Lack of Cul4b, an E3 Ubiquitin Ligase Component, Leads to Embryonic Lethality and Abnormal Placental Development

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Abstract
Cullin-RING ligases (CRLs) complexes participate in the regulation of diverse cellular processes, including cell cycle progression, transcription, signal transduction and development. Serving as the scaffold protein, cullins are crucial for the assembly of ligase complexes, which recognize and target various substrates for proteosomal degradation. Mutations in human CUL4B, one of the eight members in cullin family, are one of the major causes of X-linked mental retardation. We here report the generation and characterization of Cul4b knockout mice, in which exons 3 to 5 were deleted. In contrast to the survival to adulthood of human hemizygous males with CUL4B null mutation, Cul4b null mouse embryos show severe developmental arrest and usually die before embryonic day 9.5 (E9.5). Accumulation of cyclin E, a CRL (CUL4B) substrate, was observed in Cul4b null embryos. Cul4b heterozygotes were recovered at a reduced ratio and exhibited a severe developmental delay. The placentas in Cul4b heterozygotes were disorganized and were impaired in vascularization, which may contribute to the developmental delay. As in human CUL4B heterozygotes, Cul4b null cells were selected against in Cul4b heterozygotes, leading to various degrees of skewed X-inactivation in different tissues. Together, our results showed that CUL4B is indispensable for embryonic development in the mouse.

Introduction
Cullin-RING ligases (CRLs) complexes comprise the largest known class of ubiquitin ligases [1]. CRLs regulate diverse cellular processes, including cell cycle progression, transcription, signal transduction and development [2]. CRLs are multisubunit complexes composed of a cullin, RING protein and substrate-recognition subunit, which was linked by an adaptor. Human cullin family consists of eight members, CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7 and PARC [3], among them, CUL4A and CUL4B have the highest degree of homology, with 83% identity in protein sequences [4]. There is only one ortholog, Cul4, in lower organisms. CUL4A CRL complexes contained Rbx1 and the adaptor protein DDB1. DDB1 interact with substrate recognition subunits, which determine the substrate specificity of the CUL4A CRL complexes [5,6,7,8,9]. The substrates of CUL4A CRL complexes include CDT1, p21, p27, p53, c-Jun, HOXA9, H3 and CHK1 that play important roles in cell cycle regulation, chromosome remodeling, and differentiation [10-11].

Compared to CUL4A, CUL4B is less studied, and so far very few substrates of CUL4B CRL complexes have been identified [4,12,13,14,15]. However, mutations in human CUL4B appear to be a common cause of X-linked mental retardation (XLMR). To date, at least 12 families of XLMR have been reported to be attributable to base substitutions or deletions in CUL4B [16,17,18,19,20]. In addition to mental retardation, those patients also manifest short stature, abnormal gait, impaired speech and other abnormalities. These findings suggest that CUL4B and CUL4A do not necessarily play redundant roles during neurogenesis and other developmental processes.

While Cul4a knockout mice have been independently generated by several groups, they showed highly variable phenotypes depending on the nature of the mutation introduced. Homozygous deletion of exon 1 of Cul4a was first reported to result in embryonic lethality [21]. However, a recent study showed that deletion of exon 1 of Cul4a inadvertently deleted the essential Pud1 gene located adjacent to Cul4a on the complementary strand [22]. Mice with deletion of exons 17–19 of Cul4a, on the other hand, were viable and displayed no overt developmental abnormalities, although skin-specific Cul4a ablation rendered resistance to UV-induced skin carcinogenesis [22]. Similarly, homozygous deletion of exons 4–8 of Cul4b resulted in no gross abnormalities [23]. However, the male knockout mice were sterile and exhibit severe deficiencies in spermatogenesis [23,24]. Homozygous deletion of exons 4–8 of Cul4b is also associated with severe proliferation...
defects in embryonic fibroblasts and hepatocytes, and an increase in genome instability [25].

In this study, we generated Cul4b floxed mice and crossed it to EIIa-Cre transgenic mice to produce Cul4b null mice. We observed that Cul4b null mice are embryonic lethal. Cul4b null embryos displayed decreased proliferation and increased apoptosis. Cul4b heterozygotes were also affected, as reflected by their recovery at a reduced ratio at birth and by their developmental delay. Cells expressing Cul4b null allele in Cul4b heterozygous mice were selected against, to different degrees in different tissues, from early embryogenesis to early postnatal development. The embryonic lethality of Cul4b null mice, when compared to the lack of gross abnormalities in Cul4a null mice, indicated that Cul4b has diverged from Cul4a to carry out some essential and unique functions during embryogenesis.

Results

Generation of Cul4b floxed mice

Because CUL4B-deficient cells are strongly selected against [17], we envisaged that it might be difficult to generate Cul4b-deficient embryonic stem (ES) cells via conventional knockout technology. We therefore used the Cre/loxP strategy to generate Cul4b floxed ES cells. First, a Cul4b floxed targeting vector was constructed (Fig. 1A). In this vector, exons 3–5 were floxed by two loxP sites. As predicted, exon 2 was spliced onto exon 6 after the excision of exons 3–5. (D) Real-time RT-PCR analysis of Cul4b mRNA isolated from brain tissues of wild-type males (Cul4b+/Y; Nestin-Cre+/−), wild-type females (Cul4b+/Y; Nestin-Cre−/−), heterozygous females (Cul4b+/flox/Y; Nestin-Cre+/−), and conditional knock-out males (Cul4b+/flox/Y; Nestin-Cre+/−), and conditional knock-out females (Cul4b+/flox/flox/Y; Nestin-Cre+/−). (E) Western blot analysis of Cul4b protein isolated from brain tissues of wild-type, heterozygous and conditional knockout mice using an anti-Cul4b antibody. Gapdh was used as a loading control.

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Neither male hemizygous (Cul4bflox/Y) nor female heterozygous/homozygous (Cul4bflox/flox) for the Cul4b floxed allele showed any apparent phenotype, suggesting that the flox allele did not disturb the normal function of Cul4b gene. To verify that Cul4bfllox can be rendered nonfunctional by the expression of Cre, due to the removal of exons 3–5, we generated brain-specific knockout mice Cul4bflox/Y;Nestin-Cre mice and sequenced the cDNA of Cul4b prepared from the brain. We observed that exon 2 was indeed spliced onto exon 6, as a result of removal of exons 3–5 (Fig. 1C). The deletion would also result in a frameshift, 2 was indeed spliced onto exon 6, as a result of removal of exons 3–5 (Fig. 1C). The deletion would also result in a frameshift, suggesting that the truncation mutation also resulted in nonsense-mediated decay (NMD), as observed in patients with CUL4B.

Western blot analysis showed that Cul4b mRNA level in brains of the conditional knockout mice is much lower than that of littermate wild-type control (Fig. 1D), supporting the role of Cul4b in placental development. As expected, the embryonic part of the cylinder was not expanded and boundaries between germ layers were indistinguishable in the Cul4b null embryos (Fig. 2C), which were identified by their negative staining by anti-Cul4b antibody (see below). In addition, extraembryonic compartment in Cul4b null embryos was also underdeveloped and poorly organized (Fig. 2C). Thus, in general, the Cul4b null embryos are developmentally retarded when compared to stage-matched wild-type embryos.

To determine the exact time point when Cul4bnull/Y conceptuses die, timed mating was performed and embryos at different developmental stages were dissected. DNA was extracted from yolk sac or total embryo and used for PCR genotyping. Genotyping of E9.5, 10.5, 12.5 and 14.5 embryos showed that no Cul4bnull/Y conceptuses were recovered on 9.5 day post coitum (dpc) and beyond. Instead, a proportion of embryos, ~25%, appeared to have been absorbed and only placentas or empty deciduas remained (Fig. 2A), suggesting that these embryos implanted but died by E9.5. Cul4b null embryos were recovered at a ratio expected of Mendelian inheritance at E7.5 and 9.5, and no empty deciduas were found at these developmental stages. However, the Cul4b null embryos were much smaller (Fig. 2B). The recovery of Cul4b null embryos at the expected Mendelian ratio indicated that the mosaic females are functionally Cul4bnull in their capacity to transmit Cul4bnull allele.

To gain further insight into the anomalies in the Cul4b null embryos, we performed H&E staining of paraffin-embedded sections of E7.5 embryos. While in wild-type embryos, ectoderm, endoderm and endoderm appeared to have properly formed, the embryonic part of the cylinder was not expanded and boundaries between germ layers were indistinguishable in the Cul4b null embryos (Fig. 2C), which were identified by their negative staining by anti-Cul4b antibody (see below). In addition, extraembryonic compartment in Cul4b null embryos was also underdeveloped and poorly organized (Fig. 2C). Thus, in general, the Cul4b null embryos are developmentally retarded when compared to stage-matched wild-type embryos.

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Table 1. Distribution of Cul4b genotypes in progeny of Cul4bflox/flox/Cul4bflox/null;EIIa-Cre+/− females.  

| Group | Wild-Type | Heterozygous | Knockout | Absorbed |
|-------|-----------|--------------|-----------|----------|
| Cul4b genotype | Cul4b+/+ or Cul4b++/+ | Cul4b+/null | Cul4bnull/null | ND* |
| Expected Mendelian % | 50% | 25% | 25% | - |
| 3 weeks (%) (n = 135) | 107 (79%) | 28 (21%) | 0 (0%) | - |
| 14.5 dpc (%) (n = 61) | 31 (51%) | 11 (18%) | 0 (0%) | 19 (31%) |
| 12.5 dpc (%) (n = 39) | 17 (44%) | 9 (23%) | 0 (0%) | 13 (33%) |
| 10.5 dpc (%) (n = 38) | 19 (50%) | 12 (32%) | 0 (0%) | 7 (18%) |
| 9.5 dpc (%) (n = 31) | 15 (48%) | 7 (23%) | 0 (0%) | 9 (29%) |
| 8.5 dpc (%) (n = 28) | 15 (54%) | 6 (21%) | 7 (25%) | (0%) |

Litters were dissected at the times shown and genotyped by PCR as described in Materials and Methods.  

*ND indicates that the Cul4b genotype could not be determined by PCR.  

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tochemical staining of paraffin sections of E7.5 embryos for Ki67, a marker of proliferating cells. While there was a high level of proliferation in the wild-type embryos, proliferative cells were less abundant in \textit{Cul4b} null embryos (Fig. 3A). BrdU incorporation assay confirmed the decrease in proliferation in \textit{Cul4b} null embryos (Fig. 3B), suggesting that the proliferative activity was greatly compromised in \textit{Cul4b} null embryos.

Apoptosis in E7.5 embryos was evaluated by TdT-mediated dUTP nick end labeling (TUNEL) assay. Apoptotic cells were rarely detected in wild-type E7.5 embryos; however, the number of apoptotic cells was remarkably increased in the \textit{Cul4b} null embryos (Fig. 3C). Apoptotic cells were also observed to line along the Reichert’s membrane.

It was previously reported that CUL4B can interact with cyclin E and the CUL4B immunocomplexes can polyubiquitinate cyclin E \textit{in vitro} [26]. We previously showed that silencing of \textit{CUL4B} could lead to increased accumulation of cyclin E [4]. To determine whether deficiency of \textit{Cul4b} would also cause increased accumulation of cyclin E in the \textit{Cul4b} null embryos, the cyclin E level was examined using immunohistochemistry. Indeed, cyclin E was accumulated in \textit{Cul4b} null embryos compared to littermate wild-type embryos (Fig. 3D).
The levels of other known substrates of CRL4 complex, p27 and p53, were also examined in wild-type and Cul4b null embryos at 7.5 dpc. They were not found to differ between Cul4b null embryos and wild-type embryos (Figure S1B–S1C), suggesting that Cul4b probably does not play a critical role in the proteasomal degradation of those proteins during embryonic development. Alternatively, Cul4a may have compensated for the lack Cul4b in lowering the levels of those substrates.

Taken together, these results suggested that the developmental delay in Cul4b null embryos is attributable to a reduction in proliferation and an increase in apoptosis.

Reduced recovery and growth retardation of Cul4b heterozygous mice

While no Cul4b null conceptuses could survive to E9.5, the Cul4b heterozygous females were also recovered at a lower than expected ratio at weaning (Table 1). This deficit was probably caused by an increased prenatal lethality in Cul4b heterozygotes, because their ratio was reduced even at E14.5. For the Cul4b heterozygotes that survive to term, they were significantly smaller than their wild-type littermates. The average body weight of heterozygous newborns (1.13±0.17 g) was much smaller than that of wild-type controls (1.79±0.12 g). However, Cul4b heterozygous mice were able to gradually catch up after birth, and the body weight difference became narrow in adults (Fig. 4A). Except for growth retardation, Cul4b heterozygous mice showed no gross abnormalities for the first 18 months.

The fact that Cul4b heterozygous mice were smaller in size at birth but could catch up gradually suggests that developmental delay is primarily due to factor(s) that operate during prenatal development. Therefore, heterozygous embryos at different developmental stages were dissected and examined (Fig. 4B–4E). Cul4b heterozygous embryos were smaller than wild-type controls at all development stages examined, suggesting that growth retardation began at early developmental stage. In addition, the Cul4b heterozygous embryos appeared pale (Fig. 4D and 4E). Blood vessels on yolk sac were not well developed (Fig. 4D), and the blood flow appeared to be reduced (Fig. 4E). Poor vascularization and shortage in blood supply may have caused undernourishment in the heterozygous embryos.

Placental defects of Cul4b heterozygous embryos

To further delineate the mechanism underlying the growth retardation of Cul4b heterozygotes during prenatal development, we examined the morphology and histology of placentas at E14.5. Placentas of Cul4b heterozygous embryos appeared smaller and paler than those of wild-type (Fig. 5A). H&E staining of placental sections showed that the labyrinth layer of Cul4b heterozygous placentas was more loosely formed compared to that of wild-type controls, and was deeply invaded by the spongiosrophoblast layer, leaving the demarcation between labyrinth and spongiosrophoblast layers indiscernible (Fig. 5B, upper panels). While the network in the labyrinth layer of placentas in the wild-type was highly compacted and densely branched; the labyrinth layer in Cul4b heterozygotes was laid out very loosely (Fig. 5B, lower panels).
panels). The labyrinth and spongiotrophoblast layers were indistinguishable in the placentas of absorbed embryos. The vascular network in the placenta was further characterized by immunohistochemistry using an antibody to platelet/endothelial cell adhesion molecule-1 (PECAM), which marks endothelial cells in blood vessels. In contrast to normal placentas in which the blood vessels were well formed and uniformly distributed (Fig. 5C, left panels), PECAM staining signals in Cul4b heterozygous placentas only appeared in rather isolated regions (Fig. 5C, middle panels). Furthermore, vascular structures were not readily recognizable within the PECAM-positive islands. Only background staining of PECAM was detected in placentas of absorbed embryos (Fig. 5C, right panels).

Taken together, these results showed that the placentas of Cul4b heterozygous embryos were generally disorganized and were impaired in vascularization, which may contribute to the growth retardation during prenatal development of Cul4b heterozygous mice.

Pattern of X-chromosome inactivation (XCI) in Cul4b heterozygous mice

Because Cul4b is X-linked, Cul4b heterozygous females are functional mosaics in terms of the expression of Cul4b. The somatic cells are either Cul4b functional or null depending on the choice of the X-chromosome that becomes inactivated. In the XLMR family with CUL4B mutation, X-chromosome inactivation (XCI) was extremely skewed in peripheral blood cells of carriers, due to the selection that favors the cells in which the mutant CUL4B allele is inactivated [17,20]. We first determined the XCI pattern in 4-month-old adult Cul4b heterozygous females by immunohistochemical analysis for Cul4b expression. If XCI were balanced in Cul4b heterozygous females, the proportion of cells expressing Cul4b should be ~50% of that in wild-type females. On the other hand, if XCI is skewed toward Cul4b allele, as in human CUL4B heterozygous carriers, the percentage of cells expressing Cul4b should be closer to that in wild-type females. As shown in Fig. 6A, the percentages of cells expressing Cul4b in kidney, liver and lung were identical between Cul4b heterozygous females and wild-type females, suggesting that XCI is extremely skewed in those organs. XCI in hippocampus was also skewed, but to a lesser extent, since the percentage of Cul4b positive cells in heterozygotes remained lower than that in wild-type. Western blot assay showed a similar trend (Figure S2). While the expression levels of Cul4b in liver and lung in Cul4b heterozygous mice were comparable to those in wild type mice, the expression levels in cortex and hippocampus were decreased in Cul4b heterozygous mice compared to those in wild type mice.

The skewed XCI toward Cul4bnull-bearing chromosome could be caused either by an initiation of XCI that favors the Cul4bnull-bearing X or by a selection against Cul4bnnull-expressing cells during development. To distinguish between those two possibilities, we next examined the XCI pattern in younger Cul4b heterozygous females. As shown in Fig. 6B–C, the percentages of Cul4b-expressing cells in lung were not significantly different between Cul4b heterozygous and wild-type females even in the newborn. However, the percentages of Cul4b positive cells in kidney and liver were significantly lower in heterozygotes than in wild-type (Fig. 6B–C). These results suggest that the skewed XCI is caused by a gradual selection against the Cul4b negative cells and that different organs face differential selection pressure during prenatal and postnatal development. While Cul4b null cells in lung were nearly all eliminated before birth, those cells remained in

Figure 4. Growth retardation of Cul4b heterozygous mice during embryonic development. (A) Bodyweights of Cul4b heterozygous mice and littermate wild-type females after birth. Data were presented as mean±SD. N = 8, *: p<0.05; **: p<0.01; ***: p<0.001. (B–E) Representative photographs of Cul4b heterozygous embryos and littermate wild-type controls at 9.5 (B), 10.5 (C), 12.5 (D) and 14.5 (E) dpc. The bar represents 1 mm in (B–C) and 2 mm in (D) and (E), respectively. doi:10.1371/journal.pone.0037070.g004
hippocampus four months after birth (Fig. 6A–C, 6E). The less stringent selection against Cul4b null cells in hippocampus is consistent with the clear difference in the Cul4b expression level between Cul4b heterozygotes and wild-type in the brain (Fig. 1D and 1E, Fig. S2).

To gain insight into the XCI pattern at embryonic stage, we examined the distribution of Cul4b-positive and –negative cells in E7.5 embryos. XCI is usually completed by E6.5 in epiblasts. As shown in Fig. 6F, Cul4b-positive and –negative cells were not evenly distributed in the embryonic regions of the E7.5 heterozygous embryos. While the two types of cells appeared in an intermingled pattern and in roughly equal proportions in some areas (middle), indicative of the randomness of XCI before selection took place, Cul4b-positive cells were predominant in other areas (right), suggesting that selection for Cul4b-positive cells started before E7.5 wherein. The differential selection in different embryonic regions at early developmental stage may contribute to the distribution pattern of Cul4b-negative cells and the selection dynamics in various tissues at later developmental stages.

Figure 5. Morphology and histology of placentas of wild-type, Cul4b heterozygous and absorbed embryos at 14.5 dpc. (A) Representative photographs of placentas of wild-type, Cul4b heterozygous and absorbed embryos at 14.5 dpc. (B) H&E staining of radial sections of placentas. sp, spongiotrophoblast layer; la, labyrinthine layer. Lower panels are the higher magnification of the upper panels. (C) Immunohistochemistry of radial sections of placentas with an antibody to PECAM, an angiogenesis marker. Middle panels are the higher magnification of the upper panels, and lower panels are the higher magnification of the middle panels.

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Discussion

The cullin members are critical for the assembly of CRL complexes that play important roles in multiple cellular processes. Lack of cullin function has indeed been shown to have severe consequences in cellular function and organism development. Disrupting either Cul1 or Cul3 genes in mice caused embryonic lethality and dysregulation of cyclin E [27,28,29]. Deletion of Cul7 caused neonatal lethality, growth retardation, and vascular abnormality [30]. In this study, we showed that deletion of Cul4b resulted in embryonic lethality in null hemizygotes. This finding is consistent with the embryonic lethality of Cul4b mutant mice that were generated by gene trap technology [31]. In addition, Cul4b heterozygotes were recovered in deficit and those that survive to term exhibited a developmental delay. Underscoring the importance of Cul4b function is the observed skewedness of X-inactivation in Cul4b heterozygotes as a result of selection against Cul4b null cells. The severe phenotype of Cul4b null mice is in sharp contrast to the lack of obvious phenotype in Cul4a null mice, although the two cullin members are closely related. These findings with Cul4b mutant mice, together with the development of XLMR syndrome in humans carrying CUL4B mutation, suggest that CUL4A cannot fully compensate for the absence of CUL4B activity in mammals.

While mutations in CUL4B cause mental retardation, short stature, impaired speech, and other abnormalities, the patients usually survive to adulthood. Thus, the Cul4b mutant mice are more severely affected. The mechanism underlying this species-specific difference in phenotypic severity caused by CUL4B deficiency remains to be elucidated. It is possible that certain mutations in humans may retain residual function of CUL4B and thus have less deleterious effect than the Cul4b null mutation in the mouse. However, several mutations in human CUL4B resemble null mutations. The p.R388X mutation, for example, would have truncated the whole CULLIN domain and the rest of the C-terminus [16,17]. Moreover, this mutation caused nonsense mediated decay of CUL4B mRNA [17]. Two reported deletions in CUL4B causing XLMR would have been devoid of most of the CULLIN domain, which is essential for CRL activity [19,20]. It should be noted that the more severe phenotype in mouse mutants
of genes responsible for XLMR is not restricted to Cul4b null mice. Loss of Atrx function, which is the second most common cause of XLMR, next to fragile X gene, also resulted in early embryonic lethality in mice [32], and even deletion of Atrx just in forebrain caused perinatal lethality [33]. Targeted inactivation of Huael1 in the CNS also resulted in neonatal lethality [34]. It is also possible that certain functions of Cul4b are redundantly carried out by CUL4A in the humans, but not in the mouse. We are tempted to speculate that because extraembryonic development is particularly affected in Cul4b null and heterozygous embryos, CUL4B is probably more critical for placental development in the mouse than in the humans. Future studies employing ablation of Cul4b in embryo proper may help resolve this issue.

We previously showed that silencing of CULAB led to an increased accumulation of cyclin E and a reduced cell proliferation that was accompanied by a prolonged S phase [4]. Correspondingly, we found that Cul4b null embryonic cells are also accompanied by an increased accumulation of cyclin E. The inverse relationship between CUL4B and cyclin E was also observed in mouse liver in which Cul4b was ablated (unpublished data). While several recent studies showed that excessive cyclin E may play negative roles in cell proliferation [35,36], it remains to be determined whether the developmental arrest in Cul4b null embryos is caused by excessive cyclin E.

Unexpectedly, we observed that Cul4b heterozygous mice were also affected, as reflected by their recovery at a lower than expected ratio and by their remarkable developmental delay at birth. They began to catch up with their wild-type littermates after intron 2 to intron 5, was subcloned from a BAC clone (BMQ-455M17) into a vector which contained a TK cassette. A loxP site was inserted into intron 2 of Cul4b gene and a FRT-neo-FRT-loxP cassette was inserted into intron 5. (Fig. 1A)

The floxed targeting vector was linearized using Ncol restriction enzyme and electroporated into 129 male ES cells (Rw.4, obtained from ATCC), followed by selection in culture medium containing G418 and ganciclovir. To identify correctly targeted clones, 96 clones were selected, replicated and screened by long-range PCR using DNA isolated from each clone. Positive clones identified were further confirmed by Southern blot analysis. Southern blot of DNA digested with BamHI restriction enzyme with the 5′ probe that hybridizes with the upstream of the targeted region yielded a 14.4 kb fragment for the wild-type allele and a 10 kb fragment for the targeted allele. Analysis using XbaI restriction enzyme with the 3′ probe that hybridizes with the downstream of the targeted region revealed a 20 kb fragment for the wild-type allele and a 10 kb fragment for the targeted allele.

Correctly targeted ES cells were injected into the blastocysts of E3.5 embryos from hyperovulated C57BL/6j mice. Surviving blastocysts were transferred into the oviducts of pseudopregnant recipient females to produce chimaeric mice, which were further crossed with wild-type mice for germ line transmission to produce Cul4b floxed mice.

Generation of constitutive and brain-specific Cul4b knock-out mice

To produce mice null for Cul4b gene, Cul4b floxed mice were crossed with Elia-Cre transgenic mice [39], in which Cre transgene was under the control of the adenovirus Elia promoter that drives the expression of Cre recombinase in a wide range of tissues, including the germ cells that transmit the genetic alteration to progeny. Due to the mosaic pattern of Cre expression in Elia-Cre transgenic mice, deletion of the floxed fragment is usually not achieved in all the somatic cells, thus commonly resulting in genetically mosaic mice. To avoid mosaicism, the following strategy was used. Cul4b+/floxed females were crossed with Elia-Cre+/− males to obtain mosaic females that are Cul4b+/floxed/Elia-Cre+/−. Female mosaic mice were subsequently crossed with wild-type male mice. A germ cell produced by the mosaic may carry either Cul4b+/-cultured or Cul4b+/-alleles, but not both. The Cul4b+/-type of germ cells would give rise to progeny that were Cul4b knockout males (Cul4b+/Y) and Cul4b heterozygous females (Cul4b+/+). The Cul4b+/- type of germ cells, on the other hand, will give rise to Cul4b+/- males or Cul4b+/- females. As shown in the Results, even though the females were supposed to be Cul4b+/-/Cul4b+/-, no Cul4b+/- allele was detected in the progeny, suggesting that Cul4b+/- allele is also converted into Cul4b+/- during germ line transmission.

To produce conditional knock-out mice in which Cul4b gene was specific deleted in brain tissue, Cul4b+/- female mice were

Materials and Methods

Generation of mice with the floxed Cul4b gene

The animal work was approved by Animal Use Committee, Shandong University School of Medicine (Approval number: ECAEDUSM2008005). Cul4b floxed mice were generated at National Resource Center of Mutant Mice/Model Animal Research Center of Nanjing University. First, a Cul4b floxed targeting vector was constructed. Briefly, a 14.5 Kb DNA fragment of the mouse Cul4b gene, spanning the region from intron 2 to intron 5, was subcloned from a BAC clone (BMQ-455M17) into a vector which contained a TK cassette. A loxP site was inserted into intron 2 of Cul4b gene and a FRT-neo-FRT-loxP cassette was inserted into intron 5. (Fig. 1A)

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To produce mice null for Cul4b gene, Cul4b floxed mice were crossed with Elia-Cre transgenic mice [39], in which Cre transgene was under the control of the adenovirus Elia promoter that drives the expression of Cre recombinase in a wide range of tissues, including the germ cells that transmit the genetic alteration to progeny. Due to the mosaic pattern of Cre expression in Elia-Cre transgenic mice, deletion of the floxed fragment is usually not achieved in all the somatic cells, thus commonly resulting in genetically mosaic mice. To avoid mosaicism, the following strategy was used. Cul4b+/floxed females were crossed with Elia-Cre+/− males to obtain mosaic females that are Cul4b+/floxed/Elia-Cre+/−. Female mosaic mice were subsequently crossed with wild-type male mice. A germ cell produced by the mosaic may carry either Cul4b+/-cultured or Cul4b+/-alleles, but not both. The Cul4b+/-type of germ cells would give rise to progeny that were Cul4b knockout males (Cul4b+/Y) and Cul4b heterozygous females (Cul4b+/+). The Cul4b+/- type of germ cells, on the other hand, will give rise to Cul4b+/- males or Cul4b+/- females. As shown in the Results, even though the females were supposed to be Cul4b+/-/Cul4b+/-, no Cul4b+/- allele was detected in the progeny, suggesting that Cul4b+/- allele is also converted into Cul4b+/- during germ line transmission.

To produce conditional knock-out mice in which Cul4b gene was specific deleted in brain tissue, Cul4b+/- female mice were
cross with Nestin-Cre transgenic mice [40], in which Cre recombinase was under the control of the promoter and enhancer of rat nestin that was primarily expressed in the nervous system. These crosses would be expected to yield wild-type females (Cul4b+/−/Nestin-Cre+/−), heterozygous females (Cul4b+/−/Nestin-Cre−/−), wild-type males (Cul4b−/−/Nestin-Cre+/−), and conditional knock-out males (Cul4b−/−/Nestin-Cre−/−) in a ratio of 1:1:1:1.

The Egr2-Cre and Nestin-Cre transgenic mice were purchased as model animal research center of Nanjing University. All experiments involving animals were conducted in compliance with national regulations and by protocols approved by institutional animal care and use committee.

PCR genotyping
Genomic DNA was extracted from tails, whole embryos or yolk sac, and used for genotyping by PCR analysis. For the genotyping of Cul4b flox mice, primers p01 (5′-ACAGGTATTTG-CAGTTGCTGTG-3′) and p02 (5′-TTTCTGTTACCTTG- TACCAGAGATCT-3′) and loxP site in intron 2 were used to amplify Cul4b allele (501 bp) and wild-type allele (383 bp) (Fig. S3A). For the genotyping of Cul4b null mice, primers p03 (5′- GACCTTACAGGATTATCTGGTTG-3′) and p04 (5′- ACAAGAGGGAGATTCTCAGG-3′) were used for detection of the Cul4b null allele (498 bp), and primers p05 (5′-AGCGAGCAGGCACAATACACC-3′) and p06 (5′- CTGGAAGGCCAAGGCAGAAG-3′) were used for detection of the Cul4b wild-type allele (321 bp) (Fig. S3B).

Reverse transcription PCR and real-time RT-PCR
Total RNA from the brain tissues of 2-week mice of different genotypes was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and treated with RQ1 RNase-Free DNase (Promega) to eliminate genomic DNA contamination. Freshly isolated RNA was reverse transcribed to cDNA using Super Script first-strand synthesis system (Invitrogen) following the manufacturer’s recommendations. Cul4b gene was amplified by PCR using cDNA as template, and PCR product were sequenced. Real-time PCR was performed for quantitation of Cul4b mRNA using the TaqMan 7500 instrument (PE Applied Biosystems). The mRNA levels of Cul4b was measured by SYBR Green I assay using SYBR Green Universal PCR Master Mix (Applied Biosystems). Mice Gapdh was used as endogenous control. The sequences of the primers were for Cul4b, 5′-TATTAGTTGCGCAAGTGCTAT-3′ and 5′-CCAGTTAACCCATGTCAGAT-3′, and for Gapdh, 5′- AGGTGCGTTGGAAAGGATT-3′ and 5′-GTGAGAGCATGATGAGTCA-3′. Four independent measurements per sample were performed. The quantified individual RNA expression levels were normalized to Gapdh.

Western blot
Protein was extracted from brain tissues of 2-week mice of different genotypes. The concentration of tissue lysates was determined by using the BCA kit (Pierce, Rockford, IL, USA). Then equal amounts (50 μg) of total protein was subjected to 12% SDS-polyacrylamide gel for electrophoresis, followed by blotting onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech), and incubated with the anti-Cul4b primary antibody (Sigma; used at 1:1,000 dilution) overnight at 4°C. After washing, the membranes were incubated with a horseradish peroxidase (HRP) conjugated secondary antibody (Jackson Immunoresearch; 1:200 dilution), anti-Cul4a (Abcam, 1:200 dilution), anti-p27 (Santa Cruz, 1:50 dilution), anti-ki67 (Abcam, 1:200 dilution), anti-PECAM (Abcam, 1:50 dilution), anti-Cu4a (Abcam, 1:200 dilution), anti-p27 (Santa Cruz, 1:200 dilution), and anti-p53 (Santa Cruz, 1:200 dilution). After washing, the sections were coated with a horseradish peroxidase (HRP) conjugated second antibody (Jackson Immunoresearch; 1:200 dilution) and then incubated at 37°C for 1 hour. The DAB was used to visualize immunoreactions sites. Sections were counterstained with hematoxylin and mounted on glass slides. Negative controls were obtained by substituting the primary antibody with normal serum.

BrdU incorporation and immunofluorescence
For labeling of cells in S phase, BrdU (Sigma-Aldrich) was injected intraperitoneally into pregnant mice at 7.5 dpc, with 100 mg per Kg body weight. Animals were sacrificed after 2 hours by cervical dislocation and the embryos were recovered in ice cold PBS and were fixed in 4% paraformaldehyde. Incorporation of modified nucleotide was detected by staining with an anti-BrdU primary antibody (Abcam, 1:100 dilution) and Rhodamin-labeled secondary antibody (Jackson Immunoresearch; 1:100 dilution). After staining, the slides were counterstained with DAPI and visualized with a fluorescence microscope.

TUNEL assay
TUNEL assay was performed using the In Situ Cell Death Detection Kit, TMR red (Roche) following the manufacturer’s recommendations. After labeling, the slides were counterstained with DAPI and visualized with a fluorescence microscope.

Statistical Analysis
Data were expressed as the mean±SD. Data from the two groups were evaluated statistically by a two-tailed unpaired t test using SPSS13.0 for any significant differences. A p value of less than 0.05 was considered statistically significant.
Supporting Information

Figure S1  Expression of Cul4a, p27 and p53 in wild-type and Cul4b null embryos at 7.5 dpc. Paraffin sections of wild-type and Cul4b null embryos at 7.5 dpc were stained with an antibody against Cul4a (A), p27 (B) and p53 (C). Sections were counterstained with haematoxylin. Lower panels (100×) are the higher magnification of the upper panels (20×).

(TIFF)

Figure S2  Western blot analysis of Cul4b levels. Proteins prepared from tissues of wild-type and heterozygous mice at 4 months were subjected to Western blot analysis using an anti-Cul4b antibody. Gapdh was used as a loading control.

(TIFF)

Figure S3  PCR genotyping of Cul4b flox mice and Cul4b null mice. (A) PCR genotyping analysis of tail DNA from wild-type (WT), Cul4b flox and heterozygous (Het) mice. (B) PCR genotyping analysis of wild-type mice (Cul4b<sup>flox</sup> and Cul4b<sup>+/+</sup>), Cul4b knockout mouse mice (Cul4b<sup>+</sup>/<sup>+</sup>) and Cul4b heterozygous female mice (Cul4b<sup>+</sup>/<sup>lox</sup>). The null allele can only be amplified by primers p03 and p04 when exons 3–5 are deleted.

(TIFF)

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Author Contributions

Conceived and designed the experiments: BJ CS YG. Performed the experiments: BJ WZ JY YQ WS YZ CG BC CS. Analyzed the data: BJ WZ CS YG. Wrote the paper: BJ CS YG.

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