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Human Sir2-related protein SIRT1 associates with the bHLH repressors HES1 and HEY2 and is involved in HES1- and HEY2-mediated transcriptional repression

Takehiko Takata[a] and Fuyuki Ishikawa[a,b,*]

[a] *Laboratory of Molecular and Cellular Assembly, Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8501, Japan*
[b] *Laboratory of Cell Cycle Regulation, Department of Gene Mechanisms, Graduate School of Biostudies, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan*

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Abstract

The Hairy-related bHLH proteins function as transcriptional repressors in most cases and play important roles in diverse aspects of metazoan development. Recently, it was shown that the *Drosophila* bHLH repressor proteins, Hairy and Deadpan, bind to and function with the NAD^+-dependent histone deacetylase, Sir2. Here we demonstrate that the human Sir2 homologue, SIRT1, also physically associates with the human bHLH repressor proteins, hHES1 and hHEY2, both in vitro and in vivo. Moreover, using the reporter assay, we show that both SIRT1-dependent and -independent deacetylase pathways are involved in the transcriptional repressions mediated by these bHLH repressors. These results indicate that the molecular association between bHLH proteins and Sir2-related proteins is conserved among metazoans, from *Drosophila* to human, and suggest that the Sir2–bHLH interaction also plays important roles in human cells.

Keywords: SIR2; HDAC; Deacetylase; Hairy; HES1; HEY2; bHLH; Repression

The basic helix–loop–helix (bHLH) superfamily of transcription factors consists of a large number of proteins and plays important roles as activators or repressors in diverse aspects of development [1–4]. Hairy-related proteins comprise one subfamily of bHLH and in most cases function as transcriptional repressors [5]. The Hairy-related bHLH protein shows unique structural features that are not shared by bHLH activators: They possess a proline at a specific position in the basic region, a motif called the Orange domain immediately C-terminal to the bHLH domain, and a four-amino-acid motif, WRPW, or its related sequences at the C-terminus [6]. Besides these specific amino acid sequences, the bHLH domain of the Hairy-related proteins shows general features distinct from those of the activator bHLH. Accordingly, the preferred DNA binding sequence of the Hairy-related bHLH proteins may be different from that of activator bHLH proteins. For example, the activator bHLH, by forming either homodimers or heterodimers, binds to the E-box sequence (CANNTG) [7]. In contrast, some of the Hairy-related bHLH proteins preferentially bind to the N-box sequence (CACNAG) [8,9].

A detailed amino acid sequence comparison subdivided the Hairy-related bHLH family into four groups, namely, Hairy, E(spl) (Enhancer of split), Hey, and Stra13 [6]. The Hairy subfamily, represented by *Drosophila* Hairy and its mammalian homologue Hes1, shows typical features of the Hairy-related bHLH proteins, including the proline in the basic region and the C-terminal WRPW sequence [8,10,11]. In contrast, the Hey subfamily, represented by the mouse Hey2 and its zebrafish homologue, Gridlock (GRL), has glycine

* Corresponding author. Fax: +81-75-753-4197.
E-mail address: fishikaw@lif.kyoto-u.ac.jp (F. Ishikawa).

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instead of proline in the basic region, and a YXXW sequence at the C-terminus [12–17].

Genetic studies clearly demonstrated that the Hairy-related bHLH repressors antagonize the activator bHLH proteins, thereby preventing differentiation induced by the activators [3,5]. The repression of differentiation-inducing genes by the Hairy-related bHLH proteins appears to be achieved through multiple molecular pathways. The finding that the C-terminal WRPW tetrapeptide sequence of the Hairy and related proteins mediates repression by recruiting the co-repressor, Groucho, and its mammalian homologue, TLE, established the role of the Hairy-related bHLH protein as an autonomous transcriptional repressor [18–20]. Groucho itself has no enzymatic activity, but it was shown that Groucho directly interacts with the histone deacetylase, HDAC1/Rpd3, indicating that the repression activity in this case involves chromatin modification [21]. Similarly, Hey1 and Hey2 (alternatively called HERP2/Hesr1/HRT1/CHF2 and HERP1/HRT2/CHF1/GRL, respectively) have their own autonomous repression activities [12,22,23]. Interestingly, however, the transcriptional repression domain of Hey proteins resides not at the C-terminal tetrapeptide-related sequences, but at the bHLH domains. The bHLH domain of Hey proteins recruits the mSin3 complex that contains the histone deacetylase HDAC1 [22]. Therefore, it appears that multiple pathways, including hitherto-unknown ones, are involved in the transcriptional repression mediated by the Hairy-related bHLH proteins.

Sir2 is a novel type of histone deacetylase that requires NAD^+ as a co-factor [24–26]. Sir2 was originally isolated as one of the genes essential for the silencing of the yeast silent mating type loci [27]. Subsequent studies revealed that Sir2 is required for heterochromatin formation at yeast telomeres and the rDNA arrays and is involved in longevity [28,29]. However, the physiological function of Sir2 in higher eukaryotes remains largely uncharacterized. Recently, it was reported that *Drosophila* Sir2 interacts genetically and physically with Hairy and Deadpan (another member of the Hairy subfamily) [30], further expanding the repression mechanisms mediated by the Hairy-related bHLH proteins. In mammal, the human SIRT1 and mouse SIR2α proteins show the closest similarities to the yeast and fly proteins [24,31]. In this report, we show that human SIRT1 also physically and functionally interacts with the Hairy-related bHLH proteins. Specifically, we found that SIRT1 interacts with both human HES1 (hHES1, the Hairy homologue) and HEY2 (hHEY2). Therefore, the interaction between Sir2 and Hairy-related proteins is conserved phylogenetically and shared by at least proteins belonging to the two subfamilies of Hairy-related proteins, the Hairy and the Hey subfamilies.

Materials and methods

Cell cultures. All cells were cultured with supplementation of 10% FCS, L-glutamine, penicillin, and streptomycin and grown in 5% CO_2 at 37 °C. PBS(−) (phosphate-buffered saline) without magnesium and calcium was used in this work. FuGENE6 (Roche) was used to transfect various cell lines.

Isolation of SIRT1 gene. Human SIRT1 gene was obtained by screening the cDNA library prepared from poly(A)$^+$ RNA of PA-1 cells using the ZAP-cDNA Synthesis Kit and the lambda ZAP II Predigested *Eco*RI/CIAP-Treated Vector Kit (Stratagene) according to the manufacturer's protocol. The cDNA was synthesized using oligo(dT) primer. An *Eco*RV–*Xba*I fragment of SIRT1 full-length cDNA was cloned into pBluescript SK(−) (pBST-SIRT1). To generate the SIRT1H363Y mutant, the ExSite PCR-Based Site-Directed Mutagenesis Kit (Stratagene) was used to create the point mutation (CAT to TAT) at codon 363.

Isolation of hHES1 and hHEY2 genes. hHES1 and hHEY2 cDNAs were obtained by PCR amplification from Full-Length Clones (Invitrogen). Due to the GC-rich nature of the hHES1 cDNA, we used the Advanced-GC cDNA Kit (BD Biosciences). The PCR products were cloned into pT7Blue T-Vector (Novagen) and sequenced. A series of hHES1 derivatives were generated by PCR amplification.

Construction of expression plasmids. A *Bam*HI–*Not*I fragment of pBST-SIRT1 was used to create the expression vectors. Recombinant proteins were expressed in *Escherichia coli* using pGEX-5X-1 and pGEX-5X-2 vectors (Amersham). For the co-immunoprecipitation assay, cDNAs encoding hHES1 or hHEY2 tagged with the HA epitope at their N-termini were subcloned to pcDNA3 (Invitrogen), transfected, and expressed in 293T cells. The ORF encoding GAL4 DNA binding domain, which was obtained from pDBLeu (Invitrogen), was inserted into the *Hin*dIII–*Nhe*I site of pcDNA3-HA-hHES1 derivatives in frame (effector vector). For the luciferase assay, the reporter vectors pβA-luc and pUAS-βA-luc were used [32,33]. The pβA-luc vector was a gift from Dr. Ryoichiro Kageyama (Kyoto University).

Antibody production. Anti-SIRT1 antibody was affinity-purified from rabbit antisera directed against a synthesized oligopeptide of the carboxyl terminus of SIRT1, CLEDEPDVPERAGG (BEX, Tokyo). This anti-SIRT1 antibody did not recognize mouse Sir2α (SIRT1 homologue).

Recombinant proteins. GST-fused recombinant proteins were expressed in *E. coli* XLI-blue MRF' (Stratagene). The cells were lysed by sonication in PBS(−) supplemented with protease inhibitor cocktail (Complete, Roche). After sonication, Triton X-100 was added and the mixture was rotated at 4 °C for 30 min. Then, GST-fused recombinant proteins were purified using glutathione–Sepharose 4B (Amersham).

GST pull-down assay. Ten μg of GST-fused SIRT1 proteins or GST alone was incubated with ^{35}S-labeled in vitro translated hHES1 protein in the binding buffer (PBS with 0.1% Nonidet P40 and protease inhibitor cocktail (Complete, Roche)) for 60 min on ice. The previously prepared glutathione–Sepharose beads were added to the mixture and rotated at 4 °C for 60 min. After rotation, the beads were washed three times with the binding buffer and resolved using 12% SDS–PAGE. Bound proteins were analyzed by BAS (Fuji film) or autoradiography. Ten μg of GST-fused hHES1 or hHEY2 derivative proteins or GST alone was incubated with ^{35}S-labeled in vitro translated SIRT1 protein in the binding buffer described above.

Immunoprecipitation. Lysates of 293T cells transiently expressing HA-hHES1 (or HA-hHEY2) and SIRT1-FLAG were incubated with 5 μg of anti-HA monoclonal antibody (HA.11, BAbCO) or anti-FLAG M2 monoclonal antibody (Sigma) overnight. Fifty μl of protein G–Sepharose beads was added to the lysates and rotated at 4 °C for 2 h. The immune complex was collected, washed three to five times, and resolved using 6% or 12% SDS–PAGE.

Western blot analyses. Proteins on the gels were transferred to PVDF membranes (Immobilon, Millipore) at 15 V for 45 min. After

blotting, the membranes were blocked in Block Ace (Dainippon Pharmaceuticals) for 2 h at room temperature, incubated with anti-SIRT1 antibody, anti-FLAG monoclonal antibody or anti-HA monoclonal antibody for 1 h at room temperature, and washed thrice with TBS–T (20 mM Tris–HCl, pH 7.5, 140 mM NaCl, and 0.05% Tween 20). Anti-rabbit or mouse Ig conjugated with horseradish peroxidase (Amersham) was used as the secondary antibody. The primary and secondary antibodies in 1/10 Block Ace were incubated with the membranes. The membranes were washed four times for 10 min with TBS–T after incubation. The specific proteins were detected using ECL (Amersham) and X-ray films (Hyperfilm MP, Amersham).

Luciferase assays. NIH 3T3 cells were transfected using FuGENE6 (Roche) with the effectors (pcDNA3-GAL4-hHES1, pcDNA3-GAL4-hHEY2, pcDNA3-SIRT1-FLAG, and pcDNA3-SIRT1H363Y-FLAG) and the reporter constructs (pβA-luc and pUAS-βA-luc) and luciferase assays were performed (Promega).

Results

Physical interactions of human SIRT1 with hHES1 and hHEY2 in vivo

To investigate whether SIRT1 interacts physically with hHES1 in vivo, we performed co-immunoprecipitation experiments (Figs. 1A and B). 293T cells were transiently transfected with FLAG-tagged SIRT1 and/or HA-tagged hHES1. The cell lysates were immunoprecipitated with anti-HA or anti-FLAG antibodies and the precipitated fractions were analyzed by Western blotting using anti-FLAG or anti-HA antibodies, respectively. Human SIRT1-FLAG was co-immunoprecipitated with anti-HA antibodies only when HA-hHES1 and SIRT1-FLAG were simultaneously overexpressed (Fig. 1A, lane 12). Reciprocally, HA-hHES1 was co-immunoprecipitated with anti-FLAG antibodies only when SIRT1-FLAG was simultaneously overexpressed (Fig. 1B, lane 12). These results demonstrated that SIRT1 associates with hHES1 in vivo.

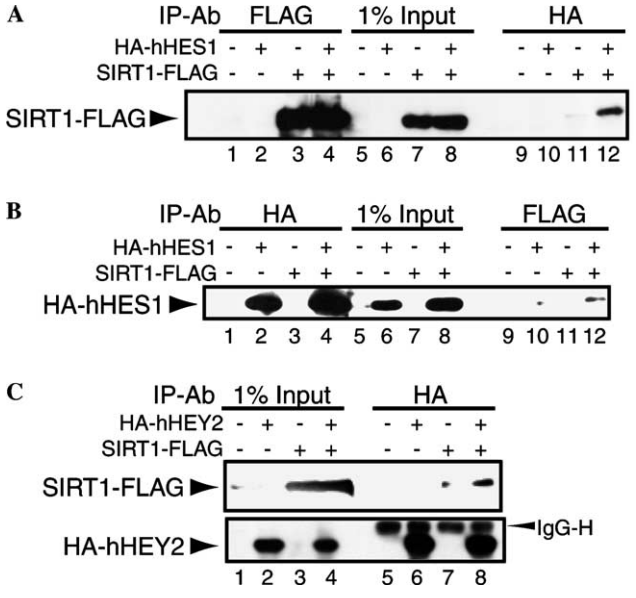

Fig. 1. SIRT1 associates with hHES1 and hHEY2 in vivo. (A) 293T cells were transfected with combinations of plasmid DNAs expressing the indicated recombinant proteins. When the plasmids were not transfected (indicated by –), empty expression vectors were transfected instead. Cell extracts were obtained after transfection for 48 h and immunoprecipitated with antibodies indicated at the top of the panel (IP-Ab). The precipitated proteins was analyzed in Western blot experiments using anti-FLAG antibodies. As control, 1% of the pre-immunoprecipitated cell extracts was also analyzed (1% input). (B) Similar experiments to A, except that anti-HA antibodies were used in Western blot experiments. (C) Similar experiments to A and B, except that HA-hHEY2-expressing plasmid was used instead of HA-hHES1-expressing plasmid.

It has been suggested that Hes1 functions by forming a heterodimer with Hey2 in vivo [22]. To test whether SIRT1 also possesses the capability to form a protein complex with hHEY2, we performed similar co-immunoprecipitation experiments (Fig. 1C). SIRT1-FLAG was co-immunoprecipitated with anti-HA antibody only when SIRT1-FLAG and HA-hHEY2 were simultaneously overexpressed. These results indicated that SIRT1 associates with both hHES1 and hHEY2 in vivo.

Human SIRT1 directly interacts with hHES1 and hHEY2 in vitro

To confirm the physical interaction of SIRT1 with hHES1 and hHEY2, we performed an in vitro pull-down assay. Purified GST-SIRT1 or GST alone was incubated with ^{35}S-labeled in vitro translated HA-hHES1 or HA-hHEY2 protein. The GST-fusion proteins as well as the associated proteins were purified using glutathione–Sepharose 4B beads and analyzed by autoradiography. The results demonstrated that both full-length HA-hHES1 and HA-hHEY2 specifically interacted with GST-SIRT1 but not with GST (Figs. 2A and B). The reciprocal experiment revealed that ^{35}S-labeled in vitro translated SIRT1-FLAG specifically bound to GST-hHES1, but not to GST (Figs. 2C, lanes 1 and 5). Because only very limited amounts of soluble GST-hHEY2 could be prepared, we could not perform a similar experiment using the GST-hHEY2. However, a truncated version, GST-hHEY2ΔC, was shown to bind to SIRT1-FLAG as described below.

To map the region in hHES1 responsible for interacting with SIRT1, we performed pull-down experiments using a series of hHES1 deletion mutants (Figs. 2C and D; see Fig. 2F for the structures of the deletion mutants). While the N-terminal deletion (hHES1ΔN) that lacked bHLH did not associate with SIRT1 (Fig. 2C, lane 4), the C-terminal deletion (hHES1ΔC) that possessed bHLH but lacked the Orange domain or its C-terminal region significantly associated with SIRT1 (Fig. 2C, lane 3). Finally, the hHES1 bHLH domain alone (hHES1-bHLH) efficiently bound to SIRT1, but the most N-terminal region that lacks bHLH (hHES1-N31) did not (Fig. 2D, lanes 4 and 3, respectively). Because of difficulty in preparing recombinant GST-hHEY2

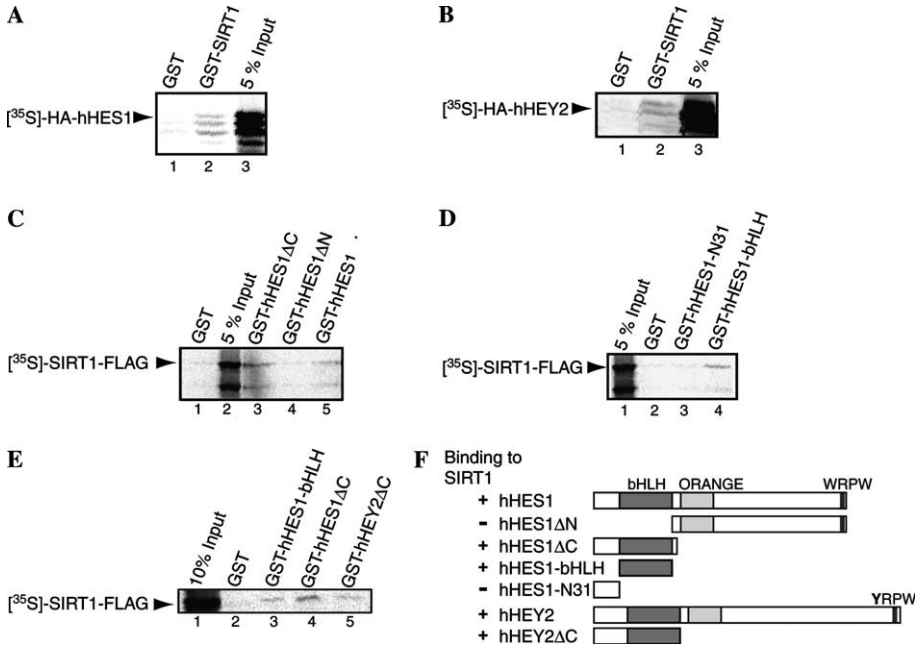

Fig. 2. SIRT1 associates with hHES1 and hHEY2 in vitro. (A–E) ^{35}S-labeled recombinant proteins were prepared using the rabbit reticulocyte lysate system. The lysates were incubated with the GST-fusion proteins as indicated. GST-fusion proteins and the associated proteins were purified by using glutathione–Sepharose 4B beads. The presence of the labeled proteins was examined by SDS–PAGE and autoradiography. As control, 5% of the input protein mixtures that were not treated with glutathione–Sepharose beads was analyzed (5% input). (F) Structures of the recombinant hHES1 and hHEY2 proteins used in (A–E). These recombinant proteins correspond to the following regions of the full-length proteins (hHES1, 280 a.a. and hHEY2, 337 a.a.): hHES1ΔN, a.a. 96–280; hHES1ΔC, a.a. 2–104; hHES1-bHLH, a.a. 32–93; hHES1-N31, a.a. 2–31; hHEY2, a.a. 2–337; and hHEY2ΔC, a.a. 2–118. The binding activity of each protein to SIRT1 is also indicated.

proteins, we performed only similar experiments using hHEY2ΔC that possessed bHLH but lacked the Orange domain or its C-terminal region. This protein also bound to SIRT1 (Fig. 2E, lane 5). Therefore, we concluded that the bHLH domain of hHES1, and most probably of hHEY2 (Fig. 2F), is responsible for binding to SIRT1, an observation consistent with the previous study on *Drosophila* Hairy [30].

Effects of SIRT1 on the transcriptional activity of hHES1 and hHEY2 proteins

The physical association of SIRT1 with hHES1 and hHEY2 suggested that SIRT1 might be involved in the gene repression mediated by hHES1 and hHEY2. To investigate this possibility, we utilized a GAL4-based reporter assay (Fig. 3A). The expression vector producing the GAL4DBD (Gal4 DNA-binding domain)-hHES1 or -hHEY2 chimeric protein (GAL4-hHES1 or GAL4-hHEY2) was cotransfected to mouse NIH 3T3 cells with the reporter plasmid pUAS-βA-luc. pUAS-βA-luc contains six copies of GAL4-binding sequence upstream of the chicken β-actin promoter that drives the transcription of the luciferase reporter gene. A plasmid expressing GAL4DBD alone was used as the control effector. GAL4DBD alone produced minimal, if any, transcriptional repression of the reporter gene (Fig. 3B, lane 2). In contrast, as shown in previous reports [20,22], GAL4-hHES1 and GAL4-hHEY2 showed significant repressive effects on the reporter gene expression from GAL4-binding sites (Fig. 3B, lanes 5 and 8). hHES1 or hHEY2 expression vector without GAL4DBD did not inhibit transcription of the reporter gene, indicating the requirement of the specific DNA binding of hHES1 or hHEY2 to the upstream GAL4-binding sequences in the transcriptional repression (data not shown).

To investigate the possible role of SIRT1 protein in the transcriptional repression mediated by hHES1 and hHEY2, we cotransfected in the reporter assay the expression vector producing the wild-type SIRT1 or the mutant SIRT1H363Y protein. SIRT1H363Y contains a base substitution that converts a conserved histidine residue at position 363 into a tyrosine residue. It is known that this amino acid substitution leads to the inactivation of deacetylase activity [24,34]. Neither wild-type nor mutant SIRT1 showed any effect on the reporter expression when GAL4DBD alone was used as the effector plasmid (Fig. 3B, lanes 3 and 4). In contrast, wild-type SIRT1 significantly augmented the repression activity mediated by hHES1 or hHEY2 (lanes 6 and 9). Interestingly, SIRT1H363Y partially relieved the transcriptional repression by hHES1 or hHEY2 (lanes 7 and 10). These results suggest that SIRT1 is involved, at least in part, in the transcriptional repression in this system.

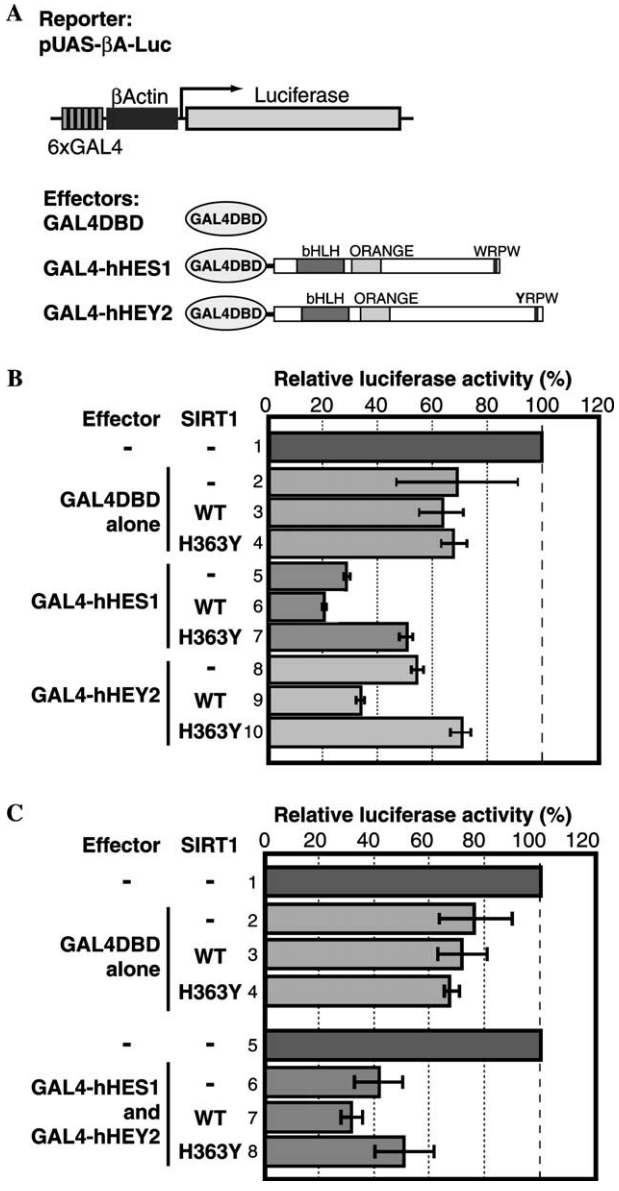

Fig. 3. SIRT1 is involved in the transcriptional repression by hHES1 and hHEY2. (A) Reporter and effector plasmid constructs used in the reporter assay. Six times GAL4, six tandem copies of the GAL4-binding sequence; β-Actin, the promoter of the chicken β-actin gene; and GAL4DBD, GAL4 DNA-binding domain. (B, C) Relative transcriptional activity of the reporter gene when combinations of effector plasmids and SIRT1-expressing plasmids were co-transfected into NIH 3T3 cells and incubated for 48 h. The transfection efficiency was calibrated based on the expression levels of the co-transfected *Renilla* luciferase-expressing plasmid. The result obtained when no effector plasmid was co-transfected (lane 1) was set as 100%. The values are means + SD of the relative activities obtained in an experiment performed in triplicate. Similar results were obtained in at least three independent experiments.

The simplest explanation for the negative effect of SIRT1H363Y on the repression is that SIRT1H363Y has a dominant-negative effect on endogenous wild-type SIRT1 by competing for hHES1 and hHEY2. Similar results were obtained when hHES1 and hHEY2 were co-expressed (Fig. 3C, lanes 6–8). Since hHES1 and hHEY2 form the hHES1–hHEY2 heterodimer preferentially to the hHES1 or hHEY2 homodimer [22], these results suggested that the repression mediated by the hHES1–hHEY2 heterodimer also involves SIRT1. Taken together, we concluded that the catalytic activity of SIRT1 is important for the hHES1- and hHEY2-mediated transcriptional repression.

Repression activity of Hes1 depends on both TSA-insensitive and -sensitive pathways

The deacetylase activity of Sir2 proteins is insensitive to the potent deacetylase inhibitor, Trichostatin A (TSA) [24,35], which places Sir2 as a unique deacetylase distinguishable from class I (e.g., HDAC1/Rpd3) and class II deacetylases. If the repression activity of Hes1 depends on the deacetylase activity of SIRT1, one would expect that it is insensitive to TSA. As expected, the repression activity of GAL4-hHES1 on the UAS-containing reporter gene was not affected by the addition of TSA to the culture (Fig. 4, lanes 4 and 5). Interestingly, we found that GAL4-hHES1ΔN, which does not bind to SIRT1, induced a significant repression that was partly derepressed by the addition of TSA, suggesting that class I or II deacetylases are involved in this case (lanes 6 and 7). Taken together, these results suggested that full-length hHES1 proteins potentially repress genes via both TSA-insensitive and -sensitive pathways. When the SIRT1-mediated TSA-insensitive pathway is proficient,

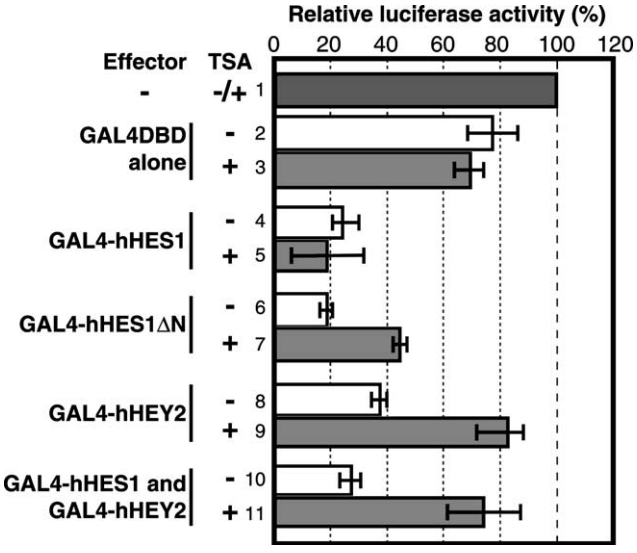

Fig. 4. Sensitivity to TSA of the transcriptional repression by hHES1 and hHEY2. Reporter assays using the indicated effector proteins with (+) or without (−) the addition of TSA (100 nM). The result obtained when no effector plasmid was co-transfected in the presence or absence of TSA (lane 1) was set as 100%. The values are means + SD of the relative activities obtained in an experiment performed in triplicate. Similar results were obtained in three independent experiments. The structure of hHES1ΔN is indicated in Fig. 2F.

this pathway may be responsible for the majority of the repression effect. However, when the SIRT1-mediated pathway is inactivated (as in the case of hHES1ΔN that does not bind to SIRT1), a TSA-sensitive backup mechanism operates to repress genes. The recruitment of HDAC1 via Groucho to the C-terminal tetrapeptide of Hes1 is a good candidate for this backup pathway. In contrast, the repression caused by GAL4-hHEY2 was markedly derepressed by the addition of TSA (lanes 8 and 9). Similar TSA-sensitive repression was also observed when hHES1 and hHEY2 were co-expressed (lanes 10 and 11). Therefore, it is suggested that the relative contribution of SIRT1 is more significant in the hHES1-mediated repression than in the hHEY2-mediated repression and most probably in the hHES1–hHEY2-heterodimer-mediated repression.

Discussion

It has been recently reported that *Drosophila* Sir2 (dSir2) physically associates in vitro with *Drosophila* Hairy and Deadpan (Dpn), but not with E(spl) proteins [30]. Genetic experiments in the same study clearly showed that these interactions have functional roles in vivo. Interestingly, dSir2-interacting Hairy and Dpn are classified as members of the Hairy subfamily and non-interacting E(spl) is classified as a member of another subfamily E(spl). Therefore, it appears that the ability of Hairy-related proteins to interact with Sir2 is restricted to some subfamilies or members of the superfamily. Here, we showed that hHES1 and hHEY2, members of the Hairy and Hey subfamilies, respectively, physically interact with SIRT1, indicating that the association with Sir2 occurs in Hairy-related proteins belonging to more than two subfamilies. Moreover, using the reporter assay, we showed for the first time that SIRT enhances the repression activities of hHES1 and hHEY2. The hHES1-mediated repression activity is TSA-insensitive, a finding consistent with the idea that the deacetylase activity of SIRT is involved in the process. These results broaden our understanding of how Hairy-related repressors function in vivo.

Although Hes and Hey proteins are members of different subfamilies of the Hairy-related superfamily, several lines of evidence suggest that they may function by forming the Hes–Hey heterodimer in a single cell. Some of the Hairy-related repressors are expressed in an oscillating manner in the presomitic mesoderm both spatially and temporally. It was found that the expression of chicken Hairy genes (*c-hairy1* and *c-hairy2*; *c-hairy2* is the chicken homologue of human *HES1*) oscillates exactly synchronously with that of chicken *Hey2* (*c-Hey2*; chicken homologue of human *HEY2*), suggesting that these genes function coordinately [36,37]. Furthermore, when both Hes1 and Hey2 are expressed, they form a heterodimer preferentially to forming cognate homodimers. Importantly, the Hes1–Hey2 heterodimer showed an elevated DNA-binding activity and a synergistic repression of the target genes, compared to the Hes1 or Hey2 homodimer [22]. It was suggested that the transcriptional repression achieved by Hes1–Hey2 is mediated by twofold recruitment of HDAC1-containing histone deacetylase complexes: the TLE-HDAC1 complex by the C-terminal tetrapeptide of Hes1 and the mSin3 complex by the bHLH domain of Hey2 [22]. We propose that another deacetylase, SIRT1, is also involved in the transcriptional repression by the Hes1–Hey2 complex as a third pathway to recruit deacetylase. The repression activities produced by the expression of hHES1 or hHEY2 alone, and by the co-expression of hHES1 and hHEY2, were increased and decreased by over-expressing wild-type and mutant SIRT1, respectively. On the other hand, while the hHEY2-mediated repression was sensitive to TSA, the hHES1-mediated repression was not. Because the bHLH domain of hHEY2 is responsible for both the interactions with the mSin3 complex [22] and with SIRT1 (this study), the mSin3 complex and SIRT1 may be recruited to hHEY2 in a competitive manner. In contrast, hHES1 interacts with HDAC1 and SIRT1 via different domains (the C-terminal tetra-peptide motif and the bHLH domain, respectively) ([19,20], and this study), making the possibility likely that hHES1 recruits HDAC1 and SIRT1 simultaneously. These different modes of interaction of hHES1 and hHEY2 to SIRT1 and HDAC1 may explain the apparently different sensitivities to TSA of these proteins. Although it is likely that the SIRT1-dependent and the HDAC1-dependent pathways operate synergistically, the relative contribution of these pathways may vary in different contexts. For example, genetic studies in *Drosophila* have shown that *hairy* exhibits stronger interaction with *dSir2* than with *Rpd3* [30].

This study did not address the issue of how SIRT1 induces transcriptional repression. One obvious scenario is that SIRT1, recruited to chromatin by the DNA-binding proteins HES1 and HEY2, modifies histones. However, Sir2 also deacetylates non-histone proteins, a remarkable example of which is the SIRT1-mediated p53 deacetylation [38–40]. Several transcription factors are known to be modified by acetylation, leading to the activation of their activity [41], among which MyoD is of interest to this study. The acetylation of MyoD by PCAF or CBP/p300 activates its activity and is required for normal muscle differentiation [42,43]. It was reported that Hey1 (also called CHF2) forms a heterodimer with MyoD, thereby repressing the activity of MyoD in undifferentiated cells [44]. Therefore, it is possible that Hey proteins repress MyoD or related activators not as the Hes–Hey heterodimer or the Hey homodimer, but as the MyoD–Hey heterodimer and by recruiting SIRT1 to deacetylate activated MyoD.

Acknowledgments

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References

[1] M.E. Massari, C. Murre, Helix–loop–helix proteins: regulators of transcription in eucaryotic organisms, Mol. Cell. Biol. 20 (2000) 429–440.
[2] H. Weintraub, The MyoD family and myogenesis: redundancy, networks, and thresholds, Cell 75 (1993) 1241–1244.
[3] R. Kageyama, S. Nakanishi, Helix–loop–helix factors in growth and differentiation of the vertebrate nervous system, Curr. Opin. Genet. Dev. 7 (1997) 659–665.
[4] J.E. Lee, Basic helix–loop–helix genes in neural development, Curr. Opin. Neurobiol. 7 (1997) 13–20.
[5] A. Fisher, M. Caudy, The function of hairy-related bHLH repressor proteins in cell fate decisions, Bioessays 20 (1998) 298–306.
[6] R.L. Davis, D.L. Turner, Vertebrate hairy and enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning, Oncogene 20 (2001) 8342–8357.
[7] T.K. Blackwell, H. Weintraub, Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection, Science 250 (1990) 1104–1110.
[8] Y. Sasai, R. Kageyama, Y. Tagawa, R. Shigemoto, S. Nakanishi, Two mammalian helix–loop–helix factors structurally related to *Drosophila* hairy and enhancer of split, Genes Dev. 6 (1992) 2620–2634.
[9] K. Tietze, N. Oellers, E. Knust, Enhancer of split^{D}, a dominant mutation of Drosophila, and its use in the study of functional domains of a helix–loop–helix protein, Proc. Natl. Acad. Sci. USA 89 (1992) 6152–6156.
[10] K. Takebayashi, Y. Sasai, Y. Sakai, T. Watanabe, S. Nakanishi, R. Kageyama, Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix–loop–helix factor HES-1. Negative autoregulation through the multiple N box elements, J. Biol. Chem. 269 (1994) 5150–5156.
[11] J.N. Feder, L. Li, L.Y. Jan, Y.N. Jan, Genomic cloning and chromosomal localization of HRY, the human homolog to the *Drosophila* segmentation gene, hairy, Genomics 20 (1994) 56–61.
[12] M.T. Chin, K. Maemura, S. Fukumoto, M.K. Jain, M.D. Layne, M. Watanabe, C.M. Hsieh, M.E. Lee, Cardiovascular basic helix loop helix factor 1, a novel transcriptional repressor expressed preferentially in the developing and adult cardiovascular system, J. Biol. Chem. 275 (2000) 6381–6387.
[13] T. Iso, V. Sartorelli, G. Chung, T. Shichinohe, L. Kedes, Y. Hamamori, HERP, a new primary target of Notch regulated by ligand binding, Mol. Cell. Biol. 21 (2001) 6071–6079.
[14] H. Kokubo, Y. Lun, R.L. Johnson, Identification and expression of a novel family of bHLH cDNAs related to *Drosophila* hairy and enhancer of split, Biochem. Biophys. Res. Commun. 260 (1999) 459–465.
[15] C. Leimeister, A. Externbrink, B. Klamt, M. Gessler, Hey genes: a novel subfamily of hairy- and enhancer of split related genes specifically expressed during mouse embryogenesis, Mech. Dev. 85 (1999) 173–177.
[16] O. Nakagawa, M. Nakagawa, J.A. Richardson, E.N. Olson, D. Srivastava, HRT1, HRT2, and HRT3: a new subclass of bHLH transcription factors marking specific cardiac, somitic, and pharyngeal arch segments, Dev. Biol. 216 (1999) 72–84.
[17] T.P. Zhong, M. Rosenberg, M.A. Mohideen, B. Weinstein, M.C. Fishman, gridlock, an HLH gene required for assembly of the aorta in zebrafish, Science 287 (2000) 1820–1824.
[18] G. Chen, A.J. Courey, Groucho/TLE family proteins and transcriptional repression, Gene 249 (2000) 1–16.
[19] Z. Paroush, R.L. Finley Jr., T. Kidd, S.M. Wainwright, P.W. Ingham, R. Brent, D. Ish-Horowicz, Groucho is required for *Drosophila* neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins, Cell 79 (1994) 805–815.
[20] A.L. Fisher, S. Ohsako, M. Caudy, The WRPW motif of the hairy-related basic helix–loop–helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain, Mol. Cell. Biol. 16 (1996) 2670–2677.
[21] G. Chen, J. Fernandez, S. Mische, A.J. Courey, A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in *Drosophila* development, Genes Dev. 13 (1999) 2218–2230.
[22] T. Iso, V. Sartorelli, C. Poizat, S. Iezzi, H.Y. Wu, G. Chung, L. Kedes, Y. Hamamori, HERP, a novel heterodimer partner of HES/E(spl) in Notch signaling, Mol. Cell. Biol. 21 (2001) 6080–6089.
[23] O. Nakagawa, D.G. Mcfadden, M. Nakagawa, H. Yanagisawa, T. Hu, D. Srivastava, E.N. Olson, Members of the HRT family of basic helix–loop–helix proteins act as transcriptional repressors downstream of Notch signaling, Proc. Natl. Acad. Sci. USA 97 (2000) 13655–13660.
[24] S. Imai, C.M. Armstrong, M. Kaeberlein, L. Guarente, Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase, Nature 403 (2000) 795–800.
[25] J. Landry, A. Sutton, S.T. Tafrov, R.C. Heller, J. Stebbins, L. Pillus, R. Sternglanz, The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases, Proc. Natl. Acad. Sci. USA 97 (2000) 5807–5811.
[26] J.S. Smith, C.B. Brachmann, I. Celic, M.A. Kenna, S. Muhammad, V.J. Starai, J.L. Avalos, J.C. Escalante-Semerena, C. Grubmeyer, C. Wolberger, J.D. Boeke, A phylogenetically conserved NAD^{+}-dependent protein deacetylase activity in the Sir2 protein family, Proc. Natl. Acad. Sci. USA 97 (2000) 6658–6663.
[27] D. Shore, M. Squire, K.A. Nasmyth, Characterization of two genes required for the position-effect control of yeast mating-type genes, EMBO J. 3 (1984) 2817–2823.
[28] L. Guarente, Sir2 links chromatin silencing, metabolism, and aging, Genes Dev. 14 (2000) 1021–1026.
[29] D. Moazed, Common themes in mechanisms of gene silencing, Mol. Cell 8 (2001) 489–498.
[30] M.I. Rosenberg, S.M. Parkhurst, *Drosophila* Sir2 is required for heterochromatic silencing and by euchromatic Hairy/E(Spl) bHLH repressors in segmentation and sex determination, Cell 109 (2002) 447–458.
[31] R.A. Frye, Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins, Biochem. Biophys. Res. Commun. 273 (2000) 793–798.
[32] K.I. Tatematsu, T. Yamazaki, F. Ishikawa, MBD2–MBD3 complex binds to hemi-methylated DNA and forms a complex containing DNMT1 at the replication foci in late S phase, Genes Cells 5 (2000) 677–688.
[33] S. Bae, Y. Bessho, M. Hojo, R. Kageyama, The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation, Development 127 (2000) 2933–2943.

[34] J.C. Tanny, G.J. Dowd, J. Huang, H. Hilz, D. Moazed, An enzymatic activity in the yeast Sir2 protein that is essential for gene silencing, Cell 99 (1999) 735–745.
[35] M. Yoshida, M. Kijima, M. Akita, T. Beppu, Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A, J. Biol. Chem. 265 (1990) 17174–17179.
[36] C. Jouve, I. Palmeirim, D. Henrique, J. Beckers, A. Gossler, D. Ish-Horowicz, O. Pourquie, Notch signalling is required for cyclic expression of the hairy-like gene HES1 in the presomitic mesoderm, Development 127 (2000) 1421–1429.
[37] C. Leimeister, K. Dale, A. Fischer, B. Klamt, M. Hrabe De Angelis, F. Radtke, M.J. Mcgrew, O. Pourquie, M. Gessler, Oscillating expression of c-Hey2 in the presomitic mesoderm suggests that segmentation clock may use combinatorial signaling through multiple interacting bHLH factors, Dev. Biol. 227 (2000) 91–103.
[38] H. Vaziri, S.K. Dessain, E. Ng Eaton, S.I. Imai, R.A. Frye, T.K. Pandita, L. Guarente, R.A. Weinberg, hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase, Cell 107 (2001) 149–159.
[39] J. Luo, A.Y. Nikolaev, S. Imai, D. Chen, F. Su, A. Shiloh, L. Guarente, W. Gu, Negative control of p53 by Sir2α promotes cell survival under stress, Cell 107 (2001) 137–148.
[40] E. Langley, M. Pearson, M. Faretta, U.M. Bauer, R.A. Frye, S. Minucci, P.G. Pelicci, T. Kouzarides, Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence, EMBO J. 21 (2002) 2383–2396.
[41] D.E. Sterner, S.L. Berger, Acetylation of histones and transcription-related factors, Microbiol. Mol. Biol. Rev. 64 (2000) 435–459.
[42] V. Sartorelli, P.L. Puri, Y. Hamamori, V. Ogryzko, G. Chung, Y. Nakatani, J.Y. Wang, L. Kedes, Acetylation of MyoD directed by PCAF is necessary for the execution of the muscle program, Mol. Cell 4 (1999) 725–734.
[43] A. Polesskaya, A. Duquet, I. Naguibneva, C. Weise, A. Vervisch, E. Bengal, F. Hucho, P. Robin, A. Harel-Bellan, CREB-binding protein/p300 activates MyoD by acetylation, J. Biol. Chem. 275 (2000) 34359–34364.
[44] J. Sun, C.N. Kamei, M.D. Layne, M.K. Jain, J.K. Liao, M.E. Lee, M.T. Chin, Regulation of myogenic terminal differentiation by the hairy-related transcription factor CHF2, J. Biol. Chem. 276 (2001) 18591–18596.