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Brief Report

Repression of hedgehog signal transduction in T-lineage cells increases TCR-induced activation and proliferation

Nicola J. Rowbotham,[1,2] Anna L. Furmanski,[1] Ariadne L. Hager-Theodorides,[1] Susan E. Ross,[1,2] Ekati Drakopoulou,[1] Costas Koufaris,[2] Susan V. Outram[1] and Tessa Crompton[1,2]

[1]Immunobiology Unit; UCL Institute of Child Health; London, United Kingdom; [2]Division of Cell and Molecular Biology; Imperial College London; London, United Kingdom

Hedgehog proteins signal for differentiation, survival and proliferation of the earliest thymocyte progenitors, but their functions at later stages of thymocyte development and in peripheral T-cell function are controversial. Here we show that repression of Hedgehog (Hh) pathway activation in T-lineage cells, by expression of a transgenic repressor form of Gli2 (Gli2ΔC2), increased T-cell differentiation and activation in response to TCR signalling. Expression of the Gli2ΔC2 transgene increased differentiation from CD4+CD8+ to single positive thymocyte, and increased peripheral T cell populations. Gli2ΔC2 T-cells were hyper-responsive to activation by ligation of CD3 and CD28: they expressed cell surface activation markers CD69 and CD25 more quickly, and proliferated more than wild-type T-cells. These data show that Hedgehog pathway activation in thymocytes and T-cells negatively regulates TCR-dependent differentiation and proliferation. Thus, as negative regulators of TCR-dependent events, Hh proteins provide an environmental influence on T-cell fate.

Introduction

The Hedgehog (Hh) family of secreted intercellular signalling molecules regulate both the embryonic development and adult homeostasis of many mammalian tissues, including the immune system.[1-4] Hh proteins bind to their cells surface receptor Patched (Ptch) releasing the signal transducer Smoothened (Smo) to transmit the Hh signal into the cell, leading to transcriptional changes mediated by transcription factors Gli1, Gli2 and Gli3.[2] Gli1 acts exclusively as an activator of Hh signal, and is neither essential for mouse development nor initiation of the Hh signal.[5] Gli2 and Gli3 are both essential for mouse development and can undergo processing to act as transcriptional activators in the presence of Hh, or repressors in its absence.[6,7] Gli2 is necessary to initiate the first transcriptional changes upon Hh signalling.[8]

In the thymus, analysis of mouse mutants of Sonic Hh (Shh), Gli3 and Smo have demonstrated an essential positive regulatory

role for the pathway in signalling for survival, proliferation and differentiation of the earliest thymocyte progenitor populations.[9-11] The influence of the pathway at later stages of T lineage differentiation and on T cell activation, however, is highly controversial, with different experimental models suggesting variously opposing effects, or no effect.[9,12-16] In vitro addition of recombinant Shh (r-Shh) to T cells has been shown to enhance T cell activation and proliferation induced by antibodies against CD3 and CD28.[13,14] In contrast, constitutive activation of Hh signalling by expression of a transgenic activator form of Gli2 (Gli2ΔN₂) in T-lineage cells inhibited T cell activation and proliferation, by repressing TCR signal transduction.[3,12] Surprisingly however, a recent study in which Smo was conditionally deleted from T-lineage cells failed to reveal any influence (either positive or negative) of loss of Hh signalling on anti-CD3 induced T cell proliferation.[9]

Here we describe a novel mouse model in which Hh signalling is repressed in T-lineage cells, by transgenic expression of the repressor form of Gli2 (Gli2ΔC2), in order to ask if T cell autonomous inhibition of Hh signalling influences the TCR dependent stages of T cell differentiation and peripheral T cell function.

Results

Transgenic *Gli2ΔC₂* expression in thymocytes leads to a repression in Hh signalling. To test the effect of T cell autonomous inhibition of Hh signalling on T cell development and T cell activation, we produced a transgenic mouse that expressed a C-terminally truncated form of Gli2 under the control of the lck promoter. Transgene expression was thus restricted to T-lineage cells from the DN2 stage onwards (with full expression achieved at DN4), including peripheral T cells, but not thymic epithelium.[17] This truncated form of Gli2 (Gli2ΔC₂) can only act as a strong repressor of transcription, thus mimicking the transcriptional events that occur in the absence of Hh signalling.[6] Two independent transgenic lines were generated with similar copy number (Fig. 1A). Both lines showed the same phenotype so we present experimental data from one line only. Transgene expression was demonstrated by quantitative RT-PCR in both thymus and periphery, with expression levels corresponding to the proportion of T-lineage cells (Fig. 1B). To confirm that the Gli2ΔC₂ transgene was functional and able to repress an exogenous Hh signal, we compared the ability of purified CD4+ T cells to upregulate the Hh target gene *Ptc1* in response to
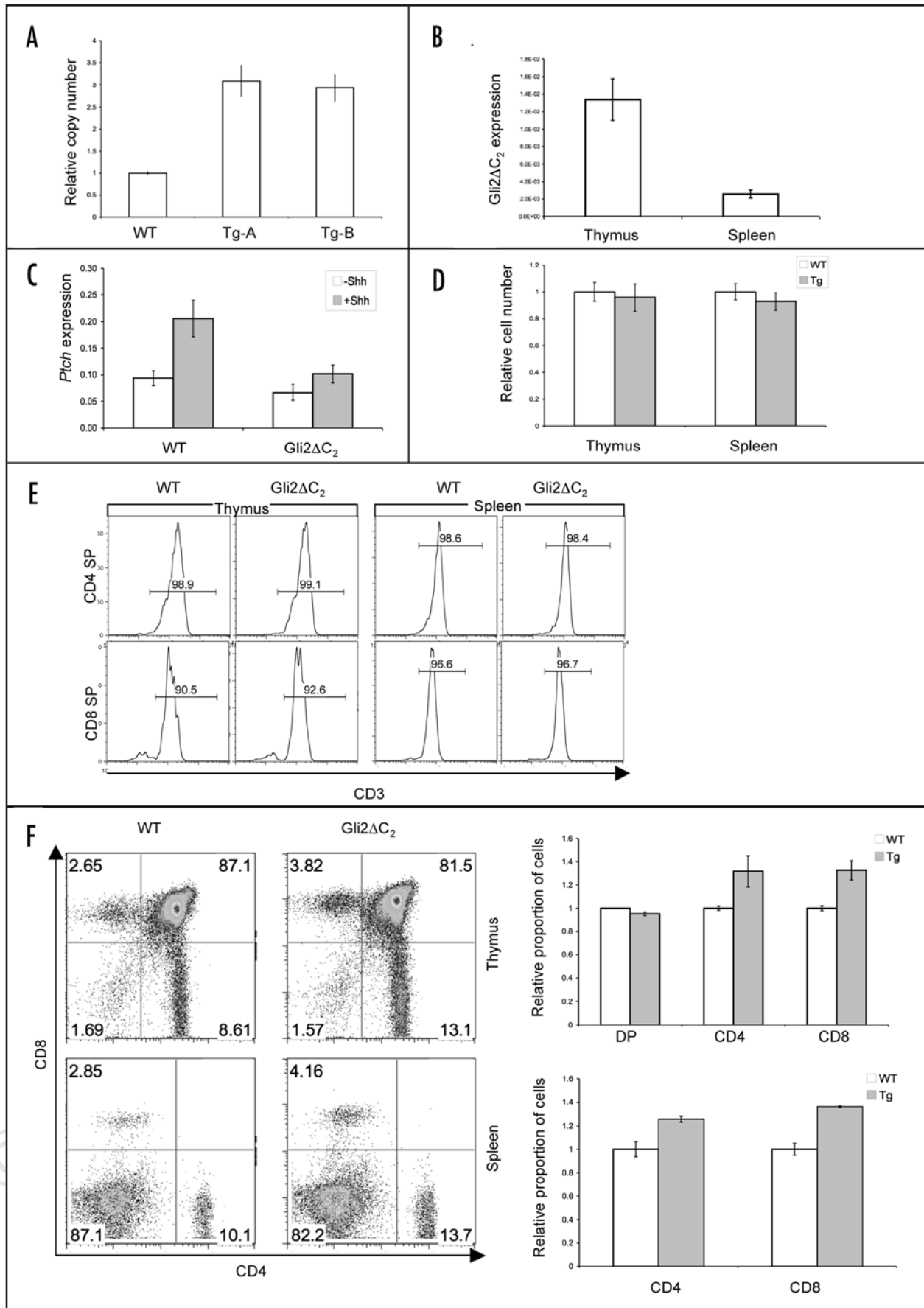
Figure 1. For figure legend, see page 906.

Figure 1. Phenotype of Gli2ΔC$_2$ mice. (A) Quantitative RT-PCR to show transgene copy number for both transgene positive lines. Error bars show standard deviation (SD). (B) Gli2 expression relative to HPRT expression by quantitative RT-PCR analysis in thymus and spleen of Gli2ΔC2 transgenic tissues. Error bars show SD. (C) Ptch expression relative to HPRT expression as assessed by quantitative RT-PCR analysis in purified CD4$^+$ Gli2ΔC2 or WT splenocytes cultured with or without 0.2 µg r-Shh for 3 hours. Gli2ΔC2 cells are less responsive to Shh signalling than WT cells. Error bars show SD. (D) Relative cell number (calculated relative to the mean of WT littermates) in thymus and spleen. (E) Histograms to show CD3 expression in thymus and peripheral CD4 and CD8 SP cells. Numbers indicate percentage of cells falling with in the marker. (F) Typical CD4 and CD8 profile of thymus (top panel) and spleen (lower panel) of a Gli2ΔC2 transgenic mouse and it's littermate. Numbers indicate the percentage of cells falling within each quadrant. Bar charts show the relative proportion of different populations. Differences were statistically significant as given: (thymus) CD4SP, p = .049; CD8SP, p = .005; DP, p = .017; and (spleen) CD4SP, p = .024; CD8SP, p = .007.

treatment with r-Shh. There was a marked decrease in the ability of the Gli2ΔC$_2$ transgenic CD4$^+$ cells to upregulate Ptc1 confirming that the Gli2ΔC$_2$ protein was a successful repressor of Hh signalling (Fig. 1C).

Inhibition of Hh signalling in the Gli2ΔC$_2$ transgenic influences the production of SP cells. Initial analysis showed that thymus and spleen were grossly normal, with no marked differences in lymphocyte numbers or TCR/CD3 expression in the thymus or spleen between WT and Gli2ΔC$_2$ (Fig. 1D and E). In the thymus there was, however, a significant increase in the proportion of SP cells, with concomitant decrease in the DP population, indicating that reduction in the ability of the thymocyte to respond to the Hh signal increases differentiation from DP to SP cell (Fig. 1E).

The effect of reduction of Hh signal at the transition from DP to SP thymocyte is controversial. In the Shh$^{-/-}$ thymus there is an increase in the production of SP cells.[12] Consistent with this, T-lineage specific constitutive activation of Hh signalling decreased DP to SP transition.[12] In contrast, in the T-lineage specific conditional Smo knock-out, no effect on this transition was described,[9] suggesting that the action of Shh on the production of SP cells could be an indirect effect through another cell type. However, the repression of Hh signalling described here is restricted to thymocytes through use of the lck transgenic cassette, demonstrating a T cell autonomous effect of Hh signalling on DP to SP differentiation.

In peripheral lymphoid organs we observed a significant increase in T cell populations (Fig. 1E).

Increased T cell activation in peripheral Gli2ΔC$_2$ transgenic T cells. To test the effect of repression of Hh signalling on T cell activation, splenocytes were activated with anti-CD3 and anti-CD28. After 24 hours we observed an increase in expression of the early activation marker CD69 and the later activation marker CD25, in both CD4$^+$ and CD8$^+$ cells from the Gli2ΔC$_2$ transgenic compared to WT littermates (Fig. 2A and B).

Enhanced proliferation of Gli2ΔC$_2$ T Cells on TCR/CD28 ligation. To assess proliferation, WT and Gli2ΔC$_2$ transgenic splenocytes were labelled with CFSE and stimulated with anti-CD3 and anti-CD28. The Gli2ΔC$_2$ T cells underwent more cell divisions than their WT counterparts (Fig. 2C) demonstrating that repression of full Hh pathway activation in T cells increases their ability to proliferate in response to TCR/CD28 ligation.

To ask if the increase in proliferation was saturated at this level of Gli2ΔC$_2$ expression, we doubled the transgene copy number by crossing the transgenic mice with each other. This further increased proliferation (Fig. 2D). The fact that we were able to increase repression demonstrates competitive inhibition of Hh-dependent transcription by the Gli2ΔC$_2$ transgene.

Discussion

Loss-of-function mutants have previously shown that Hh signalling promotes proliferation of very early thymocytes progenitors.[9,11] In contrast, here we show that constitutive repression of the Hh pathway promotes proliferation of mature T cells. The effect of Hh signalling on proliferation has also appeared ambiguous in other systems.[18] In the chick and mouse retina Hh pathway activation has been shown to promote proliferation,[19-21] whereas Hh mutant Zebrafish have prolonged retinal proliferation, due to the inability of precursor cells to exit the cell cycle.[22] Recent work has suggested that Hh signalling may effect stem, progenitor and mature cells differently, with Hh signalling either promoting cell cycle progression or pushing cells out of the cell cycle, depending on the state of differentiation of the cell.[23] The T cell lineage thus provides another example of Hh activation promoting proliferation of the progenitor cell, but limiting the proliferation of the more differentiated mature cell.

In summary, here we have shown that repression of Hh pathway activation in thymocytes and T cells increases differentiation and activation in response to TCR signalling. Thus, Hh proteins are negative regulators of TCR-dependent events in T cells, providing an environmental influence on T cell fate.

Methods

Construction of *lck*-Gli2ΔN$_2$ transgenic. Gli2ΔC$_2$ cDNA[6] was bluntend cloned into the *Bam*HI site of the lck proximal promoter cassette.[24] The 8.9 kb transgene was isolated by *Not*I digestion and purified using QIAEXII Gel Extraction Kit (Qiagen). CBA x C57BL/6 oocytes were injected, generating 2 independent transgenic lines.

Mice. *lck*-Gli2ΔC$_2$ transgene-positive mice were backcrossed for >7 generations with C57BL/6 (B&K Universal) and maintained under UK Home Office regulations.

Genotyping. DNA was extracted as described.[11] Gli2ΔC$_2$ transgene positive mice were detected by the presence of human growth hormone DNA (5'-hGH CGAACCACTCAGGGTCCTGTGG, 3'-hGH GGATTTCTGTTGTGTTTCCTCCCTG).[25]

EasySep bead purification. CD4$^+$ lymphocytes were purified from whole spleen by magnetic bead separation using the EasySep® Negative Selection Mouse CD4$^+$ T cell Enrichment kit (StemCell Technologies, UK) according to the manufacturer's instructions giving ≥95% purity.

Quantitative RT-PCR. Quantitative RT-PCR was as described.[10] Primers were Gli2ΔC$_2$F:AGAACCTGAAGACACACCTGCG, Gli2ΔC$_2$R:GAGGCATTGGAGAAGGCTTTG. Ptch1F:TGCTCT CCAGTTCTCAGACTC,Ptch1R:CCACAACCTTGGCTTTGG

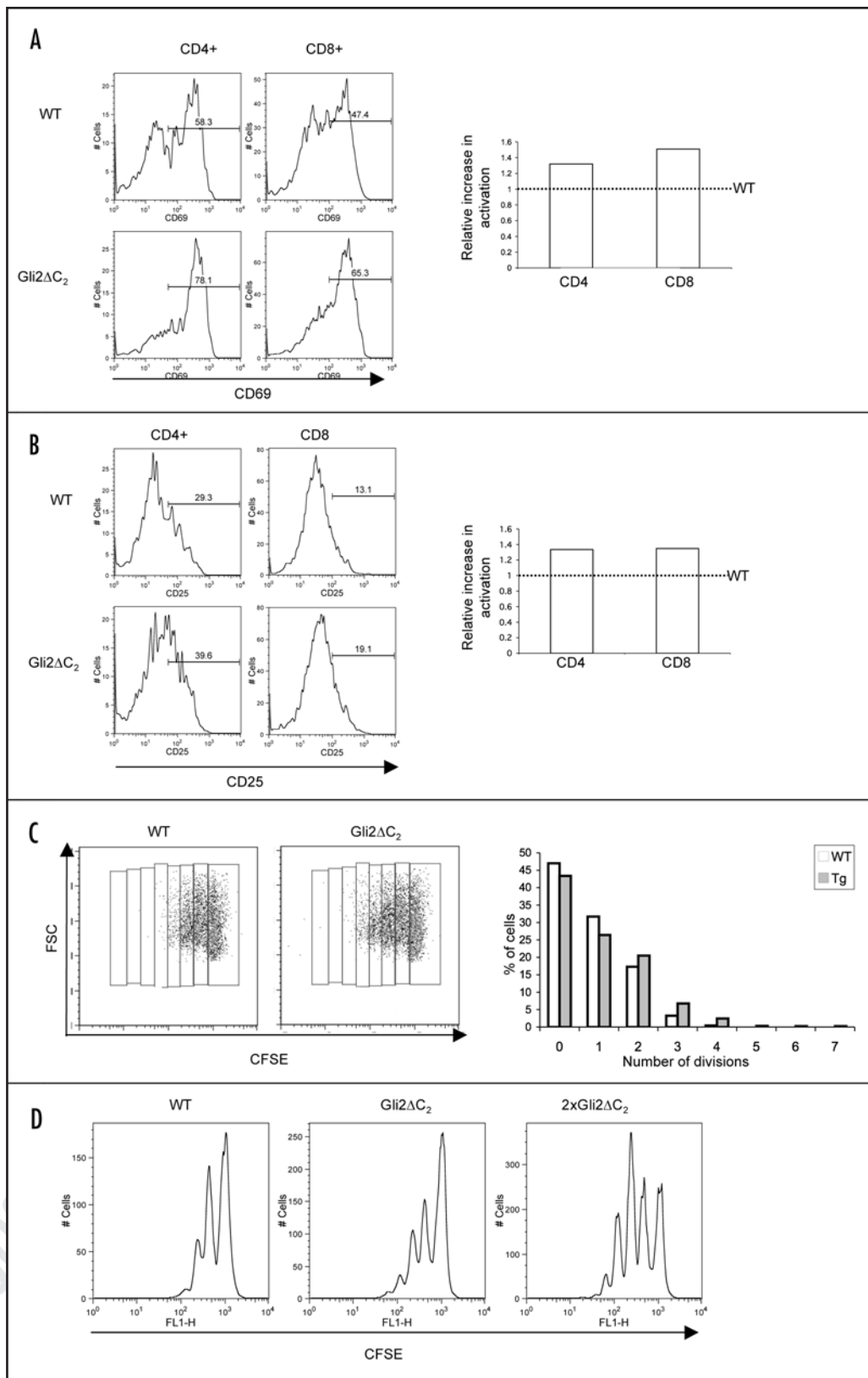**Flow Cytometry.** Cells were stained as described.[10,12] Data are representative of >3 experiments.

Figure 2. Gli2ΔC2 transgene expression improves TCR-induced activation and proliferation in peripheral T cells. (A) Histograms showing the early activation marker CD69 on CD4 and CD8 SP cells after 24 hours in culture stimulated with 0.01 μg/ml of each of anti-CD3 and anti-CD28. Numbers indicate the percentage of cells falling within the marker. Bar chart shows relative increase in activation compared to WT littermate controls. (B) Histograms showing the later activation marker CD25 on CD4 and CD8 SP cells after 24 hours in culture stimulated with 0.01 μg/ml of each of anti-CD3 and anti-CD28. Numbers indicate the percentage of cells falling within the marker. Bar chart shows relative increase in activation compared to WT littermate controls. (C) CFSE staining in peripheral T cells cultured for 72 hours with 0.01 μg/ml of each of anti-CD3 and anti-CD28. Bar graph to show the number of cell divisions that had occurred. (D) Histograms of CFSE staining to show dose effect of the Gli2ΔC2 transgene on proliferation.

In vitro T cell culture and activation. Splenic T cells were cultured and activated as described.[12] r-Shh was a gift from Curis.[15]

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