Molecular dissection of Cdc6 and the miR-148 family : two stories with common themes

Duursma, A.M.

Molecular dissection of Cdc6 and the miR-148 family; two stories with common themes

The research described in this thesis was performed at the department of Tumour Biology at the Netherlands Cancer Institute (NKI) and supported by the Dutch Cancer Society (KWF) and a European Young Investigator award (EURYI) to Reuven Agami. The printing of this thesis was supported by the Dutch Cancer Society (KWF) and Leiden University.

Cover Pictures were taken by A. D. and show HeLa cell nuclei.

Printed by PrintPartners Ipskamp, Enschede, The Netherlands

Molecular dissection of Cdc6 and the miR-148 family; two stories with common themes

proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,

volgens besluit van het College voor Promoties

te verdedigen op woensdag 14 mei 2008

klokke 13.45 uur

door

Anja Maaike Duursma

geboren te Leuven (België)

in 1978

Promotiecommissie

Promotor:	Prof. Dr. J. J. Neefjes
Co-promotor:	Dr. R. Agami Nederlands Kanker Instituut, Amsterdam
Referent:	Prof. Dr. R. Bernards Universiteit Utrecht
Overige leden:	Prof. Dr. R. H. Medema Universiteit Utrecht
	Dr. B. Blom Universiteit van Amsterdam
	Dr. A. G. Jochemsen
	Prof. Dr. L. H. Mullenders
	Prof. Dr. P. J. van den Elsen

Contents

Chapter 1

Introduction
Molecular Mechanism of cancer development

Introduction

Molecular Mechanism of cancer development

Cancer is the result of a multi-step process in which cells acquire features that enable them to divide uncontrollably and to metastasize. Crucial steps in transformation of normal cells into malignant cells are the ability of cells to be self-sufficient in growth signals and to be insensitive to growth-inhibitory signals (Hanahan and Weinberg, 2000). As a consequence the cell cycle will be deregulated in favour of continues growth. The cell cycle is the period from one cell division to the next and can be divided into four phases. In G1, the first phase, mitogenic stimulation results in activation of cell cycle dependent kinases like Cyclin D1/CDK4 and Cylin E/ CDK2, which activate proteins involved in DNA replication and inhibit proteins that retain cells in a non-dividing state (Dulic et al., 1992; Koff et al., 1992; Morgan, 1997; Quelle et al., 1993). Cells that are not stimulated to divide in G1 enter the G0 state and can remain quiescent for longer periods of time. However, activated cells will enter the second phase, S-phase, in which DNA is duplicated and here for the activity of cyclin A/CDK2 is required (Rosenblatt et al., 1992). A schematic representation of the cell cycle and in which stages these different CDK/ Cyclin complexes are active is depicted in Figure 1. In G2-phase cells ensure that the DNA is properly replicated and that the conditions are right for the final separation of sister chromatids and cytokinesis in M-phase or mitosis (Smits and Medema, 2001).

In cancer the transition from G1-phase to S-phase is often deregulated due to altered gene function. Continues growth signalling can be a consequence of mutations in extracellular receptors or intracellular signal transducers, like the EGF receptor and Ras (Riese et al., 2007; Schubbert et al., 2007). However, it can also be due to amplifications of cell cycle activating proteins such as Cyclin D1 and cyclin E or loss of negative regulators of the cell cycle such as the CDK inhibitors p21 and p27 (Malumbres and Barbacid, 2001). Also loss of a functional Retinoblastoma (Rb) protein, which inhibits cell cycle progression by inhibiting the E2F transcription factor family that activate genes that are crucial for G1/S transition, is a frequent event in human cancers (Dannenberg and te Riele, 2006).

The basis of altered gene function often lies in genetic changes, which result in activation of oncogenes or repression of tumour suppressor genes. However, it has become increasingly clear that epigenetic abnormalities can contribute to tumourigenesis as well by altering patterns of gene expression (Jones and Baylin, 2007; Lund and van Lohuizen, 2004). Epigenetics can be described as the heritable changes in gene expression that are not accompanied by changes in DNA sequence. These include covalent modification of DNA and histone proteins, such as methylation. Silencing modifications will result in recruitment of proteins, which change the chromatin structure in a densely packed transcriptional inactive state. On the other hand activating modifications will recruit proteins that open the chromatin to make it accessible for

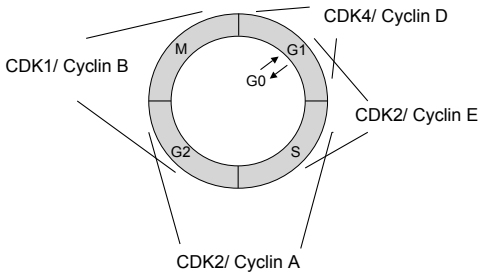


Figure 1. Schematic representation of CDK activity during different stages of the cell cycle. Lines show the overlapping activity ranges of different CDK/ Cyclin complexes.

the transcription machinery (Kouzarides, 2007). A marked example of epigenetic deregulation is the polycomb repressor gene BMI1, which overexpression in mouse cells has been shown to silence the INK4A locus that encodes for the tumour suppressor proteins p16 and p19ARF (Jacobs et al., 1999). Furthermore, BMI1 has been found overexpressed in several human tumours, suggesting that alterations in gene silencing indeed may contribute to tumourigenesis (Sparmann and van Lohuizen, 2006). The importance of epigenetic changes versus DNA mutations in cancer development was further underscribed by studies showing hypermethylation and thereby repression of promoter regions of tumour suppressor genes like Rb, VHL (von Hippel-Lindau), MLH1 and p16 (Gal-Yam et al., 2007). Based on these findings it has been proposed that promoter methylation can act in a similar way to gene mutation in cancer development.

DNA damage responses

Every cell division has a potential risk of creating a mistake in the genetic code. As a consequence the risk of developing cancer will increase with age (Serrano and Blasco, 2007). Moreover, the chance of genetic mutations is increased by exposure to DNA damage inducing agents such as UV light. Cells have evolved several mechanisms to protect themselves from DNA damage induced genetic abnormalities. Proteins like ATM and ATR will sense DNA damage and activate a genotoxic stress checkpoint that induces a cell cycle arrest or activation of repair proteins (Bartek and Lukas, 2007). Unrestorable damage will result in a permanent cell cycle arrest or apoptosis, depending on the cell type.

A central player in the DNA damage response is the transcription factor p53. In response to double strand breaks ATM will activate the CHK2 kinase (Matsuoka et al., 1998) that in turn will phosphorylate the p53 N-terminus (Chehab et al., 2000; Hirao et al., 2000; Shieh et al., 2000). This phosphorylation interferes with p53 binding to MDM2 (Chehab et al., 1999; Shieh et al., 1997; Siliciano et al., 1997). Significantly, MDM2 functions both in inhibiting p53 transcriptional activity and as a p53 E3-ligase (Michael and Oren, 2003). Therefore, phosphorylation of p53 in response to DNA damage will allow for its stabilization and activation. The key role of p53 in a DNA damage induced G1 arrest is mediated through its transcriptional target gene $p21^{Cip1}$ (el-Deiry et al., 1993),a cell cycle inhibitor that exerts its function by inhibiting CDK-cyclin complexes (Harper et al., 1993). Additionally, p53 can induce apoptosis by inducing transcription of proteins of the apoptotic machinery, such as Bax and Puma (Miyashita and Reed, 1995; Nakano and Vousden, 2001). Further, degradation of crucial cell cycle proteins can contribute to a G1 cell cycle arrest, such as the increased proteolysis of Cyclin D1, Cdc6 and Cdc25A (Agami and Bernards, 2000; Duursma and Agami, 2005; Mailand et al., 2000).

The importance of a proper DNA damage response is clear from a large number of heritable human diseases that arise from defects in checkpoints or DNA damage repair functions (Shiloh, 2003). Examples are the mutations in genes as ATM or BRCA1, which highly increase the risk of developing cancer. Patients with mutated ATM are diagnosed with the Ataxia telangiectasis syndrome and are immune deficient and are particularly predisposed to leukaemia's and lymphomas. Patients with a mutation in BRCA1 (Breast cancer 1) have an increased risk to develop breast cancer or ovarian cancer. Also in non-heritable forms of cancer genes participating in DNA damage responses are often lost. p53 is considered as one of the most important tumour suppressor proteins and its function is impaired in a large percentage of human tumours (Levine, 1997).

DNA replication

In S-phase, DNA is replicated in a tightly regulated manner to ensure proper duplication of the genome. In G1-phase of the cell cycle DNA replication is initiated at origins of replication, by binding of Cdc6 to the Origin Recognition Complex (ORC) (Liang et al., 1995) (Figure 2). This allows binding of Cdt1, which is a loading factor for the minichromosome maintenance (MCM)

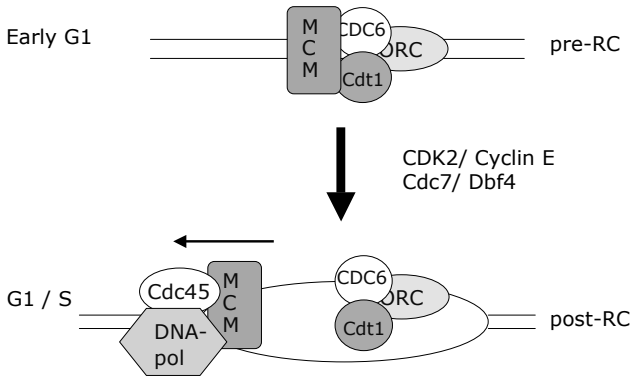


Figure 2. Schematic representation of initiation of DNA replication. CDK2/ Cyclin E as well as Cdc7/ Dbf4 activity convert a pre-RC (pre-Replication Complex) to a post-RC.

complex (Maiorano et al., 2000), a putative replicative helicase (Blow and Dutta, 2005). This prereplication complex (preRC) is triggered for replication at the onset of S-phase by binding of MCM10 (Wohlschlegel et al., 2002) and phosphorylation of preRC proteins by CDK2/Cyclin E and Cdc7/Dbf4. This facilitates the loading of Cdc45 and DNA polymerase-alpha (Arias and Walter, 2007). The activation of pre-RCs does not only account for accurate timing of replication, but also converts pre-RCs into post-RCs, which are not competent for initiation of replication.

The binding of Cdc6 and Cdt1 to the ORC complex in G1-phase is thought to be the limiting step in initiation of DNA replication and is therefore called replication licensing. To ascertain proper timing of origin licensing, both Cdt1 and Cdc6 are regulated by multiple pathways. Since both genes are targets of transcription factors of the E2F family, their expression increases in G1 when E2Fs are active (Hateboer et al., 1998; Yoshida and Inoue, 2004). To prevent re-licensing of replication origins Cdt1 is degraded in the beginning of S-phase by ubiquitin mediated proteolysis. In humans, two E3 ligases are involved in Cdt1 degradation, CUL4-DDB1CDT2 and SCFSkp2 (Li et al., 2003; Nishitani et al., 2006). In addition direct binding of Cdt1 to Geminin will inhibit its function (Wohlschlegel et al., 2000). Cdc6 protein stability has been shown to be regulated by the E3 ligase APC^{Cdh1} (Petersen et al., 2000). Interestingly, serine 54 phosphorylation of Cdc6 by CDK2/ cyclin E stabilizes the protein by interfering with APC^{Cdh1} mediated regulation (Duursma and Agami, 2005), which will be discussed in this thesis. Subsequently, it was shown by others that phosphorylation of this site by cyclin E plays a role in exit from quiescence and entrance into the cell cycle (Mailand and Diffley, 2005).

Cells have evolved several checkpoints that prevent DNA replication in case of DNA damage. One of these checkpoints inhibits origin firing by downregulating the levels of licensing proteins. Cdt1 has been described to be degraded in response to gamma-irradiation in an ATM independent manner by the Cul4-Roc1 E3 ligase (Higa et al., 2003) or in an ATM and ATR dependent manner upon treatment with both UV and gamma-irradiation by the SCF^{Skp2} E3-ligase (Kondo et al., 2004). We found that also the licensing protein Cdc6 is degraded upon gamma-irradiation in a p53-dependent manner (Duursma and Agami, 2005) (described in this thesis). It has been reported by others that Cdc6 can also be degraded in a p53 independent manner, possibly by the Huwe1 E3 ligase (Blanchard et al., 2002; Hall et al., 2007). This indicates that following DNA damage both Cdt1 and Cdc6

protein abundance are regulated by multiple pathways as well, perhaps signifying the prevention of origin licensing in cells with damaged DNA.
Deregulation of initiation of DNA replication can be linked to cancer in several ways. It was shown that rereplication of the genome can be induced by deregulation of licensing factors. Overexpression of Cdt1 and Cdc6 along with Cyclin A resulted in rereplication of human cancer cells with inactive p53 (Vaziri et al., 2003). Further, loss of Geminin was shown to induce rereplication in the presence of functional p53 (Melixetian et al., 2004).
Interestingly, a role for aberrant DNA replication was suggested in oncogene induced senescence (Bartkova et al., 2006; Di Micco et al., 2006). Oncogene activation, such as expression of active RasV12, resulted in augmented numbers of active replication origins and changes in replication fork progression (Di Micco et al., 2006). This in turn resulted in a partly replicated and rereplicated genome, which induced senescence via activation of the DNA damage checkpoint. Abrogation of this checkpoint could prevent oncogene-induced senescence and resulted in tumour growth in a mouse model (Di Micco et al., 2006).

Epigenetics
In mammals, epigenetic gene silencing can be regulated by direct modifications of DNA, but also by modifications of histones, protein complexes around which the DNA is enfolded. DNA can be modified by DNA methyltransferases (DNMTs), which transfer methyl groups from a S-adenosyl methionine (SAM) methyl donor to cytosine residues in DNA at CpG dinucleotides. These CpG dinucleotides are underrepresented in the genome apart from discrete regions with high CpG content that are called CpG islands and these occur mostly in gene promoter regions and in repetitive DNA sequences (Gal-Yam et al., 2007). Histones can be modified at many sites by many different proteins, but two important families of proteins are the Polycomb group (PcG) proteins and the Thritorax Group (TrxG). The PcG proteins are assembled in complexes that can initiate repression by methylation of lysines such as histone H3 lysine 27 (H3K27) in histone tails, and complexes that maintain silencing by facilitating additional remodelling of the chromatin (Schuettengruber et al., 2007). The activity of silencing complexes can be counteracted by complexes that promote transcriptionally active chromatin. During development the Thritorax Group (TrxG) antagonizes the effects of PcG group proteins by methylating histone H3K4 and thereby marking the chromatin as active (Schuettengruber et al., 2007). Mixed Lineage Leukemia 1 (MLL1) is a human TrxG homologue and a novel regulatory mechanism of this gene will be discussed in chapter 5.

Genomic DNA methylation of cytosines can be established by the 'de novo' methyltransferases Dnmt3a and Dnmt3b, which are able to methylate unmethylated DNA during early development and gametogenesis (Okano et al., 1999). This genomic imprinting coincides with their high expression in embryonic stem cells, whereas Dnmt3a and Dnmt3b are expressed to a low extent in differentiated somatic cells (Okano et al., 1998). On the other hand Dnmt1, the maintenance DNA methyltransferase, is merely expressed in somatic cells. It has a strong preference for hemi-methylated DNA and was shown to be the factor that preserves methylation marks on newly synthesized DNA during DNA replication (Gruenbaum et al., 1982; Leonhardt et al., 1992). Inactivation of a single Dnmt in mice results in embryonic lethality or the mice die shortly after birth, indicating that all three methyltransferases are essential for normal development (Li et al., 1992; Okano et al., 1999).
Although Dnmt3a and Dnmt3b have overlapping functions in imprinting of genes, Dnmt3b was shown to be essential for methylation of centromeric minor satellite repeats (Okano et al., 1999; Xu et al., 1999). Furthermore, inactivating mutations in the human Dnmt3b gene were linked to the Immunodeficiency, Centromeric

region instability and Facial anomalies (ICF) syndrome (Xu et al., 1999) and in all tissues of these patients reduced methylation of the minor satellite repeats was observed (Jeanpierre et al., 1993). Interestingly, lymphocytes of these patients show a high frequency of chromosomal abnormalities of chromosome 1, 9 and 16 such as centromeric decondensation and chromosome and chromatid breaks that are mostly restricted to the juxtacentromeric regions. In addition, multiradiate structures of chromosomes are observed (Maraschio et al., 1988; Sumner et al., 1998; Tuck-Muller et al., 2000). ICF patients are also characterized by low numbers of T-cells and this was recapitulated in a mouse model for the ICF syndrome. In this model an ICF-like mutation in Dnmt3b was shown to result in decreased T-cell survival (Ueda et al., 2006). From a study with conditional Dnmt3b knock-out mice it appeared that Dnmt3b is not involved in lymphoid lineage differentiation, but it does play a critical role in hematopoietic stem cell renewal (Tadokoro et al., 2007).

Initially, aberrant DNA hypomethylation was proposed to play a role in cancer, since tumour cells were shown to have a reduced content of methylated cytosines and a reduced amount of methylated genes (Feinberg and Vogelstein, 1983; Gama-Sosa et al., 1983). This hypomethylation was shown to result in reactivation of imprinted oncogenes, which in the case of Insulin Growth Factor 2 (IGF2) was associated with an increased risk for colon cancer (Cui et al., 2003; Sakatani et al., 2005). In addition, DNA hypomethylation was shown to promote chromosomal instability and it was shown to be sufficient to induce T-cell lymphomas in a mouse model (Eden et al., 2003; Gaudet et al., 2003).
In contrast, DNA hypermethylation of promoter regions was shown to promote tumourigenesis by repressing tumour suppressor gene activity. CpG islands in the promoter of the Rb tumour suppressor gene were the first discovered aberrantly methylated sequences in cancer (Greger et al., 1989). This was followed by several studies showing that promoter hypermethylation correlated with reduced expression, such as methylation of the p16 gene in bladder cancer (Gonzalez-Zulueta et al., 1995) and the mismatch repair gene hMLH1 in colon cancer (Kane et al., 1997). From the above it is clear that a tight control of DNA methyltransferase abundance is required. In chapter 4 of this thesis we describe a novel regulatory mechanism of Dnmt3b by a miRNA family.

MicroRNAs
MicroRNAs (miRNAs) are endogenous non-coding single-stranded RNAs of about 19-25 nucleotides, which function as negative regulators of protein coding mRNA sequences (Bushati and Cohen, 2007). According to computational studies each miRNA can regulate hundreds of mRNA targets and more than 30% of animal genes may be miRNA targets (Brennecke et al., 2005; Krek et al., 2005; Lewis et al., 2005; Xie et al., 2005). Therefore it is not surprising that miRNA have been implicated in regulation of many biological processes, such as differentiation, apoptosis and metabolism (Bushati and Cohen, 2007).
miRNAs are generally transcribed by RNA polymerase II into large pri-miRNA transcripts (Cai et al., 2004; Lee et al., 2004), which are processed by the RNase III enzyme Drosha and it's co-factor Pasha into a approximately 70 nucleotide pre-miRNA that is folded in the characteristic stem-loop structure (Lee et al., 2003)(Figure 3). This pre-miRNA will be exported to the cytosol by RAN-GTP and exportin 5 (Lund et al., 2004; Yi et al., 2003), where it will be processed further by Dicer, another RNase III enzyme (Hutvagner et al., 2001). Dicer cuts the loop of pre-miRNAs to generate a double stranded mature miRNA of which one strand will be loaded onto the miRNA-associated multiprotein RNA-induced silencing complex (miRISC), which directs the miRNA to its target mRNA (Carmell and Hannon, 2004). Several studies indicate that nucleotides 2-8 of the 5' end of the mature miRNA (the miRNA seed) are important in mRNA target recognition (Doench and Sharp, 2004; Lewis

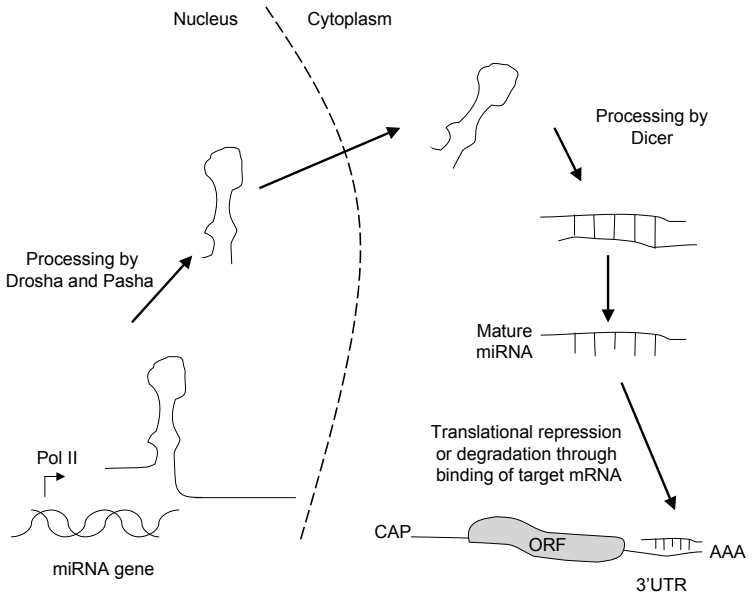


Figure 3. Schematic representation of miRNA biogenesis.

et al., 2005). In animals, miRNAs exert their function by binding with imperfect complementarity to the 3'UTR of protein coding mRNA sequences. This results in translational repression and enhanced RNA decay, possibly through reduced adenylation of the mRNA (Standart and Jackson, 2007). However, it has also been described that in case of near-perfect homology of the miRNA with the 3'UTR, the target mRNA can be cleaved in manner similar to siRNA-guided cleavage (Yekta et al., 2004). Also in plants miRNAs bind target mRNA with very high sequence complementarity and can induce both translational repression or siRNA-like RNA cleavage (Chen, 2004; Llave et al., 2002; Rhoades et al., 2002). However, in contrast to mammalian miRNAs, which target 3'UTRs, most plant miRNAs target protein coding sequences (CDS). Currently, no functional miRNA binding sites have been identified in mammalian CDS, nevertheless, computational approaches predict that CDS miRNA targets are present in mammals as well (Miranda et al., 2006). In this thesis we show for the first time an example of a human miRNA that regulates the protein abundance of Dnmt3b by binding to its coding region with high sequence complementarity.

With the possibility of miRNAs to regulate key cellular processes like cell growth and apoptosis, impaired miRNA expression has been implicated in tumourigenesis. Like protein encoding genes, deregulation of miRNA encoding genes can occur through genetic alterations such as amplifications, deletions and point mutations. One of the first examples was the frequent deletion of miR-15a and miR-16-1 in chronic lymphocytic leukemia (CLL) (Calin et al., 2002), which where shown later to negatively regulate the anti-apoptotic oncogene BCL2 (Cimmino et al., 2005). Also certain transcripts of the let-7 miRNA family were shown to be down-regulated in human lung cancer and this correlated with decreased post-operative survival (Takamizawa et al., 2004). This suggested a role for let-7 as a tumour suppressor and this was further supported by evidence that the let-7 family can negatively regulate Ras (Johnson et al.,

2005). In addition, it has been shown that let-7 can regulate the HMGA2 oncogene (Lee and Dutta, 2007; Mayr et al., 2007). Notably, HMGA2 translocates in certain leukaemia's thereby losing its 3'UTR with let-7 target sites, which renders the HMGA2 fusion protein insensitive to let-7 regulation.
Conversely, the expression of other miRNAs was found increased in tumours, such as the miR-17-92 cluster on chromosome 13 that was shown to be amplified in human B-cell lymphoma's (He et al., 2005). Remarkably, this cluster was shown to be upregulated by MYC and to modulate E2F1 expression (O'Donnell et al., 2005), but also to collaborate with MYC by accelerating tumourigenesis in a mouse B-cell lymphoma model (He et al., 2005). In addition, miR-372 and miR-373 have been identified from a genetic screen for miRNAs that cooperate with oncogenic RAS to transform primary human cells, thereby bypassing activation of the p53 pathway (Voorhoeve et al., 2006). Interestingly, these miRNAs were found to be expressed in most testicular germ cell tumours, which are wild-type for p53. This suggests a role for miR-372 and miR-373 in the development of these tumours. Finally, the p27 tumour suppressor was shown to be regulated by miR-221 and miR-222 (le Sage et al., 2007). High expression of these miRNAs was found to correlate inversely with reduced expression of p27 in human glioblastoma's and reducing the levels of miR-221 in these cells resulted in decreased proliferation.

From the above it is clear that genetic alterations of human miRNAs can play a key role in tumourigenesis. However, human miRNAs expression can also be altered by epigenetic changes such as aberrant methylation of CpG islands in promoter regions. Treatment of cancer cells with a demethylating agent resulted in increased expression of miR-127 and BCL6 was identified as a target (Saito et al., 2006). In another study CpG methylation status of miRNAs were analysed in breast tissues and here miRNA-9-1 was demonstrated to be methylated in breast tumour tissues but not in normal tissue (Lehmann et al., 2007).
Lastly, altered transcriptional activation of miRNAs might contribute to the process of tumourigenesis. This was discussed above for the miR-17-92 cluster, which is regulated by MYC. However, recent studies identified several miRNAs that are regulated by the tumour suppressor protein p53 of which miR-34 was most prominent (Bommer et al., 2007; Chang et al., 2007; Corney et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007). Significantly, p53 was shown to directly target the miR-34 promoter region and loss of p53 resulted in decreased miR-34 expression. Ectopic expression of miR-34 induced apoptosis and a cell cycle arrest, thereby suppressing tumour cell proliferation. This indicates that reduced miRNA expression due to functional inactivation of well-known tumour suppressor pathways might contribute to the process of carcinogenesis as well.

Outline of this thesis

The studies described in this thesis aimed to gain more insight in the regulation and function of the Cdc6 and the miR-148/152 family.

In Chapter 2 we studied the effect of DNA damage on Cdc6 protein stability. We identified that phosphorylation of Cdc6 at Serine 54 by CDK2/ Cyclin E stabilises the protein during normal replication. In response to DNA damage this kinase complex is inhibited in a p53 and p21-dependent manner, resulting in decreased Cdc6 protein level. In Chapter 3, the implications of these findings will be discussed in more detail.
Next, we studied several aspects of the function of the conserved miR-148/152 miRNA family. In chapter 4, we describe our finding that miR-148 regulates Dnmt3b mRNA through interacting with its protein coding sequence. Notably, all current described mammalian miRNAs target mRNAs by binding to mRNA 3'UTRs. In Chapter 5, we extended this study by exploring whether miR-148 mediated regulation of Dnmt3b plays a role in early human thymic development. Interestingly, we found differential expression of miR-148 in plasmacytoid

dendritic cells (pDCs) and its precursor cell. Moreover, exogenous expression of miR-148 resulted in a dramatic increase of pDCs in an in vitro assay, suggesting that miR-148 expression interferes with normal differentiation, survival or apoptosis. In Chapter 6, we describe a role for miR-152 in S and G2/M-phase of cell cycle progression in diploid fibroblasts. These findings will be generally discussed in Chapter 7.

References

Agami, R., and Bernards, R. (2000). Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. Cell 102, 55-66.

Arias, E. E., and Walter, J. C. (2007). Strength in numbers: preventing rereplication via multiple mechanisms in eukaryotic cells. Genes Dev 21, 497-518.

Bartek, J., and Lukas, J. (2007). DNA damage checkpoints: from initiation to recovery or adaptation. Curr Opin Cell Biol 19, 238-245.

Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L. V., Kolettas, E., Niforou, K., Zoumpourlis, V. C., et al. (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature 444, 633-637.

Blanchard, F., Rusiniak, M. E., Sharma, K., Sun, X., Todorov, I., Castellano, M. M., Gutierrez, C., Baumann, H., and Burhans, W. C. (2002). Targeted destruction of DNA replication protein Cdc6 by cell death pathways in mammals and yeast. Mol Biol Cell 13, 1536-1549.

Blow, J. J., and Dutta, A. (2005). Preventing re-replication of chromosomal DNA. Nat Rev Mol Cell Biol 6, 476-486.

Bommer, G. T., Gerin, I., Feng, Y., Kaczorowski, A. J., Kuick, R., Love, R. E., Zhai, Y., Giordano, T. J., Qin, Z. S., Moore, B. B., et al. (2007). p53-mediated activation of miRNA34 candidate tumor-suppressor genes. Curr Biol 17, 1298-1307.

Brennecke, J., Stark, A., Russell, R. B., and Cohen, S. M. (2005). Principles of microRNA-target recognition. PLoS Biol 3, e85.

Bushati, N., and Cohen, S. M. (2007). microRNA Functions. Annu Rev Cell Dev Biol 23, 175-205.

Cai, X., Hagedorn, C. H., and Cullen, B. R. (2004). Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. Rna 10, 1957-1966.

Calin, G. A., Dumitru, C. D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., et al. (2002). Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 99, 15524-15529.

Carmell, M. A., and Hannon, G. J. (2004). RNase III enzymes and the initiation of gene silencing. Nat Struct Mol Biol 11, 214-218.

Chang, T. C., Wentzel, E. A., Kent, O. A., Ramachandran, K., Mullendore, M., Lee, K. H., Feldmann, G., Yamakuchi, M., Ferlito, M., Lowenstein, C. J., et al. (2007). Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol Cell 26, 745-752.

Chehab, N. H., Malikzay, A., Appel, M., and Halazonetis, T. D. (2000). Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev 14, 278-288.

Chehab, N. H., Malikzay, A., Stavridi, E. S., and Halazonetis, T. D. (1999). Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. Proc Natl Acad Sci U S A 96, 13777-13782.

Chen, X. (2004). A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science 303, 2022-2025.

Cimmino, A., Calin, G. A., Fabbri, M., Iorio, M. V., Ferracin, M., Shimizu, M., Wojcik, S. E., Aqeilan, R. I., Zupo, S., Dono, M., et al. (2005). miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A 102, 13944-13949.

Corney, D. C., Flesken-Nikitin, A., Godwin, A. K., Wang, W., and Nikitin, A. Y. (2007). MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. Cancer Res 67, 8433-8438.

Cui, H., Cruz-Correa, M., Giardiello, F. M., Hutcheon, D. F., Kafonek, D. R., Brandenburg, S., Wu, Y., He, X., Powe, N. R., and Feinberg, A. P. (2003). Loss of IGF2 imprinting: a potential marker of colorectal cancer risk. Science 299, 1753-1755.

Dannenberg, J. H., and te Riele, H. P. (2006). The retinoblastoma gene family in cell cycle regulation and suppression of tumorigenesis. Results Probl Cell Differ 42, 183-225.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P. G., Bensimon, A., et al. (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature 444, 638-642.

Doench, J. G., and Sharp, P. A. (2004). Specificity of microRNA target selection in translational repression. Genes Dev 18, 504-511.

Dulic, V., Lees, E., and Reed, S. I. (1992). Association of human cyclin E with a periodic G1-S phase protein kinase. Science 257, 1958-1961.

Duursma, A., and Agami, R. (2005). p53-Dependent regulation of Cdc6 protein stability controls cellular proliferation. Mol Cell Biol 25, 6937-6947.

Eden, A., Gaudet, F., Waghmare, A., and Jaenisch, R. (2003). Chromosomal instability and tumors promoted by DNA hypomethylation. Science 300, 455.

el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell 75, 817-825.

Feinberg, A. P., and Vogelstein, B. (1983). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 301, 89-92.

Gal-Yam, E. N., Saito, Y., Egger, G., and Jones, P. A. (2007). Cancer Epigenetics: Modifications, Screening, and Therapy. Annu Rev Med.

Gama-Sosa, M. A., Slagel, V. A., Trewyn, R. W., Oxenhandler, R., Kuo, K. C., Gehrke, C. W., and Ehrlich, M. (1983). The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res 11, 6883-6894.

Gaudet, F., Hodgson, J. G., Eden, A., Jackson-Grusby, L., Dausman, J., Gray, J. W., Leonhardt, H., and Jaenisch, R. (2003). Induction of tumors in mice by genomic hypomethylation. Science 300, 489-492.

Gonzalez-Zulueta, M., Bender, C. M., Yang, A. S., Nguyen, T., Beart, R. W., Van Tornout, J. M., and Jones, P. A. (1995). Methylation of the 5' CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res 55, 4531-4535.

Greger, V., Passarge, E., Hopping, W., Messmer, E., and Horsthemke, B. (1989). Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. Hum Genet 83, 155-158.

Gruenbaum, Y., Cedar, H., and Razin, A. (1982). Substrate and sequence specificity of a eukaryotic DNA methylase. Nature 295, 620-622.

Hall, J. R., Kow, E., Nevis, K. R., Lu, C. K., Luce, K. S., Zhong, Q., and Cook, J. G. (2007). Cdc6 stability is regulated by the Huwe1 ubiquitin ligase after DNA damage. Mol Biol Cell 18, 3340-3350.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell 100, 57-70.

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75, 805-816.

Hateboer, G., Wobst, A., Petersen, B. O., Le Cam, L., Vigo, E., Sardet, C., and Helin, K. (1998). Cell cycle-regulated expression of mammalian CDC6 is dependent on E2F. Mol Cell Biol 18, 6679-6697.

He, L., He, X., Lim, L. P., de Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D., et al. (2007). A microRNA component of the p53 tumour suppressor network. Nature 447, 1130-1134.

He, L., Thomson, J. M., Hemann, M. T., Hernando-Monge, E., Mu, D., Goodson, S., Powers, S., Cordon-Cardo, C., Lowe, S. W., Hannon, G. J., and Hammond, S. M. (2005). A microRNA polycistron as a potential human oncogene. Nature 435, 828-833.

Higa, L. A., Mihaylov, I. S., Banks, D. P., Zheng, J., and Zhang, H. (2003). Radiation-mediated proteolysis of CDT1

by CUL4-ROC1 and CSN complexes constitutes a new checkpoint. Nat Cell Biol 5, 1008-1015.

Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S. J., and Mak, T. W. (2000). DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science 287, 1824-1827.

Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T., and Zamore, P. D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 293, 834-838.

Jacobs, J. J., Kieboom, K., Marino, S., DePinho, R. A., and van Lohuizen, M. (1999). The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature 397, 164-168.

Jeanpierre, M., Turleau, C., Aurias, A., Prieur, M., Ledeist, F., Fischer, A., and Viegas-Pequignot, E. (1993). An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome. Hum Mol Genet 2, 731-735.

Johnson, S. M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K. L., Brown, D., and Slack, F. J. (2005). RAS is regulated by the let-7 microRNA family. Cell 120, 635-647.

Jones, P. A., and Baylin, S. B. (2007). The epigenomics of cancer. Cell 128, 683-692.

Kane, M. F., Loda, M., Gaida, G. M., Lipman, J., Mishra, R., Goldman, H., Jessup, J. M., and Kolodner, R. (1997). Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res 57, 808-811.

Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S., Nishimoto, T., Morgan, D. O., Franza, B. R., and Roberts, J. M. (1992). Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. Science 257, 1689-1694.

Kondo, T., Kobayashi, M., Tanaka, J., Yokoyama, A., Suzuki, S., Kato, N., Onozawa, M., Chiba, K., Hashino, S., Imamura, M., et al. (2004). Rapid degradation of Cdt1 upon UV-induced DNA damage is mediated by SCFSkp2 complex. J Biol Chem 279, 27315-27319.

Kouzarides, T. (2007). Chromatin modifications and their function. Cell 128, 693-705.

Krek, A., Grun, D., Poy, M. N., Wolf, R., Rosenberg, L., Epstein, E. J., MacMenamin, P., da Piedade, I., Gunsalus, K. C., Stoffel, M., and Rajewsky, N. (2005). Combinatorial microRNA target predictions. Nat Genet 37, 495-500.

le Sage, C., Nagel, R., Egan, D. A., Schrier, M., Mesman, E., Mangiola, A., Anile, C., Maira, G., Mercatelli, N., Ciafre, S. A., et al. (2007). Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. Embo J 26, 3699-3708.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., and Kim, V. N. (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature 425, 415-419.

Lee, Y., Kim, M., Han, J., Yeom, K. H., Lee, S., Baek, S. H., and Kim, V. N. (2004). MicroRNA genes are transcribed by RNA polymerase II. Embo J 23, 4051-4060.

Lee, Y. S., and Dutta, A. (2007). The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. Genes Dev 21, 1025-1030.

Lehmann, U., Hasemeier, B., Christgen, M., Muller, M., Romermann, D., Langer, F., and Kreipe, H. (2007). Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer. J Pathol.

Leonhardt, H., Page, A. W., Weier, H. U., and Bestor, T. H. (1992). A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell 71, 865-873.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. Cell 88, 323-331.

Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15-20.

Li, E., Bestor, T. H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69, 915-926.

Li, X., Zhao, Q., Liao, R., Sun, P., and Wu, X. (2003). The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1

degradation. J Biol Chem 278, 30854-30858.

Liang, C., Weinreich, M., and Stillman, B. (1995). ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. Cell 81, 667-676.

Llave, C., Xie, Z., Kasschau, K. D., and Carrington, J. C. (2002). Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science 297, 2053-2056.

Lund, A. H., and van Lohuizen, M. (2004). Epigenetics and cancer. Genes Dev 18, 2315-2335.

Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E., and Kutay, U. (2004). Nuclear export of microRNA precursors. Science 303, 95-98.

Mailand, N., and Diffley, J. F. (2005). CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. Cell 122, 915-926.

Mailand, N., Falck, J., Lukas, C., Syljuasen, R. G., Welcker, M., Bartek, J., and Lukas, J. (2000). Rapid destruction of human Cdc25A in response to DNA damage. Science 288, 1425-1429.

Maiorano, D., Moreau, J., and Mechali, M. (2000). XCDT1 is required for the assembly of pre-replicative complexes in Xenopus laevis. Nature 404, 622-625.

Malumbres, M., and Barbacid, M. (2001). To cycle or not to cycle: a critical decision in cancer. Nat Rev Cancer 1, 222-231.

Maraschio, P., Zuffardi, O., Dalla Fior, T., and Tiepolo, L. (1988). Immunodeficiency, centromeric heterochromatin instability of chromosomes 1, 9, and 16, and facial anomalies: the ICF syndrome. J Med Genet 25, 173-180.

Matsuoka, S., Huang, M., and Elledge, S. J. (1998). Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science 282, 1893-1897.

Mayr, C., Hemann, M. T., and Bartel, D. P. (2007). Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science 315, 1576-1579.

Melixetian, M., Ballabeni, A., Masiero, L., Gasparini, P., Zamponi, R., Bartek, J., Lukas, J., and Helin, K. (2004). Loss of Geminin induces rereplication in the presence of functional p53. J Cell Biol 165, 473-482.

Michael, D., and Oren, M. (2003). The p53-Mdm2 module and the ubiquitin system. Semin Cancer Biol 13, 49-58.

Miranda, K. C., Huynh, T., Tay, Y., Ang, Y. S., Tam, W. L., Thomson, A. M., Lim, B., and Rigoutsos, I. (2006). A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. Cell 126, 1203-1217.

Miyashita, T., and Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80, 293-299.

Morgan, D. O. (1997). Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 13, 261-291.

Nakano, K., and Vousden, K. H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 7, 683-694.

Nishitani, H., Sugimoto, N., Roukos, V., Nakanishi, Y., Saijo, M., Obuse, C., Tsurimoto, T., Nakayama, K. I., Nakayama, K., Fujita, M., et al. (2006). Two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4, target human Cdt1 for proteolysis. Embo J 25, 1126-1136.

O'Donnell, K. A., Wentzel, E. A., Zeller, K. I., Dang, C. V., and Mendell, J. T. (2005). c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435, 839-843.

Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99, 247-257.

Okano, M., Xie, S., and Li, E. (1998). Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet 19, 219-220.

Petersen, B. O., Wagener, C., Marinoni, F., Kramer, E. R., Melixetian, M., Lazzerini Denchi, E., Gieffers, C., Matteucci, C., Peters, J. M., and Helin, K. (2000). Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. Genes Dev 14, 2330-2343.

Quelle, D. E., Ashmun, R. A., Shurtleff, S. A., Kato, J. Y., Bar-Sagi, D., Roussel, M. F., and Sherr, C. J. (1993). Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes Dev 7, 1559-1571.

Raver-Shapira, N., Marciano, E., Meiri, E., Spector, Y., Rosenfeld, N., Moskovits, N., Bentwich, Z., and Oren, M. (2007). Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. Mol Cell 26, 731-743.

Rhoades, M. W., Reinhart, B. J., Lim, L. P., Burge, C. B., Bartel, B., and Bartel, D. P. (2002). Prediction of plant microRNA targets. Cell 110, 513-520.

Riese, D. J., 2nd, Gallo, R. M., and Settleman, J. (2007). Mutational activation of ErbB family receptor tyrosine kinases: insights into mechanisms of signal transduction and tumorigenesis. Bioessays 29, 558-565.

Rosenblatt, J., Gu, Y., and Morgan, D. O. (1992). Human cyclin-dependent kinase 2 is activated during the S and G2 phases of the cell cycle and associates with cyclin A. Proc Natl Acad Sci U S A 89, 2824-2828.

Saito, Y., Liang, G., Egger, G., Friedman, J. M., Chuang, J. C., Coetzee, G. A., and Jones, P. A. (2006). Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 9, 435-443.

Sakatani, T., Kaneda, A., Iacobuzio-Donahue, C. A., Carter, M. G., de Boom Witzel, S., Okano, H., Ko, M. S., Ohlsson, R., Longo, D. L., and Feinberg, A. P. (2005). Loss of imprinting of Igf2 alters intestinal maturation and tumorigenesis in mice. Science 307, 1976-1978.

Schubbert, S., Shannon, K., and Bollag, G. (2007). Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer 7, 295-308.

Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B., and Cavalli, G. (2007). Genome regulation by polycomb and trithorax proteins. Cell 128, 735-745.

Serrano, M., and Blasco, M. A. (2007). Cancer and ageing: convergent and divergent mechanisms. Nat Rev Mol Cell Biol 8, 715-722.

Shieh, S. Y., Ahn, J., Tamai, K., Taya, Y., and Prives, C. (2000). The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. Genes Dev 14, 289-300.

Shieh, S. Y., Ikeda, M., Taya, Y., and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell 91, 325-334.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer 3, 155-168.

Siliciano, J. D., Canman, C. E., Taya, Y., Sakaguchi, K., Appella, E., and Kastan, M. B. (1997). DNA damage induces phosphorylation of the amino terminus of p53. Genes Dev 11, 3471-3481.

Smits, V. A., and Medema, R. H. (2001). Checking out the G(2)/M transition. Biochim Biophys Acta 1519, 1-12.

Sparmann, A., and van Lohuizen, M. (2006). Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer 6, 846-856.

Standart, N., and Jackson, R. J. (2007). MicroRNAs repress translation of m7Gppp-capped target mRNAs in vitro by inhibiting initiation and promoting deadenylation. Genes Dev 21, 1975-1982.

Sumner, A. T., Mitchell, A. R., and Ellis, P. M. (1998). A FISH study of chromosome fusion in the ICF syndrome: involvement of paracentric heterochromatin but not of the centromeres themselves. J Med Genet 35, 833-835.

Tadokoro, Y., Ema, H., Okano, M., Li, E., and Nakauchi, H. (2007). De novo DNA methyltransferase is essential for self-renewal, but not for differentiation, in hematopoietic stem cells. J Exp Med 204, 715-722.

Takamizawa, J., Konishi, H., Yanagisawa, K., Tomida, S., Osada, H., Endoh, H., Harano, T., Yatabe, Y., Nagino, M., Nimura, Y., et al. (2004). Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 64, 3753-3756.

Tarasov, V., Jung, P., Verdoodt, B., Lodygin, D., Epanchintsev, A., Menssen, A., Meister, G., and Hermeking, H. (2007). Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. Cell Cycle 6, 1586-1593.

Tuck-Muller, C. M., Narayan, A., Tsien, F., Smeets, D. F., Sawyer, J., Fiala, E. S., Sohn, O. S., and Ehrlich, M. (2000). DNA hypomethylation and unusual chromosome instability in cell lines from ICF syndrome patients. Cytogenet Cell Genet 89, 121-128.

Ueda, Y., Okano, M., Williams, C., Chen, T., Georgopoulos, K., and Li, E. (2006). Roles for Dnmt3b in mammalian development: a mouse model for the ICF syndrome. Development 133, 1183-1192.

Vaziri, C., Saxena, S., Jeon, Y., Lee, C., Murata, K., Machida, Y., Wagle, N., Hwang, D. S., and Dutta, A. (2003). A p53-dependent checkpoint pathway prevents rereplication. Mol Cell 11, 997-1008.

Voorhoeve, P. M., le Sage, C., Schrier, M., Gillis, A. J., Stoop, H., Nagel, R., Liu, Y. P., van Duijse, J., Drost, J., Griekspoor, A., et al. (2006). A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell 124, 1169-1181.

Wohlschlegel, J. A., Dhar, S. K., Prokhorova, T. A., Dutta, A., and Walter, J. C. (2002). Xenopus Mcm10 binds to origins of DNA replication after Mcm2-7 and stimulates origin binding of Cdc45. Mol Cell 9, 233-240.

Wohlschlegel, J. A., Dwyer, B. T., Dhar, S. K., Cvetic, C., Walter, J. C., and Dutta, A. (2000). Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science 290, 2309-2312.

Xie, X., Lu, J., Kulbokas, E. J., Golub, T. R., Mootha, V., Lindblad-Toh, K., Lander, E. S., and Kellis, M. (2005). Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. Nature 434, 338-345.

Xu, G. L., Bestor, T. H., Bourc'his, D., Hsieh, C. L., Tommerup, N., Bugge, M., Hulten, M., Qu, X., Russo, J. J., and Viegas-Pequignot, E. (1999). Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402, 187-191.

Yekta, S., Shih, I. H., and Bartel, D. P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. Science 304, 594-596.

Yi, R., Qin, Y., Macara, I. G., and Cullen, B. R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17, 3011-3016.

Yoshida, K., and Inoue, I. (2004). Regulation of Geminin and Cdt1 expression by E2F transcription factors. Oncogene 23, 3802-3812.

Chapter 2

p53-dependent regulation of Cdc6 protein stability controls cellular proliferation

Anja M. Duursma and Reuven Agami

Molecular and Cellular Biology. 2005. 25 (16): 6937-47

p53-dependent regulation of Cdc6 protein stability controls cellular proliferation

Anja M. Duursma and Reuven Agami*

The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
*Communicating author

Activation of the tumor suppressor p53 in response to genotoxic stress imposes cellular growth arrest or apoptosis. We identified Cdc6, a licensing factor of the pre-replication complex, as a novel target of the p53 pathway. We show that activation of p53 by DNA damage results in enhanced Cdc6 destruction by the anaphase-promoting complex. This destruction is triggered by inhibition of CDK2 mediated CDC6 phosphorylation at serine 54. Conversely, suppression of p53 expression results in stabilization of Cdc6. We demonstrate that loss of p53 results in more replicating cells, an effect that can be reversed by reducing Cdc6 protein levels. Collectively, our data suggest that initiation of DNA replication is regulated by p53 through Cdc6 protein stability.

Introduction

Initiation of DNA replication is a tightly regulated process, which is highly conserved in eukaryotes. Key to this process is the licensing of replication origins for DNA replication in which Cdc6 plays a major role (Pelizon, 2003). Cdc6 is recruited to the origins by the origin recognition complex (ORC) during G1 where it serves together with Cdt1 as a loading factor for the minichromosome maintenance (MCM) complex, a putative replicative helicase. Together, this pre-replication complex (pre-RC) renders the genome competent for replication, but initiation of replication only occurs after activation of the complex by cyclin-dependent kinases at the onset of S-phase. This promotes the loading of Cdc45, replication protein A and DNA polymerase-alpha onto chromatin. After initiation of DNA replication, the pre-RCs are converted to post-RCs, which are present in S, G2 and M-phase and are not competent for initiation of replication (Bell and Dutta, 2002; Diffley, 2004; Takisawa et al., 2000).

Cdc6 has been shown to be essential for loading of the MCM-complex onto chromatin and therefore for the initiation of DNA replication in several eukaryotic organisms. First, mutant studies in yeast have demonstrated that association of MCMs to origins is dependent on Cdc6 (Aparicio et al., 1997; Tanaka et al., 1997). Second, it was shown in Xenopus laevis that immuno-depletion of Cdc6 led to loss of MCM origin association (Coleman et al., 1996). Last, ectopic expression of Cdc6 was sufficient to induce stable association of endogenous MCM protein with chromatin in mammalian cells with low Cdc6 levels as a result of serum deprivation (Cook et al., 2002). In addition, immuno-depletion of Cdc6 in human cells by micro-injection of anti-cdc6 antibody could block initiation of DNA replication, which suggests that Cdc6 is also limiting for DNA synthesis in human cells (Hateboer et al., 1998; Yan et al., 1998). Conversely, recombinant Cdc6 protein induced premature entry into S phase in a mammalian cell-free system (Stoeber et al., 1998) and ectopic expression of Cdc6 together with Cdt1 induces rereplication and polyploidy (Vaziri et al., 2003). Thus, Cdc6 is crucial for MCM-loading and therefore involved in regulation of S-phase entry.

In support of an essential function of Cdc6 in initiation of DNA replication, Cdc6 protein abundance is tightly regulated during the cell cycle. In human cells, Cdc6 transcription

is regulated by the E2F transcription factors (Hateboer et al., 1998; Yan et al., 1998). In addition cdc6 is targeted for ubiquitin-mediated proteolysis in early G1 by the E3 ligase-anaphase promoting complex (APC) (Petersen et al., 2000). This is mediated by Cdh1, a protein required for both substrate recognition and activation of the APC in late mitosis/ early G1 (Pfleger et al., 2001; Reed, 2003). Typically, Cdh1 associates with its substrates via their N-termini and requires a well-defined RXXL destruction box (D box) or KEN box (Pfleger and Kirschner, 2000; Pfleger et al., 2001). APC^{Cdh1} dependent proteolysis of Cdc6 requires both a D box and a KEN box (Petersen et al., 2000).

Several studies have shown that mammalian Cdc6 can be phosphorylated in vitro by both CyclinE/CDK2 and CyclinA/CDK2 and an interaction between the cyclins and Cdc6 could be detected (Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999). Moreover, Cdc6 can be phosphorylated in vivo at three N-terminal sites (ser54, ser74 and ser106) that are phosphorylated by CDK2 in vitro (Jiang et al., 1999). Together, this strongly suggests that mammalian Cdc6 is an in vivo CDK2 substrate. However, the role of this phosphorylation in Cdc6 regulation remains controversial. Based on studies with ectopically expressed and tagged wild-type Cdc6 or Cdc6 that was mutated in several potential CDK2 phosphorylation sites, it has been proposed that phosphorylation of Cdc6 by CyclinA/CDK2 in S-phase results in translocation from the nucleus to the cytosol and subsequent degradation (Delmolino et al., 2001; Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998). Now, it has become evident that only ectopically expressed Cdc6 or the soluble endogenous form is translocated to the cytosol, whereas the chromatin bound form persist through S and G2 phase (Coverley et al., 2000; Mendez and Stillman, 2000). Furthermore, by using a phospho-specific antibody it has been shown that phosphorylated serine-54 Cdc6 maintains high affinity for chromatin during S-phase (Alexandrow and Hamlin, 2004).

The DNA-damage response is essential for maintenance of genome stability. Genotoxic stress activates checkpoints that account for a rapid and prolonged cell cycle arrest, DNA-repair or apoptosis (Iliakis et al., 2003). Primary among the checkpoint genes is the tumor suppressor p53 (Fei and El-Deiry, 2003). In response to DNA damage, such as induced by ionizing radiation (IR), the transcription factor p53 is activated by the ATM-CHK2 pathway. A rapid phosphorylation at serines 15 and 20 leads to release of Mdm2 and stabilization and activation of p53 (Shiloh, 2003). p53 is required for the maintenance of the G1 arrest through activation of its transcriptional target gene p21cip1, induction of apoptosis and for the sustained arrest of cells prior to M phase (Bunz et al., 1998; Fei and El-Deiry, 2003; Motoyama and Naka, 2004). In addition, DNA damage initiates a rapid cell cycle arrest by inactivation of proteins that promote cell cycle progression, such as Cyclin D1 and CDC25A (Agami and Bernards, 2000; Falck et al., 2001).

In this study, we determined the effect of DNA damage on Cdc6 protein levels. We observed a rapid degradation of Cdc6 in response to IR. Interestingly, this irradiation-induced Cdc6 destruction was p53-dependent. Activation of p53 caused inhibition of cyclin E/CDK2 through induction of the cell cycle inhibitor p21cip1. This resulted in loss of Cdc6-serine 54 phosphorylation, which led to accelerated proteolysis by the APC^{Cdh1}.

Results

p53 dependent down-regulation of Cdc6 following DNA damage

Since the licensing factor Cdc6 is critical for formation of the pre-RC and the initiation of DNA replication, we examined the effect of IR on the Cdc6 protein levels. Primary human BJ fibroblasts were treated with increasing amounts of IR and the cells were harvested at various time-points. We found reduced Cdc6 protein levels within 2 hours after exposure to IR and 2 Gy was sufficient to elicit this response (Fig. 1A). Control immuno-stainings showed an increase in

the p53 transcription factor and its target $p21^{Cip1}$ at these doses, demonstrating that p53 was both stabilized and activated after DNA damage in these cells. Similar down-regulation of Cdc6 was observed in other cell types, such as MCF-7 cells (Fig. 1B). However, this cancer cell line required a higher dose of 5-20 Gy to activate a DNA damage response that resulted in both Cdc6 down-regulation and stabilization of p53 (data not shown). Furthermore, Cdc6 down-regulation following irradiation in MCF-7 cells was observed 2-4 hours following IR and at these time-points no significant change in cell cycle profile was observed (Supplementary Fig. 1A). Interestingly, the reduction in Cdc6 levels following IR in MCF-7 cells was preceded the accumulation of $p21^{Cip1}$ (Fig. 1B). This is likely to be the result of p53-independent DNA damage responses, such as cyclin D1 and Cdc25A destruction, operating early after DNA damage to inhibit CDK2 activity (Agami and Bernards, 2000; Mailand and Diffley, 2005). Indeed, the reduction in Cdc6 levels following radiation of MCF-7 cells correlated well with the reduction in CDK2 activity, which was almost completed within 2 hrs after IR

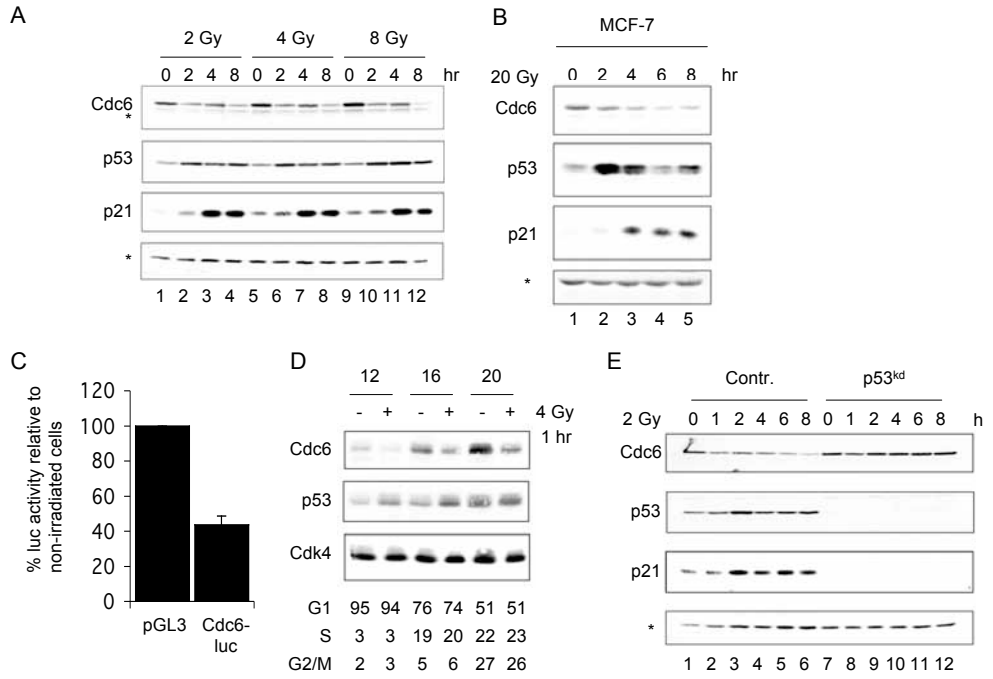


Figure 1. p53 dependent regulation of Cdc6. A Primary human BJ fibroblasts were subjected to 2, 4 and 8 Gy of IR and harvested 0, 2, 4, and 8 hours later. Whole cell extracts (WCE) were made, separated on 10% SDS-PAGE and immuno-blotted to detect Cdc6, p53 and p21 proteins. Asterisks mark background bands. **B** MCF-7 cells irradiated with 20 Gy were treated as described in A. **C** MCF-7 cells were transfected with Cdc6-Luciferase and control pGL3-promoter vector constructs (n=3). 72 hrs after transfection, cells were irradiated (20 Gy) and harvested 2 hrs later. Luciferase activities were measured, normalized to co-transfected Renilla luciferase and compared to non-irradiated cells. **D** Primary human BJ fibroblasts were serum-starved for 72 hours, released with addition of 10% FCS, irradiated (4Gy) 1 hour prior to the harvest at the indicated time points. In parallel, cells were collected for flow cytometric analysis (Supplementary Fig 1). **E** Polyclonal pools of primary human BJ fibroblasts stably carrying the retroviral vector pRETRO-SUPER (pRS) or pRS-p53kd, which mediates the inhibition of p53 expression through RNAi, were subjected to 2 Gy of irradiation. WCE were prepared at the indicated time-points and protein levels of Cdc6, p53 and p21 were examined by immuno-blotting. Contr., control.

(Supplementary Fig. 1B). To estimate the quantity of Cdc6 protein reduction following IR, we constructed a firefly Cdc6-luciferase fusion construct driven by the SV40 promoter and transfected it into MCF-7 cells together with a Renilla-luciferase control. Treatment with 20 Gy irradiation resulted in a reduction of 60% Cdc6-luciferase activity after 4 hours, whereas no change in control luciferase activity was observed (Fig. 1C). Collectively, these results indicate that Cdc6 protein level is reduced in response to DNA damage.

Next, we assessed whether Cdc6 protein levels are reduced in G1 phase of the cell cycle when pre-RCs are formed. We synchronized primary human BJ fibroblasts in G0 by serum starvation and then released them to enter synchronously G1 phase around 12 hours later and S phase around 16 and 20 hours later (supplementary Fig. 1C). Cells were irradiated (4 Gy) 12, 16 and 20 hours after the release, harvested one hour later and Cdc6 protein level was compared to untreated cells by immuno-blot analysis. Clearly, potent down-regulation of Cdc6 following IR occurred when cells were almost exclusively in G1 phase of the cell cycle (12 hr), indicating that Cdc6 protein levels are reduced when it is functional (Fig. 1D). Also here, Cdc6 down-regulation preceded any detectable cell cycle changes induced by IR, suggesting that Cdc6 down-regulation in G1 contributes to the IR-induced cellular responses (Fig. 1D and Supplementary Fig. 1C).

The p53 protein is a central player in the DNA damage response. We therefore investigated whether the decrease in Cdc6 protein level following IR is p53-dependent. We transduced primary human BJ cells with the pRS-p53^{kd} that stably suppresses p53 expression through RNA interference (RNAi) (Voorhoeve and Agami, 2003) and monitored Cdc6 protein level following IR. Interestingly, immuno-blot analysis showed that IR-induced Cdc6 down-regulation was completely abrogated in p53^{kd} cells (Fig. 1E). Control immuno-labeling showed the absence of p53 activation and induction of its transcriptional target p21^{Cip1} following IR in the p53^{kd} cells. Quantification of the Cdc6 reduction in control BJ cells revealed that Cdc6 protein level was reduced to 10-30% following 2 Gy of irradiation, whereas no reduction in cdc6 level was observed in p53kd cells (data not shown). These results demonstrate that the p53 pathway controls Cdc6 protein level in human cells in response to DNA damage.

Enhanced Cdc6 proteolysis following DNA damage

As a transcription factor, p53 can directly repress gene expression (Nakade et al., 2004) and may therefore switch-off Cdc6 transcription when activated. However, no changes in Cdc6 mRNA level after IR were found by northern blot analysis (Fig. 2A). Furthermore, Cdc6 protein expressed from a heterologous CMV promoter was reduced after DNA damage to a similar extent as the endogenous protein (data not shown). These results indicate that the IR-induced Cdc6 down-regulation occurs at the post-transcriptional level.

Next, we tested whether the rapid decrease in Cdc6 protein level after IR is a result of increased proteolysis. We used the protein-synthesis inhibitor cycloheximide (CHX) to monitor Cdc6 half-life. To allow proper cell cycle response to DNA damage, CHX was added to cells 2 hours after the IR treatment. (If CHX was added right after the radiation treatment, Cdc6 stability remained unchanged following IR, supplementary Fig. 2). In MCF-7 cells, Cdc6 protein level decreased more rapidly in irradiated cells compared to non-irradiated cells (Fig. 2B) and quantification analysis showed that the half-life of Cdc6 was reduced from 90 min to 30 min upon IR treatment (supplementary Fig. 3A). Also in synchronized G1/S primary BJ fibroblasts we found that the half-life of Cdc6 protein is reduced from 25 min to 12.5 min following DNA damage (Fig. 2C and supplementary Fig. 3B). Since Cdc6 down-regulation following IR appeared to be p53-dependent, we monitored Cdc6 protein stability in control and p53^{kd} MCF-7 cells 2 hours after IR (Fig. 2D and supplementary Fig. 3C). Indeed, Cdc6

maintained its stability following IR in p53kd MCF-7 cells (half-life 90 min) compared to control irradiated cells (half-life 25 min). The protein stability of cyclin D1 following IR was not increased by the reduced level of p53, thereby showing the specificity of Cdc6 stabilization (Fig. 2D). Last, this p53-dependent Cdc6 degradation was mediated by the proteasome since it was abrogated when MCF-7 cells were irradiated in the presence of the proteasome inhibitor MG-132 (Fig. 2E). Taken together, these results demonstrate that the proteasome machinery mediates the accelerated proteolysis of Cdc6 following genotoxic stress and that Cdc6 protein abundance following IR is mainly controlled by p53.

Enhanced destruction of Cdc6 following DNA damage is mediated by the E3-ligase APC^{Cdh1}

In early G1, the N-terminal KEN and RxxL (D-box) destruction motifs of Cdc6 (Fig. 3A) mediate its degradation by the E3-ligase anaphase-promoting complex (APC) (Petersen et al., 2000). To determine whether Cdc6 destruction following IR involves the APC, we suppressed the expression of Cdh1 and Cdc20 by RNAi. Both proteins act as substrate recognition and activating modules for the APC (Reed, 2003). Inhibition of Cdh1 expression completely abrogated IR-induced Cdc6 destruction, whereas the Cdc20 knockdown had no effect (Fig. 3B). The reduced expression of both Cdh1 and Cdc20

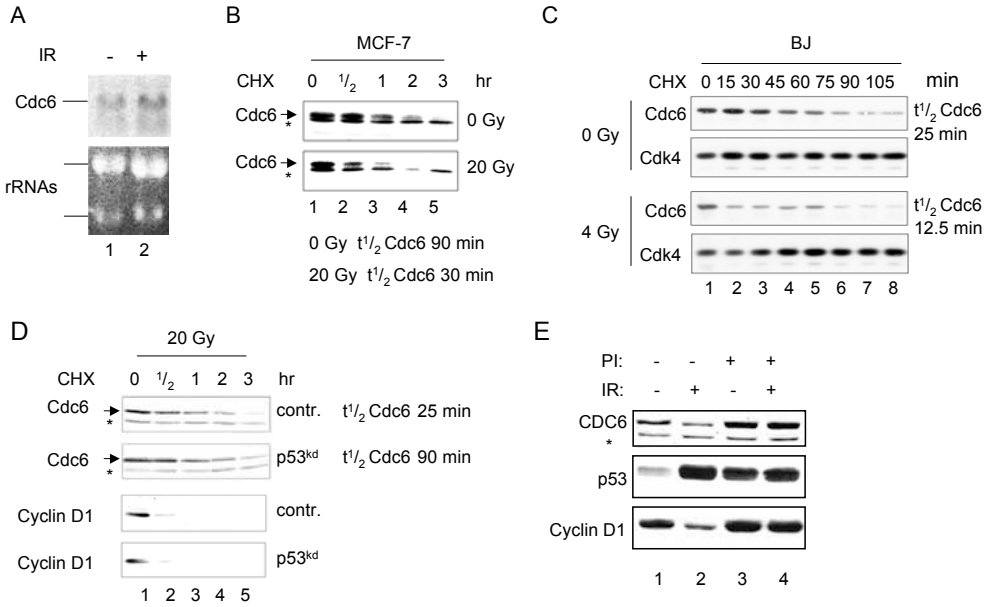


Figure 2. Increased Cdc6 proteolysis following DNA damage. A MCF-7 cells were subjected to IR treatment (20 Gy) and collected for northern blot analysis 4 hours later. Blots were hybridized with a Cdc6 cDNA probe to detect Cdc6 mRNA. **B** Irradiated (20Gy) or non-irradiated MCF-7 cells were treated with cycloheximide (CHX) 2 hours after IR. Cells were harvested at the indicated time-points after CHX addition and immuno-blotted against Cdc6. Asterisks indicate background bands. Half-life values were determined by quantification analysis **C** Serum starved BJ cells were released for 13 hours, treated as described above and immuno-blotted to detect Cdc6 and CDK4. Quantification of Cdc6 protein level was used to determine the half-life. **D** pRS and pRS-p53^{kd} cells were irradiated and treated with CHX 2 hours later. Cells were harvested at the indicated time-points and quantifications of half-life were calculated **E** MCF-7 cells were treated with or without 20 Gy irradiation (IR) in the presence or absence of proteasome inhibitors (PI). PI was added 30 min prior to the IR treatment. WCE were immuno-blotted against Cdc6, p53 and cyclin D1. A cross-reacting band (asterisk) was used as loading control.

was not accompanied with any significant change in cell cycle profile or p53 activation following IR (Supplementary Fig. 4A and data not shown).
The involvement of the APC^{Cdh1} in the p53-dependent regulation of Cdc6 suggests that also the KEN or the RxxL destruction motifs play a role in IR-induced Cdc6 destruction. Therefore, we constructed Cdc6-GFP chimeras with mutations in these destruction motifs. Similar to endogenous Cdc6, the stability of the transfected Cdc6-GFP fusion protein is reduced upon IR treatment (Fig. 3C lanes 1-4 and quantified in Fig. 3D). Mutating the KEN motif to AAA (KENmut) or the RxxL motif to AxxA (RxxLmut) partly prevented IR-induced Cdc6 destruction (lanes 5-12 and Fig. 3d). However, mutating both destruction motifs (R+K) completely abrogated Cdc6 destruction in response to IR (lanes 13-16). This demonstrates that both the KEN and the RxxL destruction motifs are involved in the p53-dependent Cdc6 destruction following IR through the APC^{Cdh1}.

Inhibition of CDK2 activity results in enhanced Cdc6 proteolysis

Activation of the p53 transcription factor following genotoxic stress results in up-regulation of the cell cycle inhibitor p21^{Cip1}. We therefore asked whether p21^{Cip1} is required for the p53-dependent destruction of Cdc6 and transfected MCF-7 cells with knockdown constructs targeting p21^{Cip1,} p27^{Kip1} (a homologue of p21^{Cip1} that is not regulated by p53) and p53. Suppression of p21^{Cip1} expression, but not p27^{Kip1}, completely abrogated Cdc6 destruction after IR (Fig. 4A lanes, 9-16). This effect was in correlation with CDK2 activity, as p21^{Cip1}, but not p27^{Kip1},

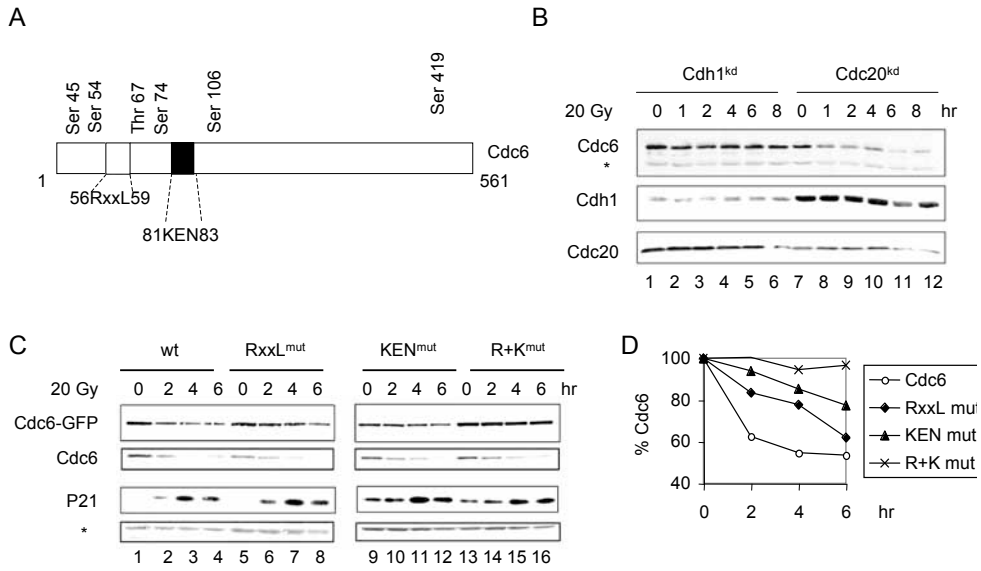


Figure 3. Enhanced destruction of Cdc6 following DNA damage is mediated by the E3-ligase APC^{Cdh1}. A A schematic representation of the Cdc6 protein with its five putative CDK2 N-terminal phosphorylation sites and the APC destruction boxes; RxxL and KEN motifs. **B** MCF-7 cells were transiently electroporated with pSUPER (pS) and RNAi constructs that target Cdh1 and Cdc20. Cells were subjected to IR treatment 60 hours after transfection and subsequently harvested for immuno-blotting analysis to detect Cdc6, Cdh1 and Cdc20 at the indicated time points. The asterisk marks a background band. **C** The same experimental settings as in b, only that cells were electroporated with Cdc6-GFP chimera (wt) or mutants of the RxxL and the KEN-box destruction motifs. R+K contains mutations in both the RxxL and KEN-box motif. WCE were immuno-blotted against Cdc6 and p21. The asterisk indicates a background band and shows equal loading. **D** Quantification analysis of the different Cdc6 proteins shown in Figure 3C. Mut., mutant.

knockdown cells maintained significant CDK2 activity following IR treatment (Supplementary. Fig. 4b). The protection from IR-induced destruction of Cdc6 in cells with suppressed expression of $p21^{Cip1}$ was comparable to that observed in $p53^{kd}$ cells (Fig. 4A, lanes 4-12). This effect was specific to Cdc6 as the enhanced degradation of cyclin D1, which is p53-independent, was still sustained in the $p21^{Cip1}$ knockdown cells (data not shown).

In response to IR, accumulation of $p21^{Cip1}$ inhibits CDK2 activity. It was described that mammalian Cdc6 is phosphorylated in vivo at three N-terminal sites (S54, S74 and S106) (Jiang et al., 1999), which can be phosphorylated by Cyclin E/CDK2 complex in vitro (Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999). To address the role of CDK2 in regulating Cdc6 stability, we treated cells expressing the Cdc6-luciferase chimera protein with increasing amounts of the CDK2 inhibitor roscovitin and monitored luciferase activity 1 hour later. Roscovitin treatment resulted in rapid reduction in luciferase activity suggesting a direct connection between CDK2 activity and Cdc6 protein stability (Fig. 4B). In a second experiment, we transfected MCF-7 cells with control, pS-p53kd or pS-$Cdh1^{kd}$ RNAi vectors, treated

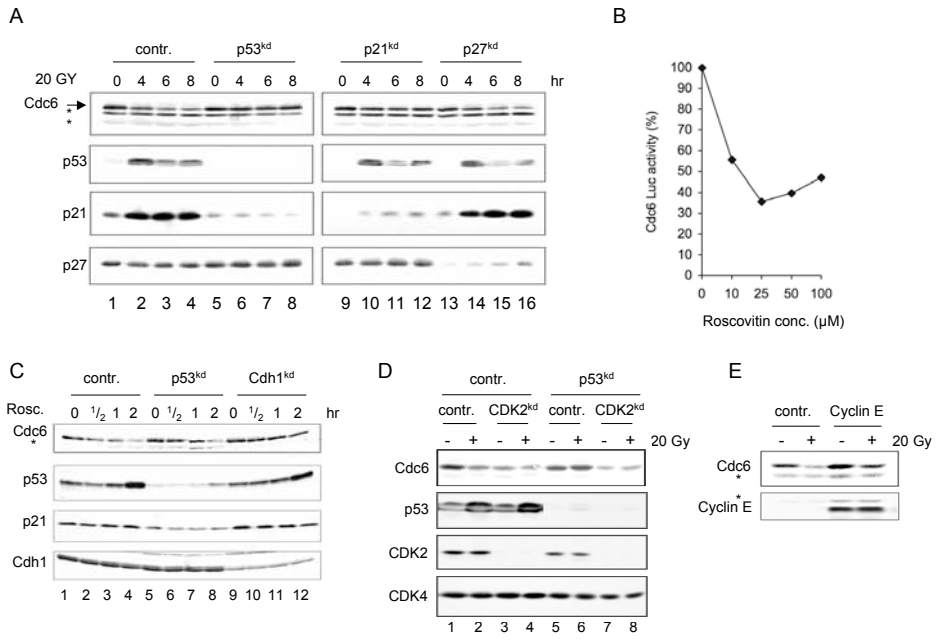


Figure 4. Inhibition of CDK2 activity results in enhanced Cdc6 proteolysis. A MCF-7 cells were electroporated with pS, pS-$p53^{kd}$, pS-$p21^{kd}$ and pS-$p27^{kd}$ and subjected to IR treatment 60 hours after transfection. WCE were prepared at indicated times-points and immuno-blot analysis was performed to detect protein levels of Cdc6, p53, p21 and p27. Asterisks indicate cross-reacting bands. **B** MCF-7 cells expressing the Cdc6-luciferase chimera protein were treated with the indicated amounts of Roscovitin and harvested for luciferase analysis 1 hour later. **C** MCF-7 cells were electroporated with a control construct and RNAi constructs targeting p53 and Cdh1 and treated with Roscovitin 60 hours later. WCE were prepared several time-points after treatment and immuno-blot analysis was performed to detect the indicated proteins. **D** MCF-7 and MCF-7-p53kd cells were electroporated with CDK2-siRNA oligos, irradiated 60 hours later (20 Gy) and harvested 4 hours later. WCE were made and immuno-blotted with the indicated antibodies. **E** MCF-7 cells were electoporated with cyclin E and control expression vectors. WCE were made and immuno-blotted to detect Cdc6 and Cyclin E proteins. Asterisks indicate cross-reacting bands.

them with roscovitin and harvested the cells for immuno-blot analyses at various time-points. Figure 4c shows that also endogenous Cdc6 was rapidly reduced upon roscovitin treatment in control-transfected cells (lanes 1-4). In addition, Cdc6 was down-regulated upon roscovitin treatment in $p53^{kd}$ cells (lanes 5-8), although these cells are impaired in their ability to enhance degradation of Cdc6 following IR (Fig. 1E and 4A). This indicates that CDK2 is downstream of p53 in the IR-induced Cdc6 degradation. In contrast, Cdc6 remained stable upon roscovitin treatment in cells with suppressed Cdh1 levels (lanes 9-12), suggesting that the APC^{Cdh1} is downstream of CDK2 in this pathway. Notably, this experiment also demonstrates that the resistance of Cdc6 to IR in $p53^{kd}$ cells is not a result of increased mRNA levels.

To further substantiate the role of CDK2 in Cdc6 destruction following DNA damage, we assessed the effect of IR on Cdc6 protein level in MCF-7 and MCF-7-$p53^{kd}$ cells transfected with control or CDK2 siRNAs. In agreement with the observed Cdc6 protein reduction upon roscovitin treatment, Cdc6 protein level was reduced in response to low CDK2 levels in both control and $p53^{kd}$ cells (Fig. 4D, lanes 1, 3, 5 and 7), Indicating that CDK2 plays an essential role in maintaining Cdc6 protein level. Furthermore, Cdc6 protein was down-regulated in control cells within 2 hours after irradiation, whereas it remained unaffected in irradiated $CDK2^{kd}$ cells (Fig. 4D, lanes 1-4). As expected, no change in Cdc6 levels was observed in $p53^{kd}$ cells after irradiation (lanes 5-8). Last, our model predicts that ectopic activation of CDK2 should confer significant resistance to Cdc6 from the enhanced destruction following DNA damage. To test this hypothesis we over-expressed cyclin E, an activator of CDK2, in cells that were treated with IR. Figure 4e shows that cyclin E over-expression results in accumulation of Cdc6 in untreated cells and significant resistance to DNA damage effects. Altogether, these results demonstrate the direct requirement of CDK2 inhibition in the p53-dependent destruction of Cdc6 protein after IR.

Phosphorylation of serine-54 by CDK2 inhibits p53-dependent Cdc6 proteolysis

It has been proposed that phosphorylation of Cdc6 results in translocation from the nucleus to the cytosol (Delmolino et al., 2001; Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998). In stark contrast, our results indicate that phosphorylation of Cdc6 by CDK2 leads to its stabilization. Therefore, we aimed to map this stabilizing phosphorylation site by mutating putative CDK2 phosphorylation sites to aspartic acid, thereby mimicking their phosphorylated state. The N-terminus of Cdc6 harbors three sites that can be phosphorylated in vivo, serines 54, 74 and 106 (Jiang et al., 1999). We chose to mutate S54 and S74, as they are the sites nearest to the destruction motifs (Fig. 3A). We made Cdc6-GFP fusion constructs that contain S54D and S74D mutations and examined them in a transient transfection assay in MCF-7 cells. Mutation of S54 to aspartic acid (S54D) completely abrogated the p53-induced Cdc6 destruction, whereas the S74D mutant was degraded to an extent that was comparable to wt Cdc6-GFP and to the endogenous protein (Fig 5A. and supplementary Fig. 4C). Importantly, the expression of the S54D mutant did not affect the upstream p53 response, as $p21^{Cip1}$ levels accumulated in these cells after DNA damage (Fig 5A). This result suggests that the phoshorylation of S54 is critical for Cdc6 stability in non-stressed cells. To examine this point we mutated serine 54 to alanine (S54A), a change mimics unphosphorylated state of Cdc6 at this position. Introduction of S54A into cells as a GFP chimera protein resulted in very little expression compared with wt Cdc6 and its stable S54D mutant (Fig. 5B, lanes 1, 3 and 5). Treatment with the proteasome inhibitor MG-123 resulted in the accumulation of both wt and S54A Cdc6 whereas S54D remained unchanged (lanes 2, 4 and 6). This result indicates that in non-stressed cells, phosphorylation of at serine 54 protects Cdc6 from rapid destruction.

To further substantiate the connection between S54 phosphorylation and the p53-

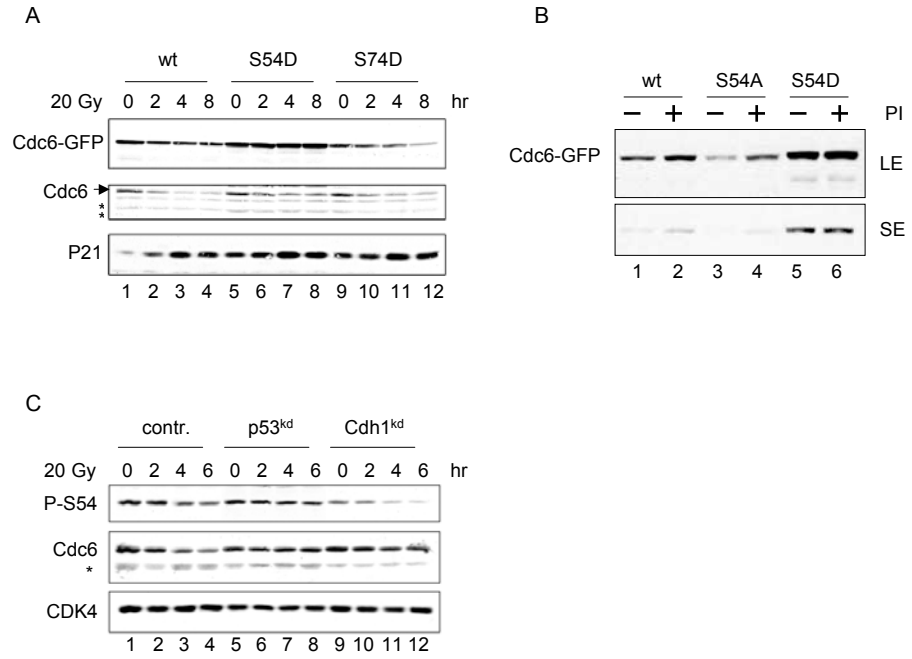


Figure 5. Phosphorylation of serine-54 by CDK2 inhibits p53-dependent Cdc6 proteolysis. A MCF-7 cells were electroporated with wt Cdc6-GFP or S54D and S74D Cdc6-mutant constructs and the experiment was performed as described in Figure 3C. **B** MCF-7 cells were electroporated with wt, S54A and S54D Cdc6-GFP constructs. Forty eight hrs after transcfection, the proteasome inhibitor MG-123 (PI) was added, WCE were separated on 10 SDS-PAGE and immuno-blotted to detect Cdc6. For comparison, long and short (LE and SE, respectively) exposures are shown. **C** MCF-7 cells were electroporated with pS, pS-p53^{kd} and pS-Cdh1^{kd}, irradiated (20 Gy) and harvested at the indicated times. WCE were immuno-blotted with anti-phospho-S54-Cdc6, Cdc6 or control Cdk4 antibodies. Asterisk marks a non-specific band.

dependent destruction of Cdc6, we used an anti-phospho-S54 specific Cdc6 antibody and monitored the phosphorylation state of endogenous Cdc6. We transfected MCF-7 cells with pS, pS-p53^{kd} or pS-Cdh1^{kd} constructs and subjected them to IR treatment. Importantly, we detected phosphorylated-S54 Cdc6 in whole cell extracts from cycling control-transfected cells and these levels were reduced following IR (Fig. 5C, lanes 1-4). In the cells transfected with either pS-p53^{kd} or pS-Cdh1^{kd}, Cdc6 protein level remained high following DNA damage, however the level of phosphorylated S54 was only stable after IR in the p53kd cells (lanes 4-12). In the cdh1^{kd} cells the level of S54 phosphorylated Cdc6 was still reduced. This result is in full agreement with a pathway consisting of upstream p53-dependent regulation of Cdc6 stability by phosphorylation and downstream APC^{Cdh1}-mediated degradation. Last, we examined the effect of cyclin E over-expression or Cdh1^{kd} on the stability of S54D-Cdc6 in the absence of DNA damage. Supplementary Fig. 4D shows that S54D mutant was unaffected by both treatments, suggesting that S54 phosphorylation is a major determinant of Cdc6 stability. Collectively, these results reveal that inhibition of S54 phosphorylation is the mechanism by which p53 regulates Cdc6 stability following DNA damage. The importance of this pathway is further extended by the fact that both Cdc6 S54 and the RxxL and KEN destruction boxes are evolutionary conserved in mice.

p53-dependent regulation of Cdc6 protein stability controls cellular proliferation

Apart from its role in DNA damage responses, p53 regulates cellular proliferation in non-stressed cells as well, since its suppression by RNAi accelerates proliferation of primary human cells (Voorhoeve and Agami, 2003) and MCF-7 cells (data not shown). Therefore, we examined the effect of reduced p53 expression on the cell cycle profile of MCF-7 cells. Also here we observed that suppression of endogenous p53 expression resulted in more proliferating cells (6 % increase in S-phase and 10% decrease in G1, Fig. 6A). This higher fraction of S-phase population could be due to earlier assembly of the pre-RC in G1. Since Cdc6 is regulated by p53 in the DNA damage response, we examined the level of Cdc6 in proliferating cells with suppressed p53 expression. Interestingly, we found a higher level of Cdc6 protein in pRS-p53^{kd} cell extracts compared to control cells (Fig. 6B). This suggests that Cdc6 is more stable in p53-knockdown cells. To test this directly, we treated MCF-7 cells with CHX and observed that the protein turnover of Cdc6 is slower in the p53^{kd} cells (115 min),

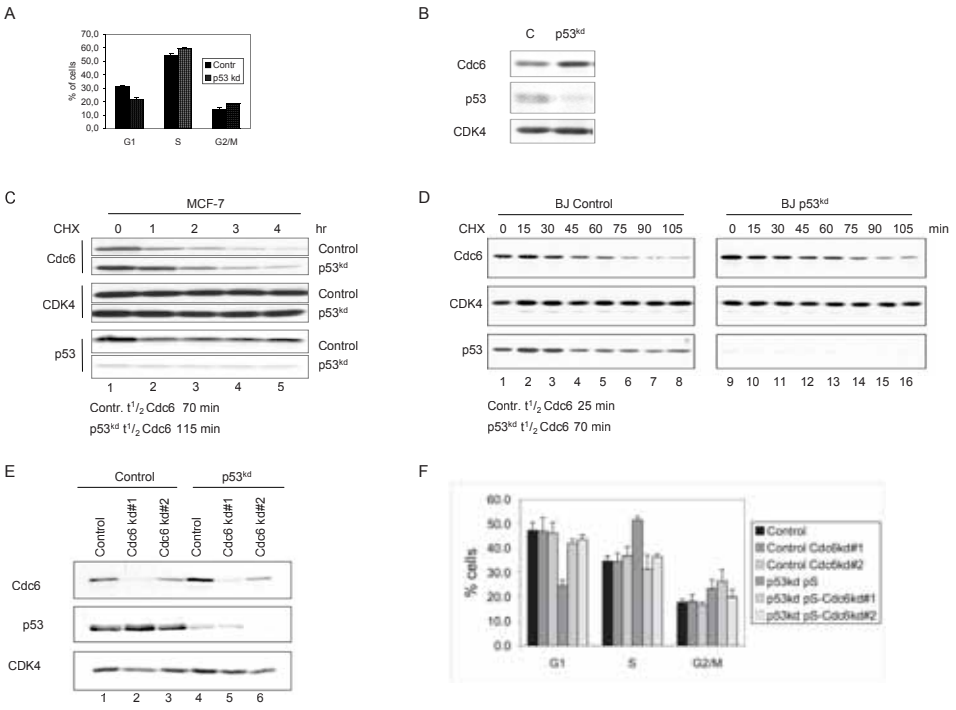


Figure 6. p53-dependent regulation of Cdc6 protein stability controls cellular proliferation. A Cell cycle analysis of polyclonal control or p53-knockdown MCF-7 cells. BrdU-labeled cells were analyzed by fluorescence activated cell sorting (FACS). The result is the mean of three independent experiments. **B** Whole cell lysates of control and p53^{kd} MCF-7 cells were immuno-blotted to detect Cdc6, p53 and CDK4 protein. **C** pRS and pRS-p53^{kd} MCF-7 cells were treated with CHX as described in Fig 2B. WCE were labeled with Cdc6, p53 and control CDK4 antibodies. **D** Serum starved BJ cells that were released for 13 hours were treated as described in Fig. 2C **E** The polyclonal pools of pRS and pRS-p53^{kd} cells were electroporated with Cdc6 knockdown constructs (#1 and #2), harvested after 72 hours and immuno-blotted to detect Cdc6, p53 and as loading control CDK4 proteins. **F** The same experimental setting as in Fig. 5E, but cells were subjected to flow cytometric analysis. The result is the mean of three independent experiments.

compared to control cells (70 min, Fig. 6C). Control immuno-blot analyses of CDK4 and p53 showed equal loading and the extent of the p53 knockdown. The same qualitative result was also seen in synchronized G1-phase primary BJ cells where Cdc6 stability was raised from 25 min in normal cells to 70 min in the BJ-p53^{kd} cells (Fig. 6D). To investigate whether Cdc6 stability is important for the increased proliferation of p53^{kd} cells, we transfected control and stable polyclonal p53^{kd} cells with two effective knockdown constructs targeting Cdc6. Immuno-blot analysis showed reduction of Cdc6 expression in pS-Cdc6^{kd} transfected cells in both cell types (Fig 6E). Notably, the Cdc6^{kd} had no significant effect on p53 levels. Flow cytometric analysis of the same cell populations repeatedly revealed that inhibition of Cdc6 expression altered the cell cycle profile of only p53^{kd} cells by increasing the fraction of cells in G1 and decreasing the fraction of cells in S phase (Fig. 6F). In contrast, no significant change was detected in the cell cycle profile of the control MCF-7 cells. This indicates that the increased Cdc6 levels observed in the absence of p53 are required for the enhanced proliferation of these cells. Collectively, our data implies that Cdc6 is part of a p53 protein-network that regulates cellular proliferation.

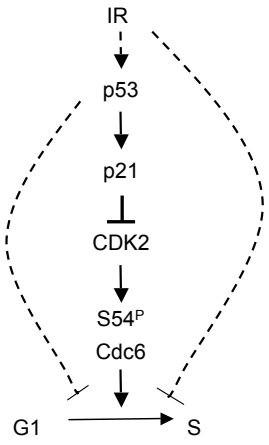


Figure 7. Model. A schematic model depicts the p53 pathway that regulates Cdc6, and thereby S-phase entry, both under normal tissue culture conditions (solid lines) and following IR (dashed lines).

Discussion

In response to genotoxic stress, cells activate checkpoints that prevent DNA replication and cell cycle progression (Bartek and Lukas, 2001). Our results establish a novel connection between DNA damage and the formation of preRCs in G1 phase (Fig 7). Cdc6, an essential protein of the preRC, is rapidly degraded upon genotoxic stress. The enhanced degradation of Cdc6 following IR is not complete, which can be a result of a small population of Cdc6 that is protected from degradation or that the enhanced is restricted to certain phases of the cell cycle. A central role in this destruction pathway is played by the p53 tumor suppressor gene, which controls the rate by which Cdc6 is degraded by the APC^{Cdh1}. Cdc6 is a licensing factor of DNA replication and once bound to the ORC-complex at origins of replication it recruits together with Cdt1 the MCM-complex. Our data implies that IR-induced down-regulation of Cdc6 contributes to a checkpoint that prevents DNA replication in cells with damaged DNA. Interestingly, DNA damage also induces the destruction of the licensing factor Cdt1, but in a p53-independent manner (Higa et al., 2003; Hu et al., 2004; Kondo et al., 2004). Therefore, we propose that the destruction of both Cdc6 and Cdt1 acts in concert to execute a tight regulation of pre-RC assembly following genotoxic stress.

We also found that p53-dependent regulation of Cdc6 protein stability plays a role in non-genotoxic stressed cells. Under normal tissue culture conditions Cdc6 protein stability is increased in p53^{kd} cells. We show that reducing Cdc6 protein level by siRNAs in p53^{kd} cells results in a reduction of replicating cells and a cell cycle distribution that is comparable to cells with functional p53. However, reducing Cdc6 protein levels in control MCF-7 cells did not affect their proliferation, which suggests that cells

without functional p53 are more susceptible to reduction in Cdc6 levels.

It is generally thought that initiation of DNA replication is regulated in a two-step process to ensure that the DNA is replicated only once in each cell cycle (Bell and Dutta, 2002). First, pre-replication complexes are assembled at origins of replication and second the origins are fired. Notably, the regulation of these events varies in different organisms. In mammals, CDKs appear to have an inhibiting role in pre-RC assembly. For both Cdt1 and Orc1 it has been proposed that they are targeted for proteolysis in a CDK-dependent manner (Liu et al., 2004; Mendez et al., 2002; Nishitani et al., 2004). In addition, it has been proposed that cyclinA/CDK2 activity results in Cdc6 translocation from the nucleus to the cytosol and subsequent proteolysis (Delmolino et al., 2001; Herbig et al., 2000; Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998). In contrast to these observations, we found that phosphorylation of Cdc6 by cyclinE/CDK2 results in stabilization of the protein. We established that one amino acid (serine 54) mediates p53-induced Cdc6 destruction. Once serine 54 is phosphorylated by CDK2, Cdc6 becomes more resistant to APC^{Cdh1} degradation. Our results are in agreement with a report showing that only ectopically expressed Cdc6 is translocated to the cytosol and that serine 54 phosphorylated Cdc6 remains chromatin bound in S-phase (Alexandrow and Hamlin, 2004). Therefore, we propose that phosphorylation of S54 by cyclinE/CDK2 is a novel stabilizing modification of the Cdc6 protein, which can be modulated by the p53 pathway.

Our data implicates that cyclinE/CDK2 activity might have a positive role in DNA replication licensing by stabilizing Cdc6. This is consistent with in vitro studies, which show that cyclin E cooperates with Cdc6 to make mammalian G1 nuclei competent for replication (Coverley et al., 2002). Furthermore, Cdc6 and cyclin E appear to have a synergistic effect on inducing S-phase entry upon co-transfection in human cells (Cook et al., 2002; Hateboer et al., 1998). In addition, it was shown that overexpression of cyclin E could stabilize Cdc6 protein during megakaryocytic endoreplication (Bermejo et al., 2002). In these cells cyclin E could not stabilize an unphosphorylatable Cdc6 mutant in which all five N-terminal putative CDK2 phosphorylation sites were mutated to alanines. Significantly, it was also shown that cyclin E ablation in mice results in DNA replication defects (Geng et al., 2003; Parisi et al., 2003). Cells that re-enter the cell cycle from quiescence fail to load MCM2-7 and endoreplication in trophoblast giant cells and megakaryocytes is impaired. In view of our results it will be interesting to examine whether these defects are a consequence of reduced Cdc6 stabilization in these cells.

Both CDKs and the APC are key regulators of initiation of DNA replication (Diffley, 2004). Intriguingly, proteolysis of both the licensing factor Cdc6 and geminin, the inhibitor of the licensing factor Cdt1 are regulated by the APC (McGarry and Kirschner, 1998). It is at present unclear how the APC-dependent degradation of an activator and an inhibitor of DNA replication are coordinated to ensure that Cdc6 and Cdt1 are present in the same time frame to allow for efficient pre-RC assembly. Our results shed new light on this. Similar to geminin, Cdc6 is degraded in the end of G2/M and in early G1 phase of the cell cycle (Petersen et al., 2000). We propose that the increased activity of cyclin E/CDK2 during G1 protects Cdc6 from APC^{Cdh1}-dependent destruction, whereas geminin is still degraded. This provides the cells with a time frame in which both Cdt1 and Cdc6 can bind to the origins and recruit the MCM-complex to assemble the pre-RC.
In conclusion, our findings give novel insights in the regulation of Cdc6 in the assembly of pre-RCs. We established that phosphorylation of Cdc6 at S54 by cyclinE/CDK2 stabilizes the protein by protecting it from APC^{Cdh1}-mediated destruction. The rapid and p53-dependent destruction of Cdc6 in response to DNA damage suggests that it guards the genome for mutations by blocking new origin firing.

Methods

Materials and antibodies

Irradiation was performed with a 2 x 415 Ci 137Cs Source. The proteasome inhibitor MG-132 was purchased from Sigma and used at a final concentration of 10 mM. Cycloheximide (Sigma) was used at a final concentration of 25 µg/ml and Roscovitin (Sigma) at 25µM. The siRNA oligos against luciferase and CDK2 were described (Elbashir et al., 2001; Tetsu and McCormick, 1999). The antibodies used in this report were directed against human Cdc6 (180.2), p-Cdc6 (Ser 54), p53 (DO-1), p21 (C-19), Cyclin D1 (M-20), CDK4 (C-22), Cdc20 (H-175) and CDK2 (M-20) from Santa Cruz. Further Kip1/p27 (Transduction Lab.), Cdh1 (Abcam) ORC1 (Abcam) and MCM2 (Abcam) antibodies were used.

Constructs

Cdc6 expression constructs were cloned by PCR in pcDNA3.1 vector (Invitrogen), pGL3 (Promega) or pEGFP-N3 (Clontech). Cdc6 mutants were generated by site directed mutagenesis using PCR and constructs were verified by DNA sequence analysis. We used pSuper (pS) constructs targeting p53, Cdh1 and Cdc20 that were described previously (Brummelkamp et al., 2002b). The other 19 nt target knockdown sequences used were:

$p21^{Cip1}$: GACCATGTGGACCTGTCAC

$p27^{Kip1}$: GGGCAGCTTGCCCGAGTTC

Cdc6#1: ATGTCCAAACCGTAACCTG

Cdc6#2: CCTATGCAACACTCCCCAT

For stable expression siRNAs constructs were cloned in the retroviral 480-puro vector (Voorhoeve and Agami, 2003).

Cell culture and transfection

MCF-7 and BJ cells were grown in DMEM and 10% fetal bovine serum. MCF-7 cells carrying the murine ecotropic receptor were infected with ecotropic retroviral supernatants as described (Brummelkamp et al., 2002a) to generate a polyclonal pool of cells. MCF-7 cells were transfected by electroporation as described (Agami and Bernards, 2000) and about 90% of transfection efficiency was obtained. Cells were electroporated and seeded, washed 16 hours later and harvested 48 hours after washing.

Western blot, cell cycle profile analysis and luciferase assay

For western blots, whole cell extracts were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Milipore). Western blots were developed using enhanced chemiluminescence (Amersham Bioscience, Inc.). Densitometric quantification of western blots was done by using Tina 2.09 software. For bromodeoxyuridine (BrdU) labeling, cells were incubated with 2.5 mM BrdU 40 min prior to harvesting. Cells were fixed ON at 4°C in ethanol. Fixed cells were treated 30 min at 37°C with RNase A (0.5 mg/ml), washed with PBS, incubated 20 min in 5M HCl/0.5% Triton solution and neutralized with 0.1 M Na2B4O7. Cells were sequentially stained with anti-BrdU antibodies (DAKO) and FITC-conjugated goat-anti-mouse antibodies (Molecular Probes). Prior to the flow cytometric analysis, cells were resuspended in 50µg/ml propidium iodide in PBS. In each assay 10,000 single cells were analyzed using the Cell Quest program (Becton Dickinson). Firefly luciferase and Renilla luciferase (internal control) activities were measured by employing the Dual Luciferase Reagent Assay Kit (Promega).

Northern blot analysis

RNA was isolated from cells using Trizol (Invitrogen) and northern blot analysis was performed exactly as described (Brummelkamp et al., 2002b) and membranes were hybridized with a probe made from the Cdc6 cDNA.

Acknowledgements

We thank Mathijs Voorhoeve for the p21 and p27 knockdown constructs and Judith Kaufmann for assistance during her student-project. We also thank Ingrid Kolfschoten, Eithan Zlotorynski, Mathijs Voorhoeve and Jacques Neefjes for critical reading of the manuscript and all members of the Agami lab for stimulating discussions. This work was supported by the Dutch Cancer Society (KWF) and the Centre for Biomedical Genetics.

Refences

Agami, R., and Bernards, R. (2000). Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. Cell 102, 55-66.

Alexandrow, M. G., and Hamlin, J. L. (2004). Cdc6 chromatin affinity is unaffected by serine-54 phosphorylation, S-phase progression, and overexpression of cyclin A. Mol Cell Biol 24, 1614-1627.

Aparicio, O. M., Weinstein, D. M., and Bell, S. P. (1997). Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell 91, 59-69.

Bartek, J., and Lukas, J. (2001). Mammalian G1- and S-phase checkpoints in response to DNA damage. Curr Opin Cell Biol 13, 738-747.

Bell, S. P., and Dutta, A. (2002). DNA replication in eukaryotic cells. Annu Rev Biochem 71, 333-374.

Bermejo, R., Vilaboa, N., and Cales, C. (2002). Regulation of CDC6, geminin, and CDT1 in human cells that undergo polyploidization. Mol Biol Cell 13, 3989-4000.

Brummelkamp, T. R., Bernards, R., and Agami, R. (2002a). Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell 2, 243-247.

Brummelkamp, T. R., Bernards, R., and Agami, R. (2002b). A system for stable expression of short interfering RNAs in mammalian cells. Science 296, 550-553.

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 282, 1497-1501.

Coleman, T. R., Carpenter, P. B., and Dunphy, W. G. (1996). The Xenopus Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts. Cell 87, 53-63.

Cook, J. G., Park, C. H., Burke, T. W., Leone, G., DeGregori, J., Engel, A., and Nevins, J. R. (2002). Analysis of Cdc6 function in the assembly of mammalian prereplication complexes. Proc Natl Acad Sci U S A 99, 1347-1352.

Coverley, D., Laman, H., and Laskey, R. A. (2002). Distinct roles for cyclins E and A during DNA replication complex assembly and activation. Nat Cell Biol 4, 523-528.

Coverley, D., Pelizon, C., Trewick, S., and Laskey, R. A. (2000). Chromatin-bound Cdc6 persists in S and G2 phases in human cells, while soluble Cdc6 is destroyed in a cyclin A-cdk2 dependent process. J Cell Sci 113 (Pt 11), 1929-1938.

Delmolino, L. M., Saha, P., and Dutta, A. (2001). Multiple mechanisms regulate subcellular localization of human CDC6. J Biol Chem 276, 26947-26954.

Diffley, J. F. (2004). Regulation of early events in chromosome replication. Curr Biol 14, R778-786.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494-498.

Falck, J., Mailand, N., Syljuasen, R. G., Bartek, J., and Lukas, J. (2001). The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature 410, 842-847.

Fei, P., and El-Deiry, W. S. (2003). P53 and radiation responses. Oncogene 22, 5774-5783.

Geng, Y., Yu, Q., Sicinska, E., Das, M., Schneider, J. E., Bhattacharya, S., Rideout, W. M., Bronson, R. T., Gardner, H., and Sicinski, P. (2003). Cyclin E ablation in the mouse. Cell 114, 431-443.

Hateboer, G., Wobst, A., Petersen, B. O., Le Cam, L., Vigo, E., Sardet, C., and Helin, K. (1998). Cell cycle-regulated expression of mammalian CDC6 is dependent on E2F. Mol Cell Biol 18, 6679-6697.

Herbig, U., Griffith, J. W., and Fanning, E. (2000). Mutation of cyclin/cdk phosphorylation sites in HsCdc6 disrupts a late step in initiation of DNA replication in human cells. Mol Biol Cell 11, 4117-4130.

Higa, L. A., Mihaylov, I. S., Banks, D. P., Zheng, J., and Zhang, H. (2003). Radiation-mediated proteolysis of CDT1 by CUL4-ROC1 and CSN complexes constitutes a new checkpoint. Nat Cell Biol 5, 1008-1015.

Hu, J., McCall, C. M., Ohta, T., and Xiong, Y. (2004). Targeted ubiquitination of CDT1 by the DDB1-CUL4A-ROC1 ligase in

response to DNA damage. Nat Cell Biol 6, 1003-1009.

Iliakis, G., Wang, Y., Guan, J., and Wang, H. (2003). DNA damage checkpoint control in cells exposed to ionizing radiation. Oncogene 22, 5834-5847.

Jiang, W., Wells, N. J., and Hunter, T. (1999). Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. Proc Natl Acad Sci U S A 96, 6193-6198.

Kondo, T., Kobayashi, M., Tanaka, J., Yokoyama, A., Suzuki, S., Kato, N., Onozawa, M., Chiba, K., Hashino, S., Imamura, M., et al. (2004). Rapid degradation of Cdt1 upon UV-induced DNA damage is mediated by SCFSkp2 complex. J Biol Chem 279, 27315-27319.

Liu, E., Li, X., Yan, F., Zhao, Q., and Wu, X. (2004). Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation. J Biol Chem 279, 17283-17288.

Mailand, N., and Diffley, J. F. (2005). CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. Cell 122, 915-926.

McGarry, T. J., and Kirschner, M. W. (1998). Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell 93, 1043-1053.

Mendez, J., and Stillman, B. (2000). Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. Mol Cell Biol 20, 8602-8612.

Mendez, J., Zou-Yang, X. H., Kim, S. Y., Hidaka, M., Tansey, W. P., and Stillman, B. (2002). Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. Mol Cell 9, 481-491.

Motoyama, N., and Naka, K. (2004). DNA damage tumor suppressor genes and genomic instability. Curr Opin Genet Dev 14, 11-16.

Nakade, K., Zheng, H., Ganguli, G., Buchwalter, G., Gross, C., and Wasylyk, B. (2004). The tumor suppressor p53 inhibits Net, an effector of Ras/extracellular signal-regulated kinase signaling. Mol Cell Biol 24, 1132-1142.

Nishitani, H., Lygerou, Z., and Nishimoto, T. (2004). Proteolysis of DNA replication licensing factor Cdt1 in S-phase is performed independently of geminin through its N-terminal region. J Biol Chem 279, 30807-30816.

Parisi, T., Beck, A. R., Rougier, N., McNeil, T., Lucian, L., Werb, Z., and Amati, B. (2003). Cyclins E1 and E2 are required for endoreplication in placental trophoblast giant cells. Embo J 22, 4794-4803.

Pelizon, C. (2003). Down to the origin: Cdc6 protein and the competence to replicate. Trends Cell Biol 13, 110-113.

Petersen, B. O., Lukas, J., Sorensen, C. S., Bartek, J., and Helin, K. (1999). Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. Embo J 18, 396-410.

Petersen, B. O., Wagener, C., Marinoni, F., Kramer, E. R., Melixetian, M., Lazzerini Denchi, E., Gieffers, C., Matteucci, C., Peters, J. M., and Helin, K. (2000). Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. Genes Dev 14, 2330-2343.

Pfleger, C. M., and Kirschner, M. W. (2000). The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. Genes Dev 14, 655-665.

Pfleger, C. M., Lee, E., and Kirschner, M. W. (2001). Substrate recognition by the Cdc20 and Cdh1 components of the anaphase-promoting complex. Genes Dev 15, 2396-2407.

Reed, S. I. (2003). Ratchets and clocks: the cell cycle, ubiquitylation and protein turnover. Nat Rev Mol Cell Biol 4, 855-864.

Saha, P., Chen, J., Thome, K. C., Lawlis, S. J., Hou, Z. H., Hendricks, M., Parvin, J. D., and Dutta, A. (1998). Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. Mol Cell Biol 18, 2758-2767.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer 3, 155-168.

Stoeber, K., Mills, A. D., Kubota, Y., Krude, T., Romanowski, P., Marheineke, K., Laskey, R. A., and Williams, G. H. (1998). Cdc6 protein causes premature entry into S phase in a mammalian cell-free system. Embo J 17, 7219-7229.

Takisawa, H., Mimura, S., and Kubota, Y. (2000). Eukaryotic DNA replication: from pre-replication complex to initiation complex. Curr Opin Cell Biol 12, 690-696.

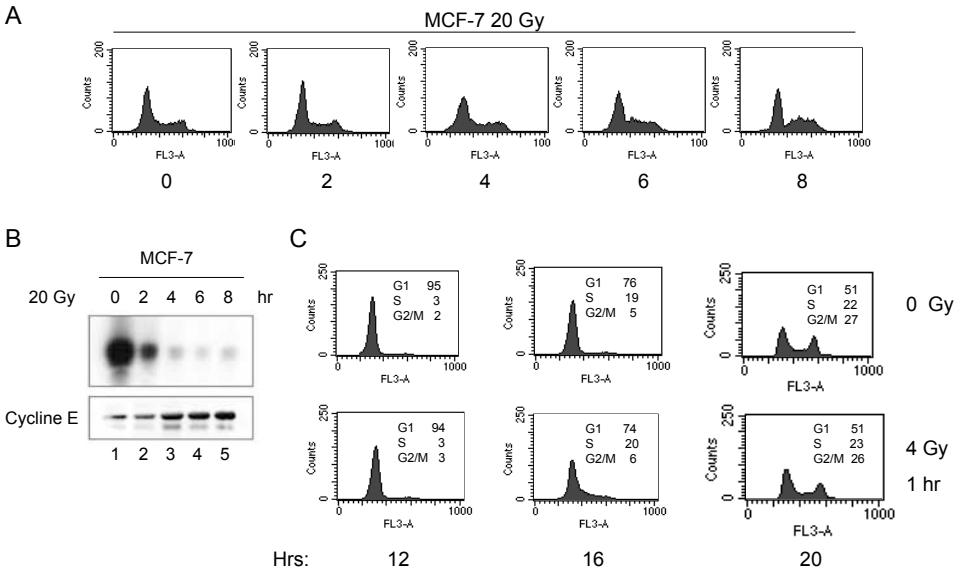
Tanaka, T., Knapp, D., and Nasmyth, K. (1997). Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. Cell 90, 649-660.

Tetsu, O., and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398, 422-426.

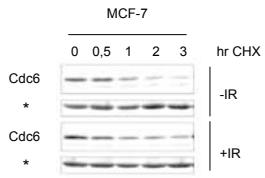
Vaziri, C., Saxena, S., Jeon, Y., Lee, C., Murata, K., Machida, Y., Wagle, N., Hwang, D. S., and Dutta, A. (2003). A p53-dependent checkpoint pathway prevents rereplication. Mol Cell 11, 997-1008.

Voorhoeve, P. M., and Agami, R. (2003). The tumor-suppressive functions of the human INK4A locus. Cancer Cell 4, 311-319.

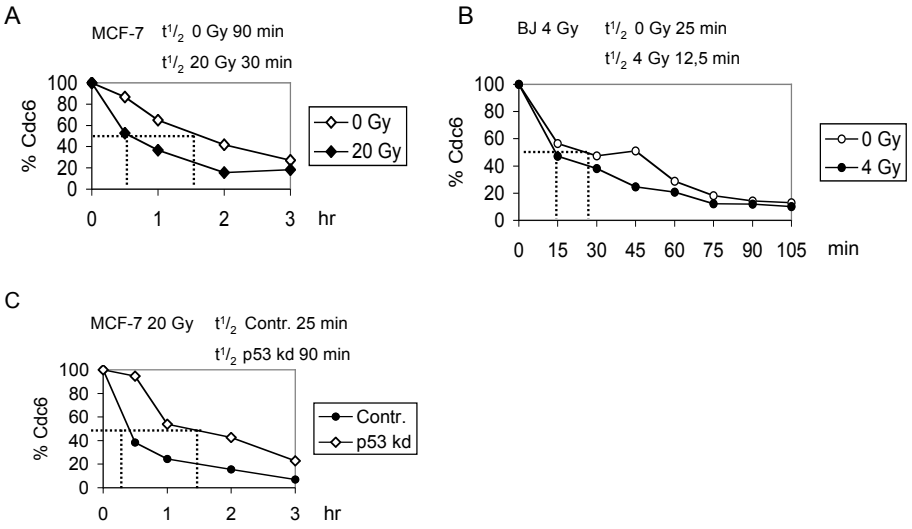
Yan, Z., DeGregori, J., Shohet, R., Leone, G., Stillman, B., Nevins, J. R., and Williams, R. S. (1998). Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells. Proc Natl Acad Sci U S A 95, 3603-3608.



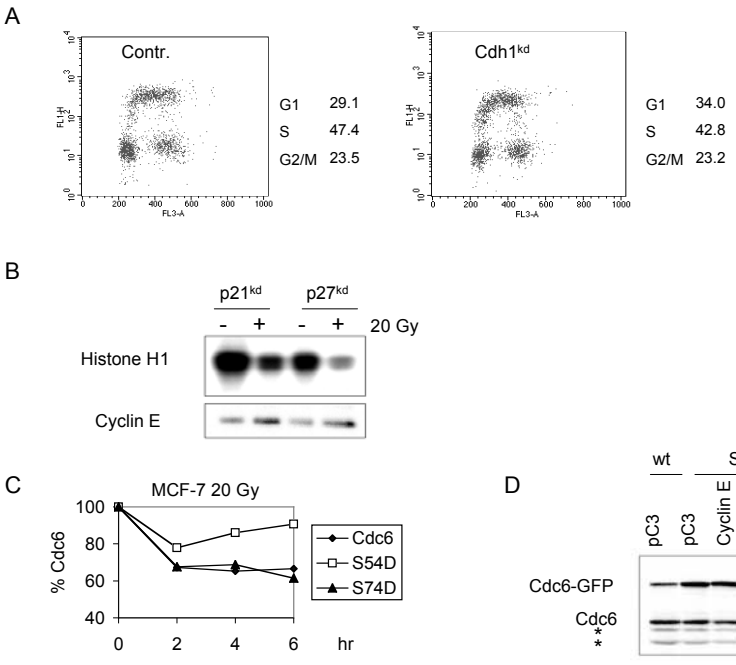
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4

Chapter 3

CDK-dependent stabilization of Cdc6: Linking growth and stress signals to activation of DNA replication

Anja M. Duursma and Reuven Agami

Cell Cycle. 2005. 4 (12): 1725-8

CDK-dependent stabilization of Cdc6: Linking growth and stress signals to activation of DNA replication

Anja M. Duursma and Reuven Agami*

The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
*Communicating author

Cyclin-dependent kinases (CDKs) play a crucial role in cell cycle progression by controlling the transition from G1 phase into S phase where DNA is replicated. Key to this transition is the regulation of initiation of DNA replication at replication origins. CDKs are thought to regulate origins of replication both positively and negatively by phosphorylating replication proteins at origins. Several replication proteins that are potentially negatively regulated upon CDK phosphorylation have been identified. However, the mechanism by which CDKs activate replication is currently less well understood. New observations revealing that the initiation protein Cdc6 is stabilized by CDK2-dependent phosphorylation may give more insight in this process.

Origin determination

Prior to cell division, the eukaryotic genome is duplicated during S-phase of the cell cycle. To ensure that only one single round of DNA replication occurs per cell cycle, a strict regulation is imposed. Replication initiates at specific sites that are distributed throughout the genome, called origins of replication (Bell and Dutta, 2002; Cvetic and Walter, 2005). These origins were initially identified in Saccharomyces cerevisiae as autonomously replicating sequences (ARS). Further characterization revealed an 11 base-pair (bp) ARS consensus sequence as the most important element of origins in S. cerevisiae. Interestingly, no elements analogous to ARS sequences were identified in Schizosaccharomyces pombe. The domains that control replication in S. pombe appear to be larger in size (500-1500 bp) and they consist of multiple elements, which contribute to origin activity. These elements are heterogeneous, although they contain an unusually high A-T content. At present, the complexity of the metazoan genomes seems to hamper the identification of replication and characterization origins and therefore no specific initiation sequence could be uncovered. However, several studies suggested that origin localization is determined by both epigenetic modifications and transcriptional activity (Antequera, 2004; MacAlpine et al., 2004). Further progress awaits a large-scale origin characterization in mammalian genomes.

Origin recognition

The lack of knowledge of metazoan replication origins is in sharp contrast to the insight gained on the proteins involved in recognizing origins and regulating DNA replication (Blow and Dutta, 2005; Takeda and Dutta, 2005). This is due to the fact that these proteins are to a large extent evolutionary conserved from yeast to mammals and most initiation factors were initially identified in yeast. Origins are marked by the origin recognition complex (ORC), which consists of multiple proteins. In mammalian cells the ORC complex appears to be bound to origins throughout the cell cycle, except Orc1 that is cell cycle regulated (Mendez et al., 2002). In G1/S phase of the cell cycle, pre-Replication Complexes (preRCs) are assembled at origins of replication. During G1 the protein levels of both Cdc6 and Cdt1 increase, and they bind

to the ORC complex. Since Cdc6 and Cdt1 are essential for the subsequent loading of the minichromosome maintenance (MCM) complex, which is a putative replicative helicase, these factors are thought to license the cell for replication. It has been established that not all origins are activated at the same time in S-phase. Some origins appear to be activated early in S-phase, which has been shown to correlate with active transcription, whereas other origins are activated late in S-phase. Therefore, in the existing model preRCs are individually activated and fired. This firing is thought to be executed through phosphorylation of the preRC components by cyclin dependent kinases (CDKs) and the Cdc7-Dbf4 kinase (Lei and Tye, 2001). Once preRCs are activated, DNA unwinding takes place and Cdc45, replication protein A and DNA polymerase-alpha are loaded onto the chromatin. Apart from its role in activating DNA replication, CDK phosphorylation inhibits the formation of new replication complexes and inactivates components of the fired preRCs. Only at the end of mitosis CDK activity decreases due to degradation of mitotic cyclins, which allows new preRC formation in the G1 phase of the next cell cycle.

Origin firing by CDK activity

How exactly CDK activity both activates the initiation of replication at origins and inhibits the firing of origins that have already been activated is at present not clear. This control mechanism has been referred to as a 'replication switch' (Jallepalli and Kelly, 1997). It has been proposed that the level of CDK activity is the primary determinant of this switch. Low kinase levels would be sufficient to trigger origin activation, whereas high CDK activity would result in disassembly of pre-RCs and inhibition of new preRC formation. However, this model would have the risk of aberrant regulation at intermediate levels of CDK activity.

Another possibility would be that the specific phosphorylation of replication proteins determines whether the origin is activated or inhibited. Whether these proteins are phosphorylated or not depends on their availability or their accessibility. In this model E3-ubiquitin ligase proteins like the anaphase promoting complex (APC) and the Skp1-cullin-F box SCF complex could play a central role in determining the availability of the initiation proteins by regulating their cell cycle dependent destruction. At present several proteins of the mammalian preRC have been described to be phosphorylated by CDKs and as a result degraded or inactivated. First, human Orc1 was shown to be phosphorylated by cyclin A/CDK2 and degraded in a SCF^{Skp2}-dependent manner (Mendez et al., 2002). Second, it was demonstrated that cyclin A-dependent Cdt1 phosphorylation in S-phase induces its SCF^{Skp2}-dependent degradation (Li et al., 2003; Liu et al., 2004; Sugimoto et al., 2004). Notably, Cdt1 activity remained regulated by geminin in G2/M and G1-phase of the cell cycle. Third, CDK phosphorylation decreases the helicase activity of the MCM-complex (Ishimi, 1997). However, phosphorylation of an initiation protein by CDK2 that is essential for activation of DNA replication has not been revealed.

CDK2 dependent stabilization of Cdc6

Intriguingly, in addition to Orc1, Cdt1 and the MCM proteins also the licensing protein Cdc6 was previously recognized as a CDK target. Cdc6 phosphorylation by Cyclin-A/CDK2 was described to occur in S-phase and to result in its translocation from the nucleus to the cytosol and subsequent degradation (Delmolino et al., 2001; Herbig et al., 2000; Jiang et al., 1999b; Petersen et al., 1999; Saha et al., 1998). However, these studies were all performed with ectopically expressed and tagged wild-type (wt) Cdc6 or Cdc6 that had been mutated in several phosphorylation sites. Later on, this model has been challenged by the finding that only ectopically expressed Cdc6 or the soluble endogenous form are translocated to the cytosol, whereas the chromatin-bound form persist through S and G2 phases (Coverley et al., 2000; Mendez and Stillman, 2000). Notably, serine 54 phosphorylated Cdc6 was also shown to remain chromatin bound in S-phase (Alexandrow and Hamlin, 2004).

Nevertheless, Cdc6 failed to be recognized as a positive regulator of DNA replication.
Interestingly, latest observations establish Cdc6 as the first example of a key replication initiation protein whose stability is increased by CDK phosphorylation. We recently observed that the licensing protein Cdc6 is phosphorylated and thereby stabilized by CDK2/cyclin E activity (Duursma and Agami, 2005). In particularly, phosphorylation of one amino acid (serine 54) protects Cdc6 from APCCdh1 mediated destruction. In line with our results, Mailand et al showed that phosphorylation of Cdc6 prevents it Cdh1-dependent ubiquitination (Mailand and Diffley, 2005).
The fact that Cdc6 stability is controlled by CDK2 implies regulation through the p53 pathway in stress responses. DNA damage induces stabilization and activation of the p53 transcription factor, which results in increased synthesis of the CDK inhibitory protein $p21^{Cip1}$ (Fei and El-Deiry, 2003). Indeed, we observed enhanced Cdc6 destruction following DNA damage in a p53 and $p21^{Cip1}$-dependent manner (Duursma and Agami, 2005). Interestingly, we also revealed that Cdc6 is regulated in a p53-dependent manner in non-stressed cells. We demonstrate that siRNA mediated reduction of p53 protein levels results in more replicating cells, an effect that can be reversed by reducing Cdc6 protein level.

Controlling the stability and activity of the licensing factors

Both Cdc6 and Cdt1 are the licensing factors of DNA replication. Therefore, this key step in initiation of DNA replication appears to be regulated by several independent pathways. Whereas the protein abundance of Cdc6 in G1/S phase is positively regulated by cyclin E/CDK2 phosphorylation through protecting it from APC^{Cdh1}-dependent degradation, Cdt1 is negatively regulated during S-phase by Cyclin A phosphorylation in a SCF^{Skp2}-dependent manner (Li et al., 2003; Liu et al., 2004; Sugimoto et al., 2004). However, this

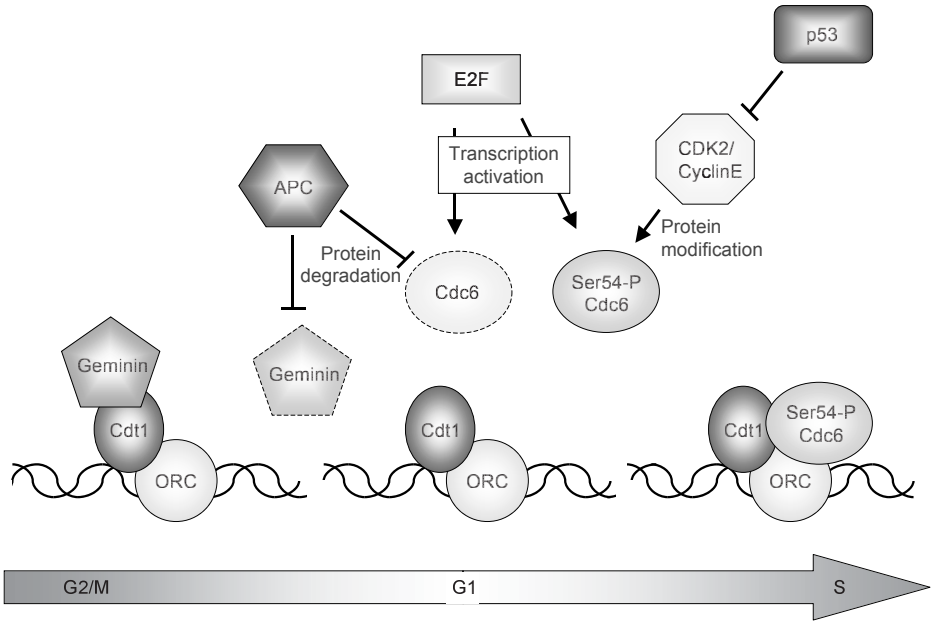


Figure 1: A schematic model of the regulation of DNA replication licensing in G1 phase of the cell cycle. Licensing of replication origins occurs in a time-frame where geminin is degraded by the APC, resulting in active Cdt1, and Cdc6 is protected from APC-dependent degradation by CDK2/ cyclin E phosphorylation of serine 54.

model is more complex since Cdt1 activity is also inhibited by geminin (Sugimoto et al., 2004). As both Cdc6 and geminin (McGarry and Kirschner, 1998) are regulated by the APC a paradox emerges. How is the APC-dependent destruction of both an activator (Cdc6) and an inhibitor (geminin) of DNA replication coordinated to ensure that Cdc6 and Cdt1 are present in the same time-frame to allow efficient pre-RC assembly? Based on our results and those of Mailand et al, we propose the following model for the assembly of preRCs (Figure 1). Geminin is degraded in an APC-dependent manner at the end of G2/M and in early G1 phase of the cell cycle. Due to the degradation of Geminin the levels of Cdt1 will rise during G1. Cdc6 transcription is regulated by E2F transcription factors (Hateboer et al., 1998; Yan et al., 1998), hence Cdc6 transcription increases in G1 phase. Yet, Cdc6 protein levels will only be stabilized in the course of G1 as the activity of CDK2/ cyclin E increases. Phosphorylation of Cdc6 at serine 54 will protect Cdc6 from APC^{Cdh1}-dependent destruction and the protein is allowed to accumulate. This provides the cells with a period in which both licensing factors Cdt1 and Cdc6 are present. Binding of these proteins to the ORC-complex at origins results in recruitment of the MCM-complex and formation of a preRC. Thus, phosphorylation of Cdc6 serine 54 could be the primary determinant of the timing of preRC formation at G1/S transition.

Another question that arises is whether the CDK2-dependent phosphorylation of Cdc6 not only stabilizes the protein but also plays a role in recruitment of the MCM-complex. Interestingly, defective MCM loading was observed in mouse embryonic fibroblasts (MEFs) lacking both cyclin E1 and E2. Cells that re-entered the cell cycle from quiescence showed chromatin bound Cdc6 but not MCM2 (Geng et al., 2003). In addition, it was shown that a phospho-mimicking mutant

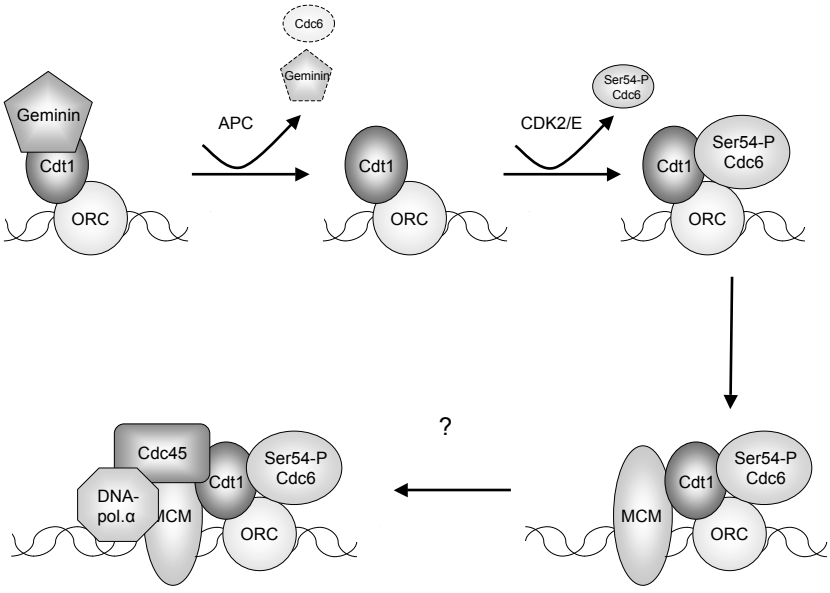


Figure 2. A proposed model for the regulation of initiation of DNA replication. Geminin and Cdc6 are both degraded by the APC, until Cdc6 is stabilized by CDK2/ Cyclin E activity in course of G1 phase. We propose that this is the step in origin firing that requires CDK activity. Once Cdc6 and Cdt1 license the chromatin, the MCM-complex will be recruited. We suggest that the subsequent loading of Cdc45 and DNA-polymerase-alpha occur in a CDK independent manner, but what is required for this loading remains to be determined.

of Cdc6 that was co-expressed with Cdt1 under conditions where the endogenous licensing proteins were absent, could enforce chromatin loading of MCM6 (Mailand and Diffley, 2005). Together, a model can be depicted whereby phosphorylation of Cdc6 plays a crucial role in MCM recruitment to the chromatin.

Controlling initiation of DNA replication: the model

We propose here a model for regulation of initiation of DNA replication in mammalian cells which is based on the finding that Cdc6 is stabilized by CDK phosphorylation and the observation that phosphorylation of Cdc6 was sufficient to load MCM6 to the chromatin (Figure 2). In this model, Cdc6 is a key CDK2/cyclin E target in the replication complex for activation of origins in G1-phase. Stabilization of Cdc6 by phosphorylation together with the destruction of geminin results in binding of both Cdc6 and Cdt1 to the ORC complex at origins of replication leading to the loading of the MCM-complex. Next, independent of further CDK activity, the MCM complex recruits Cdc45, which in turn recruits DNA polymerase. Thus, opposing the hypothesis that CDK-dependent phosphorylation of MCM proteins results in origin firing, we propose that CDK activity is crucial for activating replication origins by phosphorylating Cdc6.

This model is consistent with the fact that all CDK-phosphorylated MCM proteins identified until now are negatively regulated by this modification (Hendrickson et al., 1996; Ishimi, 1997). However, this hypothesis disagrees with the suggested requirement of CDK activity between the assembly of MCM in the preRC and the loading of Cdc45, which was mainly based on studies in yeast and in vitro studies with Xenopus egg extracts. In yeast it was shown that association of Cdc45 with chromatin correlated with activation of S-phase CDK activity at G1/S transition (Zou and Stillman, 1998). Nevertheless, chromatin binding of yeast Cdc45 in G1 was reported by others (Aparicio et al., 1997). Further, in vitro experiments with Xenopus egg extracts and sperm chromatin showed that addition of $p21^{Cip1}$ or $p27^{Kip1}$ could block Cdc45 loading whereas MCM was still recruited (Mimura and Takisawa, 1998; Walter and Newport, 2000). Thus, to our knowledge direct evidence for CDK activity in the loading process of Cdc45 in mammalian cells is lacking and therefore interesting to be determined.

CDKs were also proposed to fire individual origins, resulting in early and late replicating origins. Therefore, if CDKs do not play a role in activating individual preRCs, what then determines the activation of individual origins at different time-points during S-phase? One possibility is the activity of Cdc7-Dbf4 kinase which was shown to phosphorylate MCM2 and to be required for activation of DNA replication in mammalian cells (Jiang et al., 1999a). Moreover, it has been proposed that this kinase acts locally at individual origins (Jares et al., 2000). Therefore, it would be interesting to find out what determines the activation of individual replication origins and whether the Cdc7-Dbf4 kinase is involved.

Concluding remarks

Cdc6 has emerged as the first positively regulated target of CDK2/cyclin E activity within the initiation of DNA replication, since its phosphorylation results in stabilization of the protein. CDK2/ cyclin E activity is inhibited during stress responses due to the p53-$p21^{Cip1}$ pathway and as a consequence Cdc6 is subjected to increased degradation. However, p53-dependent regulation of Cdc6 protein abundance also appears to play a role in non-stressed cells. The increase in replicating cells due to loss of p53 can be reverted by reducing the levels of CDC6. Many tumors lost p53 activity or have elevated levels of cyclin E. Therefore it would be interesting to determine whether these cells have increased Cdc6 expression and whether this plays a role in tumorigenesis. Then, Cdc6 might emerge as a novel target in cancer therapy.

References

Alexandrow, M. G., and Hamlin, J. L. (2004). Cdc6 chromatin affinity is unaffected by serine-54 phosphorylation, S-phase progression, and overexpression of cyclin A. Mol Cell Biol 24, 1614-1627.

Antequera, F. (2004). Genomic specification and epigenetic regulation of eukaryotic DNA replication origins. Embo J 23, 4365-4370.

Aparicio, O. M., Weinstein, D. M., and Bell, S. P. (1997). Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell 91, 59-69.

Bell, S. P., and Dutta, A. (2002). DNA replication in eukaryotic cells. Annu Rev Biochem 71, 333-374.

Blow, J. J., and Dutta, A. (2005). Preventing re-replication of chromosomal DNA. Nat Rev Mol Cell Biol 6, 476-486.

Coverley, D., Pelizon, C., Trewick, S., and Laskey, R. A. (2000). Chromatin-bound Cdc6 persists in S and G2 phases in human cells, while soluble Cdc6 is destroyed in a cyclin A-cdk2 dependent process. J Cell Sci 113 (Pt 11), 1929-1938.

Cvetic, C., and Walter, J. C. (2005). Eukaryotic origins of DNA replication: could you please be more specific? Semin Cell Dev Biol 16, 343-353.

Delmolino, L. M., Saha, P., and Dutta, A. (2001). Multiple mechanisms regulate subcellular localization of human CDC6. J Biol Chem 276, 26947-26954.

Duursma, A., and Agami, R. (2005). p53-Dependent regulation of Cdc6 protein stability controls cellular proliferation. Mol Cell Biol 25, 6937-6947.

Fei, P., and El-Deiry, W. S. (2003). P53 and radiation responses. Oncogene 22, 5774-5783.

Geng, Y., Yu, Q., Sicinska, E., Das, M., Schneider, J. E., Bhattacharya, S., Rideout, W. M., Bronson, R. T., Gardner, H., and Sicinski, P. (2003). Cyclin E ablation in the mouse. Cell 114, 431-443.

Hateboer, G., Wobst, A., Petersen, B. O., Le Cam, L., Vigo, E., Sardet, C., and Helin, K. (1998). Cell cycle-regulated expression of mammalian CDC6 is dependent on E2F. Mol Cell Biol 18, 6679-6697.

Hendrickson, M., Madine, M., Dalton, S., and Gautier, J. (1996). Phosphorylation of MCM4 by cdc2 protein kinase inhibits the activity of the minichromosome maintenance complex. Proc Natl Acad Sci U S A 93, 12223-12228.

Herbig, U., Griffith, J. W., and Fanning, E. (2000). Mutation of cyclin/cdk phosphorylation sites in HsCdc6 disrupts a late step in initiation of DNA replication in human cells. Mol Biol Cell 11, 4117-4130.

Ishimi, Y. (1997). A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. J Biol Chem 272, 24508-24513.

Jallepalli, P. V., and Kelly, T. J. (1997). Cyclin-dependent kinase and initiation at eukaryotic origins: a replication switch? Curr Opin Cell Biol 9, 358-363.

Jares, P., Donaldson, A., and Blow, J. J. (2000). The Cdc7/Dbf4 protein kinase: target of the S phase checkpoint? EMBO Rep 1, 319-322.

Jiang, W., McDonald, D., Hope, T. J., and Hunter, T. (1999a). Mammalian Cdc7-Dbf4 protein kinase complex is essential for initiation of DNA replication. Embo J 18, 5703-5713.

Jiang, W., Wells, N. J., and Hunter, T. (1999b). Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. Proc Natl Acad Sci U S A 96, 6193-6198.

Lei, M., and Tye, B. K. (2001). Initiating DNA synthesis: from recruiting to activating the MCM complex. J Cell Sci 114, 1447-1454.

Li, X., Zhao, Q., Liao, R., Sun, P., and Wu, X. (2003). The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. J Biol Chem 278, 30854-30858.

Liu, E., Li, X., Yan, F., Zhao, Q., and Wu, X. (2004). Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation. J Biol Chem 279, 17283-17288.

MacAlpine, D. M., Rodriguez, H. K., and Bell, S. P. (2004). Coordination of replication and transcription along a Drosophila chromosome. Genes Dev 18, 3094-3105.

Mailand, N., and Diffley, J. F. (2005). CDKs promote DNA replication origin licensing in human cells by protecting

Cdc6 from APC/C-dependent proteolysis. Cell 122, 915-926.

McGarry, T. J., and Kirschner, M. W. (1998). Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell 93, 1043-1053.

Mendez, J., and Stillman, B. (2000). Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. Mol Cell Biol 20, 8602-8612.

Mendez, J., Zou-Yang, X. H., Kim, S. Y., Hidaka, M., Tansey, W. P., and Stillman, B. (2002). Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. Mol Cell 9, 481-491.

Mimura, S., and Takisawa, H. (1998). Xenopus Cdc45-dependent loading of DNA polymerase alpha onto chromatin under the control of S-phase Cdk. Embo J 17, 5699-5707.

Petersen, B. O., Lukas, J., Sorensen, C. S., Bartek, J., and Helin, K. (1999). Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. Embo J 18, 396-410.

Saha, P., Chen, J., Thome, K. C., Lawlis, S. J., Hou, Z. H., Hendricks, M., Parvin, J. D., and Dutta, A. (1998). Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. Mol Cell Biol 18, 2758-2767.

Sugimoto, N., Tatsumi, Y., Tsurumi, T., Matsukage, A., Kiyono, T., Nishitani, H., and Fujita, M. (2004). Cdt1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding. J Biol Chem 279, 19691-19697.

Takeda, D. Y., and Dutta, A. (2005). DNA replication and progression through S phase. Oncogene 24, 2827-2843.

Walter, J., and Newport, J. (2000). Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. Mol Cell 5, 617-627.

Yan, Z., DeGregori, J., Shohet, R., Leone, G., Stillman, B., Nevins, J. R., and Williams, R. S. (1998). Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells. Proc Natl Acad Sci U S A 95, 3603-3608.

Zou, L., and Stillman, B. (1998). Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. Science 280, 593-596.

Chapter 4

miR-148 targets human Dnmt3b protein coding region

Anja M. Duursma, Martijn Kedde, Mariette Schrier, Carlos le Sage, Reuven Agami

RNA. 2008.

miR-148 targets human Dnmt3b protein coding region

Anja M. Duursma, Martijn Kedde, Mariette Schrier, Carlos le Sage, Reuven Agami*

The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
*Communicating author

MicroRNAs (miRNAs) are small non-coding RNA molecules of 20-24 nucleotides that regulate gene expression. In animals, miRNAs form imperfect interactions with sequences in the 3' Untranslated region (3'UTR) of mRNAs, causing translational inhibition and mRNA decay. In contrast, plant miRNAs mostly associate with protein coding regions. Here we show that human miR-148 represses DNA methyltransferase 3b (Dnmt3b) gene expression through a region in its coding sequence. This region is evolutionary conserved and present in the Dnmt3b splice variants Dnmt3b1, Dnmt3b2 and Dnmt3b4, but not in the abundantly expressed Dnmt3b3. Whereas overexpression of miR-148 results in decreased DNMT3b1 expression, short-hairpin RNA mediated miR-148 repression leads to an increase in DNMT3b1 expression. Interestingly, mutating the putative miR-148 target site in Dnmt3b1 abolishes regulation by miR-148. Moreover, endogenous Dnmt3b3 mRNA, which lacks the putative miR-148 target site, is resistant to miR-148 mediated regulation. Thus, our results demonstrate that the coding sequence of Dnmt3b mediates regulation by the miR-148 family. More generally, we provide evidence that coding regions of human genes can be targeted by miRNAs and that such a mechanism might play a role in determining the relative abundance of different splice variants.

Introduction

MiRNAs are small non-coding RNAs that have been identified as post-transciptional regulators of mRNA expression in many multi-cellular organisms such as plants, insects and mammals. In animals, miRNAs have been shown to inhibit mRNA translation and to decrease mRNA stability by binding sequences in the 3'UTR (Standart and Jackson, 2007; Wu et al., 2006). Perfect binding of nucelotides 2-7, the miRNA 'seed', to the target mRNA is considered to play a key role in target recognition (Doench and Sharp, 2004; Lewis et al., 2005). Binding of other miRNA nucleotides has been suggested to be involved in mRNA repression as well, but in this instance no perfect complementarity is required (Grimson et al., 2007).

In plants, most described miRNAs bind the protein coding sequence (CDS) of their target mRNAs with very high sequence complementarity and this induces translational repression or RNA degradation in a way similar to RNA interference (Chen, 2004; Llave et al., 2002; Rhoades et al., 2002). The existence of miRNA binding sites in animal CDS has been predicted by computational approaches. By analyzing mRNAs for conserved complementarity to the miRNA seed sequence, about a thousand target sites were predicted to occur in vertebrate CDSs (Lewis et al., 2005) and a similar approach in flies predicted sites in the CDSs as well (Stark et al., 2007). Furthermore, by combining computational approaches and human mRNA expression data, effective miRNA sites were detected in human CDS (Grimson et al., 2007). In an experimental assay, let-7 mediated repression was observed in zebrafish embryos when a let-7 target site was fused

to the GFP CDS (Kloosterman et al., 2004). Furthermore, a miRNA pull-down of mRNAs and subsequent validation of predicted CDS sites of these mRNAs in reporter assays, showed that fly miRNAs can target CDSs (Easow et al., 2007). Yet, to our knowledge no functional miRNA binding sites have been described in mammalian coding regions.

DNA methylation is an epigenetic modification, which is involved in gene silencing, chromatin remodeling and genome stability (Jones and Baylin, 2007). In mammals, DNA methyltransferases attach methyl groups to cytosine residues of long stretches of CpG dinucleotides (CpG islands) that occur in repetitive sequences or in gene promoter regions (Gal-Yam et al., 2007). DNMT3a and DNMT3b have been identified as 'de novo' methyltransferase, which methylate DNA during early development and gametogenesis (Okano et al., 1999). Inactivation of *Dnmt3b* results in embryonic lethality and *Dnmt3a* knock-out mice die shortly after birth (Okano et al., 1999). Although DNMT3a and DNMT3b have overlapping functions in imprinting of genes, DNMT3b was shown to be essential for methylation of centromeric minor satellite repeats (Okano et al., 1999). Furthermore, mutations in the human *Dnmt3b* gene have been linked to the ICF (Immunodeficiency, Centromere instability and Facial anomalies) syndrome (Xu et al., 1999). Lymphocytes from these patients display hypomethylation of centromeric repeat sequences, chromatin decondensation and genomic instability (Jeanpierre et al., 1993; Tuck-Muller et al., 2000). In cancer, single nucleotide polymorphisms in the *Dnmt3b* promoter that were hypothesized to result in elevated Dnmt3b expression, have been correlated with an increased risk of lung cancer (Lee et al., 2005; Shen et al., 2002). Interestingly, a recent paper showed direct regulation of the Dnmt3a and Dnmt3b 3'UTRs by miR-29 and reduced expression of miR-29 correlated inversely with increased expression of these Dnmts in lung cancer tissue (Fabbri et al., 2007).

Dnmt3b has several splice variants of which Dnmt3b1 and Dnmt3b3 are expressed most abundantly (Robertson et al., 1999). DNMT3b1 and DNMT3b2 have been shown to be catalytically active (Okano et al., 1998), whereas the role of DNMT3b3 in DNA methylation is not fully understood. Murine DNMT3b3 is catalytically inactive both in vitro (Aoki et al., 2001) and in vivo (Chen et al., 2003), however, the DNA methyltransferase activity of human DNMT3b3 depended on which substrate was chosen (Chen et al., 2005; Soejima et al., 2003). This discrepancy might reflect a change in target preference since Dnmt3b3, but also Dnmt3b4, lack a motif that could be responsible for target recognition (Kumar et al., 1994). Interestingly, high expression of splice variant Dnmt3b4 has been associated with DNA hypomethylation at pericentromeric satellite regions (Saito et al., 2002). This could be a result of dominant negative regulation or an example of changed target preference. The latter could result in altered gene expression and this was indeed observed by micro-array analysis in cells that exogenously expressed Dnmt3b7, a splice variant that was found highly expressed in cancer cells (Ostler et al., 2007). Another possibility would be that DNMT3b3 stimulates DNMT3b1 catalytic activity in a similar way to the DNMT3b related protein DNMT3L that also lacks methyltransferase activity (Chen et al., 2005).

Here, we describe our finding that human miR-148 regulates DNMT3b expression through interaction with a conserved site in its protein coding sequence. Interestingly, the target site is absent in the abundantly expressed Dnmt3b3 splice variant, making it resistant to miRNA-mediated regulation. Thus, the expression of miR-148 changes the relative abundance of DNMT3b splice variants.

Results and Discussion

In plants, most miRNAs regulate mRNA expression by interacting with highly homologous CDS (Rhoades et al., 2002). We identified a site in the *Dnmt3b* CDS (nucleotides 2384-2411), which is highly

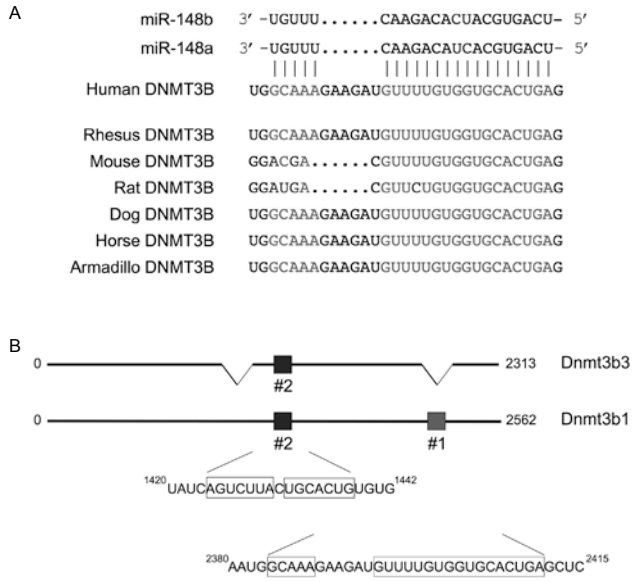


Figure 1. Schematic representation of putative miR-148 sites in the Dnmt3b1 coding region. A miR-148a and b are highly complementary to nucleotides 2382 to 2412 of human Dnmt3b. This potential miR-148 target site is conserved in rhesus, mouse, rat, dog, horse and armadillo. This site is referred to as target site #1. **B** Schematic representation of 2 possible miR-148 target sites in the Dnmt3b coding region.

homologous to the miR-148 family (Figure 1A). This region has the potential to interact with all 22 nucleotides of miR-148a and b (leaving only one gap) and therefore resembles miRNA target interactions observed in plants. Importantly, the target site (referred to as site #1) is conserved in rhesus, mouse, rat, dog, horse and armadillo *Dnmt3b1*, *Dnmt3b2*, and *Dnmt3b4*, suggesting that this sequence is retained through evolution (Figure 1A). However, this particular sequence is not present in the *Dnmt3b3* splice variant (Figure 1B and data not shown). Another site (#2) that contains complementarity to the miR-148 family is situated between nucleotides 1424 and 1439 (determined using targetscanS and RNA22)(Figure 1B). Although it has a good match of the miRNA seed sequence (nucleotides 2-7), which is thought to specify miRNA targets in 3'UTRs (Doench and Sharp, 2004; Lewis et al., 2005), extensive complementarity such as observed with site #1 is not seen.

Next, we designed experiments to study the regulation of Dnmt3b by miR-148. First, we used miR-Vec-148a, a miRNA-expression vector for ectopic expression of miR-148a (Voorhoeve et al., 2006). Using an RNase protection assay (RPA) for miR-148 in HeLa cells, which were previously shown to be negative for miR-148 (Barad et al., 2004), transient and stable introduction of miR-Vec-148a led to high expression of this miRNA (Figure 2A). Second, we determined whether we could identify cell lines that endogenously express miR-148 by quantitative real-time (qRT) PCR specific for mature miR-148a. We observed high miR-148 expression in the 2102EP testicular germ cell line, 293T cells, Jurkat T cells, but not in HeLa, MCF-7 cells and diploid BJ fibroblasts that were immortalized with telomerase (Figure 2B). Notably, the high expression observed in the transient transfected miR-148a HeLa cells was very similar to the endogenous expression of miR-148a found in Jurkat cells (2B). In contrast to miR-148a, we were unable to detect endogenous expression miR-148b (which was originally cloned from rat) in human cell lines (data not shown). Third, we co-transfected miR-Vec-148a with an expression vector containing the coding region of for *Dnmt3b1* downstream of the green fluorescent protein (*GFP-Dnmt3b1*), and performed immuno-blotting analysis for GFP. Notably, Dnmt3b was cloned in-frame with GFP, which was verified by sequencing.

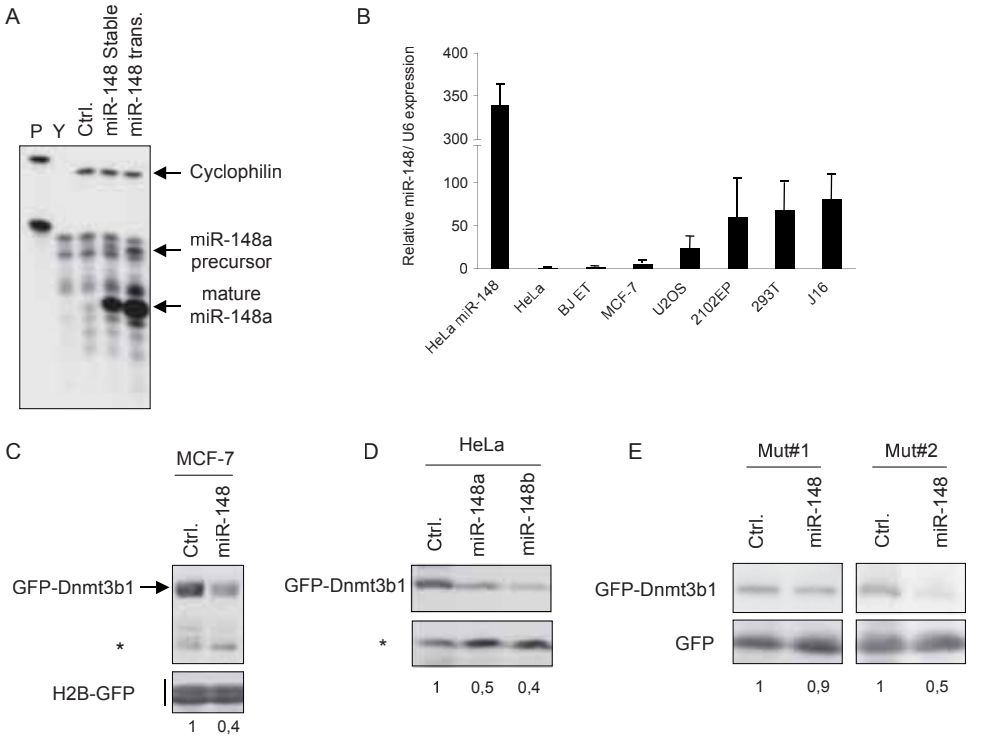


Figure 2. miR-148 regulates exogenous DNMT3b protein expression through interaction with its protein coding region. A RPA was used to detect miR-148 level in HeLa cells that were transfected with miR-148, a control miRNA (Ctrl) or in a stable polyclonal pool of miR-148a expressing cells. The protected fragments are indicated. Lane P shows the probes for cyclophilin and miR-148a. In lane Y yeast RNA was used as control. **B** qRT-PCR to detect miR-148a expression was performed using the indicated cell lines. Error bars represent standard deviation. **C** MCF-7 cells were co-transfected with either miR-Vec-148a or a miR-Vec control, together with GFP-Dnmt3b and H2B-GFP. Whole cell extracts were subjected to immunoblot analysis to detect GFP. H2B-GFP was used to demonstrate equal transfection efficiencies. Asterisk marks a non-specific band and shows equal loading. **D** and **E** As described in C. HeLa cells were transfected with the indicated constructs.

Furthermore, the translational fusion product was detected at the appropriate size by Immunoblotting analysis (data not shown). We observed a marked reduction of 60% of GFP-DNMT3b1 expression, but not of co-transfected H2B-GFP, in cells expressing miR-148 (Figure 2C). As expected from the sequence similarity between miR-148a and miR-148b, a similar reduction of GFP-DNMT3b1 expression in cells expressing miR-148b was observed (Figure 2D). These results suggest that the presence of miR-148 represses the expression of DNMT3b1.

To determine whether the observed reduction in DNMT3b1 expression by miR-148 is dependent on the putative miR-148 target sites, we mutated these regions to significantly reduce recognition by the miRNAs. Importantly, mutating these sites did not change the frame of the fusion proteins, as was verified by sequencing and Immunoblotting analysis (data not shown). Transient co-transfection experiments revealed that mutating the highly homologous target site #1 almost completely abrogated miR-148-mediated regulation (Figure 2E).

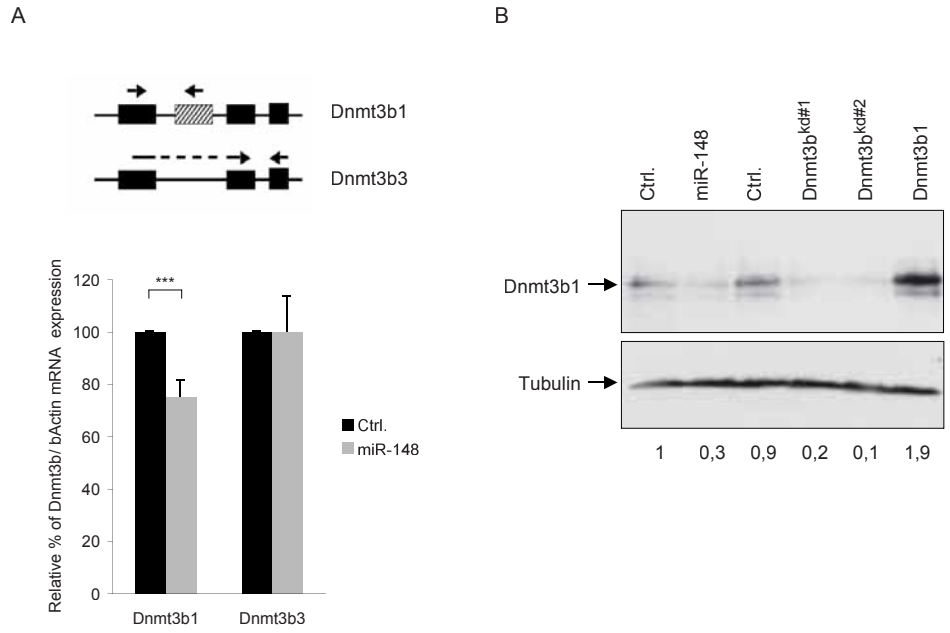


Figure 3. miR-148 reduces endogenous Dnmt3b1 mRNA and protein level. A qRT-PCR of either miR-Vec-148 or control (Ctrl.) transfected HeLa cells. Specific primers were used to detect Dnmt3b1 and Dnmt3b3 as shown schematically. Error bars represent standard deviation, n=4 and *** p<0.001. **B** 2102EP cells were electroporated with the indicated constructs and subjected to immunoblot analysis for Dnmt3b and Tubulin.

However, a mutant of target site #2, which has much less complementarity to miR-148, still retained sensitivity to miR-148. This suggests that miR-148 targets Dnmt3b1 through a highly complementary sequence in the Dnmt3b1 coding region.

The results above imply that the Dnmt3b3 splice variant, which lacks miR-148 target site #1, is resistant to miR-148 mediated regulation. To examine this issue, we designed primers for qRT-PCR that distinguish endogenous Dnmt3b3 from Dnmt3b1 and other variants that express the targeted exon such as Dnmt3b2 and Dnmt3b4 mRNA (Figure 3A). This analysis revealed that stable expression of miR-148a in HeLa cells resulted in small but significant reduction ($p<0.001$) in Dnmt3b1 mRNA levels of 25%, while no reduction in Dnmt3b3 mRNA levels was observed (Figure 3A). Thus, the expression of miR-148a affects the mRNA stability of Dnmt3b1, but not Dnmt3b3, leading to a relative increase in the abundance of Dnmt3b3 compared to Dnmt3b1 and all other Dnmt3b splice variants that express the targeted site.

DNMT3b protein is highly expressed in undifferentiated embryonic stem cells, whereas its expression level is much reduced in somatic cells (Okano et al., 1998). To study the effect of miR-148a on endogenous DNMT3b protein expression we attempted to detect it in several tumor cell lines using immuno-blot analysis. In our hands, only 2102EP testicular germ cell tumor cells expressed endogenous DNMT3b in sufficient amount to allow detection by western blot (data not shown). Furthermore, these cells express moderate level of miR-148a (Figure 2B). To assess whether increasing miR-148 level would result in reduced endogenous DNMT3b1 protein level, we electroporated the miR-148 expression vector into 2102EP cells and subjected them to immunoblotting

analysis with a specific anti-Dnmt3b antibody. In line with the results above, over-expression of miR-148a reduced DNMT3b1 protein level up to 70 % compared to two different miRNA control constructs (Figure 3B). We used two short-hairpin RNA (shRNA) constructs targeting Dnmt3b and an expression vector for *Dnmt3b1* to control for the specificity of the Dnmt3b antibody (Figure 3B).

Lastly, we asked whether inhibition of miR-148a activity would elevate DNMT3b1 expression. We designed two shRNA constructs targeting the miR-148 precursor RNA. Electroporation of 21202EP cells with these constructs showed that one construct, miR-148$^{kd\#2}$, was functional in reducing endogenous miR-148 level as determined by RPA analysis (Figure 4A). Western blot analysis of cells electroporated with miR-148$^{kd\#2}$ indeed revealed increase in the DNMT3b1 protein level (Figure 4B). We therefore conclude that endogenous DNMT3b1 expression is controlled by miR-148a in 2102EP cells.

Our data reveal a novel function of miRNA-mediated regulation. miRNA targeting of human coding regions in addition to 3'UTRs not only extends the range of potential target sites, but also allows for splice variant specific regulation. Significantly, different DNMT3b splice variants might play a role in regulating DNMT3b activity or target site preference. This could be an additional mechanism to the regulation of splice variant abundance by alternative splicing. Our results rule out the possibility that alternative splicing is induced by miR-148 targeting, since miR-148 reduced expression of exogenous DNMT3b-GFP in a site-specific manner.

It is interesting that only the high affinity miR-148 target site in Dnmt3b1 was affected by miR-148. In the future, it will be important to determine whether miRNA binding to CDS requires more complementarity with the target than interactions with 3'UTRs. Plant miRNAs interact with high sequence complementarity with their target CDS eliciting in most of the cases an RNA interference-mediated cleavage of the target mRNA (Llave et al., 2002; Rhoades et al., 2002). Although we observed reduction of Dnmt3b mRNA in the presence of miR-148, the reduction was less prominent than the observed reduction on protein level. This suggests that miR-148 induces both translational repression and mRNA degradation of Dnmt3b1. Further experiments should approach this point.

The mechanism by which miRNAs mediate repression is not completely understood. Whereas some evidence exists for interference with initiation of translation, others suggest that miRNAs prevent the 'closed loop' mRNA configuration induced by interaction of poly-A binding proteins with initiation factors at

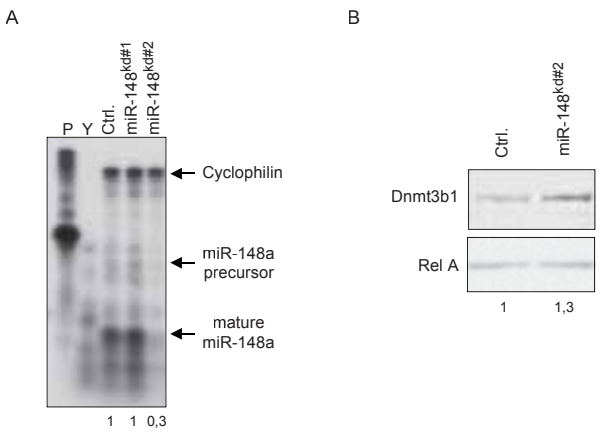


Figure 4. Suppression of endogenous miR-148 increases DNMT3b1 expression. A miR-148a level was detected by RPA as described in Figure 2A. **B** 2102EP cells were electroporated with either control (Ctrl.) or miR-148$^{kd\#2}$ constructs and subjected to immunoblot analysis with Dnmt3b and Rel A antibodies.

the 5' cap (Standart and Jackson, 2007). The latter enhances translation efficiency. miR-148 targets Dnmt3b1 approximately 150 nucleotides before the stop codon in the CDS, which is near the 3'UTR and the poly(A)-tail. Therefore, the location of miR-148 target site might allow for miRNA-mediated repression in a similar fashion as 3'UTR binding miRNAs. On the other hand, experiments with miRNA target sites cloned in the 5'UTR of a luciferase construct showed miRNA mediated repression (Lytle et al., 2007), suggesting that repression might be independent of the target site location. The exact mechanism through which miR-148 targets Dnmt3b1 remains to be explored.

In conclusion, we present evidence for a functional interaction of human miR-148 with the Dnmt3b1 CDS. It would be interesting to see whether miRNA-mediated regulation of protein coding regions turn out to be a common theme in miRNA targeting of mammalian mRNAs or the exception that confirms the rule.

Material and methods

Constructs and antibodies

Dnmt3b expression constructs were cloned by PCR in pEGFP-C2 (Clontech). Dnmt3b mutant constucts were generated by site-directed mutagenesis using PCR and constructs were verified by DNA sequence analysis. Mutant#1 1429TACTGCACTG1438 was replaced by TTCTCGTCA. Mutant#2 2398TTGTGGTGCACT2409 was replaced by TTAGCCGCGACC. All miRNAs were expressed from a retroviral miR-Vec vector as described before (Voorhoeve et al., 2006). We used pSuper short-hairpin constructs (Brummelkamp et al., 2002) targeting Dnmt3b and miR-148: Dnmt3bkd#1 AGATGACGGATGCCTAGAG Dnmt3bkd#2 (AGGTAGGAAAGTACGTCGC) miR-148kd#1 ACTCTGAGTATGATAGAAG miR-148kd#2 GTCAGTGCACTACAGAACT. Antibodies against Dnmt3b (H-230, Santa Cruz), Tubulin (YL1/2, ECACC) and NFkB p65 (C-20 Santa Cruz) and GFP (a gift from J. Neefjes) were used.

Cell culture and transfection

Hela, 2102EP, 293T, MCF-7, U2OS, BJ ET primary fibroblasts (expressing the ecotrophic receptor and human telomerase) were grown in Dulbecco Modified Eagle Medium and Jurkat cells in Iscove's Modified Dulbecco's Medium suplemented with 10% fetal bovine serum. Hela cells were transfected with Fugene (Roche) using manufacturer's protocol. 2102 EP cells were transfected by electroporation as described (Agami and Bernards, 2000). A Gene Pulser Xcell Electroporation System (Bio-Rad) was used at 120 volts, 10 times 1.5 ms burst duration and 1 s interval.

Immuno-blotting

For Western blot analysis whole-cell extracts were separated at 6% SDS-PAGE mini-gels and transferred to polyvinylidene difluoride membranes (Millipore). Western blots were developed using enhanced chemiluminescence (Amersham Biosciences). Densitometric quantification of Western blot assays was done by using Tina (version 2.09f) software.

RNA isolation, RPA and real-time RT PCR

RNA was isolated from cells using Trizol (Invitrogen). RNase protection assays (RPAs) were performed using the mirVana miRNA probe construction and detection kits (Ambion) according to manufacturers protocol. 5 µg of RNA was used per assay and a ATAGAAGTCAGTGCACTACAGAACTTTGTCTCCCTGTCTC primer was used to make a miR-148a probe. Quantification was performed by densitometry.

cDNA was prepared from 1.5 µg of RNA using random hexamer primers (Superscript III first-strand synthesis system for RT-PCR, Invitrogen). Quantitative RT-PCR was performed with a standard 2-step amplification protocol of a MiniOpticon System (Bio-Rad) apparatus using a SYBRgreen PCR master mix (Applied Biosystem) and specific primers.

Dnmt3b1 forward GCCGTTCTTCTGGATGTTTGAG
Dnmt3b1 reverse ATCCTATTGTATTCCAAGCAGTCC
Dnmt3b3 forward ATCTCACGGTTCCTGGAGTG
Dnmt3b3 reverse AAGCCAAAGATCCTGTTCATCC
Beta Actin forward CCTGGCACCCAGCACAAT
Beta Actin reverse GGGCCGGACTCGTCATACT

The mirVana qRT-PCR miRNA detection kit (Ambion) was used to detect miRNA expression by quantitative RT-PCR. Specific miR-148a and U6 RT and PCR primers were used (Ambion) and 25 ng of RNA was used per reaction.

Acknowledgements

We thank all members of the Agami lab for stimulating discussions. This work was supported by the Dutch Cancer Society (KWF) and the European Young Investigator Award (EURYI) to R. A.

References

Agami, R., and Bernards, R. (2000). Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. Cell 102, 55-66.

Aoki, A., Suetake, I., Miyagawa, J., Fujio, T., Chijiwa, T., Sasaki, H., and Tajima, S. (2001). Enzymatic properties of de novo-type mouse DNA (cytosine-5) methyltransferases. Nucleic Acids Res 29, 3506-3512.

Barad, O., Meiri, E., Avniel, A., Aharonov, R., Barzilai, A., Bentwich, I., Einav, U., Gilad, S., Hurban, P., Karov, Y., et al. (2004). MicroRNA expression detected by oligonucleotide microarrays: system establishment and expression profiling in human tissues. Genome Res 14, 2486-2494.

Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science 296, 550-553.

Chen, T., Ueda, Y., Dodge, J. E., Wang, Z., and Li, E. (2003). Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. Mol Cell Biol 23, 5594-5605.

Chen, X. (2004). A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science 303, 2022-2025.

Chen, Z. X., Mann, J. R., Hsieh, C. L., Riggs, A. D., and Chedin, F. (2005). Physical and functional interactions between the human DNMT3L protein and members of the de novo methyltransferase family. J Cell Biochem 95, 902-917.

Doench, J. G., and Sharp, P. A. (2004). Specificity of microRNA target selection in translational repression. Genes Dev 18, 504-511.

Easow, G., Teleman, A. A., and Cohen, S. M. (2007). Isolation of microRNA targets by miRNP immunopurification. Rna 13, 1198-1204.

Fabbri, M., Garzon, R., Cimmino, A., Liu, Z., Zanesi, N., Callegari, E., Liu, S., Alder, H., Costinean, S., Fernandez-Cymering, C., et al. (2007). MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci U S A 104, 15805-15810.

Gal-Yam, E. N., Saito, Y., Egger, G., and Jones, P. A. (2007). Cancer Epigenetics: Modifications, Screening, and Therapy. Annu Rev Med.

Grimson, A., Farh, K. K., Johnston, W. K., Garrett-Engele, P., Lim, L. P., and Bartel, D. P. (2007). MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 27, 91-105.

Jeanpierre, M., Turleau, C., Aurias, A., Prieur, M., Ledeist, F., Fischer, A., and Viegas-Pequignot, E. (1993). An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome. Hum Mol Genet 2, 731-735.

Jones, P. A., and Baylin, S. B. (2007). The epigenomics of cancer. Cell 128, 683-692.

Kloosterman, W. P., Wienholds, E., Ketting, R. F., and Plasterk, R. H. (2004). Substrate requirements for let-7 function in the developing zebrafish embryo. Nucleic Acids Res 32, 6284-6291.

Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R. J., and Wilson, G. G. (1994). The DNA (cytosine-5) methyltransferases. Nucleic Acids Res 22, 1-10.

Lee, S. J., Jeon, H. S., Jang, J. S., Park, S. H., Lee, G. Y., Lee, B. H., Kim, C. H., Kang, Y. M., Lee, W. K., Kam, S., et al. (2005). DNMT3B polymorphisms and risk of primary lung cancer. Carcinogenesis 26, 403-409.

Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15-20.

Llave, C., Xie, Z., Kasschau, K. D., and Carrington, J. C. (2002). Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science 297, 2053-2056.

Lytle, J. R., Yario, T. A., and Steitz, J. A. (2007). Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. Proc Natl Acad Sci U S A 104, 9667-9672.

Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99, 247-257.

Okano, M., Xie, S., and Li, E. (1998). Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet 19, 219-220.

Ostler, K. R., Davis, E. M., Payne, S. L., Gosalia, B. B., Exposito-Cespedes, J., Le Beau, M. M., and Godley, L. A. (2007). Cancer cells express aberrant DNMT3B transcripts encoding truncated proteins. Oncogene 26, 5553-5563.

Rhoades, M. W., Reinhart, B. J., Lim, L. P., Burge, C. B., Bartel, B., and Bartel, D. P. (2002). Prediction of plant microRNA targets. Cell 110, 513-520.

Robertson, K. D., Uzvolgyi, E., Liang, G., Talmadge, C., Sumegi, J., Gonzales, F. A., and Jones, P. A. (1999). The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic Acids Res 27, 2291-2298.

Saito, Y., Kanai, Y., Sakamoto, M., Saito, H., Ishii, H., and Hirohashi, S. (2002). Overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis. Proc Natl Acad Sci U S A 99, 10060-10065.

Shen, H., Wang, L., Spitz, M. R., Hong, W. K., Mao, L., and Wei, Q. (2002). A novel polymorphism in human cytosine DNA-methyltransferase-3B promoter is associated with an increased risk of lung cancer. Cancer Res 62, 4992-4995.

Soejima, K., Fang, W., and Rollins, B. J. (2003). DNA methyltransferase 3b contributes to oncogenic transformation induced by SV40T antigen and activated Ras. Oncogene 22, 4723-4733.

Standart, N., and Jackson, R. J. (2007). MicroRNAs repress translation of m7Gppp-capped target mRNAs in vitro by inhibiting initiation and promoting deadenylation. Genes Dev 21, 1975-1982.

Stark, A., Lin, M. F., Kheradpour, P., Pedersen, J. S., Parts, L., Carlson, J. W., Crosby, M. A., Rasmussen, M. D., Roy, S., Deoras, A. N., et al. (2007). Discovery of functional elements in 12 Drosophila genomes using evolutionary signatures. Nature 450, 219-232.

Tuck-Muller, C. M., Narayan, A., Tsien, F., Smeets, D. F., Sawyer, J., Fiala, E. S., Sohn, O. S., and Ehrlich, M. (2000). DNA hypomethylation and unusual chromosome instability in cell lines from ICF syndrome patients. Cytogenet Cell Genet 89, 121-128.

Voorhoeve, P. M., le Sage, C., Schrier, M., Gillis, A. J., Stoop, H., Nagel, R., Liu, Y. P., van Duijse, J., Drost, J., Griekspoor, A., et al. (2006). A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell 124, 1169-1181.

Wu, L., Fan, J., and Belasco, J. G. (2006). MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci U S A 103, 4034-4039.

Xu, G. L., Bestor, T. H., Bourc'his, D., Hsieh, C. L., Tommerup, N., Bugge, M., Hulten, M., Qu, X., Russo, J. J., and Viegas-Pequignot, E. (1999). Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402, 187-191.

Chapter 5

Differential expression of miR-148 in hematopoietic thymic subsets and its potential function

Anja M. Duursma#, Remko Schotte#, Ferenc A. Scheeren,

Bianca Blom, Ton N. Schumacher, Reuven Agami

# These authors contributed equally to this work

Differential expression of miR-148 in hematopoietic thymic subsets and its potential function

Anja M. Duursma#, Remko Schotte#, Ferenc A. Scheeren, Bianca Blom, Ton N. Schumacher, Reuven Agami

Division of Tumour Biology, Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
Department of Cell Biology and Histology of the Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands
# these authors contributed equally to this work

MicroRNAs (miRNAs) have been implicated in several cellular processes, such as cell proliferation, differentiation and apoptosis. miR-148 was found expressed in the spleen and enriched in human cell lines of hematopoietic origin. Interestingly, we identified Dnmt3b and MLL (mixed lineage leukaemia) as possible miR-148 targets and they both appear to be involved in T cell development and survival. To study whether miR-148 mediated repression of Dnmt3b and MLL plays a role in human lymphoid development, we determined the expression of miR-148, Dnmt3b and MLL in different lymphoid subsets isolated from human postnatal thymus. Whereas miR-148 is highly expressed in CD34+CD1a- early thymic progenitor cells and remains expressed in all subsets of T cell committed lineages, its expression is significantly decreased in natural killer (NK) cells and plasmacytoid dendritic cells (pDCs). However, we found that Dnmt3b and MLL mRNA levels are reduced in these subsets as well. This suggests that relieve of miR-148 mediated repression of Dnmt3b and MLL is not a key step in pDC and NK development. Instead, miR-148 might play a role in fine-tuning the expression of these targets in lymphoid development. To test whether reduction of miR-148 level is essential for pDC development we exogenously expressed miR-148 in mTPCs and performed in vitro pDC differentiation assays. Interestingly, miR-148 overexpression resulted in a dramatic increase in both percentage and number of pDCs.

Introduction

Currently, several hundreds of miRNAs have been identified, and they have been implicated in many cellular processes such as cell proliferation, apoptosis and differentiation (Carleton et al., 2007). Whereas some miRNAs are broadly expressed, others are expressed in a tissue-specific manner (Landgraf et al., 2007). To gain more insight in the role of miRNAs in development and differentiation, a number of studies examined the expression of miRNAs in cells of the immune system. In a first large-scale attempt to identify miRNAs involved in mammalian hematopoiesis approximately 100 miRNAs were cloned from mouse bone marrow (Chen et al., 2004). Three of these miRNAs showed preferential expression in hematopoietic tissue compared to tissues of other origins. Ectopic expression in a hematopoietic precursor cell of one of these miRNAs, miR-181, resulted in a significant increase in B cell development (Chen et al., 2004). In another study, several miRNAs were shown to be down-regulated following in vitro differentiation from CD34+ bone marrow cells to megakaryocytes (Garzon et al., 2006). Further, miR-155 expression was shown to regulate the germinal center reaction, a specific differentiation processes in the immune response (Thai et al., 2007).

Finally, miR-150, which is specifically expressed in mature lymphocytes and not in their progenitors was shown to control B cell differentiation by regulating its target c-Myb (Xiao et al., 2007). miR-150 overexpression in B-cell progenitors resulted in down-regulation of c-Myb and in a partial block of early B-cell development. Together, these studies indicate that miRNAs can play an important role in differentiation of hematopoietic cells.

Human miR-148a (which we will refer to as miR-148) was originally cloned from the mouse spleen. This expression appeared to be specific, since no clones were derived from heart, liver, small intestine, colon or brain tissue (Lagos-Quintana et al., 2002). In humans miR-148 was also found expressed in the spleen and enriched in human cell lines of hematopoietic origin (Landgraf et al., 2007). This is in accordance with our finding that miR-148a is expressed to the highest extent in Jurkat T cells compared to other cancer cell lines (Chapter 4). Interestingly, we identified a number of miR-148 targets that might play a role in T cell survival and development, such as Dnmt3b (Chapter 4). Dnmt3b mutations in humans were linked to the Immunodeficiency, Centromeric region instability and Facial anomalies (ICF) syndrome (Xu et al., 1999). ICF patients are characterized by low serum immunoglobulin levels and low numbers of peripheral blood B and T cells (Ehrlich, 2003). A mouse model of the ICF syndrome implied that the low number of T cells are the result of T cell death, thereby suggesting that Dnmt3b expression might play a role in T cell survival (Ueda et al., 2006). Furthermore, gene expression profiling by micro-array analysis revealed that Dnmt3b is differentially expressed in consecutive stages of T cell development. Whereas Dnmt3b was highly expressed in early thymic progenitor cells, its expression gradually decreased at more mature stages of development (Dik et al., 2005). Another possible miR-148 target mRNA is MLL (mixed lineage leukaemia) as will be shown here. MLL was identified as a common breakpoint in human leukaemia's and subsequently was shown to be involved in myeloid and lymphoid cancers through its fusion with many genes of other chromosomes (Daser and Rabbitts, 2005). Although less is known about its role in normal hematopoiesis, MLL appears to be essential, since MLL heterozygous mice displayed hematopoietic abnormalities (Yu et al., 1995). Furthermore, MLL is a major activator of class I homeobox (HOX) gene expression, by interacting directly with HOX promoter regions (Milne et al., 2002; Nakamura et al., 2002). For HOX genes also several lines of evidence exist for their involvement in hematopoiesis (Daser and Rabbitts, 2005).

Several cell subsets have been characterized in human thymic development (Figure 1). CD34+CD1a- cells are early multipotent thymic progenitor cells (mTPCs), which have the capacity to give rise to T cells, NK (natural killer) cells and pDCs (plasmacytoid dendritic cells). Upregulation of CD1a resulting in CD34+CD1a+ cells, is associated with T cell commitment, since these cells

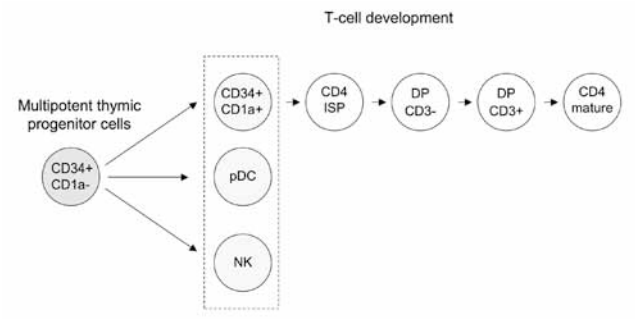


Figure 1. Schematic representation of human thymocyte differentiation.

have little capacity to develop into NK cells and completely lost their pDC developmental potential (Spits et al., 2000). CD34+CD1a+ cells develop into CD4 immature single positive (ISP) cells, CD3- double positive (DP) cells that are positive for both the CD4 and CD8 receptor, CD3+ DP cells and finally they develop into CD4 mature single positive T cells (Blom and Spits, 2006).
We were intrigued by the overlap of miR-148 expression in hematopoietic cells and the possible functions of its targets Dnmt3b and MLL in T cell development and survival. In this study we aimed to analyse the potential role of miR-148 and its targets in more detail in human thymic development.

Results and discussion

miR-148 and Dnmt3b

To study the role of miR-148 and Dnmt3b in early hematopoiesis, cells in different stages of thymic development were isolated from post natal human thymus tissue by flow cytometric sorting. The expression level of several markers was used to isolate cells from different developmental stages as depicted in Figure 1. In addition to these T cell committed cells, pDC (BDCA2+CD123hi) and NK cells (CD56+CD3-) were isolated, which both can arise from mTPCs (Figure 1). We isolated RNA from sorted cell populations, prepared cDNA and performed quantitative Real-Time (RT) PCR analysis for miR-148. We found that mTPCs contain high miR-148 levels (Figure 2A and data not shown). Moreover, the CD34+CD1a+ T cell committed cells showed an increase of miR-148 expression of 2,5 times. However, apart from a slight increase observed in DP CD3- cells compared to mTPCs, we did not observe a significant change of miR-148 expression in other analysed T cell subsets.
We previously showed that miR-148 regulates Dnmt3b1 expression through interaction with its coding region (Chapter 4). Therefore, the same RNA was subjected to quantitative RT-PCR analysis for Dnmt3b1 expression (Figure 2B). Also Dnmt3b1 was highly expressed in mTPCs, however, its expression gradually

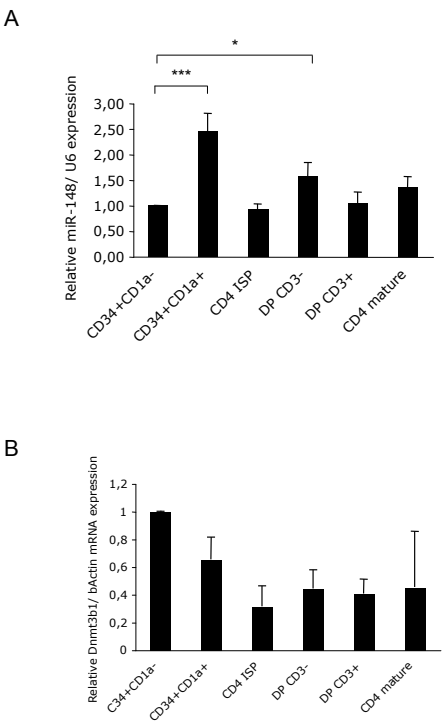


Figure 2. Relative miR-148 and Dnmt3b expression in thymic T cell subsets. A Freshly isolated thymic subsets were analyzed by quantitative RT-PCR for the presence of miR-148 (n=3). Values were calculated relative to CD34+CD1a- cells. Error bars represent standard error of the mean. **B** As descibed in A but analyzed for Dnmt3b1 mRNA expression (n=2). Error bars represent standard deviation. * P<0.05 *** P<0.001

decreased in developing T lymphocytes. This result is in line with previously published micro-array data, were similar T cell subsets from the human thymus were analysed for mRNA expression (Dik et al., 2005). Compared to CD34+CD1a- cells Dnmt3b1 mRNA level decreases in the CD34+CD1a+ cells whereas miR-148 level goes up. Yet, in other early T cell subsets no clear correlation was observed.
Next, we analysed miR-148 expression in pDC and NK cells, which arise from the same mTPC (Figure 1). Interestingly, we detected a seven fold and a five fold reduction in

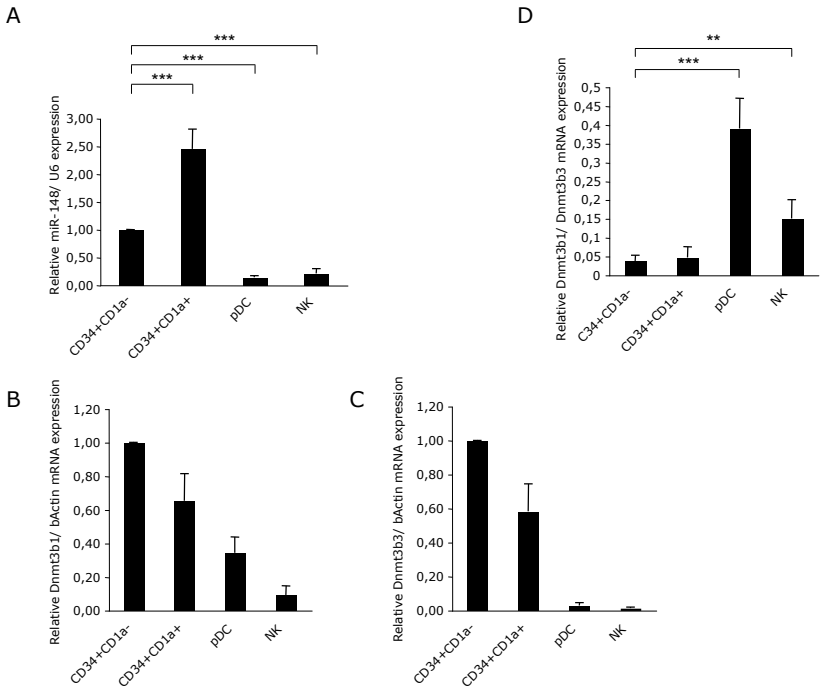


Figure 3. Relative miR-148, Dnmt3b1 and Dnmt3b3 expression in early thymic subsets. A Freshy isolated thymic subsets were analyzed by quantitative RT-PCR for the presence of miR-148 (n=3). Values were calculated relative to CD34+CD1a- cells. Error bars represent standard error of the mean. **B** and **C** As described in A but analyzed for Dnmt3b1 and Dnmt3b3 (n=2). D Relative Dnmt3b1/Dnmt3b3 ratio calculated from Dnmt3b1 and Dnmt3b3 values relative to CD34+CD1a- Dnmt3b1. ** P<0.01 *** P<0.001

miR-148 expression in pDC and NK cells, respectively (Figure 3A). Since miR-148 expression increased 2,5 times in T cell committed CD34+CD1a+ cells (Figure 2A), this result shows differential expression of miR-148 in different early thymic subsets, which all arise from the same pool of progenitor cells.

In addition, we examined Dnmt3b1 expression in the pDC and NK cells and found a reduction of its expression in both pDC and NK cells (Figure 3B). This suggests that also in pDC and NK cells Dnmt3b1 mRNA level is regulated by other means than miR-148 expression.

We previously described that miR-148 targets a site in the Dnmt3b1 coding region that is absent from the Dnmt3b3 splice variant mRNA (Chapter 4). Dnmt3b3 mRNA expression appears to be down-regulated in pDC and NK cells as well (Figure 3C). However, whereas the ratio of Dnmt3b1 and Dnmt3b3 was similar in CD34+CD1a- cells and CD34+CD1a+ cells, the relative abundance of Dnmt3b1 to Dnmt3b3 increased dramatically in pDC and NK cells (Figure 3D). This alteration in balance of Dnmt3b splice variants, could reflect the significant reduced miR-148 expression in pDC and NK cells. Yet, a role of changed splicing activity in the different hematopoietic subsets can not be excluded.

Currently it is unknown whether Dnmt3b splice variants play a role in Dnmt3b function. The role of the highly expressed and miR-148 resistant Dnmt3b3 splice variant in DNA methylation is particularly interesting. Some discrepancy has been reported on whether

human Dnmt3b3 is catalytically active or inactive depending on the substrate chosen (Chen et al., 2005; Soejima et al., 2003). This could reflect a change in target preference compared to Dnmt3b1 since Dnmt3b3 lacks a motif that could be involved in target recognition (Kumar et al., 1994). Further, it is interesting to note that overexpression of the Dnmt3b4 splice variant has been associated with dominant negative regulation, since hypomethylation of pericentromeric satellite regions was observed (Saito et al., 2002). Alternatively, this lack of satellite methylation could reflect a change in target preference. Since Dnmt3b3 and Dnmt3b4 lack the same target recognition motif, it would be interesting to determine whether Dnmt3b3 like Dnmt3b4 regulates target preference or has dominant negative activity. If so, it would be interesting to study the altered balance of Dnmt3b1 and Dnmt3b3 in more detail in pDC and NK development.

miR-148 and MLL

MLL was predicted by Targetscan 3.0 to be a likely miR-148 target. To test this experimentally, the MLL 3'UTR was cloned behind Luciferase in the pGL3 vector. Co-transfection of this vector with miR-148 resulted in a reduction of Luciferase activity of almost 50% compared to the empty pGL3 vector (Figure 4A). According to the prediction, two miR-148 target sites exist in the MLL 3'UTR. Deletion of 140 basepairs surrounding these sites of the 3 kb total MLL 3'UTR, completely abolished miR-148 dependent MLL regulation (Figure 4A). This suggests that miR-148 is indeed able to directly regulate MLL mRNA expression by interacting with at least one of these sites in its 3'UTR.

To test whether miR-148 could induce degradation of endogenous MLL mRNA, miR-148 was transfected into HeLa cells. Three days after transfection, RNA from these cells was isolated and cDNA was prepared

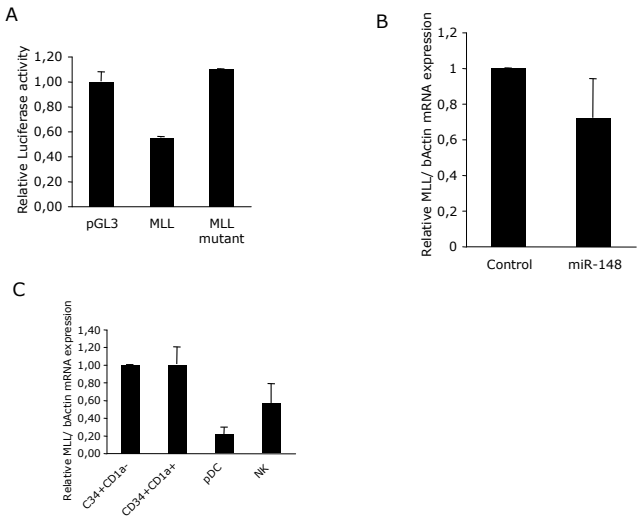


Figure 4. miR-148 mediated regulation of MLL and MLL expression in early thymic subsets. A miR-148 represses pGL3-Luc expression with the wild-type MLL 3'UTR (MLL) but not a mutant with mutated miR-148 target sites (MLL mutant). Expression was calculated relative to a co-transfection with a control miRNA. A representative experiment is shown. Error bars represent standard deviation. **B** Representative quantitative RT-PCR of endogenous MLL expression in HeLa cells transfected with a control miRNA or with miR-148. Error bars represent standard deviation. **C** Relative MLL expression was calculated in freshly isolated early thymic subsets (n=1). Error bars represent standard deviation.

for quantitative RT-PCR. The expression of MLL was 30% reduced in the presence of miR-148 suggesting that at least part of the miR-148 regulation is mediated through decreased mRNA stability (Figure 4B).
Next, we tested by quantitative RT-PCR whether MLL mRNA expression was affected in pDC or NK cells. Although miR-148 was considerably decreased in pDC and NK cells compared to mTPCs, we observed both in pDC and NK cells a down-regulation of MLL expression (Figure 4C). No change in MLL mRNA level was observed in CD34+CD1a+ cells compared to mTPCs.
The down-regulation of MLL expression in pDC and NK cells was less pronounced than the Dnmt3b down-regulation in these cells (Figure 3B). Nevertheless, it is a similar trend for both miR-148 targets. This suggests that miR-148 is highly expressed in cells with high Dnmt3b and MLL mRNA levels and low expressed in cells with low Dnmt3b and MLL mRNA levels. Notably, also human stem cell like testicular germ cells express high levels of miR-148 and these cells express high levels of Dnmt3b (Chapter 4 and data not shown).
Different kind of miRNA and mRNA interactions have been described so far (Plasterk, 2006). Some interactions might result in cell-fate switches during development and these interactions are under positive evolutionary pressure. Although the targeting of Dnmt3b and MLL by miR-148 appears to be evolutionary conserved (chapter 4 and Targetscan 3.0), these interactions are most likely not part of a switch in pDC and NK cell development from mTPCs.
Alternatively, it has been proposed that miRNAs and their targets can be induced by the same pathway. It has been suggested that this mechanism controls the fine-tuning of target gene expression (Hornstein and Shomron, 2006). An example is c-Myc that simultaneously induces the expression of E2F1 and of miR-17-5p and miR-20a that target E2F1 (O'Donnell et al., 2005). Likewise, miR-148, Dnmt3b and MLL might be co-regulated and miR-148 might play a role in fine-tuning the expression of these targets in early thymic subsets.

Exogenous miR-148 expression results in increased pDC cell numbers

Since miR-148 is down-regulated in pDC cells compared to CD34+CD1a- mTPCs, we were interested to determine the effect of miR-148 overexpression on pDC development. To study this we made use of an in vitro pDC differentiation assay (Schotte et al., 2003; Spits et al., 2000). We transduced CD34+CD1a- mTPCs derived from human post natal thymus with miR-148. The mixture of transduced and non-transduced cells were allowed to differentiate into pDC by coculturing with the murine bone marrow stromal cell line OP9 in the presence of the cytokines IL-7 and Flt3L. miR-147 and a construct containing a non-functional part of human Telomerase RNA (hTR) were used as controls and all three vectors contained a YFP marker. Transduced mTPCS were counted and analysed for YFP expression on day 0 and day 11. A slight increase in YFP positive cells was observed at day 11 in both control transduced cells. However, in the miR-148 transduced cells a dramatic increase in the percentage of YFP positive cells of almost 8 fold was observed (data not shown). A similar increase was observed for absolute cell numbers (data not shown). Staining for the pDC markers CD123high BDCA2+ showed increased percentages of pDCs (Figure 5A). Interestingly, calculating the absolute cell number of pDCs in the transduced populations revealed that forced miR-148 expression gave rise to 15-fold higher numbers of pDCs compared to the hTR and miR-147 expressing cells (Figure 5B).
miR-148 transduced YFP positive cells that were not positive for the pDC markers showed only a slight increase of four times in relative expansion (data not shown), indicating that the observed effect was most pronounced in pDC cells. Together, these results show that overexpression of miR-148 strongly increases the number of pDCs that are derived from CD34+CD1a- mTPCs.

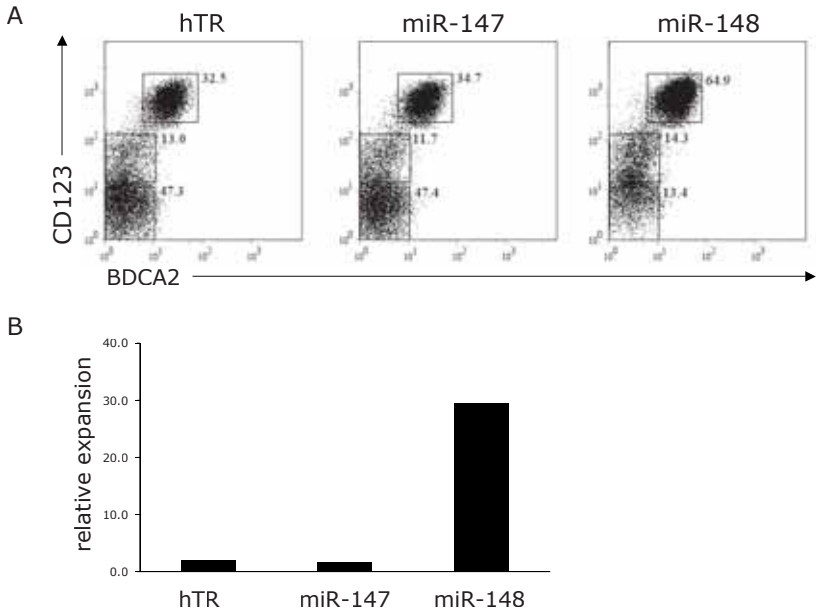


Figure 5. miR-148 overexpression results in an increased number of pDCs. A hTR, miR-147 and miR-148 transduced CD34+CD1a- cells were cocultered with OP9 cells in the presence of IL-7 and Flt3L and analysed after 11 days for the presence of pDCs (CD123highBDCA2+) **B** Relative expansion of pDC cells. Fold expansion in absolute cell numbers of the pD subset in the transduced population was calculated on basis of total numbers of cells harvested from the cultures after 11 days, percentages of transduced cells, and percentages of each population corrected for the number of input cells

Whether this effect is due to enhanced differentiation, proliferation or survival of pDCs remains to be determined. Furthermore, these results imply that miR-148 is down-regulated in pDCs to limit the number of pDCs in the human thymus. The main function of pDCs in the immune response is their capacity to produce high levels of type I interferons, which directly inhibits viral replication and triggers T cell mediated responses (Blom et al., 2002; Kadowaki et al., 2000). However, in the thymus pDC activation has recently been shown to impair thymic T cell development (Schmidlin et al., 2006). Therefore, miR-148 downregulation in pDCs could ensure proper thymic T cell development by keeping the numbers of pDCs low. In case miR-148 expression leads to increased survival and/or proliferation of pDCs it would be interesting to test pDC leukemic cells for expression of miR-148 (Chaperot et al., 2001). What remains to be determined is via which targets miR-148 exerts this function in pDCs and whether reduced Dnmt3b1 or MLL are involved. Furthermore, considering the similar expression profile of miR-148 and its targets in pDCs and NK cells it would be interesting to see whether miR-148 has a similar stimulatory effect on NK cell development as it has on pDCs.

Material and Methods

Constructs

All miRNAs were expressed from a retroviral miR-Vec vector expressing Blastacidin or YFP as described before (Voorhoeve et al., 2006). The MLL 3'UTR was cloned downstream the luciferase gene in AatII and AgeI sites generated in the pGL3 constructs. The following primers were used to clone MLL from genomic DNA:

MLL 3'UTR for
GCGCGACGTCAGCTGCTCTTCTCCCCCAGTGT
MLL 3'UTR rev
GATCACCGGTGGGGGATTCTTGGGAATGACCCATC
MLL mutant was generated by using endogenous SpeI and EcoRI sites. Digestion and religation deleted 140 nucleotides.

Cell culture and transfection

Hela cells were grown in Dulbecco Modified Eagle supplemented with 10% fetal bovine serum. Hela cells were transfected with Fugene (Roche) using manufacturer's protocol. For luciferase assays cells were cultured in 24-well plates and transfected one day after seeding with 5 ng pcDNA3-Renilla, 50 ng of the indicated pGL3 Luciferase construct and 200 ng of miR-Vec-148a or a control miRNA construct. Luciferase activity was measured 48 hours after transfection using the Dual-Luciferase reporter assay system (Promega).

RNA isolation and quantitative RT-PCR

RNA was isolated from cells using Trizol (Invitrogen) and 20 µg of glycogen was used for precipitation. Standard cDNA was prepared from 1.5 µg RNA of Hela cells, 0.5 or 1 µg RNA of T cell subsets of RNA using the standard protocol with 2 ul of random hexamer primers (Superscript III first-strand synthesis system for RT-PCR, Invitrogen). DTT was not added to the cDNA reaction to prevent interference with SYBRgreen. Real-time RT-PCR was performed with a standard 2-step amplification protocol of a MiniOpticon System (Bio-Rad) apparatus using a SYBRgreen PCR master mix (Applied Biosystem) and specific primers.
Dnmt3b1 forward GCCGTTCTTCTGGATGTTTGAG
Dnmt3b1 reverse ATCCTATTGTATTCCAAGCAGTCC
Dnmt3b3 forward ATCTCACGGTTCCTGGAGTG
Dnmt3b3 reverse AAGCCAAAGATCCTGTTCATCC
MLL forward CATTGATGCAGGTGAGATGG
MLL reverse GTGATTGATGAAGCGTGCAG
Beta Actin forward CCTGGCACCCAGCACAAT
Beta Actin reverse GGGCCGGACTCGTCATACT
The mirVana qRT-PCR miRNA detection kit (Ambion) was used to detect miRNA expression by quantitative RT-PCR. Specific miR-148a and U6 RT and PCR primers were used (Ambion) and 25 ng of RNA was used per reaction.

Reagents and monoclonal antibodies

Monoclonal antibodies to CD3, CD4, CD8, CD45RA and CD123, conjugated to FITC, PE, PeCy7, APC, or APCCy7 were purchased from Becton Dickinson (BD, San Jose, CA). CD1a-PE was obtained from Coulter/Immunotech (Luminy, France). FITC or APC conjugated anti-BDCA2 and anti-BDCA4 was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany).

Isolation of postnatal thymic subsets

The use of postnatal thymus tissue was approved by the Medical Ethical Committee of the Academic Medical Center. Thymocytes were obtained from surgical specimens removed from children up to 3 years of age undergoing open heart surgery, with informed consent from patients in accordance with the Declaration of Helsinki. The tissue was disrupted by mechanical means and pressed through a stainless steel mesh to obtain a single-cell suspension, which was left overnight at 4°C. The following day thymocytes were isolated from a Ficoll-Hypaque density gradient (Lymphoprep; Nycomed Pharma, Oslo, Norway). Subsequently, thymic subsets were isolated by MACS separation and subsequent cell sorting using a FACSAria (BD) as described below. Purity of the sorted cells in all experiments was greater than 99%.

CD34+1a- and CD34+1a+ subsets

CD34+ cells were enriched by immunomagnetic cell sorting, using a CD34 cell separation kit (varioMACS, Miltenyi Biotec). The CD34+ thymocytes were stained with antibodies against CD34, CD1a, CD56, and BDCA2. CD56-BDCA2- CD34+CD1a- (CD34+CD1a-) and CD56-BDCA2-CD34+CD1a+ (CD34+CD1a+) populations were sorted to purity.

CD4ISP and CD4SP T cells

CD4 immature single positive (CD4ISP) and mature CD4 single positive (CD4SP) T cells were sorted after CD8+ enrichment using a CD8 separation kit (Miltenyi Biotec) CD56-BDCA2-CD8-CD4+CD1a+ (CD4+CD1a+) and CD56-BDCA2-CD8-CD4+CD1a- (CD4+CD1a-) respectively.

CD3- and CD3+ DP T cells

Without prior MACS selection CD3- and CD3+ double positive T cells were sorted based on CD56-BDCA2-CD4+CD8+CD3- (CD3- DP) and CD56-BDCA2-CD4+CD8+CD3+ (CD3+ DP).

Natural Killer (NK) cells

CD56 positive cells were enriched by using the CD56 separation kit (Miltenyi Biotec) and subsequently sorted for NK cells on basis of CD56+CD3-. IL7R+ NK cells (described by Vosshenrich, Nature Immunology, 2006) were excluded by sorting negative for CD127.

Plasmacytoid Dendritic cells (pDC)

For the isolation of pDCs, BDCA4+ cells were enriched by immunomagnetic cell sorting, using a BDCA4+ cell separation kit (Miltenyi Biotec). The BDCA4+ cell fraction was labeled with anti-CD56, anti-CD123 and anti-CD45RA antibodies and CD56-CD123hiCD45RA+ cells were sorted to purity.

Retroviral transduction and differentiation assays

For transduction experiments CD34+CD1a- postnatal thymocytes were cultured overnight in Yssel medium44 with 5% NHS, 20 ng/mL SCF, and 10 ng/mL IL-7 (Yssel et al., 1984). The following day cells were incubated for 6 to 7 hours with virus supernatant in retronectin-coated plates (30 µg/mL; Takara Biomedicals, Otsu, Shiga, Japan). The development of pDCs was assessed by coculturing CD34+CD1a- (BDACA2-/CD56-/CD3-) thymic progenitor cells with 5 x 104 OP9 cells in MEM(alpha) (Invitrogen) with 20% FCS (Hyclone), 5 ng/mL IL-7, and 5 ng/mL Flt3L. At indicated timepoint the assays was analysed for the presence of pDCs by flow cytometry on a CyAn ADP analyzer (Dako, Fort Collins, Co).

References

Blom, B., Ligthart, S. J., Schotte, R., and Spits, H. (2002). Developmental origin of pre-DC2. Hum Immunol 63, 1072-1080.

Blom, B., and Spits, H. (2006). Development of human lymphoid cells. Annu Rev Immunol 24, 287-320.

Carleton, M., Cleary, M. A., and Linsley, P. S. (2007). MicroRNAs and cell cycle regulation. Cell Cycle 6, 2127-2132.

Chaperot, L., Bendriss, N., Manches, O., Gressin, R., Maynadie, M., Trimoreau, F., Orfeuvre, H., Corront, B., Feuillard, J., Sotto, J. J., et al. (2001). Identification of a leukemic counterpart of the plasmacytoid dendritic cells. Blood 97, 3210-3217.

Chen, C. Z., Li, L., Lodish, H. F., and Bartel, D. P. (2004). MicroRNAs modulate hematopoietic lineage differentiation. Science 303, 83-86.

Chen, Z. X., Mann, J. R., Hsieh, C. L., Riggs, A. D., and Chedin, F. (2005). Physical and functional interactions between the human DNMT3L protein and members of the de novo methyltransferase family. J Cell Biochem 95, 902-917.

Daser, A., and Rabbitts, T. H. (2005). The versatile mixed lineage leukaemia gene MLL and its many associations in leukaemogenesis. Semin Cancer Biol 15, 175-188.

Dik, W. A., Pike-Overzet, K., Weerkamp, F., de Ridder, D., de Haas, E. F., Baert, M. R., van der Spek, P., Koster, E. E., Reinders, M. J., van Dongen, J. J., et al. (2005). New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. J Exp Med 201, 1715-1723.

Ehrlich, M. (2003). The ICF syndrome, a DNA methyltransferase 3B deficiency and immunodeficiency disease. Clin Immunol 109, 17-28.

Garzon, R., Pichiorri, F., Palumbo, T., Iuliano, R., Cimmino, A., Aqeilan, R., Volinia, S., Bhatt, D., Alder, H., Marcucci, G., et al. (2006). MicroRNA fingerprints during human megakaryocytopoiesis. Proc Natl Acad Sci U S A 103, 5078-5083.

Hornstein, E., and Shomron, N. (2006). Canalization of development by microRNAs. Nat Genet 38 Suppl, S20-24.

Kadowaki, N., Antonenko, S., Lau, J. Y., and Liu, Y. J. (2000). Natural interferon alpha/beta-producing cells link innate and adaptive immunity. J Exp Med 192, 219-226.

Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R. J., and Wilson, G. G. (1994). The DNA (cytosine-5) methyltransferases. Nucleic Acids Res 22, 1-10.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., and Tuschl, T. (2002). Identification of tissue-specific microRNAs from mouse. Curr Biol 12, 735-739.

Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., Pfeffer, S., Rice, A., Kamphorst, A. O., Landthaler, M., et al. (2007). A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129, 1401-1414.

Milne, T. A., Briggs, S. D., Brock, H. W., Martin, M. E., Gibbs, D., Allis, C. D., and Hess, J. L. (2002). MLL targets SET domain methyltransferase activity to Hox gene promoters. Mol Cell 10, 1107-1117.

Nakamura, T., Mori, T., Tada, S., Krajewski, W., Rozovskaia, T., Wassell, R., Dubois, G., Mazo, A., Croce, C. M., and

Canaani, E. (2002). ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. Mol Cell 10, 1119-1128.

O'Donnell, K. A., Wentzel, E. A., Zeller, K. I., Dang, C. V., and Mendell, J. T. (2005). c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435, 839-843.

Plasterk, R. H. (2006). Micro RNAs in animal development. Cell 124, 877-881.

Saito, Y., Kanai, Y., Sakamoto, M., Saito, H., Ishii, H., and Hirohashi, S. (2002). Overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis. Proc Natl Acad Sci U S A 99, 10060-10065.

Schmidlin, H., Dontje, W., Groot, F., Ligthart, S. J., Colantonio, A. D., Oud, M. E., Schilder-Tol, E. J., Spaargaren, M., Spits, H., Uittenbogaart, C. H., and Blom, B. (2006). Stimulated plasmacytoid dendritic cells impair human T-cell development. Blood 108, 3792-3800.

Schotte, R., Rissoan, M. C., Bendriss-Vermare, N., Bridon, J. M., Duhen, T., Weijer, K., Briere, F., and Spits, H. (2003). The transcription factor Spi-B is expressed in plasmacytoid DC precursors and inhibits T-, B-, and NK-cell development. Blood 101, 1015-1023.

Soejima, K., Fang, W., and Rollins, B. J. (2003). DNA methyltransferase 3b contributes to oncogenic transformation induced by SV40T antigen and activated Ras. Oncogene 22, 4723-4733.

Spits, H., Couwenberg, F., Bakker, A. Q., Weijer, K., and Uittenbogaart, C. H. (2000). Id2 and Id3 inhibit development of CD34(+) stem cells into predendritic cell (pre-DC)2 but not into pre-DC1. Evidence for a lymphoid origin of pre-DC2. J Exp Med 192, 1775-1784.

Thai, T. H., Calado, D. P., Casola, S., Ansel, K. M., Xiao, C., Xue, Y., Murphy, A., Frendewey, D., Valenzuela, D., Kutok, J. L., et al. (2007). Regulation of the germinal center response by microRNA-155. Science 316, 604-608.

Ueda, Y., Okano, M., Williams, C., Chen, T., Georgopoulos, K., and Li, E. (2006). Roles for Dnmt3b in mammalian development: a mouse model for the ICF syndrome. Development 133, 1183-1192.

Voorhoeve, P. M., le Sage, C., Schrier, M., Gillis, A. J., Stoop, H., Nagel, R., Liu, Y. P., van Duijse, J., Drost, J., Griekspoor, A., et al. (2006). A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell 124, 1169-1181.

Xiao, C., Calado, D. P., Galler, G., Thai, T. H., Patterson, H. C., Wang, J., Rajewsky, N., Bender, T. P., and Rajewsky, K. (2007). MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell 131, 146-159.

Xu, G. L., Bestor, T. H., Bourc'his, D., Hsieh, C. L., Tommerup, N., Bugge, M., Hulten, M., Qu, X., Russo, J. J., and Viegas-Pequignot, E. (1999). Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402, 187-191.

Yssel, H., De Vries, J. E., Koken, M., Van Blitterswijk, W., and Spits, H. (1984). Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. J Immunol Methods 72, 219-227.

Yu, B. D., Hess, J. L., Horning, S. E., Brown, G. A., and Korsmeyer, S. J. (1995). Altered Hox expression and segmental identity in Mll-mutant mice. Nature 378, 505-508.

Chapter 6

miR-152 plays a role in S-phase and G2/M-phase cell cycle progression of diploid fibroblasts

Anja M. Duursma, Joachim Oude Vrielink, Reuven Agami

miR-152 plays a role in S-phase and G2/M-phase cell cycle progression of diploid fibroblasts

Anja M. Duursma, Joachim Oude Vrielink, Reuven Agami

The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Eukaryotic cells have evolved several mechanisms to ensure proper proliferation. Upon detection of aberrant replication or DNA damage, cells activate an intra-S phase or a G2 phase checkpoint, which arrest cells to allow for repair and to prevent entry into mitosis. MicroRNAs (miRNAs) are small RNA molecules of approximately 22 nucleotides that bind to target mRNAs and inhibit translation. miRNAs have been implicated in many cellular processes, but only a few miRNAs have been shown to be involved in cell cycle regulation. In this study we aimed to study the role of miR-152 in diploid BJ ET fibroblasts by using antagomirs, which inhibit miRNA function. Interestingly, we observed that inhibition of miR-152 resulted in an increase of the relative percentage of S-phase and G2/M–phase cells. More detailed analysis revealed that these cells arrested in G2-phase. Together these results suggest that endogenous expression of miR-152 is required for proper cell cycle progression.

Introduction

Eukaryotic cells have evolved several mechanisms to ensure proper DNA replication and to prevent genomic instability and cancer. A crucial role is played by checkpoints that delay cell cycle progression in the presence of damaged DNA (Nyberg et al., 2002). This allows time for DNA repair or when the damage is unrepairable in a permanent cell cycle arrest or apoptosis. Detection of DNA damage during DNA replication in S-phase, will activate an intra-S-phase checkpoint in early S-phase, which suppresses firing of late replication origins and stabilizes the replication fork (Bartek et al., 2004). Cells that enter G2 with incompletely replicated DNA or with damaged DNA trigger the G2/M checkpoint that arrests cells in G2 and prevents entry into mitosis (Lobrich and Jeggo, 2007; O'Connell and Cimprich, 2005).

These responses are primarily controlled by the ATM (Ataxia Telangiectasia-Mutated) and ATR (ATM and Rad-3 related) protein kinases (Lobrich and Jeggo, 2007). ATM is activated by double strand breaks (DSBs) that are often induced by ionizing radiation (IR), whereas ATR can be activated by multiple types of DNA damage. ATR appears to be triggered by single stranded DNA, which is generated in response to ultraviolet (UV) radiation, DNA replication inhibitors, but also by processed DSBs. In S-phase, stalled or collapsed replication forks activate ATR, leading to the phosphorylation of its main effector Chk1, resulting in the phosphorylation and degradation of Cdc25A (Mailand et al., 2000; Paulsen and Cimprich, 2007). This in turn prevents activation of CDK2/ Cyclin E, which inhibits initiation of new replication origins. In G2-phase, activation of both ATR and ATM, with its downstream effector Chk2, result in a similar signalling cascade towards Cdc25. Inactivation of Cdc25 phosphases inhibits CDK1/ Cyclin B activity that is required for progression into mitosis (Lobrich and Jeggo, 2007).

MicroRNAs (miRNAs) are small RNA molecules of approximately 22 nucleotides, which inhibit mRNA translation through binding to target mRNA sequences (Bushati and Cohen, 2007). Most described target sequences are located in the 3' untranslated region (3'UTR), although regulation through binding of the protein coding region has

been found as well (Llave et al., 2002) (and this thesis Chapter 4). miRNAs have been described to be involved in several processes, such as apoptosis and differentiation, but only a few have been shown to play a role in cell cycle progression (Carleton et al., 2007). Overexpression of both miR-34 and miR-16 were shown to induce a G1 arrest and were shown to regulate multiple target genes that are involved in cell cycle progression (He et al., 2007; Linsley et al., 2007). On the other hand, inhibition of miR-221 and miR-222 in cancer cell lines, resulted in a G1 arrest through relief of miR-221 and miR-222 mediated p27 repression (le Sage et al., 2007). Inhibition of another miRNA that was found widely expressed in cancer cells, miR-27a, resulted in an increase of G2/M cells (Mertens-Talcott et al., 2007). This was thought to be a result of increased expression of the miR-27a target Myt-1, which phosphorylates and inactivates Cdc2.
We detected specific expression of miR-152 in diploid BJ ET fibroblasts. To study its function we made use of antagomirs, antisense 2'-O-methylated RNA oligonucleotides that inhibit miRNA function (Krutzfeldt et al., 2005; le Sage et al., 2007). Interestingly, we observed that inhibition of miR-152 resulted in an increase in the relative number of S-phase cells and a G2 arrest.

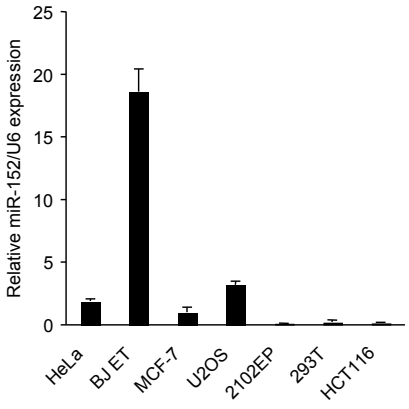


Figure 1. miR-152 is expressed in BJ ET primary fibroblasts. miR-152 expression was determined in several cell lines by quantitative RT-PCR.

Results

We analyzed the expression of miR-152 by quantitative Real-time PCR in several cell lines and detected significant expression in human immortalized diploid BJ ET fibroblasts (Figure 1). Low expression of miR-152 was detected in U2OS cells while other tested cell lines where negative. This result is in line with miR-152 expression data derived from small RNA library sequencing (Landgraf et al., 2007). Notably, BJ ET cells expressed only very low amounts of miR-148a, a conserved member of the miR-152 family, whereas miR-148b was undetectable (data not shown). This enabled us to study the role of miR-152 in fibroblasts.
To study the function of endogenous miR-152 in BJ ET fibroblasts we designed antagomirs, molecules that were previously shown to inhibit miRNA function (Krutzfeldt et al., 2005; le Sage et al., 2007). These inhibitors are 2'-O-methylated RNA oligonucleotides that are complimentary to a specific miRNA and contain a cholesterol group at the 5' or 3' end enabling cellular uptake through membranes. To test our designed miR-152 antagomiR (antagomiR-152) we made use of a luciferase assay in which the 3'UTR of Dnmt1, a verified target of miR-152, was cloned downstream luciferase. Co-transfection of HeLa cells with miR-152 and a Luciferase-Dnmt1 wild-type-3'UTR (Dnmt1 wt) resulted in 2-fold reduced Luciferase expression relative to cells transfected with a control miRNA (Figure 2A). This effect was abrogated when the miR-152 target site in the Dnmt1 3'UTR was mutated (Dnmt1 mut), demonstrating the specificity of the miR-152 Dnmt1 3'UTR interaction. To test the inhibitory effect of antagomiR-152, cells were co-transfected with miR-152 and the Luciferase-Dnmt1-3'UTR construct. 16 hours after transfection either antagomiR-152 or a scrambled control was added to the culture medium and cells were harvested 48 hours later. An almost 2-fold increase of Dnmt1-3'UTR wt Luciferase activity was detected in antagomiR-152 treated cells compared to antagomiR-control treated cells. This indicated that antagomiR-152 is a useful tool

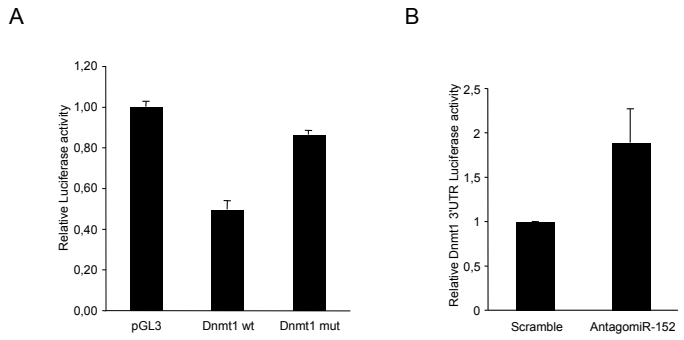


Figure 2. AntagomiR-152 inhibits miR-152 activity towards a Luciferase-Dnmt1-3'UTR. A miR-152 represses pGL3-Luc expression with the wild-type Dnmt1 3'UTR (Dnmt1 wt) but not a mutant with a mutated miR-152 target site (Dnmt1 mut). Expression was calculated relative to a co-transfection with a control miRNA. **B** AntagomiR-152 represses miR-152 mediated regulation of Dnmt1. Cells transfected with miR-152 and Dnmt1 were treated with AntagomiR-152 or a Scrambled control. Error bars represent standard deviation.

to inhibit miR-152 activity.

Interestingly, we noted a decrease in cell proliferation upon administering antagomiR-152 to BJ ET cells. To quantify this effect we seeded cells in equal amounts and treated them with either antagomiR-152 or a scramble control and counted cells on days 0, 2 and 4. Whereas control treated cells continued proliferating, we observed a complete block in proliferation after 2 days in antagomiR-152 treated cells (Figure 3A). After 4 days, cell numbers decreased compared to day 0, which is indicative of cell death.

To examine whether the block in proliferation was due to an arrest in a specific stage of the cell cycle, we determined the cell cycle profile of asynchronous dividing cells after 24 hours of antagomiR-152 treatment. Cells were fixed and labelled with propidium iodide (PI) and subsequently analyzed by flow cytometry. Interestingly, we observed a 3-fold increase in S-phase and an almost 4-fold increase in G2/M when cells were treated with antagomiR-152 (Figure 3B). No detectable effect on proliferation was seen with antagomiR-control. Further, G1-phase was significantly reduced in antagomiR-152 treated cells compared to control treated cells. Notably, the cell cycle profile of control treated cells was identical to untreated cells (data not shown), implying that the observed changes in cell cycle distribution in response to antagomiR-152 treatment were due to inhibition of endogenous miR-152 activity. Taken together, the ability of antagomiR-152 to inhibit proliferation and the dramatic increase in cells in G2/M suggest that cells lacking functional miR-152 are not able to divide.

To distinguish between G2 and M phases, we performed the same experiment 24 hours of antagomiR treatment) only in the presence of nocodazole, which inhibits mitosis. Cells were harvested and labelled with PI and the MPM-2 antibody that specifically recognizes mitotic phospho-epitopes. Flow cytometric analysis revealed separate mitotic and G2 percentages. While 41% of control treated cells accumulated in mitosis, this was reduced to 17% in antagomiR-152 treated cells. Most important, inhibition of miR-152 resulted in a more than 2-fold increase of cells in G2-phase. Whereas 13% of control cells were shown to be in G2-phase, 33% of antagomiR-152 related cells appeared in G2, suggesting that these cells are in a G2 arrest. In summary, these results illustrate the requirement of miR-152 for proper cell cycle progression through G2 phase.

The similar total percentage of cells in G2 and M-phase in cells treated with nocodazole

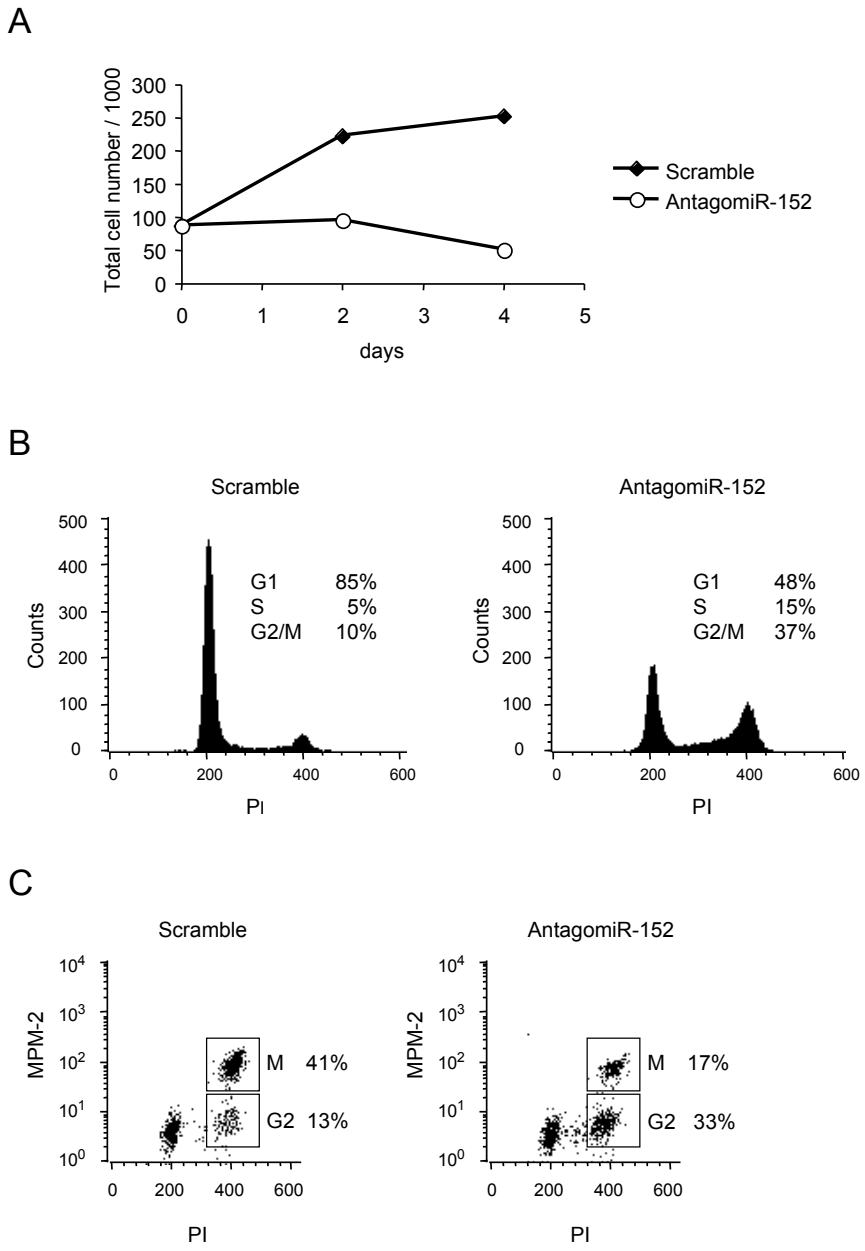


Figure 3. Inhibition of endogenous miR-152 in BJ ET diploid fibroblasts results in a G2 arrest. A Inhibition of miR-152 by AntagomiR-152 results in reduced proliferation. BJ ET cells were seeded and counted on day 0, 2 and 4 after AntagomiR-152 treatment. A Scrambled AntagomiR was used as control **B** Inhibition of miR-152 by AntagomiR-152 results in increased S-phase and G2/M. Asynchronously dividing BJ ET cells were treated 24 hours with AntagomiR-152 or a Scrambled controls and fixed and labelled with PI. Cells were analysed by flow cytometry. C Inhibition of miR-152 by AntagomiR-152 results in a G2 arrest. Cells were treated for 24 hours with AntagomiR-152 or a Scrambled control in the presence of Nocodazole. Cells were harvested and labelled with PI and MPM-2 and analysed by flow cytometry.

(scramble 54%, antagomiR-152 50%) suggests that antagomiR-152 and control treated cells divide with comparable kinetics. However, also an increase in S-phase was observed in antagomiR-152 treated cells (Figure 2B). To test whether cells entered S-phase faster in the absence of miR-152, BJ ET cells were serum starved for 48 hours to synchronize cells in G0. Cells were released in the presence of antagomiRs upon addition of serum and harvested 12, 16, and 21 hours later. Cells were labelled with PI and cell cycle distribution was analyzed. As depicted in Figure 4 no significant change in S-phase entry was observed, demonstrating that miR-152 is not involved in G1/S transition.

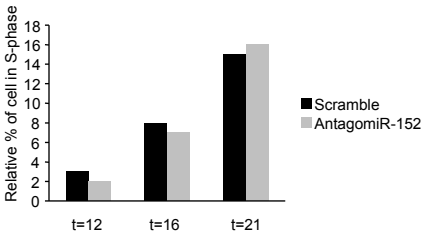


Figure 4. Inhibition of endogenous miR-152 does not result in increased S-phase entry. BJ ET cells were synchronized by serum starvation in G0 for 48 hours. Cells were released by addition of serum in the presence of AntagomiR-152 or a Scrambled control.

Discussion

Some miRNAs are widely expressed, whereas other miRNAs are only expressed in specific cell types (Landgraf et al., 2007). We observed that miR-152 was particularly expressed in BJ ET diploid fibroblasts (Figure 1). Low expression was detected in U2OS osteosarcoma cells but other tested cancer cell lines were considered negative for miR-152 expression (Figure 1). This is in line with data derived from small RNA library sequencing that also revealed high expression of miR-152 in fibroblasts and low expression in U2OS cells (Landgraf et al., 2007). This study also revealed considerably miR-152 expression in tissues from the Thyroid, Uterus, Prostate and Epididymis and Unrestricted Somatic Stem cells. Notably, this indicates that miR-152 is only expressed in a minority of the total of 250 tissues and tumour samples screened in this study. Therefore, miR-152 expression might be restricted to certain cell types and there it might have a specialised function.

To study the role of miR-152 in BJ ET fibroblast we inhibited its function by antisense antagomirs. Antagomirs were previously shown to be highly specific and to function both in vivo and in vitro in inhibiting miRNAs (Krutzfeldt et al., 2007; Krutzfeldt et al., 2005; le Sage et al., 2007). We demonstrated functionality of our designed antagomiR-152 by showing relief of miR-152 mediated repression in a luciferase reporter assay.

Interestingly, we noticed that long-term inhibition of miR-152 resulted in decreased cell proliferation and eventually in reduced number of cells, indicative of cell death (Figure 2A). Cell cycle profile analysis revealed a major increase in the number of cells in S-phase and G2/M-phase (Figure 2B). More specific analysis showed a clear increase of cells in G2-phase, suggesting that these cells are in G2 arrest.

The G2 arrest prevents cells with damaged DNA or with DNA that is not fully replicated from entering mitosis (Lobrich and Jeggo, 2007). Since we observed an increase in the percentage of cells in S-phase, this could mean that the observed G2 arrest was due to problems during S-phase. Alternatively, the increased number of S-phase could be a result of increased proliferation. To distinguish between these possibilities, cells were synchronized in G0 by serum starvation and released in the presence of a control or miR-152 antagomir. No difference in kinetics of S-phase entry was observed, suggesting that the relative increase in S-phase cells is most likely not due to more replicating cells. The increase rather reflects a slow-down of replication in the absence of miR-152 or an S-phase arrest. Activation of the

intra-S-phase checkpoint prevents firing of late replicating replication origins, therefore arresting cells in early S-phase (Bartek et al., 2004). The observed increase in S-phase appears to be predominant in late S-phase (Figure 2B), implying that it is not the intra-S-phase checkpoint that is activated. Together these data could indicate that DNA damage accumulates during S-phase and that cells slow down end S-phase and arrest in G2 to prevent entry of mitosis. However, another possibility is that cells accumulate due to aberrant activation of checkpoint proteins or due to lack of a cell cycle protein that is essential for progression to mitosis.
To clarify the function of miR-152 in S-phase and G2 progression, it is obligatory to identify the targets that are involved. We previously identified Dnmt3b, Dnmt1 and MLL as miR-152 targets. Inactivation of Dnmt1 was shown to result in mitotic catastrophe in human cancer cells (Chen et al., 2007). In future, it would therefore be interesting to determine whether increased expression of Dnmt1 or our other identified targets, have an effect on G2-phase progression.

Material and Methods

Cell culture and antagomiRs

All cells were grown in Dulbecco Modified Eagle supplemented with 10% fetal bovine serum. For serum starvation cells were sultered 72 hours in 0,5% fetal bovine serum and released in the normal medium. BJ ET cells expressing the Ecotropic Receptor and Human Telomerase were used for antagomiR assays. Cells were seeded 6 hour prior to addition of antagomiR and treated for the indicated times. AntagomiRs were used at concentrantions of 6 uM. Scramble and miR-152 antagomiRs were purchased from Dharmacon. Sequences are:
Scramble
Chl-AAUCCUCGGUCUUGCGUUAUGCGCAAUAAG
antagomiR-152
Chl-CCGGGCCCAAGUUCUGUCAUGCACUGACUGCU
Chl is the 5' cholesterol modification and all nucleosides where 2'-O-methyl modified.

RNA isolation and quantitative RT-PCR

RNA was isolated from cells using Trizol (Invitrogen) and 20 µg of glycogen was used for precipitation. The mirVana qRT-PCR miRNA detection kit (Ambion) was used to detect miRNA expression by quantitative RT-PCR. Specific miR-152 and U6 RT and PCR primers were used (Ambion) and 25 ng of RNA was used per reaction. Real-time RT-PCR was performed using the MiniOpticon System (Bio-Rad).

Luciferase assays

The Dnmt1 3'UTR was cloned from genomic DNA using the forward primer
GATCGACGTCTTCTGCCCTCCCGTCACCCCTGTTTCTG
and reverse primer
GATCACCGGTGGTTTATAGGAGAGATTTATTTGAAGAAAT.
The Dnmt1 mutant was generated by using a different foward primer:
GATCGACGTCTTCTGCCCTCCCGTCACCCCTGTTTCTGGCAC-CAGGAATCCCCAACAAAGCTTCATGTTGTGTTTTTAAC.
The Dnmt1 3'UTR was cloned downstream the luciferase gene in AatII and AgeI sites generated in the pGL3 constructs. Hela cells were used for Luciferase assays and transfected with Fugene (Roche) using manufacturer's protocol. Cells were cultured in 24-well plates and transfected one day after seeding with 5 ng pcDNA3-Renilla, 50 ng of the indicated pGL3 Luciferase construct and 200 ng of miR-Vec-152 or a control miRNA construct. Luciferase activity was measured 48 hours after transfection using the Dual-Luciferase reporter assay system (Promega).

Flow Cytometry

For cell cycle analysis cells were trypsinized, collected by centrifugation and resuspended in PBS containing 0.6% NP-40, 50 ug/ml RNaseA and 50 mg/ml propidium iodide for 10 min. For the MPM2 staining cells were cultured 24 hrs in the presence of Nocodazole (30 ng/ml). Cells were harvested and fixed in ice-cold ethanol ON. Next cells were blocked with PBS-0.05% Tween in 1% BSA (Sigma) and stained 2 hrs with MPM-2 antibody (Upstate) and Allexa 488 anti-mouse (Molecular Probes). Flow cytrometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson) and analysed with Cell Quest software (Becton Dickinson).

References

Bartek, J., Lukas, C., and Lukas, J. (2004). Checking on DNA damage in S phase. Nat Rev Mol Cell Biol 5, 792-804.

Bushati, N., and Cohen, S. M. (2007). microRNA Functions. Annu Rev Cell Dev Biol 23, 175-205.

Carleton, M., Cleary, M. A., and Linsley, P. S. (2007). MicroRNAs and cell cycle regulation. Cell Cycle 6, 2127-2132.

Chen, T., Hevi, S., Gay, F., Tsujimoto, N., He, T., Zhang, B., Ueda, Y., and Li, E. (2007). Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. Nat Genet 39, 391-396.

He, L., He, X., Lim, L. P., de Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D., et al. (2007). A microRNA component of the p53 tumour suppressor network. Nature 447, 1130-1134.

Krutzfeldt, J., Kuwajima, S., Braich, R., Rajeev, K. G., Pena, J., Tuschl, T., Manoharan, M., and Stoffel, M. (2007). Specificity, duplex degradation and subcellular localization of antagomirs. Nucleic Acids Res 35, 2885-2892.

Krutzfeldt, J., Rajewsky, N., Braich, R., Rajeev, K. G., Tuschl, T., Manoharan, M., and Stoffel, M. (2005). Silencing of microRNAs in vivo with 'antagomirs'. Nature 438, 685-689.

Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., Pfeffer, S., Rice, A., Kamphorst, A. O., Landthaler, M., et al. (2007). A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129, 1401-1414.

le Sage, C., Nagel, R., Egan, D. A., Schrier, M., Mesman, E., Mangiola, A., Anile, C., Maira, G., Mercatelli, N., Ciafre, S. A., et al. (2007). Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. Embo J 26, 3699-3708.

Linsley, P. S., Schelter, J., Burchard, J., Kibukawa, M., Martin, M. M., Bartz, S. R., Johnson, J. M., Cummins, J. M., Raymond, C. K., Dai, H., et al. (2007). Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. Mol Cell Biol 27, 2240-2252.

Llave, C., Xie, Z., Kasschau, K. D., and Carrington, J. C. (2002). Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science 297, 2053-2056.

Lobrich, M., and Jeggo, P. A. (2007). The impact of a negligent G2/M checkpoint on genomic instability and cancer induction. Nat Rev Cancer 7, 861-869.

Mailand, N., Falck, J., Lukas, C., Syljuasen, R. G., Welcker, M., Bartek, J., and Lukas, J. (2000). Rapid destruction of human Cdc25A in response to DNA damage. Science 288, 1425-1429.

Mertens-Talcott, S. U., Chintharlapalli, S., Li, X., and Safe, S. (2007). The oncogenic microRNA-27a targets genes that regulate specificity protein transcription factors and the G2-M checkpoint in MDA-MB-231 breast cancer cells. Cancer Res 67, 11001-11011.

Nyberg, K. A., Michelson, R. J., Putnam, C. W., and Weinert, T. A. (2002). Toward maintaining the genome: DNA damage and replication checkpoints. Annu Rev Genet 36, 617-656.

O'Connell, M. J., and Cimprich, K. A. (2005). G2 damage checkpoints: what is the turn-on? J Cell Sci 118, 1-6.

Paulsen, R. D., and Cimprich, K. A. (2007). The ATR pathway: fine-tuning the fork. DNA Repair (Amst) 6, 953-966.

Acknowledgements

We would like to thank Martijn Kedde and Josyanne van Duijse for many helpful discussions.

Chapter 7

General Discussion

General Discussion

Cancer is a result of multiple genetic and epigenetic alterations, which together change the balance of cellular regulatory mechanisms towards uncontrolled proliferation. Crucial mechanism to prevent cancer are cell cycle checkpoints and the DNA damage response (DDR) that prevents cell cycle progression in the presence of DNA damage or aberrantly replicated DNA. How cells protect themselves from DNA damage and how the cell cycle is regulated are consequently key questions in current cancer research. Furthermore, the role of epigenetic DNA modifications have received attention for their role in regulating gene expression and genome stability. This thesis describes several aspects of these topics by studying Cdc6 and the miR-148/152 family. In the following section I will discuss the major findings and their relevance to oncogenesis.

Dual function of Cdc6 in DNA damage checkpoints

The first study described in this thesis (Chapter 2), reveals a novel mechanism that is used by cells to prevent DNA replication in the presence of DNA damage. We demonstrated that Cdc6, an essential protein for initiation of DNA replication, is downregulated upon ionizing radiation (IR) in a p53-dependent manner. In particular, we found that serine 54 phosphorylation of Cdc6 by CDK2/ Cyclin E stabilizes the protein by inhibiting recognition by the E3-ligase APCCdh1. Yet, following DNA damage, p53 induced upregulation of p21 inhibits this kinase and as a result Cdc6 is destabilized. Since Cdc6 is essential for initiation of DNA replication, these data suggest that DNA damage-dependent regulation of Cdc6 might play a role in preventing DNA replication in cells with damaged DNA.

Also p53-independent mechanism of CDC6 destruction after DNA damage have been described. Cdc6 was found to be degraded following treatment with the alkylating agents adozelesin (Blanchard et al., 2002) methyl methane sulfonate (MMS) and ultraviolet (UV) radiation (Hall et al., 2007). This regulation was proposed to involve Cdc6 ubiquitination by Huwe1 since Cdc6 was shown to be ubiquitinated in vitro and a Huwe1 knockdown rescued Cdc6 degradation following UV (Hall et al., 2007). Since the latter was shown in cells with non-functional p53 it would be of interest to determine the relative contribution of p53 and Huwe1 to Cdc6 degradation in cells with wild-type p53.

In contrast to these findings, a positive role for Cdc6 in the DDR has been described as well. First, a role of the fission yeast Cdc6 ortholog Cdc18 was described in checkpoint activation, since its deletion not only blocked DNA replication, but also allowed cells to enter mitosis without prior DNA replication (Kelly et al., 1993; Piatti et al., 1995). More recent, several studies reported a similar role of Cdc6 in checkpoint activation in higher eukaryotes. First, a study in Xenopus egg extracts demonstrated the requirement of Cdc6 for Chk1 activation in response to replication inhibition (Oehlmann et al., 2004). Second, overexpression of Cdc6 in human G2-phase cells prevented entry into mitosis in a Chk1-dependent manner (Clay-Farrace et al., 2003). Third, RNA interference mediated depletion of Cdc6 in human cells that where synchronized in S-phase inhibited ATR-Chk1 activation, although these cells displayed inefficient DNA replication that resulted in mitotic failure (Lau et al., 2006). Thus, this positive role of Cdc6 in checkpoint activation appears conserved from yeast to human. Interestingly, recent findings in fission yeast suggested direct checkpoint activation by Cdc6, as Cdc18 was shown to anchor the ATR ortholog Rad3 to chromatin and to activate it in the absence of DNA replication

structures (Fersht et al., 2007; Hermand and Nurse, 2007). Taken together, these results strongly suggest that Cdc6 plays a role in the S/M checkpoint in addition to its role in origin licensing in G1. Thus, in separate cell cycle stages Cdc6 plays opposing roles in the DNA damage response (DDR). Whereas Cdc6 is a DNA damage checkpoint target in G1 and its degradation prevents entry into S-phase its presence in G2 plays a role in checkpoint activation. Since Cdc6 is not degraded in G2-phase of the human cell cycle but in M-phase in a APCCdh1-dependent manner (Petersen et al., 2000) it would be of interest to determine how Cdc6 activates a checkpoint in G2 in response to DNA damage. One possibility would be that Cdc6 is modified in this particular phase of the cell cycle in response to DNA damage.

The role of Cdc6 in cell proliferation and tumourigenesis

The first step in initiation of DNA replication involves the binding of Cdc6 and Cdt1 to replication origins and has been referred to as origin licensing. The Cdk2/Cyclin E dependent serine 54 phosphorylation and subsequent stabilization of Cdc6 that we identified (Chapter 2) has also implications for the licensing process. An outstanding question regarding the mechanism of origin licensing was how both an activator of licensing, Cdc6, and inhibitors of licensing, Geminin and Cyclin A, could be targeted by the same E3-ligase APC^{Cdh1}. We now demonstrated that phosphorylation of Cdc6 serine 54 by CDK2/ Cyclin E in early G1 protects the protein from negative regulation by APCCdh1(Chapter 2). This allows for a window of time where Cdc6 can accumulate before the replication inhibitors Geminin and Cyclin A, which enables licensing of replication origins by Cdc6 and Cdt1 (discussed in Chapter 3). This mechanism of Cdc6 protection was also shown to play a critical role in exit from quiescence (Mailand and Diffley, 2005).

Recently, Cdc6 caught attention by its proposed roles in oncogenesis. Cdc6 transcription is regulated by E2F transcription factors (Hateboer et al., 1998) and since inactivation of the negative regulators of E2F activity p16 and Rb are common in cancer cells, it is not surprising that increased Cdc6 levels are observed in many cancers (Borlado and Mendez, 2007). The p53 tumour suppressor is often lost in cancers as well and in line with p53-dependent regulation of Cdc6, we observed increased Cdc6 protein level in p53 knock-down (kd) primary fibroblast. Interestingly, we observed that the increase in replicating cells in p53kd primary fibroblasts could be reversed by simultaneous downregulation of Cdc6, further stressing the importance of p53-mediated regulation of CDK2/ Cyclin E in regulation of Cdc6 protein abundance (Chapter 2). Together, these results suggest that the upregulated Cdc6 protein level commonly observed in cancer cells could in part be responsible for the increased proliferation of these cells.

Another proposed role for Cdc6 in oncogenesis is that Cdc6 overexpression drives aberrant DNA replication, which results in genomic instability. The first evidence for such a mechanism came from a study that demonstrated that overexpression of Cdc6 and Cdt1 along with CDK2/ Cyclin A induced rereplication of the genome in the absence of p53 (Vaziri et al., 2003). Interestingly, it was also shown that expression of activated H-RasV12 in normal human cells, induced increased Cdc6 expression. Furthermore, these cells showed reduced inter-origin distances and rereplicated DNA, which eventually resulted in activation of the DDR and oncogene induced senescence (OIS) (Di Micco et al., 2006). A second study showed that Cdc6 overexpression in human primary fibroblasts triggered the DDR and subsequent OIS (Bartkova et al., 2006). With regards to the dual roles that have been described for Cdc6 in the DDR it would be of interest to determine in more detail whether increased Cdc6 level activates the DDR due to aberrant DNA replication or through direct activation of the checkpoint response.

The miR-148/152 family and regulation of its targets

The second part of this thesis describes the role of the miR-148/152 family in multiple processes (Chapter 4-6). We identified several target proteins of this miRNA family, respectively the DNA methyltransferases Dnmt3b and Dnmt1 and the histone methyltransferase MLL. However, many more targets might exist as a single miRNA has been predicted to regulate the expression of hundreds of mRNAs (Krek et al., 2005).

Current described mammalian miRNAs regulate their target mRNAs through binding of the 3' untranslated region (3'UTR) and thereby inhibit mRNA translation and decrease mRNA stability (Bushati and Cohen, 2007). Indeed, the miR-148/152 family regulates Dnmt1 and MLL through interaction with their 3'UTR (Chapter 5 and Chapter 6 and data not shown). However, Dnmt3b appeared to be targeted through a region with high sequence complementarity to the miRNA in its protein coding sequence (CDS). Interestingly, this is to our knowledge the first example of a mammalian miRNA that regulates its target expresssion through interaction with the CDS. Yet, targeting of the CDS by a miRNA with high sequence complementarity is the most common mechanism of miRNA mediated regulation in plants (Rhoades et al., 2002), which implies that this is a conserved regulatory mechanism. In addition, we demonstrated that a Dnmt3b splice variant lacking the target sequence, Dnmt3b3, is resistant to miR-148 mediated regulation (Chapter 4). This enables miR-148 to regulate the relative abundance of Dnmt3b splice-variants. It would be interesting to determine whether miRNA targeting of CDS in mammals, and with this the possibility to regulate splice-variants expression, is a more general mechanism of mammalian miRNA-mediated regulation.

A possible role for miR-148 in early hematopoietic development

miR-148 was shown to be predominantly expressed in cells of hematopoietic origin and by comparing the expression of multiple cancer cell lines we found the highest expression of miR-148 in Jurkat T cells (Chapter 4). Moreover, all three identified miR-148 targets were known to play a role in hematopoiesis, most notably Dnmt3b of which an inactivating mutation results in a human immunodeficiency syndrome (Xu et al., 1999). Therefore, we set out to study the role of miR-148 in early hematopoiesis and isolated cells in different stages of thymic development (Chapter 5). Indeed, we found high expression of miR-148 in early multipotent progenitor cells (mTPCs) and all subsets derived from these mTPCs that were committed to T-cell development. Intriguingly, we observed major reduction of miR-148 expression in plasmacytoid dendritic cells (pDCs) and natural killer (NK) cells, which both arise from the same mTPCs. This could indicate that increased expression of miR-148 targets is a prerequisite for pDC and NK development. Therefore, we determined the expression of Dnmt3b, MLL and Dnmt1 (Chapter 5 and data not shown) and found that these miR-148 targets were reduced in pDCs and NK cells as well. This suggests that miR-148 and its targets Dnmt3b, Dnmt1 and MLL are not part of a developmental switch in pDC and NK cell development. Nonetheless, this could point to a fine-tuning function of miR-148 towards the expression of these targets. Such a function of miRNAs has been previously described for miR-17-5p and miR-20a and their target E2F1 that are all co-regulated by c-Myc (O'Donnell et al., 2005).

Potential functions of miR-148/152 in cell proliferation

In Chapter 6 we studied the role of miR-152 in diploid fibroblasts by using antagomiRs and surprisingly we found that inhibition of miR-152 resulted in decreased cell proliferation and eventually in cell death. More detailed analysis of the cell cycle profile of antagomiR-152 treated fibroblasts showed that within 24 hours cells were arrested in G2-phase. The G2/M checkpoint arrest cells in G2 to prevent entry into mitosis of cells that have incompletely replicated or damaged DNA (O'Connell and Cimprich, 2005). Since an

increased population of late S-phase cells was observed, this suggests that cells encountered problems while replicating their DNA and subsequently arrested in G2-phase. Alternatively, inhibition of miR-152 resulted in direct activation of checkpoint proteins. In either case, these data imply that endogenous miR-152 is involved in proper S-phase progression in primary fibroblasts.
Interestingly, overexpression of miR-148 resulted in a strong increase of the number of pDCs derived from mTPCs in an in vitro pDC differentiation assay (Chapter 5). This increased number of pDC could be due to enhanced differentiation, proliferation or survival of pDCs. Yet, in light of our findings with antagomiR-152 it is tempting to speculate that miR-148 overexpression has the opposite effect and results in increased proliferation of pDCs or pDC progenitor cells. Furthermore, this would be in line with the high endogenous miR-148 expression of thymic subsets of the T-cell lineage that have a highly proliferative nature, while pDC cells that express low levels of miR-148 have a low proliferative capacity ((Kabashima et al., 2005) and data not shown).
An outstanding question is which miR-148/152 targets or combination of targets are responsible for the observed effects in S and G2-phase. It would be of interest to test whether in case of miR-152 inhibition Dnmt3b and Dnmt1 upregulation are involved. It has been reported in human cells that a Dnmt1 knock-down activated a replication stress checkpoint (Unterberger et al., 2006) and complete inactivation resulted in a G2 arrest and eventually in mitotic catastrophe (Chen et al., 2007). Although Dnmt1 would be upregulated in antagomiR-152 treated cells, these data do implicate that disturbing the balance of Dnmt1 levels in cells will result in DNA replication stress and mitotic failure. Also MLL proteins have been suggested to play a role in cell cycle progression, although it is less clear whether this would favor cell division. MLL has been associated with activation of E2F dependent genes (Takeda et al., 2006; Tyagi et al., 2007), which promotes proliferation, as well as activation of CDK inhibitors that prevent cell cycle progression (Milne et al., 2005).
Interestingly, we recently performed a micro-array study of BJ ET cells that were treated 6 hours with antagomiR-152 to reveal possible miR-152 targets among the upregulated genes. We identified significant upregulation of the ID3 helix-loop-helix protein, suggesting that it is a target of the miR-148/152 family (data not shown). ID proteins negatively regulate the function of basic-helix-loop-helix transcription factors, which are involved in cell growth and differentiation (Engel and Murre, 2001; Zebedee and Hara, 2001). Remarkably, ID3 overexpression was shown to inhibit pDC development (Spits et al., 2000) in the same in vitro pDC differentiation assay we performed for overexpression of miR-148 (Chapter 5). Thus, it would be extremely interesting to test whether ID3 is directly regulated by the miR-148/152 family and whether this regulation is involved in pDC development. Moreover, since ID proteins are involved in cell cycle proliferation it would also be of significance to determine its role in cell cycle control of human diploid fibroblasts.

Potential roles of miR-148/152 in oncogenesis

Many studies have described a correlation of Dnmt3b expression and cancer. Significantly, we demonstrated in this thesis that Dnmt3b can be regulated by the miR-148/152 family (Chapter 4). In cancer, reduced Dnmt3b expression resulted in increased oncogene expression and in genomic instability, whereas increased Dnmt3b protein level was found to repress tumour suppressor expression (Feinberg et al., 2006). In addition, a recent report indicated that alterations in the relative abundance of Dnmt3b splice-variants could be involved in oncogenesis. Dnmt3b7 was found overexpressed in human cancers and in a human cell line enhanced Dnmt3b7 expression was shown to alter gene expression (Ostler et al., 2007). Interestingly, we demonstrated that miR-148/152 can alter the relative abundance of Dnmt3b splice variants by targeting Dnmt3b1, Dnmt3b2 and Dnmt3b4 but not

Dnmt3b3 and Dnmt3b7 (Chapter 4 and data not shown).
It has been reported that the Dnmt3b4 splice-variant has dominant negative activity based on observed hypomethylation of pericentromeric satellite regions (Saito et al., 2002). However, in line with the changed gene expression in cells that overexpress Dnmt3b7 this might also reflect a changed target specificity, as Dnmt3b4 lacks a target recognition motif. Interestingly, Dnmt3b3 lacks the same motif and it has been shown that its catalytic activity depends on the substrate chosen (Chen et al., 2005; Soejima et al., 2003). Therefore, is possible that Dnmt3b3 as well as Dnmt3b4 regulate genomic methylation patterns by influencing target specificity. Thus, it would be of interest to determine whether miR-148-mediated regulation of Dnmt3b splice-variant abundance plays a role in oncogenesis.
A potential tumour suppressive role of miR-148 involves loss of miRNA-mediated repression of MLL. The MLL protein has attracted attention for its role in human leukemia of lymphoid and myeloid origin and more particularly for the many MLL-translocations that can be involved in this disease (Daser and Rabbitts, 2005). MLL translocations result in fusion proteins with up to 60 different fusion partners. Intriguingly, these partners can be of diverse origin among which transcription factors but also cytoplasmic proteins. How all these different fusion proteins can induce tumourigenesis is a subject of current research and until now not known. Significantly, the MLL 3'UTR is lost in all fusion proteins. Moreover, these translocations occur in cell types that most likely express miR-148 most likely to a high extend (Chapter 4 and data not shown). Therefore, it would be interesting to determine whether part of the oncogenic activity of the diverse MLL fusion proteins can be explained by the fact that in all cases the 3'UTR is lost and subsequently miR-148-mediated regulation. This would be similar to the mechanism that has been described for the oncogenic fusions of Hmga2 that disrupt miRNA-mediated repression of the Let-7 miRNA (Lee and Dutta, 2007; Mayr et al., 2007). In conclusion, these data suggest possible roles for miR-148/152 in oncogenesis and it would be of great interest to further explore this.

Concluding remarks
This thesis describes that Cdc6 is degraded upon ionizing radiation in a p53-dependent manner. Molecular unravelling of the pathway resulting in Cdc6 degradation revealed a novel regulatory mechanism of Cdc6 by CDK2/Cyclin E. Phosphorylation by this kinase results in stabilization of Cdc6 during the normal cell cycle, yet, upon DNA damage this activity is blocked through p53 and p21. Almost half of the human cancer cells lost p53 (Levine, 1997) indicating that Cdc6 is aberrantly regulated in these cells. Indeed, we found elevated levels of Cdc6 in p53 knock-down diploid human fibroblasts, and this increase of Cdc6 appeared to be partially responsible for the increased proliferative capacity of these cells. Taken together, these findings give insight in the essential role of Cdc6 in the regulation of initiation of DNA replication in the normal cell cycle and following DNA damage.
In the second part of this thesis, the function of the miR-148/152 family was studied. We identified three mRNA targets of this miRNA family, Dnmt3b, Dnmt1 and MLL. Significantly, Dnmt3b appeared not to be regulated through its 3'UTR but through a highly homologous target site in its CDR. This is a novel miRNA-mediated regulatory mechanism in mammalian cells. Furthermore, the target site of miR-148/152 is only present in specific Dnmt3b splice-variants, which enables miR-148/152 to regulate the relative splice-variant abundance. To further characterise the biological function of the miR-148/152 family we studied the role of miR-148 in early T cell development and miR-152 in primary fibroblast, since these are the cell types that preferentially express the seperate miRNA family members. We found that miR-148 expression was reduced in pDC cells compared to expression of its progenitor. Interestingly, we found that overexpression of miR-148 strongly increases the number of pDC derived from

mTPC in an in vitro differentiation assay. This effect could be due to enhanced proliferation, differentiation or survival. On the other hand, we found that inhibition of miR-152 in diploid fibroblasts resulted in increased numbers of late S-phase cells and a G2 block. These results suggest that miR-152 is required for accurate S-phase progression and that interfering with miR-152 results in aberrant DNA replication, DNA damage and a G2 block. Combining the results derived from miR-152 inhibition in fibroblasts and miR-148 overexpression in pDC development, makes it tempting to speculate that miR-148 overexpression results in increased proliferation of pDC cells or a progenitor. In summary, our studies on the miR-148/152 family resulted in novel insights in the function of a current uncharacterized miRNA-family. It would be of interest to further explore its potential function in regulation of cell proliferation and to determine which of its targets are involved in this process.

In conclusion, this thesis describes a molecular dissection of the function of Cdc6 and the miR-148/152 family. We demonstrated that a specific Cdc6 modification plays a key role in the regulation of initiation of DNA replication in the normal cell cycle and in response to DNA damage. Furthermore, we revealed a novel mechanism of miRNA targeting by the miR-148/152 family and found a role for these miRNAs in regulation of proper S-phase progression and proliferation as well. Thus, both studies described here impinge on cell proliferation, which is one of the most fascinating characteristics of a cell and a feature that is found deregulated in all human cancers.

References

Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L. V., Kolettas, E., Niforou, K., Zoumpourlis, V. C., et al. (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature 444, 633-637.

Blanchard, F., Rusiniak, M. E., Sharma, K., Sun, X., Todorov, I., Castellano, M. M., Gutierrez, C., Baumann, H., and Burhans, W. C. (2002). Targeted destruction of DNA replication protein Cdc6 by cell death pathways in mammals and yeast. Mol Biol Cell 13, 1536-1549.

Borlado, L. R., and Mendez, J. (2007). CDC6: from DNA replication to cell cycle checkpoints and oncogenesis. Carcinogenesis.

Bushati, N., and Cohen, S. M. (2007). microRNA Functions. Annu Rev Cell Dev Biol 23, 175-205.

Chen, T., Hevi, S., Gay, F., Tsujimoto, N., He, T., Zhang, B., Ueda, Y., and Li, E. (2007). Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. Nat Genet 39, 391-396.

Chen, Z. X., Mann, J. R., Hsieh, C. L., Riggs, A. D., and Chedin, F. (2005). Physical and functional interactions between the human DNMT3L protein and members of the de novo methyltransferase family. J Cell Biochem 95, 902-917.

Clay-Farrace, L., Pelizon, C., Santamaria, D., Pines, J., and Laskey, R. A. (2003). Human replication protein Cdc6 prevents mitosis through a checkpoint mechanism that implicates Chk1. Embo J 22, 704-712.

Daser, A., and Rabbitts, T. H. (2005). The versatile mixed lineage leukaemia gene MLL and its many associations in leukaemogenesis. Semin Cancer Biol 15, 175-188.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P. G., Bensimon, A., et al. (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature 444, 638-642.

Engel, I., and Murre, C. (2001). The function of E- and Id proteins in lymphocyte development. Nat Rev Immunol 1, 193-199.

Feinberg, A. P., Ohlsson, R., and Henikoff, S. (2006). The epigenetic progenitor origin of human cancer. Nat Rev Genet 7, 21-33.

Fersht, N., Hermand, D., Hayles, J., and Nurse, P. (2007). Cdc18/CDC6 activates the Rad3-dependent checkpoint in the fission yeast. Nucleic Acids Res 35, 5323-5337.

Hall, J. R., Kow, E., Nevis, K. R., Lu, C. K., Luce, K. S., Zhong, Q., and Cook, J. G. (2007). Cdc6 stability is regulated by the Huwe1 ubiquitin ligase after DNA damage. Mol Biol Cell 18, 3340-3350.

Hateboer, G., Wobst, A., Petersen, B. O., Le Cam, L., Vigo, E., Sardet, C., and Helin, K. (1998). Cell cycle-regulated expression of mammalian CDC6 is dependent on E2F. Mol Cell Biol 18, 6679-6697.

Hermand, D., and Nurse, P. (2007). Cdc18 enforces long-term maintenance of the S phase checkpoint by anchoring the Rad3-Rad26 complex to chromatin. Mol Cell 26, 553-563.

Kabashima, K., Banks, T. A., Ansel, K. M., Lu, T. T., Ware, C. F., and Cyster, J. G. (2005). Intrinsic lymphotoxin-beta receptor requirement for homeostasis of lymphoid tissue dendritic cells. Immunity 22, 439-450.

Kelly, T. J., Martin, G. S., Forsburg, S. L., Stephen, R. J., Russo, A., and Nurse, P. (1993). The fission yeast cdc18+ gene product couples S phase to START and mitosis. Cell 74, 371-382.

Krek, A., Grun, D., Poy, M. N., Wolf, R., Rosenberg, L., Epstein, E. J., MacMenamin, P., da Piedade, I., Gunsalus, K. C., Stoffel, M., and Rajewsky, N. (2005). Combinatorial microRNA target predictions. Nat Genet 37, 495-500.

Lau, E., Zhu, C., Abraham, R. T., and Jiang, W. (2006). The functional role of Cdc6 in S-G2/M in mammalian cells. EMBO Rep 7, 425-430.

Lee, Y. S., and Dutta, A. (2007). The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. Genes Dev 21, 1025-1030.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. Cell 88, 323-331.

Mailand, N., and Diffley, J. F. (2005). CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. Cell 122, 915-926.

Mayr, C., Hemann, M. T., and Bartel, D. P. (2007). Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science 315, 1576-1579.

Milne, T. A., Hughes, C. M., Lloyd, R., Yang, Z., Rozenblatt-Rosen, O., Dou, Y., Schnepp, R. W., Krankel, C., Livolsi, V. A., Gibbs, D., et al. (2005). Menin and MLL cooperatively regulate expression of cyclin-dependent kinase inhibitors. Proc Natl Acad Sci U S A 102, 749-754.

O'Connell, M. J., and Cimprich, K. A. (2005). G2 damage checkpoints: what is the turn-on? J Cell Sci 118, 1-6.

O'Donnell, K. A., Wentzel, E. A., Zeller, K. I., Dang, C. V., and Mendell, J. T. (2005). c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435, 839-843.

Oehlmann, M., Score, A. J., and Blow, J. J. (2004). The role of Cdc6 in ensuring complete genome licensing and S phase checkpoint activation. J Cell Biol 165, 181-190.

Ostler, K. R., Davis, E. M., Payne, S. L., Gosalia, B. B., Exposito-Cespedes, J., Le Beau, M. M., and Godley, L. A. (2007). Cancer cells express aberrant DNMT3B transcripts encoding truncated proteins. Oncogene 26, 5553-5563.

Petersen, B. O., Wagener, C., Marinoni, F., Kramer, E. R., Melixetian, M., Lazzerini Denchi, E., Gieffers, C., Matteucci, C., Peters, J. M., and Helin, K. (2000). Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. Genes Dev 14, 2330-2343.

Piatti, S., Lengauer, C., and Nasmyth, K. (1995). Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast Saccharomyces cerevisiae. Embo J 14, 3788-3799.

Rhoades, M. W., Reinhart, B. J., Lim, L. P., Burge, C. B., Bartel, B., and Bartel, D. P. (2002). Prediction of plant microRNA targets. Cell 110, 513-520.

Saito, Y., Kanai, Y., Sakamoto, M., Saito, H., Ishii, H., and Hirohashi, S. (2002). Overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis. Proc Natl Acad Sci U

S A 99, 10060-10065.

Soejima, K., Fang, W., and Rollins, B. J. (2003). DNA methyltransferase 3b contributes to oncogenic transformation induced by SV40T antigen and activated Ras. Oncogene 22, 4723-4733.

Spits, H., Couwenberg, F., Bakker, A. Q., Weijer, K., and Uittenbogaart, C. H. (2000). Id2 and Id3 inhibit development of CD34(+) stem cells into predendritic cell (pre-DC)2 but not into pre-DC1. Evidence for a lymphoid origin of pre-DC2. J Exp Med 192, 1775-1784.

Takeda, S., Chen, D. Y., Westergard, T. D., Fisher, J. K., Rubens, J. A., Sasagawa, S., Kan, J. T., Korsmeyer, S. J., Cheng, E. H., and Hsieh, J. J. (2006). Proteolysis of MLL family proteins is essential for taspase1-orchestrated cell cycle progression. Genes Dev 20, 2397-2409.

Tyagi, S., Chabes, A. L., Wysocka, J., and Herr, W. (2007). E2F activation of S phase promoters via association with HCF-1 and the MLL family of histone H3K4 methyltransferases. Mol Cell 27, 107-119.

Unterberger, A., Andrews, S. D., Weaver, I. C., and Szyf, M. (2006). DNA methyltransferase 1 knockdown activates a replication stress checkpoint. Mol Cell Biol 26, 7575-7586.

Vaziri, C., Saxena, S., Jeon, Y., Lee, C., Murata, K., Machida, Y., Wagle, N., Hwang, D. S., and Dutta, A. (2003). A p53-dependent checkpoint pathway prevents rereplication. Mol Cell 11, 997-1008.

Xu, G. L., Bestor, T. H., Bourc'his, D., Hsieh, C. L., Tommerup, N., Bugge, M., Hulten, M., Qu, X., Russo, J. J., and Viegas-Pequignot, E. (1999). Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402, 187-191.

Zebedee, Z., and Hara, E. (2001). Id proteins in cell cycle control and cellular senescence. Oncogene 20, 8317-8325.

Nederlandse samenvatting

Een gezonde cel heeft meerdere regulatiemechanismen die ervoor zorgen dat een cel zich op een gecontroleerde manier deelt. Deze regulatie ligt vast in de code van het erfelijk materiaal van een cel, het DNA. Bij elke celdeling is er een kleine kans dat het DNA niet helemaal goed gerepliceerd wordt en kan er een mutatie ontstaan. Wanneer er een mutatie ontstaat in een van de cellulaire controlemechanismen, kan het gebeuren dat cellen zich in het lichaam ongecontroleerd vermenigvuldigen. Wanneer deze cellen zich uitbreiden naar andere weefsels noemen we dit kanker.
Om te voorkomen dat er DNA-schade en dus kanker ontstaat heeft een cel meerdere overlappende controlemechanismen die DNA-schade kunnen vaststellen en hier vervolgens adequaat op reageren. Deze reactie wordt de DNA-schaderesponse genoemd, en deze response zorgt ervoor dat een cel onmiddellijk stopt met delen en begint met de reparatie van het DNA. Hierna zal een cel weer verder delen, tenzij de schade zo aanzienlijk is dat reparatie niet meer mogelijk is. In dat geval zal een cel in permanente groeiarrest blijven of dood gaan.

De genetische code van het DNA wordt in een cel vertaald naar RNA, dat weer vertaald wordt naar eiwitten. Mutaties in het DNA van een gen dat voor een bepaald eiwit codeert, kan de functie van dat eiwit veranderen, maar kan ook tot veranderde expressie (de hoeveelheid eiwit die van RNA en DNA wordt gemaakt) daarvan leiden. Ook andere veranderingen in een cel die tot veranderde eiwitexpressie leiden kunnen een rol spelen bij het ontstaan van kanker, zoals veranderingen in bepaalde modificaties van het DNA of eiwitten die aan het DNA binden, de zogenaamde epigenetische veranderingen.
Recentelijk zijn er kleine RNA moleculen van 20-25 nucleotiden ontdekt waarvan geen eiwit wordt gemaakt. Deze moleculen, de zogenaamde microRNAs (miRNAs) binden aan grotere target-RNA moleculen waarvan wel eiwit wordt gemaakt. Hierdoor kan de vertaling van RNA naar eiwit worden geremd en kan het target-RNA ook worden afgebroken. Doordat miRNAs de expressie van RNA naar eiwit reguleren, kunnen ook deze kleine regulatoren een rol spelen bij het ontstaan van kanker.

In het eerste deel van dit proefschrift (Hoofdstukken 2 en 3) wordt het effect van DNA-schade op de stabiliteit van Cdc6, een belangrijk DNA replicatie-eiwit, beschreven. Hoofdstuk 2 laat zien dat serine 54 fosforylatie van Cdc6 door CDK2/Cycline E het Cdc6 eiwit stabiliseert. Deze modificatie voorkomt herkenning door de E3-ligase APC^{Cdh1} die voor de afbraak van Cdc6 zorgt. Na DNA-schade wordt CDK2/Cycline E activiteit echter geblokkeerd op een p53-afhankelijke manier, waardoor ook Cdc6 instabiel wordt en wordt afgebroken. De belangrijke rol die Cdc6 speelt in de initiatie van DNA-replicatie suggereert dat de afbraak van Cdc6 een rol zou kunnen spelen in het voorkomen van celdeling in cellen met DNA-schade. P53 is een van de belangrijkste tumor-suppressors in een cel, en p53 is in heel veel tumoren gemuteerd. Behalve de p53-afhankelijke regulatie van Cdc6 na DNA-schade laat Hoofdstuk 2 zien dat dit regulatiemechanisme ook een rol speelt tijdens normale celdeling. Cellen met verlaagde expressie van p53 groeien sneller, een effect dat teniet gedaan kan worden door gelijktijdig ook de expressie van Cdc6 te verlagen. Deze bevindingen geven meer inzicht in de essentiële rol van Cdc6 in de initiatie van DNA-replicatie en hoe Cdc6 wordt gereguleerd in de normale celcyclus en na DNA-schade. In Hoofdstuk 3 worden deze bevindingen en de implicaties hiervan verder bediscussieerd.

In het tweede deel van dit proefschrift (Hoofdstukken 4, 5 en 6) wordt de functie

van de miR-148/152 miRNA familie ontrafeld. In Hoofdstuk 4 wordt een nieuw miRNA regulatiemechanisme beschreven. Tot nu toe werd gedacht dat miRNAs hun target-RNAs reguleren door aan de 3'UTR van dat RNA te binden, het gedeelte dat niet in eiwit vertaald wordt. Dit hoofdstuk laat zien dat in het geval van Dnmt3b en miR-148/152 deze regulatie plaatsvindt in het eiwit coderende gedeelte van het RNA. Dit heeft als belangrijk gevolg dat een Dnmt3b mRNA splice-variant die dit gedeelte niet tot expressie brengt, ook niet gereguleerd kan worden door de miR-148/152 familie. Om verder de biologische functie van de miR-148/152 familie te karakteriseren hebben we de functie van deze miRNAs onderzocht in vroege humane T-cel ontwikkeling (witte bloedcellen uit de thymus) en in primaire fibroblasten, omdat dit de celtypes zijn waar deze miRNAs met name tot expressie komen. miR-148 bleek hoog tot expressie te komen in multipotente voorlopercellen en in alle vroege T-cellen die uit deze voorlopercel ontstaan. miR-148 expressie is echter aanzienlijk verlaagd in plasmacytoide dendritische cellen (pDCs) en natural killer cellen die uit diezelfde multipotente voorlopercellen ontstaan. Wanneer miR-148 tot overexpressie wordt gebracht in voorloper cellen die vervolgens in vitro naar pDCs worden gedifferentieerd neemt het aantal pDCs dramatisch toe (Hoofdstuk 5). Aan de andere kant vonden we dat inhibitie van miR-152 in fibroblasten het doorlopen van een celdelingscyclus remde (Hoofdstuk 6). Samen suggereren deze data dat de miR-148/152 een rol zou kunnen spelen in celdeling.

In dit proefschrift getiteld "Moleculaire ontrafeling van Cdc6 en de miR-148 familie" komen twee verschillende onderzoekslijnen aan bod. In het eerste gedeelte wordt de rol van een specifieke modificatie van het DNA replicatie-eiwit Cdc6 onderzocht tijdens de normale celdelings cyclus en na DNA-schade. In het tweede gedeelte worden de functies van een bepaalde miRNA familie, de miR-148/152 familie, ontrafeld. Net als Cdc6 speelt ook deze miRNA familie mogelijk een rol in de regulatie van celdeling. Beide studies grijpen dus aan op een van de meest fascinerende regulatiemechanismen van een cel, het celdelings controlemechanisme, dat in alle humane tumoren gedereguleerd is.

Curriculum Vitae

Anja Duursma werd geboren op 17 februari 1978 te Leuven (België). Zij behaalde in 1996 haar VWO diploma op het Haags Montessori Lyceum in Den Haag. Van 1996 tot 2001 studeerde zij biologie aan de Universiteit van Utrecht. Haar eerste afstudeerstage vond plaats in de groep van Prof. Sjef Smeekens bij de vakgroep Moleculaire Plantenfysiologie van de Universiteit van Utrecht. In haar tweede stage werd zij gegrepen door het kankeronderzoek door de inspirerende begeleiding van Dr. Stephen Tait. Deze stage vond plaats in de groep van Prof. Jannie Borst op het Nederlands Kanker Instituut (NKI). Van november 2001 tot en met december 2007 werkte zij als onderzoeker in opleiding in de groep van Dr. Reuven Agami op de afdeling Tumor Biologie aan het NKI. Het resultaat hiervan is in dit promotieboekje te lezen. Sinds 1 april 2008 is zij werkzaam in de groep van Ass. Prof. Karlene Cimprich op Stanford University in Californië, waar zij haar onderzoek naar DNA-schaderesponsen zal voortzetten.

List of publications

Duursma AM, Kedde M, Schrier M, Le Sage C, Agami R. 2008.
miR-148 targets human Dnmt3b protein coding region.
RNA.

Kedde M, Le Sage C, **Duursma A**, Zlotorynski E, van Leeuwen B, Nijkamp W, Beijersbergen R, Agami R. 2006.
Telomerase-independent regulation of ATR by human telomerase RNA.
J Biol Chem (52): 40503-14.

Duursma AM, Agami R. 2005.
CDK-dependent stabilization of Cdc6: linking growth arrest and stress signals to activation of DNA replication.
Cell Cycle (12): 1725-8.

Duursma A, Agami R. 2005.
P53-Dependent regulation of Cdc6 protein stability controls cellular proliferation.
Mol Cell Biol (16): 6937-47.

Duursma AM, Agami R. 2003.
Ras interference as cancer therapy.
Semin Cancer Biol (4): 267-73.