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

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SUMMARY

Human tumors are characterized by widespread reduction in microRNA (miRNA) expression [1], 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 [2]. A defect in miRNA-processing is one possible mechanism for the global down-regulation. To explore this possibility in more detail *in vivo* we have manipulated *Dicer1* gene dosage in a mouse model of retinoblastoma. We show that while monoallelic loss of *Dicer1* does not affect normal retinal development it dramatically accelerates tumor formation on a retinoblastoma-sensitized background. Importantly, these tumors retain one wild-type *Dicer1* allele and exhibit only partial decrease in miRNA-processing. Accordingly, *in silico* analysis of human cancer genome data reveals frequent hemizygous, but not homozygous, deletions of *DICER1*. Strikingly, complete loss of *Dicer1* function in mice did not accelerate retinoblastoma formation. miRNA profiling of these tumors identified members of the let-7 and miR-34 families as candidate tumor suppressors in retinoblastoma. We conclude that *Dicer1* functions as a haploinsufficient tumor suppressor. This finding has implications for cancer aetiology and cancer therapy.

Keywords

Dicer; microRNA; retinoblastoma; tumor suppressor; haploinsufficiency

Introduction

A large body of evidence indicates that alterations in the expression of miRNAs contribute to cancer pathologies [3]. miRNAs act as agents of the RNA interference pathway to silence their cognate coding target genes either by cleaving mRNA molecules or inhibiting their translation [4]. By silencing tumor suppressive and oncogenic mRNAs, miRNAs themselves can function as oncogenes or tumor suppressors, respectively [5]. The let-7 family, for instance, limit lung tumorigenesis through inhibition of several oncogenes including members of the Ras family or HMGA2 [6,7]

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Conflicts of interest

The authors declare no conflict of interest

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 characteristic miRNA signatures of human cancers [8]. Surprisingly, they also highlighted an overall down-regulation of mature miRNAs in several types of mouse and human cancer [1]. This observation raised two important questions: (i) one of causality - is there a causative link between global down-regulation of miRNAs and cancer progression or is the down-regulation a byproduct of tumor development? (ii) and 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, demonstrating that global down-regulation of miRNA-processing increased the transforming properties of a lung adenocarcinoma cell line in *in vitro* culture assays and in xenograft experiments [2]. Widespread silencing of miR 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 in tumors have been reported [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 cause 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 down-regulation rather than its complete loss of function is selected for during tumorigenesis.

In order to directly address this question, we manipulated *Dicer1* (referred thereafter 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 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 for 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 retinæ of several *Chx10Cre; Dic^{lox/+}* mice at various stages of postnatal development did not reveal any morphological abnormalities (Supplemental Figure S1). Strikingly, however, on the retinoblastoma-sensitized background, loss of one *Dic* allele dramatically accelerated tumor formation (Figure 1A). Virtually all *Chx10Cre; Rb^{lox/lox}; p107^{-/-}; Dic^{lox/+}* mice develop aggressive and invasive intraocular retinoblastoma (Figure 1 B and 1C), with an average time to visible tumors of 180

days (Figure 1A). 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 1A). Moreover, while 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 of the eye through the optic nerve were only observed in *Chx10Cre; Rb^{lox/lox}; p107^{-/-}; Dic^{lox/+}* mice (data not shown).

We next examined retinæ 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 due to focal expansion of immature cells from the inner nuclear layer (INL) and their protrusion through the outer plexiform layer (OPL) (Figure 1C). Additionally, 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 1C). The laminar organization in *Chx10Cre; Rb^{lox/lox}; p107^{-/-}; Dic^{lox/+}* retinæ was focally severely disrupted, 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 1c, P60) and eventually invaded the anterior chamber of the eye (Figure 1C, P200). The lesions contained Homer-Wright rosettes (Figure 1C), 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 retinoblastoma [24].

This histopathological analysis indicated that the phenotype observed in *Chx10Cre; Rb^{lox/lox}; p107^{-/-}; Dic^{lox/+}* retinæ is similar in nature but significantly more severe than that observed in *Chx10Cre; Rb^{lox/lox}; p107^{-/-}* retinæ. We therefore hypothesized that the dysplasia and the partial degeneration observed in *Dic* heterozygous retinæ 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 retinæ expressed the progenitor cell markers Syntaxin and Chx10 [25–27] (Figure 2A, P45). 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 towards the amacrine cell fate. Importantly, these immature cells were GFP-positive and therefore Cre-positive (Figure 2B). Cre was fused to GFP in the *Chx10Cre* transgenic mice [15] so that Cre-positive cells can be identified using anti-GFP-antibodies.

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^{-/-}; Dic^{lox/+}* retinæ (Figure 2B). 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 2B, P14 and P21). 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^{-/-}* retinoblastoma [17]. Accordingly, active-caspase-3-positive cells could be detected in the *Chx10Cre; Rb^{lox/lox}; p107^{-/-}* retinoblastoma (Figure 2D). 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/+}* retinoblastoma (Figure 2D and 2E). However, as the number of GFP-positive cells is increased in these retinoblastoma compared to the *Chx10Cre; Rb^{lox/lox}; p107^{-/-}* retinoblastoma, the data indicate that proportionally the number of dying Cre-positive cells is reduced in the Dicer heterozygous retinoblastoma.

Collectively, these data indicate that heterozygosity for a *Dicer1* mutation promotes the switch from benign retinoma lesions to aggressive and invasive retinoblastoma. Monoallelic loss of *Dic* is sufficient to promote the expansion of retinal progenitor cells (with a bias towards 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 appears 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/+}* retinoblastoma 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 obtain evidence of Cre-mediated recombination of the conditional *Dic* allele in *Chx10Cre; Rb^{lox/lox}; p107^{-/-}; Dic^{lox/+}* normal retinae (Ctr Retinae, P20) and isolated tumors (Figure 3A). Importantly, the wild-type allele was retained in all tumors carefully dissected and genotyped (Figure 3A). Western blotting was further used to confirm that Dicer1 expression is retained in *Chx10Cre; Rb^{lox/lox}; p107^{-/-}; Dic^{lox/+}* tumors (data not shown). To ensure that the remaining *Dic* allele is 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 to the levels in *Chx10Cre; Rb^{lox/lox}; p107^{-/-}; Dic^{+/+}* (Figure 3C, left panel). Importantly, all mature miRNAs that are expressed at high levels in *Chx10Cre; Rb^{lox/lox}; p107^{-/-}; Dic^{lox/+}* P20 retinae are expressed at comparable levels in all 5 tumors (Figure 3C, 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. While we confirmed that complete ablation of Dicer is well tolerated and only leads to progressive retinal degeneration [16] our data indicate that it dramatically affects retinal formation on the retinoblastoma-sensitized background. The retina of *Chx10Cre; Rb^{lox/lox}; p107^{-/-}; Dic^{lox/lox}* is completely disorganized as early as P10 and completely degenerates soon after (Lambertz et al., manuscript in preparation). Consequently these mice are entirely protected from tumor formation (Figure 1A) but are at the same time completely blind. Together the data argue that while 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 retinae 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 up-regulated between wild-type and *Chx10Cre; Rb^{lox/lox}; p107^{-/-}; Dic^{+/+}* (Figure 4A). Most interestingly, among them are 2 members of the let-7 family (let-7c and let7-i), the up-regulation of which and of another let-7 member, let-7b, was confirmed by independent Q-RT-PCR analysis (Figure 4B). Given that this up-regulation 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 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 4A and 4B). There was also a clear upregulation of miR-34b-3p in both microarray and RT-q-PCR analyses and a moderate, but reproducible, up-regulation of miR-34a was evident from the micro-array data (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

In order to address the importance of *Dicer1* gene dosage in cancer development we used a preclinical mouse model of retinoblastoma [17]. Our data provide clear genetic evidence that *Dicer1* function as a haploinsufficient tumor suppressor *in vivo*. In keeping with this observation, information in the public domain, e.g., 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 central nervous system, lung, pancreas, soft tissues, breast, bone, haematopoietic 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, while the present study focuses on role of *Dicer* 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. 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 [2]. 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 showed 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 is therefore still formally possible that lung tumors developed in the *Dic^{lox/lox}* mice only as a result of incomplete inactivation of *Dicer* function. Data from our retinoblastoma model clearly emphasize that only partial, rather than complete, inactivation of this process enhances tumorigenesis *in vivo* (Figure 5).

It is generally accepted that complete loss of tumor suppressor function through mutations and loss of heterozygosity is a pre-requisite for tumor development. To date, only a very small number of haploinsufficient tumor suppressor genes have been identified [32], mostly through the use of genetically-engineered mouse models. Our mouse genetic data identify *DICER1* as another member of this group. As the list of haploinsufficient tumor suppressors grows hemizygous deletions at loci such as *DICER1* should be considered as potential key pro-tumorigenic lesions.

Our data further establish a causative link between global down-regulation 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 5). In

the context of retinoblastoma, our profiling data identified the let-7 family members as potential candidate tumor suppressors. Another 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 activity [29] 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. Of note, it has been shown that miR-34 family members are differentially expressed in human retinoblastoma cell lines and tumors compared to normal retina samples [33]. Exogenous miR-34a inhibited cell growth and/or increased apoptosis in retinoblastoma cells lines (Y73, Weri-Rb1) [33]. 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 retinoblastoma [34] and in approximately 10% of human retinoblastomas [35]. 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 anti-cancer drugs. Indeed, even if complete Dicer inactivation seems to be deleterious for cancer development partial inactivation, at least in the context of retinoblastoma, promotes rather than inhibits tumor formation.

Materials and Methods

Mice

All animal experiments were performed in accordance with the guidelines of the University of Gent 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, 1/10); Ki-67 (DAKO, 1/30); cleaved caspase-3 (Cell Signaling, 1/100); syntaxin (Sigma, 1/20000); calretinin (Millipore, 1/700); Chx10 (Exalpha Biologicals, 1/100).

Recombination analysis

DNA was isolated from dissected retina and isolated tumors 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. PCR conditions were as follow: 1x precycle at 94°C for 3min and 30cycles of 94°C, 30sec; 60°C, 30sec; 72°C, 45sec.

microRNA Expression analyses

Total RNA was prepared from dissected retina or isolated tumors using the miRNeasy kit (Qiagen) according to the manufacturer's instructions. Profiling of these samples was performed by both micro-array (miRCURY LNA Array, Exiqon) and qPCR. The RT-qPCR expression profiling of 522 murine miRNAs and subsequent data normalization were performed as described previously[36, 37]. Differential miRNA expression was evaluated using Student's t-test. Hierarchical clustering was performed with method Ward and distance Manhattan using R Bioconductor software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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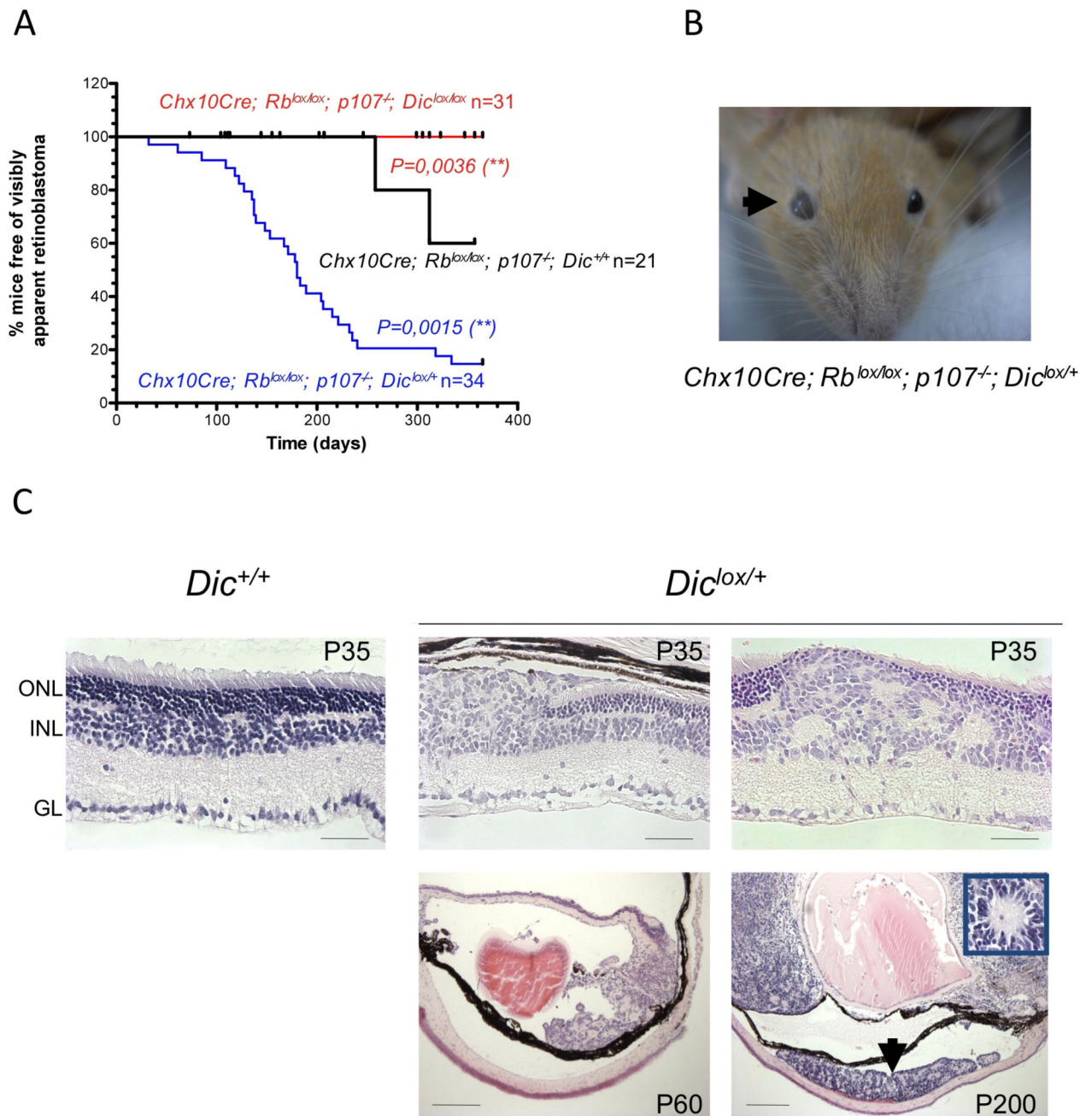


Figure 1. *Dicer1* (*Dic*) heterozygosity enhances tumorigenesis on a retinoblastoma-sensitized background

(A) Kaplan-Meier curve showing the time to first observation of externally visible retinoblastoma. This time was respectively markedly decrease 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 $P=0,0036$) relative to *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{+/+}* littermates. (B) A 3 months-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 chamber (arrow). inset: a representative Homer-Wright rosette found in *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{lox/+}* tumors. Scale bars in the top panels = 40 μ m and = 200 μ m in the lower panels.

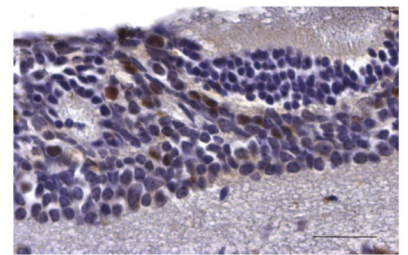
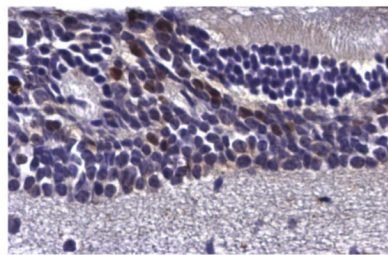
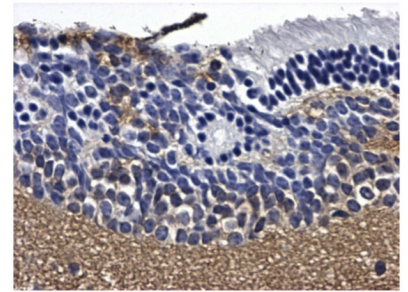
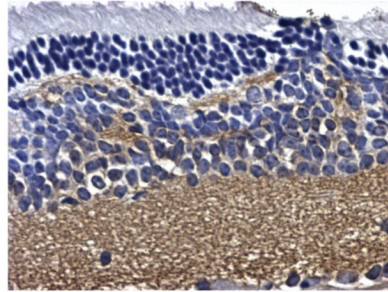
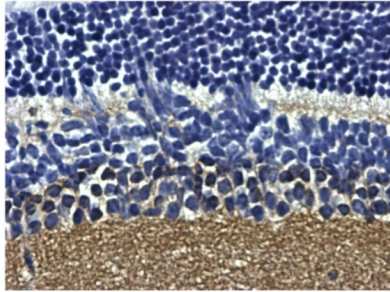
A

P45

Dic^{+/+}

Dic^{lox/+}

SYNTAXIN



CHX10

GFP

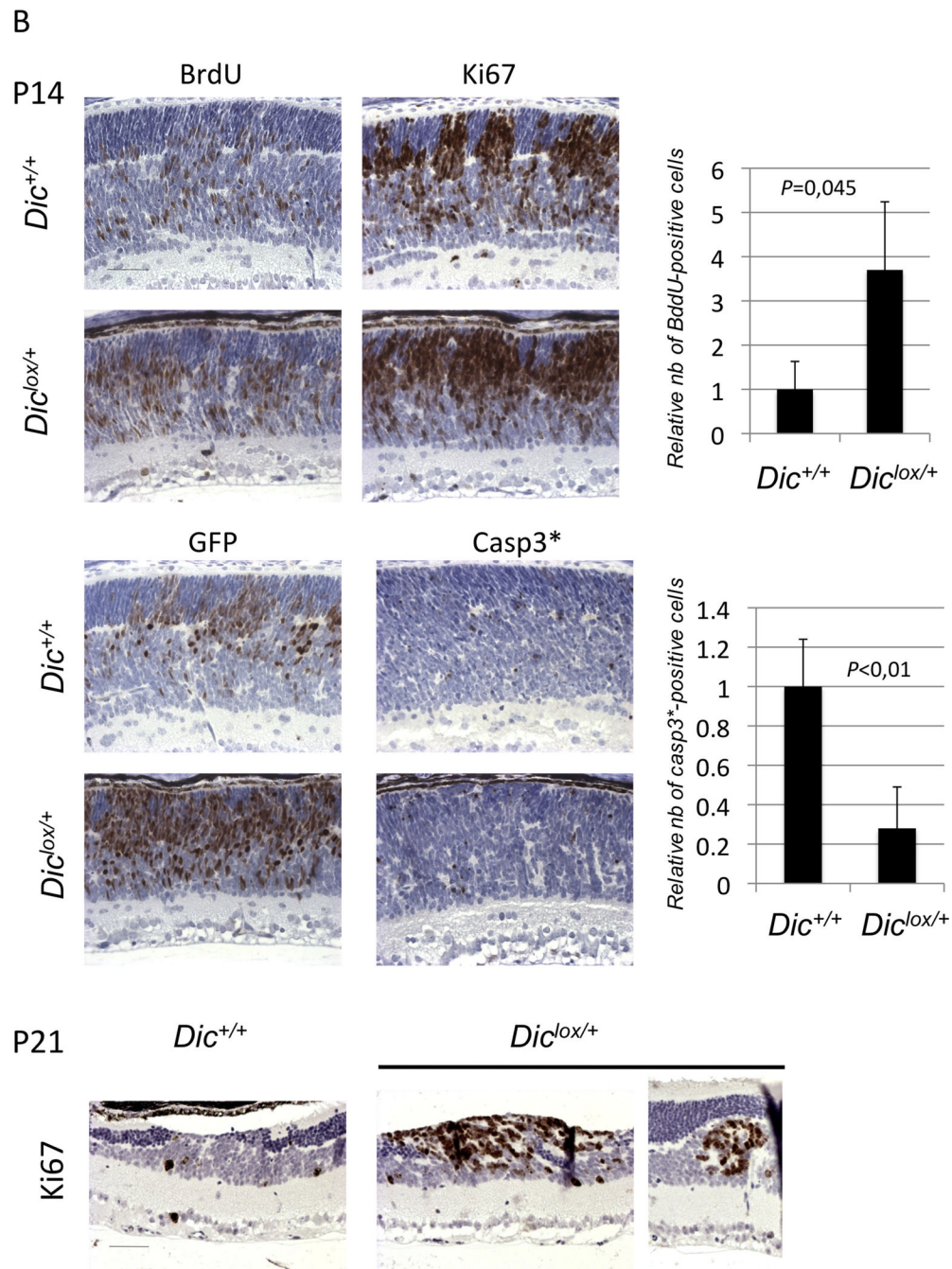


Figure 2. Immunostaining of the *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{lox/+}* retinoblastoma lesions (A) Syntaxin, Chx10 and GFP immunostaining of *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{lox/+}* (*Dic^{lox/+}*) and *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{+/+}* (*Dic^{+/+}*) retinæ at P45. (B) Proliferation and apoptosis in *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{lox/+}* (*Dic^{lox/+}*) and *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{+/+}* (*Dic^{+/+}*) at P14 and P21. BrdU incorporation, Ki67 and active Caspase3 immunostaining. Scale bars = 40µm. Quantification of BrdU and Caspase3 staining was performed on horizontal serial sections at the optic nerve level. The numbers (nb) of BrdU positive cells were normalized to the nb in *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{+/+}* (*Dic^{+/+}*), which was set 1 (top right panel). The numbers of immunoreactive cells to active caspase3 staining were normalized to the numbers of GFP-

positive cells in serial consecutive sections. The data are presented relative to the observed ratio GFP/Caspase3 in *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{+/+}* (*Dic^{+/+}*), which was set 1 (low right panel). Error bars represent standard deviation. *P*-values (Student's *t*-test; *N*=3).

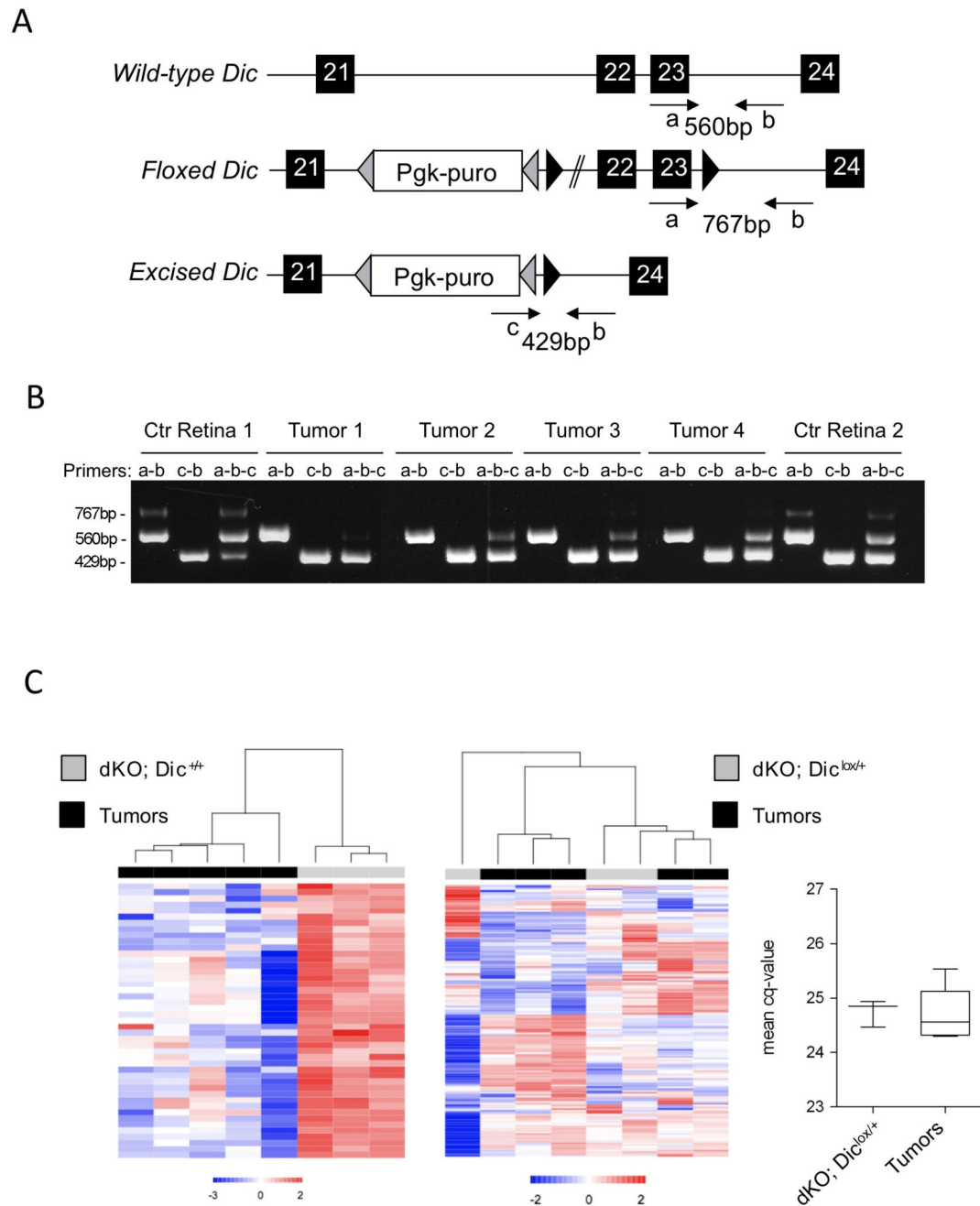


Figure 3. *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{lox/+}* retinoblastoma tumors retain a wild-type and functional *Dic* allele

(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 retinae (Ctr Retina) and 10 isolated tumors (results from 4 are shown) and examined by PCR using the primers depicted in the top panel. (B) RT-qPCR analyses of mature miRNAs expression. Left panel: hierarchical clustering of miRNAs significantly down-regulated in five *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{lox/+}* tumours (black, dKO; *Dic^{lox/+}*) compared to *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{+/+}* P20 retinae (grey, 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 retinae shows comparable expression levels in both *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{lox/+}* P20 retinae 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 retinae and tumors.

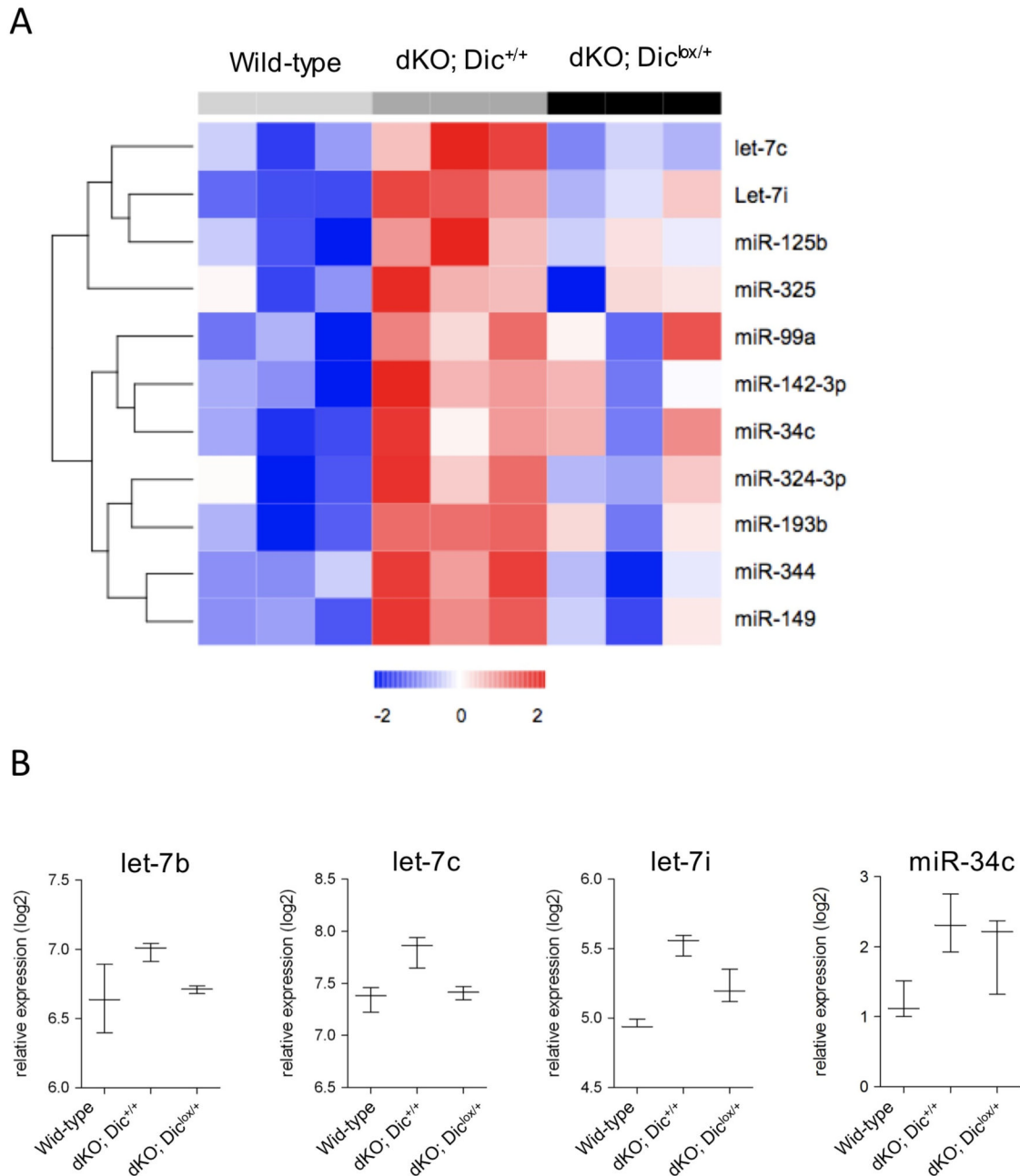


Figure 4. Search for putative tumor suppressor miRNAs in retinoblastoma

(A) Heatmap of selected differentially expressed miRNAs in *Chx10Cre*-negative mice (light grey, wild-type), *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{+/+}* (dKO; *Dic^{+/+}*) and *Chx10Cre;Rb^{lox/lox};p107^{-/-};Dic^{lox/+}* P20 retinæ (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.

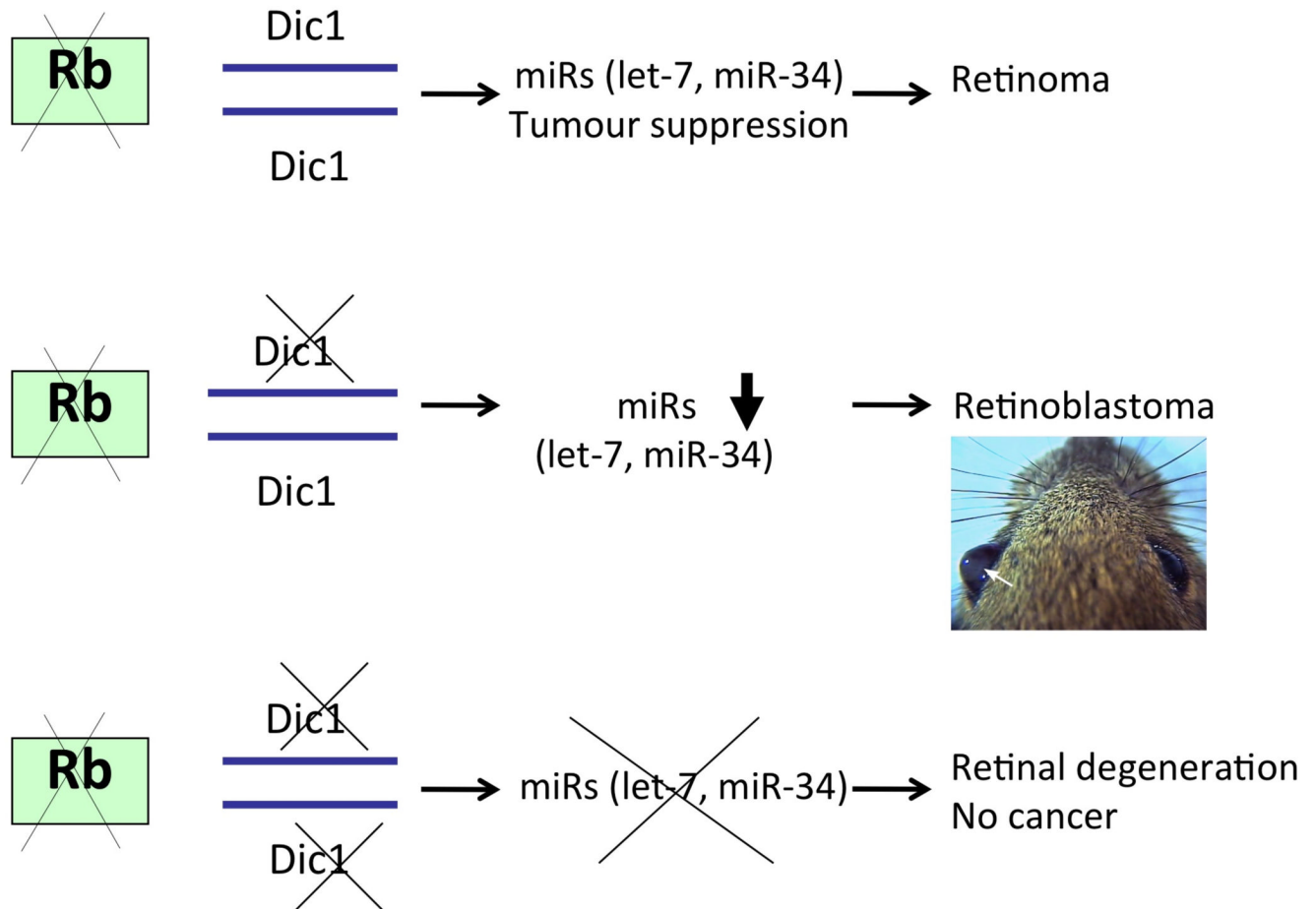


Figure 5. Monoallelic loss of Dicer enhances retinoblastoma formation

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.