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Requirement of multiple lysine residues for the transcriptional activity and the instability of Hes7

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ABSTRACT

The basic helix-loop-helix (bHLH) gene *Hes7* is expressed in an oscillatory manner and regulates the periodic somite formation. Oscillatory expression of *Hes7* depends on negative feedback and rapid degradation of the gene products, but the precise mechanisms of how the transcriptional activity and the degradation of Hes7 protein are regulated remain to be analyzed. Here, we found that lysine residues (K22, K52, and K55) in the bHLH domain are essential not only for the instability of Hes7 protein but also for the transcriptional repressor activity. Introduction of lysine-to-arginine mutations into the bHLH domain led to stabilization of Hes7 protein and to abnormalities in either the N box-binding activity or partner preference in heterodimer formation. These results indicate that common amino acid residues are involved in both the transcriptional repressor activity and the instability of Hes7 protein, suggesting of a critical link between the transcription and degradation control.

Hes7 encodes a basic helix-loop-helix (bHLH) transcriptional repressor and displays dynamic expression patterns in the presomitic mesoderm (PSM), which is located in the posterior part of embryos [1,2]. The anterior ends of the PSM are segmented every two hours in mouse embryos, thereby giving rise to a bilateral pair of somites. During formation of each pair of somites, *Hes7* expression initially occurs in the posterior PSM, propagates anteriorly, and stops at the region S-1, which is just posterior to the next forming somite (S0). Then, the expression disappears when the *Hes7*-expressing region becomes S0, but new expression occurs again in the posterior PSM. This dynamic expression is repeated every 2 h and is caused by oscillatory expression of *Hes7* in individual PSM cells. Fgf signaling regulates the initiation and Notch signaling regulates the amplification and the anterior propagation of Hes7 oscillation, while cyclic expression of Hes7 links oscillations in Fgf and Notch signaling activities [3]. Both sustained expression and inactivation of *Hes7* lead to loss of oscillations in Fgf and Notch signaling activities and to severe somite fusion, indicating that oscillatory expression of Hes7 is required for periodic somite formation [2–4].

The oscillatory expression of Hes7 is regulated by negative feedback and rapid degradation of the gene products [5], like Hes1 oscillation in fibroblasts [6]: increase in Hes7 expression leads to repression of its own expression by negative feedback and to subsequent disappearance of the gene products because of their short half-lives, resulting in relief from negative feedback and the next round of expression [1,5]. Thus, the transcriptional repressor activity and the instability of gene products are essential features for Hes7 oscillation. However, the precise mechanisms of how the transcriptional activity and the degradation of Hes7 protein are regulated remain to be analyzed.

Here, we found that lysine residues in the bHLH domain, which are well conserved among the Hes family members, are essential for both the transcriptional repressor activity and the short half-life of Hes7 protein. Thus, common amino acid residues are involved in both the transcriptional activity and the instability of Hes7, suggesting of a critical link between these two features of Hes7. We further examined why these conserved lysine residues are important for the transcriptional repressor activity and found that they are involved in either the N box-binding activity or partner preference in heterodimer formation.

Materials and methods

Construction of lysine-to-arginine mutations. Lysine-to-arginine mutants were constructed, as described previously [4]. K-less mutant (all lysine residues are changed to arginine residues) was made by site-directed mutagenesis using a QuickChange PCR-based kit (Stratagene). The mutations were confirmed by sequencing.

Transfection and luciferase assay. Transfection and luciferase assay were performed, as described previously [4].

Measurement of half-life. NIH3T3 cells were transfected with pEF-Hes7 wild-type or lysine-to-arginine mutant expression

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vectors in 6-well plates. 60 h after transfection, cells were treated with cycloheximide (Sigma) at 100 μg/ml for the indicated time and harvested for Western blot analysis. Products were detected using peroxidase-conjugated anti-HA antibody (3F10, Roche), and signals were scanned and quantified using Typhoon 9410 (GE healthcare).

Protein purification. cDNAs were subcloned into either pGEX6P1 (GE Healthcare) or pMAL (NEB) expression plasmids. Rosseta(DE3)pLys (Stratagene) cells transformed by expression plasmids were grown and treated with 0.05 mM IPTG for 3 h at 25 °C. After cell lysis with 1 mg/ml of Lysozyme and 0.5% Triton X-100, GST-fused proteins were purified with Glutathione Sepharose 4B (GE Healthcare). MBP-fused proteins were purified with Amylose resin (NEB). Purified proteins were dialyzed against 1% Triton X-100/NET-150 (50 mM Tris–HCl pH8.0, 150 mM NaCl, 1 mM EDTA).

GST pull-down assay. In vitro translated ^{35}S-labeled proteins were prepared using the TNT coupled transcription-translation system (Promega). *In vitro* translated proteins were mixed with 1 μg of purified recombinant GST-Hes7 wild-type or lysine-to-arginine mutant proteins in the binding buffer (20 mM Tris–HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% NP-40) and incubated at 4 °C for 2 h. Beads were washed with binding buffer three times, and bound proteins were eluted with 2× SDS–PAGE sample buffer and analyzed by SDS–PAGE and autoradiography.

Immunocytochemistry. Cells on chamber slides were fixed with 4% paraformaldehyde for 15 min and incubated with blocking buffer (1.5% Normal goat serum and 0.1% Triton X-100 in PBS) at room temperature for 20 min. Cells were immunostained with anti-HA rat monoclonal antibody (3F10, Roche) at 4 °C overnight. The biotin-conjugated anti-rat IgG (Vector) and FITC-conjugated avidin (Vector) were used to visualize the signals. Cells were rinsed in 0.1% TritonX-100 in PBS containing DAPI (Sigma), mounted in Fluoromount-G (Southern Biotech), and photographed with Axiophoto (Olympus).

Electrophoresis mobility shift assay (EMSA). Oligonucleotides containing the N box sequence from the mouse *Hes7* promoter (top strand 5′-TCCGGGAGCCTCGTGCCGGGGTCCTTGAGC-3′; bottom strand 5′-GCTCAAGGACCCCGGCACGAGGCTCCCGGA-3′) were annealed and labeled with [γ-^{32}P]-ATP (GE Heathcare) using T4 polynucleotide kinase. Purified proteins and labeled probes (20 fmol) were incubated for 30 min at room temperature in binding buffer (20 mM Hepes [pH 7.9], 100 mM KCl, 1 mM EDTA, 25 ng/ml poly-dI/dC, 0.5 μg/ml BSA, 10% glycerol). Samples were then electrophoresed on a 5% non-denaturing polyacrylamide gel.

Chromatin immunoprecipitation (ChIP) assay. C3H10T1/2 cells transfected with pCI, pCI-FLAG-Hes7 or pCI-FLAG-K52R were cross-linked with 1% formaldehyde at 37 °C for 10 min, sonicated, and incubated with 2 μg of anti-FLAG M2 antibody (Sigma) overnight. Immune complexes were incubated with Protein G Sepharose beads (Amersham) and then with 100 μg/ml Proteinase K for DNA extraction. DNA was analyzed by PCR and real-time PCR with SYBR® Premix Ex Taq™ (TAKARA) using 7500 real-time PCR system (Applied Biosystems). The following specific primers were used to detect the *Hes1* promoter region: 5′-GCCAGACCTTGTGCCTAGC-3′ and 5′-GGCCTCTATATATATCTGGGACTGC-3′.

MBP pull-down assay. Expression vectors were transiently transfected into C3H10T1/2 cells, and cell lysates were prepared and mixed with purified MBP, MBP-Hes7, and MBP-Hes7 K52R in the binding buffer (20 mM Tris–HCl pH8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with freshly prepared protease inhibitors. Associated proteins were affinity purified by amylose resin and eluted with 30 mM maltose. Eluted fractions were analyzed by Western blot with anti-myc HRP conjugated antibody (MC450, Nacalai Tesque), anti-FLAG M2 HRP conjugated antibody (Sigma) or anti-MBP-N antibody (Santacruz).

Immunoprecipitation assay. C3H10T1/2 cells were transfected with expression vectors, and the cell lysis was done in the binding buffer (20 mM Tris–HCl pH8.0, 150 mM NaCl, 1 mM EDTA, 0.3% NP-40) supplemented with freshly prepared protease inhibitors. Cell extracts were incubated with anti-HA affinity matrix (Roche) overnight at 4 °C, and bound proteins were eluted with 2× SDS–PAGE buffer after washing and analyzed by Western blot.

Results and discussion

Loss of repressor activity and stabilization of Hes7 protein by lysine-to-arginine point mutations

There are seven lysine residues (K14, K17, K22, K52, K55, K129, and K211) in Hes7 protein, and we previously found that some of the lysine-to-arginine point mutant proteins lose transcriptional

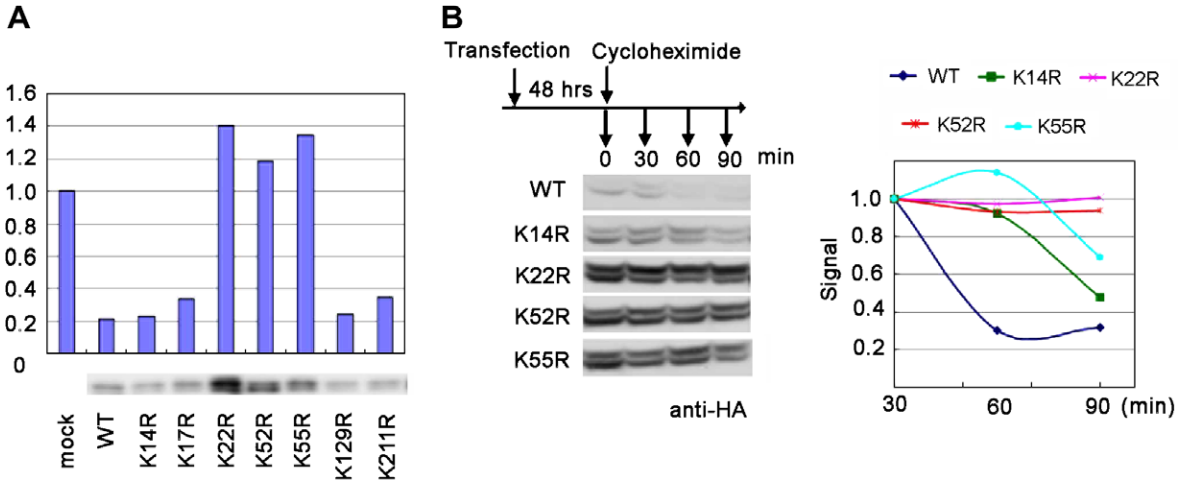

Fig. 1. Loss of transcriptional repressor activity and stabilization of Hes7 protein by lysine-to-arginine point mutations. (A) The luciferase reporter under the control of the N box-containing promoter (0.1 μg) was co-transfected with either the empty vector or the expression vector for wild-type or mutant Hes7 tagged with HA (0.5 μg each) into C3H10T1/2 cells, and luciferase activity was determined 24 h later. HA-Hes7 protein levels were determined by immunoblot analysis using anti-HA antibody (lower panel). Three independent experiments were performed, and similar results were obtained. Only one representative result is shown. (B) C3H10T1/2 cells transfected with the expression plasmid pEF-HA-Hes7 or the indicated mutants were treated with 100 μg/ml cycloheximide, and the protein levels were determined by immunoblot analysis using anti-HA antibody (left). Signals were quantified by densitometric scanning (right). Hes7 protein levels relative to the one at time = 0 were determined.

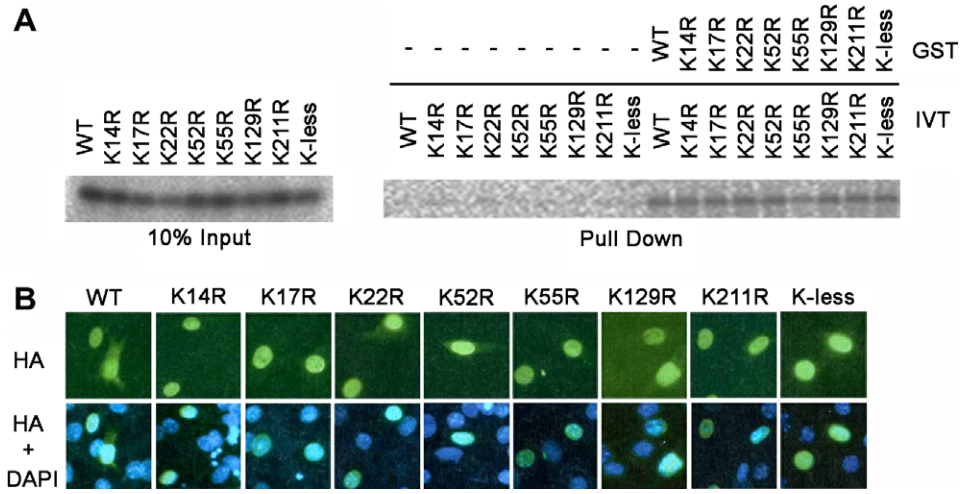

Fig. 2. Self-association activities and nuclear localization of lysine-to-arginine mutant proteins. (A) ^{35}S-labeled *in vitro* translated (IVT) proteins were incubated with or without GST-fusion proteins as indicated. Associated IVT proteins were precipitated with glutathione–Sepharose 4B beads and were examined by SDS–PAGE and autoradiography (right). As control, 10% of the input protein mixtures were applied (left). (B) C3H10T1/2 cells were transfected with plasmids expressing wild-type or mutant HA-Hes7 and visualized by anti-HA antibody. Cells were counterstained with DAPI to visualize the nuclei. Wild-type and mutant Hes7 proteins were localized to the nuclei.

repressor activity [4]. Loss of the transcriptional repressor activity could be due to low expression levels of mutant proteins, because introduction of mutations often affects the stability of the protein. To address this issue, we decided to compare the repressor activity and the protein expression levels. A lysine-to-arginine point mutation was introduced into each lysine residue, and we transfected the same amount of the expression vector for each point mutant protein together with a luciferase reporter under the control of the promoter containing the N box, a target sequence of Hes7. We found that K22R, K52R, and K55R point mutant proteins completely lost the transcriptional repressor activity, whereas the other mutant proteins maintained it (Fig. 1A). Western blot analysis showed that K22R, K52R, and K55R point mutant proteins were expressed at higher levels than the others although the same amount of each expression vector was transfected (Fig. 1A). These results indicate that loss of the transcriptional repressor activity is not due to low expression levels of mutant proteins. On the contrary, these mutant proteins seemed to be stabilized compared to others.

To analyze a possible link between the transcriptional activity and the instability of Hes7 protein, we examined the degradation time course of the mutant Hes7 proteins that lost the repressor activity. Expression vectors for these proteins were transfected into C3H10T1/2 fibroblasts, and their expression levels were measured after blockade of new synthesis of the proteins by cycloheximide treatment. The K22R, K52R, and K55R point mutant proteins were found to be significantly stabilized compared to the wild-type (Fig. 1B). They were also more stable than K14R (Fig. 1B), whose half-life was previously shown to be about 10 min longer than the wild-type [4]. These results indicate that the lysine residues K22, K52, and K55, which are located in the bHLH domain, are involved in both the transcriptional repressor activity and the instability of Hes7 protein. It has been shown that Hes7 protein is subjected to polyubiquitination- and proteasome-mediated degradation [5]. Because lysine residues are polyubiquitinated before proteasome-mediated degradation [7], it is most likely that the lysine residues K22, K52 and K55 of Hes7 are major targets for polyubiquitination.

Self-association and nuclear localization are not affected by lysine-to-arginine point mutations

We next sought to determine why the K22R, K52R, and K55R point mutants lose the repressor activity. Because ubiquitination is known to be involved not only in degradation but also in subcellular localization of target proteins [7], we examined whether lysine-to-arginine point mutations affect the nuclear localization of Hes7 protein. However, all point mutants were found to be localized to the nucleus like the wild-type Hes7 (Fig. 2B). Even the K-less mutant (all lysine residues are changed to arginine residues) was present in the nucleus (Fig. 2B). These results indicate that lysine residues are not involved in the subcellular localization of Hes7.

Hes7 represses transcription by binding to the target sequences such as the N box as a dimer [4,8]. Because the bHLH domain is involved in both dimer formation and DNA binding, K22R, K52R, and K55R point mutants could be defective in these activities. We thus examined whether lysine-to-arginine point mutations affect dimer formation. GST pull-down assay showed that all lysine-to-arginine point mutants efficiently self-associated (Fig. 2A). Furthermore, the K-less mutant efficiently self-associated, and there was no significant difference from the wild-type (Fig. 2A). These results suggest that dimer formation is not impaired by lysine-to-arginine point mutations.

The DNA-binding activity is altered by lysine-to-arginine point mutations

We next examined the DNA-binding activities of lysine-to-arginine mutants by using the N box sequence as a probe. The wild-type Hes7 specifically bound to the N box (Fig. 3A, lanes 2–6, and B) but not to the mutated sequence (data not shown). In contrast, the N box-binding activity of K22R and K55R point mutants was significantly reduced (Fig. 3A, lanes 7–11 and 17–21, and B), compared to the wild-type, suggesting that the loss of repressor activity of K22R and K55R is due to reduced N box-binding activity. However, the K52R point mutant was found to have a strong N box-binding activity (Fig. 3A, lanes 12–16, and B). Interestingly, at a lower concentration, K52R seemed to have a rather stronger N box-binding activity than the wild-type (Fig. 3A, compare lanes 3 and 13), suggesting that the DNA-binding activity could be altered by K52R point mutation. To compare the binding activity to the endogenous promoter regions, the wild-type Hes7 and K52R were overexpressed in C3H10T1/2 fibroblasts, and the binding to the N box in the endogenous *Hes1* promoter region was examined by ChIP assay. Immunoprecipitated DNA fragments were amplified by PCR (Fig. 3B, middle panel) and quantified by real-time PCR (Fig. 3C) using specific primers. These experiments showed that K52R bound to the N box more efficiently than the wild-type (Fig. 3B

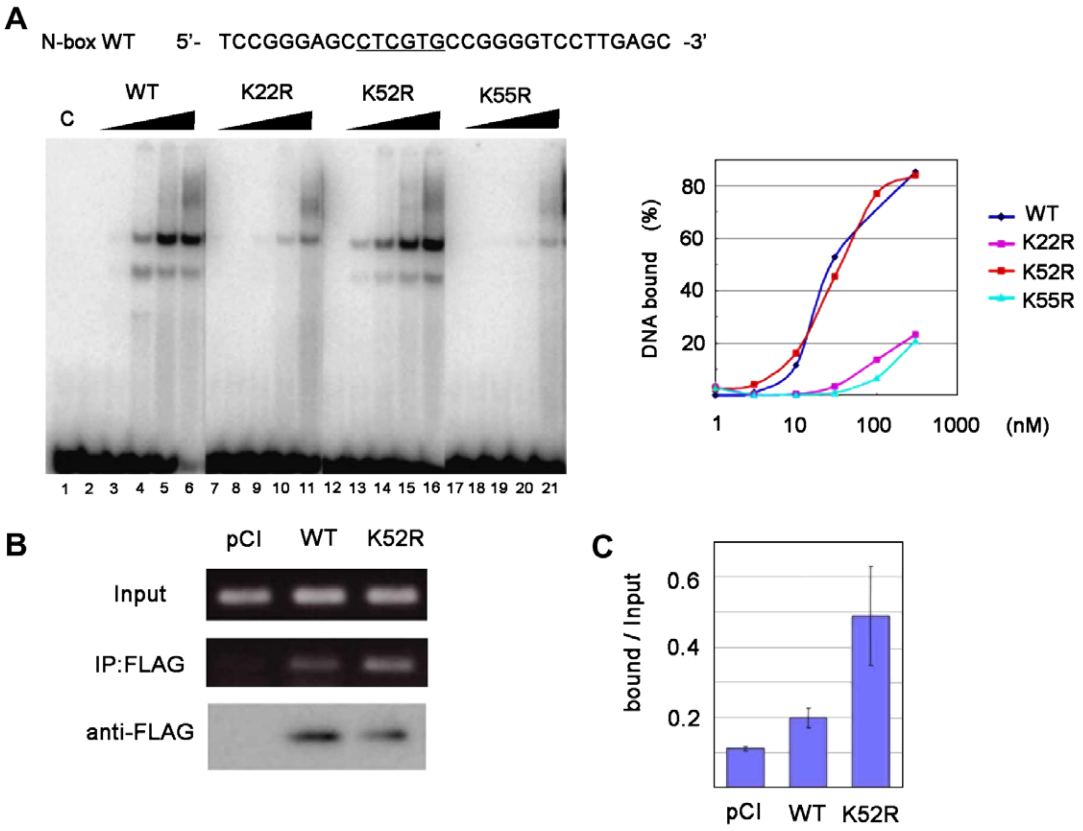

Fig. 3. The N box-binding activity of lysine-to-arginine point mutant proteins. (A) For EMSA experiments, the probe (20 fmol) was mixed with wild-type (WT) Hes7 or lysine-to-arginine point mutant proteins with the concentrations of 3 nM (lanes 2, 7, 12, and 17), 10 nM (lanes 3, 8, 13, and 18), 30 nM (lanes 4, 9, 14, and 19), 100 nM (lanes 5, 10, 15, and 20), and 300 nM (lanes 6, 11, 16, and 21) (left). The density of the shifted bands was quantified (right). The sequence of the probe is shown in the above, and the N box is underlined. The N box-binding activity of K22R and K55R was reduced whereas that of K52R was rather enhanced compared to the wild-type. (B) C3H10T1/2 cells were transfected with pCI, pCI-FLAG-Hes7 or pCI-FLAG-K52R, and interaction with the N box region in the *Hes1* promoter was examined by ChIP analysis with anti-FLAG antibody (middle panel, IP). FLAG-Hes7 and FLAG-K52R were expressed at similar levels (bottom panel). (C) The proportion (%) with a standard deviation of the precipitated band relative to the input was quantified by real-time PCR ($n = 3$). K52R interacted more efficiently with the N box region in the *Hes1* promoter than WT.

and C). These results suggest that the DNA-binding activity is altered by K52R mutation.

Interaction efficiency of Hes7 with Hes1 is altered by K52R point mutation

Although the above results suggest that the DNA-binding activity of K52R is altered, it does not explain why K52R loses the repressor activity. Because it has been shown that Hes factors repress transcription by recruiting corepressors such as TLE and Sirt1 [1,9–11], we next examined whether association of these corepressors is affected by K52R point mutation. However, TLE1, TLE3, TLE4, HDAC1, and Sirt1 interacted efficiently with both the wild-type and K52R (Fig. 4A and B), suggesting that association of corepressors is not affected by K52R point mutation.

In the PSM, both *Hes1* and *Hes7* are co-expressed in an oscillatory manner, but only *Hes7* is functionally required for periodic somite segmentation: somites are severely fused in *Hes7*-null mice [2] whereas somites are normally segmented in *Hes1*-null mice [12], suggesting that *Hes1* and *Hes7* function independently in the PSM. In agreement with this idea, in cells overexpressing both Hes1 and Hes7, the interaction between these two factors was weak (Fig. 4C, lane 2 of middle panel). In contrast, Hes7 with K52R mutation interacted with Hes1 more efficiently than the wild-type Hes7 (Fig. 4C, compare lanes 2 and 3). Thus, Hes1/Hes7 heterodimer formation could be increased by K52R point mutation in the PSM. It is most likely that Hes1/Hes7 heterodimer is non-functional because Hes1 and Hes7 have different functions and do not compensate each other in the PSM [2,12], and thus increased formation of Hes1/Hes7 heterodimer could compete for Hes7 homodimer formation or for target binding by Hes7 homodimer. These results suggest that alteration of partner preference in heterodimer formation contributes to loss of repressor activity of K52R, although further analysis will be required to determine the transcriptional activity of Hes1/Hes7 heterodimer.

Multiple lysine residues are involved in both the transcriptional activity and the instability of Hes7

In this study, we found that three lysine residues, K22, K52, and K55, are required for both the transcriptional repressor and the instability of Hes7, two important features that make the expression oscillatory. It is intriguing that common amino acid residues are involved in both repression activity and degradation of the protein. A similar feature was previously shown in transcriptional activators such as Myc: a region required for degradation of Myc is mapped to the transcriptional activation domain [13], and Skp2 functions not only as a ubiquitin ligase but also as a transcriptional coactivator for Myc [14]. It seems to be an important strategy for cells to keep critical transcriptional activators such as Myc short-lived, because sustained presence of such factors could lead to tumorigenesis. It is thus possible that both transcriptional repressor activity and degradation of Hes7 could be also regulated by common factors, although further analysis such as identification of E3 ligase for Hes7 will be required to understand the precise mechanism of these regulations.

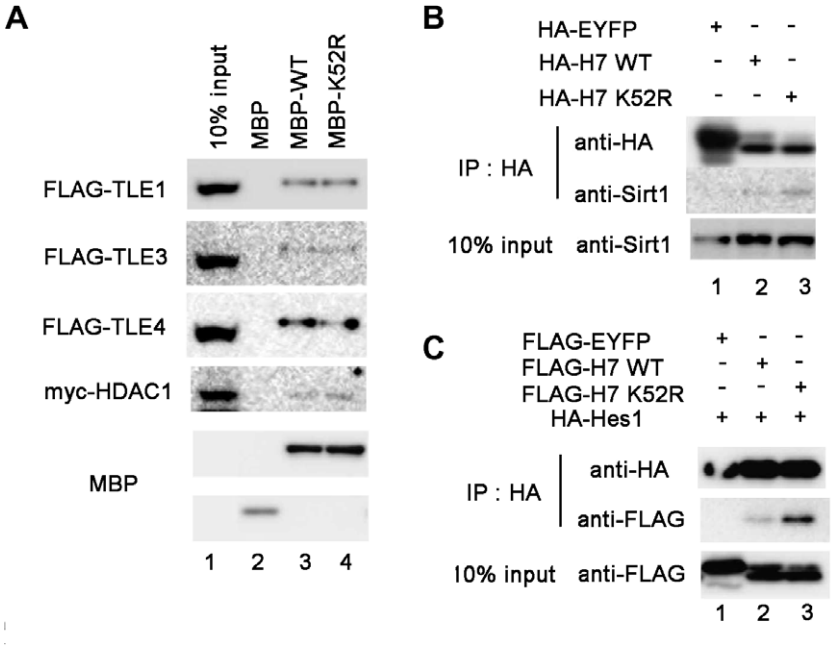

Fig. 4. Interactions of Hes7 with corepressors and Hes1. (A) MBP pull-down assay. Expression vectors indicated on the left were transfected into C3H10T1/2 cells, and cell lysates were mixed with either purified MBP, MBP-wild-type Hes7 or MBP-K52R. The associated proteins were affinity purified and eluted with 30 mM maltose. (B,C) Co-immunoprecipitation assay. Expression vectors were co-transfected in C3H10T1/2 cells, and cell lysates were immunoprecipitated using anti-HA agarose. Bound proteins were eluted with sample buffer and analyzed by Western blot. K52R interacted with Hes1 more efficiently than the wild-type Hes7 (C, compare lanes 2 and 3).

All three lysine residues are located in the bHLH domain and are conserved among Hes family members except for Hes6 [1]. Hes6 has an asparagine residue at a corresponding position of K55 and loses the transcriptional repressor activity [15]. Thus, it is possible that these lysine residues are required for the normal conformation of Hes7, although it remains to be determined how lysine-to-arginine mutations affect the tertiary structure of the protein. Another possibility is that these lysine residues could be important for other modifications than ubiquitination, such as SUMOylation and acetylation, which are known to play important roles in transcriptional activity [7,16]. For example, lysine-to-arginine mutations in the bHLH domain impair acetylation and the DNA binding of the bHLH factor BETA2 [17]. Further analysis on modifications of Hes7 protein will be important to understand the roles of conserved lysine residues.

Acknowledgments

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