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Hrt and Hes negatively regulate Notch signaling through interactions with RBP-Jκ

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Abstract

Notch signaling is central to cell differentiation, organ development, and apoptosis. Upon ligand binding, the Notch intracellular domain (NotchIC) translocates to the nucleus to interact with its DNA-binding partner, RBP-Jκ. The NotchIC-RBP-Jκ complex activates target genes, such as those encoding the Hrt and Hes families of basic-helix-loop-helix (bHLH) transcriptional repressors. Hrt transcripts are enriched in the developing cardiovascular system, and mice lacking Hrt2 have cardiac malformations. Here we show that Hrt2 and Hes1 interact with RBP-Jκ to negatively regulate Notch-dependent activation of *Hrt* and *Hes* expression. The bHLH domain of Hrt2 was necessary for this interaction, and disrupting the protein complex abrogated the negative autoregulation. The interaction did not interfere with the formation or DNA-binding of the NotchIC-RBP-Jκ complex, indicating direct inhibition by Hrt and Hes as co-repressors. These findings suggest a novel mechanism for negative feedback on Notch signaling that requires RBP-Jκ to interact physically with Hrt and Hes.

Keywords: Notch; Suppressor of Hairless; RBP-Jκ; CSL; CBF-1; Hey; Hrt; Hes

Notch, a large transmembrane protein, regulates cellular patterning and differentiation during development [1]. Initially characterized in *Drosophila*, Notch is required for development of all three germ layers in metazoans. In humans, aberrant Notch signaling is associated with several diseases, including neoplasms [2], cerebral autosomal dominant adult onset arteriopathy [3], Alagille's syndrome [4,5], and congenital heart disease [6].

Notch signaling depends on a complex balance of ligands, receptors, signal transducers, and effectors. Upon ligand binding, Notch undergoes regulated intramembrane proteolysis, and the intracellular domain (NotchIC) translocates to the nucleus to form a complex with its DNA-binding partner, RBP-Jκ (also known as CSL, Suppressor of Hairless, CBF-1, Lag-1) [7,8]. RBP-Jκ is a transcriptional repressor or activator, depending on its interaction with other co-factors [9]. RBP-Jκ partners include the general repressor proteins N-CoR, SMRT, the RBP-Jκ-specific co-repressor CIR, and adaptor proteins (e.g., SKIP) [10]. In the RBP-Jκ-dependent pathway, Notch relies on its innate transcriptional activity and concomitant masking of the repressive domains of RBP-Jκ to promote target gene activation [11]. The mechanism that results in alteration between activator and repressor function is unknown.

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Selected members of the *Hairy/Enhancer of split* (Hes) family of basic helix-loop-helix (bHLH) transcriptional repressors are downstream targets of the Notch-RBP-Jκ pathway that mediate a subset of Notch signaling [12]. We, and others, have identified a distinct sub-family of Hairy-related transcription factors Hrt (also known as Hesr, Hey, CHF, grl, and HERP) that is similar but distinct from Hes proteins [13–17]. For example, Hes family members are characterized by a highly conserved proline residue in the DNA-binding basic domain. In contrast, Hrt proteins have a glycine at the comparable position that likely underlies Hrt specificity for E box DNA-binding sites (CACGTG) over the N-box site (CACNAG) favored by Hes family members. In addition, the extreme carboxy-terminal region of Hrt proteins contains a YXPW-TEI/VGAF (Y/T) motif that is similar to but distinct from the carboxy-terminal WRPW motif of Hes1. This region is necessary for Hes1's recruitment of the co-repressor, groucho [18] but the Y/T domain of Hrt proteins does not appear to interact with groucho [19].

In vivo, Hrt proteins are essential for normal cardiovascular development. Hrt 1,-2 transcripts are co-expressed in the cardiac outflow tract and pulmonary arteries. In contrast, Hrt1 and Hrt2 are expressed in a complementary fashion in atrial and ventricular precursors, respectively, while Hrt3 is found in postnatal hearts and somitic precursors [14]. In zebrafish, mutations in the Hrt2 orthologue gridlock (*grl*) can result in a narrowing of the dorsal aorta [20]. Knockdown studies using morpholinos to grl result in alterations in arterial versus venous fate determination that phenocopy misexpression of RBP-Jκ [21], suggesting that the Hrt family may function to repress Notch1 and RBP-Jκ in vivo [22,23]. In mice, targeted deletion of *Hrt2* can result in ventricular septal defects, cardiomegaly, tetralogy of Fallot, and pulmonary stenosis, but the molecular mechanisms remain unknown [24–26].

At the *Hrt2* locus, Notch1 activates transcriptional activity that depends on an RBP-Jκ binding site 140 bp upstream of the transcription initiation site. Mutating this site or co-expressing the non-DNA-binding mutant of RBP-Jκ (RBP-Jκ R218H) results in the loss of Notch1 activation of *Hrt2* transcription [27]. Hrt2 negatively regulates Notch1-mediated transcriptional activation of the *Hrt2* promoter in a negative feedback loop [27]. The minimal region of the *Hrt2* promoter required to repress Notch activation does not contain E or N boxes, suggesting that the mode of repression by Hrt2 is not dependent on Hrt2 DNA-binding. Here, we show that Hrt2 and Hes1 associate with RBP-Jκ and that this interaction is necessary for the repression of Notch1-dependent activation of the Hrt2 promoter.

Materials and methods

Cell culture. Cells were grown under standard conditions [28].

Luciferase assay. Cells transfected with Fugene6 (Roche) were harvested 36 h after transfection, lysed, and assayed as described [27,28]. Western analysis of lysates verified expression levels of the proteins of interest.

Immunocytochemistry. HeLa cells were transiently transfected with FLAG-RBP-Jκ, myc-Hrt2, myc-Hrt2 (Δ48–61), myc-Hrt2 (Δ120–164), myc-Hrt2 (2–326), myc-Hrt2 (L68P), myc-Hes1, or myc-Hes1 (L54P) expression plasmids; 48 h later, cells were fixed in 5% paraformaldehyde and incubated with rabbit anti-myc polyclonal or murine anti-FLAG monoclonal antibody (Santa Cruz, Sigma). Secondary antibodies included FITC-conjugated anti-mouse antibodies and Cy3-conjugated anti-rabbit antibodies (Jackson ImmunoResearch).

Gel electromobility assays. Cos1 cell lysates were incubated with ^{32}P-labeled oligonucleotides encoding the RBP-Jκ cis-element 5′-GGAAACACGCCGTGGGAAAAAATTTGGG-3′ [8]. Protein expression was verified by Western blot. Retardation of oligonucleotide migration was assessed on a native 6% polyacrylamide gel.

Co-immunoprecipitation assays. After transient transfection of Cos1 cells, myc-tagged proteins were immunoprecipitated with polyclonal anti-myc antibody (Santa Cruz) and FLAG-tagged proteins were detected by Western blotting with anti-FLAG (Sigma).

Oligonucleotide precipitation. Lysates from transfected Cos1 cells were incubated with biotin-labeled oligonucleotides containing an RBP-Jκ binding site for 30 min in binding buffer (50 mM Tris–HCl, pH 8.0, 20% glycerol, 1 mM EDTA, 0.01% Triton X-100, 100 mM NaCl, 10 mM DTT, 10 mM NaF, and 1 mM Na_3VO_4). Oligonucleotide was precipitated with avidin beads, washed three times with binding buffer, and boiled for 5 min in Laemmli buffer (Bio-Rad) containing 5% β-mercaptoethanol. Samples were examined on a 10% polyacrylamide gel.

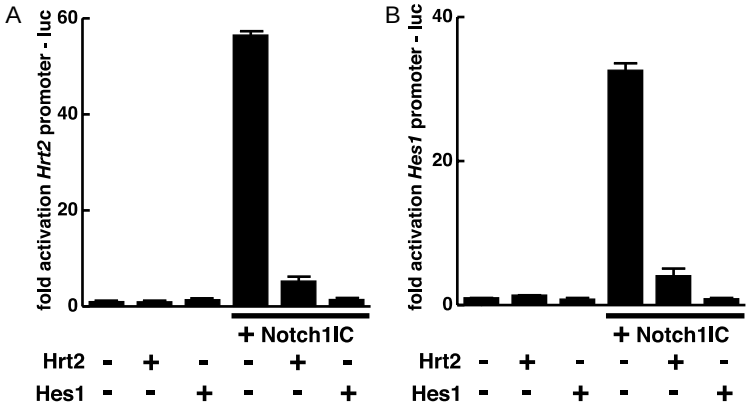

Fig. 1. Hrt2 and Hes1 repress Notch1IC transactivation. Cos1 cells were transiently transfected with Hrt2 (10-kb) (A) or Hes1 (B) promoter-driven luciferase reporters (300 ng). Notch1IC (300 ng), Hrt2 (600 ng) or Hes1 (100 ng) expression constructs were co-transfected as indicated. β-Galactosidase was used to normalize for transfection efficiency. Fold activation from representative experiments done in duplicate with standard deviation error bars is shown.

Results and discussion

Negative regulation of Hrt and Hes

To identify other bHLH family members that suppress the *Hrt2* promoter, we examined the effects of Hes1 on the *Hrt2* promoter-luciferase reporter. Hes1 blocked Notch1-dependent activation of the *Hrt2* promoter (Fig. 1A). Conversely, Hrt2 also suppressed Notch1-dependent activation of a *Hes1* promoter-luciferase reporter (Fig. 1B). Hrt2 and Hes1 proteins did not suppress basal luciferase activity without Notch activation. Northern analysis confirmed that Hrt2 and Hes1 did not downregulate endogenous RBP-Jκ expression (data not shown).

Hrt2 and Hes1 do not disrupt Notch-RBP-Jj or RBP-Jj - DNA interactions

Hrt2 and Hes1 repress Notch1-dependent transcriptional activation that relies on the RBP-Jκ site in their promot-

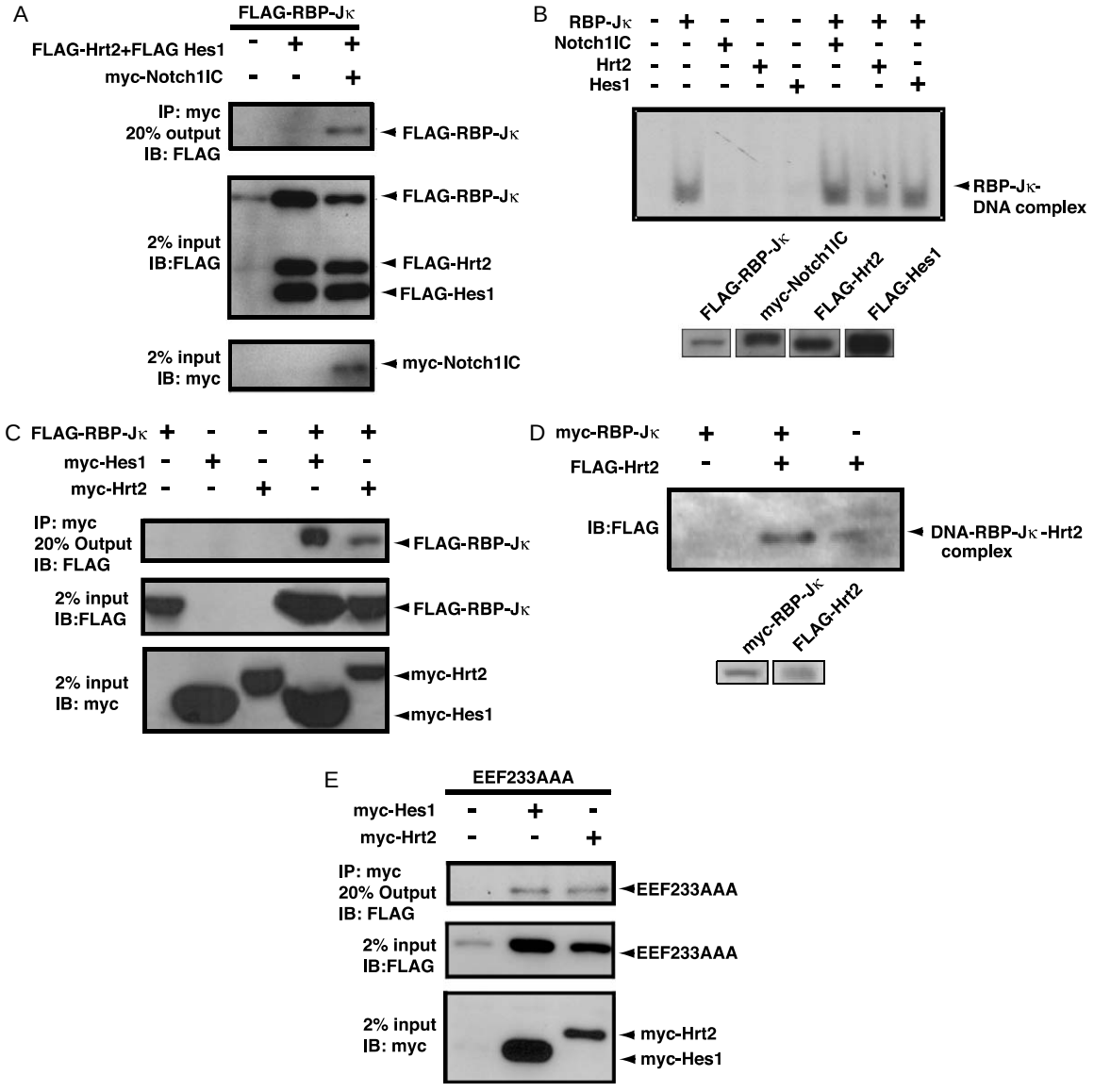

Fig. 2. Hrt2 and Hes1 interact with RBP-Jκ. (A) Cos1 cells were transiently transfected with expression constructs for FLAG-RBP-Jκ (900 ng), FLAG-Hrt2 (600 ng), FLAG-Hes1 (300 ng) or myc-Notch1IC (900 ng) and immunoprecipitated (IP) with anti-myc antibody. Immunoblot (IB) with anti-FLAG antibody revealed that RBP-Jκ could still associate with Notch1IC in the presence of Hrt2 and Hes1. (B) Cell lysates from Cos1 cells transiently transfected with RBP-Jκ (900 ng), Notch1IC (900 ng), Hrt2 (600 ng) or Hes1 (300 ng), were incubated with radiolabeled oligonucleotide containing RBP-Jκ binding site and analyzed by EMSA. RBP-Jκ DNA-binding was unaffected by the presence of Hrt or Hes1 in electromobility shift assays. Appropriate expression was confirmed by Western blot. (C) FLAG-RBP-Jκ (900 ng), myc-Hrt2 (600 ng) or myc-Hes1 (300 ng) were co-expressed in Cos1 cells and immunoprecipitated with anti-myc antibody. Hrt or Hes1 formed a complex with RBP-Jκ. (D) Oligonucleotide containing the RBP-Jκ binding site was precipitated with avidin beads in the presence of myc-RBP-Jκ and/or FLAG-Hrt2. The recovery of Hrt2 was enhanced in the presence of RBP-Jκ. (E) FLAG-RBP-JκEEF233AAA (EEF233AAA) (900 ng), myc-Hrt2 (600 ng), or myc-Hes1 (300 ng) were co-expressed in Cos1 cells and immunoprecipitated with anti-myc antibody. Input controls are shown for (A, C, and E).

ers, suggesting mediation through RBP-Jκ. Since Notch1 dissociates SMRT from RBP-Jκ to activate transcription of the *Hes1* promoter [29], we tested whether disruption of the Notch1-RBP-Jκ complex by Hrt2 or Hes1 might explain the repressive effects. However, in co-immunoprecipitation assays, co-expression of Hrt2 and Hes1 did not prevent Notch1IC association with RBP-Jκ (Fig. 2A). We compared the association of Notch1IC with RBP-Jκ in the presence and absence of Hrt2 and/or Hes1, and found that fluctuations in band intensity precluded conclusions concerning the effect of Hrt2 and Hes1 on the affinity of Notch1 for RBP-Jκ (data not shown).

To determine if Hrt2 or Hes1 disassociates RBP-Jκ from its binding site, we performed gel electromobility shift assays (EMSA) with a radiolabeled binding site for RBP-Jκ. With RBP-Jκ, migration of this oligonucleotide was retarded, indicating a protein–DNA complex (Fig. 2B). Addition of cellular extracts from transient transfections of expression plasmids for RBP-Jκ, with or without expression plasmids for Notch1IC, Hrt2, or Hes1, showed no significant difference in RBP-Jκ DNA-binding.

*Hrt2 and Hes1 physically associate with RBP-J*j

Since Hairless binds the DNA-RBP-Jκ complex and recruits *Drosophila* C-terminal binding protein [30] to repress transcription, we tested if Hrt and Hes complex with RBP-Jκ. FLAG-RBP-Jκ co-immunoprecipitated only in the presence of myc-Hrt2 or myc-Hes1, suggesting complex formation (Fig. 2C). Similar results were observed with mouse Hrt1 and Hrt3 and human HRT1, 2, and 3 (data not shown). Precipitation of an oligonucleotide containing the RBP-Jκ binding site with avidin beads in the presence of myc-RBP-Jκ and/or FLAG-Hrt2 enhanced recovery of Hrt2 in the presence of RBP-Jκ, confirming interaction of RBP-Jκ and Hrt2 (Fig. 2D).

Because SMRT complexes with both Hrt and RBP-Jκ, we performed co-immunoprecipitation experiments to determine if mutations in RBP-Jκ reported to abolish SMRT binding would influence the RBP-Jκ-Hrt interaction [10,31]. A mutant RBP-Jκ containing EEF to AAA mutations at positions 233–235 had no effect on the association with Hrt2 or Hes1 (Fig. 2E), suggesting that SMRT is not required for Hrt2/Hes1-RBP-Jκ complex formation.

*Hrt2/Hes1 bHLH domain is necessary for interaction with RBP-J*j

To map regions needed for interaction between RBP-Jj and Hrt2/Hes1, deletion constructs of Hrt2 and Hes1 were tested for RBP-Jκ binding in co-immunoprecipitation assays. Expression constructs lacking the basic, orange, and extreme C-terminal region (Y/T) of Hrt2 and successive C-terminal truncations of Hes1 were generated. All mutant proteins associated with RBP-Jκ to some degree (Fig. 3), although the interaction of the basic and orange domain deletions of Hrt2 were much weaker.

In addition, we generated mutations in the remaining area of conservation that spanned the HLH domain. To determine the necessity of the first helix in Hrt2 or Hes1 for formation of the RBP-Jκ-bHLH complex, we replaced a leucine at position 68 (Hrt2) or 54 (Hes1) with a proline predicted to perturb the alpha helical structure [32]. Hrt2 (L68P) and Hes1 (L54P) failed to interact with RBP-Jκ, indicating that the first helix is necessary for the Hrt2/Hes1-RBP-Jκ complex (Fig. 3). The proline mutants did not abrogate homo- or hetero-dimer formation between Hrt2 (L68P) and Hes1 (L54P), suggesting that the HLH

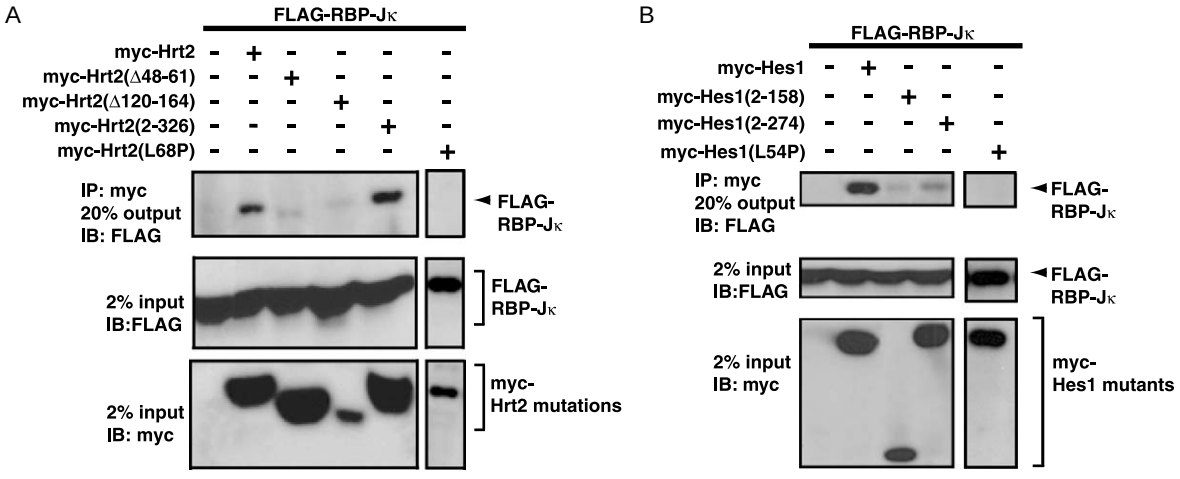

Fig. 3. The bHLH domain of Hrt2 or Hes1 is necessary for interaction with RBP-Jκ. (A) Cos1 cells were transiently transfected with FLAG-RBP-Jκ (900 ng), myc-Hrt2 (600 ng), myc-Hrt2 (Δ48–61) (600 ng), myc-Hrt2 (Δ120–164) (900 ng), myc-Hrt2 (2–326) or myc-Hrt2(L68P) (1200 ng) and immunoprecipitated (IP) with anti-myc antibody. Association with RBP-Jκ was detected by anti-FLAG immunoblot (IB). (B) Cos1 cells were transiently transfected with FLAG-RBP-Jκ (900 ng), myc-Hes1 (300 ng), myc-Hes1 (2–158) (300 ng), myc-Hes1 (2–274) (300 ng) or myc-Hes1 (L54P) (300 ng) and immunoprecipitated with anti-myc antibody. Interaction with RBP-Jκ was detected by anti-FLAG IB. Equivalent expression of the Hes1 constructs was verified by Western blot.

domain was generally intact and that the disruption of the Hrt2/Hes1-RBP-Jκ complex was specific (data not shown). Nuclear staining patterns were indistinguishable from wild-type, confirming proper intracellular trafficking (data not shown). Therefore, the integrity of the first helix of the HLH domain was necessary for RBP-Jκ-Hrt2/Hes interaction.

To further examine the helical domain, we tested if disrupting the interaction with RBP-Jκ affects Hrt2 or Hes1 repression of Notch1-dependent activation of the *Hrt2* promoter. Notch1IC was co-transfected with the *Hrt2* promoter-driven luciferase reporter and each of the Hrt2 and Hes1 mutants. The ability to repress Notch activation of the *Hrt2* promoter-luciferase reporter corresponded to the degree of interaction, with the basic domain deletion mutation disrupting all repressive activities (Figs. 3, 4, and data not shown). Although the Hrt2 mutant lacking the basic domain weakly associated with RBP-Jκ, the basic domain may be required for adequate recruitment of co-repressors. The leucine to proline mutation in the HLH region, which disrupted Hrt2/Hes1-RBP-Jκ interaction, did not repress Notch1IC activation (Figs. 3 and 4). This evidence is consistent with a role for Hrt2 and Hes1 in negative autoregulation via their interaction with RBP-Jκ through the HLH domain.

In the current work, we sought to elucidate the mechanism of negative feedback regulation of Notch1 by the bHLH transcription factors Hrt2 and Hes1. Our findings that Notch1, RBP-Jκ, and bHLH proteins do not form mutually exclusive partnerships indicate that Hrt and Hes1 may modulate rather than terminate the Notch1-RBP-Jκ signaling pathway. RBP-Jκ and Hrt2 or Hes1

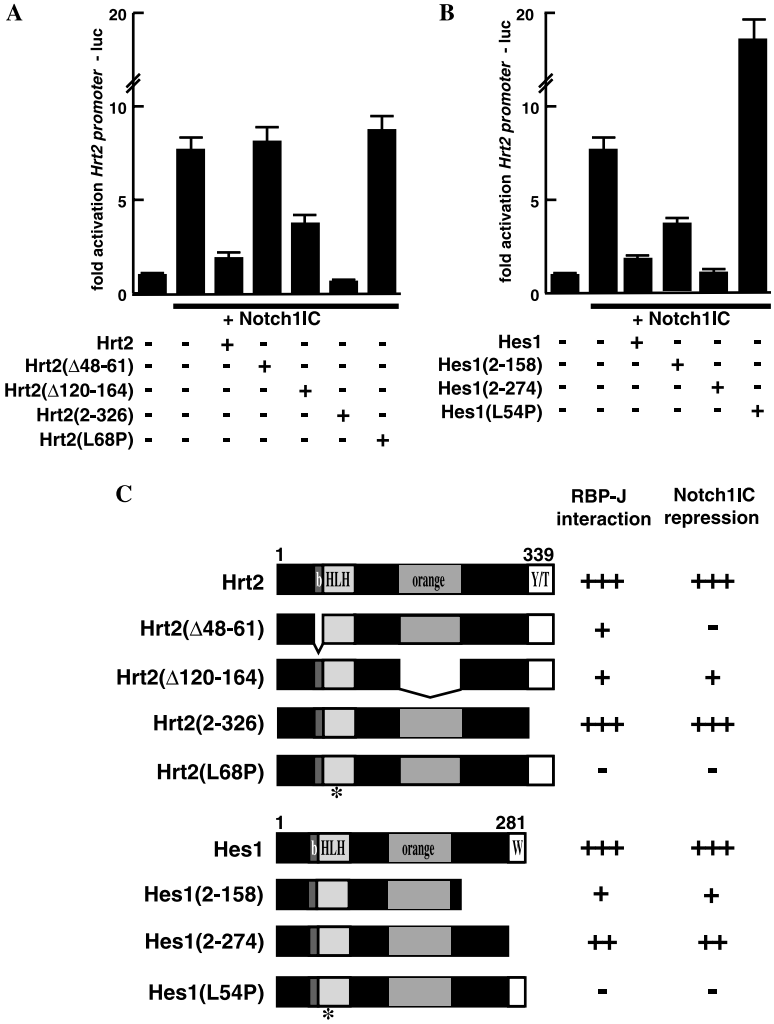

Fig. 4. The bHLH domain of Hrt2 or Hes1 is necessary for interaction with RBP-Jκ and repression of Notch1IC. (A) Fold activation of the Hrt2 enhancer-driven luciferase reporter (300 ng), in the presence of Notch1IC (150 ng), Hrt2 (600 ng), Hrt2 (Δ48–61) (600 ng), Hrt2 (Δ120–164) (900 ng), Hrt2(2–326) (300 ng) or myc-Hrt2 (L68P) (1200 ng). Equivalent expression of the Hrt2 constructs was verified by Western blot. β-Galactosidase was used to normalize for transfection efficiency. (B) Hrt2 enhancer-driven luciferase activity in the presence of Notch1IC (150 ng), Hes1 (200 ng), Hes1 (2–158) (200 ng), Hes1 (2–274) (200 ng) or myc-Hes1 (L54P) (300 ng). Equivalent expression of the Hes1 constructs was verified by Western blot. (C) Schematic of Hrt2 and Hes1 mutations and the results of the interaction with RBP-Jκ and Notch1IC repression. b, basic region; HLH, helix-loop-helix domain; Y/T, YXPW-TE/IGAF domain; W, WRPW domain. Asterisks denote positions of amino-acid substitutions.

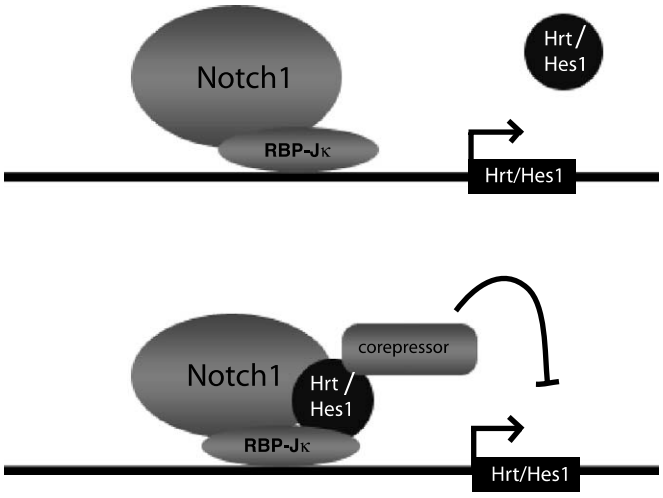

Fig. 5. Hypothetical model of the negative feedback signal of the Notch signal. Model whereby Notch1 transactivation of the Hrt/Hes1 promoter is repressed through the recruitment of Hrt/Hes1 and co-repressors to RBP-Jκ. The association of Hrt and Hes1 with their co-repressor(s) does not result in the dissociation of the Notch1-RBP-Jκ complex from DNA, nor a disassociation of Notch from RBP-Jκ.

formed a complex that depended on an intact first helix in the HLH domain: altering the secondary structure of this region abrogated its repressive properties when tested in the RBP-Jκ-dependent Notch1-signaling pathway (Fig. 5). Our findings expand the role of the bHLH region beyond DNA-binding and bHLH factor dimerization and suggest that moderate changes in the structure of the HLH domain can have significant effects on protein function. As Hrt and Hes1 are expressed in a cyclical fashion in somites [12], it is possible that this periodic expression may re-enforce, or limit, Notch1 activity in these tissues during specific temporal periods or for a subgroup of RBP-Jκ pathway dependent genes. Recognition of the importance of the Hrt2/Hes1-RBP-Jκ complex in the negative regulation of Notch signaling may lead to a greater understanding of how Notch signaling is modulated during development and help to elucidate targets of cardiovascular gene expression refined via the Notch-Hrt autoregulatory loop.

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