The "Bald Mill Hill" Mutation in the Mouse Is Associated with an Abnormal, Mislocalized HR bmh Protein

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We have previously identified a mutation in the mouse hairless locus—hairless rhino bald Mill Hill (Hr^{rhbmh}). The genetic alteration in these mice consists in a large 296 bp deletion at the 3′ part of the *hairless* gene (ID:MGI:3039558; J:89321). Here, we show that this deletion removes the stop codon and creates a new reading frame at the C terminus of the hairless protein, generating a larger mutant protein harboring an additional sequence of 117 amino acids. The mutant *hairless* gene mRNA is expressed during the embryonic and post-natal development of the hair follicle. The mutant protein is identified in bmh mouse skin at different stages of development by a specific antibody. We demonstrate that the HR bmh protein is able to interact with the vitamin D receptor (VDR), but is not able to repress VDR-mediated transactivation. Immunofluorescence analysis reveals that HR bmh protein displays an abnormal cellular localization in transfected cell lines, as well as in the epidermis and hair follicle of bmh mutant mice. We discuss the relevance of the hairless protein mis localization in cell signalling pathways and with respect to the specific skin phenotype of mouse hairless mutants.

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INTRODUCTION

The *hairless* gene in mammals encodes a nuclear factor that is highly expressed in skin and appears to control hair follicle integrity and cycling (Panteleyev *et al.*, 1998b). The *hairless* gene function is required for correct transition from hair follicle morphogenesis to entry in and maintenance of the hair cycle all along the life of a mammal. In the absence of a normal and functional Hairless protein, the hair bulb undergoes premature apoptosis during the first catagen stage of the hair cycle. The most striking effects of the mutation are loss of hair follicles and formation of epidermal utricles and dermal cysts (Panteleyev *et al.*, 1998c). The *hairless* gene expression appears to be widespread and temporally regulated. In wild-type mice, hairless mRNAs were detected in skin, brain, cartilage, gonads, thymus, and colon (Cachon-Gonzalez *et al.*, 1999). In addition to alopecia, hairless mice strains show subtle defects in the development and differentiation of various tissues and organs (Panteleyev *et al.*, 1998a; San Jose *et al.*, 2001; Zhang *et al.*, 2005).

The HR protein is localized in cell nuclei and functions as a transcriptional regulator. Although its role has not been resolved in molecular terms, it was demonstrated that HR is able to behave as a corepressor for nuclear receptors (Potter *et al.*, 2001). HR seems to be a part of a large multiprotein complex, able to repress transcription by its association to chromatin remodelling factors such as histone deacetylases (Djabali *et al.*, 2001; Potter *et al.*, 2001, 2002). In particular, recent experimental data suggest that HR binds directly to the vitamin D receptor (VDR) (Hsieh *et al.*, 2003) and that *HR* gene overexpression is able to repress vitamin D-stimulated transcriptional activity in cultured cell lines including human keratinocytes (Hsieh *et al.*, 2003; Xie *et al.*, 2006). Indeed, the phenotype of hairless mutant mice is comparable to that of VDR-null animals and human patients with mutations in the VDR gene, where hair development occurs normally, but the hair cycle is blocked at the first catagen stage (Li *et al.*, 1997; Miller *et al.*, 2001). Recently, it has been shown that HR repressor, by its ability to bind to and repress VDR action, is a key player in the sequential regulation of keratinocyte differentiation by vitamin D (Bikle *et al.*, 2007). It has also been reported that HR might be involved in cell adhesion

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Abbreviations: bmh, bald Mill Hill; HR bmh, hairless rhino bald Mill Hill; HDAC5, histone deacetylase 5; PBS, phosphate-buffered saline; VDR, vitamin D receptor

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modulation, *Hox* gene regulation, Wnt signalling, and establishment of hair follicle progenitor cell identity (Brancaz *et al.*, 2004; Beaudoin *et al.*, 2005; Thompson *et al.*, 2006). At least in the skin, but probably in other organs, the HR repressor seems to be responsible for the timing of epithelial cell differentiation. We have recently identified a mutation in the mouse hairless locus—hairless rhino bald Mill Hill (Hr^{rhbmh}). The genetic alteration in these mice consists in a large 296 bp deletion at the 3′ part of exon 19 of the hairless gene (ID:MGI:3039558; J:89321) (Brancaz *et al.*, 2004). In this paper, we show that this deletion removes the stop codon and creates a new reading frame of the hairless protein, generating a mutant product, HR bmh, harboring an additional sequence of 117 amino acids at its C-terminal end. This higher molecular weight protein is present in the embryo and at different stages of post-natal development. We show that the mutant bmh protein can interact with the VDR but is not able to repress VDR-mediated transcriptional activity. In contrast to its wild-type counterpart, HR bmh is mislocalized in the cytoplasm of transfected cell lines and epidermal and hair follicle cell populations. Our data contribute to refine current views on Hairless protein function in the molecular interactions responsible to shape morphogenesis and cycling of the hair follicle.

RESULTS

The mutant hairless gene is transcribed during development of rhino bmh mice

The molecular alteration in the hairless locus of bmh mutants consists in a 296-base-pair deletion at the 3′ end of the gene. More precisely, this deletion removes the last 6 nucleotides of exon 19 and a large part of the *hairless* gene 3′-UTR (Brancaz *et al.*, 2004; Figure 1a). This result allowed us to design specific primers for this part of the hairless locus and test the patterns of gene expression at different time points of epidermis and hair follicle development by reverse transcriptase PCR (RT-PCR). Such analysis is available for none of the dozen hairless alleles described to date in mice. Strikingly, the product of the bmh mutant locus was present at all stages of mid-embryonic to post-natal development. Figure 1b shows the RT-PCR product of the *hairless* gene expression in newborn mutants. The same bands of about 700 and 400 bp for the wild-type and mutant gene transcripts respectively were detected at embryonic day 15.5 (E15.5) and at post-partum day 10 (P10), P15, and P30 (data not shown). To validate the RT-PCR data, we investigated the tissue-specific *hairless* gene expression patterns in the mutants by non-radioactive RNA *in situ* hybridization. As shown in Figure 1c and d, the signals are

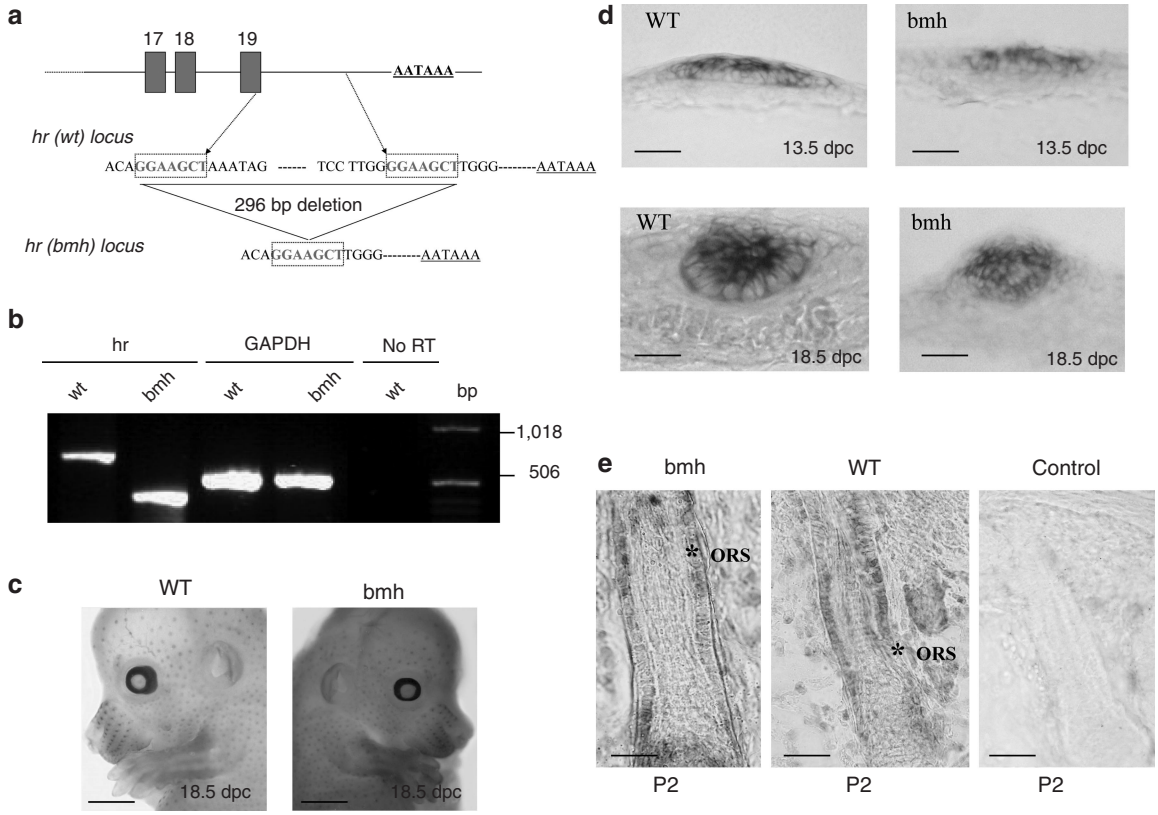

Figure 1. The hairless gene expression is unaffected in Hr^{rhbmh}/Hr^{rhbmh} mice. (**a**) Schematic representation showing the location of the deletion at the hairless locus of the bmh mice. The genomic region encompassing the previously described deletion (Brancaz *et al.*, 2004) was amplified by PCR and the products were sequenced. (**b**) RT-PCR analysis of Hr gene expression in wild-type and mutant mice. (**c**) Whole-mount *in situ* hybridization of E18.5 wild-type and Hr^{rhbmh}/Hr^{rhbmh} embryos with hairless gene-specific DIG-labelled RNA probe. (**d**) Cryostat sections of embryonic whole-mounts at E13.5 and E18.5. (**e**) *In situ* hybridization of skin sections from Hr^{rhbmh}/Hr^{rhbmh} and +/+ mice showing specific hairless gene expression in the cells of the outer root sheath of the hair follicle in mutant and wild-type mice at 2 days post-partum (P2). ORS = outer root sheath. Bars = 100 μm.

localized in the hair placode at the very beginning of its morphogenesis in the mutant embryo as well as in its wild-type counterpart. Histological analysis of E13.5–E18.5 embryos confirms the specificity of *hairless* gene expression in the epidermal thickenings at the origin of the follicular placodes. In fact, this expression is restricted to the cells of the hair primordia and is not detected in the zone of the interfollicular epidermis (Figure 1d). Moreover, *hairless* transcripts persist in the cells of the outer root sheath of the hair follicle of newborn mice and in the utricles well after the establishment of fully blown hairless phenotype 3 weeks after birth (Figure 1e and data not shown). The expression of the *hairless* gene in the mutants is therefore not different from that of their wild-type counterparts. Sequence analysis of the mutant locus has shown that the polyadenylation signal of the *hairless* gene is not affected by the deletion. This observation correlates with the presence and detection of a mature RNA of the mutant allele. The correct spatial and temporal accumulation of the mutant transcript raised the question about its fate at the level of translation.

The HR bmh mutant protein is bigger than its wild-type counterpart

Having established that *hairless* gene transcription is not affected in bmh mice, we set out to investigate the effects of this deletion on the translation and the integrity of the protein product. Using the Bioedit software, we introduced deletion in the published sequence of the mouse *hairless* gene cDNA to examine the possible products of mRNA translation. Strikingly, the mutant protein designed by the software was longer than its wild-type counterpart. Comparison of the two sequences reveals the creation of a new open reading frame. In fact, this deletion—eliminating the last six nucleotides of exon 19 of the wild-type mRNA, as well as the stop codon and a substantial part of the 3′-UTR—leads to the synthesis of a protein characterized by the absence of the last two amino acids of the normal HR protein and containing an additional motif of 117 amino acids at the C′-terminal portion of the HR bmh protein (Figure 2b and c). To verify the existence of this mutant product *in vitro* and *in vivo,* we cloned both cDNAs in eukaryotic expression vectors (see Materials and Methods). We then tested the *in vitro* protein synthesis by incubating

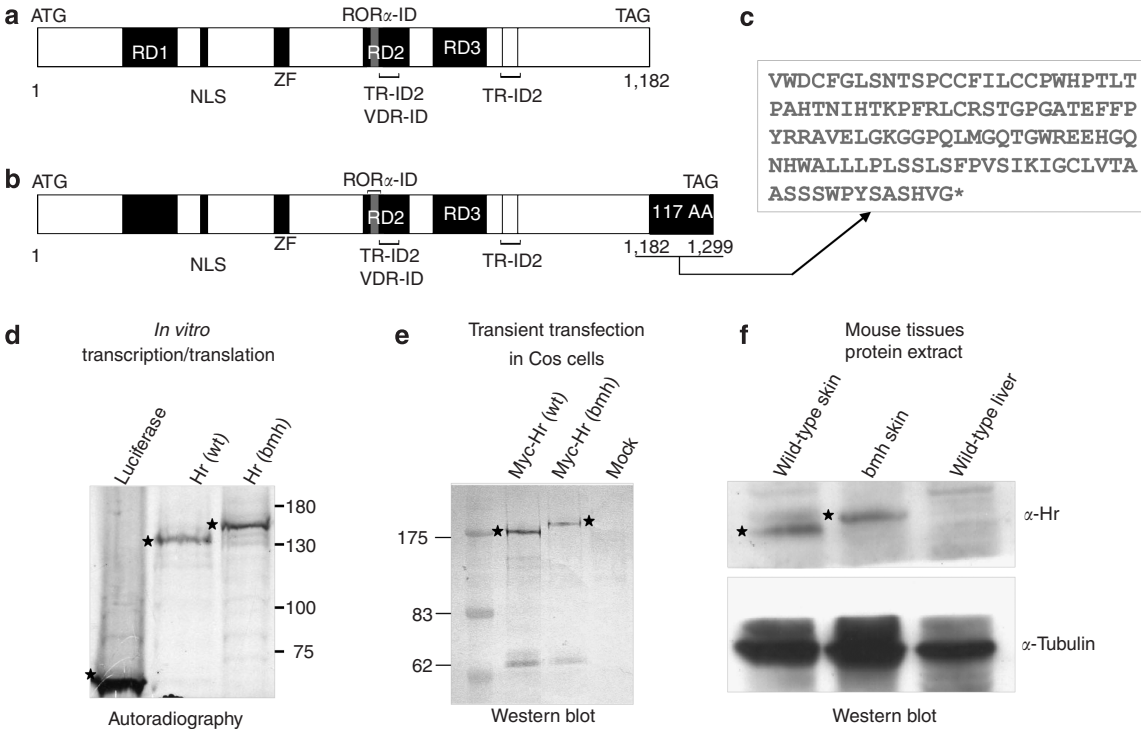

Figure 2. Deletion in the hairless gene results in loss of the termination codon and creation of a new reading frame coding for a supplementary 117-amino-acid sequence leading to a larger Hairless protein, HR bmh. (**a**) Schematic representation of wild-type Hairless protein. HR domains are defined as follows: repression domains (RD1, 2 and 3), RORa interaction domain (RORa ID), TR interaction domains (TR IDs), and VDR interaction domains (VDR IDs). The nuclear localization signal and the putative zing -inger domain are also indicated (see Nonchev *et al.*, 2006; Thompson *et al.*, 2006). (**b**) Schematic representation of the Hairless (bmh) protein. In addition to the functional domains present in the HR (wt) protein, the HR bmh protein contains a supplementary 117-amino-acid sequence (AS 117) at the C-terminus. (**c**) Amino-acid sequence of the C′-AS117. (**d**) *In vitro* transcription/translation of the Hr(wt) and Hr(bmh) cDNA. *In vitro* transcription/translation was carried out as described in Materials and Methods. (**e**) Wild-type and bmh Hr cDNA were Myc6-tagged and transiently transfected into Cos cells. Proteins were analyzed for the presence of HR(wt) and HR(bmh) by Western blotting. (**f**) HR(wt) and HR(bmh) detected by Western blot analysis with anti-HR-specific antisera in whole-cell protein extracts prepared from the skin of wild-type and bmh mice, respectively. Sizes of molecular mass markers (kDa) are indicated.

cloned cDNAs in rabbit reticulocytes transcription/translation system. The autoradiographs clearly show that *in vitro* translation of the wild-type cDNA results in the synthesis of a 130 kDa protein, which is approximately the size of the product expected for the wild-type HR protein. As anticipated, translation of the mutant cDNA generates a larger product of an apparent molecular weight of about 150 kDa, in adequacy with the putative presence of an additional sequence of 117 amino acids (Figure 2d). Then, we tested whether such HR mutant protein could well accumulate *in vivo*. To answer this question, Cos cells were transfected by vectors expressing Myc-tagged wild-type and bmh mutants cDNAs. The raw protein extracts of transfected cells were run on SDS-PAGE and the tagged proteins were revealed on Western blots by an anti-Myc antibody. As shown in Figure 2e, the mutant HR protein is well translated *in vivo* and presents a higher molecular weight than its normal counterpart. Finally, immunoblots of whole protein extracts from skins of +/+ and Hr^{rhbmh}/Hr^{rhbmh} mice at different embryonic and post-natal stages with anti-HR antibody confirmed that the higher molecular weight HR protein is produced specifically in skin development (Figure 2f). These data indicate that the 296 bp deletion at the hairless locus generates in homozygous bmh mice an HR protein whose functional properties and cellular localization are unknown.

The bmh mutant protein is still able to associate *in vivo* with the vitamin D receptor

Although the HR mutant protein is expressed *in vivo* and apparently conserves the integrity of all functional domains identified and described to date (see Figure 2b), the phenotype observed in bmh mice suggests that its function is impaired at least in epidermis and hair follicle development. To address the molecular nature of this deficiency, we started by analyzing its ability to interact with the VDR, which in the skin is a known partner of the wild-type HR protein (see Introduction). The fact that we were able to identify, clone, and translate the mutant bmh protein gave us molecular tools to explore further its functionality in cell culture experiments.

Whole-cell protein extracts from Cos cells co-transfected with Flag-tagged VDR and either wild-type hairless or bmh mutant Myc6x-tagged proteins were subjected to immunoprecipitation using a Flag antibody as described in Materials and Methods. Our immunoprecipitation results (Figure 3a) clearly show that the anti-Flag antibody, recognizing the fusion protein Flag VDR, is able to immunoprecipitate wild-type as well as bmh mutant HR proteins detected by Western blot using an anti-Myc antibody (Figure 3a, lanes 4 and 5, asterisks). No proteins were immunoprecipitated by the anti-Flag antibody when whole extracts from cells co-transfected with Flag and Myc empty vectors were used in the assay (Figure 3a, lane 6). These data strongly suggest that the fusion HR bmh co-immunoprecipitate and therefore can bind efficiently to its known VDR protein partner.

The bmh mutant protein is unable to repress VDR-mediated transactivation

The nuclear receptor corepressor HR associates with TR (Potter *et al.*, 2001), VDR (Hsieh *et al.*, 2003), and ROR (Moraitis *et al.*, 2002) in various cellular contexts, and has been shown to suppress VDR-mediated transactivation (Hsieh *et al.*, 2003). To tackle the repressor functions of the mutant bmh protein, Cos cells were co-transfected with the reporter plasmid Gal4-TK (thymidine kinase) luciferase along with hemagglutinin-VDR (HA-VDR)- and Gal4-RXR-expressing constructs in the presence of either HR wild type or HR bmh. Co-transfected cells were then cultured for 24 hours in the presence of vitamin D ($1,25(OH)_2D_3$) before measuring the luciferase activity. Our results show that wild-type HR protein represses efficiently ligand-stimulated VDR-mediated reporter expression, whereas HR bmh protein has no repressive effect on VDR-mediated transactivation (Figure 3b, compare transfections 6 and 7). The HR repression effect is VDR dependant, as both wild-type and mutant HR proteins do not affect reporter transcription when the VDR plasmid is omitted in the co-transfection assay (lanes 2–4). Taken together, these data illustrate a sharp loss in the repressive capacity of the mutant HR protein. This observation is quite unexpected, provided that HR repression domains involved in VDR interaction are not affected in hairless bmh mutant protein. To trace this loss of repressive function, we decided to look at the subcellular localization of the HR bmh mutant protein in a wide variety of cultured cell lines.

The HR bmh protein is mislocalized in the cytoplasm

The fact that the HR bmh protein was able to associate with VDR but failed to repress VDR-mediated transactivation is intriguing. This prompted us to investigate the cellular distribution of all these proteins in cell cultures. It is established that the wild-type Hairless protein is a nuclear protein and its localization is tightly associated to nuclear matrix-specific bodies (Thompson and Bottcher, 1997; Djabali *et al.*, 2001; Potter *et al.*, 2001). We therefore first tried to address the subcellular localization of the HR bmh protein. By cloning wild-type and mutant cDNAs in vectors tagged with the HA and the Flag epitopes, we generated expression constructs to transfect a variety of cell lines in culture. We then analyzed the expression and the localization of the mutant protein and its wild-type counterpart in these cell types by indirect immunofluorescence. Figure 4 shows that in all of the transfected lines the wild-type HR protein is nuclear, whereas the bmh mutant product is localized only in the cytoplasm and totally absent in the nuclei. As the transfection efficiency varies from line to line, at least 10–20 fields were analyzed for each construct and for every cell line. To confirm the authenticity of this protein mislocalization, in addition to the lines NIH3T3, 293, and Cos, we transfected mouse pituitary GHFT1 cells, mouse epidermal keratinocytes MCA-3D, and human epidermal keratinocytes HaCat. In all of the transfected lines, HR bmh protein was present in the cytoplasm. However, variations were observed in its cytoplasmic distribution pattern within the transfected lines. If in Figure 4 the mutant protein is

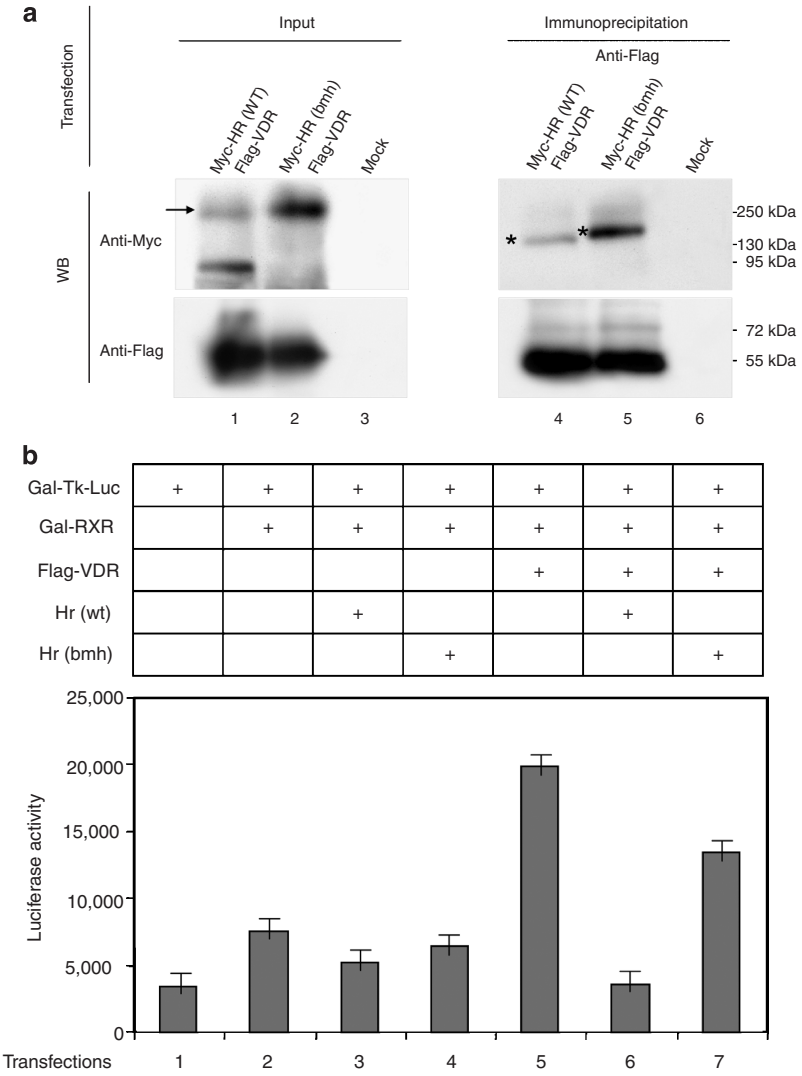

Figure 3. HR bmh binds to VDR, but is unable to repress VDR-mediated transactivation. (**a**) HR bmh co-immunoprecipitates with the nuclear receptor VDR in Cos cells. Co-immunoprecipitation experiment was carried out as described in Materials and Methods. Cos cells were transfected with HA-tagged VDR plasmid together with either Flag-tagged Hr(wt) or Flag-tagged Hr(bmh). Whole-cell extracts were immunoprecipitated with Flag antibody, subjected to Western blot analysis, and probed with anti-Flag and anti-HA antibodies. Input indicates that 1/10 of the whole-cell extract is used for immunoprecipitation. (**b**) HR bmh fails to repress VDR-mediated transactivation. Cos cells were transfected with Gal4-TK-Luc reporter, Gal4-mRXR, HA-VDR, along with either Hr(wt) or Hr(bmh) expression plasmid, as indicated. Each transfection was carried out with a normalizing reporter, pRL encoding *Renilla* luciferase. The amount of expression plasmids was kept constant by balancing the total amount with empty vector. Quantification is from a representative experiment performed in duplicate.

evenly distributed in the cytoplasm, we found a high variability in the repartition of the bmh product in the cytoplasmic compartment of the transfected cells. Indeed, in some cases, strong staining was detected in a dense cytoplasmic mass with compact or more decondensed perinuclear localization, whereas in other cells the immunofluorescence was distributed in a punctuated way, localized in compact speckles of variable size all over the cytoplasmic area (data not shown). A rough estimation indicates that in about 10–20% of the cells analyzed, HR bmh protein expression is not uniform, but rather concentrated in discrete cytoplasmic regions. Considering that the nuclear localization signal is intact in the mutant HR bmh protein, this abnormal cytoplasmic localization is likely to be associated with the presence of an additional sequence of 117 amino acids (C′-AS117). An exogenous nuclear localization signal, added at the N-terminus of the bmh mutant protein, was unable to target the protein in the nucleus (data not shown).

HR bmh is unable to sequester the VDR receptor in the cytoplasm

Next, we asked whether the association between HR bmh and VDR established in our immunoprecipitation experiment would affect the cellular distribution of these two proteins. HR bmh could well sequester the VDR in the cytoplasm, or in contrast, VDR would drag the mutant protein to the nucleus, which is less likely, as VDR is able to activate transcription despite the presence of the hairless mutant protein. Using the

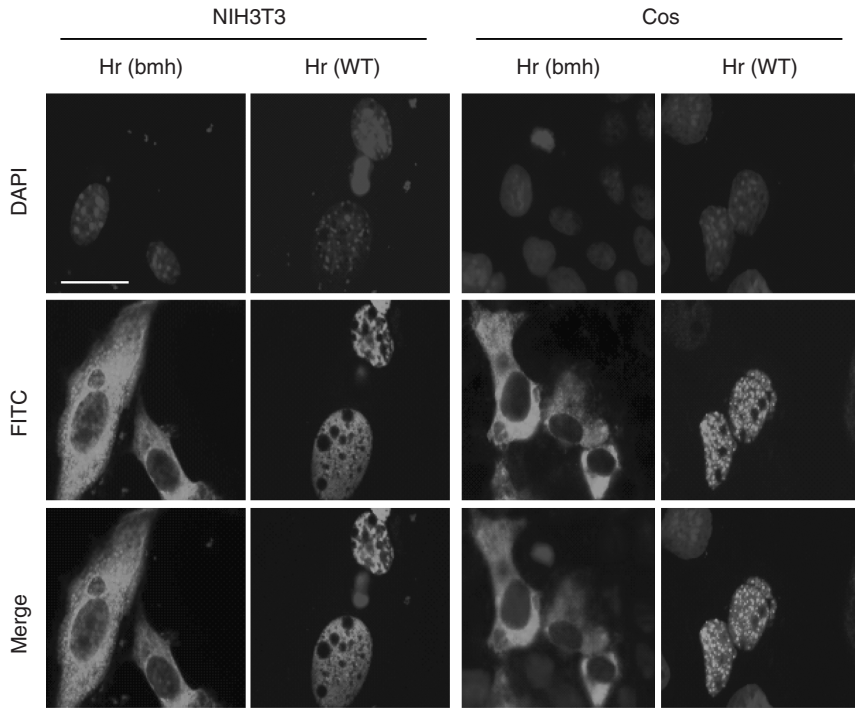

Figure 4. The additional 117-amino-acid sequence at the C-terminus (C′-AS117) abolishes HR bmh nuclear localization in mammalian cell lines. Flag-tagged Hr(wt) or Flag-tagged Hr(bmh) plasmids were transiently transfected into Cos and NIH3T3 cell lines. Subsequent to the transfection procedure (24–48 hours), cells were fixed and stained as outlined in Materials and Methods. Cells were viewed on an inverted fluorescence microscope using a magnification × 60 objective.

reagents described above, we performed co-transfections of Cos cells by HR bmh—Flag and VDR-HA constructs. Figure 5 illustrates the separate localization of both proteins: VDR (in red) remains nuclear, whereas HR bmh (in green) is more or less uniformly distributed over large cytoplasmic regions. This result indicates that the binding capacities of these two proteins are not sufficient to modify their respective cellular localizations. To further validate these observations in a more physiological context, we decided to explore the *in situ* distribution of the HR bmh and VDR proteins in embryonic and adult epidermis and hair follicles of Hr^{rhbmh}/Hr^{rhbmh} mutant mice.

The HR bmh protein is mislocalized in adult mutant skin

Immunohistochemistry experiments were first carried out on whole-mounts of E16.5 Hr^{rhbmh}/Hr^{rhbmh} mouse embryos using our anti-HR antibody. At this stage, the hair peg is well defined with epidermal cells invaginating to form all the concentric structures of the future follicle with mesodermal condensations ready to shape the dermal papillae. As shown in Figure 6a, the HR bmh protein is well detected in the mutant embryos. However, the cellular resolution of our whole-mount immunostainings does not allow defining unequivocally the nuclear *versus* cytoplasmic distribution of the protein. In sagittal and transversal sections of whole-mount preparations, the protein seems to be localized at the periphery of the majority of cells forming the placode and the hair peg. However, in other cells of the same structures, the staining does not display a privileged subcellular localization (Figure 6b). To compare the patterns of HR and VDR at this stage, we performed immunostaining with anti-VDR antibody used in cell transfection experiments. As with the anti-HR antibody, the analysis of whole-mounts and sections of VDR-stained embryos did not allow to discriminate nuclear *versus* cytoplasmic distribution at this stage (Figures 6e and f). To circumvent this lack of resolution, we studied both HR bmh and VDR localization in the adult mutant skin by immunostaining of frozen histological sections.

Histological sections of P21 Hr^{rhbmh}/Hr^{rhbmh} mouse skins were probed with the anti-HR antibody and analyzed by immunofluorescence. Figure 6c and d shows a sagittal section of an utricle with the cells of the utricle epithelium where the cell nuclei and cytoplasm are clearly distinguished. In these sections, the anti-HR staining is detected mostly and with a much higher intensity in the cytoplasm of the utricle epithelial cells. Control longitudinal sections of full-size wild-type hair follicles clearly show nuclear localization of the HR protein in most of the cell layers surrounding the hair shaft (Figure 6i–n). We then performed immunohistochemical staining, using the anti-VDR antibody, of frozen sections at the same stage. It is interesting to note that specific nuclear staining was detected with this antibody. At high magnification, one can clearly see that the nuclei of cells forming the utricle (Figure 6g) and the interutricular epithelium (Figure 6h) display few zones or spots with strong immunoreactivity, reminding a nucleolar pattern of localization. These results confirm the observations in cell transfection experiments and suggest that the cytoplasmic

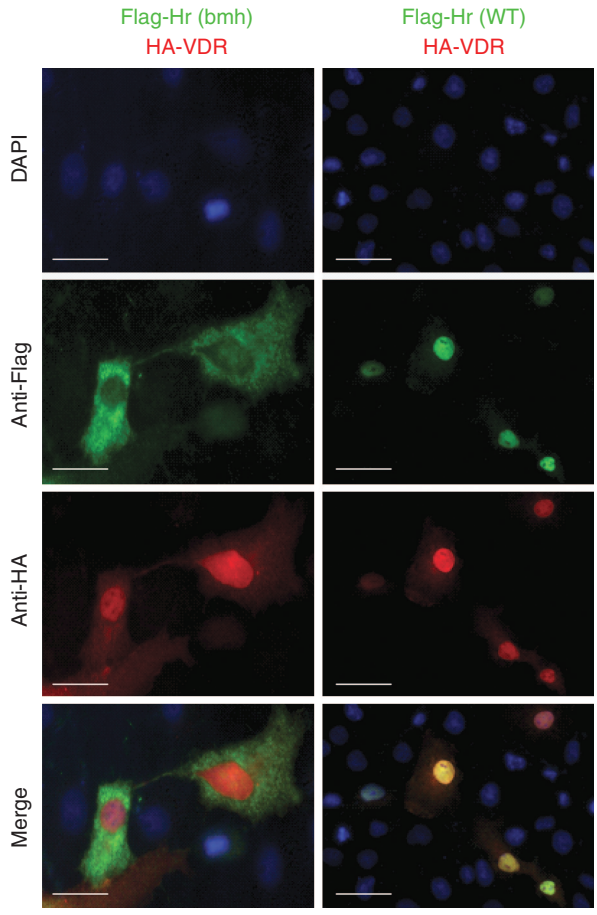

Figure 5. The cytoplasmic HR bmh protein has no influence on VDR nuclear localization. The results of immunofluorescence assay show Cos cells co-transfected with HA-tagged VDR together with Flag-tagged Hr(wt) (right) or Flag-tagged Hr(bmh) plasmid (left). After 48 hours, cells were processed with anti-Flag and anti-HA antibodies. HR wt colocalizes in the nucleus with VDR. HR bmh remains cytoplasmic, whereas VDR shows nuclear localization.

mislocalization of the HR mutant protein might be a primary event in the cascade of molecular interactions underlying the hairless bmh phenotype.

DISCUSSION

HR and VDR in anagen reinitiation

Unravelling the capacity of hair regrowth in molecular terms remains a challenge in skin biology. Among multiple candidates, *Hr* and *Vdr* genes seem to be particularly implicated in the control of hair cycle. Their mutant phenotypes specifically affecting this process have striking similarities, and the capacity of their protein products to physically interact is well documented (Li *et al.*, 1997; Hsieh *et al.*, 2003; Xie *et al.*, 2006). In a simple scenario, if the HR bmh mutant protein loses its nuclear localization, it would not be able to associate with VDR. However, the type of interaction between HR and VDR in the hair follicle remains to be elucidated. The HR bmh product is able to interact physically in transfected cells with the VDR. Despite the integrity of its RDs and VDR-IDs, it is neither able to repress ligand-dependant or ligand-independant VDR-mediated reporter transcription, nor modify the VDR nuclear localization. In the case of the bmh mutants, the fine-tuning of these activities could be much more subtle as the availability of residual nuclear HR bmh could not be ruled out, and we know very little about its ability to transit between nucleus and cytoplasm. Very recently, it has been shown that *Hr* expression is boosted in *Vdr* null mice, indicating that HR might be acting downstream of VDR (Bikle *et al.*, 2006). Our immunostaining data confirm this observation in the sense that VDR is specifically expressed in the nuclei of bmh P21 utricles and epidermis, apparently in the absence of HR. It is tempting to speculate that in the hair follicle intense HR–VDR synergy is particularly required in the process of catagen–anagen transition for a balanced control of the cell proliferation machinery. However, a detailed exploration of the *Hr* gene regulatory regions for VDRE should be performed to shed more light on protein partnerships responsible to drive this interaction and confer spatial and temporal specificity of its effects on hair cycling.

Cytoplasmic HR in the temporal and spatial control of hair cycling

According to a current model based on transgenic rescue and knockout data, high expression of *Hr* in the nuclei of the ORS in early catagen represses Wnt signalling modulators like Wise and Soggy, which is crucial for correct differentiation of progenitor keratinocytes and entry (re-entry) in anagen (O'Shaughnessy *et al.*, 2004; Zarach *et al.*, 2004; Beaudoin *et al.*, 2005). Our data contribute to refine this concept of hair follicle differentiation and cycling. Indeed, the alteration in HR subcellular localization offers a tool to challenge and test the existing hypothesis about exactly how HR interferes with Wnt signalling. Monitoring Wise and Soggy expression and distribution in bmh mutants as compared to the *Hr* −/− knockout mice would shed more light on the precise molecular way *Hr* represses Wnt signalling modulators. Transfections and gel shift experiments with the HR bmh protein would help to resolve the question of direct *versus* indirect implication of *Hr* in regulating Wnt modulators. Our previous data suggest that in mid-embryogenesis, Hr^{rhbmh} interferes with *Hox* gene regulation (Brancaz *et al.*, 2004). As some Wnts and the gene Wise itself are supposed to interact with *Hox* genes in various cell contexts (Itasaki *et al.*, 2003; Arata *et al.*, 2006; Nordstrom *et al.*, 2006), it is conceivable that different *Hr* mutations would help to reveal more factors in the cascades required for anagen re-initiation. On the other hand, bmh mutants are likely to be instrumental to tackle effectors and targets of the Wnt signalling in the cytoplasmic compartment, or to explore the perturbations in the outer root sheath (encompassing the bulge region) during the catagen phase of the first hair cycle. Our present data show that the HR bmh protein is cytoplasmic at this period of hair follicle (utricle) development. Using markers of HF stem cells, we have started analyzing the fate of the cell populations forming the bulge region in Hr^{rhbmh}/Hr^{rhbmh} mice. Our first results indicate that the formation of this territory is severely disrupted in bmh mutant mice (D. Salameire *et al.*, unpublished).

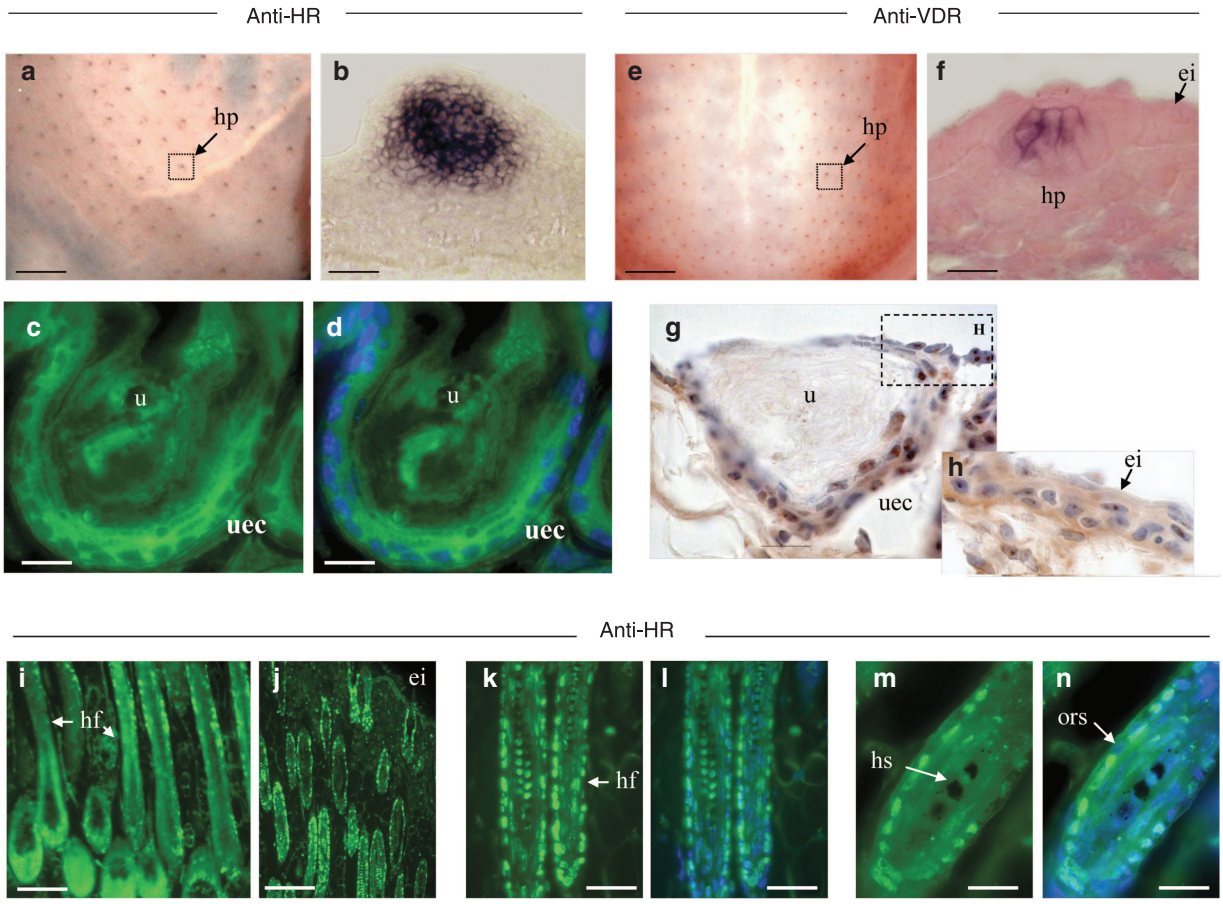

Figure 6. Immunostaining of the HR and VDR proteins in Hr^{rhbmh}/Hr^{rhbmh} embryos and adult mice. (**a** and **e**) HR (**a**) and VDR (**e**) proteins are detected in the hair pegs of E16.5 whole-mount immunostained mutant embryos. (**b** and **f**) Cross-sections through the hair pegs show that both HR and VDR are specifically expressed in the population of invaginating epidermal cells but not in the interfollicular epidermis. Note that (**b**) shows a section of optimal cutting temperature-embedded and frozen whole mount, whereas (**f**) is a section of paraffin-embedded whole mount. (**c** and **d**) At P21, HR protein is detected in the cytoplasm of the utricle epithelial cells in the skin of Hr^{rhbmh}/Hr^{rhbmh} mice. Note the strong staining of the cytoplasmic compartments in the majority of the utricle epithelial cells. Panel **c** illustrates FITC-conjugated anti-HR staining, whereas in (**d**) cell nuclei are visualized with Hoechst (Sigma). (**g** and **h**) VDR detection in the nuclei of utricle epithelial cells (**g**) and interutricular epidermis (**h**). Note the strong immunoreactivity in specific spots of the cell nuclei. (**i–m**) Longitudinal control sections of full-size hair follicles illustrating the nuclear localization of HR in wild-type mice. Abbreviations: hp = hair peg; u = utricle; uec = utricle epithelial cells; iue = interutricular epidermis; hf = hair follicle; ei = interfollicular epidermis; ors = outer root sheath; hs = hair shaft. Bars = 1 mm (**a** and **e**); 10 μm (**b** and **f**); 20 μm (**i** and **j**); 100 μm (**c**, **d**, **g**, **k–n**).

A function for mislocalized HR bmh?

The genetic alterations that have been identified to underlie most of the 13 allelic mutations reported to date at the mouse hairless locus include deletions, insertions, and point mutations supposed to abolish completely its expression, or assumed to affect specifically major functional domains of the protein (Bernard, 2002; Zhang *et al.*, 2005; Nonchev *et al.*, 2006; Thompson *et al.*, 2006). Detailed analysis of a mutant *hairless* gene product is not available in the literature. We have started *in silico* and molecular studies to check if the additional 117-amino-acid sequence (C′-AS117) is responsible for HR bmh cytoplasmic localization. Till now, we have not been able to figure out a specific motif implicated in the cytoplasmic sequestration of the mutant HR bmh protein. It remains to be determined if and how the folding of the mutant HR bmh impairs the recognition of the nuclear localization signal and whether the C′-AS117 is involved in this process.

Potter *et al.* (2001) have shown that HR repressor activity might be due to its association in multiprotein complexes with histone deacetylases, in particular the class II histone deacetylase—histone deacetylase 5 (HDAC5) (Khochbin *et al.*, 2001). Interestingly, HDAC5 activity is known to be controlled by its intracellular localization. Indeed, its cytoplasmic sequestration is thought to make it unavailable for its nuclear partners (Yang and Gregoire, 2005). HR bmh could therefore be considered as an additional HDAC5-anchoring molecule that would affect the nuclear activities of HDAC5 by favoring its cytoplasmic retention.

A layer of complexity is added by the fact that HR protein harbors a characteristic JmjC domain, a motif conserved from yeast to human, which is a signature for a large family of histone demethylases (Takeuchi *et al.*, 2006). In the bmh mutants, the very C-terminal JmjC domain lies in continuity with the C′-AS117 motif described in this paper. Trapped in the cytoplasm, HR bmh, potentially able to associate with

deacetylases, could now modify complexes involved in histone demethylation and generate variable biological consequences as far as temporal and spatial gene regulation is concerned (Klose *et al.*, 2006).

In conclusion, we have identified, cloned, and characterized a mutant hairless protein that displays abnormal molecular anatomy and cellular mislocalization. Our results suggest that both *Hr* gene integrity and HR protein nuclear localization are required for the correct morphogenesis and cycling of the hair follicle. The study presents a description and analysis of a *hairless* gene mutant product. This is a precious tool to further unravel the genetic circuitry and the molecular partnerships underlying hair follicle cycling and specific skin pathology.

MATERIALS AND METHODS

Care of mice

The mutant Hr^{rhbmh} allele has been maintained on a C57 BL/6J background for more than 20 generations in the mouse house of the Institute Albert Bonniot, Grenoble, France. The mice care and sample collection procedures were according to the guidelines of the French Comité d'Ethique. For skin whole-mount and histological analysis, embryos and mice were killed by cervical dislocation at different stages of embryogenesis and post-natal development.

Plasmid constructs

Mouse EST, clone image 3710814, containing *Hr* cDNA lacking 450 bp from the 5′ was purchased from MRC Gene Service (Cambridge, UK). The 5′ missing sequence was amplified by 5′ RACE experiment. Amplification product was inserted into the EST clone to generate full-length wild-type hairless cDNA. Full-length bmh cDNA was amplified by RT-PCR and the product was cloned into a TA cloning vector (Invitrogen, Paisly, UK). Full-length wild-type *Hr* and bmh *Hr* cDNA were subcloned into pCS2-Myc6x or pcDNA3-Flag expression vectors, and cDNA encoding the wild-type human VDR was subcloned into pcDNA3-Flag expression vector. The sequences of all clones were confirmed by sequencing. Plasmid encoding fusion protein Gal4-RXR was obtained from Hinrich Gronemeyer. Plasmids Gal4-TK-LUC and pRL (encoding *Renilla* luciferase) were obtained from Saadi Khochbin.

RT-PCR to detect Hr(wt) and Hr(bmh) transcripts

Total RNA was prepared from the skin of wild-type and bmh embryos and post-natal mice using Trizol reagent (Invitrogen) according to the manufacturer's instructions. For RT-PCR, first-strand cDNA was synthesized with SuperScript II RNase H-reverse transcriptase and the PCR was carried out with *Taq* polymerase (Qiagen, Courtaboruf, France). Primer pairs that are specific for mouse hairless (forward (exon 17): 5′-GGCAGCTGCTACTTG GATGC; and reverse (3′-UTR): 5′-CCAGTAGACCTACAGAGTCGG) were used to generate a 737 bp product for *Hr*(wt) and 441 bp product for *Hr*(bmh). The mouse GAPDH primers were used to control the quality and the amount of the reverse-transcribed RNA.

Whole-mount embryos and skin *in situ* hybridization

To analyze *hairless* gene expression during development, whole-mount *in situ* hybridization with digoxigenin-labelled RNA probes was performed on E13.5 and E18.5 embryos and skin samples of P2 and 3-week-old mice.

To generate Hr-specific probe, an EST fragment of 4.2 kb from the 3′ end of the *hairless* gene was first subcloned in pBluscript KS + (Stratagene, La Jolla, CA). The resulting plasmid was then linearized with *Hind*III or *Not*1 to produce templates for sense and antisense probes, respectively. DIG-labelled cRNA probes were made as described (Xu and Wilkinson, 1998). The stained whole mounts were then embedded in optimal cutting temperature, frozen, serially sectioned with a cryotom, and the slides mounted in epox under coverslip. Skin from P2 and P21 post-natal mice were processed for *in situ* hybridization on 5 μm sections as previously described (Wilkinson and Nieto, 1993).

Transcription–translation *in vitro*

Transcription–translation was performed *in vitro* using the Promega TNT Reticulocyte Lysate System according to the manufacturer's recommendations. Comparable amounts of wild-type and mutant proteins were analyzed by SDS-PAGE by monitoring the incorporation of [^{35}S] methionine.

Antibodies

The polyclonal rabbit anti-HR antibody was generated in rabbits against a peptide that contains amino acids 1–200 of the mouse HR protein (Figure S2). It was made by the company Covalab (Lyon, France) and purified by affinity chromatography in our laboratory. Other antibodies used in this study are the mouse monoclonal M2 anti-Flag (Sigma, Chesnes, France), the rat monoclonal 3F10 anti-HA (Roche, Meylan, France), monoclonal 9E10 anti-Myc (Roche), and anti-VDR (BIOMOL, Plymouth, MA).

Preparation of skin proteins

Freshly harvested whole skin from wild-type and bmh mice was minced and homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 0.25% sodium deoxycholate, 0.25% SDS, 1 mM EGTA, 1% Nonidet P-40, and protease inhibitors). Tissues were homogenized by repeated passing through a syringe needle and then incubated on ice for 1 hour. Debris was removed by centrifugation and the tissue extracts were analyzed by Western blot.

Cell culture and transfection experiments

Cos, NIH3T3, and 293 cells were grown in DMEM containing 10% fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin. Cells were transfected with Superfect transfection reagent (Qiagen) according to the manufacturer's instructions. Mouse pituitary GHFT1 and mouse epidermal keratinocyte MCA3D (a gift from Dr A Nieto) cells were grown in DMEM, high glucose (4.5 g/l), and Ham's F12 medium, respectively, supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin. Transfections of these cell lines were performed by the calcium phosphate precipitation method.

Co-immunoprecipitation assays and Western blot analysis

Cos cells were transfected as described above with 5 μg of expression vector encoding Myc6x-tagged wild-type and bmh *hairless* gene constructs, and 3 μg of expression vector for HA-tagged-hVDR per 10 cm dish. Cells, 48 hours after transfection, were washed with cold phosphate-buffered saline (PBS) and lysed

in immunoprecipitation buffer (10% glycerol, 150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 1% Nonidet P-40, and protease inhibitors). Protein estimation of cell lysates was determined using the Bradford reagent 5Bio-Rad. An equal amount of cellular proteins was immunoprecipitated with monoclonal anti-Flag antibodies crosslinked to protein G agarose beads (Sigma) at 4°C overnight. The beads were then washed, the bound proteins eluted with 0.1 M glycine buffer, pH 2.5, and separated by SDS-PAGE followed by transfer onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat dried milk in TBST (20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.05% Tween 20) and incubated for 2 hours with mouse mAbs against either the Flag or Myc epitope. The membranes were washed three times for 10 minutes each with Tris-bufered saline Tween and then probed with secondary (anti-mouse and anti-rat horseradish peroxidase-conjugated) antibodies, washed again with TBST, and developed with ECL Western blotting detection system (Pharmacia Biotech Inc.).

Luciferase assays

Cos cells were transfected in duplicate on 12-well plates (2×10^6 cells/well) as described above. Each transfection mixture included 500 ng of pRL reporter construct (for expression of *Renilla* luciferase), normalization control (Promega, Lyon, France), 250 ng of reporter plasmid (Gal4-TK-Luc), 250 ng of pSG4-mRXR (for expression of Gal4 mRXR), 250 ng of pcDNA3-HA-VDR (for expression of VDR-HA fusion protein), and 250 ng of either pCMV-Flag-*Hr(wt)* (for expression of wild-type HR) or pCMV-Flag-*Hr(bmh)* (for expression of HR bmh protein). The cells were cultured in the presence or absence of $1,25(OH)_2D_3$ as indicated in the figure legend and then harvested 48 hours post-transfection. The luciferase reporter assays were performed according to the manufacturer's instructions (Promega).

Immunofluorescent analysis in cell cultures

Cells were plated onto a two-wells (2×10^5 cells/well) labteck chamber slide (Nalgen Nunc International). Cells were transiently transfected with plasmid constructs as described above. Two days after transfection, cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 10 minutes. The cells were then washed three times with cold PBS and permeabilized for 5 minutes with cold PBS containing 0.2% Triton X-100. Permeabilized cells were then washed three times with PBS and blocked for 30 minutes in PBS containing 5% non-fat dried milk before incubation with primary antibodies for 1 hour at 37°C in PBS containing 1% non-fat dried milk. After three washes with PBS, cells were incubated with secondary antibodies at 37°C for 30 minutes before being washed with PBS and stained with 4,6-diamino-2-phenylindole dihydrochloride in PBS. Cells were washed once again with PBS and then mounted in fluorescent mounting medium (DAKO, Trappes, France) before being observed under an epifluorescence Zeiss Axiophot microscope.

Whole-mount immunostaining of E16.5 embryos

After dissection, E16.5 embryos were fixed in 4% cold paraformaldehyde overnight, washed in PBT (PBS plus 1% Triton-X-100) then in PBTB (PBT plus 2 mg/ml of BSA), blocked for 2 hours in 10% fetal calf serum in PBTB and incubated in primary antibody diluted in PBTB with 10% fetal calf serum (1/300 dilution for anti-HR and 1/500 dilution for anti-VDR) for 8 hours or overnight. The embryos were then washed thoroughly in PBTB at 4°C, incubated with alkaline phosphatase-labelled secondary antibody, washed, and developed for 10–30 minutes in an NBT-BCIP-Levamisole solution. The stained embryos were embedded in optimal cutting temperature or paraffin, sectioned at 10 μm, and mounted under coverslip.

Immunofluorescent and immunohistochemistry analysis of P21 frozen skin sections

For immunofluorescence, frozen sections of dorsal skin from P21 mutant and wild-type mice were refixed in 5% paraformaldehyde overnight, then incubated in blocking solution and permeabilized in a hypotonic 10 mM sodium citrate solution. The slides were then incubated with primary anti-HR antibody (1/200), washed three times in dilution buffer, and incubated in an FITC-conjugated secondary antibody (anti-rabbit Alexa 488). After washing, the slides were counterstained in Hoechst, mounted under cover slip and observed under an epifluorescence Zeiss Axiophot microscope. For immunohistochemistry, the frozen sections were refixed in cold acetone, incubated in 0.5% hydrogen peroxide to quench endogenous peroxidase activity, antigen-retrieved by heating in a microwave in citrate buffer, and blocked in 1% BSA at room temperature. The sections were then incubated with primary anti-VDR antibody (BIOMOL), washed, covered by the peroxidase-coupled streptavidin/biotin complex (Dakopatts) and revealed by the DAB reagent. The slides were counterstained with hematoxylin and mounted under coverslips.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Coexpression of both wild-type and mutant HR has no effect on their respective localization.

Figure S2. Design, purification, and characterization of the anti-HR antibody.

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