains the death domain (DD) (Itoh & Nagata, 1993; Klein *et al.*, 2002; Lorenzo & Susin, 2004; Tartaglia *et al.*, 1993). The best characterised members of this family include tumour necrosis factor-α receptor 1 (TNF-R1), CD95 (Apo-1/Fas), TRAIL (TNF-related apoptosis-inducing ligand)-R1 and -R2, DR3, DR6, as well as the p75 nerve growth factor receptor (reviewed in Debatin & Krammer, 2004). Ligands (L) for these DR, such as TNF-α, CD95L (Apo-1L/FasL/CD178) or TRAIL, are type II transmembrane proteins. They belong to the TNF/NGF (nerve growth factor) superfamily (Smith *et al.*, 1994) and play important roles in tissue homeostasis, in the immune system, in T cell mediated cytotoxicity, in deletion of activated T cells after an immune response, and in eliminating auto-reactive lymphocytes in the periphery (Ashkenazi & Dixit, 1998). For example, mice lacking functional CD95 (lpr/lpr) (lymphoproliferation) show some of the phenotypes of the autoimmune disease lupus, and similar to *lpr*$^{-/-}$ mice, mice lacking functional CD95L (gld/gld) (generalised lymphoproliferative diseases) fail to remove autoreactive lymphocytes from their immune systems appropriately (Van Parijs & Abbas, 1996). In humans a similar disease with a dysfunction of the CD95 system is found, called autoimmune lymphoproliferative syndrome (ALPS). These patients show massive, non-malignant lymphadenopathy, an altered and enlarged T cell population, and severe autoimmunity (Lenardo, 2003).

CD95 is one of the most studied death receptors and was discovered by the generation of monoclonal antibodies which induced apoptosis in various human cell lines (Trauth *et al.*, 1989; Yonehara *et al.*, 1989). CD95 is a type I transmembrane glycoprotein and has a molecular mass of approximately 45 to 52 kDa (Itoh *et al.*, 1991; Oehm *et al.*, 1992). CD95 is expressed in many tissues with the highest expression in thymus, heart, lung, and liver tissue. Under normal physiological conditions CD95-mediated apoptosis is triggered by its natural ligand, CD95L. CD95L is exclusively expressed on activated T and NK cells as well as on cells of immune privileged sites, such as the anterior chamber of the eye or the testes (Griffith *et al.*, 1995; Yu *et al.*, 1999). It is a type II membrane protein of the TNF family and has a molecular mass of 40 kDa (Suda *et al.*, 1993; Takahashi *et al.*, 1994; Yu *et al.*, 1999). Its expression can be induced in T cells through activation of the T cell receptor. Cleavage of the

transmembrane form by metalloproteases generates the soluble form of CD95L (Kayagaki *et al.*, 1995; Mariani *et al.*, 1995; Takahashi *et al.*, 1994). The function of soluble CD95L remains elusive. It was reported that the soluble ligand may only be efficient in triggering apoptosis at very high concentrations (Bouillet & O'Reilly, 2009; Schneider *et al.*, 1998).

Engagement by its ligand leads to trimerisation of CD95, which facilitates the binding of the adaptor molecule FADD (Fas-associated death domain containing protein). Following recruitment to the receptor, FADD forms higher-order oligomers, which in turn recruit *via* its death effector domain (DED) other DED-containing molecules, such as procaspase-8/10 or c-FLIP (Boldin *et al.*, 1996; Muzio *et al.*, 1996) to form the death inducing signalling complex (DISC) (Kischkel *et al.*, 1995; Peter & Krammer, 2003). At the protein level, three isoforms of the c-FLIP family have been identified: c-FLIP long, c-FLIP short and c-FLIP Raji (Krammer *et al.*, 2007; Krueger *et al.*, 2001). C-FLIP$_L$ is known to be anti-apoptotic but under certain conditions may function as a proliferation factor by activating NF-kB. In contrast, c-FLIP$_S$ is solely anti-apoptotic by itself or through sensitising c-FLIP$_L$ (Krammer *et al.*, 2007; Fricker *et al.*, 2010). Procaspase-8 normally exhibits low levels of activity, yet the DISC provides a scaffold that facilitates its self-cleavage (Chang *et al.*, 2003; Hughes *et al.*, 2009). Activated caspase-8 then functions as a heterotetramer, consisting of two small subunits (p10) and two large subunits (p18), and transmits apoptotic signalling *via* cleavage and activation of effector kinases such as caspase-3, -6, and -7. In cells that contain too low amounts of caspase-8 to activate procaspase-3 directly, signal amplification *via* caspase-8-mediated cleavage of Bid and subsequent mitochondrial permeabilisation can occur, triggering the apoptotic cascade *via* the activation of procaspase-9 (Gross *et al.*, 1999; Luo *et al.*, 1998; Korsmeyer *et al.*, 2000).

1.1.2 The intrinsic apoptotic pathway

The intrinsic apoptotic pathway is regulated by the B-cell lymphoma 2 (Bcl-2) proteins and can be induced by a variety of stimuli. This includes the deprivation of nutrients or essential survival factors, as well as upon exposure to DNA damage, toxins, irradiation, hypoxia, or oxidative stress (Erlacher *et al.*, 2005; Norbury & Zhivotovsky, 2004; Takahashi *et al.*, 2004). The founding member of this family, *Bcl-2*, was identified as an overexpressed gene found in the t(14:18)(q32;q21) translocation of follicular B-cell lymphoma (Bakhshi *et al.*, 1985). By now more than 20 Bcl-2 family members have been identified and it has become evident that they all share α-helical domains (BH1-4) homologous to those present in Bcl-2.

They can be subdivided into three groups on the basis of their structure and anti- or pro-apoptotic functions (Cory & Adams, 2002; Gross *et al.*, 1999; Strasser, 2005). Anti-apoptotic Bcl-2 family members, such as Bcl-X_L, myeloid cell leukemia sequence 1 (Mcl-1), Bcl-w, and Bcl-2, contain all four BH domains, and the BH4 domain is specifically required for their anti-apoptotic functions. The second group consists of the pro-apoptotic Bcl-2 family members Bcl-2-associated X protein (Bax), Bcl-2 homologous antagonist killer (Bak) and Bcl2-related ovarian killer (Bok), which contain BH1-3 domains. Members of both of these groups usually have a transmembrane domain in their C-terminus, and they regulate the release of calcium from the endoplasmatic reticulum (ER) and pro-apoptotic molecules such as cytochrome c (cyt c), Smac/DIABLO (Second Mitochondria-derived Activator of Caspases/Direct IAP Binding Protein with Low PI), and Omi/HtrA2 (Omi/high temperature requirement A2) from mitochondria. The third group consists of BH3-only family members, such as Bax, Bad, Bak, BH3 interacting domain death agonist (Bid), Bim, p53 upregulated modulator of apoptosis (Puma), and Noxa, among others, which function as signalling entities that tip the balance towards death in response to intrinsic cell death triggers.

Upon apoptosis induction, the transmembrane domain of Bax inserts into the mitochondrial outer membrane, and it then oligomerises to initiate membrane permeabilisation. This then leads to the release of cyt c and other pro-apoptotic proteins (Green & Kroemer, 2004). In the cytosol, cyt c triggers the formation of a large multimeric complex in an ATP-dependent manner. The apoptosome consists of the adapter protein Apaf-1, procaspase-9, and cyt c itself. Procaspase-9 is activated at the apoptosome and can therefore initiate the caspase signaling cascade by cleavage of caspase-3 (Adams & Cory, 2002; Zou *et al.*, 1999), ultimately leading to cell death induction. In addition, several other factors, such as AIF (Klein *et al.*, 2002; Lipton & Bossy-Wetzel, 2002; Susin *et al.*, 1999) or endonuclease G (Li *et al.*, 2001; Parrish *et al.*, 2001) are released from mitochondria into the cytosol, contributing to apoptosis.

The death receptor pathway and the mitochondrial apoptotic pathway are linked by the pro-apoptotic BH3 only family member, Bid. Upon stimulation, Bid is cleaved by caspase-8 to a truncated form (tBID), which translocates to the mitochondria where it acts together with the pro-apoptotic Bcl-2 family members Bax and Bak to induce the intrinsic cell death pathway (Igney & Krammer, 2002; Krammer *et al.*, 2007; Scaffidi *et al.*, 1998).

1.2 Regulation of the cell cycle

This chapter is partly adapted from (Mendelsohn *et al.*, 2008).

1.2.1 Basic principles of cell cycle progression

The essential function of cell cycle control is the regulated duplication of the cells' genetic material and its division and distribution to each daughter cell, such that one copy is provided to each cell upon cell division. The cell cycle can be divided into two sections: the interphase, during which the cell grows and duplicates its DNA, and the M (mitosis) phase, during which the cell is split into two distinct cells. M phase itself is composed of two processes: mitosis, in which the nucleus divides and the cell's chromosomes are divided between the two daughter cells, and cytokinesis, in which the cell splits into two and the cell's cytoplasm is divided into two and forms distinct cells. The interphase encompasses the remaining three phases of the cell cycle: G1 phase (G = gap), S phase (synthesis) and G2 phase (Fig. 1.2). During S phase, the cell replicates its DNA, an essential prerequisite for cell division. S phase is flanked by two phases in which the cell continues to grow. The G1 phase is the interval between the completion of M phase and the beginning of S phase. The G2 phase is the interval between the end of S phase and the beginning of M phase. G0 or quiescence occurs when cells exit the cell cycle due to the absence of growth-promoting signals or the presence of pro-differentiation signals.

To monitor and regulate proper cell cycle progression, the cell uses so called *cell cycle checkpoints* (Fig. 1.2). Three cell cycle checkpoints (G1-S, intra-S and G2-M) control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity (Elledge, 1996). Checkpoint loss results in genomic instability and has been implicated in the evolution of normal cells into cancer cells (Meeran & Katiyar, 2008).

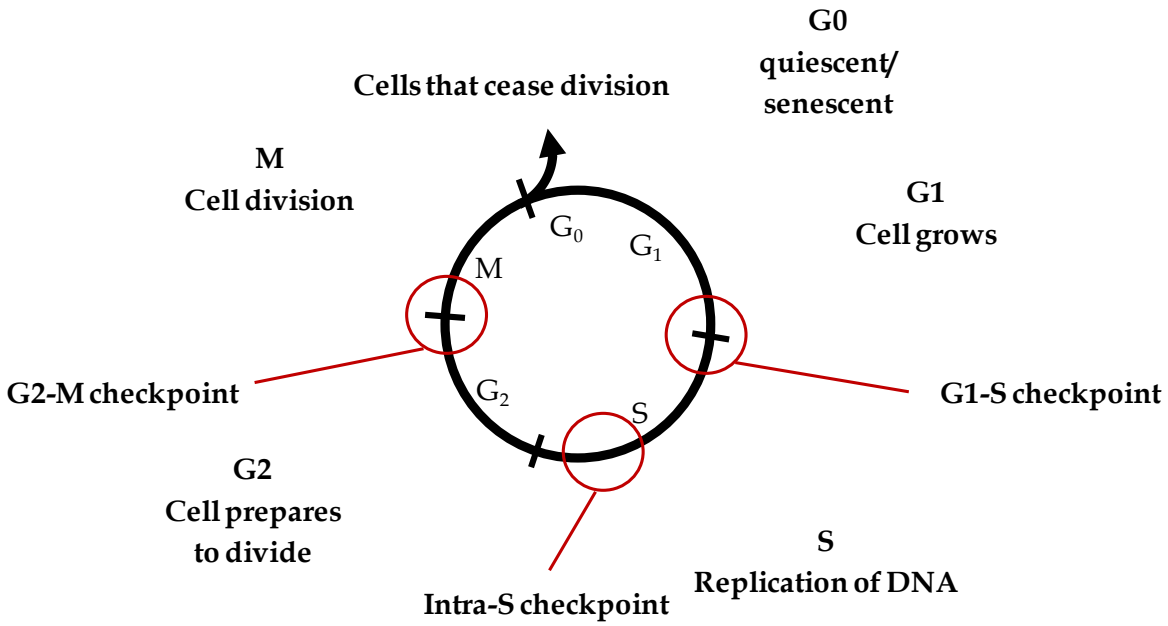


Figure 1.2 | The mammalian cell cycle.

The cell cycle consists of four distinct phases: G1 phase (G = gap), S phase (synthesis), G2 phase and M phase (mitosis). Three cell cycle checkpoints (G1-S, intra-S and G2-M) control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity.

1.2.2 Cyclin-dependent kinases

Progression through the cell cycle is tightly regulated by a set of kinases termed cyclin-dependent kinases (Cdks). Cdks are heterodimeric complexes composed of a catalytic kinase subunit and a regulatory cyclin subunit (Deep & Agarwal, 2008; Schwartz & Shah, 2005; Vermeulen *et al.*, 2003). Cdk subunits associate with specific cyclins during distinct phases of the cell cycle, by which they get activated. As active protein kinases they then trigger transition through cell cycle phases. Although some Cdks can form complexes with multiple cyclins, in most cases active complexes rely on specific cyclin-Cdk interactions.

Mammals express approximately twenty Cdk-related proteins, of which Cdk1-11 have been extensively studied (Satyanarayana & Kaldis, 2009). The associated cyclins can be divided into four major classes: D-, E-, A- and B-type cyclins (Satyanarayana & Kaldis, 2009). In mammalian cells, cyclin B resides mainly in the cytoplasm, whereas cyclins A, D and E show nuclear localisation (Ohtsubo *et al.*, 1995; Pines & Hunter, 1994; Sherr, 1993). D-type cyclins (D1, D2 and D3) are expressed in a variety of cell types and tissues with cyclin D1 being the most ubiquitously expressed (Waclaw & Chatot, 2004). The activities and functions

of cyclin/Cdk complexes are regulated by both inhibitory and activating phosphorylations at various sites, as well as by two families of cyclin-dependent kinase inhibitors (CKIs).

1.2.2.1 Regulation of Cdks by small-polypeptide inhibitors

CKI proteins consist of inhibitor of kinase 4/alternative reading frame (Ink4a/Arf) and Cdk interacting protein/Kinase inhibitory protein (Cip/Kip) family members (Deep & Agarwal, 2008; Schwartz & Shah, 2005; Shapiro, 2006). CKIs can directly bind to and inactivate cyclin/Cdk complexes. The Ink4 bind exclusively to G1 phase cyclin D/Cdk4/6 complexes and directly inhibit their activity (Sherr & Roberts, 1999). The Ink4a/Arf family of proteins consists of four members: $p16^{Ink4a}$, $p15^{Ink4a}$, $p18^{Ink4a}$, and $p19^{Ink4a}$, which are homologous in their primary structure. Further, they share similar biochemical activities and comparable tertiary structures. However, their regulation is distinct and expression can be triggered by *e.g.* Ras overexpression (Serrano *et al.*, 1997), retinoblastoma protein (pRB) inactivation or can as well be promoted by p53 (Shapiro *et al.*, 1995).

The Cip/Kip family of CKIs includes three members: $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, which can bind to and inhibit various Cdks. The proteins are highly homologous and share approximately 50% of their sequence (Mendelsohn *et al.*, 2008). In particular, in response to DNA damage and genotoxic stress $p21^{Cip1}$ levels are upregulated by p53 activation, which leads to G1 cell cycle arrest (Sherr & Roberts, 1999). Nevertheless, it has now become clear that $p21^{Cip1}$ can also be regulated *via* several p53-independent pathways, such as transcriptional regulation by *MYC* (Abukhdeir & Park, 2008; Coller *et al.*, 2000).

1.2.2.2 Positive and negative regulation of Cdks by phosphorylation

Besides direct interaction of CKIs with Cdks, the activity of Cdks can also be inhibited by phosphorylation at Thr14 and Tyr15 which is mediated by two protein kinases, namely Wee1 and Myt1 (Bartek & Lukas, 2007; Gutierrez & Ronai, 2006). Phosphorylation of Cdk1 and Cdk2 contributes to their timed activation and inactivation during normal cell cycle progression. In contrast, Cdk4 and Cdk6 protein kinases appear to be subject to this inhibitory phosphorylation only when cells are exposed to DNA damage (Terada *et al.*, 1995). Dephosphorylation of Thr14 and Tyr15 is mediated by the cell division cycle 25 (Cdc25) family of proteins, which leads to Cdk activation (see below for more details).

Activating phosphorylations (Thr161 in Cdk1, Thr160 in Cdk2 and Thr172 in Cdk4) are mediated by Cdk-activating kinase (CAK) and are required for the complete activation of Cdks (Kaldis, 1999). In mammalian cells, CAK itself is a cyclin-dependent kinase composed of cyclin H and Cdk7 (Mäkelä *et al.*, 1994). Throughout the cell cycle, CAK contributes constitutively to Cdk activation following cyclin binding. Cyclin H/Cdk7 complexes are also involved in transcriptional activation by phosphorylating multiple serine/threonine residues located in the carboxyl-terminal domain (CTD) of the largest subunit of DNA polymerase II (RNAPII) (Feaver *et al.*, 1994; Shiekhattar *et al.*, 1995).

1.2.3 Transcriptional regulation by E2F transcription factors

E2F was originally identified as a cellular DNA binding factor that regulated expression of the viral E2 promoter (Yee *et al.*, 1987; Kovesdi *et al.*, 1987). Further studies revealed that in mammals the E2F family comprises eight genes (*E2F1-8*), which give rise to nine distinct proteins (DeGregori & Johnson, 2006; Polager & Ginsberg, 2009). They can both transactivate and repress gene expression to regulate a wide range of biological processes, including mitosis, the function of DNA damage checkpoints, DNA replication and repair, differentiation, autophagy, and apoptosis (Dimova & Dyson, 2005; Polager & Ginsberg, 2008).

For transcriptional control E2F associates with the DNA as a heterodimer with its two binding partners DP1 and DP2. In addition to DP1/2, E2F complexes are further modulated by members of the pRB family of proteins (pRB, p107, p130). All proteins of this family can inhibit E2F-responsive promoters, actively repress transcription, and thereby arrest cellular growth (Claudio *et al.*, 1994; Dimova & Dyson, 2005).

1.2.4 Regulation of cell cycle progression

1.2.4.1 G1 regulation/restriction point control

During G1 phase cells prepare for DNA replication and synthesise proteins that are necessary for genome replication. Once this is finished, all necessary components of the DNA replication machinery need to be assembled on the chromatin at what are called the *origins of replication*. When cells enter from quiescence into early G1 phase to prepare for DNA replication, Cdk4 and/or Cdk6 form active complexes with D-type cyclins (Fig. 1.3). This

step is dependent on the presence of nutrient and growth factors to ensure an environment that supports cell division. In G1 phase progression pRB family proteins are key players that regulate cell cycle. In the resting or quiescent state pRB family proteins are in a hypophoshorylated, active form and bind to E2F transcription factors, thereby repressing E2F-dependent transcription. Upon growth factor signalling, cyclin D/Cdk4/6 complexes are activated and initiate the phosphorylation and inactivation of the pRB family proteins (Sherr & Roberts, 1999; 2004; Meeran & Katiyar, 2008). This leads to the release of E2F transcription factors and results in the activation and transcription of E2F responsive genes required for cell-cycle progression, such as DNA replication complexes and cyclin E and A (Dyson, 1998; Lundberg & Weinberg, 1998). In late G1 phase, Cdk2 is activated by binding to cyclin E (Sherr & Roberts, 1999; 2004; Meeran & Katiyar, 2008), thereby increasing the phosphorylation of pRB family proteins on additional sites. This leads to irreversible initiation of the gene expression program required for S phase and to passage through the G1-S restriction point, also known as the G1-S checkpoint (Fig. 1.3). At this cell cycle checkpoint the key decision is made whether the cell will divide, delay division, or enter a resting stage. It is therefore also called the *point of no return*. Alterations and mutations in the key regulatory players of G1-S transition are frequently observed in cancer, allowing cells to proliferate independently of growth factor stimuli (Mendelsohn *et al.*, 2008).

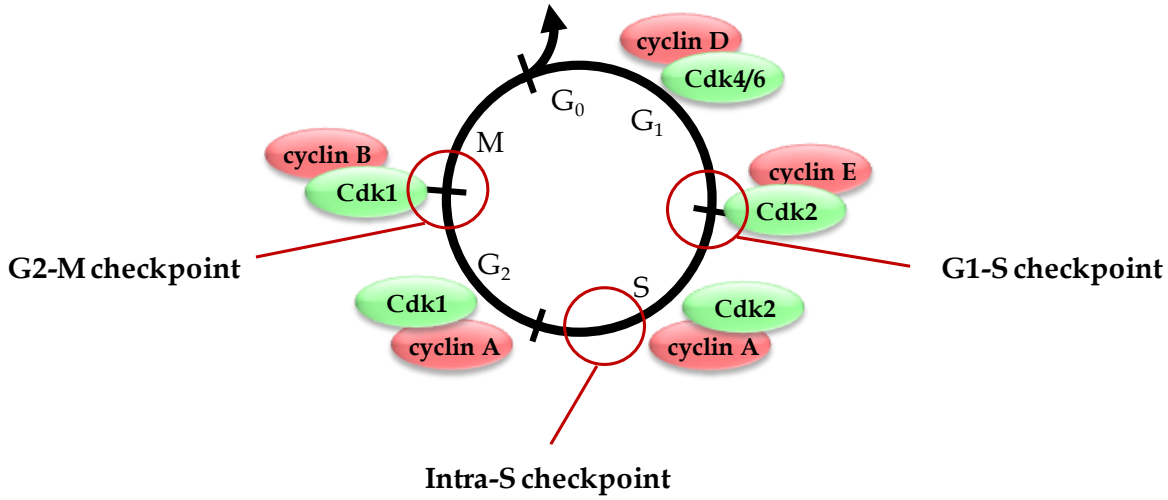


Figure 1.3 | Regulation of the cell cycle.

The cell cycle is tightly regulated by cyclin/Cdk complexes. When cells enter from quiescence into early G1 phase, Cdk4 and/or Cdk6 form active complexes with D-type cyclins. In late G1 phase, Cdk2 is activated by binding to cyclin E, which leads passage through the G1-S checkpoint. For S phase progression cyclin A/Cdk2 complexes are required to enable DNA replication. In late S and beginning of G2 phase cyclin B is expressed and associates with Cdk1, together they regulate the transition of G2 into M phase. See text for more details.

1.2.4.2 Regulation of DNA replication (S phase)

For S phase progression cyclin A/Cdk2 complexes are required to enable DNA replication (Fig. 1.3) and to create exactly two identical semi-conserved chromosomes (Schwartz & Shah, 2005; Shapiro, 2006). DNA replication is started at the origins of replication, which must first be established in G1 phase prior to S phase entry. Origin of replication complexes (ORC) are constitutively bound to DNA throughout the cell cycle and must first be associated with chromatin to then enable the formation of the pre-replication complex (pre-RC). Formation of the pre-RC involves the recruitment of Cdc6 to the ORC. Cdc6 subsequently recruits the minichromosome maintenance (MCM) complex and Cdt1 (Bell & Dutta, 2002). The MCM complex functions as the putative replicate helicase, however, MCMs are not stably bound in this step of pre-RC formation. In an ATPase-dependent manner Cdc6 loads the MCM complex stably, which results in the release of Cdt1 (Tye, 1999). Once the DNA is licensed to replicate, pre-RCs are phosphorylated by Dbf4/Cdc7 and cyclin/Cdk complexes. Additional factors are recruited, such as MCM10, that recruits Cdc45 and activates the origin. Activation of the pre-RC complex triggers unwinding of the replication origin and loading of the single-stranded DNA binding proteins Replication Protein A (RPA), DNA polymerase α and primase (Woo & Poon, 2003), which subsequently leads to the replication start.

1.2.4.3 G2-M transition regulation

In late S and beginning of G2 phase, cyclin B is expressed and associates with Cdk1, and together they regulate the transition of G2 into M phase (Fig. 1.3). However, the complexes remain inactive until late G2 when their activation is required for entry into mitosis (Matsusaka & Pines, 2004; Obaya & Sedivy, 2002). The onset of mitosis is triggered by dephosphorylation of Cdk1 by a Cdc25 isoform (see below) and includes increased nuclear transport and decreased nuclear export of cyclin/Cdk1 complexes. In addition to Cdk1, accurate mitotic progression needs a second family of kinases, termed polo-like kinases (Plks). Plks are a family of conserved serine/threonine kinases and in mammals, four Plks (Plk1-4) are known. Plks are involved in the mitotic processes of centrosome maturation, bipolar spindle formation, activation of the Anaphase-Promoting Complex (APC/C^{Cdh1}), chromosome segregation, and actin ring formation (cytokinesis) (Glover *et al.*, 1998; van de Weerdt & Medema , 2006).

The primary goal of mitosis is to ensure that each daughter cell receives one pair of chromosome after cellular division. This implicates that a cell can only divide after chromosomes are attached to the microtubules of the mitotic spindle. The mitotic checkpoint, also known as the G2-M checkpoint (Fig. 1.3), insures that cells do not initiate mitosis with damaged DNA after replication. Cells that have a defective G2-M checkpoint enter mitosis before repairing their DNA through inducing death after cell division (Mendelsohn *et al.*, 2008).

1.3 Cdc25A function and regulation during cell cycle

The Cdc25 family of proteins are highly conserved dual specificity phosphatases, which regulate progression through cell cycle by activating cyclin/Cdk complexes. Cdc25 was first identified in fission yeast as a factor required for entry into mitosis (Russell & Nurse, 1986). Subsequently, three mammalian isoforms have been identified: Cdc25A, Cdc25B and Cdc25C (Galaktionov & Beach, 1991; Nagata *et al.*, 1991; Sadhu *et al.*, 1990). Orthologues of these isoforms have been found in *Xenopus laevis* (Cdc25A and Cdc25C) and in chicken (*Gallus gallus*; Cdc25A and Cdc25B) (Bénazéraf *et al.*, 2006; Izumi & Maller, 1993; Okazaki *et al.*, 1996).

In mammalian cells, Cdc25A, Cdc25B, and Cdc25C display distinct expression patterns and specificity for cyclin/Cdk complexes (Draetta & Eckstein, 1997; Fernandez-Vidal *et al.*, 2008). While Cdc25B and Cdc25C play important roles at the G2/M transition and during mitosis by dephosphorylating and activating their respective Cdk substrates (Gabrielli *et al.*, 1996; Lammer *et al.*, 1998; Millar *et al.*, 1991), Cdc25A, apparently has a more general function. During G1–S transition Cdc25A mainly activates the cyclin E/Cdk2 and cyclin A/Cdk2 complexes (Blomberg & Hoffmann, 1999; Hoffmann *et al.*, 1994; Jinno *et al.*, 1994) but it also has a role in the G2–M transition by activating cyclin B/Cdk1 complexes (Molinari *et al.*, 2000; Zhao *et al.*, 2002). Importantly, *Cdc25A*$^{-/-}$ mice display early embryonic lethality (Ray *et al.*, 2007) indicating the absence of compensation by Cdc25B or Cdc25C isoforms and a non-redundant role in mice. It appears from these data that Cdc25A is solely indispensable to drive cell cycle progression.

Cdc25A is tightly regulated at the protein level, being periodically synthesised and degraded *via* ubiquitin-mediated proteolysis (Donzelli *et al.*, 2002). Therefore, Cdc25A is a very short-lived protein. In late G1 phase, Cdc25A accumulates as a result of E2F1- and c-Myc-mediated transcriptional activation (Galaktionov *et al.*, 1996; Jinno *et al.*, 1994; Vigo *et al.*, 1999). Subsequently, Cdc25A dephosphorylates Cdk2, which activates cyclin E/Cdk2 complex. This creates an autoamplification loop that further phosphorylates and activates Cdc25A and thereby contributes to S phase progression (Hoffmann *et al.*, 1994; Zou & Stillman, 1998).

Regulation of Cdc25A protein levels takes place by two E3 ubiquitin ligase complexes: the APC/C^{Cdh1} and the Skp1/Cullin/F-box (SCF$^{\beta TrCP}$) protein complexes, each acting at distinct stages of the cell cycle (Busino *et al.*, 2003; Donzelli *et al.*, 2002). In unperturbed cells, Cdc25A

binding to $SCF^{\beta TrCP}$ in S and G2 phase requires the phosphorylation of serine residues within a so-called DSG motif that is mediated by the checkpoint kinases Chk1 and/or Chk2 and another yet unknown kinase (Boutros *et al.*, 2007; Busino *et al.*, 2004; Busino *et al.*, 2003; Jin *et al.*, 2003). Interaction with APC/C^{Cdh1} at the late state of mitosis is dependent on a KEN-box motif (Fig. 1.4) (Boutros *et al.*, 2007; Busino *et al.*, 2004). During mitosis, Cdc25A is phosphorylated by cyclin B/Cdk1 complexes on two specific serine residues (Ser18 and Ser116), which results in its stabilisation and uncoupling from its ubiquitin-mediated turnover (Fig. 1.4) (Busino *et al.*, 2004; Mailand *et al.*, 2002). This creates a positive feedback loop that allows Cdc25A to dephosphorylate and further activate Cdk1, leading to APC/C^{Cdh1}–mediated ubiquitylation of Cdc25A and its post-mitotic degradation (Donzelli *et al.*, 2002). Now, the start is set for another round of replication when, in response to transcriptional induction, Cdc25A protein begins to accumulate again at G1-S transition.

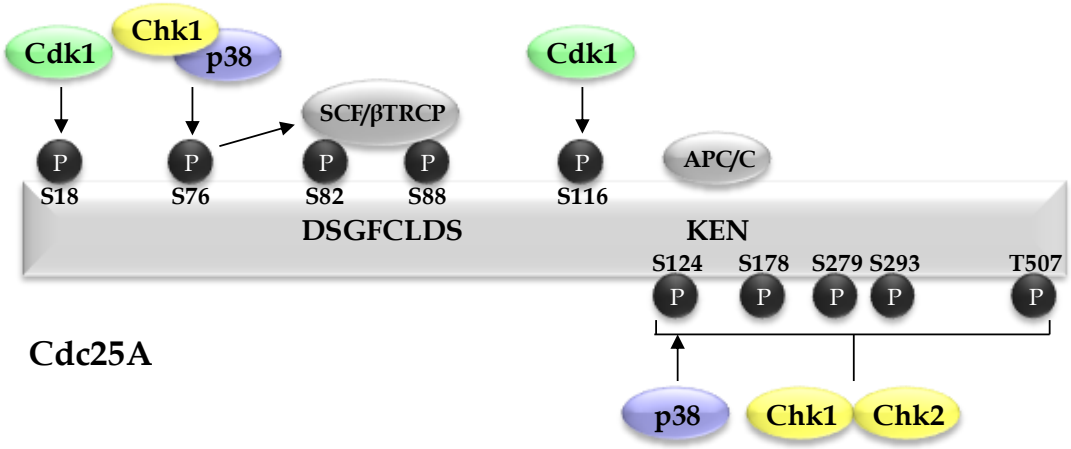


Figure 1.4 | Multiple key phosphorylation events regulate Cdc25A levels.

Cdc25A is phosphorylated at serine and threonine by multiple kinases that regulate its activity, interactions with other proteins, and intracellular localisations (S = serine, T = threonine). Degradation by the $SCF^{\beta TrCP}$ or by the APC/C–ubiquitin (Ub)-dependent degradation pathways is also controlled by the indicated phosphorylation events. In mitosis, Cdc25A is stabilised by Cdk1-mediated phosphorylation at indicated sites. Upon DNA damage or osmotic stress Cdc25A is phosphorylated by Chk1, Chk2 and/or p38 at indicated sites. Modified from (Boutros *et al.*, 2007).

1.4 The DNA damage response pathway

The DNA damage response (DDR) pathway displays a sophisticated signal transduction network that enables cells to sense DNA damage and to mount an appropriate response. It consists of sensors, mediators, and effectors (Fig. 1.5). Deregulation of the DDR leads to genomic instability and cancer. Therefore, depending on the cellular background and extent of DNA damage, the DDR triggers either cell cycle checkpoint arrest and DNA repair, or in the case of irreparable damage, inactivation of the cells by senescence or removal by apoptosis (Bitomsky & Hofmann, 2009). Cell cycle checkpoints supervise the structural integrity of chromosomes before progression through crucial cell cycle stages (Canman *et al.*, 1998; Hartwell & Kastan, 1994; Zhou & Elledge, 2000). The DDR pathway facilitates communication between damage recognition proteins and the checkpoint machinery to trigger arrest of cell cycle progression and thereby increases the opportunity for repair before activating important events, such as replication or mitosis (Löbrich & Jeggo, 2007; Terzoudi *et al.*, 2005).

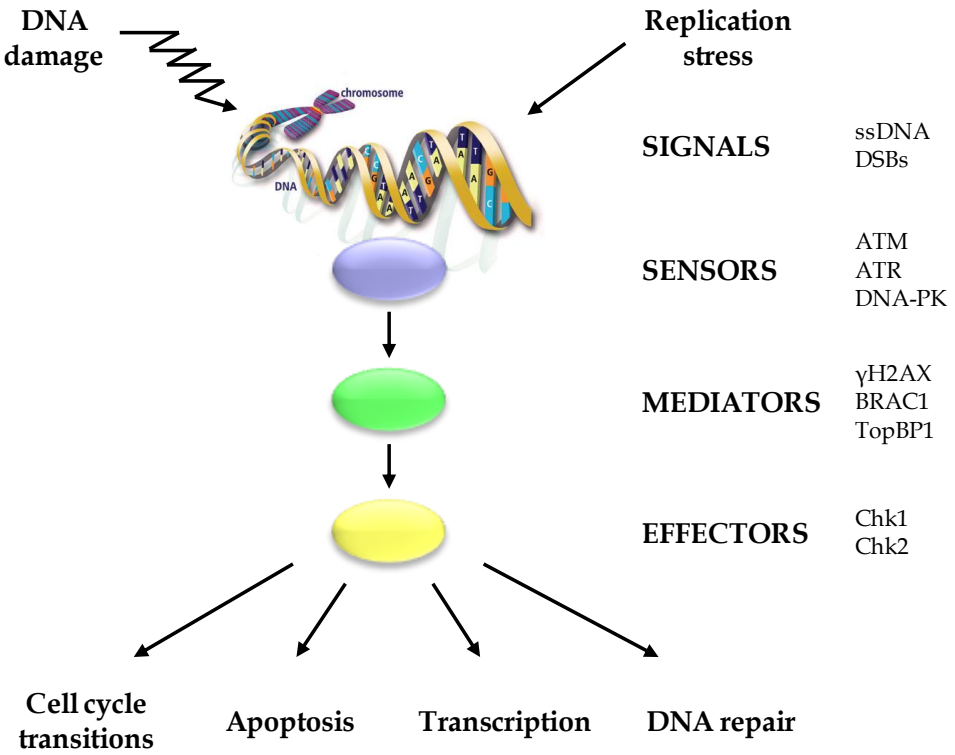


Figure 1.5 | The DNA damage response pathway.

The DNA damage response pathway is a signal transduction pathway consisting of sensors, mediators, and effectors that modulate cell cycle transitions, apoptosis, transcription, or DNA repair. See text for more details. Adapted from (Shiloh, 2003; Zhou & Elledge, 2000).

1.4.1 Activation of cell cycle checkpoints

1.4.1.1 Sensors of DNA damage

A complex cellular network of mechanisms has evolved to maintain the integrity of genetic information after genotoxic stress. Multiple checkpoint pathways are activated in response to DNA damage in order to block the G1–S or G2–M transitions or S phase progression to minimise the risks of transmitting mutations to the cells' progeny. The upstream factors that initiate a checkpoint response are the sensor phosphatidylinositol 3-kinase-related kinases (PI3Ks) ATM (ataxia-telangiectasia mutated), ATR (ataxia-telangiectasia and Rad3-related) and DNA-PK (DNA-dependent protein kinase) (Fig. 1.5). Upon sensing DNA damage, these kinases are believed to be phosphorylated (Bakkenist & Kastan, 2003; 2004; Chan *et al.*, 2003; Liu *et al.*, 2011) and subsequently to phosphorylate a large number of substrates, such as BRCA1, TopBP1 or NBS1 (Hurley & Bunz, 2007). ATM is primarily activated in response to double strand DNA breaks (DSBs), whereas ATR is mainly activated by breaks in single-stranded DNA (ssDNA) or at stalled DNA replication forks. Nevertheless, redundancy and cooperation between the ATM and ATR signalling pathways were reported *in vitro* (for review see Hurley & Bunz, 2007). DNA-PK also cooperates with ATR and ATM to phosphorylate proteins involved in DNA damage checkpoint control but has a more pronounced role in DNA repair (Meek *et al.*, 2008). Various mediators (Fig. 1.5) facilitate the activation of ATM and ATR proteins, which then in turn phosphorylate additional downstream kinases, such as checkpoint kinases Chk1 and Chk2 (Niida & Nakanishi, 2006). These modulate several downstream substrates, including Cdc25 phosphatases, that are involved in the DNA repair machinery or DNA damage checkpoint maintenance (Fig. 1.5).

DSB formation induces rapid autophosphorylation on inactive ATM homodimers at Ser1981, which results in their dissociation to form partially active monomers (Fig. 1.6) (Bakkenist & Kastan, 2003). Ser1981 was the first autophosphorylation site to be identified; however, this residue is not essential for ATM function, at least in mice (Pelligrini *et al.*, 2006), although its modification is tightly linked to ATM activation under most circumstances (Bakkenist & Kastan, 2003; Smith *et al.*, 2010). ATM monomers are then recruited to sites of DSBs with the aid of the sensor MRN complex, which is comprised of meiotic Mre11 (meiotic recombination 11), Rad50, and Nbs1 (Nijmegen breakage syndrome 1) (van den Bosch *et al.*, 2003; Berkovich *et al.*, 2007; Lee & Paull, 2005; Uziel *et al.*, 2003). In addition, DNA-PK senses DSBs, adheres to the damaged site and recruits and

activates its catalytic subunit (DNA-PKcs) *via* the Ku70-Ku80 heterodimer (Smith & Jackson, 1999). DNA-PKcs, in turn, binds and brings together the broken ends (DeFazio *et al.*, 2002). It then undergoes autophosphorylation on several serine residues that are essential for its activation (Chan *et al.*, 2002; Douglas *et al.*, 2002; Shiloh, 2003).

In contrast, replication stress- or ultraviolet (UV) light-mediated ssDNA becomes rapidly coated with the trimeric ssDNA-binding protein complex, Replication Protein A (RPA), which leads to the recruitment of ATR together with its interacting partner ATRIP (Fig. 1.6) (Zou & Elledge, 2003). Full activation of the ATR/ATRIP complex and successful checkpoint function requires loading of the sensor Rad17 and 9-1-1 (Rad9, Rad1, and Hus1) complexes, together with TopBP1 and Claspin, onto DNA (Kumagai *et al.*, 2006; Liu *et al.*, 2006; Lee *et al.*, 2002; Cimprich & Cortez, 2008). In the past it was believed that ATR kinase may be constitutively active to phosphorylate substrates but is controlled largely by its subcellular localisation upon binding of the ATR/ATRIP complex to RPA (Zou & Elledge, 2003). However, very recently it was shown that ATR, like ATM and DNA-PK, undergoes autophosphorylation at Thr1989, which is thought to be crucial for its full activation (Liu *et al.*, 2011).

The two main DNA damage sensor kinases ATM and ATR respond to very different types of stimuli and it was believed for years that they were components of independent pathways. However, several reports demonstrated that ATR also responds to DSBs in an ATM-dependent manner (Adams *et al.*, 2006; Cuadrado *et al.*, 2006; Hurley & Bunz, 2007; Jazayeri *et al.*, 2006; Myers & Cortez, 2006). These studies showed that ATM initiates the generation of local regions of RPA–coated ssDNA, thereby recruiting ATR to DSB sites. Activated by the former DNA–protein structure, ATR then phosphorylates its downstream substrates. Thus, ATM can indirectly cause ATR activation. *Vice versa*, Stiff and colleagues showed that UV-light and hydroxyurea, both potent activators of ATR signalling, also activate ATM and, importantly, that this activation is ATR-dependent (Stiff *et al.*, 2006).

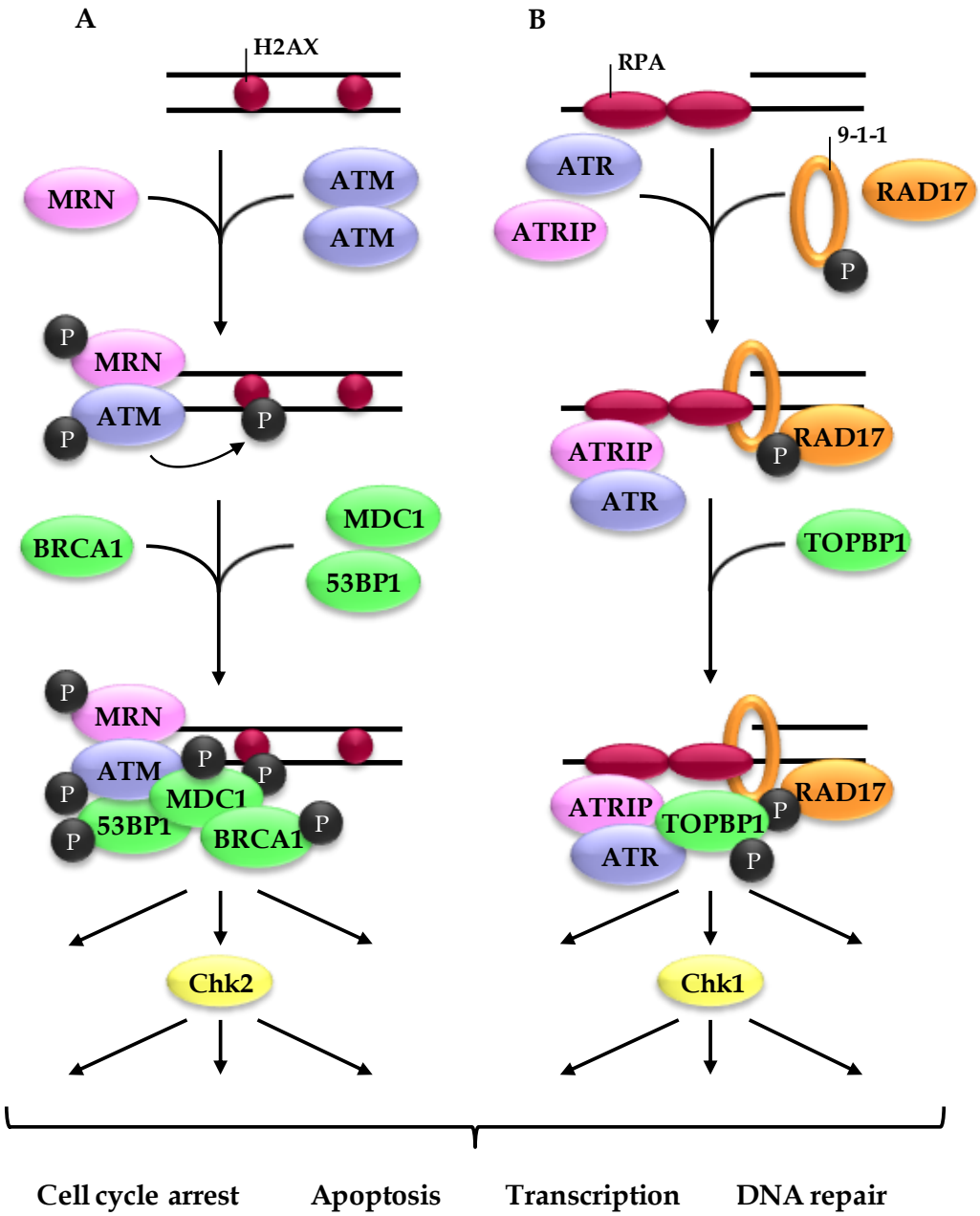


Figure 1.6 | Simple models for ATR and ATM activation.

(**A**) Formation of DSB leads to the recruitment of the MRN complex and the separation of the dimeric, inactive form of ATM to a monomeric, phosphorylated form. This monomeric form of ATM binds the MRN complex at DSB and is further activated by the DNA and MRN complex. Activated ATM then phosphorylates the C-terminal tail of the histone variant H2AX. Phosphorylated H2AX (γH2AX) binds to the mediators MDC1, 53BP1 and BRCA1, which leads to recruitment of additional ATM–MRN complexes and further H2AX phosphorylation. The activated ATM also phosphorylates downstream targets, including CHK2. Phosphorylation of downstream targets leads to cell-cycle arrest, slow down of origin firing and/or DSB repair. (**B**) Two complexes, 9-1-1 and one comprising ATR and ATRIP, are independently recruited to the junction of the 5' primer with single-stranded DNA (ssDNA). RPA binds ATRIP and directs RAD17 to load the 9-1-1 at the 5' primer junction. Loading of the 9-1-1 complex brings the ATR activator TOPBP1 to the damage site. TOPBP1 binds and activates ATR in an ATRIP-dependent manner, leading to phosphorylation of the downstream checkpoint kinase Chk1 and other ATR effectors. See text for more details. Adapted from (Cimprich & Cortez, 2008).

1.4.1.2 Mediators of checkpoint signalling

Several mediator proteins are involved in facilitating the activation of ATM and ATR downstream targets. Direct DSBs or ssDNA, which can be converted to DSBs by the action of nucleases which cleave ssDNA to yield DSBs (Cimprich & Cortez, 2008), lead to phosphorylation of DSB mediator H2AX (γH2AX) flanking the sites of DNA damage (Fig. 1.6). Phosphorylation of H2AX at Ser139 can be mediated by ATM, ATR or DNA-PK (Bonner *et al.*, 2008). To facilitate the activation of the downstream effector kinase Chk2, mediator proteins including mediator of DNA damage checkpoint 1 (Mdc1), p53-binding protein (53BP1), and breast cancer 1 (BRCA1) accumulate at γH2AX (Fig. 1.6) (Canman, 2003). To ease Chk1 activation ATR interacts with the mediator topoisomerase (DNA) 2-binding protein 1 (TopBP1) (Fig. 1.6) to phosphorylate a number of proteins, including H2AX (Liu *et al.*, 2006). The interaction of ATR with TopBP1 and its downstream mediator claspin, results in recruitment and phosphorylation of BRCA1 and subsequent activation of Chk1 (Bucher & Britten, 2008).

1.4.1.3 Chk1 and Chk2 in checkpoint signalling

Chk1 and Chk2 are structurally unrelated serine/threonine kinases which have overlapping functions in response to diverse genotoxic insults (Antoni *et al.*, 2007; Bartek & Lukas, 2003; Dai & Grant, 2010). Chk2 is a stable protein expressed throughout the cell cycle (Lukas *et al.*, 2001) and appears to be largely inactive in the absence of DNA damage. In response to DSBs it is mainly activated by ATM, leading to its phosphorylation at Thr68 (Ahn *et al.*, 2000; Lee & Chung, 2001; Matsuoka *et al.*, 2000; Melchionna *et al.*, 2000). In contrast, Chk1 protein is largely restricted to S and G2 phases (Lukas *et al.*, 2001) and it is active even in unperturbed cell cycles (Kaneko *et al.*, 1999; Sorensen *et al.*, 2003; Zhao *et al.*, 2002). Further, Chk1 is indispensable for embryonic development as *Chk1*$^{-/-}$ mice are lethal (Liu *et al.*, 2000; Takai *et al.*, 2000). The current view is that Chk1 activation does not require dimerisation or trans-autophosphorylation. Rather, ATR (predominantly) or ATM (to a lesser extent) phosphorylates Chk1 at Ser317/345, directly leading to activation (Dai & Grant, 2010).

Various reports disproved a strict dependency of Chk1 on ATR and Chk2 on ATM, demonstrated by phosphorylation/activation of Chk1 by ATM in response to ionising radiation (Gatei *et al.*, 2003; Jazayeri *et al.*, 2006; Sørensen *et al.*, 2003). However,

ATM-independent activation of Chk2 is also reported (Hirao *et al.*, 2002; Matsuoka *et al.*, 2000).

Once activated, Chk1 and Chk2 can phosphorylate and promote degradation or sequestration of effector Cdc25s (Boutros *et al.*, 2007; Gatei *et al.*, 2003; Goloudina *et al.*, 2003; Sørensen *et al.*, 2003; Xiao *et al.*, 2003; Zhao *et al.*, 2002). In addition, they induce the phosphorylation of the effector p53, and thereby increase its stability. Cdc25 inactivation and p53 accumulation together halt cell cycle progression G0/G1 or G2/M transitions.

1.4.2 The G1 and G1-S checkpoint responses

Upon exposure to genotoxic stress cells can enter into a sustained, and sometimes even permanent G1 arrest *via* the ATM(ATR)/Chk2(Chk1)-p53-p21 pathway by p53-dependent transcription of effector genes (*e.g.* $p21^{Cip1}$, Gadd45, 14-3-3σ) (Fig. 1.7) (Di Leonardo *et al.*, 1994; Kiyokawa & Ray, 2008; Kastan & Bartek, 2004). The p53 transcription factor is directly phosphorylated by ATM within its amino-terminal transactivation domain, particularly on Ser15 (Banin *et al.*, 1998; Lambert *et al.*, 1998; Dai & Grant, 2010). Chk1 and/or Chk2 phosphorylate p53 at Thr18 and Ser20 in the same domain, along with probably some additional p53 sequences (Bartek & Lukas, 2003; Craig *et al.*, 2003; Kastan & Lim, 2000; Shiloh, 2003; Wahl & Carr, 2001). In addition, the ubiquitin ligase Mdm2, which normally binds p53 and ensures rapid p53 turnover, is targeted and decreased after DNA damage by ATM (Maya *et al.*, 2001) as well as by Chk1/Chk2 (Kastan & Bartek, 2004). Together, these modifications of p53 and Mdm2 contribute to the stabilisation and accumulation of p53 protein and to its increased activity as a transcription factor.

In contrast, activation of the ATM(ATR)/Chk2(Chk1)-Cdc25A pathway leads to Cdc25A phosphorylation and degradation by the combined actions of Chk1, Chk2 and/or p38 (Fig. 1.7). It represents a rapid cellular response that induces a DNA synthesis block prior to the p53-dependent checkpoint activation. The basal turnover of Cdc25A during S and G2 phase requires phosphorylation on four different residues by Chk1 (Ser124, 178, 279 and 293) (Falck *et al.*, 2001) and is accelerated upon chemically, UV- or ionising radiation (IR)-induced DNA damage (Fig. 1.4) (Boutros *et al.*, 2006; Goloudina *et al.*, 2003; Mailand *et al.*, 2000; Xiao *et al.*, 2003; Zhao *et al.*, 2002). In addition, it has been shown that IR-induced hypophosphorylation of Cdc25A on Ser124, 178 and 293 by both Chk2 and Chk1 promotes the accelerated turnover of Cdc25A, which is mediated by $SCF^{\beta TrCP}$ (Sørensen *et al.*, 2003).

Moreover, phosphorylation on Ser76 (Goloudina *et al.*, 2003) and phosphorylations within the DSG motif of Cdc25A (Busino *et al.*, 2003) by Chk1 and a yet to be identified kinase is required for the binding of $SCF^{\beta TrCP}$ to the phosphatase and its subsequent ubiquitylation (Fig. 1.4). Furthermore, upon accelerated proteolysis of Cdc25A, the inhibitory phosphorylations on cyclin/Cdk complexes cease to be removed. This has been proposed to lead to the inactivation of the cyclin E/Cdk2 complex. Through these events cells are arrested before progression into S phase (Mailand *et al.*, 2000; Sørensen *et al.*, 2003; Mendelsohn *et al.*, 2008), which allows additional time to repair damaged DNA (Fig. 1.7) (Mailand *et al.*, 2000).

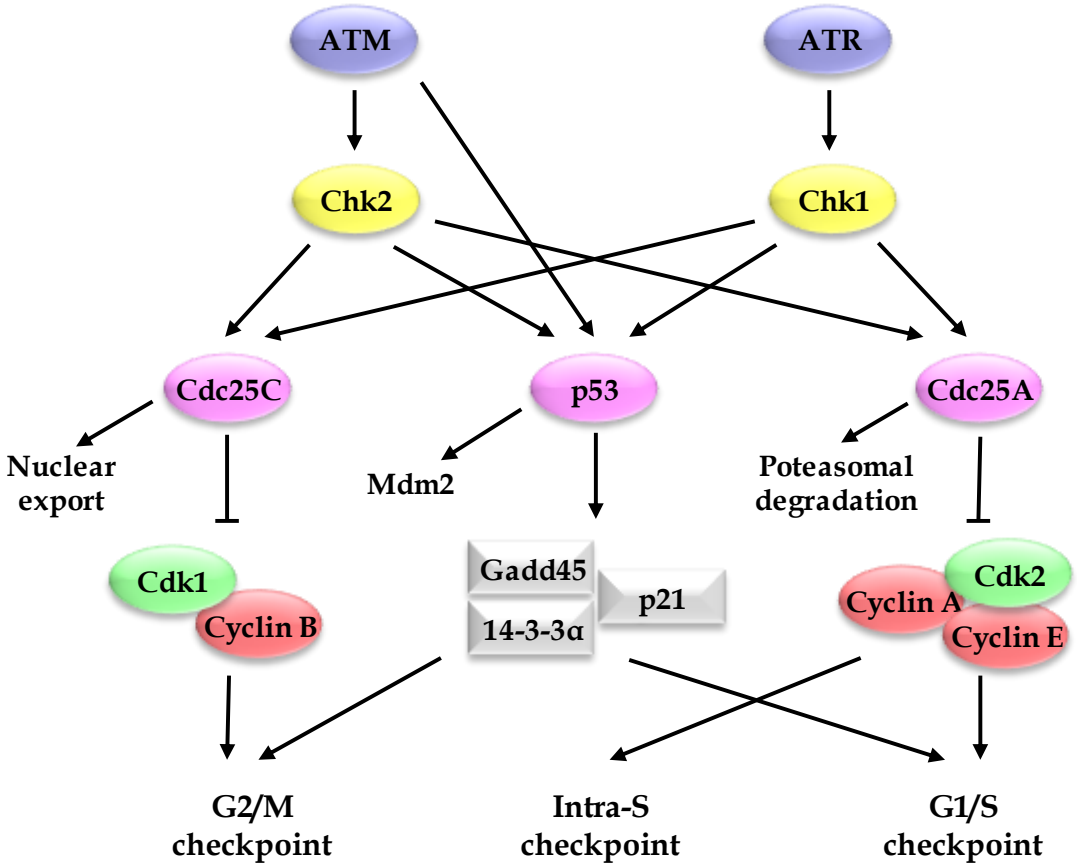


Figure 1.7 | Signalling network of DNA damage checkpoints.

DNA damage (DSB and SSB) initiate complex checkpoint signalling pathways to arrest cells in their cell cycle progression and to allow time for DNA repair. See text for details. Adapted from (Dai & Grant, 2010).

1.4.3 The S-phase checkpoint pathways

Protecting the integrity of the genome during S phase transition is of highest importance as it represents the genetically most vulnerable period during the cell cycle. S phase checkpoints are considerably more important for preventing genetic instability than the G1 or G2 checkpoints or the mitotic-spindle checkpoint (Myung *et al.*, 2001a; 2001b). During the DNA synthesis phase, cells may be exposed to genotoxic stress from either difficulties with the replication process itself or from DNA-damaging insults. This can lead to the activation of two S phase checkpoint pathways: the replication-dependent checkpoint (Fig. 1.8) or the replication-independent intra-S phase checkpoint (Fig. 1.7) (Bartek *et al.*, 2004).

1.4.3.1 Replication-dependent checkpoint

The replication checkpoint serves as protection for the integrity of stalled replication forks in response to stresses such as the depletion of deoxyribonucleotide (dNTP) pools, chemical inhibition of DNA polymerases, or as a consequence of the collision of replication forks with damaged DNA and/or aberrant DNA structures (Bartek *et al.*, 2004; Kastan & Bartek, 2004). Activation of the replication checkpoint is ATR-dependent and requires the establishment of DNA replication forks (Fig. 1.8) (Lupardus *et al.*, 2002; Stokes *et al.*, 2002; Tercero *et al.*, 2003) and the generation of ssDNA. The ssDNA binds RPA, which then triggers the activation of the checkpoint response (You *et al.*, 2002; Zou *et al.*, 2003). Besides RPA, the key components of this machinery include the ATR–ATRIP complex, the mediator protein claspin, Rad17, and the 9-1-1 complex (Bartek *et al.*, 2004; Kastan & Bartek, 2004; Segurado & Tercero, 2009). The replication checkpoint response co-ordinates DNA replication, DNA repair, and cell-cycle progression. It includes broad regulation of processes, such as the firing of replication origins, stabilisation of DNA replication forks in response to DNA damage or replicative stress, resumption of stalled DNA replication forks, transcriptional induction of DNA damage response genes, choice of the repair pathway, and inhibition of mitosis until replication is completed (for review see Segurado & Tercero, 2009).

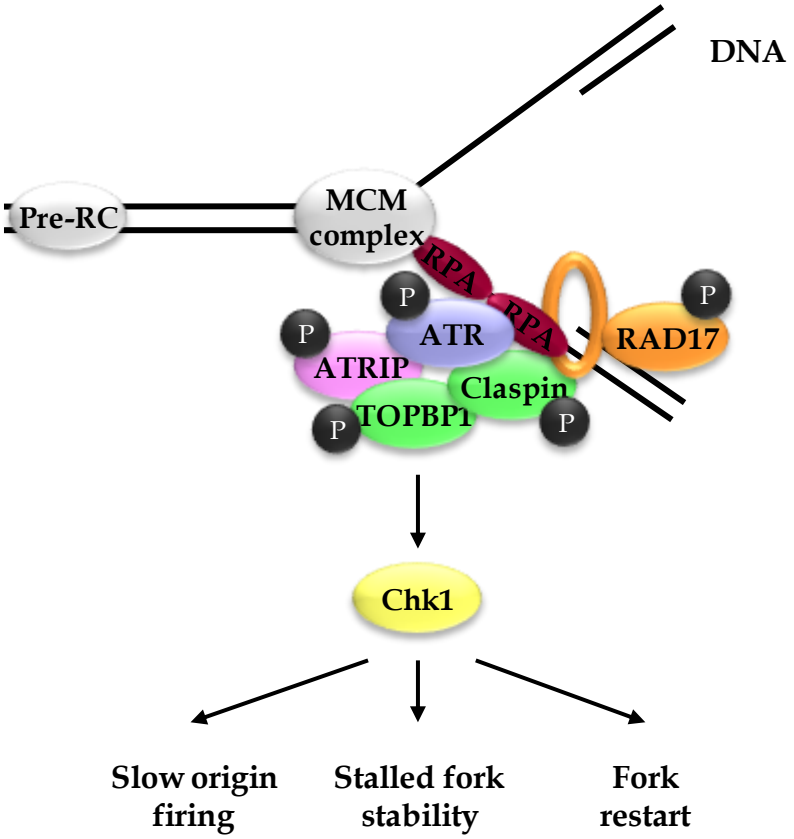


Figure 1.8 | The replication-dependent cell cycle checkpoint.

In S phase, endogenous/exogenous insults hinder replication fork progression, resulting in stalled forks that are unstable and breakage-prone. When a fork ancounters a lesion, DNA polymerase stalls whils helicase unwinds DNA, generating a large stretch of ssDNA. ssDNA lesions are then coated by RPA, redruiting ATR-ATRIP complexes via recognition and association of RPA-ssDNA by ATRIP. ATR/ATRIP activation requires Rad17/9-1-1 complex loading, which is also essential for ATR-mediated Chk1 activation. Adapted from (Cimprich & Cortez, 2008; Dai & Grant, 2010).

1.4.3.2 Intra-S phase checkpoint

The intra-S phase checkpoint serves to handle DNA damage that occurred during S phase and transduces its signals through two parallel pathways (Fig. 1.7): ATM(ATR)/Chk2(Chk1)/Cdc25A/Cdk2 and ATM/MRN complex (Bartek *et al.*, 2004; Bucher & Britten, 2008; Falck *et al.*, 2002). The first pathway includes the activation of ATM(ATR)/Chk2(Chk1) upon DNA damage, which results in Cdc25A degradation and thereby inhibits cyclin E(A)/Cdk2 activity and progression through S phase. Therefore, Chk1 is thought to be the primary S phase checkpoint kinase, whereas Chk2 plays a supportive role. This assumption is supported by studies with siRNAs targeting Chk1 and Chk2, which

demonstrate that downregulation of Chk1, but not Chk2, abrogates camptothecin- or 5-fluorouracil-induced S phase arrest (Xiao *et al.*, 2003). In the second effector branch, the sensor MRN complex recruits ATM with the help of Mdc1 to sites of DNA damage (Watrin & Peters, 2006). Once localised to damaged DNA, ATM phosphorylates Smc1, a component of the cohesin complex, which is thought to function in DNA repair (Bucher & Britten, 2008).

While p53 displays the key component of the sustained G1 cell cycle arrest, S phase checkpoints do not require p53 activity (Fig. 1.7) (Bartek & Lukas, 2001; 2003). Cells that experience genotoxic stress during DNA replication only delay their progression through S phase in a transient manner. If damage is not repaired during this delay, they exit S phase and arrest later when reaching the G2 checkpoint (Bartek *et al.*, 2004).

1.4.4 The G2 checkpoint

The G2 checkpoint (also known as the G2-M checkpoint) serves to prevent cells with genomic DNA damage from entering mitosis (M phase) (Nyberg *et al.*, 2002; Xu *et al.*, 2002). In cells exposed to DNA damage during G2, or when they progress into G2 phase with unrepaired DNA damage from previous S or G1 phases, the activation of cyclin B/Cdk1 complexes is blocked (Fig. 1.7). This prevents the initiation of mitotic events until replication is complete or DNA damage is repaired (Takizawa & Morgan, 2000). Timing of cyclin B/Cdk1 activation and the initiation or inhibition of mitosis involves the collaboration of the Cdc25 phosphatases (Kiyokawa & Ray, 2008). Genomic DNA damage at the G2 checkpoint activates ATM/ATR and Chk1/Chk2 and/or mitogen-activated protein kinase (MAPK) p38. They in turn phosphorylate Cdc25 proteins to inhibit their activity (Lopez-Girona *et al.*, 2001), to promote their degradation (Jin *et al.*, 2003; Mailand *et al.*, 2000), and/or to induce their translocation to the cytoplasm (O'Connell *et al.*, 2000). This sequence of events finally leads to inactivation of cyclin B/Cdk1 complexes and accumulation of cells in G2 phase. In addition, other upstream regulators, such as the Polo-like kinases Plk3 and Plk1, of Cdc25 and/or cyclin B/Cdk1 seem to be targeted by DNA damage-induced mechanisms (Nyberg *et al.*, 2002). By inducing the transcription of $p21^{Cip1}$ and other proteins, p53 also plays a role in the G2 checkpoint control (Fig. 1.7) (Taylor & Stark, 2001).

1.5 Agents targeting the cell cycle in anti-cancer therapy

Cancer is a major health problem and causes unbearable morbidity and mortality worldwide (Ferlay *et al.*, 2010; Parkin *et al.*, 2001). Cell cycle deregulation associated with cancer occurs through mutation of proteins important at different levels of the cell cycle (Deep & Agarwal, 2008). In cancer, mutations have been observed in genes encoding Cdks, cyclins, Cdc25 phosphatases, CKIs, Cdk substrates, and checkpoint proteins (reviewed by McDonald & El-Deiry, 2000; Sherr, 1996). Thus, deregulated cell cycle progression has been considered as one of the ten (recently) described hallmarks of cancer progression (Hanahan & Weinberg, 2011). Therefore, the development of agents targeting the deregulated cell cycle has been considered as an ideal strategy for cancer therapy in recent years (Collins & Garrett, 2005; Deep & Agarwal, 2008; Vermeulen *et al.*, 2003).

Twenty years ago, the first report by Nagata and colleagues showed the connection of Cdc25 isoform expression and cancer (Nagata *et al.*, 1991). Since then, Cdc25 phosphatases, particularly the Cdc25A and Cdc25B isoforms have been reported to be overexpressed in primary tissue samples from various human cancers (Boutros *et al.*, 2007; Kristjánsdóttir & Rudolph, 2004). In detail, for non-Hodgkin lymphoma several reports have been published revealing Cdc25A overexpression on RNA and protein levels (Aref *et al.*, 2003; Hernández *et al.*, 2000; 1998; Moreira *et al.*, 2003). In most cases, overexpression of Cdc25A (and Cdc25B) correlates with more aggressive disease and poor prognosis of patients, raising evidence that Cdc25A may act as an oncogene. Accordingly, Cdc25A overexpression has been found to lead to unsustained cell proliferation, tumour growth and resistance to chemotherapeutic drugs (Draetta & Eckstein, 1997; Boutros *et al*, 2007).

The important role of Cdc25 phosphatases as activators of Cdks makes them attractive candidates for the development of indirect inhibitors of the kinases. Their potential as anti-proliferative cancer drugs has been explored in various strategies in recent years. In-depth studies of compounds with inhibitory actions on Cdc25A, such as Flavopiridol, Roscovitine or ARQ-501, led to their engagement in phase I and II clinical trials on patients with various types of relapsed or refractory tumours, both in single or combination treatment (for review see Boutros *et al.*, 2007; Deep & Agarwal, 2008).

Besides direct inhibition of Cdc25A, the activation of the cellular DNA damage pathway displays an alternative way in anti-cancer treatment. In chemotherapy, fast-dividing malignant cells are targeted by DNA damaging agents such as alkylating agents,

antimetabolites, anthracyclines, plant alkaloids, topoisomerase inhibitors and other anti-tumour agents, all of which affect cell division or DNA synthesis and function (Takimoto & Calvo, 2009). The general purpose of these drugs is to induce sufficient amounts of DNA damage (Herr & Debatin, 2001; Stahnke *et al.*, 2001) and thereby activate p53-dependent and -independent DNA damage signalling pathways, which leads to cell cycle arrest and to the induction of cell death pathways. However, cancer cells can become resistant to chemotherapy over time through a variety of apoptotic resistance mechanisms (Igney & Krammer, 2002). Breaking apoptosis resistance and sensitising malignant cells towards cell death are crucial in cancer therapy. The exploration of new anti-cancer drugs with minimal side effects on normal cells and the development of enhanced combination therapies with cell cycle agents will support the fight against one of the major health problems of our century.

1.6 Plant extracts in anti-cancer therapy

This chapter is adapted from (Ebada *et al.*, 2011).

The study of natural products that have traditionally been used to treat human diseases throughout the ages is one of the bases for modern drug development. Analysis of medicinal plants, bioactive cultures, and increased understanding of micronutrients in the food chain has opened the door for the development of purified and defined chemical compounds as dose-controlled medicines (Rishton, 2008). The first known preserved medical records consist of cuneiform writing on clay tablets found in Mesopotamia (now Iraq) dating from about 4600 years ago (Mitscher, 2007). In the 19th century, synthetic medicinal chemistry began and drugs were derived by extracting plant, animal or marine products with varying degrees of purification (Brown, 2007). Data collected ten years ago revealed that about 120 compounds used in conventional medicine were derived from traditional medicine plant sources; 80% of these compounds were used in the same or related manner as the traditional use (Fabricant & Farnsworth, 2001). Further, an analysis of the origin of drugs revealed, that almost half of the drugs approved since 1994 were based on natural products (Harvey, 2008). Since the beginning of the 21st century, new approaches in drug discovery, such as genomics, proteomics and antibody therapy are being widely deployed. They provide new advances to drug discovery and display an important source of new drugs. However, natural compounds which have existed for thousands of years will continue to be central players in the treatment of human disease.

1.6.1 The plant genus *Aglaia* and its rocaglamide derivatives

In Traditional Chinese Medicine plant elements and extracts are by far the most common elements used (Foster & Chongxi, 1992). The family Meliaceae (= Mahogany family, order Sapindales) is an angiosperm plant family of mostly trees and shrubs together with a few herbaceous plants (Isman *et al.*, 1997). The genus *Aglaia* displays the largest genus within the family of Meliaceae comprising about 120 woody species with tree heights up to 40 meters, mainly distributed in the tropical rain forest of countries in South-East Asia. The active compounds isolated from these plant extracts are derivatives of cyclopenta[*b*]benzofuran, also called rocaglamide (King *et al.*, 1982). Plant extracts are traditionally used in folk medicine as heart stimulant, febrifuge, and for the treatment of coughs, diarrhea,

inflammation, and injuries (Proksch *et al.*, 2001). Extracts have also been used as bactericides, insecticides, and in perfumery (Janaki *et al.*, 1999). The phytochemical activities in the natural constituents of *Aglaia* have now been studied for almost 30 years, since the discovery of the first cyclopenta[*b*]benzofuran in 1982 (King *et al.*, 1982). To date, more than one hundred naturally occurring rocaglamide-type (= flavagline) compounds have been isolated from over 30 *Aglaia* species (Kim *et al.*, 2006; Proksch *et al.*, 2001; Ebada *et al.*, 2011). Rocaglamide derivatives differ from each other by the nature of their backbone structure and of their moities at positions R1 – R5 (Fig. 1.9). The activity of rocaglamide derivatives is dependent on different substituents, as analysed in previous studies (Ebada *et al.*, 2011).

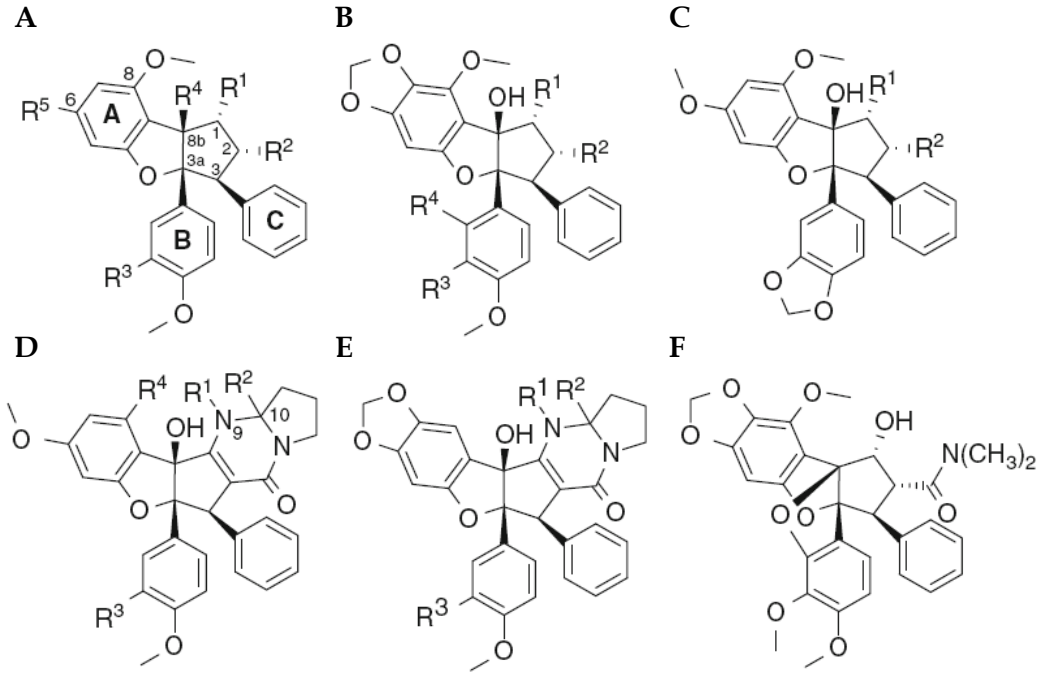


Figure 1.9 | Rocaglamide derivatives isolated from *Aglaia* species.

(**A-E**) Rocaglamide derivatives differ from each other by the nature of their backbone structures and of their moities at positions R1 – R5; the most common ones are for R1: -H, -OH, -$OCOCH_3$, - OCHO, -OCHO; R2: -H, - $CON(CH_3)_2$, -$CONHCH_3$, -$CONH_2$, -$COOCH_3$; R3: -H, -OH, -OCH_3; R4: -H, -OH, -OC_2H_5, - OCH_3; R5: - OCH_3. The structure of the rocaglamide derivative used in this study is based on (A) with R1: -OH, R2: -$CON(CH_3)_2$, R3: -H, R4: -OH and R5: - OCH_3; it is abbreviated as Rocaglamide A (Roc A). (**F**) Cyclorocaglamide. Modified from (Ebada *et al.*, 2011).

1.6.2 Pharmacological significance of rocaglamides

Since their discovery almost 30 years ago (King *et al.*, 1982), rocaglamides have been profoundly studied and besides their insecticidal activity (Bacher, 1999; Brader *et al.*, 1998; Dreyer *et al.*, 2001; Grege *et al.*, 2001; Nugroho, 1999; Schneider *et al.*, 2000) various reports described them to exhibit anti-inflammatory (Baumann *et al.*, 2002; Proksch *et al.*, 2005) and anti-cancer activities (Kim *et al.*, 2006; Proksch *et al.*, 2001; Ebada *et al.*, 2011). Tumour inhibiting activities include anti-proliferative (Hayashi *et al.*, 1982; Kim *et al.*, 2006; Lee *et al.*, 1998; Zhu *et al.*, 2009), translation inhibitory (Bleumink *et al.*, 2010; Bordeleau *et al.*, 2008; Lee *et al.*, 1998), and pro-apoptotic actions (Bleumink *et al.*, 2010; Zhu *et al.* 2007, 2009;). While the molecular mechanisms of translation inhibitory (Bleumink *et al.*, 2010; Bordeleau *et al.*, 2008; Lee *et al.*, 1998) and pro-apoptotic actions (Bleumink *et al.*, 2010; Zhu *et al.* 2007, 2009;) of rocaglamides are elucidated, how they exert their anti-proliferative activity in cancers remains subject to further investigations.

1.6.2.1 Anti-inflammatory activities of rocaglamides

Inflammatory diseases arise from improper activation of the immune system which leads to abnormal expression of pro-inflammatory cytokines, such as TNF-α, INF-γ or IL-4, and tissue-destructive enzymes (Li-Weber & Krammer, 2003; O'Shea *et al.*, 2002). Over-production of cytokines is known to be tightly associated with autoimmune and inflammatory diseases. Traditional medicine in several countries of South-East Asia uses leaves and flowers of *Aglaia* species for the treatment of asthma and inflammatory skin diseases. In addition, it has been shown by our group that rocaglamide inhibits TNF-α, INF-γ and IL-4 production in human peripheral blood cells at very low doses (25-50 nM) (Proksch *et al.*, 2005). Pro-inflammatory cytokines are transcriptional targets of the NF-κB pathway (Li-Weber & Krammer, 2003b; Macian, 2005; Yamamoto & Gaynor, 2001), which is affected by rocaglamides. They were shown to be potent inhibitors of TNF- and PMA (phorbol 12-myristate 13-acetate)-induced NF-κB activity (Baumann *et al.*, 2002). However, the conclusion from the study of Baumann and colleagues (Baumann *et al.*, 2002) could not be confirmed by later studies. We have shown that rocaglamide at concentrations of 25-100 nM, did not show inhibition of PMA-induced IκB degradation and also did not block PMA-induced nuclear translocation of p65 (a subunit of NF-κB) (Zhu *et al.*, 2007; Proksch *et al.*, 2005). Instead, at concentrations of 25-100 nM, rocaglamide even substantially increased NF-κB-mediated transcription (Proksch *et al.*, 2005). Using an enzyme-based NF-κB activity

readout it was shown that rocaglamide inhibited NF-κB activity only at very high doses (IC_{50} = 2 μM) (Salim *et al.*, 2007). Since rocaglamide also inhibits protein synthesis (see below), it is, therefore, unclear whether the observed inhibition of NF-κB activity at high concentrations of rocaglamide was due to inhibition of NF-κB activation signalling pathway or was rather the consequence of translation inhibition.

Our group has shown that rocaglamide derivatives inhibit NF-AT activity in activated T cells (Proksch *et al.*, 2005). It was demonstrated that rocaglamide derivatives selectively inhibit NF-AT-dependent gene expression of several cytokines after stimulation with anti-CD3/anti-CD28 antibodies or PMA/ionomycin in peripheral blood T cells. These data suggest that rocaglamide derivatives may function as immunosuppressive agents by targeting NF-AT activity in T cells. However, the precise molecular mechanism of the suppression of cytokines like TNF-α, INF-γ and IL-4 in normal T cells remains unknown.

1.6.2.2 Anti-tumour activities *in vivo*

As early as 1982, the first report on rocaglamides showed their potency to increase the lifetime of tumour-bearing mice in a leukaemic model using P388 murine lymphocytic leukaemia cells (King *et al.*, 1982). Several rocaglamide derivates were then extensively tested in various mouse tumour models (Cencic *et al.*, 2009; Hwang *et al.*, 2004; Lee *et al.*, 1998; Zhu *et al.*, 2009). In particular, the work done in our lab showed a significant delay in growth of the mouse lymphoma RMA after 16 days of intraperitoneal treatment with desmethyl-rocaglamide at 5mg/kg three times per week (Zhu *et al.*, 2009). Together, these mice studies prove the anti-cancer activities of rocaglamide derivatives. Importantly, no toxicity to the liver (as evaluated by glutamate pyruvate transaminase activity) and no body weight loss could be observed upon rocaglamide treatment (Zhu *et al.*, 2009).

1.6.2.3 Anti-proliferative activities of rocaglamides

A powerful action of rocaglamides is their anti-proliferative activity in cancer cells, which was observed upon their first discovery in 1982 and has been studied further since then (Hayashi *et al.*, 1982; Kim *et al.*, 2006; Lee *et al.*, 1998; Zhu *et al.*, 2009; Bleumink *et al.*, 2010).

Inhibition of tumour cell proliferation with a pronounced accumulation of cells in a specific phase of the cell cycle was first reported by Lee and colleagues (Lee *et al.*, 1998). It

was shown that 4′-demethoxy-3′,4′-methylenedioxy-methyl rocaglate inhabits the potency to inhibit tumour cell proliferation of the human lung carcinoma cells Lu1 with cell accumulation in G0/G1 phase of the cell cycle accompanied by only marginal cell death. Thereafter, other derivatives, such as didesmethyl-rocaglamide, aglaxorin D (aglaiastatin), and silvestrol were also shown to inhibit cell proliferation of different malignant cell lines with induction of cell cycle arrest in G2/M phase and negligible death (Bohnenstengel *et al.*, 1999; Hausott *et al.*, 2004; Mi *et al.*, 2006). Further, our group showed that 1-oxo-11,12-methyldioxyrocaglaol induces G0/G1 cell cycle arrest in human T cell lines infected with the human T cell lymphotropic virus type 1 (HTLV-1) (Bleumink, 2007).

Taken together, rocaglamide derivatives show anti-proliferative activity with apparent accumulation of cells in different cell cycle phases, which was observed for several human cancer cell lines *in vitro*. This potency to halt tumour cells in their cell cycle progression and force malignant cells into programmed cell death or senescence suggests rocaglamides as promising new chemotherapeutic agents. However, the mechanism(s) of rocaglamide-mediated cell cycle arrest is not yet completely understood.

1.6.2.4 Inhibition of translation

Besides its anti-proliferative activities, rocaglamide derivatives were found to strongly inhibit protein biosynthesis in tumour cells determined by ^{3}H-leucine incorporation (Lee *et al.*, 1998). However, its mode of function was only recently explored.

The rate limiting step of translation is largely controlled by binding of the initiation factor to the mRNA 5′ cap structure. The activity of eIF4E is regulated by two major signalling pathways: the Ras-Erk (extracellular signal-regulated kinase)-Mnk1 (MAP kinase interacting kinase 1) and the PI3K-mTOR pathway (Silvera *et al.*, 2010). In the former pathway, phosphorylation of eIF4E is mediated by Mnk1 and increases its affinity for the 5′ cap structure. It was recently shown by our lab that 1-oxo-11,12-methyldioxyrocaglaol strongly inhibits protein synthesis (IC_{50} = 30 nM) in HTLV-1 infected human T cells (Bleumink *et al.*, 2010). It was further shown that it does not directly inhibit the translation machinery but rather mediates its effects *via* the Ras-Erk-Mnk1 signalling pathway. Several rocaglamide derivates have been shown to suppress Erk phosphorylation and thereby inhibit Erk-Mnk1-mediated phosphorylation of eIF4E (Bleumink *et al.*, 2010; Zhu *et al.*, 2007).

Importantly, these compounds do not inhibit Erk activity in normal, primary lymphocytes (Zhu *et al.*, 2007).

In addition, a second mechanism of translation inhibition by rocaglamides was reported by Bordeleau and colleagues (Bordeleau *et al.*, 2008). Using a small molecule screening approach, two rocaglamide derivatives, 1-0-formylaglafoline and silvestrol, were found to inhibit translation by interfering with eIF4A activity. It is thought that eIF4A exists as a free form or as part of the eIF4F complex and recycles through the eIF4F complex during translation initiation. 1-0-formylaglafoline and silvestrol were shown to stimulate the RNA-binding activity of eIF4A and this action prevents incorporation of free eIF4A into the eIF4F complex.

1.6.2.5 Pro-apoptotic activities of rocaglamides

The anti-cancer activities of rocaglamides include the potency to induce programmed cell death in malignant cells. Almost ten years ago, it was observed that rocaglamide derivatives were able to induce and to enhance apoptotic cell death in cancer cells (Baumann *et al.*, 2002). Further data by our group support the role of rocaglamides as potent apoptosis inducers, as it was shown that they trigger the depolarisation of the mitochondrial membrane potential, caspase activation and Bid cleavage upon treatment (Zhu *et al.*, 2007). Importantly, rocaglamides showed no or very low toxicity to normal peripheral blood T and B lymphocytes as well as to human bone marrow stem cells. Studies of the molecular mechanisms by which rocaglamide derivates kill cancer cells but not normal cells revealed that these compounds preferentially induce apoptosis in malignant cells by differential modulation of the activities of Erk, p38, and Jnk (Zhu *et al.*, 2007). In particular, erk, p38, and Jnk activities were not affected in normal lymphocytes upon treatment of rocaglamide derivatives.

Susceptibility to apoptosis induction depends on a balanced level of pro- and anti-apoptotic proteins. Proteins with a short half-life are highly vulnerable if treated with compounds that inhibit *de novo* protein synthesis. Therefore, rocaglamide-mediated translational inhibition may cause an imbalance of pro- and anti-apoptotic protein levels in malignant cells. It was shown that upon treatment of the human breast cancer cells MDA-MB-231 with silvestrol the expression of the anti-apoptotic proteins Mcl-1 and survivin were reduced (Cencic *et al.*, 2009). Further, our group reported that treatment with different rocaglamide derivatives lead to a down-regulation of c-FLIP, the major inhibitor of caspase-8, and thereby sensitises tumour cells to programmed cell death (Bleumink *et al.*,

2010; Zhu *et al.*, 2009). We could also show the enhancement of CD95L-mediated activation induced cell death (AICD) in malignant cells by up-regulation of CD95L but down-regulation of c-FLIP expression (Zhu *et al.*, 2009). CD95L promoter is strongly regulated by Jnk/AP-1 activity (Li-Weber & Krammer, 2003a) and therefore, an increase of p38/Jnk activity leads to an increase of AP-1 activity and consequently enhances promoter activity (Zhu *et al.*, 2009). On the other hand, c-FLIP expression in T cells is strongly regulated by NF-AT (Ueffing *et al.*, 2008). Since rocaglamides are known to be potent NF-AT inhibitors (Proksch *et al.*, 2005), this may explain the downregulation of c-FLIP expression on mRNA level (Zhu *et al.*, 2009). Taken together, the capability of rocaglamide derivates to induce or sensitise programmed cell death in tumour cells but not in normal cells strongly supports the efforts undertaken to develop rocaglamide derivatives into potent anti-cancer drugs.

1.7 Aim of the study

It is believed that inhibition of translation is the key mode of action by which rocaglamides exert their anti-tumour activities (Kim *et al.*, 2006; Proksch *et al.*, 2001; Ebada *et al.*, 2011). Our lab could show that by translational suppression of c-FLIP expression, TRAIL-resistant HTLV-1-associated adult T-cell leukaemia/lymphoma cell lines could be sensitised towards cell death (Bleumink *et al.*, 2010). However, our group also suggested further molecular mechanisms which rendered malignant cells sensitive to cell death by treatment with rocaglamides, such as the preferential activation of MAPKs p38 and Jnk while suppression of survival MAPK Erk activity in malignant *vs.* normal cells (Zhu *et al.*, 2007). We have shown that due to selective activation of p38 and Jnk in tumour cells, rocaglamides can sensitise malignant T cells to undergo cell death by upregulation of CD95L but downregulation of c-FLIP expression (Zhu *et al.*, 2009).

Rocaglamides' pro-apoptotic functions as well as their anti-proliferative activities contribute to their anti-tumour properties (Lee *et al.*, 1998; Bleumink, 2007; Bohnenstengel *et al.*, 1999; Mi *et al.*, 2006; Hausott *et al.*, 2004). Whether inhibition of proliferation is solely due to inhibition of protein *de novo* synthesis has remained elusive to date. Therefore, the aim of this study was to elucidate molecular mechanisms by which rocaglamides exert their anti-proliferative activities on cancer cells, and to analyse how cell cycle arrest is induced. We hypothesised that in addition to the likely downregulation of important (short-lived) cell cycle proteins, the activation of certain cellular signalling pathways contributes to the inhibition of proliferation and arrest in a specific cell cycle phase. Several haematological cancer cell lines were analysed as model system in this study: Jurkat J16 (acute lymphoblastic leukemia (ALL)), Molt-4 (ALL), Hut-78 (cutaneous T cell lymphoma (CTCL)), HL-60 (acute myeloid leukemia (AML)), and DND-41 (ALL). Derivative Rocaglamide A (Roc A) was used as representative rocaglamide.

Understanding how rocaglamides exert their anti-proliferative activities in cancers will help to develop compounds of the Traditional Chinese Medicine plant *Aglaia* into new potent anti-cancer drugs. Also, as induction of cell cycle arrest is known to be able to trigger apoptotic cell death, it is of interest to identify the underlying molecular properties to further elucidate the cell death pathways induced by rocaglamides.

2 Materials & Methods

2.1 Materials

2.1.1 Chemicals

All chemicals, if not otherwise stated, were purchased from the companies Fluka (Neu-Ulm), Merck (Darmstadt), Serva (Heidelberg), Sigma (Munich) or Roth (Karlsruhe).

2.1.2 Instruments

Instrument	**Manufacturer**
7500 Real-Time PCR system	Applied Biosystems, Carlsbad, USA
Amaxa Nucleofector I	Lonza, Cologne, Germany
Analytical and precision balances	Mettler-Toledo, Giessen, Germany
Bacteria culture incubator/shaker	HAT Infors, Bottmingen, Switzerland
Biofuge Fresco 17	Heraeus, Hanau, Germany
Biofuge Pico	Heraeus, Hanau, Germany
Cell incubator Stericult	ThermoFisher Scientific, Langenselbold, Germany
Chemiluminescence detector Chemi-Smart 5100	Vilber Lourmat, Eberhardzell, Germany
FACS Canto II	Becton Dickinson, Heidelberg, Germany
GFL analogue orbital-rocking shaker	GFL, Burgwedel, Germany
Magnetic stirrer	IKA, Staufen, Germany
Microscope Axiovert 25	Zeiss, Jena, Germany
Mini Protean II SDS-PAGE apparatus	Bio-Rad, Munich, Germany

Instruments continued

Multifuge 3SR+	ThermoFisher Scientific, Langenselbold, Germany
NanoDrop	Peqlab, Erlangen, Germany
Neubauer cell-counting chamber	Brand, Wertheim, Germany
pH-meter Calimatic	LHD Labortechnik, Berlin, Germany
Refrigerator (-20°C)	Liebherr, Leimen, Germany
Refrigerator (-80°C)	ThermoLife Science, Egelsbach, Germany
Semi-dry or wet blotting system	Bio-Rad, Munich, Germany
Sorvall Evolution RC	ThermoFisher Scientific, Langenselbold, Germany
Spectrophotometer BioPhotometer	Eppendorf, Hamburg, Germany
Sterile bench place HeraSafe	Heraeus, Hanau, Germany
Thermocycler PTC-200 DNA engine	MJ Research, Watertown, USA
Thermomixer compact	Eppendorf, Hamburg, Germany
Tomtec multiple automated harvester	Perkin Elmer, Waltham, USA
Vortex shaker REAX1R	Heidolph Instruments, Schwabach, Germany
Wallac Microbeta Trilux scintillation counter	Perkin Elmer, Waltham, USA
Water baths	Köttermann, Uetze/Hänigsen, Germany
Zeiss LSM700	Carl Zeiss, Göttingen, Deutschland
Zeiss LSM710	Carl Zeiss, Göttingen, Deutschland

2.1.3 Reagents

Reagent	Company
β-mercaptoethanol	Neolab, Heidelberg, Germany
Bovine serum albumin (BSA)	Sigma, Munich, Germany
Chk2 II Inhibitor II	Calbiochem, Merck, Darmstadt, Germany
Complete protease inhibitor cocktail	Roche Applied Science, Mannheim, Germany
Cycloheximide	Sigma, Munich, Germany
DNA-PK Inhibitor IV	Merck, Darmstadt, Germany
Doxorubicin	Merck, Darmstadt, Germany
DRAQ5	Cell Signaling Technology, Beverly, USA
Etoposide	Sigma, Munich, Germany
ER-Tracker™ Red (BODIPY® TR glibenclamide)	Invitrogen, Darmstadt, Germany
KU-55933	Calbiochem, Merck, Darmstadt, Germany
MitoTracker® Deep Red FM	Invitrogen, Darmstadt, Germany
SB 203580	Enzo Life Sciences, Lörrach, Germany
SB 218078	Biozol Diagnostics, Eching, Germany
Rocaglamide A	Enzo Life Sciences, Lörrach, Germany
UCN-01	Calbiochem, Merck, Darmstadt, Germany
Western Lightning™ Plus-ECL	Amersham, Little Chalfont, GB

2.1.4 Buffers and Solutions

Buffer	Composition
PBS	137 mM NaCl 8.1 mM Na_2HPO_4 2.7 mM KCl 1.5 mM KH_2PO_4, pH 7.4
Lysis buffer	120 mM NaCl 50 mM Tris base/HCl (pH = 8.0) 1% NP-40 5 mM DTT 200 µM Na_3VO_4 0.02% (w/v) Complete protease inhibitor cocktail 1 mM PMSF 25 mM NaF
Stacking gel buffer (5%)	24 mM Tris base (pH = 6.8) 5% (w/v) Acrylamide/Bisacrylamide 0.1% (w/v) SDS 0.1% (w/v) Ammoniumpersulfat (APS) 0.1% (w/v) Tetramethylethylendiamine (TEMED)
Resolving gel (7.5 – 13%)	37.5 mM Tris base (pH = 8.8) 7.5 - 13% (w/v) Acrylamid/Bisacrylamid 0.1% (w/v) SDS 0.03% (w/v) APS 0.1% (w/v) TEMED
Running buffer (SDS-PAGE)	0.19 M Glycin 0.1% (w/v) SDS 25 mM Tris base (pH = 6.8)
Transfer buffer (Western Blot)	25 mM Tris base 0.19 M Glycine 20% (v/v) Methanol 0,037% (w/v) SDS

Buffer and Solutions continued

Reducing sample buffer (5x)	50% (v/v) glycerol 10% (w/v) SDS 50 mM Tris (pH 6.8) 25% (v/v) β-mercaptoethanol 0.25 mg/ml bromophenol blue
Blocking buffer	5% (w/v) non-fat dry milk in PBS-T 5% (w/v) BSA in PBS-T
Nicoletti lysis buffer	0.1% (w/v) Sodium citrate (pH 7.4) 0.1% (w/v) Triton X-100 50 µg/ml Propidium iodide
Annexin V binding buffer	10 mM HEPES 140 nM NaCl 2.5 mM $CaCl_2$

2.1.5 Eukaryotic cell lines

Cell line	**Medium**	**Characteristics**
Jurkat J16 and JE6.1	RPMI	Human acute lymphoblastoid T (ALL) cell line
Molt-4	RPMI	Human acute lymphoblastoid T (ALL) cell line
Hut-78	RPMI	Human cutaneous T cell lymphoma (CTCL) cell line
HL-60	RPMI	Human acute myeloid leukemia (AML) cell line
DND-41	RPMI	Human acute lymphoblastoid T (ALL) cell line
HT-29	DMEM	Human colorectal cancer cell line
PC-3	DMEM	Human prostate cancer cell line
Mcf-7	DMEM	Human breast cancer cell line

2.1.6 Eukaryotic cell culture media

Unless otherwise indicated, media were supplemented with 10% (v/v) heat-inactivated FCS. All media were stored at 4°C for further use.

Reagent	**Company**
Dulbecco's Modified Eagle Medium (DMEM)	Sigma, Munich, Germany
Roswell Park Memorial Institute (RPMI) 1640 medium	Sigma, Munich, Germany
Fetal calf serum (FCS)	Sigma, Munich, Germany
Penicillin/Streptomycin	Sigma, Munich, Germany
Trypsin-EDTA	Sigma, Munich, Germany
Methionine-free medium (RPMI)	Sigma, Munich, Germany

2.1.7 Antibodies

2.1.7.1 Primary Antibodies

Name	**Antigen**	**Isotype**	**Origin**
Anti-Annexin V, FITC	Annexin V	Rabbit, polyclonal	ImmunoTools
Anti-ATM (D2E2)	ATM (D2E2)	Rabbit, monoclonal	Cell Signaling Technology (CST)
Anti-phospho-ATM (10H11.E12)	Phospho-ATM (Ser1981) (10H11.E12)	Mouse IgG1, monoclonal	CST
Anti-ATR	ATR	Rabbit, polyclonal	CST

Primary antibodies continued

Anti-phospho-ATR	Phospho-ATR (Ser428)	Rabbit, polyclonal	CST
Anti-Cdc25A Ab3 (Clone DCS-120 + DCS-121)	Cdc25A	Mouse IgG2a, polyclonal	NeoMarkers
Anti-phospho-Cdc25A (Ser76)	Phospho-Cdc25A (Ser76)	Rabbit, polyclonal	Abgent
Anti-phospho-Cdc25A (Ser178)	Phospho-Cdc25A (Ser178)	Rabbit, polyclonal	Abgent
Anti-Cdc25B	Cdc25B	Rabbit, polyclonal	CST
Anti-Cdc25C (5H9)	Cdc25C (5H9)	Rabbit, monoclonal	CST
Anti-Cdk2	Cdk2	Rabbit, monoclonal	(Hoffmann *et al.*, 1994)
Anti-Cdk4	Cdk4	Mouse IgG1, monoclonal	CST
Anti-Cdk6	Cdk6	Mouse IgG1, monoclonal	CST
Anti-Chk1 (FL-476)	Chk1 (FL-476)	Rabbit, polyclonal	Santa Cruz Biotechnology (SCB)
Anti-phospho-Chk1	Phospho-Chk1 (Ser317)	Rabbit, polyclonal	CST
Anti-phospho-Chk1 (133D3))	Phospho-Chk1 (Ser345)	Rabbit, polyclonal	CST
Anti-Chk2	Chk2	Rabbit, polyclonal	CST
Anti-phospho-Chk2	Phospho-Chk2 (Th68)	Rabbit, polyclonal	CST

Primary antibodies continued

Anti-cyclin A (H-432)	Cyclin A (H423)	Rabbit, polyclonal	SCB
Anti-cyclin D_3	Cyclin D_3	Mouse IgG1, monoclonal	CST
Anti-cyclin E (HE12)	Cyclin E (HE12)	Mouse IgG1, monoclonal	CST
Anti-Erk1 (MK12)	Erk	Mouse IgG1, monoclonal	BD Biosciences
Anti-phospho-Erk (E-4)	Phospho-Erk	Mouse IgG2a, monoclonal	SCB
Anti-γH2AX, clone JBW301	γH2AX (Ser139)	Mouse IgG1, monoclonal	Millipore
Anti-γH2AX, Alexa Fluor 488	γH2AX (Ser139)	Mouse IgG2b, monoclonal	Biozol Diagnostics
Anti-p38 (5F11)	p38	Mouse IgG2b, monoclonal	CST
Anti-phospho p38 (V121A)	Phospho-p38	Rabbit, polyclonal	Promega
Anti-PHB1	PHB1	Rabbit, polyclonal	(Emerson *et al.*, 2010)
Anti-Tubulin (clone B-5-1-2)	Tubulin	Mouse IgG1, monoclonal	Sigma

2.1.7.2 Secondary Antibodies

Name	**Antigen**	**Isotype**	**Origin**
Anti-mouse IgG1, HRP	Mouse IgG1	Goat, polyclonal	SCB
Anti-mouse IgG2a, HRP	Mouse IgG2a	Goat, polyclonal	Southern Biotech
Anti-mouse IgG2b, HRP	Mouse IgG2b	Goat, polyclonal	Southern Biotech
Anti-rabbit, HRP	Rabbit	Goat, polyclonal	SCB
Anti-rabbit, FITC	Rabbit	Goat, polyclonal	Southern Biotech

2.1.8 siRNA oligonucleotides for transfection

Name of gene	**Sequence (5′→3′)**
Control non-silencing siRNA	UUCUCCGAACGUGUCACGUTT
Chk1 #1	AACTGAAGAAGCAGTCGCAGT
Chk1 #2	AAGAAAGAGATCTGTATCAAT
Chk2	CAGGATGGATTTGCCAATCTT
PHB1	CCCAGAAATCACTGTGAAATT

All siRNA oligos were purchased from Qiagen.

2.2 Methods

2.2.1 Cell biological methods

2.2.1.1 Standard procedures for eukaryotic cell cultures

All cell lines were cultured at 37°C in an atmosphere with a relative humidity of 90% and a CO_2 content of 5%. For the inactivation of complement factors, FCS was heated to 56°C for 30 min before use. Unless otherwise indicated, all media contained 10% (v/v) FCS and were supplemented with antibiotics. Cells were harvested by centrifugation for 10 min at 1500 rpm and 4°C. Cell culture work was performed under sterile conditions using a laminar flow hood.

Adherent growing cell lines PC-3, HT-29 and Mcf-7 were maintained in DMEM medium. At a confluency of about 80% cells were split in a ratio of 1:3-1:10. To this end, the supernatant was discarded and 2-5 ml of trypsin/EDTA solution was added for 3-5 min at 37°C. Detached cells were resuspended in fresh DMEM medium and seeded onto new flasks with a confluence of 10-25%.

Jurkat J16, Molt-4, Hut-78, HL-60 and DND41 were grown as suspension cultures and maintained by the replacement of RPMI medium every third day of culture. Cell density was kept between 1-8 x 10^5 cells/ml.

2.2.1.2 Storage of eukaryotic cell lines

Cells were harvested and resuspended in freezing medium containing 70% (v/v) cell culture medium, 20% FCS (v/v) and 10% (v/v) DMSO. The cell density was adjusted to 1-1.5 x 10^7 cells/ml and 1 ml cell suspension was transferred into a cryo-vial and immediately stored at -80°C (-140°C for long term storage). A slow gradient of lowering freezing temperatures was achieved by enwrapping the vials with hand tissue papers.

Frozen cells were thawed directly in a water bath at 37°C. Immediately after thawing, the cell solution was transferred into a 10 ml Falcon tube and resuspended in RPMI with 10% FCS, and centrifuged at 1500 rpm for 5 min. The supernatant was discarded and cells were resuspended in an appropriate volume of fresh culture medium.

The cell density of a culture was determined by use of a Neubauer chamber slide. If necessary, cells were diluted prior to counting.

2.2.1.3 Cell death analysis according to Nicoletti

DNA fragmentation was examined according to the method of Nicolletti (Nicoletti *et al.*, 1991). Approximately 1 x 10^6 cells were collected, lysed in 150 µl of Nicoletti-buffer and stored at 4 ^oC overnight in the dark. The propidium iodide stained DNA fragments were quantified by flow cytometry (FACSCanto II). A minimum of 10'000 cells per sample was analysed. Specific DNA fragmentation was calculated as: (percentage of experimental DNA fragmentation – percentage of spontaneous DNA fragmentation) / (100 – percentage of spontaneous DNA fragmentation) x 100%.

2.2.1.4 Annexin V staining

For analysis of the surface expression of Annexin V, 5 x 10^5 cells were collected and resuspended in 50 µl Annexin V binding buffer supplemented with 10% FCS. Then, 1 µl of Annexin V FITC labelled antibody was added and incubated for 10-20 min at 4°C. Cells were washed with Annexin V binding buffer and analysed by flow cytometry (FACSCanto II). A minimum of 10'000 cells per sample were analysed.

2.2.1.5 Cell cycle analysis

Treated cells were collected and stained with Nicoletti lysis buffer as described in 2.2.1.4. Quiescent and G1 cells have 2N DNA and will therefore have 1X fluorescence intensity. Cells in G2/M phase of the cell cycle will have 4N DNA and therefore will have twice the intensity. Since the cells in S phase are synthesising DNA, they will have fluorescence values between the 1X and 2X populations. The resulting histogram consists of three populations: two Gaussian curves (1X and 2X peaks) and the S-phase population. Adjacent populations overlap each other. Therefore, a population of cells at different cell cycle state can be examined by measuring the amount of fluorescence-labelled DNA by flow cytometry (FACSCanto II). A minimum of 10'000 cells per sample were analysed.

2.2.1.6 Intracellular FACS staining

For intracellular FACS staining of γH2AX 1 x 10^6 cells were collected, washed twice with PBS and fixed with 3% paraformaldehyde for 10 min at 37°C. Cells were then permeabilised with 90% methanol for a minimum of 30 min at 4°C or left overnight at -20°C. After fixation and permeabilisation cells were washed three times with 0.5% BSA in PBS. Afterwards, cells were blocked with 5% mouse serum (inactivated) in PBS and stained with 5 µl γH2AX Alexa Fluor 488 antibody for 1 h at RT in the dark. Cells were washed once again and co-stained with 25 µg/ml propidium iodide containing 100 ng/µl RNase A for 20 min at RT in the dark. Subsequent fluorescence intensities were examined by flow cytometry (FACSCanto II). A minimum of 10'000 cells per sample were analysed.

2.2.1.7 Proliferation analysis

For proliferation analysis 1-2 x 10^6 cells per sample were collected, washed with PBS and incubated for 10 min with 100 nM CFSE stain at RT in the dark. For inactivation, equal volumes of FCS were added, incubated for 2 min at RT in the dark. Cells were then washed twice with culture medium and were incubated as described in 2.2.1.1. Cells were then treated with Roc or DMSO control and after 24 h cells were analysed for proliferation by flow cytometry (FACSCanto II). A minimum of 10'000 cells per sample were analysed

2.2.1.8 Live cell imaging *via* confocal microscopy

To monitor living cells under the microscope, HeLa cells were grown on microscopy chamber slides. Cells were treated with respective compounds and staining solutions, washed with PBS and incubated again with culture medium. Live cell imaging was performed immediately at the Zeiss LSM700 microscope. A minimum of three pictures were taken of each sample.

2.2.1.9 Translocation studies with antibody staining *via* confocal microscopy

For PHB1 translocation studies HeLa cells were grown on microscopy chamber slides and treated appropriately. After washing twice with PBS cells were fixed with 4% paraformaldehyde for 15 min at RT in the dark. After additional washing steps cells were now subjected to permeabilisation with 0.2% Triton X-100 for 10 min at RT in the dark. Cells

were incubated with 1:1250 diluted PHB1 antibody and incubated overnight at 4°C. The next day, cells were incubated with secondary FITC labelled antibody for 2 h at RT in the dark. For microscopy approximately 10 µl Dapi-mounting medium per slide was added and cells were examined at the Zeiss LSM710 microscope. A minimum of three pictures were taken of each sample.

2.2.1.10 siRNA-mediated gene silencing

For siRNA-mediated gene silencing Jurkat JE6.1 cells were transiently transfected by nucleofection using the *Cell Line Nucleofector® Kit V* according to the manufacturer's instructions. 2 x 10^6 JE6.1 cells were resuspended in appropriate nucleofection solution containing 1-2 µM siRNA. Nucleofection was performed using program X-01 for Jurkat JE6.1 cells. After 48-72 h, knock-down efficiency was assessed and cells were used for subsequent analyses.

2.2.1.11 Metabolic labelling

Protein synthesis was estimated by measuring the amount of incorporated ^{35}S methionine. Briefly, 1 x 10^5 cells were incubated for 2 h with methionine-free medium. Then 7 µCi of ^{35}S protein-labelling mix per sample was added and cells were treated with different drugs as indicated. After incubation, cells were washed twice with PBS and lysed in ice cold lysis buffer for 15 min on ice and centrifuged (20 min, 13'000 rpm). Then, 4 µl of each lysate was incubated in 1 ml of Liquid Scintillation Cocktail solution and radioactivity was determined with Liquid Scintillation counting.

2.2.2 Biochemical methods

2.2.2.1 Cell lysis

Cells were harvested, washed with PBS and 200 µl ice-cold lysis buffer per 1 x 10^7 cells was added. The lysates were incubated on ice for at least 20 min at 4°C and were cleared from insoluble cell debris by centrifugation at 14'000 g and 4°C for 30 min. Protein concentrations of whole cell lysates were measured by Bradford according to the manufacturers' instructions and adjusted to equal levels. For gel electrophoresis lysates were mixed with 50 µl of 5x reducing SDS sample buffer per 1 x 10^7 cells and heated to 95°C for 5 min.

2.2.2.2 SDS-PAGE

For electrophoresis 7.5, 10, or 13% polyacrylamide separating gels and 5% polyacrylamide stacking gels were prepared. Polymerisation was initiated by adding 0.1% (v/v) TEMED and the polymerising solution was used immediately. The separating gel was covered with isopropanol. After 15 min the isopropanol was removed by washing with water, the gel surface was dried and the stacking gel was added. Proteins were electrophoretically separated at a constant current of 25-30 mA/gel for 1-1.5 h. To estimate apparent molecular weights of analysed proteins an appropriate molecular weight marker was used. Subsequent to SDS-PAGE, the polyacrylamide gel was subjected to Western blotting (see 2.2.2.3).

2.2.2.3 Western Blot

For Western blot analysis, proteins separated by SDS-PAGE were electrophoretically transferred onto a nitrocellulose membrane using a semi-dry-transfer system (transfer conditions: 0.8 mA/cm^2; 2 h). Gel and membrane were pre-incubated in transfer buffer. After electroblotting, the transferred proteins are bound to the membrane, which providing access for detection by specific antibodies. To avoid unspecific binding the membrane was incubated in blocking solution (5% (w/v) non-fat dried milk in PBS-T or 5% (w/v) BSA in PBS-T) at RT for 1 h on a shaker. The membrane was washed three times with PBS-T for 5 min each time. For specific detection of proteins the membrane was incubated with a primary antibody solution for at least 2 h at RT or overnight at 4°C on a shaker. After washing three times with PBS-T, the membrane was incubated with a HRP-conjugated

α-immunoglobulin secondary antibody for 1 h at RT on a shaker. Finally, the membrane was washed three times with PBS-T for 5 min each time. Detection of membrane bound HRP was performed by enhanced chemiluminescence (ECL) using the Western Lightning™ Plus-ECL reagent according to the manufacturer's instructions. Chemiluminescence was detected using a digital chemiluminescence acquisition system. Analysis was accomplished using the software Chemi-Capt. For specific detection of further proteins the enzymatic activity of the HRP was inactivated by washing with PBS-T. Thereafter, the membrane was incubated with the subsequent primary antibody and the visualisation procedure was repeated as described above.

The quantification of protein levels was performed using ImageJ 1.44p (Abràmoff *et al*., 2004) and a method described in the following Web site: http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/. For each lane, the protein/phosphorylation level was normalised to its respective loading control.

2.2.2.4 Immunprecipitation

For immunprecipiation (IP) cleared lysates of 1-6 x 10^7 cells were incubated with the desired antibody at 4°C on a rotator for 1 h. Subsequently, 40 µl of protein A-sepharose beads were added and the suspension was incubated at 4°C on a rotator for at least 3 h or overnight. The matrix was washed 1-5 times with 1 ml of ice-cold lysis buffer of PBS to remove unspecifically adsorbed proteins. Subsequently, beads were resuspended in 50 µl of lysis buffer and heated in 25 µl 3x sample buffer at 95°C for 5 min. (Co-)purified proteins were detected by SDS-PAGE and immunoblotting (see 2.2.2.2 and 2.2.2.3).

3 Results

3.1 Rocaglamide A induces cell cycle arrest

3.1.1 Rocaglamide A treatment leads to G0/G1 cell cycle arrest in haematological cancer cell lines

Rocaglamide (Roc) derivates inhibit tumour cell proliferation *in vivo,* which was reported by several groups (King *et al.*, 1982; Lee *et al.*, 1998; Hwang *et al.*, 2004; Mi *et al.*, 2006; Zhu *et al.*, 2009). Further analysis revealed that inhibition of tumour growth *in vitro* is accompanied by halt in specific phases of the cell cycle. Various reports showed cell cycle arrest in G0/G1 (Lee *et al.*, 1998; Bleumink, 2007) and G2/M phase of the cell cycle (Bohnenstengel *et al.*, 1999; Mi *et al.*, 2006; Hausott *et al.*, 2004) depending on the cell line tested and the derivative used (Ebada *et al.*, 2011). However, the molecular mechanism underlying Roc-mediated inhibition of proliferation remained elusive.

In order to elucidate the molecular mechanism(s) how rocaglamide derivatives inhibit tumour cell proliferation haematological cancer cell lines Jurkat J16 (ALL), Molt-4 (ALL), Hut-78 (CTCL), HL-60 (AML), DND-41 (ALL) were used as model cells. Thus, it was of interest whether an anti-proliferative effect and/or cell cycle arrest could be observed in these cell lines when treated with Roc A. The death dose curve for Roc A was previously determined by Zhu and colleagues (Zhu *et al.*, 2007). Therefore, the cell lines were treated with the effective concentrations of 50 nM or 100 nM Roc A, respectively, for up to 48 h. Subsequently proliferation, cell cycle and apoptosis status were analysed.

Consistent with other studies (Kim *et al.*, 2006; Proksch *et al.*, 2001; Ebada *et al.*, 2011), upon Roc A exposure Jurkat J16 cells showed a significant (p-value ≤ 0.05) inhibition of cell proliferation after 24 h in a dose-dependent manner (Fig. 3.1 A). Cell cycle analysis of J16 and other haematological cancer cell lines (Molt-4, HL-60, Hut-78, and DND-41) revealed a significant (p-value ≤ 0.05) G0/G1 cell cycle arrest accompanied by a significant (p-value ≤ 0.05) decrease of cells in S phase 24 h after Roc A treatment (Fig. 3.1 B, C). This was accompanied with only mild apoptosis induction after 24 h as measured by Nicoletti-staining and FACS analysis (Fig. 3.1 D).

The presented data show that Roc A inhibits tumour cell proliferation at G0/G1 phase of the cell cycle in haematological cancer cells.

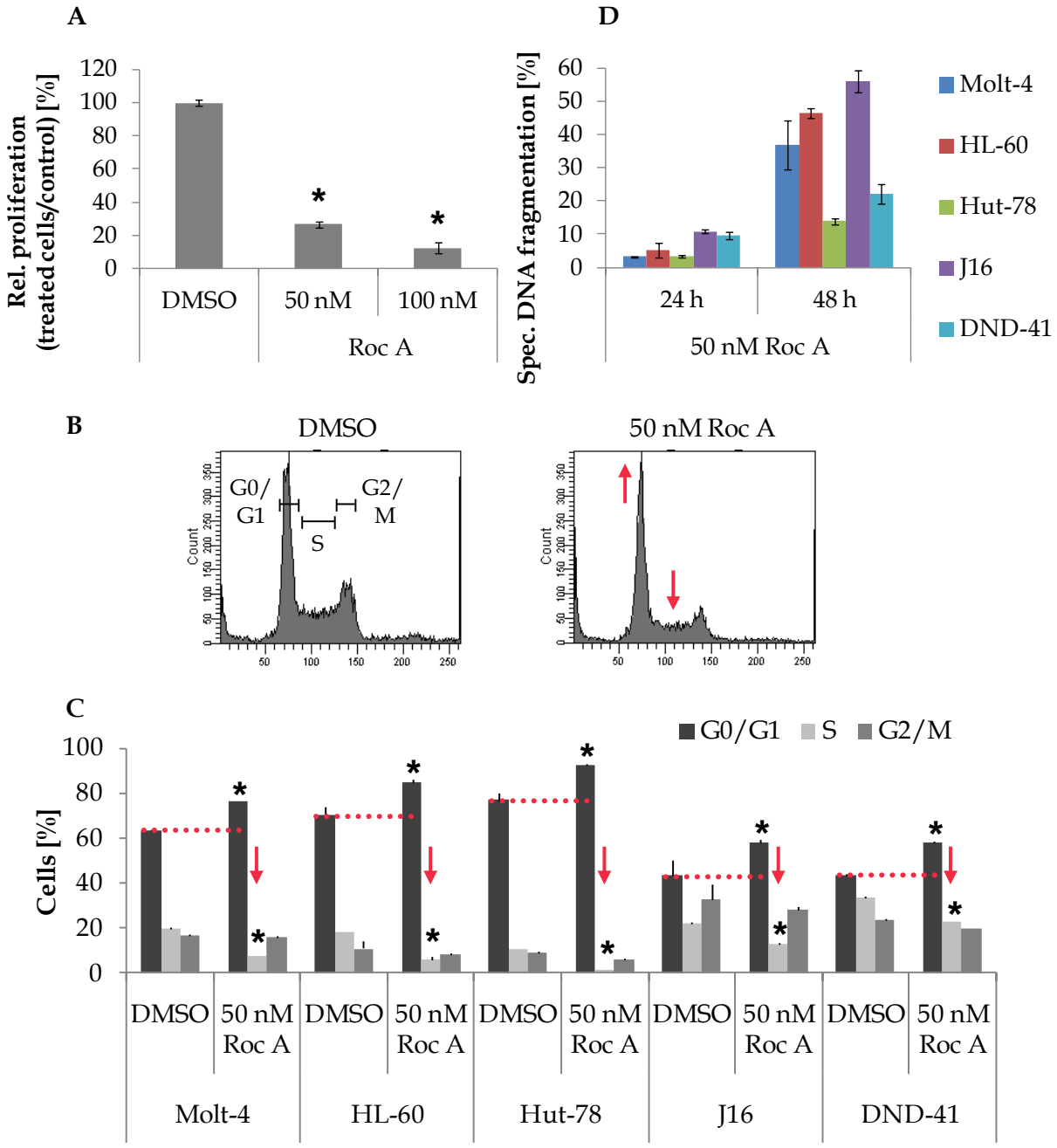


Figure 3.1 | Roc A treatment leads to G0/G1 cell cycle arrest in haematological cancer cell lines.

(**A**) J16 cells were stained with 1 µM CFSE and afterwards treated with 50 nM, 100 nM Roc A or DMSO as a control. Proliferation was measured after 24 h by FACS. Relative proliferation to start of treatment was calculated, DMSO control treated cells were set to 100% proliferation. (**B - D**) Haematological cancer cell lines Molt-4, HL-60, Hut-78, J16 and DND-41 were treated with 50 nM Roc A or DMSO as a control for up to 48 h. (**B, C**) Cell cycle was analysed by FACS after 24 h; (**B**) shows representatively J16 original data. (**D**) Apoptotic cell death was determined by specific DNA fragmentation measurement after 24 h and 48 h. Data are representative of at least two independent experiments (Error bars represent standard deviations; significance was calculated with Student's t-test, * = p-value ≤ 0.05).

3.1.2 Rocaglamide A downregulates proteins necessary for G1-S transition

To investigate further the mechanisms of Roc A-mediated G0/G1 cell cycle arrest various Cdks, cyclins and members of the Cdc25 family of dual specificity phosphatases were analysed by immunoblotting after treatment with 50 nM Roc A for different time periods (Fig. 3.2 A). As seen in Fig. 3.2 B, several proteins necessary for G1-S transition were downregulated upon Roc A treatment. Among them, Cdc25A phosphatase showed a fast decrease, which could be determined as early as 10-15 min after exposure to Roc A. Later events included the downregulation of Cdc25B, Cdk4 and cyclin D3 after 1-2 h and of Cdk6 and cyclin E after 9-12 h treatment. In contrast, Cdc25C, Cdk2 and cyclin A protein levels were not affected in the analysed time frame (Fig. 3.2 B).

Downregulation of Cdc25A was the first effect seen after Roc A treatment and it is known that Cdc25A plays a well characterised role in G1-S transition (Busino *et al.*, 2004, Boutros *et al.*, 2008). Therefore, further studies were carried out to address the molecular mechanisms of Roc A-mediated suppression of Cdc25A expression.

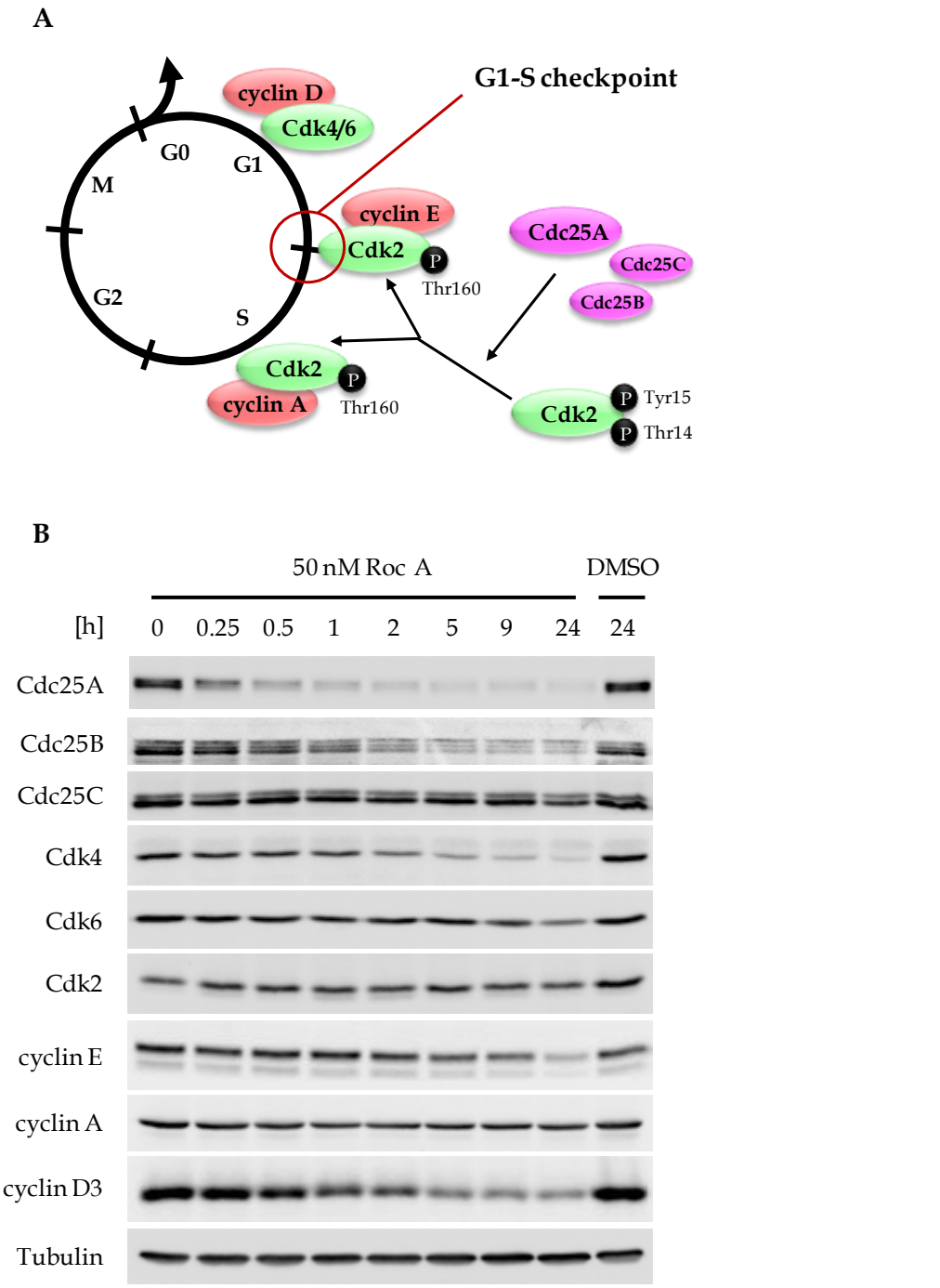


Figure 3.2 | Roc A downregulates proteins necessary for G1-S transition.

(**A**) Progression through G1-S transition is mediated by the activities of cyclin D/Cdk4/6 and cyclin E/Cdk2 complexes. S phase progression is mediated by the activities of cyclin A/Cdk2 complexes. The phosphatases Cdc25A, Cdc25B and Cdc25C regulate Cdk2 activity by removal of inhibitory phosphorylations. For more details see chapter 1.2.4. (**B**) J16 cells were treated with 50 nM Roc A or DMSO as a control for up to 24 h. Cells were lysed and protein expression was analysed by SDS-PAGE followed by immunoblotting with specific antibodies as indicated. Data are representative of at least two independent experiments.

3.2 Rocaglamide A downregulates Cdc25A

3.2.1 Rocaglamide A downregulates Cdc25A only partially *via* translation inhibition

Cdc25A is subject to multiple regulations and is a very unstable protein with basal turnover and protein half-life of 20-30 min (Falck *et al.*, 2001; Mailand *et al.*, 2000; 2002; Molinari *et al.*, 2000; Sørensen *et al.*, 2003). Since rocaglamide derivates are known to inhibit translation (Bleumink *et al.*, 2010; Lee *et al.*, 1998), it was of interest whether Roc A-mediated downregulation of Cdc25A is due to its potency to inhibit protein *de novo* synthesis.

The levels of translation inhibition of rocaglamide derivatives depend on treatment concentration and time (Bleumink *et al.*, 2010; Lee *et al.*, 1998). Therefore, the inhibitory activities of Roc A were analysed by an incorporation assay of ^{35}S-labelled methionine. J16 cells were treated with different concentrations of Roc A or with the well known protein synthesis inhibitor cyclohexamide (CHX) as positive control. Protein *de novo* synthesis was analysed after 2 and 6 h of treatment. The experiment showed that Roc A indeed inhibited protein *de novo* synthesis dependent on the concentration used (Fig. 3.3 A). Notably, the working concentration of 50 nM Roc A used in this study inhibited ^{35}S-incorporation only to about 20%.

In the next step, the kinetics of Cdc25A downregulation in J16 cells treated with Roc A was compared to the kinetics of cells treated with CHX to elucidate whether a similar pattern could be observed. Therefore, J16 cells were exposed to either 50 nM Roc A or 30 µg/ml CHX for up to 120 min. Cdc25A protein levels were analysed by immunoblotting and Western blot bands were quantified with the data imaging software ImageJ (see Materials & Methods). In CHX-treated cells 50% downregulation of Cdc25A occurred after about T_{CHX} = 25 min (Fig. 3.3 B, C). However, Roc A-treated cells showed a faster kinetics of downregulation as Cdc25A protein levels were reduced to 50% after about $T_{Roc\ A}$ = 15 min (Fig. 3.3 B, C). Also, downregulation of Cdc25A did not occur completely as compared to CHX treatment in the analysed time frame (Fig. 3.3 B, C).

Bleumink *et al.* (2010) reported that inhibition of protein *de novo* synthesis occurs *via* the Mek-Erk-Mnk1 signalling pathway. In line, analysis of J16 cells treated with 50 nM Roc A showed downregulation of Erk activity as measured by its phosphorylation after approximately 1-2 h (Fig. 3.3 D).

In summary, these data indicate that Roc A-mediated downregulation of translation occurs at later time points but to only minor extents of about 20%. Therefore, additional mechanisms apart from inhibition of protein *de novo* synthesis may be involved in Roc A-induced downregulation of Cdc25A which are analysed in the following sections.

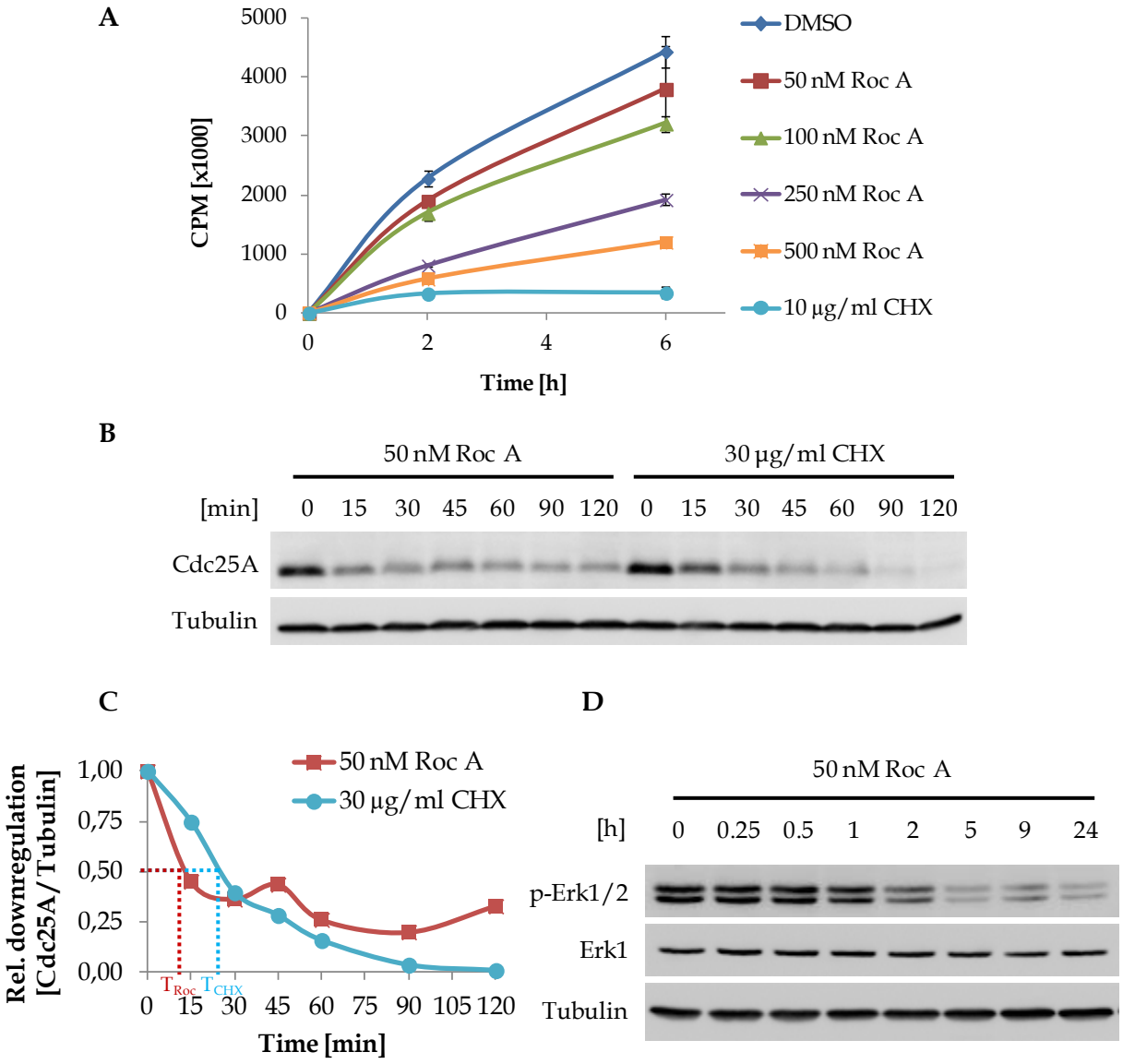


Figure 3.3 | Roc A downregulates Cdc25A only partially *via* translation inhibition.

(**A**) J16 cells were incubated with ^{35}S-labelled methionine and afterwards treated with different concentrations of Roc A (0-500 nM) or with 10 µg/ml cyclohexamide (CHX) for indicated time points. Cells were lysed and radioactivity was analysed with a beta counter. (Error bars represent standard deviations). (**B**) J16 cells were treated with either 50 nM Roc A or 30 µg/ml CHX for up to 120 min. Cells were lysed and protein expression was analysed by SDS-PAGE followed by immunoblotting with specific antibodies. (**C**) Quantification of (B) with the software ImageJ (see Materials & Methods). Cdc25A downregulation was normalised to Tubulin expression. (**D**) J16 cells were treated with 50 nM Roc A for up to 24 h. Cells were lysed and protein expression was analysed by SDS-PAGE followed by immunoblotting with specific antibodies. Data are representative of at least two independent experiments.

3.2.2 Rocaglamide A induces rapid Cdc25A phosphorylation

So far it could be shown that Roc A-mediated downregulation of Cdc25A protein levels may include other mechanisms in addition to translation inhibition. It is known that the stability of Cdc25A is under multiple regulations mediated by phosphorylation. Upon genotoxic stress the rate of phosphate incorporation into Cdc25A on Ser76, Ser124, Ser178, Ser279, Ser 293 and Thr507 increases, which leads to a stronger interaction with the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase and acceleration of Cdc25A protein turnover. Phosphorylation of these sites results in the reduction of protein half-life to up to 10 min (Busino *et al.*, 2003; Jin *et al.*, 2003; Bartek *et al.*, 2004; Sørensen *et al.*, 2003). Interestingly, such short half-life was also observed upon Roc A treatment (Fig. 3.2 and 3.3 B, C). In order to elucidate whether increased phosphorylation of Cdc25A occurs upon exposure to Roc A, J16 cells were treated with 50 nM Roc A for up to 120 min and the phosphorylation and total protein levels were analysed by immunoblotting. The experiments showed that after Roc A treatment for 15 min, an increase in phosphorylation of Cdc25A was observed on both Ser76 and Ser178 sites (Fig. 3.4). Western blot bands were quantified with the data imaging software ImageJ (see Materials & Methods), which revealed an about 6-8 fold increase in Cdc25A phosphorylation in the time frame measured(Fig. 3.4).

Above data demonstrate that upon Roc A treatment the phosphorylation of Cdc25A is increased at least on two crucial sites. Accelerated proteasomal degradation of Cdc25A, the well acknowledged event occurring upon these phosphorylations, may therefore be involved in Roc A-mediated downregulation. So far, increase of phosphate incorporation into Cdc25A was described to occur upon genotoxic stress. Therefore, it remains elusive by which mechanism(s) Roc A triggers Cdc25A phosphorylation.

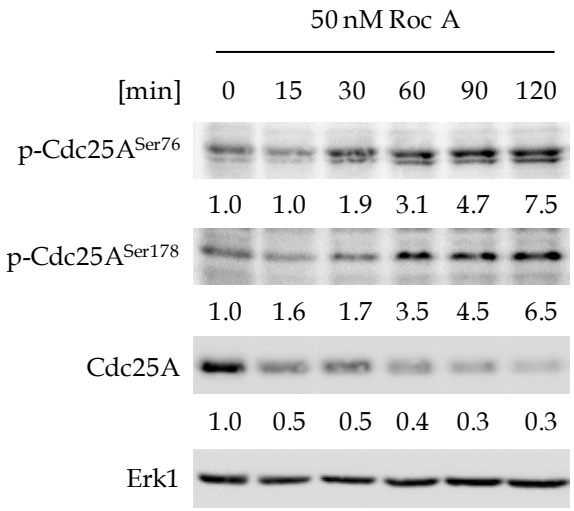


Figure 3.4 | Roc A induces rapid Cdc25A phosphorylation.

J16 cells were treated with 50 nM Roc A for up to 120 min. Cells were lysed and phosphorylation status of Cdc25A was analysed by SDS-PAGE followed by immunoblotting with specific antibodies. Quantification of the western blot bands was conducted with the software ImageJ (see Materials & Methods). Cdc25A phosphorylation was normalised to total-Cdc25A expression and total-Cdc25A downregulation to Erk1 expression. Data are representative of at least two independent experiments.

3.2.3 Rocaglamide A-mediated downregulation of Cdc25A is independent of the p53 status

Increased phosphorylation of Cdc25A upon Roc A treatment was analysed in Jurkat J16 cells, which harbour a heterozygous p53 mutation (Cheng & Haas, 1990). To explore whether the Cdc25A signalling pathway was actively chosen or whether it was only chosen as alternative pathway to mutant p53, several other cancer cell lines harbouring wild-type (wt) or mutated (mut) p53 were analysed (Table 1). The haematological cancer cell lines used before in G0/G1 cell cycle arrest measurements (Fig. 3.1 C), and three additional solid human cancer cell lines (colorectal cancer cells HT-29, prostate cancer cells PC-3 and breast cancer cells Mcf-7) were treated with 50 nM Roc A and Cdc25A protein levels were analysed by immunoblotting. The experiments showed that Cdc25A downregulation was independent from p53 (Fig. 3.5).

Collectively, these data indicate that Roc A treatment leads to the induction of p53-independent signalling pathways, which subsequently trigger Cdc25A phosphorylation, its proteasomal degradation and this eventually leads to the observed G0/G1 cell cycle arrest.

Table 1. p53 status of analysed cells.

Cell line	Cancer type	p53 status
J16	ALL	p53 mut
Molt-4	ALL	p53 wt
Hut-78	CTCL	p53 mut
HL-60	AML	p53 null
HT-29	Colorectal cancer	p53 mut
PC-3	Prostate cancer	p53 null
Mcf-7	Breast cancer	p53 wt

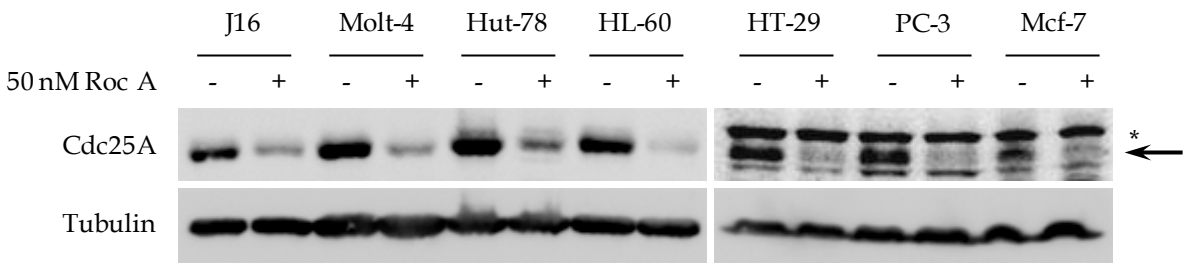


Figure 3.5 | Roc A-mediated downregulation of Cdc25A is independent of the p53 status.

Haematological cancer cell lines J16, Molt-4, Hut-78 and HL-60 and solid cancer cell lines HT-29, PC-3 and Mcf-7 were incubated with 50 nM Roc A or DMSO as a control for 2 h. Cells were lysed and protein expression was analysed by SDS-PAGE followed by immunoblotting with specific antibodies (* unspecific band). Data are representative of at least two independent experiments.

3.3 Rocaglamide A activates a signalling pathway that resembles the DNA damage response pathway

3.3.1 Rocaglamide A activates checkpoint kinases Chk1 and Chk2

Three kinases, namely the cellular checkpoint kinases Chk1 and Chk2 and the mitogen-activated protein kinase (MAPK) p38, are known to regulate Cdc25A stability and turn-over *via* phosphorylation upon exposure to genotoxic stress (Boutros *et al.*, 2007; Goloudina *et al.*, 2000; Mailand *et al.*, 2000; Mikhailov *et al.*, 2005; Xiao *et al.*, 2003; Zhao *et al.*, 2002). As it was shown that Roc A treatment triggers Cdc25A phosphorylation (Fig. 3.4), it was of interest which kinase is involved in the rapid phosphorylation of Cdc25A. To investigate which kinase is involved in Roc A mediated Cdc25A phosphorylation and downregulation, J16 cells were treated with 50 nM Roc A for up to 24 h and kinase activation was investigated as changes in their phosphorylation status by immunoblotting.

The activation of p38 MAPK upon Roc A treatment has been shown by Zhu and colleagues (Zhu *et al.*, 2007) and could be confirmed in this study (Fig. 3.6). Exposure to Roc A resulted in an activation of this kinase measured by the induction of phosphorylation at Thr180/Tyr182 as early as 15 min after treatment. Similar to p38 MAPK activation, Chk2 activation could be determined by its phosphorylation at Thr68 as early as 30 min upon Roc A treatment (Fig. 3.6). In contrast, Chk1 showed a rather transient activation as seen by its phosphorylation at Ser317 peaking at 2-4 h.

In summary, these data provide evidence of possible roles for p38 MAPK and the checkpoint kinases Chk1 and Chk2 in Roc A-mediated Cdc25A phosphorylation that is involved in the accelerated proteolysis and in stopping cell cycle progression.

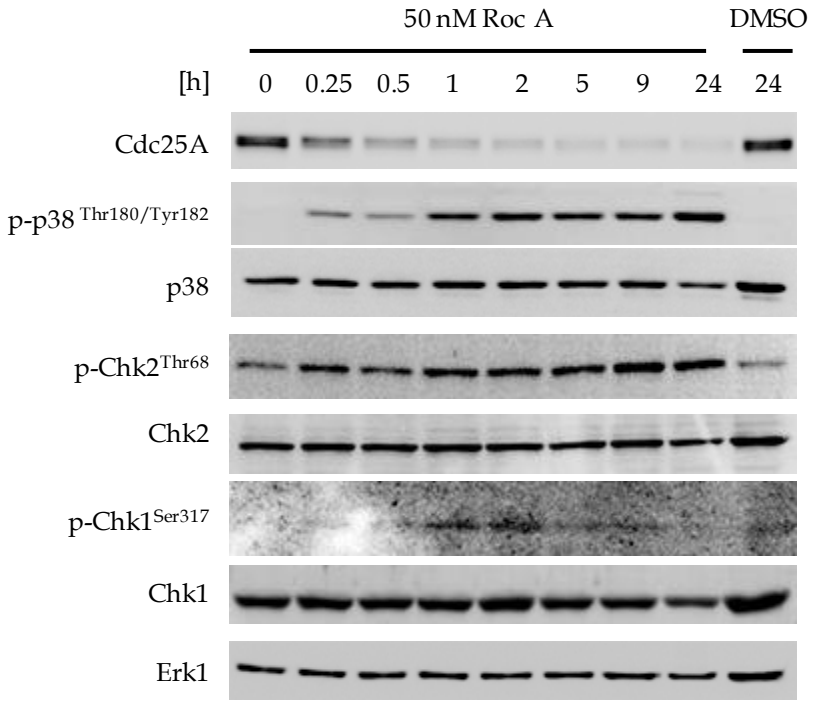


Figure 3.6 | Roc A activates p38 MAPK and checkpoint kinases Chk1 and Chk2.

J16 cells were treated with 50 nM Roc A or DMSO as a control for up to 24 h. Cells were lysed and kinase activities were analysed by SDS-PAGE followed by immunoblotting with specific antibodies. Data are representative of at least two independent experiments.

3.3.2 Rocaglamide A activates DNA damage sensor kinases

DNA damage sensor kinases ATM and ATR are the first to be activated upon exposure to genotoxic stress, which then in turn trigger activation of effector kinases Chk1 and Chk2 (Polager & Ginsberg, 2009). Chk1 is a direct target of the sensor kinase ATR, whereas Chk2 is known to be a direct target of ATM (Bartek & Lukas, 2003; Hurley & Bunz, 2007).

To analyse the upstream signals triggered by Roc A, J16 cells were treated with 50 nM Roc A for up to 33 h and the activities of ATR and ATM were analysed by immunoblotting. The experiments showed that after Roc A treatment the activation of ATM protein kinase could be identified by phosphorylation at Ser1981 (Fig. 3.7 A). In contrast to ATM, the activation status of ATR protein kinase measured by phosphorylation at Ser428 remained unchanged upon Roc A treatment (Fig. 3.7 A).

To further prove that ATM is involved in Roc A-mediated Chk2 activation, an inhibitor against ATM was used (to date, no specific ATR inhibitor exists). J16 cells were pre-treated with 10 µM of the specific ATM inhibitor KU-55933 (ATM i) and then co-incubated with 50 nM Roc A for 3 h. Since Chk2 is known to be a direct target of ATM (Bartek & Lukas, 2003; Hurley & Bunz, 2007), Chk2 activation was analysed by immunoblotting. The experiment showed that inhibition of ATM partially abrogated Roc A-mediated Chk2 activation (Fig. 3.7 B).

Collectively, these data indicate the activation of the DNA damage sensor kinase ATM but not ATR by Roc A. It could also been shown that ATM may be involved in the activation of the downstream effector kinase Chk2. However, the possible involvement of ATM in Chk1 activation remains elusive.

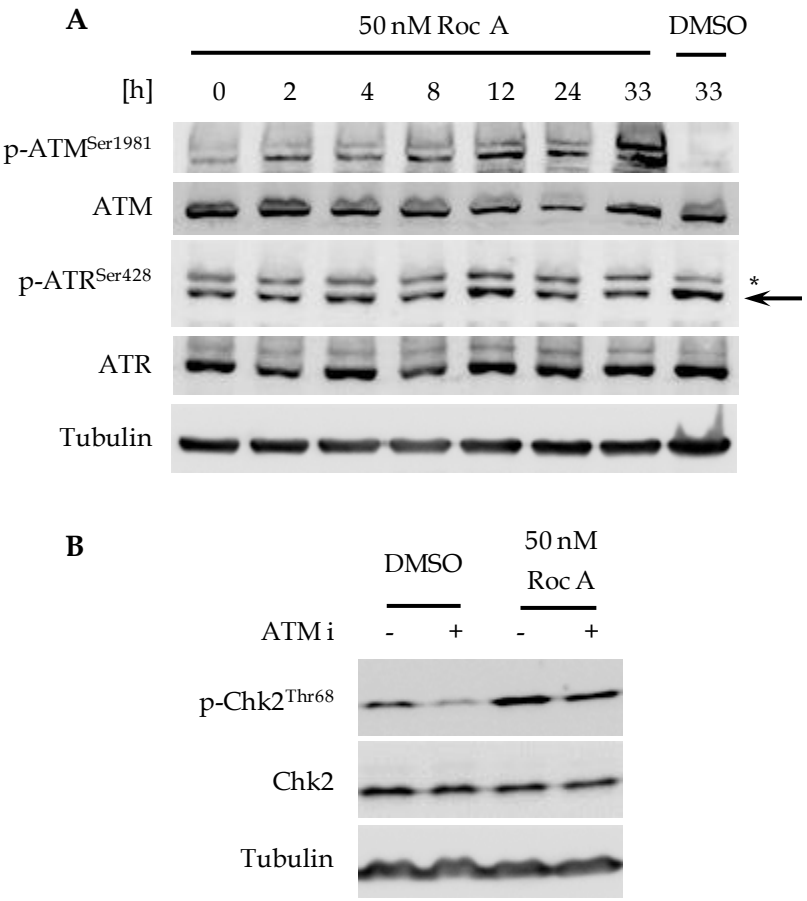


Figure 3.7 | Roc A activates DNA damage sensor kinases.

(**A**) J16 cells were treated with 50 nM Roc A or DMSO as a control for up to 33 h. Cells were lysed and kinase activities were analysed by SDS-PAGE followed by immunoblotting with specific antibodies. (**B**) J16 cells were pre-incubated for 0.5 h with 10 µM KU-55933 (ATM i) or DMSO as a control and then co-incubated with 50 nM Roc A or DMSO as a control for 8 h. Cells were lysed and protein expression was analysed by SDS-PAGE followed by immunoblotting with specific antibodies (* unspecific band). Data are representative of at least two independent experiments.

3.3.3 Inhibition of Chk1 rescues Rocaglamide A-mediated Cdc25A downregulation

To identify the kinase(s) which mediate Cdc25A phosphorylation upon Roc A treatment, in a first attempt studies with inhibitors against MAPK p38, Chk1 and Chk2 were carried out. J16 cells were pre-treated for 0.5 h with 5 µM SB 203580 (p38 i), 5 µM Chk2 inhibitor II (Chk2 i), 5 µM SB 218078 (Chk1 i) or DMSO as a control and then co-incubated with 50 nM Roc A for 4 h. The experiments showed that Roc A-mediated Cdc25A downregulation could be rescued upon treatment with Chk1 i but not with Chk2 i nor p38 i (Fig. 3.8 A).

To confirm the results obtained with the Chk1 inhibitor SB 218078, the effects of a second Chk1 inhibitor, UCN-01, were analysed. J16 cells were pre-treated for 0.5 h with different concentrations of SB 218078 (Chk1 i), UCN-01 or DMSO as a control. Cells were then co-incubated with 50 nM Roc A for 2 h and Cdc25A protein levels were analysed by immunoblotting. Consistent to the former experiment, treatment of the cells with SB 218078 showed a concentration-dependent rescue of Cdc25A (Fig. 3.8 B). However, concentrations up to 5 µM could not fully rescue Roc A-mediated Cdc25A downregulation (Fig. 3.8 B). Exposure to UCN-01 showed a similar concentration-dependent rescue of Cdc25A as SB 218078 (Fig. 3.8 C). In contrast, UCN-01 at concentrations of 500 nM could fully rescue Cdc25A downregulation after 2 h of Roc A treatment (Fig. 3.8 C). Altogether, these data strengthen the possible role of Chk1 in Roc A-mediated Cdc25A downregulation.

ATM was indicated to be an upstream kinase upon Roc A-treatment (Fig. 3.7 A) and activation of ATM has been shown to be involved in downregulation of Cdc25A (Boutros *et al.*, 2007; Shiloh, 2003). To elucidate whether ATM is involved in Cdc25A downregulation, J16 cells were pre-treated with 10 µM of the specific ATM inhibitor KU-55933 (ATM i) and DMSO as a control. Cells were then co-incubated with 50 nM Roc A for up to 120 min and Cdc25A expression levels were analysed by immunoblotting. As seen in Fig. 3.8 D, inhibition of ATM did not rescue Roc A-mediated Cdc25A downregulation. This indicates that other upstream kinases, such as ATR and/or DNA-PK, may be involved in Chk1 and Cdc25A regulation.

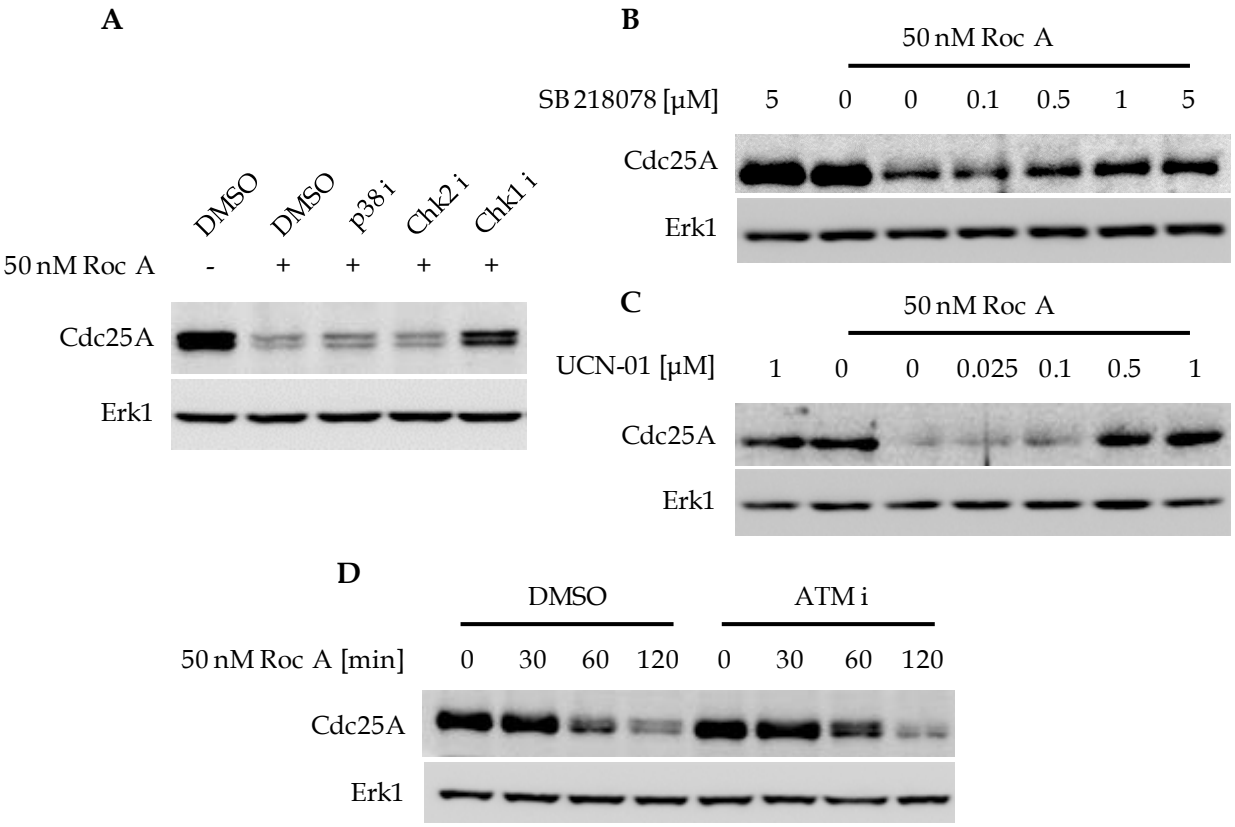


Figure 3.8 | Inhibition of Chk1 rescues Rocaglamide A-mediated Cdc25A downregulation.

(**A**) J16 cells were pre-incubated for 0.5 h with 5 µM SB 203580 (p38 i), 5 µM Chk2 inhibitor II (Chk2 i), 5 µM SB 218078 (Chk1 i) or DMSO as a control and then co-incubated for 4 h with 50 nM Roc A. Cells were lysed and protein expression was analysed by SDS-PAGE followed by immunoblotting with specific antibodies. (**B, C**) J16 cells were pre-incubated for 0.5 h with different concentrations of SB 218078 (**B**) or UCN-01 (**C**) and then co-incubated for 2 h with 50 nM Roc A. Cells were lysed and protein expression was analysed by SDS-PAGE followed by immunoblotting with specific antibodies. (**D**) J16 cells were pre-incubated for 0.5 h with 10 µM KU-55933 (ATM i) or DMSO as a control and then co-incubated with 50 nM Roc A for up to 120 min. Cells were lysed and protein expression was analysed by SDS-PAGE followed by immunoblotting with specific antibodies. Data are representative of at least two independent experiments.

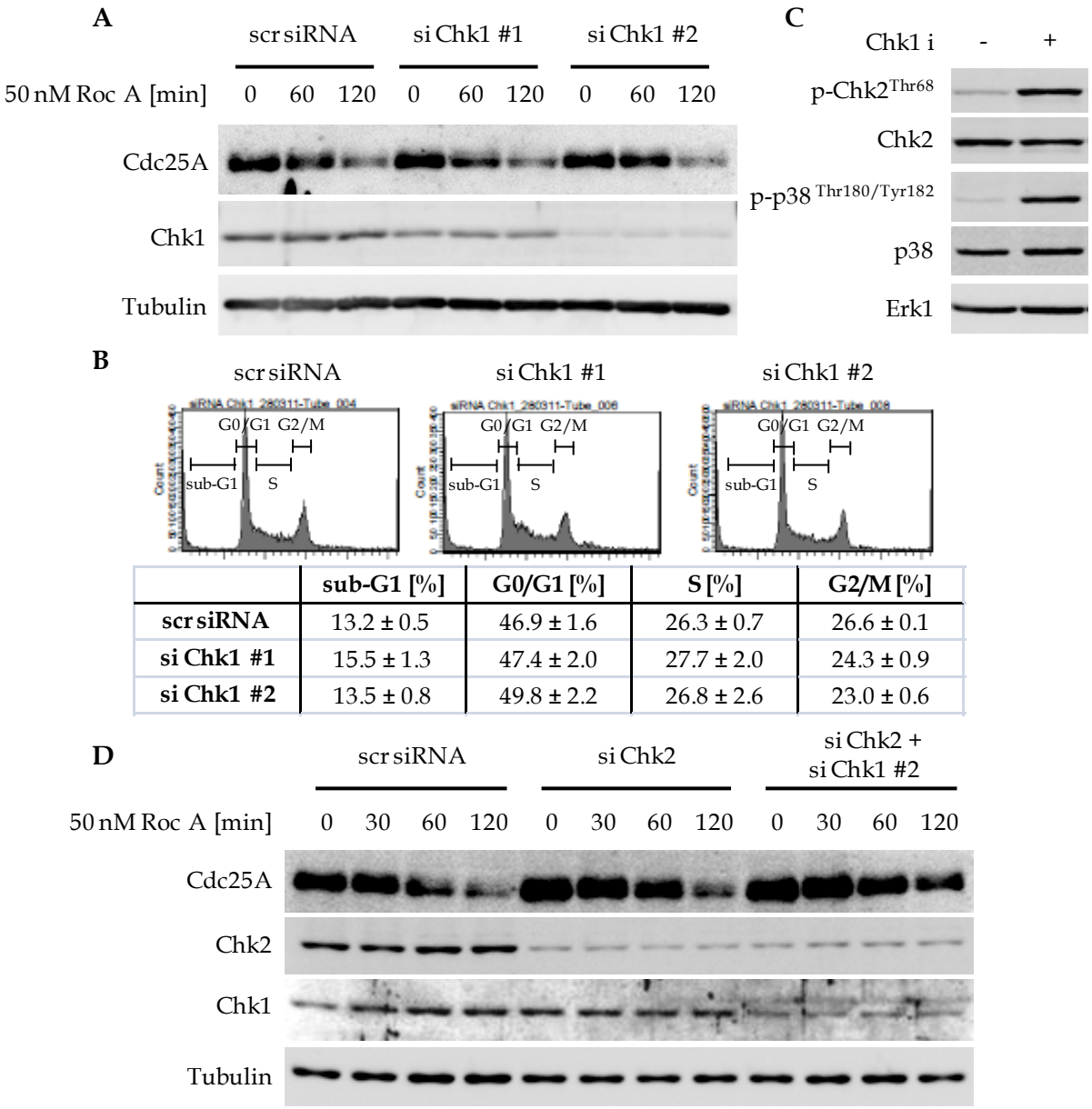
3.3.4 Combined knock-down of Chk1 and Chk2 rescues Rocaglamide A-mediated Cdc25A downregulation

The previous inhibitor studies indicate an important role of Chk1 in Roc A-mediated Cdc25A downregulation. To confirm the obtained results, Jurkat JE6.1 cells were transiently transfected with two different Chk1-specific siRNA oligonucleotides (si Chk1 #1, #2). The cells were then treated with 50 nM Roc A for up to 120 min and protein levels were analysed by immunoblotting. As seen in Fig. 3.9 A, one of the two analysed siRNAs, namely si Chk1 #2, displayed a knock-down effect. However, transient knockdown of this kinase could not rescue Roc A-mediated Cdc25A downregulation.

Interestingly, Chk1 deficient cells displayed a normal cell cycle profile after 48 h (Fig. 3.9 B), which means that the Chk2 pathway may take over functions of Chk1. Since siRNA-mediated knock-down of Chk1 was unable to rescue Cdc25A downregulation after Roc A treatment, it was hypothesised that the cells may have adapted and bypassed their Chk1 deficiency. It is known that next to Chk1, also MAPK p38 and Chk2 can modulate Cdc25A phosphorylation and can impact its protein stability (Boutros *et al.*, 2007; Goloudina *et al.*, 2000; Mailand *et al.*, 2000; Mikhailov *et al.*, 2005; Xiao *et al.*, 2003; Zhao *et al.*, 2002). Therefore, to elucidate whether inhibition of Chk1 may influence Chk2 and MAPK p38 activity, J16 cells were treated with 5 µM SB 218078 (Chk1 i) for 7 h and kinase activation was investigated as changes in the phosphorylation status by immunoblotting activity. As shown in Fig. 3.9 C, abrogation of Chk1 activity upregulated basal activity of Chk2 and MAPK p38. This indicates that possibly Chk2 and/or MAPK p38 are able to overtake Chk1 deficiency.

Several reports showed that Chk1 and Chk2 can have overlapping functions and can substitute each other as seen by common downstream targets (Bartek & Lukas, 2003; Reinhardt & Yaffe, 2009). This was not reported for MAPK p38 and therefore the focus was on Chk2 to possibly overtake Chk1 actions. To analyse whether Chk2 can overtake Chk1 functions in the Roc A-mediated Cdc25A regulation, a combined transient knock-down of Chk1 and Chk2 in JE6.1 cells was performed. The cells were transfected with the Chk1-specific siRNA oligonucleotide (si Chk1 #2) and/or a Chk2-specific siRNA oligonucleotide (si Chk2). After 48 h cells were then treated with 50 nM Roc A for up to 120 min and protein levels were analysed by immunoblotting. Whereas single Chk2 knock-down slightly rescued Roc A-mediated Cdc25A downregulation, significant rescue was obtained by combined knock-down of both Chk1 and Chk2 after 120 min of exposure to Roc A (Fig. 3.9 D).

In summary, these data demonstrate that Chk1 plays an important role in the Roc A-induced Cdc25A downregulation. However, siRNA knock-down data indicate that Chk2 may also participate in Roc A-mediated downregulation of Cdc25A protein expression.



	sub-G1 [%]	G0/G1 [%]	S [%]	G2/M [%]
scr siRNA	13.2 ± 0.5	46.9 ± 1.6	26.3 ± 0.7	26.6 ± 0.1
si Chk1 #1	15.5 ± 1.3	47.4 ± 2.0	27.7 ± 2.0	24.3 ± 0.9
si Chk1 #2	13.5 ± 0.8	49.8 ± 2.2	26.8 ± 2.6	23.0 ± 0.6

Figure 3.9 | Combined knock-down of Chk1 and Chk2 rescues Roc A-mediated Cdc25A downregulation.

(**A, B**) JE6.1 cells were transiently transfected with 1 µM scrambled (scr siRNA) or two different Chk1-specific siRNA oligonucleotides (si Chk1 #1, #2). After 48 h cells were treated with 50 nM Roc A for up to 120 min. (**A**) Cells were lysed and protein expression was analysed by SDS-PAGE followed by immunoblotting with specific antibodies. (**B**) Cells were stained according to the Nicoletti-method and subjected to cell cycle analysis. (**C**) J16 cells were pre-incubated for 0.5 h with 5 µM SB 218078 (Chk1 i) or DMSO as a control and then co-incubated for 7 h with 50 nM Roc A. Cells were lysed and protein expression was analysed by SDS-PAGE followed by immunoblotting with specific antibodies. (**D**) JE6.1 cells were transiently transfected with 1 µM scrambled (scr siRNA), Chk2-specific siRNA (si Chk2) or Chk1-specific siRNA (si Chk1 #2) oligonucleotides either alone or in combination. After 48 h cells were treated with 50 nM Roc A for up to 120 min. Cells were lysed and protein expression was analysed by SDS-PAGE followed by immunoblotting with specific antibodies. Data are representative of at least two independent experiments.

3.4 Sources of the activation of the Rocaglamide A-resembled DNA damage response pathway

3.4.1 Rocaglamide A does not induce direct DNA double strand breaks

Roc A was shown to activate ATM (Fig. 3.7 A), however, it still remains elusive how Roc A triggered its activation. Most published data show the role of ATM as primary sensor protein of DNA double-strand breaks (DSB). However, recently it was reported that ATM can be activated by oxidative stress (Guo *et al.*, 2010). Since rocaglamide derivatives do not induce oxidative stress (Kim *et al.*, 2006; Proksch *et al.*, 2001; Ebada *et al.*, 2011), the possibility of ATM activation by reactive oxygen species (ROS) upon Roc A treatment was excluded and the focus drawn to ATM activation by DSB.

Upon DSB, ATM is recruited to the DNA and leads to phosphorylation of the signal mediator Histone H2AX (γH2AX) and foci formation (Canman, 2003). Therefore, γH2AX is used as common biomarker for detection of DSB (Bonner *et al.*, 2008; Rogakou *et al.*, 1998). In order to detect any development of DSB upon Roc A treatment, the formation of γH2AX was determined by intracellular FACS-staining and confocal microscopic analysis. J16 cells were treated with 100 nM Roc A for up to 24 h and for a positive control 200 nM Doxorubicine (Dox) was used. At these concentrations, no DNA fragmentation could be seen after 8 h treatment and only 10-15% DNA fragmentation was detected after 24 h (Fig. 3.10 A). Thereby a role of γH2AX foci formation due to apoptotic DNA fragmentation could be excluded.

The cells were co-stained with a fluorescent labelled antibody against γH2AX and with the DNA staining dye propidium iodide (PI) to visualise distribution of foci formation in the different cell cycle phases. As expected, after 8 h treatment with 200 nM Dox about 15% of the cells showed γH2AX foci formation and 50% were γH2AX positive after 24 h (Fig. 3.10 B, upper panel). Using confocal microscopy analysis the foci formation after Dox treatment could be clearly visualised as increasing green *dots* that represent γH2AX foci (Fig. 3.11, upper panel). Nuclear staining *via* PI showed no apoptotic DNA fragmentation (Fig. 3.11, upper panel). This indicates that the increase in fluorescence intensity measured by FACS was indeed due to increased γH2AX foci formation in living cells.

In contrast, 100 nM Roc A treatment did not show any foci formation in living cells as measured by FACS (Fig. 3.10 B, lower panel). An increase of γH2AX fluorescent intensity could be observed in Roc A-treated cells only after 24 h (Fig. 3.11, lower panel). Consistent

with the apoptosis analysis (Fig. 3.10 A), PI-staining clearly showed apoptotic cells as visible by condensated DNA (Fig. 3.11, lower panel).

Collectively, no signs of direct DNA damaging potency of Roc A could be detected by the methods used in this study. However, a response similar to DDR occurs immediately upon Roc A treatment (Fig. 3.7). Since at this time no apoptotic DNA fragmentation is measurable, other kinds of DNA damage or DNA stress might be triggered by Roc A as an alternative mechanism.

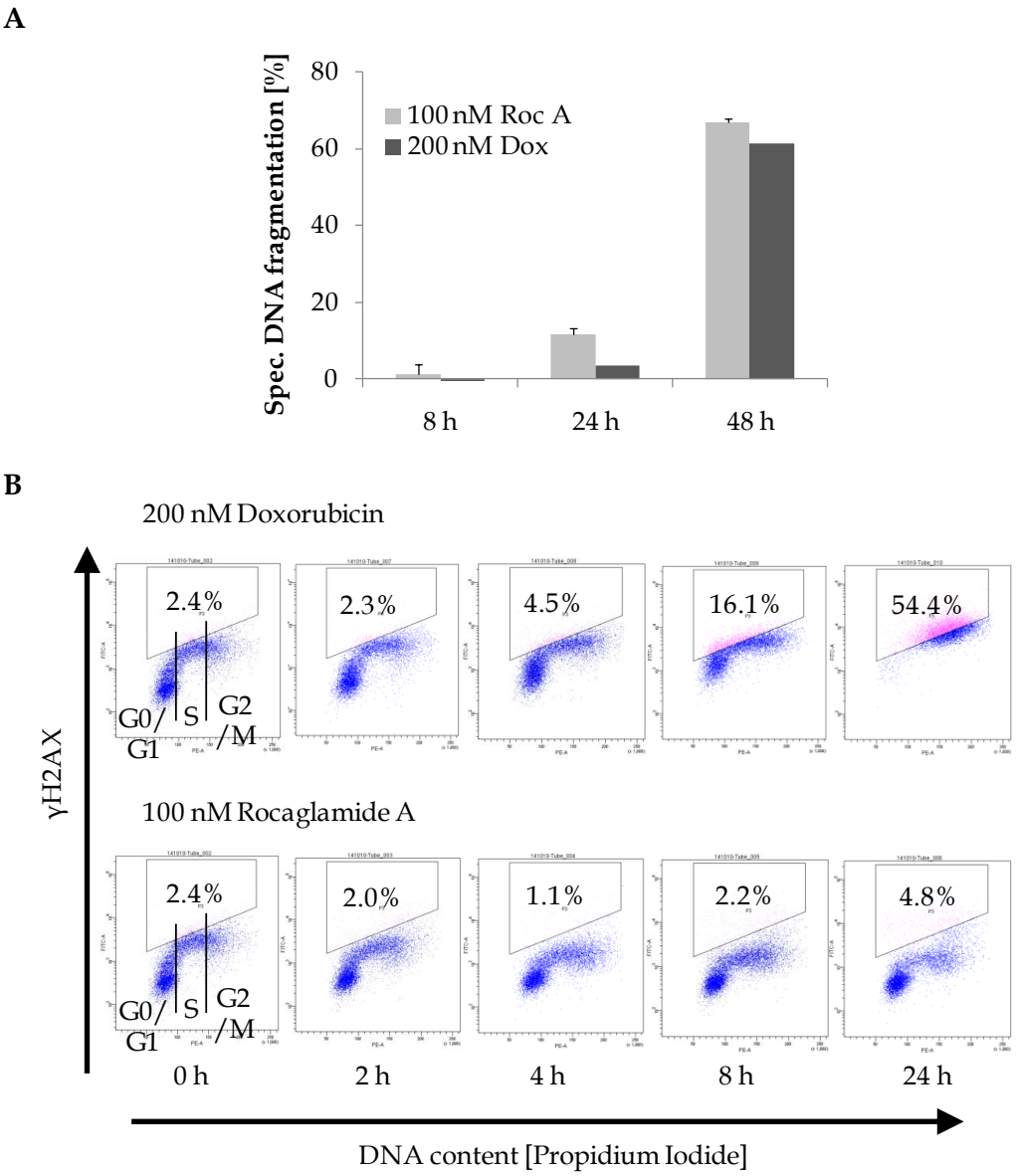


Figure 3.10 | Roc A does not induce direct DNA double strand breaks as measured by FACS.

(**A**) J16 cells were treated with 100 nM Roc A, 200 nM Doxorubicin or DMSO as a control. DNA fragmentation was measured according to the Nicoletti-method at indicated time points. (**B**) Cells were treated as in (A) and after fixation and permeabilisation of the cells intracellular γH2AX staining was performed. After co-staining with propidium iodide (PI) cells were analysed by FACS. Only living cells were analysed (blue), PI staining shows cell cycle distribution (G0/G1, S and G2/M phase). Increase of γH2AX staining is represented by increase in purple colour. Data are representative of at least two independent experiments (Error bars represent standard deviations).

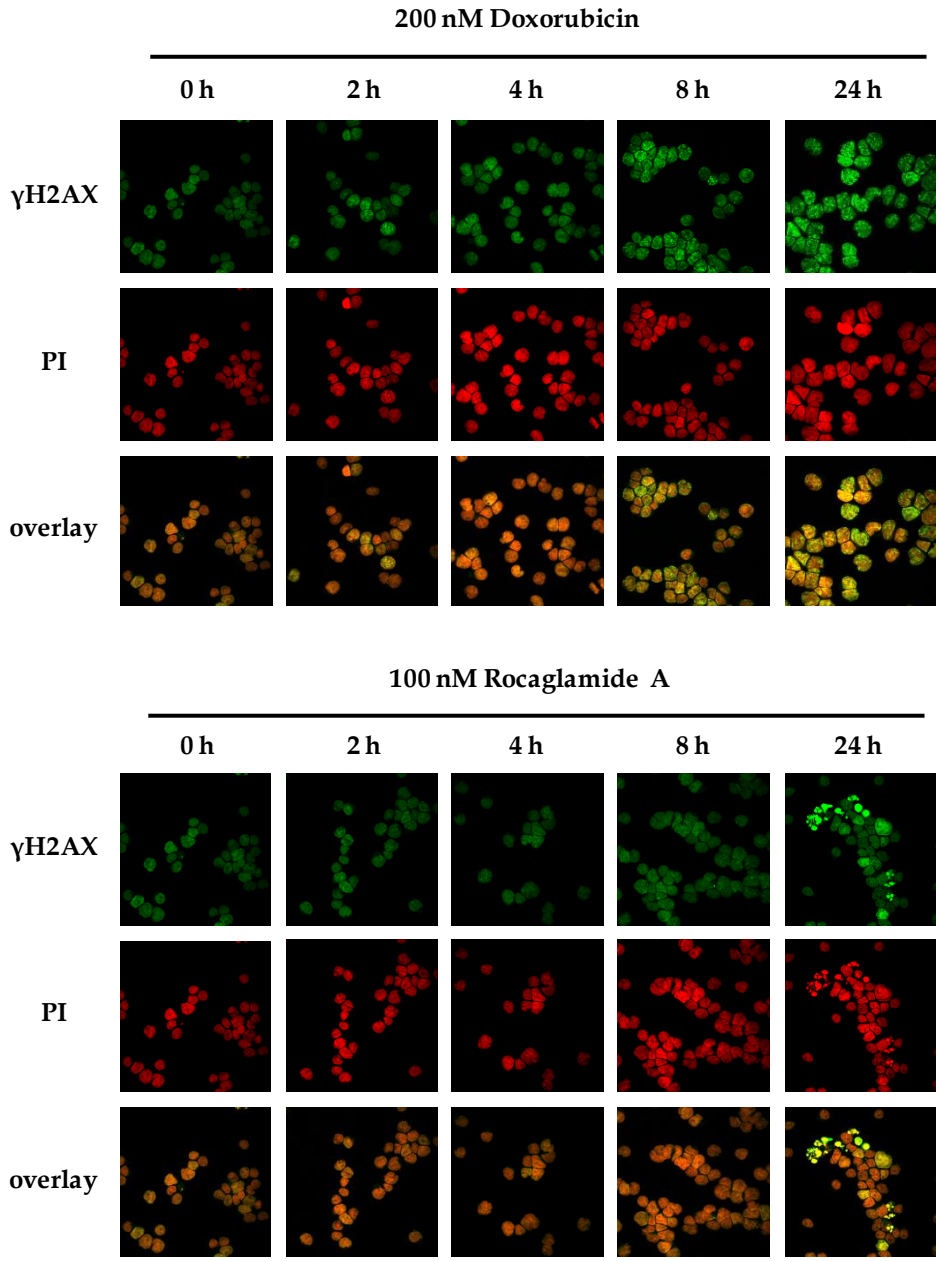


Figure 3.11 | Roc A does not induce direct DNA double strand breaks as measured by confocal microscopy.

J16 cells were treated as in Fig. 3.10 B and analysed by confocal microscopy. γH2AX staining is represented in green, increase of γH2AX foci formation can be observed as increasing in green *dots*. PI-staining shows DNA (red), overlay of γH2AX- and PI-staining is represented in orange. Data are representative of at least two independent experiments.

3.4.2 Rocaglamide is not localised in the nucleus

So far, the mechanism by which Roc A-mediated genotoxic stress in turn activates the DDR pathway remains elusive. In chemotherapy, fast-dividing malignant cells are targeted by DNA damaging agents such as alkylating agents, antimetabolites, anthracyclines, plant alkaloids, and topoisomerase inhibitors, all of these drugs affecting cell division or DNA synthesis and function (Takimoto & Calvo, 2009). To investigate the nuclear localisation of Roc A and to approach the question whether it exerts similar effects a fluorescent-labelled rocaglamide derivate was monitored for its sub-cellular localisation.

In cooperation with Thuaud and colleagues (Thuad *et al.*, 2009), who reported of a fluorescent-labelled synthetic rocaglamide derivative (FLO) with anti-proliferative activities, the same compound was analysed in our lab. To exclude artefacts due to cell fixation live cell imaging of human cervical cancer cells HeLa was performed. The cells were treated with 50 µM FLO for 1.5 h and then co-incubated with either 50 nM Mito-Tracker (mitochondrial stain), 500 nM ER-Tracker (endoplasmatic reticulum stain) or 1 µM DRAQ5 (nuclear stain) for 0.5 h. Analysis by confocal microscopy showed that FLO can be detected in the mitochondria (Fig. 3.12, upper panel) as well as in the ER (Fig. 3.12, middle panel) as seen by purple colour in the overlay. However, a nuclear localisation of FLO could be ruled out (Fig. 3.12, lower panel).

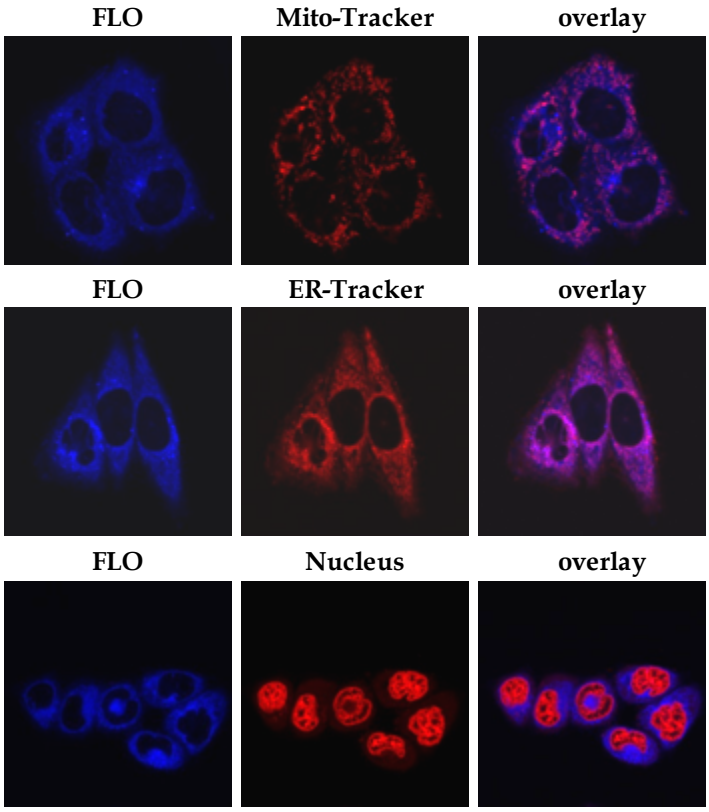


Figure 3.12 | Rocaglamide derivatives are not localised in the nucleus.

HeLa cells were treated for 1.5 h with 50 µM FLO and then co-incubated with 50 nM Mito-Tracker, 500 nM ER-Tracker or 1 µM DRAQ5 (Nucleus) for 0.5 h. The cells were washed once with PBS, incubated in fresh medium and analysed by confocal microscopy. Data are representative of at least two independent experiments.

3.5 Rocaglamide A binds to PHB1

3.5.1 Rocaglamide A induces PHB1 translocation into the nucleus

Recently, it was discovered that Roc A binds to Prohibitin (PHB1) (unpublished data). PHB1 belongs to a highly conserved and ubiquitously expressed family of proteins and is described to be involved in cell proliferation (Mishra *et al.*, 2005). It has been shown that PHB1 can translocate between nucleus, mitochondria, and cytoplasm upon various stimuli (Mishra *et al.*, 2005; Theiss & Sitaraman, 2011) and that the sub-cellular localisation of PHB1 can affect cell fate, specifically apoptosis (Rastogi *et al.*, 2006). Therefore, it was analysed whether Roc A exerts genotoxic stress *via* interaction with PHB1 and subsequent translocation of PHB1 into the nucleus.

One of the most sensitive methods to analyse protein translocation in sub-cellular compartments is by confocal analysis. Thus, HeLa cells were treated with 50 nM Roc A for 4 h and after fixation, permeabilisation, and antibody staining subjected to confocal analysis. As it is shown in Fig. 3.13 A, Roc A treatment induces translocation of PHB1 into the nucleus. For quantification, the confocal data were analysed with the image software ImageJ. A mask of the nucleus was generated and the fluorescence intensity of PHB1 in the nucleus with and without Roc A treatment was investigated. The data were calculated *via* the Student's *t*-test and visualised by box plots. Quantification of the images displayed a significant ($p<0.001$) translocation of PHB1 into the nucleus upon Roc A treatment (Fig. 3.13 B).

In summary, these data suggest that elevated levels of PHB1 in the nucleus after Roc A treatment might induce and/or contribute to genotoxic stress. This can then lead to the activation of the DDR pathway, inhibition of proliferation and induce G0/G1 cell cycle arrest.

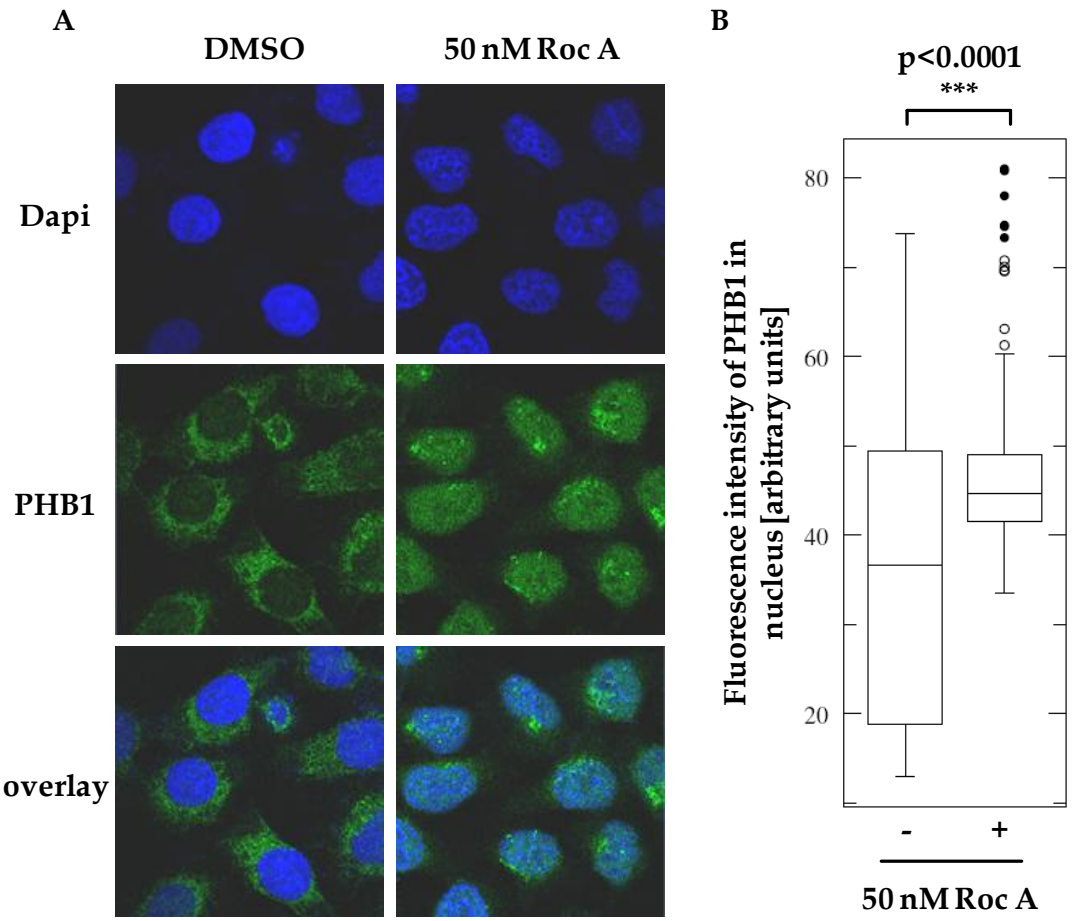


Figure 3.13 | Roc A induces PHB1 translocation into the nucleus.

HeLa cells were treated with 50 nM Roc A or DMSO as a control for 4 h. Cells were fixed for 15 min with 4% PFA and then permeabilised for 10 min with 0.2% T-100. After 1 h blocking with 5% BSA, cells were incubated with PHB1 antibody overnight. Then, cells were incubated with fluorescent labelled secondary antibody and analysed by confocal microscopy. (**A**) Representative pictures of confocal microscopic analysis. (**B**) A mask of the nucleus was generated with the software ImageJ and the fluorescence intensity of PHB1 treatment in the nucleus with and without Roc A treatment was analysed. Data sets of each experiment undertaken are graphically represented in box plots. Data are representative of at least two independent experiments.

3.5.2 PHB1 knock-down does not increase Chk1 or Chk2 activity

The previous data obtained suggest that through the Roc A-mediated translocation of PHB1 into the nucleus genotoxic stress is exerted to the cells. To test whether PHB1 is inducing this genotoxic stress, Jurkat JE6.1 cells were depleted of PHB1 and the activation status of Chk1 and/or Chk2 was analysed. Thus, the cells were transiently transfected with a PHB1-specific siRNA oligonucleotide (si PHB1) and after 16 and 24 h of knock-down the activation status of Chk1 and Chk2 was analysed by immunoblotting. As seen in Fig. 3.14, depletion of PHB1 did not lead to an increased activation of Chk1 nor Chk2 as it is seen after Roc A treatment.

In summary, these data indicate that Roc A-mediated PHB1 translocation into the nucleus does not lead to genotoxic stress by which subsequently the signalling pathway that resembles the DDR is activated. Neither these data indicate that PHB1 is responsible for the rapid downregulation of Cdc25A. Instead, the data rather suggest that Roc A triggers genotoxic stress prior to (or aside of) binding to PHB1.

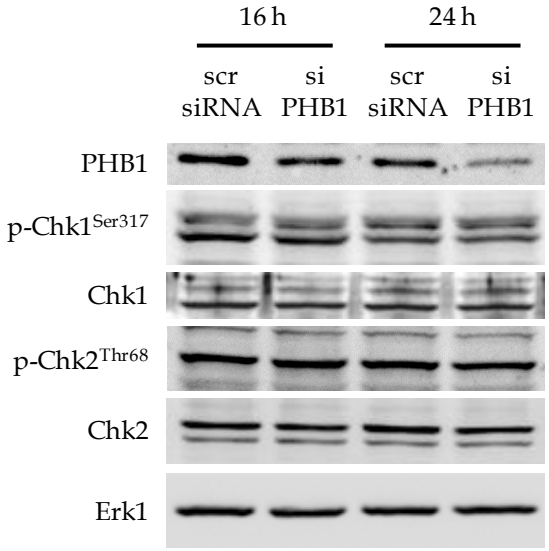


Figure 3.14 | PHB1 knock-down does not increase Chk1 or Chk2 activity.

JE6.1 cells were transiently transfected with 1 µM scrambled (scr siRNA) or PHB1-specific siRNA oligonucleotides (si PHB1). After 16 and 24 h cells were lysed and activation status was analysed by SDS-PAGE followed by immunoblotting with specific antibodies. Data are representative of at least two independent experiments.

3.5.3 PHB1 knock-down mimics the effects of Rocaglamide A on cell cycle progression

After it could be ruled out that the interaction of Roc A with PHB1 most certainly does not trigger genotoxic stress it was questioned by which other mechanism this interaction may lead to the observed effects on the cell cycle upon Roc A treatment. To analyse this, Jurkat JE6.1 cells were transiently transfected with a PHB1-specific siRNA oligonucleotide and cell cycle analysis was performed by Nicoletti staining and FACS measurements. Indeed, PHB1 knock-down leads to a significant (p-value ≤ 0.05) G0/G1 cell cycle arrest accompanied by a significant (p-value ≤ 0.05) decrease of cells in S phase (Fig. 3.15 A). The cells were also analysed by immunoblotting with respect to downregulation of cell cycle proteins necessary for G1-S transition. Interestingly, those proteins that were donwregulated after Roc A treatment (Fig. 3.2 B) showed also decreased expression in PHB1 deficient cells after 24 h, such as Cdc25A, Cdc25B, Cdk4, Cdk6 and Cyclin D3 (Fig. 3.15 B).

Since it is known that Roc A exerts translation inhibitory effects (Fig. 3.3 A) we asked the question whether PHB1 knock-down also leads to inhibition of protein *de novo* synthesis and by this to downregulation of the cell cycle proteins. Therefore, PHB1 deficient cells were analysed by an incorporation assay of ^{35}S-labelled methionine after 2 and 6 h. The experiment revealed that PHB1 deficient cells indeed showed a significant (p-value ≤ 0.05) decrease in protein *de novo* synthesis of about 20% (Fig. 3.15 C). Similar to Roc A treated cells (Fig. 3.3 D), PHB1 deficient cells also showed decreased Erk activity (Fig. 3.15 D).

In summary, these data indicate that the main mechanism of Roc A mediated cell cycle arrest and downregulation of cell cycle proteins is mediated by the interaction of Roc A with PHB1. This interaction leads to downregulation of the Erk pathway by which protein *de novo* synthesis in inhibited (Bleumink *et al*., 2010).

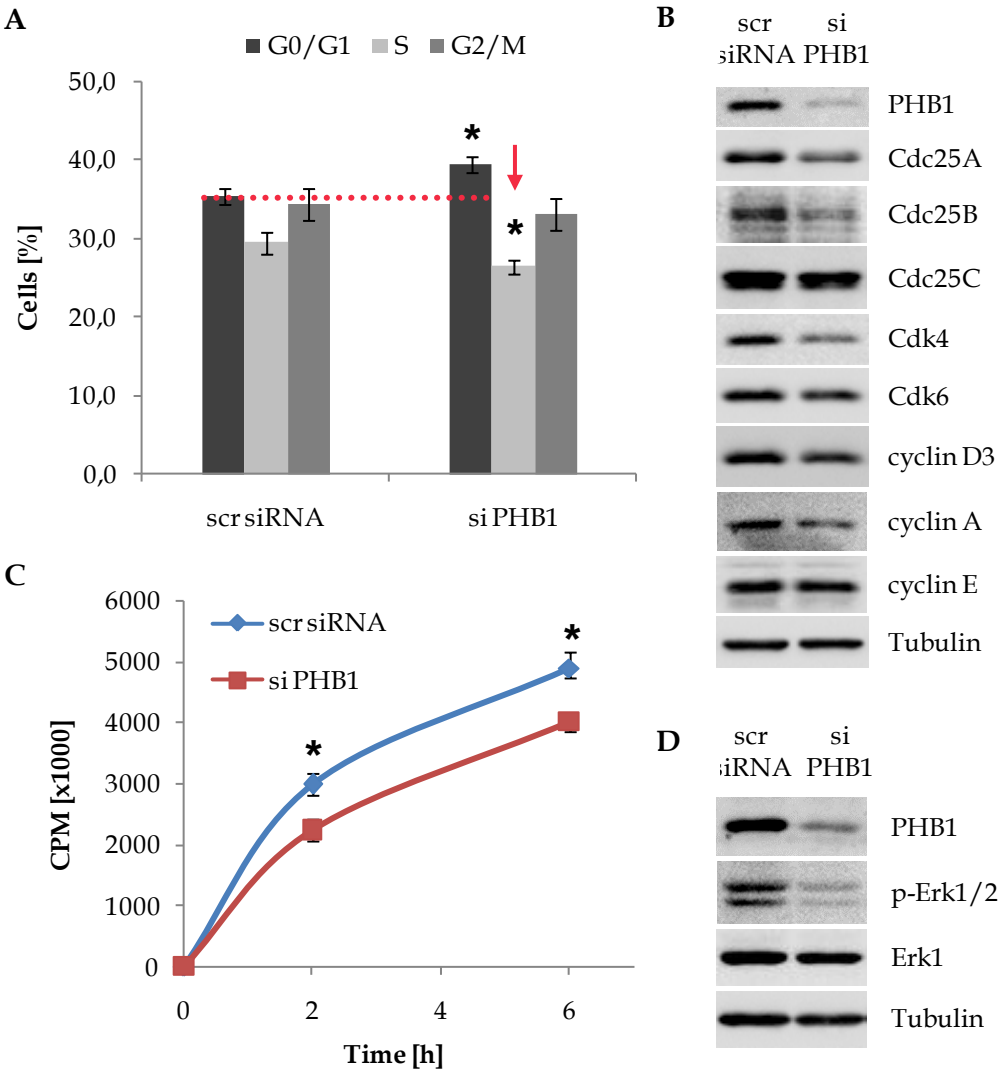


Figure 3.15 | PHB1 knock-down mimics the effects of Rocaglamide A on cell cycle progression.

(**A-D**) JE6.1 cells were transiently transfected with 1 µM scrambled (scr siRNA) or PHB1-specific siRNA (si PHB1) oligonucleotides. (**A**) After 16 h cells were stained according to the Nicoletti method and subjected to cell cycle analysis by FACS measurement. (**B**) After 24 h cells were lysed and protein expression was analysed by SDS-PAGE followed by immunoblotting with specific antibodies. (**C**) After 72 h cells were incubated with ^{35}S-labelled methionine, after the indicated time points cells were lysed and radioactivity was analysed with a beta counter. (**D**) After 72 hours cells were lysed and protein expression was analysed by SDS-PAGE followed by immunoblotting with specific antibodies. Data are representative of at least two independent experiments (Error bars represent standard deviations).

4 Discussion

4.1 Anti-proliferative activities of Rocaglamide A

Although the anti-proliferative activities of rocaglamide derivatives in cancer have been known for 30 years, the molecular mechanisms by which they exert inhibition of proliferation are largely unknown. It has been shown that 4′-demethoxy-3′,4′-methylenedioxy-methylrocaglate inhibits tumour cell proliferation with cell accumulation in the G0/G1 phase of the cell cycle in human lung carcinoma cells Lu1 (Lee *et al.*, 1998). Similarly, treatment of human HTLV1-associated T cells with 1-oxo-11,12-methylendioxyrocaglaol (Roc AR) resulted in cell accumulation in the G0/G1 phase of the cell cycle and decreased cyclin D_1 and D_2 protein expression (Bleumink, 2007). Inhibition of tumour cell proliferation with cell accumulation in the G2/M phase of the cell cycle (Bohnenstengel *et al.*, 1999; Mi *et al.*, 2006) or cell cycle block in mitosis (Hausott *et al.*, 2004) was also observed upon treatment with rocaglamide derivatives. Didesmethyl-rocaglamide was even shown to be able to induce growth arrest of human monocytic leukemia cells MONO-MAC-1 in the G2/M and probably G0/G1 phases of the cell cycle (Bohnenstengel *et al.*, 1999). For the rocaglate derivative silvestrol, it could be demonstrated that its cytotoxicity in human prostate cancer cells LNCaP was associated with a block at the G2/M cell cycle checkpoint and with a decrease in cyclin B and Cdc25C protein levels in a p53-independent manner (Mi *et al.*, 2006). Aglaiastatin, a flavonol-cinnamate-derived cyclopenta[b]benzofuran, caused a cell cycle block in colon carcinoma cells SW480 in early mitosis (Hausott *et al.*, 2004). This was accompanied with an increase in cyclin B and a decrease in cyclin A protein levels upon exposure (Hausott *et al.*, 2004). Uniformly, all active rocaglamide derivatives demonstrate anti-proliferative activities with often only minor cell death induction (Kim *et al.*, 2006). Their way to arrest cells in a specific cell cycle phase, however, is dependent on the cell line and/or rocaglamide derivative.

In line with the previous observations, this study shows that the rocaglamide derivative Rocaglamide A (Roc A) inhibits proliferation of haematological cancer cell lines with an associated G0/G1 cell cycle arrest (Fig. 3.1 A-C). Importantly, this was accompanied by little cell death induction after 24 h (Fig. 3.1 D), which gave rise to the working hypothesis of this study that Roc A treatment leads firstly to inhibition of proliferation and secondly to induction of apoptosis.

Deeper analysis of the molecular mechanism by which Roc A may have triggered stop of cell proliferation at the G0/G1 cell cycle phase led to the investigation of important key players in G1-S transition, such as Cdk2, Cdk4, Cdk6, cyclin D3, A and E and the Cdk-activating phosphatases Cdc25A, Cdc25B and Cdc25C, upon treatment (Fig. 3.2 A). Downregulation of Cdk4, Cdk6, and cyclin D3 as seen after exposure (Fig. 3.2 B) would probably diminish formation of cyclin D3/Cdk4/6 complexes. These complexes are required for progression through G1 phase and for the preparation to enter S phase. Therefore, decreased cyclin D3/Cdk4/6 levels may contribute to the G0/G1 cell cycle arrest observed in the haematological cancer cell lines tested. Consequently, deregulated phosphorylation and inactivation of the pRB family proteins, and thereby inhibition of transcription of E2F target genes, such as MCMs, cyclin D, E, and A, amongst others (Sherr & Roberts, 1999; 2004; Bracken *et al.*, 2004), may occur.

In late G1 phase, Cdk2 is activated by binding to cyclin E (Sherr & Roberts, 1999; 2004), which enhances the phosphorylation of pRB family proteins on additional sites. This is known to start the irreversible initiation of the gene expression program for the S phase and leads to passage through the G1-S restriction point. The observation that Roc A treatment led to downregulation of cyclin E after 5 h treatment (Fig. 3.2 B) hints at decreased formation of cyclin E/Cdk2 complexes and implicates a further possible contribution to G0/G1 cell cycle arrest and delay in S phase entry.

Nevertheless, activity of cyclin/Cdk complexes is regulated far beyond the abundance of certain proteins. Cdk activity is under the control of multiple regulatory proteins, namely Cdk inhibitors from the Cip/Kip and INK4 protein family and activating and inactivating phosphorylations (Sherr & Roberts, 1999; 2004). Inactivating phosphorylations are removed by the Cdc25 family of dual specificity phosphatases, which thereby activate cyclin/Cdk complexes and contribute to proper cell cycle progression and transition through cell cycle checkpoints (Boutros *et al.*, 2006). By analysing important key players in G1-S transition upon Roc A treatment, the earliest event observed after exposure was the rapid downregulation of Cdc25A and Cdc25B protein levels after 15-30 min, while Cdc25C protein levels were not changed (Fig. 3.2 B).

Cdc25A mainly activates the cyclin E/Cdk2 and cyclin A/Cdk2 complexes during G1-S transition (Blomberg & Hoffmann, 1999; Hoffmann *et al.*, 1994; Jinno *et al.*, 1994). This phosphatase is also described to play a role in G2-M transition (Molinari *et al.*, 2000; Zhao *et al.*, 2002) by activating cyclin B/Cdk1 complexes, which are thought to initiate chromosome

condensation (Boutros *et al.*, 2006; Lindqvist *et al.*, 2005; Mailand *et al.*, 2002; Molinari *et al.*, 2000). In contrast, Cdc25B and Cdc25C are primarily required for entry into mitosis (Gabrielli *et al.*, 1996; Lammer *et al.*, 1998; Millar *et al.*, 1991). Cdc25B is proposed to be responsible for the initial activation of cyclin B/Cdk1 at the centrosome during the G2-M transition (De Souza *et al.*, 2000; Gabrielli *et al.*, 1996; Lindqvist *et al.*, 2005), which is then followed by the complete activation of cyclin B/Cdk1 complexes by Cdc25C in the nucleus at the onset of mitosis (Gabrielli *et al.*, 1997). However, knockdown studies with Cdc25B or Cdc25C using antisense or interference RNA showed that these two phosphatases may also be involved in the control of S phase entry (Garner-Hamrick & Fisher, 1998; Turowski *et al.*, 2003). In general, all three Cdc25 phosphatases are reported to be central regulators of G1-S and G2-M transitions and mitosis entry and as such, are involved in spatially and temporally controlling their respective Cdk substrates (Boutros *et al.*, 2007). Since Roc A mediated a rapid downregulation of Cdc25A and Cdc25B, reduction of these two phosphatases may play a decisive role in Roc A-mediated arrest of cell cycle at G0/G1 phase.

The complex regulatory mechanisms and the overlapping functions of the Cdc25 family of dual specificity phosphatases may explain the contradictory findings of rocaglamide derivatives that induce cell cycle arrest in different phases of the cell cycle observed by other studies (Lee *et al.*, 1998; Bleumink, 2007; Bohnenstengel *et al.*, 1999; Mi *et al.*, 2006; Hausott *et al.*, 2004). Depending on the cell type and probably the activation and/or expression status of cell cycle regulatory proteins, the same compound can lead to different results. For example, Roc A treatment was also analysed in human cervical carcinoma cells HeLa, in which exposure to Roc A led to G2/M cell cycle arrest (data not shown).

Taken together, downregulation of important cell cycle proteins are the key to the anti-proliferative effects of Rocaglamide A and its derivatives, as already shown by others (Bleumink, 2007; Mi *et al.*, 2006; Hausott *et al.*, 2004). In which cell cycle phase a certain cell type will arrest may strongly depend on its basal expression status of its cell cycle regulatory proteins and its capacity to deal with interference in normal cell cycle progression.

4.2 Rocaglamide A induces rapid cell cycle arrest

4.2.1 Rapid cell cycle arrest occurs *via* downregulation of Cdc25A

Although all three Cdc25 phosphatases are reported to be involved in all cell cycle phases, Cdc25B and Cdc25C have been shown to play more pronounced roles at the G2-M transition and during mitosis, whereas Cdc25A is mainly involved in G1-S transition (Busino *et al.*, 2004, Boutros *et al.*, 2008). Over-expression of Cdc25A accelerates S phase entry, while both microinjection of anti-Cdc25A antibodies and transfection of antisense oligonucleotides inhibit DNA synthesis (Cangi *et al.*, 2000; Hoffmann *et al.*, 1994; Jinno *et al.*, 1994; Sexl *et al.*, 1999; Vigo *et al.*, 1999). Moreover, double knockout (*Cdc25b*$^{-/-}$, *Cdc25c*$^{-/-}$) mice develop normally (Ferguson *et al.*, 2005) while *Cdc25a*$^{-/-}$ knockout mice are embryonically lethal (Ray *et al.*, 2007). These studies implicate that Cdc25A is capable of performing Cdc25B and Cdc25C functions and is an essential protein in cell cycle progression. Since Roc A-mediated downregulation of Cdc25A is faster than that of Cdc25B (Fig. 3.2 B), Cdc25A downregulation is suggested to induce rapid inhibition of proliferation and cell cycle arrest, in particular at the G0/G1 phase in the haematological cancer cell lines tested.

4.2.2 Rocaglamide A downregulates Cdc25A beyond protein synthesis inhibition

Cdc25A is a very unstable protein with a half-life of 20-30 min (Falck *et al.*, 2001; Mailand *et al.*, 2000; 2002; Molinari *et al.*, 2000; Sørensen *et al.*, 2003). This can be confirmed in this study by treatment of J16 cells with the known protein synthesis inhibitor cyclohexamide (CHX) (Fig. 3.3 B, C). Since rocaglamide derivatives are known to confer translation inhibitory activities (Bleumink *et al.*, 2010; Lee *et al.*, 1998), at the first glance one would suspect that Roc A downregulates Cdc25A *via* protein synthesis inhibition. Indeed, analysis of incorporation of ^{35}S-labelled methionine in J16 cells treated with Roc A showed inhibition of protein *de novo* synthesis in a dose-dependent manner (Fig. 3.3 A). In an elegant study by Bleumink and colleagues (Bleumink *et al.*, 2010) it could be shown that inhibition of translation is mediated *via* downregulation of the Mek-Erk-Mnk1 signalling pathway. It was shown that Erk activity was downregulated in the HTLV-1-associated ATL T cell line SP as early as 15-30 min which was accompanied with immediate and complete inhibition of translation after treatment with 100 nM Roc AR (Bleumink *et al.*, 2010). But in the working concentration of 50 nM Roc A the inhibitory effect on ^{35}S incorporation on J16 cells was only

around 20% (Fig. 3.3 A) and downregulation of Erk activity occurred after approximately 1-2 h upon exposure to Roc A (Fig. 3.3 D). However, the observed downregulation of the cell cycle proteins neccesary for G1-S transition may now be explainable through the diminished protein *de novo* synthesis (Fig. 3.2 B).

Yet, it was shown that cells treated with Roc A downregulated Cdc25A protein very rapidly and even faster than CHX treatment (Fig. 3.3 B, C). Therefore, inhibition of translation may add to the downregulation of Cdc25A at later time points, but is not capable of explaining the Roc A-mediated fast downregulation after only 15 min.

4.2.3 Rocaglamide A induces Cdc25A degradation by phosphorylation of Cdc25A

As depicted earlier, the basal turnover of Cdc25A is approximately 20-30 min. Upon genotoxic stress induction, however, it can be decreased to only 10 min (Bartek *et al.*, 2004; Sørensen *et al.*, 2003). Genotoxic stress increases the rate of Cdc25A phosphorylation through the (combined) action of Chk1, Chk2 and/or MAPK p38 (Bulavin *et al.*, 2001; Busino *et al.*, 2003; Falck *et al.*, 2001; Reinhardt *et al.*, 2007; Sorensen *et al.*, 2003). Chk1 phosphorylates Cdc25A on Ser76, Ser124, Ser178, Ser279, Ser 293 and Thr507, while Chk2 phosphorylates Ser124, Ser178, Ser279 and Ser 293 (Fig. 1.4; Boutros *et al.*, 2007; Kiyokawa & Ray, 2008). In addition, MAPK p38 was reported to phosphorylate Cdc25A at Ser76 and Ser124 in response to hyperosmotic stress and cytokine withdrawal (Khaled *et al.*, 2005). Of these sites, Ser76 phosphorylation functions as priming event, initiating the process of destabilising Cdc25A protein (Goloudina *et al.*, 2003; Hassepass *et al.*, 2003). Ser178 contains a 14-3-3 docking site and phosphorylation of this site facilitates association of Cdc25A with 14-3-3 scaffold proteins (Chen *et al.*, 2003), which inactivates the enzymatic activity of Cdc25A (Hermeking & Benzinger, 2006). Increased phosphorylation at these two crucial sites was analysed and observed after Roc A treatment (Fig. 3.4).

Inactivation of Cdc25A phosphatase activity either by protein downregulation or by association with 14-3-3 scaffold proteins reduces its ability to dephosphorylate and thereby activate Cdk2 at the G1-S and Cdk1 at the G2-M boundary. This subsequently leads to halt in cell cycle progression with arrest in G0/G1 and G2/M phase, respectively, depending on the cell line or the kind of genotoxic stress (Boutros *et al.*, 2007; Busino *et al.*, 2004; Malumbres & Barbacid, 2009). However, the G0/G1 cell cycle arrest analysis in this study did not include the investigation of changes in Cdk2 or Cdk1 activity or DNA replication in S phase.

Interestingly, both inhibition (Bohnenstengel *et al.*, 1999) and no inhibition (Lee *et al.*, 1998) of DNA replication could be seen after exposure to rocaglamide derivatives. Treatment of human monocytic leukemia cells MONO-MAC-1 with didesmethyl-rocaglamide resulted in arrest in G2/M- and probably G0/G1-phase of the cell cycle and showed inhibition of DNA synthesis (Bohnenstengel *et al.*, 1999). In contrast, treatment of human lung carcinoma cells Lu1 with 4'-demethoxy-3',4'-methylenedioxy-methylrocaglate resulted in G0/G1 cell cycle arrest but showed no measurable inhibition of DNA replication (Lee *et al.*, 1998). Both groups used the same method to measure DNA replication; however, Bohnenstengel and colleagues (Bohnenstengel *et al.*, 1999) pulsed cells for 3 h with 1 μCi [methyl-^{3}H]thymidine whereas Lee and colleagues (Lee *et al.*, 1998) exposed cells for only 1 h. It remains elusive whether the discrepancies in the ability of rocaglamide derivatives in inhibiting DNA replication are due to the different techniques employed or whether they are due to the different cell lines or compounds used.

4.2.4 Rocaglamide A-mediated downregulation of Cdc25A involves activation of Chk1 and Chk2

Three kinases, Chk1, Chk2 and MAPK p38, are known to modulate phosphorylation sites on Cdc25A upon genotoxic stress induction and were shown to be activated upon Roc A treatment (Fig. 3.6). Whereas activation of Chk2 and MAPK p38 could be observed immediately upon exposure to Roc A, activation of Chk1 was very weak and rather transient between 1-2 h. As the latter is later shown to play essential roles in the downregulation of Cdc25A (Fig. 3.8 and 3.9), additional experiments are needed, in particular a Chk1 kinase assay, to confirm activation of the kinase. A kinase assay will give a definitive answer as to whether the activation of Chk1 is triggered by Roc A treatment.

Still, it could be shown that inhibition of Chk1 with a selective Chk1 inhibitor, SB 218078, abrogated phosphorylation at Ser76 and Ser178 of Cdc25A. Through this inhibition, a rescue of Roc A-mediated Cdc25A downregulation was achieved (Fig. 3.8 A, B). In contrast, Chk2 and MAPK p38 did not seem to play a role in phosphorylating Cdc25A since neither single nor combined inhibition of these kinases resulted in any rescue of phosphorylation or downregulation of the phosphatase (Fig. 3.8 A; data not shown). Abrogation of Roc A-mediated Cdc25A downregulation could be shown with a second Chk1 inhibitor, UCN-01, with which an even stronger rescue could be obtained (Fig. 3.8 C).

Both SB 218078 and UCN-01 are potent and selective inhibitors of Chk1 kinase (Jackson *et al.*, 2000; Shao *et al.*, 1997; Wang *et al.*, 1996) that displayed in *in vitro* kinase assays IC_{50} values of 15 nM and 7 nM, respectively (Jackson *et al.*, 2000). Both compounds belong to the group of staurosporines that act as inhibitors of serine/threonine protein kinases by preventing ATP binding to the kinase. Although both inhibitors show very low IC_{50} values towards Chk1, they exhibit further inhibitory activities towards other kinases, such as for example PKC, Cdk1 and Cdk2. Jackson and colleagues (Jackson *et al.*, 2000) reported for SB 218078 IC_{50} values of 6 nM for Cdk1 and of 5 nM for PKC. UCN-01 was shown to exhibit inhibitory activities with IC_{50} values of 30 nM for PKC and of 300-600 nM for Cdk1 and Cdk2 *in vitro* (Lapenna & Giordano, 2009). However, as IC_{50} values are analysed in *in vitro* kinase assays, they may not correspond to the concentrations that enter into the cells used in cell culture. In general, higher working concentrations are used when treating living and metabolising cells, such as 1-5 µM for SB 218078 and 0.5-1 µM for UCN-01 in the underlying study (Fig. 3.8 B, C). Although Chk1 and Chk2 have overlapping functions in response to diverse genotoxic insults, they are structurally unrelated serine/threonine kinases (Antoni *et al.*, 2007; Bartek & Lukas, 2003; Dai & Grant, 2010). As the tested inhibitors are serine/threonine kinase inhibitors, it cannot be excluded that they also inhibit Chk2 activity. While inhibitory activities of SB 218078 against Chk2 were not reported to be tested, UCN-01 was shown to obtain an IC_{50} value of 1040 nM against Chk2, which makes it 100-fold less potent against Chk2 than against Chk1 (Seynaeve *et al.*, 1994). Therefore, it cannot be entirely excluded that these inhibitors cause a simultaneous inhibition of Chk2.

Studies with RNAi against Chk1 could not confirm the rescue observed with the chemical inhibitors against Chk1 (Fig. 3.9 A). This could be due to the involvement of inhibition of both Chk1 and Chk2 kinases by the Chk1 inhibitors. However, one could also assume that the cells had adapted to their Chk1 deficiency and that Chk2 and/or MAPK p38 had taken over its functions. Chk1 is described to be an essential kinase during embryonic development as *Chk1*$^{-/-}$ (Liu *et al.*, 2000; Takai *et al.*, 2000) but not *Chk2*$^{-/-}$ (Hirao *et al.*, 2002; Takai *et al.*, 2002) mice are lethal. In addition, Chk1 but not Chk2 is an important kinase in normal cell cycle progression and is involved in several cellular events (Kaneko *et al.*, 1999; Sorensen *et al.*, 2003; 2004; Zhao *et al.*, 2002; Shechter *et al.*, 2004; Krämer *et al.*, 2004). Yet, complete deficiency of Chk1 in somatic cells can be tolerated and does not affect cell division (Jin *et al.*, 2008; Petermann *et al.*, 2010; Syljuasen *et al.*, 2005; Tang *et al.*, 2006; Zachos *et al.*, 2003), as shown also in this study (Fig. 3.9 B). Cells with depleted Chk1 exhibited a similar cell cycle profile with the same distribution of cells in the different cell cycle phases as

compared to control transfected cells (Fig. 3.9 B). This hints at possible overlapping functions of Chk2 and Chk1 and indeed, combined transient knockdown of Chk1 and Chk2 was found in this study to be able to delay Roc A-mediated downregulation of Cdc25A (Fig. 3.9 D).

In response to ionising radiation (IR) it was reported that Chk2 is unable to overtake Chk1 functions (Jin *et al.*, 2008). Several studies showed that Chk1 mediates phosphorylation of Cdc25A at Ser76, which promotes its ubiquitination by the $SCF^{\beta\text{-}TRCP}$ ubiquitin ligase (Busino *et al.*, 2003; Donzelli *et al.*, 2002; Jin *et al.*, 2003). Phosphorylation of Ser76 is thought to serve as essential priming phosphorylation for accelerated proteolysis (Goloudina *et al.*, 2003; Hassepass *et al.*, 2003). Chk2 was shown to be unable to phosphorylate efficiently Ser76 in Cdc25A in response to IR and to support $SCF^{\beta\text{-}TRCP}$-mediated ubiquitination of Cdc25A *in vitro* (Jin *et al.*, 2008). The authors suggested that in response to IR, Chk1 is the major checkpoint kinase controlling Cdc25A degradation and that Chk2 has only a minor contributory role. However, the data obtained in this study suggest that Roc A mediated downregulation of Cdc25A involves Chk1 as well as Chk2 kinase activities. Still, the rescue of Cdc25A was not complete and other mechanisms are assumed to take place, such as the well-known translation inhibitory effects by Roc A *via* the Mek-Erk-Mnk1 pathway.

4.3 Activation of the DNA damage response pathway by Rocaglamide A

Activation of the DNA damage sensor kinase ATM could be shown immediately after exposure to Roc A (Fig. 3.7 A). The hallmark of ATM is its rapid response to DSBs measured by increase in its kinase activity through autophosphorylation at Ser1981 (Banin *et al.*, 1998; Canman *et al.*, 1998; Driscoll & Jeggo, 2002). However, in response to UV treatment or replication fork stalling, which induces prominently ssDNA and ATR-dependent phosphorylation, activation of ATM was also reported (Stiff *et al.*, 2006). This phenomenon might be explainable by the action of nucleases, which cleave ssDNA to yield DSBs (Cimprich & Cortez, 2008), and by this mechanism ATR is activated initially and subsequently triggers ATM activation.

ATM activation upon Roc A treatment could be further confirmed by inhibitor studies with the specific ATM inhibitor KU-55933 (Fig. 3.7 B). Inhibition of ATM partially abrogated Roc A-mediated Chk2 activation. However, since inhibition of Chk2 activation was not complete, it could be assumed that other DNA damage sensor kinases are involved in the Roc A-mediated Chk2 activation, such as ATR and/or DNA-PK. While activation of DNA-PK upon Roc A treatment was not analysed, no change in the phosphorylation status of ATR at Ser428 after treatment could be observed (Fig. 3.7 A). Although several modifications of ATR, including phosphorylation at Ser428, were reported to take place upon DNA damage, none of them had been clearly demonstrated to be a reliable indicator of ATR activation (Cimprich & Cortez, 2008). It was suggested that ATR kinase may be constitutively ready to phosphorylate substrates but is mainly controlled by its subcellular localisation. Activation of ATR was thought to occur by the translocation to the site of the DNA damage and the formation of a complex with ATRIP and RPA (Kastan & Bartek, 2004; Cimprich & Cortez, 2008). However, recently it was shown that ATR, like ATM and DNA-PK, undergoes autophosphorylation at Thr1989 after translocation to the DNA damage site, which is thought to be crucial for its full activation (Liu *et al.*, 2011). Since this report was published after having conducted the experimental part of this study, it remains to analyse further whether Roc A activated ATR at Thr1989 upon exposure.

The activation of Chk1 could be shown by increased phosphorylation at Ser317 after Roc A exposure (Fig. 3.6). Next to Ser345, Ser317 is discussed to be a reliable indicator of Chk1 activation (Cimprich & Cortez, 2008). Chk1 is a known direct target of ATR but as

discussed earlier, it is so far not clear whether Roc A mediated ATR activation. Yet, it should be pointed out that Gatei and colleagues (Gatei *et al.*, 2003) reported an ATM-dependent phosphorylation of Chk1 at Ser317 after exposure to IR. This would hint at a possible Roc A-mediated Chk1 activation independent of ATR but rather through ATM. However, inhibition of the upstream kinase ATM could not rescue Cdc25A downregulation (Fig. 3.8 D). This suggests a more complex DNA damage sensing and signalling mechanism than could be shown so far in this study. Further studies with single and combined inhibition of the upstream kinases ATM, ATR and DNA-PK are needed to elucidate their involvements in Chk1 and Chk2 activation and Cdc25A downregulation.

Taken together, data in this study show that Roc A triggers a signalling pathway that resembles the DDR pathway. It could not be demonstrated in the experiments undertaken so far that the DNA damage sensing kinases are clearly involved in the Roc A-mediated activation of Chk1 and Chk2 as well as Cdc25A downregulation. Therefore, possible mechanisms of Chk1 and Chk2 activation other than *via* activation of the DDR pathway upon treatment with Roc A may be assumed.

4.4 Rocaglamide A does not induce direct DNA double strand breaks

To this point, data in this study indicated that Roc A treatment leads to ATM activation that is mainly activated by DSBs. Therefore, the possibility that Roc A induces activation of DSBs was analysed (Fig. 3.10 and 3.11). A reliable read-out and acknowledged biomarker of DSB formation is the analysis of phosphorylation of H2AX (Bonner *et al.*, 2008; Rogakou *et al.*, 1998). Immediately upon DSB formation, ATM, ATR and/or DNA-PK are activated and phosphorylate H2AX next to other DNA repair and checkpoint proteins (Bonner *et al.*, 2008). Thus, formation of γH2AX was analysed by intracellular FACS staining and confocal microscopy.

Treatment with the known direct DSB-inducing agent doxorubicine showed increasing γH2AX foci formation in living cells as analysed by intracellular FACS staining (Fig. 3.10 B, upper panel) and microscopic analysis (Fig. 3.11, upper panel). In contrast, γH2AX foci formation could not be observed upon Roc A-treatment (Fig. 3.10 B, lower panel; Fig. 3.11, lower panel). Microscopic analysis of Roc A-treated cells after 24 h showed a diffuse pattern referred to as *pan-nuclear staining* (Bonner *et al.*, 2008). In a similar manner peripheral nuclear staining and pan-staining were also observed during TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis (Solier *et al.*, 2009).

Thus, intracellular FACS and confocal microscopy analysis suggest that γH2AX formation at later time points might be due to apoptotic DNA fragmentation. But still it does not explain the early activation of Chk1 and Chk2 when Roc A-mediated apoptosis had not yet occurred. This data indicate that Roc A activates Chk1 and Chk2 through a mechanism other than DDR. However, more sensitive methods to detect low levels of DSBs and ssDNA, such as the quantitative PCR-based measurement of DNA damage (Santos *et al.*, 2005), need to be applied in further experiments.

4.5 Rocaglamide A is not localised in the nucleus

Analysis of a synthetic fluorescent labelled rocaglamide derivative (FLO) showed that FLO could be detected in the ER and mitochondria but was excluded from the nucleus (Fig. 3.12). FLO was assembled by Thuaud and colleagues (Thuaud *et al.*, 2009) by conjugating a chemically synthesised rocaglic derivative through a linker to the small molecule *N,N*-dimethyl-7-aminocoumarin (Coumarin) (Alexander *et al.*, 2006). This dye was selected because of its lack of biological activity and because it displays suitable photophysical properties for fluorescence microscopy, it easily penetrates into cells, and it does not localise preferentially in a subcellular compartment (Alexander *et al.*, 2006). Therefore, the fluorescent labelled compound should not have problems penetrating the nuclear membrane. Thus, it can be excluded that FLO was not present in the nucleus because of its incapability to enter the nucleus due to its fluorescent label.

Further, it has to be considered that FLO does not entirely resemble Roc A in its chemical structure and properties and therefore might exhibit slightly different molecular mechanisms. However, the data observed do not contradict the findings of Thuaud and colleagues (Thuaud *et al.*, 2009). They showed that FLO is located in the ER, which could also be observed in the experiments undertaken in our laboratory (Fig. 3.12, middle panel). In addition, co-localisation studies of FLO with the mitochondria showed that it is also located in this sub-cellular compartment (Fig. 3.12, upper panel).

Taken together, these data show that FLO is not located in the nucleus and thus do not support a model, in which Roc A directly interacts with the DNA in the nucleus and triggers DNA damage/stress.

4.6 Rocaglamide A binds to Prohibitin

4.6.1 Prohibitin translocation into the nucleus does not lead to genotoxic stress

In our lab it was discovered that Roc A binds to/ interacts with Prohibitin (PHB1) (unpublished data). Interestingly, it could be shown that upon Roc A treatment PHB1 translocates into the nucleus (Fig. 3.13). It was therefore hypothesised that PHB1 interacted as a mediator of some kind with the DNA, which then resulted in genotoxic stress. In unperturbed cells it was reported that PHB1 can co-localise with the transcription factors E2F1 and p53 (Fusaro *et al.*, 2003) and with the DNA replication proteins MCM (Rizwani *et al.*, 2009). Since all three proteins are closely located to the DNA, Roc A-mediated increase of PHB1 in the nucleus would lead to increased interaction with these proteins and thereby induce DNA stress.

However, siRNA-mediated knock-down of PHB1 did not show increased activity of Chk1 and/or Chk2 (Fig. 3.14) which were earlier shown to be responsible for the rapid Roc A-mediated downregulation of Cdc25A and subsequent rapid cell cycle arrest. To verify these results, more siRNA oligos against PHB1 have to be tested, and the possibility that PHB2 took over functions of PHB1 need to be validated by combined knockdown of PHB1 and PHB2.

These data demonstrate no hint of the importance of the interaction of Roc A with PHB1 regarding the induction of genotoxic stress that leads to the activation of a signalling pathway that resembles the DDR. In contrast, these findings suggest a model in which Roc A induces independently from its interaction with PHB1 genotoxic stress to the cells. This may occur prior to binding of Roc A to PHB1 or simultaneously.

4.6.2 Prohibitin knockdown mimics the effects of Rocaglamide A on cell cycle progression

Analysis of cell cycle distribution and status of cell cycle proteins in PHB1 deficient cells showed the same effects as they were seen for Roc A treated cells (Fig. 3.15 A, B). PHB1 was shown to physically interact with Raf and to be required for Ras-induced Raf-Mek-Erk activation (Rajalingam *et al.*, 2005). Consistent with this data, we could show that knock-down of PHB1 leads to a significant decrease of protein *de novo* synthesis (Fig. 3.15 C) and to reduction of Erk1 activity (Fig. 3.15 D). This is in line with data observed upon treatment with Roc A and other rocaglamide derivatives (Fig. 3.3 D; Zhu *et al.*, 2007; Bleumink *et al.*, 2010). Therefore, these data strongly imply that PHB1 deficiency leads to translation inhibition *via* the downregulation of the Raf-Mek-Erk-pathway. This mimics the effects of Roc A on cell cycle progression and thus, displays the molecular mechanism of Roc A through which cell cycle proteins are downregulated that are needed for proper cell cycle progression.

Further, E2F-mediated transcription is regulated *via* signal transduction pathways mediated by the family of Ras and Rho GTPase proteins (Coleman *et al.*, 2004). Therefore, one would assume that interaction of Roc A with PHB1 might contribute to downregulation of E2F-target genes needed for proper cell cycle transition from G1 to S phase, such as MCMs, cyclin D, E and A and Cdc25A, amongst others (Dyson, 1998; Lundberg & Weinberg, 1998). This would potentially add further evidence to explain the Roc A-mediated inhibition of proliferation and in particular the G0/G1 cell cycle arrest observed in the haematological cancer cell lines used in this study. However, this needs to be analysed in future quantitative PCR experiments.

4.7 Proposed model of anti-proliferative effects of Rocaglamide A

The data discussed above demonstrate a model of anti-proliferative effects of Roc A through two independent pathways (Fig. 4). At the first instance rapid cell cycle arrest is induced *via* the Roc A-mediated downregulation of Cdc25A. This occurs immediately after exposure of the cells to the drug which induces a signalling pathway that resembles the DNA damage pathway. However, the molecular mechanism of induction of genotoxic stress remains so far unknown.

Prolonged cell cycle arrest is induced by Roc A through its interaction with PHB1 which downregulates the Raf-Mek-Erk pathway (Fig. 4). Thereby, protein *de novo* synthesis is diminished that leads to decreased expression of cell cycle proteins.

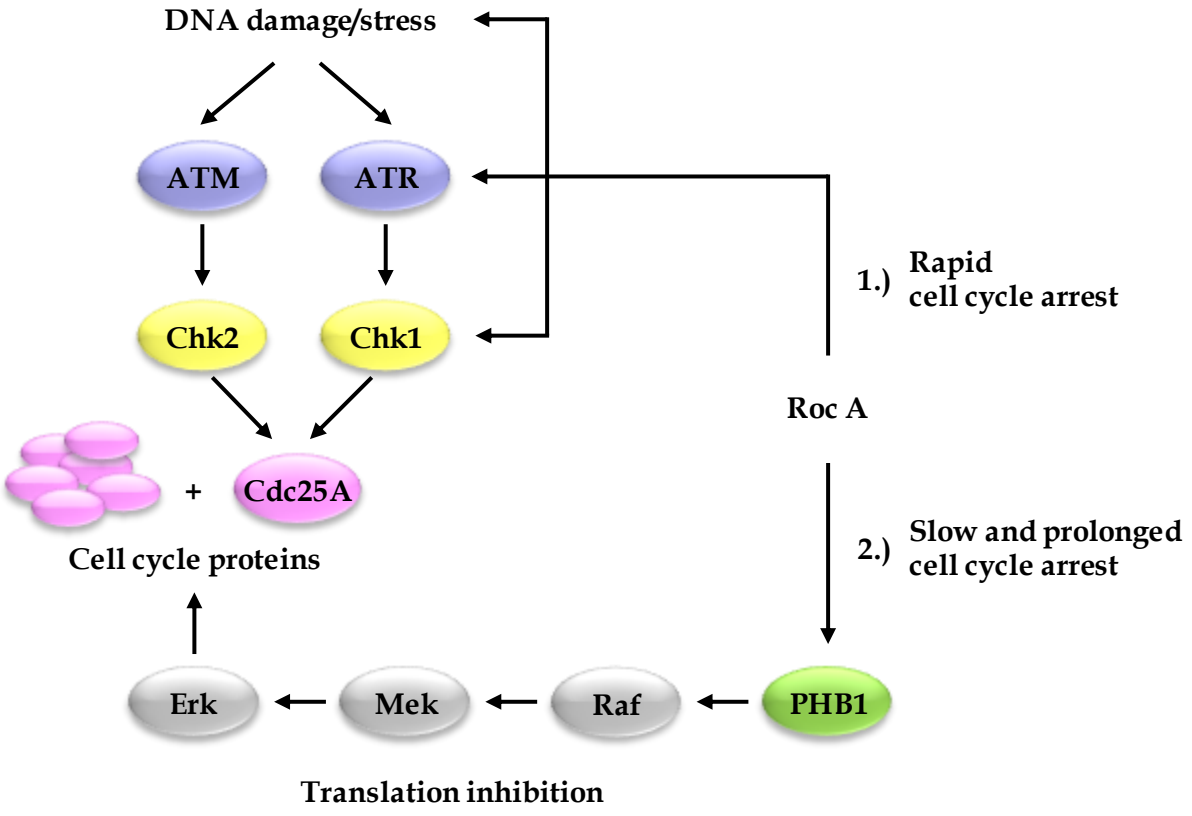


Figure 4 | Proposed model of anti-proliferative effects of Rocaglamide A.

Upon Roc A treatment rapid cell cycle arrest is induced *via* the downregulation of Cdc25A. This is mediated through a signalling pathway that resembles the DNA damage pathway. Slow and prolonged cell cycle arrest occurs through the interaction of Roc A with PHB1 that leads to downregulation of translation and cell cycle protein expression.

4.8 Outlook: Rocaglamide A in anti-cancer treatment

Many cancer cells show deregulated cell cycle progression with overexpression of positive regulators and inhibition of negative regulators, which gives them unlimited replication potential (Deep & Agarwal, 2008). In recent years, much effort has been made to develop new agents, which target the deregulated cell cycle and are considered as an ideal strategy for cancer therapy. Cell cycle based agents have been categorised as Cdk, Cdc25, checkpoint, and mitotic inhibitors. In particular, the crucial role played by Cdks in the control of cell cycle make them attractive pharmacological targets for the development of anti-proliferative cancer drugs (Senderowicz, 2004; 2005). Various strategies have been proposed to inhibit directly or indirectly the activities of these enzymes (Boutros *et al.*, 2007). Direct inhibition is based on the use of competitive ATP analogues with well known compounds such as Flavopiridol, which targets Cdk1, Cdk2, Cdk4, Cdk6, Cdk7, and Cdk9, and Roscovitine, which targets Cdk1, Cdk2, Cdk7, and Cdk9 amongst many others (Lapenna & Giordano, 2009). Both were applied in phase I and II clinical trials in patients with various types of relapsed or refractory tumours, either alone or in association with currently used chemotherapeutic agents (Benson *et al.*, 2007; Byrd *et al.*, 2007).

As activators of Cdks, Cdc25 phosphatases are obvious candidates for the development of approaches to indirectly inhibit Cdks and their associated effects on cell-cycle control (Boutros *et al.*, 2007). Several Cdc25 inhibitory compounds have been reported, which belong to various groups, including quinonoids, phosphate surrogates, and electrophilic entities. Much effort has been made in the development of Cdc25 phosphatase inhibitors, especially during the past five years, and more than 40 patents were applied for which reported new applications of natural and synthetic compounds (Lavecchia *et al.*, 2010). However, only very few Cdc25 inhibitory compounds have been shown to inhibit the proliferation of cancer cells efficiently and which are active *in vivo* on xenografted human tumours (Boutros *et al.*, 2007; Lavecchia *et al.*, 2010). Published data are limited to the BN82002 (Brezak *et al.*, 2004), BN82685 (Brezak *et al.*, 2005), IRC-083864 (Brezak *et al.*, 2009) and PM20 (Kar *et al.*, 2006) compounds, and therefore other approaches to target Cdc25 phosphatases and cell cycle progression need to be considered.

In the underlying study it could be demonstrated that Roc A treatment leads to downregulation of Cdc25A protein levels, which is assumed to lead to cell cycle arrest and inhibition of cell proliferation. Inhibition of Cdc25A activity towards Cdks was not achieved by direct inhibition but rather *via* activation of cellular signalling pathways, which in turn

mediated Cdc25A downregulation. Compared to direct Cdc25 phosphatase inhibitors, rocaglamide derivatives were shown to exhibit potent anti-proliferative activities *in vitro* (Hayashi *et al.*, 1982; Kim *et al.*, 2006; Lee *et al.*, 1998; Zhu *et al.*, 2009) and to inhibit tumour growth *in vivo* (King *et al.*, 1982; Lee *et al.*, 1998; Hwang *et al.*, 2004; Mi *et al.*, 2006; Zhu *et al.*, 2009). Importantly, rocaglamide derivatives displayed only minor cytotoxic effects towards normal, healthy cells (Zhu *et al.*, 2007), which suggests they could form the basis of new and promising anti-cancer dugs.

Treatment of haematological cancer cells with 50 nM Roc A resulted in pronounced cell death induction after 48 h (Fig. 3.1 C). In contrast, colorectal cancer cells HT-29, prostate cancer cells PC-3 and breast cancer cells Mcf-7 displayed only minor cell death after similar treatment for 48 h (data not shown). However, Cdc25A downregulation was observed in these cells after exposure to Roc A (Fig. 3.5), which indeed led to inhibition of cell proliferation (data not shown). Therefore, even growth inhibition of solid tumours can be achieved by Roc A treatment, which increases its potential to be applied in further development as a new anti-cancer drug.

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Abbreviations

4E-BP1	eIF4E-binding protein
53BP1	p53-binding protein
9-1-1	Rad9 – Rad1 - Hus1
Ab	antibody
AICD	activation-induced cell death
AIDS	acquired immunodeficiency syndrome
ALPS	autoimmune lymphoproliferative syndrome
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
Apaf-1	apoptosis protease-activating factor 1
APC	anaphase-promoting complex
APS	ammonium peroxidisulfate
Asp	cspartate
ATM	ataxia-telangiectasia mutated
ATL	adult T-cell leukemia/lymphoma
ATP	adenosine triphosphate
ATR	ataxia-telangiectasia and Rad3-related
Bcl-2	B-cell lymphoma-2
Bid	BH3 interacting domain death agonist
Bim	Bcl-2-interacting mediator of death
BH	Bcl-2 homology
bp	base pare
BRCA1	breast cancer 1
BSA	bovine serum albumin
CAK	Cdk-activating kinase
caspase	cysteine aspartate-specific protease
CD	cluster of differentiation
Cdc25	cell division cycle 25
Cdk	cyclin-dependent kinase
c-FLIP	cellular FLICE-inhibitory protein

CFSE	carboxyfluorescein succinimidyl ester
Chk	checkpoint kinases
CHX	cycloheximide
CKI	cyclin-dependent kinase inhibitors
Cip/Kip	Cdk interacting protein/Kinase inhibitory protein
DMSO	dimethyl sulphoxide
CREB	cAMP responsive element binding factor
cs	catalytic subunit
CTCL	cutaneous T cell lymphoma
CTD	carboxyl-terminal domain
DD	death domain
DDR	DNA damage response
DED	death effector domain
DISC	death-inducing 138ignalling complex
DMEM	Dulbecco's modified Eagle medium
DNA	desoxy-ribose-nucleic acid
DNA-PK	DNA-dependent protein kinase
dNTP	deoxyribonucleotide
Dox	doxorubicine
DR	death receptor
DSB	double strand DNA break
DTT	dithiotreitol
ECL	enhanced chemiluminescence
eIF	eukaryotic initiation factor
Erk	extracellular signal-regulated kinase
Etop	etoposide
FADD	Fas-associated death domain protein
FCS	fetal calf serum
FLICE	FADD-like ICE
h	hour
HDAC	histone deacetylases
HRP	horseradish peroxidase

HTLV	human T cell leukemia virus
IAP	inhibitors of apoptotic proteins
IC_{50}	inhibitory concentration 50%
IgG	immunoglobulin G class
IκB	inhibitor of κB proteins
IKK	I-κB kinase
IL	interleukine
IFN-γ	interferon γ
Ink4a/Arf	Ink4a/Arf (Inhibitor of Kinase 4/Alternative Reading Frame
IP	Immunprecipitation
IR	ionising radiation
JNK	c-Jun N-terminal Kinase
kD	kilodalton
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia sequence 1
MCM	minichromosome maintenance
Mdc1	mediator of DNA damage checkpoint 1
min	minute
Mnk1	MAP kinase interacting kinase 1
Mre11	meiotic recombination 11
MRN	Mre11 - Rad50 - Nbs1
mTOR	mammalian target of rapamycin
Nbs1	Nijmegen breakage syndrome 1
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor κB
NGF	nerve growth factor
O/N	over night
ORC	origin of replication complexes
p-	phospho-
PAGE	polyacrylamide gel-elektrophoresis
PBS	phosphate buffered saline

PCD	programmed cell death
PI3K	phosphoinositide-3 kinase
Plk	polo-like kinases
PMA	phorbol 12-myristate 13-acetate
RNA	ribonucleic acid
Roc A	Rocaglamide A
RPA	replication protein A
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute medium
pRB	retinoblastoma protein
pre-RC	pre-replication complex
RT	room temperature
$SCF^{\beta TrCP}$	Skp1/Cullin/F-box
Ser	serine
SDS	natriumdodecylsulfate
siRNA	small interfering RNA
Smac	second mitochondria-derived activator of caspases
Smc1	structural maintenance of chromosome 1
ssDNA	single-stranded DNA
TCR	T cell receptor
TEMED	tetramethylethyl endiamin
Thr	threonine
TNF	Tumor Necrosis Factor
TopBP1	topoisomerase (DNA) 2-binding protein 1
TRAIL	TNF-related apoptosis-inducing ligand
Tyr	tyrosine
UV	ultraviolet
V	Volt
WB	western blot

Declaration

Herewith, I declare that I wrote this thesis independently under supervision, and no other sources and aids than those indicated were used. Furthermore, my submission as a whole is not substantially the same as any that I have previously made or I am currently making, whether in published or unpublished form, for a degree, diploma, or similar qualification at any university or similar institution.

Heidelberg, _____ _____

Jennifer Neumann