iRhom2 Mutation Leads to Aberrant Hair Follicle Differentiation in Mice

Yang Leilei1*, Liu Bing2, Li Yang1, Wang Shaoxia1, Xu Yuan2, Wang Dongping2, Ye Huahu2, Shang Shichen2, Zhang Guangzhou2, Peng Ruiyun1, Zeng Lin2, Li Wenlong2*

1. Beijing Institute of Radiation Medicine, Beijing 100850, China, 2. Institute of JingFeng Medical Laboratory Animal, Beijing 100071, China
*leileiyang@126.com (YL); liwl@bmi.ac.cn (WL)

Abstract

iRhom1 and iRhom2 are inactive homologues of the rhomboid intramembrane serine proteases lacking essential catalytic residues, which are necessary for the maturation of TNFα-converting enzyme (TACE). In addition, iRhoms regulate epidermal growth factor family secretion. The functional significance of iRhom2 during mammalian development is largely unclear. We have identified a spontaneous single gene deletion mutation of iRhom2 in Uncv mice. The iRhom2Uncv/Uncv mice exhibit hairless phenotype in a BALB/c genetic background. In this study, we observed dysplasia hair follicles in iRhom2Uncv/Uncv mice from postnatal day 3. Further examination found decreased hair matrix proliferation and aberrant hair shaft and inner root sheath differentiation in iRhom2Uncv/Uncv mutant hair follicles. iRhom2 is required for the maturation of TACE. Our data demonstrate that iRhom2Uncv cannot induce the maturation of TACE in vitro and the level of mature TACE is also significantly reduced in the skin of iRhom2Uncv/Uncv mice. The activation of Notch1, a substrate of TACE, is disturbed, associated with dramatically down-regulation of Lef1 in iRhom2Uncv/Uncv hair follicle matrix. This study identifies iRhom2 as a novel regulator of hair shaft and inner root sheath differentiation.

Introduction

iRhom1 and iRhom2, which are inactive homologues of the rhomboid intramembrane serine proteases that lack the essential catalytic residues [1]. iRhom1 and iRhom2 regulate the secretion of the epidermal growth factor (EGF) family by endoplasmic reticulum-associated degradation [2]. iRhom2 is required for the release of tumor necrosis factor α (TNFα) in macrophages by controlling...
the maturation of TNFα-converting enzyme (TACE, also called ADAM17) [3–5]. Moreover, iRhom2 controls the activation and substrate selectivity of TACE-dependent shedding events [6]. Dominant mutations of iRhom2 is the cause of human tylosis esophageal cancer [7, 8]. In addition, iRhom2 plays an important role in inflammatory arthritis [9]. iRhom2 knockout mice could survive in a lethal lipopolysaccharide dose [5]. However, the functional significance of iRhom2 during mammalian skin development is unclear.

In the developing hair follicle, signals from adjacent mesenchymal dermal papilla cells instruct the overlying epithelium to form hair placodes [10, 11]. The placode proliferates to form a larger bulb (matrix) and further differentiates into a central hair shaft consisting of the medulla, cortex and hair shaft cuticle surrounded by the inner root sheath (IRS), which consists of the inner root sheath cuticle and Huxley’s and Henle’s layers. The outer root sheath (ORS) is outside the IRS, is contiguous with the interfollicular epidermis and contains a reservoir of quiescent SCs that are known as the bulge. The bone morphogenetic protein (BMP), Notch and Wnt/β-catenin signaling pathways allow the normal differentiation of matrix cells into the hair shaft and the IRS envelope [12–15]. Gata3 is expressed in IRS, the Gata3 mutant mice generate primary IRS defects, which lead to alterations in the shaft [16, 17]. Foxn1 and Hoxc13, which are important hair shaft gene regulators, both of which cause hair defects when mutated [18, 19].

Here, we report a role for iRhom2 in mouse skin development. In a BALB/c genetic background, homozygous uncovered (Uncv, MGI: 1261908) mice have a hairless phenotype [20, 21]. We identified a spontaneous non-frameshift deletion mutation in the N-terminal cytoplasmic domain of iRhom2 (iRhom2Uncv) in Uncv mice by sequence capture array and sequencing platform. iRhom2Uncv could not induce the maturation of TACE, and decrease the level of NICD and Lef1. This study suggests that iRhom2 regulates hair shaft and IRS differentiation by specifically modulating Notch1 and Wnt signaling pathway which maybe mediated by TACE.

Results

Pattern of iRhom2 expression in the mouse
In a BALB/c genetic background, homozygous Uncv mice are hairless; heterozygous Uncv mice have a sparse hair coat (Figure A in S1 Fig.). A previous study demonstrated that the Uncv mouse hair abnormalities were linked to a single autosomal gene mutation and incomplete dominant inheritance [20]. Uncv mice with heterozygous parents exhibited the expected Mendelian ratio (wild-type:heterozygous:homozygous =105:191:103). These results show that Uncv/ Uncv mice only have a single gene mutation. Using a genetic linkage analysis of Uncv mice, the mutated gene was mapped to a region between markers D11mit338 and D11mit337 on mouse chromosome 11 [20, 22]. We identified a 309 bp spontaneous non-frameshift deletion mutation in the N-terminal
cytoplasmic domain of iRhom2 (iRhom2Uncev) in Uncev mice by sequence capture array and sequencing platform (Figure B in S1 Fig.). PCR analysis showed that each hairless mouse’s genotype is iRhom2Uncev/Uncev, each sparse mouse’s genotype is iRhom2Uncev/+. The hair phenotype of mouse is depended on the dose of iRhom2. All the results indicated that iRhom2 mutation leads to the hairless in mice.

The pattern of iRhom2 mRNA expression was analyzed in 8-week-old mice. iRhom2 was expressed at high levels in the lung and spleen and at moderate levels in the skin (Fig. 1A). Next, we examined the expression of iRhom2 mRNA during skin development. iRhom2 was found to be specifically expressed at high levels on postnatal days (P) 2–15 and P28–35 (Fig. 1B), which corresponds well with the hair follicle growth phase (anagen). The expression of iRhom2 was detected at much lower levels in the hair follicle morphogenesis stage [embryonic day (E) 13.5-P0] and telogen stage (P21) (Fig. 1B). The cyclic expression pattern of iRhom2 implies that it maybe play a role in the progression of anagen in hair follicles. The strong ubiquitous cellular expression of iRhom2 is detected in the hair follicles (Fig. 1C–E and Figure B in S2 Fig.). Double immunofluorescence staining demonstrated that iRhom2 and K14 (marker for the ORS), AE13 (marker for the hair shaft cuticle and cortex keratins) or AE15 (marker for the IRS and medulla of the hair shaft) were colocalized in the hair follicles (Fig. 1C–E). iRhom2 was not expressed in dermal papilla cells (Fig. 1E). Moreover, iRhom2 was also expressed in basal layer of epidermis (Figure C in S2 Fig.). There is a non-frameshift deletion mutation of iRhom2 in Uncev mice, the antibody we used could not discriminate iRhom2 and iRhom2Uncev, so iRhom2 expression could still be detected in iRhom2Uncev/Uncev mice (Figure E in S2 Fig.). However, we performed real-time PCR analysis of iRhom2 and iRhom2# (The PCR primers were located in the deletion region of iRhom2Uncev) in wild type and iRhom2Uncev/Uncev mice, the results showed that iRhom2# could not be detected in iRhom2Uncev/Uncev mice (Figure A in S2 Fig.). To confirm whether iRhom2 was expressed in the dermis, the expression of iRhom2 was separately assessed in the epidermis and dermis at P5 using real-time PCR. The results indicated that the dermis expresses very little iRhom2 (Fig. 1F).

Aberrant hair shaft and inner root sheath differentiation in iRhom2Uncev/Uncev mice

In mice, the development of the primary hair follicles is initiated at approximately E13 and extends to P16 [10]. To determine whether the deletion mutation in iRhom2 affects hair follicle morphogenesis, we examined the dorsal skin histology of iRhom2Uncev/Uncev and wild-type mice at E15.5, E17.5, P0, P3 and P9. At hair follicle morphogenesis stage (E15.5, E17.5 and P0), the follicles of iRhom2Uncev/Uncev were histologically similar to wild-type mice (Fig. 2A–F). Skin follicle density was not significantly different in iRhom2Uncev/Uncev mice compared to the wild-type at E17.5 (12 ± 2 compared to 12 ± 1 follicles per mm, respectively; P=0.543, n=8, Fig. 2S). At P3, the differentiating stage, iRhom2Uncev/Uncev mice showed slightly shorter hair follicles (Fig. 2G and H). However, the skin of iRhom2Uncev/Uncev mice
displayed striking defects in hair follicle morphology at later postnatal stages. By P9, the hair follicles of wild-type mice were in mid-anagen become fully differentiated with large hair bulbs that had descended deep into the fat layer of the skin. In contrast, the hair follicles of \(iRhom2^{Uncv/Uncv}\) mice were smaller and misshapen, and the majority of follicles failed to produce hair shafts (Fig. 2I and J). Few apoptotic cells were detected by TUNEL staining in wildtype and \(iRhom2^{Uncv/Uncv}\) hair follicle matrix of P9 (2.94 ± 1.12 vs. 3.49 ± 1.49, \(P=0.42\), \(n=8\), Fig. 2K and L). At P18, hair follicles of both wildtype and mutant mice entered catagen [23] (Figure A and B in S3 Fig.), and subsequently entered
At the telogen stage, the hair follicles shortened and condensed in the dermis (Figure C and D in S3 Fig.). At P32, the second anagen, both wildtype and mutant hair follicles elongated and reentered subcutis, however, hair follicles of \( iRhom2^{Uncv/Uncv} \) mice were still misshapen compared with the wildtype mice (Figure E and F in S3 Fig.). Thus the \( iRhom2^{Uncv/Uncv} \) hair
follicle cycled normally and the marked shrinkage of the hair follicle matrix in mutant mice is not due to premature catagen development.

During follicle maturation, hair matrix keratinocytes rapidly propagate and differentiate, forming columns of cells that become the hair shaft and IRS [17]. To investigate hair follicular proliferation in iRhom2Uncv/Uncv mice, we performed Ki67 immunofluorescence staining. In normal hair follicles, a large number of Ki67-positive cells was concentrated in the hair matrix at P0 and P3. A similar number of Ki67-positive cells was observed in iRhom2Uncv/Uncv mouse follicles at P0 (21.13 ± 2.38 in wild-type follicles compared to 20.75 ± 3.19 in mutant follicles; P=0.82, n=8, Fig. 2M, N and T) and P3 (43.88 ± 9.03 in wild-type follicles compared to 38.13 ± 8.72 in mutant follicles; P=0.22, n=8, Fig. 2O, P and T). However, compared to P9 wild-type mice, the number of Ki67-positive cells was significantly lower in iRhom2Uncv/Uncv mice (40.50 ± 7.39 in wild-type follicles compared to 15.38 ± 11.45 in mutant follicles, P=1.31E-4, n=8, Fig. 2Q, R and T).

Next, we examined the expression of several markers of differentiation in the hair follicle at P3 and P9. The ORS expresses keratin (K) 14. In iRhom2Uncv/Uncv mouse follicles, K14 was expressed at normal levels (Fig. 3A, B, I and J). K6 was expressed in the companion layer of the hair follicle [24]. The expression of K6 was increased in iRhom2Uncv/Uncv mouse hair follicles (Fig. 3C, D, K and L). In contrast, AE15, which is normally expressed in the IRS and medulla of the hair shaft [25], was markedly reduced in the majority of iRhom2Uncv/Uncv mouse follicles and was absent from the most distorted follicles, indicating defective IRS differentiation and an absence of the hair shaft medulla (Fig. 3E, F, M and N). AE13, a specific marker for the hair shaft cuticle and cortex keratins [26], was absent in the majority of iRhom2Uncv/Uncv mouse follicles (Fig. 3G, H, O and P). We confirmed these results by real-time PCR, and the results showed that the expression of IRS markers (K71 and K72) and the hair shaft marker (K85) was significantly decreased in iRhom2Uncv/Uncv mouse follicles and that the expression of K6 was increased (Fig. 3Q). These results indicate that the hair matrix cells failed to differentiate toward the hair shaft and the IRS in iRhom2Uncv/Uncv mouse follicles, suggesting a crucial role for iRhom2 in hair follicle differentiation.

The iRhom2Uncv mutant protein cannot induce the maturation of TACE

iRhom2 inhibits secretion of EGF family ligands by inducing degradation of epidermal growth factor receptor (EGFR) ligands in mammalian cells [2]. Therefore, we analyzed the expression of phospho-EGFR in P3 and P5 mouse skin. The expression of phospho-EGFR was similar in the iRhom2Uncv/Uncv and the wild-type hair follicles (Fig. 4A-E), so was the expression of phospho-ERK (Fig. 4E). These data indicated that during hair follicle maturation the expression of phospho-EGFR and phospho-ERK were not altered in iRhom2Uncv/Uncv mice. Furthermore, an in vitro EGF degradation assay showed that both wild-type iRhom2 and iRhom2Uncv could promote EGF degradation (Fig. 4F). This
degradation is dependent on the proteasome; the proteasome inhibitor MG132 could partly restore the expression of EGF (Fig. 4F). Therefore, the deleted region in the iRhom2Uncv is not necessary for the iRhom2-mediated degradation of EGF.

It was reported that iRhom2 interacts with TACE and promotes its maturation in macrophages [3]. Immunoprecipitation of iRhom2-overexpressing 293T cells followed by immunoblotting revealed that iRhom2Uncv did not affect it’s
interaction with TACE (Fig. 5D). We expressed wild-type iRhom2 and iRhom2Uncv in 293T cells and assayed its effect on the maturation of TACE. Consistent with previous reports [3], the overexpression of wild-type iRhom2 caused the excessive maturation of TACE; however, exogenous iRhom2Uncv could not induce the maturation of TACE compared with the empty vector (Fig. 5A).

Moreover, exogenous iRhom2Uncv does not significantly affect the maturation of TACE that is induced by exogenous wild-type iRhom2 (Fig. 5A). In western blots of iRhom2UncvUncv mouse skin, mature TACE was significantly reduced at P3, P5.

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**Fig. 4.** The expression of phospho-EGFR is not affected during follicle differentiation in the iRhom2Uncv mutation. (A–D) Immunohistochemistry for phospho-EGFR in the dorsal skin of P3 and P5 wild-type and iRhom2UncvUncv mice. (E) Western blot analysis of phospho-EGFR and phospho-ERK expression in the dorsal skin of P3 wild-type and iRhom2UncvUncv mice. (F) 293T cells were transfected with the indicated constructs, and the cell lysates were then probed with anti-Flag, anti-Myc and anti-β-actin antibodies (white arrowhead is a non-specific band). Transfected cells were treated with 10 μM MG132 for 12 hours. Scale bars: (A–D), 25 μm.
and P9 (Fig. 5B). The mRNA of TACE was not significantly different between the iRhom2Un cv/Un cv and wild-type mice at P5 (Fig. 5C). Double immunofluorescence staining revealed that TACE and iRhom2 were colocalized in the hair follicles of wild-type and iRhom2Un cv/Un cv mice (Figure D and E in S2 Fig.). These results indicate that iRhom2Un cv cannot induce the maturation of TACE in vitro or in vivo.

Expression levels of regulators of inner root sheath and hair shaft differentiation in iRhom2Un cv/Un cv follicles

Notch1 and BMP signaling play critical roles in the differentiation of the hair shaft and the IRS [12, 13, 15, 27]. Wnt/β-catenin signaling controls the differentiation of matrix cells along the hair shaft lineage [14]. TACE participates in the activation of the Notch pathway [28–31]. The expression of Notch intracellular
Fig. 6. Expression levels of regulators of inner root sheath and hair shaft differentiation in iRhom2\(^{Uncv/Uncv}\) follicles. (A, B) Immunofluorescence staining of NICD in the dorsal skin of P9 wild-type and age-matched iRhom2\(^{Uncv/Uncv}\) mice. (C–J) Immunohistochemistry for Lef1 in the dorsal skin of E17.5, P0, P3 and P9 wild-type and iRhom2\(^{Uncv/Uncv}\) mice. (K) Western blot analysis of NICD protein expression in the dorsal skin of wild-type and iRhom2\(^{Uncv/Uncv}\) mice at P0, P3, P5 and P9. (L) Notch signaling transcriptional activity affected by Flag-iRhom2 or Flag-iRhom2\(^{Uncv}\) mutation in HeLa cells. NICD serves as a positive control. (M) Western blot analysis of Lef1 protein expression in the dorsal skin of wild-type and iRhom2\(^{Uncv/Uncv}\) mice at P0, P2, P3, P5 and P9. (N) Real-time PCR analysis of Notch1, Hes1, Hey1, Hey2, Bmp2, Bmp4, Lef1, Foxn1, Gata3, Ctnnb1, Tcf3, Dact1, Wif1 and Daam2 mRNA expression in the dorsal skin of wild-type and iRhom2\(^{Uncv/Uncv}\) mice at P5. n=3, *P<0.05, **P<0.01. Scale bars: (A, B), 12.5 μm; (C, D), 50 μm; (E–J), 25 μm.
domain (NICD) was dramatically reduced in \textit{iRhom2}\textsuperscript{Uncv/Uncv} mouse hair follicles from P3-P9 (Fig. 6A, B and K); however, the expression of Notch1 mRNA was not significantly different from the wild-type (Fig. 6N), indicating that the post-translational maturation of the Notch1 protein was inhibited in \textit{iRhom2}\textsuperscript{Uncv/Uncv} mouse skin. The Notch1 target genes \textit{Hes1}, \textit{Hey1} and \textit{Hey2} \cite{27} were also downregulated in \textit{iRhom2}\textsuperscript{Uncv/Uncv} mouse skin (Fig. 6N). To determine whether \textit{iRhom2} affects Notch signaling transcriptional activity, HeLa cells were co-transfected with Notch-dependent CSL luciferase reporter (CSL-Luc) and increasing amounts of Flag-\textit{iRhom2} or Flag-\textit{iRhom2}\textsuperscript{Uncv} mutation. As shown in Fig. 6L, Notch transcriptional activation activated by \textit{iRhom2} in a dose-dependent manner, but \textit{iRhom2}\textsuperscript{Uncv} was not able to activate Notch transcriptional activation. It was reported that expression of \textit{Lef1}, the Wnt/\beta\,-\,catenin pathway transcriptional effector, could be regulated by Notch1 \cite{32, 33}. The expression of \textit{Lef1} was similar at E17.5 (Fig. 6C, D and M), dramatically reduced at P3 and P5 (Fig. 6E–H and M), and absent at P9 in \textit{iRhom2}\textsuperscript{Uncv/Uncv} mouse follicles compared to wild-type follicles (Fig. 6I, J and M). The expression of \textit{Foxn1}, \textit{Gata3} and \textit{Hoxc13}, which are implicated in hair follicle differentiation \cite{34}, were dramatically reduced in \textit{iRhom2}\textsuperscript{Uncv/Uncv} mouse follicles (Fig. 6N, Figure E and F in S4 Fig.). The nuclear localization of \beta\,-\,catenin, which complexes with \textit{Lef1} to activate the transcription of Wnt target genes, was normal in \textit{iRhom2}\textsuperscript{Uncv/Uncv} mice (Figure A and B in S4 Fig.). Other genes involved in the Wnt pathway, including \textit{Ctnmb1} (\beta\,-\,catenin), \textit{Tcf3}, \textit{Dact1}, \textit{Wif1} and \textit{Daam2} mRNA, were normally expressed in the dorsal skin of wild-type and \textit{iRhom2}\textsuperscript{Uncv/Uncv} mice at P5 (Fig. 6N). The levels of phospho-Smad1/5/8 were comparable between \textit{iRhom2}\textsuperscript{Uncv/Uncv} mouse follicles and the wild-type (Figure C and D in S4 Fig.), as were the expression levels of \textit{Bmp2} and \textit{Bmp4} mRNA (Fig. 6N). These results indicated that \textit{iRhom2} is involved in hair follicle differentiation by controlling the Notch and Wnt/\beta\,-\,catenin signaling pathways.

\section*{Discussion}

In this study, we discovered that \textit{iRhom2} regulate murine hair follicle differentiation by specifically modulating Notch1 and Wnt signaling pathway which maybe mediated by TACE.

Our study demonstrated that the \textit{iRhom2}\textsuperscript{Uncv/Uncv} mouse has hair differentiation abnormalities and displays a hairless phenotype in a BALB/c genetic background. The \textit{iRhom2}\textsuperscript{Uncv/Uncv} mouse showed decreased hair matrix proliferation and could not differentiated into IRS and hair shaft. In P9, the hair follicles of wild-type and mutant mice were in mid-anagen and had descended deep into the subcutis. However, the hair matrix of mutant follicles were less proliferative than that of wildtype. Comparable and few apoptotic cells were detected in both wildtype and \textit{iRhom2}\textsuperscript{Uncv/Uncv} hair matrix of P9, implying that mutant mice did not entered into catagen stage prematurely. Further examination showed that hair follicle cycling of mutant mice was normal. So, the marked
shrinkage of the hair follicle matrix in mutant mice is not due to premature catagen development. The \(iRhom2^{Uncv/Uncv}\) hair matrix failed to differentiate into IRS and hair shaft which resulted in the hairless phenotype of \(iRhom2^{Uncv/Uncv}\) mouse. However, the hair follicle phenotype was not reported in \(iRhom2\) knockout C57BL/6 mouse [3, 5]. We noticed that \(cub/cub\) mice display a hairless or a wavy-coated phenotype depending on the modifier gene \(mcub\) [35]. When homozygous for the recessive \(mcub\) allele, \(cub/cub\) mice appear hairless. A single copy of the dominant \(Mcub\) allele confers a full, curly coat to \(cub/cub\) mice. The hairless phenotype in \(cub/cub, mcub/mcub\) mice resemble the \(Uncv/Uncv\) mice. The mapping regions of \(cub\) and \(Uncv\) cover a large overlapping region including the \(iRhom2\) gene. We speculate that \(cub\) and \(Uncv\) are the same gene, \(iRhom2\). The modifier gene, \(Mcub\) or \(mcub\), maybe affect hair follicle phenotype of \(iRhom2\) knockout mouse.

\(iRhom2\) is necessary for the maturation of TACE. The \(Adam17\) knockout mice displayed a disorganized distribution and structure of hair follicles [36]. Consistent with phenotype in hair follicle of \(Adam17\) knockout mice, the \(iRhom2^{Uncv/Uncv}\) mice showed irregularly positioned and oriented hair follicles with abnormal structure. \(Adam17\) conditional knockout mice induced by \(K14-Cre\) showed delayed hair outgrowth, shortened and disorganized hair follicles, abnormal epidermal proliferation and there was a dramatically increased infiltration of inflammatory macrophages [37]. \(iRhom2^{Uncv/Uncv}\) mice showed defects in hair development and macrophages infiltration (Figure A, B and C in S5 Fig.) resembling those in \(Adam17\) conditional knockout mice. There are some disparity between \(iRhom2^{Uncv/Uncv}\) mice and \(Adam17\) knockout mice. The majority of Adam17 knockout mice died between E17.5 and the first day after birth, those few mice that survived for several weeks had 20 to 40% body weights loss than those of littermates [36], while \(iRhom2^{Uncv/Uncv}\) mice could survive up to 12 months and had 20% body weights loss at 4 weeks after birth. It was reported that there is some redundancy between them \(iRhom1\) and \(iRhom2\) [38], this provides some explanation \(iRhom2^{Uncv/Uncv}\) mice do not show the severe defects seen in \(Adam17\) knockouts. We noticed that \(Adam17\) knockouts have perturbed hair coats and curly vibrissae [36], however, \(iRhom2^{Uncv/Uncv}\) mice have hairless phenotype. This discrimination implies that \(iRhom2\) has additional physiologically substrates other than \(Adam17\).

\(iRhom2\) is required for controlling the activation of TACE-dependent shedding events [6]. Consistently, \(iRhom2^{Uncv}\) cannot induce the maturation of TACE in vitro, and mature TACE was also significantly reduced in \(iRhom2^{Uncv/Uncv}\) mouse skin. Furthermore, \(iRhom2^{Uncv}\) does not affect the maturation of TACE induced by wild-type \(iRhom2\). These findings indicated that \(iRhom2^{Uncv}\) is a loss of function with respect to the maturation of TACE. However, the deleted region in the \(iRhom2^{Uncv}\) mutant is not necessary for the degradation of EGF. So, the \(iRhom2^{Uncv}\) mutant is not a simple loss of function mutation, which selectivity affects the client protein.

Our results indicated that the deleted region in the \(iRhom2^{Uncv}\) is necessary for the maturation of TACE, which is required for the normal processing of Notch
The Notch precursor protein is cleaved by furin to produce a bipartite heterodimeric molecule. Notch ligand-receptor interactions induce S2 and S3 proteolytic cleavage. S2 cleavage within the extracellular domain is mediated by TACE. Subsequently, S3 cleavage by the γ-secretase releases NICD, which translocates to the nucleus \cite{39,40}. We demonstrated that the \textit{iRhom2}^\textit{Uncv} mutation affects TACE activity and inhibits the activation of Notch1. It was reported that Notch and WNT signaling exists cross-talk \cite{32,33,41}. Moreover, the Notch intracellular domain can function as a coactivator for Lef1 and regulator for expression of Lef1 \cite{32,41}. We observed that the expression of NICD and Lef1 were dramatically reduced from P3, which is the initial stage of hair follicle differentiation and the numbers of hair matrix cells were comparable between the wild-type and the \textit{iRhom2}^\textit{Uncv/Uncv} mice, indicating that the loss of NICD and Lef1 is not due to loss of the matrix cells. Therefore, the reduction in NICD and Lef1 expression in the \textit{iRhom2}^\textit{Uncv/Uncv} mouse hair matrix appeared before the change in hair bulb morphology, which resulted in disorders of follicle differentiation. This study identifies that \textit{iRhom2} regulates hair shaft and IRS differentiation by specifically modulating Notch1 and Wnt signaling pathway which maybe mediated by TACE.

\section*{Materials and Methods}

\subsection*{Mouse strains and genotyping}

\textit{Uncv} mice were maintained in a BALB/c background in a specific pathogen-free environment. \textit{Uncv} mice were bred in a heterozygous mating heterozygous format. The genotyping primers were \textit{iRhom2} fwd, 5’-CACAGCCCAGTGGTTGGGGTCA -3’, and \textit{iRhom2} rev, 5’-GAGGGCGCGGCTGCTGAAGCT -3’. In wild-type mice, \textit{iRhom2} was amplified by standard PCR to yield a 469 bp fragment. In \textit{Uncv/Uncv} mutant mice, \textit{iRhom2} was amplified to yield a 160 bp fragment. Wild-type mice littermates were used as controls. All animal studies were approved by the Review Board of the Institute of Radiation Medicine, Beijing, China.

\subsection*{Plasmid construction}

Full-length and mutant \textit{iRhom2}^\textit{Uncv} were individually cloned into the pcDNA3.0-Flag vector using the primers \textit{iRhom2} fwd, 5’-cccaagcttatggcctcagctgacaagaatggcagcaacctccca-3’, and \textit{iRhom2} rev, 5’-ccggaattcttagtgtagcacctggtctagctcg-3’. Myc-tagged mouse EGF in pcDNA3.1 plasmid was a kind gift from Dr. Matthew Freeman \cite{2}.

\subsection*{Cell culture, transfection and immunoprecipitation}

293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37°C in a humidified incubator with 5% CO\textsubscript{2}. \textit{iRhom2} plasmids and the pcDNA3.0-Flag
vector were transfected into 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The immunoprecipitation was performed as described previously [42].

**Histological analysis, immunohistochemistry and immunofluorescence**

Dorsal skin tissue samples were fixed in 4% paraformaldehyde at 4°C overnight, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin for histological analysis. Immunohistochemistry and immunofluorescence was performed as described previously [43, 44]. For the iRhom2 antibody-blocking experiment, iRhom2 antibody was pre-mixed with iRhom2 antibody (N-terminus) blocking peptides (Abgent) before incubation with the sections.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay**

The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed on 4-μm-thick sections of dorsal skin using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Roche) following the manufacturer’s directions.

**RNA isolation and real-time PCR**

Total RNA was isolated from the dorsal skin using TRIzol reagent (Life Technologies) following the manufacturer’s protocol, and cDNA was then prepared using the Prime Script RT reagent kit (TaKaRa) according to the manufacturer’s instructions with oligo-dT primers. SYBR Premix Ex Taq (TaKaRa) was used for real-time quantification, and gene expression was normalized to GAPDH using the ΔΔcycle threshold method. Primer sequences are available upon request. To assess the iRhom2 expression separately in the epidermis and dermis, dorsal skin was incubated in 0.25% trypsin at 4°C for 24 to 48 hours; the epidermis was removed as soon as it could be separated from the dermis as an intact sheet.

**Luciferase assays**

HeLa cells were transfected with Notch-dependent CSL luciferase reporter containing CSL binding sites, Renilla luciferase reporter, iRhom2 and iRhom2Uncev plasmids. Renilla luciferase was used as a transfection control and signals are given as fold Firefly/Renilla corrected for background. 48 h after transfection, luciferase-reporters activity was measured. Datas are representative of at least three independent experiments.
Statistical analysis
All results are presented as the means ± SE. All statistical analyses were performed using the SPSS software. The significance of the differences between groups was determined using Student’s t-test; P<0.05 was considered significant.

Supporting Information
S1 Fig. Genotyping of wild-type, iRhom2Uncv/+ and iRhom2Uncv/Uncv mice. (A) All homozygous Uncv/Uncv mice are hairless, and all heterozygotes (HE) are sparsely coated. (B) The PCR genotyping of iRhom2 revealed a 469 bp wild-type PCR product and a 160 bp mutant product. Heterozygous mice produced both the 469 bp and 160 bp products.
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S2 Fig. Expression of iRhom2 in mouse skin. (A) Real-time PCR analysis of iRhom2, iRhom2# (The PCR primers were located in the deletion region of iRhom2Uncv), mRNA expression in the dorsal skin of wild-type and iRhom2Uncv/Uncv mice at P5. n=3, **P<0.01. (B, C) Immunofluorescence staining of iRhom2 at P9 mouse dorsal skin from wild-type mice. (D, E) Immunofluorescence staining of iRhom2 and TACE in the dorsal skin of P9 wild-type and iRhom2Uncv/Uncv mice. Scale bars: (B–E), 12.5 μm.
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S3 Fig. Histology of the dorsal skin from wild-type and iRhom2Uncv/Uncv mice. (A–F) Histology of the dorsal skin from wild-type and iRhom2Uncv/Uncv mice at P18, P22 and P32, respectively. Scale bars: (A–F), 100 μm.
doi:10.1371/journal.pone.0115114.s003 (TIF)

S4 Fig. Expression levels of β-catenin, phospho-Smad1/5/8 and Hoxc13 in iRhom2Uncv/Uncv follicles. (A–F) Immunofluorescence staining of β-catenin, phospho-Smad1/5/8 and Hoxc13 in the dorsal skin of P9 wild-type and iRhom2Uncv/Uncv mice. Scale bars: (A–F), 12.5 μm.
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S5 Fig. Excessive macrophages infiltration in iRhom2Uncv/Uncv mice skin. (A, B) Immunohistochemistry staining of skin with anti-F4/80 antibodies to detect macrophages in wild-type and iRhom2Uncv/Uncv mice at P5. (C) Number of F4/80-positive cells per 20× field in the dermis of P9; n=5, **P<0.01. Scale bars: (A, B), 25 μm.
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Author Contributions
Conceived and designed the experiments: LW YL. Performed the experiments: LW YL LB XY WS. Analyzed the data: LW YL LY PR ZL. Contributed reagents/materials/analysis tools: WD SS YH ZG. Wrote the paper: LW YL.

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