From LPA signaling to polycomb: oncogenic cooperations revealed by genetic screens and dynamics and recruitment of polycomb group proteins

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Chapter 1

General Introduction

Cooperative Oncogenic Alterations Involved in Tumorigenesis and Bmi1, the Founding Member of Mammalian Polycomb Group Proteins
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Malignant transformation is a multistep process during which normal cells acquire abilities that allow uncontrolled cellular proliferation and escape from inhibitory mechanisms, which guard their genomic integrity. During this process, cells are subject to genetic mutations and continuous selection in interplay with their microenvironment. To specifically eradicate tumor cells during therapeutic intervention, it is of extreme importance to find cooperating oncogenic lesions and to identify the mechanism by which they direct oncogenic transformation.

Cancer cells arise from normal cells that have undergone a series of genetic alterations and consequently have acquired all the properties that confer a selective advantage during their micro-evolution towards malignancy (Hanahan and Weinberg, 2000). Therefore, cancer is a heterogeneous disease and the oncogenic process engages many different proteins and pathways that regulate tumor initiation, progression and metastasis. During the past few decades, we have been able to gather a great deal of knowledge on pathways and mechanisms involved in cancer. Yet many anticancer compounds used in the clinic to date still lack specificity and only a handful of designed targeted drugs are developed so far. The reason for this is that identification of molecular alterations driving cancer, which eventually prove to be suitable as drug targets, remains an extremely challenging task. However, many already known proteins and cancer-relevant pathways could still be verified as drug targets and >40% of human open reading frames have not yet been given a function. This emphasizes that there are still numerous potentially “druggable” targets to be discovered. Carefully designed genetic screens based on phenotypes associated with different aspects of cancer (from tumor initiation to metastasis) are one of the most powerful tools in identifying new proteins acting in certain pathways (Brummelkamp et al., 2003; Douma et al., 2004; Jacobs et al., 2000; Uren et al., 2008).

This introductory chapter will give an overview concerning the role of the Myc and Ras oncogenes and the Arf tumor suppressor pathway in oncogenesis. These pathways are affected in many types of human cancers. We will focus on the search for novel oncogenic partners cooperating with c-Myc or a constitutively active mutant of Ras, RasV12, and their potential targets in tumorigenesis (chapters 2 and 3). Moreover, we will discuss Polycomb group (PcG) proteins in order to introduce our study (chapter 4) on dynamics and recruitment of Bmi1, which is one of the oncogenic collaborators of c-Myc and a major chromatin-associated repressor of the Ink4a-Arf tumor suppressors.
Search for novel cooperating genetic alterations involved in tumorigenesis

Cooperating genetic events
Genetic and epigenetic mutations responsible for the deregulated growth of cancer cells can be simplified as alterations that activate oncogenes and/or inactivate tumor suppressor genes. Although mutations occur randomly, there is a selective advantage for those mutations and eventually cooperative pathways that confer the tumor cell a more malignant phenotype. One model example is the gradual development of colorectal cancer, where specific genetic lesions are associated with distinct stages of tumor initiation and progression (Kinzler and Vogelstein, 1996). Likewise, normal human epithelial cells and fibroblasts need at least four distinct genetic alterations to become fully transformed in vitro (Hahn et al., 1999). The idea that genetic lesions cooperate to induce transformation, “the oncogenic cooperation model”, was generated when the addition of only two oncogenes (Myc and Ras) was sufficient to transform normal rodent cells (Land et al., 1983).

Immortalization and transformation
Normal cells need to battle a number of anticancer defense mechanisms to become fully malignant. Tumor initiation requires proliferation without growth factor stimulation, denial of growth inhibitory signals and escape from apoptosis. Tumor maintenance and progression necessitate sustained angiogenesis, invasive potential and final metastasis (Hanahan and Weinberg, 2000).

Here, we discuss events that accompany the initiating steps of tumorigenesis i.e. immortalization and transformation. When normal primary human or rodent cells are expanded in vitro, they will undergo an irreversible G1 cell cycle arrest and will no longer be sensitive to growth factor stimulation after a finite number of passages. This phenomenon was observed many years ago and is now known as replicative senescence (Hayflick, 1965). From current studies we know that senescence is not only induced after extensive culturing, but also following various stresses such as oxidative damage, telomere dysfunction, oncogene activation and DNA damage (Campisi and d’Adda di Fagagna, 2007). More recently, studies have shown that senescence in vivo is not only associated with aging but also functions as a tumor suppressive mechanism (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005). Senescence inhibits proliferation of premalignant lesions in response to oncogene activation or when malignant tumor cells are treated with cytotoxic agents (Collado and Serrano, 2006; Shay and Roninson, 2004). Thus, senescence seems to be a common mechanism in aging and cancer, simply preventing cells from proliferating indefinitely, while accumulating genetic errors.

Lately, oncogene induced senescence (OIS) has caught a lot of attention since many groups have confirmed its relevance in premalignant lesions in vivo, and
revealed its possible mechanisms of induction (Acosta et al., 2008; Kuilman et al., 2008; Sharpless and DePinho, 2005; Wajapeyee et al., 2008). OIS was first observed when introduction of RasV12 in human and rodent cells, albeit through slightly different mechanisms, induced a premature cell cycle arrest (Palmero et al., 1998; Serrano et al., 1997). Soon, oncogenic versions of other members of the Ras pathway (Raf, B-Raf and Mek) were also shown to evoke premature senescence (Lin et al., 1998; Michaloglou et al., 2005; Zhu et al., 1998). Senescence is thus an important defense mechanism against excessive proliferation induced by activated oncogenes.

Normal primary cells must escape the senescence crisis in order to proliferate indefinitely and to become so called “immortal”. Senescence is mainly induced by activation of the Arf-p53 and Ink4a-pRb tumor suppressor pathways. Genetic or epigenetic inactivation of any member of these pathways can lead to immortalization. Immortal cells are not transformed per se. Many primary human cell types can be immortalized, for instance by expression of the enzyme that protects telomere caps (telomerase), without presenting the phenotypic traits of malignant transformation (Hahn et al., 1999). One of the hallmarks of transformation is anchorage-independent growth, which is strongly associated with in vivo tumor formation. Immortalized cells can become transformed and thus tumorigenic upon additional oncogenic hits like oncogene activation and tumor suppressor inactivation. Hence, immortalization can be considered as one of the initiating steps towards malignant transformation.

The vicious and the evil: Ras and Myc

Ras and Myc oncogenes are found mutated or overexpressed in a wide spectrum of human tumors and have consequently been the subject of intense research during the past decades (Adhikary and Eilers, 2005; Karnoub and Weinberg, 2008).

H-Ras, K-Ras and N-Ras are the founding members of a large family of Ras GTPases consisting of 39 members (Wennerberg et al., 2005). Ras proteins are GTP-regulated molecular switches associated with the inner face of the plasma membrane, where they propagate a varied collection of extracellular signals (Barbacid, 1987). There is also evidence for Ras signaling from intracellular membranes such as the Golgi apparatus and the endoplasmic reticulum (Chiu et al., 2002). Ras modulates diverse signaling pathways that affect cell proliferation, differentiation, motility, adhesion, cytoskeletal rearrangements and apoptosis (Campbell and Der, 2004; Lowy and Willumsen, 1993). Therefore, Ras proteins play crucial roles in diverse aspects of normal development and malignant transformation. In more than 25% of all human cancers, Ras related pathways are activated either by activating mutations of Ras itself or alterations of upstream or downstream pathway members (Downward, 2003). Deregulated Ras activity is not only involved in oncogenesis but has also been suggested to have essential developmental consequences. A number of congenital developmental disorders
known as cardio-facio-cutaneous disorders have been linked to abnormal activity of the Ras pathway (Karnoub and Weinberg, 2008).

Ras is activated and able to engage downstream signaling pathways when it is bound to GTP. It is inactivated when it is bound to GDP. Ras activity is tightly regulated by guanine nucleotide exchange factors (GEFs), and GTPase activating proteins (GAPs). GEFs stimulate Ras activity by promoting the nucleotide exchange rate and GAPs inactivate Ras by accelerating the intrinsic nucleotide hydrolysis rate (GTP to GDP) and thereby stimulate the formation of the inactive, GDP-bound form (Mitin et al., 2005). Mutated variants of Ras that are particularly affected at residues 12, 13 or 61 are found in many human cancers. These mutants are insensitive to GAP activity and are consequently locked in a constitutively, GTP-bound, activated state (Malumbres and Barbacid, 2003). The function of Ras is in addition regulated by the posttranslational processing of its C-terminal end. This is

![Figure 1. Major Ras signaling pathways. An example of Ras activation through stimulation of a receptor tyrosine kinase (RTK) or a G protein-coupled receptor (GPCR). The Ras cycle starts with its posttranslational modification (farnesylation) that targets it to the plasma membrane. At the plasma membrane, receptor stimulation leads to the recruitment of Ras specific GEF, son of sevenless (Sos), and subsequent Ras activation. Activated Ras induces many different signaling pathways that, depending on the cellular context, could either result in cellular survival and cell cycle progression (through PI3K, Mapk and RalGDS cascade) or induce cell cycle arrest and apoptosis (through Ink4a and Arf tumor suppressor pathways).](image-url)
an essential step guiding Ras to specific locations of the plasma membrane, where it translates extracellular signals to downstream pathway activation (Downward, 2003).

Mitogenic signals elicited by growth factors and cytokines activate Ras proteins by regulating the activity of GEFs and GAPs. One of the best studied examples involves the activation of the Ras specific GEF, son of sevenless (Sos) in response to epidermal growth factor (EGF) stimulation (Figure 1). Sos is recruited to the plasma membrane through adaptor proteins Grb2 and Shc upon EGF receptor tyrosine kinase activation and autophosphorylation (Mitin et al., 2005). Once Ras is switched on, it activates a variety of pathways. The most intensively studied effectors of Ras are depicted in Figure 1. Ras triggers the mitogen-activated protein (Map) kinase cascade through interaction with and activation of Raf (a Map kinase kinase kinase, Mekk). Activated Raf phosphorylates downstream Map kinase kinases (Mek), which in turn activate extracellular signal-regulated kinases (Erk1/2). Erk1/2 phosphorylate a variety of the cytosolic and nuclear proteins that are necessary to promote cell cycle progression. In addition to the Map kinase pathway, Ras can directly interact with and activate the catalytic subunit of phosphatidylinositol 3-kinase (PI3K), leading to the induction of the best characterized anti-apoptotic pathway of the cell. Both of these pathways are sufficient and necessary in Ras-induced transformation (Karnoub and Weinberg, 2008). Activation of RalGDS-Ral pathway by Ras also leads to cell survival and proliferation (Hamad et al., 2002). Furthermore, activation of Rac and Rho-dependent pathways downstream of Ras lead to cytoskeletal rearrangements inducing changed morphology and altered migrative capacity (Downward, 2003).

In addition to promoting cell proliferation, Ras also induces tumor suppressor pathways (Figure 1), which induce cell cycle arrest and counteract the proliferative effects of Ras. Ras activates pRb and p53 tumor suppressors through induction of Ink4a and Arf respectively, possibly via p38, to trigger a premature senescence response (Karnoub and Weinberg, 2008). Ras can also induce Ink4b, which is expressed from a locus in close proximity to the Ink4a-Arf locus. Ink4b can, similar to Ink4a, trigger cell cycle arrest through activation of pRb (Malumbres et al., 2000). Induction of these tumor suppressor pathways partly explains the mechanism of oncogenic cooperation between Ras and oncogenes such as E1A and SV40, which inhibit p53 and pRb pathways (Figure 2).

There are distinct cell type- and species-specific differences in the molecular mechanisms that induce senescence. It is generally accepted that Ras induces cell cycle arrest through p19Arf in mouse cells, whereas p16Ink4a fulfills a more important role in Ras-induced senescence in human cells (Gil and Peters, 2006). Furthermore, expression levels of Ras seem to differentiate between a proliferative and a senescence response to Ras activation. Moderate expression of activated Ras, comparable with endogenous expression levels, leads to Ras-induced transformation. In contrast, strong overexpression of Ras triggers an OIS
response to protect the cells against oncogenic transformation (Johnson et al., 2001; Tuveson et al., 2004).

Another oncogene which is of special interest in this thesis is c-Myc. c-Myc is a nuclear protein that is induced as an early response gene by many types of signals. It is a potent regulator of cell proliferation, differentiation and apoptosis. Although its expression is normally tightly regulated (Sears, 2004), genetic alterations resulting in overexpression of c-Myc are found in many human cancers (Nesbit et al., 1999). c-Myc is a member of the Myc-family of bHLH/LZ transcription factors including L-Myc and N-Myc and acts upon heterodimerization with its partner Max. c-Myc is not only involved in transactivation of its target genes but also in transcriptional repression. Max can in addition heterodimerize with Mad-family transcription factors. Mad-Max heterodimers antagonize Myc-Max heterodimers (Grandori et al., 2000).

Figure 2. Schematic outline of p16ink4a-pRb and p19Arf-p53 tumor suppressor pathways. Oncogenic activation or cellular stress induce p16ink4a and p19Arf leading to downstream activation of pRb and p53 through inhibition of Cdk4/6 and Mdm2, respectively, resulting in cell cycle arrest or apoptosis. Oncogenic cooperations between different oncogenes can be partly explained based on their opposite regulatory effects on these tumor suppressors and their downstream effectors. Oncogenes inducing a given tumor suppressor can cooperate with oncogenes inhibiting the same tumor suppressor, a simplified explanation for some oncogenic cooperations. Oncogenes inhibiting the tumor suppressors are depicted in red circles and oncogenes activating tumor suppressors are shown in the green circle.
The mechanism by which c-Myc is induced in an immediate response to external stimuli remains unclear. However, more is known about the regulation of c-Myc protein stability, which indirectly influences c-Myc protein level. Many growth factors and cytokines seem to regulate the abundance of the c-Myc protein rather than its direct transcriptional induction (Eisenman, 2001; Sears, 2004). One example is the regulation of c-Myc protein stability by the Ras signaling pathway which partly underlies the oncogenic cooperation between c-Myc and activated Ras. Ras controls c-Myc stability through regulating phosphorylation at Serine 62 and Threonine 58. The phosphorylation of Serine 62 by activated Erk stabilizes c-Myc by inhibiting its ubiquitin mediated degradation. Phosphorylation of Threonine 58 by GSK3β destabilizes c-Myc by facilitating dephosphorylation at Serine 62. Ras activation inhibits GSK3β through induction of PI3K and thereby stabilizes c-Myc (Figure 3) (Sears, 2004). Regulating stabilization of c-Myc protein is not the only mechanism underlying the oncogenic cooperation between activated Ras and c-Myc. FoxO transcription factors, which counteract c-Myc transactivation are inactivated by PI3K, which in turn is activated downstream of Ras. In addition, PI3K activation has been shown to be required for transcriptional repression by c-Myc (Figure 3) (Adhikary and Eilers, 2005).

Unlike Ras, the molecular mechanism by which c-Myc promotes tumorigenesis is largely unknown. The reason for this is that identification of specific target genes, which could explain the diverse biological effects of c-Myc has remained elusive. In the classical “gene specific” model, c-Myc was shown to control the cell cycle by directly activating cyclin D2 and Cdk4 and inhibiting p21 and p27, which are known cell cycle regulators. In addition, many other putative target genes were identified (eIF4E, hTert, p53 and Arf) that could partially explain some functions of c-Myc in cell cycle regulation, immortalization and apoptosis (Grandori et al., 2000). These

![Figure 3. Oncogenic collaboration between Myc and Ras. Ras activation leads to downstream induction of Map kinase and PI3K pathways, which both support Myc-induced oncogenesis. 1) Activated Erk and PI3K both lead to Myc protein stabilization through stimulation of Ser62 and inhibition of Thr58 phosphorylation 2) Activated PI3K pathway additionally cooperates with Myc through activation and repression of common target genes.](image-url)
initial attempts of hunting Myc-target genes were mainly based on the function of putative targets or the presence of potential c-Myc binding sites in the promoter regions of these genes. The validation of the ever-accumulating putative c-Myc target genes proved to be a difficult task because of its weak transactivation and transcriptional repression capacity. Recently, high throughput screens and chromatin binding profiling combined with target gene expression profiling of Myc and its partner Max have revealed that c-Myc can bind up to 15% of all genes in different model organisms (Fernandez et al., 2003; Guo et al., 2000; Li et al., 2003; Mao et al., 2003; O’Connell et al., 2003; Orian et al., 2003; Schuhmacher et al., 2001). However, the transcription of only a subset of these putative loci seems to be regulated by c-Myc thus far. c-Myc affects transcription mainly, through recruitment of chromatin remodeling proteins such as histone acetyltransferases, histone kinases, DNA methyltransferases and other basic co-transcription factors to target loci, by opening or condensing local chromatin structure (Knoepfler et al., 2006). Hence, c-Myc can be considered as a global regulator of the transcriptome, modulating target gene expression rather than being a target specific transcription factor with a limited number of defined downstream targets, which corroborate its biological function (Dang et al., 2006; Knoepfler, 2007). Nevertheless, so far, c-Myc target genes seem to be involved in cell cycle regulation, metabolism, ribosome biogenesis, general translation, mitochondrial function, apoptosis, immortalization and genetic instability. In addition, c-Myc seems to downregulate genes involved in cell cycle inhibition and cellular adhesion (Dang et al., 2006; Grandori et al., 2000). Thus, c-Myc affects many diverse biological processes that can explain its prominent role in tumorigenesis.

Activation of Ras or enhanced expression of c-Myc by itself is not sufficient to transform primary cells and drive tumorigenesis. Additional lesions are required to cooperate with each of these oncogenes to drive transformation. As mentioned earlier, the Ink4a- and Arf-mediated tumor suppressor pathways are crucial fail safe mechanisms that inhibit transformation upon abnormal mitogenic stimuli by triggering cell cycle arrest or apoptosis. Immortalization and subsequent transformation is therefore possible through removal of these tumor suppressors either directly by locus deletion or inactivating mutations or indirectly via activation of oncogenes that attenuate these protective pathways. In addition to the cooperating oncogenes mentioned above (E1A and SV40), Ras and Myc are capable of collaborating with Tbx2, Twist and Bmi1 (Figure 2). These oncogenes are capable of counteracting Ras- and Myc-induced senescence and apoptosis respectively (Ansieau et al., 2008; Jacobs et al., 1999b; Jacobs et al., 1999a; Jacobs et al., 2000; Maestro et al., 1999). Such oncogenes synergize with Ras or c-Myc by suppressing Ink4a and/or Arf, thereby facilitating immortalization or transformation of primary cells.
The gatekeepers: Ink4a and Arf

The evolutionary developed response to the action of onco-proteins is the fail-safe mechanism induced by tumor suppressors. pRb and p53 are the founding members of a large group of functionally related proteins that protect the cells against inadequate proliferation. They do so through the induction of cell cycle arrest or apoptosis when cells commit to division at an inappropriate time and place with an inapt genetic content. Hence, many pathways activated by tumor suppressors are involved in maintaining genomic integrity and guiding normal cell division in response to cellular microenvironment.

One of the major tumor suppressor pathways involves pRb. pRb is part of a family of transcriptional corepressors including p107 and p130, which inhibit the expression of genes required for cell cycle progression. The pRb pathway regulates the cell cycle mainly by repressing transcription of E2F target genes, which are required for cell cycle entry. pRb binds to E2F transcription factors that are located at the promoters of target genes and recruits chromatin remodeling complexes. These multimeric complexes contain histone deacetylases (Hdacs) and DNA methyltransferases (Dnmts), which are known to induce transcriptional silencing (Burkhart and Sage, 2008). pRb has in addition been implicated in lineage specific commitment of precursor cells during differentiation through global transcriptional regulation (Hansen et al., 2004). This is recently more emphasized by studies revealing its interaction with chromatin modifiers such as Rbp2 (Lopez-Bigas et al., 2008; Pasini et al., 2008).

Another crucial tumor suppressor pathway is directed by p53, which is a transcription factor that when activated, coordinates a wealth of downstream events. This occurs through protein modifications, protein-protein interactions and transactivation via DNA sequences of p53-response-elements. The global transcriptional change induced by p53 activates specific pathways, which result in cell cycle arrest, DNA repair, senescence or apoptosis (Levine et al., 2006). These two major tumor suppressive pathways are activated by many kinds of intrinsic and extrinsic stress signals including oxidative stress, DNA damage, hypoxia, replicative stress and aberrant mitogenic signaling (Lowe et al., 2004). These signals are sensed by as yet unknown mechanisms, which in turn activate upstream core regulators of pRb and p53: Ink4a and Arf.

Ink4a and Arf take centre stage when it comes to the cell’s defense mechanisms against inappropriate growth signals. They do so mainly by modulating the activity of negative regulators of p53 and pRb. Ink4a and Arf are unique tumor suppressors, because they can register and respond to culture stress and mitogenic stimuli specifically. Although they share the same genetic locus, they do not have protein sequence homology, as they are efficiently encoded from alternative reading frames. Given the crucial role of pRb and p53 in tumor suppression, it is conceivable that their upstream regulators are also targeted for mutations in many cancers. In fact, inactivation of the Ink4a-Arf and pRb-p53 tumor suppressive
network occurs in almost all human cancers in a mutually exclusive fashion (Kim and Sharpless, 2006). While this combined genetic locus seems to create a weak point in tumorigenesis, a coupled response of the encoded tumor suppressors to common cues must have endured a stronger selective pressure during normal development (Sherr, 2004).

Ink4a (p16Ink4a) belongs to the class of cyclin-dependent kinase (Cdk) inhibitors, which act upstream of pRb. Interaction of Ink4 proteins (Ink4a and Ink4b) with Cdk4 and Cdk6 hinders their binding to D-type cyclins and consequently inhibits phosphorylation of pRb proteins by Cdk4/6. Hypophosphorylated pRb binds to E2F and inhibits transcription from its target loci resulting in cell cycle arrest, senescence or cell death (Burkhart and Sage, 2008; Gil and Peters, 2006). Arf (p19Arf in mouse and p14Arf in human), binds to and inactivates Mdm2, which in turn inactivates p53 by targeting it for degradation (Figure 2). Although Arf-dependent tumor suppressor activity is for the most part attributed to its key regulation of p53, numerous p53-independent functions of Arf have been also reported (Sherr et al., 2005). Additionally, there is reciprocal regulation between the Arf-p53 and the Ink4a-pRb axis. The Cdk inhibitor p21 is induced downstream of p53 and activates the pRb pathway through inhibition of Cdk2/CycE (Sherr, 2004). Activated E2F can in part counteract its own action by inducing Arf (Figure 2) (Aslanian et al., 2004; Bates et al., 1998). Hence, inactivation of pRb leads to derepression of E2F target genes and subsequent activation of Arf-p53 pathway, linking pRb directly to p53.

The importance of Ink4a and Arf as tumor suppressors in human cancers has been confirmed by the frequently found genetic or epigenetic inactivation of their genomic location (Cdkn2a) on chromosome 9p21 in many tumor types. In close proximity to this genomic locus lies Cdkn2b, which encodes Ink4b. Recent studies have shown clear tumor suppressive functions for Ink4b in the absence of Ink4a, suggesting that Ink4b acts as a back-up mechanism that functionally substitutes for Ink4a (Krimpenfort et al., 2007).

The relative importance and the species- and cell type-specificity of these tumor suppressors are only recently being elucidated. This is achieved through studies using mouse models and by searching for specific mutations in different human tumor types that inactivate one member of the locus while leaving the others functional. It has been proposed that Ink4a plays a relatively more important role in tumor suppression in human cells, whereas Arf has a more prominent function in mouse cells (Gil and Peters, 2006). For instance, although Myc and Ras are both capable of inducing Ink4a and Arf, oncogene induced apoptosis and senescence in mouse cells are mechanistically connected to Arf induction.

How the upstream oncogenic signaling is distinguished from normal growth signaling is not entirely clear. It is thought that perceiving at least two interrelated signals such as stress signals and proliferative signals simultaneously is required to induce a response such as OIS (Kim and Sharpless, 2006). The molecular
The machinery underlying senescence is not only involved in protecting against cancer, but it is also involved in aging, since it limits the regenerative potential of aging tissues. Expression of Ink4a and Arf is significantly increased in almost all tissues of aging mice and humans (Kim and Sharpless, 2006; Krishnamurthy et al., 2004). Considering the crucial functions of these gatekeeping tumor suppressors, the expression of Ink4a and Arf is subject to tight regulation. Many transcriptional regulators that are found to (co-)regulate the expression or the function of these tumor suppressors (Gil and Peters, 2006) may cooperate and fulfill crucial roles in cancer formation and progression and are therefore of special interest (Figure 2). In this thesis, we will only focus on the role of c-Myc, Ras, Tbx2 and Bmi1 in tumorigenesis with regard to Arf regulation in mouse cells. Oncogenic signaling by c-Myc or activated Ras in mouse fibroblasts leads to upregulation of p19Arf and subsequent induction of oncogene-induced apoptosis or oncogene-induced senescence, respectively. Tbx2, a T-box transcription factor, represses p19Arf and thereby collaborates with c-Myc or RasV12 to immortalize primary cells (Figure 2) (Jacobs et al., 2000). Bmi1 is a member of the Polycomb Group (PcG) of transcriptional repressors. One of the most extensively studied targets of these repressive protein complexes is the Ink4b-Arf-Ink4a locus which explains the oncogenic collaboration between Bmi1 and c-Myc or RasV12 (Adhikary and Eilers, 2005; Grandori et al., 2000; Jacobs et al., 1999a).

**Bmi1; the founding member of mammalian Polycomb group proteins and a prominent regulator of Cdkn2a locus**

Loss-of-function of the Ink4a and Arf tumor suppressors, which is commonly involved in the development of many human malignancies, not only occurs through genetic changes such as mutations and deletions of the Cdkn2a locus, but also happens through epigenetic silencing of the locus.

DNA and its associated proteins are packaged into chromatin. This highly organized structure not only functions to compress and protect DNA, but it also regulates transcription. Epigenetic transcriptional regulation relates to mechanisms that affect gene expression by altering higher order chromatin structure rather than changes in DNA sequence. These mechanisms range from DNA methylation and histone modifications to nucleosome compaction and chromatin fiber organization, determining the accessibility of chromatin to the transcriptional machinery. This so-called “epigenetic code” is maintained and inherited through cell divisions, creating a cellular transcriptional memory.

One of the most important building blocks of chromatin are histones, around which DNA is wrapped. Posttranslational modifications (acetylation, methylation, ubiquitination, sumoylation, phosphorylation and ADP-ribosylation) of histone tails directly influence the epigenetic state. Transcriptionally active genes contain
relatively more acetylation on lysine 14 of histone H3 (H3K14ac) and trimethylation on lysine 4 of histone H3 (H3K4me3) on their promoter regions, whereas silent genes are more associated with H3K9me3 and H3K27me3 and are generally deacetylated. Except for acetylation, other covalent modifications of histone tails were believed to be stable and irreversible. However, recent studies have revealed the possibility of erasing histone modifications through identification of deubiquitinating and demethylating enzymes that are capable of removing these marks, adding an additional level of complexity to the epigenetic code (Agger et al., 2008).

Bmi1, an important regulator of the Cdkn2a locus, is a member of the PcG proteins. PcG proteins belong to a large family of epigenetic transcriptional repressors, which were originally identified in Drosophila to regulate appropriate expression of homeotic (Hox) genes responsible for the establishment of the body plan during early embryonic development. The mammalian counterparts of PcG proteins are now known to function as global regulators of gene expression, responsible for maintenance and inheritance of silent chromatin states. PcG proteins are involved in many processes, including stem cell renewal, differentiation, cellular proliferation and X-chromosome inactivation (Masui and Heard, 2006; Sparmann and van Lohuizen, 2006).

PcG proteins act in multimeric protein complexes, which, based on biochemical purifications and genetic experiments, are roughly classified into initiation and maintenance complexes (Table 1). Polycomb repressive complex 2 (PRC2),
consisting of Ezh2, Eed and Suz12, is responsible for initiation of repression. PRC1, containing the core components Hpc/Cbx, Hph/Edr, Bmi1 and Ring1b, is involved in the maintenance of transcriptional repression. This is a simplified classification since many of these proteins exist in a number of isoforms and homologues, creating many variants of each complex. Additional PRCs (PRC3 and 4) in flies and mammals have been identified, including subtle changes in complex composition and consequent functional specificity, confirming a high degree of flexibility in fine-tuning epigenetic regulation (Klymenko et al., 2006; Kuzmichev et al., 2004).

PcG proteins take part in histone tail modifications either by catalyzing them directly or by interaction with specific enzymes. PRC2 contains histone methyltransferase activity through Ezh2, which catalyzes H3K27me³ and to a lesser extend H3K9me³ (Cao et al., 2002; Czermin et al., 2002; Muller et al., 2002). Ezh2 is also able to catalyze H1K26 methylation depending on interaction with distinct Eed isoforms (Kuzmichev et al., 2004). In addition, PRC2 has been linked to histone deacetylase (Hdac) activity and to H3K4 demethylase activity through interaction with Rbp2 (Pasini et al., 2008). PRC1 has E3 ubiquitin ligase activity through Ring1b. Ring1b is able to monoubiquitinate histone H2A (H2AK119Ub¹) which is stimulated by its interaction with cofactors Bmi1 and Mel18 (Cao et al., 2005; Elderkin et al., 2007). Although PRC1 has been found to recruit additional H3K4 demethylases through Mblr (Lee et al., 2007), no histone methyltransferase activity has been associated with PRC1 yet. Furthermore, recent studies have shown

Figure 4. A basic model for PcG complex composition and action. The initial recruitment of PRC2 to target loci inhibits acetylation and H3K4me³ and induces H3K27me³ of the histone tails. This is recognized by PRC1 like complexes which are subsequently recruited to these loci. PRC1 catalyzes an additional histone tail modification that is linked to transcriptional silencing, H2A119Ub¹. PRC2 and PRC1 are thought to initiate and sustains transcriptional silencing respectively.
Chromatin immunoprecipitation (ChIP) profiling experiments in mouse and human cells have shown that the repression of the Cdkn2a locus depends on binding of the PRC2 members, Ezh2 and Suz12 and the subsequent appearance of the H3K27me3 mark on this locus. Furthermore, binding of the PRC1 proteins Bmi1 and Cbx8 to this locus increases concomitantly, leading to repression of Ink4a and Arf, confirming PRC1 recruitment by PRC2. Corroborating this, Ezh2 expression levels decrease during replicative- and stress-induced senescence, leading to downregulation of H3K27me3 mark, displacement of Bmi1 from the locus and derepression of the encoded tumor suppressors (Bracken et al., 2007). In addition to Bmi1, the other PRC1 core members Cbx7 and Cbx8 have been shown to regulate Ink4a-Arf and thereby influence cell cycle progression. Ectopic expression of these genes was shown to postpone the onset of senescence in mouse and human cells (Dietrich et al., 2007; Gil et al., 2004).

As mentioned earlier in this introductory chapter, Cdkn2a encodes bona fide tumor suppressors with a prominent role in cancer development and their direct repression connects PcG activity to tumorigenesis. Although Ink4a-Arf repression
significantly contributes to the oncogenic capacity of Bmi1, this locus is certainly not the sole target of Bmi1 and other PcG protein members in cancer. Several studies have established genome wide maps for binding profiles of PcG proteins and for histone modifications linked to PcG silencing (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006). These studies show that next to Hox genes, PcG proteins are involved in the repression of many developmentally regulated genes that are necessary for differentiation of stem cells and progenitors into various cell types. This suggests that PcG silencing is part of a program that directs cell fate decisions from pluripotent stem cells to differentiated cells, keeping the genes that induce differentiation repressed. These data confirm genetic studies in which targeted disruption of PcG proteins revealed a crucial role, for instance for Bmi1 and Ring1b, in the proliferation and self renewal of embryonic and adult stem cell pools (Sparmann and van Lohuizen, 2006; Valk-Lingbeek et al., 2004; van der Stoop et al., 2008).

Even though there is a direct link between PcG proteins and tumorigenesis through Ink4a-Arf, other potential targets have been identified which could contribute to the Ink4a-Arf independent oncogenic effects of PcG proteins (Bracken et al., 2006; Bruggeman et al., 2007; Dietrich et al., 2007; Squazzo et al., 2006). Furthermore, since PcG proteins regulate stem cell pluripotency, they could influence tumor cell malignancy through establishing a stem cell-like transcriptional program that maintains the self renewal capacity and inhibits differentiation. This has been postulated to contribute to cancer development and tumor recurrence after treatment, which is proposed to be attributed to “cancer stem cells” or tumor re-initiating cells (Pardal et al., 2003).

Moreover, Ink4a-Arf levels rise during aging, but Bmi1 levels do not change. However, Ezh2 levels decrease during senescence, indicating that H3K27me3 mark is limiting to recruit Bmi1 and maintain repression. Therefore, Ezh2 levels may be regulated with aging (Bracken et al., 2007). Finally, in this context, the upregulation of Bmi1 and Ezh2 in many cancers could inhibit oncogene-induced and/or stress-induced senescence in tumor initiating cells, through repression of Ink4a and Arf, extending time to accumulate additional mutations that select for a more malignant phenotype.

The scope and aim of this thesis

*Genetic transformation screens*

Overexpression or aberrant activation of Myc and Ras are found in most human cancers. Given the critical functions of these proto-oncogenes in cellular proliferation, we were interested in finding new oncogenes that could cooperate with Myc or Ras. *Chapters 2 and 3* of this thesis are devoted to our search for novel genes that are able to collaborate with c-Myc or Ras in oncogenic transformation,
using genetic screens.

Immortalized mouse cells can easily be transformed by addition of a single oncogene. In addition, primary mouse fibroblasts can be transformed by introduction of only two cooperating oncogenes such as c-Myc and activated Ras (RasV12). In contrast, in primary human cells, genetic events necessary to induce transformation are far more complicated. Therefore, primary mouse cells constitute a suitable cellular system to search for cooperating oncogenes using forward genetic screens.

Activation of Ras alone in primary mouse fibroblasts (MEFs) leads to p19Arf induction and subsequently to activation of p53-dependent senescence. Likewise, overexpression of c-Myc induces p53-dependent apoptosis through upregulation of p19Arf (Figure 5). Inactivation of p19Arf or p53 cooperates with c-Myc or RasV12 to immortalize primary MEFs. The p19Arf-p53 pathway plays an essential role in replicative senescence and oncogene-induced responses such as senescence or apoptosis in mouse cells. MEFs, overexpressing Ras or c-Myc, can sporadically

![Figure 5. p19Arf-p53 pathway induction by Ras and Myc in mouse cells.](image)

Ras activation leads to premature senescence through p53-dependent cell cycle arrest branch, while Myc overexpression triggers p53-dependent apoptosis branch. Modulating p19Arf expression levels upstream of p53 can bypass senescence and apoptosis and induce immortalization of primary mouse cells. This model has been used, in the studies described in this thesis, to set up genetic screens to find genes that cooperate with Ras or Myc in tumorigenesis when p19Arf levels are reduced. Adapted from (Lowe et al., 2004)
escape either senescence or apoptosis by random genetic lesions which usually result in p53-pathway inactivation. In order to immortalize MEFs overexpressing Ras or Myc in a controlled manner, we used exogenous overexpression of the transcription factor Tbx2. Tbx2 suppresses p19Arf to a threshold level, which is enough to bypass Ras-induced senescence and c-Myc-induced apoptosis. Thereby, Tbx2 efficiently cooperates with Ras or Myc to immortalize MEFs, yet does not transform them (Figure 5). We have taken advantage of this cooperation and made use of MEFs that either co-expressed c-Myc and Tbx2, or RasV12 and Tbx2. Under these conditions, MEFs are immortalized and prone to transformation upon additional genetic lesions that collaborate with c-Myc or Ras, when p19Arf is suppressed.

In chapter 2, we have performed a genetic transformation screen in MEFs overexpressing c-Myc and Tbx2 using retroviral based cDNA libraries. We aimed to find novel genes that cooperate with c-Myc in transformation, which, in vitro, is measured by the ability of cells to grow detached form a substrate using anchorage-independent growth assays.

In chapter 3, we searched for genes that cooperate with Ras in tumorigenesis, when p19Arf is repressed. Accordingly, we have performed two independent genetic transformation screens in MEFs overexpressing Ras and Tbx2 using various retroviral cDNA expression libraries and anchorage-independent growth assays.

**Dynamics and recruitment of Polycomb group protein Bmi1 to PcG bodies**
PcG complex composition and target gene binding must be regulated in a highly flexible and dynamic manner. The action of PcG proteins is essential for normal development and maintenance of embryonic and adult stem cells. In addition to cellular differentiation, PcG proteins are involved in cell cycle regulation. Moreover, chromatin remodeling by PcG proteins should act in direct response to physiological stimuli and external signals to allow cellular survival and adaptation. Therefore, PcG complexes are thought to be highly plastic. Initial evidence came from studies in *Drosophila*, where alternative PcG complexes were discovered and where activators and repressors were found on the same target loci, indicating that PcG-mediated silencing is more dynamic than was thought at the time (Breiling et al., 2001; Dellino et al., 2004; Klymenko and Muller, 2004; Ringrose et al., 2003) Recently, PcG complexes were found to target specific sets of genes that change during differentiation from embryonic stem cells to lineage specific progenitors and terminally differentiated cells. These loci, although transcriptionally inactive, contain the active H3K4me³ mark in addition to the PRC2-specific silent H3K27me³ mark. These so called “bivalent loci”, although silenced, are prone to become activated when proper signals are received before proceeding to the subsequent differentiation step. At each stage of differentiation, PcG proteins are thought to keep genes “off” that promote progression to the next differentiated stage and thereby define the identity of the differentiated cell (Mikkelsen et al., 2007; Mohn
et al., 2008; Pietersen and van Lohuizen, 2008). This emphasizes the dynamics and flexibility of PcG-mediated transcriptional silencing. Although PcG proteins are supposed to act in a fairly dynamic manner, little is known about their actual mobility at target loci.

As mentioned earlier, the recruitment of PcG proteins to their target loci is proposed to start by recruitment of PRC2, which catalyzes the H3K27me\(^3\) mark. This methylated histone mark is recognized and bound by the Cbx proteins, which are PRC1 core components, leading to recruitment of PRC1-like complexes (Figure 4). This sequential recruitment theory is controversial and has so far been confirmed for only a number of PcG target genes (Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007). This model necessitates the initial recruitment of PRC2 to PcG target loci, the mechanism of which still needs to be elucidated. Although regulatory DNA elements (Polycomb response elements) and sequence-specific DNA binding proteins seem to be responsible for recruitment of PcG-specific complexes and silencing in flies, no such mechanism has been delineated in mammalian systems. Moreover, biochemical studies have shown that binding of Cbx proteins to the H3K27me\(^3\) mark is rather weak and PRC1 recruitment to the inactive X-chromosome has been reported in the absence of PRC2 (Masui and Heard, 2006; Ringrose et al., 2004). Finally, even though DNA elements and the H3K27me\(^3\) mark are sufficient for PcG recruitment, other mechanisms such as RNAi machinery have been recently linked to PcG recruitment and transcriptional silencing. Altogether, there is enough evidence suggesting that PRCs exist in many different complexes and independent mechanisms could direct PcG recruitment to different loci depending on cellular context.

Bmi1 is one of the most extensively studied members of the PcG proteins and is implicated in stem cell identity and cancer. In chapter 4, we have tried to address two major questions in this field by studying specific, presumably clustered, PcG target loci, namely PcG bodies. These are stable subnuclear foci containing repetitive DNA sequences from some pericentric heterochromatic domains (Saurin et al., 1998; Voncken et al., 1999). These foci were originally observed in many transformed cell lines and few primary cell types and are distinct from hP1-related constitutive pericentric heterochromatin. They can be visualized in mammalian cells and are therefore suitable for studying physical aspects of PcG-target regions. First, we have studied the mobility of Bmi1 interaction with PcG bodies. We have used fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) to test Bmi1 mobility during G1 and G2 of the cell cycle, when recruited to PcG bodies. Secondly, we have investigated the recruitment of Bmi1 (PRC1) by Ezh2/Eed (PRC2) and the maintenance DNA methyltransferase (Dnmt1) to PcG bodies.

Finally, in chapter 5, we summarize the preceding experimental chapters and briefly discuss the obtained results and future perspectives with regard to recent studies in the field.
References


Chapter 1


Chapter 1


Chapter 1


Chapter 1
