



## Mutations in Col4a1 Cause Perinatal Cerebral Hemorrhage and Porencephaly

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these somatic factors or other, unrelated layers of gene regulation.

The microRNA pathway and RNAi share an overall mechanistic framework. However, to date, DCR-1 is the only identified component shared between the two related small-RNA pathways. Inactivation by RNAi of each of the 90 RNAi candidates did not affect precursor microRNA processing (25), underscoring the lack of overlap between RNAi and the microRNA pathways at this step. We also examined phenotypes associated with defects in the heterochronic pathway controlled by the *let-7* microRNA (5). Mutations in *let-7* cause supernumerary (>16) seam cells (26). Of the 90 RNAi factors, inactivation of six genes, including *dcr-1*, caused an increased number of seam cells and, of those, only three (*dcr-1*, *pop-1*, and *kin-10*) also significantly enhanced the weak *let-7* protruding vulva phenotype (tables S3 and S5); furthermore, *pop-1* RNAi may cause cell fate transformations rather than microRNA defects. These findings indicate little molecular overlap between the new RNAi factors and factors required for the microRNA pathway.

Protein-protein interaction maps ("interactomes") can facilitate the identification of complex molecular networks. Interrogating the Worm Interactome map (WI5) (27) and our screening of four additional factors provided protein interaction data for 42 of the 90 RNAi factors, giving a total of 161 interactions (table S6). We then tested and found that 21 of these interactors not identified by the initial RNAi screen were nonetheless required for transgene silencing, thus supporting the validity of many of the interactions for the RNAi pathway (fig. S4). The interaction map provides a useful tool from which to postulate connections among the new RNAi factors that were not predicted a priori. For example, the interactome map links the NMD factor, SMG-2, with T25G3.3; the known RNAi factors, RSD-2 and RSD-6 (28); and a cleavage and polyadenylation component, F56A8.6 (CPF-2).

The new factors we have identified suggest new steps in dsRNA-triggered gene silencing, including nuclear import/export and downstream stages that use NMD and mRNA polyadenylation/cleavage factors. We also found a near-complete overlap among factors required for RNAi and those required for transgene silencing in somatic tissues. Further, we showed that many of these factors are required for silencing in the germ line, possibly contributing to the maintenance of germ line-soma distinctions, genome integrity, and protection from parasitic genetic elements. Overall, these findings provide a global view of how the machinery of RNAi is integrated into RNA-mediated cellular processes.

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## Supporting Online Material

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Materials and Methods

Figs. S1 to S4

Tables S1 to S6

References

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# Mutations in *Col4a1* Cause Perinatal Cerebral Hemorrhage and Porencephaly

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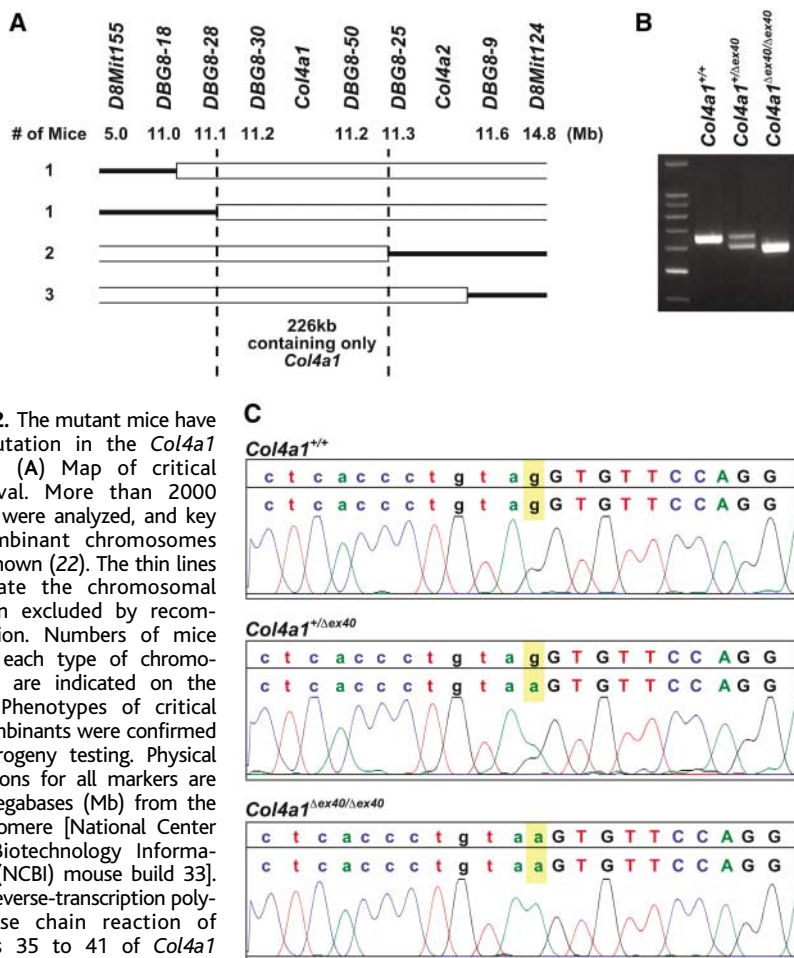
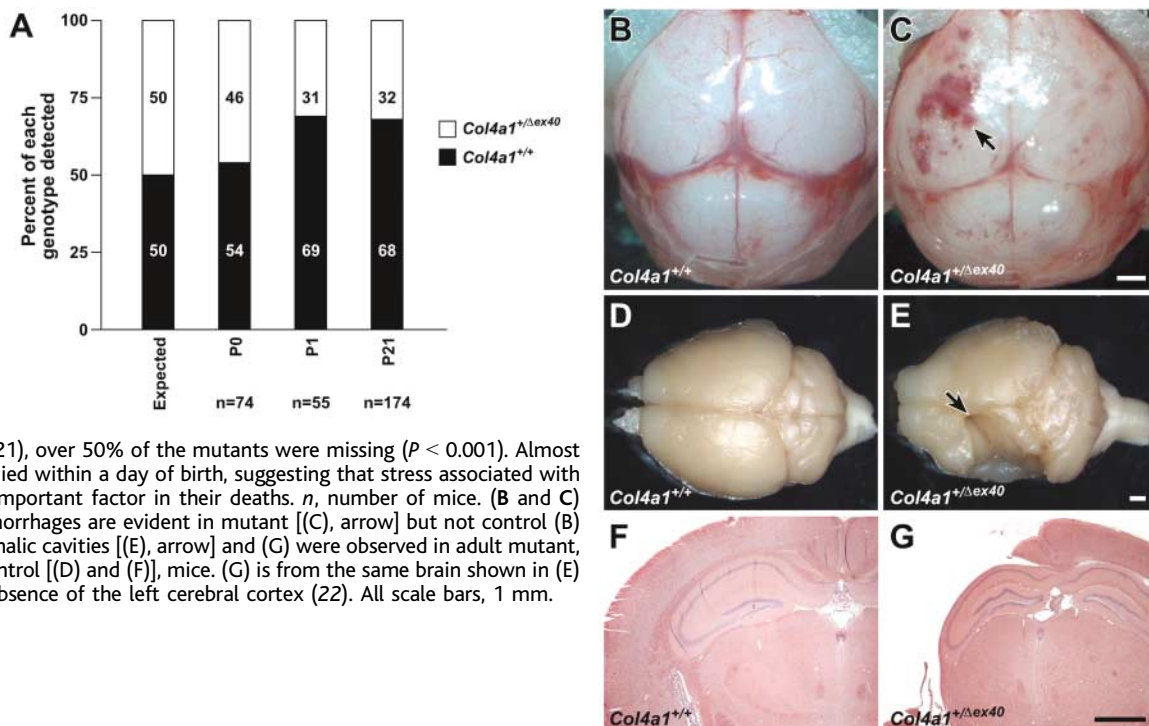
Porencephaly is a rare neurological disease, typically manifest in infants, which is characterized by the existence of degenerative cavities in the brain. To investigate the molecular pathogenesis of porencephaly, we studied a mouse mutant that develops porencephaly secondary to focal disruptions of vascular basement membranes. Half of the mutant mice died with cerebral hemorrhage within a day of birth, and ~18% of survivors had porencephaly. We show that vascular defects are caused by a semidominant mutation in the procollagen type IV  $\alpha 1$  gene (*Col4a1*) in mice, which inhibits the secretion of mutant and normal type IV collagen. We also show that *COL4A1* mutations segregate with porencephaly in human families. Because not all mutant mice develop porencephaly, we propose that *Col4a1* mutations conspire with environmental trauma in causing the disease.

Porencephaly [Online Mendelian Inheritance in Man (OMIM) record 175780] is a rare central nervous system disease usually diagnosed in infants. Type I or encephaloclastic porencephaly is characterized by cerebral white-matter lesions and degenerative cavities. Severe cases have drastic consequences, including profound disability and death. Infants who survive are often diagnosed with poor or absent speech development, epilepsy, hydrocephalus, seizures, mental retardation, and cerebral palsy. It has been suggested that porencephalic cavities in humans result from focal cerebral degeneration involving hemorrhages (1). Association studies

suggest that clotting-factor genes may contribute to genetic susceptibility by predisposing to thrombophilia (2). Despite these associations, the genetic and environmental etiology of familial cases is not established (3–10), and it seems reasonable that a distinct mechanism involving primary defects of vasculature could predispose to hemorrhage and porencephaly.

To advance the understanding of porencephaly, we have identified and characterized a new mouse mutant (generated by random mutagenesis) with severe perinatal cerebral hemorrhage. In addition to cerebral hemorrhage, mutant mice are smaller than control littermates

**Fig. 1.** Mutant mice have cerebral hemorrhage, reduced viability, and porencephaly. (A) Mutant mice (*Col4a1*<sup>+/ $\Delta$ ex40</sup>, see Fig. 2) have reduced perinatal viability. *Col4a1*<sup>+/ $\Delta$ ex40</sup> mice were bred to wild-type C57BL/6J mice, and the expected proportion of *Col4a1*<sup>+/ $\Delta$ ex40</sup> progeny was 50%. *Col4a1*<sup>+/ $\Delta$ ex40</sup> pups were present at the expected frequency on the day of birth (P0). By weaning age (P21), over 50% of the mutants were missing ( $P < 0.001$ ). Almost all of the missing pups died within a day of birth, suggesting that stress associated with parturition may be an important factor in their deaths. *n*, number of mice. (B and C) After birth, cerebral hemorrhages are evident in mutant [(C), arrow] but not control (B) pups. (D to G) Porencephalic cavities [(E), arrow] and (G) were observed in adult mutant, but not age-matched control [(D) and (F)], mice. (G) is from the same brain shown in (E) and demonstrates the absence of the left cerebral cortex (22). All scale bars, 1 mm.

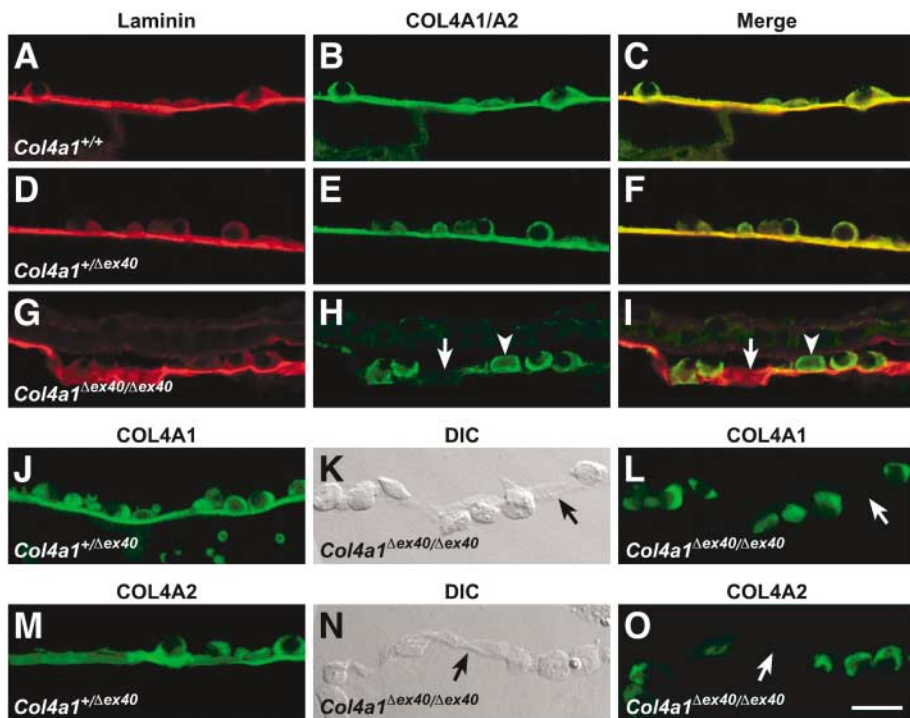


**Fig. 2.** The mutant mice have a mutation in the *Col4a1* gene. (A) Map of critical interval. More than 2000 mice were analyzed, and key recombinant chromosomes are shown (22). The thin lines indicate the chromosomal region excluded by recombination. Numbers of mice with each type of chromosome are indicated on the left. Phenotypes of critical recombinants were confirmed by progeny testing. Physical locations for all markers are in megabases (Mb) from the centromere [National Center for Biotechnology Information (NCBI) mouse build 33]. (B) Reverse-transcription polymerase chain reaction of exons 35 to 41 of *Col4a1* revealed a smaller amplicon (transcript) in mutant embryos as compared with controls. The mutant transcript lacks exon 40. (C) Sequence analysis of genomic DNA revealed a G-to-A transition in the splice acceptor site of exon 40. The intron sequence is shown in lowercase and the exon 40 sequence is shown in uppercase.

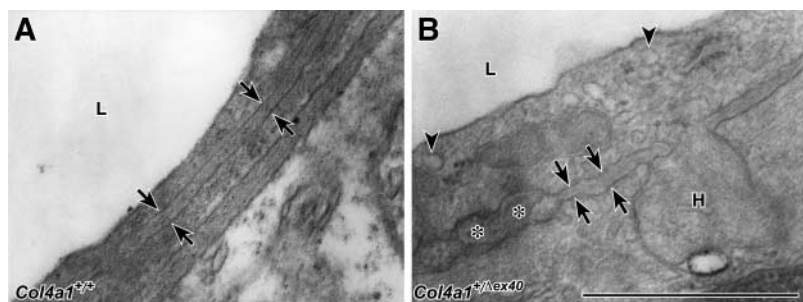
and can have multiple pleiotropic phenotypes (including ocular abnormalities, mild renal abnormalities, and reduced fertility) that appear to be influenced by genetic context (11). Homozygous mutant mice are not viable after mid-embryogenesis, and about 50% of heterozygous mice die within a day of birth (Fig. 1A). Reduced viability may be explained by variability in the severity and/or location of cerebral hemorrhages that are externally visible in most mutant pups. Detailed analysis of a subset of postnatal day 0 (P0) pups identified cerebral hemorrhages in 12 out of 12 mutant pups but in 0 out of 9 littermate controls (Fig. 1, B and C). About 18% of adult heterozygous mutant mice had obvious porencephalic lesions (6 out of 33) that were not observed in wild-type controls (0 out of 17, Fig. 1, D to G). We mapped the causative gene to a 226-kilobase region on chromosome 8 that contains

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**Fig. 3.** The COL4A1 mutant inhibits collagen secretion into the BMs. (A to C) *Col4a1*<sup>+/+</sup>, (D to F, J, and M) *Col4a1*<sup>+/Δex40</sup>, and (G to I, K, L, N, and O) *Col4a1*<sup>Δex40/Δex40</sup> mice are shown. In (A), (D), and (G), immunolabeling for laminin demonstrates the presence of RMs in embryos of each genotype (22). In (B) and (E), an antibody that recognizes both COL4A1 and COL4A2 shows the presence of these proteins in RMs of *Col4a1*<sup>+/+</sup> (B) and *Col4a1*<sup>+/Δex40</sup> (E) embryos, which is confirmed by colocalization with laminin, shown in (C) and (F), respectively. (G) to (I) show that in contrast, *Col4a1*<sup>Δex40/Δex40</sup> embryos do not have collagen staining in the RM (arrow) and instead show nonsecreted mutant protein in the parietal endoderm cells (arrowheads). In (J) and (M), specific monoclonal antibodies show that COL4A1 (J) and COL4A2 (M) are present in both the parietal endoderm cells and the RM of *Col4a1*<sup>+/Δex40</sup> embryos. (L) and (O) show that in contrast, no COL4A1 or COL4A2 is present in the RM of *Col4a1*<sup>Δex40/Δex40</sup> embryos (arrows). Differential interference contrast (DIC) images showing RMs are shown in (K) and (N). Scale bar, 0.2 μm.



**Fig. 4.** *Col4a1*<sup>+/Δex40</sup> mutant mice have structural defects in the cerebral vasculature. Electron micrographs of cerebral vessels of mice of the indicated genotypes are shown (22). (A) The vascular BMs of all analyzed *Col4a1*<sup>+/+</sup> mice had well-defined edges, uniform density, and consistent thickness (between arrows). (B) In contrast, the vascular BMs of *Col4a1*<sup>+/Δex40</sup> mice often had uneven edges, inconsistent density, and highly variable thickness (between arrows). Focal disruption (asterisks) and herniation (H) of BMs were specific to mutant mice and probably represent damage due to a weaker BM. Disruptions were observed in about 20% of vessels, whereas qualitative differences such as variable thickness and inconsistent and rough edges existed in all mutant vascular BMs analyzed. Mutant endothelial cells appear enlarged with an accumulation of vesicles, a characteristic of deficient secretion (arrowheads). Further analysis of phenotypes observed in noncerebral tissue reveals that BM defects are not restricted to the brain (11). L indicates vessel lumen. Scale bar, 0.5 μm.

a single gene encoding procollagen type IV α 1 (*Col4a1*) (Fig. 2A and fig. S1). It has previously been suggested that a large undefined

region including this locus contains a gene involved in cerebral hemorrhage in mice (12). Control and mutant embryos expressed *Col4a1*

transcripts of distinct size (Fig. 2B). In mutant transcripts (hereafter called *Col4a1*<sup>Δex40</sup>), exon 39 was spliced directly to exon 41 because of a mutation in the splice-acceptor site of exon 40 (Fig. 2C).

Type IV collagens are basement membrane (BM) proteins (13–15) that are expressed in all tissues, including the vasculature (16). COL4A1 and COL4A2 are the most abundant type IV collagens. COL4A1 and COL4A2 form heterotrimers with a 2:1 stoichiometry, respectively (17, 18). Assembly of the heterotrimers is initiated by the C-terminal non-collagenous domains, and the heterotrimer forms a triple helix along the length of the collagenous domains (fig. S2B). The skipped exon in *Col4a1*<sup>Δex40</sup> mice is in the triple helix-forming domain and codes for exactly 17 amino acids. One mechanistic hypothesis is that after normal heterotrimer initiation, the mutant proteins alter triple helix formation or structure and thus inhibit heterotrimer secretion into the BM. The triple helix domain consists of long stretches of Gly-Xaa-Yaa repeats (where Xaa and Yaa are amino acids), with frequent interruptions in the repeats. These interruptions are thought to be important for the flexibility of BM collagens (19–21). Because the skipped exon contains a repeat imperfection whose position is highly conserved across species (19, 20), an alternative hypothesis is that the mutant protein is assembled and secreted, but its structural properties in the vascular BMs are altered.

To distinguish between these hypotheses, we assessed the effect of the mutation on the secretion of COL4A1 and COL4A2 into BMs. Because *Col4a1*<sup>Δex40/Δex40</sup> embryos are not viable after mid-gestation, we assayed for COL4A1 and COL4A2 expression in the Reichert’s membrane (RM), a BM of embryonic origin) by immunolabeling embryonic day 9.5 (E9.5) embryos. *Col4a1*<sup>+/+</sup> embryos showed robust labeling of COL4A1 and COL4A2 in the RM (Fig. 3B). Similarly, both secreted and intracellular COL4A1 and COL4A2 were present in *Col4a1*<sup>+/Δex40</sup> embryos (Fig. 3, E, J, and M). In contrast, although the RM of *Col4a1*<sup>Δex40/Δex40</sup> embryos labeled robustly for laminin (Fig. 3G), there was no evidence of COL4A1/A2 heterotrimer secretion into this BM (Fig. 3, H, L, and O). Instead, the proteins appeared to accumulate within the parietal endoderm cells. These findings are consistent with the hypothesis that heterotrimers form but are not secreted (22). Similar to our results, a mutation of the nematode *Caenorhabditis elegans* ortholog of *Col4a1* (*emb-9*) results in the impaired secretion and intracellular accumulation of the products of both *emb-9* and the *Col4a2* ortholog *let-2* (23).

In further support for a dominant role of the mutant collagen in pathogenesis, mice heterozygous for null alleles of *Col4a1* and *Col4a2*

are normal (24). Presumably, these heterozygous mice produce fewer COL4A1/2 heterotrimers than do control mice, but all of the heterotrimers should have normal structure and be secreted. Although *Col4a1*<sup>+/ $\Delta$ ex40</sup> mice may secrete even less normal collagen [possibly only 25% (fig. S2)], the absence of a phenotype in mice with a null allele is consistent with a requirement for mutant proteins to induce disease. Analysis of mutations in the *C. elegans Col4a2* ortholog *let-2* supports this notion (25). Consistent with a potential pathogenic role of nonsecreted proteins, we observed swollen vascular endothelial cells with prominent vesicles in *Col4a1*<sup>+/ $\Delta$ ex40</sup> mice (Fig. 4).

COL4A1 and COL4A2 give strength to BMs (24, 26). Mouse embryos homozygous for null alleles of both *Col4a1* and *Col4a2* (24), or for null alleles of type IV collagen-processing enzymes (26, 27), die around mid-gestation with disrupted embryonic BMs. Similarly, BMs are weak in *C. elegans* type IV collagen mutants (23, 28). To investigate whether the inhibition of heterotrimer secretion in *Col4a1*<sup>+/ $\Delta$ ex40</sup> mice compromises the structural integrity of the vascular BMs, we assessed the BMs of cerebral vessels by electron microscopy. Compared with controls, *Col4a1*<sup>+/ $\Delta$ ex40</sup> mice had uneven BMs with inconsistent density and focal disruptions (Fig. 4). Although detailed studies are needed, we found that BMs in other tissues also were affected. However, the major site of hemorrhage was the brain. This may be explained by tissue-specific compositional differences in the vascular BMs or the vascular wall (16). Alternatively or additionally, the mutant BMs may greatly predispose to hemorrhage at times of stress. Substantial stress on the head during birth could explain the substantial cerebral hemorrhage.

To determine whether *COL4A1* mutations in humans also cause porencephaly, we assessed two families with autosomal dominant porencephaly (6, 10) for mutations in *COL4A1* (GenBank accession number 000013) on chromosome 13q34 (Fig. 5). The first family (6) has a Gly<sup>1236</sup> to Arg<sup>1236</sup> (G1236R) mutation that segregates with the porencephaly but was not present in 192 ethnically and geographically matched Dutch control chromosomes. The second family (10) has a Gly<sup>749</sup> to Ser<sup>749</sup> (G749S) mutation that also segregates with the phenotype but was not present in 192 ethnically and geographically matched Italian control chromosomes. Both mutations change conserved Gly residues within Gly-Xaa-Yaa repeats in the triple helix domain (fig. S2). Glycine has a single hydrogen-atom side chain, and there is little tolerance for amino acids with larger side chains that likely disrupt the triple helix during collagen assembly (29). Gly-to-Arg and Gly-to-Ser mutations have been shown to be pathogenic in the *C. elegans Col4a1* ortholog (23, 25) and in human *COL4A5*, respectively (30).

We have shown that mutations in *Col4a1* can lead to perinatal cerebral hemorrhage and can predispose to porencephaly. Because not all mice of a defined genetic background develop porencephaly, the *COL4A1* mutations in humans may conspire with environmental factors (for example, birth trauma) to cause the disease. In addition to having porencephaly, one adult patient from family 1 suffered from recurrent hemorrhagic strokes, which were caused by his weakened vessels (fig. S3). Thus, it is possible that mutations in *COL4A1* could contribute to hemorrhagic stroke in the absence of porencephaly. In

addition to *COL4A1*, alleles of *COL4A2* or other genes encoding BM or BM-associated proteins (including laminins, entactin/nidogen, perlecan, and integrins) also may be important predisposing factors for cerebral hemorrhage with or without porencephaly.

Our findings may have important implications for disease prevention in families with porencephaly resulting from vascular defects caused by mutations in *COL4A1* or other BM genes. For at-risk individuals, preemptive measures could be taken to reduce stress on the abnormal cerebral vessels that may in turn reduce the neurological deficits. For example, cesarean delivery of at-risk babies may increase the likelihood of survival and the possibility of a healthy life by decreasing the severity of cerebral hemorrhage and its sequelae.

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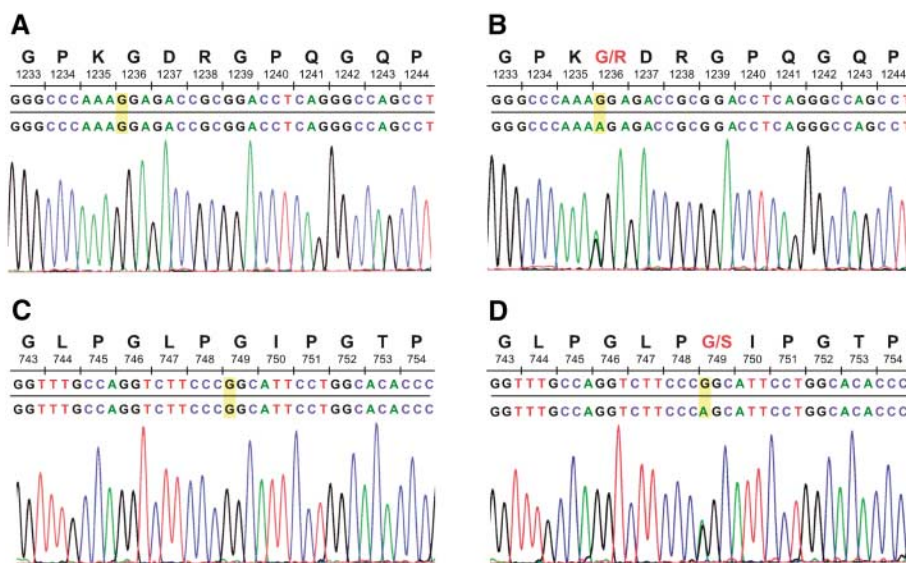


Fig. 5. *COL4A1* mutations in two human families with porencephaly. (A and C) Sequence chromatograms for unaffected members from family 1 and family 2, respectively. (B) Chromatogram for an affected patient from family 1, showing the G3706A transition mutation. (D) Chromatogram for an affected patient from family 2, showing the G2245A transition mutation.

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Table S1  
References and Notes

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# Clonal Dominance of Hematopoietic Stem Cells Triggered by Retroviral Gene Marking

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Gene marking with replication-defective retroviral vectors has been used for more than 20 years to track the *in vivo* fate of cell clones. We demonstrate that retroviral integrations themselves may trigger nonmalignant clonal expansion in murine long-term hematopoiesis. All 29 insertions recovered from clones dominating in serially transplanted recipients affected loci with an established or potential role in the self-renewal or survival of hematopoietic stem cells. Transcriptional dysregulation occurred in all 12 insertion sites analyzed. These findings have major implications for diagnostic gene marking and the discovery of genes regulating stem cell turnover.

Because of their unspecific insertion properties, replication-defective retroviral vectors represent unique tools for genetic marking studies, enabling the numbers and progeny of transplanted (stem) cells to be determined (1, 2). However, retrovirally marked cells can only be expected to behave normally if vector insertion itself does not confer a selective advantage or disadvantage.

Most marking studies published to date have used vectors based on murine leukemia virus (MLV), which is a simple gamma-retrovirus with strong enhancer-promoters in the long terminal repeats (LTRs). In replication-competent retroviruses, these sequences may trigger up-regulation of randomly hit proto-oncogenes (3, 4). Given the multistep nature of oncogenesis (5), the accumulation of several such events in single cells potentially results in malignant transformation (3). Retrovirally marked genes that have been repeatedly associated with experimentally induced murine tumors have been summarized in a database of ~300 common insertion sites (CISs), representing proposed or established cellular proto-oncogenes (4). Besides these,

there may be many other genes with the potential to influence stem cell kinetics when up-regulated or disrupted. The identification of such genes is of great interest for regenerative medicine, as exemplified by the powerful effect of *Hoxb4* on the self-renewal of hematopoietic stem cells (HSCs) (6).

In light of the recent demonstration that MLV vectors preferentially target the promoter regions of active genes (7), proto-oncogene activation might be more frequent (>1% of all insertions) than previously anticipated (8). However, in the numerous studies performed with retroviral vectors in human subjects, non-human primates, dogs, and mice (9), few cases have been identified in which malignant complications were caused by insertional mutagenesis. We previously observed an acute myeloid leukemia after insertion of a vector expressing an engineered low-affinity nerve growth factor receptor (dLNGFR) into the *Evi1* proto-oncogene (10). *Evi1* encodes a transcription factor with a role in both self-renewal and transformation of HSCs (11). Because dLNGFR was not associated with side effects in other studies (12), it remained unclear whether vector insertion into *Evi1* was sufficient for leukemogenesis in mice. Similarly, unusual lymphatic leukemias occurred after insertional up-regulation of the *LMO2* proto-oncogene by a vector expressing the interleukin-2 receptor common  $\gamma$ -chain, in a clinical trial performed to correct inborn severe combined immunodeficiency (13). We have also shown that leukemias reproducibly evolve in mice if vector insertions occur in independent proto-oncogenes of a single clone (14), consistent with the hypothesis that at

least two collaborating signal alterations are required for leukemogenesis (15).

To examine the effect of retroviral gene hits in normal HSCs, we analyzed cohorts of healthy mice in which a single or very few clones dominated hematopoiesis after serial bone marrow transplantation (BMT) (16, 17) (Fig. 1A). The vectors expressed either a signal-deficient variant of human CD34 (tCD34) or its full-length variant (fCD34); there was no evidence of a selective advantage related to transgene expression (16, 18). Using ligation-mediated polymerase chain reaction (LM-PCR), which introduces a bias for dominant clones (19, 20), we detected several clones contributing to hematopoiesis in each transplant recipient of the first cohort (after 28 weeks observation) (Fig. 1B). We mapped 22 different insertions in the six primary recipients of fCD34-marked cells (mice f1 to f6) and 19 insertions in the six primary recipients of tCD34-marked cells (mice t1 to t6) (Table 1 and table S1).

In the tCD34 primary cohort, we observed four insertions in CIS/proto-oncogenes (Table 1), three of which occurred in *Evi1* (Fig. 2A). We have seen a similar incidence of *Evi1* insertions in primary recipients after retroviral expression of the human multidrug resistance 1 (MDR1) cDNA (14). Such a recovery of independent hits in identical loci, or pathways, strongly suggested *in vivo* selection (4). Another hit was located between *Hoxb5* and *Hoxb4* (Fig. 2B), marking a clone that we could detect even in two independent primary recipients by locus-specific PCR (Table 1 and table S2).

In the fCD34 primary cohort, LM-PCR showed no insertions in CIS/proto-oncogenes and also fewer insertions within or close to signaling genes (Table 1 and table S1). Among the latter, *Map3k5* is highly related to *Map3k14* detected in primary tCD34 recipients, and both *Stxbp4* (fCD34) and *Igfbp4* (tCD34) map to insulin pathways. Even in this small set of dominant clones from primary fCD34 recipients, we found two hits belonging to the same growth factor pathway (*Vegfa*, *Shb*) (21). Vascular/endothelial growth factor encoded by *Vegfa* enhances HSC self-renewal (22), and again, the affected clone was detected in two independent primary recipients (Table 1).

To further determine the fate of marked clones, we examined 16 secondary recipients (11 for tCD34, 5 for fCD34) observed for 22 weeks after receiving bone marrow cells pooled from the primary recipients (Fig. 1A). This analysis thus focused on true HSCs, charac-

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