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LABORATORY INVESTIGATION

Functional relevance of genes predicted to be affected by epigenetic alterations in atypical teratoid/rhabdoid tumors

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Abstract

Purpose Atypical teratoid/rhabdoid tumor (ATRT) is a highly malignant brain tumor predominantly arising in infants. Mutations of SWI/SNF chromatin remodeling complex members SMARCB1/INI1 or (rarely) SMARCA4/Brg1 are the sole recurrent genetic lesions. Epigenetic studies revealed a large number of genes predicted to be affected by differential histone modifications in ATRT, but the role of these genes in the biology of ATRT remains uncertain. We therefore aimed at exploring the role of these genes in the detrimental effects of SMARCB1-deficiency.

Methods The functional relevance of 1083 genes predicted to be affected by epigenetic alterations in ATRT was examined in vivo using a *Drosophila melanogaster* model of SMARCB1-deficiency. Human orthologues of genes whose knockdown modified the phenotype in the Gal4-UAS fly model were further examined in ATRT samples and SMARCB1-deficient rhabdoid tumor cells.

Results Knockdown of *Snr1*, the fly orthologue of *SMARCB1*, resulted in a lethal phenotype and epigenetic alterations in the fly model. The lethal phenotype was shifted to later stages of development upon additional siRNA knockdown of 89 of 1083 genes screened in vivo. These included TGF-beta receptor signaling pathway related genes, e.g. *CG10348*, the fly orthologue of transcriptional regulator *PRDM16*. Subsequently, *PRDM16* was found to be over-expressed in ATRT samples and knockdown of *PRDM16* in SMARCB1-deficient rhabdoid tumor cells reduced proliferation.

Conclusions These results suggest that a subset of genes affected by differential histone modification in ATRT is involved in the detrimental effects of SMARCB1-deficiency and also relevant in the biology of ATRT.

Keywords *Drosophila melanogaster* · Malignant rhabdoid tumor · Histone modifications · SMARCB1 · TGFbeta signaling · PRDM16

Introduction

Atypical teratoid/rhabdoid tumor (ATRT) is a highly aggressive brain tumor predominantly arising in infants [1]. Inactivation of SWI/SNF chromatin remodeling complex members SMARCB1 (also known as INI1/hSNF5) [2] or (rarely)

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SMARCA4 (Brg1) [3] are the sole recurrent genetic lesions [4–6]. Despite this apparent genetic homogeneity, several studies have independently shown that ATRT represents an epigenetically heterogeneous disease and can be divided into three molecular subgroups based on gene expression and DNA methylation profiles [6, 7]. While ATRT-SHH is characterized by NOTCH/SHH signaling, ATRT-TYR and ATRT-MYC share activation of BMP and PDGFRB pathways, but also show subgroup-specific differences, e.g. MYC/HOX being higher expressed in ATRT-MYC tumors [6, 7]. Interestingly, chromatin-immunoprecipitation sequencing (ChIP-seq) of activating histone mark H3K27ac yielded clear differences between molecular subgroups, pointing towards subgroup-specific regulatory networks and potential therapeutic targets [6]. Even though biostatistical analysis of ChIP-seq data resulted in a large number of candidate genes potentially affected by differential histone modification, their functional relevance in the detrimental effects of SMARCB1-deficiency and the biology of ATRT remains uncertain.

Drosophila melanogaster allows for high throughput in vivo screening at a rate unmatched by current mammalian models [8] and histone modifications play an important role throughout all stages of fly development [9]. Taking advantage of the high evolutionary conservation of the SWI/SNF chromatin remodeling complex, we already have developed a fly model of SMARCB1-deficiency. Here, ubiquitous and glial-specific siRNA-mediated knockdown of *Snr1*, the fly orthologue of *SMARCB1*, causes a lethal phenotype [10, 11]. Taking advantage of the versatility of this fly model, we aimed at exploring the functional relevance of a large number of candidate genes predicted to be affected by differential histone modification in ATRT.

Materials and methods

Fly stocks

Flies were maintained on *Drosophila* standard food at 25 °C. For crossing experiments, virgin females were mated with male flies of different ages. Strains used were obtained from the Vienna *Drosophila* Resource Center (VDRC, Vienna Austria), the Bloomington *Drosophila* stock collection (BDSC, Bloomington, IN) or kindly provided by C. Klämbt (Institute of Neurobiology, University of Münster, Münster, Germany):

- *UASSnr1*$^{\text{dsRNA}}$ (VDRC, KK108599)
- *UASbrm*$^{\text{dsRNA}}$ (VDRC, GD37721)
- *UASGFP*^{dsRNA} (BDCS 9331)
- *tubulin-Gal4/TM6B* (kindly provided by C. Klämbt)
- *repo-Gal4/repo-Gal4;repo-Gal4/TM6B* (kindly provided by C. Klämbt)
- w^{1118} (kindly provided by C. Klämbt)

ChIP-Seq analysis and bioinformatics

ChIP analysis was performed using *D. melanogaster* chromatin of 2nd instar larvae with ubiquitous knockdown of *Snr1* (tubulin-Gal4/TM6B x *UASSnr1*^{dsRNA}) and controls (*tubulin-Gal4/TM6B x w1118*). The GAL4/upstream activating sequence (UAS) system enables ectopic gene expression in specific tissues or developmental stages. Expression of yeast *Gal4* is activated by a tissue- or developmental specific promotor. The GAL4 transcription factor binds to UAS *cis*-regulatory sites of a target gene and thus activates its transcription. When *tubulin-Gal4/TM6B* flies are crossed to flies carrying *UASSnr1*^{dsRNA} construct, *Snr1* is silenced in all cells in the next generation. For chromatin preparation, 350 mg 2nd instar larvae were collected and stored on − 80 °C. HistonePath ChIP-Seq and TranscriptionPath ChIP-Seq were completed by Active Motif (Carlsbad, CA) by performing the H3K27ac and RNA Pol II ChIP reactions using 4 µg of *D. melanogaster*, 2nd instar larvae chromatin and 4 µg of H3K27ac antibody (Active Motif, cat # 39133) and 20 µl of RNA Pol II antibody (Active Motif, cat # 39097). Library preparation, single read sequencing (NextSeq500 system; high-*output* 75 cycles v2 sequencing reagent kits, Illumina San Diego, CA) were performed at the Core Facility Genomics of the Medical Faculty, University of Münster.

Fly genetic modifier screens

All siRNA lines used for the modifier screen were obtained from VDRC or BDSC (Supplementary Table 1). Candidate genes for the modifier screen were chosen based on epigenetic data generated in ATRT samples [6]. In brief, of 42,619 active enhancers (H3K27ac), 1749 could be aligned to specific genes. For 984 of these genes, one or several *Drosophila* orthologues were identified using the BioMart tool of Ensembl [12], resulting in a total of 1486 *Drosophila* genes. For 1083 of these genes, siRNA strains were available. The fly strain with the genotype *repo-Gal4,UASSnr1*^{dsRNA}*/repo-Gal4,UASSnr1*^{dsRNA}*;repo-Gal4/tubulin-Gal80* and the strain with the genotype *repo-Gal4,UASbrm*^{dsRNA}*/repo-Gal4,UASbrm*^{dsRNA}*;repo-Gal4/tubulin-Gal80* were used to perform the genetic modifier screens. Single knockdown of either *Snr1* or *brm* caused pupal lethality when kept at 25 °C and 29 °C, respectively. When these flies are crossed to flies carrying a second UAS-dsRNA constructs, *Snr1* or *brm* and the additional genes are silenced in glial cells in the F1 generation. The progeny was scored by counting the number of hatched animals in comparison to the whole number of affected pupae. To be classified as a modifier gene,

at least 20% of flies needed to hatch in three independent experiments. Crossings with UASGFP^{dsRNA} served as control. Gene ontology (GO) analyses were performed using the Functional Annotation Tool by DAVID Bioinformatics Resources 6.8 [13, 14].

Human cell culture

The human ATRT cell line BT16 was obtained from ATCC (Manassas, VA) and molecularly characterized in our laboratory. On immunohistochemistry of formalin-fixed paraffin-embedded cell pellets (anti-SMARCB1, 1:200; #612110, BD Biosciences, Heidelberg, Germany) BT16 cells showed lack of nuclear SMARCB1 immunoreactivity. On FISH and *SMARCB1* sequencing [5] an underlying small truncating *SMARCB1* deletion (c.177_178delGA) was identified. DNA was also subjected to methylation profiling using the Infinium Methylation EPIC BeadChip (Illumina). After quality controls, bisulfite conversion and DNA restore according to the manufacturer´s recommendations, data was analyzed using the Brain Tumor Methylation Classifier (v11b4, DKFZ Heidelberg, Germany) and the DNA methylation profile matched best with that of ATRT. Furthermore, a copy number profile derived from 450 k intensity values showed no major chromosomal losses or gains. Cells were cultured under standard conditions (37 °C in a 5% CO_2 humidified atmosphere) in Dulbecco's modified Eagle's minimal essential medium (DMEM, high Glucose) supplemented with 10% fetal calf serum, 1% L-glutamine (100 ×) and 1% Penicillin/Streptomycin (100 ×; all from Thermo Fisher Scientific, Waltham, MA).

Human siRNA transfection

siRNA oligonucleotides were obtained from Qiagen (Hilden, Germany): Hs_LOC100510662_1 (Hs_PRDM16_1) FlexiTube siRNA (SI05656826), Hs_LOC100510662_3 (Hs_PRDM16_3) FlexiTube siRNA (SI05656840), Hs_ITM2B_3 FlexiTube siRNA (SI00132083), Hs_ITM2B_6 FlexiTube siRNA (SI03084648). As transfection control served a non-silencing negative control (Ctrl_AllStars_1 (SI03650318); Qiagen). 0.5×10^5 BT16 cells in 500 ml antibiotic-free media were plated on 24-well dishes and transfected with siRNA at a concentration of 10 nM using 4.5 ml HiPerFect Transfection Reagent (Qiagen).

MTT assay

The MTT assay was performed as described previously [15]. siRNA-mediated knockdown was conducted as described above and cells were subsequently seeded into 96-well plates at 5000 cells per 60 ml. Cell viability was measured after 48 h, 72 h or 96 h. Three independent experiments were performed, each with eight technical replicates.

BrdU assay

Proliferation was assessed by colorimetric BrdU assay (11647229001, Roche). According to the manufacturer's protocol, 6000 cells (with and without siRNA) per well were seeded into 96-well plate and incubated for 48, 72 and 96 h. Incubation time with BrdU labelling solution was applied for 4 h and for anti-BrdU-POD working solution 90 min. Three independent experiments were performed, each with eight technical replicates.

SMAD reporter assay

To quantity the activity of the TGF beta pathway, the Cignal SMAD ReporterAssay Kit (#336841, Qiagen) was used in combination with the Dual-Glo Lucerferase Assay System (E2920, Promega) according to the manufacturer´s protocols. 40,000 cells per well were seeded into 96-well plate and incubated for 72 h. Signal negative controls were subtracted as background. Three independent experiments were performed, each with six technical replicates.

Cytotoxicity assay

Cytotoxicity was assessed by CytoTox-Glo Cytotoxicity Assay (Promega; Madison, WI). Cells were transfected with siRNA as described above and 5000 cells were seeded out per 96-well followed by an incubation time of 96 h. Cytotoxicity was measured according to manufacturer's protocol. Three independent experiments were performed, each in triplicate.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from *Drosophila* 2nd instar larvae or BT16 cells using the Maxwell® 16 LEV simplyRNA Tissue Kit (Promega) according to manufacturer's protocol. cDNA was generated using the High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) following the manufacturer's instructions. Quantitative RT–PCR was performed in triplicate using TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA) for monoplex reaction according to standard protocols. The transcription levels of mRNA were analyzed using comparative Ct method. The following primers were obtained for TaqMan Gene Expression Assays (Life Technologies): Snr1 (Dm02147905_g1), Act5c (Dm02361909_s1), PRDM16 (Hs00223161_m1) and GAPDH (Hs02758991_g1).

Gene expression profiling in *Drosophila*

Following RNA isolation from larval tissue, gene expression profiling was performed at the Core Facility Genomics (University of Münster) using the *Drosophila* Genome 2.0 Array (Affymetrix, Santa Clara, CA). Data were analyzed using the Transcriptome Analysis Console (TAC) package (Version 2.0, Affymetrix).

Gene expression profiling of human samples

For gene expression profiling, data of 83 ATRT samples, 442 medulloblastoma samples and 169 normal brain control samples from public sources deposited in GEO (http://www.ncbi.nlm.nih.gov/geo); GSE10327 [16], GSE37418 [17], GSE12992 [18], GSE3526 [19], GSE35493 [20] or data which had been generated at the German Cancer Research Center DKFZ in Heidelberg were used. The MAS5.0 algorithm of the GCOS program (Affymetrix) was used for normalization of the expression data. Data were analyzed using the R2 program for analysis and visualization of microarray data (http://R2.amc.nl). For generation of a heat map, gene expression data of human orthologues of functional relevant genes identified in the fly model, samples were sorted according to molecular subgroup and gene expression data was clustered by unsupervised hierarchical clustering (Pearson correlation and average linkage clustering) using the TMEV program [21].

Immunohistochemistry of human samples

Tissue sections from formalin-fixed paraffin embedded samples of 15 AT/RT with known molecular subgroup status (ATRT-TYR = 5, ATRT-SHH = 6 and ATRT-MYC = 4) were stained using an antibody directed against PRDM16 (1:500, ABE543, Merck) on an automated staining system (Dako, Glostrup, Denmark). Staining results were scored as 1 (weak), 2 (present) and 3 (strong). Samples had been obtained in the context of the European Rhabdoid Tumor Registry EU-RHAB. EU-RHAB has received ethical committee approval (Ethics committee of the University Hospital Münster, 2009-532-f-S) and all parents had given informed consent for scientific use of the archival samples.

Statistical analysis

Data were analyzed using ANOVA followed by t-test with Bonferroni correction (cell culture data) or Dunn's test (gene expression data) using GraphPad Prism version 5.01 for Windows, (GraphPad Software, La Jolla, CA).

Results

Knockdown of Snr1 results in a lethal phenotype and epigenetic alterations of histone H3K27 in *Drosophila*

Ubiquitous and glial-specific knockdown of *Snr1* in the fly model (Fig. 1a) as described previously resulted in a lethal phenotype at the larval and pupal stage of development, respectively [10, 11]. Ubiquitous knockdown of *Snr1* was effective (Fig. 1b) and ChIP-seq using chromatin extracted from 2nd instar larvae resulted in an increase in acetylation of histone H3 on lysine 27 (H3K27ac) affecting 4922 out of all 13,931 fly genes covered by the Berkley *Drosophila* Genome Project (35%), while a decrease in H3K27ac was noted for 3050 out of 13,931 genes (22%, Fig. 1c). Differential binding of RNA Polymerase II (RNA Pol II) was also encountered, 65% of genes affected by increases in H3K27ac showing concurrent increases in RNA Pol II binding. Furthermore, genes showing increased RNA Pol II binding were associated with increased mRNA expression (> two-fold; Chi-Square 6.498, df = 1, p = 0.01). Taken together, these results indicate that the detrimental effects of *Snr1* knockdown are associated with dynamic alterations of activating histone mark H3K27ac and gene expression. These results further qualify this fly model of SMARCB1-deficiency as a screening tool for the functional relevance of genes affected by epigenetic alterations in ATRT.

Modifier screens performed in a fly model of SMARCB1-deficiency result in the identification of functional relevant genes

Of 42,619 active enhancers (H3K27ac) identified in human ATRT samples [6], 1749 could be aligned to specific genes. For 984 of these genes, one or several *Drosophila* orthologues were identified, resulting in a total of 1486 *Drosophila* genes. For 1083 of these genes, siRNA strains were available (Supplementary Table 1, for further details see also materials and methods) and the effect of siRNA knockdown of these genes on the lethal phenotype associated with glial-specific *Snr1* knockdown was examined using Gal4-UAS modifier screens [10]. Knockdown of 89/1083 genes shifted the pupal lethality to later stages of development (≥ 20% of animals hatching; Fig. 1d; Table 1), suggesting a role of these genes in the detrimental effects of *Snr1* knockdown. These included genes known to play a role in ATRT biology such as *cycD* (*CCND1*) [22] and *lin-28* (*LIN28A*) [23], but also genes involved in epigenetic regulation, such as polycomb

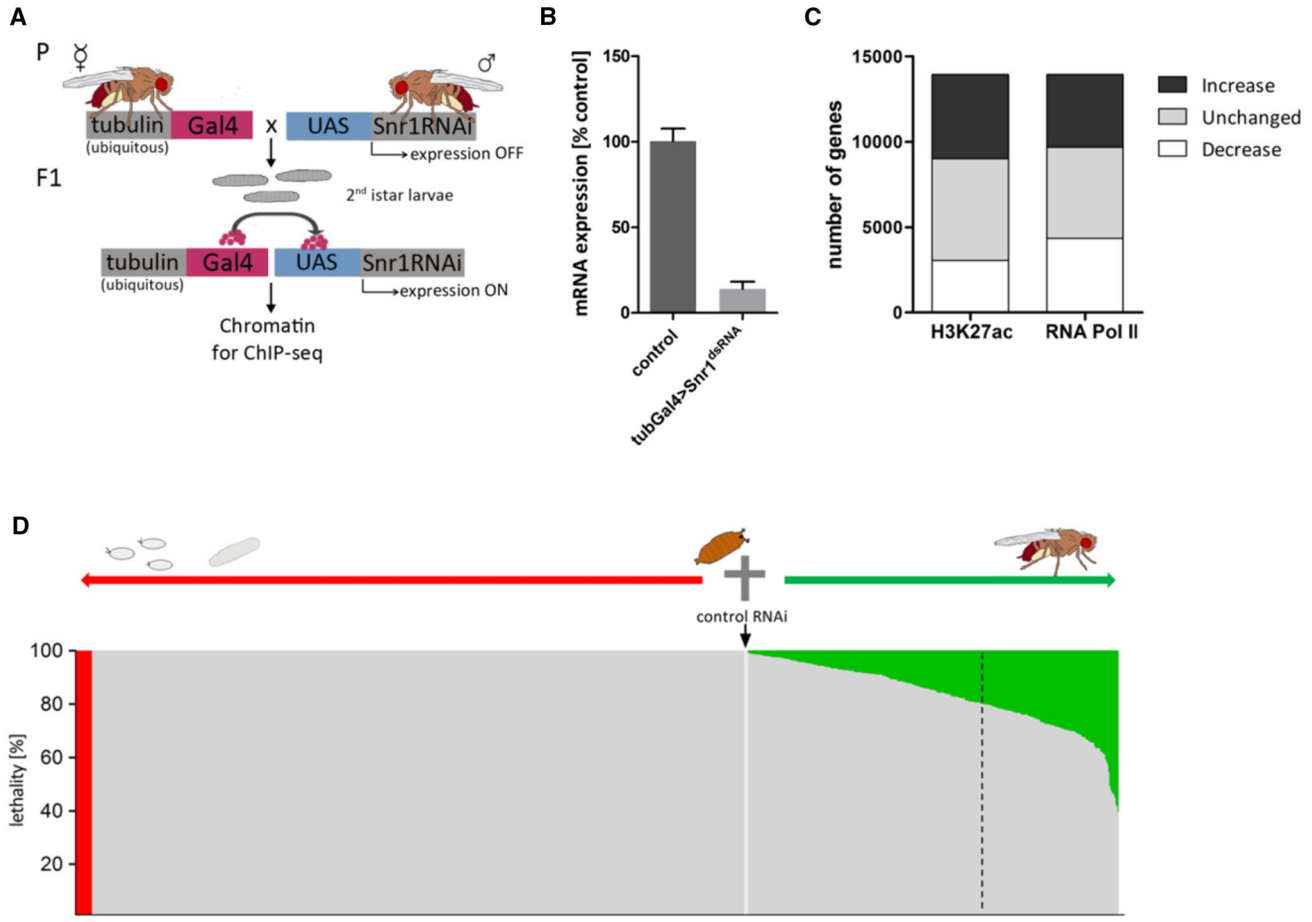

Fig. 1 Fly model of SMARCB1-deficiency. Overview (**a**) of the GAL4-UAS system allowing efficient knockdown of *Snr1* in the F1 generation by crossing flies carrying a ubiquitous Gal4-driver with flies carrying a suitable UAS-RNAi construct. Chromatin from 2nd instar larvae of F1 generation was used to perform ChIP-seq. Expression of *Snr1* mRNA in 2nd instar larvae under these conditions (**b**) and Chip-seq performed on DNA from 2nd instar larvae (**c**) showing global changes in H3K27ac and RNA Pol II upon *Snr1* knockdown. Crossing *Snr1* knockdown flies with strains expressing specific RNA interference (RNAi) shifted the pupal lethality associated with glial specific *Snr1* knockdown to later stages of development in 89 out of 1083 screened candidate genes (D).Shown is the percentage of embryonal (red), larval and pupal lethality (grey) as well as the percentage hatched animals (green) for 1083 screened candidate genes. For list of the 89 genes resulting in ≥20% hatched flies (dotted line) see Table 1, for complete list of all 1083 candidate genes see Supplementary Table 1

repressive complex 1 (PRC1) member *ph-d (PHC2)*. None of the hatched animals showed gross alterations of phenotype.

Biological processes over-represented among these 89 genes based on gene ontology (GO) analysis included GO:0000122 (negative regulation of transcription from RNA Pol II promotor; $P < 0.001$), GO:0006351 (transcription, DNA templated; $P < 0.01$) and GO:0007179 (TGF-beta receptor signaling pathway; $P < 0.01$). TGF-beta signaling pathway members included *dpp* (*BMP4*), *vis* (*TGIF2*), *Smox* (*SMAD3*) and *daw* (*TGFB2*), but also *CG10348*, whose human orthologue *PRDM16* (also known as *MEL1*) has been shown to be involved in transcriptional regulation [24] and TGF-beta signaling [25]. Since rare ATRTs show retained SMARCB1 protein expression, but inactivating mutations of *SMARCA4*, we also established a fly model of SMARCA4 deficiency for comparison. Here, ubiquitous and glial-specific knockdown of *brm*, the fly orthologue of *SMARCA4*, also resulted in pupal lethality and a comparable number of candidate genes shifted lethality to later stages of development (76/1083), suggesting a role of these genes in the detrimental effects of *brm* knockdown (Supplementary Table 2). There was substantial overlap with genes involved in the detrimental effects of *Snr1* knockdown, including over-representation of TGF-beta receptor signaling pathway members (GO:0007179; $P < 0.05$) and also *CG10348* (Supplementary Fig. 1). Taken together, fly modifier screens of 1083 candidate genes predicted to be affected by epigenetic alterations in atypical teratoid/rhabdoid tumors resulted in the identification of a number of genes of functional relevance, which

Table 1 Genes of functional relevance in a fly model of SMARCB1-deficiency

Drosophila gene	Human gene	Gene function	Mean hatching rate (%)	Standard deviation (%)
ph-d	*PHC2*	PRC1-like complex	62	19
hb	*ZNF462*	Positive regulation of transcription from RNA polymerase II promoter	59	14
Optix	*SIX3*	Regulation of cell cycle phase transition	56	5
CG10348	*PRDM16*	TGF-beta signaling, histone-lysine *N*-methyltransferase activity	54	12
CG8243	*ADAP2*	GTPase-activating protein	53	14
CG3662	*ITM2B*	Nervous system development	46	16
CG9265	*RDH10, DHRS3*	Retinol metabolic process	44	2
Bub1	*BUB1B*	Mitotic cell cycle	42	23
sli	*SLIT1, SLIT2, SLIT3*	Axon guidance, cell migration	40	9
vis	*TGIF2*	Positive regulation of neuron differentiation, regulation of transcription, TGF-beta signaling	40	12
Cct2	*PCYT1B*	Phosphatidylcholine biosynthetic process	39	7
Ptr	*PTCHD1*	Smoothened signaling pathway	39	13
RpS5b	*RPS5*	Defense response to bacterium	37	12
CG7656	*CDC34*	DNA replication initiation	37	9
Rab6	*RAB6A*	Protein targeting to Golgi	37	22
NAA20	*NAA20*	N-terminal peptidyl-methionine acetylation	37	25
CG40006	*SCARB1*	Endothelial cell proliferation	36	7
ninaD	*SCARB1*	Endothelial cell proliferation	35	11
Rgk2	*GEM, RRAD*	Small GTPase mediated signal transduction	35	3
CG10185	*NWD1*	Negative regulation of cell cycle arrest, TGF-beta signaling	35	18
ECSIT	*ECSIT*	Regulation of oxidoreductase activity	34	4
dpp	*BMP4*	BMP signaling pathway, activation of MAPKK activity	34	20
CG42541	*GEM, RRAD*	Small GTPase mediated signal transduction	34	15
sob	*OSR1*	Protein serine/threonine kinase, intracellular signal transduction	33	11
pdm2	*POU2F1*	Negative regulation of transcription	33	10
NetA	*NTN4, NTN1, NTNG1, NTNG2*	Axon guidance, axonogenesis	33	5
NetB	*NTN4, NTN1, NTNG1, NTNG2*	Axon guidance, axonogenesis	33	11
nos	*NANOS3*	Negative regulation of translation, germ cell development	32	2
CG9386	*TRMT44*	tRNA methylation	32	23
CG44836	*NSUN7*	Methyltransferase activity	32	8
Mmp2	*MMP24, MMP7, MMP17, MMP15*	Aging, cellular response to mechanical stimulus, glial cell differentiation	32	8
Zasp52	*PDLIM5*	Heart development	31	8
Eip75B	*PPARG*	DNA binding	31	10
Sod2	*SOD2*	Negative regulation of cell proliferation negative regulation of neuron apoptotic process	31	5
lin-28	*LIN28A*	Regulation of transcription	31	10
dlp	*GPC4, GPC6*	Co-receptor activity involved in Wnt signaling pathway, cell proliferation	31	9
Atf6	*ATF6*	Regulation of transcription by RNA polymerase II	31	7
CG40160	*PRSS54*	Serine-type endopeptidase activity	30	6
daw	*TGFB2*	TGF-beta signaling, cell cycle arrest, cell death, cell development ,cell growth, cell migration	30	1
bi	*TBX2*	Transcriptional repressor activity, RNA polymerase II core promoter proximal region sequence-specific binding heart development	30	8

Table 1 (continued)

Drosophila gene	Human gene	Gene function	Mean hatching rate (%)	Standard deviation (%)
CG9279	*DCTN1*	Microtubule binding, nervous system development	29	15
Fs	*FST*	Negative regulation of transcription from RNA polymerase II promoter	29	8
CG7987	*ZNF606*	DNA binding	29	9
a	*PDZD2*	Cell adhesion	29	3
oc	*OTX2*	Transcriptional activator activity	28	12
tsg	*TWSG1*	BMP signaling pathway	28	4
slgA	*PRODH*	Proline catabolic process	28	8
lqfR	*CLINT1*	Endocytosis	28	19
LpR1	*VLDLR*	Lipid transport	28	7
CG42271	*INPP4B*	Inositol phosphate metabolic process	27	6
CG5334	*MKRN3*	Protein ubiquitination	26	8
Oaz	*ZNF521*	Involved in BMP signaling	26	10
GluRIIA	*GRIK3*	G-protein coupled glutamate receptor signaling pathway	26	6
Osbp	*OSBP*	Sterol transporter activity	25	24
CycD	*CCND1*	Negative regulation of apoptotic process, positive regulation of cell proliferation	25	7
CG15803	*INADL*	Tight junction assembly, intracellular signal transduction	25	15
Sema-1b	*SEMA6A, SEMA6D*	Axon guidance, cell surface receptor signaling pathway	25	13
Hs3st-B	*HS3ST3B1, HS3ST3A1*	Glycosaminoglycan biosynthetic process	24	8
mfr	*MYOF*	Muscle contraction myoblast fusion	24	8
pyd	*TJP2*	Hippo signaling	24	8
CG6752	*RNF123*	Protein ubiquitination	24	6
dia	*DIAPH2*	Cytokinesis	24	10
Pi3K21B	*PIK3R3*	Insulin receptor signaling pathway	24	11
GluRIB	*GRIA1, GRIK3*	Glutamate receptor	23	6
CAH1	*CA14*	Carbonate dehydratase activity	23	5
Spn43Ab	*SERPINB9, SERPINB1*	Serine-type endopeptidase	23	22
robo2	*ROBO1*	Receptor for SLIT1 and SLIT2, axon guidance	23	3
Socs44A	*SOCS3*	Negative regulation of apoptotic process	23	6
Spn88Eb	*SERPINI1*	Nervous system development	23	9
bowl	*OSR1*	Protein serine/threonine kinase activity	22	4
l(3)72Dr	*GGH*	Metabolic processes	22	11
CG41378	*IFI30*	Antigen processing	22	7
CG5214	*DLST*	Metabolic processes	21	4
GluRIIB	*GRIK3*	Glutamate receptor signaling pathway	21	13
dpn	*HES4*	BMP signaling pathway	21	10
CG3077	*SNX20*	Protein transport	21	2
CG6805	*INPP5K*	Inositol 5-phosphatase	21	9
Bteb2	*KLF15*	Transcriptional activator activity	21	6
CG31902	*SERPINB1, SERPINB9*	Nervous system development	21	10
Ndae1	*SLC4A10*	Nervous system development	21	8
CG31637	*CHST7*	Metabolic processes	20	9
CG15012	*TMEM50B*	Transmembran protein	20	7
CG4968	*OTUB1*	DNA repair	20	7
scramb1	*PLSCR1*	Positive regulation of gene expression	20	5
CG11151	*HSD17B4*	Fatty acid beta-oxidation	20	14
Rip11	*RAB11FIP1*	Endosomal recycling	20	5

Table 1 (continued)

Drosophila gene	Human gene	Gene function	Mean hatching rate (%)	Standard deviation (%)
Smox	*SMAD3*	TGF-beta-mediated transcription	20	4
Sik2	*SIK1*	Serine/threonine-protein kinase	20	8
CycG	*CCNG2*	Regulation of cell cycle progression	20	3

Knockdown of 89 genes out of 1083 genes predicted to be affected by epigenetic alterations shifted pupal lethality associated with glial-specific *Snr1* knockdown to later stages of development (≥20% animals hatching). Mean hatching rate as well as standard deviation from three independent experiments are presented

included TGF-beta receptor signaling pathway members and *CG10348*, the fly orthologue of *PRDM16*.

A cluster of human orthologues of identified functional relevant genes is specifically over-expressed in human ATRT samples

Gene expression data of 98 human orthologues of *Drosophila* genes identified in the fly model were examined in human ATRTs (n = 83), medulloblastomas (n = 442) and normal brain (n = 169). On unsupervised hierarchical clustering, a cluster of 14 genes, including *PRDM16*, was found to be over-expressed in ATRT as compared to medulloblastoma and normal brain samples (Fig. 2).

PRDM16 is over-expressed in ATRT and has a functional role in human rhabdoid tumor cells

Because a role of *PRDM16* has not yet been described in ATRT biology, we examined *PRDM16* expression and its functional impact in ATRT in more detail. On gene expression profiling, over-expression of *PRDM16* was observed in ATRT of all three molecular subgroups as compared to medulloblastomas and normal brain (ANOVA $P < 0.0001$; Fig. 3a). Among the three ATRT subgroups, *PRDM16* expression was higher in ATRT-TYR as compared to ATRT-SHH ($P < 0.05$) and ATRT-MYC ($P < 0.001$). In an unrelated cohort of 15 ATRT, PRDM16 expression was also demonstrated on protein level (Fig. 3b), the majority of ATRT showing high staining scores (Fig. 3c). Transient silencing of *PRDM16* expression in the ATRT cell line BT16 using two different siRNAs was effective (Fig. 4a) and did not cause cytotoxicity (Supplementary Fig. 2), but resulted in reduced metabolic activity in the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (Fig. 4b) as well as proliferation in the BrdU assay (Fig. 4c). Furthermore, knockdown of

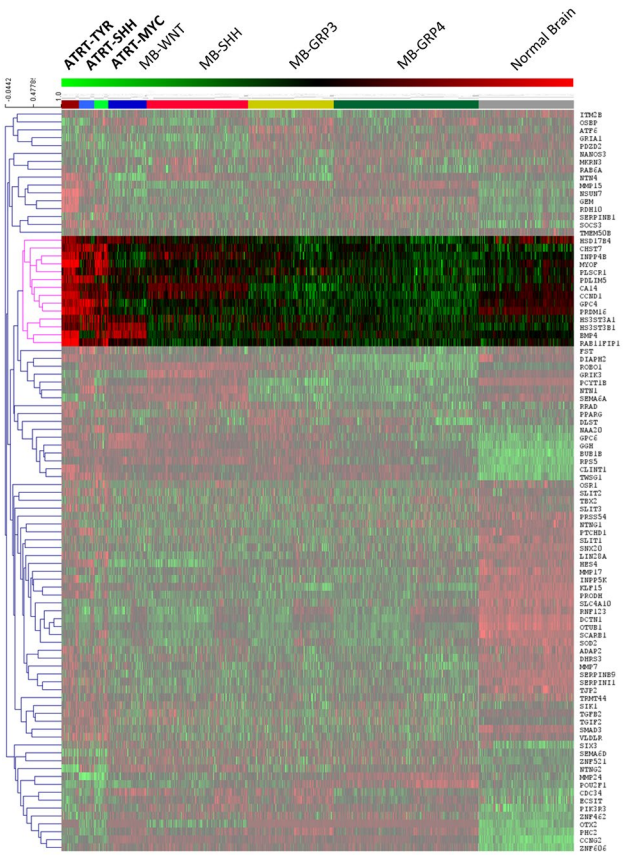

Fig. 2 Expression of human orthologues of identified functionally relevant genes in human ATRT samples. Gene expression data of 98 human orthologues of the 89 *Drosophila* genes identified in the fly model were examined in human ATRT samples of the molecular subgroups ATRT-TYR, ATRT-SHH and ATRT-MYC, medulloblastoma samples of all four molecular subgroups (MB-WNT, MB-SHH, MB-GRP3 and MB-GRP4) and normal brain samples. On unsupervised hierarchical clustering, a cluster of 14 genes including *PRDM16* (highlighted) was found to be over-expressed in ATRT samples as compared to medulloblastoma and normal brain samples

PRDM16 reduced TGFbeta signaling activity as assessed by SMAD reporter assay (Fig. 4d).

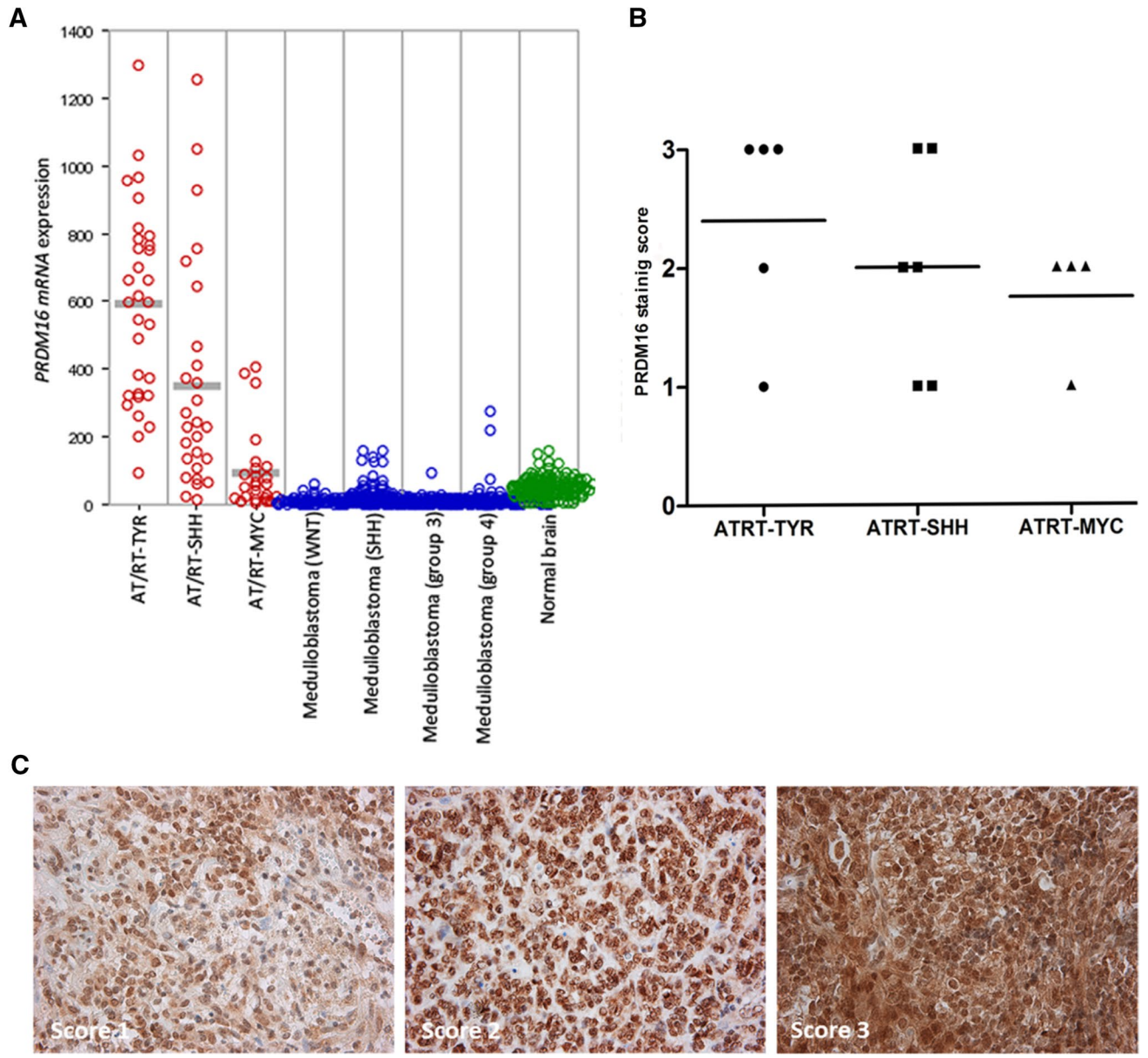

Fig. 3 Expression of *PRDM16* in ATRT. In human tumor samples (**a**) mRNA Expression of *PRDM16* was increased in 83 ATRTs of the three molecular subgroups (ATRT-TYR, ATRT-SHH, ATRT-MYC) as compared to 442 medulloblastomas of all four molecular subgroups (WNT, SHH, Group 3, Group 4) as well as normal CNS controls (N = 169; P < 0.0001). In an unrelated cohort of 15 ATRT, PRDM16 expression was also demonstrated on protein level (**b**), the majority of ATRT showing high staining scores [staining scores 2 and 3 (**c**)]

Discussion

In this study we have shown that a number of genes predicted to be affected by epigenetic alterations in human ATRT samples are functionally involved in the lethal effect observed upon *Snr1* knockdown in a fly model of SMARCB1-deficiency in vivo. These included genes known to play a role in ATRT biology as well as genes involved in epigenetic regulation. TGF-beta signaling pathway members were also over-represented, which is well in line with a recent study implying an important role of TGF-beta signaling in the biology of ATRT [11].

Of note, *CG10348* (*PRDM16*) which has also been shown to be involved in TGF-beta signaling [26, 27] was among the identified genes of functional relevance. The PRDM proteins belong to the SET domain family of histone methyltransferases, which play an important role in the regulation of gene expression [28, 29]. *PRDM16* had first been described

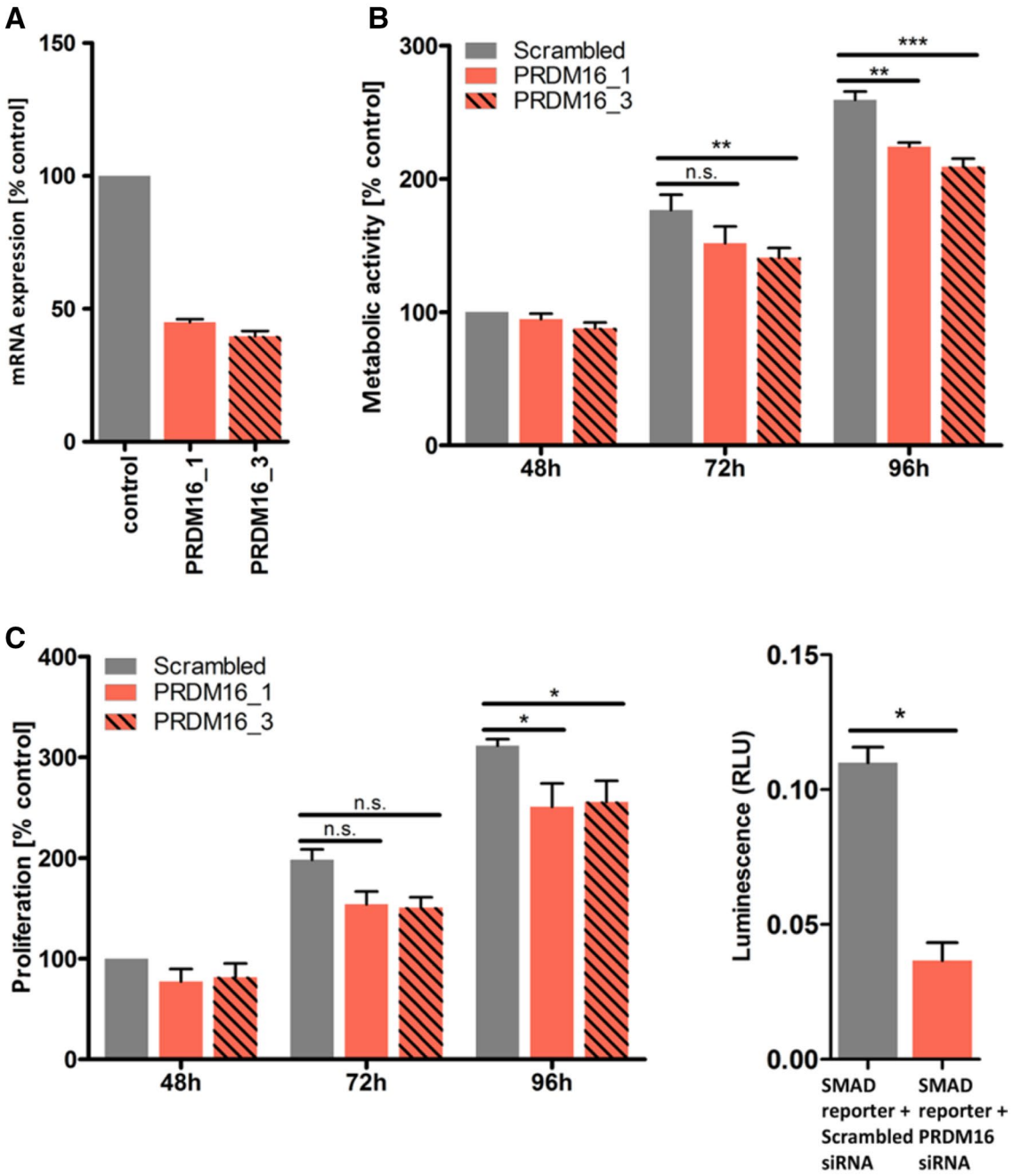

Fig. 4 Functional relevance of *PRDM16* in human ATRT cells. In ATRT tumor cells BT16, silencing of *PRDM16* using two different siRNAs was effective (**a**) and resulted in reduced metabolic activity (**b**, MTT-assay) as well as reduced proliferation (**c**, BrdU assay) as compared to control (non-target siRNA***P < 0.001 vs. control, **P < 0.01 vs. control, *P < 0.05 vs. control). Furthermore, TGFbeta signaling activity as assessed by SMAD reporter assay was reduced (**d** *P < 0.05 vs. control)

in brown adipose tissue [30] and encodes a zinc finger protein that is highly homologous to MDS1/EVI1, a protein encoded by a splice variant of the *EVI1* gene [31]. Subsequently, overexpression in acute myeloid leukemia (AML) [32] and a role in the induction of myeloid leukemia in mice [33] was reported, leading to the assumption of *PRDM16* being an oncogene. Little is known about the role of PRDM proteins in the biology of brain tumors, but PRDM13 has been described as an antigen in medulloblastoma [34]. Among the genes predicted to be affected by epigenetic alterations in ATRT, *PRDM16* was highly overexpressed, suggesting that *PRDM16* over-expression is tightly linked to activating histone mark histone H3K27ac. In rhabdoid tumor cells, *PRDM16* knockdown resulted in decreased

proliferation, which was comparable to that previously observed upon knockdown of *SMAD3* and *SMAD6* [11] and further suggests a functional role of TGFbeta signaling in the detrimental effects of SMARCB1-deficiency. Specific PRDM16 inhibitors that could be tested for treatment of children with AT/RT are not yet available. In brown adipose tissue, however, PRDM16 has been shown to interact with histone deacetylase (HDAC) 3 and the effect of an HDAC inhibitor was blunted in the absence of PRDM16 [35]. In AT/RT, HDAC1 and HDAC2 are overexpressed [36] and the role of PRDM16 in the effects of HDAC inhibitor treatment remains to be determined. One limitation of the present study is the fact that cell culture data could only be obtained in one SMARCB1-deficient ATRT cell line. Further work investigating the role of *PRDM16* and other candidate genes in additional cell lines and in vivo models will be desirable.

The majority of ATRT is characterized by *SMARCB1* mutations causing loss of functional SMARCB1/INI1 protein, but rare ATRT rather show *SMARCA4* mutations [37]. In contrast to SMARCB1-deficient ATRT, whose gene expression profiles, genetic alterations and epigenetic profiles are well characterized [6, 38], much less is known about the biology of SMARCA4-deficient ATRTs [39, 40]. Of note, DNA methylation profiles of three SMARCA4-deficient ATRT grouped with ATRT-SHH, one of three molecular subgroups of SMARCB1-deficient ATRT [6], rather suggesting similarities than differences with SMARCB1-deficient ATRT. Our finding of comparable phenotypes in the fly models of SMARCB1- and SMARCA4-deficiency as well as a substantial overlap of genes involved in the detrimental effects of *Snr1* and *brm* knockdown, respectively, suggests that impaired SWI/SNF chromatin remodeling complex function results in comparable downstream events and points towards a potential functional role of TGF-beta receptor signaling pathway activation and PRDM16 also in SMARCA4-deficient ATRT. However, in the absence of gene expression data and SMARCA4-deficient ATRT cell lines, expression and functional role of *PRDM16* in the detrimental effects of SMARCA4-deficiency require further investigation.

Because in ATRT recurrent genetic alterations are restricted to mutations affecting members of the evolutionarily highly conserved SWI/SNF complex, this tumor entity is ideally suited for modelling in the fruit fly. Moreover, our results suggest that *Drosophila* could also be suitable for high throughput functional screening in other brain tumor entities characterized by mutations in evolutionarily conserved genes and pathways such as pediatric high-grade gliomas, which are driven by specific histone mutations [41, 42].

In conclusion, our results suggest that a subset of genes affected by differential histone modification in ATRT is involved in the detrimental effects of SMARCB1-deficiency and also relevant in the biology of ATRT.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or vertebrates performed by any of the authors. Human tumor samples for immunohistochemistry had been obtained in the context of the European Rhabdoid Tumor Registry EU-RHAB. EU-RHAB has received ethical committee approval (Ethics committee of the University Hospital Münster, 2009-532-f-S) and all parents had given informed consent for scientific use of the archival samples.

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