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DNA-binding and transcriptional activities of human HSF4 containing mutations that associate with congenital and age-related cataracts

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**Abstract**

Heat shock transcription factor HSF4 is necessary for ocular lens development and fiber cell differentiation. Mutations of the human *HSF4* gene have been implicated in congenital and age-related cataracts. Here, we show that HSF4 activates transcription of genes encoding crystallins and beaded filament structural proteins in lens epithelial cells. Five missense mutations that have been associated with congenital cataract inhibited DNA binding of HSF4, which demonstrates the relationship between *HSF4* mutations, loss of lens protein gene expression, and cataractogenesis. However, two missense mutations that have been associated with age-related cataract did not or only slightly alter HSF4 activity, implying that other genetic and environmental factors affect the functions of these mutant proteins.

Keywords: HSF4, congenital cataract, age-related cataract, crystallin, beaded filament structural protein

## 1. Introduction

Cataracts are defined as lens opacities. Cataractogenesis has multiple causes and is often associated with a breakdown of the lens microarchitecture [1]. Lens proteins undergo a wide variety of alterations with age, and many of these are accelerated by oxidative, osmotic, and other stresses. In addition to environmental risk factors, genetic factors are also important in age-related cataract formation [2, 3]. Congenital cataract is likely to cause childhood blindness. It is known that mutations in genes encoding two groups of proteins cause congenital cataract formation. The first group consists of structural proteins in the lens such as lens crystallins, beaded filaments unique to lens fiber cells, gap junction connexins important for intercellular communication in the avascular lens, and membrane water transporter aquaporin. The second group consists of transcription factors e.g. HSF4, MAF, FOXE3, and PITX3 that regulate lens development and fiber cell differentiation [4].

HSF4, despite being a member of the heat shock transcription factor (HSF) family, plays a role in cell growth and differentiation instead of stress-induced transcription of genes encoding heat shock proteins [5, 6]. The N-terminal half of HSF4 contains a DNA-binding domain (DBD) with a 'winged' helix-turn-helix motif and hydrophobic repeat (HR-A and -B) regions with coiled-coil structures [7]. The HR-A/B regions mediate HSF4 trimerization. HSF4 trimers bind to the consensus heat shock element (HSE), made up of inverted repeats of the 5-bp sequence nGAAn, and to HSE-like sequences [7-9]. There are two HSF4 isoforms; HSF4b has the ability to activate transcription but not HSF4a [10]. Expression of HSF4 is restricted to the brain and lung [7, 10]. *HSF4* knockout mice exhibit reduced expression of crystallins and beaded filaments in lens and develop cataracts [11-13].

Mutations of human *HSF4* have been associated with congenital cataract [14-18]. Furthermore, two missense mutations were identified by screening *HSF4* in 150 age-related cataract patients [19]. However, how these mutations affect HSF4 function remains unclear.

In this study, we introduced missense mutations into *HSF4b* cDNA and characterized the molecular properties of the mutant proteins.

## 2. Materials and methods

### 2.1. In vitro polypeptide synthesis, chemical cross-linking, and electrophoretic mobility shift assay

Plasmid pcDNA-*HSF4b* was a derivative of pcDNA3.1(+) containing *HSF4b* cDNA [9]. Nucleotide changes were introduced into *HSF4b* by site-directed mutagenesis. HSF4b polypeptides were synthesized in vitro using the TNT T7 Coupled Reticulocyte Lysate System (Promega). The amounts of synthesized polypeptides were determined by immunoblot analysis with an anti-HSF4 antibody using purified recombinant HSF4b as a reference [9].

For the chemical cross-linking analysis, HSF4b polypeptides (0.4 ng) in 4  $\mu$ l of 25 mM HEPES-KOH, pH 7.6, 70 mM NaCl, 2 mM EDTA, and 5% glycerol were incubated at 37 °C for 20 min, treated with EGS (ethylene glycol bis-(succinimidylsuccinate)) at room temperature for 20 min, electrophoresed on a SDS-polyacrylamide gel, and subjected to immunoblot analysis [9]. The experiments were performed at least three times with similar results.

For the electrophoretic mobility assay, the binding reaction was carried out in an 8- $\mu$ l mixture containing 0.5 ng of HSF4b polypeptides, 25 mM HEPES-KOH, pH 7.6, 90 mM NaCl, 2 mM EDTA, 5% glycerol, 100 ng of poly(dI-dC), and 0.01 ng of <sup>32</sup>P-labeled HSE oligonucleotide at 37 °C for 20 min. The samples were electrophoresed and subjected to phosphorimaging [9, 20]. The experiments were performed at least four times with similar results.

## 2.2. Cell culture and transfection

HeLa cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and 2.5 µg/ml Fungizone (Invitrogen) at 37 °C in a 5% CO<sub>2</sub> atmosphere [9, 20]. SRA01/04 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, 10 µg/ml gentamycin, and 2.5 µg/ml Fungizone at 37 °C in a 5% CO<sub>2</sub> atmosphere [21]. To express the HSF4b-VP16 fusion protein in HeLa cells, the activation domain of herpes simplex virus VP16 (amino acid residues from 413 to 490) was fused in-frame to the C-terminus of HSF4b in the pcDNA3.1(+) vector [9]. Transient transfection was performed using HilyMax reagent (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. Expression of HSF4b-VP and HSF4b was verified by immunoblot analysis using anti-VP16 and anti-HSF4 antibodies, respectively [9].

## 2.3. Luciferase assay

The HSE-SV40p-*LUC* reporter genes contained 3P-, gap-, and step-type HSE oligonucleotides inserted upstream of the SV40 promoter fused to the firefly luciferase gene [9, 20]. The luciferase reporter genes *BFSP1-LUC*, *BFSP2-LUC*, *CRYAB-LUC*, and *CRYGC-LUC* contained the following promoter regions respectively: -1044 to -43 of *BFSP1*, -998 to -48 of *BFSP2*, -586 to +3 of *CRYAB*, and -734 to +3 of *CRYGC* (relative to the translation initiation site) [9]. Luciferase assays were performed as previously described [9, 20]. Experiments were carried out at least eight times, and statistical significance was determined using the Student's *t*-test.

## 2.4. Reverse transcription (RT)-PCR analysis

SRA01/04 cells grown in 60-mm dishes were transfected with a DNA mixture containing 1.1 µg of pcDNA-*HSF4b* and 7.7 µg of carrier pcDNA3.1(+) DNA. After 15 h,

total RNA was isolated from the cells using the FastPure RNA Kit (TAKARA BIO). RNA (1.6 µg) was reverse transcribed with oligo-dT primer (1 µM) using AMV reverse transcriptase (Promega) in a 20-µl reaction mixture for 1 h at 42 °C. Two microliters of sample was used for PCR (25-µl mixture) with gene-specific primers. The PCR products were separated by polyacrylamide gel electrophoresis and visualized by staining with ethidium bromide. The amounts of PCR products were compared after normalizing each sample to the amount of *GAPDH* PCR product [9, 20]. The experiments were performed at least three times with similar results.

### 3. Results

#### 3.1. Oligomerization and HSE-binding of HSF4b in vitro

*HSF4* mutations associated with congenital cataract formation are located in the DBD (A20D, R74H, and I87V), linker region (L115P and R120C), and HR-A (R176P) (Fig. 1A) [14, 16, 17]. Two mutations identified in age-related cataract patients, Q62R and R117H, are located in the DBD and linker region, respectively [19]. The effects of these mutations on HSF4b oligomerization were analyzed using in vitro-synthesized polypeptides (Fig. 1B). Wild-type HSF4b polypeptides treated with EGS, a chemical cross-linker, were detected as two bands on SDS-polyacrylamide gels (Fig. 1C). The faster migrating band with a molecular size of 60 kDa was a monomeric form of HSF4b. The slower migrating band had a molecular size of 180 kDa, which corresponds to the size of an HSF4b trimer. The Q62R, R74H, I87V, and R117H mutations did not affect the formation of HSF4b trimers. However, oligomerization was markedly inhibited in the A20D, L115P, and R120C mutant proteins and completely inhibited in the R176P mutant protein under the assay conditions. Therefore, HSF4b oligomerization is modulated by the DBD, linker region, and HR region.

Electrophoretic mobility shift assays were conducted to determine the HSE-binding

ability of HSF4b polypeptides. Probe oligonucleotides were synthetic model HSEs consisting of three inverted repeats of nGAAn that were either continuous (3P), containing one (gap), or two (step) skips between three repeats (Fig. 1D). As shown previously [9], wild-type HSF4b polypeptides were capable of binding to continuous 3P-type HSE and discontinuous gap- and step-type HSEs (Fig. 1E). HSF4b containing the A20D mutation bound to continuous HSE, but not to discontinuous HSEs. In contrast, HSF4b-Q62R exhibited slightly higher binding affinity for discontinuous HSEs than wild-type HSF4b. The I87V and R117H mutations did not significantly affect binding of HSF4b to the three HSE types; however, the R74H, L115P, R120C, and R176P mutations completely inhibited binding. These results show that the eight mutant HSF4b polypeptides exhibit different abilities to oligomerize or bind to HSEs in vitro.

### 3.2. Expression of model reporter genes by HSF4b-VP

It has been shown that the DBD of *Saccharomyces* HSF regulates not only the DNA-binding ability but also the transactivating ability of the activation domain [22]. To further analyze the effects of HSF4 mutations on protein-DNA interaction, HSF4b fused to the constitutively active VP16 activation domain was cotransfected with reporter gene into HeLa cells. HSF4b-VP fusions induce expression of HSE-SV40p-*LUC* reporter genes, which contain model HSEs upstream of the SV40 promoter-luciferase gene fusion, depending on the DNA-binding ability, even though the mutations may affect the function of the HSF4b activation domain [9]. Expression of HSF4b-VP was shown by immunoblot analysis (Fig. 3A). Cotransfection of wild-type HSF4b-VP caused a 9.4-, 4.7-, and 4.1-fold increase in luciferase activity of reporter genes that contained HSE3P, HSEgap, and HSEstep, respectively (Fig. 3B). Consistent with the results of the electrophoretic mobility shift assays, HSF4b-VP containing the A20D mutation was capable of inducing expression of reporter gene containing continuous 3P-type HSE, but not discontinuous gap- and step-type HSEs.

Three mutations slightly affected reporter gene activity: the Q62R mutation appeared to enhance expression of reporter genes containing discontinuous HSEs, while the I87V and R117H mutations appeared to inhibit expression of all three reporters. Other mutations almost completely inhibited the transcription activating ability of HSF4b-VP. Note that, despite the same amount of plasmid DNA being used for transfection, protein levels of the transcriptionally active HSF4b-VP derivatives (WT, Q62R, I87V, and R117H) were reduced compared with those of the inactive derivatives (A20D, R74H, L115P, R120C, and R176P) (see Fig. 2A), implying that expression of HSF4b-VP is negatively regulated by an unknown feedback mechanism. Whatsoever the reason, the induction of expression by mutant HSF4b-VP fusions acting on model HSEs varied according to their DNA-binding abilities.

### 3.3. Transcription of lens protein genes by HSF4b

To examine the transcriptional activity of HSF4b, wild-type and mutant proteins were expressed in the human lens epithelial cell line SRA01/04 (Fig. 3A). The mRNA levels of *CRYGC* encoding  $\gamma$ C-crystallin were significantly increased by wild-type HSF4b, as determined by RT-PCR (Fig. 3B). HSF4b containing the A20D, R74H, L115P, R120C, and R176P mutations, but not Q62R, I87V, and R117H, failed to cause an increase in *CRYGC* mRNA levels. Similar results were obtained with reporter gene assays of SRA01/04 cells transfected with HSF4b derivatives and *CRYGC-LUC* (luciferase reporter gene fused to the *CRYGC* promoter) (Fig. 3C). It is noteworthy that wild-type HSF4b failed to induce expression of luciferase reporter genes driven by the  $\gamma$ A-,  $\gamma$ B-,  $\gamma$ D-crystallin promoters (< 2.0-fold). Expression of the  $\alpha$ B-crystallin (*CRYAB*) gene and *CRYAB-LUC* reporter gene was activated by HSF4b, but the R74H, L115P, R120C, and R176P mutations inhibited the transcription activation ability (Fig. 3B and C). The differential effects of the A20D mutation on the *CRYGC* and *CRYAB* expression could be due to differences in HSE structure: the

*CRYGC* promoter contains an atypical HSE consisting of multiple GAA-like sequences, whereas the *CRYAB* promoter contains a 3P-type HSE [9, 23]. The *BFSP1* and *BFSP2* genes encoding beaded filament structural proteins contain atypical HSEs consisting of three GAA-like sequences at the promoters [13]. Wild-type HSF4b induced expression of the *BFSP2* gene, *BFSP1-LUC*, and *BFSP2-LUC*, but HSF4b containing the A20D, R74H, L115P, R120C, and R176P mutations did not (Fig. 3C). Constitutive mRNA levels of heat shock protein genes encoding HSP27, HSP70, and HSP90 were not significantly (<1.2-fold) affected by wild-type HSF4b (data not shown). These results show that five out of eight mutant HSF4b proteins are defective for activating transcription of *CRYGC*, *CRYAB*, *BFSP1*, or *BFSP2* in SRA01/04 cells.

#### 4. Discussion

In this study, we show that HSF4b is a transcriptional activator of lens protein genes such as *CRYAB*, *CRYGC*, *BFSP1*, and *BFSP2*. Five missense mutations (A20D, R74H, L115P, R120C, and R176P) that have been associated with congenital cataracts inhibit the transcription activation ability of HSF4b. The transcriptional defect of these mutant proteins is due to a defect in HSE-binding. Mutations in *CRYAB*, *CRYGC*, *BFSP1*, and *BFSP2* cause congenital cataracts [24-28]. A 5-bp insertion in the *CRYGC* gene that disrupts the open reading frame is associated with dominant congenital cataract, suggesting that reduction of the normal  $\gamma$ C-crystallin level or accumulation of the truncated protein induces cataract [29]. Lenses of mice lacking the *BFSP1* and *BFSP2* genes develop opacities with increasing age [30, 31]. Therefore, reduced expression of these lens protein genes caused by mutations in *HSF4* could lead to the formation of cataracts. HSF4a repressor, an alternatively spliced form of HSF4b activator, shares the DBD and HR-A/B with HSF4b [10], suggesting that mutant proteins defective in HSE-binding are unable to function properly as transcriptional activators

or repressors.

The R74H mutation in the third helix of the DBD did not affect HSF4b trimerization but did inhibit HSE-binding. This is consistent with observations that the same arginine residue in *Kluyveromyces* HSF makes bidentate hydrogen bonds to the guanine base of the nGAA sequence [32] and the same residue in *Saccharomyces* HSF is essential for HSE binding [33]. The A20D mutation in the first helix of the DBD markedly inhibited HSF4b oligomerization. The first helix of the human HSF1 DBD is important for oligomerization and HSE-binding [34]. Interestingly, HSF4b-A20D was capable of binding to continuous HSE but not discontinuous HSEs. The cross-linking assay was performed in the absence of DNA, while in the DNA-binding experiments protein-DNA interaction would facilitate protein-protein interaction, and trimeric HSF4b-A20D bound to continuous HSE. However, efficient oligomerization is a prerequisite for binding to discontinuous HSEs in human HSF1 and HSF4b [9, 20], suggesting that the A20D mutant protein will not bind to discontinuous HSEs. The L115P and R120C mutations in the linker region markedly inhibited HSF4b oligomerization and completely inhibited binding to various HSE types. In *Saccharomyces* HSF and human HSF1, mutations in the linker region deregulate oligomerization [35, 36]. It is possible that the L115P and R120C mutations change the configuration of the DBD and inhibit its DNA-binding ability. The R176 residue is located in the HR-A, and a substitution with a helix-destabilizing proline residue would disrupt the coiled-coil structure and result in a loss of its ability to oligomerize and bind to HSE.

The Q62R, I87V, and R117H mutations had marginal effects on lens protein gene expression by HSF4b in lens epithelial cells (Fig. 3). The I87V mutation was identified in an individual with infantile cataract. This individual's father carried the same mutation and showed a mild cataract similar to senile cataracts [14]. The Q62R and R117H mutations were identified in age-related cataract patients [19]. Although R117 in the linker region is near

L115 and R120, L115 and R120 are highly conserved in mammalian and chicken HSFs while R117 is not – the same position in chicken HSF2 is histidine [5]. Similarly, I87 in the wing region of the HSF4b DBD is a valine residue in chicken HSF2 [5]. The Q62 residue in the turn of the helix-turn-helix motif changes to basic arginine, and the corresponding residue is basic lysine in human HSF1 and HSF2 [5]. Therefore, it is likely that the Q62R, I87V, and R117H mutations do not affect HSF4b function significantly. It should be noted, however, that the Q62R mutation slightly enhanced binding of HSF4b to discontinuous HSEs (Figs. 1E and 2B), and that the I87V mutation inhibited binding to step-type HSE (Fig. 2B). The possibility remains that slight changes in the transcriptional activity of HSF4 cause age-related cataract. These mutations could alter expression of other HSF4 target genes. Genetic and environmental factors could affect the function of these mutant HSF4 proteins. Hence, studies on *HSF4* mutations and the development of cataract should be carried out with this in mind.

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### Figure Legends

**Fig. 1.** Characterization of mutant HSF4b polypeptides in vitro.

(A) Mutation sites. The DBD, linker, and HR-A/B are shown. The DBD consists of three helices (H1, H2, and H3), four sheets (S1, S2, S3, and S4), and a wing region. Numbers indicate amino acid positions in HSF4b.

(B) SDS-polyacrylamide gel electrophoresis of HSF4b. In vitro-synthesized polypeptides (0.3 ng) were electrophoresed on a SDS-polyacrylamide gel and subjected to immunoblot analysis with an anti-HSF4 antibody. The positions of molecular mass markers are shown in kilodaltons (left).

(C) Chemical cross-linking of HSF4b. Polypeptides were incubated at 37 °C for 20 min, treated with 0, 0.5, 1.5, and 4.5 mM EGS for 20 min, and subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Open and closed arrowheads indicate the positions of HSF4b monomers and trimers, respectively.

(D) Nucleotide sequences of three HSE types. The GAA and inverted TTC sequences are indicated by bold uppercase letters with arrows.

(E) Electrophoretic mobility shift assay of HSF4b-HSE complexes. Polypeptides were incubated with <sup>32</sup>P-labeled HSE oligonucleotides of the 3P- (P), gap- (G), and step- (S) types at 37 °C for 20 min. Samples were subjected to polyacrylamide gel electrophoresis and phosphorimaging. Open and closed circles indicate the positions of free probe DNAs and protein-DNA complexes, respectively. The binding abilities relative to wild-type HSF4b are shown below each lane.

**Fig. 2.** Transcriptional regulation by HSF4b-VP in HeLa cells.

(A) Immunoblot analysis of HSF4b-VP. Cells were transfected without (indicated by -) or with the indicated HSF4b-VP derivatives (10 ng). Extracts prepared from the cells were subjected to immunoblot analysis using an anti-VP16 antibody. Position of HSF4b-VP is shown on the right. NS: non-specific band.

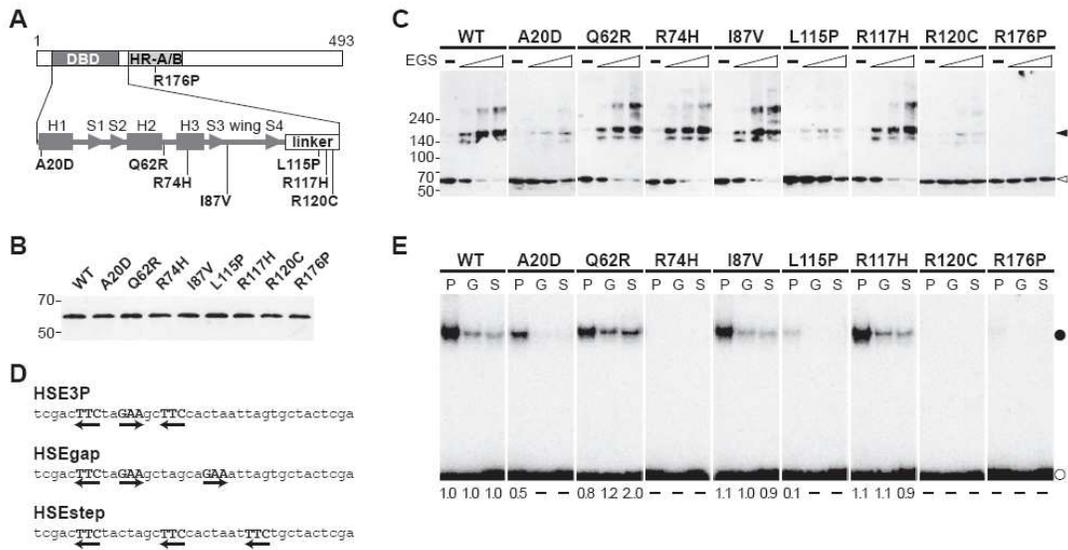
(B) Expression of HSE-SV40p-*LUC* reporter genes by HSF4b-VP. Cells were transfected with 100 ng of reporter plasmid containing the indicated HSE and 10 ng of HSF4b-VP-expression plasmid. Data are expressed as fold-induction compared to luciferase activity in cells transfected with empty expression vector. Asterisks indicate significant differences ( $p < 0.01$ ).

**Fig. 3.** Transcriptional regulation by HSF4b in SRA01/04 cells.

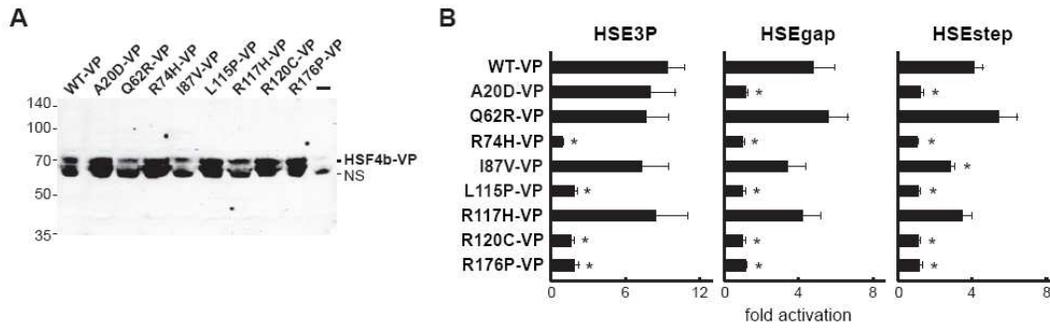
(A) Immunoblot analysis of HSF4b. Cells were transfected without (indicated by -) or with the indicated HSF4b derivatives (200 ng). Extracts prepared from the cells were subjected to immunoblot analysis using an anti-HSF4 antibody.

(B) RT-PCR analysis of *CRYGC*, *CRYAB*, and *BFSP2*. Total RNA was prepared from cells transfected without (indicated by -) or with the indicated HSF4b derivatives, and was subjected to RT-PCR analysis of *CRYGC*, *CRYAB*, *BFSP2*, and control *GAPDH*.

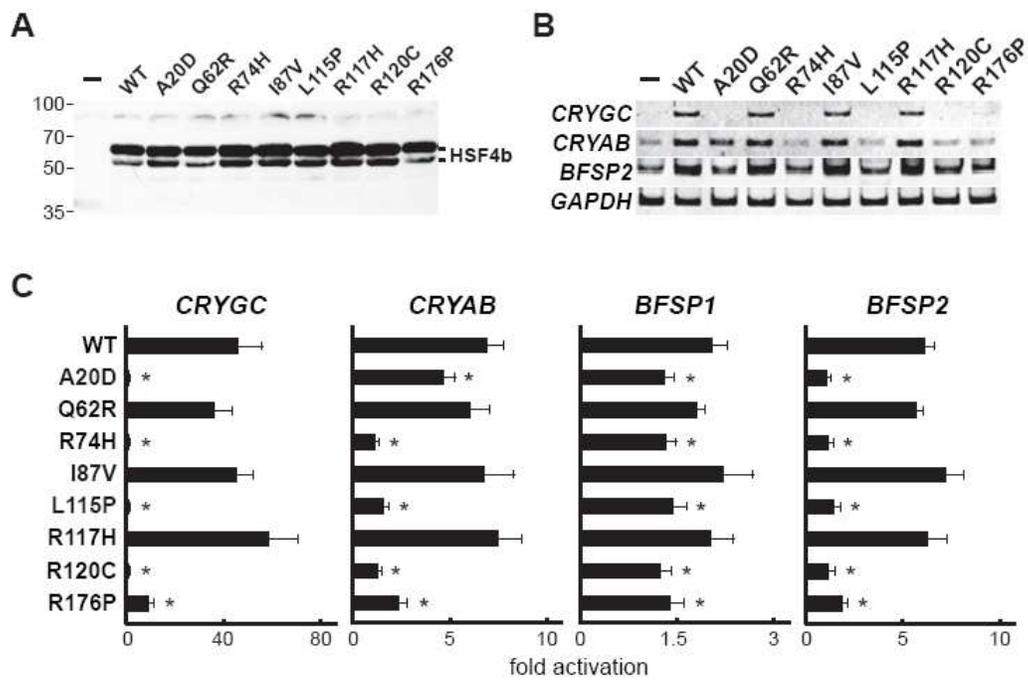
(C) Expression of various reporter genes by HSF4b. Cells were transfected with *CRYGC-LUC* (400 ng), *CRYAB-LUC* (200 ng), *BFSP1-LUC* (500 ng), or *BFSP2-LUC* (500 ng) reporter plasmid and 200 ng of HSF4b-expression plasmid. Data are expressed as fold-induction compared to luciferase activity in cells transfected with empty expression vector. Asterisks indicate significant differences ( $p < 0.01$ ).



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