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, organelar polarity disruption, and disorganized apical polarity complex and junction proteins such as Crumbs, Palβ1, 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.

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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...
constitute progenitor cells (Cvekl and Duncan, 2007; Graw, 2010; Lovicu and McAvoy, 2005; Martínez and de Jongh, 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 models in which to study growth, development, and differentiation, it was 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, 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 phosphorylation, 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, Yapf/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. 1I, 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
and histological analysis of Yap CKO clearly demonstrates the absolute requirement of Yap function in normal lens development, including growth and patterning.

Yap is essential for the maintenance of lens progenitor state

Our expression and localization studies demonstrated the presence of Yap in the nucleus of lens progenitor cells in the LE, which is defined as total cells in the epithelium excluding cells undergoing cell cycle exit at TZ. However, soon after LE cells undergo cell cycle exit in TZ and prior to differentiating into LF cells, Yap’s major localization was switched from the nucleus to the cytoplasm and junction (Fig. 1A–D). In addition, early-stage differentiating LF cells located posterior to the equator sharply attenuated the level of Yap. Along with specific expression and nuclear localization in WT progenitors, the thinning and hypocellularity in Yap CKO LE suggest Yap functions in controlling the proliferation of lens progenitor cells. In order to address this, we first determined the total number of LE cells in the Yap CKO and compared it to that of WT. We examined LE at E14.5 and E16.5 because the severity of the above-mentioned phenotypes dramatically increased between these two days. As shown in Fig. 2, total LE cell numbers, determined by counting total Hoechst dye (+) cells in E14.5 and E16.5 Yap CKO lenses, respectively (Fig. 2C, D, G, H and J). When we used phospho-Histone H3 (pH3) antibody to mark the subset of cells undergoing mitosis, we obtained similar progressive reductions (70.94% (p < 0.05) and 100% (p < 0.005)) at E14.5 and E16.5, respectively (Fig. 2C, D, G, H and J). In addition, we observed an increase of cleaved caspase (CC) 3 (+) cells specifically in Yap CKO lenses at E14.5, indicating that at least some portion of the hypocellularity seen in Yap CKO lens is caused by caspase-mediated apoptosis (Fig. 2K and L). In summary, the defective self-renewal capability and increased apoptosis in Yap-deficient lens progenitor cells are the main contributors to the development of hypocellularity in Yap CKO lenses.

Yap prevents precocious cell cycle exit, premature differentiation, and is essential for normal physiology

Next, we reasoned that the reduced pool of lens progenitor cells in Yap CKO might have resulted from precocious differentiation of LE progenitor cells. To test this possibility, we examined cell cycle exit markers such as Prox1 and p57. In WT, Prox1 is strongly expressed in TZ cells undergoing cell cycle exit and differentiating LF cells at E16.5 (Fig. 3A). Similarly, p57 is also highly expressed in TZ cells although p57 was further downregulated in mature LF cells (Fig. 3C). Intriguingly, weak levels of Prox1 and p57 were also observed in subsets of
WT LE cells (Fig. 3A, A1, C and C1). In Yap CKO lens, the increased level of both Prox1 and p57 were clearly demonstrated in LE (white arrows) and LF (white arrowheads), respectively, are marked for comparison with those from CKO (red arrows). (E–F) Analysis of LF marker, β-Crystallin (red), in WT and Yap CKO lenses at E16.5. Asterisks indicate the equators. Dotted lines indicate the LE layer. β-Catenin and Pax6 (green, A–B1 and E and F, respectively) stainings are provided to identify the cellular and global morphology of the LE and lens. (G–H1) IF analysis of extracellular matrix protein, Nidogen, in LE (arrows) and LC (arrowheads) in WT and Yap CKO lenses, respectively at E16.5. Dotted line indicates the apical margin of LE. Scale bars; 50 μm.

WT LE cells (Fig. 3A, A1, C and C1). In Yap CKO lens, the increased level of both Prox1 and p57 were clearly demonstrated in LE (Fig. 3B, B1, D and D1). The expression level of Prox1 and p57 in Yap CKO LE was comparable to that of WT TZ cells undergoing cell cycle exit. We then investigated whether Yap-deficient LE cells show any signs of premature differentiation using antibody against β-Crystallin, a gene normally expressed in fully differentiated LF cells (Shaham et al., 2009). In WT lens, β-Crystallin is exclusively expressed in all mature LF cells and not in immature LF or LE cells (Fig. 3E and E1). Consistent with the notion that Yap is a guardian of premature differentiation, Yap-deficient LE cells exhibited precocious β-Crystallin expression in both immature LF and LE cells (Fig. 3F and F1). LE and LF cells produce and secrete basement membrane proteins, including Nidogen, to the basal surface of the cell, forming a ring-like layer called the lens capsule (LC) that surrounds the entire lens (Fig. 3G and G1; (Danysh and Duncan, 2009; Dong et al., 2002)). In the Yap CKO lens, Nidogen production in LE cells was greatly reduced and, likewise, its distribution in the anterior LC was prominently diminished, providing a
further evidence of LE cell abnormality (Fig. 3H and H1). In summary, Yap in the LE is required to prevent precocious differentiation of LE cells into LF cells via suppression of cell cycle exit genes such as Prox1 and p57.

Yap is indispensable for correct localization of polarity proteins, the maintenance of cell shape and organellar polarity

It has been proposed that epithelial junctions control the localization of Yap through direct interaction with Crb polarity complex proteins including Pals1, Patj and Amot. These interactions via the WW-domain and PDZ binding motif of Yap are important for epithelial cell density sensing at the apical junction of high-density Eph4 cell, a mouse mammary epithelial cell line. Interestingly, our Yap expression study yielded unexpected apical junction localization where Crb apical polarity complex proteins are localizing in the developing LE (Fig. 1). In order to investigate a potential interaction between Yap and Crb polarity complex in the developing lens, we first examined whether the localization of Yap protein using mouse monoclonal Yap antibody (ab56701; Abcam) overlaps with that of apical junctional complex proteins such as Pals1 and ZO-1 along the apical surface of the LE, most prominently in the TZ (Fig. 4A–B3).

Next, we examined whether Yap controls the localization of Crb and Par polarity complex proteins. Normal LE cells adopt a simple cuboidal epithelial shape (Fig. 4C), which later acquire a thin, elongated morphology as differentiation to LF cells proceeds from the TZ. In Yap CKO lens, LE cells were generally flattened compared to WT (Fig. 4D, I–P). Although cell shape change might have occurred due to hypocellularity in Yap CKO lens, it also may have resulted from the loss of epithelial polarity. In order to investigate the effects of Yap deletion on the polarity complex and cell adhesion proteins, we used IF assays using specific antibodies for apical proteins associated with adherens and tight junctions (ZO-1, Crbs, Pals1 and Par3). In WT lens, ZO-1 strongly localizes to the apical surface of LE, TZ and LF cells (Fig. 4C, C1 and C2; (Nielsen et al., 2003)). Consistent with our prediction, Yap-deleted LE cells lost the apical localization of ZO-1, a tight junction associated protein, throughout the lens (Fig. 4D–D2). In addition, the apical localization of Par and Crb polarity complex proteins (Par3, Crbs and Pals1) was partially disrupted or randomized (Fig. 4E–H2 and data not shown). In addition to the cell shape change in Yap CKO LE (Fig. 4I–L), we also noticed the change of nuclear shape and size in Yap CKO lens. Hoechst dye (+) nuclei of WT LE cells are oval while those of Yap CKO are clearly flattened (Fig. 4I, J, M and N).

Fig. 4. Yap is necessary for the establishment and/or maintenance of apical polarity complex, and cell and nuclear shape. (A and B) Double antibody staining of Yap (green) with ZO-1 (red, A–A3) and Pals1 (red, B–B3) in WT developing LE at E14.5 and E13.5, respectively. (C–H2) Antibody staining of junctional polarity proteins, ZO-1 (C–D2), Par3 (E–F2) and pan-Crb (G–H2) in WT and Yap CKO lenses at E16.5. Insets represent the magnified images of TZ and LE regions of the lens. Dotted lines in C2–H2 indicate the putative border between LE and LC. α-Catenin and N-Cadherin staining (green) is provided to visualize the global morphology of the lens. (I–P) Representative images of WT (I) and Yap CKO (J) LE showing cell shape (green, α-Catenin) and apical junction (red, ZO-1). Schemes illustrating cell shape change (K and L), nuclear shape change (M and N), and altered localization of the apical junction complex proteins (O and P). Scale bars; 50 μm.
Defects in the morphology and physiological function of LE cells in Yap CKO prompted us to examine the effects on the intracellular structures such as subcellular or cellular landmarks. First we examined the cellular organization of Golgi apparatus. In WT, Golgi, stained by cis-Golgi matrix protein GM130 (Jechlinger et al., 2009), was located apical to the nucleus stained with Hoechst33258 (Fig. 5A, A1 and A2). In Yap CKO LE, however, apical restriction of GM130 staining was severely disturbed. Notably, GM130 was also detected in the area basal to the nucleus (Fig. 5B, B1 and B2). Pericentrin, a centrosomal protein that localizes to the base of primary cilium in embryonic tissues and a microtubule-organizing center (Delaval and Duxey, 2009), was strictly detected apical to the LE and TZ nuclei where the centrosomes are located (Fig. 5C, C1 and C2) (Sugiyama et al., 2011; Sugiyama and McAvoym, 2012). In E16.5 Yap CKO lens, the Pericentrin localization pattern was altered at both LE and TZ levels (Fig. 5D, D1 and D2). Apical primary cilium/microtubule-organizing center pattern was randomized and apical TZ pattern was significantly reduced and disrupted. Consistently, adenylyl cyclase III, a marker for primary cilium (Chizhikov et al., 2007), was also similarly reduced and randomized in Yap-deficient LE and TZ (Fig. 5E–F2).

Histological analysis of Yap CKO lens revealed disorganized LF cells (Fig. 1). Although primary LF cells are thought to be exempt from Yap gene deletion (see above), not only the secondary but also primary LF cells show abnormal appearance in addition to the extensive vacuolization in Yap CKO lens. IF staining with α-Catenin, β-Catenin, N-Cadherin and F-Actin (via Phalloidin) showed irregular packing/assembly of LF cells in Yap CKO (Fig. 5G–H3 and data not shown). Tracing of the individual LF cell in WT lens shows fully extended, thin apical termini (Fig. 5H–H3). Basal termini also attached to the posterior pole or forming suture of the lens (asterisk). Arrows and arrowheads indicate normal and mislocalized array of organelles in WT and CKO, respectively. (C–D2) Tight junction-associated protein ZO-1, which normally localizes at the apical interface of LF and LE cells. In WT lens, ZO-1 localizes to the apical termini of both LE and LF cells forming an interfacing layer, regardless of primary and secondary LF cells (Fig. 5G and G1). In Yap CKO, ZO-1 (+) apical endings of secondary LF cells are scattered and ectopically located in the middle of the lens supporting the interpretation that secondary LF cells are shorter and have defective in apical termini (Fig. 5H–H3). Together, impaired LE polarity results in disruption of apical attachment of LF, ultimately leading to disorganization of lens structure and degeneration of LF cells.

In summary, Yap proteins colocalize with Crb polarity complex proteins at the apical junction of developing LE and our data support the notion that Yap is essential for the proper organization of the lens cells by establishing the apical polarity complex.

Discussion

Precise regulation of Yap expression and localization is essential for self-renewal and timely differentiation in the developing lens

Yap’s role in the self-renewal of progenitor/stem cells has been demonstrated in other tissues including liver, pancreas and neural progenitors. In the liver and pancreas, the Hippo pathway functions

![Fig. 5](image-url). Yap is essential for the maintenance of subcellular organellar orientation and polarity maintenance in E16.5 LE and LF cells, respectively. (A–F2) IF analysis of Golgi apparatus (A–B2), basal body (C–D2) and primary cilia (E–F2) in WT and Yap CKO lenses. Insets show magnified images of TZ and LE regions. Arrows and arrowheads indicate normal and mislocalized array of organelles in WT and CKO, respectively. (C–H3) Tight junction-associated protein ZO-1 is mislocalized in Yap CKO lens. α-Catenin (green) was co-stained to visualize the morphology of LE cells and some of LF cells were traced to visualize the defect in elongated morphology (white lines). Putative suture is developing in the posterior lens (asterisk). Arrows in H1–H3 indicate the ectopically localized apical termini of LF cells (ZO-1) in CKO. Dotted lines demark the apical and basal margins of LE. Nuclear counterstaining was done with Hoechst33258 staining. Scale bars; 50 μm.
as a critical negative regulator of tissue growth or tumor formation by controlling phosphorylation of Yap (George et al., 2012; Lu et al., 2010). In developing dorsal telencephalon, NF2 also regulates the size of neural progenitor pools by inhibiting transcriptional coactivators Yap/Taz (Lavado et al., 2013). Furthermore, the Hippo-Yap signaling cascade, in conjunction with other signaling pathways including Wnt, plays essential roles in the production of constituent cardiomyocytes during development. In Sav, Lats2, and Mst1/2 CKO mouse models, where Yap phosphorylation is decreased, similar cardiomyocyte expansion resulted from nuclear Yap and β-Catenin complex formation and subsequent activation of Wnt target genes (Heallen et al., 2011). In developing retina, Yap is expressed in retinal progenitors and its activity correlates with stimulation of proliferation and inhibition of differentiation (Zhang et al., 2011).

Consistent with Yap’s known roles in transcriptional activation and promotion of cell proliferation in normal and cancer cells, our study confirmed a positive function of Yap in lens progenitor cell proliferation. First, Yap expression is restricted to LE where lens progenitor cells reside. Second, Yap localizes in the nucleus of LE and co-labels with BrdU. Third, Yap localization pattern changes in such way that anterior progenitor cells show dominant nuclear localization while TZ cells exiting the cell cycle exclude Yap from the nucleus. Fourth, Yap expression disappears in differentiating LF cells. Lastly and most importantly, Yap deleted LE was not competent to maintain a pool of progenitor cells. As a result, LE becomes extremely hypocellular as development proceeds.

Yap is essential for the maintenance of epithelial polarity

In addition to the defective lens progenitor phenotypes seen in the Yap CKO mice, we also observed unexpected changes in LE morphology. These included alterations of cellular and nuclear shape, misorientation of intracellular organelles, and nearly-randomized junctional and polarity complex proteins. At E14.5, when the disappearance of Yap protein is clear, LE cell and nuclear flattening is obvious throughout the LE layer while LE proliferation is not severely affected. It would be intriguing to investigate whether cell and nuclear shape change precede the other lens phenotypes, including the self-renewal defect, or vice versa. With the current analyses, however, it is difficult to decisively determine such sequences of cellular and molecular events. Nevertheless, this study clearly demonstrates Yap’s novel and indispensable function in maintaining cellular, nuclear and organellar polarity. In this regard, it is noteworthy that a protein-protein interaction between Yap and polarity complex has been demonstrated in cell culture. Based on a biochemical interaction study, it was proposed that Crb polarity complex proteins, Crb, Pals1 and Patj, localize to the apical side of cells and form a complex with Yap (Varelas et al., 2010). Therefore, it is possible that the mislocalization and/or randomization of polarity complex proteins in Yap-deficient lens may be due to a disruption of the physical interaction between Yap and Crb polarity complex proteins. Although it is not clear whether the Yap-Crb complex protein interaction in the lens is direct or indirect, it has been proposed that Drosophila Crumbs directly binds to Ex (Expanded), a FERM domain containing Hippo upstream regulator. This Yap localization is altered due to the basal mislocalization of Ex in Crumbs mutant cells (Genevet and Tapon, 2011; Grzeschik et al., 2010).

Although additional studies are needed to characterize the nature of potential protein-protein interactions between Yap and/or pYap and Crb polarity complex proteins in the context of developing lens tissue, it is known that Yap interacts with another PDZ domain containing protein, EBP50, at the apical membrane of airway epithelia and cultured bronchial epithelial cells (Mohler et al., 1999). Similarly, our co-localization study using confocal microscopy supports this idea as Yap apical junctional localization overlaps with that of Pals1, Crbs and ZO-1. As illustrated in Fig.6, Yap may help establish or structurally stabilize assembles apical polarity complex proteins at epithelial cell apical junctions. Alternatively, Yap may coordinate the transmission of extracellular signals. For example, Yap may translocate from the nucleus to the cytoplasm to the apical junction depending on the cellular context, such as cell density differences or other exogenous properties such as stiffness of matrix (Dupont et al., 2011; Halder et al., 2012; Piccolo et al., 2013) that promote differentiation rather than proliferation. The latter idea is favored as Yap interaction with Crb polarity complex proteins is proposed to be cell density dependent (Varelas et al., 2010). Yap’s strong apical junctional association in TZ compared to weak localization in anterior LE further support this interpretation.

Hippo-Yap signaling in lens development

NF2, mammalian homolog of Drosophila Merlin and an upstream negative regulator of Yap, has been implicated in the control of lens development. Conditional deletion of NF2 from the murine lens causes incomplete differentiation of LF cells along with morphological immaturity, demonstrating that NF2 is specifically required for normal cell cycle exit at TZ (Wiley et al., 2010). Based on the suppressive relationship between NF2 and Yap in the canonical Hippo-Yap signal pathway (Zhang et al., 2010), we hypothesized that Yap CKO would induce opposite defects in cell cycle progression. Consistent with this prediction, Yap CKO exhibited phenotypes that can be explained by down-regulation of lens progenitor cell proliferation and simultaneous precocious withdrawal from the cell cycle. Shared phenotypes between NF2 and Yap CKOs include the disruption of apical-basal polarity as shown by ZO-1, indicating the importance of coordinated activities of Yap and upstream suppressors in normal development. Furthermore, the enriched expression of NF2 in TZ and concurrent nuclear exclusion and near complete suppression of Yap in TZ and differentiating LF cells supports a NF2-Yap antagonistic role during lens progenitor cell cycle exit (Genepaint.com: Genepaint SetID MH805). This implies that the interplay between Yap and its upstream repressor NF2 may play an important role in pivotal decision-making processes in TZ during lens development (Fig. 6). Although it is still unknown whether NF2 acts directly on Yap or acts through canonical Hippo upstream kinases like Mst1/2 and Lats1/2 in the lens, NF2 mediated inhibition of Yap activity in the developing lens is likely a major upstream regulator as NF2 mutant lens phenotype is ameliorated by reducing Yap (Zhang et al., 2010). Similarly, abnormalities in hippocampus development of the NF2 mutant were phenocopied by Yap overexpression and upon removal of Yap, normal hippocampus development is largely restored (Lavado et al., 2013). Therefore it is likely that NF2-Yap interplay directly regulates cell cycle exit of LE cells in TZ. Upon premature exit of cell cycle in Yap CKO, LE cells preciously
activate genes involved in differentiation into LF cells, such as β-Crystallin.

Use of Nestin-Cre for our CKO of Yap is fortuitous as Nestin is specifically expressed in cells derived from neuroectoderm. The Nestin-Cre line used in this study directs Cre expression in the LE starting after primary LF is formed, thus allowing conditional Yap gene deletion during lens growth after lens vesicle formation. Therefore, our study is not aimed to address any role of Yap during the early stages of lens development such as lens vesicle induction. After the lens vesicle stage, LE greatly contributes to lens growth and maturation by continuously providing lens progenitor cells for the production of LF cells. During this process, Yap plays an indispensable role in maintaining lens progenitor cells. In the absence of Yap, LE cells fail to extensively self-renew, prematurely exit the cell cycle, and precociously differentiate into LF cells. As a result, Yap-null LE cells no longer perform normal physiological functions including production and secretion of extracellular matrix proteins like Nidogen. Although it is conceivable that the LE and LF cell phenotypes of Yap CKO can be interpreted as secondary to the potential retinal defects caused by Nestin-Cre mediated gene deletion in retinal progenitor cells, we prefer to interpret that Yap CKO lens phenotypes are autonomous because general retinal morphology was not altered in Yap CKO at or around P0, when lenses are often almost completely absent.

Our data not only confirm the essential engagement of canonical Hippo-Yap signaling in the crucial decision making process in lens progenitor cells, but also suggest mechanistic collaboration with additional regulatory pathways. The near-complete loss of the lens seen in early neonatal Yap CKO mice is reminiscent of reported phenotypes mediated by decreased proliferation in several mouse mutants including Pax6, β-Catenin, Notch, Jagged1, FoxE3, FGFR2 and β-integrin (Ashery-Padan and Gruss, 2001; Blixt et al., 2000; Cain et al., 2008; Garcia et al., 2005; Le et al., 2009; Saravanamuthu et al., 2011). This suggests a possible higher-order interaction at both extra- and intracellular levels (Graw, 2010). For example, B1-integrin ablation in LE layer induced similar phenotypes including loss of LE and increased vacuolization of LF cells (Simirskii et al., 2007). In particular, a homozygous mutation of LE-specific transcription factor FoxE3 in humans causes congenital primary aphakia (Valleix et al., 2006). Furthermore, a classic lens-defective mouse model which was shown to possess mutated FoxE3 exhibited phenotypes that significantly overlap with those of our Yap CKO including loss of LE, precocious induction of cell cycle exit/differentiation genes such as Proxl and β-Crystallin, and subsequent activation of apoptosis program (Blixt et al., 2000). At TZ, NF2 and Yap may interact with FGF signaling via FGFR2 to guarantee precise coordination of LE cell cycle exit and subsequent differentiation of LF (Garcia et al., 2005).

Based on the observations from this and previous works, it is plausible that Hippo-Yap signaling plays an important role in orchestrating the size control by multiple aspects of cellular properties including proliferation, cell death, differentiation and polarity. During these processes, Yap is thought to be a central player in integrating extracellular events (such as contact inhibition) and transmitting information to the nucleus to elicit context dependent activity (either proliferation or differentiation). Signaling initiated by unidentified ligand and receptor interactions at the cellular membrane may initiate the stimulation of Hippo signaling downstream players including Yap. Alternatively, proteins associated with adherens junction (E-Cadherin) and/or tight junction (Crb polarity complex proteins, Crbs, Pals1 and Patj/Amot/Amotl) may participate at this initial step, linking cellular density information to cellular proliferative activity in the nucleus (Kim et al., 2011; Varelas et al., 2010). This interpretation is supported by the observation showing the link between cell-to-cell contact and TAZ, a closely related transcription cofactor of Yap. In retinal pigmented epithelial cells, nuclear translocation of Taz and concomitant transcriptional activation of target gene Zeb1 was induced by the removal of cell-to-cell contact inhibition (Liu et al., 2010). LE, a sheet of epithelial cells, is an important regulator of osmolality in the lens (Candia, 2004). These cells also permit lifelong growth of the lens by serving as progenitors for LF cells which are continuously added to the existing lens outer cortex (Augusteyn, 2007). Constant lens growth during development and continuous lens growth throughout life require fine control of LF cell generation, which is possibly regulated by both intrinsic and extrinsic mechanisms. Intrinsic regulatory factors may respond to extracellular changes, such as cell crowdedness in a given space and/or accessibility to essential nutrients, growth factors or oxygen availability. In this respect, recent findings suggest that the age-dependent HIF-1 regulation by oxygen could be one of the mechanisms directly controlling the growth of the adult lens, which is normally in a hypoxic state (Shui et al., 2008; Shui and Beebe, 2008). Although the mechanistic mode that Yap is engaging in lens growth is not well understood, it is likely that Yap serves as a linking factor that relays exogenous modulations to the nuclear outputs.

Materials and methods

Animals, genotyping and handling of Yap CKO mice

All animal housing and handling were approved and conducted in accordance with the guidelines of the Temple University institutional animal care and use committee. Nestin-Cre line (Jackson Lab #003771) and Yapf/f allele were described previously (Xin et al., 2011); and genotyping was done by PCR analysis using two primers (Yapf+: CCACACAGCTCTATGATGG and Yap f-: ACCTAGTAACAGGTTTCCCAGT). Typically, Yapf/+; Nestin-Cre/+ animals were crossed with Yapf/f; Nestin-Cre/++; heterozygote (Yapf/+; Nestin-Cre/+) and WT control littermates (no Nestin-Cre).

Histology, immunofluorescence assays and ISH

The entire embryo heads and enucleated eyes of postnatal mice were fixed with 4% parafomaldehyde, dehydrated, embedded in paraffin, sectioned and stained with H&E, using standard methods. IF antibody staining procedures were described previously (Cho et al., 2012). Visualization and imaging were achieved using Axioplan 2 (Carl Zeiss Microimaging GmbH, Germany) or confocal microscopes (TCS SP5 and SP8, Leica Microsystems GmbH, Germany and Nikon Eclipse Ti). Yap in situ hybridization was done as described previously (Cho and Cepko, 2006). RNA probes were made from the 3′-terminal 335 bp of Yap cDNA corresponding to 1291 nucleotide to 1625 nucleotide on Yap cDNA (Accession number NM_009534). Immunohistochemical staining of Yap was done using a Yap antibody following manufacturer’s protocol (Vectastain Elite ABC kit, Vector).

Antibodies

Two antibodies against-Yap (#4912; 1:200; Cell Signaling Technology and ab56701; 1:200; Abcam) and anti-pYap(#4911; 1:200; CST) antibodies were used in this study. Other primary antibodies used were: p57 (sc-8298; 1:200; Santa Cruz Biotechnology), Adenylyl Cyclase III (sc-588; 1:200; SCB), BrdU (347580; 1:50; BD); E-Cadherin (610404; 1:200; BD), N-Cadherin (610920; 1:200; BD), α-Catenin (610193; 1:200, BD), β-Catenin (610153; 1:200; BD), CC3 (9661; 1:200; CST), pan-Crb (1:300; Dr. Jarema Malicki), β-Crystallin (sc-22745; 1:200; SCB), GM130
(610822; 1:200; BD), pH3 (06570; 1:200; Millipore), Pals1 (07-708; 1:200; Millipore), Par3 (07330; 1:100; Millipore), Pax6 (RBP-278P; 1:200; Covance or 1:100; DSHB), Perciprin (PRB-432C; 1:200; Covance), Proxl (PRB-238C; 1:200; Covance), and ZO-1 (610966; 1:200; BD).

Quantification of S- and M-phase lens progenitor cells

For the analysis of cells undergoing cell division, BrdU (50 mg/kg body weight, Sigma-Aldrich) was injected intraperitoneally to timed-pregnant females harboring E14.5 and E16.5 embryos and tissues were harvested 30 min later for tissue fixing and further processing. For S- and M-phase analysis, slides were stained for BrdU or pH3. Quantification was performed on more than three lenses from both WT and Yap CKO mice. Cell counting using Hoechst33342 (+) and BrdU (+) or pH3 (+) staining was described previously (Cho and Cepko, 2006). Apoptotic cell staining was done at E14.5 using anti-C3 antibody.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.12.037.

References


