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Targeted Deletion of Hsf1, 2, and 4 Genes in Mice

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Abstract

Heat-shock transcription factors (Hsfs) regulate transcription of heat-shock proteins as well as other genes whose promoters contain heat-shock elements. There are at least five Hsfs in mammalian cells, Hsf1, Hsf2, Hsf3, Hsf4, and Hsfy (Wu, *Ann Rev Cell Dev Biol* 11: 441–469, 1995; Morimoto, *Genes Dev* 12: 3,788–3,796, 1998; Tessari et al., *Mol Human Reproduction* 4: 253–258, 2004; Fujimoto et al., *Mol Cell Biol* 21: 106–116, 2010; Nakai et al., *Mol Cell Biol* 17: 469–481, 1997; Sarge et al., *Genes Dev* 5: 1,902–1,911, 1991). To understand the physiological roles of Hsf1, Hsf2, and Hsf4 *in vivo*, we generated knockout mouse lines for these factors (Zhang et al., *J Cell Biochem* 86: 376–393, 2002; Wang et al., *Genesis* 36: 48–61, 2003; Min et al., *Genesis* 40: 205–217, 2004). In this chapter, we describe the design of the targeting vectors, the plasmids used, and the successful generation of mice lacking the individual genes. We also briefly describe what we have learned about the physiological functions of these genes *in vivo*.

Keywords

Hsf1; Hsf2; Hsf4; Knockout mice; Targeting vector; Hsf4-EGFP

1. Introduction

1.1. Mammalian Cells Possess Multiple Hsfs with Diverse Functions

Transcription of the genome is controlled by a class of proteins known as transcription factors. Transcription factors bind to specific DNA sequences and enhance (or repress) expression of specific genes. These factors usually share a high level of overlap in their DNA recognition sequence. The heat-shock transcription factor (Hsf) family members (Hsf1, 2, and 4) bind to heat-shock elements (HSEs) (5′-nGAAn-3′ units) and regulate transcription of Hsps and other molecular chaperones (1, 2). Comparisons between Hsf protein sequences between different organisms indicate the presence of a conserved DNA-binding domain and three hydrophobic heptad repeats known as the trimerization domain. These domains are located within the amino-terminal portion of the protein. The transcriptional activation domain is located toward the carboxyl-terminus end of the molecule. The intramolecular interactions between the amino- and carboxyl-terminal domains keep Hsf1 in an inactive state under nonstress conditions (1). The expression of Hsf2 and Hsf4 in the cell correlates with their increased DNA-binding activity (2–4).

Gene targeting in mice by homologous recombination in embryonic stem (ES) cells has become a routine procedure (5–8). Gene targeting alters the mouse genome at specific selective locus. A targeting vector that carries a specific portion of the gene to be targeted is normally flanked by a neomycin gene, most often containing its own promoter sequence. Other strategies, where neomycin expression is under the control of an endogenous gene, have also been used. The neomycin gene is used as a positive selectable marker for isolation of embryonic stem cells that carry the targeted allele. Neomycin gene may be flanked by loxP sites so that it can be removed following generation of the knockout mouse line (7, 8). The targeting vector may also contain one or two thymidine kinase (TK) genes that can be used as a negative selectable marker. Following electroporation of the targeting vector into

the ES cells, if the targeting vector is randomly inserted into the genome by nonhomologous recombination, the TK genes will also be inserted and the gene is expressed. Treatment of the ES cells with ganciclovir ensures removal of the cells containing the random integration of the targeting constructs. In contrast, if the targeting vector is inserted into the genome by homologous recombination, the TK genes will not be inserted into the genome and the ES clones will survive the treatment with ganciclovir. The strategy we used was to generate mutant *hsf1*, *hsf2*, and *hsf4* targeting vectors containing a neomycin gene flanked by two loxP sites and two TK genes.

In this chapter, we describe the targeting vectors and generation of *hsf1*-, *hsf2*-, and *hsf4*-deficient mouse lines. We also briefly describe the phenotype of the *hsf* knockout mice generated in our laboratory.

2. Materials

2.1. Genomic DNA Identification, Isolation, and Analyses

At the time when we began constructing targeting vectors for the *hsf* genes, the mouse genome had not been entirely sequenced. Therefore, we cloned these genes as described in the following sections. However, currently, a BAC clone has been identified that encodes the murine *hsf* genes. To identify the BAC clone number that contains specific gene, one can go to the NCBI Web site “Clone Registry” and search for the gene of interest. After clicking the BAC clone number, the Web sites for the *hsf1*, 2, and 4 clones are indicated.

The BAC clone containing the *hsf1* gene can be found at: <http://www.ncbi.nlm.nih.gov/genome/clone/clname.cgi?stype=Name&list=RP23-266H9>. The BAC clone containing the *hsf2* gene can be found at: <http://www.ncbi.nlm.nih.gov/genome/clone/clname.cgi?stype=Name&list=RP23-212L2>, and the BAC clone containing the *hsf4* gene can be found at: <http://www.ncbi.nlm.nih.gov/genome/clone/clname.cgi?stype=Name&list=RP23-118P12>.

Once the clone is identified, the clone can be obtained from <http://bacpac.chori.org>.

One method of using a BAC clone to construct the targeting vector is from the available sequencing information, first design the targeting vector, and then fragments that are needed can be amplified by PCR. Any fragment that is amplified via PCR must be sequenced entirely to detect the presence of any errors. For PCR of genomic DNA, use of a high-fidelity TAQ polymerase is highly recommended. For the conventional knockout strategy, presence of more than one or two base pair (bp) differences per kb pairs of DNA may reduce homologous recombination. For the conditional cre-loxP techniques, even one change in the DNA bases may be detrimental in proper expression of the gene under study and should be avoided.

2.2. Plasmids and Phages

Conventional plasmids can be used for manipulation of the DNA fragments. For the final targeting vector, we used Lamda DASH II-254-2TK Phage (Stratagene, and a modified version was provided by Drs. NR Manley and B. Condi, Department of Genetics, University of Georgia, Athens, GA 30602, USA). A map of Lamda DASH II-254-2TK has been provided in Fig. 1. It contains stuffer sequences that can be removed by restriction enzyme (XhoI) digestion, and the gene fragments (in two or three pieces) of interest can then be inserted. If three fragments are inserted into the final phage, it is advisable to use different restriction enzymes for each fragment to avoid excessive self-ligation.

2.3. ES Electroporation, Southern Blotting of ES Clones, and Identification of Positively Targeted ES Clones

For identification of ES clones with correct targeting, Southern blotting from a small amount of DNA (may be as low as 2 μ g) is essential. For Southern blotting, we follow the procedure in the Molecular Cloning, Laboratory Manual (9). Figure 2 represents Southern blotting of 22 clones of ES cells obtained for targeting of the *hsf4* gene. As the data indicate from 22 clones, 6 clones were found to be positive. Other methods of this section have been briefly described for each knockout mouse line; however, the methodology can be found in detail in the following references (5–8, 10).

2.4. ES Cell Microinjection into Blastocysts, Generation of Chimeras, and Germline Transmission

This section has been briefly described for each knockout mouse line; however, the methodology can be found in more detail in the following references (5–8, 10).

3. Methods

3.1. Knockout of *hsf1* Gene

Hsf1 gene structure: The mouse Hsf1 gene (*hsf1*) is located on chromosome 15 and encodes an Hsf1 protein that contains 503 amino acids (11, 12). The mouse *hsf1* gene has 12 exons. Exon 1 contains 274 bp, and the start codon (ATG) is located at 158 bp, i.e., exon 1 encodes 39 amino acids. The distance between exon 1 and exon 2 (intron 1) is 18 kb (18, 254 bp). The remaining exons (exon 2 through exon 12) are located compactly in a 4.6-kb region. The entire *hsf1* gene is 23.5 kb.

Design of the targeting vector to delete *hsf1*: To target the *hsf1* gene, the cloned fragment containing *hsf1* was sequenced and analyzed for the presence of unique restriction enzyme sites. In our targeting strategy, we selected to delete a portion of exon 2 of the *hsf1* gene for the reasons provided here: (1) In the *hsf1* gene, exon 1 is located 18 kb apart from other exons. (2) Intron 1 encodes the promoter of another gene (known as Bop1), as we have previously reported (12). Disruption of exon 1 (plus insertion of neomycin gene in this exon) could potentially disrupt the expression of the Bop1 gene. (3) Since the distance between exon 1 and exon 2 is 18 kb, deletion of both exons would have been impossible. (4) If only exon 1 was deleted, there would be a possibility that a truncated Hsf1 mRNA and protein encoded by other exons (from exon 2 to exon 12) would be generated. This truncated Hsf1 would potentially contain amino acid residues 40–503 due to the presence of an ATG at amino acid 40. (5) For the *hsf1* targeting construct, we planned that a LacZ gene could be inserted under control of the *hsf1* promoter. The best strategy would have been to insert the LacZ gene at the first ATG. However, for the reasons noted above, we inserted the LacZ gene into the *hsf1* exon 2.

The amino acid sequence encoded by exon 2 is critical for the DNA-binding domain of Hsf1 protein. If exon 2 was deleted, the Hsf1 DNA-binding domain would be disrupted and Hsf1 could not bind to the DNA. Furthermore, if exon 2 was deleted, the *hsf1* open reading frame (i.e., a cDNA-encoding exon 1 and exons 3–12) will shift and a stop codon will appear immediately at amino acid residue 49 in exon 2. Three more stop codons are also present within the next 100 bp. Therefore, deletion of exon 2 completely disrupts the Hsf1 protein structure and function. According to these criteria, we designed an *hsf1* targeting vector with deletion of 55 bp of exon 2. The final targeting vector contains a 3.2 kb proximal fragment with homology to *hsf1*, a 3.2 kb internal ribosomal entry site (IRES)-LacZ-neomycin fragment, and a 3.7 kb distal fragment with homology to *hsf1* (Fig. 3) (11).

Targeting vector: Cloning of the *hsf1* gene: An 18 kb clone containing a portion of the *hsf1* gene fragment (exon 2–exon 12) was isolated following screening a 129/SvJ mouse genomic library in Lambda Fix II vector (Stratagene) using Hsf1 cDNA as a probe (12). This 18 kb DNA fragment was used as a template for constructing the *hsf1* targeting vector.

External probe for detection of correct targeting: To identify homologous recombination, an external probe was designed to screen the *hsf1* mutant ES clones and eventually the *hsf1*-deficient mice. The external probe should hybridize to the DNA region that is close to, but not included in the targeting vector. According to our targeting strategy, we designed a 1 kb probe that hybridized to the 5' region of the targeting vector as presented in Fig. 3a. The probe was tested on genomic DNA prepared from wild-type mouse tail that was digested by the restriction enzyme PstI. PstI was selected because of its suitability of detecting the correct targeting of ES cell clones.

Vector construct: A 3.2 kb proximal fragment with homology to *hsf1* was amplified by PCR using the isolated 18 kb *hsf1* clone as a template using forward primer: 5'-CTG CAG AAC CAA TGC ATT GGC GGC CGC TCG AGA ACA CAG CAT TC TTG AAA GAA A-3' that included BstXI, NotI, EagI, and XhoI restriction enzyme sites and a reverse primer: 5'-GAA TCG GCC GTG GTC AAA CAC GTG GAA GCT GTT-3' that included an EagI restriction enzyme site. The PCR product was sequenced to confirm DNA sequence fidelity. The PCR product was digested by EagI for subcloning.

A plasmid containing an IRES-lacZ-neomycin cassette was used to insert an IRES-lacZ-neomycin fragment in the targeting vector (Fig. 3a) (11). The neomycin gene (used as a positive selectable marker) was driven by the phosphoglycerate kinase (PKG) promoter and contained an SV40 poly-(A) signal and a stop codon. The neomycin gene was flanked by Cre recombinase recognition sequences (loxP) to allow removal of the neomycin gene in the mutant mice. The lacZ gene contained sequences of the picornaviral IRES at its 5'-end and a poly-(A) signal and a stop codon at its 3'-end (clones encoding sequences for lacZ and IRES were the gift of Dr. A. Smith (Univ. of Edinburgh, Scotland)). Since the decision was made to insert the lacZ gene (containing its own ATG) into exon 2, it was possible that Hsf1 transcripts that start from exon 1 would interfere with the lacZ expression. Therefore, an IRES was inserted before the lacZ gene to direct translation of the reporter gene. However, as we have noted in our previous publication (11), in *hsf1*-deficient mice, the lacZ gene (plus the entire exon 2) is spliced out, fusing exon 1 directly to exon 3 and making a shorter transcript that excluded exon 2. Since we had predicted this may occur in vivo, we still went with such a design since fusion of exon 1 to exon 3 would generate an Hsf1 transcript that would be out-of-frame and no protein could be generated from this transcript (as noted above). As such, cells deficient in the *hsf1* gene do not express the lacZ gene (11).

The proximal 3.2 kb fragment was subcloned into the IRES-lacZ-neomycin plasmid. The resulting plasmid was then digested with XhoI/NruI to release the 7.8 kb proximal fragment. For the distal 3.7 kb fragment with homology to *hsf1*, the 18 kb genomic clone was digested with NheI to release a 7.5 kb fragment, which was subcloned into plasmid pBlueScript at an EcoRV site. This plasmid was then digested with HindIII to remove a 3.8 kb fragment. The remaining 3.7 kb fragment (portion of exons 2–9) was subsequently released by SmaI/XhoI digestion.

The proximal fragment containing the IRES-lacZ-neomycin cassette and the 3.7 kb distal fragment were then ligated into the phage DNA vector λ DASHIII-254-2TK at an XhoI site (11). The targeting construct is flanked by two TK genes, which are used as a negative selectable marker. The vector was packaged into phage, and the positive phage clones were selected by PCR and restriction enzyme digestion. Several positive phage DNA clones were

digested by NotI (see Fig. 1) to release the final vector that contained the targeting vector and the two TK genes. This final vector can be recircularized into a plasmid that could be amplified in bacteria. After amplification, the final vector was linearized with NotI digestion for ES cell transfection.

ES cell electroporation: ES (D3; Incyte Genomics, St. Louis, MO) cells were cultured as described previously (10, 11). ES cells were electroporated (BioRad Gene Plus, 250 V, 950 μ F) with the linearized targeting vector and cultured in the presence of neomycin (200 μ g/ml) and ganciclovir (2 μ M) for 10 days. Following Southern blotting using the external probe, two doubly resistant ES cell clones (Fig. 3b) (from 167 clones tested) were selected and expanded. Generation of *hsf1* mutant mice: The two ES clones that were found to be positive by Southern blotting were microinjected into C57BL/6 blastocysts, and germline-transmitting chimeric mice were obtained. The chimeric mice were then crossed with C57BL/6 mice to obtain *hsf1*^{+/-} mice which were interbred to generate *hsf1*^{-/-} mice.

Genotyping of mutant mice: Southern blotting: ES clones, germline-transmitting chimeric mice, and the first several litters of mice were genotyped by Southern blotting. Mouse genomic DNA was isolated and digested with the restriction enzyme PstI. Southern blotting was performed using an external probe (indicated in Fig. 3b) (11). This generates a 7.7 kb fragment for the targeted locus and a 4.3 kb fragment for the wild-type locus.

PCR: When the *hsf1* mutant mouse line was established after screening by Southern blotting (Fig. 3b), the mice were subsequently routinely genotyped by PCR (Fig. 3c). Genomic DNA isolated from mouse tail was used as template. Two sets of primers were used to identify wild-type and mutant alleles: for wild type, forward primer (P1): 5'-GAG ATG ACC AGA ATG CTG TGG GTG-3' and reverse primer (P2): 5'-GCA AGC ATA GCA TCC TGA AAG AG-3'; for mutant alleles (the primers to amplify IRES region): forward primer (P3): 5'-ACT GGC CGA AGC CGC TTG GAA TAA-3' and reverse primer (P4): 5'-ATA CAC GTG GCT TTT GGC CGC AGA-3'. These PCR reactions generated a 400 bp fragment for wild type and a 285 bp fragment for mutants (Fig. 3) (11).

Figure 3d–g shows analyses of *hsf1*^{-/-} tissues confirming that no Hsf1 protein was produced.

3.2. Knockout of *hsf2* Gene

Hsf2 gene structure: The mouse *hsf2* gene (*mhsf2*) is located on chromosome 10 and encodes Hsf2 protein that contains 517 amino acids. The mouse *hsf2* gene contains 12 exons. Exon 1 contains 117 bp and the start codon (ATG) begins at 25 bp. Exon1 and exon 2 are separated by a 9.4 kb intron (13).

Design of the targeting vector *to delete hsf2*: To disrupt the mouse *hsf2* gene, we designed a targeting vector in which 67 bp from the start codon were deleted. An EGFP reporter gene with a start and stop codon were inserted into this region of the *hsf2* gene. Removal of the first 67 bp from exon I of the *hsf2* gene results in an out-of-frame shift in the cDNA. Since EGFP inserted in exon 1 of the *hsf2* gene contained a stop codon, it is therefore unlikely that the truncated Hsf2 cDNA could be translated. Therefore, deletion of 67 bp from exon I of the *hsf2* gene completely disrupts the *hsf2* gene. The final targeting vector contained a 2.8 kb proximal fragment with homology to the *hsf2* gene, a 2.2 kb EGFP-neomycin fragment, and a 6.1 kb distal fragment with homology to *hsf2* (Fig. 4) (13).

Targeting vector: Isolation of the *hsf2* gene: A 22 kb DNA fragment containing 3.8 kb of the promoter region and the first 6 of the 12 exons of the murine *hsf2* gene were isolated from a 129/SvJ mouse genomic library in Lambda FixII vector (Stratagene, La Jolla, CA) by

hybridization with a mouse *hsf2* exon 1 cDNA as a probe. This genomic clone was used to construct a targeting vector (13).

External probe for detection of correct targeting: To identify the homologous recombination, a 500 bp external probe was generated by PCR using primers: 5'-GTT TCT GCA CTG AGC CCT TG-3' and 5'-CAA GGA TTC AAT AAT CGT GAC AC-3'. This probe hybridizes to a fragment of the *hsf2* gene that is located in the 5' region of the targeting vector (Fig. 4a). The probe was tested on wild-type genomic DNA digested with PvuII restriction enzyme.

A 2.8 kb proximal fragment, including part of the *hsf2* promoter and the *hsf2* start codon, was amplified by PCR using the following primers: 5-AGT CCG CTC GAG GAG AGG TGG TAT ACA TAA ACA AGG (included a *XhoI* site, underlined) and 5-GAA CTC GGA TCC ATT GTT AGC CCG GTG CAG GGA TTC CAA ATT CTA CTA CCG AAC GCG GAG GTC GCA GCG GCG GCG G (included a *BamHI* site, underlined). This fragment lacks an unwanted PvuII site present 99 bp upstream of the start codon. This PCR fragment was digested by *XhoI* and *BamHI* and cloned into pBluescript II KS plasmid for amplification, and was sequenced to confirm the DNA sequence fidelity. The 2.8 kb proximal fragment was released by *XhoI* and *BamHI* digestion for subcloning into the final targeting vector.

A plasmid containing the 2.2 kb EGFP-neo cassette was digested with *BamHI* and *ClaI* to release the EGFP-neo fragment. The *EGFP* gene with the poly(A) signal was driven by the *hsf2* promoter (EGFP was from Clontech; neomycin was modified by the addition of two loxP sites by Dr. M. Capecchi's laboratory) (14, 15). Neomycin gene was driven by the TK promoter with a simian virus 40 poly(A) signal.

A 6 kb distal fragment, including the C-terminal 26 bp of exon 1 extending into the first intron, was PCR-amplified using the following primers: 5'-CCA TCG ATC CAA CGA GTT CAT CAC CTG GAG TC (included a *ClaI* site) and 5'-CTC ATA CTC GAG TTA ACT AAA CCA ATG CAT TCA ACTG-3' (included *XhoI* site).

The 2.8 kb proximal fragment, 2.2 kb EGFP-neo fragment, and 6 kb distal fragment were ligated to phage DNA vector IDASHII-254-2TK at an *XhoI* site flanked by two TK genes (Fig. 1). The vector was then packaged into phage, and the positive phage clones were selected by PCR and restriction enzyme digestion. The positive phage DNA was digested by *NotI* to release the final targeting vector containing two TK genes. This final vector could be recircularized into a plasmid and could be amplified in bacteria. After amplification, the final vector was linearized with *NotI* and purified for transfection into ES cells.

ES cell electroporation: ES (D3; Incyte Genomics, St. Louis, MO) cells were cultured as described previously. ES cells were electroporated (BioRad Gene Plus, 250 V, 950 μ F) with the linearized targeting vector and double selected by G418 (200 μ g/ml) and ganciclovir (2 μ M) (10, 13). Double-resistant ES cell clones were selected and expanded. Genomic DNA was isolated as described in Molecular Cloning (9).

Generation of mutant mice: Positive ES clones were selected by Southern blotting (Fig. 4b). The positive clones were microinjected into C57BL/6 blastocysts and germline-transmitting chimeric mice were obtained. The chimeric mice were then crossed with the wild-type mice to obtain *hsf2*^{+/-} mice, which were crossed to generate *hsf2*^{-/-} mice (13).

Genotyping of mutant mice: Southern blotting: ES clones, germline-transmitting chimeric mice, and first several litters of mice were genotyped by Southern blotting. Mouse genomic DNA was isolated (9) and digested with PvuII. Southern blotting was performed using an

external probe (Fig. 4b) (13). This generates a 5 kb fragment for the targeted locus and a 3.5 kb fragment for the wild-type locus. PCR: When the *hsf2* mutant mouse line was established after Southern blotting, mice were genotyped routinely by PCR (Fig. 4c). Genomic DNA isolated from mouse tail was used as a template. PCR was performed using one common (i.e., recognized by both wild type and mutant) primer (P1; 5'-GTGGTGTGCGTTCCCCGGAG-3'), a primer located in the wild-type locus (P2; 5'-TGACTCCAGGTGATGAACTC-3') to identify the untargeted allele, and a primer (P3; 5'-CTTCGGGCATGGC GGAAGTTG-3') located in the EGFP gene to identify the mutant gene. The locations of the primers P1, P2, and P3 are indicated by arrows in Fig. 4a. The expected PCR products for wild-type and targeted *hsf2* loci are fragments of 200 and 406 bp, respectively (Fig. 4c).

Figure 4d and e shows that cells deficient in Hsf2 do not express Hsf2 mRNA or protein.

3.3. Knockout of *hsf4* Gene

Hsf4 gene structure: The mouse *hsf4* (*mhsf4*) gene is located on chromosome 8 and contains 13 exons (Fig. 5a) (16).

Design of the targeting vector *to delete hsf4*: To disrupt the *hsf4* gene, we designed a targeting vector by inserting the *EGFP-neo* cassette after the start codon. Ligation of the *EGFP-neo* fragment with the proximal fragment of the *hsf4* gene would result in an out-of-frame cDNA product for the *hsf4* gene by disruption of the gene at the ATG. The final targeting vector contains a 2.8 kb proximal fragment with homology to *hsf4*, a 2.2 kb *EGFP-neo* cassette (14, 15), and a 5.5 kb distal fragment with homology to *hsf4* (16).

Targeting vector: To generate the *hsf4* targeting vector, a 129/SvJ mouse genomic DNA phage library (Lambda FixII vector, Stratagene, La Jolla, CA) was used to identify clones containing the *hsf4* gene using a mouse *hsf4* cDNA probe. The isolated *hsf4* gene contained 13 exons within a 5.9 kb fragment, as well as several kb flanking sequences at both 5' and 3' regions.

The proximal 3.2 kb region was amplified by PCR using the following primers: 5'-TTCCCACGCGTCGACCCCTCCAGTCC CATTCTTTTGTTG-3' and 5'-GAAGATCTGCCATGGCGCAGTCTCGGCCGGCCGG-3' (included BglII site, underlined). Because of the existence of an Sal I site within the amplified product, digestion with Sal I and Bgl II gives the final 2.8 kb proximal gene product.

A plasmid containing a 2.2 kb *EGFP-neo* cassette was digested with BamHI and ClaI to release the *EGFP-neo* fragment. The *EGFP* gene with the poly(A) signal was driven by the *hsf4* promoter, followed by the neomycin resistance gene, which was flanked by two loxP sites to allow its removal by cre recombinase, and was driven by the TK promoter with a simian virus 40 poly(A) signal.

The distal 5.5 kb was PCR-amplified by using the following primers: 5'-CCATCGATGGCAGGAAGCGCCAGCTGCGCTG CC-3' (included ClaI site, underlined) and 5'-CCGCTCGAGCGGGCAGGGTCTTGTTG CATAGCCT-3' (included Xho I site, underlined).

The 2.8 kb proximal fragment, 2.2 kb *EGFP-neo* fragment, and the 5.5 kb distal fragment were subcloned into phage DNA vector (λ DASHII-254-2TK) at the XhoI sites to flank the targeting construct with two TK genes. The vector was then packaged into phage, and the positive phage clones were selected by PCR and restriction enzyme digestion. The positive phage DNA was digested by NotI to release the final targeting vector that contained the two

TK genes. This vector could be circularized into a plasmid and amplified in bacteria. After amplification, the final vector was linearized by NotI digestion and used for electroporation of 129/SvJ ES cells (16).

ES cell electroporation: ES cells (D3; Incyte Genomics, St. Louis, MO) were electroporated with the linearized targeting vector. ES cells were then selected by G418 (200 $\mu\text{g/ml}$) and ganciclovir. Double-resistant ES cell clones were selected and expanded for screening by Southern blotting (9).

Genotyping of mutant mice: Southern blotting: Mouse genomic DNA from ES cells was isolated and digested with BgIII. Restriction enzyme-digested genomic DNA was then hybridized with an external probe located upstream of the targeting vector, yielding 5 and 7.2 kb fragments for wild-type and *hsf4*-targeted alleles, respectively. From the 141 isolated ES clones that were analyzed by Southern blotting, 33 clones contained the correctly targeted allele (Fig. 2) (16). Two positive ES clones were injected into C57BL/6J blastocysts, and the resulting chimeric male mice were crossed with C57BL/6J females to generate germline transmission. Homozygous mice were obtained by interbreeding of F1 heterozygous mice (Fig. 5b).

Primers used for genotyping: For routine genotyping of mice, DNA extracted from tail was used for PCR analysis to verify a 260 bp wild type and a 600 bp targeted *hsf4* fragments using the following primers: P1: 5'-GCAAACGCAGCACTTTCGCG-3'; P2: 5'-CGGATCTTGAAGTTCACCTTGAT-3'; P3: 5'-TGGACAGGGGTGTTACACACA-3' (Fig. 5c).

Immunoblotting of lens extracts of *hsf4*-deficient mice showed no Hsf4 expression (Fig. 5d).

3.4. Physiological Function of Mice with a Targeted Disruption of the *hsf1*, *hsf2*, or *hsf4* Gene

In this section, we briefly describe the major phenotypes of the *hsf1*-, *hsf2*-, and *hsf4*-deficient mice generated in our laboratory. Additionally, since some of our *hsf*-deficient mouse lines encode a reporter gene and we have also generated *hsp25*^{-/-}-lacZ and *hsp70.3*^{-/-}-lacZ (17, 18) reporter genes, we briefly describe the beneficial uses of knockout mice containing reporter genes in investigating the effects of hsf deletions on expression of their downstream target gene in vivo.

In addition to the *hsf* knockout mice that we have generated (11, 13, 16), there are two other *hsf1* (19, 20), two other *hsf2* (21, 22), and one other *hsf4* (23) mutant mouse lines that have been generated in other laboratories. The phenotypes of all mouse lines are almost comparable with each other. *Hsf1*-deficient mice generated in our laboratory exhibit complete female infertility (unpublished data). This phenotype was reported by Christians et al. (24), and the cause appears to be the inability of the *hsf1*^{-/-} zygote to undergo zygotic gene activation following fertilization (24). Another major phenotype of *hsf1*^{-/-} mice is their inability to mount a heat-shock response in every tissue that has been tested using immunoblotting or following crossing of *hsf1*^{-/-} mice with *hsp70.3*^{-/-}-lacZ reporter mice (11). *Hsf1*^{-/-} mice exhibit an age-dependent demyelinating disease (25), and cells deficient in *hsf1* exhibit accumulation of ubiquitinated proteins, including wild-type and mutant p53 (25, 26). *Hsf1*^{-/-} mice in p53-deficient background (*hsf1*^{-/-}*p53*^{-/-}) exhibit a delay in development of lymphomas compared to *p53*^{-/-} mice. There is a change in tumor spectrum that is observed in *hsf1*^{-/-}*p53*^{-/-} mice compared to *p53*^{-/-} mice, as double-knockout mice exhibit reduced lymphomas (7.9% in *hsf1*^{-/-}*p53*^{-/-} mice versus 72.2% in *p53*^{-/-} mice) while they exhibit increased levels of solid tumors (27). *Hsf2*^{-/-} mice exhibit defects in spermatogenesis and males exhibit reduced fertility a few months after birth and in the

background of *hsf1* deficiency, all males are infertile due to complete disruption in spermatogenesis (28). Hsf2 also expresses at high levels in the brain, and *hsf2*^{-/-} mice exhibit developmental defects in the central nervous system (CNS) (13). Further studies on these mice are needed to reveal additional functions of Hsf2 in mammalian organisms. Before we generated *hsf4*^{-/-} mice, there was no information on how Hsf4 becomes transcriptionally activated or in which tissues or cells it expresses. Previous reports indicated that the *hsf4* gene was mutated in humans, and humans who carry the mutation exhibit lamellar and marnier cataracts (29). Interestingly, *hsf4*^{-/-} mice exhibit developmental defects in fiber cell differentiation in the lens which leads to blindness in 100% of the progeny (16). Hsf4 activity was, for the first time, detected in the lens epithelial cells at 3 days postnatally (16). The activation of Hsf4 leads to expression of Hsp25, which was 1,000-fold lower in *hsf4*^{-/-} lens (16).

Hsf4-EGFP is expressed in many tissues. Addition of a reporter gene (EGFP) under control of the *hsf2* and *hsf4* promoters has been a powerful means of revealing in which cell types these transcription factors express in vivo. As we already have reported, Hsf2-EGFP expression can be detected in the testis during spermatogenesis using flow cytometry (13). Flow cytometry and immunoblotting experiments also show that *hsf4-EGFP* (knockout/knock-in mice) is expressed in a number of adult tissues (Fig. 6). The expression of *hsf4-EGFP* was analyzed in spleen and found to be expressed in mature CD4⁺, CD8⁺, and CD3⁺ (T cell receptor) thymocytes, GR-1-positive granulocytes (not presented), neutrophils/macrophages (CD11b⁺), and dendritic cells (CD11c⁺) (Fig. 6). Interestingly, Hsf4 does not express in B cells or immature CD4⁻CD8⁻ or CD4⁺CD8⁺ T cells (Fig. 6, thymus), but it is expressed in spleen and peripheral lymph nodes (Fig. 6a and b). Interestingly, Hsf4-EGFP expression can be detected only once T cells leave the thymus and enter the periphery.

Hsf4 regulation of Hsp25 expression in vivo: Another unique method of analyzing knockout mice expressing a reporter gene is the use of intercrossing Hsfs with their downstream target genes that express LacZ (or EGFP when possible) under their endogenous promoters to determine the extent that they regulate each downstream target gene in vivo. In one study, we crossed *hsf4*-deficient mice with *hsp25*^{-/-}-*lacZ* reporter mice (18). We found that *hsp25-lacZ* is a downstream target of the *hsf4* gene in the lens. As we described earlier, Hsf4 DNA-binding and transcriptional activity was demonstrated in developing lens epithelial cells (16). Using gel mobility shift assays, Hsf4 DNA-binding activity could be detected between postnatal days P1–P5 lens extracts (data not presented, please see ref. 16). Using *hsp25*^{+/-}-*LacZ* knock-in mice, we were able to demonstrate that Hsp25-lacZ expression coincides with the onset of Hsf4 activity in the lens epithelium and fiber cells (Fig. 7, upper panel, X-gal staining). Crossing *hsf4*^{-/-} mice with *hsp25*^{-/-} mice, we were able to completely eliminate the Hsp25 promoter-driven β-galactosidase (β-gal) expression, suggesting that Hsp25 is a downstream target of the *hsf4* gene during lens epithelial cell differentiation.

4. Notes

General considerations to knock out *hsf1*, *hsf2*, or *hsf4* genes.

The following points are important considerations for successful gene targeting:

1. The genomic DNA source must be the same as the ES cells to be used. This facilitates homologous recombination.
2. Purchase a BAC clone containing the gene of interest (<http://bacpac.chori.org>).
3. For designing the targeting vector, the length of the 5' and 3' fragments is important in facilitating homologous recombination. Sizes between 0.8 and 5 kb have been successfully used. The total size of the fragment that can be inserted into

the final vector needs to be considered before constructing the vector. The λ Dash II-254-2TK phage used by our laboratory can accommodate approximately 14 kb. Therefore, the sizes of the 5' and 3' fragments plus the neomycin gene that is required for positive selection of ES cells and a reporter gene (such as green fluorescent protein (GFP) or β -gal) should not exceed more than 14 kb pairs. Before attempting to construct the targeting vector, it is best to schematically draw the entire plan for the construct to be made. This should include all the restriction enzymes to be used.

4. It is best to insert the EGFP or LacZ genes at the ATG of the gene that is targeted. This design ensures that expression of EGFP or LacZ is under the direct control of the promoter of the gene to be targeted.
5. Although insertion of the EGFP or LacZ gene at the ATG interrupts the gene of interest, the design of the targeting construct could be such that a portion of the gene to be targeted is also deleted to ensure complete disruption of the gene.
6. Two probes need to be designed for detection of ES cell clones following electroporation of the targeting vector into ES cells. The outside probe is located outside of the 5' and 3' fragments. A restriction enzyme must be selected so that the outside probe can detect correct targeting into the intended locus. Sometimes, creating a unique restriction enzyme before the EGFP (or LacZ genes) or between the EGFP and neomycin genes (or after neomycin) is an option. Another consideration is that the fragment size created following restriction enzyme digest is not larger than 12–15 kb since large fragments are more difficult to detect by Southern blotting. Once the outside probe (best size is 500 bp–1 kb) is selected, it is best to perform Southern blotting using the genomic DNA following digestion with one or two restriction enzymes to ensure that the probe generates a predicted band for the wild-type locus.
7. It is best that the neomycin gene is flanked by two loxP sites so that it can be removed following generation of the knockout mouse by crossing with transgenic female mouse expressing Cre recombinase (Splicer mice (30), Jackson laboratory).
8. The identity of all the fragments that have been amplified by PCR needs to be verified by DNA sequencing. The ligation sites of the final targeting vector also need to be confirmed by sequencing since deletions may occur during the ligation.

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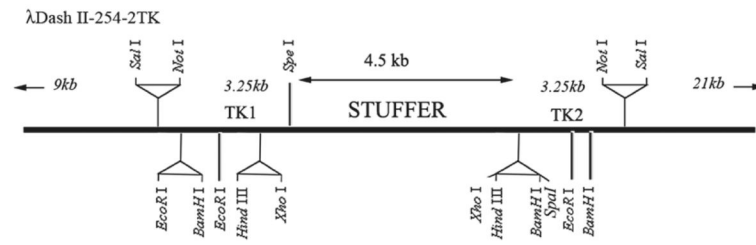


Fig. 1. Schematic presentation of Lambda Dash II-254-2TK Vector. The portion of the map of lambda-Dash II-254-2TK is presented. The stuffer sequence can be removed and the targeting vector can be ligated into the phage DNA in two or three fragments.

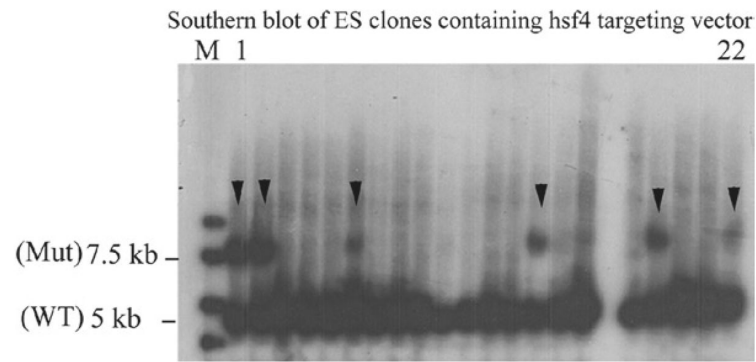


Fig. 2. Southern blot of ES cells electroporated with *hsf4* targeting constructs. From 22 clones presented, 6 clones carried the correct targeting for the *hsf4* mutant (*arrow* heads). WT is wild-type band (5 kb), and Mut is the *hsf4* mutant band at 7.5 kb.

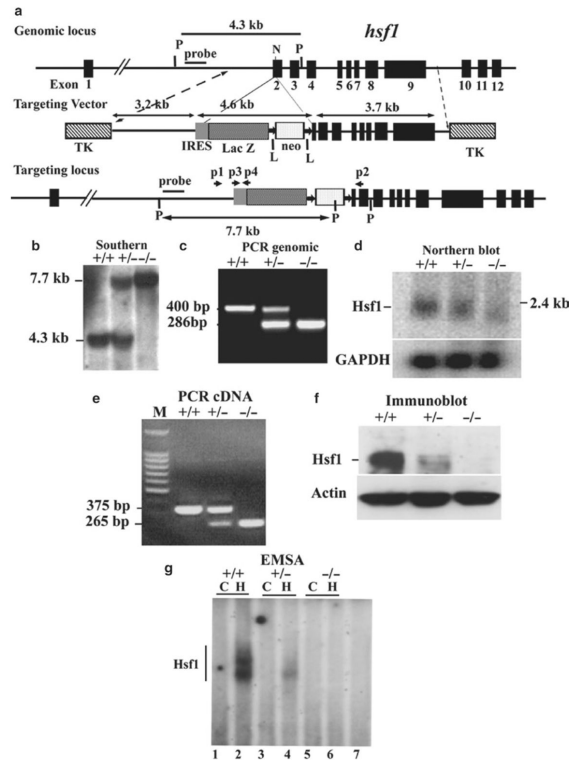


Fig. 3. Targeted disruption of *hsf1* by homologous recombination. **(a)** Schematic of segment of the *hsf1* locus, targeting construct, and targeted locus. Coding exons are *boxes in black*, beginning with exon 2 (12). Targeting construct replaces the coding region for 55 bp of exon 2. *LoxP*-flanked, PKG-neomycin cassette with upstream *IRES-LacZ* is indicated. Two TK genes were used for negative selection. “Outside probe” was used for screening ES cell clones to distinguish between endogenous and targeted alleles. The 3.2 and 3.7 kb fragments are the proximal and distal *hsf1* homologous segments in the targeting vector (11). The final insert between the two TK genes is 13 kb. N and P represent *NheI* and *PstI* sites, respectively. Primers p2, p2, p3, and p4 are used for genotyping. **(b)** Southern blot analysis of genomic DNA prepared from tails of wild-type (+) and targeted mutant mice (-). The 7.7 and 4.3 kb bands are *PstI*-digested fragments corresponding to the targeted (-) and wild-type (+) alleles, respectively. **(c)** PCR analysis of tail DNA derived from wild-type (+) and targeted mutant mice (-) showing 420 and 890 bp fragments derived from wild-type and targeted alleles, respectively. **(d)** Northern blot analysis of total RNA derived from mouse embryo fibroblasts (MEFs) of wild-type (+) or mutant (-) mice. Full-length murine *hsf1* cDNA was used as a probe. Hsf1 generates an approximately 2.4 kb fragment. GAPDH is shown for equal loading. **(e)** cDNA from wild-type (+) and *hsf1* mutant (-) mice were amplified using forward primer located in exon 1 and reverse primers located in exon 3. Sequencing the 375 bp (+) and 265 bp (-) fragment indicated normal splicing of exons 1, 2, and 3 and splicing of exon 1 to exon 3, respectively. **(f)** Immunoblot analysis of extracts of MEFs derived from wild-type (+) or mutant (-) *hsf1* analyzed by SDS-PAGE using antibody to Hsf1. Actin is shown as an indicator of loading. **(g)** Electrophoretic mobility shift assays (EMSAs) (11). Nuclear extracts of wild-type (+) or *hsf1* mutant (-) MEFs were prepared from untreated control (C) or heated (43°C for 20 min plus 30 min recovery at 37°C to ensure Hsf1 activation). Lanes 1 and 2, 3 and 4, 5 and 6 represent untreated control

(C) and heated (H) samples, respectively. *Lane 7* is the same extract as in *lane 2*, but with 200× excess cold HSE to show specificity.

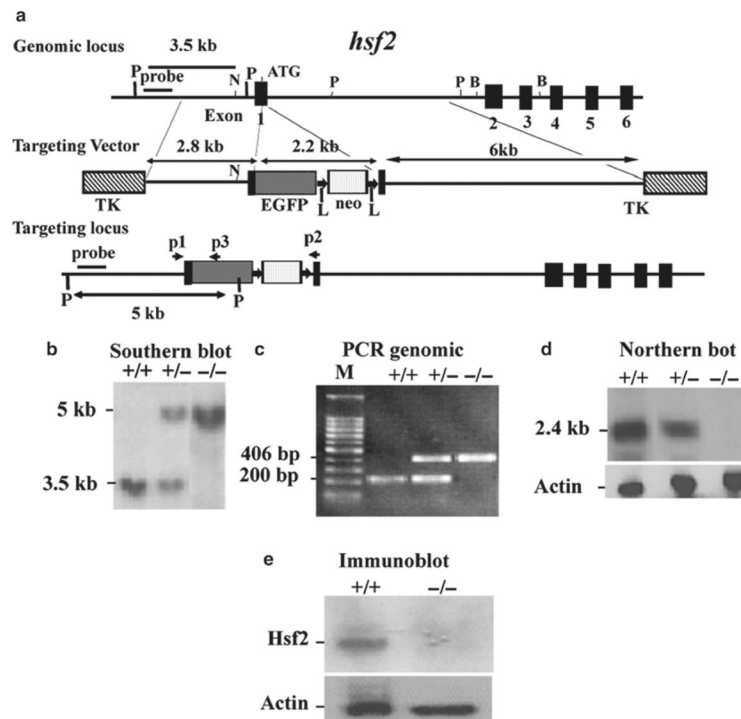


Fig. 4. Targeting strategy for the *hsf2* genomic locus and generation of *hsf2*-deficient mice. **(a)** Restriction map of the *hsf2* gene, showing the wild-type allele (*top*), targeting vector (*middle*), and the predicted targeted allele following homologous recombination (*bottom*). The ATG indicates the start codon (*top*). The position of the *EGFP-neo* and TK cassettes, probes for Southern blotting, and PCR primers P1, P2, and P3 are indicated. The vectors were designed so that the promoter of *hsf2* gene drives *EGFP* expression. Note that the Pvu II restriction enzyme site located upstream of exon 1 was destroyed in the targeting vector. The restriction enzymes are designated: P, PvuII; N, NheI; B, BamHI (13). **(b)** Southern blotting analysis of tail DNA derived from wild-type (+/+), heterozygous (+/-), or homozygous (-/-) *hsf2* mice. PvuII-digested genomic DNA was hybridized with an external probe to yield bands of 3.5 and 5 kb for the *hsf2* wild-type and targeted loci, respectively. **(c)** PCR-based genotyping assay amplifies wild-type and targeted *hsf2* locus fragments of 200 and 406 bp, respectively. **(d)** Northern blotting analysis. Total RNA extracted from the livers of 8-weeks-old mice of wild type (+/+), heterozygous (+/-), or homozygous (-/-) for the targeted *hsf2* allele adult mice was hybridized with a full-length murine *hsf2* cDNA probe. The expected 2.4 kb *hsf2* transcript was present in the wild-type and heterozygous mice, but absent in mice homozygous for the targeted *hsf2* allele. The level of actin mRNA is shown to indicate equal loading of RNA. **(e)** Western blot analysis. Equal amount of protein from cell extracts from the livers of 8-weeks-old mice of wild type (+/+) or homozygous (-/-) for the targeted *hsf2* allele adult mice was analyzed using SDS-PAGE followed by immunoblotting using antibody to Hsf2. The level of actin is shown to indicate equal loading of protein.

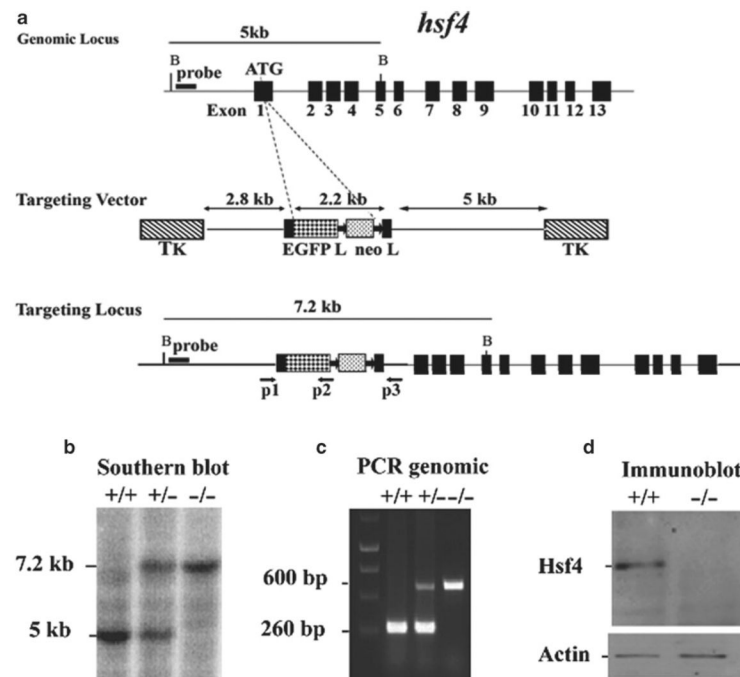


Fig. 5.

Targeted disruption of the *hsf4* gene. (a) Wild-type *hsf4* locus, targeting vector, and the predicted targeted allele following homologous recombination are shown. Exons are presented by *black boxes*. The probe used for Southern blotting and PCR primers P1, P2, and P3 are indicated by *arrows* below the targeted allele (16). (b) Bgl II-digested tail DNA (10 μ g) from wild-type (+/+), *hsf4*^{+/-} (+/-), or *hsf4*^{-/-} (-/-) mice was hybridized with an external probe to yield bands of 5 and 7.2 kb for the wild-type and targeted *hsf4* loci, correspondingly. (c) PCR-based genotyping assay amplifies fragments of 260 and 600 bp for wild-type and targeted *hsf4* allele, respectively. (d) 50 μ g protein from lens extracts of wild-type (+/+) or *hsf4*^{-/-} (-/-) mice at P28 was analyzed by Western blotting using antibody specific to Hsf4b. As a control for equal protein loading, the blot was probed for β -actin.

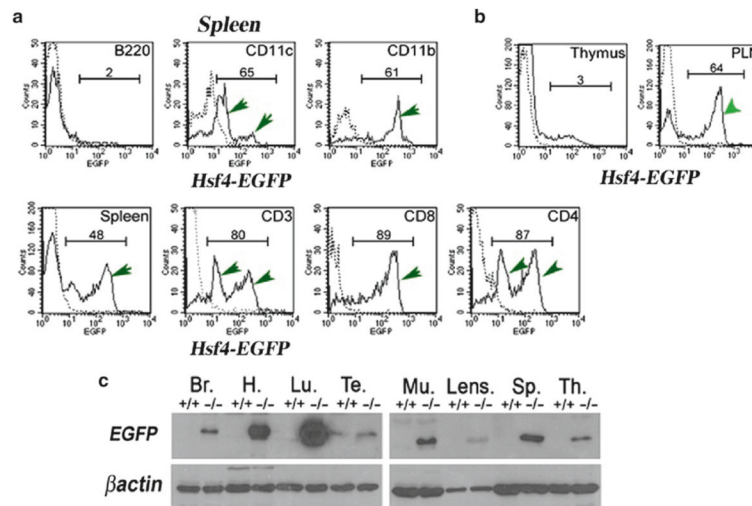


Fig. 6. Hsf4-EGFP is expressed in adult normal tissues. **(a)** Cells from *hsf4*^{-/-} spleen were immunostained to detect a specific cell population expressing Hsf4-EGFP as indicated. B220 detects B cell population; CD11c detects dendritic cells; CD11b detects neutrophils/macrophages; CD3⁺, CD4⁺, or CD8⁺ are T cell-specific markers. *Dotted lines* are immunostained +/+ cells (no EGFP). **(b)** Cells from *hsf4*^{-/-} thymus or peripheral lymph nodes (PLNs) were analyzed by flow cytometry for expression of EGFP. In **(a)** and **(b)**, *numbers* represent percentages of cells expressing EGFP, and these populations are indicated by *green arrows*. **(c)** 30 μ g of indicated adult tissue extracts from wild-type or *hsf4*^{-/-} mice were used in immunoblotting experiments using antibody to EGFP. Hsf4-EGFP expression can be detected in the brain (Br), heart (H), lung (Lu), testis (Te), muscle (Mu), lens, spleen (Sp), and thymus tissue (Th). Wild type (+/+), *hsf4*^{-/-} (-/-). Note that EGFP expression is an indication of Hsf4 expression.

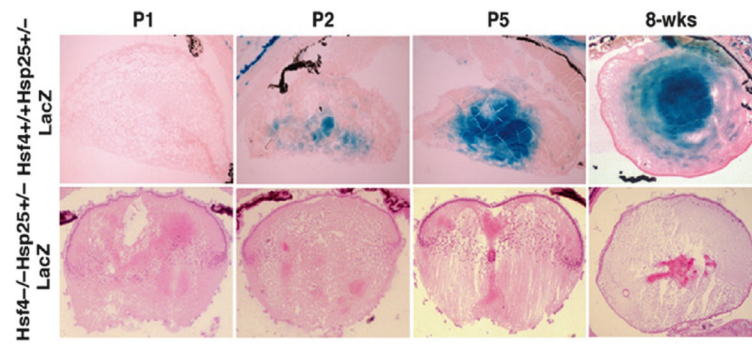


Fig. 7. Hsf4 controls the expression of Hsp25 in the lens during development. Histological analyses of *hsf4*^{+/+}*hsp25*^{+/-}-*LacZ* lens at P1 to 8-weeks to show the expression of β -gal in developing and mature lens. Note the positive X-gal staining at P2 *hsf4*^{+/+}*hsp25*^{+/-}-*lacZ* while no X-gal staining can be detected in *hsf4*^{-/-}*hsp25*^{+/-}-*lacZ*.