Association with the Nuclear Matrix and Interaction with Groucho and RUNX Proteins Regulate the Transcription Repression Activity of the Basic Helix Loop Helix Factor Hes1*

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Hairy/Enhancer of split 1 (Hes1) is a mammalian transcriptional repressor that plays crucial roles in the regulation of several developmental processes, including neuronal differentiation. The aim of this study was to elucidate the molecular mechanisms that regulate the transcription repression activity of Hes1. It is shown here that Hes1 associates with the nuclear matrix, the ribonucleoprotein network of the nucleus that plays important roles in transcriptional regulation. Nuclear matrix binding is mediated by the same Hes1 C-terminal domain that is also required for transcriptional repression. This domain contains the WRPW motif that acts as a binding site for the transcriptional corepressor Groucho, which also localizes to the nuclear matrix. Both the nuclear matrix association and transcription repression activity of Hes1 are inhibited by deletion of the WRPW motif, indicating that Groucho acts as a transcriptional corepressor for Hes1. This corepressor role is not modulated by the Groucho-related gene product Grg5. In contrast, the Runt-related protein RUNX2, which localizes to the nuclear matrix and interacts with Groucho and Hes1, can inhibit both the Groucho-Hes1 interaction and the transcription repression ability of Hes1. Together, these observations suggest that transcriptional repression by Hes1 requires interactions with Groucho at the nuclear matrix and that RUNX proteins act as negative regulators of the repressive activity of Groucho-Hes1 complexes.

In the mammalian central nervous system, progenitor cells located in the ventricular zone of the neural tube undergo proliferation and ultimately differentiate into neurons in response to intrinsic and extrinsic cues. The mechanisms regulating the commitment of these progenitor cells to the neuronal fate are controlled by either positive or negative regulators belonging to separate families of transcription factors containing the basic helix loop helix (bHLH) motif. Proteins that promote neuronal differentiation include a number of evolutionarily conserved transcriptional activators, generally referred to as the proneural proteins (reviewed in Refs. 1 and 2). These include several related molecules belonging to the Neurogenin, Ash, Ath, and NeuroD families (3–8). Ectopic expression of proneural proteins results in the differentiation of supernumerary neurons, whereas disruption of their functions causes neuronal loss (4, 5, 8). Factors that antagonize neuronal differentiation include bHLH proteins which mediate transcriptional repression rather than transactivation. These molecules are homologous to the Drosophila Hairy/Enhancer of split (Hes) proteins, which control insect neuronal development by negatively regulating the functions of the neurogenic genes in response to activation of the cell surface receptor, Notch (9–13).

The best characterized member of the mammalian Hes family is the Hes1 gene (14). In the nervous system, Hes1 is highly expressed during the progenitor-to-neuron transition, and its expression then decreases during the developmental maturation of postmitotic neurons (14). Persistent expression of Hes1 inhibits neuronal differentiation in the developing telencephalon (15). Conversely, targeted disruption of Hes1 causes premature neuronal differentiation and up-regulation of proneural genes, in addition to other developmental defects (16–18). Similar to Drosophila Hes proteins, Hes1 is a transcriptional repressor that acts as a nuclear effector of the Notch signaling pathway during mammalian neurogenesis (14, 18, 19). Together, these findings demonstrate that Hes1 is a crucial negative regulator of neuronal differentiation in mammals and underscore the importance of understanding how its cellular functions are regulated.

Although little is presently known about the molecular mechanisms that underlie transcriptional repression by Hes1, studies in Drosophila show that invertebrate Hes proteins interact with the general transcriptional corepressor, Groucho (11, 20, 21). Mutations that inhibit the Groucho-Hes interaction interfere with the ability of Drosophila Hes proteins to repress transcription, suggesting that Groucho acts as a transcriptional corepressor for these factors (11, 20, 21). These findings first raised the possibility that mammalian homologs of Drosophila Groucho, designated as transducin-like Enhancer of split (TLE) or Groucho-related genes (Grg) 1 through 4 (hereafter referred to as TLE1–4) (22, 23), may be involved in transcriptional repression by Hes1. Consistent with this notion, Hes1 and TLE

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¶ The abbreviations used are: bHLH, basic helix loop helix; AES, amino-terminal Enhancer of split; Ash, achaete-scute homolog; Ath, atonal homolog; GAL4bd, DNA binding domain of GAL4; Grg, Groucho-related gene; GST, glutathione S-transferase; Hes, Hairy and Enhancer of split; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TLE, transducin-like Enhancer of split; WDR, WD40 repeat; kb, kilobase(s).
genes are coexpressed in the nervous system (14, 24–26), and perturbations that force a persistent expression of Hes1 or TLE1 in the mammalian forebrain result in similar phenotypes characterized by neuronal loss (15, 27). In addition, Hes1 physically interacts with TLE1 and TLE2 in vitro (28, 29). Taken together with the demonstration that TLE proteins provide a transcriptional corepressor function to a number of different DNA-binding factors (30–33), these observations suggest that transcriptional repression by Hes1 is regulated by interactions with TLE family members. It remains to be determined, however, whether Hes1 and TLE proteins functionally interact in vivo and, if so, how their transcriptional functions are regulated.

Here we describe experiments that demonstrate that Hes1 interacts with TLEs in mammalian cells and localizes to the nuclear matrix, where TLEs are also found. Both the nuclear matrix association and transcription repression activity of Hes1 are inhibited by deletion of the same C-terminal domain, indicating a correlation between nuclear matrix binding and transcriptional repression. This observation is in agreement with previous studies showing that the nuclear matrix is functionally involved in the regulation of gene expression by concentrating and localizing transcription factors and/or facilitating the formation of appropriate chromatin structures (reviewed in Ref. 34). Our results also show that the ability of Hes1 to interact with TLEs is required for both nuclear matrix association and transcriptional repression, indicating that TLEs act as transcriptional corepressors for Hes1. The involvement of TLEs in Hes1 function is not regulated by the AESI/Grg5 (hereafter referred to as Grg5) protein. Grg5 is a naturally occurring factor related to the first 200 amino acids of Groucho/TLEs and has been regarded as a dominant-negative regulator of at least certain TLE transcriptional corepressor functions (33, 35–38). In contrast, our findings suggest that the corepressor role of TLEs for Hes1 is negatively regulated by members of the RUNX family of transcription factors, which are coexpressed, and interact, with both Hes1 and TLE proteins (14, 24–26, 30, 31, 39–41). Taken together, these findings suggest that interactions among Hes1, TLE, and RUNX proteins at the nuclear matrix regulate the transcriptional activity of Hes1.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The following is a summary of the names and origins of the constructs used in these studies. Additional information on cloning strategies and oligonucleotide primers used in PCR experiments is available upon request. Constructs pcEBG-Hes1, pCMV2-FLAG-Hes1, pCMV2-FLAG-Hes1-(1–275), pcDNA3-TLE1, pCMV5-RUNX2, pGEX3-TLE3/WDR, pRec/CMV-Hes1, pBluescript-RUNX2, and p6N-fAct-luc have been described previously (14, 28, 29, 31). Plasmid pEBG-Hes1-(1–275) was obtained by subcloning the appropriate PCR product into the filled-in BamHI site of pEBG. pCMV2-FLAG-Hes1-(1–275) was generated by subcloning the appropriate PCR product into the filled-in BamHI site of pCMV2-FLAG. Plasmid pcDNA3-GAL4bd-Hes1-(193–281) was obtained by digesting the pcRec/CMV-Hes1 DNA with Smal and then subcloning the fragment encoding the last 88 amino acids of Hes1 (plus ~1.2 kb of 3′-untranslated region) into the EcoRV site of pcDNA3-GAL4bd. Construct pCMV-Grg5 (42) was kindly provided by Dr. T. Okamoto (Nagoya University).

**Interaction Assays in Transfected Cells and Western Blotting Analysis**—Human 293 embryonic kidney or rat ROS17/2.8 osteoblastic cells were grown and transfected using the SuperFect reagent (Qiagen) as described previously (31). Coprecipitation assays using plasmids pEBG-Hes1 and pEBG-Hes1-(1–275) (or pEBG-Act as control) and immunoprecipitation experiments with anti-FLAG epitope antibodies (Sigma) were performed exactly as described previously (31, 43). Western blotting studies were performed with either panTLE (22, 44), anti-GST (Santa Cruz Biotechnology), anti-FLAG, anti-histone H3 (a kind gift of Dr. C. D. Allis, University of Rochester), anti-RUNX2 (31), anti-GAL4bd (Santa Cruz Biotechnology), or anti-lamin B1 (Chemicon) antibodies as described previously (22, 31, 43, 44).

Isolation of Nuclear Matrix Proteins and Preparation of Nuclear Extracts—ROS17/2.8 cells were subjected to sequential extraction and solubilization steps to isolate nuclear matrix proteins exactly as described by Merriman et al. (45). High salt-resistant and -soluble nuclear fractions were prepared by subjecting isolated nuclei to incubation in the presence of 0.5 M NaCl as described previously (48).

**Transient Transfection/Transcription Assays**—Human 293 cells were transfected using the SuperFect reagent. The amount of DNA transfected was adjusted using pcDNA3 plasmid so that the total amount of DNA used in each transfection was the same (3.0 μg). Twenty-four hours later, transcription assays were performed as described (29, 31), using a reporter plasmid p6N-fAct-luc in the absence or presence of pcMV2-FLAG-Hes1, pcMV2-FLAG-Hes1-(1–227), pcDNA3-TLE1, pCMV-Grg5, or pCMV5-RUNX2 as indicated in the figure legends. In each case, 0.25 μg of pCMV-β-galactosidase plasmid DNA was cotransfected to provide a means of normalizing the assays for transfection efficiency. Results were expressed as mean ± S.D. and were tested for statistical significance by the one-tailed Student’s t test for paired differences.

**RESULTS**

**Hes1 Interacts with TLE in a WRWP Motif-dependent Way**—Hes1 is coexpressed with TLE proteins in a number of mammalian cell types (14, 24–26, 29) and physically interacts with TLE1 and TLE2 in vitro (20, 28, 29). These findings suggest that TLEs may form transcription repression complexes with Hes1 in vivo. To begin to examine this possibility, we first asked whether Hes1 and TLE proteins would interact in cultured mammalian cells. Rat ROS17/2.8 osteoblastic cells, which express high levels of endogenous TLEs (31), were transfected with an expression construct for a fusion protein of GST and Hes1. After isolation of GST-Hes1 on glutathione-Sepharose beads, Western blotting analysis with panTLE monoclonal antibodies (22, 43) revealed that endogenous TLE proteins coprecipitated with GST-Hes1 (Fig. 1A, lane 2). In contrast, TLEs did not coprecipitate with a fusion protein of GST and Hes1-(1–275), which lacks the last six amino acids containing the WRWP motif (Fig. 1A, lane 4). GST-Hes1 and GST-Hes1-(1–275) were expressed at equivalent levels (Fig. 1B, lanes 2 and 4). These results show that TLEs and Hes1 interact with each other in mammalian cells and that this interaction requires the WRWP motif of Hes1.

**Hes1 Associates with the Nuclear Matrix**—To determine whether Hes1 and TLE proteins might colocalize to a common nuclear compartment, it was then asked whether Hes1 could associate with the nuclear matrix, where TLE proteins are known to localize (46). Other Hes1-binding proteins, like RUNX family members, also associate with the nuclear matrix (45, 47, 48). This filamentous ribonucleoprotein network of the nucleus is thought to play important roles in the regulation of gene expression by mediating the colocalization and interaction of transcription factors and/or facilitating the establishment of appropriate chromatin structures. ROS17/2.8 cells were transfected with FLAG epitope-tagged Hes1 and then subjected to two different kinds of biochemical fractionation to determine the subnuclear localization of Hes1. In one procedure, nuclei were isolated and high salt-soluble and -insoluble nuclear fractions were obtained as described previously (43). Hes1 was poorly extracted from nuclei by high ionic strength conditions (Fig. 2A, cf. lanes 1 and 2). This behavior is similar to that of other nuclear matrix proteins, including RUNX family members (45, 47), and suggests that Hes1 is tightly associated with internal nuclear compartments. To confirm this possibility, the nuclear matrix isolation protocol described previously (45) was utilized to test the possibility that Hes1 might associate with this compartment. Cytoplasmic proteins were extracted, and the remaining insoluble fraction was treated with DNase I and RNase A, followed by ammonium sulfate to release chromatin components and nuclear proteins that are not bound to the nuclear matrix. The remaining pellet was then extracted in...
disassembly buffer to yield nuclear matrix proteins (45). The effectiveness of this fractionation procedure was confirmed by Western blotting analysis of cytoplasmic, nuclease-treated, and nuclear matrix fractions. The chromatin component, histone H3 was present only in the nuclease-treated fraction (Fig. 2B, lane 2), whereas the nuclear matrix protein, lamin B1 was only recovered in the nuclear matrix fraction (Fig. 2C, lane 3). A significant amount of Hes1 immunoreactivity was found in both the nuclease-treated and nuclear matrix fractions (Fig. 2D, lanes 2 and 3), in agreement with the tight association of Hes1 with the high salt-resistant nuclear fraction described above. Because TLE proteins have been shown previously to be associated with both chromatin and the nuclear matrix (44, 46), these findings show that Hes1 has a subnuclear distribution similar to that of TLEs.

Elimination of the TLE binding ability of Hes1 by removal of the last six amino acids containing the WRPW motif (resulting in the truncated protein Hes1-(1–275)) almost completely abolished the nuclear matrix association of Hes1 (Fig. 2E, lane 3). This situation was correlated with an increase in the amount of Hes1 found in the cytoplasmic fraction (Fig. 2E, lane 1). A smaller form of Hes1-(1–275), retaining the amino-terminal FLAG epitope, was also observed under the experimental conditions used to prepare non-nuclear and nuclear fractions (Fig. 2E, lane 1). This faster-migrating species, likely representing the product of a C-terminal proteolytic degradation, was predominantly found in the cytoplasmic fraction. These findings demonstrate a correlation between the ability of Hes1 to associate with the nuclear matrix and its ability to interact with TLEs, suggesting that the latter mediate the nuclear matrix association of Hes1. A larger C-terminal deletion that removed the last 54 amino acids of Hes1, resulting in the truncated protein Hes1-(1–227), completely abolished the association of Hes1 with the nuclear matrix (Fig. 2F, lane 3) and greatly reduced its association with the nuclease-treated fraction (Fig.
2F, lane 2). This was correlated with the accumulation of Hes1-(1–227) in the cytosolic fraction (Fig. 2F, lane 1). Together, these results show that Hes1 can associate with the nuclear matrix and that its C-terminal domain is necessary for this association.

To examine these possibilities further, we asked whether the C-terminal region of Hes1 was sufficient to mediate the nuclear matrix binding of a heterologous protein. ROS17/2.8 cells were transfected with either the DNA-binding portion of the yeast nuclear protein GAL4 (which does not associate with the nuclear matrix (48)) or a fusion protein of GAL4bd and the last 88 amino acids of Hes1. GAL4bd-Hes1-(193–281) was able to associate with the nuclear matrix (Fig. 3G, lane 3), whereas GAL4bd alone did not bind to this compartment (Fig. 3H, lane 3), indicating that the last 88 amino acids of Hes1 can mediate nuclear matrix binding. These results are consistent with previous studies showing that GAL4bd-Hes1-(193–281) interacts with TLEs, whereas GAL4bd alone does not (28). Taken together, these findings demonstrate that the C-terminal domain of Hes1 mediates the association of this protein with the nuclear matrix.

**TLE Proteins Act as Transcriptional Corepressors for Hes1**—Based on the physical interaction and subnuclear colocalization of Hes1 and TLEs, we next asked whether TLEs are required for the transcription repression function of Hes1. Human 293 cells, which express endogenous TLEs (31), were transfected for the transcription repression function of Hes1. Human 293 cells were transfected with the p6N-βAct-luc reporter construct (2.0 μg) with or without the following expression vectors (ng/ transfection): pFLAG-Hes1 (100 ng), pFLAG-Hes1-(1–227) (100 ng), or pcDNA3-TLE1 (75 ng). The activity of the reporter constructs in the absence of any expression plasmid was considered as 100%. Luciferase activities were expressed as the average ± S.D. of four independent experiments performed in duplicates. TLE1 enhanced transcriptional repression by Hes1 (lane 3; *p = 0.021) but had no effect on Hes1-(1–227) (lane 5).

**Grg5 Is Not Involved in the Regulation of Transcriptional Repression by Hes1**—To elucidate the molecular mechanisms that regulate the corepressor activity of TLEs for Hes1, we asked whether Grg5 might act as a negative regulator of this function. This hypothesis was suggested by previous studies showing that Grg5 can heterodimerize with TLE proteins (29, 33, 37) and, in some cases, have a dominant-inhibitory effect on the corepressor activity of TLEs (33, 38). To examine whether Hes1 might interact with Grg5 and/or TLE-Grg5 multimers, ROS17/2.8 cells were transfected with GST-Hes1 in the presence or absence of a FLAG epitope-tagged Grg5 protein. After cell lysis and immunoprecipitation of Grg5 with anti-FLAG antibodies (Fig. 4A, lane 2, arrow), GST-Hes1 did not coimmunoprecipitate with Grg5 (Fig. 4B, cf. lanes 1 and 2). In contrast, endogenous TLE proteins coimmunoprecipitated with Grg5 (Fig. 4C, cf. lanes 1 and 2). TLEs were not coimmunoprecipitated from cells transfected with the empty FLAG vector (Fig. 4C, lane 4). In converse experiments in which GST-Hes1 was isolated on glutathione-Sepharose beads, Grg5 did not co-isolate with GST-Hes1 (Fig. 4D, cf. lanes 1 and 2), whereas endogenous TLE proteins coprecipitated with GST-Hes1 (Fig. 4E, lane 2). These results suggest that Hes1 interacts with neither Grg5-Grg5 oligomers nor TLE-Grg5 oligomers. To examine this possibility further, transient transfection assays were performed to determine whether Grg5 might negatively regulate transcriptional repression by Hes1 in cells expressing endogenous TLEs. Transfection of increasing amounts of a Grg5 expression plasmid had no effect on the repressor activity of Hes1 (Fig. 4F). Expression of the Grg5 protein was confirmed by Western blotting analysis (Fig. 4G). Taken together, these studies demonstrate that Grg5 does not associate with Hes1 and does not act as a negative regulator of the corepressor effect of TLE on Hes1.

**Hes1-mediated Transcriptional Repression Is Inhibited by RUNX2**—To examine further the mechanisms involved in the regulation of Hes1 activity, we asked whether the Runt-related protein RUNX2 might modulate the transcription repression ability of Hes1. This possibility was suggested by the demonstration that both Hes1 and TLE bind to RUNX1 and RUNX2 (30, 31). Moreover, Hes1 can inhibit the TLE-RUNX interaction and potentiate transactivation by RUNX2 (31). Our studies showed that the ability of Hes1 to mediate transcriptional repression was completely inhibited when it was coexpressed with RUNX2 (Fig. 5A, cf. lanes 2 and 3). RUNX2 alone had no detectable effect on reporter gene expression (Fig. 5A, lane 4).

The inhibition of Hes1-mediated repression did not appear to be due to an effect of RUNX2 on the stability of this protein, because Hes1 was equally stable in the presence of RUNX2 (Fig. 5B). We therefore asked whether the inhibitory effect of RUNX2 might be due to an inhibition of the interaction of Hes1 with TLEs, which are required for Hes1-mediated repression. Based on the previous observation that the C-terminal WDR domain of Groucho/TLEs is involved in Hes protein binding (21), interaction assays were set up to determine whether increasing amounts of RUNX2 would reduce binding of a fixed amount of Hes1 to the TLE WDR domain. Binding of in vitro translated Hes1 (shown in Fig. 5C, lane 1) to a GST-TLE(WDR) fusion protein was compared in the absence or presence of in vitro translated RUNX2 (shown in Fig. 5C, lane 5). Because RUNX2 also interacts with the TLE WDR domain (31), these assays were performed using very small, limiting amounts of GST-TLE(WDR) substrate to prevent having an excess of binding sites for both Hes1 and RUNX2. Hes1 bound to the WDR...
expression plasmid was considered as 100%. Luciferase activities were expressed as the average or presence of increasing amounts of a FLAG-Grg5 expression construct (lane 1–3) transfected in the absence (lane 1) or presence (lanes 3–6) of the indicated amounts of a FLAG-Grg5 expression construct. The activity of the reporter gene in the absence of any ligand was considered as 100%. Luciferase activities were expressed as the average ± S.D. of at least three independent experiments performed in duplicates. Grg5 has no effect on transcriptional repression by Hes1. GST-TLE(WDR) (not shown). Taken together with the observation that RUNX2 does not inhibit the DNA binding ability of Hes1 in electrophoretic mobility shift assays,2 these findings suggest that RUNX2 may act as a negative regulator of the transcription repression ability of Hes1 by interfering with the interaction of Hes1 with the TLE corepressors.

**DISCUSSION**

**Nuclear Matrix Association of Hes1 and Transcriptional Repression**—The present studies have provided the first demonstration that Hes1 associates with the nuclear matrix, a nuclear compartment where TLE and RUNX proteins, both of which interact with Hes1, are also found (45–48). The ability of Hes1 to associate with the nuclear matrix is mediated by the same C-terminal domain that is also required for transcriptional repression, showing a correlation between transcription repression ability and nuclear matrix targeting. No sequences resembling consensus nuclear matrix targeting signals found in other proteins (47) were identified within the C-terminal domain of Hes1. However, this region contains binding sites for TLEs, suggesting that these proteins are involved in the nuclear matrix association of Hes1. Consistent with this possibility, the nuclear matrix binding of Hes1 is almost completely abolished by deletion of the WRPW motif that mediates TLE binding. Because the cells used in our nuclear matrix preparations express high levels of endogenous TLEs, these findings strongly suggest that TLEs mediate the nuclear matrix localization of Hes1. The residual nuclear matrix association observed after deletion of the WRPW motif alone may be the result of the ability of Hes1 to interact with RUNX proteins, which are also expressed in ROS17/2.8 cells and localize to the nuclear matrix. This possibility is consistent with the observation that the nuclear matrix binding of Hes1 is blocked by removal of the last 54 amino acids, which are part of the domain (Fig. 5C, lane 2) and this interaction was gradually decreased by the addition of increasing amounts of RUNX2 (Fig. 5C, lanes 3 and 4). At lower RUNX2 concentrations, this decrease was not correlated with detectable binding of RUNX2 to the TLE WDR domain (Fig. 5C, lane 3). This result suggests that reduced Hes1 binding was due to the formation of RUNX2:Hes1 complexes at the expense of TLE:Hes1 complexes and is in agreement with the previous observation that RUNX2 has a higher affinity for Hes1 than for TLE (31). At higher RUNX2 concentrations, binding of RUNX2 to the TLE WDR domain was also observed, as described previously (31) (Fig. 5C, lane 4). The addition of equal amounts of unprogrammed rabbit reticulocyte lysate had no effect on Hes1 binding to GST-TLE/WDR (not shown). Taken together with the observation that RUNX2 does not inhibit the DNA binding ability of Hes1 in electrophoretic mobility shift assays,2 these findings suggest that RUNX2 may act as a negative regulator of the transcription repression ability of Hes1 by interfering with the interaction of Hes1 with the TLE corepressors.

Regulation of Hes1 Function

FIG. 5. Inhibition of Hes1-mediated transcriptional repression by RUNX2. A, transient transfection/transcription assays. 293 cells were cotransfected with the p6N-βAct-luc reporter construct (2.0 μg) and a Hes1 expression plasmid (20 ng) in the absence (lane 2) or presence (lane 3) of a RUNX2 expression construct (400 ng). In lane 4, the reporter plasmid was cotransfected with the RUNX2 construct alone. Luciferase activity in the absence of any expression plasmid was considered as 100%. RUNX2 inhibited transcriptional repression by Hes1 but had no effect on the basal expression of the reporter gene. B, Western blotting analysis. 293 cells were transfected with a fixed amount (20 ng) of a FLAG-Hes1 expression plasmid in the absence (lane 1) or presence (lanes 2–4) of 100, 200, or 400 ng, respectively, of a RUNX2 expression construct. Cell lysates were collected and subjected to Western blotting with either anti-FLAG or anti-RUNX2 antibodies. C, competition binding assays. In vitro translated 35S-labeled Hes1 (lane 1, one-fourth the amount used in each binding assay) was incubated with 50 ng of GST-TLE(WDR) fusion protein in the absence (lane 2) or presence of either a 5-fold (lane 3) or a 25-fold (lane 4) volume excess of in vitro translated 35S-labeled RUNX2 (lane 5, one-tenth of the amount used in lane 4). Due to the low amount of fusion protein used, the pull-down efficiency was low and lanes 2–4 were exposed longer than lanes 1 and 5. The positions of migration of M, standards are indicated.

C-terminal domain previously shown to mediate interaction with RUNX factors (31).

We also found that TLE proteins promote transcriptional repression by Hes1 and that inhibition of the TLE-Hes1 interaction, either by deleting the TLE binding domain of Hes1 or overexpressing the RUNX2 protein, impairs the ability of Hes1 to mediate transcriptional repression. These findings support a model in which Hes1 and TLEs form transcription repression complexes whose gene regulatory functions may be regulated by association with the nuclear matrix. It must be emphasized, however, that the transient nature of the transfection/transcription assays that were performed does not allow the determination of whether the reporter gene targeted by Hes1 was associated with the nuclear matrix. Future studies using stably integrated reporter genes or in vivo targets of Hes1 will be needed to investigate further the role played by the nuclear matrix in the transcription repression activity of Hes1. Although much remains to be learned about the properties of the nonchromatin elements of the internal nuclear structure, there is increasing evidence that the nuclear matrix may be involved in the regulation of gene expression by supporting the formation of transcriptionally competent nuclear domains and/or facilitating the assembly of active transcription complexes. Thus, it is possible that nuclear architecture may play a role in regulating the transcription functions of complexes containing Hes1 and TLE proteins.

In the future, it will also be important to elucidate the molecular mechanisms that underlie the repressor activity of TLE-Hes1 complexes. Previous studies have shown that Groucho/TLEs can interact with histone proteins and histone deacetylases (32, 44). Moreover, transcriptional corepression by Groucho/TLEs can be inhibited by the histone deacetylase inhibitor, trichostatin A (32). These observations suggest that at least one of the mechanisms utilized by Groucho/TLEs to mediate transcriptional repression is to recruit histone deacetylases to specific DNA sites through interaction with selected DNA-binding proteins. Our preliminary studies suggest that Hes1-mediated transcriptional repression also involves the activity of histone deacetylases (3). However, it is also possible that Groucho/TLE proteins interact with components of the basal transcriptional machinery. This is suggested by the finding that the yeast protein, TUP1, a general transcriptional corepressor that is often regarded as a functional analog of Groucho/TLEs, interacts with subunits of the yeast RNA polymerase II holoenzyme and can repress transcription even in the absence of chromatin components (49, 50). Thus, both histone deacetylase-dependent and-independent mechanisms may underlie transcriptional repression by TLE-Hes1 complexes.

Grg5 Is Not Involved in the Regulation of the Transcriptional Repression Ability of TLE-Hes1 Complexes—Our investigations have also shown that the naturally occurring Grg5 protein, which can heterodimerize with full-length TLEs and has been regarded as a possible dominant-inhibitory regulator of TLE functions, does not associate with Hes1 and does not regulate transcriptional repression by Hes1. This is consistent with the demonstration that the C-terminal WDR domain of Groucho/TLEs, which is missing in Grg5, is involved in Hes protein binding. These findings suggest that Hes1 only recruits full-length TLE proteins to repress transcription and that neither Grg5 nor Grg5-TLE complexes are involved in the functions of Hes1. Importantly, because Grg5 does not inhibit transcriptional repression by TLE-Hes1, our observations also suggest that Grg5 does not act as a dominant-negative regulator of the corepressor function that TLE proteins provide to Hes1. In turn, this suggests that Grg5 is not a general negative regulator of TLE activity. It is possible that Grg5 can exert a dominant-inhibitory effect on TLEs only when it shares with the latter the ability to interact directly with specific DNA-binding proteins, like Tcf/LEF family members (38) or the PRDI-BF1/Blimp1 factor (33). In this case, Grg5 may compete with full-length TLE proteins for binding to Tcf or PRDI-BF1/Blimp1
without being able to provide a transcription repression function, thereby inhibiting the repressive effect of TLEs. Such a dominant-inhibitory function is unlikely to be observed when the DNA-binding protein that requires the TLE corepressor activity binds to neither Grg5 itself nor to TLE-Grg5 complexes, as appears to be the case for Hes1.

Involvement of RUNX2 in Transcriptional Repression by Hes1—In both invertebrates and vertebrates, Hes- and Runt-related proteins contribute to the regulation of common genes (40, 46, 51) and interact with Groucho/TLEs (30, 31, 52). Moreover, Hes1 binds to both RUNX1 and RUNX2 and promotes transcriptional repression by Hes1. Our results show that RUNX2 can inhibit the TLE-Hes1 interaction in vitro and Hes1-mediated transcriptional repression in transfected cells. Taken together with the observation that the DNA-binding ability of Hes1 does not appear to be inhibited by RUNX2, these findings suggest that RUNX2 acts as a negative regulator of Hes1-mediated repression by antagonizing the corepressor function provided by TLEs. This model is consistent with the observation that a number of genes whose promoters contain binding sites for both RUNX and Hes family members are antagonistically regulated by these proteins. For instance, Hes1 is a negative regulator of the expression of the osteopontin gene in osteoblasts (40), whereas RUNX2 positively regulates this gene (46). It is possible that TLE-Hes1 complexes repress the osteopontin gene and that RUNX2 contributes to its activation both indirectly, by interfering with the TLE-Hes1 interaction, and directly, by providing a transactivating function. A similar situation may occur in the case of the Drosophila gene, Sex- lethal, which is positively regulated by the Runt protein (53) and negatively regulated by the Hes family member Deadpan in combination with Groucho (11). The further elucidation of the molecular mechanisms that regulate the interactions of Hes1 with TLE and RUNX proteins will be the subject of future studies and will help clarify the cell differentiation functions of these factors.

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