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DISSECTING THE P53 PATHWAY BY MEANS OF SMALL MOLECULE-MEDIATED REACTIVATION AND COMPUTATIONAL BIOLOGY

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Cover Illustration: An example of a model that captures the essense of a noisy system. The figure depicts a cut-out model of my writing desk since several years back. Importantly, the final model does not fully depict the compexity of the underlying system, but captures enough detail to build a basic understanding that can be extended by conventional laboratory methods, such as paying me a visit.

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ABSTRACT

The p53 tumor suppressor is a highly connected signaling molecule with paramount importance to tumor suppression. It is activated by diverse stress signals and in response activates the transcription of affector genes that can induce several cellular processes, most importantly cell-cycle arrest and apoptosis. Consequently, the p53 tumor suppressor pathway is inactivated in a large proportion of cancers, across almost all tumor types. In up to 50% of tumors, inactivation is achieved by point mutations in the p53 gene. In tumors that retain wild-type p53, mechanisms of inactivation mainly converge on de-regulation of MDM2, the E3 ligase and the main destructor of p53.

This thesis presents the identification and further investigations of the precise mechanism of action of a novel p53-reactivating compound dubbed RITA (Reactivation of p53 and Induction of Tumor cell Apoptosis). RITA was identified in a cell-based screening assay, using a pair of isogenic colon cancer cell-lines that differ only in the status of p53. Thus, RITA was selected based upon its ability to selectively inhibit the growth of the p53-carrying cell line without toxicity on the p53 null derivative. RITA was shown to bind directly to p53 and disrupt its complex with MDM2, thereby stabilizing the protein. Consequently, p53 levels increased in cells leading to massive programmed cell death. Furthermore, RITA inhibited tumor growth in a mouse xenograft model without apparent toxicity to the animals.

In order to further characterize the p53 response induced by RITA, we investigated the global transcriptional response upon RITA treatment by oligonucleotide microarrays. Pathway analysis revealed an unusually selective induction of the apoptotic branch of the p53 pathway. Further investigation revealed that hnRNP-K, a transcriptional co-activator of p53, was down-regulated after RITA treatment causing deficient cell-cycle arrest and thereby guiding the p53 response towards induction of apoptosis. p21, the major cell-cycle arrest target gene of p53 was shown not only to be weakly induced due to hnRNP-K reduction, but also degraded on protein level. We present evidence for a model where the cause of both hnRNP-K and p21 degradation is traced back to MDM2, which, after being displaced from p53 by RITA and thereby unable to degrade its primary target, instead induced degradation of these two proteins. In the context of pharmacological reactivation of p53 by RITA, it therefore seems that MDM2 goes out of character and actually co-operates with p53 to boost the apoptotic response.

In the third paper we introduce a method, called revarray (from Reverse-engineering Microarray), for analyzing the transcriptional program that underlies the changes observed in an oligonucleotide microarray experiment. The underlying transcriptional model is based on combinations of modules of either single or clusters of Transcripion Factors (TFs), and properly takes into account whether the module is an activator, a repressor or an unspecific enhancer. We apply the method on the p53 response upon treatment with three p53-activating compounds – 5-Fluorouracil, RITA and Nutlin. The results suggest that the FOX family of transcription factors could be important co-factors of p53 and that repression of genes by p53 seems to play an important role in apoptosis induction.

LIST OF PUBLICATIONS

I. Issaeva N, Bozko P, **Enge M**, Protopopova M, Verhoef LG, Masucci M, Pramanik A, Selivanova G, .Small-molecule RITA binds to p53, blocks p53-HDM2 interaction and activates p53 function in tumors. Nat Med. 2004 Dec;10(12):1321-8. Epub 2004 Nov 21.

II. **Enge M**, Bao W, Hedström E, Jackson SP, Moumen A, Selivanova G., MDM2-Dependent Downregulation of p21 and hnRNP-K Provides a Switch between Apoptosis and Growth Arrest Induced by Pharmacologically Activated p53. Cancer Cell. 2009 Mar 3;15(3):171-83.

III. **Enge M**, Nikulenkov F, Turunen M, Taipale J, Kel A and Selivanova G., Reverse-engineering microarray data applied to the p53 transcriptional response. Manuscript.

ASSOCIATED PUBLICATIONS

IV. Grinkevich V, Nikulenkov F, Shi Y, **Enge M**, Bao W, Maljukova A, Sangfelt O, Gluch A, Kel A, Selivanova G. Ablation of key oncogenic pathways by RITA-reativated p53 is required for efficient apoptosis. Cancer Cell May 2009.

V. Hedström E, Issaeva N, **Enge M**, Selivanova G .Tumor-specific induction of apoptosis by a p53-reactivating compound. Exp Cell Res. 2009 Feb 1;315(3):451-61. Epub 2008 Dec 3.

CONTENTS

LIST OF ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
BCL2	B-cell lymphoma 2
CDK	Cyclin-dependent kinase
ChIP	Chromatin Immunoprecipitation
DNA	Deoxyribonucleic acid
Mcl-1	Myeloid cell leukemia sequence 1
MDM2	Mouse Double Minute 2
Myc	Myelocytomatosis
NF-kB	Nuclear factor kappa B
PML	Promyelocytic leukemia
PTEN	Phosphatase and tensin homolog
Ras	Rat sarcoma viral oncogene homolog
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
siRNA	Small interfering RNA
QPCR	Quantitative real-time PCR
shRNA	Short haipin RNA.
Skp2	S-phase kinase-associated protein 2
TP53	Tumor protein p53
HDAC	Histone deacetylases
FDR	False discovery rate
SAM	Significance analysis of microarrays
GSEA	Gene set enrichment analysis
RMA	Robust multiarray analysis
TF	Transcription factor
APC	Adenomatous polyposis coli
EGF	Epidermal growth factor
IGF1R	Insulin-like growth factor 1 receptor
ATR	ATM and Rad3-related
Bax	Blc-2 associated X protein
EGFR	Epidermal growth factor receptor
GADD45	Growth arrest and DNA damage gene 45
Her2/Neu	Heregulin/neuregulin receptor/ErbB2
RITA	Reactivation of p53 and induction of tumor cell apoptosis

1 CONTEXT

1.1 CANCER AS A MICROEVOLUTIONARY PROCESS.

During the life-cycle of all higher metazoans most cells that they are composed of go through a big number of life-cycles. As cells in the body die they are replaced by new ones because the cells of each organ in the body carries the capacity to divide and proliferate. While being a prerequisite of multi-cellular life this capacity to proliferate also forms the foundation for tumorigenesis. Tumors arise from normal cells of the body by an evolutionary process, whereby mutations in the genes of somatic cells that happen to be advantageous to the growth of the cell, will lead to larger progeny or bigger chance of survival. The proliferation of cells is tightly controlled by mechanisms active both within the cell and between cells or even between organs, so every mutation advantageous to the individual cell removes some part of this control.

Tumors from the different organs of the body (or even substructures withing these organs) retain characteristics from their tropism of origin, which is one reason why the origin of a tumor can often be deduced by inspection of the morphology of the tumor, and in some cases even characteristics of single cells within the tumor, even when the tumor is growing as a metastasis far removed from its origin. Another reason for the differences observed between tumor types is that the type of control mechanisms that have to be overcome vary between cell-types. For example, the APC gene is mutated in more than 80% of all colon cancers (Rowan et al., 2000). APC is a scaffolding protein that brings together β-Catenin with GSK-3-β, which leads to phosphorylation and subsequent degradation of the former. Because of signalling by Wnt from the stroma, resulting in inhibition of GSK-3-β, β-Catenin is usually active in the colonic crypts and is a potent activator of cell proliferation. Further towards the intestinal lumen, these stromal cells are no longer present and Wnt signalling is diminished, resulting in degradation of β-Catenin and cessation of proliferation. If, however, APC is non-functional, GSK-3-β cannot degrade β-Catenin and the colonic epithelial cells retain their proliferative capacity, which greatly increases the risk of tumorigenesis. Control exerted by the localization of the stroma is unique to epithelial colon cells, and consequently mutations to APC are a good indicator that the origin of a tumor is the colon.

On the other hand, many control mechanisms are shared between cell-types. The cell-cycle is tightly controlled by sequential activation of cyclin/Cyclin-dependent kinase complexes. An important level of control is through the pRb protein, which binds and inhibits members of the E2F-family transcription factors that are needed for the cell to go through a round of cell-division. Mitogenic signals lead to hyperphosphorylation of pRb resulting in its dissociation from E2F and cell division. Consequently, inactivating pRb would be predicted to be advantageous to any cell type. As predicted, tumors from diverse origins carry inactivating mutations of pRb to circumvent the need for mitogenic signals.

Examples of both tissue-specific and general traits abound, although in most cases the division is not absolute but rather along a sliding scale from specific to general. Of course, the analogy between tumorigenesis and evolution does not hold on every level

– tumorigenesis is of much more limited scope, for example. Also, the mechanisms that underly phenotypic alterations of the tumor cells are only partially the same. Epigenetic alterations have a huge impact on tumor formation, with tumor suppressors such as p16 commonly silenced by promoter methylation, for example. Although epigenetic changes are inherited and probably play a role in evolution of species, the relative importance of these changes in tumorigenesis is probably much bigger than in the evolution of species.

Molecularly designed drugs have targeted both tumor-specific traits and general ones. Examples of drugs that target tumor-specific traits include Imatinib mesylate (also called Gleevec or STI-571), which targets the Bcr-Abl fusion protein in Chronic Myelogenous Leukemia (Wisniewski et al., 2002) and Herceptin, which is an inactivating antibody that targets the mitogenic HER2/neu receptor amplified in breast cancer (Baselga et al., 1996; Slamon et al., 1989), while some more tumor-agnostic drugs are for example Iressa (gefitinib, ZD1839; AstraZeneca, Wilmington, DE), which inhibits the EGF-Receptor - a receptor for mitogenic signals that is aberrantly activated in many tumor types, and derivatives of the natural compound Rapamycin (CCI-779, RAD-001) which inhibits mTOR, an activator of the mitogenic Akt pathway that is commonly hyper-activated by loss of its natural suppressor PTEN in many tumors (LoPiccolo et al., 2008).

1.2 THE P53 TUMOR SUPPRESSOR PATHWAY.

p53 is a transcription factor located in the center of the cells' general (as opposed to tissue/tumor specific) tumor-suppressive network. A wide variety of stresses activate p53 by stabilizing it, thereby activating its downstream pathways. The two main outcomes of p53 activation are programmed cell-death and cell-cycle arrest, which can be either transient, or permanent (senescence). Since its discovery more than 25 years ago, p53 has been the subject of an immense body of work, including a fair amount of contradictory studies. Any review is bound to be a simplification, to the point of inaccuracy. The following text is therefore very general, except when touching upon matters directly related to the work in my thesis.

1.2.1 Activation of p53.

In the unstressed cell, the p53 protein is highly unstable, with a half-life of typically less than 20 minutes. Protein levels are kept low because of continued proteasomal degradation. Several E3 ligases have been shown to ubiquitinate p53, tagging it for degradation, including PIRH1, COP9 and MDM2 (Dornan et al., 2004; Iwakuma and Lozano, 2003; Leng et al., 2003). The most widely researched antagonist of p53 is MDM2, which is also a target of p53 transactivation (reviewed in (Iwakuma and Lozano, 2003)), creating a negative feedback loop. The importance of MDM2 and its close association with p53 is perhaps best illustrated by the fact that knock-down of MDM2 is embryonically lethal in mice, which is rescued by simultaneous knock-down of p53 (Jones et al., 1995; Mendrysa et al., 2003; Montes de Oca Luna et al., 1995).

The main processes that activate p53 converge on the p53/MDM2 complex. These processes can be divided into two distinct types – post-translational modifications and

protein-protein interactions (Fig.1, top panel). First, in addition to negative regulation by ubiquitination, the p53 protein can be activated by post-translational modifications, mainly by kinases and acetylases (reviewed in (Appella and Anderson, 2001)). For example, upon DNA damage Chk1, Chk2, ATM and ATR can all phosphorylate p53 in the MDM2-binding region, thereby abolishing the interaction between p53 and its destructor to stabilize the p53 protein (Xu, 2003). Phosphorylation of MDM2 may also abolish its interaction with p53 with similar results (Appella and Anderson, 2001; Olsson et al., 2007). However, despite extensive research over two decades on post-translational modification of p53, both the precise nature and relative importance of the modifications remain elusive. Biochemical evidence about the importance of modifications to the stability of p53 obtained *in vitro* has in many cases been clearly contradicted by *in vivo* studies in mice, which found little or no effect of using mutants lacking the modification sites (Chao et al., 2003; Toledo et al., 2007; Wu et al., 2002). It seems clear that the modifications are meaningful but probably they can only be definitively understood within a larger cellular context, probably they also need to be studied in groups rather than on-by-one (Olsson et al., 2007).

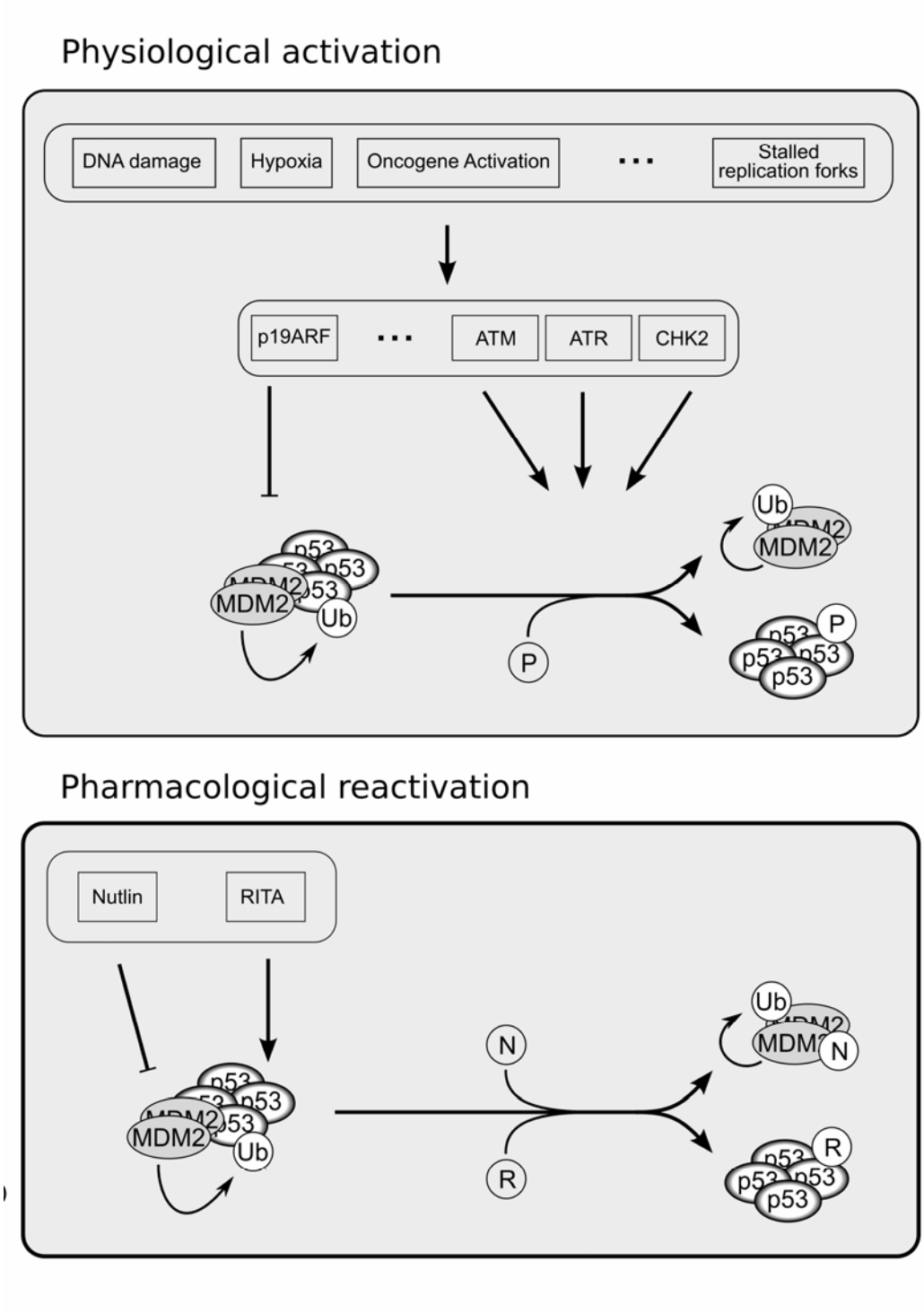


Figure 1. Activation of p53.
A diverse collection of stress conditions such as DNA damage or hypoxia, which activate transducer kinases and co-factors that either modify p53 or bind to MDM2. This result in disruption of the p53/MDM2 complex with concomitant stabilization of p53 and de-stabilization of MDM2 (top panel).
Pharmacological reactivation (see section below) is analogous to physiological reactivation. Here, the p53/MDM2 complex is disrupted by small molecules binding to either p53 (for RITA) or MDM2 (for nutlin), with the same result as above (bottom panel).

1.2.2 Protein interactions dictate stability and specific response of p53

The second mechanism of p53 activation is through protein-protein interactions. The most well-known interaction is binding of p19ARF to MDM2 which abolishes the negative regulation of p53 (Pomerantz et al., 1998). p19ARF is transcriptionally induced following oncogene activation (de Stanchina et al., 1998; Inoue et al., 1999; Inoue et al., 2000; Palmero et al., 1998). The importance of p14ARF for tumor suppression is corroborated by a tumor susceptibility phenotype of p14ARF NULL mice (Kamijo et al., 1999).

While p19ARF could be said to work on the same axis as p53/MDM2 in increasing the stability/activation of p53 under stress, other factors have been shown to specifically induce a certain response, rather than simply increasing the intensity of the response. Of course, this division is slightly artificial since most factors that activate a certain response also probably increase the intensity of the p53 response. However, it is a useful abstraction for our discussion on regulation of the vastly different outcomes of p53 activation, and one commonly used in scientific literature. Three members in the ASPP family – ASPP1, ASPP2 and iASPP influence the ability of p53 to induce the proapoptotic Bax protein. iASPP binds to the proline-rich domain of p53 and inhibits induction of Bax along with inhibiting apoptosis induction. ASPP1 and ASPP2, disrupt the interaction between iASPP and p53, resulting in increased binding to the Bax promoter (Bergamaschi et al., 2003; Samuels-Lev et al., 2001).
Brn-3a and Brn-3b both regulate survival of neuronal cells, in opposing ways. While Brn-3a promotes cell survival and inhibits p53-dependent transactivation of the proapoptotic Bax and Noxa genes, Brn-3b-/- neurons display deficient Bax activation and apoptosis induction (Budhram-Mahadeo et al., 2006; Hudson et al., 2005). p300 is a ubiquitous co-activator of transcription and, together with auxiliary factors it is involved in p53 transcriptional regulation. Thus, JMY has been shown to form a complex with p300 and p53 to increase acetylation (Shikama et al., 1999), resulting in transcriptional activation and induction of apoptosis. SKP2, also an auxiliary factor to p300, prevents the interaction between p53 and p300 resulting in inhibition of transcriptional activation (Kitagawa et al., 2008).

In addition, several groups have reported that pro-apoptotic Bcl2 family members (that are targets of p53 transactivation and that were previously thought to be exclusively downstream of p53) can physically interact with p53, resulting in induction of transcription-independent apoptosis (Chipuk et al., 2005; Chipuk et al., 2004; Marchenko et al., 2000). The mechanism proposed involves cytoplasmic p53, held in check by associating with the anti-apoptotic Bcl-Xl or Bcl-2 and released upon stress when PUMA interferes with the interaction. Cytoplasmic p53 then activates Bax and Bak, resulting in mitochondrial membrane permeabilisation and apoptosis. The hypothesis couples the transacriptional and non-transcriptional modes since PUMA is a target gene of p53 transactivation. Even with more than 60 papers reporting transcriptionaly independent functions of p53, the field is still somewhat sceptical to these ideas, and some observations are in direct disagreement with them. In particular, overexpression of PUMA triggers cytochrome c release irrespective of the status of p53 – also p53-/- MEFs go into apoptosis (Kim et al., 2006). Deletion of the p53 response

element in the PUMA promoter has also been reported to abrogate apoptosis in DLD-1 and HCT116 cell-lines (Wang et al., 2007).

1.2.3 Transcriptional control by p53.

The major mechanism by which p53 orchestrates the response to stress is through transcriptional regulation of target genes from the diverse cellular pathways it controls. Sequence-specific DNA binding is mediated through the central "core" domain of tetrameric p53, which preferentially binds to palindromic pairs of the sequence RRRCWWGYYY, separated by a variable-length spacer of up to 13 bases (although global analysis of binding sites using Chromatin Immunoprecipitation have found a strong preference for none or a very short spacer (Wei et al., 2006)). p53 can act both as a transcriptional activator and repressor (Fig.2). The mechanism of transcriptional activation is not controversial – p53 binds a specific DNA sequence and recruits co-factors from the general transcriptional machinery (TATA-binding protein-associated factors, TAFs) to initiate transcription as well as chromatin-modifying factors such as histone acetyltransferases, p300 and PCAF (Farmer et al., 1996; Gu and Roeder, 1997; Gu et al., 1997; Thut et al., 1995). The mechanism of repression by p53, however, has been the subject of some dispute. It has been suggested to be an artifact, or to be a downstream effect of well-known activities such as p21 activation negatively regulating E2F1 activity (Lohr et al., 2003). Although this is certainly true for some genes, recent global binding data strongly argues against it being a dominant mechanism, since p53 is clearly found in the promoter region of repressed genes ((Wei et al., 2006), and Selivanova group, unpublished data). Other methods of repression are steric interference owing to overlapping binding sites – p53 has for example been shown to interfere with the binding of POU4F1 to the Bcl2 promoter and the binding of FOXA1 to the AFP promoter (Budhram-Mahadeo et al., 1999; Lee et al., 1999), and inactivation of transcriptional transactivators by binding and inactivating the actual proteins (not necessarily on the promoters) – examples include Sp1 activation of cyclin A2, TERT and IGF-1R and AP1 activation of MMP1 (Innocente and Lee, 2005; Kanaya et al., 2000; Ohlsson et al., 1998; Sun et al., 2004). Finally, the nature of complexes of p53 with other sequence specific DNA binding factors, where both bind to DNA and thereby stabilize the complex, might dictate the outcome of p53 activation (Riley et al., 2008).

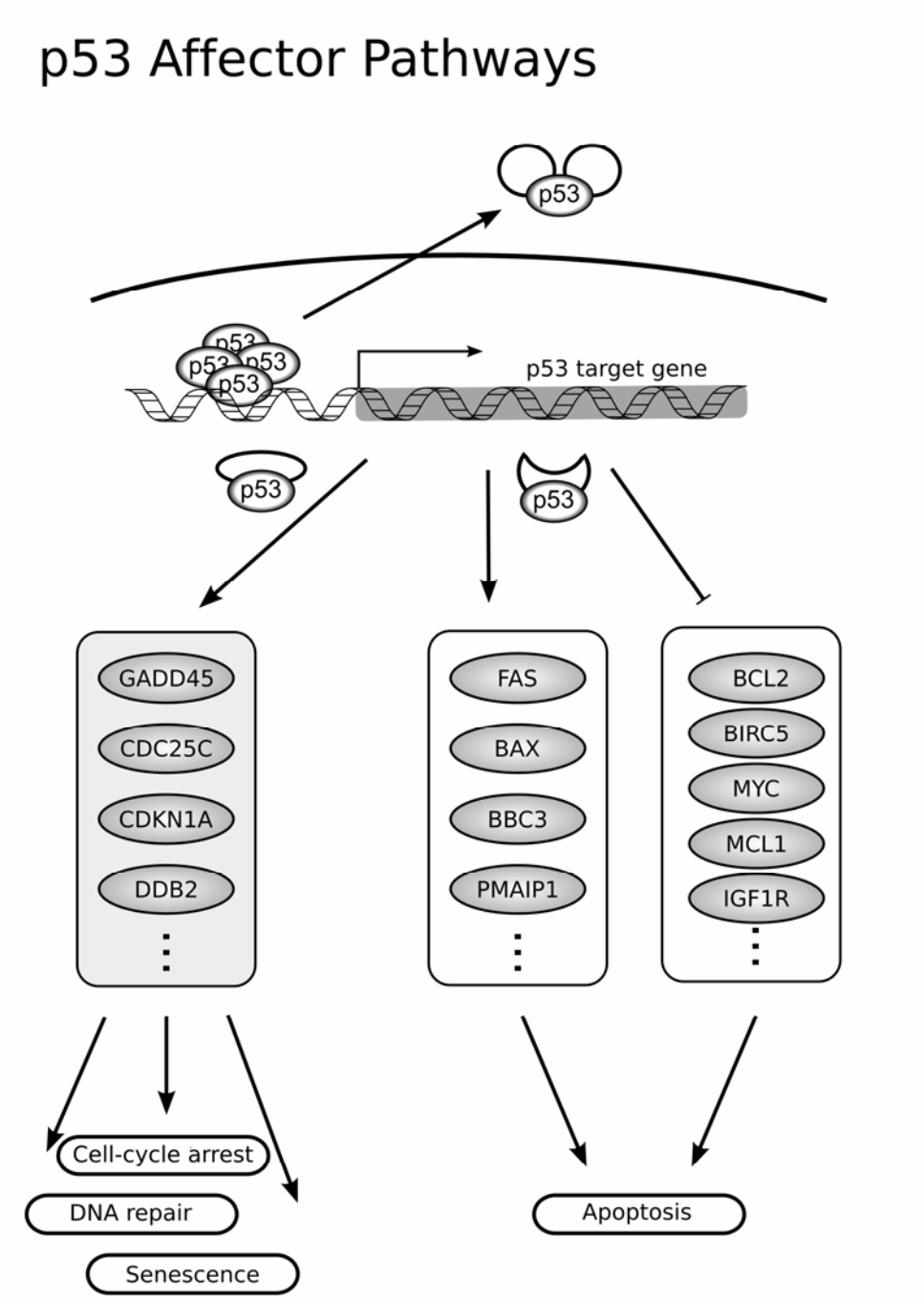


Figure 2. p53 affector pathways.
p53 binds DNA of the promoter regions of its targets as a tetramer. Several classes of p53 target genes can be discerned, such as cell-cycle arrest genes and pro-apoptotic genes, and the hoice of targets is dictated by which co-factors are in complex with p53 and which post-translational modifications are affecting p53. Genes related to the cell-cycle are mainly induced by p53 (left panel). Genes involved in apoptosis, however, are either induced (pro-apoptotic genes, middle panel) or repressed (right panel).

1.2.4 MDM2

Although the main tumor-promoting potential of MDM2 is clearly tied to its ability to inactivate p53, several lines of evidence indicate the existence of p53-independent functions of MDM2. MDM2 is overexpressed in human tumors and whereas concomitant mutation of p53 is very rare, underlining the importance of MDM2 as a p53 inhibitor, the patients where it is found have poorer prognosis than either alone

(Cordon-Cardo et al., 1994; Lu et al., 2002). Recently, MDM2 was also shown to modulate the p53 response by targeting several proteins involved in p53 pathway, as well as cofactors of p53 (Legube et al., 2002; Moumen et al., 2005; Rinaldo et al., 2007). The Moumen et al. study (Moumen et al., 2005) was followed-up in paper 2 of this thesis. Table 1 summarizes some of the binding partners and associated activities of MDM2.

Table. 1 Summary of MDM2-interacting molecules (adapted and extended from (Iwakuma and Lozano, 2003).

Protein	**Consequence of interaction**	**p53 dependency**	**Physiological Interaction measured**	**References**
p53	Inhibition and degradation of p53.	-	Yes	(Chen et al., 1993; Haines et al., 1994; Momand et al., 1992; Oliner et al., 1992)
p73	Inhibition of p73 without degradation.	No	No	(Balint et al., 1999; Dobbelstein et al., 1999; Ongkeko et al., 1999; Zeng et al., 1999)
p63	Stabilization and activation of p63.	No	No	(Calabro et al., 2002)
pRB	Suppression of pRB inhibition of E2F1.	No	Yes	(Hsieh et al., 1999; Xiao et al., 1995)
MTBP	Inhibition of MTBP-induced G1 arrest.	No	No	(Boyd et al., 2000)
PML	Inhibition of PML-dependent transactivation, nuclear exclusion.	No	No	(Wei et al., 2003)
E2F/DP1	Activation of E2F/D1 transactivation, inhibition of pro-apoptotic E2F activity.	No	Yes	(Loughran and La Thangue, 2000; Martin et al., 1995)
Sp1	Inhibition of Sp1 DNA binding.	No	Yes	(Guo et al., 2003; Johnson-Pais et al., 2001)
Numb	Altered localization and increased degradation of Numb.	?	No	(Juven-Gershon et al., 1998; Yogosawa et al., 2003)
AR	Increased ubiquitination and degradation by MDM2/Akt	?	Yes	(Lin et al., 2002)
p300/CBP	Disruption of the complex between p300 and p53.	No	Yes	(Gu and Roeder, 1997; Kobet et al., 2000; Wadgaonkar and Collins, 1999)
PCAF	Inhibition of PCAF-mediated p53 acetylation/activation.	?	No	(Jin et al., 2002)
HDAC	HDAC/MDM2 complex deacetylates p53, to induce degradation.	No	Yes	(Ito et al., 2002)
L5/RNA	May affect translation.	Yes	Yes	(Elenbaas et al., 1996; Guerra and Issinger, 1998; Marechal et al., 1994)
DNA pol. ε	Activation of DNA pol. ε activity	No	No	(Asahara et al., 2003; Vlatkovic et al., 2000)
TAFII250	Transcriptional activation of Cyclin A.	?	No	(Leveillard and Wasylyk, 1997;

				Wasylyk and Wasylyk, 2000)
TBP/TFIIE	Interaction with the small subunit of TBP/TFIIE might inhibit transcription.	?	No	(Thut et al., 1997)
hnRNP-K	Degradation of hnRNP-K causes deficiency in cell-cycle arrest, preferential induction of apoptosis.	No	Yes	(Enge et al., 2009; Moumen et al., 2005)
Nutlin	Inhibits binding between MDM2 and p53, activates p53.	No	Yes	(Vassilev et al., 2004)
ARF-peptide	p19ARF-derived peptide that mimics ARF-mediated inhibition of MDM2. Activates p53.	No	Yes	(Midgley et al., 2000)
p53-peptide	Peptide derived from the MDM2 binding site on p53. Activates p53.	No	Yes	(Chene et al., 2000)
p21	Proteasome-independent degradation.	No	Yes	(Zhang et al., 2003; Zhang et al., 2004)
HIPK2	Degradation, leading to deficient Ser46 acetytlation of p53 and repression of apoptosis (favoring cell-cycle arrest).	No	Yes	(Rinaldo et al., 2007)
Tip60	Proteasomal degradation of Tip60.	No	Yes	(Legube et al., 2002)

1.2.5 Pharmacological reactivation of p53

Inactivation of p53 overcomes several of the common obstacles for tumor development. Therefore it comes as no surprise that the TP53 gene is one of the most frequently mutated tumor suppressors in a wide array of cancers. In a recent exon-wide screen of mutations in breast and colon cancer, TP53 was the only gene which scored equally high in both malignancies at a level similar to the APC in colon cancer (Sjoblom et al., 2006). In fact, p53 has been found mutated in a significant fraction of most tumor types (Petitjean et al., 2007). In tumors that retain wild-type p53, it has often been shown to be inactivated by other means, such as inactivation and polyubiquitination by deregulated MDM2. It might well be the case that the p53 pathway is inactivated in all cancers. Clearly, inactivation of p53 represents a common trait in most tumor types and the p53 pathway is thus a part of the fundamental cellular machinery to prevent uncontrolled growth. Such a broad penetrance in human cancer not only proves the importance of p53 as a tumor suppressor, but also suggests that p53 might be a good candidate for targeted therapy.

Reactivation of mutant p53 has been attempted before, using antibodies, small peptides and small molecules with varying success (Bykov et al., 2002; Foster et al., 1999; Friedler et al., 2002; Hupp et al., 1995). Strategies to reactivate wild-type p53 have focused on disrupting the interaction between p53 and MDM2, its main destructor. There is clearly a potential for activation since antisense inhibition of MDM2 results in tumor inhibition *in vivo* (Wang et al., 2001; Wang et al., 1999), and since the binding interface between p53 and MDM2 is quite small and well defined - MDM2 binds to a

15-amino acid alpha helix at the N-terminus of p53 (Bottger et al., 1996; Picksley et al., 1994) several efforts have been made towards this goal (reviewed in (Zheleva et al., 2003)). The viability of using a pharmacological reactivation approach was demonstrated using peptides derived from either the MDM2-binding surface of p53 or from ARF, which were shown to disrupt the interaction and induce apoptosis in human cell lines (Bottger et al., 1997; Chene et al., 2000; Midgley et al., 2000). While disruption of the interaction is often achieved, most compounds have had modest effect in cells (reviewed in (Klein and Vassilev, 2004)). Nutlin, however, which binds to the p53 binding pocket of MDM2 (Fig.1, bottom panel), is active in the high-nanomolar range and has an antitumor effect in a mouse xenograft model (Vassilev et al., 2004). RITA (Fig.1, bottom panel), identified in paper 1, binds to p53 instead, and gives rise to a different type of p53 response (see paper 2).

1.3 MASSIVELY PARALLEL AND HYPOTHESIS-FREE METHODS

"Global" or "Genome-wide" analysis methods could be loosely defined as any method allowing the simultaneous evaluation of all (or at least a major part) of the observable hypotheses, which varies depending on the type of assay employed – for transcriptome-wide analysis it might mean simultaneously measuring all known transcripts. Most methods used in biological research are sequential in nature, i.e one hypothesis is tested at a time. This inevitably leads to almost exclusively hypothesis -driven research. While hypothesis-driven research has resulted in impressive advances of science, it introduces a bias simply owing to the choice of hypothesis selected for a study. Also, this type of research is very dependent on the accuracy of published material used to construct hypotheses, which has been called into question (Young et al., 2008). I have divided my discussion into two sections covering the methodology relevant to the work presented in this thesis. Thus, the present review does not attempt to fully summarize all "global" methods and many important parts are missing, most notably proteomic methods.

Of course, no method is truly hypothesis-free (and if such a method existed, it would probably be pointless anyway), and few methods are truly global. But these terms still represent something more than buzzwords for new techniques, because they represent an attitude and an endpoint to work towards. The most important contribution of the new massively parallel methods is that they offer a much less biased scientific method in areas where earlier no alternative to introducing a selection bias existed.

1.3.1 DNA microarrays

Although DNA arrays was introduced as early as in the seventies, as dot-blots and slot-blots (Kafatos et al., 1979), reaching 10.000 spots on a single membrane in the eighties owing to the introduction of robotics (Lennon and Lehrach, 1991), high-density array based methods as we know them today were introduced in the 90s. Two popular approaches are discernible (although features from the two have been mixed-and-matched by researchers) – two-color spotted cDNA arrays and oligonucleotide chips. Two-color arrays are typically long sequences (complete cDNA clones) spotted on glass slides and competitively hybridized with two samples labeled with different fluorophores. Oligonucleotide chips (pioneered by Affymetrix) are very high-density slides containing short sequences where the DNA sequence is exactly known. To obtain

precise control and high density, the arrays are produced with optical laser lithography (a similar technique to those used to manufacture miniature circuit boards), making in-house manufacture impossible. The spotted arrays, in contrast, are relatively simple to produce in-house – one of the main advantages of the method. The flip-side of the flexibility of spotted arrays, however, is that standardization and comparison of results between platforms is difficult. To many researchers (including the author) the difficulties in standardization and comparison with existing results deposited in public databases is the reason to ultimately favor oligonucleotide arrays when possible.

1.3.2 Massively Parallel DNA Sequencing

Even the most recent arrays, however, cannot capture the whole complexity of the genome or transcriptome. Estimates of the number of functional genes in the human genome suggest a number between 25.000 and 30.000 (Lander et al., 2001; Pennisi, 2003; Venter et al., 2001) – a low enough number for array-based methods. Recent estimates of the number of alternative spliced transcripts based on deep-sequencing data estimate that there are around 100.000 medium- to high-abundance transcripts in total, already too much for efficient detection by array-based methods (Pan et al., 2008). Further, alternative splicing might result in very similar transcripts, which is problematic when selecting sequences for array-based methods, and out of the 100.000 transcripts many are expressed at quite low level, putting additional stress on the dynamic range of detection methods.

Fortunately, a new class of unbiased methods has been developed that promises to better handle these shortcomings. Massively parallel DNA sequencing (MPS, or simply "mass-sequencing") was introduced as late as 2005 with the first 454 sequencer. Currently, four different MPS platforms are available from different companies, the most widely used being 454 sequencing and Illumina sequencing. The common technological feature is to sequence a huge number of DNA fragments that are spatially separated in a flow cell. The 454 platform uses PCR amplification of individual transcripts in emulsions of water in oil, where each droplet is deposited in its own well in a "picotiter plate" flowcell containing up to 3.4E6 wells. Then, many rounds of elongation is performed using luminescent dNTP bases – after each stage the luminescence is recorded and translated into sequence output. 454 sequencing enables the collection of around 1E6 sequence reads of up to 400 bases. In Illumina sequencing the picotiter plate is replaced with a glass slide coated with oligonucleotide anchors. DNA to be sequenced is applied to the surface and allowed to hybridize to the anchors, and a first round of sequencing is performed. Then, amplification is performed by the replicated (attached) sequence "bridging" over to adjacent anchors in successive rounds of replication forming a "cluster" of replicated sequences around the original one. Illumina sequencing produces many more reads than 454 sequencing (up to 50E6 reads), and has considerably lower cost. The read length is less than 50 bases making whole-genome sequencing and assembly inefficient. For experiments where the composition of a selection of sequences from a known genome is under analysis (for example when sequencing chromatin immunoprecipitated together with a transcription factor), the method is ideal – especially when strategies to amplify the ends of a sequence "paired-end sequencing" are used (Campbell et al., 2008; Korbel et al., 2007). The remaining platforms are SOLiD, where the amplification is similar to 454 but the

sequencing interrogates dinucleotide combinations according to a more complicated scheme, and HELICOS, which promises single-molecule sequencing without amplification. Interestingly, the Church lab where SOLiD originates also offer do-it-yourself mass-sequencing (Shendure et al., 2005), http://www.polonator.org.

1.3.3 Cell-based screening methods for hypothesis-free discovery.

The second major part of massively parallel methods is an even more heterogenous group of screening methods. The basic premise, again, is parallelism - to cull out factors that are important in a process under investigation, from a large collection (library) of factors. If selection is efficient we can increase the size of the library to a global scale. Early methods used cDNA libraries packaged into phage particles, which allows a big variety of screenings for DNA or protein binding. Using this family of methods allowed the identification of among others the scrapie gene (Li et al., 1995; Oesch et al., 1985). Similarly, yeast cells can be used as hosts for cDNAs for different forms of screening procedures like yeast two-hybrid screens for protein interactions (Fields and Song, 1989).

In human cells, functional screening where the "factors" are exogenous genetic material expressed under the control of a constitutively active promoter has been particularly successful. DNA is introduced either by transfection or, more recently, by retroviral transduction. If the screen is constructed in a way so that interesting factors will confer a growth advantage, a mixed library can be used. The "interesting" DNA will be enriched in the population and its identity can be determined by sequencing single clones or measuring enrichment by microarray, analogous to differential expression. For experiments where the outcome is not translatable into a growth advantage arrayed libraries are usually used. Factors are investigated individually, but in parallel, and when the readout can be automated high-enough throughput can be achieved. For example, activators of a known transcription factor can be discovered by using a reporter plasmid harboring transcriptional regulatory sequences of that factor in combination with an arrayed library. The reporter plasmid readout is some bioluminescence marker such as luciferase which can be analyzed in a high-throughput manner, see for example Irelan et al. (Irelan et al., 2009). Recently, loss-of-function screens have been made easier by the introduction of siRNA – based protocols, which in addition allows for massively parallel screening of under arguably more physiological conditions than overexpression screens. Using state-of-the art retroviral transduction of siRNA combined with mass sequencing and array-CGH has even allowed for negative screening for factors that confer growth disadvantage (Zender et al., 2008).

Chemical libraries screens for small-molecule inhibitors or binding factors of a known target are traditionally made under very controlled conditions using purified proteins *in vitro*. We and others, however, have used cell-based methods also for screens of chemical libraries (Bykov et al., 2002; Torrance et al., 2001). The rationale is to move the conditions of the screen closer to the physiological conditions under which the chemicals are expected to work. Indeed, cell-based screens do provide some very attractive features; for example, molecules that do not cross the plasma membrane are

immediately discarded, as well as molecules that display general toxicity to cells. Chemical screens using isogenic cell-lines differing in a gene of interest can be used to select target-specific factors when the readout is easy to automate, such as with a growth-suppression assay (Gudkov and Komarova, 2003; Gudkov, 2004). The main drawback is that the specificity is purely functional, there is no selection for actual binding to the target. Also, given the complexity of the cell there is no guarantee that the selected factors are actually the primary targets. Therefore extensive experimentation is required to verify the results. On the other hand, screens based on binding strength disregard this complexity to an even greater extent since only binding to the intended target is explored while small-molecule inhibitors usually have multiple targets. In a cell-based screening, we do not know if the intended target is directly affected, but we have a much stronger indication that this target is *functionally* affected. Also, in many cases the endpoint that is measured can be more meaningful – for example in the case of p53 activation we directly measure the desired effect, which is growth suppression, rather than some intermediate effect (such as dissociation of p53 and MDM2 or transcriptional activation).

1.3.4 Hypothesis testing in massively parallel experiments.

With genome-wide methods now accessible to any research laboratory, the field of biology is going through a major technological revolution. While acquisition of the data is now cheap and relatively easy, interpretation still presents considerable difficulties. The following discussion mainly concerns array data, but many principles apply to any massively parallel experiment. First, the simple fact that we move from testing only a few hypotheses to testing tens of thousands creates a huge multiple hypothesis testing problem. The problem is often overlooked by researchers not mathematically inclined, but at its core it is very simple and intuitive. *Any* method occasionally produces false-positive results. If the rate of false-positive discovery is, say, 1 in 1000, you would hardly need to think to take the possibility into account if performing a single experiment. If you were to conduct 20.000 experiments, however, you would on average find 20 false positives and be practically guaranteed to find at least around ten ($p \sim 5E\text{-}3$). The significance estimates can be adjusted to account for multiple hypothesis testing with for example Bonferroni or Holm-Bonferroni correction, but they are usually too conservative for microarray experiments with few replicates. The problem is compounded by the fact that genome-wide methods are still quite expensive so replicates are usually kept to a minimum, and because the data quality is not always as easy to control as in traditional methods (for example see (Dai et al., 2005)). None of the traditional multiple hypothesis testing adjustments can account for the combination of lack of replicates and large number of hypothesis under testing.

To ameliorate the problem an improved class of statistics was developed (Smyth, 2004; Tusher et al., 2001). These methods are designed for DNA microarray experiments and all take advantage of the parallel nature of the experiments using an empirical bayes approach, although not all authors explicitly use bayesian terminology. Thus, a prior describing the expected parameters (such as standard deviation, degrees of freedom) is constructed from the entire set of genes, and is then used together with the usual per-gene estimation to obtain the marginal parameters. For the two-sample case (chosen for

illustration), these methods are similar to a modified t-statistic $T = \frac{x - y}{s + s_0}$ where x and y are the log2 expression levels in the samples, s is the standard deviation, and s0 is a constant (usually small) value which can be either arbitrarily chosen or derived from data as above. This has the important consequence that for small values of s or large values of s0, the statistic T approaches a measure of the fold-change. Thus the statistic can be seen as a mix between a traditional t statistic and a fold-change, making it more relevant for biological interpretation where minute differences might not be of interest.

Together with better methods to obtain accurate measures of the statistical significance of findings – including ways to calculate false discovery rate in a manner that is statistically sound but not as conservative as traditional methods (Qian and Huang, 2005), this type of approach has made inferences about differential expression stable enough for everyday use and has been instrumental in the success of chip-based methods.

1.3.5 Data, Information and knowledge.

Even with statistical methods that properly stabilize the gene-wise error rate, interpreting the resulting gene-lists presents a significant challenge. A typical microarray experiment will result in hundreds of clearly differentially expressed genes and thousands of genes that are weakly significant. This is clearly too much information to handle. An efficient researcher might investigate all the clearly significant genes, whereas an unscrupulous researcher would pick his way through the list of thousands of genes. Obviously neither approach is ideal.

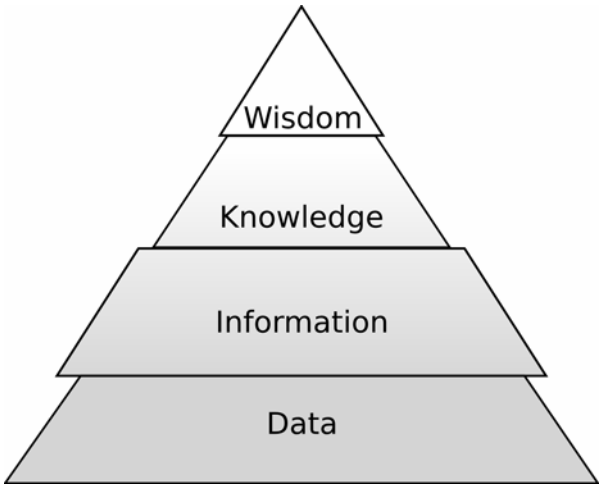


Figure 3: The Wisdom hierarchy.
Higher abstractions are further up in the hierarchy, and includes the levels below them.

The fundamental problem is not only one of scale, but also of the type of information provided. Within the research fields of information management and information systems, related problems are commonly discussed within the context of the DIKW (Data, Information, Knowledge, Wisdom) hierarchy (figure 3), usually attributed to Russel Ackoff (Russel, 1989), reviewed in (Rowley, 2007). The metaphor of a pyramid suggests that the higher levels are more abstract or refined and each level includes all the categories below it. At the base of the hierarchy is data, which are the raw observations – useless on their own. The next level, information, is inferred from data and can be thought of as answers to simple questions (who? what? when?) or descriptions. Knowledge goes beyond the descriptive and includes contextual information, rules – while data and information both are properties of things, knowledge is a property of the people who study them, "know-how". Wisdom, finally, is probably the most weakly defined, and least widely discussed (information systems do not deal with wisdom) but represents the highest level of abstraction.

Now let us transpose these ideas to microarray data. At first glance the raw expression values are clearly data, and per-gene hypothesis testing provides information. In many cases, however, one could argue that the per-gene hypothesis is more similar to data than information. Especially when considering the big amounts of marginally significant genes that usually heavily outnumber the clear cases. This is important on a conceptual level: when we want to refine information into knowledge, we need to decide on a hypothesis and then investigate it fully, because otherwise it is still information, not knowledge (and good journals do not publish information). This means that the "hypothesis-free" domain extends to the per-gene hypothesis testing, but no further – however the level of abstraction is still too low to make an "undisputed" or "impartial" decision about which direction to take in further investigation. What started out as well-behaved unbiased experiment turns out to be open to heavy selection bias.

1.3.6 Using prior knowledge to interpret massively parallel experiments.

The problem of raising the level of abstraction is actually a widely recognized one and many solutions have been proposed. The goal is to raise the level of abstraction to "information" or "knowledge" using an unbiased method. A popular way to do this is to take information about which genes are already known to belong together in functional categories, and then test for differential regulation of these groups instead of individual genes. Since the categories are created according to biologically relevant functions, they readily meet the criteria of information (who? what? when?) and some might even be said to hover in between information and knowledge. For example, the Gene Ontology project collects a huge collection of hierarchically organized categories, such as "induction of apoptosis" or "Nitric Oxide metabolism" (Ashburner et al., 2000). If we know one of these is highly differentially regulated, we are already quite close to be able to formulate a testable hypothesis. Moving to the level of categories also solves the scale problem, since typically the huge number of genes is distilled into a fairly small number of categories.

This type of procedure, sometimes called "pathway analysis" has gained momentum with the increasing popularity of microarray analysis. Simple methods usually compare the list of differentially expressed genes to lists of each of the categories, looking for over-representation using some test such as binomial, hypergeometric or fisher tests (see for example (Beissbarth, 2006; Curtis et al., 2005)). The procedures disregard the strength of the evidence and reduce it to a binary outcome, discarding a big part of the information content from the original data.

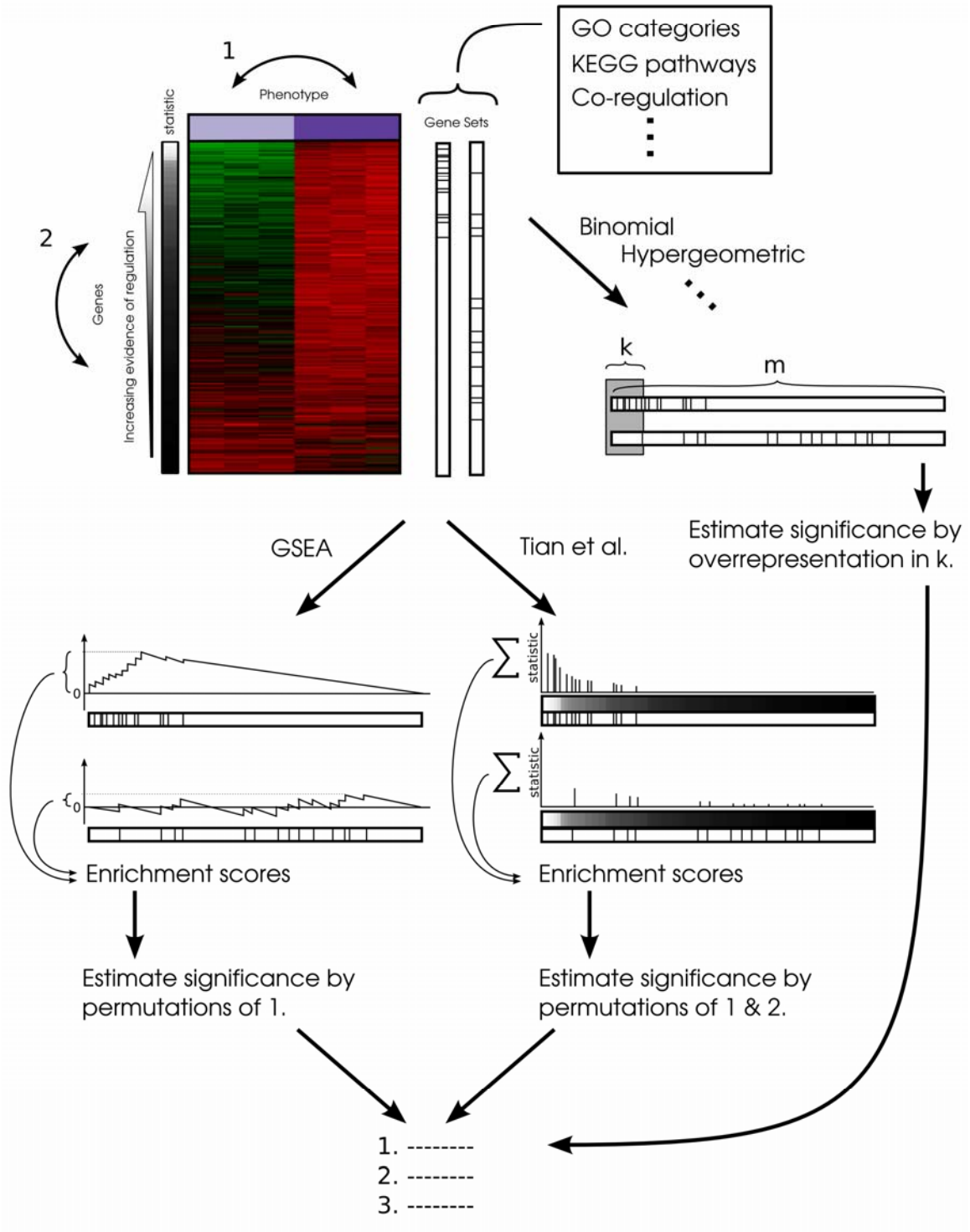
Figure 4. Summary of pathway analysis methods.
We start from a microarray experiment (shown as a red-green heatmap, center, with each row representing a gene and each column an array), with associated phenotype (bar above heatmap with shades of blue – two classes), and statistics for differential regulation (shades of grey in the vertical bar left, with lighter colors representing higher/better statistics). Gene sets can be taken from diverse sources, and are shown as black-white bar codes, where black signifies that the corresponding gene is part of the set.

Basic approaches like binomial or hypergeometric tests simply use a group of genes deemed significant according to some criteria like a p-value cutoff (area under shaded box), and test whether genes from each gene set is enriched within this group.

Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) uses a non-parametric test where a running score is computed on the sorted list of genes, adding to the score if the gene is in the group and subtracting if it is not (the amount added/subtracted is weighted by the size of the group). The enrichment score is the maximum deviation from zero of the running sum. Statistical significance is computed by comparing the score to an empirical distribution of scores constructed by permutations of phenotype (1).

The approach of Tian et al (Tian et al., 2005) uses the sum of the raw statistics for the genes in a set as the enrichment score. Statistical significance is estimated by permutations of 1 and 2, and the final list is ranked by the sum of ranks.

Pathway analysis methods



A more recent approach is Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005), which uses a Kolmogorov-Smirnov statistic on the ordered list of genes as the "enrichment score", thereby conserving the strength of evidence as a rank within the list of all investigated genes (Fig.4). Thus, the genes are ordered according to decreasing evidence of differential regulation and for each category under study a running sum is calculated, starting at zero, by incrementing if the next gene is part of the category, and decrementing if it is not. The enrichment score is the maximum deviation from zero, corresponding to a Kolmogorov-Smirnov statistic. Significance is assessed by constructing an empirical null distribution by permutation of the class labels of arrays.

Another approach by Tian et al. (Tian et al., 2005) addresses two potential shortcomings of GSEA. First, the power of the non-parametric Kolmogorov-Smirnov statistic is quite low, requiring more data than a test that makes some distributional assumptions. Second, permutation of class labels of arrays to assess significance can sometimes be problematic when the patterns of gene expression are highly correlated, i.e when a big part of the interrogated genes are differentially expressed (Figure.4). Strictly speaking, this null distribution addresses the question of whether the category being examined contains differentially regulated genes, not whether it does so to a higher extent than random sets. Tian et al., therefore propose to use the sum of the modified t-statistics as an "enrichment score" and then estimate significance twice, both by random permutations of class-labels *and* gene-labels. The pathways are then ranked according to each significance assessment, with the final rank being the sum of ranks (Tian et al., 2005).

One fundamental problem with these knowledge-based methods is that the categories usually are collected from data gathered using sequential discovery. Therefore they suffer from the same shortcomings as these – the collection will be biased towards certain topics, and the accuracy of each source can be radically different (a consistent measure of noise or quality is not possible). Common solutions to this problem involve using unbiased measures to create groups, such as co-regulation or recurring motifs (Subramanian et al., 2005; Xie et al., 2005). Such solutions, however, might remove the bias but in return sacrifice ease of interpretation, since these categories usually do not correspond to a specific hypothesis. Since improved interpretability was one of our initial goals, another solution is needed.

1.3.7 Promoter analysis.

Transcription factors (TFs) bind DNA in a sequence-specific manner, dictated by the protein structure of the binding interface on the TF. The binding specificity (binding motif) can be determined by a multitude of methods. Common methods are to first locate binding sites either on chromatin (Elnitski et al., 2006) or by in vitro selection (SELEX) (Klug and Famulok, 1994), followed by alignment of the retrieved sequences and recovery of the motif by computational means (e.g Gibbs sampling). In this way we can obtain a motif in the form of a Positional Weight Matrix (PWM), a matrix of scores for each base at each position in the motif. To score a PWM against a substring (subsequence) s of DNA, both of the same length, one simply sums up the scores of the bases of s at each position. To put it another way, s is a string of length N and m is the motif – a matrix with N columns and 4 rows (corresponding to the four bases). Then the final score is defined as $\sum_{j=1}^{N} m_{s_j,\,j}$, where j is the index of the substring, s_j is the base in the substring at position j, and $m_{a,j}$ is the score of the PWM at row a, column j. The scores usually represent either a log-likelihood, or log odds (if a generative model is used). Sometimes the scores are interpreted as physical binding energies of the individual bases. For searching sequences for binding sites, a log-odds model is the most appropriate, at least theoretically.

Much research has been devoted to obtaining PWMs for Transcription factors, and large collections are easily accessible in the form of databases such as Transfac (Matys et al., 2006) and Jaspar (Bryne et al., 2008). Therefore, an attractive alternative to the pathway analysis approaches outlined above is to use PWMs to search the promoters of differentially regulated genes. The pathways deal with the activity of proteins and how they functionally interact; in order to use transcriptome data to test hypothesis about protein networks one has to make quite a few assumptions, some of which are essentially always false. For example, due to differences in translation/degradation of the protein the raw amounts of proteins in a cell are not necessarily correlated to mRNA (p53 is a good example), and even if they are, post-translational modifications are often more important to the activity of a protein than the amount (for example, the Akt serine/threonine kinase, a key signaling factor in cancer and many other processes, is activated by phosphorylation, reviewed in (Franke, 2008). In comparison, transcriptional activation seems more closely tied to the levels of mRNA because of the functional link between TF binding and modulation of mRNA production. The idea here is to use PWMs to search the promoters of co-regulated genes for binding sites that occur more frequently than expected by chance, thereby inferring which TFs are likely to be responsible for the transcriptional changes. The hypothesis testing is analogous to pathway analysis, so the same methods can be used.

The main problem at the conceptual level is how to handle processes that circumvent the simple rules of TF activation/repression, such as promoter methylation and epigenetic changes in general. At a more practical level, the sheer size of the promoters present a problem – enhancers can be located several tens of thousands of bases away from transcription start site (Hallikas et al., 2006; Lavenu et al., 1994). Several approaches for prediction of functional binding sites have been proposed, either by concentrating on the promoter regions proximal to the transcript (Suzuki et al., 2004;

Xie et al., 2005) or by focusing on phylogenetically conserved clusters of binding sites (Hallikas et al., 2006).

2 RESULTS

The thesis is based on three papers, exploring different aspects of the p53 pathway. Although they span quite a wide methodological space they are connected by the use and advancement of global or hypothesis-free methods. In paper one we use a cell-based screen to identify a novel wild-type p53-reactivating compound, RITA. In paper two, we use oligonucleotide microarray followed by pathway analysis to establish that RITA is indeed p53 specific, and furthermore it elicits a focused apoptotic response. We propose a hypothesis for the selective induction of apoptosis. In paper three, we introduce a novel method of promoter analysis that amalgamates modern methods of pathway analysis with an advanced model of transcriptional regulation. We apply the method to cell cycle re-entry and to the p53 response.

The aims of the thesis were

- To discover wild-type p53 reactivating compounds.
- To dissect the molecular mechanisms of p53 activation by these compounds.
- To use these compounds to advance the understanding of p53 biology.
- To advance the methodology for dealing with the analysis of microarray data, from the perspective of transcriptional regulation and especially with respect p53 as a transcription factor.

2.1 SMALL MOLECULE RITA BINDS TO P53, BLOCKS P53–HDM-2 INTERACTION AND ACTIVATES P53 FUNCTION IN TUMORS.

In paper one we report the identification of a small, cell-permeable and target-specific chemical ligand, which can re-activate the tumor-suppressive function of wt p53. We used a screening assay based on a pair of isogenic cell-lines, HCT116 colon carcinoma cell line and HCT116 TP53-/-, its isogenic derivative where p53 has been knocked-out by homologous recombination. The selected compound 2,5-bis(5-hydroxymethyl-2-thienyl)furan (NSC652287) was dubbed RITA (Reactivation of p53 and Induction of Tumor cell apoptosis). RITA induced massive apoptosis in p53-containing cells in a dose-dependent manner, with almost no effects on p53 null cells.

We observed disruption of p53/MDM2 interaction in tumor cells grown in culture, *in vitro* using purified proteins and *in vivo* in a mouse xenograft model. Taking advantage of the natural fluorescent properties of RITA, we used Fluorescence Correlation Spectroscopy (FCS) to directly observe the association of RITA to p53. Consequently, we detected the inhibition of ubiquitination of p53 in cell culture and xenografts followed by p53 stabilization with an increase of the half-life of the protein. The binding site of RITA was mapped to within the N-terminal transactivation domain. Interestingly, we also observed dissociation of other negative regulators of p53, iASPP and PARC, neither of which bind to the N-terminal domain and suggesting that RITA binding induces a conformational shift in p53 that affects the overall folding of the protein.

RITA stabilized p53 and suppressed the growth of a panel of tumor cell lines harboring wt p53. In a panel of cell lines with known p53 status, RITA showed strong p53-dependency. The p53 transcriptional response was restored, with induction of p53 reporter activity and strong induction of p53 apoptotic genes. p21 was induced, but to a lower degree than initially expected. This aspect was further explored in paper number two.

Importantly, while we observed disruption of the p53/MDM2 complex in human diploid fibroblasts and lymphoblasts, similar to in transformed cells, induction of p53 was only transient and the growth suppressive effect was very weak. Overexpression of the Myc oncogene, however, sensitized the cells to RITA. Also, although no formal toxicity study was performed, mice treated with high dose (10mg/kg) of RITA had no weight loss. This is important since a major concern of using RITA or any other wild-type p53-reactivating compounds in the clinic is unspecific toxicity elicited by activation of p53 in normal cells.

In a follow-up study, we used substitution and truncation mutants in a binding assay with C-14 labeled RITA to map precisely the binding site of RITA to amino acids 32-37 of p53. In the same study, we determine that p53 is the major RITA-binding protein in cells grown in culture (Zawacka-Pankau et al., submitted).

The main findings in paper one are:

- RITA, a low-molecular-weight compound, suppresses tumor cell proliferation in a p53-dependent manner.
- RITA binds to p53 and disrupts the interaction between p53 and its main destructor, MDM2.
- RITA re-activates transcriptional activity by p53 and induces potent apoptosis in a p53-dependent manner.
- Growth suppression was also observed in a mouse xenograft model, without any apparent toxicity.

2.2 MDM2-DEPENDENT DOWNREGULATION OF P21 AND HNRNP K PROVIDES A SWITCH BETWEEN APOPTOSIS AND GROWTH ARREST INDUCED BY PHARMACOLOGICALLY ACTIVATED P53.

In paper two we further investigated the mechanism of action of RITA-reactivated p53. We performed oligonucleotide microarray analysis on wt-p53 and p53-null isogenic cell-lines treated with RITA, showing massive changes in gene expression in p53-carrying cells but little or no transcriptional effect on p53-null cells. Furthermore, we found a strong preference for apoptosis-associated genes upon RITA treatment, while the cell-cycle arrest associated genes were not significantly affected. A comparison of the transcriptional profile of RITA to that of known p53-dependent stresses corroborated the observation – H_2O_2, Hypoxia, Hydroxyurea and Nitric oxide all

induced cell-cycle arrest genes to a greater extent than pro-apoptotic genes. Although 5-Fluorouracil (5-FU) treatment induced pro-apoptotic genes to a greater extent than cell-cycle arrest genes, both categories were potently induced. However, RITA treatment failed to induce cell-cycle arrest genes almost completely. Also, comparison of genes affected by the p53-reactivator nutlin, which binds the p53 binding pocket of MDM2 disrupting its complex with p53, indicated that nutlin also induces mainly cell-cycle arrest genes.

We hypothesized that the difference in response could be caused by differential activity of a transcriptional cofactor of p53. Further, in spite of the similar mechanism of action of nutlin and RITA (disruption of p53/MDM2 complex) the type of p53 response is different, i.e., induction of growth arrest and apoptosis, respectively. This prompted us to explore the mechanistic difference between the two. Although both target the p53/MDM2 interface, they actually target different proteins – RITA binds to p53, whereas nutlin binds to MDM2. Thus, we investigated the effect of both compounds on hnRNP-K - a transcriptional cofactor of p53 that leads to the induction of cell-cycle arrest genes and that is a target of MDM2 E3-ligase activity.

hnRNP K was indeed down-regulated upon RITA treatment, which was reversed by neutralizing MDM2 through overexpression of a dominant-negative MDM2 mutant or by expression of siRNA specific for MDM2. In line with this being an effect of E3 ligase activity of MDM2, an increase in the direct interaction of MDM2 and hnRNP K was observed. Furthermore, the effect was independent of p53, since reconstitution of MDM2 in MDM2-/-;TP53-/- cells also lead to reduction of hnRNP K protein levels. Using Chromatin Immunoprecipitation (ChIP) we established that while p53 binding to the promoters of both cell-cycle arrest gene p21 and pro-apoptotic gene PMAIP1 (Noxa) was increased to the same extent upon RITA and nutlin treatment, hnRNP K binding was increased on the p21 promoter exclusively upon nutlin treatment. Consequently, induction of p21 mRNA level was impaired after RITA treatment.

However, p21 protein level was more severely affected than the mRNA levels, actually showing a reduction upon RITA treatment. Also, p21 half-life was markedly reduced and inhibition of the proteasome partially rescued this reduction. Since p21 has previously been implicated as an anti-apoptotic factor, we investigated the impact of p21 on the apoptotic response by RITA and nutlin. Indeed, removing p21 by either siRNA or homologous recombination increased the apoptosis induction by both RITA and nutlin. In a long-term growth suppression assay, however, growth suppression by nutlin was impaired after removing p21, indicating that inhibition of proliferation by p21 has a major contribution to growth suppression by nutlin (in agreement with earlier studies). Importantly, knock-down of MDM2 did not enhance apoptotic response by RITA, whereas concomitant knock-down of MDM2 and p21 enhanced apoptosis in a synergistic way.

The main findings in paper two are:

- In the model system used, RITA is entirely p53-specific.
- The transcriptional profile upon RITA treatment involves induction of apoptosis genes, with weak induction of growth-arrest genes.
- hnRNP-K, a transcriptional cofactor of p53, is degraded upon RITA treatment

in an MDM2-dependent manner and acts as an outcome-specific factor .
- Upon RITA treatment, p21 protein is antagonized on both the transcriptional and protein levels.
- p21 acts as an anti-apoptotic factor (in agreement with earlier studies) and dampens the response to RITA.
- In spite of its anti-apoptotic activity, long-term growth suppression upon treatment with nutlin is dependent on p21, indicating that cell-cycle arrest plays a major role in the functional mechanism of action of nutlin.

2.3 REVERSE-ENGINEERING MICROARRAY DATA APPLIED TO THE P53 TRANSCRIPTIONAL RESPONSE.

In paper three, we report a method to analyze microarray data with respect to analysis of the promoters of the investigated genes, called revarray (from *rev*erse-engineering micro*array*s). Contrary to earlier methods revarray uses the actual statistics of differential expression in conjunction with transcription factors binding site data. Thus, both clearly differentially regulated genes and mores subtle effects on gene expression are captured. Revarray predicts a model of transcriptional regulation as a set containing Transcription Factors (TFs) and clusters of adjacent TFs with an associated mode – activation of transcription, repression of transcription or general enhancer activity (either activating or repressing). Statistical significance is estimated using a permutation-based approach, which takes the modes into account. Model optimization is achieved through a genetic algorithm, resulting in fast convergence.

We first used simulated data to asses the robustness of the method in a controlled setting. We determined the robustness to noise ratio in the form of increasing false-positive binding sites to be excellent, with good success rates even when the true positive sites constitute less than 10% of the total number. The method also showed good properties with high numbers of simulated non-functional TFs.

Thereafter, we tested the method on a dataset of cells re-entering the cell cycle. We predicted binding sites using 210 Positional Weight Matrices (PWMs) from the Transfac database, containing all known vertebrate transcription factors. The output predicts activation of among others E2F, EGR1 and NFKB, and repression of CEBP-α, all in agreement with current knowledge in the field.

Finally, we applied the revarr procedure to cells upon reactivation of p53. Again, we used the full set of vertebrate PWMs from Transfac to predict TF binding sites. To overcome the problems of inaccurate binding site predictions caused by the highly degenerate p53 PWM, we used *in vivo* ChIP binding data on p53/DNA interactions upon 5-FU treatment quantified by mass sequencing (ChIP-seq) to accurately determine the binding sites of p53. We analyzed oligonucleotide microarray data from cells treated with RITA, nutlin and 5-FU. Taking advantage of our background knowledge of the experiment, we used revarray in semi-supervised mode, specifically looking for regulatory complexes with p53. Our analysis predicts p53 to form regulatory complexes with members of the FOX family of transcription factors. Also, the analysis suggests the existence of different “modes” of p53 binding. Low-amplitude peaks from ChIP-seq experiment tend to correspond to repressed genes, whereas high-amplitude peaks tend to correspond to activated genes. Interestingly,

repressor complexes were predicted as part of the transcriptional model for the apoptosis-inducing treatments 5-FU and RITA, but for not for nutlin. This latter finding is in agreement with our previous data which indicates that down-regulation of genes belonging to oncogenic pathways is necessary for the induction of apoptosis (Grinkevich et al., 2009). More research is needed to validate the results.

The main findings in paper three are:

- Revarray is a novel method for determining the nature of the transcriptional response by "reverse-engineering" microarray data together with transcription factors binding sites in the promoters of investigated genes.
- The procedure uses a statistic that takes into account both clearly differentially regulated genes and subtle changes in gene expression.
- Revarray was validated on simulated data and on data of cells re-entering the cell-cycle.
- The p53 response upon treatment with 5-FU, RITA and nutlin was analyzed, suggesting that:
  + p53 is active as a single factor upon nutlin treatment.
  + Repression of genes is associated with apoptosis induction.
  + One or more members of the FOX family of transcription factors act in complex with p53.

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