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# Rap1 GTPase is required for mouse lens epithelial maintenance and morphogenesis

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ABSTRACT

Rap1, a Ras-like small GTPase, plays a crucial role in cell – matrix adhesive interactions, cell – cell junction formation, cell polarity and migration. The role of Rap1 in vertebrate organ development and tissue architecture, however, remains elusive. We addressed this question in a mouse lens model system using a conditional gene targeting approach. While individual germline deficiency of either Rap1a or Rap1b did not cause overt defects in mouse lens, conditional double deficiency (Rap1 cKO) prior to lens placode formation led to an ocular phenotype including microphthalmia and lens opacification in embryonic mice. The embryonic Rap1 cKO mouse lens exhibited striking defects including loss of E-cadherin- and ZO-1-based cell – cell junctions, disruption of paxillin and β1-integrin-based cell adhesive interactions along with abnormalities in cell shape and apical – basal polarity of epithelium. These epithelial changes were accompanied by increased levels of α-smooth muscle actin, vimentin and N-cadherin, and expression of transcriptional suppressors of E-cadherin (Snai1, Slug and Zeb2), and a mesenchymal metabolic protein (Dihydropyrimidine dehydrogenase). Additionally, while lens differentiation was not overtly affected, increased apoptosis and dysregulated cell cycle progression were noted in epithelium and fibers in Rap1 cKO mice. Collectively these observations uncover a requirement for Rap1 in maintenance of lens epithelial phenotype and morphogenesis.



## 1. Introduction

Morphogenesis of vertebrate organs is critically dependent on temporally and spatially coordinated events including specialized cell – cell junction formation, cell – extracellular matrix (ECM) adhesive interactions, establishment of cell polarity and migration (Boettner and Van Aelst, 2009; Bulgakova et al., 2012; Franke, 2009; Nelson, 2009). Importantly, defects in these cellular characteristics can lead to various phenotypes and diseases (Franke, 2009; Halbleib and Nelson, 2006). Identifying the molecular mechanisms regulating cell adhesive interactions, cell polarity and migration is therefore not only necessary for disease understanding, but is also expected to provide novel insights for therapeutic manipulation of structural and functional defects in certain tissues and organs. Rap1 GTPase, a Ras-like small GTPase, is considered to be a crucial player in controlling formation and stability of E-cadherin- and ZO-1-based adherens junctions (AJ) and tight junctions (TJ), respectively, and integrin-mediated cell – ECM adhesion, cell polarity and migration (Asha et al., 1999; Boettner and Van Aelst, 2009; Glading et al., 2007; Hogan et al., 2004; Knox and Brown, 2002; Kooistra et al., 2007; Severson et al., 2009). Rap1 is a widely expressed small GTPase that integrates signals from external cues and receptors to regulate cell adhesive interactions, actin cytoskeletal reorganization and intracellular regulatory pathways (Boettner and Van Aelst, 2009; Kooistra et al., 2007). Analogous to other small GTPases, Rap1 acts as a molecular switch by cycling between inactive GDP-bound and active GTP-bound forms, with the transition between these two conformations being tightly controlled by specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. Several multi-domain GEFs which activate Rap1 and cell junction formation have been identified and characterized,

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including C3G, Epacs, PDZ-GEFs, RapGAPs and DOCK4 (Gloerich and Bos, 2011). Likewise, a number of downstream effector proteins work in concert with Rap1 to regulate cell adhesive interactions, including afadin/AF6, KRIT/CCM1 and RIAM (Boettner et al., 2000; Glading et al., 2007; Liu et al., 2011; Mandai et al., 2013). Inhibition of Rap1 has been demonstrated to impair AJs formation and disrupt integrin-based cell adhesive interactions, and conversely, activation of Rap1 strengthens cell – cell junctions and cell adhesion (Kooistra et al., 2007). Cell – cell junctions (TJs, AJs) and integrin-linked focal adhesions are considered to be important sites for anchoring and organization of the actin cytoskeleton (Meng and Takeichi, 2009; Rikitake et al., 2012; DeMali et al., 2003; Mitic and Anderson, 1998), and Rap1 has been demonstrated to regulate actin cytoskeletal organization at cell – cell junctions by controlling the activities of different Rho GTPases and myosin II (Ando et al., 2013; Arthur et al., 2004; Boettner and Van Aelst, 2007; Fukuyama et al., 2006; Jeon et al., 2007; Ogita and Takai, 2006). In our previous studies, although cell – cell junctions and cell adhesive interactions regulated by E-cadherin and N-cadherin (Lang et al., 2014; Pontoriero et al., 2009), and Rho GTPases were found to be crucial for lens development and morphogenesis (Chauhan et al., 2011; Maddala et al., 2011a; Maddala et al., 2008), the role of Rap1 in lens morphogenesis and architecture remains unexplored.

Moreover, although a vast amount of knowledge is available regarding the mechanisms by which Rap1 regulates cellular processes (Boettner and Van Aelst, 2009; Choi et al., 2013; Kooistra et al., 2007; Liu et al., 2011; O'Keefe et al., 2012; Sawyer et al., 2009), little is known at large about Rap1 function in vertebrate organ development, morphogenesis and tissue architecture. Additionally, functional redundancy of the two isoforms of Rap1 (Rap1a and Rap1b) poses a major impediment for the ability to thoroughly delineate the biological role of Rap1 (Chrzanowska-Wodnicka, 2013; Frische and Zwartkruis, 2010; Li et al., 2007). Early attempts to explore Rap1 role in vertebrate morphogenesis and tissue architecture were also thwarted by embryonic lethality noted in model systems lacking both isoforms of Rap1 (Chrzanowska-Wodnicka et al., 2008; Li et al., 2007). Therefore, it is necessary to design a model system presenting conditional deficiency of Rap1 to determine how double deficiency of Rap1a and Rap1b might impact organ morphogenesis and tissue architecture. In this study we targeted Rap1 (Rap1a and Rap1b) expression in a conditional manner to identify the definitive and mechanistic role of Rap1 in ocular lens morphogenesis and architecture.

Vertebrate lens morphogenesis, which is initiated from a single cell type (Chow and Lang, 2001; Cvekl and Ashery-Padan, 2014; McAvoy et al., 1999), provides a unique model system to investigate the role of Rap1 regulated cell – cell junctions and cell adhesive interactions in the specification, polarization and proliferation of epithelial cells, their differentiation into fiber cells, and in the migration and arrangement of mature fiber cells. The ocular lens is an avascular transparent tissue with no innervation, comprised only of epithelial cells that continuously proliferate and differentiate into specialized fiber cells, all encased by a thick basement membrane, the lens capsule. The lens develops from ectoderm that overlies the optic vesicle, a process that involves transition of the surface ectoderm through the lens placode, pit and vesicle stages (McAvoy et al., 1999). At the lens vesicle stage, the posterior half of the cells elongate and differentiate into primary fibers, whereas cells in the anterior half differentiate into epithelial cells that cover the apical ends of the fiber cells and develop lens distinctive polarized structure. Proliferation in the adult lens is restricted to the epithelium, mostly in the germinative zone above the lens equator, and progeny migrate below the equator where they elongate and differentiate into secondary fiber cells that progressively become added to the primary fiber mass. Like primary fibers, secondary fibers are also highly polarized with their apical ends associated with the overlying epithelium and the posterior terminals attaching to the inner surface of the extracellular matrix-enriched elastic capsule. Since we possess a good understanding of the temporal sequence of events in lens morphogenesis (Chow and Lang, 2001; Cvekl and Ashery-Padan, 2014; Lovicu and McAvoy, 2005), here we sought to determine the role of Rap1 in lens development and architecture, using the developing lens as a model system for organogenesis. Rap1 deficient conditional mice (Rap1 cKO) generated in this study exhibit a lens phenotype at embryonic stages, epithelial plasticity and mesenchymal transition accompanied with loss of AJs and ZO-1-based cell junctions in conjunction with upregulation of E-cadherin suppressing transcription factors, demonstrating the requirement of Rap1 in the establishment and maintenance of epithelial phenotype and morphogenesis of lens.

## 2. Materials and methods

### 2.1. Animal Maintenance and Use

Animals were maintained in a pathogen-free vivarium under a 12 h dark and light cycle with ad libitum food and water. All experiments using mice were carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Association for Research in Vision and Ophthalmology. The protocol was approved by the Committee for Ethics of Animal Experiments at the Duke University School of Medicine. At required gestational ages, fetuses were removed by hysterectomy after the dams had been anesthetized with Euthasol.

### 2.2. Rap1a and Rap1b null mouse eyes

Eyes from adult Rap1a$^{-/-}$ (Li et al., 2007) and Rap1b$^{-/-}$ (Chrzanowska-Wodnicka et al., 2008) null mice on a C57BL/6 genetic background, fixed in 3.7% buffered formalin, were generously provided by the laboratories of Magdalena Chrzanowska-Wodnicka from the Blood Research Institute, Blood Center of Wisconsin, Milwaukee, WI, and Lawrence Quilliam from the Department of Biochemistry and Molecular Biology, Indiana University of School of Medicine, Indianapolis, IN, respectively.

### 2.3. Generation of Rap1 (Rap1a/Rap1b) GTPase conditional deficient mice

To generate Rap1a/Rap1b double conditional deficient mice (Rap1 cKO), the well characterized Rap1a and Rap1b homozygous double floxed mice (Rap1a$^{ff}$/Rap1b$^{ff}$ with 129S/C57BL6 mixed background; Listed in the Jackson Laboratory as strain B6;129S-*Rap1a*$^{tm1Morz}$*Rap1b*$^{tm1Morz}$/J) described earlier (Pan et al., 2008) were mated with Le-Cre transgenic mice (C57BL6 background). The resulting heterozygous offspring were inbred for 4 generations, until we could generate the progeny carried with Rap1a$^{ff}$/Rap1b$^{ff}$/Cre genotype. The homozygous double floxed Rap1a$^{ff}$/Rap1b$^{ff}$ mice used in this study contain loxp sites flanking exon 2-3 (in Rap1a) and exon-1 (in Rap1b) and are viable, fertile and do not display any abnormalities (Pan et al., 2008). Le-Cre transgenic mice used in this study express Cre recombinase at embryonic day 8.75 under the control of a Pax6 P0 enhancer/promoter, with Cre being expressed in lens epithelium and fiber cells as well as other surface ectoderm-derived eye structures (Ashery-Padan et al., 2000). The Le-Cre mice also express a Cre-activatable GFP reporter that is incorporated into the Cre transgene. For comparison with Rap1 cKO mice, we used age-matched