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Analysis of Hairless Corepressor Mutants to Characterize Molecular Cooperation with the Vitamin D Receptor in Promoting the Mammalian Hair Cycle

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Abstract

The mammalian hair cycle requires both the vitamin D receptor (VDR) and the hairless (Hr) corepressor, each of which is expressed in the hair follicle. Hr interacts directly with VDR to repress VDR-targeted transcription. Herein, we further map the VDR-interaction domain to regions in the C-terminal half of Hr that contain two LXXLL-like pairs of motifs known to mediate contact of Hr with the RAR-related orphan receptor alpha and with the thyroid hormone receptor, respectively. Site-directed mutagenesis indicates that all four hydrophobic motifs are required for VDR transrepression by Hr. Point mutation of rat Hr at conserved residues corresponding to natural mutants causing alopecia in mice (G985W and a C-terminal deletion ΔAK) and in humans (P95S, C422Y, E611G, R640Q, C642G, N988S, D1030N, A1040T, V1074M and V1154D), as well as alteration of residues in the C-terminal Jumonji C domain implicated in histone demethylation activity (C1025G/E1027G and H1143G) revealed that all Hr mutants retained VDR association, and that transrepressor activity was selectively abrogated in C642G, G985W, N988S, D1030N, V1074M, H1143G and V1154D. Four of these latter Hr mutants (C642G, N988S, D1030N and V1154D) were found to associate normally with histone deacetylase-3. Finally, we identified three regions of human VDR necessary for association with Hr, namely residues 109–111, 134–201, and 202–303. It is concluded that Hr and VDR interact via multiple protein-protein interfaces, with Hr recruiting histone deacetylases and possibly itself catalyzing histone demethylation to effect chromatin remodeling and repress the transcription of VDR target genes that control the hair cycle.

Keywords

calcitriol receptors; histone deacetylase; histone demethylase; human HR protein; rat hr protein; Jumonji domain

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The hairless protein (Hr), a 130 kDa nuclear transcription factor, is mutated in at least two forms of human alopecia: alopecia universalis congenita and atrichia with papular lesions [Ahmad et al., 1998; Ahmad et al., 1999]. Also, mouse models in which *hr* gene expression has been reduced or eliminated [Zarach et al., 2004] display a phenotype of hair loss, hyperproliferation of skin and dermal cysts. However, the molecular mechanisms by which Hr exerts its effects on the skin and on hair growth/maintenance are still being elucidated.

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Hr, which is also highly expressed in brain, has been shown to interact with the thyroid hormone receptor (TR) [Potter et al., 2001] and with the RAR-related orphan receptors (RORs), especially RORα [Moraitis et al., 2002]. In addition, some of the functional domains in Hr mediating these interactions have been mapped (Fig. 1D and Fig. 2A). Hr possesses a nuclear localization signal (NLS) from residues 437–454 [Djabali et al., 2001] and a single zinc-finger motif (amino acids 620–645) [Cachon-Gonzalez et al., 1994] (see also Fig. 2A), as well as four motifs of hydrophobic amino acids, two of the form LXXLL (where L = leucine and X = any amino acid residue) and two of the form ΦXXΦΦ (where Φ = any one of the hydrophobic amino acids leucine, isoleucine or valine) [Moraitis et al., 2002; Potter et al., 2001; Potter et al., 2002]. These hydrophobic motifs are also referred to as interaction domains (IDs) and are illustrated schematically in Fig. 1D. Similar hydrophobic motifs (especially of the LXXLL type) are known from various coactivator families, such as the histone acetyltransferases, and other nuclear receptor interacting proteins, including the TRIP (thyroid receptor interacting protein) and DRIP (D-receptor interacting protein) families of nuclear receptor comodulators, where they serve as nuclear receptor (NR) boxes [Djabali et al., 2001]. Hr has been shown to interact with the RORα and TR nuclear receptors via the LXXLL motif pair and the ΦXXΦΦ motif pair, respectively [Moraitis et al., 2002; Potter et al., 2002]. Through its interactions with TR, Hr has been implicated as playing a role in mammalian CNS development [Potter et al., 2001]. Similarly, Hr interactions with RORα have been shown to be important in cerebellar development [Moraitis et al., 2002]. Hr appears to function as a corepressor when interacting with TR and RORα. Consistent with a repressive role, Hr has been demonstrated to interact with histone deacetylases (HDACs), which modify chromatin structure to silence gene transcription [Djabali and Christiano, 2004; Malloy et al., 2009; Potter et al., 2001; Potter et al., 2002; Wang et al., 2007]. Three repressor domains (RD1, RD2 and RD3) that are essential for the repressive function of Hr have been mapped (see [Potter et al., 2001], and Figs. 1D and 2A). However, with the exception of the Hr-HDAC association, protein interactions that might underlie the role of Hr in the mammalian hair cycle have not as yet been characterized.

The mammalian nuclear vitamin D receptor (VDR) has also been demonstrated to have an important role in hair growth, specifically in the mammalian hair cycle. VDR-null mice [Sakai et al., 2001] and a subset of human patients with inactivating VDR mutations [Malloy et al., 2004] display a complete lack of hair (alopecia) in addition to rickets. The retinoid X receptor alpha (RXRα) is a third protein required for normal hair cycling, given that temporal RXRα ablation in the skin of mice causes irreplaceable hair loss in the first hair cycle [Li et al., 2000]. Since VDR and RXR function as a heterodimer in gene regulation, it might be predicted that VDR's main physiologic ligand, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), would also be involved in hair cycling. However, humans who are deficient in, or cannot synthesize, 1,25$(OH)_2D_3$ do not have alopecia [Malloy et al., 2004]. Additional studies also demonstrate that, whereas mutations in the C-terminal activation function-2 (AF-2) domain of VDR or in regions of VDR involved in binding the 1,25$(OH)_2D_3$ ligand cause rickets, they do not elicit the alopecic phenotype [Malloy et al., 2002; Skorija et al., 2005]. These observations suggest that the mechanism by which the VDR-RXRα heterodimer facilitates normal hair cycling is functionally different from its participation in bone and calcium metabolism, where the 1,25$(OH)_2D_3$-liganded VDR-RXRα heterodimer activates or, in some cases, represses target genes in a ligand-dependent manner.

Two specific subtypes of alopecia that have been associated with mutation or ablation of Hr in mice and humans are alopecia universalis congenita (ALUNC, OMIM #203655) and atrichia with papular lesions (APL, OMIM #209500). Animals with mutations in Hr display a stalling of the first hair cycle, such that it cannot progress from telogen to anagen for regrowing hair after the first shedding [Sakai et al., 2001]. As indicated above, Hr has been identified as a corepressor of TR [Potter et al., 2001] and RORα [Moraitis et al., 2002].

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However, mice in which genes encoding either TR or RORα have been ablated do not replicate the atrichia phenotype. A TR knockout in mice causes wrinkling of the skin [Zarach et al., 2004] but not alopecia, while a RORα knockout in mice causes the "staggerer" phenotype, which includes only partial hair loss [Steinmayr et al., 1998]. On the other hand, specific mutations in the VDR gene result in a virtual phenocopy of atrichia caused by mutations in the *hr* gene [Bergman et al., 2005]. These observations suggest that VDR and Hr act in the same genetic pathway to control hair follicle cycling in mammals.

We have demonstrated previously that Hr not only interacts physically with VDR, but also dramatically represses VDR-mediated transactivation [Hsieh et al., 2003]. In this previous study, domains in both Hr and VDR that mediate their interaction were mapped to general regions of the respective proteins. For VDR, one major region appeared to reside in the N-terminal portion of the ligand binding domain corresponding to helices 3–6 and, for Hr, the C-terminal half of the macromolecule (residues 568–1207) was shown to be capable of binding VDR and repressing its transactivation effect on a 1,25$(OH)_2D_3$-responsive reporter gene. Herein we extend the studies of VDR-Hr interactions by: a) further mapping the C-terminal half of Hr and examining the functional role of individual ID motifs by site-directed mutagenesis, b) probing the molecular effects of natural point mutations in Hr which confer the alopecic phenotype on the interaction of this corepressor with VDR and with HDAC3, c) providing evidence that a Jumonji C-like domain is crucial for Hr action and may serve as a histone demethylase in gene repression, and d) reporting that the C-terminal extension of the zinc finger DNA binding domain, as well as the hinge region of VDR, constitute two additional docking sites for Hr on VDR.

MATERIALS AND METHODS

PLASMID CONSTRUCTS

The human VDR cDNA cloned into the pSG5 vector (pSG5hVDR) has been described previously [Hsieh et al., 1991]. VDR truncations (Δ403, Δ303, Δ202 and Δ134) and VDR point mutations E98K/E99K; R102A/K103A/R104A (RKR→AAA); K109A/R110A/K111A (KRK→AAA) and I238D were previously generated in the pSG5 vector as described in Hsieh et al. [1995; 1999]. Expression vectors for wild-type rat Hr (pRK5myc-rhr), containing the coding sequence of rHr fused to a myc tag, and similar expression plasmids expressing rHr fragments were described previously [Potter et al., 2001]. Full-length rHr point mutants 1–9 (see Fig. 2A) and twelve rHr point mutants corresponding to the natural mutations in hHr were created in the pRK5 expression vector by site-directed mutagenesis utilizing the Quickchange XL Mutagenesis Kit (Stratagene, La Jolla, CA). An expression vector for human Hr (hHr) was kindly provided by Dr. A. Hillmer (Rheinische Friedrich-Wilhelms-Universität, Germany). The construction of a human TR-β1 expression plasmid in the vector pSG5 was described previously [Thompson et al., 1999]. The reporter construct utilized in this study for assaying VDR signaling, p24OHaseLuc, contains 5500 bp of the natural promoter from the human 24OHase gene upstream of the firefly luciferase gene [Jin et al., 1996] and was kindly provided by Drs. J.W. Pike (University of Wisconsin) and S. Christakos (New Jersey Medical School). The reporter construct utilized for assaying TR signaling was constructed by inserting the double-stranded oligonucleotide CTGGG<u>AGGTGA</u>CAGG<u>AGGACA</u>CGAGCTGGG<u>AGGTGA</u>CAGG<u>AGGACA</u>CGAG, with a BglII overhang on the 5' end and a HindIII overhang on the 3' end, upstream of a minimal promoter in the luciferase vector pLUC-MCS (Stratagene Corp., La Jolla, CA). This oligonucleotide contains two copies of the thyroid hormone responsive element (half sites are underlined) from the rat myosin heavy chain gene [Tsika et al., 1990]. The following plasmids were obtained from commercial sources: pRL-CMV (Promega, Madison, WI), which expresses Renilla luciferase driven by a constitutive CMV promoter, and pTZ18U (Promega, Madison, WI), which was used as carrier DNA for transfections.

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GST fusion proteins were cloned as described in Hsieh et al. [2003] using parent vectors obtained from GE Healthcare Biosciences, Piscataway, NJ.

GST PULL-DOWN ASSAYS

Bacteria harboring the GST protein and the pGEX-4t-2 fusion constructs were grown in 500 mL cultures containing tetracycline and ampicillin. Fusion proteins GST-VDR and GST-(568–1207)Hr were extracted from the cultures and linked to glutathione-sepharose beads, according to the protocol of the manufacturer (Amersham Biosciences, Piscataway, NJ). GST glutathione-sepharose beads were prepared for use as negative controls. The various glutathione-sepharose beads served as an immobilized protein matrix to recruit ^{35}S-labeled proteins (rHr, hVDR or hRXRα) in the pull-down assays described below.

Expression plasmids (pSG5hVDR, pRK5myc-rhr and the indicated hVDR and rHr mutations – see figure legends for point and deletion mutants) were used as templates for *in vitro* transcription and translation (IVTT), utilizing a TNT coupled rabbit reticulocyte lysate kit (Promega, Madison, WI) in the presence of ^{35}S-labeled methionine (PerkinElmer Life And Analytical Sciences, Inc, Waltham MA). Each of the IVTT reactions included template DNA (0.5–2.0 μg), lysate, amino acids and RNA polymerase in a total volume of 42 μL, and were incubated for 2 hours at 30°C. For each pull-down reaction, 25 μL of GST or 25 μL of GST-fusion protein beads were preblocked in KETZD buffer (0.15 M KCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.6, 0.3 mM ZnCl, 1 mM DTT containing 0.1% Tween-20, 150 mM KCl, 1 mg/mL BSA and protease inhibitors aprotinin, leupeptin, pefabloc SC and pepstatin (protease inhibitors obtained from Roche Applied Science, Indianapolis, IN)) for one hour at 4°C. Forty μL aliquots from each IVTT reaction were then incubated with the preblocked beads in a 500 μL solution of KETZD buffer for one hour at 4°C in the absence or presence of 10^{-8} M hormone. The GST or GST-fusion protein beads were then washed four times with 1 mL of KETZD to release any nonspecifically bound proteins. After removal of wash buffer, 40 μL of 2X final sample buffer (FSB) (4% SDS, 10% 2-mercaptoethanol, 0.125M Tris-HCl and 20% glycerol) were added to each sample of beads, followed by boiling in a hot water bath for 2 minutes to elute the bound, ^{35}S-labeled proteins. Eluates, along with IVTT input samples (1.2 μL of IVTT reaction diluted with 18.8 μL of KETZD, 15 μL of which was aliquoted and mixed with 15 μL 2X FSB), were resolved by 5–15% gradient SDS polyacrylamide gel electrophoresis (SDS-PAGE). Autoradiography of Kodak XAR-5 film at −80°C allowed visualization of the protein bands.

COTRANSFECTIONS OF CELLS WITH EFFECTENE TRANSFECTION REAGENT

In the transfections using COS-7 monkey kidney cells and human KERTr-1106 skin cells (keratinocytes), 24 well plates were employed at a density of 80,000 cells per well. COS-7 cells were cultured in 1 mL of 10% FBS DMEM per well, and keratinocytes were grown in 2 ml per well of keratinocyte serum free media (SFM) obtained from Invitrogen Corp., Carlsbad CA. Six hours (COS-7) or 18 hours (keratinocytes) following plating, the cells were cotransfected using Effectene transfection reagent (Qiagen, Valencia, CA) with the natural VDRE-reporter p24OHaseLuc (250 ng), pRL-CMV (10 ng) and pSG5hVDR (250 ng) along with the indicated pRK5myc-rhr or p3XFLAG-CMV-hHr constructs (125 ng). For each well, the amounts of each plasmid indicated above were combined with 1.33 μL of Enhancer buffer and a volume of buffer EC to bring the mixture to a total of 100 μL per well. The DNA, Enhancer buffer and buffer EC mixtures were vortexed for 10 seconds, and 1.33 μL per well of Effectene reagent was immediately added. Each mixture was incubated for 10 minutes at room temperature to allow DNA and liposome complexing. During this incubation, all wells were aspirated, washed with 0.5 mL of medium, and supplied with 0.5 mL of fresh medium. Twenty-five μL of medium per well was added to each master mix, and the resulting mixture was equally distributed among six similar wells. The cells were

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incubated either overnight (COS-7) or for three hours (keratinocytes) at 37°C. After the indicated incubation times, the cells were supplied with 1 mL fresh 10% FBS DMEM media (COS-7) or 1.5 mL SFM (keratinocytes) per well and treated with either ethanol or 10^{-8} M of $1,25(OH)_2D_3$. After a 24 hour incubation at 37°C, the cells were washed twice with PBS and lysed in 150 μL of PLB in a 37°C shaker for 10 minutes. The cell lysates were collected by scraping and frozen at −80°C. A dual luciferase assay system (Promega, Madison, WI) and a Sirius Luminometer (Pforzheim, Germany) were used to sequentially measure firefly and Renilla luciferase activities in 10 μL of COS-7 or keratinocyte cell lysate combined with 50 μL of LARII reagent for the firefly reading, followed by 50 μL of Stop and Glo reagent for the Renilla reading. The ratio of firefly to Renilla luciferase activity was calculated for each sample. Results are expressed as the average of triplicate samples ± the standard deviation.

COTRANSFECTIONS OF CELLS WITH TRANSIT TRANSFECTION REAGENT

Human KERTr-1106 skin cells (keratinocytes) were plated at a density of 80,000 cells per well in 24-well plates. The cells were cultured in 2 mL of keratinocyte SFM and incubated overnight at 37°C. The morning after the day of plating, keratinocytes were cotransfected using TransIT transfection reagent (Mirus, Madison, WI) with the natural VDRE-reporter p24OHaseLuc (375 ng), pRL-CMV (1 ng) and pSG5hVDR (100 ng) with the indicated pRK5myc-rhr constructs (50 ng). For each well, 3 μL of the TransIT reagent was mixed with 33.3 μL of SFM. The plasmids listed above were then added to the liposome mixture and incubated for twenty minutes to allow the DNA to penetrate the liposomes. During this incubation, all wells were aspirated, washed with 0.5 mL of PBS, and supplied with 0.5 mL of fresh SFM. DNA and liposome mixtures were added to the wells and the cells were incubated for three hours at 37°C. Following this incubation, the cells were supplied with 1.5 mL fresh SFM per well and treated with either 2 μL of ethanol or ethanol containing 10^{-5} M of $1,25(OH)_2D_3$ for a final concentration of 10^{-8} M. After a 36 hour incubation at 37°C, the cells were washed twice with PBS and lysed in 150 μL of PLB in a 37°C shaker for 10 minutes. The cell lysates were collected and assayed using a luminometer as described above.

COIMMUNOPRECIPITATION ASSAY

Rat Hr and mutants thereof were produced in an *in vitro* transcription-translation system (see "GST Pull-down Assay" section above), then incubated with an HDAC3 protein containing a FLAG epitope at its N-terminus overnight at 4°C (this FLAG-HDAC3 construct was a kind gift from W.-M. Yang and E. Seto, Moffitt Cancer Center and Research Institute, Tampa FL). Immunocomplexes were formed using either non-specific IgG or α-Flag antiserum, then incubated with Protein A-Sepharose beads (Sigma) for 3 h at 4°C. Beads were washed 3 times with ice-cold IP buffer (see [Potter et al., 2002] for further details). Immunoprecipitates were fractionated by SDS-PAGE and analyzed by Western blotting. Western analysis was performed with the indicated antisera and detected by use of enhanced chemiluminescence (ECL) under conditions specified by the manufacturer (Amersham). Antisera (α-FLAG-, HDAC3-specific and preimmune IgG) were obtained from Sigma.

RESULTS

THE C-TERMINAL HALF OF RAT HR POSSESSES SEVERAL EPITOPES FOR INTERACTION WITH VDR

Utilizing coimmunoprecipitation methodology, we previously observed and reported [Hsieh et al., 2003] that full-length rat Hr (residues 1–1207) interacts physically with VDR, and showed that this interaction with VDR is concentrated in the C-terminal half (residues 568–1207) of the Hr macromolecule. Fig. 1D reiterates these previous results in the context of a

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schematic representation of Hr and its relevant deletion constructs, and recapitulates that only the C-terminal half of Hr and its residue 750–1084 and 750–864 fragments retain significant VDR corepressor functional activity. As a prelude to point mutagenesis studies of rat Hr, we repeated Hr-VDR association experiments employing the independent technique of pull-down of Hr deletion mutants with immobilized GST-VDR. The results in Fig. 1B verify that whereas the N-terminal half of Hr (31–568) does not bind VDR specifically compared to the GST alone control (Fig. 1C), the C-terminal half (568–1207) and all C-terminal subfragments tested retained specific binding to VDR. We conclude that there exist multiple regions in the C-terminal half of Hr for VDR association, and Fig. 1D illustrates that all VDR-interacting Hr fragments identified by pull-down assay (PDA), with the exception of the 864–981 fragment, contain at least one of the four LXXLL-like interaction domains (IDs) identified in Hr known to mediate interaction with other nuclear receptors, namely RORα [Moraitis et al., 2002] shown in green in Fig. 1D and TR [Potter et al., 2002] shown in blue in Fig. 1D. However, Hr segment 864–981 neither coimmunoprecipitates with VDR nor retains corepressor activity (Fig. 1D), which is more consistent with the lack of a nuclear receptor ID. Notably, Hr fragment 980–1084 also neither coimmunoprecipitates with VDR nor retains corepressor activity (Fig. 1D), suggesting that TR ID2 may be the least important of the four IDs with respect to the functioning of the VDR-Hr repressor complex.

TESTING OF KNOWN NUCLEAR RECEPTOR INTERACTION DOMAINS FOR THEIR INVOLVEMENT IN BINDING TO VDR

In order to determine which of the four Hr ID motifs are crucial for VDR interaction and repressor function, nine point mutants were constructed within these ID motifs, denoted Hr mutants 1–9 (Fig. 2A). Each constructed mutant contained at least one altered ID, and several possess dual ID mutations. Tests of the abilities of these nine ID mutants to bind immobilized VDR are illustrated in Fig. 2B. The inputs of wild type Hr and the majority of the Hr ID mutants (abbreviated m1–m9) exhibited significant expression in the IVTT system (Fig. 2B, left panels); only mutants 4, 5 and 9 showed reduced expression. Hr mutant 5 was eliminated from the study because of especially poor expression (not shown in Fig. 2B).

The right panels of Fig. 2B show the results of pull-down experiments with either GST-bound beads or with beads bearing a full-length VDR protein fused to GST. Protein bands do not appear in the control GST pull-downs, indicating a lack of nonspecific binding to GST by wild-type Hr or any tested Hr mutants (Fig. 2B, lanes labeled "GST"). The Hr bands observed in the GST-VDR pull-downs of wild-type Hr and mutants 1–4 and 6–9 show that all of these mutants retain their ability to bind immobilized VDR to some extent. Closer observation of multiple pull-down results (Fig. 2B and data not shown) revealed that wild-type rHr and mutants 2, 8 and 9 had the strongest association to GST-VDR. Mutants 1, 3, 4 and 7 had slightly weaker associations to immobilized VDR, and mutant 6 had the weakest association to GST-VDR relative to its IVTT expression (Fig. 2B, compare Hr m6 input to Hr m6 pull-down lanes), indicating that it is the most compromised Hr mutant in its ability to bind VDR.

As depicted in Fig. 2A, each of the nine Hr mutants are altered in only one or two of the four IDs. The slight decrease in intensity of binding to VDR by mutants 1, 3, 4, and 7, and the even more significant decrease in binding to VDR by mutant 6 suggest that some Hr nuclear receptor IDs contribute to VDR association more than others. Thus, it appears that mutation of both RORα IDs (mutant 6) is more damaging to Hr-VDR interaction than mutation of both TR IDs (mutant 9). Taken together, however, these results indicate that all four known interaction domains (RORα ID1, RORα ID2, TR ID1 and TR ID2) participate to some degree in Hr association with VDR. Evidently, the presence of remaining intact nuclear

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receptor IDs can compensate for the mutations of one or two IDs to allow detectable VDR association.

ALL FOUR NUCLEAR RECEPTOR INTERACTION DOMAINS OF HR ARE NECESSARY TO REPRESS TRANSACTIVATION BY VDR

To assess which of the known RORα and TR nuclear receptor interaction domains of Hr are of primary importance in functional repression of VDR signaling, the nine point mutants examined in GST pull-downs were also tested in transfection studies using the p24OHaseLuc reporter (Fig. 2C). Both monkey kidney (COS-7) and human keratinocyte (KERTr-1106) cell lines were used in this study. The COS-7 transfection shows a significant loss of repression of VDR transactivation by all rHr point mutants, except for Hr mutant 1 (Fig. 2C, left panel). The KERTr-1106 transfection also shows that all rHr point mutants, again excepting Hr mutant 1, have lost some or all of their ability to act as VDR corepressors (Fig. 2C, right panel). Together, these results indicate that point mutation of L586A in the N-terminal RORα ID is not sufficiently damaging to compromise the repressive function of Hr. However, mutation of two other leucines in this same RORα ID (mutant 2) does compromise the repressor function of rHr. These results therefore suggest that, unlike the GST pull-down results in which loss of one or two ID domains can be compensated for by other intact IDs, the integrity of all four nuclear receptor interaction domains is necessary for the rHr repression of VDR transcriptional activation to occur.

SITE-DIRECTED MUTATION OF RAT HR CDNA BASED ON ALOPECIC MOUSE MODELS

Fig. 3A illustrates the location of two Hr residues found to be mutated in strains of alopecic mice. The first mutation, Gly-960 to Trp, was found in homozygous offspring of mice originally mutagenized with N-ethyl-N-nitrosourea [Nam et al., 2006]. In homozygous mice, the G960W Hr mutation leads to a phenotype of irreversible hair loss soon after birth, wrinkled skin and long, curved nails. This glycine residue is positionally conserved, not only in the hairless genes from other mammals (shown are mouse, rat and human), but also in the JmJD1A, JmJD1B and JmJD1C proteins that are related to hairless (Fig. 3A, lower left). We generated the corresponding mutation (G985W) in rat Hr for functional testing.

The second mutation was based on an early report of the "bald Mill Hill" mouse [Brancaz et al., 2004]. This mutation, which spontaneously arose in a mouse colony in Mill Hill, London in 1998, also causes irreversible loss of hair in homozygous mice. The original report described a 296 bp deletion at the 3' end of the gene that "removed the last two amino acids of the C-terminal region of the hairless protein and a large part of the 3'-UTR" [Brancaz et al., 2004]. The terminal two amino acids of all known mammalian Hr proteins to date are either Ala-Lys (all eutherial mammals) or Ser-Lys (the marsupial opossum (Fig. 3A, lower right)). We therefore created a rat Hr expression plasmid by site-directed mutagenesis with a premature stop codon that terminates translation at the codon prior to Ala-Lys, thus producing a rat Hr lacking the terminal two amino acids (designated ΔAK).

GST pull-down experiments were performed with the G985W and ΔAK rat Hr mutants (Fig. 3B). As can be seen in the lanes containing the GST-VDR fusion protein, both mutants interact relatively normally with VDR. We then proceeded to test whether these mutation might disrupt the ability of rHr to repress VDR and/or TR signaling. As illustrated in Fig. 4A, the G985W mutant, despite its ability to interact near normally with VDR in a GST pull-down, has largely lost its ability to repress VDR signaling when assayed using a VDRE-containing p24OHaseLuc reporter plasmid. Further, G985W rHr is also impaired in its ability to repress TR signaling as assayed using a thyroid hormone-responsive reporter construct, $(rMHC)_2$pLucMCS (Fig. 4B). In contrast, the terminal Ala-Lys residues appear to

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be essential for the ability of Hr to repress TR signaling (Fig. 4D), but are not required for repressing VDR signaling (Fig. 4C), at least in the context of the present assay systems.

EVALUATION OF RAT HR POINT MUTANTS CORRESPONDING TO HUMAN HR MUTATIONS CAUSING ALOPECIA

Twelve naturally-occurring Hr missense mutations associated with human alopecia were separately introduced into positionally conserved residues in rHr cDNA and subjected to functional analysis similar to that carried out on the mouse alopecia-derived G985W and ΔAK mutants shown in Fig. 3 and Fig. 4. For the sake of brevity, functional data for only two of the rat Hr mutants are illustrated in Fig. 5A; rat Hr V1154D and D1030N exhibit a striking loss of their ability to repress VDR signaling.

A complete compilation of our analysis of the twelve Hr mutants appears in Table 1, along with a comparison to published work from another laboratory [Wang et al., 2007] (shaded portion of Table 1). Each laboratory evaluated a similar set of Hr mutations: the mutations we studied were in the context of rat Hr, whereas the mutations probed by Wang et al. were generated in human Hr. In our experiments, most mutants were expressed at levels similar to that of wild-type rat Hr in transfected COS-7 cells; however, two mutants created in rat Hr, namely A596V and E603V, appeared to be degraded in transfected cells (data not shown). Wang et al. also experienced poor expression of one human Hr mutant, P69S. Curiously, however, the equivalent rat mutant, P95S, was well expressed in our hands. Conversely, the human mutants corresponding to the poorly expressed rat mutants A596V and E603V, namely A576V and E583V, were well expressed in the studies of Wang et al. The explanation for these species differences in stability of certain expressed Hr mutants is unclear.

Both laboratories tested the ability of each mutant to repress VDR signaling using reporter gene assays in transfected COS-7 cells. As is evident in Table 1, the results are very comparable between the human Hr mutants probed by Wang et al. and the corresponding rat Hr mutants tested in our laboratory. In both cases, wild-type Hr shows a robust ability to repress signaling by VDR, and this ability was shown by Wang et al. to extend to signaling by RORα as previously reported by Moraitis and colleagues [2002]). This repressive ability of Hr was reduced or abolished by some but not all Hr mutants. A comparison of our results with those of Wang et al. show that certain mutants, notably rat C642G (human C622G), rat N988S (human N970S), and rat D1030N (human D1012N), lose the ability to repress VDR signaling in the context of Hr from both species (see Fig. 5A for results with D1030N). In the case of rat V1154D, our results show essentially a complete loss of VDR repression (Fig. 5A), whereas the corresponding human mutant (V1136D) still retained partial activity [Wang et al., 2007]. Conversely, the rat V1074 mutant was only partially impaired in its capacity to repress VDR signaling in our experiments, but the corresponding human mutant (V1056M) displayed no VDR-repressive activity when tested by Wang et al. These minor discrepancies could be because of species difference, or the single dose of $1,25(OH)_2D_3$ chosen for our assays, which is in contrast to the several concentrations of $1,25(OH)_2D_3$ employed by Wang et al. to construct dose-response curves.

It is interesting to note that the same Hr mutations identified above that affect VDR repression also affect RORα repression [Wang et al., 2007]. One might therefore expect that these mutations would affect the ability of Hr to interact with VDR and/or RORα. However, as summarized in Table 1, both our data and the results of Wang et al. demonstrate that the strength of interaction between a given Hr mutant and either VDR or RORα is not a reliable predictor of the ability of that mutant to repress transactivation by VDR or RORα. For example, we were able to detect some degree of VDR interaction by every rat Hr mutant we tested, even those that had lost the ability to repress VDR signaling. Likewise, Wang et al.

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reported some degree of VDR interaction with all human Hr mutants, including the five which exhibited low to absent VDR repression. The fact also that the methodologies used by the two laboratories to assess interaction of Hr with VDR differed (GST-pull-down vs. co-immunoprecipitation) indicates that this result is not an artifact of the specific method used, and suggests that these Hr mutants must be defective in some other activity related to transcriptional corepression.

As mentioned above, Hr has been reported to interact with histone deacetylases [Potter et al., 2001; Potter et al., 2002; Djabali and Christiano, 2004]. Therefore, CoIP experiments were undertaken to determine whether individual Hr mutations might compromise the ability of Hr to interact with HDACs. Results in Fig. 5B indicate that all four rat Hr mutants tested (C642G, N988S, D1030N and V1154D) were able to interact with HDAC3, although the extent of interaction may be slightly reduced in the case of C642G and V1154D (note however that the inputs are also reduced for these two mutants). The human panel of Hr mutants tested by Wang et al. (Table 1) revealed strongly reduced HDAC1 interaction by C622G (the equivalent of rat C642G) and moderately reduced interaction by mutants N970S, D1030N, T1022A, V1056M and V1136D. The reasons for the differences between the present results with HDAC3 and the findings of Wang et al. with HDAC1, as well as the relevance of either set of results to the mechanism of VDR-Hr in the mammalian hair cycle, are discussed below.

POINT MUTAGENESIS OF THE JUMONJI DOMAIN IN HR

We next mutated three residues in the conserved Jumonji C domain of rat Hr (Fig. 6A, grey shaded residues) to determine if this alteration would influence the ability of the Hr protein to repress VDR signaling. These three residues have been proposed to play crucial roles in histone demethylation by other Jumonji C containing proteins (see [Trewick et al., 2005] and Discussion). As illustrated in Fig. 6B, mutation of one of these residues, a highly conserved histidine at position 1143 near the C-terminus of the Jumonji C domain, abolished the ability of the Hr protein to repress VDR signaling from a reporter construct. However, mutation of the other two residues, a cysteine and a glutamate, had no discernible effect on Hr activity (Fig. 6C). It should be noted that unlike H1143, neither the cysteine nor the glutamate are positionally conserved in traditional Jumonji proteins (Fig. 6A), suggesting that loss of function in the H1143 mutant may be more informative.

A CLUSTER OF POSITIVELY-CHARGED RESIDUES C-TERMINAL TO THE VDR ZINC FINGER REGION IS IMPORTANT FOR HR ASSOCIATION

Utilizing primarily coimmunoprecipitation of full-length Hr and VDR, we previously identified the helix 3–6 region of VDR as a major domain that mediates interaction with rat Hr [Hsieh et al., 2003]. As summarized in Fig. 7A, this docking region for Hr on VDR is designated Hr Site #1, and is flanked on the N-terminal side by hVDR residues 134–202 comprising the hinge domain of VDR which contains a second weaker interface for Hr (cross-hatched at the top of Fig. 7A). We performed independent GST-pull-down experiments, utilizing the immobilized C-terminal half of rat Hr (residues 568"1207) which possesses all VDR-interaction domains (Fig. 1), to reexamine C-terminal truncations of hVDR for their ability to complex with Hr. As expected, interaction with Hr was dramatically reduced upon truncation of VDR from residue 303 to residue 201, however, there still existed a significant signal for Hr binding to hVDR truncated to residue 201 (data not shown), confirming that a second site of Hr contact lies N-terminal of residue 202 in hVDR, likely in the unconserved “hinge” region of the receptor. Moreover, because the truncation strategy used to map these sites does not exclude a role for other, even more N-terminal VDR domains in interacting with Hr to create the binary complex, we examined the potential role of highly conserved clusters of charged residues located between hVDR

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residues 89 (the C-terminus of the zinc finger motif) and 134 (the N-terminus of the “hinge”). As depicted in Fig. 7A, several of these clusters of charged amino acids occur just C-terminal of the zinc finger DNA binding domain (DBD) which are fully conserved in mammalian VDR proteins, and are partially conserved in the closely related nuclear receptors, the pregnane X receptor (PXR, also called NR1I2) and the constitutive androstane receptor (CAR, also called NR1I3).

A GST-Hr pull-down assay was employed using the following IVTT-generated hVDR point mutants: E98K/E99K, RKR → AAA102-104, KRK → AAA109-111 and I238D (see Methods for construction of these mutants). E98/E99 and the RKR triplet (residues 102"104) VDR mutations are located in the “tandem” T-box region of VDR just C-terminal of the second zinc finger. This T-box region provides a dimerization interface for contact with the first zinc finger of the RXR coreceptor and thus mediates VDR heterodimerization and binding to direct repeat VDREs [Hsieh et al., 1995] with spacers of 3 residues. The KRK triplet (residues 109–111) is positioned more C-terminally in the A-box region in hVDR that participates in DNA binding and transactivation by VDR [Hsieh et al., 1999]. Although these three mutations do not lie within the 134–303 interval in the hVDR amino acid sequence, they may still participate in interaction with Hr because they are part of the residue 89–427 fragment of hVDR which was demonstrated by coimmunoprecipitation experiments to associate with Hr [Hsieh et al., 2003]. Another pertinent observation is that a P160R point mutation in TR renders this nuclear receptor incapable of binding to rHr [Potter et al., 2001]; the Pro-160 residue in TR corresponds to position 128 in hVDR, N-terminal to amino acid 134 which marks the N-terminal boundary of Hr contacts in the hinge and helix 3–6 domains of hVDR. Finally, the I238D hVDR point mutant was chosen because it lies within the 134–303 interval, although it is also required for the transactivation function of VDR as a residue in helix #3 that generates a platform for intramolecular interaction with helix 12/AF-2. The inputs of hVDR and the four hVDR point mutants show a strong ^{35}S signal at the same apparent molecular mass as wild type VDR, indicating significant expression of intact proteins in the IVTT system (Fig. 7B). The control GST pull-downs of hVDR and mutants E98K/E99K, RKR→AAA, KRK→AAA and I238D show a lack of nonspecific binding to GST (Fig. 7C, left lanes in each set). IVTT-generated wild-type hVDR, E98K/E99K, RKR→AAA, and I238D retain the ability to bind immobilized GST-Hr (Fig. 7C, right lanes in the first, second, third and fifth sets), whereas Hr binding by the KRK→AAA mutant at residues 109–111 is essentially abolished (Fig. 7C, right lane, fourth set). These results provide evidence that the hVDR A-box is yet a third potential contact site for Hr binding to VDR. However, neither Ileu 238 in helix 3 (Fig. 7C) nor Glu 420 in helix 12 [Hsieh et al., 2003], both of which are required for VDR-mediated transactivation, are contacts for rHr.

In summary, combined analysis of the interaction of deletion and point mutants of hVDR with rHR, as assessed by GST pull-down and coimmunoprecipitation, indicates that rHr does not dock on either the VDRE recognition zinc fingers or on the helix 12/AF-2 site that binds LXXLL motifs in coactivators such as SRC-1 [Skorija et al., 2005]. Instead, Hr apparently associates with the 3-dimensional structure of an array of hVDR amino acids in the central portion of the primary sequence (89–303), ranging from a KRK cluster at residues 109–111 in the A-box in the C-terminal extension of the DNA-binding domain (Fig. 7A, CTE) through the unconserved “hinge” region (Fig. 7A, hatched region at top of figure) to helices 3–6 of the ligand binding domain (Fig. 7A, Hr site #1).

DISCUSSION

The interaction between VDR and Hr has many implications for the control of hair cycling, but also for other differentiation and/or proliferation processes in skin and possibly other

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tissues. We have therefore attempted to further characterize this interaction at the molecular level, using not only natural alopecic mutations, but also mutated residues in highly conserved regions of either VDR or Hr. Our results reveal a site of contact in human VDR in a cluster of three basic residues (109–111) in the C-terminal extension of the DNA-binding domain (Fig. 7), in addition to two interaction sites located in the ligand binding domain [Hsieh et al., 2003] (Fig. 8). Interestingly, this same positively charged cluster of amino acids (residues 109–111) is required for DNA binding and transactivation by VDR [Hsieh et al., 2003]. Whether this domain could simultaneously mediate both DNA binding and interaction with Hr is not clear, and it is conceivable that Hr binding to this basic cluster might be part of the mechanism by which Hr interferes with transactivation by VDR. Regardless, one might expect DNA binding by VDR to be affected by Hr, as has been suggested by Xie et al. [2006]. However, these authors drew their conclusion indirectly from the effect of an anti-Hr antibody, which intensified VDRE gel mobility shifts using 24OHase, PLC-γ1 and involucrin VDREs. This result was interpreted to mean that the anti-Hr antibody was relieving inhibition by Hr of VDR binding to these VDREs [Xie et al., 2006]. Although this interpretation is plausible, direct evidence to prove that this simple model explains the ability of Hr to inhibit VDR signaling is still lacking.

We also investigated a diverse group of mutations in Hr for their influence on the ability of Hr either to interact with VDR or to repress transactivation by VDR. One group of mutations was created in the four previously described "interaction domains" (IDs). The two LXXLL domains reported to mediate interaction of Hr with RORα are highly conserved among seven eutherian, one marsupial and one monotreme mammal for which Hr sequences could be located in GenBank. RORα ID1, with the sequence LCRLL, shows only one conservative replacement (LCRVL) in cow and a non-conservative, but still hydrophobic, replacement (FCRLL) in opossum. RORα ID2, LCELL, is completely conserved in all nine species (see legend to Fig. 3 for the sources of these sequences, except for platypus, which was taken from ENSOANP00000001824 in the ENSEMBL database at http://www.ensembl.org/Ornithorhynchus_anatinus/index.html). This high level of conservation occurring with the RORα IDs may be related to the current observation that mutation of these two IDs (mutant 6 in Fig. 2) had an especially deleterious effect on Hr interaction with VDR.

The first TR ID, with the sequence LDSII, is also highly conserved, with the only deviation being a conservative replacement (LESII) in the platypus. In contrast, the VSDLI sequence of TR ID2 present in the rat possesses many variations in other species (VTSLV in the pig, VTDLV in the cow and dog, VTDLI in opossum; VADLV in the human, rhesus macaque and horse, and VTDFV in the platypus).

It should be noted that an oligopeptide based on the sequence of RORα ID2 (CPSLSELLASTVKL), with the Cys residue in the LCELL motif changed to Ser, was recently shown to bind to VDR with high affinity [Teichert et al., 2009]. In the same study, an oligopeptide containing TR ID1 (CNILDSIIAQVVERK) bound to VDR with much lower affinity [Teichert et al., 2009] in spite of its high level of conservation as noted above. The other two IDs examined in the present study, namely RORα ID1 and TR ID2, were not investigated by Teichert and colleagues. The results of Teichert et al. generally support the conclusion herein that the RORα IDs play a more important role in interacting with VDR than do the TR IDs; however, the binding of such ID motifs in the context of full-length Hr must be evaluated to further delineate which IDs are of greatest relevance in the corepression of VDR.

We conclude from the data shown in Fig. 2 that all four of the ID domains in Hr are required for the functional interaction of Hr to repress VDR signaling (as diagrammed in Fig. 8).

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Nevertheless, some level of interaction between the proteins occurs when one, or as many as two IDs are altered as indicated by the GST pull-down results in Fig. 2. The precise role of TR ID2, however, is still questionable given its poor level of conservation among mammals and also the fact that we have not been able to test a mutation that alters only this ID motif. How all of these interaction domains in Hr and VDR might combine in three dimensions to form a functional interface is not easily visualized in the absence of complete x-ray crystal structures for Hr and/or full-length VDR.

Another pair of mutations tested herein were based on published alopecic Hr mutants in mice. These mutations were recreated in rat Hr, which is 93% identical to mouse Hr at the amino acid level. The current results indicate that both rat mutations, G985W and the C-terminal deletant ΔAK, render Hr unable to repress TR signaling from a myosin heavy chain TRE. However, only the G985W mutant impaired the ability of Hr to repress VDR signaling, as the ΔAK mutant retains its capacity to repress VDR transactivation normally. It should be noted that, according to a more recent report on the bald Mill Hill mouse [Brancaz-Bouvier et al., 2008], the ΔAK mutation does not accurately recapitulate the defect in this alopecic mouse model. The more recent report describes the bald Mill Hill mutation as a 296 bp deletion leading to the loss of only a single residue at the C-terminus, but also the loss of the stop codon, leading to the introduction of 117 amino acids by read-through to the next in-frame stop codon [Brancaz-Bouvier et al., 2008]. Thus, the bald Mill Hill mutant Hr is different than the C-terminal truncation evaluated in the current experiments, although it may be significant that the ΔAK mutant tested herein displays a unique impairment in Hr's ability to repress TR, but not VDR, signaling, perhaps indicating that the functional interactions of Hr with TR vs. VDR differ qualitatively.

Twelve additional rHr mutations evaluated in the present report, as summarized in Table 1, correspond to human mutations originally reported as being alopecic (see [Wang et al., 2007] for citations on each of these alopecic alterations). All of these Hr mutations were tested by another laboratory as human Hr alterations in transfected COS-7 cells [Wang et al., 2007]. It is significant to note that all three of the mutations that severely reduced or abolished the ability of Hr to repress VDR signaling in both rat and human Hr (listed with rat/human numbering: C642/622G, N988/970S and D1030/1012N) have been positively identified to cause alopecia in human patients [Aita et al., 2000;Klein et al., 2002;Kruse et al., 1999]. Two other mutations (V1074/1056M and V1154/1136D) were reported to be alopecic in humans [Zlotogorski et al., 2002; Chichon et al., 1998], but each showed severe reduction in Hr repressive ability only in one species (V1074/1056M in human Hr and V1154/1136D in rat Hr). Finally, the alopecic E603/583V mutant [Paradisi et al., 2003], which was only tested in its human version, showed essentially a normal ability to repress VDR signaling, indicating that some other function of Hr must be affected in order to explain the alopecic phenotype in this case.

One obvious candidate function of Hr that might be impaired in certain alopecic mutants is the ability to interact with HDACs [Djabali and Christiano, 2004; Potter et al., 2001; Potter et al., 2002]. We therefore examined the ability of selected rHr mutants to interact with an HDAC. Our choice of HDAC3 was based on colocalization studies of Hr and Hr fragments with HDAC3 in brain tissue [Potter et al., 2002] and in mouse NIH3T3 cells [Djabali and Christiano, 2004]. HDAC3 has also been reported to specifically interact with VDR [Dong et al., 2005] to mediate transcriptional repression. However, both VDR and Hr have also been shown to interact with other HDACs, most notably HDACs 1 [Dong et al., 2005; Huang and Hung, 2006; Potter et al., 2002], 2 [Kim et al., 2007] and 5 [Potter et al., 2002], raising the possibility that VDR and Hr may associate with distinct HDACs depending on the cellular, or possibly the promoter, context [Huang and Hung, 2006]. Wang et al. chose HDAC1 for their studies based on analysis in transfected COS-7 cells, which indicated that

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the addition of exogenous HDAC1 (and also HDAC2, but not HDAC3) was able to potentiate the repressive effect of Hr on VDR signaling as measured from a reporter construct containing the 1,25$(OH)_2D_3$-responsive CYP24A1 promoter [Wang et al., 2007].

The four rat Hr mutants (C642G, N988S, D1030N, V1154D) corresponding to human alopecic alterations probed herein via a coimmunoprecipitation experiment using anti-FLAG antiserum all appeared to interact well with FLAG-tagged HDAC3, comparably to wild type rHr (Fig. 5 and Table 1). Using a very similar co-immunoprecipitation protocol (but with FLAG-tagged VDR), Wang et al. found that six hHr mutants were impaired in their ability to interact with HDAC1 ([Wang et al., 2007] and Table 1). These six hHr mutants included five alopecic mutants (C622G, N970S, D1012N, V1056M and V1136D), which was interpreted by these authors to indicate that the reduced ability of these mutants to repress signaling by VDR and RORα might be explained by a defect in HDAC1 interaction [Wang et al., 2007]. This could indeed be the case; however, it should be borne in mind that the HDAC form(s) that are actually involved in Hr action during the hair cycle are not yet known and could conceivably be different from either HDAC1 or HDAC3. Also, the fact that the T/A Hr variants, the predominant form of which is A1040 in rat and T1022 in human, also showed reduced interaction with HDAC1 is problematic, since the association of this genetic variant with human alopecia has been questioned [Cichon et al., 1998;Kruse et al., 1999]. This observation adds a cautionary note to the extrapolation of the HDAC1 interaction results in the context of artificial reporter systems to the poorly understood mechanism of control of the mammalian hair cycle.

The presence of a conserved Jumonji C domain in mammalian Hr proteins has led to intriguing possibility that Hr proteins possess intrinsic chromatin-modifying activity [Clissold and Ponting, 2001; Trewick et al., 2005]. This supposition has intensified with recent reports that a total of ten mammalian Jumonji-C containing proteins are capable of demethylating various methylated histone residues [Tsukada et al., 2006; Whetstine et al., 2006]. Hr proteins share particular similarity with one group of the newly recognized histone demethylases, namely the JmJD1A, JmJD1B and JmJD1C proteins, all of which have a similar domain structure to Hr, including a zinc finger domain, and a Jumonji domain that is 33–34% identical to that of Hr (data not shown). Also of interest, the JmJD1A and JmJD1C proteins have both been shown to interact with nuclear receptors [Lee et al., 1995; Wolf et al., 2009]. Comparisons of the amino acids sequences among Jumonji C-containing proteins have revealed the presence of three conserved residues, highlighted in Fig. 6A. These three residues consist of a triad of two histidines and an acidic residue of the form –Hx(D/E)---(n=69-126)---H- in all Jumonji C containing proteins known to date to be histone-modifying enzymes [Tsukada et al., 2006; Whetstine et al., 2006]. The initial Hx(D/E)-motif is believed to coordinate a metal atom for catalysis, as has been shown in a crystal structure of the Jumonji C domain from the factor-inhibiting hypoxia-inducible factor 1 protein, which is a known asparaginyl hydroxylase [Dann et al., 2002]. This same structure indicates that the conserved histidine in the final position of this motif serves to bind an oxoglutarate cofactor [Dann et al., 2002]. Although the N-terminal histidine of the triad is not conserved in Hr proteins (Fig. 6A), we reasoned that the cysteine residue present in Hr at this position (C1025) might be able to function as a histidine-like, metal-coordinating residue, as it often does in zinc finger proteins, for example in nuclear receptors such as VDR [Krishna et al., 2003].

However, as shown in Fig. 6C, mutation of this cysteine (C1025) and its flanking acidic residue (E1027) to glycine appeared to have no effect on the ability of Hr to repress VDR signaling. One interpretation of this observation is that Hr is therefore not a histone-modifying enzyme. An additional argument in favor of this interpretation is that this cysteine is not totally conserved among Hr proteins, being a serine in platypus Hr (not

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shown). Nevertheless, mutation of the third residue in the Jumonji C catalytic triad, the C-terminal histidine 1143 (which is conserved in all Hr proteins, including platypus) exhibits a complete loss in the ability of Hr to repress VDR signaling (Fig. 6B). This latter observation likely has relevance to the function of Hr, and we favor the interpretation that Hr acts as a corepressor in part by catalyzing histone demethylation and/or to modify chromatin in a fashion that is not revealed by functional testing using an artificial, plasmid-based reporter gene construct. It is important to emphasize that five of the eight alopecic mutations in conserved residues described herein for either mouse or human Hr occur in, or closely flank, the Jumonji C domain. Clearly, this novel domain in Hr which we have highlighted for the first time in the present communication is crucial to the direction of the mammalian hair cycle by the VDR-Hr complex.

The original paper describing the cloning of human Hr reported the existence of an Hr isoform in which exon 17 is absent (as illustrated in Fig. 8, upper right), presumably due to alternative splicing of the Hr mRNA in certain human tissues, resulting in the in-frame deletion of codons 1072 to 1126 in hHr (corresponding to 1090–1144 in rHr) [Cichon et al., 1998]. This deletion removes a C-terminal portion of the Jumonji C domain and specifically eliminates H1125, the human equivalent of rat Hr H1143, which is the proposed oxoglutarate-binding residue that could potentially participate in enzymatic activity by Hr [Dann et al., 2002] and, most important, we have demonstrated as essential for the VDR corepressor activity of Hr (Fig. 6B). A study of the tissue distribution of the isoform lacking exon 17 (designated isoform b), along with the full-length isoform, was reported in this same publication, with the result that the isoform missing exon 17 was the predominant, if not sole, isoform found in human skin [Cichon et al., 1998]. Human Hr isoform b, lacking residues 1072 to 1126, was tested by Wang et al. and more recently by Malloy et al. [Malloy et al., 2009], with the result that this Hr isoform is incapable of repressing signaling either by RORα [Wang et al., 2007] or by VDR, and is also incapable of interacting with HDAC1 [Malloy et al., 2009], suggesting that perhaps this isoform is biologically inactive. Why the predominant isoform of Hr in human skin should be inactive is unclear, although regulation of hHr mRNA splicing would be one mechanism of generating a gradient of functional Hr to create the cyclical nature of hair growth. Also, information about the existence of an Hr isoform b in any species other than human appears to be lacking. It should be noted, however, that none of the naturally occurring alopecic mutants described so far occur in this deleted part of the Hr sequence in isoform b. Thus, the significance of isoform b, if any, in the bioactions of Hr remains unclear.

Fig. 8 illustrates our current understanding of Hr structure and function. Specific residues or sequence motifs that have a demonstrated role in Hr function are largely confined to the C-terminal two-thirds of the protein. The zinc finger domain, repression domains 2 and 3, and the Jumonji C domain are all found in the C-terminal half of the protein, and it is significant to note that point mutations leading to alopecia in either human patients or rodent models are found in, or immediately adjacent to, each of these domains. The C-terminus of Hr (ending in Ala-Lys) also may be important for Hr function, at least for the repression of TR signaling (Fig. 4D and Fig. 8). Our understanding of the N-terminal portion of Hr is limited to the original description of repression domain 1 (RD1) [Potter et al., 2001] and of a nuclear localization domain [Djabali et al., 2001] at the C-terminal boundary of RD1. No alopecic mutants have been reported in either of these domains, although a polymorphic variant in RD1 (C422/397Y) has been reported [Hillmer et al., 2002]. However, testing by both Wang et al. and by our laboratory of the human and rat versions of this variant, respectively, failed to show any significant reduction in the ability to repress VDR signaling relative to wild type (see Table 1 and [Wang et al., 2007]).

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As suggested in Fig. 8, the current concept of Hr action in the hair cycle is that Hr acts in concert with VDR and likely also with the VDR coreceptor RXRα, based on the fact that inactivating mutations or deletions in all three of these genes give a similar alopecic phenotype [Li et al., 2000;Malloy et al., 2004;Sakai et al., 2001]. The mechanism by which this occurs remains to be elucidated, although, as discussed above, the $1,25(OH)_2D_3$ ligand for VDR appears to play no role [Malloy et al., 2004]. The involvement of HDACs is suggested not only by studies demonstrating an interaction between Hr and certain HDACs [Djabali et al., 2004;Potter et al., 2001;Potter et al., 2002], but also by the results of Wang et al. that certain alopecic mutants in Hr are deficient in their interactions with HDAC1 [Wang et al., 2007]. However, at least one alopecic mutant, E583V, is not deficient in HDAC interaction as tested by Wang et al. (Table 1). It therefore appears that other interactions may be important for biological functioning of Hr, such as an as-yet-uncharacterized role for the well-conserved Jumonji C domain. We theorize that the Jumonji C domain in Hr, which contains the highest concentration of naturally occurring alopecic mutations of any domain, and is strikingly compromised by the alteration of absolutely conserved H1143, performs repressive modifications of chromatin such as histone demethylation. Further studies into the structure function relationships of the Hr protein will likely shed light not only on the control of the mammalian hair cycle, but also may provide general insights into the mechanisms by which nuclear receptors interact with transcriptional repressors.

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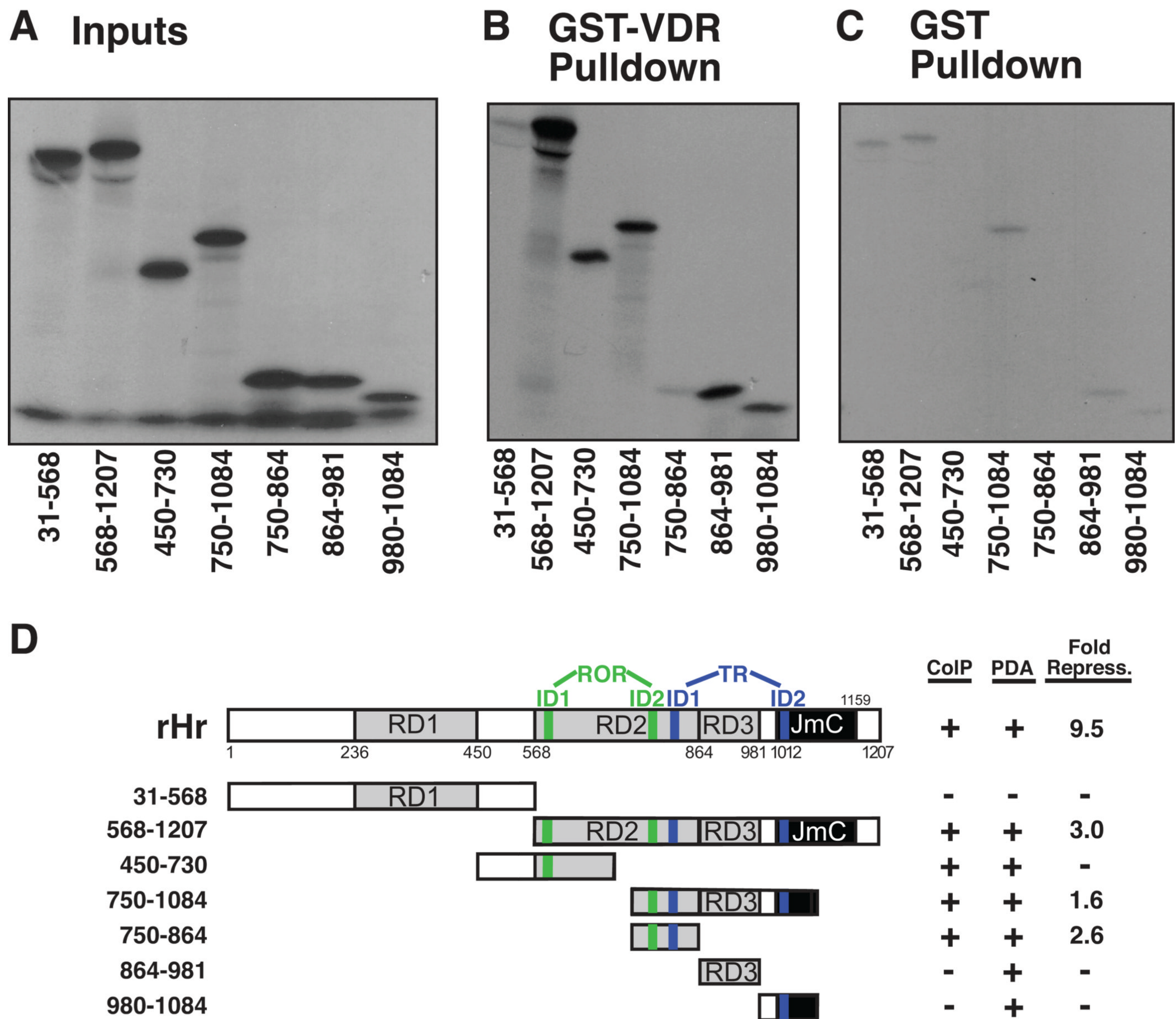

Fig. 1. C-terminal fragments of Hr associate with immobilized VDR

GST pull-downs were carried out as described in Methods. The following radiolabeled rHr fragments were generated by IVTT: an N-terminal portion with residues 31–568, a C-terminal portion containing 568–1207, and C-terminal fragments 450–730, 750–1084, 750–864, 864–981 and 980–1084. A: Inputs shown are 6.9% of the amounts used in the pull-down reactions. B: Pull-downs in which the IVTT-synthesized Hr fragments described above were incubated with immobilized GST-VDR, containing wild-type VDR. C: As a negative control, the radiolabeled Hr fragments were also incubated with immobilized GST alone. To allow for direct comparisons, the gels shown in panels A–C were exposed to film for the same amount of time. D: Tabular summary of pull-down (PDA) results in A–C for each rHr fragment appears to the right of a schematic diagram of rHr and its fragments. The ability of each Hr fragment to interact with VDR by coimmunoprecipitation (CoIP) [Hsieh et al., 2003] or by repressing (Fold Repress.) VDR signaling [Hsieh et al., 2003] also is listed to the right of the schematic. The location of the following Hr functional domains is

depicted in the schematic: three repression domains (RD1-3) [Potter et al., 2001], interaction domains 1 and 2 (ID1, ID2) mediating interaction between Hr and the RAR-related orphan receptor-alpha (RORα) [Moraitis et al., 2002] or with the thyroid hormone receptor (TR) [Potter et al., 2002], and a Jumonji C-like domain (abbreviated JmC) [Clissold and Ponting, 2001].

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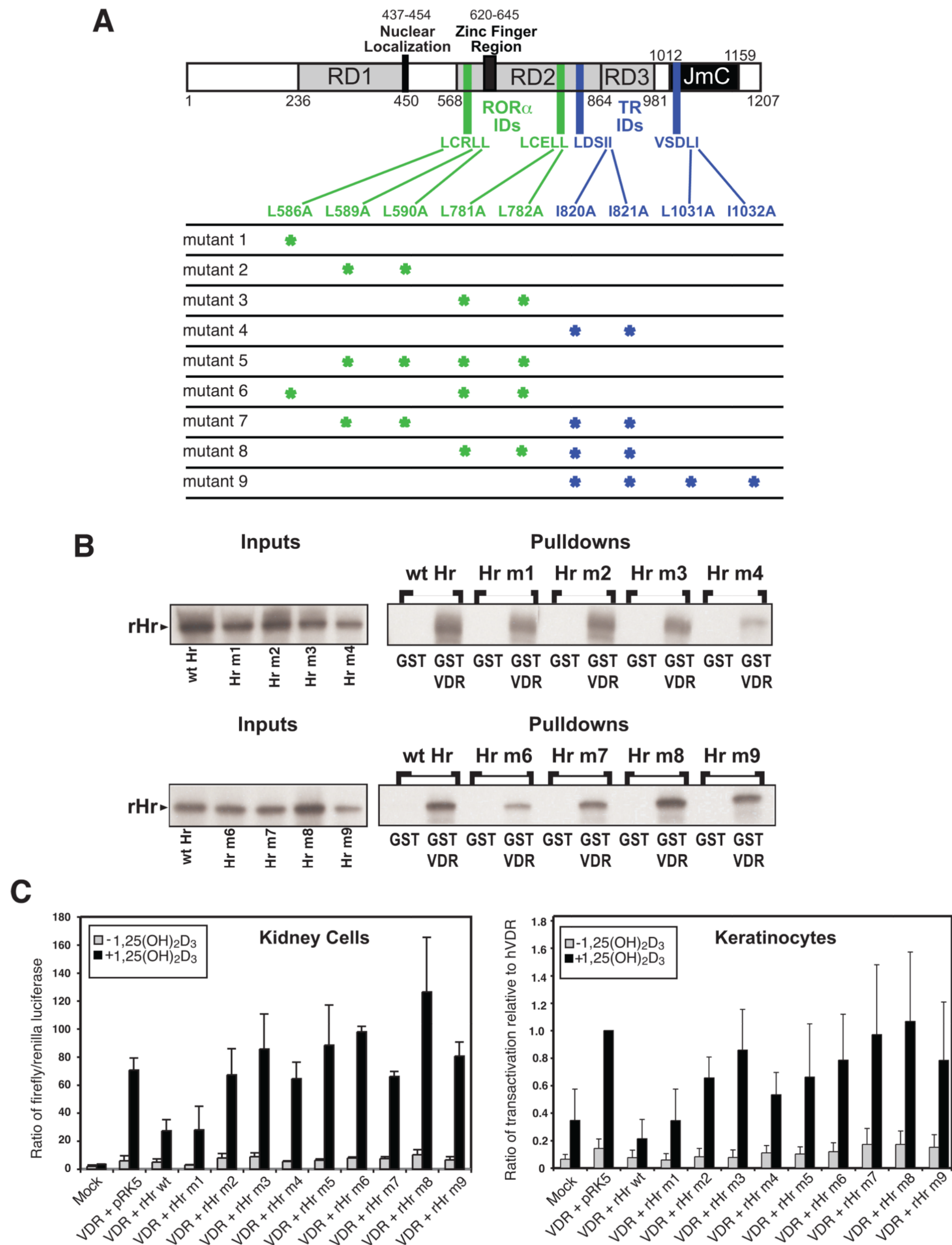

Fig. 2. Functional analysis of rat Hr point mutations in the interaction domains for RORα and TR

A: Various combinations of ID point mutations were used to create nine rHr mutants for functional testing, designated mutant 1 through mutant 9. Altered amino acids (all to alanine) are indicated by asterisks (*) and color-coded to match their corresponding interaction domains. Functional domains, in addition to those shown in Fig. 1D, are a nuclear localization domain [Djabali et al., 2001] and a zinc-finger domain [Cachon-Gonzalez et al., 1994]. B: GST pull-down results for the association of nuclear receptor binding domain mutants of Hr with immobilized VDR. The left panels of B show 4.5% of the inputs of ^{35}S-labeled wild type (wt) or mutant (m1-m9) Hr proteins produced in an *in*

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vitro transcription-translation system (see Methods). The right panels of B illustrate pull-down experiments using labeled Hr proteins incubated with either immobilized GST protein (negative control) or GST-VDR fusion protein containing full-length human VDR. C: Ability of each mutant to repress VDR signaling. Expression plasmids encoding all nine mutants were transfected into COS-7 monkey kidney cells (left panel in C) or into human KERTr-1106 skin cells (right panel in C) as described in Methods. Mock transfected wells received reporter plasmid but no expression plasmid for VDR. Cells were treated with ethanol or 10 nM $1,25(OH)_2D_3$ and allowed to incubate for 24 hours (COS-7) or 36 hours (KERTr-1106). Transcriptional activities were quantified by a luciferase assay and are normalized to the expression of Renilla luciferase, a constitutively expressed reporter gene used to monitor transcription efficiency. Error bars represent standard deviations for triplicate analyses. To obtain relative values for plotting of the KERTr-1106 results (right panel of C), transactivation units (ratios) obtained with wild type VDR and pRK5 (empty vector) in the presence of $1,25(OH)_2D_3$ and the absence of Hr were arbitrarily set to 1.0 in six independent experiments (thus lacking an error bar); results for all Hr mutants are expressed in comparison to this arbitrary standard. The values are shown as an average of six determinations ± the standard deviation.

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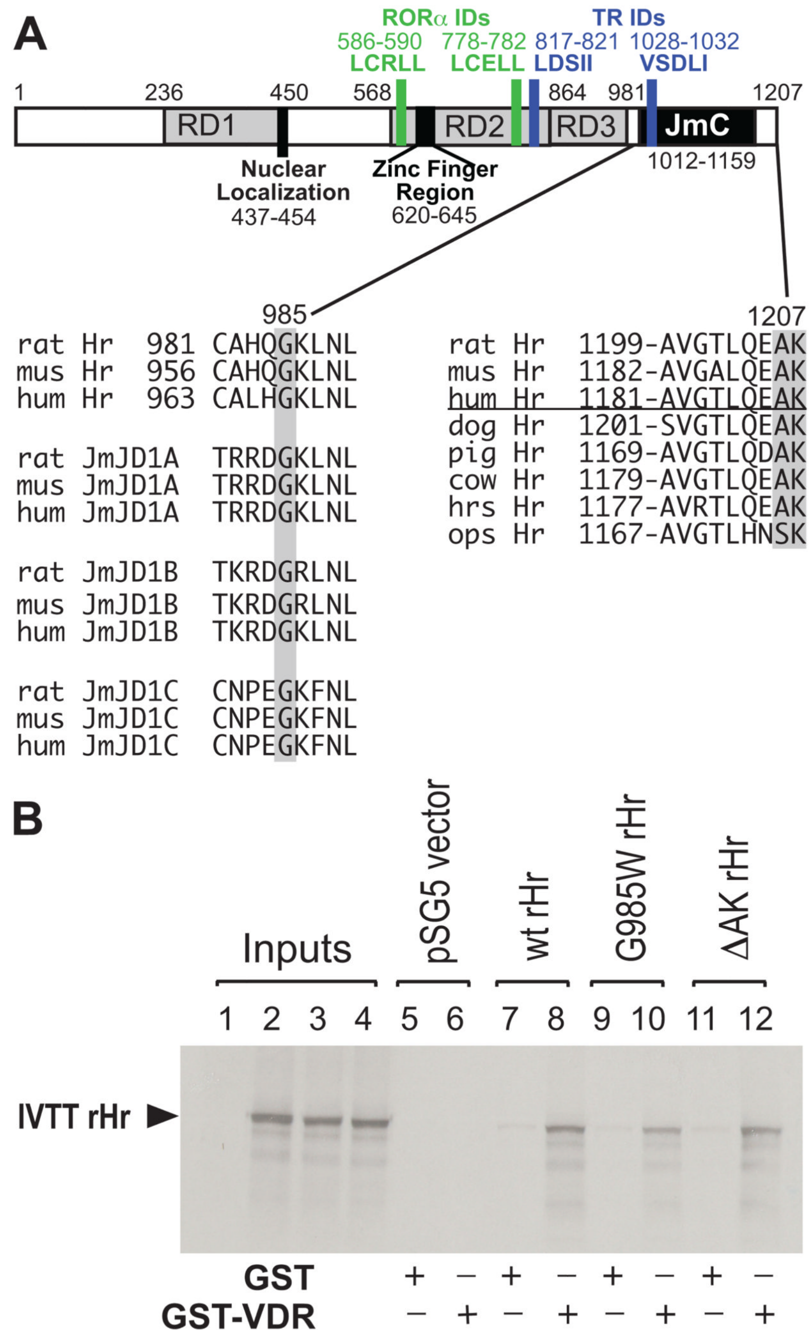

Fig. 3. Testing of two alopecic mouse mutations in the context of rat Hr

A: The location of the two mouse alopecic mutations on either side of the conserved Jumonji C domain. The two mutations, originally described in mice [Brancaz et al., 2004; Nam et al., 2006], were recreated in rHr as G985W and Δ1206-1207 (designated ΔAK to denote the loss of the two terminal amino acids Ala-Lys). The conservation of the residues affected by these mutations, along with immediately flanking residues, is shown in the lower portion of panel A. Species abbreviations are: human (hum), mouse *Mus musculus* (mus), horse, *Equus caballus* (hrs), and opossum *Monodelphis domestica* (ops). Accession numbers for these sequences are as follows: rHr NP_077340.2; mouse Hr NP_068677.2; hHr NP_005135.2; dog (*Canis lupus familiaris*) Hr XP_543256.2 (predicted); pig (*Sus scrofa*) Hr

NP_001077399.1; cow (*Bos taurus*) Hr NP_001096005.1; horse (*Equus caballus*) Hr XP_001490941.1; opossum Hr XP_001381979.1 (predicted); rat JmJD1a, also known as JmjC-containing histone demethylase 2A (JHDM2A) or as testis-specific gene A (TSGA), NP_786940; mouse JmJD1a, NP_766589; human JmJD1a, NP_060903.2; rat JmJD1b, also known as nuclear protein 5qNCA or as JHDM2B, XP_001061636 (predicted); mouse JmJD1b, NP_001074725; Human JmJD1b, NP_057688; rat JmJD1c, also called thyroid receptor interacting protein 8 (TRIP8) or JHDM2C, XP_001080424 (predicted); Mouse JmJD1c XP_980927 (predicted); and human JmJD1c NP_004232. B: GST-VDR pull-down analysis of the G985W and ΔAK rHr mutants using radiolabeled rHr proteins generated in an *in vitro* transcription-translation system as described in Methods. Beads contained either GST alone or full-length hVDR fused to GST (GST-VDR). Input lanes 1–4 correspond to, and represent 5% of the amount used for respective pull-down lanes 5/6, 7/8, 9/10, and 11/12.

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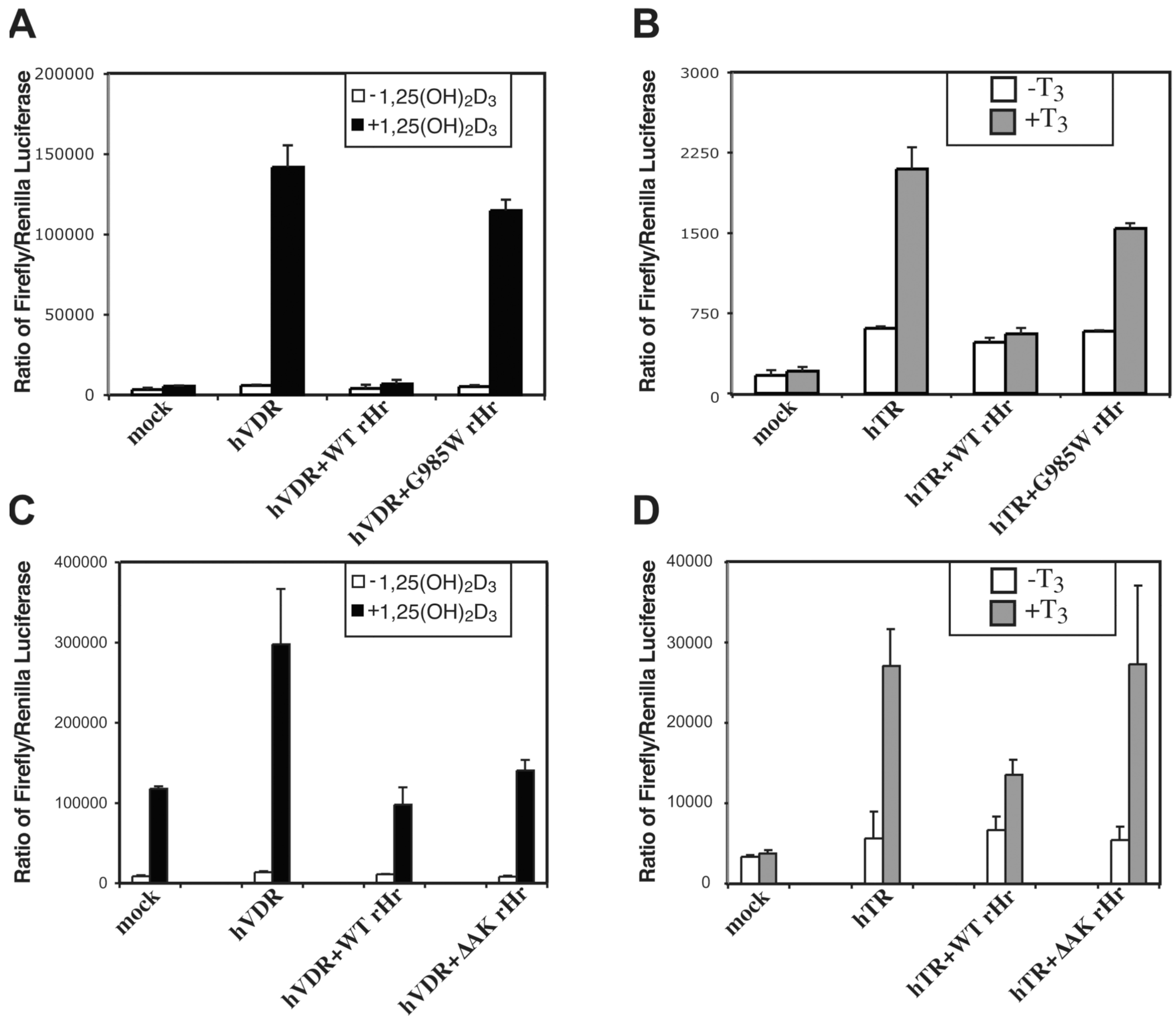

Fig. 4. Ability of the G985W and ΔAK rat Hr mutants to repress VDR- and TR-mediated transactivation of reporter genes

Expression plasmids for wild-type and mutant rHrs were cotransfected into COS-7 cells along with, if indicated, a reporter plasmid for VDR (the p24OHaseLuc vector) and the pSG5-hVDR expression plasmid (panels A and C) or a reporter plasmid for TR, $(rMHC)_2$pLucMCS, and the pSG5-hTRB1 expression plasmid for human TRβ1 (B, D). Transfected cultures were treated ± 10 nM $1,25(OH)_2D_3$ or ± 10 nM tri-iodothyronine (T_3) as indicated. Luciferase assays were performed as described in Methods and the results shown represent the average of six replicates ± the standard deviation.

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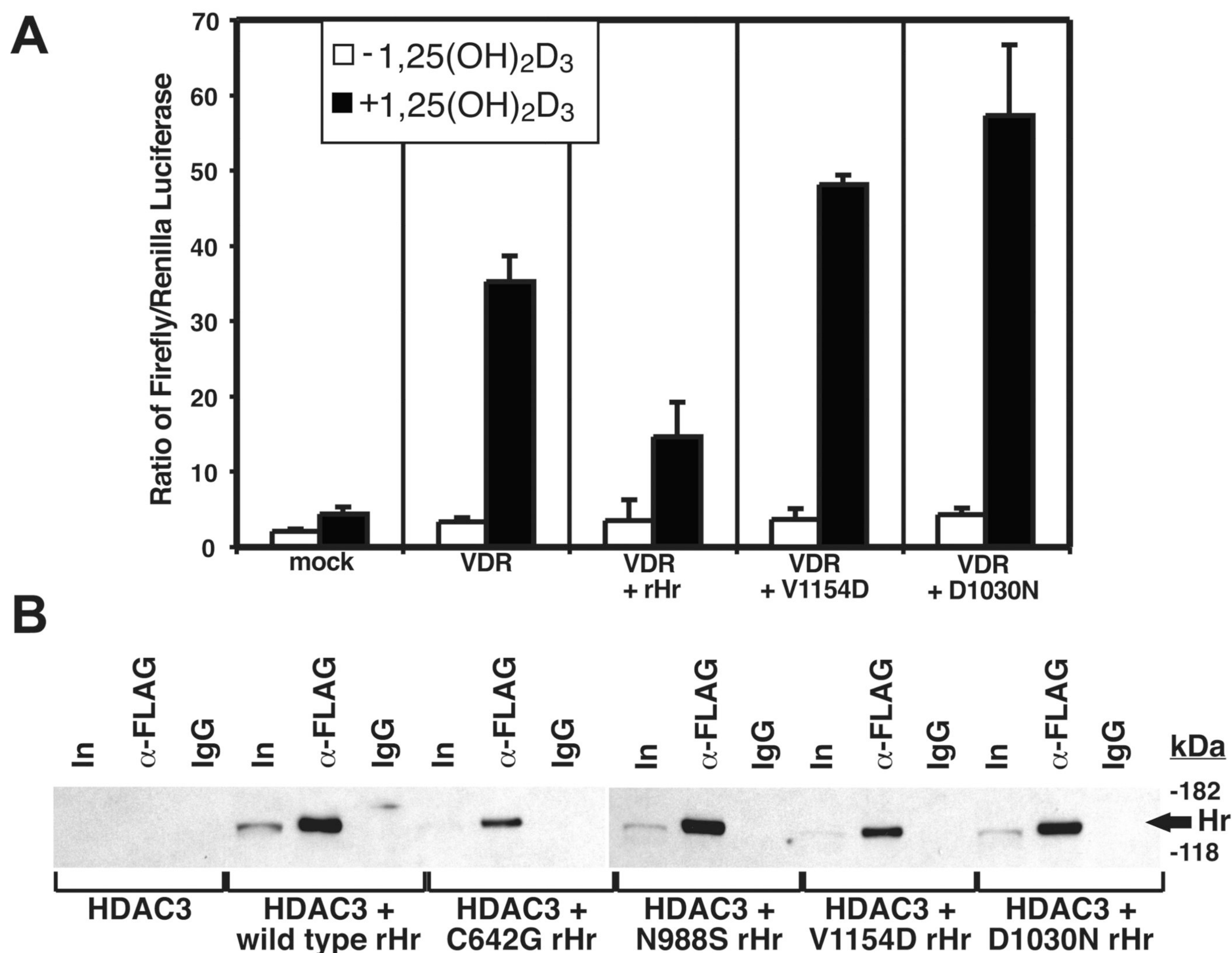

Fig. 5. Functional characterization of selected rat Hr mutants which recapitulate naturally occurring point mutations in human Hr that confer alopecia

A: Loss of VDR corepressor activity by rHr mutants D1030N and V1154D. COS-7 cells were transfected with the natural VDRE-reporter plasmid p24OHaseLuc, pSG5-hVDR and an expression plasmid for the indicated rHr mutant as described in Methods. Cells were treated for 24 hrs ± 10 nM $1,25(OH)_2D_3$ as indicated, and transcriptional activity was quantified via luciferase assay as described in Methods and the legend to Fig. 2C. Error bars represent standard deviations for triplicate wells. B: Radiolabeled rHr mutants were produced in an in vitro transcription-translation system, incubated with FLAG-tagged HDAC3 [Wen et al., 2000], immunoprecipitated with the indicated antisera, and subjected to SDS-PAGE and autoradiography as described in Methods. A small aliquot of radiolabeled input was electrophoresed in lanes indicated by “In”. The appearance of a labeled rHr band in the α-Flag lane, but not the non-specific IgG lane, is taken as evidence of a direct interaction between the rHr mutant and HDAC3.

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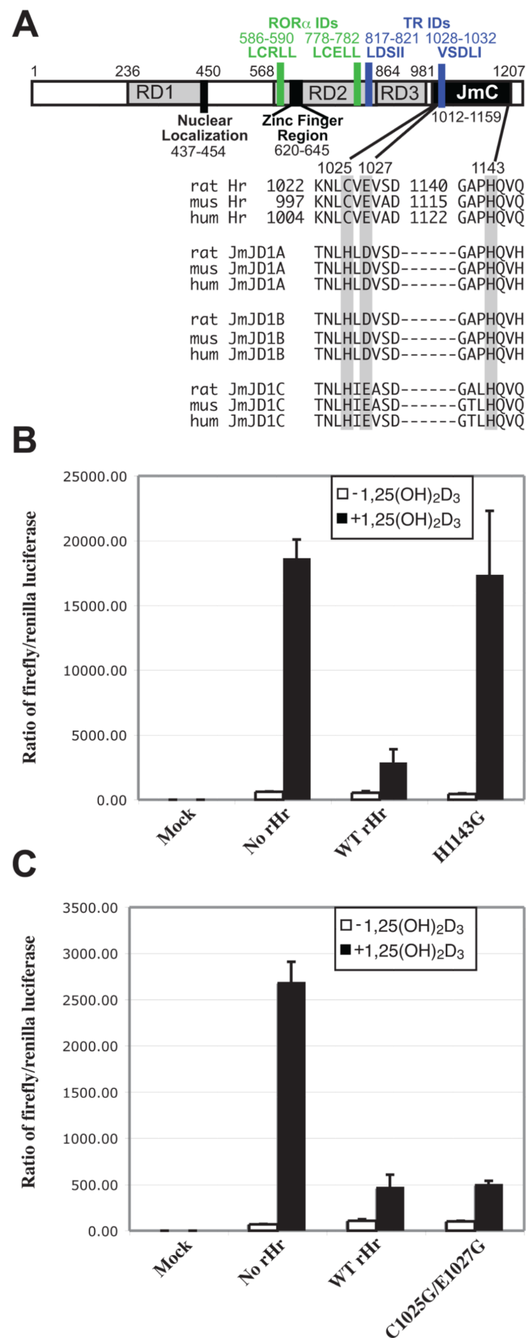

Fig. 6. Mutation of conserved residues in the Jumonji C domain of rat Hr and the functional consequence of these alterations on the VDR corepressor activity of Hr

A: Conservation of the three mutated residues which are postulated to confer enzymatic activity, such as histone demethylase, onto the Hr protein (see text). Sources for sequence data are given in the legend to Fig. 3. B: Effect of mutating residue H1143 to glycine in rat Hr. Cells were transfected, and cell lysates were assayed for luciferase activity as described in Methods and the legend to Fig. 2C; "No rHr" wells received reporter plasmid and the pSG5-hVDR expression plasmid, but no expression plasmid for rHr. C: Similar to panel B, except that the double mutant C1025 and E1027 (each to glycine) rHr was investigated.

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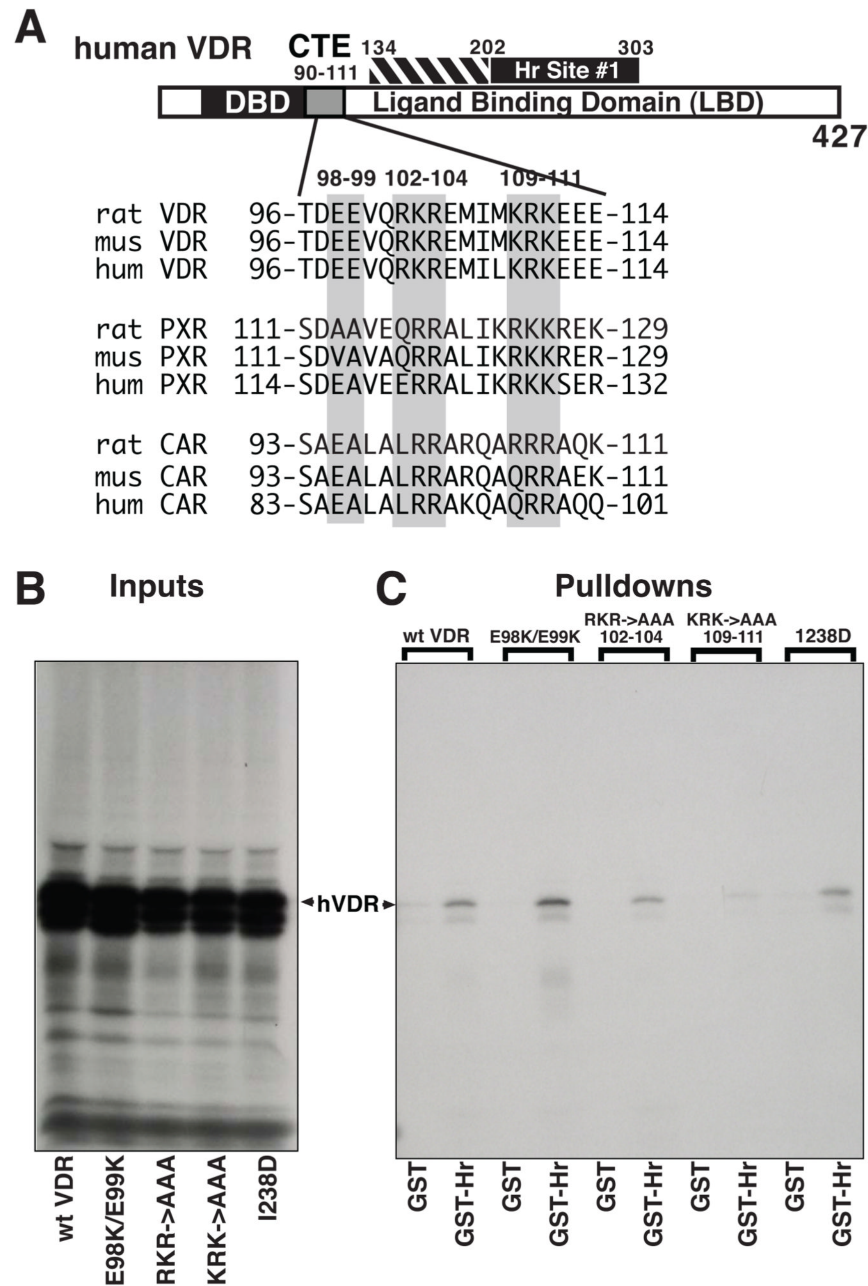

Fig. 7. Evidence for a third site in VDR that is required for optimal Hr interaction

A: Schematic diagram of full-length hVDR showing the DNA-binding domain (DBD), the C-terminal extension of the DNA-binding domain (CTE) [Hsieh et al., 1999], and the ligand-binding domain (LBD) of intact VDR. Two Hr interaction domains are located in the ligand binding domain of hVDR between residues 134 and 303. Major Hr site #1 (202–303) was revealed by immunoprecipitation [Hsieh et al., 2003] and minor Hr site #2 (cross-hatched; 134–201) was deduced from GST pull-down results (data not shown). The lower portion of panel A depicts the location of conserved clusters of charged residues, which were mutated previously [Hsieh et al., 2003] and in the present study in an attempt to determine if a third Hr binding site exists in VDR, one more proximal to the DNA binding

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domain. In the first mutant, glutamate residues at positions 98 and 99 in human VDR were changed to lysines to produce E98K/E99K. Next, clusters of basic residues at positions 102–104 and at 109–111 were altered to alanines to produce RKR->AAA 102–104 and KRK->AAA 109–111, respectively. The lower portion of panel A illustrates conservation of these residues among VDRs (NR1I1) and also among the related PXR (NR1I2) and CAR (NR1I3) receptors. Sequence sources are as follows: rat VDR NP_058754.1; mouse VDR NP_033530.2; human VDR NP_000367.1; rat PXR NP_443212.1; mouse PXR NP_035066.1; human PXR NP_003880.3; rat CAR NP_075230.1; mouse CAR NP_033933.2; human CAR NP_001070950.1. Human VDR proteins containing mutations in each of the charged clusters were separately expressed in an *in vitro* transcription-translation system along with wild-type VDR (see Methods). B and C: A representative GST pull-down experiment designed to assess the ability of these mutant proteins to bind immobilized rat Hr. B: Display of 4.5% of the inputs of ^{35}S-labeled wild type (wt) or mutant VDRs used in the pull-down reactions. C: Autoradiograph of a gel containing point-mutated hVDR pull-down reactions using either immobilized GST protein (negative control) or GST-Hr fusion protein containing full-length rat Hr.

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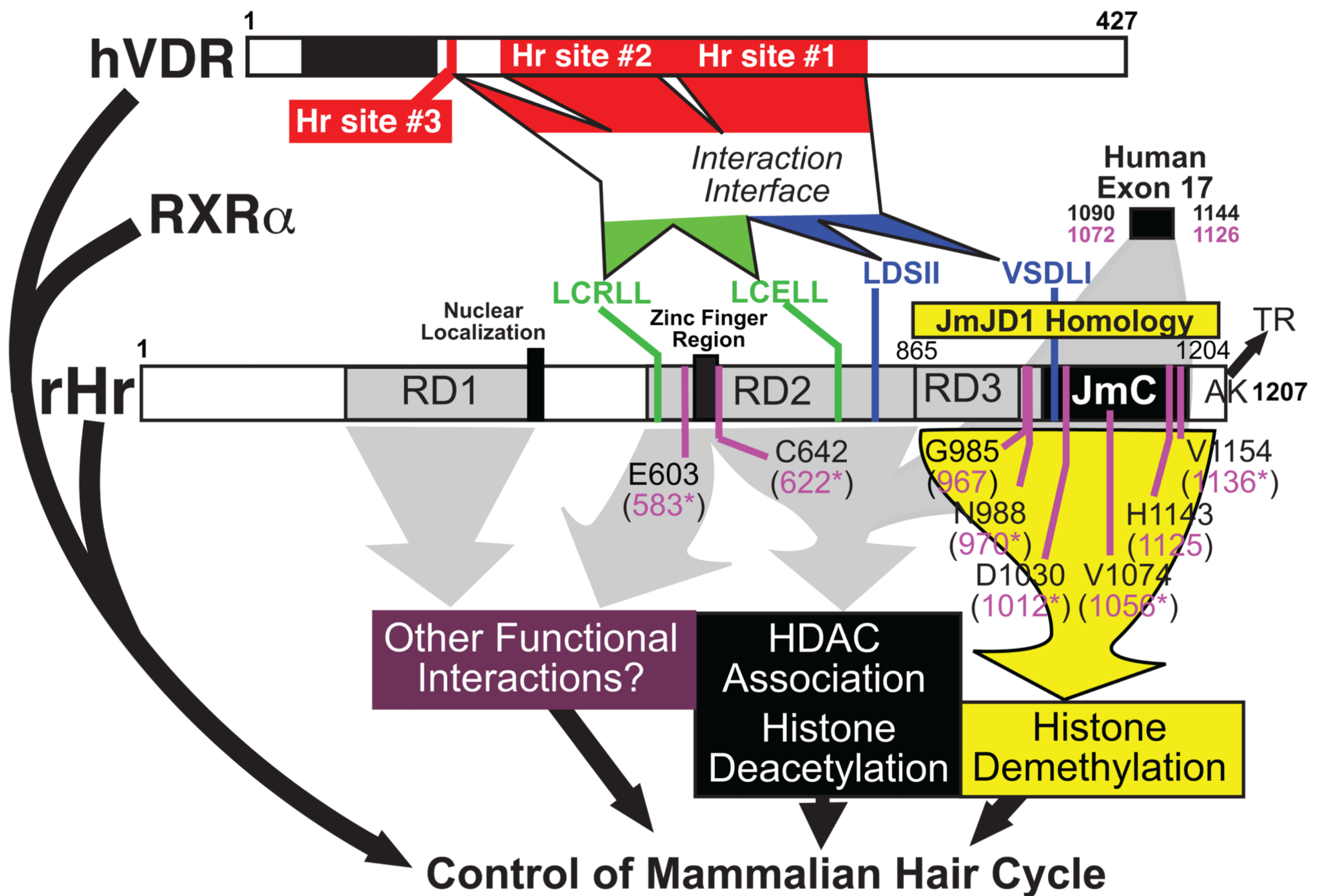

Fig. 8. Integrated model for the functional interactions of VDR and Hr in controlling gene expression to drive the mammalian hair cycle

Functional domains in VDR and Hr are defined in the legends to Fig. 1D, Fig. 2A, and Fig. 7, and in the text. The upper portion depicts schematically the interaction interface between VDR and Hr, which appears to consist of three domains in VDR (denoted in red as Hr sites #1 – #3) and four domains in Hr, previously described [Potter et al., 2001;Moraitis et al., 2002;Potter et al., 2002] as LXXLL-like nuclear receptor interaction domains for either RORα (shaded in green: LCRLL and LCELL) or TR (shaded in blue: LDSII and VSDLI). The central portion illustrates the position in Hr of a set of naturally occurring mutations which confer the alopecic phenotype and were evaluated experimentally in the present study and/or by Wang and coworkers [Wang et al., 2007]. Residue numbers shown in black are for rat Hr; human Hr numbering is shown in magenta underneath the rat numbering. Mutated amino acids in human patients that display alopecia with papular lesions (APL, OMIM #209500) are designated with an asterisk; the corresponding residue in rHr that was evaluated in the current study is shown above the human residue number. A single alopecic Hr mutation from mice, G985W, is represented by the designation G985 with the corresponding human Hr residue number shown in parentheses below. The region of hHr that is encoded by exon 17 and that is missing in a human variant of Hr (isoform b) [Cichon et al., 1998] is shown at upper right with both rat (black) and human (magenta) residue numbering (note that is this isoform is only found in the human). Potential functional interactions between these residues and either HDACs or other unknown nuclear factors are depicted as large arrows in the lower portion and are discussed in the text. Residues 865 to

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1204 in rat Hr show high homology to the Jumonji domain-containing protein JmJD1 (34% homology to rat JmJD1B and 33% each to rat JmJD1A and JmJD1C; for accession numbers used in these comparisons see legend to Fig. 3); this region of rat Hr is shaded yellow. Most of the alopecia conferring mutants occur in this region of Hr, as discussed in the text, and lead us to propose that the C-terminal JmJD1-homologous domain in Hr is enzymatically active in histone demethylation, yielding chromatin modifications that repress the expression of genes to control the mammalian hair cycle. The proposed histone demethylase activity of Hr likely operates in concert with histone deacetylases recruited by the JmJD1-homologous domain as well as other repressive domains of Hr to complete a constellation of chromatin repressive alterations required to silence RXR-VDR-Hr target genes. Potential interactions between these residues or regions and either HDACs or other unknown nuclear factors are discussed in the text.

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Table 1

Summary of evaluation of rat Hr mutations in the current study compared to previously published (Wang et al., 2007) results with human Hr (shaded in grey)

Rat Hr Mutants	VDR Repression (reporter)	VDR Association (GST)	HDAC3 Association (CoIP)	Human Hr Mutants*	VDR Repression (reporter)	RORα Repression (reporter)	VDR Association (CoIP)	HDAC1 Association (CoIP)
wtVDR	++++	yes	++++	wtVDR	++++	++++	+++	++
P95S	+++	yes	Not tested	P69S	(degraded)	(degraded)	(degraded)	(degraded)
C422Y	+++	yes	Not tested	C397Y	+++	++	+	++
A596V	(degraded)	(degraded)	(degraded)	A576V	++	++	+	++++
E603V	(degraded)	(degraded)	(degraded)	E583V†	++++	++++	+	++++
E611G	++++	yes	Not tested	E591G	++++	++++	+	++
R640Q	++	yes	Not tested	R620Q	++++	+++	++	++
C642G	–	yes	+++	C622G†	+/–	–	+++	+/–
N988S	–	yes	++++	N970S†	–	+/–	+++	+
D1030N	–	yes	+++	D1012N†	–	–	++	+
A1040T	++	yes	Not tested	T1022A	++	–	+++	+
V1074M	++	yes	Not tested	V1056M †	–	++	++++	+
V1154D	–	yes	++++	V1136D†	++	+	+++	+

* Wang et al. (Wang et al., 2007) human Hr mutants

† Hr mutants reported to cause alopecia in humans