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# Dual function of Yap in the regulation of lens progenitor cells and cellular polarity

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ABSTRACT

Hippo-Yap signaling has been implicated in organ size determination via its regulation of cell proliferation, growth and apoptosis (Pan, 2007). The vertebrate lens comprises only two major cell types, lens progenitors and differentiated fiber cells, thereby providing a relatively simple system for studying size-controlling mechanisms. In order to investigate the role of Hippo-Yap signaling in lens size regulation, we conditionally ablated Yap in the developing mouse lens. Lens progenitor-specific deletion of Yap led to near obliteration of the lens primarily due to hypocellularity in the lens epithelium (LE) and accompanying lens fiber (LF) defects. A significantly reduced LE progenitor pool resulted mainly from failed self-renewal and increased apoptosis. Additionally, Yap-deficient lens progenitor cells precociously exited the cell cycle and expressed the LF marker, β-Crystallin. The mutant progenitor cells also exhibited multiple cellular and subcellular alterations including cell and nuclear shape change, organellar polarity disruption, and disorganized apical polarity complex and junction proteins such as Crumbs, Pals1, Par3 and ZO-1. Yap-deficient LF cells failed to anchor to the overlying LE layer, impairing their normal elongation and packaging. Furthermore, our localization study results suggest that, in the developing LE, Yap participates in the cell context-dependent transition from the proliferative to differentiation-competent state by integrating cell density information. Taken together, our results shed new light on Yap's indispensable and novel organizing role in mammalian organ size control by coordinating multiple events including cell proliferation, differentiation, and polarity.



## Introduction

One of the intriguing questions in organogenesis is how cells constituting an organ know when to either divide or stop proliferating in order for them to achieve a particular organ size and maintain a steady-state number of cells within the cell population. The Hippo-Yap (Yes-associated protein) signaling pathway has been shown to regulate cell proliferation and apoptosis during development (Edgar, 2006; Harvey and Tapon, 2007). Core components of the signaling pathway comprising two serine/threonine kinases, Mst1/2 (*Hippo*) and Lats1/2 (*Warts*), negatively regulate transcriptional cofactor Yap (*Yorkie*) by phosphorylating and sequestering it in the cytoplasm (Zhao et al., 2007). In the absence of Hippo upstream signaling, hypophosphorylated Yap translocates to the nucleus where it binds to DNA with sequence-specific transcription factor TEAD (*Scalloped*) and activates the transcription of target genes such as cyclin E and Diap, which stimulate cell proliferation and prevent apoptosis, respectively (Vassilev et al., 2001). Yap also contains multiple protein-protein interaction domains including PDZ- and SH3-binding, coiled-coil and WW, suggesting pleiotropic functions (Sudol et al., 2012). More recent findings implicate the Hippo-Yap pathway in cell-cell contact-mediated control of proliferation in cancer cells and normal developing tissues (Varelas et al., 2010; Zeng and Hong, 2008; Zhao et al., 2007). In addition to regulating proliferation via cell density-dependent nuclear localization, Yap also physically interacts with adherens and tight junction associated proteins including α-Catenin, E-Cadherin, NF2 (*Merlin*), Amot (Angiomotin) and Crb (*Crumbs*). Based on these observations, Yap has been proposed to play major roles in conveying contact inhibition signals from the cell surface to the nucleus via Hippo pathway regulation (Kim et al., 2011; McClatchey and Fehon, 2009; Schlegelmilch et al., 2011; Varelas et al., 2010)

The lens is composed of two populations of cells: anteriorly-located LE and posterior LF cells. LE cells form a thin layer, secrete extracellular matrix proteins which surround the entire lens, and

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constitute progenitor cells (Cvekl and Duncan, 2007; Graw, 2010; Lovicu and McAvoy, 2005; Martinez and de Iongh, 2010; Sue Menko, 2002). LF cells constitute the majority of the lens and are thin, transparent, fully differentiated, and firmly packed cells. Primary LF cells derive from the posterior end of the lens vesicle epithelium. Secondary LF cells are generated by lens progenitor cells in LE, which undergo extra cell divisions at germinative zone (GZ) followed by cell cycle exit at the transition zone (TZ). Cells in GZ comprise transient amplifying 5-bromo-2′-deoxyuridine (BrdU) (+) progenitor cells, which then exit the cell cycle at TZ as indicated by the expression of, p57 and Prox1, two postmitotic markers. During development, the entire LE serves as GZ, and narrows down into a smaller area located just anterior to the TZ. Differentiating LF cells generated from TZ undergo dramatic cellular changes including bi-directional elongation, production of massive amount of proteins such as Crystallins, and degradation of cellular organelles (Andley, 2007). These new-born secondary LF cells constitute the majority of the lens cells by a mechanism that involves their successive addition to the preexisting LF layer while the primary LF cells form a centrally located nucleus of the lens.

Owing to its simple and unique anatomical nature and well-established, easily traceable sequences of cellular events, including transcriptional networks driving cell proliferation or differentiation (Ogino et al., 2012), the lens serves as one of the best tissue models in which to study growth, development, and differentiation mediated by the Hippo-Yap pathway. Based on the earlier observation that NF2 is crucial for cell cycle exit regulation in TZ of developing lens along with Yap′s enriched expression in LE, we hypothesized that Yap may play an essential function in the proliferation of lens progenitors and differentiation. In order to test this, we have deleted Yap in the early developing lens, when major lens growth is occurring but after the lens vesicle has formed. Our results demonstrate that Yap activity is essential for the maintenance of progenitor status in LE through preserving self-renewal and inhibiting apoptosis and precocious differentiation. Unexpectedly, we also found that Yap plays a crucial role in maintaining lens epithelial and fiber morphology via stabilizing apical polarity complex and junction proteins. Furthermore, the Yap localization results showing elevated apical junctional association in a cell proliferation to differentiation dependent manner in the developing lens may suggest Yap′s pivotal role in regulating lens growth via cell-cell contact inhibition.

In summary, our genetic study revealed Yap′s critical function in maintaining the lens progenitor pool and polarized architecture as a potential mechanism of organ size regulation

## Results

### *Yap expression and localization demarcate lens progenitor and early post-mitotic cells*

Expression and cellular localization of Yap and its Ser127-phosphorylated form (pYap) during lens development were determined by immunofluorescence (IF) staining and in situ hybridization (Fig. 1 and Supplementary Fig. S1). Anti-Yap antibody recognizes all forms of Yap including pYap, while anti-pYap antibody is specific to Yap proteins phosphorylated at Serine 127 in human Yap (equivalent to Ser112 in mouse), a target site of the Hippo kinase cascade. Upon Serine 127 phospholylation, cytoplasmic retention of pYap is facilitated by 14-3-3 binding (Zhao et al., 2010). At mouse developmental stage embryonic day 11.5 (E11.5), Yap proteins in the optic vesicle exclusively localized to the nucleus in the majority of LE cells (located in the anterior side of the lens vesicle) and was excluded from the primary LF cells (Supplementary Fig. S1). This pattern of expression is maintained throughout the embryonic stages (Fig. 1A and Supplementary Fig. S1). In late postnatal lenses, Yap expression in the LE gradually decreases while it is maintained in the TZ, suggesting an essential function involving lens progenitor cells and cell cycle exit in TZ (Supplementary Fig. S1). At E15.5, Yap proteins are localized in both the nucleus and cytosol of LE cells, as demonstrated by anti-Yap and anti-pYap antibodies, respectively (Fig. 1A–D, F and G). Nuclear Yap localization in lens progenitor cells is further supported by the partial co-localization with BrdU, a marker for S-phase cells (Fig. 1C–E). pYap proteins largely did not co-localize with BrdU, validating the specificity for detecting non-nuclear Yap (Fig. 1F–H). However in the TZ, where BrdU staining is absent, nuclear staining is nearly completely lost although cytoplasmic staining is clearly enriched (Fig. 1A–H). In addition to the expected nuclear and cytoplasmic localization of the Yap, we noticed some Yap and pYap proteins preferentially localized at the apical surface of the LE and TZ cells (Fig. 1A–D, F and G). In summary, Yap is expressed exclusively in LE and TZ, and repressed in differentiating LF cells throughout the lens development. Within progenitor cells in LE, Yap localizes to three distinctive compartments: nucleus, cytosol and apical surface.

### *Conditional Yap ablation disrupts normal lens growth and differentiation*

In order to directly address the function of Yap in lens development and growth, we took a conditional knock out (CKO) approach using Nestin-Cre, where Cre proteins are expressed in the developing LE starting at/around E12 when lens vesicle formation is almost complete (Cammas et al., 2012; Cang et al., 2006; Yang et al., 2000). Because primary LF cells are unlikely to be targeted by this approach and LE cells normally differentiate into secondary LF cells after cell cycle exit in TZ, the central phenotypes are expected to be observed in LE and secondary LF cells. Outside of the lens, $Yap^{f/f}$; Nestin-Cre (Yap CKO) litters did not display any distinctive abnormality except diminished eye pigmentation at P0 (Supplementary Fig. S1). The lens size was most severely reduced while the eyeball was often slightly smaller than that of wild type (WT) littermates (Supplementary Fig. S1). We then determined whether (and when) Nestin-Cre mediated Yap gene ablation sufficiently eliminates Yap proteins in the lens. As shown in Fig. 1I and J, immunohistochemical staining of Yap clearly demonstrated the absence or severe reduction of Yap proteins specifically in Yap CKO LE and TZ as early as E14.5. When anti-pYap antibody was used, the striking reduction of pYap staining was also observed (Supplementary Fig. S1), indicating the absence of both forms of Yap proteins in Yap CKO lenses. To examine the global and stage-specific abnormalities of Yap CKO lenses, histological analysis was performed with Hematoxylin and Eosin (H&E) staining. The Yap-deleted lens was generally unaffected before E14.5 except minor thinning of the LE and slight reduction of the total lens area (20%, $n=4$) was observed (Fig. 1K and L). At E16.5, the lens was smaller than in WT (57%, $n=4$) and the structural deformation expanded to the LF layer in addition to the severe thinning of LE (Fig. 1M and N). Compared to that of control littermates, cell density in LE was sparse and cell shape was altered from cuboidal to near-squamous (Fig. 1L, N, and P). The LF layer also appeared disorganized and degenerative, including the formation of vacuoles. Secondary LF cells failed to pack around primary LF cells, as these cells formed a sublayer in the posterior side of the lens (Fig. 1N arrows). At E18.5, LE cell density was further decreased as cell flattening intensified (Fig. 1O and P). Vacuolization in LF also became more evident. In addition, the overall lens was much smaller. At P0 and later, we also frequently observed the eyeball without any noticeable lens. Collectively, our gross morphological