

RESEARCH ARTICLE

Chromatin remodeling enzyme Snf2h regulates embryonic lens differentiation and denucleation

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ABSTRACT

Ocular lens morphogenesis is a model for investigating mechanisms of cellular differentiation, spatial and temporal gene expression control, and chromatin regulation. Brg1 (Smarca4) and Snf2h (Smarca5) are catalytic subunits of distinct ATP-dependent chromatin remodeling complexes implicated in transcriptional regulation. Previous studies have shown that Brg1 regulates both lens fiber cell differentiation and organized degradation of their nuclei (denucleation). Here, we employed a conditional *Snf2h^{lox}* mouse model to probe the cellular and molecular mechanisms of lens formation. Depletion of Snf2h induces premature and expanded differentiation of lens precursor cells forming the lens vesicle, implicating Snf2h as a key regulator of lens vesicle polarity through spatial control of *Prox1*, *Jag1*, *p27^{Kip1}* (*Cdkn1b*) and *p57^{Kip2}* (*Cdkn1c*) gene expression. The abnormal *Snf2h^{-/-}* fiber cells also retain their nuclei. RNA profiling of *Snf2h^{-/-}* and *Brg1^{-/-}* eyes revealed differences in multiple transcripts, including prominent downregulation of those encoding Hsf4 and DNase II β , which are implicated in the denucleation process. In summary, our data suggest that Snf2h is essential for the establishment of lens vesicle polarity, partitioning of prospective lens epithelial and fiber cell compartments, lens fiber cell differentiation, and lens fiber cell nuclear degradation.

KEY WORDS: Lens, Terminal differentiation, Smarca4, Brg1, Smarca5, Snf2h, Denucleation, Cataract

INTRODUCTION

ATP-dependent chromatin remodeling is required for transcription, DNA replication, DNA repair and genetic recombination (de la Serna et al., 2006). At least four families of multiprotein chromatin remodeling complexes have been identified in mammalian cells, including SWI/SNF, ISWI, CHD and INO80 (Ho and Crabtree, 2010; Sharma et al., 2010). Twenty-seven genes encode unique DEAD/H-box helicases [e.g. Brg1 (Smarca4), Brm (Smarca2),

Snf2h (Smarca5) and Snf2l (Smarca1)] of these complexes. For example, ISWI/Snf2h plays roles in nucleosome sliding and assembly, while Brg1-containing SWI/SNF complexes regulate nucleosome sliding and disruption (Cairns, 2007).

Genetic studies in mice have demonstrated crucial roles for *Brg1* (Bultman et al., 2000) and *Snf2h* (Stopka and Skoultchi, 2003) in blastocyst formation and peri-implantation development, consistent with their functions in embryonic stem cells (Ho et al., 2011; Kidder et al., 2009). Tissue-specific inactivation of *Brg1* demonstrated a range of functions in multiple tissues and organs, including blood, brain, eye, lens, muscle and skin. Brg1 controls the proliferation of T-cells (Gebuhr et al., 2003), terminal differentiation in erythrocytes (Griffin et al., 2008), keratinocytes (Indra et al., 2005), lens fibers (He et al., 2010), cardiomyocytes (Hang et al., 2010), Schwann cells (Weider et al., 2012) and adult neural progenitors (Matsumoto et al., 2006; Ninkovic et al., 2013). Brg1 also controls apoptosis in T-cells (Gebuhr et al., 2003) and erythrocytes (Griffin et al., 2008). Two specific *Brg1* mutant alleles were identified in model organisms. In mouse, a hypomorphic mutation in the ATPase domain was used to probe β -globin chromatin structure and expression (Bultman et al., 2005). In zebrafish, a nonsense mutation in one of two duplicated *brg1* genes abrogates retinal development (Gregg et al., 2003). Compared with Brg1, less is known about the role(s) of Snf2h and of Snf2h-containing complexes (ACF, CHRAC, ISWI and WICH) during organogenesis. Snf2h regulates erythropoiesis (Stopka and Skoultchi, 2003) and neuronal progenitor cell formation and their subsequent differentiation (Alvarez-Saavedra et al., 2014).

Mammalian lens development is an advantageous system with which to study the molecular mechanisms of cellular differentiation, including the regulation of cell cycle exit, chromatin dynamics and elimination of subcellular organelles (Bassnett, 2009; Cvekl and Ashery-Padan, 2014). The lens is composed of a layer of epithelial cells that overlie a bulk of differentiated fiber cells. The mature fiber cells express and accumulate crystallin proteins, acquire a highly elongated cellular morphology, and degrade endoplasmic reticulum (ER), Golgi apparatus, mitochondria and nuclei. Lens compartmentalization into the epithelium and fibers originates from the early transitional structure termed the lens vesicle (~E11.5 in mouse embryos). The lens vesicle is polarized. Its posterior cells exit the cell cycle in response to the BMP and FGF growth factors produced by the retina and ciliary body, and differentiate into the primary lens fiber cells (Griep and Zhang, 2004; Gunhaga, 2011). The anterior cells differentiate into a sheet of single-layered lens epithelial cells (Martinez and de Jongh, 2010). Lens epithelial cells close to the lens equator divide continually. Following cell cycle exit, these cells subsequently differentiate into secondary lens fiber cells. Between E16.5 and E18, lens fiber cell nuclei are degraded to produce an organelle-free zone (OFZ) at the center of the lens (Bassnett, 2009). DNase II-like acid nuclease DNase II β (Dnase2b)

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plays an essential role in this process. Expression of *Dnase2b* is downstream of transcription factors including AP-2 α (Tfap2a), FoxE3, Hsf4 and Pax6 (Blixt et al., 2007; Fujimoto et al., 2004; Medina-Martinez et al., 2005; West-Mays et al., 2002; Wolf et al., 2009). Our previous studies showed that Brg1 is required for lens fiber cell differentiation, expression of DNase II β , and the degradation of lens nuclei (He et al., 2010).

Genetic studies have implicated retinoblastoma protein (Rb1), E2Fs and the cell cycle inhibitors p27^{Kip1} (Cdkn1b) and p57^{Kip2} (Cdkn1c) in the regulation of cell cycle exit in lens (Chen et al., 2000; McCaffrey et al., 1999; Morgenbesser et al., 1994; Wenzel et al., 2011; Zhang et al., 1998). BMP, FGF and Notch signaling pathways regulate lens fiber cell differentiation in conjunction with DNA-binding transcription factors, including FoxE3 (Blixt et al., 2007; Brownell et al., 2000; Medina-Martinez et al., 2005), Gata3 (Maeda et al., 2009), Pax6 (Shaham et al., 2009), Pitx3 (Ho et al., 2009; Medina-Martinez et al., 2009), Prox1 (Duncan et al., 2002; Wigle et al., 1999), Hey1 (Herp2) and Rbpj (Jia et al., 2007; Rowan et al., 2008). Although little is known about links between BMP and FGF signaling and these factors, disruption of Prox1 blocks expression of p27^{Kip1} and p57^{Kip2} in the posterior part of the lens vesicle, followed by arrested lens fiber cell elongation (Wigle et al., 1999). Loss of FoxE3 abrogates Prox1 expression and consequently dysregulates expression of p57^{Kip2} (Medina-Martinez et al., 2009). Hey1 and Rbpj DNA-binding proteins directly control p27^{Kip1} and p57^{Kip2} expression (Jia et al., 2007). Taken together, perturbation of cell cycle exit in the lens is linked to abnormal fiber cell differentiation. To expand our knowledge of chromatin remodeling during mammalian embryogenesis, we have investigated whether Snf2h regulates lens development in mouse.

RESULTS

Conditional inactivation of Snf2h disrupts lens differentiation

To understand the function of Snf2h in lens development, we first determined the Snf2h expression pattern during mouse eye development by immunofluorescence. We found expression of Snf2h throughout embryonic lens development (E11.5 to E16.5) (Fig. 1A–D). The data show similar levels of Snf2h expression in the anterior and posterior parts of the lens vesicle (Fig. 1B), the lens epithelium, and the primary and secondary lens fibers (Fig. 1C,D). The cornea and both inner and outer nuclear layers of the retina also express Snf2h (Fig. 1B–D). At postnatal stages, Snf2h expression continues in the lens epithelium and the differentiating secondary lens fiber cells (Fig. 1H; data not shown).

To investigate the roles of Snf2h in mouse lens development, we inactivated Snf2h in the surface ectoderm-derived tissues using the *Le-Cre* transgene. The *Le-Cre* mouse is a transgenic line in which a 6.5 kb genomic fragment from the mouse *Pax6* gene (Fig. 1E) drives the expression of Cre recombinase and green fluorescent protein (GFP) from between E8.5 and E9 (Ashery-Padan et al., 2000). Genotyping of genomic DNA samples showed bands corresponding to the *Snf2h* wild-type (*wt*), flox (*fl*) and null (deletion of exons 5–9) alleles (Fig. 1F). The newborn *Snf2h* heterozygous mice (*Snf2h*^{fl/+}) appeared normal. Depletion of Snf2h proteins was confirmed in the E14.5 and newborn lens of *Snf2h*^{fl/fl}; *Le-Cre* (Fig. 1G,H). *Snf2h*^{fl/-}; *Le-Cre*^{wt/+} (referred to as Snf2h cKO) exhibited a wide spectrum of ocular defects (see Fig. 2). However, their littermates were normal and served as controls in the comparative experiments.

The specific eye defects of the Snf2h cKO were first characterized by histology (Fig. 2). At E11.5, although the mutant lens vesicle

separated normally from the surface ectoderm, a number of posterior cells started to differentiate prematurely, as evidenced by their elongation (compare Fig. 2A,B). At E12.5, both the Snf2h cKO and control lenses underwent primary lens fiber cell differentiation. However, the Snf2h cKO lens was surrounded by a hypertrophic hyaloid vasculature, leaving a narrower vitreous space between the lens and retina (compare Fig. 2C,D). Compared with the control, the Snf2h cKO lens was reduced in size at E14.5, when primary lens fiber cells normally reach the lens epithelium (compare Fig. 2E,F). The elongation of primary lens fiber cells was disturbed in Snf2h cKO lenses, as indicated by abnormal formation of transitional zones (marked by the nuclei of cells that exited the cell cycle) at the lens equator (Fig. 2E,F). In addition, the lens epithelium of the Snf2h cKO was thinner and its cuboidal morphology was compromised (Fig. 2G,H). At E17.5, the growth deficiency of the Snf2h cKO lens was very pronounced (Fig. 2I,J). At postnatal stages (P1 and P14) we detected progressive deterioration and cataractogenesis in the mutant lenses (compare Fig. 2K,M,O with L,N,P). The cornea in the Snf2h cKO failed to differentiate into its stratified layers (compare Fig. 2I,J with M,N), probably owing to loss of Snf2h from the presumptive corneal epithelial cells, which also express the *Le-Cre* transgene (Ashery-Padan et al., 2000). At E17.5 and P1, the abnormal lens fiber cell mass protruded towards the cornea and eventually formed iridocorneal adhesion masses at the anterior segment (Fig. 2J–L), raising questions concerning the presence of lens epithelial cells and/or their ability to establish proper contacts with the elongating lens fiber cell mass to control lens shape (see below). Notably, lens fiber cell nuclei were retained in the presumptive OFZ in the Snf2h cKO lenses (Fig. 2K,L). Taken together, deletion of Snf2h in the mouse embryo does not appear to affect early stages of lens formation; however, it leads to arrested lens growth, aberrant lens compartmentalization, perturbed fiber cell differentiation, and marked defects in lens fiber cell denudation.

To aid data interpretation, expression of the related protein Snf2l was assessed in the mouse eye. Expression of Snf2l protein in the eye is mostly restricted to the retina, as described previously at the RNA level (Magdaleno et al., 2006). By immunofluorescence, additional expression of Snf2l was detected in the lens transitional zone (Fig. S1A–C). At the RNA level, the expression of *Snf2h* is much higher than that of *Snf2l* in both the E15.5 and newborn lens (Fig. S1D). Interestingly, depletion of Snf2h in mouse cerebral extracts was followed by increased levels of Snf2l protein (Alvarez-Saavedra et al., 2014). By contrast, western immunoblotting data showed no upregulation of Snf2l in *Snf2h* mutant lens/eyes (Fig. S1E).

Cellular and molecular characterization of lens differentiation defects in the Snf2h cKO model

To explain the disrupted lens growth and differentiation of Snf2h cKO embryos, we focused on lens size reduction and aberrant morphogenesis following the completion of primary lens fiber cell elongation (Fig. 2E,F). Microphthalmia suggested reduced cell growth in the anterior part of the lens vesicle/prospective lens epithelium due to the deletion of Snf2h. It has been shown previously that ISWI chromatin remodeling complexes control proliferation via the rate of S-phase progression (Arancio et al., 2010; Collins et al., 2002). To test this possibility, we evaluated cell proliferation by analysis of BrdU (5-bromo-2'-deoxyuridine) incorporation and expression of the Ki67 protein, a marker of dividing cells, in E14.5 lenses. We found that the number of proliferating presumptive lens epithelial cells was reduced in the

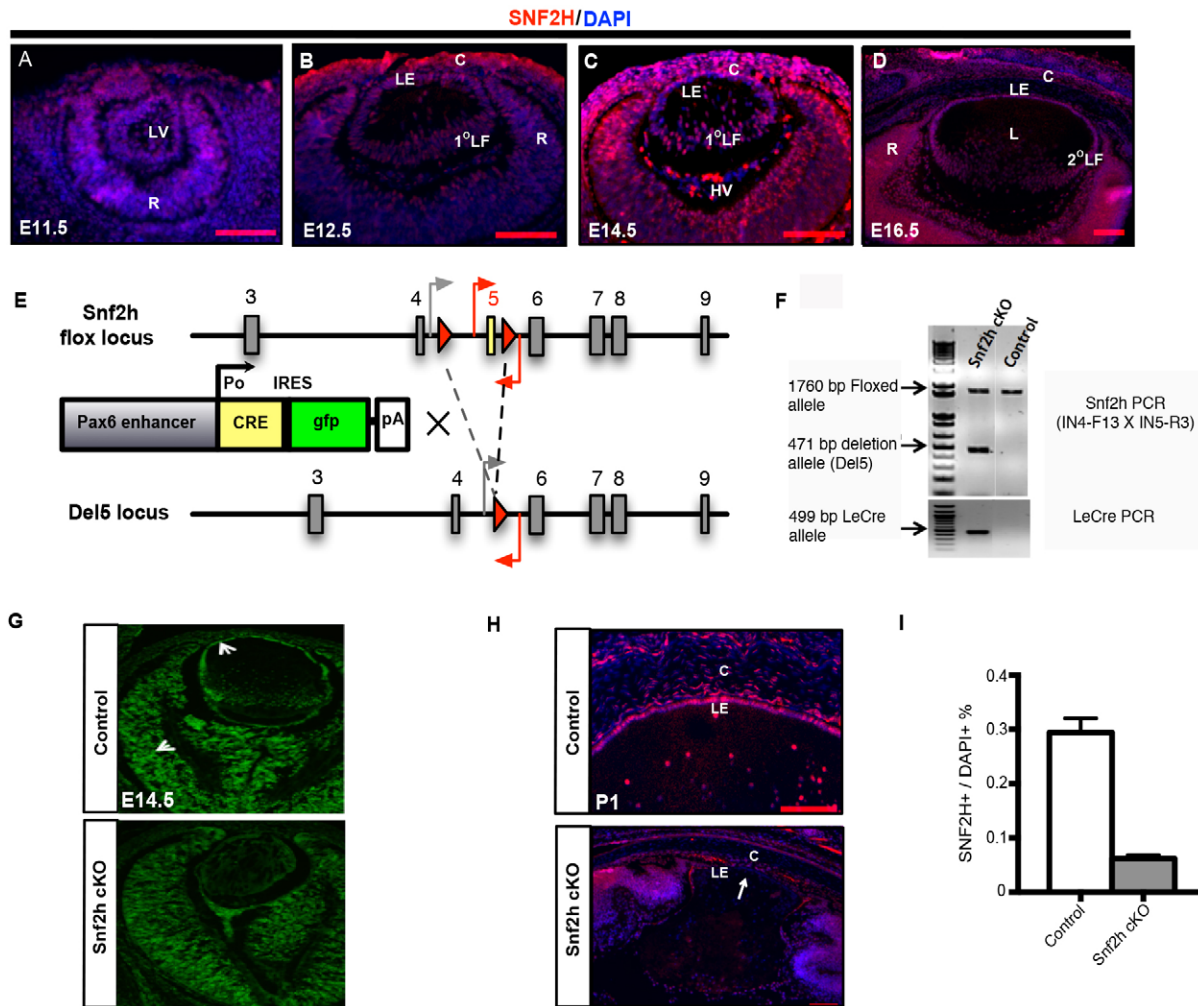


Fig. 1. Expression of *Snf2h* and initial analysis of the *Snf2h* lens conditional knockout (*Snf2h* cKO) mouse. (A–D) Localization of *Snf2h* proteins (red) during embryonic mouse eye development as assessed by immunofluorescence. Nuclei were counterstained with DAPI (blue). HV, hyaloid vasculature; L, lens; LE, lens epithelium; 1°LF and 2°LF, primary and secondary lens fibers; C, cornea; R, retina. (E) Schematic representation of the *Snf2h* floxed allele (showing exons 3 to 9 as gray boxes), the *Le-Cre* driver construct and the resulting deletion of exon 5 (*Del5* locus). Two loxP sites (red triangles) were inserted to flank exon 5 (yellow box). Primers used for PCR genotyping are indicated by gray and red arrows. (F) *Le-Cre*-mediated recombination of the *Snf2h* floxed locus. The 1760, 471 and 499 bp bands correspond to the *Snf2h*^{fl}, *Snf2h* null (*Del5*) and *Le-Cre* transgene alleles analyzed with lens/anterior segment genomic DNA, respectively. (G) Immunofluorescence analysis of *Snf2h* expression (green) in E14.5 control and *Snf2h* cKO embryos. Arrows indicate positive staining of *Snf2h* in the ocular tissues. (H) Immunofluorescence of *Snf2h* expression (red) in newborn (P1) control and *Snf2h* cKO eyes. Arrow indicates reduction of *Snf2h* expression in mutant lens but not in other regions of the eye. (I) Quantification of *Snf2h*-positive cells in control and *Snf2h* cKO P1 lenses. Error bars indicate s.d. of three different animals. There is a marked reduction of *Snf2h* expression in mutant lens but not in other regions of the eye (G–I). Scale bars: 100 μ m.

Snf2h cKO (Fig. S2). The bilateral lens germinative zones, where active proliferating lens epithelial cells reside, normally increase in cell number towards the lens equator, where cells exit from the cell cycle and differentiate (Fig. S2A–C). However, in the *Snf2h* cKO lenses, reduced numbers of dividing cells were found around the lens equator at E14.5. In addition, the lens transitional zones moved anteriorly, and hence the size of the presumptive lens epithelial region was reduced (Fig. S2D–F). Quantitative analysis of BrdU-positive and Ki67-positive cells (Fig. S2G,H) confirmed these staining patterns. We conclude that the *Snf2h*-deficient lens cells exhibit a reduced number and disturbed pattern of dividing cells in the presumptive lens transitional zone.

The histological analysis of P1 lenses (compare Fig. 2M,N) raised a major question regarding the status of lens epithelium in *Snf2h* cKO lenses. Lens epithelium is marked by expression of FoxE3 and E-cadherin, and higher levels of Pax6 expression are found in lens epithelium than in lens fibers. In E12.5–E14.5 lens,

expression of FoxE3 is confined to the nascent lens epithelium (Blixt et al., 2000; Medina-Martinez et al., 2005), and its inactivation accounts for the *dysgenetic lens* (*dyl*) mutant phenotype (Blixt et al., 2000; Medina-Martinez et al., 2005), which is characterized by abnormal lens fibers, defects in denucleation, vacuolization, and the structural collapse of the lens. The *dyl* defects are directly comparable to the present abnormalities in *Snf2h* mutant lens (Fig. 2). In wild-type (WT) E12.5 embryos, FoxE3 expression was found in the anterior portion of the forming lens (Fig. 3A). By contrast, expression of FoxE3 was significantly reduced in the *Snf2h* cKO as early as E12.5 (Fig. 3B). In E14.5 WT lens, expression of FoxE3 continued in the lens epithelial cell layer (Fig. 3C), whereas FoxE3 expression was strongly reduced in E14.5 *Snf2h* cKO lenses (Fig. 3D). Lens-specific expression of FoxE3 never reappeared at subsequent stages examined: E16.5 and P1 (data not shown). The DNA-binding transcription factor Pax6 is a key regulator of

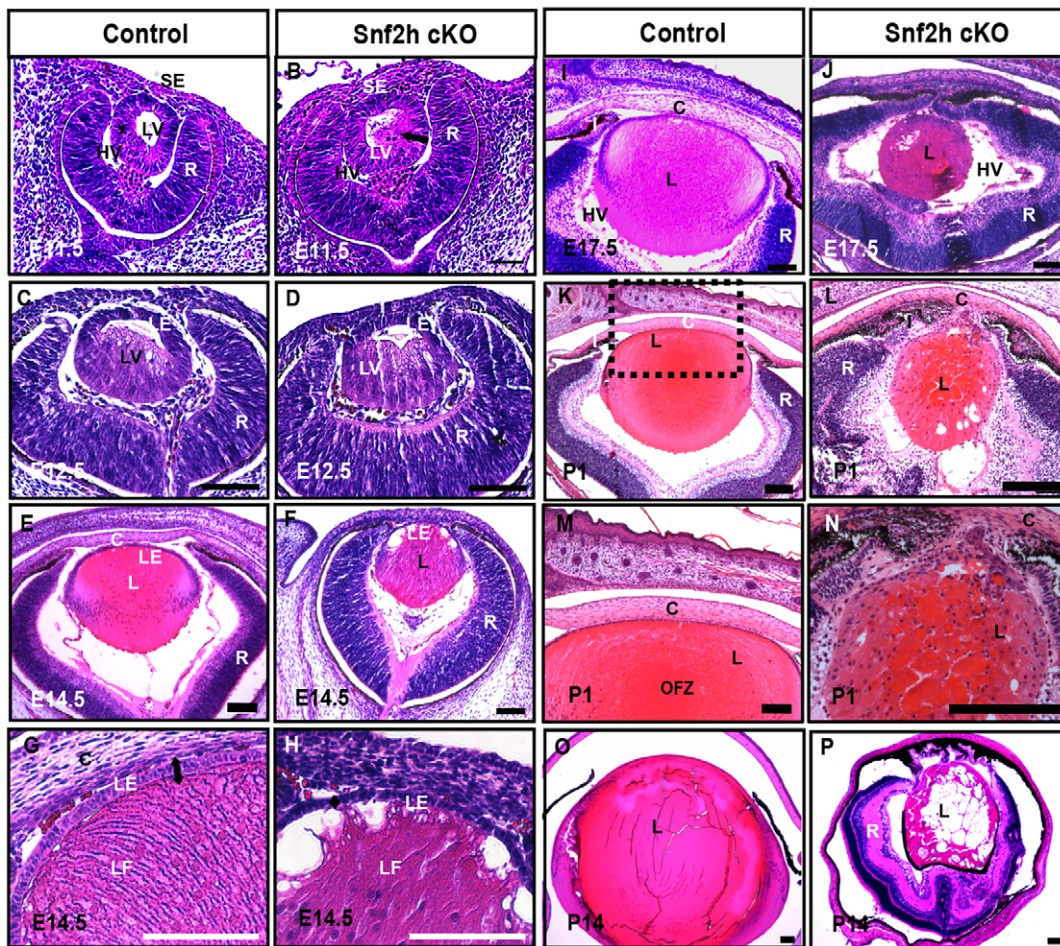


Fig. 2. *Snf2h* is necessary for mouse lens development. (A,B) Lens vesicle is separated from the surface ectoderm in the *Snf2h* cKO at E11.5. Note that a number of cells at the posterior of the lens vesicle (LV) in the *Snf2h* cKO already appeared to elongate (arrow in B). (C,D) Primary lens fiber cells are elongated within the lens vesicle at E12.5. The prominent hyaloid vasculature (HV) occupies the space between the lens (L), cornea (C) and retina (R) in the *Snf2h* cKO eyes. (E–H) At E14.5, lens fiber cells in the *Snf2h* cKO were unable to form the bow region/transitional zone and the fiber-like morphology of these cells deteriorated at the anterior of the lens. (I,J) The E17.5 *Snf2h* cKO shows a series of defects in lens, cornea, iris (I) and retina. (K–N) The newborn *Snf2h* mutant lens does not form the presumptive organon free zone (OFZ). The boxed region in K is magnified in M. (O,P) Profound ocular defects in *Snf2h* cKO are found at P14, including the absence of lens anterior chamber, lens vacuoles, and disorganization of the lens fiber cells. LE, lens epithelium; LF, lens fibers; SE, surface ectoderm. Scale bars: 100 μ m.

multiple stages of lens development (Shaham et al., 2012). At E14.5, discontinuous and moderately reduced Pax6 expression was found at the anterior of the *Snf2h* cKO lenses (Fig. S3C). At P1, expression of Pax6 was further reduced in mutant lenses (compare Fig. S3B,D). Thus, expression of Pax6 and its downstream target FoxE3 (Blixt et al., 2007) are reduced in lens following *Snf2h* depletion.

The defects in *Snf2h* mutant lens can also be assessed through expression of E-cadherin [cadherin 1 (Cdh1)], a cell-cell adhesion glycoprotein specific to epithelial cells and required for lens morphogenesis (Pontoriero et al., 2009). At E12.5 in the control, strong expression of E-cadherin was restricted to the prospective lens epithelium at the anterior of the developing lens vesicle (Fig. 3E). By contrast, in the *Snf2h* cKO, E-cadherin expression was reduced and its distribution was expanded towards the primary lens fiber cell compartment (Fig. 3F). Importantly, at E14.5 the *Snf2h* cKO lenses lost E-cadherin expression and did not establish any morphologically discernible lens epithelium (compare Fig. 3G,H). Taken together, these results show that depletion of *Snf2h* disrupts lens differentiation through downregulation of Pax6, FoxE3 and E-cadherin expression, and

by reducing the number of cells from which the lens epithelium is normally formed.

Dysregulation of cell cycle exit control genes in *Snf2h* mutant lens

To probe the disrupted lens growth and differentiation we analyzed the expression of genes involved in cell cycle exit control. Prox1, regulated by FGF signaling (Zhao et al., 2008), controls expression of the cyclin kinase inhibitors p27^{Kip1} and p57^{Kip2} in differentiating lens cells (Wigle et al., 1999). In parallel, Notch signaling, as probed through conditional inactivation of the Notch2 receptor (Saravanamuthu et al., 2012), the jagged 1 (Jag1) ligand (Le et al., 2012) and the downstream Rbpj DNA-binding transcription factor (Jia et al., 2007; Rowan et al., 2008) were shown to act upstream of p27^{Kip1} and/or p57^{Kip2}. We examined the expression of Prox1, Jag1, p27^{Kip1} and p57^{Kip2}.

In WT E14.5 and P1 newborn lens, abundant expression of Prox1 was found in the transitional zones (Fig. 4A,C). By contrast, in *Snf2h* cKO lenses the expression of Prox1 was expanded into regions that included the presumptive lens epithelium (Fig. 4B,D). The Jag1 expression pattern shifted from the equatorial zone towards the lens anterior in the *Snf2h* cKO (Fig. 4E–H). In WT

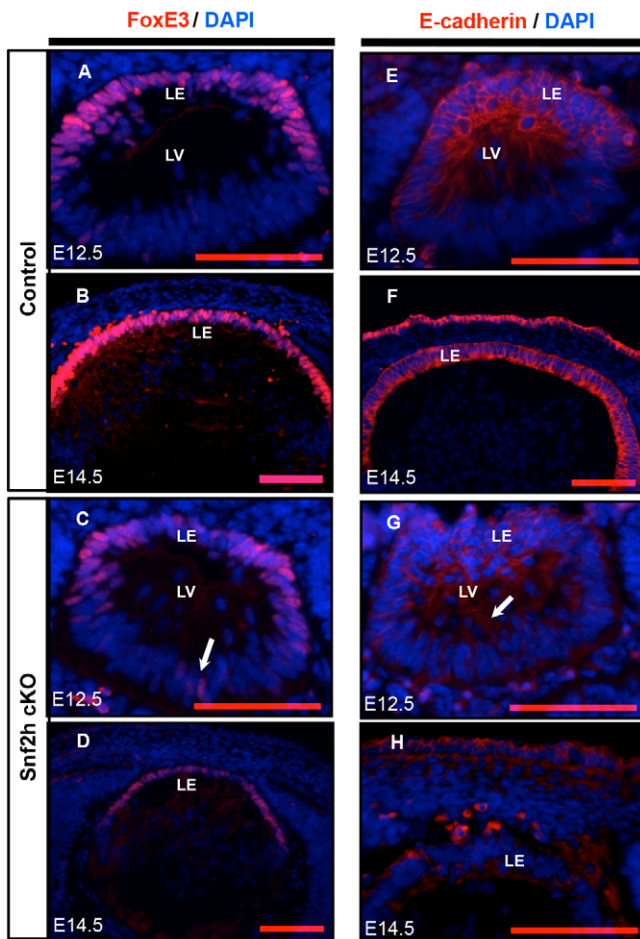


Fig. 3. Depletion of *Snf2h* results in a disrupted presumptive lens epithelial compartment. (A–D) Downregulation of FoxE3 (red) in *Snf2h* mutant lenses. Arrow (C) indicates sparse FoxE3 protein at the posterior cells undergoing lens fiber cell elongation. (E–H) Reduced and disorganized expression of E-cadherin (red) in wild-type and *Snf2h* cKO lenses. Arrow (G) indicates dislocation of E-cadherin in the E12.5 *Snf2h* cKO lens vesicle. The nuclei were counterstained with DAPI (blue). LV, lens vesicle; LE, lens epithelium. Scale bars: 100 μ m.

lenses, p27^{Kip1} and p57^{Kip2} (Zhang et al., 1998) were only expressed in cells localized in the lens equator transitional zone that are undergoing cell cycle exit (Fig. 4I,K,M,O). In *Snf2h* cKO E14.5 lenses, cells expressing p27^{Kip1} (Fig. 4J,L) and p57^{Kip2} (Fig. 4N,P) did not form the ‘compact’ transitional zone but were instead found in many abnormal positions. In P1 *Snf2h* cKO lenses, transitional zones were not established and the expression of p27^{Kip1} was found in the lens anterior compartment (Fig. 4L). These data suggest that *Snf2h* first regulates spatial aspects of *Prox1* and *Jag1* expression, and directly and/or indirectly affects the expression of p27^{Kip1} and p57^{Kip2}. Hence, inactivation of *Snf2h* promotes cell cycle exit and thus the ‘borderline’ dividing proliferating from differentiating cells shifts towards the anterior of the lens, and cells in this region differentiate prematurely. We propose that these changes deplete the lens epithelium of progenitor cells and arrest lens growth.

Retention of lens fiber cell nuclei, normal mitochondrial degradation, and disrupted expression of autophagy regulatory proteins in the *Snf2h* cKO lens

Lens fiber cell denucleation and the degradation of other subcellular organelles, including mitochondria and ER, mark the terminal

differentiation of lens fibers (Bassnett et al., 2011). The persistence of nuclei in *Snf2h* mutant lens fibers (Fig. 2) was further examined through the detection of free 3′-OH DNA double-strand ends, which are generated by the lens-specific enzyme DNase II β . In the *Snf2h* cKO lenses, both a higher density of lens fiber nuclei and a reduced number of TUNEL-positive nuclei were observed, particularly in the lens cortical area (Fig. S4A–I). This suggested that retention of nuclei in the *Snf2h* cKO lens fiber cells could be caused by reduced expression and/or activity of DNase II β (see below).

Lens organelle degradation has recently been linked to autophagy-related processes (Basu et al., 2014) and mitophagy (Costello et al., 2013). We first examined the degradation of mitochondria (visualized by Tomm20 antibodies) and of ER (visualized by PDI antibodies) in control and *Snf2h*-depleted lenses. We found that both processes occurred normally in the mutant lenses (Fig. 5A,B; data not shown).

Next, we evaluated the expression of the serine/threonine kinase mechanistic target of rapamycin (Mtor) and microtubule-associated protein 1 light chain 3 beta (LC3b; also known as Map11c3b) autophagy proteins in control and *Snf2h* cKO lenses at E16.5, i.e. ~48 h prior to the formation of the OFZ in WT mouse lens (Bassnett, 2009), as well as in newborn lens. In E16.5 control lenses, mTOR was predominantly localized near the basal ends of lens fibers (Fig. 5E). In the corresponding *Snf2h*-depleted lenses, expression of this kinase was both reduced and spatially perturbed (Fig. 5G). In control P1 lenses, mTOR protein was found throughout the lens fibers excluding the OFZ (Fig. 5F). By contrast, in *Snf2h* cKO lenses (Fig. 5H) mTOR protein was unevenly distributed throughout the entire lens fiber cell compartment, including the presumptive nuclear-free zone (NFZ). In E16.5 control lenses, LC3b was found throughout the entire lens (Fig. 5I), whereas in the *Snf2h* cKO there was a notable reduction of LC3b proteins in the lens (Fig. 5K). In control P1 lenses, LC3b proteins were predominantly expressed outside of the NFZ (Fig. 5J). In the absence of NFZ in *Snf2h* mutant lenses, LC3b proteins displayed a disorganized spatial distribution throughout the lens fiber cell mass (Fig. 5L). Finally, western immunoblotting was used to evaluate expression levels of these proteins in lens-containing cellular extracts. Less LC3b protein was present in extracts prepared from mutant tissues than from controls (Fig. 5M). Notably, expression of LC3b form II, which is associated with the autophagosome (Kabeya et al., 2000), was not found in extracts prepared from mutant newborn lens and surrounding tissues. By contrast, expression of mTOR1 appeared to be increased in the *Snf2h*-depleted tissues (Fig. 5M).

Taken together, these data show normal degradation of mitochondria in *Snf2h*-depleted lenses in the presumptive OFZ. However, degradation of nuclei in the presumptive NFZ is not executed and the autophagic flux in the *Snf2h*-depleted lens fiber cell compartment is disrupted.

Molecular analysis of the lens fiber cell denucleation pathway in *Brg1*^{-/-} and *Snf2h*^{-/-}

The SWI/SNF and ISWI complexes regulate gene expression through distinct molecular mechanisms (Kadam and Emerson, 2003; Narlikar et al., 2002; Tang et al., 2010). Nevertheless, similar defects in nuclear degradation were observed in both *Brg1* (He et al., 2010) and *Snf2h* (Figs 2 and 5) cKO lenses. To clarify this, we examined differential gene expression in *Snf2h* cKO eyes using high-density oligonucleotide microarray hybridizations and compared the results with the earlier *Brg1* null lens studies (He et al., 2010). We found 1461 differentially expressed transcripts in the *Snf2h* cKO

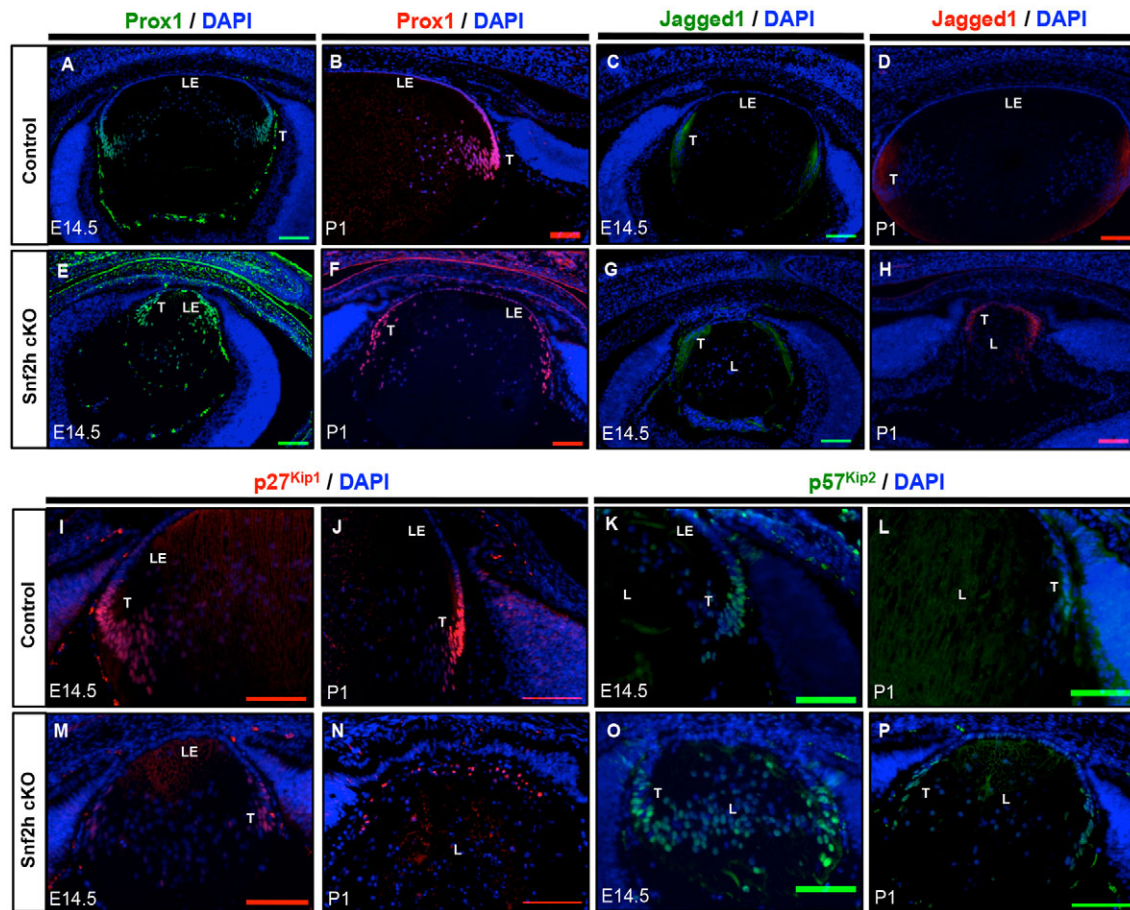


Fig. 4. Depletion of Snf2h disrupts expression of Prox1, Jag1, p27^{Kip1} and p57^{Kip2} in the lens. (A,B,E,F) In the control, Prox1 is expressed in cells undergoing cell cycle exit and at the onset of lens secondary fiber cell differentiation at the transitional zone (T) (A,B). In Snf2h cKO, Prox1 staining is expanded towards the lens anterior, including the middle of the presumptive lens epithelial layer (LE) (E,F). (C,D,G,H) Jag1 is present in cells that are starting to elongate and differentiate. Cells expressing p27^{Kip1} in E14.5 or P1 animals are shown in red (I,J,M,N). Cells expressing p57^{Kip2} in E14.5 or P1 animals are shown in green (K,L,O,P). In the control, the Jag1 staining pattern initiated at the lens equator and expanded towards the posterior of the lens (C,D). In the mutant lens, localization of Jag1 was shifted from posterior to the anterior of the lens (G,H). (I-P) Distribution of the cell cycle inhibitors p27^{Kip1} and p57^{Kip2} in control and Snf2h mutant lenses. Note disorganized localization of both p27^{Kip1} and p57^{Kip2} in the mutated lenses. Nuclei were counterstained with DAPI (blue). L, lens. Scale bars: 100 μm.

eyes, including 902 upregulated and 559 downregulated genes (Fig. 6A). These genes were mostly classified into expected categories such as DNA replication, DNA damage repair, cell cycle control, transcription, and growth signaling response (Table S1). Next, we compared the Snf2h cKO differentially expressed genes with genes differentially expressed in *Brg1* mutant eyes. Using our earlier data (He et al., 2010), re-analyzed using identical statistical criteria ($P < 0.05$ and at least 1.5-fold change), we found a total of 798 differentially expressed transcripts in the *Brg1* mutant eyes. The majority (96%) of the differentially expressed genes were unique to each system. Nevertheless, 92 transcripts were identified as regulated in both systems, including 88 individual genes. In this group, we found downregulation of *Hsf4* and *Dnase2b* mRNAs (Fig. 6B,C), both of which are implicated in lens fiber cell denudation (Cui et al., 2013; Fujimoto et al., 2004; Nishimoto et al., 2003). In addition, retention of nuclei in *Foxe3* mutant lens (Medina-Martinez et al., 2005) is consistent with the reduced expression of FoxE3 observed at both the protein (Fig. 3) and mRNA (Fig. 6C) levels in Snf2h mutant lenses. Among the 1461 differentially expressed genes, dysregulated expression of p27^{Kip1}, p57^{Kip2}, *Cdh1*, *Jag1*, *Prox1* and *Rbpj* in Snf2h cKO eye tissues was independently confirmed by qRT-PCR (Fig. S5).

Transcription of *Dnase2b* could be directly regulated by the transcription factors Hsf4 (Cui et al., 2013) and Pax6 (He et al.,

2010). Hsf4 and Pax6 binding sites are located around positions –160 and –60, respectively, of the *Dnase2b* promoter (Fig. 6D). To test whether they directly regulate the *Dnase2b* promoter in cultured lens cells, we cotransfected the mouse *Dnase2b* promoter fragment (–580 to +180) with cDNAs encoding Hsf4 and Pax6. We found over 3-fold transcriptional activation by both factors in lens cells; however, no transcriptional synergism between these factors was detected (Fig. 6E).

Taken together, the current data show that depletion of either *Brg1* or Snf2h results in the retention of nuclei in lens fiber cells, fewer TUNEL-positive nuclei, and reduced expression of *Hsf4* and of *Dnase2b*, which is a direct target of Hsf4 and Pax6.

DISCUSSION

The goal of the present study was to dissect the *in vivo* roles of Snf2h during lens development using conditional gene targeting, and to compare functions of Snf2h and *Brg1* in this system. We show two major roles of Snf2h in lens formation: (1) Snf2h regulates lens differentiation through maintaining the balance between epithelial and fiber cell differentiation; and (2) Snf2h and *Brg1* are both independently required for lens fiber cell denudation through regulation of at least two common genes essential for this process, namely *Hsf4* and *Dnase2b* (Fig. 7). Comparison of the Snf2h and

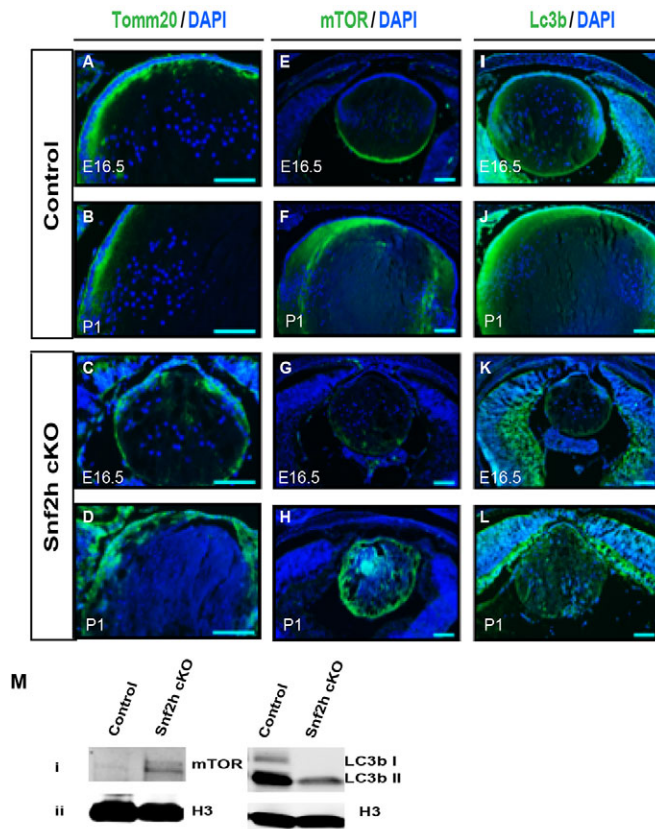


Fig. 5. Degradation of mitochondria and expression of autophagy proteins mTOR and LC3b in E16.5 and P1 control and Snf2h cKO lenses. (A–L) Immunofluorescence analysis of Tomm20, mTOR and LC3b in control and Snf2h cKO eyes. Nuclei were counterstained with DAPI (blue). Scale bars: 100 μ m. (M) Western blot analysis of mTOR and LC3b in extracts prepared from P1 tissues. LC3b I and LC3b II bands represent cytoplasmic and autophagosome forms of the protein, respectively. Histone H3 was used as a loading control.

Brg1 loss-of-function studies in the lens demonstrate that these two chromatin remodeling enzymes play mostly distinct roles (He et al., 2010). This is consistent with the distinct biochemical modes of action of Brg1 and Snf2h ATPases (Erdel and Rippe, 2011; Kadam and Emerson, 2003; Khavari et al., 1993; Narlikar et al., 2002; Tang et al., 2010; Toiber et al., 2013). The role of Snf2h in lens differentiation is related to its recently established function in the control of Purkinje and granule cell progenitor proliferation during cerebellar development (Alvarez-Saavedra et al., 2014). In contrast to the neuronal progenitor cells, loss of Snf2h in lens was not compensated by the induction of Snf2l expression. Thus, our data demonstrate tissue-specific molecular responses following Snf2h depletion, and establish its role as a gatekeeper to assure the timely differentiation of lens fibers.

The earliest morphological abnormality that we found was disrupted polarity of the lens vesicle in Snf2h cKO embryos, as inferred from subsequent differentiation defects (Fig. 7). At E14.5 there are notable differences between control and Snf2h cKO lenses, including reduced size, vacuolization, and disruption of primary lens fiber cell differentiation in mutant lenses. In newborn lens and in the absence of the anterior epithelium, the primary lens fiber cell mass penetrates anteriorly through the bulk lens mass and reaches the cornea. This mass is distinct from corneal-lenticular bridges that originate from incomplete separation of the lens vesicle from the

surface ectoderm, as caused by mutations in genes including *Pax6*, *Foxe3* and *AP-2 α* (Cvekl and Ashery-Padan, 2014). It is noteworthy that reduction of Snf2h expression in *Xenopus* by morpholinos caused similar lens growth and differentiation defects (Dirscherl et al., 2005). Analysis of lens morphology coupled with expression analysis of epithelial markers shows that the presumptive lens epithelial cell layer is markedly reduced at E14.5, and later eliminated due to the premature terminal differentiation of lens precursor cells in Snf2h mutant lenses. The ‘earlier’ cells detected at the anterior pole of the lens vesicle at E14.5 do not display the cuboidal morphology characteristic of the WT lens epithelium. Although these cells initially express E-cadherin, the expression of this crucial structural protein of lens epithelium (Pontoriero et al., 2009) is reduced at E14.5 and abolished by E16.5. In Snf2h cKO lenses, expression of FoxE3 was reduced at E12.5 and E14.5, with no detectable expression of this protein at E16.5. These findings support the idea that the lens precursor cells at the anterior portion of the Snf2h cKO lens vesicle do not differentiate properly into mature lens epithelium. Instead, these anterior cells are converted into abnormal lens fibers.

In WT lenses, the regulatory proteins Prox1 and Jag1, and their targets cyclin kinase inhibitors p27^{Kip1} and p57^{Kip2}, are upregulated in the cells undergoing cell cycle exit and in the early stages of secondary lens fiber cell formation. Their unique temporal/spatial expression patterns are completely disrupted in Snf2h cKO lenses. Expression of p27^{Kip1} and p57^{Kip2} proteins is detected in scattered cells around the anterior pole of the mutant lenses. Interestingly, many features of the Snf2h cKO lenses are comparable to defects found in *Rbpj* lens cKO mutants (Jia et al., 2007; Rowan et al., 2008). These similarities include a disrupted lens polarization/differentiation zone boundary, loss of cell type identity of the presumptive lens epithelium, and perturbed spatial expression of p27^{Kip1} and p57^{Kip2}. Since Snf2h inactivation produced more significant spatial changes in the expression of these genes than *Rbpj* mutants, and expression of *Rbpj* is strongly reduced in the Snf2h mutants, it is possible that Snf2h is genetically upstream of one or more genes encoding components of Notch signaling. It is noteworthy that retention of nuclei and downregulation of *Dnase2b* were also reported in *Notch2* lens mutants (Saravanamuthu et al., 2012).

Our data suggest that Snf2h is required for the denucleation process. Degradation of nuclei is a process unique to lens fibers, erythrocytes and skin keratinocytes. Erythrocytes extrude their nuclei from the individual cells, which are then engulfed and degraded by macrophages (Yoshida et al., 2005). Skin keratinocytes lose their nuclei by a caspase-independent apoptosis-like process (Lippens et al., 2009). The first possibility to consider is that the abnormal differentiation in Snf2h-deficient lens fibers disrupts various ‘late’ differentiation events, including nuclear degradation. We indeed observed downregulation of *Dnase2b* mRNA in both *Brg1* (He et al., 2010) and *Snf2h* (present study) mutant lenses and in *Pax6*^{+/-} lenses (Wolf et al., 2009) and activation of the *Dnase2b* promoter by Hsf4 and Pax6 in cotransfections. Additional experiments are needed to probe the transcriptional control of *Hsf4* and *Dnase2b*, as well as other genes (e.g. p27^{Kip1} and p57^{Kip2}), by Brg1 and Snf2h via ChIP-seq.

The observation that ER and mitochondria are degraded ‘normally’ in Snf2h mutant lenses suggests that degradation of mitochondria, initiated prior degradation of nuclei (Bassnett and Beebe, 1992), is not a prerequisite for denucleation. Degradation of mitochondria in Snf2h cKO lenses indicates that mitophagy (Costello et al., 2013) is active in Snf2h mutant lens fibers. However, in Snf2h-deficient lenses the presumptive NFZ is not established,

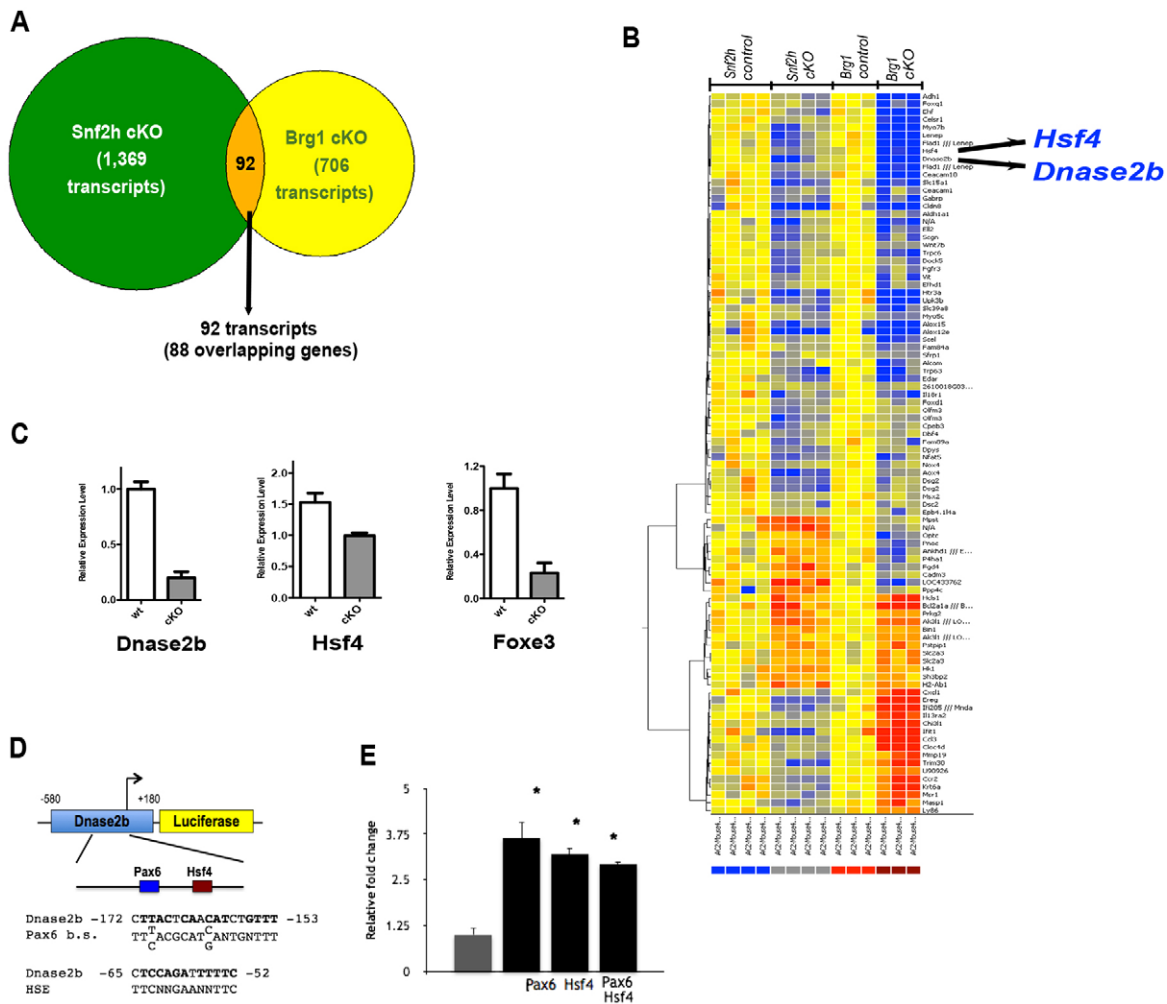


Fig. 6. Comparative analysis of Snf2h-dependent and Brg1-dependent transcriptomes in the eye and transcriptional regulation of *Dnase2b* by Hsf4 and Pax6. (A) Venn diagram comparing RNA profiling of *Brg1* (1461 dysregulated transcripts) versus *Snf2h* (798 dysregulated transcripts) mutant mouse lens. (B) The list of 92 (~4%) common transcripts regulated by both *Snf2h* and *Brg1* contains 88 unique genes, including *Dnase2b* and *Hsf4*. Note that only 62 genes (52 downregulated and 10 upregulated) out of the 92 found were regulated similarly in *Brg1* and *Snf2h* mutant eyes. (C) Reduced expression of *Foxe3*, *Hsf4* and *Dnase2b* mRNAs in *Snf2h* mutant tissues analyzed by qRT-PCR. (D) The *Dnase2b* promoter (–580 to +180) showing Hsf4 and Pax6 binding sites (b.s.), HSE, heat shock element-Hsf4 binding site. (E) Hsf4 and Pax6 activate the *Dnase2b* promoter in cultured lens cells. Data are shown as mean±s.d. (n=3) normalized to Renilla-TK luciferase internal control, and relative fold change in luciferase activity was calculated using the empty cDNA control value set at 1 (gray bar). *P<0.05 (paired Student's *t*-test), cDNA control vector versus Pax6 and/or Hsf4 cDNA.

pointing to disrupted autophagy and/or autophagy-related processes (Basu et al., 2014). The reduction in phosphorylated LC3b proteins supports this possibility. In addition, lamin B phosphorylation mediated by Cdk1, a process that occurs during normal mitosis, is required for denucleation (Caceres et al., 2010; Chaffee et al., 2014). Further experiments are required to probe signaling upstream of JNK/mTOR, the phosphorylation of lamin B by Cdk1, and yet to be

identified steps in the cascade of cellular and molecular events leading to nuclear degradation in lens fibers (Morishita and Mizushima, 2016). For example, a recent study has revealed a novel role of p27^{Kip1}, upstream of Cdk1, in lens fiber cell denucleation (Lyu et al., 2016).

It is important to note that both *Brg1* and *Snf2h* might have additional roles in the denucleation processes. ATP-dependent

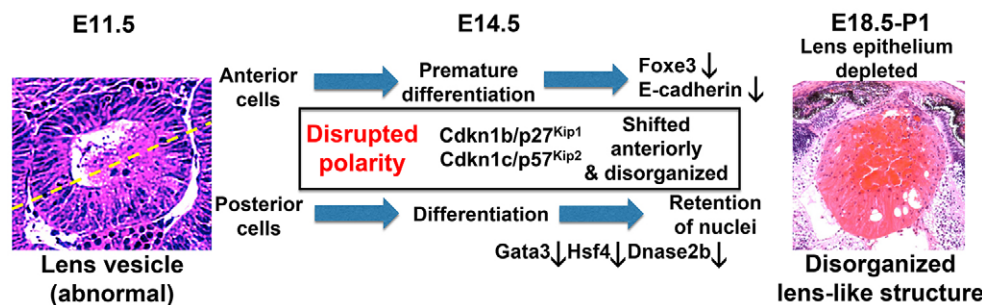


Fig. 7. A multitude of Snf2h functions in lens development. The model proposes that, in the absence of Snf2h, disrupted polarity of the lens vesicle triggers a cascade of events resulting in abnormal lens fiber cell differentiation and premature differentiation of anterior lens epithelial cells. Yellow dashed line indicates division between the anterior and posterior compartment of the lens vesicle.

chromatin remodeling participates in DNA repair (Erdel and Rippe, 2011; Lans et al., 2012; Zhang et al., 2009). SWI/SNF complexes are known to be recruited to phosphorylated H2AX via the interaction between the Brg1 bromodomain and acetylated lysines in histone tails (Lee et al., 2010). Studies of the canonical DNA repair protein Nbs1 (nibrin) in lens (Park et al., 2006), DNA repair-associated proteins Ddb1 (Cang et al., 2006) and Nco6 (Wang et al., 2010), the identification of DNA repair foci in lens fiber cell chromatin through phosphorylated H2AX outside of the OFZ (Wang et al., 2010), and the retention of nuclei in *p53* (*Trp53*) null lenses (Wiley et al., 2011), raise the intriguing possibility that specific components of the DNA repair machinery participate in some aspects of this process using their ‘non-canonical’ activities adopted for the lens environment. Finally, it is possible that Brg1- and Snf2h-containing complexes assist in chromatin degradation in parallel with DNase II β .

MATERIALS AND METHODS

Antibodies

Primary antibodies used for immunofluorescence were anti- α A-crystallin (Santa Cruz Biotechnology, sc-22743, 1:1000), anti-BrdU (BD Biosciences, 347580, 1:500), anti-E-cadherin (BD Biosciences, 610181, 1:200), anti-FoxE3 (a gift from Dr Peter Carlsson, Goteborg University, Goteborg, Sweden; 1:200), anti-histone H3 (Abcam, ab1791, 1:200), anti-jagged 1 (Santa Cruz Biotechnology, sc-8303, 1:200), anti-Ki67 (Abcam, ab15580, 1:200), anti-LC3b (Sigma-Aldrich, L7543-100UL, 1:500), anti-mTOR (Cell Signaling Technology, 7C10, 1:400), anti-p27^{Kip1} (Santa Cruz Biotechnology, sc-528, 1:200), anti-p57^{Kip2} (Santa Cruz Biotechnology, sc-8298, 1:200), anti-PDI (protein disulfide isomerase; Sigma-Aldrich, P7122-200UL, 1:100), anti-Snf2h (Bethyl Laboratories, A301-017A, 1:500), anti-Snf2l (Bethyl Laboratories, A301-086A, 1:500), anti-Pax6 (Covance, PRB-278P-100, 1:500), anti-Prox1 (Abcam, ab37128, 1:500) and anti-Tomm20 (Santa Cruz Biotechnology, sc-11415, 1:100). Secondary antibodies were Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 568 goat anti-rabbit IgG, Alexa Fluor 568 rabbit anti-mouse IgG (A11008, A11011, A11061, respectively, Invitrogen, 1:250) and biotin-conjugated secondary anti-rabbit IgG (Dako, E0466, 1:500).

Conditional inactivation of *Snf2h* in the presumptive lens ectoderm

The *Snf2h* flox allele (in C57BL/6 background) was created through homologous recombination as described elsewhere (Alvarez-Saavedra et al., 2014). The *Snf2h* null allele was obtained by deletion of exons 5 to 9. *Snf2h* cKO (*Snf2h*^{fl/fl}; *Le-Cre*⁺) and their control littermates were generated by crossing the *Snf2h*^{fl/fl} with the *Snf2h*^{+/-}; *Le-Cre*⁺ mice. Primers used for genotyping were (5'-3'): IN4-F13, GTGCAAAGCCAGAGACGATGGTATG; IN4-F14, ACTGAGGACTCTGATGCAAACAGTCAAG; IN5-R3, TACACAATAAGGCAGTGGGTTATAGTGC; IN9A-R35, TCACTAT-ATTTAGAGTCAGATGATCAACTGGTCC. The PCR cycle was as follows: initial denaturation step at 95°C for 3 min; then 35 cycles at 95°C for 40 s, primer annealing at 60°C for 40 s, polymerization at 72°C for 50 s; and a final extension at 72°C for 10 min. The *Le-Cre* transgenic mouse and genotyping are described elsewhere (Ashery-Padan et al., 2000; Wolf et al., 2013). Animal husbandry and experiments were conducted in accordance with the approved protocol of the Institutional Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Noon of the day the vaginal plug was detected and was considered E0.5.

Histological analysis, immunofluorescence, immunohistochemistry and immunoblotting

Animals were euthanized by CO₂ and mouse embryos were dissected from pregnant females. In some cases, whole eyeballs were removed from the postnatal animals. Tissues were then fixed in 10% neutral buffered paraformaldehyde overnight at 4°C, processed and embedded in paraffin. Serial sections were cut at 5 μ m thickness through the mid section of the

lens. Slides were stained with Hematoxylin and Eosin, or used for subsequent experiments. Immunohistochemistry was performed as described elsewhere (He et al., 2010).

Immunofluorescence was performed following standard procedures. Tissues were incubated with primary antibodies overnight at 4°C in a humidified chamber and with the secondary antibody for 1 h at room temperature. Sections were mounted with VECTASHIELD Antifade Mounting Medium (Vector Laboratories). The nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Whole-cell extracts were prepared from lens and surrounding remnants of the anterior segment (it was impossible to isolate the mutant lens) in homogenization buffer [10 mM Tris pH 7.9, 1 mM EDTA, 0.1% SDS and protease inhibitors (Roche)] followed by sonication. The supernatants were analyzed by SDS-PAGE and 4-15% gradient gel (Bio-Rad). Proteins were then transferred to a nitrocellulose membrane. Membranes were blocked using Odyssey blocking buffer in PBS (Li-Cor) for 1 h and incubated with primary antibody overnight at 4°C. The membrane was then washed with TBS (Tris-buffered saline) containing 0.1% Tween 20. Secondary antibody was anti-rabbit IRDye 800 CW (Li-Cor). Bound antibody was imaged using a Li-Cor Odyssey imager. For each experiment, at least two extracts from control and *Snf2h* mutant tissues were analyzed.

RNA expression profiling

Collection of lens and surrounding tissues from P1 eye and total RNA isolation are described elsewhere (He et al., 2010). Four biological replicates of RNAs from different *Snf2h* cKO embryos and their littermates were used. cDNA synthesis and amplifications were performed with Ovation RNA Amplification System V2 (Nugen) using 50 ng total RNA per sample. Amplified cDNAs were cleaned and purified with the DNA Clean & Concentrator-25 Kit (Zymo Research). Fragmentation and labeling were performed using the FL Ovation cDNA Biotin Module V2 (Nugen). The four sets of samples were subsequently hybridized on Mouse Genome 430A 2.0 Arrays (Affymetrix).

Bioinformatic tools and statistical filtering of RNA microarray results

Differentially regulated genes/mRNAs between *Snf2h* knockout and control littermates were identified using biological quadruplicate sets of robust multichip average (RMA)-normalized Affymetrix CEL files (Irizarry et al., 2003) by a combination of Student's *t*-test ($P < 0.05$) and significance analysis of microarrays (SAM; false discovery rate set to <1%), using the TIGR Multiexperiment Viewer of the TM4 microarray software suite (Saed et al., 2003). A similar strategy was used to identify differentially regulated genes/mRNAs in *Brg1* null lenses in newborn eyes (biological triplicates, RMA normalization, by applying Student's *t*-test $P < 0.05$) (He et al., 2010). Primary data were deposited in the NCBI Gene Expression Omnibus database under accession numbers GSE41608 (the *Snf2h* cKO part) and GSE25168 (the *Brg1* cKO part). The GO and KEGG pathway functional annotations were performed using the NIH web-based Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009).

qRT-PCR

cDNA was diluted 10-fold and qRT-PCR was performed with the Applied Biosystems (ABI) 7900HT fast real-time PCR system with Power SYBR Green PCR Master Mix (ABI) as described elsewhere (He et al., 2010). Primers, and genes used for normalization (*B2m*, *Sdha* and *Hprt*), are listed in Table S2.

Transient transfections and reporter gene analysis

A conserved region (-580 to +180) of the mouse *Dnase2b* promoter was synthesized by GenScript and cloned into the pGL3-Basic vector (Promega). *Pax6* and *Hsf4* cDNAs were obtained in the CMV vectors pKW10 and pCMV6-XL6, respectively (Yang et al. 2006; OriGene). Transient co-transfections were conducted in mouse lens epithelial α TN4 cells (Yang and Cvekl, 2005). The cells were transfected using Lipofectamine 2000 (Life Technologies) with 0.5 μ g firefly luciferase reporter plasmid, 200 ng empty control vector (pKW10/pCMV6-XL6) or expression plasmids. As an internal control, 0.25 ng Renilla-TK luciferase

plasmid was included. The promoter activity was measured using the Dual-Luciferase Reporter Assay System (Promega) 30 h following transfection. The experiments were performed in triplicate with two independent repeats.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.H., S.L., R.S.M., Q.X., W.L., J.Z., M.K., A.I.S., T.S. and A.C. designed the experiments. S.H., S.L., R.S.M., Q.X., L.A.B., W.L.K. and J.Z. performed experiments and analyzed data. J.K., R.M., H.H., W.E., R.A.-P., A.I.S. and T.S. developed the mouse models. S.H., R.A.-P., A.I.S., T.S. and A.C. conceived the project and wrote the manuscript. S.H., S.L., R.S.M., Q.X. and J.Z. prepared figures.

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Data availability

Primary microarray data have been deposited in NCBI Gene Expression Omnibus under accession numbers GSE41608 (Snf2h cKO) and GSE25168 (Brg1 cKO).

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.135285/-DC1>

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