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

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Chapter 7

General Discussion

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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.

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