

Monoallelic but not biallelic loss of *Dicer1* promotes tumorigenesis *in vivo*

I Lambertz¹, D Nittner¹, P Mestdagh², G Denecker¹, J Vandesompele², MA Dyer³ and J-C Marine^{*1}

Human tumors are characterized by widespread reduction in microRNA (miRNA) expression, although it is unclear how such changes come about and whether they have an etiological role in the disease. Importantly, miRNA knockdown has been shown to enhance the tumorigenic potential of human lung adenocarcinoma cells. A defect in miRNA processing is one possible mechanism for global downregulation. To explore this possibility in more detail *in vivo*, we have manipulated *Dicer1* gene dosage in a mouse model of retinoblastoma. We show that although monoallelic loss of *Dicer1* does not affect normal retinal development, it dramatically accelerates tumor formation on a retinoblastoma-sensitized background. Importantly, these tumors retain one wild-type *Dicer1* allele and exhibit only a partial decrease in miRNA processing. Accordingly, *in silico* analysis of human cancer genome data reveals frequent hemizygous, but not homozygous, deletions of *DICER1*. Strikingly, complete loss of *Dicer1* function in mice did not accelerate retinoblastoma formation. miRNA profiling of these tumors identified members of the let-7 and miR-34 families as candidate tumor suppressors in retinoblastoma. We conclude that *Dicer1* functions as a haploinsufficient tumor suppressor. This finding has implications for cancer etiology and cancer therapy.

Cell Death and Differentiation (2010) 17, 633–641; doi:10.1038/cdd.2009.202; published online 18 December 2009

A large body of evidence indicates that alterations in the expression of microRNAs (miRNAs) contribute to cancer pathologies.¹ miRNAs function as agents of the RNA interference pathway to silence their cognate coding target genes either by cleaving mRNA molecules or by inhibiting their translation.² By silencing tumor-suppressive and oncogenic mRNAs, miRNAs themselves can function as oncogenes or tumor suppressors, respectively.³ The let-7 family, for instance, limits lung tumorigenesis through inhibition of several oncogenes including members of the Ras family or HMGA2.^{4,5}

Initial efforts to link miRNAs with cancer were based on expression analyses in which tumors were compared with normal tissues. Such expression profiling analyses revealed the characteristic miRNA signatures of human cancers.⁶ Surprisingly, they also highlighted an overall downregulation of mature miRNAs in several types of mouse and human cancers.⁷ This observation raised two important questions: (i) one of causality – is there a causative link between global downregulation of miRNAs and cancer progression or is the downregulation a byproduct of tumor development? and (ii) one of mechanism – what are the molecular mechanisms and the genetic events underlying such widespread changes in tumors? The first question was addressed by RNAi targeting of factors involved in miRNA maturation, such as DICER1 and DROSHA, showing that global downregulation of miRNA processing increased the transforming properties of a lung adenocarcinoma cell line in *in vitro* culture assays and in

xenograft experiments.⁸ Widespread silencing of miRNA expression was proposed to be, at least partly, a consequence of Myc-mediated transcriptional repression;⁹ however, the data also raised the possibility that *DICER1* might be a target of genetic disruption in human cancers. Surprisingly, however, although reduced levels of DICER1 have been reported in tumors,^{10,11} no loss-of-function mutations in *DICER1* have been reported to date. There have, however, been reports of truncating mutations in *TARBP2*, encoding an integral component of a DICER1-containing complex, in sporadic and hereditary carcinomas with microsatellite instability.¹² Frameshift mutations in *TARBP2* diminished TRBP protein expression and caused a partial defect in the processing of miRNAs. Importantly, the TRBP impairment is associated with a partial destabilization of the DICER1 protein. These data raised the possibility that Dicer downregulation rather than its complete loss of function is selected for during tumorigenesis.

To directly address this question, we manipulated *Dicer1* (referred hereafter as *Dic*) gene dosage in a mouse model of retinoblastoma. As germline inactivation of *Dic* in mice causes an early embryonic lethal phenotype,¹³ we specifically inactivated *Dic* in retinoblasts by combining a conditional floxed allele of *Dic*¹⁴ with the retinal *Chx10Cre* transgenic line.¹⁵ We chose this genetic model system for two main reasons. First, *Chx10Cre*-mediated inactivation of one *Dic* allele decreases the production of mature miRNAs without affecting retinogenesis.¹⁶ Second, the *Chx10Cre* mice have

¹Laboratory for Molecular Cancer Biology, VIB-UGent, Ghent B9052, Belgium; ²Center for Medical Genetics, Ghent University Hospital, Ghent B9000, Belgium and

³Department of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA

*Corresponding author: J-C Marine, Laboratory for Molecular Cancer Biology, Flanders Interuniversity Institute for Biotechnology, 927 Technologiepark, Ghent B-9052, Belgium. Tel: + 32 09 33 13640; Fax: + 32 09 33 13516; E-mail: chris.marine@dmb.ugent.be

Keywords: Dicer; microRNA; retinoblastoma; tumor suppressor; haploinsufficiency

Abbreviations: miRNA, microRNA

Received 13.11.09; accepted 23.11.09; Edited by G Melino; published online 18.12.09

been used to create the first preclinical mouse model of retinoblastoma.^{17,18} *Chx10Cre*-mediated inactivation of the *Retinoblastoma* (*Rb*) gene leads to inappropriate exit from the cell cycle of retinal progenitor cells and a block in rod maturation.¹⁹ These mice do not develop retinoblastoma as expression of another Rb family member, p107, increases in a compensatory manner.¹⁸ However, on a p107-null background, retinal Rb inactivation (*Chx10Cre; Rb^{lox/lox} p107^{-/-}*) leads to the formation of early hyperproliferative lesions,¹⁷ which are often referred to as retinomas.²⁰ Importantly, these lesions rarely go on to become aggressive and invasive tumors and only do so in older animals, suggesting that additional oncogenic lesions are required for full-blown tumorigenesis. For example, conditional inactivation of p53 in mice is one mechanism through which these lesions can progress into aggressive retinoblastoma.¹⁷ Accordingly, amplification and overexpression of MDMX, a key negative regulator of p53, is a frequent event that is selected during human retinoblastoma formation.^{21,22}

Results

We first confirmed that specific loss of one *Dic* allele in retinal progenitor cells does not affect normal retinal development. As expected, histological analysis of the retinae of several *Chx10Cre; Dic^{lox/+}* mice at various stages of postnatal development did not reveal any morphological abnormalities (Figure 1). Strikingly, however, on the retinoblastoma-sensitized background, loss of one *Dic* allele dramatically accelerated tumor formation (Figure 2a). Virtually all *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* mice develop aggressive and invasive intraocular retinoblastoma (Figure 2b and c), with an average time to visible tumors of 180 days (Figure 2a). Half of these mice had anterior chamber invasion that was clearly visible by 10 weeks of age. By contrast, *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{+/+}* developed retinoblastoma only with slow and inconsistent kinetics (Figure 2a). Moreover, although more than 30% of *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* mice had developed bilateral retinoblastoma with clear evidence of

anterior chamber invasion, *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{+/+}* mice only ever developed unilateral tumors (data not shown). Finally, metastatic tumors that had invaded local tissues outside the eye through the optic nerve were only observed in *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* mice (data not shown).

We next examined the retinae histologically at postnatal days P35 and onwards. As previously described,¹⁸ the retinal cytoarchitecture of *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{+/+}* at P35 is slightly disrupted because of the focal expansion of immature cells from the inner nuclear layer (INL) and their protrusion through the outer plexiform layer (OPL) (Figure 1c). In addition, defects in the maturation of the rod photoreceptors lead to a hypocellular outer nuclear layer (ONL), although the three nuclear layers are still detected in these mice. In contrast, the three nuclear layers can no longer be distinguished in the *Dic* heterozygous mutants (Figure 2c). The laminar organization in *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* retinae was focally disrupted severely, with immature cells from the INL invading the OPL and extending up to the apical surface of the retina. This resulted in the disruption of the interaction between photoreceptor outer segments and the retinal pigment epithelium (RPE) and focally to dramatic ONL hypocellularity, presumably as a consequence of photoreceptor cell death. The focal nature of the phenotype is consistent with the previously reported mosaic expression pattern of Cre in *Chx10Cre* transgenic mice.^{15,23} Beyond P35, larger dysplastic lesions, found mainly at the periphery (6/7 eyes analyzed), seeded the vitreous (Figure 2c, P60) and eventually invaded the anterior chamber of the eye (Figure 2c, P200). The lesions contained Homer–Wright rosettes (Figure 2c), which consist of a radial arrangement of cells around a central tangle of neuronal processes. Interestingly, these histological structures are often found in a subset of human retinoblastomas.²⁴

This histopathological analysis indicated that the phenotype observed in *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* retinae is similar in nature but is significantly more severe than that observed in *Chx10Cre; Rb^{lox/lox} p107^{-/-}* retinae. We therefore hypothesized that the dysplasia and the partial degeneration observed in *Dic* heterozygous retinae might result from severe expansion of a pool of retinal progenitor cells that normally reside in the INL. Accordingly, immunostaining showed that the cells that disrupted synaptogenesis in the OPL and extended all the way to the apical surface of the retinae expressed the progenitor cell markers Syntaxin and Chx10^{25–27} (Figure 3). The early tumors also stained for Calretinin, which labels a subset of amacrine and ganglion cells. However, Calretinin expression was less abundant than Syntaxin, more scattered and variable from animal to animal (data not shown). Calbindin, which labels horizontal cells and a subset of amacrine cells weakly, was either expressed at very low levels or undetectable in *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* early lesions (data not shown). Together, these data indicate that the lesions are composed of retinal progenitor cells biased toward the amacrine cell fate. Importantly, these immature cells were GFP-positive and therefore Cre-positive (Figure 3). Cre was fused to GFP in the *Chx10Cre* transgenic mice,¹⁵ so that Cre-positive cells can be identified using anti-GFP antibodies.

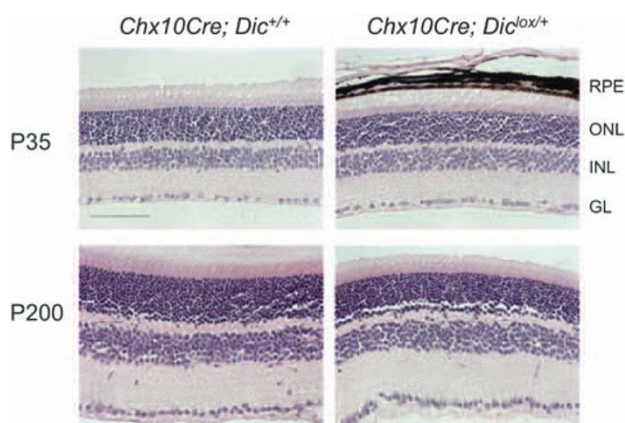


Figure 1 Loss of one copy of *Dicer1* does not affect retinogenesis. Hematoxylin–eosin stain of P35 and P200 retinae show normal retinal lamination in *Chx10Cre; Dic^{lox/+}* mice. Abbreviations: ONL, outer nuclear layer; RPE, retinal pigment epithelium; INL, inner nuclear layer; GL, ganglion layer. Scale bars = 40 μ m

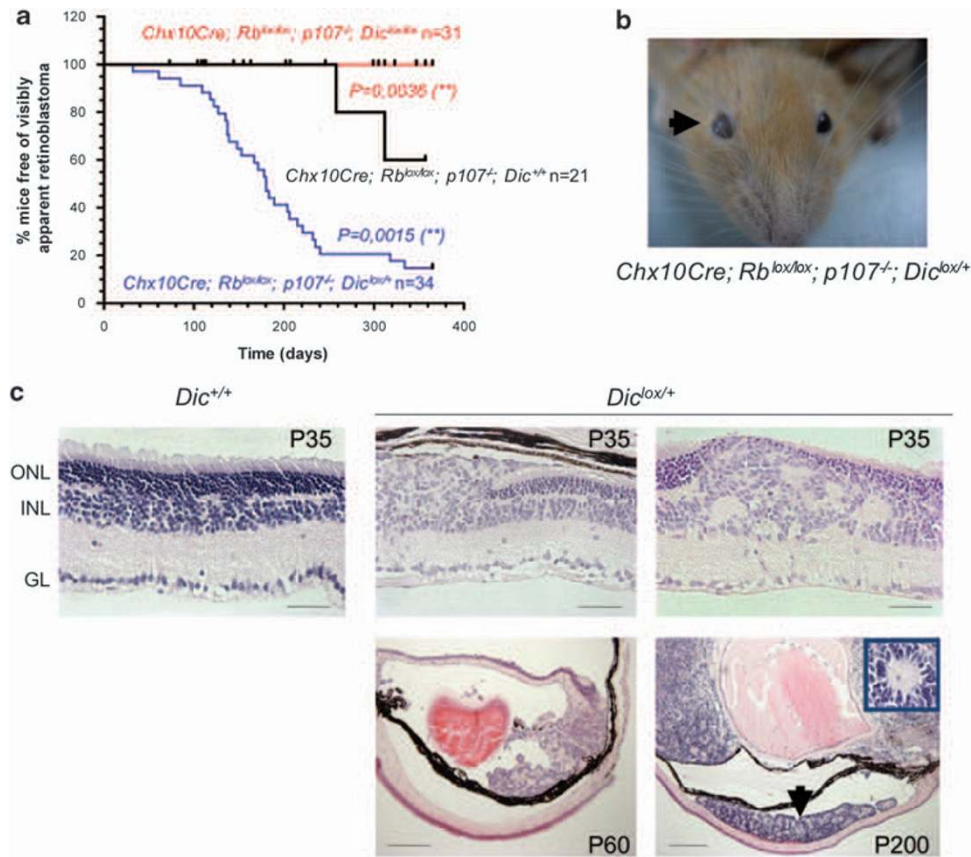


Figure 2 Dicer1 (*Dic*) heterozygosity enhances tumorigenesis on a retinoblastoma-sensitized background. (a) Kaplan–Meier curve showing the time to first observation of externally visible retinoblastoma. This time was respectively markedly decreased in *Chx10Cre; Rb^{lox/lox}p107^{-/-}; Dic^{lox/+}* mice and increased in *Chx10Cre; Rb^{lox/lox}p107^{-/-}; Dic^{lox/lox}* mice (log rank test, $P=0.0015$ and 0.0036) relative to *Chx10Cre; Rb^{lox/lox}p107^{-/-}; Dic^{+/+}* littermates. (b) A 3-month-old *Chx10Cre; Rb^{lox/lox}p107^{-/-}; Dic^{lox/+}* mouse with aggressive retinoblastoma. (c) Hematoxylin and eosin stain with the three retinal nuclear layers (GL: ganglion layer; INL: inner nuclear layer; ONL: outer nuclear layer) indicated. Early retinoblastoma lesions at P35 and invasive tumors seeding the vitreous at P60 are found in *Chx10Cre; Rb^{lox/lox}p107^{-/-}; Dic^{lox/+}* mice. P200 shows late stage retinoblastoma that had filled the vitreous and the anterior chambers (arrow). Inset: a representative Homer–Wright rosette found in *Chx10Cre; Rb^{lox/lox}p107^{-/-}; Dic^{lox/+}* tumors. Scale bars in the top panels = 40 and = 200 μ m in the lower panels

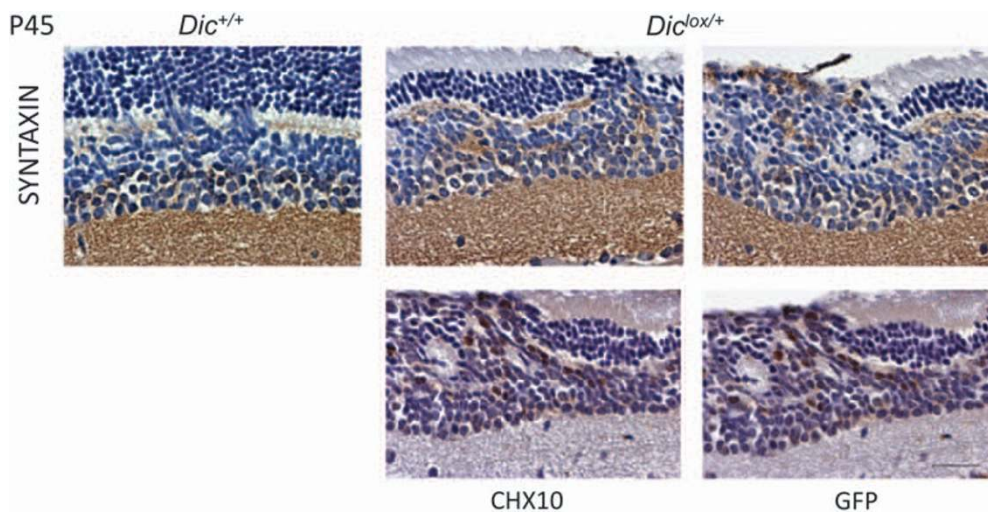


Figure 3 Immunostaining of the *Chx10Cre; Rb^{lox/lox}p107^{-/-}; Dic^{lox/+}* retinoblastoma lesions. Syntaxin, *Chx10* and GFP immunostaining of *Chx10Cre; Rb^{lox/lox}p107^{-/-}; Dic^{lox/+}* (*Dic^{lox/+}*) and *Chx10Cre; Rb^{lox/lox}p107^{-/-}; Dic^{+/+}* (*Dic^{+/+}*) retinae at P45

As inappropriate expansion of the immature retinal cells could be a consequence of increased cell proliferation and/or survival, we measured cell proliferation and apoptosis using immunohistochemical assays. As previously reported, we found extensive BrdU incorporation in *Chx10Cre; Rb^{lox/lox} p107^{-/-}* retinæ at P14, a time when retinogenesis is normally complete. This phenotype was significantly exacerbated in *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dicer^{lox/+}* retinæ (Figure 4a and b). Importantly, BrdU-positive cells were

localized to the regions containing the GFP immunopositive cells and to where lamination was disrupted. These regions were also strongly positive for the proliferation marker Ki67 (Figure 4a and c). BrdU- and Ki67-positive cells were found in all early dysplastic lesions and late (i.e., P200) retinoblastoma tumors (data not shown). Inappropriate cell proliferation leads to increased apoptosis in *Chx10Cre; Rb^{lox/lox} p107^{-/-}* retinæ.¹⁷ Accordingly, active caspase-3-positive cells could be detected in the *Chx10Cre; Rb^{lox/lox} p107^{-/-}* retinæ

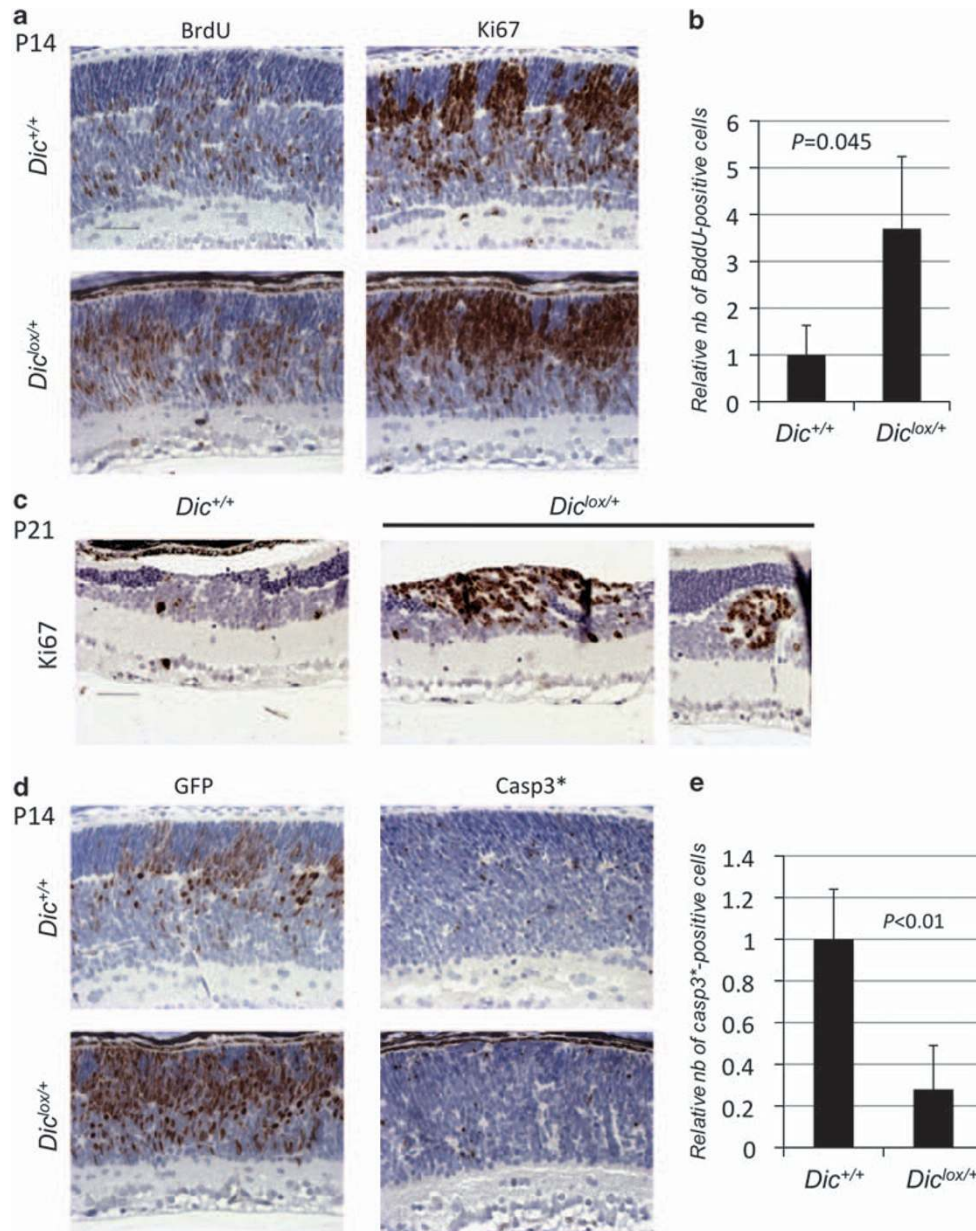


Figure 4 Proliferation and apoptosis in *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dicer^{lox/+}* (*Dicer^{lox/+}*) and *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dicer^{+/+}* (*Dicer^{+/+}*) at P14 and P21. (a, c and d) BrdU incorporation, Ki67 and active Caspase3 immunostaining. Scale bars = 40 μ m. Quantification of BrdU (b) and Caspase3 (c) staining was performed on horizontal serial sections at the optic nerve level. (b) The number (nb) of BrdU-positive cells were normalized to the nb in *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dicer^{+/+}* (*Dicer^{+/+}*), which was set at 1. (e) The number of immunoreactive cells to active Caspase3 staining were normalized to the number of GFP-positive cells in serial consecutive sections. The data are presented relative to the observed ratio of GFP/Caspase3 in *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dicer^{+/+}* (*Dicer^{+/+}*), which was set at 1. Error bars represent standard deviation. *P*-values (Student's *t*-test; *N*=3)

(Figure 4d). There was no significant decrease *per se* in the number of cleaved caspase-3-positive cells in the *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* retinæ. However, as the number of GFP-positive cells is increased in these retinæ compared with the *Chx10Cre; Rb^{lox/lox} p107^{-/-}* retinæ, the data indicate that the number of dying Cre-positive cells is reduced proportionally in the *Dicer1* heterozygous retinæ (Figure 4d and e).

Collectively, these data indicate that heterozygosity for a *Dicer1* mutation promotes the switch from benign retinoma lesions to aggressive and invasive retinoblastomas. Mono-allelic loss of *Dic* is sufficient to promote the expansion of retinal progenitor cells (with a bias toward the amacrine cell fate), which ultimately leads to the formation of early neoplastic lesions that progress into aggressive and metastatic tumors. The ability of these progenitor cells to form

these aggressive tumors seems to be a consequence of both increased cell proliferation potential and resistance to apoptosis.

These data strongly support the view that *Dicer1* functions *in vivo* as a haploinsufficient tumor suppressor gene. Considering that the dysplastic lesions in *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* retinæ are observed as early as P35, it is very unlikely that active selection against complete loss of the remaining wild-type *Dic* allele is required for tumorigenesis. However, this possibility had to be formally excluded experimentally. As expected, we obtained evidence of Cre-mediated recombination of the conditional *Dic* allele in *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* normal retinæ (Ctr Retinæ, P20) and isolated tumors (Figure 5a and b). Importantly, the wild-type allele was retained in all tumors carefully dissected and genotyped (Figure 5b). To ensure that

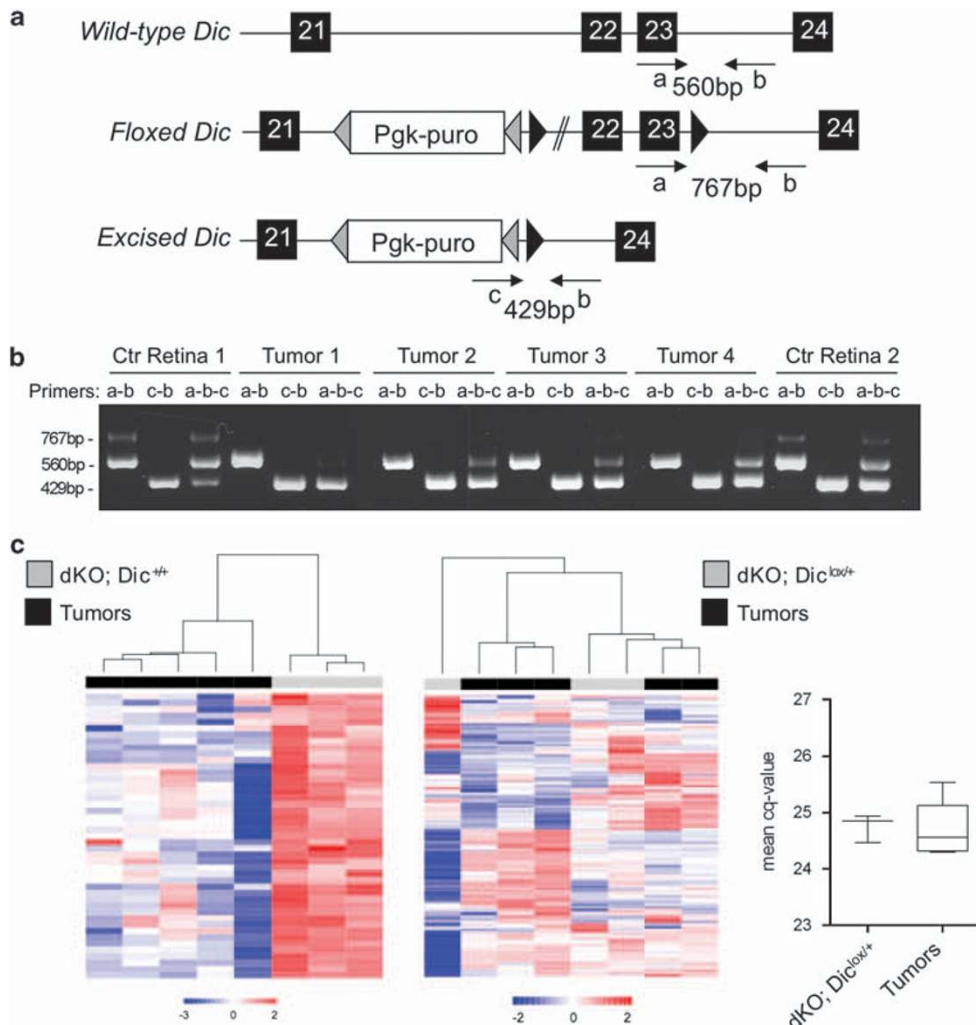


Figure 5 *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* retinoblastoma tumors retain a wild-type and functional *Dic* allele. (a) Schematic representation of the *Dic* wild-type, floxed and Cre-excised alleles. (b) DNA was prepared from two *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* P20 retinæ (Ctr Retina) and 10 isolated tumors (results from 4 different tumors are shown) and examined by PCR using the primers depicted in the top panel. (c) RT-qPCR analyses of mature miRNA expression. Left panel: hierarchical clustering of miRNAs significantly downregulated in five *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* tumors (black, dKO; *Dic^{lox/+}*) compared with *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{+/+}* P20 retinæ (gray, dKO; *Dic^{+/+}*). Middle panel: heat map of all miRNAs expressed at higher levels than the mean in *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* P20 retinæ and tumors. Right panel shows the mean cq values for all miRNAs analyzed (522 different murine miRNAs) in both *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* P20 retinæ and tumors

the remaining *Dic* allele was not functionally inactivated through mutations, we assessed the expression levels of all mature miRNAs in five different *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* tumors by RT-qPCR analysis. Consistent with a decrease of Dicer1 function, we observed a global decrease in steady-state miRNA levels in all five tumors analyzed compared with the levels in *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{+/+}* (Figure 5c, left panel). Importantly, all mature miRNAs that are expressed at high levels in *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* P20 retinæ are expressed at comparable levels in all five tumors (Figure 5c, right panels). These analyses indicate that microRNA processing is only partly impaired, but not completely disrupted, in these tumors. Finally, we directly assessed the consequences of complete Dicer1 inactivation in the retinoblastoma mouse model. We confirmed that complete ablation of Dicer1 in the retina is well tolerated and only leads to progressive retinal degeneration.¹⁶ Similar results were obtained on the retinoblastoma-sensitized background. The retinæ of adult *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/lox}* were partly dis-organized (Lambertz *et al.*, manuscript in preparation). Surprisingly, these mice were protected from tumor formation (Figure 2a). Together, the data argue that although partial loss of Dicer function favors tumor formation, complete loss of Dicer is deleterious to retinoblastoma development, further arguing in favor of a haploinsufficient tumor suppressor function of Dicer1.

These data also imply that a set of miRNAs, the function of which requires Dicer1 function, might act to prevent full-blown retinoblastoma formation on the *Chx10Cre; Rb^{lox/lox} p107^{-/-}* background. To identify such candidate tumor suppressor miRNAs, we determined the expression profile of the entire miRNome in P20 retinæ from wild-type (Cre-negative), *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{+/+}* and *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{lox/+}* using LNA-based microarray and RT-qPCR approaches. Both types of analyses identified a common set of 11 miRNAs that are consistently upregulated between wild-type and *Chx10Cre; Rb^{lox/lox} p107^{-/-}; Dic^{+/+}* (Figure 6a). Most interestingly, among them are two members of the let-7 family (let-7c and let-7i), the upregulation of which and of another let-7 member, let-7b, was confirmed by independent Q-RT-PCR analysis (Figure 6b). Given that this upregulation was significantly attenuated in the *Dic* heterozygous retinæ and the recognized role of let-7 family members in tumor suppression,²⁸ this observation raises the possibility that let-7 family members have a causal role in retinoblastoma formation as critical regulators of the switch from retinomas to retinoblastoma. Our list of 11 differentially expressed miRNAs also included miR-34c (Figure 6a and b). There was also a clear upregulation of miR-34b-3p in both microarray and RT-q-PCR analyses, and a moderate, but reproducible, upregulation of miR-34a was evident from the microarray data (data not shown). Interestingly, the miR-34 family members have been identified as p53 targets and key mediators of its tumor suppressor function.²⁹

Discussion

To address the importance of *Dicer1* gene dosage in cancer development, we used a preclinical mouse model of retino-

blastoma.¹⁷ Our data provide clear genetic evidence that Dicer1 functions as a haploinsufficient tumor suppressor *in vivo*. In keeping with this observation, information in the public domain, for example, Cancer Genome Project at the Sanger Institute,³⁰ indicates that hemizygous deletions of *DICER1* occur in 27% (207/761) of tumors derived from tissues of diverse origins, such as the central nervous system, lung, pancreas, soft tissues, breast, bone, hematopoietic or lymphoid. Importantly, consistent with our findings, homozygous deletions have never been observed in any of these 761 tumors. Very recently, heterozygous point mutations in *DICER1* were reported in patients with pleuropulmonary blastoma.³¹ However, *DICER1* expression was retained in the mesenchymal tumor cells from these patients, again arguing against strong selective pressure for complete loss of Dicer function in human tumors. Thus, although this study focuses on the role of Dicer1 in a mouse model of retinoblastoma, there is evidence supporting a broad role for *DICER1* as a haploinsufficient tumor suppressor in human cancer.

Kumar *et al.* had attempted to investigate an etiological role for Dicer1 in a mouse model of cancer, in which *Dicer1* was inactivated in a K-Ras-induced mouse model of lung cancer using intranasal infection with adenovirus expressing Cre.⁸ Although it highlighted an important role for *Dicer1* in tumor suppression, this elegant study did not resolve the important issue of gene dosage. The authors had shown that tumorigenesis was enhanced on both *Dic^{lox/+}* and *Dic^{lox/lox}* genetic backgrounds, but they did not analyze the extent of Cre-mediated *Dicer1* inactivation in these tumors. It therefore remained formally possible that lung tumors developed in the *Dic^{lox/lox}* mice only as a result of incomplete inactivation of Dicer1 function. This possibility has just very recently been confirmed in a study that was published while our manuscript was being processed.³² This last report, together with the data presented herein, clearly emphasizes that only partial, rather than complete, inactivation of Dicer1 enhances tumorigenesis *in vivo* (Figure 7).

It is generally accepted that complete loss of tumor suppressor function through mutations and loss of heterozygosity is a prerequisite for tumor development. To date, however, several haploinsufficient tumor suppressor genes have been identified,³³ mostly through the use of genetically engineered mouse models. Our mouse genetic data identify *DICER1* as yet another member of this group. A general theme in haploinsufficient genes is that tumors generated in a haploinsufficient context are frequently of later onset and less severe than the corresponding tumor carrying the homozygously mutated gene. This is not the case for *Dicer1*. Complete loss of *Dicer1* did not enhance but rather inhibited retinoblastoma formation. In agreement with this, biallelic loss of *Dicer1* was strongly selected against in K-Ras-induced lung tumors.³² To our knowledge, *Dicer1* is therefore the first example of a gene that functions as a tumor suppressor only in the context of haploinsufficiency and not on homozygous loss of function. Regardless, as the list of haploinsufficient tumor suppressors grows, hemizygous deletions at loci such as *DICER1* should be considered as potential key pro-tumorigenic lesions.

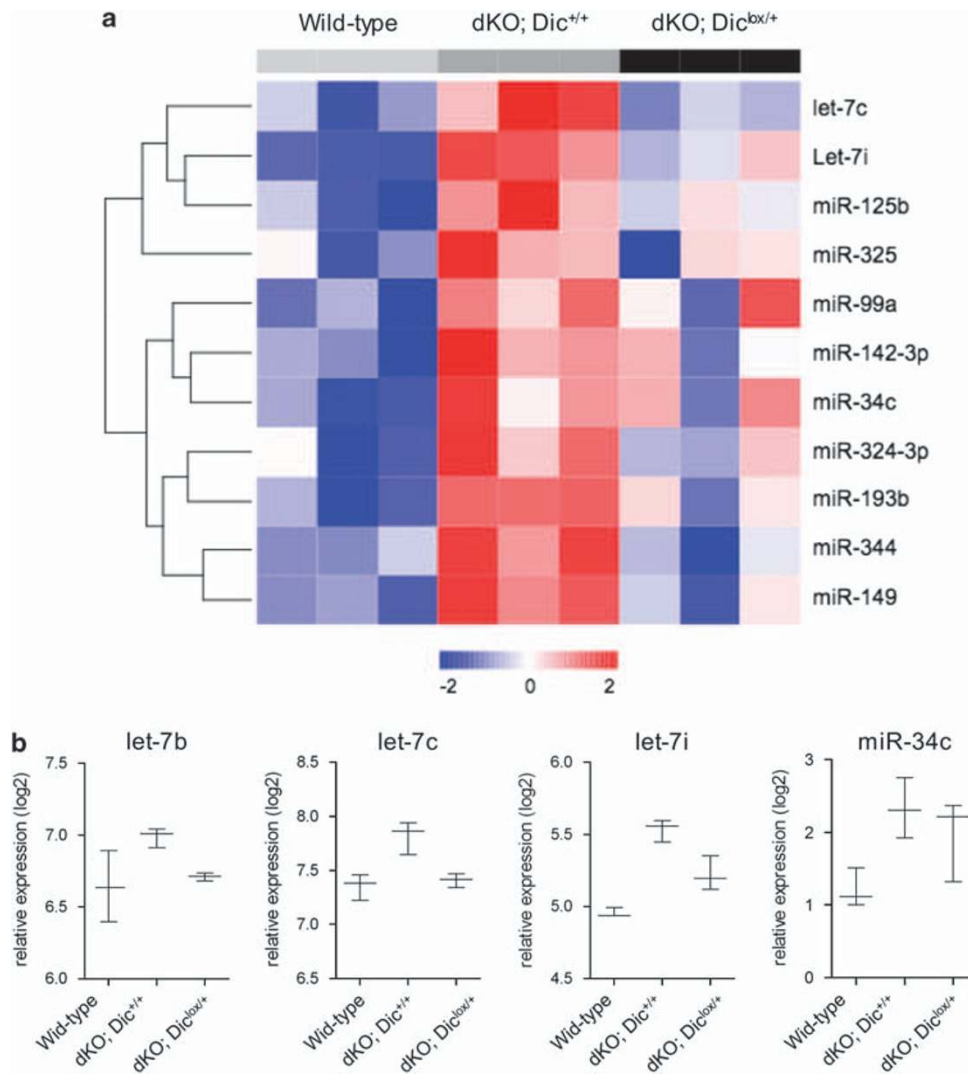


Figure 6 Search for putative tumor suppressor miRNAs in retinoblastoma. (a) Heat map of selected differentially expressed miRNAs in *Chx10Cre*-negative mice (light gray, wild-type), *Chx10Cre; Rb^{lox/lox}p107^{-/-}; Dic^{+/+}* (dKO; *Dic^{+/+}*) and *Chx10Cre; Rb^{lox/lox}p107^{-/-}; Dic^{lox/+}* P20 retinae (black, dKO; *Dic^{lox/+}*). (b) Expression analysis by RT-qPCR of let-7 family members (let-7b, let-7c and let-7i) and miR-34c

Our data further establish a causative link between global downregulation of miRNA-processing and cancer development. It is possible that the reduction of expression of only a subset of tumor suppressor miRNAs is the event that promotes tumorigenesis (Figure 7). In the context of retinoblastoma, our profiling data identified the let-7 family members as potential candidate tumor suppressors. It may be noted that let-7b has previously been shown to be downregulated in human retinoblastoma.³⁴ Other sets of miRNAs that can account for the observed tumor-suppressive activity of *Dicer1* in retinoblastoma are the miR-34 family members. Given that these genes have been identified as p53 targets and are critical mediators of its activity²⁹ and the importance of the p53 tumor suppressor function in retinoblastoma,^{17,21} it will be interesting to further assess genetically their functional relevance in the retinoblastoma mouse model. It has been shown that miR-34 family members are differentially expressed in human retinoblastoma cell

lines and tumors compared with normal retina samples.³⁵ Exogenous miR-34a inhibited cell growth and/or increased apoptosis in retinoblastoma cell lines (Y79, Weri-Rb1).³⁵ Together, these data identify the miR-34 family members as potential therapeutic targets for retinoblastoma. Interestingly, among the common putative mRNA targets of both let-7 and miR-34 family members is the N-myc oncogene. N-myc is indeed a validated miR-34a target (<http://mirecords.umn.edu/miRecords/>) and is a predicted target of let-7i (<http://www.targetscan.org/>). This gene is amplified in another mouse model of retinoblastoma³⁶ and in approximately 10% of human retinoblastomas.³⁷ These observations raise the possibility that members of both let-7 and miR34 families cooperate to restrain N-Myc oncogenic function in retinoblastoma.

Finally and importantly, our data argue against the development of DICER1-inactivating molecules as anticancer drugs. Indeed, even if complete *Dicer1* inactivation seems to

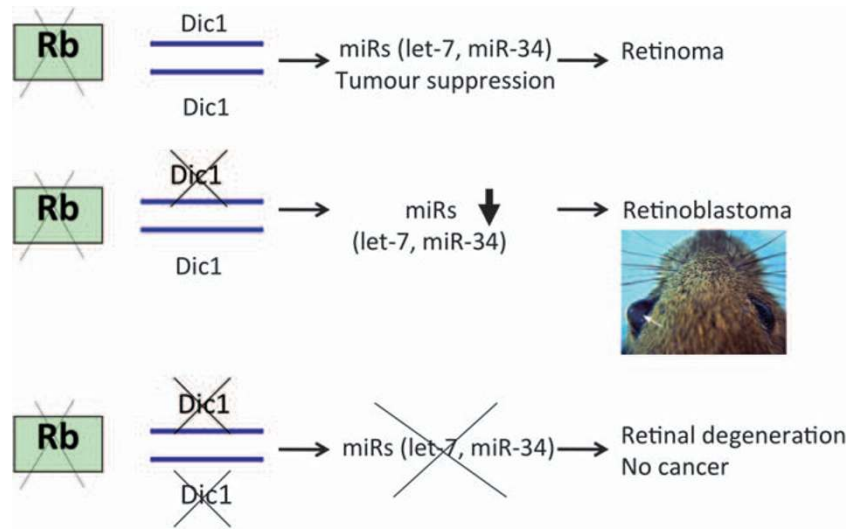


Figure 7 Monoallelic loss of Dicer enhances retinoblastoma formation. Our data support a model in which monoallelic loss of *Dicer1* cooperates with Rb inactivation in the formation of aggressive and invasive retinoblastoma. In contrast, biallelic loss of *Dicer1* leads to retinal degeneration on a retinoblastoma-sensitized background and inhibition of tumor formation

be deleterious for cancer development its partial inactivation promotes, rather than inhibits, tumor formation.

Materials and Methods

Mice. All animal experiments were performed in accordance with the guidelines of the University of Ghent Animal Care and Use Ethics Committee. BrdU (100 μ g/g of body weight) was injected intraperitoneally 1 h before killing.

Immunohistochemistry. Eyes were fixed overnight in 4% paraformaldehyde/PBS, and paraffin embedded. Sections of 5 μ m thickness were immunostained with the following antibodies: GFP (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1/100); BrdU (BD Pharmingen, San Jose, CA, USA, 1/10); Ki-67 (DAKO, Glostrup, Denmark, 1/30); cleaved caspase-3 (Cell Signaling, Danvers, MA, USA, 1/100); syntaxin (Sigma, St Louis, MO, USA, 1/20 000); calretinin (Millipore, Billerica, MA, USA, 1/700); Chx10 (Exalpha Biologicals, Shirley, MA, USA, 1/100).

Recombination analysis. DNA was isolated from dissected retinæ and isolated tumors using the DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA). *Dicer1* recombination was analyzed by PCR using the following primers: primer a 5'-ATTGTTACCAGCGCTTAGAATTCC; primer c 5'-TCG GAATAGGAACCTCGTTTAAAC; and the reverse b primer 5'-GGGAGGT GTACGTCTACAATT. PCR conditions were as follows: 1 \times precycle at 94 $^{\circ}$ C for 3 min and 30 cycles of 94 $^{\circ}$ C, 30 s; 60 $^{\circ}$ C, 30 s; 72 $^{\circ}$ C, 45 s.

microRNA expression analyses. Total RNA was prepared from dissected retinæ or isolated tumors using the miRNeasy kit (Qiagen) according to the manufacturer's instructions. Profiling of these samples was performed by both microarray (miRCURY LNA Array, Exiqon, Vedbaek, Denmark) and qPCR. The RT-qPCR expression profiling of 522 murine miRNAs and subsequent data normalization were performed as described previously.^{38,39} Differential miRNA expression was evaluated using Student's *t*-test. Hierarchical clustering was performed using Ward's method and Manhattan distance using R Bioconductor software (Oxford, UK).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements. We thank Natalie Farla and Rose Van Isacker for their excellent technical assistance. We thank G Hannon for providing the *Dic* floxed mince. J-C Marine received support from the EMBO Young Investigator program. This work was supported in part by Geconcerteerde Onderzoek Aangelegenheden (GOA, University Ghent, Belgium) and EU (FP7 Program, ONCOMIRs, Contract no. 201102). This publication reflects only the authors' views. The commission is not liable for any use that may be made of the information herein.

- Croce CM. Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet* 2009; **10**: 704–714.
- Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009; **136**: 215–233.
- Ventura A, Jacks T. MicroRNAs and cancer: short RNAs go a long way. *Cell* 2009; **136**: 586–591.
- Esquela-Kerscher A, Trang P, Wiggins JF, Patrawala L, Cheng A, Ford L *et al.* The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle* 2008; **7**: 759–764.
- Kumar MS, Erkeland SJ, Pester RE, Chen CY, Ebert MS, Sharp PA *et al.* Suppression of non-small cell lung tumor development by the let-7 microRNA family. *Proc Natl Acad Sci USA* 2008; **105**: 3903–3908.
- Esquela-Kerscher A, Slack FJ. Oncomirs – microRNAs with a role in cancer. *Nat Rev Cancer* 2006; **6**: 259–269.
- Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D *et al.* MicroRNA expression profiles classify human cancers. *Nature* 2005; **435**: 834–838.
- Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nat Genet* 2007; **39**: 673–677.
- Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM *et al.* Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet* 2008; **40**: 43–50.
- Pampalakis G, Diamandis EP, Katsaros D, Sotiropoulou G. Down-regulation of dicer expression in ovarian cancer tissues. *Clin Biochem* 2009; e-pub ahead of print 25 September 2009.
- Karube Y, Tanaka H, Osada H, Tomida S, Tatematsu Y, Yanagisawa K *et al.* Reduced expression of Dicer associated with poor prognosis in lung cancer patients. *Cancer Sci* 2005; **96**: 111–115.
- Melo SA, Ropero S, Moutinho C, Aaltonen LA, Yamamoto H, Calin GA *et al.* A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. *Nat Genet* 2009; **41**: 365–370.
- Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ *et al.* Dicer is essential for mouse development. *Nat Genet* 2003; **35**: 215–217.
- Murchison EP, Partridge JF, Tam OH, Cheloufi S, Hannon GJ. Characterization of Dicer-deficient murine embryonic stem cells. *Proc Natl Acad Sci USA* 2005; **102**: 12135–12140.
- Rowan S, Cepko CL. Genetic analysis of the homeodomain transcription factor Chx10 in the retina using a novel multifunctional BAC transgenic mouse reporter. *Dev Biol* 2004; **271**: 388–402.
- Damiani D, Alexander JJ, O'Rourke JR, McManus M, Jadhav AP, Cepko CL *et al.* Dicer inactivation leads to progressive functional and structural degeneration of the mouse retina. *J Neurosci* 2008; **28**: 4878–4887.

17. Zhang J, Schweers B, Dyer MA. The first knockout mouse model of retinoblastoma. *Cell Cycle* 2004; **3**: 952–959.
18. Donovan SL, Schweers B, Martins R, Johnson D, Dyer MA. Compensation by tumor suppressor genes during retinal development in mice and humans. *BMC Biol* 2006; **4**: 14.
19. Zhang J, Gray J, Wu L, Leone G, Rowan S, Cepko CL *et al.* Rb regulates proliferation and rod photoreceptor development in the mouse retina. *Nat Genet* 2004; **36**: 351–360.
20. DiCiommo D, Gallie BL, Bremner R. Retinoblastoma: the disease, gene and protein provide critical leads to understand cancer. *Semin Cancer Biol* 2000; **10**: 255–269.
21. Laurie NA, Donovan SL, Shih CS, Zhang J, Mills N, Fuller C *et al.* Inactivation of the p53 pathway in retinoblastoma. *Nature* 2006; **444**: 61–66.
22. Marine JC, Dyer MA, Jochemsen AG. MDMX: from bench to bedside. *J Cell Sci* 2007; **120** (Pt 3): 371–378.
23. Dyer MA, Cepko CL. p27Kip1 and p57Kip2 regulate proliferation in distinct retinal progenitor cell populations. *J Neurosci* 2001; **21**: 4259–4271.
24. Yuge K, Nakajima M, Uemura Y, Miki H, Uyama M, Tsubura A. Immunohistochemical features of the human retina and retinoblastoma. *Virchows Arch* 1995; **426**: 571–575.
25. Alexiades MR, Cepko CL. Subsets of retinal progenitors display temporally regulated and distinct biases in the fates of their progeny. *Development* 1997; **124**: 1119–1131.
26. Liu IS, Chen JD, Ploder L, Vidgen D, van der Kooy D, Kalnins VI *et al.* Developmental expression of a novel murine homeobox gene (Chx10): evidence for roles in determination of the neuroretina and inner nuclear layer. *Neuron* 1994; **13**: 377–393.
27. Burmeister M, Novak J, Liang MY, Basu S, Ploder L, Hawes NL *et al.* Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. *Nat Genet* 1996; **12**: 376–384.
28. Bussing I, Slack FJ, Grosshans H. let-7 microRNAs in development, stem cells and cancer. *Trends Mol Med* 2008; **14**: 400–409.
29. He L, He X, Lowe SW, Hannon GJ. microRNAs join the p53 network—another piece in the tumour-suppression puzzle. *Nat Rev Cancer* 2007; **7**: 819–822.
30. Forbes SA, Bhamra G, Bamford S, Dawson E, Kok C, Clements J *et al.* The catalogue of somatic mutations in cancer (COSMIC). *Curr Protoc Hum Genet* 2008; **Chapter 10** Unit 10 11.
31. Hill DA, Ivanovich J, Priest JR, Gurnett CA, Dehner LP, Desruisseau D *et al.* DICER1 mutations in familial pleuropulmonary blastoma. *Science* 2009; **325**: 965.
32. Kumar MS, Pester RE, Chen CY, Lane K, Chin C, Lu J *et al.* Dicer1 functions as a haploinsufficient tumor suppressor. *Genes Dev* 2009; **23**: 2700–2704.
33. Santarosa M, Ashworth A. Haploinsufficiency for tumour suppressor genes: when you don't need to go all the way. *Biochim Biophys Acta* 2004; **1654**: 105–122.
34. Huang JC, Babak T, Corson TW, Chua G, Khan S, Gallie BL *et al.* Using expression profiling data to identify human microRNA targets. *Nat Methods* 2007; **4**: 1045–1049.
35. Dalgard CL, Gonzalez M, deNiro JE, O'Brien JM. Differential microRNA-34a expression and tumor suppressor function in retinoblastoma cells. *Invest Ophthalmol Vis Sci* 2009; **50**: 4542–4551.
36. MacPherson D, Conkrite K, Tam M, Mukai S, Mu D, Jacks T. Murine bilateral retinoblastoma exhibiting rapid-onset, metastatic progression and N-myc gene amplification. *EMBO J* 2007; **26**: 784–794.
37. Lee WH, Murphree AL, Benedict WF. Expression and amplification of the N-myc gene in primary retinoblastoma. *Nature* 1984; **309**: 458–460.
38. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F *et al.* A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol* 2009; **10**: R64.
39. Mestdagh P, Feys T, Bernard N, Guenther S, Chen C, Speleman F *et al.* High-throughput stem-loop RT-qPCR miRNA expression profiling using minute amounts of input RNA. *Nucleic Acids Res* 2008; **36**: e143.