SCREENING FOR NOVEL DRUG TARGETS IN CANCER

Jeroen Hendrik Nijwening

ISBN: 978-94-6182-062-4

Layout & printing: Off Page, Amsterdam

Cover: Yggdrasil. Designed by Elke Sillen

The research in this thesis is supported by Top Institute Pharma.

SCREENING FOR NOVEL DRUG TARGETS IN CANCER

SCREENEN VOOR NIEUWE DOELWITTEN VOOR GENEESMIDDELEN IN KANKER

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op dinsdag 10 januari 2012 des middags te 12.45 uur

door

Jeroen Hendrik Nijwening
geboren op 25 oktober 1979 te Heerlen

Promotor:	Prof.dr. R. Bernards
Co-promotor:	Dr. R.L. Beijersbergen

voor mijn ouders, Henk en Marion

CONTENTS

1

GENERAL INTRODUCTION

CANCER

Cancer has been the leading cause of death in the western world since 2008 (www.who.int). One of the main reasons for its current position is that significant success has been made in development of cholesterol-lowering drugs and antihypertensives, leading to a reduction in the incidence of cardiovascular diseases, the former primary cause of death in the western world. These, and other, medical breakthroughs extend our lifespan and therefore may ultimately lead to an increase in cancer-related deaths, which correlates with age. Most likely, the global rate of cancer incidence will only increase. Particularly at the present time, life expectancy of humans living in the non-Western world is expected to increase by an improving global welfare. In recent years, there have been some major successes in cancer treatment and current data shows a modest decrease in cancer-related death incidents (www.cancer.org). However, this slight reduction can likely be attributed to early detection of tumors by population based screening for, for example, breast and colon cancers, by preventative measures such as reduction in the use of postmenopausal estrogen replacement, by educating the public about the hazards of smoking, and by banning the use of materials that contain carcinogens. Therefore, the war on cancer as declared by Richard M. Nixon in 1971 can be considered as an ongoing battle.

Curing cancer is quite challenging however, given that cancer is an extremely complex disease. Approximately 110 distinct types of human cancers have been described to date. In addition, each individual tumor tends to so complex in structure that it might even exceed the complexity of healthy tissues. Moreover, tumors have the ability to drive rampant genetic diversification, generating genetically distinct subpopulations. High-resolution sequencing of cancer cells microdissected from different parts of an identical tumor has revealed remarkable intratumoral genetic heterogeneity (Navin et al 2011). This genetic diversification may allow for functional specialization, generating subpopulations of cancer cells within the tumor that produce distinct and complementary competences that in a Darwinian process contribute to overall tumor survival and progression. This characteristic could very well be the main reason that many tumors relapse after therapy. Nonetheless, most tumor cells seem to display the same set of specific characteristics that distinguishes them from normal, healthy cells, the so-called hallmarks of cancer (Hanahan and Weinberg 2000).

The common characteristics of tumors

The discovery of the first oncogene by Varmus and Bishop (Stehelin et al 1975) in 1975 caused a breakthrough in cancer research and radically changed the way we look at cancer: a tumor could develop because of a defect in a normal cellular proto-oncogene (Hunter 1984). Only 25 years after the discovery of the first oncogene a clear and comprehensive view about cancer was summarized in the seminal review by Hanahan and Weinberg (Hanahan and Weinberg 2000). Herein they claimed that six traits exist, that are universal in all cancers. These hallmarks of cancer are: self-sufficiency in

proliferation signaling, insensitivity to anti-proliferation signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and metastasis.

Ten years later, after extensive research and an explosion in knowledge about cancer, six additional common tumor traits have been added to this list: the presence of (cancer) stem cells within the tumor population (Trosko et al 2004), the stress phenotype of cancer cells (Luo et al 2009), tumor-promoting inflammation (Colotta et al 2009), the mutator phenotype of cancer cells (Loeb 1991, Loeb 2001, Negrini et al 2010, Salk et al 2010), escape from immuno-surveillance (Kroemer and Pouyssegur 2008), and deregulation of tumor cell metabolism (Koppenol et al 2011, Kroemer and Pouyssegur 2008). Most of these additional hallmarks have been reviewed by the same authors of the original cancer hallmarks paper (Hanahan and Weinberg 2011). The original six hallmarks of cancer have been described previously and are widely accepted by the cancer research community; therefore four of the six novel features mentioned above will be further discussed below as they are specifically relevant to the topic of this thesis.

Cancer stem cells

Cancer stem cells (CSCs) are a subclass of neoplastic cells within tumors that have the ability to self-renew and differentiate to generate the heterogeneity of the developing tumor (reviewed in (Vermeulen et al 2008). CSCs have been described in hematopoietic malignancies (Bonnet and Dick 1997) and in solid tumors (Al-Hajj et al 2003). The current practical definition of CSCs is that a subset of tumor cells with a distinct cell surface antigen profile (often in CD receptors) have the ability to efficiently seed new tumors after injection into recipient host mice, reviewed in (Cho and Clarke 2008). But whether it is an intrinsic stem cell that may receive the first oncogenic mutation (Jamieson et al 2004), as is the case for crypt stem cells in intestinal cancer (Barker et al 2009), or a cancer cell that is induced into endothelial-to-mesenchymal (EMT) transdifferentiation (Singh and Settleman 2010), or a neoplastic cell that de-differentiated and acquired stem-cell like properties (Sell 1993), giving rise to cancer stem cells, still remains unresolved. Despite the lack of solid experimental data, CSCs may prove to be a common driving element of most, if not all, tumors.

The stress phenotype

The stress phenotype of cancer cells is another hallmark of cancer and was proposed by Stephen Elledge and colleagues (Luo et al 2009, Solimini et al 2007). Although the stress phenotype is not responsible for initiating tumorigenesis, it is a common feature of many tumor types. The stress phenotype can be subdivided into DNA damage stress, oxidative stress, mitotic stress, proteotoxic stress and metabolic stress. The hypothesis is that the dramatic rewiring of the genetic network that cancer cells undergo to become neoplastic, also causes potentially deleterious circumstances and even vulnerabilities (Luo et al 2009). In other words, cancer cells depend on (stress supporting) pathways that on their own are non-oncogenic. This dependency is also called non-oncogene

addiction, an extenstion of the term oncogene addiction, a high dependency of cancer cells on the oncogene that drives their proliferation and / or survival (Weinstein 2002). A similar phenomenon is "tumor suppressor gene hypersensitivity", a dependence of cancer cells on loss-of a specific tumor suppressor (Weinstein and Joe 2008). It is believed that distinct proteins in the pathways supporting non-oncogene addiction are highly attractive drug targets. Theoretical proof of a dependence of cancer cells on non-oncogenes stems from the observation that oncogenes induce strong and opposing pro-survival and pro-apoptotic signals in cancer cells. The net result of these conflicting signaling pathways in cancer cells is in favor of proliferation and survival due to intratumoral selection mechanisms. Direct inhibition of the driving oncogene shifts this balance towards cell death (Sharma and Settleman 2007). Two salient examples of non-oncogene addiction are heatshock factor 1 (HSF1) dependency of tumor cells and PARP-dependency of BRCA-deficient cells, described below. Lindquist and colleagues (Dai et al 2007) show that HSF1 is activated by a range of cellular stresses including hypoxia and heat. In *in vivo* experiments in mice they show that loss of *HSF1* protects mice against tumor formation driven by mutant p53. In *in vitro* experiments, they illustrate that knockdown of *HSF1* decreases the viability of multiple cancer cell lines, but not primary cell lines. The challenge in the case of HSF1 is that it is a transcription factor and therefore not readily targetable by small molecule inhibitors, hampering its development as a therapeutic target. Another example of non-oncogene addiction which is more clinically relevant is the BRCA and PARP dependency. Individuals that carry germline mutations in BRCA genes, BRCA1 or BRCA2, have a defect in homologous recombination (HR), needed for double-strand break repair, and are therefore prone to breast, ovarian and prostate cancer (Lord and Ashworth 2008). Poly(ADP-ribose) polymerase (PARP), involved in base excision repair that is required for repair of single strand breaks (SSBs), but when depleted lethal double strand breaks (DSBs) can occur when HR is lacking. As a result, targeting of PARP in tumor cells with deficiencies in BRCA1 or BRCA2 renders these cells over 1,000 fold more sensitive to PARP inhibition than normal BRCA-proficient cells (Farmer et al 2005). This observation has so far lead to successful clinical trials with PARP inhibitors in BRCA mutation carriers (Fong et al 2009).

Tumor promoting inflammation

Over the last decade the knowledge of the biology of tumors has been shifted from a multicellular mass that proliferates and metastasizes to a concept that most solid tumors are sustained by the so-called tumor microenvironment. This concept further expands the complexity of cancer biology. Cytokines, chemokines and cellular effectors from the immune system constitute the local environment of tumors. It has been known for decades that tumors are infiltrated by immune inflammatory cells (Dvorak 1986, Kinzler and Vogelstein 1998). In contrast, it has been demonstrated that the cells of mostly the innate immune system are actually tumor-promoting (Colotta et al 2009, Grivennikov et al 2010, Qian and Pollard 2010). In addition, transformed

cells can produce inflammatory mediators, which can polarize the immune response towards a tumor supporting microenvironment, inducing proliferation and survival of malignant clones, and in a later stage, promoting angiogenesis and metastasis (Biswas and Mantovani 2010). On the other hand it is known that chronic inflammations are associated with a higher risk of developing cancer (Terzic et al 2010). Although a comprehensive description of the tumor microenvironment and its tumor promoting inflammation exceeds the scope of this introduction, the fact that multiple treatment strategies which attack the microenvironment are currently being developed, underscores the importance of this tumor-supportive structure.

The mutator phenotype

The genome is instable in the great majority of human cancer cells (Negrini et al 2010). The pervasive genomic aberrations detected in cancer cells can greatly enable mutations in favor of proliferation, adaptation to the microenvironment, metastasis and, importantly, lead to resistance of therapeutic treatments. This common trait seen in most tumor cells is also called the mutator phenotype of cancer (Loeb 2001). Evidence for genomic instability in cancer cells comes, for example, from comparative genomic hybridization (CGH) studies that measure the gains and losses of gene copy number across the whole genome. CGH analysis of tumor samples has shown that many cancers have specific deletions and amplifications in their genome, indicating that those loci harbor genes whose loss or overexpression favors tumor development (Mattison et al 2009). Notably, genomic instability, driven by loss-of function in genes that detect, activate, and repair DNA damage (Ciccia and Elledge 2010), is often exploited in cancer treatment. Many current therapeutic strategies make use of the fact that tumor cells do not respond effectively to DNA damage. Thus, genomic instability lies at the basis of multistep tumor progression, increasing the heterogeneity of the tumor population, in most cases leading to the high versatility seen in tumors.

These twelve hallmarks of the majority, if not all, tumors have been a seminal contribution of fundamental research to cancer biology. These hallmarks are an excellent conceptual framework that distinguishes cancer cells from normal cells and one that could ultimately lead to improvement in rationally designed cancer therapeutics. In addition to these hallmarks, some major conceptual changes and further insights into fundamental processes have been made in the last decade. Not surprisingly, every new cellular mechanism discovered often impinges on tumor development when deregulated. Some of these mechanisms involved are: epithelial to mesenchymal transition (EMT) (Singh and Settleman 2010), autophagy (Kroemer and Jaattela 2005), the tumor microenvironment (Sneddon and Werb 2007), senescence (Campisi and d'Adda di Fagagna 2007, Lowe et al 2004), miRNA expression (Garzon et al 2010), pseudogenes expression (Poliseno et al 2010), chromatin remodeling (Kouzarides 2007), nuclear lamina-mediated silencing (Kalverda et al 2008, Kind and van Steensel 2010), and expression of non-coding RNAs (Lee and Calin 2011). Given the relevance to this thesis, cancer related senescence is further discussed below.

Senescence

Cells that are senescent are characterized by a stable and long-term loss of proliferative capacity, despite measurable metabolic activity and continued viability (Hayflick and Moorhead 1961). Two decades ago, senescence was demonstrated to be induced upon oncogenic activation (Serrano et al 1997) and senescence was thought to be involved in blocking proliferation (reviewed in (Hayflick 1997). However, at the time, the concept of senescence met with much criticism and debate with some renowned scientists suggesting that it was more likely to be an artifact of cell culture (Hanahan and Weinberg 2000). Today, senescence is widely accepted as a cell-intrinsic stress phenotype that suppresses tumorigenesis and acts in conjunction with cell death programs. Particularly since 2005 senescence has gained much more credibility following the observation of senescence markers in pre-malignant tumors (Braig et al 2005, Chen et al 2005, Collado et al 2005, Lazzerini Denchi et al 2005, Michaloglou et al 2005). Senescence can be induced by oncogene activation (like RAS, RAF, AKT), loss-of tumor suppressor gene activity (PTEN, NF1), telomere dysfunction, or extensive DNA damage (Courtois-Cox et al 2008, Kuilman et al 2010). These stimuli support the hypothesis that the induction of senescence is similar to induction of apoptosis; senescence requires a programmed cellular response triggered by external and internal factors.

Senescent cells can be recognized by several distinct markers, such as clear morphological alterations resulting in a large and flat phenotype *in vitro*, induction of expression of tumor suppressors ($p14^{ARF}$, $p16^{INK4A}$, $p15^{INK4B}$ and p21), decreased expression of so-called cell cycle genes (*CCNE1A*, *MCM3* and *PCNA*) (Ben-Porath and Weinberg 2005). Senescent cells stain positive for senescence-associated acidic β-galactosidase (SA-β-GAL) (Dimri et al 1995), and secrete proteins which direct autocrine and paracrine signaling (Acosta et al 2008, Kuilman et al 2008, Wajapeyee et al 2008) also called the senescence-mediating secretome (SMS) (Kuilman and Peeper 2009). In addition, senescent cells express several biomarkers including PAI1 (Kortlever and Bernards 2006), DEC1 and DCR2 (Collado et al 2005), although no general and context-independent molecular markers exist to identify senescent cells *in vitro* and *in vivo* (Cichowski and Hahn 2008). Some senescent cells display senescence-associated heterochromatin foci (SAHF) (Narita et al 2003). SAHFs are dramatic changes in chromatin structure visualized by a punctuate pattern of DNA staining dyes that cannot be found in cycling or quiescent cells. These foci are enriched for H3K9 methylation (a transcriptionally suppressive mark) and depleted for histone 3 lysine 9 (H3K9) acetylation and H3K4 methylation (both markers of transcriptional activation) (Narita et al 2003). It is believed that SAHFs attract heterochromatin binding and stabilizing proteins surrounding the promoters of several E2F target genes (Chicas et al 2010, Narita et al 2003, Narita et al 2006, Zhang et al 2007). SAHF formation is suppressed by inhibition of the $p16^{INK4A}$-RB pathway. Not surprisingly, several chromatin modifying and maintaining proteins have been identified that are recruited by RB to E2F binding sites in senescent cells (Bandyopadhyay et al 2007)(see chapter 6 of this thesis). Another heavily regulated locus during senescence is the *INK4A-ARF* locus, encoding

$p16^{INK4A}$ and $p14^{ARF}$, which regulated the two canonical tumor suppressors, RB and p53, respectively. The *INK4A-ARF* locus is repressed by the polycomb group proteins (Gil et al 2004, Jacobs et al 1999) (Bracken et al 2007, Dietrich et al 2007) and activated by JMJD3, a H3K27 demethylase (Agger et al 2009, Barradas et al 2009). Although the presence and function of SAHFs are controversial (Di Micco et al 2011), the dramatic changes (chromatin-driven or not) in gene expression observed during senescence and the fact that E2F target loci as well as the *INK4A-ARF* locus are accessed by chromatin modifying enzymes are proof that an intrinsic mechanism protecting cells from oncogenic progression exists. Thus, senescence may be as important as apoptosis, one of the alternative intrinsic protective mechanisms.

Future research will without doubt elucidate more intricacies of senescence, given that many observations still remain. For example, it has been shown that senescent cells can induce their own clearance (Xue et al 2007). In contrast, it has been reported that senescent cells induce a cell non-autonomous effect on the tumor stroma, thereby enhancing proliferation of cancer cells (Krtolica et al 2001). In addition, senescent cells in naevi can turn malignant when expression of their tumor suppressor JARID1B is lost (Roesch et al 2010).

Functional genomics

To comprehend the nature of complex systems, like organisms, one could reduce them to the interactions and understandings of their parts. Many of the components and interactions of a biological system have been unraveled by the reductionist approach, where single genes were studied for their function, their probable corresponding phenotype, interaction domain, and interaction partners. However, reductionism offers little understanding of a functioning, whole organism, which is probably better comprehended by a holistic approach that combines the research efforts on the individual parts. Functional genomics is the effort that focuses on the functional aspects of genetic interactions at the level of genes, RNA transcripts, and protein products. It often integrates information from other "omics" approaches like genomics, proteomics and genome sequencing efforts. Functional genomic studies often use a genome-wide unbiased approach, applying high-throughput methods, quantitative measures, data integration, statistical and mathematical models to integrate and describe gene and protein functions as well as their interactions.

To map gene function and genetic interactions in a systematic, unbiased and comprehensive fashion, and in the context of a biological system, scientists have turned to easy-to breed and easy-to handle model organisms, like bacteria, yeast, worms, fruit flies and mammalian cell cultures. In order to study functional genomics in these model systems one requires large collections of defined mutant alleles or genetic reagents that can inhibit gene function (like libraries of knockout strains or larger collections of reagents that interfere with mRNA, as is discussed later). In addition, studying functional genomics in model organisms requires an easy to score phenotype that can

be detected in parallel in a large number of samples in order to perform genetic studies in a high-throughput and unbiased manner.

A vast amount of genetic information comes from the model organism budding yeast (*Saccharomyces cerevisiae*), where large collections of knockout strains were generated and genes were functionally annotated matching to their corresponding knockout phenotype. In addition, knockout strains were combined with one another to identify synthetic lethal interactions, defined as the condition in which two auxiliary genes become essential when both are mutated. This knockout collection contributed to a rich source of genetic interaction networks and to a large dataset of gene classification (Costanzo et al 2010, Dixon et al 2009) that quite resembles the 18th century deterministic approach of Linnaean taxonomy. For example, the deletion collection of each known or putative open reading frame of *S. cerevisiae* contains around 1000 essential genes. Moreover, *S. cerevisiae* was utilized in a big effort to construct a large-scale genetic interaction map of a single cell (Tong et al 2004). Within this network 132 genome-wide synthetic genetic arrays were used and around 4000 genetic interactions among around 1000 genes were discovered (Tong et al 2004). Analysis of the interaction data suggested that the complete network may contain approximately 100,000 genetic interactions, illustrating the challenge of a genome-wide analysis of all possible combinations of genetic interactions.

Despite its clear utility, many genes that are involved in human disease are not found in yeast or bacteria, but only exist in metazoans. Therefore, a large scientific effort has been invested in multicell model organisms represented by the nematode worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and cultured mammalian cells. Although large collections of deletion strains exist in these models, studying functional genomics in a high throughput manner in these model systems was greatly improved by the discovery of RNA-interference (RNAi) recognized by the award of a Nobel Prize.

RNAi

The discovery of RNAi in *C. elegans* (Fire et al 1998), as a direct extrapolation from an identical mechanism in plants (Napoli et al 1990, van der Krol et al 1990) propelled the efforts of studying gene function in model organisms. In particular the discovery that RNAi is also effective in mammalian cells (Elbashir et al 2001) enabled the study of loss-of function phenotypes in mammalian cells. Gene regulation by RNAi can be based on the micro-RNA (miRNA) pathway or siRNA pathway. The miRNA pathway is an endogenous pathway where pri-miRNAs are produced by transcription and within the nucleus cleaved by the RNase III enzyme Drosha to small, doublestrand RNAs with secondary loop structures, the so called pre-miRNAs. Pre-miRNAs are exported to the cytoplasm and subsequently recognized by DICER, a ribonuclease that cleaves the pre-miRNAs into around 21-22 basepair fragments that are incorporated into the RNA-induced silencing complex (RISC) to target and downregulate the expression of genes. This process is mediated by the seed sequence, base-pairs 2-8 on the 21

bp miRNA, that specifically recognize highly conserved target sites in the 3'UTR of mRNAs. miRNAs can trigger translational silencing of mRNAs or mRNA-directed cleavage depending on the binding properties of the non-seed region of the miRNA. In general, strong binding leads to cleavage of the target mRNA, while weak binding leads to initial suppression and subsequent processing in the P-bodies (Carthew and Sontheimer 2009). Unlike miRNAs, siRNAs are generated from exogenous sources to target the transcripts they are derived from. An example of this is the host defense of lower organisms against the expression of invading viral genes. In this manner, siRNAs are produced from double-stranded RNAs (dsRNAs) that arise during viral replication; these dsRNAs are recognized by DICER and processed in a way similar to miRNAs (Rana 2007). Since the siRNAs bind with full length complimentarity to their target mRNAs, these are cleaved and degraded (Carthew and Sontheimer 2009).

The similarity between the miRNA and siRNA pathway enables scientists to introduce exogenous small interfering RNAs (siRNAs) into cells to target a gene of interest by transient transfection or vector-driven endogenous expression.

RNAi reagents come in different varieties: small interfering RNAs (siRNAs), doublestrand RNAs (dsRNAs), short hairpin RNAs (shRNAs), miRNA-driven RNAs (shRNAmirs), and endoribonuclease-prepared siRNAs (esiRNAs). siRNAs are synthetically produced 21-mer double strand RNAs that are transfected into cells and directly integrated into the RISC complex (Rana 2007). dsRNAs are double stranded RNAs used to transfect *C. elegans* or *D. melanogaster*, for example. These dsRNAs are endogenously processed into many different siRNAs that together silence their target genes. Short-hairpin RNAs (shRNAs) are vector driven siRNAs that after transcription form a hairpin-like structure, a 19- to 22 base-pair stem with a 4- to 12 base-pair loop (Brummelkamp et al 2002). These hairpins are processed by DICER and RISC in the same way as miRNAs. shRNAmirs are also vector based siRNAs that are embedded in a miRNA-like structure that in most designs is derived from miRNA-30 (Stegmeier et al 2005). It is believed that this resemblance to miRNAs enhances siRNA processing and therefore improves knockdown (Gregory et al 2005). esiRNAs are, like dsRNAs, double strand RNAs that are processed into siRNAs *in vitro* by RNAses, and can subsequently be transfected into mammalian cells (Kronke et al 2004). Vector-based RNAi systems are delivered by viruses and can be retroviral, adenoviral, or lentiviral. These systems are developed as single-well reagents or can be applied in a pooled format to perform functional genomic screening efforts (Arts et al 2003, Berns et al 2004, Paddison et al 2004). The different siRNA systems mentioned above have their advantages and disadvantages when applied in screening efforts, as discussed by (Boutros and Ahringer 2008, Mohr et al 2010).

Initially, RNAi was used to study the function of individual genes after knockdown; now it is widely applied in whole-genome screening efforts, enhancing the field of functional genomics. RNAi screening efforts for specific phenotypes or context-dependent drug targets have become more feasible with improved siRNA design rules (Patzel et al 2005, Reynolds et al 2004) and increasing numbers of RNAi libraries

(Boutros and Ahringer 2008). However, hits from RNAi screens should be validated properly (Echeverri et al 2006) and interpreted according to the context they have been identified in, since the RNAi technologies still have their drawbacks and can lead to many false positive and false negative hits (Jackson and Linsley 2010).

False positives and false negatives

False positive and false negative hits resulting from RNAi screens are very common. False positives are of two types: they can be dependent on the context of the screening conditions, for example, a specific cell line, or they can be caused by so called off-target effects. The source of off-target effects is a natural consequence of the RNAi mechanism, where an exogenously introduced siRNA in the cell is recognized and treated as a miRNA (Doench et al 2003). miRNAs recognize their target transcripts by means of their seed region and therefore reduce the expression of many target genes. It has been described that some miRNAs can have hundreds of different targets (Xie et al 2005). Consequently, siRNAs could have hundreds of targets as well (Birmingham et al 2006). For example, in one study almost one third of randomly selected siRNAs induced an effect on cell viability that did not correlate with knockdown (Fedorov et al 2006). False positives through off-target effects are a significant limitation to the use of RNAi as a screening method and could require extensive validation and follow-up resulting in significant delays of experiments and consumption of resources (Ma et al 2006). However, proper design of siRNAs by improved predictive algorithms that exclude miRNA-like target sites (Rajewsky 2006), multiple individual siRNAs per gene, good quality control of screens (Boutros and Ahringer 2008), as well as appropriate statistical methods and hit selection methods (Birmingham et al 2009) can reduce the time and effort lost by pursuing false positives.

In contrast, false negatives often pose the question why certain genes that are indeed involved in a phenotype of interest do not appear in the hit list of an RNAi screen. There could be many reasons for this observation, including incomplete knockdown of the mRNA or functionally redundant mechanisms existing in the cell. These false negatives could impose serious limitations to the interpretation of screening results. Nevertheless, validated hits resulting from RNAi screens for drug targets are likely to be therapeutically relevant because of their non-redundant nature in key nodes of cellular pathways.

Genetic dependency

High-throughput RNAi screens are a powerful research tool in fundamental biology, but can also be easily applied for the identification of novel drug targets, especially by using synthetic lethal screening approaches (Described in Chapter 2 of this thesis). In brief, a synthetic lethal interaction in the context of cancer is a type of genetic interaction in which one mutation (specific for cancer cells) is only lethal to a cell in combination with another mutation (or molecular inhibition) of a second gene. The term synthetic lethality was first coined in 1946 by Dobzhansky (Dobzhansky 1946).

1

Synthetic lethal screens have been in use for almost 100 years by using model systems that could be genetically modified. In mammalian cells, this was not possible because of their diploid or polyploid genomes. Friend and colleagues described the use of synthetic lethal screens in yeast to extrapolate interactions identified as potential cancer drug targets (Hartwell et al 1997). When high-throughput RNAi screening became feasible, Brummelkamp (Brummelkamp and Bernards 2003) and Tucker (Tucker and Fields 2003) proposed the exploitation of RNAi for the identification of synthetic lethal interactions in relation to drug target discovery.

When applying synthetic lethal screening to identify cancer-specific drug targets, one searches for genetic vulnerabilities of cancer cells, which do not appear in normal cells. Ultimately, small molecule inhibitors can be designed against the drug targets resulting from RNAi screens. These small molecule inhibitors could be applied in clinical studies and tested for their ability to specifically attack the vulnerabilities in a genetically-defined type of cancer. These cancer-specific vulnerabilities are also called genetic dependencies, that is, the reliance of cancer cells on a specific event (mostly the presence (or absence) of a protein) driven by a specific gene. Genetic dependencies can be classified into two groups: gene addiction and functional redundancy.

Genetic dependency:
1. Gene addiction
   a. Oncogene addiction
   b. Addiction to loss-of tumor suppressor genes (TSG) / TSG-hypersensitivity.
   c. Non-oncogene addiction.
2. Functional redundancy

The classification of gene addiction can be subdivided into three situations. First, cancer cells can be addicted to a genetic event that in most cases drives the tumor progression, known as oncogene addiction (Jonkers and Berns 2004, Weinstein 2002). There are numerous examples in the literature describing examples of addiction to oncogenes like EGFR, RAS, BCR-ABL (Chin et al 1999, Felsher and Bishop 1999, Pelengaris et al 1999, Sharma et al 2006b) and logically these oncogenes could serve as drug targets for cancer treatment. The term "oncogenic shock" is a variation on oncogene addiction (Sharma et al 2006a) that states that oncogenes induce both pro-survival and pro-apoptotic signals. Removal of the survival signal leaves the apoptotic signal to linger on, resulting in cell death (Sharma et al 2006a). An example of oncogenic shock is v-SRC, an oncoprotein which induces both apoptosis and cell survival (Johnson et al 2000). Second, tumor cells can be addicted to loss-of tumor suppressor genes (TSGs) that are inactivated by mutations or deletion. These cells show TSG-hypersensitivity as is demonstrated by restoring the lost TSG, like p53 (Harris et al 1996, Xue et al 2007). Third, tumor cells can be addicted to non-oncogenes, termed non-oncogene addiction, that make the corresponding proteins excellent drug targets (Luo et al 2009). This phenomenon is also called functional buffering or genetic buffering and several examples have been shown (Dai et al 2007, Scholl et al 2009). The classification

of hits from RNAi screens for novel drug targets that fall into functional redundancy are classical synthetic lethal hits and some of these interactions have overlap with non-oncogene addiction. Synthetic lethal interactions can exist intrinsically in cells or can be created by an oncogenic event like the BRCA-PARP relationship. The goal of most of the work described in this thesis was to identify synthetic lethal interactions with oncogenic events.

OUTLINE OF THIS THESIS

The research performed and described in this thesis is focused on three of the most frequently mutated genes in human cancer: the archetypal tumor suppressors p53 and RB and the oncoprotein RAS. An aspect that these genes have in common is that they have met little success with targeted in cancer therapy. Loss-of-function screening using RNAi screening is the method applied to identify genes in an unbiased approach involved in these pathways or genes involved in parallel pathways that support neoplastic progression. The RNAi screening approach is the central method used to explore drug targets related to the chemotherapy cisplatin, the oncogene RAS and the tumor suppressor RB.

Chapter one is a general introduction to the chapters in this thesis and briefly describes several aspects of cancer biology and the oncogenes and tumor-suppressor genes studied in herein.

Chapter two describes how RNAi can contribute to the identification of novel drug targets in cancer.

Chapter three describes the results of an RNAi screen for enhancement of cisplatin-induced lethality.

Chapter four elucidates a synthetic lethal interaction between KIF18A and the oncogene $HRAS^{G12V}$ identified by synthetic lethal screening.

Chapter five describes that Jarid1b is a novel component of the Rb pathway in Rb-mediated senescence induction.

Chapter six discusses our findings. This chapter explains what connects the pathways studied in the technical chapters and how they contribute to the challenges that lay ahead in the treatment of cancer.

REFERENCES

Acosta JC, O'Loghlen A, Banito A, Guijarro MV, Augert A, Raguz S *et al* (2008). Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell* **133:** 1006-1018.

Agger K, Cloos PA, Rudkjaer L, Williams K, Andersen G, Christensen J *et al* (2009). The H3K27me3 demethylase JMJD3 contributes to the activation of the INK4A-ARF locus in response to oncogene- and stress-induced senescence. *Genes Dev* **23:** 1171-1176.

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003). Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* **100:** 3983-3988.

Arts GJ, Langemeijer E, Tissingh R, Ma L, Pavliska H, Dokic K *et al* (2003). Adenoviral vectors expressing siRNAs for discovery and validation of gene function. *Genome Res* **13:** 2325-2332.

Bandyopadhyay D, Curry JL, Lin Q, Richards HW, Chen D, Hornsby PJ *et al* (2007). Dynamic assembly of chromatin complexes during cellular senescence: implications for the growth arrest of human melanocytic nevi. *Aging Cell* **6:** 577-591.

Barker N, Ridgway RA, van Es JH, van de Wetering M, Begthel H, van den Born M *et al* (2009). Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* **457:** 608-611.

Barradas M, Anderton E, Acosta JC, Li S, Banito A, Rodriguez-Niedenfuhr M *et al* (2009). Histone demethylase JMJD3 contributes to epigenetic control of INK4a/ARF by oncogenic RAS. *Genes Dev* **23:** 1177-1182.

Ben-Porath I, Weinberg RA (2005). The signals and pathways activating cellular senescence. *Int J Biochem Cell Biol* **37:** 961-976.

Berns K, Hijmans EM, Mullenders J, Brummelkamp TR, Velds A, Heimerikx M *et al* (2004). A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature* **428:** 431-437.

Birmingham A, Anderson EM, Reynolds A, Ilsley-Tyree D, Leake D, Fedorov Y *et al* (2006). 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat Methods* **3:** 199-204.

Birmingham A, Selfors LM, Forster T, Wrobel D, Kennedy CJ, Shanks E *et al* (2009). Statistical methods for analysis of high-throughput RNA interference screens. *Nat Methods* **6:** 569-575.

Biswas SK, Mantovani A (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* **11:** 889-896.

Bonnet D, Dick JE (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* **3:** 730-737.

Boutros M, Ahringer J (2008). The art and design of genetic screens: RNA interference. *Nat Rev Genet* **9:** 554-566.

Bracken AP, Kleine-Kohlbrecher D, Dietrich N, Pasini D, Gargiulo G, Beekman C *et al* (2007). The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. *Genes Dev* **21:** 525-530.

Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AH, Schlegelberger B *et al* (2005). Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* **436:** 660-665.

Brummelkamp TR, Bernards R, Agami R (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296:** 550-553.

Brummelkamp TR, Bernards R (2003). New tools for functional mammalian cancer genetics. *Nat Rev Cancer* **3:** 781-789.

Campisi J, d'Adda di Fagagna F (2007). Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* **8:** 729-740.

Carthew RW, Sontheimer EJ (2009). Origins and Mechanisms of miRNAs and siRNAs. *Cell* **136:** 642-655.

Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M *et al* (2005). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* **436:** 725-730.

Chicas A, Wang X, Zhang C, McCurrach M, Zhao Z, Mert O *et al* (2010). Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence. *Cancer Cell* **17:** 376-387.

Chin L, Tam A, Pomerantz J, Wong M, Holash J, Bardeesy N *et al* (1999). Essential role for oncogenic Ras in tumour maintenance. *Nature* **400:** 468-472.

Cho RW, Clarke MF (2008). Recent advances in cancer stem cells. *Curr Opin Genet Dev* **18:** 48-53.

Ciccia A, Elledge SJ (2010). The DNA damage response: making it safe to play with knives. *Mol Cell* **40:** 179-204.

Cichowski K, Hahn WC (2008). Unexpected pieces to the senescence puzzle. *Cell* **133:** 958-961.

Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M *et al* (2005). Tumour biology: senescence in premalignant tumours. *Nature* **436:** 642.

Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A (2009). Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* **30:** 1073-1081.

Costanzo M, Baryshnikova A, Bellay J, Kim Y, Spear ED, Sevier CS *et al* (2010). The genetic landscape of a cell. *Science* **327:** 425-431.

Courtois-Cox S, Jones SL, Cichowski K (2008). Many roads lead to oncogene-induced senescence. *Oncogene* **27:** 2801-2809.

Dai C, Whitesell L, Rogers AB, Lindquist S (2007). Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. *Cell* **130:** 1005-1018.

Di Micco R, Sulli G, Dobreva M, Liontos M, Botrugno OA, Gargiulo G *et al* (2011). Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. *Nat Cell Biol* **13:** 292-302.

Dietrich N, Bracken AP, Trinh E, Schjerling CK, Koseki H, Rappsilber J *et al* (2007). Bypass of senescence by the polycomb group protein CBX8 through direct binding to the INK4A-ARF locus. *EMBO J* **26:** 1637-1648.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C *et al* (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A* **92:** 9363-9367.

Dixon SJ, Costanzo M, Baryshnikova A, Andrews B, Boone C (2009). Systematic mapping of genetic interaction networks. *Annu Rev Genet* **43:** 601-625.

Dobzhansky T (1946). Genetics of Natural Populations. Xiii. Recombination and Variability in Populations of Drosophila Pseudoobscura. *Genetics* **31:** 269-290.

Doench JG, Petersen CP, Sharp PA (2003). siRNAs can function as miRNAs. *Genes Dev* **17:** 438-442.

Dvorak HF (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* **315:** 1650-1659.

Echeverri CJ, Beachy PA, Baum B, Boutros M, Buchholz F, Chanda SK *et al* (2006). Minimizing the risk of reporting false positives in large-scale RNAi screens. *Nat Methods* **3:** 777-779.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411:** 494-498.

Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB *et al* (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* **434:** 917-921.

Fedorov Y, Anderson EM, Birmingham A, Reynolds A, Karpilow J, Robinson K *et al* (2006). Off-target effects by siRNA can induce toxic phenotype. *RNA* **12:** 1188-1196.

Felsher DW, Bishop JM (1999). Reversible tumorigenesis by MYC in hematopoietic lineages. *Mol Cell* **4:** 199-207.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391:** 806-811.

Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M *et al* (2009). Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med* **361:** 123-134.

Garzon R, Marcucci G, Croce CM (2010). Targeting microRNAs in cancer: rationale, strategies and challenges. *Nat Rev Drug Discov* **9:** 775-789.

Gil J, Bernard D, Martinez D, Beach D (2004). Polycomb CBX7 has a unifying role in cellular lifespan. *Nat Cell Biol* **6:** 67-72.

Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R (2005). Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* **123:** 631-640.

Grivennikov SI, Greten FR, Karin M (2010). Immunity, inflammation, and cancer. *Cell* **140:** 883-899.

Hanahan D, Weinberg RA (2000). The hallmarks of cancer. *Cell* **100:** 57-70.

Hanahan D, Weinberg RA (2011). Hallmarks of cancer: the next generation. *Cell* **144:** 646-674.

Harris MP, Sutjipto S, Wills KN, Hancock W, Cornell D, Johnson DE *et al* (1996). Adenovirus-mediated p53 gene transfer inhibits growth of human tumor cells expressing mutant p53 protein. *Cancer Gene Ther* **3:** 121-130.

Hartwell LH, Szankasi P, Roberts CJ, Murray AW, Friend SH (1997). Integrating genetic approaches into the discovery of anticancer drugs. *Science* **278:** 1064-1068.

Hayflick L, Moorhead PS (1961). The serial cultivation of human diploid cell strains. *Exp Cell Res* **25:** 585-621.

Hayflick L (1997). Mortality and immortality at the cellular level. A review. *Biochemistry (Mosc)* **62:** 1180-1190.

Hunter T (1984). The proteins of oncogenes. *Sci Am* **251:** 70-79.

Jackson AL, Linsley PS (2010). Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nat Rev Drug Discov* **9:** 57-67.

Jacobs JJ, Kieboom K, Marino S, DePinho RA, 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.

Jamieson CH, Ailles LE, Dylla SJ, Muijtjens M, Jones C, Zehnder JL *et al* (2004). Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N Engl J Med* **351:** 657-667.

Johnson D, Agochiya M, Samejima K, Earnshaw W, Frame M, Wyke J (2000). Regulation of both apoptosis and cell survival by the v-Src oncoprotein. *Cell Death Differ* **7:** 685-696.

Jonkers J, Berns A (2004). Oncogene addiction: sometimes a temporary slavery. *Cancer Cell* **6:** 535-538.

Kalverda B, Roling MD, Fornerod M (2008). Chromatin organization in relation to the nuclear periphery. *FEBS Lett* **582:** 2017-2022.

Kind J, van Steensel B (2010). Genome-nuclear lamina interactions and gene regulation. *Curr Opin Cell Biol* **22:** 320-325.

Kinzler KW, Vogelstein B (1998). Landscaping the cancer terrain. *Science* **280:** 1036-1037.

Koppenol WH, Bounds PL, Dang CV (2011). Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer* **11:** 325-337.

Kortlever RM, Bernards R (2006). Senescence, wound healing and cancer: the PAI-1 connection. *Cell Cycle* **5:** 2697-2703.

Kouzarides T (2007). Chromatin modifications and their function. *Cell* **128:** 693-705.

Kroemer G, Jaattela M (2005). Lysosomes and autophagy in cell death control. *Nat Rev Cancer* **5:** 886-897.

Kroemer G, Pouyssegur J (2008). Tumor cell metabolism: cancer's Achilles' heel. *Cancer Cell* **13:** 472-482.

Kronke J, Kittler R, Buchholz F, Windisch MP, Pietschmann T, Bartenschlager R *et al* (2004). Alternative approaches for efficient inhibition of hepatitis C virus RNA replication by small interfering RNAs. *J Virol* **78:** 3436-3446.

Krtolica A, Parrinello S, Lockett S, Desprez PY, Campisi J (2001). Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A* **98:** 12072-12077.

Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ *et al* (2008). Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell* **133:** 1019-1031.

Kuilman T, Peeper DS (2009). Senescence-messaging secretome: SMS-ing cellular stress. *Nat Rev Cancer* **9:** 81-94.

Kuilman T, Michaloglou C, Mooi WJ, Peeper DS (2010). The essence of senescence. *Genes Dev* **24:** 2463-2479.

Lazzerini Denchi E, Attwooll C, Pasini D, Helin K (2005). Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. *Mol Cell Biol* **25:** 2660-2672.

Lee SK, Calin GA (2011). Non-coding RNAs and cancer: new paradigms in oncology. *Discov Med* **11:** 245-254.

Loeb LA (1991). Mutator phenotype may be required for multistage carcinogenesis. *Cancer Res* **51:** 3075-3079.

Loeb LA (2001). A mutator phenotype in cancer. *Cancer Res* **61:** 3230-3239.

Lord CJ, Ashworth A (2008). Targeted therapy for cancer using PARP inhibitors. *Curr Opin Pharmacol* **8:** 363-369.

Lowe SW, Cepero E, Evan G (2004). Intrinsic tumour suppression. *Nature* **432:** 307-315.

Luo J, Solimini NL, Elledge SJ (2009). Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell* **136:** 823-837.

Ma Y, Creanga A, Lum L, Beachy PA (2006). Prevalence of off-target effects in Drosophila RNA interference screens. *Nature* **443:** 359-363.

Mattison J, van der Weyden L, Hubbard T, Adams DJ (2009). Cancer gene discovery in mouse and man. *Biochim Biophys Acta* **1796:** 140-161.

Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM *et al* (2005). BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* **436:** 720-724.

Mohr S, Bakal C, Perrimon N (2010). Genomic screening with RNAi: results and challenges. *Annu Rev Biochem* **79:** 37-64.

Napoli C, Lemieux C, Jorgensen R (1990). Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell* **2:** 279-289.

Narita M, Nunez S, Heard E, Lin AW, Hearn SA, Spector DL *et al* (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* **113:** 703-716.

Narita M, Krizhanovsky V, Nunez S, Chicas A, Hearn SA, Myers MP *et al* (2006). A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation. *Cell* **126:** 503-514.

Navin N, Kendall J, Troge J, Andrews P, Rodgers L, McIndoo J *et al* (2011). Tumour evolution inferred by single-cell sequencing. *Nature* **472:** 90-94.

Negrini S, Gorgoulis VG, Halazonetis TD (2010). Genomic instability--an evolving hallmark of cancer. *Nat Rev Mol Cell Biol* **11:** 220-228.

Paddison PJ, Silva JM, Conklin DS, Schlabach M, Li M, Aruleba S *et al* (2004). A resource for large-scale RNA-interference-based screens in mammals. *Nature* **428:** 427-431.

Patzel V, Rutz S, Dietrich I, Koberle C, Scheffold A, Kaufmann SH (2005). Design of siRNAs producing unstructured guide-RNAs results in improved RNA interference efficiency. *Nat Biotechnol* **23:** 1440-1444.

Pelengaris S, Littlewood T, Khan M, Elia G, Evan G (1999). Reversible activation of c-Myc in skin: induction of a complex neoplastic phenotype by a single oncogenic lesion. *Mol Cell* **3:** 565-577.

Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP (2010). A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature* **465:** 1033-1038.

Qian BZ, Pollard JW (2010). Macrophage diversity enhances tumor progression and metastasis. *Cell* **141:** 39-51.

Rajewsky N (2006). microRNA target predictions in animals. *Nat Genet* **38 Suppl:** S8-13.

Rana TM (2007). Illuminating the silence: understanding the structure and function of small RNAs. *Nat Rev Mol Cell Biol* **8:** 23-36.

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A (2004). Rational siRNA design for RNA interference. *Nat Biotechnol* **22:** 326-330.

Roesch A, Fukunaga-Kalabis M, Schmidt EC, Zabierowski SE, Brafford PA, Vultur A *et al* (2010). A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. *Cell* **141:** 583-594.

Salk JJ, Fox EJ, Loeb LA (2010). Mutational heterogeneity in human cancers: origin and consequences. *Annu Rev Pathol* **5:** 51-75.

Scholl C, Frohling S, Dunn IF, Schinzel AC, Barbie DA, Kim SY *et al* (2009). Synthetic lethal interaction between oncogenic KRAS dependency and STK33 suppression in human cancer cells. *Cell* **137:** 821-834.

Sell S (1993). Cellular origin of cancer: dedifferentiation or stem cell maturation arrest? *Environ Health Perspect* **101 Suppl 5:** 15-26.

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88:** 593-602.

Sharma SV, Fischbach MA, Haber DA, Settleman J (2006a). "Oncogenic shock": explaining oncogene addiction through differential signal attenuation. *Clin Cancer Res* **12:** 4392s-4395s.

Sharma SV, Gajowniczek P, Way IP, Lee DY, Jiang J, Yuza Y *et al* (2006b). A common signaling cascade may underlie "addiction" to the Src, BCR-ABL, and EGF receptor oncogenes. *Cancer Cell* **10:** 425-435.

Sharma SV, Settleman J (2007). Oncogene addiction: setting the stage for molecularly targeted cancer therapy. *Genes Dev* **21:** 3214-3231.

Singh A, Settleman J (2010). EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* **29:** 4741-4751.

Sneddon JB, Werb Z (2007). Location, location, location: the cancer stem cell niche. *Cell Stem Cell* **1:** 607-611.

Solimini NL, Luo J, Elledge SJ (2007). Non-oncogene addiction and the stress phenotype of cancer cells. *Cell* **130:** 986-988.

Stegmeier F, Hu G, Rickles RJ, Hannon GJ, Elledge SJ (2005). A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc Natl Acad Sci U S A* **102:** 13212-13217.

Stehelin D, Varmus HE, Bishop JM (1975). Detection of nucleotide sequences associated with transformation by avian sarcoma viruses. *Bibl Haematol***:** 539-541.

Terzic J, Grivennikov S, Karin E, Karin M (2010). Inflammation and colon cancer. *Gastroenterology* **138:** 2101-2114 e2105.

Tong AH, Lesage G, Bader GD, Ding H, Xu H, Xin X *et al* (2004). Global mapping of the yeast genetic interaction network. *Science* **303:** 808-813.

Trosko JE, Chang CC, Upham BL, Tai MH (2004). Ignored hallmarks of carcinogenesis: stem cells and cell-cell communication. *Ann N Y Acad Sci* **1028:** 192-201.

Tucker CL, Fields S (2003). Lethal combinations. *Nat Genet* **35:** 204-205.

van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR (1990). Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* **2:** 291-299.

Vermeulen L, Sprick MR, Kemper K, Stassi G, Medema JP (2008). Cancer stem cells--old concepts, new insights. *Cell Death Differ* **15:** 947-958.

Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green MR (2008). Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. *Cell* **132:** 363-374.

Weinstein IB (2002). Cancer. Addiction to oncogenes--the Achilles heal of cancer. *Science* **297:** 63-64.

Weinstein IB, Joe A (2008). Oncogene addiction. *Cancer Res* **68:** 3077-3080; discussion 3080.

Xie X, Lu J, Kulbokas EJ, Golub TR, Mootha V, Lindblad-Toh K *et al* (2005). Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. *Nature* **434:** 338-345.

Xue W, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovsky V *et al* (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* **445:** 656-660.

Zhang R, Chen W, Adams PD (2007). Molecular dissection of formation of senescence-associated heterochromatin foci. *Mol Cell Biol* **27:** 2343-2358.

2

USING LARGE-SCALE RNAI SCREENS TO IDENTIFY NOVEL DRUG TARGETS FOR CANCER

Jeroen H Nijwening & Roderick L Beijersbergen

Netherlands Cancer Institute, Division of Molecular Carcinogenesis and NKI Robotics and Screening Center, Amsterdam, the Netherlands

Published in IDrugs 2010 Nov;13(11):772-7

2

ABSTRACT

In recent years, the development and clinical implementation of targeted therapeutics have progressed significantly. The specific inhibition of components of signal transduction pathways controlling proliferation and survival has been a highly successful research strategy. However, cancer is a heterogeneous disease and, even within one type of cancer, different genetic alterations are associated with identical phenotypes. To advance the use of targeted therapeutics, it is not only essential to identify the crucial factors in the signal transduction networks that control cell proliferation and survival, but also to classify individual tumors according to genetic alterations that correlate with pathway activation. RNAi screening technologies have become established as an important strategy both to identify novel targets and to provide novel biomarkers that are crucial for the further development of personalized medicine. This feature review discusses different RNAi screening strategies and their contribution to the rapidly evolving field of targeted therapeutics.

Keywords Biomarker, cancer, drug target, pathway activation, RNAi, screening, synthetic lethality, targeted therapeutic

INTRODUCTION

Most conventional cancer therapies target global tumor cell characteristics, represented by a high proliferation rate; this proliferation often occurs from the inability of cells to respond effectively to DNA damage. Although current treatments, such as surgery and chemotherapy, are often highly effective, recurrence of disease and acquired resistance to therapy remain major factors in consolidating cancer as the main cause of death in Western countries. However, in recent years, an increased understanding of the molecular alterations that occur in cancer cells has yielded a novel class of therapeutics targeting specific genetic abnormalities that are unique to tumor cells, resulting in improved outcomes for certain types of cancer. One example of such a successful therapeutic is the kinase inhibitor imatinib, which targets the constitutively active Bcr-Abl kinase that is essential for the development of chronic myeloid leukemia; other examples include inhibitors that target EGFR and HER2 signaling in lung and breast cancers, respectively. Thus, not only is a detailed understanding of the molecular networks underlying cancer pathogenesis required to extend the clinical benefit of targeted therapeutics in many cancers, but such knowledge is also needed to determine which treatments are likely to be most effective for each individual patient. Therefore, an increase in the discovery rate of novel drug targets, as well as novel biomarkers, is indispensable for the further development of targeted therapeutics and personalized medicine.

Traditionally, novel targets in cancer have been identified by analyzing the genomic alterations present in tumor cells, followed by experimental validation of the specific gene product in the tumorigenic process and in tumor cell behavior. The increase in technological developments to analyze complete genomes has resulted in a massive amount of genomic data covering genetic alterations and gene expression characteristics in tumors. This information has been critical in advancing classification tools for tumor subtypes, as well as in the creation of prognostic tools for the prediction of cancer progression. However, these efforts have been limited in their ability to identify novel cancer drug targets. Tumors occurring in humans can harbor hundreds of genetic alterations, the majority of which are unlikely to be required for the initiation and maintenance of the tumor. Thus, identifying mutations in those genes that are essential for the transformation of normal cells into tumor cells can be difficult. Equally challenging is the identification of genes in which mutations and the associated continuous aberrant activities are required for tumor cell survival, and that therefore representing promising targets for therapy. Finally, genomic data do not provide an understanding of the role of specific genes in tumor development. Ideally, targets would be identified based on their functional relevance in the tumorigenic process. For most targets that are currently in clinical use, elaborate experimentation has established their role in cancer, underscoring their relevance as therapeutic targets. More recently, the development of RNAi-based tools has provided the important combination of target identification both in a functional manner and on a genome-

wide scale. This feature review discusses different RNAi screening strategies and their contribution to the rapidly evolving field of targeted therapeutics.

2

RNAi SCREENING TECHNOLOGY

The discovery of RNAi in mammalian cells has stimulated the functional interrogation and validation of the role of individual proteins in cell function and in signal transduction pathways, and as potential drug targets. RNAi is based on inhibiting gene expression through the introduction or expression of small (~ 21 bp) RNA fragments (ie, siRNAs) that are incorporated into an RNA-induced silencing complex (RISC). The RISC complex cleaves specific mRNAs that are complementary to the siRNA, causing degradation of the mRNA and, consequently, inhibiting protein production. The net result of this approach is the sequence-specific suppression of gene expression. The decrease in gene expression is, in most cases, not complete and is termed knockdown rather than knockout. In essence, RNAi mimics the pharmacological (partial) inhibition of a target protein. This property allows RNAi to be an effective tool for the interrogation of causal relationships between protein function and phenotype, and can provide an indication of the feasibility of therapeutic intervention by small-molecule inhibitors. However, pharmacological inhibition does not necessary result in an identical phenotype observed with RNAi-mediated knockdown. Whereas RNAi-mediated knockdown causes the absence of the protein, small molecule inhibition results in the formation of an inactive complex. This complex can still have functions in the cell that would be absent upon RNAi-mediated knockdown. Another complicating factor in the interpretation of RNAi results is the occurrence of off-target effects, defined as the consequence of alterations in gene expression or protein function (other than alterations of the intended, specific target) on the cellular phenotype or a related assessed parameter. A large proportion of off-target effects can be attributed to microRNA (miRNA)-mediated effects. In these cases, a short 'seed' sequence present in the siRNA can function as an miRNA seed, binding to miRNA target sites and, consequently, inhibiting the translation of a group of mRNAs. The exact requirements for miRNA target sites are unknown, but hundreds of such sites likely exist in the human transcriptome. Furthermore, one seed sequence can target a large number of transcripts, making the interpretation of a single, individual siRNA result challenging. As a result of these complications requirements have been defined to determine whether a gene can be established as an on-target hit: multiple, independent siRNAs for each gene should confer the same phenotype; there should be a strong correlation between the decrease in gene expression and the phenotype observed; and, when possible, the siRNA effect should be rescued by expression of the target gene in a form that is insensitive to the siRNA (eg, by introducing a silent mutation into the target cDNA sequence or, in the case of a 3' untranslated region (UTR)-targeting siRNA, by deleting the 3' UTR). Given this complicated and labor-intensive validation strategy, a limited number of siRNA screens have been completed in mammalian cells to a level

that provides a validated on-target gene list. However, despite these limitations, several examples have been described that clearly demonstrate the capacity of RNAi screening to identify important proteins in cancer-relevant phenotypes.

RNAI SCREENING FOR THE IDENTIFICATION OF NOVEL DRUG TARGETS AND BIOMARKERS

A challenge in the effective targeted treatment of cancer involves identifying those targets that are essential for the survival of tumor cells, but that are generally redundant in normal cells. The most successful targeted therapies to date have been directed against proteins ('drivers') that are directly responsible for the malignant phenotype. However, the list of targets fulfilling this criterion is limited, and many of these drivers are able to escape small-molecule inhibition (eg, RAS). Conversely, the loss of tumor suppressor genes cannot, by definition, be targeted directly. Thus, in cases in which tumor suppressors are non-functional, other cancer drug targets should be identified.

Targets, essential for the survival of tumor cells with specific genetic alterations can potentially be identified in lateral signaling pathways to those controlled by oncogenes or tumor suppressor genes. However, novel targets identified in this manner are not obvious, and cannot be easily predicted or extrapolated from model organisms. The implementation of RNAi screening technologies allows for systematic interrogation of the human genome for such gene targets and their therapeutic potential. Although there is a significant need for novel cancer drug targets, an equal challenge is the selection of appropriate treatments for each individual tumor – a challenge that exists even for current therapies. In order to facilitate treatment selection, the identification and validation of appropriate biomarkers that can predict the probable effectiveness of a therapy for an individual patient is essential.

The identification of novel cancer drug targets can be based on various strategies. One approach involves identifying novel components in important signal transduction pathways that are relevant for tumorigenesis, based either on pathway activation (eg, reporter gene expression) or the associated phenotypic consequences (eg, cell cycle arrest, apoptosis or differentiation). RNAi screens can also be conducted to identify components that enhance or sensitize cells to a specific treatment (eg, chemical-genetic screens), as measured by an increase or decrease in cell survival after exposure to a given drug. Importantly, those hits could also be explored as biomarkers for treatment prediction. Additionally, synthetic lethal screens performed in cell lines or tumor cell line panels with specific genetic alterations can lead to the discovery of genotype-specific drug targets. Finally, RNAi screens can be conducted *in vivo* in the context of a complete organism. Although there is substantial expectation that these RNAi screening strategies will identify new and highly relevant cancer drug targets, this possibility has generally been unfulfilled to date. However, several unbiased large-scale screens have resulted in the identification of genes with potential as novel drug targets (Table 1).

Table 1. RNAi screening strategies to identify novel drug targets.

Strategy	Typical readout	Examples of identified pathways	Examples of potential drug targets
Signal transduction and molecular pathway screens	Reporter based High-content read-out* Phenotypic selection	WNT/β- catenin pathway	CDK8
Chemical-genetic screens	Viability	PI3K-AKT pathway	PI3K
Synthetic lethal screens	Differential viability	RAS-PI3K-mTORC1 pathway	STK33
In vivo screens	Viability Phenotypic selection	DNA damage response	Rad17

*: High-content read-out: automated microscopy and image acquisition to measure morphology, localization, movement, structures and organization between and within single cells

Functional interrogation of cancer-specific pathways

One strategy that can be used to identify novel drug targets for cancer therapeutics is the functional interrogation of cancer-specific pathways that have already been established as important for tumor formation and maintenance, such as the p53 or WNT pathway. A salient examples of such a screen is one of the first described RNAi screens (*Nature* (2003) **424**(6950):797-801). In this approach, a small, but specific, shRNA library targeting 50 ubiquitin-specific proteases (deubiquitinating enzymes) was used in a reporter gene assay to identify repressors of the NFκB pathway. *CYLD*, the familial cylindromatosis tumor suppressor gene, was identified as an inhibitor of NFκB signaling; loss of CYLD increased the activity of the NFκB pathway. NFκB signaling was observed to correlate with cylindromatosis, suggesting a novel approach for treating such tumors. Indeed, treating patients with cylindromatosis with salicylic acid, a known NFκB inhibitor, caused a significant decrease in the size of small tumor lesions (*Br J Dermatol* (2006) **155**(1):182-5). This study is an excellent example demonstrating the use of an unbiased RNAi screen for the development of a novel therapeutic strategy. Another important example of novel target identification is based on a study of the WNT/β-catenin signaling pathway in colon cancer (*Nature* (2008) **455**(7212):547-51). Two loss-of-function RNAi screens were conducted, one aimed at the identification of regulators of β-catenin activity and one for the assessment of colon cancer cell survival. Copy number alterations in colon cancer specimens were analyzed for overlapping genes identified in both screens, and led to the identification of the gene *CDK8*. Inhibition of *CDK8* was demonstrated to reduce β-catenin-induced gene expression and to block transformation, establishing CDK8 as potential novel target for the treatment of colon cancer. Notably, the integration of other genomic data sets (e.g. copy number variation) and in-depth follow-up research were required to identify this single target, highlighting the limited value of using only a primary hit list derived from individual screens.

Chemical-genetic screening

A major challenge in the treatment of cancer is the development of resistance to therapy. After prolonged exposure to chemotherapeutic agents or small-molecule inhibitors, additional genetic and epigenetic changes can occur that render tumor cells insensitive to treatment. Although such acquired resistance represents a major barrier in the successful treatment of cancer, the increase in treatment options available for cancer also creates a challenge in the need for methods that can effectively determine the intrinsic sensitivity of tumors to a specific treatment. Chemical-genetic screening is one method to identify functional correlations between the characteristics of tumor cells and sensitivity to a drug (Figure 1). Several chemical-genetic screening strategies are available, including drug enhancer screens and drug resistance screens.

In drug enhancer screens, a cell line is either transfected with siRNAs or infected with vector-driven short-hairpin RNAs (shRNAs) in a single-well format. Following gene knockdown, the cells are treated with a suboptimal dose of a given drug and, after several days, viability is determined. Genes that, upon knockdown, render cells more sensitive to the drug are considered positive hits. Genes identified with this approach can be used to develop small molecules that sensitize cells to a drug response. If this type of screen is conducted using normal and tumor cells, then genes with inhibited expression that consequently enhance the drug response of tumor cells, without affecting normal cells, could potentially be identified. Additionally, a drug that causes various side effects could be used at reduced concentrations when administered in combination with another drug that uniquely modulates the sensitivity of the tumor

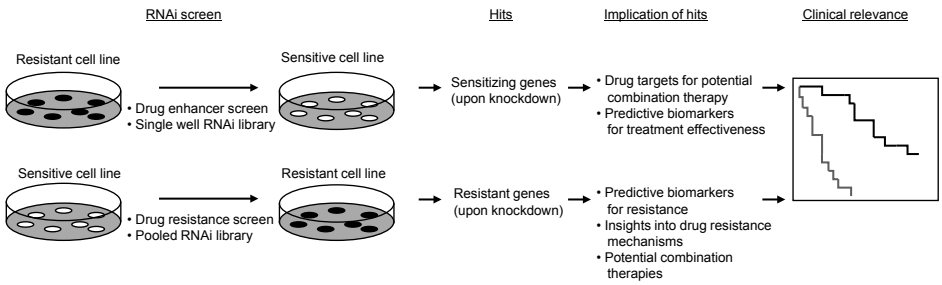


Figure 1. Chemical-genetic screening to identify novel drug targets and novel biomarkers. Typical RNAi screening approaches for drug enhancer and resistance screens are shown. Enhancer screens are generally conducted in a single-well format; drug resistance screens generally use a pooled library approach. Following transfection or infection of cells with an RNAi library, a drug is added; after 3 to 5 days, the viability of cells in each assay well is measured. Drug-resistant genes in pooled screens are identified by the selective enrichment of vector-driven short-hairpin RNAs (shRNAs) in the resistant population, a process that can require up to several weeks to complete. The enriched shRNAs are identified by DNA microarray hybridization or deep sequencing. Validated shRNA hits can yield insights into the mechanism of action of the drug, and can provide potential targets for enhancing drug response. Moreover, expression of the selected genes can be examined in clinical samples and correlated to treatment outcome in patients. This approach can result in the identification of powerful predictive biomarkers that enable an evaluation of the potential effectiveness of specific treatments.

to its effects. The identified genes can also be valuable as biomarkers of drug response for individual tumors, based on the tumor-specific expression or mutational status of the genes. For the validation of potential biomarkers, it is essential to have access to a large number of patient samples that have been treated with a specific compound and for which detailed clinical follow-up has been documented.

Drug resistance screens not only can identify genes that may have a role in the reversal of drug resistance, but can also be used to identify biomarkers that may predict the resistance of tumors to specific therapies. A powerful strategy to identify biomarkers of drug resistance involves screening pooled libraries of vector-based shRNAs. In this approach the relative abundance of each specific shRNA is measured in the population treated or untreated with the specified drug. The shRNA cassettes are recovered from genomic DNA and hybridized to a DNA microarray containing all complementary shRNA sequences of the library. By doing so, one can identify those shRNA sequences that are enriched in the drug-treated population and thus represent shRNAs that cause resistance against the drug treatment. Using this method, large numbers of shRNA vectors can be screened simultaneously in one population of cells that is exposed to the same drug concentration, providing the possibility of identifying genes that, when partially inhibited, also cause the resistance phenotype. This possibility is particularly important when complete knockdown of an individual resistance gene is lethal. Using shRNA screens the drug response of the cells can be monitored for up to several weeks due to continuous knockdown of the target genes. Single-well assays, on the contrary, are mainly restricted to several days, since in single-well screens mostly siRNAs are used that have a limited life-span within cells or due to the proliferation limitations of cells within a well. An example of an shRNA barcode screen involved the identification of genes that confer resistance to trastuzumab (an antibody that blocks the HER2 receptor) in breast cancer cells (*Cancer Cell* (2007) **12**(4):395-402). This screen identified PTEN as a critical determinant for the response to trastuzumab. Subsequent analysis of the PI3K-PTEN pathway status in a group of patients (n = 55) with breast cancer treated with trastuzumab demonstrated the clinical value of this pathway as a biomarker to predict the response to the drug for this disease. Furthermore, the resulting hypothesis that inhibition of PI3K could restore sensitivity to trastuzumab was demonstrated *in vitro* with a PI3K inhibitor combined with trastuzumab (*Cancer Cell* (2009) **15**(5):353-5).

Synthetic lethal screens

As noted, many cancers are driven by molecular events that cannot be targeted directly. As a consequence, investigators may aim to inhibit other targets that have a lethal effect only on tumor cells with specific genetic alterations, and not on normal cells. The concept that a particular mutation has deleterious consequences under specific conditions is known as synthetic lethality. Two genes are defined as being synthetic lethal genes if cells die when both genes are mutated, but can survive if only one gene is mutated. The approach of exploring synthetic lethal gene-gene interactions is attractive because a

hallmark of cancer cells (ie, the mutation of specific genes) becomes a weakness that can be exploited therapeutically. However, predicting how cancer-associated mutations might make tumor cells more vulnerable to therapy is difficult. The use of a genome-wide RNAi approach for a systematic examination of the consequences of inactivating large numbers of individual genes with defined tumor-specific genetic alterations in tumor cell lines could yield novel synthetic lethal targets. An example of this approach is the identification of STK33 in a synthetic lethal screen with oncogenic Ras. It was found that STK33 is selectively required for the survival and proliferation of tumor cells that are driven by an oncogenic *KRAS* gene (*Cell* (2009) **137**(5):821-34). Although most screens described to this date lack sufficient validation in large panels of tumor cell lines to exclude context dependency, or lack extensive *in vivo* validation of their identified hits, this strategy can clearly yield interesting individual drug targets.

In-vivo screening

A bottleneck in RNAi screens remains an ongoing issue in the development of relevant screening models. Results obtained with genetically modified cell lines or panels of tumor cell lines do not necessarily recapitulate *in vivo* consequences or, more importantly, clinical response. Several research groups have embarked on RNAi screening *in vivo* (Table 1). Although these screens are limited by the number of genes that can be interrogated, they have yielded promising results. An example is the introduction of a small library of shRNAs into a transplantable model of lymphoma. After tumor collection, shRNAs were identified that accelerated lymphomagenesis. Hits of this in vivo screen that have lost expression in human patient samples were associated with poor prognosis (*Cancer Cell* (2009) **16**(4):324-35). A variation of this approach involves direct screening in primary tumor cells isolated from a patient-derived tumor sample, termed 'direct functional *ex vivo* screening'. In a recent example of this approach, the vulnerabilities of primary leukemia cells from patients (n = 30) were investigated with a siRNA library selectively targeting tyrosine kinases (*Proc Natl Acad Sci USA* (2009) **106**(21):8695-8700). The JAK2 kinase was identified as being critical for the survival of tumor cells from ten of the patients. Moreover, inhibiting JAK2 kinase with a chemical compound affected the *in vitro* viability of tumor cells derived from these patients. Thus, direct functional *ex vivo* screening of tumor material from patients could theoretically, and within several days, indicate susceptibilities that can be readily targeted in personalized cancer therapy.

Validation of RNAi screening hits

RNAi screening technologies clearly have the capability to provide novel, important insights into genes and molecular networks that can be explored for therapeutic intervention. However, because of limitations, both of a technical nature as well as because of the biological model systems used in the screens, hits still require extensive validation and follow-up to establish that the biological effect is independent of the cellular context and that the effect will occur under the influence of a complex *in vivo*

environment. As a first step in the validation process, the expression levels of selected genes can be correlated either to treatment response or to disease outcome. Although this correlation does not replace the extensive follow-up required, it can establish the potential relevance of a particular gene in a clinical setting more rapidly.

CANCER PATHWAY-SPECIFIC VULNERABILITIES

The rapid development of targeted therapies has been enabled by significant efforts in the pharmaceutical industry to discover and develop therapeutics (eg, small molecules or biologicals) that target proteins that are important for the proliferation and survival of tumor cells. Targets are selected based on biological knowledge of signal transduction pathways and biological networks, in combination with genetic information obtained from patient-derived tumor samples. As a result of these efforts, many 'pathway-drugs' are progressing into the clinic. However, to identify those patients who would most benefit from a specific treatment, the pathway activation status of each individual tumor has to be established. Currently, cancers are generally defined and treated according to their organ or tissue of origin. Although different types of cancers are driven by diverse sets of mutations, an identical signaling pathway is often activated. However, the components of the pathway that are mutated can differ, and may occur either upstream or downstream of the implicated pathway. These differences can have significant effects on the response to specific therapeutics. For example, mutations in K-Ras are associated with non-responsiveness to the inhibition of its upstream regulator, EGFR, in colon cancer. It is therefore important to refine the classification of tumors for targeted therapies. Although there have been initial attempts to define pathway activation based on gene expression signatures, creating predictive gene expression profiles remains challenging, and these profiles often cannot discriminate the point at which the pathway is activated. Therefore, additional techniques capable of detecting the activation status of individual components of signaling pathways will be required. One such approach is the analysis of protein lysates of patient samples with phopho-specific antibodies directed at components of the signaling network. Results of these analyses will provide insight in the activation status of the components of a network and define a deregulated pathway or network. If cancer can be successfully classified into a limited number of activated pathways, then the next step in the research process is the identification of vulnerabilities per category of cancer type (ie, pathway-specific vulnerabilities). RNAi screening can be used to identify genetic vulnerabilities associated with the activation of aberrant pathways involved in cancer; these genes and their associated networks can then be explored further as potential drug targets. Although this concept remains at an early stage of development, the use of RNAi technologies is expected not only to broaden knowledge of the control of regulatory networks, but also to provide the means to stratify patients for the best treatment options directly.

CONCLUSION

The development of new therapeutics, as well as new tools to enable patient selection for existing drugs, has continued to be challenging. With the development of RNAi screening technologies, it is possible to identify novel targets and biomarkers that can predict treatment responses. Some major advantages of RNAi screening are the direct functional role that can be ascribed to the identified proteins, and the use of this strategy to establish the potential of these proteins as targets for drug development. RNAi screening approaches still require extensive follow-up and validation. However, significant benefit can be gained from an integrated approach in which additional large-scale data sets (eg, gene expression, genomic alterations and large-scale sequencing) are combined at an early stage with the hit lists derived from primary RNAi screens. Using this strategy enables the selection of the most promising hits for follow-up, and also directs the selection of hits as clinically useful targets.

FURTHER READING

Bernards R: **It's diagnostics, stupid.** *Cell* (2010) **141**(1):13-17.

Hyduke DR, Palsson BO: **Towards genome-scale signalling-network reconstructions.** *Nat Rev Genet* (2010) **11**(4):297-307.

Chang JT, Carvalho C, Mori S, Bild AH, Gatza ML, Wang Q, Lucas JE, Potti A, Febbo PG, West M, Nevins JR: **A genomic strategy to elucidate modules of oncogenic pathway signaling networks.** *Mol Cell* (2009) **34**(1):104-114.

Iorns E, Lord CJ, Turner N, Ashworth A: **Utilizing RNA interference to enhance cancer drug discovery.** *Nat Rev Drug Discov* (2007) **6**(7):556-568.

Jänne PA, Gray N, Settleman J: **Factors underlying sensitivity of cancers to small-molecule kinase inhibitors.** *Nat Rev Drug Discov* (2009) **8**(9):709-723.

Luo J, Solimini NL, Elledge SJ: **Principles of cancer therapy: Oncogene and non-oncogene addiction.** *Cell* (2009) **136**(5):823-837.

Mullenders J, Bernards R: **Loss-of-function genetic screens as a tool to improve the diagnosis and treatment of cancer.** *Oncogene* (2009) **28**(50):4409-4420.

3

SCREENING FOR MODULATORS OF CISPLATIN SENSITIVITY: UNBIASED SCREENS REVEAL COMMON THEMES

Jeroen H. Nijwening[1,*], Hendrik J. Kuiken[1,*] and Roderick L. Beijersbergen[1]

[1]: Division of Molecular Carcinogenesis, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands

*: These authors contributed equally to this work

Published in Cell Cycle 2011 Feb1;10(3):380-6

3

ABSTRACT

Cisplatin is a widely used chemotherapeutic agent to treat a variety of solid tumors. The cytotoxic mode of action of cisplatin is mediated by inducing conformational changes in DNA including intra- and inter-strand crosslink adducts. Recognition of these adducts results in the activation of the DNA-damage response resulting in cell cycle arrest, repair, and potentially, apoptosis. Despite the clinical efficacy of cisplatin, many tumors are either intrinsically resistant or acquire resistance during treatment. The identification of cisplatin drug response modulators can help us understand these resistance mechanisms, provide biomarkers for treatment strategies, or provide drug targets for combination therapy. Here we discuss functional genetic screens, including one performed by us, set up to identify genes whose inhibition results in increased sensitivity to cisplatin. In summary, the validated genes identified in these screens mainly operate in DNA-damage response including nucleotide excision repair, translesion synthesis, and homologous recombination.

Keywords: Cisplatin, RNAi, screen, sensitivity, DDR

INTRODUCTION

Cisplatin (*cis*-diammine-dichloro-platinum) is a commonly used chemotherapeutic agent displaying clinical activity against a wide variety of solid tumors. However, the response to cisplatin is variable, tumors can be either intrinsically resistant or acquire resistance to platinum drugs. Besides such resistance, side effects like nephrotoxicity seriously limit the therapeutic efficacy of platinum drugs in the clinic. Cisplatin is particularly active against germ cell tumors, epithelial ovarian cancer, and is considered to be a curative treatment for testicular cancer (Kelland 2007). Although different types of tumors initially respond very well to cisplatin, the majority of patients experience incurable recurrences. In ovarian cancer for example, initial response rate can be up to 70% but this results in a 5-year patient survival rate of only 15-20%. In other tumor types, like small-cell lung cancers, the relapse rate can be as high as 95% (Ozols 2005). To further advance the clinical utility of cisplatin, it is crucial to increase our understanding of the mechanisms underlying the response to the drug. Of interest are those mechanisms that can enhance the response to platinum drugs in a tumor-specific manner. A striking example of the latter is the high sensitivity of tumors with impaired function of BRCA1 or BRCA2 proteins to drugs that generate DNA inter-strand cross-links (ICLs). The BRCA proteins are essential for the homology-directed DNA repair (HR) that allows error-free repair of the duplex breaks caused by the excision of platin-DNA adducts. In addition to HR, nucleotide excision repair (NER) is required for adduct excision preceeding homology-directed DNA repair. As a consequence, tumor cells with impaired HR or NER have an increased sensitivity to platinum drugs. These examples illustrate the importance of the identification of additional components of pathways crucial for the response to cisplatin. It is anticipated that large-scale functional genomic screening would provide us with these novel components. Here, we will discuss the current understanding of modulators of the cisplatin response and the results and interpretation of screening efforts aimed at the identification of genes involved in cisplatin response.

CISPLATIN AND DNA-DAMAGE RESPONSE

The cytotoxic mode of action of platinum drugs is predominantly mediated by the interaction with DNA to form DNA adducts. Upon cell entry, cisplatin becomes aquated and positively charged. In this state cisplatin is able to interact with nucleophilic molecules like DNA, RNA and methallothioneins. Over the years many mechanisms that modulate cisplatin drug response have been described, such as transporters that control the cellular accumulation of cisplatin by influx or efflux, and methallothioneins that form complexes with cisplatin, thereby limiting the pool of free cisplatin that can interact with DNA (Borst et al 2008). However, their clinical significance remains speculative, and in addition, recent studies suggest that the responsiveness to cisplatin is mostly determined by the activity of DNA damage repair in cells.

Cisplatin preferably interacts with guanosine and at a lower frequency with adenosine. It thereby creates intra-strand cross-links, and, to a lesser extent mono-adducts and inter-strand cross-links (Ahmad 2010, Kelland 2007, Rabik and Dolan 2007). These cisplatin-induced conformational changes of DNA can be recognized resulting in activation of ATR-CHEK1 and ATM-CHEK2 DNA damage signal transduction pathways(Bartek et al 2007, Ciccia and Elledge 2010). These pathways orchestrate a cell cycle arrest to allow DNA repair or the clearance of damaged cells through induction of apoptosis.

The repair of intra-strand cross-links, the major lesion (80-90%) caused by cisplatin, occurs primarily through the nucleotide excision repair (NER) pathway (Kelland 2007, Rabik and Dolan 2007). The NER pathway includes proteins that are mutated in Xeroderma Pigmentosum (XP) and Cockayne Syndrome (CS) patients. Indeed, defects in the NER pathway (XPA, ERCC2, ERCC4, ERCC5, ERCC6 and ERCC8) result in hypersensitivity to platinating agents, and restoration of NER integrity correlates with rescue of the cisplatin sensitivity of these cells (Furuta et al 2002). Key components of NER include the proteins Excision Repair Cross Complementing (ERCC1) and Xeroderma Pigmentosum F (XPF, also known as ERCC4). ERCC1-ERCC4 cleaves the damaged strand 5' of the DNA lesion and the resulting gap is filled in by DNA polymerases in the presence of replication factors. Increased expression of ERCC1-ERCC4 is associated with cisplatin resistance in ovarian and gastric tumors (Rabik and Dolan 2007).

The DNA distortions caused by cisplatin are also recognized by protein complexes such as the MSH2-MSH6 component of the mismatch repair (MMR) system and the high mobility group proteins HMG1 and HMG2 .The MMR pathway is involved in replacing mispaired bases in the DNA duplex with correct bases after replication (Hoeijmakers 2001). Interestingly, MMR proteins are able to recognize cisplatin-induced intra-strand cross-links, but are not involved in the actual repair of the damage. However, its is thought that upon crosslink binding they do give rise to activation of apoptotic signaling (Siddik 2003). Hence tumors that are MMR-deficient can continue to proliferate in spite of the cisplatin induced DNA-damage (Vaisman et al 1998) (Geisler et al 2003). Like MMR proteins, high-mobility group (HMG) proteins are able to bind cisplatin-induced intra-strand cross-links, but are not involved in their repair. It has been suggested that binding of these HMG proteins can conceal the DNA damage from DNA damage repair pathways (He et al 2000).

Instead of initiating repair, DNA-damage can also be ignored by a cell. When the replication machinery runs into a platinum-DNA-adduct it will stall due to the inability of replicative polymerase to move across the lesion. However the replicative polymerase can be exchanged for translesion polymerases that can proceed across the DNA adduct, a process called translesion synthesis (TLS). Human cells expressing reduced levels of REV3L, an essential component of the translesion polymerase DNA Polζ, are more sensitive to cisplatin (Wu et al 2004a). The strong cisplatin sensitization by downregulation of the components of TLS can also be the result of their involvement

in the repair of both cisplatin-induced intra- and inter-strand crosslinks. The function of Polζ (REV3L-REV7), REV1, Polη, and RAD18 are all required for replicative bypass of cisplatin intra-strand cross-links (Hicks et al 2010). Although this form of DNA-damage tolerance is associated with cell viability, TLS is error-prone and accumulation of mutations will be detrimental to cells over prolonged periods of time (Livneh 2006).

The inter-strand cross-links (ICLs) induced by cisplatin occur less frequent (2%) than the intra-strand cross-links (80-90%). However, they impose an extreme danger to the cell (Scharer 2005). Recently it was shown that the exceptional sensitivity of testicular germ cell tumours (TGCT) to cisplatin is associated with a decreased ability to repair ICLs. It appears that low levels of ERCC1-ERCC4 are rate limiting in this response, suggesting that ERCC1-ERCC4 could be used as a target to enhance the response of tumors to ICL-inducing drugs (Usanova et al 2010). The repair of ICLs is complex and involves a series of DNA-damage repair pathways including NER, TLS, homologous recombination (HR), and the Fanconi anemia (FA) proteins. Although our knowledge of ICL repair has increased significantly over the past years, a comprehensive understanding of the mechanism is far from complete (Ciccia and Elledge 2010, Moldovan and D'Andrea 2009).

It is clear that several different mechanisms are involved in the recognition of DNA-damage induced by platinating agents and the efficient repair of these lesions. Mutations that affect DDR result in increased sensitivity to cisplatin. To further improve cisplatin therapy, it is important to identify essential components of the cellular pathways involved in cisplatin response, as they may represent suitable biomarkers for patient stratification or targets for combination therapy (Bernards 2010, Iorns et al 2007). To this date, several screens for modulators of cisplatin drug response have been performed with the use of model organisms *S. cerevisiae*. In addition, RNA-interference (RNAi) screens for modulators of cisplatin response have been performed in several human cancer cell lines (Table 1). Although it remains questionable whether drug response mechanisms identified in vitro significantly contribute to resistance observed in the clinical setting (Borst et al 2008), screens for genes that modulate cisplatin response seem to recover genes strongly implicated in the high sensitivity to platinum drugs. It can be concluded that functional genomic screens, although still in infancy, show promise in the identification of components playing crucial roles in the sensitivity of cells towards cisplatin based therapies. Here we will compare the outcome of several screening efforts, including our own, and discuss the results in a wider perspective.

SCREENS FOR MODULATORS OF CISPLATIN SENSITIVITY

Lessons learned from yeast

Several screens for modulators of cisplatin sensitivity were performed in the budding yeast S. *cerevisiae*. As the function of the genes identified by these screens is highly conserved between *S. cerevisiae* and man, results of these screens are valuable for the understanding of the cisplatin response in human cells. A collection of yeast deletion

Table 1. Genetic screens for modulators of cisplatin sensitivity in *S. cerevisiae* and human cells.

Reference	Screen	Library	Screening model	Screen setup	Viability assay	Validation	Modulators of cisplatin response
Simon et al., 2000, Cancer Research	Multi-drug screen in yeast mutants	22 *S. cerevisiae* DDR-mutants	S. cerevisiae	18h drug treatment, serial dilutions	Colony formation assay and OD measurement	None	RAD1 RAD6 RAD14 RAD18 RAD50 RAD51 RAD52 REV1 REV3
Lum et al., 2004, Cell	Multi-drug screen in yeast heterozygotes	Genome-wide *S. cerevisiae* heterozygotes	S. cerevisiae	15h drug treatment, serial dilutions	Barcode readout	only compounds	VPS65 PSY2 ATP4
Wu et al., 2004, Cancer Research	Sensitizer screen for cisplatin, oxaliplatin, mitomycin C in yeast mutants	4728 homozygous deletion strains of *S. cerevisiae*	S. cerevisiae	4h drug treatment, additional 16h w/o drug	Barcode readout	clonogenic survival of positive outliers from barcode readout	RAD2, 5, 10 REV1 REV3 MUS81 MMS4 RAD57 SAE2 PSO2 IMP2 PPH3 PSY1 PSY2 PSY3 YKL076C YNL201C YIL132C YLR376C

Table 1. Continued.

Reference	Screen	Library	Screening model	Screen setup	Viability assay	Validation	Modulators of cisplatin response
Bartz et al., 2006, Molecular and Cellular Biology	RNAi screen for cisplatin, gemcitabine, paclitaxel sensitizers	20.000 genes (Sigma, 3 siRNAs/gene)	HeLa	FW transfection, 4h later 30 μM cisplatin (EC_{10}), 68h later endpoint assay	Alamar blue	2 independent siRNAs kd-phenotype correlation	ATR CHEK1 BRCA1 BRCA2 RAD51 SHFM1 RAD18 BRIP1 REV1L REV3L
Swanton et al., 2007, Cancer Cell	RNAi screen for paclitaxel, doxorubicin, 5-FU, cisplatin sensitizers	779 kinases, 50 ceramides (Dharmacon, 4 siRNAs/gene)	A549	FW transfection, 48h later 12.5 μM cisplatin, 48h later endpoint assay	Cell Titer Glo	> 2 independent siRNAs kd-phenotype correlation	CERT
Arora et al., 2010, Gynecologic Oncology	RNAi screen for cisplatin sensitizers	572 kinases (Qiagen, 2 siRNAs/gene)	SKOV3	RV transfection, 24h later 2 μM ($EC_{10\text{-}20}$), 72h later endpoint assay	Cell Titer Glo	2 independent siRNAs kd-phenotype correlation	ATR CHEK1 PRKAB1
Nijwening et al., 2010, this Extra View	RNAi screen for cisplatin sensitizers	7000 genes (druggable genome, Dharmacon, 4 siRNAs/gene)	BJET-p53KD	RV transfection, 24h later 0.4 μM cisplatin (EC10), 72h later endpoint assay	Nuclei count	2 independent siRNAs kd-phenotype correlation	ATR BRCA1 BRCA2 REV3L

strains with defined genetic alterations in DDR elements was used to determine whether these confer a specific vulnerability to a panel of chemotherapeutic agents, including cisplatin (Simon et al 2000). The drug sensitivity profiles showed that yeast strains deleted for genes in the key-pathways for cisplatin-induced DNA-damage repair including the post-replication repair pathway (*RAD6* and *RAD18*), recombinational repair (*RAD50*, *RAD51* and *RAD52*), and TLS (*REV1*, *REV3*) are more sensitive to cisplatin treatment. In a similar screening model, using a panel of 4728 homozygous yeast deletion strains of nonessential genes, sensitivity profiles were determined for cisplatin, oxaliplatin and mitomycin C (MMC) (Wu et al 2004b). The identified genes, that upon deletion confer sensitivity to these anticancer agents, include those operating in NER (e.g. *RAD1, RAD2, RAD4, RAD10 and RAD14*), postreplication repair (*RAD5, MMS2*), TLS (*REV1* and *REV3*), recombinational repair (*MMS4, RAD54, RAD55, RAD57, RAD59 and SAE2*), and ICL repair (*PSO2*) (Table 1). Interestingly, ES cells with a homozygous deletion of the mouse ortholog of *PSO2, mSNM1*, display increased sensitivity to MMC, but not to cisplatin (Dronkert et al 2000). In both screening models, no genes were identified operating in the non-homologous end joining (NHEJ), DSB repair pathway, the base excision repair (BER) pathway, or in DNA-damage checkpoints (Simon et al 2000, Wu et al 2004b). However, Brown et al., did identify novel cisplatin-sensitizers including *IMP2, PPH3, PSY1, PSY2,* and *PSY3. PSY2* was previously identified by Shoemaker and colleagues, in a screen of 78 therapeutically active chemical agents in a genome-wide screen of yeast heterozygotes (Lum et al 2004). Heterozygous deletion of *PSY2* (platinum sensitivity 2) caused increased sensitivity towards cisplatin, (Lum et al 2004). The human ortholog of *PSY2, PP4R3*, is part of the protein phosphatase 4 cisplatin sensitive complex (PP4cs). Taken together, screens for modulators of cisplatin sensitivity performed in *S. cerevisiae* led to the identification of genes mainly involved in the DDR.

Towards understanding the platinum response in human cancer

A genome-wide screen to identify modifiers of cisplatin sensitivity was performed by Linsley and colleagues (Bartz et al 2006). Their validated hits are shown in Table 1 and show a considerable overlap with the orthologs identified in *S. cerevisiae*. These genes mainly operate in pathways of the DDR that are activated by stalled replication forks (*ATR*, *CHEK1* and *RAD18*) and HR-directed repair (*BRCA1, BRCA2, RAD51, SHFM1, REV1L*, and *REV3L*). In a smaller scaled siRNA screen comprising 2400 pools of siRNAs, sensitivity to cisplatin, gemcitabine or paclitaxel was tested. The results show unique drug response profiles for the genes tested, similarly to the yeast screen described above (Simon et al 2000). In another study, Azorsa and colleagues performed a screen for cisplatin drug response modulators in an ovarian cancer cell line SKOV3, using an RNAi library targeting the kinome (Arora et al 2010). Knockdown of three validated genes, *ATR, CHEK1*, and *PRKAB1*, were validated that upon knock-down enhance the cisplatin response. PRKAB1 is a subunit of the adenosine monophosphate-activated protein kinase (AMPK) that has been implicated in cisplatin drug response

previously. Follow-up studies on *CHEK1* show that inhibition of CHEK1 by a small molecule inhibitor indeed results in increased sensitivity towards cisplatin treatment (Weng et al 2008).

A targeted siRNA screen was performed by Nicke and colleagues using a small siRNA library targeting the kinome and ceramidome (reviewed by Reynolds (Reynolds et al 2004) to study drug response to several chemotherapeutics including paclitaxel, doxorubicin, 5-FU and cisplatin (Swanton et al 2007). One of the hits was *COL4A3BP* (*CERT*), a ceramide transporting protein, and knockdown of this gene sensitized diverse cell types to the chemotherapeutics tested. Many chemotherapeutic agents, as well as knockdown of CERT, cause ceramide accumulation in cells, which is toxic. Hence the sensitizing effect of CERT knockdown on cisplatin treatment might be explained by a ceramide dependent additive or synergistic effect.

To explore the possibility of identifying additional modulators of sensitivity to cisplatin, we performed a screen with an RNAi library consisting of 7000 siRNA pools targeting the kinome and druggable genome (see Table 1 for screening details). In contrast to previously described screens, we used primary human fibroblasts (BJs) that were immortalized by stable introduction of the catalytic subunit of human telomerase (hTERT), and which stably expresses a p53-knockdown vector (BJET-p53KD) leading to a p53null phenotype (data not shown). We chose a primary cell line, because most of the DNA-damage response pathways, except those relying on p53, are still intact in these cells. Distinctive to other drug modulator screens in mammalian cells, we used a viability assay based on counting nuclei by staining DNA with DAPI, high content image acquisition and automated image analysis. An advantage of this approach is that nuclei counts, in contrast to metabolic assays, are not affected by changes in metabolism caused by knockdown of genes involved in these processes. However, a reduction in nuclei count is not associated with reduced cell viability per se, as it can also be caused by a proliferative arrest.

Based on the original screen data we selected 37 genes, which upon knockdown selectively reduced the nuclei count (>30%) in BJET-p53KD cells treated with cisplatin, and which only show a modest toxicity (<25%) in untreated BJET-p53KD cells (Fig. 1A). To confirm the observed phenotypes, we retested the siRNA smartpools and of 37 smartpools tested, 7 (19%) gave reproducible results (Fig. 1B). To exclude possible off-target effects, we subsequently tested the 4 individual siRNAs of the regular siGenome smartpools, and 4 On-Target Plus (OTP) modified siRNAs. OTP siRNAs carry a base modification in the antisense strand to reduce binding through seed-sequences to prevent miRNA mediated effects. For 4 (57%) of the 7 genes tested, we were able to reproduce the results with 3 or more unique siRNAs (Fig. 1C). With this approach and selection criteria, we were able to validate *ATR, BRCA1, BRCA2*, and *REV3L* as hits. These four genes are components of DNA repair pathways previously implicated in cisplatin sensitivity. Importantly, the results of our screen show an almost complete overlap with the results described by Linsely and colleagues (Bartz et al 2006). However several of the genes identified by Linsley and coworkers were not selected for validation in our study

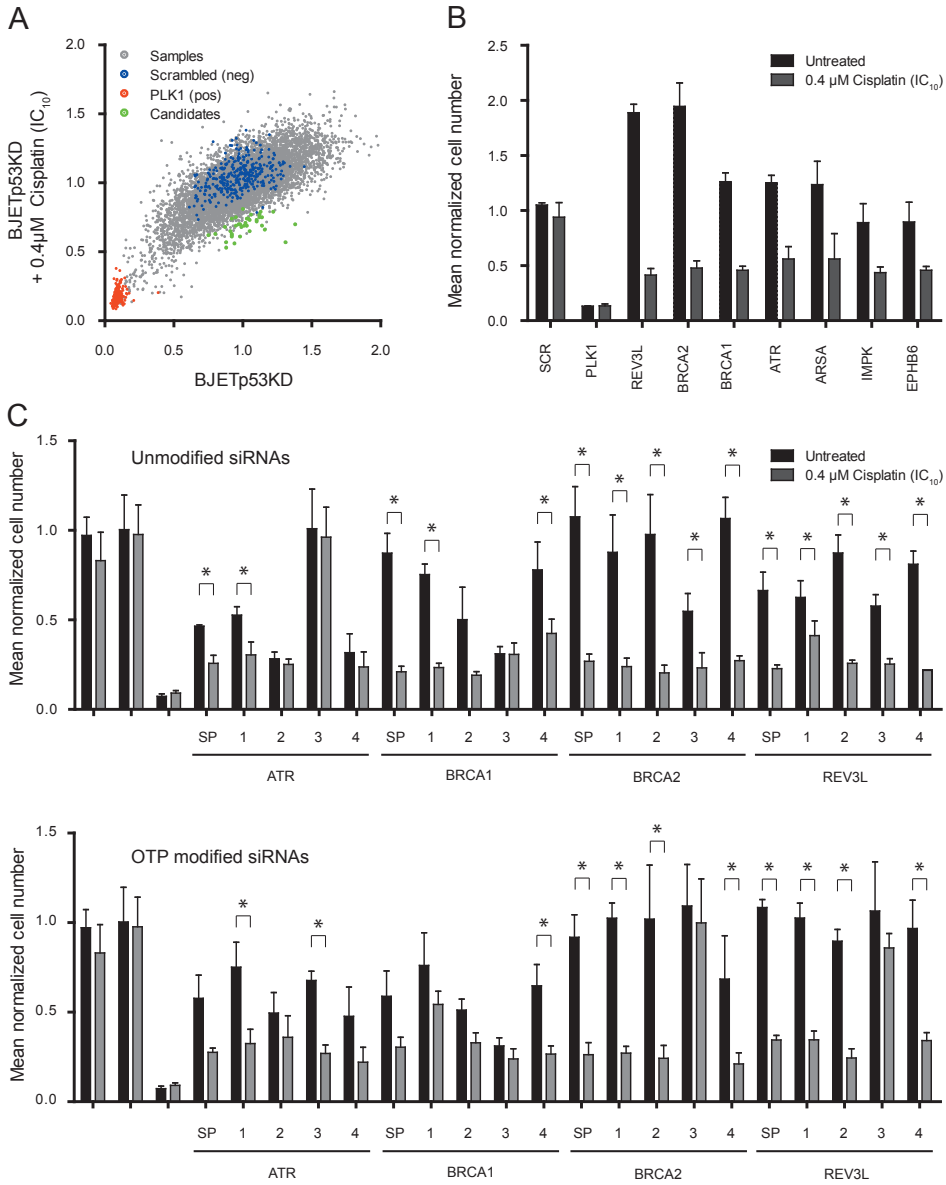


Figure 1. A siRNA screen for drug modulators of cisplatin in primary human fibroblasts. (A) Data from the screen presented in a scatter plot with the mean normalized nuclei counts for every siRNA smartpool in both the untreated as well as the cell line treated with 0.4μM cisplatin (IC_{10}). Grey: experimental samples, blue: negative controls, red: positive controls, green: selected candidates. In total 37 candidates were selected based on the following criteria: mean normalized nuclei count in untreated setting between 0.75 and 1.50, >25% reduction in nuclei count in the treated setting, and a relative reduction in nuclei count of >30% in the treated versus the untreated setting. (B) A validation experiment with siRNA smartpools targeting the candidate genes to determine the reproducibility of the phenotypes observed in the screen. Only those that showed a reproducible phenotype are shown. Error-bars represent standard deviation of three measurements. (C) Summary of a validation experiment with siRNA smartpools and individual siRNAs from the siGenome and on-target plus (OTP) collection of ThermoFischer Dharmacon. Only data for the validated genes, *ATR, BRCA1, BRCA2*, and *REV3L* are shown. Error-bars represent standard deviation of three independent experiments. Asterisks denote significant (student t-test, $p < 0.05$) differences between the treated and untreated settings.

despite their selective enhanced cisplatin cytotoxicity (*CHEK1, RAD51*, and *SHFM1*). These genes were not selected in our screen because knockdown resulted already in a significant reduction in nuclei count in cells in the absence of cisplatin treatment. One explanation for the difference in the effects on cell viability could be the difference in cell types. We have used primary human fibroblasts in contrast to HeLa cells, a human cervical carcinoma cell line, in which confounding mutations can mask the consequences of knockdown of these genes. We were unable to validate other genes such as BARD1, or these did not meet our selection criteria because they did not show a significant selective effect towards cisplatin treatment in our screen (*REV1, BRIP1*). In addition, members of the RAD family, that were identified in the mammalian and yeast screens described above, were not selected in our screens because they influenced cell viability of untreated cells in a positive or negative way, more than our stringent selection criteria permitted.

Overlapping hits in common pathways

Despite differences in the size of the libraries and the genes they target, the screening models, treatment schedules, and assays used to readout cell viability, most of the screens described above led to similar results. The majority of the identified genes, which upon knockdown enhance cisplatin cytotoxicity, share overlapping functions in TLS, HR and ICL repair. Although the screens are based on in vitro models, many of the genes and their associated pathways have been confirmed in *in vivo* models and tumors carrying genetic alterations in these genes have shown to be highly sensitive to cisplatin treatment (Doles et al 2010). No genes were identified, acting in signal transduction pathways such as MEK/ERK or PI3K, inhibitors of apoptosis or other stress sensors. The predominant modulators of cisplatin response are part of the DNA damage sensing signaling and repair pathways. However, some genes were identified which are not yet implicated in DDR, such as a subunit of AMPK and CERT in human cell lines. Although available data suggest a mechanism for AMPK and CERT in modulation of cisplatin drug response, it is of interest to further elucidate the role of non-DDR related proteins, and explore whether they have clinical significance. Interestingly, none of the screens led to the identification of genes in the expected NER or Fanconi anemia pathways despite their well established involvement in the DDR to cisplatin. However, as RNAi results in suppression of protein levels rather than complete ablation of protein function, remnant levels of NER activity or FA proteins might be sufficient for the cell to deal with the DNA damage induced by cisplatin treatment. The same argument could be used against the notion that saturation in screening for modulators of cisplatin sensitivity has been reached based on the highly correlative results from the RNAi screens conducted. It is also noteworthy that the libraries used are based on distinct reagents and show differences in their coverage.

In conclusion, the sensitized screens with RNAi or yeast mutants both yielded novel chemical-genetic interactions and highlight the importance of NER, and especially HR, TLS, ICL repair, and the ATR-CHEK1 DNA-damage signal transduction pathway in response to cisplatin induced DNA-damage.

THE NEXT STEPS

The major task at hand is exploration of the clinical significance of the modulators of cisplatin sensitivity that have been described. For example, it will be interesting to await the results of the clinical trials with cisplatin in combination with a compound targeting one of the identified drug response modulators, CHEK1 (Sharma et al 2009). In addition, it is attractive to investigate whether tumors that show the BRCAness phenotype, meaning wild type BRCA-genes but yet showing similar defects as those with BRCA-mutations (Darzynkiewicz et al 2009, Turner et al 2004), bear (epi) genetic alterations in one of the genes listed in Table 1. The presence thereof could be associated with a better response to cisplatin treatment (Cass et al 2003, D'Andrea 2003, Majdak et al 2005). It will also be interesting to screen for genes that sensitize cells to cisplatin in cellular models that are highly resistant to cisplatin. Hits identified with this approach could be potential drug targets for combination therapy in patients that show no response to cisplatin. Of equal importance, the identification of cisplatin drug response modulators could guide the stratification of patients that might benefit most from treatment (Bernards 2010). In conclusion, the results of specific sensitized RNAi screens, as discussed in this Extra View, could yield important insights that can be explored to enhance the treatment of cancer in a personalized manner.

ACKNOWLEDGEMENTS

We thank R. Bernards, P. Borst, S. Rottenberg and C. Guyader for critical reading of the manuscript. This work was supported by Top Institute Pharma (project T3-105) and Merck & Co., Inc.

ABBREVIATIONS

5-FU	5-fluoro-1H-pyrimidine-2, 4-dione, 5-fluorouracil
BER	Base excision repair
DAPI	4', 6-diamidino-2-phenylindole
DDR	DNA damage response
DSB	Double strand break
HR	Homologous recombination
ICL	Inter-strand cross-link
MMC	Mitomycin C
MMR	Mismatch repair
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
OTP	On-Target Plus, modified siRNA
RNAi	RNA interference
TLS	Translesion synthesis

REFERENCES

Ahmad S (2010). Platinum-DNA interactions and subsequent cellular processes controlling sensitivity to anticancer platinum complexes. *Chem Biodivers* **7:** 543-566.

Arora S, Bisanz KM, Peralta LA, Basu GD, Choudhary A, Tibes R *et al* (2010). RNAi screening of the kinome identifies modulators of cisplatin response in ovarian cancer cells. *Gynecol Oncol* **118:** 220-227.

Bartek J, Lukas J, Bartkova J (2007). DNA damage response as an anti-cancer barrier: damage threshold and the concept of 'conditional haploinsufficiency'. *Cell Cycle* **6:** 2344-2347.

Bartz SR, Zhang Z, Burchard J, Imakura M, Martin M, Palmieri A *et al* (2006). Small interfering RNA screens reveal enhanced cisplatin cytotoxicity in tumor cells having both BRCA network and TP53 disruptions. *Mol Cell Biol* **26:** 9377-9386.

Bernards R (2010). It's diagnostics, stupid. *Cell* **141:** 13-17.

Borst P, Rottenberg S, Jonkers J (2008). How do real tumors become resistant to cisplatin? *Cell Cycle* **7:** 1353-1359.

Cass I, Baldwin RL, Varkey T, Moslehi R, Narod SA, Karlan BY (2003). Improved survival in women with BRCA-associated ovarian carcinoma. *Cancer* **97:** 2187-2195.

Ciccia A, Elledge SJ (2010). The DNA damage response: making it safe to play with knives. *Mol Cell* **40:** 179-204.

D'Andrea AD (2003). The Fanconi Anemia/BRCA signaling pathway: disruption in cisplatin-sensitive ovarian cancers. *Cell Cycle* **2:** 290-292.

Darzynkiewicz Z, Traganos F, Wlodkowic D (2009). Impaired DNA damage response--an Achilles' heel sensitizing cancer to chemotherapy and radiotherapy. *Eur J Pharmacol* **625:** 143-150.

Doles J, Oliver TG, Cameron ER, Hsu G, Jacks T, Walker GC *et al* (2010). Suppression of Rev3, the catalytic subunit of Pol{zeta}, sensitizes drug-resistant lung tumors to chemotherapy. *Proc Natl Acad Sci U S A* **107:** 20786-20791.

Dronkert ML, de Wit J, Boeve M, Vasconcelos ML, van Steeg H, Tan TL *et al* (2000). Disruption of mouse SNM1 causes increased sensitivity to the DNA interstrand cross-linking agent mitomycin C. *Mol Cell Biol* **20:** 4553-4561.

Furuta T, Ueda T, Aune G, Sarasin A, Kraemer KH, Pommier Y (2002). Transcription-coupled nucleotide excision repair as a determinant of cisplatin sensitivity of human cells. *Cancer Res* **62:** 4899-4902.

Geisler JP, Goodheart MJ, Sood AK, Holmes RJ, Hatterman-Zogg MA, Buller RE (2003). Mismatch repair gene expression defects contribute to microsatellite instability in ovarian carcinoma. *Cancer* **98:** 2199-2206.

He Q, Liang CH, Lippard SJ (2000). Steroid hormones induce HMG1 overexpression and sensitize breast cancer cells to cisplatin and carboplatin. *Proc Natl Acad Sci U S A* **97:** 5768-5772.

Hicks JK, Chute CL, Paulsen MT, Ragland RL, Howlett NG, Gueranger Q *et al* (2010). Differential roles for DNA polymerases eta, zeta, and REV1 in lesion bypass of intrastrand versus interstrand DNA cross-links. *Mol Cell Biol* **30:** 1217-1230.

Hoeijmakers JH (2001). Genome maintenance mechanisms for preventing cancer. *Nature* **411:** 366-374.

Iorns E, Lord CJ, Turner N, Ashworth A (2007). Utilizing RNA interference to enhance cancer drug discovery. *Nat Rev Drug Discov* **6:** 556-568.

Kelland L (2007). The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer* **7:** 573-584.

Livneh Z (2006). Keeping mammalian mutation load in check: regulation of the activity of error-prone DNA polymerases by p53 and p21. *Cell Cycle* **5:** 1918-1922.

Lum PY, Armour CD, Stepaniants SB, Cavet G, Wolf MK, Butler JS *et al* (2004). Discovering modes of action for therapeutic compounds using a genome-wide screen of yeast heterozygotes. *Cell* **116:** 121-137.

Majdak EJ, Debniak J, Milczek T, Cornelisse CJ, Devilee P, Emerich J *et al* (2005). Prognostic impact of BRCA1 pathogenic and BRCA1/BRCA2 unclassified variant mutations in patients with ovarian carcinoma. *Cancer* **104:** 1004-1012.

Moldovan GL, D'Andrea AD (2009). How the fanconi anemia pathway guards the genome. *Annu Rev Genet* **43:** 223-249.

Ozols RF (2005). Treatment goals in ovarian cancer. *Int J Gynecol Cancer* **15 Suppl 1:** 3-11.

Rabik CA, Dolan ME (2007). Molecular mechanisms of resistance and toxicity associated with platinating agents. *Cancer Treat Rev* **33:** 9-23.

Reynolds CP, Maurer BJ, Kolesnick RN (2004). Ceramide synthesis and metabolism as a target for cancer therapy. *Cancer Lett* **206:** 169-180.

Scharer OD (2005). DNA interstrand crosslinks: natural and drug-induced DNA adducts that induce unique cellular responses. *Chembiochem* **6:** 27-32.

Sharma S, Freeman B, Turner J, Symanowski J, Manno P, Berg W *et al* (2009). A phase I trial of PTK787/ZK222584 in combination with pemetrexed and cisplatin in patients with advanced solid tumors. *Invest New Drugs* **27:** 63-65.

Siddik ZH (2003). Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* **22:** 7265-7279.

Simon JA, Szankasi P, Nguyen DK, Ludlow C, Dunstan HM, Roberts CJ *et al* (2000). Differential toxicities of anticancer agents among DNA repair and checkpoint mutants of Saccharomyces cerevisiae. *Cancer Res* **60:** 328-333.

Swanton C, Marani M, Pardo O, Warne PH, Kelly G, Sahai E *et al* (2007). Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs. *Cancer Cell* **11:** 498-512.

Turner N, Tutt A, Ashworth A (2004). Hallmarks of 'BRCAness' in sporadic cancers. *Nat Rev Cancer* **4:** 814-819.

Usanova S, Piee-Staffa A, Sied U, Thomale J, Schneider A, Kaina B *et al* (2010). Cisplatin sensitivity of testis tumour cells is due to deficiency in interstrand-crosslink repair and low ERCC1-XPF expression. *Mol Cancer* **9:** 248.

Vaisman A, Varchenko M, Umar A, Kunkel TA, Risinger JI, Barrett JC *et al* (1998). The role of hMLH1, hMSH3, and hMSH6 defects in cisplatin and oxaliplatin resistance: correlation with replicative bypass of platinum-DNA adducts. *Cancer Res* **58:** 3579-3585.

Weng SC, Kashida Y, Kulp SK, Wang D, Brueggemeier RW, Shapiro CL *et al* (2008). Sensitizing estrogen receptor-negative breast cancer cells to tamoxifen with OSU-03012, a novel celecoxib-derived phosphoinositide-dependent protein kinase-1/Akt signaling inhibitor. *Mol Cancer Ther* **7:** 800-808.

Wu F, Lin X, Okuda T, Howell SB (2004a). DNA polymerase zeta regulates cisplatin cytotoxicity, mutagenicity, and the rate of development of cisplatin resistance. *Cancer Res* **64:** 8029-8035.

Wu HI, Brown JA, Dorie MJ, Lazzeroni L, Brown JM (2004b). Genome-wide identification of genes conferring resistance to the anticancer agents cisplatin, oxaliplatin, and mitomycin C. *Cancer Res* **64:** 3940-3948.

KIF18A DISPLAYS A SYNTHETIC LETHAL INTERACTION WITH ONCOGENIC RAS

Jeroen H. Nijwening*, Hendrik J. Kuiken*, Wouter van Zon, Erik Voets, Rob Wolthuis, Rene Bernards, Roderick L. Beijersbergen

The Netherlands Cancer Institute, Division of Molecular Carcinogenesis, Amsterdam, The Netherlands

* These authors contributed equally to this work

4

ABSTRACT

Members of the RAS family are frequently mutated in human cancers and tumors driven by oncogenic RAS are mostly aggressive and have a poor response to regular therapies. Although many cancers carrying oncogenic RAS display addiction to this oncogene, targeting RAS itself has met little clinical success. A complementary approach for therapeutically targeting oncogenic RAS is to identify genes that upon inhibition only result in decreased viability in the presence of oncogenic, but not wild type, RAS. Here we applied a high-throughput RNAi screening approach to identify synthetic lethal interactions with oncogenic RAS. We identified the Kinesin protein KIF18A to be synthetic lethal with H, K, and NRAS. Time-lapse imaging of mitotic events of KIF18A-depletion in oncogenic RAS cells shows an increase in mitotic duration, often paired with aberrant exit of mitosis or cell death. We suggest that KIF18A could be a potential drug target in cells driven by oncogenic RAS.

INTRODUCTION

Genes of the RAS family of small GTPases are among the most frequently mutated in human cancer; approximately a quarter of human tumors harbor activating point mutations in the *KRAS*, *NRAS* or *HRAS* oncogenes (Karnoub and Weinberg 2008). These mutations give rise to constitutive active RAS proteins contributing to several aspects of the malignant phenotype including an increase in proliferation rate, pro-survival signaling, invasiveness, and angiogenesis (Downward 2003, Roberts and Der 2007). Moreover, mutations in *KRAS* have been associated with resistance to radiation therapy (Cengel et al 2007), chemotherapy (De Roock et al 2010), and targeted therapeutics like the EGF-receptor inhibitors gefitinib and erlotinib (Massarelli et al 2007, Pao et al 2005, Van Cutsem et al 2009).

The importance of sustained oncogenic RAS signaling is clear from experiments that showed that repression of the *RAS* oncogene in mouse models or human tumor cell lines results in tumor regression or apoptosis, respectively (Chin et al 1999, Shirasawa et al 1993), a phenomenon shared by other oncogenes and defined as oncogene addiction (Weinstein 2002). These observations support the notion that RAS is an attractive target for cancer therapy, however targeting RAS proteins with small molecule inhibitors has proven a daunting task. Attempts have been made to selectively target RAS through inhibition of RAS farnesylation, a posttranslational modification involved in localization of RAS to the plasma membrane where RAS proteins transfer signals from membrane receptors. Preclinical studies with farnesyltransferase inhibitors (FTIs) in mouse models with oncogenic RAS-driven tumors showed potent tumor reduction (Kohl et al 1995). However, in patients with solid tumors, FTIs have failed to show objective responses (Adjei et al 2003, Agrawal and Somani 2009, Macdonald et al 2005). Alternative targeting approaches include targets downstream in the RAS effector pathways. Inhibition of RAF, MEK, and/or the PI3K/mTOR pathway could prove efficacious in treating oncogenic RAS-driven tumors, as shown, for example, by the tumor-regressive effect of combined application of MEK and PI3K inhibitors in a mouse model of oncogenic RAS-driven lung cancer (Engelman et al 2008).

In the search for therapeutic targets specific to oncogenic *RAS* driven cancers, expression studies and unbiased screening approaches have been explored. Tumors addicted to the expression of oncogenic *KRAS* display a distinct gene expression signature. It is conceivable that this signature includes genes whose expression is required for the survival of KRAS tumors. These could represent potential therapeutic targets in RAS mutant tumors (Singh and Settleman 2010). In addition, it is possible to identify dependencies associated with oncogenic RAS by high-throughput (un) biased RNAi screening approaches (Kaelin 2005, Kuiken and Beijersbergen 2010). Several synthetic lethal screens have led to the identification of genes that operate in downstream or parallel pathways of the RAS pathway. For example, it has been shown that oncogenic RAS associated dependencies include the kinases STK33 and TBK1 (Barbie et al 2009, Scholl et al 2009). It was suggested that these kinases deliver critical pro-survival signals in cancer cell lines driven by oncogenic RAS, STK33 indirectly

targets the pro-apoptotic protein BAD1 for destruction, whereas TBK1 activates the NF-κB driven survival signaling pathway. However, the reproducibility of the synthetic lethal effect observed with STK33 is controversial (Babij et al 2011). Another example of a synthetic lethal interaction with mutant RAS is SNAIL2, a zinc finger transcriptional repressor induced by active RAS and involved in epithelial-mesenchymal transition (EMT). SNAIL2 was found to be selectively required for the long-term survival of KRAS mutant cancer cells that have undergone epithelial-mesenchymal transition (Wang et al 2010). Furthermore, others found that cells expressing oncogenic *RAS* display an increased dependency on the anaphase-promoting complex / cyclosome (APC/C), and the proteasome (Luo et al 2009).

Different screening models have been used for the identification of synthetic lethal interactions with *RAS* including panels of tumor cell lines and pairs of isogenic (tumor) cell lines, differing in the RAS mutation status (Kuiken and Beijersbergen 2010). Although the use of a panel of tumor cell lines allows for the identification of dependencies in a broader context, a small size panel does not provide evidence that the dependency is associated with the mutation of interest, because of possible confounding mutations. In pairs of isogenic cell lines, the association between the mutation status and dependency can be more reliable, but might yield context dependent addictions (e.g. cell type dependency) that are of limited clinical interest. Isogenic cell line models come in two flavors; those generated by introducing a mutation in a cell line wild type for the gene of interest, or by reversal of a mutation of interest in a mutant cell line. As cells can become dependent on expression of oncogenic RAS, they can be applied in screening efforts to identify pathways that these oncogene-addicted cells need to maintain their oncogenic state (Solimini et al 2007)

Here we describe the results of a synthetic lethal screen for the identification of dependencies associated with the expression of oncogenic RAS in a screening model of genetically engineered fibroblasts that are isogenic in their expression of constitutive active RAS. We have identified a number of synthetic lethal interactions. Knockdown of one of those, Kinesin-like protein KIF18A, causes a delay in mitotic progression and increased cell death in cells expressing oncogenic HRAS.

RESULTS

A genome-wide siRNA screen for synthetic lethal interactions with oncogenic HRAS

We generated oncogenic RAS isogenic cell lines by introducing the $HRAS^{V12}$ mutant in primary human fibroblasts (BJs). First we introduced human telomerase (hTERT) rendering these primary cells immortal allowing for continuous propagation. Second, we introduced a *Tp53* shRNA construct resulting in stable knockdown of *Tp53* preventing oncogene induced senescence upon expression of the $H\text{-}RAS^{V12}$ oncogene (Voorhoeve and Agami 2003). Finally, we introduced an $HRAS^{V12}$ expression construct resulting in the isogenic cell line pair: BJETp53kd and BJETp53kd-$HRAS^{V12}$. To confirm

that these cells have constitutive active RAS signaling as a result of the (over)expression of the *HRAS^{V12}* oncogene, we serum starved the isogenic cell line for 48h and assessed HRAS and phosphor-ERK (pERK) expression by Western blot analysis (Fig. 1). Despite the serum starvation the BJETp53kd-HRAS^{V12} cell line maintained downstream ERK activation, unlike its isogenic counterpart (Fig. 1A). In addition, to test whether the BJETp53kd-HRAS^{V12} cell line became addicted to oncogenic RAS we knocked down *HRAS* and measured the effect on cell viability after 96 hours. We detected a decrease in cell number in the BJETp53kd-HRAS^{V12} cell line upon knockdown of *HRAS* with siRNAs (Fig. 1B).

To identify dependencies associated with expression of oncogenic *HRAS*, we screened a siRNA library consisting of pools of 4 unique siRNAs (smartpools) targeting a single gene, encompassing 6801 genes in total. The isogenic cell lines BJETp53kd and BJETp53kd-HRAS^{V12} were transfected with these siRNAs pools in a 384-well format and left to propagate for 96 hours (Fig. 1C). To determine the effect of the siRNA smartpools on cell viability, cells were fixed, DNA content was stained with DAPI, representative images were acquired using an automated microscope, and CellProfiler Software (Carpenter et al 2006, Kamentsky et al 2011) was used to quantify the number of nuclei per image as previously described (Nijwening et al 2011). Nuclei counts were normalized to the sample median per plate and three technical replicates were summarized by median +/- standard deviation (Fig. 1D). Importantly, non-targeting siRNAs (applied as negative controls) might affect biological processes through off-target effects and therefore remain to be validated in the context of a large number of siRNAs to be justifiably called as a negative control and used in subsequent experiments. In our screen, the negative control, a non-targeting siRNA smartpool (siScr) showed similar counts compared to the median of all samples, a normalized nuclei count score of approximately 1, which is in agreement with the assumption that the majority, and therefore the median, of all samples represents the true H_0-hyphothesis (Fig. 1D). As positive controls, siRNA smartpools targeting *PLK1* or *KIF11*, both genes are essential for cell division (reviewed by (Lens et al 2010)), were used, both showing a strong reduction in cell number in both cell lines (Fig. 1D). The selection criteria of siRNA smartpools for follow-up are represented by a median normalized nuclei count score of 0.75 – 1.50 in BJETp53kd, < 0.75 in BJETp53kd-HRAS^{V12}, and a relative reduction in the normalized nuclei count score of > 50% for BJETp53kd-HRAS^{V12} *versus* BJETp53kd (Fig. 1E). Among the 98 selected genes for follow up is *HRAS* itself. This is not surprising as expression of oncogenic *HRAS* led to the addiction on its introduction in the BJETp53kd cell line (Figure 1B).

To determine the reproducibility of the selected siRNA smartpools, they were re-screened as three independent biological replicates each performed in triplicate. Out of the 98 synthetic lethal candidates tested, 14 siRNA smartpools met the aforementioned criteria, now with a less stringent > 30% cut-off for relative reduction in cell number (Fig. 2A). Because individual siRNAs can be selected based on their off-target effects (Jackson and Linsley 2010, Sigoillot and King 2011), we tested the individual siRNAs

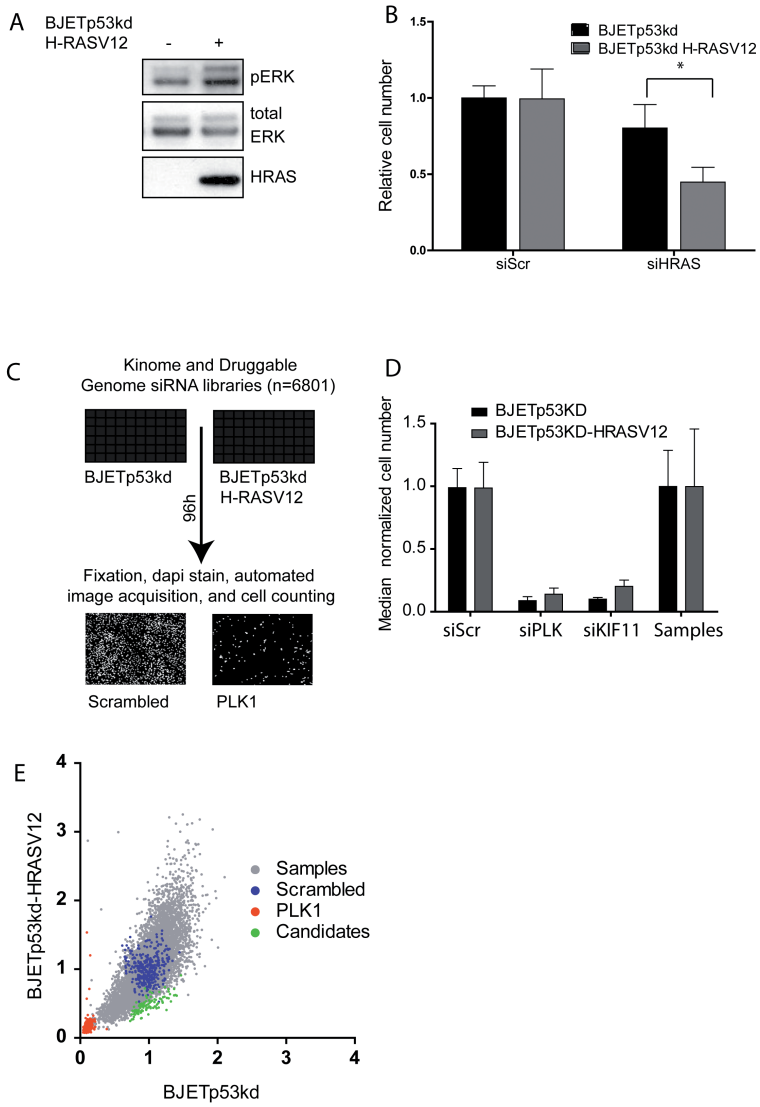


Figure 1. An RNAi synthetic lethal screen against oncogenic HRAS. (A) Western blot analysis of HRAS expression and pERK levels in the isogenic BJ cell line pair. (B) Relative and normalized cell number as determined by DAPI-stained nuclei count in the RAS isogenic BJ cell line pair transfected with scrambled non-targeting siRNAs (siScr) or siRNAs against *HRAS*. (* $p<0.05$) (C) Schematic representation of the screen, see text for details. (D) Medians of normalized cell number within wells transfected with siRNA pools directed against the positive controls: *PLK1* (siPLK) and *KIF11* (siKIF11), negative (non-targeting) controls (siScr), and all genes comprising the kinome and druggable genome (Samples). Error bars denote standard deviations of the screen performed in triplicate. (E) Screen results represented as a scatter plot. Each dots represents the effect on normalized and relative cell viability of a single pool of siRNAs targeting one target in the isogenic cell line pair. Grey dots represent pools of siRNAs, blue dots are the negative controls, red dots the positive controls and green dots are the selected synthetic lethal candidates.

constituting the siRNA smartpools in parallel with the siRNA smartpools. Genes for which 2 or more individual siRNAs met the selection criteria for differential killing were regarded as validated synthetic lethal hits with fibroblasts expressing oncogenic *HRAS*. In total 7 genes, including *HRAS*, were selected based on these two validation rounds (Table 1).

KIF18A has a synthetic lethal interaction with oncogenic RAS

Among the three *RAS* family members *KRAS* is the most frequently mutated in human cancer. In contrast, only a specific subset of human tumors harbor mutations in the *HRAS* or *NRAS* genes, for example in melanoma and bladder cancer (Downward 2003). We determined whether the identified dependencies were *HRAS* specific or shared among the *RAS* family members. In order to test this we generated isogenic cell lines, BJETp53kd-KRAS^{V12} and BJETp53kd-NRAS^{K61}, expressing oncogenic *KRAS^{V12}* or *NRAS^{K61}*, respectively. Out of the 7 genes for which an increased dependency is observed in cells expressing oncogenic *HRAS*, knockdown of some of these genes also impaired proliferation in cells expressing either oncogenic *KRAS* or *NRAS* (Table 2). Why the synthetic lethal interaction with HRAS^{V12} in some instances did not extend to KRAS^{V12} or NRAS^{K61} remains to be investigated. Among the genes that are synthetic lethal with all RAS family members is Kinesin-like protein KIF18A, a protein involved in chromosome segregation during mitosis (Mayr et al 2007) (Fig. 3A). Previous work looking for synthetic lethal interactions with *RAS* led to the identification of genes involved in mitotic progression such as *PLK1* and members of the APC/C, *ANAPC1* and *ANAPC4* (Luo et al 2009) suggesting an increased dependency on processes involved in mitosis associated with the expression of oncogenic *RAS*. The identification of *KIF18A* in our screen would fit with this model and we decided to investigate the mechanism behind the observed RAS associated dependency.

First, to confirm that the siRNAs used indeed target *KIF18A* we measured expression with RT-qPCR. As expected, transfection of the individual siRNAs

Table 1. List of candidate synthetic lethal genes with HRASV12 that with two or more individual siRNAs gave a reproducible phenotype of at least 30% increased killing in the RAS mutant cell line in three independent technical triplicates ($p<0.05$, Paired t-test).

Gene	Accession number	Description
HRAS	NM_005343	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
KIF18A	NM_031217	kinesin family member 18A
P2RX5	NM_002561	purinergic receptor P2X, ligand-gated ion channel, 5
RAI1	NM_030665	retinoic acid induced 1
STK39	NM_013233	serine threonine kinase 39 (STE20/SPS1 homolog, yeast)
TNFRSF17	NM_001192	tumor necrosis factor receptor superfamily, member 17
YWHAE	NM_006761	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein

Table 2. Candidate synthetic lethal genes with $HRAS^{V12}$ display differential synthetic lethality with $KRAS^{V12}$ or $NRAS^{K61}$. The synthetic lethal interaction of the Smartpool (Pool) or the individual siRNAs with $HRAS^{V12}$ (H), $KRAS^{V12}$ (K), or $NRAS^{K61}$ (N) is indicated with the corresponding letter. Shown is the result of three independent technical triplicates where the difference in mean viability was larger than 30% ($p<0.05$, Paired *t*-test).

Gene	Pool	siRNA #1	siRNA #2	siRNA #3	siRNA #4
HRAS	H / N	H		H	
KIF18A	H / K / N	H / K / N	H / K / N	H / N	H / K / N
P2RX5	H / N		H / N		H / N
RAI1	H / N		H / N	H	N
STK39	H / N	H / K / N	H / K / N	H / N	
TNFRSF17	H / K / N	H / K / N	N	H / N	
YWAHE	H	H / K / N		H / N	H / K

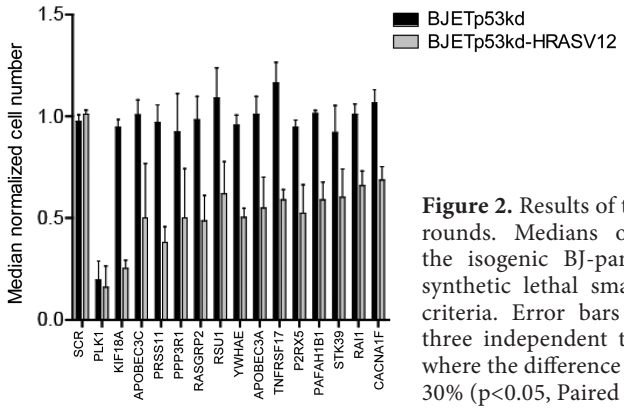


Figure 2. Results of the validation and deconvolution rounds. Medians of normalized cell number of the isogenic BJ-panel re-screened with candidate synthetic lethal smartpools that past the selection criteria. Error bars denote standard deviations of three independent technical triplicates. Only genes where the difference in mean viability was larger than 30% ($p<0.05$, Paired *t*-test) are shown.

targeting *KIF18A* results in a strong decrease of *KIF18A* mRNA levels compared to the non-targeting siRNA control (Fig. 3B). In an independent experiment we tested knockdown efficiency on protein level by Western blot analysis, and in agreement with the reduction in mRNA expression, all four individual siRNAs caused depletion of KIF18A protein expression (Fig. 3C). These data also show that there is no differential expression of *KIF18A* mRNA and protein expression between the cell line expressing oncogenic *HRAS* compared to its wild type counterpart. This result makes it unlikely that the observed synthetic lethal interaction is due to differential expression of a dosage dependent essential gene. The observed synthetic lethal phenotype could be due to cell cycle specific expression of KIF18A. To confirm that the protein expression of KIF18A is comparable in the isogenic cell line pair during mitosis, we performed Western blot analysis for KIF18A protein levels of cells that are arrested in mitosis. The BJETp53kd and BJETp53kd-$HRAS^{V12}$ cell lines were treated overnight with either

nocodazole, taxol, or left untreated. Next the mitotic fraction, cells arrested in mitosis by nocodazole or taxol treatment, or the asynchronous cells were used for analysis of protein expression. Western blot analysis of these samples showed that KIF18A protein levels are higher in the mitotic fraction compared to the asynchronous populations, and that, in line with the RT-qPCR and WB data, KIF18A levels are comparable between cell line expressing oncogenic *HRAS* compared to its wild type counterpart (Fig. 4A). Together these results support the conclusion that the synthetic lethal interaction is not due to differences in expression levels or timing or KIF18A.

Knockdown of KIF18A induces a mitotic arrest

It has been described that KIF18A functions in mitotic progression (Stumpff et al 2008). Therefore, we determined the cell cycle profiles of our isogenic cell lines, with or without silencing of *KIF18A*. FACS analysis of propidium iodide (PI) stained cells showed that knockdown of *KIF18A* results in a G2/M arrest independent of the *RAS* mutation status (Fig. 4B). Furthermore, visual inspection showed that, irrespective of

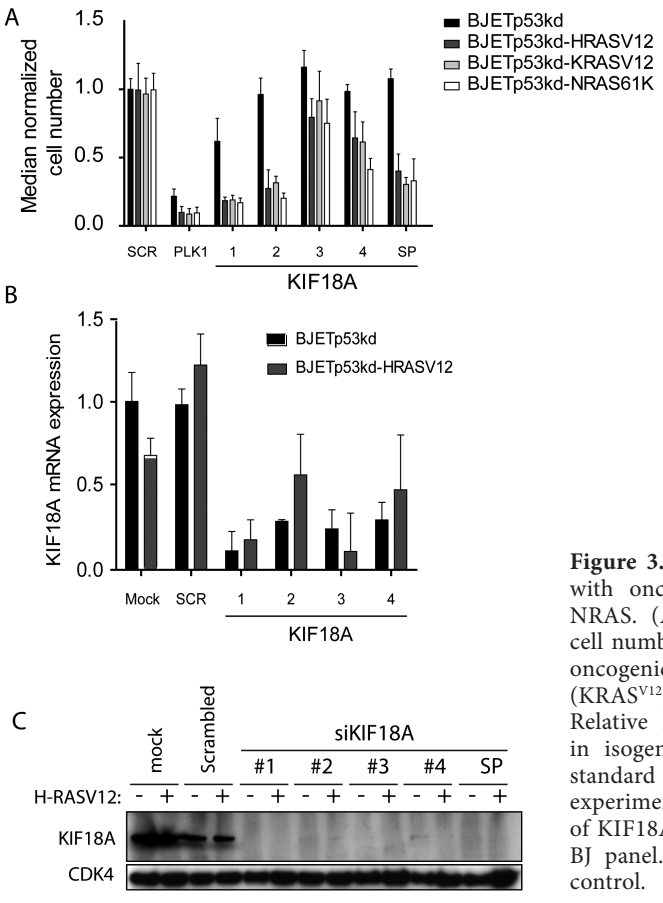


Figure 3. KIF18A is synthetic lethal with oncogenic HRAS, KRAS and NRAS. (A) Medians of normalized cell number of BJ panels isogenic for oncogenic HRAS ($HRAS^{V12}$), KRAS ($KRAS^{V12}$), and NRAS ($NRAS^{S61K}$). (B) Relative *KIF18A* mRNA expression in isogenic BJs. Error bars denote standard deviations of a triplicate experiment. (C) Western blot analysis of KIF18A expression in the isogenic BJ panel. CDK4 is used as loading control.

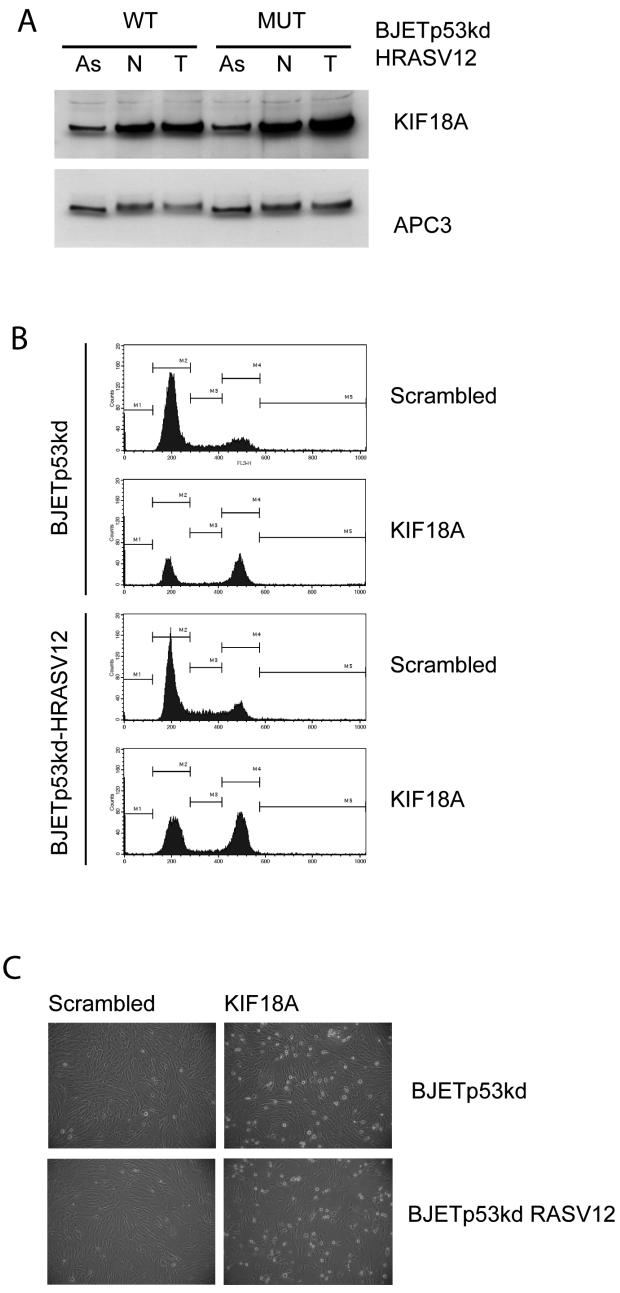


Figure 4. Knockdown of KIF18A results in a mitotic arrest. (A) Western blot analysis of KIF18A and APC3 expression in cell fractions of asynchronously growing cells arrested with nocodazole, or arrested with taxol. (B) Cell cycle profiles as determined by flow cytometry. Samples were harvested 48 hours after siRNA transfection. Peaks corresponding to G1 and G2/M are indicated. (C) Brightfield images of HRAS wild-type (WT) or HRAS mutant (MUT) cells 48 hours after transfection of non-targeting siRNAs (Scrambled), or siRNAs against *KIF18A* (KIF18A).

the *RAS* mutation status, cell populations transfected with *KIF18A* siRNAs display an increased fraction of cells with a rounded up morphology, a phenotype associated with mitosis (Fig. 4C). Together these findings suggest that *KIF18A* knockdown results in a mitotic arrest. However, as both wild-type and RAS mutant cells undergo the mitotic arrest, this observation does not explain the synthetic lethal effect of *KIF18A* depletion in cells expressing constitutive active *RAS*.

Mutant RAS cells are more susceptible to a mitotic arrest after KIF18A knockdown, resulting in cell death

To further explore the observed mitotic arrest, we analyzed the isogenic cell line pair, with or without *KIF18A* knockdown, by live cell imaging using phase contrast time lapse microscopy. For every cell that underwent mitosis, the duration of the time spent in mitosis as well as its fate was recorded. From this analysis we could define 2 major phenotypes: "death in mitosis" and "failure of mitosis". Cells that clearly died in mitosis (all after a pronounced mitotic delay, >300minutes) were grouped as "death in mitosis". For the other group of cells it was unclear whether they died in mitosis or aberrantly left mitosis into the next G1-phase after a pronounced mitotic delay. These cells were grouped as "failure of mitosis". RAS mutant cells transfected with a non-targeting siRNA showed an increased duration of mitosis over its wild-type counterparts, 78 minutes vs. 38 minutes, respectively (Fig. 5A). This prolongation of mitosis in oncogenic RAS cells could be explained by the elevated mitotic stress these cells experience (Luo et al 2009). In addition, *KIF18A* knockdown results in a mitotic

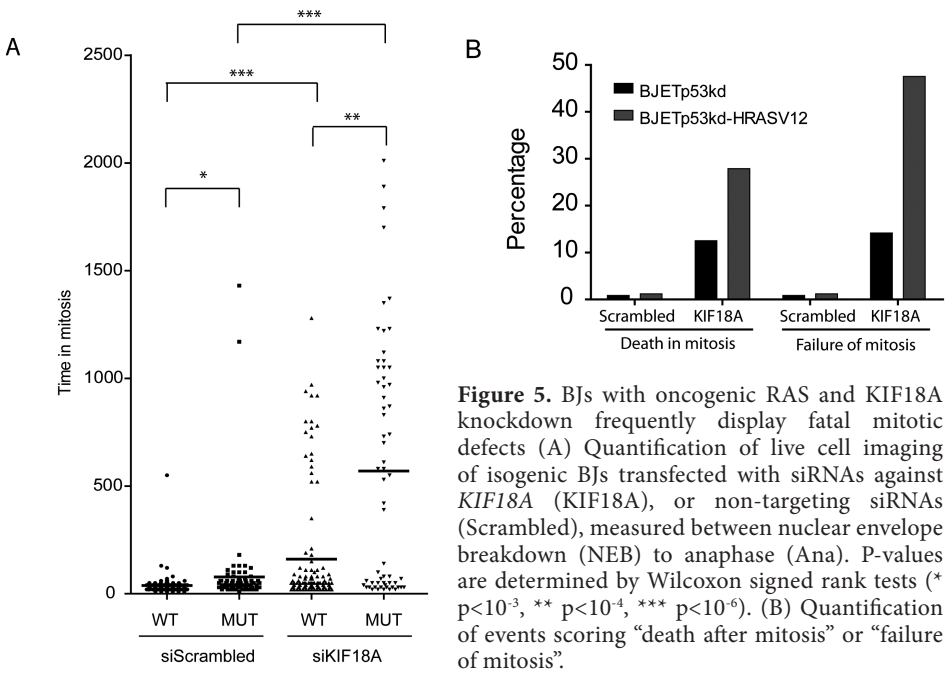


Figure 5. BJs with oncogenic RAS and KIF18A knockdown frequently display fatal mitotic defects (A) Quantification of live cell imaging of isogenic BJs transfected with siRNAs against *KIF18A* (KIF18A), or non-targeting siRNAs (Scrambled), measured between nuclear envelope breakdown (NEB) to anaphase (Ana). P-values are determined by Wilcoxon signed rank tests (* $p<10^{-3}$, ** $p<10^{-4}$, *** $p<10^{-6}$). (B) Quantification of events scoring "death after mitosis" or "failure of mitosis".

arrest in a substantial fraction of BJs with wild type RAS and mutant RAS, where the fraction of arrest in mitosis (time > 100 minutes) is larger in the mutant RAS (58%), compared to the wild type RAS (24%) (Fig. 5A). Upon *KIF18A* knockdown, the duration of the mitotic arrest increases to an average of 161 minutes for the wild type RAS cells and an average of 569 minutes for mutant RAS cells when measured over the whole population (Fig. 5A). The increased duration of the mitotic arrest is mostly correlated with death in mitosis, or failure of mitosis (Fig. 5B). Transfection of *KIF18A* siRNAs increases these events over transfection of Scrambled siRNAs in both cell lines. In addition, knockdown of KIF18A increases the percentages of death in mitosis, or failure of mitosis, in BJs with mutant RAS (Fig. 5B). Cells that are arrested in mitosis for over 100 minutes have a ~50% chance of undergoing cell death, irrespective of RAS status or *KIF18A* knockdown (data not shown); implicating that mitotic arrest above a certain threshold in BJs leads to cell death. Combined, these results show that BJs with oncogenic RAS and KIF18A knockdown display an increased duration of mitosis and that mitosis in these cells is often incomplete or results in cell death, which could explain the increased dependency on *KIF18A* observed in cells expressing oncogenic RAS. Importantly, these results also demonstrate that the increased dependency on *KIF18A* in cells expressing oncogenic RAS is not due to differences in intrinsic proliferation rates between the isogenic cell lines.

DISCUSSION

Using a single-well siRNA screen in genetically engineered human fibroblasts that are isogenic for the expression of oncogenic HRAS, we identified 15 candidate genes that are synthetic lethal with constitutive active RAS. Of these, 9 genes show a synthetic lethal interaction between oncogenic H, K, or NRAS in BJ fibroblasts. We observe that in cells with *KIF18A* knockdown the duration of mitosis is significantly prolonged, in particular in cells that express an oncogenic mutant of *HRAS*. Moreover, the RAS mutant cells depleted for *KIF18A* display impaired mitosis and increased cell death after mitosis, possibly explaining the observed synthetic lethal phenotype.

KIF18A is a member of the kinesin-8 family of molecular motors associated to microtubules (also known as microtubule-associated proteins or MAPs). Microtubules are dynamic cytoskeletal filaments that have a key function in cell division and intracellular organization (Nogales 2000). Microtubules coordinate chromosome congression during metaphase by growing and shortening before onset into anaphase is induced, a process tightly monitored by proteins of the spindle assembly checkpoint (reviewed in (Walczak et al 2010)). Kinesin-8 members have been implicated in chromosome congression, based on the observation that motor deletion mutants in yeast and drosophila cause unusually long microtubules (Gatt et al 2005, Gupta et al 2006, Varga et al 2006). A similar phenotype has been observed in mammalian cells depleted for *KIF18A* (Mayr et al 2007, Stumpff et al 2008). KIF18A differs from other MAPs because of its dual function, it is both a plus end-directed motor and a plus

end-specific depolymerase (Mayr et al 2007). This dual function suggests that KIF18A moves to the poles of microtubules and depolymerizes them in a concentration-dependent manner, hereby dampening the amplitude of chromosome oscillations during metaphase (Stumpff et al 2008).

It has been shown that *KIF18A* knockdown with siRNAs in HeLa cells caused a mitotic arrest due to unaligned chromosomes (Huang et al 2009). The authors stated (but did not show) that these HeLa cells eventually underwent mitotic catastrophe, similar to our observations. Moreover, they showed that knockdown of *KIF18A* reduced both centromere protein E (CENP-E) protein expression and its localization to kinetochores. Overexpression of the c-terminal tail domain of CENP-E, however, partially restored the chromosomal congression effects caused by depleted KIF18A (Huang et al 2009). In line with these observations, previous work shows that inhibition or knockdown of *CENPE* (coding for CENP-E) causes a mitotic arrest due to defects in chromosome alignment at the metaphase plate similar to depletion of *KIF18A* (Putkey et al 2002, Schaar et al 1997). However, we did not identify *CENPE* in our screen. Though, this could be explained by insufficient knockdown or insufficient CENP-E protein degradation after knockdown of *CENPE*.

It has been described that breast cancer cells show increased levels of *KIF18A* expression (Zhang et al 2010). Depletion of *KIF18A* with siRNAs in MDA-MB-231 cells (that harbor a $KRAS^{G12D}$ mutant) inhibits proliferation *in vitro* and in a mouse xenograft (Zhang et al 2010). In addition, increased expression of *KIF18A* has been observed in colorectal cancer (CRC) (Nagahara et al 2011). Here, the authors assessed expression of *KIF18A* mRNA in tumors of patients with CRC and found that high *KIF18A* expression was associated with a poorer overall survival rate than patients with low *KIF18A* expression (Nagahara et al 2011). However, it remains to be investigated whether the correlation between low expression of *KIF18A* and good prognosis was a result of the cycling status of the tumor cells. In addition, it will be interesting to determine the prevalence of RAS mutations in these CRC samples to confirm the hypothesis that RAS-driven tumors with low *KIF18A* expression have a reduced proliferative capacity.

An explanation for the deleterious effect of depleted KIF18A on cancer cells could be that *KIF18A* knockdown causes a prolonged activation of the MAD2-dependent spindle assembly checkpoint (Mayr et al 2007). MAD2 is a critical component of the spindle assembly checkpoint by inhibiting the activity of the APC/C when chromosomes are not properly aligned, thereby blocking onset into anaphase (Walczak et al 2010). Benezra and colleagues have demonstrated that tumor cells driven by oncogenic RAS depend on *Mad2l1* (coding for Mad2) upregulation, by showing that growth of allograft fibrosarcomas derived from $HRas^{V12}$ transformed MEFs that are deficient of all three pocket proteins (TKO MEFs) is delayed by knockdown of *Mad2* to endogenous levels (Schvartzman et al 2011). This observation is in line with a previous study from the same lab where they showed that tumor reduction by oncogene withdrawal is reversed by *Mad2* overexpression (Sotillo et al 2010). The observed synthetic lethal phenotype could possibly be explained by increased expression of MAD2 upon expression of

HRAS^{V12}, leading to delayed mitotic progression, which is fatal to cells when mitosis is even further delayed by depletion of *KIF18A*.

Several other attempts to identify synthetic lethal interactions with oncogenic RAS have been performed in the isogenic DLD-1 cell line pair where the mutant *KRAS* allele was deleted by targeted homologous integration (Shirasawa et al 1993). Interestingly, most of the candidate synthetic lethal interactors identified in these cells function in the mitotic pathway (Luo et al 2009, Morgan-Lappe et al 2007, Sarthy et al 2007). By using a hairpin library targeting more than 32000 human transcripts, Elledge and colleagues identified many synthetic lethal interactors that function in mitotic progression including components of the APC/C, PLK1 and the proteasome (Luo et al 2009). These hits were validated in additional isogenic cell lines, a xenograft model and a panel of NSCLC cell lines. However, most of these genes are also required for survival of normal cells and therefore the synthetic lethal effects observed can be considered more as dosage dependent effects rather than genotype specific lethality. Although KIF18A was selected as a synthetic lethal hit with oncogenic RAS, we also observe a reduction in cell number in normal cells upon very efficient knockdown of KIF18A. However, this is by far not comparable to the effects seen with PLK1 (Fig. 3A). In mice studies, it has been shown that *Kif18a* knockout mice are viable and male mice, but not female mice, show infertility due to severe testis atrophy (Liu et al 2010). This data suggests that *KIF18A* is not an essential gene and that KIF18A could be further explored as a drug target in oncogenic RAS driven tumors.

Here, we describe a novel and possible potent synthetic lethal interaction with oncogenic RAS-driven BJ fibroblasts. Future research should confirm this synthetic lethal interaction in a broader and context-independent perspective. In addition, the data described here suggest that prolongation of mitosis can be a promising strategy to kill cancer cells with oncogenic RAS.

METHODS

Generation of cell lines, cell culture, and siRNA transfection

BJ primary human fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum. To generate the RAS isogenic cell line pair used in the screen BJ cells were infected with lenti virus containing the pBabe-EcoR-Zeo construct resulting in stable expression of the ecotropic receptor. Subsequent retroviral transductions with pBABE-hTERT-Hygro, pRS-p53, and pBABE-HRAS^{V12}-Puro gave rise to the final RAS isogenic cell line pair, BJETp53KD and BJETp53KD-HRASV12.

BJ cells were transfected using a reverse transfection protocol and the Dharmafect 3 (Thermo Scientific) or RNAiMAX (Invitrogen) transfection agents. Medium was refreshed 24 hours after transfection and cells were left to propagate for given time periods prior to analyses. Controls for siRNA experiments included a non-targeting siRNA smartpool (Scrambled) as negative control, and siRNA smartpools targeting PLK1 or KIF11 as positive controls.

Screen

The Kinome and Druggable Genome siRNA libraries (Thermo Scientific) were aliquoted in µClear 384 well plates (BD Biosciences). Each well contained 5 µl siRNA smartpool encompassing 4 unique siRNAs targeting a single gene at a totel concentration of 500 nM. The RAS isogenic cell line pair was transfected using a reverse transfection protocol. 24 hours after transfection medium was refreshed and the cells were left to propagate for an additional 72 hours prior to overnight fixation with 3.7% formaldehyde PBS buffer. Cells were washed with PBS, permeabilized with 0.2% Triton-X PBS buffer, and DNA was stained with 0.5µg/mL DAPI for 10 minutes. Next, cells were washed with PBS and plates were sealed and kept in dark and at 4°C till analysis. For every cell line the screen included three technical replicates.

Nuclei count assay

Representative images, a 2x2 montage, were acquired from wells containing fixed, DAPI stained cells, at 10x with the BD Pathway 855 High Content Imager (BD Biosciences) and analysed by Cell Profiler image analysis software (Carpenter et al 2006, Jones et al 2008). The DAPI channel was used for nuclei segmentation allowing for counting of the number of nuclei in the representative areas.

Data analysis, hit selection, and validation

For the screen and subsequent validation experiments, the nuclei counts were normalized to the median of nuclei counts of all the samples per plate or the median of the nuclei counts of the negative control wells respectively. *P*-values were computed from unpaired two-tailed *t*-tests. Candidates of interest were selected from the screen by the following these selection criteria: a median normalized nuclei count between 0.75 and 1.5 for the reference cell line, BJETp53kd, a median normalized nuclei count below 0.75 for the BJETp53kd-HRAS^{V12} cell line, and a relative decrease of nuclei count with 30% in BJETp53kd-HRAS^{V12} *versus* BJETp53kd. Candidate siRNA smartpools were subjected to deconvolution when they met the selection criteria in 2 out of the three siRNA smartpool validation experiments. Finally, only those genes for which 2 or more independent siRNAs reproducibly mediated a significant phenotype ($P<0.05$ were considered as validated synthetic lethal hits.

RT-qPCR

RNA was isolated using either Trizol (Invitrogen) or RNAeasy mini kit (Qiagen). Subsequently, 1 µg RNA was used for generating cDNA with the SuperScript II kit (Invitrogen). Using qPCR primers listed below, and the FastStart MasterPLUS SYBR Green kit (Roche), relative mRNA levels for genes of interest were measured. The relative levels were normalized to that of RPL13 or RPL4 control genes for every sample. mRNA levels were determined in three independent experiments.

Antibodies and Immunoblotting

Antibodies used in this study were anti-p21 (C-19), anti-p53 (DO-1), anti-HRAS (F235), and anti-CDK4 (C-22) from Santa Cruz Biotechnology, and anti-KIF18A (ab72417) from Abcam anti-ERK from Santa Cruz Biotechnology and pERK (#3179) from Cell Signaling Technology. Western blots were performed using whole cell extracts, separated on 8-10% SDS-PAGE gels or pre-cast gradient gels (Invitrogen) and transferred to polyvinylidine difluoride membranes or nitro cellulose membranes (Millipore). Western blots were probed with the indicated antibodies.

Filming of mitotic events

Cells were reverse transfected with siRNAs (50 nM) in a 12-well format and medium was replaced after 24 hours. Culture plates were transferred to a temperature and CO2 controlled chamber (5% CO2 at 37C) on a microscope (Axiovert 200M; Carl Zeiss, Inc.) equippted with an NA 0.55 condenser and a 40x NA 1.3 Plan Neo differential interference contrast objective using a charge-coupled device camera (CoolSNAP HQ; Photometrics). Images were taken after 100-ms exposure time and quantified and processed using MetaMorph software (Universal Imaging) and Excel (Microsoft). The acquired images were used to track cells entering mitosis, as observed by nuclear envelope breakdown (NEB) and the time spend in mitosis was measured till they entered anaphase (Ana), showed failure to complete mitosis correctly, or underwent cell death.

ACKNOWLEDGEMENTS

The authors would like to thank Mathijs Voorhoeve and Janneke Ogink for technical assistance and members of the Beijersbergen and Bernards labs for useful discussions. This work has been supported by Top Institute Pharma (project T3-105).

REFERENCES

Adjei AA, Mauer A, Bruzek L, Marks RS, Hillman S, Geyer S *et al* **(2003).** Phase II study of the farnesyl transferase inhibitor R115777 in patients with advanced non-small-cell lung cancer. *J Clin Oncol* **21:** 1760-1766.

Agrawal AG, Somani RR (2009). Farnesyltransferase inhibitor as anticancer agent. *Mini Rev Med Chem* **9:** 638-652.

Babij C, Zhang Y, Kurzeja RJ, Munzli A, Shehabeldin A, Fernando M *et al* **(2011).** STK33 Kinase Activity Is Nonessential in KRAS-Dependent Cancer Cells. *Cancer Res* **71:** 5818-5826.

Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF *et al* **(2009).** Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature* **462:** 108-112.

Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O *et al* **(2006).** CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol* **7:** R100.

Cengel KA, Voong KR, Chandrasekaran S, Maggiorella L, Brunner TB, Stanbridge E *et al* **(2007).** Oncogenic K-Ras signals through epidermal growth factor receptor and wild-type H-Ras to promote radiation survival in pancreatic and colorectal carcinoma cells. *Neoplasia* **9:** 341-348.

Chin L, Tam A, Pomerantz J, Wong M, Holash J, Bardeesy N *et al* (1999). Essential role for oncogenic Ras in tumour maintenance. *Nature* **400:** 468-472.

De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G *et al* (2010). Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol* **11:** 753-762.

Downward J (2003). Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer* **3:** 11-22.

Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, Upadhyay R *et al* (2008). Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. *Nat Med* **14:** 1351-1356.

Gatt MK, Savoian MS, Riparbelli MG, Massarelli C, Callaini G, Glover DM (2005). Klp67A destabilises pre-anaphase microtubules but subsequently is required to stabilise the central spindle. *J Cell Sci* **118:** 2671-2682.

Gupta ML, Jr., Carvalho P, Roof DM, Pellman D (2006). Plus end-specific depolymerase activity of Kip3, a kinesin-8 protein, explains its role in positioning the yeast mitotic spindle. *Nat Cell Biol* **8:** 913-923.

Huang Y, Yao Y, Xu HZ, Wang ZG, Lu L, Dai W (2009). Defects in chromosome congression and mitotic progression in KIF18A-deficient cells are partly mediated through impaired functions of CENP-E. *Cell Cycle* **8:** 2643-2649.

Jackson AL, Linsley PS (2010). Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nat Rev Drug Discov* **9:** 57-67.

Jones TR, Kang IH, Wheeler DB, Lindquist RA, Papallo A, Sabatini DM *et al* (2008). CellProfiler Analyst: data exploration and analysis software for complex image-based screens. *BMC Bioinformatics* **9:** 482.

Kaelin WG, Jr. (2005). The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer* **5:** 689-698.

Kamentsky L, Jones TR, Fraser A, Bray MA, Logan DJ, Madden KL *et al* (2011). Improved structure, function and compatibility for CellProfiler: modular high-throughput image analysis software. *Bioinformatics* **27:** 1179-1180.

Karnoub AE, Weinberg RA (2008). Ras oncogenes: split personalities. *Nat Rev Mol Cell Biol* **9:** 517-531.

Kohl NE, Omer CA, Conner MW, Anthony NJ, Davide JP, deSolms SJ *et al* (1995). Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. *Nat Med* **1:** 792-797.

Kuiken HJ, Beijersbergen RL (2010). Exploration of synthetic lethal interactions as cancer drug targets. *Future Oncol* **6:** 1789-1802.

Lens SM, Voest EE, Medema RH (2010). Shared and separate functions of polo-like kinases and aurora kinases in cancer. *Nat Rev Cancer* **10:** 825-841.

Liu XS, Zhao XD, Wang X, Yao YX, Zhang LL, Shu RZ *et al* (2010). Germinal Cell Aplasia in Kif18a Mutant Male Mice Due to Impaired Chromosome Congression and Dysregulated BubR1 and CENP-E. *Genes Cancer* **1:** 26-39.

Luo J, Emanuele MJ, Li D, Creighton CJ, Schlabach MR, Westbrook TF *et al* (2009). A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. *Cell* **137:** 835-848.

Macdonald JS, McCoy S, Whitehead RP, Iqbal S, Wade JL, 3rd, Giguere JK *et al* (2005). A phase II study of farnesyl transferase inhibitor R115777 in pancreatic cancer: a Southwest oncology group (SWOG 9924) study. *Invest New Drugs* **23:** 485-487.

Massarelli E, Varella-Garcia M, Tang X, Xavier AC, Ozburn NC, Liu DD *et al* (2007). KRAS mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. *Clin Cancer Res* **13:** 2890-2896.

Mayr MI, Hummer S, Bormann J, Gruner T, Adio S, Woehlke G *et al* (2007). The human kinesin Kif18A is a motile microtubule depolymerase essential for chromosome congression. *Curr Biol* **17:** 488-498.

Morgan-Lappe SE, Tucker LA, Huang X, Zhang Q, Sarthy AV, Zakula D *et al* (2007). Identification of Ras-related nuclear protein, targeting protein for xenopus kinesin-like protein 2, and stearoyl-CoA desaturase 1 as promising cancer targets from an RNAi-based screen. *Cancer Res* **67:** 4390-4398.

Nagahara M, Nishida N, Iwatsuki M, Ishimaru S, Mimori K, Tanaka F *et al* (2011). Kinesin 18A expression: Clinical relevance to colorectal cancer progression. *Int J Cancer*.

Nijwening JH, Kuiken HJ, Beijersbergen RL (2011). Screening for modulators of cisplatin sensitivity: unbiased screens reveal common themes. *Cell Cycle* **10:** 380-386.

Nogales E (2000). Structural insights into microtubule function. *Annu Rev Biochem* **69:** 277-302.

Pao W, Wang TY, Riely GJ, Miller VA, Pan Q, Ladanyi M *et al* (2005). KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med* **2:** e17.

Putkey FR, Cramer T, Morphew MK, Silk AD, Johnson RS, McIntosh JR *et al* (2002). Unstable kinetochore-microtubule capture and chromosomal instability following deletion of CENP-E. *Dev Cell* **3:** 351-365.

Roberts PJ, Der CJ (2007). Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* **26:** 3291-3310.

Sarthy AV, Morgan-Lappe SE, Zakula D, Vernetti L, Schurdak M, Packer JC *et al* (2007). Survivin depletion preferentially reduces the survival of activated K-Ras-transformed cells. *Mol Cancer Ther* **6:** 269-276.

Schaar BT, Chan GK, Maddox P, Salmon ED, Yen TJ (1997). CENP-E function at kinetochores is essential for chromosome alignment. *J Cell Biol* **139:** 1373-1382.

Scholl C, Frohling S, Dunn IF, Schinzel AC, Barbie DA, Kim SY *et al* (2009). Synthetic lethal interaction between oncogenic KRAS dependency and STK33 suppression in human cancer cells. *Cell* **137:** 821-834.

Schvartzman JM, Duijf PH, Sotillo R, Coker C, Benezra R (2011). Mad2 is a critical mediator of the chromosome instability observed upon Rb and p53 pathway inhibition. *Cancer Cell* **19:** 701-714.

Shirasawa S, Furuse M, Yokoyama N, Sasazuki T (1993). Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. *Science* **260:** 85-88.

Sigoillot FD, King RW (2011). Vigilance and validation: Keys to success in RNAi screening. *ACS Chem Biol* **6:** 47-60.

Singh A, Settleman J (2010). EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* **29:** 4741-4751.

Solimini NL, Luo J, Elledge SJ (2007). Non-oncogene addiction and the stress phenotype of cancer cells. *Cell* **130:** 986-988.

Sotillo R, Schvartzman JM, Socci ND, Benezra R (2010). Mad2-induced chromosome instability leads to lung tumour relapse after oncogene withdrawal. *Nature* **464:** 436-440.

Stumpff J, von Dassow G, Wagenbach M, Asbury C, Wordeman L (2008). The kinesin-8 motor Kif18A suppresses kinetochore movements to control mitotic chromosome alignment. *Dev Cell* **14:** 252-262.

Van Cutsem E, Kohne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A *et al* (2009). Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* **360:** 1408-1417.

Varga V, Helenius J, Tanaka K, Hyman AA, Tanaka TU, Howard J (2006). Yeast kinesin-8 depolymerizes microtubules in a length-dependent manner. *Nat Cell Biol* **8:** 957-962.

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

Walczak CE, Cai S, Khodjakov A (2010). Mechanisms of chromosome behaviour during mitosis. *Nat Rev Mol Cell Biol* **11:** 91-102.

Wang Y, Ngo VN, Marani M, Yang Y, Wright G, Staudt LM *et al* (2010). Critical role for transcriptional repressor Snail2 in transformation by oncogenic RAS in colorectal carcinoma cells. *Oncogene* **29:** 4658-4670.

Weinstein IB (2002). Cancer. Addiction to oncogenes--the Achilles heal of cancer. *Science* **297:** 63-64.

Zhang C, Zhu C, Chen H, Li L, Guo L, Jiang W *et al* (2010). Kif18A is involved in human breast carcinogenesis. *Carcinogenesis* **31:** 1676-1684.

5

THE HISTONE DEMETHYLASE Jarid1b (Kdm5b) IS A NOVEL COMPONENT OF THE Rb PATHWAY AND ASSOCIATES WITH E2f-TARGET GENES IN MEFs DURING SENESCENCE

Jeroen H. Nijwening*, Ernst-Jan Geutjes*,
Rene Bernards, Roderick L. Beijersbergen

Division of Molecular Carcinogenesis,
The Netherlands Cancer Institute, Amsterdam,
The Netherlands

*J.H.N. and E.J.G. contributed equally to this work

Published in PLoS ONE 2011:6(9):e25235

ABSTRACT

Senescence is a robust cell cycle arrest controlled by the p53 and Rb pathways that acts as an important barrier to tumorigenesis. Senescence is associated with profound alterations in gene expression, including stable suppression of E2f-target genes by heterochromatin formation. Some of these changes in chromatin composition are orchestrated by Rb. In complex with E2f, Rb recruits chromatin modifying enzymes to E2f target genes, leading to their transcriptional repression. To identify novel chromatin remodeling enzymes that specifically function in the Rb pathway, we used a functional genetic screening model for bypass of senescence in murine cells. We identified the H3K4-demethylase Jarid1b as novel component of the Rb pathway in this screening model. We find that depletion of *Jarid1b* phenocopies knockdown of *Rb1* and that Jarid1b associates with E2f-target genes during cellular senescence. These results suggest a role for Jarid1b in Rb-mediated repression of cell cycle genes during senescence.

Keywords Jarid1b / Rb / senescence / demethylase / RNAi

INTRODUCTION

Senescence is a robust cell cycle arrest that can be triggered by various stress signals such as telomere attrition, oncogene activation or DNA damage, which functions to protect cells against malignant transformation [1],[2]. Senescent cells undergo a series of events leading to marked morphological changes, the expression of senescence-associated β-galactosidase (SA-β-gal) and profound changes in gene expression, including activation of the *INK4A-ARF* locus. The *INK4A-ARF* locus is a potent activator of the p53 and RB tumor suppressor networks that enforce an intricate program including the repression of E2F-target genes required for proliferation [3,4]. Not surprisingly, the p53 and RB proteins are commonly inactivated by viral oncoproteins such as E1A or SV40LT thereby contributing to cellular transformation. In human fibroblasts it has been found that senescence induction is associated with dramatic changes in chromatin organization and several chromatin modifying enzymes have been identified that modulate the senescence response [5]. Both the *INK4A–ARF* locus and genes controlled by RB and E2F are major targets of epigenetic regulation during senescence. The *INK4A-ARF* locus is repressed by concerted action of polycomb group proteins (PcG), which impose trimethylation of histone H3 Lysine 27 (H3K27me3) and histone demethylases JARID1A (KDM5A) and NDY1 (KDMB2B) that remove H3K4me3 and H3K36me3 from this locus respectively [6,7,8,9,10]. PcG-mediated repression of the *INK4A-ARF* locus is counteracted by JMJD3 which actively removes methylation on H3K27 [11,12]. In addition, the promoter regions of E2F-target genes become enriched for H3K9me3 and depleted for H3K4me3 during senescence, which is important for gene silencing and correct execution of the senescence response by the RB tumor suppressor network [13]. RB can be regarded as an adaptor protein that recruits several histone modifiers to create a repressive complex to silence E2F-target genes during senescence [5]. For example, RB has been shown to recruit HDAC1, DNMT1, SUV39H1 and the SWI/SNF complex to E2F-target gene promoters [5,14,15]. It has been reported that inactivation of *Suv39h1* prevents induction of oncogene-induced senescence, which underscores H3K9 trimethylation as a critical feature of senescence [16]. These observations suggest a role for RB in heterochromatinization of E2F-target genes in senescent cells. Concordantly, RB depletion prevents heterochromatin formation in human diploid fibroblasts [13]. Recently, it has been found that RB has a specific and non-redundant role during senescence in the repression of transcription of E2F-target genes involved in DNA replication [17]. Moreover, an RB mutant unable to associate with chromatin modifying enzymes could not repress DNA replication during oncogene-induced senescence [18]. However, this RB mutant was not compromised in its ability to repress DNA replication during quiescence or differentiation, underscoring the significant role of chromatin modifying enzymes in repression of DNA replication during senescence.

Based on the observations described above and the association of Rb with several different chromatin remodeling enzymes, we argued that Rb may recruit additional chromatin remodeling enzymes that contribute to the suppression of E2f-target genes. The identification of such enzymes is potentially compromised by the notion

that inactivation of the RB pathway only is not sufficient to bypass senescence in both murine and human cells [1]. Using a functional genetic screen in murine models in which abrogation of the Rb pathway is sufficient to bypass senescence we discovered that the histone demethylase Jarid1b (Kdm5b) is a critical component of the Rb-E2f pathway. In addition, we found that Jarid1b (Kdm5b) associates with E2f-target genes during senescence, suggesting it may contribute to the repression of E2f-target genes during senescence.

RESULTS

A screen for bypass of senescence in MN-tsLT cells identifies Jarid1b

To identify novel chromatin remodeling enzymes that specifically cooperate with Rb in tumor suppression, we used a senescence model in which abrogation of the Rb pathway is sufficient to bypass senescence (Figure 1A). The primary mouse striatum cell line MN-tsLT has been conditionally immortalized through the expression of a temperature-sensitive mutant (tsA58) of SV40 large-T antigen (tsLT) [19]. At the permissive temperature MN-tsLT cells proliferate rapidly but they enter into a synchronous senescence-like arrest when shifted to the non-permissive temperature (39°C). MN-tsLT cells arrested at 39°C display several hallmarks of cellular senescence including SA-β-gal positivity, senescent morphology, decreased expression of E2f-target genes and activation of the p53 target gene and cell cycle inhibitor *Cdkn1a* ($p21^{cip1}$) (Figure 2C and D, Supplementary Figure S1B-E). However, similar to murine embryonic fibroblasts (MEFs) and in contrast to human cells [13], senescence-associated heterochromatin foci (SAHF) cannot be detected in MN-tsLT cells. It has been shown previously that inhibition of the $p19^{ARF}$-p53 pathway is sufficient to bypass senescence in this model [20,21,22](Figure 1A). We tested whether loss of *Rb1* expression in MN-tsLT cells was sufficient to bypass senescence. As can be seen in Figure 1, the expression of an shRNA targeting *Rb1* (Supplementary Figure S1A) results in the rescue of the senescence phenotype analogous to inactivation of the *Ink4a-Arf* locus or knockdown of *p53*. As such, the dependency on either p53 or Rb in MN-tsLT cells offers an opportunity to find novel components of the $p16^{INK4A}$-Rb pathway.

For this purpose we constructed a retroviral shRNA library consisting of multiple independent shRNAs directed against 50 known and putative chromatin binding and modifying enzymes: Jumonji C (JmjC)-domain-containing proteins, the lysine specific demethylase 1 (LSD1)-like family members, methyl CpG binding proteins and DNA methylases [23,24]. The shRNAs were pooled in 50 sets of 4 vectors, in which each set of vectors was designed to target a single transcript (Supplementary Table 1).

MN-tsLT cells were transduced at 32 °C with the 50 individual sets of shRNAs in a single-well format and seeded for long term clonogenic outgrowth assays (Figure 1B). As a positive control we used a functional shRNA targeting *p53* that was used in previous studies [21,22]. We used an shRNA targeting green fluorescent protein (*GFP*) as a negative control throughout this study. As expected, knockdown of *p53* prevented

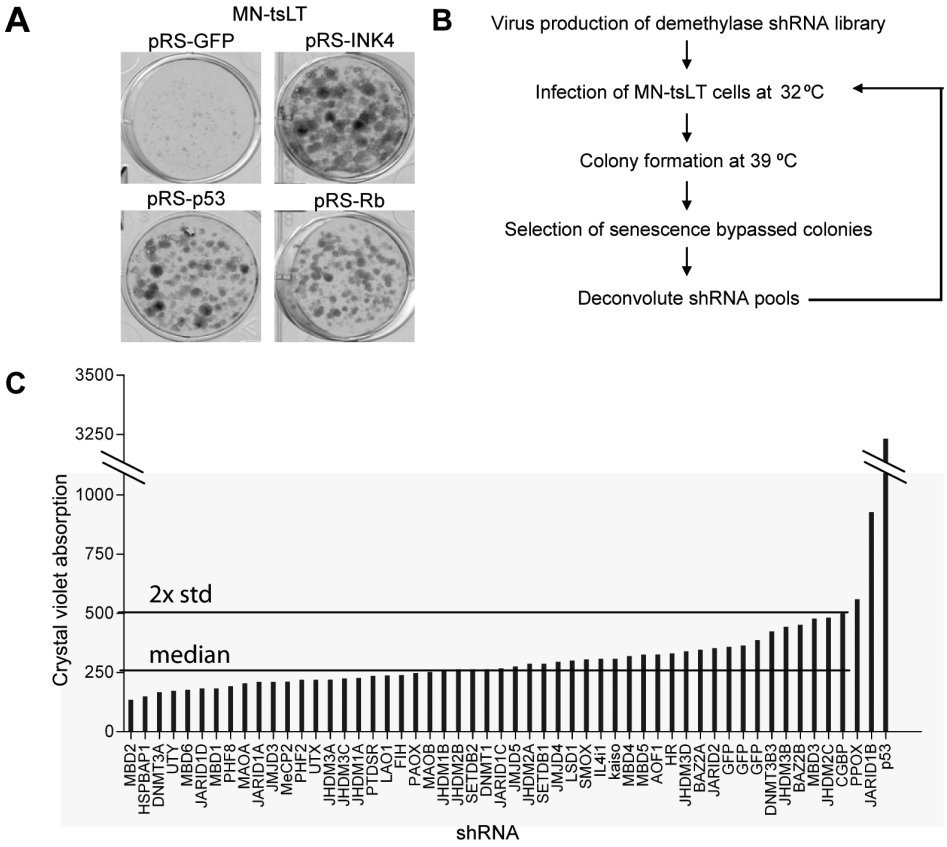


Figure 1. A functional shRNA screen in conditionally immortalized cells. (A) Colony formation assay at 39ºC of MN-tsLT cells transduced with pRS-*GFP*, pRS-*Ink4a* (targeting both *Ink4a* and *Arf*), pRS-*p53* and pRS-*Rb* (B) Schematic outline of the senescence bypass screen using MN-tsLT cells. Cells were transduced at the permissive temperature (32ºC) with 50 pools of retroviral knockdown vectors targeting candidate chromatin binding and modifying enzymes. Each pool contains 4 unique shRNAs targeting a single transcript. The transduced cells were seeded at the non-permissive temperature (39ºC) for a colony formation assay. After 2 weeks cells were fixed and stained with crystal violet. (C) Quantification of the colony formation assay of the shRNA screen in MN-tsLT cells shown are the average absorption and 2x standard deviations (SD) from the median of the samples.

senescence induction of MN-tsLT cells (Figure 1A and 1C). Clonogenic outgrowth was quantified by measuring crystal violet absorption. Only wells with an absorption value greater than the median plus 2x standard deviation were considered as hits (Figure 1C). Except for the positive control, only the shRNA pool targeting *Jarid1b (Kdm5b, Plu-1, Rbp2-h1)* [25,26] fitted these criteria. Jarid1b is a member of the Jarid1 family of H3K4 demethylases [27,28,29,30,31]. This family encompasses four members (Jarid1a-d) with a high degree of homology [32], all capable of demethylating tri- and di-methylated H3K4 and function as transcriptional repressors. Although shRNA

pools against Jarid1 family members a, c and d were present in the library they did not score as hits. On one hand, this could be due to inefficient knock-down of their respective targets but, in contrast to Jarid1b, we did not detect expression of Jarid1a, c or d in MN-tsLT cells (data not shown).

To rule out off target effects [33], each of the individual knockdown vectors of the *Jarid1b* shRNA pool were introduced into MN-tsLT cells and tested for their ability to bypass senescence and their efficiency of knocking down *Jarid1b*. We found two independent shRNAs targeting *Jarid1b* (pRS-*Jarid1b*#1 and #3) that allowed bypass of senescence in MN-tsLT cells. Both shRNAs reduced *Jarid1b* mRNA levels, confirming Jarid1b as an on-target hit (Figures 2A and B). In addition, we found that *Jarid1b* mRNA expression is highly induced when MN-tsLT cells are shifted to the non-permissive temperature, suggesting a role for Jarid1b in the execution of senescence (Figure 2B). Importantly, the expression of *Jarid1b* is not a surrogate marker for the absence of

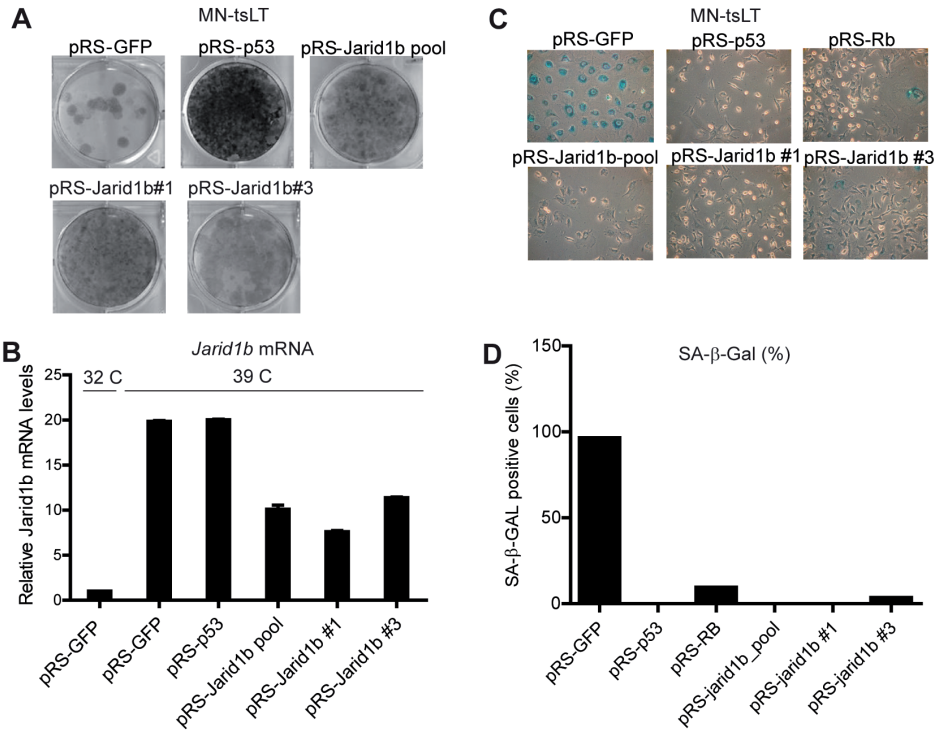


Figure 2. Multiple *Jarid1b*-targeting shRNAs prevent senescence induction of MN-tsLT cells. (A) Colony formation assay of MN-tsLT transduced with two independent *Jarid1b* shRNAs derived from the pool (#1 and #3). pRS-*GFP* was used as a negative control, pRS-*p53* was used as a positive control. (B) Relative *Jarid1b* mRNA expression levels determined by RT-qPCR. Samples are derived from cells shown in (A) and mRNA values were normalized to *Gapdh* and proliferating cells at 32 °C. (C) β-galactosidase assay of MN-tsLT cells from (A). (D) Quantification of β-galactosidase positive cells from (C).

cellular proliferation as MN-tsLT cells that express knockdown vectors against *p53*, and cycling at 39 ºC, retain high levels of *Jarid1b*.

Next, we analyzed MN-tsLT cells transduced with the two functional *Jarid1b*-knockdown vectors for typical senescence markers [1]. Whereas the negative control vector transduced cells stained highly positive for β-galactosidase, cells expressing the functional *Jarid1b*-knockdown vectors did not or stained weak for β-galactosidase (Figures 2C and 2D). Moreover, *Jarid1b*-knockdown cells did not show a typical senescent morphology observed in the control vector-transduced cells (Supplementary Figure S1B). Expression of two *bona fide* cell cycle markers *Ccna1* and *Pcna* was restored in *Jarid1b* knockdown cells (Supplementary Figure S1C and S1D). Remarkably, levels of *Cdkn1a*, a marker of slowly cycling and senescent cells, remained high in proliferating *Jarid1b*-knockdown cells (Supplementary Figure S1E). Taken together, these data demonstrate that MN-tsLT cells with *Jarid1b* knockdown do not undergo senescence when shifted to the restrictive temperature.

Jarid1b functions in the Rb pathway

Suppression of either the $p16^{INK4A}$-Rb or the $p19^{ARF}$-p53-$p21^{cip1}$ pathways can mediate bypass of senescence in MN-tsLT cells (Figure 1A). To determine in which of these two pathways Jarid1b operates, we examined gene expression profiles of senescent MN-tsLT cells and MN-tsLT cells with knockdown of *p53*, *Rb1*, *Ink4a* (*Ink4a-Arf* locus) or the *Jarid1b* shRNA pool. Unsupervised hierarchical clustering of mRNA expression profiling revealed that the transcriptional profiles of *Jarid1b*-knockdown and *Rb*-knockdown cells were highly similar (Figure 3A), suggesting that Rb and Jarid1b may operate in the same pathway. Concordantly, expression of established E2f-target genes was downregulated in senescent cells but restored in *Rb1* and *Jarid1b*-knockdown cells similar to *p53*-knockdown cells (Figure 3B). To ask whether Jarid1b also functions in the p53 pathway we looked for the expression of *bona fide* p53-target genes in our micro-array data sets. As expected, p53-target genes were upregulated in senescent cells and downregulated in p53-knockdown cells (Figure 3C). In contrast, p53-target genes were induced in both *Rb1*-knockdown and *Jarid1b*-knockdown cells to a similar extent as in senescent MN-tsLT cells. These data may indicate that Jarid1b does not function in the $p19^{ARF}$-p53-$p21^{cip1}$ pathway. Moreover, *Jarid1b* is not a transcriptional target of p53 as knockdown of *p53* does not affect the expression of *Jarid1b* in MN-tsLT cells (Figure 2B).

Interestingly, it was previously reported that the protein product of a *JARID1B* splice variant binds to RB in co-immunoprecipitation experiments in MCF7 human breast cancer cells [34]. However, the functional significance of JARID1B in RB-mediated suppression of E2F-target genes was not explored. This is not a trivial question as over 150 proteins are known to interact with RB (www.hprd.org) but many of those do not modulate E2F-target gene expression. To further substantiate the interaction between Jarid1b and Rb, we performed a co-immunoprecipitation experiment in senescent MN-tsLT cells using an antibody against Jarid1b. Indeed we were able to

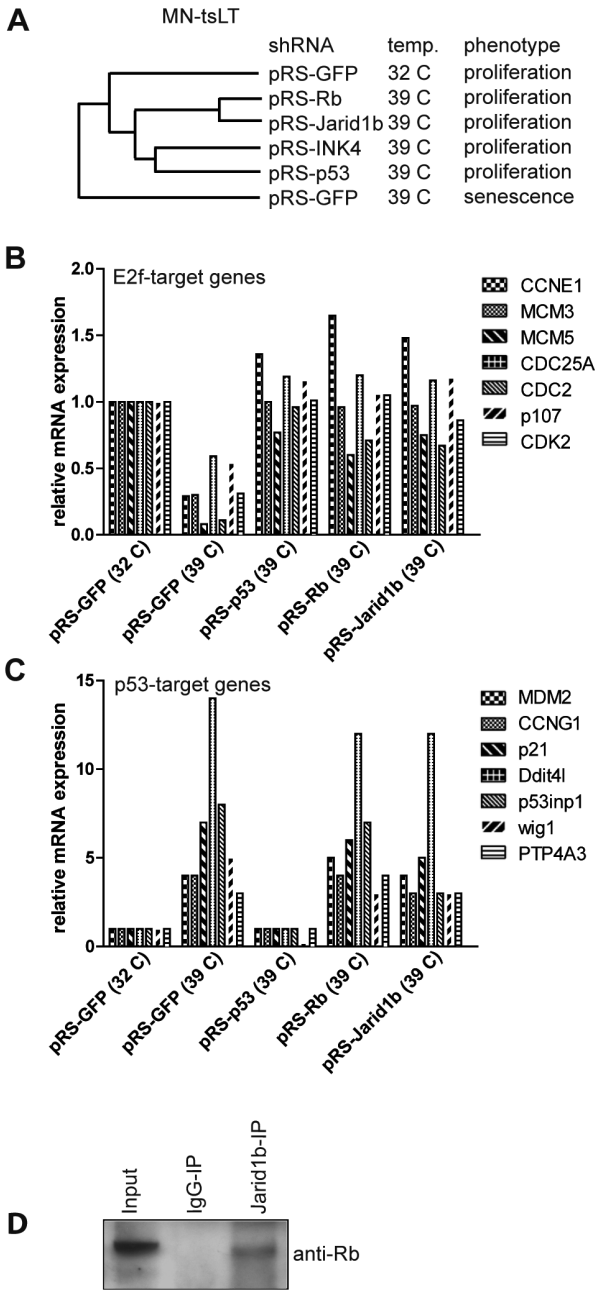


Figure 3. Jarid1b operates in the Rb pathway. (A) Unsupervised hierarchical clustering of mRNA expression of MN-tsLT cells stably transduced with the indicated knockdown vectors grown under the permissive or non-permissive temperature. (B) Relative expression of E2f-target genes from expression profiles from (A). (C) Relative expression of *p53* target genes from expression profiles from (A). Values were normalized to MN-tsLT cells cycling at 32 °C. (D) Co-immunoprecipitation of Rb and Jarid1b. Protein fractions were isolated from MN-tsLT cells cultured for 4 days at 39 °C followed by immunoprecipitation of Jarid1b. Immunoprecipitated samples were analyzed by western blot and probed with anti-Rb antibody.

detect endogenous Rb in the Jarid1b immunoprecipitation by western blotting using an Rb antibody, demonstrating that Jarid1b physically interacts with Rb in senescent MN-tsLT cells (Figure 3D). The expression data together with the interaction of Jarid1b and Rb may suggest that Jarid1b is involved in Rb-, but not p53, mediated execution of senescence in MN-tsLT cells.

Jarid1b knockdown phenocopies loss of Rb in Rb-dependent senescence models

To confirm that Jarid1b functions in the Rb pathway we tested whether loss of *Jarid1b* could bypass senescence in another senescence model in which abrogation of the Rb pathway is sufficient for bypass. Primary MEFs with knockdown of *p53* are unable to undergo senescence whereas knockdown of *Rb1* does not result in bypass of senescence. Transduction of primary MEFs with the *Jarid1b* shRNA pool did not result in bypass of senescence (Supplementary Figure S2A). It has been shown previously that MEFs deficient for all three pocket proteins *Rb1*, *Rbl1* and *Rbl2* are unable to undergo senescence [35,36]. In contrast, MEFs only deficient for *Rbl1* and *Rbl2* retain the ability to undergo senescence [37], suggesting that in these double knockout MEFs (DKO MEFs) Rb is the only retinoblastoma gene family member that executes the senescence program. We subsequently tested whether our knockdown vectors against *Jarid1b* could replace knockdown of *Rb1* to override cellular senescence in these DKO MEFs. Indeed, depletion of *Jarid1b* or *Rb1* prevented cellular senescence in DKO MEFs (Figure 4A). Unlike senescent DKO MEFs, *Rb1* and *Jarid1b*-knockdown cells did not stain positive for β-galactosidase (Figure 4B and Supplementary Figure S2B) and did not show a senescent morphology (Supplementary Figure S2C). Mutations in *Ink4a*, *Arf* and *p53* can lead to spontaneous immortalization of MEFs [1]. To exclude that *Jarid1b*-knockdown DKO MEFs were spontaneously immortalized (SPi), we assessed the status of the p53 pathway by treating cells with the DNA-damaging agent cisplatin and subsequently analyzed the expression of the p53 target gene *Cdkn1a* (Figure 4C). In contrast to SPi colonies derived from pRS-GFP transduced DKO MEFs, *Cdkn1a* expression was potently induced in *Jarid1b*-knockdown DKO MEFs after treatment with cisplatin. Collectively, these results show that *Jarid1b*-knockdown can phenocopy *Rb1*-knockdown in the bypass of cellular senescence in both MN-tsLT cells and DKO MEFs.

Jarid1b associates with E2f-target genes during senescence

Using chromatin immunoprecipitation (ChIP) with an RB antibody followed by deep sequencing it was shown that RB associates with E2F-target genes involved in DNA replication and cell cycle progression in senescent diploid human fibroblasts [17]. RB orchestrates the senescence response by recruiting chromatin modifying enzymes to induce and maintain a repressive state of heterochromatin surrounding E2F-target genes [38,39]. JARID1B has been shown to function as a transcriptional repressor by demethylating the active transcription mark H3K4me3 [40,41]. We hypothesized that

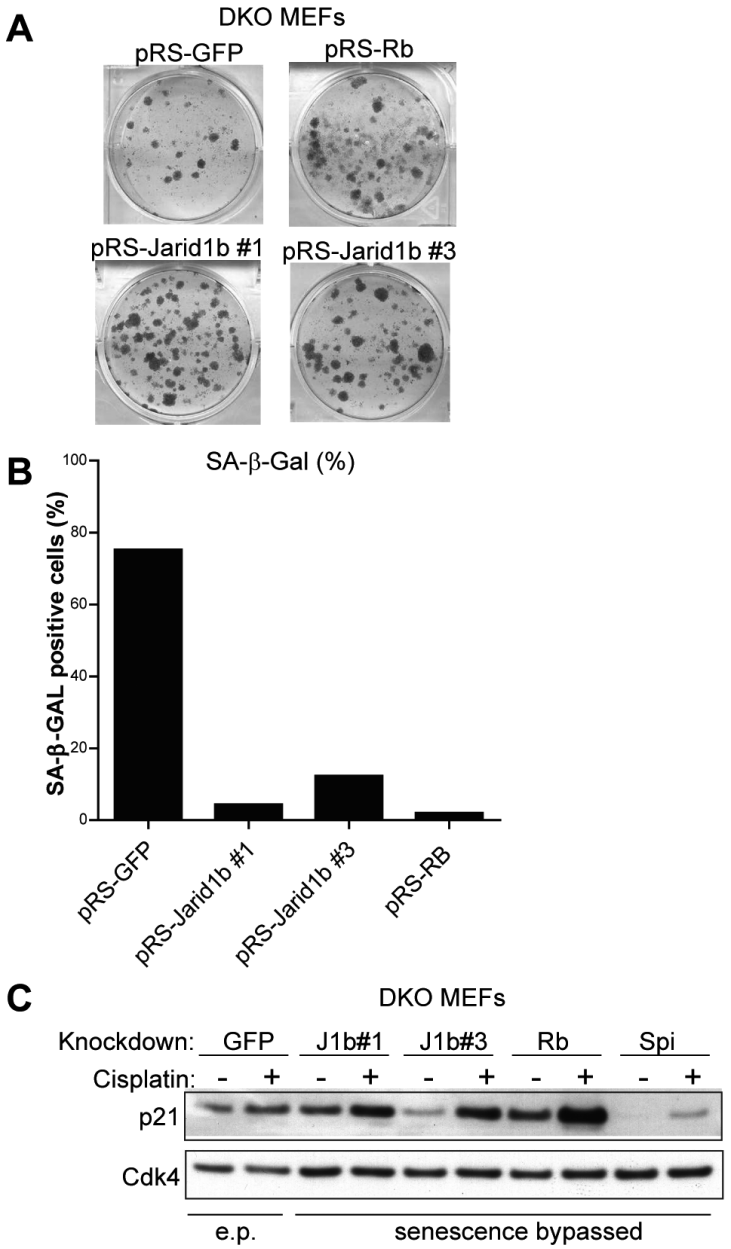


Figure 4. *Jarid1b*-knockdown is able to bypass cellular senescence in MEFs deficient for *Rbl1* and *Rbl2*. (A) MEFs, deficient for *Rbl1* and *Rbl2* (DKO MEFs), were transduced with functional *Jarid1b* or *Rb1* retroviral knockdown vectors and seeded for a colony formation assay. pRS-*GFP* was used as a negative control. (B) β-galactosidase quantification of DKO MEFs from (S2B). (C) Western blot analysis of $p21^{cip1}$ in DKO MEFs from (A) treated with cisplatin o/n. pRS-*GFP* sample in first two lanes are early passage (e.p.) DKO MEFs. Spontaneously expanded colony outgrowths of pRS-*GFP* (SPi: spontaneously immortalized) transduced DKO MEFs from (A) were used as a control. Cdk4 staining serves as a loading control.

Jarid1b associates with Rb during senescence to remove the activating H3K4-trimethyl mark at promoters of E2f-target genes. To test whether Jarid1b associates with E2f-target genes during senescence we determined Jarid1b occupancy at E2f binding sites of E2f-target gene promoters in cycling and senescent MEFs by performing a ChIP experiment with an antibody specific for Jarid1b. We confirmed that MEFs at passage 8 (P8) were senescent as they displayed hallmarks of senescence that were not observed in passage 5 (P5) MEFs, such as positive staining for β-galactosidase, induction of senescence-associated tumor suppressor genes *Ink4a*, *Arf* and *Cdkn1a*, and downregulation of E2f-target genes *Ccne1*, *Mcm3*, *Pcna* and *Rbl1* (Figures 5A and B). In support of our hypothesis, we found an increased association of Jarid1b with promoters of E2f-target genes but not at promoters of control genes in senescent MEFs (Figure 5C). Next, we tested whether Jarid1b occupancy at E2f-target gene promoters was correlated with decreased H3K4 methylation at these promoters, by performing a ChIP with an antibody specific for H3K4me3 in the same chromatin factions. Indeed, we found that H3K4me3 was severely depleted at promoters of E2f-target genes in senescent cells (Figure 5D). Similar to MEFs, we observed an enhanced occupancy of Jarid1b at E2f-target gene promoters in senescent MN-tsLT cells associated with depletion of H3K4me3 levels (Supplementary Figure S3A and S3B). Taken together, these results demonstrate that there is increased binding of Jarid1b to E2f-target genes during senescence, which is correlated with a strong reduction of H3K4me3 of these E2f-target genes.

DISCUSSION

Chromatin is extensively modified during senescence to allow selective repression of E2F-target genes that control cellular proliferation. E2F-target gene promoters become targets for heterochromatin formation that are enriched for H3K9 methylation but depleted in H3K4 methylation [3,13]. H3K4me3 is exclusively associated with the 5' regions of practically all active genes whereas H3K9me3 is invariably enriched in transcriptionally silent regions [42,43]. Several studies suggest that the formation of an epigenetic landscape that induces silencing of E2F-target genes during senescence is orchestrated by RB. In contrast to proteins responsible for H3K9 methylation of E2F-target genes, it is unknown which enzymes selectively demethylate H3K4me3 of E2F-target genes. Our data suggest that Jarid1b functions in a repressive complex with Rb to remove the H3K4 activation mark from E2f-target genes, a process that could contribute to their stable silencing during senescence in murine cells.

Recently, Lowe and colleagues, identified a non-redundant role for RB, but not p107 and p130, in promoting senescence by specifically repressing E2F-target genes involved in DNA replication [17], providing a rationale for why RB, but not its family members p107 and p130, is disabled in many, if not all, tumor cells [44]. Although near complete loss of RB may delay senescence induction [17], inactivation of Rb is not sufficient to bypass senescence in almost all models of senescence [4,45]. We find here that suppression of *Jarid1b* can substitute for *Rb1* loss in override of senescence in

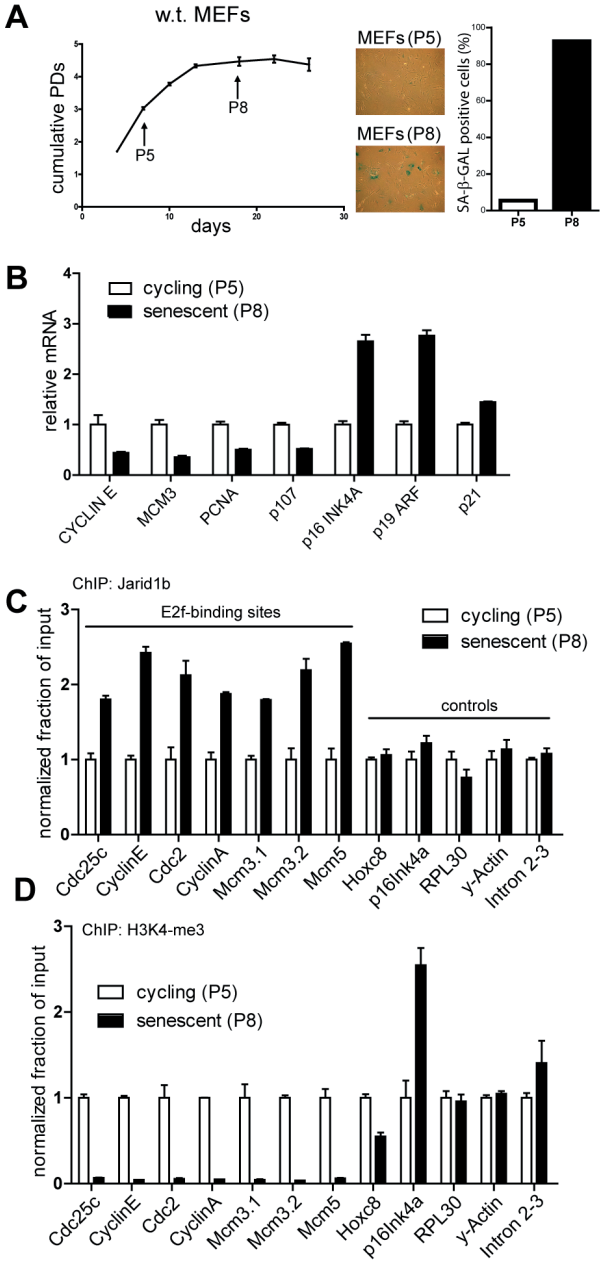


Figure 5. Jarid1b associates with the promoters of E2f-target genes during senescence. (A) Analysis of cycling, passage 5 (P5) and senescent, passage 8 (P8), primary MEFs for hallmarks of senescence. Shown are a proliferation curve of MEFs according to the 3T4 protocol (left panel, results shown as means ± SD), β–galactosidase staining of P5 and P8 MEFs (center panel) and quantification of β–galactosidase positive cells (right panel). (B) Analysis of mRNA expression of the indicated genes from P5 and P8 MEFs. Values are normalized to P5 and shown as means ± SD. (C) Jarid1b ChIP in P5 MEFs and P8 MEFs. The degree of enrichment at indicated promoters of E2f-target genes and control genes was measured by qPCR, non-specific binding of rabbit IgG controls was subtracted and results are presented as percentage of bound / input normalized to P5 samples for each gene. (D) H3K4me3 ChIP in P5 MEFs and P8 MEFs, performed as in (C). Non-specific binding of rabbit IgG controls was subtracted and quantification of H3K4me3 samples was normalized to H3-immunoprecipitations in the same experiment. PD: passage doubling, P: passage

mouse fibroblasts that can be bypassed by knockdown of *Rb1* alone, indicating a role for Jarid1b in the Rb pathway.

JARID1B has been implicated as an oncogene in breast and prostate cancer but as a tumor suppressor in melanoma, which may be attributed to tissue-specific regulation of genes that control carcinogenesis by JARID1B. For example, JARID1B was reported to transcriptionally regulate *BRCA1* in breast cancer, via direct interaction with promoter sites [40,41,46]. *JARID1B* is highly expressed in benign human melanocytic nevi, which invariably harbor oncogenic mutations but are protected from progressing into malignant tumors by oncogene-induced senescence [47,48]. Importantly, it was found that the RB tumor suppressor network and not the $p14^{ARF}$-p53-$p21^{cip1}$ axis has a key role in the induction of senescence in naevi [48]. This study provided a rationale for the frequent genetic alterations in the $p16^{INK4A}$-RB pathway in melanoma and the genetic predisposition of patients with germline mutations of the $p16^{INK4A}$-RB tumor suppressor network to melanoma [49]. It was reported that RB recruits HDAC1, HP1β and SUV39H1 to induce senescence in naevi [39]. We speculate that JARID1B assists RB in senescent naevi to aid in the execution of senescence. Indeed, *JARID1B* is downregulated in malignant melanoma that progressed from a senescent naevus, while restoration of *JARID1B* expression in malignant melanoma inhibits proliferation [50]. It was recently found that in contrast to the bulk of melanoma tumor cells expressing very low levels of *JARID1B*, a small slow-growing subpopulation expresses high levels of *JARID1B*. The *JARID1B* expressing subpopulation was found to act as tumor-initiating cells, giving rise to highly proliferative progeny with low *JARID1B* expression [47]. We speculate that the high proliferation rate of melanoma cells with low *JARID1B* expression may be caused by depression of E2F-target genes and the consequential activation of the cell cycle.

In conclusion, we identified a novel component of the Rb-repressor complex that associates with E2f-target genes during senescence correlating with a strong decrease of H3K4me3 at the same promoters. Jarid1b binds to Rb in senescent cells and *Jarid1b*-knockdown can substitute for *Rb1*-knockdown in senescence models that are solely dependent on functional Rb. We speculate that one of the functions of Jarid1b is to repress E2f-target genes, providing a possible explanation for the differential expression of *JARID1B* in distinct tumors although additional research is needed to dissect the functional role of the plasticity in *JARID1B* expression in different tumor types.

MATERIALS AND METHODS

shRNA Library Design

Design of oligonucleotides was done as previously described [51]. Multiple independent oligonucleotides (4 oligos/transcript) were designed to target the Jumonji C (JmjC)-domain-containing proteins, the lysine specific demethylase 1 (LSD1)-like family members, methyl CpG binding proteins and DNA methylases (see Supplemental table 1 for sequences). The oligonucleotides were pooled in 50 sets of 4 vectors, where each

set of vectors was designed to target a single transcript, and cloned into the pRISC retroviral vector as previously described [51,52]. More information and protocols on the oligo design and vector can be found at: http://www.screeninc.nl. (see Supplementary Table 1 for sequences).

Cell Lines and Cell Culture

All cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. MN-tsLT mouse striatum cells express a mutant version of the huntingtin protein with an expanded polyglutamine repeat from a knock-in MN-tsLT allele and a stably introduced temperature sensitive mutant (tsA58) of SV40T antigen [19]. Primary MEFs deficient for the pocket proteins encoding genes *Rbl1* and *Rbl2* (DKO MEFs) were obtained from Dannenberg [37].

Viral Transduction

Retroviral supernatants for each shRNA-pool were produced by transfection of phoenix packaging cells using the calcium-phosphate precipitation method. Forty-eight hours post-transfection, supernatants were harvested, filtered through a 0.45-µm filter and used for infection of target cells.

shRNA library Screen, Colony Formation and Proliferation Assays

MN-tsLT cells grown at the permissive temperature (32°C) were seeded at a density of $2x10^5$ cells per well of a 6-well dish and used for viral infection the next day. After infection cells were selected with puromycin (1 µg/ml) for 2 days. The puromycin selected MN-tsLT cells were seeded at a density of $1x10^4$ cells per well of a 6-well dish and 6hrs after plating shifted to the non-permissive temperature 39°C. After 2 weeks, cells were fixed with 4% formaldehyde, stained with 0.1% crystal violet and photographed. Crystal violet was extracted using 10% acetic acid and quantified at OD 590 nm.

Primary MEFs of FVB genetic background were transduced with retroviral shRNA constructs at passage 3, selected for two passages and subsequently seeded at a density of $1x10^4$ per 10 cm Ø dish for a colony formation assay. Cells were fixed and stained after 3 weeks. Growth curves were performed according to the 3T4 protocol and counted every 4 days. DKO MEFs (passage 2) were transduced with retroviral shRNA constructs at passage 3 and selected for two more passages with puromycin (1 µg/ml). Passage 5 DKO MEFs were seeded $1x10^4$ cells per well of a 6-well plate for a colony formation assay. Cells were fixed and stained after 2 weeks.

Chromatin Immunoprecipitation

ChIP assays were performed using a commercially available ChIP assay kit (Simple ChIP Cell Signaling Technology, #9002) following the manufacturer's instructions. In short, MEFs and MN-tsLT cells were cultured in 15 cm Ø dishes and fixed with 1% formaldehyde (Sigma-Aldrich) for 10 min, followed by 2 washes with ice-cold

PBS containing 1 mM PMSF. For each sample $4x10^7$ isolated nuclei were resuspended in 1 ml buffer B and treated with 6 µl micrococcal nuclease (2000 gel units/µl) for 20 min at 37°C, followed by sonication with a Branson Sonifier 250 for 3 times 10 s with 30 s-off interval times at output setting 2 for MEFs, and 5 times 15 s with 30 s-off interval times at output setting 2 for MN-tsLT cells. DNA was recovered from immune complexes on protein A-agarose beads with the following antibodies: Jarid1b (#3273, Cell Signaling Technology), H3K4me3 (ab1012, Abcam), H3 (#4620, Cell Signaling Technology) and normal rabbit IgG (#2729, Cell Signaling Technology). Real-time qPCR was performed using FastStart Universal SYBR Green Master (Roche) in a 7500 Fast Real-Time PCR System (Applied Biosystems). ChIP primers used are derived from Blais [53], Rowland [54] and Barradas [12] and listed in Supplementary Table 1. Data are presented as percentage of bound minus IgG controls divided by input and normalized to the proliferating condition (P5 for MEFs and 32°C for MN-tsLT cells) of 3 independent ChIPs on a single chromatin fraction. For MN-tsLT cells, the experiments were performed in 3 biological triplicates and for the MEFs in biological duplicates.

Protein Extraction, Co-Immunoprecipitation and Western Blotting

Cells were lysed on ice with RIPA (150 mM NaCl, 50 mM Tris pH 8.0, 1% NP40, 0.5% sodium deoxycholate (DOC), 0.1% Sodium Dodecyl Sulfate (SDS)). Total protein (20-40µg) was used for Western analysis with antibodies for p21 (F-5, Santa Cruz Biotechnology) and CDK4 (C-220, Santa Cruz Biotechnology). Co-immunoprecipitation was performed using total cell lysate of $8x10^6$ senescent MN-tsLT cells after culture for 4 days at 39°C. Binding of Jarid1b to Rb, was analyzed by immunoprecipitation with 8 µl Jarid1b (#3273, Cell Signaling Technology) in ELB (250 mM NaCl, 50 mM Hepes pH 7.3, 0.1% NP40 and Complete protease inhibitor cocktail from Roche) and subsequent Western analysis with an antibody against Rb (#554136, BD Pharmingen).

Quantitative RT-qPCR

RNA was extracted using TriZOL (Invitrogen) according to the manufacture's protocol. cDNA synthesis and subsequent quantitative RT-PCR assays were performed to measure mRNA levels of genes using the 7500 Fast Real-Time PCR System as previously described [52]. Relative mRNA levels of each gene shown were normalized to the expression of *Rpl4*, *Gapdh*, *Actin* or *Rpl13* as indicated. The sequences for the primers for assays using SYBR Green master mix are listed in Supplementary Table 1.

β-Galactosidase Staining

Cells were washed with PBS and fixed with 0.5% gluteraldehyde (in PBS) for 15' at RT. Fixed cells were washed with PBS supplemented with 1 mM $MgCL_2$. Cells were subsequently incubated 4-6 hrs (for MN-tsLT cells) or 10-12 hrs (for MEFs) at 37°C in staining solution (PBS pH 6.0, 5 mM $K_4Fe(CN)^6$ *$3H_2O$, 5 mM $K_3Fe(CN)^6$, 1 mM

$MgCl_2$, 1 mg/ml X-Gal). All cells were processed simultaneously to allow comparison. A total of 1000 cells were counted per plate and scored for SA-β -Gal positive cells. For all SA-β -Gal stainings, the representative of at least two independent experiments is shown.

Unsupervised Hierarchical Clustering

MN-tsLTcells were transduced with the indicated shRNAs, puromycin selected and shifted to the non-permissive temperature as indicated. RNA samples were made in TriZOL (Invitrogen) according to the manufacturer's instructions, RNA was cleaned with the RNeasy mini kit (#74106, Qiagen) and DNase treated with the RNase-free DNase Set (#79254, Qiagen) according to the manufacturer's protocols. RNA was amplified using the Illumina TotalPrep RNA amplification Kit (Part Number AMIL 1791) and subsequently hybridized to an Illumina HumanWG-6 V3 beadchip (BD-101-0603). Unsupervised hierarchical clustering analysis was performed after background subtraction and normalization with BeadStudio analysis software from Illumina.

ACKNOWLEDGEMENTS

We thank Menno Creyghton for design of the demethylase RNAi library, Prasanth Kumar, Sidong Huang and Undine Hill for technical assistance, Tinke Vormer for DKO MEFs and Ron Kerkhoven and Marja Nieuwland for supporting gene-expression studies. This work was supported by Top Institute Pharma (project T3–105) and by the Netherlands Organisation for Scientific Research Spinoza award (to R.B.).

REFERENCES

1. Campisi J, d'Adda di Fagagna F (2007) Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 8: 729-740.
2. Prieur A, Peeper DS (2008) Cellular senescence in vivo: a barrier to tumorigenesis. Curr Opin Cell Biol 20: 150-155.
3. Adams PD (2007) Remodeling of chromatin structure in senescent cells and its potential impact on tumor suppression and aging. Gene 397: 84-93.
4. Courtois-Cox S, Jones SL, Cichowski K (2008) Many roads lead to oncogene-induced senescence. Oncogene 27: 2801-2809.
5. Macaluso M, Montanari M, 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.
6. Bracken AP, Kleine-Kohlbrecher D, Dietrich N, Pasini D, Gargiulo G, et al. (2007) The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. Genes Dev 21: 525-530.
7. Dietrich N, Bracken AP, Trinh E, Schjerling CK, Koseki H, et al. (2007) Bypass of senescence by the polycomb group protein CBX8 through direct binding to the INK4A-ARF locus. EMBO J 26: 1637-1648.
8. Pasini D, Hansen KH, Christensen J, Agger K, Cloos PA, et al. (2008) Coordinated regulation of transcriptional repression by the RBP2 H3K4 demethylase and Polycomb-Repressive Complex 2. Genes Dev 22: 1345-1355.

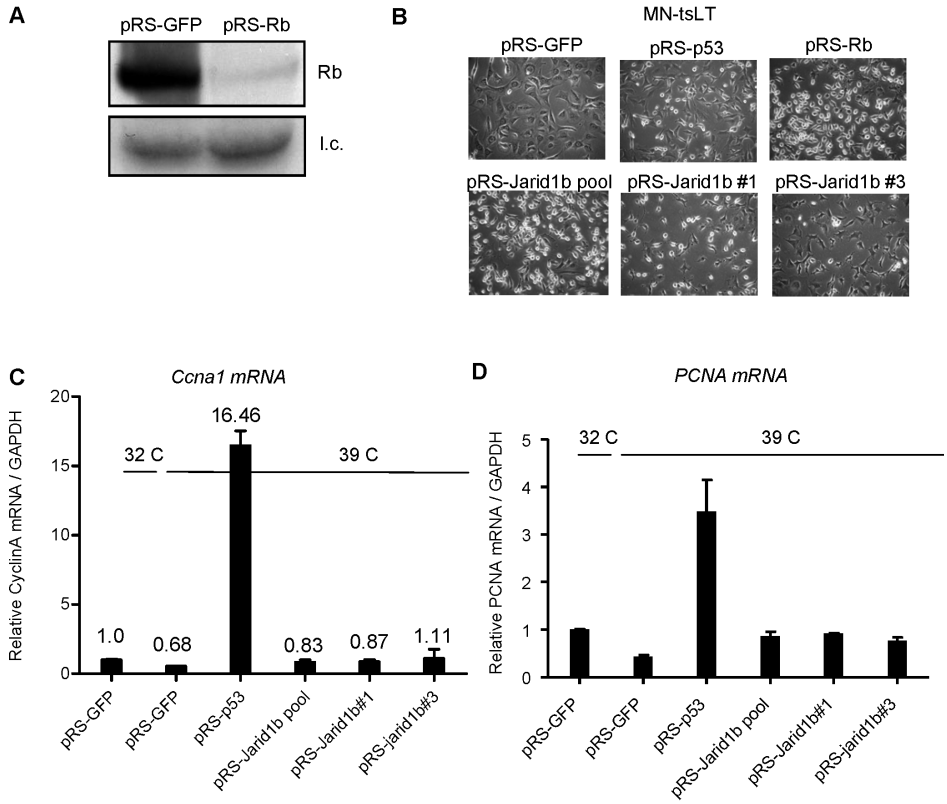
9. Tzatsos A, Pfau R, Kampranis SC, Tsichlis PN (2009) Ndy1/KDM2B immortalizes mouse embryonic fibroblasts by repressing the Ink4a/Arf locus. Proc Natl Acad Sci U S A 106: 2641-2646.
10. Pfau R, Tzatsos A, Kampranis SC, Serebrennikova OB, Bear SE, et al. (2008) Members of a family of JmjC domain-containing oncoproteins immortalize embryonic fibroblasts via a JmjC domain-dependent process. Proc Natl Acad Sci U S A 105: 1907-1912.
11. Agger K, Cloos PA, Rudkjaer L, Williams K, Andersen G, et al. (2009) The H3K27me3 demethylase JMJD3 contributes to the activation of the INK4A-ARF locus in response to oncogene- and stress-induced senescence. Genes Dev 23: 1171-1176.
12. Barradas M, Anderton E, Acosta JC, Li S, Banito A, et al. (2009) Histone demethylase JMJD3 contributes to epigenetic control of INK4a/ARF by oncogenic RAS. Genes Dev 23: 1177-1182.
13. Narita M, Nunez S, Heard E, Lin AW, Hearn SA, et al. (2003) Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell 113: 703-716.
14. Ferreira R, Naguibneva I, Pritchard LL, Ait-Si-Ali S, Harel-Bellan A (2001) The Rb/chromatin connection and epigenetic control: opinion. Oncogene 20: 3128-3133.
15. Kuilman T, Michaloglou C, Mooi WJ, Peeper DS (2010) The essence of senescence. Genes Dev 24: 2463-2479.
16. Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AH, et al. (2005) Oncogene-induced senescence as an initial barrier in lymphoma development. Nature 436: 660-665.
17. Chicas A, Wang X, Zhang C, McCurrach M, Zhao Z, et al. (2010) Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence. Cancer Cell 17: 376-387.
18. Talluri S, Isaac CE, Ahmad M, Henley SA, Francis SM, et al. (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.
19. Trettel F, Rigamonti D, Hilditch-Maguire P, Wheeler VC, Sharp AH, et al. (2000) Dominant phenotypes produced by the HD mutation in STHdh(Q111) striatal cells. Hum Mol Genet 9: 2799-2809.
20. Brummelkamp TR, Kortlever RM, Lingbeek M, Trettel F, MacDonald ME, et al. (2002) TBX-3, the gene mutated in Ulnar-Mammary Syndrome, is a negative regulator of p19ARF and inhibits senescence. J Biol Chem 277: 6567-6572.
21. Dirac AM, Bernards R (2003) Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. J Biol Chem 278: 11731-11734.
22. Epping MT, Meijer LA, Krijgsman O, Bos JL, Pandolfi PP, et al. (2011) TSPYL5 suppresses p53 levels and function by physical interaction with USP7. Nat Cell Biol 13: 102-108.
23. Shi Y, Whetstine JR (2007) Dynamic regulation of histone lysine methylation by demethylases. Mol Cell 25: 1-14.
24. Kouzarides T (2007) Chromatin modifications and their function. Cell 128: 693-705.
25. Vogt T, Kroiss M, McClelland M, Gruss C, Becker B, et al. (1999) Deficiency of a novel retinoblastoma binding protein 2-homolog is a consistent feature of sporadic human melanoma skin cancer. Lab Invest 79: 1615-1627.
26. Lu PJ, Sundquist K, Baeckstrom D, Poulsom R, Hanby A, et al. (1999) A novel gene (PLU-1) containing highly conserved putative DNA/chromatin binding motifs is specifically up-regulated in breast cancer. J Biol Chem 274: 15633-15645.
27. Christensen J, Agger K, Cloos PA, Pasini D, Rose S, et al. (2007) RBP2 belongs to a family of demethylases, specific for tri- and dimethylated lysine 4 on histone 3. Cell 128: 1063-1076.
28. Iwase S, Lan F, Bayliss P, de la Torre-Ubieta L, Huarte M, et al. (2007) The X-linked mental retardation gene SMCX/JARID1C defines a family of histone H3 lysine 4 demethylases. Cell 128: 1077-1088.
29. Klose RJ, Yan Q, Tothova Z, Yamane K, Erdjument-Bromage H, et al. (2007) The

retinoblastoma binding protein RBP2 is an H3K4 demethylase. Cell 128: 889-900.

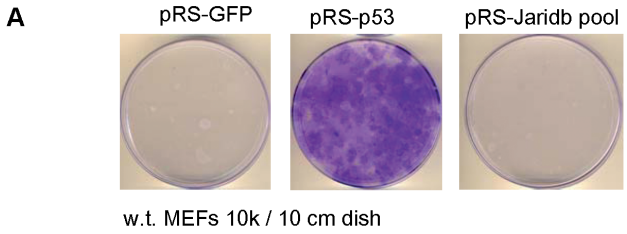
30. Seward DJ, Cubberley G, Kim S, Schonewald M, Zhang L, et al. (2007) Demethylation of trimethylated histone H3 Lys4 in vivo by JARID1 JmjC proteins. Nat Struct Mol Biol 14: 240-242.
31. Xiang Y, Zhu Z, Han G, Ye X, Xu B, et al. (2007) JARID1B is a histone H3 lysine 4 demethylase up-regulated in prostate cancer. Proc Natl Acad Sci U S A 104: 19226-19231.
32. Cloos PA, Christensen J, Agger K, Helin K (2008) Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. Genes Dev 22: 1115-1140.
33. Echeverri CJ, Beachy PA, Baum B, Boutros M, Buchholz F, et al. (2006) Minimizing the risk of reporting false positives in large-scale RNAi screens. Nat Methods 3: 777-779.
34. Roesch A, Becker B, Meyer S, Wild P, Hafner C, et al. (2005) Retinoblastoma-binding protein 2-homolog 1: a retinoblastoma-binding protein downregulated in malignant melanomas. Mod Pathol 18: 1249-1257.
35. Dannenberg JH, van Rossum A, Schuijff L, 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.
36. Sage J, Mulligan GJ, Attardi LD, Miller A, Chen S, et al. (2000) Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. Genes Dev 14: 3037-3050.
37. Dannenberg JH, Schuijff L, Dekker M, van der Valk M, te Riele H (2004) Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev 18: 2952-2962.
38. Narita M, Krizhanovsky V, Nunez S, Chicas A, Hearn SA, et al. (2006) A novel role for high-mobility group a proteins in cellular senescence and heterochromatin formation. Cell 126: 503-514.
39. Bandyopadhyay D, Curry JL, Lin Q, Richards HW, Chen D, et al. (2007) Dynamic assembly of chromatin complexes during cellular senescence: implications for the growth arrest of human melanocytic nevi. Aging Cell 6: 577-591.
40. Scibetta AG, Santangelo S, Coleman J, Hall D, Chaplin T, et al. (2007) Functional analysis of the transcription repressor PLU-1/JARID1B. Mol Cell Biol 27: 7220-7235.
41. Yamane K, Tateishi K, Klose RJ, Fang J, Fabrizio LA, et al. (2007) PLU-1 is an H3K4 demethylase involved in transcriptional repression and breast cancer cell proliferation. Mol Cell 25: 801-812.
42. Ruthenburg AJ, Allis CD, Wysocka J (2007) Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. Mol Cell 25: 15-30.
43. Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, et al. (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448: 553-560.
44. Burkhart DL, Sage J (2008) Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nat Rev Cancer 8: 671-682.
45. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88: 593-602.
46. Tan K, Shaw AL, Madsen B, Jensen K, Taylor-Papadimitriou J, et al. (2003) Human PLU-1 Has transcriptional repression properties and interacts with the developmental transcription factors BF-1 and PAX9. J Biol Chem 278: 20507-20513.
47. Roesch A, Fukunaga-Kalabis M, Schmidt EC, Zabierowski SE, Brafford PA, et al. (2010) A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. Cell 141: 583-594.
48. Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, et al. (2005) BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature 436: 720-724.

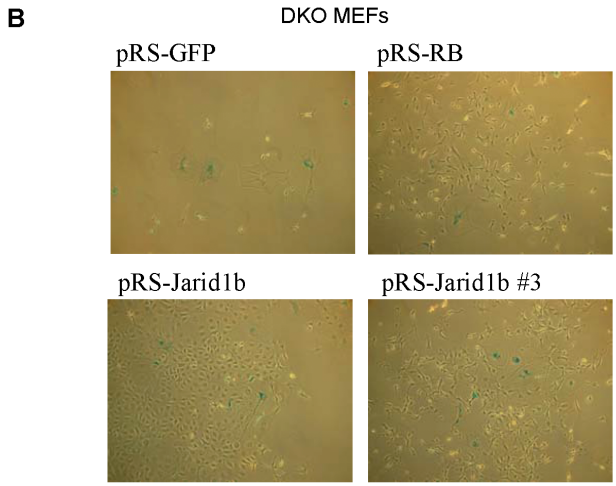
49. Halaban R (2005) Rb/E2F: a two-edged sword in the melanocytic system. Cancer Metastasis Rev 24: 339-356.
50. Roesch A, Becker B, Schneider-Brachert W, Hagen I, Landthaler M, et al. (2006) Re-expression of the retinoblastoma-binding protein 2-homolog 1 reveals tumor-suppressive functions in highly metastatic melanoma cells. J Invest Dermatol 126: 1850-1859.
51. Berns K, Hijmans EM, Mullenders J, Brummelkamp TR, Velds A, et al. (2004) A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature 428: 431-437.
52. Huang S, Laoukili J, Epping MT, Koster J, Holzel M, et al. (2009) ZNF423 is critically required for retinoic acid-induced differentiation and is a marker of neuroblastoma outcome. Cancer Cell 15: 328-340.
53. Blais A, van Oevelen CJ, Margueron R, Acosta-Alvear D, Dynlacht BD (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.
54. Rowland BD, Denissov SG, Douma S, Stunnenberg HG, Bernards R, et al. (2002) E2F transcriptional repressor complexes are critical downstream targets of p19(ARF)/p53-induced proliferative arrest. Cancer Cell 2: 55-65.

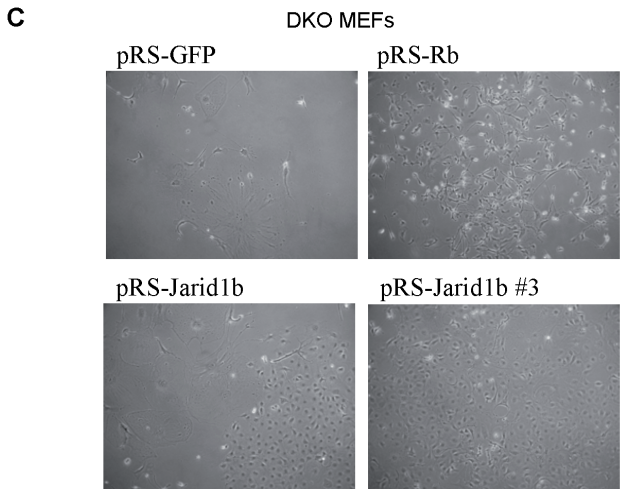
SUPPLEMENTARY MATERIAL



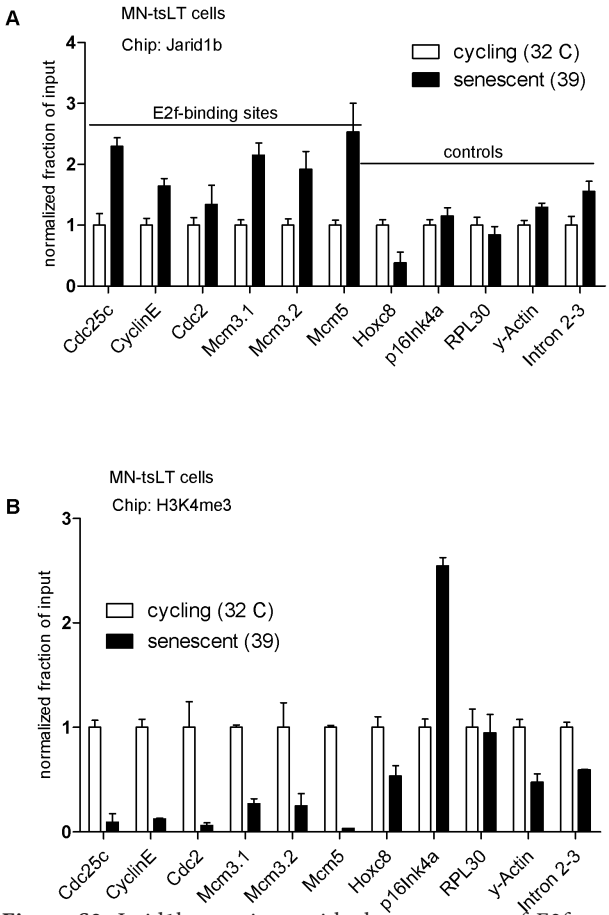
Supplementary Figure S1. *Jarid1b*-knockdown prevents senescence in MN-tsLT cells without affecting the induction of CDKNA1 expression. (A) Protein expression of Rb in senescent MN-tsLT cells transduced with a control vector (pRS-*GFP*) or an *Rb1*-knockdown vector (pRS-*Rb*). l.c.: loading control. (B) Brightfield images of MN-tsLT cells transduced with the indicated knockdown vectors and cultured at 39°C. pRS-*GFP* was used as a negative control. pRS-*p53*, and pRS-*Rb* were used as positive controls. The shRNA pool targetting *Jarid1b* and the two most potent *Jarid1b* knockdown vectors (#1 and #3) were tested. Cells transduced with negative control vectors show a typical round and flat morphology, characteristic of senescent cells. (C, D and E) RT-qPCR analysis shows relative mRNA expression of *Ccna1* (cyclin A) (C), *PcnA* (D) and *Cdkn1a* (E) as described in figure 2B.







Supplementary Figure S2. *Jarid1b*-knockdown can replace *Rb1*-knockdown to prevent cellular senescence in *Rb1*^{wt}, *Rbl1*$^{-/-}$, *Rbl2*$^{-/-}$ (DKO) MEFs. (A) Colony formation assay of primary MEFs transduced with the indicated knockdown vectors. Late passage infected MEFs were seeded at low density in a 10 cm dish allowed for colony formation for 2 weeks and colonies were visualized by crystal violet. (B) β–galactosidase staining of DKO MEFs from Figure 4A. (C) Brightfield images of DKO MEFs transduced with the indicated constructs. As a negative control pRS-*GFP* was used. The negative control shows a round and flat morphology, which is typical of senescent cells.



Supplementary Figure S3. Jarid1b associates with the promoters of E2f-target genes during senescence. (a) Jarid1b ChIP in MN-tsLT cells when cycling (32°C) or in senescence (39°C). The degree of enrichment at indicated promoters of E2f-target genes and control genes was measured by qPCR, non-specific binding of rabbit IgG controls was subtracted and results are presented as percentage of bound / input normalized to 32°C samples. (b) H3K4me3 ChIP in MN-tsLT cells when cycling (32°C) or in senescence (39°C), performed as in (a). Non-specific binding of rabbit IgG controls was subtracted and quantification of H3K4me3 samples was normalized to H3-immunoprecipitations performed in the same experiment on the same samples.

Supplementary Table 1. Chromatin modifiers knockdown library. Sequences of knockdown vectors and sequences of primers are depicted. **This table can be downloaded** from the PLoS ONE website: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0025235

6

GENERAL DISCUSSION

The aim of the research described in this thesis is to identify novel drug targets against cancer that are effective within a defined, genetic context. Cancer-incidences are increasing, around 12.7 million new cancer cases were diagnosed worldwide in 2008, in the same year an estimated 7.6 million people died from cancer worldwide (www.cancerresearch.uk.org). The treatment of cancer in recent years has been shifted from classical chemotherapy and irradiation to more specific cancer drugs (targeted therapeutics). However, a large portion of cancer remains refractory to effective treatment, therefore, novel and specific drugs that only and effectively target the tumor cells, and are non-toxic to normal and healthy cells, are needed.

High-throughput RNAi screening is the main research tool applied in the studies here to identify specific vulnerabilities of cancer cells or novel members of cancer-relevant pathways. Hits from such RNAi screens could potentially be novel drug targets (described in chapter 2). For most of the screens performed in this thesis, primary human diploid fibroblasts (BJs) were used. These BJs were genetically modified to create isogenic cell lines for synthetic lethal screening. The rationale behind this approach is to start with a cell line that has no transforming mutations and has intact pathways required for induction of, for example, apoptosis, senescence, and the DNA-damage response. Sets of isogenic cell lines were applied in high-throughput RNAi screening with libraries against the human kinome (779 genes) and the druggable genome (6022 genes). By comparing siRNA-pools that induce an anti-proliferative phenotype in cells that are untreated or treated with cisplatin, specific cisplatin-sensitizing drug targets could be identified (described in chapter 3). For this screen, BJs with p53 knockdown were used, since cisplatin is mostly applied in p53-negative tumors (Kelland 2007). Tumors often relapse after initially successful treatment with cisplatin (Borst et al 2008) and cisplatin treatment is often associated with serious side effects. Identified drug targets of this screen could be explored for their potential to efficiently sensitize cells to cisplatin thereby reducing their capability to develop resistance. In addition, the identified hits could be used in combination therapy with a reduced dose of cisplatin, thereby reducing the side effects frequently observed in patients.

The RAS oncogenes are among the most mutated drivers of tumor progression. By comparing the results of RNAi screens performed in fibroblasts with or without expression of an oncogenic mutant of HRAS, synthetic lethal interactions were identified that eventually could be clinically exploited (described in chapter 4). The results and implications of this screen are discussed below.

In addition to the synthetic lethal or sensitizing approaches, discussed in chapters 3 and 4 in this thesis, another screening model was applied to gain more insight into the tumor suppressor RB whose pathway is frequently deregulated in many cancer types (described in chapter 5).

SYNTHETIC LETHAL INTERACTORS WITH ONCOGENIC RAS FREQUENTLY OPERATE IN MITOSIS

In the synthetic lethal screen in genetically engineered human fibroblasts isogenic for the expression of oncogenic HRAS, 7 synthetic lethal candidates were identified. Among these is the microtubule associated and depolymerizing enzyme KIF18A (Mayr et al 2007, Stumpff et al 2008). *KIF18A* knockdown prolongs the duration of mitosis, in particular in cells that express an oncogenic mutant of *HRAS*. Moreover, in cells driven by oncogenic RAS, knockdown of *KIF18A* leads to impaired mitotic events and aberrant exit of mitosis possibly explaining the observed synthetic lethal phenotype.

It has been described that breast cancer cells have increased levels of *KIF18A* expression (Zhang et al 2010). Depletion of *KIF18A* with siRNAs in MDA-MB-231 cells (that harbor a $KRAS^{G12D}$ mutant) inhibits proliferation *in vitro* and in mouse xenografts (Zhang et al 2010). In addition, increased expression of *KIF18A* has been observed in colorectal cancer (CRC) (Nagahara et al 2011). Here, the authors assessed expression of *KIF18A* mRNA in tumors of patients with CRC and found that high *KIF18A* expression was associated with a poorer overall survival rate than patients with low *KIF18A* expression (Nagahara et al 2011). However, it remains to be investigated whether the correlation between low expression of *KIF18A* and good prognosis was a result of the cycling status of the tumor cells. In addition, it will be interesting to determine the prevalence of RAS mutations in these CRC samples to confirm our hypothesis that RAS-driven tumors with low *KIF18A* expression display a reduced proliferative capacity.

We and others (Huang et al 2009) show that depletion of KIF18A causes a mitotic arrest. In addition, we show that the duration of this mitotic arrest is increased in cells that express oncogenic RAS. We also show that cells that have a 2-3 times increased duration of mitosis show mitotic failures and cell death. The reason for the observed cell death upon a prolonged arrest in mitosis is not known. It has been shown that a 2-hour delay in mitosis induces p53 stabilization (Uetake and Sluder 2010). However, the cell line we used is p53-negative, suggesting that additional mechanisms exist that induce cell death after prolonged duration of mitosis. It has been described that *KIF18A* knockdown causes a prolonged activation of the MAD2-dependent spindle assembly checkpoint (Mayr et al 2007). MAD2 is a critical component of the spindle assembly checkpoint by inhibiting the activity of the APC/C when chromosomes are not properly aligned. This delay of mitotic progression in combination with continuous oncogenic signaling from constitutive active RAS can become deleterious for cells.

Interestingly, a role for the RAS pathway in mitotic progression has been suggested, highlighted by several studies that describe an interaction of the RAS-pathway with spindle assembly formation. Activated downstream mediators of the RAS-pathway, ERK1/2 kinases, localize to the kinetochores and associate with the motor protein CENP-E (Zecevic et al 1998). During development, the mitotic spindle orientation is controlled by RAS-regulated ERK1/2 to determine lung tube shape (Tang et al 2011). In yeast, the RAS effector Scd1 interacts with Moe1 to induce proper spindle formation

(Chen et al 1999). Raf kinase inhibitory protein (RKIP), which is downstream in the RAS-pathway, regulates the spindle assembly checkpoint in mammalian cells (Eves et al 2006). In addition, RAS-association domain family (RASSF) members (reviewed in (van der Weyden and Adams 2007)) have been implicated in spindle assembly in several ways: knockdown of RASSF7 abolished spindle formation and chromosomal congression (Recino et al 2010, Sherwood et al 2008). RASSF1A associates with the microtubules and depletion of RASSF1A causes mitotic abnormalities (Dallol et al 2007, Song et al 2005). In addition, RASSF1A has been found to negatively regulate the APC/C in mammalian cells (Whitehurst et al 2008). The close connection between RAS and mitotic progression could cause cells expressing oncogenic RAS to require additional time in mitosis to rewire damaged signaling cascades. In our study, we indeed show that BJs expressing oncogenic RAS remain longer in mitosis, which is also in line with the work of Elledge and colleagues (Luo et al 2009). Prolonging the time of mitotic progression in RAS-driven tumor cells could lead to an increased activation of the mitotic spindle checkpoint that eventually can lead to mitotic cell death.

TARGETING GENES THAT OPERATE IN THE SPINDLE ASSEMBLY CHECKPOINT

Most cancer cells have difficulties with mitotic progression, perhaps explaining why compounds that alter microtubule function have proved to be quite effective in treating cancer (reviewed in (Dumontet and Jordan 2010)), or why several mitotic proteins are upregulated in various cancers (Janssen and Medema 2011, Rhodes et al 2007, Strebhardt 2010). Consequently, inhibition of the spindle assembly checkpoint has been proposed as a strategy to kill tumor cells (Janssen and Medema 2011). Several studies that inhibit targets specifically operating in mitosis, like Eg5, Aurora A, CENP-E and PLK1, are currently in phase I and II clinical trials (reviewed in (Janssen and Medema 2011, Lens et al 2010). Furthermore, inhibition of Mps1, a protein specifically functioning in the spindle assembly checkpoint and responsible for accurate segregation of chromosomes during mitosis, sensitized diploid cells to clinically relevant levels of taxol (Janssen et al 2009) and inhibition by small molecules partially inhibited tumor growth in mouse models of cancer (Colombo et al 2010). Together these findings suggest that the inhibition of mitotic genes in cells driven by oncogenes could be an improved strategy to treat oncogene-driven tumors.

ADDITIONAL SYNTHETIC LETHAL INTERACTIONS WITH ONCOGENIC RAS

Besides the identification of mitotic proteins as potential synthetic lethal interactions with constitutive active RAS, additional pathways that are synthetic lethal have been identified. Stockwell and colleagues identified in a series of high-throughput compound screens that cells with oncogenic RAS display enhanced sensitivity to compounds

that inhibit voltage-dependent anion channels (VDACs) (Dolma et al 2003, Yagoda et al 2007, Yang and Stockwell 2008). Inhibition of VDACs leads to oxidative stress-induced cell death, which is in part mediated by RAS-induced increase of intracellular iron content (Yang and Stockwell 2008). Interestingly, a recent compound screen in genetically engineered MEFs, isogenic for oncogenic KRas, identified a compound, lanperisone (LP), that induces oxidative stress and selectively kills KRas-mutant cells (Shaw et al 2011). LP is an approved muscle relaxant and it will be interesting to see the results of phase I clinical trials in RAS-driven tumors with this drug.

A similar type of synthetic lethal interaction is described in mutant *Kras/p53*-driven lung cancer and *Nf1*-deficient malignancies (De Raedt et al 2011). A combination of two different inhibitors against heat shock protein 90 (Hsp90) and the mammalian target of rapamycin (mTOR) caused catastrophic endoplasmic reticulum (ER) stress by increasing the pool of reactive oxygen species (ROS) that is deleterious to oncogenic RAS-driven cells (De Raedt et al 2011). The availability of drugs targeting these pathways (IPI-504 (an HSP90-inhibitor) and mTOR-inhibitors) should enable the study of these drug combinations in a clinical setting. In the screen described in chapter 3 in this thesis, we also identified several ion-channels (P2RX5, TMP21, and TRPM8). The effectiveness of inhibiting these channels can be context dependent, since hits related to oxidative stress identified by Stockwell and colleagues were also found using BJs as a model system.

Settleman and colleagues identified candidate genes required in RAS-dependent cell lines, through comparative gene expression arrays of RAS-dependent – and independent cell lines (Singh et al 2009). This comparison revealed a gene expression signature in KRAS-dependent cells, associated with a well-differentiated epithelial phenotype. The authors found that EMT in epithelial KRAS dependent cell lines leads to KRAS independency (Singh et al 2009). These findings are in agreement with the synthetic lethal screen performed by Downward and colleagues (Wang et al 2010). Here, SNAIL2 knockdown reduced cell viability in RAS-mutant cells that had undergone EMT. This experiment supports the observation that mesenchymal cells with mutant RAS do not show RAS oncogene addiction but upon reversal of EMT induced by loss of SNAIL2, become RAS dependent again. Interestingly, response to tyrosine kinase receptor inhibition is also affected by an epithelial to mesenchymal transition of cancer cells (Fuchs et al 2008, Thomson et al 2005) suggesting a change in dependency on growth factor signaling pathways, in part wired through the RAS pathway. It has also been shown that mesenchymal tumors are more invasive, are generally more drug resistant, and are associated with poorer prognosis (Singh and Settleman 2010).

The cell cycle gene CDK4 has also been identified to be synthetic lethal with KRas-driven tumors in a mouse model (Puyol et al 2010). In this study they found that depletion of *CDK4* induces an immediate senescence response in lung tumors that are driven by a constitutive active, endogenous KRas (Puyol et al 2010). Remarkably, CDK4 was not identified in the studies mentioned above, or in our synthetic lethal screen.

The identified RAS synthetic lethal hits are associated with different cellular pathways and functions. In addition, there seems to be quite a high degree of context dependency. This could mean that for therapeutic exploitation a more precise stratification of patients will be needed. This lack of context independency is also called "soft synthetic lethality" (Ashworth et al 2011), meaning that these hits should be extensively tested in multiple genetic backgrounds. An alternative term would be a context-dependent hit that can be exploited as a drug target within a specific context. An exception to most of the hits so far identified could be TBK-1, which has been identified by screening a large panel (19 in total) of cell lines from different origins (Barbie et al 2009). TBK1 is a component of the NF-κB signaling network and the authors showed that oncogenic RAS induces pro-survival signals via the NF-κB pathway. This observation illustrates that the induction of pro-survival pathways is required to sustain oncogenic signaling. In addition, two studies confirmed the dependency of RAS-driven tumors on the NF-κB pathway in mouse models of lung tumorigenesis (Basseres et al 2010, Meylan et al 2009). We identified *TNFRSF17*, which functions upstream of the NF-κB pathway. It will be interesting to test if inhibition of this receptor reduces pro-survival signaling in cells that are stressed by oncogenic RAS. In conclusion, RAS synthetic lethal hits identified by us and others should extensively be studied in different contexts and exploited according to their clinical context.

JARID1B AS A POTENTIAL DRUG TARGET

Senescence can function as a barrier against oncogenic transformation and is often induced by oncogenes. Therefore, most cancers have lost the ability to undergo senescence by loss-of senescence-inducing pathways, like the RB pathway (Chicas et al 2010). In senescent cells E2F-target genes are exclusively occupied by RB, but not by other members of the pocket protein family (Chicas et al 2010). In addition, it has been shown that sites of E2F-target genes in senescent cells show decreased histone 3 lysine 4 (H3K4, a transcriptionally active mark) methylation and increased H3K9 (a transcriptionally repressive mark) methylation (Narita et al 2003). In an RNAi screen against demethylases for bypass of senescence in murine cells, we identified Jarid1b (chapter 5). Jarid1b demethylates the transcriptionally active mark H3K4 and thereby functions as a transcriptional repressor. We show that Jarid1b specifically associates with E2f-target genes in senescent murine cells, suggesting that Jarid1b is responsible for the hypomethylated state of H3K4. It was reported that RB recruits HDAC1, HP1β and SUV39H1 to induce senescence in naevi (Bandyopadhyay et al 2007). We hypothesize that JARID1B assists RB in senescent naevi to aid in the execution of senescence and therefore prevent malignant progression into melanoma. Indeed, *JARID1B* is downregulated in malignant melanoma that progressed from a senescent naevus, while restoration of *JARID1B* expression in malignant melanoma inhibits proliferation (Roesch et al 2006). It was also found that in contrast to the majority of melanoma tumor cells expressing low levels of *JARID1B*, a small slow-growing subpopulation

expresses high levels of *JARID1B*. The *JARID1B* expressing subpopulation was found to act as tumor-initiating cells, giving rise to highly proliferative progeny with low *JARID1B* expression (Roesch et al 2010). We speculate that this subpopulation of JARID1B expressing cells reflects a distinct epigenetic state induced by JARID1B-mediated E2F-target gene expression. Especially after treatment with chemotherapy, this subpopulation might be left unaffected. Subsequently, this subpopulation may dedifferentiate into progenitor cells, which function as intermediates between slowly proliferating stem cells and the fully differentiated descendents by loss-of *JARID1B* expression causing derepression of E2F-target genes. This observation is in line with a recent study that showed that a population of transient and drug-resistant cells in melanomas but also other tumors is dependent on JARID1A, the closest JARID1B homolog (Sharma et al 2010).

In conclusion we suggest that JARID1A and JARID1B should be explored as therapeutic targets by inhibiting the demethylase functions of JARID1A and JARID1B, thereby preventing their indirect tumorigenic effects by inducing a slow-cycling and chemotherapy resistant sub-population of cells.

Ten years after the fist application of mRNA silencing in mammalian cells, drug targets that specifically operate in a distinct genetic background begin to emerge. The logical next step is to design small molecules against these targets that can subsequently be tested in properly diagnosed and stratified patients. The work described in this thesis illustrates how RNAi screening approaches can contribute to identify drug targets that are specific in a distinct genetic background and therefore are potentially very effective in killing cancer cells

REFERENCES

Ashworth A, Lord CJ, Reis-Filho JS (2011). Genetic interactions in cancer progression and treatment. *Cell* **145:** 30-38.

Bandyopadhyay D, Curry JL, Lin Q, Richards HW, Chen D, Hornsby PJ *et al* (2007). Dynamic assembly of chromatin complexes during cellular senescence: implications for the growth arrest of human melanocytic nevi. *Aging Cell* **6:** 577-591.

Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF *et al* (2009). Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature* **462:** 108-112.

Basseres DS, Ebbs A, Levantini E, Baldwin AS (2010). Requirement of the NF-kappaB subunit p65/RelA for K-Ras-induced lung tumorigenesis. *Cancer Res* **70:** 3537-3546.

Borst P, Rottenberg S, Jonkers J (2008). How do real tumors become resistant to cisplatin? *Cell Cycle* **7:** 1353-1359.

Chen CR, Li YC, Chen J, Hou MC, Papadaki P, Chang EC (1999). Moe1, a conserved protein in Schizosaccharomyces pombe, interacts with a Ras effector, Scd1, to affect proper spindle formation. *Proc Natl Acad Sci U S A* **96:** 517-522.

Chicas A, Wang X, Zhang C, McCurrach M, Zhao Z, Mert O *et al* (2010). Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence. *Cancer Cell* **17:** 376-387.

Colombo R, Caldarelli M, Mennecozzi M, Giorgini ML, Sola F, Cappella P *et al* (2010). Targeting the mitotic checkpoint for cancer therapy with NMS-P715, an inhibitor of MPS1 kinase. *Cancer Res* **70:** 10255-10264.

Dallol A, Cooper WN, Al-Mulla F, Agathanggelou A, Maher ER, Latif F (2007). Depletion of the Ras association domain family 1, isoform A-associated novel microtubule-associated protein, C19ORF5/MAP1S, causes mitotic abnormalities. *Cancer Res* **67:** 492-500.

De Raedt T, Walton Z, Yecies JL, Li D, Chen Y, Malone CF *et al* (2011). Exploiting cancer cell vulnerabilities to develop a combination therapy for ras-driven tumors. *Cancer Cell* **20:** 400-413.

Dolma S, Lessnick SL, Hahn WC, Stockwell BR (2003). Identification of genotype-selective antitumor agents using synthetic lethal chemical screening in engineered human tumor cells. *Cancer Cell* **3:** 285-296.

Dumontet C, Jordan MA (2010). Microtubule-binding agents: a dynamic field of cancer therapeutics. *Nat Rev Drug Discov* **9:** 790-803.

Eves EM, Shapiro P, Naik K, Klein UR, Trakul N, Rosner MR (2006). Raf kinase inhibitory protein regulates aurora B kinase and the spindle checkpoint. *Mol Cell* **23:** 561-574.

Fuchs BC, Fujii T, Dorfman JD, Goodwin JM, Zhu AX, Lanuti M *et al* (2008). Epithelial-to-mesenchymal transition and integrin-linked kinase mediate sensitivity to epidermal growth factor receptor inhibition in human hepatoma cells. *Cancer Res* **68:** 2391-2399.

Huang Y, Yao Y, Xu HZ, Wang ZG, Lu L, Dai W (2009). Defects in chromosome congression and mitotic progression in KIF18A-deficient cells are partly mediated through impaired functions of CENP-E. *Cell Cycle* **8:** 2643-2649.

Janssen A, Kops GJ, Medema RH (2009). Elevating the frequency of chromosome mis-segregation as a strategy to kill tumor cells. *Proc Natl Acad Sci U S A* **106:** 19108-19113.

Janssen A, Medema RH (2011). Mitosis as an anti-cancer target. *Oncogene* **30:** 2799-2809.

Kelland L (2007). The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer* **7:** 573-584.

Lens SM, Voest EE, Medema RH (2010). Shared and separate functions of polo-like kinases and aurora kinases in cancer. *Nat Rev Cancer* **10:** 825-841.

Luo J, Emanuele MJ, Li D, Creighton CJ, Schlabach MR, Westbrook TF *et al* (2009). A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. *Cell* **137:** 835-848.

Mayr MI, Hummer S, Bormann J, Gruner T, Adio S, Woehlke G *et al* (2007). The human kinesin Kif18A is a motile microtubule depolymerase essential for chromosome congression. *Curr Biol* **17:** 488-498.

Meylan E, Dooley AL, Feldser DM, Shen L, Turk E, Ouyang C *et al* (2009). Requirement for NF-kappaB signalling in a mouse model of lung adenocarcinoma. *Nature* **462:** 104-107.

Nagahara M, Nishida N, Iwatsuki M, Ishimaru S, Mimori K, Tanaka F *et al* (2011). Kinesin 18A expression: Clinical relevance to colorectal cancer progression. *Int J Cancer*.

Narita M, Nunez S, Heard E, Lin AW, Hearn SA, Spector DL *et al* (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* **113:** 703-716.

Puyol M, Martin A, Dubus P, Mulero F, Pizcueta P, Khan G *et al* (2010). A synthetic lethal interaction between K-Ras oncogenes and Cdk4 unveils a therapeutic strategy for non-small cell lung carcinoma. *Cancer Cell* **18:** 63-73.

Recino A, Sherwood V, Flaxman A, Cooper WN, Latif F, Ward A *et al* (2010). Human RASSF7 regulates the microtubule cytoskeleton and is required for spindle formation, Aurora B activation and chromosomal congression during mitosis. *Biochem J* **430:** 207-213.

Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Varambally R, Yu J, Briggs BB *et al* (2007). Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. *Neoplasia* **9:** 166-180.

Roesch A, Becker B, Schneider-Brachert W, Hagen I, Landthaler M, Vogt T (2006). Re-expression of the retinoblastoma-binding protein 2-homolog 1 reveals tumor-suppressive functions in highly metastatic melanoma cells. *J Invest Dermatol* **126:** 1850-1859.

Roesch A, Fukunaga-Kalabis M, Schmidt EC, Zabierowski SE, Brafford PA, Vultur A *et al* (2010). A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. *Cell* **141:** 583-594.

Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S *et al* (2010). A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* **141:** 69-80.

Shaw AT, Winslow MM, Magendantz M, Ouyang C, Dowdle J, Subramanian A *et al* (2011). Selective killing of K-ras mutant cancer cells by small molecule inducers of oxidative stress. *Proc Natl Acad Sci U S A* **108:** 8773-8778.

Sherwood V, Manbodh R, Sheppard C, Chalmers AD (2008). RASSF7 is a member of a new family of RAS association domain-containing proteins and is required for completing mitosis. *Mol Biol Cell* **19:** 1772-1782.

Singh A, Greninger P, Rhodes D, Koopman L, Violette S, Bardeesy N *et al* (2009). A gene expression signature associated with "K-Ras addiction" reveals regulators of EMT and tumor cell survival. *Cancer Cell* **15:** 489-500.

Singh A, Settleman J (2010). EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* **29:** 4741-4751.

Song MS, Chang JS, Song SJ, Yang TH, Lee H, Lim DS (2005). The centrosomal protein RAS association domain family protein 1A (RASSF1A)-binding protein 1 regulates mitotic progression by recruiting RASSF1A to spindle poles. *J Biol Chem* **280:** 3920-3927.

Strebhardt K (2010). Multifaceted polo-like kinases: drug targets and antitargets for cancer therapy. *Nat Rev Drug Discov* **9:** 643-660.

Stumpff J, von Dassow G, Wagenbach M, Asbury C, Wordeman L (2008). The kinesin-8 motor Kif18A suppresses kinetochore movements to control mitotic chromosome alignment. *Dev Cell* **14:** 252-262.

Tang N, Marshall WF, McMahon M, Metzger RJ, Martin GR (2011). Control of mitotic spindle angle by the RAS-regulated ERK1/2 pathway determines lung tube shape. *Science* **333:** 342-345.

Thomson S, Buck E, Petti F, Griffin G, Brown E, Ramnarine N *et al* (2005). Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition. *Cancer Res* **65:** 9455-9462.

Uetake Y, Sluder G (2010). Prolonged prometaphase blocks daughter cell proliferation despite normal completion of mitosis. *Curr Biol* **20:** 1666-1671.

van der Weyden L, Adams DJ (2007). The Ras-association domain family (RASSF) members and their role in human tumourigenesis. *Biochim Biophys Acta* **1776:** 58-85.

Wang Y, Ngo VN, Marani M, Yang Y, Wright G, Staudt LM *et al* (2010). Critical role for transcriptional repressor Snail2 in transformation by oncogenic RAS in colorectal carcinoma cells. *Oncogene* **29:** 4658-4670.

Whitehurst AW, Ram R, Shivakumar L, Gao B, Minna JD, White MA (2008). The RASSF1A tumor suppressor restrains anaphase-promoting complex/cyclosome activity during the G1/S phase transition to promote cell cycle progression in human epithelial cells. *Mol Cell Biol* **28:** 3190-3197.

Yagoda N, von Rechenberg M, Zaganjor E, Bauer AJ, Yang WS, Fridman DJ *et al* (2007). RAS-RAF-MEK-dependent oxidative cell death involving voltage-dependent anion channels. *Nature* **447:** 864-868.

Yang WS, Stockwell BR (2008). Synthetic lethal screening identifies compounds activating iron-dependent, nonapoptotic cell death in oncogenic-RAS-harboring cancer cells. *Chem Biol* **15:** 234-245.

Zecevic M, Catling AD, Eblen ST, Renzi L, Hittle JC, Yen TJ *et al* (1998). Active MAP kinase in mitosis: localization at kinetochores and association with the motor protein CENP-E. *J Cell Biol* **142:** 1547-1558.

Zhang C, Zhu C, Chen H, Li L, Guo L, Jiang W *et al* (2010). Kif18A is involved in human breast carcinogenesis. *Carcinogenesis* **31:** 1676-1684.

SUMMARY

NEDERLANDSE SAMENVATTING

CURRICULUM VITAE

LIST OF PUBLICATIONS

DANKWOORD

SUMMARY

The research summarized in this thesis describes the identification of novel drug targets that are only toxic in a cancer-specific context, thereby leaving normal cells unharmed. The applied research tool is RNA-interference (RNAi), a technique that enables knockdown of expression of specific genes. RNAi can be applied in high-throughput screening efforts to identify genes that are causally related to distinct phenotypes. These genes could represent potential novel drug targets. Chapter two elaborates on the application of RNAi screening efforts to identify novel drug targets in cancer. Three different studies are described in this thesis that identified cancer-specific drug targets.

Chapter three describes the results of a drug-sensitization screen. The identification of drug targets that, upon inhibition, sensitize cells to an already clinically applied drug can be beneficial in two ways. First, the combination of a sensitizing drug with a regular drug allows administration of a lower amount of the regular drug, thereby potentially reducing side effects. Secondly, a small fraction of less sensitive tumor cells can be selected for under drug treatment, due to high heterogeneity of the tumor population. This could lead to recurrence of the tumor, often seen in cancer. Sensitization of the tumor with an additional compound could rapidly kill all tumor cells, preventing the selection of cells that are resistant to the original compound. Cisplatin, a DNA-crosslinking agent, is widely used as chemotherapy in the clinic, however, often tumors recur after treatment with this drug. We used cisplatin in an RNAi sensitization screen and showed that knockdown of *ATR*, *REV3L*, *BRCA1*, or *BRCA2*, genes that function in the DNA-damage response, greatly sensitizes primary cells that are depleted for the tumor suppressor p53 to suboptimal doses of cisplatin. The next step will be to validate compounds against these targets in mouse models of cancer.

Chapter four describes a synthetic lethal screening approach to identify novel drug targets that are specifically required for oncogenic HRAS-driven cancer. Synthetic lethality means that mutation or depletion of a gene, which is non-lethal to a cell, is lethal in the context of mutation or depletion of a second (non-lethal) gene. Since tumor cells have specific hallmarks (see introduction of this thesis), they potentially have a rewired genetic interaction network. Synthetic lethal screening allows for the identification of genes essential for tumor cells but not to normal cells. For this synthetic lethal screen we used a pair of isogenic cell lines, of which one cell line expressed $HRAS^{V12}$ an oncogenic mutant of RAS, the most frequently mutated oncogene in pancreas, lung, and colon cancer. A library targeting 6800 individual genes was screened and we identified 7 synthetic lethal genes. These genes were not only lethal with the expression of $HRAS^{V12}$ but also in cell lines expressing oncogenic mutants of the K-and N-RAS family members, but not normal cell lines. For one of these genes, *KIF18A*, we showed that its knockdown prolonged mitotic progression in RAS-mutant cells leading in most cases to cell death. The next step will be to validate this finding in panels of tumor cell lines and mouse models of oncogenic RAS-driven tumors.

Chapter five describes an RNAi screen for bypass of senescence. Senescence is one of the cell-intrinsic mechanisms that protects against oncogenic transformation.

Senescent cells show a dramatic change in gene expression together with extensive chromatin modification. Here, we used a family of chromatin modifying enzymes in an RNAi screen in a mouse cell line model for senescence. We identified Jarid1b as being required for the induction of senescence in this model. We further demonstrate that Jarid1b through binding to Rb, contributes to the suppression of E2f-target genes specific for DNA replication and cell cycle progression during senescence. Senescent cells could require Jarid1b, like other chromatin modifiers, to remain a repressive induction of E2f-target genes. On the other hand, cells that have repressed expression of genes involved in DNA replication and cell cycle progression regularly escape toxicity induced by chemotherapy. Therefore, these escaped cells could seed a new tumor after treatment. In this context, Jarid1b can be applied as a drug target, since its inhibition by small molecules could kill slowly or non-dividing cells in a tumor mass. However, this hypothesis should be further explored in preclinical models.

The work described in this thesis has led to the identification of potential novel drug targets that are specific for a defined (genetic or therapeutic) context. The next steps will be to test these drug targets with compounds in more advanced models of cancer. If successful, the drug targets described here could be exploited to attack tumors in a more specific manner and thereby contributing to patient-specific therapy.

NEDERLANDSE SAMENVATTING

Sinds 2008 is kanker doodsoorzaak nummer één in de westerse wereld. In dat jaar alleen werden er wereldwijd 12,7 miljoen mensen gediagnosticeerd met kanker en stierven er wereldwijd 7,6 miljoen mensen aan deze aandoening. Kanker is een verzamelnaam voor ongeveer 120 zeer complexe ziektes die zich uiten in een ongeremde proliferatie van cellen op een of meerdere locaties in het lichaam. Kanker kan in een vroeg stadium goed behandeld worden, maar in een (ver)gevorderd stadium leiden behandelingen met chemotherapie en straling in veel van de gevallen tot een terugkeer van de tumor, vaak met dodelijke afloop vanwege de uitzaaiingen die dan optreden. Daarnaast brengt chemotherapiebehandeling zeer vervelende bijwerkingen teweeg. Een nieuwe generatie medicijnen tegen kanker, de zogenaamde 'targeted therapeutics' (gerichte medicijnen), vallen tumorspecifieke doelwitten (dit zijn in de meeste gevallen eiwitten, zie kader) aan, waardoor de tumorcellen doodgaan en de normale lichaamscellen intact blijven. Het doel van het onderzoek beschreven in dit proefschrift is het vinden van nieuwe kankerspecifieke doelwitten. Voor deze doelwitten kunnen dan medicijnen ontwikkeld worden, een taak voor de farmaceutische industrie, die zeer specifiek kunnen zijn voor het bestrijden van bepaalde types kanker. De methode die voor dit onderzoek gebruikt wordt is 'RNAi-screening' (screenen op basis van RNA-interferentie). Dat is een techniek waarmee men op een lopendebandachtige manier de expressie, en dus voor een groot deel de functie, van genen kan uitschakelen.

In de introductie van dit proefschrift (hoofdstuk een) wordt beschreven wat kankercellen anders maakt dan normale cellen. Deze eigenschappen kunnen dus uitgebuit worden om kankercellen te onderscheiden en zo specifiek die cellen aan te vallen.

In hoofdstuk twee wordt beschreven hoe de hierboven genoemde techniek RNAi-screening kan bijdragen aan de nieuwe generatie kankerspecifieke medicijnen.

In hoofdstuk drie wordt een onderzoek beschreven dat nieuwe doelwitten voor medicijnen zoekt die mogelijk kankercellen nóg gevoeliger maakt voor cisplatina, een veelgebruikte chemotherapie in de kliniek. Dit kan twee positieve gevolgen hebben voor de patiënt: ten eerste hoeft er nu minder cisplatina in de behandeling gebruikt te worden, waardoor de patiënt minder last van bijwerkingen heeft. Ten tweede zou de tumor nu nog beter behandeld kunnen worden, want behandeling met cisplatina heeft vaak cisplatinaresistente tumorcellen tot gevolg. Een combinatie van twee medicijnen die tegelijkertijd de tumor aanvallen, zou een hogere slagingskans van de behandeling kunnen betekenen.

Twee van de meest voorkomende types kanker bij zowel vrouwen als mannen zijn long- en darmkanker. Opmerkelijk genoeg is één gen het meest gemuteerd in deze types kanker. Dit gen heet K-RAS. Patiënten die een tumor hebben die is gediagnosticeerd met een mutatie in K-RAS, worden vaak uitgesloten van behandeling met zogenaamde gerichte medicijnen omdat men uit resultaten uit het verleden weet dat deze behandeling niet zal aanslaan. De studie beschreven in hoofdstuk vier van dit proefschrift heeft als onderwerp het identificeren van een gen door screenen middels RNA-interferentie dat mogelijk als doelwit van een medicijn (dat door dit onderzoek

hopelijk ontwikkeld gaat worden) kan dienen om specifiek cellen met een gemuteerd K-RAS aan te vallen. Als er een medicijn ontwikkeld kan worden tegen het eiwit dat wij ontdekt hebben, zouden in de toekomst patiënten met een tumor die een mutatie hebben in het K-RAS-gen beter behandeld kunnen worden.

Onze lichaamscellen hebben een intrinsiek verdedigingsmechanisme tegen het transformeren van een normale cel in een kankercel. Dit mechanisme heet senescentie, van het Latijnse woord senescentia, wat 'oud worden' betekent. Cellen kunnen dus snel oud worden, of deze verschijnselen vertonen, onder condities die tumorveroorzakend zijn. Hoofdstuk vijf in dit proefschrift beschrijft een RNA-interferentiescreen die genen ontdekt die betrokken zijn bij senescentie. In dit hoofdstuk wordt een studie beschreven die een gen ontdekt, Jarid1b, dat betrokken is bij het onderdrukken van expressie van genen die betrokken zijn bij DNA-replicatie en voortgang in celdeling. Beide zijn processen die stilgelegd dienen te worden om senescentie te induceren. Ironisch genoeg kan het stilleggen van DNA-replicatie en voortgang in de celdeling ook betrokken zijn bij het herbevolken van een tumor door individuele cellen. Chemotherapie is namelijk gericht op het doden van sneldelende cellen. Cellen die langzaam of niet delen, bijvoorbeeld cellen die in senescentie gaan, worden niet beschadigd door de chemotherapie. Na behandeling kunnen het juist deze cellen zijn die verantwoordelijk zijn voor de hergroei van een tumor. Daardoor kan Jarid1b een zeer interessant doelwit zijn dat wellicht in combinatie met een ander medicijn tegen kanker gebruikt kan worden. Deze hypothese dient zeer zeker nader onderzocht te worden.

Dit proefschrift beschrijft het ontdekken van genen die als nieuwe doelwitten voor medicijnen kunnen dienen om zo de ziekte kanker nog beter te kunnen behandelen. Vervolgonderzoek zal moeten uitwijzen of deze ontdekte doelwitten ook klinisch toepasbaar zijn.

Verklarende woordenlijst:

DNA: Het cellulaire opslagmechanisme van erfelijke informatie en componenten die een organisme nodig heeft om normaal te kunnen functioneren. Specifieke, gedefinieerde stukken informatie worden genen genoemd.

Gen: Een specifieke eenheid van erfelijke en/of functionele informatie dat codeert voor een eiwit.

RNA: Het tussenproduct van een gen en een eiwit. Voordat genetische informatie vertaald wordt in een product (het eiwit), wordt het gen gekopieerd als 'RNA'.

Eiwit: Componenten in een organisme die verantwoordelijk zijn voor het omzetten (katalyseren) van chemische reacties, de communicatie tussen cellen, het doorgeven van signalen in en tussen individuele cellen, het structureren van cellen en weefsels en intra- en transcellulair transport van chemische stoffen.

RNAi: Een techniek om RNA van een specifiek gen af te breken, zodat er geen eiwit meer geproduceerd kan worden en dus een specifieke functie wordt stilgelegd. Dit kan grote gevolgen hebben voor het functioneren van het hele organisme.

CURRICULUM VITAE

Jeroen Nijwening was born on October 25th, 1979 in Heerlen. He graduated from the Bernardinuscollege gymnasium Heerlen in 1998. In that same year he started to study Biology at Utrecht University. After his propedeuse in 1999 he continued to study Fundamental Biomedical Sciences at the faculty of Biology at Utrecht University. During his studies he performed several research internships. At 2002 he started an internship at the Hubrecht Institute in Utrecht, where he worked under the supervision of Jeroen den Hertog on receptor protein tyrosine phosphatise alpha (RPTP-α). At the end of 2002 until the end of 2003 he worked as a research assistant at Phenomix Corporation in San Diego, California, where he was involved in phenotype characterization of mouse models created by insertional mutagenesis. Subsequently, he wrote his doctoral thesis under the supervision of Tony Hunter at the Salk Institute of Biological Studies in San Diego, California and Rene Bernards at the Netherlands Cancer Institute in Amsterdam. In November 2004 he started his PhD in the labs of Roderick Beijersbergen and Rene Bernards at the Netherlands Cancer Institute. During his PhD training he was involved in the set-up and performance of high-throughput RNAi screening efforts to identify novel drug targets in cancer. The results of this research are described in this thesis.

LIST OF PUBLICATIONS

Nijwening JH, Kuiken HJ, van Zon W, Voets E, Wolthuis RM, Bernards R, Beijersbergen RL (2011). KIF18A displays a synthetic lethal interaction with oncogenic $HRAS^{V12}$ (Manuscript in preparation)

Nijwening JH, Geutjes EJ, Bernards R, Beijersbergen RL (2011). The Histone Demethylase Jarid1b (Kdm5b) Is a Novel Component of the Rb Pathway and Associates with E2f-Target Genes in MEFs during Senescence. *PLoS One* **6:** e25235.

Nijwening JH, Kuiken HJ, Beijersbergen RL (2011). Screening for modulators of cisplatin sensitivity: unbiased screens reveal common themes. *Cell Cycle* **10:** 380-386.

Nijwening JH, Beijersbergen RL (2010). Using large-scale RNAi screens to identify novel drug targets for cancer. *IDrugs* **13:** 772-777.

Kortlever RM, **Nijwening JH**, Bernards R (2008). Transforming growth factor-beta requires its target plasminogen activator inhibitor-1 for cytostatic activity. *J Biol Chem* **283:** 24308-24313.

DANKWOORD

Promoveren doe je niet alleen. Sterker nog, mijn promotieonderzoek had niet eens afgerond kunnen worden zonder de enorme hoeveelheid hulp en steun van collega's, vrienden en familie wier onmisbare bijdrage hier wordt gereduceerd tot luttele pagina's. Voordat je verder leest, heb je de samenvatting eigenlijk al gelezen? Of misschien de discussie? Beloof me dat je deze zult lezen na dit laatste hoofdstuk.

Roderick, het is een voorrecht om jou als begeleider te mogen hebben. Door het zelfstandig laten werken van je OIO's en je ongebreidelde kritiek op andermans, maar vooral ook je eigen, resultaten waren mijn jaren onder jouw vleugels zeer leerzaam. Je blijft hameren op de wetenschappelijke methode en steeds weer weet je je personeel scherp te houden door projecten een nieuwe richting te geven. Ik ben benieuwd wat je de wereld gaat brengen als je op de top van de Maslow piramide staat.

Rene, bedankt dat je mij een positie in je lab hebt aangeboden. Je heldere visie, bijdehante scherpte en je onmeetbare intelligentie werken zeer inspirerend. Waren er maar wat meer wetenschappers zoals jij, dan was kanker zo opgelost. Niks dan lof over jou!

Ernst, het was zeer prettig om met je samen te werken. Ik bewonder professionele en gedreven houding, de rust die je uitstraalt en je doorzettingsvermogen. De vele nachten samen doorwerken en ploegendiensten in de weekenden hebben zeker mooie wetenschappelijke resultaten opgeleverd. Toch beschouwde ik ons vaak als broers van Tantalus, waar high-impact papers binnen handbereik lagen, verdwenen deze weer als we ernaar reikten. Buiten het lab, heb ik ook veel plezier beleefd met jou, Mr. Hyde. Succes Ernst, jouw glanzende carrière is al begonnen!

Johan, collega, vriend, Nemesis. Waar ik doorschoot in mijn holistische benadering op ons onderzoek en vaak vooral door wilde gaan, trok jij mij altijd weer terug naar de realiteit met je voor mij onbegrijpelijke statistische analyses. Dat had echter ook zo zijn voordelen, als ik niet zeker wist of mijn resultaten significant waren, wist jij er altijd wel de juiste toets op los te laten! Je was mijn ideale sparringpartner en je bent de gangmaker op B7. Zonder jou, geen festiviteiten of gezelligheden. Ik heb je eigenlijk nooit kunnen bedanken voor je enorme hoeveelheid hulp aan het einde van mijn promotie.

Klaas, ik moet altijd flink lachen om je niets ontziende relativeringsvermogen. Je bent een ware aanvulling voor de Beijersbergen groep. Bedankt dat je mijn paranimf wilt zijn en ik wens je zeer veel succes in dit lab waar alle kansen liggen. Denk eraan, screenen is net als goud zoeken; voordat je een ader aanboort, stuit je eerst op lagen tin en zink.

Een geslaagde promotie is het proces waarin orde uit chaos ontstaat en dit zou zich best kunnen laten modelleren door een van de componenten uit de chaostheorie: het juiste duwtje van een collega kan je onderzoek een enorme impuls geven. Er zijn vele collega's van de Beijersbergen en Bernards labs die mij meerdere malen hebben geholpen door (nogmaals) een protocol uit te leggen, of door het doneren van construct, of het geven

van een cellijn. Ik wil de volgende mensen vooral bedanken omdat ze gezellige en kleurrijke collega's zijn waar ik veel leuke momenten mee heb beleefd gedurende heel wat dagen in Amsterdam, tot diep in de nacht in Lausanne, en tot de ochtend in Boedapest: Guus (voor ons geen intelligent design of schepper), Bram (mijn persoonlijke Go sensei), Marielle (je was een zeer gezellige en behulpzame collega, ik ga je missen!), Katrien (ik beschouwde jou toch altijd als de meest normale persoon in het lab), Astrid en Annemieke (gezellige dames en het labgeheugen), Annette (forever young), Floris, Cor, Rianne (respect, een gezin, oncoloog en OIO tegelijkertijd) en Wouter (nog even door de zure appel heen bijten).

I know that Dutch is a difficult language for most of you guys, and that learning Dutch will not be prioritized over successful experiments. By the time of writing these acknowledgements, I counted 12 different nationalities in my lab. That is the reason why I included this English section.

Sid, my dear friend, we started at the same day and it is amazing to see how many high-quality papers you managed to publish while you were taking care of such a beautiful family. It is a true honor to have you as my paranimf! Prasanth, thanks for your amazing help with the ChIPs, you are the best! I will miss the political discussions we had. Chong and Anirudh, you guys will translate the discus of Phaistos one day. Pasi and Ben, thank you so much for all your help with our screens, I will miss you guys. Jordi and Kathy, you are really fun to hang out with. Winny, keep an eye on Klaas for me. Ian, Lorenza, Valentina, Gandhi once had an advice for scientists within the NKI (that is at least how I interpret his quote): "First they ignore you, then they laugh at you, then they fight you, then you win". There were many non-Dutch PhD students and postdocs outside my department that contributed to this thesis in one way or the other: Izhar (I hope to continue seeing you after my NKI-era), Ewald, Eitan, Jens, Joanna, Shalin (I remember me introducing you to a famous drink of Gerard Adriaan upon your completely jetlagged arrival in Amsterdam), Dominika, Celia, Sedef, Christelle, Francesca, Anke, Baoxu Xanthippi, and of course Andrej my constant influx of positive energy.

Er is ook een groeiend groepje gezellige en hardwerkende mensen op mijn afdeling waardoor ik mij altijd stiekem geïntimideerd voelde door de schone Western blotjes die ze wisten te produceren: De Wolthuisjes. Bedankt Janneke voor je hulp tijdens het FACSen, Linda altijd feest met jou, Wouter en Erik voor het filmen van onze mitoses, Michiel en Bas. Rob, natuurlijk, voor de begeleidende discussies over mijn synthetic lethal project.

De afdeling wordt af en toe opgevrolijkt door de aanwezigheid van studenten die ons weer even jong laten voelen. Ten eerste wil ik mijn eigen studenten Undine en Eva bedanken voor hun inzet en vertrouwen in mijn aanpak. Rogier (de sterkste man die ik ken), Karla, Donna, Esther, Bart, Ellen en Laura, bedankt voor alle gezellige momenten.

Toen ik jaren geleden als PhD-student begon in het Nederlands Kanker Instituut, liepen er op mijn afdeling al heel wat topwetenschappers in spé rond. Deze "oude

garde" wil ik nog speciaal bedanken: Roderik ik verwacht veel van je intellect, op zijn minst consilience. Ik waardeer onze zeer plezierige discussies die gelukkig afgewisseld worden met de nodige dosis humor, ik hoop dat wij elkaar nog vaak zullen zien. Mandy, Jasper en Armida, bedankt dat jullie mij hebben opgepakt en verzorgd, toen ik als baby in het Beijersbergen / Bernards lab kwam aankruipen. Veel van mijn dank gaat uit naar de ex-leden van mijn afeling die mijn onderzoek op de rails hebben gezet met veel nuttig advies of mij op andere wijze hebben gesteund: Thijn, Sebastian, Menno, Luis, Piet, Mirjam, Miguel, Benjamin, Linda, Miranda, Helena, Sue-Ellen, Michael, Michiel "gezellig rechts" en Franciska mijn Krav Maga buddy.

Ook wil ik een paar regels wijden aan het bedanken van Principal Investigators die mijn onderzoek middels kritische opmerkingen in de juiste richting hebben geloosd: Jos, Hein, Daniel, Bas, Maarten, Titia, Piet, Jacques, Reuven, Huib en Tassos.

Dan zijn er nog de nodige (ex-)collega's buiten de afdeling Moleculaire Carcinogenese met wie ik veel leuke momenten heb gehad (al dan niet geholpen door spiritualiën), interessante discussies mee heb gevoerd, of wetenschappelijke kennis heb uitgewisseld: Chiel (al heel wat jaren lopen onze carrières parallel, nu sla ik echter een nieuwe weg in), Maaike (ik hoop dat wij nog lang vrienden zullen blijven!), Martijn, Marieke, Bernike, Thomas, Liesbeth, Joanna, Henne, Puck, Pim (de vriendelijkheid zelve), Valerie, Kitty, Carlos, Sietske, Bastiaan, Niek (mijn politieke evenbeeld), Rogier, Dieuwke, Jolita, Mike, Daoud, Mark, Alex, Christian, Britta, Victor, Marlieke, Petra, Inge, Joke, Ulrich, Wilbert, Marjon, Boris, Peter, Camiel, Marleen, Tinke, Tanja, Mathijs Voorhoeve, Jarno, Dalila, Anna, Richard, Evert, Artur, Rik, Vincent, Marit, Yme, Henri en Patty. Van Top Institute Pharma wil ik Jorg en Karin nog speciaal bedanken.

Zonder de kundige faciliteiten in het NKI waren wij veel tijd en resources kwijt geweest aan het steeds opnieuw uitvinden van het wiel, daarom ben ik deze collega's zeer dankbaar: Roelof (gewoon bedankt voor alles), Anita, Frank, Lenny, Lauran, Ron -gecensureerd-, Marja, Wim, Arno en Minze.

OIO zijn is geen opleiding en niet alleen een beroep, maar vooral een levensstijl. De hoge werkdruk en slopende internationale competitie die daarmee gepaard gaan, konden in mijn geval alleen goed verdragen worden door de niet aflatende steun van veel vrienden buiten mijn leven in het lab. Tjakko, mijn wetenschappelijke carrière is begonnen door jou. Ik herinner me onze discussies nog levendig die begonnen op de faculteit Biologie, doorgingen tot diep in de nacht in café de Flater en eindigden op Black's beach. Veel succes met het opzetten van je eigen research groep en bedankt voor de onvergetelijke tijd in La Jolla (work hard, play hard) en Phenomix (thank you Laura Shawver and good luck with the Clearity Foundation!).

Niels, hoe kan het dat jij mij toch altijd verslaat met squashen, schaken of golfen? Maar misschien zegt dat meer over mij dan over jou. Jorrit, "homie", jammer dat mijn deelname aan de surftrips inverse gecorreleerd was met het naderen van mijn promotiedatum. Maarten en Nick, ik bewonder jullie discipline en ambities. Foreno

en Irmo bedankt voor de avonturen diep in de grotten of afdrijvend op zee. Gerben, wanneer gaan we weer merovingische relikwieën zoeken?

Dan wil ik nog enkele hele speciale vrienden bedanken voor de buitengewone interesse die ze in mijn onderzoek tonen, de wijnproeverijen en de hedonistische weekenden in Grasse: Jeroen, Mary, Marlies, Rutger, Evelien, Sophie, Martin, Laura, Christian, Mimi, Beathe, Anna en Douwke. Vrienden, ik kijk uit naar de vele leuke momenten die ik nog met jullie ga beleven!

Aisun, Matthijs, Orlo, Erika, Jan-Joost, Ginny, Rob en Damay, succes met jullie specialisaties en eigen praktijken. Cor, Suzanne (bedankt voor het verbeteren van de Nederlandse Samenvatting). Ferdinand en Luc, bedankt voor de hang-loose momenten in de States. Arnoud en Bas, Hubrecht-buddies. Louis & Heike, bedankt. Jan-Frens, onze paden blijven elkaar nog wel kruisen binnen de Life Sciences.

&

Arnout, eeuwige vriend, wij delen veel passies, variërend van Wushu tot whisky. Ik waardeer je om je diversiteit, om alles wat je hebt opgebouwd en je vermogen om je in diverse kringen te bewegen. Hopelijk gaan wij nog vaak samen duiken en eilanden bezoeken.

Martijn, er is niemand meer genetisch verwant aan mij dan jij bent. Toch uiten onze fenotypes zich zeer verschillend: ik bioloog, jij psycholoog. Aardig voer voor onze never ending nature versus nurture discussies. Ik vind het mooi om te zien hoeveel wij van elkaar (hebben) kunnen leren. Het gaat je goed, broertje.

Marion en Henk, lieve ouders, al vroeg hebben jullie mijn interesse in de wetenschap gewekt. Ik kreeg een microscoop voor mijn 8e verjaardag en toen ik 12 was, bouwde ik het tuinhuis om tot laboratorium. Jullie hebben mij gestimuleerd om mijn kennis te verbreden en te verdiepen. Bedankt dat jullie er altijd voor mij waren en mij gesteund hebben in alles wat ik deed.

Estimados Carlos y Norma, quisiera expresar mi agradecimiento hacia ustedes por su inmenso apoyo, cariño y respeto.

Querida Valeria, estoy muy felíz con vos. Somos una pareja de estudiantes de doctorado con una vida muy ocupada, pero así y todo hemos encontrado mucha diversíon en cada momento que pasamos juntos. Admiro tu habilidad de hablar con todo el mundo, tu inteligencia y tu actitud positiva. Cada vez que llegas el sol brilla. Espero disfrutar el mundo juntos.

Jeroen