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Deletion of *Atoh1* disrupts Sonic Hedgehog signaling in the developing cerebellum and prevents medulloblastoma

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Abstract

Granule neuron precursors (GNPs) are the most actively proliferating cells in the post-natal nervous system and mutations in pathways controlling their cell cycle can result in medulloblastoma. The transcription factor *Atoh1* has been suspected to contribute to GNP proliferation, but its role in normal and neoplastic post-natal cerebellar development remains unexplored. We show that *Atoh1* regulates the signal transduction pathway of Sonic Hedgehog, an extracellular factor that is essential for GNP proliferation, and we demonstrate that deletion of *Atoh1* prevents cerebellar neoplasia in a mouse model of medulloblastoma. Our data shed light on the function of *Atoh1* in post-natal cerebellar development and identify a new mechanism that can be targeted to regulate medulloblastoma formation.

Keywords

Atoh1; medulloblastoma; cerebellar granule neuron precursors; Sonic Hedgehog

Disruption of the delicate balance between proliferation and differentiation in cerebellar granule neuron precursors (GNPs) underlies medulloblastoma, the most common pediatric tumor of the nervous system (1, 2). Interestingly, a class of particularly aggressive medulloblastomas associated with very poor prognosis show high expression of *Atoh1* (3), a transcription factor highly expressed in GNPs also known as *Math1* (4), and recent *in vitro* studies proposed that *Atoh1* might be involved in neoplastic proliferation (5, 6). Given the fact that deletion of *Atoh1* in the mouse results in perinatal death (7), the function of this transcription factor in the developing post-natal cerebellum has remained opaque.

To delete *Atoh1* in the post-natal developing cerebellum we crossed *Atoh1*^{flox/flox} mice (8) with mice carrying the gene coding for a tamoxifen-inducible Cre recombinase in the *Rosa* locus (R26CreER) (9) and a null allele of *Atoh1* (10). Following activation of Cre by tamoxifen, *Rosa*^{CreER}; *Atoh1*^{+/flox} animals (designated herein as *Atoh1*^{wt}) maintain one functional allele of *Atoh1*, whereas *Rosa*^{CreER}; *Atoh1*^{-flox} mice (designated herein as *Atoh1*^Δ) lose *Atoh1* expression. We injected post-natal day 3 (P3) animals and analyzed their cerebella three days later. Nissl staining of matching sections of the external granule

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The transcription factor *Atoh1* regulates sonic hedgehog signaling in the developing cerebellum and is required for medulloblastoma formation.

layer (EGL), the neuroepithelium formed by GNPs, revealed that *Atoh1*^Δ animals had a much thinner EGL than their *Atoh1*^{wt} littermates (Fig. 1S). Using phospho-histone H3 staining to visualize the M phase of the cell cycle and Tuj1 for neural differentiation, we found that the EGL of *Atoh1*^Δ mice had been depleted of cycling immature precursors (Fig. 1A, B and 2S). Staining for active caspase 3 did not reveal any apoptosis in the EGL of *Atoh1*^Δ mice (not shown). We thus investigated whether deletion of *Atoh1* triggers the GNP differentiation to granule neurons or induces these cells to trans-differentiate to other cell types. Figure 2S shows that cells still populating the surface of *Atoh1*^Δ cerebellum that had deleted *Atoh1* still expressed *Zic1*, a marker of differentiating post-mitotic EGL cells and mature granule neurons, making trans-differentiation unlikely. Proliferating GNPs express *Pax6* at low levels, whereas differentiating post-mitotic precursors show high expression of *Pax6* and turn on the neural differentiation marker NeuN. The cells residing on the surface of the cerebellum of *Atoh1*^Δ expressed high levels of *Pax6* and NeuN (Fig 1C and D), again suggesting that deletion of *Atoh1* in the post-natal cerebellum activates the differentiation process of cerebellar granule neurons.

To investigate the molecular effects of *Atoh1* deletion, we isolated GNPs from *Atoh1*^{flox/flox} P5 animals and infected them with adenoviruses expressing either the GFP or the Cre recombinase gene. We cultured the transduced cells in the presence of Shh for three days (11), and evaluated their proliferative status by BrdU and phospho histone H3 staining. *Atoh1* deletion led to a sharp decrease in cell proliferation (Fig. 3S A and B), suggesting that GNPs are unable to respond to Shh stimulation in the absence of *Atoh1*. We then purified total RNA from the transduced GNPs and performed quantitative RT-PCR analysis of the Shh target genes cyclin D1 and D2 (*Ccnd1*, *Ccnd2*), *Mycn* and *Gli1* (Fig. 2A and data not shown). Surprisingly, all of these genes were down-regulated, as was *Gli2*, the main transcriptional effector of Shh signaling in GNPs (12, 13) (11, 14).

To determine whether *Gli2* down-regulation was responsible for the failure to proliferate in the absence of *Atoh1*, we infected GNPs with a retroviral vector expressing *Gli2* before deleting *Atoh1*. Cells infected with the *Gli2*-expressing retrovirus were still able to proliferate in response to Shh (Fig. 2B and C), showing that down-regulation of *Gli2* following *Atoh1* deletion plays a major role in GNP withdrawal from the cell cycle. Since *Gli2* transcription does not require Shh (15), we reasoned that down-regulation of its mRNA is unlikely to be a secondary effect of the blockade of the Shh signal and that *Gli2* might be a direct transcriptional target of *Atoh1*—which would explain how deletion of *Atoh1* inhibits Shh signaling.

Atoh1 activates its target genes through binding to regions enriched in specific sites called E-boxes (16). Computational scanning identified a DNA sequence highly enriched in E-boxes (eight E-boxes) in the second intron of *Gli2* (Fig. 4S A). We designed an oligonucleotide spanning two E-boxes in the cluster that are conserved in mouse and human and used it as a probe for electrophoretic mobility shift assay (EMSA) experiments (See Figure 4S A, boxed sequence). After incubating the probe with nuclear extracts of Neuro2a cells transfected with an *Atoh1*-Flag expression plasmid, we observed the appearance of a specific complex which was supershifted by an anti-Flag antibody (Fig. 4S B, lane 2, arrow and 3, arrowhead) and competed by the wild type (wt) unlabelled oligonucleotide but not by an oligonucleotide bearing a point mutation in each of the two E-boxes (lanes 4 and 5). Next, we cloned the entire sequence spanning the E-box cluster in front of a minimal promoter driving the expression of the luciferase gene. Co-transfection of Neuro2a cells with the *Gli2* reporter construct and a vector expressing *Atoh1* resulted in strong luciferase expression (Figure 2D), showing that *Atoh1* acts as a positive regulator through binding to this sequence. The control reporter vector containing only the minimal promoter had no effect.

To verify our findings *in vivo*, we took advantage of a knock-in mouse model in which the endogenous *Atoh1* protein is tagged with three flag peptides on its C-terminus (Fig. 5S). *Atoh1*^{flag/flag} P5 cerebella were collected and subjected to Chromatin Immuno-Precipitation (ChIP). As a negative control, we took cortex (which does not express *Atoh1*) from the same animals. As shown in figure 2E, we obtained a strong amplified band in the cerebellar sample but virtually no signal in the negative control, implying that *Atoh1* binds to this DNA region during cerebellum development. To confirm that this sequence is a transcriptional enhancer, we carried out additional chromatin IP experiments using an antibody recognizing the monomethylated form of histone H3 on lysine 4 (me1-H3), a well-defined marker of enhancers (17). An antibody recognizing the trimethylated form of histone H3 on lysine 4 (me3-H3), a marker of promoter sequences (17), served as a negative control. As expected, we recovered the DNA sequence corresponding to the *Gli2* intergenic region only when we used anti me1-H3 antibody, confirming that the region bound by *Atoh1* in the *Gli2* gene is an active transcriptional enhancer. Finally, using an unbiased approach (ChIP followed by deep sequencing) we identified the sites bound by *Atoh1* *in vivo* in GNPs. Analysis of the results confirmed that *Atoh1* binds to the intergenic *Gli2*E-box cluster in the developing cerebellum (T.J.K. and H.Y.Z., manuscript in preparation). We next evaluated whether the two genes are coexpressed in the neural progenitors that give rise to GNPs. Starting at E14.5 we observed strong expression of *Gli2* and *Atoh1* in the cells that are populating the surface of the developing cerebellum (Fig 6S A). To visualize the *Atoh1* protein, we used a mouse model expressing an *Atoh1*-GFP fusion protein from the endogenous *Atoh1* locus (18). At E18.5, a stage of cerebellar development that immediately precedes the post-natal expansion of the GNP population in the EGL, the cells expressing *Atoh1* in the EGL also expressed *Gli2*. This is consistent with the possibility that *Gli2* is a transcriptional target of *Atoh1* (Fig 6S B).

If *Atoh1* plays a central role in the regulation of *Gli2*, its expression might be required for the genesis of medulloblastomas induced by constitutive activation of the Shh pathway. To test this hypothesis, we used a mouse model bearing an inducible allele coding for a mutant form of the *Smoothened* protein, an activator of the Shh pathway, fused with YFP (*Rosa*^{SmoM2}). The expression of the mutant *Smoothened* allele can be activated by Cre recombinase, resulting in the formation of medulloblastomas in the developing cerebellum (19). We generated *Rosa*^{SmoM2/CreER}; *Atoh1*^{+/flox} animals (designated *SmoAtoh1*^{wt}) and *Rosa*^{SmoM2/CreER}; *Atoh1*^{lacZ/flox} mice (designated *SmoAtoh1*^Δ). Cre-mediated recombination activates the Shh pathway in all of the animals by inducing expression of the *Smoothened* gene, but *SmoAtoh1*^{wt} mice still express *Atoh1* from the functional allele, whereas *SmoAtoh1*^Δ lack *Atoh1* expression. After injecting P4 animals, we harvested the brains ten days later. Non-injected animals served as controls.

In normal P14 animals the vast majority of GNPs have already stopped cycling and the EGL is reduced to a few rows of cells (Fig. 3A), whereas *SmoAtoh1*^{wt} mice (15/15 animals) showed clearly hypertrophic EGLs (compare Fig3A and 3B). All these animals developed pre-neoplastic lesions in the external layer of the cerebellum (Fig. 3B, middle and right panels), where the EGL lost the typical layered organization (20, 21). Strikingly, none of the *SmoAtoh1*^Δ animals showed any evidence of over-proliferation in the EGL (0/12 animals) and had atrophic cerebella (Fig 3C), strongly suggesting that *Atoh1* deletion blocks the mitogenic activity of *Smoothened* in GNPs. Moreover, the EGL of *SmoAtoh1*^Δ animals was much thinner than that of control littermates (compare Figure 3A and C, right panels, arrows). We investigated the proliferative status of the GNPs of *SmoAtoh1*^{wt} and *SmoAtoh1*^Δ animals by phospho-histone H3 staining. The ectopic cell masses in *SmoAtoh1*^{wt} mice showed numerous mitotic cells (Figure 4A), whereas the EGL of *SmoAtoh1*^Δ animals was negative for the presence of cycling cells (Fig. 4B). Staining for caspase 3 revealed no sign of apoptosis in *SmoAtoh1*^Δ (not shown). As expected, the

outgrowths in *SmoAtoh1^{fl}* mice were positive for the YFP staining, an indication of *SmoM2* expression, and showed high levels of *Gli2* (Fig. 7S, arrow and 4C). We also observed strong staining with the YFP antibody in *SmoAtoh1^{fl}* animals (Fig. 7S B), but *Gli2* levels were drastically reduced (Fig. 4D), confirming that *Atoh1* expression is required for *Gli2* expression in the EGL even in the presence of constitutively active Shh signaling. *Zic1* staining showed that the cells that underwent recombination in *SmoAtoh1^{fl}* activated the granule neuron differentiation program, despite the constitutive activation of the Shh pathway (Fig. 4F). Once again, these data confirmed that *Gli2* expression in GNP requires *Atoh1* and that its absence abolishes the response to Shh. Moreover, the fact that deletion of *Atoh1*, a gene expressed only in GNPs, is able to prevent medulloblastoma formation strongly suggests that these cells are the cell-of-origin of tumors caused by constitutive activation of Shh signaling in the developing cerebellum. We provide genetic and molecular evidence that *Atoh1* regulates proliferation of GNPs in the post-natal cerebellum by controlling the activity of the Shh pathway expression through the direct transcriptional regulation of *Gli2*. Consistent with this role, *Atoh1* is also required for the formation of medulloblastomas induced by constitutive activation of the Shh pathway. A recent report suggests that *Atoh1* acts as a tumor suppressor in colorectal cancer and Merkel cell carcinomas by inducing cell differentiation and apoptosis through expression of tyrosine kinase receptor type I (*Ntrk1*), which is opposite the function we describe in this paper (22). We propose that *Atoh1* might activate the transcription of both *Gli2* and *Ntrk1*. The activation of *Gli2* makes the cells competent to transduce the mitogenic signal of Shh, whereas expression of *Ntrk1* allows the cells to respond to differentiative or apoptotic stimuli. If the cells are exposed to Shh, as in the case of GNPs in the EGL, the outcome of *Atoh1* expression is proliferation. On the other hand, if the cells are exposed to a differentiative signal, such as factors that activate tyrosine kinase receptors in the gut, *Atoh1* expression allows the cells to respond by exiting the cell cycle and differentiating.

The central role of *Atoh1* in the regulation of *Gli2* has important implications for the modulation of normal and neoplastic proliferation in the developing cerebellum. The possibility to exploit this developmental mechanism to control Shh-induced proliferation makes *Atoh1* a possible target for therapeutic strategies to inhibit medulloblastomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. *Atoh1* deletion disrupts GNP proliferation and induces differentiation. **A, B)** Phosphohistone H3 staining of cerebella of animals injected with tamoxifen. The arrow in B indicates a single cycling cell in the *Atoh1*^Δ EGL. **C and D)** Pax6 and NeuN staining shows the immature GNPs (white bar in G) not expressing NeuN and the differentiating population (between the yellow dotted lined in G and H) co-expressing both markers. The colocalization pattern is shown on the bottom right. Scale bars are shown.

Figure 2.

Atoh1 controls GNP proliferation through regulation of *Gli2* expression. **A)** QRT-PCR on RNA extracted from transduced GNPs cultured in presence of Shh for 3 days. The genes tested are shown below the bar graph. The amount of RNA for each gene was normalized over the GAPDH RNA level in each sample, and expressed as a percentage over the level of RNA of the GFP-transduced cells. **B)** Purified GNPs were infected either with a control retrovirus or a *GLI2*-expressing retrovirus and transduced with an Adenovirus expressing GFP or Cre. Cycling cells were labeled by BrdU and visualized by the red fluorescence signal. DAPI counterstaining (blue) reveals the nuclei. **C)** Quantification of the rescue effect by *GLI2* on proliferation after deletion of *Atoh1* in GNPs (BrdU positive cells/field). Student's T-test results are shown. **D)** Luciferase assay showing that Atoh1 can activate transcription when it interacts with the *Gli2* intronic region. Results are expressed as fold induction over the cells transfected with the empty expression vector. The reporter plasmids used in the experiments are shown below the graph. **E)** Chromatin-IP experiment indicating that Atoh1 is bound to the *Gli2* genomic region in the postnatal cerebellum and that this sequence has the epigenetic hallmark of an active transcriptional enhancer (see text for details). Sample identities are shown above and the origin of the chromatin used in the reactions is indicated below the gel.

Figure 3.

Atoh1 deletion prevents tumor formation in a mouse model of Shh-induced medulloblastoma. Pictures of **A**) P14 cerebella of a control animal that did not receive tamoxifen, and thus doesn't express the activated form of Smoothened; of **B**) an *Atoh1*^{wt} and of **C**) an *Atoh1*^Δ animal injected with tamoxifen to activate the expression of the constitutively active Smoothened (see text). The treatment is shown on the side of each image series. The cerebellum is outlined with a dotted line in the left panels; the arrowheads point out two neoplastic outgrowths. A sagittal section around the midline is shown in the

middle panels; neurons and neuronal precursors are visualized by Nissl staining. The boxed regions are shown at high magnification in the right panels; arrows indicate the EGL.

Figure 4.

Disruption of *Atoh1* blocks Shh-induced proliferation and *Gli2* expression in a mouse model of medulloblastoma. **A and B)** The M phase of the cell cycle was visualized by phosphohistone H3 staining in the cerebellum of *Atoh1*^{wt} (A) or *Atoh1*^Δ (B) animals injected with tamoxifen. The red signal shows actively cycling cells, whereas the blue signal reveals cell nuclei. The boxed portion of the left panels is magnified at the right. The scale and genotype are shown. **C)** Zic1 staining of a cerebellar sample from an *Atoh1*^{wt} animal and **D)** from an *Atoh1*^Δ mouse. The cells above the white line in E are the Zic1-negative undifferentiated cells of the neoplastic tissue.