PROJECT FINAL REPORT

Grant Agreement number: 201102

Project acronym: ONCOMIRS

Project title: miRs and Cancer: from bench to bedside

Funding Scheme: Collaborative projects, small or medium-scale focused research projects

Period covered: from 01/02/2008 to 31/01/2013

Name of the scientific representative of the project's co-ordinator[1], Title and Organisation:

Jean-Christophe Marine, PhD

VIB

Tel: +32 9 33 13640

Fax: +32 9 33 13512

E-mail: chris.marine@dmbr.ugent.be or JeanChristophe.Marine@cme.vib-kuleuven.be

Project website Error! Bookmark not defined. **address:**

http://med.kuleuven.be/cme-mg/oncomirs/index

---

[1] Usually the contact person of the coordinator as specified in Art. 8.1. of the Grant Agreement.

4.1   Final publishable summary report

- Executive summary.

ONCOMIRS focused on the identification and characterization of ncRNAs, including microRNAs (miRs) and long non-coding RNAs, that play a key role in the aetiology and/or progression of human cancer.

The sequential processing of primary miR transcripts into the mature miRs depends on two main protein complexes, the nuclear microprocessor complex and the Dicer complex. One of the project objectives was the identification of novel key components of the miR-processing machine and examination of their role in carcinogenesis. Through protein complex purification and spectrometry (MS) analyses many factors involved in this process have been identified and reported. Importantly, truncating mutations in TARBP2, encoding for the Dicer-binding protein TRBP, was shown to occur in human cancer. These mutations were shown to affect microRNA processing, dicer function and promote tumorigenesis. In addition, PRS14, EDC4 and FXR2 have been identified as AGO2-binding proteins and shown to modulate the transforming capacity of various human cancer cell lines. Consistent with the notion that an overall down-regulation of miR levels in cancer contributes significantly to the progression of the disease we showed that while a decrease in Dicer expression increase the transforming potential of cancer cell lines complete silencing is not tolerated by most cancer cells. These data were further supported by genetic experiments in mice. We indeed showed that Dicer functions as a haploinsufficient tumor suppressor gene in a pre-clinical mouse model of retinoblastoma but that it is in the same time required for tumorigenesis. Together these observations have opened exciting new avenues for anticancer therapy. Another important objective is the identification of novel putative cancer-causing ncRNAs. On a technological point of view, in order to further facilitate and improve sensitivity and accuracy of genome-wide ncRNA profiling, we generated new microarray platforms based on the LNA technology. In addition, promiscuous LNA oligos have been designed in attempts to facilitate the detection or inhibition of entire miRNA families rather than single members of a given family as well as long non-coding RNAs. We also explored the possibility of validating single miRNA-mRNA interactions using LNA oligonucleotides that bind specifically to sequences encompassing and surrounding miRNA target sites within mRNAs. These so-called target site blockers have given very promising results and are now commercially available at Exiqon. Assessment of the biological functions of selected oncomiRs by direct experimental identification of target mRNAs is another objective of this project. In order to define the role of selected oncomiRs, lncRNAs and components of the miR-processing machinery in the genesis and progression of cancer, classical gain and loss of function approaches in vivo, using the mouse as a model system were performed. We for instance showed that complete loss of miR17-92 completely prevents retinoblastoma formation in mice (Nittner et al., 2012). In contrast loss of miR17-92 did not affect normal retinogenesis; an observation that has important therapeutic implications. In contrast, however, although upregulated in mouse and human melanoma the miR17-92 cluster was shown to be dispensable for NRas-induced melanoma formation in mice. These data highlighted important differences between tumor types and their dependency to specific oncomiRs. Assessment of the therapeutic potential of miR (over)expression and/or inhibition in preclinical mouse models of human cancers was another goal. A new generation of miR mimics and antisense therapeutics have developed. We have tested and optimized the biostability, cytotoxicity and electropulsation-based delivery in vivo of such molecules and demonstrated that electrotransfer of miR inhibitors offers a new avenue for anti-cancer (retinoblastoma and glioblastoma) therapy.

- Summary description of project context and objectives (not exceeding 4 pages).

The objectives of the ONCOMIRs' project are presented as they were introduced at the beginning of each period. They remained consistent throughout the whole duration of the project and by and large all objectives have been reached/met.

Period 1 (First)

Recent experiments have indicated that alterations in miR levels in cancer could be partly the result of aberrant post-transcriptional processing of precursor miR transcripts. In the last few years, Partner 2 has isolated two complexes required for the sequential processing of primary miR transcript into the mature miR in human cells. While the initial processing of primary miR into precursor miR requires the complex composed of RNase III, Drosha, and the double stranded RNA-binding domain protein DGCR8, the formation of the mature miR requires the action of Dicer/TRBP complex. We proposed to actively persue the identification of additional components involved in the regulation of silencing by miRs, through protein complexes purification. One of our main objectives was to assess whether or not components of the Drosha and Dicer complexes are either mutated and/or aberrantly expressed in human cancers.

We proposed to implement the LNA technology present in the group (partner 6) on high-density flexible array platforms to facilitate ncRNA expression analyses. Once this technology will be available, a more long-term goal will be the identification of novel small non-coding RNAs (ncRNAs, including miRs) whose expression is dysregulated in human cancers. In the meantime, we proposed to establish ncRNA gene expression profiles using currently available technologies with the aim to identify genes that are regulated by and therefore act as downstream mediators of the p53 tumor suppressor pathway.

We also proposed to set up/use various gain and loss of function genetic screens with the aim to identify new putative oncomiRs based on their abilities to modulate key cellular pathways.

Only a limited number of oncomiR-target pairs have been identified to date. Partner 4 has recently developed a new approach for miR targets identification. We proposed to use this approach in combination with genome-wide gene expression profiling in order to identify the primary (and secondary) targets of a number of selected oncomiRs.

One main objective of this application was to study the physiological relevance of a few selected ncRNAs and demonstrate/dissect their active role in cancer formation and progression *in vivo*. To this end, we proposed to generate classical gene targeting vectors that disrupt expression of the miR(s) of interest in mice. For conditional gain of function studies, we will also knock-in miR gene(s) into the ROSA26 locus by gene targeting. The newly generated alleles will be combined with tumour-causing mutations to study the consequence of aberrant expression of a particular miR in relevant tumor models of human cancers.

RNA interference represents a potential strategy for *in vivo* target validation, therapeutic product development and clinical new technologies. It is expected to be particularly useful to silence cancer-causing genes that encode targets that are not amenable to conventional therapeutics. Moreover, there is evidence that miR-based molecules enter the RNAi pathway through a more natural route and yield more effective silencing with reduced toxicity and off-target effects. miR delivery *in vivo* in tumors is achievable by using new physical methods (electropulsation) developed by partner 5 and prone for clinical applications. One of our main objectives is to design miR-based molecules (miR mimics) that either allow potent activation of a "dormant" tumor suppressor pathway (for instance p53) or alter expression of specific oncogenes. The molecules will be designed to allow efficient delivery, high biostability and low toxicity. Similarly miR antagonists will be designed to target specific oncomiRs. The LNA technology developed by partner 6 will be particularly useful in this context. We first planned to test the efficacy and toxicity of these compounds in culture cells.

Period 2
Recent experiments have indicated that alterations in miR levels in cancer could be partly the result of aberrant post-transcriptional processing of precursor miR transcripts. We proposed to actively persue the identification of additional components involved in the regulation of silencing by miRs, through protein

complexes purification. One of our main objectives is to assess whether or not components of the Drosha and Dicer complexes are either mutated and/or aberrantly expressed in human cancers. To this end we proposed to generate monoclonal antibodies against a number of proteins, involved in miRNA function such as Ago proteins, Drosha, DGCR8 and exportin 5.

Human tumors are characterized by widespread reduction in microRNA (miRNA) expression, although it is unclear how such changes come about and whether they have an etiological role in the disease. A defect in miRNA-processing is one possible mechanism for the global down-regulation. To explore this possibility in more detail *in vivo* we proposed to manipulate *Dicer1* gene dosage in a mouse model of retinoblastoma and identify miRs that can either function as tumor suppressors or oncogenes in such a model.

We proposed to implement the LNA technology present in the group (partner 6) on high-density flexible array platforms to facilitate ncRNA expression analyses. A more long-term goal was the identification of novel small non-coding RNAs (ncRNAs, including miRs) whose expression is dysregulated in human cancers. In the meantime, we proposed to establish ncRNA gene expression profiles using currently available technologies with the aim to identify genes that are regulated by and therefore act as downstream mediators of the p53 tumor suppressor pathway.

We also proposed to set up/use various gain and loss of function genetic screens with the aim to identify new putative oncomiRs based on their abilities to modulate key cellular pathways.

Only a limited number of oncomiR-target pairs have been identified to date. Partner 4 has recently developed a new approach for miR targets identification. We proposed to use this approach in combination with genome-wide gene expression profiling in order to identify the primary (and secondary) targets of a number of selected oncomiRs.

One main objective of this application is to study the physiological relevance of a few selected ncRNAs and demonstrate/dissect their active role in cancer formation and progression *in vivo*. To this end, we proposed to generate classical gene targeting vectors that disrupt expression of the miR(s) of interest in mice. For conditional gain of function studies, we will also knock-in miR gene(s) into the ROSA26 locus by gene targeting. The newly generated alleles will be combined with tumour-causing mutations to study the consequence of aberrant expression of a particular miR in relevant tumor models of human cancers.

RNA interference represents a potential strategy for *in vivo* target validation, therapeutic product development and clinical new technologies. It is expected to be particularly useful to silence cancer-causing genes that encode targets that are not amenable to conventional therapeutics. Moreover, there is evidence that miR-based molecules enter the RNAi pathway through a more natural route and yield more effective silencing with reduced toxicity and off-target effects. miR delivery *in vivo* in tumors is achievable by using new physical methods (electropulsation) developed by partner 5 and prone for clinical applications. One of our main objectives is to design miR-based molecules (miR mimics) that either allow potent activation of a "dormant" tumor suppressor pathway (for instance p53) or alter expression of specific oncogenes. The molecules will be designed to allow efficient delivery, high biostability and low toxicity. Similarly miR antagonists will be designed to target specific oncomiRs. The LNA technology developed by partner 6 will be particularly useful in this context. We first planned to test the efficacy and toxicity of these compounds in culture cells.

Period 3

Recent experiments have indicated that alterations in miR levels in cancer could be partly the result of aberrant post-transcriptional processing of precursor miR transcripts. We proposed to actively persue the identification of additional components involved in the regulation of silencing by miRs, through protein complexes purification. One of our main objectives is to assess whether or not components of the Drosha and Dicer complexes are either mutated and/or aberrantly expressed in human cancers.

Human tumors are characterized by widespread reduction in microRNA (miRNA) expression, although it is unclear how such changes come about and whether they have an etiological role in the disease. A defect in miRNA-processing is one possible mechanism for the global down-regulation. To explore this possibility in more detail *in vivo* we proposed to manipulate *Dicer1* gene dosage in a mouse model of retinoblastoma and identify miRs that can either function as tumor suppressors or oncogenes in such a model.

We proposed to implement the LNA technology present in the group (partner 6) on high-density flexible array platforms to facilitate ncRNA expression analyses. Another goal is the identification of novel non-coding RNAs (ncRNAs, including miRs) whose expression is dysregulated in human cancers.

We also proposed to set up/use various gain and loss of function genetic screens with the aim to identify new putative oncomiRs based on their abilities to modulate key cellular pathways.

Only a limited number of oncomiR-target pairs have been identified to date. Partner 4 and 7 have recently developed new approaches for miR targets identification. We proposed to use this approach in combination with genome-wide gene expression profiling in order to identify the primary (and secondary) targets of a number of selected oncomiRs.

One main objective of this application is to study the physiological relevance of a few selected ncRNAs and demonstrate/dissect their active role in cancer formation and progression *in vivo*. To this end, we proposed to generate miRKO and transgenic mouse lines and combined these alleles with tumour-causing mutations to study the consequence of aberrant expression of selected miRs in relevant tumor models of human cancers.

RNA interference represents a potential strategy for *in vivo* target validation, therapeutic product development and clinical new technologies. It is expected to be particularly useful to silence cancer-causing genes that encode targets that are not amenable to conventional therapeutics. Moreover, there is evidence that miR-based molecules enter the RNAi pathway through a more natural route and yield more effective silencing with reduced toxicity and off-target effects. miR delivery *in vivo* in tumors is achievable by using new physical methods (electropulsation) developed by partner 5 and prone for clinical applications. One of our main objectives is to design miR-based molecules (miR mimics) that either allow potent activation of a "dormant" tumor suppressor pathway (for instance p53) or alter expression of specific oncogenes. The molecules will be designed to allow efficient delivery, high biostability and low toxicity. Similarly miR antagonists will be designed to target specific oncomiRs. The LNA technology developed by partner 6 will be particularly useful in this context.

Period 4 (Last)
Recent experiments have indicated that alterations in miR levels in cancer could be partly the result of aberrant post-transcriptional processing of precursor miR transcripts. We proposed to actively search for additional components involved in the regulation of silencing by miRs, through protein complexes purification.

Human tumors are characterized by widespread reduction in microRNA (miRNA) expression, although it is unclear how such changes come about and whether they have an etiological role in the disease. A defect in miRNA-processing is one possible mechanism for the global down-regulation. To explore this possibility in more detail *in vivo* we proposed to manipulate *Dicer1* gene dosage in a mouse model of retinoblastoma and identify miRs that can either function as tumor suppressors or oncogenes in such a model.

We proposed to implement the LNA technology present in the group (partner 6) as a tool to knock-down expression of selected non-coding RNAs. Another goal was the identification of novel non-coding RNAs (ncRNAs, including miRs) whose expression is dysregulated in human cancers.

We also proposed to set up/use various gain and loss of function genetic screens with the aim to identify new putative oncomiRs based on their abilities to modulate key cellular pathways.

Only a limited number of oncomiR-target pairs have been identified to date. Partner 4 and 7 have recently developed new approaches for miR targets identification. We proposed to use this approach in combination with genome-wide gene expression profiling in order to identify the primary (and secondary) targets of a number of selected oncomiRs.

One main objective of this application was to study the physiological relevance of a few selected ncRNAs and demonstrate/dissect their active role in cancer formation and progression *in vivo*. To this end, we generated KO and transgenic mouse lines for miRs and lncRNAs and combined these alleles with tumour-causing mutations to study the consequence of aberrant expression of selected miRs in relevant tumor models of human cancers.

RNA interference represents a potential strategy for *in vivo* target validation, therapeutic product development and clinical new technologies. It is expected to be particularly useful to silence cancer-causing genes that encode targets that are not amenable to conventional therapeutics. Moreover, there is evidence that miR-based molecules enter the RNAi pathway through a more natural route and yield more effective silencing with reduced toxicity and off-target effects. miR delivery *in vivo* in tumors is achievable by using new physical methods (electropulsation) developed by partner 5 and prone for clinical applications. One of our main objectives was to design miR-based molecules (miR mimics) that either allow potent activation of a "dormant" tumor suppressor pathway (for instance p53) or alter expression of specific oncogenes. Such molecules have be designed to allow efficient delivery, high biostability and low toxicity. Similarly miR antagonists were designed to target specific oncomiRs. The LNA technology developed by partner 6 has been particularly useful in this context.

- Description of the main S&T results/foregrounds

WP2: miR Biogenesis and Cancer

MiRNAs are transcribed as primary miRNA transcripts (pri-miRNAs), which serve as substrates for the nuclear microprocessor complex. This multi-protein complex contains the RNase III enzyme Drosha, its co-factor DGCR8 as well as a number of so far uncharacterized components. Drosha processing yields miRNA precursors (pre-miRNAs), which are subsequently transported to the cytoplasm by the export receptor exportin 5. In the cytoplasm, the RNase III enzyme Dicer further processes pre-miRNAs yielding mature miRNAs. MiRNAs are incorporated into miRNA-protein complexes (miRNPs), where they interact with a member of the Argonaute (Ago) protein family. In human somatic cells, Ago1, Ago2, Ago3 and Ago4 are expressed. It is likely that all four Ago proteins are incorporated into similar protein complexes. In order to function in gene silencing, miRNPs interact with a member of the GW protein family. In human, the GW proteins TNRC6A, B and C have been implicated in miRNA function. One of our objectives was the identification of novel components of the miR-processing machinery, through protein complexes purification. Partner 2 identified the huntingtin (Htt) protein as a component of the Argonaute complex. Colocalization studies demonstrated Htt and Ago2 to be present in P bodies, and depletion of Htt showed compromised RNA-mediated gene silencing. Huntington's disease is a dominant autosomal neurodegenerative disorder caused by an extension of polyglutamines in the Htt protein. The data therefore suggest that this disease may be attributed in part to mutant Htt's role in post-transcriptional processes (Savas et al., 2008). A possible role for components of effector complexes such as Ago1-4-containing miRNPs or TNRC6A-C-containing complexes in cancer will also be studied. In close collaboration with Elisabeth Kremmer (Helmholtz center, Munich), beneficiary 7 generated monoclonal antibodies against a number of proteins, involved in miRNA function. Antibodies against individual human Ago proteins have thus been generated. One of these antibodies allows detection of endogenous Ago2 expression on tissue sections. In addition, antibodies against Drosha, DGCR8 and exportin 5 have been generated. Beneficiary 7 isolated a number of interesting Drosha protein partners among them EWS. Partner 7 also established a list of Dicer-dependent and -independent AGO2-binding proteins (Frohn et al., 2012). Beneficiary 7 has confirmed the presence of the helicase p68 in the DROSHA complex; an observation which is consistent with previously published studies. As expected, they also found the Dicer cofactor TARBP2 (TRBP) and the RNA helicase A/DHX9, which has been implicated in siRNA-loading. Proteins such as YBX1, Gemin4, Gemin5, HNRNPC, HNRNPUL1, the ARE binding protein ELAVL1/HuR, the poly-A binding proteins PABPC1 and 4, the mitochondrial protein Matrin3 and the Fragile X mental retardation protein paralog FXR2 have been found in Ago complex purifications before. In addition, they identified the PABPC1-binding protein LARP1, the mRNA binding proteins FAM120A/Ossa and CSDA, which have not been implicated in Ago2 function before. In contrast to the GW protein family members TNRC6A and B, TNRC6C interacts with Ago2 only in the presence of Dicer, suggesting that it requires Ago2 to be loaded onto miRNA target mRNAs. Moreover they found that a high number of proteins associate with Ago2 in a RNA-dependent manner even in the absence of miRNAs. In this group, they found many RNA binding proteins including IGF2BP1–3, DHX36, DHX30, HNRNPL, and the ribosomal protein RPS14. Strikingly, genetic data in C. elegans demonstrated that PRS14 modulates mature let-7 tumor suppressor. A set of proteins associates with Ago2 in an mRNA- independent manner. This includes the TNRC6 proteins and Dicer. Other miRNA-independent protein-protein interactors of Ago2 are the HSP90 alpha and beta proteins with their cochaperones PTGES and FKBP5. Among the interactors preferentially binding in the absence of Dicer and miRNAs only Ago3, CLTC and ZNF521 were identified in the datasets and they show a direct binding behavior. Taken together, this mass spectrometry approach revealed that Ago2 associates with larger RNA species even in the absence of small RNAs. Furthermore, several mRNA-binding proteins are specific to miRNA-free and miRNA-containing Ago2-mRNA complexes. To functionally validate the interaction data, Partner 7 used luciferase-based miRNA reporters. The 3'-UTR of Hmga2, a well-characterized let-7a target, was fused to firefly luciferase and transfected into HeLa, HCT116 and MCF-7 cells in which ZNF521, CSDA and EDC4 were depleted by RNAi. In addition, we employed a reporter containing the Hmga2 3'-UTR with mutated let-7a target sites and normalized the data against each other. As expected, Ago2 knock down led to increased luciferase activity. Knock down of EDC4, which has been implicated in miRNA function in Drosophila, resulted in specific luciferase up-regulation as well, suggesting that EDC4 is indeed involved in silencing of the Hmga2 reporter construct. Similar results were obtained for

the mRNP component CSDA. ZNF521, however, is not involved in miRNA-guided gene silencing. Since ZNF521 is a putative transcription factor, it is possible that it cooperates with Ago2 in nuclear Ago functions. The previously recognized role of let-7 family members as tumor suppressors prompted us to assess the role of PRS14 and EDC4 in cellular transformation by performing colony and soft agar assays. Moreover we have also assessed the role of FXR2 in cellular transformation, as other Fragile X mental retardation proteins have recently been implicated in cancer development. These experiments have confirmed that all these factors are able to modulate cellular transformation in these assays. Consistently, depletion of these factors by RNAi in HCT116, MCF-7 and BJ-cells had profound phenotypic consequences on the ability of these cells to grow in vitro and their transforming potential. Further experiments aimed at dissecting the mechanisms underlying these phenotypic consequences are ongoing in the laboratory of Partner 1 and 7.

Overall down-regulation of miR levels in cancer contributes significantly to the progression of the disease. Since this overall-down-regulation is likely to be, at least partly, a consequence of aberrant processing of their precursor transcripts, we proposed to examine the expression levels of Drosha/DGCR8 and Dicer/TRBP components, in cancer cell lines and human primary tumors and assess the contribution of aberrant expression and/or altered function in cellular transformation. Beneficiary 2 -in collaboration with Pr Manel Esteller (CNIO, Madrid, Spain)- has screened various cancer cell lines with microsatellite instability for the presence of mutations in all exonic mononucleotide repeats present in the coding sequences of eight established members of the miRNA processing machinery: Dicer and Drosha, DGCR8, TRBP, PACT and AGO1, AGO2 and AGO4. Wild-type sequences were found for all genes with the exception of TARBP2 encoding for TRBP. Two heterozygous frame-shift mutations were identified in two different cell lines. These mutations were found to cause a significant decrease in TRBP expression. Since TRBP stabilizes DICER, these mutations also affected Dicer expression levels and consequently caused a drastic reduction in the efficiency of endogenous processing of miRs. Importantly, ectopic expression of wild-type TRBP, but not of the naturally occurring mutants, induced tumor suppressor-like features in colorectal cancer cell lines. Notably, Dicer overexpression also showed tumor suppressor properties as determined using classical tissue culture assays (MTT and colony assays) and mouse xenograph models (nude mice). Finally, TARBP2 mutations were detected in about 25% of a panel of 282 human primary tumors (Melo et al., 2009). The above findings provide further evidence of a role of loss of function events in the regulation of miRNA processing machinery during tumorigenesis. However, the molecular mechanisms that promote tumorigenesis as a consequence of global repression of miRNA maturation remain elusive. Data obtained by beneficiary 1 suggest that the p53 tumor suppressor pathway is compromised in cancer cells harbouring hypomorphic Dicer expression.

Given the results of the mutational analyses of the various components of the miR-processing machinery in cancer cell lines and primary tumors, it was decided to slightly deviate from the initial plans. Instead of constructing artificial TRBP mutants lacking the dsRNA-binding domains, it was preferred to assess the oncogenic properties of the naturally occurring TRBP mutants. To add relevance to our structure function analysis, the experiments were conducted in colorectal cancer cell lines, instead of NIH-3T3, since these mutations were found to occur in this particular tumor context. Moreover, since no mutations were found in Dicer and AGO2 in human tumors, construction of the initially proposed Dicer and AGO2 mutants has been cancelled. Instead, we have already tested the role of TRBP and the influence of the naturally occurring mutations to cell transformation. In this context, the role of Dicer in cell transformation was also assessed. Moreover, we have demonstrated that the mutations in TRBP affect Dicer levels and consequently the integrity of the RNAi machinery. These data, recently reported in Melo et al., 2009, indicate that (i) TRBP and DICER exhibit tumor suppressive activities and (ii) mutations that ultimately affect miR-processing occur in human cancer.

Human tumors are characterized by widespread reduction in microRNA (miRNA) expression, although it is unclear how such changes come about and whether they have an etiological role in the disease. Importantly, miRNA-knockdown has been shown to enhance the tumorigenic potential of human lung adenocarcinoma cells. A defect in miRNA-processing is one possible mechanism for the global down-regulation. To explore this possibility in more detail *in vivo* we have manipulated *Dicer1* gene dosage in a mouse model of retinoblastoma. We show that while monoallelic loss of *Dicer1* does not affect normal retinal development it dramatically accelerates tumor formation on a retinoblastoma-sensitized background. Importantly, these tumors retain one wild-type *Dicer1* allele and exhibit only partial decrease in miRNA-processing. Accordingly, *in silico* analysis of human cancer genome data reveals frequent hemizygous, but not

homozygous, deletions of *DICER1*. Strikingly, complete loss of *Dicer1* function in mice did not accelerate retinoblastoma formation. miRNA profiling of these tumors identified members of the let-7 and miR-34 families as candidate tumor suppressors in retinoblastoma. We conclude that Dicer1 functions as a haploinsufficient tumor suppressor (Lambertz et al., 2010).
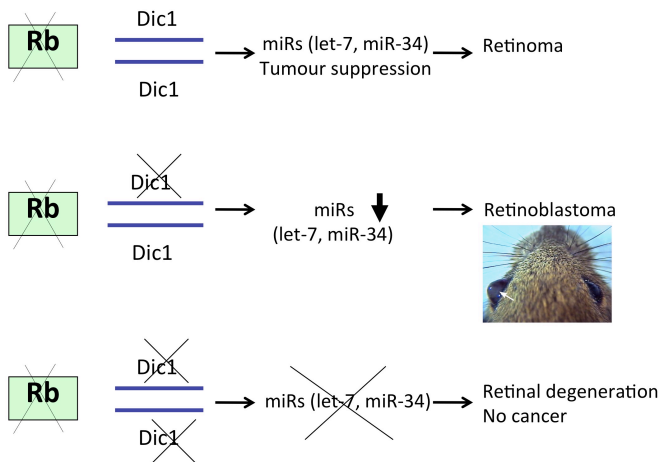
The above finding provides genetic evidence for a causative link between down-regulation of miRNA-processing and cancer progression.

      Interestingly, although reduced levels of DICER1 in tumors have been reported, no loss-of-function mutations in *DICER1* have been reported to date. Moreover, homozygous loss of *Dicer1* appears to be strongly selected against in a K-Ras-induced mouse model of lung cancer and Myc-induced mouse model of B-cell lymphoma. These data raise the possibility that *Dicer1* is required for tumour formation. Beneficiary 1's goup has recently demonstrated that targeted homozygous loss of *Dicer1* completely prevents the formation of retinoblastoma in mice in which the Rb and p53 tumour suppressor pathways are inactivated. Strikingly, it was found that *Dicer1* deficiency selectively kills Rb-deficient retinal cells in which p53 is inactivated while sparing cells that retain functional p53. miRNA profiling of mouse and human primary retinoblastomas showed dramatic overexpression of the pro-oncogenic miR17-92 cluster in all samples analyzed. High-resolution array-CGH indicates that in ~20% of human retinoblastoma patients overexpression of miR-17-92 results from copy number alterations. Crucially, functional inactivation of the miRNAs encoded by the miR-17-92 cluster is sufficient to decrease the viability of human retinoblastoma cells and to prevent retinoblastoma formation in mice. The data therefore provide genetic evidence of a synthetic lethal interaction between Dicer and p53 and designate members of the miR-17-92 cluster as a highly selective therapeutic target for the treatment of retinoblastoma (Nittner et al., 2012).
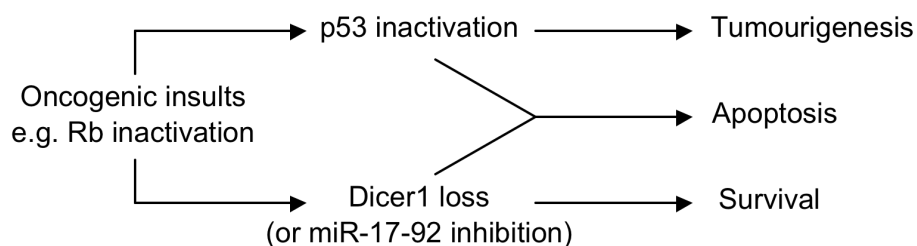
WP3: Identification of novel OncomiRs (and long non-coding RNAs)

The data described above imply that a set of miRNAs might act to prevent full-blown retinoblastoma formation on the *Chx10Cre;Rb$^{lox/lox}$;p107$^{-/-}$* background. To identify such candidate tumor suppressor miRNAs beneficiary 1 determined the expression profile of the entire miRNome in P20 retinae from wild-type (Cre-negative), *Chx10Cre; Rb$^{lox/lox}$; p107$^{-/-}$; Dic$^{+/+}$* and *Chx10Cre;Rb$^{lox/lox}$;p107$^{-/-}$;Dic$^{lox/+}$* using LNA-based microarray (in collaboration with Patrner 6) and RT-qPCR approaches. Both types of analyses identified a common set of 11 miRNAs that are consistently up-regulated between wild-type and *Chx10Cre; Rb$^{lox/lox}$; p107$^{-/-}$; Dic$^{+/+}$*. Most interestingly, among them are 2 members of the let-7 family (let-7c and let7-i), the up-regulation of which and of another let-7 member, let-7b, was confirmed by independent Q-RT-PCR analysis. Given that this up-regulation was significantly attenuated in the *Dic* heterozygous retinae and the recognized role of let-7 family members in tumor suppression, this observation raises the possibility that let-7 have a causal role in retinoblastoma formation as critical regulators of the switch from retinomas to retinoblastoma. Our list of 11 differentially expressed miRNAs also included miR-34c. There was also a clear upregulation of miR-34b-3p in both microarray and RT-q-PCR analyses and a moderate, but reproducible, up-regulation of miR-34a was evident from the micro-array data. Interestingly, the miR-34 family members have been identified as p53 targets and key mediators of its tumor suppressor function.

The data from WP2 also implied that a set of miRNAs is required for retinoblastoma formation despite the combined mutation in Rb, p107 and p53. In search of such pro-oncogenic miRNAs we profiled the miRNome by stem-loop RT-qPCR in P21 retinae from wild-type (Cre-negative), *Chx10Cre; Rb$^{lox/lox}$; p107$^{-/-}$* and from Laser-Captured-Microdissected tumor materials from 4 *Chx10Cre;Rb$^{lox/lox}$;p107$^{-/-}$;p53$^{lox/lox}$* (TKO) mice. This analysis identified a set of 102 miRNAs that are significantly up-regulated in the *TKO* tumours. To find correlates of the mouse data in human tumours, we also profiled miRNA expression in 30 different human primary retinoblastomas. 68 miRNAs were significantly up-regulated in retinoblastoma compared to normal human retinae. Cross-species comparison identified 25 miRNAs that were up-regulated in both mouse and human tumours. Strikingly, 12 of them are members of the known oncogenic miR-17-92 and 106b-25/miR-106a-92 paralogue clusters. Consistently, hierarchical clustering of all RB cases and normal retinae based on miRNA expression singled out all members of these clusters as being dramatically up-regulated in all mouse and human tumours analyzed. To explore the potential causes of miRNA deregulation in human retinoblastoma beneficiary 1 looked for genomic aberrations using a 44K oligonucleotide array, which was specifically designed to include regions harbouring miRNA genes. In addition to identifying previously reported retinoblastoma-associated genomic aberrations (1q gain and 6p22 gain were frequently seen in our cohort) focal amplification of the miR-17-92 locus, which lies on chromosome 13, was found in one patient. Another patient had a whole chromosome 13 gain and 3 patients had copy number gains including the miR-17-92 cluster but, importantly, not the closely linked *Rb-1* locus. miR-17-92 copy number gains were found in 17% of the patients (5 out of 29 cases analysed). Moreover, while the *Rb-1* locus was deleted in 21% of cases (6/29) this deletion never included the closely linked miR-17-92 locus. This analysis therefore indicates that up-regulation of the miR-17-92 cluster is, at least in a proportion of retinoblastoma cases, a direct consequence of increased gene copy number. Since transcription of miR-17-92 is positively regulated by the E2Fs and negatively regulated by p53, deregulation of their transcriptional activities may also account for miR-17-92 overexpression in retinoblastoma. Regardless of the underlying mechanism, our data demonstrate that the miR17-92 cluster is overexpressed in 100% of retinoblastomas analysed. Together these data raise the possibility that up-regulation of expression of the miR17-92 cluster is a critical event for the development retinoblastoma; this was later shown to be case (see WP6).

We also proposed to establish miR gene expression profiles using currently available technologies with the aim to identify genes that are regulated by and therefore act as downstream mediators of the p53 tumor suppressor pathway. In a first set of experiments, we have activated the p53 pathway chemically using the Mdm2-inhibitor Nutlin3a. BJ primary human diploid fibroblasts undergo p53-mediated irreversible cell cycle arrest or senescence upon such treatment. Using a Q-RT-PCR-based profiling method, we have identified miRs that are induced and repressed under these conditions. As control, quiescent –serum starved-cells were also profiled. MiR-34a, b and c were all specifically induced in senescent cells. In addition, miR-24, miR-23b and miR-27b, all encoded by the same locus, were also found induced in Nutlin-treated cells. In addition, miR-195, miR-7 and miR-452# were also all found dramatically up-regulated whereas miR-302b and miR-586 were both strongly down-regulated in senescence cells but not in quiescent cells.

Beneficiary 3 identified a panel of miRs whose expression is repressed by p53 during replicative senescence of human diploid fibroblasts; this study has now been published (Brosh et al, 2009). In subsequent work, the regulation of miR-372 and miR-373 during oncogene-induced senescence has been addressed. Specifically, it was found that these two miRNAs are downregulated in response to overexpression of oncogenic H-Ras. This correlates with the upregulation of the Lats2 gene by oncogenic Ras. Of note, Lats2 is required for p53 activation in human fibroblasts in response to oncogenic Ras, and is thus a critical mediator of oncogene-induced senescence in this system. These observations are consistent with a potential role for the miRNAs in inhibition of oncogene-induced senescence, and provide a mechanistic explanation for earlier observations of Agami and co-workers (Aylon et al, 2010). Beneficiary 3 also investigated the notion that some p53-regulated miRNAs may be epigenetically silenced in tumors that retain wild-type p53, rendering these tumors less susceptible to killing by genotoxic chemotherapy. To that end, conditions for the synergistic killing of A549 lung cancer cells by a combination of cisplatin and agents that "erase" epigenetic silencing marks (TSA+ 5-Aza-cytidine) have been optimized. miRNA profiling from such cells with and without various treatment combinations will be performed by beneficiary 6. The data will be subjected to validation by qRT-PCR in the subsequent months. To identify miRNAs that are regulated by p53 *in vivo*, possibly in a tissue-specific manner, beneficiary 2 carried out miRNA profiling on different tissues of wild type and p53 knockout mice. Following validation of the most promising hits by qRT-PCR, it was decided to focus on miR-223 and miR-449. Both of these miRNAs are positively regulated by p53 in some cultured cell types but not in others. miR-223 was previously reported to play a role in the regulation of differentiation in the myeloid lineage. Interestingly, using a myeloid leukemia cell line carrying an inducible (temperature sensitive) p53, a modest but reproducible increase in miR-223 levels upon p53 induction was observed. The effect of p53 activation on the differentiation of those cells (which can be induced by either IL6 or dexamethazone) and the contribution of miR-223 to this effect will be further investigated. Beneficiary 2 has also performed miRNA profiling using an experimental array developed by beneficiary 6, which combines probe sets for mature miRNA and for pre-miRNA. The input RNA was derived from H1299 lung cancer-derived cells containing a temperature sensitive p53 (the same experimental model that was employed earlier to discover that miR-34a was a p53 target). Surprisingly, the results suggested that there may be profound differences between the impact of p53 activation on some mature miRNAs relative to their precursors. The data still need to be validated by more quantitative methods; if validated, this may suggest an effect of p53 on the processing of specific pre-miRNAs by Dicer, and will merit further investigation. An additional observation provided by this miRNA profiling experiment was that p53 seemed to up-regulate the levels of miR-372 and miR-373*; subsequent preliminary data suggest that these miRNAs may also be positively regulated by p53 in A549 cells, which harbor endogenous wtp53. Given that the expression of miR-372 was found by us earlier to be negatively regulated by Ras, it will be interesting to investigate the cross-talk between p53 and Ras in regulation of the expression of the miR-372/373 cluster. In parallel, we initiated a new line of investigation, not included in the original workplan. This is based on the recent realization that p53 plays a role in regulation of autophagy, a process that is turning out to be of great importance in cancer biology. In a collaborative study with Prof. Z. Elazar at the Weizmann Institute, we searched for miRNAs whose expression is induced in HeLa cells under conditions of nutrient starvation that trigger autophagy. One of the top hits was miR-492, primate-specific miRNA that highly expressed in retinoblastoma. Interestingly, miR-492 was also one of the top hits in another screen carried out by beneficiary 3, which looked for miRNAs whose expression is altered in a p53-dependent manner in HCT116 colorectal cancer cells treated with the mitotic spindle poison nocodazole. Follow-up experiments validated that miR-492 is induced by nutrient starvation (24-48h) in p53-proficient HCT116 cells but not in their derivative p53 knockout cells (HCT116 p53-/-):
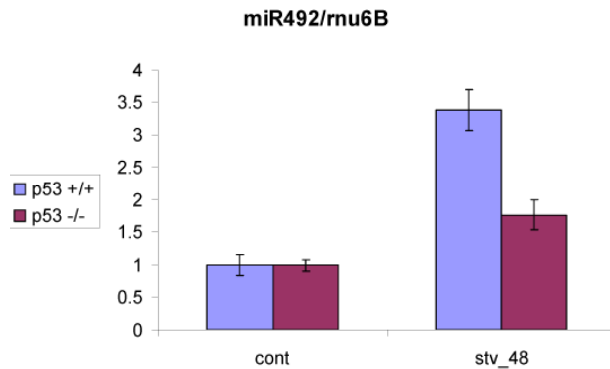
miR492/rnu6B

Figure legend: miR-492 levels are induced by nutrient starvation (48 hours) in a p53-dependent manner. Cont- cells growing in full medium; stv – starved cells.

We further found that HCT116 p53-/- cells have an increased propensity to become polyploid upon nutrient starvation, a feature not seen in wt HCT116 cells and thus indicative of a role of p53 in preventing starvation-induced polyploidization. On note, overexpression of miR-492 in the p53-/- cells could reduce significantly this starvation-induced polyploidy.

Beneficiary 7 searched for miRNAs whose expression is dependent on the p53 family members p63 and p73. A list of validated p63- and p73-regulated miRNAs has been available to the members of the consortium.

The ultra conserved regions (UCRs) are a subset of conserved regions that are absolutely conserved (100%) between orthologous regions of the human, rat and mouse genomes and are longer than 200bp (Bejerano et al., 2004). Because of the very high degree of conservation, the UCRs are likely to have fundamental functional importance for the ontogeny and normal physiological functioning of cells and tissues, including gene regulation and chromosome biology of the organisms in which they are found. The majority of UCRs (about 90%) are transcribed (T-UCRs) in normal human tissues. While a minority is expressed ubiquitously, most are tissue specific (Calin et al., 2007). As with miRs (Liu et al., 2004), hierarchical clustering of T-UCR expression in various hematopoietic and non-hematopoietic tissues from different individuals suggests that T-UCRs can be used as tissue specific markers. The same types of tissues from different individuals clustered as closest neighbours. Transcription of UCRs may be regulated by specific transcription factors and/or initiated from polyadenine rich genomic regions, as was recently proposed for several long ncRNAs in the mouse (Furuno et al., 2006). Importantly, levels of UCRs may be regulated post-transcriptionally by miRs. Calin et al. (2007) has indeed provided evidence that specific miRs interact with complementary T-UCRs and that this interaction results in down-regulation of T-UCRs expression. T-UCRs are frequently located at fragile sites and other genomic regions that have been implicated in cancers (Calin et al., 2007). Genome-wide profiling revealed that UCRs, just like miRs, have distinct signatures in human leukemias and carcinomas and therefore can be used to differentiate between human cancers (Calin et al., 2007). Moreover, about 10% of T-UCRs are differentially expressed in at least one type of tumor cells when compared to normal cells of the same origin. Among the most significant differentially expressed T-UCRs in leukemias and carcinomas are uc.29, uc.73 (high in colorectal cancers), uc111, uc135 (low in CLL), … Interestingly, a significant negative correlation between 87 miRs (out of 285 analyzed) and T-UCRs expression levels was found at a genome-wide level in 50 patients with leukemias (CLL) (Calin et al., 2007). This observation is consistent with the possibility that T-UCRs are targeted by miRs and these interactions may have biological and prognostic significance for cancer patients. Strikingly, preliminary functional studies suggest that uc.73 (P), which is significantly up-regulated in colon cancers, behaves like an oncogene in tissue culture by promoting both proliferative and anti-apoptotic activities (Calin et al., 2007). We have therefore decided – even if not planned originally – to profile the T-UCRnome (481 different UCRs) using a Q-RT-PCR-based approach in senescent and quiescent BJ cells. A list of UCRs specifically induced and repressed in senescent cells is now available. Of note, among the most differentially regukated genes are uc135 (up 30-fold) and uc73 (down more than 100-fold). Equally interesting is the observation that uc.177, which is drastically down-regulated in senescent cells is putatively regulated by all miR-34 family members. Further experiments are currently ongoing in the laboratory of partner 1 to test this hypothesis and assess the

relevance of both miRs and UCRs that are specifically induced in Nutlin-treated cells for the senescence phenotype.

Partner 3 has identified a panel of miRs whose expression is altered during replicative senescence of human diploid fibroblasts. A substantial fraction of those miRs were found to belong to 3 paralogous polycistronic clusters, including the miR17-92 cluster, the miR-106b/93/25 cluster and the miR-17-5p cluster; levels of many miRs encoded within these clusters were shown to decrease markedly in senescent cells. Furthermore, this downregulation was p53-dependent, whereas the expression of these miRs in non-senescent cells was largely driven by E2F. Further analysis revealed a feed-forward loop that drives E2F-dependent transcription of those miRs. Functionally, overexpression of these miRs could promote excessive proliferation and delay senescence, consistent with a proposed oncogenic role of at least some of them. Indeed, expression of many of those miRs was found to be upregulated in breast cancer tumors that carry p53 mutations. These findings have recently been published (Brosh et al., 2008).

Partner 4 has identified a set of miRNAs regulated by oncogenic B-RAF in TIG3 primary human fibroblasts. Of the ~10 miRNAs significantly altered following 4 days of B-RAF induction miR-34a and miR-146a were the most significantly upregulated. We focused on miR-34a, as the absolute level of this miRNAs was much higher. Using a combination of siRNA experiments, chromatin-immunoprecipitations and heterologous promoter assays we have identified ELK1 as the transcription factor responsible for miR-34a upregulation downstream B-RAF. In addition, we have established that p53 plays little or no role in miR-34a induction during oncogene-induced senescence.

Partner 2 has recently compared induction of miRs in different mouse organs following exposure of wild type and p53 knock-out mice to whole body ionizing radiation (IR). We observed substantial differences between different organs: while some miRs (e.g. miR-34a) were more widely induced following p53 activation by IR, others were induced only in one or two organs (e.g. miR-223 and miR-449a), attesting to the tissue-specificity of p53-dependent miR induction. The results of this analysis, obtained by miR array hybridization, are presently being subjected to validation by qPCR-RT-PCR analysis.

We explored the regulation of miR199a by p53. Initial results suggested that this particular microRNA is selectively induced by several p53 mutants. Further analysis revealed a more complex picture: depending on cell type, miR-199a could be induced by either mutant p53 and/or WT p53. However, we could not find any experimental model in which the extent of induction was more than 2-3 fold, rendering the subsequent functional analysis insufficiently definitive. This line of research was therefore discontinued. In parallel, we sought to identify more comprehensively the spectrum of miRs regulated by mutant p53. To that end, we performed miR expression profiling on DU-145 cells (human prostate cancer, harboring 2 mutant p53 alleles) before and after knockdown of their endogenous mutant p53 proteins. The results are still being validated, but overall the differences were rather minimal and disappointing, suggesting that this cell line is probably not an optimal choice for discovery of mutant p53-regulated miRs. We are presently investigating the possible regulation of miR-214 by WT and mutant p53. Preliminary results suggest that WT p53 can repress the expression of this miR, whose excessive expression has been implicated in ovarian cancer. Further analysis will therefore focus on cell lines derived from this type of cancer.

The setting up of several automated forward genetic screens as a mean to identify cancer-causing miRs is in progress. To this end, Partner 6 (Exiqon) has optimized Tm and LNA spiking pattern of LNA oligos to obtain miRNA inhibitors with increased potency and stability relative to previous versions. LNA based miRNA inhibitors have been designed for all human miRNAs listed in miRBASE 11.0 and the inhibitors have been assembled in a library consisting of 96-well microtiter plates with the inhibitors. In addition to these, oligos have been designed in attempts to facilitate miRNA family specific inhibition. The inhibitor oligos target three families: 1) miR-20a-b, -18a-b, -17, -106, -93, 2) miR-34a-c, and 3) miR-25,-92, -363.

Towards setting up functional screens for miRNA in various cellular pathways, we optimized the protocols needed for efficient reverse transfections of different cell lines including HeLa, Nmumg and TIG3/hTERT, for automated immunostainings of endogenous proteins and for visualization and enzymatic readouts using reporters such as GFP and luciferase. Several successful screenings have been conducted by partner 3. miR101 was shown to modulate autophagy (Frenkel et al., 2011). miR-339 was for instance identified as a modulator of p53 transcriptional activity and overexpression of miR-339 was shown to induce

senescence in BJ-cells. In collaboration Partner 3 and 4 have further dissected the miR-339 mechanism of action. Partner 3 and 4 also collaborated on the functional characterization of miR-661, which also regulates p53 functionality. miR-661 expression was shown to decrease in response to DNA damage and Nutlin-3-induced p53. Expression of both miR-661 and miR-339 enhance p53 levels and activity and sensitize cancer cell lines to DNA-damaging agents. Several putative binding sites for both of these miRs had been identified in the 3'-UTR of MDM2 and MDMX. Recent biochemical data confirm the targeting of MDM2 and MDM2 and MDMX by miR-339 and miR-661, respectively.

Interestingly, the predicted targets of miR-661 within the mRNAs of both Mdm2 and Mdm4 are mainly located within Alu repeats. This prompted us to study the functionality of miR targets within human Alu repeats, as described below. In the case of Mdm2 and Mdm4, we are still in the process of determining which of the several potential miR-661 binding sites within each transcript are actively employed in miR-661 binding and are required for the ability of that miRNA to suppress the expression of the relevant protein.

As regards cancer, one would suspect that a miRNA such as miR-661, which positively regulates the stability and function of p53 by suppressing its two main inhibitors, will be selected against in tumors as a means to quench the tumor suppressor activity of wild type p53. In contrast, in tumors that harbor p53 mutations, particularly mutations that can confer upon the mutant p53 protein an oncogenic gain-of-function, miR661 expression will actually benefit the tumor by upregulating the levels of the oncogenic mutant p53, and therefore will be selected for rather than against. Indeed, analysis of available cancer-derived expression data revealed that in estrogen receptor-positive breast cancer, where p53 is usually wild type, low levels of miR-661 (which are expected to result in attenuation of the resident wild type p53) are a predictor of bad prognosis. In contrast, in high grade serous ovarian cancer, where p53 is almost unanimously mutant and p53 mutations are perhaps the earliest genetic alteration, high miR-661 is associated with bad prognosis.

Prompted by the realization that some of the predicted miR-661 targets are hosted within Alu repeats, we undertook a computational analysis of the functionality of putative miRNA targets located within Alu repeats throughout the human transcribed genome. Using a comprehensive dataset of miRNA overexpression assays, we could show that mRNAs with miRNA targets within Alus are significantly less responsive to the miRNA effects as compared to mRNAs that have the same targets outside Alus. Using Ago2-binding mRNA profiling data, we confirmed that the miRNA machinery avoids miRNA targets within Alus, as opposed to the highly efficient binding of targets outside Alus. Based on our analysis, we proposed three features that prevent potential miRNA sites within Alus from being recognized by the miRNA machinery: (i)Alu repeats that contain miRNA targets and genuine functional miRNA targets appear to reside in distinct mutually-exclusive territories within 3'UTRs, (ii)Alus have tight secondary structure that may limit access to the miRNA machinery, (iii)A-to-I editing of Alu-derived mRNA sequences may divert miRNA targets. The combination of these features is proposed to allow toleration of Alu insertions into mRNAs. Nonetheless, a subset of miRNA targets within Alus appear not to possess any of the above features, and thus may represent cases where Alu insertion in the genome has introduced novel functional miRNA targets, as probably exemplified by Mdm2 and Mdm4 mRNA in the context of miR-661 (Hoffman et al., 2013).

Partner 1 has recently focused his attention to miR-605 which is also predicted to target MDM2 and for which evidence of a role in the regulation of p53 has been obtained. A recently published paper by a competitor group indicated that miR-605 is regulated by p53 and in turn directly targets MDM2 for degradation. Although we could confirm that miR-605 regulates p53 function, we could not confirm MDM2 as a bone fide target. Target identification experiments have been performed to resolve this conundrum.

Through in silico and expression profiling experiments Partner 1 identified miR-29 as putative modulators of melanomagenesis. The miR-29 family of microRNAs has three mature members, miR-29a, miR-29b, and miR-29c. miR-29s are encoded by two gene clusters. The miR-29 family members share a common seed region sequence and are predicted to target largely overlapping sets of genes. miR-29s directly target at least 16 extracellular matrix genes, providing a dramatic example of a single microRNA targeting a large group of functionally related genes. miR-29s have also been shown to be proapoptotic and involved in the regulation of cell differentiation. The physiological and pathological roles of miR29s has been further explored using mouse models.

WP4: OncomiRs Targets Identification

Only few oncomiR-target pairs have been identified to date. Beneficiary 4 has developed an approach for miR targets identification. This technique has already been used to, in a first step, predict targets for the cancer-causing miRs miR10a, miR21 and miR34a and, in a second step, validate several of these targets. Some of these data have been published (Ørom et al., Mol Cell, 2008, Christoffersen et al., 2010). We proposed to use this approach in combination with genome-wide gene expression profiling in order to identify the primary (and secondary) targets of a number of selected oncomiRs.

miR-145 is reported to be down-regulated in several cancers, but knowledge of its targets in colon cancer remains limited. To investigate the role of miR-145 in colon cancer, beneficiary 4 has employed the microarray-based approach to identify miR-145 targets. Based on seed site enrichment analyses and unbiased word analyses, they found a significant enrichment of miRNA binding sites in the 3'-untranslated regions (UTRs) of transcripts down-regulated upon miRNA overexpression. Gene Ontology analysis showed an overrepresentation of genes involved in cell death, cellular growth and proliferation, cell cycle, gene expression and cancer. A number of the identified miRNA targets have previously been implicated in cancer, including YES, FSCN1, ADAM17, BIRC2, VANGL1 as well as the transcription factor STAT1. Both YES and STAT1 were verified as direct miR-145 targets. The study identifies and validates new cancer-relevant direct targets of miR-145 in colon cancer cells and hereby adds important mechanistic understanding of the tumor-suppressive functions of miR-145. This work has recently been published (Gregersen et al., 2010).

miR-34a is an established p53-target gene and a large body of evidence suggests a critical role for this particular miR in tumor suppression. Consistent with this notion miR34a was found up-regulated in senescence cells and thus both in Nutlin-treated BJ cells or following RAF activation in TIG3 fibroblasts. Even if in the latter, induction appears largely p53-independent. To further gain insight into the mode of action of miR-34a, partner 4 searched for putative miR-34a targets and identified a number of putative miR-34a targets. Among this list is the prominent proto-oncogene Myc.

Partner 4 has isolated and identified targets for **miR-10a** from mouse embryonic stem cells. Interestingly, the majority of the targets encode proteins involved in translational regulation, most notably ribosomal protein mRNAs. Through a series of genetic and biochemical analyses we have demonstrated that miR-10a (and other miRNA from the miR-10 family) can bind to the 5'-UTR of ribosomal protein mRNAs. Surprisingly, miR-10a binding at the 5'-UTR mediates translational enhancement rather than repression. Furthermore, translational induction is dependent on the presence of the canonical 5'-TOP motif involved in the translational regulation of many mRNAs encoding ribosomal proteins. Importantly, overexpression of miR-10a mediates a global increase in protein production, which may explain why miR-10 family members have been found overexpressed in several cancers. Towards this we have demonstrated that overexpression of miR-10a results in increased transformation in cell culture models, whereas miR-10a inhibition confers the opposite effect. This work has recently been published (Ørom et al., 2008).

Several reports identified **miR-21** as a key cancer-causing miR. To identify functionally important targets for miR-21 we used the affinity purification method described above and performed Affymetrix array analyses following miR-21 inhibition. Through a series of genetic analyses and reporter studies we validated a set of bone fide miR-21 targets (Frankel et al., 2008).

MDM4 (or MDMX) is amplified and over-expressed in various human cancers. Evidence suggests that MDM4 contributes to tumor formation and/or progression via its ability to bind and inactivate the p53 tumor suppressor protein. A single nucleotide polymorphism (SNP34091) in the 3'-UTR of MDM4 that creates a new illegitimate target site for **miR-191**, a microRNA (miR) that is highly expressed in normal and tumor tissues. We obtained evidence that miR191 binds and regulates the 3'-UTR of MDMX-C but not of MDMX-A allele and that in agreement with that, MDMX protein expression is significantly higher in ovarian cancer patients with the A/A genotype. Importantly, we show that the presence of the C-allele significantly delays

ovarian carcinoma progression and increases sensitivity to chemotherapy. Based on these data, we propose that micro-RNA-mediated downregulation of MDMX-C has a significant impact on cancer progression and chemoresistance by affecting the functionality of p53 tumor suppressor pathway (Wynendaele et al., 2010).

A recent genetic screen identified **miR-372** and **miR-373** as miRs able to neutralize the p53 pathway in cells expressing oncogenic RAS. This effect was reported to be partly dependent on the down-regulation of the mRNA encoding for the LATS2 kinase. However, the reported data suggest that additional targets are likely to play a role in the observed miRs-mediated phenotype. SuV39H1 was identified as another putative target of miR-372 and miR-373. Nevertheless, these pairs were not validated because the implication of Suv39H1 in the regulation of the p53 pathway has not been established. In the meantime, partner 1 has obtained data indicating that SuV39H1 is a new physical p53-binding partner that may play a critical role in p53-induced senescence. In light of these data, we decided to assess experimentally the ability of miR-372 and miR-373 to target SuV39H1 expression. However, our experiments failed to support a regulation of Suv39H1 by miR372-373.

Finally, Partner 4 is currently optimizing the affinity-purification technique by combining in in vivo cross-linking between miRNA and targets and Solexa mass parallel sequencing. This revised method facilitated the identification of both target and miRNA binding site.

miR-449: Invitrogen NCode miRNA microarrays identified miR-449 to be decreased in 1-year-old Gastrin KO mice and in H. Pylori infected gastric tissues compared to tissues from wild type animals. Growth rate of gastric cell lines over-expressing miR-449 was inhibited by 60% compared to controls. FACS cell cycle analysis of miR-449 over-expressing cells showed a significant increase in the sub-G1 fraction indicative of apoptosis. ß-Gal assays indicated a senescent phenotype of gastric cell lines over-expressing miR-449. Affymetrix 133v2 arrays identified GMNN, MET, CCNE2, SIRT1 and CDK6 as miR-449 targets. Luciferase assays were used to confirm GMNN, MET, CCNE2 and SIRT1 as direct targets. Partner 4 also showed that miR-449 over-expression activated p53 and its downstream target p21 as well as the apoptosis markers cleaved CASP3 and PARP. Importantly, qPCR analyses showed a loss of miR-449 expression in human clinical gastric tumours compared to normal tissues (Bou Kheir et al., 2011).

Two studies –one in the lab of partner 4 and one in the lab of Partner 7- focused on **miR-9**:

Cancer stem cells or cancer initiating cells are believed to contribute to cancer recurrence after therapy. MicroRNAs (miRNAs) are short RNA molecules with fundamental roles in gene regulation. The role of miRNAs in cancer stem cells is only poorly understood. Partner 7 has recently reported miRNA expression profiles of glioblastoma stem cell-containing CD133(+) cell populations. They find that miR-9, miR-9(*) (referred to as miR-9/9(*)), miR-17 and miR-106b are highly abundant in CD133(+) cells. Furthermore, inhibition of miR-9/9(*) or miR-17 leads to reduced neurosphere formation and stimulates cell differentiation. Calmodulin-binding transcription activator 1 (CAMTA1) is a putative transcription factor, which induces the expression of the anti-proliferative cardiac hormone natriuretic peptide A (NPPA). They identify CAMTA1 as an miR-9/9(*) and miR-17 target. CAMTA1 expression leads to reduced neurosphere formation and tumour growth in nude mice, suggesting that CAMTA1 can function as tumour suppressor. Consistently, CAMTA1 and NPPA expression correlate with patient survival. The findings could provide a basis for novel strategies of glioblastoma therapy (Schraivogel et al., 2011).

miR-9 is overexpressed in several cancer forms, including brain tumours, hepatocellular carcinomas, breast cancer and Hodgkin lymphoma (HL). Partner 4 has recently demonstrated a relevance for miR-9 in HL pathogenesis and identified two new targets Dicer1 and HuR. HL is characterized by a massive infiltration of immune cells and fibroblasts in the tumour, whereas malignant cells represent only 1% of the tumour mass. These infiltrates provide important survival and growth signals to the tumour cells, and several lines of evidence indicate that they are essential for the persistence of HL. We show that inhibition of miR-9 leads to derepression of DICER and HuR, which in turn results in a decrease in cytokine production by HL cells followed by an impaired ability to attract normal inflammatory cells. Finally, inhibition of miR-9 by a systemically delivered antimiR-9 in a xenograft model of HL increases the protein levels of HuR and DICER1 and results in decreased tumour outgrowth, confirming that miR-9 actively participates in HL pathogenesis and points to miR-9 as a potential therapeutic target (Leucci et al., 2012).

In addition, partner 4 has more recently obtained evidence that MALAT1 a lon non-coding RNA is a target of miR-9. The data indicate that miR-9-mediated regulation of MALAT1 impact on the splicing of several ribosomal genes and eventually on overall efficiency of protein translation (Leucci et al., manuscript re-submitted for publication in 2013).

Finally, MDM2 has been validated as a direct target of miR-339 and miR-661 but not of miR-605 (as previously reported in the literature). In contrast, COP9 and TSN were identified as direct miR-605 targets (Wynendaele et al., manuscript in preparation 2013).

WP5: Expression of miRs and miR-processors in cancer

To further facilitate and improve sensitivity and accuracy of genome-wide ncRNA profiling, we proposed to generate new microarray platforms based on the LNA technology developed by partner 6. In order to incorporate LNA monomer into the synthesis of oligonucleotides on the array, the LNA phosphoramidites have to have appropriate protecting groups for the light directed synthesis. This means a protecting group, which can be cleaved by light at the 5'-position. The protecting groups on the nucleobase should be photo-stable, but easy to cleave off by hydrolysis. The standard protected LNA amidites do not fulfill these requirements; therefore LNA phopshoramidites with new protecting groups were synthesized. The initial strategy for synthesizing the desired LNA phoshphoramidites was to use the same synthetic pathways as for the DNA counterparts. This proved to be unsuccessful, due to slightly different reactivity of the hydroxyl groups of the LNA compared with the DNA counterparts. A different synthetic strategy was therefore developed, which is a bit longer but requires less cumbersome purification steps than for the DNA counterparts. The synthesis of the LNA mC was, however, inherently difficult to synthesize, purify and store due to its instable character. The first LNA-NimbeGen slides were produced with the LNA amidites and slides were tested using Exiqon standard Lung vs. Lung (+validation pools) and with synthetic miR pools. So far the results indicate that there are some issues to be solved concerning variations between different batches of produced slides. The issues are related to capture probe discrimination, which was good on the first set of arrays but less good on the second batch. Furthermore signal/sensitivity was also lower (approximately 5-fold) on the second batch relative to the first batch of slides and lower than identical hybridization signals obtained on the Exiqon miRCURY slides. In contrast the second batch of slides showed less background hybridization than the first batch. At this point the cause of the observed batch variations is unknown and therefore further work is needed to elucidate whether variation is caused by amidites, capture probe synthesis, post-synthesis handling of slides or caused by inappropriate conditions for hybridization and washing. Work will continue to resolve these issues.

We have set up a system for fast and flexible design of LNA capture probe for any set of non-coding RNAs. The system takes advantage of several in house software tools, consisting primarily of an LNA probe design tool and several probe property prediction tools. It also takes experimental probe property data into account, in order to re-use existing experimentally verified optimal probes whenever possible and discard any probes previously demonstrated to posses bad properties. Basically, the design process is split into a number of 'steps' that are automatically carried out consecutively. By treating the target sequence dataset as a whole, the system also design probes that are as specific as possible (i.e. they are able to distinguish similar target sequence).

Subsequently, LNA amidites with new protective groups had been developed to allow light directed synthesis for use on the NimbleGen flexible microarray platform. Last year beneficiary 6 reported the development of an LNA capture probe design algorithm for ncRNAs. Building on results from last year, miRNA capture probes were synthesised on the NimbleGen platform in an initial feasibility study. To examine coupling efficiencies of the new amidites, the amidites were added to the linkers in varying numbers. Oligo signals were found to be unaffected by the number of LNA amidites in the linkers, and all 4 amidites could be synthesized with same coupling efficiencies as their DNA counterparts. Next, a round of optimisation experiments were carried out to optimise the experimental conditions of the flexible arrays towards the LNA spiked oligos, including determining optimal hybridisation temperature, linker, oligo concentration, and probe spot size. Finally, a round of experiments was performed to measure within-slide and between-slide

reproducibility, sensitivity, as well as absolute and relative quantification. The flexible microarray platform was found to correlate well with the Exiqon's miRCURY LNA™ microRNA array platform, and overall performance was comparable. Importantly, relative quantification typically used for expression profiling was on par with the commercial miRCURY LNA array. The primary drawbacks were higher background levels and a more cumbersome (and risky) protocol. In conclusion, our results suggest that de novo design and implementation of LNA ncRNA flexible arrays are feasible, although additional specificity and stability experiments are required.
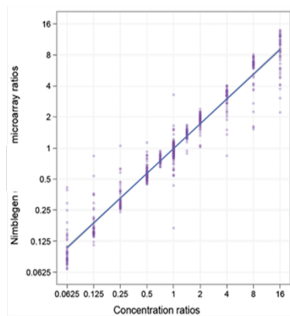
Fig Legend: Relative concentration correlation on NimbleGen arrays (R=0.95)

Improved procedures for rapid integration of public database (e.g. miRBase) updates were implemented. During the course of period 2 beneficiary 6 has uploaded to the database 366 snoRNAs, 35 snRNAs, and 481 ultra-conserved regions based on a review of different classes of ncRNAs. In addition, quantitative real-time PCR assays against this new set of ncRNAs based on Exiqon's Universal Probe Library were designed. Also, in silico investigations suggest that array capture probe design against the new set of ncRNAs using LNA technology was shown to be feasible.

An internal RNA sequence database has been created. The system is based on PostgreSQL and supporting Ruby scripts. Effort has been put into ensuring data accuracy by the use of foreign keys, user education, limited access rights and strict procedures for data entry. Novel miRNAs have been predicted from high throughput sequencing data. These predicted miRNAs have been uploaded to the database system. miRNA upload from mirbase has been improved by uploading the sequences directly from the mirbase SQL interface rather than using traditional flat files thereby allowing us to get much more context information in a structured way. This procedure has also enabled us to upload not only the mature miRNA but also the pre-miRNA sequences easily. Predicted published miRNAs that were not deposited in mirbase have been identified and uploaded to our database. A review of different classes of ncRNAs have identified novel ultra-conserved regions, predicted antisense RNA and piRNAs.

Using LNA-based microarray analysis, Beneficiary 6 has generated a genome-wide miRNA profile for 59 cell lines held within the NCI60 panel. The NCI60 panel is a reference panel of cell lines for which a large body of data concerning, among others gene expression and drug response, is publically available. The panel is generally used as a starting point for global evaluation of promising new anti-cancer drugs, both with respect to their activity but also in relation to the discovery of predictive biomarkers for activity. We have generated a miRNA profile for the cell lines in the panel and performed a meta-analysis incorporating both our profile, and similar profiles performed on other platforms, to show excellent concordance between the platforms. This may form a basis for the experimental validation of novel oncomirs and tumour suppressor miRNAs, and may help in identifying miRNAs that predict response to certain chemotherapeutic drugs (Søkilde et al, 2011).

Partner 1 and 4 (in collaboration with partner 6) have profiled the expression of a series of miRs, their targets and more than 2000 long non-coding RNAs in the NCI60 cancer cell lines, normal mouse and human tissues and cell types and selected human primary tumors (lymphoma, bladder cancer, prostate cancer, melanoma and

mouse Eu-Myc B-cell lymphoma). The data are available to the consortium (Deliverables 38 and 42). Interestingly, a number of long non-coding RNAs that are specifically expressed in cancer cells (as opposed to their normal counterparts), or regulated by p53 or MYC have thus been identified. A set of 10 ncRNAs that are highly and specifically expressed in melanomas but not in other tumor types will be particularly studied in the laboratory of Partner 1. Their functional characterization is ongoing using, among other tools, gapmers provided by Partner 6 for their functional downregulation. Their putative oncogenic or tumor suppressor roles will be explore in cell-based assays and possibly in mice.

The expression levels of NF90 and EDC4 (see WP2) in the NCI60 cancer cell lines, various human tissues and cell types and selected human primary tumors (lymphoma, bladder cancer, prostate cancer, melanoma) is available at Partner 7 (Deliverable 40). Both of these genes are downregulated in a number of cancer cell lines and primary tumors.

Partner 1 and 4 (in collaboration with partner 6) have profiled the expression of a series of miRs, their targets and more than 30.000 long non-coding RNAs in the NCI60 cancer cell lines, normal mouse and human tissues and cell types and selected human primary tumors (lymphoma, bladder cancer, prostate cancer, melanoma and mouse Eu-Myc B-cell lymphoma). The data are available to the consortium (Deliverables 38 and 42). Interestingly, a number of long non-coding RNAs that are specifically expressed in cancer cells (as opposed to their normal counterparts), or regulated by p53 or MYC have thus been identified. A set of 7 ncRNAs that are highly and specifically expressed in melanomas and melanoma cell lines (from the NC60 panel; NC60x) but not primary normal human melanocytes (PMx) are being studied in the laboratory of Partner 1.
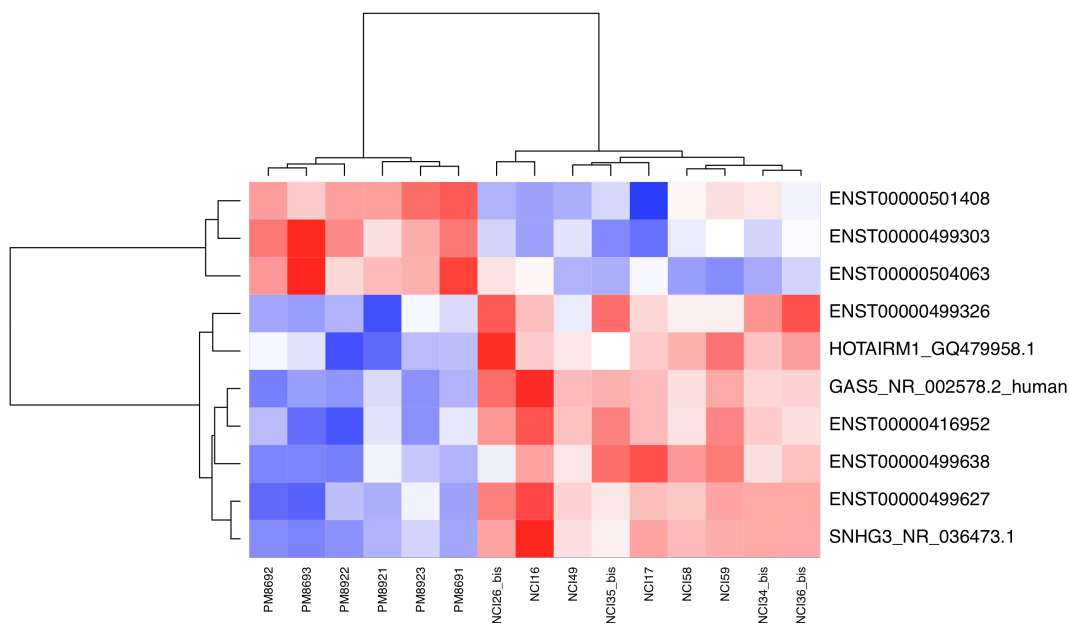
Their functional characterization is ongoing using, among other tools, LNA-based gapmers provided by Partner 6 for their functional downregulation. Their putative oncogenic or tumor suppressor roles will be explore in cell-based assays and possibly in mice. A p53-regulated non-coding RNA has also been identified, its role in p53-mediated tumor suppression and reprogramming is currently being investigated using LNA-gapmers and in mice.

WP6: OncomiR Mouse Models

In order to define the physiological function and assess the role of selected ncRNAs in the genesis and progression of cancer, we proposed to perform classical gain and loss of function experiments in cultured cells and in vivo, using the mouse as a model system. For conditional gain of function studies, we knock-in the gene of interest into the ROSA26 locus by gene targeting. The newly generated alleles are combined with tumour-causing mutations to study the consequence of aberrant expression of a particular ncRNA or component of the miR-machinery in relevant tumor models of human cancers. Moreover, given the data obtained in the context of WP2, we decided to analyze the consequences of aberrant expression of Dicer in a relevant preclinical mouse model of cancer.

We decided not to invest efforts and resources in the generation of two of the initially proposed mutant mouse lines: miR34a KO and miR199a-transgenic mice. miR34a KO mice (D44) had already been generated by a competitor laboratory. Moreover, we also decided not to generate miR-199a transgenic mice (D46) since we could not obtain reliable and reproducible data on the regulation of miR-199a by wild-type and mutant p53. As a consequence D49 and D51 have not be achieved. However, this is largely compensated by the involvement of partner 1 in the characterization of Dicer loss of function in the retinoblastoma mouse model and the generation of other ncRNA mutant mouse lines as described below.

Dicer function has thus been genetically inactivated in a mouse model of retinoblastoma (Chx10-cre;Rb $^{lox/lox}$; p170$^{-/-}$). The currently available data suggest that loss of one **Dicer1** allele accelerates significantly tumorigenesis. The data therefore suggested that Dicer is a haplo-insufficient tumor suppressor gene on this sensitized tumor genetic background. The molecular mechanisms that promote tumorigenesis as a consequence of global repression of miRNA maturation remain elusive. Beneficiary 1 has recently obtained evidence that p53 tumor suppressor function is compromised in cancer cells harbouring hypomorphic Dicer expression. Interestingly, loss of p53 was previously shown to dramatically enhance retinoblastoma formation and progression. Importantly we showed that Dicer1 ablation completely prevents tumorigenesis (Nittner et al., 2012).

P68 (DDX5) was identified by partner 7 as part of the DROSHA complex, thus confirming previously published data. In order to study its physiological function and role in cancer development partner 1 has generated a conditional floxed allele for p68 (DDX5). This gene encodes a DEAD-box-containing RNA helicase, which associates with the DROSHA processing complex to facilitate the processing of primary miRNAs to precursor miRNAs. Importantly p68 has been shown to interact with p53 to modulate its transcriptional activity. Vis-versa p53, through its association with attenuates microRNA-processing activity of the DROSHA complex. Using this genetic tool we could not confirm an important role for p68 in the regulation of p53 activity in MEFs and in vivo (thymocytes following whole body irradiation), however, we could confirm an implication in miR biogenesis. We have also recently obtained evidence that, just like Dicer, Chx10-Cre-mediated mono-allelic as well as bi-allelic loss of p68 in retinoblasts do not affect normal retinogenesis. Interestingly, monoallelic loss of p68 accelerates retinoblastoma development. In contrast of Dicer loss, however, complete ablation of p68 even further accelerated cancer development on this background. These data identify a classical tumor suppressor function of p68 in tumorigenesis. The underlying mechanism of p68-mediated tumor suppression in currently under investigation in the laboratory of partner 1.

The **miR-17-92** floxed mice generated by A Ventura and T Jacks were imported in the laboratory of beneficiary 1. This allele has been transferred into (Tyr::NRas) melanoma and retinoblastoma genetic backgrounds in order to test the importance of this microRNA oncogenic cluster for the development of these diseases. Strikingly, we showed that complete loss of miR17-92 phenocopied Dicer loss on the retinoblastoma background and completely prevents cancer formation (Nittner et al., 2012). In contrast loss of miR17-92 did not affect normal retinogenesis; an observation that has important therapeutic implications. In sharp contrast, although upregulated in mouse and human melanoma this cluster appears dispensable for melanogenesis and NRas-induced melanoma formation in mice

(Nittner et al., manuscript in preparation 2013). The importance of the miR-17-92 cluster for Myc-induced lymphomagenesis had been demonstrated by a competitor group (Ventura lab).

The microRNA (miRNA) miR-10 family has attracted attention because of its conservation and the position of the miR-10 genes within the Hox clusters of developmental regulators. In several species, miR-10 is co-expressed with a set of Hox genes and has been found to regulate the translation of Hox transcripts. In addition, members of the miR-10 family are de-regulated in several cancer forms. Aside from acting in translational repression, miR-10 was recently found to bind a group of transcripts containing a terminal oligo-pyrimidine (TOP) motif and to induce their translation, thereby adding a new function to the miRNA repertoire (Lund, 2010). **miR-10a** KO mice have been generated by partner 4 and are currently being analyzed. In particular these mice have been crossed with the tumor prone APC-min mice. Preliminary observations indicate that loss of miR-10a significantly increase the number of adenomas in female mice. The mechanism underlying this observation is currently under investigation. miR-10a KO MEFs have been characterized with respect to proliferation, senescence and transformation capacity. Interestingly, miR-10a KO MEFs transform less efficiently than wild-type when transduced with RAS and MYC oncogenes. The mechanism responsible for this phenotype is also under investigation.

miR-101a and miR-101b have been identified as key regulators of autophagy by partner 4. Partner 4 is in the process of creating KO mouse strains for both of these miRs using the ZNF technology.

miR-29a, miR-29b and miR-29c were identified by partner 1 as putative modulator of melanomagenesis and in particular of metastasis. We have generated Rosa26-targeted conditional miR-29 transgenic mice and a triple KO (double targeting) mouse line. These mice allowed us to study the biological consequences of miR-29 gain and loss of function in the context of melanomagenesis and retinoblastoma development. The data indicate that miR-29a, b and c are completely dispensable for both melanoma and retinoblastoma development; however – interestingly, enforced expression of miR-29s dramatically enhances metastasis formation on the melanoma background. The underlying mechanism, and in particular the miR-29 targets that account for the invasive phenotype is still under investigation.

miR-605 transgenic and KO mice have been generated in the laboratory of Partner 1. While miR-605 KO mice did not exhibit any obvious phenotype as evidences by extensive histopthalogical analyses, miR-605 overexpression led to an embryonic lethality, which could be rescued on the p53-deficient background. This observation confirms our biochemical data and further establish a genetic link between miR-605 and the p53 network. Additional experiments are on going in the laboratory of partner 1 in order to further dissect the molecular basis underlying this genetic link.

WP7: miR-based therapy

RNA interference represents a potential strategy for in vivo target validation, therapeutic product development and clinical new technologies. It is expected to be particularly useful to silence cancer-causing genes that encode targets that are not amenable to conventional therapeutics. Moreover, there is evidence that miR-based molecules enter the RNAi pathway through a more natural route and yield more effective silencing with reduced toxicity and off-target effects. miR delivery in vivo in tumors is achievable by using new physical methods (electropulsation) developed by partner 5 and prone for clinical applications. Towards this goal, a new generation of miR mimics and antisense therapeutics have been developed and tested. The synthesis of chemically modified LNA-based oligos as miR inhibitors and miR-like oligos is in progress. Initiatives made in collaboration with Partner 3 continue to evaluate performance of both miRNA specific and miRNA family specific inhibitor oligos.

Very little was known about the basic processes supporting the electrotransfer of RNA molecules and LNA-based oligonucleotides. Partner 5 tested and optimized the biostability, cytotoxicity and electropulsation-based delivery in cultured cells and in transgenic mice of such molecules. Unlabelled and cy5 labelled-antisens LNAs were designed to inhibit miR34a. A scrambled LNA was used as negative control. LNAs were electrotransferred into HCT116 cells (Human Colorectal Tumor Cell Lines) or CHO cells (Chinese Hamster Ovary Cells). A Jouan square wave electropulsator was used with parallel plate electrodes. The pulses were monitored on line. Four parameters were analyzed: Feasibility and optimization of LNA delivery by electropulsation, Visualization of electrotransferred LNA, Mechanism of LNA delivery by electropulsation, electrotransferred LNA functionality. HCT116 cells (500 000 cells/point) were electropulsed with the cy5-LNA (250nM) using several distinct electrical parameters. FACS analyses were performed 24 hours later in order to determine which protocol is the most efficient for LNA delivery. Three electrical parameters which were: 8 and 4 pulses with an intensity of 700 V/cm and 8 pulses with 600 V/cm (duration of each pulse was 5ms and delay between pulses was 1 s), were more efficient for LNA delivery. Cell viability was then tested for these 3 parameters. When 8 pulses were applied (for 700 or 600 V/cm), cell viability was only around 50% whereas with 4 pulses it was almost 90%. Considering these results, the optimal electrical parameter is: 4 pulses of 5 ms with an intensity of 700 V/cm. The delay was kept at 1 s. HCT116 cells (500 000 cells/point) were then electropulsed using the optimal electrical parameters as determined above in presence of cy5-LNA (750nM). Cells were then plated on lab-tek slides. The next day, LNA delivery was visualized by fluorescence microscopy. We observed a slight fluorescence when cells are in presence of 750nM cy5-LNA even without electrical pulses application (NP), meaning that there was a passive diffusion of LNA into cells. However, when cells were electropulsed a strong cy5 staining was observed within the cells. This staining was restricted to the cytoplasm; no staining was seen in the nucleus. Thus, electropulsation is an efficient technique for cytoplasmic LNA delivery. To assess th emechanism of entry, CHO cells (500 000 cells/point) were plated on lab-tek and electropulsed (8 pulses of 5 ms with an intensity of 700 V/cm and a delay between pulses of 1 s).in presence of 750nM Cy5 LNA directly under a fluorescence confocal microscope (zeiss 510) allowing us to visualize in live the LNA entry. During pulses application, LNA which is negatively charged, penetrated into the cytoplasm only on the side of the cell facing the cathode [-] and then moved towards the anode [+]. This behaviour is experimental evidence that LNA entry is driven by electrophoretic forces. They next compared electropulsation with other classical transfection methods. Cy5-LNA (250nM) was electrotransferred using the optimal electrical parameters as determined above and with the polymer-based DNA transfection reagent; Jet PEI according to manufacturer instructions. Cells were then plated on lab-tek slides. The next day, LNA delivery was visualized by fluorescence microscopy. Quantifications were done with the Image J software. Cy5 staining is almost 2.5 fold higher when cells were electrotransfected in comparison with chemical transfection. Thus, electropulsation method was shown to be much more efficient for LNA delivery than other transfection methods.

NP	Pulses	JetPEI
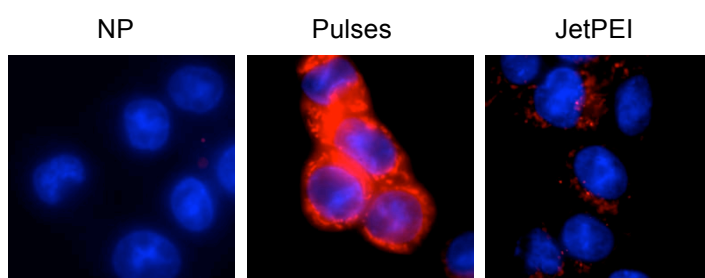
Cy5-LNAs staining visualized by fluorescence microscopy

To further assess the elecrotransfer efficacy HCT116 cells were pulsed in presence of unstained antagomiR34a LNA (250nM) using optimal electrical parameter as described above (4x, 700 V/cm, 5ms, 1 s). In order to activate the p53 and so the miR34a pathway, adriamycin (0.2μg/mL) was added to the pulsed cells for 48 hours. Apoptosis analysis was performed by flow cytometry. We observed a decrease in adriamycin induced apoptosis when cells were electrotransferred with antagomiR34a LNA. This is encouraging preliminary data need however to be confirmed. In conclusion, we demonstrated that LNA can be

electrotransferred into cells. The delivery, which is driven by the electrophoretic forces during pulses application, is restricted to the cytoplasm. We determined the optimal electrical parameters for LNA delivery in accordance with good cell viability. The electropulsation method can deliver LNA with higher efficient than classical chemical reagent for LNA transfection. Moreover we showed that electrotransferred LNA are biologically active.

Electropermeabilization is one of the nonviral methods successfully used to transfer small molecules such as oligos including siRNA, miR mimics or anti-miRs into living cells in vitro and in vivo. Although this approach is effective in the field of gene silencing by RNA interference, very little is known about the basic processes supporting the transfer. Partner 5 has recently investigated, by direct visualization at the single-cell level in vitro and in vivo, the efficiency and mechanism of delivery of Alexa Fluor 546-labeled siRNA into murine melanoma cells stably expressing the enhanced green fluorescent protein (EGFP) as a target gene (Deliverable 59). The electrotransfer of siRNA was quantified by time-lapse fluorescence microscopy and was correlated with the silencing of egfp expression. A direct transfer into the cell cytoplasm of the negatively charged siRNA was observed across the plasma membrane exclusively on the side facing the cathode. When added after electropulsation, the siRNA was inefficient for gene silencing because it did not penetrate the cells. Therefore, an electric field acts on both the permeabilization of the cell plasma membrane and on the electrophoretic drag of the negatively charged siRNA molecules from the bulk phase into the cytoplasm. The transfer kinetics of siRNA are compatible with the creation of nanopores, which are described with the technique of synthetic nanopores. The mechanism involved was clearly specific for the physico-chemical properties of the electrotransferred molecule and was different from that observed with small molecules or plasmid DNA (Paganin-Gioanni et al., PNAS 2011). Fluorescence uptake of Cy5 labeled oligonucleotides was observed by whole body fluorescence imaging. Different amounts (10 and 30 mM concentration) were injected in subcutaneous HCT tumors expressing GFP implanted in Nude mouse model. Only a slight improvement in uptake was detected just after the electrodelivery (under EGT protocols). This was not statistically significant. On the long-term post electropulsation, whole body imaging results were confirmed by FACS after tumor dissociation and histological slices. No enhanced uptake induced by electropulsation was detected with Cy5 tagged siLNA and siRNA. GFP silencing was nevertheless present with siRNA electrodelivery as already observed (Paganin-Gioanni et al., PNAS 2011). The lack of enhancement in uptake suggests that the presence of Cy 5 creates an artefactual endocytotic pathway in HCT tumors. This was not the case for delivery in muscles where an enhanced uptake was brought by the electrical treatment.

Cy5.5 and Cy7 labeled miR34a-based LNA oligos with an exciting and emission wavelengths in the near infrared were synthesized by partner 6 and electrotransferred into HCT116 cells (Human Colorectal Tumor Cell Lines) or CHO cells (Chinese Hamster Ovary Cells). A Jouan square wave electropulsator was used with parallel plate electrodes. FACS analyses were performed 24 hours later. Three electrical parameters which were: 8 and 4 pulses with an intensity of 700 V/cm and 8 pulses with 600 V/cm (duration of each pulse was 5ms and delay between pulses was 1 s), were more efficient for LNA delivery. Cell viability was then tested for these 3 parameters. When 8 pulses were applied (for 700 or 600 V/cm), cell viability was only around 50% whereas with 4 pulses it was almost 90%. The optimal electrical parameters were comparable to the ones obtained earlier using similar oligos labeled with cy5 (4 pulses of 5 ms with an intensity of 700 V/cm). These oligos were then electrotransferred into murine melanoma cells in vivo as described above. Live Whole body imaging using the spectral CRI Nuance 2 camera and the appropriate filter set confirmed efficient delivery of the molecules into the cancer cells in vivo.

A major issue for successful human gene therapy or genetic vaccination is a safe high-transgene expression level. Plasmid-based (non-viral) physical methods of gene transfer offered attracting approaches but their low efficiencies have limited their use in human pre-clinical trials. One of the limits appears to be the size of the plasmid that must be transferred across the cell membrane to the nucleus for its processing. In the present work to enhance gene transfer and expression, we evaluated a new generation of DNA vector; the minicircle, combined with the electropulsation technique. Minicircle is a doubled-stranded circular DNA with reduced size as it is devoid of bacterial sequences. We showed that electrotransferred minicircle encoding green fluorescent protein had higher in vitro transfection level compared with full-length plasmid. We demonstrated that minicircle great efficiency was not because of cellular toxicity decrease but was correlated to more efficient vector uptake by cells. Vector electrotransfection was operated in vivo and, using fluorescence

imaging, minicircle electrotransfer was shown to enhance the efficiency and duration of tissue-targeted gene delivery and expression. By combining powerful expression and delivery systems, we have provided a valuable method for new approaches in gene therapy and genetic vaccination (Chabot et al., 2012). This method could be used in the future for the delivery of anti-miR molecules (such as sponges).

Mouse genetic data obtained in the laboratory of partner 1 indicate that while miR-17~92 is dispensable for normal retinogenesis, just like *Dicer1*, it is required for the formation of aggressive and invasive retinoblastoma by synthetic lethality with p53. To investigate whether the survival function of miR-17~92 revealed with the mouse model was applicable to human tumours that harbour RB1 mutations we inactivated all components of the miR-17~92 cluster using miRNA-inhibitors in the human retinoblastoma cell lines Rb15, WERI-Rb1 and Y-79 (Deliverable 63). We confirmed that the miR-17~92 cluster is expressed at very high levels in these cells. Electrotransfer of all 6 miR-17~92 members did not affect cell growth significantly, however, we observed a consistent and reproducible decrease in cell growth upon transfection of a mixture of miR-17/miR-20a inhibitors in the Y79 parental cell line or in Y79 expressing a control shRNAs. The growth inhibitory effect was also observed in WERI-RB1. Because the p53 tumour suppressor pathway is compromised in retinoblastoma through overexpression of negative regulators of p53 these data indicate that the synthetic lethal interaction between members of the miR-17~92 and p53 may be conserved in pre-formed retinoblastoma. To further test this possibility we further silenced p53 artificially by stably expressing shRNAs targeting p53 in Y79 cells. Transfection of the anti-miR-17/20a mixture in these cells resulted in an even greater growth defect than the one observed in cells expressing the control shRNAs. These data therefore indicate that retinoblastoma cells are addicted to high levels of miR-17-20a expression as a result of a synthetic lethal interaction with p53. Targeting these microRNAs should therefore be explored as a new therapeutic approach for the selective treatment of retinoblastoma (Nittner et al., 2012). Anti-miR-17 and anti-miR20a have more recently been electroporated in the eye of retinoblastoma-bearing mice; preliminary data indicate that this treatment (as opposed to electroporation on non-targeting anti-miR) has a profound impact on the survival and growth of these tumors. Further experiments are required to confirm this exciting finding.

Moreover, miR-9 and miR-9* are highly expressed in human glioblastomas. 5.10^6 Luc expressing glioblastoma cells were subcutaneously injected in Nude mice. Luciferase activity was observed following intraperitoneal injection of luciferin. AntagomiR electrotransfer was performed 7 days after cell implantation when tumors reached ≈3mm^3. Briefly, 4 nmoles in 20μL PBS of each antagomiR (9 or 9* or 122 as control) were injected directly into the tumors. Thirty seconds after injection, plate parallel electrodes were fitted around the tumor and electric pulsation (8 pulses lasting 5ms at 600V/cm, 1Hz) was applied. Mice received 3 treatments at days 0, 2 and 5. As tumor growth is quite heterogeneous between mice, results were expressed relative to the luciferase activity at Day 0 for each tumor. While we observed no tumor growth inhibition with the antagomiR9 tumors electrotransferred with the antagomiR9* showed a decrease in growth following the third treatment:
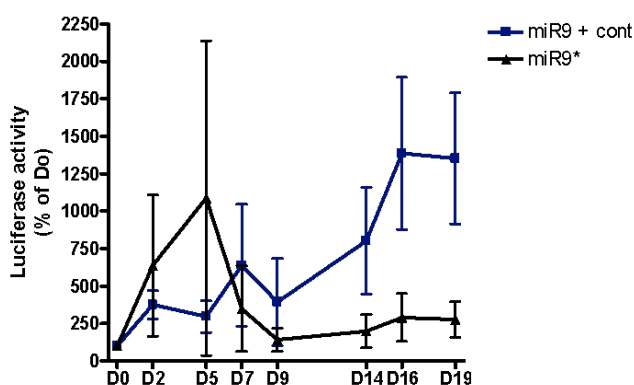
Glioblastoma growth following antagomiR electrotransfer

These data therefore indicate that electrotransfer of antagomiRs offers a new avenue for anti-glioblastoma therapy.

For an updated list of ONCOMIRS' publications see:
http://med.kuleuven.be/cme-mg/oncomirs/Publications.html

- The potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results.

Scientific impact

The importance of non-coding RNA on normal development and cellular processes such as proliferation, differentiation and apoptosis can no longer be under appreciated. The results from the efforts of this consortium unveiled novel functions of non-coding RNAs in normal and pathological processes.

The experiments proposed for the **ONCOMIRS** consortium followed a natural succession through four experimental strategies. In the work packages WP1, WP2 and in part WP4 we **DISCOVERED** and unveiled novel non-coding RNAs and investigated links between ncRNAs - and the processing machinery involved in their biogenesis - and human cancers. In the work packages WP2, WP3 and WP5 we **CONNECTED** the information obtained on deregulated ncRNAs, in particulat miRs, with known cellular pathways involved in cancer, such as the p53 tumour suppressor pathway. We experimentally identified a number of miR aetiologically related to cancer. To establish a causal relationship between dysregulated ncRNAs and cancer development we **MODELED** the tumorigenic processes in mice lacking or overexpressing particular ncRNAs (WP5). These mouse models have been used in conjunction with other cancer models in WP6 to explore **therapeutic** strategies and develop delivery protocols for modified oligonucleotides mimicking or inhibiting endogenous ncRNAs.

In particular we have:

- Identified ncRNAs deregulated in cancer
- Unveiled links between ncRNAs and cancer-related processes
- Unveiled links between ncRNAs and known tumour suppressors or oncogenes
- Explored the significance of the miR biogenesis machinery in cancer
- Identified target mRNAs for ncRNAs deregulated in cancer
- Established mouse models for deregulated ncRNAs
- Developed chemically modified ncRNA mimicks and inhibitors
- Developed advanced preclinical treatment and monitoring protocols for ncRNA mimicks and inhibitors

The ONCOMIRS consortium created a large amount of knowledge, knowhow and scientific data, most of which ended up being published in high impact factor journals such as Nature Cell Biology, Nature genetics, Molecular Cell, Cell Death and Differentiation, Oncogene, ... This was possible thanks to the good synergy between the 6 complementary groups involved in this project; all of which joining forces in a concerted effort towards knowledge-based cancer therapeutics.

Community impact

The relevance and impact for the European community is evident at several levels. **Scientifically**: The research in the consortium is of outmost importance for understanding the roles of ncRNAs in pathological processes such as cancer. **Educationally**: The consortium created a perfect environment for research training, both at PhD and post-doc levels. These PhDs and post-docs will continue being very attractive for biotech and pharmaceutical companies, as well as other research institutions. **Economically**: The research led to commercially interesting results and thus enable patent applications covering novel targets for anticancer therapy. Moreover, the consortium attracted international competitive funding and high-level non-EU researchers, thereby boosting European research. Finally, the research led to the identification of <u>novel targets for cancer therapy and development of novel drugs or clinical tools for diagnostics and cancer treatment</u>. This will ultimately lead to better treatment of cancer and possible other serious diseases and therefore **improve the health** of the European population.

The ONCOMIRS consortium bridged ongoing independent research efforts in 6 distinct laboratories and created synergy by joining hitherto unconnected research groups encompassing biophysical, biochemical, biological and genetic expertise at the highest level. Importantly, the research required the combined scientific expertise of each of the laboratories. The research plan and the scientific objectives were very ambitious and could only been met with the establishment of a committed research consortium and long-term financial support from the EU.

The ONCOMIRS consortium built and extended upon national research initiatives and strategies from the 5 countries involved. Accordingly, via extended collaborations and matching of complementary capacities at the EU level this synergistic FP7 network added significant additional value to existing national research initiatives. Importantly, this ambitious scientific bench-to-bedside pipeline was not possible on a national scale.

An important goal for this research consortium was to foster and develop research talent. The research centre had more than 40 scientists and technicians. We applied for salaries and funding (EC and non EC contribution) for 10 PhD students and 15 post-docs per year in the outlined program. The research environments participating in this consortium were excellent, and the PhD students and post-docs, in addition to the challenging research projects, were embedded in these superb research-training environments. The consortium furthermore mediated increased internationalisation of European research environments.

Contributions to standards:

THE PROJECT FOCUSSED ON THE PRE-CLINICAL STAGE OF DEVELOPING MOLECULAR DIAGNOSTICS AND THERAPEUTICS. THEREFORE, IT WAS NOT ANTICIPATED TO AFFECT THE CURRENT CLINICAL STANDARDS FOR CANCER TREATMENT ON A SHORT-TERM BASIS. HOWEVER, THE IDENTIFICATION OF MOLECULAR SIGNATURES WITHIN TUMOR TISSUE WILL FACILITATE THE CLASSIFICATION OF PATIENTS INTO PROGNOSTIC GROUPS. ACCORDING STRATIFICATION OF PATIENTS FOR TREATMENT IS THEREFORE ANTICIPATED TO BECOME EUROPEAN STANDARD IN THE FORESEEABLE FUTURE.

Contribution to policy developments

THIS PROJECT DID NOT HAVE AN INTEGRAL COMPONENT THAT SHOULD AFFECT RESEARCH POLICY. HOWEVER, IN BRINGING TOGETHER 6 INDEPENDENT RESEARCH GROUPS FROM 5 COUNTRIES, THE PROJECT REPRESENTS AN EXAMPLE FOR INTEGRATING COMPLEMENTARY RESEARCH CAPABILITIES ALL OVER EUROPE. THE SUCCESS OF THIS PROJECT IS LIKELY TO ENCOURAGE ADDITIONAL ATTEMPTS TO PROMOTE CO-ORDINATED MULTIDISCIPLINARY, MULTINATIONAL EFFORTS TO FIGHT CANCER IN PARTICULARLY AND RELATED HEALTH PROBLEMS IN GENERAL.

Dissemination of results

The primary channel of dissemination of the research results has been through publication in peer-reviewed international scientific journals. At the same time, the technology transfer division of the host institutions rigorously screened all outgoing communications of research results, in order to secure intellectual property rights when applicable. This was done trough patent applications.

Online publication of the research respected international copyright law, and the host institutions followed the current discussions on 'free access' in the life sciences. The most suitable journals for the results offered the possibility of paying a publication fee to ensure free access to all, this option has always been chosen.

The VIB research website (www.vib.be/research) and the "ONCOMIRS" website has been adapted in order to provide dedicated space to present the activities and up-to-date research results of the research project in a dynamic way. Reference was made to Framework 7 of the European Union. In addition, these activity-specific website pages were fully integrated into the existing VIB website in order to demonstrate the integration of this new project into the host insitution's and the team leader's strategy.

The results obtained by the consortium were presented at various national and international conferences throughout the world by the PI, postdocs and PhD students involved in this project.
.

Finally, the coordination office collaborated with the host's communication team, which has a high track record in successfully organising activities to increase the understanding of life science in the general population. (www.vib.be/education).

Intellectual Property Management

In keeping with all the relevant legislation, intellectual property has been protected through the filing of international patent applications. VIB (www.vib.be), has a technology transfer team in place consisting of experienced invention analysts, patent lawyers and licensing managers specialized in the field of life sciences.

- The address of the project public website, if applicable as well as relevant contact details.

Website:
http://med.kuleuven.be/cme-mg/oncomirs/index.html

Beneficiaries:

Flanders Institute for Biotechnology, Belgium

Jean-Christophe Marine
Flanders Institute for Biotechnology (VIB)
CME-KuLeuven
Herestraat, 49
3000 Leuven, Belgium
e-mail: jeanchristophe.marine@cme.vib-kuleuven.be

Weizmann Institute, Israel

Moshe Oren
Department of Molecular Cell Biology
Weizmann Institute of Science,
Rehovot, 76100 Israel
e-mail: moshe.oren@weizmann.ac.il

University of Regensburg, Germany

Gunter Meister
RNA Biology - Biochemistry I
Universitaet Regensburg
Universitaetsstrasse, 31
93053 Regensburg, Germany
e-mail: meister@biochem.mpg.de

Exiqon, Denmark

Peter Mouritzen
Exiqon
Skelstedet 16
DK-2950 Vedbaek , Denmark
e-mail: pm@exiqon.com

University of Copenhagen, Denmark

Anders H. Lund
Københavns Biocenter, BRIC
Ole Maaløes Vej 5
2200 København N, Denmark
e-mail: Anders.Lund@bric.ku.dk
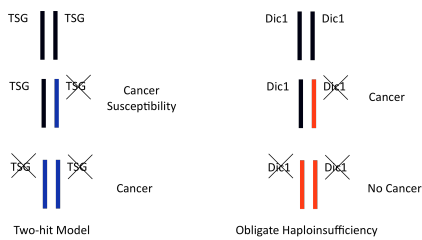
Centre National de la Recherche Scientifique, France

Justin Teissie
IPBS, CNRS
205 route de NARBONNE
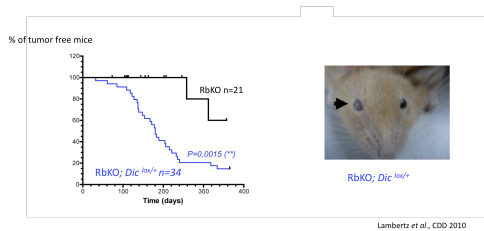31077 TOULOUSE, France
e-mail: justin.teissie@ipbs.fr

Logo:

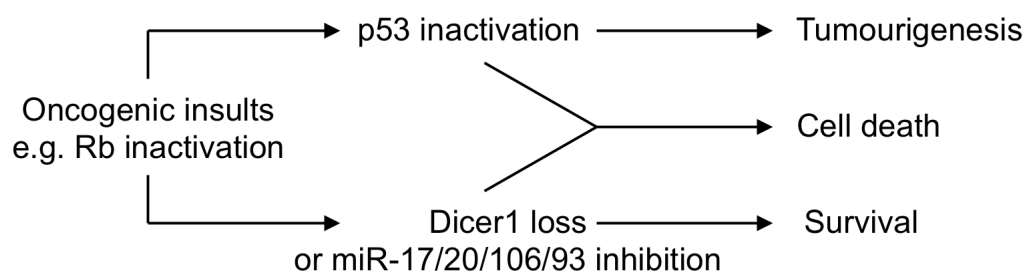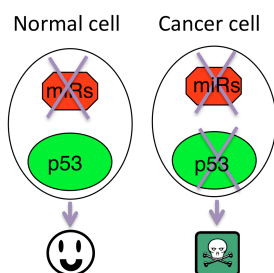# Some of the most important results of Oncomirs are illustrated below:

Dicer1 is an obligate haploinsufficient tumor suppressor gene

TSG ‖ TSG	Dic1 ‖ Dic1
TSG ‖ TSG Cancer Susceptibility	Dic1 ‖ Dic1 Cancer
TSG ‖ TSG Cancer	Dic1 ‖ Dic1 No Cancer
Two-hit Model	Obligate Haploinsufficiency

Mono-allelic loss of *Dicer* enhances retinoblastoma formation

% of tumor free mice

RbKO n=21

RbKO; *Dic* ^inv/+ n=34

P=0,0015 (**)

Time (days)

RbKO; *Dic* ^lox/+

Lambertz *et al.*, CDD 2010

Targeting miRs for cancer therapy

Normal cell   Cancer cell

miRs   miRs

p53   p53

Oncogenic insults
e.g. Rb inactivation

p53 inactivation ⟶ Tumourigenesis

⟶ Cell death

Dicer1 loss
or miR-17/20/106/93 inhibition ⟶ Survival

4.2   Use and dissemination of foreground

A plan for use and dissemination of foreground (including socio-economic impact and target groups for the results of the research) shall be established at the end of the project. It should, where appropriate, be an update of the initial plan in Annex I for use and dissemination of foreground and be consistent with the report on societal implications on the use and dissemination of foreground (section 4.3 – H).

The plan should consist of:

- Section A

This section should describe the dissemination measures, including any scientific publications relating to foreground. **Its content will be made available in the public domain** thus demonstrating the added-value and positive impact of the project on the European Union.

- Section B

This section should specify the exploitable foreground and provide the plans for exploitation. All these data can be public or confidential; the report must clearly mark non-publishable (confidential) parts that will be treated as such by the Commission. Information under Section B that is not marked as confidential **will be made available in the public domain** thus demonstrating the added-value and positive impact of the project on the European Union.