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RESEARCH LETTER

Brain Pathology

Generation of new transgenic SMARCA4-deficient mouse models results in neuromuscular weakness and paralysis of limbs

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The SWItch/sucrose non-fermentable (SWI/SNF) complex is an ATP-dependent chromatin-remodeling complex, which is crucially involved in the regulation of gene expression. Many physiological processes during the development of the nervous system essentially rely on transcriptional regulation through the SWI/SNF complex, such as the differentiation of Schwann cells, which are required for the myelination of peripheral nerves [1]. Besides its regulatory role in normal cellular development, the SWI/SNF complex is known to be deregulated in various human cancers, either by mutations in genes encoding the subunits of the complex or by overexpression of these genes. In fact, alterations in at least one of the genes that make up the complex are observed in a large number of malignancies [1, 2]. Mutations in *SMARCA4*, which is one of the two ATPase subunits of the SWI/SNF complex, are not only frequently found in various carcinoma types of adult patients, but have also been described as driver mutations in the development of a subset of pediatric rhabdoid tumors (RT) [3]. RT are rare, highly aggressive embryonal tumors mostly affecting young children, with a poor prognosis, and an overall survival of 17 months. They comprise RT of the central nervous system (CNS), which are known as atypical teratoid/rhabdoid tumors (AT/RT), RT of the kidney (RTK), and malignant RT of the soft tissue (MRT). All RT show a biallelic inactivation of the SWI/SNF subunits SMARCB1 or SMARCA4 [2, 3]. Mutations in *SMARCB1* leading to the development of RT have been characterized by various mouse models, whereas, to date, SMARCA4 deficiency has never caused RT development in vivo. So far, there is no cell line for SMARCA4-deficient RT. Therefore, in vitro and in vivo models for SMARCA4-deficient RT are urgently needed to better understand the oncogenesis and to develop potential treatment options.

Recently, neural crest cells were suggested as one potential cellular origin of SMARCB1-deficient RT [2]. Such cells express the glycoprotein myelin protein zero (MPZ, P0), which is also known to be the major structural protein in peripheral myelin. In a previous study, *P0-Cre* mice were used to conditionally delete *Smarcb1*, which resulted in the development of RT [4]. In addition, genomic and single-cell transcriptomic analyses of human RT-derived organoids revealed a shared cellular lineage of *SMARCB1*-mutated RT cells and neural crest-derived Schwann cells [2]. To examine, whether P0 also marks cells of origin of SMARCA4-deficient RT, we crossed

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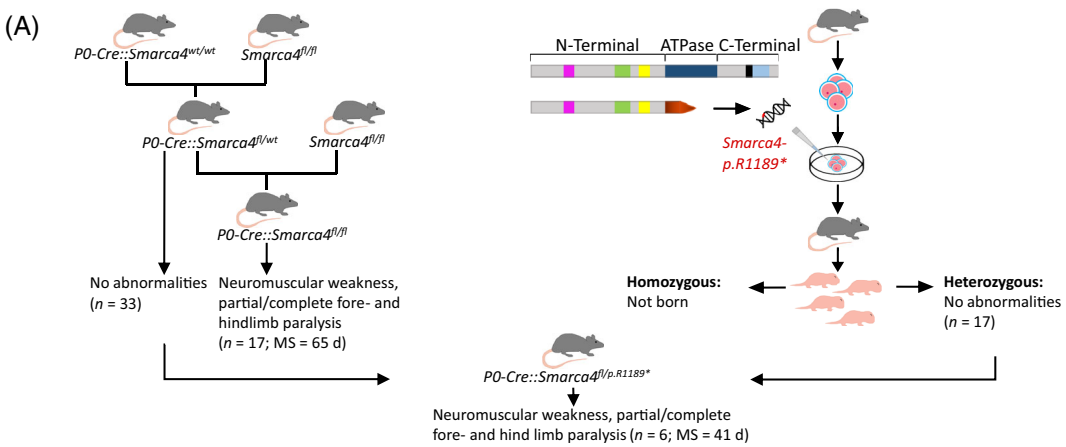
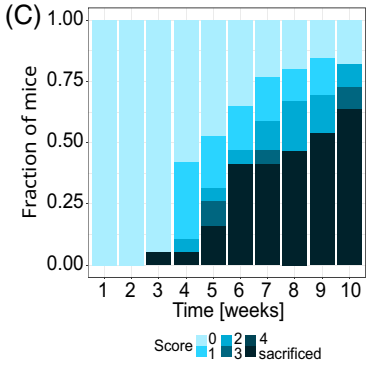


(B)

Score	Name	Gait	Grid
0	No symptoms	Normal	Normal
1	Neuromuscular weakness	Mildly abnormal gait	No difficulty to hold on grid
2	Partial hind limb paralysis	Abnormal gait with intermitted dragging of both hind limbs	Mild difficulty to hold on grid, slipping of the grid
3	Complete hind limb paralysis	Abnormal gait with permitted dragging of both hind limbs	Inability to hold on grid
4	Partial fore- and hind limb paralysis	Abnormal gait with permitted dragging of both hind limbs	Difficulty to hold on grid with fore limbs



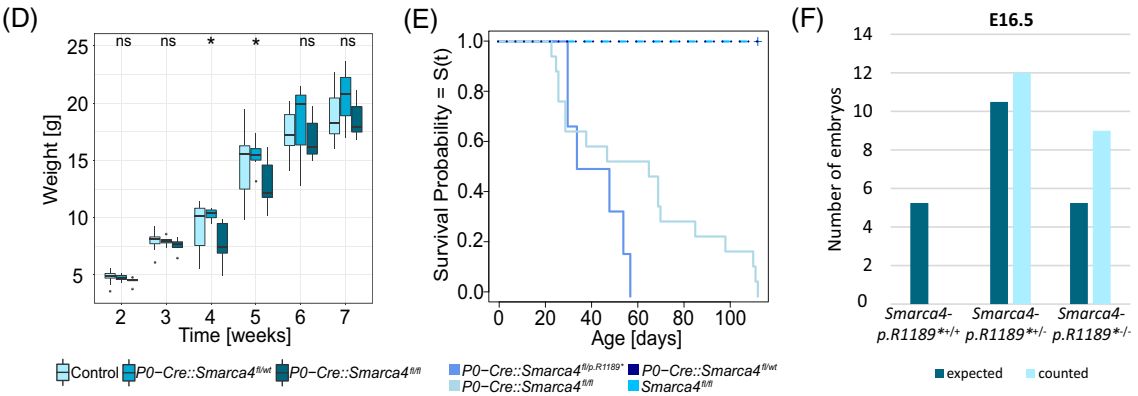


FIGURE 1 Development and characterization of SMARCA4-deficient mouse models. (A) *P0-Cre* mice were crossed with *Smarca4*$^{fl/fl}$ mice. A second mouse model was created by insertion of the *Smarca4* point mutation c.3565C>T (p.R1189*), which is a nonsense mutation leading to a truncated protein with impaired function, by pronuclear injection into 1-cell stage embryos in vitro using CRISPR/Cas9. The third mouse model *P0-Cre::Smarca4*$^{fl/p.R1189*}$ was created by crossing *P0-Cre::Smarca4*$^{fl/wt}$ with *Smarca4-p.R1189**$^{+/-}$ mice. MS, median survival; d, days. (B) Score for the analysis of symptoms of *P0-Cre::Smarca4*$^{fl/fl}$ mice adapted from Weider et al. (C) Symptoms of *P0-Cre::Smarca4*$^{fl/fl}$ mice ($n = 19$, according to B) during an observation period of 10 weeks. (D) Body weight of *P0-Cre::Smarca4*$^{fl/fl}$, *P0-Cre::Smarca4*$^{fl/wt}$, and control mice ($n_{total} = 33$) over 7 weeks. (E) Kaplan Meier plot of *P0-Cre::Smarca4*$^{fl/fl}$ mice (MS = 65 d), *P0-Cre::Smarca4*$^{fl/wt}$ mice, *P0-Cre::Smarca4*$^{fl/p.R1189*}$ mice (MS = 41 d), and *Smarca4*$^{fl/fl}$ control mice. (F) Expected numbers of each point mutation genotype were calculated according to Mendel and compared to the counted mice of each genotype at E16.5.

P0-Cre mice [5] with *Smarca4*$^{fl/fl}$ mice [6] to generate *P0-Cre::Smarca4*$^{fl/wt}$ ($n = 33$) and *P0-Cre::Smarca4*$^{fl/fl}$ mice ($n = 17$). As a control, *P0-Cre::Smarcb1*$^{fl/lf}$ mice ($n = 23$) were produced by crossing *P0-Cre* mice [5] with *Smarcb1*$^{fl/fl}$ mice [7]. All mice were bred on a C57BL/6J background and mice of both sexes were used for the study.

No abnormalities were observed in heterozygous mice (*P0-Cre::Smarca4*$^{fl/wt}$), but all homozygous mice developed neuromuscular weakness and partial or complete fore- and hind limb paralysis within 10 weeks (Figure 1A). To further analyze and classify this phenotype, a score for the symptoms of *P0-Cre::Smarca4*$^{fl/fl}$ mice was adapted from Weider et al. [8] (Figure 1B). Symptoms started at 3 weeks of age and increased continuously as visualized in Figure 1C. Body weight control revealed that homozygous mice were significantly lighter compared to heterozygous

and control mice after 4 ($p = 0.018$) and 5 weeks ($p = 0.028$, Figure 1D). Mice were sacrificed based on the severity of paralysis and weight loss. The median overall survival was 65 days (Figure 1E). However, none of these mice developed tumors, as shown by thorough macroscopic inspection of all mice and histological analyses of the brains and kidneys in a subset of mice. Additionally, control mice with the genotype *P0-Cre::Smarcb1*$^{fl/fl}$ ($n = 23$) developed the same neuromuscular phenotype after the age of 10 weeks without any clinical or macroscopic signs of tumor development.

Since SMARCA4 loss in other cell types than neural crest cells previously failed to model RT development as well, our goal was to develop a mouse model that mimics the human situation more specifically. Therefore, we next generated *Smarca4-p.R1189** knock-in mice carrying a constitutive point mutation. The same mutation had previously been described in the germline of a family with RT predisposition syndrome leading to the development of RT during infancy of two siblings [9]. Knock-in mice were generated by pronuclear injection in 1-cell stage embryos after induction of a c.3565C>T (p.R1189*) point mutation via CRISPR/Cas9.

In human patients, this mutation leads to a nonsense-mediated decay of the transcript [9] (Figure 1A). Mice carrying a heterozygous *Smarca4-p.R1189** point mutation ($n = 17$) did not show any abnormalities over the observation period of 1 year. However, none of the 75 mice that were born after further crossing of heterozygous mice and none of the embryos genotyped at embryonal day 10.5 (E10.5) and E16.5 had a homozygous genotype, suggesting an early embryonal death of mice homozygous for *Smarca4-p.R1189** (Figure 1F).

Finally, *P0-Cre::Smarca4*$^{fl/wt}$ mice were crossed with *Smarca4-p.R1189**$^{+/-}$ mice for the generation of *P0-Cre::Smarca4*$^{fl/p.R1189*}$ mice. In this way, a constitutive mutation–similar to a human germline mutation - and a conditional mutation in neural crest cells were combined to recapitulate the potential situation in pediatric patients with RT predisposition syndrome and to potentially avoid embryonic death of the mice. All *P0-Cre::Smarca4*$^{fl/p.R1189*}$ mice ($n = 6$) showed a uniform phenotype similar to *P0-Cre::Smarca4*$^{fl/fl}$ mice, with neuromuscular weakness and paralysis of the fore- and hind limbs (Figure 1A) and a median overall survival of 41 days (Figure 1E). These results indicate that a homozygous *Smarca4-p.R1189** mutation is impairing SMARCA4 function in mice, but they also indicate that a recombination of *Smarca4* through the above described *P0-Cre* strain is still insufficient for tumor development, even if the other allele carries a constitutive loss-of-function mutation.

In conclusion, homozygous *Smarca4* mutations introduced in the *P0-Cre* mouse line used in this study cause neuromuscular weakness and paralysis of the fore- and hind limbs. A similar phenotype was previously observed in Schwann cell-specific transgenic *DHH-Cre::Smarca4*$^{fl/fl}$ [8] and *DHH-Cre::Smarcb1*$^{fl/fl}$ mice [4]. In both *DHH-Cre* mouse lines, the observed phenotype could be attributed to an impaired myelination of peripheral nerves. Further analyses of our *P0-Cre::Smarca4*$^{fl/fl}$ mouse model confirmed genomic recombination of *Smarca4*$^{fl/fl}$ alleles (Figure S1A) and loss of SMARCA4 (Figure S1D,E) together with *Cre*-expression (Figure S1A) in the peripheral nerves of *P0-Cre::Smarca4*$^{fl/fl}$ mice. Here, we could prove that myelination of peripheral nerves is also impaired in our *P0-Cre::Smarca4*$^{fl/fl}$ mouse model. Histopathological assessment using toluidine blue staining (TBO) revealed thinner and impaired myelin sheaths surrounding axons of sciatic nerves of *P0-Cre::Smarca4*$^{fl/fl}$ mice (Figure S1H,I) compared to *Smarca4*$^{fl/fl}$ control mice (Figure S1F,G). Histological assessment of thigh muscles of *P0-Cre::Smarca4*$^{fl/fl}$ mice (Figure S1L,M) also revealed abnormalities in contrast to *Smarca4*$^{fl/fl}$ control mice (Figure 1J,K) showing a neurogenic image of muscle tissue with angular atrophic myocytes (Supplementary Figure 1M) in muscles that were innervated by impaired sciatic nerves. No histological abnormalities were detected in the spinal cord of *P0-Cre::Smarca4*$^{fl/fl}$ mice (Figure 1P,Q) compared to *Smarca4*$^{fl/fl}$ control mice (Figure S1N,O).

The similarity of phenotypes caused by a *Smarca4*-knockout in *DHH-Cre*, which starts to be expressed at E12.5 [4], and our *P0-Cre*, which is presumably expressed from E13.5 onwards [10] indicates that both *Cre*-lines target a cellular compartment, which requires a functional SWI/SNF complex for proper myelination of peripheral nerves. However, these results highlight that the choice of the *Cre*-driver is critical for targeting the exact cellular population of origin for a tumor type of interest. For example, previous tumor generation in a *P0-Cre::Smarcb1*$^{fl/fl}$ mouse strain, in which *P0-Cre* has been shown to be expressed at E9.5 [4], could not be reproduced in another *P0-Cre* mouse strain, for which the expression first starts at E13.5 [10]. Overall, our results indicate that the initiation of RT from neural crest-derived cells might require an inactivation of *Smarca4* earlier than E13.5 or that RT originate from a different cell type. While such early time points have previously been identified for the development of *Smarcb1*-mutated RT [4], the exact temporal window for the initiation of SMARCA4-deficient RT and the cells of origin remain to be determined. In fact, the developmental time point of origin may even be earlier than for SMARCB1-mutated RT, since the median age of diagnosis is significantly lower in patients with SMARCA4-deficient AT/RT [3].

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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