RESEARCH PAPER

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RNA editing of the GLI1 transcription factor modulates the output of Hedgehog signaling

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The Hedgehog (HH) signaling pathway has important roles in tumorigenesis and in embryonal patterning. The Glioma-associated oncogene 1 (GLI1) is a key molecule in HH signaling, acting as a transcriptional effector and, moreover, is considered to be a potential therapeutic target for several types of cancer. To extend our previous focus on the implications of alternative splicing for HH signal transduction, we now report on an additional post-transcriptional mechanism with an impact on GLI1 activity, namely RNA editing. The GLI1 mRNA is highly edited at nucleotide 2179 by adenosine deamination in normal cerebellum, but the extent of this modification is reduced in cell lines from the cerebellar tumor medulloblastoma. Additionally, basal cell carcinoma tumor samples exhibit decreased GLI1 editing compared with normal skin. Interestingly, knocking down of either ADAR1 or ADAR2 reduces RNA editing of GLI1. This adenosine to inosine substitution leads to a change from Arginine to Glycine at position 701 that influences not only GLI1 transcriptional activity, but also GLI1-dependent cellular proliferation. Specifically, the edited GLI1, GLI1-701G, has a higher capacity to activate most of the transcriptional targets tested and is less susceptible to inhibition by the negative regulator of HH signaling suppressor of fused. However, the Dyrk1a kinase, implicated in cellular proliferation, is more effective in increasing the transcriptional activity of the non-edited GLI1. Finally, introduction of GLI1-701G into medulloblastoma cells confers a smaller increase in cellular growth relative to GLI1. In conclusion, our findings indicate that RNA editing of GLI1 is a regulatory mechanism that modulates the output of the HH signaling pathway.

Introduction

Post-transcriptional modifications, exemplified by alternative splicing, are regulatory mechanisms of biological processes that create enormous diversity in the proteins derived from a single gene.[1,2] RNA editing is an additional post-transcriptional mechanism that also generates RNA and protein diversity. In mammals, the most common form of RNA editing is substitution of adenosine (A) to inosine (I). Edited RNAs have consequently a sequence that is different from genomic DNA. RNA editing can selectively alter either coding or noncoding sequences in nuclear transcripts, including pre-mRNAs. A-to-I editing is catalyzed by the adenosine deaminases acting on RNA (ADARs) class of enzymes, which recognize unique double-stranded RNA structures containing bulges and loops. RNA editing may cause changes in alternative splicing patterns, microRNA interaction sites or even amino acids.[3] Several reports highlighted that RNA editing can frequently occur in the brain, however in tumors, these post-transcriptional events were found to be less pronounced compared with normal tissues.[4-6]

The Hedgehog (HH) signaling pathway is known to have important roles in diverse biological contexts, including embryonic development, stem cell maintenance and tumorigenesis. Deregulation of HH signaling results in developmental defects and different types of tumors. In these tumors, the pathway is abnormally upregulated, and effective signaling inhibition can repress tumor growth and induce apoptosis.[7] The Glioma-associated oncogene 1 (GLI1) is a transcription factor, which acts as a terminal effector of the HH signaling pathway, in addition to being a target gene. GLI1 has been characterized as an oncogene and its overexpression leads to basal cell carcinoma in transgenic mice.[8] Recently, several reports highlighted that GLI1 acts as a key molecule in the regulation of tumor growth and the self-renewal of cancer stem cells.[9,10]

GLI1 expression and activity are known to be regulated at different levels and include both transcriptional and

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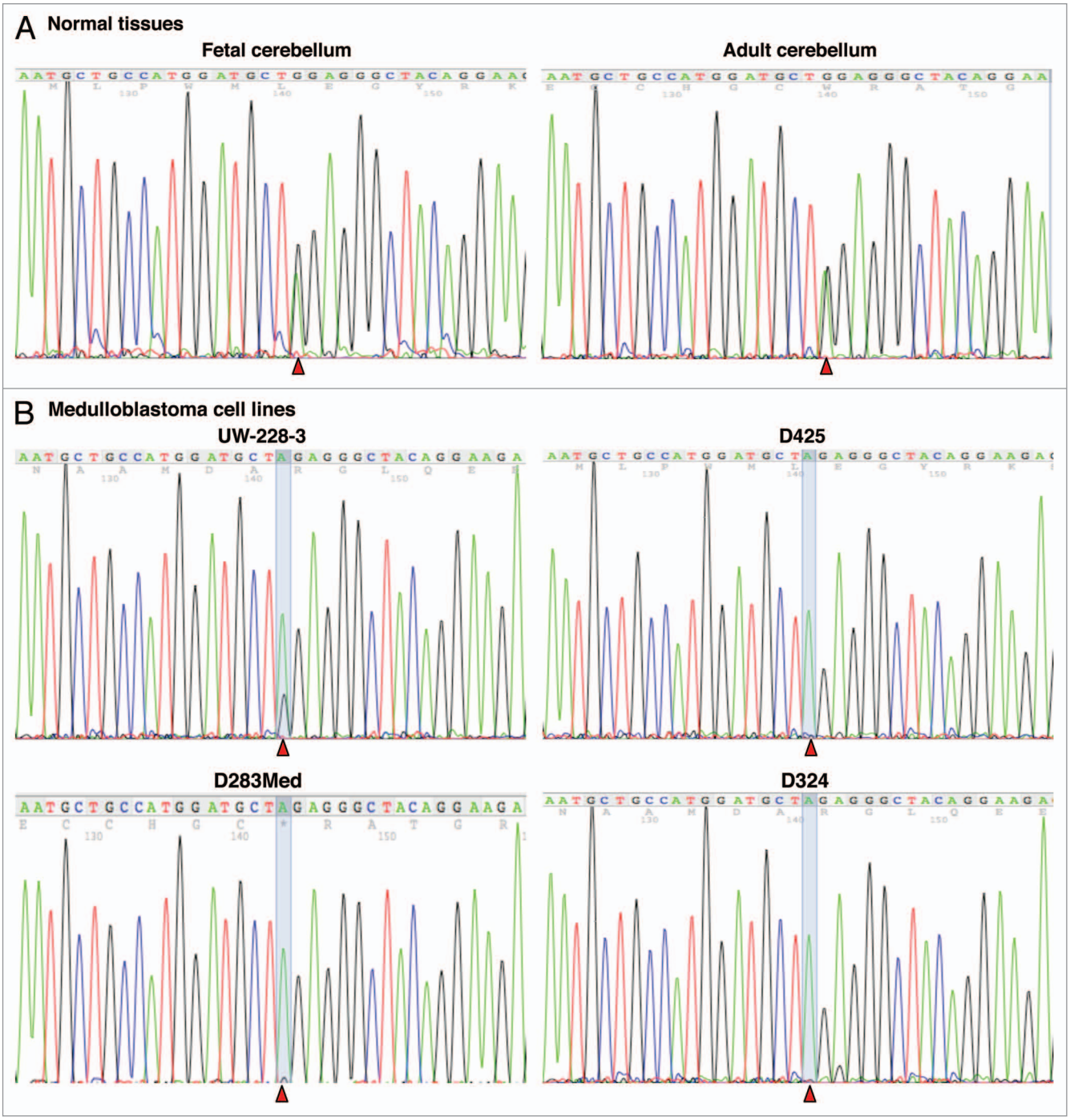

Figure 1A–B. Identification of RNA editing in GLI1. Electropherograms of the sequence analysis of the RNA-edited region in GLI1. mRNAs from normal tissues (**A**) or medulloblastoma cell lines (**B**) were RT-PCR amplified and the products were analyzed by direct sequencing. Red triangles indicate the target nucleotide of RNA editing.

post-transcriptional processes.[11-15] These post-transcriptional mechanisms increase the functional diversity of the GLI1 protein and have an impact on the biological activity of GLI1, exemplified by the role of a GLI1 splice variant for glioblastoma and breast cancer cell migration and invasion.[16,17] Moreover, GLI1 has been the focus of several recent attempts on cancer therapy as a novel molecular target.[9,18-21] Consequently, to fully understand the functional implications of GLI1 for HH signaling activation, it is important to clarify the regulatory mechanisms that act on the GLI1 transcriptional activity.

In this study, we demonstrate that the GLI1 mRNA is also under the regulation of the post-transcriptional process of RNA editing, resulting in an amino acid change of the GLI1 protein with an impact on GLI1 activity. This editing event is prevalent in a number of tissues; however, in the corresponding tumors, the extent of GLI1 editing is reduced. These findings suggest

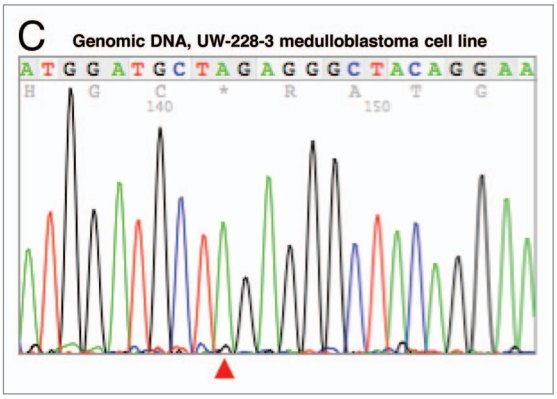

Figure 1C. Genomic DNA sequence analysis of the GLI1 edited region in the UW-228-3 medulloblastoma cell line. The red triangle indicates the target nucleotide of RNA editing. Note the presence of a single A peak.

that novel post-transcriptional mechanisms are involved in controlling the functional impact of the terminal effector of HH signaling, the transcription factor GLI1.

Results

Identification of human GLI1 modification by RNA editing. A recent report on whole genome screening of RNA editing revealed that this post-transcriptional event is more frequent than previously anticipated. These observations highlighted that a large number of mRNAs, including GLI1, are edited in the brain.[22] To confirm this finding, we analyzed the GLI1 mRNA from human cerebellum and medulloblastoma cell lines (**Fig. 1A and B**). Our data revealed that the GLI1 mRNAs are highly edited by conversion of A to I at nucleotide position 2179 (accession number NM_005269) in both fetal and adult cerebellum. However, in cell lines from the cerebellar tumor medulloblastoma, and in agreement with the postulate that tumors are characterized by hypoediting,[4] this A to I substitution was almost negligible. Interestingly, the RNA editing of GLI1 was detected not only in brain but also in other tissues, including lung, colon, pancreas and ovary. In tumor cell lines though, originating from these tissues, the extent of editing was severely reduced (**Fig. S1**). Additionally, a single A peak was observed when genomic DNA from the medulloblastoma cell line that revealed detectable levels of RNA editing, the UW-228-3, was sequenced, ruling out the presence of a gene polymorphism (**Fig. 1C**). This is in agreement with the A/A genotype of more than 400 analyzed chromosomal samples from individuals of European, Asian and Sub-Saharan African descent (www.ncbi.nlm.nih.gov/snp). Moreover, to check whether the reduction in RNA editing of GLI1 is not limited to cancer cell lines but also occurs in biopsy tumor specimen, basal cell carcinomas and control skin were also analyzed. In line with the observations described above, the extent of GLI1 editing was reduced in the tumor relative to the control samples (**Fig. 1D**).

To determine the role of the ADAR1 and ADAR2 enzymes in the adenosine deamination of nucleotide 2179, four neuroblastoma cell lines that exhibit detectable levels of GLI1 editing were analyzed. Interestingly, knocking down of either ADAR1 or ADAR2 in SK-N-AS, SH-SY5Y, SK-N-BE(2) or SK-N-SH cells resulted in a reduction of adenosine 2179 deamination in the cell lines examined (**Fig. 2A–D**).

Adenosine 2179 of human GLI1 is highly conserved in mammals, but the surrounding sequence has certain variations (**Fig. 3A**). These nucleotide substitutions could have an impact on the secondary structure of the mRNA and, thus, on RNA editing. The predicted secondary structures according to Mfold (www.mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) highlight similar stem-loop patterns among the chimpanzee, the orangutan and the human GLI1 (**Fig. 3B**). However, in the marmoset, a more evolutionary distant primate, and in the mouse, different stem-loop patterns are observed (**Fig. S2**). In fact, we analyzed mouse Gli1 mRNAs from various sources but could not detect any evidence of RNA editing (**Fig. S3**). These observations may indicate that RNA editing of GLI1 could only occur in some primates.

The majority of A-to-I editing events have been predicted to occur in the UTR regions or in the introns, however there are also numerous cases that affect coding exons, including the glutamate receptor and serotonin receptor mRNAs.[23,24] The editing of GLI1 was found in the coding region, namely in the sequence encoding the C-terminal half of the protein (**Fig. 3C**). This base substitution results in an amino acid change at position 701, from Arginine to Glycine, which may influence the secondary structure of the GLI1 protein (**Fig. 3D**). In fact, prediction programs (the Consensus Secondary Structure Prediction www.npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html) indicate that this editing shortens the length of the α helix structure. This finding suggests that RNA editing could influence the biochemical properties of GLI1.

Biochemical characterization of the RNA-edited GLI1. To clarify the functional implications on GLI1 elicited by RNA editing, the edited GLI1, named GLI1-701G, was cloned into the mammalian expression vector, pCMV5. Transfection of the cloned construct into HEK293 cells revealed that the GLI1-701G protein was expressed at similar levels as the non-edited GLI1 (**Fig. 4A**). Moreover, the transcriptional activity, as determined by the 12xGLIBS-luc reporter (**Fig. 4B**) and the mPtch1-1B-luc reporter (**Fig. 4C**), was slightly higher for GLI1-701G than for GLI1 at the range of 10–30 ng of transfected construct. However, with the use of the mGli1-luc reporter (**Fig. 4D**), the activity of GLI1-701G and GLI1 was equivalent. These findings indicate that GLI1-701G and GLI1 can have different transcriptional activities depending on the target gene or the DNA-binding sequence.

The RNA-edited target site exchanges an amino acid within a domain in the GLI1 C-terminal half that binds to the negative regulator of HH signaling, suppressor of fused (SUFU)[25] (**Fig. 3C**). This implies that GLI1-701G may have a different susceptibility to inhibition by SUFU. To verify this possibility, we analyzed the GLI activity in the HH signaling constitutively active cell lines, *Ptch1-/-* and *Sufu-/-* mouse embryonic fibroblasts (MEFs) (**Fig. 4E**). In *Sufu*$^{-/-}$ MEFs, GLI1-701G had a similar activity as GLI1. On the other hand, GLI1 showed reduced activity compared with

Figure 1D. mRNAs from six basal cell carcinoma and four control skin samples were RT-PCR amplified and the products were analyzed by direct sequencing. Red triangles indicate the target nucleotide of RNA editing.

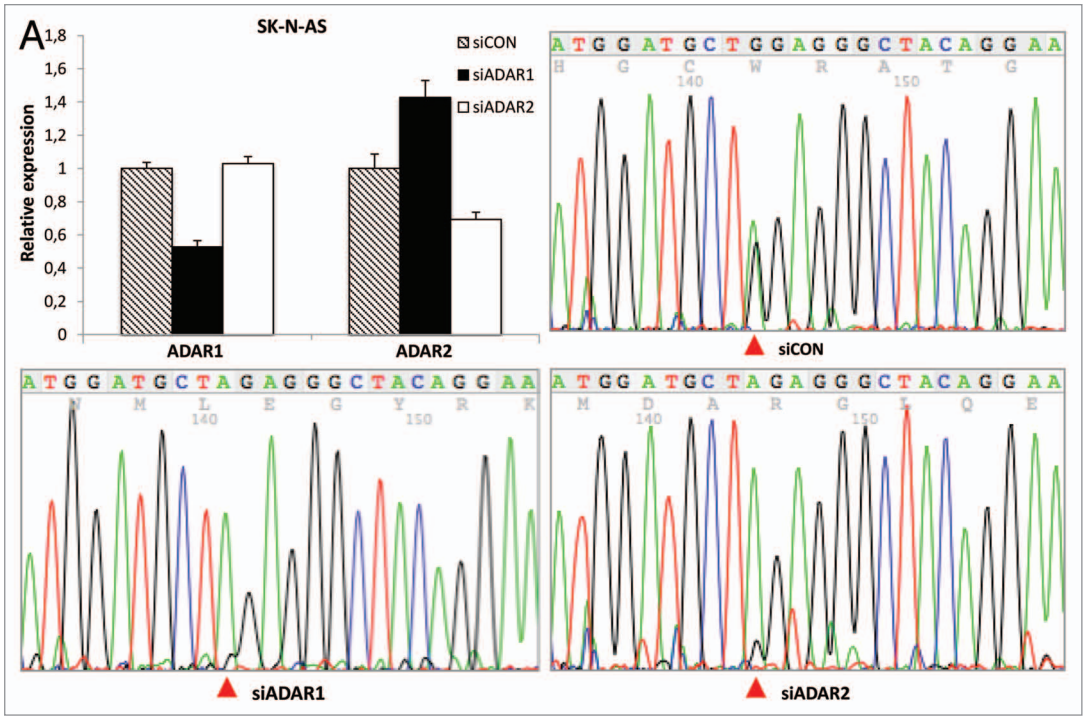

Figure 2A. Knockdown of ADAR1 or ADAR2 affects RNA editing of GLI1. Electropherograms of the sequence analysis of the RNA-edited region in GLI1 in the four neuroblastoma cell lines SK-N-AS (**A**), SH-SY5Y (**B**), SK-N-BE(2) (**C**) or SK-N-SH (**D**), following knockdown of ADAR1 or ADAR2. Note that knocking down of either ADAR1 or ADAR2 results in reduced RNA editing of GLI1. The effectiveness and specificity of the ADAR1 and ADAR2 knockdowns, determined by real-time RT/PCR analysis, is shown for all four neuroblastoma cell lines.

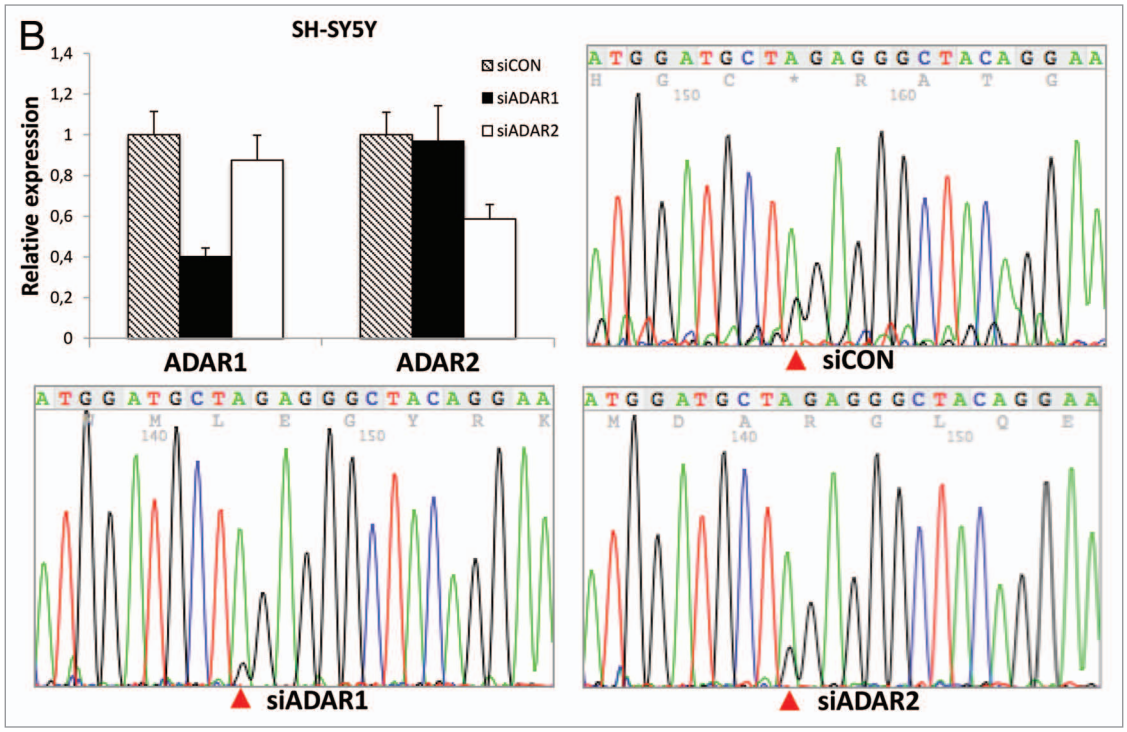

Figure 2B. Knockdown of ADAR1 or ADAR2 affects RNA editing of GLI1. Electropherograms of the sequence analysis of the RNA-edited region in GLI1 in the four neuroblastoma cell lines SK-N-AS (**A**), SH-SY5Y (**B**), SK-N-BE(2) (**C**) or SK-N-SH (**D**), following knockdown of ADAR1 or ADAR2. Note that knocking down of either ADAR1 or ADAR2 results in reduced RNA editing of GLI1. The effectiveness and specificity of the ADAR1 and ADAR2 knockdowns, determined by real-time RT/PCR analysis, is shown for all four neuroblastoma cell lines.

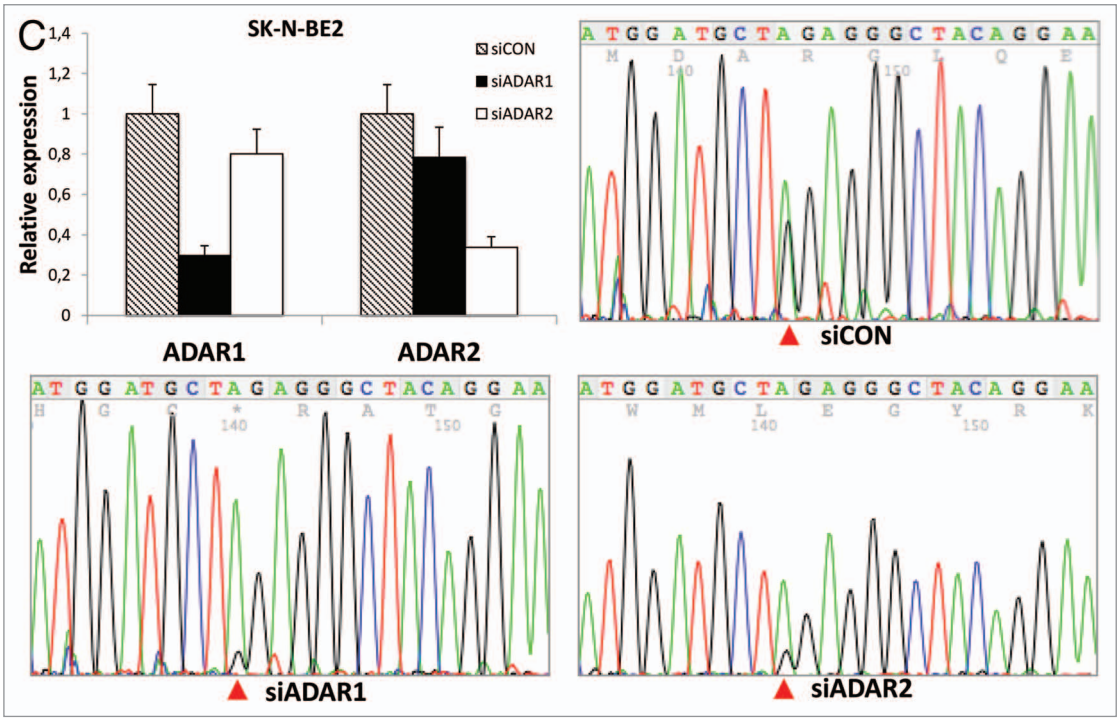

Figure 2C. Knockdown of ADAR1 or ADAR2 affects RNA editing of GLI1. Electropherograms of the sequence analysis of the RNA-edited region in GLI1 in the four neuroblastoma cell lines SK-N-AS (**A**), SH-SY5Y (**B**), SK-N-BE(2) (**C**) or SK-N-SH (**D**), following knockdown of ADAR1 or ADAR2. Note that knocking down of either ADAR1 or ADAR2 results in reduced RNA editing of GLI1. The effectiveness and specificity of the ADAR1 and ADAR2 knockdowns, determined by real-time RT/PCR analysis, is shown for all four neuroblastoma cell lines.

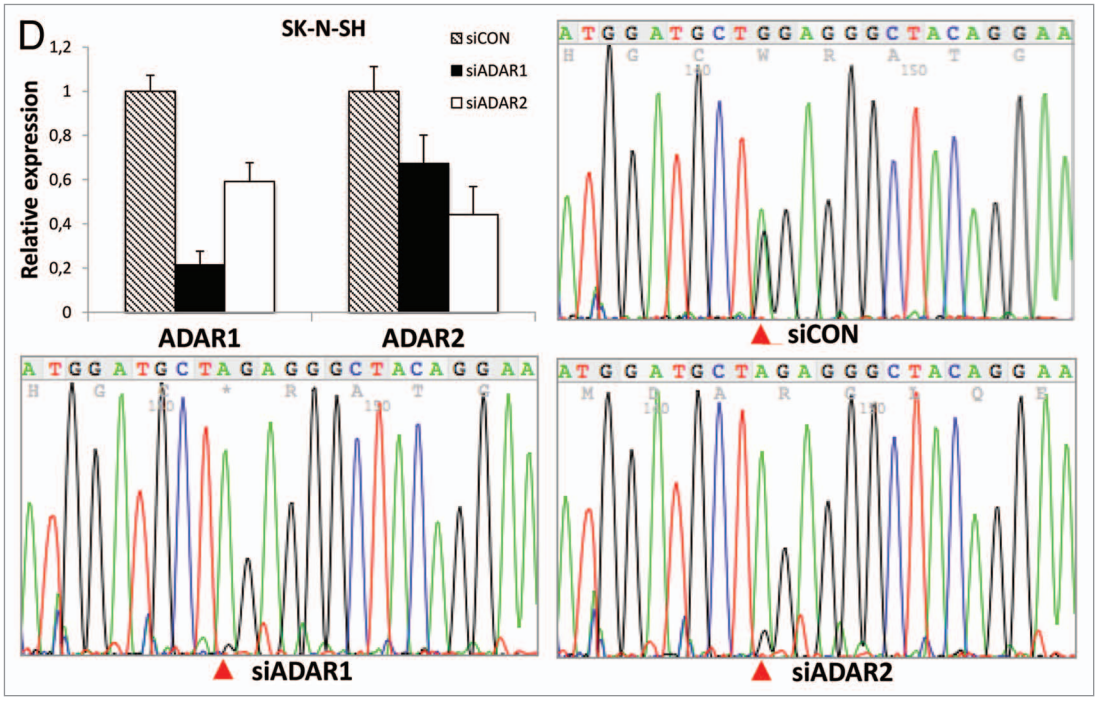

Figure 2D. Knockdown of ADAR1 or ADAR2 affects RNA editing of GLI1. Electropherograms of the sequence analysis of the RNA-edited region in GLI1 in the four neuroblastoma cell lines SK-N-AS (**A**), SH-SY5Y (**B**), SK-N-BE(2) (**C**) or SK-N-SH (**D**), following knockdown of ADAR1 or ADAR2. Note that knocking down of either ADAR1 or ADAR2 results in reduced RNA editing of GLI1. The effectiveness and specificity of the ADAR1 and ADAR2 knockdowns, determined by real-time RT/PCR analysis, is shown for all four neuroblastoma cell lines.

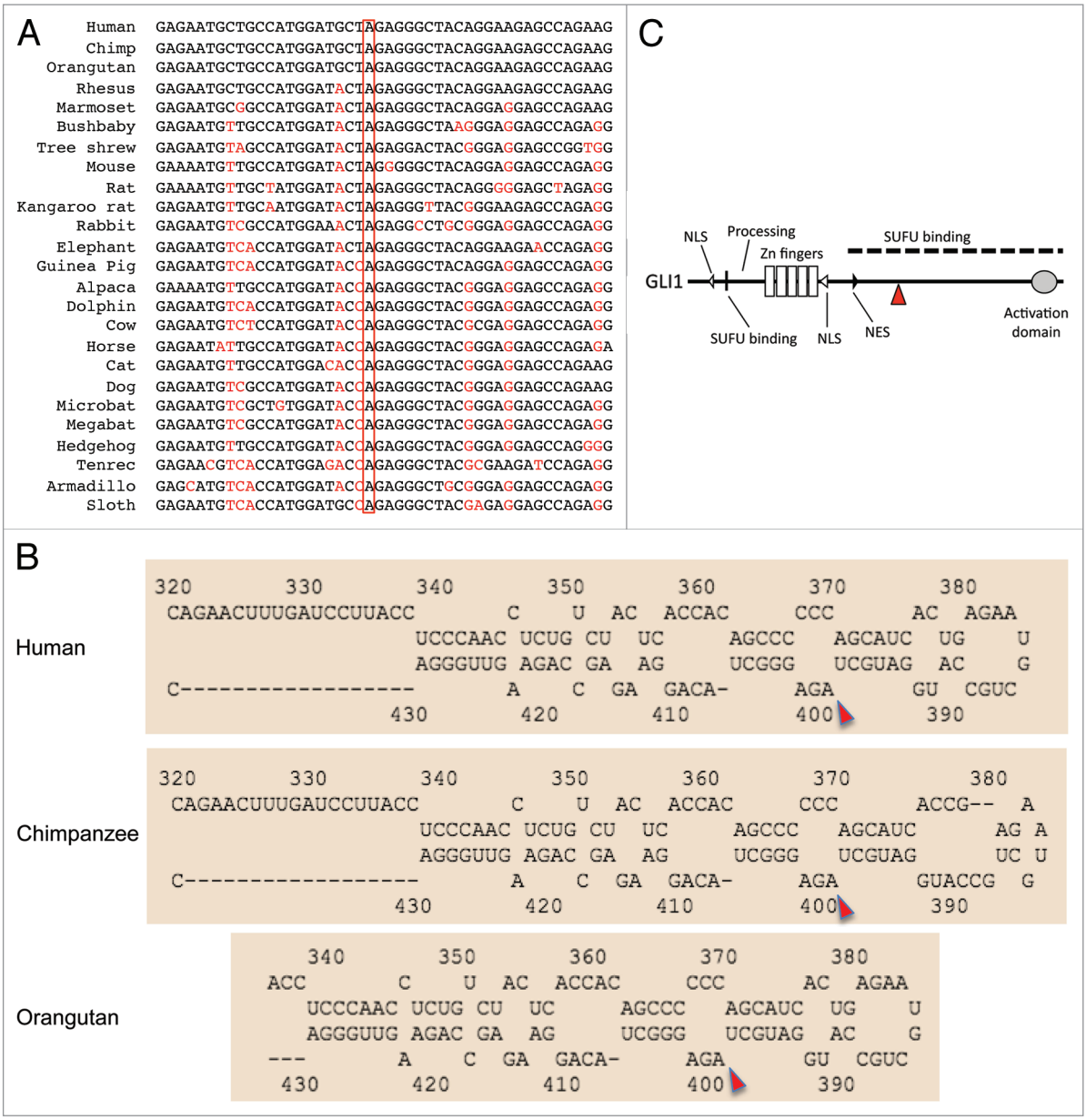

Figure 3A–C. Impact of RNA editing on GLI1 protein structure. (**A**) Comparison of the sequence of the RNA-edited region in GLI1 among different mammalian species. The target nucleotide of RNA editing in GLI1 and its orthologs is highlighted by a red box. Nucleotides in red indicate differences from the human sequence. (**B**) Secondary structures of the RNA-edited region in human GLI1 (upper panel) and the corresponding ones in the chimpanzee (middle panel) and the orangutan (lower panel). The edited A nucleotide in human and the equivalent in the other two primates are highlighted by red triangles. Note that the edited nucleotide is positioned within the terminal exon 12 of the human GLI1, 524 nucleotides 3' of the splice junction. (**C**) Predicted protein domains of GLI1. The protein processing site, SUFU-binding sites, zinc finger domains, activation domain, nuclear localization signals (NLS) and nuclear export signal (NES) are shown. A red triangle indicates the target position of RNA editing.

GLI1–701G in *Ptch1*$^{-/-}$ MEFs. Additionally, co-transfection with a SUFU expression construct in C3H10/T1/2 cells demonstrated that GLI1-701G is less susceptible to inhibition by SUFU relative to GLI1 (**Fig. 4F**). These differences were not large, yet represented significant changes that may be relevant for endogenous GLI1 expression levels, which are usually very low.

Subcellular localization of GLI1-701G. Previous reports have shown that the subcellular localization of GLI1 can be critical for its capacity to act as a transcription factor.[26] To examine whether RNA editing has an impact on the subcellular distribution of GLI1, the GLI1 and GLI1-701G constructs were transfected into C3H10/T1/2 cells followed by immunofluorescence analysis (**Fig. 5**). Both GLI1 variants were found to be localized in the cytoplasm and the nucleus. This result highlights that RNA editing does not confer a major change on GLI1 subcellular localization and the differences in the transcriptional activities of GLI1-701G / GLI1 are not likely to be due to alterations in cytoplasmic/nuclear distributions.

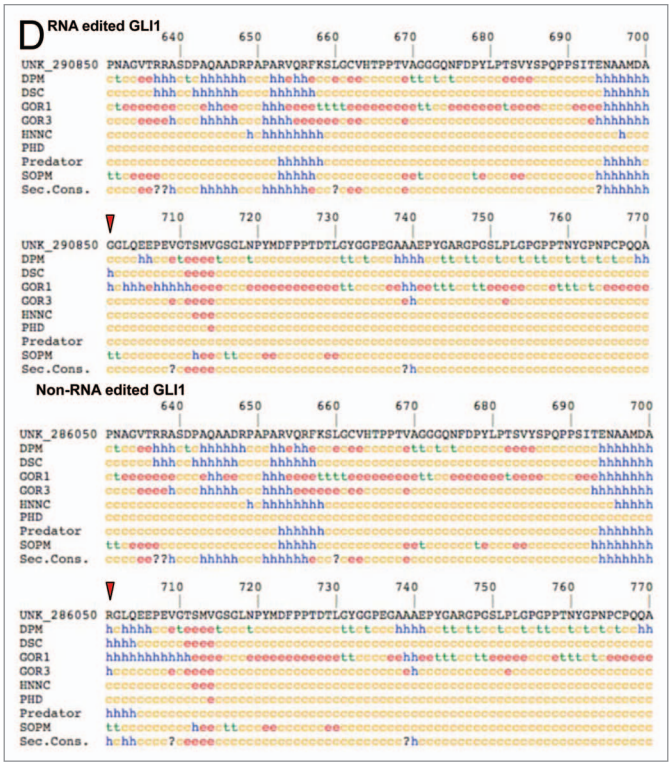

Figure 3D. (**D**) Influence of RNA editing on the GLI1 protein structure. The predicted secondary structure of the GLI1 protein was calculated by the use of several algorithms. The R-to-G amino acid substitution at position 701 that is elicited by RNA editing is shown by red triangles. The abbreviations are: h, α helix; e, extended strand; c, random coil; t: β turn; ?, ambiguous state. Note the decreased α helix structure in the 690–710 region of the edited GLI1.

Biological significance of RNA editing. GLI1 is also known to be regulated by phosphorylation. Dyrk1a is reported to be a kinase, with an apparent role in neuroblast proliferation,[27] which modifies the GLI1 activity.[28] To examine the impact of RNA editing of GLI1 on the Dyrk1a effects, we co-transfected Dyrk1a with GLI1 or GLI1-701G. In C3H10/T1/2 cells, Dyrk1a activated only GLI1, while in NIH3T3 cells, the activity of both variants was increased, but to a lesser extent for GLI1-701G (**Fig. 6A**). Thus, in two cellular contexts, RNA editing of GLI1 reduces its responsiveness to the Dyrk1a kinase.

GLI1 functions as an oncogene, and inhibition or knockdown of GLI1 negatively affects tumor cell growth.[18-21] However, our data suggest that GLI1 editing is frequently observed in normal cells but not in tumor cells. Therefore, we hypothesized that RNA editing of GLI1 might reduce the impact of this oncogenic factor on cellular growth. To test this hypothesis, we introduced the GLI1 or GLI1-701G expression constructs, together with pEGFP, into the medulloblastoma cell line D283Med. As anticipated, GLI1-701G compared with GLI1, resulted in a decreased percentage of EGFP-positive cells after 3 d of cellular growth (**Fig. 6B**).

Taken together, these results indicate that RNA editing acts as a novel modifier of the HH signaling pathway via its impact on the GLI1 transcription factor.

Discussion

Post-transcriptional biological processes are key regulatory events that increase the diversity of the transcriptome and the proteome. RNA editing is a post-transcriptional mechanism that alters nucleotide residues, with its most prevalent form in mammals being the A-to-I conversion. Double-stranded RNA structures harboring stem-loops are the targets of A-to-I editing, with most of these modifications occurring in untranslated regions, introns or pre-miRNAs. However, RNA editing can also actively change nucleotides in the coding sequence of mRNAs and consequently expand the variability of the encoded proteins. Recent reports have demonstrated that thousands of human genes are subjected to A-to-I RNA editing. This nucleotide modification is most abundantly observed in brain tissues, and dysfunction of RNA editing has been related to neurological disorders. Accumulated evidence is therefore suggesting that RNA editing is an important regulatory mechanism with an impact on diverse biological processes. However, the specific roles and outcomes of individual RNA editing events have been analyzed only in a limited number of genes or cellular contexts.[3,22,29,30] In this study, we demonstrate abundant A-to-I RNA editing in the coding region of the GLI1 oncogene in normal human tissues, reduced editing in the corresponding cancer cells and provide biochemical evidence for the role of this post-transcriptional modification as a novel regulatory mechanism that modulates GLI1 activity.

Worth noting is that knocking down of either ADAR1 or ADAR2 reduces the deamination of adenosine 2179 in all four cell lines analyzed (**Fig. 2**). This observation highlights the requirement of both ADAR1 and ADAR2 for effective editing of the GLI1 mRNA. One scenario that could provide a mechanistic interpretation of this finding is the formation of ADAR1/ADAR2 heterodimers[31] that are catalytically active on this substrate.

Recently, Joost et al. showed that low level of GLI1 expression in pancreatic cancer cells is related to epithelial-to-mesenchymal transition, while high expression promotes epithelial differentiation.[32] Consequently, GLI1 may have different functions depending on its expression level. Our data indicate that in normal tissues, where GLI1 expression is usually very low, GLI1 is frequently modified by RNA editing. Moreover, when GLI1 was overexpressed in non-cancer cell lines, no major differences in the transcriptional activities of GLI1 and GLI1-701G were observed at higher levels of expression. However, we clearly detected that GLI1 and GLI1-701G have distinct transcriptional capacities at lower levels of expression. Thus, it is likely that in the context of normal adult tissues, characterized by low GLI1 expression, RNA editing of GLI1 may be functionally significant.

A different scenario is probably taking place in GLI1-dependent cancer cells. RNA editing of GLI1 is very low, GLI1 expression is relatively high and the non-edited GLI1 has been shown to be the major target of the GLI1 activating Dyrk1a kinase. The proliferative advantage elicited by overexpression of GLI1 compared with GLI1-701G is also in-line with the impact of the non-edited GLI1 in cancer cells. Moreover, the inverse correlation between GLI1 levels and the extent of RNA editing

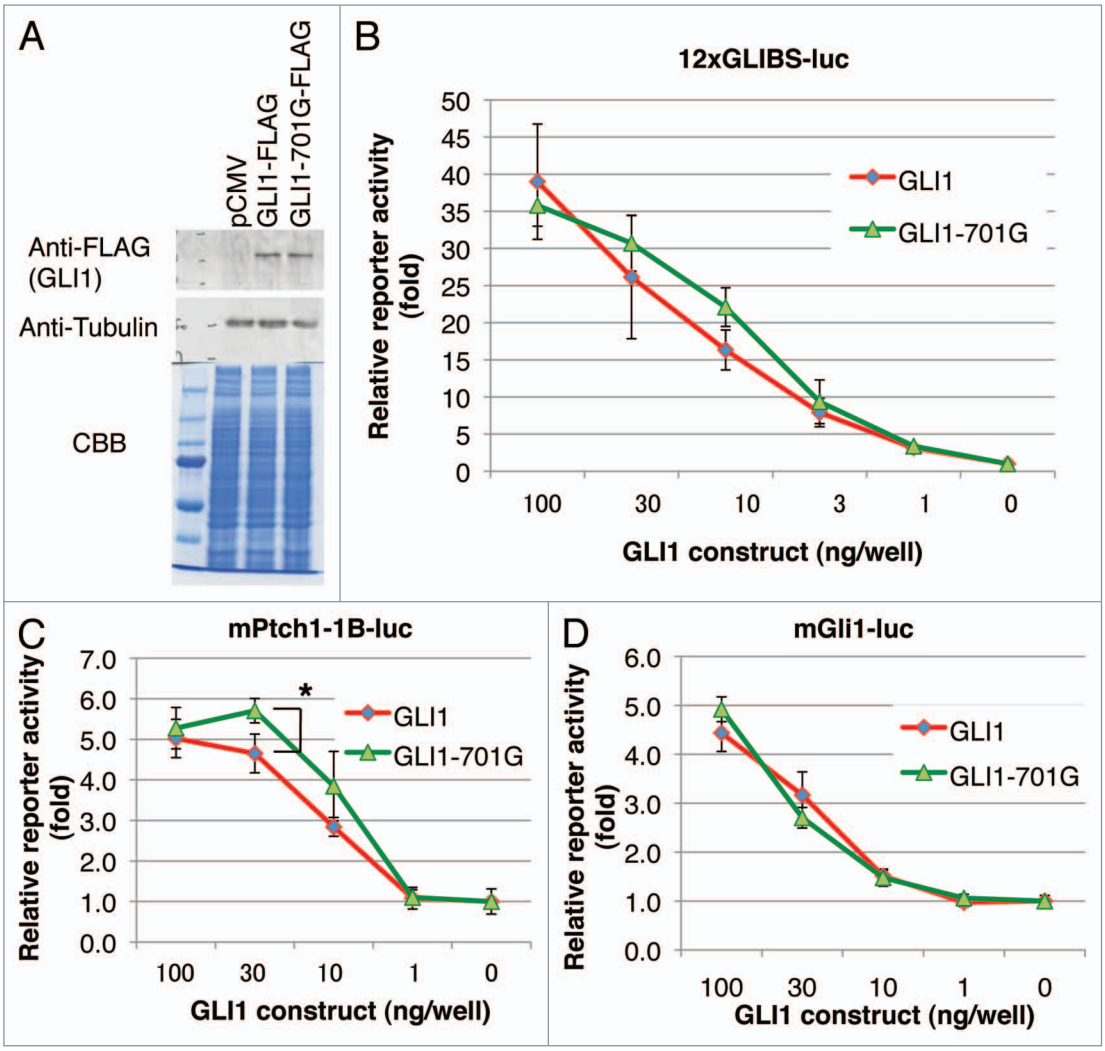

Figure 4A–D. (**A–D**) Biochemical characterization of the RNA edited GLI1. (**A**) Protein expression following transfection of pCMV5, FLAG-tagged GLI1 and FLAG-tagged GLI1-701G into HEK293 cells. Western blotting was performed using an anti-FLAG antibody. α/β-Tubulin expression and coomassie brilliant blue staining (CBB) are also shown as loading controls. (**B–D**) Luciferase activity of the GLI1 and GLI1-701G constructs in C3H10/T1/2 cells. One to 100 ng of the GLI1 expression constructs were transfected with 200 ng of 12xGLIBS-luc (**B**), mPtch1 promoter-luc (**C**) or mGli1 promoter-luc (**D**). The error bars indicate the standard deviation, with the experiment done in triplicate.

in normal and tumor cells implies that this post-transcriptional modification may act as a "protective barrier" that has to be overcome in the process of tumorigenesis. Whether efforts to increase the frequency of GLI1 editing in tumors can have a therapeutic significance remains to be seen.

In summary, our findings demonstrate a novel regulatory mechanism that acts on the GLI1 oncogene, as summarized in the model of **Figure 7**. RNA editing of GLI1 alters its susceptibility to both the negative HH signaling regulator SUFU and the positive regulator Dyrk1a. This post-transcriptional modification of GLI1 may therefore affect the biological outcomes of the HH signal transduction cascade.

Materials and Methods

Validation of GLI1 RNA editing. cDNAs and genomic DNA was amplified by initial and then nested primers, designed within GLI1 exon 10, exon 11 and exon 12 (**Table S1**). Multiple tissue and cancer cell line cDNA panels from BD Biosciences were also used as a template. Each PCR reaction consisted of 1x standard *Taq* buffer, 0.2 mM for each dNTP, 1 μM forward primers, 1.0 μM reverse primer, 0.5 μl of *Taq* DNA polymerase (5 units/μl, New England Biolabs) and 1 ng of cDNA in a total volume of 25 μl. Twenty cycles with 20 sec at 95°C, 20 sec at 66°C and 1 min at 72°C were performed on a Perkin-Elmer thermocycler. For nested PCR, 0.5 μl of the initial amplification products was used. Thirty cycles with 20 sec at 95°C, 20 sec at 66°C and 45 sec at 72°C were performed on a Perkin-Elmer thermocycler. The PCR products from the cDNAs (698 bp) and the genomic DNA (1,291 bp) were isolated by agarose gel electrophoresis, and then purified products were sequence-verified.

Knockdown of ADAR1 and ADAR2. Predesigned siRNAs targeting human ADAR1 and ADAR2 (ADARB1)

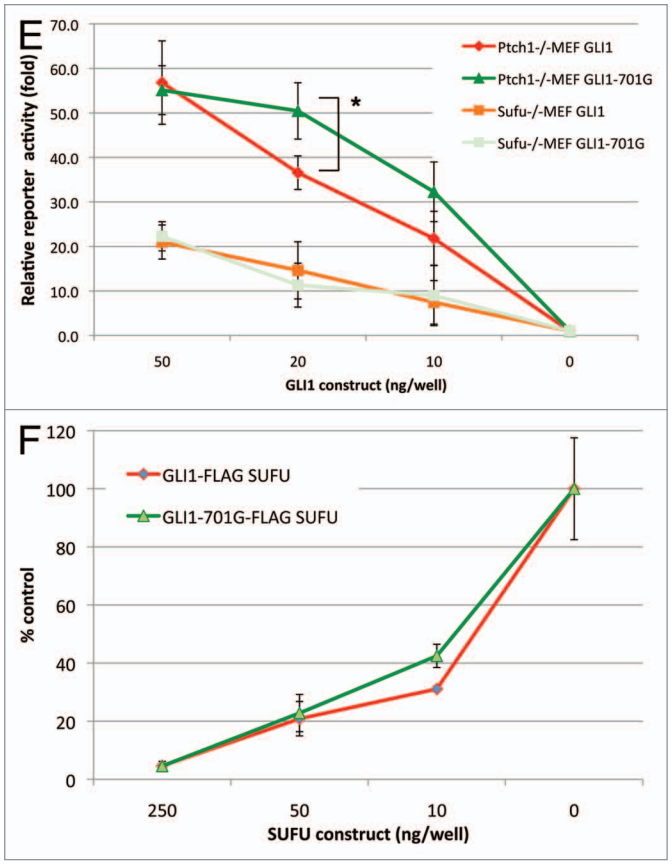

Figure 4E–F. (**E**) GLI activities in HH signaling active cell lines. The GLI1 or GLI1-701G expression constructs (10–50 ng) were transfected with 12xGLIBS-luc (200 ng) into Ptch1-/- MEFs or Sufu-/- MEFs. The error bars indicate the standard deviation, with the experiment replicated four times. (**F**) Inhibition of GLI1 activities by SUFU. The GLI1 or GLI1–701G expression constructs (50 ng) were transfected into C3H10T1/2 cells with a SUFU expression construct (10–250 ng) and 12xGLIBS-luc (200 ng), and the luciferase activity of the 12xGLIBS-luc reporter was measured. The control values represent GLI1 activities without SUFU. The error bars indicate the standard deviation, with the experiment done in duplicate. In the figure panels, the asterisks represent statistical significance calculated by the Welch's t-test ($p < 0.05$).

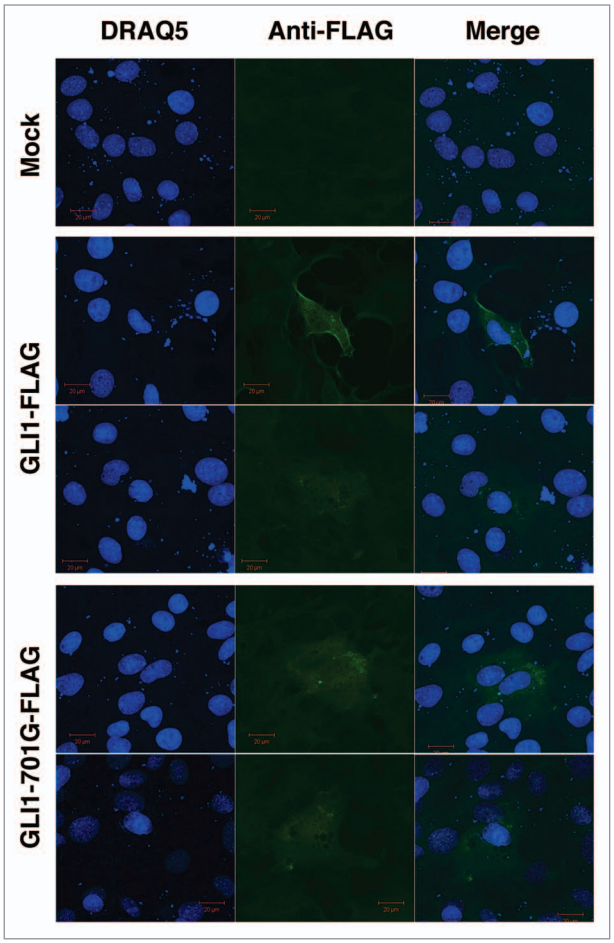

Figure 5. Subcellular localization of the RNA-edited GLI1. The FLAG-tagged GLI1-701G and GLI1 constructs were transfected into C3H10/T1/2 cells and expressed proteins (green signal) and the nuclei stained with the marker DRAQ5 (blue signal) were visualized by immunofluorescence microscopy. Note the similar subcellular localization in both the cytoplasm and nucleus of GLI1-701G and GLI1.

were purchased from Dharmacon (SiGenome SMART pools, Thermo Scientific). As controls, non-targeting siRNA pools from Dharmacon (SiGenome Non-Targeting siRNA control pools, Thermo Scientific) were used. Cells were plated in 6-well dishes at 50–60% confluency, and transfections were performed with Lipofectamine 2000 (Invitrogen) and 150 pmol siRNA. After 48 h, total RNA was prepared with the RNeasy kit (Qiagen) followed by cDNA synthesis with random (N6) primers (New England Biolabs) and Superscript II (Invitrogen). Real-time RT-PCR was performed with Power SYBR Green (Applied Biosystems) on a 7,500 fast real-time PCR system (Applied Biosystems) with the primers used shown in **Table S2**. mRNA expression levels were normalized against the housekeeping genes RPLPO and TBP, and the expression relative to the control siRNA sample, which is assigned to 1, is shown.

Subcloning of expression and reporter constructs. For construction of the 3'-FLAG-tagged GLI1-701G expression plasmid, we first performed a PCR amplification using the ThermoPol Reaction buffer (New England Biolabs) on a full-length 3'-FLAG-tagged human GLI1 expression plasmid in pCMV5, with a GLI1 primer (5'- CACTGAGAATGCTGC CATGGATGCTG) and a vector primer (5'- ACAAGGCTG GTGGGCACT). The PCR product and the FLAG-tagged GLI1 expression construct were digested with *SalI* and *BsmI* restriction enzymes and then ligated together. To make the mouse *Gli1*-luc reporter construct (mGli1-luc), the mouse *Gli1* promoter region was first cloned into the pGL4.21 vector (Promega) and then the -572 to +1,346 region was subcloned into the pGL3-basic reporter vector.

Cell culture and reporter assays. The human embryonic kidney cell line Hek293, the murine fibroblast cell lines NIH3T3 and C3H10T1/2 and the MEF cell lines with the negative regulators of HH signaling Ptch1 and Sufu genetically eliminated, *Ptch1*$^{-/-}$ [33] and *Sufu*$^{-/-}$ [34] MEFs were cultured as described before.[35,36] The human medulloblastoma cell line D283Med was cultured according to the ATCC-LGC Promochem

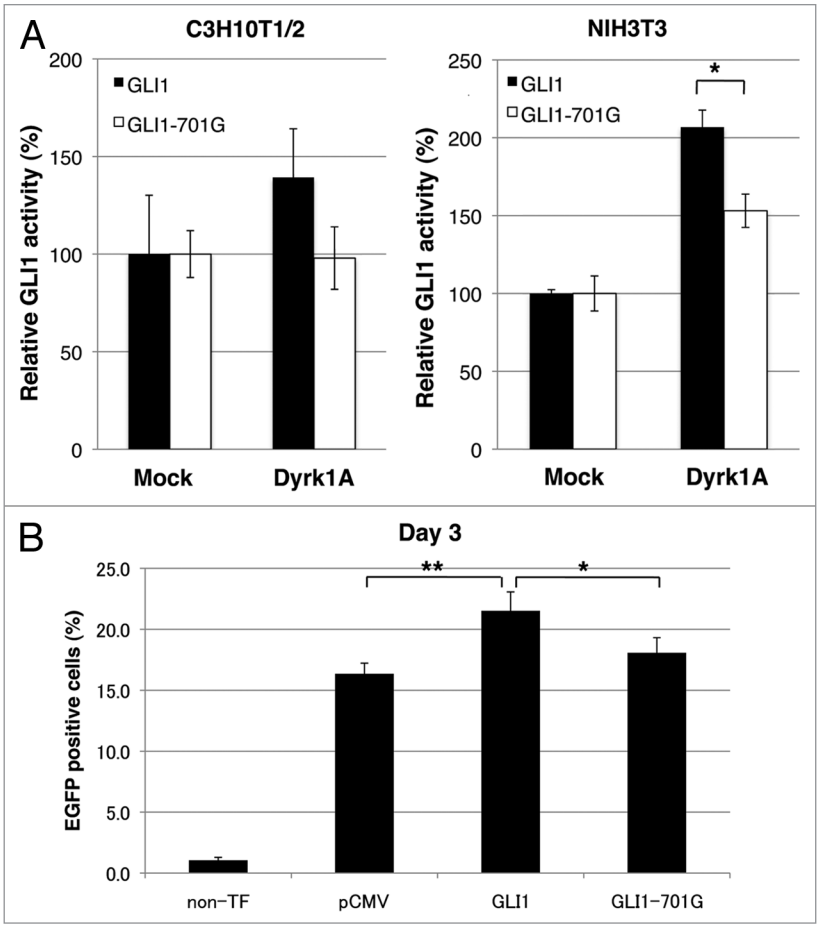

Figure 6. Biological effects of the RNA edited GLI1. (**A**) Regulation of GLI1 by Dyrk1a. The FLAG-tagged GLI1-701G or GLI1 expression constructs were co-transfected with a Dyrk1a expression construct into C3H10/T1/2 and NIH3T3 cells. The luciferase activity of the 12xGLIBS-luc reporter was measured. The error bars indicate the standard deviation, with the experiment done in triplicate. The asterisk represents statistical significance calculated by the Student's t-test ($p < 0.01$). (**B**) Effect of GLI1 on the proliferation of medulloblastoma cells. pEGFP (0.5 μg) vector and FLAG-tagged GLI1-701G or GLI1 expression constructs (1.5 μg) were transfected into the D283Med medulloblastoma cell line. After 3 d of incubation, EGFP-positive cells were measured by flow cytometry. The error bars indicate the standard deviation, with the experiment done in triplicate. The asterisks represent statistical significance calculated by the ANOVA Bonferroni's multiple t-test (*:$p < 0.05$, **:$p < 0.01$). Non-TF, non-transfected cells.

recommendation. GLI1 activity was measured by the dual-luciferase assay kit (Promega), as described before.[13] Expression constructs for GLI1,[13] SUFU[25,26] or Dyrk1a,[28] and the appropriate reporter construct, the 12xGLIBS-luc,[28] the mouse *Ptch1*-1B-luc (mPtch1-1B-luc)[13] or the mGli1-luc, together with the renilla luciferase pRL-SV construct, were transfected into cultured cells using FuGENE 6 (Roche Diagnostics). All experiments were performed at least three times.

Western blotting. Forty-eight hours after transfection, proteins were extracted by lysis buffer [50 mM Tris (pH 7.4), 1% SDS, 250 mM NaCl, 2 mM dithiothreitol, 0.5% Nonidet P-40, 1% phosphatase inhibitor mixture 1 (Sigma), and 1% mammalian protease inhibitor mixture (Sigma)]. After normalization based on protein concentration measured by the DC protein assay kit (Bio-Rad), samples were run on a SDS-acrylamide gel. Thereafter, proteins were transferred onto Hybond-ECL nitrocellulose membrane (GE Healthcare). GLI1 variants or α/β-tubulin as internal control were determined by the use of anti-FLAG monoclonal (Stratagene) or α/β-tubulin rabbit polyclonal antibodies (Cell Signaling Technology). Horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare) was used as the secondary antibody, followed by detection of these proteins with the Western Lightning western blot Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). The ImageJ software was used for quantitation.

Immunofluorescence microscopy. Transfected C3H/10T1/2 cells were plated into the Lab-tec chamber slide (Nalge Nunc International). The cells were fixed in 4% paraformaldehyde for 15 min and cold methanol for 10 min. After blocking, the cells using a solution of phosphate-buffered saline with 5% normal goat serum for 60 min, anti-FLAG M2 mouse monoclonal antibody (1:800, Sigma) in 0.3% Triton X-100/phosphate-buffered saline was added and incubated overnight at 4°C. Then the cells were washed and fluorescent-tagged secondary antibody, the Alexa Fluor 488-goat anti-mouse IgG (1:6,000, Invitrogen) was applied. Three phosphate-buffered saline washes (5 min) were also used after each treatment. The nuclei were stained with 5 μM DRAQ5 (Alexis Biochemicals). Slides were mounted

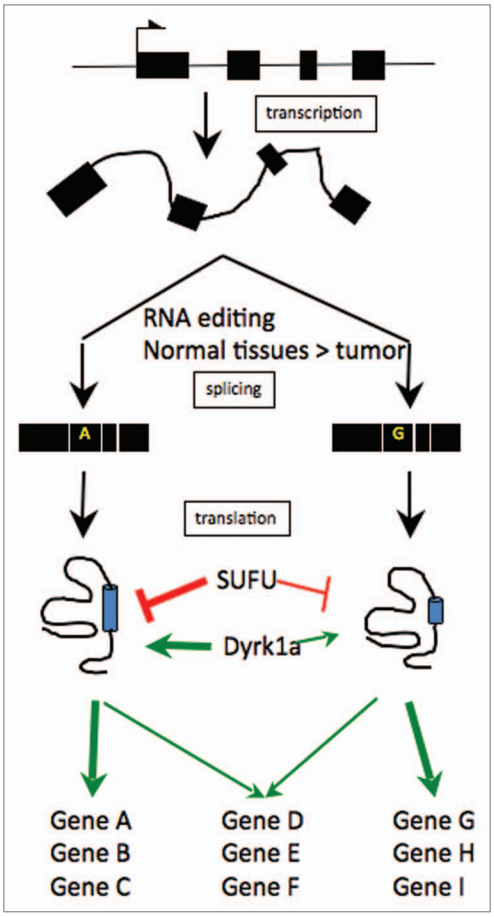

Figure 7. Model for modification of GLI1 transcriptional activity by RNA editing. GLI1 pre-mRNAs are transcribed from genomic DNA, edited by ADARs and then subjected to splicing. The edited and non-edited mature mRNAs are translated into the GLI1-701G and GLI1 proteins. The amino acid change elicited by RNA editing influences the secondary structure of GLI1. Consequently, the edited and non-edited GLI1 variants have different susceptibilities to the negative and positive regulators of HH signaling, SUFU and Dyrk1a. This may contribute to the targeting of a differential set of genes by the GLI1-701G and GLI1 transcription factors.

in FluorSave reagent (Carbiochem). Fluorescence images were collected using a LSM510 (Carl Zeiss) confocal laser-scanning microscope with a Plan APOCHROMAT 63/1.4 OilDIC objective lens.

Cell proliferation assay. D283Med cells were co-transfected with pEGFP and pCMV, FLAG-tagged GLI1 or FLAG-tagged GLI1-701G. After 3 d of incubation, the EGFP-positive cells were detected by flow cytometry. The assay was performed independently, with triplicate samples, twice.

Clinical samples. Skin biopsies were taken, after obtaining informed consent, from healthy individuals and from patients with basal cell carcinoma, at the Dermatology and Venerology Unit, Karolinska University Hospital. The clinical diagnosis was made by a dermatologist and was confirmed by histopathological evaluation. All studies were approved by the Regional Committees of Ethics. Isolation of total RNA was performed as described.[37]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental materials may be found here:
www.landesbioscience.com/journals/rnabiology/article/23343

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