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 G1 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 G1 phase. Consistently, loss of pocket proteins was found to induce uncontrolled proliferation in vitro and to bypass G1 arrest in response to various growth-inhibitory signals.

The expression of constitutively active RAS (RASV12) is an oncogenic signal that, strikingly, induces a pocket protein-dependent G1 arrest in Mouse Embryonic Fibroblasts (MEFs). We have previously found that bypass of RASV12-induced cell cycle arrest by loss of pocket proteins was not sufficient to support RASV12-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 RASV12-induced transformation.

In Chapter 2, we performed a gain-of-function screen to identify genetic events that enabled RASV12/pocket protein-deficient MEFs to grow anchorage independently, a hallmark of transformation. We found that expression of TBX2 in RASV12/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 RASV12 expression were required to induce oncogenic transformation in MEFs.

TBX2 is a transcriptional regulator that inhibits the p19ARF/p53/p21CIP1 pathway. Consistently, we found that downregulating the p53 pathway by RNA interference supported anchorage-independent growth of RASV12/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$^{N750F/wt}p130^{-/-}$ 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 RASV12.