

Loss of *SUFU* Function in Familial Multiple Meningioma

Mervi Aavikko,^{1,2,8} Song-Ping Li,^{1,8} Silva Saarinen,^{1,2,8} Pia Alhopuro,^{1,3} Eevi Kaasinen,^{1,2} Ekaterina Morgunova,⁴ Yilong Li,^{1,2} Kari Vesanen,^{1,2} Miriam J. Smith,⁵ D. Gareth R. Evans,⁵ Minna Pöyhönen,³ Anne Kiuru,⁶ Anssi Auvinen,⁷ Lauri A. Aaltonen,^{1,2} Jussi Taipale,^{1,4} and Pia Vahteristo^{1,2,*}

Meningiomas are the most common primary tumors of the CNS and account for up to 30% of all CNS tumors. An increased risk of meningiomas has been associated with certain tumor-susceptibility syndromes, especially neurofibromatosis type II, but no gene defects predisposing to isolated familial meningiomas have thus far been identified. Here, we report on a family of five meningioma-affected siblings, four of whom have multiple tumors. No *NF2* mutations were identified in the germline or tumors. We combined genome-wide linkage analysis and exome sequencing, and we identified in suppressor of fused homolog (*Drosophila*), *SUFU*, a c.367C>T (p.Arg123Cys) mutation segregating with the meningiomas in the family. The variation was not present in healthy controls, and all seven meningiomas analyzed displayed loss of the wild-type allele according to the classic two-hit model for tumor-suppressor genes. In silico modeling predicted the variant to affect the tertiary structure of the protein, and functional analyses showed that the activity of the altered *SUFU* was significantly reduced and therefore led to dysregulated hedgehog (Hh) signaling. *SUFU* is a known tumor-suppressor gene previously associated with childhood medulloblastoma predisposition. Our genetic and functional analyses indicate that germline mutations in *SUFU* also predispose to meningiomas, particularly to multiple meningiomas. It is possible that other genic mutations resulting in aberrant activation of the Hh pathway might underlie meningioma predisposition in families with an unknown etiology.

Meningiomas are the most common primary tumors of the CNS and account for more than one-third of all CNS tumors.^{1,2} Meningiomas originate from the arachnoidal cells of the leptomeninges, and slowly growing benign tumors comprise the great majority (95%) of them. Depending on the location, meningiomas can cause significant neurological deficits, but they can also be asymptomatic. Imaging and autopsy studies have shown that subclinical meningiomas occur in up to 3% of the population.^{3,4} Although meningiomas are most commonly observed as solitary sporadic tumors, 1%–5% are familial and fewer than 10% of individuals have multiple lesions.⁵ The environmental risk factors for meningiomas, except for ionizing radiation, are unclear.¹ An increased risk of meningiomas has been associated with tumor-susceptibility syndromes such as neurofibromatosis type II (*NF2* [MIM 101000]), Cowden syndrome (*CS* [MIM 601728]), and Werner syndrome (*WRN* [MIM 277700]), which are caused by mutations in *NF2* (MIM 607379), *PTEN* (MIM 601728), and *RECQL2* (MIM 604611), respectively. Also, a germline *SMARCB1* (MIM 601607) mutation, causing predisposition to schwannomatosis and rhabdoid tumors, was recently identified in a family affected by multiple cranial and spinal meningiomas and schwannomas.⁶ Gene defects predisposing merely to meningiomas have not been previously reported.

Here, we report on a family of five meningioma-affected siblings, four of whom were diagnosed with multiple

tumors. We utilized genome-wide linkage analysis and exome sequencing to identify the genetic predisposition in this family. The index case (III-4, Figure 1) was referred to genetic counseling after being operated on for meningiomas at different sites at the ages of 44, 48, and 59 years. Multiple meningiomas had also been removed from his three sisters: III-3 at the ages of 43 (single) and 54 (multiple) years, III-2 at the ages of 58 (single) and 64 (multiple) years, and III-1 at the age of 72 years (multiple). His fourth sister (III-5) had been operated on for a single meningioma when she was 60 years old. In addition to meningiomas, a benign myoma had been removed from III-3 at the age of 42, and III-5 was diagnosed with immature teratoma in both ovaries when she was 16 years old. Other siblings in the family had been diagnosed with lymphoma, colon adenocarcinoma, squamous cell carcinoma of the facial skin (III-9), and facial basaloma (III-11). Their cousin's child (IV-1) had died of medulloblastoma at the age of 5 years. The father and mother of the siblings (II-1 and II-2) had died of chronic lymphocytic leukemia at the age of 58 years and of coronary thrombosis at the age of 57 years, respectively. They had not been examined for meningiomas. The index case (III-4) was screened at the clinic for *NF2* mutations by direct sequencing and multiplex ligation-dependent probe amplification, but no mutations were detected. We also confirmed with array comparative genomic hybridization (CGH) [Human Genome CGH Microarray 105A platform, Agilent, Santa

¹Genome-Scale Biology Research Program, University of Helsinki, 00014 Helsinki, Finland; ²Department of Medical Genetics, University of Helsinki, 00014 Helsinki, Finland; ³Department of Clinical Genetics, Helsinki University Central Hospital, 00029 Helsinki, Finland; ⁴Department of Biosciences and Nutrition, Science for Life Center, Karolinska Institutet, 14183 Stockholm, Sweden; ⁵Department of Genetic Medicine, St. Mary's Hospital, Manchester Academic Health Sciences Centre, University of Manchester, Manchester M13 9WL, UK; ⁶Department of Research and Environmental Surveillance, Radiation and Nuclear Safety Authority, 00881 Helsinki, Finland; ⁷Tampere School of Public Health, University of Tampere, 33014 Tampere, Finland

⁸These authors contributed equally to this work

*Correspondence: pia.vahteristo@helsinki.fi

<http://dx.doi.org/10.1016/j.ajhg.2012.07.015>. ©2012 by The American Society of Human Genetics. All rights reserved.

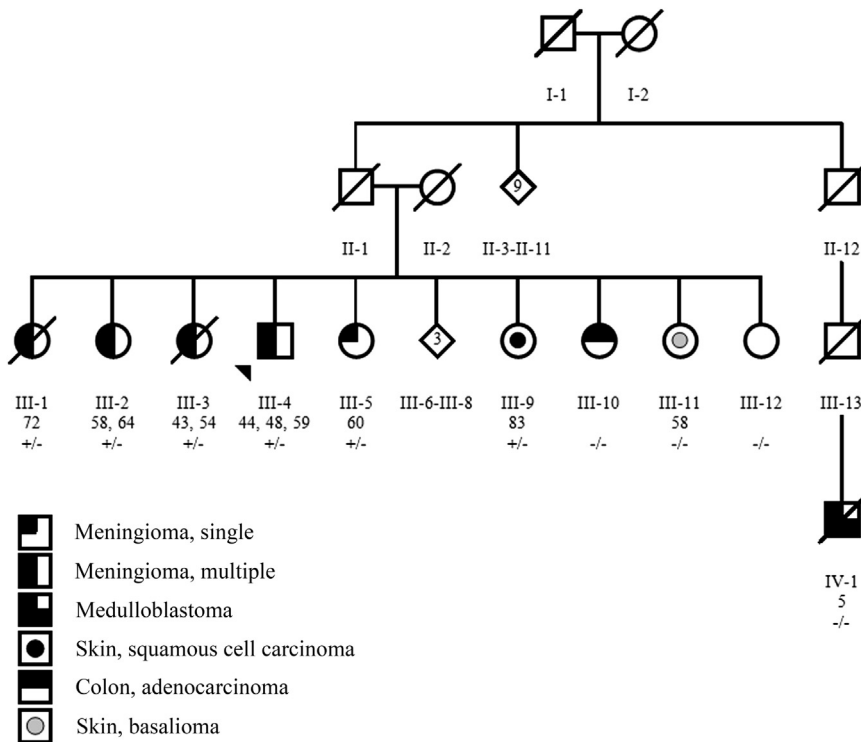


Figure 1. Pedigree of Family 1 with Five Siblings with Intracranial Meningiomas
The ages at meningioma operations and the c.367C>T (p.Arg123Cys) *SUFU* mutation status of the individuals are shown. A plus sign denotes a mutation, and a minus sign denotes a wild-type allele. The pedigree has been slightly modified for confidentiality.

unknown, and the sibling with no meningioma was marked as unaffected (Figure 1). In total, 174 chromosomal regions (1.3 Gb all together) with positive LOD scores were identified (Table S1, available online).

To study the protein-coding variants residing in the linked regions, we used the SureSelect Human All Exon Kit (v.1) (Agilent) to capture the germline exomes from III-1, III-2, and III-4. Paired-end short read sequences covering ~38 Mb of the human genome were sequenced with Illumina Genome Analyzer II at

Clara, CA) and transcriptome analysis (GeneChip Human Exon 1.0 ST Array, Affymetrix, Santa Clara, CA) that the affected family members did not carry any larger chromosomal alterations at the *NF2* locus and that their *NF2* expression was not altered (fold change = 0.99). The seven available tumor samples from the affected individuals were screened by direct sequencing for somatic *NF2* mutations, but none were identified.

Samples were obtained from four affected (III-1, III-2, III-4, and III-5) and four other (III-10, a child of III-1, a child of III-3, and a child of III-5) family members from Family 1 (Figure 1) after written informed consent was acquired. Genomic DNAs were extracted from blood and were genotyped with Illumina's Human610-Quad DNA analysis BeadChips (Illumina, San Diego, CA). The hybridization and scanning were performed according to the manufacturer's protocol at the Finnish Institute for Molecular Medicine (FIMM) Genome and Technology Centre. Genotype calling and quality analyses were done in BeadStudio (Illumina), and genotypes with a GenCall score less than 0.15 were excluded as unreliable.

Nonparametric linkage analysis was performed with Merlin⁷ (v.1.1.2) and for X chromosome with MINX. Unlikely genotypes calculated with pedwipe in MERLIN were removed before the analyses. Allele frequencies were obtained from genotypes from members of family 1 and 265 healthy Finnish controls provided by the Nordic Center of Excellence in Disease Genetics consortium. In the analysis, the family members with multiple meningiomas (III-1, III-2, and III-4) were marked as affected, the sibling with a single meningioma (III-5) and the three children (children of III-1, III-3, and III-5) were marked as

FIMM Genome Technology Centre. NextGENe (v.2.1) software (Softgenetics, State College, PA) was used for removing bad-quality reads, for mapping the reads to human reference genome version NCBI37/Hg19, and for calling the variants. All synonymous variants, known polymorphisms obtained from dbSNP (v.132), variants present in 78 control exomes available from other projects in the laboratory, and variants residing outside the linked regions were excluded from further analyses.

After the exome data were combined with the linkage data, only 20 candidate variants remained (Table S2). These were further directly sequenced in all affected individuals in family 1, and seven were shown to segregate with the meningiomas in the family (Table S2). All seven variants were then sequenced in a set of 188 Finnish blood donor samples obtained through the Finnish Blood Transfusion Service. Three of the variants were found in the controls with a low frequency, and two were present in the screened unaffected siblings, from whom we had available DNA (Table S3).

The two remaining variants were heterozygous missense mutations c.1990A>G (p.Ser664Pro) (rs202247760) in amino adipate-semialdehyde synthase (*AASS* [MIM 605113; RefSeq accession number NM_005763.3]) and c.367C>T (p.Arg123Cys) (rs202247756) in suppressor of fused homolog (*Drosophila*), *SUFU* (MIM 607035; RefSeq NM_016169.3). In silico analyses with PolyPhen2⁸ and SIFT⁹ predicted only the *SUFU* mutation to be damaging. Both variants were sequenced in the seven available formalin-fixed paraffin-embedded (FFPE) meningiomas (one from III-1 and III-5 each, two from III-3, and three from III-4) from the members of family 1. Loss of

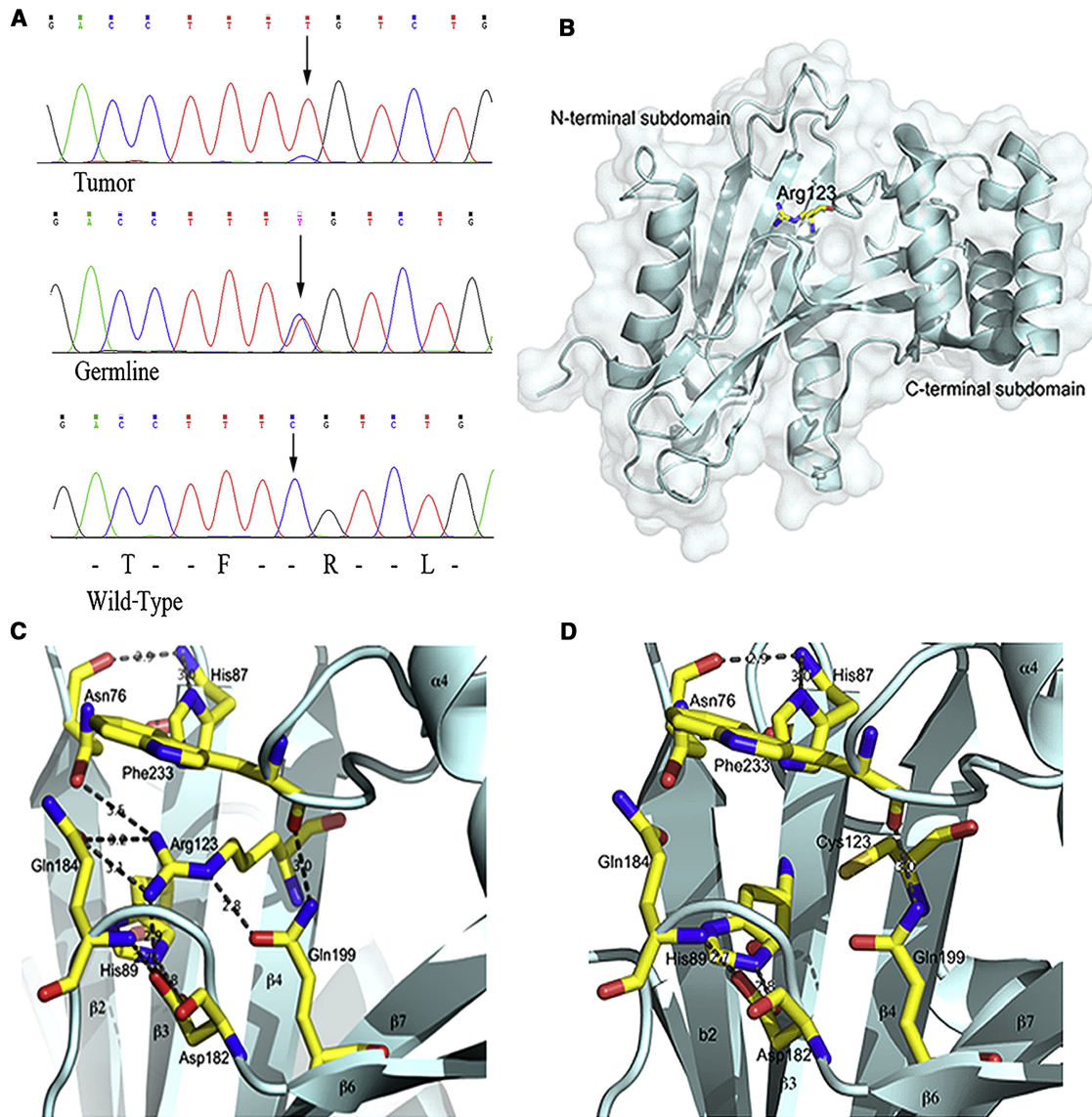


Figure 2. Loss of Heterozygosity and In Silico Modeling of the *SUFU* Mutation

(A) Heterozygous germline *SUFU* mutation c.367C>T (p.Arg123Cys) and loss of heterozygosity in a meningioma tumor tissue. The site of the mutation is indicated by an arrow.

(B–D) Surface representation of the N-terminal part of *SUFU* modeled in COOT.¹²

(B) Cartoon of the secondary structure. The N- and C-terminal subdomains are marked, and Arg123 is represented by a ball-and-stick model.

(C) Ribbon representation of the wild-type N-terminal part of *SUFU*. Secondary structure elements are labeled in accordance with Merchant et al.¹¹ Arg123 and residues involved in interactions are represented by ball-and-stick models. Dashed lines represent hydrogen bonds and ionic contacts.

(D) The same orientation for the p.Arg123Cys altered *SUFU*. All contacts formed by Arg123 disappear, and loops are connected rather weakly.

heterozygosity (LOH) was detected at the site of the *SUFU* mutation (Figure 2A) in all the tumors, whereas the AASS variant site remained heterozygous in all of the seven tumors. Because *SUFU* mutations are found in a fraction of familial medulloblastomas, we screened the c.367C>T mutation in a distant relative (IV-1, Figure 1) who had died of medulloblastoma at 5 years of age. We utilized DNA extracted from two different FFPE samples and performed multiple sequencing reactions, but no mutations were detected. *SUFU* mutations have also been observed

in a subset of skin tumors, and we acquired the FFPE blocks of the facial-skin lesions from the two siblings (III-9 and III-11). The sister with basaliooma (III-11) did not harbor the mutation, whereas the other sister with facial-skin squamous cell carcinoma (III-9) did, but no LOH was observed in the lesion.

To elucidate the properties of the p.Arg123Cys altered *SUFU*, we modeled it with the program COOT¹² (Figure 2B–2D). Arg123 resides inside the core of the N-terminal subdomain of *SUFU* (Protein Data Bank entry 1M1L)¹³

(Figure 2B), and it forms a very intensive network of hydrogen bonds and ionic interactions with His89, Asp182, Gln184, and Gln199 (Figure 2C). These residues are central in the loop structures of the N-terminal subdomain of *SUFU*. An alteration in Arg123 might increase the flexibility of the loops and affect the formation of proper tertiary structure of the protein (Figure 2D).

SUFU is the major negative regulator of the Hh-signaling pathway in humans.^{10,11,14} The pathway is essential in embryogenesis, and in adult tissues, it is involved in the control of stem cell proliferation.¹⁵ Its activation has also been linked to the development of medulloblastomas, glioblastomas, and some basal cell carcinomas.¹⁵ The effect of *SUFU* on Hh signaling is mainly mediated by its binding to GLI1, GLI2, and GLI3 transcription factors. *SUFU* has also been shown to prevent the nuclear transportation of GLIs,^{16,17} as well as to promote their processing.

To study the effect of the observed *SUFU* variant on GLI function in vitro, we generated the c.367C>T mutation in a human *SUFU* cDNA construct cloned in a pCMV5_Myc backbone vector (Clontech, Mountain View, CA) by utilizing the QuikChange Site-Directed mutagenesis kit (Agilent). We directly sequenced the mutated clones to ensure that only the correct mutation was created. The primers used in the site-directed mutagenesis are listed in Table S3. FuGENE HD (Roche, Basel, Switzerland) was used for transfecting human rhabdomyosarcoma cells (CRL-2061) with Myc-tagged wild-type or mutant *SUFU* constructs, an Hh-pathway-specific reporter plasmid "SASAKI COOPER,"¹⁸ and a control reporter *Renilla* luciferase phRL-TK¹⁹ (Promega, Fitchburg, WI). CRL-2061 cells are known to have constitutively activated Hh signaling as a result of a 30-fold amplification of *GLI1*.²⁰ Cells were incubated for 2–3 days before the luciferase activity was measured with the Dual-Reporter Luciferase system (Promega). The luminescence was detected by the Digene DCR-1 luminometer (Digene Diagnostics/QIAGEN, Hilden, Germany). The assays were conducted with and without ShhN-conditioned medium in duplicate and were repeated at least three times. We calculated the relative luciferase activity by dividing the firefly luciferase counts by the *Renilla* luciferase counts. The same experiments were also conducted in *Sufu*-deficient (*Sufu*^{-/-}) mouse embryonic fibroblasts (MEFs), and the results from both cell lines indicated that compared with the wild-type transfected cells, the altered *SUFU* had significantly reduced ability to suppress GLI1 activity (Figure 3A and 3B).

To study whether this was due to reduced binding of altered *SUFU* to GLI, we studied the interaction of *SUFU* and endogenous GLI1 by pull-down immunoblot analysis in CRL-2061 cells. The cells were lysed and clarified by centrifugation, and the supernatant was removed and incubated with anti-GLI1 (H-300) agarose (Santa Cruz, Santa Cruz, CA). The agarose beads were washed three times with the lysis buffer without protease inhibitors before the SDS sample buffer was added. The samples

were resolved on SDS-PAGE gels, transferred to polyvinylidene-fluoride membranes, and incubated with the following antibodies: goat anti-Myc HRP (ab1261, Abcam, Cambridge, UK) and rabbit anti-Gli1 (ab92611, Abcam). Goat anti-rabbit HRP was used as a secondary antibody. The protein level of the p.Arg123Cys altered *SUFU* was clearly reduced, and the altered protein bound less GLI1 than did the wild-type *SUFU* (Figure 3C).

To assess the effect of the p.Arg123Cys altered *SUFU* on the subcellular localization of GLI1, we seeded *Sufu*^{-/-} MEFs on human-fibronectin-coated glass coverslips (BD, Franklin Lakes, NJ) and transfected them with the Myc-tagged wild-type and c.367C>T mutant *SUFU* expression plasmids. Cells were incubated for 2 days in low-serum medium with and without ShhN-conditioned medium (Figure S1) and were stained as described previously.¹⁰ Specific anti-Myc, anti-Gli1 (ab49314, Abcam), Alexa-594-conjugated anti-mouse, and Alexa-488-conjugated anti-rabbit (Molecular Probes/Life Technologies, Carlsbad, CA) antibodies were used for staining. This showed that the altered *SUFU* was unable to relocalize GLI1 into the cytoplasm (Figure 3D). Taken together, the functional experiments indicate that the c.367C>T (p.Arg123Cys) mutation in *SUFU* causes a partial loss of its repressor activity and leads to aberrant activation of the Hh-signaling pathway.

After identification of the *SUFU* c.367C>T (p.Arg123Cys) mutation in family 1, the coding exons and exon-intron boundaries of *SUFU* were sequenced in the DNA samples from a total of 162 meningioma-affected individuals (four Finnish individuals who had multiple meningiomas and from whom *NF2* mutations had been screened but not identified, 77 Finnish simplex cases who had solitary meningiomas and who were enrolled in the Interphone case-control study,²¹ 40 UK individuals with multiple meningiomas,²² and 41 FFPE meningiomas collected through the Finnish Cancer Registry [FCR] [Table 1]). Informed consent was obtained from the Interphone study subjects and from the four Finnish individuals with multiple meningiomas. The use of 41 FCR-retrieved FFPE samples was approved by the National Supervisory Authority for Welfare and Health. The study was approved by the Ministry of Social Affairs and Health in Finland, and the usage of the samples was approved by the local ethics review committees in Finland and the UK.

The primers used for blood and FFPE-tissue-derived DNAs were designed with Primer3 (Table S3). Sequences were analyzed manually and with Mutation Surveyor software v.3.30 (SoftGenetics) with an NCBI reference sequence (RefSeq NM_016169.3).

In total, 162 individuals with meningioma were screened for additional *SUFU* mutations, but no pathogenic variants were detected (Table S4). Of note, only 11 of the cases had a confirmed family history of the disease. Furthermore, in family 1, four of the affected individuals had been diagnosed with multiple tumors, which are usually seen in fewer than 10% of the cases. The absence

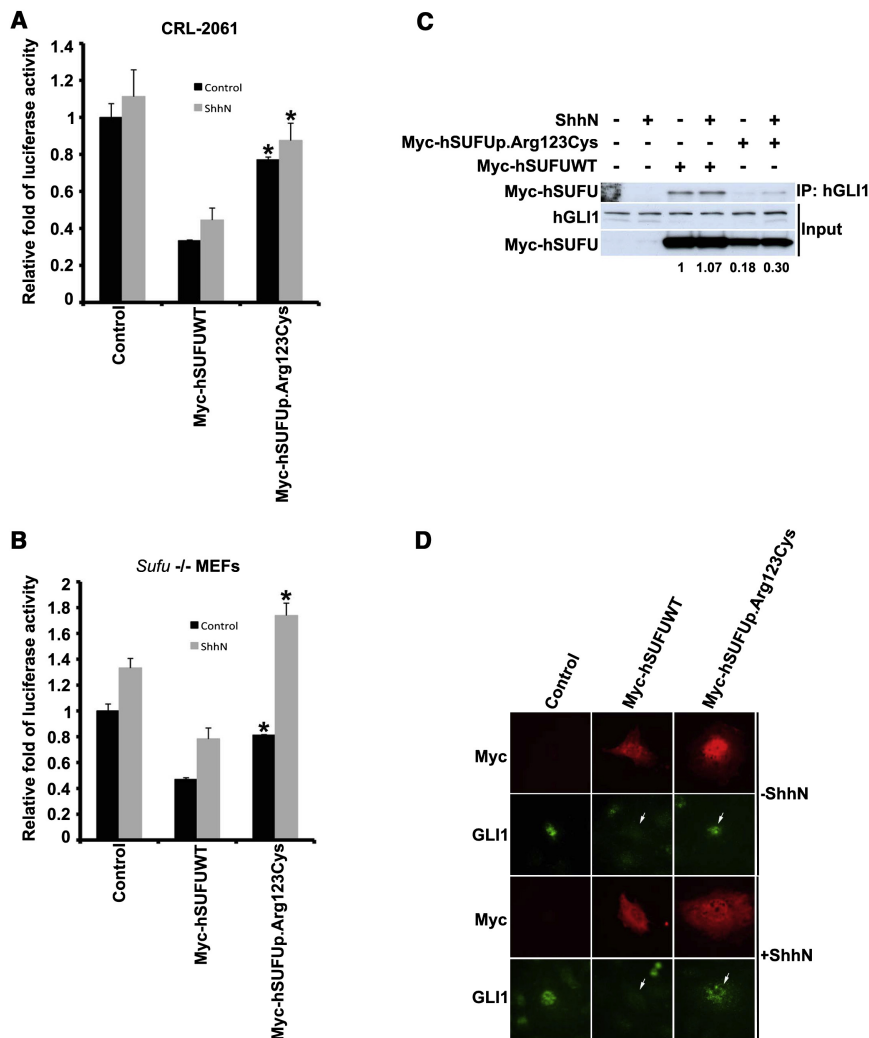


Figure 3. The Effect of the p.Arg123Cys Altered SUFU on Hedgehog Signaling Activity

(A and B) The p.Arg123Cys altered SUFU has decreased activity. Human rhabdomyosarcoma cells (CRL-2061) and *Sufu*-deficient (*Sufu*^{-/-}) MEFs were transfected with Myc-tagged wild-type (Myc-SUFUWT) or altered (Myc-SUFUp.Arg123Cys) SUFU constructs, an Hh-pathway-specific reporter, and a control reporter. The cells were incubated with and without ShhN-conditioned medium, and luciferase activity was measured. Note that Myc-SUFU suppresses Hh-pathway activity in both human and mouse cell lines. **p* < 0.05 compared with Myc-hSUFUWT (statistical analysis was done with a *t* test). Error bars indicate one standard deviation (duplicate samples).

(C) The p.Arg123Cys altered SUFU binds less GLI1 than does the wild-type SUFU. CRL-2061 cell lysates used in luciferase reporter assay were subjected to pull-down immunoblot analysis (IP: GLI1; WB: Myc and GLI1). In the top panel, Myc-SUFU is pulled down with antibodies against GLI1. Input of GLI1 and Myc-tagged SUFU constructs is presented in the middle and bottom panels, respectively. Quantification of GLI1 binding relative to the Myc-SUFU protein level is shown below the panel (quantitative protein analysis was performed with the Single Dimensional Electrophoretic Gel Analysis program from the ImageJ software package v.1.46g).

(D) Subcellular localization of p.Arg123Cys altered SUFU in *Sufu*^{-/-} MEFs. Note that the Gli1 nuclear localization was not affected by the human SUFU mutant (Myc-SUFUp.Arg123Cys) compared with the wild-type SUFU (Myc-SUFUWT), as indicated by arrows.

of additional mutation-positive individuals might indicate the very specific nature of the mutation—which provides a “just right” level of SUFU dysfunction—and the resulting small mutational target.

Mutations in *SUFU* predispose to childhood medulloblastomas, and somatic mutations have been observed in medulloblastomas, as well as in a small subset of basal cell skin cancers.²³ A splice-site mutation has also been reported in a family affected by basal cell nevus syndrome (BCNS [MIM 109400]) without the presence of medulloblastomas.²⁴ The germline *SUFU* mutations have been predominantly truncating, and the tumors have typically shown loss of the wild-type allele, indicating that *SUFU* is a tumor suppressor. This notion has been validated in knockout mice experiments.^{14,25} In contrast to the previously reported truncating mutations, the mutation identified in the meningioma-affected family is a missense change converting arginine to cysteine. Functional analyses indicate that the activity of the altered protein is significantly reduced but not completely lost. Because SUFU is known to regulate Hh

signaling in a concentration-dependent manner,¹⁶ we hypothesize that the remaining activity of the altered protein might explain the phenotypic difference between aggressive childhood medulloblastomas and meningiomas. This, however, remains to be validated in further studies.

SUFU is located in chromosomal region 10q24.32. The long arm of chromosome 10 is frequently deleted in meningiomas, especially in tumors with anaplastic, atypical, and malignant morphology. This deleted region contains various known tumor-suppressor genes, among which are *PTEN* and *SUFU*. Boström et al. (1998) studied chromosome arm 10q in glioblastomas and meningiomas and found *PTEN* to be mutated and deleted in a subset of gliomas but not in meningiomas, suggesting that *PTEN* is not the major tumor-suppressor gene at 10q in meningiomas.²⁶ Of note, Laurendeau et al. (2010) showed that 23 of 32 (72%) genes in the Hh-signaling pathway were differentially expressed in human meningiomas compared to normal tissue, suggesting an important role for Hh-pathway activation in meningiomas.²⁷ A germline

Table 1. Meningioma Cases and Healthy Controls Used in the Study

Description of the Cases and Controls	Number of Cases	Family History of Meningiomas	Lymphocyte DNA	FFPE Tumor-Tissue DNA
Family 1, meningioma cases	5	yes	4	7
Family 1, other family members	11	yes	11	2
Finnish meningioma cases recruited from the Interphone study ^a	77	not determined	77	0
Finnish multiple-meningioma cases ^b	4	no	4	0
UK multiple-meningioma cases ^b	35	no	35	0
Familial ^c UK meningioma cases	5	yes	5	0
Healthy blood donors	181	not determined	181	0
Meningioma Cases Identified from the Finnish Cancer Registry				
Familial ^c Finnish meningioma cases	6	yes	1	5
Putatively familial meningioma cases ^d	20	possibly	0	20
Meningioma cases from the same geographical region as family 1	6	not determined	0	6
Early-onset (≤ 35 years) meningioma cases	9	not determined	0	9

The following abbreviation is used: FFPE, formalin-fixed paraffin-embedded.

^aINTERPHONE Study Group; Bethke et al.²¹

^bThese cases have been screened for *NF2* mutations, but no mutations were detected.

^cDefined as \geq two first-degree relatives with meningioma.

^dCollected on the basis of the family name at birth and birth municipality through a search into the Finnish Cancer Registry.

mutation in *PTCH1* (MIM 601309), another key player in Hh signaling, has also been reported in an individual with BCNS with multiple basal cell carcinoma and meningioma.²⁸

Meningiomas are common tumors, and a subset of them displays familial aggregation. In some familial cases, the genetic predisposition is caused by an inherited syndrome, and the individuals display other syndromic manifestations as well. In other familial cases, however, the predisposing factor remains unclear, particularly if additional clinical signs pointing toward a well-characterized syndrome are lacking. Here, we report on a germline *SUFU* c.367C>T (p.Arg123Cys) mutation segregating with the meningiomas in a family of five affected siblings. The mutation was absent from the healthy controls, and all available tumors from the family displayed clear loss of the wild-type allele. We also showed that the altered protein's ability to suppress Hh signaling was significantly reduced. This suggests that *SUFU* mutations predispose to meningiomas in addition to medulloblastomas. Additional mutations were not detected in other meningioma cases studied, and this intuitively interesting finding thus awaits confirmation in an independent material. It is also possible that other genic mutations resulting in an abnormal activation of the Hh pathway might underlie the meningioma susceptibility in some families with yet unknown etiology.

Supplemental Data

Supplemental Data include one figure and four tables and can be found with this article online at <http://www.cell.com/AJHG>.

Acknowledgments

This study was supported by the Academy of Finland (Center of Excellence in Cancer Genetics and grant 21290), the Cancer Society of Finland, and the Sigrid Juselius Foundation. We would like to thank all the individuals who participated in the study. We thank Riitta Herva and Hannu Tuominen for expertise in pathology and Sini Nieminen, Mairi Kuris, Iina Vuoristo, and Inga-Lill Svedberg for excellent technical assistance. We acknowledge the Finnish Institute for Molecular Medicine Genome and Technology Centre for service, the CSC-IT Center for Science for the allocation of computational resources, and the Nordic Center of Excellence in Disease Genetics for providing the Finnish control SNP data.

Received: March 28, 2012

Revised: May 14, 2012

Accepted: July 5, 2012

Published online: September 6, 2012

Web Resources

The URLs for data presented herein are as follows:

National Center for Biotechnology Information (NCBI) database, <http://www.ncbi.nlm.nih.gov/>

Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org>

PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>

Primer3, <http://frodo.wi.mit.edu/>

SIFT, <http://sift.jcvi.org/>

Accession Numbers

The dbSNP accession numbers for the sequences reported in this paper are available in Tables S2 and S4.

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