Functional identification of novel modulators of the p53 and TGFβ signaling networks

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Functional identification of novel modulators of the p53 and TGFβ signaling networks

Functionele identificatie van nieuwe componenten van de p53 en TGFβ signaaltransductie netwerken

(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. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op vrijdag 22 januari 2010 des middags te 12.45 uur

door

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Chapter I

Introduction

Characteristics and treatment of cancer

The fight against cancer has been an ongoing struggle even before Richard Nixon officially declared "war" against cancer in 1971. But, despite all efforts this disease is currently the leading cause of death in the western world. Although much progress has been made in extending the life of cancer patients, in about half of the cases a cure is not available.

Cancer is a very heterogeneous disease that is defined by specific characteristics, or hallmarks. These include the ability to evade apoptosis, to be self-sufficient in growth signalling, the insensitivity for anti-growth signals, and a limitless replicative potential. Intrinsic alterations also underly the spreading of cancer by stimulating angiogenesis, invasion and metastatic capacity [1]. The discovery of novel anticancer drugs remains a tantalizing task with a very low success rate despite the investment of billions of dollars in basic and clinical research. However, the progress that has been made in understanding the molecular basis of cancer has led to a shift in cancer drug development strategy. With this knowledge, rational, hypothesis-driven, mechanism-based molecular therapeutics can be developed. An example of this approach is the anticancer drug imatinib (Gleevec), which targets the BCR-ABL fusion protein in chronic myeloid leukemia (CML). In contrast to normal tissue CML cells are dependent on BCR-ABL for their survival. This has created a therapeutic window where Imatinib (Gleevec) inhibits CML without serious side effects [2]. The success of this targeted therapeutic has fuelled expectations about increased specificity and efficacy of the next generation cancer drugs.

Identification of cancer drug targets

The study of the genetic and molecular basis of cancer has yielded detailed knowledge on signal transduction pathways, regulatory networks and protein-protein interactions. Many of the proteins involved in these networks have altered activity in cancer, with the consequence being uncontrolled growth and survival.

Large-scale sequencing efforts and other genomic approaches have identified many mutations, deletions and amplifications in human tumours. In addition, large-scale efforts using gene-expression profiling have led to identification of oncogenic pathway signatures as potential guides for therapy. An important goal of cancer research will be to prioritize and select targets based on their functional involvement in tumour onset and progression.

Functional genomics

The functional role of genes and their products, which are potential drug targets, can be identified by experimental approaches that are collectively termed functional genomics. In contrast to many other techniques, which identify correlations, the aim of functional genomics is to identify causal relationships. Functional genomics is a technique that uses genetic perturbations to study biological problems [3]. Examples of perturbation technologies are loss-off-function genetic screens, cDNA overexpression screens and insertional mutagenesis screens, among many others. In this chapter, I will mainly focus on loss-of-function genetic screens. Loss-of-function genomics was traditionally a technique that was exclusively used in model organisms. For functional genomic screening, yeast was the initial model of choice for drug testing because of the well-known genetics and ease of manipulation. Full genome collections of yeast knockout strains were constructed [4]

and libraries of knockouts have been screened in a variety of assays i.e. lethality caused by simultaneous knockout of two different genes [5] and drug sensitivity [6, 7]. The data from these screens have been very informative in understanding the mode of action and possible resistance mechanism of drugs. In addition, these screens can provide a framework in which human biology and disease may be understood [4]. However, translation of yeast data to mammalian systems is not always possible since a number of yeast genes have no human orthologue and the increased complexity of mammalian cell signalling [8].

The discovery of RNA interference (RNAi) has allowed researchers to perform loss-of-function screens in higher organisms. A mayor step forward was made in 2001 when it was shown that 21-nt long RNAs mediate RNAi without eliciting an antiviral response in mammalian cells [9]. More recently, expression vectors have been developed that direct the synthesis of short hairpin RNAs (shRNAs) [10, 11]. These shRNAs then act as small interfering RNA (siRNA) molecules that can suppress gene expression. In addition to plasmid based shRNA libraries, large sets of synthetic small interfering RNAs have become available in the last couple of years (for available RNAi libraries see reviews; [12-15]).

Until now, functional genetic siRNA screens have predominantly contributed to a better understanding of cellular pathways and enabled the identification of modulators of drug response. As example, a genome wide siRNA screen was performed with the goal to identify genes that after silencing sensitised to PARP inhibition [16]. Modulators of response to Poly ADP-Ribose Polymerase (PARP) inhibitors are investigated in order to identify drug targets for combinatorial therapy. PARP is a nuclear enzyme that recognizes and binds to DNA breaks facilitating DNA break repair. PARP inhibitors are currently in clinical trials, since cells that have defects in homologous recombination pathways are highly sensitive for PARP inhibition. Cyclin dependent kinase 5 (CDK5) was identified by a functional RNAi screen as an important determinant for the sensitivity to PARP inhibition [16]. The same approach has been taken to identify genes that after ablation can enhance sensitivity to cancer therapeutics such as cisplatin [17], paxlitaxel [18], gemcitabine [19] and tamoxifen [20].

Finally, functional genomics siRNA screens are an effective tool to identify tumour specific vulnerabilities. Identification of a drug target that only causes lethality in tumour cells is the starting point for development of tumour-specific anti-cancer drugs. The specificity of such targets is based on an dependency of a specific genetic alteration. This can be the oncogene itself (oncogene addiction) or other targets whose activity is required to allow oncogenic mutation to persist in tumour cells (non-oncogene addiction). The latter is also described as synthetic lethality, a situation in which loss of a gene is only lethal in the background of another, pre-existing, genetic alteration [21-23].

CANCER SPECIFIC SYNTHETIC LETHAL SCREENS

RNAi in mammalian systems paved the road for cancer target identification through functional genomics using cell-based assays with human cancer cell lines. Specific lethal interactions can be identified in isogenic cell-line pairs. Recently, three siRNA screens have been published that identified synthetic lethal genes in combination with oncogenic *KRAS*. Activating mutations in the *KRAS* gene are found in approximately 30% of human cancer cells. Importantly these tumours depend on the continious activation of the RAS pathway for their survival. However, attempts to target the RAS pathway, either directly

or indirectly have remained largely unsuccessful. It is therefore of great interest to identify genes or cellular pathways that can be used to specifically kill RAS transformed cells. By using large scale RNAi screens, either using siRNA or shRNA technologies, several genes were identified displaying a synthetic lethal phenotype with RAS. Among those are multiple components of the mitotic checkpoint suggesting increased mitotic stress as consequence of RAS activation. This could be explored for therapeutic strategies in RAS mutant tumours [24-26]. In addition, a small shRNA library screen identified synthetic lethal interactions with *VHL* tumour suppressor [27]. Synthetic lethal screening in mammalian cells is rapidly emerging as a promising approach to identify new drug targets for cancer therapy.

In this thesis I will describe functional genomic siRNA screens that we have performed in two important biological pathways; the p53 pathway and the TGFβ pathway. Functional studies in the p53 and TGFβ pathway will be discussed in separate chapters of this introduction. As introduction, a general overview of these signalling pathways will be given and their various functions in cancer will be discussed.

FUNCTIONAL GENOMICS AND P53

THE P53 PATHWAY

The p53 tumour suppressor is a transcription factor that responds to a variety of cellular stresses. Under normal conditions p53 protein is kept inactive through rapid degradation mediated by MDM2. MDM2 binds to p53, inhibits its transactivation, causes nuclear export and acts as an E3-ligase leading to ubquitination and proteosomal degradation of p53 [28]. Upon cellular stress, such as DNA damage, oncogenic stress or nutrient deprivation p53 is activated. This activation is either through activation of p53 by ATM and CHK2 or by inhibition of MDM2 by ARF (also known as p14ARF in humans, see Figure 1). As a result p53 protein accumulates and activates a large number of target genes, including genes involved in apoptosis induction and cell cycle control and arrest [28, 29]. It is believed that cellular signals originating from DNA damage or activated oncogenes are the most physiological stress responses that lead to p53 activation. These two stimuli use different mediating factors to activate p53. The DNA damage signal is transmitted through a cascade of kinases, the ATM, ATR and CHK proteins, the end result being posttranslational modifications and activation of p53 [30]. As a consequence cells will undergo a cell cycle arrest, allowing repair of the DNA damage or if this damage is beyond repair, they will undergo apoptosis [31]. Consequently, p53 has an important role in preventing the accumulation of genomic alterations leading to tumourigenic transformation. On the other hand, p53 can be activated as a result of oncogene activation. Oncogenes activation occurs either through mutation or through increased expression as a result of gene amplification. This oncogenic signalling activates p53 through induction of ARF, which inhibits the ubiquitin ligase MDM2, resulting in stabilization and increased activity of p53. [28, 29, 32, 33]. Alternatively, oncogene activation can also lead to the induction of DNA damage and subsequent p53 activation [34].

P53 IS A VERY POTENT TUMOUR SUPPRESSOR

The p53 pathway is often inactivated during human tumourigenesis. Most, if not all, solid human cancers lack a functional p53 response. In about 50% of all human cancers p53 is mutated by missense mutations, which leads to expression of inactive

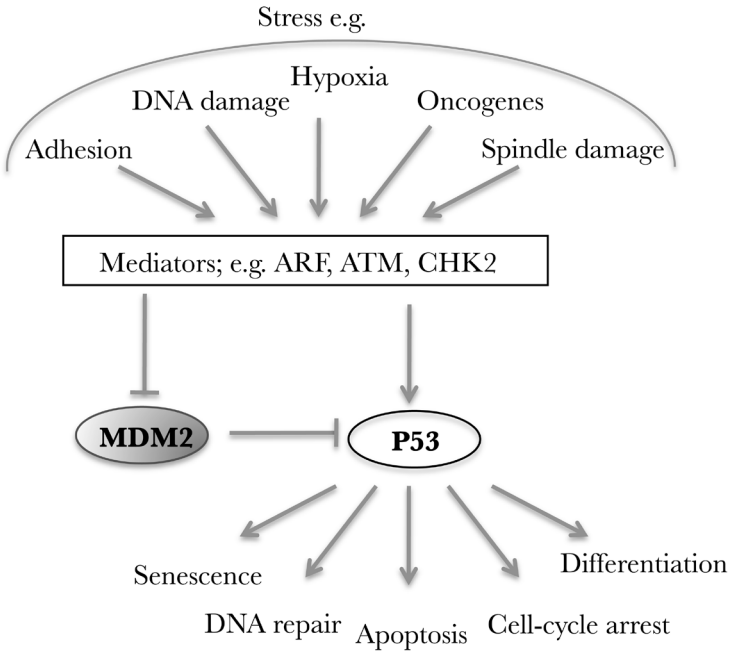

Figure 1. The p53 pathway
The p53 pathway is activated in response to different kinds of stress. This activation is carried out by mediators that either activate p53 or inhibit MDM2. Possible responses to this activation are the induction of a cell cycle arrest, apoptosis, DNA repair, senescence or differentiation.

p53 protein [35]. In the remaining half of human cancers retaining wild type *TP53*, the p53 pathway is often inactivated through alterations in the *MDM2* or *ARF* genes.

The importance of p53 as a tumour suppressor is illustrated by a group of patients carrying a germline mutation in the *TP53* gene. As a consequence these patients display the Li-Fraumeni syndrome which is characterized by the occurence of many different types of tumours. Among the tumours seen in Li-Fraumeni patients are breast carcinoma, soft tissue sarcomas, osteosarcomas, brain tumours, adrenocortical carcinoma, Wilms' tumour and phyllos tumours [36].

The experimental validation of p53 as a major tumour suppressor has been provided over the years by the use of many different mouse models. These models consist of mice that have a complete knockout of the *Trp53* gene or knock-in models that recapitulate *TP53* mutations found in human cancers. Although these different models display different phenotypes they unequivocally demonstrate the essential role of p53 in tumour suppression.

As most tumours lack a functional p53 pathway, it has been proposed that reactivation of the p53 pathway may be used as a potential anti-cancer therapy. To investigate p53 pathway activation, mouse models have been developed which make use of a genetic switch enabling restoration of p53 expression. These studies have shown that, restoration of the p53 pathway in established tumour cells leads to regression of tumours and increased survival of the animal [37, 38]. Different strategies must be designed to reactivate the pathway both in tumours that have lost p53 expression, express an inactive mutant form of p53 or have sustained mutations or aberrations in upstream components required for p53 activation.

Restoration of the p53 pathway in mutant p53 tumours

The most frequent mutations in the *p53* gene are clustered in the DNA binding region [39]. These mutations result in distortion of the DNA binding domain and as a consequence such mutant proteins are unable to activate p53 target gene expression [39]. These mutated forms of p53 are in general highly expressed and can act as dominant negatives. However, it has been shown that the misfolding of mutant p53 protein is reversible and restoration of mutant p53 protein into wild type conformation can result in reactivation of p53 signalling [40]. Various strategies have been explored to restore the function of mutant p53. The design and use of small molecules is most attractive because these easily can enter tumour cells. An example of a low molecular weight compound that can reactivate the p53 pathway in mutant p53 tumour cells is PRIMA (p53 reactivation and induction of massive apoptosis) [41, 42]. PRIMA restores DNA binding potential and active conformation of mutant p53 proteins. This leads to an apoptotic response in mutant p53 tumour cells but not in wt p53 cells. Additionally, *in vivo* anti-tumour activity without apparent toxicity has been shown for PRIMA [41, 42]. Although PRIMA can rescue both p53 DNA binding mutants as well as other structural mutants, it will probably not do so for all p53 mutations.

Restoration of the p53 pathway in wt p53 tumours

Besides the many tumours that have an inactivating mutation in the p53 coding sequence, some tumours do contain a wild-type *TP53* gene. It is thought that these wild-type p53 tumours contain genetic alterations that prevent activation of the p53 protein. One observed mechanism to inactivate p53 function is amplification of its negative regulator MDM2. Amplification of *MDM2* is found in ~7% of all human cancers. Although amplification of *MDM2* is observed both in wt p53 and mutant p53 mutant tumours, a significant correlation is found in tumours with p53 wt status [43]. In addition to *MDM2* amplification, deletion of *ARF* also results in elevated levels of MDM2, thereby reducing p53 function [28]. One strategy to reactivate p53 in these tumour types is to force the stabilization of p53 protein by liberating it from the negative control of MDM2.

Reintroduction of wild-type p53 protein expression in tumours that don't express any p53 protein can be accomplished through adenoviral gene therapy. Gene therapy uses viruses to insert a wt copy of *p53* in tumour cells. The approach is promising but main the challenges remain efficient delivery and expression in all tumour cells [44-46]. In addition this technique is not expected to be efficacious in p53 mutant tumours as most mutations in p53 are thought to yield a dominant negative protein.

Reactivation of the p53 pathway in tumours that have epigenetically silenced ARF can potentially be done through reversion of the silencing. DNA methyl transferase inhibitors can reverse this silencing and are currently used in the clinic [47, 48].

Small molecule p53 activators

A clinically more feasible approach to activate p53 is the disruption of the MDM2-p53 interaction by small molecules. A variety of MDM2 inhibitors have been developed that target different aspects of MDM2 function. Both inhibitors that block binding of MDM2 to p53 as well as inhibitors of MDM2's E3-ligase activity have been developed.

The binding of p53 to MDM2 is mediated by a hydrophobic pocket of MDM2

and several essential residues in p53; Phe19, Trp23 and Leu26. Although the disruption of protein-protein interactions by small molecules is considered difficult, the MDM2-p53 binding is a positive exception. Different classes of small molecules have been identified that disrupt binding of p53 to MDM2 (reviewed in [49]). Exposure of cells to these MDM2 inhibitors results in stabilization of p53 and thereby activation of the p53 pathway [50, 51].

HLI98 is an example of a small molecule that inhibits MDM2's E3 ligase activity, resulting in p53 activation and apoptosis. However, HLI98 does not seem to be very specific for MDM2 and acts on other E3-ligases as well (Yang, cancer cell, 2005). As a result, the clinical application of HLI98 seems limited.

RITA is a compound that was described to block the p53-MDM2 interaction through binding to the N-terminus of p53. RITA has a p53-dependent anti-tumour effect *in vitro* and *in vivo* [52]. However, binding to the N-terminus of p53 could not be confirmed by NMR experiments [53]. This has shed doubt on the mechanism of action of RITA for several years but recently RITA was described to modulate p53 dependent transcription. Reactivation of p53 by RITA is thought to lead to transcriptional repression of anti-apoptotic proteins and to downregulation of oncogenes such as *c-MYC*, *Cyclin E* and *b-catenin*. This leads to a reduction in the ability to buffer pro-apoptotic signals which then subsequently can result in robust apoptosis [54].

Nutlin-3 is a compound that specifically blocks the MDM2-p53 interaction by mimicking residues of p53 that are essential for the interaction with MDM2. Exposure of tumour cells to Nutlin-3 results in the stabilization of p53 and activation of the p53 pathway. As a result these cells undergo cell cycle arrest or apoptosis. The effect of Nutlin-3 is dependent on the presence of WT p53. The application of Nutlin-3 to animals with human cancer xenografts resulted in inhibition of tumour growth with remarkable absence of toxic side effects [50, 55].

Table 1 Compounds that activate p53

Compound	Specificity	Action	Evidence	References
PRIMA	Mutant p53	Restores active conformation p53	*in vitro* *in vivo*	[41], [42]
MIRA	Mutant p53	Restores active conformation p53	*in vitro* *in vivo*	[59]
Nutlin-3	WT p53	Binds to MDM2	*in vitro* *in vivo*	[50], [55]
RITA	WT p53	Modulation of p53 dependent transcription	*in vitro* *in vivo*	[52], [53], [54]
HLI98	WT p53	Inhibits MDM2 autoubiquitination	*in vitro*	[60]
MI-219	WT p53	Binds to MDM2	*in vitro* *in vivo*	[23]

Ref #	Shows
[41]	*In vivo* activity PRIMA
[42]	PRIMA is converted into adducts which covalently form adducts with thiols in mutant p53
[59]	Identification of MIRA, activity *in vitro* and *in vivo*
[50]	Nutlin-3 binds in the hydrophobic MDM2 pocket that normally binds to p53, Nutlin-3 activity *in vitro* and *in vivo*
[55]	*In vitro* and *in vivo* activity Nutlin-3 also in tumours with wt MDM2 levels
[52]	Identification RITA, *in vitro* and *in vivo* activity
[53]	RITA does not inhibit p53-MDM2 interaction *in vitro*
[54]	Modulation of p53-dependent transcrition by RITA
[60]	Identification HLI98, *in vitro* action but *in vivo* also p53 independent apoptosis
[23]	Identification MI-219 *in vitro* and *in vivo* activity

Complete loss of *MDM2* expression is embryonic lethal [56, 57]. However, the *in vivo* situation that matches more closely to MDM2 inhibition is the hypomorph *MDM2* mouse model. Mice with this hypomorph *MDM2* allele have reduced MDM2 levels but this is not embryonic lethal. However, these mice are resistant for tumour formation presumably through increased levels of active p53 [58]. Interference with the p53-MDM2 interaction suggests a potential strategy for cancer therapy. At this moment, different components targeting the p53 pathway are tested (see Table 1 for an overview of compounds that can activate p53 in mutant and wt tumours).

As discussed above, the strategy to reactivate the p53 pathway in p53 wt tumours seems a viable approach in cancer treatment. However, to increase our understanding and to extend possible strategies to achieve this, we need a more comprehensive overview of genes that are causally involved in regulating the p53 pathway and this reactivation. The use of functional genomic screens can contribute to this understanding.

Insight in the p53 pathway through functional genomic screens

De Leo and colleagues identified p53 thirty years ago as an oncogene. p53 forms a complex with SV40 T antigen and was found to be low in normal cells and increased in SV40 transformed cells [61]. Only ten years later the labs of Moshe Oren and Arnold Levine showed that the initially identified form of p53 is a mutant form. Moreover, both labs showed that overexpression of wt p53 protein inhibits transformation and that p53 is not an oncogene but a tumour suppressor gene [62, 63].

Over the last 30 years, since the discovery of the p53 gene, a detailed understanding of the p53 pathway has been obtained. The complexity of the different pathways and downstream targets of p53 makes it difficult to pinpoint those pathways in which p53 is causally required for tumour growth and survival. The use of functional genomics enables us to identify genes that are causally involved in tumour growth and survival. Using this technology several functional components of the p53 pathway have been identified. Retroviral cDNA expression libraries and RNAi technologies have been used in combination

with genetically engineered cell lines to probe p53 function [64-66]. In cDNA over-expression screens components involved in the prevention of a senescence-like cell cycle arrest have been identified such as *TBX-2* and *-3* and *BCL6*. TBX2 was identified in a retroviral cDNA screen using *BMI1* knockout mouse embryonic fibroblasts. These *BMI1*-null cells enter a premature cell cycle arrest due to increased expression of p19ARF and p16INK4a both encoded by the *INK4A/ARF* locus. Over-expression of TBX2 down-regulates p19ARF, thereby inhibiting p53 activation [65]. Brummelkamp and colleagues used conditionally immortalized mouse neuronal cells with a temperature sensitive mutant of SV40 Large T antigen that undergo a cell cycle arrest under restrictive temperature [64]. This arrest is also associated with upregulation p19ARF and activation of p53. Brummelkamp et al identified that TBX3, a family member of TBX2, was also able to bypass the cell cycle arrest through suppression of p19ARF expression. Importantly, both TBX2 and TBX3 have also been implicated in cancer. *TBX2* is found amplified in a subset of human breast cancer cell lines [65] and *TBX3* mutations cause Ulnar Mammary syndrome [67]. Both screens described above, identified components upstream of p53 whose over-expression prevent activation of p53 and a subsequent cell cycle arrest. In addition, a protein acting downstream of p53, *BCL6* has been identified through a cDNA expression screen. The mechanism by which BCL6 rescues the cell cycle arrest is through up-regulation of cyclin D1, thereby preventing the growth inhibitory function of p21, an essential downstream target of p53 [66].

The first large RNAi screen in the p53 pathway was performed with a set 23,742 shRNA vectors (the NKI shRNA library). This set of shRNA vectors targets 7,914 different human genes (3 shRNA vectors are designed per gene) [68]. By using this technology, five new modulators of the p53 pathway were identified namely HDAC4, CCT2, RSK4, KIA0828 and TIP60. Suppression of these genes by two independent short hairpin vectors conferred resistance to a p53 induced senescence-like arrest in human fibroblasts. Subsequently, it was found that inactivation of these genes resulted in an over-ride of a DNA damage induced p53 dependent G1 arrest and a p19ARF induced cell cycle arrest, which further substantiated the implication of these genes in the p53 pathway. Interestingly, it was shown by Tang and colleagues that TIP60, a histone acetylase transferase, plays a role in posttranslational modification of p53. TIP60 is instrumental for p53-dependent apoptosis and cell cycle arrest through acetylation of p53 at lysine 120. This lysine120 is a recurrent site for mutation in human cancer and analysis of K120R mutants revealed that this acetylation site is necessary for stimulation of p53-induced apoptosis but not for cell cycle arrest [69]. Moreover, TIP60 is a haplo insufficient tumour suppressor and mono-allelic loss of TIP60 is observed in human lymphomas, mammary and head-and –neck carcinomas [70].

Recently Llanos and colleagues also used the NKI shRNA library for a screen in which the transcriptional activity of p53 was assayed with a p53- responsive luciferase reporter. They identified four novel inhibitors of p53 mediated transcriptional activation [71].

These studies illustrate that functional genetic screens can identify novel genes that play a role in the p53 pathway, and subsequent studies can further elucidate the role of such new members in the p53 network.

Functional genomics and TGFβ

The TGFβ pathway

The TGFβ pathway has a dual role in cancer progression. In normal epithelium and early stage tumours the TGFβ pathway was reported to have an inhibitory effect on cell proliferation. This is mainly achieved by inducing a G1 cell cycle arrest through up-regulation of the CDK inhibitors p21 [72] and p15 [73] and downregulation of the c-Myc proto-oncogene [74]. However, cells can become insensitive to this proliferative control by loss of expression of the TGFβ receptor or SMADs and the pathway subsequently promotes cancer progression through various routes such as the induction of EMT, promoting angiogenesis and prometastatic cytokine secretion. Furthermore, TGFβ can act as a tumour-derived immunosupressor, an inducer of tumour mitogens and a promoter of carcinoma invasion [75, 76] (see Figure 2).

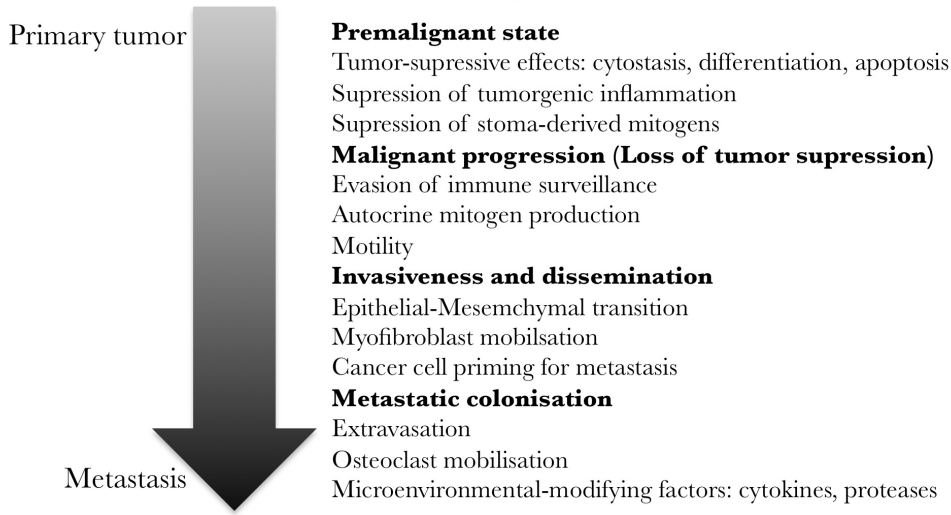

Figure 2. Roles of TGFβ in cancer
Figure adapted from; *Massague et al.*, Cell, 2008

The TGFβ pathway regulates an extensive transcriptional program. The canonical TGFβ signalling cascade is stimulated by its ligands TGFβ1, TGFβ2 and TGFβ3 that bind to the TGFβ receptors, TGFBR1 and TGFBR2. In turn, the TGFBR2 phosphorylates TGFBR1, which subsequently phosphorylates the regulatory SMADs (R-Smads); SMAD2 and SMAD3. The regulatory SMADs consecutively translocate to the nucleus and form a trimeric complex with SMAD4, also called the Co-SMAD. This trimeric complex binds to the DNA using SMAD binding elements. In addition, co-activators (i.e.; p300, CBP) and co-repressors (i.e. SNON, SKIL) also bind to this complex on the DNA. Such assembled complexes bind to SMAD binding elements and orchestrate an extensive transcriptional program involving around 500 different target genes [77, 78]. This transcriptional program also includes the induction of inhibitory SMADs (SMAD6 and SMAD7) and SMURF ubiquitin ligase proteins (SMURF1 and SMURF2). SMAD7 is then recruited to the

TGFβ receptor complex leading to inhibition of SMAD2 and SMAD3 phosphorylation. Furthermore, SMAD7 induces the catalytic activity of SMURF ubiquitin ligases leading to the degradation of TGFBR1 [79], thus providing a negative feedback loop. Cell lines derived from human colon [80] and lung [81] cancers are often resistant to the anti-proliferative effect of TGFβ signalling. This suggests that components of the pathway are inactivated these cancers. Moreover, *SMAD4* was already proposed as a candidate tumour suppressor in 1996 [82]. Since then, several mechanisms of inactivation in cancer have been indentified for *TGFBR1*, *TGFBR2*, *SMAD2* and *SMAD4* [83]. No evidence for mutation of *SMAD3* in cancer has been reported, but loss of expression has been observed in gastric cancer [84]. In concordance, important inhibitors of the TGFβ pathway such as *SMAD7* [85], *SMURF2* [86] and the transcriptional repressors *SNON* and *SKI* [87] have been found amplified or over-expressed in cancer. (see Figure 3).

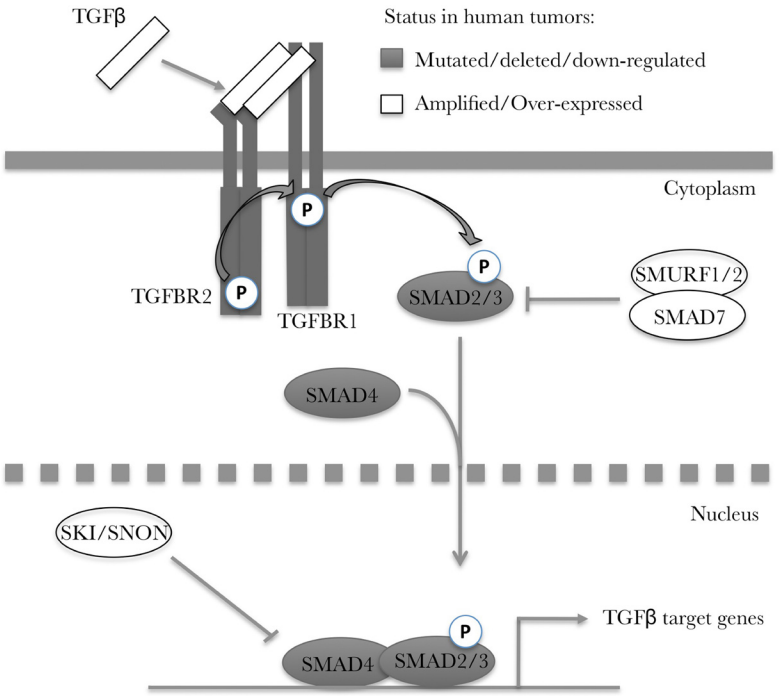

Figure 3. TGFβ pathway alterations in cancer
In grey are the genes that are found mutated, deleted or down-regulated in cancer and in white are the genes that are found amplified or over-expressed in cancer. Figure adapted from; Levy and Hill, Cytokine and growth factor reviews, 2006

As discussed earlier, if cells become insensitive to the anti-proliverative effects of TGFβ, it can act as a tumour promoter. The TGFβ ligand is found overexpressed in many cancers [83]. High expression levels are associated with poor prognosis, enhanced tumour vascularisation and metastasis [88]. Moreover, an activated TGFβ pathway correlates with poor prognosis in general [89, 90]. In spite of the pleiotropic effect of TGFβ on cell proliferation diverse TGFβ inhibitors have been developed as cancer therapy. Clinical trials have been

performed with TGFβ antisense oligonucleotides either delivered directly to tumours or indirectly by engineering an antisense TGFβ-2 RNA expressing vector into immune cells (TGFβ vaccin) [91]. In addition, TGFβ antibodies and small molecule TGFBR1 inhibitors have entered clinical trials [92, 93] (Table 2).

Table 2 TGFβ inhibitors in cancer therapy

Type	Target	Action	Stage	References
TGFβ antisense oligos	TGFβ-2	Inhibitors of TGFβ production	Phase I, II, III	[94], [95]
TGFβ antibodies	All TGFβ isoforms	TGFβ neutralizing antibody	Phase I	[96]
TGFβ vaccine	TGFβ-2	TGFβ-2 antisense gene modified allogeneic tumour vaccine	Phase II	[91]
Small molecule inhibitor of TGFBR1	TGFBR1	Block the phosphorylation domain of TGFBR1	Phase II	[97], [98]

Ref#	Shows
[94]	Phase I/II studies in glioma with AP 12009 a TGFβ-2 inhibitor
[95]	Phase III clinical trial with AP 12009 TGFβ-2 antisense inhibitor in RRA
[96]	Phase I trials with GC-1008 Antibody specific for all three TGFβ forms in MM, FSGS, RCC
[91]	Phase II clinical trial in NSCLC patients with Belagenpumatucel-L
[97]	*In vitro* action of TGFBR1 inhibitor LY573636 in various hematopoietic malignant cells
[98]	LY573636 clinical trials. Phase I; AML. Phase II; MM, sarcoma, NSCLC, ovarian cancer
Abbrevations	MM; malignant Melanoma, NSCLC; non-small cell lung cancer, RRA; Refractory Anaplastic Astrocytoma, RCC; Renal cell carcinoma, FSGS; Focal Segmental Glomerulosclerosis.

The effect of TGFβ inhibitors in glioma, melanoma and renal carcinoma is thought to depend on activation of the immune system. These tumours are highly dependent on the immuno suppressive action of TGFβ [76]. Inhibition of TGFβ presumably leads to a subsequent immune response targeting the tumour cells. A potential danger of TGFβ inhibition is an over-activation of the immune system and subsequent chronic inflammatory and auto-immune reactions. However, so far this has not been observed in patients treated with TGFβ inhibitors.

Furthermore, theoretically TGFβ inhibition leads to an increase in premalignant lesions through loss of TGFβ's cytostatic action. However, the benefit of eradication

of existing tumours is probably bigger and until now, no spontaneous increase in de novo tumour formation is observed in mouse model systems upon TGFβ ablation [76].

Insight in the TGFβ pathway through functional genomic screens

Several approaches have been taken to identify new players in the TGFβ pathway. For example protein-protein interaction screens have been used to extend the knowledge about the TGFβ pathway [99, 100]. However, contrary to functional genetic screens, protein interactions do not provide information about the functional role of proteins in the pathway. Functional genetic screens in the TGFβ pathway have been performed in *C. elegans* and in *Drosophila*. In *C. elegans* Tewari and colleagues first performed a protein-protein interaction screen to create a TGFβ interactome (the homologue in *C. elegans* is called *DAF-7*). The functional role of the proteins in this network was subsequently established by a RNAi screen with this network of proteins in both wildtype and *DAF7* mutant animals. Nine modulators of the TGFβ signalling network in *C. elegans* were identified and one of the human orthologues of these genes is already implicated in the mammalian TGFβ system. It is possible that other orthologues also play a role in the mammalian TGFβ system [101]. In *Drosophila* cells, two RNAi screens have been performed to identify new modulators of TGFβ signalling. In the first RNAi screen a phosphatase library was screened to identify the phosphatase of mothers against decapentaplegic (MAD), an orthologue of the mammalian regulatory SMADs. It must be noted that dMAD is orthologous with both mammalian TGFβ regulated SMADs (2 and 3) and Bone Morphogenic Protein (BMP) regulated SMADs (1,5,8). It was subsequently shown that the identified pyruvate dehydrogenase phosphatase (PDP) dephosphorylates SMAD1 [102]. In the second screen, a whole genome dsRNA library was screened to identify genes that modulate nuclear accumulation of MAD. Through this screen Moleskin (MSK) was identified as a protein important for MAD import into the nucleus. The translation to a mammalian system is also made in this screen. The human orthologues of MSK, importin 7 and 8 were tested in TGFβ and BMP signalling. Indeed, knockdown of importin 7 and 8 also impaired the nuclear translocation of SMAD1 in BMP signalling and of SMAD2/3 in TGFβ signalling [103].

Until now, two labs have identified new modulators of the TGFβ network through siRNA screens directly in mammalian systems. Dupont and colleagues performed a screen in human keratinocytes (HaCat cells) with a siRNA library against deubiquitinating enzymes (DUBs). They identified Fat Facets in Mammals (FAM) as a DUB essential for TGFβ signalling. More in depth analysis showed that FAM controls SMAD4 monoubiquitination [104]. In an alternative approach Levy and colleagues describe the screening of a siRNA library targeting the ubiquitin E3-ligase gene-family in a cell line with an integrated TGFβ responsive reporter. Through this approach they identified ARKADIA as a positive regulator of the pathway [105].

Scope of this thesis

In this introduction I have outlined the p53 and the TGFβ signalling pathways, their roles in cancer, and the functional genetic screens that have been performed in these pathways. The four experimental chapters in this thesis describe functional genetic screens in both pathways. In chapter II, a follow-up on the work of Berns and colleagues (Berns et al, nature, 2004) is presented. We performed a large scale RNA interference screen in human fibroblasts and

investigated which shRNAs cause an override of the p53 dependent cell cycle arrest. We identify a regulator of the circadian clock, ARNTL. In chapter III, the mechanism of action the MDM2 inhibitor Nutlin-3 is investigated. A large-scale RNA-interference screen in wt p53 tumour cells identified genes that mediate cancer cell specific vulnerability to MDM2 inhibition. Reactivation of the p53 pathway by Nutlin-3 can be prevented through ablation of 53BP1, which is an upstream component of the DNA damage signalling pathway that leads to p53 activation. Chapter IV and V describe single well siRNA screens aiming to identify new modulators of the TGFβ pathway. In chapter IV we use a siRNA library directed against kinases and kinase related proteins, and in chapter V we use a library directed against the so-called "druggable" genome. With these screens we have identified IRAK2 (chapter IV) and NUP98 (chapter V) as modulators of the TGFβ pathway.

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Chapter II

An shRNA barcode screen provides insight into cancer cell vulnerability to MDM2 inhibitors

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An shRNA barcode screen provides insight into cancer vulnerability to MDM2 inhibitors

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Abstract

The identification of the cellular targets of small molecules with anticancer activity is crucial to their further development as drug candidates. Here, we present the application of a large-scale RNA interference–based short hairpin RNA (shRNA) barcode screen to gain insight in the mechanism of action of Nutlin-3 (Compound [1]). Nutlin-3 is a small-molecule inhibitor of MDM2, which can activate the p53 pathway. Nutlin-3 shows strong antitumor effects in mice, with surprisingly few side effects on normal tissues [1]. Aside from p53, we here identify 53BP1 as a critical mediator of Nutlin-3–induced cytotoxicity. 53BP1 is part of a signaling network induced by DNA damage that is frequently activated in cancer but not in healthy tissues [2]. Our results suggest that Nutlin-3's tumor specificity may result from its ability to turn a cancer cell–specific property (activated DNA damage signaling [3]) into a weakness that can be exploited therapeutically.

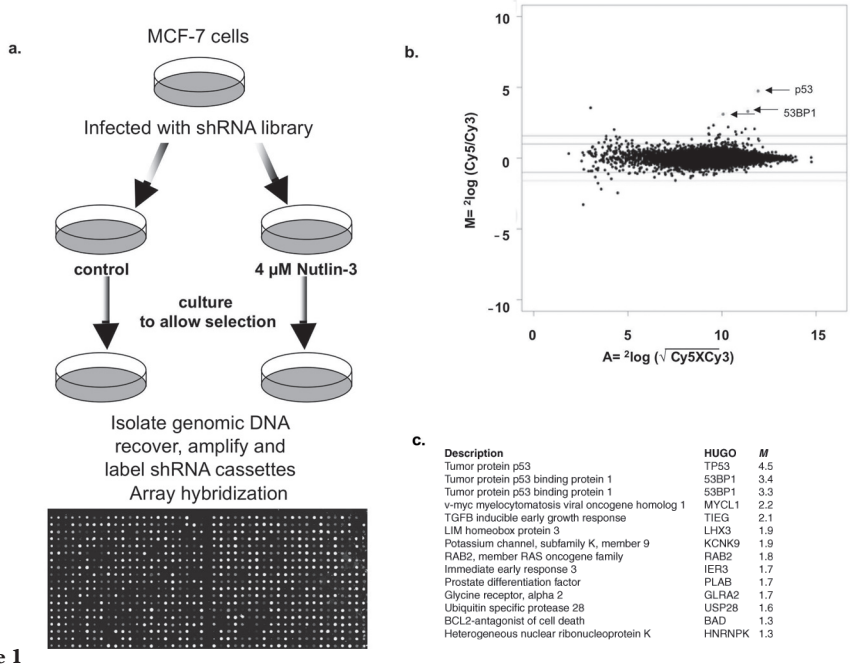
Results and Discussion

The tumor-suppressor gene TP53 (also called p53 and encoding the protein p53) is the gene most frequently mutated in human cancer. However, approximately 50% of all human tumors retain normal p53 [4]. Direct activation of p53 in these tumors could in principle be used as a means to eradicate tumor cells. The activity of p53 is tightly regulated. p53 is activated in response to a variety of stresses, such as DNA damage, nutrient deprivation or oncogene activation, resulting in the transcriptional activation of target genes involved in growth arrest and apoptosis [5]. To protect healthy cells from the deleterious effects of uncontrolled p53 activation, p53 is subject to a negative feedback loop activated by the protein product of one of its target genes, MDM2. The protein MDM2 binds to p53, inhibits transcriptional activation, causes nuclear export and acts as an E3 ligase to target p53 for proteasomal degradation [6, 7].

One potential approach for activating p53 in tumor cells is to disrupt the interaction between MDM2 and p53 with the small molecule Nutlin-3 [1, 8]. Nutlin-3 binds to MDM2, thereby preventing the interaction with p53 and causing p53 to be stabilized. Nutlin-3 has strong antitumor effects *in vivo* but, notably, few toxic effects in normal mice [1]. In theory, activation of p53 could be at least as deleterious to normal cells as to tumor cells, because the former are not attenuated in their ability to undergo apoptosis and are more obedient to growth-inhibitory signals than are cancer cells. However, tumor

cells that have lost the *Tp53* gene through mutation seem to be exquisitely sensitive to reintroduction of the wild-type gene [9]. This phenomenon has never been understood in detail, and has often been attributed to the presence of other oncogenic mutations or to a differential wiring of the p53 network in tumor cells as compared to normal cells [9]. We report here a loss-of-function genetic screen designed to identify important components of the p53 network mediating the cytotoxic effect of Nutlin-3 in human tumor cells.

A variety of approaches can be used for the identification of genes essential for a drug-induced phenotype [9-16], including the recently developed RNAi barcode screening technology [10-13]. Barcode screens use DNA microarrays to follow the relative abundance, under particular conditions, of each shRNA vector in a large population of cells infected with an shRNA vector library (Fig. 1a). Using retroviral infection, we introduced a collection of 23,742 different pRETRO-SUPER vectors designed to target 7,914 human genes for suppression by RNA interference (RNAi) into MCF-7 cells (which have wild-type p53 [10]). The infected cells were split into two pools, one of which was left untreated and was used as a reference while the other was exposed to 4 μM Nutlin-3. MCF-7 cells respond to this concentration of Nutlin-3 predominantly by undergoing cell-cycle arrest rather than apoptosis



c.

Description	HUGO	*M*
Tumor protein p53	TP53	4.5
Tumor protein p53 binding protein 1	53BP1	3.4
Tumor protein p53 binding protein 1	53BP1	3.3
v-myc myelocytomatosis viral oncogene homolog 1	MYCL1	2.2
TGFB inducible early growth response	TIEG	2.1
LIM homeobox protein 3	LHX3	1.9
Potassium channel, subfamily K, member 9	KCNK9	1.9
RAB2, member RAS oncogene family	RAB2	1.8
Immediate early response 3	IER3	1.7
Prostate differentiation factor	PLAB	1.7
Glycine receptor, alpha 2	GLRA2	1.7
Ubiquitin specific protease 28	USP28	1.6
BCL2-antagonist of cell death	BAD	1.3
Heterogeneous nuclear ribonucleoprotein K	HNRNPK	1.3

Figure 1

shRNA barcode screen identifies 53BP1 as a mediator of the antiproliferative effects of nutlin-3.

(a) Schematic outline of the nutlin-3 barcode experiment in MCF-7 cells. Identical populations of MCF-7 cells infected with the NKI shRNA library were either left untreated or treated with 4 μM nutlin-3. After 14 d the shRNA cassettes were recovered and labeled10, 11. The labeled shRNA probes were hybridized to DNA microarrays containing the sequences complementary to the hairpin oligos. (b) Analysis of the relative abundance of shRNAs recovered from the nutlin-3 barcode experiment. Data are normalized and depicted as M, the ^{2}log (ratio Cy5/Cy3), versus A (^{2}log (radicintensity Cy3 times Cy5)). The data are the average of two independent hybridization experiments performed in duplicate with reversed color. (c) List of genes targeted by the shRNA vectors from b that were significantly enriched ($P < 0.01$) in nutlin-3–treated cells. The vectors for TP53 and both vectors for 53BP1 have been validated for knockdown and rescue of nutlin-3–induced cell-cycle arrest. Column M depicts the ^{2}log ratio of the abundance of barcodes present in cells treated with nutlin-3 (Cy5) as compared to untreated cells (Cy3).

(data not shown and Fig. 2c), possibly because the cells have a deficiency in caspase-3 [14]. After 14 d of selection with Nutlin-3, we recovered shRNA cassettes by PCR amplification and analyzed them by hybridization to DNA microarrays as described [10, 15]. A small number of shRNA vectors were consistently enriched in the population treated with Nutlin-3 (Fig. 1b). As expected, one of these vectors that was positively selected in the presence of Nutlin-3 as compared to untreated control cells is directed against p53 which validates the shRNA barcode approach used here. Loss of p53 confers resistance to Nutlin-3 by removing the effector of MDM2 [1]. In addition, several of the shRNA vectors we identified target proteins with known or predicted functions in p53-mediated pathways (Fig. 1c). Two of these shRNA vectors target p53-binding protein-1 (53BP1), through independent sequences. 53BP1 has been implicated in DNA damage sensing leading to p53 activation. In addition, we found an shRNA vector targeted against protein heterogeneous nuclear ribonucleoprotein K (hnRNPK), which has recently been identified as an MDM2 target and transcriptional coactivator of p53 in response to DNA damage [16]. The observation that depletion of hnRNPK abrogates the transcriptional induction of p53 target genes is consistent with an override of a Nutlin-3–mediated cell-cycle arrest upon hnRNPK inactivation. We also

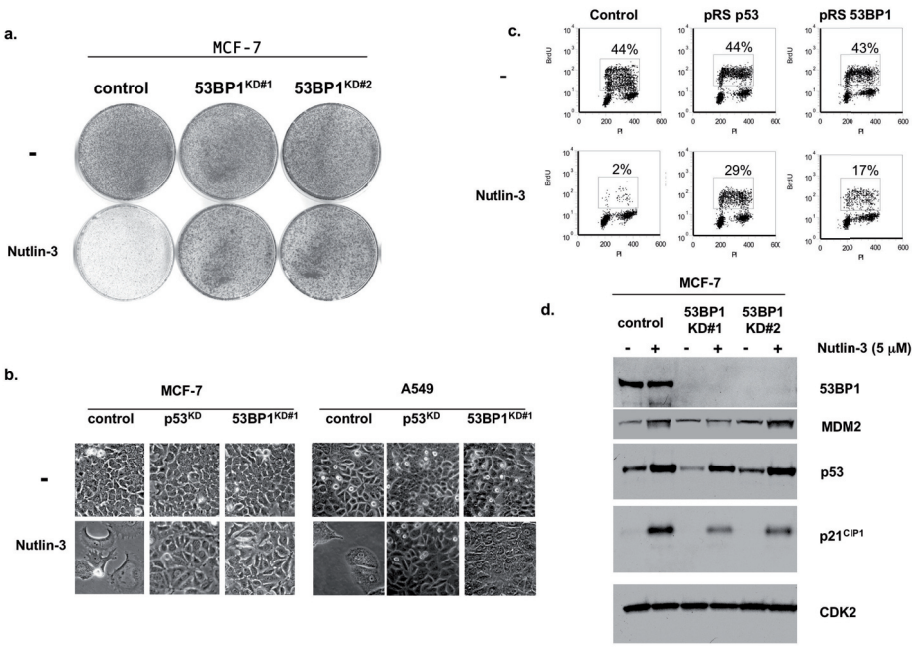


Figure 2
53BP1 modulates the cellular cytotoxicity induced by nutlin-3 in MCF-7 cells.
(a) MCF-7 cells infected with shRNAs against GFP or two independent shRNAs directed against 53BP1 (knockdown vector is labeled KD), untreated or treated with 4 μM nutlin-3 for 14 days. Cell density is visualized by Coomassie staining of the cells. (b) MCF-7 cells and A549 cells infected with shRNAs directed against GFP, p53 or 53BP1, untreated or treated with nutlin-3.(c) Cell-cycle profile and BrdU incorporation of MCF-7 infected with shRNAs against GFP (control), p53 or 53BP1. Percentages of BrdU-positive cells are shown. (d) Western blot analysis of the MCF-7 cells expressing either shRNA against GFP (control) or one of the two shRNAs against 53BP1, 53BP1#1 or 53BP1#2. The cells were analyzed for the expression levels of 53BP1, the stabilization of p53 and the induction of p21 and MDM2.

identified three vectors targeting genes encoding known p53 transcriptional targets: BCL2-antagonist of cell death (BAD), placental bone morphogenic protein (PLAB) and immediate-early response 3 (IER3, also called IEX-1). Both BAD and IEX-1 have roles in the regulation of apoptosis. IEX-1 can block the PI3K/AKT pathway, resulting in increased sensitivity to apoptosis [17]. PLAB is also implicated as an important downstream mediator of DNA damage signaling [18]. Other shRNA vectors we identified in our screen target genes that are not implicated in the regulation of p53 or p53-dependent pathways. The presence of the genes encoding TGFbeta-inducible early growth response-1 (TIEG1, also called KLF10) and PLAB among the targets of the vectors identified suggests that the TGFbeta pathway is involved in the cell-cycle arrest induced through the activation of the p53 pathway by Nutlin-3 treatment. Notably, TIEG1 expression is reduced in invasive breast cancer cells [19]. The shRNA barcode screen did not identify genes that enhance the cytotoxic effects of Nutlin-3 in MCF-7 cells—that is, there were no shRNA vectors that were significantly depleted from the population as a result of Nutlin-3 treatment. This may either be a consequence of the biological system or reflect a technical limitation of the current barcode protocol. In addition, we cannot exclude the possibility that some vectors are selected in this screen as a result of effects on cellular components other than the intended targets.

Our identification of two independent shRNA vectors targeted against 53BP1 in our screen greatly reduces the possibility that the observed effects are due to 'off-target' effects of the RNAi vectors. Moreover, this indicates that the observed phenotype is due to the reduced expression of the intended target. We therefore focused our subsequent validation on the effect of 53BP1 on Nutlin-3 cytotoxicity. To validate the selection of shRNAs against 53BP1 in our barcode experiment, we performed a long-term growth assay with MCF-7 cells expressing the two different shRNA vectors against 53BP1. MCF-7 cells exposed to Nutlin-3 undergo a proliferation arrest and acquire a flattened phenotype (Fig. 2a,b, left). In contrast, MCF-7 cells transduced with shRNA encoding retroviruses against p53 or 53BP1 and exposed to Nutlin-3 show a cellular morphology resembling that of untreated MCF-7 cells. This effect is not unique to MCF-7 breast cancer cells, as inhibition of 53BP1 also alleviates the cytotoxic effects of Nutlin-3 in A549 (human lung cancer) cells (Fig. 2b, right). We tested the effect of the inactivation of either p53 or 53BP1 on the cell-cycle progression of MCF-7 cells exposed to 4 μM Nutlin-3 for 48 h. The percentage of MCF-7 cells in S-phase, as determined by BrdU incorporation, was reduced from 44% for control cells to 2% in cells exposed to Nutlin-3, whereas knockdown of p53 or 53BP1 largely prevented this reduction, resulting in S-phase percentages of 29% and 17%, respectively (Fig. 2c). To further validate the efficacy of the shRNA vectors against 53BP1, we tested their effects on the expression of 53BP1, the stabilization of p53 by Nutlin-3, and the activation of a p53 target gene, *CDKN1A* (also called *p21* and encoding p21cip1). Both shRNA vectors targeting 53BP1 were active in inhibiting 53BP1 expression (Fig. 2d). The induction of p53 by Nutlin-3 treatment is not altered in the presence or absence of 53BP1 expression. Notably, the shRNA-mediated knockdown of 53BP1 impairs the activation of the p53 target gene *CDKN1A* by Nutlin-3. These results demonstrate that, beside p53, 53BP1 also has an essential role in mediating the cytotoxic effects of Nutlin-3 in MCF-7 cells.

53BP1 is a component of the ATM-CHK-53BP1 pathway that is activated by double-stranded DNA breaks or shortened telomeres, resulting in the induction of p53 [20]. Notably,

this ATM-CHK-53BP1 DNA damage checkpoint-signaling pathway is often constitutively activated in human cancers and premalignant lesions, but not in normal, healthy tissues [2, 3, 21]. Based on the role of 53BP1 in mediating the antiproliferative effect of Nutlin-3 identified here, we hypothesized that intrinsic DNA damage signaling in MCF-7 cells is required for Nutlin-3 cytotoxicity. The presence of activated DNA damage signaling in MCF-7 cells was demonstrated by the detection of nuclear foci containing 53BP1, a characteristic of DNA damage–induced activation of 53BP1 [22]. Indeed, a substantial fraction of MCF-7 cells show localization of 53BP1 in nuclear foci. Normal BJ fibroblasts have diffuse nuclear staining, although some 53BP1 foci are present (Fig. 3a, above). The presence of DNA damage foci in normal human fibroblasts in culture is in agreement with previous studies describing the presence of H2AX DNA damage foci and p53 activation in primary cells cultured *in vitro* [23]. MCF-7 cells also show strong nuclear staining with an antibody directed against phosphorylated CHK-2, even in the absence of ionizing radiation (Fig. 3a, below). In contrast, normal BJ fibroblasts show very little immunoreactivity, whereas BJ fibroblasts treated with ionizing radiation show strong nuclear staining (Fig. 3a, below). Together these data indicate that in MCF-7 cells, in the absence of ionizing irradiation, activation of DNA damage checkpoint signaling involving 53BP1 occurs, reflecting DNA damage signaling seen in many human tumors [2]. The presence of activated DNA damage checkpoints in MCF7 cells suggests that DNA damage–induced 'stress signals' could be important in sensitizing MCF-7 cells to MDM2 inhibition. To test this, we inhibited DNA damage signaling in MCF-7 cells through inhibition of ATM/ATR-

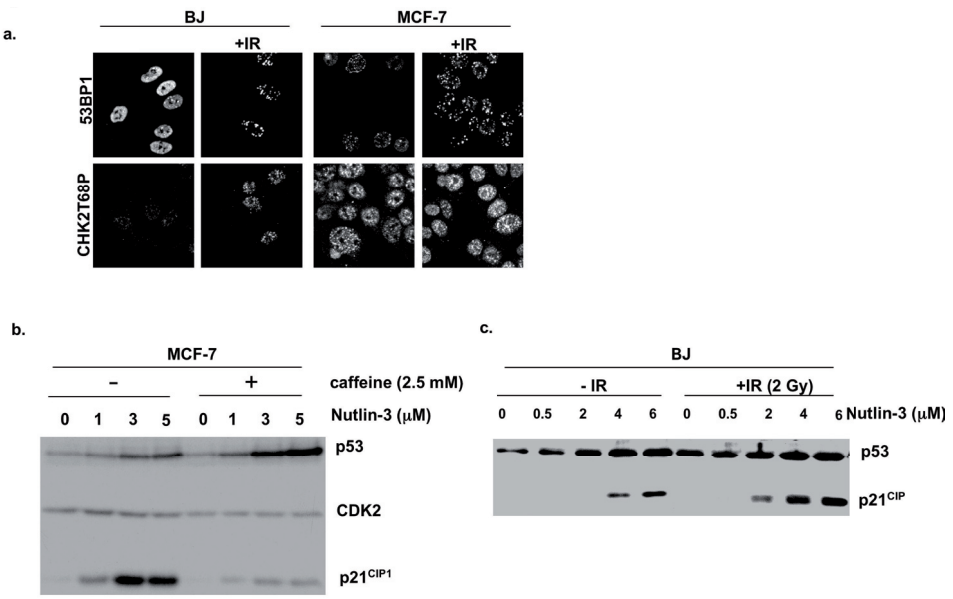


Figure 3

Intrinsic DNA damage signaling contributes to the cytotoxic effect of nutlin-3 in MCF-7 cells.

(a) Immunolocalization of p53BP1 (above) and CHK2 (below) in human primary fibroblasts (left) and MCF-7 cells (right) before and after irradiation. (b) Western blot analysis of the stabilization of p53 and induction of p21 in MCF-7 cells treated with increasing concentrations nutlin-3 for 18 h in the absence or presence of 2.5 mM caffeine. The cells were pretreated with 2.5 mM caffeine for 12 h.(c) Western blot analysis of the stabilization of p53 and the induction of p21 in normal BJ fibroblasts treated with increasing concentrations nutlin-3 without or with exposure to 2 Gy irradiation.

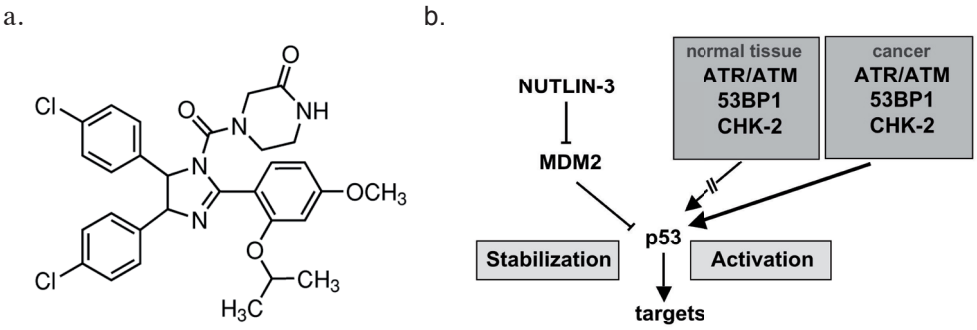


Figure 4
The cytotoxic effect of nutlin-3 in tumor cells is enhanced by intrinsic DNA damage signaling.
(a) Chemical structure of nutlin-3.(b) Schematic representation of a putative model reflecting the intrinsic DNA damage signaling (involving 53BP1) present in tumor cells (but absent in normal cells) increasing the cytotoxicity associated with nutlin-3 treatment.

dependent checkpoints by caffeine (Compound 2) treatment [24-26]. The inhibition of ATM/ATR kinases in MCF-7 cells resulted in a decrease in the transcriptional activation by p53 in response to Nutlin-3 exposure, as evidenced by induction of p21cip1 (Fig. 3b). The inhibition of ATM/ATR kinases does not prevent p53 accumulation in response to Nutlin-3 exposure, indicating that p53 stabilization and transactivation are separate events (Fig. 3b). To further test whether DNA damage signaling enhances the effects of Nutlin-3, we treated normal BJ fibroblasts with increasing concentrations of Nutlin-3 in the absence or presence of a low level of DNA damage induced by ionizing radiation. A more robust activation of the p53 target gene p21cip1 was seen in response to Nutlin-3 treatment in the presence of DNA damage (Fig. 3c). From these results we conclude that inhibition of intrinsic DNA damage signaling by 53BP1 knockdown prevents full activation of p53 after stabilization in response to MDM2 inhibition.

Genomic instability is a well-recognized property of the vast majority of human cancers. This labels cancer tissues with a feature not seen in normal tissues, and it has therefore been suggested that genetic instability is an Achilles heel of tumor cells that could be exploited by future anticancer therapeutics [27]. Recent experiments have indicated that activation of DNA damage signaling (involving H2AX, 53BP1, ATR and ATM) occurs very early during tumorigenesis [3, 21]. Our data indicate that this DNA damage signaling enhances the effects of the MDM2 inhibitor Nutlin-3 (Fig. 4). The absence of such signals in normal cells *in vivo* may well explain their reduced sensitivity to Nutlin-3 [2]. The consequences of partial inactivation of MDM2 by small-molecule inhibitors resemble the phenotype seen in mice carrying a hypomorphic allele of *MDM2*. These mice are viable but show severe radiosensitivity [28].

Our data suggest that MDM2 inhibitors would be most effective for tumors that have both wild-type p53 and activated DNA damage signaling, which is a substantial fraction of all human tumors. However, a combination of MDM2 inhibitors with DNA-damaging treatments may be harmful to normal cells and would cause undesired side effects in patients. In conclusion, the present study illustrates that shRNA barcode screens can be used to reveal cellular components that mediate drug cytotoxicity, resulting in a more complete understanding of drug action and enabling more rational decisions to be made in regard to drug application.

Methods

Cell culture

MCF-7 breast cancer cells and BJ primary human fibroblasts, both expressing the ecotropic receptor, were cultured in DMEM supplemented with 10% fetal calf serum and penicillin and streptomycin. Cells were grown at 37 °C in 5% CO2. Phoenix cells were used to produce ecotropic retroviruses as described previously11. Nutlin-3 ((+/-)-4-[4,5-bis(4-chlorophenyl)-2-(2-isopropoxy-4-methoxyphenyl)-4,5-dihydroimidazol-1-carbonyl]piperazin-2-one) was obtained from Cayman Chemical. Caffeine pretreatment of MCF-7 cells was performed by incubation with 2.5 mM caffeine (Sigma-Aldrich) for 12 h.

Barcode screen

MCF-7 cells were infected with retroviruses representing the complete Netherlands Cancer Institute (NKI) shRNA library described previously10. Infected cells were selected on puromycin (2.0 μg/ml) for 4 d and split into two populations. One population was left untreated, while the other population was exposed to 4 μM Nutlin-3. After 14 d, genomic DNA was isolated with DNAzol (Invitrogen) and shRNA inserts were amplified from genomic DNA by PCR using the primers pRS-T7-fw, 5'-GGCCAGTGAATTGTAATACGAC-TCACTATAGGGAGGCGGC CCTTGAACCTCCTCGTTCGACC-3', containing a T7 RNA polymerase promoter sequence, and pRS8-rev, 5'-TAAAGCGCATGCTCCAGACT-3'. After purification (QIAquick PCR purification kit, Qiagen), PCR products were used for linear RNA amplification using the Megascript T7 kit (Ambion), and purified RNA probes (RNeasy, Qiagen) were labeled with cyanine-3 (Cy3) or cyanine-5 (Cy5) fluorescent groups using ULS (Kreatech) and purified over a KreaPure (Kreatech) spin column as described [29]. Labeled RNA probes from untreated and Nutlin-3–treated cells were combined and hybridized to oligonucleotide arrays in 40 mul of hybridization mixture (25% formamide, 5times SCC, 0.01% SDS, 25% Kreablock (Kreatech)). Samples were heated to 100 °C for 5 min and applied to the array. Microarrays were hybridized for 18 h at 42 °C, washed and scanned using an Agilent microarray scanner. Quantification of the resulting fluorescent images was performed with Imagene 5.6 (BioDiscovery), local background was subtracted, and the data were normalized [30] and 2log transformed.

Plasmids

The shRNA sequences used to target p53 and p53BP1 were as follows: p53, 5'-GACTCCAGTGGTAATCTAC-3'; 53BP1#1, 5'-GATACTGCCTCATCACAGT-3'; 53BP1#2, 5'-GAACGAGGAGACGGTAATA-3'. The shRNA sequences were cloned into pRETROSUPER as described previously [10].

Cell-cycle analysis

MCF-7 cells infected with shRNA vectors against GFP (control), p53 and 53BP1 were treated for 48 h with Nutlin-3 or left untreated. The cells were labeled with 10 μM BrdU for 30 min before harvesting. Cells were fixed in 70% ethanol and permeabilized in HCl-Triton solution (2 M HCl, 0.5% Triton X-100). The samples were neutralized in borate solution (0.1 M Na2B4O7dot10H2O) and labeled with antibody to BrdU (Dako; 1% BSA in PBS containing 0.5% Tween-20) in combination with FITC-conjugated secondary antibody (Dako; goat anti–mouse IGM–FITC). Samples were incubated with 10 mug ml-1 propidium iodine and 250 mug ml-1 RNase. In each assay 10,000 cells were collected by FACScan (Becton Dickinson) and analyzed using the FCS Express program (De Novo Software).

Western blotting

Protein lysates were separated on SDS-PAGE gels, blotted onto nitrocellulose membranes and incubated with antibodies directed against MDM2 (2A10 and SMP14, Santa Cruz), 53BP1 (Novus Biologicals), p53 (DO-1), p21 (C19, Santa Cruz), CDK4 (C-22, Santa Cruz) and CDK2 (M2, Santa Cruz).

Immunofluorescence

Cells grown on coverslips were washed with PBS, fixed and permeabilized in 3% paraformaldehyde and 0.1% Triton X-100, and washed three times with PBS containing 0.05% saponin. Slides were blocked with 10% normal goat serum in PBS with 0.05% saponin. Cells were stained with antibodies directed against 53BP1 (1:100, Novus Biologicals) and against phosphorylated CHK-2 (p-T68-CHK2; 1:100, Cell Signaling Technologies); FITC-conjugated goat anti-rabbit antibodies were used as secondary antibodies. All antibodies were diluted in PBS containing 10% normal goat serum and 0.05% saponin.

Microarray data

Microarray datasets for the barcode experiments can found at the authors' supporting website (http://www.screeninc.nl).

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Chapter III

A large scale shRNA barcode screen identifies the circadian clock component ARNTL as putative regulator of the p53 tumor suppressor pathway

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A large scale shRNA barcode screen identifies the circadian clock component ARNTL as putative regulator of the p53 tumor suppressor pathway.

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Abstract

Background: The p53 tumor suppressor gene is mutated in about half of human cancers, but the p53 pathway is thought to be functionally inactivated in the vast majority of cancer. Understanding how tumor cells can become insensitive to p53 activation is therefore of major importance. Using an RNAi-based genetic screen, we have identified three novel genes that regulate p53 function. Results: We have screened the NKI shRNA library targeting 8,000 human genes to identify modulators of p53 function. Using the shRNA barcode technique we were able to quickly identify active shRNA vectors from a complex mixture. Validation of the screening results indicates that the shRNA barcode technique can reliable identify active shRNA vectors from a complex pool. Using this approach we have identified three genes, *ARNTL, RBCK1* and *TNIP1*, previously unknown to regulate p53 function. Importantly, *ARNTL (BMAL1)* is an established component of the circadian regulatory network. The latter finding adds to recent observations that link circadian rhythm to the cell cycle and cancer. We show that cells having suppressed ARNTL are unable to arrest upon p53 activation associated with an inability to activate the p53 target gene $p21^{CIP1}$. Conclusions: We identified three new regulators of the p53 pathway through a functional genetic screen. The identification of the circadian core component ARNTL strengthens the link between circadian rhythm and cancer.

Introduction

The *TP53* gene product is important in the cellular response to different types of stress [1,2]. A major physiological stress is DNA damage. DNA damage leads to activation of the ATM/ATR, CHK1/2 cascade, which in turn activates p53. Activation of p53 is achieved by increased stability and post translational modifications of the p53 protein. These modifications include phosphorylation, methylation [3], ubiquitination [4,5] and acetylation [6] leading to enhanced transcriptional activity of p53. Furthermore, oncogene activation can also lead to p53 activation through activation of the $p19^{ARF}$ protein. $p19^{ARF}$ inhibits MDM2, the major E3 ubiquitin ligase for p53 [7] leading to stabilization and activation of p53. Activation of p53 leads to transcriptional activation of a large set of p53 target genes, which in turn causes cell cycle arrest or apoptosis [8].

The p53 pathway is inactivated in almost all human cancers [9]. In about half of human cancers this is due to mutation or deletion of the *TP53* gene itself. However in a significant fraction of human tumors, the p53 pathway is inactivated through alteration in cellular

components acting up- or down-stream of p53. For example, amplification of the negative regulator of p53, *MDM2*, leads to accelerated degradation and inactivation of p53 [2,10].

As a model to screen for genes that modulate p53 function, we previously developed a human fibroblast cell line named BJtsLT [11]. These cells express a temperature-sensitive mutant of the SV40 large T antigen, which allows the cells to proliferate at the permissive temperature (32ºC). However, when the cells are shifted to 39ºC, the large T antigen is degraded and the cells enter a stable p53-dependent cell cycle arrest.

We and others have previously described the construction and initial screening of shRNA libraries using the barcode technique [11-17]. The barcode technique allows the rapid identification of individual shRNA vectors from a large pool of shRNA vectors that produce a specific phenotype. This approach takes advantage of the fact that each shRNA vector contains a unique 19-mer sequence as part of the shRNA cassette, which can serve as a molecular "barcode" identifier. Briefly, cells are infected with the pooled shRNA library of some 24,000 vectors. The population of cells is then split into two separate populations. One population is used as a reference sample while the other sample is subjected to a selective treatment. Knockdown of a specific gene by the shRNA vector can lead to three possible cellular responses to this treatment. First the cells can remain unaffected identical to control cells, second the cells can become more sensitive to the treatment and third the cells can acquire resistance to the treatment. As a consequence of the differential response to the treatment, the relative number of cells that harbor a specific shRNA can increase, decrease or remain the same. The relative abundance of each shRNA cassette can be determined by the isolation of the shRNA cassettes from the population, labeling of the barcode identifiers with different fluorescent dyes and subsequent hybridization to DNA microarrays representing all shRNA sequences. By comparing the relative abundance of all shRNAs against the reference population, shRNAs responsible for the three possible phenotypes can be identified.

The shRNA barcode technique can be used for different screening approaches, most notably the identification of genes that are involved in drug resistance. For instance, we have used the barcoding approach to identify genes that are involved in the resistance to the cytotoxic effects of Nutlin-3, a drug that activates p53 by acting as an inhibitor of MDM2 [13]. This led to the identification of 53BP1, a p53 binding protein, as a critical modulator of the effect of Nutlin-3. More recently, we found the tumor suppressor *PTEN* as a gene, which upon decreased expression confers resistance to trastuzumab in breast cancer [12]. Significantly, the PI3K pathway, which is negatively regulated by PTEN, was also shown to be a major regulator of trastuzumab sensitivity in the clinic, underscoring the utility of the *in vitro* genetic screens to identify drug modulators.

In the first screen applying the NKI shRNA library, Berns et al. used BJtsLT cells to identify 5 new players in the p53 pathway [11]. This screen did not take advantage of the barcode technology. Rather, it was performed by the conventional method of isolating and expanding colonies that are resistant to p53-mediated growth arrest. Subsequent isolation of the shRNA inserts and sequence analysis was required to identify the shRNA responsible for the bypass of the p53 mediated cell cycle arrest. This method is rather laborious and such an approach may not uncover all active shRNAs in a library. Moreover, due to the labour-intensive nature of the screen, only part of the 24,000 vector NKI shRNA library was covered in this initial approach. Here we describe the screening of the entire NKI

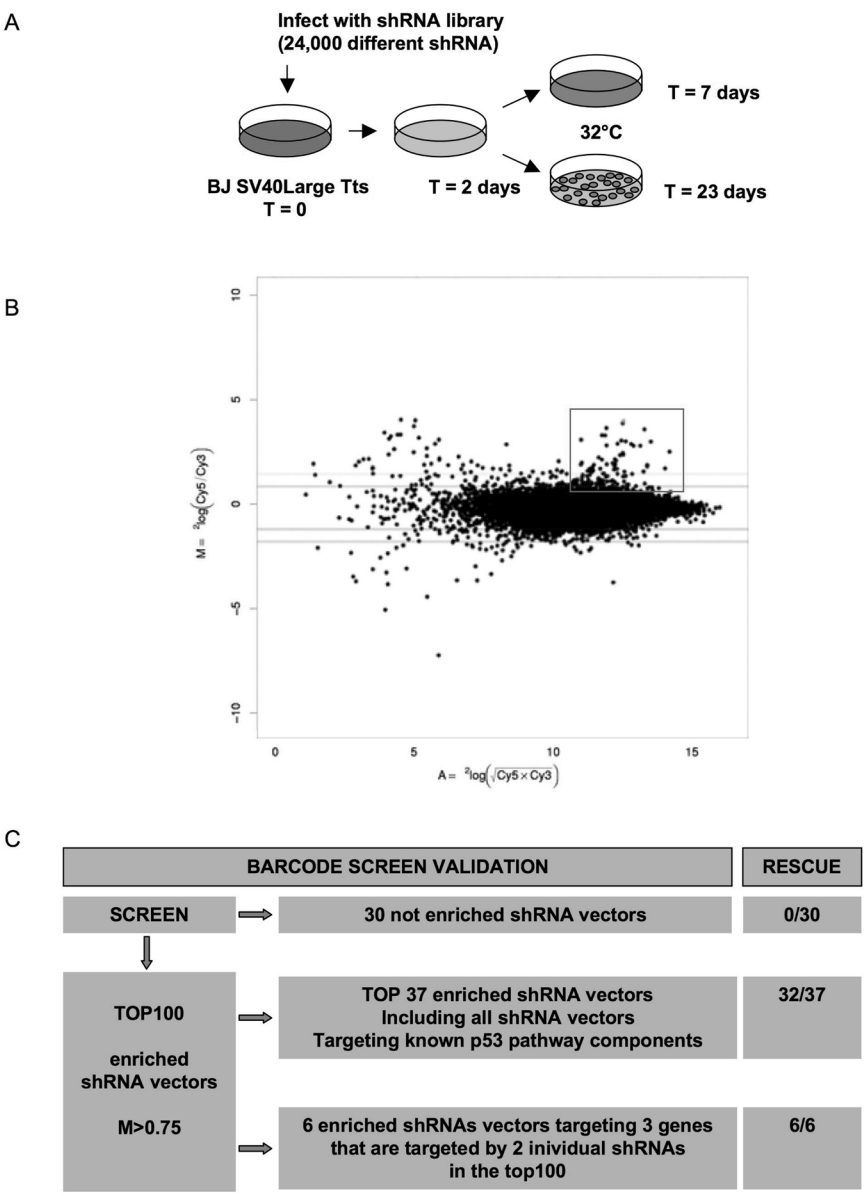

Figure 1

shRNA barcode screen identifies mediators of the p53 dependent cell cycle arrest.

(a) Schematic outline of the BJtsLT genetic screen. BJtsLT cells were infected with the NKI shRNA library and were either left at 32°C or shifted to 39°C. After 7 days the cells at 32°C had reached confluency and were harvested. Cells at 39°C were harvested after 23 days after which they had formed visible colonies. (b) Analysis of the relative abundance of shRNAs recovered from the BJtsLT barcode experiment. Data are normalized and plotted as M, the ^{2}log (ratio Cy5/Cy3), versus A (^{2}log(√intensity Cy3 × Cy5)). The data are the average of two independent hybridization experiments performed in duplicate with reversed colour. A box is drawn around the top 100 enriched shRNAs at 39°C. (c)Schematic overview of selection criteria used to select hits from the shRNA barcode screen for further validation.

shRNA library for modulators of p53 activity using the barcode technology. We find three additional genes whose suppression causes resistance to p53-dependent proliferation arrest.

RESULTS

SCREENING OF THE BJTSLT CELLS

To identify shRNA vectors that can modulate the activity of the p53 pathway, we performed a shRNA bar code screen in BJtsLT cells infected with the NKI shRNA library (See Figure 1a). These cells proliferate at 32°C but enter into a p53-dependent proliferation arrest at 39°C [11]. The infected cells were cultured for three days at 32°C to allow retroviral integration and for gene knockdown to become effective. At day 4 the cells were split into two populations; one was kept at 32°C and the other was shifted to 39°C. The BJtsLT at 32°C were cultured until the population reached confluency and genomic DNA was isolated. The BJtsLT cells grown at 39°C cease proliferation unless a shRNA is expressed that inactivates the p53-dependent anti-proliferative response. Such cells will continue to proliferate and give rise to a colony. In addition to infection with the NKI shRNA library, we also used a shRNA vector targeting p53 as a positive control for colony outgrowth of the BJtsLT cells at 39°C. After 3 weeks of culturing at 39°C, the control plates infected with p53 shRNA vector contained large numbers of colonies consisting of rapidly proliferating cells (data not shown). The plates infected with the shRNA library also contained several colonies. Colonies from the library-infected plates were pooled and genomic DNA was isolated. The shRNA cassettes were recovered from the genomic DNA using PCR. The recovered shRNA inserts from both the control population and the colonies that continued proliferation at 39°C following infection with the shRNA library, were used to hybridize DNA microarrays containing all 24,000 19-mer sequences of the NKI shRNA library. The hybridization was performed for each of the replicate experiments, after which the results were combined to increase the statistical significance of the enriched shRNA vectors identified. Those shRNA vectors that confer resistance to the p53 mediated cell cycle arrest are enriched in the population cultured at 39°C and can be detected as outliers in a MA-plot representation of the barcode microarray experiment (figure 1b).

From this MA-plot we generated a "hit list" of shRNA vectors that are specifically enriched in cell cultured at 39°C. The following considerations were used to produce this hit list (summarized in figure 1c). We excluded all shRNAs that had an intensity (A-value) lower than 10, as spots with a relative low intensity are likely to be "noise" and as a consequence can have aberrantly high "M" ratios. In addition we only included shRNAs if their ratio ($M=^2$log ratio cy5/cy3) was >0.75, this effectively means the top 100 most enriched shRNAs on the micro-array.

To shorten the list of shRNA vectors to be validated, we first asked where shRNA vectors targeting known components of the p53 pathway were positioned in the top 100. In total, five shRNA vectors targeting known components of the p53 pathway were present in the top 100 and their distribution was as follows (Table 1): one shRNA vector targeting p53 (position #8), two shRNA vectors targeting $p21^{cip1}$ (#7 & #37) and two shRNA vectors targeting 53BP1 (#5 & #36). As we identified shRNA vectors targeting 53BP1 and $p21^{cip1}$ at positions 36 and 37 on the hit list, we decided to individually test all shRNA vectors on positions 1-37 in a second round selection. Included in this set are vectors targeting 3 out of 5 genes that were previously identified and validated by Berns et al., (2004): *HDAC4* (#2) *KIA0828* (#13), *HTATIP* (#25). The identification of these shRNA vectors provides further support for the

Rank	M	A	Rescue	HUGO	RefSeq	19mer_start	19mer_sequence	Remarks
1	4.07	12.24	+	KCNK9	NM_016601	217	GTACAACATCAGCAGCGAG	
2	3.95	12.22	+	HDAC4	NM_006037	3751	GCATGTGTTTCTGCCTTGC	Berns et al
3	3.73	11.65	+	ABHD6	NM_020676	374	GGATATGTGGCTCAGTGTG	
4	3.67	12.98	+	MYCL1	NM_005376	933	GAGACACTCCAAACCTGAA	
5	3.39	11.49	+	TP53BP1	NM_005657	657	GATACTGCCTCATCACAGT	Known p53 pathway
6	3.39	11.60	+	XRCC1	NM_006297	258	GGAGGAGCAGATACACAGT	
7	3.18	10.76	+	CDKN1A	NM_078467	919	CTAGGCGGTTGAATGAGAG	Known p53 pathway
8	3.14	12.17	+	TP53	NM_000546	1026	GACTCCAGTGGTAATCTAC	Known p53 pathway
9	3.09	13.20	+	CALCA	NM_001741	428	AGGGATATGTCCAGCGACT	
10	3.00	12.03	+	RBCK1	NM_031229	1389	GTCAGTACCAGCAGCGGAA	this manuscript
11	2.99	12.52	+	INSRR	J05046	2402	GAACAGTGTCCTTCTGCGC	
12	2.92	11.65	+	SSR4	NM_006280	377	GAGTCCTACAGCCTCCTCA	
13	2.91	12.60	+	KIAA0828	NM_015328	3945	GAGTACATTCTGCCTTGCT	Berns et al
14	2.91	12.83	+	PTPRN2	NM_002847	2968	GAGATTGATATCGCAGCGA	
15	2.63	13.86	+	TCEAL1	NM_004780	386	GGACCTGTTTGAGGTTCGC	
16	2.56	12.22	+	LHX3	AF156888	266	GTGTCTCAAGTGCAGCGAC	
17	2.47	12.12	+	NR2E3	AF148128	852	GTGGGCCAAGAACCTGCCT	
18	2.35	11.37	+	LOC90925	BC002792	226	AGAACTGGAGTGGATGGGG	
19	2.28	11.94	+	MPZ	NM_000530	793	GGATAAGAAATAGCGGTTA	
20	2.26	11.49	+	NR2E3	NM_014249	889	GTGGGCCAAGAACCTGCCT	
21	2.21	11.98	+	PENK	NM_006211	779	GATACGGAGGATTTATGAG	
22	2.12	10.78	+	SLIT2	NM_004787	490	AGAGGAGCATTCCAGGATC	
23	2.09	11.87	+	TRAR3	NM_175057	52	GTGAACGAATCCTGCATTA	
24	2.04	11.66	–	CDKN2A	NM_000077	745	GAACCAGAGAGGCTCTGAG	
25	2.04	11.96	+	HTATIP	NM_006388	1045	GTACGGCCGTAGTCTCAAG	Berns et al
26	2.02	11.21	+	PRPF18	NM_003675	228	AGAGGAGGACCAGAAACCA	
27	2.02	11.93	–	GOLGA5	NM_005113	2209	GATACCCCATAGCGCGAGT	
28	2.01	13.08	–	TIEG	NM_005655	2179	GAATTGGAATCCTCCTTAA	
29	1.97	11.92	–	COL12A1	NM_004370	1844	GGATGCCGTTCGCTCAGAA	
30	1.94	13.07	+	RAD51C	NM_058216	263	GATATGCTGGTACATCTGA	
31	1.85	13.70	–	TLR4	NM_003266	2180	GACCATCATTGGTGTGTCG	
32	1.83	11.43	+	RAB2	NM_002865	725	GAAGGAGTCTTTGACATTA	
33	1.83	11.05	+	ZNF347	NM_032584	387	GAGTAATACAGGAGAAGTA	
34	1.82	12.01	+	HSD17B4	NM_000414	142	AGAGGAGCGTTAGTTGTTG	
35	1.80	11.91	–	GCGR	NM_000160	547	AGTGCAACACCGCTTCGTG	
36	1.75	11.19	+	TP53BP1	NM_005657	387	GAACGAGGAGACGGTAATA	Known p53 pathway
37	1.72	11.09	+	CDKN1A	NM_078467	560	GACCATGTGGACCTGTCAC	Known p53 pathway
40	1.67	11.13	+	RPS6KA6	NM_014496		GATTATCCAAAGAGGTTCT	Berns et al
46	1.49	10.96	+	RBCK1	NM_031229	710	GGGGATGAACAGGTGGCAA	this manuscript
51	1.31	10.57	+	TNIP1	NM_006058	1718	GGAAGAGCTGAAGAAGCAA	this manuscript
59	1.23	11.67	+	TNIP1	NM_006058	408	GAGTCCCAGATGGAAGCGA	this mansucript
74	1.00	10.42	+	ARNTL	NM_001178	1468	GAACTTCTAGGCACATCGT	this manuscript
99	0.76	11.55	+	ARNTL	NM_001178	590	GGGAAGCTCACAGTCAGAT	this manuscript

Table 1

List of shRNA vectors that were selected by the criteria as in figure 1c. Depicted are shRNAs that were used in validation experiments. Under rescue; + means a validated shRNA, 2 means not validated. M indicates the 2log (ratio Cy5/Cy3), A the 2log(!intensity Cy36Cy5).

notion that the barcode method enables both fast and reliable screening of shRNA libraries.

In addition, we selected 3 genes that were represented by two independent shRNAs in the top 100 (of which one is present in the 37 already selected shRNAs), bringing the total number of shRNAs to be tested to 42. We included these 3 additional genes in the validation, because if two independent shRNAs targeting the same transcript are enriched in the shRNA screen, this gives higher level of confidence to that specific hit. This is because it is less likely to be "off target" when two independent shRNAs yield the same phenotype and such off target effects of shRNAs are a common problem in these types of genetic screens [18,19]. The other

three genes, *ARNTL* [20], *RBCK1* [21] and *TNIP1* [22] have not been linked to p53 before.

To prove that the shRNA barcode technique specifically identifies shRNAs that are enriched in the experiment we also tested 30 randomly-selected shRNAs not enriched in the experiment. All shRNAs were re-tested in the BJtsLT cells. Cells were infected with individual shRNAs, shifted to 39°C and incubated for 3 weeks. When colonies were observed the cells were fixed and stained. As expected, none of the 30 randomly-selected shRNAs was able to produce colonies at 39°C (data not shown). However, for 37 of the 42 enriched shRNA vectors, we could clearly demonstrate that they allowed the cells to proliferate at 39°C. Among the genes targeted by these 37 shRNA vectors were all known p53 pathway components. In addition, we also observed rescue of growth arrest by all three genes that were targeted by two individual shRNAs: *ARNTL*, *RBCK1* and *TNIP1* (Figure 2). Therefore we decided to focus on these three newly identified genes for which we identified two independent shRNAs.

To show the sensitivity and selectivity of the shRNA barcode technique, we decided to test all three shRNA vectors targeting *ARNTL*, *RBCK1* and *TNIP1* that are present in the library. As mentioned before, for these three genes we found only two of the three shRNAs to be enriched in the screen, whereas one shRNA vector was not enriched. When we infected all three shRNA vectors independently into BJtsLT cells we found that only shRNAs enriched in the shRNA barcode screen gave rise to colonies (Figure 2).

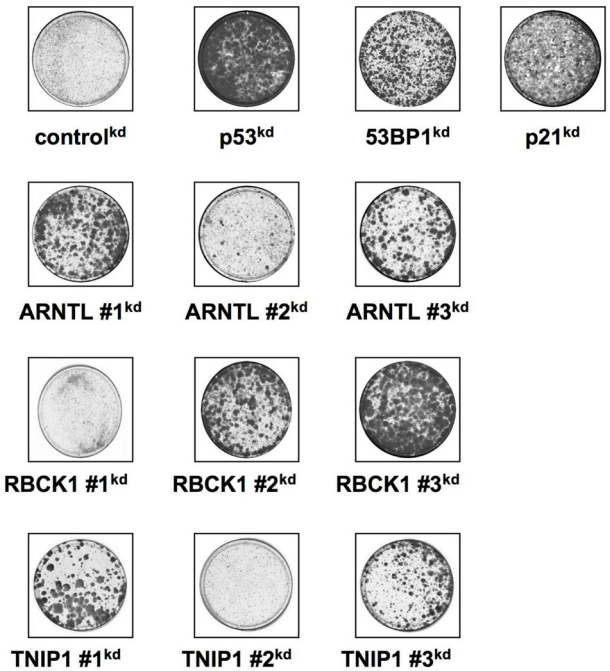

Figure 2
Colony formation ARNTL, RBCK1 and TNIP1 shRNA vectors.
Cells were infected with shRNA vectors targeting ARNTL, RBCK1 and TNIP1 and control shRNA vectors targeting GFP, p53, 53BP1 and p21. Cells were infected at 32°C and shifted to 39°C 2 days after infection. After three weeks culture at 39°C, the cells were fixed and stained.

Knockdown of target genes by shRNA vectors

Next, we investigated if the shRNA vectors targeting *ARNTL*, *TNIP1* and *RBCK1* also reduced mRNA levels of their cognate target genes. BJtsLT cells were infected at 32°C and shifted to 39°C 3 days after infection. When colonies appeared, RNA was isolated and subjected to quantification by QRT-PCR. The result from the QRT-PCR showed that enriched shRNA vectors targeting *ARNTL*, *RBCK1* and *TNIP1* were more potent in decreasing target mRNA than the shRNA vectors that were not enriched (Figure 3a, 3b, 3c). In addition, we tested for both ARNTL and TNIP1 if protein levels were also affected by the shRNA vectors. For ARNTL we co-expressed the three shRNA vectors together with a cDNA encoding hARNTL

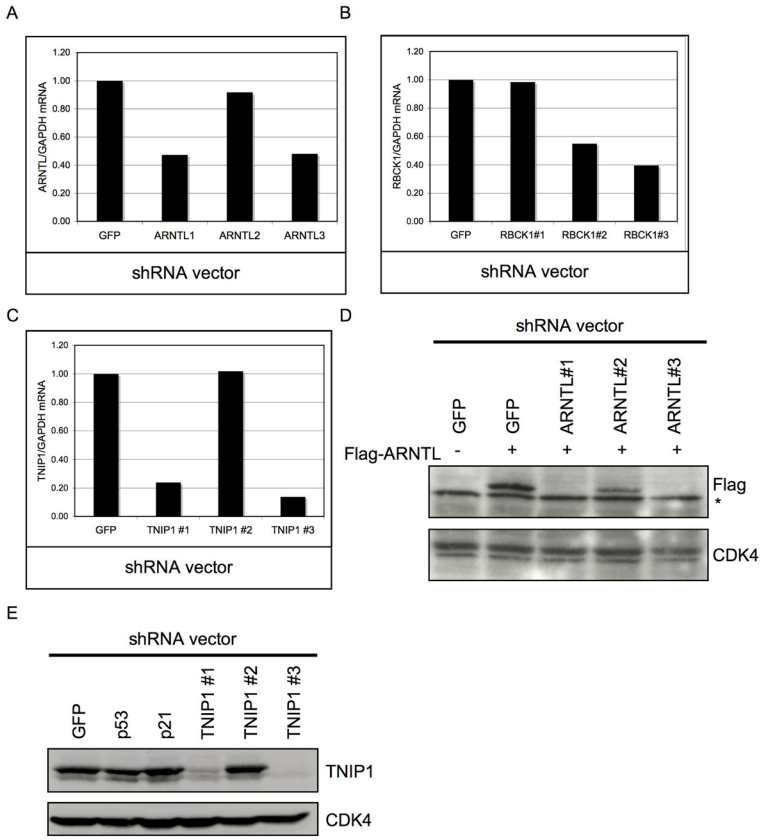

Figure 3

Barcode identified shRNA vectors suppress protein and mRNA levels of their targets.

(a) QRT-PCR for ARNTL in BJtsLT cells. BJtsLT cells were infected with indicated shRNA vectors. Samples for RNA isolation were taken 8 days after shift to 39°C. (b) QRT-PCR for RBCK1 in BJtsLT cells. BJtsLT cells were infected with indicated shRNA vectors. Samples for RNA isolation were taken 8 days after shift to 39°C. (c) QRT-PCR for TNIP1 in BJtsLT cells. BJtsLT cells were infected with indicated shRNA vectors. Samples for RNA isolation were taken 8 days after shift to 39°C. (d) Flag-ARNTL together with the shRNA vectors targeting ARNTL were transiently transfected in Phoenix cells. Extracts were immunoblotted using Flag and CDK4 (control) antibodies. (e) BJ cells were infected with the indicated shRNA vectors and Extracts were immunoblotted using TNIP and CDK4 (control) antibodies.

in Phoenix cells. From the western blot analysis it was clear that only the vectors that could produce colonies also induced potent knockdown of protein expression, thus linking gene knockdown to the p53 growth arrest bypass phenotype (Figure 3d). Knockdown of TNIP1 was determined by analyzing endogenous protein levels in BJtsLT cells (Figure 3e). As can be seen only the vectors that are enriched in the barcode screen and validated to enable colony growth at 39°C were able to reduce endogenous TNIP1 protein levels. We conclude that by limiting the hit selection to genes that are targeted by two independent shRNAs we have only selected 'on-target' hits from a complex library.

Knockdown of ARNTL, RBCK1 and TNIP1 in BJtsLT cells leads to reduced p21^{CIP1} levels

p21^{CIP1} is one of the critical effectors of p53 to induce a cell cycle arrest [23]. This is further supported by our identification of two shRNAs targeting p21^{CIP1} in the list of outliers of the BJtsLT screen. We therefore examined the effect of knockdown of *ARNTL*, *RBCK1* and *TNIP1* on *p21^{CIP1}* induction. We tested the effects on p21^{CIP1} in the BJtsLT system that we used for the initial screen. When BJtsLT cells are shifted to 39°C, a rapid increase in p21^{CIP1} protein levels is observed (Figure 4a). As expected, this p21^{CIP1} induction is attenuated in cells infected with shRNA vectors targeting *p53, p21^{CIP1}* or *53BP1* (Figure 4a). When we used shRNA vectors targeting ARNTL, RBCK1 and *TNIP1* we observed a decrease in p21^{CIP1} levels for those shRNA vectors that produced colonies at 39°C, but not for shRNA vectors that failed to produce colonies (Figure 4a-c).

To be sure that the decrease in p21^{CIP1} protein levels were caused by decreased p21^{CIP1} transcription we also measured *p21^{CIP1}* mRNA levels by QRT-PCR (Figure 4d). All shRNAs that could produce colonies at 39°C also showed a decrease in p21^{CIP1} mRNA. This result suggests that the knockdown of *ARNTL*, *RBCK1* and *TNIP1* leads to a decreased transcriptional activity of p53 towards its target *p21^{CIP1}*.Recently multiple reports have discussed the relationship between cancer and circadian rhythm [24-26]. ARNTL is a core component of circadian rhythm transcriptional machinery [20,27]. ARNTL binds to CLOCK and together they regulate expression of 1,000s of genes in a circadian timing [28,29]. Genes regulated in a circadian fashion are involved in cell cycle, detoxification and other processes [30]. Therefore we decided to test if ARNTL is involved in the regulation of p21^{CIP1} expression in other cell systems.

Reduced p21^{CIP1} activation after DNA damage in HCT116 cells with ARNTL knockdown

Normal human cells arrest either in G1 or S phase of the cell cycle after encountering DNA damage to repair the DNA, thereby preventing accumulation of mutations in the genome of daughter cells. The G1 phase cell cycle arrest is p53 dependent and mainly executed by the CDK inhibitor p21^{CIP1} [31,32].

To investigate if ARNTL is also required for the *p21^{CIP1}* activation after DNA damage, we infected U2OS osteosarcoma derived cells with different shRNAs targeting ARNTL. The cells were incubated to allow knockdown to take affect, after which cells were irradiated to inflict DNA damage and monitored for *p21^{CIP1}* activation. When we compared cells that were infected with a shRNA vector targeting p53 to cells infected with a control shRNA vector, we observed lower p53 and p21^{CIP1} levels after γ-radiation. In the cells infected with ARNTL

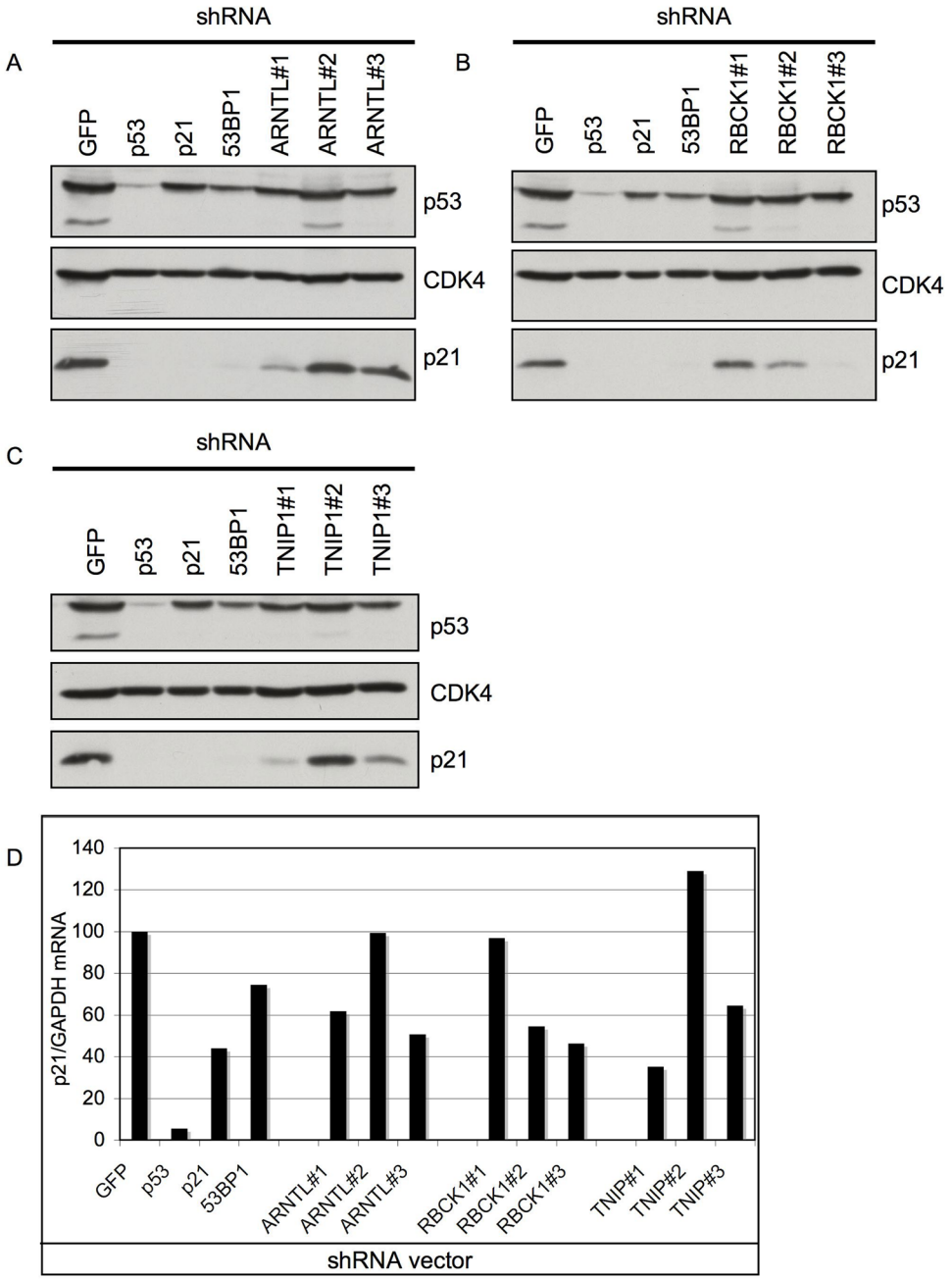

Figure 4
Knockdown of ARNTL, TNIP1 and RBCK1 prevents $p21^{CIP1}$induction in BJtsLT cells
(a-c) BJtsLT cells were infected at 32°C and shifted to 39°C for colony formation. After 14 days of culturing at 39°C cells were harvested, protein lysates were prepared and subjected to western blot for p53, CDK4 (control) and $p21^{CIP1}$. (d) Additionally, total RNA was isolated and used for QRT-PCR for $p21^{CIP1}$.

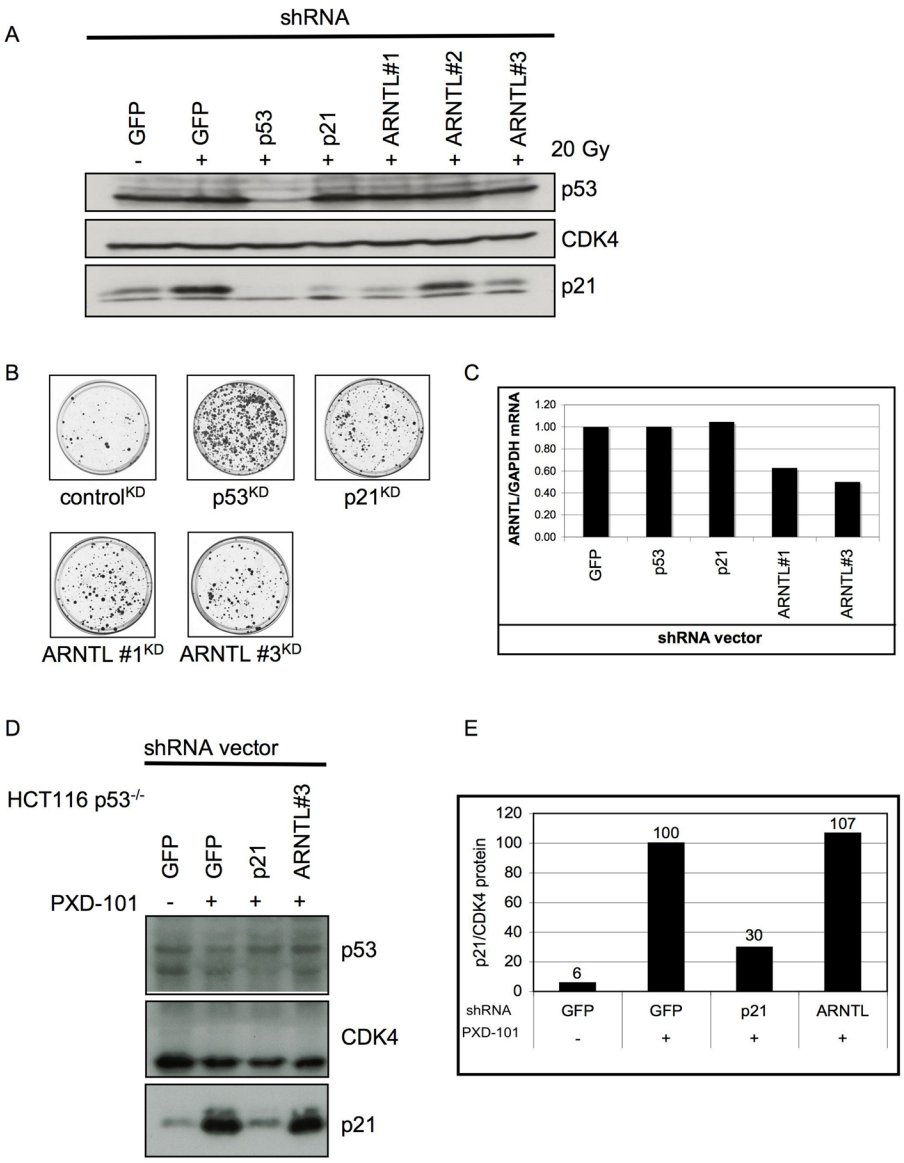

Figure 5
ARNTL regulates p21^{CIP1} expression.
(a) Knockdown of ARNTL inhibits radiation induced p21^{CIP1} induction. U2OS cells were infected with the shRNA vectors as indicated. Cells were seeded and irradiated with 20 Gy of γ-radiation. After o/n incubation cells were lysed and lysates were subjected to western blot using antibodies for p53, CDK4 (control) and p21^{CIP1}. (b) Knockdown of ARNTL can also rescue a p19-induced cell cycle arrest. U2OS cells were infected with the indicated shRNA vectors followed by a super-infection with p19^{ARF}-RFP virus. Cells were seeded and incubated for three weeks. After three weeks the infected cells were fixed and stained. (c) Knockdown of ARNTL in U2OS cells (Fig 5b) was quantified by QRT-PCR. (d) ARNTL knockdown is not involved in p53 independent p21^{CIP1} induction. HCT116 wt and p53-/- cells were infected with knockdown vectors targeting p53, p21^{CIP1} and ARNTL. Cells were treated with 0.5 μM PXD101 for 16 hrs. Cells were then lysed and lysates were subjected to western analysis for p53, CDK4 (control) and p21^{CIP1}. (e) Quantification of p21 protein levels in the western blot in figure 5c using IMAGE J software.

knockdown vectors we also observed lower $p21^{CIP1}$ protein levels, but p53 protein levels were unaffected (Figure 5a). This observation suggests that ARNTL can modulate the activity of p53 towards its target *p21CIP1*. However, we cannot distinguish between a specific effect of ARNTL on $p21^{CIP1}$ and a more general effect of ARNTL on p53 transcriptional activity.

ARNTL knockdown also allows bypass a $p19^{ARF}$ induced growth arrest

As the BJtsLT cells are quite artificial due to the presence of the SV40 T viral oncogene, we also investigated if ARNTL knockdown could bypass a more physiological p53-induced cell cycle arrest. To address this, we used cells in which we can activate p53 by over-expression of $p19^{ARF}$. $p19^{ARF}$ inhibits MDM2 function thereby leading to an increase of p53 protein and activation of target genes [33]. Activation of $p19^{ARF}$ leads to a stable p53-dependent cell cycle arrest [34]. To test if ARNTL knockdown can also rescue a $p19^{ARF}$-induced cell cycle arrest, we infected U2OS cells with the shRNAs targeting *ARNTL*. After knockdown had taken effect the cells were super-infected with a $p19^{ARF}$ encoding retrovirus. We observed that cells with knockdown of *p53* or *p21CIP1* continue proliferation after the forced expression of *p19ARF* (Figure 5b), knockdown of *ARNTL* also allows cells to proliferate after p53 activation by $p19^{ARF}$ (for knockdown see figure 5c). This result suggests that *ARNTL* expression is required for the anti-proliferative response of 19^{ARF} activation. When *ARNTL* levels are low, the cells escape this arrest.

P53 independent $p21^{CIP1}$ activation does not require ARNTL

The results described above suggest a role for ARNTL in the regulation of *p21CIP1* expression by p53. However they do not rule out that ARNTL controls $p21^{CIP1}$ activation in a general fashion, independent of p53. To test this possibility we made use of small molecule HDAC inhibitors (HDACi). These HDACi cause induction of $p21^{CIP1}$ in both a p53 dependent and p53 independent manner [35-38]. In order to study the effect of ARNTL on p53 independent activation of $p21^{CIP1}$, we made use of a HCT116 p53 knockout cell-line (HCT116 p53-/-) and the HDACi PXD101 (Belinostat®) [32]. When these HCT116 p53-/- cells are treated with PXD-101, a strong induction of $p21^{CIP1}$ is observed (Fig 5d and e). HCT116 p53-/- cells infected with a shRNA vector against *p21CIP1* show reduced $p21^{CIP1}$ protein levels after PXD-101 treatment. However, cells infected with a shRNA targeting *ARNTL* do not show any alteration in the induction of $p21^{CIP1}$ protein levels following HDACi treatment. Thus the p53-independent induction of $p21^{CIP1}$ by HDACi is not dependent on ARNTL. From this we conclude that ARNTL is not generally required for *p21CIP1* induction, but does affect the capacity of p53 to activate *p21CIP1* expression.

Discussion

The screening of large-scale RNAi libraries has been used increasingly over the last years to identify the specific functions of genes in cellular pathways, networks and mechanisms. Here we describe the screening of a complex RNAi library to identify genes that were previously unknown to regulate a p53-dependent cell cycle arrest.

We have used the RNAi barcode technique to screen a human shRNA library containing ~24,000 vectors targeting ~8,000 genes. Using this approach, we were able to

rapidly identify shRNAs that allow bypass of a p53 dependent cell cycle arrest. In total we confirmed that 32 out of the
37 genes that were identified by the barcode screen could indeed prevent cells from entering into a p53 dependent cell cycle arrest. However, only 5 of these 32 genes were targeted by two independent shRNAs. Two out of these five genes (*TP53BP1* and *p21^{CIP1}*) are well-known to be involved in p53 signaling. However the other three genes (*TNIP1, RBCK1* and *ARNTL*) were previously not known to be involved in the p53 pathway.
The three newly identified genes all affect the induction of the p53 target gene p21^{CIP1} but no change in p53 protein stability is observed after ARNTL, TNIP1 or RBCK1 knockdown. Importantly, p21^{CIP1} knockdown alone is sufficient to rescue cells from the p53 induced cell cycle arrest. This observation indicates that the rescue of the p53 induced cell cycle arrest by ARNTL, RBCK1 or TNIP1 knockdown is the result of a lack of p21^{CIP1} induction by p53.

The activity of p53 has been mainly attributed to its role as transcription factor with tumor suppressive capacities. Therefore, we assessed if any of the genes identified in our screen had been linked to transcription before. TNIP1 was originally identified as an inhibitor of NF-κB signaling [22,39]. Although it was shown that TNIP1 over-expression inhibits the transcriptional activity of the NF-κB heterodimer it is believed that this is an indirect effect through an currently unknown mechanism. The ubiquitin E3 ligase RBCK1 has been reported to regulate and ubiquitinate several proteins [21,40-42]. Although experiments have been performed that suggest a role for RBCK1 in transcription [43] a clear role for RBCK1 in regulating transcription has not been reported up till now. This picture is different for ARNTL which is known to be the central transcription factor in regulating circadian rhythm. The critical role of ARTNL in circadian rhythm was demonstrated by the construction of the knockout mouse. Mice that are deficient for *ARNTL* are unable to maintain a circadian rhythm in constant darkness [20]. In addition, the *ARNTL* knockout mouse also suffers from premature aging [44]. In recent years, many other processes have been shown to be regulated in a circadian fashion. Most importantly it was shown that the mammalian cell cycle is controlled by circadian rhythm [45]. The possible involvement of circadian rhythm in cancer results from studies of the *Period 2* knockout mouse.This mouse is prone to develop tumors after radiation. Later it was shown that also the Period 1 protein can regulate cell cycle checkpoints [24-26]. Interestingly both *Period 1* and *2* are bona-fide transcriptional targets of ARNTL.

For the induction of target genes ARNTL must form a heterodimer with the CLOCK protein [28]. Target genes of the CLOCK/ARNTL heterodimer include the *Period 1, 2* and *3* and *Cryptochromes (Cry 1 & 2)* [46]. The increased abundance of Period and Cryptochrome proteins [47] induces a negative feedback loop that ultimately shuts down transcription by the CLOCK/ARTNL heterodimer. When the concentration of Period and Cryptochrome decreases due to proteasomal degradation the CLOCK/ARNTL complex can initiate another round of transcription thereby completing a cycle of circadian rhythm.

Another transcriptional target of the CLOCK/ARNTL is the CDK inhibitor *p21^{CIP1}* which is also regulated in a circadian manner [48]. We show here that *ARNTL* knockdown in human cells can abrogate induction of *p21^{CIP1}* after p53 activation and overrides a p53-dependent cell cycle arrest. The effect on the induction of *p21^{CIP1}* is in contrast with previous reports on the *p21^{CIP1}* regulation in ARNTL knockout mice [48]. In these animals ARNTL

is required for the circadian expression of $p21^{CIP1}$. This discrepancy might be explained by differential regulation of the $p21^{CIP1}$ promoter in mice or man. In particular, this difference may arise from stress signals differences from *in vitro* versus *in vivo* conditions. Nevertheless, our data clearly indicate that there is a link between the regulation of circadian rhythm and the control of p53 activity in human cells.

Conclusions

By screening a large scale RNAi library in human cells we have identified three novel genes that can regulate p53 function. Loss of expression for each of three genes results in a decreased ability of p53 to activate *$p21^{CIP1}$* expression. Importantly, we showed that ARNTL is required for the p53-dependent induction of *$p21^{CIP1}$* in two additional cell types using different ways to activate p53: a $p19^{ARF}$-induced cell cycle arrest and a DNA damage mediated cell cycle arrest. We conclude that *ARNTL* suppression affects the ability of p53 to induce a cell cycle arrest upon cellular stress signals such as DNA damage.

Materials and Methods

Cell lines & culture conditions

BJtsLT cells were cultured in medium that consisted of DMEM 75% / M199 25% supplemented with 10% FCS, Penicillin and Glutamine. BJtsLT cells were cultured at 32°C in 5% CO2. U2OS and Phoenix cells were cultured in DMEM supplemented with 10% FCS, Penicillin and Glutamine. U2OS and Phoenix cells were cultured at 37°C in 5% CO2.

Plasmids and library

Expression plasmid for ARNTL was generated by PCR from a cDNA library and subsequent cloning the PCR product into pCR3-Flag. The P19-RFP construct was described previously [11]. The construction of the library was described previously [11]. Briefly, the NKI shRNA library was designed to target 7914 human genes, using three shRNA vectors for every targeted gene, cumulating in a total of 23,742 shRNA vectors. The shRNAs are cloned into a retro-viral vector to enable infection of target cells.

Retroviral infection

Phoenix cells were transfected using calcium phosphate method. Viral supernatant was cleared through a 0.45 μM filter. Cells were infected with the viral supernatant in presence of polybrene (8 μg/ml). The infection was repeated twice.

shRNA barcode screen

To screen the NKI shRNA library we reasoned that we would need 100 fold coverage of the library to get a good representation of all 23,742 shRNA vectors present in the library. BJtsLT cells were infected with the NKI shRNA library. Two days after infection cells were plated at 150,000 cells/15 cm dish. In total 2 x 106 cells were shifted to 39°C, equal number of cells were kept at 32°C. Cells at 32°C were harvested after 5 days. Cells at 39°C were harvested at 21 days after shift. From both populations gDNA was isolated using DNAzol (Invitrogen). The shRNA cassettes were amplified by PCR. The PCR product was used for *in vitro* RNA synthesis. RNA was labeled with Cy3 or Cy5 (Kreatech) and hybridized on a microarray. Quantification of the resulting fluorescent images was performed with Imagene 5.6 (BioDiscovery), local background was subtracted, and the data were normalized and 2log transformed. Additional information on barcode screens can be found at http://www.screeninc.nl.

Colony formation assay

Cells were infected with retroviral supernatant. Two days after infection the cells were seeded at 50,000/10 cm dish and shifted to 39°C. Cells were cultured at 39°C for approx 21 days. When colonies appeared cells were fixed in MeOH/HAc (3:1) and subsequently stained (50% MeOH/10% HAc/0.1% Coomassie).

Western blotting

Cell lysates were separated using 10% SDS-PAAGE. Proteins were transferred to PVDF membrane and incubated with primary antibody as indicated. Primary antibodies were detected using a secondary HRP-conjugated antibody.

Antibodies used for these studies: Flag (M2; Sigma), TNIP1 (1A11E3; Zymed), CDK4 (C-22; Santa Cruz), p53 (DO-1; Santa Cruz) and p21CIP1 (C-19; Santa Cruz).

QRT-PCR

Total RNA was isolated using TRIzol (Invitrogen). From the total RNA cDNA was generated using Superscript II (Invitrogen) using random primers (Invitrogen). cDNA was diluted and QRT reaction was performed using Taqman probes (Applied Biosystems). All QRT reactions were run in parallel for GAPDH to control of for input cDNA. The QRT reactions were run at a AB7500 Fast Real Time PCR system (Applied Biosystems). Results shown are a representative of three independent experiments.

$p21^{CIP1}$ induction by g-radiation and PXD101

For the $p21^{CIP1}$ induction by radiation 50,000 cells were seeded per 6-well. HCT116 p53-/- cells were irradiated with 20 Gy g-radiation from a Cs-137 source. Cells were incubated for 16 hrs and lysed. For the $p21^{CIP1}$ induction by PXD101 50,000 cells were seeded/6-well and treated with 0.5 μM PXD101 for 16 hrs after which the cells were lysed and protein lysates were subjected to western analysis.

AUTHOR CONTRIBUTIONS

JM and AWMF performed the experiments, wrote the manuscript. MM performed the microarray hybridizations. RB and RLB supervised the research and corrected the manuscript.

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Chapter IV

IRAK2 is a novel modulator of the TGFβ signaling cascade

submitted to Molecular Cancer Research

IRAK2 is a novel modulator of the TGFβ signaling cascade

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Abstract

The TGFβ pathway orchestrates an extensive transcriptional program that is important for many processes in the cell. For example TGFβ regulates cell cycle, migration and epithelial-to-mesenchymal transition (EMT). The TGFβ pathway has a dual role in cancer: it is involved in early stage tumor suppression, but also contributes to tumor progression by promoting invasion. To identify novel genes involved in TGFβ pathway signaling, we have performed a functional genetic loss-of-function screen. We screened a siRNA library targeting 700 kinases and kinase related genes in a TGFβ responsive reporter assay. Several genes were identified that upon knockdown could repress the reporter signal; among these are the two cellular receptors for TGFβ. In addition to these two known components of the TGFβ pathway, several genes were identified that were previously not linked to the TGFβ signaling. Knockdown of one of these genes, the *IRAK2* kinase, resulted not only in an impaired TGFβtarget gene response but also in a reduction of the nuclear translocation and phosphorylation of SMAD2. In addition, suppression of *IRAK2* expression led to a partial override of a TGFβ induced cell cycle arrest. Our data demonstrate that IRAK2 is a novel and critical component of TGFβ signaling.

INTRODUCTION

The TGFβ pathway is an important signaling pathway that regulates many different processes such as cell cycle, epithelial to mesenchymal transition (EMT), migration and angiogenesis [1, 2]. Stimulation of the TGFβ pathway is initiated by binding of the TGFβ cytokine to the TGFβ receptors (TGFBR1 and TGFBR2). This leads to TGFβ receptor complex formation, which results in the phosphorylation of TGFBR1 by TGFBR2. Subsequently, SMAD2 and SMAD3 are phosphorylated by the TGFBR1, which leads to their translocation to the nucleus. In the nucleus the activated SMADs can form a complex with SMAD4. This activated SMAD complex recruits transcriptional co-factors which assist in regulating transcription of genes whose promoters contain SMAD binding DNA elements [3].

The TGFβ pathway plays a dual role in cancer pathogenesis. In normal epithelium and early stage tumors, the TGFβ pathway was reported to have an inhibitory effect on cell proliferation [4]. This is achieved mainly through induction of a G1 cell cycle arrest through up-regulation of the CDK inhibitors *CDKN1A* (encoding p21) and *CDKN2B* (encoding p15) and downregulation of the *MYC* proto-oncogene [5-7]. However, cancer cells can become insensitive to this proliferation control by loss of expression of the TGFβ receptors or SMADs. In addition, it has been observed that some tumors have specifically inactivated the cytostatic response to TGFβ while retaining normal SMADs and receptors. In these tumors, the TGFβ pathway subsequently promotes cancer progression through induction of EMT, angiogenesis and evasion of immune surveillance [1, 2]. Furthermore, it was recently shown that patients carrying tumors with an activated TGFβ pathway have a worse prognosis than patients carrying tumors without activation of the TGFβ pathway [8, 9]. For this reason different anti-cancer strategies are developed to inhibit the TGFβ pathway. Clinical trials have been performed with TGFβ antisense oligonucleotides, TGFβ antibodies and a TGFBR1 small molecule inhibitor [10].

Several approaches have been taken to identify new players in the TGFβ pathway. For example protein-protein interaction screens have been used to extend the knowledge about the TGFβ pathway [11, 12]. In addition, a functional genetic screen to identify new players in the mammalian TGFβ network has been performed by Levy and colleagues. They describe the screening of a siRNA library targeting the ubiquitin E3-ligase gene-family in a cell line with an integrated TGFβ responsive reporter. Through this approach they identified Arkadia as a positive regulator of the pathway [13].

We describe here a functional genetic screen aimed at the identification of novel kinases that function in the TGFβ pathway. We identify here an unexpected new kinase that contributes to TGFβ signaling.

RESULTS

THE SCREENING OF A TGFβ REPORTER CELL LINE WITH A HUMAN KINOME SIRNA LIBRARY

To screen siRNA libraries in high throughput format for novel modulators of TGFβ signaling, we generated an U2OS osteosarcoma cell line with a stably integrated pCAGA$_{12}$-Luciferase TGFβ responsive reporter (this cell lines was named U2OS-CAGA). This reporter has been described to be primarily responsive to SMAD3/4 dependent transcription [14]. To test the responsiveness of the integrated reporter in the U2OS-CAGA cell line, we transfected siRNAs

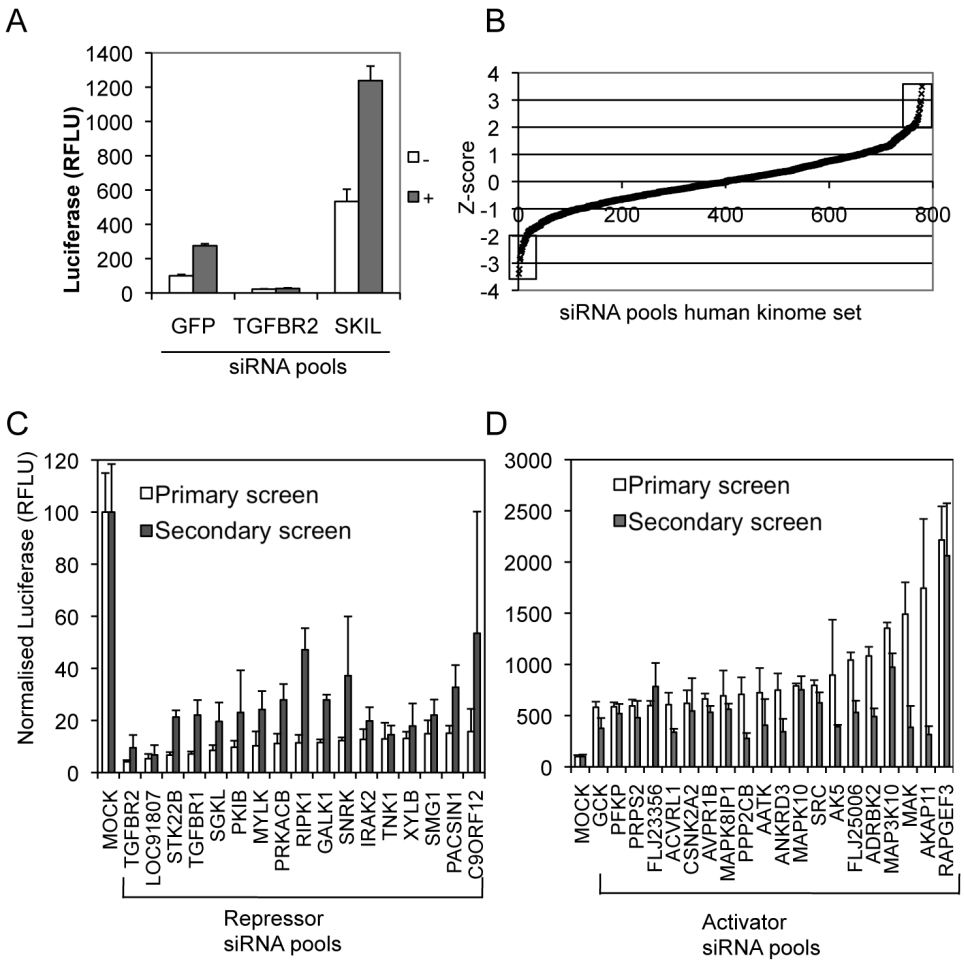

Figure 1
Screen with TGFβ responsive reporter identifies modulators of the TGFβ pathway
U2OS-CAGA cells harboring a stable TGFβ responsive luciferase reporter were transfected with siRNAs against GFP, TGFBR2 or SKIL and treated with 200 pM TGFβ (+) for 14 hrs or left untreated (-) (a). Results from the kinase siRNA screen, Z-scores were calculated and plotted in ascending order. Boxed areas indicate the siRNA pools selected for follow-up which repress or activate the TGFβ responsive reporter (b). Seventeen siRNA pools that repress the reporter (c) and twenty siRNAs that activate the reporter (d) were tested in a follow-up experiment. The normalized luciferase counts from the screen (white bars) and follow-up (grey bars) are plotted.

targeting a positive and a negative regulator of TGFβ signaling. As expected, ablation of the positive regulator *TGFBR2* by RNAi led to complete abrogation of both basal and TGFβ induced reporter activity (fig. 1a). Moreover, knockdown of a negative regulator of the TGFβ signaling cascade, *SKIL* caused the opposite effect, showing increased reporter activity. It is worth noting that in absence of exogenously added TGFβ knockdown of SKIL still causes enhanced signal of the TGFβ responsive reporter (fig.1a). We also observed a robust TGFβ dependent

induction of the TGFβ reporter in cells transfected with siRNAs targeting GFP (fig. 1a).

These initial experiments showed that the integrated TGFβ reporter in the U2OS-CAGA cell line behaved as expected. Therefore, we used this cell line to screen a siRNA library targeting 700 kinases and kinase related proteins. The siRNA library that we used consists of siRNA pools; each gene is targeted by 4 separate siRNAs. The U2OS-CAGA cells were transfected in triplicate with siRNA pools of the human kinome library and the entire screen was performed in presence of TGFβ. To identify siRNAs that modulate the reporter, we measured both luciferase activity and cell viability. The cell viability was measured to exclude siRNAs that cause a decrease in cell viability as these might be identified as false positive repressors in the screen. Data was normalized and a hit list was generated by calculating a Z-score for every tested siRNA pool (fig. 1b).

Only siRNA pools that produced a Z-score greater than 2 (20 genes) or lower than -2 (17 genes) were selected for further analysis (Supplementary table 1). Among the genes targeted by siRNA pools that repressed the reporter were the positive controls: *TGFBR1* and *TGFBR2*. In addition, we identified the siRNA pool against *ALK1 (ACVRL1)* as an activator of the TGFβ responsive reporter. ALK1 was previously identified as a TGFβ superfamily receptor type I

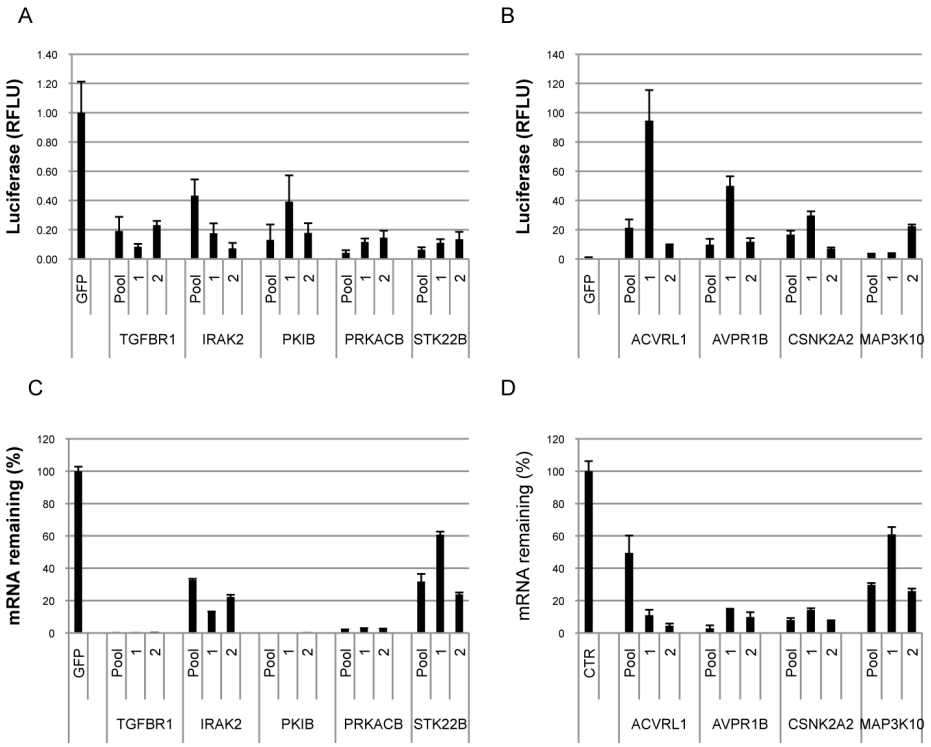

Figure 2
Nine hits from the screen are 'on-target'
Individual siRNAs that form the siRNA pool (P) were tested separately in the U2OS-CAGA cell line. A GFP siRNA was taken along as negative control. Two individual siRNAs and the pool that could either repress (a) or activate (b) the reporter are shown. Knockdown for these individual siRNAs was determined by quantitative PCR (c and d). One-tailed t-tests determined that all values are significantly different from the control siRNA (p-value < 0.05).

protein that can form a complex with TGFBR2 and directly antagonizes TGFβ transcriptional activation mediated by TGFBR1 [15, 16]. The fact that the screen was able to identify these genes that are part of the canonical TGFβ signaling network supports the notion that at least some of the other genes in the hit list are potential genuine players in the TGFβ pathway.

As a first step to validate our screening results we re-tested the same siRNA pools that were identified by the screen to verify that they could indeed reproduce the phenotype from the primary screen. Therefore, we transfected 37 siRNA pools into the U2OS-CAGA cell line and measured the luciferase signal. For 36 of the 37 siRNA pools we could show significant reproduction of the phenotype as measured in the primary screen, indicating that the initial screen with the siRNA pools was reproducible (fig. 1c and 1d).

One of the major drawbacks of using RNAi as a screening tool is that some observed phenotypes can potentially be caused by 'off-target' effects [17, 18]. Therefore we performed a second round of validation for a subset of our hits. We selected hits based on the position in the primary hit list. In addition, protein-protein interaction databases were used to preferentially select hits that interact with known TGFβ pathway members. Sixteen hits (eight siRNA pools that could activate the reporter and eight that repressed) were

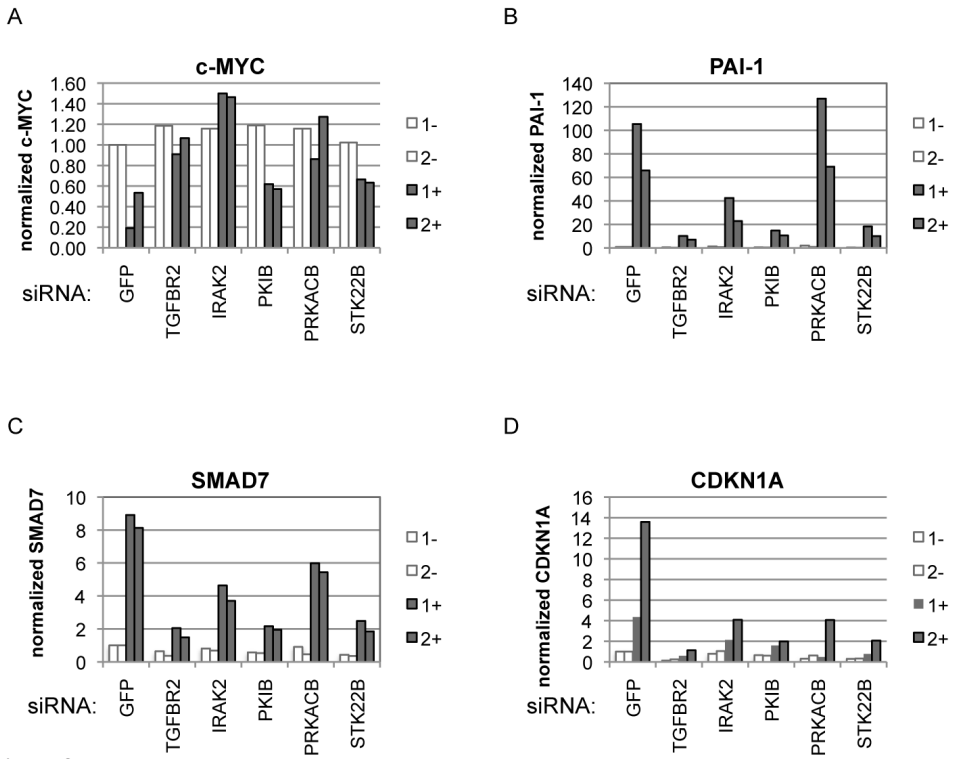

Figure 3

Endogenous TGFβ target gene regulation

The 'on target' siRNA pools that repress the reporter were transfected in PC3 cells and target gene expression was measured in the absence (in duplicate; 1- and 2-) or presence of TGFβ; 2 hrs for c-MYC (a) and CDKN1A (d) or 8 hrs for PAI-1 (b) and SMAD7 (c) (in duplicate; 1+ and 2+). The mRNA levels of the target genes were determined by quantitative PCR and normalized to the reference gene RPL13.

selected for a second round of validation. In this second round validation the four siRNAs of each siRNA pool were transfected separately, to test if they could reproduce the originally observed phenotype. For nine of the sixteen hits tested, we identified two individual siRNAs that could reproduce the original phenotype (fig. 2a and b). In addition, all siRNAs that could either repress or activate the reporter could also significantly repress the intended target mRNA. Therefore we consider the genes targeted by these siRNAs to be 'on target' [19].

Effect of knockdown of 'on-target' hits on endogenous TGFβ signaling

Since the reporter system used here is only an indirect measurement of TGFβ pathway activity, we set out to test if the validated hits could also affect endogenous TGFβ target gene expression. Many different genes are directly regulated by TGFβ through the SMADs; among these genes are *CDKN1A, SMAD7, PAI-1* and *c-MYC*. As a model for endogenous TGFβ signaling we used the prostate cancer cell line PC3, in these cells transcription of *CDKN1A, SMAD7, PAI-1* is upregulated while *c-MYC* is transcriptionally repressed after addition of TGFβ. Because we identified a relative large number (11) of hits that proved to be 'on-target', we decided to proceed with only the genes whose downregulation impaired activation of the TGFβ reporter (4 in total) as these genes are potential novel targets for therapy in cancer. These four hits, together with *TGFBR2* as a control, were tested for their ability to modulate endogenous TGFβ target gene expression. Indeed, all hits tested showed impaired target gene regulation for two or more endogenous TGFβ targets (fig. 3a-d).

Identified hits are involved in SMAD2/3 cytoplasmic to nuclear translocation

TGFβ target gene activation is a downstream event in the TGFβ signaling cascade, which is preceded by activation and the translocation of SMAD2/3 complexes from the cytoplasm to the nucleus. For this reason we tested if the genes that could repress TGFβ target gene activation also impaired SMAD2/3 translocation. As a control, we measured the effect of the knockdown of TGFBR1 on TGFβ induced SMAD2/3 nuclear translocation by immunohistochemistry. We quantified the effect by determining the ratio of SMAD2/3 in the nucleus versus the cytoplasm using Cellprofiler software [20] (fig. 4a). PC3 cells were transfected with siRNAs targeting the 'on target' hits in order to determine the nuclear/cytoplasmic ratio of SMAD2/3 in presence of TGFβ. A clear reduction in SMAD2/3 nuclear accumulation was measured in cells transfected with siRNAs against *TGFBR1*. In addition, we measured a lower TGFβ induced SMAD2/3 nuclear/cytoplasmic ratio for cells transfected with siRNA pools against IRAK2, PKIB and STK22B (fig. 4b). This suggests that, like the *TGFBR1, IRAK2, PKIB* and *STK22B* function upstream or at the level of SMAD2/3 translocation in the TGFβ pathway.

IRAK2 is a genuine player in the TGFβ signaling cascade

As can be seen in figure 3, of all genes tested only knockdown of IRAK2 expression affected all bona fide TGFβ target genes tested, which is accompanied by reduced nuclear translocation of SMAD2/3. Therefore, we decided to perform additional experiments to find out if IRAK2 can modulate the TGFβ pathway in different cell lines. In addition we set out to investigate the exact mechanism of inhibition of the TGFβ pathway by IRAK2

knockdown. Previously, IRAK2 (IL-1R-associated kinase) was described to play a role in Toll like receptor signaling [21]. As shown before, knockdown of IRAK2 impaired the TGFβ target gene activation of *c-MYC, PAI-1, CDKN1A* and *SMAD7* (fig. 3). At least three of these genes are involved in cell cycle regulation (*c-MYC, PAI-1 and CDKN1A*) and it has

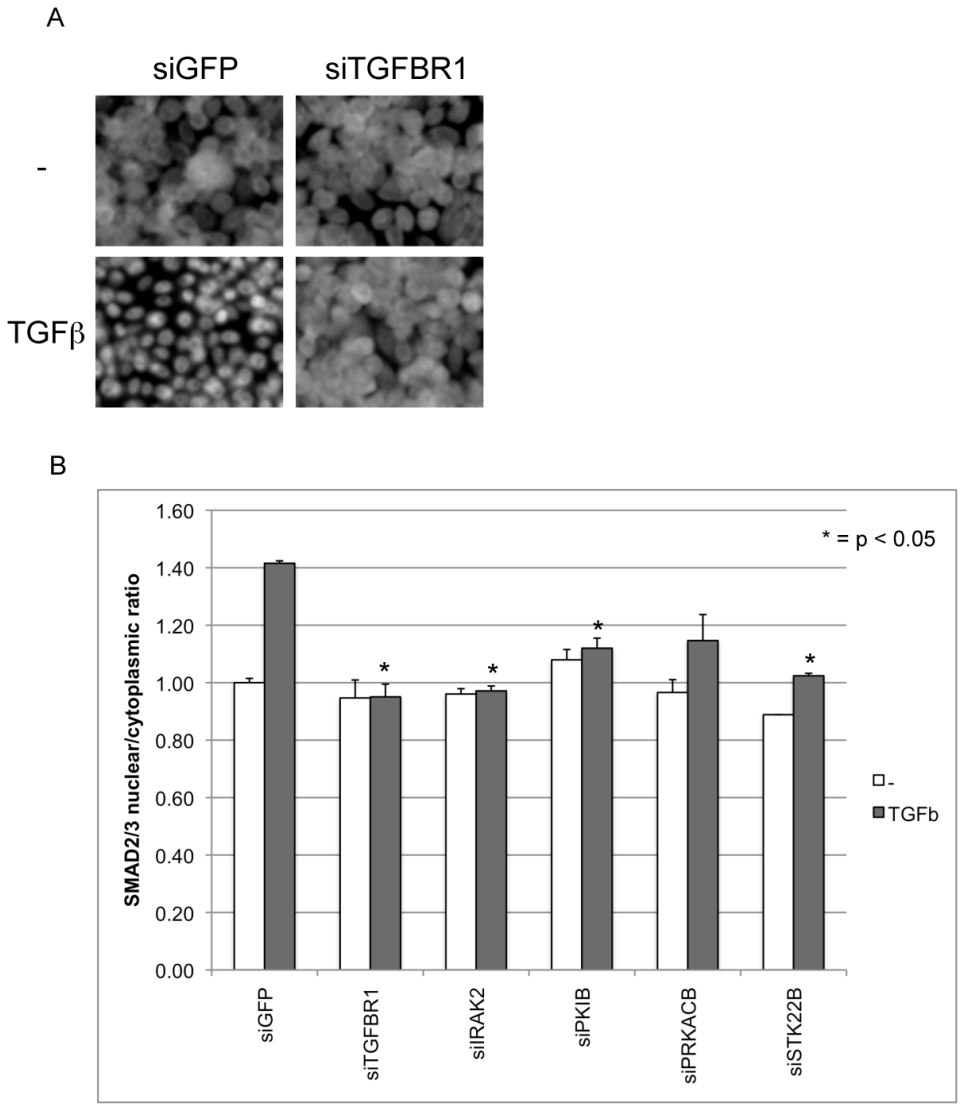

Figure 4

Effect of knockdown of the hits on SMAD2/3 translocation

PC3 cells were transfected with siRNAs against GFP or TGFBR1 and 48 hours later treated with TGFβ (+) for 1 hr or left untreated (-). Immunohistochemistry was performed with an antibody specific for SMAD2/3. (a) PC3 cells were transfected with four siRNA pools identified in the screen together with negative (GFP) and positive control (TGFBR1). 48 hours after transfection cells were treated with TGFb for 1 hr. Cells were fixed and stained with an anti-SMAD2/3 antibody and cells were counterstained with DAPI to determine the position of the nuclei. The ratio of nuclear and cytoplasmic SMAD2/3 is quantified with Cellprofiler.(b). P-values were calculated for the samples treated with TGFβ using a paired one-tailed t-test, observations that are statistically significant ($p < 0.05$) are indicated by asterisks.

been shown that at least p21 and PAI-1 are required for a TGFβ induced proliferative arrest [22, 23]. For this reason we setup an experiment to test if IRAK2 is also required for a TGFβ induced cell cycle arrest. TGFβ is known to induce a G1 cell cycle arrest and consequently fewer cells will enter the DNA replication phase (S phase). This arrest can be clearly measured in the HaCat cell-line which is derived from human keratinocytes. As expected a TGFBR2 targeting siRNA pool can block the proliferative arrest. Moreover, we also observed that knockdown of *IRAK2* can also partly block the G1 cell cycle arrest (fig. 5).

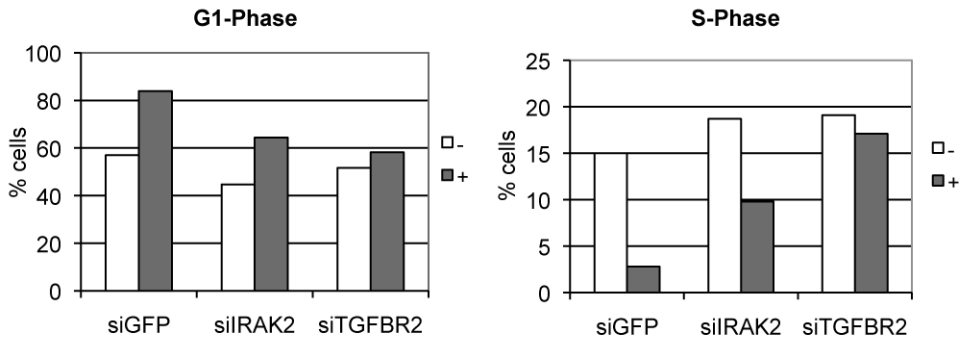

Figure 5
IRAK2 is a genuine regulator of TGFβ signaling
HaCaT cells were transfected with GFP, TGFBR2 or IRAK2 siRNAs (siRNA pool) and cells were treated with TGFβ (+) for 24 hrs or left untreated (-). The cells were fixed and stained with propidium iodide. Cell cycle profiles were measured and the amount of cells in G1 phase and S phase is plotted.

IRAK2 knockdown affects SMAD2, but not SMAD3, translocation to the nucleus

As shown earlier knockdown of the identified hits has a clear effect on the TGFβ induced translocation of SMAD2/3. However, it must be noted the antibody used in the immunofluorescence assay (fig. 4) detects both the SMAD2 and SMAD3 proteins. This can potentially lead to misinterpretation of the results as the individual contributions of SMAD2 and SMAD3 cannot be determined. For this reason we measured the effect of IRAK2 knockdown on SMAD2 and SMAD3 individually. Therefore, we transfected PC3 cells with either control or IRAK2 siRNAs and after incubation treated the cells with TGFβ for 1 hour. Nuclear and cytoplasmic extracts were prepared and the lysates were analyzed by western blot using antibodies that can specifically detect either SMAD2 or SMAD3 (fig. 6a). In control transfected cells (siRNA GFP) a regular translocation of SMAD2 and SMAD3 can be observed after treatment with TGFβ. Strikingly, cells that were transfected with IRAK2 siRNAs showed a normal translocation of SMAD3, but an impaired translocation of SMAD2. This points into a specific role for IRAK2 in the activation and therefore translocation of SMAD2.

IRAK2 knockdown impairs the SMAD2 but not the SMAD3 phosphorylation

SMAD2/3 translocation is preceded by a phosphorylation by the TGFβ receptors [24-26]. For this reason we measured the level of phosporylation of both SMAD2 and

SMAD3 upon *IRAK2* knockdown. PC3 cells were transfected with siRNAs targeting *GFP* and *IRAK2* and incubated for 48 hrs. Transfected cells were subsequently treated with TGFβ for 1 hour. Protein lysates were prepared and analyzed by western blot. No change in total levels of either SMAD2 or SMAD3 could be detected while a reduction in phosphorylated SMAD2, but not SMAD3, was observed. This supports the notion that IRAK2 is specifically required for SMAD2 activation by TGFβ (fig. 6b).

SMAD2 is required for TGFβ target gene induction

We have shown that *IRAK2* knockdown impairs phosphorylation and translocation of SMAD2 to the nucleus. Therefore we assessed the effect of SMAD2 knockdown on the TGFβ responsive reporter and on endogenous TGFβ target genes. First, we tested if knockdown of SMAD2 just like SMAD3 represses the TGFβ responsive reporter. We used the U2OS-CAGA cells and transfected control (GFP), SMAD2 or SMAD3 siRNAs (fig. 7a). From this experiment we conclude that a SMAD2 siRNA is indeed able to repress the TGFβ responsive reporter. Furthermore, we could show that endogenous TGFβ target gene induction is also partly impaired in PC3 cells transfected with SMAD2 siRNAs (fig.7b-e). To control the efficacy of the knockdown of SMAD2 and SMAD3 by the used siRNAs we performed QRT-PCR. The result from this experiment showed that both used siRNAs give more than 80% knockdown of SMAD2 and SMAD3 mRNA levels (fig. 7f and 7g). The observed repression of the TGFβ responsive reporter and TGFβ dependent target genes by knockdown of SMAD2 supports the finding that IRAK2 exerts its function on the TGFβ pathway through an effect on SMAD2.

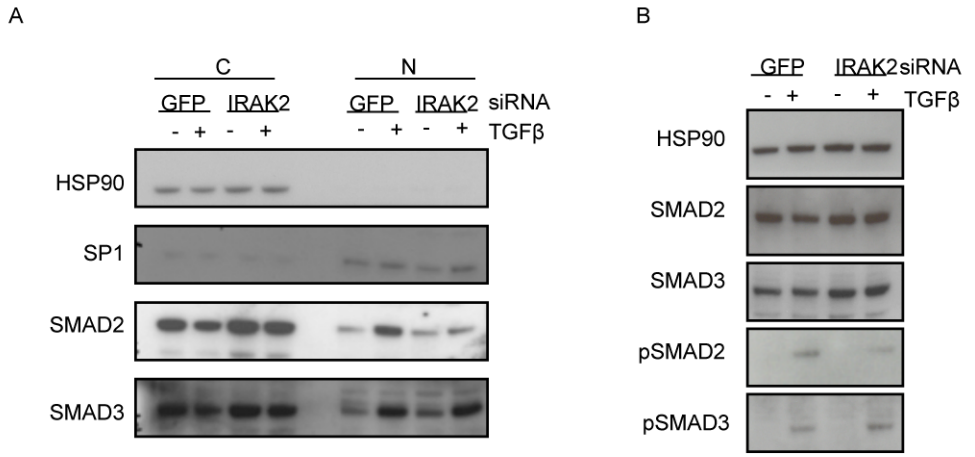

Figure 6
IRAK2 knockdown impairs SMAD2 and SMAD3 translocation and phosphorylation
PC3 cells were transfected with GFP and IRAK2 siRNAs (siRNA pool) and cells were treated with TGFβ (+) for 1 hr or left untreated (-). Nuclear and cytoplasmic fractions were immunoblotted for HSP90; cytosolic loading control, SP1; nuclear loading control and antibodies specific for SMAD2 and SMAD3 (a). To study the SMAD2 and 3 phosphorylation whole cell lysates were immunoblotted for HSP90 (loading control), total SMAD2 and SMAD3 protein and with phospo-SMAD2 (Ser465/476) and phospho-SMAD3 (Ser423/425) antibodies (b).

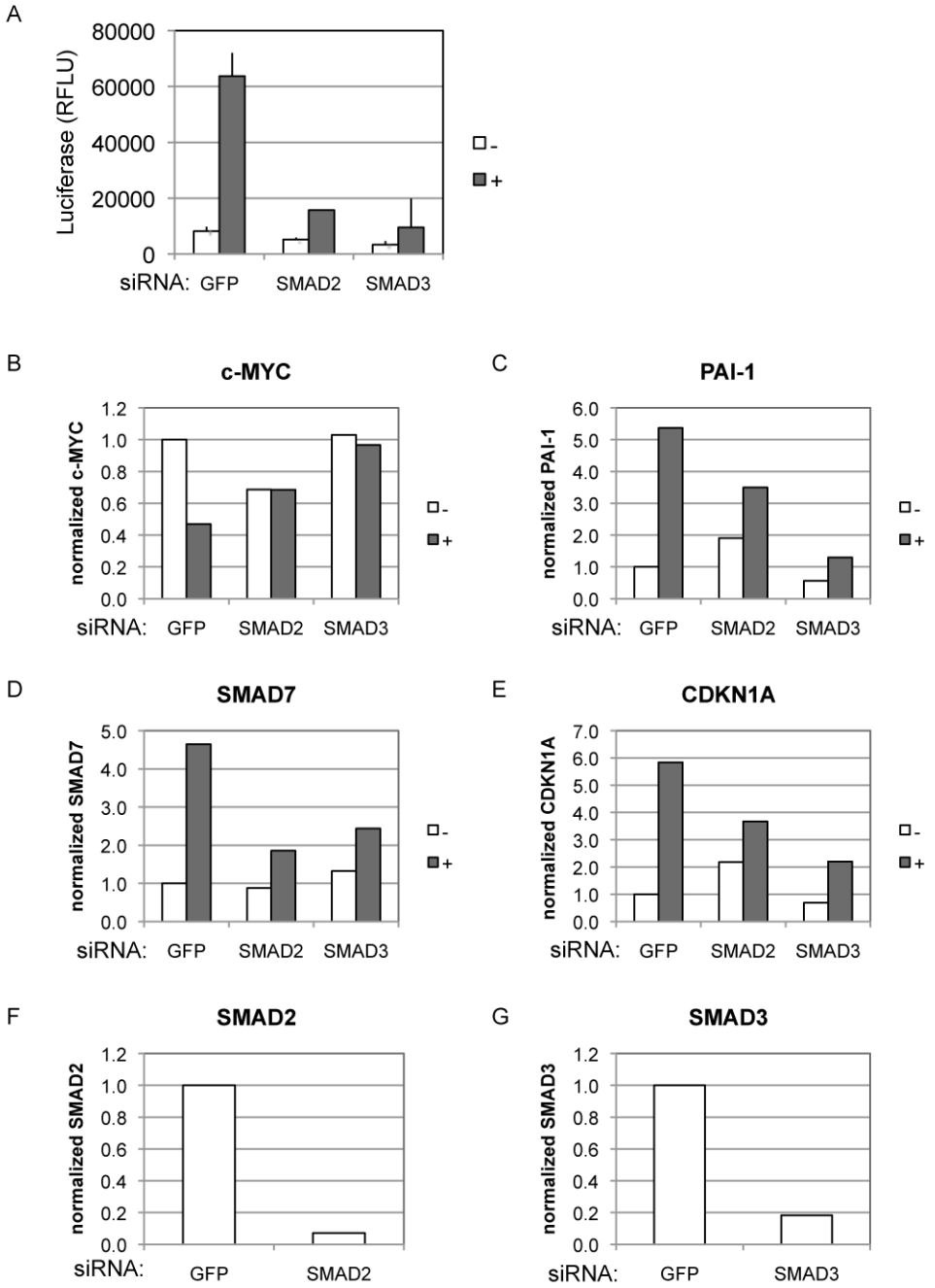

Figure 7
SMAD2 is required for TGFβ target gene regulation
U2OS-CAGA cells were transfected with siRNAs against GFP, SMAD2 or SMAD3 and treated with 200 pM TGFβ (+) for 14 hrs or left untreated (-) (a). PC3 cells were transfected and target gene expression was measured in absence or presence of TGFβ (8 hrs) for c-MYC (b), PAI-1 (c), SMAD7 (d) and CDKN1A (e). Knockdown of SMAD2 (f) and SMAD3 (g) was determined by measuring SMAD2 and SMAD3 mRNA levels.

Discussion

In this study we describe the identification of new modulators of the TGFβ pathway through the screening of a siRNA library targeting kinases and kinase related genes. Data analysis of the screen revealed a relatively large number of genes that could either repress or activate the TGFβ responsive reporter. Somewhat surprising was the fact that the data-distribution is exponential instead of linear. However, this was also observed in two previously performed reporter based RNAi screens [13, 27]. Some RNAi screening efforts have reported high levels of 'off-target' effects caused by aspecific suppression of mRNAs [28, 29]. Therefore we were pleased to find that about 70% (11 out of 16 genes) of the hits tested could be classified as 'on target' [19]

Observations made in reporter assays are not necessarily reflecting endogenous pathway activation. Therefore, we tested if the 5 'on target' hits that repressed the reporter also repress endogenous target gene induction. This experiment was performed in a different cell line than the original U2OS-CAGA to exclude cell type specificity of the identified hits. For all 5 'on target' hits impaired activation of two or more endogenous target genes was observed. The fact that not all 5 hits show a full repression on all TGFβ target genes can be explained by various previously reported observations. For instance the fact that the ratio of SMAD2, 3 and 4 dictates the selectivity for certain target genes [30-33]. In addition the availability of SMAD co-factors can also determine the specific regulation of subsets of TGFβ target genes [3]. The fact that the newly identified hits can regulate the TGFβ dependent transcription of certain TGFβ targets suggests that these genes indeed have a role in the TGFβ pathway.

The TGFβ/SMAD signaling pathway can be regulated at many different levels, co-factors that regulate SMAD dependent transcription, nucleoporin proteins assist in SMAD nuclear accumulation and of course the phosphorylation by the TGFβ receptors. Strikingly for three out of four of the newly identified modulators of TGFβ signaling (IRAK2, PRKACB and STK22B) we observed a defective SMAD2/3 translocation. This points into the direction that these genes function upstream of SMAD translocation.

IRAK2 is specifically required for SMAD2 activation

When we tested the effects of all newly identified genes on endogenous TGFβ target genes only *IRAK2* knockdown impaired the regulation of all tested TGFβ target genes. Further analysis showed that IRAK2 seems specifically required for the TGFβ dependent phosphorylation and subsequent translocation of SMAD2. We subsequently showed that *SMAD2* knockdown impairs activation of a TGFβ responsive reporter and induction of TGFβ dependent target genes. This observation was unexpected since the TGFβ responsive reporter has been described to be dependent on SMAD3/4 dependent transcription. However, this conclusion was based on overexpression experiments that showed that SMAD3 or SMAD4 overexpression activate this TGFβ responsive reporter while SMAD2 overexpression did not [14]. Several studies have reported that SMAD3 is responsible for the majority of the TGFβ induced phenotypes and that SMAD2 is often dispensable [32-34]. More recently it was reported that under some conditions SMAD2 indeed plays a critical role in transmitting TGFβ signals from the cell membrane to the nucleus [35]. More experiments are required to fully understand the mechanism of the loss of SMAD2-phosphorylation in cells with knockdown of *IRAK2*.

IRAK2 and SMAD2: connecting IL-1 and TGFβ signaling

IRAK2 is part of a family of four IL-1R-associated kinases (IRAKs), despite its name it was predicted that IRAK2 does not contain an active kinase domain [36, 37]. One of the first publications on IRAK2 described its function as a downstream mediator of the IL-1R in a complex with TRAF6 and MyD88 [38]. It was only recently unveiled that IRAK2 also has a functional role in Toll like receptor signaling [21, 39]. Our observation that IRAK2 ablation leads to an impaired TGFβ response suggests a possible interaction between TGFβ and IL-1/TLR signaling. This is supported by various previously performed studies. For example the fact that IRAK2 was found in a luminescence based proteomics screen to interact with both SMAD2 and SMURF1 [12]. In addition it was recently shown that TRAF6, which interacts with IRAK2, mediates TGFβ dependent activation of JNK and p38. This effect was reported to be SMAD independent, proven by the fact that TRAF6 knockdown does not lead to activation of a TGFβ responsive reporter [40]. Finally it was shown that stimulation of cells with IL-1β leads in many cases to phosphorylation of SMAD2 and subsequent target gene activation [41]. Taken together our and other observations indicate the existence of cross talk between two important cellular signaling pathways involved in immunity and other processes.

Materials & methods

Cell lines & culture conditions

U2OS, HaCaT and PC3 cells were cultured in DMEM supplemented with 10% FCS, penicillin, streptomycin and glutamine. All cells were cultured at 37°C in 5% CO_2.

Generation of stable reporter cell line

U2OS cells were transfected using calcium phosphate with the TGFb responsive reporter pGL3-$CAGA_{12}$-Luc and a puromycin resistant plasmid pBabe-puro in a ratio 10:1. Next the cells were sparsely seeded and selected with 2 μg/ml puromycin. Subsequently, colonies were picked and tested for TGFb dependent induction of luciferase signal.

siRNA transfection

Cells were reverse transfected with siRNAs according to manufacturers instructions (Dharmacon) (Day 1). Dharmafect 1 (Dharmacon) was used for U2OS cells and Dharmafect 2 (Dharmacon) for HaCaT and PC3 cells. At day two penicillin and streptomycin was added. TGFb (R&D systems, Minneapolis, MN, human platelet derived, 200 pM in all experiments) was added overnight on day 3 (U2OS) or for 24 hrs (HaCaT). For PC3 cells TGFb was added on day 4 for 2 or 8 hrs in order to assay target gene activation or 1 hr for the SMAD2/3 translocation assay.

Human kinase siRNA screen

Cells with stably integrated TGFb responsive reporter were reverse transfected in 384 well plates in triplicate with the human kinome library of Dharmacon. Dharmafect 1 (0.1 μl per 384-well) was used as transfection reagent and the siRNA pools were transfected at a concentration of 50 nM. 3000 cells were seeded per well with a cell dispenser (Wellmate, Matrix). The transfection was performed in absence of antibiotics. Some 18 hrs after the transfection we added the penicillin and streptomycin. 48 hrs after transfection cells were stimulated with TGFb for 14 hrs. Cell titer blue reagent (Promega) was used in order to determine the cell viability. After 1 hr incubation $560_{EX}/590_{EM}$ fluorescence was measured with a plate reader (Envision multilabel reader 2101, Perkin Elmer). Subsequently, medium was aspirated with a robot (STAR liquid handling workstation, Hamilton) and luminescence was measured with the same plate reader using Steady-Glo Luciferase (Promega).

The cell viability counts were used to calculate a viability score and siRNA pools that reduce cell viability by more than 25% were omitted from further validation rounds. The luciferase counts were corrected for cell viability giving the normalized luciferase counts (NORM LUC). Subsequently, the NORM LUC counts were LOG2 transformed in order to get a normal distribution. The transformed NORM LUC counts are used to calculate the Z-score (difference of the sample luciferase measurement and the average luciferase of the population divided by the standard deviation of the population) per plate in order to be able to compare the different plates with each other. Further validation was performed on the siRNA pools with Z-scores <-2 or >2.

Validation of single siRNAs
The four separate siRNAs and the siRNA pool were tested in the U2OS-CAGA cell-line. As a control we used a siRNA targeting GFP. All single siRNA validation experiments were performed at least three times using quadruplicate transfection and one representative experiment is shown. To confirm that the observations obtained with the single siRNAs were statistically significant we calculated the p value using a paired one-tailed t-test. All reporter experiments shown that include single siRNAs produced effects on the reporter with a p-value < 0.05.

Immunohistochemistry to quantify SMAD2/3 translocation
PC3 cells were cultured in 96 or 384 well plates. Formaldehyde was used for fixation of the cells and we permeabilized with 0.2% Triton X-100. Subsequently, samples were blocked with 5% BSA in PBST (0.05% Tween 20 in PBS). A SMAD2/3 (BD Transduction Laboratories, 610842) specific primary antibody was used and subsequently a mouse ALEXA 488 conjugated (Alexa Fluor 488, Invitrogen) secondary antibody. The cells were counterstained with DAPI in order to define the position of the nuclei. Images were acquired with a high content imager (BD Pathway Bioimager 855, BD biosciences) using a 20x objective (Olympus). CellProfiler software [20] was used to quantify the nuclear/cytoplasmic SMAD2/3 ratio of all individual cells per well.

Western blotting
Cell lysates were separated using 4-12% Bis-Tris gels (Nupage, Invitrogen). Proteins were transferred to PVDF membrane and incubated with primary antibody as indicated. Primary antibodies were detected using a secondary HRP-conjugated antibody. Antibodies used for these studies: Hsp90 (Santa Cruz H-114, sc-7947), SP1 (Santa Cruz PEP 2, sc-59), SMAD2 (Cell Signaling (L16D3) #3103), SMAD3 (Cell Signaling (C67H9) #9523), p-SMAD2 ser465/467 (Cell Signaling #3101), p-SMAD3 ser423/425 (Cell Signaling (C25A9) #9520).

Quantitative PCR
Total RNA was isolated using TRIzol (Invitrogen). From the total RNA cDNA was generated using Superscript II (Invitrogen) using random primers (Invitrogen). cDNA was diluted and QRT reaction was performed using FAST Cyber green (Invitrogen) with specific primers (Supplementary table 2). All QRT reactions were run in parallel with RPL13 or GAPDH to control for input cDNA. The QRT reactions were perfromed using a fast Real Time PCR system (AB7500, Applied Biosystems).

Cell-cycle analysis
For fluorescence-activated cell sorting (FACS) analysis, siRNA transfected HaCaT cells that were TGFb treated for 24 hrs are fixed, stained and assayed as described previously [42].

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siRNA POOL	NCBI RefSeq	AVG COUNTS	STDEV	Z score	Hitsort and number
TGFBR2	NM_003242	445	64	-3.4	repressor reporter 1
LOC91807	NM_182493	570	195	-3.2	repressor reporter 2
STK22B	NM_053006	714	122	-2.9	repressor reporter 3
TGFBR1	NM_004612	765	101	-2.8	repressor reporter 4
SGKL	NM_013257	908	214	-2.7	repressor reporter 5
PKIB	NM_032471	1037	267	-2.5	repressor reporter 6
MYLK	NM_005965	1098	590	-2.4	repressor reporter 7
PRKACB	NM_002731	1191	403	-2.4	repressor reporter 8
RIPK1	NM_003804	1219	329	-2.3	repressor reporter 9
GALK1	NM_000154	1233	123	-2.3	repressor reporter 10
SNRK	NM_017719	1315	124	-2.2	repressor reporter 11
IRAK2	NM_001570	1359	418	-2.2	repressor reporter 12
TNK1	NM_003985	1379	660	-2.2	repressor reporter 13
XYLB	NM_005108	1396	276	-2.2	repressor reporter 14
SMG1	NM_014006	1594	546	-2.0	repressor reporter 15
PACSIN1	NM_020804	1612	311	-2.0	repressor reporter 16
C9ORF12	NM_022755	1677	935	-2.0	repressor reporter 17
RAPGEF3	NM_006105	236250	35033	3.5	activator reporter 1
AKAP11	NM_016248	185965	72124	3.2	activator reporter 2
MAK	NM_005906	158950	33275	3.0	activator reporter 3
MAP3K10	NM_002446	144278	5978	2.9	activator reporter 4
ADRBK2	NM_005160	115431	9590	2.7	activator reporter 5
FLJ25006	NM_144610	111246	7961	2.6	activator reporter 6
AK5	NM_012093	95504	57637	2.5	activator reporter 7
SRC	NM_005417	84798	5495	2.3	activator reporter 8
MAPK10	NM_002753	84297	2516	2.3	activator reporter 9
ANKRD3	NM_020639	79917	17361	2.3	activator reporter 10
AATK	XM_375495	77166	25618	2.2	activator reporter 11
PPP2CB	NM_004156	75428	17818	2.2	activator reporter 12
MAPK8IP1	NM_005456	73987	26314	2.2	activator reporter 13
AVPR1B	NM_000707	70746	5557	2.1	activator reporter 14
CSNK2A2	NM_001896	66154	13509	2.1	activator reporter 15
ACVRL1	NM_000020	64707	12487	2.0	activator reporter 16
FLJ23356	NM_032237	63775	4867	2.0	activator reporter 17
PRPS2	NM_002765	63347	6626	2.0	activator reporter 18
PFKP	NM_002627	62607	4563	2.0	activator reporter 19
GCK	NM_000162	62005	5789	2.0	activator reporter 20
		10666		AVG all values	

Supplementary table 1
Overview of hits from primary screen
Overview of the top siRNA pools that repress or activate the TGFβ responsive reporter with a Z score <-2 or >2, respectively.

Gene	Seq	Primer name	Refseq
IRAK2	GCAACTTGTGGACCTCCTGT	IRAK2-Forward	NM_001570
IRAK2	TGGAATGGGACACCTGATTT	IRAK2-Reverse	NM_001570
c-MYC	CAGCTGCTTAGACGCTGGATT	c-MYC-Forward	NM_002467
c-MYC	GTAGAAATACGGCTGCACCGA	c-MYC-Reverse	NM_002467
CDKN1A	GTCCACTGGGCCGAAGAG	CDKN1A-Forward	NM_078467
CDKN1A	TGCGTTCACAGGTGTTTCTG	CDKN1A-Reverse	NM_078467
PAI-1	AGCTCCTTGTACAGATGCCG	PAI-1-Forward	NM_000602
PAI-1	ACAACAGGAGGAGAAACCCA	PAI-1-Reverse	NM_000602
SMAD7	CCAGGCTCCAGAAGAAGTTG	SMAD7-Forward	NM_005904
SMAD7	CCAACTGCAGACTGTCCAGA	SMAD7-Reverse	NM_005904
Actin B	CCTGGCACCCAGCACAA	Actin B-Forward	NM_001101
Actin B	GCCGATCCACACGGAGTACT	Actin B-Reverse	NM_001101
RPL13	GAGACAGTTCTGCTGAAGAACTGAA	RPL13-Forward	NM_000977
RPL13	TCCGGACGGGCATGAC	RPL13-Reverse	NM_000977
IRAK2	GCAACTTGTGGACCTCCTGT	IRAK2-Forward	NM_001570
IRAK2	TGGAATGGGACACCTGATTT	IRAK2-Reverse	NM_001570
PKIB	AGAAGCAGAAAACCCTGTGC	PKIB-Forward	NM_032471
PKIB	GAAGATGCAAAATTGGCGAC	PKIB-Reverse	NM_032471
PRKACB	CTGACCCCTTCTTGCCATC	PRKACB-Forward	NM_002731
PRKACB	AAGTCTTCTTTGGCTTTGGC	PRKACB-Reverse	NM_002731
STK22B	CATCAGGAAGATGCTGCGTA	STK22B-Forward	NM_053006
STK22B	AGCATGCGGTAGATGAGGTC	STK22B-Reverse	NM_053006
TGFBR1	ACGGCGTTACAGTGTTTCTG	TGFBR1-Forward	NM_004612
TGFBR1	GCACATACAAACGGCCTATCT	TGFBR1-Reverse	NM_004612
ACVRL1	GACTGACATCTGGGCCTTTG	ACVRL1-Forward	NM_000020
ACVRL1	TTGGGCACCACATCATAGAA	ACVRL1-Reverse	NM_000020
AVPR1B	GCTCCCTTCTTCAGTGTCCA	AVPR1B-Forward	NM_000707
AVPR1B	GAGGTTGCCCAAAAGCATAG	AVPR1B-Reverse	NM_000707
CSNK2A2	TCACCAACAATGAGAGAGTGG	CSNK2A2-Forward	NM_001896
CSNK2A2	GTTCCACCACGAAGGTTCTC	CSNK2A2-Reverse	NM_001896
MAP3K10	GGAGGTTATCCGTCTCTCCC	MAP3K10-Forward	NM_002446
MAP3K10	GATCTCACGGTAGGGGACCT	MAP3K10-Reverse	NM_002446

Supplementary table 2
Overview of QRT primers used in this study

Chapter V

Large scale siRNA screen to identify modulators of the TGFβ signaling cascade

Large scale siRNA screen to identify modulators of the TGFβ signaling cascade

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Abstract

The TGFβ pathway orchestrates an extensive transcriptional program that regulates many processes in the cell. In normal cells the TGFβ pathway can inhibit the cell cycle and thereby acts primarily as a tumor suppressor. In contrast, tumor cells are often insensitive to the cytostatic actions of TGFβ, and are able to benefit from the tumor promoting actions caused by TGFβ stimulation. Examples of these oncogenic activities of TGFβ include the induction of epithelial to mesenchymal transition, invasion and immune evasion. Therefore, therapies have been developed to inhibit the TGFβpathway in cancer cells. To identify new modulators of the TGFβ pathway, we performed a functional genetic loss-of-function screen. 6,000 genes were individually suppressed by RNA interference and the effect on a TGFβ responsive reporter system was determined. Apart from several well-established components of the TGFβ signaling network, we identified the nucleoporin protein NUP98 as a potential novel modulator of the TGFβ pathway. Consistent with this, we find that ablation of NUP98 activates TGFβ dependent target genes.

INTRODUCTION

The canonical TGFβ pathway activates an extensive transcriptional program that regulates many processes in the cell [1, 2]. The TGFβ pathway plays an important but dual role in cancer. It exerts a tumor suppressive effect on epithelial cells through induction of a proliferative arrest [3-5]. However, tumor cells are often insensitive to the proliferative control of TGFβ. Therefore, other actions of the TGFβ pathway can instead promote tumor progression. Examples of these other actions are the induction of epithelial to mesenchymal transition (EMT), promotion of angiogenesis and evasion of tumour surveillance [6, 7].

Mechanistically, the canonical signaling cascade is stimulated by TGFβ, which serves as a ligand for the TGFβ receptor II (TGFBR2). Upon ligand binding, the TGFBR2 phosphorylates TGFBR1, which in turn phosphorylates the regulatory SMADs; SMAD2 and SMAD3. Subsequently, the regulatory SMADs translocate to the nucleus and form a complex with the co-SMAD, SMAD4. This nuclear SMAD complex binds to specific DNA- binding sites and associates with co-activators (i.e.; p300 and CBP) and co-repressors (i.e.; SKI and SNON) in order to regulate an array of TGFβ target genes.

Recently, it has been shown that tumours with an activated TGFβ pathway have a worse outcome compared to tumours without TGFβ pathway activation [8, 9]. In spite of the pleiotropic effect of TGFβ on cell proliferation, several TGFβ inhibitors have been developed as cancer therapy. Clinical trials have been performed with antisense TGFβ oligonucleotides, TGFβ antibodies and small molecule inhibitors of TGFBR1 [10]. We set out to identify new genes that regulate TGFβ signaling, since this would expand the number of potential targets for therapy in this signaling cascade.

Several approaches have already been taken to identify new players in the TGFβ pathway. For example protein-protein interaction screens have identified genes that interact with members of the TGFβ signaling network [11, 12]. A genome wide functional siRNA screen has been performed in the TGFβ signaling cascade in *Drosophila* [13]. Furthermore, functional siRNA screens have been performed in mammalian cells, but this screening effort was limited to smaller gene families such as the deubiquitinating enzymes [14] and the ubiquitin E3 ligase genes [15].

Here we describe a functional genomic siRNA screen performed in a cell line with an integrated TGFβ responsive reporter using a siRNA library that targets around 6000 different genes. The results of this screen, the validation and follow-up are reported here.

RESULTS

SCREENING OF A TGFβ REPORTER CELL LINE WITH A LARGE SIRNA LIBRARY

To screen siRNA libraries in high throughput format for novel modulators of TGFβ signaling, we have generated an osteosarcoma cell line with a stably integrated $pCAGA_{12}$-Luciferase TGFβ responsive reporter (U2OS-CAGA) [16]. This cell line was validated in an earlier screen where we searched for novel kinases in the TGFβ pathway. We identified the kinase IRAK2 as a novel modulator of TGFβ signaling. Here, we use the U2OS-CAGA cell line to perform a screen with a siRNA library that targets around 6000 genes in the human genome with a pool of four different siRNAs. The screen was performed by transfecting the U2OS-CAGA cell-line in triplicate with all siRNAs pools of the library. To stimulate the TGFβ response three days post transfection,

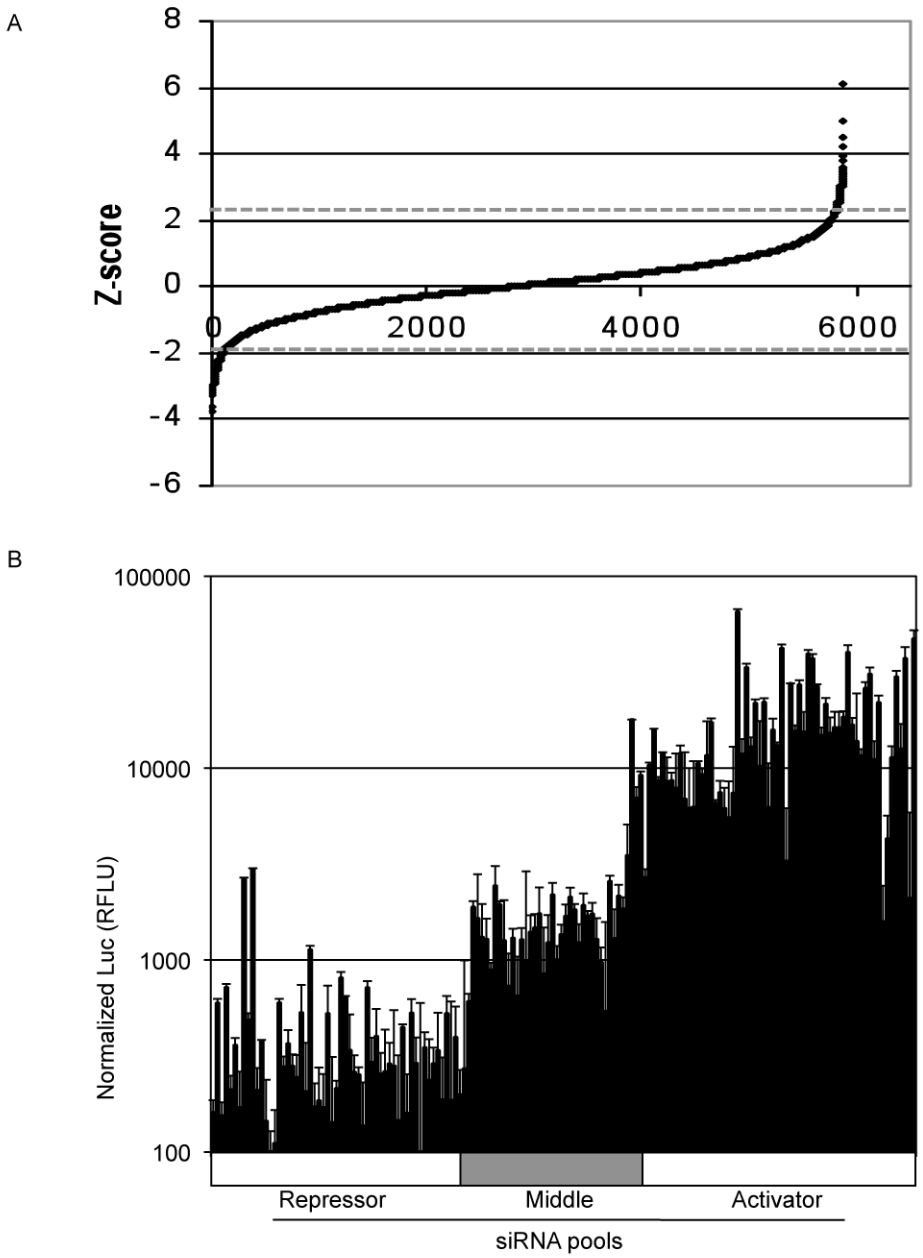

Figure 1
Screen with siRNAs targeting ~6000 genes in a TGFb responsive reporter identifies modulators of the TGFb pathway
U2OS cells harboring a stable TGFb responsive luciferase reporter were transfected with siRNAs directed against ~6000 different genes. The screen was performed in triplicate and Z-scores were calculated and plotted in ascending order. The outliers respectively below the lower and above the upper dahed line were selected for follow-up (a). In a secondary screen siRNA pools with a Z<-2 (55 repressor siRNA pools) or Z>2.3 (65 activator siRNA pools) were rescreened. In addition, 35 siRNA pools with a Z-score around 0 in the primary screen were also screened as reference (b).

TGFβ was added for 14 hours. Both CAGA-luciferase activity and cell viability were measured in triplicate and after normalization, statistical analysis was performed. Knockdown of genes that lead to cell death without influencing TGFβ activity can result in false positive repressive hits. Therefore, we excluded siRNA pools with 50% or more lethality from further analysis. In total, 199 siRNA pools conferred more than 50% lethality. Genes were sorted based on Z-score (fig. 1a) and a tentative hit list was generated. Only siRNA pools that had a Z-score higher than 2.3 (65) or lower than -2.0 (55) were selected for a secondary screening round. In addition to the 120 siRNA pools that were selected for a secondary screening round, we also took 35 siRNA pools along that scored with a Z-score around 0 in the primary screen as controls for the validation experiment. We transfected these 155 siRNA pools in the U2OS-CAGA cell-line and measured cell viability and the raw luciferase signal. From these results we calculated the normalized LUC score for each siRNA pool. In 86% of the cases re-testing of the siRNA pools confirmed the phenotype found in the primary screen. For the remaining 14% of the hits we could not confirm the phenotype of the primary screen and we considered these as false positives in the primary screen (fig. 1b). For further follow-up, we selected the top 15 for both the repressors and activators of the secondary screening round. In addition, we used protein-protein interaction databases [17-19] to identify genes among the selected hits that interact with each other or genes whose encoded proteins interact with other components of the TGFβ signaling network (n=10). Together these 40 hits were selected for deconvolution to eliminate "off target effects" [20-22]. We next tested the four individual siRNAs for each hit for their ability to confer the phenotype on the TGFβ responsive reporter. Only if two or more siRNAs confer the phenotype from the primary screen the gene is considered "on target" (Supplementary table 1). Sixty percent (n=24) of the genes tested have two or more siRNAs that conferred the desired phenotype and were therefore considered "on target" (fig. 2a). The "on target" hits were confirmed by four (n=14), three (n=7) or two (n=3) separate siRNAs (fig. 2b). For the hits that are considered "on target", the results of the individual siRNA experiment are plotted in a heat map [23] (fig. 2c). In total we identified 5 genes that upon knock-down repress TGFβ mediated transcription and 19 genes that activate TGFβ mediated transcription. Among the genes that repressed the reporter are *SMAD3* and *SMAD4*, confirming that this screen can identify genuine players of the TGFβ signaling network. The NUP98 siRNA pool was one of the top outliers enhancing TGFβ mediated transcription, that could be validated with all 4 siRNAs. The NUP98 protein is part of the nucleopore protein complex (NPC), a large protein complex with multiple subunits that facilitates nuclear import of proteins and export of mRNAs and proteins. NUP98 is a nucleoporin that contains phenylalanine-glycine (FG) rich repeats also found in around 30% of the other NPC proteins. Previously, other FG nucleoporins have been found to be involved in the nuclear import and export of SMAD2 and import of SMAD3 and SMAD4 [24, 25]. This could suggest that NUP98 also plays a role in the nucleoplasmic localization of SMADs. Interestingly, the gene encoding NUP98 is also a frequent translocation partner in leukemia. We therefore further focused on the role of NUP98 in the TGFβ pathway.

The TGFβ pathway is activated in NUP98 ablated cells

We first tested if the 4 siRNAs that enhanced TGFβ pathway activation also conferred knockdown of *NUP98*. Indeed the siRNA pool and the 4 individual siRNAs against

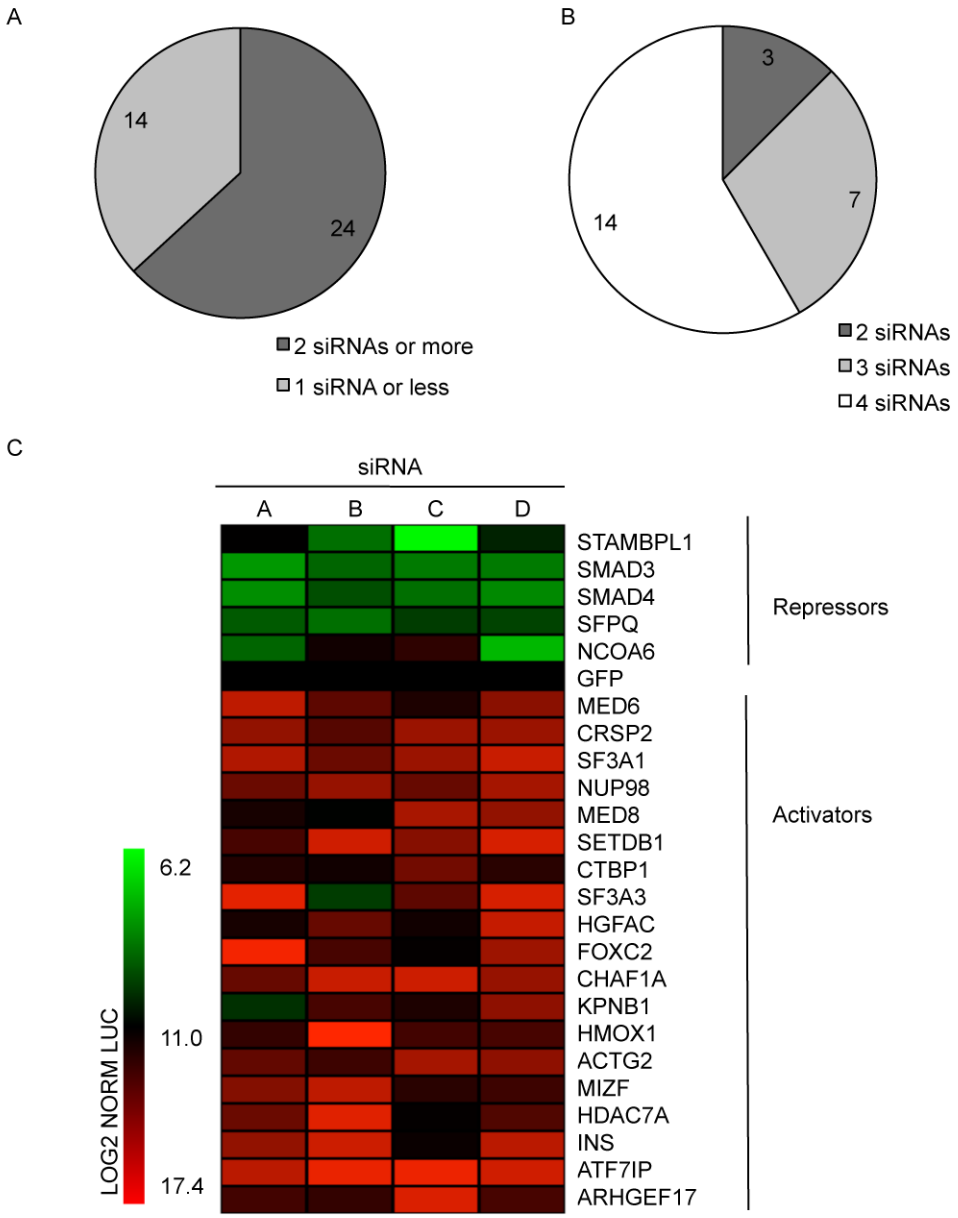

Figure 2
"On-target" validation of the hits
The four siRNAs that form the siRNA pool were tested separately (A, B, C, D) for 40 hits in the U2OS-CAGA cell line. A siRNA targeting GFP was taken along as negative control. The validation rate of the tested hits is plotted in a pie diagram. Hits are classified "on target" when 2 or more siRNAs against a hit confer the phenotype as in the screen (a). The hits that are "on target" validate with 2, 3 or 4 siRNAs (b). LOG2 normalized luciferase counts are plotted in a heatmap for the "on target" hits (c).

NUP98 gave around 95% knockdown of *NUP98* mRNA in U2OS cells (fig. 3a). Secondly, we investigated whether TGFβ pathway activation can be measured in the U2OS-CAGA cells after NUP98 ablation, both in absence and in presence of TGFβ. We took SnoN knockdown along in this experiment as positive control. SnoN is an inhibitor of the TGFβ pathway and ablation of SnoN (Chapter IV) gives TGFβ pathway activation under basal and stimulated conditions. The pool and four individual siRNAs against *NUP98* also showed TGFβ pathway activation in absence and in presence of TGFβ (fig. 3b).

NUP98 IS PART OF THE NPC COMPLEX BUT ABLATION DOES NOT CAUSE GENERAL EXPORT INHIBITION

NUP98 is part of the NPC complex which is assembled from more than 30 different proteins [26]. The individual role of each of these proteins is largely unknown. Most nucleoporin proteins are not essential for export as ablation of these proteins does not inhibit general export of proteins. However, depletion of NUP214 by RNAi did inhibit general protein export [27]. Therefore, we tested if NUP98 plays an essential role in general protein export. To investigate general protein export, we made use of an U2OS cell line that stably expressed a fluorescent protein fused to a general nuclear export tag (U2OS REV-GFP). We subsequently knocked down *NUP98* or *CRM1*, a general nuclear export protein, which functions as positive control. As expected, ablation of *CRM1* leads to nuclear retention of REV-GFP. In contrast, in cells ablated for NUP98 no nuclear retention of the REV-GFP protein was observed (fig. 3c). Thus we conclude that NUP98 does not play a role in the general export of nuclear proteins.

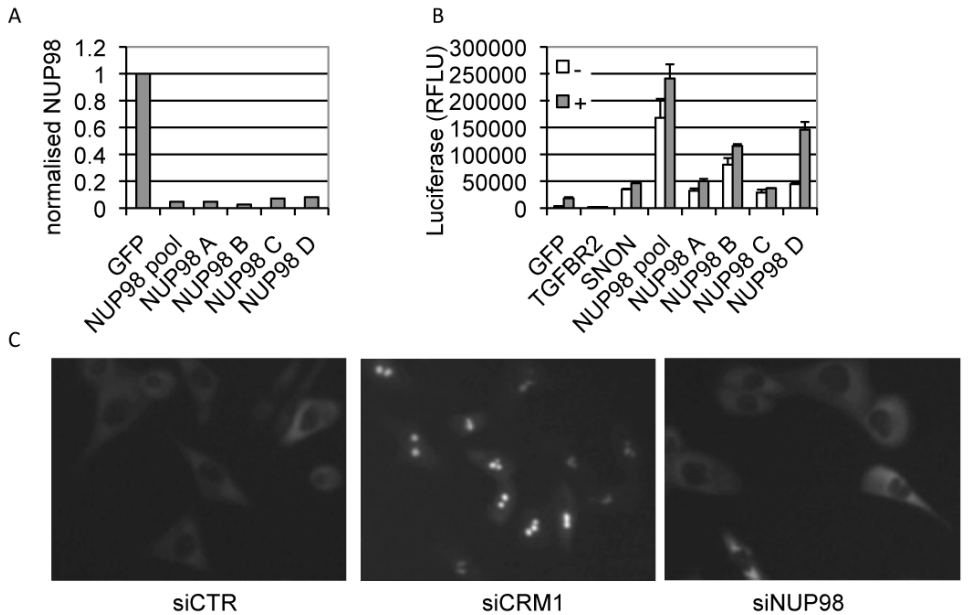

Figure 3

Activation of the TGFb pathway and no general export inhibition by NUP98 ablation

U2OS cells were transfected with a GFP siRNA, the NUP98 siRNA pool or the 4 separate siRNAs against NUP98. The mRNA levels of the NUP98 were determined by quantitative PCR and were normalized against the reference gene RPL13 (a). The stable U2OS-CAGA cell line is transfected with the siRNAs as in (a) and an siRNA against SNON as positive control. Cells were treated with TGFb or left untreated, luciferase counts are plotted on the y-axis (bU2OS-REV-GFP cells were transfected with CTR, CRM1 or NUP98 siRNAs and pictures were taken 48 hrs later (c).

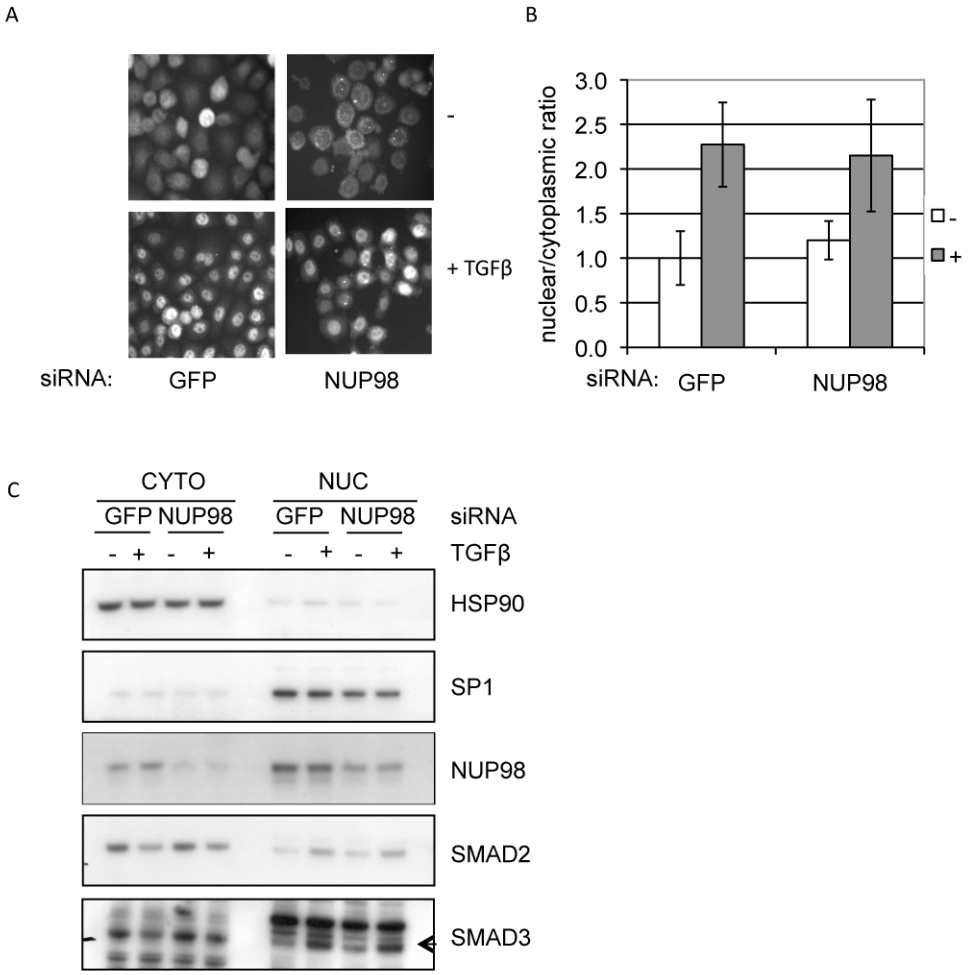

Figure 4

NUP98 knockdown does not impair SMAD2 or SMAD3 translocation to the nucleus

PC3 cells were transfected with GFP and NUP98 siRNAs and cells were treated with TGFb (+) for 1 hr or left untreated (-). Immunohistochemistry was performed with an antibody specific for smad2/3 and (a) cells were counterstained with DAPI to determine the position of the nuclei. The ratio of nuclear and cytoplasmic SMAD2/3 is quantified with Cellprofiler (b). PC3 cells were transfected with GFP and NUP98 siRNAs and cells were treated with TGFb (+) for 1 hr or left untreated (-). Nuclear and cytoplasmic fractions were immunoblotted for HSP90; cytosolic loading control, SP1; nuclear loading control and antibodies specific for NUP98, SMAD2 and SMAD3 (c).

Effect of NUP98 on SMAD2/3 nucleocytoplasmic shuttling

The nucleoporin proteins NUP214 and NUP153 have been reported to be important nucleocytoplasmic shuttling of SMAD2 [24, 25]. Furthermore, NUP214 in also involved in nuclear translocation of SMAD3 and SMAD4 [24, 25]. Therefore, we speculated that NUP98 might also play a role in nucleocytoplasmic shuttling of SMAD2/3 proteins. Knockdown of *NUP98* leads to transcriptional activation of TGFβ signaling, we tested if this effect was exerted by increased SMAD2/3 levels in the nucleus. We examined the localization of SMAD2/3 using immunohistochemistry (fig. 4a and 4b) in the prostate

cancer derived PC3 cell line. These cells were transfected with siRNAs against *NUP98* or *GFP* and TGFβ induced SMAD2/3 translocation was measured. The ratio of SMAD2/3 in the nucleus versus the cytoplasm was determined from images that were quantified with Cellprofiler software [28]. Upon TGFβ stimulation we observed a strong increase in nuclear staining indicative of the translocation of SMAD2/3 to the nucleus (Fig. 4a and 4b). However we did not observe a difference in the nuclear-cytoplasmic ratio of SMAD2/3 between siGFP and siNUP98 transfected cells either in absence or presence of TGFβ.

In the immunohistochemistry experiments described above, we measured the protein localization of SMAD2 and SMAD3 together, as the antibody is both SMAD2 and SMAD3 reactive. To investigate the translocation of SMAD2 and SMAD3 separately, we determined the subcellular localization of SMAD2 and SMAD3 protein by immunoblotting of nuclear and cytoplasmic cell fractions. To do this, PC3 cells were transfected with GFP or NUP98 siRNAs. Subsequently, the cells were treated with TGFβ or left untreated and cytoplasmic and nuclear extracts were prepared. Again, we did not observe a difference between GFP control or NUP98 ablated cells (fig. 4c). Therefore, we conclude that NUP98 does not play a role in the nucleocytoplasmic shuttling of SMAD2 or SMAD3.

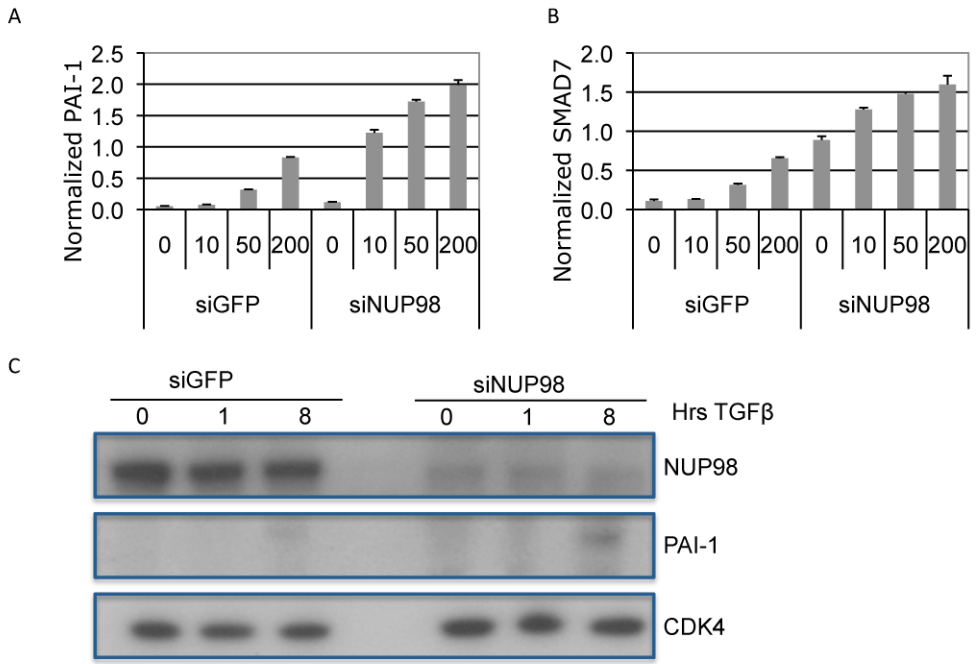

Figure 5
Endogenous TGFb target gene regulation
PC3 cells transfected with NUP98 or GFP siRNAs were treated for 8 hrs with different concentrations of TGFb (0, 10, 50 and 200pM). The mRNA levels of the TGFb target genes PAI-1 and SMAD7 were determined by quantitative PCR and were normalized for the reference gene RPL13 (a and b). PC3 cells transfected with siRNAs against GFP or NUP98 were treated for 0,1 or 8 hrs with 200 pM of TGFb. Whole cell lysates were immunoblotted for NUP98, PAI-1 and CDK4 (loading control) (c).

Effect of NUP98 ablation on endogenous TGFβ signaling

Transcriptional activation of TGFβ target genes is downstream of SMAD2/3 translocation to the nucleus in the TGFβ signaling cascade. We therefore we investigated whether knockdown of NUP98 also affects endogenous TGFβ target gene transcription. As a model for endogenous TGFβ signaling we used PC3 cells. In these cells transcription of *SMAD7* and *PAI-1* is upregulated after addition of TGFβ. We added different concentrations of TGFβ (0 to 200 pM) to PC3 cells transfected with either control GFP siRNAs or NUP98 siRNAs. As can be seen in Figure 5a and 5b, the expression of both *PAI-1* and *SMAD7* was increased upon TGFβ stimulation of PC3 cells. We also measured PAI-1 induction at the protein level. Increased PAI-1 protein levels were observed in NUP98 ablated cells after 8 hrs of TGFβ treatment compared to siGFP transfected cells (fig. 5c). This is consistent with the higher *PAI-1* mRNA levels seen in NUP98 ablated cells.

Discussion

In this study we describe the identification of new modulators of the TGFβ pathway through the screening of a large siRNA library. We identified 24 hits with 2 or more independent siRNAs including the canonical TGFβ pathway components SMAD3 and SMAD4. Associations with TGFβ signaling were found for several of the other genes identified. Three of these hits; MED6, MED8 and CRSP2 are components of the mediator (MED) complex [29]. This complex transmits information from transcription factors to RNA polymerase II to regulate transcription. Whether distinct MED protein are involved in transcription stimulated by specific signaling8 pathways is still unclear. However, the mediator complex component ARC105 (human homologue is MED15) was found to play an essential role in TGFβ signaling in *Xenopus laevis* [29]. Depletion of ARC105 inhibits the TGFβ signaling cascade. Surprisingly, in our system, depletion of MED6, MED8 or CRSP2 activated TGFβ signaling. This would indicate an opposite role of mediator proteins in the stimulation of transcription upon signaling pathway activation or an indirect effect on TGFβ mediated transcription.

Another hit that activates TGFβ mediated transcription, STAMBPL1, was identified earlier as an activator of TGFβ signaling. STAMBPL1 (AMSH-2) binds directly to SMAD2 and SMAD7 and might potentiate the TGFβ pathway through these proteins [30]. For the other hits identified in this screen, the interaction with the TGFbeta pathway remains unclear.

We studied NUP98 in more detail. Knockdown of *NUP98* enhances expression of some TGFβ induced genes. For example, we were able to show that *SMAD7* and *PAI-1* mRNA levels are elevated after ablation of NUP98. However, for some other TGFβ target genes such as *p21* we were not able to measure enhanced expression after NUP98 suppression. This can be explained by various previously reported observations. For instance the fact that the different combination of SMAD2, 3,4 can regulate the expression of different sets of target genes [31-34]. In addition the availability of SMAD co-factors can also determine the specific regulation of subsets of TGFβ target genes [1].

As NUP98 is part of the nucleopore complex, we speculated that this protein might play a role in the translocation of SMAD2 or SMAD3. However cells transfected with NUP98 siRNAs did not show any difference in SMAD2 or SMAD3 nuclear translocation. It remains possible that NUP98 is required for transport of TGFβ pathway inhibitors (such as SnoN or SMAD7) to the nucleus or other

TGFβ activators to the cytoplasm thereby affecting TGFβ mediated transcription.

Around 25 different NUP98 oncogenic fusion proteins have been identified in various forms of leukemia [26]. NUP98 fusion proteins play a direct role in transcription, typically the NUP98 N-terminus is fused to a variety of C-terminal partner proteins [26]. These NUP98 oncogenic fusion proteins play a role in transcriptional activation through binding to CBP/p300 histone acetyltransferase [35] and transcriptional inhibition via histone deacetylase HDAC1 [36]. It has been shown that the FG repeat domain in NUP98 is necessary for binding of the fusion protein to CBP/p300 and HDAC1 [35, 36]. However, the basis for target gene selectivity by these fusion proteins is not known [26]. Most, but not all, of these proteins have DNA binding or chromatin modifying activities [26]. Therefore, NUP98 alone might play a direct role in the control of TGFβ transcriptional program.

In conclusion, we identified several novel modulators of the TGFβ pathway. We show that NUP98 ablation activates endogenous TGFβ dependent target gene activation. However, at this moment a mechanistic explanation for the effects of NUP98 is still lacking. Future work is needed to establish a direct role of NUP98 in the regulation of TGFβ signaling.

MATERIALS & METHODS

Cell lines & culture conditions

U2OS and PC3 cells were cultured in DMEM supplemented with 10% FCS, penicillin, streptomycin and glutamine. All cells were cultured at 37°C in 5% CO_2. Stable U2OS TGFβ responsive reporter pGL3-CAGA$_{12}$-Luc cell line as described in chapter IV

siRNA transfection

Cells were reverse transfected with siRNAs according to manufacturers instructions (Dharmacon) (Day 1). Dharmafect 1 (Dharmacon) was used for U2OS cells and Dharmafect 2 (Dharmacon) for PC3 cells. At day two penicillin and streptomycin was added. For U2OS cells TGFβ (200 pM) (R&D systems, Minneapolis, MN, human platelet derived) was added on day 3 and incubated overnight. For PC3 cells TGFβ was added on day 4 for 8 hrs in order to assay target gene activation or 1 hr for the SMAD2/3 translocation assay.

siRNA screen targeting ~6000 human genes

Cells with stably integrated TGFβ responsive reporter were reverse transfected in 384 well plates in triplo with a human siRNA library of Dharmacon (~6000 genes). Dharmafect 1 (0.1 µl per 384-well) was used as transfection reagent and the siRNA SMART pools were transfected at a concentration of 50 nM. 3000 cells were seeded per well with a cell dispenser (Wellmate, Matrix). The transfection was performed in the absence of antibiotics. Some 18 hrs after the transfection we added the penicillin and streptomycin. 48 hrs after transfection cells were stimulated with TGFβ (200pM is used in all experiments unless indicated) for 14 hrs. Cell titer blue reagent (Promega) is used in order to determine the cell viability. After 1 hr incubation $560_{EX}/590_{EM}$ fluorescence is measured with a plate reader (Envision multilabel reader 2101, Perkin Elmer). Subsequently, medium was aspirated with a robot (STAR liquid handling workstation, Hamilton), cells were lysed in Steady-Glo Luciferase (Promega) and luminescence was measured with the same plate reader.

The cell viability counts were used to calculate a viability score and the siRNA pools that are less than 50% viable are not taken along in the further validation rounds. Subsequently, the LUC counts were LOG2 transformed in order to get a normal distribution. The transformed LUC counts (x) were used to calculate the Z-score ((X –Average X of plate)/ (STDEV X of plate)) per plate in order to be able to compare the different plates with each other. In the secondary screen, luciferase counts were divided by the cell viability score resulting in the normalized LUC value . The repressor and activator siRNA pools only validated in this secondary screen when the normalized LUC counts were 2 standard deviations away from the median normalized LUC of the middle siRNA pools.

Validation single siRNAs

The four separate siRNAs were tested in quadruplicate in the U2OS cell-line with stable TGFβ reporter, GFP is taken along as control. For the heatmap representation the luciferase counts were LOG2 transformed, the heatmap was created with TM4 software [23].

General export assay
The general export was investigated with a U2OS cell-line that stably harbors a REV-GFP protein (kind gift of Johan Kuiken). REV proteins are recognized in the nucleus and are exported into the cytoplasm. The U2OS-REV-GFP cell-line is transfected with siRNAs and after 48 hrs images were taken using a 10x objective (Olympus)

Immunohistochemistry to quantify SMAD 2/3 translocation
Cells were cultured in 96 well plates. Formaldehyde was used for fixation of the cells and we permeabilized with 0.2% Triton X-100. Subsequently, samples were blocked with 5% BSA in PBST (0.05% Tween 20 in PBS). A SMAD2/3 (BD Transduction Laboratories, 610842) specific first antibody was used and subsequently a mouse FITC 488 conjugated (Alexa Fluor 488, Invitrogen) secondary antibody. The cells were counterstained with DAPI in order to define the position of the nuclei. Readout was performed with the high content imager Pathway 855, BD biosciences, using a 20x objective (Olympus). CellProfiler software [28] was used to quantify the nuclear/cytoplasmic SMAD2/3 ratio of all individual cells per well.

Western blotting
Cell lysates were separated using 4-12% Bis-Tris gels (Nupage, Invitrogen). Proteins were transferred to PVDF membrane and incubated with primary antibody as indicated. Primary antibodies were detected using a secondary HRP-conjugated antibody. Antibodies used for these studies: Hsp90 (santacruz H-114, sc-7947), SP1 (santacruz PEP 2, sc-59), SMAD2 (cell signaling (L16D3) #3103), SMAD3 (cell signaling (C67H9) #9523). NUP98 (cell signaling (L205) #2288).

Quantitative PCR
PC3 cells were transfected with siRNAs against *NUP98* or against *GFP*. Sixty hours after transfection the cells were treated with different concentrations of TGFβ; 0, 10, 50 and 200 pM. Eight hours later cells were harvested. Total RNA was isolated using TRIzol (Invitrogen). From the total RNA cDNA was generated using Superscript II (Invitrogen) using random primers (Invitrogen). cDNA was diluted and QRT reaction was performed using FAST Cyber green (Invitrogen) with specific primers (Supplementary table 2). All QRT reactions were run in parallel for RPL13 to control for input cDNA. The QRT reactions were run at a fast Real Time PCR system (AB7500, Applied Biosystems).

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Gene	Identified as;	Number of siRNAs^	"On target"
ACTG2	activator#	4	yes
AKR1A1	repressor*	1	no
ARHGEF17	activator#	4	yes
ARHGEF6	repressor*	0	no
ATF7IP	activator#	4	yes
CHAF1A	activator#	4	yes
CRSP2	activator#	4	yes
CTBP1	activator#	3	yes
CTNNB1	repressor*	0	no
CTNND1	repressor*	0	no
EEF2	repressor*	1	no
FOXC2	activator#	3	yes
GRIK5	repressor*	0	no
HDAC7A	activator#	3	yes
HGFAC	activator#	2	yes
HMOX1	activator#	4	yes
INS	activator#	3	yes
ITGB1	repressor*	1	no
ITGB3	repressor*	0	no
KIR3DL3	repressor*	0	no
KPNB1	activator#	2	yes
MED6	activator#	3	yes
MED8	activator#	4	yes
MIZF	activator#	4	yes
NCOA6	repressor*	2	yes
NUP98	activator#	4	yes
PDPR	repressor*	1	no
PECAM1	repressor*	0	no
RHAG	repressor*	1	no
SETDB1	activator#	4	yes
SF3A1	activator#	4	yes
SF3A3	activator#	3	yes
SFPQ	repressor*	2	yes
SMAD3	repressor*	4	yes
SMAD4	repressor*	3	yes
SMARCD3	repressor*	1	no
STAMBPL1	repressor*	2	yes
TCF8	repressor*	1	no
THY1	repressor*	0	no
TIMP2	repressor*	0	no

* siRNA represses the reporter
\# siRNA activates reporter
^ siRNAs (average value triplicate experiment) that score significantly different from GFP (2x stdev)

Supplementary table 1
Overview of the hits tested in the "on target" validation.

Gene	Seq	Primer name	Refseq
NUP98	GGACTCCTGACACTTCCCCT	NUP98-114F	NM_005387
NUP98	CCAAAGGGTGTTCCAAATGA	NUP98-209R	NM_005387
PAI-1	AGCTCCTTGTACAGATGCCG	SERPINE1-192R	NM_000602
PAI-1	ACAACAGGAGGAGAAACCCA	SERPINE1-92F	NM_000602
SMAD7	CCAGGCTCCAGAAGAAGTTG	SMAD7-174R	NM_005904
SMAD7	CCAACTGCAGACTGTCCAGA	SMAD7-68F	NM_005904
RPL13	GAGACAGTTCTGCTGAAGAACTGAA	RPL13-457F	NM_000977
RPL13	TCCGGACGGGCATGAC	RPL13-522R	NM_000977

Supplementary table 2
Overview of all QRT primers pairs used in this study.

Chapter VI

Discussion

Modulators of cancer pathways

The heterogeneity of cancer makes that individualized approaches need to be developed in order to kill cancer cells efficiently. Currently, a new era with targeted therapeutics fuels the expectations that cancer drugs will become more specific and efficacious. However, insight in modulators of these cancer pathways is essential to be able to identify responders to such targeted therapeutics. Loss-of-function functional genetic screens have identified regulators of cancer pathways and are therefore a valuable tool in the fight against cancer. For example the cylindromatosis *(CYLD)* tumor suppressor gene has been identified in a loss-of function screen as modulator of NFκB activity [1].

Activation of the p53 protein by small molecule drugs

In this thesis I describe a functional genetic loss-of-function screen in which genes were identified that modulate the response to MDM2 inhibitor Nutlin-3 (chapter II). We found that shRNAs against *p53* and *53BP1* are able to override a Nutlin-3-induced cell cycle arrest in p53 wild type breast cancer cells (MCF-7). 53BP1 has been implicated in DNA damage sensing, leading to p53 pathway activation. DNA damage signaling is often constitutively activated in cancer cells and premalignant lesions, but not in healthy tissues [2-4]. We showed that inhibition of DNA damage signaling in MCF-7 cells prevents activation of p53. These observations led to the hypothesis that stabilization of p53, by inhibition of MDM2, by itself is not sufficient to activate downstream targets and consequential cellular responses. In addition to stabilization, an activating signal is also required. Posttranslational modifications associated with p53 activation have been described. These include phosphorylation, ubiquitination, sumoylation and lysine methylation [5-7]. Phosphorylation of p53, on multiple residues, is observed after DNA damage [8]. However, phosphorylation of p53 seems to be dispensable for p53 function *in vivo*. Knockin mice carrying mutations to analogous of human p53 Ser18/Ser23 or p53 Ser392 have been described. These mice are phenotypically normal and p53 activation is only partly compromised [6]. It has been shown that Nutlin-3 treatment does not result in p53 phosphorylation. However, p53 target genes are nevertheless activated [9, 10]. This suggests that the signal required for p53 activation after Nutlin-3 mediated stabilization is of different nature. One of the posttranslational modifications seen on p53 after DNA damage is the presence of dimethylation of p53 on lysine 370 (K370me2). This modification facilitates the binding of p53 to 53BP1 and subsequent p53 target activation [11]. We find that cells escape from a p53 induced cell cycle arrest in absence of 53BP1 suggesting that the K370me2 is the signal required for the activation of p53 by Nutlin-3.

Our work suggests that DNA damage signaling synergizes with Nutlin-3 to activate p53 target genes. Constitutive DNA damage signaling is observed in cancers and premalignant tissues but not in normal healthy tissues [2-4]. This could explain the apparent absence of Nutlin-3 mediated the effect in normal tissues [9]. Coll-Mulet and colleagues also showed that Nutlin-3 synergizes with DNA damaging drugs as doxorubicin, chlorambucil and fludarabine in chronic B-cell lymphocytic leukemia (B-CLL) [12]. However, they also reported that a low dosage of DNA damage agents caused a synergistic effect for B-CLL cells, but not for normal human T cells [12]. This illustrates that there might be a window for combination of genotoxic drugs combined with Nutlin-3.

In addition to 53BP1 and p53 shRNAs we also identified additional shRNAs that

were able to override the Nutlin-induced cell cycle arrest, among them an shRNA vector against *USP28*. Recently, USP28 was implicated in control of the p53 dependent DNA damage response where USP28 directly regulates the protein stability of 53BP1 [13]. In addition an shRNA vector against *hnRNPK* was also among the top hits identified in this screen. It has been described that hnRNPK plays a critical role in coordinating the p53 dependent transcriptional response after DNA damage [14] although the exact nature of this signal remains unclear.

The identification of several components of the DNA damage signaling complex strengthens the conclusion that activated DNA damage signaling is a critical factor required for the cellular response to Nutlin-3 [14].

Different types of compounds have been developed that activate the p53 pathway in p53 wt tumors. Nutlin-3 is an example, of a compound that disturbs the interaction between MDM2 and p53 [9], while RITA directly modulates p53 dependent transcription [15]. P53 pathway activation by RITA mostly leads to apoptosis, while Nutlin-3 treated cells often undergo a cell cycle arrest [16, 17]. These differences in response have been attributed to altered regulation of the proapototic gene *HIPK2* [17] and the cell cycle arrest inducing gene *hnRPNK* [16] by RITA and Nutlin-3.

Not only the mode of p53 pathway activation determines the cellular response, but variables between cell lines can also result in different responses to Nutlin-3. The p53 wt tumor cell lines MCF-7, U2OS and HCT116 cells [17] enter a cell cycle arrest in response to Nutlin-3 treatment, whereas SJSA-1 [9], ML-1 and RKO cells [18] mainly undergo apoptosis. Recently, Vaseva et al. showed that the mitochondrial pathway is the main determinant for Nutlin-3 induced p53 dependent apoptosis [19]. Nutlin-3 treatment causes in addition to nuclear, also cytoplasmic accumulation of p53. This cytoplasmic p53 is still normally ubiquitinated and is able to translocate to the mitochondria were it induces transcription-independent apoptosis through cytochrome-C release [18].

Although differential responses can be observed in different cell types upon exposure to Nutlin-3, the strategy to use inhibitors of the MDM2-p53 interaction remain promising. Currently a clinical trail has started with the MDM2-p53 inhibitor R7112 [20].

Barcode screen versus colony formation screen

In chapter III, a large-scale RNA interference screen in human fibroblast is described. An identical screen, using the same shRNA collection was performed previously [21], however this effort did not take advantage of the barcode technology. Using the barcode technology we identified shRNA vectors against 4 of the 5 genes identified and validated in the previous screen [21]. Among the overlapping genes are *HTATIP, HDAC4, AHCYL2 (KIAA0828), RSP6KA6*. It was shown that mRNA expression levels of *HTATIP, HDAC4, AHCYL2, RSP6KA6* were significantly decreased in colon carcinoma samples compared to normal tissue [22]. The downregulation of these genes might contribute to malignant transformation *in vivo*.

P53 signaling and the circadian clock

In chapter III we focused on three genes, which were identified with two or more shRNA vectors in the screen; *ARNTL, TNIP1* and *RBCK1*. We were able to show that knockdown of these genes results in a decreased ability of p53 to activate $p21^{CIP1}$ expression, none of the identified genes affect p53 levels. TNIP1 has been described to inhibit NF-κB [23, 24] and RBCK1 [25-27] is

an ubiquitin E3 ligase which ubiquitinates several proteins. ARNTL is a transcription factor that controls circadian rhythm. Circadian rhythm is an internal timing system that regulates for example the sleep-wake cycle in mammals [28, 29]. The circadian clock is regulated centrally by suprachiasmatic nucleus (SCN) (master clock) that is located in the hypothalamus but also intrinsically in the different organs (peripheral clock) [28]. The circadian clock also regulates cell cycle control [29] and circadian rhythm can also be measured in *in vitro* cultured cell-lines [30].

The identification of ARNTL in a screen for genes that upon inactivation can bypass a p53 mediated cell cycle arrest suggest a potential role of ARNTL in tumorigenesis. The transcription factor ARNTL is key regulator of circadian rhythm. ARNTL forms a heterodimer with CLOCK [31] to activate circadian target genes. This heterodimer drives the expression of Cryptochrome (*CRY*) and Period (*PER*) genes. The increase in concentration of the CRY and PER proteins subsequently leads to the transcriptional inhibition of ARNTL. Subsequently, proteasomal degradation decreases the level of CRY and PER proteins, which allows the start of a new circadian cycle [32].

Several lines of evidence suggest a role for circadian rhythm in cancer development and progression. Destruction of the SCN in mice leads to the accelerated growth of two types of tumors in mice [33]. Furthermore, mice that are exposed to a chronic jetlag show a dampened circadian clock and faster tumor progression [34]. However, the data on mouse models for circadian clock components is not unambigous. Lack of some of circadian clock components like ARNTL or CLOCK (mutant) can cause early ageing phenotypes making these models less suitable for tumor onset experiments. [35, 36] Furthermore, not all mutants have lost their circadian rhythm completely; some mutants have only lost regulation by the master clock or the peripheral clock [37]. In addition, the loss of *CRY1*$^{-/-}$*CRY2*$^{-/-}$ does not lead to abnormalities with respect to tumor onset compared to wild-type mice [38]. A clear effect on development of lymphomas has only been shown for Per2 mutations [39].

Besides evidence from *in vivo* studies that link circadian rhythm with cancer, several epidemiological studies have been performed. One study, performed in nightshift workers, reported an increased risk on breast cancer in women [40-42]. A disruption in the circadian clock results in lower melatonin levels [43-45]. It has been suggested that higher cancer risk due to circadian disruptions is related to the lower melatonin levels [46-48]. Melatonin has been found to increase p53 and p21 levels *in vitro* in breast cancer cells [49]. From our results it was clear that *ARNTL* knockdown led to an override of a p53 arrest, but we have not yet obtained insight in a possible mechanism explaining this observation.

New modulators of TGFβ signaling

In chapter IV and V the identification of new modulators of the TGFβ pathway is described. In both screens, using a kinase siRNA library or a large siRNA library targeting around 6000 genes, known TGFβ components were identified that either activate or inhibit TGFβ signaling.

In chapter IV we identified IRAK2 as new modulator of the TGFβ pathway. Knockdown of IRAK2 inhibited the induction of TGFβ target genes and the induction of a TGFβ dependent proliferative arrest. Furthermore, ablation of IRAK2 impaired translocation of SMAD2, but not SMAD3 to the nucleus. This observation was unexpected since the TGFβ responsive reporter has been described to be dependent on SMAD3/4 dependent transcription. Moreover, SMAD3 or SMAD4 overexpression

activated this TGFβ dependent reporter while SMAD2 overexpression did not [50].

In chapter V, a more detailed study of NUP98 is described in which we demonstrated that NUP98 ablation activates endogenous TGFβ signaling. NUP98 is a nucleoporin protein that contains phenylalanine-glycine (FG) rich repeats. About 30% of the nucleoporin proteins have these repeats. Two other nucleorporin proteins with a FG domain have earlier been implicated earlier in the nuclear export of SMAD2 and the nuclear import of SMAD2, SMAD3 and SMAD4 [51, 52]. We investigated if NUP98 plays a role in nucleocytoplasmic shuttling of SMAD2 and SMAD3 but we did not find evidence for this hypothesis. NUP98 oncogenic fusion proteins are found in leukemia where these fusion proteins play a role in transcriptional activation through binding of the NUP98 FG domain to CBP1/p300 and HDAC1 [53, 54].

Therefore, it is possible that NUP98 alone directly affects SMAD dependent transcriptional activation. However, at this moment mechanistic insight on how NUP98 interferes with the TGFβ pathway is lacking. More work is needed to establish if NUP98 plays a direct role in TGFβ signaling.

In this thesis identification of functional modulators of the p53 and the TGFβ signaling networks are described. These findings can contribute to a better understanding of TGFβ and p53 signaling. A better understanding of these signaling networks will ultimately lead to the development of better cancer therapeutics.

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Summary

Cancer is a heterogeneous disease that must be attacked from multiple angles. A better understanding of the signaling networks that are involved in cancer development is the basis for the development of targeted cancer therapeutics. In this thesis we describe functional genomic loss-of function approaches in order to place genes in pathways, which are involved in cancer development.

We focus on two signaling networks important for the development of cancer, the p53 and TGFβ pathway. In **chapter I** these two signaling networks, their roles in cancer and functional genetic screens related to these pathways are outlined.

In **chapter II** and **chapter III** we use two different cell based models to activate the p53 pathway for the identification of genes that override a p53 dependent cell cycle arrest. These genes are identified through a loss-of function screen using a shRNA library that targets 8,000 human genes. In **chapter II** we used Nutlin-3, a small molecule inhibitor of MDM2, to activate the p53 pathway leading to a cell cycle arrest. Reactivation of the p53 pathway by Nutlin-3 in p53 wt tumors leads to tumor eradication in mice, an analogue of Nutlins is currently in clinical trials. We performed a large-scale RNA interference-based short hairpin (shRNA) barcode screen to gain insight in the mechanism of action of Nutlin-3. We found that activation of the p53 pathway and the resulting cell cycle arrest by Nutlin-3 could be prevented by ablation of 53BP1. 53BP1 is an upstream component of the DNA damage signaling network, which signals to p53 in response to stress. The DNA damage network is frequently activated in cancer but not in healthy tissues. Our data suggest that Nutlin's tumor specificity results from the activated DNA damage signaling in tumor cells. In **chapter III** we identified additional genes that could modify p53 function. Among these genes is ARNTL, a component of the circadian clock. Knockdown of ARNTL led to an override of a p53 and a $p19^{ARF}$ induced cell cycle arrest. Our findings are in accordance with recent observations, which link the circadian clock to cell cycle and cancer.

In **chapter IV** and **chapter V** we describe single well siRNA screens that are performed to identify new modulators of the TGFβ pathway. We used a TGFβ responsive reporter system to assess the modulation of the TGFβ pathway.

In **chapter IV** we used a siRNA library directed targeting 700 kinases and kinase related proteins. We found that depletion of the IRAK2 kinase, which is a component of the toll like receptor signaling pathway, inhibits TGFβ signaling. Inhibition of IRAK2 impaired SMAD2 phosphorylation and translocation to the nucleus. Our data suggest that IRAK2 is involved in the TGFβ signaling cascade, providing a possible link to the interplay between TGFβ signaling and TLR signaling that has been reported in literature.

In **chapter V** we use a larger siRNA library that targets 6,000 different genes. We identify the nucleoporin protein NUP98 as modulator of the TGFβ signaling cascade. Ablation of NUP98 leads to the activation of TGFβ dependent target genes.

The screens performed in this thesis have lead to the identification of genes that are functionally involved in the p53 and TGFβ signaling cascade. This novel insight potentially contributes to a better understanding of cancer development and can therefore help to gain territory in the 'war' against cancer.

SAMENVATTING

Het lichaam bestaat uit miljarden cellen. Beschadigde en verouderde cellen worden voordurend vervangen door nieuwe cellen. Nieuwe cellen ontstaan door celdeling, een proces waarin een cel zijn genetisch materiaal dupliceert en twee nieuwe cellen vormt. Dit is normaliter een heel goed gecontroleerd proces, dat miljoenen keren per dag in het lichaam plaatsvindt. Het genetisch materiaal (de genen) codeert voor heel veel verschillende eiwitten die een groot aantal functies in de cel reguleren. "Reparatie eiwitten" zorgen ervoor dat eventuele fouten tijdens de duplicatie van het genetisch materiaal worden hersteld. Dit proces kan echter verstoord worden door mutaties in het genetisch materiaal, die er voor kunnen zorgen dat deze "reparatie-eiwitten" hun werk niet meer uitvoeren. Fouten in het genetisch materiaal kunnen er dan toe leiden, dat cellen onbeheerst gaan delen en een tumor vormen. Een gemeenschappelijk kenmerk van alle soorten kanker is dat er ongeremde celdeling plaatsvindt. Er zijn wel 100 verschillende soorten kanker en in 2008 was kanker doodsoorzaak nummer één in Nederland.

Eiwitten communiceren met elkaar in zogenoemde signaleringscascades. Dit zijn een soort kettingreacties waarbij het ene eiwit een signaal doorgeeft naar het volgende eiwit. Uiteindelijk worden hierdoor vele verschillende processen in de cel geregeld. De p53 en de TGFβ signaleringscascades spelen een belangrijke rol bij de ontwikkeling van kanker. P53 is erg belangrijk voor het controleren van de celdeling, verder reguleert deze cascade dat cellen die teveel beschadigd zijn, een gecontroleerde celdood ondergaan. In kankercellen wordt de p53 signaleringscascade vaak uitgeschakeld door een mutatie in het p53 gen of door remming van het p53 eiwit door andere eiwitten. Ook genen uit de TGFβ signaleringscascade zijn vaak gemuteerd in kankercellen. Hierdoor kunnen tumoren bijvoorbeeld makkelijker uitzaaien.

In de proeven beschreven in dit proefschrift wordt gebruik gemaakt van RNA interferentie (RNAi), een techniek die elk gen apart kan uitschakelen. We hebben gekeken met RNAi welke genen een effect hebben op de p53 of TGFβ signaleringscascade. Van een aantal van die genen was nog niet eerder bekend dat ze een rol speelden bij deze signaleringscascades. Vervolgonderzoek naar de aanwezigheid van mutaties in deze genen en hun rol in de ontwikkeling van kanker kan helpen om het ontstaan van kanker beter te begrijpen. Dit kan dan op den duur weer leiden tot een betere kankerbestrijding.

Curriculum vitae

Armida Fabius werd geboren op 30 september 1979 te Amsterdam. Na het behalen van het VWO diploma in 1997 aan het Amsterdams Lyceum te Amsterdam is zij in 1998 begonnen met de studie biologie aan de Universiteit van Amsterdam. Tijdens deze studie heeft zij stage gelopen bij het Sanquin te Amsterdam op de afdeling plasma-eiwitten. Hier deed zij in groep van Dr. Jan Voorberg onderzoek naar de moleculaire achtergrond van hoge factor VIII niveaus in mensen met veneuze trombose. Haar tweede stage was bij IsoTis te Bilthoven, zij bestuurde hier het mechanisme van chondrogenese. In 2002 behaalde zij Cum Laude haar Master in de biologie. In 2003 vertrok zij naar Boston in de Verenigde Staten om bij de groep van Prof. Dr. Nick Dyson onderzoek te doen naar regulatoren van de celcyclus in *Drosophila*. In 2004 is zij gestart met haar promotieonderzoek bij de afdeling Moleculaire Carcinogenese van het NKI-AvL te Amsterdam. Hier heeft zij onder leiding van Dr. Roderick Beijersbergen en Prof. Dr. Rene Bernards onderzoek gedaan. De resultaten van dit onderzoek zijn in dit proefschrift te lezen. In 2010 zal zij in New York starten met haar postdoctorale onderzoek bij het toonaangevende Memorial Sloan-Kettering Cancer Center.

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Furthermore, I would like a to go a little further back in time. Jan Voorberg, Nick Dyson, and Dessy Dimova were among the ones that gave me enthusiasm to pursue a scientific career, thank you very much.

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Myrna, Jet en Sal, we gaan gewoon weer verder waar we gebleven waren, maakt niet uit of daar een dag, een maand of een jaar tussen heeft gezeten, heel fijn is dat!

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Pau & Steef… Jullie zijn de eerste stop onderweg naar Jordanië, wordt zeker gezellig! Ducktape en trouwen horen nu voor altijd bij elkaar. Lieve Lot; nog lekker mooi van het badderen geworden? Christine, speciaal voor jou, je lievelings NY gekozen!

Nina, Lone, Yara en Roos, ons meer of minder sporadisch, contact is eigenlijk ook altijd leuk. Wim, Erica, Martijn, Roos, Fabje, Jimmy en Susanne erg fijn om jullie te hebben leren kennen de afgelopen tijd!

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Paps en mam, heel cliché maar jullie zijn de liefste pap en mam van de wereld. Liesje jij bent ook de liefste gekke zus. Niet je ticket nog een keer omboeken hè... 22 Januari in Nederland zijn lijkt me veel gezelliger dan op een tropisch eiland!

Lieve Jasper, wij komen er wel, ook als de Suzuki niet meer rijdt heb ik er vertrouwen in, dat we dan ook nog steeds vooruit gaan!!! Heb heel erg veel zin in ons nieuwe avontuur en in alle jaren die daarna volgen.

Armida