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(54) **Title**: INHIBITION OF DICER FUNCTION FOR TREATMENT OF CANCER

(57) **Abstract**: The present application relates to the field of cancer, particularly cancers wherein p53 tumour suppression function is lost or impaired. It is shown herein that Dicer is a synthetic lethal partner of p53, allowing the selective targeting and killing of cancer cells. The effects of Dicer on survival on cancer cells are mediated through the miR17-92 cluster and inhibition of members of this miRNA cluster is an attractive treatment strategy in cancer. Most particularly, these findings are of importance in the field of retinoblastoma.

INHIBITION OF DICER FUNCTION FOR TREATMENT OF CANCER

Field of the invention

The present application relates to the field of cancer, particularly cancers wherein p53 tumour suppression function is lost or impaired. It is shown herein that Dicer is a synthetic lethal partner of p53, allowing the selective targeting and killing of cancer cells. The effects of Dicer on survival on cancer cells are, at least partly, mediated through the miR17-92 cluster and inhibition of members of this miRNA cluster is an attractive treatment strategy in cancer. Most particularly, these findings are of importance in the field of retinoblastoma.

Background

A large body of evidence indicates that alterations in the expression of miRNAs contribute to cancer pathologies (1). This is at least partly the consequence of reduced stability and/or activity of DICER, an RNAse III endonuclease playing a critical role in miRNA biogenesis (2-6). Consistently, *Dicer1* is a haploinsufficient tumour suppressor in mice (7,8), supporting the hypothesis that a decrease in Dicer1 function leads to a global downregulation of miRs and promotes tumorigenesis. Surprisingly, however, homozygous deletions or loss-of-function mutations in *DICER1* do not occur in human tumours and homozygous loss of *Dicer1* appears to be selected against in a K-Ras-induced mouse model of lung cancer (7) and Myc-induced mouse model of B-cell lymphoma (9).

A vast majority of human cancers are also characterized by the loss of p53 tumour suppression function (29). Therefore, identification of synthetic lethal interactors of p53 should lead to conceptually simple and attractive approaches to selective targeting of cancer cells. Synthetic lethality has been proposed as an interesting concept in the context of anticancer therapy (30). Two genes are synthetic lethal if mutation of either alone is compatible with viability but mutation of both leads to death. ("Synthetic" is thus used in the sense of synthesis, or coming together.) So, targeting a gene that is synthetic lethal to a cancer-relevant mutation, like for instance in p53, should kill only cancer cells and spare normal cells. Synthetic lethality therefore provides a conceptual framework for the development of cancer-specific cytotoxic agents. Although it has been shown to work for cells that have lost BRCA1 or BRCA2 (31, 32), no genetic/in vivo evidence for a synthetic lethal interaction with p53 tumour suppressor has been described to date.

Of note, also retinoblastoma (Rb) mutations are found in a majority of human cancers (33, 34). The Rb gene was initially identified as a genetic locus associated with the development of an inherited eye

tumour. The realization that it was a loss of function of Rb that was associated with disease established the tumour suppressor paradigm. Mutations in Rb have also been seminal for the "two-hit hypothesis" of cancer, which states that cancer is the result of accumulated mutations to a cell's DNA.

Apart from its role in eye tumours, loss of Rb has for instance been demonstrated to increase the risk of osteosarcoma development in children and teenagers. In adults, human papillomavirus (HPV) is thought to initiate cervical carcinoma and squamous cell carcinoma of the head and neck in part by inactivating Rb through expression of the E7 oncoprotein, and similar mechanisms are possibly involved in virus-induced liver cancers. Rb is inactivated in more than 90% of human small-cell lung carcinomas (SCLC), and mouse genetic studies have confirmed that Rb is crucial in preventing the initiation of this lung cancer subtype. For an overview, see ref. 34, particularly Table 1 of this reference, incorporated herewith.

The disease retinoblastoma, affecting approximately 1 in 15,000 live births, is a rapidly developing cancer which develops in the cells of retina, the light detecting tissue of the eye. Both genetic and sporadic forms of retinoblastoma exist, and loss of Rb has been implicated in both. Moreover, it has recently been shown that, contrary to earlier suggestions, both the Rb and p53 pathways are inactivated – although not necessarily mutated - in retinoblastoma (13).

It would be advantageous to identify a synthetic lethal partner for p53, which would provide a new therapeutic target in cancer. It would be particularly advantageous if this allows the selective targeting of cancer cells in which more than one tumor suppressor pathway is compromised, such as for instance retinoblastoma.

Summary

The data presented herein show that *Dicer1* is required for tumour formation. It is 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. Unexpectedly, this shows 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 miR17-92 results from copy number alterations. Crucially, functional inactivation of the miRNAs encoded by the miR17-92 cluster is sufficient to induce apoptotic death of human retinoblastoma cells. Our data identify Dicer as the first synthetic lethal partner of p53 and designate members of the miR-17-92 cluster as a highly selective therapeutic target for the treatment of retinoblastoma.

Accordingly, it is an object of the invention to provide methods of inducing cell death in a cell where p53 function is compromised, comprising inhibiting the function of Dicer. According to particular embodiments, cell death is due to synthetic lethality.

According to particular embodiments, in addition to the compromised function of p53, the cell is further characterized by activation of an oncogene or inhibition of a tumor suppressor gene (such as e.g. Rb).

According to specific embodiments, the cell wherein p53 function is compromised is a tumour cell. Most particularly, the tumour is a retinoblastoma (and the tumour cell thus is a retinoblastoma cell).

As will be described herein, inhibiting the function of Dicer can be done in different ways. It is particularly envisaged that the function of Dicer1 is inhibited by inhibiting one or more of the miRNAs that are upregulated in the cell where p53 function is impaired. These miRNAs are listed in the application (e.g. in the tables provided herein). According to particular embodiments, the one or more miRNAs that are inhibited (i.e. that are upregulated in the cell wherein p53 function is impaired) are selected from the miR 17-92 cluster or a paralog thereof (such as the mir-106a-363 and mir-106b-25 cluster). According to further particular embodiments, the one or more miRNA is selected from the miR 17-92 cluster, most particularly selected from miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92.

As will be described herein, inhibition of miRNAs can be done in several ways. According to particular embodiments, inhibition of the miRNAs is with an LNA or an antagomir.

According to alternative embodiments, inhibiting the function of Dicer is done by inhibition of Dicer itself, i.e. by inhibiting the Dicer1 gene, the Dicer1 mRNA or the Dicer protein.

P53 function in the cell wherein p53 function is impaired can be impaired in different ways. According to particular embodiments, p53 function is impaired by functional dysregulation but not mutation. According to alternative embodiments, p53 function is impaired by at least one mutation.

According to a further aspect, an inhibitor of Dicer function is provided for use in treatment of cancer. In particular embodiments, the cancer is retinoblastoma.

According to specific embodiments, the inhibitor of Dicer function is an inhibitor of one or more of the miRNAs that are upregulated in the cancer cells. More particularly, the miRNA is selected from the miR 17-92 cluster or a paralog thereof (such as the mir-106a-363 and mir-106b-25 cluster). According to

further particular embodiments, the one or more miRNA is selected from the miR 17-92 cluster, most particularly selected from miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92.

According to alternative embodiments, the inhibitor is an inhibitor of the Dicer1 gene, the Dicer1 mRNA or the Dicer protein.

Brief description of the Figures

Figure 1. *Dicer1* is required for Retinoblastoma formation. (A) Kaplan-Meier curve showing the time to first observation of externally visible retinoblastoma. This time was markedly decreased in *Chx10Cre;Rb$^{lox/lox}$;p107$^{-/-}$;p53$^{lox/lox}$* (TKO, blue line at bottom of graph) mice relative to *Chx10Cre;Rb$^{lox/lox}$;p107$^{-/-}$* (DKO, black line in middle of graph) littermates. *Chx10Cre;Rb$^{lox/lox}$;p107$^{-/-}$; p53$^{lox/lox}$; Dic$^{lox/lox}$* (QKO, red line at top of graph) mice did not develop tumours (log rank test, *P*<0,0001). (B) Invasive tumours that fill the vitreous and the anterior chamber are found in *TKO* mice as early as P60. Hematoxylin and eosin stain with the three retinal nuclear layers (GL: ganglion layer; INL: inner nuclear layer; ONL: outer nuclear layer) indicated. (C) KI67 and Chx10, Syntaxin, and Calbindin immunostaining of *DKO and QKO* retinae at P14 and P45. Black Scale bars are = 40µm and the red scale bar in panel B is = 400µm.

Figure 2. Chx10/Rb/p107-mutant cells are lost upon concomitant inactivation of *Dicer1* and *p53*. (A) Chx10 and GFP immunostaining of *Chx10Cre;Rb$^{lox/lox}$;p107$^{-/-}$*;Dicer$^{lox/lox}$;*p53$^{+/+}$* versus *Chx10Cre;Rb$^{lox/lox}$;p107$^{-/-}$*;Dicer$^{lox/lox}$; *p53$^{lox/lox}$* retinae at P48. GFP-positive cells are only detected in p53 wild-type mice. (B) AP-stained transverse retinal sections from *Chx10Cre;Rb$^{lox/lox}$;p107$^{-/-}$*;Dicer$^{lox/lox}$;*p53$^{+/+}$* and *Chx10Cre;Rb$^{lox/lox}$;p107$^{-/-}$*;Dicer$^{lox/lox}$;*p53$^{lox/lox}$* adult mice (P82). Regions of AP reporter activity are only detected in p53 wild-type mice. (A) and (B) Scales bars =40µm. (C) Schematic representation of the *Dicer1 and p53* wild-type, floxed and Cre-excised alleles (Top panels). DNA was prepared from P21 retinae of at least 5 mice with the indicated genotypes and examined by PCR using the primers depicted in the top panels. Representative PCRs are shown in the lower panels.

Figure 3. The miRNA-17-92 cluster is overexpressed in retinoblastoma and required for survival of established retinoblastoma cell lines. (A) Heatmap of the miRNA-17-92 and paralogue clusters in normal mouse retina (*Chx10Cre*-negative mice, light green), normal human retina (dark green), 4 mouse *TKO* tumours (light blue) and 30 different primary human retinoblastoma (dark blue). (B) Expression analysis by RT-qPCR of miR-17 in normal human retina and the established retinoblastoma cell lines WERI-Rb1 and Y79. Data represents the mean of three independent experiments ±SD. (C) Transfection of miR17-92 specific inhibitors affect the survival of the retinoblastoma cell line WERI-RB1

as assessed by MTT assay. The Y axis represents the relative percentage of viable cells following transfection of the miRNA-inhibitors. The data are normalized to the percentage of viable cells following transfection of a scramble control oligonucleotide. Data represents the mean of three independent experiments ±SD. (D) Loss of *Dicer1* protects against the formation of aggressive and invasive retinoblastoma by selective killing of the Rb-deficient cells in which the p53 pathway is inactivated.

Figure 4. Dicer loss does not affect retinogenesis. Chx10 and GFP immunostaining indicate the presence of Dicer1-deficient cells in adult retina of *Chx10Cre;* Dicer$^{lox/lox}$ mice. (A) construct used for GFP immunostaining. (B) Retinal lamination is normal in these mice. ONL, outer nuclear layer; RPE, retinal pigment epithelium; INL, inner nuclear layer; GL, ganglion layer. Scale bars = 40µm.

Detailed description

Definitions

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. Any reference signs in the claims shall not be construed as limiting the scope. The drawings described are only schematic and are non-limiting. In the drawings, the size of some of the elements may be exaggerated and not drawn on scale for illustrative purposes. Where the term "comprising" is used in the present description and claims, it does not exclude other elements or steps. Where an indefinite or definite article is used when referring to a singular noun e.g. "a" or "an", "the", this includes a plural of that noun unless something else is specifically stated.

Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

The following terms or definitions are provided solely to aid in the understanding of the invention. Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Plainsview, New York (1989); and Ausubel et al., Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons,

New York (1999), for definitions and terms of the art. The definitions provided herein should not be construed to have a scope less than understood by a person of ordinary skill in the art.

As used herein, the term "inducing cell death" refers to a process that results in the killing of cells. Most particularly, as defined herein, the cell death is selective, i.e. cell death is induced in cells in which p53 function is compromised (thus, those cells die) and not induced in cells wherein p53 function is normal (those cells stay alive). According to particular embodiments, the term "cell death" refers to apoptotic cell death.

The term "p53 function" as used herein, refers to the tumor suppressor function exerted by the p53 protein encoded by the TP53 gene (Gene ID: 7157 in humans). The tumor suppressor function of p53 involves one or more of the following: activating DNA repair proteins when DNA has sustained damage; inducing growth arrest by holding the cell cycle at the G1/S regulation point on DNA damage recognition (if DNA repair proteins fix the damage, the cell will typically be allowed to continue the cell cycle); and/or initiating apoptosis if DNA damage proves to be irreparable.
"Compromised function" or "impaired function" as used throughout the application, particularly in the context of p53, refers to a reduced or absent functionality of this tumor suppressor function. The function can be compromised because one or both copies of the TP53 gene are mutated or absent in the cells (i.e. at the DNA level), and/or because the gene is not correctly transcribed or translated (i.e. at the RNA or protein level, respectively), and/or because no or mutant (non-functional) p53 protein is expressed in the cell, and/or because lower levels of functional p53 protein are expressed in the cells. "Lower levels" as used herein means lower levels than those observed in a suitable population of control cells, particularly 25% lower, 50% lower or 75% or more lower. For instance, 50% lower expression of functional p53 protein may arise from the loss of one functional p53 allele. However, a decrease in functional p53 levels may also be the consequence of inactivation of components of the p53 pathway or overexpression of other components, such as the p53 binding protein MDMX (13).

"Dicer" as used herein refers to the protein product of the DICER1 gene (Gene ID: 23405 in humans). This gene encodes a protein possessing an RNA helicase motif containing a DEXH box in its amino terminus and an RNA motif in the carboxy terminus. The encoded protein functions as a ribonuclease (ribonuclease type III) and is required by the RNA interference and small temporal RNA (stRNA) pathways to produce the active small RNA component that represses gene expression. In humans, two transcript variants encoding the same protein have been identified for this gene. The "function of Dicer" as used herein is the processing of microRNAs or miRNAs (35, 36), and "inhibiting the function of

Dicer" consequently means inhibiting the function of correctly processed miRNAs, be it by inhibiting their processing (e.g. by directly interfering with Dicer) or by inhibiting the miRNAs themselves (e.g. via LNAs or antagomirs). According to particular embodiments, "inhibiting the function of Dicer in a cell where p53 function is compromised" means inhibiting the miRNAs that are upregulated in cells where p53 function is compromised, wherein upregulation should be compared to suitable control cells wherein p53 function is not compromised. In particular embodiments, upregulation of miRNAs may also mean that they are expressed in cells wherein p53 function is compromised, whereas they are not expressed in control cells. According to very particular embodiments, "inhibiting the function of Dicer" means "inhibiting at least one miRNA from the miR 17-92 cluster".

According to the definitions herein, two genes are said to be in a "synthetic lethal" relationship or "synthetic lethal partners" or interactors if a mutation in, or downregulation or knockout of, either gene alone is not lethal but mutations/downregulation/knockout in or of both cause the death of the cell. Note that, according to this definition, genes can be synthetically lethal if e.g. a mutation in one gene is combined with e.g. downregulation of the other gene. In cancer research, a synthetic lethal partner is a gene that, when mutated or otherwise inhibited, kills cells that harbor a specific cancer-related alteration, such as a mutated tumor-suppressor gene or an activated oncogene, but spares otherwise identical cells lacking the cancer-related alteration (30). According to very particular embodiments, the synthetic lethal partner is synthetically lethal with mutations in, or functional dysregulation of, p53. According to even more specific embodiments, the synthetic lethal partner of p53 is Dicer or an effector of Dicer function, such as a specific miRNA, particularly one of the miR 17-92 cluster.

An "oncogene" as defined herein is a gene that has the potential to cause cancer. In tumor cells, they are often mutated or expressed at high levels. Typically, an oncogene is the result of changes (i.e. mutations, overexpression) of a normal gene, termed proto-oncogene. Proto-oncogenes typically code for proteins that help to regulate cell growth and differentiation. The proto-oncogene can become an oncogene by a relatively small modification of its original function, such as a mutation (e.g. leading to increase in protein or enzyme activity or loss in regulation), increase in protein concentration (e.g. by protein overexpression, increase in mRNA stability or gene duplication), or chromosomal translocation (leading to e.g. aberrant expression or constitutively active fusion genes encoding hybrid proteins). Conversion of proto-oncogenes in oncogenes can be quantitative or qualitative. Non-limiting examples of oncogenes (or proto-oncogenes that can become oncogenes upon activation) are listed further in the detailed description.

A "tumor suppressor gene", or "anti-oncogene", as herein defined is a gene that protects a cell from one step on the path to cancer. When this gene is mutated to cause a loss or reduction in its function, the cell can progress to cancer, usually in combination with other genetic changes. Tumor-suppressor genes, or more precisely, the proteins for which they code, either have a dampening or repressive effect on the regulation of the cell cycle or promote apoptosis, and sometimes do both. Well-known examples of tumor suppressors are the p53 and retinoblastoma (pRb) proteins. The cell cycle may be coupled to DNA damage by tumor suppressors (i.e., as long as there is damaged DNA in the cell, it should not divide. If the damage can be repaired, the cell cycle can continue). Indeed, increased mutation rate from decreased DNA repair leads to increased inactivation of other tumor suppressors and activation of oncogenes (Markowitz, J Clin Oncol. 2000; 18(21 Suppl):75S-80S). Accordingly, in particular embodiments, DNA repair proteins are included in the definition of tumor suppressors. Non-limiting examples of such DNA repair proteins whose mutation leads to increased cancer risk include HNPCC, MEN1 and BRCA.

The disease "retinoblastoma" as used herein refers to an embryonic malignant neoplasm of retinal origin (OMIM +180200).

The "miR 17-92 cluster" as used herein is a polycistronic cluster consisting of different miRNAs that are processed from a common precursor transcript. The precursor transcript derived from the mir-17-92 gene contains six tandem stem-loop hairpin structures that ultimately yield six mature miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1 (18, 37, 38). The six miRNAs encoded by mir-17-92 can be categorized into three separate miRNA families according to their seed sequences: the miR-17 family (including miR-17, miR-20, and miR-18), the miR-19 family (miR-19a and miR-19b), and the miR-92 family (18). It is worth noting that miR-18 exhibits a significant sequence homology with miR-17 and

miR-20, despite one nucleotide difference within the seed regions. Ancient gene duplications have given rise to two mir-17-92 cluster paralogs in mammals: mir-106a-363 and mir-106b-25, each of which only contains homologous miRNAs to a subset of mir-17-92 components (18, 37, 38), also referred to as paralogs or paralog clusters herein. The sequences of the miRNAs (including seed regions) and organization of the different clusters can also be found in these references.

microRNAs (miRNAs) are short (typically 20-24 nt) non-coding RNAs that are involved in post-transcriptional regulation of gene expression in multicellular organisms by affecting both the stability and translation of mRNAs. miRNAs are transcribed by RNA polymerase II as part of capped and

polyadenylated primary transcripts (pri-miRNAs) that can be either protein-coding or non-coding. The primary transcript is cleaved by the Drosha ribonuclease III enzyme to produce an approximately 70-nt stem-loop precursor miRNA (pre-miRNA), which is further cleaved by the cytoplasmic Dicer ribonuclease to generate the mature miRNA and antisense miRNA star (miRNA*) products. The mature miRNA is incorporated into a RNA-induced silencing complex (RISC), which recognizes target mRNAs through imperfect base pairing with the miRNA and most commonly results in translational inhibition or destabilization of the target mRNA.

According to a first aspect, methods are provided for inducing cell death in a cell where p53 function is compromised. These methods involve the inhibition of the function of Dicer. Accordingly, it can be said that an inhibitor of Dicer function is provided for use in inducing cell death in a cell where p53 function is compromised.

According to specific embodiments, the cell death that is induced is apoptotic cell death.

According to particular embodiments, inhibiting the function of Dicer in a cell where p53 function is compromised will result in synthetic lethality. I.e., inhibiting Dicer function in cells wherein p53 function is not compromised will not kill the cells, but only when both p53 and Dicer function are compromised, the cells will die. As p53 function is most typically compromised in tumor cells, it is particularly envisaged that the method can be used to kill tumor cells. (In other words, inhibitors of Dicer function are provided for use in treatment of cancer). Moreover, the killing is selective, as cell death will not be induced in cells where p53 function is normal.

In cancer, it is often seen that the function of more than one tumor suppressor is compromised. Thus, cell death may also be induced in cells wherein, in addition to impaired p53 function, Rb function is compromised, and/or p107 function is compromised. However, the methods provided herein require defective p53 function for cell death to be induced, as inhibiting Dicer function in a cell with compromised Rb function but wherein p53 functions normally will not result in cell death. In other words, impaired Dicer function is synthetically lethal with compromised p53 function, but not with other tumor suppressors.

Nonetheless, as cancer cells typically undergo many genetic changes, according to particular embodiments, it is envisaged that the cell(s) to be killed are characterized by impaired function of another tumor suppressor gene (in addition to compromised function of p53), and/or activation of one or more (proto-)oncogenes. The compromised function or inhibition of the tumor suppressor gene may be through mutation of that gene (e.g. in the case of BRCA), or as a result of lower expression/stability of the gene product, or through genetic deletion. The activation of one or more oncogenes (or

conversion of proto-oncogenes in oncogenes) may occur through mutation, gene amplification/overexpression, or chromosomal rearrangements.

In addition to TP53, tumor suppressors that may also be impaired in the cells to be killed include, but are not limited to, Rb, APC, CD95, ST5, YPEL3, ST7, and ST14. As mentioned above, tumor suppressors may also include DNA repair proteins such as HNPCC, MEN1 and BRCA genes.

A non-limiting list of (proto-)oncogenes that may be activated or overexpressed in the cells to be killed includes: regulatory GTPases such as RAS; cytoplasmic Serine/threonine kinases or regulatory subunits thereof, such as Raf kinases (e.g. B-Raf, C-Raf), AKT1, cyclin-dependent kinases (typically activated through overexpression); cytoplasmic tyrosine kinases such as the Src-family, Syk-ZAP-70 family, and BTK family of tyrosine kinases, or fusion genes like Nup-Abl, Bcr-Abl; receptor tyrosine kinases such as EGFR, PDGFR, VEGFR, Her2-Neu, Trk receptors or their ligands; growth factors such as c-Sis; Transcription factors such as Myb or Myc; Extracellular signal-regulated kinases (ERK or MAPK); and Wnt signaling proteins.

The further requirement (i.e. in addition to impaired p53 function) for oncogene activation or tumour suppressor inhibition ensures that only cells which truly undergo oncogene activation (i.e. tumour formation) are targeted for cell death.

According to most particular embodiments, the tumour or cancer to be treated is retinoblastoma. Thus, inhibitors of Dicer function are provided for use in treatment of retinoblastoma.

Although the methods can be used *in vitro*, e.g. to induce cell death in a cell line, it is particularly envisaged that they are applied *in vivo*, by inhibiting Dicer function in a subject in need thereof. Most particularly, this will be done by administering an inhibitor of Dicer function to a subject in need thereof, but gene therapy is also envisaged.

Most typically, the "subject" as used herein will be an animal, more particularly a mammal (e.g., cats, dogs, horses, cows, pigs, sheep, goats, llamas, monkeys, mice, rats, ...), most particularly a human.

Inhibiting Dicer function can be done in many ways. This can for instance be done by inhibiting functional expression of the Dicer1 gene itself. With "functional expression" of the Dicer1 gene, it is meant the transcription and/or translation of functional Dicer1 gene product. "Inhibition of functional expression" can be achieved at three levels. First, at the DNA level, e.g. by removing or disrupting the Dicer1 gene, or preventing transcription to take place (in both instances preventing synthesis of the Dicer1 gene product). Second, at the RNA level, e.g. by preventing efficient translation to take place –

this can be through destabilization of the mRNA so that it is degraded before translation occurs from the transcript, or by hybridizing to the Dicer mRNA. Third, at the protein level, e.g. by binding to the Dicer protein, inhibiting its function, and/or marking the protein for degradation.

If inhibition is to be achieved at the DNA level, this may be done using gene therapy to knock-out or disrupt the Dicer1 gene. As used herein, a "knock-out" can be a gene knockdown or the gene can be knocked out by a mutation such as, a point mutation, an insertion, a deletion, a frameshift, or a missense mutation by techniques known in the art, including, but not limited to, retroviral gene transfer. Another way in which genes can be knocked out is by the use of zinc finger nucleases. Zinc-finger nucleases (ZFNs) are artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain. Zinc finger domains can be engineered to target desired DNA sequences, which enables zinc-finger nucleases to target unique sequence within a complex genome. By taking advantage of endogenous DNA repair machinery, these reagents can be used to precisely alter the genomes of higher organisms.

In such embodiments, it will be particularly envisaged that the knock-out of the Dicer1 gene is limited to the tissue where the tumour is located, and most particularly, the knock-out is limited to the tumour itself, and Dicer1 is not inhibited in the host subject.

Apart from tissue-specific inhibition of Dicer1 gene product function, the inhibition may also be temporary (or temporally regulated).

Temporally and tissue-specific gene inactivation may for instance also be achieved through the creation of transgenic organisms expressing antisense RNA, or by administering antisense RNA to the subject. An antisense construct can be delivered, for example, as an expression plasmid, which, when transcribed in the cell, produces RNA that is complementary to at least a unique portion of the cellular Dicer mRNA.

A more rapid method for the inhibition of gene expression is based on the use of shorter antisense oligomers consisting of DNA, or other synthetic structural types such as phosphorothiates, 2'-0-alkylribonucleotide chimeras, locked nucleic acid (LNA) (see further in the application for a more detailed discussion of this technology), peptide nucleic acid (PNA), or morpholinos. With the exception of RNA oligomers, PNAs and morpholinos, all other antisense oligomers act in eukaryotic cells through the mechanism of RNase H-mediated target cleavage. PNAs and morpholinos bind complementary DNA and RNA targets with high affinity and specificity, and thus act through a simple steric blockade of the RNA translational machinery, and appear to be completely resistant to nuclease attack. An "antisense oligomer" refers to an antisense molecule or anti-gene agent that comprises an oligomer of

at least about 10 nucleotides in length. In embodiments an antisense oligomer comprises at least 15, 18 20, 25, 30, 35, 40, or 50 nucleotides. Antisense approaches involve the design of oligonucleotides (either DNA or RNA, or derivatives thereof) that are complementary to an mRNA encoded by polynucleotide sequences of Dicer1. Antisense RNA may be introduced into a cell to inhibit translation of a complementary mRNA by base pairing to it and physically obstructing the translation machinery. This effect is therefore stoichiometric. Absolute complementarity, although preferred, is not required. A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense polynucleotide sequences, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense polynucleotide sequence. Generally, the longer the hybridizing polynucleotide sequence, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex. Oligomers that are complementary to the 5' end of the message, e.g., the 5' untranslated region (UTR) up to and including the AUG translation initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' UTR of mRNAs have recently been shown to be effective at inhibiting translation of mRNAs as well (Wagner, R. (1994) Nature *372*, 333-335). Therefore, oligomers complementary to either the 5', 3' UTRs, or non-coding regions of a Dicer1 gene could be used in an antisense approach to inhibit translation of said endogenous mRNA encoded by Dicer1 polynucleotides. Oligomers complementary to the 5' UTR of said mRNA should include the complement of the AUG start codon. Antisense oligomers complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5', 3' or non-coding region of a said mRNA, antisense oligomers should be at least 10 nucleotides in length, and are preferably oligomers ranging from 15 to about 50 nucleotides in length. In certain embodiments, the oligomer is at least 15 nucleotides, at least 18 nucleotides, at least 20 nucleotides, at least 25 nucleotides, at least 30 nucleotides, at least 35 nucleotides, at least 40 nucleotides, or at least 50 nucleotides in length. A related method uses ribozymes instead of antisense RNA. Ribozymes are catalytic RNA molecules with enzyme-like cleavage properties that can be designed to target specific RNA sequences. Successful target gene inactivation, including temporally and tissue-specific gene inactivation, using ribozymes has been reported in mouse, zebrafish and fruitflies. RNA interference (RNAi) is a form of post-transcriptional gene silencing. The phenomenon of RNA interference was first observed and described in *Caenorhabditis elegans* where exogenous double-stranded RNA (dsRNA) was shown to specifically and potently disrupt the

activity of genes containing homologous sequences through a mechanism that induces rapid degradation of the target RNA. Several reports describe the same catalytic phenomenon in other organisms, including experiments demonstrating spatial and/or temporal control of gene inactivation, including plant (*Arabidopsis thaliana*), protozoan (*Trypanosoma bruceii*), invertebrate (*Drosophila melanogaster*), and vertebrate species (*Danio rerio* and *Xenopus laevis*). The mediators of sequence-specific messenger RNA degradation are small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from longer dsRNAs. Generally, the length of siRNAs is between 20-25 nucleotides (Elbashir et al. (2001) Nature *411*, 494-498). The siRNA typically comprise a sense RNA strand and a complementary antisense RNA strand annealed together by standard Watson Crick base pairing interactions (hereinafter "base paired"). The sense strand comprises a nucleic acid sequence that is identical to a target sequence contained within the target mRNA. The sense and antisense strands of the present siRNA can comprise two complementary, single stranded RNA molecules or can comprise a single molecule in which two complementary portions are base paired and are covalently linked by a single stranded "hairpin" area (often referred to as shRNA). The term "isolated" means altered or removed from the natural state through human intervention. For example, an siRNA naturally present in a living animal is not "isolated," but a synthetic siRNA, or an siRNA partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated siRNA can exist in substantially purified form, or can exist in a non native environment such as, for example, a cell into which the siRNA has been delivered.

The siRNAs of the invention can comprise partially purified RNA, substantially pure RNA, synthetic RNA, or recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non nucleotide material, such as to the end(s) of the siRNA or to one or more internal nucleotides of the siRNA, including modifications that make the siRNA resistant to nuclease digestion.

One or both strands of the siRNA of the invention can also comprise a 3′ overhang. A "3′ overhang" refers to at least one unpaired nucleotide extending from the 3′ end of an RNA strand. Thus, in one embodiment, the siRNA of the invention comprises at least one 3′ overhang of from one to about six nucleotides (which includes ribonucleotides or deoxynucleotides) in length, preferably from one to about five nucleotides in length, more preferably from one to about four nucleotides in length, and particularly preferably from about one to about four nucleotides in length.

In the embodiment in which both strands of the siRNA molecule comprise a 3′ overhang, the length of the overhangs can be the same or different for each strand. In a most preferred embodiment, the 3′

overhang is present on both strands of the siRNA, and is two nucleotides in length. In order to enhance the stability of the present siRNAs, the 3′ overhangs can also be stabilized against degradation. In one embodiment, the overhangs are stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides.

Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g., substitution of uridine nucleotides in the 3′ overhangs with 2′ deoxythymidine, is tolerated and does not affect the efficiency of RNAi degradation. In particular, the absence of a 2′ hydroxyl in the 2′ deoxythymidine significantly enhances the nuclease resistance of the 3′ overhang in tissue culture medium.

The siRNAs of the invention can be targeted to any stretch of approximately 19 to 25 contiguous nucleotides in any of the target Dicer1 mRNA sequences (the "target sequence"), of which examples are given in the application. Techniques for selecting target sequences for siRNA are well known in the art. Thus, the sense strand of the present siRNA comprises a nucleotide sequence identical to any contiguous stretch of about 19 to about 25 nucleotides in the target mRNA.

The siRNAs of the invention can be obtained using a number of techniques known to those of skill in the art. For example, the siRNAs can be chemically synthesized or recombinantly produced using methods known in the art. Preferably, the siRNA of the invention are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. The siRNA can be synthesized as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions. Commercial suppliers of synthetic RNA molecules or synthesis reagents include Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, Colo., USA), Pierce Chemical (part of Perbio Science, Rockford, Ill., USA), Glen Research (Sterling, Va., USA), ChemGenes (Ashland, Mass., USA) and Cruachem (Glasgow, UK).

Alternatively, siRNA can also be expressed from recombinant circular or linear DNA plasmids using any suitable promoter. Suitable promoters for expressing siRNA of the invention from a plasmid include, for example, the U6 or H1 RNA pol III promoter sequences and the cytomegalovirus promoter. Selection of other suitable promoters is within the skill in the art. The recombinant plasmids of the invention can also comprise inducible or regulatable promoters for expression of the siRNA in a particular tissue or in a particular intracellular environment. The siRNA expressed from recombinant plasmids can either be isolated from cultured cell expression systems by standard techniques, or can be expressed intracellularly, e.g. in breast tissue or in neurons.

The siRNAs of the invention can also be expressed intracellularly from recombinant viral vectors. The recombinant viral vectors comprise sequences encoding the siRNAs of the invention and any suitable

promoter for expressing the siRNA sequences. Suitable promoters include, for example, the U6 or H1 RNA pol III promoter sequences and the cytomegalovirus promoter. Selection of other suitable promoters is within the skill in the art. The recombinant viral vectors of the invention can also comprise inducible or regulatable promoters for expression of the siRNA in the tissue where the tumour is localized.

As used herein, an "effective amount" of the siRNA is an amount sufficient to cause RNAi mediated degradation of the target mRNA, or an amount sufficient to inhibit the progression of metastasis in a subject. RNAi mediated degradation of the target mRNA can be detected by measuring levels of the target mRNA or protein in the cells of a subject, using standard techniques for isolating and quantifying mRNA or protein as described above.

One skilled in the art can readily determine an effective amount of the siRNA of the invention to be administered to a given subject, by taking into account factors such as the size and weight of the subject; the extent of the disease penetration; the age, health and sex of the subject; the route of administration; and whether the administration is regional or systemic. Generally, an effective amount of the siRNA of the invention comprises an intracellular concentration of from about 1 nanomolar (nM) to about 100 nM, preferably from about 2 nM to about 50 nM, more preferably from about 2.5 nM to about 10 nM. It is contemplated that greater or lesser amounts of siRNA can be administered.

Recently it has been shown that morpholino antisense oligonucleotides in zebrafish and frogs overcome the limitations of RNase H-competent antisense oligonucleotides, which include numerous non-specific effects due to the non target-specific cleavage of other mRNA molecules caused by the low stringency requirements of RNase H. Morpholino oligomers therefore represent an important new class of antisense molecule. Oligomers of the invention may be synthesized by standard methods known in the art. As examples, phosphorothioate oligomers may be synthesized by the method of Stein et al. (1988) Nucleic Acids Res. *16*, 3209-3021), methylphosphonate oligomers can be prepared by use of controlled pore glass polymer supports (Sarin et al. (1988) Proc. Natl. Acad. Sci. USA. *85*, 7448-7451). Morpholino oligomers may be synthesized by the method of Summerton and Weller U.S. Patent Nos. 5,217,866 and 5,185,444.

The Dicer gene product inhibitor may also be an inhibitor of Dicer protein. A typical example thereof is an anti-Dicer antibody.

The term 'antibody' or 'antibodies' relates to an antibody characterized as being specifically directed against Dicer or any functional derivative thereof, with said antibodies being preferably monoclonal antibodies; or an antigen-binding fragment thereof, of the $F(ab')_2$, F(ab) or single chain Fv type, or any

type of recombinant antibody derived thereof. These antibodies of the invention, including specific polyclonal antisera prepared against Dicer or any functional derivative thereof, have no cross-reactivity to other proteins. The monoclonal antibodies of the invention can for instance be produced by any hybridoma liable to be formed according to classical methods from splenic cells of an animal, particularly of a mouse or rat immunized against Dicer or any functional derivative thereof, and of cells of a myeloma cell line, and to be selected by the ability of the hybridoma to produce the monoclonal antibodies recognizing Dicer or any functional derivative thereof which have been initially used for the immunization of the animals. The monoclonal antibodies according to this embodiment of the invention may be humanized versions of the mouse monoclonal antibodies made by means of recombinant DNA technology, departing from the mouse and/or human genomic DNA sequences coding for H and L chains or from cDNA clones coding for H and L chains. Alternatively the monoclonal antibodies according to this embodiment of the invention may be human monoclonal antibodies. Such human monoclonal antibodies are prepared, for instance, by means of human peripheral blood lymphocytes (PBL) repopulation of severe combined immune deficiency (SCID) mice as described in PCT/EP 99/03605 or by using transgenic non-human animals capable of producing human antibodies as described in US patent 5,545,806. Also fragments derived from these monoclonal antibodies such as Fab, $F(ab)'_2$ and scFv ("single chain variable fragment"), providing they have retained the original binding properties, form part of the present invention. Such fragments are commonly generated by, for instance, enzymatic digestion of the antibodies with papain, pepsin, or other proteases. It is well known to the person skilled in the art that monoclonal antibodies, or fragments thereof, can be modified for various uses. The antibodies involved in the invention can be labeled by an appropriate label of the enzymatic, fluorescent, or radioactive type. In a particular embodiment said antibodies against Dicer or a functional fragment thereof are derived from camels. Camel antibodies are fully described in WO94/25591, WO94/04678 and in WO97/49805. Processes are described in the art which make it possible that antibodies can be used to hit intracellular targets. Since Dicer is an intracellular target, the antibodies or fragments thereof with a specificity for Dicer must be delivered into the cells. One such technology uses lipidation of the antibodies. The latter method is fully described in WO94/01131 and these methods are herein incorporated by reference. Another method is by fusing the antibody to cell-penetrating peptides (Chen and Harrison, Biochem Soc Trans. 2007).

Antibodies binding to Dicer are commercially available, e.g. from Abcam, Santa Cruz biotechnology, Sigma-Aldrich and the like.

If the tumour is located in the brain, the inhibitor should be able to pass the blood-brain barrier. Technologies of modifying antibodies to pass the blood-brain barrier are well known to the skilled person.

Other inhibitors of Dicer include, but are not limited to, peptide inhibitors of Dicer, peptide-aptamer (Tomai et al., J Biol Chem. 2006) inhibitors of Dicer, and protein interferors as described in WO2007/071789, incorporated herein by reference.

Small molecule inhibitors, e.g. small organic molecules, and other drug candidates can be obtained, for example, from combinatorial and natural product libraries.

According to particularly envisaged embodiments, however, Dicer function is inhibited downstream of Dicer, by inhibiting one or more miRNAs that are upregulated by Dicer in cells where p53 function is impaired. As will be detailed in the examples section, specific miRNAs that fall under this category are the members of the polycistronic miR 17-92 cluster. In some settings (like for instance retinoblastoma) upregulation of these miRNAs means that they are present in p53 deficient cells, whereas they are not expressed in control cells (e.g. retinoblasts). Thus, inhibition of these miRNAs can be done in tissues where they are not normally expressed, thereby reducing the risk of side effects.

Inhibition of one or more of the miRNAs upregulated by Dicer in cells where p53 is compromised can be done at the DNA or RNA level, as described for Dicer above. (Inhibition at the protein level is not feasible since miRNAs are non-protein coding RNAs). Particularly suited for inhibition of miRNAs are locked nucleic acids (LNAs) or antagomirs.

A locked nucleic acid, often referred to as inaccessible RNA, is a modified RNA nucleotide. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo (North) conformation, which is often found in the A-form of DNA or RNA. LNA nucleotides can be mixed with DNA or RNA bases in the oligonucleotide whenever desired. Such oligomers are commercially available (e.g. from Exiqon). The locked ribose conformation enhances base stacking and backbone pre-organization. This significantly increases the thermal stability (melting temperature) of oligonucleotides. Importantly, LNA incorporation generally improves mismatch discrimination compared to unmodified reference oligonucleotide, and LNA mediates high-affinity hybridization by using the Watson-Crick rules without compromising base pairing selectivity.

LNA oligonucleotides are readily transfected into cells using standard techniques: they are sequence-specific and non-toxic, and show improved nuclease resistance, which make them highly useful for

powerful and selective antisense-based silencing. Hence, LNA oligonucleotides are uniquely suited for mimicking RNA structures and for miRNA targeting both in vivo and in vitro. Such LNA-based RNA antagonists have unusually high potency, biostability, and duration of action. See *Nature Methods* - 4, (2007) for more background on miRNA knockdown using LNA probes.

Antagomirs are another example of chemically engineered oligonucleotides that can be used to silence endogenous microRNA. An antagomir is a small synthetic RNA that is perfectly complementary to the specific miRNA target with either mispairing at the cleavage site of Ago2 or some sort of base modification to inhibit Ago2 cleavage. Usually, antagomirs have some sort of modification to make it more resistant to degradation. It is unclear how antagomirization (the process by which an antagomir inhibits miRNA activity) operates, but it is believed to inhibit by irreversibly binding the miRNA. Antagomirs are now used as a method to constitutively inhibit the activity of specific miRNAs (39). One clear advantage with respect to siRNA technology is that antagomirs did not induce an immune response.

According to a specific embodiment, inhibition of Dicer function means inhibition of one or more of the following miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1. Although inhibition of one of these miRNAs in cells in which p53 function is impaired already results in killing of these cells, it is envisaged that more than one of these miRNAs is inhibited, e.g. all members of the same miRNA family (see above), or also members of a paralog gene cluster, other family members of this gene cluster, and so on, may be inhibited as well. Inhibitors of these miRNAs may be used in treatment of cancer, particularly in retinoblastoma.

Although full inhibition of a given miRNA is particularly envisaged, partial inhibition may have beneficial effects as well (e.g. at least 25% inhibition, at least 50% inhibition or at least 75% inhibition).

As Dicer function (and miRNA control) is important, inhibition of Dicer function is particularly envisaged to be temporally and/or spatially regulated, rather than just systemic inhibition. According to particular embodiments, inhibition will not be done during prenatal development. According to further particular embodiments, inhibition of Dicer function will be restricted in time: after the cells in which p53 function is compromised have died, Dicer function will no longer be inhibited.

According to other particular embodiments, inhibition of Dicer function will only be done in the tissue where cells with compromised p53 function are located (in practice: the tumour itself or the tissue where a tumour is located). A non-limiting example hereof is in the case of retinoblastoma, where inhibitors of Dicer function can be administered directly into the eye. A further benefit hereof is that

this direct administration approach facilitates inhibition at the RNA level – indeed, in the case of systemic inhibition, stability of RNA inhibitors is often an issue, but if time and location of inhibition can be restricted, this allows more efficient inhibition.

As mentioned, the methods provided herein induce cell death in cells wherein p53 function is compromised. The way in which p53 function is compromised is in fact not essential to the invention. In many tumours, for instance, p53 function is compromised as a result of one or more mutations. However, it is particularly envisaged herein that p53 function may also be compromised by functional dysregulation that is not the result of mutation in p53. "Functional dysregulation" as used herein typically means that p53 function is impaired as the result of downregulation of levels of functionally active p53 protein. This may for instance be the result of mutations in other components of the p53 pathway that ultimately result in lowering of the levels of functional p53. As a non-limiting example, p53 function is compromised in retinoblastoma as a result of amplification of the MDMX gene, and not due to mutations in p53 itself (13).

It is to be understood that although particular embodiments, specific configurations as well as materials and/or molecules, have been discussed herein for cells and methods according to the present invention, various changes or modifications in form and detail may be made without departing from the scope and spirit of this invention. The following examples are provided to better illustrate particular embodiments, and they should not be considered limiting the application. The application is limited only by the claims. Note that, due to the requirements of black and white reproducibility of the figures, some experimental data are not shown in the figures, but can be obtained upon request.

Examples

Example 1. *Dicer1* is a synthetic lethal partner of p53

In order to test whether Dicer is required for retinoblastoma formation we conditionally inactivated *Dicer1* in mice that develop aggressive and invasive retinoblastoma (10). *Chx10Cre; $Rb^{lox/lox}$; $p107^{-/-}$* mice (thereafter referred to as DKO mice) develop early hyperproliferative lesions (11), which only rarely go on to become aggressive and invasive tumours. Accordingly, these mice develop only retinoblastoma with delayed and variable kinetics (Figure 1A). As previously reported (10;12), conditional inactivation of p53 on this sensitized background (*Chx10Cre; $Rb^{lox/lox}$; $p107^{-/-}$; $p53^{lox/lox}$*, referred to as the TKO mice) leads to rapid formation of visible retinoblastoma in virtually all mice

analyzed (122 out of 129). On average it takes 100 days for these mice to develop visible tumours (Figure 1A). Moreover, while DKO mice only ever develop unilateral tumours more than 80% of TKO mice (97 out of 122) develop bilateral retinoblastoma with clear evidence of anterior chamber invasion. In addition, metastatic tumours that had invaded local tissues outside of the eye through the optic nerve are only observed in *TKO* mice (data not shown). In sharp contrast to this very aggressive and nearly 100% penetrant phenotype, none of the *Chx10Cre; $Rb^{lox/lox}$; $p107^{-/-}$*; $p53^{lox/lox}$; $Dicer1^{lox/lox}$* (referred to as QKO) mice analyzed (19 out of 19) developed visible retinoblastoma within their first year of life (Figure 1A). This striking result argues in favour of a critical role for Dicer in the formation of retinoblastoma.

Unexpectedly, retinae of *QKO* mice have a normal cytoarchitecture (Figure 1B). The retinal laminar organization of *DKO* mice is slightly disrupted due to focal expansion of immature cells from the inner nuclear layer (INL) and their protrusion through the outer plexiform layer (OPL), in agreement with a previous report (13). Additionally, defects in the maturation of the rod photoreceptors lead to a hypocellular outer nuclear layer (ONL) (Figure 1B). This phenotype is exacerbated upon loss of p53, and tumours rapidly develop in retinae of *TKO* mice. In contrast, no evidence of tumour formation was seen in any of the *QKO* retinae examined. More surprisingly, dysplastic lesions were absent in these retinae and the ONL appeared intact (Figure 1B). We assessed cell proliferation in retinae at P14, by which time retinogenesis is normally complete. As previously reported (11;8), we found extensive BrdU incorporation and numerous Ki67-positive cells in *DKO* retinae (Figure 1C). This phenotype was significantly exacerbated in *TKO* retinae and BrdU- and Ki67-positive cells were found in all early dysplastic lesions and late (i.e. P200) retinoblastoma tumours (data not shown). Consistent with the absence of dysplastic lesions we found no evidence of ectopic cell proliferation in *QKO* retinae from P14 onwards. In fact, the retinal cytoarchitecture of *QKO* mice was indistinguishable from wild-type retinae at all stages and in all mice analyzed. The integrity of the retinal cytoarchitecture was further confirmed by analyzing the proportion and distribution of the retinal cell types in the *QKO* retinas and controls (Figure 1C and data not shown).

As it is unlikely that loss of *Dicer1* completely reverts the transformed Rb1/p107/p53-deficient retinoblasts into phenotypically normal cells, we hypothesized that biallelic loss of *Dicer1* compromises the survival of the tumour initiating cells. Because of the mosaicism exhibited in the *Chx10Cre* transgenic line (14), there remained a possibility that Cre-positive cells, and therefore the *Dicer1* deficient cells, might be eliminated and/or outcompeted by the remaining *Dicer1* wild-type Cre-negative cells without causing any obvious retinal morphological defects. In order to fate map the *Dicer1* deficient cells we took advantage of the green fluorescent protein (GFP) and alkaline

phosphatase (AP) reporter genes present in the BAC Chx10 transgenic construct (14;15). GFP expression accurately marks subsets of Chx10-positive cells that underwent Cre-mediated recombination (15). In adult retinae GFP is detected in a subset of postmitotic bipolar and Müller cells, which are located within the INL, at the interface between the INL and OPL (Figure 2A). Importantly, we found that Dicer is dispensable for the expansion, cell fate specification and differentiation of retinal progenitor cells since GFP-positive cells were identified in the INL of *Chx10Cre; Dicer1*$^{lox/lox}$ retinae; moreover, these retinae were indistinguishable from those of wild-type littermates (Figure 4). Consistent with a previous study (16) focal and progressive retinal degeneration was observed in a few older mice suggesting that Dicer might be required for the survival of some terminally differentiated neuronal cell populations. However, the penetrance of this phenotype was extremely low (1 out of 11 mice examined). Critically, GFP-positive cells could also be identified in retinae of *Chx10Cre; Rb*$^{lox/lox}$*; p107*$^{-/-}$; *Dicer1*$^{lox/lox}$ (Figure 2A) indicating that Dicer deficiency does not compromise the viability of the retinal progenitors on either wild-type or Rb/p107-deficient backgrounds.

To further demonstrate the presence of Dicer-deficient cells in *Chx10Cre; Dicer1*$^{lox/lox}$ and *Chx10Cre; Rb*$^{lox/lox}$*; p107*$^{-/-}$; *Dicer1*$^{lox/lox}$ retinae we determined mature miRNAs expression levels in FACS-sorted GFP-positive cells from three retinae of each genotype. Consistent with the loss of Dicer function we observed a dramatic global shut-down/down-regulation in steady-state miRNA levels in all samples analyzed compared to the levels in *Chx10Cre; Dicer1*$^{+/+}$ and *Chx10Cre; Rb*$^{lox/lox}$*; p107*$^{-/-}$; *Dicer1*$^{+/+}$ retinae (data not shown). This analysis supports the presence of Dicer-deficient cells in these retinae.

In sharp contrast, GFP-positive cells could not be identified in *QKO* adult retinae (Figure 2A), indicating that the Chx10Cre/GFP-positive cells are specifically eliminated and/or segregated out during development upon inactivation of both *Dicer1* and *p53*. To substantiate this observation we dissociated *QKO* and control adult retinae and scored individual GFP-positive cells in 3 independent samples for each genotype by FACS. This analysis confirmed the presence of GFP-positive cells in *Chx10Cre; Dicer1*$^{lox/lox}$ and *Chx10Cre; Rb*$^{lox/lox}$*; p107*$^{-/-}$; *Dicer1*$^{lox/lox}$ but not in *QKO* retinae (data not shown). Consistently, AP reporter activity was detected in all *Chx10Cre; Rb*$^{lox/lox}$*; p107*$^{-/-}$; *Dicer1*$^{lox/lox}$ adult retinae analyzed (Figure 2B). In contrast, no activity could be detected in 3 out of 4 *QKO* adult retinae analyzed and very few positive-cells were identified in the remaining sample, most likely due to incomplete Cre-mediated recombination of at least one of the floxed loci (Figure 2B). In keeping with the above observations, PCR-based genotyping confirmed *Cre*-mediated recombination of the conditional *Dicer1* allele in *Chx10Cre; Rb*$^{lox/lox}$*; p107*$^{-/-}$; *Dicer1*$^{lox/lox}$ but not in *QKO* adult retinae (P20) (Figure 2C). Consistent with the mosaicism exhibited in the *Chx10Cre* transgenic line the non-recombined *Dicer1* allele remained detectable in all *Dicer1*$^{lox/lox}$ samples analyzed.

To exclude that the lack of Cre-positive cells in *QKO* adult retinae is due to compromised Cre expression we performed whole-mounts AP staining on E11.5 mutant and control embryos, a developmental stage at which the Chx10-positive retinal progenitor cells are first detected. AP reporter activity was detected in the retina of all *Chx10Cre*-positive embryos, including *QKO*, but as expected not in *Chx10Cre*-negative embryos (data not shown). The presence of the mutant cells in the retina of *QKO* E11.5 embryos was further confirmed using the GFP reporter (data not shown).

As mutant cells are detected in *QKO* retinae at E11.5 but not in adult mice, we searched for evidence of apoptotic cell death as a possible cause for their elimination from E11.5 onwards (data not shown). All GFP-positive cells were negative for the activated-form of caspase-3 (casp-3*) in *Chx10Cre; $Rb^{lox/lox}$; $p107^{-/-}$; $Dicer1^{lox/lox}$* retinae at all stages analyzed (E11.5, E13.5 and E14.5). In contrast, GFP/Casp-3*-double positive cells were detected as early as E11.5 in *QKO* retinae (data not shown). At E14,5, GFP-positive cells (as well as AP-reporter activity) were only detected in *Chx10Cre; $Rb^{lox/lox}$; $p107^{-/-}$; $Dicer1^{lox/lox}$*, but not QKO, retinae (data not shown). These findings indicate that apoptosis contributes to the rapid elimination of Rb/p107/Dicer/p53-mutant cells during development and that Dicer1 is required for the survival of Rb1/p107-deficient retinal progenitor cells in which p53 function is disabled but not in cells with an intact p53 pathway. Collectively, our results demonstrate that inactivation of Dicer and p53 is synthetically lethal to susceptible (Rb/p107-deficient) cells. To the best of our knowledge, this is the first genetic evidence of an in vivo synthetic lethal interaction with p53.

Example 2. Dicer effects on survival mediated via miR 17-92 cluster

These data indicate that pharmacological inactivation of Dicer enzymatic activity may represent an attractive therapeutic modality, at least in the context of retinoblastoma. However, we have previously shown that partial *Dicer1* inactivation enhances rather than inhibits retinoblastoma formation (8). We therefore set out to investigate the underlying molecular basis for tumour suppression elicited by complete *Dicer1* inactivation in order to explore more appropriate therapeutic approaches. We reasoned that a subset of miRNAs, the maturation of which depends on Dicer function, is likely to promote Dicer1-mediated survival of Rb/p107/p53-deficient retinoblastoma cells. To search for such miRNAs we profiled miRNA expression in P21 retinae from wild-type (Cre-negative), *DKO* and dissected tumour material from *TKO* mice. This analysis identified a set of 102 miRNAs that are significantly up-regulated in the *TKO* tumours (Table 1 and data not shown). 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 (Table 2 and data not shown). Cross-species comparison identified 25 miRNAs that were

up-regulated in both mouse and human tumours (Table 3). Strikingly, 12 of them are members of the known oncogenic miR-17-92 and 106b-25/miR-106a-92 paralogue clusters (17,18). 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 (Figure 3A and data not shown). Sporadic retinoblastomas are often more advanced than tumours from patients with germline *RB1* mutations, mainly due to the fact that they tend to be diagnosed significantly later (19). Interestingly, miR-17-92 expression levels are significantly higher in sporadic retinoblastoma samples (heatmap data not shown; Mann-Whitney P<0,05).

To explore the potential causes of miRNA deregulation in human retinoblastoma we 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 (data not shown). 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 (20) and negatively regulated by p53 (21), 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.

The miR17-92 cluster is also expressed at very high levels in the human retinoblastoma cell lines Rb15, WERI-Rb1 and Y-79 in which both the Rb and p53 tumour suppressor pathways are inactivated and/or compromised (12). Data for miR-17 are shown in figure 3B. To explore if the viability of the retinoblastoma cells is dependent on functional miR-17-92 expression, each miRNA of the cluster was inhibited by transient transfection of miRNA-inhibitors. Inhibition of all individual miRNAs induced a significant decrease in cell viability as measured by MTT (Figure 3C) and caspase-glow (data not shown) assays. The apoptotic effects of miR17-92 knockdown were evident in the two cell lines tested, Y79 and WERI-RB1 (Figure 3C and data not shown).

Conclusion

These results lead us to propose the following working model for the role of Dicer inactivation-induced tumour suppression (Figure 3D). First, complete *Dicer1* inactivation does not affect survival

and/or differentiation of retinal progenitor cells, even in cells experiencing oncogenic stress elicited as a result of Rb inactivation. Second, Dicer and p53 inactivation are synthetically lethal to cells harbouring a deficient Rb pathway. Given that the Rb and p53 pathways are inactivated in most human cancers, our observations (i) provide a rational explanation for the selection against homozygous loss of *DICER1* in human cancer (22) and (ii) identify a novel pharmacological mode of tumour-type-specific intervention. Third, inactivation of members of the miR-17-92 cluster is sufficient to kill human retinoblastoma cells and, importantly, we show that it does so in a selective manner. Indeed, inactivation of Dicer, and consequently processing of the pre-miRs, in normal retinoblasts does not affect their survival and function. The miR-17-92 cluster is in fact not normally expressed in these cells. Therapeutic silencing of another pro-oncogenic miR, miR10b, was recently shown to successfully suppress metastasis in a mouse mammary tumour model (23). Our results call for the development and optimization of miR17-92 inhibitors for the treatment of Retinoblastoma patients. Importantly, in the context of retinoblastoma there will be no need for systemic exposure to the miR-inhibitors thus reducing the potential side effects of such treatment in other tissues/organs. Retinoblastomas could be simply treated by sub-conjuctival injection of the miRNA-inhibitory molecules.

Table 1

Differential miRNA expression in mouse tumors

	Tumour vs. normal retina		**Tumour vs. DKO**	
miRNA	**fold change**	p value	**fold change**	p value
let-7a	0.734301438	0.273072124	0.895076406	0.691557694
let-7a*	5.500790963	0.01960028	5.415980315	0.021881848
let-7b	3.535319648	0.010370294	2.780131413	0.044361147
let-7c	3.359480225	0.029237804	2.438476587	0.068784999
let-7c-1*	4.741319935	0.12659217	3.893437575	0.169852634
let-7d	1.234074148	0.297825824	1.172828306	0.456789172
let-7d*	0.369079986	0.088943386	0.530989595	0.119000858
let-7e	1.284143189	0.16447827	1.396422488	0.14130197
let-7f	0.944737925	0.776895779	1.00984217	0.97561554
let-7g	1.135094436	0.376289911	1.315125134	0.258536706
let-7g*	2.633175622	0.103630898	4.144327923	0.072340757
let-7i	1.946962814	0.047999992	1.303389019	0.239329412
let-7i*	1.393543863	0.564728821	2.186352712	0.272723927
miR-1	0.793125549	0.828543422	0.953113488	0.97561554
miR-100	2.12461644	0.299729218	1.60608008	0.514352631
miR-101	2.426418798	0.028992766	2.840896611	0.132338746
miR-101b	2.288168687	0.031233542	2.551209713	0.150229027
miR-103	1.304877384	0.395284529	1.114962608	0.755335075
miR-106a	51.88732466	0.009211953	22.434866	0.044361147
miR-106b	6.475493206	0.008756512	5.22690581	0.037187565
miR-106b*	9.844896779	0.005663749	7.645337351	0.093681498
miR-107	0.516511513	0.076016013	0.489836945	0.074673743
miR-10a	3.007375768	0.109853962	3.007375768	0.120591568
miR-122	2.411861573	0.068257972	1.876264315	0.193963821
miR-124*	0.385164461	0.092967467	2.18350663	0.234819943
miR-124a	0.201127661	0.013065457	0.422317566	0.090516995
miR-125a-3p	0.934740784	0.868020022	0.878067241	0.755335075

miRNA	fold change	p value	fold change	p value
miR-125a-5p	1.596537378	0.081265395	1.390863409	0.169906827
miR-125b	1.088496483	0.886038235	0.818446203	0.769067534
miR-125b-1*	1.770265293	0.457427124	0.831135341	0.830086967
miR-125b*	5.297581936	0.06229364	4.625811288	0.084606159
miR-126	2.66222575	0.029237804	4.879164064	0.044174334
miR-126*	1.811762582	0.088943386	3.263671823	0.048681312
miR-127	0.721441505	0.456333899	0.70710633	0.514352631
miR-127-5p	0.650588359	0.370899476	1.057760247	0.939920721
miR-128a	0.682300808	0.388937085	0.654503545	0.398452113
miR-129	0.112886294	0.009114302	0.16193731	0.031840324
miR-129-3p	0.096027627	0.131817586	0.137454408	0.184663709
miR-130a	1.714212739	0.009211953	1.992597859	0.008284863
miR-130b	0.584223581	0.218057936	0.76690631	0.545897969
miR-130b*	0.648138518	0.35055361	0.928990552	0.884258382
miR-132	1.610414979	0.071921844	1.995821337	0.045032645
miR-133a	1.209649917	0.85756787	1.341565656	0.784560308
miR-133b	0.819424977	0.816788557	0.913055327	0.927148592
miR-134	2.533648981	0.082752178	2.182816839	0.13242275
miR-135a	0.947012745	0.896990567	0.711812659	0.514352631
miR-135b	2.695144104	0.056906094	2.055214293	0.101841409
miR-136	1.454762185	0.369116137	1.824672752	0.339420243
miR-136*	0.888645206	0.86965276	1.195340751	0.846473322
miR-137	4.292698447	0.029314715	3.841261671	0.062517807
miR-138	0.774582196	0.404055957	0.734488596	0.399768272
miR-138*	1.066972749	0.896990567	1.277755155	0.688990152
miR-139-5p	0.931787036	0.868020022	0.797496216	0.65840633
miR-140	2.735000626	0.108061678	3.059077333	0.093644648
miR-140-3p	2.747517645	0.110169748	3.598233545	0.081908469
miR-141	1.910039874	0.22554139	1.910039874	0.241987116
miR-141*	0.739060349	0.338434293	0.820596646	0.305973764
miR-142-3p	16.58444981	0.009211953	4.825204192	0.049181724
miR-142-5p	1.764389659	0.251822108	1.764389659	0.26818725
miR-143	0.944622517	0.867361688	1.985466353	0.272723927
miR-145	0.874432289	0.437037218	2.019517987	0.272723927
miR-146a	11.11818391	0.002195316	3.384791564	0.169906827
miR-146b	7.609411663	0.025658662	3.48580934	0.156890504
miR-146b*	5.421234813	0.034057514	3.357236673	0.272723927
miR-148a	1.419358221	0.172214176	1.550539374	0.170193706
miR-148b	0.496591566	0.019160538	0.701865297	0.26072901
miR-149	1.413545671	0.553677869	1.070146451	0.927148592
miR-150	1.077543601	0.850026351	1.605402811	0.421756911
miR-151	1.443399753	0.120638185	1.95622999	0.045032645
miR-152	1.8558237	0.077264211	2.100334454	0.07594739
miR-153	0.973132222	0.918184799	1.012256895	0.97561554
miR-154	0.413246188	0.350076141	0.269319659	0.048681312
miR-154*	1.463869297	0.350076141	1.409677841	0.42548775
miR-155	5.933582476	0.015061298	5.046847506	0.13464374
miR-15a	0.79465405	0.365620036	1.596374249	0.140793064
miR-15a*	6.458406117	0.015061298	7.178543943	0.032975304
miR-15b	5.453081609	0.042476325	4.601895305	0.119290587
miR-15b*	63.19234564	0.004880222	54.2892756	0.071472927
miR-16	3.03333113	0.031145366	5.157747255	0.021881848
miR-16*	33.00514612	0.004880222	18.22452737	0.068784999
miR-17	47.58305778	0.003001541	21.55266196	0.048681312
miR-17*	7.05075498	0.028402843	11.1288388	0.021881848
miR-181a	0.389439038	0.033900585	0.37898382	0.045032645
miR-181c	0.541217538	0.149815607	0.545253329	0.169906827
miR-182	0.048317007	0.172214176	0.275678755	0.514352631
miR-183	0.01589759	0.113284896	0.106294068	0.297958323

miRNA	fold change	p value	fold change	p value
miR-183*	0.055440811	0.19087909	0.266099108	0.514352631
miR-184	0.702490237	0.518954901	0.709519298	0.585727041
miR-185	1.293797425	0.218834142	1.097829361	0.664490289
miR-186	7.494097062	0.002195316	6.952665003	0.008284863
miR-186*	8.533299375	0.015061298	9.996246045	0.016693972
miR-187	1.027614389	0.980198027	1.008589011	0.993935867
miR-188-5p	0.667991583	0.469533282	1.01831524	0.977738661
miR-18a	72.27794902	0.002806999	20.79573463	0.086537932
miR-18a*	7.461352046	0.023256995	5.850095442	0.03182938
miR-190	0.608081033	0.456333899	0.642448143	0.615865824
miR-190b	1.50113164	0.868020022	1.896581777	0.813855461
miR-191	1.517794263	0.003001541	2.736758872	0.001019177
miR-191*	1.033571955	0.816788557	1.895671408	0.031840324
miR-192	2.614714927	0.034279558	2.872128728	0.070328146
miR-193*	1.154246211	0.818492942	1.444852692	0.621086022
miR-193a-3p	3.384089903	0.126516653	2.558701214	0.22188577
miR-193b	2.205458831	0.104792913	1.333084513	0.456789172
miR-194	1.527037153	0.176652403	1.687718055	0.239130081
miR-195	3.362819079	0.007422321	2.881220841	0.021881848
miR-197	1.171123896	0.456333899	1.171123896	0.514352631
miR-199a-3p	3.291284517	0.092894954	3.076816693	0.104739682
miR-199b	2.038745576	0.177764775	2.402577967	0.14130197
miR-19a	9.927933234	0.010215094	13.18852662	0.021881848
miR-19a*	1.670580162	0.19087909	1.670580162	0.208481167
miR-19b	10.94909897	0.009211953	13.99269546	0.021881848
miR-200b	2.76435281	0.299729218	2.76435281	0.32227898
miR-200c	1.550928585	0.331120109	1.550928585	0.358211754
miR-202	1.350339304	0.504186314	1.261854694	0.664490289
miR-203*	1.563018795	0.224988858	1.563018795	0.241480188
miR-204	0.720597805	0.307830726	0.793270468	0.552196956
miR-205	15.45435663	0.031233542	10.87447684	0.045032645
miR-206	0.214325624	0.131817586	0.220662084	0.14130197
miR-20a	36.49125415	0.009367224	14.18148047	0.082523815
miR-20a*	12.64508979	0.031145366	12.64508979	0.045032645
miR-20b	45.10422985	0.009778614	17.69737212	0.072340757
miR-21	1.530207798	0.241105342	2.11482555	0.098003228
miR-21*	2.178372553	0.273587458	2.178372553	0.289295829
miR-210	1.068042579	0.868020022	1.898411281	0.15264265
miR-211	0.101938501	0.00269215	0.184349707	0.148151099
miR-212	2.095296625	0.121654045	2.929655266	0.032615321
miR-214	1.249048156	0.338434498	1.249048156	0.368787367
miR-214*	6.444509298	0.087108433	8.736090098	0.086350539
miR-215	5.7967875	0.057413845	5.772739652	0.021881848
miR-216b	0.449015451	0.746146781	0.456152379	0.76428247
miR-217	0.917777961	0.933056246	1.095641499	0.955360333
miR-218	3.359471008	0.123495218	4.383785358	0.111992679
miR-218-1*	1.216832772	0.299729218	1.039949396	0.856459229
miR-218-2*	5.394466383	0.126740139	5.394466383	0.14130197
miR-22	0.726056134	0.243229591	1.468079822	0.549700638
miR-22*	0.439713022	0.053252118	0.714149279	0.517563385
miR-221	1.439574378	0.404055957	1.098312104	0.857942498
miR-222	8.233459518	0.002477929	5.760855863	0.02196098
miR-224	1.616805345	0.128099157	2.424040963	0.283239022
miR-23b	0.549417777	0.131817586	0.591267648	0.184663709
miR-24	2.935123408	0.004880222	3.008964137	0.031840324
miR-24-2*	8.262577106	0.009778614	7.379976617	0.021881848
miR-25	3.301359495	0.009211953	3.953525313	0.147449509
miR-26a	0.955412338	0.868020022	1.122090337	0.755335075
miR-26b	0.622394369	0.187404783	1.383676479	0.39095235

miRNA	fold change	p value	fold change	p value
miR-26b*	1.57311448	0.16447827	2.513156903	0.030023219
miR-27a	2.128180224	0.029237804	1.854173712	0.086001101
miR-27a*	14.50243499	0.010592223	8.470060818	0.045703577
miR-27b	0.564000917	0.210139343	0.73676401	0.494743354
miR-27b*	2.019547735	0.19087909	3.221104938	0.089837184
miR-28	2.53628581	0.011718545	2.18151906	0.021881848
miR-28*	3.334422074	0.010296625	2.616597308	0.093681498
miR-292-3p	0.756824001	0.462734767	1.467777214	0.289229344
miR-294	0.284616114	0.025036412	0.460831722	0.445352694
miR-296	0.57961935	0.339739106	0.553266603	0.336484438
miR-296-3p	2.793743218	0.141726173	2.739625394	0.156890504
miR-297a*	1.971740006	0.113284896	2.010202832	0.12049269
miR-298	4.078869679	0.101060762	2.662832771	0.209875402
miR-29a	1.106416311	0.864949181	1.046986915	0.961527386
miR-29a*	0.982755597	0.975686838	1.106716723	0.920800726
miR-29b	0.532832996	0.338434293	0.645308267	0.613669566
miR-29b*	1.128941383	0.404392937	1.119747668	0.689327143
miR-29c*	0.63573551	0.350076141	1.275140009	0.709363957
miR-300*	0.70568922	0.428115117	0.875143602	0.846473322
miR-301	4.171971098	0.025036412	4.175698588	0.037187565
miR-301b	3.731903884	0.025648621	3.878303154	0.032975304
miR-30a-3p	1.181700893	0.568071703	1.51203164	0.209875402
miR-30a-5p	0.764071999	0.370256504	1.112925416	0.793409148
miR-30b*	0.743174547	0.233382276	1.559178475	0.398452113
miR-30c	1.186731174	0.38520049	1.733321867	0.127055907
miR-30c-2*	0.288265326	0.370256504	0.680034832	0.779398143
miR-30d	0.75400365	0.265997027	0.990977927	0.977738661
miR-30e	1.046184279	0.801569724	1.685014195	0.17380784
miR-30e-3p	1.312239675	0.299729218	1.762942388	0.090516995
miR-31	1.258277385	0.339739106	0.882067364	0.735171366
miR-31*	1.302160544	0.362179657	0.997946753	0.992428383
miR-32	1.968680846	0.128099157	2.787876058	0.083290936
miR-320	1.68260058	0.121654045	1.549664415	0.169906827
miR-322*	3.186523933	0.058839215	4.027983886	0.048681312
miR-323-3p	1.385764477	0.311612691	1.126559487	0.719103365
miR-324-3p	1.693398183	0.151319454	1.346138634	0.374315528
miR-324-5p	1.386265489	0.370899476	1.367864504	0.427515762
miR-326	1.339559268	0.412198934	1.048609239	0.690257512
miR-328	0.401820455	0.121654045	0.341491126	0.101075499
miR-329	0.392018347	0.01960028	0.392297259	0.021881848
miR-330	0.376728552	0.047566241	0.410819253	0.086001101
miR-330-5p	0.208501643	0.021084568	0.244828159	0.06894565
miR-331	1.215237109	0.395284529	1.156358906	0.555544935
miR-331-5p	1.329401064	0.200967878	1.097067146	0.433778552
miR-335	0.34794996	0.091473239	0.60235664	0.363569096
miR-335*	0.492432367	0.081265395	1.127428997	0.726765601
miR-337-3p	0.922626731	0.747038992	0.987637197	0.977738661
miR-337-5p	1.135879266	0.550334698	0.975404531	0.942290692
miR-338-3p	1.264343814	0.481069555	1.047223646	0.927148592
miR-339-3p	1.512618669	0.105930341	3.051383536	0.021881848
miR-339-5p	0.718037823	0.199777922	0.909743133	0.71427332
miR-33a*	2.581623585	0.046809672	3.978300936	0.037187565
miR-340	3.188345552	0.071604623	3.084818792	0.083302341
miR-340*	2.667250664	0.068172479	2.610031398	0.076261141
miR-342-3p	3.997027494	0.017397038	3.753691047	0.021881848
miR-342-5p	2.500670539	0.066726255	2.487495781	0.081726277
miR-344	1.655242867	0.176101917	1.285483116	0.448692586
miR-345-3p	0.92216192	0.82850515	1.135903869	0.76428247
miR-345-5p	0.687386734	0.299729218	0.932411689	0.846473322

miRNA	fold change	p value	fold change	p value
miR-34b-3p	10.55748467	0.001159773	5.124725257	0.083290936
miR-34b-5p	7.063390153	0.002195316	3.821921762	0.008284863
miR-34c	5.707106215	0.004880222	2.629549816	0.048681312
miR-34c*	3.72344702	0.004880222	3.304202056	0.044361147
miR-350	2.207086231	0.029314715	2.065103962	0.081908469
miR-361	0.552326147	0.065612423	0.585848584	0.12135725
miR-362-3p	0.633463031	0.037408149	1.195985373	0.720252214
miR-362-5p	2.221917366	0.091171643	2.71380723	0.045032645
miR-365	0.824623509	0.595041808	0.783795889	0.608286912
miR-369-3p	0.819879142	0.652767457	0.684251965	0.419175589
miR-369-5p	0.661743335	0.336087302	0.524967159	0.204276192
miR-370	1.793307426	0.172214176	1.49546994	0.321170272
miR-374-5p	14.90076157	0.00269215	18.4683778	0.008284863
miR-375	0.026637006	0.046666911	0.030708812	0.048681312
miR-376a	1.329384861	0.529884042	1.274801167	0.664490289
miR-376a*	1.488607762	0.329228454	1.745751338	0.36261197
miR-376b	1.642789821	0.368672372	1.253600212	0.71364656
miR-376b*	1.527510888	0.350076141	1.613538586	0.398452113
miR-376c	4.800449482	0.025036412	4.883448374	0.030023219
miR-379	2.591977294	0.029314715	2.166626535	0.045703577
miR-380-5p	1.858433626	0.068257972	1.804782827	0.14130197
miR-381	0.840433066	0.783714269	0.837869954	0.793409148
miR-382	0.680076053	0.377542333	0.664518105	0.405733666
miR-383	0.604287357	0.570538299	0.657994796	0.692629097
miR-384-3p	0.830275623	0.477475762	0.749497028	0.514504677
miR-384-5p	1.263544957	0.356435352	1.256898147	0.555544935
miR-409-3p	1.668592979	0.086848647	1.411265241	0.187850527
miR-409-5p	0.263755713	0.030591879	0.233114933	0.031840324
miR-410	0.741091926	0.4093458	0.808024249	0.596983315
miR-411	3.542896933	0.013065457	2.935804524	0.021881848
miR-411*	2.480316104	0.006051333	2.509954849	0.045032645
miR-412	0.76903269	0.469533282	0.729580249	0.458576975
miR-423-5p	0.487791913	0.273296662	0.742411259	0.647239401
miR-424	3.428612823	0.023285286	2.594904442	0.048995445
miR-425	1.385804901	0.19087909	2.243967185	0.044361147
miR-431	2.883067265	0.051828341	2.914596654	0.13912127
miR-433	0.393321988	0.151319454	0.360022629	0.14130197
miR-434-3p	0.90344285	0.767783897	0.893649494	0.782556356
miR-434-5p	1.613209246	0.310939846	1.394632488	0.489816324
miR-448	0.422943062	0.121654045	0.248566155	0.049181724
miR-449	102.6824215	0.004880222	56.61076425	0.088862403
miR-449b	128.9933081	0.004880222	44.61289388	0.116681589
miR-450a	1.381592985	0.183220355	0.830173285	0.585232362
miR-465a-3p	1.99424507	0.273296662	1.99424507	0.289229344
miR-466d-3p	1.585382229	0.233382276	1.585382229	0.249458962
miR-467*	1.723813659	0.036500299	1.922333409	0.037187565
miR-467b	1.54226347	0.148331233	1.505981445	0.08226263
miR-467c	5.252469016	0.003001541	3.688157233	0.101841409
miR-467d	4.959036126	0.01960028	3.490504124	0.1249962
miR-467e	1.14489331	0.545017463	0.964910193	0.91993227
miR-470*	0.461332125	0.271593853	0.821692068	0.76428247
miR-483*	41.75718578	0.154057991	16.19056621	0.274864415
miR-484	2.600402741	0.004880222	2.25064809	0.02196098
miR-485-3p	1.224395796	0.491579492	1.200872579	0.57670453
miR-485-5p	0.758859203	0.456333899	0.721217957	0.552196956
miR-487b	1.068581679	0.903647597	0.51057984	0.492132068
miR-488*	2.399873308	0.057413845	2.039168975	0.086350539
miR-489	2.237001632	0.439867626	2.015723732	0.552196956
miR-491	1.668119812	0.121654045	1.526443999	0.187741925

miRNA	fold change	p value	fold change	p value
miR-493	2.288713925	0.218610127	2.239356065	0.242136244
miR-494	1.919773463	0.065556364	1.490057663	0.176394532
miR-495	0.764449222	0.439496494	0.672112821	0.374327019
miR-496	0.434944607	0.124402182	0.369536813	0.090087879
miR-497	5.181492004	0.009211953	2.7617805	0.090087879
miR-500	0.821677071	0.425002275	1.072299931	0.814600896
miR-501*	0.596201536	0.139840583	1.206867544	0.675993427
miR-503	1.711918045	0.180312483	1.711918045	0.193963821
miR-503*	3.126537158	0.046809672	3.243329079	0.048681312
miR-504	0.358906171	0.2919671	0.507132848	0.552196956
miR-532	2.121351093	0.046666911	2.280616436	0.045032645
miR-532-3p	1.782203809	0.01102795	1.607152405	0.03182938
miR-541	2.942632062	0.077241102	2.491735463	0.1249962
miR-542-3p	0.855281405	0.477475762	1.306195185	0.40382326
miR-542-5p	0.950842963	0.902032977	1.424200536	0.254267886
miR-543	0.524686562	0.087217891	0.446754499	0.076261141
miR-544	0.345383264	0.088943386	0.350655233	0.14130197
miR-547	2.541613068	0.08836386	2.217181941	0.125039073
miR-551b	0.28349257	0.287355951	0.277024071	0.292826252
miR-574-3p	2.973537623	0.007422321	2.515454473	0.045032645
miR-582-3p	4.165620504	0.057413845	4.138268237	0.070133539
miR-582-5p	1.399029044	0.456333899	1.17434746	0.755335075
miR-592	2.289089699	0.271593853	1.778765192	0.433778552
miR-598	0.426074317	0.068257972	0.408875563	0.088773549
miR-652	0.636293739	0.194070228	0.700346562	0.29089308
miR-665	1.700791643	0.19087909	1.035713632	0.955360333
miR-666	0.473046844	0.116648667	0.407443158	0.08761121
miR-667	0.711059691	0.336087302	0.642043519	0.258536706
miR-668	0.290739651	0.178598651	0.246709498	0.153516058
miR-669a	1.449460227	0.166039541	1.222400887	0.494586921
miR-671-3p	1.374227314	0.143397391	1.305526804	0.209875402
miR-672	8.983491367	0.001838729	4.031887344	0.076261141
miR-673	2.492276551	0.020873863	2.043056703	0.060357995
miR-673-3p	1.635569434	0.369116137	1.635569434	0.404595183
miR-674	1.155102262	0.594497898	1.118446046	0.755335075
miR-674*	2.226094873	0.028402843	2.175479108	0.028237922
miR-676	1.583016083	0.288734017	0.989037638	0.97786865
miR-676*	1.931133627	0.121914991	1.401418726	0.374327019
miR-677	2.166066503	0.013845756	1.268218255	0.675993427
miR-678	0.951977191	0.828543422	0.92431852	0.735171366
miR-680	1.995818651	0.159229059	1.995818651	0.170193706
miR-682	2.011180321	0.273296662	1.684415297	0.406256121
miR-684	1.792610927	0.238613487	1.139238088	0.880698728
miR-685	2.325010927	0.38520049	2.644261601	0.374315528
miR-690	2.338722322	0.01960028	2.733449386	0.028768204
miR-694	1.839427985	0.029314715	1.40766756	0.088210532
miR-699	1.029833303	0.886038235	1.383595518	0.205791448
miR-7*	2.095520783	0.057413845	3.162943047	0.045032645
miR-700	1.177692243	0.545017463	1.350393622	0.13464374
miR-701	8.360447968	0.016106684	7.559666875	0.021881848
miR-702	0.295973602	0.035503778	0.367094184	0.086001101
miR-704	0.498610757	0.076092689	0.617381002	0.076261141
miR-706	3.315589915	0.034057514	3.212761001	0.048681312
miR-708	1.862467279	0.547038088	1.662053508	0.675993427
miR-709	2.41065996	0.01960028	2.279881086	0.032975304
miR-720	3.141664044	0.046255445	3.112153735	0.090516995
miR-721	1.012018813	0.932335465	1.028011824	0.927148592
miR-741	1.197128719	0.356435352	1.197128719	0.393791022
miR-744	1.408439893	0.251822108	1.416693905	0.26072901

miRNA	fold change	p value	fold change	p value
miR-744*	1.729464216	0.299729218	2.368809057	0.070772723
miR-760	1.477122606	0.091171643	1.350277872	0.082523815
miR-764-5p	0.848747797	0.370256504	0.752519347	0.184272096
miR-770-3p	0.679425174	0.299729218	0.524444615	0.123910683
miR-770-5p	0.205624489	0.091783392	0.167023585	0.08455984
miR-7b	0.698605261	0.429744436	0.582983448	0.412944332
miR-802	0.484868745	0.377542333	0.683187703	0.674028334
miR-804	1.832576274	0.43434326	2.249789457	0.22743239
miR-805	7.454970171	0.01102795	8.344999686	0.021881848
miR-872	1.34031196	0.389235666	2.617246084	0.077926571
miR-872*	1.653821653	0.143397391	3.620843307	0.008284863
miR-873	0.479110688	0.298230697	0.964124373	0.977738661
miR-875-5p	0.50208087	0.120638185	0.847081339	0.689327143
miR-877*	0.653592007	0.123495218	0.70658629	0.209875402
miR-878-3p	1.092119012	0.464148893	0.763159963	0.65946088
miR-879	1.117983007	0.868020022	1.645889118	0.358211754
miR-879*	2.832452305	0.063455872	2.752840607	0.032975304
miR-881*	1.379222746	0.464148893	1.379222746	0.520838834
miR-9	1.103277147	0.878261768	0.925655215	0.927148592
miR-9*	1.869502085	0.448244867	2.642650853	0.31397158
miR-92	2.935771459	0.063681353	5.773853137	0.070020922
miR-93	30.58766513	0.002195316	9.491046446	0.12573963
miR-93*	7.585371012	0.001838729	5.86831438	0.083302341
miR-96	0.010674683	0.068893257	0.092353946	0.209875402
miR-99a	1.974761714	0.34280185	1.537660844	0.552196956
miR-99b	0.573127422	0.028147949	0.69967891	0.14130197
miR-99b*	2.242795888	0.019861482	2.247101168	0.044361147

Table 2

Differential miRNA expression in human tumors

	tumour vs. normal retina	
miRNA	**fold change**	p value
hsa-let-7a	0.35537902	0.00013862
hsa-let-7b	0.09604549	7.16E-06
hsa-let-7c	0.08579614	7.16E-06
hsa-let-7d	0.2603711	0.00013862
hsa-let-7e	1.07286876	0.77502568
hsa-let-7f	0.34865756	0.00043067
hsa-let-7g	0.25157949	7.16E-06
hsa-let-7i	0.31234196	4.01E-05
hsa-mir-1	0.05903321	0.00049403
hsa-mir-100	0.08595542	7.16E-06
hsa-mir-101	0.34705063	0.00033484
hsa-mir-103	2.72037506	7.16E-06
hsa-miR-105	1.57465508	0.09943833
hsa-mir-106a	22.4668082	7.16E-06
hsa-mir-106b	10.6402635	7.16E-06
hsa-mir-10a	2.19201791	0.04611805
hsa-mir-10b	1.34390722	0.74156615
hsa-mir-124a	0.0900642	0.00043067
hsa-mir-125a	0.52319617	0.00226454
hsa-mir-125b	0.07878721	7.16E-06
hsa-miR-126	0.45730233	0.24270864
hsa-mir-126*	0.62578168	0.25602782
hsa-mir-127	0.0342777	7.16E-06

miRNA	fold change	p value
hsa-mir-128a	0.78245662	0.13861277
hsa-mir-128b	0.32953805	0.00043067
hsa-mir-129	0.28183933	7.16E-06
hsa-mir-130a	2.25433284	0.01339514
hsa-mir-130b	21.4342907	7.16E-06
hsa-mir-132	1.49485219	0.0054634
hsa-mir-133a	0.0436029	0.00018537
hsa-mir-133b	0.06463161	0.00033484
hsa-mir-134	0.04372136	7.16E-06
hsa-mir-135a	0.68609293	0.32682787
hsa-mir-135b	2.46777872	0.01539956
hsa-mir-136	0.20587978	4.14E-05
hsa-mir-137	0.11902455	0.03203347
hsa-mir-139	0.41085904	0.0013856
hsa-mir-140	0.44076828	0.00897368
hsa-mir-142-3p	1.00447986	0.85372115
hsa-mir-142-5p	1.1893089	0.48954435
hsa-mir-143	0.41216203	0.00025116
hsa-mir-145	1.21216392	0.26976018
hsa-mir-146a	0.68463308	0.19350179
hsa-miR-146b	0.49382848	0.05480526
hsa-mir-147	1.02106039	0.72621669
hsa-mir-148a	0.43564789	0.00152628
hsa-mir-148b	0.80433376	0.30889578
hsa-mir-149	1.01129007	0.90770942
hsa-mir-150	0.50410072	0.0227267
hsa-mir-151	1.07359588	0.46559024
hsa-mir-152	0.2517561	7.16E-06
hsa-mir-153	0.39547506	0.0117662
hsa-mir-155	2.13489084	0.01749556
hsa-mir-15a	2.06363552	0.00900422
hsa-mir-15b	26.1777272	7.16E-06
hsa-mir-16	7.00699074	7.16E-06
hsa-mir-17-3p	2.9472995	0.00025116
hsa-mir-17-5p	26.3119159	0.00043067
hsa-mir-181a	0.41250441	0.0018715
hsa-mir-181b	0.4559957	0.00418778
hsa-mir-181c	0.38846465	0.00018537
hsa-mir-181d	0.70318804	0.12703441
hsa-mir-182	0.35232871	0.00043067
hsa-mir-182*	0.42557789	0.01749556
hsa-mir-183	0.5743967	0.02859318
hsa-mir-184	0.06398151	7.16E-06
hsa-miR-185	1.54155538	0.12703441
hsa-mir-186	1.85438899	0.00124058
hsa-mir-187	0.46481307	0.39367912
hsa-mir-188	0.88806239	0.72621669
hsa-mir-189	0.16893503	0.00030233
hsa-mir-18a	79.0815882	7.16E-06
hsa-mir-18a*	63.9114703	7.16E-06
hsa-mir-190	1.64013997	0.2889012
hsa-mir-191	1.10477886	0.26976018
hsa-mir-192	0.14622399	7.16E-06
hsa-mir-193a	1.16789826	0.67465852
hsa-mir-193b	4.2090245	0.00013862
hsa-mir-194	0.20907205	7.16E-06
hsa-mir-195	2.85739458	0.00018537
hsa-mir-196a	16.7342353	0.00043067
hsa-mir-196b	17.7581272	0.00043067

miRNA	fold change	p value
hsa-mir-197	3.3116927	7.16E-06
hsa-mir-199a*	0.55000083	0.10843751
hsa-mir-19a	6.88751333	7.16E-06
hsa-mir-19b	4.49373357	1.30E-05
hsa-mir-200b	0.42791773	0.00378473
hsa-mir-200c	1.41579868	0.01749556
hsa-mir-202	1.1245744	0.26368061
hsa-mir-203	0.83558823	0.48954435
hsa-mir-204	0.03706516	7.16E-06
hsa-mir-205	0.53675893	0.07581168
hsa-mir-206	0.95807252	0.77502568
hsa-mir-20a	26.4811479	7.16E-06
hsa-mir-20b	14.1612372	7.16E-06
hsa-mir-21	0.57656504	0.01339514
hsa-mir-210	0.96285721	0.99060345
hsa-mir-211	0.02482795	0.00043067
hsa-mir-213	0.48361999	0.24766578
hsa-mir-214	1.38943797	0.12703441
hsa-mir-216	2.32107449	0.00939182
hsa-mir-217	7.81322416	1.30E-05
hsa-mir-218	0.25179093	0.00762549
hsa-mir-219	0.94631518	0.960105
hsa-mir-22	0.05253163	7.16E-06
hsa-mir-221	1.40947664	0.5272094
hsa-mir-222	1.09339696	0.67465852
hsa-mir-223	0.89534602	0.85372115
hsa-mir-224	291.864825	7.16E-06
hsa-mir-23b	0.42004824	2.44E-05
hsa-mir-24	0.63896374	0.04906702
hsa-mir-25	15.6401857	7.16E-06
hsa-mir-26a	0.59553671	0.04427405
hsa-mir-26b	0.5583957	0.03203347
hsa-mir-27a	0.3573996	0.00025116
hsa-mir-27b	0.36225236	7.16E-06
hsa-mir-28	1.03190904	0.64778272
hsa-mir-296	2.13514657	0.00013862
hsa-mir-29a	0.0421722	7.16E-06
hsa-mir-29b	0.0482461	0.00021017
hsa-mir-29c	0.02886594	7.16E-06
hsa-mir-301	7.42502783	0.00043067
hsa-mir-302a	0.83938937	0.56829947
hsa-mir-302b	0.67342364	0.52689892
hsa-mir-302c	1.19328061	0.77502568
hsa-mir-30a-3p	0.14800778	7.16E-06
hsa-mir-30a-5p	0.53136762	0.00063136
hsa-mir-30b	0.54588527	0.01339514
hsa-mir-30c	0.55939359	0.00391308
hsa-mir-30d	0.7681248	0.53670222
hsa-mir-30e-3p	0.21060566	4.01E-05
hsa-mir-31	0.02101799	0.00043067
hsa-mir-32	1.76548942	0.04427405
hsa-mir-320	1.12000353	0.3483077
hsa-mir-323	0.33369345	0.00141352
hsa-mir-324-3p	1.26345004	0.25054001
hsa-mir-324-5p	1.93278119	0.00226454
hsa-mir-328	0.33475394	0.00013862
hsa-mir-329	0.05237916	0.0007761
hsa-mir-33	0.48968232	0.091318
hsa-mir-330	0.09526962	0.00043067

miRNA	fold change	p value
hsa-mir-331	1.20513488	0.06785334
hsa-mir-335	0.31105792	6.49E-05
hsa-mir-337	0.20862313	0.00900422
hsa-mir-338	0.95192321	0.72621669
hsa-mir-339	0.97027964	0.99060345
hsa-mir-340	4.3996595	4.01E-05
hsa-mir-342	1.20499242	0.26976018
hsa-mir-345	2.23359741	0.00063136
hsa-mir-34a	3.29325121	0.00251841
hsa-mir-34b	2.18090789	0.00404264
hsa-mir-34c	11.480094	0.00043067
hsa-mir-361	1.64485503	0.00018537
hsa-mir-362	4.66675665	9.72E-05
hsa-mir-363	0.76690276	0.26976018
hsa-mir-365	3.18513195	0.00043067
hsa-mir-367	0.78848663	0.70717062
hsa-mir-368	0.2349716	0.00010121
hsa-mir-369-3p	0.02751693	0.00091092
hsa-mir-369-5p	0.08361946	0.00251841
hsa-mir-370	0.07476827	0.00060692
hsa-mir-371	0.48418781	0.10843751
hsa-mir-372	0.54306705	0.04415163
hsa-mir-373	0.95097536	0.62116166
hsa-mir-374	1.99163251	0.08189477
hsa-mir-375	1.25035362	0.85372115
hsa-mir-376a	0.07683527	0.00049403
hsa-miR-376a*	0.09681493	9.39E-06
hsa-mir-378	0.12962734	7.16E-06
hsa-mir-379	0.03956945	0.00025116
hsa-miR-380-5p	0.03800462	0.00069548
hsa-mir-381	0.46185069	0.0254578
hsa-mir-382	0.0307304	0.00017972
hsa-mir-383	0.0283853	0.00049403
hsa-mir-409-5p	0.3510641	0.00048429
hsa-mir-410	0.05228181	0.00124058
hsa-mir-411	0.0505212	0.0022499
hsa-mir-422a	0.19101909	9.72E-05
hsa-mir-422b	0.12338888	0.00055578
hsa-mir-423	1.28423439	0.00939182
hsa-mir-424	0.37428883	0.02624396
hsa-mir-425	1.28014247	0.0244766
hsa-miR-425-5p	1.62536378	0.03203347
hsa-mir-429	0.3929063	0.00994593
hsa-mir-432	0.0528755	0.00332382
hsa-mir-432*	0.27657592	0.00163922
hsa-mir-433	0.03069235	0.00031742
hsa-mir-449	155.434676	0.00043067
hsa-mir-449b	16.6971009	0.00043067
hsa-mir-451	0.46332132	0.01339514
hsa-mir-452*	50.0864717	0.00043067
hsa-mir-455	0.7951811	0.62116166
hsa-miR-484	1.17789333	0.52511306
hsa-mir-485-3p	0.11089838	0.00063136
hsa-mir-486	5.9867152	7.16E-06
hsa-mir-487a	0.34346089	1.49E-05
hsa-mir-487b	0.03597225	0.00074741
hsa-miR-488	0.20122425	9.72E-05
hsa-mir-489	0.31905186	0.00900422
hsa-mir-491	0.36527896	0.00081334

miRNA	fold change	p value
hsa-mir-493-3p	0.68599194	0.00259778
hsa-mir-495	0.03849728	0.00076348
hsa-mir-496	0.3300278	0.00479033
hsa-mir-497	0.2185559	0.00074164
hsa-mir-500	3.25977843	0.01446879
hsa-mir-501	4.79990937	0.00404264
hsa-mir-502	4.99722754	0.00378473
hsa-miR-504	0.48939463	0.01584247
hsa-mir-511	1.99855529	0.03799159
hsa-mir-516-3p	2.69457618	0.0007761
hsa-mir-517c	0.69861325	0.25602782
hsa-mir-518b	0.68684019	0.24796386
hsa-mir-520b	1.62964764	0.02510968
hsa-mir-520c	0.70751266	0.32020428
hsa-mir-520d	1.14690339	0.93441565
hsa-mir-520f	1.11158693	0.90770942
hsa-mir-520g	1.3021177	0.90770942
hsa-mir-524	1.43984302	0.1241732
hsa-mir-526b*	1.0973129	1
hsa-miR-532	3.49359963	0.00098366
hsa-mir-539	0.05218903	0.0007761
hsa-mir-542-3p	0.14402793	0.00124243
hsa-mir-544	2.62052266	0.02211232
hsa-miR-545	1.01146185	0.90770942
hsa-miR-548a	2.05655775	0.02510968
hsa-miR-548c	1.45298408	0.49035982
hsa-mir-548d	67.9436778	0.00043067
hsa-mir-550	7.76782145	7.16E-06
hsa-mir-551b	1.89296142	0.07482871
hsa-miR-556	1.40849181	0.10164507
hsa-mir-563	1.83609752	0.22335188
hsa-mir-564	0.80404032	0.69033352
hsa-mir-565	1.86022235	0.04427405
hsa-mir-572	1.57248444	0.11788226
hsa-mir-574	1.7388258	0.0400753
hsa-miR-576	3.46042567	0.00939182
hsa-miR-579	1.45550865	0.17876651
hsa-mir-580	1.67404523	0.07581168
hsa-mir-586	3.5635258	0.00416508
hsa-miR-589	2.73919005	0.02246324
hsa-miR-591	1.78203829	0.32614379
hsa-miR-592	3.68492668	0.00391308
hsa-miR-594	5.95325565	0.0018715
hsa-miR-597	1.07407362	0.93441565
hsa-mir-601	1.56904541	0.20907235
hsa-mir-604	0.89158812	0.85372115
hsa-miR-606	0.93037864	0.83163543
hsa-mir-610	1.67142973	0.04906702
hsa-mir-616	1.13999295	0.46559024
hsa-miR-617	0.99644562	1
hsa-miR-618	0.55607267	0.11788226
hsa-mir-624	0.71607099	0.38454635
hsa-mir-627	1.17089243	0.85372115
hsa-miR-628	1.76076528	0.06916151
hsa-miR-629	13.7301513	7.16E-06
hsa-mir-630	0.91056169	0.64778272
hsa-mir-632	2.19061098	0.02859318
hsa-miR-638	1.36058072	0.43080058
hsa-mir-639	1.28944024	0.7278872

miRNA	**fold change**	p value
hsa-miR-641	1.17976993	0.77502568
hsa-miR-642	0.67712899	0.85372115
hsa-mir-643	1.35673645	0.15056971
hsa-miR-645	0.65285887	0.0400753
hsa-mir-650	1.3372344	0.4175414
hsa-mir-651	2.83286228	0.00569964
hsa-miR-653	0.33088794	0.00250862
hsa-mir-655	0.06240409	0.00200424
hsa-mir-656	0.13595654	0.00017353
hsa-mir-660	1.75285267	0.0610666
hsa-mir-7	42.6616607	7.16E-06
hsa-mir-9	0.26396093	6.49E-05
hsa-mir-9*	0.19311894	2.44E-05
hsa-mir-92	6.40573466	7.16E-06
hsa-mir-93	23.3811787	7.16E-06
hsa-mir-95	2.18070927	0.03239561
hsa-mir-96	0.15148064	1.30E-05
hsa-mir-98	0.350188	0.03239561
hsa-mir-99a	0.09033381	7.16E-06
hsa-mir-99b	1.46567717	0.00762549

Table 3

List of miRNAs that are up-regulated both in mouse and human tumours
Column 1: miRNAs; column 2: Murine tumour vs. normal retina; column 3: Human tumour vs. normal retina; column 4: Mouse Validated targets according to http://mirecords.biolead.org/; column 5: Human Validated targets according to http://mirecords.biolead.org/

miR-449b	128.9933081	16.69710093	-	-
miR-449	102.6824215	155.4346758	**E2F5**	-
miR-18a	72.27794902	79.08158818	-	-
miR-106a	51.88732466	22.46680818	**MYLIP, ARID4B, LOC100048439**	**VEGFA**, RB1, **RUNX1**, APP
miR-17-5p	47.58305778	26.31191595	RB2 (p130)	NCOA3, **VEGFA, RUNX1, CCND1**
miR-20b	45.10422985	14.16123719	**MYLIP, ARID4B, LOC100048439**	**VEGFA**
miR-20a	36.49125415	26.4811479	ZBTB7A, **STAT3**	**VEGFA, E2F1, RUNX1, CCND1**
miR-93	30.58766513	23.3811787	**STAT3**	**E2F1, VEGFA, CDKN1A (p21Cip1)**
miR-19b	10.94909897	4.493733566	**MYLIP, LOC100048439**	-
miR-19a	9.927933234	6.887513332	-	**PTEN**
miR-186	7.494097062	1.854388991	-	-
miR-18a*	7.461352046	63.91147033	-	-
miR-34b	7.063390153	2.180907894	-	-
miR-17*	7.05075498	2.947299501	-	-
miR-106b	6.475493206	10.64026349	-	**E2F1, VEGFA, CDKN1A (p21Cip1), ITCH**
miR-155	5.933582476	2.134890842	SFPI1, MYB, RHEB, BAT5, JARID2, **TRP53INP1**, IKBKE, FADD, RIPK1, MAF, AICDA, SOCS1	AGTR1, BACH1 (FANCJ), LDOC1, MATR3, TM6SF1, RHOA, ETS1, MEIS1
miR-34c	5.707106215	11.48009405	-	-
miR-15b	5.453081609	26.17772719	-	BCL2, **CCNE1**
miR-301	4.171971098	7.425027828	-	-
miR-195	3.362819079	2.85739458	-	-
miR-25	3.301359495	15.64018573	-	-
miR-16	3.03333113	7.00699074	-	**CCND1**
miR-92	2.935771459	6.405734661	**MYLIP, LOC100048439**	-
miR-532	2.121351093	3.493599628	-	-
miR-130a	1.714212739	2.254332844	-	TAC1, CSF1, MAFB, **MEOX2**, HOXA5

miRNAS in red belong to the miR-17-92 or the two paralogue clusters; targets found more than once are highlighted in bold

Materials and Methods

Mice

All animal experiments were performed in accordance with the guidelines of the University of Leuven Animal Care and Use ethical Committee. BrdU (100 µg/g of body weight) was injected intraperitoneally 1hr prior to sacrifice.

Immunohistochemistry

Eyes were fixed overnight in 4% paraformaldehyde/PBS, and paraffin embedded. 5µm sections were immunostained with the following antibodies: GFP (Santa Cruz Biotechnology, 1/100); BrdU (BD Pharmingen); Ki-67 (DAKO cytomation); cleaved Caspase-3 (Cell Signaling); GFP (Santa Cruz Biotechnology); Chx10 (Exalpha Biologicals); syntaxin (Sigma); calbindin (Abcam); Calretinin (Millipore).

AP staining

Dissected retinae were fixed for 1h in 4% paraformaldehyde/PBS on ice, heated to 65°C for 30min and embedded in 4% agarose/PBS. 40µm sections were rinsed once in AP detection buffer (100mM Tris pH9.5, 50mM $MgCl_2$, 100 mM NaCl) before developing in Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP Ready-to-use tablets, Roche) for 4h.

Recombination analysis

DNA was isolated from dissected retinae and isolated tumours using DNeasy Blood&Tissue Kit (Qiagen). *Dicer1* recombination was analyzed by PCR using the following primers: a 5'-ATTGTTACCAGCGCTTAGAATTCC; c 5'-TCGGAAT AGGAACTTCGTTTAAAC and the reverse b primer 5'-GGGAGGTGTACGTCTA CAATT. *P53* recombination was analyzed by PCR using the following primers: d 5'-CACAAAAACAGGTTAAACCCAG and the reverse primers f 5'-AGCACATAGGAGGCAGAGAC and e 5'-GAAGACAGAAAAGGGGAGGG. PCR conditions were as follow: 1x precycle at 94°C for 3min and 30cycles of 94°C, 30sec; 60°C, 30sec; 72°C, 45sec.

Retinoblastoma tumour samples and RNA isolation

Immediately following enucleation, dissected retinae or tumour samples were removed from the mouse eyes under the binocular using forceps. The specimens were placed on ice and immediately processed for RNA or DNA isolation. Before tumour samples were collected from human retinoblastoma samples, serial cryosections where obtained from all tumours. The first and last cryosection of each series were H&E stained for tumour cell content verification. 3-5mm^3 samples were placed on ice and immediately processed for RNA and DNA isolation. Total RNA and genomic DNA were isolated using the miRNeasy kit (Qiagen) and the QIAmp mini kit (Qiagen), respectively, according to manufacturer's instructions. Written informed consent was obtained from patients and/or their

parents. All procedures have been approved by the institutional review board of the Children's University Hospital of Essen.

microRNA Expression analyses

For human samples, miRNA expression profiling was performed as described previously (24). For murine samples, 60 ng of total RNA was reverse transcribed using the murine stem-loop megaplex pool A and B followed by limited cycle pre-amplification (Applied Biosystems). Expression of 430 human and 509 murine miRNAs was profiled using Taqman miRNA assays on a 7900HT detection system (Applied Biosystems). Data were normalized using the global mean (25). miRNA expression data are available as RDML-files upon request (26). Differentially expressed miRNAs were identified using the Mann-Whitney test followed by multiple testing correction according to the Benjamini-Hochberg algorithm. Hierarchical clustering was performed using method Ward and distance Manhattan. All statistical analyses were performed using R Bioconductor software.

Array CGH

Samples were profiled on a custom designed 44K/60K array (Agilent Technologies) enriched for miRNA and T-UCR regions and regions around cancer gene census genes. Utilizing random prime labelling (BioPrime ArrayCGH Genomic Labeling System, Invitrogen), 150 ng of test and control DNA (DNA from an EBV cell line if cell lines were tested or male control DNA, Promega if tumour samples were tested) was labeled with Cy3 and Cy5 dyes (GE healthcare). Slides were scanned using an Agilent scanner (Agilent Technologies) an in-house developed visualisation software program arrayCGHbase (http://medgen.ugent.be/arrayCGHbase) (27). Array CGH profiles were evaluated by using the circulary binary segmentation (CBS) algorithm.

Cell culture and Inhibition of miRNAs

Retinoblastoma cell lines Weri and Y79 were authenticated by DNA fingerprinting (DMSZ, Braunschweig, Germany). Cells were cultured in suspension in Dulbecco's Modified Eagles's Medium (DMEM) (Invitrogen), containing 15% FCS, Penicillin/Streptomycin, 4mM L-Glutamin, 50µM ß-Mercaptoethanol and 0.1% Insulin (all from Invitrogen). $1x10^4$ Weri and Y79 cells / well were seeded on 24-well plates and transfected with specific antagomirs or scrambled Cy3-labelled control oligos (all from Ambion) at a final concentration of 100nM using NeoFx transfection agent (Ambion) according to the manufacturers recommendations. Further, antisense inhibitors were designed against all members of the miR-17/92 cluster using the locked nucleic acid (LNA) technology. The inhibitors were synthesized as fully phosphorothiolated DNA/LNA mixmers and purified by preparative HPLC before

use. The number and position of LNA nucleotides was chosen in each case in order to maximize affinity and selectivity towards the specific miRNA target.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay MTT assays were performed as previously described (28). Briefly, after the addition of 200 µL MTT solution (6 mg/mL in PBS, Roche, Germany), cells were incubated for 1h and then solubilized by the addition of 1 mL stop solution (10% SDS, 5% acetic acid in dimethyl sulfoxide). Absorbance at 570 nm was measured using a GloMax®-Multi Microplate Multimode Reader (Promega).

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Claims

1. A method of inducing cell death in a cell where p53 function is compromised, comprising inhibiting the function of Dicer.

2. The method according to claim 1, wherein the cell death is due to synthetic lethality.

3. The method according to claim 1 or 2, wherein the cell is further characterized by activation of an oncogene or inhibition of a tumor suppressor gene.

4. The method according to any one of claims 1 to 3, wherein the cell is a tumour cell.

5. The method according to claim 4, wherein the tumour is a retinoblastoma.

6. The method according to claim 5, wherein the function of Dicer1 is inhibited by inhibiting one or more of the miRNAs that are upregulated in the cell where p53 function is impaired.

7. The method according to claim 5, wherein the miRNA is selected from the miR 17-92 cluster, particularly selected from miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92.

8. The method according to claim 6 or 7, wherein inhibition of the miRNAs is with an LNA or an antagomir.

9. The method according to any one of claims 1 to 5, wherein inhibiting the function of Dicer is done by inhibition of the Dicer1 gene, the Dicer1 mRNA or the Dicer protein.

10. The method according to any one of claims 1 to 9, wherein p53 function is impaired by functional dysregulation but not mutation.

11. The method according to any one of claims 1 to 9, wherein p53 function is impaired by at least one mutation.

12. An inhibitor of Dicer function for use in treatment of cancer.

13. The inhibitor according to claim 12, wherein the cancer is retinoblastoma.

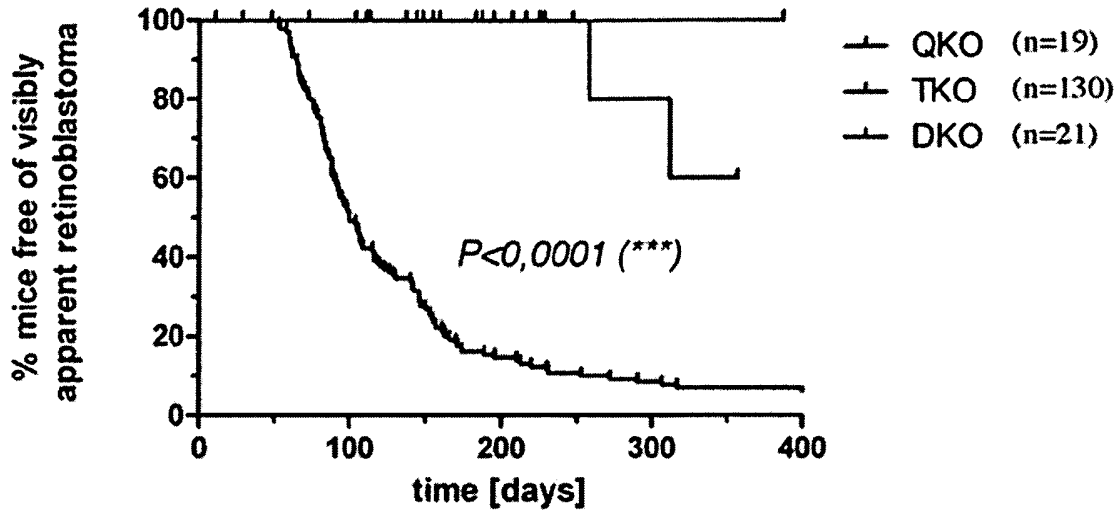
14. The inhibitor according to claim 13, which is an inhibitor of one or more of the miRNAs that are upregulated in the cancer cells.

15. The inhibitor according to claim 14, wherein the miRNA is selected from the miR 17-92 cluster, particularly selected from miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92.

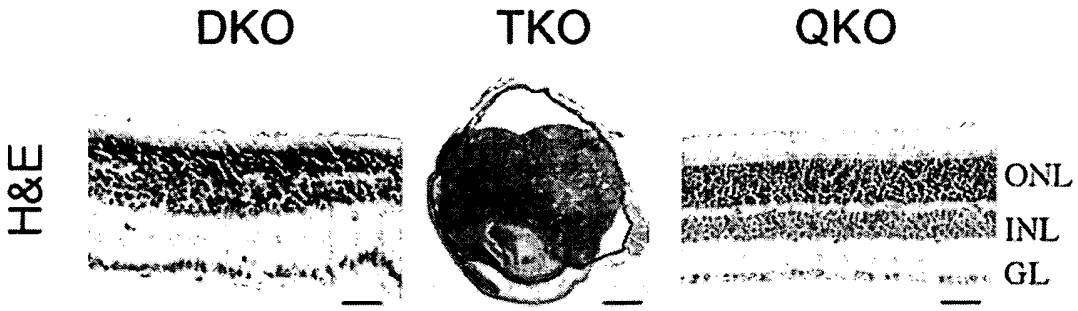
16. The inhibitor according to claim 12 or 13, which is an inhibitor of the Dicer1 gene, the Dicer1 mRNA or the Dicer protein.

Figure 1

A


% mice free of visibly apparent retinoblastoma
100
80
60
40
20
0
0
100
200
300
400
time [days]
P<0,0001 (***)
QKO (n=19)
TKO (n=130)
DKO (n=21)

B


DKO
TKO
QKO
H&E
ONL
INL
GL

c

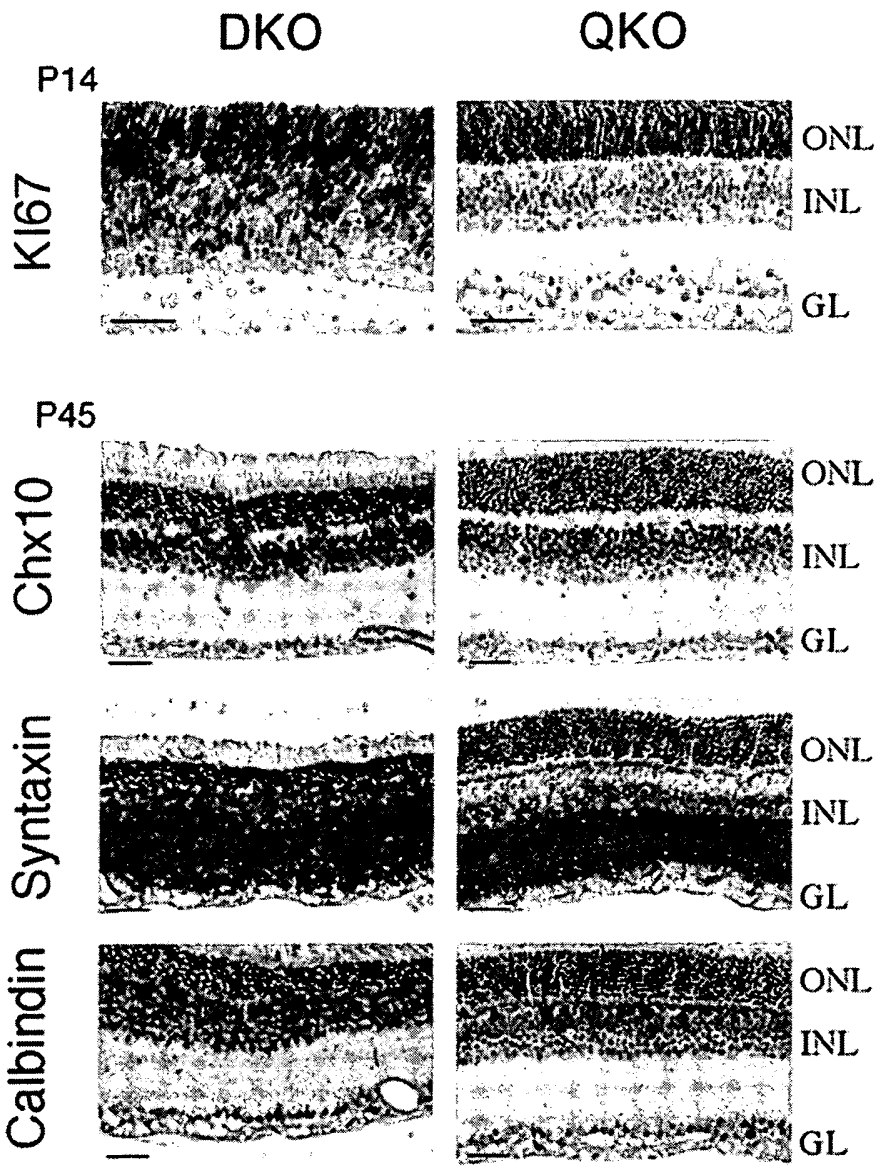
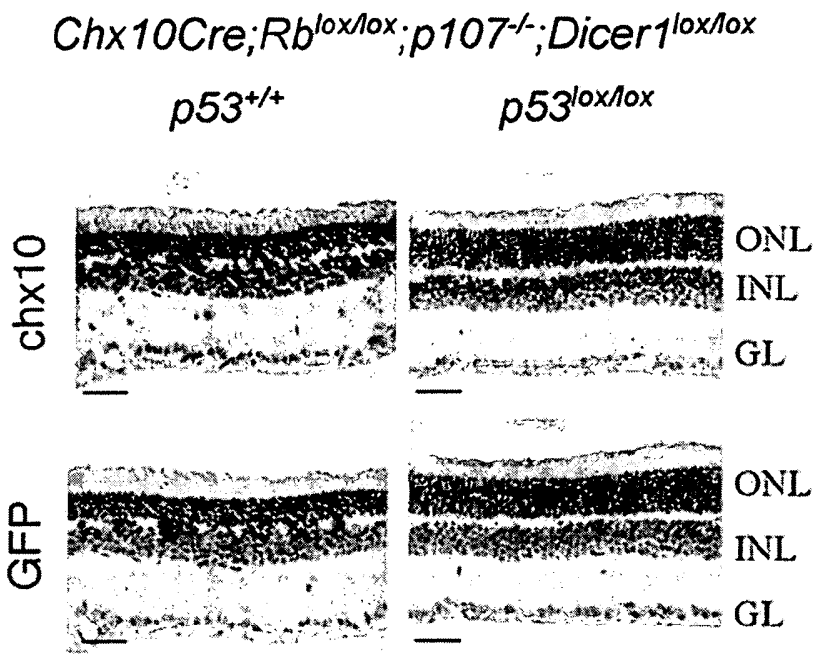
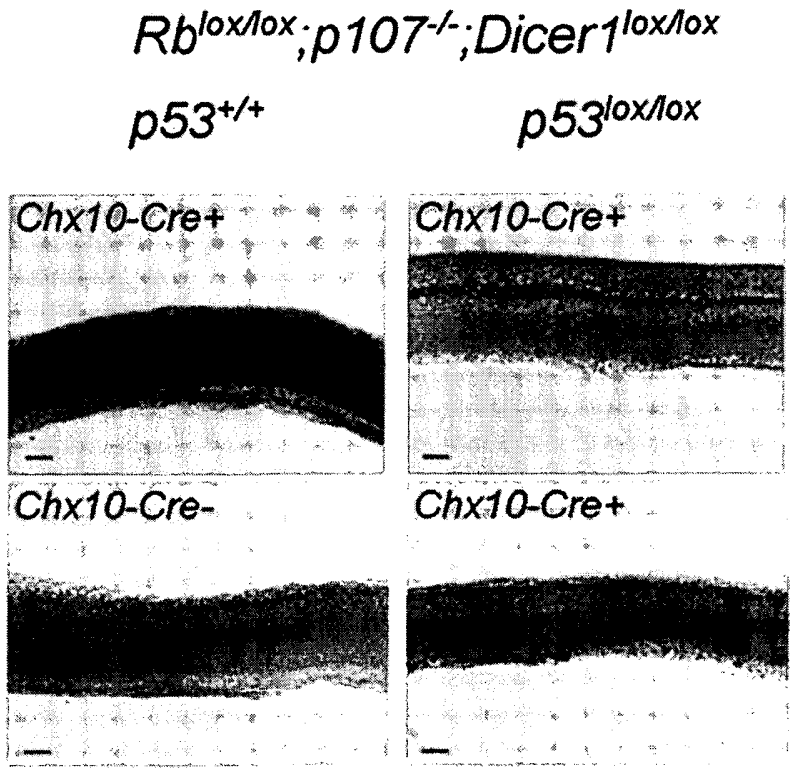

DKO
QKO
P14
KI67
ONL
INL
GL
P45
Chx10
ONL
INL
GL
Syntaxin
ONL
INL
GL
Calbindin
ONL
INL
GL

Figure 2

A


Chx10Cre;Rb^lox/lox;p107^-/-;Dicer1^lox/lox
p53^+/+
p53^lox/lox
chx10
GFP
ONL
INL
GL
ONL
INL
GL

B


Rb^lox/lox;p107^-/-;Dicer1^lox/lox
p53^+/+
p53^lox/lox
Chx10-Cre+
Chx10-Cre+
Chx10-Cre-
Chx10-Cre+

C

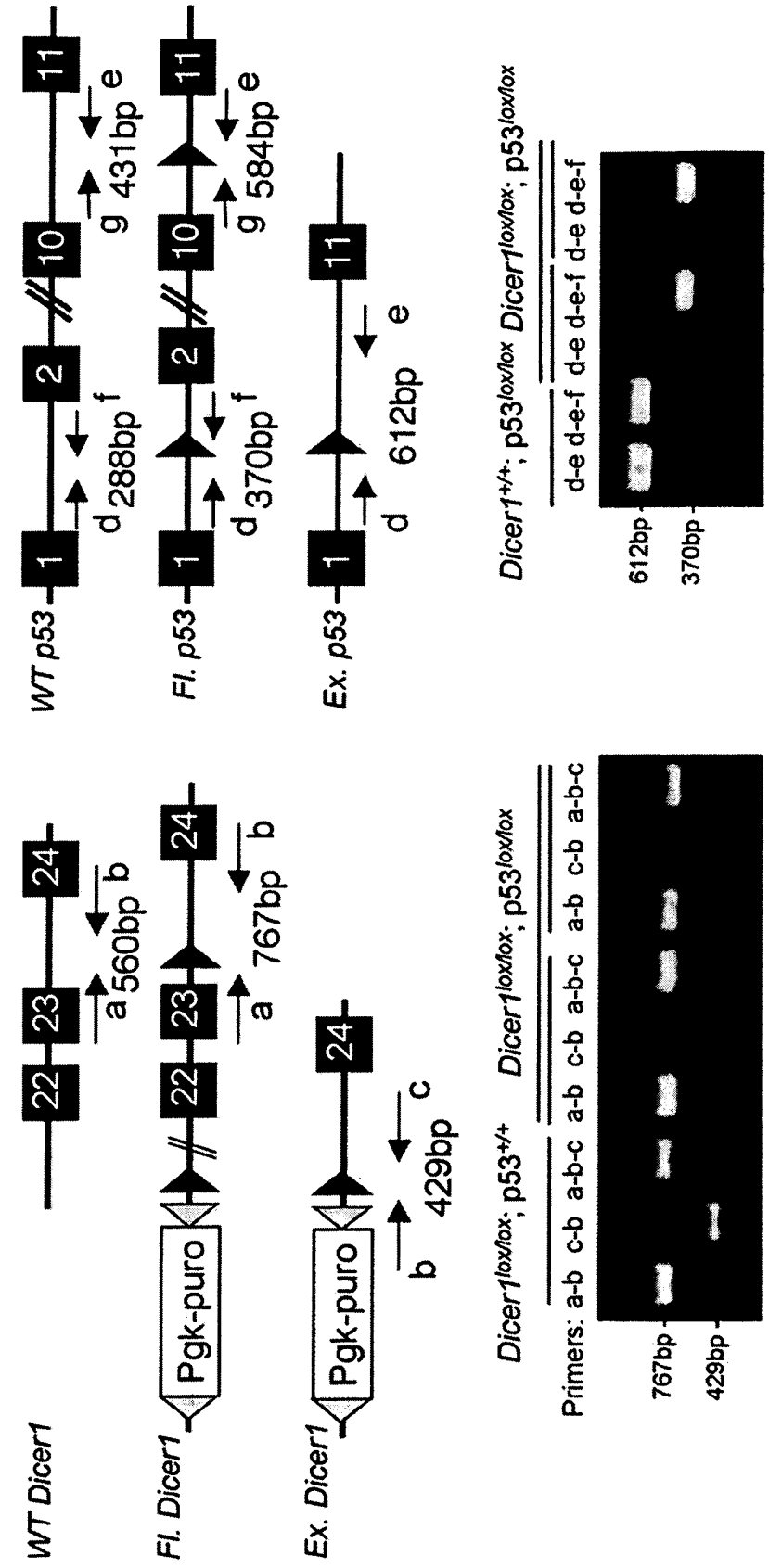
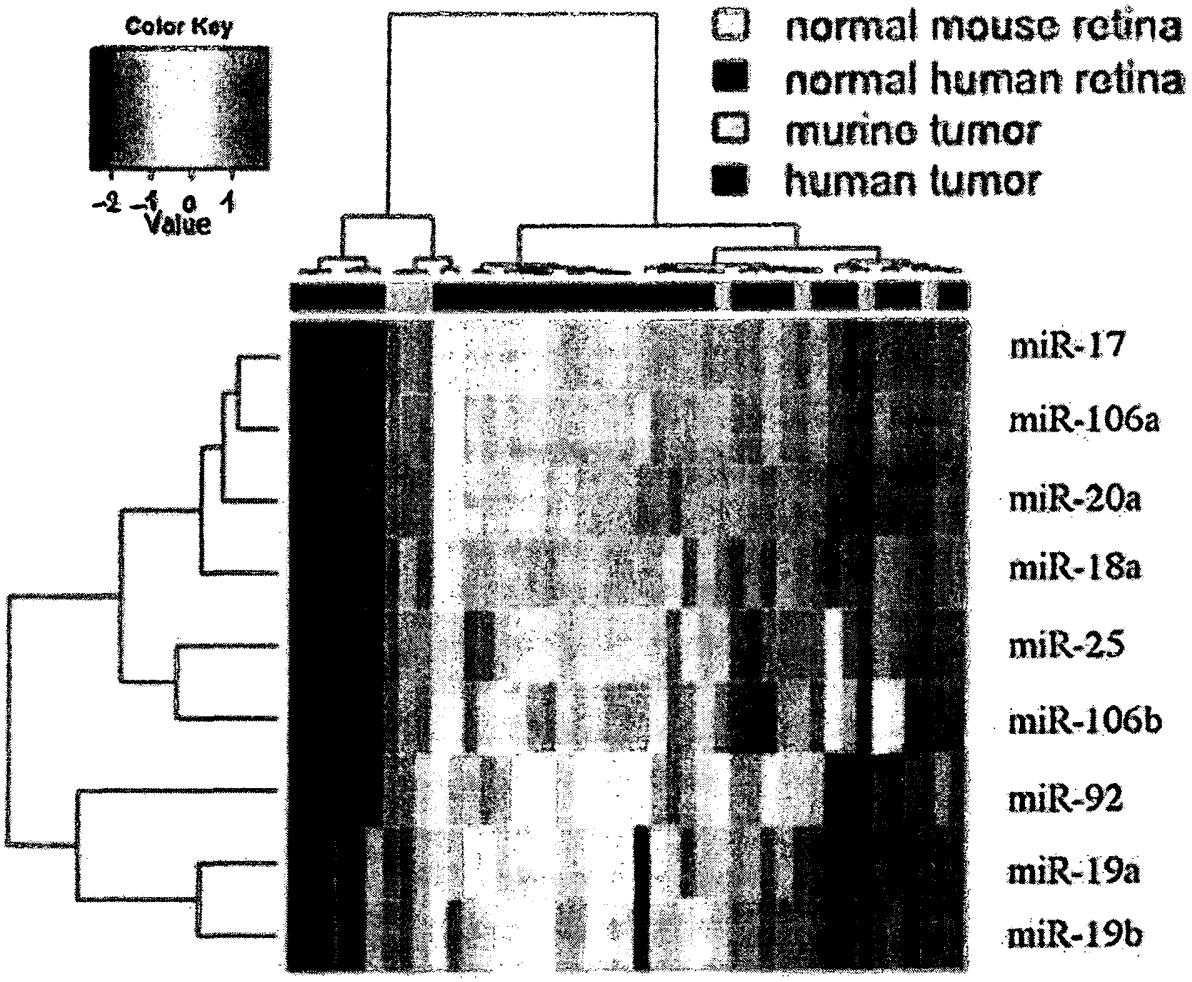
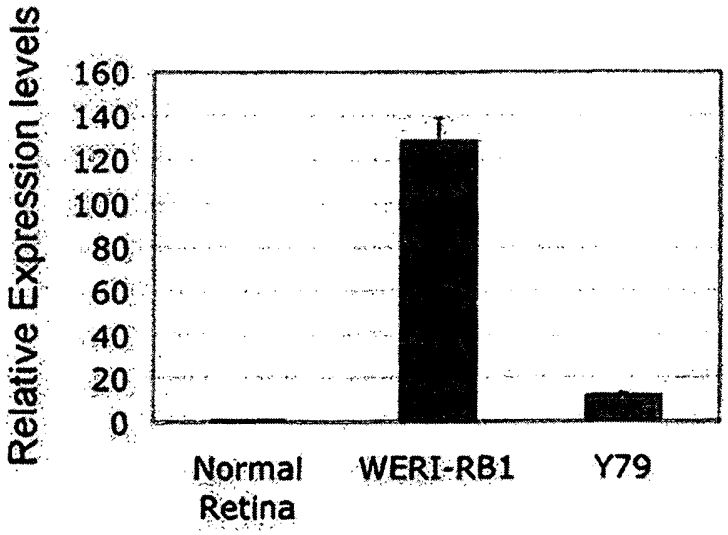

WT p53
Fl. p53
Ex. p53
1
2
10
11
d 288bp f
g 431bp e
d 370bp f
g 584bp e
d 612bp e
Dicer1+/+; p53lox/lox
Dicer1lox/lox; p53lox/lox
d-e d-e-f d-e d-e-f d-e d-e-f
612bp
370bp
WT Dicer1
Fl. Dicer1
Ex. Dicer1
22
23
24
a 560bp b
a 767bp b
b 429bp c
Pgk-puro
Dicer1lox/lox; p53+/+
Dicer1lox/lox; p53lox/lox
Primers: a-b c-b a-b-c a-b c-b a-b-c a-b c-b a-b-c
767bp
429bp

Figure 3

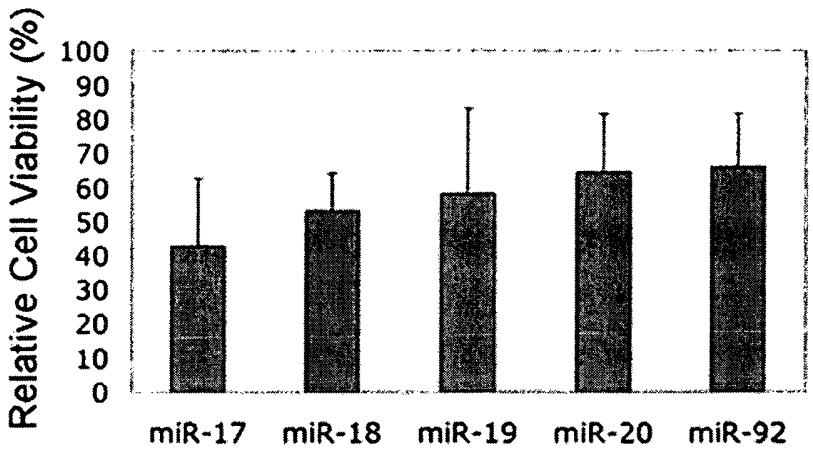
A


Color Key
-2 -1 0 1
Value
normal mouse retina
normal human retina
murine tumor
human tumor
miR-17
miR-106a
miR-20a
miR-18a
miR-25
miR-106b
miR-92
miR-19a
miR-19b

B


Relative Expression levels
160
140
120
100
80
60
40
20
0
Normal Retina
WERI-RB1
Y79

C


Relative Cell Viability (%)
100
90
80
70
60
50
40
30
20
10
0
miR-17
miR-18
miR-19
miR-20
miR-92

D

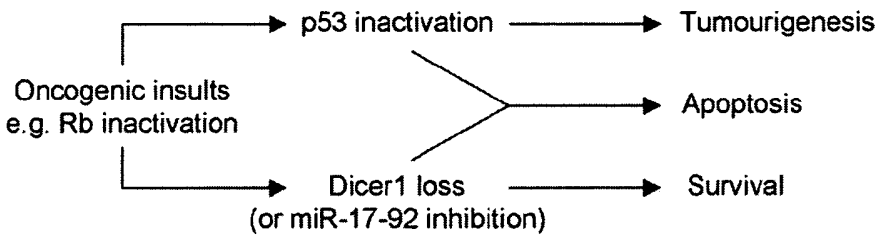
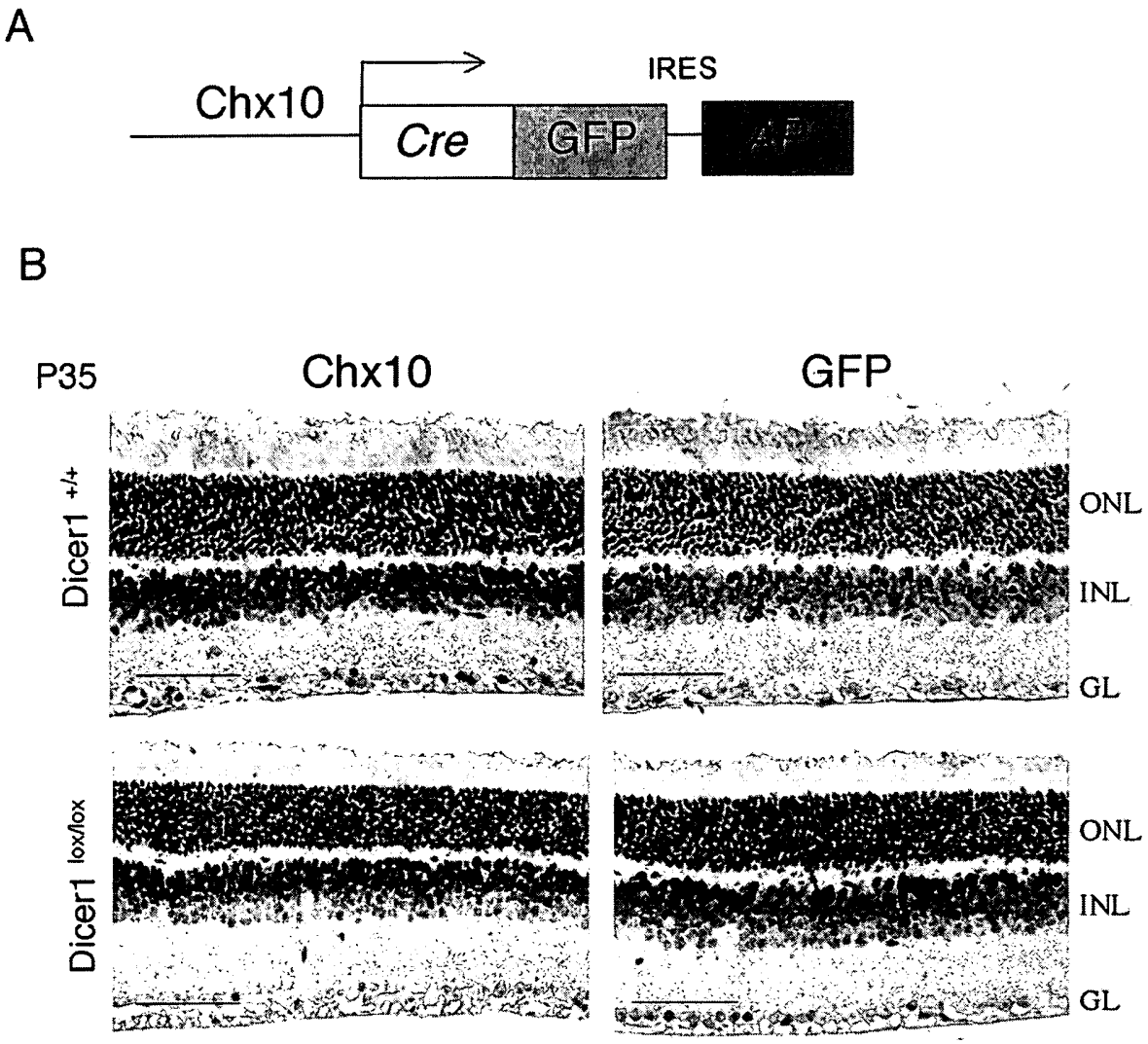

Oncogenic insults
e.g. Rb inactivation
p53 inactivation
Tumourigenesis
Apoptosis
Dicer1 loss
(or miR-17-92 inhibition)
Survival

Figure 4


A
Chx10
IRES
Cre
GFP
B
P35
Chx10
GFP
Dicer1 +/+
Dicer1 lox/lox
ONL
INL
GL
ONL
INL
GL

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/063233

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113 A61K31/7088
ADD. A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NITTNER D. ET AL.: "Dicer1 is a synthetic lethal partner of tumour suppressor p53", EJC SUPPLEMENTS, vol. 8, no. 5, June 2010 (2010-06), page 98, XP002661054, & 21ST MEETING OF THE EUROPEAN-ASSOCIATION-FOR-CANCER-RESEARCH; OSLO, NORWAY; JUNE 26 -29, 2010 ISSN: 1359-6349 the whole document ----- -/--	1-16

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 October 2011	07/11/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Macchia, Giovanni

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LAMBERTZ I. ET AL.: "Monoallelic but not biallelic loss of Dicer1 promotes tumorigenesis in vivo", CELL DEATH AND DIFFERENTIATION, vol. 17, no. 4, 1 April 2010 (2010-04-01), pages 633-641, XP055008885, ISSN: 1350-9047, DOI: 10.1038/cdd.2009.202 cited in the application the whole document -----	1-16
A	YAN LI ET AL: "Targeted deletion of Dicer disrupts lens morphogenesis, corneal epithelium stratification, and whole eye development", DEVELOPMENTAL DYNAMICS, vol. 238, no. 9, 1 September 2009 (2009-09-01), pages 2388-2400, XP055008881, ISSN: 1058-8388, DOI: 10.1002/dvdy.22056 the whole document -& YAN LI ET AL: "Supporting Information: Targeted deletion of Dicer disrupts lens morphogenesis, corneal epithelium stratification, and whole eye development", DEVELOPMENTAL DYNAMICS , vol. 238, no. 9 September 2009 (2009-09), XP002661055, Retrieved from the Internet: URL:http://onlinelibrary.wiley.com/doi/10.1002/dvdy.22056/suppinfo [retrieved on 2011-10-06] the whole document -----	1-16
A	WO 2009/102225 A2 (INSTYTUT CHEMII BIOORGANICZNEJ PAN [PL]) 20 August 2009 (2009-08-20) the whole document -----	1-16

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2011/063233

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009102225 A2	20-08-2009	EP 2255002 A2	01-12-2010
		US 2011207197 A1	25-08-2011