TUMOR SUPPRESSION BY RETINOBLASTOMA PROTEINS: INTERACTING PROTEINS AND COOPERATING PATHWAYS

Author: Tinke Lizbet Vormer
Cover: Cooperation of the eyes to form one image, design: Suzanne Vormer
Pinted by: Gildeprint drukkerijen, Enschede, The Netherlands
ISBN: 9789461080981

The research described in this thesis was performed at the Division of Molecular Biology of the Netherlands Cancer Institute - Antoni van Leeuwenhoek Hospital, Amsterdam, the Netherlands and was financially supported by the Dutch Cancer Society. Publication of this thesis was performed with financial support of the Dutch Cancer Society and the Netherlands Cancer Institute.

VRIJE UNIVERSITEIT

TUMOR SUPPRESSION BY RETINOBLASTOMA PROTEINS: INTERACTING PROTEINS AND COOPERATING PATHWAYS

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. L.M. Bouter,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Geneeskunde
op vrijdag 26 november 2010 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Tinke Lizbet Vormer

geboren te Utrecht

promotor: prof.dr. H.P.J. te Riele

Contents

Chapter 1	General introduction	7
Chapter 2	Anchorage-independent growth of pocket protein-deficient murine fibroblasts requires bypass of G_2 arrest and can be accomplished by expression of TBX2	33
Chapter 3	Mapkapk3 is a suppressor of anchorage-independent growth	61
Chapter 4	The pRB-LxCxE interaction is critical for RAS^{V12}- and γ-irradiation induced cell cycle arrest	75
Chapter 5	Overlapping roles of pRB-LxCxE interactions and p130 functions in mouse development and viability	105
Chapter 6	General discussion	129
Appendices	English summary	141
	Nederlandse samenvatting	145
	List of publications	149
	Curriculum Vitae	150
	Dankwoord	151

CHAPTER 1

General introduction

Oncogenic transformation, the gradual change of a normal cell into a cancerous cell, requires sequential alterations in several cellular processes and involves both activation of oncogenic pathways and inhibition of tumor suppressor pathways (reviewed by Hanahan and Weinberg, 2000). The retinoblastoma (pRB) pathway is a well known tumor suppressor pathway that is deregulated in the majority of (human) cancers (reviewed by Burkhart and Sage, 2008; Sherr, 1996). pRB and its two close homologs, p107 and p130, comprise the family of so-called pocket proteins and are essential for regulation of the cell cycle, differentiation and apoptosis. This thesis focuses on the role of the retinoblastoma proteins in tumor suppression: we have identified events that collaborate with pocket protein ablation during *in vitro* transformation and we have studied the role of the interaction between pRB and proteins containing an LxCxE motif during cell cycle arrest and tumor suppression.

The mammalian cell cycle and regulation by cyclin-cdk complexes

The mammalian cell cycle generates two virtually identical daughter cells and can be divided into four sequential phases: G_1, S, G_2 and M phase. DNA duplication occurs in the Synthesis or S phase, whereas chromosome segregation and cell division occur in Mitosis or M phase. G_1 and G_2 comprise gap phases, during which both stimulatory and inhibitory signals of proliferation are integrated into the cell cycle machinery, resulting in cell cycle progression or arrest in G_1 or G_2 phase. Cells that are withdrawn from the cell cycle, for example during serum starvation or differentiation, are defined to be in G_0.

Progression through the cell cycle is driven by different cyclin-cdk complexes (Fig. 1). Full activation of cyclin dependent kinases, cdks, requires both binding to a cyclin protein and phosphorylation by a cyclin activating kinase, CAK. The expression and nuclear localization of cyclins fluctuates during the cell cycle, resulting in the timed activation of different cyclin-cdk complexes. During early G_1, Cyclin D-CDK4/6 complexes are activated, followed by Cyclin E-CDK2 complexes at the G_1/S transition and Cyclin A-CDK1/2 complexes during S phase. Cyclin B1-CDK1 complexes enter the nucleus at the G_2/M transition, and are, together with Cyclin A-CDK1 complexes, required for G_2/M transition. Cyclin B1-CDK1 is subsequently required for M phase progression (Katsuno et al., 2009; Ciemerych and Sicinski, 2005; Sherr and Roberts, 2004; Pines and Hunter, 1992). The activity of cyclin-cdks is inhibited by the family of inhibitors of cdk4 (INK4) proteins, which inhibit Cyclin D-CDK4/6 kinase activity, and by the family of cdk-interacting protein/ kinase-inhibitory protein (CIP/KIP) proteins, which inhibit CDK1- and CDK2-associated kinase activities. Notably, the CIP/KIP proteins play a stimulatory role in the activation of Cyclin D-CDK4/6 complexes (reviewed by Berthet and Kaldis, 2007; Ciemerych and Sicinski, 2005; Sherr and Roberts, 1999).

The pocket proteins play a key role in cell cycle regulation by maintaining the

G_0/G_1 state in the absence of proliferative stimuli. Under growth stimulating conditions, Cyclin D-CDK4/6 and Cyclin E-CDK2 complexes phosphorylate the pocket proteins in G_1, which causes pocket protein inactivation and subsequent S phase entry (reviewed by Cobrinik, 2005).

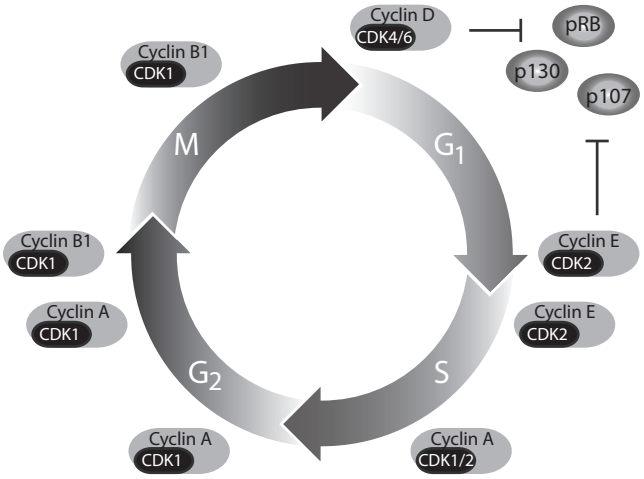


Figure 1: Schematic representation of the mammalian cell cycle. Progression through the cycle is driven by the activity of Cyclin-CDK complexes. The pocket proteins, pRB, p107 and p130, play an inhibitory role during G_1, which can be reverted by the activity of CyclinD-CDK4/6 and CyclinE/A-CDK2 complexes.

Interaction of E2Fs and pocket proteins in the cell cycle

An important function of the pocket proteins in G_1 consists of binding and inhibiting the family of E2F transcription factors, which are essential for S phase entry. The E2F transcription factor family consists of at least 11 members and is classically divided into activator E2Fs (E2F1, E2F2 and E2F3a) and repressor E2Fs (E2F3b, E2F4, E2F5, E2F6a, E2F6b, E2F7a, E2F7b and E2F8). E2F1-6 require the interaction with a DP protein for proper binding to the DNA (reviewed by DeGregori and Johnson, 2006). The division between 'activator' and 'repressor' E2Fs is not black and white. The E2F3b, E2F4 and E2F5 'repressors' contain a transcriptional activation domain (reviewed by Dimova and Dyson, 2005), and both E2F3b and E2F4 have been shown to function as transcriptional activators (Chong et al., 2009a; Tsai et al., 2008; Kinross et al., 2006; Wu et al., 2001; Muller et al., 1997). Additionally, the 'activator' E2Fs were recently shown to perform a 'repressor' function as well (Sahin and Sladek, 2010; Chong et al., 2009b).

The importance of E2Fs in S phase induction was first of all demonstrated by the presence of E2F-binding sites in various genes encoding proteins involved in DNA replication and cell cycle progression, such as PCNA, DNA polymerase α, Cyclin E and Cyclin A (reviewed by Lavia and Jansen-Durr, 1999). In line with this, ectopic expression of E2F1, -2, or -3 drove quiescent cells into S phase (DeGregori et al., 1997; Lukas et al., 1996; Johnson et al., 1993). Conversely, fibroblasts deficient for E2F1, -2 and -3 were unable to proliferate in culture and displayed impaired induction of several, but not all, E2F target genes (Wu et al., 2001). E2Fs have also been suggested to function beyond S phase, as they bind and regulate promoters of genes involved in DNA repair, DNA damage checkpoints, chromatin assembly and condensation, chromosome segregation, the mitotic spindle checkpoint, differentiation, development and apoptosis (Ren et al., 2002; Muller et al., 2001).

The different members of the pocket protein family interact with the different E2Fs with varying affinities: pRB interacts with E2F1-4 (Moberg et al., 1996; Lees et al., 1993), whereas p130 and p107 interact with E2F4 and E2F5. E2F6 and E2F7 do not interact with the pocket proteins, as they lack the pocket protein binding domain (reviewed by Dimova and Dyson, 2005; Frolov and Dyson, 2004). It should be noted that the identity of the pocket protein-E2F complexes can shift upon ablation of components, as combined depletion of pRB and E2F4 promoted binding of p130 and p107 to E2F1 and E2F3 (Lee et al., 2002).

Both the presence and promoter binding of the different pocket protein-E2F complexes fluctuates during the cell cycle. During G_0/G_1, E2F4 was found to primarily complex with p130 and to a lower extent with pRB and p107. At this stage E2F4, p130 and p107 were detected at the promoter regions of repressed E2F target genes. At the G_1/S transition, E2F4-p130 complexes were replaced by E2F4-p107 and E2F4-pRB complexes. Simultaneously, free E2Fs, not in complex with pocket proteins, became visible. Moreover, E2F1-3 were detected at the promoter regions of E2F target genes, which correlates with the induction of transcription (Macaluso et al., 2006; Balciunaite et al., 2005; Rayman et al., 2002; Takahashi et al., 2000; Hurford, Jr. et al., 1997; Moberg et al., 1996; Cobrinik et al., 1993).

Transcriptional repression and silencing via the pRB-LxCxE interaction

Pocket proteins can inhibit E2F-mediated transcription in several ways (Fig. 2). First, pocket protein binding masks the E2F transactivation domain, abrogating its activity. Second, pocket proteins can simultaneously bind to both E2Fs and chromatin remodeling proteins, resulting in the recruitment of these remodeling proteins to E2F-regulated promoters (reviewed by Dick, 2007). Thus, the transactivation function of the activators E2Fs 1, 2 and 3a can be inhibited by pRB binding, whereas pocket protein-E2F-chromatin

remodeling complexes, which promote a chromatin state incompatible with transcription, can be formed by pRB-E2F3b, pRB-E2F4, p130/p107-E2F4 and p130/p107-E2F5.

Various chromatin remodeling proteins that have been associated with transcriptional repression, contain an 'LxCxE like' motif through which they can bind to pRB and to the other pocket proteins. Among LxCxE-containing proteins are Class I histone deacetylases (HDACs), heterochromatin protein 1 (HP1), brahma (BRM) and brahma-related gene 1 (BRG1) ATPases of the mating-type switch (SWI)/sucrose non-fermenting (SNF) nucleosome remodeling complex, the C-terminal binding protein (CtBP)/CtBP-interacting protein (CtIP) co-repressor, the retinoblastoma binding proteins RBP1 and RBP2, and possibly others (reviewed by Dick, 2007).

The interaction between pocket proteins and HDACs in transcriptional repression

The three pocket proteins interact with Class I HDACs, which include HDAC1, 2 and 3. Since de-acetylated chromatin is more condensed and therefore less accessible to transcription factors than acetylated chromatin, the recruitment of HDACs to the promoter region is considered to actively repress transcription. HDAC1 and HDAC2 both contain an LxCxE-like motif, which is required for formation of pRB-HDAC1/2 and p107-HDAC1 complexes: mutating the LxCxE-like sequence in HDAC1 abrogated binding to pRB/p107 and additionally, mutating the LxCxE binding site in pRB abrogated binding to HDAC1/2 (Chen and Wang, 2000; Dahiya et al., 2000; Ferreira et al., 1998; Magnaghi-Jaulin et al., 1998). Although these observations are suggestive for direct binding between

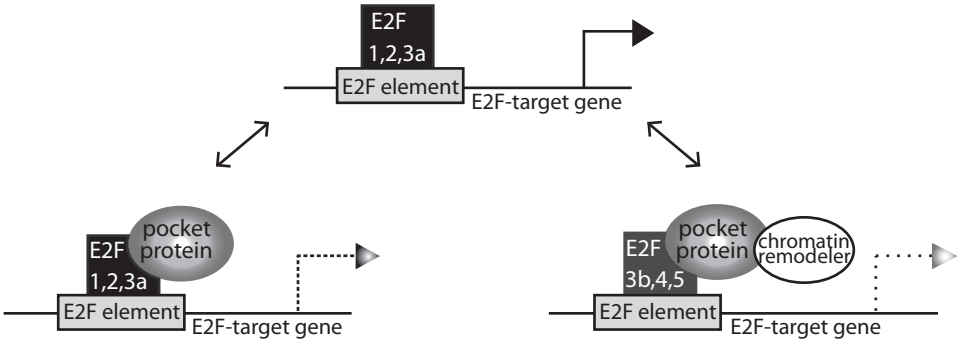


Figure 2: Model showing the role of E2Fs and pocket proteins in transcriptional regulation of E2F-target genes. Binding of 'activator' E2Fs (E2F1-3a) to an E2F element in the promoter region induces transcriptional activation (upper picture, visualized by the bold arrow). E2F-mediated transactivation can be inhibited via binding of pocket proteins to the transactivation domain of E2Fs (lower left). Active repression of E2F-target genes is achieved via the recruitment of complexes containing 'repressor' E2Fs (E2F3b-5), pocket proteins and chromatin remodeling proteins (lower right). Note that E2F4 and E2F3b, which are generally viewed as ´repressor´ E2Fs, can also function in transactivation and that complexes between pRB and the E2F1-3a 'activators' can also repress transcription of E2F target genes.

pRB/p107 and HDACs, experiments performed by Lai and co-workers indicated that this interaction requires bridging by the LxCxE-containing protein RBP1. In a similar way, RBP1 could possibly enable binding of pocket proteins to HDAC3, which lacks an LxCxE-like motif (Lai et al., 2001; Lai et al., 1999). Importantly, pRB-, p107- or p130-mediated repression of E2F-inducible reporter constructs could (partially) be relieved by the HDAC inhibitor Trichostatin A (TSA). Additionally, pRB-mediated repression of several endogeneous E2F target genes was sensitive to TSA treatment, indicating that pocket protein-mediated repression indeed involves HDACs (Siddiqui et al., 2003; Chen and Wang, 2000; Dahiya et al., 2000; Brehm et al., 1998; Ferreira et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998).

Furthermore, various groups have demonstrated binding of pocket proteins, HDACs and/or E2Fs to repressed promoters *in vitro*: pRB and HDAC1 were recruited to the repressed *Cyclin E* promoter in serum-starved fibroblasts (Morrison et al., 2002), pRB, p130 and HDAC1 were recruited to the *Cyclin E* promoter in $p16^{INK4A}$-arrested U2OS cells (Dahiya et al., 2001) and p107, p130, E2F4, HDAC1 and mSin3B were recruited to several E2F-regulated promoters in mouse embryonic fibroblasts arrested in G_0 (Rayman et al., 2002). Notably, TSA treatment relieved repression of Cyclin E under serum starved conditions (Morrison et al., 2002). Furthermore, Rayman and colleagues (2002) demonstrated that combined ablation of p107 and p130 caused de-repression and loss of recruitment of E2F4, HDAC1 and mSin3B to the E2F-regulated *B-Myb* promoter. However, for most E2F promoters tested, p107 and p130 ablation caused de-repression and loss of recruitment of E2F4 and HDAC1, but not mSin3B. This implicates that p107/p130-E2F4-HDAC and p107/p130-E2F4-mSin3B-HDAC1 complexes are involved in repression of different E2F-target genes. In conclusion, a great body of evidence points to the involvement of complexes containing pocket proteins and HDACs in transcriptional repression.

The interaction between pocket proteins and Histone Methyl Transferases (HMTases) in transcriptional repression and silencing

The site in pRB that binds to the LxCxE motif has also been suggested to mediate binding to the Suv39h1 HMTase and to HP1. Suv39h1 specifically methylates Lysine 9 of Histone 3, H3K9 (Rea et al., 2000), which creates a binding site for the heterochromatin protein HP1 (Lachner et al., 2001). Both H3K9 tri-Methylation (H3K9-triM) and the presence of HP1 are characteristics of transcriptionally inactive heterochromatin, suggesting a role for pRB in gene silencing via recruitment of these proteins.

All three pocket proteins can bind Suv39h1 and contain HMTase activity when immunoprecipitated from nuclear extracts. In reporter assays, Suv39h1 enhanced repression by pRB, p107 or p130, indicating that recruitment of Suv39h1 can be involved

in pocket protein-mediated repression (Nicolas et al., 2003; Nielsen et al., 2001; Vandel et al., 2001). Interestingly, in MEFs, pRB was required for the recruitment of HP1 and methylated H3K9 to the *Cyclin E* promoter, indicating the involvement of both HP1 and Suv39h1 in pRB-mediated transcriptional repression of *Cyclin E* (Nielsen et al., 2001). In overexpression studies, the pRB-Suv39h1 and p107-Suv39h1 interaction could be inhibited by an LxCxE-containing peptide, indicating the involvement of the LxCxE binding site of pocket proteins in contacting Suv39h1 (Nicolas et al., 2003; Vandel et al., 2001). Additionally, HMTase activity of pulled down GST-pRB could be inhibited by an LxCxE-containing competitor peptide during precipitation (Nielsen et al., 2001). However, no LxCxE-like sequence has been detected in Suv39h1 (Vandel et al., 2001), suggesting that the interaction is indirect, and might for example involve the LxCxE-containing protein HP1. Indeed, pRB, Suv39H1 and HP1 bound simultaneously to a peptide mimicking methylated H3K9 (Nielsen et al., 2001). Additionally, pRB, HP1 and methylated H3K9 were detected at the *Cyclin A* and *PCNA* promoters in RAS^{V12}-induced senescent cells (Narita et al., 2003). It should be noted that p107, immuno-precipitated from *Suv39h1*$^{-/-}$*Suv39h2*$^{-/-}$ double knockout MEFs still contained HMTase activity, pointing to the involvement of an HMTase other than Suv39h1/h2. Indeed, ectopically expressed p107 could interact with the EMT1 HMTase, that is also known to specifically methylate H3K9 (Nicolas et al., 2003). To further complicate matters, Suv39h1 can interact with HDAC1, 2 and 3. Moreover, Suv39h1-mediated repression involved HDAC activity and Suv39h1 could only methylate H3K9 when this residue was not acetylated (Vaute et al., 2002; Rea et al., 2000), suggesting a cooperation between pRB, Suv39h1, HDACs and HP1 in silencing.

In line with the involvement of pocket proteins in recruiting HMTases to the chromatin, Gonzalo and co-workers (2005) showed that pocket protein-deficient MEFs displayed reduced H4K20-triM in both centromeric and telomeric heterochromatin. No defects in H3K9-triM could be detected in this study, although a recent study reported a mild reduction in total H3K9-triM upon acute pRB loss (Siddiqui et al., 2007). H4K20-triM is performed by the Suv4-20h1 and -h2 HMTases (Schotta et al., 2004), which can bind pRB, p107 and p130 (Isaac et al., 2006; Gonzalo et al., 2005). Strikingly, disruption of E2F-pocket protein complexes in wild-type MEFs did not abrogate H4K20-triM (Gonzalo et al., 2005), suggesting that H4K20-triM is dependent on pocket proteins, but independent of E2Fs.

Similar to pocket protein-deficient MEFs, MEFs expressing a pRB mutant deficient in binding LxCxE-containing proteins also displayed aberrant patterns of H4K20-triM in the heterochromatin. Surprisingly, the mutant pRB protein, defective in binding LxCxE-containing proteins, could still interact with Suv4-20h1/h2 HMTases (Isaac et al., 2006). Additionally, ectopically expressed Suv4-20h1/h2 was correctly

targeted to the chromatin in pocket protein-deficient MEFs and was able to rescue H4K20-triM (Gonzalo et al., 2005). The latter results argue against a role for the pocket proteins in recruiting Suv4-20h1/h2 to the chromatin. However, ablation of the LxCxE-binding site in pRB did cause aberrant patterns of H4K20-triM (Isaac et al., 2006). This could imply that an LxCxE-containing protein is recruited to the chromatin by pRB and is required for stabilization of Suv4-20h1/h2 and subsequent induction of HMTase activity. Upon overexpression of Suv4-20h1/h2, this stabilization would not be required to obtain sufficient levels of HMTase activity. Alternatively, one can envision that, in addition to Suv4-20h1/h2, a yet unidentified LxCxE-containing HMTase is responsible for the pRB-dependent methylation of H4K20.

In conclusion, whereas the pocket protein-LxCxE interaction has been implicated in silencing via the establishment of heterochromatic marks, the exact mechanism remains obscure and might involve yet unspecified proteins.

The interaction of pocket proteins with the CtIP/CtBP and SWI-SNF complexes in transcriptional repression

The pocket proteins also interact with the CtBP/CtIP repressor complex. CtBP functions as a transcriptional repressor via binding and inhibiting several transcriptional factors, whereas CtIP interacts with CtBP and probably enhances repression (reviewed by Wu and Lee, 2006). Both pRB and p130 were reported to bind CtIP, and additionally, pRB was reported to bind CtBP (Dahiya et al., 2001; Meloni et al., 1999). CtBP/CtIP have been suggested to repress transcription in both an HDAC-dependent and -independent manner. HDAC independently, CtBP/CtIP is possibly involved in pocket protein-mediated repression of *Cyclin A* and *Cdk1* via recruitment of polycomb group proteins (Chinnadurai, 2002; Dahiya et al., 2001). CtIP contains an LxCxE sequence and deletion of the LxCxE and neighbouring sequence disrupted CtIP-p130 binding (Meloni et al., 1999). Additionally, a pRB protein deficient in binding LxCxE-containing proteins, could no longer interact with CtBP1, indicating the involvement of the LxCxE binding site in contacting the CtBP/CtIP co-repressor (Isaac et al., 2006).

In addition, pRB interacts with the BRG1 and BRM ATPases of the SWI-SNF nucleosome remodeling complex (Dahiya et al., 2001; Strober et al., 1996; Singh et al., 1995). The SWI-SNF complex is thought to function in both transcriptional activation and repression. Also, complex formation between p107 and BRG1, p107 and BRM and, to a lower extent, p130 and BRG1 was demonstrated in a yeast two-hybrid system (Strober et al., 1996). Both BRG1 and BRM1 contain an LxCxE sequence and deletion of the LxCxE plus the neighbouring sequence in BRG or BRM1 ablated formation of pRB-BRG1, pRB-BRM, p107-BRG1 and p107-BRM complexes (Strober et al., 1996; Singh et al., 1995; Dunaief et al., 1994). LxCxE-mediated binding was, however, strongly questioned

by the observation that a pRB protein with a mutated LxCxE binding site was still able to bind BRG1, although unable to bind HDAC1 or HDAC2 (Dahiya et al., 2000; Zhang et al., 2000). An indication for the involvement of BRG1 in pRB-mediated repression was provided by the observation that dominant-negative BRG1 counteracted pRB-mediated repression of the E2F-target gene *Cyclin A* (Siddiqui et al., 2003). Additionally, repression of the *polo-like kinase 1* gene by pRB depended on SWI/SNF activity, and could be reversed by TSA (Gunawardena et al., 2004). Since a tri-molecular complex containing BRG1, pRB and HDAC1 could be formed upon ectopic expression of these proteins (Zhang et al., 2000), this again points to an interplay of various chromatin remodeling activities during pRB-mediated transcriptional repression.

Extra-cellular signaling to the cell cycle machinery extends beyond G_1/S

As described above, pocket proteins function in inhibiting E2F target gene expression, which involves the recruitment of LxCxE-containing proteins. The pocket proteins are active during G_1, where they function as effectors of various extra-cellular stimuli to induce cell cycle progression under growth stimulating conditions, and conversely, to inhibit cell cycle progression under growth restricting conditions. Well-known examples of extra-cellular stimuli are signaling from the extra-cellular matrix (ECM) to the cell via integrin receptors, growth factor stimulation via Receptor Tyrosine Kinases (RTKs), or cell-cell signaling, which induces contact inhibition. Classically, extra-cellular signaling was suggested to convey the decision between proliferation or arrest during the G_1 phase, specifically, before the Restriction point in mid G_1 (Pardee, 1974). However, our recent results have indicated that external stimuli can also interfere with cell cycle progression beyond S phase, *i.e.*, independently of pocket proteins.

Requirements for anchorage-induced signaling during G_1/S progression

Untransformed cells require combined signaling via anchorage and growth factors to stimulate cell cycle progression. Signaling via anchorage is mediated by integrin receptors, whereas growth factor signaling is mediated by RTKs.

Integrins are transmembrane glycoproteins that are composed of an α and β subunit and mediate binding between the ECM and the cell. Contact with the ECM induces integrin clustering at defined places (focal contacts or focal adhesions) and induces a signaling cascade in the cell that promotes proliferation. In addition, the cytoplasmic part of the integrin receptor binds to the actin cytoskeleton, which influences cell shape and migration (reviewed by Hood and Cheresh, 2002).

Integrins and RTKs cooperate to stimulate cell cycle progression by simultaneously inducing the RAS/RAF/MEK/ERK pathway. Integrin stimulation probably converges at different points in this pathway, as both RAS-dependent and RAS-independent

stimulation of ERK was reported upon adhesion-induced stimulation. Upstream in the pathway, integrins have been suggested to enhance activation of the RAS/RAF/MEK/ERK pathway by inducing RTK-mediated signaling. This occurs via both association of integrins with RTKs and via inducing the presence of certain RTKs. Downstream, integrins were reported to stimulate transport of activated ERK into the nucleus (Aplin et al., 2002; Aplin et al., 2001; Schwartz and Assoian, 2001).

Adhesion and growth factors are simultaneously required for the induction of Cyclin D1 protein and of Cyclin D1-associated kinase activity via the RAS/RAF/MEK/ERK pathway. Although the RAS pathway could be stimulated by the individual actions of either integrins or RTKs, combined stimulation was required to induce sufficiently high levels of activated ERK to induce Cyclin D1 (Roovers and Assoian, 2000; Roovers et al., 1999). In addition to inducing Cyclin D1-associated kinase activity, integrin signaling promoted the proteosomal degradation of the cdk-inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ (Bao et al., 2002), which led to a rise in Cyclin E/A-CDK2 kinase activity. Together, the induced Cyclin D-CDK4/6 and Cyclin E/A-CDK2 kinase activities promote phosphorylation of the pocket proteins and entry into S phase.

Conversely, detachment from the ECM caused downregulation of Cyclin D1, via both inhibition of *Cyclin D1* transcription and inhibition of protein translation (Zhu et al., 1996). Additionally, the cdk inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ were induced upon loss of anchorage. Since integrin signaling promoted proteosomal degradation of these inhibitors (Bao et al., 2002), the absence of integrin signaling likely stabilizes $p21^{CIP1}$ and $p27^{KIP1}$ via inhibition of proteosomal degradation. Additionally, loss of anchorage induced $p21^{CIP}$ at the transcriptional level, possibly via activation of p53 (Wu and Schonthal, 1997). Importantly, cells cultured in the absence of anchorage displayed increased association of $p21^{CIP1}$ and $p27^{KIP1}$ with Cyclin E/A-CDK2 complexes, which correlated with downregulation of CDK2 kinase activity. As a result, cells cultured without anchorage contain active, hypo-phoshorylated pRB and arrest in the G_1 phase of the cell cycle (Wu and Schonthal, 1997; Fang et al., 1996; Zhu et al., 1996; Guadagno et al., 1993).

Anchorage signaling beyond S phase; requirements for transformation of pocket protein deficient MEFs

Consistent with a role for pocket proteins in G_1 arrest upon loss of anchorage, ablation of pocket proteins in MEFs bypassed G_1 arrest under non-adherent conditions. Additionally, pocket protein-deficient MEFs bypassed G_1 arrest induced by prolonged culturing, expression of RAS^{V12}, cell-cell contact, growth factor depletion and DNA damage (Foijer et al., 2005; Dannenberg et al., 2004; Peeper et al., 2001; Dannenberg et al., 2000; Sage et al., 2000). Specifically, MEFs deficient for pRB *and* p107, pRB *and* p130 (double knockout, DKO MEFs) or all three pocket proteins (triple knockout, TKO MEFs) were

refractory to both replicative senescence and senescence induced by constitutively active RAS, RAS^{V12}, demonstrating the crucial role of pocket proteins in G_1 control (Dannenberg et al., 2004; Peeper et al., 2001; Dannenberg et al., 2000; Sage et al., 2000).

In line with the integrin-dependent induction of the RAS pathway, we have found that expression of RAS^{V12} was required for anchorage-independent growth of DKO or TKO MEFs (Vormer et al., 2008, Chapter 2). Interestingly, RAS^{V12} expression was not sufficient to induce anchorage-independent growth of these MEFs, but required additional events. Chapters 2 and 3 of this thesis focus on the additional requirements for anchorage-independent growth of pocket protein-deficient MEFs. In Chapter 2, we show that dependent on the level of pocket proteins, loss of adhesion induced both G_1 and G_2 arrest, demonstrating that anchorage signaling is required for cell cycle progression beyond S phase. Similarly, we have previously shown that serum stimulation was required beyond S phase (Foijer et al., 2005). Our present results show that a combination of pocket protein loss and either TBX2 overexpression or p53 downregulation induces G_1 and G_2-associated kinase activities under non-adherent conditions, resulting in the induction of anchorage-independent growth and transformation.

Moreover, the results described in Chapter 3 point to an involvement of the p38/Mapkapk3 pathway in inhibiting anchorage-independent growth. The p38 MAPK, which induces several cell cycle inhibitors, is known to be activated upon stress signaling, but has also been placed downstream of the RAS/RAF/MEK/ERK pathway. Furthermore, the p38 pathway is suggested to activate the p53 tumor suppressor pathway. Mapkapk3 functions downstream of p38, but also of MEK/ERK (Han and Sun, 2007; Zakowski et al., 2004; Ludwig et al., 1996). We show that downregulation of p38 or of Mapkapk3 induced anchorage-independent growth in RAS^{V12}/pocket protein-deficient MEFs. It is likely that upon loss of anchorage, the p38 pathway plays an important role in tumor suppression via activation of p53.

In vitro transformation requirements for human and mouse fibroblasts

As mentioned above, we have found that transformation of MEFs requires expression of RAS^{V12} plus downregulation of both the pocket protein and the p53 pathways. In contrast to our findings, others have claimed previously that RAS^{V12}-induced transformation of murine fibroblasts could be accomplished by ablation of *either* the pocket protein *or* the p53 pathway (Rangarajan et al., 2004; Sage et al., 2000). Since RAS^{V12}-induced transformation of human fibroblasts has been reported to require ablation of *both* pathways (Rangarajan et al., 2004; Voorhoeve and Agami, 2003), a widely held view has emerged that RAS^{V12}-induced transformation of human and murine fibroblasts meets different requirements.

Although oncogenic, expression of constitutively active RAS, RAS^{V12}, induces

G_1 arrest in wild-type fibroblasts, a phenomenon known as oncogene-induced senescence (Serrano et al., 1997). In murine fibroblasts, ablation of either the pocket protein or the p53 pathway was sufficient to bypass RAS^{V12}-induced senescence (Dannenberg et al., 2004; Rangarajan et al., 2004; Peeper et al., 2001; Sage et al., 2000; Kamijo et al., 1997; Serrano et al., 1997). Concerning human fibroblasts, several studies implied that downregulation of both the pocket protein and p53 pathways was required to bypass RAS^{V12}-induced senescence (Wei et al., 2003; Hahn et al., 2002; Serrano et al., 1997). In contrast, others claimed that downregulation of either p53 or pocket proteins suffices to bypass RAS^{V12}-induced senescence in human fibroblasts (Rangarajan et al., 2004; Voorhoeve and Agami, 2003), implying that bypass of RAS^{V12}-induced senescence actually meets similar requirements in human and mouse fibroblasts.

RAS^{V12}-induced transformation clearly meets different requirements in human and mouse fibroblasts: expression of hTERT and SV40 small t antigen (st) are required for transformation of human fibroblasts (Voorhoeve and Agami, 2003; Hahn et al., 2002; Hahn et al., 1999), whereas they are dispensable for transformation of murine fibroblasts (Vormer et al., 2008; Rangarajan et al., 2004). Murine fibroblasts contain long telomeres (Prowse and Greider, 1995; Kipling and Cooke, 1990) and consequently do not require expression of hTERT. The requirement for st in transformation has been linked to the inactivation of PP2A (Hahn et al., 2002), which possibly causes downregulation of PTEN (Boehm et al., 2005) and stabilization of c-Myc (Yeh et al., 2004). Why st is dispensable for transformation of murine fibroblasts remains currently unknown. As already mentioned, a view has emerged that RAS^{V12}-induced transformation of human fibroblasts requires ablation of both the pocket protein and p53 pathways, whereas transformation of murine fibroblasts requires ablation of only one of the two pathways (Rangarajan et al., 2004; Voorhoeve and Agami, 2003; Sage et al., 2000). This contrasts sharply with our findings in murine fibroblasts, showing that ablation of the pocket protein and the p53 pathway synergistically supports RAS^{V12}-induced transformation. These results, described in Chapter 2, imply that transformation requirements for murine and human fibroblasts are not as different as previously claimed.

The role of the pocket proteins in development and tumorigenesis

In line with their key role in cell cycle control, differentiation and apoptosis, pocket proteins are essential for both tumor suppression and embryonic development. $Rb^{-/-}$ mouse embryos died between embryonic day 13.5 and 15.5 with defects in the nervous system, lens, placenta, liver and erythroid lineage. Elevated levels of both proliferation and apoptosis were detected in the lens and in both the central and peripheral nervous system. The presence of a wild-type placenta suppressed apoptosis in the central nervous system and rescued survival of $Rb^{-/-}$ embryos to term. However, the increased levels of

proliferation in the central nervous system and of both proliferation and apoptosis in the lens were maintained, demonstrating that these abnormalities were not caused by placental malfunctioning. Rescued *Rb*$^{-/-}$ animals died shortly after birth with a collapsed alveolar space and severe defects in the skeletal muscle (de Bruin et al., 2003; Wu et al., 2003; Tsai et al., 1998; Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). *p130*$^{-/-}$ and *p107*$^{-/-}$ animals displayed normal survival, however, the combined loss of p130 and p107 resulted in early neonatal death, probably caused by increased proliferation of chondrocytes, resulting in aberrant bone development (Cobrinik et al., 1996).

Loss of pocket proteins strongly predisposes to tumorigenesis. In human cancer, mutations in the *Rb* gene were first identified in the early childhood tumor retinoblastoma, and were subsequently found in various tumor types, including osteocarcoma, small cell lung cancer as well as breast- and bladder carcinomas (reviewed by Burkhart and Sage, 2008; Sherr and McCormick, 2002; Nevins, 2001; Sherr, 1996; Weinberg, 1995). In addition, *p130* was found mutated in a variety of tumor types including retinoblastoma and Burkitt's lymphoma (De Falco G. et al., 2007; Tosi et al., 2005; Cinti et al., 2000), whereas an intragenic deletion in *p107* was detected in a B-cell lymphoma cell line (Ichimura et al., 2000). Importantly, components of the pocket protein pathway were frequently found mutated in human cancer: loss of p16^{INK4A} or overexpression of Cyclin D1 or CDK4, which all promote inactivation of the complete pocket protein family, were detected in melanoma, non-small cell lung cancer, mantle cell lymphoma, sarcomas, pancreatic- and breast cancers, and others. In conclusion, the pocket protein pathway has been suggested to be deregulated in the majority of human tumors (reviewed by Sherr and McCormick, 2002; Nevins, 2001; Sherr, 1996; Weinberg, 1995).

In mice, *Rb* loss clearly predisposes to tumorigenesis: *Rb*$^{+/-}$ germline and *Rb*$^{-/-}$ chimeric mice died of pituitary tumors at early age and additionally developed hyperplasia in the thyroid and pre-neoplastic lesions in the adrenals (Harrison et al., 1995; Maandag et al., 1994; Williams et al., 1994). The wild-type *Rb* allele was lost in pituitary tumors recovered from *Rb*$^{+/-}$ germline mice, further emphasizing the role of *Rb* in counteracting tumorigenesis (Maandag et al., 1994). The tumor spectrum, induced by *Rb* loss, was extended by the additional loss of either *p130* or *p107*, which drove the formation of various tumors, including retinoblastoma, osteocarcoma, lymphosarcoma, pheochromocytoma and adenocarcinoma in the coecum (Dannenberg et al., 2004; Robanus-Maandag et al., 1998).

Implications for the role of pocket protein-chromatin remodeling complexes in tumorigenesis

Whereas the importance of loss of pocket proteins in tumor development has been clearly established, the contribution of pocket protein-E2F-chromatin remodeling complexes in

this process remains elusive. A view has emerged that mainly loss of control of 'activator' E2Fs is involved in tumorigenesis. Specifically, tumorigenesis in *Rb*$^{+/-}$ mice could be counteracted by the additional loss of the activator E2F1 or by loss of E2F4; the latter caused a redistribution of pocket protein-E2F complexes resulting in complex formation between p107/p130 and E2F1/E2F3, presumably inhibiting these activator E2Fs (Lee et al., 2002; Yamasaki et al., 1998). However, the interpretation of these experiments is hampered by the fact that the division between 'activator' and 'repressor' E2Fs is far from absolute. Recent studies have suggested that the 'activator' E2Fs also contain a repressor function (Sahin and Sladek, 2010; Chong et al., 2009b). Moreover, the 'repressor' E2Fs have been implicated in transcriptional activation. Both E2F3b and E2F4 could transactivate an E2F-luciferase-reporter *in vitro* (Chong et al., 2009a; Chapter 4 of this thesis) and overexpression of E2F4 in the nucleus could induce E2F target gene expression and cell cycle progression (Muller et al., 1997). Furthermore, free, unbound E2F4 was strongly implicated in the induction of E2F target genes and S phase progression in fetal erythrocytes (Kinross et al., 2006). Similarly, E2F3b was suggested to function as an activator in MEFs, as it could induce proliferation of the otherwise proliferation deficient *E2f1*$^{-/-}$*E2f2*$^{-/-}$*E2f3*$^{-/-}$ MEFs (Tsai et al., 2008; Wu et al., 2001). Finally, E2F3b was also implicated to function as an activator in the lens and possibly also in the central nervous system (Chong et al., 2009a).

Importantly, a great body of evidence points to an involvement of pocket protein-chromatin remodeling complexes in processes critical for tumor suppression, such as (irreversible) cell cycle arrest and senescence. This is described in detail in Chapter 5 of this thesis. p130, p107 and the 'repressor' E2F4 were recruited to repressed promoters in G_0-arrested mouse fibroblasts (Rayman et al., 2002) and in G_0/G_1-arrested human T98G cells (Takahashi et al., 2000). Additionally, E2F-repressor complexes were shown to be involved in contact inhibition, replicative senescence and RAS^{V12}-induced senescence (Rowland et al., 2002; Zhang et al., 1999). Consistent with a role for LxCxE-containing proteins in executing pRB-mediated silencing, active repression of E2F target genes involving pRB and LxCxE-containing proteins was detected under various growth inhibitory conditions, such as serum starvation (Isaac et al., 2006; Morrison et al., 2002), RAS^{V12}-induced senescence (Narita et al., 2003) and cell cycle arrest induced by $p16^{INK4A}$ (Dahiya et al., 2001). Additionally, pRB-mediated silencing and cell cycle arrest occurred during differentiation of various cell types (Guo et al., 2009; Blais et al., 2007; Khidr and Chen, 2006; Novitch et al., 1996; Gu et al., 1993). During muscle differentiation, the LxCxE-containing Suv39H1 was involved in establishing terminal silencing (Ait-Si-Ali et al., 2004) and moreover, pRB-mediated silencing of cell cycle genes correlated with the presence of repressive chromatin marks (Blais et al., 2007). Together, these studies imply an involvement for the recruitment of LxCxE-containing proteins by pocket proteins in

cell cycle arrest and tumor suppression.

To determine the role of the interaction between pocket proteins and LxCxE-containing proteins in tumorigenesis, we have generated mice and MEFs expressing a mutant pRB protein, pRB^{N750F}, from the endogenous locus. This protein was unable to interact with LxCxE-containing proteins and was thus impaired in recruiting chromatin-remodeling proteins via the LxCxE binding site. Given the compensatory role of p130 and p107 in binding LxCxE-containing proteins, we combined this mutation with loss of p130 or p107 and studied both cell cycle arrest *in vitro* and tumor predisposition *in vivo*. The results of these experiments are described in Chapter 4 and 5 of this thesis. Interestingly, we found that the pRB-LxCxE interaction contributed to cell cycle arrest in response to γ-irradiation or expression of RAS^{V12}. Surprisingly, mice homozygously expressing the pRB^{N750F} protein were not tumor prone under the conditions tested. By combining the pRB^{N750F} mutation with loss of p130 or p107, we show that reducing pocket protein-E2F chromatin remodeling complexes to a level that is compatible with embryonic development, did not ablate the tumor suppressor function of the pocket proteins. As discussed in Chapter 4, we hypothesize that the pRB-LxCxE interaction might play a role during transcriptional silencing and tumor suppression *in vivo*, however, the presence of the remaining pocket proteins and/or of chromatin-remodeling proteins interacting independently of the LxCxE binding site, prevents a full relieve of transcriptional silencing *in vivo* and subsequently inhibits tumor formation.

Outline thesis

This thesis focuses on the role of the pocket proteins during *in vitro* transformation and *in vivo* tumorigenesis. The first part of this thesis describes the transformation requirements for pocket protein-deficient MEFs. In Chapter 2, we show that a combination of pocket protein loss and either TBX2 overexpression or p53 downregulation is required for the induction of G_1- and G_2-associated kinase activities under non-adherent conditions, resulting in the induction of anchorage-independent growth and transformation. In Chapter 3, we describe the involvement of the p38 pathway and one of its downstream effectors, Mapkapk3, in counteracting transformation of pocket protein-deficient MEFs. The second part of this thesis focuses on the interaction between pRB and proteins containing an LxCxE motif during cell cycle arrest *in vitro* and tumorigenesis *in vivo*. In Chapter 4, we show that the pRB-LxCxE interaction contributes to cell cycle arrest in response to γ-irradiation or overexpression of RAS^{V12}, whereas the interaction is dispensable for arrest in response to serum deprivation or cell-cell contact. Chapter 5 describes the effect of ablating the pRB-LxCxE interaction in mice. Surprisingly, we found that ablation of this interaction did not predispose mice to tumor formation, implying that the pRB-LxCxE interaction does not comprise a major tumor suppressor role of pRB.

References

Ait-Si-Ali,S., Guasconi,V., Fritsch,L., Yahi,H., Sekhri,R., Naguibneva,I., Robin,P., Cabon,F., Polesskaya,A., and Harel-Bellan,A. (2004). A Suv39h-dependent mechanism for silencing S-phase genes in differentiating but not in cycling cells. EMBO J. *23*, 605-615.

Aplin,A.E., Hogan,B.P., Tomeu,J., and Juliano,R.L. (2002). Cell adhesion differentially regulates the nucleocytoplasmic distribution of active MAP kinases. J. Cell Sci. *115*, 2781-2790.

Aplin,A.E., Stewart,S.A., Assoian,R.K., and Juliano,R.L. (2001). Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. J. Cell Biol. *153*, 273-282.

Balciunaite,E., Spektor,A., Lents,N.H., Cam,H., te Riele,H., Scime,A., Rudnicki,M.A., Young,R., and Dynlacht,B.D. (2005). Pocket protein complexes are recruited to distinct targets in quiescent and proliferating cells. Mol. Cell Biol. *25*, 8166-8178.

Bao,W., Thullberg,M., Zhang,H., Onischenko,A., and Stromblad,S. (2002). Cell attachment to the extracellular matrix induces proteasomal degradation of p21(CIP1) via Cdc42/Rac1 signaling. Mol. Cell Biol. *22*, 4587-4597.

Berthet,C., Kaldis,P. (2007) Cell specific responses to loss of cyclin-dependent kinases. Oncogene *26,* 4469-4477.

Blais,A., van Oevelen,C.J., Margueron,R., costa-Alvear,D., and Dynlacht,B.D. (2007). Retinoblastoma tumor suppressor protein-dependent methylation of histone H3 lysine 27 is associated with irreversible cell cycle exit. J. Cell Biol. *179*, 1399-1412.

Boehm,J.S., Hession,M.T., Bulmer,S.E., and Hahn,W.C. (2005). Transformation of human and murine fibroblasts without viral oncoproteins. Mol. Cell Biol. *25*, 6464-6474.

Brehm,A., Miska,E.A., McCance,D.J., Reid,J.L., Bannister,A.J., and Kouzarides,T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature *391*, 597-601.

Burkhart,D.L. and Sage,J. (2008). Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nat. Rev. Cancer *8*, 671-682.

Chen,T.T. and Wang,J.Y. (2000). Establishment of irreversible growth arrest in myogenic differentiation requires the RB LXCXE-binding function. Mol. Cell Biol. *20*, 5571-5580.

Chinnadurai,G. (2002). CtBP, an unconventional transcriptional corepressor in development and oncogenesis. Mol. Cell *9*, 213-224.

Chong,J.L., Tsai,S.Y., Sharma,N., Opavsky,R., Price,R., Wu,L., Fernandez,S.A., and Leone,G. (2009a). E2f3a and E2f3b contribute to the control of cell proliferation and mouse development. Mol. Cell Biol. *29*, 414-424.

Chong,J.L., Wenzel,P.L., Saenz-Robles,M.T., Nair,V., Ferrey,A., Hagan,J.P., Gomez,Y.M., Sharma,N., Chen,H.Z., Ouseph,M., Wang,S.H., Trikha,P., Culp,B., Mezache,L., Winton,D.J., Sansom,O.J., Chen,D., Bremner,R., Cantalupo,P.G., Robinson,M.L., Pipas,J.M., and Leone,G. (2009b). E2f1-3 switch from activators in progenitor cells to repressors in differentiating cells. Nature *462*, 930-934.

Ciemerych,M.A. and Sicinski,P. (2005). Cell cycle in mouse development. Oncogene *24*, 2877-

2898.

Cinti,C., Claudio,P.P., Howard,C.M., Neri,L.M., Fu,Y., Leoncini,L., Tosi,G.M., Maraldi,N.M., and Giordano,A. (2000). Genetic alterations disrupting the nuclear localization of the retinoblastoma-related gene RB2/p130 in human tumor cell lines and primary tumors. Cancer Res. *60*, 383-389.

Clarke,A.R., Maandag,E.R., van Roon,M., van der Lugt,N.M., van der Valk,M., Hooper,M.L., Berns,A., and te Riele,H. (1992). Requirement for a functional Rb-1 gene in murine development. Nature *359*, 328-330.

Cobrinik,D. (2005). Pocket proteins and cell cycle control. Oncogene *24*, 2796-2809.

Cobrinik,D., Lee,M.H., Hannon,G., Mulligan,G., Bronson,R.T., Dyson,N., Harlow,E., Beach,D., Weinberg,R.A., and Jacks,T. (1996). Shared role of the pRB-related p130 and p107 proteins in limb development. Genes Dev. *10*, 1633-1644.

Cobrinik,D., Whyte,P., Peeper,D.S., Jacks,T., and Weinberg,R.A. (1993). Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes Dev. *7*, 2392-2404.

Dahiya,A., Gavin,M.R., Luo,R.X., and Dean,D.C. (2000). Role of the LXCXE binding site in Rb function. Mol. Cell Biol. *20*, 6799-6805.

Dahiya,A., Wong,S., Gonzalo,S., Gavin,M., and Dean,D.C. (2001). Linking the Rb and polycomb pathways. Mol. Cell *8*, 557-569.

Dannenberg,J.H., Schuijff,L., Dekker,M., van der Valk,M., and te Riele,H. (2004). Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev. *18*, 2952-2962.

Dannenberg,J.H., van Rossum,A., Schuijff,L., and te Riele,H. (2000). Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. Genes Dev. *14*, 3051-3064.

de Bruin,A., Wu,L., Saavedra,H.I., Wilson,P., Yang,Y., Rosol,T.J., Weinstein,M., Robinson,M.L., and Leone,G. (2003). Rb function in extraembryonic lineages suppresses apoptosis in the CNS of Rb-deficient mice. Proc. Natl. Acad. Sci. U. S. A *100*, 6546-6551.

De Falco G., Leucci,E., Lenze,D., Piccaluga,P.P., Claudio,P.P., Onnis,A., Cerino,G., Nyagol,J., Mwanda,W., Bellan,C., Hummel,M., Pileri,S., Tosi,P., Stein,H., Giordano,A., and Leoncini,L. (2007). Gene-expression analysis identifies novel RBL2/p130 target genes in endemic Burkitt lymphoma cell lines and primary tumors. Blood *110*, 1301-1307.

DeGregori,J. and Johnson,D.G. (2006). Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis. Curr. Mol. Med. *6*, 739-748.

DeGregori,J., Leone,G., Miron,A., Jakoi,L., and Nevins,J.R. (1997). Distinct roles for E2F proteins in cell growth control and apoptosis. Proc. Natl. Acad. Sci. U. S. A *94*, 7245-7250.

Dick,F.A. (2007). Structure-function analysis of the retinoblastoma tumor suppressor protein - is the whole a sum of its parts? Cell Div. *2*, 26.

Dimova,D.K. and Dyson,N.J. (2005). The E2F transcriptional network: old acquaintances with new faces. Oncogene *24*, 2810-2826.

Dunaief,J.L., Strober,B.E., Guha,S., Khavari,P.A., Alin,K., Luban,J., Begemann,M., Crabtree,G.R., and Goff,S.P. (1994). The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. Cell *79*, 119-130.

Fang,F., Orend,G., Watanabe,N., Hunter,T., and Ruoslahti,E. (1996). Dependence of cyclin E-CDK2 kinase activity on cell anchorage. Science *271*, 499-502.

Ferreira,R., Magnaghi-Jaulin,L., Robin,P., Harel-Bellan,A., and Trouche,D. (1998). The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase. Proc. Natl. Acad. Sci. U. S. A *95*, 10493-10498.

Foijer,F., Wolthuis,R.M., Doodeman,V., Medema,R.H., and te Riele,H. (2005). Mitogen requirement for cell cycle progression in the absence of pocket protein activity. Cancer Cell *8*, 455-466.

Frolov,M.V. and Dyson,N.J. (2004). Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. J. Cell Sci. *117*, 2173-2181.

Gonzalo,S., Garcia-Cao,M., Fraga,M.F., Schotta,G., Peters,A.H., Cotter,S.E., Eguia,R., Dean,D.C., Esteller,M., Jenuwein,T., and Blasco,M.A. (2005). Role of the RB1 family in stabilizing histone methylation at constitutive heterochromatin. Nat. Cell Biol. *7*, 420-428.

Gu,W., Schneider,J.W., Condorelli,G., Kaushal,S., Mahdavi,V., and Nadal-Ginard,B. (1993). Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell *72*, 309-324.

Guadagno,T.M., Ohtsubo,M., Roberts,J.M., and Assoian,R.K. (1993). A link between cyclin A expression and adhesion-dependent cell cycle progression. Science *262*, 1572-1575.

Gunawardena,R.W., Siddiqui,H., Solomon,D.A., Mayhew,C.N., Held,J., Angus,S.P., and Knudsen,E.S. (2004). Hierarchical requirement of SWI/SNF in retinoblastoma tumor suppressor-mediated repression of Plk1. J. Biol. Chem. *279*, 29278-29285.

Guo,J., Longshore,S., Nair,R., and Warner,B.W. (2009). Retinoblastoma protein (pRb), but not p107 or p130, is required for maintenance of enterocyte quiescence and differentiation in small intestine. J. Biol. Chem. *284*, 134-140.

Hahn,W.C., Counter,C.M., Lundberg,A.S., Beijersbergen,R.L., Brooks,M.W., and Weinberg,R.A. (1999). Creation of human tumour cells with defined genetic elements. Nature *400*, 464-468.

Hahn,W.C., Dessain,S.K., Brooks,M.W., King,J.E., Elenbaas,B., Sabatini,D.M., DeCaprio,J.A., and Weinberg,R.A. (2002). Enumeration of the simian virus 40 early region elements necessary for human cell transformation. Mol. Cell Biol. *22*, 2111-2123.

Han,J. and Sun,P. (2007). The pathways to tumor suppression via route p38. Trends Biochem. Sci. *32*, 364-371.

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

Harrison,D.J., Hooper,M.L., Armstrong,J.F., and Clarke,A.R. (1995). Effects of heterozygosity for the Rb-1t19neo allele in the mouse. Oncogene *10*, 1615-1620.

Hood,J.D. and Cheresh,D.A. (2002). Role of integrins in cell invasion and migration. Nat. Rev.

Cancer *2*, 91-100.

Hurford,R.K., Jr., Cobrinik,D., Lee,M.H., and Dyson,N. (1997). pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. Genes Dev. *11*, 1447-1463.

Ichimura,K., Hanafusa,H., Takimoto,H., Ohgama,Y., Akagi,T., and Shimizu,K. (2000). Structure of the human retinoblastoma-related p107 gene and its intragenic deletion in a B-cell lymphoma cell line. Gene *251*, 37-43.

Isaac,C.E., Francis,S.M., Martens,A.L., Julian,L.M., Seifried,L.A., Erdmann,N., Binne,U.K., Harrington,L., Sicinski,P., Berube,N.G., Dyson,N.J., and Dick,F.A. (2006). The retinoblastoma protein regulates pericentric heterochromatin. Mol. Cell Biol. *26*, 3659-3671.

Jacks,T., Fazeli,A., Schmitt,E.M., Bronson,R.T., Goodell,M.A., and Weinberg,R.A. (1992). Effects of an Rb mutation in the mouse. Nature *359*, 295-300.

Johnson,D.G., Schwarz,J.K., Cress,W.D., and Nevins,J.R. (1993). Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature *365*, 349-352.

Kamijo,T., Zindy,F., Roussel,M.F., Quelle,D.E., Downing,J.R., Ashmun,R.A., Grosveld,G., and Sherr,C.J. (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell *91*, 649-659.

Katsuno,Y., Suzuki,A., Sugimura,K., Okumura,K., Zineldeen,D.H., Shimada,M., Niida,H., Mizuno,T., Hanaoka,F., and Nakanishi,M. (2009). Cyclin A-Cdk1 regulates the origin firing program in mammalian cells. Proc. Natl. Acad. Sci. U. S. A *106*, 3184-3189.

Khidr,L. and Chen,P.L. (2006). RB, the conductor that orchestrates life, death and differentiation. Oncogene *25*, 5210-5219.

Kinross,K.M., Clark,A.J., Iazzolino,R.M., and Humbert,P.O. (2006). E2f4 regulates fetal erythropoiesis through the promotion of cellular proliferation. Blood *108*, 886-895.

Kipling,D. and Cooke,H.J. (1990). Hypervariable ultra-long telomeres in mice. Nature *347*, 400-402.

Lachner,M., O'Carroll,D., Rea,S., Mechtler,K., and Jenuwein,T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*, 116-120.

Lai,A., Kennedy,B.K., Barbie,D.A., Bertos,N.R., Yang,X.J., Theberge,M.C., Tsai,S.C., Seto,E., Zhang,Y., Kuzmichev,A., Lane,W.S., Reinberg,D., Harlow,E., and Branton,P.E. (2001). RBP1 recruits the mSIN3-histone deacetylase complex to the pocket of retinoblastoma tumor suppressor family proteins found in limited discrete regions of the nucleus at growth arrest. Mol. Cell Biol. *21*, 2918-2932.

Lai,A., Lee,J.M., Yang,W.M., DeCaprio,J.A., Kaelin,W.G., Jr., Seto,E., and Branton,P.E. (1999). RBP1 recruits both histone deacetylase-dependent and -independent repression activities to retinoblastoma family proteins. Mol. Cell Biol. *19*, 6632-6641.

Lavia,P. and Jansen-Durr,P. (1999). E2F target genes and cell-cycle checkpoint control. Bioessays *21*, 221-230.

Lee,E.Y., Cam,H., Ziebold,U., Rayman,J.B., Lees,J.A., and Dynlacht,B.D. (2002). E2F4 loss

suppresses tumorigenesis in Rb mutant mice. Cancer Cell *2*, 463-472.

Lee,E.Y., Chang,C.Y., Hu,N., Wang,Y.C., Lai,C.C., Herrup,K., Lee,W.H., and Bradley,A. (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature *359*, 288-294.

Lees,J.A., Saito,M., Vidal,M., Valentine,M., Look,T., Harlow,E., Dyson,N., and Helin,K. (1993). The retinoblastoma protein binds to a family of E2F transcription factors. Mol. Cell Biol. *13*, 7813-7825.

Ludwig,S., Engel,K., Hoffmeyer,A., Sithanandam,G., Neufeld,B., Palm,D., Gaestel,M., and Rapp,U.R. (1996). 3pK, a novel mitogen-activated protein (MAP) kinase-activated protein kinase, is targeted by three MAP kinase pathways. Mol. Cell Biol. *16*, 6687-6697.

Lukas,J., Petersen,B.O., Holm,K., Bartek,J., and Helin,K. (1996). Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. Mol. Cell Biol. *16*, 1047-1057.

Luo,R.X., Postigo,A.A., and Dean,D.C. (1998). Rb interacts with histone deacetylase to repress transcription. Cell *92*, 463-473.

Maandag,E.C., van der Valk,M., Vlaar,M., Feltkamp,C., O'Brien,J., van Roon,M., van der Lugt,N., Berns,A., and te Riele,H. (1994). Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. EMBO J. *13*, 4260-4268.

Macaluso,M., Montanari,M., and Giordano,A. (2006). Rb family proteins as modulators of gene expression and new aspects regarding the interaction with chromatin remodeling enzymes. Oncogene *25*, 5263-5267.

Magnaghi-Jaulin,L., Groisman,R., Naguibneva,I., Robin,P., Lorain,S., Le Villain,J.P., Troalen,F., Trouche,D., and Harel-Bellan,A. (1998). Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature *391*, 601-605.

Meloni,A.R., Smith,E.J., and Nevins,J.R. (1999). A mechanism for Rb/p130-mediated transcription repression involving recruitment of the CtBP corepressor. Proc. Natl. Acad. Sci. U. S. A *96*, 9574-9579.

Moberg,K., Starz,M.A., and Lees,J.A. (1996). E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. Mol. Cell Biol. *16*, 1436-1449.

Morrison,A.J., Sardet,C., and Herrera,R.E. (2002). Retinoblastoma protein transcriptional repression through histone deacetylation of a single nucleosome. Mol. Cell Biol. *22*, 856-865.

Muller,H., Bracken,A.P., Vernell,R., Moroni,M.C., Christians,F., Grassilli,E., Prosperini,E., Vigo,E., Oliner,J.D., and Helin,K. (2001). E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes Dev. *15*, 267-285.

Muller,H., Moroni,M.C., Vigo,E., Petersen,B.O., Bartek,J., and Helin,K. (1997). Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. Mol. Cell Biol. *17*, 5508-5520.

Narita,M., Nunez,S., Heard,E., Narita,M., Lin,A.W., Hearn,S.A., Spector,D.L., Hannon,G.J., and Lowe,S.W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during

cellular senescence. Cell *113*, 703-716.

Nevins,J.R. (2001). The Rb/E2F pathway and cancer. Hum. Mol. Genet. *10*, 699-703.

Nicolas,E., Roumillac,C., and Trouche,D. (2003). Balance between acetylation and methylation of histone H3 lysine 9 on the E2F-responsive dihydrofolate reductase promoter. Mol. Cell Biol. *23*, 1614-1622.

Nielsen,S.J., Schneider,R., Bauer,U.M., Bannister,A.J., Morrison,A., O'Carroll,D., Firestein,R., Cleary,M., Jenuwein,T., Herrera,R.E., and Kouzarides,T. (2001). Rb targets histone H3 methylation and HP1 to promoters. Nature *412*, 561-565.

Novitch,B.G., Mulligan,G.J., Jacks,T., and Lassar,A.B. (1996). Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. J. Cell Biol. *135*, 441-456.

Pardee,A.B. (1974). A restriction point for control of normal animal cell proliferation. Proc. Natl. Acad. Sci. U. S. A *71*, 1286-1290.

Peeper,D.S., Dannenberg,J.H., Douma,S., te Riele,H., and Bernards,R. (2001). Escape from premature senescence is not sufficient for oncogenic transformation by Ras. Nat. Cell Biol. *3*, 198-203.

Pines,J. and Hunter,T. (1992). Cyclins A and B1 in the human cell cycle. Ciba Found. Symp. *170*, 187-196.

Prowse,K.R. and Greider,C.W. (1995). Developmental and tissue-specific regulation of mouse telomerase and telomere length. Proc. Natl. Acad. Sci. U. S. A *92*, 4818-4822.

Rangarajan,A., Hong,S.J., Gifford,A., and Weinberg,R.A. (2004). Species- and cell type-specific requirements for cellular transformation. Cancer Cell *6*, 171-183.

Rayman,J.B., Takahashi,Y., Indjeian,V.B., Dannenberg,J.H., Catchpole,S., Watson,R.J., te Riele,H., and Dynlacht,B.D. (2002). E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. Genes Dev. *16*, 933-947.

Rea,S., Eisenhaber,F., O'Carroll,D., Strahl,B.D., Sun,Z.W., Schmid,M., Opravil,S., Mechtler,K., Ponting,C.P., Allis,C.D., and Jenuwein,T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature *406*, 593-599.

Ren,B., Cam,H., Takahashi,Y., Volkert,T., Terragni,J., Young,R.A., and Dynlacht,B.D. (2002). E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. Genes Dev. *16*, 245-256.

Robanus-Maandag,E., Dekker,M., van der Valk,M., Carrozza,M.L., Jeanny,J.C., Dannenberg,J.H., Berns,A., and te Riele H. (1998). p107 is a suppressor of retinoblastoma development in pRb-deficient mice. Genes Dev. *12*, 1599-1609.

Roovers,K. and Assoian,R.K. (2000). Integrating the MAP kinase signal into the G1 phase cell cycle machinery. Bioessays *22*, 818-826.

Roovers,K., Davey,G., Zhu,X., Bottazzi,M.E., and Assoian,R.K. (1999). Alpha5beta1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth

factor-treated cells. Mol. Biol. Cell *10*, 3197-3204.

Rowland,B.D., Denissov,S.G., Douma,S., Stunnenberg,H.G., Bernards,R., and Peeper,D.S. (2002). E2F transcriptional repressor complexes are critical downstream targets of p19(ARF)/p53-induced proliferative arrest. Cancer Cell *2*, 55-65.

Sage,J., Mulligan,G.J., Attardi,L.D., Miller,A., Chen,S., Williams,B., Theodorou,E., and Jacks,T. (2000). Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. Genes Dev. *14*, 3037-3050.

Sahin,F. and Sladek,T.L. (2010). E2F-1 has dual roles depending on the cell cycle. Int. J. Biol. Sci. *6*, 116-128.

Schotta,G., Lachner,M., Sarma,K., Ebert,A., Sengupta,R., Reuter,G., Reinberg,D., and Jenuwein,T. (2004). A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev. *18*, 1251-1262.

Schwartz,M.A. and Assoian,R.K. (2001). Integrins and cell proliferation: regulation of cyclin-dependent kinases via cytoplasmic signaling pathways. J. Cell Sci. *114*, 2553-2560.

Serrano,M., Lin,A.W., McCurrach,M.E., Beach,D., and Lowe,S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593-602.

Sherr,C.J. (1996). Cancer cell cycles. Science *274*, 1672-1677.

Sherr,C.J. and McCormick,F. (2002). The RB and p53 pathways in cancer. Cancer Cell *2*, 103-112.

Sherr,C.J. and Roberts,J.M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. *13*, 1501-1512.

Sherr,C.J. and Roberts,J.M. (2004). Living with or without cyclins and cyclin-dependent kinases. Genes Dev. *18*, 2699-2711.

Siddiqui,H., Fox,S.R., Gunawardena,R.W., and Knudsen,E.S. (2007). Loss of RB compromises specific heterochromatin modifications and modulates HP1alpha dynamics. J. Cell Physiol *211*, 131-137.

Siddiqui,H., Solomon,D.A., Gunawardena,R.W., Wang,Y., and Knudsen,E.S. (2003). Histone deacetylation of RB-responsive promoters: requisite for specific gene repression but dispensable for cell cycle inhibition. Mol. Cell Biol. *23*, 7719-7731.

Singh,P., Coe,J., and Hong,W. (1995). A role for retinoblastoma protein in potentiating transcriptional activation by the glucocorticoid receptor. Nature *374*, 562-565.

Strober,B.E., Dunaief,J.L., Guha, and Goff,S.P. (1996). Functional interactions between the hBRM/hBRG1 transcriptional activators and the pRB family of proteins. Mol. Cell Biol. *16*, 1576-1583.

Takahashi,Y., Rayman,J.B., and Dynlacht,B.D. (2000). Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. Genes Dev. *14*, 804-816.

Tosi,G.M., Trimarchi,C., Macaluso,M., La Sala D., Ciccodicola,A., Lazzi,S., Massaro-Giordano,M., Caporossi,A., Giordano,A., and Cinti,C. (2005). Genetic and epigenetic alterations of

RB2/p130 tumor suppressor gene in human sporadic retinoblastoma: implications for pathogenesis and therapeutic approach. Oncogene *24*, 5827-5836.

Tsai,K.Y., Hu,Y., Macleod,K.F., Crowley,D., Yamasaki,L., and Jacks,T. (1998). Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos. Mol. Cell *2*, 293-304.

Tsai,S.Y., Opavsky,R., Sharma,N., Wu,L., Naidu,S., Nolan,E., Feria-Arias,E., Timmers,C., Opavska,J., de,B.A., Chong,J.L., Trikha,P., Fernandez,S.A., Stromberg,P., Rosol,T.J., and Leone,G. (2008). Mouse development with a single E2F activator. Nature *454*, 1137-1141.

Vandel,L., Nicolas,E., Vaute,O., Ferreira,R., it-Si-Ali,S., and Trouche,D. (2001). Transcriptional repression by the retinoblastoma protein through the recruitment of a histone methyltransferase. Mol. Cell Biol. *21*, 6484-6494.

Vaute,O., Nicolas,E., Vandel,L., and Trouche,D. (2002). Functional and physical interaction between the histone methyl transferase Suv39H1 and histone deacetylases. Nucleic Acids Res. *30*, 475-481.

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

Vormer,T.L., Foijer,F., Wielders,C.L., and te Riele,H. (2008). Anchorage-independent growth of pocket protein-deficient murine fibroblasts requires bypass of G2 arrest and can be accomplished by expression of TBX2. Mol. Cell Biol. *28*, 7263-7273.

Wei,W., Jobling,W.A., Chen,W., Hahn,W.C., and Sedivy,J.M. (2003). Abolition of cyclin-dependent kinase inhibitor p16Ink4a and p21Cip1/Waf1 functions permits Ras-induced anchorage-independent growth in telomerase-immortalized human fibroblasts. Mol. Cell Biol. *23*, 2859-2870.

Weinberg,R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323-330.

Williams,B.O., Schmitt,E.M., Remington,L., Bronson,R.T., Albert,D.M., Weinberg,R.A., and Jacks,T. (1994). Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. EMBO J. *13*, 4251-4259.

Wu,G. and Lee,W.H. (2006). CtIP, a multivalent adaptor connecting transcriptional regulation, checkpoint control and tumor suppression. Cell Cycle *5*, 1592-1596.

Wu,L., de Bruin,A., Saavedra,H.I., Starovic,M., Trimboli,A., Yang,Y., Opavska,J., Wilson,P., Thompson,J.C., Ostrowski,M.C., Rosol,T.J., Woollett,L.A., Weinstein,M., Cross,J.C., Robinson,M.L., and Leone,G. (2003). Extra-embryonic function of Rb is essential for embryonic development and viability. Nature *421*, 942-947.

Wu,L., Timmers,C., Maiti,B., Saavedra,H.I., Sang,L., Chong,G.T., Nuckolls,F., Giangrande,P., Wright,F.A., Field,S.J., Greenberg,M.E., Orkin,S., Nevins,J.R., Robinson,M.L., and Leone,G. (2001). The E2F1-3 transcription factors are essential for cellular proliferation. Nature *414*, 457-462.

Wu,R.C. and Schonthal,A.H. (1997). Activation of p53-p21waf1 pathway in response to disruption of cell-matrix interactions. J. Biol. Chem. *272*, 29091-29098.

Yamasaki,L., Bronson,R., Williams,B.O., Dyson,N.J., Harlow,E., and Jacks,T. (1998). Loss of E2F-1 reduces tumorigenesis and extends the lifespan of Rb1(+/-)mice. Nat. Genet. *18*, 360-364.

Yeh,E., Cunningham,M., Arnold,H., Chasse,D., Monteith,T., Ivaldi,G., Hahn,W.C., Stukenberg,P.T., Shenolikar,S., Uchida,T., Counter,C.M., Nevins,J.R., Means,A.R., and Sears,R. (2004). A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. Nat. Cell Biol. *6*, 308-318.

Zakowski,V., Keramas,G., Kilian,K., Rapp,U.R., and Ludwig,S. (2004). Mitogen-activated 3p kinase is active in the nucleus. Exp. Cell Res. *299*, 101-109.

Zhang,H.S., Gavin,M., Dahiya,A., Postigo,A.A., Ma,D., Luo,R.X., Harbour,J.W., and Dean,D.C. (2000). Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell *101*, 79-89.

Zhang,H.S., Postigo,A.A., and Dean,D.C. (1999). Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFbeta, and contact inhibition. Cell *97*, 53-61.

Zhu,X., Ohtsubo,M., Bohmer,R.M., Roberts,J.M., and Assoian,R.K. (1996). Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein. J. Cell Biol. *133*, 391-403.

CHAPTER 2

Anchorage-independent growth of pocket protein-deficient murine fibroblasts requires bypass of G_2 arrest and can be accomplished by expression of TBX2

Tinke L. Vormer, Floris Foijer, Camiel L.C. Wielders and Hein te Riele

Molecular and Cellular Biology 2008 Dec;28(24):7263-73.

Abstract

Mouse embryonic fibroblasts (MEFs) deficient for pocket proteins (*i.e.*, pRB/p107-, pRB/p130-, or pRB/p107/p130-deficient MEFs) have lost proper G_1 control and are refractory to RAS^{V12}-induced senescence. However, pocket protein-deficient MEFs expressing RAS^{V12} were unable to exhibit anchorage-independent growth or to form tumors in nude mice. We show that depending on the level of pocket proteins, loss of adhesion induces G_1 and G_2 arrest, which could be alleviated by overexpression of the TBX2 oncogene. TBX2-induced transformation occurred only in the absence of pocket proteins and could be attributed to downregulation of the $p53/p21^{CIP1}$ pathway. Our results show that a balance between the pocket protein and p53 pathways determines the level of transformation of MEFs by regulating cyclin-dependent kinase activities. Since transformation of human fibroblasts also requires ablation of both pathways, our results imply that the mechanisms underlying transformation of human and mouse cells are not as different as previously claimed.

Introduction

Deregulation of the pRB tumor suppressor pathway is a frequent event in the development of cancer (reviewed by Nevins, 2001; Hanahan and Weinberg, 2000). pRB and its close homologs p107 and p130 comprise the family of so-called pocket proteins and are widely known for their role in cell cycle regulation, especially during G_1 phase. In their active, hypophosphorylated form, pocket proteins restrict cell cycle progression by binding to E2F transcription factors. E2F-pocket protein complex formation inhibits the expression of E2F target genes both by blocking E2F's ability to induce transcription and by active repression. As a result, initiation of S phase is inhibited. Upon cell cycle stimulation, Cyclin D-CDK4/6 (Cyclin-dependent kinase 4 or 6) and Cyclin E-CDK2 complexes are activated and hyperphosphorylate the pocket proteins, resulting in liberation and activation of E2F transcription factors and initiation of S phase. Further cell cycle progression requires Cyclin A-CDK1/2 activity in S phase and Cyclin A-CDK1/2 and Cyclin B1-CDK1 activities in G_2/M phase. The activity of cyclin-cdk complexes can be inhibited by the INK4a (inhibitor of cyclin-dependent kinase 4a) and the CIP/KIP families of cdk inhibitors (reviewed by Berthet and Kaldis, 2007).

Consistent with a role for pocket proteins in G_1 control, we and others have shown that complete ablation of pocket proteins in mouse embryonic fibroblasts (MEFs) abrogated G_1 arrest in response to growth-inhibitory signals, such as cell-cell contact, growth factor depletion and DNA damage. Additionally, upon prolonged culturing or expression of constitutively active RAS (RAS^{V12}), wild-type MEFs arrested in G_1 and displayed hallmarks of senescence, while MEFs deficient for both pRB *and* p107 or both pRB *and* p130 (double-knockout, DKO MEFs) or all three pocket proteins (triple-

knockout, TKO MEFs) were refractory to replicative and RAS^{V12}-induced senescence (Dannenberg et al., 2004; Peeper et al., 2001; Dannenberg et al., 2000; Sage et al., 2000). Similar to ablation of pocket proteins, ablation of the tumor suppressor p53 or its upstream regulator $p19^{ARF}$ also bypassed replicative and RAS^{V12}-induced senescence in MEFs (Kamijo et al., 1997). However, while p53- or $p19^{ARF}$-deficient MEFs could easily be transformed by RAS^{V12}, pocket protein-deficient MEFs expressing RAS^{V12} were unable to exhibit anchorage-independent growth and did not form tumors in nude mice (Peeper et al., 2001; this communication). This demonstrates that the loss of pocket proteins in primary MEFs is not sufficient for RAS^{V12}-induced transformation.

In the present study, we performed a cDNA screen aimed at identifying oncogenes that collaborate with the loss of pocket proteins and RAS^{V12} in transformation of MEFs. We identified the oncogene TBX2 as a cooperating factor in transformation of pocket protein-deficient MEFs. TBX2 downregulates the $p53/p21^{CIP1}$ pathway both in wild-type and pocket protein-deficient MEFs, and indeed, downregulating the $p53/p21^{CIP1}$ pathway by RNA interference transformed pocket protein-deficient MEFs. In contrast to previous reports, our results demonstrate that RAS^{V12}-induced transformation of murine fibroblasts requires downregulation of *both* the pocket protein and p53 pathways. Since downregulation of both pathways is also required for transformation of human fibroblasts (Boehm et al., 2005; Rangarajan et al., 2004; Voorhoeve and Agami, 2003), our results indicate that the mechanisms underlying RAS^{V12}-induced transformation of human and mouse fibroblasts are not fundamentally different. Furthermore, we show that anchorage-independent growth not only requires bypass of G_1 control but also requires bypass of G_2 control.

Results

Gain-of-function screen for anchorage-independent growth of RAS^{V12}-expressing pocket protein-deficient MEFs

We have previously shown that ablation of pRB and p107 rendered MEFs insensitive to replicative and RAS^{V12}-induced senescence but was not sufficient for RAS^{V12}-induced transformation: RAS^{V12}-expressing DKO MEFs were unable to exhibit anchorage-independent growth in culture and did not form tumors in nude mice (Peeper et al., 2001; Dannenberg et al., 2000). To identify events enabling RAS^{V12}-induced transformation of pocket protein-deficient MEFs, we performed a gain-of-function screen using $Rb^{-/-}p130^{-/-}$ (DKO) MEFs expressing RAS^{V12}. In short, DKO MEFs were retrovirally transduced with pBABE-RAS^{V12} and subsequently with the pEYK-MCF7 cDNA library or with pEYK-GFP. Transduced cells were then seeded in soft agar. Although some colonies appeared in the control vector-infected cells, infection with the pEYK-MCF7 library increased the number of colonies three times. Colonies were picked, and the presence

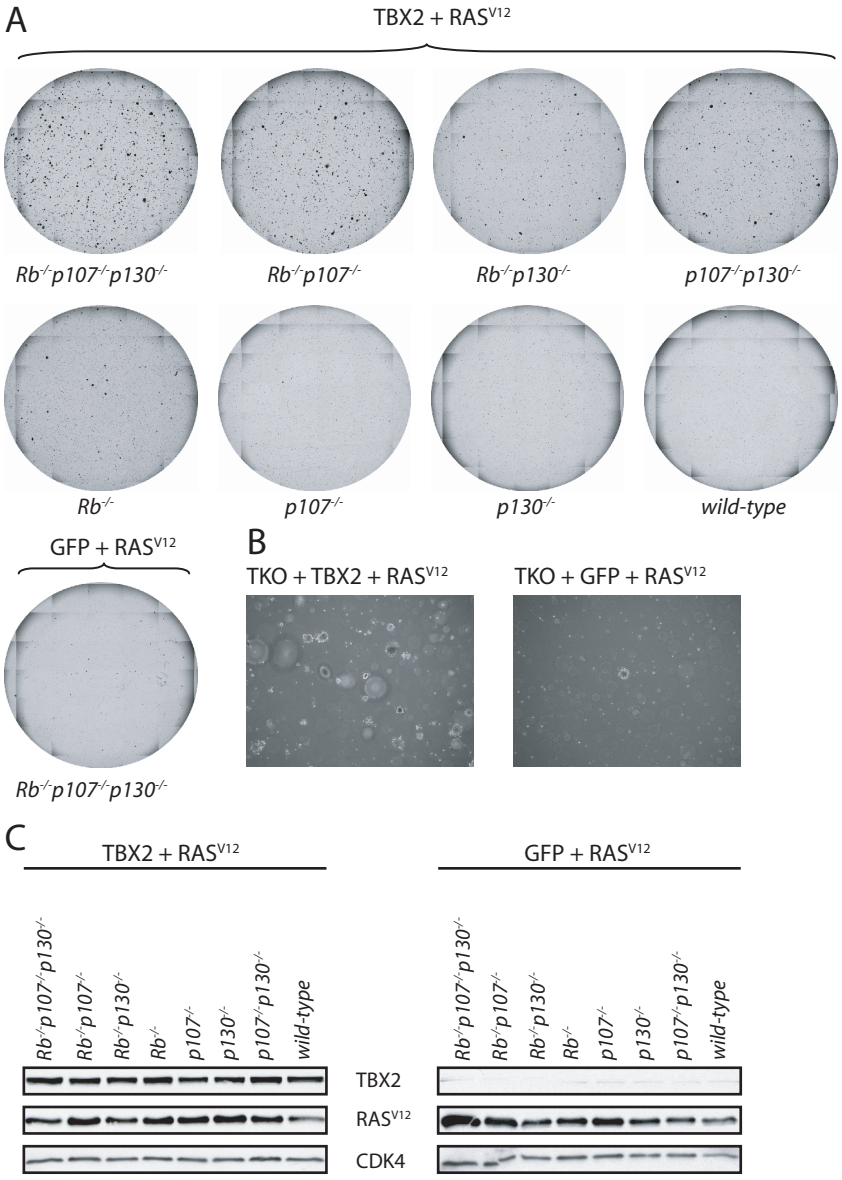


Figure 1: TBX2 specifically transforms RAS^{V12}/pocket protein-deficient MEFs. (A) Primary MEFs of the indicated genotypes were first transduced with pEYK-TBX2 and subsequently with pBABE-RAS^{V12} (TBX2 + RAS^{V12}) and plated in soft agar. The left bottom dish shows Rb$^{-/-}$p107$^{-/-}$p130$^{-/-}$ MEFs infected with pEYK-GFP and pBABE-RAS^{V12} (GFP + RAS^{V12}). Pictures of soft agar plates were taken using a non-phase-contrast lens (×2.5 magnification). (B) Detailed pictures of Rb$^{-/-}$p107$^{-/-}$p130$^{-/-}$ (TKO) MEFs transduced with either pEYK-TBX2 or pEYK-GFP and subsequently with pBABE-RAS^{V12}. Pictures show the same cells as in panel A, taken using a phase-contrast lens (×5 magnification). (C) RAS^{V12} and TBX2 protein levels in the cell lines depicted in panels A and B. (Left) MEFs infected with pEYK-TBX2 and pBABE-RAS^{V12}. (Right) MEFs infected with pEYK-GFP and pBABE-RAS^{V12}.

of a pEYK-cDNA integration was determined by both PCR analysis and by a shuttling method that enabled excision of the integrated pEYK-cDNA from the genomic DNA and its propagation as a plasmid in *Escherichia coli* (Koh et al., 2002). Sequencing of recovered pEYK vectors revealed many fragmented cDNA sequences in the sense or antisense orientation. Three independent colonies contained a full cDNA encoding the TBX2 oncogene. Only reintroduction of the TBX2-expressing vector into pocket protein-deficient MEFs expressing RAS^{V12} caused robust colony formation in soft agar (Fig. 1A, compare left top and left bottom dishes).

TBX2 transforms pocket protein-deficient MEFs expressing RAS^{V12}

To determine whether transformation by TBX2 required the loss of specific pocket proteins, we retrovirally transduced MEFs deficient for all different combinations of pocket proteins with either pEYK-TBX2 or pEYK-GFP, followed by pBABE-RAS^{V12}, and tested their ability to form colonies in soft agar. In all the cell lines tested, expression of RAS^{V12} alone hardly induced anchorage-independent growth (see Fig. 1A and B for TKO MEFs; also data not shown). Concomitant expression of TBX2 induced colony formation in TKO, DKO, and $Rb^{-/-}$ MEFs (Fig. 1A and B). The strongest colony formation was observed with TKO MEFs, followed by $Rb^{-/-}p107^{-/-}$ MEFs and then by $Rb^{-/-}p130^{-/-}$, $p107^{-/-}p130^{-/-}$, and $Rb^{-/-}$ MEFs. In contrast, expression of TBX2 and RAS^{V12} did not support anchorage-independent growth of $p107^{-/-}$, $p130^{-/-}$, and wild-type MEFs, the latter being consistent with previous results (Jacobs et al., 2000). Note that RAS^{V12} and TBX2 were expressed to comparable levels in all cell lines (Fig. 1C). Expression of TBX2 alone in pocket protein-deficient MEFs did not induce colony formation (data not shown). Together, these results indicate that the combination of pocket protein ablation, RAS^{V12} expression, and TBX2 expression is required for transformation of primary MEFs.

Transformation of TBX2/RAS^{V12}-expressing MEFs requires ablation of pocket proteins

To provide independent proof for collaboration between TBX2 and the loss of pocket proteins, we made use of $Rb^{F/F}p130^{-/-}$ MEFs. In these cells, exon 19 of both *Rb* alleles is flanked by LoxP sites and can therefore be deleted by Cre-mediated recombination, which inactivates the gene. $Rb^{F/F}p130^{-/-}$ MEFs were transduced with retroviral vectors expressing TBX2 and RAS^{V12} and subsequently with either pMSCV-Cre-ERT2 or empty pMSCV. Even without the addition of tamoxifen, transduction of Cre-ERT2 in $Rb^{F/F}p130^{-/-}$ MEFs already resulted in nuclear Cre activity, yielding a mixed population of cells containing active (Rb^{F}) and/or inactive ($Rb^{\Delta 19}$) *Rb* alleles (Fig. 2A). Expression of Cre-ERT2 in TBX2/RAS^{V12}/$Rb^{F/F}p130^{-/-}$ MEFs increased colony formation in soft agar, indicating that inactivation of the *Rb* allele promoted anchorage-independent growth (Fig. 2B). Conversely, expression of TBX2, RAS^{V12}, and Cre-ERT2 in $Rb^{+/+}p130^{-/-}$ MEFs did

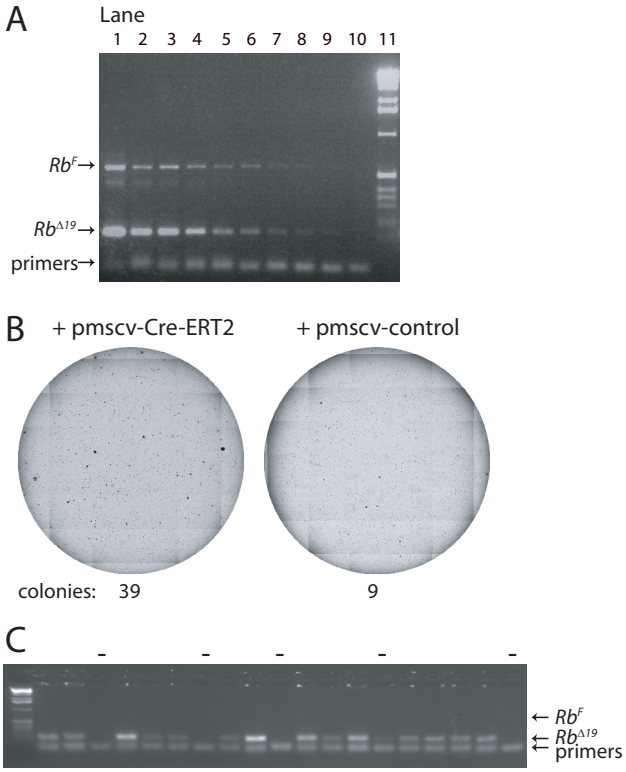


Figure 2: Transformation by TBX2 and RAS^{V12} requires ablation of pocket proteins. (A) Expression of Cre-ERT2 yields a mixed population of cells containing active (Rb^F) and/or inactive ($Rb^{\Delta 19}$) Rb alleles. $Rb^{F/F}p130^{-/-}$ MEFs were transduced with TBX2, RAS^{V12}, and pMSCV-Cre-ERT2 viruses. Genomic DNA (gDNA) was isolated and analyzed for the presence of active and inactive Rb by PCR. Lane 1, 100 ng of input gDNA; lanes 2 to 10, genomic DNA diluted 2-, 4-, 8-, 16-, 32-, 64-, 128-, 256-, and 512-fold, respectively, showing that both alleles could be detected at a low input concentration of gDNA; lane 11, 1-kilobase ladder. (B) Expression of Cre-ERT2 enhances colony formation in $Rb^{F/F}p130^{-/-}$ MEFs. $Rb^{F/F}p130^{-/-}$ MEFs infected with TBX2 and RAS^{V12} and either pMSCV-Cre-ERT2 (left) or pMSCV-empty (right) viruses were plated in soft agar. Pictures were taken using a non-phase-contrast lens (×2.5 magnification). (C) Nineteen soft agar colonies derived from $Rb^{F/F}p130^{-/-}$ MEFs expressing TBX2, RAS^{V12}, and Cre-ERT2 were picked and immediately analyzed for the presence of active and inactive Rb by PCR. Each lane represents one soft agar colony; the leftmost lane contains a 1-kilobase ladder. Five colonies (-) failed to produce PCR fragments. The positions of active and inactive Rb alleles are indicated by Rb^F and $Rb^{\Delta 19}$.

not enhance colony formation (data not shown). Note that some colonies appeared in the control vector-infected cells (Fig. 2B, right part), which was probably due to the prolonged culturing required for transduction with three retroviral vectors, creating a window for the selection for additional growth-stimulating mutations. Soft agar colonies derived from $Rb^{F/F}p130^{-/-}$ MEFs transduced with TBX2, RAS^{V12}, and Cre-ERT2 were picked and analyzed for *Rb* status by PCR. Strikingly, while the starting population of cells consisted

of a mixed population carrying active and inactive *Rb* alleles (Fig. 2A), all 14 soft agar colonies exclusively carried inactive *Rb* alleles (Fig. 2C; note that 5 colonies failed to produce PCR fragments, probably due to the loss of cells during picking). Together with the results presented in Fig. 1, these experimental results show that transformation of MEFs by RAS^{V12} requires both ablation of pocket proteins and expression of TBX2.

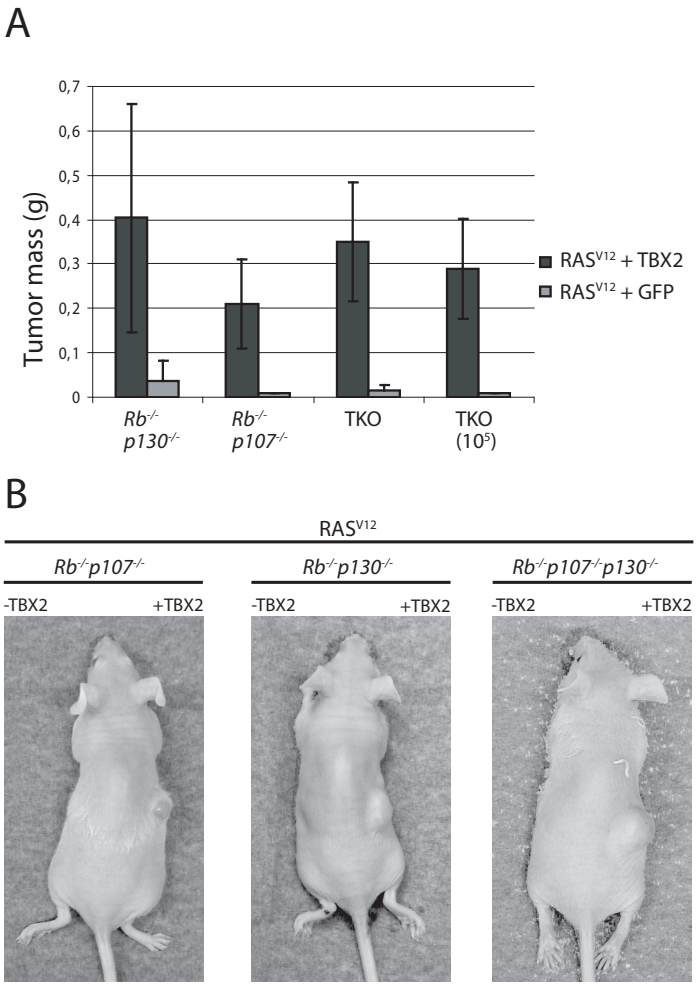


Figure 3: Expression of TBX2 promotes tumor formation of pocket protein-deficient MEFs expressing RAS^{V12}. Immunocompromised mice (nude mice) were injected with MEFs of the indicated genotypes, which had been transduced with either pBABE-RAS^{V12} and pEYK-GFP (RAS^{V12} + GFP) (injected into the left flanks) or pBABE-RAS^{V12} and pEYK-TBX2 (RAS^{V12} + TBX2) (injected into the right flanks). Unless indicated, 10^6 MEFs were injected per flank. Three mice were injected per cell line; two additional mice were injected with 10^5 $Rb^{-/-}p107^{-/-}p130^{-/-}$ (TKO) MEFs. (A) Graphical representation of tumor mass. Error bars denote standard deviations. (B) Pictures of immunocompromised mice injected with MEFs of the indicated genotypes. MEFS were transduced with either pEYK-TBX2 (+TBX2) or with pEYK-GFP (-TBX2).

Requirements for growth of MEFs in nude mice

To study transformation in an *in vivo* system, *Rb*$^{-/-}$*p107*$^{-/-}$, *Rb*$^{-/-}$*p130*$^{-/-}$, and *Rb*$^{-/-}$*p107*$^{-/-}$ *p130*$^{-/-}$ MEFs were retrovirally transduced with either RAS^{V12} and TBX2 or RAS^{V12} and green fluorescent protein (GFP) and injected into immunocompromised mice (nude mice) (Fig. 3). TBX2 and RAS^{V12} expression levels were comparable in the different cell lines (data not shown). *Rb*$^{-/-}$*p107*$^{-/-}$*p130*$^{-/-}$ MEFs expressing RAS^{V12} and TBX2 formed tumors within a shorter time window than *Rb*$^{-/-}$*p107*$^{-/-}$ or *Rb*$^{-/-}$*p130*$^{-/-}$ MEFs expressing RAS^{V12} and TBX2; mice injected with TKO MEFs had to be sacrificed between 16 and 21 days, while mice injected with DKO MEFs were sacrificed after 26 days. This suggests, consistent with the soft agar experiments (Fig. 1), that MEFs deficient for all three pocket proteins were more easily transformed than MEFs partially deficient for pocket proteins. DKO and TKO MEFs expressing RAS^{V12} and TBX2 formed robust tumors, weighing between 0.1 and 0.7 g upon dissection. In contrast, MEFs expressing RAS^{V12} and GFP formed very small clumps of cells, in 9 out of 11 cases weighing less than 0.01 g. In the other two cases, small tumors were formed; these tumors were 10 and 4 times smaller than the corresponding tumors expressing RAS^{V12}/TBX2. These results demonstrate that also *in vivo*, expression of TBX2 strongly supports transformation of RAS^{V12}-expressing DKO and TKO MEFs.

Downregulating the p53 pathway transforms pocket protein-deficient MEFs

Overexpression of TBX2 induces bypass of both replicative and RAS^{V12}-induced senescence. This has been attributed to downregulation of p19^{ARF}, and as a consequence, to downregulation of p53 (Dobrzycka et al., 2006; Jacobs et al., 2000). Furthermore, TBX2 has been reported to directly repress the promoter of the *p53* target gene *p21*^{CIP1} (Vance et al., 2005; Prince et al., 2004). Given the importance of p53 in transformation, we investigated whether TBX2-induced transformation could be attributed to downregulation of the p53/p21^{CIP1} pathway. To this aim, we expressed a pBABE-p21^{CIP1} vector in TKO MEFs expressing TBX2 and RAS^{V12}. Ectopic expression of p21^{CIP1} dramatically reduced the number of soft agar colonies induced by TBX2 (Fig. S1), indicating that TBX2-induced transformation involves downregulation of p21^{CIP1}.

To further address the involvement of downregulating the p53/p21^{CIP1} pathway in transformation, we used pRetroSuper (pRS) vectors to suppress expression of p53 or p21^{CIP1} in DKO MEFs by RNA interference. Similar to overexpression of TBX2, downregulation of either p53 or p21^{CIP1} supported anchorage-independent growth of RAS^{V12}-expressing *Rb*$^{-/-}$*p107*$^{-/-}$ MEFs (Fig. 4A). Knockdown of p53 was more potent in inducing anchorage-independent growth than knockdown of p21^{CIP1} was. Residual levels of p21^{CIP1} were comparable or even somewhat higher in p53 knockdown cells (see Fig. 6D), suggesting that p53 protects against anchorage-independent growth only

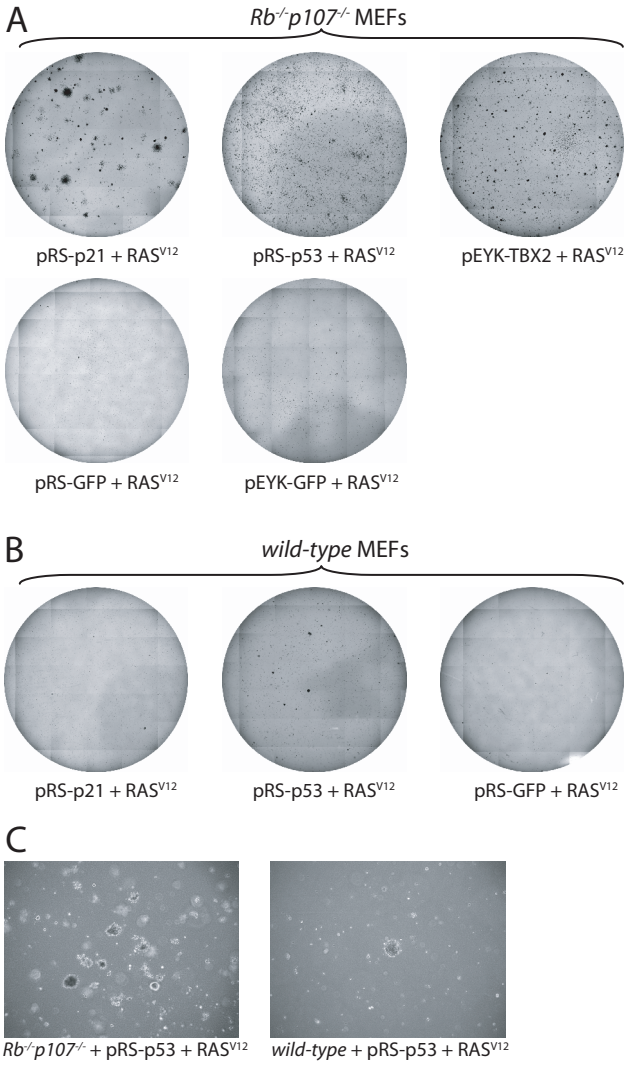


Figure 4: Similar to TBX2 overexpression, downregulation of either p21^{CIP1} or p53 specifically transforms pocket protein-deficient MEFs. (A) Rb$^{-/-}$p107$^{-/-}$ MEFs were transduced with the indicated constructs and plated in soft agar. Pictures were taken using a non-phase-contrast lens (×2.5 magnification). (B) Wild-type MEFs were transduced with the indicated constructs and plated in soft agar. Pictures were taken using a non-phase-contrast lens (×2.5 magnification). (C) Detailed pictures of the indicated cell lines, plated in soft agar. Pictures show the same cells as in panels A and B, taken using a phase-contrast lens (×5 magnification).

partially via $p21^{CIP1}$. As observed for transformation by TBX2, transformation by $p21^{CIP1}$ knockdown required pocket protein loss; $p21^{CIP1}$ downregulation and RAS^{V12} expression in wild-type MEFs did not induce colony formation in soft agar (Fig. 4B). Although p53 downregulation and RAS^{V12} expression in wild-type MEFs induced a small number of colonies (Fig. 4B), this sharply contrasted to the robust colony formation observed for RAS^{V12}/*Rb*$^{-/-}$*p107*$^{-/-}$ MEFs (Fig. 4C). These results show that in a RAS^{V12} transformation assay, downregulation of the p53 pathway mimics overexpression of TBX2. Similar as observed for TBX2, transformation by downregulation of the p53 pathway requires the loss of pocket proteins.

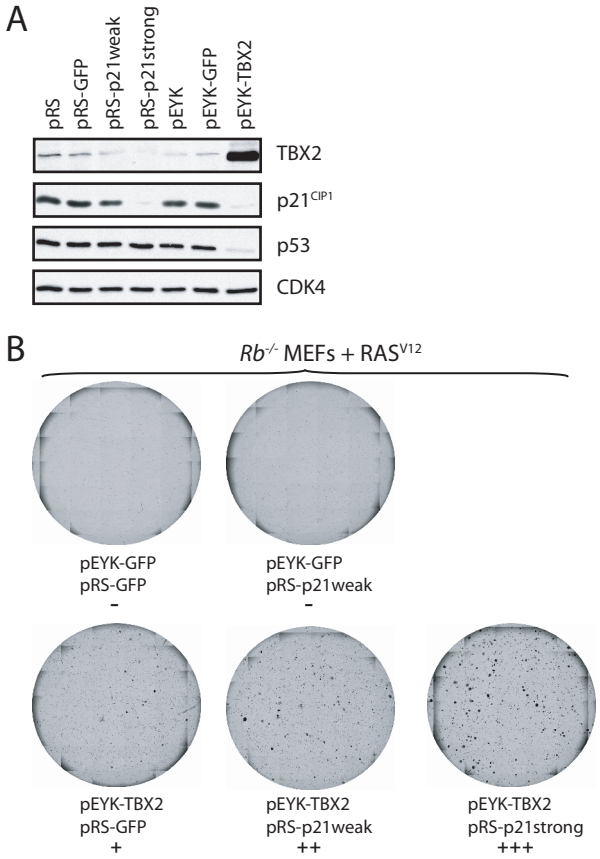


Figure 5: A balance between the activities of the pocket protein and $p21^{CIP1}$ pathways determines the level of transformation of MEFs. (A) Downregulation of $p21^{CIP1}$ using various constructs. $Rb^{-/-}p107^{-/-}$ MEFs were infected with the indicated constructs and plated in suspension culture 6 days postinfection. After 24 h, cells were harvested, lysed, and immunoblotted for the indicated proteins. (B) $Rb^{-/-}$ MEFs were first infected with pEYK-GFP or pEYK-TBX2, next with pRS-GFP or a pRS-p21 vector, and last with pBABE-RAS^{V12}. Forty-eight hours postinfection, cells were plated in soft agar. Pictures were taken using a non-phase-contrast lens (×2.5 magnification). + and - symbols refer to the quantification of colony formation as depicted in Table 1.

A balance between the p53 pathway and the pocket protein pathway determines the level of transformation of MEFs

Since colony formation in TBX2/RAS^{V12}-expressing MEFs was more robust upon the loss of two or three pocket proteins compared to the loss of pRB alone (Fig. 1) and transformation by TBX2 could be attributed to downregulation of the p53/p21^{CIP1} pathway (Fig. 4), we hypothesized that a balance between the activities of the pocket protein and the p53/p21^{CIP1} pathways determines the rate of transformation of MEFs. We therefore used pRS vectors downregulating p21^{CIP1} to different levels, referred to as pRS-p21weak and pRS-p21strong. Figure 5A shows that infection with pRS-p21weak in *Rb*$^{-/-}$*p107*$^{-/-}$ MEFs caused a mild decrease of p21^{CIP1} levels compared to infection with control vectors (pRS or pRS-GFP), while infection with pRS-p21strong caused a strong decrease. We downregulated p21^{CIP1} to different degrees by combined expression of the pRS-p21 vectors and the pEYK-TBX2 expression vector in MEFs deficient for different combinations of pocket proteins. Briefly, TKO MEFs, *Rb*$^{-/-}$*p107*$^{-/-}$ MEFs, *Rb*$^{-/-}$ MEFs, and wild-type MEFs were first infected with pEYK-GFP or pEYK-TBX2, subsequently with pRS-GFP or a pRS-p21 vector, and finally with pBABE-RAS^{V12}. The colony-forming capacity in soft agar was monitored, and the results are summarized in Table 1. Expression of RAS^{V12} and TBX2, without additional p21^{CIP1} downregulation, induced colony formation most robustly in TKO MEFs, followed by *Rb*$^{-/-}$*p107*$^{-/-}$ and *Rb*$^{-/-}$ MEFs, but expression of RAS^{V12} and TBX2 did not induce colony formation in wild-type MEFs (Table 1 and Fig. 1). Strikingly, in *Rb*$^{-/-}$ MEFs, coexpression of the pRS-p21

Summary of growth of the cell lines in soft agar

genotype of cell line	growth[a] in soft agar of cell line infected with the following construct:				
	Control vector	pRS-p21weak	pEYK-TBX2	pEYK-TBX2 + pRS-p21weak	pEYK-TBX2 + pRS-21strong
wild-type	-	-	-	-	+/-
Rb$^{-/-}$	-	-	+	++	+++
Rb$^{-/-}$*p107*$^{-/-}$	-	-	++++	++++	++++
TKO	+/-	+/-	+++++	+++++	+++++

Table 1: Summary of growth of the cell lines in soft agar. A balance between the activities of the pocket protein and p21^{CIP1} pathways determines the level of transformation of MEFs. Cells were infected as described in the legend to Fig. 5, plated in soft agar, and monitored for anchorage-independent growth. [a]Symbols: –, no growth; +/–, less than 20 colonies; +, ++, +++, ++++, and +++++, indicate the relative capacity for growth in soft agar, where each additional + indicates an approximately twofold increase. +, ++, and +++ correspond to the levels of soft agar growth shown in Fig. 5B.

vectors enhanced TBX2-induced colony formation (Fig. 5B). These results show that relatively mild $p21^{CIP1}$ downregulation was sufficient to induce transformation of MEFs with complete pocket protein loss (pEYK-TBX2 expression in RAS^{V12}/TKO MEFs), while strong $p21^{CIP1}$ downregulation was required to induce transformation of MEFs partially deficient for pocket proteins (pEYK-TBX2 plus pRS-p21strong in $RAS^{V12}/Rb^{-/-}$ MEFs). Thus, a balance between pocket protein and $p21^{CIP1}$ levels determines the rate of transformation of RAS^{V12}-expressing MEFs.

RAS^{V12}-induced transformation of pocket protein-deficient MEFs requires rescue of G_2 arrest

Whereas under adherent conditions, the loss of pocket proteins turned RAS^{V12} expression from a growth-inhibiting signal to a growth-promoting signal, RAS^{V12}/TKO MEFs were largely unable to form colonies in soft agar. To determine why proliferation ceased upon removal of anchorage, we analyzed the cell cycle profile of anchorage-deprived DKO and TKO MEFs by FACS. For this experiment, we cultured cells in methylcellulose, which like soft agar, prohibits attachment but enables easy recovery of nonproliferating cells. Upon removal of anchorage, wild-type fibroblasts arrest in G_1 (Fang et al., 1996; Guadagno et al., 1993; Guadagno and Assoian, 1991). Here, we show that the majority of $RAS^{V12}/Rb^{-/-}p107^{-/-}$ MEFs arrested in both G_1 and G_2 upon removal of anchorage (Fig. 6A). Knockdown of $p21^{CIP1}$ reduced the fraction of G_2 cells (Fig. 6A). As the number of S-phase cells remained low, this indicates that a large fraction of the cells had arrested in G_1. In contrast, knockdown of p53 increased the number of S-phase cells, indicating rescue of both G_1 and G_2 arrest in non-adherent cells (Fig. 6A). RAS^{V12}/TKO MEFs predominantly arrested in G_2 upon removal of anchorage, which could be alleviated by knockdown of $p21^{CIP1}$ or p53 (Fig. 6B) or by overexpression of TBX2 (Fig. 6C). Note that G_2 arrest under non-adherent conditions in RAS^{V12}/TKO MEFs was not induced by expression of RAS^{V12}, as TKO MEFs also arrested in G_2 upon removal of anchorage (Fig. S2). Similar to knockdown of p53 or $p21^{CIP1}$, knockdown of $p19^{ARF}$ also rescued G_2 arrest in non-adherent RAS^{V12}/TKO MEFs (Fig.7C; note that while the vector used targets the INK4A/ARF locus, the effect was due to knockdown of $p19^{ARF}$, as downregulation of $p16^{INK4A}$ alone did not rescue G_2 arrest of RAS^{V12}/TKO MEFs). As TBX2 expression downregulated $p19^{ARF}$ (Fig. 7A), these results suggest that transformation by TBX2 involved downregulation of $p19^{ARF}$, and subsequently p53 and $p21^{CIP1}$. In conclusion, our results demonstrate that the loss of anchorage induces checkpoint activation in G_1 and G_2, the latter becoming more prominent upon the loss of pocket proteins. Transformation requires rescue of cell cycle arrest, which can be achieved by expression of TBX2 or downregulation of the $p19^{ARF}$/p53/$p21^{CIP1}$ pathway.

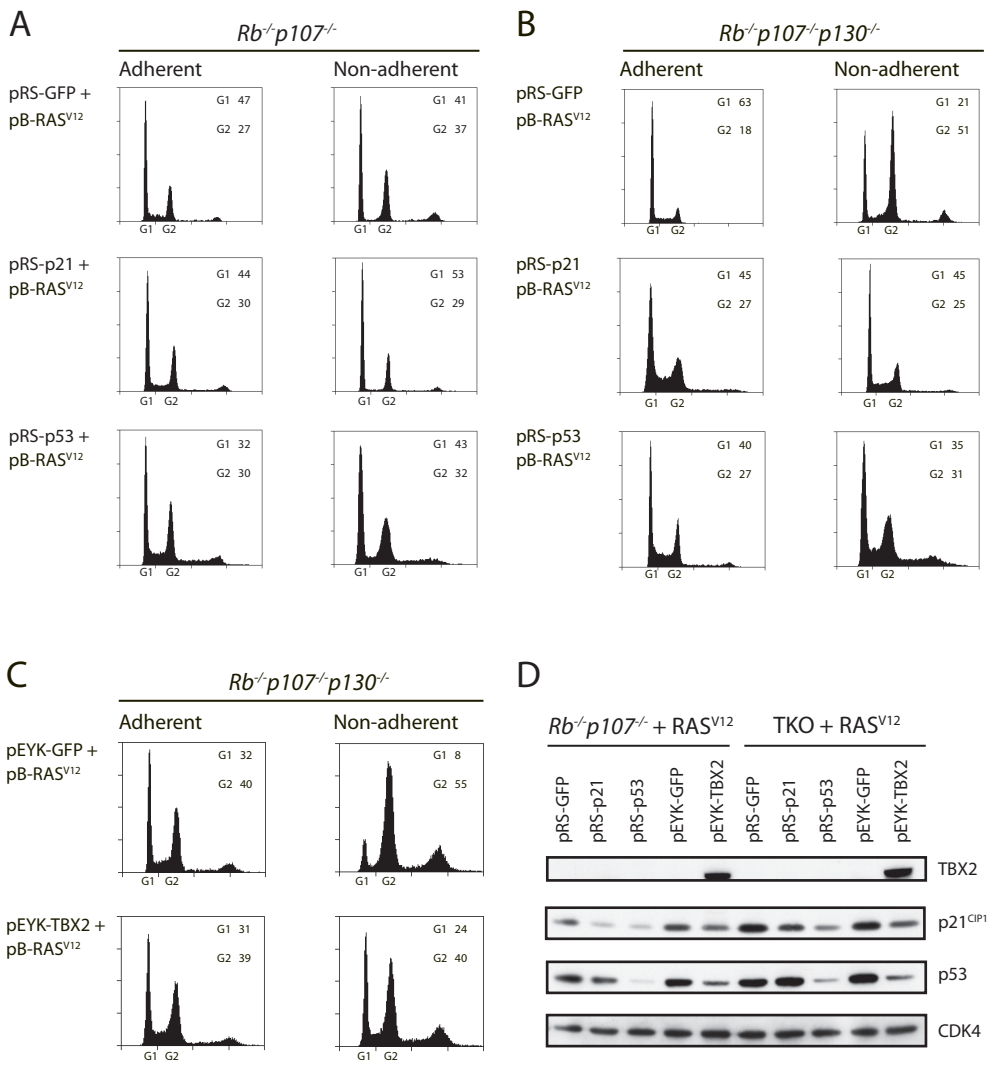


Figure 6: Anchorage-independent growth of RAS^{V12}/pocket protein-deficient MEFs requires rescue of G_2 arrest. Rb$^{-/-}$p107$^{-/-}$ MEFs (A) or Rb$^{-/-}$p107$^{-/-}$p130$^{-/-}$ (TKO) MEFs (B and C) were infected with the indicated constructs and cultured either under adherent conditions or for 6 days in methylcellulose. Cells were harvested, stained with propidium iodide, and analyzed by FACS. The percentages of cells in G_1 and G_2 are shown in the top right corners of graphs. (D) Downregulation of p21^{CIP1} and p53 in RAS^{V12}/Rb$^{-/-}$p107$^{-/-}$ and RAS^{V12}/TKO MEFs upon infection with the indicated constructs. Cells were plated in suspension culture for 24 h, lysed, and immunoblotted for the indicated proteins.

Downregulating the p53/p21^{CIP1} pathway by TBX2 expression or RNA interference rescues cyclin-dependent kinase activities under non-adherent conditions

As the G_1/S and G_2/M transitions require Cyclin E-CDK2 and Cyclin B1-CDK1, respectively, we analyzed the activity of these complexes under non-adherent conditions. Figure 8A shows that expression of TBX2 induced Cyclin B1- and CDK2-associated kinase activities in RAS^{V12}-expressing TKO cells under non-adherent conditions (Fig. 8A, panels 1 and 2). Increased kinase activity in TBX2-expressing cells was related to reduced association of p21^{CIP1} with Cyclin B1 and CDK2 (Fig. 8A, panels 3 and 4). This is most likely due to reduction of p53 levels by TBX2 (Fig. 8A, panels 5, 6, and 7). These results indicate that expression of TBX2 relieves inhibition of cyclin-dependent kinase activity by p21^{CIP1}, leading to rescue of cell cycle arrest and anchorage-independent growth.

Downregulating the p53/p21^{CIP1} pathway transformed DKO and TKO MEFs more effectively than *Rb*$^{-/-}$ MEFs (Fig. 1 and Table 1). We therefore compared the regulation of cyclin-dependent kinase activities in *Rb*$^{-/-}$ and *Rb*$^{-/-}$*p107*$^{-/-}$ MEFs under both adherent and non-adherent conditions. Both cell lines were infected with either pRS-p21 or pRS-GFP and then with pBABE-RAS^{V12} and plated either in methylcellulose or under adherent conditions. Cyclin B1- and CDK2-associated kinase activities in *Rb*$^{-/-}$*p107*$^{-/-}$ MEFs were higher than in *Rb*$^{-/-}$ MEFs, which was particularly visible under adherent conditions (Fig. 8B, compare lanes 1 and 3 and lanes 5 and 7). The loss of anchorage caused inhibition of kinase activities in *Rb*$^{-/-}$ and *Rb*$^{-/-}$*p107*$^{-/-}$ MEFs (Fig. 8B, compare lanes 1 and 5 and lanes 3 and 7). Knockdown of p21^{CIP1} induced kinase activities under both adherent and non-adherent conditions, and as expected, no association of p21^{CIP1} with CDK2 could be detected (Fig. 8B, lanes 2, 4, 6, and 8). However, in non-adherent RAS^{V12}/*Rb*$^{-/-}$ MEFs (Fig. 8B, lane 6), the increase in CDK2 and Cyclin B1-associated kinase activity was minor and apparently not sufficient to support anchorage-independent growth. In contrast, knockdown of p21^{CIP1} in RAS^{V12}/*Rb*$^{-/-}$*p107*$^{-/-}$ MEFs (Fig. 8B, lane 8) raised kinase activities to much higher levels than observed in RAS^{V12}/*Rb*$^{-/-}$ MEFs (lane 6). Note that knockdown of p21^{CIP1} induced Cyclin B1-associated kinase activity more robustly than CDK2-associated kinase activity. This fits with the observation that knockdown of p21^{CIP1} was more potent in rescuing G_2 arrest than in rescuing G_1 arrest (Fig. 6). We conclude that downregulation of p21^{CIP1} sufficiently rescued the decrease in kinase activities induced by the loss of adhesion only in RAS^{V12}/*Rb*$^{-/-}$*p107*$^{-/-}$ MEFs. This allowed bypass of G_2 arrest and, to a lower extent, G_1 arrest, enabling anchorage-independent growth.

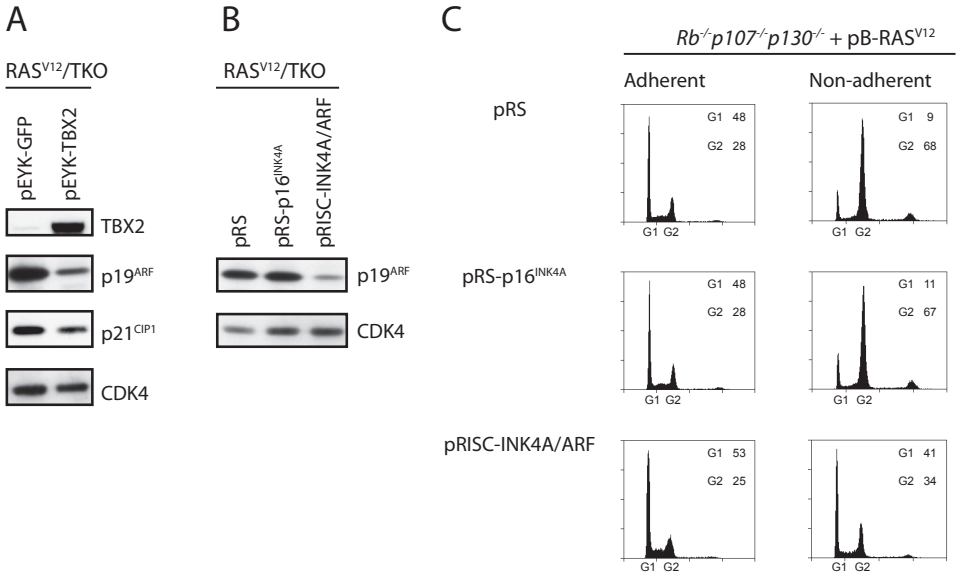
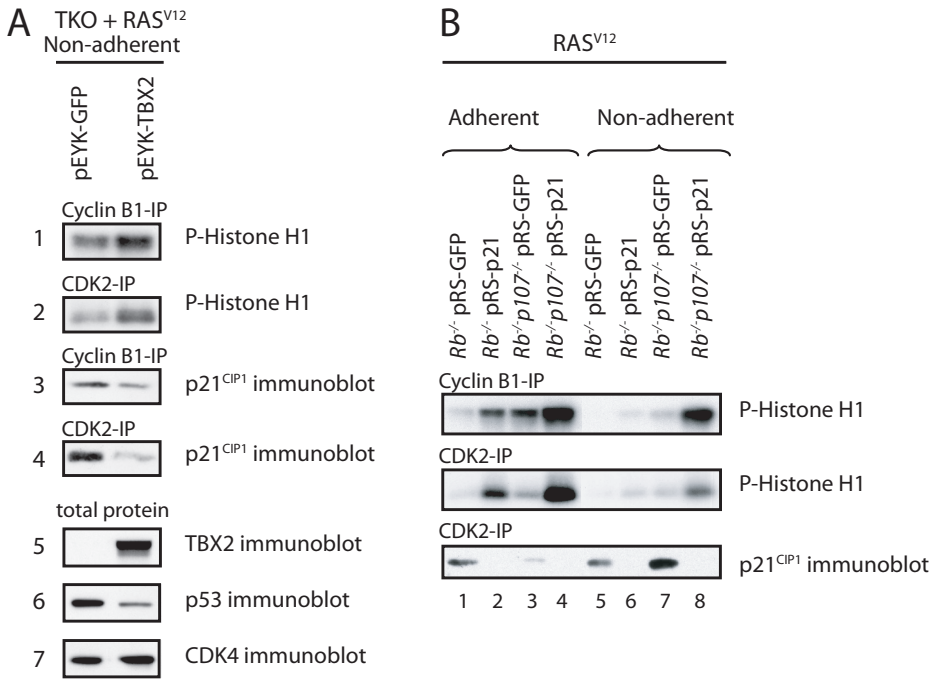


Figure 7: Transformation by TBX2 involves downregulation of $p19^{ARF}$. (A) TBX2 downregulates $p19^{ARF}$ in RAS^{V12}/TKO MEFs. (B) Downregulation of $p19^{ARF}$ by a knockdown vector targeting the INK4A/ARF locus, but not by a vector targeting $p16^{INK4A}$. (C) $p19^{ARF}$ downregulation rescues G_2 arrest in non-adherent RAS^{V12}/TKO MEFs. Cells were infected with the indicated constructs and cultured under adherent conditions or for 5 days in methylcellulose. Cells were harvested, stained with propidium iodide, and analyzed by FACS. The percentages of cells in G_1 and G_2 are shown in the top right corners of graphs.



Discussion

Pocket protein depletion is not sufficient for RAS^{V12}-induced transformation of MEFs

Functional inactivation of the pocket protein and p53 tumor suppressor pathways is one of the most frequent events in the development of cancer. Consistent with their importance in G_1 control, the loss of either p53 or pocket proteins bypasses replicative senescence in primary murine embryonic fibroblasts (Dirac and Bernards, 2003; Dannenberg et al., 2000; Sage et al., 2000; Harvey et al., 1993). Furthermore, inactivation of the p53 pathway or the loss of pRB and at least one other pocket protein reversed the growth-inhibitory effect of RAS^{V12} into a proliferative stimulus (Peeper et al., 2001; Serrano et al., 1997). However, unlike abrogation of p53, the loss of pocket proteins was not sufficient to support oncogenic transformation by RAS^{V12} (Peeper et al., 2001). This observation contradicts a report by others (Sage et al., 2000) but is strongly confirmed in the present study; RAS^{V12}-expressing MEFs deficient for both pRB and p130, both pRB and p107, or all three pocket proteins were largely unable to exhibit anchorage-independent growth or to form tumors in nude mice. By screening a cDNA library, we have identified a missing link: overexpression of the TBX2 oncogene strongly induced transformation of pocket protein-deficient MEFs by RAS^{V12}, as evidenced by anchorage-independent growth *in vitro* and *in vivo* (Fig. 1 and 3). Consistently, we have shown that transformation of MEFs overexpressing TBX2 and RAS^{V12} required the loss of pocket proteins (Fig. 2). These results show that the loss of pocket proteins and overexpression of TBX2 strongly act synergistically in RAS^{V12}-induced transformation of primary murine embryonic fibroblasts.

The pRB and p53 pathways cooperate in suppression of transformation

TBX2 overexpression is known to attenuate the $p53/p21^{CIP1}$ tumor suppressor pathway by downregulating transcription of the Mdm2 inhibitor $p19^{ARF}$. Consistently, we show that partial RNA interference-mediated knockdown of $p19^{ARF}$, p53, or $p21^{CIP1}$ strongly supported RAS^{V12}-induced transformation of pocket protein-deficient MEFs (Fig. 4A, 6, and 7). Moreover, expression of $p21^{CIP1}$ inhibited TBX2-induced transformation (Fig.

> Figure 8: A combination of pocket protein loss and downregulation of the $p53/p21^{CIP1}$ pathway rescues cyclin-dependent kinase activities under non-adherent conditions. (A) $Rb^{-/-}p107^{-/-}p130^{-/-}$ (TKO) MEFs infected with pBABE-RAS^{V12} (RAS^{V12}) and either pEYK-TBX2 or pEYK-GFP were cultured in methylcellulose for 6 days. (Panels 1 and 2) Cyclin B1- and CDK2-associated kinase activities as determined by in vitro phosphorylation of histone H1. IP, immunoprecipitate; P-Histone H1, phosphorylated histone H1. (Panels 3 and 4) Cyclin B1 and CDK2 immunoprecipitates were immunoblotted for $p21^{CIP1}$. (Panels 5, 6, and 7) Total protein levels of TBX2, p53, and CDK4 as loading control. (B) $Rb^{-/-}$ and $Rb^{-/-}p107^{-/-}$ MEFs infected with either pRS-p21 or pRS-GFP and subsequently with pBABE-RAS^{V12} were cultured under adherent conditions or in methylcellulose for 5 days. Cyclin B1- and CDK2-associated kinase activities as well as association of $p21^{CIP1}$ with CDK2 are shown in the top, middle, and bottom panels, respectively.

S1). The complete loss of p53 was sufficient for RAS^{V12}-induced transformation of wild-type MEFs (Peeper et al., 2001). Here we show that partial knockdown of p53 induced anchorage-independent growth only to a very limited degree in wild-type MEFs but was very effective in pocket protein-deficient MEFs (Fig. 4B and C). Furthermore, we found that anchorage-independent growth upon RAS^{V12} expression could be achieved by either a combination of moderate $p21^{CIP1}$ downregulation and severe pocket protein loss or strong $p21^{CIP1}$ downregulation and mild pocket protein loss (Fig. 5 and Table 1). Apparently, the p53 and pRB pathways act synergistically in preventing anchorage-independent growth.

The complete loss of pocket proteins did not support anchorage-independent growth upon expression of RAS^{V12}, although it should be noted that a small number of colonies appeared in the soft agar culture. This may be explained by downregulation of the p53/$p21^{CIP1}$ pathway in a subset of the cells. Indeed, Sage and coworkers (2000) reported that two out of five TKO MEF cultures displayed downregulation of p53 and $p21^{CIP1}$ during culturing under adherent conditions.

Regulation of cell cycle arrest and cyclin-dependent kinase activity by pRB, TBX2, and p53

Anchorage-independent growth is a hallmark of transformed cells. Untransformed cells are dependent on anchorage via integrin signaling. Upon attachment to the extracellular matrix, integrin signaling induces *Cyclin D1* and inhibits $p21^{CIP1}$ and $p27^{KIP1}$ expression, thereby authorizing cell cycle progression (Walker et al., 2005; Bao et al., 2002; Roovers and Assoian, 2000). Conversely, detachment from the extracellular matrix is associated with downregulation of *Cyclin D1*, induction of $p21^{CIP1}$ and $p27^{KIP1}$, and increased association of $p21^{CIP1}$ and $p27^{KIP1}$ with Cyclin-CDK complexes (Wu and Schonthal, 1997; Fang et al., 1996; Zhu et al., 1996). As a result, cells deprived of attachment downregulate CDK2 activity and arrest in G_1 (Fang et al., 1996; Guadagno et al., 1993). We show here that in anchorage-deprived RAS^{V12}/DKO MEFs, there was only partial G_1 arrest, as a substantial fraction of cells arrested in G_2 (Fig. 6A). In RAS^{V12}/TKO MEFs, which have completely lost the G_1 restriction point, G_2 arrest predominated (Fig. 6B). Thus, anchorage dependence is regulated via both G_1 and G_2 checkpoints. Our results therefore indicate that the transforming activity of TBX2 not only relies on override of the G_1 checkpoint but also on override of the G_2 checkpoint and involves downregulation of the p53 pathway (Fig. 6).

Are G_2-arrested cells senescent? We were unable to perform a senescence-associated β-galactosidase staining on arrested cells, as both DKO and TKO MEFs were targeted with a lacZ reporter gene. However, we have strong indications that the arrest is reversible, which according to the current definition excludes senescence. To address this issue, we centrifuged RAS^{V12}/TKO MEFs harvested from methylcellulose at low speed to

separate the low number of growing colonies from G_2-arrested cells. By FACS, we found that the population of cells in the supernatant was enriched for G_2-phase cells. These cells entered the cell cycle upon reattachment, as observed by growth of the cells and by FACS profiles (data not shown).

The level of $p21^{CIP1}$ knockdown required to induce transformation was dependent on the extent of pocket protein ablation: relatively moderate $p21^{CIP1}$ knockdown was sufficient to induce anchorage-independent growth in TKO MEFs, whereas strong $p21^{CIP1}$ knockdown was required to transform *Rb*-deficient MEFs. Our results indicate that these different requirements for anchorage-independent growth are due to different levels of kinase activities in pocket protein-ablated cells. Despite high levels of cyclins A, E, and B1 in $Rb^{-/-}$ MEFs (Dannenberg et al., 2004; Gad et al., 2004; Lukas et al., 1999; Hurford, Jr. et al., 1997), their associated kinase activities were lower than in $Rb^{-/-}p107^{-/-}$ MEFs (Fig. 8), and this may be caused by E2F-dependent induction of p107 (Williams et al., 2006). As Rodier and coworkers (2005) suggested that overexpression of p107 reduces the half-life of Skp2 protein, this could lead to downregulation of Skp2 in $Rb^{-/-}$ MEFs and subsequently, inhibition of $p27^{KIP1}$ degradation. However, we did not observe different $p27^{KIP1}$ levels in $Rb^{-/-}$ and $Rb^{-/-}p107^{-/-}$ MEFs (data not shown). An alternative may be that high p107 levels in $Rb^{-/-}$ cells, together with p130, inhibit kinase activities in $Rb^{-/-}$ MEFs via direct binding to Cyclin E-A/CDK2 (Coats et al., 1999; Grana et al., 1998). This mechanism may explain why the extent of $p21^{CIP1}$ knockdown needed to achieve sufficient cyclin-dependent kinase activity to support anchorage-independent growth was lower in DKO and TKO MEFs than in $Rb^{-/-}$ cells. In conclusion, we propose that the pocket protein and p53 pathways synergistically protect against anchorage-independent growth by their shared ability to regulate cyclin-dependent kinase activities.

Roles of pRB, TBX2, and p53 in tumorigenesis

We identified TBX2 as a transforming oncogene in pocket protein-deficient cells. Several lines of evidence point to a role for TBX2 during *in vivo* tumorigenesis. TBX2 was found amplified in a range of human tumor samples and cancer cell lines of different developmental origin, such as melanoma cell lines (Vance et al., 2005), a subset of human breast tumors (Sinclair et al., 2002; Jacobs et al., 2000), and pancreatic cancer cell lines (Mahlamaki et al., 2002). The combined loss of components of the pRB and p53 pathways cooperates in tumorigenesis *in vivo*. The loss of $p16^{INK4A}$ accelerated tumorigenesis in $p53^{-/-}$ mice (Sharpless et al., 2002), and similarly, INK4A/ARF knockout mice developed tumors with a shorter latency than $p16^{INK4A-/-}$ or $p19^{ARF-/-}$ mice (Sharpless et al., 2004). In a $p16^{INK4A}$ null background, the loss of one $p19^{ARF}$ allele accelerated tumorigenesis, while strikingly, the wild-type allele was retained in a subset of the tumors (Krimpenfort et al., 2001). This indicates that also *in vivo*, tumor development by abrogation of the pRB

pathway can be accelerated by partial ablation of the p53 pathway. Moreover, various mouse tumor models have been generated by combined deletion of *p53* and *Rb*, including tumors of the lung (Meuwissen et al., 2003), central nervous system (Marino et al., 2000), breast (Simin et al., 2004), and pineal and pituitary glands (Vooijs et al., 2002). Finally, simultaneous mutations in both pathways have been found in a variety of mouse and human tumor samples (Miwa et al., 2006; Burke et al., 2005). Thus, the synergism between the loss of the pocket protein and p53 pathways, as observed in this study during transformation of MEFs, also applies to tumorigenesis in both mice and humans. While this synergism has been explained by alleviation of apoptosis by p53, our previous (Foijer et al., 2005) and present findings indicate that the loss of p53 may also be required for alleviation of cell cycle arrest of cells with abrogated pocket protein function.

Comparison of the requirements for transformation of human and murine fibroblasts

By using variants of simian virus 40 large T antigen (LT) that specifically target p53 *or* the pRB family, Rangarajan and co-workers (2004) suggested that in both human and mouse cells, the loss of p53 *or* the loss of pocket proteins is sufficient for bypassing RAS^{V12}-induced senescence. However, RAS^{V12}-induced transformation met different requirements in human and murine fibroblasts. First, transformation of human fibroblasts required expression of hTERT and simian virus 40 small t antigen (ST) (Hahn et al., 1999), which was dispensable for transformation of murine fibroblasts. Since murine cells contain long telomeres due to active telomerase (Prowse and Greider, 1995; Kipling and Cooke, 1990), mTERT overexpression is not required for transformation. The requirement for ST in transformation of human fibroblasts is caused by its interaction with protein phosphatase 2A (Hahn et al., 2002), which possibly downregulates PTEN (phosphatase and tensin homolog) (Boehm et al., 2005). Why ST expression is not required for murine transformation is currently unknown. Second, LT-mediated ablation of either the pRB or the p53 pathway appeared sufficient for RAS^{V12}-induced transformation of mouse embryonic fibroblasts, while ablation of both pathways was required for transformation of human fibroblasts (Rangarajan et al., 2004). Similarly, Boehm et al. (2005) claimed that expression of a dominant-negative version of p53, c-Myc, and RAS^{V12} was sufficient for transformation of MEFs, whereas human fibroblasts needed expression of ST and suppression of pRB. Also, RAS^{V12}- and hTERT-expressing BJ primary human fibroblasts could exhibit anchorage-independent growth only upon concomitant knockdown of p53 and pRB (Voorhoeve and Agami, 2003). Taken together, a widely held view has emerged that suppression of both the p53 and pRB pathways is mandatory to transformation of human cells while either one of these events is sufficient for transformation of murine cells. This view was supported by Sage et al. (2000), who reported anchorage-independent growth of TKO MEFs upon expression of RAS^{V12}. However, our previous (Peeper et al.,

2001; Dannenberg et al., 2000) and present results sharply contrast with this view: partial or complete pocket protein-deficient MEFs were largely unable to exhibit anchorage-independent growth upon RAS^{V12} expression. Furthermore, we found that partial ablation of the p53 pathway strongly stimulated anchorage-independent growth of pocket protein-deficient MEFs. We therefore conclude that in murine cells, ablation of the p53 and pRB pathways synergistically supports oncogenic transformation and that in this respect, mouse fibroblasts are not fundamentally different from human fibroblasts.

Materials and Methods

MEF isolation, cell culture, and retroviral infections

Rb$^{-/-}$, *p107*$^{-/-}$*p130*$^{-/-}$, *Rb*$^{-/-}$*p107*$^{-/-}$, *Rb*$^{-/-}$*p130*$^{-/-}$, and *Rb*$^{-/-}$*p107*$^{-/-}$*p130*$^{-/-}$ MEFs were isolated from chimeric embryos, which were generated by injection of mutant embryonic stem cells into blastocysts as previously described (Dannenberg et al., 2000). *p107*$^{-/-}$ and *p130*$^{-/-}$ MEFs were isolated from embryos which were generated by intercrossing *p107*$^{+/-}$ and *p130*$^{+/-}$ mice, respectively. MEFs were cultured in Glasgow minimal essential medium (GMEM) (Invitrogen/Gibco) containing 10% fetal calf serum, 1 mM nonessential amino acids (Invitrogen/Gibco), 1 mM sodium pyruvate (Invitrogen/Gibco), 100 units of penicillin/ml (Invitrogen/Gibco), 100 µg streptomycin/ml (Invitrogen/Gibco), and 0.1 mM β-mercaptoethanol and incubated at 37°C in the presence of 5% CO_2. Retroviral supernatants were produced by calcium phosphate transfection (Invitrogen) of phoenix cells with 16 µg of the desired construct and 4 µg pCL-eco. Forty-eight hours posttransfection, retroviral supernatant was filtered using 0.45-µm filters (mixed cellulose ester membrane; Millipore) and immediately frozen using a dry ice-ethanol bath and stored at −80°C. After viral supernatant was harvested, phoenix cells were supplemented with GMEM containing medium supplements as described above, and supernatant was again harvested using the same procedure, with an interval of at least 7 h. MEFs were twice infected with viral supernatant, supplemented with Polybrene to a concentration of 4 µg/ml, during a time span of at least 7 h per infection. For serial infections, MEFs were cultured in non-virus-containing medium for 48 h between infections and reseeded before infection to obtain optimal cell density. After the last infection, MEFs were cultured in non-virus-containing medium for at least 48 h.

Constructs

The pEYK-MCF7 library and pEYK-GFP were a gift of G. Q. Daley. pCl-Eco was kindly provided by D. Peeper. pBABE-RAS^{V12} was kindly provided by T. Brummelkamp, pRetroSuper-p53 and pRetroSuper-$p16^{INK4A}$ by A. Dirac, and pBABE- $p21^{CIP1}$ by J. Dannenberg. The pRetroSuper-p21strong vector was previously generated and contains the following 19-mer $p21^{CIP1}$ targeting sequence: GCCCTCACTCTGTGTGTCT (Foijer et al., 2005). The 19-mer $p21^{CIP1}$ targeting sequence in pRetroSuper-p21weak is ACAGGAGCAAAGTGTGCCG. The pRISC-$p16^{INK4A}$/$p19^{ARF}$ vector was a gift of S. Huang; this vector is a variant of pRetroSuper (pRS) containing an additional chloramphenicol resistance marker under the control of a TET promoter and contains the following targeting sequence: ATCAAGACATCGTGCGATA.

Soft agar and methylcellulose assays

For soft agar assays, 6×10^4 MEFs were suspended in 2 ml of a 37°C, 0.35% soft agar solution (low gelling agarose type VII from Sigma) in GMEM containing 10% fetal calf serum, the same medium supplements as mentioned above plus gentamicin to a concentration of 0.02 mg/ml (Invitrogen/

Gibco) and plated in one well of a six-well plate. To prevent cells from attaching to the bottom of the well, the 0.35% soft agar solution was poured into an ultra-low-attachment surface plate (catalog no. 3471; Corning Incorporated) coated with a 1% soft agar layer. To allow solidification of the agar, plates were incubated at 4°C for 30 min. Subsequently, cells were incubated at 37°C in the presence of 5% CO_2 for 2 to 4 weeks. A few drops of fresh medium were added twice a week. Pictures were taken using a non-phase-contrast lens (×2.5 magnification) and assembled using Axiovision 4.5. Detail images were taken using a phase-contrast lens (×5 magnification). For methylcellulose assays, 3 × 105 MEFs were suspended in 4 ml of a 37°C, 1.3% methylcellulose solution in GMEM supplemented with fetal calf serum to a concentration of 5%, penicillin to a concentration of 100 units/ml (Invitrogen/Gibco), streptomycin to a concentration of 100 μg/ml (Invitrogen/Gibco), and gentamicin to a concentration of 0.02 mg/ml (Invitrogen/Gibco) and plated in one well of a six-well ultra-low-attachment surface plate (catalog no. 3471; Corning Incorporated). A 2.6% methylcellulose stock solution was obtained from Stem Cell Technologies (catalog no. H4100). Cells were harvested by suspending 4 ml of methylcellulose culture with 40 ml ice-cold phosphate-buffered saline (PBS) (Invitrogen/Gibco), followed by centrifugation and aspiration of methylcellulose-PBS.

Recovery of retroviral integrations from soft agar colonies

Soft agar colonies were isolated using sterilized glass pipettes and propagated under attached conditions. Cells were lysed overnight (O/N) at 55°C in lysis mix containing 0.1 M Tris HCl (pH 8.5), 5 mM EDTA, 0.2 M NaCl, 0.2% sodium dodecyl sulfate (SDS), and 100 μg/ml proteinase K. Genomic DNA was isolated by phenol-chloroform-isoamyl alcohol extraction, precipitated using isopropanol, and dissolved O/N at 37°C in 10 mM Tris HCl (pH 8.0)-0.1 mM EDTA. Recovery of retroviral inserts was performed using a shuttle strategy modified from the method of Koh et al. (2002). Five micrograms of genomic DNA was digested using 50 units of either NotI (Roche) or AscI (New England Biolabs) O/N in 100 μl, followed by an additional 3 h digestion upon the addition of 25 units. For each analyzed colony, both digestions were performed. Subsequently, fragments were purified using phenol-chloroform-isoamyl alcohol extraction and ethanol precipitated in the presence of 200 μg of type VII mussel glycogen (catalog no. G1508; Sigma) at -80°C. Ligation was performed O/N at 16°C in a total volume of 200 μl using 2 units of T4 DNA ligase (Roche) and in the presence of extra ATP to a concentration of 0.05 mM. An extra 3 h ligation was performed the next day upon the addition of 2 units of T4 DNA ligase. Ligated plasmids were purified as described above, electroporated in 5 μl of DH10B electromax competent cells per reaction (Invitrogen/Life Technologies), and plated on LB plates containing 5 μg of NaCl/liter and 100 μg of zeocin/ml (Invitrogen/Life Technologies). Colonies were propagated, and plasmids were isolated according to standard protocols. Sequencing was performed using the following primers: FW (5′ CAC CCC CAC CGC CCT CAA AGT AG 3′) and RV (5′ GGA ACG GCA CTG GTC AAC TTG G 3′).

MEF injections in immunocompromised mice

A total of 1 × 10^6 MEFs were suspended in 200 μl PBS and injected into immunocompromised BALB/c nude mice. Mice were inspected twice a week.

Protein isolation, immunoblot, immunoprecipitation, and in vitro kinase assays

For protein isolations, cells were lysed for 30 min on ice in lysis buffer containing 150 mM NaCl, 50 mM HEPES (pH 7.5), 5 mM EDTA, 0.1% NP-40, 5 mM NaF, 0.5 mM vanadate, 20 mM β-glycerolphosphate, and 1 tablet complete protease inhibitor cocktail (Roche) per 50 ml. After centrifugation, protein concentration was determined using Bio-Rad protein assay. For immunoblot

analysis, 20 to 30 μg protein was separated on 12% SDS-polyacrylamide gels. Blotting was performed by using standard protocols. For immunoprecipitation reactions, lysates were incubated with antibody and 50% beads in lysis buffer (either protein A [Pharmacia] or protein A/G [Santa Cruz Biotechnology], depending on the antibody used) and incubated O/N at 4°C while rotating. Prior to immunoblot analysis, lysates were washed five times in lysis buffer and loaded on 12% SDS-polyacrylamide gels. Prior to kinase assays, lysates were washed three times in lysis buffer and then washed two times in kinase buffer containing 50 mM HEPES (pH 7.5), 5 mM MgCl2, 2.5 mM MnCl2, and 1 mM dithiothreitol. Kinase assays were performed by incubating 50 μg protein (CDK2 assay) or 20 μg protein (Cyclin B1 kinase assay) with 5 μg histone H1 (Roche) and 2.5 μCi [γ-32P]ATP in kinase buffer for 30 min at 37°C while shaking. After the reaction was stopped by adding protein loading buffer, one-third of this mixture was separated on 10% SDS-polyacrylamide gels, blotted by using standard protocols, and exposed to Kodak BioMax XAR films. The antibodies used for immunoblot analysis were mouse monoclonal anti- $p21^{CIP1}$ (F-5, SC-6246; Santa Cruz), mouse monoclonal anti-RAS (catalog no. 610002; BD Transduction Laboratories), sheep polyclonal anti-p53 (Ab-7, PC35; Calbiochem), rabbit anti-TBX2 (kindly provided by M. van Lohuizen), rabbit polyclonal anti-$p19^{ARF}$ (ab80; Abcam), and goat polyclonal anti-CDK4 (C-22, SC-260-G; Santa Cruz Biotechnology). Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, and rabbit anti-goat antibodies (DakoCytomation). The antibodies used for immunoprecipitation reactions were rabbit anti-CDK2 (M2; Santa Cruz), mouse anti-Cyclin B1 (GNS1; Santa Cruz) for kinase assays, and rabbit anti-Cyclin B1 (H433; Santa Cruz) for immunoblots. Immunoprecipitation reactions for Cyclin B1 kinase assay were performed using protein A/G beads (Santa Cruz). All other immunoprecipitation reactions were performed using protein A beads (Pharmacia).

FACS analysis

For fluorescence-activated cell sorting (FACS) analysis, cells were trypsinized and fixed in 70% ethanol in PBS at 4°C. Subsequently, cells were washed in PBS, suspended in PBS containing 200 μg/ml RNase A and 20 μg/ml propidium iodide, incubated at 37°C for 15 min, and analyzed using Cell Quest and Summit software.

Acknowledgements

We thank George Q. Daley for the pEYK-MCF7 library, Daniel Peeper and Theo van Laar for help with recovery of cDNA vectors, Anja van der Wal for generation of MEFs, Tanja van Harn for kinase assays, Elly Delzenne-Goette and Sjaak Greven for nude mice injections and tumor analyses, Lauran Oomen and Lenny Brocks for help with digital microscopy, and Frank van Diepen and Anita Pfauth for help with FACS. We are grateful to Marieke Aarts, Jan-Hermen Dannenberg, Tanja van Harn, and Rob Wolthuis for helpful discussions and critically reading the manuscript.
This work was financially supported by the Dutch Cancer Society (NKI 2002-2634).

References

Bao,W., Thullberg,M., Zhang,H., Onischenko,A., and Stromblad,S. (2002). Cell attachment to the extracellular matrix induces proteasomal degradation of p21(CIP1) via Cdc42/Rac1 signaling. Mol. Cell Biol. *22*, 4587-4597.

Berthet,C. and Kaldis,P. (2007). Cell-specific responses to loss of cyclin-dependent kinases. Oncogene *26*, 4469-4477.

Boehm,J.S., Hession,M.T., Bulmer,S.E., and Hahn,W.C. (2005). Transformation of human and murine fibroblasts without viral oncoproteins. Mol. Cell Biol. *25*, 6464-6474.

Burke,L., Flieder,D.B., Guinee,D.G., Brambilla,E., Freedman,A.N., Bennett,W.P., Jones,R.T., Borkowski,A., Caporaso,N.A., Fleming,M., Trastek,V., Pairolero,P., Tazelaar,H., Midthun,D., Jett,J.R., Liotta,L.A., Travis,W.D., and Harris,C.C. (2005). Prognostic implications of molecular and immunohistochemical profiles of the Rb and p53 cell cycle regulatory pathways in primary non-small cell lung carcinoma. Clin. Cancer Res. *11*, 232-241.

Coats,S., Whyte,P., Fero,M.L., Lacy,S., Chung,G., Randel,E., Firpo,E., and Roberts,J.M. (1999). A new pathway for mitogen-dependent cdk2 regulation uncovered in p27(Kip1)-deficient cells. Curr. Biol. *9*, 163-173.

Dannenberg,J.H., Schuijff,L., Dekker,M., van der Valk,M., and te Riele,H. (2004). Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev. *18*, 2952-2962.

Dannenberg,J.H., van Rossum,A., Schuijff,L., and te Riele,H. (2000). Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. Genes Dev. *14*, 3051-3064.

Dirac,A.M. and Bernards,R. (2003). Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. J. Biol. Chem. *278*, 11731-11734.

Dobrzycka,K.M., Kang,K., Jiang,S., Meyer,R., Rao,P.H., Lee,A.V., and Oesterreich,S. (2006). Disruption of Scaffold Attachment Factor B1 Leads to TBX2 Up-regulation, Lack of p19ARF Induction, Lack of Senescence, and Cell Immortalization. Cancer Res. *66*, 7859-7863.

Fang,F., Orend,G., Watanabe,N., Hunter,T., and Ruoslahti,E. (1996). Dependence of cyclin E-CDK2 kinase activity on cell anchorage. Science *271*, 499-502.

Foijer,F., Wolthuis,R.M., Doodeman,V., Medema,R.H., and te Riele,H. (2005). Mitogen requirement for cell cycle progression in the absence of pocket protein activity. Cancer Cell *8*, 455-466.

Gad,A., Thullberg,M., Dannenberg,J.H., te Riele,H., and Stromblad,S. (2004). Retinoblastoma susceptibility gene product (pRb) and p107 functionally separate the requirements for serum and anchorage in the cell cycle G1-phase. J. Biol. Chem. *279*, 13640-13644.

Grana,X., Garriga,J., and Mayol,X. (1998). Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. Oncogene *17*, 3365-3383.

Guadagno,T.M. and Assoian,R.K. (1991). G1/S control of anchorage-independent growth in the fibroblast cell cycle. J. Cell Biol. *115*, 1419-1425.

Guadagno,T.M., Ohtsubo,M., Roberts,J.M., and Assoian,R.K. (1993). A link between cyclin A expression and adhesion-dependent cell cycle progression. Science *262*, 1572-1575.

Hahn,W.C., Counter,C.M., Lundberg,A.S., Beijersbergen,R.L., Brooks,M.W., and Weinberg,R.A. (1999). Creation of human tumour cells with defined genetic elements. Nature *400*, 464-468.

Hahn,W.C., Dessain,S.K., Brooks,M.W., King,J.E., Elenbaas,B., Sabatini,D.M., DeCaprio,J.A., and Weinberg,R.A. (2002). Enumeration of the simian virus 40 early region elements necessary for human cell transformation. Mol. Cell Biol. *22*, 2111-2123.

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

Harvey,M., Sands,A.T., Weiss,R.S., Hegi,M.E., Wiseman,R.W., Pantazis,P., Giovanella,B.C., Tainsky,M.A., Bradley,A., and Donehower,L.A. (1993). In vitro growth characteristics of embryo fibroblasts isolated from p53-deficient mice. Oncogene *8*, 2457-2467.

Hurford,R.K., Jr., Cobrinik,D., Lee,M.H., and Dyson,N. (1997). pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. Genes Dev. *11*, 1447-1463.

Jacobs,J.J., Keblusek,P., Robanus-Maandag,E., Kristel,P., Lingbeek,M., Nederlof,P.M., van Welsem,T., van de Vijver,M.J., Koh,E.Y., Daley,G.Q., and van Lohuizen,M. (2000). Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19(ARF)) and is amplified in a subset of human breast cancers. Nat. Genet. *26*, 291-299.

Kamijo,T., Zindy,F., Roussel,M.F., Quelle,D.E., Downing,J.R., Ashmun,R.A., Grosveld,G., and Sherr,C.J. (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell *91*, 649-659.

Kipling,D. and Cooke,H.J. (1990). Hypervariable ultra-long telomeres in mice. Nature *347*, 400-402.

Koh,E.Y., Chen,T., and Daley,G.Q. (2002). Novel retroviral vectors to facilitate expression screens in mammalian cells. Nucleic Acids Res. *30*, e142.

Krimpenfort,P., Quon,K.C., Mooi,W.J., Loonstra,A., and Berns,A. (2001). Loss of p16Ink4a confers susceptibility to metastatic melanoma in mice. Nature *413*, 83-86.

Lukas,C., Sorensen,C.S., Kramer,E., Santoni-Rugiu,E., Lindeneg,C., Peters,J.M., Bartek,J., and Lukas,J. (1999). Accumulation of cyclin B1 requires E2F and cyclin-A-dependent rearrangement of the anaphase-promoting complex. Nature *401*, 815-818.

Mahlamaki,E.H., Barlund,M., Tanner,M., Gorunova,L., Hoglund,M., Karhu,R., and Kallioniemi,A. (2002). Frequent amplification of 8q24, 11q, 17q, and 20q-specific genes in pancreatic cancer. Genes Chromosomes Cancer *35*, 353-358.

Marino,S., Vooijs,M., van Der Gulden,H., Jonkers,J., and Berns,A. (2000). Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. Genes Dev. *14*, 994-1004.

Meuwissen,R., Linn,S.C., Linnoila,R.I., Zevenhoven,J., Mooi,W.J., and Berns,A. (2003). Induction of small cell lung cancer by somatic inactivation of both Trp53 and Rb1 in a conditional mouse model. Cancer Cell *4*, 181-189.

Miwa,S., Uchida,C., Kitagawa,K., Hattori,T., Oda,T., Sugimura,H., Yasuda,H., Nakamura,H., Chida,K., and Kitagawa,M. (2006). Mdm2-mediated pRB downregulation is involved in carcinogenesis in a p53-independent manner. Biochem. Biophys. Res. Commun. *340*, 54-61.

Nevins,J.R. (2001). The Rb/E2F pathway and cancer. Hum. Mol. Genet. *10*, 699-703.

Peeper,D.S., Dannenberg,J.H., Douma,S., te Riele,H., and Bernards,R. (2001). Escape from premature senescence is not sufficient for oncogenic transformation by Ras. Nat. Cell Biol. *3*, 198-203.

Prince,S., Carreira,S., Vance,K.W., Abrahams,A., and Goding,C.R. (2004). Tbx2 directly represses the expression of the p21(WAF1) cyclin-dependent kinase inhibitor. Cancer Res. *64*, 1669-1674.

Prowse,K.R. and Greider,C.W. (1995). Developmental and tissue-specific regulation of mouse telomerase and telomere length. Proc. Natl. Acad. Sci. U. S. A *92*, 4818-4822.

Rangarajan,A., Hong,S.J., Gifford,A., and Weinberg,R.A. (2004). Species- and cell type-specific requirements for cellular transformation. Cancer Cell *6*, 171-183.

Rodier,G., Makris,C., Coulombe,P., Scime,A., Nakayama,K., Nakayama,K.I., and Meloche,S. (2005). p107 inhibits G1 to S phase progression by down-regulating expression of the F-box protein Skp2. J. Cell Biol. *168*, 55-66.

Roovers,K. and Assoian,R.K. (2000). Integrating the MAP kinase signal into the G1 phase cell cycle machinery. Bioessays *22*, 818-826.

Sage,J., Mulligan,G.J., Attardi,L.D., Miller,A., Chen,S., Williams,B., Theodorou,E., and Jacks,T. (2000). Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. Genes Dev. *14*, 3037-3050.

Serrano,M., Lin,A.W., McCurrach,M.E., Beach,D., and Lowe,S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593-602.

Sharpless,N.E., Alson,S., Chan,S., Silver,D.P., Castrillon,D.H., and DePinho,R.A. (2002). p16(INK4a) and p53 deficiency cooperate in tumorigenesis. Cancer Res. *62*, 2761-2765.

Sharpless,N.E., Ramsey,M.R., Balasubramanian,P., Castrillon,D.H., and DePinho,R.A. (2004). The differential impact of p16(INK4a) or p19(ARF) deficiency on cell growth and tumorigenesis. Oncogene *23*, 379-385.

Simin,K., Wu,H., Lu,L., Pinkel,D., Albertson,D., Cardiff,R.D., and Van Dyke,T. (2004). pRb inactivation in mammary cells reveals common mechanisms for tumor initiation and progression in divergent epithelia. PLoS. Biol. *2*, E22.

Sinclair,C.S., Adem,C., Naderi,A., Soderberg,C.L., Johnson,M., Wu,K., Wadum,L., Couch,V.L., Sellers,T.A., Schaid,D., Slezak,J., Fredericksen,Z., Ingle,J.N., Hartmann,L., Jenkins,R.B., and Couch,F.J. (2002). TBX2 is preferentially amplified in BRCA1- and BRCA2-related breast tumors. Cancer Res *62*, 3587-3591.

Vance,K.W., Carreira,S., Brosch,G., and Goding,C.R. (2005). Tbx2 is overexpressed and plays an important role in maintaining proliferation and suppression of senescence in melanomas. Cancer Res. *65*, 2260-2268.

Vooijs,M., te Riele,H., van der Valk,M., and Berns,A. (2002). Tumor formation in mice with somatic inactivation of the retinoblastoma gene in interphotoreceptor retinol binding protein-expressing cells. Oncogene *21*, 4635-4645.

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

Walker,J.L., Fournier,A.K., and Assoian,R.K. (2005). Regulation of growth factor signaling and cell cycle progression by cell adhesion and adhesion-dependent changes in cellular tension. Cytokine Growth Factor Rev. *16*, 395-405.

Williams,J.P., Stewart,T., Li,B., Mulloy,R., Dimova,D., and Classon,M. (2006). The retinoblastoma protein is required for Ras-induced oncogenic transformation. Mol Cell Biol *26*, 1170-1182.

Wu,R.C. and Schonthal,A.H. (1997). Activation of p53-p21waf1 pathway in response to disruption of cell-matrix interactions. J. Biol. Chem. *272*, 29091-29098.

Zhu,X., Ohtsubo,M., Bohmer,R.M., Roberts,J.M., and Assoian,R.K. (1996). Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein. J. Cell Biol. *133*, 391-403.

Supplemental figures

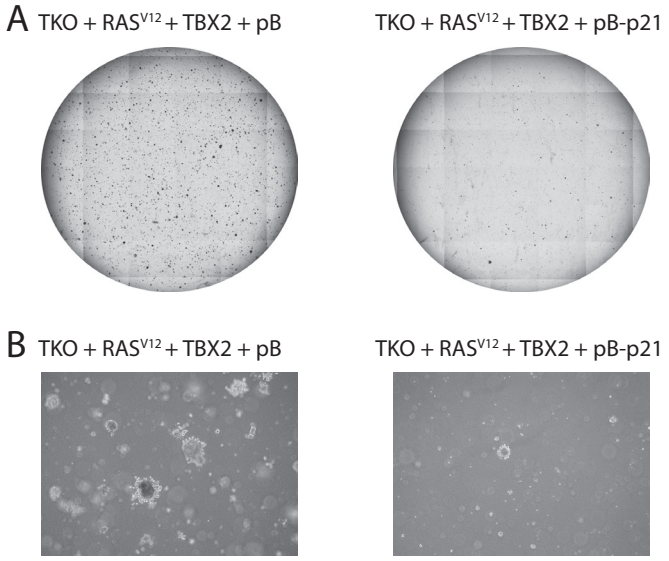


Figure S1: Ectopic expression of p21^{CIP1} inhibits TBX2-induced transformation of RAS^{V12}/ Rb$^{-/-}$p107$^{-/-}$p130$^{-/-}$ (TKO) MEFs. Cells were transduced with pBABE-RAS^{V12}, pEYK-TBX2 and either a pBABE control vector or pBABE-p21^{CIP1}. Infected cells were plated in soft agar and pictures were taken after 11 days using a non-phase contrast lens (2.5x magnification) (A) or a phase contrast lens (5x magnification) (B).

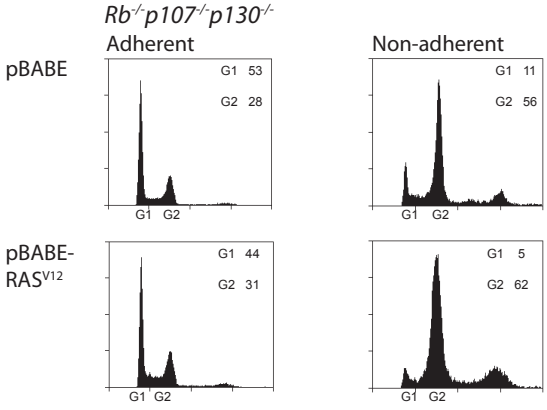


Figure S2: Both TKO and RAS^{V12}/TKO MEFs arrest in G$_2$ upon loss of adhesion. Cells were infected with the indicated constructs and cultured under adherent conditions or in methylcellulose for 6 days, stained with propidium iodide and analyzed by FACS.

CHAPTER 3

Mapkapk3 is a suppressor of anchorage-independent growth

Tinke L. Vormer, Camiel L.C. Wielders, Marije Scholte and Hein te Riele

Manuscript in preparation

Abstract

Ablation of the pocket proteins pRB, p107 and p130 in mouse embryonic fibroblasts (MEFs) abrogated RAS^{V12}-induced senescence, but was not sufficient to support RAS^{V12}-induced transformation. To identify events that support transformation, we performed an insertional mutagenesis screen in RAS^{V12}/*Rb*$^{-/-}$*p130*$^{-/-}$*p107*$^{-/-}$ MEFs. As insertional mutagen we used the ERM vector, which was specifically designed to enhance gene function, and scored for colony formation in soft agar. In one of the colonies, we identified an ERM integration in *Mapkapk3*, however, this integration appeared to abrogate rather than to enhance *Mapkapk3* expression. Consistently, we found that shRNA-mediated down regulation of Mapkapk3 or chemical inhibition of the upstream Map kinase p38 promoted anchorage-independent growth.

Introduction

The pocket proteins pRB, p107 and p130 play a key role during cell cycle regulation by promoting the G_0/G_1 state. Consistently, our previous experiments in mouse embryonic fibroblasts (MEFs) showed that G_1 arrest in response to various growth inhibitory signals was abrogated by loss of either pRB and p130, pRB and p107 or all three pocket proteins simultaneously. *E.g.*, whereas wild-type MEFs entered premature senescence upon expression of RAS^{V12}, *Rb*$^{-/-}$*p130*$^{-/-}$ (DKO), *Rb*$^{-/-}$*p107*$^{-/-}$ (DKO) and *Rb*$^{-/-}$*p130*$^{-/-}$*p107*$^{-/-}$ (TKO) MEFs continued to proliferate. Strikingly though, these pocket-protein deficient MEFs could not grow anchorage independently upon expression of RAS^{V12}, demonstrating that transformation requires additional events (Vormer et al., 2008; Dannenberg et al., 2004; Peeper et al., 2001; Dannenberg et al., 2000).

In Chapter 2, we showed that transformation of RAS^{V12}-expressing DKO or TKO MEFs could be achieved by expression of TBX2. The TBX2 transcription factor, which can both activate and repress transcription of a variety of target genes (Paxton et al., 2002; Chen et al., 2001), can downregulate well-known cell cycle inhibitors such as $p19^{ARF}$ and, to a lower extent, $p16^{INK4A}$ (Lingbeek et al., 2002; Jacobs et al., 2000). As $p16^{INK4A}$ functions upstream of the pocket proteins, TBX2-mediated downregulation of $p16^{INK4A}$ is unlikely to contribute to the transformation of pocket protein-deficient MEFs. In line with this, we found that downregulation of $p19^{ARF}$, p53 or $p21^{CIP1}$ contributed to anchorage-independent growth of RAS^{V12}/TKO MEFs, whereas downregulation of $p16^{INK4A}$ did not (Vormer et al., 2008). Interestingly, downregulation of p53 more robustly induced soft agar colony formation than downregulation of $p21^{CIP}$, indicating that additional p53 effectors contributed to the induction of transformation. Similarly, one may envision that targets of TBX2 functioning in distinct tumor suppressor pathways could contribute to TBX2-induced transformation of pocket protein-deficient MEFs. We therefore aimed to identify additional inducers of anchorage-independent growth. For this purpose, we performed

an insertional mutagenesis screen in RAS^{V12}/TKO MEFs and found that an inactivating proviral insertion in the *Mapkapk3* (mitogen-activated-protein-kinase-activated protein-kinase 3) gene promoted anchorage-independent growth. Consistently, shRNA-mediated down-regulation of Mapkapk3 induced anchorage-independent growth of RAS^{V12}/pocket protein-deficient MEFs.

Results

A retroviral insertional mutagenesis screen to identify mediators of anchorage-independent growth

To identify promoters or inhibitors of anchorage-independent growth, we performed a retroviral insertional mutagenesis screen in RAS^{V12}/TKO MEFs using the Enhanced Retroviral Mutagen (ERM) vector (Liu et al., 2000). This retroviral vector is a derivative of pBABE and was designed to enhance gene transcription upon integration into the genomic DNA. When the ERM virus integrates upstream of an endogenous promoter, transcription can be increased via enhancer elements present in ERM. Additionally, a splice donor site at the 3' end of ERM enables splicing towards a splice acceptor site of an endogenous exon, resulting in a fusion transcript under the control of the ERM-promoter. The latter can occur when integration occurs either upstream or within a genetic locus. RAS^{V12}/TKO MEFs were infected with ERM, plated under non-adherent conditions (soft agar) and monitored for colony formation. Infection with ERM caused a four-fold increase in the number of soft agar colonies compared to non-infected RAS^{V12}/TKO MEFs. Soft agar colonies produced by ERM/RAS^{V12}/TKO MEFs were isolated and the position of the ERM integration in the genomic DNA was determined using splinkerette PCR. This analysis identified an ERM-integration in intron 2 of *Mapkapk3* in one of the colonies (Fig. 1A). The position of the ERM-integration into *Mapkapk3* was confirmed by PCR analysis using genomic DNA (Fig. 1B) and by RT-PCR analysis. Sequencing of the RT-PCR product revealed that the ERM vector had spliced to exon 3 of *Mapkapk3* but that the ERM-*Mapkapk3* fusion was out of frame (Fig. 1C). This suggested that transformation was not due to increased expression of *Mapkapk3*. To study the effect of this integration, we generated cDNA from the ERM-*Mapkapk3* out-of-frame fusion and inserted this into pBABE. Additionally, full-length wild-type *Mapkapk3* cDNA was inserted into pBABE. Infection of RAS^{V12}/TKO MEFs with these vectors and subsequent soft agar plating showed that pBABE-*Mapkapk3* inhibited the number of background colonies produced by RAS^{V12}/TKO MEFs, whereas pBABE-ERM-*Mapkapk3* had no effect (data not shown). These results suggest that overexpression of Mapkapk3 suppressed anchorage-independent growth and furthermore, that inactivation of one allele of *Mapkapk3* by ERM insertion had induced transformation.

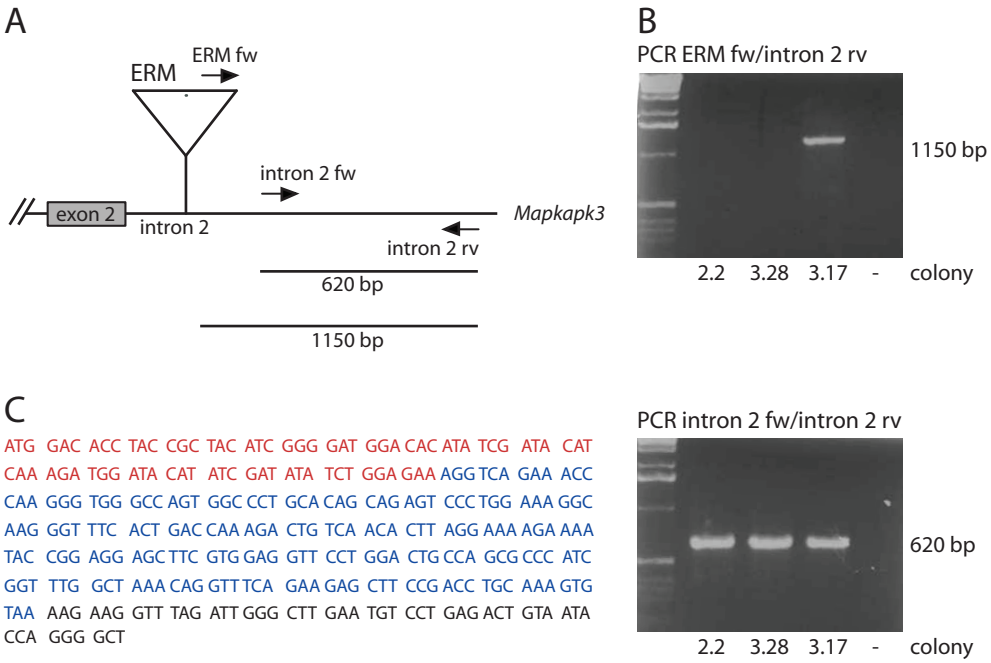


Figure 1: An insertional mutagenesis screen for anchorage-independent growth of RAS^{V12}/TKO MEFs reveals integration of ERM into Mapkapk3. (A) Genomic organization of the Mapkapk3 locus in a RAS^{V12}/TKO soft agar colony (number 3.17). ERM is integrated into intron 2 of Mapkapk3. (B) PCR analysis of 3 different soft agar colonies, demonstrating the presence of the ERM-Mapkapk3 fusion in colony 3.17. The positions of the used primers are depicted in (A). (C) Sequence of the ERM-Mapkapk3 RT-PCR product that was derived from colony 3.17. Reverse transcription and amplification of ERM-Mapkapk3 was performed as described in the Materials and Methods section. Red bases represent the last part of the ERM sequence, blue bases represent the out-of-frame Mapkapk3 sequence, which is the resultant of splicing of ERM towards exon 3 of Mapkapk3. After reaching a stop codon, the sequence is shown in black.

RNAi-mediated downregulation of Mapkapk3 promotes anchorage-independent growth

To verify that downregulation of Mapkapk3 promotes anchorage-independent growth, we generated five different pRetroSuper (pRS) vectors targeting Mapkapk3. Upon infection of RAS^{V12}/*Rb*$^{-/-}$*p107*$^{-/-}$ MEFs, 2 out of 5 pRS-Mapkapk3 vectors induced colony formation in methylcellulose (Fig. 2A). Additionally, we investigated whether downregulation of the p38 MAPK pathway, which is an upstream regulator of Mapkapk3, could support anchorage-independent growth. Indeed, treatment with the p38 inhibitor SB203580 induced colony formation in RAS^{V12}/*Rb*$^{-/-}$*p107*$^{-/-}$ MEFs, cultured in methylcellulose (Fig. 2B). In conclusion, downregulation of p38/Mapkapk3 induced anchorage-independent growth in RAS^{V12}/pocket protein-deficient MEFs.

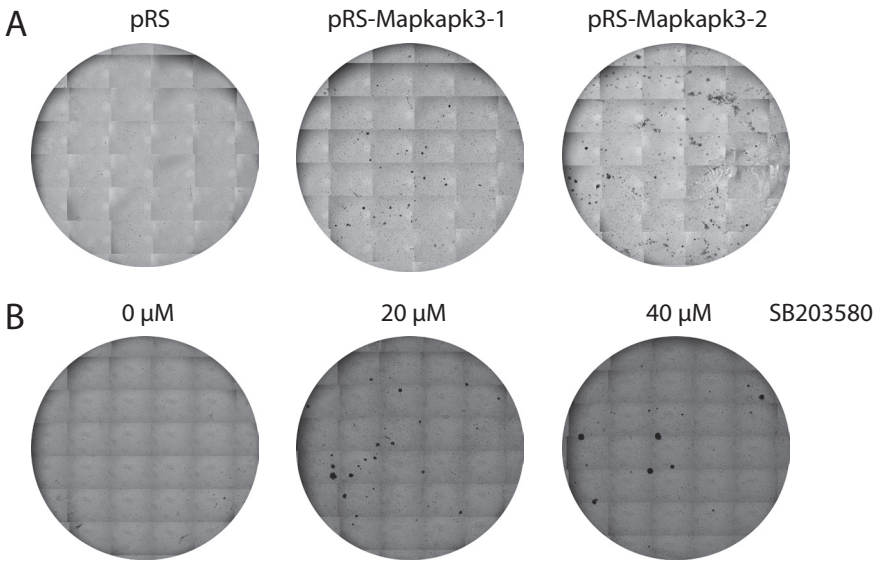


Figure 2: Downregulation of Mapkapk3 or p38 MAPK promotes anchorage-independent growth. (A) $Rb^{-/-}p107^{-/-}$ MEFs were infected with pBABE-RAS^{V12} plus pRS-Mapkapk3 or empty pRS and cultured in methylcellulose for 3 weeks. (B) $Rb^{-/-}p107^{-/-}$ MEFs were infected with pBABE-RAS^{V12}, treated with the indicated concentrations of the p38 inhibitor SB203580 and subsequently cultured in methylcellulose for 3 weeks. Pictures were taken using a non-phase-contrast lens (2.5x magnification).

Mapkapk3 regulation by TBX2 and anchorage

Since TBX2 over-expression induced anchorage-independent growth, we performed micro-array analyses of $RAS^{V12}/Rb^{-/-}p107^{-/-}$ MEFs to identify mRNAs that were regulated by loss of anchorage or the presence of TBX2. Strikingly, *Mapkapk3* appeared to be one of the regulated genes. Fig. 3A shows that non-adherent $RAS^{V12}/Rb^{-/-}p107^{-/-}$ MEFs displayed a two-fold increase in *Mapkapk3* mRNA level compared to their adherent counterparts. Expression of TBX2 in non-adherent $RAS^{V12}/Rb^{-/-}p107^{-/-}$ MEFs reverted *Mapkapk3* expression to the level observed in adherent $RAS^{V12}/Rb^{-/-}p107^{-/-}$ MEFs (Fig. 3A). These results suggest that the induction of *Mapkapk3* upon loss of anchorage functions as a growth suppressor mechanism that can be abrogated by expression of TBX2. Of note, the related *Mapkapk2*, which was previously identified as a downregulated TBX2-target (Chen et al., 2001), was not transcriptionally induced upon loss of anchorage, and was only slightly inhibited by TBX2. For comparison, loss of anchorage also caused a two- to three-fold increase in $p21^{CIP1}$ and $p27^{KIP1}$ mRNA levels, which could be inhibited by expression of TBX2 (Fig. 3B). This is in line with our previous studies showing that downregulation of $p21^{CIP1}$ could promote anchorage-independent growth of RAS^{V12}/pocket protein-deficient MEFs (Vormer et al., 2008). Taken together, our results identify

Mapkapk3 as a haploinsufficient suppressor of anchorage-independent growth and suggest that the transforming activity of TBX2 is at least partially mediated by down regulation of *Mapkapk3*.

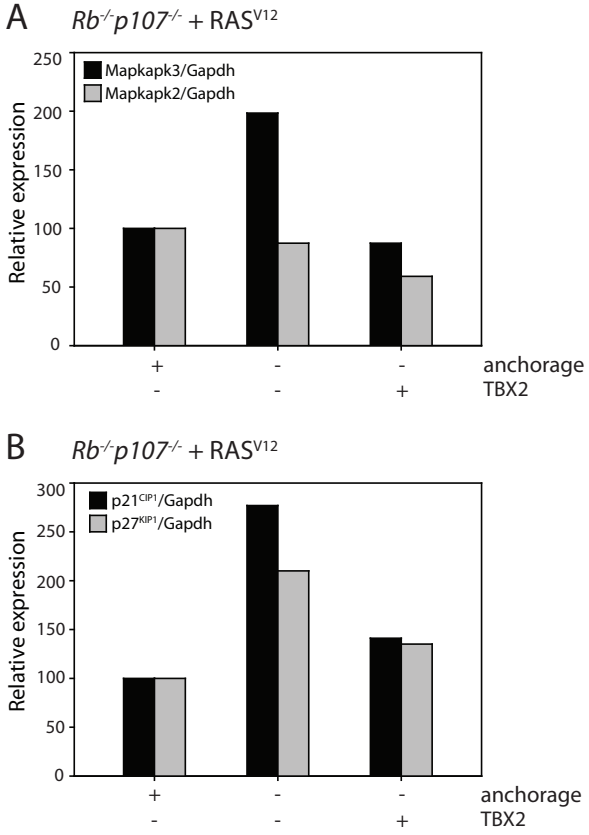


Figure 3: Regulation of Mapkapk3, $p21^{CIP1}$ and $p27^{KIP1}$ by TBX2 and anchorage. (A, B) $RAS^{V12}/Rb^{-/-}p107^{-/-}$ MEFs were cultured in the presence of TBX2 (cells were infected with pEYK-TBX2) or in the absence of TBX2 (cells were infected with pEYK-GFP), under adherent conditions (+ anchorage) or in methylcellulose (- anchorage). cDNA was generated from these cells and analyzed on micro-array. The relative expression was calculated by dividing the signal produced by the gene of interest by the signal produced by Gapdh.

Discussion

In this study, we have identified *Mapkapk3* as a suppressor of anchorage-independent proliferation of pocket protein-defective cells. *Mapkapk3* was found to be transcriptionally induced upon anchorage deprivation and downregulation of *Mapkapk3* enabled anchorage-independent growth: inactivation of one allele of *Mapkapk3* by retroviral insertion or shRNA-mediated downregulation of *Mapkapk3* promoted proliferation under non-adherent conditions of RAS^{V12}/TKO and RAS^{V12}/DKO MEFs. Furthermore, we found that

the oncogene TBX2 can downregulate *Mapkapk3*. Thus, downregulation of *Mapkapk3* is likely to be one of the mechanisms through which TBX2 exerts its transforming activity.

Mapkapk3 can be activated by three different mitogen-activated-protein-kinases (MAPK): p38, extracellular-signal-regulated kinase (ERK) and Jun-N-terminal kinase (JNK) (Ludwig et al., 1996; Zakowski et al., 2004). These three MAPK pathways can all become activated by oncogenic RAS, although ERK and JNK are generally more strongly activated than p38 (Chen et al., 2000). The ERK pathway is well known for its stimulatory effect on cell cycle progression, however, high levels of activated ERK can induce cell cycle arrest. This controversy is illustrated by ERK's ability to induce both Cyclin D1 and $p21^{CIP1}$ (Sebolt-Leopold and Herrera, 2004; Roovers and Assoian, 2000) The stress-activated p38 pathway functions in inhibiting proliferation and counteracting transformation (Loesch and Chen, 2008; Han and Sun, 2007). Of relevance, various ways of cross talk have been reported between the different MAPK pathways. Particularly, active MEK could induce p38 and additionally, activation of p38 by oncogenic RAS required the RAF/MEK/ERK pathway (Han and Sun, 2007; Wang et al., 2002; Chen et al., 2000). On the other hand, activated p38 could inhibit MEK/ERK (Aguirre-Ghiso et al., 2003; Li et al., 2003) and has also been reported to inhibit the proliferation-stimulating JNK pathway (Loesch and Chen, 2008; Chen et al., 2000).

Given the reported growth inhibitory effect of the p38 pathway, we wondered whether down-regulation of p38 could promote transformation of RAS^{V12}/*Rb*$^{-/-}$*p107*$^{-/-}$ MEFs. Indeed, treatment with the p38 inhibitor SB203580 supported anchorage-independent growth of these cells (Fig. 2B). A tumor suppressor role for p38 could simply be explained by the aforementioned inhibition of the proliferation- and survival-stimulating ERK pathway (Sebolt-Leopold and Herrera, 2004; Li et al., 2003). Along these lines, Aguirre-Ghiso and co-workers (2003) suggested that a high p38/ERK ratio correlated with cell cycle arrest in cancer cell lines. Additionally, experiments performed by Wang and colleagues (2002) pointed to a role for p38 in cell cycle arrest: chemical inhibition of p38 or chemical inhibition of MEK, which in turn inhibited p38, counteracted RAS^{V12}-induced senescence. Thus, their results suggest that RAS^{V12}-induced senescence is mediated via MEK/p38.

In addition to inhibiting MEK/ERK, p38 can induce several cell cycle inhibitors and tumor suppressors, including $p19^{ARF}$, p53, $p21^{CIP1}$, $p27^{KIP1}$ and $p16^{INK4A}$ (Han and Sun, 2007), which can all contribute to growth inhibition. In line with this, Bulavin and co-workers (2004) showed that ablation of the WIP1 phosphatase in MEFs raised the level of activated p38, which correlated with inhibition of tumor formation in nude mice via the $p16^{INK4A}$/$p19^{ARF}$ pathways, however, this effect was independent of p53. In contrast, Dolado and co-workers (2007) suggested that p38 could inhibit RAS^{V12}-induced transformation independently of $p16^{INK4A}$/$p19^{ARF}$ and also independently of affecting ERK. In fact, they

correlated p38 with the induction of apoptosis in response to reactive oxygen species (ROS), which were produced in response to RAS^{V12} signaling. Similarly, Nicke and co-workers (2005) suggested that ROS, induced by RAS^{V12}, caused activation of p38. In conclusion, several different mechanisms could contribute to the tumor suppressor role of p38 in our system.

We consider it likely that p38 suppresses transformation via the induction of cell cycle inhibitors, since (1) our previous studies clearly demonstrated that downregulation of the $p19^{ARF}$/p53/$p21^{CIP1}$ pathway induced transformation of RAS^{V12}/pocket protein-deficient MEFs and (2) Mapkapk3 is possibly involved in regulation of $p19^{ARF}$. Experiments by Voncken et al. (2005) showed that Mapkapk3 can phosphorylate the Polycomb group protein BMI1, resulting in its release from the chromatin. BMI1 is part of the Polycomb-Repressive Complex which represses the INK4A/ARF locus (Bracken et al., 2007; Jacobs et al., 1999). Together, this suggests that Mapakpk3 might inhibit transformation via phosphorylation and displacement of BMI1 upon loss of anchorage, resulting in the induction of $p19^{ARF}$. Future studies are required to address this possibility.

Whereas Mapkapk3 is relatively uncharacterized, Mapkapk2 is a well established target of the p38 pathway. A growth suppressive role for p38/Mapkapk2 was demonstrated by the observation that expression of p38 or of Mapkapk2 inhibited RAS^{V12}-induced S-phase entry in serum-deprived NIH3T3 cells. Strikingly, both Mapkapk2 and p38 could inhibit RAS-induced transcription in *in vitro* reporter assays (Chen et al., 2000). Additionally p38/Mapkapk2 was implicated in DNA-damage-induced cell cycle arrest in the absence of p53 (Reinhardt et al., 2007; Manke et al., 2005). We did not detect transcriptional induction of *Mapkapk2* upon loss of anchorage, in contrast to *Mapkapk3*, which was clearly induced. However, since Mapkapk proteins are activated by phosphorylation, the possibility remains that Mapkapk2 is activated upon loss of anchorage and performs a similar role as Mapkapk3.

In conclusion, we identified downregulation of Mapkapk3 or p38 as an additional event that could promote anchorage-independent growth of RAS^{V12}-expressing, pocket-protein-deficient MEFs. These results point to a tumor suppressor role for Mapkapk3 signaling upon loss of anchorage. Future experiments are required to determine the upstream and downstream mechanisms that mediate this growth-inhibitory role, and whether a similar role can be performed by the related Mapkapk2.

Materials and Methods

Cell culture

MEFs were cultured in GMEM (Invitrogen/Gibco), containing 10% fetal calf serum, 1 mM nonessential amino acids (Invitrogen/Gibco), 1 mM sodium pyruvate (Invitrogen/Gibco), 100 units/ml penicillin (Invitrogen/Gibco), 100 µg/ml streptomycin (Invitrogen/Gibco) and 0.1 mM β-mercaptoethanol and incubated at 37 °C in the presence of 5% CO_2.

Retroviral supernatants were produced by calcium phosphate transfection (Invitrogen) of phoenix cells with 16 µg of the desired construct and 4 µg pCL-Eco. Forty-eight h post transfection, retroviral supernatant was filtered using 0.45 µm filters (MCE membrane, Millipore) and either used directly or immediately frozen using a dry-ice ethanol bath and stored at -80 °C. After harvesting viral supernatant, phoenix cells were supplemented with GMEM containing media supplements as described above, and supernatant was again harvested using the same procedure, with an interval of at least 6 h. Subconfluent cell cultures were infected twice with retroviral supernatants, supplemented with polybrene to a concentration of 4 µg/ml during a time-span of at least 6 h per infection. For serial infections, MEFs were cultured in non-virus containing media for at least 36 h between infections and reseeded before infection to obtain optimal cell density.

Culturing without anchorage was performed in either soft agar or in methylcellulose-containing medium, as described in Vormer et al. (2008). In short, MEFs were suspended in a 37 °C, 0.35% soft agar solution (low gelling agarose type VII from Sigma) in GMEM containing 10% fetal calf serum, the same medium supplements as mentioned above plus gentamicin to a concentration of 0.02 mg/ml (Invitrogen/Gibco) and plated on top of a pre-casted 1% soft agar layer. For methylcellulose assays, MEFs were suspended in a 37 °C, 1.3% methylcellulose solution (diluted from 2.6% methylcellulose medium, Stem Cell Technologies, catalog no. H4100) in GMEM supplemented with fetal calf serum to a concentration of 10%, penicillin to a concentration of 100 units/ml (Invitrogen/Gibco), streptomycin to a concentration of 100 µg/ml (Invitrogen/Gibco) and gentamicin to a concentration of 0.02 mg/ml (Invitrogen/Gibco) and plated in ultra-low-attachment surface plates (catalog no. 3471; Corning incorporated). Pictures were taken using a non-phase-contrast lens (2.5x magnification) and assembled using 'Axiovision 4.5'. Cells were harvested by suspending 4 ml of methylcellulose culture with 40 ml ice-cold phosphate-buffered saline (PBS) (Invitrogen/Gibco), followed by centrifugation and aspiration of methylcellulose-PBS.

Constructs and PCR analysis

The ERM vector was kindly provided by Dr. Z. Songyang, pBABE-RAS^{V12} by Dr. T Brummelkamp and pCL-Eco by Dr. D. Peeper. The pEYK-TBX2 vector was previously isolated from MEFs infected with the pEYK-MCF7 library (Vormer et al., 2008), which was a gift of Dr. G.Q. Daley.

PCR analysis (Fig. 1B) which demonstrated the ERM-*Mapkapk3* fusion in a soft agar colony isolated from the insertional mutagenesis screen was performed with the following primers: ERM forward (fv): 5' GGC CAT GGA CAC CTA CCG CTA CAT CG 3'; Intron 2 fv: 5' CTC ATA AGC TGA CCC ACC CT 3'; Intron 2 reverse (rv): 5' CCT TGA CTC ACC TTC ATG ATC C 3'.

For analysis of the ERM-*Mapkapk3* fusion at the mRNA level (Fig. 1C), reverse transcription was performed using a primer annealing to the 3' end of *Mapkapk3*, downstream of the stopcodon (5' CCC CAA GTT CAA TGT GAC AC 3'). The RT fragment was subsequently amplified using the same primer and a primer annealing to the myristylation signal of ERM (5' ACC ATG GGG AGC AGC AAG AGC AAA CCA AAA GAC CCC AGC CAA CGC 3' (Liu et al., 2000)). The resulting RT-PCR product was cloned into pGEM-Teasy and sequenced using primers annealing to the T7 or Sp6 sequence present in pGEM-Teasy. Next, the ERM-*Mapkapk3* RT-PCR product was cloned from

pGEM-Teasy into pBABE-bleomycin using the EcoRI site, generating pBABE-ERM-*Mapkapk3*.

Full-length, wild-type *Mapkapk3* was reverse transcribed using the primer annealing to the 3' end of *Mapkapk3* (see above), amplified using this primer and a primer located upstream of the *Mapkapk3* start codon (5' GCT GTA CGT GCC TCT GGA C 3'), cloned into pGEM-Teasy and sunsequently into pBABE-bleomycin using the EcoRI site, generating pBABE-*Mapkapk3*.

Targeting sequences in pRetroSuper-Mapkapk3 vectors are: GCT CCT CAG CCT CAC AAG G (pRS-Mapkapk3-1) or GGA AAA AGC AGG CAG GCA GC (pRS-Mapkapk3-2).

Insertional mutagenesis screen

Rb$^{-/-}$*p107*$^{-/-}$*p130*$^{-/-}$ MEFs were retrovirally infected with pBABE-RAS^{V12} and ERM and subsequently cultured in 0.35% soft agar. Soft agar colonies were isolated using sterilized glass pipettes and propagated under adherent conditions, after which genomic DNA (gDNA) was isolated by lysis in 0.1 M TrisHCl pH 8.5, 5 mM EDTA, 0.2 M NaCl, 0.2 % SDS and 100 µg/ml Proteinase K (O/N at 55 ºC) and subsequent ethanol precipitation. gDNA was dissolved in Milli-Q water and purified using phenol/chloroform extraction. The position of the ERM integrations in the genomic DNA was determined using splinkerette PCR conform Mikkers et al. (2002), followed by sequencing.

Micro-array analysis

RNA was isolated using the RNeasy mini kit (Qiagen, catalog nr 74106). mRNA amplification using the Superscript RNA Amplification System (Invitrogen, catalog no. L1016-01), labeling and hybridization to MouseWG-6 v2.0 Expression BeadChip (Illumina) was performed as described on: http://microarray.nki.nl/download/protocols.html.

Acknowledgements

We thank Ron Kerkhoven and the members of the central microarray facility of the Netherlands Cancer Institute for microarray analysis and advice. This work was financially supported by the Dutch Cancer Society, grant nr NKI 2002-2634.

References

Aguirre-Ghiso,J.A., Estrada,Y., Liu,D., and Ossowski,L. (2003). ERK(MAPK) activity as a determinant of tumor growth and dormancy; regulation by p38(SAPK). Cancer Res. *63*, 1684-1695.

Bracken,A.P., Kleine-Kohlbrecher,D., Dietrich,N., Pasini,D., Gargiulo,G., Beekman,C., Theilgaard-Monch,K., Minucci,S., Porse,B.T., Marine,J.C., Hansen,K.H., and Helin,K. (2007). The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. Genes Dev. *21*, 525-530.

Bulavin,D.V., Phillips,C., Nannenga,B., Timofeev,O., Donehower,L.A., Anderson,C.W., Appella,E., and Fornace,A.J., Jr. (2004). Inactivation of the Wip1 phosphatase inhibits mammary tumorigenesis through p38 MAPK-mediated activation of the p16(Ink4a)-p19(Arf) pathway. Nat. Genet. *36*, 343-350.

Chen,G., Hitomi,M., Han,J., and Stacey,D.W. (2000). The p38 pathway provides negative feedback for Ras proliferative signaling. J. Biol. Chem. *275*, 38973-38980.

Chen,J., Zhong,Q., Wang,J., Cameron,R.S., Borke,J.L., Isales,C.M., and Bollag,R.J. (2001).

Microarray analysis of Tbx2-directed gene expression: a possible role in osteogenesis. Mol. Cell Endocrinol. *177*, 43-54.

Dannenberg,J.H., Schuijff,L., Dekker,M., van der Valk,M., and te Riele,H. (2004). Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev. *18*, 2952-2962.

Dannenberg,J.H., van Rossum,A., Schuijff,L., and te Riele,H. (2000). Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. Genes Dev. *14*, 3051-3064.

Dolado,I., Swat,A., Ajenjo,N., De,V.G., Cuadrado,A., and Nebreda,A.R. (2007). p38alpha MAP kinase as a sensor of reactive oxygen species in tumorigenesis. Cancer Cell *11*, 191-205.

Han,J. and Sun,P. (2007). The pathways to tumor suppression via route p38. Trends Biochem. Sci. *32*, 364-371.

Jacobs,J.J., Keblusek,P., Robanus-Maandag,E., Kristel,P., Lingbeek,M., Nederlof,P.M., van Welsem,T., van de Vijver,M., Koh,E.Y., Daley,G.Q., and van Lohuizen,M. (2000). Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19(ARF)) and is amplified in a subset of human breast cancers. Nat. Genet. *26*, 291-299.

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.

Li,S.P., Junttila,M.R., Han,J., Kahari,V.M., and Westermarck,J. (2003). p38 Mitogen-activated protein kinase pathway suppresses cell survival by inducing dephosphorylation of mitogen-activated protein/extracellular signal-regulated kinase kinase1,2. Cancer Res. *63*, 3473-3477.

Lingbeek,M.E., Jacobs,J.J., and van Lohuizen,M. (2002). The T-box repressors TBX2 and TBX3 specifically regulate the tumor suppressor gene p14ARF via a variant T-site in the initiator. J. Biol. Chem. *277*, 26120-26127.

Liu,D., Yang,X., Yang,D., and Songyang,Z. (2000). Genetic screens in mammalian cells by enhanced retroviral mutagens. Oncogene *19*, 5964-5972.

Loesch,M. and Chen,G. (2008). The p38 MAPK stress pathway as a tumor suppressor or more? Front Biosci. *13*, 3581-3593.

Ludwig,S., Engel,K., Hoffmeyer,A., Sithanandam,G., Neufeld,B., Palm,D., Gaestel,M., and Rapp,U.R. (1996). 3pK, a novel mitogen-activated protein (MAP) kinase-activated protein kinase, is targeted by three MAP kinase pathways. Mol. Cell Biol. *16*, 6687-6697.

Manke,I.A., Nguyen,A., Lim,D., Stewart,M.Q., Elia,A.E., and Yaffe,M.B. (2005). MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation. Mol. Cell *17*, 37-48.

Mikkers,H., Allen,J., Knipscheer,P., Romeijn,L., Hart,A., Vink,E., and Berns,A. (2002). High-throughput retroviral tagging to identify components of specific signaling pathways in cancer. Nat. Genet. *32*, 153-159.

Nicke,B., Bastien,J., Khanna,S.J., Warne,P.H., Cowling,V., Cook,S.J., Peters,G., Delpuech,O.,

Schulze,A., Berns,K., Mullenders,J., Beijersbergen,R.L., Bernards,R., Ganesan,T.S., Downward,J., and Hancock,D.C. (2005). Involvement of MINK, a Ste20 family kinase, in Ras oncogene-induced growth arrest in human ovarian surface epithelial cells. Mol. Cell *20*, 673-685.

Paxton,C., Zhao,H., Chin,Y., Langner,K., and Reecy,J. (2002). Murine Tbx2 contains domains that activate and repress gene transcription. Gene *283*, 117-124.

Peeper,D.S., Dannenberg,J.H., Douma,S., te Riele,H., and Bernards,R. (2001). Escape from premature senescence is not sufficient for oncogenic transformation by Ras. Nat. Cell Biol. *3*, 198-203.

Reinhardt,H.C., Aslanian,A.S., Lees,J.A., and Yaffe,M.B. (2007). p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage. Cancer Cell *11*, 175-189.

Roovers,K. and Assoian,R.K. (2000). Integrating the MAP kinase signal into the G1 phase cell cycle machinery. Bioessays *22*, 818-826.

Sebolt-Leopold,J.S. and Herrera,R. (2004). Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat. Rev. Cancer *4*, 937-947.

Voncken,J.W., Niessen,H., Neufeld,B., Rennefahrt,U., Dahlmans,V., Kubben,N., Holzer,B., Ludwig,S., and Rapp,U.R. (2005). MAPKAP kinase 3pK phosphorylates and regulates chromatin association of the polycomb group protein Bmi1. J. Biol. Chem. *280*, 5178-5187.

Vormer,T.L., Foijer,F., Wielders,C.L., and te Riele,H. (2008). Anchorage-independent growth of pocket protein-deficient murine fibroblasts requires bypass of G2 arrest and can be accomplished by expression of TBX2. Mol. Cell Biol. *28*, 7263-7273.

Wang,W., Chen,J.X., Liao,R., Deng,Q., Zhou,J.J., Huang,S., and Sun,P. (2002). Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced premature senescence. Mol. Cell Biol. *22*, 3389-3403.

Zakowski,V., Keramas,G., Kilian,K., Rapp,U.R., and Ludwig,S. (2004). Mitogen-activated 3p kinase is active in the nucleus. Exp. Cell Res. *299*, 101-109.

CHAPTER 4

The pRB-LxCxE interaction is critical for RAS^{V12}- and γ-irradiation induced cell cycle arrest

Tinke L. Vormer[1], Jacob B. Hansen[1], Marleen Dekker,
Sandra de Vries, Jan-Hermen Dannenberg and Hein te Riele

[1] These authors contributed equally to this work

Manuscript in preparation

Abstract

Members of the pocket protein family (pRB, p130 and p107) play a key role in G_1 by binding and inhibiting the activity of E2F transcription factors via blockage of E2F's transactivation domain and via the recruitment of chromatin remodeling proteins. The latter occurs via binding of the pocket region to an LxCxE motif in the target protein. In this study, we generated mouse embryonic fibroblasts (MEFs) carrying a mutation in pRB, pRB^{N750F}, which abrogated binding of pRB to LxCxE-containing proteins, while maintaining the ability to inhibit E2F-mediated transactivation. We show that $Rb^{N750F/N750F}$ and $Rb^{N750F/N750F}p130^{-/-}$ MEFs arrested efficiently in G_1 in response to growth factor deprivation or cell-cell contact. Strikingly, $Rb^{N750F/N750F}$ MEFs were impaired in arresting in response to γ-irradiation. Additionally, $Rb^{N750F/N750F}p130^{-/-}$ MEFs, but not $p130^{-/-}$ MEFs, were impaired in arresting in response to expression of RAS^{V12}. In conclusion, our results show that the pRB-LxCxE interaction is critical for G_1 arrest in response γ-irradiation or expression of RAS^{V12}, thereby possibly contributing to the tumor suppressor activity of pRB.

Introduction

A frequent event towards the development of cancer is loss of the retinoblastoma tumor suppressor gene, *RB1*, or one of its upstream regulators. pRB and its close homologs p130 and p107 form the family of pocket proteins and play a key role during cell cycle regulation. They collectively regulate the family of E2F transcription factors, whose activity is essential for progression of the cell cycle from G_1 into S phase. To date, eleven E2F transcription factors have been identified, which are generally classified as activator E2Fs (E2F1, E2F2 and E2F3a) and repressor E2Fs (E2F3b, E2F4, E2F5, E2F6a, E2F6b, E2F7a, E2F7b and E2F8). Whereas pRB can interact with E2F1-4 (Moberg et al., 1996), p130 and p107 interact with E2F4 and E2F5. E2F6-8 do not bind pocket proteins and thus function independently (reviewed by DeGregori and Johnson, 2006; Dimova and Dyson, 2005; Frolov and Dyson, 2004).

Pocket protein-E2F complexes fluctuate during the cell cycle. E2F4 is present at all cell cycle stages, though binding to promoters mainly occurs in G_0 and early G_1, and is correlated with repression of E2F target genes. At this stage, E2F4 primarily complexes with p130 and to a lower extent with pRB and p107. At the G_1/S transition, E2F4-p130 complexes are replaced by E2F4-p107 and E2F4-pRB complexes. Simultaneously, free, unbound E2Fs become visible at the G_1/S transition, coinciding with transcription of E2F target genes (Balciunaite et al., 2005; Rayman et al., 2002; Takahashi et al., 2000; Moberg et al., 1996; Cobrinik et al., 1993). The balance between pocket protein-bound and free E2Fs is regulated by the Cyclin-dependent kinases, which phosphorylate the pocket proteins, causing disruption of pocket protein-E2F binding. This enables activating E2Fs

to induce transcription and to drive cell cycle progression (reviewed by Macaluso et al., 2006; Frolov and Dyson, 2004).

Expression of E2F target genes, which is required for cell cycle progression, is inhibited by the formation of pocket protein-E2F complexes. Pocket protein-binding blocks E2F's transactivation domain, resulting in direct inhibition of E2F-mediated transcription. Additionally, pocket proteins can recruit chromatin-remodeling complexes to promoters of E2F target genes by their capacity to simultaneously bind to E2Fs and to proteins containing an LxCxE-like motif (x encoding any amino acid) (reviewed by Dick, 2007).

The LxCxE motif was originally identified as the motif present in viral oncoproteins that is used to bind and inactivate pRB. The LxCxE-binding site in pRB is highly conserved and a variety of proteins use an LxCxE motif to interact with pRB and the other pocket proteins (reviewed by Dick, 2007). Many LxCxE-containing proteins have been implicated in chromatin remodeling and transcriptional repression, indicating that pocket protein-E2F complexes can favor a chromatin state incompatible with transcription. Examples of chromatin remodeling proteins containing an LxCxE-like sequence are histone deacetylase 1 (HDAC1), HDAC2, heterochromatin protein 1 (HP1) and the CtBP-interacting protein (CtIP) (reviewed by Dick, 2007). HDAC1/2-pocket protein binding was shown to involve the LxCxE binding site (Chen and Wang, 2000; Dahiya et al., 2000; Ferreira et al., 1998; Magnaghi-Jaulin et al., 1998) and pocket protein-mediated repression of E2F-reporter constructs involved HDAC activity (Brehm et al., 1998; Ferreira et al., 1998; Magnaghi-Jaulin et al., 1998). Moreover, pocket proteins and HDACs could be detected at the promoter regions of E2F-regulated genes during cell cycle arrest (Morrison et al., 2002; Rayman et al., 2002; Dahiya et al., 2001), pointing to the involvement of the pocket protein-LxCxE interaction in HDAC-mediated transcriptional repression. In addition to HDACs, the Suv39h1 histone methyltransferase enhanced pocket protein-mediated repression of reporter constructs *in vitro*, however, no LxCxE-like sequence was detected in Suv39h1. Strikingly, pRB/p107-Suv39h1 complex formation and histone methyl transferase activity of pulled down GST-pRB could be inhibited by an LxCxE-containing competitor peptide (Nicolas et al., 2003; Nielsen et al., 2001; Vandel et al., 2001). Since a pRB-Suv39h1-HP1 complex could be formed *in vitro* (Nielsen et al., 2001), this suggests that pocket protein-Suv39h1 binding possibly involves the LxCxE-containing protein HP1. In conclusion, the pocket protein-LxCxE interaction has been implicated in both HDAC- and Suv39h1-mediated transcriptional repression.

Given the involvement of LxCxE-mediated interactions between pocket proteins and chromatin remodeling proteins in transcriptional repression, we wondered whether these interactions are crucial for pRB's tumor suppressor function. To this aim, we

generated MEFs carrying a mutation in the LxCxE binding site of pRB (*Rb*$^{N750F/N750F}$ MEFs). As the induction of G_1 arrest upon growth inhibitory signals is an important aspect of pRB's tumor suppressor function, we analyzed the response of *Rb*$^{N750F/N750F}$ MEFs to various growth inhibitory signals in tissue culture. Importantly, loss of pRB can be compensated for by expression of p130: while ablation of pRB had no or a minor effect on G_1 arrest induced by serum deprivation, prolonged culturing or RAS^{V12} expression, the additional loss of p130 bypassed cell cycle arrest and stimulated proliferation (Dannenberg et al., 2004; this communication). Therefore, we generated MEFs mutant for pRB and deficient for p130 (*Rb*$^{N750F/N750F}$*p130*$^{-/-}$ MEFs) and analyzed the response of these MEFs to various growth inhibitory signals in tissue culture. Our results show that the pRB-LxCxE interaction contributes to cell cycle arrest in response to expression of RAS^{V12} or γ-irradiation.

Results

Mutating the LxCxE binding site of pRB impairs transcriptional repression

The crystal structure of human pRB bound to a human papillomavirus-16 E7 LxCxE peptide revealed that LxCxE-mediated binding occurs via a hydrophobic groove in the B domain of the pRB pocket. Whereas the A domain is required for stable folding of the B domain, binding to the LxCxE peptide involves four conserved residues in the B domain: Asn 757, Tyr 756, Lys 713 and Tyr 709 (Lee et al., 1998). Mutating Asn 757 to phenylalanine in human pRB is predicted to cause steric hindrance and to disrupt hydrogen bonding with the LxCxE motif. Indeed, this mutation disrupted binding to LxCxE-containing proteins. Importantly, binding to E2F1, which does not contain an LxCxE sequence, remained intact (Chen and Wang, 2000; Dahiya et al., 2000). To investigate whether binding of LxCxE containing proteins to pRB is crucial for pRB's role in cell cycle regulation, we mutated Asn 750 in murine pRB, which corresponds to Asn 757 in human pRB, to phenylalanine. Co-immunoprecipitation experiments showed that the N750F substitution abrogated the interaction of pRB with the LxCxE containing protein SV40 large T antigen (TAg) (Fig. 1A). This confirms disruption of the LxCxE binding site in murine pRB^{N750F}, as was described for human pRB^{N757F} (Chen and Wang, 2000; Dahiya et al., 2000).

To test the ability of pRB^{N750F} to repress transcription, we introduced a reporter construct containing 6 E2F binding sites upstream of the luciferase gene into mouse *Rb*$^{-/-}$ 3T9 cells. Luciferase activity was induced by ectopic expression of E2F1 (Fig. 1B, lanes 1 and 2) and could be efficiently reversed by co-expression of either wild-type pRB or pRB^{N750F} (Fig. 1B, compare lanes 3 to 5 with lane 6 to 8). As a control, we used pRB^{C699F}, the murine variant of human pRB^{C706F}, which is considered a null mutant: the C706F mutation disrupted binding to E2Fs (Otterson et al., 1997) and LxCxE-containing

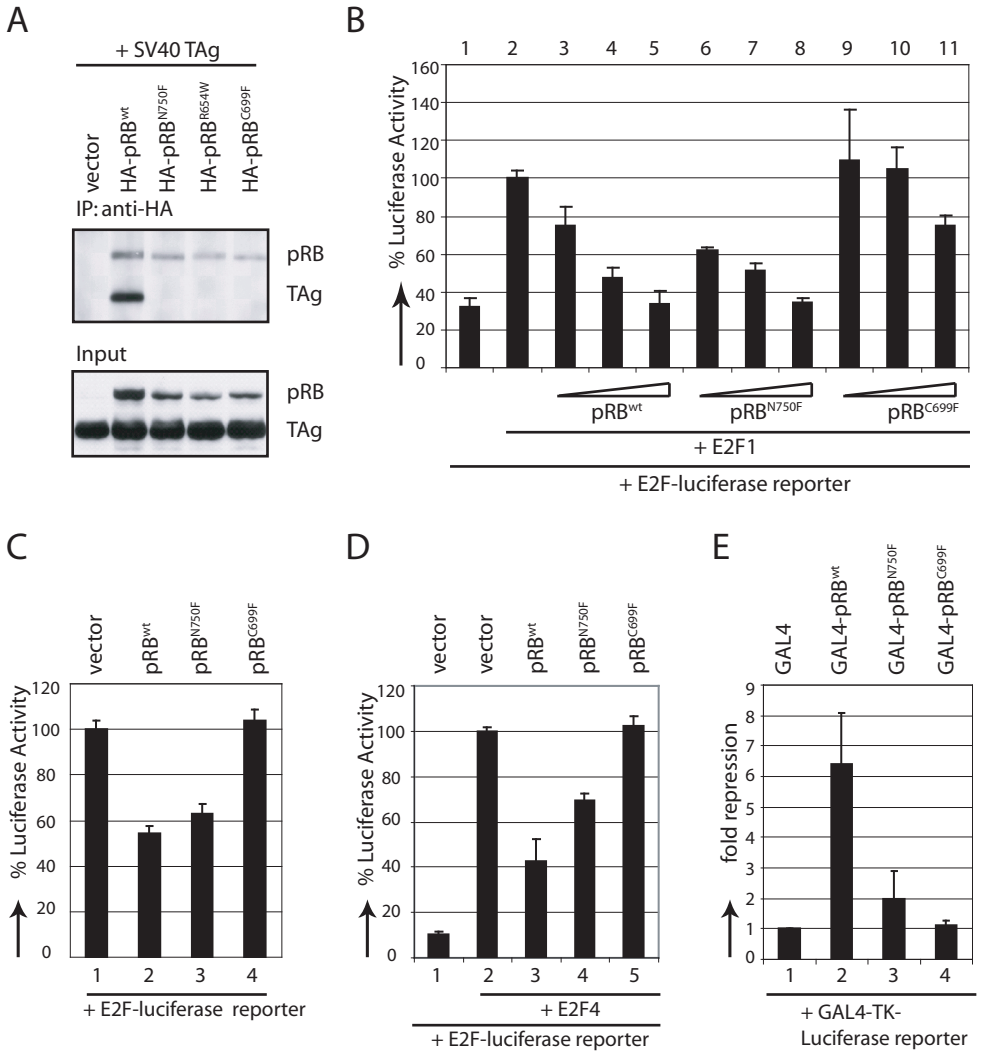


Figure 1: Characterization of pRB^{N750F}. (A) pRB^{N750F} does not interact with SV40 TAg. Rb$^{-/-}$ C33A cells were transfected with pSG5-TAg plus empty pcDNA3.1(-) (vector) or pcDNA3.1(-) encoding HA-tagged wild-type or mutant versions of murine pRB. Upper panel: protein extracts were immunoprecipitated using anti-HA antibody and immunoblotted using anti-TAg and anti-HA, the latter visualizing HA-pRB. Lower panel: total protein extracts (input) were immunoblotted for TAg and HA. (B-E) Transcriptional repression by wild-type and mutant pRB in various luciferase reporter assays. Rb$^{-/-}$ 3T9 cells were transfected with the indicated constructs, using the following amounts of plasmid per well of a 12-wells plate: 6xE2F-luciferase reporter or GAL4-TK-luciferase reporter: 200 ng; CMV-HA-E2F1: 0.8 ng; CMV-HA-E2F4: 20 ng; pcDNA3.1(-)-mRb wild-type or pcDNA3.1(-)-mRb mutant: 3.2, 8 and 20 ng in (B), 3.2 ng in (C) and 140 ng in (D); pM encoding GAL4-pRB wild-type or GAL4-pRB mutant: 20 ng; CMV-Renilla: 4 ng. Luciferase/Renilla luminescence is plotted in B-D and set to 100% in lane 2 (B and D) or lane 1 (C). Fold repression, defined as (Luciferase/Renilla luminescence)$_{GAL4\text{-}pRB\ wildtype\ or\ mutant}$ / (Luciferase/Renilla luminescence)$_{GAL4}$ is plotted in E.

proteins (Kratzke et al., 1992; Bignon et al., 1990; Kaye et al., 1990), caused impaired transcriptional repression in reporter assays (Sellers et al., 1995) and was unable to inhibit proliferation of SAOS2 cells (Otterson et al., 1997). Indeed, pRB^{C699F} was severely impaired in repressing E2F1-induced transcription (Fig. 1B, lanes 9 to 11). In the absence of ectopically-expressed E2F1, pRB^{N750F} repressed the luciferase reporter with similar efficiency as wild-type pRB, whereas pRB^{C699F} did not repress the reporter at all (Fig. 1C). These experiments demonstrate the ability of pRB^{N750F} to bind and inhibit E2F transcription factors, probably by masking E2F's transactivation domain.

In contrast to the results obtained with E2F1, pRB^{N750F} could only partly repress the E2F-luciferase reporter upon expression of E2F4 (Fig. 1D, compare lanes 2, 3 and 4). Recruitment of E2F4 to promoters generally correlates with transcriptional repression (Rayman et al., 2002; Takahashi et al., 2000), most likely via the formation of E2F4-pocket protein-chromatin remodeling complexes. Alternatively, E2F4 can also function as a transcriptional activator (Kinross et al., 2006; Muller et al., 1997) and indeed, we observed E2F4-mediated induction of the E2F-luciferase reporter (Fig. 1D, compare lanes 1 and 2). We propose that pRB^{N750F} can inhibit E2F4-mediated transactivation, but is impaired in the formation of E2F4-pRB-chromatin remodeling complexes, resulting in the observed partial inhibition of E2F4-mediated transcription.

To specifically analyze repressor activity of pRB, we fused pRB^{wt}, pRB^{N750F} and pRB^{C699F} to a GAL4-DNA binding motif, targeting pRB to a co-transfected GAL4-TK-luciferase reporter construct. Compared to pRB^{wt}, both pRB^{N750F} and pRB^{C699F} were severely impaired in repressing transcription of this reporter (Fig. 1E). All together, we conclude that pRB^{N750F} is able to interact with E2F transcription factors, as it can efficiently inhibit E2F1-induced transcription of the E2F-luciferase reporter. However, pRB^{N750F} is unable to establish complete transcriptional repression, as it is impaired in (1) repressing E2F4-induced transcription of the E2F-luciferase reporter and (2) repressing transcription when targeted to the GAL4-TK-luciferase reporter.

Generation of $Rb^{N750F/N750F}$ and $Rb^{N750F/N750F}p130^{-/-}$ MEFs

To test the impact of mutating the LxCxE binding site in pRB on cell cycle control, we generated MEFs homozygous for the Rb^{N750F} mutation. As the tumor suppressor function of pRB is believed to rely on establishing cell cycle arrest under growth inhibitory conditions, the generated MEFs were tested for their ability to arrest in response to various inhibitory signals in culture (see below). We and others have previously shown that loss of pRB alone did not bypass cell cycle arrest in MEFs, due to compensation by p130 and p107. Importantly, while ablation of pRB had no or a minor effect on G_1 arrest in response to various inhibitory signals, the additional loss of either p130 or p107 bypassed cell cycle arrest and stimulated proliferation (Dannenberg et al., 2004; Dannenberg et al., 2000). As

p130 is the main pocket protein recruited to repressed promoters during G_0 and G_1, we additionally generated the Rb^{N750F} mutation in a p130 null background.

To generate MEFs containing the Rb^{N750F} mutation, we made use of $Rb^{N750F/wt}$ mice that were generated by Aarts et al. (2006) using oligonucleotide-directed modification of the endogenous *Rb* gene in murine embryonic stem cells (Fig. S1A). These mice were crossed with $p130^{wt/-}$ mice and subsequent intercrossings enabled the isolation of $Rb^{N750F/N750F}p130^{-/-}$ and $Rb^{N750F/N750F}p130^{wt/wt}$ MEFs. Additionally, $Rb^{-/-}p130^{-/-}$, $Rb^{-/-}p130^{wt/wt}$, $Rb^{wt/wt}p130^{-/-}$ and $Rb^{wt/wt}p130^{wt/wt}$ MEFs were isolated from inter- and intra-crosses of $Rb^{wt/-}p130^{wt/wt}$ and $Rb^{wt/wt}p130^{wt/-}$ mice.

When compared to $Rb^{wt/wt}p130^{-/-}$ MEFs, $Rb^{N750F/N750F}p130^{-/-}$ MEFs expressed similar levels of pRB (Fig. S1B, compare lanes 1 and 3, 4 and 6, and 7 and 8). Strikingly, $Rb^{N750F/N750F}p130^{-/-}$ MEFs displayed elevated expression of Cyclin E and p107 during asynchronous proliferation (Fig. S1B, lanes 1 and 3), upon expression of RAS^{V12} (Fig. S1B , lanes 4 and 6) and after serum starvation (Fig. S1B , lanes 7 and 8). Additionally, increased expression of Cyclin A was observed in $Rb^{N750F/N750F}p130^{-/-}$ MEFs upon expression of RAS^{V12} (Fig. S1B, lanes 4 and 6). These observations demonstrate the involvement of the pRB-LxCxE interaction in repression of E2F target genes. In $Rb^{-/-}p130^{-/-}$ MEFs the levels of Cyclin E, Cyclin A and p107 were higher than in $Rb^{N750F/N750F}p130^{-/-}$ MEFs (Fig. S1B, compare lanes 2 and 3, 5 and 6). These results are consistent with the relieve of pRB-mediated repression of E2F-target genes in $Rb^{N750F/N750F}p130^{-/-}$ MEFs, while the regulation of E2F transactivation activity was still intact.

Response of $Rb^{N750F/N750F}$ and $Rb^{N750F/N750F}p130^{-/-}$ MEFs to growth inhibitory signals in tissue culture

We tested the ability of $Rb^{N750F/N750F}$ and $Rb^{N750F/N750F}p130^{-/-}$ MEFs to arrest in response to the following growth inhibitory signals: growth factor deprivation, cell-cell contact, expression of constitutively active RAS (RAS^{V12}), γ-irradiation, and anchorage deprivation. We have previously found that (partial) override of cell cycle arrest in response to serum deprivation, cell-cell contact or expression of RAS^{V12} could be achieved by loss of both pRB and p130, whereas loss of pRB only had a very minor effect (Foijer et al., 2005; Dannenberg et al., 2004; Dannenberg, unpublished). In contrast, G_1 arrest in response to γ-irradiation was predominantly dependent on functional pRB (Brugarolas et al., 1999; Dannenberg, unpublished). Concerning anchorage deprivation, we have found that $Rb^{-/-}p130^{-/-}$ MEFs arrested efficiently in the absence of anchorage, but this could be overcome by the combined expression of RAS^{V12} and TBX2 (Vormer et al., 2008).

Growth factor deprivation

Serum deprivation of wild-type MEFs causes an arrest in both the G_1 and G_2 phases of the cell cycle. Consistent with their role during G_1, loss of all three pocket proteins bypassed G_1 arrest and shifted the arrest towards G_2 (Foijer et al., 2005). Table 1 shows the response of $Rb^{N750F/N750F}$, $Rb^{N750F/N750F}p130^{-/-}$ and wild-type MEFs to serum deprivation. The strength of the arrest is depicted by the $(G_1+G_2)/S$ ratios in serum starved versus serum stimulated cells. Consistent with previous results (Foijer et al., 2005; Dannenberg, unpublished), the single loss of either pRB or p130 did not affect cell cycle arrest upon serum deprivation, whereas the combined loss of pRB and p130 resulted in an impaired arrest (compare ratios of wild-type, $Rb^{-/-}$, $p130^{-/-}$ and $Rb^{-/-}p130^{-/-}$ MEFs). In contrast to $Rb^{-/-}p130^{-/-}$ MEFs, $Rb^{N750F/N750F}p130^{-/-}$ MEFs arrested as efficiently as wild-type and $p130^{-/-}$ MEFs. Additionally, $Rb^{N750F/N750F}p130^{-/-}$ MEFs arrested efficiently in G_1, whereas arrest in $Rb^{-/-}p130^{-/-}$ MEFs had shifted slightly towards G_2 (Fig. S2). These results confirm that bypass of G_1 arrest upon serum deprivation requires loss of at least two pocket proteins, and furthermore, demonstrate that the interaction of pRB with LxCxE-containing proteins is not required for G_1 arrest upon serum deprivation.

Cell line	Condition	(G1 + G2)/S	Fold increase in (G1+G2)/S upon serum starvation
wild-type	+ serum	2,4	11,6
	7 days serum starved	27,6	
$Rb^{-/-}$	+ serum	1,6	9,6
	7 days serum starved	15,5	
$p130^{-/-}$	+ serum	1,8	10,0
	7 days serum starved	18,2	
$Rb^{-/-}p130^{-/-}$	+ serum	1,6	6,3
	7 days serum starved	10,2	
$Rb^{N750F/N750F}$	+ serum	1,7	11,8
	7 days serum starved	20,0	
$Rb^{N750F/N750F}p130^{-/-}$	+ serum	1,7	10,9
	7 days serum starved	18,7	

Table 1: $Rb^{N750F/N750F}p130^{-/-}$ MEFs arrest efficiently in response to growth factor deprivation, which is in contrast to $Rb^{-/-}p130^{-/-}$ MEFs and demonstrates that the pRB-LxCxE interaction is not required for G_1 arrest under these conditions. MEFs were cultured in the presence or absence of serum, stained for BrdU and propidium iodide incorporation and analyzed by FACS. G_1- and G_2-phase populations were defined as the populations of cells with a 2N or 4N DNA content as apparent in the propidium iodide profile. The S-phase population was defined as the BrdU-positive population of cells with a DNA content ranging from 2N to 4N.

It still remained possible that, in response to serum deprivation, pRB^{N750F} induced a less stringent arrest than pRB^{wt}. To test this possibility, we analyzed the rate of cell cycle re-entry of serum-starved *Rb$^{N750F/N750F}$p130$^{-/-}$* MEFs upon serum re-addition. As shown in Fig. 2, *Rb$^{N750F/N750F}$p130$^{-/-}$* MEFs re-entered the cell cycle with similar kinetics as *p130$^{-/-}$* MEFs. In contrast, release from G_1 arrest occurred slightly earlier in *Rb$^{-/-}$p130$^{-/-}$* MEFs compared to *Rb$^{N750F/N750F}$p130$^{-/-}$* or *p130$^{-/-}$* MEFs (Fig. 2, compare lane 4 of the upper, middle and lower panels: a reduction in G_1 phase and increase in S-phase is observed in *Rb$^{-/-}$p130$^{-/-}$*, but not in *Rb$^{N750F/N750F}$p130$^{-/-}$* or *p130$^{-/-}$* MEFs). We conclude that ablation of the pRB-LxCxE interaction did not affect G_1 arrest in response to serum deprivation.

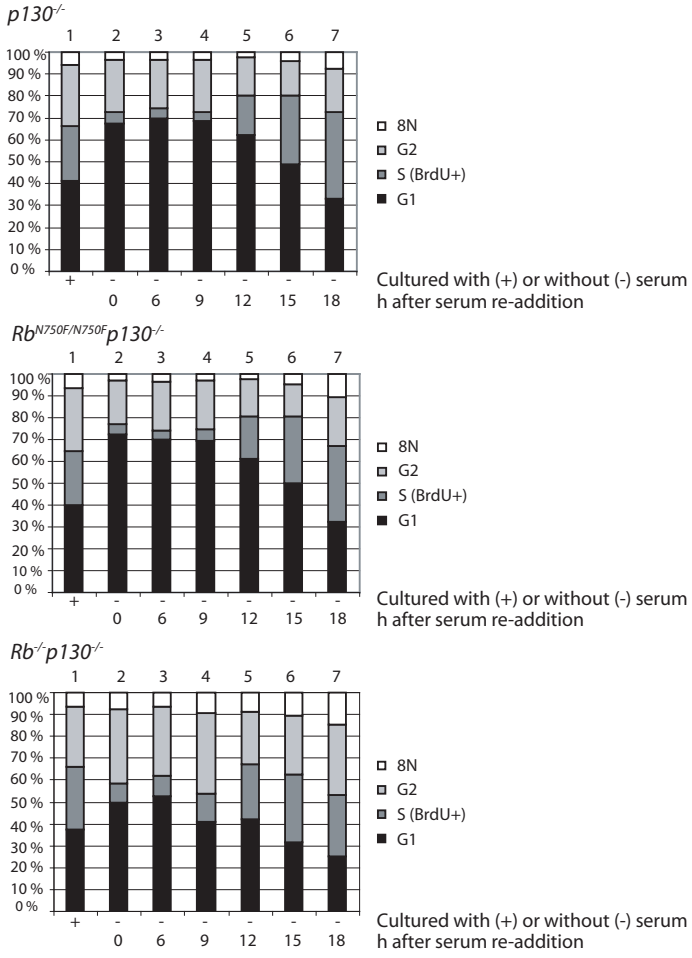


Figure 2: Similar kinetics of cell cycle re-entry after growth factor deprivation for Rb$^{N750F/N750F}$p130$^{-/-}$ and p130$^{-/-}$ MEFs. p130$^{-/-}$, Rb$^{N750F/N750F}$p130$^{-/-}$ and Rb$^{-/-}$p130$^{-/-}$ MEFs were either cultured in 10% serum or in 0% serum for 7 days, after which they were provided with 10% serum for the indicated time points. BrdU was added 1 h before harvesting. Harvested cells were stained for BrdU and propidium iodide incorporation and analyzed by FACS. G_1-, G_2- and S-phase populations were defined conform the legend of Table 1.

Cell-cell contact

Similar to serum deprivation, cell-cell contact induced arrest of wild-type MEFs in both G_1 and G_2 phase. Ablation of all three pocket proteins resulted in complete override of cell cycle arrest (Table 2 and Dannenberg et al., 2000). Analyzing the $(G_1+G_2)/S$ ratio in contact inhibited versus subconfluent cultures demonstrated a small but reproducible reduction of cell cycle arrest in $Rb^{-/-}p130^{-/-}$, but not in $Rb^{N750F/N750F}p130^{-/-}$ MEFs (Table 2). This indicates that in a *p130* null background, loss of pRB, but not abrogation of the pRB-LxCxE interaction, partially bypassed cell cycle arrest (Table 2).

Cell line	Condition	(G1 + G2)/S	Fold increase in (G1+G2)/S upon contact inhibition
wild-type	subconfluent contact inhibited	6,2 34,1	5,5
$Rb^{-/-}$	subconfluent contact inhibited	6,8 27,0	4,0
$p130^{-/-}$	subconfluent contact inhibited	6,4 26,1	4,1
$Rb^{-/-}p130^{-/-}$	subconfluent contact inhibited	8,2 23,1	2,8
$Rb^{N750F/N750F}$	subconfluent contact inhibited	4,7 35,6	7,6
$Rb^{N750F/N750F}p130^{-/-}$	subconfluent contact inhibited	5,3 33,0	6,3
$Rb^{-/-}p130^{-/-}p107^{-/-}$	subconfluent contact inhibited	6,2 7,8	1,2

Table 2: $Rb^{N750F/N750F}p130^{-/-}$ MEFs arrest efficiently in response to cell-cell contact, demonstrating that the pRB-LxCxE interaction is not required for G_1 arrest under these conditions. Subconfluent or contact-inhibited MEFs were stained with propidium iodide and analyzed by FACS to determine G_1-, G_2- and S-phase populations.

Ionizing radiation

Also γ-irradiation-induced cell cycle arrest occurs in G_1 and G_2. However, G_1 arrest in response to γ-irradiation was primarily dependent on functional pRB (Brugarolas et al., 1999; Dannenberg, unpublished). Indeed, we found that $Rb^{-/-}$ MEFs were severely impaired in arresting the cell cycle in response to γ-irradiation: in response to irradiation with 5.5 Gy, wild-type and $p130^{-/-}$ MEFs displayed a 45-50% reduction in S phase cells, whereas $Rb^{-/-}$ MEFs displayed a reduction of only 25% (Fig. 3A, lanes 1-6). Similarly, the fold increase in $(G_1+G_2)/S$ ratio of irradiated versus untreated cells revealed an impaired arrest in $Rb^{-/-}$ MEFs (Fig. 3B, compare fold increase of wild-type and $Rb^{-/-}$ MEFs, lanes 1 and 2).

In addition to $Rb^{-/-}$ MEFs, we found that also $Rb^{N750F/N750F}$ MEFs were impaired in arresting in response to irradiation: $Rb^{N750F/N750F}$ MEFs treated with 5.5 Gy of γ-irradiation

displayed a reduction in S phase cells of only 35% (Fig. 3A, lanes 9 and 10) and the fold increase in (G_1+G_2)/S ratio upon irradiation was very similar for *Rb*$^{N750F/N750F}$ and *Rb*$^{-/-}$ MEFs (Fig. 3B, compare lanes 2 and 5). Thus, *Rb*$^{N750F/N750F}$ MEFs were almost as impaired as *Rb*$^{-/-}$ MEFs in irradiation-induced cell cycle arrest. These results are consistent with recent findings of Talluri and co-workers (2010), who showed that MEFs with an impaired pRB-LxCxE interaction arrested less stringently in G_1 upon γ-irradiation. Furthermore, we found that p130 did not affect the cellular response to irradiation, as *Rb*$^{N750F/N750F}$*p130*$^{-/-}$ MEFs displayed a similar fold increase in (G_1+G_2)/S ratio as *Rb*$^{N750F/N750F}$ MEFs upon irradiation (Fig. 3B, compare lanes 5 and 6).

Treatment with higher doses of irradiation demonstrated that *Rb*$^{N750F/N750F}$ MEFs displayed an intermediate phenotype: *Rb*$^{N750F/N750F}$ MEFs arrested less efficiently than wild-type MEFs, but more efficiently than *Rb*$^{-/-}$ MEFs upon irradiation with 10 or 15 Gy (Fig. 3C, lanes 4 to 6 and 7 to 9). Interestingly, irradiation with 10 or 15 Gy induced a shift from G_1 towards G_2 arrest in both *Rb*$^{N750F/N750F}$ MEFs and *Rb*$^{-/-}$ MEFs (Fig. S3). These results demonstrate that both *Rb*$^{N750F/N750F}$ and *Rb*$^{-/-}$ MEFs were impaired in arresting in G_1 upon irradiation, resulting in an increase in G_2 arrest.

To study the stringency of the residual G_1 arrest in *Rb*$^{N750F/N750F}$ MEFs in response to γ-irradiation, we analyzed S phase entry of synchronized, γ-irradiated MEFs: serum starved *Rb*$^{N750F/N750F}$, *Rb*$^{-/-}$ and *Rb*$^{wt/wt}$ MEFs were re-stimulated with serum and subsequently irradiated 7 h later. As shown in Fig. 3D, both *Rb*$^{N750F/N750F}$ and *Rb*$^{-/-}$ MEFs entered S phase slightly earlier compared to *Rb*$^{wt/wt}$ MEFs, with *Rb*$^{-/-}$ MEFs again displaying a more severe phenotype than *Rb*$^{N750F/N750F}$ MEFs. In conclusion, our results point to a small but reproducible involvement of the pRB-LxCxE interaction in inducing G_1 arrest in response to γ-irradiation.

Expression of RAS^{V12}

We have previously shown that loss of pRB only was not sufficient for bypass of RAS^{V12}-induced senescence in MEFs. However, senescence bypass could be accomplished by loss of pRB and p130, pRB and p107, or all three pocket proteins (Dannenberg et al., 2004; Peeper et al., 2001). To test whether the pRB-LxCxE interaction is involved in G_1 arrest in response to RAS^{V12}, we analyzed proliferation rates of *Rb*$^{N750F/N750F}$*p130*$^{-/-}$, *Rb*$^{-/-}$*p130*$^{-/-}$ and *p130*$^{-/-}$ MEFs upon expression of RAS^{V12} (Fig. 4). Strikingly, we found that similar to *Rb*$^{-/-}$*p130*$^{-/-}$ MEFs, *Rb*$^{N750F/N750F}$*p130*$^{-/-}$ MEFs maintained a high proliferation rate in the presence of RAS^{V12}, whereas proliferation of *p130*$^{-/-}$ MEFs almost ceased. These results show that the pRB-LxCxE interaction is critical for the establishment of cell cycle arrest in response to RAS^{V12}.

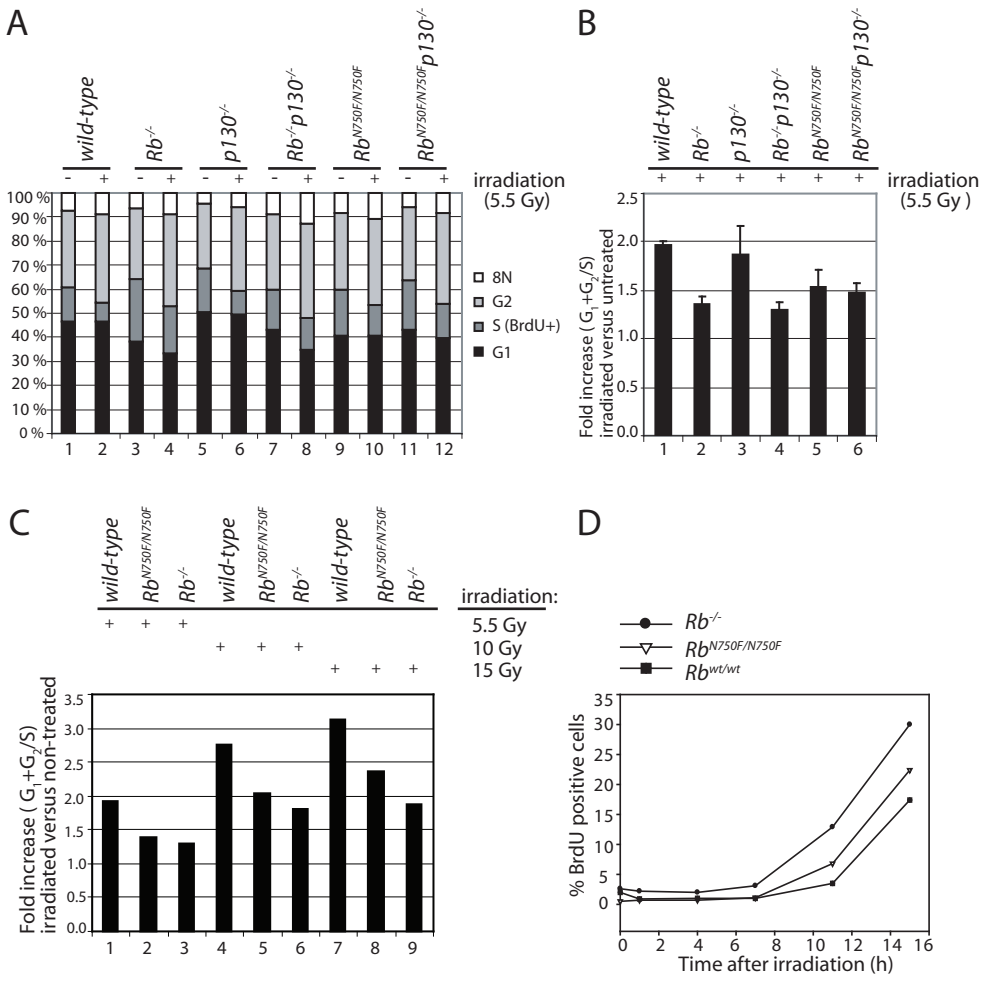


Figure 3: The pRB-LxCxE interaction is involved in cell cycle arrest after γ-irradiation. (A) MEFs of the indicated genotypes were either untreated or irradiated with 5.5 Gy and harvested 16 h post irradiation. BrdU was added 1 h before harvesting. Harvested cells were stained for BrdU and propidium iodide incorporation and analyzed by FACS. (B) Fold increase in (G_1+G_2)/S in 5.5 Gy-irradiated versus untreated MEFs. Depicted is the average fold increase, calculated from 2 independent experiments. One of these experiments is shown in (A). (C) Fold increase in (G_1+G_2)/S in response to the indicated doses of irradiation. (D) Cell cycle re-entry of synchronized, irradiated MEFs. Cells were serum-starved for 7 days and subsequently stimulated with 10% serum. Seven h after serum stimulation, cells were irradiated with 15 Gy and fixed at the indicated time after irradiation. BrdU was again added 1 h before harvesting and cells were analyzed by FACS. G_1-, G_2- and S-phase populations were defined conform the legend of Table 1.

RAS^{V12}-induced transformation

Cell cycle arrest upon loss of anchorage is a characteristic of non-transformed cells. We have previously shown that *Rb$^{-/-}$p130$^{-/-}$* MEFs could be induced to proliferate under non-adherent conditions by expression of RAS^{V12} and TBX2 and that this anchorage-independent growth was strictly dependent on the absence of both pRB and p130 (Vormer et al., 2008). We therefore tested whether also *Rb$^{N750F/N750F}$p130$^{-/-}$* MEFs could proliferate under these conditions. Surprisingly, we found that in contrast to *Rb$^{-/-}$p130$^{-/-}$*, *Rb$^{N750F/N750F}$p130$^{-/-}$* MEFs could not grow anchorage-independently upon expression of RAS^{V12} and TBX2 (Fig. 5). These results demonstrate that ablation of the pRB-LxCxE interaction did not render cells susceptible to transformation. Moreover, our results show that bypass of RAS^{V12}-induced senescence was not sufficient to support TBX2-induced transformation.

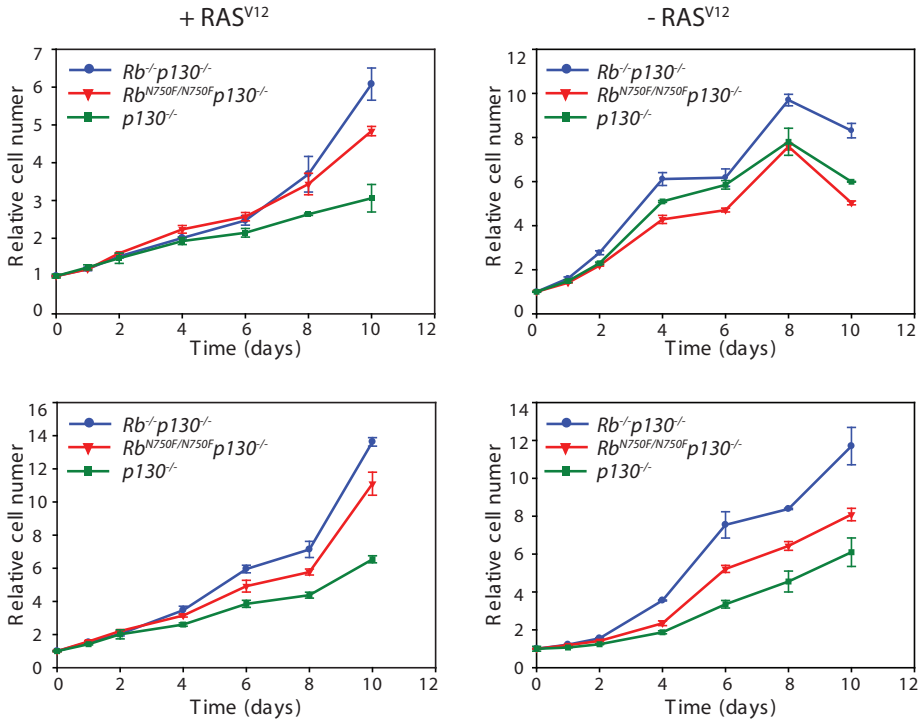


Figure 4: The pRB-LxCxE interaction is involved in cell cycle arrest in response to expression of RAS^{V12}. p130$^{-/-}$, Rb$^{N750F/N750F}$p130$^{-/-}$ and Rb$^{-/-}$p130$^{-/-}$ MEFs were infected with pBABE-RAS^{V12}-puro (+ RAS^{V12}) or pBABE-puro (- RAS^{V12}) and plated for proliferation assays 4 days after infection. Cells were fixed and stained with crystal violet at the indicated time points, the relative cell number was determined by dividing OD$_{590nm}$ at time point x by OD$_{590}$ at time point 0. Two independent experiments, performed in triplicate, are shown.

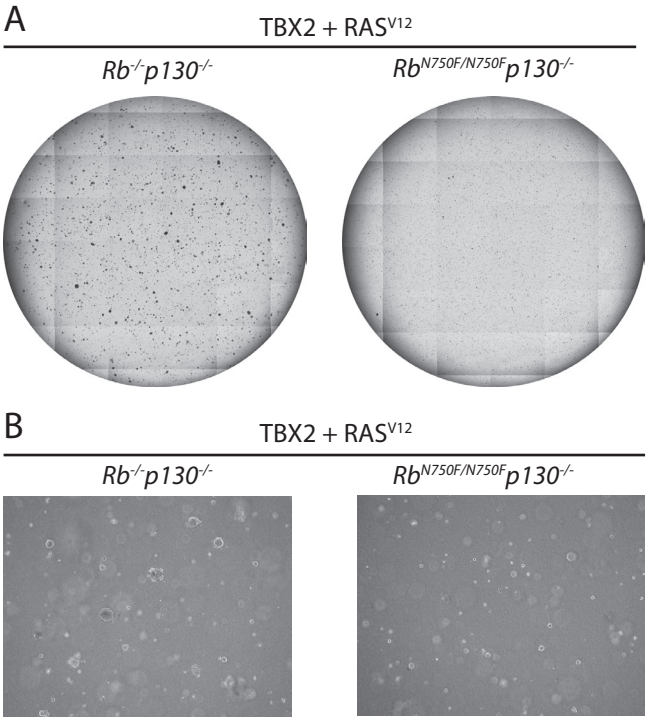


Figure 5: Ablation of the pRB-LxCxE interaction in a p130 null background is not sufficient to induce anchorage-independent growth upon expression of TBX2 and RAS^{V12}. $Rb^{N750F/N750F}p130^{-/-}$ and $Rb^{-/-}p130^{-/-}$ MEFs were infected with pEYK-TBX2 and subsequently with pBABE-RAS^{V12}-puro and plated in soft agar. Pictures were taken after 2 weeks using a non-phase-contrast lens (x2.5 magnification) in (A) and a phase-contrast lens (x5 magnification) in (B). RAS^{V12} and TBX2 were expressed to comparable levels in the 2 cell lines (not shown).

Discussion

The pRB tumor suppressor protein is primarily known for binding and inhibiting E2F transcription factors. Besides E2Fs, pRB can simultaneously interact with a wide variety of proteins via a site that recognizes an LxCxE motif in the target protein. Many of these interacting proteins are involved in chromatin remodeling and transcriptional repression (examples are HDAC1 and -2, Suv39h1 and HP1). Thus, tri-molecular complexes containing pocket proteins, E2Fs and LxCxE-containing proteins can promote a chromatin state incompatible with transcription, resulting in active repression of E2F target genes (reviewed by Dick, 2007). Whereas chromatin remodeling complexes containing p130 and p107 are thought to function in G_0/G_1, pRB-containing chromatin remodeling complexes have been implicated in irreversible gene repression during terminal cell cycle exit (Balciunaite et al., 2005; Frolov and Dyson, 2004; Narita et al., 2003; Rayman et al., 2002; Dahiya et al., 2001). *E.g.*, association of pRB and HP1 with stably repressed E2F-

regulated promoters could specifically be detected in RAS^{V12}-induced senescent cells, but not in reversibly arrested, quiescent cells (Narita et al., 2003). However, pRB and HDAC1 were recruited to the *Cyclin E* promotor in cells arrested by $p16^{INK4A}$ (Dahiya et al., 2001) and in G_0 arrested MEFs (Morrison et al., 2002). Furthermore, the pRB-LxCxE interaction was required for the establishment of irreversible cell cycle arrest during myogenic differentiation (Chen and Wang, 2000). These results led to the view that the interaction of pRB with chromatin remodeling proteins mainly plays a role during terminal cell cycle exit.

Given the proposed role of the pRB-LxCxE interaction in gene repression and cell cycle exit, we asked whether this interaction is essential for pRB's role in cell cycle arrest under growth inhibitory conditions. To this aim, we generated a mutation in the LxCxE binding site of murine pRB at position 750: pRB^{N750F}. This mutant protein retained the ability to efficiently inhibit an E2F1-induced E2F-reporter, but was unable to bind the LxCxE-containing protein SV40 large T antigen. Moreover, pRB^{N750F} was impaired in repressing an E2F4-induced E2F-reporter and in repressing a GAL4-TK-luciferase reporter when fused to the GAL4 DNA binding domain (Fig. 1). These results are consistent with the view that LxCxE-containing proteins are involved in pRB-dependent transcriptional repression. Concerning repression of E2F1-induced transcription and repression of a GAL4-reporter construct, results similar to ours were obtained with human pRB-LxCxE mutants: pRB^{N757F}, the human equivalent of murine pRB^{N750F}, and other LxCxE mutants could efficiently repress an E2F1-induced E2F-reporter, but were impaired in repressing a GAL4-reporter when targeted via fusion to the GAL4 DNA binding domain (Chan et al., 2001; Dahiya et al., 2000). In line with the involvement of the pRB-LxCxE interaction in gene repression and chromatin remodeling, Isaac et al. (2006) observed aberrant methylation patterns in the pericentric heterochromatin of MEFs expressing a pRB protein deficient in binding LxCxE-containing proteins. This further underlines the proposed role for the pRB-LxCxE interaction in permanent gene silencing.

To study the involvement of the pRB-LxCxE interaction in cell cycle arrest, we generated MEFs in which both *Rb* alleles encode the mutant pRB^{N750F} protein ($Rb^{N750F/N750F}$ MEFs) and analyzed the ability of these MEFs to bypass cell cycle arrest in response to growth inhibitory signals. We generated this mutation both in a wild-type and a $p130^{-/-}$ background, since bypass of G_1 arrest in response to various inhibitory signals in culture required loss of pRB plus loss of either p130 or p107 (Dannenberg et al., 2004; this communication). We did not find a contribution of the pRB-LxCxE interaction in the induction of cell cycle arrest in response to serum deprivation, contact inhibition or loss of anchorage (Tables 1 and 2; Fig. 2 and 5). However, the pRB-LxCxE interaction was critical for G_1 arrest in response to γ-irradiation and expression of RAS^{V12} (Fig. 3 and 4).

Growth factor deprivation

The normal cell cycle arrest of $Rb^{N750F/N750F}p130^{-/-}$ MEFs that we observed upon growth factor deprivation was somewhat surprising, since active repression of E2F target genes was shown to occur during serum starvation (Isaac et al., 2006; Morrison et al., 2002). Morrison and co-workers (2002) detected recruitment of pRB and HDAC1 to the *Cyclin E* promoter upon serum starvation and showed that repression of the *Cyclin E* gene under these conditions depended on pRB, could be relieved by the HDAC inhibitor trichostatin A, and involved pRB dependent de-acetylation of a part of the *Cyclin E* promotor. Moreover, Isaac and co-workers (2006) detected increased expression of *Cyclin E* and other E2F regulated genes during serum starvation in MEFs carrying a mutation in the LxCxE binding site of pRB. This suggests that, during serum starvation, active repression of E2F target genes might contribute to cell cycle arrest. However, we found that $Rb^{N750F/N750F}p130^{-/-}$ MEFs arrested normally in response to serum starvation, whereas $Rb^{-/-}p130^{-/-}$ MEFs displayed an impaired arrest (Table 1). Note that both $Rb^{N750F/N750F}$ and $Rb^{-/-}$ MEFs arrested efficiently in G_1 upon serum deprivation, which is in line with our previous findings that pRB loss was not sufficient to bypass G_1 arrest upon serum deprivation (Table 1, Figure S1, and Foijer et al., 2005). Furthermore, we found that serum-starved $Rb^{N750F/N750F}p130^{-/-}$ MEFs re-entered the cell cycle upon serum re-stimulation with similar kinetics as $Rb^{wt/wt}p130^{-/-}$ MEFs (Fig. 2), demonstrating that cell cycle arrest induced by the pRB^{N750F} protein, which is still able to inhibit E2F transcription factors, was as stable as arrest induced by pRB^{wt}. Apparently, relieve of repression by mutation of the LxCxE binding site in pRB plus loss of p130 was not sufficient to bypass cell cycle arrest upon serum starvation. Therefore, although serum deprivation may induce pRB-dependent chromatin remodeling, our results imply that bypass of cell cycle arrest after serum withdrawal requires loss of pocket protein-mediated inhibition of the transactivation activity of E2Fs.

Ionizing radiation

In response to γ-irradiation, cell cycle arrest enables repair of DNA damage, after which cell cycle progression can be resumed. Because of the transient nature of this arrest we intuitively expected pRB-chromatin remodeling complexes, and thus pRB-LxCxE interactions, not to play a major role. We found that cell cycle arrest after γ-irradiation critically depends on pRB and, strikingly, involved its interaction domain with LxCxE-containing proteins. Thus, $Rb^{N750F/N750F}$ MEFs were impaired in the establishment of cell cycle arrest after γ-irradiation (Fig. 3) and additionally, in response to higher doses of irradiation, residual cell cycle arrest in $Rb^{N750F/N750F}$ MEFs had shifted to G_2 (Fig. S3). These results clearly point to an involvement of pRB-LxCxE interactions in DNA-damage-induced G_1 arrest. In agreement with our data, Pennaneach and co-workers

(2001) reported that ectopically expressed human pRB^{N757F} protein could not induce cell cycle arrest in UV-irradiated *Rb*$^{-/-}$ C33a cells. Moreover, Talluri et al. (2010) reported recently that MEFs expressing a pRB protein with a mutated LxCxE-binding site arrested less stringently in G_1 in response to γ-irradiation.

Previous work has shown that cell cycle arrest upon γ-irradiation coincided with reduced CDK2 kinase activity (Brugarolas et al., 1999). This may point to pRB-HDAC-mediated repression of the CDK2 regulator *Cyclin E*. Besides HDACs, several other LxCxE containing proteins are candidates for mediating pRB-induced cell cycle arrest after γ-irradiation. First, Pennaneach and co-workers (2001) reported that the LxCxE containing protein RF-Cp145 enhanced survival after DNA damage in the presence of pRB. RF-Cp145 is a component of the replication factor C (RF-C) complex that is involved in DNA replication. Strikingly, RF-C can also function in transcriptional repression: RF-C enhanced pRB-mediated repression of a p73-luciferase reporter (Pennaneach et al., 2004). Therefore, a possible role for RF-Cp145 in inducing cell cycle arrest could involve direct inhibition of DNA replication and/or transcriptional repression. Second, members of the p200 family, which contain an LxCxE motive and bind to the pocket proteins, have been reported to inhibit S phase entry in a pRB proficient background (Hertel et al., 2000). As the p205 protein, a member of the p200 family, contains a putative ATM phosphorylation site (Dermott et al., 2004), it is possible that p200 proteins are activated by ATM/ATR signaling after DNA damage. These proteins can then interact with pRB via the LxCxE binding site and inhibit S phase entry, for example by stabilization of the hypo-phosphorylated form of pRB. Of interest, a differential sensitivity to phosphorylation of wild-type and mutant pRB was found during muscle differentiation: whereas wild-type pRB was stabilized in the hypo-phosphorylated form and could no longer be phosphorylated by serum stimulation, the pRB^{N757F} protein remained sensitive to serum-induced phosphorylation (Chen and Wang, 2000). Possibly, a similar mechanism occurs after irradiation, involving the stabilization of pRB^{wt} in the active, hypo-phosphorylated state, while the pRB^{N750F} protein is inactivated by Cdk-dependent phosphorylation, causing aberrant S phase entry. Finally, an interesting observation was made by Binne and colleagues (2007), who showed that pRB could bind to APC-cdh1 in an LxCxE-dependent manner and suggested that the pRB-APC interaction in G_1 downregulates Skp2 and stabilizes p27^{KIP1}. Thus, reduced p27^{KIP1} levels in *Rb*$^{N750F/N750F}$ MEFs could contribute to bypass of G_1 arrest in response to irradiation.

RAS^{V12}-induced senescence

Previously, we found that bypass of RAS^{V12}-induced senescence could be accomplished by combined ablation of pRB and p130 (Dannenberg et al., 2004). Strikingly, we have now found that not only *Rb*$^{-/-}$*p130*$^{-/-}$, but also *Rb*$^{N750F/N750F}$*p130*$^{-/-}$ MEFs were refractory

to the growth inhibitory effect of RAS^{V12} (Fig. 4). Thus, the interaction between pRB and LxCxE-containing proteins is critical for the establishment of cell cycle arrest in response to RAS^{V12}. This is in agreement with the view that cell cycle arrest induced by RAS^{V12} is irreversible and involves transcriptional silencing via the recruitment of chromatin remodeling proteins. A role for E2F-repressor complexes during RAS^{V12}-induced senescence was previously suggested by Rowland et al. (2002), who showed that expression of the DNA binding domain of E2F1 (E2F-DB) could bypass RAS^{V12}-induced senescence. Since E2F-DB was unable to activate or repress transcription and could displace endogenous E2Fs from promoter regions, these results implied that relieve of E2F-mediated transcriptional repression can overcome RAS^{V12}-induced senescence. Additionally, Narita and colleagues (2003) detected recruitment of HP1γ and the presence of methylated H3K9 to promoter regions specifically in RAS^{V12}-induced senescent cells, and not in confluent quiescent cells, pointing to the involvement of chromatin remodeling proteins during RAS^{V12}-induced senescence. Our present results using $Rb^{N750F/N750F}p130^{-/-}$ MEFs clearly point to an essential role for the pRB-LxCxE interaction during RAS^{V12}-induced senescence and suggest that E2F-pRB-chromatin remodeling complexes are critical for the establishment of RAS^{V12}-induced cell cycle arrest.

RAS^{V12}-induced transformation

Loss of anchorage generally induces cell cycle arrest and bypass of this arrest is a hallmark of transformation. Our previous studies showed that expression of RAS^{V12} and TBX2 bypassed cell cycle arrest in $Rb^{-/-}p130^{-/-}$ MEFs under non-adherent conditions and induced tumor formation in nude mice (Vormer et al., 2008). We wondered whether $Rb^{N750F/N750F}p130^{-/-}$ MEFs could also be induced to proliferate anchorage-independently by expression of RAS^{V12} and TBX2. To our surprise, we found that, in contrast to $Rb^{-/-}p130^{-/-}$ MEFs, $Rb^{N750F/N750F}p130^{-/-}$ MEFs could not grow anchorage-independently upon expression of RAS^{V12} and TBX2 (Fig. 5). Since $Rb^{N750F/N750F}p130^{-/-}$ MEFs were refractory to RAS^{V12}-induced senescence, but were not transformed by RAS^{V12} and TBX2, these results demonstrate that bypass of RAS^{V12}-induced senescence meets lower requirements than transformation induction by RAS^{V12} and TBX2. Interestingly, we found that $Rb^{N750F/N750F}p130^{-/-}$ MEFs displayed higher Cyclin E protein levels compared to $p130^{-/-}$ MEFs, but lower levels compared to $Rb^{-/-}p130^{-/-}$ MEFs (Fig. S1). Thus, pocket protein-mediated suppression of *Cyclin E* transcription in wild-type MEFs occurs via both recruitment of chromatin remodeling proteins, as was previously suggested (Morrison et al., 2002; Dahiya et al., 2001; Nielsen et al., 2001) and via inhibition of E2F-mediated transactivation. Elevated expression of *Cyclin E* is expected to induce Cyclin E-CDK2 kinase activity. Possibly, derepression of *Cyclin E* in $Rb^{N750F/N750F}p130^{-/-}$ MEFs induces Cyclin E-CDK2 kinase activity to a level sufficient to bypass RAS^{V12}-induced senescence,

but insufficient to counteract the dramatic drop in kinase activities, which occurs upon loss of anchorage (Vormer et al., 2008).

In summary, we have shown that the pRB-LxCxE interaction is not involved in cell cycle arrest upon serum starvation or cell-cell contact, suggesting that blocking the transactivation function of E2F is the main mechanism by which pRB establishes cell cycle arrest under these conditions. The pRB-LxCxE interaction is critical for cell cycle arrest in response to γ-irradiation and expression of RAS^{V12}. However, ablation of the pRB-LxCxE interaction was not sufficient to induce anchorage-independent growth upon additional ablation of p130 plus expression of RAS^{V12} and TBX2. We conclude that, whereas ablation of the pRB-LxCxE interaction in MEFs bypasses some barriers involved in counteracting transformation, it is not sufficient to bring cells beyond the verge of transformation. We are currently investigating whether the pRB^{N750F} mutation in a *p130* null background predisposes to tumorigenesis *in vivo*.

Materials and Methods

Constructs

The 6xE2F-luciferase reporter, CMV-HA-E2F1, CMV-HA-E2F4, CMV-Renilla and pJ3-mRb were kindly provided by Dr. R. Bernards, GAL4-TK-luciferase was obtained from Dr. R. Medema and pECE-pRbΔcdk-HA was from Dr. J. Lukas. An N-terminal HA-tag was added to pRB by replacing the *Eco*RI/*Cla*I fragment from pJ3-mRb with the *Eco*RI/*Cla*I fragment from pECE-pRbΔcdk-HA, thereby creating pJ3-HA-mRb. Mutation of the Rb cDNA was done by subcloning the *Bgl*II fragment (~1 kb) from pJ3-Rb into the *Bgl*II site of LITMUS 28, followed by mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene 200518). Regeneration of full-length Rb in an expression vector was achieved by co-ligation of the *Eco*RI/*Bgl*II fragment from pJ3-HA-mRb and the wild-type or mutated *Bgl*II fragment from LITMUS 28 into the *Eco*RI/*Bam*HI site of pcDNA3.1(-), thereby creating pcDNA3.1(-)-HA-mRb, pcDNA3.1(-)-HA-mRb N750F, pcDNA3.1(-)-HA-mRb C699F and pcDNA3.1(-)-HA-mRb R654W. Generation of vectors encoding GAL4-pRB fusion proteins was achieved by PCR cloning of an Rb fragment (encoding amino acid residues 372-921) from the pcDNA3.1(-)-HA-mRb series using 5' and 3' primers encompassing *Eco*RI and *Xba*I sites, respectively. These *Eco*RI/*Xba*I fragments were inserted in-frame into the *Eco*RI/*Xba*I site of pM (Clontech). pSG5-Tag was kindly provided by Dr. J. A. DeCaprio, pBABE-RAS^{V12}-puro by Dr. T. Brummelkamp and pCL-Eco by Dr. D. Peeper. The pEYK-TBX2 vector was previously isolated from MEFs infected with the pEYK-MCF7 library (Vormer et al., 2008), which was a gift of Dr. G. Q. Daley.

Reporter assays

Subconfluent *Rb* $^{-/-}$ 3T9 cells were transfected one day after seeding using FuGENE6 Transfection Reagent (Roche) according to the manufacturer's protocol. Transfections were performed using 0.4 µg DNA per well of a 12-well plate, in a DNA-FuGENE ratio of 1 to 3 (µg and µl respectively). All transfections included a luciferase reporter plasmid (6xE2F-luciferase or GAL4-TK-luciferase) and a Renilla luciferase expression plasmid. Empty pcDNA3.1 or empty pM plasmids were added to each reaction to reach the total amount of 0.4 µg DNA/well. Twenty-four h post transfection, cells were washed in PBS and lysed in 100 µl lysis buffer (provided with the Dual-Luciferase Reporter Assay

System (Promega E1910). Lysates were measured for Luciferase and Renilla luminescence activity by using the Dual-Luciferase Reporter Assay System (Promega E1910) according to the manufacturer's protocol. For each sample, luciferase luminescence was divided by Renilla luminescence. All transfection experiments were carried out at least in duplicate.

MEF isolation

We previously generated $Rb^{N750F/wt}$ mice by injecting an $Rb^{N750F/wt}$ embryonic stem cell clone into B6 blastocysts. The $Rb^{N750F/wt}$ embryonic stem cell clone was generated by oligonucleotide-directed modification of the endogenous *Rb* gene in 129OLA embryonic stem cells (Aarts et al., 2006). The resulting $Rb^{N750F/wt}$ chimeras were backcrossed to FVB. Subsequently, $Rb^{N750F/wt}$ mice were crossed with previously generated $p130^{wt/-}$ FVB mice (Dannenberg et al., 2000), generating $Rb^{N750F/wt}p130^{wt/-}$ mice. These animals were intercrossed enabling the isolation of $Rb^{N750F/N750F}p130^{-/-}$ and $Rb^{N750F/N750F}p130^{wt/wt}$ MEFs at embryonic day 14.5 (day of vaginal plug was defined as day 0.5). Additionally, we generated $Rb^{wt/-}p130^{wt/-}$ animals by crossing $Rb^{wt/-}$ FVB animals (Dannenberg et al., 2000; te Riele et al., 1992) with $Rb^{wt/wt}p130^{wt/-}$ mice, which had been derived from $Rb^{N750F/wt}p130^{wt/-}$ intercrossings. Subsequent intercrossings of $Rb^{wt/-}p130^{wt/-}$ animals enabled isolation of $Rb^{-/-}p130^{-/-}$ MEFs. Lastly, intercrossing $Rb^{wt/-}p130^{wt/wt}$ animals enabled isolation of $Rb^{-/-}p130^{wt/wt}$ MEFs, whereas intercrossing $Rb^{wt/wt}p130^{wt/-}$ animals was used to isolate $Rb^{wt/wt}p130^{-/-}$ and $Rb^{wt/wt}p130^{wt/wt}$ MEFs. Due to embryonic lethality, $Rb^{-/-}p130^{-/-}$ MEFs were isolated at embryonic day 13.5, all other MEF isolations were performed at embryonic day 14.5. $Rb^{-/-}p130^{-/-}p107^{-/-}$ MEFs were previously generated by (Dannenberg et al., 2000).

Cell culture

MEFs were cultured in GMEM (Invitrogen/Gibco), containing 10% fetal calf serum, 1 mM nonessential amino acids (Invitrogen/Gibco), 1 mM sodium pyruvate (Invitrogen/Gibco), 100 units/ml penicillin (Invitrogen/Gibco), 100 µg/ml streptomycin (Invitrogen/Gibco) and 0.1 mM β-mercaptoethanol and incubated at 37 °C in the presence of 5% CO_2.

For serum starvation experiments, cells were seeded at a density of 8 x 10^5 per 10- cm dish. Three h after plating, cells were washed in PBS and medium as described above was added, but containing 0% fetal calf serum. Cells were harvested for FACS at the indicated time points and BrdU was added to a concentration of 10 µM 1 h before harvesting.

For contact inhibition experiments, cells were plated at a density of 8 x 10^5 per 10-cm dish and analyzed 10 days after plating. Cells were given fresh medium every 2 or 3 days.

For γ-irradiation experiments, cells were plated at a density of 5 x 10^5 per 10-cm dish, irradiated the next day with 5.5, 10 or 15 gray and analyzed 16 h after irradiation. One h before harvesting, BrdU was added to a concentration of 10 µM.

For retroviral infections, cells were plated at a density of 8.5 x 10^5 per 10-cm dish and infected twice the next day with retroviral supernatants, supplemented with polybrene to a concentration of 4 µg/ml during a time-span of at least 6 h per infection. For serial infections, MEFs were cultured in non-virus containing media for at least 36 h between infections and reseeded before infection to obtain optimal cell density. Retroviral supernatants were produced by calcium phosphate transfection (Invitrogen) of phoenix cells with 16 µg of the desired construct and 4 µg pCL-Eco. Forty-eight h post transfection, retroviral supernatant was filtered using 0.45 µm filters (MCE membrane, Millipore) and either used directly or immediately frozen using a dry-ice ethanol bath and stored at -80 °C. After harvesting viral supernatant, phoenix cells were supplemented with GMEM containing media supplements as described above, and supernatant was again harvested using the same procedure, with an interval of at least 6 h.

Soft agar plating was performed as previously described (Vormer et al., 2008): 6 x 10^4 MEFs were suspended in 2 ml of a 37 °C, 0.35% soft agar solution (low gelling agarose type VII from Sigma) in GMEM containing 10% fetal calf serum, the same medium supplements as mentioned above plus gentamicin (Invitrogen/Gibco) to a concentration of 0.02 mg/ml and plated in one well of a six-well plate. To prevent cells from attaching to the bottom of the well, the 0.35% soft agar solution was poured into an ultra-low-attachment surface plate (catalog no. 3471; Corning Incorporated) coated with a 1% soft agar layer. To allow solidification of the agar, plates were incubated at 4°C for 30 minutes. Subsequently, cells were incubated at 37°C in the presence of 5% CO2 for 2 weeks. Pictures were taken using a non-phase-contrast lens (x2.5 magnification) and assembled using Axiovision 4.5. Detail images were taken using a phase-contrast lens (x5 magnification).

Proliferation curves: crystal violet assays

For proliferation curves, cells were seeded in triplicate at a density of 2.5 x 10^4 cells per well of a 12-well plate (Corning Incorporated, 3512). At the indicated time points (time point 0 was defined as 3 h after seeding), cells were fixed for 5 minutes in 4% formaldehyde in PBS, which was freshly made from a 37% formaldehyde stock. Subsequently, cells were washed three times in demineralized water and stained with 0.1% crystal violet solution for 30 minutes. Again, cells were washed three times in demineralized water and allowed to dry in the dark. Dye was extracted by adding 1 ml 10% acetic acid per well. 100 µl of this solution was used to determine the optical density at 590 nm using a microplate reader (M200 Tecan).

BrdU staining and FACS

For BrdU labeling, BrdU was added to the culture medium 1 h before harvesting to a concentration of 10 µM. After this incubation, cells were trypsinized and fixed in 70% ethanol in PBS at 4 ºC. To detect incorporated BrdU, cells were first washed in cold PBS and then suspended in 5 M HCl/0.5% Triton and incubated at room temperature for 20 minutes. Next, cells were washed in 1 M TrisHCl pH 7.5 and subsequently in PBS/0.5% Tween 20. Cells were thereafter incubated with mouse anti-BrdU Antibody (DAKO, clone BU20A M0744) in a concentration of 1:40 in PBS/0.5% Tween 20/1% BSA for 30 minutes at room temperature. Next, cells were washed twice in PBS/0.5% Tween 20 and incubated with goat anti-mouse immunoglobulins/FITC (DAKO F0479) in a concentration of 1:20 in PBS/0.5% Tween 20/1% BSA for 30 minutes at room temperature in the dark. After washing twice with PBS/0.5% Tween 20, cells were suspended in PBS containing 200 µg/ml RNase A and 20 µg/ml propidium iodide, incubated at 37 ºC for 15 min, measured in FL1 for FITC and FL3 for propidium iodide, and analyzed using 'Cell Quest' and 'Summit' software.

Cells that were analyzed for propidium iodide only were fixed overnight in 70% ethanol in PBS at 4 ºC, washed in PBS and directly suspended in PBS containing 200 µg/ml RNase A and 20 µg/ml propidium iodide, incubated at 37 ºC for 15 min and measured in FL3.

Protein isolation, immunoprecipitation and immunoblot

For protein isolations, cells were lysed for 30 minutes on ice in lysis buffer containing 150 mM NaCl, 50 mM Hepes pH 7.5, 5 mM EDTA, 0.1% NP-40 and 1 tablet complete protease inhibitor cocktail (Roche) per 50 ml. After centrifugation, protein concentration was determined using Bio-Rad protein assay (Bio-Rad). For immunoblot analysis, protein was separated on 3-8% Tris-Acetate and 4-12% Bis-Tris NuPage gradient gels (Invitrogen). Blotting was performed using standard protocols.

For immunoprecipitation experiments, $Rb^{-/-}$ C33A cells were co-transfected with pSG5-TAg and pcDNA3.1(-)-HA-mRb (wild-type, N750F, R654W or C699F) or empty pcDNA3.1(-)

using calcium phosphate (Invitrogen). Protein was isolated using the lysis buffer described above plus 5 mM NaF, 0.5 mM vanadate, 20 mM β-glycerolphosphate and 1mM PMSF. Lysates were incubated O/N at 4 °C with anti-HA antibody and 50 % protein A/G beads (Santa Cruz) in lysis buffer while rotating. Subsequently, lysates were washed 5 times in lysis buffer, separated on a 12% polyacrylamide gel and immunoblotted. The membrane was probed with mouse anti-TAg and mouse anti-pRB and next with HRP-conjugated secondary antibody.

Used primary antibodies were: mouse monoclonal anti pRB (554136, BD Biosciences Pharmingen), rabbit polyclonal anti-p107 (C18, SC-318, Santa Cruz), rabbit polyclonal anti-cyclin E (M20, SC-481, Santa Cruz), rabbit polyclonal anti-cyclin A (C19, SC-596, Santa Cruz) and goat polyclonal anti-cdk4 (C22-G, SC260-G, Santa Cruz). Secondary antibodies were: HRP conjugated goat anti mouse, goat anti rabbit and rabbit anti goat (DakoCytomation).

Acknowledgments

We thank Anja van der Wal and Elly Delzenne-Goette for MEF isolations and Tanja van Harn, Rob Dekker, Marieke Aarts and Eva Wielders for helpful discussions. This work was financially supported by the Dutch Cancer Society, grant nr. NKI 2002-2634.

References

Aarts,M., Dekker,M., de,V.S., van der Wal,A., and te Riele,H. (2006). Generation of a mouse mutant by oligonucleotide-mediated gene modification in ES cells. Nucleic Acids Res. *34*, e147.

Balciunaite,E., Spektor,A., Lents,N.H., Cam,H., te Riele,H., Scime,A., Rudnicki,M.A., Young,R., and Dynlacht,B.D. (2005). Pocket protein complexes are recruited to distinct targets in quiescent and proliferating cells. Mol. Cell Biol. *25*, 8166-8178.

Bignon,Y.J., Shew,J.Y., Rappolee,D., Naylor,S.L., Lee,E.Y., Schnier,J., and Lee,W.H. (1990). A single Cys706 to Phe substitution in the retinoblastoma protein causes the loss of binding to SV40 T antigen. Cell Growth Differ. *1*, 647-651.

Binne,U.K., Classon,M.K., Dick,F.A., Wei,W., Rape,M., Kaelin,W.G., Jr., Naar,A.M., and Dyson,N.J. (2007). Retinoblastoma protein and anaphase-promoting complex physically interact and functionally cooperate during cell-cycle exit. Nat. Cell Biol. *9*, 225-232.

Brehm,A., Miska,E.A., McCance,D.J., Reid,J.L., Bannister,A.J., and Kouzarides,T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature *391*, 597-601.

Brugarolas,J., Moberg,K., Boyd,S.D., Taya,Y., Jacks,T., and Lees,J.A. (1999). Inhibition of cyclin-dependent kinase 2 by p21 is necessary for retinoblastoma protein-mediated G1 arrest after gamma-irradiation. Proc. Natl. Acad. Sci. U. S. A *96*, 1002-1007.

Chan,H.M., Smith,L., and La Thangue,N.B. (2001). Role of LXCXE motif-dependent interactions in the activity of the retinoblastoma protein. Oncogene *20*, 6152-6163.

Chen,T.T. and Wang,J.Y. (2000). Establishment of irreversible growth arrest in myogenic differentiation requires the RB LXCXE-binding function. Mol. Cell Biol. *20*, 5571-5580.

Cobrinik,D., Whyte,P., Peeper,D.S., Jacks,T., and Weinberg,R.A. (1993). Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes Dev. *7*, 2392-2404.

Dahiya,A., Gavin,M.R., Luo,R.X., and Dean,D.C. (2000). Role of the LXCXE binding site in Rb function. Mol. Cell Biol. *20*, 6799-6805.

Dahiya,A., Wong,S., Gonzalo,S., Gavin,M., and Dean,D.C. (2001). Linking the Rb and polycomb pathways. Mol. Cell *8*, 557-569.

Dannenberg,J.H., Schuijff,L., Dekker,M., van der Valk,M., and te Riele,H. (2004). Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev. *18*, 2952-2962.

Dannenberg,J.H., van Rossum,A., Schuijff,L., and te Riele,H. (2000). Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. Genes Dev. *14*, 3051-3064.

DeGregori,J. and Johnson,D.G. (2006). Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis. Curr. Mol. Med. *6*, 739-748.

Dermott,J.M., Gooya,J.M., Asefa,B., Weiler,S.R., Smith,M., and Keller,J.R. (2004). Inhibition of growth by p205: a nuclear protein and putative tumor suppressor expressed during myeloid cell differentiation. Stem Cells *22*, 832-848.

Dick,F.A. (2007). Structure-function analysis of the retinoblastoma tumor suppressor protein - is the whole a sum of its parts? Cell Div. *2*, 26.

Dimova,D.K. and Dyson,N.J. (2005). The E2F transcriptional network: old acquaintances with new faces. Oncogene *24*, 2810-2826.

Ferreira,R., Magnaghi-Jaulin,L., Robin,P., Harel-Bellan,A., and Trouche,D. (1998). The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase. Proc. Natl. Acad. Sci. U. S. A *95*, 10493-10498.

Foijer,F., Wolthuis,R.M., Doodeman,V., Medema,R.H., and te Riele,H. (2005). Mitogen requirement for cell cycle progression in the absence of pocket protein activity. Cancer Cell *8*, 455-466.

Frolov,M.V. and Dyson,N.J. (2004). Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. J. Cell Sci. *117*, 2173-2181.

Hertel,L., Rolle,S., De Andrea M., Azzimonti,B., Osello,R., Gribaudo,G., Gariglio,M., and Landolfo,S. (2000). The retinoblastoma protein is an essential mediator that links the interferon-inducible 204 gene to cell-cycle regulation. Oncogene *19*, 3598-3608.

Isaac,C.E., Francis,S.M., Martens,A.L., Julian,L.M., Seifried,L.A., Erdmann,N., Binne,U.K., Harrington,L., Sicinski,P., Berube,N.G., Dyson,N.J., and Dick,F.A. (2006). The retinoblastoma protein regulates pericentric heterochromatin. Mol. Cell Biol. *26*, 3659-3671.

Kaye,F.J., Kratzke,R.A., Gerster,J.L., and Horowitz,J.M. (1990). A single amino acid substitution results in a retinoblastoma protein defective in phosphorylation and oncoprotein binding. Proc. Natl. Acad. Sci. U. S. A *87*, 6922-6926.

Kinross,K.M., Clark,A.J., Iazzolino,R.M., and Humbert,P.O. (2006). E2f4 regulates fetal erythropoiesis through the promotion of cellular proliferation. Blood *108*, 886-895.

Kratzke,R.A., Otterson,G.A., Lin,A.Y., Shimizu,E., Alexandrova,N., Zajac-Kaye,M., Horowitz,J.M., and Kaye,F.J. (1992). Functional analysis at the Cys706 residue of the retinoblastoma protein. J. Biol. Chem. *267*, 25998-26003.

Lee,J.O., Russo,A.A., and Pavletich,N.P. (1998). Structure of the retinoblastoma tumour-suppressor pocket domain bound to a peptide from HPV E7. Nature *391*, 859-865.

Macaluso,M., Montanari,M., and Giordano,A. (2006). Rb family proteins as modulators of gene expression and new aspects regarding the interaction with chromatin remodeling enzymes. Oncogene *25*, 5263-5267.

Magnaghi-Jaulin,L., Groisman,R., Naguibneva,I., Robin,P., Lorain,S., Le Villain,J.P., Troalen,F., Trouche,D., and Harel-Bellan,A. (1998). Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature *391*, 601-605.

Moberg,K., Starz,M.A., and Lees,J.A. (1996). E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. Mol. Cell Biol. *16*, 1436-1449.

Morrison,A.J., Sardet,C., and Herrera,R.E. (2002). Retinoblastoma protein transcriptional repression through histone deacetylation of a single nucleosome. Mol. Cell Biol. *22*, 856-865.

Muller,H., Moroni,M.C., Vigo,E., Petersen,B.O., Bartek,J., and Helin,K. (1997). Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. Mol. Cell Biol. *17*, 5508-5520.

Narita,M., Nunez,S., Heard,E., Narita,M., Lin,A.W., Hearn,S.A., Spector,D.L., Hannon,G.J., and Lowe,S.W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell *113*, 703-716.

Nicolas,E., Roumillac,C., and Trouche,D. (2003). Balance between acetylation and methylation of histone H3 lysine 9 on the E2F-responsive dihydrofolate reductase promoter. Mol. Cell Biol. *23*, 1614-1622.

Nielsen,S.J., Schneider,R., Bauer,U.M., Bannister,A.J., Morrison,A., O'Carroll,D., Firestein,R., Cleary,M., Jenuwein,T., Herrera,R.E., and Kouzarides,T. (2001). Rb targets histone H3 methylation and HP1 to promoters. Nature *412*, 561-565.

Otterson,G.A., Chen,W., Coxon,A.B., Khleif,S.N., and Kaye,F.J. (1997). Incomplete penetrance of familial retinoblastoma linked to germ-line mutations that result in partial loss of RB function. Proc. Natl. Acad. Sci. U. S. A *94*, 12036-12040.

Peeper,D.S., Dannenberg,J.H., Douma,S., te Riele,H., and Bernards,R. (2001). Escape from premature senescence is not sufficient for oncogenic transformation by Ras. Nat. Cell Biol. *3*, 198-203.

Pennaneach,V., Barbier,V., Regazzoni,K., Fotedar,R., and Fotedar,A. (2004). Rb inhibits E2F-1-induced cell death in a LXCXE-dependent manner by active repression. J. Biol. Chem. *279*, 23376-23383.

Pennaneach,V., Salles-Passador,I., Munshi,A., Brickner,H., Regazzoni,K., Dick,F., Dyson,N., Chen,T.T., Wang,J.Y., Fotedar,R., and Fotedar,A. (2001). The large subunit of replication factor C promotes cell survival after DNA damage in an LxCxE motif- and Rb-dependent manner. Mol. Cell *7*, 715-727.

Rayman,J.B., Takahashi,Y., Indjeian,V.B., Dannenberg,J.H., Catchpole,S., Watson,R.J., te Riele,H., and Dynlacht,B.D. (2002). E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. Genes Dev. *16*, 933-947.

Rowland,B.D., Denissov,S.G., Douma,S., Stunnenberg,H.G., Bernards,R., and Peeper,D.S. (2002). E2F transcriptional repressor complexes are critical downstream targets of p19(ARF)/p53-induced proliferative arrest. Cancer Cell *2*, 55-65.

Sellers,W.R., Rodgers,J.W., and Kaelin,W.G., Jr. (1995). A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. Proc. Natl. Acad. Sci. U. S. A *92*, 11544-11548.

Takahashi,Y., Rayman,J.B., and Dynlacht,B.D. (2000). Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. Genes Dev. *14*, 804-816.

Talluri,S., Isaac,C.E., Ahmad,M., Henley,S.A., Francis,S.M., Martens,A.L., Bremner,R., and Dick,F.A. (2010). A G1 checkpoint mediated by the retinoblastoma protein that is dispensable in terminal differentiation but essential for senescence. Mol. Cell Biol. *30*, 948-960.

te Riele,H., Maandag,E.R., and Berns,A. (1992). Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. Proc. Natl. Acad. Sci. U. S. A *89*, 5128-5132.

Vandel,L., Nicolas,E., Vaute,O., Ferreira,R., it-Si-Ali,S., and Trouche,D. (2001). Transcriptional repression by the retinoblastoma protein through the recruitment of a histone methyltransferase. Mol. Cell Biol. *21*, 6484-6494.

Vormer,T.L., Foijer,F., Wielders,C.L., and te Riele,H. (2008). Anchorage-independent growth of pocket protein-deficient murine fibroblasts requires bypass of G2 arrest and can be accomplished by expression of TBX2. Mol. Cell Biol. *28*, 7263-7273.

Supplemental figures

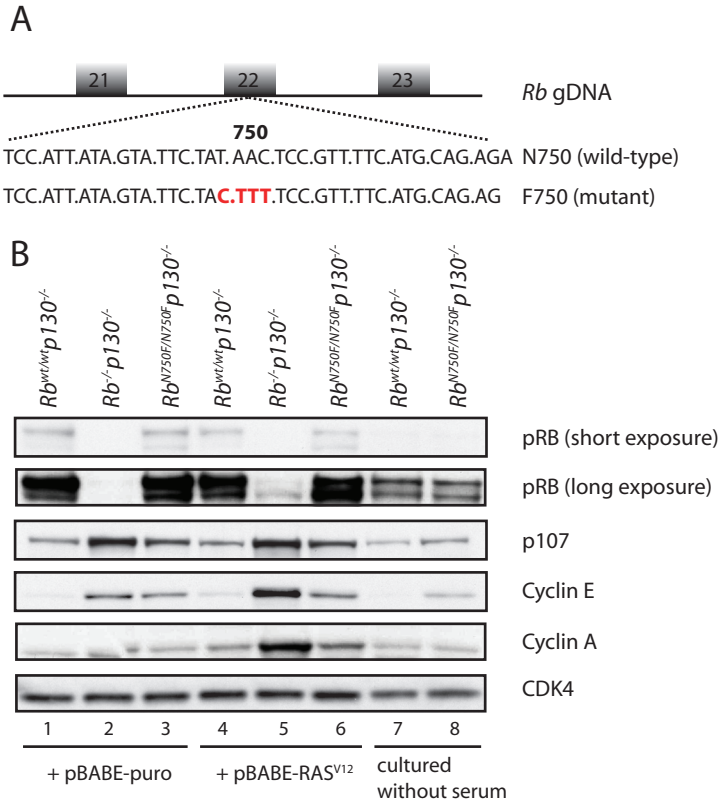
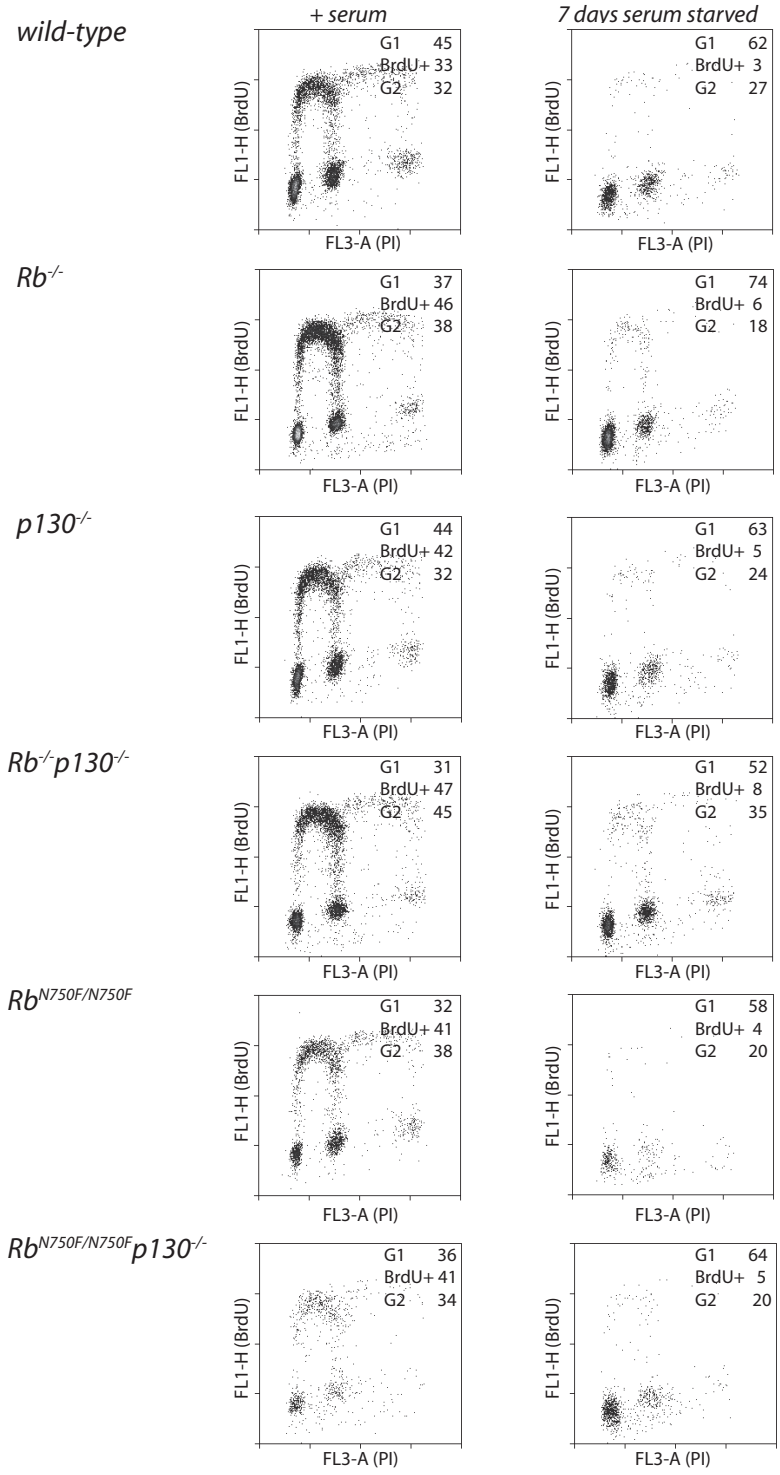


Figure S1: $Rb^{N750F/N750F}p130^{-/-}$ MEFs express wild-type levels of pRB, but increased levels of E2F-target genes. (A) Oligonucleotide-directed modification of exon 22 of Rb in murine ES cells resulted in the substitution of asparagine at position 750 with phenylalanine. The upper sequence shows a part of wild-type Rb encoding asparagine at position 750. The lower sequence depicts the oligonucleotide which was used for modification of the Rb locus, resulting in incorporation of phenylalanine at position 750. The part of the oligonucleotide that differs from the wild-type sequence is shown in red. A detailed description of the procedure can be found in (Aarts et al., 2006). (B) MEFs of the indicated genotypes were infected with pBABE-puro (lanes 1 to 3) or pBABE-RAS^{V12} (lanes 3 to 6) and cultured in the presence of serum. Additionally, non-infected MEFs were cultured in the absence of serum (lanes 7 and 8). Protein extracts were immunoblotted using the depicted antibodies.


+ serum
7 days serum starved
wild-type
Rb-/-
p130-/-
Rb-/-p130-/-
RbN750F/N750F
RbN750F/N750Fp130-/-
G1 45
BrdU+ 33
G2 32
G1 62
BrdU+ 3
G2 27
G1 37
BrdU+ 46
G2 38
G1 74
BrdU+ 6
G2 18
G1 44
BrdU+ 42
G2 32
G1 63
BrdU+ 5
G2 24
G1 31
BrdU+ 47
G2 45
G1 52
BrdU+ 8
G2 35
G1 32
BrdU+ 41
G2 38
G1 58
BrdU+ 4
G2 20
G1 36
BrdU+ 41
G2 34
G1 64
BrdU+ 5
G2 20
FL1-H (BrdU)
FL3-A (PI)

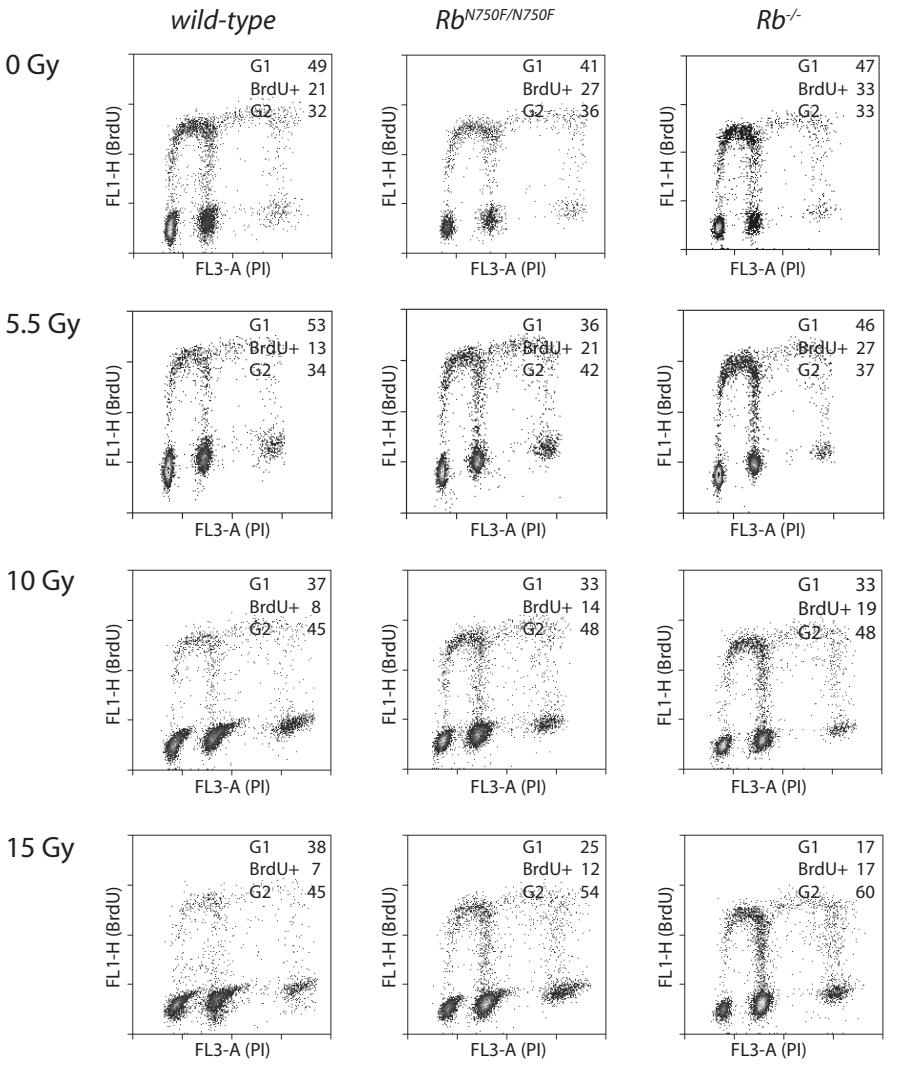


Figure S3: Cell cycle arrest in $Rb^{N750F/N750F}$ and $Rb^{-/-}$ MEFs shifts towards G_2 in response to high doses of γ-irradiation. MEFs of the indicated genotypes were treated with γ-irradiation, harvested after 16 h and stained for BrdU and propidium iodide incorporation. Shown are cell cycle profiles of the MEFs depicted in Fig. 3C. The depicted numbers of G_1, G_2 and S phase cells were determined as described in the legend of Fig. S2.

>Figure S2: $Rb^{N750F/N750F}p130^{-/-}$ MEFs arrest efficiently in G_1 upon serum starvation. MEFs of the indicated genotypes were cultured in the presence or absence of serum, stained for BrdU and propidium iodide incorporation and analyzed by FACS. Shown are cell cycle profiles of the MEFs depicted in Table 1. The depicted numbers of G_1- and G_2–phase cells represent the percentages of cells with a 2N or 4N DNA content based on propidium iodide incorporation (FL3-A). The depicted number of S-phase cells represent the BrdU-positive population of cells with a DNA content ranging from 2N to 4N.

CHAPTER 5

Overlapping roles of pRB-LxCxE interactions and p130 functions in mouse development and viability

Tinke L. Vormer, Anja van der Wal, Elly Delzenne-Goette, Marleen Dekker, Ji-Ying Song, Sjalin Naik, Jacob B. Hansen and Hein te Riele

Manuscript in preparation

Abstract

Loss of the retinoblastoma gene *Rb* can initiate tumorigenesis in both humans and mice. pRB and its two close homologs, p107 and p130, form the family of pocket proteins and collectively regulate the activity of E2F transcription factors during G_1. pRB-E2F interaction blocks E2F's transactivation domain but can also actively repress E2F target genes through the recruitment of proteins containing an LxCxE-motif. This motif is present in many proteins involved in chromatin remodeling and transcriptional repression. To investigate whether active repression is involved in pRB's role in development and tumor suppression, we generated mice expressing a variant pRB protein in which the asparagine at position 750 was replaced for phenylalanine. pRB^{N750F} is abrogated in binding LxCxE-containing proteins while still able to inhibit E2F-induced transactivation. In sharp contrast to $Rb^{+/-}$ mice and $Rb^{-/-}$ chimeras, $Rb^{N750F/N750F}$ mice had a normal life span and were not predisposed to tumorigenesis, demonstrating that ablation of the pRB-LxCxE interaction was not sufficient to induce tumor formation. Because of the compensatory role of other pocket proteins in binding LxCxE-containing proteins and in inhibiting E2F-induced transcription, we combined the Rb^{N750F} mutation with loss of *p130*. Interestingly, the $Rb^{N750F/N750F}p130^{-/-}$ genotype caused embryonic lethality, indicating a role for LxCxE-containing proteins in embryonic development. Furthermore, $Rb^{N750F/N750F}p130^{+/-}$ and $Rb^{N750F/wt}p130^{-/-}$ mice were viable but showed a reduced lifespan. However, these genotypes did not predispose to tumorigenesis, indicating that inhibiting the transactivation function of E2Fs is the dominant mechanism of tumor suppression by the pocket proteins.

Introduction

The retinoblastoma tumor suppressor pathway plays a major role in the inhibition of human and mouse tumorigenesis. The retinoblastoma protein, pRB, has two homologs, p130 and p107, which together form the family of pocket proteins. Pocket proteins are essential for regulation of the cell cycle, differentiation and apoptosis.

Pocket proteins play a key role in the G_1 phase of the cell cycle, where they inhibit the activity of E2F transcription factors. Binding to pocket proteins blocks E2F's transactivation domain, resulting in inhibition of E2F-dependent transcription. In addition to binding E2Fs, pocket proteins can simultaneously bind to proteins containing an LxCxE motive (x encoding any amino acid). Since many LxCxE-containing proteins have been implicated in chromatin remodeling and transcriptional repression, the recruitment of complexes containing pocket proteins, E2Fs and LxCxE-containing proteins may result in active repression of E2F target genes. Examples of transcriptional repressors that interact with pRB via the LxCxE binding site are histone deacetylase 1 (HDAC1), HDAC2, heterochromatin protein 1 (HP1) and the histone methyltransferase Suv39h1 (Dick, 2007; Frolov and Dyson, 2004; Lai et al., 2001; Nielsen et al., 2001; Vandel et al.,

2001; Chen and Wang, 2000; Dahiya et al., 2000; Magnaghi-Jaulin et al., 1998).

The E2F transcription factor family is classically divided into activator E2Fs (E2F1, E2F2 and E2F3a) and repressor E2Fs (E2F3b, E2F4, E2F5, E2F6a, E2F6b, E2F7a, E2F7b and E2F8). pRB interacts with E2F1-4 (Moberg et al., 1996), whereas p130 and p107 interact with E2F4 and E2F5. E2F6-8 function independently of the pocket proteins (DeGregori and Johnson, 2006; Dimova and Dyson, 2005; Frolov and Dyson, 2004). Thus, whereas the transactivation function of activator E2Fs is simply inhibited by pRB binding, pocket protein-E2F-chromatin remodeling complexes can be formed by pRB-E2F3b, pRB-E2F4, p130/p107-E2F4 and p130/p107-E2F5. Of relevance, the division between activator and repressor E2Fs is far from absolute. The E2F3b, E2F4 and E2F5 'repressors' contain a transcriptional activation domain, and both E2F3b and E2F4 have been shown to function as transcriptional activators *in vitro* and *in vivo* (Chong et al., 2009a; Tsai et al., 2008; Kinross et al., 2006; Wu et al., 2001; Muller et al., 1997). Furthermore, E2F1-3a were recently shown to form pRB-E2F repressor complexes in differentiating cells (Sahin and Sladek, 2010; Chong et al., 2009b).

Loss of pocket proteins can intitiate tumorigenesis: in mice, *Rb* loss induces pituitary and thyroid tumors, and the tumor spectrum is extended by the additional loss of *p130* or *p107* (Dannenberg et al., 2004; Harrison et al., 1995; Maandag et al., 1994; Williams et al., 1994). However, it is unclear which of the E2F-regulating activities of pocket proteins is critical for tumor suppression. On the one hand, a view has emerged that mainly regulation of the 'activator', and not the 'repressor' E2Fs is involved in Rb-mediated tumor suppression (Parisi et al., 2009; Lee et al., 2002; Yamasaki et al., 1998). On the other hand, pocket protein-chromatin remodeling complexes have been implicated in processes that are considered critical for tumor suppression, such as (irreversible) cell cycle arrest and senescence. The recruitment of p130 and E2F4 to silenced promoters in G_0-arrested mouse fibroblasts (Rayman et al., 2002) and in G_0-and G_1-arrested human T98G cells (Takahashi et al., 2000) is suggestive for active repression. Furthermore, repression of E2F target genes involving pRB and LxCxE-containing proteins was detected under various growth inhibitory conditions, including serum starvation (Isaac et al., 2006; Morrison et al., 2002), RAS^{V12}-induced senescence (Narita et al., 2003) and cell cycle arrest induced by $p16^{INK4A}$ (Dahiya et al., 2001). Additionally, involvement of E2F-repressor complexes was demonstrated during contact inhibition and replicative or RAS^{V12}-induced senescence (Rowland et al., 2002; Zhang et al., 1999). pRB was also shown to be required for the establishment of irreversible cell cycle arrest during differentiation, via both silencing of cell cycle genes and the induction of differentiation specific genes. Interestingly, pRB-mediated silencing during differentiation is associated with the presence of repressive chromatin marks (Guo et al., 2009; Blais et al., 2007; Khidr and Chen, 2006; Thomas et al., 2001; Chen et al., 1996; Novitch et al., 1996; Gu

et al., 1993). Furthermore, a mutant form of human pRB that was deficient in binding LxCxE-containing proteins, was unable to establish irreversible cell cycle arrest during myogenic differentiation (Chen and Wang, 2000). Taken together, these studies indicate that the binding of pRB to LxCxE-containing proteins is involved in the establishment of cell cycle arrest under various conditions and is therefore predicted to function in tumor suppression.

Although LxCxE-containing proteins, recruited by pRB, have been implicated in processes counteracting transformation, the requirement for these proteins to suppress tumorigenesis *in vivo* remains elusive. We aimed to dissect the involvement of pocket protein-chromatin remodeling complexes during tumor formation and therefore made use of a mutant form of pRB: pRB^{N750F}. This mutant protein was able to inhibit E2F-induced transactivation, but was impaired in binding LxCxE-containing proteins (Chapter 4). Consequently, pRB^{N750F} was unable to recruit chromatin remodelers to E2F regulated promoters via the LxCxE binding site. Surprisingly, we found that mice homozygously mutant for pRB^{N750F} (*Rb*$^{N750F/N750F}$ mice) were viable, had a normal lifespan and were not predisposed to tumor formation. Thus, the ablation of complexes consisting of pRB and LxCxE-containing proteins was not sufficient to drive tumorigenesis. Given the role of p130 in binding 'repressor' E2Fs and recruiting LxCxE-containing proteins, we combined the *Rb*^{N750F} mutation with loss of *p130*. We found that homozygous loss of *p130* completely abolished live birth of *Rb*$^{N750F/N750F}$ mice whereas *Rb*$^{N750F/N750F}$*p130*$^{+/-}$ and *Rb*$^{N750F/wt}$*p130*$^{-/-}$ animals were viable. Surprisingly, these animals had a reduced lifespan but did not show increased tumor incidence. Thus, ablation of LxCxE-dependent interactions did not promote tumor formation, indicating that pRB's tumor suppressor activity mainly operates via inhibition of activator E2Fs.

Results

p130 functionally compensates for loss of the pRB-LxCxE interaction

To investigate whether the pRB-LxCxE interaction is critical for pRB's role in embryonic development, we have generated *Rb*$^{N750F/N750F}$ mice. As described before (Vormer et al., Chapter 4), the pRB^{N750F} protein was deficient in binding LxCxE-containing proteins, whereas inhibition of E2F-induced transcription remained intact. *Rb*$^{N750F/wt}$ murine embryonic stem cells, previously generated by oligonucleotide-directed gene modification as described in Aarts et al. (2006), were used to generate *Rb*$^{N750F/wt}$ mice. These animals were intercrossed and the resulting progeny was genotyped (Table 1). In contrast to full ablation of *Rb*, which was embryonic lethal (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992), *Rb*$^{N750F/N750F}$ animals were born according to mendelian ratio and survived into adulthood.

Rb	*N750F/N750F*	*N750F/wt*	*wt/wt*	*total*
Expected	16	32	16	64
Observed	16	31	17	64

Table 1: Genotypic analysis of life born animals derived from $Rb^{N750F/wt}$ intercrosses. 64 F1 animals were analyzed at post natal day 21. Expected: number of animals expected based on mendelian inheritance. Observed: observed number of animals.

Since the recruitment of chromatin-remodeling proteins may also be effectuated by other pocket proteins, we wondered whether the additional loss of *p107* or *p130* in $Rb^{N750F/N750F}$ mice would cause embryonic or post natal death. $Rb^{N750F/wt}$ mice were crossed with $p130^{+/-}$ or $p107^{+/-}$ animals and the resulting double heterozygotes were intercrossed. The F2 progeny was genotyped at postnatal day 21. $Rb^{N750F/N750F}p130^{-/-}$ animals were completely absent in the F2 cohort of 226 animals, whereas 14 animals were expected (Table 2). In contrast, $Rb^{wt/wt}p130^{-/-}$ animals were present at normal ratio, showing that loss of *p130* did not negatively influence survival in an *Rb* wild-type background (12 $Rb^{wt/wt}p130^{-/-}$ animals were detected, whereas 14 were expected). $Rb^{N750F/N750F}p130^{+/-}$ animals appeared to be underrepresented (13 observed; 28 expected) suggesting that two functional p130 alleles were required to fully compensate for the loss of the pRB-LxCxE interaction. However, the significance of this observation is doubtful as in this cross also $Rb^{N750F/N750F}p130^{+/+}$ mice were slightly underrepresented (8 observed; 14 expected).

To determine whether the $Rb^{N750F/N750F}p130^{-/-}$ genotype led to embryonic lethality, we crossed $Rb^{N750F/wt}p130^{+/-}$ females with $Rb^{N750F/wt}p130^{-/-}$ males and analyzed 43 embryos at E18.5. We found three $Rb^{N750F/N750F}p130^{-/-}$ embryos, whereas five were expected. Two of the $Rb^{N750F/N750F}p130^{-/-}$ embryos had an abnormal appearance compared to control embryos (one was small and one was pale). Within the group of control embryos, only 3 out of 40 embryos had an abnormal appearance (one being small and two being small plus pale). Histologically, we did not detect abnormalities in the $Rb^{N750F/N750F}p130^{-/-}$ embryos, except for degeneration of the myocardium in one of the three embryos. It has previously been

Rb	*N750F/N750F*			*N750F/wt*			*wt/wt*			*total*
p130	-/-	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	
Expected	14	14	28	28	28	56	14	14	28	226
Observed	0	8	13	38	32	77	12	15	31	226

Table 2: Genotypic analysis of life born animals derived from $Rb^{N750F/wt}p130^{+/-}$ intercrosses. 226 F2 animals were analyzed at post natal day 21. Expected: number of animals expected based on mendelian inheritance. Observed: observed number of animals.

shown that combined ablation of *Rb* and *p130* in cardiac myocytes resulted in increased proliferation in the neonatal and adult myocardium, possibly contributing to the early death of these mice. Accordingly, pRB, p130 and E2F4 were suggested to repress the E2F-regulated *Myc*-promoter in cardiac myocytes *in vitro* (MacLellan et al., 2005). Our results indicate that $Rb^{N750F/N750F}p130^{-/-}$ animals died at or shortly after E18.5. This suggests that pRB-E2F4/E2F3b and p130-E2F4/E2F5 chromatin remodeling complexes are required for late embryonic development or perinatal survival.

Similarly, we investigated the combination of Rb^{N750F} and *p107*-null alleles by intercrossing $Rb^{N750F/wt}p107^{+/-}$ animals and genotyping the resulting F2 progeny at postnatal day 21 (Table 3). $Rb^{N750F/N750F}p107^{-/-}$ animals were completely absent from this cohort of 130 animals, however also $Rb^{N750F/wt}p107^{-/-}$ and $Rb^{wt/wt}p107^{-/-}$ animals were absent, whereas 16 and 8 were expected, respectively. Apparently, the FVB genetic background of mice was not compatible with p107 ablation and therefore the effects of the pRB^{N750F} mutation in the absence of p107 could not be studied. However, similar to $Rb^{N750F/N750F}p130^{+/-}$, $Rb^{N750F/N750F}p107^{+/-}$ animals were viable.

In conclusion, these analyses show that p130 is essential for the survival of $Rb^{N750F/N750F}$ mice. In the absence of p130, loss of the pRB-LxCxE interaction causes pre- or perinatal death. $Rb^{N750F/N750F}p130^{+/-}$ and $Rb^{N750F/wt}p130^{-/-}$ animals were viable and survived into adulthood.

Loss of the pRB-LxCxE interaction in combination with loss of p130 or p107 is not sufficient to drive tumorigenesis

$Rb^{N750F/N750F}$ animals had a normal lifespan with a mean survival of approximately 75 weeks, which was similar to that of $Rb^{N750F/wt}$ and $Rb^{wt/wt}$ mice (Fig. 1). We did not detect any macroscopic or microscopic abnormalities in $Rb^{N750F/N750F}$ mice (described below and data not shown). Seven sacrificed $Rb^{N750F/N750F}$ and 19 sacrificed $Rb^{N750F/wt}$ animals were microscopically analyzed for abnormalities in the lungs: lung adenomas had developed in 29% of analyzed $Rb^{N750F/N750F}$ and in 26% of analyzed $Rb^{N750F/wt}$ mice. The frequency and latency of lung adenomas was similar as in $Rb^{wt/wt}$ mice indicating lung tumor development

Rb	*N750F/N750F*			*N750F/wt*			*wt/wt*			*total*
p107	-/-	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	
Expected	8	8	16	16	16	32	8	8	16	130
Observed	0	11	13	0	28	56	0	7	15	130

Table 3: Genotypic analysis of life born animals derived from $Rb^{N750F/wt}p107^{+/-}$ intercrosses. 130 F2 animals were analyzed at post natal day 21. Expeted: number of animals expected based on mendelian inheritance. Observed: observed number of animals.

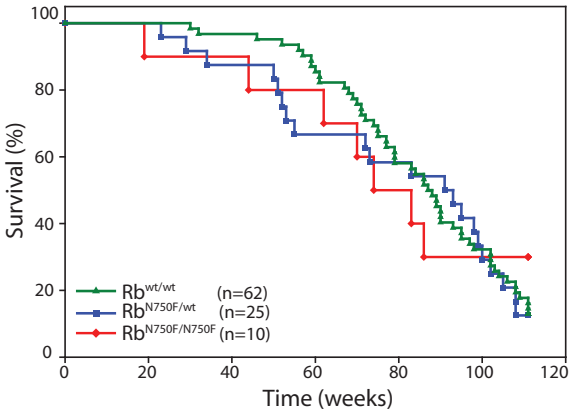


Figure 1: $Rb^{N750F/N750F}$ mice have a normal life span. Animals of the indicated genotypes were followed during 111 weeks and sacrificed when ill. Shown is the percentage of surviving animals per time point.

to be aging-related background pathology. We therefore conclude that ablation of the LxCxE-interacting domain in pRB did not cause tumor predisposition nor a decreased lifespan.

The absence of tumor development in $Rb^{N750F/N750F}$ mice sharply contrasts with the strong predisposition to pituitary and thyroid tumors in chimeric $Rb^{-/-}$ mice and $Rb^{+/-}$ mice (Harrison et al., 1995; Maandag et al., 1994; Williams et al., 1994). Our previous studies showed that the tumor spectrum induced by *Rb* loss, was broadened by the additional loss of *p130* or *p107*, and included retinoblastoma, osteocarcoma, lymphosarcoma, pheochromocytoma and adenocarcinoma in the coecum (Dannenberg et al., 2004; Robanus-Maandag et al., 1998). We therefore wondered whether tumor development in $Rb^{N750F/N750F}$ mice was suppressed by compensatory activities of p130 and p107. Several reports have shown that p130/p107 efficiently interacted with E2F4, bound chromatin remodeling proteins via their LxCxE binding sites (Nicolas et al., 2003; Ferreira et al., 1998), and were present at repressed, de-acetylated promoters in G_0 (Balciunaite et al., 2005; Rayman et al., 2002; Takahashi et al., 2000). Since $Rb^{N750F/N750F}p130^{-/-}$ and $Rb^{N750F/N750F}p107^{-/-}$ animals were not viable, we analyzed the lifespan and tumor incidence in mice with three mutated alleles, *i.e.*, $Rb^{N750F/N750F}p130^{+/-}$, $Rb^{N750F/wt}p130^{-/-}$ and $Rb^{N750F/N750F}p107^{+/-}$. Similar to previous observations in $Rb^{+/-}p107^{-/-}$ chimeric mice, tumor development may ensue from loss of the remaining wild-type allele (Dannenberg et al., 2004). Animals were followed during 70 weeks and sacrificed when ill. The survival of $Rb^{N750F/N750F}p130^{+/-}$ and $Rb^{N750F/wt}p130^{+/-}$ animals (Fig. 2A) was approximately 80% at 50 weeks of age, which was similar to that of $Rb^{N750F/N750F}p130^{+/+}$ mice (Fig. 1). After 70 weeks, the survival of $Rb^{N750F/N750F}p130^{+/-}$ animals appeared lower: 40% of $Rb^{N750F/N750F}p130^{+/-}$ animals was alive in contrast to 70% of $Rb^{N750F/wt}p130^{+/-}$ animals (Fig. 2A). Strikingly however, of the five sacrificed $Rb^{N750F/N750F}p130^{+/-}$ animals, only one was diagnosed with

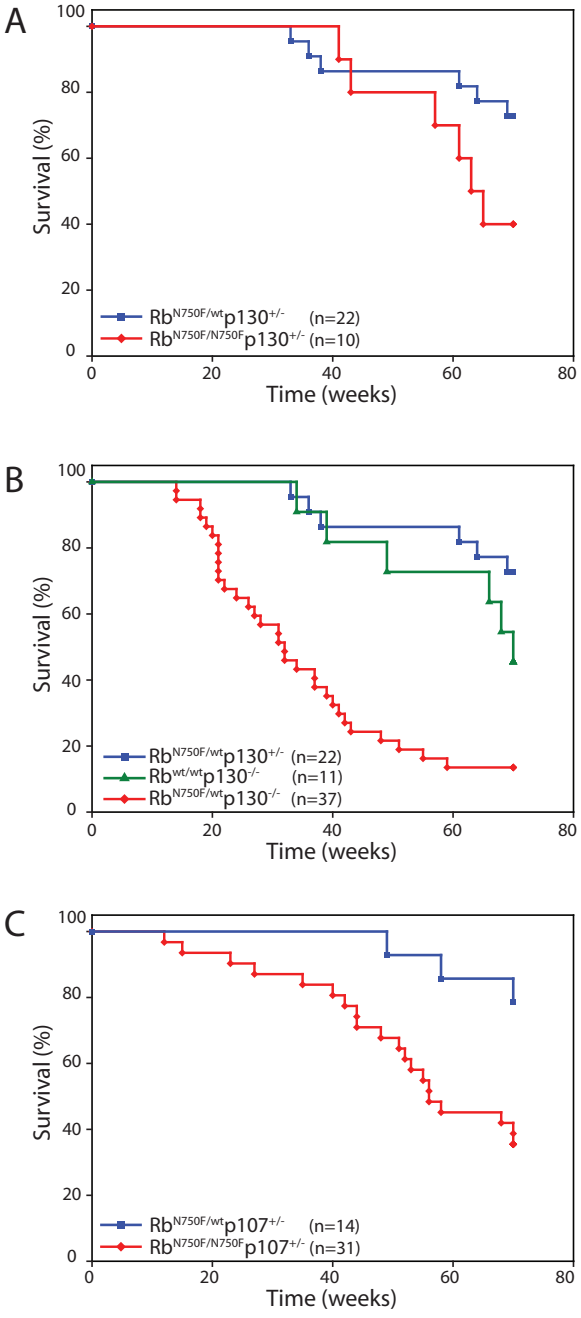


Figure 2: $Rb^{N750F/N750F}p130^{+/-}$ mice (A), $Rb^{N750F/wt}p130^{-/-}$ mice (B) and $Rb^{N750F/N750F}p107^{+/-}$ mice (C) have a decreased lifespan. Animals of the indicated genotypes were followed during 70 weeks and sacrificed when ill. Shown is the percentage of surviving animals per time point.

a tumor: an alveolar-bronchiolar adenoma in an animal sacrificed at 61 weeks (Table 4). In comparison, two adenomas (one in the lung and one in the harderian gland) and one sarcomatoid neoplasia were detected in six sacrificed $Rb^{N750F/wt}p130^{+/-}$ animals (Table 4). In conclusion, although $Rb^{N750F/N750F}p130^{+/-}$ mice showed a slightly reduced life span, we did not detect increased tumor formation in these animals before 70 weeks of age.

A severely reduced survival was seen in the cohort of $Rb^{N750F/wt}p130^{-/-}$ animals (Fig. 2B). Fifty percent of these animals had died by 32 weeks of age, and only 13.5% of the cohort was alive after 60 weeks. This was in sharp contrast to $Rb^{N750F/wt}p130^{+/-}$ and $Rb^{wt/wt}p130^{-/-}$ animals, of which approximately 75% was still alive at 60 weeks. $Rb^{N750F/wt}p130^{-/-}$ animals generally appeared unhealthy (most were skinny and had an irregular structure of the coat) and rapidly became very ill requiring them to be sacrificed. Of the cohort of 37 animals, 24 were sacrificed due to ill appearance and 8 animals had suddenly died before 60 weeks of age. In none of these animals we could detect overt macroscopical defects upon autopsy. Only limited microscopic analysis was possible for the animals that had suddenly died: we did not find indications for tumor formation or any other abnormalities in these animals. Tissues of the sacrificed 24 animals were elaborately analyzed at the microscopic level: pituitary (n=10), thyroid (n=16), brain (n=24), eyes (n=23), digestive system (stomach, intestines, liver, pancreas; n=22), testes (n=7), ovary (n=15), heart (n=15), lungs (n=22), adrenals (n=16), spleen (n=22), thymus (n=19), and bone marrow (sternum, hind leg, head; n=16). Except for some abnormalities in the spleen (see below) and some rare cases of tumor formation, all these tissues appeared generally normal. Remarkably, only 3 of these animals were diagnosed with tumor(s): 1 alveolar-bronchiolar adenoma, 2 adenomas in the pituitary gland and 1 lymphoma. All tumors were detected in animals older than 50 weeks (Table 4). For comparison, previous studies demonstrated that $Rb^{+/-}$ animals had to be sacrificed between 32 and 36 weeks of age due to pituitary tumors (Maandag et al., 1994; Williams et al., 1994), and in $Rb^{+/-}p130^{-/-}$ chimeric mice, 5 out of 15 animals developed a tumor (Dannenberg et al., 2004). Taken together, we conclude that $Rb^{N750F/wt}p130^{-/-}$ animals were not predisposed to tumorigenesis.

We also analyzed the survival of $Rb^{N750F/N750F}p107^{+/-}$ animals (Fig. 2C). Forty percent of these animals were still alive at 70 weeks of age, which was lower than in the cohorts of $Rb^{N750F/wt}p107^{+/-}$ and $Rb^{N750F/N750F}p107^{+/+}$ animals, of which 70% and 60% survived till week 70, respectively (Figs. 1 and 2C). Tumors were detected in 2 of the 18 sacrificed $Rb^{N750F/N750F}p107^{+/-}$ animals, at an age of 51 and 58 weeks, however, tumors were also detected in 2 $Rb^{N750F/wt}p107^{+/-}$ animals that were sacrificed at 58 and 70 weeks of age (Table 4). Therefore, also $Rb^{N750F/N750F}p107^{+/-}$ animals were not predisposed to tumorigenesis before 70 weeks of age.

Genotype: *Rb*$^{N750F/N750F}$*p130*$^{+/-}$ *1

Tumor type/pre-neoplastic lesion	Number of cases detected	Age at sacrifice (weeks)
alveolar/bronchiolar adenoma	1	61

Genotype: *Rb*$^{N750F/wt}$*p130*$^{+/-}$ *2

Tumor type/pre-neoplastic lesion	Number of cases detected	Age at sacrifice (weeks)
alveolar/bronchiolar adenoma	1	33
nodular hyperplasia in adrenal-cortex	1	61
sarcomatoid neoplasia	1	61
papillary adenoma harderian glands	1	64

Genotype: *Rb*$^{N750F/wt}$*p130*$^{-/-}$ *3

Tumor type/pre-neoplastic lesion	Number of cases detected	Age at sacrifice (weeks)
alveolar/bronchiolar adenoma	1	51
lymphoma	1	55
adenoma pars distalis pituitary	2	51 and 59

Genotype: *Rb*$^{wt/wt}$*p130*$^{-/-}$ *4

Tumor type/pre-neoplastic lesion	Number of cases detected	Age at sacrifice (weeks)
adenomatous hyperplasia in pars distalis pituitary	2	66 and 70

Genotype: *Rb*$^{N750F/N750F}$*p107*$^{+/-}$ *5

Tumor type/pre-neoplastic lesion	Number of cases detected	Age at sacrifice (weeks)
alveolar/bronchiolar adenocarcinoma	1	51
adenomatous hyperplasia in mucosa epithelia ileum	1	51
adenoma in mucosa ileum	1	58

Genotype: *Rb*$^{N750F/wt}$*p107*$^{+/-}$ *6

Tumor type/pre-neoplastic lesion	Number of cases detected	Age at sacrifice (weeks)
papillary adenoma in lung	1	58
adenomatous hyperplasia in alveolar/bronchiolar epithelium	1	58
sarcomatoid neoplasia	1	70

Table 4: Overview of tumors and pre-neoplastic lesions detected in mice of various genotypes, which were sacrificed when ill. Shown are mice sacrificed before or at 70 weeks of age.
**1: 5 animals out of this cohort (n=10) were sacrificed before 70 weeks of age, 1 additional animal was found dead*
**2: 6 animals out of this cohort (n=22) were sacrificed before 70 weeks of age*
**3: 24 animals out of this cohort (n=37) were sacrificed before 70 weeks of age, 8 additional animals were found dead*
**4: 6 animals out of this cohort (n=11) were sacrificed at or before 70 weeks of age*
**5:18 animals out of this cohort (n=31) were sacrificed at or before 70 weeks of age, 2 additional animals were found dead*
**6: 2 animals out of this cohort (n=14) were sacrificed at or before 70 weeks of age, 1 additional animal was found dead*

In conclusion, we have found that $Rb^{N750F/N750F}p130^{+/-}$, $Rb^{N750F/wt}p130^{-/-}$ and $Rb^{N750F/N750F}p107^{+/-}$ animals were not predisposed to tumorigenesis, although these genotypes did affect survival.

Possible causes of increased mortality in $Rb^{N750F/wt}p130^{-/-}$ animals

In an attempt to explain the strongly reduced lifespan of $Rb^{N750F/wt}p130^{-/-}$ animals we looked for defects in the hematopoietic compartment. Defective hematopoiesis was suggested by the reduced number of hematopoietic cells in the red pulp of the spleen (referred to as 'reduced cellularity') in 24% of sacrificed $Rb^{N750F/wt}p130^{-/-}$ animals (n=21; included are the animals described above that were analyzed for the spleen, minus the animal that was diagnosed with lymphoma). Reduced cellularity was not detected in spleens of sacrificed $Rb^{N750F/wt}p130^{+/-}$ and $Rb^{wt/wt}p130^{-/-}$ animals (both n=6). Increased break down of red blood cells (hemosiderosis) in the spleen was detected in 38% of $Rb^{N750F/wt}p130^{-/-}$ mice, but also in $Rb^{N750F/wt}p130^{+/-}$ and $Rb^{wt/wt}p130^{-/-}$ mice (30 and 50%, respectively). Although the pocket proteins have been reported to function in controlling proliferation and mobilization of hematopoietic stem cells in the bone marrow (Viatour et al., 2008; Walkley et al., 2007), we were unable to link the defects in the spleen to defects in the bone marrow. First, we microscopically analyzed bone marrow in hind leg, head and/or sternum. Table 5 summarizes this analysis, which was performed on 8 ill $Rb^{N750F/wt}p130^{-/-}$ animals with abnormalities in the spleen (reduced cellularity and/or hemosiderosis), and on 5 ill $Rb^{N750F/wt}p130^{-/-}$ animals, in which no abnormalities in the spleen were detected. Reduced cellularity in the red pulp of the spleen was in 3 out of 5 cases accompanied with hemosiderosis but 3 cases of hemosiderosis were seen with normal cellularity, indicating that these two abnormalities were unrelated. Furthermore, reduced cellularity in the red pulp of the spleen was accompanied by reduced cellularity in the white pulp in 2 out of 5 cases and by increased apoptosis in the white pulp in 2 of 5 cases (Table 5). Additionally, in two out of five cases, hematopoietic defects in the spleen coincided with reduced hematopoiesis in the bone marrow (animals 1 and 2 in Table 5, both animals displayed reduced cellularity in both red and white pulp). In conclusion, reduced cellularity in the spleen could not be explained by increased breakdown of red blood cells in the red pulp or increased apoptosis in the white pulp. Furthermore, defective production of hematopoietic cells in the bone marrow could not account for the observed reduced cellularity in the red pulp of the spleen. Strikingly though, reduced cellularity in the bone marrow was observed in the 2 animals that displayed reduced cellularity in *both* the red and the white pulp of the spleen.

Finally, we determined red and white blood cell counts in 5 ill $Rb^{N750F/wt}p130^{-/-}$ animals (Table 6). Compared to the reported minimum values in healthy mice (Carpenter, 2005; Quesenberry and Carpenter, 2003), red blood cell count was low in 2 of the 5

Animal	Cellularity in hematopoietic compartment in red pulp of spleen	Cellularity in white pulp of spleen	Levels of apoptosis in white pulp of spleen	Bone marrow defects in sternum	Bone marrow defects in bones of hind limb	Bone marrow defects in bones of the head
1	reduced, hemosiderosis is present	reduced	normal	reduced hematopoiesis	reduced hematopoiesis	no
2	reduced	reduced	high	nd	reduced hematopoiesis	no
3	reduced, hemosiderosis is present	normal	normal	no	no	no
4	reduced, hemosiderosis is present	normal	high	nd	no	no
5	reduced	normal	normal	no	no	no
6	normal, hemosiderosis is present	normal	normal	no	no	no
7	normal, hemosiderosis is present	normal	normal	no	no	no
8	normal hemosiderosis is present	normal	high	no	nd	nd
9	normal	normal	normal	no	no	no
10	normal	normal	normal	no	nd	nd
11	normal	normal	normal	no	nd	nd
12	normal	normal	normal	no	nd	nd
13	normal	normal	normal	nd	no	no

Table 5: Microscopic analysis of spleen and bone marrow in ill $Rb^{N750F/wt}p130^{-/-}$ animals (n=13).

Animal	RBC count in peripheral blood/ minimum value in healthy mice	WBC count in peripheral blood/ minimum value in healthy mice
1	1,17	1,25
2	1,54	0,45
3	0,60	0,25
7	0,51	0,15
13	1,34	1,90

Table 6: Red and white blood cell count in ill $Rb^{N750F/wt}p130^{-/-}$ animals. Animal numbering corresponds to that in Table 5. Depicted is the red or white blood cell count in the peripheral blood divided by the minimum reported value in healthy mice.

animals (mouse 3 and 7), whereas white blood cell count was low in 3 of the 5 mice (mouse 2, 3 and 7). Thus, reduced cellularity in the spleen did not correlated with reduced red blood cell count (compare Tables 5 and 6) and therefore the cause and significance of this observation remain elusive.

To investigate the hematopoietic system in more detail, FACS analysis was performed for spleen, bone marrow, peripheral blood and thymus in four $Rb^{N750F/wt}p130^{-/-}$ animals, of which two where severely ill, and in two healthy $Rb^{N750F/wt}p130^{+/-}$ and two healthy $Rb^{wt/wt}p130^{-/-}$ animals (Table S1 and data not shown). Consistent with the lack of abnormalities in the microscopic analysis (Table 5), we detected normal percentages of the following populations in the bone marrow of $Rb^{N750F/wt}p130^{-/-}$ animals: LSK, LT-HSC, ST-HSC, myeloid progenitors, lymphoid progenitors, erythroid progenitors, monocytes and granulocytes (data not shown). Two of the four $Rb^{N750F/wt}p130^{-/-}$ animals had a decreased population of cells with intermediate levels of staining for CD19 and B220, which possibly reflects a defective pre-B cell population (mouse a and c in Table S1; 0.75 and 1.7% respectively, versus on average 6.6% in $Rb^{N750F/wt}p130^{+/-}$ and $Rb^{wt/wt}p130^{-/-}$ control animals). However, mature B cells, characterized by high levels of CD19 and B220, where only mildly reduced (1.7 and 2.1%, versus on average 3.3% in control animals, see Table S1 for the percentages in the individual animals). Additionally, the percentage of B and T cells in spleen, peripheral blood and thymus were normal (data not shown), arguing against a B cell defect in $Rb^{N750F/wt}p130^{-/-}$ mice. In conclusion, both the microscopic and the FACS analyses argue against a major bone marrow defect in $Rb^{N750F/wt}p130^{-/-}$ animals. Microscopic analysis did show defects in the spleen, which may have contributed to the early death of $Rb^{N750F/wt}p130^{-/-}$ animals. However, as only 24% of $Rb^{N750F/wt}p130^{-/-}$ animals displayed these defects, it is likely that other defects, which were not detected in our study, had contributed to the reduced life span of these mice.

Discussion

LxCxE-containing proteins are good candidates to execute pRB's tumor suppressor role. The recruitment of LxCxE-containing proteins by chromatin-bound pocket protein-E2F complexes has been associated with silencing of E2F target genes, and importantly, repression of E2F target genes involving pRB and LxCxE-containing proteins was detected under various growth inhibitory conditions, such as serum starvation (Isaac et al., 2006; Morrison et al., 2002), RAS^{V12}-induced senescence (Narita et al., 2003) and cell cycle arrest induced by $p16^{INK4A}$ (Dahiya et al., 2001). Additionally, pRB is required for the establishment of irreversible cell cycle arrest during differentiation (Guo et al., 2009; Blais et al., 2007; Novitch et al., 1996), which possibly involves silencing of cell cycle genes by LxCxE-containing proteins. For example, pRB-mediated silencing of cell cycle genes during muscle differentiation correlated with the presence of repressive chromatin marks (Blais et al., 2007) and additionally, the Suv39H1 methylase, which binds to the pocket proteins at the LxCxE-binding site, has been implicated in establishing terminal silencing of cell cycle genes during muscle differentiation (Ait-Si-Ali et al., 2004).

We aimed to determine whether the capacity of pRB to recruit LxCxE-containing proteins is critical for mouse development and survival and for suppression of tumorigenesis. Therefore, we generated $Rb^{N750F/N750F}$ mice, which expressed the pRB^{N750F} protein from the endogenous locus. The pRB^{N750F} protein retained the ability to inhibit E2F-induced transactivation, but was impaired in binding LxCxE-containing proteins. pRB^{N750F} was impaired in repressing transcription in various reporter assays and was unable to bind the LxCxE-containing protein SV40 large T antigen (Chapter 4). Because of the compensatory role of p130 and p107, which could bind chromatin remodeling proteins via their LxCxE binding sites (Nicolas et al., 2003; Ferreira et al., 1998) and were detected at repressed, de-acetylated promoters in G_0 (Balciunaite et al., 2005; Rayman et al., 2002; Takahashi et al., 2000), we combined the Rb^{N750F} mutation with loss of either *p130* or *p107*.

Embryonic development

In sharp contrast to the embryonic lethal phenotype of $Rb^{-/-}$ mice (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992), $Rb^{N750F/N750F}$ mice were born alive and had a normal lifespan (Table 1 and Fig. 1). Strikingly though, $Rb^{N750F/N750F}p130^{-/-}$ animals could not be recovered at postnatal day 21 (Table 2), and we have strong indications that these animals had died during late embryonic development (post E18.5). Unfortunately, the effect of p107 ablation on survival of $Rb^{N750F/N750F}$ mice could not be assessed as p107 deficiency alone already caused perinatal death in FVB mice (Table 3). Our results imply that recruitment of chromatin remodeling complexes is essential for completion of embryonic development and that this can be achieved by either pRB-E2F4/E2F3b or p130-E2F4/

E2F5 complexes. The importance of LxCxE-mediated interactions during development is consistent with previous studies on *p130*$^{-/-}$*p107*$^{-/-}$ and *E2F4*$^{-/-}$*E2F5*$^{-/-}$ mice, which could not be recovered as life newborns (Gaubatz et al., 2000; Cobrinik et al., 1996). Thus, partial ablation of pocket protein-E2F-chromatin remodeling complexes is not compatible with postnatal survival.

Tumorigenesis

Rb$^{N750F/N750F}$ mice had a normal lifespan and where not tumor prone (Fig. 1). Since *Rb*$^{+/-}$ mice and chimeric *Rb*$^{-/-}$ mice were strongly predisposed to pituitary and thyroid tumors (Harrison et al., 1995; Maandag et al., 1994; Williams et al., 1994), this implies that, in a p130/p107-proficient background, regulation of E2F transactivation activity by pRB is sufficient for pRB's tumor suppressive activity. To our surprise, also *Rb*$^{N750F/N750F}$*p130*$^{+/-}$, *Rb*$^{N750F/wt}$*p130*$^{-/-}$ and *Rb*$^{N750F/N750F}$*p107*$^{+/-}$ mice did not show increased tumor susceptibility, although these animals, in particular *Rb*$^{N750F/wt}$*p130*$^{-/-}$ mice, did show a reduced lifespan (Fig. 2). Our previous studies demonstrated that *Rb*$^{+/-}$*p107*$^{-/-}$ chimeric mice developed a wide spectrum of tumors. Importantly, the wild-type *Rb* allele was lost in 100% of the detected pituitary tumors and in 70% of the other tumors (osteosarcomas, lymphosarcomas, adenocarcinomas in the coecum, and others). Also *Rb*$^{+/-}$*p130*$^{-/-}$ chimeric mice developed tumors, although with lower incidence (5 tumors were detected in 15 chimeras (Dannenberg et al., 2004)). The absence of tumor susceptibility in *Rb*$^{N750F/N750F}$ *p130*$^{+/-}$, *Rb*$^{N750F/wt}$*p130*$^{-/-}$ and *Rb*$^{N750F/N750F}$*p107*$^{+/-}$ mice implies that *Rb*$^{N750F/N750F}$*p130*$^{-/-}$ and *Rb*$^{N750F/N750F}$*p107*$^{-/-}$ cells, which are expected to frequently arise by spontaneous loss of heterozygosity, do not readily undergo oncogenic transformation. Our results therefore argue against a major role for LxCxE-containing proteins in tumor suppression and rather suggest that inhibiting the transactivation function of E2Fs is the main mechanism of tumor suppression by pRB. This finding is surprising in view of the importance of LxCxE-containing proteins in transcriptional regulation and cell cycle arrest. Moreover, the human mutant protein pRB^{R611W} causes retinoblastoma with reduced penetrance, although it is impaired in binding E2Fs (Sellers et al., 1998; Otterson et al., 1997). It has therefore been suggested that, next to E2Fs, other pRB interactors are involved in pRB-mediated tumor suppression. LxCxE-containing proteins were considered good candidates, but this is now questioned by our observations.

There are several explanations for the absence of tumor predisposition in mice with an ablated pRB-LxCxE interaction. First, chromatin remodelers that interact with pRB independent of the LxCxE-binding site might contribute to pRB-mediated silencing. Via its LxCxE binding site, pRB interacts with chromatin remodelers, such as Suv39h1, HP1 and HDAC1 and -2, which are co-operatively involved in silencing. For example, Suv39h1, which enhances pRB-mediated repression (Nielsen et al., 2001) and creates a

binding site for HP1 (Lachner et al., 2001), can only methylate H3K9 when this residue is not acetylated (Rea et al., 2000), suggesting a cooperation between pRB, Suv39h1, HDACs and HP1 in silencing. Indeed, Suv39h1 was shown to interact with HDAC1, 2 and 3 and Suv39h1-mediated repression required HDAC activity (Vaute et al., 2002). Moreover, both pRB, Suv39h1 and HP1 bound to a methylated H3K9 peptide (Nielsen et al., 2001). However, also LxCxE-independent interactions between pRB and chromatin remodelers have been described, such as the interaction between pRB and Suv4-20h1 and Suv4-20h2 (Isaac et al., 2006). It is therefore possible that silencing can also be achieved by chromatin remodelers that interact with pRB outside the LxCxE-binding domain.

Second, we haven't studied the effect of *complete* ablation of the pocket protein-LxCxE interaction, so possibly, the remaining pocket protein forms sufficient LxCxE dependent interactions to inhibit tumor formation. Embryonic lethality of $Rb^{N750F/N750F}$ $p130^{-/-}$ and $Rb^{N750F/N750F}p107^{-/-}$ mice complicated our analysis but it may be possible to analyze tumor predisposition in chimeras generated with $Rb^{N750F/N750F}p130^{-/-}p107^{-/-}$ ES cells.

Third, one can envision that, although we did not detect tumor predisposition in this study, loss of the pRB-LxCxE interaction may stimulate tumor formation under specific conditions. Interestingly, we have found that $Rb^{N750F/N750F}p130^{-/-}$ MEFs were impaired in arresting the cell cycle upon γ-irradiation or expression of oncogenic RAS^{V12} (Chapter 4). This demonstrates that G_1 arrest after DNA damage involves the interaction between pRB and LxCxE-containing proteins, suggesting that mice deficient for the pRB-LxCxE interaction might be susceptible to tumor formation upon DNA damage.

In conclusion, we have shown that ablating the interaction between pocket proteins and LxCxE-containing proteins was not sufficient to initiate tumorigenesis. This may be explained by a compensatory role of a remaining pocket protein or non-LxCxE-containing chromatin remodelers that still interact with mutant pRB. Nonetheless, as the pRB-LxCxE interaction was involved in establishing cell cycle arrest in MEFs in response to expression of oncogenic RAS or ionizing radiation (Chapter 4), it is of interest to assess the susceptibility of $Rb^{N750F/N750F}$ mice to radiation- or carcinogen-induced tumorigenesis.

Materials and Methods

Mice

$Rb^{N750F/wt}$ mice were previously generated by injecting an $Rb^{N750F/wt}$ embryonic stem cell clone into B6 blastocysts. The $Rb^{N750F/wt}$ embryonic stem cell clone was generated by oligonucleotide-directed modification of the endogenous *Rb* gene in 129OLA embryonic stem cells (Aarts et al., 2006). The resulting $Rb^{N750F/wt}$ chimeras were backcrossed to FVB. Intercrossing the resulting $Rb^{N750F/wt}$ mice generated both $Rb^{N750F/N750F}$ and $Rb^{N750F/wt}$ mice, which were used to study tumor predisposition. $Rb^{N750F/wt}$ mice were also crossed with previously generated $p130^{wt/-}$ FVB mice (Dannenberg et al., 2000), generating $Rb^{N750F/wt}p130^{wt/-}$ mice. Intercrossing these animals generated $Rb^{N750F/N750F}p130^{wt/-}$, $Rb^{N750F/wt}p130^{-/-}$ and $Rb^{N750F/wt}p130^{wt/-}$ mice, which were used to study tumor predisposition. Additionally, $Rb^{N750F/wt}$ mice were crossed with $p107^{wt/-}$ FVB mice, which were previously generated by injecting $p107^{-/-}$ ES cells (Robanus-Maandag et al., 1998) into B6 blastocysts and subsequent backcrossing of the resulting chimeras with FVB mice. $Rb^{N750F/wt}p107^{wt/-}$ mice were intercrossed, generating $Rb^{N750F/N750F}p107^{wt/-}$ mice $Rb^{N750F/wt}p107^{wt/-}$ mice, which were used to study tumor predisposition. Mice were inspected twice a week and sacrificed when ill.

Cellular preparations and FACS analysis

All cellular preparations were performed at 4°C, except where indicated. *Splenocytes:* spleens were chopped with scissors and digested for 20 min at room temperature in a mixture of 6 mL RPMI-2% containing 7 mg Collagenase (121 U/mg - Type III, Worthington Biochemicals) with a further addition of 1 mL of DNase (1mg/mL) (Roche). Pipetting the tissue through a wide bore pipette tip repetitively during digestion facilitated dispersion to single cell suspension. EDTA (600 mL of 0.1 M solution) was then added and mixed by pipetting for a further 5 min to ensure separation of dendritic cells and T cells. Undigested fragments were removed by using a 70 µM filter. *Bone marrow:* bone marrow was extracted from the tibia and femur with RMPI-2% using a 3 mL syringe and 21-gauge needle. This single cell suspension was then centrifuged and the pellet was resuspended in a small volume (0.5-2 mL) RCRB for 1 minute to lyse erythrocytes. Subsequently, cells were diluted up to 10 mL in RPMI-2% and washed 2-3 times. Pellets were resuspended in RPMI-2% and passed through a sieve to remove bone fragments and dead cell clumps. *Blood leukocytes:* blood was collected by lumbar puncture into heparinized syringes using a 25-gauge needle and collected directly into 1mL of RCRB, mixed and kept on ice. Cells were centrifuged, washed in medium and resuspended in FACS buffer. *Thymus:* thymus cells were isolated by mashing through a 70 µM filter. *Staining:* cells were stained in the antibody cocktails for 15-20 min on ice at a concentration of 1 x 10^6 per 10 µL in FACS buffer containing 2mM EDTA and 2% BSA in PBS. Cells were subsequently washed in 10 volumes of FACS buffer and subsequently suspended in FACS buffer plus propidium iodide. FACS analysis was performed using a Cyan flow cytometer (Dako). Stains used to identify the different cell populations were as follows: LSK: c-kit high/sca1 high; LT-HSC: pre-gate: c-kit high/sca1 high gate: CD150-slam high/CD48 low; ST-HSC: pre-gate: c-kit high/sca1 high gate: CD150-slam low/CD48 high; myeloid progenitors: pre-gate (1): lin negative/c-kit positive; pre-gate (2): TER119 negative; gate: FC receptor high/IL7 receptor low; lymphoid progenitors: pre-gate (1): lin negative/c-kit positive; pre-gate (2): TER119 negative; gate: FC receptor low/IL7 receptor high; erythroid progenitors: pre-gate: lin negative/c-kit positive; gate: TER119 high; monocytes: Ly6Chi ^high/Gr1^int; granulacytes: Ly6Chi^intermediate/Gr1^hi; B-cells: CD19 high/CD3 low and CD19 high/B220 high; T-cells: CD19 low/CD3 high and B220 low/CD3 high.

Determination of red and white blood cell count in peripheral blood

Blood was collected into heparinized tubes and diluted 2x in PBS. Red- and white blood cell counts were determined using a Coulter-counter.

Acknowledgements

We thank Tanja van Harn, Sandra de Vries, Sietske Bakker and Camiel Wielders for helpful discussions and members of the animal and histology facilities of the NKI for techical assistance. This work was supported by the Dutch Cancer Society, grant nr. NKI 2002-2634.

References

Aarts,M., Dekker,M., de,V.S., van der Wal,A., and te Riele,H. (2006). Generation of a mouse mutant by oligonucleotide-mediated gene modification in ES cells. Nucleic Acids Res. *34*, e147.

Ait-Si-Ali,S., Guasconi,V., Fritsch,L., Yahi,H., Sekhri,R., Naguibneva,I., Robin,P., Cabon,F., Polesskaya,A., and Harel-Bellan,A. (2004). A Suv39h-dependent mechanism for silencing S-phase genes in differentiating but not in cycling cells. EMBO J. *23*, 605-615.

Balciunaite,E., Spektor,A., Lents,N.H., Cam,H., te Riele,H., Scime,A., Rudnicki,M.A., Young,R., and Dynlacht,B.D. (2005). Pocket protein complexes are recruited to distinct targets in quiescent and proliferating cells. Mol. Cell Biol. *25*, 8166-8178.

Blais,A., van Oevelen,C.J., Margueron,R., costa-Alvear,D., and Dynlacht,B.D. (2007). Retinoblastoma tumor suppressor protein-dependent methylation of histone H3 lysine 27 is associated with irreversible cell cycle exit. J. Cell Biol. *179*, 1399-1412.

Carpenter,J.W. (2005). Exotic Animal Formulary, third edition. Elsevier Saunders).

Chen,P.L., Riley,D.J., Chen,Y., and Lee,W.H. (1996). Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes Dev. *10*, 2794-2804.

Chen,T.T. and Wang,J.Y. (2000). Establishment of irreversible growth arrest in myogenic differentiation requires the RB LXCXE-binding function. Mol. Cell Biol. *20*, 5571-5580.

Chong,J.L., Tsai,S.Y., Sharma,N., Opavsky,R., Price,R., Wu,L., Fernandez,S.A., and Leone,G. (2009a). E2f3a and E2f3b contribute to the control of cell proliferation and mouse development. Mol. Cell Biol. *29*, 414-424.

Chong,J.L., Wenzel,P.L., Saenz-Robles,M.T., Nair,V., Ferrey,A., Hagan,J.P., Gomez,Y.M., Sharma,N., Chen,H.Z., Ouseph,M., Wang,S.H., Trikha,P., Culp,B., Mezache,L., Winton,D.J., Sansom,O.J., Chen,D., Bremner,R., Cantalupo,P.G., Robinson,M.L., Pipas,J.M., and Leone,G. (2009b). E2f1-3 switch from activators in progenitor cells to repressors in differentiating cells. Nature *462*, 930-934.

Clarke,A.R., Maandag,E.R., van,R.M., van der Lugt,N.M., van der Valk,M., Hooper,M.L., Berns,A., and te Riele,H. (1992). Requirement for a functional Rb-1 gene in murine development. Nature *359*, 328-330.

Cobrinik,D., Lee,M.H., Hannon,G., Mulligan,G., Bronson,R.T., Dyson,N., Harlow,E., Beach,D.,

Weinberg,R.A., and Jacks,T. (1996). Shared role of the pRB-related p130 and p107 proteins in limb development. Genes Dev. *10*, 1633-1644.

Dahiya,A., Gavin,M.R., Luo,R.X., and Dean,D.C. (2000). Role of the LXCXE binding site in Rb function. Mol. Cell Biol. *20*, 6799-6805.

Dahiya,A., Wong,S., Gonzalo,S., Gavin,M., and Dean,D.C. (2001). Linking the Rb and polycomb pathways. Mol. Cell *8*, 557-569.

Dannenberg,J.H., Schuijff,L., Dekker,M., van der Valk,M., and te Riele,H. (2004). Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev. *18*, 2952-2962.

Dannenberg,J.H., van Rossum,A., Schuijff,L., and te Riele,H. (2000). Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. Genes Dev. *14*, 3051-3064.

DeGregori,J. and Johnson,D.G. (2006). Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis. Curr. Mol. Med. *6*, 739-748.

Dick,F.A. (2007). Structure-function analysis of the retinoblastoma tumor suppressor protein - is the whole a sum of its parts? Cell Div. *2*, 26.

Dimova,D.K. and Dyson,N.J. (2005). The E2F transcriptional network: old acquaintances with new faces. Oncogene *24*, 2810-2826.

Ferreira,R., Magnaghi-Jaulin,L., Robin,P., Harel-Bellan,A., and Trouche,D. (1998). The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase. Proc. Natl. Acad. Sci. U. S. A *95*, 10493-10498.

Frolov,M.V. and Dyson,N.J. (2004). Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. J. Cell Sci. *117*, 2173-2181.

Gaubatz,S., Lindeman,G.J., Ishida,S., Jakoi,L., Nevins,J.R., Livingston,D.M., and Rempel,R.E. (2000). E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control. Mol. Cell *6*, 729-735.

Gu,W., Schneider,J.W., Condorelli,G., Kaushal,S., Mahdavi,V., and Nadal-Ginard,B. (1993). Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell *72*, 309-324.

Guo,J., Longshore,S., Nair,R., and Warner,B.W. (2009). Retinoblastoma protein (pRb), but not p107 or p130, is required for maintenance of enterocyte quiescence and differentiation in small intestine. J. Biol. Chem. *284*, 134-140.

Harrison,D.J., Hooper,M.L., Armstrong,J.F., and Clarke,A.R. (1995). Effects of heterozygosity for the Rb-1t19neo allele in the mouse. Oncogene *10*, 1615-1620.

Isaac,C.E., Francis,S.M., Martens,A.L., Julian,L.M., Seifried,L.A., Erdmann,N., Binne,U.K., Harrington,L., Sicinski,P., Berube,N.G., Dyson,N.J., and Dick,F.A. (2006). The retinoblastoma protein regulates pericentric heterochromatin. Mol. Cell Biol. *26*, 3659-3671.

Jacks,T., Fazeli,A., Schmitt,E.M., Bronson,R.T., Goodell,M.A., and Weinberg,R.A. (1992). Effects

of an Rb mutation in the mouse. Nature *359*, 295-300.

Khidr,L. and Chen,P.L. (2006). RB, the conductor that orchestrates life, death and differentiation. Oncogene *25*, 5210-5219.

Kinross,K.M., Clark,A.J., Iazzolino,R.M., and Humbert,P.O. (2006). E2f4 regulates fetal erythropoiesis through the promotion of cellular proliferation. Blood *108*, 886-895.

Lachner,M., O'Carroll,D., Rea,S., Mechtler,K., and Jenuwein,T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*, 116-120.

Lai,A., Kennedy,B.K., Barbie,D.A., Bertos,N.R., Yang,X.J., Theberge,M.C., Tsai,S.C., Seto,E., Zhang,Y., Kuzmichev,A., Lane,W.S., Reinberg,D., Harlow,E., and Branton,P.E. (2001). RBP1 recruits the mSIN3-histone deacetylase complex to the pocket of retinoblastoma tumor suppressor family proteins found in limited discrete regions of the nucleus at growth arrest. Mol. Cell Biol. *21*, 2918-2932.

Lee,E.Y., Cam,H., Ziebold,U., Rayman,J.B., Lees,J.A., and Dynlacht,B.D. (2002). E2F4 loss suppresses tumorigenesis in Rb mutant mice. Cancer Cell *2*, 463-472.

Lee,E.Y., Chang,C.Y., Hu,N., Wang,Y.C., Lai,C.C., Herrup,K., Lee,W.H., and Bradley,A. (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature *359*, 288-294.

Maandag,E.C., van der Valk,M., Vlaar,M., Feltkamp,C., O'Brien,J., van Roon,M., van der Lugt,N., Berns,A., and te Riele,H. (1994). Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. EMBO J. *13*, 4260-4268.

MacLellan,W.R., Garcia,A., Oh,H., Frenkel,P., Jordan,M.C., Roos,K.P., and Schneider,M.D. (2005). Overlapping roles of pocket proteins in the myocardium are unmasked by germ line deletion of p130 plus heart-specific deletion of Rb. Mol. Cell Biol. *25*, 2486-2497.

Magnaghi-Jaulin,L., Groisman,R., Naguibneva,I., Robin,P., Lorain,S., Le Villain,J.P., Troalen,F., Trouche,D., and Harel-Bellan,A. (1998). Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature *391*, 601-605.

Moberg,K., Starz,M.A., and Lees,J.A. (1996). E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. Mol. Cell Biol. *16*, 1436-1449.

Morrison,A.J., Sardet,C., and Herrera,R.E. (2002). Retinoblastoma protein transcriptional repression through histone deacetylation of a single nucleosome. Mol. Cell Biol. *22*, 856-865.

Muller,H., Moroni,M.C., Vigo,E., Petersen,B.O., Bartek,J., and Helin,K. (1997). Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. Mol. Cell Biol. *17*, 5508-5520.

Narita,M., Nunez,S., Heard,E., Narita,M., Lin,A.W., Hearn,S.A., Spector,D.L., Hannon,G.J., and Lowe,S.W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell *113*, 703-716.

Nicolas,E., Roumillac,C., and Trouche,D. (2003). Balance between acetylation and methylation of histone H3 lysine 9 on the E2F-responsive dihydrofolate reductase promoter. Mol. Cell Biol. *23*, 1614-1622.

Nielsen,S.J., Schneider,R., Bauer,U.M., Bannister,A.J., Morrison,A., O'Carroll,D., Firestein,R., Cleary,M., Jenuwein,T., Herrera,R.E., and Kouzarides,T. (2001). Rb targets histone H3 methylation and HP1 to promoters. Nature *412*, 561-565.

Novitch,B.G., Mulligan,G.J., Jacks,T., and Lassar,A.B. (1996). Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. J. Cell Biol. *135*, 441-456.

Otterson,G.A., Chen,W., Coxon,A.B., Khleif,S.N., and Kaye,F.J. (1997). Incomplete penetrance of familial retinoblastoma linked to germ-line mutations that result in partial loss of RB function. Proc. Natl. Acad. Sci. U. S. A *94*, 12036-12040.

Parisi,T., Bronson,R.T., and Lees,J.A. (2009). Inhibition of pituitary tumors in Rb mutant chimeras through E2f4 loss reveals a key suppressive role for the pRB/E2F pathway in urothelium and ganglionic carcinogenesis. Oncogene *28*, 500-508.

Quesenberry,K.E. and Carpenter,J.W. (2003). Ferrets, Rabbits and Rodents: Clinical Medicine and Surgery. Saunders).

Rayman,J.B., Takahashi,Y., Indjeian,V.B., Dannenberg,J.H., Catchpole,S., Watson,R.J., te Riele,H., and Dynlacht,B.D. (2002). E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. Genes Dev. *16*, 933-947.

Rea,S., Eisenhaber,F., O'Carroll,D., Strahl,B.D., Sun,Z.W., Schmid,M., Opravil,S., Mechtler,K., Ponting,C.P., Allis,C.D., and Jenuwein,T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature *406*, 593-599.

Robanus-Maandag,E., Dekker,M., van der Valk,M., Carrozza,M.L., Jeanny,J.C., Dannenberg,J.H., Berns,A., and te Riele,H. (1998). p107 is a suppressor of retinoblastoma development in pRb-deficient mice. Genes Dev. *12*, 1599-1609.

Rowland,B.D., Denissov,S.G., Douma,S., Stunnenberg,H.G., Bernards,R., and Peeper,D.S. (2002). E2F transcriptional repressor complexes are critical downstream targets of p19(ARF)/p53-induced proliferative arrest. Cancer Cell *2*, 55-65.

Sahin,F. and Sladek,T.L. (2010). E2F-1 has dual roles depending on the cell cycle. Int. J. Biol. Sci. *6*, 116-128.

Sellers,W.R., Novitch,B.G., Miyake,S., Heith,A., Otterson,G.A., Kaye,F.J., Lassar,A.B., and Kaelin,W.G., Jr. (1998). Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth. Genes Dev. *12*, 95-106.

Takahashi,Y., Rayman,J.B., and Dynlacht,B.D. (2000). Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. Genes Dev. *14*, 804-816.

Thomas,D.M., Carty,S.A., Piscopo,D.M., Lee,J.S., Wang,W.F., Forrester,W.C., and Hinds,P.W. (2001). The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol. Cell *8*, 303-316.

Tsai,S.Y., Opavsky,R., Sharma,N., Wu,L., Naidu,S., Nolan,E., Feria-Arias,E., Timmers,C., Opavska,J., de,B.A., Chong,J.L., Trikha,P., Fernandez,S.A., Stromberg,P., Rosol,T.J., and Leone,G.

(2008). Mouse development with a single E2F activator. Nature *454*, 1137-1141.

Vandel,L., Nicolas,E., Vaute,O., Ferreira,R., it-Si-Ali,S., and Trouche,D. (2001). Transcriptional repression by the retinoblastoma protein through the recruitment of a histone methyltransferase. Mol. Cell Biol. *21*, 6484-6494.

Vaute,O., Nicolas,E., Vandel,L., and Trouche,D. (2002). Functional and physical interaction between the histone methyl transferase Suv39H1 and histone deacetylases. Nucleic Acids Res. *30*, 475-481.

Viatour,P., Somervaille,T.C., Venkatasubrahmanyam,S., Kogan,S., McLaughlin,M.E., Weissman,I.L., Butte,A.J., Passegue,E., and Sage,J. (2008). Hematopoietic stem cell quiescence is maintained by compound contributions of the retinoblastoma gene family. Cell Stem Cell *3*, 416-428.

Walkley,C.R., Shea,J.M., Sims,N.A., Purton,L.E., and Orkin,S.H. (2007). Rb regulates interactions between hematopoietic stem cells and their bone marrow microenvironment. Cell *129*, 1081-1095.

Williams,B.O., Schmitt,E.M., Remington,L., Bronson,R.T., Albert,D.M., Weinberg,R.A., and Jacks,T. (1994). Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. EMBO J. *13*, 4251-4259.

Wu,L., Timmers,C., Maiti,B., Saavedra,H.I., Sang,L., Chong,G.T., Nuckolls,F., Giangrande,P., Wright,F.A., Field,S.J., Greenberg,M.E., Orkin,S., Nevins,J.R., Robinson,M.L., and Leone,G. (2001). The E2F1-3 transcription factors are essential for cellular proliferation. Nature *414*, 457-462.

Yamasaki,L., Bronson,R., Williams,B.O., Dyson,N.J., Harlow,E., and Jacks,T. (1998). Loss of E2F-1 reduces tumorigenesis and extends the lifespan of Rb1(+/-)mice. Nat. Genet. *18*, 360-364.

Zhang,H.S., Postigo,A.A., and Dean,D.C. (1999). Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFbeta, and contact inhibition. Cell *97*, 53-61.

Supplemental Table

Animal	Genotype	Sex	Macroscopic appearance	Age (months)	% of cells expressing intermediate levels of CD19/B220 in bone marrow	% of cells expressing high levels of CD19/B220 in bone marrow
a	$Rb^{N750F/wt}p130^{-/-}$	female	ill	5	0,75	1,7
b	$Rb^{N750F/wt}p130^{-/-}$	female	ill	5	7,3	0,8
c	$Rb^{N750F/wt}p130^{-/-}$	female	relatively healthy	9	1,7	2,1
d	$Rb^{N750F/wt}p130^{-/-}$	female	relatively healthy	6	3,3	6
e	$Rb^{N750F/wt}p130^{+/-}$	female	healthy	6	7,5	4,1
f	$Rb^{N750F/wt}p130^{+/-}$	female	healthy	5	5,6	2,5
g	$Rb^{wt/wt}p130^{-/-}$	female	healthy	9	7	2,7
h	$Rb^{wt/wt}p130^{-/-}$	female	healthy	7	6,2	4

Table S1: FACS analysis of bone marrow of mice with the indicated genotypes shows that 2 out of 4 $Rb^{N750F/wt}p130^{-/-}$ mice display a decreased amount of cells with an intermediate level of staining for CD19 and B220 (possibly reflecting a pre-B cell population).

CHAPTER 6

General discussion

Tumorigenesis is a gradual process, which includes multiple alterations in both tumor suppressor- and oncogenic pathways. The retinoblastoma pathway comprises a major tumor suppressor pathway and is often found deregulated in both human and mouse cancer (Hanahan and Weinberg, 2000; Sherr, 1996). Ablation of the retinoblastoma proteins pRB, p107 and p130, also known as 'pocket proteins', bypassed cell cycle arrest that is normally induced by several growth inhibitory signals *in vitro*, but was not sufficient to establish full transformation. We focused on the identification of genetic events, cooperating with loss of pocket proteins in transformation. Additionally, we analyzed the role of the interaction between pRB and proteins containing and LxCxE motif during cell cycle arrest and tumorigenesis.

Tumor suppression mechanisms in mouse embryonic fibroblasts (MEFs)

The family of pocket proteins plays a key role in cell cycle regulation by inhibiting E2F transcription factors during G_1 (Frolov and Dyson, 2004). Consistently, ablation of pocket proteins in MEFs abrogated G_1 arrest in response to various inhibitory signals, including the expression of constitutively active (ca) RAS, RAS^{V12}. However, our previous experiments demonstrated that pocket protein-ablation was not sufficient for RAS^{V12}-induced transformation and required additional events (Dannenberg et al., 2004; Dannenberg et al., 2000; Peeper et al., 2001). The first part of this thesis is focused on the identification of genetic events that in collaboration with loss of pocket proteins and expression of RAS^{V12} promote transformation of MEFs.

Requirements for RAS^{V12}-induced transformation

In a genetic screen we found that RAS^{V12}-induced transformation of pocket protein-deficient MEFs could be accomplished by expression of TBX2. Importantly, transformation was only achieved upon *concomitant* ablation of pocket proteins, expression of RAS^{V12} and over-expression of TBX2. Thus, expression of RAS^{V12} and TBX2 in the presence of pocket proteins was not sufficient for transformation. In line with the down-modulating role of TBX2 on the p53 pathway, we subsequently found that down-regulation of p53 or $p21^{CIP}$ by RNA interference was sufficient to promote anchorage-independent growth of RAS^{V12}/pocket protein-deficient MEFs (Vormer et al., 2008; **Chapter 2**). Our results demonstrate that MEFs were protected from transformation by the synergistic activities of the pocket protein- and the p53 pathways. This could be explained by their synergistic role in regulating the activity of cyclin-dependent kinases (cdks). Loss of pocket proteins induced both G_1- and G_2-associated cdk activities, which was dramatically counteracted by loss of anchorage. Dependent on the level of pocket proteins present, this resulted in cell cycle arrest in G_1 and G_2 phase. Loss of pocket proteins *plus* down-regulation of $p21^{CIP1}$ caused an additive rise in cdk activity, which was sufficient to overcome the suppression

of cdk activities by loss of anchorage, and could therefore induce transformation.

In **Chapter 3**, we describe a second genetic screen aimed to identify inducers of anchorage-independent growth of RAS^{V12}/pocket protein-deficient MEFs. In this screen, we identified Mapkapk3 as a suppressor of anchorage-independent growth of RAS^{V12}/pocket protein-deficient MEFs. Interestingly, Mapkapk3 was transcriptionally induced upon loss of anchorage, which could be counteracted by expression of TBX2. These results suggest that part of the transforming activity of TBX2 was mediated by down-regulation of Mapkapk3. Mapkapk3 has been linked to the induction of $p19^{ARF}$ and $p16^{INK4A}$ via release of BMI1 from the chromatin (Voncken et al., 2005), again pointing to the necessity of down-regulating both the pocket protein and the $p19^{ARF}$/p53 pathway during transformation.

In conclusion, the results described in **Chapters 2** and **3** point to the compensatory role of the pocket protein- and the p53 pathways during tumor suppression. Others have repeatedly claimed that down-regulation of either the pocket protein- or the p53/$p21^{CIP1}$ pathway was sufficient for RAS^{V12}-induced transformation of MEFs (Rangarajan et al., 2004; Sage et al., 2000). Our results, however, clearly demonstrate that ablation of both pathways is required to bypass cell cycle arrest under growth-inhibiting conditions. Both pathways regulate cdk activity and various combinations of disturbances to different degrees can add up to a situation enabling tumorigenesis.

In line with the results described above, the results described in **Chapter 4** further demonstrate that transformation of MEFs requires a very specific combination of events. Specifically, we found that MEFs, expressing a mutant form of pRB and deficient for p130 ($Rb^{N750F/N750F}p130^{-/-}$ MEFs), bypassed RAS^{V12}-induced senescence, but could not grow anchorage independently upon expression of RAS^{V12} and TBX2. This is in contrast to $Rb^{-/-}p130^{-/-}$ MEFs, which bypassed RAS^{V12}-induced senescence and were able to grow anchorage independently upon expression of RAS^{V12} and TBX2. Thus, pocket protein-ablation to a level sufficient to bypass RAS^{V12}-induced senescence, was not sufficient to support RAS^{V12}/TBX2-induced transformation. These results underline the stringent requirements for transformation. At the molecular level, this can possibly be explained by control of the E2F-target gene *Cyclin E*, which is known to involve the activity of chromatin remodeling proteins (Morrison et al., 2002; Dahiya et al., 2001; Nielsen et al., 2001). $Rb^{N750F/N750F}p130^{-/-}$ MEFs were impaired in the formation of pRB-chromatin remodeling complexes, whereas still able to inhibit E2F-mediated transactivation (see below). As a result, these MEFs expressed increased levels of Cyclin E compared to wild-type or $p130^{-/-}$ MEFs, but decreased levels compared to $Rb^{-/-}p130^{-/-}$ MEFs (**Chapter 4**). Possibly, the Cyclin E level in $Rb^{N750F/N750F}p130^{-/-}$ MEFs was sufficiently high to bypass RAS^{V12}-induced senescence, but insufficient to support TBX2/RAS^{V12}-induced transformation.

Senescence and DNA damage signaling pathways

Similar to expression of TBX2, we found that downregulation of Mapkapk3 or of p38 MAPK promoted anchorage-independent growth in RAS^{V12}/$Rb^{-/-}p107^{-/-}$ MEFs (**Chapter 3**). Previous studies have designated p38 as a mediator of RAS^{V12}-induced senescence, since chemical inhibition of p38 bypassed senescence that was induced by either RAS^{V12}, caMEK1 or caMKK3/6 (both upstream inducers of p38) (Wang et al., 2002; Han and Sun, 2007). This raises the possibility that despite bypass of RAS^{V12}-induced senescence under adherent conditions, pocket protein-deficient MEFs are still sensitive to some of the inhibitory effects of RAS^{V12}, which becomes apparent upon removal of anchorage. Loss of anchorage might induce p38/Mapkapk3, which needs to be inhibited in order to stimulate anchorage-independent growth of pocket protein-deficient cells.

As observed for loss of anchorage, the removal of growth factors induced G_2 arrest in $Rb^{-/-}p107^{-/-}p130^{-/-}$ (TKO) and TKO MEFs expressing Bcl2 (TKO-Bcl2 MEFs) (Foijer et al., 2005). Strikingly, growth factor deprivation in TKO-Bcl2 MEFs induced DNA double stranded breaks and Rad51/γH2AX foci. Moreover, inhibition of the DNA damage response accelerated cell cycle re-entry of mitogen re-stimulated TKO-Bcl2 MEFs (van Harn, Foijer and te Riele, in preparation). In line with this, Cremona and Lloyd (2009) suggested recently that culturing without anchorage could induce DNA damage. Specifically, large T-expressing rat Schwann cells were impaired in cell cycle progression upon removal of anchorage, which was linked to the formation of giant nuclei and an increase in the percentage of cells containing more than 42 chromosomes. In contrast, we were unable to detect DNA damage in arrested, anchorage deprived, RAS^{V12}/TKO MEFs (see below), although we did observe an increase in the percentage of cells with an 8N DNA content, which is indicative of endoreduplication. Several explanations can be envisaged. During a normal cell cycle, entry into M phase is induced by high CDK1 activity, whereas exit from M is driven by APC/C-induced proteolysis of Cyclin B1, resulting in down-regulation of CDK1 activity. Additionally, APC/C induces the proteolysis of Geminin, a protein that inhibits the assembly of pre-replication complexes. A combination of low CDK activity and proteolysis of Geminin during late M/early G_1 enables the assembly of pre-replication complexes at replication origins (Porter, 2008). Anchorage-deprived RAS^{V12}/TKO MEFs have low CDK1 activity due to high levels of $p21^{CIP1}$ and $p27^{KIP1}$. This prevents entry into M phase, but possibly renders them more sensitive to the premature assembly of pre-replication complexes. The latter may be achieved by aberrant down-regulation of Geminin or over-expression of factors promoting the assembly of pre-replication complexes, which would result in entry into a next G_1 phase without progression through M and cytokinesis. Alternatively, a subset of the anchorage-deprived cells may slip through G_2 and enter M phase in the absence of anchorage, but then encounter problems with cytokinesis. Both mechanisms would lead

to replication of a 4N genome causing the appearance of cells with an 8N DNA content in the absence of anchorage.

As mentioned above, we could not detect DNA damage in anchorage-deprived, RAS^{V12}/TKO MEFs. Specifically, RAS^{V12}/TKO MEFs were cultured without anchorage for 4 days and analyzed for the presence of γH2AX/Rad51 double foci after 2 and 4 hours of re-attachment. In striking contrast to the situation upon growth factor withdrawal, we only observed background levels of γH2AX/Rad51 double foci in re-attached RAS^{V12}/TKO MEFs (Vormer and van Harn, unpublished results). It remains possible that a high level of DNA damage prevented re-attachment, and thus prevented the detection of DNA damage in our set-up. Therefore, further study is required to determine whether loss of anchorage can promote the acquisition of DNA damage. Our preliminary results indicate that, if present, the magnitude of DNA damage is much lower in anchorage-deprived cells compared to growth factor-deprived cells. We speculate that growth factor deprivation in TKO MEFs interferes with DNA replication, which is likely to contribute to tumorigenesis via the promotion of genomic instability. In contrast, anchorage deprivation does not interfere with DNA replication and hence under these circumstances, G_2 arrest may act as a bona fide tumor suppressor mechanism.

Involvement of pocket protein-chromatin remodeling complexes in tumor suppression?

In the second part of this thesis, we aimed to determine the role of pocket protein-bound, LxCxE-containing proteins in tumor suppression.

During G_1, pocket proteins inhibit the activity of E2F transcription factors via binding and masking E2F's transactivation domain and via binding proteins containing an LxCxE- motif. Since many LxCxE-containing proteins function in chromatin remodeling and transcriptional repression, the formation of such complexes is thought to actively repress E2F target genes via the promotion of a chromatin structure that is unfavorable for transcription (Dick, 2007). Importantly, many studies have pointed to the involvement of pocket protein-chromatin remodeling complexes in (irreversible) cell cycle arrest and senescence (Narita et al., 2003; Morrison et al., 2002; Rayman et al., 2002; Dahiya et al., 2001; Chen and Wang, 2000).

To study the involvement of pocket protein-bound, LxCxE-containing proteins, we generated mice and MEFs expressing a mutant form of pRB (pRB^{N750F}). The pRB^{N750F} protein was unable to bind LxCxE-containing proteins, whereas inhibition of E2F-mediated transactivation remained intact (**Chapters 4** and **5**). Previous studies have suggested that mainly deregulation of 'activator' E2Fs contributed to the tumor susceptibility of $Rb^{+/-}$ mice (Lee et al., 2002; Yamasaki et al., 1998). However, the classical division between 'activator' and 'repressor' E2Fs seems incorrect, and a view is now emerging that individual E2Fs can perform both functions (Chong et al., 2009a;

Chong et al., 2009b; Kinross et al., 2006). The benefit of the current approach is that we studied exclusively the involvement of pocket protein-bound, LxCxE-containing proteins. Therefore, the interpretation of our data is independent of which E2F family member, 'activator 'or 'repressor', was bound by pRB.

In **Chapter 4**, we demonstrate that the pRB-LxCxE interaction was not required for cell cycle arrest in response to growth factor deprivation or contact inhibition. However, the pRB-LxCxE interaction was critical for cell cycle arrest in response to γ-irradiation or expression of RAS^{V12}, indicating that LxCxE-mediated repression of E2F target genes was involved during these types of cell cycle arrest. Strikingly, experiments by Avni and co-workers (2003) demonstrated that γ-irradiation caused recruitment of pRB to origins of replication. Together, this raises the possibility that upon DNA damage, pRB might directly inhibit DNA replication via the recruitment of an LxCxE-containing protein. It remains the subject of further study to determine which LxCxE-containing protein(s) is involved in cell cycle arrest under these conditions.

Although we found that ablation of the pRB-LxCxE interaction did alleviate RAS^{V12}-induced senescence, it did not promote *in vitro* transformation induced by RAS^{V12} and TBX2. Consistently, we found that $Rb^{N750F/N750F}$ mice had a normal lifespan and were not predisposed to tumorigenesis. Even upon concomitant inactivation of *p130* or *p107*, we could not detect tumor susceptibility. *I.e.*, $Rb^{N750F/N750F}p130^{+/-}$, $Rb^{N750F/wt}p130^{-/-}$ and $Rb^{N750F/N750F}p107^{+/-}$ mice were not tumor prone, although they had a (slightly) reduced lifespan (**Chapter 5**). To exclude that the absence of tumor predisposition was caused by the recruitment of LxCxE-containing proteins via the remaining pocket protein, it will be helpful to study tumor predisposition in chimeric $Rb^{N750F/N750F}p130^{-/-}p107^{-/-}$ mice. Nevertheless, the sharp contrast between strong tumor predisposition in $Rb^{+/-}$ mice and $Rb^{-/-}$ chimeras (Maandag et al., 1994; Williams et al., 1994) and the complete absence of tumor predisposition in $Rb^{N750F/N750F}$ mice, suggests that inhibition of E2F-mediated transactivation is the main tumor suppressor mechanism of pRB. Since we did observe an involvement of the pRB-LxCxE interaction during cell cycle arrest upon γ-irradiation or expression of RAS^{V12}, it remains possible that mice with an ablated pRB-LxCxE interaction display increased tumor susceptibility in response to for example irradiation or upon chemical activation of RAS signaling.

Lastly, we note that our experiments using pRB^{N750F} focused exclusively on the involvement of pRB-bound, LxCxE-containing proteins. As described in **Chapter 5**, pRB-mediated repression probably involves many different chromatin remodeling proteins, and moreover, complex formation between these remodelers and the pocket proteins has not been completely elucidated. Thus, ablation of the interaction between pRB and LxCxE-containing proteins might not be sufficient to completely block pRB-

mediated recruitment of chromatin remodeling complexes. Therefore, our study can not exclude that pocket protein-chromatin remodeling complexes could still contribute to tumor suppression. Importantly, our study does exclude a main involvement of the pRB-LxCxE interaction in tumor suppression *in vivo*.

References

Avni,D., Yang,H., Martelli,F., Hofmann,F., ElShamy,W.M., Ganesan,S., Scully,R., and Livingston,D.M. (2003). Active localization of the retinoblastoma protein in chromatin and its response to S phase DNA damage. Mol. Cell *12*, 735-746.

Chen,T.T. and Wang,J.Y. (2000). Establishment of irreversible growth arrest in myogenic differentiation requires the RB LXCXE-binding function. Mol. Cell Biol. *20*, 5571-5580.

Chong,J.L., Tsai,S.Y., Sharma,N., Opavsky,R., Price,R., Wu,L., Fernandez,S.A., and Leone,G. (2009a). E2f3a and E2f3b contribute to the control of cell proliferation and mouse development. Mol. Cell Biol. *29*, 414-424.

Chong,J.L., Wenzel,P.L., Saenz-Robles,M.T., Nair,V., Ferrey,A., Hagan,J.P., Gomez,Y.M., Sharma,N., Chen,H.Z., Ouseph,M., Wang,S.H., Trikha,P., Culp,B., Mezache,L., Winton,D.J., Sansom,O.J., Chen,D., Bremner,R., Cantalupo,P.G., Robinson,M.L., Pipas,J.M., and Leone,G. (2009b). E2f1-3 switch from activators in progenitor cells to repressors in differentiating cells. Nature *462*, 930-934.

Cremona,C.A. and Lloyd,A.C. (2009). Loss of anchorage in checkpoint-deficient cells increases genomic instability and promotes oncogenic transformation. J. Cell Sci. *122*, 3272-3281.

Dahiya,A., Wong,S., Gonzalo,S., Gavin,M., and Dean,D.C. (2001). Linking the Rb and polycomb pathways. Mol. Cell *8*, 557-569.

Dannenberg,J.H., Schuijff,L., Dekker,M., van der Valk,M., and te Riele,H. (2004). Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev. *18*, 2952-2962.

Dannenberg,J.H., van Rossum,A., Schuijff,L., and te Riele,H. (2000). Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. Genes Dev. *14*, 3051-3064.

Dick,F.A. (2007). Structure-function analysis of the retinoblastoma tumor suppressor protein - is the whole a sum of its parts? Cell Div. *2*, 26.

Foijer,F., Wolthuis,R.M., Doodeman,V., Medema,R.H., and te Riele,H. (2005). Mitogen requirement for cell cycle progression in the absence of pocket protein activity. Cancer Cell *8*, 455-466.

Frolov,M.V. and Dyson,N.J. (2004). Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. J. Cell Sci. *117*, 2173-2181.

Han,J. and Sun,P. (2007). The pathways to tumor suppression via route p38. Trends Biochem. Sci. *32*, 364-371.

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

Kinross,K.M., Clark,A.J., Iazzolino,R.M., and Humbert,P.O. (2006). E2f4 regulates fetal erythropoiesis through the promotion of cellular proliferation. Blood *108*, 886-895.

Lee,E.Y., Cam,H., Ziebold,U., Rayman,J.B., Lees,J.A., and Dynlacht,B.D. (2002). E2F4 loss suppresses tumorigenesis in Rb mutant mice. Cancer Cell *2*, 463-472.

Maandag,E.C., van der Valk,M., Vlaar,M., Feltkamp,C., O'Brien,J., van Roon,M., van der Lugt,N., Berns,A., and te Riele,H. (1994). Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. EMBO J. *13*, 4260-4268.

Morrison,A.J., Sardet,C., and Herrera,R.E. (2002). Retinoblastoma protein transcriptional repression through histone deacetylation of a single nucleosome. Mol. Cell Biol. *22*, 856-865.

Narita,M., Nunez,S., Heard,E., Narita,M., Lin,A.W., Hearn,S.A., Spector,D.L., Hannon,G.J., and Lowe,S.W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell *113*, 703-716.

Nielsen,S.J., Schneider,R., Bauer,U.M., Bannister,A.J., Morrison,A., O'Carroll,D., Firestein,R., Cleary,M., Jenuwein,T., Herrera,R.E., and Kouzarides,T. (2001). Rb targets histone H3 methylation and HP1 to promoters. Nature *412*, 561-565.

Peeper,D.S., Dannenberg,J.H., Douma,S., te Riele,H., and Bernards,R. (2001). Escape from premature senescence is not sufficient for oncogenic transformation by Ras. Nat. Cell Biol. *3*, 198-203.

Porter,A.C. (2008). Preventing DNA over-replication: a Cdk perspective. Cell Div. *3*, 3.

Rangarajan,A., Hong,S.J., Gifford,A., and Weinberg,R.A. (2004). Species- and cell type-specific requirements for cellular transformation. Cancer Cell *6*, 171-183.

Rayman,J.B., Takahashi,Y., Indjeian,V.B., Dannenberg,J.H., Catchpole,S., Watson,R.J., te Riele,H., and Dynlacht,B.D. (2002). E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. Genes Dev. *16*, 933-947.

Sage,J., Mulligan,G.J., Attardi,L.D., Miller,A., Chen,S., Williams,B., Theodorou,E., and Jacks,T. (2000). Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. Genes Dev. *14*, 3037-3050.

Sherr,C.J. (1996). Cancer cell cycles. Science *274*, 1672-1677.

Voncken,J.W., Niessen,H., Neufeld,B., Rennefahrt,U., Dahlmans,V., Kubben,N., Holzer,B., Ludwig,S., and Rapp,U.R. (2005). MAPKAP kinase 3pK phosphorylates and regulates chromatin association of the polycomb group protein Bmi1. J. Biol. Chem. *280*, 5178-5187.

Vormer,T.L., Foijer,F., Wielders,C.L., and te Riele,H. (2008). Anchorage-independent growth of pocket protein-deficient murine fibroblasts requires bypass of G2 arrest and can be accomplished by expression of TBX2. Mol. Cell Biol. *28*, 7263-7273.

Wang,W., Chen,J.X., Liao,R., Deng,Q., Zhou,J.J., Huang,S., and Sun,P. (2002). Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced premature senescence. Mol. Cell Biol. *22*,

3389-3403.

Williams,B.O., Schmitt,E.M., Remington,L., Bronson,R.T., Albert,D.M., Weinberg,R.A., and Jacks,T. (1994). Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. EMBO J. *13*, 4251-4259.

Yamasaki,L., Bronson,R., Williams,B.O., Dyson,N.J., Harlow,E., and Jacks,T. (1998). Loss of E2F-1 reduces tumorigenesis and extends the lifespan of Rb1(+/-)mice. Nat. Genet. *18*, 360-364.

APPENDICES

English summary

Nederlandse samenvatting

List of publications

Curriculum Vitae

Dankwoord

English summary

Cancer is the result of a multistep process involving the gradual change of a normal cell into a cancerous cell. This process is called oncogenic transformation and requires the perturbation of essential cellular processes such as cell cycle regulation and apoptosis. Oncogenic transformation can be accomplished by multiple alterations in oncogenic and tumor suppressor pathways. The retinoblastoma pathway comprises a major tumor suppressor pathway that is found deregulated in the majority of human and mouse cancers. However, perturbation of this pathway is not sufficient for oncogenic transformation. This thesis describes the identification of genetic events that together with loss of the retinoblastoma suppressor pathway are required for oncogenic transformation. Furthermore, the involvement of the different activities of the retinoblastoma protein in tumor suppression has been studied.

pRB belongs to the retinoblastoma protein family, also called pocket protein family, which also includes p130 and p107. These proteins inhibit the progression from G_1 into S phase and as such play an essential role during cell cycle regulation. In response to growth inhibitory signals, pocket proteins are stabilized in their active, hypophosphorylated form, and subsequently induce an arrest in G_1 phase. Consistently, loss of pocket proteins was found to induce uncontrolled proliferation *in vitro* and to bypass G_1 arrest in response to various growth-inhibitory signals.

The expression of constitutively active RAS (RAS^{V12}) is an oncogenic signal that, strikingly, induces a pocket protein-dependent G_1 arrest in Mouse Embryonic Fibroblasts (MEFs). We have previously found that bypass of RAS^{V12}-induced cell cycle arrest by loss of pocket proteins was not sufficient to support RAS^{V12}-induced transformation. To gain insights into the mechanisms of oncogenic transformation, Chapters 2 and 3 of this thesis are focused on the identification of genetic events that in collaboration with loss of pocket proteins support RAS^{V12}-induced transformation.

In Chapter 2, we performed a gain-of-function screen to identify genetic events that enabled RAS^{V12}/pocket protein-deficient MEFs to grow anchorage independently, a hallmark of transformation. We found that expression of TBX2 in RAS^{V12}/pocket protein-deficient MEFs induced anchorage-independent growth *in vitro* and enabled tumor formation upon injection of cells into nude mice. Our experiments show that the combined actions of pocket protein-loss, TBX2 expression and RAS^{V12} expression were required to induce oncogenic transformation in MEFs.

TBX2 is a transcriptional regulator that inhibits the $p19^{ARF}$/p53/$p21^{CIP1}$ pathway. Consistently, we found that downregulating the p53 pathway by RNA interference supported anchorage-independent growth of RAS^{V12}/pocket protein-deficient MEFs.

Our results demonstrate that the pocket protein and p53 pathways were co-operatively involved in counteracting anchorage-independent growth in MEFs. This can be explained by the role of these pathways in the inhibition of cyclin-dependent kinases (cdks). In the absence of anchorage, G_1- and G_2-associated cdks were dramatically downregulated. Dependent on the level of pocket proteins present, this resulted in an arrest in G_1 or G_2 phase. Although loss of pocket proteins induced both G_1- and G_2-associated cdk activities and resulted in a (partial) override of the G_1 arrest, it was not sufficient to counteract the major reduction in cdk activities in response to loss of anchorage. Downregulating the p53 pathway in pocket protein-deficient MEFs caused an additive rise in cdk activities that was sufficient to support anchorage-independent proliferation, and thus oncogenic transformation. Since transformation of human fibroblasts also requires ablation of both the pocket protein and p53 pathways, our results demonstrate that in this aspect, transformation of MEFs is not fundamentally different from transformation of human fibroblasts, as was previously claimed by others.

By performing an insertional mutagenesis screen, we found in Chapter 3 that, similar to overexpression of TBX2, downregulation of the p38/Mapkapk3 pathway supported RAS^{V12}-induced transformation of pocket protein-deficient MEFs. Strikingly, we found that loss of anchorage induced Mapkapk3 in RAS^{V12}/pocket protein-deficient MEFs and could be reverted by expression of TBX2. Experiments by others have linked activation of Mapkapk3 to the release of BMI1 from the chromatin and the subsequent induction of $p19^{ARF}$. Together, this implies that the oncogenic effect of TBX2 might be partly explained by downregulation of Mapkapk3 resulting in downregulation of the $p19^{ARF}$/p53 pathway.

Our results provide a rationale for the frequent loss of pRB in tumors. The second part of this thesis is focused on identifying which of the different activities of pRB are required for tumor suppression.

pRB interacts with many cellular proteins, including the family of E2F transcription factors, which promote S phase entry and subsequent cell cycle progression. Binding of pRB to E2F blocks E2F's transactivation domain and inhibits transcription of E2F target genes, thereby inhibiting S phase entry. In addition to binding E2F, pRB can simultaneously bind to proteins containing an LxCxE motif (x encoding any amino acid). Since many LxCxE-proteins function in chromatin remodeling and transcriptional repression, the pRB-mediated recruitment of such complexes to E2F target sites favors a chromatin state incompatible with transcription.

We wondered whether the recruitment of chromatin remodeling proteins by pRB is essential for pRB's tumor suppressor role. To this aim, we made use of a mutant form

of pRB, pRB^{N750F}. This mutant protein was impaired in binding proteins containing an LxCxE motif, but was still able to inhibit E2F-mediated transactivation.

First, we analyzed whether ablation of the pRB-LxCxE interaction affected the induction of cell cycle arrest in response to growth inhibitory signals, an essential component of pRB's tumor suppressor function. In Chapter 4, we describe that $Rb^{N750F/N750F}$ MEFs were impaired in arresting in G_1 in response to γ-irradiation. Additionally $Rb^{N750F/N750F}p130^{-/-}$ MEFs were impaired in arresting in response to RAS^{V12}. This is in contrast to $Rb^{+/+}p130^{-/-}$ MEFs, which entered RAS^{V12}-induced cell cycle arrest with similar kinetics as wild-type MEFs. These results demonstrate that the interaction between pRB and LxCxE-containing proteins is critical for the induction of cell cycle arrest in response to γ-irradiation or expression of RAS^{V12}.

Second, we studied whether ablation of the interaction between pRB and LxCxE-containing proteins promoted oncogenic transformation in mice. Surprisingly, we found that $Rb^{N750F/N750F}$ mice had a normal lifespan and were not prone to tumorigenesis (Chapter 5). This is in sharp contrast to $Rb^{+/-}$ mice and $Rb^{-/-}$ chimeras, which were highly prone to the formation of tumors in the pituitary and thyroid gland.

The pRB homologues p130 and p107 can also bind LxCxE-containing proteins and might therefore compensate for the ablated pRB-LxCxE interaction in $Rb^{N750F/N750F}$ mice. Moreover, our previous studies showed that loss of p130 or p107 extended the tumor spectrum induced by loss of pRB only. We therefore wondered whether the ablation of p130 or p107 in $Rb^{N750F/N750F}$ mice would cause increased tumor susceptibility. To this aim, we combined *p130* or *p107* ablation with Rb^{N750F} mutation in mice. Strikingly, the results showed that the $Rb^{N750F/N750F}p130^{-/-}$ phenotype caused embryonic lethality around day 18.5. This suggests that the interaction between pocket proteins and LxCxE-proteins is required for embryonic development.

To study whether $Rb^{N750F/N750F}$ mice become prone to tumorigenesis upon ablation of p130 or p107, we analyzed mice with three mutated alleles: $Rb^{N750F/wt}p130^{-/-}$, $Rb^{N750F/N750F}p130^{+/-}$ and $Rb^{N750F/N750F}p107^{+/-}$ mice. In these animals, $Rb^{N750F/N750F}p130^{-/-}$ or $Rb^{N750F/N750F}p107^{-/-}$ cells are expected to arise frequently due to spontaneous loss of heterozygosity. Mice with three mutated alleles displayed a reduced survival, which was most prominent in $Rb^{N750F/wt}p130^{-/-}$ animals. Upon detailed macroscopic and microscopic analysis, we were however unable to detect increased tumor formation in these animals.

In conclusion, the second part of this thesis shows that the interaction between pRB and LxCxE-containing proteins was critical for the induction of cell cycle arrest in response to γ-irradiation or expression of RAS^{V12}. This suggested that the pRB-LxCxE interaction contributes to the tumor suppressor function of pRB. However, ablation of the pRB-LxCxE interaction did not promote spontaneous tumorigenesis in mice. These results suggest that under the tested conditions, inhibition of the transactivation function

of E2Fs was the dominant mechanism of tumor suppression by pRB. It remains the subject of further study to determine whether ablation of the pocket protein-LxCxE interaction causes increased tumor susceptibility in response to oncogenic stimuli, such as γ-irradiation or expression of RAS^{V12}.

Nederlandse samenvatting

De retinoblastoom eiwitten in tumor suppressie: interacterende eiwitten en samenwerkende signaleringsroutes

In een gezond lichaam is er sprake van gereguleerde celdeling. Celdeling is nodig om weefsels te vernieuwen of te herstellen wanneer er een beschadiging is opgetreden. Een cel ontvangt continu signalen die celdeling stimuleren en tegelijkertijd signalen die celdeling remmen. Of en hoe snel een cel deelt wordt bepaald door het totaal van deze signalen en door de reactie van de cel op deze signalen. Bij kanker is de natuurlijke regulatie van celdeling volledig verstoord. Cellen delen ongecontroleerd en reageren niet meer op signalen die normaal gesproken de celdeling remmen en het evenwicht bewaren.

De celdeling wordt gecontroleerd door twee soorten genen: oncogenen die de celdeling stimuleren en tumorsuppressorgenen die de celdeling remmen. Een gen is een stukje van het genetisch materiaal van de cel, het DNA, dat codeert voor een eiwit. Wanneer er een verandering in een gen optreedt, kan dit er toe leiden dat er een eiwit in de cel gevormd wordt dat niet meer normaal functioneert. Om de celdeling volledig te verstoren zijn er meerdere veranderingen nodig in oncogenen en tumorsuppressorgenen. Dit resulteert erin dat er meerdere eiwitten in de cel aanwezig zijn die niet meer functioneren zoals ze dat in de gezonde situatie deden, waardoor de celdeling niet meer goed gecontroleerd wordt.

Een belangrijke groep van tumorsuppressorgenen die een essentiële rol spelen tijdens het reguleren van celdeling zijn de retinoblastoom genen. Deze coderen voor de retinoblastoom eiwitten, pRB, p130 en p107, die ook wel de ‘pocket eiwitten’ worden genoemd. Wanneer een cel delingsremmende signalen ontvangt, worden de retinoblastoom eiwitten geactiveerd en wordt de celdeling geremd. In bijna alle vormen van kanker functioneren de retinoblastoom eiwitten niet meer goed, wat er aan bijdraagt dat cellen ongecontroleerd delen.

Om het ontstaan van kanker te bestuderen worden zowel muizen als cellen uit muizen gebruikt. In het eerste deel van dit proefschrift zijn cellen uit muizen embryo’s, embryonale fibroblast cellen, gebruikt waarin de retinoblastoom eiwitten (gedeeltelijk) zijn uitgeschakeld. Wanneer deze cellen in kweek worden gebracht, delen ze ongecontroleerd als gevolg van de afwezigheid van de retinoblastoom eiwitten. Ook wanneer in deze cellen een grote hoeveelheid RAS eiwit wordt gebracht, blijven de cellen delen. Dit laatste is opvallend, aangezien cellen mèt retinoblastoom eiwitten stoppen met delen wanneer er veel RAS aanwezig is. RAS is een oncogen, een gen dat het ontstaan van kanker bevordert. Door te stoppen met delen beschermt de cel zich tegen de kankerbevorderende

eigenschappen van RAS. Als de retinoblastoom eiwitten echter zijn uitgeschakeld, kan de cel in een reactie op RAS niet meer stoppen met delen. Hieruit concluderen we dat de retinoblastoom eiwitten betrokken zijn bij de bescherming tegen het kankerbevorderende eiwit RAS.

Echter, cellen zónder retinoblastoom eiwitten gedragen zich nog niet als kankercellen. Dit blijkt wanneer je de cellen kweekt onder een conditie waarbij ze niet kunnen hechten aan een ondergrond. Net als normale cellen stoppen in dit geval de cellen zonder retinoblastoom eiwitten met delen. De cellen reageren dus nog steeds normaal op het weghalen van hechting, een groeiremmend signaal. Kankercellen kunnen onder deze condities daarentegen wèl delen. Hoewel dus ten gevolge van het verlies van retinoblastoom eiwitten bepaalde beschermingsmechanismen tegen kanker niet meer goed werken, is voor de volledige ontwikkeling tot kankercellen méér nodig. In hoofdstuk 2 en 3 van dit proefschrift worden genetische veranderingen beschreven die *samen* met het verlies van de retinoblastoom genen ervoor zorgen dat cellen zonder hechting kunnen delen en dus een stap verder zijn in de ontwikkeling tot kankercellen.

In hoofdstuk 2 laten we zien dat overexpressie (= de aanwezigheid van een grote hoeveelheid) van TBX2 één van deze genetische veranderingen is die ervoor zorgt dat cellen zonder retinoblastoom eiwitten kunnen delen in de afwezigheid van een ondergrond om aan te hechten. Cellen zonder pRB *en* p130 (*Rb*$^{-/-}$*p130*$^{-/-}$ fibroblasten), cellen zonder pRB *en* p107 (*Rb*$^{-/-}$*p107*$^{-/-}$ fibroblasten) en cellen zonder pRB *en* p130 *en* p107 (*Rb*$^{-/-}$*p130*$^{-/-}$*p107*$^{-/-}$ fibroblasten) kunnen delen in de afwezigheid van hechting wanneer zowel TBX2 als RAS tot overexpressie worden gebracht. Wanneer deze cellen met TBX2 en RAS in muizen worden geïnjecteerd, vormen ze bovendien tumoren.

TBX2 is een oncogen dat een effect heeft op een grote hoeveelheid andere genen. Zo zorgt TBX2 er onder andere voor dat de activiteit van de groeiremmende eiwitroute p19^{ARF}/p53/p21^{CIP1} wordt geremd. In hoofdstuk 2 hebben we getest of het remmen van deze route leidt tot hechtingsonafhankelijke groei van cellen zonder retinoblastoom eiwitten. Dit blijkt het geval: het remmen van òfwel p19^{ARF} òfwel p53 òfwel p21^{CIP1} in deze cellen zorgt ervoor dat ze kunnen delen in de afwezigheid van hechting. Het feit dat het remmen van deze route leidt tot hechtingsonafhankelijke groei kan verklaard worden door het effect van deze route op de zogenoemde cycline-afhankelijke-kinases. Deze kinases vormen een groep eiwitten die de celdeling juist stimuleren. Door het remmen van de p19^{ARF}/p53/p21^{CIP1} route valt de remming van deze route op de cycline-afhankelijke-kinases weg en zijn de kinases vrij om de celdeling te stimuleren.

Samenvattend kan geconcludeerd worden dat cellen zonder retinoblastoom eiwitten niet delen in de afwezigheid van een ondergrond om aan te hechten. Het tot overexpressie brengen van TBX2 òf het remmen van de p19^{ARF}/p53/p21^{CIP1} route leidt tot het stimuleren van de cycline-afhankelijke-kinases. Dit zorgt ervoor dat de celdeling

geïnduceerd wordt en dat de cellen nu wel kunnen delen in de afwezigheid van hechting. Dit wijst erop dat de cellen een stap verder zijn in de ontwikkeling naar kankercellen en dat deregulatie van de TBX2/p19^{ARF}/p53/p21^{CIP1} route *in combinatie* met verlies van de retinoblastoom eiwitten belangrijk is voor het ontstaan van kankercellen. Dit wordt bevestigd door het feit dat *Rb$^{-/-}$p130$^{-/-}$* cellen die TBX2 en RAS tot overexpressie brengen, tumoren vormen wanneer ze in muizen geïnjecteerd worden.

In hoofdstuk 3 beschrijven we een andere genetische verandering die er toe leidt dat cellen zonder retinoblastoom eiwitten kunnen delen in de afwezigheid van een ondergrond om aan te hechten. We hebben gevonden dat remming van de p38/Mapkapk3 signaleringsroute cellen zonder retinoblastoom eiwitten stimuleert tot hechtingsonafhankelijke groei. Opvallend genoeg vinden we in hoofdstuk 3 dat TBX2 een effect heeft op Mapkapk3: in aanwezigheid van TBX2 wordt Mapkapk3 onderdrukt (Mapkapk3 wordt 'uitgezet'). Recente experimenten door anderen laten zien dat Mapkapk3 bovendien een effect heeft op p19^{ARF}, een onderdeel van de p19^{ARF}/p53/p21^{CIP1} route. Dus wellicht draagt het effect van TBX2 op Mapkapk3 en vervolgens op de p19^{ARF}/p53/p21^{CIP1} route bij aan het feit dat TBX2 hechtingsonafhankelijke groei, en dus kanker, stimuleert.

Het verlies van de retinoblastoom eiwitten, pRB, p130 en p107, draagt bij aan het ontstaan van kanker. Het tweede deel van dit proefschrift is gericht op de vraag welke van de specifieke functies van het pRB eiwit nodig is voor het onderdrukken van kanker. Dit eiwit kan met zeer veel verschillende eiwitten een interactie aangaan, o.a. met de E2F transcriptiefactoren, maar ook met eiwitten met een zogenoemd LxCxE motief die een belangrijke rol spelen bij de structuur van het chromatine (= het complex van DNA en eiwitten). Het effect en de significantie van vele van deze interacties is echter tot nog toe onduidelijk. Een complex bestaande uit pRB, een LxCxE-eiwit en een E2F eiwit, kan binden aan het DNA. Het LxCxE-eiwit zorgt vervolgens voor een meer 'gesloten' secundaire structuur van het DNA. Dit heeft tot gevolg dat een gen, dat ligt op de plek waarop het complex gebonden is, minder efficiënt kan worden afgelezen voor de vorming van een eiwit. Met andere woorden, de pRB-LxCxE interactie zorgt voor langdurige remming van de eiwitproductie van bepaalde genen.

Hoofdstuk 4 beschrijft het effect van het verbreken van de interactie tussen pRB en eiwitten met een LxCxE motief op de capaciteit van pRB om de celdeling te remmen. Hiertoe hebben we gebruik gemaakt van een veranderde vorm van pRB, namelijk pRB^{N750F}. In tegenstelling tot het onveranderde pRB eiwit (wild-type pRB), kan pRB^{N750F} geen interactie aangaan met eiwitten die het LxCxE motief bevatten. Daarentegen is de interactie van pRB^{N750F} met de E2F eiwitten intact gebleven.

Uit de experimenten is gebleken dat in tegenstelling tot cellen met wild-type

pRB, cellen met pRB^{N750F} niet goed kunnen stoppen met delen wanneer ze behandeld worden met γ-straling. Verder is gebleken dat cellen met pRB^{N750F} waarin bovendien p130 is uitgeschakeld, niet goed kunnen stoppen met delen wanneer er een grote hoeveelheid RAS in de cel wordt gebracht. Zoals hierboven beschreven wordt het stoppen met delen in reactie op RAS gezien als een beschermingsmechanisme tegen de kankerbevorderende eigenschappen van RAS. Samengevat laat hoofdstuk 4 zien dat het verbreken van de interactie tussen pRB en eiwitten met een LxCxE motief leidt tot een verminderde bescherming tegen signalen die kanker kunnen stimuleren, zoals γ-straling en het RAS oncogen.

In hoofdstuk 5 analyseren we het effect van het verbreken van de pRB-LxCxE interactie op de vorming van kanker in muizen. Hiertoe hebben we muizen gemaakt die alleen het mutante pRB^{N750F} eiwit kunnen maken. Verassend genoeg hebben deze $Rb^{N750F/N750F}$ muizen een normale levensduur en vinden we geen verhoogde ontwikkeling van kanker in deze muizen. Aangezien de familieleden van pRB (p130 en p107) zouden kunnen compenseren voor de afwezigheid van de pRB-LxCxE interactie, hebben we in de muizen met het pRB^{N750F} eiwit ook p130 of p107 (gedeeltelijk) uitgeschakeld. Deze dieren hebben wel een verkorte levensduur, maar ook hier vinden we geen aanwijzingen voor een verhoogde ontwikkeling van kanker. Uit het tweede deel van dit proefschrift kunnen we concluderen dat het verbreken van de interactie tussen pRB en LxCxE-eiwitten bepaalde beschermingsmechanismen tegen kanker uitschakelt. Desalniettemin hebben we geen aanwijzingen gevonden voor een verhoogde spontane kankerontwikkeling in muizen met een verbroken pRB-LxCxE interactie. Echter, het is zeer goed mogelijk dat onder specifieke omstandigheden, bijvoorbeeld in combinatie met andere genetische veranderingen of na bestraling, muizen met een verbroken pRB-LxCxE interactie *wel* gevoeliger zijn voor kanker.

List of publications

Vormer, T.L., Foijer, F., Wielders, C.L., te Riele, H. (2008). Anchorage-independent growth of pocket protein-deficient murine fibroblasts requires bypass of G_2 arrest and can be accomplished by expression of TBX2. Mol. Cell. Biol. *28*, 7263-7273

Vormer, T.L*., Hansen, J.B.*, Dekker, M., de Vries, S., Dannenberg, J.H. and te Riele, H. The pRB-LxCxE interaction is critical for RAS^{V12}- and γ-irradiation induced cell cycle arrest
(Manuscript in preparation) * equal contribution

Vormer, T.L., van der Wal, A, Delzenne-Goette, E., Dekker, M, Song, J., Naik, S., Hansen, J.B. and te Riele, H. Overlapping roles of pRB-LxCxE interactions and p130 functions in mouse development and viability
(Manuscript in preparation)

Wielders, C.L, van Nierop, P., **Vormer, T.L**., Foijer, F., te Riele, H. Enzymatic production of LEGO RNAi libraries to identify genetic differences between cell line counterparts (Manuscript in preparation)

Curriculum Vitae

Tinke Lizbet Vormer werd op 16 augustus 1979 geboren te Utrecht. In 1997 behaalde zij haar VWO diploma aan het Minkema College te Woerden, waarna ze biologie ging studeren aan Wageningen Universiteit. Tijdens haar studie raakte Tinke al snel gefascineerd door de moleculaire biologie. In 2002 doorliep zij een stage onder begeleiding van Dr. T. van der Meulen en Dr. H.W.J. Stroband in de leerstoelgroep Experimentele dierkunde aan Wageningen Universiteit. Zij deed hier onderzoek naar de ontwikkeling van bot en kraakbeen in de zebravis. Vervolgens ging zij stage lopen aan het Center for Animal Transgenesis and Germ Cell Research aan de University of Pennsylvania, Philadelphia, USA. Onder begeleiding van Dr. S. Eckardt en Dr. K.J. McLaughlin deed zij hier onderzoek naar het herprogrammeren van genexpressie tijdens klonen. Vervolgens deed Tinke een afstudeerproject in de groep van Prof. Dr. H.P.J. te Riele en onder supervisie van Dr. J. B. Hansen bij het Nederlands Kanker Instituut- Antoni van Leeuwenhoek ziekenhuis te Amsterdam. In 2003 behaalde zij haar doctoraal diploma en startte met een promotieonderzoek in de groep van Prof. Dr. H.P.J. te Riele; de resultaten van dit onderzoek zijn beschreven in dit proefschrift. Sinds oktober 2009 is Tinke werkzaam als post-doctoraal onderzoeker in de groep van Prof. Dr. S. Repping bij het Amsterdam Medisch Centrum, Nederland, waar zij onderzoek doet naar stamcel ontwikkeling en differentiatie in de testis.

Tinke Lizbet Vormer was born on August 16th 1979 in Utrecht, the Netherlands. In 1997, she finished her pre-university education at the Minkema College, Woerden. Subsequently, she started with her Biology Education at Wageningen University, and she was soon intrigued by molecular biology. She performed her first internship at the department of Experimental zoology at Wageningen University under supervision of Dr. T. van der Meulen en Dr. H.W.J. Stroband where she studied bone and cartilage development in zebrafish. The second internship was performed at the Center for Animal Transgenesis and Germ Cell Research, University of Pennsylvania, Philadelphia. Under supervision of Dr. S. Eckardt and Dr. K.J. McLaughlin, she studied reprogramming of gene expression during cloning. Next, she performed a graduation project in the group of Prof. Dr. H.P.J. te Riele and under supervision of Dr. J. B. Hansen at the Netherlands Cancer Institute-Antoni van Leeuwenhoek hospital, Amsterdam, The Netherlands. In 2003, she obtained her MSc degree and started as a PhD student in the group of Prof. Dr. H.P.J. te Riele; the results of this research are described in this thesis. Since October 2009, Tinke holds a position as postdoctoral researcher in the group of Prof. Dr. S. Repping at the Amsterdam Medical Center, the Netherlands, where she studies stem cell development and differentiation in the human testis.

Dankwoord

Nou, daar is ie dan, mijn proefschrift!
Tijdens de lange weg hiernaartoe hebben mensen me regelmatig gevraagd waarom ik toch altijd tot ’s avonds laat en in het weekend op het NKI zat en of ik daar niet helemaal gek van werd. Tegen de tijd dat ik avonden en weekenden aan het lay-outen was, heb ik deze vraag trouwens niet meer gekregen, dus ik vrees dat mijn omgeving het opgegeven heeft…. Toch ging ik zelden met tegenzin naar het NKI. Dat kwam voor een groot deel omdat het altijd (ja zelfs in het weekend!) zo gezellig en inspirerend was! Daarom wil ik hier al mijn collega’s bedanken voor de collegialiteit en voor alle leuke momenten!

Maar allereerst bedank ik natuurlijk jou, Hein, voor het gestelde vertrouwen in mij. Ik vond het altijd knap hoe je via het stellen van vragen, het gedachteproces een andere kant op probeerde te krijgen om tot nieuwe inzichten te komen. Ongelofelijk hoe je met zoveel verschillende onderwerpen in de groep, altijd weer een interessante vraag weet te stellen of inzicht weet te brengen! Ook je filosofische kijk op de dingen was vaak verrassend, en je rust om een hele middag alle discussiepunten van een project door te nemen vond ik altijd erg positief.

Dan mijn drie (!) paranimfen Elske, Tanja en Angela, wat leuk dat jullie naast me willen staan! Elske, vanaf het eerste college in Wageningen hebben we onze studie- en promotietijd samen gedeeld, en ik ga ervan uit dat we in de toekomst nog veel meer dingen te delen hebben! Of we elkaar nu veel of weinig zien, het gevoel is altijd hetzelfde, wat toont hoe goed we elkaar kennen en hoezeer we onszelf zijn als we elkaar spreken. Bedankt voor je vriendschap en ik hoop dan ook echt dat je er 26 november bij kunt zijn! Angela, ik ben blij dat ook jij mijn paranimf wilt zijn, en ik zal het erg leuk vinden als je op die dag naast me staat! Fijn dat we de promotieperikelen hebben kunnen delen, en dat we ook altijd over andere zaken kunnen bijkletsen.

Tanja, jouw komst naar het NKI heeft er voor gezorgd dat ik het onderzoek weer een stuk leuker ging vinden! Nadat Floris naar Boston was gegaan, vond ik het erg saai om als enige in de groep aan pRB te werken en jouw komst heeft me zeker opgevrolijkt. Helaas heb ik jou daarna als enige pRB-er achtergelaten…. Ik vond het erg leuk om samen over resultaten en papers te discussiëren en daarnaast hebben we ook heel wat andere dingen besproken. En geloof me, ik heb jouw humoristische directheid altijd erg handig en grappig gevonden! In bepaalde situaties (bv als mensen me vragen of ik op de fiets ben, of als ik uit moet leggen dat ik om 8 uur ’s ochtends de celkweek schoonmaken toch echt een slecht idee vind) denk ik met een glimlach aan jouw commentaar dat hier ongetwijfeld op gevolgd zou hebben! Bedankt voor de leuke tijd!

Marieke, we hebben inderdaad heel wat afgekletst in de celkweek! We hebben lang samen op het NKI gezeten en hebben wel het een en ander aan ups en downs gedeeld.

Jammer dat ik je via de Thai weer hielp herinneren aan waar je allergisch voor bent… En trouwens, ik snap nog steeds niet hoe jij op een feestje later wegging dan ik, en de volgende ochtend alweer fris en fruitig proeven aan het doen was!

Eva, ik vind het een eer dat ik maar liefst twee keer een chocoladetaart op mijn bureau vond! Het gebaar maakte uiteraard mijn dag goed, en dan waren ze natuurlijk ook nog eens héééél erg lekker. Als ik een recept zie, denk ik nog regelmatig, 'tja het lijkt me erg veel gedoe, maar Eva zou er vast niet voor terug schrikken!' Dank voor je gezelligheid en luisterend oor, en ik wens je natuurlijk veel succes bij het afronden van je proefschrift.

Sietske, ook wij hebben heel wat besproken in de kweek en ook het doen van honderden staart-PCRs schept een band! Je betrokkenheid, zowel werkgerelateerd als prive, heb ik altijd erg gewaardeerd. Succes met het schrijven van je proefschrift!

Rob, jij als AMC-er die naar het NKI ging, en ik als NKI-er die naar het AMC ging, konden heel goed samen ervaringen uitwisselen! Ook was je een goede adviseur bij problemen op het lab, en jouw suggestie om survival curves in sigmaplot te maken, maakte mijn leven een stuk aangenamer!

Camiel, we zaten zowel op de kamer als op het lab naast elkaar, en ik vind het leuk dat we jouw screens en mijn cellen hebben kunnen combineren! Als ik Fokke en Sukke voorbij zie komen, hoor ik in gedachten jouw gegrinnik naast me!

Anja, wat een heftige jaren zijn het geweest! We hebben heel wat gedeeld, zowel op het werk als privé. Bedankt voor alle organisatorische zaken op het lab, het muizenwerk, je bereidheid om bij te springen als het nodig was, en voor het geven van peptalks wanneer ik het niet meer zag zitten. Ik vrees dat ik het vaker zeg, maar het wordt tijd dat we weer eens wat gezelligs gaan doen!

Elly, jouw humoristische verhalen zorgden altijd voor een vrolijke noot! Bedankt voor het zorgen voor de muizen, het organiseren van alle kruisingen, en voor je coulance wanneer die muizen van mij toch nog niet weg mochten (want wie weet zouden we ze later eventueel misschien toch nog nodig kunnen hebben!). Tja, wat er nou mis was met die mottige kwakkel muizen, weten we helaas nog steeds niet!

Marleen en Sandra, wie had nu gedacht dat jullie pRB proefjes voor me zouden gaan doen? Jullie volgens mij niet, maar jullie waren wel de redder in de nood! Wel jammer Marleen, dat je, zoals je zelf ooit zei, jouw geluk niet op het project hebt kunnen overbrengen! Ik hoop dat ik je niet al te erg geïrriteerd heb met het laten rondslingeren van troep, de vele lades met spullen die toch echt niet weggegooid mochten worden en waar ik tegelijkertijd geen tijd voor had om uit te zoeken, en het te laat schoonmaken van de stoof… Bedankt allebei voor jullie betrokkenheid en natuurlijk voor alle gezelligheid!

Floris, jouw eeuwige enthousiasme voor de wetenschap heb ik altijd erg motiverend gevonden. Vooral toen je weg was realiseerde ik me hoe saai het was om jouw visie te moeten missen op een arrest van de cellen of op een dubbele band op een blot

(weet je nog, die RAS blot die ik helemaal in het begin gedaan heb?) waar we volgens jou toch echt wel een Nature paper van zouden kunnen brouwen! Helaas dus…. Ook als ik je iets over de mail stuurde had je altijd enthousiast en opbeurend commentaar, dank hiervoor.

Jacob, I started my time at NKI under your supervision, and I couldn't have wished for a better supervisor! I learned a lot from you on how to set up experiments and of course about pRB. Your enthusiastic comments on results or papers in the field were always motivating. I wish you good luck in personal life and of course in science.

Marije, we zijn samen aan het project begonnen maar hebben het helaas niet samen afgemaakt. Leuk dat je weer in de wetenschap werkt en het zou leuk zijn als we samen met Camiel nog wat van het Mapkap verhaal kunnen maken!

Jan-Hermen, jouw proefschrift heeft heel vaak binnen handbereik op mijn bureau gelegen! Ik ben blij dat je weer terug was op het NKI toen ik met de Rb^{750} mutant bezig was, en ik je kon lastigvallen met vragen over hoe je die TKO proeven destijds nu precies gedaan had. Leuk dat je in de commissie zit!

Mijn studenten, Judith en David, wil ik bedanken voor het analyseren van een hele berg soft agar kolonies, niet het meest dankbare werk…

En dan zijn er natuurlijk nog de Wolthuisjes; Rob, Wouter, Linda, Erik, Bas en Janneke, die voor een hoop levendigheid zorgden op het lab en die altijd klaar stonden om advies te geven over blots en IPs, of om de geheimen van Mitose nog eens te benadrukken! Ook de groepen van Jos Jonkers, Piet Borst en in de H8-tijd René Medema, zorgden altijd voor de nodige gezelligheid en interactie. En niet te vergeten de groep van Daniel Peeper, waar ik helemaal in het begin van Theo het shuttelen leerde, en wat later mijn eerste stop werd op een 'shop-ronde' wanneer ik op zoek was naar antilichamen of constructen.

Tom en Linda, bedankt voor de organisatorische zaken, het was altijd gezellig om even langs te lopen! Frank en Anita wil ik bedanken voor al het FACS advies, Lauran en Lenny voor de hulp bij het fotograferen van celkweek-platen, en Minze en Erwin voor het opslaan en organiseren van alle cellen. Wat ben ik blij dat ik dat niet hoefde te doen! Ji-Ying en Martin, bedankt voor de pathologische analyse. Ji-Ying, thanks for looking through an enormous amount of slides and for your enthusiastic involvement in the project. En natuurlijk alle medewerkers van het muizenhuis voor het verzorgen en controleren van de muizen!

Also, I would like to thank my new colleagues at the AMC for listening to my stories and complains about lay-outing, thesis printing and planning a date for the defence. After a period of ´solitary confinement' to write my thesis, I was very relieved to be able to share scientific and non-scientific things with you!

Harriet en Victor, bedankt voor de dansinspiratie en het voorkomen van tunnelvisie!

Stefanie, bedankt voor het jarenlange aanhoren van mijn verhalen, waarvan ik vrees dat ze met de jaren steeds langer en klaaglijker werden… En natuurlijk voor alle gezelligheid en voor het feit dat je me voorzien hebt van een groot aantal maaltijden, waarbij je nooit boos werd dat ik weer eens (veel te) laat was omdat die proef toch weer wat langer duurde dan ik had gedacht….

Suzanne, bedankt voor de mooie cover die je voor me gemaakt hebt! Ik vond het leuk om mijn onderzoek aan je uit te leggen en om samen te brainstormen over een manier om dit symbolisch weer te geven.

Frizo, mijn lieve broer, wat fijn dat je altijd zo met me meeleeft. Je weet altijd verstandige adviezen te geven waar ik van opknap en je laat me ook vaak dingen inzien die ik zelf niet zag. Veel geluk met Vanessa en Femka Amelie, en ik kom jullie zeker stalken in Australië! Jammer van de afstand, maar ik denk wel dat we ons daardoor beter realiseren hoe goed onze band is!

Lieve pap en mam! Wat had ik zonder jullie moeten beginnen? Tijdens het schrijven van dit dankwoord realiseer ik me dat hoe meer mensen voor me betekenen, hoe moeilijker het is om iets pakkends te schrijven, want waar zal ik eens beginnen? Bij het aanhoren van mijn verhalen, het meedenken over problemen, het personal-assistent-aspect, de gezelligheid? Jullie hebben me altijd gesteund en gezelligheid gebracht. Dank voor al jullie liefde en steun door de jaren!

Tinke