The survival gene MED4 explains low penetrance retinoblastoma in patients with large RB1 deletion

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Retinoblastoma is a non-hereditary as well as an inherited pediatric tumor of the developing retina resulting from the inactivation of both copies of the RB1 tumor suppressor gene. Familial retinoblastoma is a highly penetrant genetic disease that usually develops by carrying germline mutations that inactivate one allele of the RB1 gene, leading to multiple retinoblastomas. However, large and complete germline RB1 deletions are associated with low or no tumor risk for reasons that remain unknown. In this study, we define a minimal genomic region associated with this low penetrance. This region encompasses few genes including MED4 a subunit of the mediator complex. We further show that retinoblastoma RB1−/− cells cannot survive in the absence of MED4, both in vitro and in orthotopic xenograft models in vivo, therefore identifying MED4 as a survival gene in retinoblastoma. We propose that the contiguous loss of the adjacent retinoblastoma gene, MED4, explains the low penetrance in patients with large deletions that include both RB1 and MED4. Our findings also point to another synthetic lethal target in tumors with inactivated RB1 and highlight the importance of collateral damage in carcinogenesis.

INTRODUCTION

Retinoblastoma (Rb) is an inherited as well as a non-hereditary disease. It represents the most common pediatric intraocular neoplasm, which in virtually every case results from the inactivation of both alleles of the RB1 tumor suppressor gene (MIM *614041) in the developing retina (1–4).

In non-hereditary Rb, both somatic mutations in RB1 take place in a single retinal cell that develops into a tumor. In contrast, in hereditary Rb germline mutations of one RB1 allele are associated with predisposition to Rb. Tumor development is initiated by a second somatic inactivating mutation in retinal cell(s) and usually consists of loss of the second allele, demonstrated by loss of heterozygosity (LOH) studies. Predisposition to Rb usually segregates as an autosomal dominant trait with high penetrance (90%), and it is widely recognized that germline carriers develop unilateral multifocal or bilateral Rb. However, rare instances of familial Rb displaying low penetrance (LP) and variable expressivity have been described (5). In such families, mutation carriers develop unilateral, multifocal Rb (reduced expressivity) or remain unaffected (reduced penetrance). The underlying molecular mechanisms leading to LP in Rb are poorly understood. In addition, the existence of modifier factors influencing the clinical expression must be considered, given that an identical mutation can

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result in heterogeneous phenotypes within a single family. The most famous example was first described a quarter of a century ago by Bunin et al. (6), who found that patients with germline chromosomal deletions of RB1 were more prone to unilateral Rb compared with other patients with hereditary Rb that usually developed bilateral Rb. Later studies demonstrated that although Rb arises as a result of loss-of-function mutations, large germline deletions encompassing the entire RB1 locus could be associated with low or no tumor risk. This paradox has never been explained. One hypothesis is that large deletions may encompass an unknown contiguous gene (called ‘survival gene’ hereafter) absolutely required for Rb progression. As the second hit involves very often LOH, this will result on bi-allelic loss of the RB1 gene as well as of this putative ‘survival gene’ preventing Rb development (hence low expressivity and penetrance). In families bearing such large deletions, Rb could only arise from a second hit involving RB1 point mutations or intragenic deletions but excluding this potential ‘survival gene’.

To test the ‘survival gene’ hypothesis, we focused on a family presenting LP, with an unaffected father and daughter but a bilaterally affected son, all three of whom presented a complete and large deletion of the RB1 gene. Following deletion mapping approaches and functional testing, MED4 (MIM *605718), a key subunit of the mediator complex, was identified as the ‘survival gene’ explaining the low disease penetrance observed in this family. The demonstration that Rb RB1 homozygous cells cannot survive in the absence of MED4 solves an old medical enigma and extends well beyond the Rb field as this ‘survival gene’ mechanism echoes with synthetic lethality and collateral damage processes recently demonstrated in glioblastoma (7,8). Our findings also provide with additional gene or drug targets for the treatment of Rb.

RESULTS

The Institut Curie is a reference center for Rb management in France, providing remarkable homogeneity in the clinical and genetic management of patients with familial Rb. All patients were identified and followed exclusively at this center, thereby offering reliable clinical and biological information. Familial and non-familial cases of Rb harboring a complete RB1 deletion were collected. We focused on a particular Rb family (Fig. 1) because it underlines the importance of genetic modifiers in the development of Rb, and also illustrates how LP associated to complete RB1 deletions might occur. Three individuals in this family carry the same alteration of the RB1 gene, a complete deletion, while only one individual developed Rb. Fundus examination in the two unaffected relatives showed no sign of regressed Rb or retinoma. We characterized the second hit mutation in this family patient; the bilaterally affected child did not display LOH nor a large deletion of the RB1 gene but a distinct second causative mutation, an intragenic RB1 nonsense point mutation p.(Arg556Ter). As this family is characterized by a complete RB1 gene deletion and two relatives present no expression of the disease (no tumor) while the affected son displays an intragenic mutation as a second hit, the deleted interval in this family might contain a putative ‘survival gene’ that explains the low familial penetrance.

We mapped and sequenced the breakpoints of the deleted interval in this family using a combination of high-resolution comparative genomic hybridization (CGH) arrays and multiplex PCR/liquid chromatography assays, focusing on the RB1 locus. The same approach was performed in six bilaterally affected Rb patients harboring a complete germline RB1 deletion. Comparisons of the deleted intervals between affected and unaffected cases helped to define the critical germline interval containing the putative ‘survival gene’. By definition, the putative ‘survival gene’ needs to be expressed for the tumor to develop; hence, at least one copy of the gene must be retained in the tumor. We therefore examined six tumors carrying two full RB1 deletions in order to refine the location of the gene by subsequent CGH array analysis (Fig. 2). We compared the mapped germline and somatic intervals that included or excluded the gene, respectively. The telomeric interval was completely lost in two tumors (3 and 5) and was thereby excluded, whereas the centromeric interval was refined to genomic coordinates 47 573 600–45 962 864 due to the large deletion observed in tumor 3 (Fig. 2). The centromeric interval contained six genes: ESD, SUCLA2, NUDT15, MED4, HTR2A and LRCH1, none of which represented an obvious candidate for the ‘survival gene’.

To further narrow down the number of candidate genes, we studied the expression of each gene in the Y79 Rb cell line, using normal retina and other tissues (Burkitt’s lymphoma, thyroid) as controls. The rationale was that the putative ‘survival gene’ should be expressed in the Rb cell line. All genes were expressed in the normal retina; however, HTR2A was eliminated due to its lack of expression in Y79 cells. Considering the available literature reporting genotype-phenotype correlations in Rb patients with 13q deletions (9), MED4 and NUDT15 stood out as potential candidates as the other genes were apparently not included in the deletion intervals linked to a LP phenotype.

To determine whether Rb requires one of these two candidate genes for its growth, we conducted loss-of-function experiments using RNA interference in Rb cell lines. We successfully identified short hairpin RNAs (shRNAs) (two for each gene) that efficiently downregulated the expression of MED4 (Fig. 3A) and
NUDT15 (Supplementary Material, Fig. S1A and B) as measured by reverse transcription-quantitative real-time PCR (RT–qPCR) and western blot (WB). The lack of a suitable MED4 commercial antibody precluded the analysis of MED4 protein levels by WB. Knockdown of NUDT15 in Y79 Rb cells did not induce apoptosis (Supplementary Material, Fig. S1C) or affect proliferation (Supplementary Material, Fig. S1D), indicating that NUDT15 was not essential for Rb development under our assay conditions. Therefore, we excluded NUDT15 as a potential ‘survival gene’ in Rb. In contrast, shRNA-mediated MED4 downregulation significantly induced apoptosis both in Y79 and WERI-Rb-1RB cell lines compared with shRNA controls (Fig. 3B), as measured by FACS analysis of cleaved Caspase-3. Accordingly, we also observed a marked decrease in cell proliferation in MED4 knockdown cells using both shMED4 (Fig. 3C). We then performed clonogenic assays under semi-solid conditions. While Y79 and WERI-Rb-1 control cells formed many colonies, MED4 knockdown cells formed significantly fewer, if any colonies (Fig. 3D), indicating that MED4 is necessary for Rb anchorage independent growth, a major characteristic of transformed cells.

To verify and test in a more physiologically relevant assay our in vitro results we performed in vivo orthotopic grafting experiments using 15 non-obese diabetic severe-combined immunodeficient mice (NOD-SCID). The right eye of each mouse was injected with control Y79 (shControl) or MED4 knockdown Y79 (shMED4#1 and #2) cells. Ophthalmic examination showed that Y79 xenografts started to grow at Week 3 after injection (Fig. 4). At Week 7, retinal tumors with or without vitreous involvement were present in all mice bearing control Y79 xenografts (ShCtl; 5/5). In contrast, vitreous involvement was present only in one mouse bearing the shMED4#1 xenograft cells (1/5) and in none of the mice bearing the shMED4#2 xenograft cells (Table 1). Fibrosis without any sign of active tumor was noted in 3/10 mice bearing shMED4 xenografts (two in shMED4 #1 and one in shMED4 #2).

Histologic analysis confirmed the ophthalmic examination results (Supplementary Material, Fig. S2). All mice injected with Y79 control xenografts developed tumors in their eye. Retinal and vitreous infiltration was composed of small to medium size cells with scanty cytoplasm, and hyperchromatic nuclei with mitotic cells. No rosette or fleurette were observed. More than 95% of cells were Ki positive. For mice bearing shMED4 xenografts, histological examination revealed eyes free of tumors in 4/5 mice for shMED4#1 and 5/5 mice for shMED4#2.

Overall, our results show that Rb RB1−/− cells cannot survive in the absence of MED4 in vitro and in orthotopic xenograft models in vivo, therefore identifying MED4 as a survival gene in Rb.

DISCUSSION

Although Rb remains the prototype of inherited cancer, the molecular bases of LP are yet to be explained. LP mutations appear to reduce, but not eliminate pRB activity (i.e. so-called weak alleles) (10,11). In addition, other factors such as the MDM2 gene and imprinting have been described to modulate the Rb phenotype (12,13). Identifying these modifiers is of utmost importance, as it will clearly help elucidate prognosis for individual mutation carriers, and allow their optimal genetic counseling, and planning of ophthalmologic surveillance. At last, therapeutic opportunities may emerge from these efforts.

![Figure 2. Definition of the centromeric critical interval in Rb patients. CGH array profiles are shown for six tumors containing a full RB1 deletion. X-axis: genomic location; y-axis: signal intensity. Copy numbers are indicated above the signal traces. Location of RB1, MED4 and NUDT15 is indicated by a box (Hg18 build). The critical germline interval defined in the LP family (see text) is indicated by two vertical lines. The centromeric critical interval was defined by subtracting the homozygous deleted parts of the locus (0 copy) from the germline interval.](https://academic.oup.com/hmg/article-abstract/23/19/5243/2900957)
Hence, deciphering the link between LP and complete RB1 deletions should not only solve an old medical enigma but also provide major clues in genotype/phenotype correlations, tumor progression, and possibly point to novel therapeutic targets.

In this study, we moved from clinical observations to functional approaches. Based on an Rb family with LP ascertained in the clinics, germline and tumoral mapping studies identified two adjacent ‘survival’ candidates, MED4 (as cited above) and NUDT15. Following functional testing we demonstrated that Rb RB1 −/− cells cannot tolerate the homozygous loss of MED4, thereby explaining the LP observed in patients with large RB1 deletions. MED4, also known as TRAP36 and/or DRIP36, is a subunit of the mediator complex that connects gene-specific transcription factors and the polymerase II machinery (14). MED4 exhibits strong conservation across species, suggestive of an important but yet to be characterized biological function within the mediator complex. Together with other subunits, MED4 constitutes the middle part of the complex (15).
which is found in nearly all mediator preparations. It is thus possible that MED4 plays a structural role within this complex, and its loss may affect all the activities of the complex. Previously, several mediator subunits were shown to be involved in cancer development. For example, MED1 contributes to ERα-dependent breast cancer by bridging ERα nuclear receptors to the Pol-II machinery. Additionally, MED1 overexpression was also observed in this type of cancer due, in some cases, to its amplification (16). Accordingly, silencing MED1 in breast cancers decreased tumor growth and sensitized tumor cells to anti-estrogen treatment (17). Interestingly, MED13L is required for Rb/E2F control cell growth and cell cycle inhibition (18). Little is known about MED4 involvement in tumorigenesis; one study associated its loss and downregulation with poor clinical outcome in cervical cancer and proposed MED4 as a driver of chemoradioreistance possibly via its transactivation of the vitamin D receptor (19). Similarly, it has been demonstrated that the vitamin D active metabolite (1,25-dihydroxyvitamin D3) induces apoptosis in Rb cell lines, albeit at high doses, precluding its use in clinical practice (20). As MED4 might be a core subunit of the mediator complex, one might wonder if the survival mechanism is specific to RB1 KO cells or a general survival mechanism (i.e., MED4 expression would be necessary for any cell to survive). MED4 is indeed ubiquitously expressed to varying degrees in cells and tissues and appears highly expressed in several types of cancer (21). However, a germline homozygous frameshift mutation was reported in 16 individuals among 6259 genotypes from the Exome Variant Server (http://evs.gs.washington.edu/EVS/). This suggests that MED4 is not a ‘global survival gene’ and may indicate that in some contexts cells can survive the absence of MED4. Regardless, our results show that MED4 is required for Rb development.

Recently, a subset of genes that are essential to all cells named CYCLOPS (Copy number alterations Yielding Cancer Liabilities Owing to Partial losS) was described (22). Partial but not complete suppression of CYCLOPS genes is compatible with cancer cell viability. This study and our findings strongly suggest that this survival mechanism is a general concept. Also interesting is the close localization of a recessive tumor suppressor gene and a survival gene such as the one demonstrated in our study. We wonder if this closeness occurred by chance alone or by a common, possibly protective, mechanism. The latter hypothesis might be supported by a recent study demonstrating that most somatic homozygous deletions encompassing recessive cancer genes (such as RB1) are composed of a large deletion on one parental chromosome and a small deletion on the other (23). The size of these small deletions might actually be dictated by the presence of neighboring survival genes. In other words, homozygous deletions in cancer extend until involvement of survival genes, which might in turn represent one of the shaping forces of the tumor genome.

Our findings underline the importance of collateral damage in cancer, further highlighting that passenger deleted genes encoding essential activities could be a therapeutic target of choice (8). In this line, MED4 is another synthetic lethal target in tumors with inactivated RB1. Importantly, our shRNA inhibition experiments

![Figure 4](https://academic.oup.com/hmg/article-abstract/23/19/5243/2900957)

**Figure 4.** Loss of MED4 in Y79 xenograft cells reduces their tumorigenicity. Ophthalmic examination of mice subjected to intraocular injection was performed every week until their sacrifice. The graph shows the percentage of tumor free mice injected with control cells (ShCtl; black) or MED4 knockdown cells (shMED4 #1 and #2; grey).

| Table 1. Ophthalmological examination of 15 NOD-SCID immunodeficient mice following orthotopic grafting experiments | Tumor localization |
|---|---|---|---|---|---|---|---|
| No. mouse | Week | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| shCtl | | | | | | | | |
| 1 | – | – | – | + | + | + | + | + |
| 2 | – | – | – | – | + | + | + | + |
| 3 | – | – | + | – | – | – | – | – |
| 4 | – | – | – | – | – | – | – | – |
| 5 | – | – | – | – | – | – | – | – |
| 6 | – | – | – | – | – | – | – | – |
| 7 | – | – | – | – | – | – | – | – |
| 8 | – | – | – | – | – | – | – | – |
| 9 | – | – | – | – | – | – | – | – |
| 10 | – | – | – | – | – | – | – | – |
| shMED4 #1 | | | | | | | | |
| 11 | – | – | – | – | – | – | – | – |
| 12 | – | – | – | – | – | – | – | – |
| 13 | – | – | – | – | – | – | – | – |
| 14 | – | – | – | – | – | – | – | – |
| 15 | – | – | – | – | – | – | – | – |

Tumor involvement is described as (+) = mild, (++) = moderate to severe and (+++) = massive; (−): no tumor.
demonstrated growth arrest in our mouse model, paving the way for future clinical trials. Lastly, it should be determined if MED4 germline mutations are involved in Rb phenotypic variability and eventually be used as prognostic biomarkers.

MATERIALS AND METHODS

Patients

Diagnosis of Rb was established on the basis of examinations by an ophthalmologist and by histopathological criteria when the tumor was available. Rb patients were offered genetic counseling and individual written consent was obtained from all sampled individuals or their legal guardians. In this series of 1210 consecutively ascertained cases, a total of 11 and 30 patients were diagnosed with a complete deletion of the RB1 gene at the germline or tumor only levels, respectively. The six tumors described are from unilateral sporadic cases and were selected for the study as they showed the largest homozygous deleted intervals among our series.

CGH array and multiplex PCR/liquid chromatography

A dedicated CGH-array focusing on the RB1 locus was used to restrict the extent of deletions to ~100 kb, according to the manufacturer’s instructions (Roche Nimblegen, Madison, WI, USA). The multiplex PCR/liquid chromatography method was used to restrict the size of breakpoint regions. Multiple small genomic regions of 200–300 bp were co-amplified using unlabeled primers, and PCR products were then separated by ion-pair reversed-phase high-performance liquid chromatography and quantified by fluorescent detection using a post-column intercalation dye. The relative peak intensities for each target directly reflect the copy number of each region studied. By choosing, step by step, a set of 20 couples of primers to study the breakpoint regions, their size was able to be restricted to ~5 kb. A long-range PCR was performed in each case, using the forward primer of the most centromeric undeleted PCR fragment of the centromeric breakpoint region, and the reverse primer of the most telomeric undeleted PCR fragment of the telomeric breakpoint region. Both fragments were directly sequenced both strands in order to define the deletion breakpoints at the nucleotide level (24,25).

Expression studies

Expression levels of the six candidate genes were studied in the Y79 Rb cell line and control RNAs. As a preliminary step, we first verified that Y79 and human Rb tumors presented similar CGH pangenomic profiles and identified the two RB1 loss-of-function mutations in Y79 cells (c.2106+1G>A and a large deletion from exons 2 to 6) in order to validate the model. Control RNAs were chosen in order to display a high level of expression of the candidate genes and were derived from a Burkitt’s lymphoma (GA-10- ATCC number CRL-2392, ATCC, Manassas, VA, USA), human thyroid (Human Thyroid polyA RNA no. 636128 batch 7110100, Clontech, Mountain View, CA, USA) and human retina (Human Retina total RNA no. 3636579 batch 1005010, Clontech). TaqMan probes (assays on demand, Applied Biosystems, Carlsbad, CA, USA) were designed for each gene and two housekeeping genes (TBG and POLG). RT–PCRs were performed in duplicate with the applied PCR core kit and random hexamers on a Taqman 7900 (Applied Biosystems, Carlsbad, CA, USA). Relative expression (r) was calculated as follows r = 2 [−(ΔCt Gene of interest−ΔCt Reference Gene)].

Cell culture

The Y79 and WERI-Rb-1 Rb cell lines (ATCC) were cultured in RPMI 1640 medium (GIBCO), supplemented with 10% (WERI-Rb-1) or 20% (Y79) fetal bovine serum (GIBCO), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen), 1.25 μg/ml fungizone (Invitrogen), at 37 °C in a humidified atmosphere containing 5% CO₂. As WERI-Rb-1 cell line is known to carry a whole RB1 deletion, we demonstrated by multiplex ligation probe amplification and CGH analyses that the other copy was retained in the tumor. As a result, at least one MED4 copy also remains, in accordance with our model.

shRNA and lentiviral production and infection

Lentiviral pLKO vectors encoding shRNA targeting NUDT15 (NM_018283, clones TRCN0000289456, TRCN0000289457) and MED4 (NM_014166, clones TRCN0000353703, TRCN0000330941) were obtained from Sigma-Aldrich. Lentiviruses were produced in 293T cells, by co-transfecting pLKO-derived vectors and the packaging plasmids pS-PAX2 and pMD2-VSVG, using lipofectamine 2000 (Invitrogen). Lentiviral particles were harvested 48, 60 and 72 h post-transfection. 2 × 10⁶ Y79 and WERI (26)-Rb-1 cells were infected and selected with puromycin (1 μg/ml).

Real-time RT–PCR

Total RNA, extracted using RNeasy Plus mini kit (Qiagen), was reversely transcribed with the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). Quantitative real-time PCR assays were conducted using SYBR Green real-time PCR Master Mix and real-time PCR amplification equipment (Applied). The primer sequences were: sense, 5′-ATTGCCAGATGTCTTGTCTC-3′ and antisense, 5′-CCAGGGGTCTCCAACTCAAAA-3′ for MED4 (105 bp); sense, 5′-AAGTTGGGAGTGGGTTCCCT-3′ and antisense, 5′-TCGGCCACCTAGAGATGATT-3′ for NUDT15 (150 bp); sense, 5′-GGTCTCCTTCTGAACCTCA-3′ and antisense, 5′-AGCCAATTCTTGTGTCATAC-3′ for GAPDH (116 bp).

Western blotting and antibodies

Cell extracts and WBs were performed as previously described (26). Membranes were probed with anti-NUDT15 (HPA038968, SIGMA, 1/1000), anti-β-actin (A1978, Sigma, 1/5000) antibodies, at 4 °C overnight. Signals were acquired using a CCD camera (G:BOX, Syngene).

Cell growth curves and soft agar colony formation assays

For growth curves, cells were seeded into 12-well plates at a density of 3 × 10⁵ cells/well. Trypan blue staining was performed and viable cells were counted for 9 consecutive days.
The soft agar colony formation assay was performed as previously described (27).

**Apoposis by flow cytometry**

Cells (5 × 10^5 cells/well) were grown in 6-well plates for 72 h. Apoptosis was measured using the Caspase-3, Active Form, mAb Apoptosis Kit, FITC (BD Biosciences). The signals were detected by a FACScalibur cytometer (BD Biosciences). The percentage of apoptotic cells was determined using the FlowJo software (Tree Star).

**Mice experiments**

Adult immunodeficient NOD-SCID mice were used for the in vivo experiments. Animals were handled in accordance to the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in Ophthalmology and Research in Vision. Infected Y79 cells (shcontrol or shMED4) were used for orthotopic xenografts of the right eye. Under intraocular general anesthesia with sodium pentobarbital, the pupil was dilated with 1 drop of tropicamide and then a drop of topical anesthetic proparacaine hydrochloride (0.5%) was applied. Injections were performed using a surgical microscope; 2 μL of cell suspension (10 000 cells/μL) was injected into the subretinal space of the right eye of the three groups of mice using a Hamilton syringe with a 33-gauge needle. Special care was taken to prevent lens damage. After subretinal injection, ophthalmic examination of the mice was performed weekly after local anesthesia with a naso-fibroscope. Clinical findings regarding the presence of a retinal tumor or the presence of a vitreous invasion were recorded. When tumor cells invaded the vitreous cavity, the mice were sacrificed by cervical dislocation for histological analyses. The eyes were immediately fixed in 4% formaldehyde, and then embedded in paraffin using conventional systems. The blocks were sectioned to obtain 5 μm sections and stained with conventional hematoxylin-eosin; a second section was stained for KI67 (Novo-castraTM Lyophilized Rabbit Polyclonal Antibody Ki67 Antigen, Leica Biosystems).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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**Conflicts of Interest statements.** None declared

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