From LPA signaling to polycomb: oncogenic cooperations revealed by genetic screens and dynamics and recruitment of polycomb group proteins
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Chapter 5
Summary and General Discussion
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Cancer is a highly complex and heterogeneous disease and most probably unique in each individual patient. Numerous genetic lesions are required to turn a normal cell into a cancer cell. It is crucial to uncover cooperations between the causal lesions and to understand the mechanisms by which they synergize and induce tumorigenesis in order to uncover common cancer-related pathways that could be targeted for therapy. Research described in this thesis focuses partly on our search for novel oncogenes that cooperate with c-Myc or Ras in tumorigenesis, and partly on the mechanism of action of one of the oncogenic collaborators of c-Myc, Bmi1.

Although, c-Myc and Ras are affected in many human cancers, aberrant activation of each of them alone is not sufficient to induce transformation. These oncogenes always act in combination with other activated oncogenes or inactivated tumor suppressors. One of the tumor suppressors, which can protect the cell against the oncogenic activity of c-Myc or Ras is p19Arf. In chapters 2 and 3, we have described different genetic gain-of-function screens set up to find oncogenes that can cooperate with c-Myc or RasV12 under conditions in which p19Arf levels are compromised. We have taken advantage of Tbx2, a known repressor of p19Arf, to modulate Arf levels in MEFs. Co-expression of either c-Myc/Tbx2 or RasV12/Tbx2 immortalizes MEFs and renders them prone to transformation. The addition of a single hit is then enough to transform these cells.

In chapter 2, we report on genes that we have found to cooperate with c-Myc and Tbx2. Among 12 genes belonging to different cellular pathways, we found LPA receptor 2 (LPA2). LPA is a lipid growth factor inducing many growth stimulatory pathways through its receptors, which trigger diverse biological processes (Moolenaar et al., 2004). When extending our study to other LPA receptor family members, we found that in addition to LPA2, LPA1 and LPA4, but not LPA3, are able to induce transformation in collaboration with c-Myc and Tbx2 in vitro and in vivo. We showed that this transformation is mechanistically dependent on the Gi-linked ERK and PI3K signaling pathways. Altered expression of LPA receptors and deregulated LPA signaling have been implicated in tumor progression and metastasis in various human malignancies (van Meeteren and Moolenaar, 2007). However, our study is the first to reveal a direct role for LPA receptor signaling in tumor initiation arguing against deregulated LPA signaling being a secondary event, which only contributes to tumor progression (Murph and Mills, 2007). This could be of particular interest for research on ovarian cancer, in which aberrant LPA signaling is known to contribute to tumor progression. Notably, Myc overexpression and p53 ablation belong to the frequently found genetic lesions in these tumors (Ozols et al., 2004).

A great deal of recent research on LPA signaling has been focused on the role of autotaxin (ATX), the LPA-generating exo-enzyme (Tokumura et al., 2002; Umezuk
Goto et al., 2002). Much evidence is accumulating linking ATX to angiogenesis and tumor progression (van Meeteren and Moolenaar, 2007). Since ATX is responsible for the production of LPA in vivo, it is important to test whether this enzyme contributes to tumor formation and transformation as well. Although our in vitro experiments suggest that an excess of LPA is not sufficient to induce transformation in combination with c-Myc and Tbx2 in MEFs, we can not exclude that hyperactivation or overexpression of ATX in vivo is tumorigenic by itself or in combination with other lesions. Future research using inducible transgenic mouse models could help us to understand the contribution of ATX at different stages of cancer ranging from tumor initiation to metastasis. In addition to ATX, it is important to extend our interest to the newly discovered LPA receptors, GPR92 (LPA5), GPR87 and P2Y5, and examine their oncogenic potential (Lee et al., 2006a; Murph et al., 2006; Pasternack et al., 2008). Finally, the study we described in chapter 2 brings us one step further towards gathering direct evidence that link LPA signaling to initiation of cancer. Therefore, our findings emphasize the possibility for developing specific targeted drugs to interfere with this pathway (Murph and Mills, 2007).

For most of the additional genes found in this screen, such as Actn4, Dbs, FosB, Hrs, Map3k3 and PdgfB, there have been reports linking their action directly or indirectly to tumorigenesis. It nevertheless remains interesting to explore the molecular mechanisms by which these genes induce transformation in specific combination with c-Myc and Tbx2. Notably, 4 out of 12 genes found in this screen (Actn4, Agrn, Dbs and Flnc) are either members of cytoskeleton proteins or are involved in regulating cytoskeletal rearrangement (Bowen and Fallon, 1995; Feng and Walsh, 2004; Honda et al., 1998; Schmidt and Hall, 2002). Cytoskeletal changes are directly linked to altered cellular morphology and increased motility, which are in turn associated with aberrant migrative and invasive characteristics of cancer cells. Hence, it is interesting that our unbiased genetic screen has revealed these genes to induce transformation.

Furthermore, for some genes found in this screen, clearly more extensive research is needed to reveal their contribution to tumorigenesis. This holds true for Grhl1, that is originally identified as a laminin binding protein. Grhl1 is now recognized as a member of the CP2 transcription factor family, which is conserved from fly to human. Grh transcriptional activators in flies have been reported to participate in cell cycle regulation and development (Kokoszynska et al., 2008). In mammals, recent studies have revealed their key role during embryonic development in particular in neuronal tube closure (Auden et al., 2006; Ting et al., 2003). Grhl transcription factors are known to regulate the cadherin gene family and steroid biosynthesis and are linked to the PI3K-PKC pathway (Henderson et al., 2007; Wilanowski et al., 2008). To our knowledge, not much is known about Grhl transcription factors in tumorigenesis, a subject which evidently deserves more investigation. Another interesting gene found in our screen is Nek6, a serine/
threonine kinase that is reported to be involved in cell cycle progression and DNA-damage-induced cell cycle arrest. Inhibition of Nek6 decreases cell cycle rate and induces apoptosis in cancer cells (Lee et al., 2008; O’regan et al., 2007; Yin et al., 2003). Although there is evidence to support its involvement in cell cycle progression and possibly cancer (Lee et al., 2008; Rapley et al., 2008; Takeno et al., 2008; Yin et al., 2003), our study provides a first direct link between Nek6 and transformation. Clearly, extensive future studies are required to confirm our findings in vivo and clarify the mechanism through which transformation and tumorigenesis is mediated by these potential oncogenes.

**Chapter 3** of this thesis is focused on our search for new oncogenic partners of Ras when p19Arf levels are reduced. We performed two independent genetic screens in different genetic backgrounds. We only found one gene (ILF1) to cooperate with RasV12 and Tbx2 in transformation. ILF1 was able to induce transformation only when it was fused to the viral Gag gene in the original library retroviral construct. Hence, even though ILF1, a member of the Fox transcription factors, seemed an appropriate candidate to be involved in cellular transformation at first, it proved to be a false positive hit. The high background problem, which we unfortunately encountered during screening, is most likely the main reason for the low success rate of this screen.

Although we were not able to find novel genes cooperating with RasV12 in transformation in this study, it nevertheless remains interesting to continue searching for such oncogenic cooperations. Activation of Ras leads to the downstream activation of the Raf-Mek-Erk pathway, which is one of the most important regulatory pathways controlling cellular proliferation and differentiation (Shields et al., 2000). Myc is known to act in a complementary pathway, which cooperates not only with activated Ras, but also with Ras-pathway members, such as activated Raf or Mek (Adhikary and Eilers, 2005; Taghavi et al., 2008; chapter 2). Therefore, screening for genes that cooperate with the Ras pathway in oncogenesis could still hold interesting outcomes since screening for Ras collaborators could possibly unravel Myc-pathway components. This is of special interest since a lot of knowledge has been gathered on the role of classic oncogenes such as Ras or c-Myc in tumorigenesis, while little is known about pathways activating Myc or targets activated downstream of Myc, that are specifically involved in this process. Of note, finding single oncogenes acting downstream of Myc will prove to be challenging, as Myc is now known to globally regulate transcription rather than affecting single genes (Knoepfler, 2007). These screens could, however, provide us with more insight into factors acting upstream of Myc.

Using activated Ras in our screen induced stronger transformation than we expected, resulting in a vast number of background colonies. To solve this problem and yet to be able to screen in a similar pathway, it might be more feasible to use conditionally activated Raf-1 (ΔRaf-1) or Mek1 (ΔMek1 ΔN3-S218E-S222D), which are likely to be weaker transforming genes than RasV12 (Bosch et al.,
Moreover, using an inducible expression system would allow modulating the background, if necessary.

Finally, it is worth to mention that recent developments in the RNA interference field allow us to design loss-of-function screens in order to identify tumor suppressors associated with certain pathways using shRNA-, siRNA- or miRNA-based retroviral libraries (Brummelkamp and Bernards, 2003; Voorhoeve et al., 2006). These new tools could be applied in transformation screens similar to the screens performed in chapters 2 and 3 of this thesis.

In addition to searching for new oncogenes and genetic lesions that cooperate in tumorigenesis, it is also crucial to understand the mechanism by which these oncogenes act. In chapter 4, we described our study on association dynamics of Bmi1 and its recruitment to PcG-related pericentric heterochromatin with the ultimate goal to provide more insight into its function. Bmi1 is one of the most extensively studied members of the PcG proteins and was originally identified to collaborate with c-Myc in tumorigenesis through transcriptional repression of the Ink4a-Arf tumor suppressors (Jacobs et al., 1999; van Lohuizen et al., 1991). PcG proteins form different multimeric protein complexes that are functionally and biochemically divided into initiation- (PRC2) and maintenance- (PRC1) complexes (Schwartz and Pirrotta, 2007). Although PcG-related transcriptional silencing was considered more transient and dynamic than stable repression of HP1-related constitutive heterochromatin, little was reported on the actual kinetics of PcG proteins within the PRCs on chromatin.

Using confocal microscopy imaging and photobleaching, we studied the stability and dynamic properties of Bmi1-GFP association with PcG bodies during G1 and G2 of the cell cycle. PcG bodies are defined subnuclear foci, where some PcG-related pericentric heterochromatic regions are clustered. Although these loci were shown to represent repetitive DNA sequences homologous to chromosome region 1q12 (Saurin et al., 1998; Voncken et al., 1999), it is not entirely clear if other chromosomal domains or protein complexes are included. Moreover, it remains intriguing that these foci are mainly found in cancer cell lines rather than in primary cells. It is therefore interesting to explore the content and the function of these so called “silencing bodies” in cancer cells, which may presumably involve coordinated transcriptional silencing of tumor suppressor genes.

Our results indicated that the Bmi1 pool that is associated with these target sites is exchangeable and is composed of at least two distinct mobile fractions. FRAP experiments examined the association rate of Bmi1 and revealed a highly mobile fraction and a less mobile fraction. FLIP experiments demonstrated the dissociation rate of the slow mobile fraction. This supports the notion that Bmi1 possibly acts in functionally distinct PcG complexes with different dynamics. The highly mobile fraction could be related to transient interactions, whereas the slow mobile fraction could represent the relatively stable and static interactions. PcG proteins are involved in maintenance and inheritance of transcriptional states
through cell divisions, hence changes in complex composition and dynamics of these proteins are possibly coupled to the cell cycle (Voncken et al., 1999). Our study reveals that the Bmi1 pool associated with PcG bodies during the G2 phase of the cell cycle is significantly more mobile than during the G1 phase. This could allow more flexibility and dynamic interactions during G2 after the DNA content is duplicated and when the epigenetic transcriptional state should be propagated before committing mitosis. Likewise, changes in PcG protein mobility has been reported for the Cbx family members during embryonic stem (ES) cell differentiation very recently (Ren et al., 2008). The Cbx proteins become more mobile at the onset of differentiation, but the mobility decreases as differentiation progresses. This reflects the necessity of more dynamic and transient interactions during initiation of differentiation, when PcG proteins should be relocating to new target genes, thereby directing differentiation and determining the transcriptional identity of the differentiated cell (Pietersen and van Lohuizen, 2008). Altogether, we and others show that PcG-related repression is flexible and based on continuous exchange. This reflects a dynamic competition model, where PcG proteins must compete with other chromatin binding proteins for interaction with chromatin and argues against heterochromatin composing a stable and static structure with immobilized components.

In addition to the dynamic properties of Bmi1, we have investigated factors involved in its recruitment to PcG-associated heterochromatic target loci. Although many studies in the fly have investigated the recruitment of PRC1 by PRC2 to polycomb response elements through H3K27 methylation (Ringrose and Paro, 2004), at the time only a limited number of studies were reported confirming this model in mammalian systems (Cao et al., 2002; Kirmizis et al., 2004; Kuzmichev et al., 2002). We have provided further evidence that Ezh2 and Eed, the core components of PRC2, are crucial for Bmi1 (PRC1) recruitment to PcG bodies in a human cell line. This Bmi1 recruitment is partly dependent on the histone methyltransferase activity of Ezh2. We found that the accumulation of the Ezh2-dependent H3K27me3 mark on PcG bodies is cell cycle regulated, reaching a peak during very early S phase. These results confirm the conservation of the PRC1 recruitment mechanism by PRC2-dependent H3K27me3 from fly to human. Moreover, they suggest the involvement of additional recruitment mechanisms, given the transient and partial colocalization of the H3K27me3 mark with Bmi1 on PcG-related heterochromatin loci. Although more recent studies reinforce the link between PRC2-dependent H3K27me3 and the recruitment of PRC1 to a subset of PcG target loci in flies and mammals (Boyer et al., 2006; Bracken et al., 2006; Bracken et al., 2007; Lee et al., 2006b; Schwartz and Pirrotta, 2007; Tolhuis et al., 2006), accumulating evidence argues for additional recruitment pathways in particular in mammalian systems (Francis et al., 2004; Klymenko et al., 2006; Papp and Muller, 2006; Pasini et al., 2007; Puschendorf et al., 2008; Ren et al., 2008; Schoeftner et al., 2006; Shao et al., 1999; Vincenz and Kerppola, 2008).
When searching for alternative recruitment mechanisms for PRC1, we found that the DNA methyltransferase Dnmt1 is also required for proper Bmi1 and Ring1b localization to heterochromatin domains without affecting the Ezh2-dependent H3K27 methylation. Our study was the first to couple PcG proteins to the DNA methylation machinery. Interestingly, a functional link between Ezh2 and Dnmts was reported shortly hereafter, proposing that Ezh2 is required for the recruitment of Dnmts (Dnmt1, Dnmt3a and Dnmt3b) to certain Ezh2 target loci. This recruitment seems to be essential for subsequent DNA methylation and silencing of these loci (Vire et al., 2006). Although this study suggests an appealing mechanism which directly links PcG-mediated repression to DNA methylation, it remains rather controversial. It is surprising that Ezh2 is able to directly interact with all three Dnmts equally in vitro and in vivo, while the different Dnmts fulfill distinct roles in development and cellular viability (Li, 2002). Moreover, no other studies have found Dnmts in purified PcG complexes from flies to mammals so far. Clearly, this direct interaction between Dnmts and PRC2 should be explored more thoroughly in the future, particularly since only very few PcG targets have been shown to be effected by this mechanism. Nevertheless, Dnmt recruitment by Ezh2 can stabilize the repressed transcriptional state by CpG methylation of the surrounding DNA, but it can also function as an intermediary sequential recruitment mechanism for PRC1 (Taghavi and van Lohuizen, 2006).

However, more recent reports do verify a connection between the DNA methylation machinery and PcG-mediated gene silencing. For instance, PRC1 proteins are frequently found associated with DNA methylation pathway members, such as Dnmt-associated proteins (Dmap) or methylated DNA binding domain (MBD) proteins, and thereby cooperate with maintenance and/or de novo DNA methylation in gene silencing (NegishI et al., 2007; Sakamoto et al., 2007; Xi et al., 2007). Moreover, other studies demonstrate a mutually reinforcing interaction between PcG-mediated chromatin remodeling and DNA methylation in gene silencing in cancer cells (Gal-Yam et al., 2008; Ohm et al., 2007; Schlesinger et al., 2007; Tanay et al., 2007; Widschwendter et al., 2007; Wu et al., 2008). Although there seems to be a formal link between PcG-mediated transcriptional repression and DNA methylation, the exact molecular mechanism coupling these two silencing machineries needs to be investigated more carefully.

The connection between PcG transcriptional repression and DNA methylation also introduces a mechanism for the propagation of PcG-related expression patterns through cell divisions by maintenance Dnmts in order to preserve cellular identity (Taghavi and van Lohuizen, 2006). Another interesting mechanism for transmission of the PcG-mediated epigenetic mark through the cell cycle was proposed recently. The maintenance of the H3K27me3 mark seems to occur by PRC2 self sufficiently in proliferating cells (Hansen et al., 2007). Although this model explains the inheritance of established PRC2-mediated H3K27 methylation in proliferating differentiated cells, it remains unclear if the PRC1-mediated inactive H2AUb1191
mark is co-conducted during this process and how these marks are relocated and transmitted during differentiation.

It is worth mentioning that PRC1 and PRC2 seem to function independently on occasions. PRC1 can be recruited to chromatin independent from PRC2 and/or H3K27me3. Likewise, PRC2 might not need PRC1 for maintenance of repression, given the recruitment of other repression mechanisms such as DNA methylation (Pasini et al., 2007; Schoeftner et al., 2006; Schuettengruber et al., 2007; Vincenz and Kerppola, 2008; Vire et al., 2006).

While much has been said about the sequential recruitment of PRC1 by PRC2 and other potential intermediary recruitment mechanisms, the initial recruitment of PRC2 to PcG target loci in mammalian systems is still a black box. DNA sequence specific transcription factors, although appealing, have not been found in mammals to direct PcG proteins to target loci, which is perhaps reflected in the nonredundant nature of PcG binding sites (Schuettengruber et al., 2007). Initial recruitment and residence of PcG proteins at the Ink4b-Arf-Ink4a locus and the transcriptional silencing of Ink4a and Ink4b were recently proposed to be caused by the absence of the Swi/Snf chromatin remodeling complex at the same locus. This study indicates that PcG proteins can be indirectly targeted to loci where and when transcriptional activators are absent (Kia et al., 2008).

In addition to this, non-coding RNA (ncRNA) is a suitable candidate to direct PcG recruitment (Zaratiegui et al., 2007). In fact, many PcG proteins, such as Ezh2, Eed, Suz12, Sop-2/Hph1 and Cbx7, have been found to encompass RNA binding potential (Bernstein et al., 2006; Hall, 2005; Rinn et al., 2007; Zhang et al., 2004; Zhao et al., 2008). Notably, small regulatory ncRNA-related pathways, such as the RNAi and miRNA machinery, have been connected to PcG-mediated transcriptional gene silencing recently (Grimaud et al., 2006; Kim et al., 2006; Kim et al., 2008). A prominent example of long ncRNA-associated epigenetic silencing by PcG proteins is the mammalian X chromosome inactivation instigated by Xist RNA (Masui and Heard, 2006). Although the detailed mechanism by which Xist induces silencing remains unclear, this ncRNA is linked to PRC2 recruitment and H3K27 methylation during X chromosome inactivation (Plath et al., 2003). Just recently a repetitive RNA sequence, RepA, which is encoded by Xist, was discovered to interact with PRC2 and was shown to be required for PRC2-dependent H3K27 methylation and X chromosome inactivation (Zhao et al., 2008). Another elegant study strengthened the link between ncRNAs and PcG recruitment and transcriptional silencing by identifying the ncRNA, HOTAIR, within the human HoxC locus. HOTAIR RNA interacts with PRC2 and is required for PRC2 recruitment, H3K27 methylation and the subsequent silencing of the human HoxD locus in trans (Rinn et al., 2007). Additionally, two recent studies demonstrate an important role for another regulatory ncRNA, Kcnq1ot1, in recruitment and establishment of PcG-induced gene silencing of imprinted Kcnq locus in placenta (Pandey et al., 2008; Terranova et al., 2008). Altogether, ncRNAs appear to be suitable as recruiting factors for
PcG proteins and the PcG-mediated transcriptional silencing and therefore are an important area of future research.

ncRNAs are of particular interest due to the possibility of the combination of variable DNA sequence recognition and many tertiary structure-conformation possibilities, making it more probable to adapt to and interact with diverse protein complexes and DNA at the same time. This highly adaptable recruitment mechanism can possibly explain the current non conserved interactions of PcG proteins with chromatin and the regulatory diversity and complexity of PcG-mediated epigenetic programming.

Finally, various recruitment possibilities, protein complex diversity and the dynamic association of PcG proteins with chromatin, render the PcG-mediated transcriptional program extremely flexible. This flexibility is prerequisite to the concerted epigenetic regulation during embryogenesis but also to maintain stable transcriptional states, while staying receptive and adaptable to environmental changes in adult life.

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