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Loss of Sip1 leads to migration defects and retention of ectodermal markers during lens development

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ABSTRACT

SIP1 encodes a DNA-binding transcription factor that regulates multiple developmental processes, as highlighted by the pleiotropic defects observed in Mowat–Wilson syndrome, which results from mutations in this gene. Further, in adults, dysregulated SIP1 expression has been implicated in both cancer and fibrotic diseases, where it functionally links TGFβ signaling to the loss of epithelial cell characteristics and gene expression. In the ocular lens, an epithelial tissue important for vision, Sip1 is co-expressed with epithelial markers, such as E-cadherin, and is required for the complete separation of the lens vesicle from the head ectoderm during early ocular morphogenesis. However, the function of Sip1 after early lens morphogenesis is still unknown. Here, we conditionally deleted *Sip1* from the developing mouse lens shortly after lens vesicle closure, leading to defects in coordinated fiber cell tip migration, defective suture formation, and cataract. Interestingly, RNA-Sequencing analysis on *Sip1* knockout lenses identified 190 differentially expressed genes, all of which are distinct from previously described Sip1 target genes. Furthermore, 34% of the genes with increased expression in the *Sip1* knockout lenses are normally downregulated as the lens transitions from the lens vesicle to early lens, while 49% of the genes with decreased expression in the *Sip1* knockout lenses are normally upregulated during early lens development. Overall, these data imply that Sip1 plays a major role in reprogramming the lens vesicle away from a surface ectoderm cell fate towards that necessary for the development of a transparent lens and demonstrate that Sip1 regulates distinctly different sets of genes in different cellular contexts.

1. Introduction

The ZEB transcription factors – Smad interacting protein 1 (Sip1, ZEB2) and δ-crystallin enhancer binding factor 1 (δEF1, ZEB1) – are characterized by their centrally located homeodomain and two separate clusters of DNA binding zinc-fingers at the N-terminus and C-terminus (Verschueren et al., 1999; van Grunsven et al., 2001; Nelles et al., 2003; Vandewalle

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et al., 2009; Grabitz and Duncan, 2012). Both Sip1 and δEF1 directly bind to 5′-CACCT(G) sequences (found in E2 box elements) with both zinc-finger hands, thus competing with basic helix-loop-helix activators for these sites (Sekido et al., 1994; Remacle et al., 1999; Verschueren et al., 1999). The vast majority of research concerning this gene family focuses on its involvement in epithelial-to-mesenchymal transition (EMT) occurring during wound healing, cancer progression, and fibrosis (Vandewalle et al., 2009).

During EMT, ZEB proteins repress the expression of E-cadherin, P-cadherin, Claudin 4, Connexin 26, and other epithelial specific genes (Vandewalle et al., 2005, 2009; Bindels et al., 2006; Xia et al., 2009). In contrast, Fibronectin, Vimentin, N-cadherin, and other mesenchymal genes are upregulated by the overexpression or induction of ZEB protein expression. The transcriptional changes mediated by the ZEB proteins in this context occur through both direct binding to gene promoters and indirect mechanisms. The myriad of downstream genes controlled by Sip1 contribute to the cytoskeletal changes and increased cell motility that are characteristic of EMT, making Sip1 a critical regulator of this process. However, it is not clear if the functional consequence of altered ZEB expression in pathological situations reflects the entire functional repertoire of these proteins during normal embryonic development. This is particularly relevant for tissues that do not utilize EMT as a means of cellular remodeling during development.

During mammalian embryonic development, Sip1 is first expressed in the gastrula (E8), primarily being detected in the early neural plate, neural crest, and paraxial mesoderm (Espinosa-Parrilla et al., 2002; Van de Putte et al., 2003). Homozygous germ line loss of Sip1 protein function leads to a lack of neural tube closure and neural crest migration leading to death by E9.5 in the mouse (Van de Putte et al., 2003). Conditional deletion of *Sip1* in mice later in development has revealed important roles for Sip1 in the development of hematopoetic stem cells, motor neurons, oligodendrocytes, neocortical neurons, the hippocampus, and pain transmission by dorsal root ganglia neurons (Seuntjens et al., 2009; Jeub et al., 2011; Goossens et al., 2011; Weng et al., 2012; Miquelajauregui et al., 2007). Notably, while heterozygous *Sip1* null mice appear normal, heterozygous mutations in the human *SIP1* gene result in Mowat–Wilson syndrome, a pleiotropic developmental disorder typified by mental retardation coupled to diverse developmental defects with variable penetrance, including a lack of intestinal innervation (Hirschsprung's disease), heart malformations, urogenital defects, and eye defects, including micropthalmia and cataract (Garavelli et al., 2003; Mowat et al., 2003; Bassez et al., 2004; Adam et al., 2006; Garavelli and Mainardi, 2007; Ariss et al., 2012; Zweier et al., 2005).

Consistent with the eye defects seen in Mowat–Wilson syndrome patients, *Sip1* mRNA is detected in the lens at E9.5, shortly after lens induction (Yoshimoto et al., 2005), and continues to be expressed in all the cells of the lens vesicle, becoming localized mainly to the lens epithelium and young fiber cells as the lens matures. In adult mice, Sip1 protein is detected in both the peripheral lens epithelium and cortical fibers as well as in the inner nuclear layer and occasional ganglion cells in the adult retina (Grabitz and Duncan, 2012). Notably, while the lens does not undergo EMT during normal development, conditional deletion of the *Sip1* gene when the early lens is specified from the head ectoderm results in primary defects in lens vesicle closure associated with defects in FoxE3 expression and subsequent defects in fiber cell differentiation (Yoshimoto et al., 2005). However, it is unclear from these data if the fiber cell differentiation defects are secondary to the lack of vesicle closure and if Sip1 has distinct regulatory roles in these two separate events. Further, how the requirement for Sip1 in lens development relates to its function in other developmental contexts or in diverse pathologies, including cancer, also remains elusive. Here, we delete *Sip1* from the lens shortly after lens vesicle closure, and find that Sip1 regulates multiple genes that are generally distinct from those regulated by Sip1 during cancer and fibrosis, including those whose expression is prominent in the early head ectoderm as well as the corneal epithelium, conjunctiva, and epidermis later in development. This implies that Sip1 is a multi-faceted transcription factor that utilizes specific cues to regulate its function in different cellular contexts.

2. Results

2.1. *Sip1 protein is expressed in the developing lens epithelium*

Sip1 mRNA is expressed in the mouse lens placode starting at E9.5 with maintained lens expression until E13.5 (Yoshimoto et al., 2005) and we have shown that Sip1 protein is expressed in the lens epithelium and transition zone of adult mice (Grabitz and Duncan, 2012). Here, we show that that Sip1 protein is not detectable by immunostaining in the lens placode at E9.5 (Fig. 1A), but becomes easily detectable in the posterior aspect of the lens vesicle at E10.5 in cells fated to become primary lens fiber cells (Fig. 1B). As the lens vesicle matures, Sip1 is lost in the most central lens fiber cells beginning at E12.5 (Fig. 1C) and becomes uniformly expressed in the lens epithelium by E14.5 (Fig. 1D). By E16.5, however, Sip1 protein is no longer found in the central epithelium, but is maintained in the peripheral epithelium (Fig. 1E). Restriction of Sip1 expression to this region of the epithelium continues in later post-natal time points (Fig. 1F). In the adult, Sip1 remains localized in the peripheral epithelium and cortical fibers (Fig. 1G and H), with lower levels found in the central epithelium (Fig. 1I).

2.2. *Deletion of Sip1 in the lens results in cataract formation and abnormalities in fiber cell organization due to early defects in coordinated cell migration*

In order to clarify the function of Sip1 in lens fiber cell differentiation, we used a previously described conditional allele (Fig. 2A) (Higashi et al., 2002) to remove *Sip1* from the developing lens using MLR10 Cre, which is first active in the lens vesicle at E10.5 (Zhao et al., 2004). PCR analysis confirmed that *Sip1* exon 7 was not detectable in adult lens DNA (Fig. 2B) and immunohistochemistry showed that Sip1 protein expression was significantly reduced by E10.5 (Fig. 2D).

In the adult, *Sip1* cKO lenses are opaque (Fig. 3A and B) and dark field analysis shows profound defects in lens shape and size (Fig. 3C and D). The fiber cell structure of these lenses is abnormal (Fig. 3E and F) with major disruptions in the actin