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MUTANT P53 INHIBITS microRNA BIOGENESIS BY INTERFERING WITH THE MICROPROCESSOR COMPLEX

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1 ABSTRACT

A decrease of mature microRNAs is often observed in human malignancies giving them potential to act as tumor suppressors. Thus, microRNAs may be potential targets for cancer therapy. In agreement, inhibition of microRNA biogenesis tends to enhance tumorigenesis, and alterations in the microRNA biogenesis machinery impact on the establishment and development of cancer programs. Moreover, accumulation of pri-microRNAs and corresponding depletion of mature microRNAs occurs in human cancers compared to normal tissues, strongly indicating an impairment of crucial steps in microRNA biogenesis. Still, the mechanisms through which microRNAs are regulated in cancer remain poorly understood. Therefore, the understanding of mechanisms that drive the alterations in the microRNA biogenesis is a cardinal question in cancer biology. The TP53 tumor suppressor gene is mutated in half of human tumors resulting in an oncogene with Gain-Of-Function activities. Numerous microRNAs can be regulated by wtp53 at transcriptional and post-transcriptional level but very few data about mutant p53-dependent microRNA expression are available yet. Here we demonstrate that mutant p53 oncoproteins modulate the biogenesis of a subset of microRNAs in cancer cells inhibiting their post-transcriptional maturation. Interestingly, among these microRNAs several are also downregulated in human tumors. By confocal, coimmunoprecipitation and RNA-ChIP experiments, we show that endogenous mutant p53 binds and sequesters RNA helicases p72/82 from the Microprocessor complex, interfering with the Drosha-pri-microRNAs association. In agreement with this, the overexpression of p72 leads to an increase of mature microRNAs levels. Finally, we demonstrate that 4 of post-transcriptionally regulated microRNAs display tumor suppressive properties related to apoptosis, cell cycle arrest, epithelial-mesenchymal transition repression and migration inhibition, strongly indicating that these microRNAs are downregulated by mutp53 in order to de-activate tumor suppressive pathways. Our study highlights for the first time a novel GOF activity of mutant p53 proteins responsible, at least in part, for the microRNA deregulation observed in cancer.

2 INTRODUCTION

2.1 MicroRNAs.

With the advent of next generation sequencing techniques a previously unknown world of non-coding RNA molecules have been discovered. The best characterized non-coding RNA family consists in microRNAs. MicroRNAs (miRNAs) are small non coding single-stranded RNAs of about 20–25 nucleotides in length that regulate gene expression by binding to complementary target mRNAs and promoting their decay or inhibiting their translation (1-3). More than 2000 miRNA genes and an even greater number of predicted miRNA targets have been identified in the human genome. Thus, miRNAs are potent regulators of gene expression involved in diverse physiological processes, such as normal development, differentiation, growth control, apoptosis, and in human diseases, particularly in cancer where they act as regulators of key cancer-related pathways (4-6).

Moreover, an aberrant miRNA levels reflect the physiological state of cancer cells and can be detected by miRNA expression profiling and harnessed for the purpose of diagnosis and prognosis (7, 8). In agreement, miRNA processing defects enhance tumorigenesis (9). Although insights into the regulatory function of miRNAs are beginning to emerge, much less is known about the deregulation of miRNA in cancer.

2.2 MicroRNA biogenesis.

The expression level of biologically active mature miRNAs is the result of a fine mechanism of biogenesis, carried out by different enzymatic complexes that exert their function at transcriptional and post-transcriptional levels.

MiRNAs sequences are distributed all throughout the genome, being localized in exonic or intronic regions, as well as intergenic locations (10). The biogenesis of miRNAs starts with their transcription by RNA polymerase II (11) although some other miRNAs are transcribed by RNA polymerase III (12) resulting in a primary transcript known as pri-miRNA which contains a 33 nucleotides hairpin stem, a terminal loop and a flanking single stranded sequence of hundreds of bases or even several kilobases. In general, pri-miRNAs are capped at the 5´end and polyadenylated at the 3´ end. The canonical miRNA biogenesis pathway is characterized by two subsequent central steps utilizing ribonuclease reactions (Figure 1B). In the nucleus, pri-miRNAs are recognized and cropped into hairpin-structured precursor miRNAs (pre-miRNAs) by the Drosha complex (also known as Microprocessor complex).

Drosha, an RNase III enzyme, and DGCR8 (DiGeorge critical region 8), a double-stranded RNA-binding domain (dsRBD) protein, are two essential components of the Microprocessor complex. Drosha liberates the stem_loop pre-miRNAs from pri-miRNAs in cooperation with DGCR8-mediated recognition of the junctional region between the single-stranded and double-stranded portions of pri-miRNAs (13, 14). The Drosha complex also contains several auxiliary factors including the DEAD-box RNA helicases p68 (DDX5) and p72/p82 (DDX17) witch promote the fidelity and activity of Drosha processing (15). miRNA biogenesis is homeostatically controlled by an auto-feedback loop between Drosha and DGCR8 (16), in which DGCR8 stabilizes Drosha protein level, while Drosha destabilizes DGCR8 mRNA through cleavage of the hairpin structures in the DGCR8 mRNA. p68 and p72/p82 are prototypic members of the DEAD box protein family of RNA helicases (see section 2.4.3). Alternatively, some non-canonical biogenesis pathways may occur during mRNA splicing, giving rise to "miRtrons". MiRtrons are in fact, the spliced-out introns of mRNAs, which constitute functional pre-miRNAs. Therefore, production of miRtrons is independent of Drosha digestion (17). The pre-miRNAs of approximately 70 nucleotides in length are transported from the nucleus to the cytoplasm by Exportin-5 (XPO5) together with Ran-GTP (Figure 1B)(18). Exportin-5 can also protect pre-miRNAs against nuclear degradation (19). In the cytoplasm, Dicer, another RNase III, digests the pre-miRNA into a 20-25 nucleotides mature duplex miRNA. During this process, Dicer is associated with other proteins like TAR RNA binding protein (TRBP) and kinase R–activating protein (PACT) to increase its stability and its processing activity (20, 21). The miRNA duplex is comprised of two miRNA strands, with one strand loaded onto the RNA-induced silencing complex (RISC), which contains the Argonaute (Ago) family protein as a core component (Figure 1B). In these processes, another strand (miRNA* strand) is usually degraded, while miRNA* strands are retained and function in some cases (22). Mature miRNAs serve as guides directing RISC to target mRNAs, which are degraded, destabilized or translationally inhibited by the Ago proteins (23).

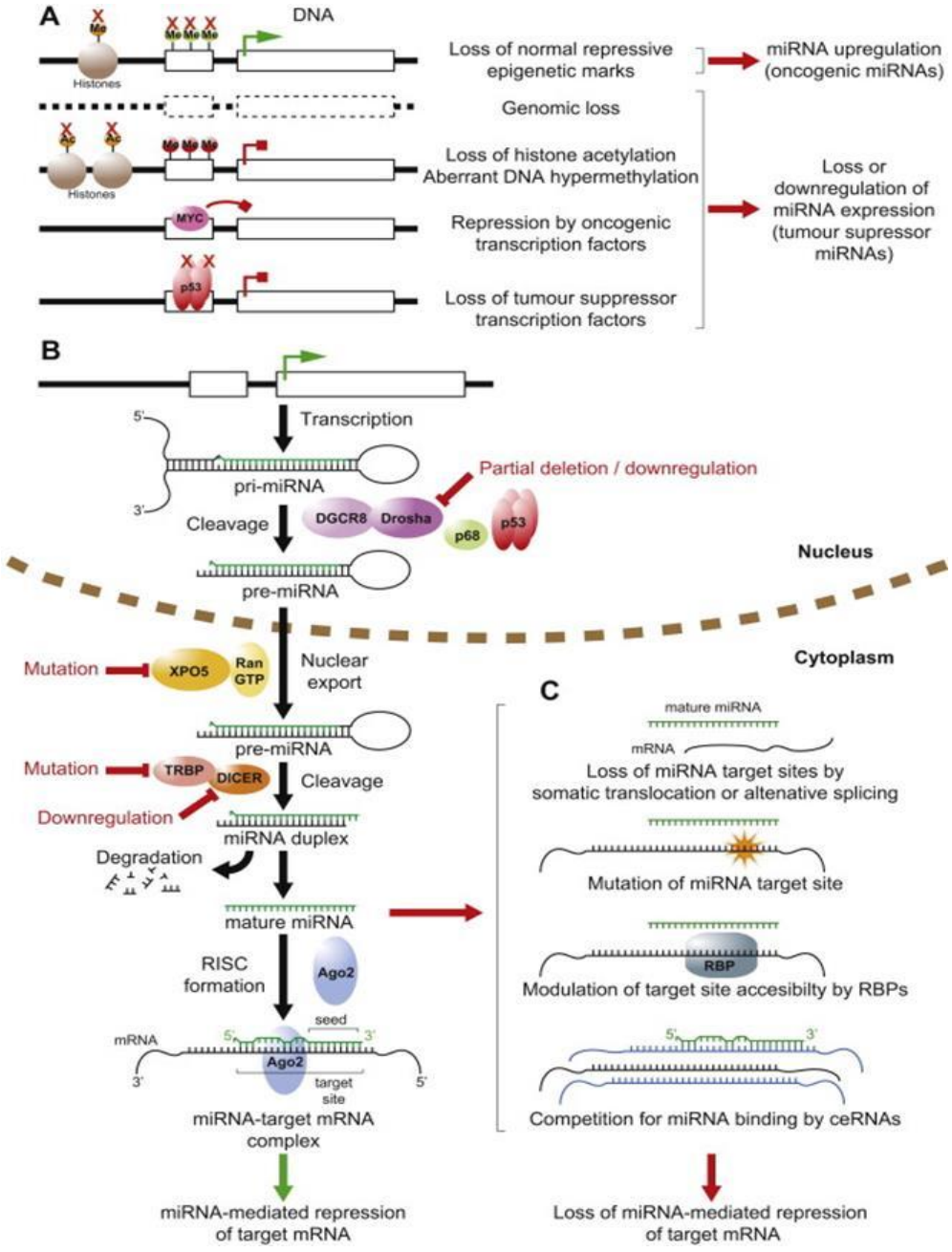

Figure 1. A schematic representation depicting the canonical miRNA biogenesis pathway and the general mechanisms whereby normal miRNA expression and function can be deregulated in cancer (ref. 23). *(*A*) Deregulation of miRNA gene transcription in cancer through genetic, epigenetic and transcriptional mechanisms. Active transcription is indicated by green arrow, blocked transcription by red block arrow. Red crosses indicate loss of gene, epigenetic mechanism or transcription factor. Me, methylation; Ac, acetylation. (*B*) Simplified canonical pathway of miRNA biogenesis and processing. Steps commonly deregulated in cancer indicated in red. (*C*) Mechanisms prevalent in cancer allowing mRNAs to escape regulation by miRNAs. RNA binding protein abbreviated as RBP. ceRNAs represented in blue.*

2.3 MicroRNAs are cancer genes.

Downregulation of miRNAs is commonly observed in human cancers (23), suggesting that miRNAs are primarily tumor suppressor genes. Microarray expression data from a wide spectrum of cancer diseases have since evidenced that aberrant miRNA expression is the rule rather than the exception in cancer (24-26). Importantly, mouse models featuring miRNA overexpression or ablation have demonstrated causal links between miRNAs and cancer development and miRNAs are rapidly entering the clinic as biomarkers and putative therapeutic targets (27). Indeed, aberrant miRNA levels reflect the physiological state of cancer cells and can be detected by miRNA expression profiling and harnessed for the purpose of diagnosis and prognosis (28, 29). In fact, miRNA profiling can be more accurate at classifying tumors than mRNA profiling because miRNA expression correlates closely with tumor origin and stage, and can be used to classify poorly differentiated tumors that are difficult to identify using a standard histological approach. The special features of miRNAs make them potentially useful for detection in clinical specimens. For example, miRNAs are relatively resistant to ribonuclease degradation, and they can be easily extracted from small biopsies, frozen samples and even formalin-fixed, paraffinembedded tissues. Furthermore, relatively simple and reproducible assays have been developed to detect the abundance of individual miRNAs, and methods that combine small RNA isolation, PCR and next-generation sequencing, allow accurate and quantitative assessment of all the miRNAs that are expressed in a patient specimen, including material that has been isolated by laser capture microdissection. Perhaps the most appealing application of miRNAs as a cancer diagnostic tool comes from the discovery of circulating miRNAs in serum. For example, miR-141 expression levels in serum were significantly higher in patients with prostate cancer than in healthy control individuals (30). Although the analysis of circulating miRNAs is only just beginning, the successful advancement of this technology could provide a relatively non-invasive diagnostic tool for single-point or longitudinal studies. With such diagnostic tools in place, miRNA profiling could be used to guide cancer classification, facilitate treatment decisions, monitor treatment efficacy and predict clinical outcome.

As a single miRNA may target up to several hundred mRNAs, aberrant miRNA expression may affect a multitude of transcripts and profoundly influence cancer-related signaling pathways. Functional studies show that miRNAs that are affected by somatic alterations in tumors can affect cancer phenotypes directly, therefore confirming their driver function in malignancy. As drivers of malignancy, mechanistic studies show that these miRNAs interact

with known cancer networks; hence, tumor-suppressor miRNAs can negatively regulate protein-coding oncogenes, whereas oncogenic miRNAs often repress known tumor suppressors. Importantly, mouse models featuring miRNA overexpression or ablation have demonstrated causal links between miRNAs and cancer development and miRNAs are rapidly entering the clinic as putative therapeutic targets (27).

Expression of miRNAs is mainly downregulated in tumor tissues, as compared to corresponding healthy tissues, which supported the role of miRNAs as primarily tumor suppressors such as miR-10b, -34a, -let-7c, 145 well described on review 31. Along with the dominance of tumor suppressor miRNAs, several well-characterized oncogenic miRNAs have been reported in tumors. Perhaps the best example of this is the oncogenic miR-17-92 cluster, which is amplified in some human B cell lymphomas, cooperates with Myc to promote B-cell lymphoma in mice. Transgenic mice overexpressing miR-17-92 developed lymphoproliferative disorders (32) and retroviral overexpression of the cluster accelerated lymphoma formation in collaboration with MYC (33). Two groups have independently dissected the cluster using knockout mice and demonstrated that loss of miR-17-92 leads to reduced tumourgenicity and increased cell death (34, 35). Another example is miR-155 overexpression in the lymphoid compartment, which triggers B-cell leukaemia or a myeloproliferative disorder depending on the system used to drive expression of the transgene; this was the first example of a miRNA that initiates cancer in a transgenic setting (36, 37). In addition to promoting cancer initiation, miRNAs can modulate processes that support cancer progression, including metastasis (38-41). For example, in breast cancer, miR-10b and miR-9 can induce metastasis, whereas miR-126, miR-335 and miR-31 act as suppressors. The miR-200 family inhibits epithelial-to-mesenchymal transition, which influences one aspect of the metastatic process (42). However, miR-200 could also promote the colonization of metastatic cells in breast cancer, which provides an example of the opposing activities of some miRNAs (43).

Because many downregulated miRNAs function as tumor suppressors, better understanding of the biological mechanisms underlying their modulation will likely enable new strategies for prevention, early detection and therapy of cancer.

Since the discovery of miRNAs in model organisms, miRNAs have emerged as key regulators of normal development and a diversity of normal cellular processes. Given what we know now, it is not surprising that perturbations in miRNA biogenesis or expression can contribute to disease. In cancer, the effects of miRNA alteration can be widespread and profound, and they touch on virtually all aspects of the malignant phenotype. The mechanisms of

modification, turnover, stabilization and reduction of miRNAs are largely unknown. Further characterization of the regulatory elements of miRNA biosynthesis and function will provide new insights yielding comprehensive understanding of the complex gene-regulatory networks governed by miRNAs and the involvement of miRNAs in cancer. These findings will also offer a molecular basis for diagnostic and therapeutic strategies based on miRNA biology.

2.4 Defects of microRNA biogenesis in cancer.

As described above, although the expression of some miRNAs is increased in malignant cells, the widespread under expression of miRNAs is a more common phenomenon. Different main mechanisms have been proposed as the underlying cause of the global downregulation of miRNAs in cancer cells: genetic alterations, transcriptional regulation (epigenetic mechanisms, miRNA suppression by oncogenic transcription factors, miRNA downregulation by loss of tumour suppressor transcription factors), post-transcriptional regulation. Collectively, the global changes in miRNA expression that are seen in cancer cells probably arise through multiple mechanisms; the combined small changes in the expression of many miRNAs seem to have a large impact on the malignant state.

2.4.1 Genetic alterations.

In 2004 it was reported that around 50% of miRNAs are located at fragile sites and cancer susceptibility loci (44). However since then, many more miRNAs have been identified and the relationship between site fragility and miRNA density seems far more complex than previously thought. More complete mapping of the human miRNA genes on fragile sites, cancer-specific translocation breakpoints, repetitive sequences and CpG islands has since been conducted and reveals that miRNA genes are indeed associated with fragile sites (45). Aside from structural genetic changes, somatic translocations of miRNA target sites have also been documented resulting in escape from regulation of an mRNA target by a specific miRNA (46). Potentially, mutations that alter a miRNA seed sequence could ablate target repression by tumor-suppressive miRNAs or allow for altered target selection, which could contribute to oncogenesis. Whereas naturally occurring sequence variations, such as SNPs (Single Nucleotide Polymorphism), have been shown to influence miRNA targeting in cancer-related pathways (47), tumor-specific mutations seem to be infrequent.

2.4.2 Transcriptional regulation.

Epigenetic mechanisms are also important for miRNA transcriptional regulation. Different approaches have shown that DNA methylation and histone deacetylase inhibitors can modify the expression of several miRNAs. The identification of miRNAs undergoing DNA methylation in a broad set of tumors, pointed out the importance of this process in miRNA downregulation and in the establishment of cancer programs. miR-124 and miR-34, well defined tumor suppressors, are subject to epigenetic silencing by aberrant DNA hypermethylation affecting cell cycle pathways in tumors (48). Furthermore, DNA methylation profiles in miRNA promoter regions can be useful as a diagnostic and prognostic marker. For example, miR-23b, a miRNA with tumor suppressor activity in prostate cancer, is downregulated through DNA hypermethylation of its promoter region and its expression level is correlated with overall survival and recurrence-free survival (49). Deregulated expression of miRNAs in cancer is also a consequence of alteration in histone marks (Figure 1A), which occur primarily due to the aberrant action of histone deacetylases and the Polycomb repressor complex (PRC2). For example, over expression of PRC2 in prostate cancer contributes to the repression of miR-101 and miR-205 by increasing the levels of H3K27me3 at their promoters. These alterations result in an increased rate of cell proliferation. In colorectal cancer, chromatin at promoter regions of tumor suppressor miRNAs show a closed configuration, producing a repressed transcriptional state (50). CTCF, another epigenetic factor, acts as a border that delimits the propagation of DNA methylation and histone repressive marks over different regulatory regions controlling gene expression. In different cancers, CTCF is lost, promoting repressive epigenetic mechanisms. Recent studies have shown that CTCF regulates miRNAs such as the tumor suppressor miR-125b1 and the oncogenic miR-375 in breast cancer cells (51). Finally, the same transcription factors that control mRNA expression can also regulate miRNA transcription (Figure 1A). For instance, the proto-oncogene *MYC* activates the miR-17–92 cluster and causes widespread downregulation of miRNA genes including tumor suppressor miRNAs such as miR-15a/16–1, miR-34a, and let-7 family members (52). It appears that the MYC-regulated miRNAs affect virtually all aspects of the MYC oncogenic program, including proliferation, survival, metabolism, angiogenesis, and metastasis (53). Besides MYC, other key proteins involved in transcriptional regulation, including E2F1 (54), ZEB1 (55) and p53 (56, 57) can also regulate the transcription of several miRNAs.

2.4.3 Post-transcriptional regulation.

The expression level of miRNA can also be modified as a result of defects in the miRNA biogenesis pathway. Deregulation of enzymes and cofactors involved in these pathways can affect the levels of mature miRNAs and have important biological ramifications.

In vivo studies have provided the most direct evidence of an active role for miRNA downregulation in at least some types of cancer. For example, analysis of mouse models in which the core enzymes of miRNA biogenesis have been constitutively or conditionally disrupted by different mechanisms suggests that these molecules function as haploinsufficient tumour suppressors. Thus, the repression of miRNA processing by the partial depletion of Dicer1 and Drosha accelerates cellular transformation and tumorigenesis *in vivo* (64). Furthermore, deletion of a single Dicer1 allele in lung epithelia promotes Kras-driven lung adenocarcinomas, whereas complete ablation of Dicer1 causes lethality because of the need for miRNAs in essential processes (58). Consistent with the potential relevance of these mechanisms, reduced Dicer1 and Drosha levels have been associated with poor prognosis in the clinic (59). In addition to the core machinery, modulators of miRNA processing can also function as haploinsufficient tumour suppressors. Hence, point mutations that affect TARBP2 or exportin-5 are correlated with sporadic and hereditary carcinomas that have microsatellite instability (60, 61). Moreover, accumulation of pri-miRNAs and corresponding depletion of mature miRNAs occurs in human cancers compared to normal tissues (62), strongly indicating an impairment of crucial steps in miRNA biogenesis.

Other miRNA modulators that influence the processing of only a subset of miRNAs could also be important. p68 (DDX5) and p72/p82 (DDX17) are prototypic members of the DEAD box protein family of RNA helicases. The isoforms p72 and p82 are transcribed from the *ddx17* gene and share identical RNA helicases functional properties (63). P68 and p72/82 have been shown to interact with each other and form heterodimers and although they share some functional overlaps, it is clear that they have also distinct roles (64). Both p68 and p72 are responsible for the processing of a subset of pri-miRNAs acting as a bridge between Drosha and other proteins. Indeed, several molecules, involved in different signalling pathways, (wtp53, SMAD, BRCA1, and YAP) have been described to bind p68 and/or p72/82 regulating Drosha/DGCR8 mediated miRNAs processing (Figure 2)(65-68). Interestingly, there are indications that miRNA processing can also be regulated by other miRNAs. The miR-103/107 family has been shown to target Dicer thereby reducing global miRNA levels (69). Elevated levels of miR-103/107 are seen in aggressive breast cancers and correlates with

increased metastatic potential. Mechanistically this is thought to occur by induction of epithelial-to-mesenchymal transition (EMT) through downregulation of miR-200 levels (69).

During carcinogenesis, point mutations can alter the function of protein coding genes resulting, for example, in activated oncogenes or deactivated tumour suppressors. This type of mechanism, being mutation of the seed sequence in the case of mature miRNAs, appears rare. However, sequence variation in the miRNA target sites present in the mRNA seemingly does occur allowing mRNAs to avoid binding and consequent negative regulation by miRNAs (Figure 1C). Indeed, there is evidence that this mechanism plays a role in tumorigenesis. Bioinformatic analysis of expressed sequence tag and single nucleotide polymorphism (SNP) databases has demonstrated differing allele frequencies of miRNA-binding sites in cancers compared to normal tissues (23). Specific incidences of this have been demonstrated experimentally, such as the case of let-7 and its oncogenic target HMGA2. In several tumours, the open reading frame and the 3′UTR that contains let-7 target sites have been found separated by chromosomal rearrangements at the *HMGA2* locus resulting in escape of HMGA2 from let-7 regulation, overexpression of the protein and promotion of tumor formation (reviewed in ref. 23). Alternative splicing or polyadenylation site usage represent other mechanisms that can generate alternative 3′UTRs in mRNAs, altering the occurrence of miRNA target sites (Figure 1C). Functionally, the truncated isoform of the proto-oncogene IGF2BP1 was shown to increase the frequency of oncogenic transformation more than expression of the full-length isoform (reviewed in ref. 23).

Accessibility of miRNA binding sites can also be regulated by the secondary structure of the mRNA molecule, which can be modulated through association of RNA binding proteins (Figure 1C). Interestingly, sometimes sequences in the vicinity of a miRNA target site are highly evolutionarily conserved. This led to the hypothesis that these sequences represent docking platforms for RNA binding proteins to act as modulators of miRNA activity. Finally, the emergence of other functional non-coding RNAs (ncRNA) has hinted at the possible complexity of the RNA-ome as a network of inter-communicating regulatory molecules.

An early study reporting the existence of a connection between other ncRNAs and miRNAs involved in cancer observed that a significant proportion of ultraconserved genomic regions code for specific set of ncRNAs whose expression is perturbed in human cancers and also regulated by aberrantly expressed miRNAs (70). Subsequently, the Competing Endogenous RNA (ceRNA) hypothesis was introduced, proposing that endogenous RNAs, for example protein coding and non-coding transcripts containing common miRNA recognition elements (MREs), can compete for the binding of a limited pool of miRNAs thereby co-regulating one

another (Figure 1C)(71). This hypothesis is exemplified experimentally by work on the tumour suppressor PTEN mRNA, where MREs are conserved in its related pseudogene PTENP1 (72). Overexpression of the PTENP1 3′UTR increased levels of PTEN and resulted in growth inhibition in a DICER-dependent manner. Interestingly,PTENP1 may be a tumour suppressor gene since copy number losses at the *PTENP1* locus were found to occur in sporadic colon cancer (72). Similar regulation by pseudogenes was observed for other important cancer genes like KRAS and its pseudogene *KRAS1P* (72).

As a consequence of these and other studies, more in-depth analysis of the role of pseudogenes and lncRNAs in cancer is being carried out since they can potentially act as potent tumour suppressors and oncogenes.

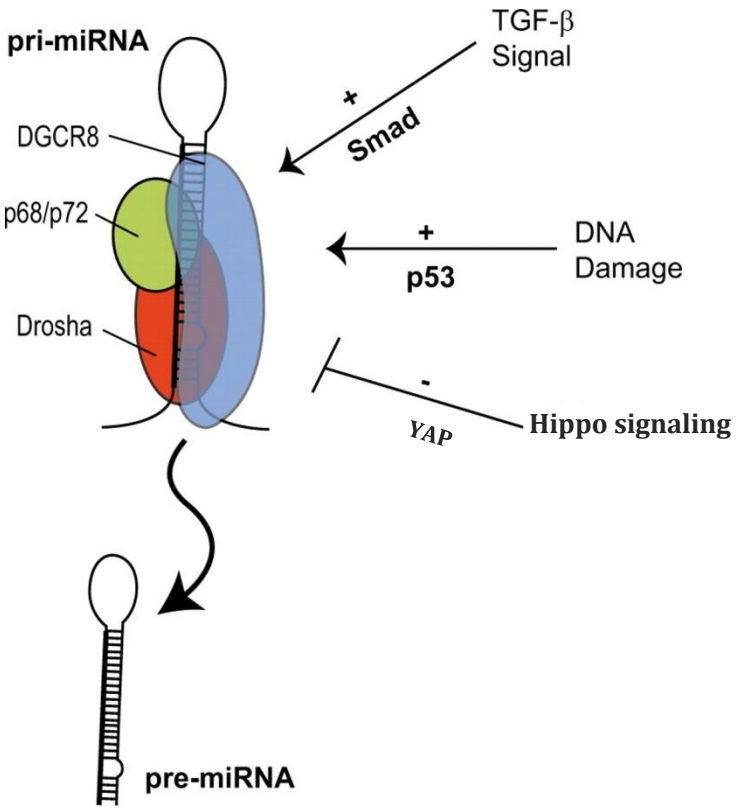

Figure 2. Post-transcriptional regulation of miRNA biogenesis in response to cellular signals.

(A) DNA damage (p53), Smads and BRCA, promote miRNA processing enhancing pre-miRNA production. Conversely, the Hippo signaling (YAP), in a cell density dependent manner, prevents the transition between pri-miRNA to pre-miRNA of a subset of miRNAs.

2.5 The tumor suppressor p53 is a master regulator of microRNAs.

The TP53 tumour suppressor is perhaps the most important and well-studied cancer gene, and it is not surprising that several studies have suggested that miRNA biology can have a role in its regulation and activity. The p53 protein acts as a sequence-specific DNA binding factor that can activate and repress transcription. Although there is no doubt that most of the actions of p53 can be explained by its ability to control canonical protein-coding targets such as CDKN1A and PUMA, it can also transactivates several miRNAs. Indeed, in the p53 tumor suppressor network, many of the functions normally associated with p53 may also be executed by miRNAs. As a transcription factor, p53 directly regulates the transcription of a growing number of miRNAs, acting both as a transactivator of tumor-suppressive miRNAs and a repressor of some oncogenic miRNAs. MiRNAs up-regulated by p53 often target anti-apoptotic and pro-proliferative genes, thus reinforcing the function of p53, or they may even feedback to regulate p53 itself (Figure 3). One of the best studied classes is the miR-34 family, which represses genes that can promote proliferation and apoptosis, plausible targets in a p53-mediated tumour-suppressor response (73). The discovery of additional p53-regulated miRNAs, and the targeting of p53 or its pathway by other miRNAs, has provided general insights into the miRNA-mediated control of gene expression and the potential therapeutic opportunities for targeting the p53 network. Several p53-activated miRNAs, such as miR-192, miR-194, miR-215 and miR-605, can target MDM2, which is a negative regulator of p53 and a therapeutic target. These potentially relevant miRNAs can be epigenetically silenced in some types of cancer; however, their reactivation or reintroduction offers an intriguing therapeutic opportunity for inhibiting MDM2 in tumours that harbour wild-type p53 (74, 75). Similarly, p53 can also activate miR-107, miR-200 or miR-192, which are miRNAs that inhibit angiogenesis and epithelial-to-mesenchymal transition (76-78). Conversely, p53 can be repressed by certain oncogenic miRNAs including miR-380-5p, which is upregulated in neuroblastomas with MYCN amplification, or miR-504, which decreases p53-mediated apoptosis and cell-cycle arrest and can promote tumorigenesis (79, 80).

Most research on p53 and miRNA focuses on the transcriptional effects of p53 on miRNA expression. However, p53 also has transcription-independent functions (Figure 2 and 3). Indeed, a recent study (68) suggests that p53's influence on miRNA might extend beyond transcriptional regulation. In HCT116 cells and human fibroblasts, p53 immunoprecipitated with the miRNA-processing protein Drosha through the DEAD-box RNA helicase p68. The

observed association of p53 with p68 correlated with an increase in the levels of several different mature and precursor miRNAs after DNA damage, including miR-16–1, miR-143, miR-145 and miR-206; primary transcript levels were unchanged. These miRNAs modulate the expression of cell proliferation and stemness associated genes, and their regulation by p53 suggests a role for p53 controlling global gene expression and cell fate. A similar post-transcriptional regulation of miRNA biogenesis involving p68/p72 has also been shown to be mediated by other nuclear proteins such as SMAD, BRCA1, and YAP (as described in section 2.4.3). In sum, these findings suggest a novel function of p53 in miRNA maturation and suggest that several transcription factors interact with the Drosha processing machinery to regulate miRNA expression.

2.6 MicroRNAs and mutant p53: more questions than answers.

More than 50% of human cancers carry mutations within the p53 locus (81). Mutant p53 (mutp53) proteins can acquire novel oncogenic functions known as gain of function activities (GOF) favouring *in vivo* tumor induction, maintenance and spreading in mouse models (82-84). GOF mutp53 proteins can exert their activities through both transcriptional regulation of target genes and by binding, sequestration and inactivation of tumor suppressor proteins (81, 85, 86). Numerous miRNAs are transcriptionally modulated by wtp53 (see section 2.6). In addition, wtp53 is able to interact with the Microprocessor complex and facilitates the post-transcriptional maturation of miRNAs with growth suppressive function (68). Recently, some miRNAs were described as transcriptional targets of mutp53 (87) whereas it is not yet clear if mutp53 plays a role in the regulation of miRNA processing.

Since tumors are characterized by downregulation of miRNA expressions and high incidence of mutp53 proteins, we asked whether a new GOF of mutp53 resides in its ability to inhibit expression of miRNAs at transcriptional and/or biogenesis level.

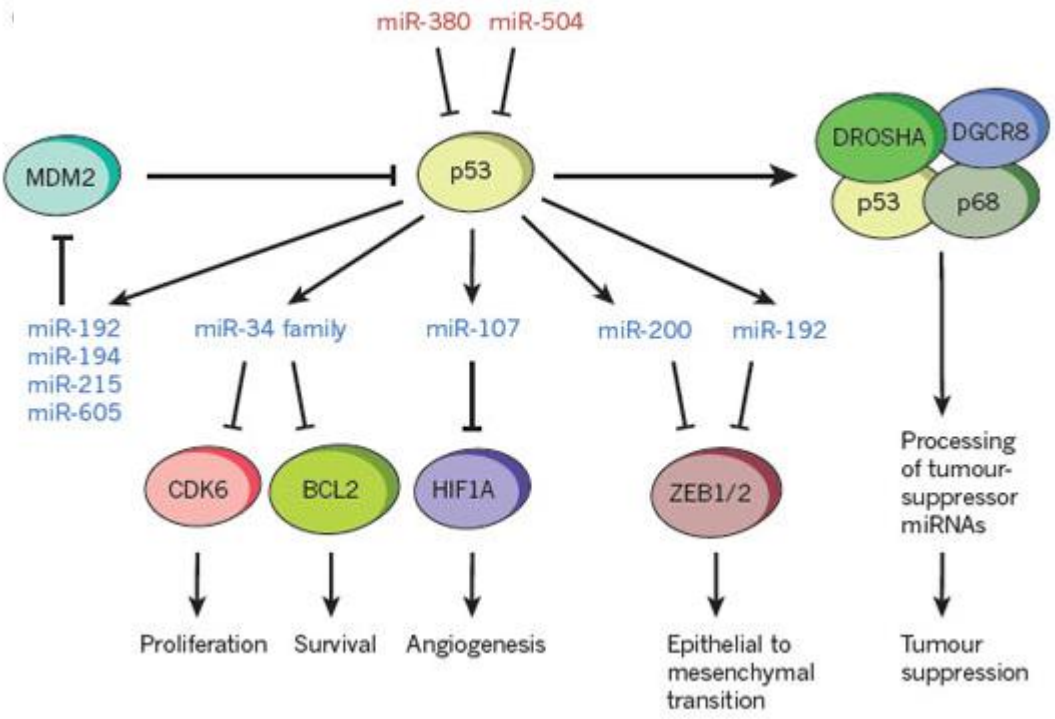

Figure 3. Contribution of miRNAs to cancer pathways (ref. 5).

Tumour suppressor p53 can regulate several tumour suppressor miRNAs (blue), activating different antitumoral pathways. The regulation of MDM2 by some of these miRNAs leads to interesting feedforward loops. At the same time, p53 can be negatively regulated by oncogenic miRNAs (in red). In addition, p53 is involved in the biogenesis of several tumour suppressormi RNAs.

3 RESULTS

3.1 Mutp53 inhibits mature miRNAs expression.

To identify miRNAs regulated by mutp53 we performed a genome wide expression analysis of 376 mature miRNA in SW480 cells before and after constitutive depletion of the endogenous mutp53 R273H. The cells were transduced with lentiviral vectors carrying either shRNAs specific for p53 (sh-p53) or shRNA scrambled control (sh-scr), and the modulation of mature miRNAs expression was investigated. The viral infection efficiently depleted the expression of mutp53 protein as measured by western blot (Figure 4A). The results revealed that 33 out of 376 miRNAs were upregulated (≥ 2 fold) after mutp53 depletion and only 4 downregulated (≥ 1,5 fold) (Figure 4B, 4C, 4D), strongly indicating, for the first time, that mutp53 may be responsible at least in part for the miRNAs downregulation observed in cancer. By qRT-PCR we validated the downregulation of 16 of these miRNAs (Figure 4E) in SW480 cells (miR-648 was non responsive). Some of the most strongly downregulated miRNAs in SW480 cells were also downregulated in another colon cancer cell line, HT29, harboring the same mutp53-R273H protein (Figure 5A, 5B). To investigate the role of different mutp53 proteins on miRNA expression, we also examined the effects of endogenous mutp53-R175H through its depletion on breast cancer cells SKBR3 (Figure 5A). Among the 16 miRNAs downregulated by mutp53-R273H in colon cancer cells, 9 were also downregulated in breast cancer cells in a mutp53-R175H dependent manner (Figure 5C). Interestingly, our analysis of miRNA expression (next-generation sequencing data from The Cancer Genome Atlas (TCGA))(88), in a large dataset of breast cancer samples (BRCA) expressing mutp53 with missense mutation, revealed that several miRNAs are downregulated in human breast cancer (Figure 5D), including 4 mutp53-dependent miRNA found on SKBR3 (miR-517a, -1, -143, -133b). To test the specificity of the observed effects, we restored mutp53 (R273H) expression on SW480-sh-p53 cells (Figure 5E) and observed a partial rescue of the downregulation of 10 miRNAs (Figure 5F). Consistent with the oncogenic role of mutp53, it has been demonstrated that several of the 33 miRNAs are downregulated in different types of human solid cancer and have tumor suppressor activity (miR-143, -218, -1, -517a, -519c, -515, 518b, 142-3p) (89-103). Of note few data are available on their role in colon cancer and their expression has not yet been associated with mutp53.

Taken together, our data demonstrate that different mutp53 proteins inhibit expression of a subset of miRNAs in several cancer cell lines. Since some of these miRNAs are already

described as oncosuppressors, these data also indicate that mutp53 GOF activity impacts on downregulation of their oncosuppressive function.

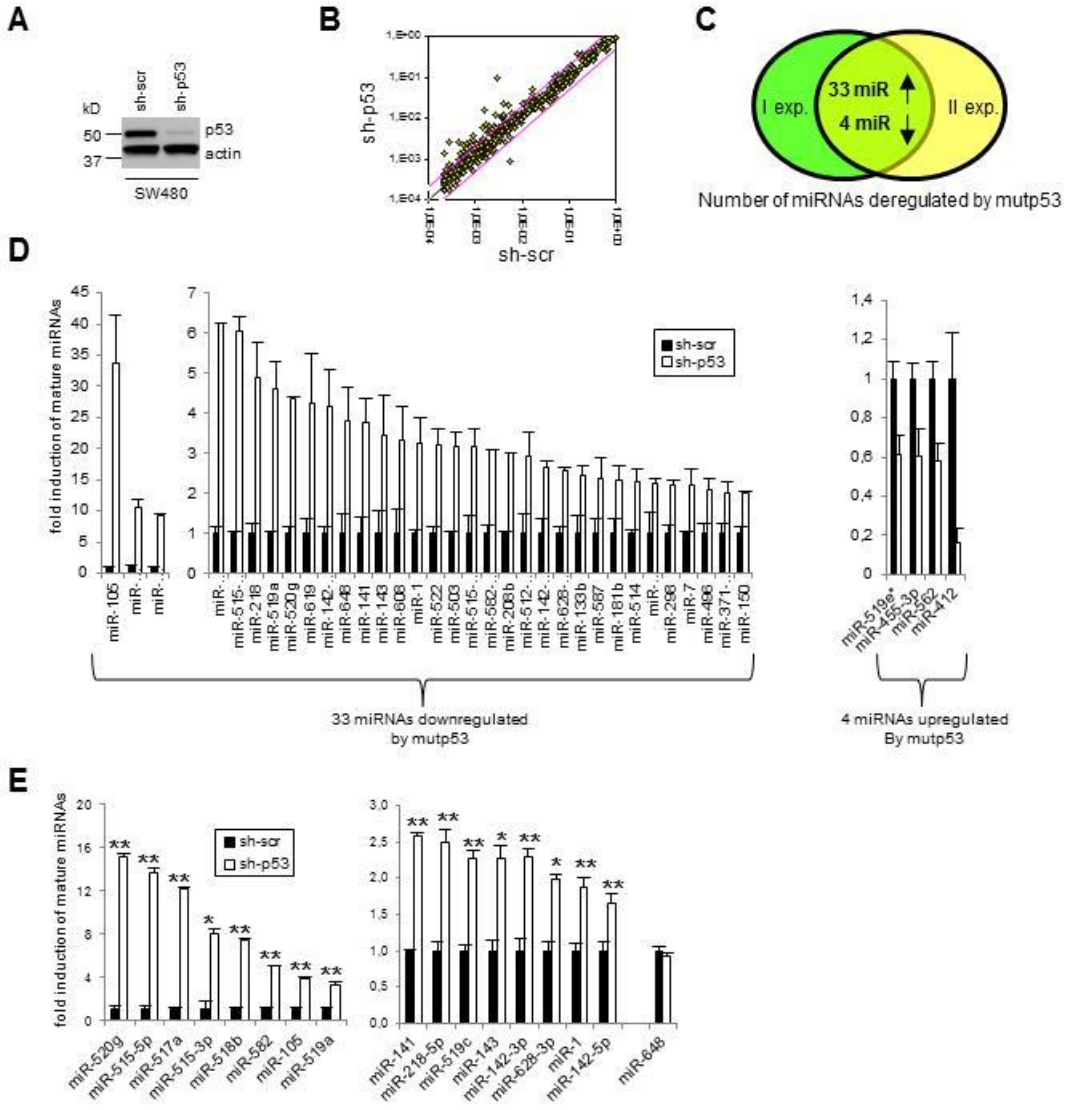

Figure 4. Widespread downregulation of miRNA by mutp53.

A)Western blot analysis performed on total lysates from SW480 (mutp53-R273H) after stable infection with either sh-p53 or control lentiviruses (sh-scr). B) Mean of 2 independent experiments of genome wide qRT-PCR array analysis of miRNA (super-array plate, SABiosciences technologies) in colorectal adenocarcinoma cell lines SW480 (mutp53-R273H/P309S) before (sh-scr) and after depletion of mutp53 (sh-p53) is showed in this

*scatter plot. The endogenous mutp53 expression was silenced through constitutive RNA interference (lentiviral infections). The results of 2 independent experiments are represented. Pink lines indicate threshold of 2. C) Number of miRNAs upregulated (≥ 2 fold) and downregulated (≥ 1,5 fold) by mutp53 in both array analysis are depicted in Venn diagram. I exp. = first experiment, II exp. = second experiment. D) qRT-PCR analysis of 33 miRNAs downregulated and 4 miRNAs upregulated by mutp53 on SW480 cells. Mean of 2 independent experiments of genome wide analysis of miRNA expression (super-array plate, SABiosciences technologies) is shown. The 37 miRNAs were divided into three graphs based on the levels of expression. E) Validation by qRT-PCR analysis of 17 mature miRNAs expression in SW480. Means of 6 independent experiments (infections) performed in triplicate are represented as fold induction where mature-miRNAs in sh-scr cells are = 1. miR-648 was non responsive. The 17 miRNAs were divided into two graphs based on the levels of expression. On figure 4D, E data were normalized to U6. *p≤0.05, **p≤0.005 student's t test.*

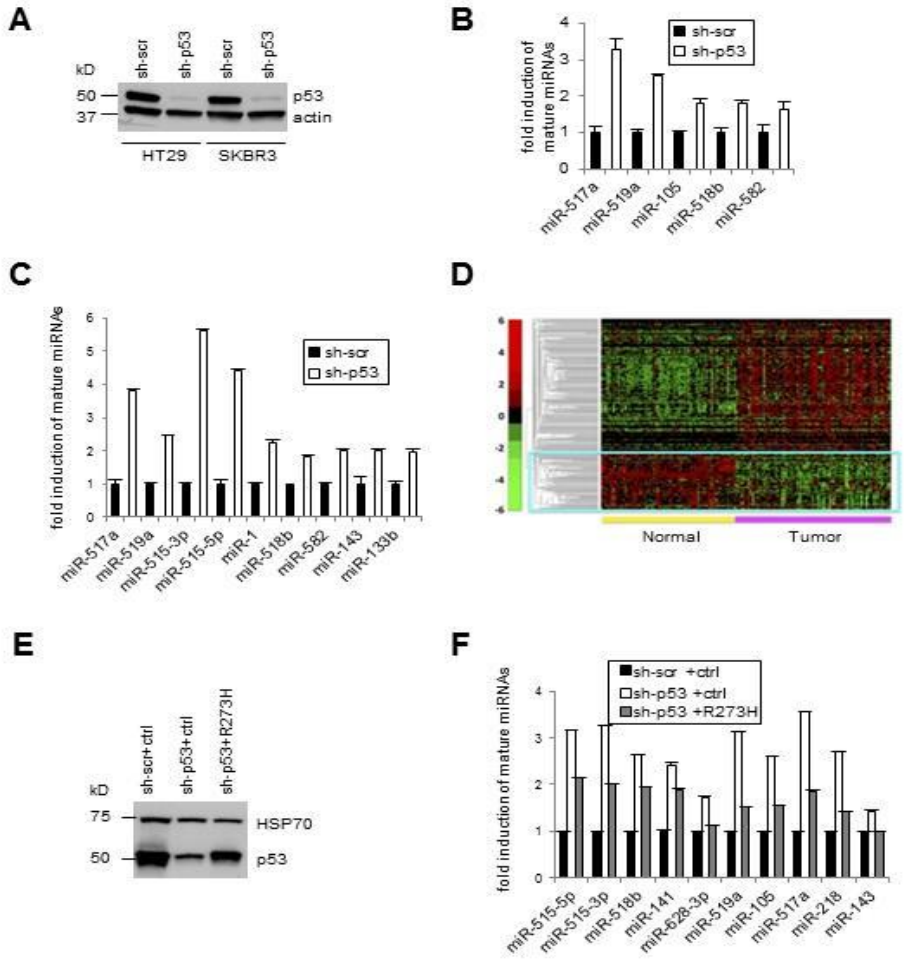

Figure 5. Widespread downregulation of miRNA by mutp53.

A)Western blot analysis performed on total lysates from HT29 (mutp53-R273H) and SKBR3 cells (mutp53-R175H) before (sh-scr) and after depletion of mutp53 (sh-p53). qRT-PCR analysis of 5 mature miRNAs expression in HT29 (B) and 8 mature miRNAs expression in SKBR3(C) cells before (sh-scr) and after depletion of mutp53 (sh-p53). n=2. qRT-PCR performed in triplicate. D) Hierarchical clustering of miRNA differentially expressed in breast carcinoma samples with missense mutation in the p53 gene (N=91) versus normal breast samples (N=80) obtained from TCGA. Relative miRNA expression values across samples are graphically rendered using a green-to-red scale for lowest-to-highest. E) Western blot analysis performed on total lysates from control cells (sh-scr+ctrl), cells stably depleted of mutp53 and transfected with control (sh-p53+ctrl) or mutp53-R273H (sh-p53+R273H) vectors. F) qRT-PCR analysis of 10 mature miRNAs in SW480 cells described on figure 5E. Means of 2 experiments performed in triplicates are presented as fold induction where mature miRNAs in SW480 cells transfected with control vector (sh-scr+ctrl) are = 1. On figure 5B, C, F data were normalized to U6.

3.2 Mutp53 negatively regulates miRNA biogenesis at transcriptional and post-transcriptional level.

To investigate the molecular mechanism by which mutp53 orchestrates miRNA expression, we measured the expression levels of pri-miRNAs and pre-miRNAs of the 16 most downregulated mutp53-dependent miRNAs identified above.

As shown in figure 6A and 6B some miRNAs were upregulated both at pri- and pre-miRNA levels after mutp53 depletion (left panels)(corresponding to mature miR-520g, -518b, -582, -141, -519c, -143, -142-5p, -142-3p) demonstrating a transcriptional level of regulation. However, several miRNAs showed no significant change in primary transcripts (Figure 6A, right panel)(corresponding to mature miR-517a, -519a, -105, -628, -1, -218, -515-5p, -515-3p), thus demonstrating that mutp53 regulates miRNA expression not only at transcriptional but also at post-transcriptional level. Moreover, the increase of pre-miR-517a, -519a-1, -105-1/2, -628, -1-2, -218-1 expression suggest that the mutp53 impacts on miRNA biogenesis at Drosha level (Figure 6B, righ panel). Interestingly the two pre-miR-218-2 and pre-miR-515-1/2 were not upregulated after mutp53 knockdown. Since their mature forms increase after mutp53 depletion this result suggests that these 2 miRNAs are mainly regulated at the level of Dicer processing. The analysis of pri- and pre-miRNA levels of miRNAs differentially expressed on SKBR3 and HT29 after mutp53 depletion identify, also in these systems, a post-transcriptional mechanism of regulation (Figure 6C, 6D).

Taken together, our results support a new key role of mutp53 in post-transcriptional regulation of diverse miRNAs.

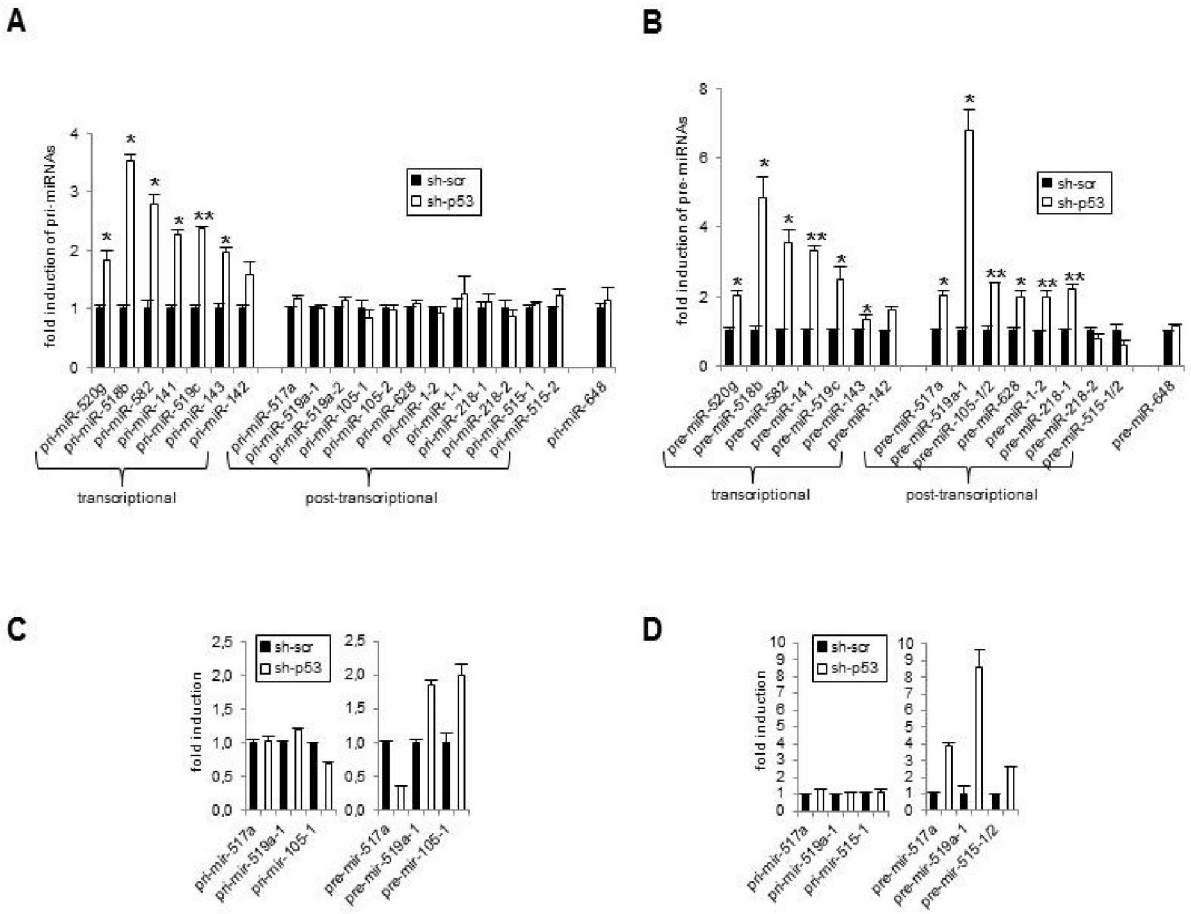

Figure 6. mutp53 plays a role both at transcriptional and post-transcriptional level.

qRT-PCR analysis of 20 pri-miRNAs (A) and 16 pre-miRNAs (B) expression, corresponding to 16 mature miRNAs of figure 4C, was performed in SW480 cells before (sh-scr) and after depletion of mutp53 (sh-p53). Means of 5 independent experiments (infections) performed in triplicate are represented as fold induction where pri-miRNAs and pre-miRNAs in sh-scr cells are = 1. Pre-miR-105-1/105-2 and pre-miR-515-1/515-2, transcribed from different genes, show a similarity in DNA sequences of 96% and 100% respectively, so it was not possible to discriminate. miR-648 is a negative control, it was non responsive to mutp53 depletion (figure 4E).

*qRT-PCR analysis of pri-miRNAs and pre-miRNAs expression in HT29 (C) and in SKBR3 cells (D), before (sh-scr) and after depletion of mutp53 (sh-p53). n=2, qRT-PCR performed in triplicate. *p≤0.05, **p≤0.005 student's t test.*

3.3 Mutp53 binds and sequesters p72/82 from Microprocessor causing an attenuation of miRNA maturation.

Since a mutp53-dependent regulation of pri-miRNAs processing has not been characterized yet, we decided to gain insight of this new mechanism. As mutp53 has an established role in the transcriptional control of genes, we first asked whether it regulates Drosha or DGCR8 mRNA and protein levels. We found that expression of Drosha and DGCR8 were not altered by mutp53 (Figure 7A, 7B), suggesting that the activity, not the quantity, of Microprocessor components may underlie the altered miRNA biogenesis. Thus, we addressed whether mutp53 modulates the *in vivo* binding of Drosha with pri-miRNAs. RNA-ChIP analysis shows that Drosha-pri-miRNAs association significantly increases after mutp53 depletion (pri-mir-517a, -519a1, -519a2, -105-1, -218-1) (Figure 7C), indicating that mutp53 inhibits the recruitment of Drosha to target pri-miRNAs and consequently leads to a downregulation of mature miRNA. To elucidate the molecular mechanism, we tested whether mutp53 might physically interact with Microprocessor components. We did not detect an association between the endogenous mutp53 and Drosha in co-immunoprecipitation experiments (Figure 7D) and confocal analysis (Figure 7F) in the nucleus. Interestingly, we found that mutp53 interferes with the assembly between endogenous Drosha complex and the DEAD-box RNA helicases p72/82. This interference is specific since it doesn't affect the Drosha-p68 binding (Figure 7D). It has been demonstrated that p72/82 and p68 are co-factor of the Microprocessor complex and are able to affect the regulation of processing of a subset of pri-miRNAs in response to different cellular signaling pathways (63-68). Therefore, we considered that mutp53 might bind p72/82 subtracting these proteins from the Drosha complex. By co-immunoprecipitation (Figure 7E) and confocal analysis (Figure 7F), we found that the endogenous mutp53 binds p72/82 in the nucleus. Consistent with this, RNA-ChIP analysis revealed that p72/82-pri-miRNAs binding significantly increases after mutp53 depletion (pri-miR-517a, -519a1, -519a2) (Figure 8A). Interestingly, mutp53 doesn't bind to pri-miRNAs indicating that mutp53-p72/82 complex is pri-miRNA independent. We further examined the role of p72 on mutp53-dependent miRNA repression testing whether forced expression of p72 could rescue the expression of these miRNAs (Figure 8A). Our results reveal that the overexpression of p72 in SW480 cells (Figure 8B), harboring the mutp53, leads to an increase of mature miRNAs (miR-517a, -519a, -218, -105) (Figure 8C) and a decrease of pri-miRNAs (pri-mir-517a, -519a1, -105-1, -218-1) (Figure 8D), demonstrating

that p72/82 positively regulates the processing of miRNAs downregulated at post-transcriptional level by mutp53.

Altogether, these results reveal a previously unrecognized function of mutp53 in miRNA processing in which mutp53, sequestering p72/82, interferes with a functional assembly between Drosha complex and these RNA helicases, causing a widespread downregulation of miRNAs.

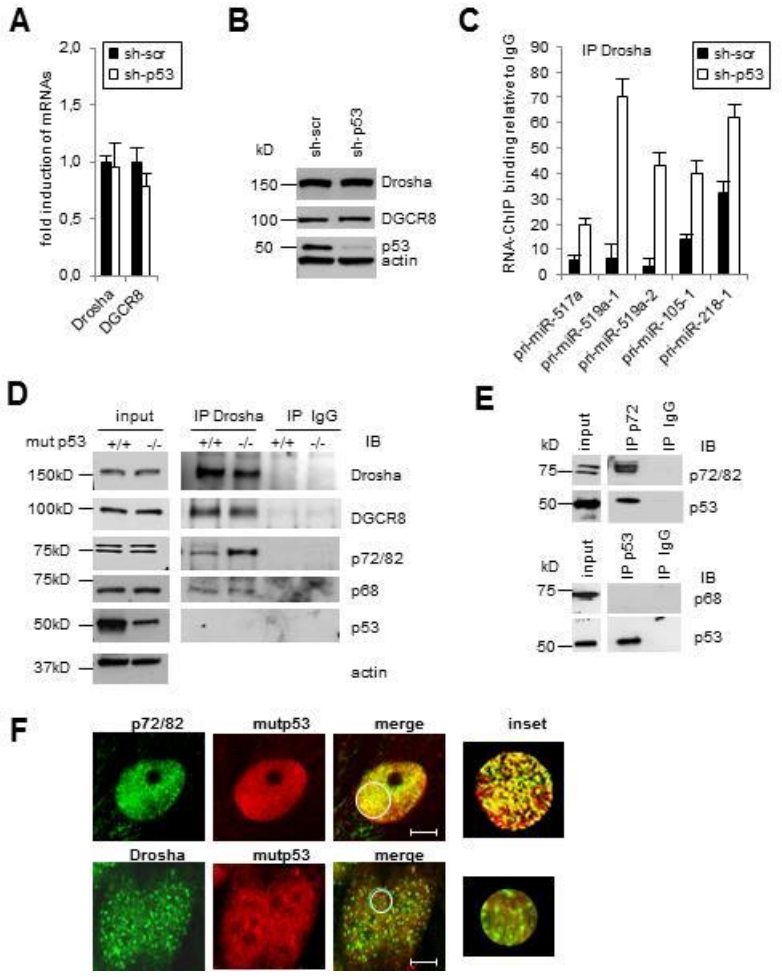

Figure 7. Drosha-pri-miRNAs association increases after mutp53 depletion.

Relative expression of Drosha and DGCR8 mRNAs (A) and protein levels (B) in SW480 cells (sh-scr) and (sh-mutp53). qRT-PCR and WB data were normalized to actin; C) RNA-ChIP analysis for the association between pri-miRNAs and Drosha in SW480 cells (sh-scr) and (sh-mutp53). Endogenous Drosha was immunoprecipitated followed by qPCR amplification with pri-miRNAs primers. One of 3 independent experiments performed in triplicate is represented as fold induction to control IgG. ***Mutp53 binds and sequesters p72 from Microprocessor complex.*** *Immunoprecipitation (IP) assays were performed with nuclear extracts of SW480 cells before (sh-scr) and after depletion of mutp53 (sh-p53) using an antibody against*

endogenous Drosha (D) and p72/p82 (E) proteins. IB, immunoblot. F) Colocalization (yellow) of endogenous mutp53 (red) with p72/p82 (green) and with Drosha (green) was analyzed by indirect immunofluorescence combined with Confocal Scanning Laser Microscopy. Confocal analysis of single optical section is shown. Bars 5 µm. The images have been collected with a 60x oil objective. One experiment of several is represented.

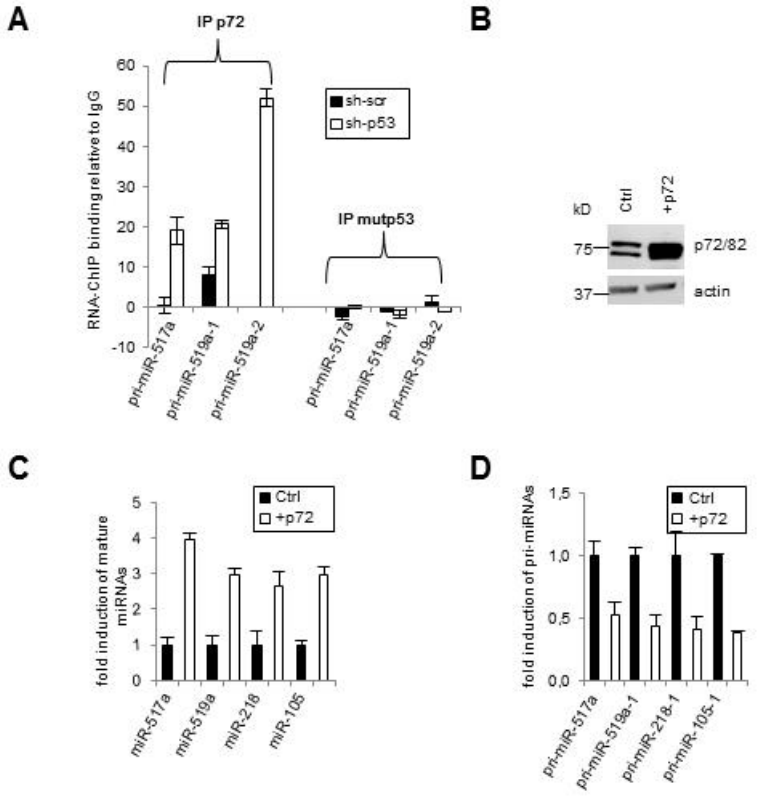

Figure 8. Overexpression of p72 induces mutp53-dependent miRNAs levels.

A) RNA-ChIP analysis for the association between pri-miRNAs, p72/p82 and mutp53 in SW480 cells before (sh-scr) and after depletion of mutp53 (sh-p53). Endogenous p72/p82 and mutp53 were immunoprecipitated followed by qPCR amplification with pri-miRNAs primers. One of 3 independent experiments performed in triplicate is represented as fold induction to control IgG. B) Western blot analysis performed on total lysates from SW480 cells after overexpression with either p72 expression vector (+p72) or control vector (ctrl). qRT-PCR analysis of mature miRNAs (C) and pri-miRNAs (D) expression in SW480 cells after transfection with control or p72 vectors. Means of 3 experiments performed in triplicates are presented as fold induction where mature miRNAs and pri-miRNAs in SW480 cells transfected with control vector are = 1. Data were normalized to U6.

3.4 Mutp53-regulated miRNAs inhibit cell growth and migration.

Our results show that, in mutp53-expressing cells a subset of miRNAs is downregulated, suggesting their tumor suppressor activity/ies. To investigate whether the above identified miRNAs have a negative effect on typical tumorigenic features such as cell cycle progression and migration, we transiently transfected SW480 cells with synthetic miRNAs (miRNA-517a,-519a, -218, -105) and with a negative control miRNA (miR-NC). Subsequently, we analyzed samples daily for cell number, cell viability and cell cycle profiles. Expression of miR-517a, -519a, -105, but not of miR-218 (data not shown), impaired, although to different extents, SW480 cell survival as indicated by the reduction of viable cell number and by the increase of trypan blue positive cells (Figure 9A, 9B, 9C). Moreover, the consistent reduction of the total cell number induced by the overexpression of miR-517a (Figure 9A, 9B) suggests that this miRNA impairs also the cell cycle progression. Cytoflurimetric analysis of cell cycle profiles (Facs) reveals a significant cell accumulation in S and G2/M phases induced by miR-517a (Figure 9D). In particular we observed a 3 fold-increase of cells in G2/M phase at 48 hours, and an accumulation of cells in S phase of 1,46 fold at 72 hours and 1,9 fold at 96h respect to control samples (Figure 9D, Figure 10A). On the contrary miR-519a and miR-105 had no appreciable effect on cell cycle (Figure 10B). Of note, the reduction of cell viability and cell cycle perturbation upon miR-517a overexpression is accompanied by a time dependent increase of cells in SubG1 peak, indicative of apoptosis (Figure 10A). We identified as an apoptotic event the cell death induced by miR-517a and miR-519a, as demonstrated by the induction of cleaved PARP, an hallmark of apoptosis and caspase activation (Figure 9E). These results are particularly significant for miR-517a, since they confer to this miRNA important potential tumor suppressive properties in terms of induction of apoptosis, yet unknown in colon cancer cells.

We also investigated the effect of these miRNAs on the migratory capability of SW480 cells, that are characterized by a marked mesenchymal phenotype important for tumor progression and metastasis. Interestingly, the transient overexpression of miR-519a and miR-218**,** individually**,** resulted in a decreased cell migration in a wound-healing assay (Figure 11A). Consistent with this, miR-519a and, to a lesser extent, miR-218 caused a reduction of Zeb1 and a concomitant induction of E-cadherin protein level (Figure 11B), thus suggesting a critical role of both miRNAs in colon cancer mesenchymal-epithelial transition (MET). miR-218 is a well known inhibitor of migration and invasion of metastatic cells in diverse kinds of solid tumors (101), though no evidence has emerged until now regarding colon cancer. Still,

any kind of oncosuppressor activity has been described for miR-519a until now. In our experimental condition transient overexpression of miR-517a and miR-105 doesn't affect cell migration (data not shown). Taken together, our data strongly indicate that these miRNAs are downregulated by mutp53 in order to de-activate tumor suppressive pathways.

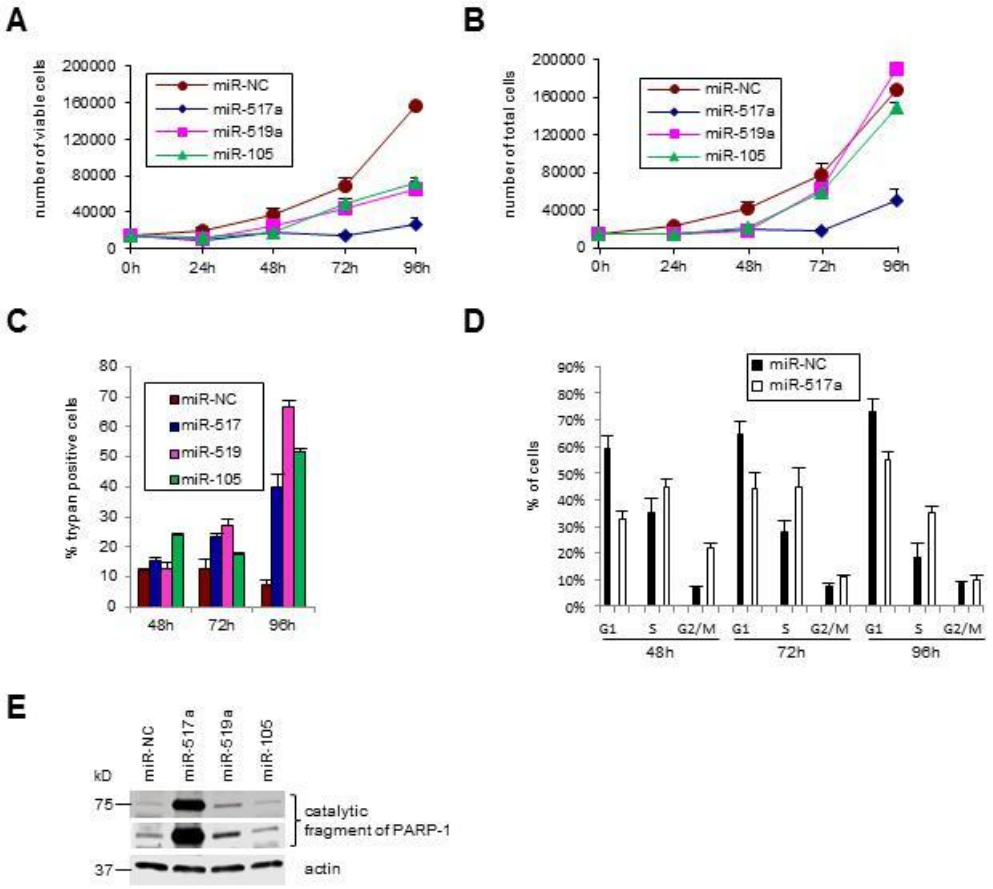

Figure 9. miR-517a impairs cell proliferation and survival.

SW480 cells transfected with the indicated synthetic miRNAs or a negative control miRNA (miR-NC) were plated in triplicate; viable cell number (A), total cell number (B) and % of trypan blue positive cells (C) were counted daily. Mean of 2 independent experiments are reported. D) FACS analysis of miR-517a or negative control miRNA (miR-NC) transfected SW480 cells. Mean of 3 independent experiments are reported. E) Protein extracts derived from SW480 cells at 96h after transfection with the indicated syntethic miRNA, were subjected to western blot analysis with the indicated antibodies. Two different exposure times are represented.

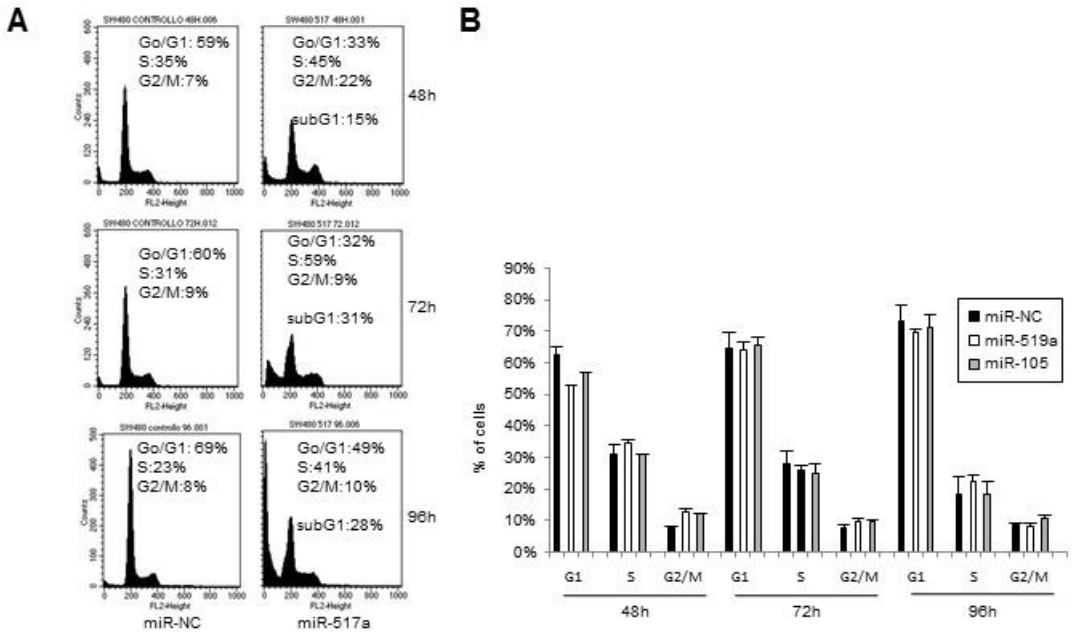

Figure 10. miR-517a impairs cell proliferation and survival.

A) FACS analysis of SW480 cells transfected with of miR-517a or negative control miRNA (miR-NC) in 3 indipendent experiments. B) FACS analysis of SW480 cells transfected with miR-519a, miR-105 or negative control miRNA (miR-NC). Mean of 3 independent experiments are reported.

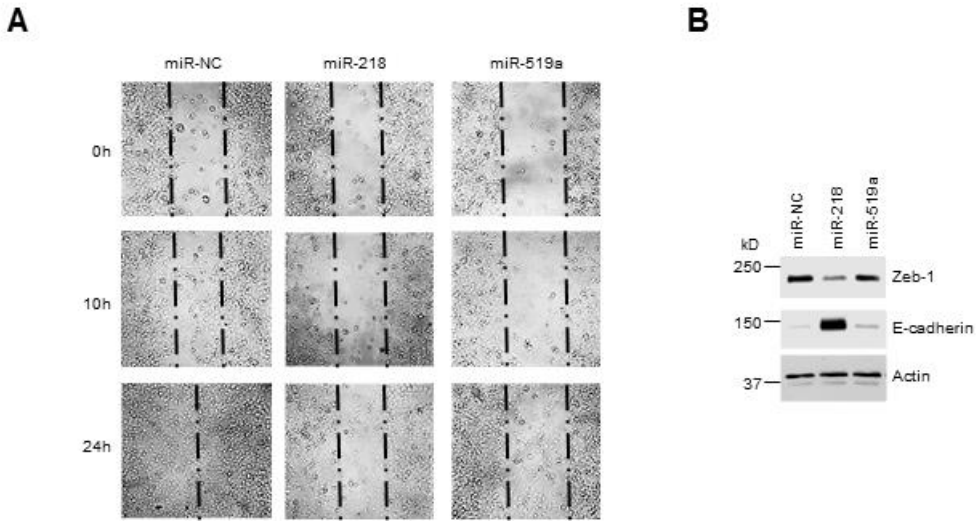

Figure 11. miR-519a and miR-218 inhibit cell migration.

A) Wound healing assay showing the reduced migratory capability of SW480 cells transfected with synthetic miR-519a and miR-218. 1 of 3 independent experiments is shown. B) Protein extracts derived from SW480 cells at 96h after transfection with the indicated syntethic miRNAs, were subjected to western blot analysis with the indicated antibodies.

4 CONCLUSIONS AND DISCUSSION

Here we reveal a previously unrecognized function of mutp53 in the regulation of miRNA processing, and provide, in part, a mechanistic explanation of the widespread downregulation of miRNAs observed in cancers. Our data of a genome wide analysis of miRNA expression revealed that mutp53 isoform R273H, which is frequently present in human tumors, downregulates 33 of 376 miRNAs analyzed in colon cancer cells. Moreover, we found that another missense mutp53, R175H, inhibits the expression of several of these miRNAs in breast cancer. These observations strongly point to a general mechanism that involves different p53 proteins with missense mutations and also indicate that the signature of miRNAs downregulated by mutp53 proteins in different solid tumors has, at least in part, common members. Further studies on a larger scale are required to identify miRNAs negatively downregulated by different mutp53 proteins in different tumor models. Interestingly, 4 of the miRNAs identified by us on SKBR3 cells are among those downregulated in the TCGA dataset of human breast cancers carrying missense mutations of p53, as compared to the normal samples. This last result strongly highlights the clinical relevance of our studies. Unfortunately, it was not possible to perform a statistically significant analysis with TCGA miRNA dataset of human colon cancers because only 8 normal tissues are profiled for miRNA and none of them is matched with the 107 tumor samples expressing p53 protein with missense mutations.

From a mechanistic point of view, we found that mutp53 downregulates miRNAs not only at transcriptional but also at post-transcriptional level. Indeed, endogenous mutp53 directly binds p72/82 hindering the association of this DEAD-box with the Microprocessor complex and pri-miRNAs, resulting in an inhibition of miRNA biogenesis (Figure 12). Moreover our analysis of pre-miRNA levels strongly suggests that mutp53 regulates miRNA biogenesis also downstream of the pri-miRNA maturation step, at least for pre-miR-515 and pre-miR-218-2. In this regard, it has been shown that mutp53 R273H can downregulate Dicer expression at protein level (102). Finally, we found that 4 mutp53-dependent miRNAs exhibit tumor suppressor functions such as cell death, cell cycle arrest and inhibition of cell migration, suggesting that mutp53 regulates proliferation and migration also through miRNAs inhibition. These data are in agreement with the literature and our previous results demonstrating that depletion of mutp53 reduces cell proliferation and *in vivo* tumorigenicity (103, 104). A miR-517a-dependent induction of apoptosis has been identified in bladder cancer cell line (105), whereas Rui-Fang Liu and colleagues described a G2/M cell cycle arrest induced by miR-

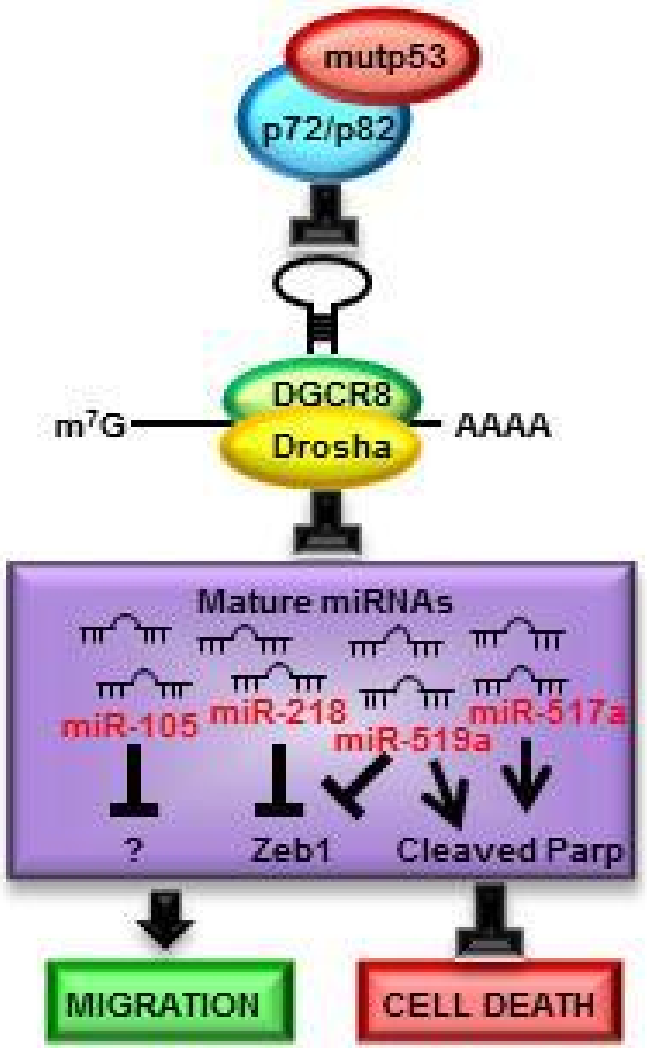

Figure 12. Proposed model.

Mutp53 binds and sequesters p72/82 from the Microprocessor complex, and inhibits the processing of tumor suppressor miRNAs.

517a in several hepatocellular carcinoma cell lines (94). Here we provide strong evidence that miR-517a suppresses cell growth, promotes apoptosis and induces a significant cell accumulation in S and G2/M phase in colon cancer cell line. Moreover we found important potential tumor suppressive properties of miR-519a yet unknown. Lastly, we found that both miR-519a and miR-218 inhibit EMT downregulating Zeb1 and subsequently upregulating E-cadherin protein levels. Interestingly, we identified 2 putative binding sites for mir-519a and 1 for miR-218 on the 3'UTR of Zeb1 gene, suggesting that this mutp53-dependent miRNA could regulate directly Zeb protein level. Our data strongly suggests that mutation of p53 can promote EMT and aggressive potential of tumor cells by inhibiting miR-519a and miR-218.

Mutp53 promotes various processes such as cell proliferation, chemoresistance, cholesterol metabolism, invasion, metastasis and various other tumour promoting events (81-83). Several effects of mutp53 proteins are mediated through their interaction and subsequent regulation of other factors such as TAp63, NF-Y, SP1 (18-25). Here we have found a new interaction between mutp53 and p72 that leads to an inhibition of miRNAs biogenesis and in turn the deregulation of gene expression. Interestingly, Mori and colleagues have recently demonstrated in an elegant paper, that the protein YAP, with oncogenic potential, regulates miRNAs biogenesis through sequestering p72 in a cell-density-dependent manner in cancer cells (67).

In conclusion, collectively our data demonstrate a global impact of mutp53 on miRNA biogenesis and suggest that miRNAs are downregulated by mutp53 in order to inactivate tumor suppressive pathways. Since mutations in TP53 occur at high frequency in human cancers, developing strategies to block the oncogenic effects of mutp53 will be an important step for their treatment. Moreover, miRNAs are powerful regulators of gene expression, thus the effects of miRNA downregulation by mutp53 are profound. A deeper understanding of the complex effects of mutp53-regulated miRNA overexpression in mouse models of cancers will be essential for deciphering the physiological function of miRNAs in mutp53 signaling and to allow new advances for therapeutic manipulation of miRNA regulated by mutp53.

5 MATERIALS AND METHODS

5.1 Cell culture and transfection.

Colon adenocarcinoma SW480 (mutp53R273H), colon adenocarcinoma HT29 (mutp53R273H) and breast carcinoma SKBR3 (mutp53R175H) human cell lines were grown in Dulbecco's modified Eagle's medium (GIBCO-BRL), supplemented with 10% FBS (GIBCO-BRL), L-glutamine (2mM), Penicillin (100 U/ml)/Streptomycin (100 ug/ml) (Life Technologies Inc.). PGS5-DDX17 vector, expressing p72 protein, was kindly provided by V. Sartorelli. SW480 were transiently transfected with Lipofectamine LTX following the manufacturer's instructions (Invitrogen).

5.2 Lentiviral vectors.

pLV-THM (sh-scr), pLV-THsi/p53 (sh-p53), were produced by transient trasfection in 293T cells according to standard protocols. Briefly, subconfluent 293T cells were cotransfected with 20 μg of a plasmid vector, 15 μg of pAX2 and 6 μg of pMD2G-VSVG by calcium phosphate precipitation (GIBCO-BRL). After 6–8 hours medium was replaced with fresh medium (6.0 ml/plate) supplemented with 1.0 mM Sodium Pyruvate (GIBCO-BRL). Lentiviruses were harvested 48 h later, centrifuged 5 min at 3,000 RPM, aliquoted and stored at -80°C. Lentiviral stocks were titered following standard protocols and routinely a viral titer of 100 transducing units per ml (TU/ml) was achieved.

5.3 RNA extraction, cDNA synthesis and RT-qPCR.

qRT-PCR assays were performed to measure the expression levels of pri-miRNAs, pre-miRNAs, mature miRNAs and mRNAs. Total RNA was extracted using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. Reverse Transcription of mature miRNAs expression was performed by TaqMan MicroRNA RT assay. For pri-miRNAs, pre-miRNAs, and mRNA expression, RNA was retro-transcribed with Moloney-Murine-Leukemia virus reverse-transcriptase (M-MLV-RT, Invitrogen) following the manufacturer's instruction. Quantitative PCR, determined on an ABI Prism 7900 apparatus (Applied Biosystems), was performed using SYBR FAST UNIVERSAL READYMIX (Kapa) for pri-miRNAs, pre-miRNAs, and mRNA; KAPA PROBE FAST QPCR UNIVERSAL MASTER

MIX (Kapa) was used for mature miRNAs reactions. Experiments were done on triplicate and the results were estimated based on the comparative threshold ($2^{-\Delta Ct}$). RNU6 was used as endogenous control to standardize mature miRNA, pri-miRNAs and pre-miRNAs expression, beta-actin was used for mRNA normalization.

The primers sequences of pri-miRNAs, designed within 200bp upstream the pre-miRNA sequence, were:

pri-miR-520g forward 5'-CCCTTGATGAACAAGGCTAACC-3'; pri-miR-520g reverse 5'-CCCTGAAAGGACCATCTCCAATT-3'; pri-miR-515-1 forward 5'-AGGATGGTCGTGGTCTCTTGAC-3'; pri-miR-515-1 reverse 5'-TGGCTCACGCCTGGAATC-3'; pri-miR-515-2 forward 5'-GTCTCTTGATGAACAAGGCTAACCT-3'; pri-miR-515-2 reverse 5'-CCCTAAAAGGACCATCTCCAGTT-3'; pri-miR-517a forward 5'-TGGTGGTGGGTGCCTCTAAT-3'; pri-miR-517a reverse 5'-TGGGTTCAAGCGATTCTTCTG-3'; pri-miR-518b forward 5'-GCCTTGATGAACAAGGCTAACC-3'; pri-miR-518b reverse 5'-CCCTAAAAGGACCATCTCCAGTT-3'; pri-miR-582 forward 5'-GTCATTCATGCACACATTGAAGAG-3'; pri-miR-582 reverse 5'-TCTACTAGAGAGAGATTTGCTAGTGGTGTT-3'; pri-miR-519a-1 forward 5'-ACAGTCATGGTTTCACCATGTTG-3'; pri-miR-519a-1 reverse 5'-GGCGGGAGGATCACTTGAG-3'; pri-miR-519a-2 forward 5'-TGGAAATGGTCCTTTTAGGGATT-3'; pri-miR-519a-2 reverse 5'-GGCCCTGTTTGCGTGTTT-3'; pri-miR-105-1 forward 5'-CATGATTTTTTGTCATA CAGTGTGTTTG-3'; pri-miR-105-1 reverse GAAGCTGACATCCAATATATAGGAGTAGATA-3'; pri-miR-105-2 forward 5'-TTTCTACACCATGTCTTTCACTATTGTG-3'; pri-miR-105-2 reverse 5'-GATACCAGGTTATAGGGAGTAGACAGATG-3'; pri-miR-141 forward 5'-CCACCCAGTGCGATTTGT-3'; pri-miR-141 reverse 5'- CCAACCCCAAGCTCAAGGT-3';

pri-miR-218-1 forward 5'-CGGGAAGAATGCATGTCATG -3'; pri-miR-218-1 reverse 5'-GGAGGGAAGGAGGAGAAAAGC-3'; pri-miR-218-2 forward 5'-GCAATCTTCGGAAGTGTTCCA-3'; pri-miR-218-2 reverse 5'-CCCCCACTGAGCGTGATTAG-3'; pri-miR-519c forward 5'-CTGGAGATGGCCCTGATAGG-3'; pri-miR-519c reverse 5'-

CATTTATTGGCCCTGTTTGCA-3'; pri-miR-143 forward 5'-TTGGTCCTGGGTGCTCAAAT-3'; pri-miR-143 reverse 5'-GGAGGCTGTTGTAATTCCTCACA-3'; pri-miR-142 forward 5'-GAAGAGGGAACTGAAGAGGAAGTGG-3'; pri-miR-142 reverse 5'-TGGGCGGTGACTCAGCAT-3'; pri-miR-628 forward 5'-TTCCTGGACTTGGGATTTCTTG-3'; pri-miR-628 reverse 5'-TCTGGTGCTGCTCCTTTATGAA-3'; pri-miR-1-1 forward 5'-GAGGGCTCCGGCAGTAGACT-3'; pri-miR-1-1 reverse 5'-TGACCCCACTGCGATGTAGA-3'; pri-miR-1-2 forward 5'-TGTCTATGTCCACCAAGAATATGGA-3'; pri-miR-1-2 reverse 5'-TTTGCAGAGACTAATTTATCGAAGACTAA-3'; pri-miR-648 forward 5'-TGTGTGCTGCATATCTTTTATATCAGTAA-3'; pri-miR-648 reverse 5'-TGCTGGCAAATGCTTAACAGA-3'; U6 forward 5'-CTCGCTTCGGCAGCACA-3'; U6 reverse 5'-AACGCTTCACGAATTTGCGT-3'.

The primers sequences of pre-miRNAs were:

pre-miR-520g forward 5'-AGAGGAAGCACTTTCTGTTTGTTGT-3'; pre-miR-520g reverse 5'-AAGGGAAGCACTTTGTTTTTTCTC-3'; pre-miR-515-1/2 forward 5'-TCATGCAGTCATTCTCCAAAAGA-3'; pre-miR-515-1/2 reverse 5'-AAAAGAAGGCACTCTGCTTTCAG-3'; pre-miR-517a forward 5'-CCTCTAGATGGAAGCACTGTCTGTT-3'; pre-miR-517a reverse 5'-GGGATGCACGATCTTTTCTTTT-3'; pre-miR-518b forward 5'-GCTGTGGCCCTCCAGAG-3'; pre-miR-518b reverse 5'-GGGAGCGCTTTGTTTTCTTT-3'; pre-miR-582 forward 5'-CTCTTTGATTACAGTTGTTCAACCAGTT-3'; pre-miR-582 reverse 5' -AATGTTTCTACTTTGCACCC-3'; pre-miR-519a-1 forward 5'-GCTGTGACACTCTAGAGGGAAG-3'; pre-miR-519a-1 reverse 5'-TCAAACAGTAACACTCTAAAAGGATGCA-3'; pre-miR-105-1/2 forward 5'-TGTGCATCGTGGTCAAATGCT-3'; pre-miR-105-1/2 reverse 5'-CCGTAGCACATGCTCAAACATC-3'; pre-miR-141 forward 5'-GGTCCATCTTCCAGTACAGTGTTG-3'; pre-miR-141 reverse 5'-CCGGGAGCCATCTTTACCA-3'; pre-miR-218-1 forward 5'-GCGAGATTTTCTGTTGTGCTTG-3'; pre-miR-pre218-1 reverse 5'-ACTCATACCTCGCAACCACATG-3'; pre-miR-218-1 forward 5'GGCTTTCCTTTGTGCTTGATCT-3'; pre-miR-218-2 reverse 5'-

TTCCGTTTCCATCGTTCCAC-3'; pre-miR-519c forward 5'-TCTCAGCCTGTGACCCTCTA-3'; pre-miR-519c reverse 5'-TTCAGACAACAGAAAGCGCT-3'; pre-miR-143 forward 5'-CTGTCTCCCAGCCTGAGGTG-3'; pre-miR-143 reverse 5'-AGACTCCCAACTGACCAGAGATG-3'; pre-miR-142 forward 5'-CAGTGCAGTCACCCATAAAGTAGAA-3'; pre-miR-142 reverse 5'-CACCCTCCAGTGCTGTTAGTAGTG-3'; pre-miR-628 forward 5'-CACTTCCTCATGCTGACATATTTACTAGA-3'; pre-miR-628 reverse 5'-CACTCTTACTAGAAGGTTATTAATTTTACCCTCT-3'; pre-miR-1-2 forward 5'-ACCTACTCAGAGTACATACTTC-3'; pre-miR-1-2 reverse 5'-GCATTCTATGTTCATATGGGTAC-3'; pre-miR-648 forward 5'-ATCACAGACACCTCCAAGTG-3'; pre-miR-648 reverse 5'-TCACTTCCGACTAAGTGCCA-3'

The primers sequences of mRNAs were:

hDrosha forward 5'-TGGATGCGCTTGAAAAATATA-3'; hDrosha reverse 5'-TCGATGAACCGCTTCTGATG-3'; h-beta-actin forward 5'-TCACCCACACTGTGCCCATCTACGA-3'; h-beta-actin reverse 5'-CAGCGGAACCGCTCATTGCCAATGG-3'; hDGCR8 forward 5'-GGAAAGGGAGGAGACTCGAAA -3'; hDGCR8 reverse 5'- GCGGACGCCACAATGG -3'.

5.4 Western blotting.

Cells were washed twice in ice-cold PBS, harvested by scraping with 1X RIPA buffer (150 mMNaCl, 1 % TritonX100, 0.25 % Sodium deoxycholate, 0.1 % SDS, 50 mM Tris/HCl pH 8.0, 20 mM EDTA) supplemented with 1X protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Lysates were incubated 30 min in ice, clarified by centrifugation 20 min at 14000 RPM, and resolved onto SDS-PAGE. Blotting was performed according to standard protocols and PVDF filter was immuno-reacted with following antibodies: mouse monoclonal anti-p53 (DOI, Blandino et al., 1999), rabbit anti-Drosha (Cell Signaling, D28B1), rabbit anti-DGCR8 (Abcam, ab90579), mouse anti-p68 (Millipore, clone PAb204), rabbit anti-DDX17 (Abcam, ab 24601), mouse anti-HSP70 (StressGen, N27F34), mouse anti-actin (Ab-1, Calbiochem), rabbit anti-TCF8/ZEB1 (Cell Signaling, D80D3), mouse anti-E-Cadherin

(Trasduction Laboratories, 610182). Signals were detected by ECL detection reagents (Pierce).

5.5 Co-immunoprecipitation.

Cells were lysed on ice with Yoshi A buffer (10mM Tris HCl pH8; 1,5 mM MgCl2; 10mM KCl, 0,5 mM DTT), supplemented with protease inhibitors (Roche) by passing cell suspension through a 26-G needle several times. After 30 min on ice and centrifugation (3000 rpm, 5 min at 4 °C), the nuclei fraction was re-suspended in nuclei lysis buffer (50mM Tris HCl pH8, 15mM MgCl2, 5mM KCl, 5mM EGTA, 150mM NaCl, 2mM EDTA, 0,1% NP40). Lysates (1 mg/sample) were clarified (13000 rpm, 10 min at 4 °C), precleared with Pierce Protein-G Agarose (Pierce) and incubated overnight at +4 °C with antibody-protein-G complexes previously crosslinked by DMP Dimethyl Pimelimidate Dihydrochloride (Sigma). 4ug of the following antibodies were used: rabbit anti-Drosha (ab12286, Abcam), mouse anti-DDX17 (C-9) (Santa Cruz, sc-271112), sheep polyclonal serum anti-p53 Ab7 (PC35, Calbiochem), polyclonal anti rabbit (Millipore, # 12370) and polyclonal anti mouse igG (Millipore, # 12371). After washes (5x) in cold lysis buffer, samples were analyzed by Western Blot, with the antibodies described in western blotting section.

5.6 RNA-ChIP.

SW480 cells were crosslinked for 15 min with 1% formaldehyde, the cell pellet was resuspended in lysis buffer (5mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40), incubated in ice for 10 min, homogenized, and pelleted by centrifugation. The nuclei fraction was then resuspended in sonication buffer (50 mM Tris at pH 8.0, 10 mM EDTA, 1% SDS, protease inhibitors). After, nuclei were disrupted by sonication, the lysates were diluted 10 times, precleared by adding protein G for 1 h at 4°C, and then incubated with 5ug rabbit polyclonal anti-Drosha (ab12286, Abcam) and mouse monoclonal anti-DDX17 (C-9) (Santa Cruz, sc-271112) antibodies overnight at 4°C with mild shaking. Immunoprecipitation was performed with protein G-agarose (Pierce), previously blocked with 1 μg/μl sheared herring sperm DNA and 1 μg/μl bovin serum albumin (BSA), for 3h at 4°C. After stringent washing and elution, the samples were heated at 65°C for only 2h to reverse formaldehyde cross-links, and then ethanol precipitated. The precipitated DNA/RNA was treated with 10mM, 40mM

Tris-HCl pH6.5, 20μg of proteinase K (Roche Diagnostics), and then extracted with Trizol reagent and ethanol precipitated. The RNA pellets were resuspended in 20 μl of RNase free water and digested with DNAse (Sigma) for 45 min at 37°C. 20μl of RNA was used for a 30 μl cDNA synthesis reaction. Quantitative PCR reactions were then performed by real-time PCR machine (Applied Biosystem). The primers sequences of pri-miRNAs are shown above, in RNA extraction, cDNA synthesis and RT-qPCR section.

5.7 Transfection of miRNA mimics.

For miRNA overexpression, SW480 cells were transiently transfected with synthetic miRNA mimics -517a, -519a, -218, -105 or miRNA mimic negative control (miR-NC), that contains a scrambled sequence and does not specifically target any human gene products (Dharmacon), at a final concentration of 20 nM using Lipofectamine RNAimax Transfection Reagent (Life Technologies).

5.8 Cell growth assay and flow cytometry.

SW480 cells were transfected with the indicated miRNA mimics (Dharmacon) and after 24h were plated in 24 well plates at low density (15000 cells per well). Cells were harvested at the indicated time points and cell number in triplicate was determined in duplicate, with a Thomas hemocytometer. Lethality was determined by counting the number of cells stained with trypan blue. Cell-cycle was monitored at the same time points by FACS analysis: cells were harvested, washed in PBS, and fixed in MetOH:acetic acid solution (4:1) for 60 minutes at +4°C. Cells were then incubated in 500 μL of staining solution (50 μg/mL of propidium iodide, 50 μg/mL of RNAase, 0.1% Triton X-100 in PBS 1×) for 1 hour at 4°C and analyzed by flow cytometry. 4 indipendent experiments were performed in triplicates.

5.9 Wound-healing assay.

For the wound healing assays, SW480 cells were plated in 35 mm plates and transfected with the indicated miRNA mimics. After 48h cell monolayers were wounded with a sterile tip and maintained in 10% serum medium. Images were taken after 0, 10 and 24 hours after wounding. 3 independent experiments were performed.

5.10 Double Immunofluorescence labeling.

Cells were fixed for 10 minutes with 2% (w/v) formaldehyde in PBS, then permeated with 0,05% Triton X-100 and blocked 1h with 5% bovin serum albumin (BSA). The following primary antibodies (diluted in 1% BSA) were used: rabbit anti-DDX17 (Abcam, ab 24601) and rabbit anti-Drosha (ab12286, Abcam) overnight, sheep polyclonal serum anti-p53 Ab7 (PC35, Calbiochem) 1h at room temperature. The following secondary antibodies (diluted in 1% BSA) were used: Cy3-conjugated donkey anti-mouse and Cy2-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories). Each secondary antibody was incubated for 1h at room temperature. The DNA was counterstained with 0.4 mg/ml 49, 69-diamidino-2-phenylindole (DAPI) (Sigma) (data not shown). Slides were mounted in 50% glycerol and analyzed within 24 h. As control, single immunofluorescence labeling for each antibody, and immunofluorescence labeling where the primary antibody was omitted were performed (data not shown).

5.11 Confocal Scanning Laser Microscopy.

All experiments were performed at least 4 times in duplicate. For each experiment, 100 nuclei were visualized. Images were recorded by using a Zeiss LSM 510 Meta confocal laser scanning microscope equipped with a 60X/1.23 NA oil immersion objective. Ar laser (488 and 514 nm), and HeNe laser (543 nm) were used to excite the fluorophores. Emitted fluorescence was detected with a 505- to 530-nm bandpass filter for the green signal and a 560-nm long-pass filter for the red signal. The LSM 510 R. 3.2 META (Zeiss) image analysis software was used.

5.12 miRNA PCR array analysis.

Human RT2 miRNA PCR Arrays (MAH-3100) (http://www.sabiosciences.com/mirna_pcr_product/HTML/MAH-3100A.html) were obtained from SA Biosciences. Two hundred ng of isolated RNA, from SW480 (sh-scr) and SW480 (sh-p53), were used for reverse transcription and the entire first strand cDNA was diluted and distributed amongst the 384 wells of the super-array plate. The reactions were performed with RT² SYBR Green / ROX PCR Master Mix (SABiosciences). Results were analyzed by the vendor .

5.13 Hierarchical clustering of breast cancer samples based on miRNA expression data.

We first downloaded miRNA sequencing data as well as clinical data of breast carcinoma (BRCA) and normal breast samples from TCGA data portal (https://tcga-data.nci.nih.gov/tcga/). Next, we selected 91 BRCA samples, carrying missense mutation in the p53 gene, and 80 normal breast samples and we analyzed the expression profile of 1047 miRNAs across these samples. We found 475 miRNAs as differentially expressed between the two groups (474 miRNAs, Student's t-test pvalue < 0.05; miR-517a, Student's t-test pvalue = 0.075, after correction for multiple testing performed with False Discovery Rate method). In order to facilitate interpretation of the data structure, we used the hierarchical clustering expression profiles of 475 miRNAs. We used the Euclidean distance as a measure of dissimilarity between the expression profiles of miRNAs. Then, we used a dendogram (or clustering tree) as a graphical representation of the resulting matrix of distances and a heatmap where different colors represent different values of the pair distances. The final dendrogram is displayed in supplementary figure 1D and represents a compact visualization of the dissimilarity matrix between miRNA expression profiles. We can see that there are two main clusters: in one cluster the miRNAs appear generally downregulated in normal tissues and upregulated in breast cancer tissues, while the other cluster encompasses miRNAs that show an opposite trend.

5.14 Statistical analysis.

Numerical data were reported as means of results of separate experiments. Significance

was assessed by Student’s t test analysis. Values at $p \leq 0,05$ were considered to indicate significant differences.

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