Dysregulation of microRNA biogenesis in cancer: the impact of mutant p53 proteins

33

Francesca Garibaldi[1], Carmen Greco[1], D Trisciuoglio[2], Teresa Colombo[3], Kamil Lisek[4], Dawid Walerych[4], Giannino Del Sal[4], Paola Paci[3], Gianluca Bossi[5] ,Giulia Piaggio[1], Aymone Gurtner[1]

[1]IRCCS Regina Elena National Cancer Institute, UOSD SAFU, Rome Italy.[2]IBPM, National Research Council, Rome, Italy. [3]Institute for Computing Applications "Mauro Picone", National Research Council, Rome, Italy.[4]Laboratorio Nazionale CIB, Area Science Park Padriciano, Trieste, Italy.[5]IRCCS Regina Elena National Cancer Institute, UOSD Medical Physics, Rome Italy.

Abstract

The TP53 tumor suppressor gene is mutated in half of human tumors resulting in an oncogene with Gain-Of-Function (GOF) activities. The global miRNA deregulation observed in human cancers is often the result of defects in the miRNA biogenesis pathway. Only a handful of miRNAs have been identified as direct targets of mutant p53 protein (mutp53) at transcriptional level, and very few data about the role of mutp53 on deregulation of miRNA biogenesis in cancer are available yet (Falcone et al., 2016; Gurtner et al., 2017).We have previously unveiled a new mechanism by which mutp53 regulates miRNA biogenesis at posttranscriptional level impacting on microprocessor complex activity and inhibiting the processing of pri-miRNA in pre-miRNA. Briefly, our data of a genome wide analysis of miRNA expression in colon cancer cell line, before and after depletion of mutp53 (R273H), revealed that this oncogene downregulates 33, and upregulates 4 of 376 miRNAs analyzed (**Figure 1)**. Several of these miRNAs were validated in breast cancer cell lines. Interestingly, interrogating the TCGA database about miRNA expression levels in samples of breast cancer patients, we found that our mutp53-dependent miRNAs are downregulated in cancer samples expressing mutp53, too (**Figure 1C**). From a mechanistic point of view, by confocal analysis, co-immunoprecipitation experiments (**Figure 2A,B,C**) and RNA-IPs (**Figure 2D,E**) we demonstrate for the first time that endogenous mutp53 directly binds to the RNA helicase p72 in the nucleus of colon and breast cancer cells thus preventing its association to Drosha and pri-miRNA targets, resulting in the inhibition of miRNA biogenesis (**Figure 3**). Thus the mechanism we found in the cells could also be relevant in human tumors (Garibaldi et al., 2016). We have now evidences of a new transcriptionally independent function of mutp53 in miRNA biogenesis that, impacting on Dicer activity, inhibits the processing of pre-miRNA in mature miRNAs. In details, by co-immunoprecipitation experiments (**Figure 4A**), confocal analysis **(Figure 4B**), RNA-ChIPs (**Figure 4C**) and Dicer overexpression **(Figure 4D,E**) we demonstrate that mutp53 directly binds Dicer affecting its interaction with pre-miRNAs. Of note, we have designed and setup an in vivo pre-miRNA processing assay to monitor mutp53 dependent Dicer activity (**Figure 5A**). Using this system we demonstrate that mutp53 impairs Dicer-mediated pre-miRNA processing (**Figure 5, 6**) (unpublished data). The relevance of the above identified molecular mechanisms resides on the fact that the identified mutp53-dependent miRNAs have oncosuppressor functions such as the induction of cell death, inhibition of proliferation and EMT has demonstrated by literature and our unpublished data (**Figure 7, 8**). Finally, the KEGG pathway (**Table 1**) and GO analyses (**Table 2**) of putative target genes of the mutp53-dependent miRNAs identifies several pathways and genes related to cancer among which EGF and FGF signaling, cell cycle, mitosis, cell death and stress response. Interestingly, many of the genes belonging to these categories have oncogenic potential and are known as key regulators of altered molecular pathways in tumors. Moreover, for many of these there are drugs already approved by the FDA or in clinical trials (unpublished data). Therefore, our data support the idea that mutp53, inhibiting the expression of specific miRNAs, upregulates a subset of genes involved in tumor progression, including genes already targeted by molecular drugs.

Mutant p53 inhibits pri-miRNA biogenesis by interfering with the microprocessor complex

-Garibaldi et al. Oncogene 2016
-Gurtner et al. Biochim Biophys Acta. 2017

33 mature miRNAs are down-regulated by mutp53

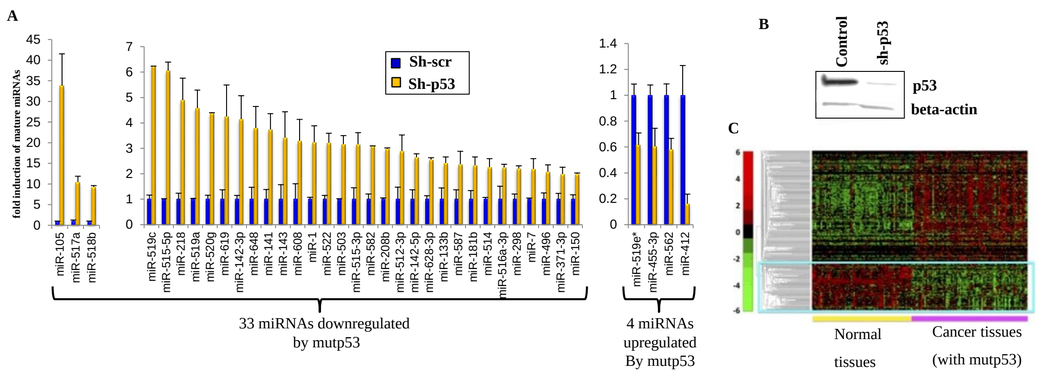

Figure 1. A) RT-qPCR analysis of 33 miRNAs downregulated and 4 miRNAs upregulated by mutp53 on SW480 cells. Mean ± s.d. of 2 independent experiments of genome wide analysis of miRNA expression (super-array plate, SABiosciences technologies) is shown. The 37 miRNAs were divided into three graphs based on the levels of expression. B) Western blot analysis performed on total lysates from SW480 after stable infection with either sh-p53 or control lentiviruses. C) 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.

Mutp53 binds and sequesters p72 from Microprocessor complex

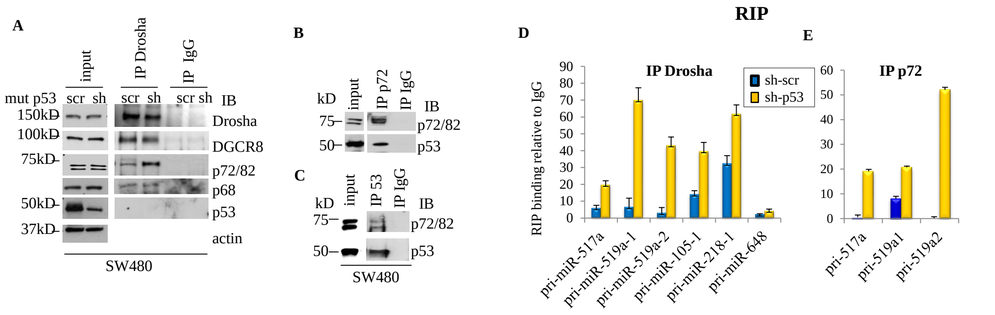

Figure 2. Immunoprecipitation (IP) assays performed with nuclear extracts of SW480, before (scr) and after (sh) depletion of mutp53, with anti-Drosha (A), anti-p72 (B), anti-p53 (C) antibodies . RIP analysis for association between pri-miRs and Drosha (D) and p72 (E) in SW480 cells before and after depletion of mutp53. Drosha and p72 were immunoprecipitated and subjected to RT–PCR analysis. As controls, RNA samples imunoprecipitated with non-specific IgG (IgG) was subjected to PCR . 1 of 3 experiments is represented.

Proposed model

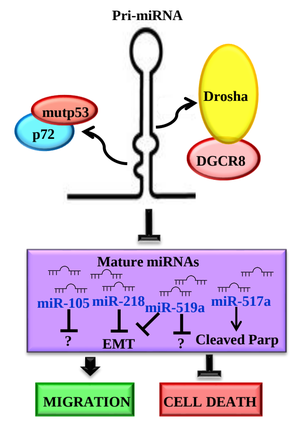

Figure 3. Proposed moldel

Mutant p53 inhibits pre-miRNA biogenesis by interfering with Dicer activity (unpublished)

Mutant p53 interacts with Dicer and affects Dicer interaction with pre-miRNA

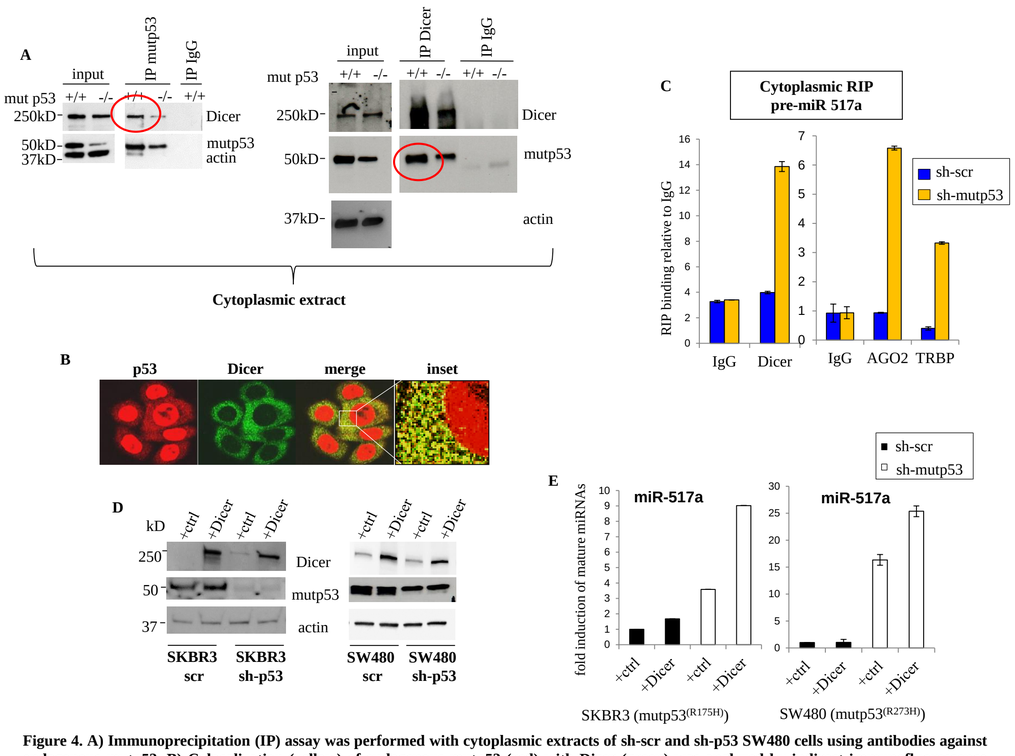

Figure 4. A) Immunoprecipitation (IP) assay was performed with cytoplasmic extracts of sh-scr and sh-p53 SW480 cells using antibodies against endogenous mutp53. B) Colocalization (yellow) of endogenous mutp53 (red) with Dicer (green) was analyzed by indirect immunofluorescence combined with Confocal Scanning Laser Microscopy. Confocal analysis of single optical section is shown. C) RIP analysis for pre-miRNA-Dicer association in sh-scr and sh-p53 SW480 cells performed with Cytoplasmic extracts. D) WB analysis performed on total lysates from sh-scr SW480 transfected with control vector (sh-scr+ctrl), sh-scr SW480 transfected with Dicer (sh-scr+Dicer), sh-p53 SW480 transfected with control vector (sh-p53+ctrl) or sh-p53 SW480 transfected with Dicer (sh-p53+Dicer). E) RT-qPCR analysis of 2 mature miRNAs in SW480 cells described above (4D).

Mutp53 inhibits Dicer-mediated pre-miRNA processing

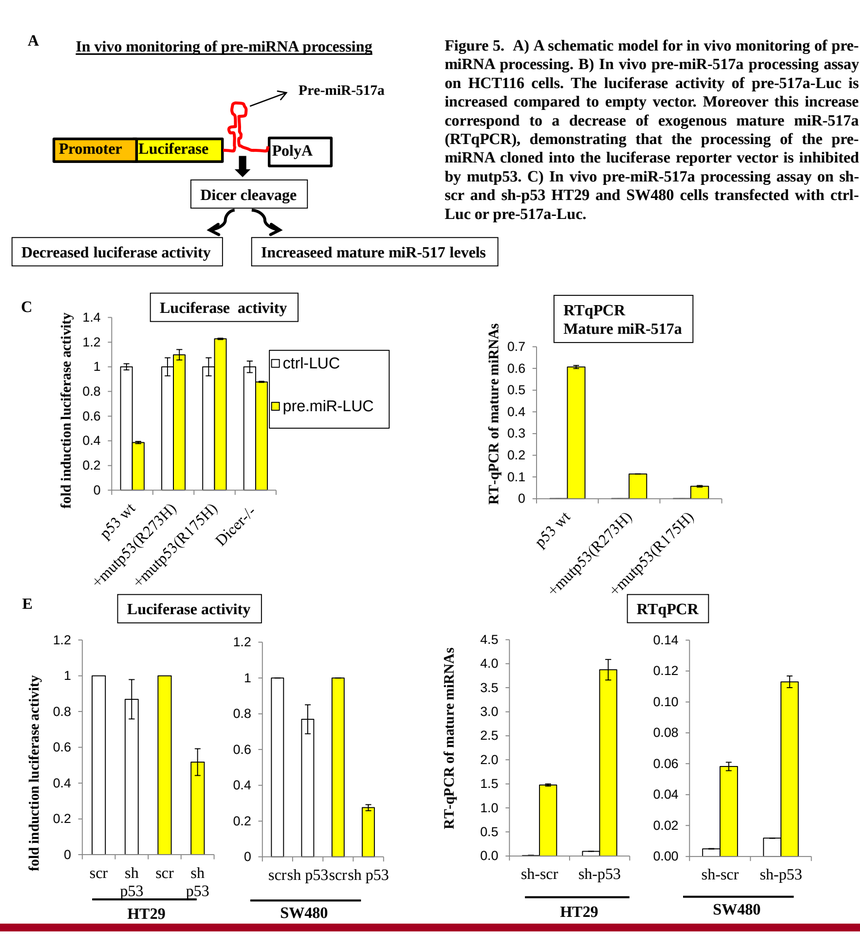

Figure 5. A) A schematic model for in vivo monitoring of pre-miRNA processing. B) In vivo pre-miR-517a processing assay on HCT116 cells. The luciferase activity of pre-517a-Luc is increased compared to empty vector. Moreover this increase correspond to a decrease of exogenous mature miR-517a (RTqPCR), demonstrating that the processing of the pre-miRNA cloned into the luciferase reporter vector is inhibited by mutp53. C) In vivo pre-miR-517a processing assay on sh-scr and sh-p53 HT29 and SW480 cells transfected with ctrl-Luc or pre-517a-Luc.

Proposed model

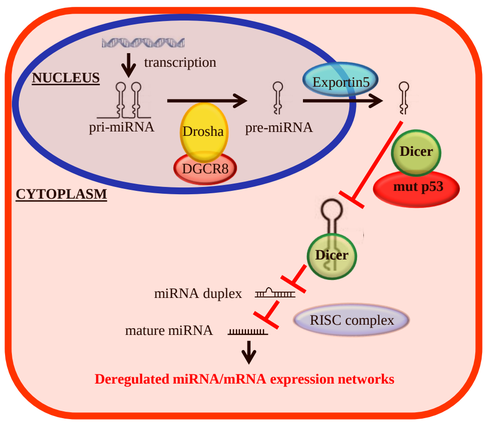

Figure 6. Proposed moldel

Functional role of mutp53-regulated miRNAs (unpublished)

miR-517a impairs cell proliferation and survival on colon cancer cell lines and inhibits colon tumor growth in mice

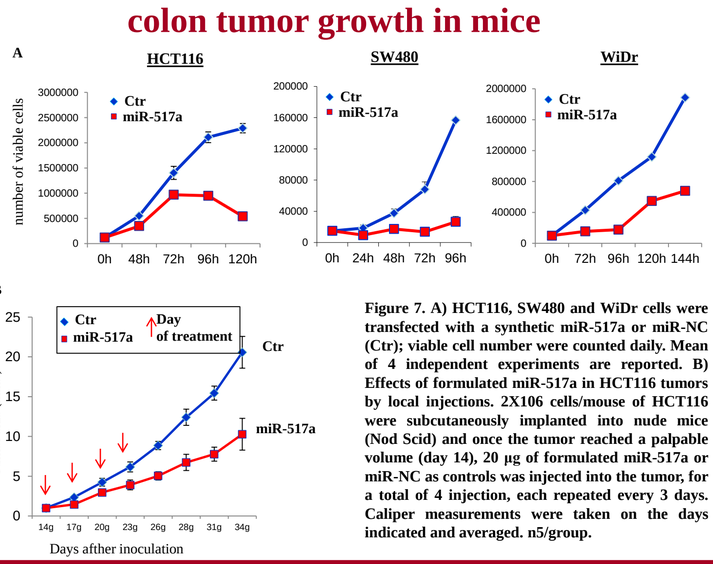

Figure 7. A) HCT116, SW480 and WiDr cells were transfected with a synthetic miR-517a or miR-NC (Ctr); viable cell number were counted daily. Mean of 4 independent experiments are reported. B) Effects of formulated miR-517a in HCT116 tumors by local injections. 2X106 cells/mouse of HCT116 were subcutaneously implanted into nude mice (Nod Scid) and once the tumor reached a palpable volume (day 14), 20 µg of formulated miR-517a or miR-NC as controls was injected into the tumor, for a total of 4 injection, each repeated every 3 days. Caliper measurements were taken on the days indicated and averaged. n5/group.

SRSF1/SF2 is down-regulated upon miR-517a over-expression

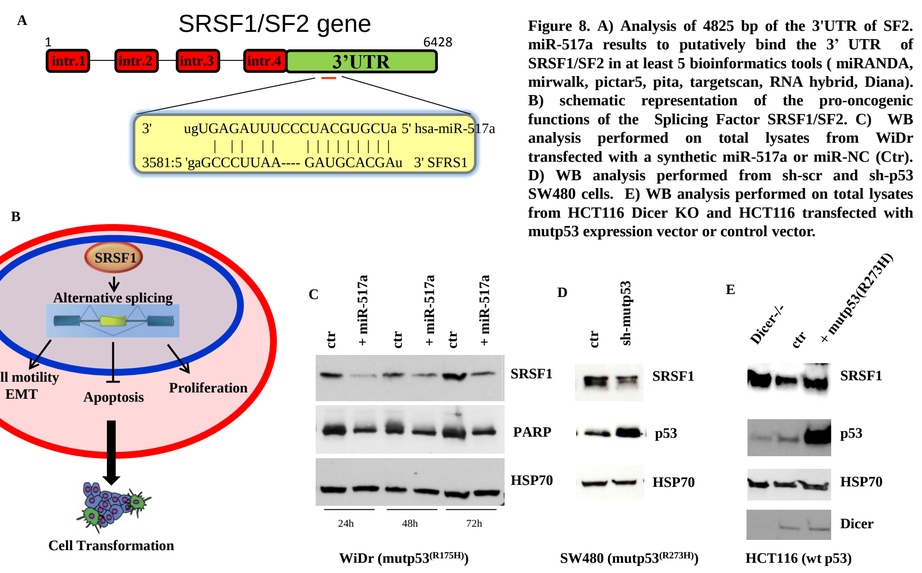

Figure 8. A) Analysis of 4825 bp of the 3'UTR of SF2. miR-517a results to putatively bind the 3' UTR of SRSF1/SF2 in at least 5 bioinformatics tools (miRANDA, mirwalk, pictar5, pita, targetscan, RNA hybrid, Diana). B) schematic representation of the pro-oncogenic functions of the Splicing Factor SRSF1/SF2. C) WB analysis performed on total lysates from WiDr transfected with a synthetic miR-517a or miR-NC (Ctr). D) WB analysis performed from sh-scr and sh-p53 SW480 cells. E) WB analysis performed on total lysates from HCT116 Dicer KO and HCT116 transfected with mutp53 expression vector or control vector.

Table 1
KEGG pathway enrichment analysis for predicted mutp53 dependent miRNA target genes

KEGG pathway	p-value
TGF-beta signaling pathway	4,55E-06
Hippo signaling pathway	4,55E-06
Glioma	7,59E-06
ErbB signaling pathway	1,68E-05
Adherens junction	0,000522
Wnt signaling pathway	0,000522
Ubiquitin mediated proteolysis	0,0008
Pathways in cancer	0,001113
PI3K-Akt signaling pathway	0,001146
Cell cycle	0,001518
Prostate cancer	0,001635
Signaling pathways regulating pluripotency of stem cells	0,001635
Transcriptional misregulation in cancer	0,001908
Pancreatic cancer	0,00321
Melanoma	0,008619
Ras signaling pathway	0,010518
mTOR signaling pathway	0,012188
Colorectal cancer	0,012225
Non-small cell lung cancer	0,015084
HIF-1 signaling pathway	0,016208
Acute myeloid leukemia	0,016208
Endometrial cancer	0,016594
Renal cell carcinoma	0,016594
Focal adhesion	0,017082

Table 2
Gene ontology (GO) category analysis for predicted mutp53 dependent miRNA target genes

GO Category	p-value
mitotic cell cycle	<1E-325
Transcription	<1E-325
cellular component assembly	<1E-325
small molecule metabolic process	<1E-325
catabolic process	<1E-325
epidermal growth factor receptor signaling pathway	<1E-325
fibroblast growth factor receptor signaling pathway	<1E-325
gene expression	<1E-325
response to stress	4,90474E-12
membrane organization	2,72238E-08
cell death	4,74561E-08
DNA metabolic process	1,5239E-05
cellular protein metabolic process	0,00032493
cellular lipid metabolic process	0,000351895
transcription initiation from RNA polymerase II promoter	0,001035799
cell-cell signaling	0,002144716
post-translational protein modification	0,004886914
G2/M transition of mitotic cell cycle	0,006198217
mRNA metabolic process	0,006932925
RNA splicing	0,01293825
RNA metabolic process	0,03795545

IRE ISTITUTO NAZIONALE TUMORI REGINA ELENA

15th Nov 2018

ISAB IOVS