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How p53 wields the scales of fate

Arrest or death?

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Abbreviations: EGFP, enhanced green fluorescent protein; PUMA, p53-upregulated modulator of apoptosis; ASPP, apoptosis-stimulating proteins of p53; ChIP, chromatin immunoprecipitation; CTCF, CCCTC-binding factor; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; FACT, FAcilitates Chromatin Transcription; HPRT, hypoxanthine phosphoribosyltransferase; HZF, hematopoietic zinc-finger; TAD, transactivation domain

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Understanding how the tumor suppressor p53 induces cell cycle arrest or apoptosis is critical for developing chemotherapeutic strategies. We have generated targeted transgenic reporter mice with which we can study p53 activity at specific promoters, and propose a model in which p53 protein conformation is key to target gene selection.

The p53 pathway is inactivated in many cancers, thereby disabling an important signaling node that is crucial for the preservation of genomic integrity. p53 protects cells from damage by mediating a reversible growth arrest to allow repair of said damage, or by inducing senescence or apoptosis. Given its critical role, p53 is often targeted in cancer therapy, which seeks to eliminate tumor cells.[1] But to effectively harness its tumor suppressive power, one must understand how p53 determines the fate of cells in which it has been activated.

p53 is a transcription factor whose levels are usually suppressed. When a cell is subject to stress, such as DNA damage or oncogene activation, p53 protein accumulates, is activated and regulates the expression of its target genes. Depending on the transcription program induced, the cell then undergoes cell cycle arrest, senescence or apoptosis, all with the ultimate aim of tumor suppression.[1] Notable p53 target genes include the cyclin-dependent kinase inhibitor *p21*, which plays a significant role in blocking cell proliferation,[2] and *PUMA* (p53-upregulated modulator of apoptosis), which is crucial for p53-induced cell death.[3]

The response to p53 activation varies depending on the cell type and the stimulus used.[4,5] For instance, treating mice with ionizing radiation causes extensive p53-dependent apoptosis in tissues such as the small intestine, spleen and thymus, but not in the liver,[6] while microarray analysis of tissue culture cells revealed that different stimuli induce distinct sets of p53 target genes.[5] These cell- and drug-specific responses must be characterized and considered during the design of chemotherapeutic regimens, in order to maximize efficacy while minimizing toxic side effects.

Despite extensive effort, the processes driving these cell fate decisions are still not well understood. Many different research groups have employed a wide variety of approaches to address this question, including structural studies of p53 itself, in vitro biophysical assays, and experiments with tissue culture cells. We have recently added to this toolbox, by generating targeted transgenic reporter mice that enable us to monitor p53 activity at specific p53 response elements.[7]

Using Targeted Transgenic Reporter Mice to Study p53 Target Gene Selection

We used targeted transgenesis to generate reporter mice in which enhanced green fluorescence protein (EGFP) provides a direct readout of p53 transcriptional activity. The *Egfp* gene is downstream of a Δ*fos* minimal promoter and a single p53 response element from either the *p21* or *PUMA* promoter. EGFP expression should thus be driven solely by p53 binding to this response element. We performed in vitro experiments to demonstrate that EGFP expression correlates

with p53 activity in a concentration-dependent manner.[7]

One copy of each reporter construct was integrated into the locus of the housekeeping gene hypoxanthine phosphoribosyltransferase (*Hprt*), which is permissive for gene targeting and in a region of transcriptionally accessible chromatin. This stable integration ensures that these mice can be bred without the reporter becoming epigenetically silenced, which may have been a problem for other transgenic reporter mice.[8-10] The key difference between our two strains of reporter mice is the sequence of the p53 response element used in the reporter construct. One can thus directly compare p53 activity at these two promoters. Using the reporter mice, we could detect and quantify variations in the p53 response that were dependent on cell type and stimulus.[7] Our data are consistent with previous reports[4,6,8,10] and validate the use of these mice as a model to study p53 activity. Using EGFP as a reporter allows us to exploit advances in microscope technology. For example, confocal microscopy analysis of embryos from our reporter mice showed that p53 is active during early embryonic development, including in the blastocyst cells from which embryonic stem cells are derived.[7]

To demonstrate that EGFP expression is p53-specific, we generated p53-null reporter mice by mating the reporter mice with p53-knockout mice.[7] Even though we adopted a reductionist approach that should minimize the contribution of other factors affecting p53 target gene selection, given cell- and tissue-specific differences, it is important to verify that the EGFP expression observed in one's experimental system of interest is indeed p53-dependent.

Studying p53 response elements in the context of reporter constructs allows us to focus on how p53 itself contributes to target gene expression, but this strategy does have caveats because it eliminates elements within the native promoter that would normally affect p53 target gene selection. These include enhancers, promoters, binding sites for other transcription factors as well as effects arising from the position of the particular p53 response element relative to the genes around it. The epigenetic landscape and quaternary structure of the DNA, including nucleosome occupancy and positioning, are also different in the reporter constructs and the native promoters, as is the core promoter architecture. Furthermore, the *p21* and *PUMA* promoters each contain more than one p53 response element. In our reporter construct, we incorporated the p53 binding site from the *PUMA* promoter that is primarily accountable for p53 responsiveness.[3] Both response elements used in our reporter constructs have similar affinities for p53 in vitro,[11] so we do not anticipate that binding affinity contributes significantly to differences observed between the reporter constructs. Nonetheless, it is possible that post-translational modifications and interacting proteins modify p53 binding in vivo.

In summary, this reporter system was designed specifically to analyze p53 activity and how one might manipulate it. Both its strengths and its limitations should be taken into consideration when using it.

A Model for Target Gene Selection that is Dependent on P53 Conformation

In our p53 reporter mice, we observed that EGFP expression varied depending on the response element, even within the same cells and tissue, indicating that p53 activity differs at each response element.[7] Given that the core promoter architecture and epigenetic regulation of the two reporters are identical, these results indicate that the primary DNA sequence of the p53 response element is sufficient to significantly affect target gene selection. Taking these and other published data into consideration, we propose a model that emphasizes the importance of p53 protein conformation with respect to its interaction with different response elements, and discuss its implications for target gene selection (**Fig. 1**).

The conformation of p53 may be determined either by post-translational modifications that occur before DNA-binding (**Fig. 1A**) or by DNA-binding itself (**Fig. 1B**). In the first part of our model, posttranslational modifications, such as phosphorylation, methylation and acetylation, dictate the conformation of each free p53 molecule (**Fig. 1A**). These modifications are mediated by enzymes such as kinases, phosphatases, methylases, acetylases and deacetylases. As p53 conformation determines its affinity for each response element, target gene expression depends on the relative abundance of p53 molecules adopting the relevant conformation. Therefore, p53 target gene selection is ultimately dependent on the tissue-specific activity of the enzymes responsible for p53 post-translational modifications.

The second part of our model posits that the conformation of the p53 molecule is determined by the response element where it is bound. Each conformation incorporates different binding sites for various transcription cofactors (represented by molecules X and Y in **Fig. 1B**) that must be recruited in order for gene expression to proceed. If a tissue has high levels of cofactor X but little of cofactor Y, p53 activity at response element 1 would be higher than that at response element 2, and vice versa. If levels of both cofactors were similar, then p53 activity at both response elements would be the same. Therefore, target gene selection is dependent on the tissue-specific expression of these transcription cofactors.

Support for our model comes from a recent publication describing different quaternary structures of p53 when bound to DNA.[12] Given our incomplete understanding of p53 protein structure (as discussed in detail below), these are tantalizing data indeed. They are nonetheless consistent with both scenarios of our model, whether the p53 adopts the conformation specific to each response element before DNA-binding (**Fig. 1A**) or after DNA-binding (**Fig. 1B**).

As described below, we also find evidence for our model in other published reports, whether they examine p53 target gene regulation at the DNA level or at the protein level.

p53 Target Gene Selection at the DNA Level

p53 binds response elements which are extremely diverse. A p53-binding consensus sequence has been defined as two 10-nucleotide motifs, specifically three purines followed by C(A/T)(T/A)G and

then three pymidines, which are separated by a linker that is 0–13 bases in length.[13] p53 affinity for each response element depends on the primary DNA sequence,[11] the linker length within the response element[14] and DNA topology.[15] In vitro analysis revealed that genes involved in cell cycle arrest generally have response elements with a higher affinity for p53 than apoptosis genes.[11] Cell cycle arrest genes thus generally require a lower threshold level of p53 for transcriptional activation. Indeed, p53 protein levels within a cell have been shown to affect target gene selection.[5]

However, the physiological significance of p53 promoter occupancy is controversial. Analysis of primary peripheral blood mononuclear cells revealed that p53 normally binds only a few target promoters, and that the diversity of promoter recruitment increases significantly upon stress,[16] an observation that supports the importance of the p53-DNA interaction as the rate-determining step in target gene expression (**Fig. 1A**). However, chromatin immunoprecipitation (ChIP) 'ChIP-on-chip' analysis of cell lines subject to genotoxic damage found no correlation between p53 localization to promoters and physiological outcome.[16] This result is consistent with part B of our model, in which cofactor expression and recruitment play major regulatory roles (**Fig. 1B**).

The conflicting data from these two reports suggest that the regulation of p53 may be different in primary cells and in cancer cells. Additionally, even conventional tissue culture growth conditions subject cells to stress that may activate p53[17] and potentially complicate gene expression analysis. Our reporter mouse system enables us to take all of the above into consideration. We can derive primary cells for use in in vitro cell culture systems as well as study p53 activity in vivo in wild type mice. By mating our reporter mice with those of the appropriate genotype, we can also examine p53 activity in different mouse models of cancer. ChIP analysis of our reporter mice would enable us to examine how p53 binding to its response elements correlates with p53 activity and target gene expression in a variety of tissues, thereby addressing this controversy.

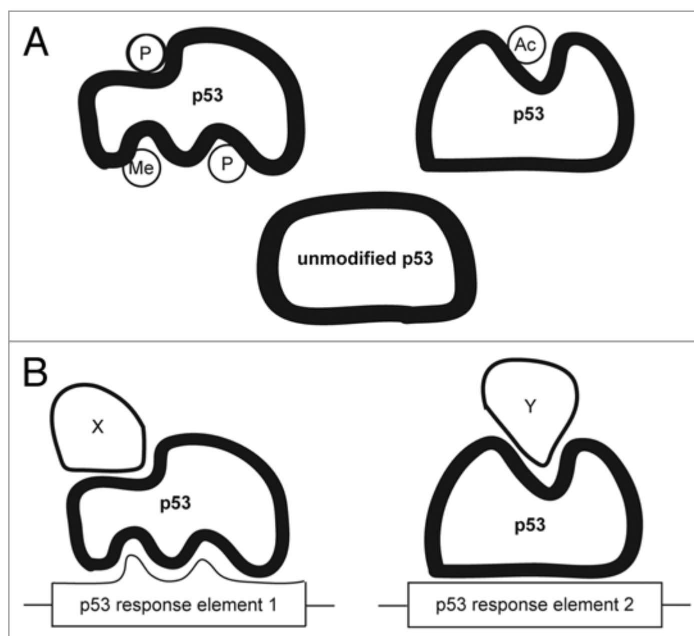

Figure 1. Model for p53 conformation-dependent target gene selection. (**A**) p53 conformation is determined by post-translational modifications prior to DNA-binding. These modifications include phosphorylation (P), methylation (Me) and acetylation (Ac). (**B**) The conformation of p53 changes upon DNA-binding and varies depending on the response element where it is bound. Each conformation has different binding sites for assorted transcription factors (represented by molecules X and Y).

Target Gene Selection at the Level of the p53 Protein

The conformation of the p53 protein plays a major role in regulating activity, although it has proven to be recalcitrant to structural study. p53 is very unstable and has multiple possible conformations.[18] Each p53 molecule comprises two stably folded domains flanked by disordered linkers. The central DNA-binding domain confers sequence specificity in terms of DNA-binding while the C-terminal domain is responsible for oligomerization.[14] The intrinsically unstructured N-terminal region contains two transactivation domains (TAD) that fold upon binding to specific protein partners. For example, TAD1 at residues 18–25 adopts a helical conformation upon binding to the transcriptional co-activator p300.[19] Interactions between the different domains modulate oligomerization as well as DNA binding and release.[20] Therefore, p53 transcriptional activity is regulated by different conformational changes, which is a key feature of our model (**Fig. 1**).

p53 binds DNA as a tetramer, specifically as a dimer of dimers. Its interaction with DNA occurs in a cooperative manner that contributes to target gene selection, as cooperativity-enhancing mutations have been shown to favor apoptosis.[21] p53 oligomerization may be affected by protein partners such as the S100 family of proteins and 14-3-3, which promote monomer and tetramer formation respectively.[14] Each p53 molecule is also subject to a multitude of posttranslational modifications, such as phosphorylation, acetylation, methylation, ubiquitination, sumoylation and neddylation. These modifications may affect p53 conformation, its affinity for DNA as well as its interactions with other proteins.[22] Accordingly, the first part of our model emphasizes their importance (**Fig. 1A**). Taken together, p53 target gene selection is heavily influenced by the complexity of p53 protein structure and the many factors that regulate it.

Furthermore, p53-binding proteins may drive p53 localization to specific DNA response elements or contribute to

the activation of transcription. For example, hematopoietic zinc-finger (HZF) promotes cell cycle arrest over apoptosis by increasing p53 binding to the *p21* promoter while decreasing p53 association with the promoters of apoptosis-associated genes such as *Bax* and *Noxa*.[23] Conversely, apoptosis-stimulating proteins of p53 (ASPP), ASPP1 and ASPP2, promote the transcription of apoptosis-associated genes such as *Bax* and *Pig3*.[24] It has also been shown that p53 must recruit its own transcription cofactors to certain promoters. For instance, p300 must be recruited to the *p21* promoter to acetylate nucleosomes before transcription can be activated.[25] Therefore, as described in the second part of our model (**Fig. 1B**), transcription cofactors that interact with p53 contribute significantly to the regulation of target gene expression.

p53-Independent Regulation of Target Gene Expression

Processes governing general transcription exert fundamental control over the gene expression, including that of p53 target genes. For example, insulator proteins such as CCCTC-binding factor (CTCF) delineate chromatin boundaries and separate regions of active and condensed chromatin. They can also interfere with the interaction between enhancer and promoter elements. CTCF can bind the *PUMA* promoter and constitutively repress *PUMA* expression even in the presence of activating signals.[26] Similarly, DNA methylation of the 14-3-3σ locus in some cancer cell lines significantly reduces the recruitment of RNA Polymerase II, resulting in constitutive gene silencing.[27] Therefore, chromatin structure and modification determine the accessibility of genes to transcription factors and machinery, and generally have a dominant effect on p53's ability to regulate gene expression.

Given the essential nature of the core transcription machinery, the architecture of the core promoter is surprisingly diverse. As a result, RNA Polymerase II is recruited to varying extents to different promoters in unstimulated cells, with there being higher levels at the promoters of genes mediating cell cycle arrest than at those of apoptosis-inducing genes. Upon stimulation, the transcription of cell cycle arrest genes is rapid but brief, while that of apoptosis genes is slower but sustained.[28] These differential kinetics indicate that cell cycle arrest, but not apoptosis, genes are primed for expression,[29] and that the core transcription machinery can control the kinetics of gene expression independently of p53.

Events downstream of transcription may also have a dominant effect on the regulation of p53 target gene expression. For example, in BV173 leukemia cells, p53 cannot effectively induce p21 expression because of the high turnover rate of *p21* mRNA in these cells.[27] Furthermore, the small non-coding RNAs known as microRNAs (miRNAs), which modulate gene expression at the posttranscriptional level, regulate and are regulated by the p53 pathway. miRNAs have been shown to also play an important role in tumorigenesis, illustrating the significance of their complex interactions with p53.[1]

Interestingly, a few p53 target genes can escape regulation by the general transcription machinery. Drugs such as 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) inhibit global mRNA synthesis, but *p21* and *PUMA* are expressed nonetheless because their expression does not require all the components of the canonical transcription process, such as phosphorylation of the C-terminal domain of RNA Polymerase II or recruitment of transcription elongation factors such as FACT (FAcilitates Chromatin Transcription).[30] This feature is important for the ability of p53 to respond to the stress exerted by the disruption of transcription itself.

Concluding Remarks

Given the mindboggling complexity of p53 regulation, the different models describing the selection of p53 target gene expression are unlikely to be mutually exclusive. It is important for us to continue investigating these questions with the most advanced technology that the fields of molecular and cell biology, biophysics, and genetics can offer. We now have better tools to study endogenous p53 in the context of non-transformed cells, as well as an ever-increasing selection of mouse models to study p53 in vivo, including our targeted transgenic reporter mice. With this multidisciplinary approach, combined with systems biology, we can strive to construct a "Grand Unified Theory of p53" that will aid us in our fight against cancer.

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