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HSF4 regulates DLAD expression and promotes lens de-nucleation

Xiukun Cui [a,1], Lei Wang [a,1], Jing Zhang [a,b,1], Rong Du [c], Shengjie Liao [a], Duanzhuo Li [a], Chang Li [a], Tie Ke [a], David Wan-Cheng Li [d,e], Hua Huang [f], Zhan Yin [b], Zhaohui Tang [a,*], Mugen Liu [a,**]

[a] Key Laboratory of Molecular Biophysics of Ministry of Education, College of Life Science and Technology, Center for Human Genome Research, Huazhong University of Science and Technology, Wuhan, Hubei 430074, PR China
[b] Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei 430072, PR China
[c] Union Hospital, Huazhong University of Science and Technology, Wuhan, Hubei 430022, PR China
[d] Department of Biochemistry & Molecular Biology, College of Medicine, University of Nebraska Medical Center, 985870 Nebraska Medical Center, Omaha, NE 68198-5870, USA
[e] Department of Ophthalmology and Visual Science, College of Medicine, University of Nebraska Medical Center, 985870 Nebraska Medical Center, Omaha, NE 68198-5540, USA
[f] Affiliated Hospital, Hubei University for Nationalities, Enshi, Hubei 445000, PR China

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ABSTRACT

HSF4 mutations lead to both congenital and age-related cataract. The purpose of this study was to explore the mechanism of cataract formation caused by HSF4 mutations. The degradation of nuclear DNA is essential for the lens fiber differentiation. DNase 2β (DLAD) is highly expressed in lens cells, and mice with deficiencies in the DLAD gene develop nuclear cataracts. In this study, we found that HSF4 promoted the expression and DNase activity of DLAD by directly binding to the DLAD promoter. In contrast, HSF4 cataract causative mutations failed to bind to the DLAD promoter, abrogating the expression and DNase activity of DLAD. These results were confirmed by HSF4 knockdown in zebrafish, which led to incomplete de-nucleation of the lens and decreased expression and activity of DLAD. Together, our results suggest that HSF4 exerts its function on lens differentiation via positive regulation of DLAD expression and activity, thus facilitating de-nucleation of lens fiber cells. Our demonstration that HSF4 cataract causative mutations abrogate the induction of DLAD expression reveals a novel molecular mechanism regarding how HSF4 mutations cause cataractogenesis.

1. Introduction

Heat shock transcription factors (HSFs) are major regulators mediating heat shock responses. Upon heat shock or under some stress conditions, these regulators would bind to the heat shock elements (HSEs) of the heat shock protein (HSP) gene promoters, regulating their expressions [1–3]. HSF4, a member belonged to HSF genes, is among the common genes linked to cataractogenesis, and now, it has been considered as a causative gene for congenital cataract. Bu et al. have identified four mutations in the DNA binding domain (A20D, I87V, L115P, and R120C) of HSF4 that cause autosomal dominant cataract [4,5]. We also previously identified a mutation (R74H) in the same domain, which causes congenital autosomal dominant cataract in a Chinese family [6]. Interestingly, several studies mentioned that mutations located in the other domain of HSF4 are responsible for the autosomal recessive cataract [7–9]. In addition, a recent study revealed that HSF4 mutations might also be associated with age-related cataract [10].

Mouse HSF4 (mHSF4) is prominently expressed in the lens compared with in other tissues. In principle, the mHSF4 is initially expressed at embryonic stage (E13.5), and its expression reaches a peak shortly after birth [11]. To investigate the molecular mechanisms by which HSF4 mutations lead to cataract, three groups generated mHSF4 knockout mice, and in all cases, mHSF4 null mice developed cataract phenotypes [11–13]. Histological studies on these cataract mice demonstrated defects in lens development. It was also found that the mHSF4$^{-/-}$ lenses were abnormal, and contained non-degenerated nuclei in the secondary fiber cells of the center regions of the embryonic, young and adult mouse eye lenses. These studies suggested that HSF4 plays a critical role in the lens fiber cell differentiation. At the molecular level, HSF4 seems to regulate lens fiber cell differentiation by modulating expression of some lens structural proteins, such as lens specific crystallins, beaded filament proteins (Bfsp1/2) and FGFs [14,15]. Despite these studies, however, the pathogenic mechanisms regarding how HSF4 mutations lead to cataractogenesis still remain elusive.

During lens development, lens transparency is ensured by the programmed elimination of nuclei and other organelles in lens fiber cells differentiation [16,17]. The degradation of nuclear DNA during lens denucleation is mediated by acid DNase. DNase 2β is encoded by *DLAD* gene and is found to exist as the primary acid DNase in the lens.

* Correspondence to: Z. Tang, Center for Human Genome Research, Huazhong University of Science and Technology, 1037 Luoyu Rd., Wuhan, Hubei 430074, PR China.
** Correspondence to: M. Liu, Key Laboratory of Molecular Biophysics of Ministry of Education, College of Life Science and Technology, Center for Human Genome Research, Huazhong University of Science and Technology, 1037 Luoyu Rd., Wuhan, Hubei 430074, PR China. Tel.: +86 27 87792649; fax: +86 27 87794549.
E-mail addresses: zh_tang@mail.hust.edu.cn (Z. Tang), lium@mail.hust.edu.cn (M. Liu).
[1] These authors contributed equally to this work.

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The DNase 2β expression level increased dramatically during the mouse lens fiber cells differentiation. Targeted knockout of the *DLAD* gene in mice retarded the nuclear DNA degradation of lens and caused cataract formation of nucleus lentis [18–20].

To explore the pathologic mechanism of how HSF4 mutations cause cataract, we conducted HSF4 knockdown by injecting morpholino into zebrafish. Uncompleted degradation of nuclear DNA and decreased DLAD expression were observed in the lens of zebrafish injected with HSF4 morpholino oligos. More importantly, our results demonstrate that HSF4 cataract causative mutations lose the ability to bind to the *DLAD* gene promoter, thus were incapable of promoting DLAD expression and DNase activity. Together, our results may reveal a novel mechanism towards better understanding the cataractogenetic mechanisms caused by HSF4 mutations.

2. Materials and methods

2.1. Plasmids, cell culture, and antibodies

Human lens epithelial cell (HLEC) lines were cultured in Dulbecco's modified Eagle's medium (GBICO) supplemented with 10% fetal calf serum (GBICO). The whole length of HSF4 cDNA was amplified from HLECs by RT-PCR. Wild-type and mutated HSF4 were cloned into pEGFP-N1 plasmids (Invitrogen). HLEC cells were cultured in the plates with 70%–80% confluency for 24 h. The plasmids were mixed with Lipofectamine® 2000 Reagent (0.5 μg/μl) in Opti-MEM® I Reduced Serum Medium (Invitrogen) and added into cells for 4–6 h. Then cells were cultured with DMEM for 36 h. Monoclonal antibody α-tubulin was purchased from Millipore and DLAD antibody from Abcam. Polyclonal antibody HSF4 was purified by Abmart Inc. [21].

2.2. In situ hybridization and promoter functional assay

For in situ hybridization, zebrafish HSF4 (ZHSF4) probes were amplified by the following primers: forward, 5′-tctgcccaaatacttcaaacacaac-3′ and reverse, 5′-tttctgtggatggcgattggatatag-3′. The amplified fragments were cloned into pGEM-T easy vectors, and then were linearized by Sac1 and Sal1. T7 and sp6 polymerases were used to synthesize the antisense and sense probes. Whole mount in situ hybridization was performed as described previously [22]. For promoter function assay, the CMV promoter of pEGFP-N1 plasmid was replaced with ZHSF4 promoter using the following primers: forward, 5′-acgtattaatggctatccaca ttcttcaaagtatc-3′ and reverse, 5′-acgtggatccccccatccacacctatagag-3′. Then the vectors were injected into one- and two-cell stage embryos. To detect the efficiency of ZHSF4 morpholino, the GFP-N1–ZHSF4 ATG vector was constructed with the primer: forward, 5′-aattccaagtccaagtg cagtcatgcaggagaacccaggctctataggtgtggatggg-3′ and reverse, 5′-gatcccc atccacacctatagagcctgggttctcctgcatgactgcacttggacttgg-3′. Then GFP-N1–ZHSF4 ATG vectors were injected into one- and two-cell stage embryos with ZHSF4 MO or control MO.

2.3. Morpholino and histology

Morpholino antisense oligonucleotides (MOs) target ZHSF4 translation start site was designed according to the sequence: ZHSF4 MO: 5′ CTGGGTTCTCCTGCATGACTGCACT 3′ (Gene Tools, Philomath OR). The one and two cell stage embryos were prepared for MO injection (1 nl per embryo at the indicated concentration). For the HE staining, 72 h post-fertilization (hpf) embryos were fixed with 4% paraformaldehyde overnight at 4 °C. After that, the embryos were sequentially embedded in 2% agarose and paraffin. 5 μm sections were cut and stained with hematoxylin–eosin (HE). For DAPI staining, 72 hpf embryos were also fixed in 4% paraformaldehyde at 4 °C overnight. Then the staged embryos were socked with 15% sucrose for several hours and then 30% sucrose for dehydration. 7 μm frozen sections were cut and stained with DAPI.

2.4. Luciferase activity assay and ChIP assays

The DLAD promoter (pGL3–DLAD) was amplified using the primers containing two restriction sites in both ends (MluI and XhoI): forward, 5′-gcatACGCGTtgggcaggtggtacagagtcc-3′; reverse, 5′-gcatCTCGAGggcg ctctcaggctgtgct-3′. The primers for DLAD promoter deletions were as follows: DEL-forward: tacaacaatcagcacaaaggcatcc; DEL-reverse: ctttgt gctgattgttgtatgaaattc; and DEL1-reverse: gcatCTCGAGgggcagggggactt cattttaat. The PCR products were sub-cloned into pGL3-basic (Promega) vectors and verified by direct DNA sequencing. Then the DLAD luciferase reporters were co-transfected with wild-type or mutated HSF4 expression vector into HLECs. Thirty-six hours after transfection, the cell lysates were prepared and analyzed with Dual-Luciferase Assay system (Promega). The relative luciferase activity was normalized with the activity of pRL-TK renilla.

The chromatin-immunoprecipitation-linked PCR (ChIP) assays were conducted as described previously [21]. Cross-linked chromatin was extracted from HLECs with GFP–HSF4 transfected and immunoprecipitated by anti-GFP antibodies. Anticipated HSF4-binding sites in DLAD and HSP70 (positive control) promoters as determined from sequence analysis were examined by the following primers: DLAD (−312 to −56), forward, 5′-ccccctgcccaatgaatttc-3′ and reverse, 5′-ggcgctctcaggctgtgct-3′; DLAD (−1.4 k to −1 k), forward, 5′-gcctggg caacaagagcaaaac-3′ and reverse, 5′-ggactctgtaccacctgcccaatta-3′; and HSP70, forward, 5′-ccgccactccccttcctc-3′ and reverse, 5′-ccgccttttccc ttctgagc-3′.

2.5. Quantitative real time polymerase chain reaction

The quantitative real time polymerase chain reaction (qRT-PCR) was conducted as described previously [21]. HLECs were transfected by earlier mentioned plasmids. The total RNAs were then isolated from various transfected cells, and 1 μg of RNA from each sample was used for RT-PCR measurements. The obtained cDNAs were analyzed with the Applied Biosystems 7500 Real-Time PCR System using the Power SYBR® Green PCR Master Mix (ABI). The assays were performed in triplicate and normalized against beta-actin. Primers were designed using the Primer Express® Software (ABI) and listed below: forward: 5′-gcaggctcctcaccacacttca-3′ and reverse: 5′-caggctgcaaagatgtcgtcaag-3′ for human *DLAD*.

2.6. Protein preparation, Western blot analysis, and statistical analysis

Preparation of total proteins from various types of cells as well as relevant western blot analysis was conducted using the same protocol described in our previous report. The statistical significance ($p < 0.05$) of the comparison among multiple samples was determined by SPSS version 17.0 (SPSS Inc., Chicago, IL) as previously described [23,24].

2.7. DNase activity assay

To measure the DNase activity of DLAD protein (Dnase2β), cell lysates were extracted from ZHSF4 knockdown zebrafish and HLECs that were transfected with wild-type or mutated HSF4 expression vector. Then, 2 μg of plasmid DNA were incubated with the above cell extracts in 300 μl of reaction buffer (10 mM Tris–HCl, 10 mM EDTA, pH 5.7) for 0.5 h at 37 °C. Then the integrity of the digested DNA was examined with 1% agarose gel electrophoresis following a reported method [25].

3. Results

3.1. HSF4 directly binds to the promoter of DLAD

To determine whether the induction of DLAD expression by HSF4 is dependent on binding to the DLAD promoter, luciferase assay was performed. The DLAD promoter fragment (named pGL3–DLAD) was

subcloned into pGL3-basic vector and the reporter constructs were co-transfected with HSF4 expression vector into HLE cells. Results for luciferase activity demonstrated that HSF4 was able to elevate the activity of the DLAD promoter luciferase reporter (Fig. 1A). As the HSF4 binding sequence was more ambiguous than the classical heat shock element (HSE) composed of the consensus sequence nGAAn, we identified two HSF4 binding elements (from −281 to −230 and from −1183 to −1133, respectively) in the DLAD promoter by TFSEARCH (Fig. 1B) [26]. To further confirm the regulation of the DLAD promoter by HSF4, chromatin immunoprecipitation (ChIP) was applied, and our results support that HSF4 strongly binds to the element from −281 to −230, and to a lesser degree to the element from −1183 to −1133 (Fig. 1B). Then the DLAD promoter luciferase reporters whose HSE was deleted from −282 to −204 and from −300 to −55 (named pGL3–DLAD–DEL and pGL3–DLAD–DEL1, respectively) were constructed. Our results showed that the deletion of such HSE sequence in DLAD promoter obviously decreased the activity of DLAD promoter reporters (Fig. 1C).

We next investigated if HSF4 cataract causative mutations could disrupt the binding ability of wild-type HSF4 to the DLAD promoter. The luciferase reporter pGL3–DLAD was co-transfected with wild-type and mutated HSF4 (A20D, R74H, L115P and R120C) expression vectors into HLEC cells for 36 h. The results obtained from luciferase activity

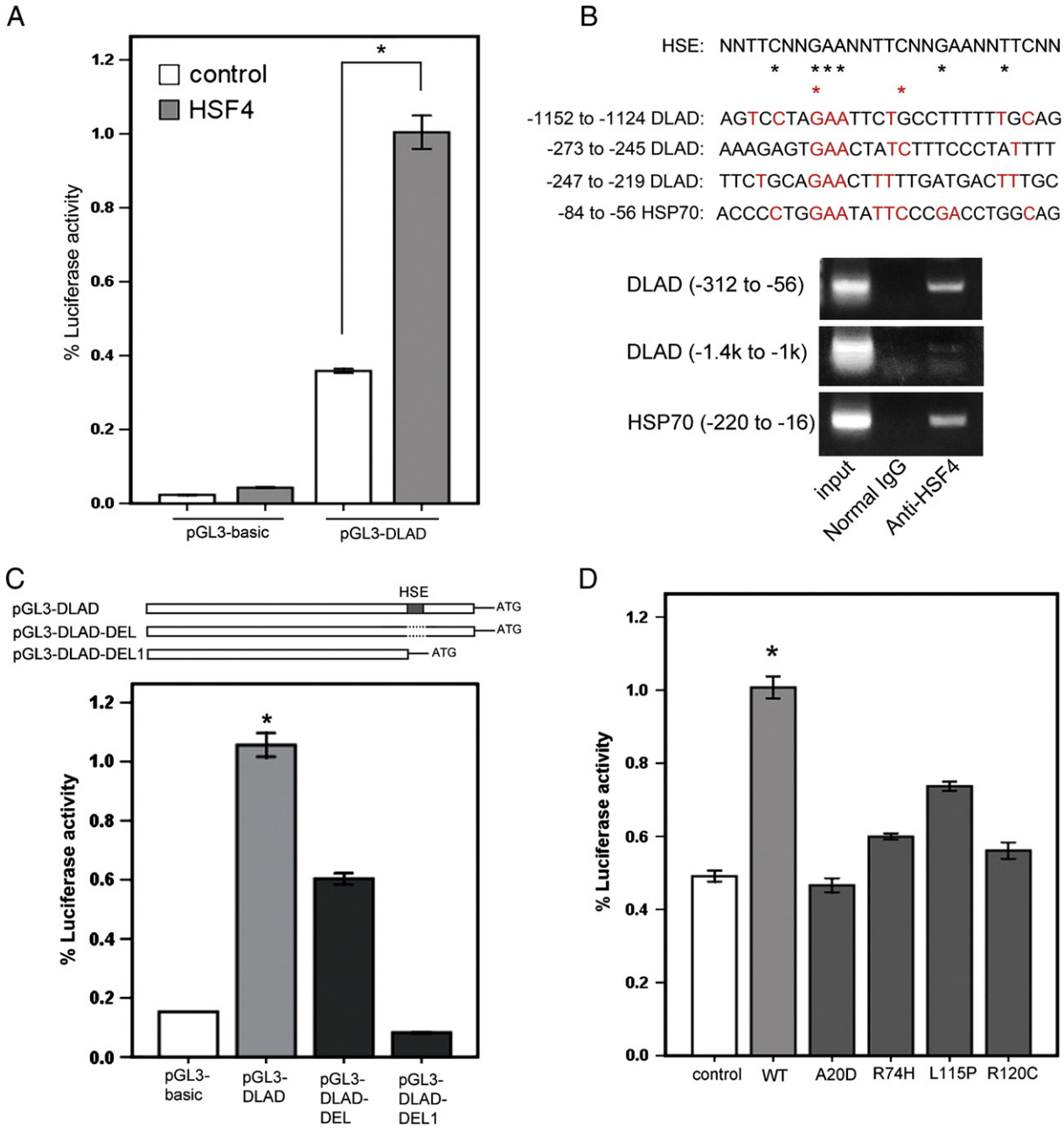

Fig. 1. HSF4 directly binds to the promoter of DLAD. (A) The DLAD promoter luciferase reporters were co-transfected with HSF4 into HLEC cells and the whole cell lysates were prepared 36 h later. (B) Schematic representation of the presumed HSF4 binding elements of DLAD and HSP70. Black asterisks indicate the key nucleotides of standard heat shock element sequence (nGAAn) Red asterisks indicate the key nucleotides essential for HSF4 binding. Nucleotides that matched the HSE sequence are shown in red characters. DLAD promoters were amplified with PCR from Chip-enriched DNA, which was precipitated by anti-HSF4 antibody from HLEC cells. The HSP70 promoter was amplified as positive control. (C) DLAD promoter luciferase reporters with HSE deleted were transfected into HLEC cells with HSF4 and the luciferase activities were measured 36 h later. (D) HSF4 cataract mutations (A20D, R74H, L115P and R120C) were co-transfected into HLEC cells with DLAD promoter reporter (pGL3–DLAD). The luciferase activity was measured 36 h later. Data are the average of three replicate experiments and error bars show +/−1.00 SD. The asterisk indicates a significant difference.

unveiled that HSF4 mutations significantly attenuated the binding activity of HSF4 to DLAD promoter (Fig. 1D).

3.2. HSF4 cataract causative mutations fail to induce the DLAD expression

To explore whether HSF4 cataract causative mutations (A20D, R74H, L115P and R120C) affect the DLAD expression patterns, wild-type and mutated HSF4 expression vectors were transfected into HLEC cells for 36 h. Total RNA was extracted and the DLAD mRNA levels in the two types of cells were compared using qRT-PCR. As shown in Fig. 2A, qRT-PCR examination showed that the mRNA level of DLAD was apparently induced by wild-type HSF4. However, HSF4 cataract causative mutations were incapable of enhancing expression of DLAD mRNA levels (Fig. 2A). Then, immunoblot was performed to examine the DLAD protein (Dnase2β) levels in HSF4 and HSF4 mutations (A20D, R74H, L115P and R120C)-transfected HLEC cells. Our data showed that wild-type HSF4 remarkably activated Dnase2β expression levels while HSF4 mutations failed to do so (Fig. 2B).

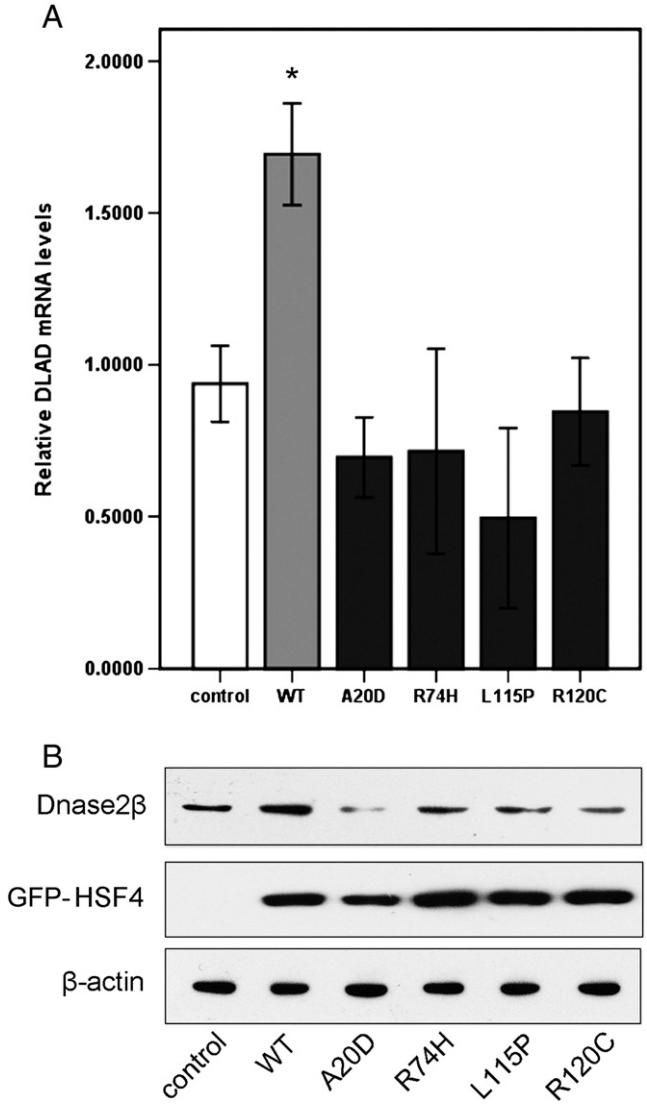

Fig. 2. HSF4 induced the expression of DLAD that could be repressed by HSF4 cataract mutations. (A) HLECs were transfected with wild-type or mutated HSF4 (A20D, R74H, L115P and R120C) and then harvested for preparation of total RNA that were subjected to qRT-PCR to detect DLAD mRNA levels. (B) Detection of HSF4 and DLAD protein levels by western blot analysis in the above clones as described in Fig. 4A. The levels of β-actin were detected as control. Error bars show +/−1.00 SD.

3.3. HSF4 cataract causative mutations decrease the DNase activity of DLAD

Finally we examined if the amounts of Dnase2β regulated by HSF4 and its mutant proteins affect the DNase activity of DLAD. Cell lysates were harvested from HLEC cells transfected with expression vectors of HSF4 and HSF4 cataract mutations (A20D, R74H, L115P and R120C). The cell lysates from the transfected cells were incubated with plasmid DNA under acid conditions. Based on agarose gel electrophoresis analysis, our results demonstrated that the augment of Dnase2β by wild-type HSF4 facilitated the DLAD DNase activity while the HSF4 mutations disrupted the ability (Fig. 3). These observations were consistent with the results that HSF4 mutations reduce the expression levels of DLAD.

3.4. Knockdown of zebrafish HSF4 leads to defects in lens fiber cells de-nucleation

We first determined the distribution of zebrafish HSF4 (ZHSF4) during the lens development. Whole mount in situ hybridization using a ZHSF4 antisense probe and results revealed that expression of zebrafish HSF4 mRNA is restricted to the ocular lens since 24 hpf (Fig. 4A). In the case of promoter function assays, EGFP-N1 plasmids with ZHSF4 promoter were injected into zebrafish embryos. GFP signals were observed and the results showed that ZHSF4 specially expressed in the lens since 24 hpf (Fig. 4B).

We next conducted morphilino oligo (MO)-mediated ZHSF4 knockdown in zebrafish. To test the efficiency of antisense morpholino oligo designed to target translation initiation sites of ZHSF4, the eGFP-N1–ZHSF4 ATG vector was constructed and injected into embryos together with ZHSF4 morpholino. The GFP signals were observed 24 h after Mo-injection. Our data showed that the morpholino suppressed the expression of the ZHSF4 efficiently 24 h after Mo-injection (Fig. 5A). The morpholino oligo-mediated ZHSF4 knockdown led to incomplete lens differentiation, which is reflected by the presence of nuclear DNA in the differentiating fiber cells of both hematoxylin–eosin (HE) and DAPI-stained samples. Our results demonstrate a de-nucleation defect in lens fiber cells of ZHSF4 knockdown fish when compared to the zebrafish injected with negative control morpholino (Fig. 5B).

3.5. Knockdown of ZHSF4 in zebrafish decreased expression levels and DNase activity of ZDLAD

To explore the molecular event mediating the de-nucleation defect, immunoblot was carried out to detect the expression of zebrafish DLAD (zDnase2β, ZDLAD). As shown in Fig. 6A, the expression level of ZDLAD was significantly decreased in ZHSF4 knockdown animals. Meanwhile, cell lysates were prepared from ZHSF4 knockdown zebrafish for DNase activity assay. Our results showed that knockdown of ZHSF4 resulted in a decrease of DNase activity of ZDLAD (Fig. 6B).

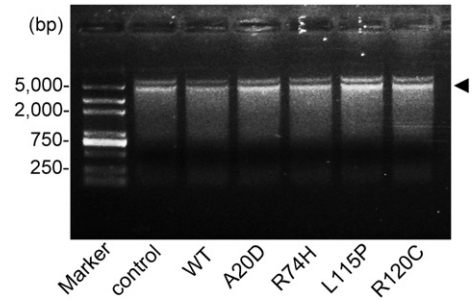

Fig. 3. HSF4 mutations attenuate the DNase activity of DLAD. Wild-type and mutated HSF4 (A20D, R74H, L115P and R120C) were transfected into HLEC cells for 36 h. Cell lysates were extracted and incubated with plasmid DNA under acidic conditions. The black arrow indicates the band of undigested plasmid DNA.

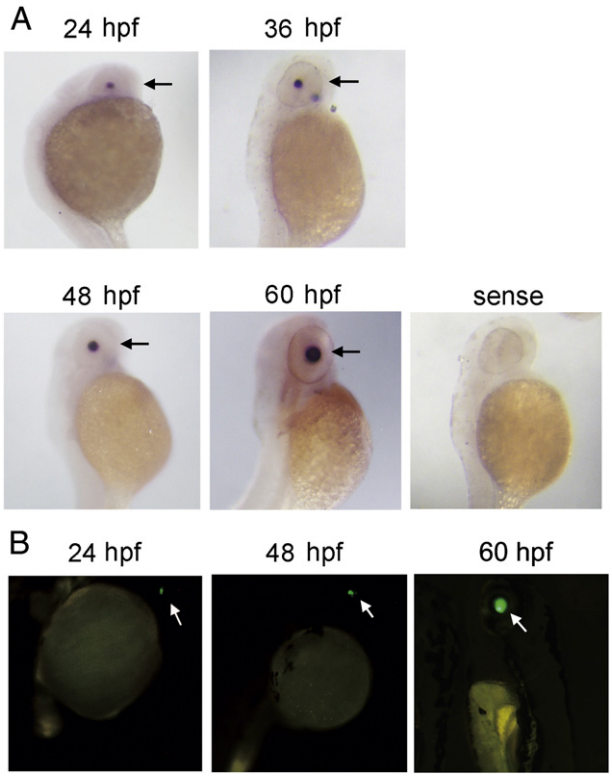

Fig. 4. Zebrafish HSF4 expresses specially in the lens. (A) In situ hybridization analysis of ZHSF4 displayed a special expression pattern since 24 hpf lens. The sense probe against HSF4 was as a negative control. (B) The eGFP-N1 plasmids with ZHSF4 promoter only expressed in zebrafish lens since 24 hpf. CMO, control morphants; HSF4 MO, ZHSF4 morphants.

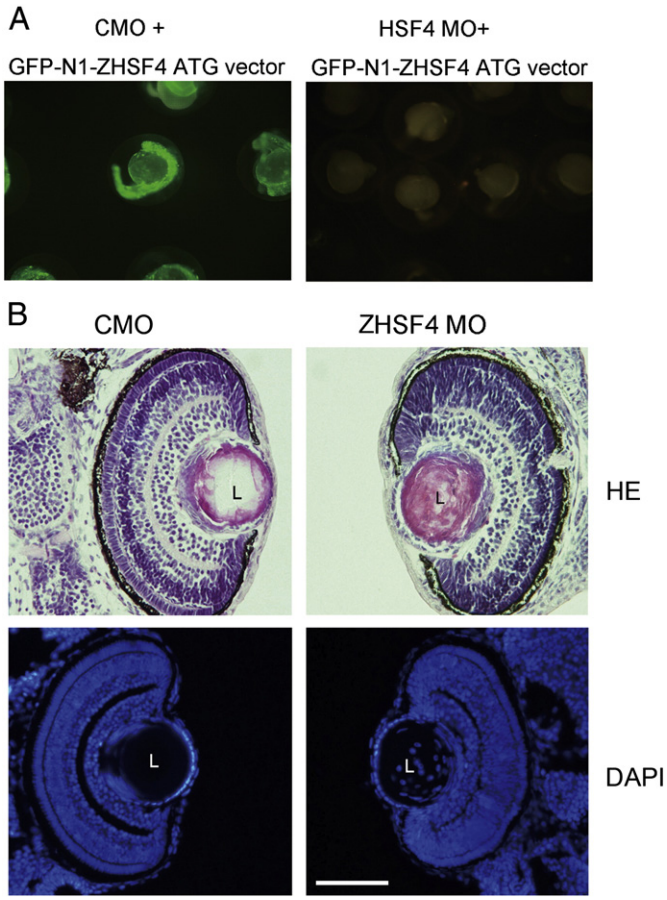

Fig. 5. Defects in lens fiber cells de-nucleation of ZHSF4 morphants. (A) The morpholino can efficiently suppress the expression of ZHSF4. (B) At 72 hpf, lens histology of ZHSF4 and control morphants were performed with hematoxylin–eosin (HE) and DAPI staining. L, lens. CMO, control morphants; HSF4 MO, ZHSF4 morphants.

4. Discussion

Several lines of evidences demonstrate that elaborate controls are necessary for the differentiation from lens epithelial cells into lens fiber cells, which require inhibition of proliferation, cell cycle exit, expression of specific structural proteins and degradation of nuclei and other cellular organelles in the lens fiber cells [27,28].

Brahma-related gene 1(Brg1) encodes the ATPase subunit of SWI/SNF chromosome remodeling complexes, which are implicated in control of cell proliferation. HSF4 can interact with Brg1 during the G1 phase of cell cycles, and thus can regulate the expression of heat shock proteins [29]. Recent studies revealed that lens-specific deletion of mice Brg1 led to cataract formation because of abnormal lens fiber cell terminal differentiation and defects of de-nucleation [30]. During lens differentiation, HSF4 may be recruited to the chromosome via interacting with Brg1, and then regulate the expression of targeted genes, such as DLAD. As HSF4 pathogenic mutations affected its DNA binding and transcriptional activity, it will be interesting to investigate how the HSF4 cataract mutations affect the interaction with Brg1, and the chromatin remodeling and expression of the differentiation related proteins during lens development [31].

In a previous study, Swan et al. analyzed the function of zebrafish HSF4 during embryonic eye development. Their data pointed out that ZHSF4 mRNA was not able to be detected prior 60 hpf, and knockdown of ZHSF4 led to no lens and eye development abnormalities at 48 hpf, based on the ratios between body to eye length, body length to lens diameter, or eye surface area to body length [32]. However, our results suggested that the promoter of ZHSF4 promotes the expression of GFP proteins in lens starting from 24 hpf and ZHSF4 mRNA could be determined since 24 hpf analyzed with In situ hybridization (Fig. 4A, B) and qRT-PCR (data not shown). ZHSF4 knockdown zebrafish displayed a defect in lens de-nucleation at 72 hpf while it

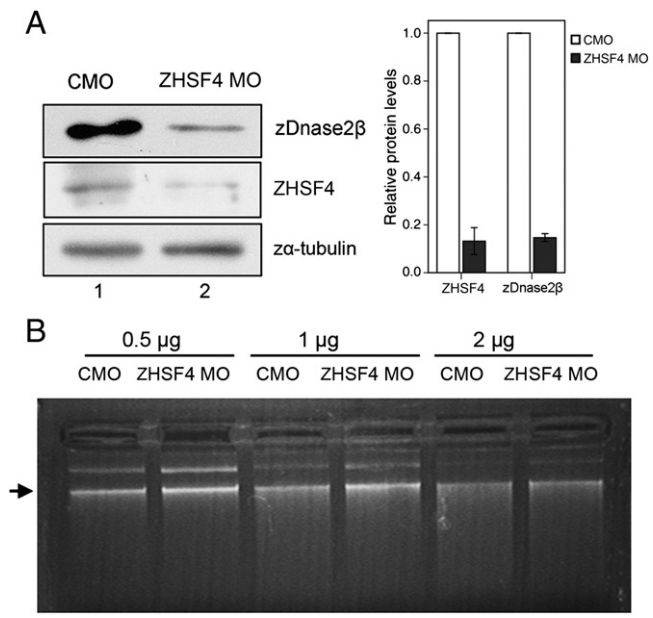

Fig. 6. ZHSF4 knockdown reduced the expression levels and DNase activity of ZDLAD. (A) Immunoblot was carried out to detect the ZDLAD and Zl protein levels of ZHSF4 and control morphants at 72 hpf. The α-tubulin protein levels were as the control. Error bars show +/−1.00 SD. (B) The DNase activity of ZDLAD from ZHSF4 knockdown morphants was estimated. CMO, control morphants; HSF4 MO, ZHSF4 morphants. The black arrow indicates the band of undigested plasmid DNA.

could not be observed apparently at 48 hpf [21]. Due to the reduced efficiency of morpholino, we did not obtain a significant phenotype in ZHSF4 knockdown zebrafish at 96 hpf (data not shown). These results support an idea that HSF4 controls the lens de-nucleation and differentiation in a time-elaborate manner.

We also found that the retina appears to be affected in HSF4 morphants, with poor lamination in the ventral retina and lack of morphological differentiation of some retinal neurons in inner nuclear layer. Several lines of evidence support a plausible association between HSF4 and retinal development. Though expressed predominantly in the lens, HSF4 could also be detected in the rat retina [33]. A thicker retina in HSF4 knockout mice has been reported in a previous study [14]. Dysfunction of Brg1 in mice and zebrafish resulted in impairment of retinal cell differentiation and development [30,34]. We infer from these data that HSF4 may be required for the normal retinal development in zebrafish.

In summary, by studying the molecular mechanism for the cataract caused by HSF4 mutations, some achieved evidences support that HSF4 plays a pivotal role in the de-nucleation of lens fiber cells through regulating DNase 2β expression level and DNase activity. Further analysis of detailed functions of HSF4, especially how HSF4 is regulated during lens development, is essential for us to better understand lens fiber cell differentiation and cataractogenesis.

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