RBL1 (p107) functions as tumor suppressor in glioblastoma and small-cell pancreatic neuroendocrine carcinoma

Thomas Naert^{1,2,&}, Dionysia Dimitrakopoulou^{1,2,&}, Dieter Tulkens^{1,2}, Suzan Demuynck¹, Rivka Noelanders^{1,2}, Liza Eeckhout¹, Gert van Isterdael³, Dieter Deforce⁴, Christian Vanhove^{2,5}, Jo Van Dorpe^{2,6}, David Creytens^{2,6}, Kris Vleminckx^{1,2,7,§}.

¹ Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

² Cancer Research Institute Ghent, Ghent, Belgium

³ Inflammation Research Center, VIB, Ghent, Belgium

⁴ Laboratory for Pharmaceutical Biotechnology, Ghent University, B-9000 Ghent, Belgium

⁵ Infinity lab, Ghent University Hospital, Ghent, Belgium

⁶ Department of Pathology, Ghent University and Ghent University Hospital, Ghent, Belgium

⁷ Center for Medical Genetics, Ghent University, Ghent, Belgium

& Contributed equally to this work

§ Corresponding author

Kris Vleminckx, Ph.D.
Dept. for Biomedical Molecular Biology
Ghent University
Technologiepark 927
B-9052 Ghent (Zwijnaarde)
Tel +32-9-33-13760, Fax +32-9-221 76 73,
E-mail : kris.vleminckx@irc.UGent.be

Competing interests

The authors declare no competing interests.

Abstract

Alterations of the retinoblastoma and/or the p53 signaling network are associated with specific cancers such as high-grade astrocytoma/glioblastoma, small cell lung cancer (SCLC), choroid plexus tumors and small-cell pancreatic neuroendocrine carcinoma (SC-PaNEC). However, the intricate functional compensation between RB1 and the related pocket proteins RBL1/p107 and RBL2/p130 in suppressing tumorigenesis remains poorly understood. Here we performed lineage-restricted parallel inactivation of *rb1* and *rbl1* by multiplex CRISPR/Cas9 genome editing in the true diploid *Xenopus tropicalis* to gain insight into these *in vivo* compensatory mechanisms. We show that while *rb1* inactivation is sufficient to induce choroid plexus papilloma, combined *rb1* and *rbl1* inactivation is required and sufficient to drive SC-PaNEC, retinoblastoma and astrocytoma. Further, using a novel Li-Fraumeni syndrome-mimicking *tp53* mutant *X. tropicalis* line, we demonstrate increased malignancy of retinoblastoma-mutant neural malignancies upon concomitant inactivation of *tp53*. Interestingly, although clinical SC-PaNEC samples are characterized by abnormal p53 expression or localization, in the current experimental models, the *tp53* status had little effect on the establishment and growth of SC-PaNEC, but may rather be essential for maintaining chromosomal stability. SCLC was only rarely observed in our experimental set-up, indicating requirement of additional or alternative oncogenic insults. In conclusion, we used CRISPR/Cas9 to delineate the tumor suppressor properties of Rbl1 and generate new insights in functional compensation within the retinoblastoma protein family in suppressing pancreatic and specific neural cancers.

Keywords

p107, p53, CRISPR/Cas9, Pancreatic neuroendocrine carcinoma, choroid plexus, retinoblastoma, *Xenopus tropicalis*

Introduction

The interplay of the signaling networks controlling cell cycle (*e.g.* Retinoblastoma (RB)) and cell death (*e.g.* p53) in suppressing the development of cancers including, amongst others, glioblastoma, choroid plexus carcinoma, pancreatic neuroendocrine carcinoma and small-cell lung cancer was previously demonstrated by clinical and animal modeling studies¹⁻⁶. Unsatisfactory, the median survival prospects for patients diagnosed with p53 and RB1 deficient cancers are extremely dismal, *e.g.* for small cell lung cancer (SCLC) (stage IV: 8-10 months⁷), small cell pancreatic neuroendocrine carcinoma (SC-PaNEC) (11 months⁸), choroid plexus tumors (*tp53*^{mutated} WHO grade III: 2-4 months⁹) and glioblastoma (12-18 months¹⁰). As such, continued generation of novel and short latency preclinical models for these highly aggressive cancers remains necessary to fuel rational design of molecular targeted drug therapies.

Intriguingly, the RB signaling network entails a family of three pocket proteins (RB1, RBL1, RBL2), which in union tightly regulate G1/S cell cycle progression, and whose differential expression may underlie cell-type specific functional compensation in suppressing tumorigenesis^{11,12}. An example of this compensation occurs in the mouse and *Xenopus* retina where RB1-deficiency fails to initiate retinoblastoma, and simultaneous inactivation of *Rbl1* (*p107*) is required to initiate tumorigenesis¹³⁻¹⁵. In an alternative case, high-grade astrocytoma and choroid plexus tumors have been previously induced in murine models by overexpression of T₁₂₁, a truncated SV40 T antigen mutant that binds and inhibits RB1, RBL1 and RBL2 proteins^{16,17}. In order to investigate the selective tumor suppressor properties of the Rbl1 protein, thus dissecting compensation on a genetic level, we performed concomitant inactivation of the *rb1* and *rb1* tumor suppressor genes, leaving *rb2* unaltered. For this we used CRISPR/Cas9 gRNA ribonucleoproteins (RNPs) delivered by microinjection in embryos of *Xenopus tropicalis*, an aquatic amphibian with an unique true diploid genome. By targeting the injections to specific blastomeres we were able to modify the retinoblastoma signaling network in different tissue lineages, including the neural and pancreatic lineages^{18,19}. As such, we aimed to obtain a deeper understanding of tissue-specific compensation between RB1 and RBL1 in cancer development²⁰.

Next to manipulating the RB pathway, we also generated a *tp53* knockout *Xenopus* line as a stable genetic background with a twofold aim. We wanted to explore whether Li-Fraumeni syndrome (LFS) could be recapitulated in *Xenopus tropicalis*. In addition, we wanted to investigate whether *tp53* deficiency sensitizes the animals to tumor initiation or progression upon additional genetic oncogenic insults, as has been extensively documented in the mouse. LFS is a cancer syndrome defined by either incapacitating germline *TP53* mutations or *de novo* mutations occurring during early embryogenesis^{21,22}. LFS patients are at risk for development of (osteo-)sarcomas, central nervous system (CNS) tumors,

breast cancer and adrenocortical carcinoma, as well as other less frequent cancers²³. Previously, *Tp53* knockout mouse models have been generated and were shown to be prone to the development of a variety of cancers, most frequently lymphomas and sarcomas^{24–26}. Additionally, LFS was modeled in *tp53* mutant zebrafish spontaneously developing malignant peripheral nerve sheath tumors (MPNST) and angiosarcoma, next to other tumor types^{27,28}.

As mentioned before, p53 is an established gatekeeper for numerous tumor types. Hence, experimental oncogenic insults, either genetic or environmental, are frequently employed in a *Tp53* mutant background because it increases tumor penetrance and reduces tumor latency. As such, we also performed parallel perturbation of the retinoblastoma and p53 signaling network, by multiplexing *rb1*, *rb1* CRISPR/Cas9 and inactivating *tp53* by either CRISPR/Cas9 multiplexing or breeding efforts. We investigated whether sensitizing *Xenopus tropicalis* to cancer development by perturbing p53 functions could alter the tumor spectrum occurring upon perturbations in the retinoblastoma signaling network. In this regard, previous work also described that concomitant perturbation of the retinoblastoma and p53 signaling network generates chromosomal instability (CIN), possibly sensitizing to cancer development^{29–31}.

By our *in vivo* gene editing strategy we identify novel cell-type specific requirements for inactivation of *rb1*, in order to bypass compensation in the native and immunocompetent *Xenopus* brain and pancreas upon inactivation of *rb1*, across different *tp53* genotypes. By combinatorial CRISPR/Cas9 editing of *rb1* and *rb1* we demonstrate highly penetrant development of SC-PaNEC, glioblastoma, choroid plexus tumors, next to other cancer types. Interestingly, we provide genetic evidence establishing *rb1* as a genuine tumor suppressor in SC-PaNEC and glioblastoma.

We believe our novel genetic *X. tropicalis* genetic cancer models have the potential to generate important molecular insights and could emerge as promising preclinical cancer models³². Namely, these *Xenopus* cancer models are uniquely positioned as an alternative to viral vector-mediated CRISPR/Cas9 mice models, mainly due to the relative ease of CRISPR/Cas9 delivery and the short latency and high penetrance of cancer development³³.

Results

***tp53* mutant *X. tropicalis* develop hematological malignancy and sarcomas reminiscent of Li-Fraumeni syndrome**

In order to generate a *tp53* mutant *X. tropicalis* line, we unilaterally injected *tp53* coding region 1 (*tp53^{cr1}*) gRNA precomplexed with recombinant Cas9 protein in two-cell *X. tropicalis* embryos. Mosaic mutant F0 animals, further called crispants, were raised until sexual maturity and out-crossed with wild-type animals. Upon targeted amplicon sequencing and BATCH-GE analysis³⁴, we selected two types of F1 *tp53* heterozygotes, each with a distinct variant of a $\Delta 4$ deletion (*tp53 ^{$\Delta 4$ var1/+}* and *tp53 ^{$\Delta 4$ var2/+}*) (Table S1A). Both of these deletions are expected to give rise to nonsense-mediated decay since due to the frame-shifting deletion, the pre-mRNA contains a premature stop codon (*tp53^{168STOP}*). These heterozygote mutants (F1) were subsequently intercrossed (*tp53 ^{$\Delta 4$ var1/+}* x *tp53 ^{$\Delta 4$ var2/+}*) to obtain F2 homozygous mutant animals (*tp53 ^{$\Delta 4$ var1/ $\Delta 4$ var2}*) (Table S1B). Both the hetero- and homozygous mutant animals were monitored for morbidity and euthanized at ethical endpoint defined by distended abdomen reminiscent of ascites, lethargy, or decreasing body weight. Of note, *X. tropicalis* has a significantly longer lifespan than murine animal models and can reach over ten years of age in experimental housing conditions.

Kaplan-Meier analysis over a period of 900 days revealed faster occurrence of morbidity in two clutches of *tp53 ^{$\Delta 4$ var1/ $\Delta 4$ var2}* (F2) nullizygous animals when compared to *tp53* heterozygotes (F1) ($p < 0.01$; Table S2A) (Fig. 1A). We investigated moribund mutant *X. tropicalis* by X-ray techniques, revealing calcified ectopic structures in 66% (n=3) of F1 *tp53* heterozygous animals (Fig. 1B).

In order to investigate the possible presence of hematological malignancy, we performed immunohistological analysis of the spleen in moribund *tp53* mutant animals. In the wild type *Xenopus* spleen, CD3⁺ cells organize in a ring-like structure surrounding the white pulp, which contains the B-cells and displays pronounced PCNA immunoreactivity (Fig. 1C-left)³⁵. However, this CD3⁺ ring-like structure was disrupted in 75% (n=8) of the moribund *tp53 ^{$\Delta 4$ var1/ $\Delta 4$ var2}* animals (Fig. 1C-right; Fig. 1D). In 25% (n=8) of these animals we also observed that PCNA immunoreactivity expanded into red pulp areas not contiguous with CD3 immunoreactivity. As such, we conclude that the observed splenic architectural disruptions are reflecting the presence of different hematologic malignancies either or not of the T-cell lineage, which in one case lead to profound cleaved caspase-3 immunostaining in splenic white pulp (Fig. S1A). Interestingly, both malignancies of the B- and T-cell lineages have been described in murine *tp53* mutant animals models³⁶.

In order to further investigate the presence of T-cell-related hematological malignancy in *tp53 ^{$\Delta 4$ var1/ $\Delta 4$ var2}* animals, peripheral blood of remaining non-moribund littermates (n=6) was collected by toe clipping and subjected to flow cytometry to investigate the proportion of cells of the T-cell lineage. Enrichment of CD3⁺ (cytoplasmic or membrane located) and CD8⁺ cells could be demonstrated in one out of six of the non-moribund animals (Fig. 1F; Fig. S2-3). An increased leukocyte count was subsequently

validated by Natt-Herrick's method in blood obtained by cardiac bleed in this animal, when compared to an age-matched control ($p < 0.01$; Table S2B) (Fig. 1E; Fig. S1B). Furthermore, in this *tp53*^{-/-} animal, CD3⁺ cells were also disorganized in the spleen and diffuse dissemination of CD3⁺ T-lymphocytes could be observed in the liver (Fig. 1G; Fig. S1C). Furthermore, we also observed the presence of liposarcoma in one *tp53*^{-/-} animal (Fig. S1D).

Finally, one moribund *tp53* heterozygous animal presented with a large intraperitoneal lobular neoplasm, classified by histopathology as a high-grade undifferentiated spindled and round cell sarcoma with profound PCNA proliferation marker immunoreactivity (Fig. 1H).

Taken together, we demonstrate development of a LFS-type tumor spectrum with hematological malignancy and sarcoma in *tp53* mutant *X. tropicalis*, in line with well-characterized *Tp53* mutant mouse models^{24,25}. The latency to neoplasm-related morbidity (> 1 year) is relatively long in the *tp53* mutant *X. tropicalis*, but can likely be attributed to the relative long lifespan (~ 10 years) of *Xenopus*. In any case, these animals constitute a valuable platform for evaluating the role of the *tp53* tumor suppressor gene inactivation in combination with other oncogenic insults. Hence, we continued on to target retinoblastoma-family tumor suppressor genes in this *tp53* mutant line.

Induction of small-cell pancreatic neuroendocrine carcinoma (SC-PaNEC) by CRISPR/Cas9 genome editing

In order to investigate the functional consequences of inactivating *rb1* and *rb1l1*, under differential *tp53* genetic backgrounds, we performed multiplex CRISPR/Cas9-mediated inactivation of these genes in *tp53* mutant and wild-type *X. tropicalis*. Note that the *rb1* and *rb1l1* gRNAs used throughout this study have previously been reported to induce retinoblastoma in *X. tropicalis*¹⁴. These gRNAs were shown to induce retinoblastoma independent from CRISPR/Cas9 off-target effects, as in that study another pair of *rb1/rb1l1* gRNAs with a differential genomic targeting site similarly induced this tumor type. For targeting *tp53*, we also included a gRNA that was different from the one used to make the KO line, i.e. *tp53*^{coding region 2} or *tp53*^{cr2}.

We intercrossed *tp53*^{Δ4var1/+} with *tp53*^{Δ4var2/+}, generating embryos with either *tp53*^{+/+}, *tp53*^{Δ4var1/+}, *tp53*^{Δ4var2/+} or *tp53*^{Δ4var1/Δ4var2} genotype, while simultaneously intercrossing wild-type (WT) *X. tropicalis* to obtain WT embryos. We subsequently injected *tp53* mutant or WT embryos in the eight-cell stage in a single vegetal-dorsal blastomere with distinct combinations of tumor suppressor-targeting CRISPR/Cas9 RNPs (Fig. 2A). As thus we performed primarily genome editing of the anterior endoderm, including the pancreas. Genome editing was confirmed by targeted amplicon sequencing and BATCH-GE analysis

(Table S1C). Animals were euthanized when presenting with distended abdomen, lethargy and decreasing bodyweight or at end-of-experiment (97 days). Necropsy revealed no gross pancreatic abnormalities in animals resulting from *rb1* CRISPR/Cas9 injection in embryos obtained from a *tp53*^{Δ4var1/+} x *tp53*^{Δ4var2/+} intercross (hereafter termed single mosaic knockout (**smKO**) at 97 days (n=13). However, we observed pancreatic dysmorphology in 86% (n=29) of the animals resulting from multiplexed *rb1/rbl1* CRISPR/Cas9 injection in embryos obtained from the same intercross (hereafter termed double mosaic knockout (**dmKO**)) and in 77% (n=22) of the animals resulting from *rb1/rbl1/tp53*^{cr2} CRISPR/Cas9 injection in WT embryos (hereafter termed triple mosaic knockout (**tmKO**)). Both for dmKO and tmKO, these pancreatic dysmorphologies were observed prior to the 70 day timepoint (Fig. 2B-C; Table S2C). Histologically, we observed cellular, poorly differentiated malignant tumors with a ‘small blue round cell’ morphology, cellular pleomorphism, high mitotic activity and foci of hemorrhage and necrosis, compatible with the histology of SC-PaNEC (Fig. 2D) ³⁷. A high proliferative index was demonstrated further by both anti-proliferating cell nuclear antigen (PCNA) and anti-Ser10 phosphorylated histone H3 (pHH3) immunostaining (Fig. 2E).

***Tp53* mutations are not essential for small-cell pancreatic neuroendocrine carcinoma (SC-PaNEC) formation**

The near-complete penetrance of SC-PaNEC development in dmKO animals, which are derived from a *tp53*^{+/-} intercross wherein 75% of the individuals are expected to have at least one wild-type copy of *tp53*, provided preliminary evidence that *tp53* does not have an essential gatekeeper function in preventing SC-PaNEC tumorigenesis. In order to validate this further, we first performed genotyping of *tp53* in each dmKO animal and showed that indeed the clutch followed expected Mendelian ratios (H_0 = non-Mendelian; $p = 0.63$) (Table S1D and S2D) ³⁸. We observed no correlation between *tp53* genotype and incidence of SC-PaNEC development ($p > 0.05$; Table S2E) or between SC-PaNEC size and *tp53* genotype ($p > 0.05$; Fig. S4A; Table S2E). Furthermore, no obvious differences in cell death between *tp53*^{-/-} or *tp53*^{+/-} SC-PaNECs could be demonstrated by TUNEL staining (Fig. S4B). In fact, SC-PaNEC cells do not show TUNEL positivity, in stark contrast to the immediately surrounding normal pancreatic cells, possible due to cellular stress from the adjacent neoplasm. Finally, investigation of SC-PaNEC proliferation characteristics across different *tp53* genotypes, by quantification of pHH3 positive cells in tissue sections, did not reveal any genotype-specific differences (Fig. S4C; Table S2F).

Interestingly, the genetic make-up of the *tp53* nullizygous mutant animals, with a distinct Δ4 variant on each chromosome, allows assessment of genomic instability on chr3.p. For this, we performed laser

capture microdissection (LCM) and *tp53* genotyping of SC-PaNECs from the dmKO. We observed chromosomal instability (aneuploidy) involving chr3.p in 66% (n=3) of *tp53*^{-/-} SC-PaNECs in the dmKO setup (Fig. 2F; Table S1E; Fig. S5). In contrast, *tp53*^{+/-} SC-PaNEC (n=3) in the dmKO retained the expected allelic ratios, demonstrating that *tp53* LOH is not occurring and that SC-PaNECs can initiate without biallelic germline mutations in *tp53* (Fig. 2F; Fig. 2G - blue ovals). Confirming this further, we showed by similar LCM and genotyping methodology that two tumors in the tmKO setup (n=7) developed without biallelic CRISPR/Cas9-mediated inactivation of the *tp53* gene (red ovals – Fig. 2G). Taken together, we found that inactivation of *tp53* is not essential for SC-PaNEC tumor initiation, but provide, albeit preliminary, evidence that its inactivation might drive genomic instability³¹.

***Rbl1* functions as a tumor suppressor in SC-PaNEC**

Given that we could not detect any development of SC-PaNECs in smKO (*rb1* mosaic mutant) animals, but SC-PaNECs were readily detected in *rb1/rbl1* double mosaic mutants (dmKO and tmKO animals), we hypothesized that *rbl1* is functioning as a genuine tumor suppressor gene in SC-PaNEC.

Previously, multiplex CRISPR/Cas9 technology has been described as a platform enabling *in vivo* functional interrogation of genes in pancreatic ductal carcinoma^{39,40}. Namely, genes functioning as genuine tumor suppressors are found to be mutated in tumors due to positive selection for inactivating genomic alterations. We had also previously demonstrated similar positive selection pressure on combined *rb1* and *rbl1* inactivation in a pediatric *X. tropicalis* retinoblastoma model¹⁴.

We performed LCM of SC-PaNECs, followed by both *rb1* and *rbl1* genotyping. We could demonstrate fully penetrant positive selection pressure for both *rb1* and *rbl1* mutations in SC-PaNECs from tmKO (100%; n=7) animals (Table S1F). Similarly, positive selection for simultaneous *rb1* and *rbl1* mutations was found in SC-PaNECs from dmKO (100%; n=6) animals (Fig. 2G). Overall, our data shows that *rb1* inactivation alone, irrespective of *tp53* genotype, is not sufficient to initiate SC-PaNEC in *X. tropicalis*. This appears to be due to redundancy with the *rbl1* gene, since we demonstrate that inactivation of the retinoblastoma pathway by multiplex *rb1/rbl1* inactivation results in highly penetrant SC-PaNEC formation with short latency.

Highly penetrant induction of central nervous system tumors in *rb1* and *rbl1* double mosaic mutants

Given the non-necessity for *tp53* inactivation in SC-PaNEC development, we further aimed to provide a proof-of-principle that p53 can indeed have tumor suppressive functions in other *X. tropicalis* Rb1-deficient cancers. We performed ectoderm-specific CRISPR/Cas9-mediated genome editing of *rb1* and

rb1 in embryos obtained from a *tp53*^{Δ4var1/+} and *tp53*^{Δ4var2/+} intercross, via injection of an animal-dorsal blastomere (Fig. 3A) (Table S1G). Animals were euthanized at metamorphic climax (69 days post-injection) and necropsy revealed that 75% (n=16) of the animals presented with externally visible retinoblastoma development (Fig. 3B). Furthermore, 44% (n=16) of the animals had excessive black skin pigmentation, indicative of a disturbed neuroendocrine reflex in the pituitary or hypothalamus for the regulation of pigment dispersion in dermal melanophores (Fig. 3B-C)⁴¹. This prompted us to investigate the animals for CNS abnormalities. Genotyping of *tp53* was performed by targeted amplicon sequencing for each animal within the setup (Table S1H). Histopathology revealed presence of retinoblastoma and brain-located poorly differentiated and highly malignant small blue round cell tumors (SBRCTs), in respectively 75% and 43% (n=16) of the animals, closely recapitulating previously reported retinoblastoma incidences in *rb1/rb1* crispants (Fig. 3D)¹⁴. The SBRCTs (Fig. 3E – white arrows) likely correspond to either invasive retinoblastoma, pinealoblastoma (trilateral retinoblastoma) or possibly medulloblastoma⁴². In any case, SBRCTs presented with aggressive growth characteristics as indicated by PCNA immunoreactivity (Fig. S6A). Comparison of retinoblastoma and SBRCTs incidences across *tp53* genotype did not reveal any clear correlation ($p > 0.05$; Table S2G). Furthermore, in line with previously published studies in the mouse, histopathological hallmarks of *rb1/rb1* inactivated retinoblastoma did not change upon *tp53* pathway inactivation⁴³. Interestingly however, we also observed penetrant induction of choroid plexus neoplasms (Fig. 3E – black arrow) and glioblastoma (Fig. 3E – red arrow) in the CNS of *tp53*^{Δ4var1/Δ4var2} animals (Fig. S6B). Therefore, we first performed a differential analysis comparing the grade and incidence of choroid plexus tumors across *tp53* genotypes.

***Tp53* mutational status underlies retinoblastoma-mutant choroid plexus tumor progression**

Having observed the presence of choroid plexus tumors (CPT) in *rb1* and *rb1* double crispant tadpoles, we aimed to investigate whether, in line with the clinical data, *tp53* inactivation is linked with an increased CPT grade⁹. For this, anonymized sections from choroid plexi (n=15) were analyzed by a pathologist and classified according to clinical WHO grading criteria in one of three categories: normal, grade 1 (G1) (choroid plexus papilloma) or >G1 (atypical choroid plexus papilloma and choroid plexus carcinoma)⁴⁴. Normal amphibian choroid plexus closely resembles the mammalian structural organization, where ependymal cells, capillaries and pia mater can be readily discriminated at high magnification (Fig. 4A). G1 neoplasms were characterized by cellular disarray, (pseudo-)stratification and loss of polarization (Fig. 4A –arrow), while mitotic figures were relatively rare. In higher grade (>G1) neoplasms, pronounced cellular disarray and severely increased mitotic figures could be observed. Clear

continuity between normal choroid plexus (CP) and neoplasms was also demonstrated (Fig. 4B). By this subclassification, a correlation between *tp53* genotype and choroid plexus neoplasm grade could be demonstrated ($p < 0.01$; Table S2H) (Fig. 4C). Furthermore, immunofluorescence, in a *tp53* ^{$\Delta 4var1/\Delta 4var2$} animal, further revealed profound PCNA immunoreactivity in a >Grade 1 neoplasm, demonstrating high proliferative activity (Fig. 4D – white arrow).

Finally, we performed LCM and targeted amplicon sequencing of the *tp53* locus in choroid plexus tumors and control tissue (Fig S7; Table S1I). As described previously for PaNECs, the genetic make-up of the *tp53* homozygous mutant animals allows assessment of genomic instability on chr3.p. In *tp53* ^{$\Delta 4var1/\Delta 4var2$} , the >G1 choroid plexus neoplasms exhibited either loss of compound heterozygosity or hyperploidy of one $\Delta 4$ -variant, while control tissue (normal brain, bone) exhibited the expected normal allelic ratios (50% each variant) (Fig. 4E - left). Furthermore, in a high-grade CP lesion in a heterozygous *tp53* mutant animal, we could demonstrate LOH of the remaining WT *tp53* allele (Fig. 4E – right). We found lack of positive selection pressure for *rb1* mutations, as we observed that the *rb1* allele remained wild-type in *tp53* nullizygous CPTs ($n = 3$) (Table S1J). As such, *rb1* does not appear to function as a tumor suppressor gene in CPTs.

***Rb1* functions as a tumor suppressor in glioma, while *tp53* inactivation underlies malignant progression**

Further analyzing the CNS of *rb1* and *rb1* multiplex CRISPR/Cas9-engineered animals obtained from a *tp53* ^{$\Delta 4var1/+$} and *tp53* ^{$\Delta 4var2/+$} intercross, we also observed the occurrence of glioma with pronounced intratumoral heterogeneity in 81% ($n=16$) of these animals. A significant correlation between *tp53* genotype and glioma grade was present, with high-grade glioblastoma lesions only seen in *tp53* ^{$\Delta 4var1/\Delta 4var2$} animals ($p < 0.001$) (Fig. 5A,B; Fig. S8A) (Table S2I). In line with murine *Rb1*-mutant glioblastoma, high-grade lesions were characterized by the presence of pleomorphic giant cells, mitotic defects and massive nuclear aneuploidy (Fig. 5A, B) (Supplemental Movie 1)^{5,45}. In depth microscopic analysis of these nuclear abnormalities showed some to be interconnected across distances spanning tens of micrometers (Supplemental Movie 2). In the higher grade lesions, the giant cells were also interspersed with smaller rounder cells with dense nuclei. Here, both cell-types possessed a high proliferative index as demonstrated by PCNA immunostaining (Fig. 5E). Furthermore, we showed heterogeneous immunoreactivity to a GFAP antibody throughout these actively cycling cells, in line with literature and validating astrocytic origin (Fig. S8C)¹⁰. Glioblastoma cells were observed predominantly in areas contiguous with normal proliferative areas (subventricular zone), especially in the forebrain (Fig. 5B-C),

but also in regions not immediately spatially associated with such proliferative zones. Areas of pseudopallisading necrosis or hemorrhaging were absent. We found profound EZH2 immunoreactivity in forebrain lesions associated with the SVZ, pointing to a proneural glioblastoma, at least at this anatomical location (Fig. 5E; Fig. S8B)^{46,47}. Unfortunately, further attempts to perform glioblastoma subtyping were unsuccessful, due to lack of suitable antibodies in *Xenopus tropicalis*.

The presence of astrocytoma in animals exclusively mutant in *rb1* and *rb1l* (Fig. 5A, D), pruned us to investigate whether *rb1l* compensates for the loss of *rb1*, under *tp53* wild-type conditions. For this, we performed a retrospective analysis of brain sections from our previously published retinoblastoma study in animals unilaterally targeted at the two-cell stage with *rb1* and *rb1l* gRNA¹⁴. We detected the presence of astrocytoma in 100% (n=9) of the animals injected with the *rb1/rb1l* gRNAs (Fig. S9). Astrocytoma was also found in animals injected with another pair of *rb1/rb1l* gRNA, ruling out potential CRISPR/Cas9 off-target effects as contributing to the observed phenotype. Interestingly, some astrocytoma lesions in this cohort had spontaneously progressed to high-grade glioblastoma, demonstrating that *rb1/rb1l*-deficient glioma progression is indeed possible *in vivo* (Fig. S9 - black arrow).

We further wanted to investigate whether the requirement for *rb1l* inactivating mutations can be overruled by additional oncogenic insults. Co-inactivation of *pten* was chosen as this gene has been widely linked to glioblastoma malignancy^{5,48}. Therefore, we performed ectoderm-specific CRISPR/Cas9-mediated genome editing of *rb1* and *pten*^{cr1} in embryos obtained from a *tp53*^{Δ4var1/+} and *tp53*^{Δ4var1/+} intercross, thus leaving *rb1l* intact (Table S1K). Animals were euthanized at day 42 and histopathology revealed absence of any glioma (0%; n=14) in either of the three *tp53* genotypes, including four *tp53*^{Δ4var1/Δ4var1} animals (Fig. 5D, Table S1L). In contrast, animals injected with *rb1*, *rb1l*, *tp53*^{cr2} and *pten*^{cr2} in the ectodermal lineage demonstrated complete penetrance in high-grade glioblastoma development (100%; n=13) at day 42 (Fig. 5C-D; Table S1M).

Collectively, our experiments provide the direct genetic evidence that *rb1l* functions as a tumor suppressor compensating for loss of *rb1* in *rb1*-mutant astrocytes and that concomitant inactivation of *rb1/rb1l* is sufficient for glioma initiation. Further, and in line with literature, we demonstrate that *tp53* inactivation facilitates progression of retinoblastoma-deficient glioma to high-grade glioblastoma⁵.

Discussion

Members of the retinoblastoma pocket protein family (RB1, RBL1, RBL2) play a pivotal role in the control of cellular proliferation and their direct inactivation by mutational events has been previously

implicated in cancers, such as small-cell lung carcinoma (SCLC) and retinoblastoma^{13,49}. However, contemporary approaches for understanding and modeling cancer has conceptualized that signaling networks can be disturbed at diverse nodes, converging on similar downstream phenotypical consequences. For instance, within the RB signaling network, the ability of RB1 and RBL1 to regulate the cell cycle can be bypassed by events altering their common upstream cyclin dependent kinase regulators (*i.e* CDK4, CDKN2A/p16). Such direct genetic deregulation of the RB signaling network by either *CDK4* amplification, or inactivation of *CDKN2A/B* or *RB1*, had been previously shown in human SC-PaNEC and glioblastoma^{4,50–52}. In contrast, direct genetic inactivation of *RBL1* (*p107*) remains rare (<0.5%) in clinical glioma samples. However, reanalyzing cancer genomic data for combined cohort of 8 glioma studies validated mutual exclusivity between *CDKN2A/B* and *CDK4* alterations ($p < 0.001$), but established significant co-occurrence of *RB1* and *RBL1* alterations ($p = 0.034$) (Table S3)^{53,54}. As such we postulate that directly inactivating *rb1* and *rb1* could contribute to cancer initiation, similar to indirect simultaneous inactivation of RB1 and RBL1 protein function due to prolonged phosphorylation by upstream Cyclin/CDK complexes. This hypothesis is further supported by reports that RBL1 is downregulated in glioma tumors and cell lines⁵⁵. Furthermore, *Rb1* deficiency alone fails to initiate tumorigenesis in astrocytes, while transgenic *in vivo* expression of a truncated SV40 large T antigen (T₁₂₁), which binds and inhibits all three retinoblastoma family members, is sufficient for tumorigenesis⁵⁶. Additionally, a *Rb1/Rbl1/Tp53/Pten* conditional murine glioblastoma model was previously generated and used as a platform for preclinical studies^{47,57}. However because the full characterization of this line is, up to this date, not reported we cannot delineate the exact impact of *Rbl1* inactivation. Finally, it was shown that *Rbl1*-deficient mice exhibit expanded neural stem cell population and impaired commitment of neuronal progenitors to a neuronal fate, showing previously underappreciated functions of Rbl1^{58,59}. In fact, to our knowledge the definitive genetic *in vivo* elucidation of *RBL1* as a glioma tumor suppressor has not been reported before. Here we showed that *rb1* inactivation is a cooperative event in the rapid establishment of glioma in immunocompetent *rb1*-deficient *Xenopus tropicalis*. Furthermore, we believe that while direct genetic inactivation of the *RBL1* gene is rare (0.3%) in glioma, additional mechanisms are at play in which the RBL1 protein could in fact be a major player in suppressing tumorigenesis, but has had its role previously seen minimized due to its indirect inactivation at a functional level.

We also demonstrate that concomitant inactivation of *rb1* and *rb1* is sufficient to induce SC-PaNEC. Unfortunately, as a consequence of the low incidence of SC-PaNEC, no large sets of whole-exome or whole-genome sequencing data are available to address the status of the *RBL1* gene in clinical SC-PaNEC. Nevertheless, previous studies already suggested functional compensation between pocket proteins in

the endocrine pancreas, where inactivation of *Rb1* has little effect on β -cell replication, while concomitant *Rb1/Rbl1* and *Rb1/Rbl2* inactivation has profound impact on proliferation and apoptosis^{60–62}.

In contrast to these newly discovered *rb1* tumor suppressor roles, we also show that choroid plexus tumorigenesis does not critically depend on *rb1* inactivation. Additionally, while we had previously and incidentally discovered a small cell lung carcinoma in a *rb1/rbl1* crispant¹⁴, we fail to identify these in larger cohorts of *rb1/rbl1* crispants, across different *tp53* genotypes. Interestingly, this is in line with mice modeling data placing *Rbl2* (*p130*), and not *Rbl1*, at the center of tumor suppression in *rb1*-deficient endocrine lung cells⁴⁹. This reflects, cell-type specific responses to inactivation of distinct retinoblastoma network elements, whose interplay could be further dissected using CRISPR/Cas9 in *Xenopus*¹¹.

Furthermore, we also co-interfered with the cell signaling death network by targeting *tp53*, in order to gain insight in how co-occurring p53 aberrations influences *rb1/rbl1*-deficient cancers. In contrast to the accepted paradigm that concomitant p53 and retinoblastoma signaling network abnormalities are required to initiate SC-PaNEC, we demonstrate unexpected low impact of *tp53* inactivation in *rb1/rbl1*-inactivated SC-PaNEC². In line with our results, the implication of p53 in clinical SC-PaNEC is predominated by abnormal immunostaining of the p53 protein, rather than absence of reactivity, which would be indicative for p53 loss-of-expression^{63,64}. We speculate that *TP53* mutational events could be dispensable in early SC-PaNEC tumorigenesis, but that *TP53* alterations and their consequent chromosomal instability could eventually leads to highly malignant and metastatic SC-PaNEC²⁹. Unfortunately SC-PaNEC is considered to be unresectable and mostly metastatic at the time of diagnosis and hence patients are almost immediately treated with chemotherapy, precluding clinical assessment of *TP53* alterations in early stages of SC-PaNEC development. Regrettably, the tumors in our *Xenopus* model are too fast growing, leading to morbidity and ethical endpoint prior to metastasis formation, impeding assessment of this hypothesis.

In contrast to the above and closely recapitulating the known roles of p53, we demonstrate that in CPTs and glioblastoma, wild-type *X. tropicalis* p53 is able to limit tumor progression. Namely, *tp53* nullizygous choroid plexus tumors and gliomas demonstrate more malignant histological characteristics, higher proliferation and increased chromosomal instability, in line with the published clinical human and mice experimental data^{3,9,65}.

In this paper we also describe the first *tp53* nullizygous *X. tropicalis* line and demonstrate that *Xenopus tp53* behaves, analogous to *TP53* in Li-Fraumeni, as a tumor suppressor gene for syndromic *Xenopus* cancer development. We demonstrate that *tp53* mutations are predisposing for a Li-Fraumeni

tumor spectrum, namely hematological malignancies and sarcomas, admittedly with a long latency of >1 year. We believe the latter to be attributable to the life-span of *X. tropicalis* that is substantially longer (>3x) than mice and may also underlie interspecies physiological differences. Interestingly, our *tp53*^{Δ4var1/Δ4var2} compound heterozygote line enables easy identification of each *tp53* allele. This allows tracking of local chromosomal instability during cancer initiation and progression by straightforward amplicon sequencing of both distinct *tp53* CRISPR/Cas9 deletion scars, permitting rapid assessment of *tp53* focal amplification, aneuploidy events or loss of heterozygosity.

When comparing our novel *X. tropicalis* cancer models to established zebrafish models, neither *tp53* nor *rb1* mutant zebrafish have been reported to develop Li-Fraumeni-related hematological malignancies, SC-PaNEC or CPT²⁷. In fact, in contrast to the clinical situation, *tp53* mutant fish develop predominantly malignant peripheral nerve sheath tumors and *rb1* TALENs F0 edited *tp53* mutant fish develop primitive neuro-ectodermal tumors and medulloblastoma^{66,67}.

In the comparison to established mammalian cancer models, we believe that *Xenopus* holds unique experimental advantages such as extremely straightforward tissue-restrictive CRISPR/Cas9 delivery and multiplexing in externally developing embryos¹⁸. Additionally, the *X. tropicalis* SC-PaNEC, glioblastoma and choroid plexus cancer models we report here are competitive with or outperform the existing *Rb1/tp53*-inactivated mice models in terms of latency and penetrance of tumor development (Table S4).

In synopsis, we demonstrate the use of tissue-restricted multiplexed inactivation in *Xenopus* embryos to functionally identify novel tumor suppressor combinations *in vivo*. This nicely complements AAV-mediated *in vivo* murine CRISPR screens^{14,45}. We established novel highly penetrant and short latency genetic *X. tropicalis* cancer models upon multiplex *in vivo* CRISPR/Cas9-mediated inactivation of the *rb1*, *rb1* and *tp53* tumor suppressor genes. These models will generate novel opportunities for gene function interrogation, therapeutic target identification and pre-clinical drug studies. Finally, showcasing the possibilities of multiplexed CRISPR/Cas9 in delineating novel driver mutations in cancer, we establish formal proof of *rb1* as a tumor suppressor in glioblastoma and small-cell pancreatic neuroendocrine carcinoma.

Material and Methods

Generation of *X. tropicalis* mosaic mutants by CRISPR/Cas9

All gRNAs were designed with the CRISPRScan algorithm (<http://www.crisprscan.org/>)⁶⁸. In this study following sequences were targeted. *Rb1* 5'-GCTGTATGATTGTGCTGTACCGG-3', *rb1*

TGGGCTTGC GCGCTGATGTGGGG, *tp53*^{cr1} 5'-CCTCAACTGAGGATTACGCAGGG-3', *tp53*^{cr2} 5'-
GAAGAGCTTGTGAGGTCGGTGG-3', *pten*^{cr1} 5'-GCGCTTGGGACCTGCTGTTGAGG-3', *pten*^{cr2} 5'-
GAGTTACAATTCCCAGCCAAAGG-3'. gRNAs were generated by *in vitro* transcription, employing the oligos
shown in supplementary table 5A, and quality control and quantification was performed as described
before^{69,70}. Recombinant NLS-Cas9-NLS protein was generated as previously described¹⁴. Natural *X.*
tropicalis matings were performed and embryos were microinjected in either the two- or the eight-cell
stage with injection mixes containing precomplexed gRNA and Cas9 protein as shown in supplementary
table 5B. Approval was obtained from the Ethical Committee for Animal Experimentation, Ghent
University, Faculty of Science and VIB-Site Ghent (EC2018-079). All methods were carried out in
accordance with the relevant guidelines set out by this committee.

Next-generation amplicon sequencing

Targeted amplicon sequencing followed by BATCH-GE analysis was performed for quantitative
assessment of genome editing, with the primer pairs shown in supplementary table 5C, as described
before⁷¹⁻⁷³. For *tp53* genotyping of animals after necropsy, spinal cord (ectoderm targeted) or heart
(anterior endoderm targeted) tissue were lysed overnight in lysis buffer (50 mM Tris pH 8.8, 1 mM EDTA,
0.5% Tween-20, 200 µg/ml proteinase K) and targeted amplicon sequencing of *tp53* was performed
identically as above. For next-generation amplicon sequencing from laser-capture microdissected
tumors, PCR input DNA was generated as previously described⁶⁹. PCR amplification relevant genomic
regions was performed with the (nested) primers shown in table 5D. All quantitative genome editing
efficiencies and INDEL variants, with their relative frequencies, in this study are shown in supplementary
Table 1.

Ex-vivo micro-CT imaging

For ex-vivo micro-CT imaging of *tp53* heterozygous animals, cone-beam micro-CT was employed
(Triumph-II, TriFoil Imaging, Northridge, CA, USA). A high-resolution micro-CT imaging protocol was used
with the following acquisition parameters: one bed position (90 mm field of view), circular trajectory
with continuous rotation over 360 degrees, 50 µm focal spot size, 50 µm detector pixel size, 1.3 times
magnification, 512 projections, 370 µA tube current and 50 kVp tube voltage, resulting in a total
acquisition time of 14 minutes. The acquired projection data were reconstructed into a three-
dimensional image with 512x512x1024 matrix and 100 µm voxel size using the Feldkamp-Davis-Kress
algorithm.

Flow Cytometry and Natt-Herrick leukocyte counting

Peripheral blood was collected from wild-type or mutant *X. tropicalis* by toe clipping and immediately suspended in cold 0.66x PBS (amphibian PBS or APBS). Cells were fixed for 20 minutes using fresh 2% paraformaldehyde, blocked/permeabilized in 2% Natural Goat Serum, 1% BSA (VWR) and 0.1% Tween 20 (Sigma-Aldrich) in APBS solution. After 10 minutes of blocking and permeabilization, cells were incubated for one hour either with a 1:5 dilution of Rat anti Human CD3:Alexa Fluor®647 (0.05 mg/mL) (clone CD3-12; Bio-Rad) or with undiluted CD8 (AM22) mouse hybridoma supernatant (0.05 mg/mL). Cells incubated with hybridoma supernatant were additionally incubated with DyLight® 488 secondary antibody for one hour. Finally, cells were acquired on a 5 laser-BD LSR Fortessa flow cytometer (BD Biosciences) and analyzed using the FlowJo software (Tree Star). For validation, blood was collected by cardiac bleed from euthanized animals and diluted 1:50 in Natt-Herrick (NH) staining solution, prepared as described before⁷⁴. NH preperates were loaded in a Buerker hemocytometer (Marienfeld) where after leukocytes and red blood cells (RBC) were counted. For each data-point in the statistical analysis, leukocytes and RBC were counted in twelve 0.04 mm² regions. Percentage leukocytes was calculated as (#leukocytes/(#leukocytes+#RBC)).

Histology, immunohistochemistry and nuclear modeling of glioblastoma cells

All macroscopic pictures were taken with a Carl Zeiss StereoLUMAR.V12 stereomicroscope. For quantification of SC-PaNEC sizes, ImageJ was used. For histology, tissues were fixed overnight in 4% paraformaldehyde (PFA) at 4°C. Bone-containing tissues (*e.g.* cranial structures) were decalcified by Morse's solution (10% sodium citrate and 22.5% formic acid) for 6 hours at room temperature. All tissue samples were dehydrated, imbedded in paraffin and 5 µM tissue sections were made by microtomy. For classical histopathology, slides were stained with hematoxylin and eosin. Choroid plexuses were analyzed by a clinical pathologist (D. C.) and assigned a WHO grade. Immunohistochemistry was performed as previously described with primary antibodies: anti-PCNA antibody (PC10; Dako), anti-pHH3 antibody (IHC-00061; Bethyl laboratories), anti-CD3 antibody (clone CD3-12; Bio-Rad), anti-GFAP (Z0334; DAKO), anti-cleaved-caspase3 (9661; CST) and anti-EZH2 antibody (A304-197A-T; Bethyl Laboratories)⁷⁵. TUNEL was performed using the In Situ Cell Death Detection Kit, AP (11684809910, Roche) according to manufacturer's instructions. In all immunohistochemical experiments, omission of either the primary antibody or the TUNEL labeling mix served as a negative control. For immunofluorescent (IF) goat anti-mouse DyLight-633 (ThermoFisher) and goat anti-rabbit DyLight-633 (ThermoFisher) was used. All IF and TUNEL samples were counterstained with Hoechst-33342. SC-PaNEC PCNA IF was captured with a Leica

TCS LSI zoom confocal microscope. Choroid plexus tumor PCNA IF and Hoechst-33324 for glioma nuclear modeling were collected on an LSM880 Airyscan (Carl Zeiss, Jena, Germany) using either a LD LCI Plan-Apochromat 25x/0.8 Imm Korr DIC M27 or a 63x PlanApo NA:1.4 oil immersion DIC M27 objective. The operating software was ZEN blue 2.3. The Airyscan detector was used in both the fast and full superresolution mode. A pixel reassignment algorithm in combination with a Wiener filter were carried out post acquisition. In order to gain nuclear 3D information, z-stacks were recorded and a 3D reconstruction of relevant z-stacks was made in Volocity 6.3.0 (Perkin Elmer). Furthermore, nuclear material from glioblastoma cells was segmented, employing MIB, and a 3D model of the nuclear membrane was build using Imaris (Bitplane)⁷⁶. TUNEL, pHH3 and GFAP IF was captured on a Leica TCS SP5 confocal microscope using the tile scan and image stitching function. For IHC with DAB detection of EZH2, PCNA, CD3 and Cleaved-Caspase 3, Goat Anti-Rabbit IgG (H+L) (VEC.BA-1000; Vector laboratories) was used. Signal was developed using the VECTASTAIN Elite ABC HRP Kit (PK-6100; Vector laboratories) and ImmPACT DAB Peroxidase (SK-4105; Vector laboratories). Samples were counterstained 1 minute with hematoxylin. Imaging of H&E stained and DAB/hematoxylin stained sections was performed with a Zeiss Axio Scan.Z1 equipped with a 20X Plan-Apochromat 0.8 NA dry objective, using a Hitachi HV-F202SCL camera. High-magnification photomicrographs of glioblastoma cells were taken with an Zeiss Axioscope using a 100x oil objective. To quantify cell proliferation in tumors, we performed automated counting of pHH3⁺ cells using ImageJ, normalizing pHH3⁺ cells over tumor section volume.

Acknowledgments

Research in the authors' laboratory is supported by the Research Foundation – Flanders (FWO-Vlaanderen) (grants G0A1515N and G029413N), by the Belgian Science Policy (Interuniversity Attraction Poles - IAP7/07) and by the Concerted Research Actions from Ghent University (BOF15/GOA/011). Further support was obtained by the Hercules Foundation, Flanders (grant AUGÉ/11/14) and the Desmoid Tumor Research Foundation. T.N. is funded by “Kom op tegen Kanker” (Stand up to Cancer), the Flemish cancer society and previously held PhD fellowship with VLAIO-HERMES during the course of this work. We thank the *Xenopus laevis* Resource for Immunobiology (Rochester, NY, NIH R24 AI059830) for the kind gift of monoclonal antibody AM20 (10A91, CD8). We are indebted to Tim Deceuninck for animal care, Kelly Lemeire for technical assistance with TUNEL staining and Marjolein Carron for BATCH-GE analysis of sequencing data. We would like to thank the VIB BioImaging Core, and in particular Chris Guerin, Eef Parthoens and Anneke Kremer, for access to the instrument park, training and support. In

conclusion, we would also like to acknowledge Marjolein Carron for critical proof-reading of this manuscript.

Author information

Affiliations

Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

Thomas Naert, Dionysia Dimitrakopoulou, Dieter Tulkens, Suzan Demuynck, Liza Eeckhout, Rivka Noelanders, Kris Vleminckx

Cancer Research Institute Ghent, Ghent, Belgium

Thomas Naert, Dionysia Dimitrakopoulou, Dieter Tulkens, Christian Vanhove, Jo Van Dorpe, David Creytens, Kris Vleminckx

Center for Medical Genetics, Ghent University, Ghent, Belgium

Kris Vleminckx

VIB Center for Inflammation Research, Ghent, Belgium

Gert Van Isterdael

Laboratory for Pharmaceutical Biotechnology, Ghent University, B-9000 Ghent, Belgium.

Dieter Deforce

Department of Pathology, Ghent University and Ghent University Hospital, Ghent, Belgium

David Creytens, Jo Van Dorpe

Infinity lab, Ghent University Hospital, Ghent, Belgium.

Christian Vanhove

Contributions

T.N, D.Di. and K.V. designed the study. D.Di., T.N, D.T. and R.N. were involved in generation and phenotyping of the *tp53* mutant *X. tropicalis*. T.N. performed genome engineering and phenotyping of all *retinoblastoma* and *retinoblastoma/tp53* mutants. T.N., L.E. and D.De were involved in laser-capture microdissection and downstream analysis. D.C. and J.v.D performed pathological analysis. D.T., D.Di. and G.v.I performed flow cytometry experiments. T.N. performed cancer genomic database mining. C.V. performed X-ray imaging. S.D. provided technical assistance throughout the project. T.N. and K.V. wrote the manuscript.

References

1. Konukiewitz, B. *et al.* Pancreatic neuroendocrine carcinomas reveal a closer relationship to ductal adenocarcinomas than to neuroendocrine tumors G3. *Hum. Pathol.* **77**, 70–79 (2018).
2. Glenn, S. T. *et al.* Conditional deletion of p53 and Rb in the renin-expressing compartment of the pancreas leads to a highly penetrant metastatic pancreatic neuroendocrine carcinoma. *Oncogene* **33**, 5706–15 (2014).
3. Tong, Y. *et al.* Cross-Species Genomics Identifies TAF12, NFYC, and RAD54L as Choroid Plexus Carcinoma Oncogenes. *Cancer Cell* **27**, 712–727 (2015).
4. McLendon, R. *et al.* Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* **455**, 1061–1068 (2008).
5. Chow, L. M. L. *et al.* Cooperativity within and among Pten, p53, and Rb pathways induces high-grade astrocytoma in adult brain. *Cancer Cell* **19**, 305–16 (2011).
6. George, J. *et al.* Comprehensive genomic profiles of small cell lung cancer. *Nature* **524**, 47–53 (2015).
7. Hann, C. L. & Rudin, C. M. Management of small-cell lung cancer: incremental changes but hope for the future. *Oncology (Williston Park)*. **22**, 1486–92 (2008).
8. Basturk, O. *et al.* The High-grade (WHO G3) Pancreatic Neuroendocrine Tumor Category Is Morphologically and Biologically Heterogenous and Includes Both Well Differentiated and Poorly Differentiated Neoplasms. *Am. J. Surg. Pathol.* **39**, 683–690 (2015).
9. Tabori, U. *et al.* TP53 alterations determine clinical subgroups and survival of patients with choroid plexus tumors. *J. Clin. Oncol.* **28**, 1995–2001 (2010).
10. Chow, R. D. *et al.* AAV-mediated direct in vivo CRISPR screen identifies functional suppressors in glioblastoma. *Nat. Neurosci.* **20**, 1329–1341 (2017).
11. Dannenberg, J.-H., Schuijff, L., Dekker, M., Van Der Valk, M. & Te Riele, H. Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. (2004). doi:10.1101/gad.322004
12. Costa, C., Paramio, J. M. & Santos, M. Skin Tumors Rb(eing) Uncovered. *Front. Oncol.* **3**, 307 (2013).
13. Robanus-Maandag, E. *et al.* p107 is a suppressor of retinoblastoma development in pRb-deficient mice. *Genes Dev.* **12**, 1599–609 (1998).
14. Naert, T. *et al.* CRISPR/Cas9 mediated knockout of rb1 and rbl1 leads to rapid and penetrant retinoblastoma development in *Xenopus tropicalis*. *Sci. Rep.* **6**, (2016).
15. MacPherson, D. *et al.* Murine bilateral retinoblastoma exhibiting rapid-onset, metastatic progression and N-myc gene amplification. *EMBO J.* **26**, 784–94 (2007).
16. Xiao, A., Wu, H., Pandolfi, P. P., Louis, D. N. & Van Dyke, T. Astrocyte inactivation of the pRb

582 pathway predisposes mice to malignant astrocytoma development that is accelerated by PTEN
583 mutation. *Cancer Cell* **1**, 157–68 (2002).

584 17. Lu, X. *et al.* Selective Inactivation of p53 Facilitates Mouse Epithelial Tumor Progression without
585 Chromosomal Instability. *Mol. Cell. Biol.* **21**, 6017–6030 (2001).

586 18. Naert, T., Van Nieuwenhuysen, T. & Vleminckx, K. TALENs and CRISPR/Cas9 fuel genetically
587 engineered clinically relevant *Xenopus tropicalis* tumor models. *genesis* **55**, e23005 (2017).

588 19. DeLay, B. D. *et al.* Tissue-specific Gene Inactivation in *Xenopus laevis* : Knockout of *lhx1* in the
589 Kidney with CRISPR/Cas9. *Genetics* genetics.300468.2017 (2017).
590 doi:10.1534/genetics.117.300468

591 20. Wirt, S. E. & Sage, J. p107 in the public eye: an Rb understudy and more. *Cell Div.* **5**, 9 (2010).

592 21. Li, F. P. & Fraumeni, J. F. Soft-tissue sarcomas, breast cancer, and other neoplasms. A familial
593 syndrome? *Ann. Intern. Med.* **71**, 747–52 (1969).

594 22. Malkin, D. *et al.* Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and
595 other neoplasms. *Science* **250**, 1233–8 (1990).

596 23. Bougeard, G. *et al.* Revisiting Li-Fraumeni Syndrome From *TP53* Mutation Carriers. *J. Clin. Oncol.*
597 **33**, 2345–2352 (2015).

598 24. Donehower, L. A. *et al.* Mice deficient for p53 are developmentally normal but susceptible to
599 spontaneous tumours. *Nature* **356**, 215–221 (1992).

600 25. Jacks, T. *et al.* Tumor spectrum analysis in p53-mutant mice. *Curr. Biol.* **4**, 1–7 (1994).

601 26. Purdie, C. A. *et al.* Tumour incidence, spectrum and ploidy in mice with a large deletion in the p53
602 gene. *Oncogene* **9**, 603–9 (1994).

603 27. Ignatius, M. S. *et al.* tp53 deficiency causes a wide tumor spectrum and increases embryonal
604 rhabdomyosarcoma metastasis in zebrafish. *Elife* **7**, (2018).

605 28. Berghmans, S. *et al.* tp53 mutant zebrafish develop malignant peripheral nerve sheath tumors.
606 *Proc. Natl. Acad. Sci.* **102**, 407–412 (2005).

607 29. Manning, A. L., Benes, C. & Dyson, N. J. Whole chromosome instability resulting from the
608 synergistic effects of pRB and p53 inactivation. *Oncogene* **33**, 2487–94 (2014).

609 30. Levine, A. J. p53, the Cellular Gatekeeper for Growth and Division. *Cell* **88**, 323–331 (1997).

610 31. Eischen, C. M. Genome Stability Requires p53. *Cold Spring Harb. Perspect. Med.* **6**, a026096
611 (2016).

612 32. Naert, T. & Vleminckx, K. CRISPR/Cas9 disease models in zebrafish and *Xenopus*: The genetic
613 renaissance of fish and frogs. *Drug Discov. Today Technol.* **28**, 41–52 (2018).

614 33. Lau, C.-H. & Suh, Y. In vivo genome editing in animals using AAV-CRISPR system: applications to
615 translational research of human disease. *F1000Research* **6**, 2153 (2017).

616 34. Boel, A. *et al.* BATCH-GE: Batch analysis of Next-Generation Sequencing data for genome editing

617 assessment. *Sci. Rep.* **6**, 30330 (2016).

618 35. Neely, H. R., Guo, J., Flowers, E. M., Criscitiello, M. F. & Flajnik, M. F. 'Double-duty' conventional
619 dendritic cells in the amphibian *Xenopus* as the prototype for antigen presentation to B cells. *Eur.*
620 *J. Immunol.* **48**, 430–440 (2018).

621 36. Ward, J. M. *et al.* Splenic marginal zone B-cell and thymic T-cell lymphomas in p53-deficient mice.
622 *Lab. Invest.* **79**, 3–14 (1999).

623 37. Wang, Z. *et al.* Pseudo-hemorrhagic region formation in pancreatic neuroendocrine tumors is a
624 result of blood vessel dilation followed by endothelial cell detachment. *Oncol. Lett.* **15**, 4255–
625 4261 (2018).

626 38. Montoliu, L. Mendel: a simple excel workbook to compare the observed and expected
627 distributions of genotypes/phenotypes in transgenic and knockout mouse crosses involving up to
628 three unlinked loci by means of a χ^2 test. *Transgenic Res.* **21**, 677–681 (2012).

629 39. Chiou, S.-H. *et al.* Pancreatic cancer modeling using retrograde viral vector delivery and in vivo
630 CRISPR/Cas9-mediated somatic genome editing. *Genes Dev.* **29**, 1576–85 (2015).

631 40. Maresch, R. *et al.* Multiplexed pancreatic genome engineering and cancer induction by
632 transfection-based CRISPR/Cas9 delivery in mice. *Nat. Commun.* **7**, (2016).

633 41. van Zoest, I. D., Heijmen, P. S., Crujisen, P. M. J. M. & Jenks, B. G. Dynamics of background
634 adaptation in *Xenopus laevis*: Role of catecholamines and melanophore-stimulating hormone.
635 *Gen. Comp. Endocrinol.* **76**, 19–28 (1989).

636 42. Marino, S., Vooijs, M., van Der Gulden, H., Jonkers, J. & Berns, A. Induction of medulloblastomas
637 in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the
638 cerebellum. *Genes Dev.* **14**, 994–1004 (2000).

639 43. McEvoy, J. *et al.* Coexpression of normally incompatible developmental pathways in
640 retinoblastoma genesis. *Cancer Cell* **20**, 260–75 (2011).

641 44. Louis, D. N. *et al.* The 2016 World Health Organization Classification of Tumors of the Central
642 Nervous System: a summary. *Acta Neuropathol.* **131**, 803–820 (2016).

643 45. Chow, R. D. *et al.* AAV-mediated direct in vivo CRISPR screen identifies functional suppressors in
644 glioblastoma. *Nat. Neurosci.* **20**, 1329–1341 (2017).

645 46. Jin, X. *et al.* Targeting glioma stem cells through combined BMI1 and EZH2 inhibition. *Nat. Med.*
646 **23**, 1352–1361 (2017).

647 47. Steed, T. C. *et al.* Differential localization of glioblastoma subtype: implications on glioblastoma
648 pathogenesis. *Oncotarget* **7**, 24899–24907 (2016).

649 48. Koul, D. PTEN signaling pathways in glioblastoma. *Cancer Biol. Ther.* **7**, 1321–5 (2008).

650 49. Schaffer, B. E. *et al.* Loss of p130 accelerates tumor development in a mouse model for human
651 small-cell lung carcinoma. *Cancer Res.* **70**, 3877–83 (2010).

652 50. Yachida, S. *et al.* Small Cell and Large Cell Neuroendocrine Carcinomas of the Pancreas are

653 Genetically Similar and Distinct From Well-differentiated Pancreatic Neuroendocrine Tumors. *Am.*
654 *J. Surg. Pathol.* **36**, 173–184 (2012).

655 51. Abou-El-Ardat, K. *et al.* Comprehensive molecular characterization of multifocal glioblastoma
656 proves its monoclonal origin and reveals novel insights into clonal evolution and heterogeneity of
657 glioblastomas. *Neuro. Oncol.* **19**, 546–557 (2017).

658 52. Wiedemeyer, W. R. *et al.* Pattern of retinoblastoma pathway inactivation dictates response to
659 CDK4/6 inhibition in GBM. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 11501–6 (2010).

660 53. Cerami, E. *et al.* The cBio Cancer Genomics Portal: An Open Platform for Exploring
661 Multidimensional Cancer Genomics Data. *Cancer Discov.* **2**, 401–404 (2012).

662 54. Gao, J. *et al.* Integrative Analysis of Complex Cancer Genomics and Clinical Profiles Using the
663 cBioPortal. *Sci. Signal.* **6**, pl1-pl1 (2013).

664 55. Liu, F. *et al.* MicroRNA-106b-5p boosts glioma tumorigenesis by targeting multiple tumor
665 suppressor genes. *Oncogene* **33**, 4813–4822 (2014).

666 56. Vitucci, M. *et al.* Genomic profiles of low-grade murine gliomas evolve during progression to
667 glioblastoma. *Neuro. Oncol.* **19**, 1237–1247 (2017).

668 57. Wojton, J. *et al.* Systemic Delivery of SapC-DOPS Has Antiangiogenic and Antitumor Effects
669 Against Glioblastoma. *Mol. Ther.* **21**, 1517–1525 (2013).

670 58. Vanderluit, J. L. *et al.* p107 regulates neural precursor cells in the mammalian brain. *J. Cell Biol.*
671 **166**, 853–863 (2004).

672 59. Vanderluit, J. L. *et al.* The Retinoblastoma family member p107 regulates the rate of progenitor
673 commitment to a neuronal fate. *J. Cell Biol.* **178**, 129–139 (2007).

674 60. Harb, G., Vasavada, R. C., Cobrinik, D. & Stewart, A. F. The retinoblastoma protein and its
675 homolog p130 regulate the G1/S transition in pancreatic beta-cells. *Diabetes* **58**, 1852–62 (2009).

676 61. Cai, E. P. *et al.* Rb and p107 are required for alpha cell survival, beta cell cycle control and
677 glucagon-like peptide-1 action. *Diabetologia* **57**, 2555–2565 (2014).

678 62. Vasavada, R. C., Cozar-Castellano, I., Sipula, D. & Stewart, A. F. Tissue-specific deletion of the
679 retinoblastoma protein in the pancreatic beta-cell has limited effects on beta-cell replication,
680 mass, and function. *Diabetes* **56**, 57–64 (2007).

681 63. Konukiewitz, B. *et al.* Somatostatin receptor expression related to TP53 and RB1 alterations in
682 pancreatic and extrapancreatic neuroendocrine neoplasms with a Ki67-index above 20%. *Mod.*
683 *Pathol.* **30**, 587–598 (2017).

684 64. Konukiewitz, B. *et al.* Pancreatic neuroendocrine carcinomas reveal a closer relationship to ductal
685 adenocarcinomas than to neuroendocrine tumors G3. *Hum. Pathol.* **77**, 70–79 (2018).

686 65. Merino, D. M. *et al.* Molecular Characterization of Choroid Plexus Tumors Reveals Novel Clinically
687 Relevant Subgroups. *Clin. Cancer Res.* **21**, 184–192 (2015).

688 66. Solin, S. L., Shive, H. R., Woolard, K. D., Essner, J. J. & McGrail, M. Rapid tumor induction in

- zebrafish by TALEN-mediated somatic inactivation of the retinoblastoma1 tumor suppressor rb1.
Sci. Rep. **5**, 13745 (2015).
67. Shim, J. *et al.* Development of zebrafish medulloblastoma-like PNET model by TALEN-mediated somatic gene inactivation. *Oncotarget* **8**, 55280–55297 (2017).
68. Moreno-Mateos, M. A. *et al.* CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. *Nat. Methods* **12**, 982–8 (2015).
69. Naert, T. & Vleminckx, K. *CRISPR/cas9-mediated knockout of RB1 in xenopus tropicalis*. *Methods in Molecular Biology* **1726**, (2018).
70. Naert, T. & Vleminckx, K. Methods for CRISPR/Cas9 *Xenopus tropicalis* Tissue-Specific Multiplex Genome Engineering. *Methods Mol. Biol.* **1865**, 33–54 (2018).
71. Steyaert, W., Boel, A., Coucke, P. & Willaert, A. BATCH-GE: Analysis of NGS Data for Genome Editing Assessment. *Methods Mol. Biol.* **1865**, 83–90 (2018).
72. Naert, T. & Vleminckx, K. Genotyping of CRISPR/Cas9 Genome Edited *Xenopus tropicalis*. *Methods Mol. Biol.* **1865**, 67–82 (2018).
73. Szenker-Ravi, E. *et al.* RSPO2 inhibition of RNF43 and ZNRF3 governs limb development independently of LGR4/5/6. *Nature* **557**, 564–569 (2018).
74. Maxham, L. A., Forzán, M. J., Hogan, N. S., Vanderstichel, R. V. & Gilroy, C. V. Hematologic reference intervals for *Xenopus tropicalis* with partial use of automatic counting methods and reliability of long-term stored samples. *Vet. Clin. Pathol.* **45**, 291–299 (2016).
75. Naert, T. & Vleminckx, K. Cancer Models in *Xenopus tropicalis* by CRISPR/Cas9 Mediated Knockout of Tumor Suppressors. *Methods Mol. Biol.* **1865**, 147–161 (2018).
76. Belevich, I., Joensuu, M., Kumar, D., Vihinen, H. & Jokitalo, E. Microscopy Image Browser: A Platform for Segmentation and Analysis of Multidimensional Datasets. *PLOS Biol.* **14**, e1002340 (2016).

Figure Legends

Figure 1. *Tp53* mutant *X. tropicalis* develop hematological malignancy and sarcomas. (A) Kaplan-Meier survival analysis on cohorts consisting of two clutches of *tp53* homozygous knockout (n=6; red and n=7; blue) and one clutch of heterozygous *tp53* knockout (n=14; black) *X. tropicalis*. Statistical analysis was done using the Prism Mantel-Cox test (ns – not significant; ** $p < 0.01$; *** $p < 0.001$). Chi-Squared values and the Hazard ratios are listed in Supplementary table 2A. **(B)** X-ray imaging of a 28-month old *tp53*^{+/Δ4var2} demonstrating ectopic calcified structures (white arrows). **(C)** (Left panels) In the wild-type spleen, CD3⁺ T-cells form a ring-like structure around the PCNA⁺ B-cells located in the white pulp. (Right panels) Disruption of a normal CD3⁺ ring-like structure observed in a *tp53*^{Δ4var2/Δ4var2} animal is indicative of hematological malignancy. **(D)** Pie chart summarizing the observed splenic immunostaining for CD3 and PCNA in *tp53*^{Δ4var1/Δ4var2} animals. Staining patterns being either normal or with loss of the typical CD3⁺ ring structure with or without ectopic staining of PCNA in the red pulp. **(E)** Photomicrograph of Natt-Herrick stained blood demonstrating a cluster of leukocytes and a normal nucleated erythrocyte. Inset demonstrates a high magnification photomicrograph of a lymphoblast. **(F)** Flow cytometry analysis reveals an increased number of both CD3⁺ (left) and CD8⁺ lymphoblasts (right) in peripheral blood of a *tp53*^{Δ4var1/Δ4var2} animal, when compared to an age-matched control. **(G)** Histopathology of the liver reveals diffuse infiltration of T-lymphoblasts, also enriched within liver capillary (white arrow; top inset) and in between the liver parenchymal cells (black arrow). **(H)** Sarcoma in a *tp53*^{+/Δ4var2} animal observed upon gross examination, with associated histopathology and demonstration of malignant nature by PCNA proliferation staining. White scale bar is 100 μm and black scale bar is 5 μm.

Figure 2. Small-cell pancreatic neuroendocrine carcinoma (SC-PaNEC) in *X. tropicalis* upon mosaic CRISPR/Cas9 genome editing of *rb1* and *rb1l* in the anterior endoderm. (A) Breeding and CRISPR/Cas9 injection scheme demonstrating the generation of the single mutant KO (smKO -orange) (*rb1* CRISPR/Cas9 injection in embryos obtained from a *tp53*^{Δ4var1/+} x *tp53*^{Δ4var2/+} intercross), double mutant KO (dmKO - blue) (*rb1/rb1l* CRISPR/Cas9 injection in embryos obtained from a *tp53*^{Δ4var1/+} x *tp53*^{Δ4var2/+} intercross) and triple mutant KO (tmKO - purple) (*rb1/rb1l/tp53* CRISPR/Cas9 injection in embryos obtained from intercrossing WT animals). **(B)** Typical external pathology of a pancreatic tumor occurring in a tmKO animal. The inset shows an age-matched control pancreas. **(C)** Tumor incidence curves and Kaplan-Meier analysis comparing smKO (n=13), dmKO (n=29) and tmKO (n=22) genotypes. Statistical analysis, done using the Prism Mantel-Cox test, revealed that SC-PaNEC incidences were significantly different across the experimental setups ($p < 0.0001$). Chi-Squared values and the Hazard ratios are listed

in supplementary table 2C. **(D)** H&E stain of pancreatic tumors shows recurrent histological features of SC-PaNEC with necrotic foci (nec). The black arrow indicates non-neoplastic pancreatic tissue. The inset shows, under higher magnification, diffuse sheets of poorly differentiated cells with small blue round morphology, nuclear pleomorphism and hemorrhages (yellow arrows). **(E)** SC-PaNEC sections immunostained for pHH3 or PCNA, counterstained with Hoechst-33342, reveal high proliferative capacity. **(F)** Laser-capture microdissection (LCM) of dmKO SC-PaNEC reveals chromosomal instability in *tp53*^{-/-} tumors. In *tp53*^{+/-del4} tumors the allelic ratio of the mutant allele remains around 50% (right graph). In contrast, two out of three *tp53*^{-/-} tumors show substantial deviation from the expected allelic ratios (50% each variant) (left graph). **(G)** Percentage of mutant *rb1* and *tp53* reads observed in LCM-derived SC-PaNECs sampled from either tmKO (left) or dmKO (right) animals. Red bullets and blue bullets demarcate SC-PaNECs with monoallelic inactivating *tp53* mutations in the tmKO and dmKO setup, respectively. White scale bar is 500μm, black scale bar is 50μm.

Figure 3. *Rb1* and *rb1* crispants (ectodermal targeted) develop retinoblastoma, excessive black skin pigmentation and a spectrum of brain tumors. **(A)** CRISPR/Cas9 targeting of *rb1* and *rb1* in ectodermal lineage via unilateral injection of an animal-dorsal blastomere. **(B)** Unilateral ectodermal targeting of *rb1/rb1* CRISPR/Cas9, in *tp53* wild-type, heterozygous or compound heterozygous background, results in crispants developing externally visible retinoblastoma (75%; n=16) and excessive skin pigmentation (44%; n=16). Inset shows an animal from the same clutch without external symptoms. **(C)** H&E stained section illustrating increased pigment deposition internal to the stratified epithelium (black arrow). Inset is an animal from the same clutch showing normal pigment deposition. **(D)** H&E stained section of the animal shown in panel A. Unilateral retinoblastoma (black arrow) can clearly be distinguished. Inset shows higher magnification of the retinoblastoma illustrating the classic histopathological characteristics (homer-wright rosettes). **(E)** H&E stained horizontal brain section (anterior side of the animal to the left) revealing multiple neoplasms. Two distinct poorly differentiated, highly malignant, small-blue round cell tumors of unknown origin are shown, most likely representing trilateral retinoblastoma and medulloblastoma (white arrow). Furthermore, a choroid plexus neoplasm can clearly be distinguished within an expanded ventricle (black arrow) and the forebrain harbors a glioblastoma lesion (red arrow). Inset shows the normal *X. tropicalis* brain architecture. White scale bar is 1mm, black scale bar is 100 μm.

Figure 4: Choroid plexus (CP) tumors arising in *rb1/rb1* crispants differ in grade according to their *tp53* genotype. **(A)** H&E stained sections of choroid plexuses representative for the CPT tumor grades

observed within the clutch. (Top panels) Normal choroid plexus architecture consists out of a layer of
ependymal cells, underlying capillaries and pia mater. Insets demonstrate both gross (left) and
histological (right) normal CP architecture. Note that in *Xenopus*, the erythrocytes are nucleated. (Middle
panels) WHO grade 1 (G1) CP neoplasm with cellular disarray, (pseudo)-stratification, atypia and loss of
polarization (black arrow). (Bottom panels) WHO > G1 CP neoplasm with pronounced atypia and
aggressive growth characteristics. **(B)** Choroid plexus tumor in a *tp53*^{+/-} animal showcasing clear
continuity between normal CP and neoplastic tissue. **(C)** Association between *tp53* genotype and grade
of choroid plexus lesions ($p < 0.01$; Table S2H). **(D)** >G1 CP neoplasm immunostained for PCNA (red),
counterstained with Hoechst-33342, reveals high proliferative capacity within highly atypical tumor areas
(white arrow). **(E)** Laser-capture microdissection (LCM) of CP tumors (CPT) reveals chromosomal
instability, as can be appreciated by the differential *tp53* allelic ratios between control and CPT tissues.
Left (*tp53*^{Δ4/Δ4}) control bar show the average of LCM control tissues (n = 2). Right (*tp53*^{Δ4/+}) control is the
allelic ratio in spinal cord control tissue from the animal carrying CPT-5. Gray scale bar is 500 μM, white
scale bar is 100 μM, black scale bar is 50 μM. White scale bar in panel D is 10 μM.

Figure 5: *Rb1* functions as a tumor suppressor in glioblastoma, while *tp53* inactivation underlies progression. **(A)** H&E stained sections of *Xenopus* forebrains containing representative histopathology of
low-grade glioma (left-middle) and high-grade glioblastoma (right) occurring across different
experimental conditions. **(B)** (Left panel) Low grade glioma with the occasional multinucleated giant cell
in *tp53* wild-type and heterozygous animals, targeted in the ectodermal lineage with *rb1* and *rb1*
CRISPR/Cas9. (Right panel) However, when similarly targeting *rb1* and *rb1* in a *tp53* nullizygous animals,
higher-grade glioblastoma lesions develop. **(C)** Quadruple multiplex CRISPR/Cas9-mediated targeting of
rb1, *rb1*, *tp53* and *pten* in the ectodermal lineage leads to fully penetrant (100%; n=13) development of
high-grade glioblastoma lesions at day 42. **(D)** Bar plot representing absence or presence of either low-
grade glioma or high-grade glioblastoma lesions across the performed experimental setups. Genes
shown in bold were edited in the ectodermal lineage by CRISPR/Cas9, while *tp53* genotypes, shown in
grey, were germline inherited. **(E)** (top) PCNA immunostaining reveals high proliferative index in
glioblastoma cells. (bottom) EZH2 immunostaining reveals EZH2 expression in glioblastoma cells, but
absence of expression in the subventricular zone (black arrow). Black scale bar is 100 μM and white scale
bar is 20 μM.

**Supplemental movie 1: Central glioblastoma cell with nuclear aneuploidy and increased number of
nucleoli, surrounded by normal neurons demonstrating two nucleoli.** 3D-reconstruction of Hoechst-

33342 stained nuclei followed by segmented 3D-model of glioblastoma nuclear membrane. This movie demonstrates heavily increased number of nucleoli within the glioblastoma cell.

Supplemental movie 2: Massive interconnected nuclear abnormalities in glioblastoma. 3D-reconstruction of Hoechst-33342 stained nuclei followed by segmented 3D-model of glioblastoma nuclear membrane. This movie demonstrates that nuclear material in seemingly disconnected glioblastoma cells can in fact be interconnected.

Supplementary data: Tables

Table S1: Genotyping by PCR amplification, sequencing (MiSeq) and BATCH-GE analysis. Genotyping performed throughout this study after BATCH-GE processing with genome editing efficiencies and variant calls, subdivided in letter-coded sheets.

Table S2: Statistical Analyses. Raw data and statistical tests performed throughout this study, subdivided in letter coded sheets.

Table S3: Analysis of co-occurrence of RB1 and RBL1 alterations in human glioma.

Table S4: Comparison between established, closest prior art, mice cancer models and the novel *Xenopus* models presented in this study in terms of time-to-onset and penetrance of cancer phenotypes.

Table S5: Primer sequences used for PCR amplification, oligo sequences used for gRNA synthesis and concentrations of injected CRISPR/Cas9 ribonucleoprotein complexes.

829 Figure 1

830

Figure 2

Figure 3

Figure 4

Figure 5