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Review

Mutant p53 exerts oncogenic effects through microRNAs and their target gene networks

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ABSTRACT

MicroRNAs are potent regulators of gene expression and modulate multiple cellular processes including proliferation, differentiation and apoptosis. A number of microRNAs have been shown to be regulated by p53, the most frequently mutated gene in human cancer. It is has been demonstrated that some mutant p53 proteins not only lose tumor suppressor activity, but also acquire novel oncogenic functions that are independent of wild-type p53. In this review, we highlight recent evidences suggesting that some mutant p53 proteins regulate the expression of specific microRNAs to gain oncogenic functions and identify a gene network regulated by the microRNAs downstream of mutant p53.

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1. Introduction

MicroRNAs (miRNAs) are an abundant class of small ~22 nucleotides (nt) endogenous non-coding RNAs. Mammalian miRNAs mostly act to post-transcriptionally inhibit gene expression by binding to the 3′ untranslated region (UTR) of target mRNAs, typically inhibiting mRNA stability and/or translation. Because the binding of mammalian miRNAs to target mRNAs occurs via partial complementarity, a single miRNA can regulate the expression of hundreds of mRNAs [1,2]. Moreover, a given mRNA can be concurrently regulated by multiple miRNAs, and an estimated 60% of the human genome is regulated by miRNAs [3].

A majority of miRNAs are transcribed by RNA pol II producing a primary transcript (pri-miRNA) [4,5]. The pri-miRNA is cleaved by Drosha, a double-stranded RNA endonuclease, to generate a precursor miRNA (pre-miRNA). Pre-miRNAs (~70 nt) are exported to the cytoplasm via Exportin 5 and processed by the RNase III enzyme Dicer to generate a ~22 nt RNA duplex consisting of the mature miRNA and the passenger strand [6]. The passenger strand is usually degraded and the mature miRNA is incorporated into the RNA-induced silencing complex (RISC) which binds to target mRNAs. The regulatory activity of miRNAs is necessary for numerous cellular processes, and, moreover, perturbation of miRNA expression often has pathological consequences [7–9].

Genome-wide down-regulation of miRNA expression is frequently observed in human cancers suggesting that most miRNAs may function as tumor suppressors. Examples of tumor suppressor miRNAs include the let-7 family, the miR-15-16 cluster, and the p53-target miR-34a. Expression of multiple let-7 family members is down-regulated in several cancers including breast, ovarian, lung and colon cancer, often due to chromosomal deletion of the let-7 loci [10]. let-7 inhibits proliferation and tumorigenesis by repressing a number of oncogenes such as *RAS*, *MYC* and *HMGA2*,

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and is itself negatively regulated by MYC [11–15]. The miR-15-16 cluster has potent anti-proliferative effects, and its locus at 13q14 is frequently deleted in chronic lymphocytic leukemia and prostate cancer [16,17]. The miR-34 family is transcriptionally regulated by p53 [18–20]. Like p53 itself, miR-34 exerts tumor suppressive functions by inhibiting cell proliferation and inducing apoptosis, and miR-34 expression is silenced in some tumors due to homozygous deletion or loss of p53 signaling [21–24].

Although most miRNAs act as tumor suppressors, some including the miR-17∼92 cluster, miR-21 and miR-155 are up-regulated in cancer, suggesting oncogenic functions [9,25,26]. The expression of the miR-17∼92 polycistron is transcriptionally up-regulated by MYC and this cluster promotes cell proliferation and survival [14,27]. The expression of this cluster of miRNAs is significantly increased in several cancer types such as lung cancer and lymphomas due to genomic amplification of its locus at 13q31 [28–30].

Wild-type p53 functions as a sequence-specific transcription factor that is activated during the stress response such as DNA damage [31–34]. Activation of p53 leads to transcriptional up-regulation of a battery of genes that control cell cycle progression, senescence, apoptosis and metabolic adaptation [35,36]. The *TP53* gene, which is located on chromosome 17p13.1, is the most frequently mutated gene in human cancer with more than 50% of all tumors exhibiting mutation at this locus [37,38]. Unlike most tumor suppressor genes that are inactivated through biallelic deletion or truncation mutations, a majority of mutations in *TP53* are missense mutations resulting in the production of a full-length mutant p53 protein [39]. Because these missense mutations are mainly located in the DNA binding domain of p53, the mutant p53 protein is unable to transactivate most of its target genes resulting in loss of tumor suppressor functions. However, it is also known that in addition to loss of tumor suppressor activity, mutant p53 proteins acquire novel oncogenic functions to modulate a wide variety of phenotypes such as increase cell growth, migration, invasion, metastasis, genomic instability and chemoresistance [40–46].

Interestingly, soon after the discovery of the *TP53* gene p53 was believed to function as an oncogene [47–52]. Ten years later, it was found that the early studies demonstrating oncogenic functions of p53 were actually performed with mutated versions of p53 that were isolated from tumor cells [53,54]. In other words, the first 10 years on p53 accidentally described the function of mutant p53 in tumor biology instead of wild-type p53. More recently, convincing evidence in support of mutant p53's oncogenic functions has come from studies in mouse models. Mice harboring tumor derived hot spot p53 mutants develop more invasive tumors than p53 null mice [55–57]. In addition to these studies that established the gain-of-function properties of mutant p53, several reports demonstrated oncogenic functions of mutant p53 including activation of growth promoting genes such as *EGFR*, *MDR1*, *MYC* and *PCNA* [46,58–63].

Although knowledge of the mechanisms of mutant p53 gain-of-function remains incomplete, mutant p53 activity has been attributed to diverse mechanisms, including both transcriptional and post-transcriptional mechanisms through interactions with other cellular proteins [33,64]. The most studied mutant p53 interacting proteins are the p53 family members, p63 and p73. Like wild-type p53, p63 and p73 regulate tumor biology by regulating transcription of effector genes [65–68]. In addition to p63 and p73, mutant p53 has also been shown to regulate gene transcription by interacting with other sequence-specific transcription factors including NF-Y [69], E2F1 [70] and VDR [71]. Finally, although most p53 hot spot mutations abolish the ability of p53 to bind DNA, some gain-of-function p53 mutations result in a protein with an intact but structurally altered DNA binding domain, causing changes in sequence specificity and transactivation of non-canonical target genes [72,73].

MiRNAs have been shown to regulate all the biological processes regulated by mutant p53. Therefore, connecting the miRNA pathway with the mutant p53-regulated pathways is important from a cancer perspective. Indeed, recent studies have shown that mutant p53 can regulate gene expression and exert oncogenic effects through specific miRNAs. Here, we review the mechanisms by which mutant p53 gains diverse oncogenic functions through miRNAs.

2. The following sections discuss the regulation of miRNA expression by mutant p53 and how miRNAs function downstream of mutant p53 to mediate oncogenic functions

2.1. miRNAs up-regulated by mutant p53

2.1.1. miR-128-2

Two recent studies have shown that mutant p53 transcriptionally up-regulates the expression of specific miRNAs. In the first study, the expression of ectopic mutant p53R175H was induced in H1299 cells (p53 null) and the effect on the abundance of select miRNAs was examined by RT-qPCR [74]. This study showed that mutant p53R175H increased the expression of miR-128-2 by activating transcription of its host gene, *ARPP21*. Chromatin immunoprecipitation assays (ChIP) suggested that mutant p53 was recruited to the promoter of *ARPP21*. The authors identified *E2F5*, a transcription repressor, as a direct target of miR-128-2 and showed that miR-128-2 binds to the *E2F5* 3′UTR to inhibit its expression. Because E2F5 represses p21 (*CDKN1A*) transcription, down-regulation of E2F5 through miR-128-2 causes an increase in p21 protein levels and cytoplasmic accumulation. Although nuclear p21 is strongly associated with growth arrest, cytoplasmic p21 exhibits anti-apoptotic effects by binding to and preventing the cleavage of pro-caspase 3. Thus, the anti-apoptotic functions of miR-128-2 were shown to be mediated by repression of E2F5, which results in cytoplasmic p21 accumulation, leading to the anti-apoptotic inhibition of pro-caspase 3 cleavage. In sum, this study demonstrated that up-regulation of miR-128-2 by mutant p53 contributes to mutant p53 associated chemoresistance by inhibiting apoptosis.

2.1.2. miR-155

In another study, mutant p53 was shown to induce EMT through miR-155. Neilsen et al. [75] used RT-qPCR to identify miRNAs over-expressed in mesenchymal breast cancer cell lines and found a significant relationship between miR-155 and epithelial-mesenchymal transition (EMT). Expression of miR-155 induced cell migration, invasion and associated significantly with up-regulation of EMT-promoting genes. Over-expression of the p53 mutants p53R248Q or p53R282W enhanced miR-155 levels while knockdown of endogenous mutant p53 in BT-549 (p53R249S) down-regulated miR-155, suggesting that miR-155 is a mutant p53 target in breast cancer cells. The authors went on to show that in the absence of mutant p53, p63 binds directly to the consensus p63-response element in the promoter of miR-155 host gene to negatively regulate miR-155 expression, indicating that mutant p53 enhances miR-155 expression by inhibiting p63. Furthermore, they identified ZNF652, a zinc-finger DNA-binding transcription repressor, as a downstream target of the miR-155/mutant-p53 axis. miR-155 negatively regulated *ZNF652* expression by binding to its 3′UTR. ZNF652 is an epithelial marker and plays an important role in suppressing cell invasion. The authors hypothesized that ZNF652 suppresses invasion and metastasis by down-regulating genes involved in EMT. Using ChIP assays, the authors demonstrated that ZNF652 was recruited to regulatory elements upstream of a subset of EMT-related genes, including *TGFB1*, *TGFB2*, *TGFBR2*, *EGFR*, *SMAD2* and *VIM*. Silencing ZNF652 increased the

expression levels of these genes, suggesting that their expression is repressed by ZNF652. Importantly, loss of ZNF652 expression was found to be associated with increased cancer invasion and metastasis in breast cancer in vivo. Taken together, this study demonstrated that mutant p53 up-regulates miR-155 to enhance invasion and metastasis through repression of ZNF652. This study therefore suggests that miR-155 targeted therapies can be utilized to inhibit the metastatic potential of mutant p53-expressing breast cancers.

2.2. miRNAs down-regulated by mutant p53

2.2.1. miR-223

In addition to up-regulating miRNA expression, we and others have shown that mutant p53 down-regulates select miRNAs to promote oncogenic characteristics. In a recent study [76], miR-223 was identified as a mutant p53-repressed miRNA through RT-qPCR screening for miRNAs that had previously been shown to belong to a cancerous gene-expression signature [7]. The authors induced mutant p53R175H in H1299 cells and showed that miR-223 was down-regulated by mutant p53. Regulation of miR-223 by mutant p53 was also examined in a more physiological setting following knockdown of mutant p53 in cell lines that express endogenous mutant p53. Both transient and stable knockdown of endogenous mutant p53 in SW480 (colorectal cancer) or MDA-MB-468 and MDA-MB-231 cells (breast cancer) resulted in increased miR-223 expression. ChIP assays at the miR-223 promoter confirmed direct regulation of miR-223 by mutant p53. Because mutant p53 does not bind DNA in a sequence-specific manner the authors performed a bioinformatic analysis for other transcription factors that could bind to the miR-233 promoter region recognized by mutant p53. This in silico analysis identified various binding sites for ZEB1, a transcription repressor that plays an important role in EMT. Consistent with this finding, the authors provided evidence that ZEB1 binding to miR-223 promoter was necessary for p53 binding to the same region.

As a downstream target of mutant p53, miR-223 antagonizes mutant p53 oncogenic functions and chemoresistance in particular. Over-expression of miR-223 sensitized cells to apoptosis in response to treatment with the chemotherapeutic drugs cisplatin and 5-flourouracil. Over-expression of miR-223 significantly reduced the protein levels of STMN1 (a key microtubule-regulating gene) in multiple cell lines. In addition, siRNA knockdown of STMN-1 increased cell death, sensitizing mutant p53 expressing cells to DNA damage. These results suggest that a mutant p53/ZEB1 axis represses miR-223, causing derepression of the miR-223 target STMN1, resulting in increased chemoresistance.

2.2.2. miR-130b

In a recent study, Dong et al. [77] found that forced expression of some hot spot p53 mutants induced EMT in HEC-50 cells (endometrial cancer, p53-null). Conversely, stable knockdown of mutant p53 in HEC-1 cells, which express endogenous mutant p53R248Q, resulted in a shift to an epithelial-like morphology, pointing to a role for mutant p53 in inducing EMT. Significantly, the authors found that mutant p53 correlated with increased ZEB1 expression while silencing mutant p53 resulted in decreased ZEB1 levels. In addition to the aforementioned interaction with mutant p53 at the miR-223 promoter, ZEB1 is a well known effector of EMT, suggesting that ZEB1 may be important for multiple facets of mutant p53 gain-of-function, including EMT induction.

In order to determine whether increased ZEB1 expression was due to altered miRNA regulation, the authors used an array-based method to investigate changes in miRNA expression following over-expression of the p53 mutants p53R273H, p53R175H and p53C135Y in the p53-null HEC-50 cells. Out of 188 miRNAs assayed, 23 were significantly down-regulated by mutant p53. Of these 23 candidate miRNAs, the authors focused on miR-130b because it was predicted to bind to the 3′UTR of *ZEB1* mRNA. MiR-130b inhibited *ZEB1* via its 3′UTR. Additionally, ChIP-PCR assays confirmed direct regulation of miR-130b by mutant p53 in EC cells. Introduction of miR-130b decreased invasion of HEC-50 cells and concomitantly reduced the mRNA levels of EMT-related genes including *SNAI1*, *BM1-1*, *KLF4*, while increasing E-cadherin expression. Conversely, in HEC-1 cells, depletion of miR-130b enhanced cell invasion. Interestingly, the authors found that wild-type p53 could also bind to the miR-130b promoter to up-regulate its expression. Thus, wild-type p53 and mutant p53 exert opposite effects on miR-130b expression, suggesting that missense mutations in p53 may play an important role in EC tumorigenesis, in part, by dysregulating miR-130b expression. This study demonstrates that in wild-type p53 expressing cells, miR-130b directly represses ZEB1, opposing EMT and invasive phenotypes. However, in the context of gain-of-function p53 mutations, mutant p53 triggers EMT by indirectly inducing ZEB1 expression through negative regulation of miR-130b.

2.2.3. miR-27a

Using an inducible mutant p53R273H system and miRNA microarrays, a recent study [78] identified miR-27a as a direct transcriptional target of mutant p53 in H1299 cells. Mutant p53 directly binds to the promoter of miR-27a and represses miR-27a transcription. *EGFR* 3′UTR was identified as a downstream target of miR-27a. The authors showed that induction of mutant p53R273H caused a prolonged activation of ERK1/2 phosphorylation following stimulation with EGF. This effect depended on the negative regulation of EGFR by miR-27a. Using over-expression and knockdown approaches, they demonstrated that mutant p53 promoted cell growth in vitro and tumorigenesis in vivo by the regulation of miR-27a and EGFR expression.

2.2.4. let-7i

Recently, we have identified the tumor suppressor miRNA let-7i as a downstream target of mutant p53 [79]. Using small RNA deep sequencing from H1299 cells stably expressing mutant p53R273H and the empty vector transfected cells, we found 38 up-regulated and 3 down-regulated miRNAs. let-7i was abundant in the control cells and significantly down-regulated in p53R273H-expressing H1299 cells. We hypothesized that let-7i is a potential effecter of mutant p53. Stable knockdown of endogenous mutant p53 increased let-7i levels in MDA-MB-231, HT-29 and DLD1 cells. The derepression of let-7i expression suggests that regulation of let-7i by mutant p53 is physiologically relevant. Moreover, let-7i expression negatively correlated with mutant p53 status in the NCI-60 panel and in breast cancer patients suggesting clinical relevance and role of mutant p53/let-7i axis in many types of cancers. Using ChIP-PCR and promoter luciferase assays, we found that mutant p53 associates with p63 at the let-7i promoter to inhibit transactivation of let-7i by p63. Over-expression of let-7i significantly decreased migration and invasion of mutant p53 cells in vitro, and reintroduction of let-7i dramatically reduced metastatic colonization of MDA-MB-231 cells. Using microarray and 3′UTR luciferase analysis, we identified the genome-wide mRNAs directly down-regulated by let-7i. A subset of let-7i targets included the oncogenes *E2F5*, *LIN28B*, *MYC* and *NRAS* and these were up-regulated upon let-7i knockdown and down-regulated when mutant p53 was knocked down in multiple cell lines. Taken together, this study demonstrates that mutant p53 acquires novel oncogenic functions by negatively regulating let-7i expression and modulating the expression levels of a subset of oncoproteins.

2.2.5. miR-205

A recent study identified miR-205 as a downstream target of mutant p53 through inhibition of p63 [80]. Over-expression of the metastasis suppressor p63 dramatically increased the expression of miR-205 whereas p63 knockdown reduced miR-205 levels in prostate cancer cells. Promoter luciferase and ChIP assays demonstrated that p63 directly binds to the miR-205 promoter to up-regulate its transcription. The author showed that up-regulation of miR-205 by p63 was essential for the inhibition of EMT associated genes such as *ZEB1* and *VIM*. Mutant p53 inhibited expression of both p63 and miR-205. Re-introducing miR-205 or silencing mutant p53 in a cell line that expresses endogenous mutant p53 reduced cell migration. Consistent with their results from cell lines, the authors found that the expression of p63 (ΔNp63) or miR-205 inhibited lung metastasis in vivo in mice. Importantly, loss of the p63/miR-205 axis was associated with increased risk of metastasis and poor clinical outcome in human prostate cancer.

Collectively, the studies described above suggest that miRNAs are key downstream effectors of mutant p53. Gene expression changes mediated by mutant p53-regulated miRNAs contribute to mutant p53 gain-of-function properties such as chemoresistance and induction of EMT (Table 1).

2.3. Global regulation of miRNA biogenesis by mutant p53

Two recent studies have shown that mutant p53 can also regulate global miRNA biogenesis. In the first study [81], the authors showed that in response to DNA damage, wild-type p53 post-transcriptionally enhances the expression of several miRNAs including miR-16-1, miR-143, and miR-145. Wild-type p53 associates with the microprocessor complex by interacting with the DEAD-box RNA helicase p68, which in turn, promotes the processing of pri-miRNAs in the nucleus. In contrast, tumor-derived p53 mutants p53R273H, p53R173H and p53C135Y ablate the ability of Drosha to bind to p68. RNA-ChIP assays revealed that

Table 1
MiRNAs regulated by mutant p53.

Name	Mechanisms	Targets	Biological activities	References
Up-regulated				
miR-128-2	Direct binding to promoter	E2F5	Chemoresi stance Anti-apoptosis	Donzelli et al. (2012) [74]
miR-155	Releases repression by p63	ZNF652	Cell invasion Metastasis	Neilsen et al. (2013) [75]
Down-regulated				
miR-223	Interacts with ZEB1 and associates with promoter	STMN-1	Chemoresi stance	Masciarelli et al. (2014) [76]
miR-130b	Direct binding to promoter	ZEB1, Snai1, BMI-1 and KLF4	EMT, cell invasion	Dong et al. (2013) [77]
miR-27a	Direct binding to promoter	EGFR	Cell growth, tum ori genesis	Wang etal. (2013) [78]
let-7i	Interacts with p63 and associates with promoter	E2F5, LIN28B, MYC and NRAS	Cell invasion, migration and tum ori genesis	Subramanian et al. (2014) [79]
miR-205	Inhibit p63 transcriptional activity	ZEB1	EMT	Tucci et al. (2012) [80]

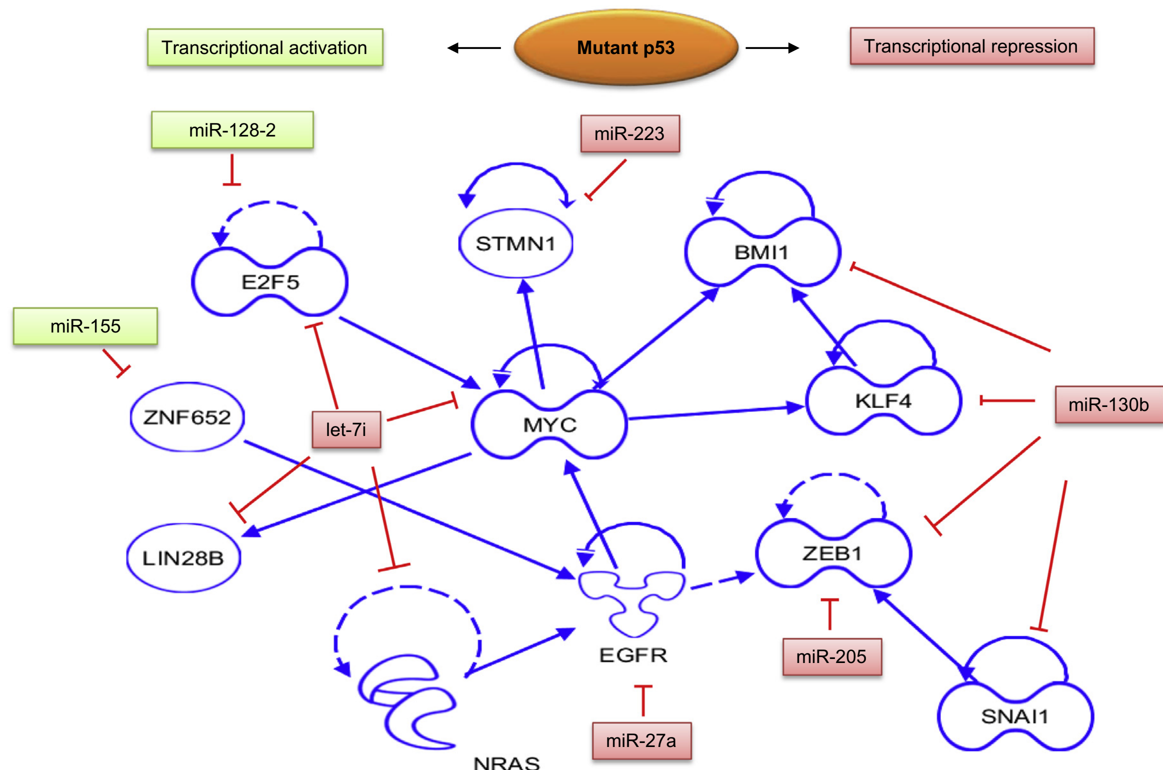

Fig. 1. Mutant p53 regulates miRNAs to regulate a gene network. Mutant p53 activates or inhibits the expression of distinct miRNAs either directly or indirectly through interacting partners such as p63 or ZEB1. Altered expression of these miRNAs results in decreased expression of genes that play key roles in tumor progression, EMT and chemoresistance including *MYC*, *LIN28B*, *NRAS*, *ZEB1* and *E2F5*.

over-expression of mutant p53 inhibits the interaction between Drosha, p68 and primary miRNAs, which leads to inhibition of miRNA biogenesis.

In another recent study [45], the authors found that depletion of p63 decreased Dicer expression. However, over-expression of p63 did not promote Dicer expression. Because of the well-known relationship between mutant p53 and p63, the authors hypothesized that Dicer down-regulation may be a consequence of mutant p53 gain-of-function. Indeed, over-expression of p53R273H decreased Dicer mRNA and protein levels in H1299 cells. Conversely, siRNA knockdown of endogenous mutant p53 up-regulated Dicer in HT29 (colorectal cancer) and A431 (squamous cell carcinoma) cells, suggesting that mutant p53 negatively regulates Dicer by inhibiting p63. They also provided evidence that p53R273H over-expression phenocopied loss of Dicer by reducing the expression of several miRNAs including miR-203, miR-130b, and miR-206, indicating that mutant p53 impairs the production of mature miRNAs by reducing the level of Dicer.

As summarized (Fig. 1), miRNAs regulated by mutant p53 modulate a network of gene products. The genes regulated by mutant p53-regulated miRNAs interact with each other, either directly or indirectly. Mutant p53 can up-regulate oncogenic miRNAs and down-regulate tumor suppressive miRNAs by directly binding to the miRNA promoters, as well as through other mutant p53-interacting proteins. Collectively, these studies suggest that miRNAs are important effectors of mutant p53 and play a role in mediating mutant p53 gain-of-function.

2.4. Future perspectives

Accumulating evidence in vitro and in vivo has established that some p53 mutant proteins exert oncogenic functions. This gain-of-function not only involves the direct, transcriptional regulation of a subset of tumor-associated genes distinct from the set of canonical p53 targets, but it also entails regulation of miRNA expression and biogenesis. Only a handful of miRNAs have so far been identified as direct targets of mutant p53, but as miRNAs are themselves powerful regulators of gene expression, the effects of miRNA dysregulation by mutant p53 are profound. Several miRNAs have shown to be necessary for the oncogenic activity of mutant p53, while other miRNAs are down-regulated in order to de-activate tumor suppressive pathways. Because missense mutations in *TP53* occur at a high frequency in human cancers, developing strategies to block the oncogenic effects of mutant p53 will be an important step for the treatment of human cancers. Although the mutant p53 protein is expressed in many cancers, the effects of mutant p53 proteins are mediated through their interaction with other cellular proteins. Therefore, depending on these interactions, a specific mutant p53 protein may have oncogenic functions in one cell-type but not in another cell-type. Another possibility is that the same mutant p53 protein may exert oncogenic functions through different interacting partners in a cell-type dependent manner. In order to achieve this goal, an improved understanding of the complex effects of miRNAs on mutant p53 gain-of-function in mutant p53 mouse models and mutant p53-regulated miRNA knockouts in cell lines is essential in understanding the physiological function of miRNAs in mutant p53 signaling and to allow new advances for therapeutic manipulation of miRNA regulation regulated by mutant p53. In sum, the mutant p53-regulated transcriptome is complex and a consequence of direct regulation by mutant p53, indirect regulation through specific mutant p53-interacting proteins at target gene promoters and post-transcriptional regulation through miRNAs. It is likely that the recently discovered long non-coding RNAs shown to act as potent regulators of gene expression in the Rb and p53 pathways [82–85] may also play a role in mutant p53 gain-of-function.

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