SKAP2, a novel target of HSF4b, associates with NCK2/F-actin at membrane ruffles and regulates actin reorganization in lens cell

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Received: November 18, 2009; Accepted: February 23, 2010

Abstract

In addition to roles in stress response, heat shock factors (HSFs) play crucial roles in differentiation and development. Heat shock transcription factor 4 (HSF4) deficiency leads to defect in lens epithelial cell (LEC) differentiation and cataract formation. However, the mechanism remains obscure. Here, we identified Src kinase-associated phosphoprotein 2 (SKAP2) as a downstream target of HSF4b and it was highly expressed at the anterior tip of lens elongating fibre cells in vivo. The HSF4-deficient lenses showed reduced SKAP2 expression and defects in actin reorganization. The disassembly of stress fibres and formation of cortical actin fibres are critical for the initiation of LEC differentiation. SKAP2 localized at actin-rich ruffles in human LECs (SRA01/04 cells) and knockdown SKAP2 using RNA interference impaired the disassembly of cellular stress fibres in response to fibroblast growth factor (FGF)-b. Overexpression of SKAP2, but not the N-terminal deletion mutant of SKAP2, induced the actin remodelling. We further found that SKAP2 interacted with the SH2 domain of non-catalytic region of tyrosine kinase adaptor protein 2 (NCK2) via its N-terminus. The complex of SKAP2-NCK2-F-actin accumulated at the leading edge of the lamellipodium, where FGF receptors and focal adhesion were also recruited. These results revealed an essential role for HSF4-mediated SKAP2 expression in the regulation of actin reorganization during lens differentiation, likely through a mechanism that SKAP2 anchors the complex of NCK2/focal adhesion to FGF receptors at the lamellipodium in lens epithelial cells.

Keywords: lens cell differentiation • actin reorganization • SKAP2 • NCK2 • HSF4b

Introduction

The lens starts development during the embryonic stage and continues to grow after birth, with the new secondary fibres being added from the outer mono-layer epithelium. Lens epithelial cells (LECs) start to differentiate, elongate, express lens fibre cell specific genes, lose contact with the capsule and epithelium, and finally lose their organelles as they become mature lens fibres [1]. Various signalling proteins are involved in this process, including fibroblast growth factor (FGF)-b, insulin-like growth factor (IGF)-1, transforming growth factor-β, N-cadherin and integrin [2–4]. Actin reorganization, which is profoundly affected by those signals, is essential for the differentiation induction and survival of LECs [5], and precise LEC differentiation is important for the transparency of the lens.

Previously we have reported that a DNA-binding domain mutant allele of heat shock factor (HSF)4 is associated with autosomal dominant lamellar and Marner cataracts [6]. Then, it has been reported that HSF4 knockout mice have defects in LECs differentiation and form cataracts [7, 8]. HSF4 belongs to the transcriptional regulator family of heat shock proteins (HSPs), and it binds to the heat shock element (HSE) that is composed of consensus inverted repeats of nGAAn [9]. Apart from the HSP
genes. HSFs may also regulate distinct, non-HSP genes. Candidate targets of HSF4b include Crygf, Fgf7, Hspb2 and Bfsp2 [7, 8, 10]. There are two splicing isoforms of HSF4: HSF4a and HSF4b. However, only HSF4b is highly expressed in mouse lens. Although HSF4 knockout mice have obvious defects in lens development, the role of HSF4 in lens development remains elusive. To understand the role of HSF4 in lens development, it is imperative to identify its downstream targets.

In searching for the downstream targets of HSF4, we compared two independent sets of microarray expression data from HSF4 knockout mice with different backgrounds (C57BL/6-129/Ss and C57BL/6-129/SvJ) and found that the Src kinase-associated phosphoprotein 2 (SKAP2) gene is down-regulated in both HSF4−/− mouse lenses [8, 10].

SKAP2 (also called SCAP2, RA70, SKAP-HOM and SKAP55R) is a homolog of SKAP55 (encoded by Skapf). Unlike SKAP55, which is expressed exclusively in the thymus and in T lymphocytes, SKAP2 is expressed ubiquitously [11, 12]. The protein structure of SKAP2 is similar to that of SKAP55, both containing a pleckstrin homology (PH) domain, which can bind to membrane lipids [13], and both having a carboxyl-terminal Src homology-3 (SH3) domain. However, SKAP2 has a unique N-terminal coiled-coil (CC) domain and tyrosine phosphorylation sites. The amino acid sequence of mouse SKAP2 is 90% identical to human SKAP55 and C57BL/6-129/SvJ) and found that the Src kinase-associated phosphoprotein 2 (SKAP2) gene is down-regulated in both HSF4−/− mouse lenses [8, 10].

SKAP2 (also called SCAP2, RA70, SKAP-HOM and SKAP55R) is a homolog of SKAP55 (encoded by Skapf). Unlike SKAP55, which is expressed exclusively in the thymus and in T lymphocytes, SKAP2 is expressed ubiquitously [11, 12]. The protein structure of SKAP2 is similar to that of SKAP55, both containing a pleckstrin homology (PH) domain, which can bind to membrane lipids [13], and both having a carboxyl-terminal Src homology-3 (SH3) domain. However, SKAP2 has a unique N-terminal coiled-coil (CC) domain and tyrosine phosphorylation sites. The amino acid sequence of mouse SKAP2 is 90% identical to human SKAP2, suggesting functional conservation of this protein. Previous studies on haematopoietic and immune systems have shown that SKAP2 binds to FYB (the Fyn binding protein) via its SH3 domain and is a substrate of Fyn kinase, which suggests a role of SKAP2 in T-cell receptor signalling similar to that of SKAP55 [12, 14]. It has also been reported that adhesion of activated B cells to fibronectin and to ICAM-1 is strongly reduced in the SKAP2−/− mouse, implying that SKAP2 might also be involved in the B-cell adhesion processes by coupling the B-cell receptor with the activation of integrin [15]. However, the mechanism of how SKAP2 is involved in integrin adhesion remains unclear, and much less is known for the function of SKAP2 beyond the immune system. Here, our results illustrate an essential role for SKAP2, a downstream target of HSF4b, in actin reorganization, providing a potential explanation for the cataract formation in HSF4 knockout mice.

### Material and methods

#### Cell culture and LEC differentiation induction

Cells from the human lens epithelial cell line SRA01/04 (a gift from Zhejiang University, China) were cultured at 37°C in low-glucose DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 15% FBS (GIBCO, Invitrogen, Grand Island, NY, USA) and 1× penicillin/streptomycin antibiotics (PAA Labs, Pasching, Austria). For the in vitro differentiation assays, the cells were starved for 24 hrs in DMEM with 0.15% FBS before treatment with 20 ng/ml of human recombinant basic FGF-b (ProSpec-Tany TechnoGene, Rehovot, Israel) to induce differentiation [16].

#### Plasmid transfection and antibodies used

Full-length SKAP2 was cloned into pcDNA3.1-triHA-5’ [the trihemagglutinin (HA) sequence was inserted into pcDNA3.1 (Invitrogen)], while full-length NCK2 or NCK1 from mouse lens were cloned into pcDNA3.1-myc-3’ [the myc sequence was inserted into pcDNA3.1 (Invitrogen)]. The Y to F mutation at position 75 of SKAP2 was made using a site-directed mutation kit (Sai Bai Sheng, Shanghai, China). The N-terminal 106 amino acids deletion mutant of SKAP2 (SKAP2A106aa) plasmid was also inserted into pcDNA3.1-triHA5’. The myc-tagged SH2 domain of NCK2 comprises residues 284-380. For knockdown assays using SRA01/04 cells, two duplexes that target different regions of hSKAP2 (5’-GATCCGCAAGGAA-GATGAGTC GGTTCCAAGAGACTG ACTCATCTCTCCTGTGTTTTGGAAA-3’ and 5’-GATCTGCT CAGACCAACAGTCTC GATTCAAGATGGAAACTGTTGCT CACTGATTGGGAAA-3’; referred to as shRNA #1 and shRNA #2, respectively) were cloned into Psilencer 3.0 (Applied Biosystems/Ambion, Austin, TX, USA). SRA01/04 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Mouse anti-HA, rabbit anti-myc, rabbit anti-FAK and the monoclonal mouse anti-Phospho-fibroblast growth factor receptor (FGFR) antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA). The primary anti-SKAP2 antibody was from Abcam (Cambridge, UK), and the rabbit anti-SKAP2 antibody was from Proteintech Group, Inc. (Chicago, IL, USA). The rabbit anti-HA antibody and the monoclonal mouse NCK antibody were from BD Biosciences (Franklin Lakes, NJ, USA). Phallolidin Alexa Fluor555, Alexa Fluor568-conjugated rabbit anti-goat IgG and goat anti-rabbit Alexa Fluor488 IgG were from Invitrogen/Molecular Probes. The goat antimonuse CY3, donkey antimonuse CY5, donkey anti-rabbit CY2 and donkey anti-goat CY3 secondary antibodies were from Jackson ImmunoResearch Labs (West Grove, PA, USA). The anti-actin antibody and Phallolidin-fluorescein isothiocyanate (FITC) were from Sigma-Aldrich (St. Louis, MO, USA).

#### Preparation and treatment of primary lens culture

The primary lens cell cultures from neonatal HSF4+/+ or HSF4−/− mice were prepared as described previously [5, 17]. Briefly, lenses were isolated from three HSF4+/+ or HSF4−/− mice at postnatal day 3, respectively. Lenes were then trypsinized in 2× trypsin- ethylenediaminetetraacetic acid (EDTA)/PBS buffer (GIBCO, Invitrogen) at 37°C for 5 min. and agitation. The collected lens cells were plated on 48-well dish (Greiner Bio-one, Stuttgart, Germany) and cultivated in M199 media supplemented with 20% FBS and 1× penicillin/streptomycin. The primary lens had well-spread epithelial morphology after 1 week culture. For the in vitro differentiation assays, the lens cells were treated with 40 ng/ml of FGF-b for 36 hrs after serum starvation in M199 supplemented with 0.15% FBS for 24 hrs.

#### Quantitative PCR

RNA from mouse lens or the SRA01/04 cells was extracted using Trizol (Invitrogen) and reverse transcribed using the MLV Transcription Kit (Invitrogen). Quantitative PCR was performed with the SYBR Green PCR kit (Applied Biosystems, Streetsville, ON, Canada) and the sequence detection system (ABI 7900HT). The following primers were used: 5’- ACCAGTTTCTC CCATTGCA-3’ and 5’-CCATTCAAAACCCCAGAAAGC-3’.

#### Chromatin immunoprecipitation

Lenses were isolated from postnatal, day-9 mice and treated as described previously [7]. Briefly, after cross-linking, the lens cells were lysed in cell
lysis buffer [5 mM N-2-hydroxyethylpiperazine-N-ethane-sulfonic acid (HEPES) including 85 mM KCl, 0.5% NP40, PMSF (phenylmethylsulfonylfluoride) and protease inhibitor cocktail] and centrifuged. The precipitated fractions were then lysed in nuclear lysis buffer (50 mM Tris-HCl including 10 mM EDTA, 1% SDS, PMSF and protease inhibitor cocktail) and sonicated. The cross-linked chromatin fragments were immunoprecipitated using the anti-HSF4b antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or Normal IgG, following the Upstate manufacturer’s instructions. We determined the enrichment of specific DNA sequences in the immunoprecipitated material using PCR with the following primers: 5’-TGGGACTCTGGTTA CCTTC-3’ and 5’-TTGCGTAGACCTTGCT TT-3’.

Immunostaining

The cultured cells were fixed with 4% paraformaldehyde in PBS for 10 min., permeabilized with 0.2% Triton X-100 in PBS for 10 min. and then blocked in 1% bovine serum albumin in PBS for 1 hr, all at room temperature. The cells were then incubated overnight at 4°C with the primary antibody. After washing, the cells were incubated with the secondary antibody in the dark or counterstained for polymerized actin (F-actin) using phalloidin for 1 hr at room temperature. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI), and cover slips were mounted on glass slides using the antifade mounting medium from Sigma. Images were taken using a fluorescence microscope (ECLIPSE 80i, Nikon, Tokyo, Japan) or a confocal microscope (TCS sp5, Leica, Wetzlar, Germany). Quantification of the cells with actin remodelling (as visualized using a microscope) was described in [18].

Mouse eyes were isolated and fixed in 4% paraformaldehyde in PBS and embedded in paraffin slides using the antifade mounting medium from Sigma. Images were taken using a fluorescence microscope (ECLIPSE 80i, Nikon, Tokyo, Japan) or a confocal microscope (TCS sp5, Leica, Wetzlar, Germany). Quantification of the cells with actin remodelling (as visualized using a microscope) was described in [18].

Co-immunoprecipitation (co-IP) and immunoblotting

The cells were lysed in ice-cold lysis buffer (KangCheng, Shanghai, China) containing a protease inhibitor cocktail (Sigma). Normalized cell lysates were mixed with the primary antibodies at 4°C overnight and 30 μL of M-280 sheep anti-rabbit IgG paramagnetic Dynabeads (Dynal Biotech, Hamburg, Germany) was added following the manufacturer’s instructions. After vigorous washing, the immunoprecipitated samples were boiled for 10 min. in Laemmli buffer, separated on 4–12% NUPAGE Bis-tris gels (Invitrogen) and transferred to the immunoprecipitated samples were boiled for 10 min. in Laemmli buffer, was added following the manufacturer's instructions. After vigorous washing, the cells were incubated with the secondary antibody in the dark or counterstained for polymerized actin (F-actin) using phalloidin for 1 hr at room temperature. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI), and cover slips were mounted on glass slides using the antifade mounting medium from Sigma. Images were taken using a fluorescence microscope (ECLIPSE 80i, Nikon, Tokyo, Japan) or a confocal microscope (TCS sp5, Leica, Wetzlar, Germany). Quantification of the cells with actin remodelling (as visualized using a microscope) was described in [18].

The expression of SKAP2 correlates with the differentiation of LECs

To understand the function of SKAP2 in lens development, we examined two independent sets of microarray expression data from HSF4 knockout mice with different backgrounds (C57BL/6-129/SJ and C57BL/6-129/SvJ). We found that SKAP2 was down-regulated in both date sets [8, 10]. To determine the magnitude of SKAP2 repression in the HSF4−/− mouse lens cells, we extracted the mRNA from mouse lenses and analysed it by quantitative PCR. Compared to the wild-type mouse, the level of mSKAP2 mRNA was reduced more than 10-fold in the newborn HSF4−/− mouse lens (Fig. 1A, left part), and approximately 2-fold in the lens cells of 8-week-old adult knockout mouse lens (Fig. 1A, right part). Then we confirmed this result by immunofluorescent staining on lens sections (Fig. 1B) or by Western blot on proteins of lens at different developmental stages (P1, P5, P10, and 4 weeks old, Fig. 1D). The SKAP2 protein was barely detectable in the lens of HSF4 knockout mice (Fig.1B, part c and 1D). Taken together, these results indicate that SKAP2 is truly down-regulated in the HSF4 knockout mouse lens. Furthermore, the expression level of SKAP2 was higher in the lens of neonatal mice than adult mice (Fig. 1A and D), synchronous with the expression time of HSF4b in the lens [8].

We next investigated whether HSF4b regulates SKAP2 through binding to the promoter region of SKAP2. The nuclear proteins were extracted from both HSF4−/− and wild-type mouse lens cells and subjected to chromatin immunoprecipitation (ChiP) assays using the anti-HSF4b antibody or IgG control. Primers that targeted the promoter region of SKAP2 (from -100 to -500) were used in the PCR reaction of ChiP precipitates. HSF4b was co-precipitated with the promoter of SKAP2 from wild-type mouse lenses, but not from HSF4−/− mouse lens or from the negative control (Fig. 1C). This finding suggests that HSF4 can bind to the promoter region of Skap2.

Results

SKAP2 is a downstream target of HSF4b in lens cells

HSF4b is expressed mainly in the lens, and it plays an important role in lens development [8]. To identify its downstream target, we
transition (EMT) mimics the LEC differentiation in vivo [16, 17]. The levels of SKAP2 mRNA were significantly increased in response to FGF-b. The increase in SKAP2 mRNA after FGF-b treatment was correlated with increased expression levels of /H9251-smooth muscle actin/, which is a marker for EMT cells [22, 23] (Fig. S1). These results imply that SKAP2 probably plays an important role in the FGF-b induced lens cell differentiation.

Actin reorganization in primary HSF4 /H11002-/- mouse lens cell cultures is inhibited during differentiation

As shown in Fig. 1B, SKAP2 concentrated at the anterior tip of elongating fibre cells, where cortical actin fibres were also assembled [24]. Indeed, SKAP2 colocalized with F-actin at the apical ends of the elongating fibre cells in wild-type mouse lens (Fig. 2A, white arrows). In contrast, not only was SKAP2 barely detectable...
in the HSF4<sup>−/−</sup> mouse lens, but also there were fewer cortical actin fibres in the anterior tip of the elongating fibre cells indicated by phalloidin staining (Fig. 2A, yellow arrows). The primary lens culture system has been proved to be an ideal model to examine the mechanisms of differentiation [5, 17, 25]. Primary LECs cultured in medium with high serum have well-spread epithelial morphology [5, 17]. When the cells are starved with serum-free medium or treated with FGF-b, they begin to transdifferentiate and elongate; withdraw from the cell cycle, express differentiation-specific markers and assemble stable N-cadherin cell–cell junctions, similar to what happens in vivo [4, 17, 26]. At the beginning of lens differentiation, the disassembly of centre stress fibres and reorganization to cortical actin filaments provide an initiation signal for lens differentiation [5]. We used primary LECs culture to examine the effect of SKAP2 on actin reorganization in HSF4<sup>−/−</sup> lens cells during differentiation. The primary actin filament structures in the undifferentiated lens cells are actin stress fibres (Fig. 2B, parts a, c), as previously reported in studies of the LEC cultures [5]. After FGF-b treatment, LECs from wild-type mice started transdifferentiation, which was accompanied by the centre actin stress fibres disassembly and cell elongation (Fig. 2B, part b). In contrast, HSF4<sup>−/−</sup> LECs still contained strong actin stress fibres and had no morphological change after starvation and FGF-b treatment (Fig. 2B, part d). These results suggest that HSF4<sup>−/−</sup> LECs fail to reorganize their actin cytoskeleton during differentiation.

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**Fig. 2** Defective reorganization of the actin cytoskeleton in HSF4<sup>−/−</sup> mouse lens cells. (A) SKAP2 was colocalized with F-actin at the anterior tip of elongating fibre cells in HSF4<sup>+/+</sup> lens (white arrows), but not in HSF4<sup>−/−</sup> lens (yellow arrows). Mid-sagittal lens sections that were prepared from wild-type or HSF4<sup>−/−</sup> mice at postnatal day 4 were stained with phalloidin-Alexa Fluor 555. The images were taken under a confocal microscope. The bar represents 50 μm. ep: epithelium; tz: transition zone; ef: elongating fibre cell; A: anterior; P: posterior. (B) The HSF4<sup>−/−</sup> cells failed to reorganize their actin cytoskeleton in vitro. The primary undifferentiated lens cells (parts a, c) were cultured in medium with serum. To induce transdifferentiation in vitro, the primary lens cells were starved for 24 hrs and subsequently treated with 40 ng/ml of FGF-b for 36 hrs (parts b, d). The cells were fixed and stained with phalloidin-FITC. Bar 100 μm.
Next we tried to overexpress SKAP2 in the primary HSF4−/−cells for rescue experiment. Due to the low expression level in primary cells, we observed partial disassembly of centre stress fibre in the cells overexpressing SKAP2 after starvation and FGF-b treatment (Fig. S2, white arrowheads). In contrast, there are still plenty of centre stress fibres in the control HSF4−/−cells expressing GFP alone (Fig. S2, top parts) or in the untransfected cells nearby (Fig. S2, yellow arrows in lower parts). These results suggest that SKAP2 plays an important role in the remodelling of the actin cytoskeleton during the differentiation of lens cells.

**SKAP2 associates with cortical actin fibres at the lamellipodium and is essential for the disassembly of centre stress fibre in SRA01/04 cells**

Bourette et al. found that phosphorylated SKAP2 can be precipitated with α-actin in myeloid cells treated with M-CSF [27]. To confirm that SKAP2 localized in actin-rich membrane ruffles, we carried co-IP and immunostaining experiments in SRA01/04 LECs treated by FGF-b. Indeed, α-actin could be co-IPed with HA-SKAP2 in SRA01/04 cells (Fig. 3A). We also observed the subcellular colocalization of HA-SKAP2 and cortical actin fibres at the lamellipodium (Fig. 3B, row 1). The FGF-b induced human LECs differentiation in vitro is also accompanied by the disassembly of actin stress fibres and the formation of cortical actin fibres (Fig. 3D, row 1). The function of the actin-binding
protein SKAP2 in actin reorganization was then examined. In fact, overexpressing SKAP2 alone in the SRA01/04 cells could cause a clear disassembly of the centre stress fibres (Fig. 4A, top part), similar to that seen in differentiated LECs that had been induced by FGF-b (Fig. 3D, row 1).

To confirm the effect of SKAP2 on actin reorganization, we knocked down SKAP2 expression in human LECs with shRNA and then examined the remodelling of actin during the initiation of LEC differentiation induced by FGF-b. The knockdown efficiency is shown in Fig. 3C. The SRA01/04 LECs were transfected with control shRNA, SKAP2 shRNA#1, SKAP2 shRNA#2, respectively. They were next treated by FGF-b to induce differentiation. In the control cells, the cellular actin stress fibres were disassembled as expected (Fig. 3D, parts a, b, c). The cells that had been transfected with SKAP2 shRNA had different knockdown efficiency. This turned out to be a very useful tool to analyse the effect of SKAP2 on the dynamics of actin. The cells with down-regulated SKAP2 (Fig. 3D, white arrows in parts f, i) exhibited clear cellular actin stress fibres. However, nearby cells, that had a higher level of SKAP2, disassembled their centre actin stress fibres in response to FGF-b (Fig. 3D, yellow arrows in parts f, i). These results suggest that SKAP2 is essential for actin reorganization during lens differentiation.

Fig. 4 The N-terminus of SKAP2 is necessary for the disassembly of centre stress fibres. (A) The full length SKAP2 but not the N-terminal truncated form of SKAP2 induced the disassembly of actin stress fibres. After parallel transfection with full-length HA-SKAP2 or HA-SKAP2Δ106aa (106DEL), the cells were fixed and stained with anti-HA antibody (red), phalloidin-FITC (green) and DAPI. Bar 10 μm. (B) The percentage of transfected SRA01/04 cells that exhibit the disassembly of cellular F-actin was quantified. The bars represent the mean ± S.E.M. of two different experiments.
The N-terminal 106 amino acids of SKAP2 are necessary for actin remodelling

To understand the mechanism of SKAP2 in actin reorganization, we first compared the two SKAP homologues. SKAP2 has unique tyrosine phosphorylation sites in the N-terminal region. To investigate the function of the N-terminal 106 amino acids, especially in the SKAP2-dependent reorganization of actin, we then transfected SRA01/04 cells with plasmids encoding either HA-tagged full-length SKAP2 or HA-tagged SKAP2Δ106aa (the N-terminal 106 amino acids deletion mutant of SKAP2). The cells were subsequently fixed and stained with anti-HA antibody and phalloidin-FITC. Both the full-length SKAP2 and SKAP2Δ106aa localized at membrane ruffles (Fig. 4A, arrows). Centre actin stress fibres were absent in the cells overexpressing full-length SKAP2 (Fig. 4A, top part), whereas the cells overexpressing SKAP2Δ106aa exhibited obvious cellular stress fibres without actin reorganization (Fig. 4A, bottom part). The efficiency of cellular F-actin disassembly in SKAP2-expressing cells was much higher than that in the SKAP2Δ106aa cells (Fig. 4B). These results suggest that the N-terminal fragment of SKAP2 has an important role in actin remodelling.

Identification of the interaction between SKAP2 and NCK2 at membrane ruffles

SKAP2 PH domain can bind directly to membrane lipids [13]. However the precise ruffle-targeting signal for SKAP2 remains unclear. We next examined the downstream proteins of SKAP2 that play a role in the actin dynamics. We observed two conserved tyrosine-based motifs (Y75DDP and Y93DKD) within the N-terminus of SKAP2 (Fig. 5A). Previous analysis showed that the consensus
motif phospho(p)-Y-D-E/D/K-V/P is a binding site for the NCK SH2 domain [29]. Thus, the Y75DDP motif is a potential binding site for the NCK adaptor proteins. There are two NCK proteins in mammals, NCK1 and NCK2, each of which contains three consecutive SH3 domains and a C-terminal SH2 domain. NCK2 is highly expressed in the mouse lens as we cloned full length NCK2 from lens sample. It has been shown that NCK2 functions in coupling phosphotyrosine (pTyr) signals to actin cytoskeletal reorganization, through a mechanism that the SH2 domain binds to phosphotyrosine (pTyr) signals, the second SH3 domain interacts with PAK (p21-activated kinase) that leads to the disassembly of stress fibre and the formation of lamellipodia upon Rac activation, and the third SH3 domain recruits focal adhesions [30–35] (Fig. 7). Therefore, it is probable that SKAP2 signals to actin via NCK2/PAK pathway.

We then examined the interaction between SKAP2 and NCK1 or NCK2 by co-IP experiments. As shown in Fig. 5C, HA-SKAP2 was co-precipitated with full-length NCK2 and the SH2 domain of

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Fig. 6 SKAP2 accumulates with NCK2, FAK and FGF receptor at the leading edges. (A) Endogenous SKAP2, NCK2 and the cortical actin fibres colocalized at the leading edge of the lamellipodia. The SRA01/04 cells were stained with rabbit anti-SKAP2 antibody, monoclonal mouse anti-NCK antibody and Phalloidin Alexa Flour 555. The arrow indicates the lamellipodia. Bar 25 μm. (B) The cells expressing HA-SKAP2 were stained with mouse anti-HA and rabbit anti-FAK antibodies. The accumulation of SKAP2 and FAK at the leading edge is indicated by arrows. Bar 10 μm. (C) Colocalization of SKAP2 and phospho-FGFR in the SRA01/04 cells. The cells were starved and treated with FGF-b. Endogenous SKAP2 and the phospho-FGFR receptor (Tyr653/654) were visualized with rabbit anti-SKAP2 antibody and monoclonal mouse anti-Phospho-FGFR antibody. Bar 50 μm.
NCK2 (Fig. 5B), but not with NCK1. This indicates different binding properties between NCK1 and NCK2. To investigate whether the Y75DDP motif in the N-terminal domain of SKAP2 mediates the association with NCK2, we introduced a tyrosine (Y) to phenylalanine (F) mutation into the full-length SKAP2 at position 75 (designated hereafter as Y75F) (Fig. 5B). Plasmids containing either full length HA-tagged SKAP2, mutant SKAP2 Y75F or SKAP2Δ106aa were each co-transfected into SRA01/04 cells with myc-tagged NCK2. The cells were then lysed and precipitated with the anti-HA antibody or the anti-myc antibody. The interaction of NCK2 was reduced with SKAP2 Y75F and was virtually undetectable with SKAP2Δ106aa (Fig. 5D). We also confirmed the interaction between endogenous SKAP2 and NCK2 by co-IP using antibodies against endogenous SKAP2 or NCK2 (Fig. 5E). Together, the N-terminal domain of SKAP2 interacts with the SH2 domain of NCK2, giving the potential explanation for the different effects between SKAP2 and SKAP2Δ106aa.

We further determined the subcellular localization of SKAP2 and NCK2. Interestingly, the endogenous SKAP2, NCK2 and the cortical actin fibres were clearly co-localized at the lamellipodial leading edge (indicated by the white arrows in Fig. 6A). Taken together, these results suggest that SKAP2 associates exclusively with the SH2 domain of NCK2 via its N-terminal domain at the leading edge.

Previous studies have identified that the third SH3 domain of NCK2 interacts with PINCH (particularly interesting new Cys-His protein 1, also named as LIMS1, LIM and senescent cell antigen-like domains 1), a linker between integrin and focal adhesions [36]. NCK2 has also been proven to interact with focal adhesion kinase (FAK) directly at cell periphery and to be involved in the formation of dorsal ruffles [35, 37–39]. It has been known that focal adhesions are sites where integrin adhesion complexes link to the actin cytoskeleton. There are various components of focal adhesions, including scaffolding molecules, GTPases, kinases and phosphatases [40]. Therefore, it is possible that SKAP2 locates with NCK2 at focal adhesions. To address this point, we transfected HA-SKAP2 into SRA01/04 cells and then stained with anti-HA antibody or anti-FAK antibody. FAK was concentrated at cell periphery when the cells were overexpressed with SKAP2. SKAP2 accumulated with FAK particularly at the leading edge (Fig. 6B, arrowheads).

Integrin adhesion complexes usually recruit actin cytoskeleton regulatory proteins that initiate or terminate actin polymerization. This event also requires other membrane receptors activation [41]. FGF receptors can activate NCK signal, however there is no evidence showing the direct interaction between NCK2 and FGFR [42]. Thus, there might be a connection between SKAP2 and FGF receptor signalling. Indeed, SKAP2 and FGFR were also co-localized at membrane (Fig. 6C).

In summary, our data provides a potential explanation on how SKAP2 regulates actin reorganization in LECs. SKAP2 might function as the link between membrane receptors and the ‘NCK-PINCH-FAK’ focal adhesion complexes at the membrane protrusions (Fig. 7).

**Discussion**

Lens cells are an ideal cell type to study the mechanisms of differentiation. During the process of differentiation into lens fibre cells, LECs undergo dramatic morphological changes including cell elongation, membrane remodelling and cell polarization. These are concomitant with migration towards the interior of the lens. Most of these events are influenced in large part by the dynamic reorganization of the actin cytoskeleton [5, 43]. HSF4 knockout mouse, which display abnormal differentiation of LECs, is a good
model for identifying novel target genes that are essential for the differentiation of LECs.

In our study, we identified a downstream target of HSF4b, SKAP2. ChiP assay revealed that HSF4 could bind to the promoter region of Skap2 (Fig. 1C). The perfect HSF binding site is composed of at least three inverted repeats of nGnnn (nTTCnGAAAnTTCn) [9] and the atypical HSEs contain gap-type HSE (nTTCnGAAAn (5 bp)nGAAAn), step-type HSE (nTTCn(5 bp)nTTCn(5 bp)nTTCn) and DR-type HSE (nGAAAnnnnnGAAAn) [44]. Besides, Fujimoto et al. have reported that the motif with at least three inverted repeats of nGnnn is also HSF4 binding site [45]. Sequence analysis on mSkap2 promoter region (from –650 to 0) suggests that there are nine putative HSEs in this region, seven of which are motifs with at least three inverted repeats of nGnnn, one is DR-type HSE and the other is gap-type HSE (Fig. S3). Further experiments will be needed to identify which motif is essential for HSF4 binding.

Previous studies have shown that SKAP2 is involved in the differentiation of myeloid cells and in cell cycle arrest [27, 46]. Consistent with that finding, expression of SKAP2 in the lens also correlated with the differentiation of LECs, indicating that SKAP2 may be involved in some process of lens cell differentiation. The facts that SKAP2 was phosphorylated after stimulation by MCS-F and that it was co-immunoprecipitated with nGnnn is also HSF4 binding site [45]. Sequence analysis on mSkap2 promoter region (from –650 to 0) suggests that there are nine putative HSEs in this region, seven of which are motifs with at least three inverted repeats of nGnnn, one is DR-type HSE and the other is gap-type HSE (Fig. S3). Further experiments will be needed to identify which motif is essential for HSF4 binding.

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Fig. S1 SKAP2 is associated with lens epithelial cell differentiation. Semi-quantitative RT-PCR of 1 μg of polyA⁺ mRNA from the SRA01/04 cells was performed with primers for the genes that are specified. The cells were incubated in the presence of FBS (+), serum-free DMEM− or 20 ng/ml FGF-b for different amounts of time.

Fig. S2 Overexpression of SKAP2 in HSF4−/− lens cells partially causes the disassembly of centre stress fibres. The primary HSF4−/− lens culture cells were transfected with SKAP2-GFP or pEGFP vectors. After 24 hrs, the cells were starved for 24 hrs and then treated with 40 ng/ml FGF-b for 12 hrs. Finally, the cells were fixed and stained with phalloidin Alexa Fluor 555 to visualize F-actin. The magnified images were from the region pointed by the white arrows. Bar 50 μm.

Fig. S3 The nucleotide sequences of putative HSEs in the promoter region of mouse Skap2. The sequences consisting of at least three inverted repeats of nGnnn include #2, #3, #4, #6, #7, #8, #9 sequences. Among these, #2 sequence has two perfect inverted repeats of nGAAn; #3 sequence has one perfect repeat of nGAAn. #1 sequence is DR-type HSE (nGAAnnnnn nGAAn) and #5 sequence is the gap-type HSE (nTTCnnGAAn(5 bp)nGAAn) . The inverted repeats of nGnnn or nGAAn sequences are shown in red.

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