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Biotin attenuates heat shock factor 4b transcriptional activity by lysine 444 biotinylation

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ABSTRACT

Genetic mutations in HSF4 cause congenital cataracts. HSF4 exhibits both positive and negative regulation on the transcription of heat shock and non-heat shock proteins during lens development, and its activity is regulated by posttranslational modifications. Biotin is an essential vitamin that regulates gene expression through protein biotinylation. In this paper, we report that HSF4b is negatively regulated by biotinylation. Administration of biotin or ectopic bacterial biotin ligase BirA increases HSF4b biotinylation at its C-terminal amino acids from 196 to 493. This attenuates the HSF4b-controlled expression of αB-crystallin in both lens epithelial cells and tested HEK293T cells. HSF4b interacts with holocarboxylase synthetase (HCS), a ubiquitous enzyme for catalyzing protein biotinylation in mammal. Ectopic HA-HCS expression downregulates HSF4b-controlled αB-crystallin expression. Lysine-mutation analyses indicate that HSF4b/K444 is a potential biotinylation site. Mutation K444R reduces the co-precipitation of HSF4b by streptavidin beads and biotin-induced reduction of αB-crystallin expression. Mutations of other lysine residues such as K207R/K209R, K225R, K288R, K294R and K355R in HSF4's C-terminal region do not affect HSF4's expression level and the interaction with streptavidin, but they exhibit distinct regulation on αB-crystallin expression through different mechanisms. HSF4/K294R leads to upregulation of αB-crystallin expression, while mutations K207R/K209R, K225R, K288R, K255R and K435R attenuate HSF4's regulation on αB-crystallin expression. K207R/K209R blocks HSF4 nuclear translocation, and K345R causes HSF4 destabilization. Taken together, the data reveal that biotin maybe a novel factor in modulating HSF4 activity through biotinylation.

1. Introduction

Heat shock factor 4 （HSF4） is a member of the heat shock transcription factor family that can respond to a variety of environmental and biophysical stresses in cells and drive the expression of heat shock proteins [1]. It performs the latter function by acting as molecular chaperones to maintain cell homeostasis by folding, assembling, sorting, and degrading proteins. This adaptive mechanism, which is known as the heat shock response [2], is conserved from yeast to humans [3]. HSF4 shares the conserved DNA binding domain and N-terminal hydrophobic region with other heat shock factor members like HSF1 and HSF2 with distinct transcription activities [3–5]. HSF4 is an essential regulator for lens development [6]. Loss-of function in HSF4 causes congenital cataracts in humans and animals [7,8]. HSF4 exerts both active and negative transcription activities during lens development. However, the molecular mechanism underlying HSF4 regulation remains largely unclear.

The *HSF4* gene encodes two protein isoforms, HSF4a and HSF4b, due

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to alternate splicing between exon 8 and exon 9, resulting in an addition of 43 amino acids in the regulatory region of HSF4b and the different transcriptional activity between the two isoforms [4]. Missense mutations in *HSF4* are associated with hereditary autosomal dominant and recessive cataracts [6]. HSF4b but not HSF4a is the dominant variant expressed in lens tissue. During lens development, HSF4b positively regulates the expression of small heat shock proteins (such as HSP25, αB-crystallin) and lens skeletal proteins (e.g., FBSP1) and simultaneously negatively regulates the expression of HSP70, FGF1, vimentin [7–9]. Its divergent transcriptional activities are regulated by post-translational modifications (e.g. phosphorylation and sumoylation) [10, 11] as well as other associated proteins such as BCSA2 [12] and dual specific protein phosphatase 26 (DUSP26) [13]. HSF4b associates with and is phosphorylated by ERK1/2 and P38, which results in Hsf4b protein stabilization. ERK1/2-mediated phosphorylation can be inhibited by DUSP26 via an ERK1/2-Hsf4b-DUSP26 complex [13]. The growth factor FGF2, which is important for early lens development, increases Hsf4b protein stabilization by activating ERK1/2-mediated phosphorylation and/or sumoylation [14,15]. EGF activates ERK-mediated phosphorylation of HSF4 at S299, which attenuates HSF4b-controlled aB-crystallin expression in lens in vitro [11]. The mutation analysis indicates that phosphorylation of S299 increases HSF4 interaction with transcriptional repressor Daxx [16]. S299 and K294 of HSF4b constitute the PDSM domain (phosphorylation-dependent sumoylation motif, φKxxP), which is conserved in most transcription factors. Phosphorylation of S299 induces K294 sumoylation, which downregulates HSF4b activity in the Gal4-reporter system [15]. In addition, HSF4/T472 is phosphorylated by MEK6, which regulates the nuclear translocation of HSF4 by modulating its interaction with importin 1β, an important component of the nuclear transporter complex [10]. These studies suggest that posttranslational modification is important for HSF4 transcription activity.

Biotin, known as vitamin H or B7, is absorbed from foods or from turnover of biotin-dependent enzymes. Biotin is transported into cells by its transporters such as the sodium-dependent multivitamin transporter (SMVT), the monocarboxylate transporter, and the solute carrier family member 3(SLC19A3) [17,18]. Biotin serves as a cofactor for biotin-dependent carboxylases (e.g. Acetyl-CoA-carboxylase, pyruvate-carboxylase, Methyl-carboxylase), where it is required for the transfer of carbon dioxide from bicarbonate to organic acid metabolites [19]. In addition, biotin also regulates mechanisms such as fatty acid synthesis, gluconeogenesis and leucine metabolism. Biotinylation of transcription factors, chromatin-remodeling proteins, Histones and chaperones can regulate gene transcription. Biotinylation of Histone H3 (at K4, K9 and K18), H4 (at K8, K12 and K16) and H2A is associated with transcription repression [20]. Biotinylation of H4/K16 is over-represented in repeat regions (pericentromeric alpha satellite repeats and long terminal repeats (LTR)) compared with euchromatin promoters, and inhibits interleukin-2 gene promoter activity in human lymphoid cells [21]. Biotin also activates the expression of holocarboxylase synthetase (HCS), an enzyme that catalyzes the covalent attachment of biotin to lysine residues of target proteins [22]. HCS and BirA are the enzymes that are responsible for catalyzing protein biotinylation in mammalian and bacteria respectively. HCS is unique because it can not only catalyzes the biotinylation of CoA-carboxylases in mammalian tissues but can also regulate gene expression through biotinylation-dependent or independent manner in other organisms. In C. elegans, HCS suppresses gene expression by interacting with histone-lysine N-methyltransferase EHMT1 to facilitate gene suppression marker H3K9 methylation. Biotinylated histones are over-represented in repressed loci including peri-centromeric alpha satellite repeats, long-terminal repeats, telomeres, and the promoters of the biotin transporter gene SMVT and interlieukin-1 gene [23]. HCS-catalyzed biotinylation also regulate gene transcription by modulating transcription factor abundancy. HCS occupies the core promoter region of the transcriptionally inactive Hsp70 gene. Upon heat shock, HCS is displaced and the promoter region becomes enriched with TFIIH subunits XPD and XPB and elongating RNA polyII [23]. These studies suggest that biotinylation is involved in regulating the transcriptional expression of heat shock proteins.

In this paper, we studied the regulation of biotin on HSF4-mediated transcription. We found that administration of biotin down-regulated HSF4-controlled HSP25 and aB-crystallin expression at both mRNA and protein levels in vitro. Ectopic expression of humanized bacterial BirA or mammalian HCS (Holocarboxylase synthetase) downregulated HSF4b's activity. Using lysine-mutation analysis, we found that K444 is a potential biotinylating site in HSF4b. In addition, we found that the mutations of K207R/K209K, K 225R, K228R, K355R and K435R in HSF4b exhibited to some extent downregulation of αB-crystallin expression. K207R/K209R mutation blocks HSF4's nuclear translocation. The K435R mutation causes HSF4 protein destabilization, and the K294R mutation increases HSF4 mediated aB-crystallin expression. The K225R, K288R and K345R mutations did not affect HSF4's nuclear localization. These data highlight that biotinylation is a novel mechanism in the regulation of HSF4 transcriptional activity.

2. Materials and methods

2.1. Cell lines and plasmids

mLEC/hsf4−/−, mLEC/HA-Hsf4b [14], HEK293 and HLE-B3 were cultured in DMEM media containing 10% FBS, 100 μg/ml streptomycin and 100u/ml penicillin G. Cells were passed every two days. The site-directed mutations were performed on the p3xFlag-Hsf4b template by following the protocol provided by the kit (Invitrogene, USA). The mutation sites and relevant primers are listed in Table 1. All of the constructs were confirmed by DNA-sequencing.

2.2. Immunoblotting, immunoprecipitation and streptavidin-pull down assay

For immunoblotting, the cells were collected and lysed in NP-40 lysis buffer (50 mM Tris.Cl, pH7.4, 150 mM NaCl, 1% NP-40) containing 1x protease inhibitor cocktail and 1x phosphatase inhibitor cocktail (Sigma, USA) for 30 min. The proteins were separated by SDS-PAGE electrophoresis and transferred onto PVDF membrane. After being blocked in 5% fat-free milk for 1hr, the membrane was incubated overnight with primary antibody at dilution of 1000:1. The membrane was washed in PBST buffer three times and then incubated with horseradish peroxidase-conjugated secondary antibody for 1hr. After washing in PBST buffer, the membrane was developed on x-ray film. For the immunoprecipitation assay, 0.8–1 mg of cell lysis protein was precleaned with proteinA/G agarose beads for 30 min. The supernatants were incubated with primary antibody overnight. The protein A/G agarose beads were added to the cell lysates for 2 h. The beads were pelleted and washed 4 times with lysis buffer. The samples were subjected to immunoblotting as described above.

For the *streptavidin*-pull down assay, the cells were transiently transfected with p3xflag-empty vector or p3xflag-Hsf4b (or Hsf4b mutants). The cells were treated with media containing sham (PBS) or 50 μM Biotin for 24 h. After this, the cells were lysed in NP-40 lysis buffer and cell lysates were incubated with streptavidin-conjugated beads for 2–3 h. The beads were pelleted and washed 4 times with NP-40 lysis buffer and once with 1% SDS buffer. The beads were boiled in the loading buffer for SDS-PAGE gel electrophoresis and subsequently subjected to immunoblotting as described above.

For the *in vivo* GST-pull down assay, the cells were transiently transfected with PEBG empty vector or PEBG-Hsf4b mutants. The cells were lysed in NP-40 lysis buffer and cell lysates were incubated overnight with glutathione-Sepharose 4b beads. The beads were pelleted and washed 4 times with NP-40 lysis buffer. The samples were subjected to immunoblotting as described above.

Table 1
The primers used in this paper.

Gene/mutation	Upper Primer	Reverse Primer
HSF4	CCCAAGCTTCAGGAAGCGCCAGCTGCGCTGCC	CCGGAATTCTTAGGGGGAGGGACTGGCTTCC
HSF4/K207R/K209R	CCGAGCAATGCAGGAGGCAGGAGAAGGCTGTCCCTGATGCTGGAT	ATCCAGCATCAGGGACAGCCTTCTCCTGCCTCCTGCATTGCTCGG
HSF4/K225R	TGCCCAACACCTGCCAGGTTCAACACCTGCCCT	AGGGCAGGTGTTGAACCTGGCAGGTGTTGGGCA
HSF4/K288R	GATGGCAGGAGGGAGAGGGGCCTGGCACTGCTC	GAGCAGTGCCAGGCCCCTCTCCCTCCTGCCATC
HSF4/K294R	GGCCTGGCACTGCTCAGAGAAGAGCCGGCCAGT	ACTGGCCGGCTCTTCTCTGAGCAGTGCCAGGCC
HSF4/K355R	GCCATCCTGGAAGGGAGAGGGAGCTTCAGCCCC	GGGGCTGAAGCTCCCTCTCCCTTCCAGGATGGC
HSF4/K435R	CCTGAGCTGGCGGTCAGGGGGTTAAATTCTCCA	TGGAGAATTTAACCCCCTGACCGCCAGCTCAGG
HSF4/K444R	TCTCCAAGCCCAGGGAGGGACCCCACGCTCGGG	CCCGAGCGTGGGGTCCCTCCCTGGGCTTGGAGA
Pbabe-puro/HA-HCS	TTCTCTAGGCGCCGGCCGAATTCATGTACCCATACGATGTGCCTGACTATGCTGAAGATAGACTCCACATGG	CCGGTCGACCGGTTACCGCCGTTTGGGGAGGATGAG

2.3. *Semi-quantitative RT-PCR and qRT-PCR*

The total RNA was extracted with Trizol buffer following the kit protocol. 1 μg of total RNA was used to synthesize the first strand of cDNA with the kit (Promega, USA). The primers for amplifying the expression of mouse Hsp25 and αB-crystallin are: forward: 5'-CAG-GACGAACATGGCTACA-3', reverse: 5'-AGAGCGCACAGATTGACAG-3 (for Hsp25) and Forward 5'-AAGAACGCCAGGACGAACAT-3', reverse 5'- GAGAGGATCCACATCGGCTG-3' (for αB-crystallin). 18S RNA was used as the internal control. For the semi-quantitative PCR, the samples were pre-denatured at 94 °C for 3 min and then subjected to 25 cycles of 94 °C 30s, 56 °C 30s and 72 °C 50s. The PCR products were separated on an agarose gel. For the qPCR, the samples were mixed with SYBR green and run on the qPCR machine. The sample values were generated against the standard curve created by the same gene primer pair and normalized to the values of GAPDH mRNA. The data is presented as relative fold change with respect to the controls. The Student t-tests performed account for three independent experiments.

2.4. *Immunofluorescence staining*

For immunofluorescence staining, cells were grown on coverslips and fixed in 3.7% paraformaldehyde for 20 min. The cells were washed with PBST buffer and permeabilized with 0.2% NP-40 buffer for 5 min. After being blocked in 5% BSA buffer for 1hr, the cells were incubated with primary antibody for 1 h and Alexa fluor-secondary antibody for 1hr. The nucleus was stained with Dapi. The fluorescent signals were photographed with the fluorescence microscope Zeiss 540.

2.5. *The luciferase assay*

HEK293 cells were transiently co-transfected with pGL-p21-luciferase and pcDNA-renilla or pGL-p21-luciferase and pcDNA-renilla together with p3xFlag-HSF4b, pcDNA-HA-HCS or p3Flag-HSF4b plus pcDNA-HA-HCS respectively. Luciferase and renilla activities were measured and calculated following the manufacturer's protocol (Promega, WI, USA). The graph bar represent data from three independent repeats.

3. Results

3.1. *Biotin down-regulates HSF4-controlled HSP25 and αB-crystallin expression*

HSF4 plays a critical role during postnatal lens development by regulating a number of genes including HSP25 and αB-crystallin, and its transcription activity is modulated by the posttranslational modification. Biotin has been shown to inhibit gene expression through biotinylating of Histones. To determine whether biotin regulates HSF4's activity, we treated mouse lens epithelial cell line mLEC cells with 50 μM biotin for 24 h. The immunoblotting results showed that biotin reduced HSP25 and αB-crystallin protein expression (Fig. 1, A, lanes 1 and 2). To further determine whether the biotin induced inhibition of HSP25 and αB-crystallin is mediated through HSF4b, mELC/Hsf4−/− and mLEC/HA-Hsf4b cells (mLEC/Hsf4−/− cells were reconstituted by infection of recombinant retrovirus expressing HA-Hsf4b) [10] were incubated with media containing 50 μM biotin for 24 h. The immunoblotting results showed that no detectable HSP25 and αB-crystallin proteins in mLEC/Hsf4−/− cells regardless of biotin-treatment (Fig. 1, B, lanes 1 and 2). Ectopic HA-HSF4 upregulated HSP25 and αB-crystallin expression (Fig. 1, B, lane 3), but this upregulation is significantly attenuated in the presence of biotin (Fig. 1, B, lanes 3 and 4, and Fig. 1C). The qPCR results showed that biotin downregulated the HSF4b-controlled mRNA expression of HSP25 and αB-crystallin (Fig. 1, D). Moreover, we found ectopic expression Flag-HSF4b in HEK293 cells increased HSP25 and αB-crystallin protein expression (Fig. 1, E, lane 1 and 3). Administration

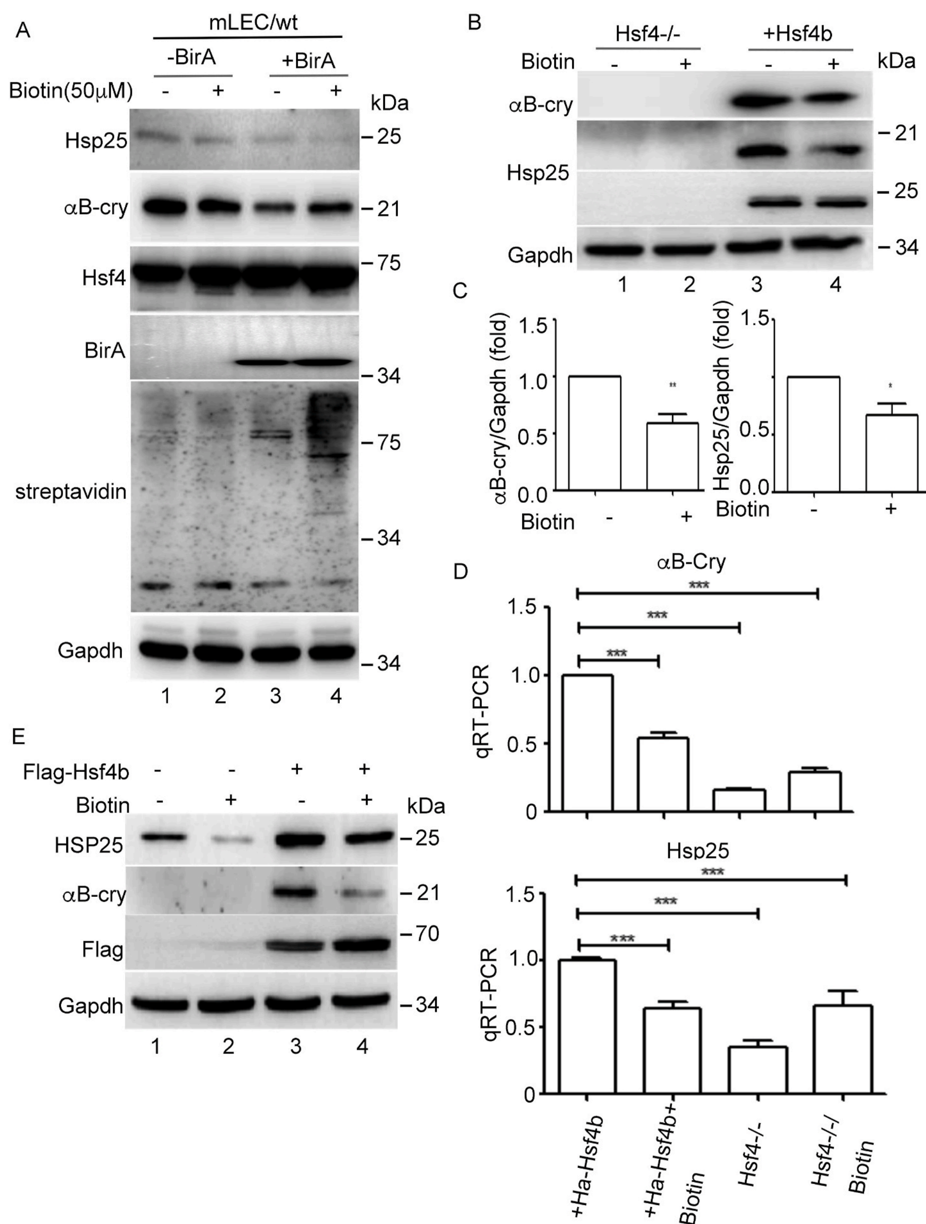

Fig. 1. Biotin downregulates HSF4-controlled HSP25 and αB-crystallin expression. A, immunoblot of the expression of HSP25, αB-crystallin, HSF4, HA-BirA, biotinylated proteins and GAPDH in mLEC/wt (lanes 1 and 2) and mLEC/wt/HA-BirA cells (lanes 3 and 4) that were treated with media containing PBS(sham, lanes 1 and 3) or 50 μM Biotin (lanes 2 and 4) for 24 h. B, immunoblot of HSP25 and αB-crystallin proteins in mLEC/Hsf4−/− and mLEC/HA-Hsf4 cells that were treated with media containing PBS (sham, lanes 1 and 3) and 50 μM biotin (lanes 2 and 4) for 24 h. C, relative quantitation of HSP25 and αB-crystallin expression in B by measuring the densitometry of HSP25, αB-crystallin and GAPDH using the Image J software. The bar graph represents data from three independent experiments (mean ± SD). The unpaired 2-tailed *t*-test was used for statistical analysis. *P < 0.05, **, p < 0.01. D. qPCR to measure the mRNA expression of αB-crystallin and HSP25 in mELC/Hsf4−/− and mELC/HA-Hsf4b cells that were treated with or without Biotin. The bar graph represents the data from four repeated experiments (n = 4). The unpaired 2-tailed t-test was used for statistical analysis, *P < 0.05, **P < 0.01. E. immunoblot of the expression of HSP25, αB-crystallin (αB-cry), Flag-HSF4, GAPDH in ectopic Flag-HSF4 expressed HEK293 cells that were treated with sham (PBS) and 50 mM Biotin for 24 h.

of biotin attenuated HSP25 expression in HEK293 cells (Fig. 1, E, lane 2) and HSF4-mediated induction of HSP25 and αB-crystallin (Fig. 1, E, lanes 3 and 4). These results suggested that ectopic biotin down-regulated HSF4b-mediated HSP25 and αB-crystallin expression at both mRNA and protein levels.

Biotin regulates gene expression through protein biotinylation. The bacterial BirA is a protein biotin ligase that has been used to study the protein biotinylation in mammal tissues. To determine whether increase biotinylation by ectopic BirA affects HSP25 and αB-crystallin expression, we transiently transfected a construct expressing HA-BirA into mLEC cells followed by treatment with media containing sham (PBS) or 50 μM biotin for 24 h (Fig. 1, A, lanes 3 and 4). The ectopic BirA, which increased the biotinylation, down-regulated HSP25 and αB-crystallin, and this downregulation was enhanced by biotin. Taken together, these results suggested that the biotin-mediated downregulation of HSP25 and αB-crystallin expression is associated with HSF4.

3.2. HSF4b is regulated by biotinylation

Because HSF4b is involved in the biotin-mediated reduction of HSP25 and aB-crystallin expression, we postulated that HSF4b is regulated by biotinylation. Streptavidin is a bacterial peptide that specifically binds to biotin residues with high affinity, and has been used to detect biotin-modified proteins, DNA and RNA [24]. To determine whether HSF4b is modified by biotin, we ectopically expressed pcDNA-HA-BirA and p3xflag-Hsf4b in HLE-B3 (human lens epithelial cell line) cells individually or in combination. The cells were then treated with media containing sham (PBS) or 50 μM biotin for 24 h. The cell lysates were incubated with and precipitated by streptavidin-conjugated beads followed by immunoblotting with antibodies against Flag-Hsf4b, HA-BirA and GAPDH. The results showed that streptavidin weakly co-precipitated Flag-Hsf4b in the cells expressing Flag-Hsf4b alone (Fig. 2, A, lane 7), and biotin increased the amount of coprecipitated Flag-Hsf4b (Fig. 2, A, lane 8). In the cells co-expressing both Flag-Hsf4b and Ha-birA, Biotin significantly enhanced the streptavidin-precipitated Flag-Hsf4b protein (Fig. 2, A,

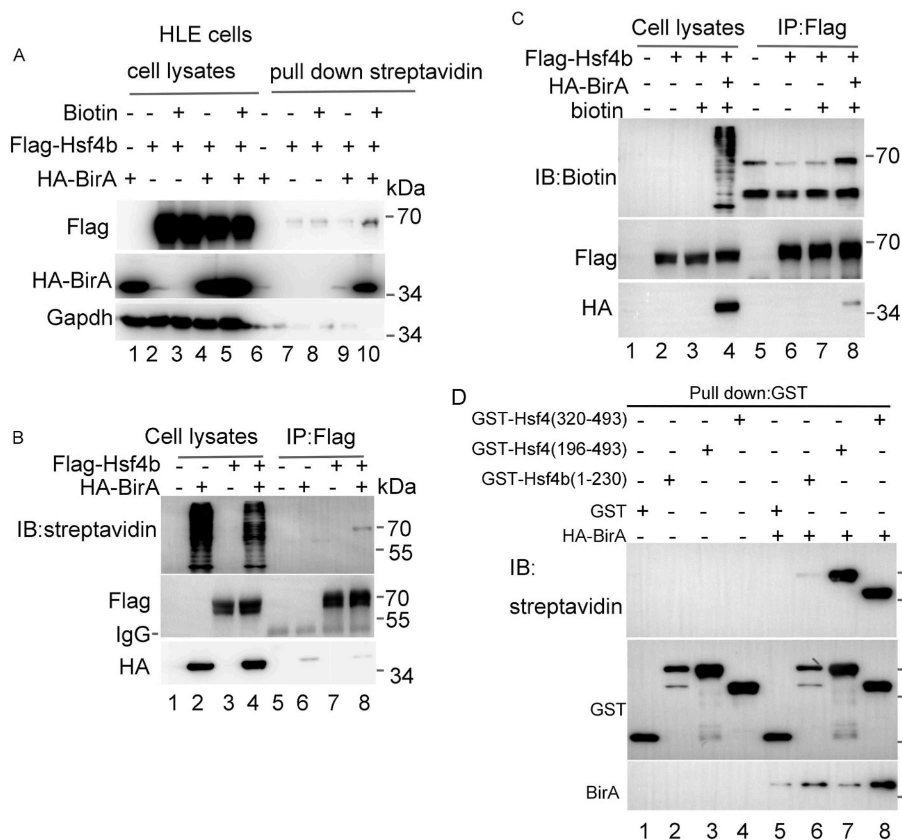

Fig. 2. HSF4 is regulated by biotinylation. A, streptavidin pull down assay. HLE-B3 cells were transiently transfected with p3x-flag vector (lane 1), p3xFlag-Hsf4b (lanes 2, 3, 4 and 5) and HA-BirA (lane 4 and 5). The cells were treated with biotin for 24 h. The cell lysates were pulled down with streptavidin-beads followed by immunoblotting with antibodies against Flag-HSF4b, HA-BirA and GAPDH (lanes 6–10). The expression of Flag-Hsf4b, HA-BirA and GAPDH was immunoblotted (lanes 1–5). B, immunoprecipitation assay to determine HSF4 biotinylation. HLE-B3 cells were transiently transfected with Flag-Hsf4b and HA-BirA. Flag-Hsf4b was immunoprecipitated with antibody against Flag followed by immunoblotting with antibodies against streptavidin-HRP, Flag-HSF4b and HA-BirA (lanes 5–8). The expression of Flag-HSF4b, HA-BirA and GAPDH in cell lysates were immunoblotted (lanes 1–4). C, immunoprecipitation assay. The cells were transfected with the plasmids in B, and then treated with biotin for 24 h. Flag-Hsf4b was immunoprecipitated followed by immunoblotting with antibody against Biotin. D. GST-pull down assay to determine the biotinylating amino acids in HSF4b protein. HLE-B3 cells were cotransfected with HA-BirA in combination with pEBG, pEBG-Hsf4(1–230), pEBG-Hsf4 (196–493) or pEBG-Hsf4 (320–493). The GST and GST-Hsf4b mutants were pull down by glutathione-Sepharose beads followed by immunoblotting with antibodies against streptavidin-HRP, GST and HA-BirA. Lanes (1–4): cell lysates; lanes 5–8: pull down assay.

lanes 9 and 10). No Flag-Hsf4b was coprecipitated by streptavidin-conjugated beads in the cells expressing HA-BirA alone (Fig. 2, A, lane 6). HA-BirA but not GAPDH was co-precipitated with streptavidin-beads (Fig. 2, A, lanes 9 and 10). The ectopic expression of Flag-Hsf4b and HA-BirA in the cell lysates were immunoblotted (Fig. 2, A, lanes 1–5). These results suggested that HSF4b is either directly modified by biotin or is associated with biotinylated proteins. To further determine whether HSF4b is directly modified by biotin, we performed an immunoprecipitation assay. HLE-B3 cells were transiently co-transfected p3xflag-Hsf4b together with either empty vector or pcDNA-HA-BirA, and the cells were treated with media containing 50 μM biotin for 24 h. The Flag-Hsf4b proteins were immunoprecipitated with anti-flag antibodies followed by immunoblotting with HRP-conjugated streptavidin, which specifically recognizes biotin residues. The results showed that the precipitated Flag-Hsf4b proteins were recognized by streptavidin-HRP in the cells expressing both Flag-Hsf4b and HA-BirA (Fig. 2, B, lane 8), but not expressing Flag-Hsf4b or HA-BirA alone (Fig. 2, B, lanes 6 and 7). Ectopic HA-BirA catalyzed the biotinylation of multiple proteins in HLE-B3 cells (Fig. 2, B, lanes 2 and 4). Taken together, we proposed that HSF4 could be modified by biotinylation directly or interacted with other biotinylated protein (Fig. 2, A). To further confirm HSF4b' biotinylation, we used anti-biotin antibody to probe the immunoprecipitated Flag-Hsf4b protein in the cells expressing both HA-BirA and Flag-Hsf4b that were treated with or without biotin. The results showed that Biotin antibody reacted with the immunoprecipitated Flag-HSF4b in HA-BirA-expressed cells but not with that in cells expressed Flag-Hsf4b alone (Fig. 2, C, lanes 7 and 8). Biotin antibody reacted with an unspecific proteins coprecipitated with anti-Flag-HSF4b antibody (Fig. 2, C, lanes 5–8). We tested several anti-biotin antibodies in this study, all of them produced unspecific bands (Fig. 2, C, lanes 5–8). The amount of biotinylated HSF4b protein was much less than the total amount of immunoprecipitated HSF4b protein (Fig. 2B and C, lane 8, the upper and middle panels). These results implied that HSF4 is modified partially by biotinylation.

HSF4 protein is functionally divided into the DNA binding domain, N-terminal hydrophobic region, regulatory domain, and C-terminal transactivation domain [4]. To determine the location of biotinylated lysine residues in HSF4b proteins, the constructs expressing GST or GST-Hsf4 mutants were transiently transfected (Fig. 2, D) into HLE-B3 cells alone (Fig. 2, D, lanes 1–4) or together with HA-BirA (Fig. 2, D, lanes 5–8). The GST and GST-Hsf4 mutants were first precipitated and then followed by immunoblotting with streptavidin-HRP. The results showed that HA-BirA increases the biotinylation of GST-Hsf4(196–493) and GST-Hsf4(320–493), but not GST-Hsf4b (1–230) protein or GST protein alone. No biotinylation was detected in GST or GST-Hsf4b mutants when they were expressed alone (Fig. 2, D, lanes 1–4). Interestingly, HA-BirA was coprecipitated with GST-Hsf4b (1–230), GST-Hsf4b (196–493) or GST-Hsf4b (320–493) (Fig. 2, D, lanes 5–8, low panel). These results suggested that HSF4's C-terminal sequences from amino acids 196 to 493 contains biotinylating amino acid residues.

3.3. *HSF4b interacts with and is regulated by holocarboxylase synthetase*

Holocarboxylase synthetase (HCS) is a unique enzyme catalyzing biotinylation in mammalian tissues. We further studied whether HCS is involved in regulating HSF4's transcriptional activity through biotinylation. To do this, we ectopically expressed HA-HCS and Flag-HSF4b individually or in combination in HEK293 cells. We used HEK293 cells here because of high transfection efficiency and Hsf4b's could uniquely upregulation of αB-crystallin expression (Fig. 1, E). Here we found that ectopic Flag-HSF4b induced αB-crystallin protein expression (Fig. 3, A, lane 1), which is consistent with the results of Fig. 1E, but HA-HCS did not (Fig. 3, A, lane 2). However, ectopic HA-HCS attenuated Flag-HSF4b-induced αB-crystallin expression (Fig. 3, A, lane 3, and low panel). Results of the luciferase assay showed that HA-HSF4b increased the promoter activity of αB-crystallin gene by 3 fold in HEK293T cells

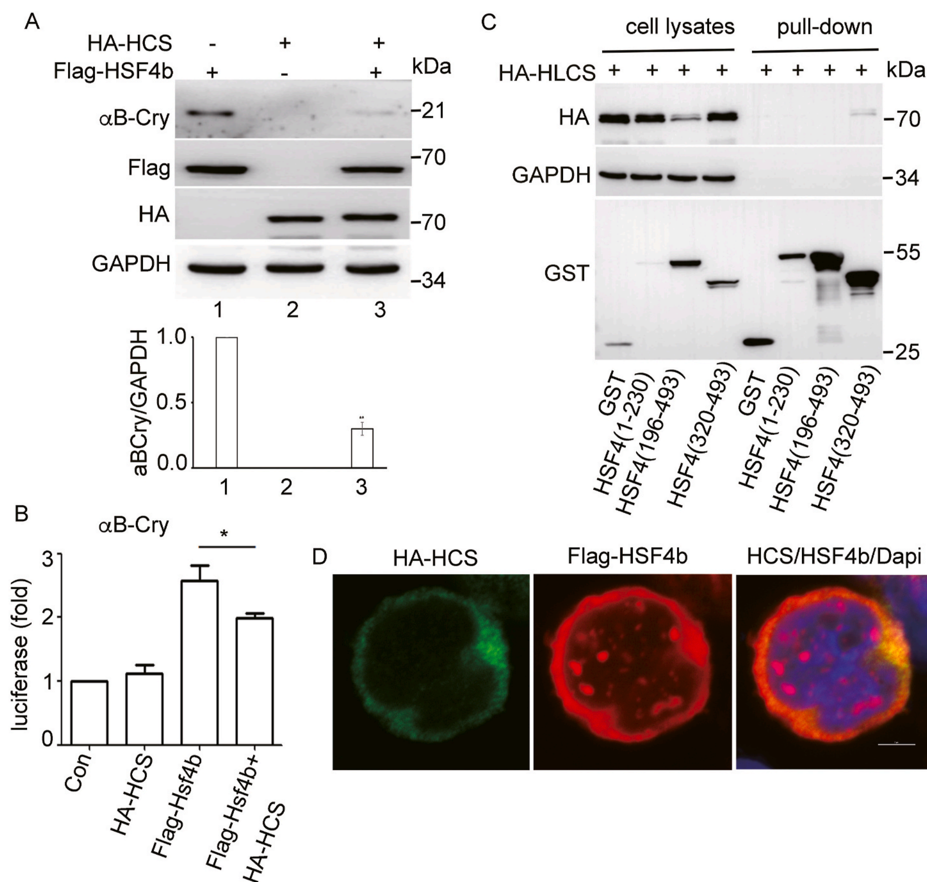

Fig. 3. HSF4b interacts with and regulated by Holocarboxylase synthetase in HEK 293 cells. A, immunoblot of the expression of αB-crystallin, ectopic HA-HCS, Flag-Hsf4b and GAPDH in HEK293 cells expressing Flag-Hsf4 (lane 1), HA-BirA (lane 2) and Flag-Hsf4b and HA-BirA (lanes 3). The low bar graph is the quantitation of αB-crystallin densitometry normalized by that of GAPDH in A. The data were mean ± SD (n = 3). The unpaired 2-tailed *t*-test was used for statistical analysis. *<0.05. B, luciferase assay to determine the biotin's regulation on Hsf4 transcription activity. The αB-crystallin promoter-drived luciferase was transiently transfected into HEK293 cells together with HA-HCS, Flag-Hsf4b or Flag-Hsf4b + HA-HCS. The construct expressing Renilla luciferase was used as internal control. The data were mean ± SD (n = 4). The unpaired 2-tailed *t*-test was used for statistical analysis. C, GST-pull-down to determine the interaction between HA-HCS and HSF4b. HEK293 cells were transfected with vector expressing HA-HCS together with GST, GST-HSF4 (1–230), GST-HSF4(196–493) or GST-HSF4 (320–493). The expression of HA-HCS, GAPDH, GST and GST-Hsf4 mutants in cell lysates (lanes 1–4) and pull-down fractions (lanes 5–8) were immunoblotted. D, immunofluorescence assay to determine the colocalization of Hsf4b and HCS. Flag-Hsf4b and HA-HCS were expressed in HEK 293 cells followed by immunochemistry staining.

(Fig. 3, B) and this increase was attenuated by ectopic HA-HCS (Fig. 3, B). To determine if HSF4 interacts with HCS, we performed a GST pull down assay. The plasmid expressing HA-HCS was transiently co-transfected together with GST, GST-Hsf4b (1–230), GST-Hsf4b (196–493) or GST-Hsf4b (320–493) into HEK293 cells. The results showed that HA-HCS protein was precipitated by GST-(320–493), but not by GST alone and nor by GST-Hsf4b (1–230) and GST-Hsf4b (196–493 (Fig. 3, C). The lack of interaction between HA-HCS and GST-Hsf4b (196–493) may be due to improper conformation of GST-Hsf4(196–493) or the transient nature of the interaction between HSF4 and HCS. The immunofluorescence staining results showed that HA-HCS was expressed predominantly in the cytoplasm. HSF4 was in both the cytoplasm and nucleus. HA-HCS and Flag-HSF4b partially colocalized in the cytoplasmic fraction (Fig. 3, D). We failed to detect an interaction between endogenous HCS and HSF4 in lens epithelial cells by using immunoprecipitation assay. This may implied that HSF4 might transiently interact with HCS in lens epithelial cells. The above results suggested that ectopic HCS can transiently interact with HSF4b and down-regulate HSF4b-mediated αB-crystallin expression.

3.4. Lysine (K)444 is a potential biotinylating amino acid in HSF4b

Protein biotinylation usually occurs on the epsilon amine of lysine. There are a number of lysine residues in HSF4 peptide sequences with unknown function. The results in Fig. 2 implied that biotinylation may occur in the C-terminal region of HSF4 from amino acids 320 to 493. To determine the biotinylated amino acid, we performed a site-mutation assay by using the p3xflag-Hsf4b construct as a template, and the amino acids K207/K209, K225, K288, K294, K335, K435, K444 were mutated to arginine (R) respectively (Fig. 4, A). Exception of K435R, these HSF4b mutants did not alter HSF4 expression (Fig. 4B and C). Ectopic Flag-Hsf4b/K435R predominantly expressed a 20 kDa fragment in addition to a weak full-length Hsf4b (68 kDa) (Fig. 5C, lanes 2 and 3 to lane 3). Hsf4b/K435R was largely cytoplasmic (Fig. 4E), and lost its ability to regulate aB-crystallin expression (data not showed). These results suggested that K435 is involved in regulating HSF4b protein stability, but underlying mechanism remains unclear. Compared to wild-type HSF4b, mutation of HSF4 at K207R/K209R, K225R, K288R and K355R weakened HSF4's regulation on aB-crystallin expression (Fig. 4, D, lanes 2–5, and Fig. 5B and C), HSF4/K294R mutation increased the αB-crystallin expression, and HSF4b/K444R weakly upregulated αB-crystallin expression (Fig. 4, D, lanes 6 and 8), when they were expressed in HEK293 cells. The immunofluorescence results indicated that Flag-Hsf4b is predominantly localized in the nucleus as scattered granules. Mutation K255R, K288R and K294R and K355R did not affect HSF4's nuclear localization and granule formation. But mutations K207R/K209R blocked Hsf4's nuclear localization, suggesting K207/K209 is an important component of the nuclear translocation signal. HSF4 with the K444R mutation localized in the nucleus but did not form granules (Fig. 4, E). Moreover, Biotin does not change HSF4's nuclear localization (Fig. 4, F). These results indicated that the different lysine residues play distinct roles on HSF4b's transcriptional regulation.

To determine which lysine residue is biotinylated, HEK293 cells were transiently transfected with constructs expressing Flag-Hsf4b or Flag-Hsf4b mutants followed by streptavidin-mediated co-precipitation. The results of the pull down assay indicated that streptavidin-beads can co-precipitate equal amounts of Flag-Hsf4b/wt, HSF4b/K255R, HSF4b/K288R, and HSF4b/K355R (Fig. 5, A, upper panel). Mutations K207R/K209R increased the amount of coprecipitated HSF4b protein (Fig. 5, A, lane 3). K444R mutation reduced streptavidin-precipitated HSF4b (Fig. 5, A, lane 8). The expression of Flag-Hsf4b mutants used for streptavidin-pull down assay were immunoblotted in Fig. 5, B, upper panel). These Flag-Hsf4b mutants exhibited distinct regulation on aB-crystallin expression (Fig. 5B and C), which is consistent with the

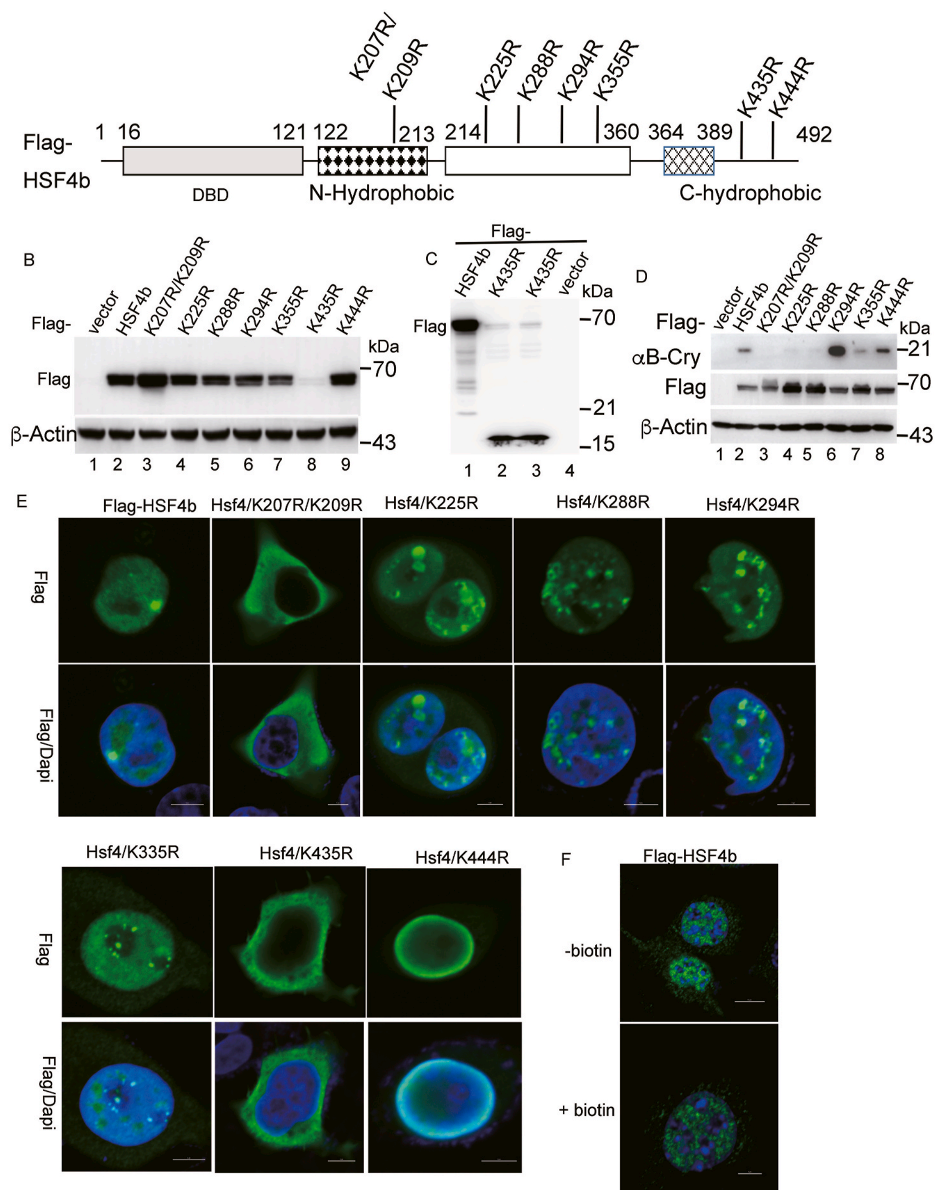

Fig. 4. The regulation of HSF4 transcription activity by different lysine residues. A, schematic map of lysine mutations in HSF4b protein. B and C, immunoblot of the ectopic expression of Flag-Hsf4b and Flag-Hsf4b mutants in HEK293T cells. D, immunoblot of the expression of αB-crystallin, Flag-Hsf4/Hsf4 mutants and GAPDH in HEK293T cells that express empty vector (lane 1), Flag-Hsf4b (lane 2), Flag-Hsf4b/K207R/K209R (lane 3), Flag-Hsf4b/K225R (lane 4), Flag-Hsf4b/K288R (lane 5), Flag-Hsf4b/K294R (lane 6), Flag-Hsf4b/K255R (lane 7), Flag-Hsf4b/K444R (lane 8). E, the immunochemistry staining of the expression of Flag-Hsf4b or Flag-Hsf4b/mutants in HLE-B3 cells. The cell nuclei were stained with Dapi. The scale bar is 10 μm. F, immunochemistry staining of Flag-HSF4b in HLE-B3 cells that were treated with or without Biotin. The scale bar is 10 μm.

results of Fig. 4, D. Since biotin increased HSF4b biotinylation (Fig. 2), we further treated the cells expressing Flag-Hsf4b or Flag-Hsf4b/K444R with Biotin for 24 h followed by streptavidin beads precipitation. The results showed that streptavidin-beads co-precipitated Flag-HSF4b protein and this interaction is enhanced in presence of biotin (Fig. 5, D, lanes 9 and 10). Mutation of Hsf4/K444R reduced the interaction of HSF4 with streptavidin-beads regardless of Biotin (Fig. 5, D, lanes 11 and 12). Biotin did not alter the ectopic expression of Flag-HSF4b and Flag-HSF4b/K444R (Fig. 5, D, lanes 3, 4, 5 and 6). Taken together, these data suggested that K444 is a potential lysine residue that undergoes biotinylation in HSF4b.

4. Discussion

HSF4b is a key regulator of postnatal lens development, and its transcription activity is developmentally regulated [7]. In this paper, we found that biotin partially down-regulates HSF4 transcriptional activity. Exogenously increasing biotin concentration in the cell culture media or ectopic expression of bacterial protein biotin ligase BirA downregulates HSF4b-controlled HSP25 and αB-crystallin expression at both mRNA and protein levels. HSF4 interacts with holocarboxylase synthetase (HCS), and ectopic expression of HCS down-regulates HSF4-controlled αB-crystallin expression. Biotin regulates HSF4 through biotinylation. Mutation analysis indicates that HSF4b/K444 is a potential lysine residue for biotinylation. This is the first report to uncover that biotin, or vitamin H or B7, can modulate HSF4's transcriptional activity.

HSF4 exerts diverse transcriptional regulation during lens development [6,7]. It activates the expression of small heat shock proteins (e.g., HSP25 and αB-crystallin) and simultaneously inhibits the expression of vimentin, FGF and HSP70 in lens epithelial cells [7,25,26]. Post-translational modifications, such as phosphorylation and sumoylation, play important roles on HSF4's divergent transcriptional activities [15]. The phosphorylation of S299 is responsible for tuning down HSF4 transcriptional activity [10]. The EGF-EGFR-ERK1/2 pathway is able to phosphorylate K299, resulting in the downregulation of αB-crystallin and HSP25 expression in mouse lens [11]. The amino acids S299 and K294 make up the PDSM motif in HSF4b, and the phosphorylation of S299 can trigger K294 sumoylation in vitro [15]. Mutations S299A and K294R in HSF4b lead to upregulation of the HSE-Gal4 reporter in Cos-7 cells [15] and HSP25 expression in lens epithelial cell line [11]. These

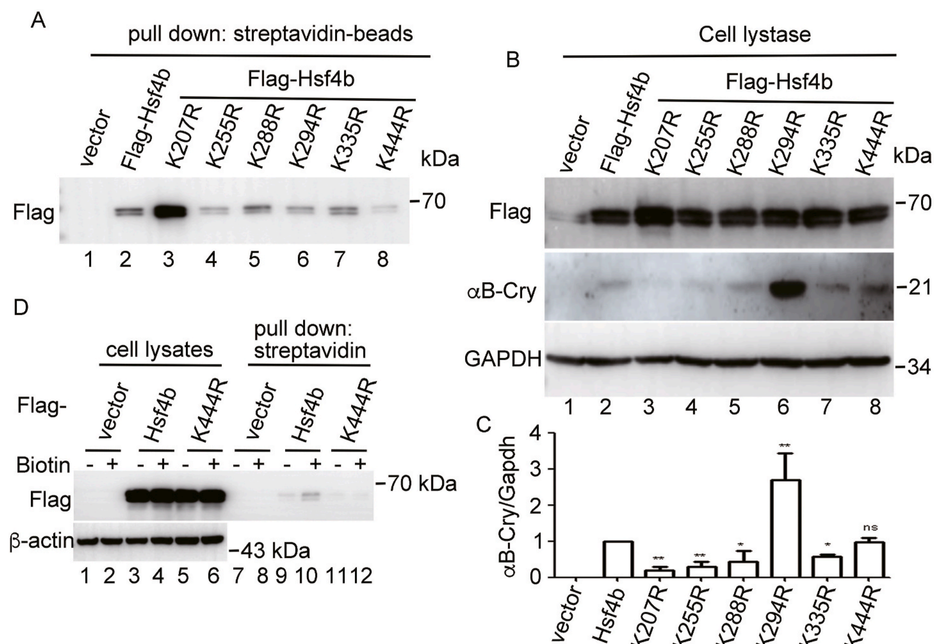

Fig. 5. The interaction between HSF4 lysine mutants and streptavidin beads. A, pulldown assay to identify biotinylating lysine residues in HSF4b protein. HEK293 cells were transfected with plasmids expressing Flag-Hsf4b or Flag-HSF4b mutants. The cells were pull down with streptavidin-magnetic beads followed by immunoblotting with antibody against Flag. B, immunoblot of the expression of Flag-Hsf4b and mutants, αB-crystallin and GAPDH in cell lysates that were used in A. C, densitometry quantitation of αB-crystallin expression in B. The densitometry of αB-crystallin were normalized by that of GAPDH. The fold induction was calculated by dividing the normalized αB-crystallin value in Hsf4b/mutants groups by that in Hsf4b/wt group. The unpaired 2 tailed *t*-test was used for statistical analysis. Data were mean ± SD ($n = 3$). *<0.05, **<0.01, ns, no statistical significance. D, Streptavidin pulldown assay demonstrating Hsf4b/K444 as the biotinylated residue in Hsf4b protein. HEK293T cells that expressed p3xFlag vector, p3xFlag-Hsf4b and p3xFlag-Hsf4b/K444R were treated with media containing PBS (sham, lanes 1, 3, 5,7,9 and 11) or Biotin (lanes 2, 4, 6, 8, 12). The cell lysates were incubated and pulled down with streptavidin-beads followed by immunoblotting with antibody against Flag (lanes 7–12). The expression of Flag-Hsf4b, Flag-Hsf4b/K444R and β-actin were immunoblotted (lanes 1–6).

studies demonstrate that posttranslational modification of K299 down-regulates HSF4's activity. Consistently, the results in Fig. 4A indicate that mutation K294R in HSF4b enhance αB-crystallin expression compared to HSF4b/wt, but this mutation does not alter HSF4's interaction with streptavidin-beads (Fig. 5, A). These results suggest that K294 is not a biotinylation site. Using the GST-pull down assay, we narrowed down the biotinylating lysine residues to HSF4's C-terminus from amino acids of 320–493 (Fig. 2D). Using lysine mutation screening assay, we found that mutation of K444 to R reduces HSF4's binding to streptavidin beads (Figs. 4 and 5) without affecting αB-crystallin expression. These results suggest that HSF4b/K444 is a potential biotinylating lysine residue, and biotin residue of biotinylated K444 of HSF4b may be important for biotin-induced reduction of αB-crystallin expression. In addition, we found that mutations of other lysine residues such as K207R/K209R, K255R, K288R, K294R, K355R do not impact HSF4's binding to streptavidin beads compared to HSF4b wild type (Fig. 5, A), implying that these lysine residues are not regulated by biotinylation. However, these mutations exhibit distinct transcriptional activities. Mutation K207R/K209R blocks HSF4b from entering the nucleus and causes loss of regulation on αB-crystallin expression (Fig. 4, B and 5, B and C), suggesting that K207 and K209 may be the important components of the nuclear translocation signal in HSF4b. Our previous studies showed that HSF4b enters the nucleus by interacting with importin 1beta, and this interaction is regulated by phosphorylation of T472 [10]. Whether K207 and K209 is involved in regulating HSF4's interaction with importin 1beta is still under investigation in our lab. In addition, mutations K255R, K288R and K355R do not disturb HSF4b's nuclear localization, but reduce to some extent the transcriptional activity compared to wild-type HSF4b (Figs. 4 and 5). We found that ectopic expression of HSF4/K435R generated a predominant protein band about 20 kDa in size and a weak band similar in size to HSF4b/wt in SDS-PAGE gel. This suggest that K435 is involved in regulating HSF4 protein stability, and the mechanism is still under investigation.

Biotinylation has been found to regulate gene expression through modifying Histones [27–29]. Histone biotinylation markers are enriched in repressed loci and repeat regions [30]. Protein biotinylation is catalyzed by HCS, a unique protein biotin ligase in mammal tissues [29]. HCS interacts with and catalyzes the biotinylation of histone lysine N-methyltransferase EHMT1 at K161, and this association is important for methylation of H3K9 an important marker for gene repression [31]. HCS represses gene expression by interacting with transcription repression complexes such as N-Cor and HDAC1 [32]. In *C. elegans*, HCS localizes in the promoter of transcriptionally inactivity HSP70. Upon heat shock, HCS is released from the promoter, allowing for the activation of HSP70 transcription [23]. HCS interacts with and biotinylates extracellular HSP70 at multiple lysine residue enhancing eHSP70 -regulated RANTES expression in HEK293 cells [33]. HCS also suppresses gene expression by interacting with HDAC1, HDAC 2 and HDAC7, and this suppression is independent of its biotin-ligase activity in HepG2 cells [23]. In this manuscript, we report that biotin inhibits the expression of HSP25 and αB-crystallin in both lens epithelial cell lines (mLEC and HLE-B3) and HEK293 cells, and this inhibitory effect is associated with the biotinylation of HSF4b (Figs. 1 and 4). The data shows that HSF4's C-terminal amino acids interact with HCS (Fig. 3), and ectopic expression of HCS reduces HSF4b-controlled αB-crystallin expression (Fig. 4). These results suggest that HCS or HCS-mediated biotinylation is involved in regulating HSF4b's transcriptional activity. Moreover, the results show that only a small amount of HSF4b protein is biotinylated (Fig. 2), suggesting that biotinylation regulates only a subset of HSF4b's functions such as tuning down HSF4b's regulation on αB-crystallin expression (Fig. 5B and C).

In conclusion, we demonstrate that HSF4b transcription activity is regulated in part by biotinylation. Our data revealed that Biotin down-regulates HSF4b-mediated expression of small heat shock proteins such as HSP25 and αB-crystallin through the biotinylation of K444. In addition, the data demonstrate that HSF4b/K207/K209 is involved in regulating HSF4 nuclear translocation and HSF4b/K435 is important for HSF4 protein stability.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101227.

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