MicroRNA Biogenesis and Hedgehog-Patched Signaling Cooperate to Regulate an Important Developmental Transition in Granule Cell Development

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ABSTRACT The *Dicer1*, *Dcr-1* homolog (*Drosophila*) gene encodes a type III ribonuclease required for the canonical maturation and functioning of microRNAs (miRNAs). Subsets of miRNAs are known to regulate normal cerebellar granule cell development, in addition to the growth and progression of medulloblastoma, a neoplasm that often originates from granule cell precursors. Multiple independent studies have also demonstrated that deregulation of Sonic Hedgehog (Shh)-Patched (Ptch) signaling, through miRNAs, is causative of granule cell pathologies. In the present study, we investigated the genetic interplay between miRNA biogenesis and Shh-Ptch signaling in granule cells of the cerebellum by way of the *Cre/lox* recombination system in genetically engineered models of *Mus musculus* (mouse). We demonstrate that, although the miRNA biogenesis and Shh-Ptch-signaling pathways, respectively, regulate the opposing growth processes of cerebellar hypoplasia and hyperplasia leading to medulloblastoma, their concurrent deregulation was nonadditive and did not bring the growth phenotypes toward an expected equilibrium. Instead, mice developed either hypoplasia or medulloblastoma, but of a greater severity. Furthermore, some genotypes were bistable, whereby subsets of mice developed hypoplasia or medulloblastoma. This implies that miRNAs and Shh-Ptch signaling regulate an important developmental transition in granule cells of the cerebellum. We also conclusively show that the *Dicer1* gene encodes a haploinsufficient tumor suppressor gene for *Ptch1*-induced medulloblastoma, with the monoallelic loss of *Dicer1* more severe than biallelic loss. These findings exemplify how genetic interplay between pathways may produce nonadditive effects with a substantial and unpredictable impact on biology. Furthermore, these findings suggest that the functional dosage of *Dicer1* may nonadditively influence a wide range of Shh-Ptch-dependent pathologies.

KEYWORDS Dicer1 protein; mouse; genes; tumor suppressor; Hedgehog proteins; heterozygote; medulloblastoma; nervous system malformations

MicroRNAs (miRNAs) are a class of ~22-nucleotide noncoding RNA molecules that function as sequence-specific guides. They direct an Argonaute (Ago) protein-containing RNA-induced silencing complex (RISC) to target messenger RNAs (mRNAs) (Hutvagner and Zamore 2002; Mourelatos *et al.* 2002) by way of Watson–Crick pairing of the miRNA seed sequence to complementary elements usu-

ally found within the 3' untranslated region of target mRNAs (Bartel 2009). This results in translational repression and/or mRNA destabilization and decay (see Iwakawa and Tomari 2015 for a recent review). MicroRNAs are first transcribed as long primary transcripts (pri-miRNAs) with a characteristic hairpin-like secondary structure (Mourelatos *et al.* 2002). In the canonical bioprocessing pathway, this hairpin-like structure is recognized by the microprocessor–enzyme complex, through Ribonuclease 3 (protein Drosha) and Microprocessor complex subunit DGCR8 (Lee *et al.* 2003; Denli *et al.* 2004; Han *et al.* 2004), which cleaves the pri-miRNA into an ~70-nucleotide precursor miRNA (pre-miRNA). The pre-miRNA is further processed, once exported to the cytoplasm (Bohnsack *et al.* 2004; Lund *et al.* 2004), by the RNase III enzyme Dicer, into a mature ~22-nucleotide RNA duplex

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with 2-nucleotide overhangs at both 3' hydroxyl termini (Blaszczyk *et al.* 2001; Hutvagner *et al.* 2001). The endoribonuclease Dicer, in addition to cleaving pre-miRNAs, participates in the loading of miRNAs into RISC (Chendrimada *et al.* 2005). Both the endoribonuclease Dicer and protein Drosha are thought to cleave double-stranded RNAs by forming an intramolecular pseudodimer between two tandem RNase III domains (a/b) (Han *et al.* 2004; Zhang *et al.* 2004). In mice, a lysine residue (K1790) within the ribonuclease (RNase) IIIb domain of endoribonuclease Dicer appears critical for double-stranded RNA cleavage (Du *et al.* 2008).

MicroRNAs, and by extension endoribonuclease Dicer function, are implicated in virtually every biological process in a wide range of organisms, and changes in their expression are associated with a plethora of human pathologies. In the cerebellum, miRNA dysfunction, via ablation of endoribonuclease Dicer function, has been linked to movement disorders (Lee *et al.* 2008; Constantin and Wainwright 2015), neurodegeneration (Schaefer *et al.* 2007), and the malignant neoplasm, medulloblastoma (Zindy *et al.* 2015). MicroRNAs play a prominent role in medulloblastoma, given that distinct miRNA expression signatures are able to classify the molecular (Cho *et al.* 2011) and histopathological (Ferretti *et al.* 2009) subtypes of human medulloblastoma, and dozens of independent studies have identified the functional networks by which specific miRNAs contribute to the growth and progression of this disease. For example, the *miR-17~92* cluster causally contributes to medulloblastoma and granule cell precursor proliferation through Sonic Hedgehog (Shh)-Patched (Ptch) signaling (Northcott *et al.* 2009; Uziel *et al.* 2009). Similarly, *miR-106b* has been shown to promote the proliferation, migration, and invasion potential of medulloblastoma (Li *et al.* 2015), presumably via the principal mediator of the transcriptional response of the Shh-Ptch-signaling pathway, *GLI-Kruppel family member GLI2 (Gli2)* (Constantin and Wainwright 2015).

The cerebellum is located anteriorly on the brainstem, close to the midbrain-hindbrain boundary. It is essential for the fine motor control of movement and posture and, on the basis of human lesion and functional neuroimaging studies, appears to contribute to a broad range of high-order non-motor functions (see Buckner 2013 for review). A principal cell type of the cerebellum, and the most numerous in the central nervous system, is the granule cell. Granule cells are of critical importance to cerebellar function, as exemplified by the severity of motor coordination, language, and cognitive deficits observed in patients with congenital granule cell degeneration (Pascual-Castroviejo *et al.* 1994). Precursors of granule cells arise from the rhombic lip, a thin strip of neuroepithelium that borders the non-neuronal roof plate of the fourth ventricle (Alder *et al.* 1996). During embryogenesis, granule cell precursors leave the rhombic lip and migrate anteriorly over the outer surface of the cerebellum to form a second germinal structure, termed the "external germinal layer" (see Hatten and Roussel 2011 for review). After birth, granule cell precursors undergo rapid proliferation in the

external germinal layer. Proliferation is maintained until perinatal life by way of multiple symmetric mitoses in single-fated germinal cells (Espinosa and Luo 2008). Once granule cells become postmitotic, they extend parallel fiber axons that synapse to the dendrites of GABAergic inhibitory Purkinje neurons, while the soma of the granule cells migrate interiorly to their final destination in the internal granular layer (Komuro and Rakic 1998). Multiple mitogenic pathways are known to drive the rapid expansion of granule cell precursors in the external germinal layer, the most potent of which is Shh (Dahmane and Ruiz i Altaba 1999; Lewis *et al.* 2004). In the central nervous system, the binding of Shh to the transmembrane protein Ptch relieves the inhibitory action of Ptch on the transmembrane protein Smoothened (Johnson *et al.* 2000; Briscoe *et al.* 2001). Active-state Smoothened is then able to translocate to the primary cilium (Corbit *et al.* 2005), where it promotes the formation of the active Gli family of transcription factors, which in turn induces the expression of target genes such as *Gli1*, *Ptch1*, and *Nmyc* (Humke *et al.* 2010; Tukachinsky *et al.* 2010). Therefore, the removal of Ptch from the plasma membrane, or the binding of Ptch to Shh, activates Shh-Ptch signaling.

Although some miRNAs are known to interact with Hedgehog-Ptch signaling, the magnitude and directionality of this interaction is not well understood, even within the cerebellum. In the present study, we investigated the gene dose-dependent interaction between miRNA biogenesis and Shh-Ptch signaling in cerebellar granule cell precursors. The inactivation of *Dicer1* in granule cell precursors is hypoplastic (Constantin and Wainwright 2015; Zindy *et al.* 2015). Alternatively, constitutive activation of Shh-Ptch signaling in granule cell precursors is well known to produce hyperplasia that resembles human medulloblastoma (Goodrich *et al.* 1997; Pietsch *et al.* 1997; Raffel *et al.* 1997). In fact, the most widely used animal model for medulloblastoma is the targeted mutation of the *Ptch1* gene, either heterozygous null (Goodrich *et al.* 1997; Hahn *et al.* 1998; Zibat *et al.* 2009) or conditional knockouts associated with the granule neuron lineage (Ellis *et al.* 2003; Yang *et al.* 2008). Given that the two biological processes of hypoplasia and hyperplasia are on opposing growth spectrums, we hypothesized that the co-deregulation of miRNA biogenesis and Shh-Ptch-signaling pathways would attenuate overall growth and lead to reduced severity of murine medulloblastoma. To inactivate miRNA biogenesis, we used the conditional *Dicer1* allele (*Dicer1^{flox}*) with the knock-in mouse model, *Atonal homolog 1-Cre recombinase (Atoh1-Cre, also known as Math1-Cre)* to confer specificity to the granule cell lineage. Similarly, we constitutively activated Shh-Ptch signaling through the conditional *Ptch1* allele (*Ptch1^{flox}*), together with *Atoh1-Cre*. Indeed, we established that a strong genetic interaction exists between miRNA biogenesis and Shh-Ptch signaling; however, this interaction did not bring the growth phenotypes toward equilibrium. Instead, combined deregulation exasperated hypoplasia in a subset of animals and exasperated medulloblastoma in the remaining subset. These findings are of interest because they

suggest that the miRNA biogenesis and Shh-Ptch-signaling pathways positively cooperate with one another to combinatorially regulate an important developmental transition in granule cell development.

Materials and Methods

Mice

Dicer1flox, *Ptch1flox*, and *Gt(ROSA)26Sor;lacZ* mice have previously been described (Soriano 1999; Ellis *et al.* 2003; Harfe *et al.* 2005). *Atoh1-Cre* mice (line 8) were generated in the Rowitch laboratory at the Institute for Regeneration Medicine (San Francisco) and are modified from *Atoh1-Cre* mice previously described (Schuller *et al.* 2007).

Atoh1-Cre and *Gt(ROSA)26Sor;lacZ* mice were interbred to test the recombination efficiency and expression pattern of the *Atoh1-Cre* transgene. *Dicer1flox/wt;Ptch1flox/flox* and *Atoh1-Cre;Dicer1flox/wt;Ptch1flox/wt* mice were interbred to generate the experimental cohort with littermate controls. Experimental cohorts were monitored daily for signs of disease. Adult and neonatal mice with a severe disease score in any one of the following phenotypes—head swelling, respiratory changes, weight loss, unresponsiveness, poor grooming, loss of balance and/or correct posture, or abnormal movements—were euthanatized by decapitation, and immediately tissues of interest were harvested. All procedures involving animals were approved by the University of Queensland Animal Ethics Committee and were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Tissue processing

Mouse brains were dissected in ice-cold phosphate-buffered saline using a stereomicroscope. One-half of the brain, in the midsagittal plane, was immersed in 4% paraformaldehyde for 12–16 hr at 4° and then processed and embedded in paraffin. From the remaining half of the brain, a 2- to 3-mm² biopsy of the superficial-medial tumor mass or cerebellum was removed for protein harvest; the remaining tumor mass or cerebellum was stored in RNAlater reagent (Qiagen) for downstream DNA and RNA analyses. For β -galactosidase staining, embryonic tissues derived from *Atoh1-Cre;Gt(ROSA)26Sor;lacZ* mice were gently fixed in 2% formaldehyde and 0.2% glutaraldehyde for 2.5 hr at 4° with agitation. Following fixation, tissues were washed in phosphate-buffered saline, incubated with agitation in 30% sucrose in phosphate-buffered saline overnight at 4–10°, and then embedded in Tissue-Tek O.C.T. compound (Sakura).

To extract genomic DNA, a 2- to 3-mm² biopsy was removed from RNAlater-preserved tumors or cerebellums. Biopsies were homogenized with a sterile razor, digested with 500 μ g/ml of proteinase K in 60 mM Tris, 100 mM ethylenediaminetetraacetic acid, and 0.5% sodium dodecyl sulfate at 55° for 4 hr and then were purified using a standard phenol/chloroform method. The miRNeasy mini kit (Qiagen) was used to extract total RNA from a 2- to 3-mm² biopsy of RNAlater-preserved tissue.

Polymerase chain reaction analyses

All polymerase chain reaction (PCR) products were amplified using standard touchdown cycling conditions with a final annealing temperature of 55° and Taq DNA polymerase (Life Technologies). Cre-mediated recombination of genomic *Dicer1flox* was measured using the following primers: forward (5'-CCT GAC AGT GAC GGT CCA AAG-3') and reverse (5'-CCT GAG TAA GGC AAG TCA TTC-3'). The wild-type allele produced an ~1200-nt PCR product, the undisrupted *loxP* allele produced an ~1400-nt PCR product, and the recombined gene produced an ~350-nt PCR product. *LoxP* recombination of genomic *Ptch1* was measured using the following primers: forward (5'-TGG TTG TGG GTC TCC TCA TAT T-3') and reverse (5'-TGA CTG CAA ACT TTC CCA TCT-3'). The undisrupted gene was too large to amplify (and was therefore undetectable), while the recombined gene produced a 499-nt PCR product.

LoxP recombination of *Dicer1* complementary DNA (cDNA) was measured using the following primers: forward (5'-AGA CGC AGA GAA AAC CCT CA-3') and reverse (5'-TCT CCG CTG GGC TAA ACT T-3'). The undisrupted gene produced a 656-nt PCR product while the recombined gene produced a 387-nt PCR product. Alternatively, *loxP* recombination of *Ptch1* cDNA was measured using the following primers: forward (5'-TGG TTG TGG GTC TCC TCA TAT T-3') and reverse (5'-CAC CGT AAA GGA GGC TTA CCT A-3'). The undisrupted gene produced a 463-nt product while the recombined gene produced a 273-nt PCR product.

Complementary DNA was synthesized from 10 ng/ μ l of total RNA primed with Oligo(dT) (Life Technologies) by way of the SuperScript III system (Life Technologies) following the manufacturer's instructions. MicroRNA cDNA was synthesized using a TaqMan miRNA reverse transcription kit (Applied Biosystems). Each RNA sample (5 ng/ μ l) was reverse-transcribed in quadruplicate with specific miRNA stem-loop primers (Applied Biosystems), including the control gene RNA, *U6 small nuclear 6*, *pseudogene (Rnu6-2)*. Quantitative reverse transcriptase PCR was performed using the TaqMan universal PCR master mix with no AmpErase UNG (Applied Biosystems), in technical triplicate, following the manufacturer's instructions. An ABI Prism 7000 Sequence Detection system with SDS version 1.2.3 software was used to detect and analyze relative expression levels. Analysis was performed using the relative threshold cycle (CT) method.

Histology and imaging

Whole-mount dorsal views of the mouse cerebellum were captured during dissection using the Olympus SZX12 stereomicroscope. Gross histological analyses were performed using standard (Luna *et al.* 1968) hematoxylin and eosin Y (H&E) staining for 3.5 min and 45 sec, respectively, on 7- μ m-thick midsagittal sections of paraffin-preserved mouse brains. Paired box protein Pax-6 (Pax6) and neuronal nuclei (NeuN) co-immunolabeling was performed sequentially. Specifically, 7- μ m-thick midsagittal sections of paraffin-preserved mouse

brains were boiled for 3 min in citric acid-based Antigen Unmasking Solution (Vector Laboratories). Sections were blocked in 10% animal serum, 2% bovine serum albumin, and 0.025% Triton X-100 in phosphate-buffered saline for 1 hr and then incubated overnight at 4–10° with the first primary antibody, Pax6 (1:200, Covance), and then with secondary antibody conjugated to Alexa Fluoro 555 (1:250, Invitrogen) for 1 hr at room temperature. Sections were again blocked in 10% animal serum, 2% bovine serum albumin, and 0.025% Triton X-100 in phosphate-buffered saline for 1 hr at room temperature and incubated for 2 hr at room temperature with the second primary antibody NeuN (1:200, Merck/Millipore) and then with secondary antibody conjugated to Alexa 488 (1:250, Invitrogen) for 1 hr at room temperature. Sections were counterstained with 0.1 µg/ml of 4',6-diamidino-2-phenylindole, mounted in Fluorescent Mounting (Dako) medium, and visualized using the LSM 510 Meta UV (Zeiss) confocal microscope.

For β-galactosidase (lacZ) staining, 7-µm-thick midsagittal cryopreserved sections were incubated in 0.2% glutaraldehyde, 50 mM ethylene glycol tetraacetic acid, and 100 mM magnesium chloride in phosphate-buffered saline for 10 min at 4° and were then washed in lacZ wash (2 mM magnesium chloride, 0.1% sodium deoxycholate, 0.02% NP40 in phosphate-buffered saline). Subsequently, sections were incubated at room temperature with 0.5 mg/ml X-gal, 5 mM potassium ferricyanide, and 5 mM potassium ferricyanide in lacZ wash until color development. Sections were counterstained with Nuclear Fast Red (Sigma-Aldrich) for 4 min.

Western blot

Directly after dissection, tissue biopsies were homogenized with a 27G 1/2-inch needle in 20 mM Tris–hydrochloride (pH 7.5) with protease inhibitors. Protein concentrations were estimated using the BCA (Pierce) protein assay kit. Five micrograms of denatured protein were separated on a 4.5% resolving gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were then transferred to a 0.45-µm Immobilon-P (Merck Millipore) polyvinylidene fluoride membrane by electroblotting at 30 mA for 16 hr at 4°. Membranes were blocked in 5% skim milk in TBS-Tween for 1 hr, incubated with the primary antibodies Dicer 349 (1:5000; kind gift from Witold Filipowicz) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:7000, Trevigen), and then incubated with IgG and HRP-conjugated antibodies (1:5000, Sigma) for 2 hr at room temperature. Proteins were visualized with ECL Plus (GE Healthcare) Western blotting detection reagents and imaged with the GS-800 Calibrated Densitometer (Bio-Rad). Theoretical molecular weights were calculated using the ExPASy server and known amino acid sequence.

In situ hybridization

Fourteen-micrometer-thick RNase-free sagittal sections of the central nervous system, medial to the vermis, were labeled using 500 ng of the *Atoh1* riboprobe as previously described

(Adolphe *et al.* 2004). The *Atoh1* riboprobe was generated using the primers SP6 forward (5'-GCG ATT TAG GTG ACA CTA TAG GAC CAC CAT CAC CTT CGC-3') and T7 reverse (5'-GCG TGT ATT CTG GGT CCG CCC TAT AGT GAG TCG TAT TAA CAT CG-3') to create a 877-nt product.

DICER1 expression vector

Inverse PCR was performed on pBluescript II SK(+)-*DICER1* (Zhang *et al.* 2002) with KOD hot start DNA polymerase (Merck) and primers oriented in the reverse direction of, yet flanking, exons 24 and 25 of human *DICER1* to amplify the entire construct but without exons 24 and 25 (Δ ex24-25). Primers used were exon 26 forward [5'(P)-AAA AGT TTT CTG CAA ATG TACC-3'] and exon 23 reverse (5'-CAG TGA TAG TAT TGT AGT GG-3'). Modifications to the manufacturer's guidelines were the addition of 1.5 mM of magnesium sulfate, an annealing temperature of 52°, and an extension of 5 m and 30 sec over 20 cycles. *DICER1* Δ ex24-25 vs. *DICER1* wild-type products were screened by *Xba*I and *Not*I restriction enzyme digest to identify 3229- vs. 3661-nt DNA products, respectively, and then sequencing of the described fragment was performed. To reduce the number of potential mutations outside of the region sequenced, a confirmed *Xba*I- and *Not*I-digested *DICER1* Δ ex24-25 fragment was subcloned into the original pBluescript II SK(+)-*DICER1* construct. To measure transfection efficiency with an enhanced GFP (eGFP), wild-type and mutant *DICER1* were excised from pBluescript II SK(+) using the restriction enzyme *Kpn*I and *Not*I, blunted using T4 DNA polymerase (New England Biolabs), and then ligated into an *Eco*RV restriction site of the pCIG2-IRES-eGFP vector (Grainger *et al.* 2010).

Tissue culture

Control and *Dicer1* null sarcoma (Ravi *et al.* 2012) and mesenchymal stem [American Type Culture Collection (ATCC) CRL-3221] cells were kindly donated by the Phillip Sharp laboratory (Massachusetts Institute of Technology). These cells were maintained in culture as previously described (Ravi *et al.* 2012; Gurtan *et al.* 2013). COS-7 cells (ATCC CRL-1651) were maintained as described by the ATCC. For transfection experiments, cell lines were seeded in a 24-well plate and transfected at ~70% confluence with Lipofectamine LTX and Plus reagents (Life Technologies) and 500 ng of pCIG2-IRES-eGFP, wild-type pCIG2-*DICER1*-IRES-eGFP, or mutant pCIG2-*DICER1* Δ ex24-25-IRES-eGFP. Total RNA was harvested from COS-7 and sarcoma cells 24 hr after transfection and 36 hr following transfection in mesenchymal stem cells.

Statistics

Tumor-free survival of *Atoh1*-Cre; *Ptch1**fl*ox/*fl*ox; *Dicer1**fl*ox/*wt* and *Atoh1*-Cre; *Ptch1**fl*ox/*fl*ox; *Dicer1**fl*ox/*fl*ox, when compared to *Atoh1*-Cre; *Ptch1**fl*ox/*fl*ox mice, was statistically significant according to both the log-rank (Mantel–Cox) and Gehan–Breslow–Wilcoxon tests. Deaths not resulting from medulloblastoma were censored in Kaplan–Meier survival estimates (see Supporting Information, Table S1).

Figure 1 Genomic *Ptch1flox* and *Dicer1flox* are inactivated in medulloblastoma. Semiquantitative PCR analyses of genomic DNA extracted from *Atoh1-Cre;Ptch1flox* tumors confirmed the efficient inactivation of *Ptch1flox* (A) and *Dicer1flox* (C) by Cre-mediated recombination. Specifically, *Ptch1* recombination produced an ~500-nucleotide (nt) product, while the *loxP* and wild-type alleles, respectively, produced ~350- and ~300-nt products (A). Furthermore, *Dicer1* inactivation of the *loxP* allele (~1400 nt) produced an ~350-nt product, while the wild-type allele generated an

~1200-nt product (C). The location of primer binding (F, forward primer; R, reverse primer) relative to exons (numerical) is depicted schematically underneath representative images of DNA agarose gel electrophoresis (A and C). *Atoh1-Cre* is expressed uniformly in the external germinal layer (solid arrowhead) from embryonic day 18.5 (B), as demonstrated by β -galactosidase staining (blue precipitate) in *Atoh1-Cre;Gt(ROSA)26Sor;lacZ* mice. Sections were counterstained with nuclear fast red. Bars in B: top, 1 mm; bottom, 50 μ m.

Results

Dicer1 function was efficiently disrupted in *Atoh1-Cre;Ptch1flox/lox* mice

To investigate the dose-dependent interactions between miRNA biogenesis and Shh-Ptch signaling in cerebellar granule cells, *Atoh1-Cre* mice were interbred with mice carrying the *Dicer1flox* and *Ptch1flox* alleles. The most widely used animal model for medulloblastoma involves the targeted mutation of the *Ptch1* gene, either heterozygous null (Goodrich *et al.* 1997; Hahn *et al.* 1998; Zibat *et al.* 2009) or conditional knockouts associated with the granule cell lineage (Yang *et al.* 2008; Li *et al.* 2013), which leads to the constitutive activation of Shh-Ptch signaling in the cerebellum. In these mouse models, including *Ptch1* heterozygous null mutants, the absence of *Ptch1* mRNA (and ensuing Shh-Ptch-pathway activation) is critical for both the initiation and growth of medulloblastoma. For example, Hedgehog pathway inhibitors cause tumor regression in mouse models of medulloblastoma (Yauch *et al.* 2009; Buonamici *et al.* 2010; Lee *et al.* 2012; Rohner *et al.* 2012), and the emergence of resistance to these inhibitors is always linked to downstream activation of Shh-Ptch signaling (Yauch *et al.* 2009; Buonamici *et al.* 2010; Dijkgraaf *et al.* 2011). Although the *Atoh1-Cre;Ptch1flox/lox* mouse model used in the present study has previously been characterized (Yang *et al.* 2008), *Ptch1* recombination in the presence of the *Dicer1flox* allele was measured by semiquantitative PCR on genomic DNA isolated from *Ptch1flox* and *Atoh1-Cre;Dicer1flox/lox* tumors. Indeed, *Ptch1* recombination was detected (by the presence of a 499-nt PCR product) in tumors with homozygous *Dicer1* inactivation (Figure 1A).

Unlike the *Ptch1* mouse model, *Atoh1-Cre;Dicer1flox/lox* mice are not well characterized in the literature. Briefly, the *Dicer1flox* mouse possesses *loxP* sites that flank exon 24 of the *Dicer1* gene (Harfe *et al.* 2005). Exon 24 encodes the N-terminal quarter of the RNase IIIb domain and a critical lysine residue (K1790) required for the 5' phosphate cleavage of pre-miRNA duplexes (Du *et al.* 2008). The removal of exon

24 is predicted to inactivate endoribonuclease Dicer function by producing a nonsense transcript with a premature stop codon in the second amino acid after the recombination site. Other widely used *Dicer1flox* mouse models possess *loxP* sites flanking exons 22–23 (Murchison *et al.* 2005) and exons 15–17 (Mudhasani *et al.* 2008). To better characterize *Atoh1-Cre;Dicer1flox/lox* mice, the recombination efficiency and expression pattern of Cre recombinase under the *Atoh1* promoter was investigated by crossing *Atoh1-Cre* transgenic mice with mice carrying the *Gt(ROSA)26Sor;lacZ* allele. Homozygous *Gt(ROSA)26Sor;lacZ* mice contain a *loxP*-flanked DNA stop sequence that prevents the downstream expression of *lacZ* (Soriano 1999). When these mice are crossed with a Cre transgenic line, the DNA stop sequence is removed such that *lacZ* is expressed in the Cre-positive tissues only. Beta-galactosidase staining of *Atoh1-Cre;Gt(ROSA)26Sor;lacZ* mice confirmed the efficient Cre-mediated recombination in the external germinal layer of the cerebellum from embryonic day 18.5 (Figure 1B). Next, the level of *Dicer1* recombination was investigated by semiquantitative PCR on genomic DNA derived from *Atoh1-Cre;Ptch1flox/lox* tumors. Indeed, a high level of *Dicer1* recombination was detected in tumors isolated from *Atoh1-Cre;Ptch1flox* mice (Figure 1C) by comparing the relative intensity of the *Dicer1* deleted band (~350 nt) to the *Dicer1flox* band (~1400 nt) (Figure 1C). Together, these data verify that genomic *Dicer1* and *Ptch1* are efficiently recombined in medulloblastoma.

The expression levels of recombined *Ptch1* mRNA (Figure 2, A and B) and *Dicer1* mRNA (Figure 2, C and D) were next assessed by reverse transcriptase PCR. Specifically, recombination of *Ptch1* transcripts were classified as incomplete (open), partial (shaded), or near-complete (solid) based on the relative levels of *Ptch1* reverse-transcribed PCR products missing *loxP*-flanked exon 3 (273 nt) compared to PCR products with exon 3 (463 nt) (Figure 2, A and B). Near-complete *Ptch1* recombination was detected in medulloblastoma, regardless of whether the tumors originated from a *Atoh1-Cre;Ptch1flox/wt* or *Atoh1-Cre;Ptch1flox/lox* tumor or the status of *Dicer1* (Figure 2E). This is consistent with previous reports

Figure 2 *Ptch1*^{flox} and *Dicer1*^{flox} transcripts are inactivated in medulloblastoma and hypoplastic cerebellums. Semiquantitative PCR analyses of reverse-transcribed RNA extracted from the cerebellums of mice, prior to medulloblastoma or hypoplasia-induced death, were examined for the comparative levels of *Ptch1*^{flox} (A and B) and *Dicer1*^{flox} (B–D) Cre-mediated recombination. Schematic diagrams (A and C) depict the variety of splice and recombined transcripts observed in cerebella, with the predicated size of PCR products in nucleotides (nt). Cre-mediated recombination of *Ptch1* (*Ptch1*^{Δex3}) produced a 273-nt product (C and D). Cre-mediated recombination of *Dicer1* (*Dicer1*^{Δex24}) produced a 387- or 224-nt product (A and B). The 224-nt product was produced because of an endogenously expressed, alternatively spliced exon 25 variant (*Dicer1*^{Δex25}; 387 nt) that also underwent recombination (*Dicer1*^{Δex24-25}). The comparative levels of recombination for *Ptch1* and *Dicer1* mRNA were classified as the following: incomplete (open box), partial (shaded box), or near-complete (solid box) recombination, as shown in the representative images of DNA agarose gel electrophoresis (B and D). Information was tabulated according to genotype, cause of death, and days alive (E). H, hypoplasia; Hh, hypoplasia with hydrocephalus; MB, medulloblastoma. Schematic diagrams (A and C) are drawn to scale.

(Berman *et al.* 2002) that silencing of wild-type *Ptch1* is necessary for the initiation of Shh-Ptch signaling and medulloblastoma growth in *Ptch1* mouse models. On the other hand, there was an incomplete or partial loss of *Ptch1* in *Atoh1-Cre; Ptch1*^{flox/wt};*Dicer1*^{flox/flox} mice that developed hypoplasia (Figure 2E). Similarly, recombination of *Dicer1* transcripts were classified as incomplete (open), partial (shaded) or near-complete (solid) based on the relative levels of *Dicer1* reverse-transcribed PCR products missing loxP-flanked exon 24 (387 and 224 nt) compared to PCR products with exon 24 (656 and 493 nt) (Figure 2, C and D). In *Atoh1-Cre; Ptch1*^{flox/wt};*Dicer1*^{flox/flox} and *Atoh1-Cre; Ptch1*^{flox/flox};*Dicer1*^{flox/flox} mice, *Dicer1* in most cases was completely, or partially, recombined, regardless of the cause of death (by medulloblastoma or hypoplasia) (Figure 2E). However, *Atoh1-Cre; Ptch1*^{flox/flox};*Dicer1*^{flox/wt} mice were incompletely or partially recombined (Figure 2E). These data suggest that *Ptch1* mRNA is not under the same selective pressure for loss of expression in hypoplastic cerebella as it is in medulloblastoma, and that wild-type *Dicer1* mRNA is not under selective pressure for loss in *Atoh1-Cre; Dicer1*^{flox/wt} tumors with *Ptch1* loss.

Endogenous *Dicer1* splice variants do not affect microRNA bioprocessing

An endogenously expressed exon 25 splice variant was identified in medulloblastoma and hypoplastic cerebella based on

amplicon size (494 nt vs. wild type of 657 nt) (Figure 2, C and D) and sequencing (data not shown). Interestingly, the exon 25 splice variant underwent simultaneous Cre-mediated recombination to produce an exon 24–25 deletion (225-nt PCR product) (Figure 2, C and D). Furthermore, Cre-mediated recombination appeared to promote exon 25 skipping, as evidenced by a relatively higher proportion of *Dicer1* exon 24–25 deleted transcripts compared to *Dicer1* exon 25 alone being deleted (Figure 2D). *Dicer1* has previously been demonstrated to undergo exon 25 alternative splicing in human primary neuroblastic tumors that originate from neural crest tissues (Potenza *et al.* 2010). A potential mechanism to promote exon skipping may involve the presence of somatic single-base substitutions in the RNase IIIb domain that introduce a splicing silencer (Wu *et al.* 2013). The excision of exons 24 and 25 is predicted to keep the transcript in-frame, but remove the majority (~80%) of the RNase IIIb domain. Alternative splicing of exon 25 would affect the *Dicer1* flox mouse model used in the present study (Harfe *et al.* 2005), but not the other widely used *Dicer1*^{flox} mouse models by Murchison *et al.* (2005) and Mudhasani *et al.* (2008), given that their loxP sites do not immediately flank exon 25.

To determine whether *Dicer1* lacking exons 24–25 was functional, sarcoma and mesenchymal stem cells deficient in *Dicer1* were transfected with full-length *DICER1* or *DICER1* lacking exons 24–25 (*DICER1*^{Δex24-25}). Sarcoma and

Figure 3 Excision of exon 24–25 of *DICER1* inactivated microRNA bioprocessing. Quantitative PCR of reverse-transcribed RNA verified that the protein products of the *DICER1* Δ ex24 (medium shading) and *DICER1* Δ ex24-25 (light shading) were unable to bioprocess three highly expressed microRNAs in *Dicer1* null sarcoma cells (A) and *Dicer1* null mesenchymal stem cells (B), while the protein product from *DICER1* (solid bar) was able to. Quantitative real-time PCR analyses were calculated using the relative threshold cycle (CT) method (A and B) relative to the housekeeping gene *Rnu6-2*. Stable expression of the endoribonuclease Dicer splice and recombination variants was confirmed by Western blot analyses of COS-7 cells transfected with various *DICER1* constructs (C). Furthermore, Western blot analysis of endogenous endoribonuclease Dicer protein levels was performed on tumors derived from *Atoh1-Cre;Ptch1flox/flox* mice (D). Protein levels were substantially reduced in tumors derived from *Atoh1-Cre;Ptch1flox/flox;Dicer1flox/flox* mice. GAPDH was used as a housekeeping protein (D). Representative Western blot images are shown (C and D).

mesenchymal stem cells were specifically chosen because they are able to tolerate null mutations in *Dicer1*, while most other cell types cannot, as exemplified by the requirement for *Dicer1* in early mouse development (Bernstein *et al.* 2003). Exon 24–25 exclusion did not alter basal miRNA biogenesis in either of the *Dicer1* null cell lines investigated, as illustrated by the comparable relative expression level of miRNAs between empty vector and *DICER1* Δ ex24-25 transfected cells (Figure 3, A and B). These data demonstrate that exon 24–25 exclusion produces a loss-of-function mutation equivalent to the recombination of *Dicer1flox* (*i.e.*, exon 24 excision) alone. To further validate that the various *DICER1* constructs lacking exons 24 and/or 25 produced stable protein products, Western blot analyses were performed. Indeed, all constructs produced protein products of the expected size that was based on the predicted amino acid sequence of the endoribonuclease Dicer: 217 kDa for *DICER1* (estimated 216 kDa), 192 kDa for *DICER1* Δ ex24 (estimated 206 kDa), 207 kDa for *DICER1* Δ ex25 (estimated 210 kDa), and 212 kDa for *DICER1* Δ ex24-25 (estimated 200 kDa) (Figure 3C). Interestingly, in primary *Atoh1-Cre;Ptch1flox/flox* tumors, regardless of the status of *Dicer1flox*, splice variants for exon 24 and/or 25 were not detected (Figure 3D), even though they were detectable at the transcript level (Figure 2, C–E). This does not preclude the possibility that low levels of alternatively spliced endoribonuclease Dicer would be able to influence tumor growth; however, given that *DICER1* Δ ex24-25 is functionally equivalent to *Dicer1flox* (*i.e.*, exon 24 excision), low undetectable levels of endoribonuclease Dicer lacking exons

24–25 would still produce a loss-of-function mutation. Therefore, these data collectively demonstrate that endoribonuclease Dicer function, regardless of exon 25 skipping, is efficiently disrupted in *Ptch1*-induced cerebellar tumors.

***Dicer1* positively corroborates with Sonic Hedgehog-Patched signaling in granule cell precursors**

To understand how loss of *Dicer1* function influences Shh-Ptch signaling, the allelic frequency of *Dicer1* was incrementally altered within granule cell precursors of *Atoh1-Cre;Ptch1flox/flox* mice. Given that homozygous and heterozygous deletion of *Ptch1* (to activate Shh-Ptch signaling in granule cell precursors) induces hyperplasia that histologically resembles human medulloblastoma (Yang *et al.* 2008) and, conversely, that the homozygous deletion of *Dicer1* in granule cell precursors produces mild hypoplasia (Constantin and Wainwright 2015) (while heterozygous deletion has no effect), these two opposing growth phenotypes of hyperplasia and hypoplasia were used as a measure of pathway activity for Shh-Ptch signaling and endoribonuclease Dicer biogenesis pathways, respectively. The severity of medulloblastoma was measured as a function of tumor-free survival (Figure 4A) with the heterozygous inactivation of *Dicer1* most severe, followed by homozygous inactivation. More specifically, mono-allelic inactivation of *Dicer1* reduced tumor-free survival to a median of 25 days (Figure 4, B and E), while homozygous inactivation reduced tumor-free survival to a median of 62 days (Figure 4, B and F), compared to control *Atoh1-Cre;Ptch1flox/flox* mice, which developed medulloblastoma in a

Figure 4 *Dicer1* inactivation reduced tumor-free survival or induced hypoplasia in *Atoh1-Cre;Ptch1*^{flox/flox} mice. The percentage of frequencies and severity of medulloblastoma (positive scale) and hypoplasia (negative scale) was substantially altered by the allelic dose of *Dicer1* (A) inactivation in *Atoh1-Cre;Ptch1*^{flox/flox} mice. Relative disease severity was ranked from least (light shading) to most severe (solid) for medulloblastoma and, similarly for hypoplasia, for the genotypes graphed only. Tumor-free percentage of survival (B) was reduced by the heterozygous (shaded solid line) and homozygous (shaded dashed line) inactivation of *Dicer1* in *Atoh1-Cre;Ptch1*^{flox/flox} mice. Deaths not caused by tumor load were censored (solid vertical line). Tumor-free survival in *Dicer1*^{flox} heterozygous and homozygous *Atoh1-Cre;Ptch1*^{flox/flox} mice was statistically significant compared to *Atoh1-Cre;Ptch1*^{flox/flox} mice (**** $P \leq 0.0001$). For each of the disease phenotypes depicted in the graph (A), the following representative images (C–G) were captured: whole-mount analysis of the cerebellum from the ventral-posterior aspect (left top), H&E staining (right top), immunofluorescence staining for neuronal differentiation by Pax6 and NeuN costaining (left bottom), and *in situ* hybridization of *Atoh1* (right bottom). Some genotypes developed one of the two opposing phenotypes: hyperplasia leading to medulloblastoma (E) or hypoplasia leading to a severely underdeveloped cerebellum (F). Dashed white line marks the cerebellar boundary, and an asterisk denotes hydrocephalus. Bars in C–G: top left, 2 mm; top right, 500 μ m; bottom, 200 μ m.

median of 145.5 days (Figure 4, B and D). These data demonstrate that *Dicer1* loss, whether monoallelic or biallelic, substantially and significantly promoted tumor progression in granule cell precursors by collaborating with Shh-Ptch signaling. Therefore, *Dicer1* functions as a tumor suppressor gene, given that loss of expression enhanced tumor severity. Further to this, monoallelic *Dicer1* loss exerted an effect, which indicates that *Dicer1* is also a haploinsufficient tumor suppressor gene. These findings are consistent with the role of *Dicer1* in other tumor types (Arrate *et al.* 2010; Lambertz *et al.* 2010; Nittner *et al.* 2012; Yoshikawa *et al.* 2013; Zhang *et al.* 2014).

The severity of hypoplasia was calculated based on the size and fissure formation of the cerebellum at the time of death. Approximately 39% ($n = 7/18$) of *Atoh1-Cre;Ptch1*^{flox/flox} mice with a homozygous loss of *Dicer1* developed severe

hypoplasia with hydrocephalus (Figure 4, A and G). More specifically, cerebella were of an embryonic appearance based on fissure formation (Figure 4G) and size ($1.69 \text{ mm} \pm 0.030$; mean $\pm$ SEM; $n = 4$), and mice died at a median of 14 days (Table S1). The presence of the completing growth phenotype of hypoplasia in biallelically inactivated (but not in monoallelically inactivated) *Dicer1* mice could explain why haploinsufficiency was more severe than near complete loss of function of *Dicer1*. More specifically, hypoplasia involves cellular growth processes that are in direct opposition to hyperplasia; therefore, the coexistence of these two phenotypes (such as observed in biallelically inactivated *Dicer1* mice) would subdue the severity of both phenotypes compared to monoallelically inactivated *Dicer1* mice that present with medulloblastoma only.

In addition to gross histological analyses of these phenotypes through H&E staining, cerebella were costained with antibodies against the granule cell lineage marker Pax6 and the neuronal differentiation marker NeuN. By identifying Pax6-immunopositive and NeuN-immunonegative cells vs. Pax6-immunopositive and NeuN-immunopositive cells, the populations of undifferentiated granule cell precursors vs. differentiated granule cells could, respectively, be determined. Pax6 and NeuN colocalization has previously been used to examine changes in the population of proliferating and differentiating granule cell precursors (Flora *et al.* 2009; Constantin and Wainwright 2015). As expected, there was a large number of Pax6-immunopositive and NeuN-immunonegative cells in all tumors (regardless of the allelic frequency of *Dicer1*), which demarcate precursors of the granule lineage (Figure 4, D–F). On the other hand, the hypoplastic phenotype was composed of Pax6-immunopositive and NeuN-immunopositive cells, which indicates the presence of differentiated cells in the majority of the hypoplastic structure with a thin layer of precursor cells superficially (Figure 4G). This suggests that the hypoplasia is a developmental defect rather than a small tumor mass. High *Atoh1* expression is consistently and exclusively associated with the SHH subtype of medulloblastoma (Swartling *et al.* 2012). All tumors, regardless of the allelic frequency of *Dicer1*, were positive for *Atoh1* (Figure 4, D–F), which indicates that these tumors likely belong to the Shh subtype.

Hedgehog-Patched signaling collaborates with microRNA biogenesis in granule cell precursors

To understand how constitutive activation of Shh-Ptch signaling (via *Ptch1* loss) influences the *Dicer1* biogenesis pathway, the allelic frequency of *Ptch1* was incrementally altered in granule cell precursors of *Atoh1-Cre;Dicer1^{flox/flox}* mice. Again, the severity of medulloblastoma was measured as a function of tumor-free survival. In this case, *Ptch1* inactivation promoted tumor severity and penetrance stepwise, with homozygous inactivation the most severe (Figure 5A). More specifically, control *Atoh1-Cre;Dicer1^{flox/flox}* mice did not develop medulloblastoma (Figure 5, B and C), which validates previous findings that *Dicer1* loss is insufficient for *de novo* tumor formation (Zindy *et al.* 2015). Instead, these mice developed anteriorly confined hypoplasia with complete penetrance (Figure 5C) that did not impact survival (Constantin and Wainwright 2015). Monoallelic *Ptch1* inactivation reduced survival to a median of 58 days at 19% penetrance ($n = 5/26$) (Figure 5, B and D), while biallelic loss reduced survival to a median of 62 days with complete penetrance (Figure 5B and Figure 4E). These data indicate that Shh-Ptch signaling also positively collaborates with *Dicer1* biogenesis (especially considering that *Atoh1-Cre;Ptch1^{flox/flox}* mice develop medulloblastoma by a median of 145.5 days) (Figure 4A) and suggest that Shh-Ptch and miRNA biogenesis pathways bilaterally cross-talk in medulloblastoma.

Similarly, allelic inactivation of *Ptch1* to activate Shh-Ptch signaling increased the relative severity of hypoplasia, based

on the calculated developmental attenuation of the cerebellum at the time of death, and also penetrance. For example, the monoallelic deletion of *Ptch1* resulted in a developmentally attenuated cerebellum that resembled that of a postnatal day 2 structure in size and foliation with 19% penetrance (Figure 5E), even though mice survived to a median of 14 days (Figure 5E). When *Ptch1* was biallelically inactivated, the cerebellum resembled an embryonic structure as previously described (Figure 4F) with a penetrance of 39% ($n = 7/18$). Therefore, Shh-Ptch signaling collaborates with *Dicer1* biogenesis to promote the developmental attenuation of the cerebellum.

To confirm the pathologic phenotypes of hypoplasia and medulloblastoma in the present study, cerebella were sequentially costained with antibodies against Pax6 and NeuN. Again, tumors were composed of a large number of undifferentiated precursors of the granule lineage (Pax6-immunopositive; NeuN-immunonegative) (Figure 5D), while the hypoplasia contained no precursor populations but Pax6-positive and NeuN-immunopositive cells or Pax6-immunonegative cell populations (Figure 5E). These data confirm that the hypoplasia is not a small tumor mass, but most likely resulted from a developmental delay. Similarly, through *Atoh1 in situ* hybridization, we were able to confirm that the tumors likely belong to the Shh subtype (Figure 5, D and E).

Discussion

In the present study, the dose-dependent roles of miRNA biogenesis and Shh-Ptch signaling were investigated through the monoallelic and biallelic inactivation of *Dicer1* and *Ptch1*. Consistent with previous reports, homozygous inactivation of *Dicer1* in granule cells produced mild cerebellar hypoplasia (Constantin and Wainwright 2015), while conditional hemizygous and homozygous *Ptch1* inactivation induced medulloblastoma (Yang *et al.* 2008). However, when different allelic frequencies of *Dicer1* and *Ptch1* were together perturbed, three new observations were made. First, only genotypes with biallelic *Dicer1* inactivation and either partial or complete *Ptch1* inactivation presented with hypoplasia and medulloblastoma. Second, concurrent inactivation of *Dicer1* and *Ptch1* led to more severe phenotypes for medulloblastoma and hypoplasia than the inactivation of either gene alone, which indicates that these pathways corroborate in *Atoh1*-positive granule cell precursors. Third, hypoplasia was progressively more severe with the dose-dependent inactivation of *Ptch1*, while monoallelic *Dicer1* inactivation had a more detrimental effect than biallelic *Dicer1* loss in medulloblastoma. *Dicer1* haploinsufficiency in *Ptch1*-induced medulloblastoma may have resulted from the coexistence of the two opposing growth processes of hyperplasia and hypoplasia in tumors, which occurred in mice with biallelic *Dicer1* loss. This would placate the severity of either phenotype, but not in tumors with monoallelic *Dicer1* loss, given that these mice develop only medulloblastoma and therefore genetic suppression of the hyperplasia (by the opposing growth effect of hypoplasia) would not take place.

Figure 5 *Ptch1* inactivation reduced tumor-free survival or promoted hypoplasia in *Atoh1-Cre;Dicer1*^{flox/flox} mice. The percentage of frequencies and severity of medulloblastoma (positive scale) and hypoplasia (negative scale) were substantially altered by the allelic dose of *Ptch1* (A) inactivation in *Atoh1-Cre;Dicer1*^{flox/flox} mice. Relative disease severity was ranked from least (light shading) to most severe (solid) for medulloblastoma and, similarly for hypoplasia, for the genotypes graphed only. Tumor-free percentage of survival (B) was reduced by the heterozygous (shaded line) and homozygous (shaded dashed line) inactivation of *Ptch1* in *Atoh1-Cre;Dicer1*^{flox/flox} mice. Deaths not caused by tumor load were censored (solid line). Tumor-free survival in *Ptch1* heterozygous and homozygous *Atoh1-Cre;Dicer1*^{flox/flox} mice was statistically significant compared to *Atoh1-Cre;Dicer1*^{flox/flox} mice (*P = 0.0251; ****P ≤ 0.0001). For each of the disease phenotypes depicted in the graph (A), the following representative images (C–E) were captured: whole-mount analysis of the cerebellum from the ventral-posterior aspect (left top), H&E staining (right top), immunofluorescence staining for neuronal differentiation by Pax6 and NeuN costaining (left bottom), and *in situ* hybridization of *Atoh1* (right bottom). Some genotypes developed one of the two opposing phenotypes: hyperplasia leading to medulloblastoma (D) or hypoplasia leading to a severely underdeveloped cerebellum (E). Dashed white line marks the cerebellar boundary. Bars in C–E: top left, 2 mm; top right, 500 μm; bottom, 200 μm.

MicroRNA biogenesis and Hedgehog-Patched-signaling pathways genetically interact to accentuate either medulloblastoma or hypoplasia

Several miRNA subsets are known to regulate components of Shh-Ptch signaling in the cerebellum. For example, *miR-106b* positively regulates *Gli2* transcription to promote granule cell expansion (Constantin and Wainwright 2015). The *miR-183~96~182* cluster synergizes with exogenous Shh ligand to promote the proliferation of *Ptch1*-induced medulloblastoma (Zhang *et al.* 2013); similarly, the *miR-17~92* cluster is necessary for *Ptch1*-induced medulloblastoma (Zindy *et al.*

2014) through N-myc (Northcott *et al.* 2009). Conversely, *miR-125b*, *miR-326*, and *miR-324-5p* antagonize the pathway activators Smoothened and/or Gli1 to inhibit granule cell precursor growth (Ferretti *et al.* 2008). Therefore, it follows that the deletion of *miR-106b*, *miR-183~96~182*, and *miR-17~92* likely induced hypoplasia in *Atoh1-Cre;Ptch1*^{flox/flox}; *Dicer1*^{flox/flox} mice, while the loss of *miR-125b*, *miR-326*, and *miR-324-5p* promoted medulloblastoma. Given that *Dicer1* regulates the bioprocessing of hundreds of miRNAs, and in turn each miRNA may regulate hundreds of targets, *Dicer1* ablation would also affect many other pathways in

addition to Shh-Ptch signaling. Also, *Dicer1* ablation may influence Shh-Ptch signaling noncanonically, for example, by way of transforming growth factor β (Dennler *et al.* 2009). Although this body of work did not specify how or what the nature of the interactions between the endoribonuclease Dicer and Shh-Ptch signaling were, it did characterize the broader effects of this genetic interaction. Through doing so it established that Dicer bioprocessing and Shh-Ptch signaling, together, have a substantial and unexpected biological impact on the developing cerebellum (as measured by tumor latency and growth attenuation) and that this interaction is a principal driving event during cerebellar development.

Bistability of phenotypes suggests that the associated pathways regulate an important developmental transition

A bistable phenotype was induced in *Atoh1-Cre;Ptch1flox/flox;Dicer1flox/flox* mice, which strongly suggests that miRNA biogenesis and Hedgehog-Patched signaling together regulate an important developmental transition in granule cell precursors. Based on the developmental stage of cerebellar attenuation in *Atoh1-Cre;Ptch1flox/flox;Dicer1flox/flox* mice, such an event likely occurred in late embryonic development. Further support of the molecular switch phenomenon includes previous findings that miRNAs regulate transcription factors associated with Hedgehog-Patched signaling. More specifically, interactions between miRNAs and transcription factors permit feedback and feed-forward loops to be established, which are required in genetic switches (Tsang *et al.* 2007). A similar phenomenon may occur in granule cell precursors, whereby miRNA-mediate control of Shh-Ptch transcription factors, such as Gli1/2 and N-myc, allows feedback and feed-forward loops to be produced that in turn mediate hypoplasia over medulloblastoma, or vice versa. The next step would be to identify the specific miRNAs and intracellular components involved in regulating such a developmental transition. This would not only cultivate a more thorough understanding of early granule cell precursor development by, for example, shedding light on relatively unstudied molecular processes involved in establishing the developmental compartment-like units of the cerebellum (Hutvagner and Zamore 2002), but may also yield novel insights into the molecular basis of Shh-induced medulloblastoma.

Dicer1 functions as a haploinsufficient tumor suppressor gene in Ptch1-induced medulloblastoma

DICER1 has previously been reported to function as a haploinsufficient tumor suppressor gene in human tumors (Karube *et al.* 2005; Chiosea *et al.* 2007; Merritt *et al.* 2008; Grelier *et al.* 2009; Faggad *et al.* 2010; Lin *et al.* 2010; Khoshnaw *et al.* 2012; Jafarnejad *et al.* 2013) and genetically engineered mouse models (Kumar *et al.* 2009; Lambertz *et al.* 2010), with only a handful of exceptions (Arrate *et al.* 2010; Sabbaghian *et al.* 2012; Zhang *et al.* 2013). The precise role of *DICER1* in medulloblastoma is less clear. For example, subsets of miRNAs have been shown as either growth-inhibitory

(Ferretti *et al.* 2008; Li *et al.* 2009; Venkataraman *et al.* 2013; Jin *et al.* 2014; Hemmesi *et al.* 2015) or oncogenic (Grunder *et al.* 2011; Weeraratne *et al.* 2012; Li *et al.* 2015). In particular, the *miR-17~92* cluster has been shown as both necessary and sufficient to initiate medulloblastoma (Zindy *et al.* 2014), which suggests an oncogenic role for *Dicer1* on the basis of *miR-17~92* alone. On the other hand, the global reduction of miRNAs in medulloblastoma (Ferretti *et al.* 2008), together with the identification of a germline *DICER1* truncation mutation in a medulloblastoma cohort (Slade *et al.* 2011), suggests that *DICER1* is tumor-suppressive and haploinsufficient.

Recently, *Dicer1* loss was shown to accelerate tumor growth in a genetically engineered mouse model of *Ptch1*-induced medulloblastoma (Zindy *et al.* 2015); however, biallelic loss was never investigated because of the widespread apoptosis and ensuing perinatal lethality induced by *Dicer1* ablation in the Murchison *et al.* (2005) mouse model. In the present study, we conclusively demonstrate that both the heterozygous and homozygous loss of *Dicer1* dramatically decreases the latency of tumor-free survival in a genetically engineered mouse model of *Ptch1*-induced medulloblastoma—different from that used by Zindy *et al.* (2015)—and that heterozygous *Dicer1* inactivation is most severe. The data presented here are consistent with the role of *DICER1* in the majority of human tumors. Interestingly, to date, only a single germline mutation (of 86 cases) has been reported for *DICER1* in medulloblastoma and infratentorial primitive neuroectodermal tumors (Slade *et al.* 2011), which suggests that the genetic interaction between miRNAs and Shh-Ptch signaling is perhaps more modest in humans or that mutations may affect this pathway downstream/post-transcriptionally to *DICER1* (mRNA). Further to this, our data imply a mechanism as to why monoallelic *Dicer1* inactivation is more severe than biallelic inactivation. That is, given that hypoplasia is on the opposite growth spectrum to medulloblastoma, and that only homozygous *Dicer1* inactivation induces hypoplasia, the biallelic loss of *Dicer1* function would genetically suppress the severity of hyperplasia, leading to medulloblastoma. This would result in the less severe tumor phenotype in mice with biallelic *Dicer1* loss, as was observed. This may also explain haploinsufficiency in other developmental pathologies.

Tumor suppressor genes can be designated as gatekeeper genes, for those involved in the control of cell proliferation or cell death, or as caretaker genes, for those involved in the control of DNA integrity, as reviewed by Kinzler and Vogelstein (1997). The inactivation of a caretaker gene often does not initiate tumor formation, but fosters transformation by rendering the cell genetically unstable. The endoribonuclease Dicer shares similarities with the majority of caretaker genes in that it cannot initiate tumors *de novo*, as illustrated here by the absence of medulloblastoma in *Atoh1-Cre;Dicer1flox/flox* mice. Furthermore, the endoribonuclease Dicer has recently been shown to maintain genomic stability in *Schizosaccharomyces pombe* by promoting transcription termination at sites associated with replication stress and DNA damage (Castel

et al. 2014). This is likely to occur in higher eukaryotes. Therefore, we extrapolate that the tumor suppressor function of *Dicer1* would be complex and would not involve the classical gatekeeper approach of directly regulating tumors by targeting effects that inhibit growth or promote cell death.

We propose that more studies that characterize the genetic interplay between principal pathways, such as here, should be conducted, given that pathway interactions may produce non-additive phenotypes that substantially differ from deregulation of a pathway in isolation. Here, we successfully demonstrated that the two principal pathways of miRNA biogenesis and Shh-Ptch signaling combinatorially and nonadditively influence the latency and severity of cerebellar hypoplasia and medulloblastoma. Novel insights were reached on the basis of these observations, such as a potential mode of action for *Dicer1* haploinsufficiency in *Ptch1*-induced medulloblastoma, and indications that miRNAs and Shh-Ptch signaling together regulate an early developmental transition in granule cells.

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Supporting Information

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MicroRNA Biogenesis and Hedgehog-Patched Signaling Cooperate to Regulate an Important Developmental Transition in Granule Cell Development

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Table S1. Cause of death and days alive in *Atoh1-Cre;Dicer1^{flox};Ptch1^{flox}* mice. e, euthanized at end of trial; h, hydrocephalus; H, hypoplasia; Hh, hypoplasia accompanied with hydrocephalus; MB, hyperplasia that manifested as medulloblastoma; O, other/unrelated.

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