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MiR-34a deficiency accelerates medulloblastoma formation *in vivo*

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Abbreviations: ESC: embryonic stem cells; GNPs: granule neuron progenitors; LCA: large cell anaplastic; MTT: 3-[4,5-dimethylthiazol-3-yl]-2,5-diphenyltetrazolium; Neo = neomycin; Puro: puromycin; tg: transgenic; TK: thymidine kinase

Additional Supporting Information may be found in the online version of this article.

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Previous studies have evaluated the role of miRNAs in cancer initiation and progression. MiR-34a was found to be downregulated in several tumors, including medulloblastomas. Here we employed targeted transgenesis to analyze the function of miR-34a *in vivo*. We generated mice with a constitutive deletion of the miR-34a gene. These mice were devoid of mir-34a expression in all analyzed tissues, but were viable and fertile. A comprehensive standardized phenotypic analysis including more than 300 single parameters revealed no apparent phenotype. Analysis of miR-34a expression in human medulloblastomas and medulloblastoma cell lines revealed significantly lower levels than in normal human cerebellum. Re-expression of miR-34a in human medulloblastoma cells reduced cell viability and proliferation, induced apoptosis and downregulated the miR-34a target genes, *MYCN* and *SIRT1*. Activation of the Shh pathway by targeting SmoA1 transgene overexpression causes medulloblastoma in mice, which is dependent on the presence and upregulation of *Mycn*. Analysis of miR-34a in medulloblastomas derived from ND2:SmoA1(tg) mice revealed significant suppression of miR-34a compared to normal cerebellum. Tumor incidence was significantly increased and tumor formation was significantly accelerated in mice transgenic for SmoA1 and lacking miR-34a. Interestingly, Mycn and Sirt1 were strongly expressed in medulloblastomas derived from these mice. We here demonstrate that miR-34a is dispensable for normal development, but that its loss accelerates medulloblastomagenesis. Strategies aiming to re-express miR-34a in tumors could, therefore, represent an efficient therapeutic option.

What's new?

MicroRNAs (miRNAs) play an important role in cancer initiation and progression. An miRNA called "miR-34a" is downregulated in a variety of human cancers. In this study, the authors found that miR-34a acts as a tumor suppressor in genetically engineered mice, and that it appears to regulate medulloblastoma formation *in vivo*. Restoring expression of miR-34a in medulloblastomas or other human cancers with deregulated miR-34a might, therefore, be a promising therapeutic strategy.

MicroRNAs (miRNAs) are a class of small endogenous RNAs that inhibit translation or initiate degradation of target mRNAs by sequence-specific interaction. They are involved in regulating important functions in development and differentiation, including cell cycle control and apoptosis.[1,2] Previous studies have evaluated the role of miRNAs in cancer initiation and progression.[3–5] Not only are miRNAs differentially expressed in tumors as compared with normal tissues, but also in the primary tumor.[6] Several reports point to the fact that miR-34a is frequently deleted or absent in human cancers, including medulloblastomas.[7–10] In mice, miR-34a is ubiquitously expressed, with the highest levels occurring in the brain.[11] MiR-34a is a direct target of the tumor suppressor p53, and ectopic miR-34a expression inhibits invasion and migration and induces apoptosis, senescence and cell cycle arrest.[10,12–15]. These p53-mediated effects are due to the repression of specific targets, including MYCN, SIRT1, E2F3, cyclin D1, DLL1, CDK4/6, cyclin E2, MET and BCL2.[10,14,16–18] However, it was most recently reported that mice deficient for miR-34a and/or its homolog, miR-34b/c, present no obvious phenotype.[19–21] In contrast to what was expected, miR-34a deficiency also did not accelerate lymphoma formation in Eμ-Myc mice.[20,21]

Medulloblastoma is the most common malignant and highly invasive embryonal brain tumor in childhood.[22] In children, medulloblastoma occurs at any age, and histological as well as molecular variants are associated with prognosis.[23–26] Despite multimodal therapy, one-third of patients with medulloblastoma succumb to the disease and survivors often suffer from treatment-related side-effects, including neurocognitive deficits related to craniospinal irradiation.[27,28]

Medulloblastomas arise either from a population of cells that have self-renewal capacity and the potential to differentiate in cells of all neuronal lineages, such as neural stem cells,[29,30] or from granule cell progenitors (GNPs).[31] MYCN drives the proliferation of GNPs during normal cerebellar development and during medulloblastomagenesis.[31–33] *MYCN* amplification or overexpression occurs in Sonic hedgehog signaling activated (Shh) medulloblastomas, while *MYC* is frequently amplified or overexpressed in Group 3 medulloblastomas, which are often associated with large cell anaplastic (LCA) or classical pathologies.[34] MYCN overexpression in GNPs from transgenic mice induced aggressive medulloblastoma with LCA histology.[35] These findings indicate that MYCN can drive medulloblastoma independently of Shh and could play an important role in medulloblastoma initiation.

The specific role of miRNAs in medulloblastoma pathogenesis has not been completely elucidated to date, although differential expression patterns in normal cerebellum and medulloblastomas, as well between different medulloblastoma subgroups have been reported.[36,37] Both the MYCN-regulated mir-17–92 cluster,[37,38] and coexpressed miR-182 and miR-183 were shown to play an oncogenic role in medulloblastoma,[36,39] by increasing proliferation and dissemination. In contrast, miR-34a has been reported to have a tumor suppressive function by downregulating MAGE antigens or proteins involved in NOTCH signaling in medulloblastoma.[40,41]

However, the role of miR-34 in the initiation and progression of medulloblastoma *in vivo* or its effect on MYCN expression in medulloblastoma cells has not yet been investigated.

MYCN is a direct target of miR-34a and could be an initiator of medulloblastomagenesis. We investigated the role of miR-34a in medulloblastoma tumor biology *in vitro* and *in vivo* by re-expressing miR-34a in medulloblastoma cell lines and analyzing the relevance of this tumor suppressive miRNA for medulloblastoma progression by cell viability, cell proliferation and apoptosis. We generated miR-34a knockout mice to assess the physiological role of the miR-34a during development and in the adult organism. To further characterize the importance and the function of miR-34a during tumor initiation and progression, we crossbred the miR-34a knockout mice with the well-established ND2:SmoA1(tg) mice with a penetrance for developing medulloblastoma. Our

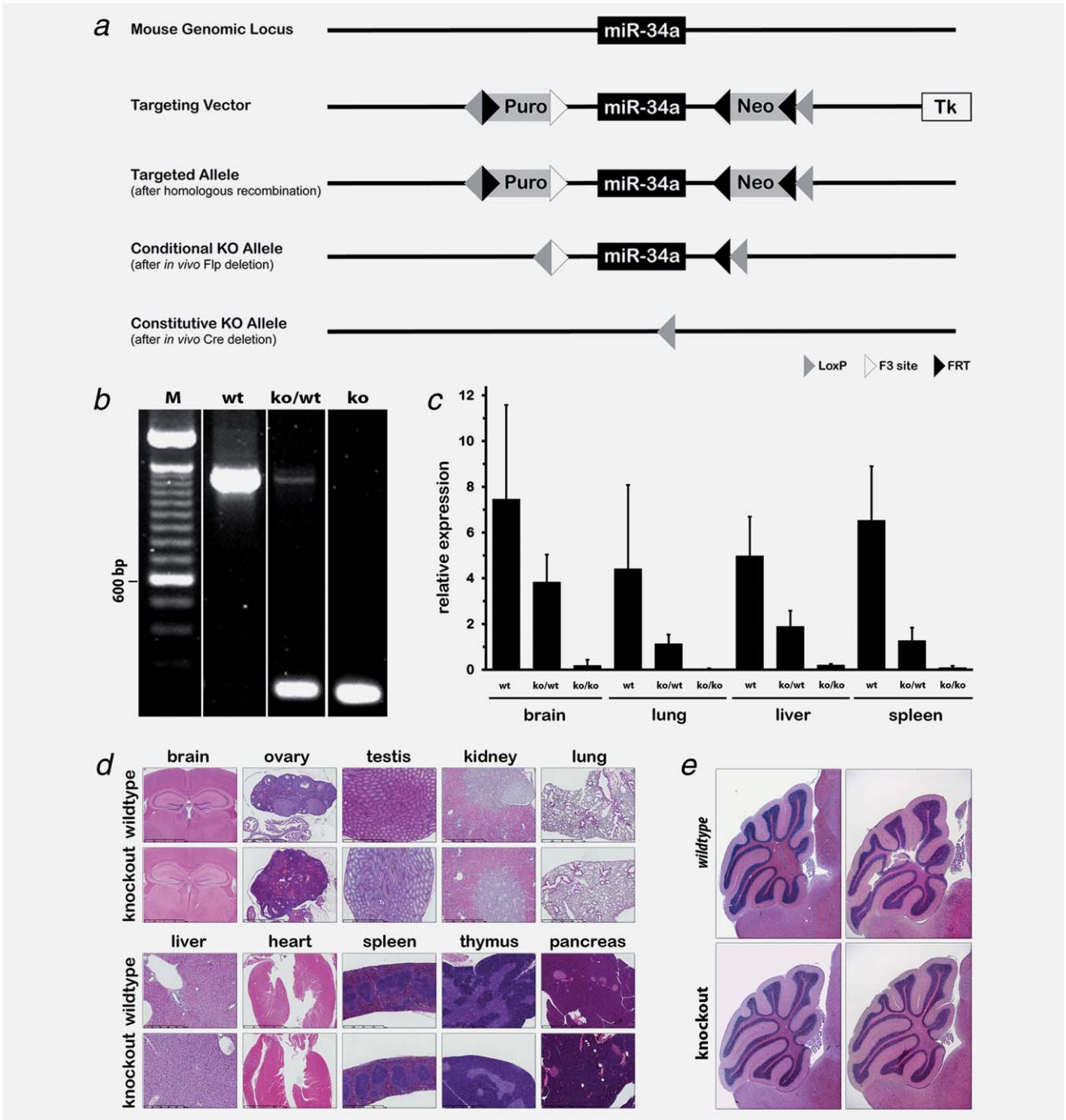

Figure 1..

results indicate a relevant role of miR-34a as a regulator of medulloblastoma formation.

Material and Methods

Generation of miR-34a mutant mice

The transgenic miR-34a knockout mouse was generated as previously described[42,43] and is outlined in Figure 1*a* (Taconic Artemis, Cologne, Germany). The targeting vector was derived from a C57Bl/6 bacterial artificial chromosome clone that includes the miR-34a gene. The first *loxP* site and the F3-flanked selection marker (Puro) were inserted in nonconserved sequence at about 0.8 kb upstream of the mir-34a gene. The second *loxP* site and the FRT-flanked Neomycin cassette (Neo) were inserted in a nonconserved sequence stretch at about 0.4 kb downstream of miR-34a. A counterselection cassette encoding thymidine kinase (TK) was inserted at the 3′ end of the vector. C57Bl/6N embryonic stem cells (ESCs) were electroporated and selected on d5 after electroporation by adding G418 (positive selection) and gancyclovir (negative selection) and by adding puromycin on d2. Correct recombination events were verified by Southern blot analysis of genomic DNA digested with BglII, AflII and HpaI, using both internal and external probes. The targeting strategy allowed generation of conditional and constitutive knockout alleles. After Flp-mediated removal of selection markers, the conditional knockout allele was generated. Crossing mice carrying mir-34a$^{fl/fl}$ to mice ubiquitously expressing Cre recombinase generated a miR-34a constitutive (conv) allele in which the mir-34a gene is deleted. Mouse genotyping was performed by PCR on tail tips using PCR Mix (Peqlab) as shown in Figure 1*b*. Specific detection of the miR-34a conv allele was achieved using the primers, CTTCAGCCATCCTGTTGAGG and CTTCAGGCAGAGTTGCATGG. Mouse experiments were carried out in accordance with the German Animal Welfare Act and approved by the local animal ethics committees.

Phenotyping of mice

The primary cohort for the phenotyping screen consisted of 36 miR-34a knockout mice and 37 wildtype control littermates. All animals were analyzed in the German Mouse Clinic (GMC) for signs of dysmorphology, behavioural and neurological peculiarities, eye properties, nociception, energy metabolism, clinical chemistry, immunology, allergy, steroid metabolism, cardiovascular and lung function and pathology. In addition, molecular profiling of selected organs was performed. The general setup of these standardized screens was previously described.[44] A secondary cohort was analyzed to confirm findings observed in the primary screen. In this second cohort, approximately half of the control animals were littermates of the mutants, as in the primary screen cohort, and the other half was substituted by independently bred control animals of the same genetic background. Most males of the secondary cohort were single-housed in contrast to the primary cohort, in which most males were group-housed.

Breeding of SmoA1-transgenic mice

Genotyping of the ND2:SmoA1(tg) mice was performed as described previously.[45] No crossbreeding of two mice transgenic for the ND2:SmoA1(tg) allele was allowed, ensuring that all ND2:SmoA1(tg) mice used in this study were hemizygous for the ND2:SmoA1 allele.

Histological analysis

Organs were fixed in 4% neutral buffered formalin for at least 24 hr. For histology, 4 μm sections were cut from paraffin blocks and stained with hematoxylin and eosin (H&E). Pictures were taken using the NanoZoomer 2.0HT (Hamamatsu Photonics Deutschland GmbH, Herrsching am Ammersee, Germany).

Nucleic acid isolation

DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions.

Figure 1. Generation and phenotypic analysis of miR-34a knockout mice. (*a*) Schematic representation of the miR-34a targeting construct. The first loxP site and the F3-flanked selection marker (Puro) have been inserted in a nonconserved sequence region ~0.8 kb upstream of miR-34a. The second loxP site and the FRT-flanked neomycin cassette have been inserted a nonconserved sequence region at ~0.4 kb downstream of miR-34a. The TK expression cassette is inserted downstream of the target as a negative selection marker. The targeting strategy allows generation of conditional and constitutive knockout alleles. After Flp-mediated removal of the selection marker the conditional ko allele is generated. *In vivo* Cre-mediated deletion of miR-34a resulted in the constitutive ko allele. (*b*) PCR of mouse genomic DNA from miR-34a homozygous mice (ko), wildtype mice (wt) and heterozygous mice (ko/wt). A single 257 bp fragment is indicative of miR-34a ko mice, a single 1379 bp fragment indicates wildtype, while both fragments are detectable in heterozygous mice (M, marker). (*c*) Detection of miR-34a deficiency in various organs. RT-qPCR was performed on RNA extracted from miR-34a$^{ko/wt}$, miR-34a$^{ko/ko}$ and wildtype brains, lungs, livers and spleens. RT-qPCR confirmed gene dose-dependent miR-34a expression in all organs. The loss of genetic information for miR-34a in the homozygous knockout consequently led to a complete loss of miR-34a expression. Data were normalized to RNU6B expression. (wt, wildtype; ko/wt, heterozygous; ko/ko, homozygous). (*d*) Hematoxylin-eosin (H&E) staining of various organs from wildtype, heterozygous and homozygous miR-34a deficient mice. H&E stained histological slides of coronal sections of brain, ovary, testis, kidney and lung revealed no difference in the organ histology between the wildtype control animals and the miR-34a mutants. Magnifications: brain, testis = 2.5×; ovary, kidney, lung = 5×. H&E staining was performed on sagittal sections of heart and coronal sections of liver, spleen, thymus and pancreas. No difference in the organ histology between control and mutant animals was detected. Magnifications: liver = 10×; heart, spleen, thymus, pancreas= 5×.

Gene expression analysis

Total RNAs from five human nonmalignant cerebellar samples derived from adult donors (age range 21–29 years) were purchased from Biocat [Human Adult Normal Tissue Total RNA-Brain-Cerebellum(Cat#: R1234040-10-3(5)-BC); Biocat GmbH, Heidelberg]. The primary medulloblastoma tumors used for the miRNA analysis ($n = 21$) were collected from the initial biopsies at diagnosis and selected from the local tumor bank in Essen and from the tumor bank of the German Society for Pediatric Oncology and Hematology. The tumor subgroup information for the histopathological subgrouping is listed in Supporting Information Table 3. MiRNA was isolated from cells or primary medulloblastomas or normal cerebellum samples using the miRNeasy® Micro Kit (Qiagen), and cDNA reversetranscribed by miScript Reverse Transcription Kit (Qiagen). Real-time PCR was performed using the StepOnePlus real-time PCR system (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Expression of human miR-34a (Qiagen, assay no. MS00003318) and mouse miR-34a (Qiagen, assay no. MS00001428) were normalized to RNU6B expression. Data analysis and error propagation was performed using qbase^{PLUS} software 1.5 (http://www.biogazelle.com).

Cell lines and cell culture

The ONS-76, HD-MB3, UW-228 and DAOY human medulloblastoma cell lines were grown in RPMI 1640 supplemented with 10% FCS, L-glutamine and antibiotics, as described previously.[46] Medium for HD-MB3 was also supplemented with 1% nonessential amino acids. The identity of all cell lines was verified by the German Collection of Microorganisms and Cell Cultures using STR genotyping (DSMZ, Braunschweig, Germany).

Transient transfection of precursor miRNA

HiPerfect transfection reagent (Qiagen, Hilden, Germany) was used to transiently transfect 250 ng of miExpress precursor miRNA expression clones (Genecopoeia, Rockville, MD) into medulloblastoma cell lines according to the manufacturer's instructions.

Cell viability, proliferation and apoptosis analysis

One day before transfection, 4,000 cells were seeded in triplicate into 96-well plates to permit surface adherence and then transfected with 0.25-μg plasmid DNA. Cells were collected after 120 hr for cell viability, proliferation and apoptosis assays. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay was performed according to the manufacture's protocol (Roche, Penzberg, Germany). Cell proliferation was analyzed using the BrdU ELISA (Roche) and apoptosis was assessed using the Cell Death ELISA (Roche), both performed according to the manufacturer's protocols.

Western blotting

Cell pellets and lysates were harvested according to standard protocols. After blotting, membranes were blocked 1 hr with 5% nonfat dry milk in TBS-$T_{0,1}$, and incubated with antibodies against MYCN (Santa Cruz sc53993, 1:200) or SIRT1 (Cell Signaling #2310 and #2028, 1:1,000) overnight at 4°C, washed twice and then incubated 1 hr with horseraddish peroxidase-conjugated (HRP) anti-rabbit IgG (GE Healthcare, 1:2,000, room temperature) or HRP anti-mouse IgG (GE Healthcare NA9340V) at room temperature as previously described.[47] Proteins were visualized using the ECLplus western blotting detection kit (Amersham) and analyzed on a FusionFX7 detection device (Vilber Lourmat, Eberhartzell, Germany). GAPDH expression (α-GAPDH, Millipore) served as a loading control.

Statistical analysis

A student's two-sided *t*-test was used for the comparison of all interval variables. GraphPad Prism 5.0 was used to perform Kaplan–Meier survival analysis with log-rank statistics on mouse cohorts.

Results

Generation and phenotypic characterization of miR-34a knockout mice

To model and to characterize the function of miR-34a *in vivo*, we used targeted transgenesis in mice. Mice lacking the miR-34a gene were generated by tetraploid blastocyst complementation from the recombinant ES cells (Fig. 1*a*). Intercrossing of heterozygous mice produced homozygous miR-34a$^{ko/ko}$ offspring that were viable, fertile and obtained in the expected Mendelian ratio (chi-square = 2.2, $n = 149$, $p = 0.3$). The lifespan of the observed mice was 15–17 months. Mouse genotypes were confirmed using PCR specific for miR-34a (Fig. 1*b*). We next aimed to analyze miR-34a mRNA expression in a panel of adult murine tissues from wildtype and mutant mice. The expression levels of miR-34a in the different organs derived from wildtype mice was highly variable. It ranged from low expression in the lung up to very high expression in the brain (Fig. 1*c*). Analysis of miR-34a transcript expression in the knockout mice confirmed efficient silencing in all murine organs analyzed compared to miRNA levels in the control mice (Fig. 1*c*). Expression of miR-34a was dependent on zygosity, with virtually no detectable miR-34 transcript expression in tissues from mice with constitutive homozygous deletion of the miR-34a gene (Fig. 1*c*). To determine the physiological role of miR-34a during development and in the adult organism, a cohort of miR-34a knockout mice underwent a standardized and comprehensive phenotypic analysis at the GMC. The analysis comprises several hundred parameters to assess dysmorphology, behavior, neurology, eye development and vision, nociception, energy metabolism, clinical chemistry, immunology, allergy, steroid

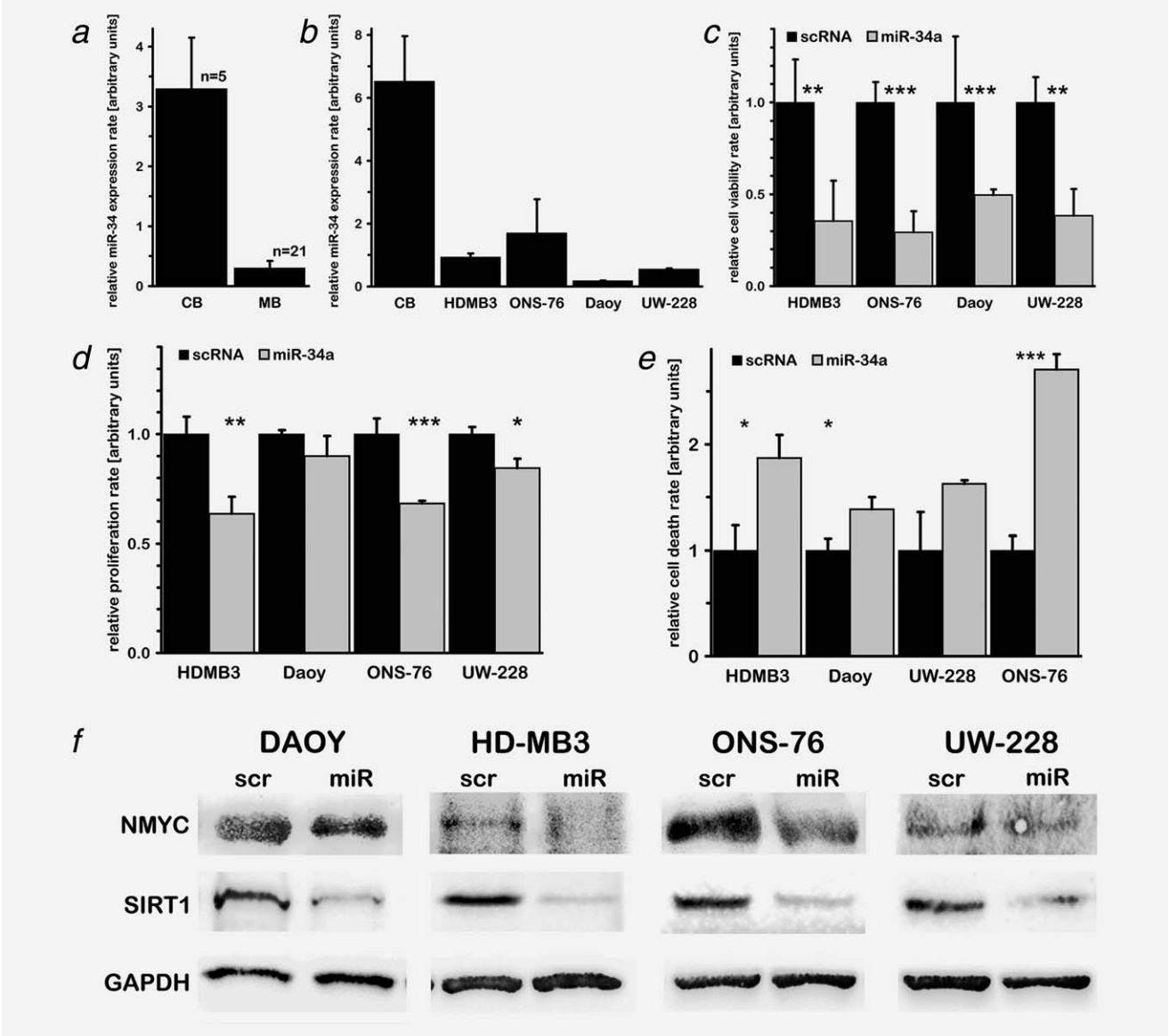

Figure 2. MiR-34a is expressed at low levels in medulloblastoma and its re-expression reduces viability of medulloblastoma cell lines. (*a*) MiR-34a expression was analyzed in 21 medulloblastomas and five samples of normal cerebellum by RT-qPCR. MiR-34a expression levels was significantly decreased in primary medulloblastomas. Data were normalized to RNU6B. (CB, cerebellum; MB, medulloblastoma). (*b*) MiR-34a expression was analyzed in four human medulloblastoma cell lines. Mir-34a mRNA expression was reduced in medulloblastoma cell lines in comparison to the control tissue. Data were normalized to RNU6B. (*c*) MiR-34a re-expression significantly reduced viability of medulloblastoma cells in the MTT assay. scRNA = scrambled RNA, $^{**}p < 0.005$, $^{***}p < 0.0005$. (*d*) MiR-34a transfection of medulloblastoma cells reduced proliferation after 120h using the BrdU ELISA, as compared to the controls scRNA. $^{*}p < 0.05$; $^{**}p < 0.005$, $^{***}p < 0.0005$. (*e*) Fractions of apoptotic cells were elevated in miR-34a transfected medulloblastoma cells using the Cell Death ELISA. $^{*}p < 0.05$; $^{***}p < 0.0005$. (*f*) Western blotting for MYCN and SIRT1 of whole-cell lysates of medulloblastoma cell lines with and without miR-34a (miR) re-expression confirmed reduced protein expression.

metabolism, cardiovascular analysis, lung function, molecular phenotyping and pathology.[44] This highly standardized analysis enables the detection of phenotypes that would otherwise not be detected, and also allows to exclude the presence of phenotype abnormalities in case of negative results. In summary, no differences between miR-34a homozygous knockout mice and their wildtype littermates were detected that could be validated in secondary screens for the analyzed parameters. Some differences were observed only in the primary screens of neurological and behaviour parameters (results of phenotyping summarized in Supporting Information Tables 1 and 2 and Supporting Information Figs. 1 and 2). No histological differences between the organs from miR-34a knockout mice and those from control animals (Figs. 1*d* and 1*e*) were observed also in regard to the cerebellum, and no increase in tumor incidence was observed in aged miR-34a knockout mice. These results are in line with previous reports.[20,21] Taken together, miR-34a deficient mice are

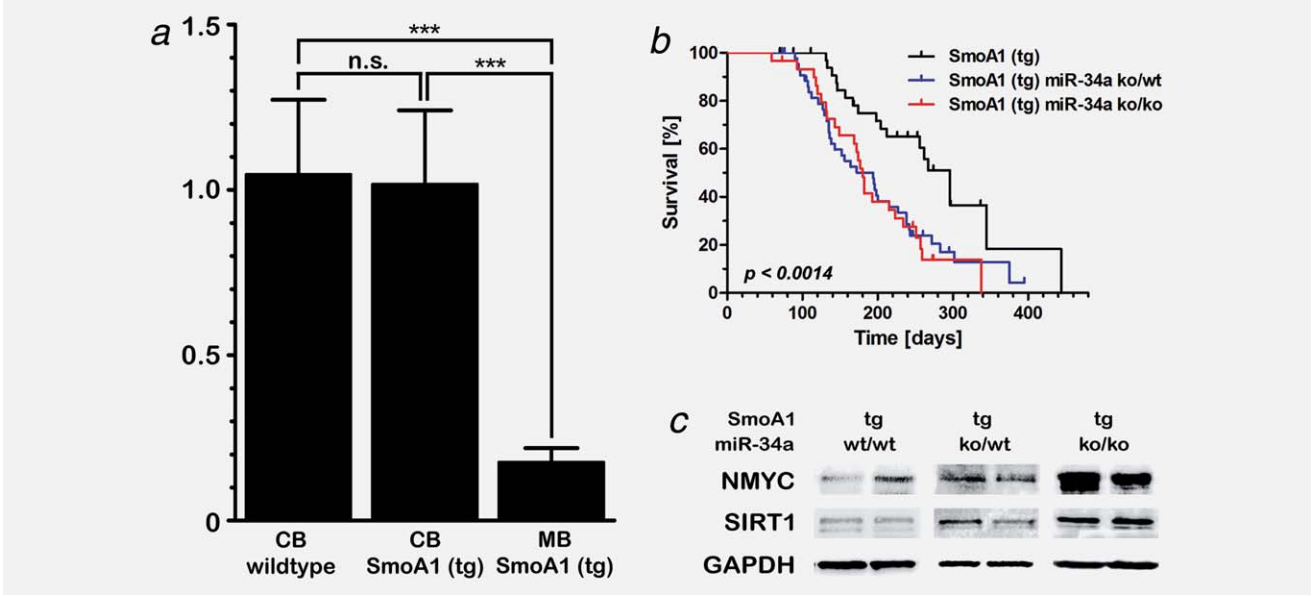

Figure 3. MiR-34a knockout accelerates tumor formation in a mouse model of medulloblastoma. (*a*) Expression of miR-34a was analyzed by RT-qPCR in the cerebella from wildtype mice and non-tumor-bearing ND2:SmoA1(tg) mice, as well as in medulloblastomas that arose in ND2:SmoA1(tg) mice. Data were normalized to RNU6B. (CB wildtype, cerebella from wildtype mice; CB SmoA1 (tg), cerebella from non-tumor bearing ND2:SmoA1(tg) mice; MB SmoA1 (tg), medulloblastomas derived from ND2:SmoA1(tg) mice). (*b*) Kaplan-Meier analysis of transgenic mice with the endpoint defined as detection of symptomatic appearance of medulloblastoma. Mice double-transgenic for ND2:SmoA1(tg) and miR-34a$^{ko/wt}$ (blue curve) or ND2:SmoA1(tg) and miR-34a$^{ko/ko}$ (red curve) showed a significantly shorter time to tumor formation than mice expressing the SmoA1 transgene alone (black curve). (*c*) Western blot of whole-tissue extracts from tumors arising in ND2:SmoA1(tg) mice and ND2:SmoA1(tg);miR-34a$^{ko/wt}$ or ND2:SmoA1(tg);miR-34a$^{ko/wt}$ mice. Mycn and Sirt1 expression was strongly increased in tumors derived from SmoA1;miR-34a double-transgenic mice. (wt/wt, tumor-bearing ND2:SmoA1(tg) mice; wt/ko, double-transgenic ND2:SmoA1(tg);miR-34a$^{ko/wt}$ mice; ko/ko, double transgenic ND2:SmoA1(tg);miR-34a$^{ko/ko}$ mice). For each genotype, tumors derived from two different mice are displayed.

viable, fertile and show no major phenotypic abnormalities despite some behavioral and neurological changes.

The role of miR-34a in human medulloblastoma

To investigate and define the effects of miR-34a overexpression on tumor biology (cell viability, proliferation and apoptosis) and on the expression of their targets, we first analyzed the miR-34a mRNA expression in 21 primary medulloblastomas compared to five cerebellum samples (Fig. 2*a*). Tumor samples expressed threefold less miR-34a. We also detected lower miR-34a expression in four medulloblastoma cell lines compared to normal cerebellum (Fig. 2*b*). Because miR-34a is a putative tumor suppressor, we first assessed the effect of miR-34a re-expression on cell viability and proliferation. We observed inhibition of cell growth (Fig. 2*c*) and proliferative capacity (Fig. 2*d*) in all four medulloblastoma cell lines after ectopic expression of miR-34a using a pEZX-based plasmid (Genecopoeia). This effect was independent of the presence of functional TP53, since *TP53* is mutated in DAOY (C242F) and UW-228 (T155N) medulloblastoma cell lines,[48] while HD-MB3 and ONS-76 cells possess no inactivating p53 mutations, but the R72P SNP in *TP53*, which affects neither TP53 expression nor function.[49] Additionally, the fraction of apoptotic cells was significantly lower in nontransfected medulloblastoma cells than in cells ectopically expressing miR-34a (Fig. 2*e*). In line with the previously published data and based on our results, we conclude that miR-34a has tumor suppressive effects *in vitro*.

MiR-34a is involved in multiple tumor suppressive pathways by directly or indirectly inhibiting the expression of numerous proteins. Therefore, and as further support of the relevance of miR-34 in the formation and progression of human medulloblastoma, we investigated the expression of its downstream targets, SIRT1 and MYCN, on protein level in medulloblastoma cell lines. SIRT1 and MYCN expression were significantly downregulated by enforced miR-34a expression in all four medulloblastoma cell lines in contrast to cells transfected with a control plasmid (Fig. 2*f*).

Taken together, we detected decreased miR-34a expression in a panel of human medulloblastoma cell lines and in primary medulloblastomas. Furthermore, our results indicate that miR-34a re-expression induces apoptosis while also inhibiting proliferation of medulloblastoma cell lines.

Absence of miR-34a accelerates tumor formation in murine medulloblastoma

As we found miR-34a expression to be downregulated in primary human medulloblastomas and in medulloblastoma cell lines, we proceeded to address the impact of miR-34a expression on medulloblastoma tumorigenesis in a mouse model. We used the established medulloblastoma model, ND2:SmoA1 transgenic (tg) mice,[45] which model the Shh

Table 1. observed mortality for the ND2:SmoA1 mouse line and the double transgenic ND2:SmoA1;miR-34a mouse lines

mouse line	Number of mice developing tumors	Time (months)			tumor incidence
		up to 4	up to 8	up to 16	
SmoA1 +/-	18 out of 47	2	11	18	38%
SmoA1;miR-34a ko/wt	36 out of 47	12	32	35	75%
SmoA1;miR-34a ko/ko	25 out of 31	8	22	25	81%

The total number of mice observed in each time period is given. Death in all cases was a result of tumor formation.

medulloblastoma subtype. Mice hemizygous for the ND2:SmoA1 allele start developing medulloblastomas at ~4 months of age and at 8 months, 50% of all ND2:SmoA1(tg) mice had to be sacrificed due to tumor burden (Fig. 3*b*).

The miR-34a levels in the tumor tissue of ND2:SmoA1(tg) mice with established medulloblastomas were significantly lower than in the cerebella from wildtype mice ($p < 0.0001$) or in the cerebella of nontumor-bearing ND2:SmoA1(tg) mice ($p < 0.0001$) (Fig. 3*a*). To elucidate the function of miR-34a on tumor formation and progression, we crossbred miR-34a knockout mice with the ND2:SmoA1(tg) mice to produce double-transgenic mice. To confirm the efficient knock-out of miR-34a transcript expression in ND2:SmoA1(tg) mice with miR-34a knock-out (ND2:SmoA1(tg);miR-34a$^{ko/wt}$ and ND2:SmoA1(tg);miR-34a$^{ko/ko}$), we analyzed miR-34a expression in these mice in comparison to miR-34a levels in the ND2:SmoA1(tg) control mice (with wildtype miR-34a). In ND2:SmoA1(tg);miR-34a$^{ko/ko}$ mice, miR-34a expression was virtually not detectable in neither cerebellum nor tumor tissue. In cerebella derived from ND2:SmoA1(tg);miR-34a$^{ko/wt}$ mice, expression of miR-34a was significant lower than in cerebella derived from ND2:SmoA1(tg) control mice. Interestingly, miR-34a was also not detectable in tumors derived from ND2:SmoA1(tg);miR-34a$^{ko/wt}$ mice.

Mice expressing the SmoA1 transgene and deficient for the miR-34a gene displayed significantly accelerated medulloblastoma formation compared to mice expressing SmoA1 alone ($p < 0.0014$, Fig. 3*b*). Both tumor incidence and time to tumor formation in double-transgenic mice were significantly different from mice expressing the SmoA1 transgene alone (Table 1 and Supporting Information Fig. 4). To further characterize the functional role of miR-34a as a regulator of medulloblastoma formation, expression of the miR-34a target genes, *Sirt1* and *Mycn*, were analyzed in the resulting tumors. Levels for both Sirt1 and Mycn proteins were elevated in tumors from double-transgenic mice compared to tumors from SmoA1 mice, and target protein expression levels were associated with the miR-34a gene dosage in the mice (Fig. 3*c*). Taken together, our results indicate an important and relevant role of miR-34a in medulloblastoma formation and progression *in vivo*.

Discussion

In several tumor entities miRNAs are deregulated. A miRNA can act as an oncogene by inhibiting the expression of a tumor suppressor gene or as a tumor suppressor by inhibiting the expression of an oncogene.[4] Our analysis of miR-34a expression in medulloblastoma cell lines and primary human medulloblastoma showed a significant downregulation of this miRNA compared to normal cerebellar tissue, confirming previous findings that miR-34a is a prominent tumor-suppressive miRNA, which is downregulated in different tumors. The aim of our *in vitro* studies was to explore the function of the miR-34a in medulloblastoma cell lines. Re-expression of miR-34a, which was present only at low levels in all human medulloblastoma cell lines analyzed, induced apoptosis and reduced the viability and proliferative capacity of the cells. These data confirm previously published reports of miR-34a tumor-suppressive functions detected in other tumor entities[12,13,50,51] and characterize the phenotypic effect of miR-34a re-expression in medulloblastoma cells.[40,41]

Next, we analyzed miR-34a function *in vivo* by targeted transgenesis in mice. For this purpose, we generated a transgenic mouse strain harboring a deletion of the miR-34a gene and, thus, lacking functional miR-34a. Using this strain, we comprehensively assessed the overall picture of miR-34a functionality in adult mice. For this purpose, 80 mice underwent an extensive standardized screen interrogating ~300 physiological parameters that revealed no major phenotypical abnormalities in the miR-34a deficient mice. This comprehensive screen, however, revealed some neurological and behavioral changes in mutant mice that have not been previously assessed or reported, including a decrease in prepulse inhibition and the acoustic startle response (Supporting Information Figs. 1 and 2). In a subsequent validation cohort these behavioral phenotypes could not be reproduced, possibly due to the inclusion of nonlittermate controls. Despite these putative behavioral and neurological changes, miR-34a deficient mice are viable, fertile and show no major phenotypic abnormalities. Our results are in line with and extend the previously reported work of Choi *et al.* and Concepcion *et al.*[20,21] Choi *et al.* described the generation of a miR-34 knockout mice born with an expected Mendelian ratio and no visible abnormalities in development or pathology. Overexpression of miR-34a, miR-34b and miR-34c in ESCs led to reductions in Nanog, Sox2 and Mycn expression. Vice versa, Choi *et al.* showed that induced pluripotent stem cells deficient in miR-34a and miR-34b/c had elevated Mycn expression.[21] Concepcion *et al.* reported no hematopoietic abnormalities or abnormalities of the several organs that they analyzed histologically.[20] It remains to be explored whether challenging the mice with

different stimuli or aging alone might reveal phenotypic abnormalities resulting from the miR-34 knockout. Indeed, Boon *et al.* found reduced age-associated decline in cardiac function in miR-34a deficient mice.[19] Taken together, our comprehensive phenotypic screen corroborates and extends previous reports and strongly points to a nonessential role of miR-34a in normal murine development.

One prominent target of miR-34a is the *MYCN* oncogene, which is amplified in 4–10% of medulloblastoma patients.[52] Overexpression of MYCN has been observed in 55–88% human medulloblastomas.[53,54] MYCN is an important and direct target of the Shh signaling pathway promoting cell cycle progression in the developing cerebellum. MYCN upregulation is observed in medulloblastomas associated with activated Shh signaling[55,56] and is correlated with unfavorable outcome. Mycn is required for normal cerebellar development, but much less is known about Mycn function in the mouse hindbrain. Numerous investigators have studied the mechanisms how MYCN alters the stages of medulloblastoma formation or progression using different mouse models for medulloblastoma.[33,57] Aberrant activation of Mycn expression in the developing mouse cerebellum initiates a variety of medulloblastomas, including both classical and LC/A tumors.[58] Hallahan *et al.* developed the well-established mouse model, ND2:SmoA1(tg). This model is based on activation of the Shh pathway by transgenic expression of a constitutively active form of smoothened in mouse cerebellar granule neuron precursors. Transgene expression causes early cerebellar granule cell hyperproliferation and ~50% incidence of medulloblastoma formation.[45] In these ND2:SmoA1(tg) mice, medulloblastomas arise depending on the presence and upregulation of Mycn.[32] We detected a tumor specific suppression of miR-34a in tumors arising in ND2:SmoA1(tg) mice. Crossing of SmoA1 mice with miR-34a knockout mice significantly shortened the time to tumor formation, increased tumor incidence and reduced survival of double-transgenic ND2:SmoA1(tg);miR-34a$^{ko/ko}$ mice. Interestingly, Sirt1 and Mycn expression was inversely correlated with miR-34a gene dosage in SmoA1-induced tumors. As the highest miR-34a target gene levels were observed in double transgenic ND2:SmoA1(tg);miR-34a$^{ko/ko}$ mice, miR-34a seems to act as a repressor of oncogenes in a dose dependent manner. These results implicate that miR-34a is involved in tumor incidence and survival in the ND2:SmoA1(tg) medulloblastoma model, and this is also reflected by increased expression of miR-34a target genes with oncogenic functions.

The mechanism, by which miR-34a is downregulated during the process of tumorigenesis in the ND2:SmoA1(tg) model remains elusive. It is tempting to speculate that activation of Shh-signaling pathway itself downregulates miR-34a in a feedback loop. Indeed, Shh-induced Gli1 has recently been shown to downregulate p53 transcription,[59] while Shh-induced MYCN could inhibit p53 activity via induction of MDM2.[60] Both mechanisms reducing p53 activity would thereby impair p53-driven miR-34a expression. An alternative explantion for the loss of miR-34a expression in the course of medulloblastoma development would be a selective pressure favoring cells having gradually downregulated or even silenced miR-34a not as a (direct) effect of Shh signaling.

Here, we demonstrate that miR-34a deficiency accelerates tumor formation in genetically engineered mice, thus, that miR-34a exerts a tumor-suppressive function *in vivo*. This has been anticipated by many investigators based on *in vitro* data, but is in contrast to a recent report by Concepcion *et al.*, who were unable to detect an effect of miR-34 deficiency on lymphoma formation in Eμ-Myc transgenic mice. Binding sites for miR-34a are present in the 3′-UTR of both MYCN and cMYC. However, tumors in Eμ-Myc mice are driven by transgenic *cMyc* cDNA overexpression. The *cMyc* cDNA expressed in the Eμ-Myc mice lacks the natural cMYC 3′-UTR which harbors miRNA binding sites, and therefore is not regulated by miR-34a in the Eμ-Myc model. If *cMyc* is a relevant target of miR-34 in lymphoma, as *MYCN/Mycn* most likely is in medulloblastoma, the missing effect of miR-34a on lymphoma development in the Eμ-Myc model may have resulted from intrinsic Eμ-Myc model properties, such as missing regulatory miR-34a binding motifs in the ectopically expressed *Myc* cDNA (Supporting Information Fig. 5). Thus, the findings for lymphoma in addition to those described here for medulloblastoma point to a tumor-suppressive role of miR-34a by regulating myc family protein expression.

The results presented here provide important clues into the functional roles of miR-34a and its relation to the mechanisms and oncogenic signaling pathways involved in medulloblastoma formation. In most cases multiple signaling pathways are simultaneously regulated by modulation of a single miRNA. Thus, therapy targeting a specific miRNA may represent a more effective cancer treatment option than the pharmacological elimination of a single gene function.[61] Comprehensive knowledge of the targets, the underlying mechanisms and the estimation of the effects of miR-34 are of clinical relevance for the assessment and prediction of possible side effects of miR-34a-based therapy. One advantage of a miR-34a-based therapy in medulloblastoma is that by overexpressing miR-34a, several targets and signaling pathways can be influenced. Re-expressing miR-34a could simultaneously downregulate several target genes, including MYCN, SIRT1, NOTCH pathway genes[21] and MAGE,[41] and signaling pathways that impede development of resistance to therapy. Effective miR-34a re-expression in human medulloblastoma cells *in vivo* using adenovirus as a vehicle has already been demonstrated

by de Antonellis *et al.*[40] Thus, strategies aiming to re-express miR-34a in tumors could be a promising and effective therapeutic strategy for the future, not only against medulloblastoma but also other tumor types.

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