

Taz-Tead1 Links Cell-Cell Contact to Zeb1 Expression, Proliferation, and Dedifferentiation in Retinal Pigment Epithelial Cells

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PURPOSE. The Hippo signaling pathway imposes the cell contact inhibition that establishes organ size and tissue topology from *Drosophila* to mammals. This pathway regulates activity of the Yap and Taz transcription factors, which link epithelial-mesenchymal transition (EMT) to cell proliferation. Here, the authors provide evidence that Taz and its coactivator, Tead1, regulate expression of the EMT transcription factor Zeb1 to control RPE cell proliferation and differentiation.

METHODS. Real-time PCR was used to examine mRNA expression during RPE dedifferentiation in primary cultures of RPE cells and after knockdown of Yap and Taz by lentivirus shRNA. Immunofluorescence was used to follow subcellular localization of proteins in cells. Chromatin immunoprecipitation was used to detect Taz at the *Zeb1* promoter in vivo.

RESULTS. Zeb1 is overexpressed during RPE dedifferentiation, leading to cell proliferation, EMT, and repression of the RPE specification transcription factor gene *Mitf*. Taz-TEAD1 translocation to the nucleus coincides with loss of cell-cell contact and with onset of Zeb1 expression in the nucleus. shRNA knockdown of Taz prevented the overexpression of Zeb1 and, in turn, prevented proliferation, repression of *Mitf* and *Mitf* target genes, and EMT when RPE cells were placed in primary culture. Taz binds to the *Zeb1* promoter in vivo, suggesting that it directly induces *Zeb1* transcription.

CONCLUSIONS. These results provide evidence of a molecular mechanism linking cell-cell contact to cell proliferation and dedifferentiation in RPE cells. (*Invest Ophthalmol Vis Sci*. 2010;51:3372-3378) DOI:10.1167/iops.09-4321

Organ size and tissue topology are controlled from *Drosophila* to mammals by the Hippo signaling pathway.¹⁻³ Ligation of cadherins on the cell surface leads to activation of a kinase cascade that phosphorylates two closely related transcription factors, yes-associated protein (Yap) and transcriptional coactivator with PDZ binding motif (Taz), and this phos-

phorylation produces a 14-3-3 binding site, leading to the retention of the transcription factors in an inactive form in the cytoplasm.¹⁻⁵ These transcription factors form a complex with members of the Tead family, and this complex is required for targeting Yap and Taz to gene promoters and thus for their function as transcription factors.^{1,3,5,6} Yap and Taz activate genes important for cell cycle progression. Their retention in the cytoplasm on cell-cell contact leads to the arrest of proliferation, thereby defining organ size and tissue topology as proliferating cells come in contact during development. Additionally, Yap and Taz induce the expression of genes such as snail, twist, and zinc finger E-box binding protein (Zeb), which regulate epithelial-mesenchymal transition (EMT).^{4,7} The Hippo signaling pathway through Yap and Taz then links cell-cell contact to cell proliferation to epithelial phenotype. Mutation of Tead1, which blocks binding to Yap and Taz, is responsible for Sveinsson's chorioretinal atrophy (helicoid peripapillary chorioretinal degeneration).⁸⁻¹² Histologic assessment suggests this progressive disease, which ultimately leads to loss of photoreceptors, initiates with the loss of the RPE and choroid, implying an important role for Yap/Taz-TEAD1 and, in turn, the Hippo signaling pathway in RPE viability in vivo.

Our previous studies demonstrated that the EMT transcription factor Zeb1 is required for maintaining epithelial versus mesenchymal balance in vivo in the mouse,^{1,3} and overexpression of Zeb1 and the other EMT transcription factors in cancer drive EMT and a metastatic phenotype in cancer cells.¹⁴⁻¹⁶ Furthermore, induction of the EMT transcription factors can initiate reprogramming of differentiated somatic cells to cells with properties of cancer stem cells,^{17,18} and we have recently found that overexpression of Zeb1 has an important role in cancer stem cell generation.¹⁷ Additionally, we found recently that Zeb1 is overexpressed as retinal pigment epithelial cells initiate proliferation and undergo EMT dedifferentiation when placed in primary culture.¹⁹ Zeb1 is a transcriptional repressor,²⁰ and we demonstrated that one of its targets is *Mitf*, which is required for RPE specification.^{19,21,22} Even heterozygous mutation of Zeb1 is sufficient to prevent loss of *Mitf* expression, onset of proliferation, and initiation of EMT in the cultured RPE cells. A similar result was seen with shRNA knockdown of Zeb1. In these studies we found that Zeb1 expression in the RPE was dependent on cell-cell contact, and only cells in culture that lost such cell-cell contact induced Zeb1, initiated proliferation, and underwent EMT. Furthermore, when dedifferentiated proliferating RPE cells expressing a high level of Zeb1 were forced to reform cell-cell only contacts, Zeb1 expression was silenced.¹⁹ This loss of Zeb1 in turn led to reexpression of *Mitf*, restoration of epithelial morphology and pigment synthesis, and loss of proliferation. These results suggested that induction of Zeb1, as a result of loss of cell-cell contact, is important for the dedifferentiating EMT that

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Supported in part by American Health Assistance Foundation; National Institutes of Health Grants P20 RR018733, EY015636, and EY017869; Research to Prevent Blindness; and the Commonwealth of Kentucky Research Challenge.

Submitted for publication July 15, 2009; revised November 6 and December 9, 2009; accepted January 18, 2010.

Disclosure: Y. Liu, None; Y. Xin, None; F. Ye, None; W. Wang, None; Q. Lu, None; H.J. Kaplan, None; D.C. Dean, None

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occurs in RPE cells in culture and that this dedifferentiation can be reversed if Zeb1 expression is downregulated.

Taken together, these results led us to hypothesize that Zeb1 expression and EMT in RPE cells in culture may be regulated by Yap and Taz by cell-cell contact through the Hippo signaling pathway. Here, we show that Yap is expressed in a low level in the RPE but that Taz is abundantly expressed. In addition, Zeb1 expression indeed depends on Taz, which binds the *Zeb1* promoter in vivo. Knockdown of Taz prevented Zeb1 overexpression when RPE cells were placed in primary culture; this knockdown of Taz prevented onset of proliferation, loss of Mitf expression, loss of pigment synthesis, and EMT in the cultured cells. These studies link Taz to Zeb1 expression and to proliferation and differentiation of RPE cells.

MATERIALS AND METHODS

Primary Cell Culture

Mouse RPE cells were isolated from mice at postnatal day (P)3, as described previously.^{19,23} Eyes were removed from C57BL/6 mice at P1 and were washed with Ca/Mg-free HBSS. The anterior segment and lens were removed, and RPE sheets were dissected away from the posterior segment and digested with 0.25% trypsin for 10 minutes at 37°C before plating in DMEM with 10% fetal bovine serum at 5% CO₂. Cells were passaged 1:2 using trypsin/EDTA once they became confluent. All animals were handled according to the regulations of the Institutional Animal Care and Use Committee, and all procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Sorting

Lentiviral-infected RPE cells on day 2 after infection were trypsinized, and 50,000 cells were sorted for GFP using a cell sorter (MoFlo; Beckman Coulter, Fullerton, CA), as described.¹⁷

Immunostaining

Cells were immunostained as described previously.¹⁹ Antibodies to Taz (H-70) and Tead1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Biosystems (Franklin Lakes, NJ), respectively, and were used at 1:50 dilution. Zeb1 antibody was described previously.¹⁵ Sheep anti-mouse and goat anti-rabbit secondary antibodies were purchased from Molecular Probes and were used at 1:500 dilution.

RNA Extraction and Real-time PCR

Real-time PCR was performed as described.¹³ The sequence and melting temperature of PCR primers is shown in Supplementary Table S1 (all supplementary material is available at <http://www.iovs.org/cgi/content/full/51/7/3372/DC1>).

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were described previously.^{13,19} Polyclonal antiserum for Yap, Taz, and histone 3 were used for immunoprecipitation. Primer sequences are shown in Supplementary Table S1.

Lentivirus shRNA

shRNA oligomers used for Yap and Taz silencing were described previously.⁴ The control sequence was 5'-CAACAAGATGAAGACACCAATCTCTTGAAT TGGTGCTCTTCATCTTGTG, which was blasted against all mouse RNA sequences to ensure that it did not target any mRNA. Lentiviral construction was described in detail previously.^{19,24,25}

RESULTS

Taz and Tead1 Are Cytoplasmic, and Zeb1 Is Not Expressed when RPE Cells Are Initially Placed in Primary Culture

Because of the link between cell-cell contact, EMT, proliferation, and Yap and Taz transcription factors in the Hippo signaling pathway, we examined a potential role for Taz/Yap in the regulation of Zeb1 expression, EMT, and proliferation of RPE cells in culture. We began by examining Taz and Yap subcellular location as a function of cell-cell contact in RPE cells. Little immunostaining for Yap was evident, and real-time PCR detected little Yap mRNA in the RPE cells at either day 1 in culture or in cells in culture for 2 weeks (results not shown). Nevertheless, Yap was detected by immunostaining in mouse embryo fibroblasts and in primary cultures of mouse retinal neurons (Supplementary Fig. S1 and results not shown). However, intense immunostaining for Taz was evident in the RPE cells. It was sequestered in the cytoplasm initially in the primary cultures (day 1), where most cells showed cell-cell contact (Figs. 1A, 1B). Both Yap and Taz heterodimerize with the Tead1; this transactivation complex with Tead is required for Yap/Taz function in the cell-cell contact inhibition pathway and in the regulation of proliferation and EMT.¹⁻⁶ Tead1 colocalized with Taz in the cytoplasm at day 1 in primary culture (Fig. 1A). Zeb1 expression was not detected in the cells at this time (Fig. 1B). Lower-power views of cells immunostained for Taz, Tead1, and Zeb1 are shown in Figures 1C-E.

Loss of Cell-Cell Contact Leads to Nuclear Translocation of Taz and TEAD1, Nuclear Expression of Zeb1, and EMT

After 1 week in culture, RPE cells maintaining epithelial morphology, pigment, and cell-cell contact retained Taz and Tead1 in the cytoplasm, and these cells did not express Zeb1 (Figs. 2A-D; Supplementary Fig. S2). However, as cells lost cell-cell contact and pigment, both Taz and Tead1 translocated to the nucleus, and this nuclear translocation was accompanied by Zeb1 expression in the nucleus (Figs. 2A, 2B; Supplementary Fig. S2). This nuclear translocation and Zeb1 expression persisted at 2 weeks in culture, when RPE cells had lost pigment and many of the cells had become fibroblastic (Fig. 2C; Supplementary Fig. S2). Nuclear translocation of Taz and Tead1 was an early event in RPE cells, which lost cell-cell contact in culture. Nuclear translocation of both factors occurred in cells lacking cell-cell contact by day 2 in culture (Fig. 2D; Supplementary Fig. S2). These cells retained pigment and epithelial morphology. Quantification of nuclear versus cytoplasmic localization of Taz, Tead1, and Zeb1 is shown in Figure 2E.

The Zeb1 Promoter Contains Conserved Consensus Taz-Tead1 Binding Sites, and Taz Binds to the Zeb1 Promoter In Vivo

Our previous studies demonstrated that onset of Zeb1 expression is required to initiate the proliferation of cultured RPE cells, their loss of pigment, and EMT.¹⁹ We showed that Zeb1 expression was closely linked to cell-cell contact, and the correlation of Zeb1 expression to nuclear translocation of Taz and Tead1 shown above suggested that Zeb1 may be a target of Taz transactivation. Inspection of the *Zeb1* promoter showed consensus binding sites for Taz/Tead (TGTAAGGA/T; though this sequence varies somewhat in different promoters),²⁶ and these sites are conserved in human, dog and mouse (Fig. 3A). We then used ChIP assays to determine whether Yap and Taz are bound directly to the *Zeb1* promoter in vivo. Indeed, we found both Yap and Taz bound to the *Zeb1* promoter, and,

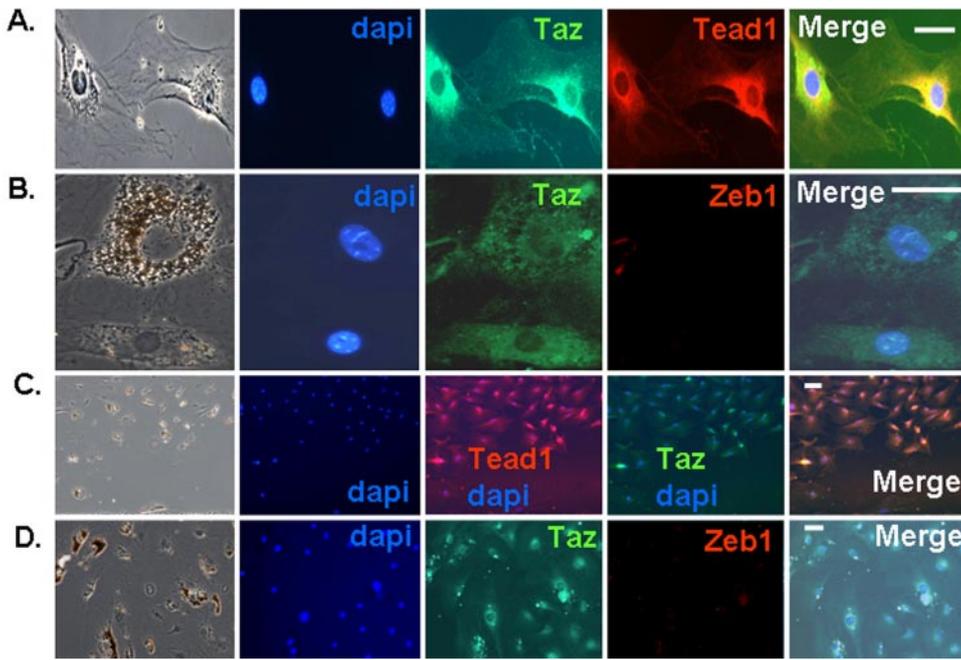


FIGURE 1. (A, B) Taz and Tead1 are cytoplasmic, and Zeb1 is not expressed in RPE cells on day 1 in culture. Immunostaining of primary culture cells is shown. (C, D) Lower-power views. Scale bars, 25 μ m.

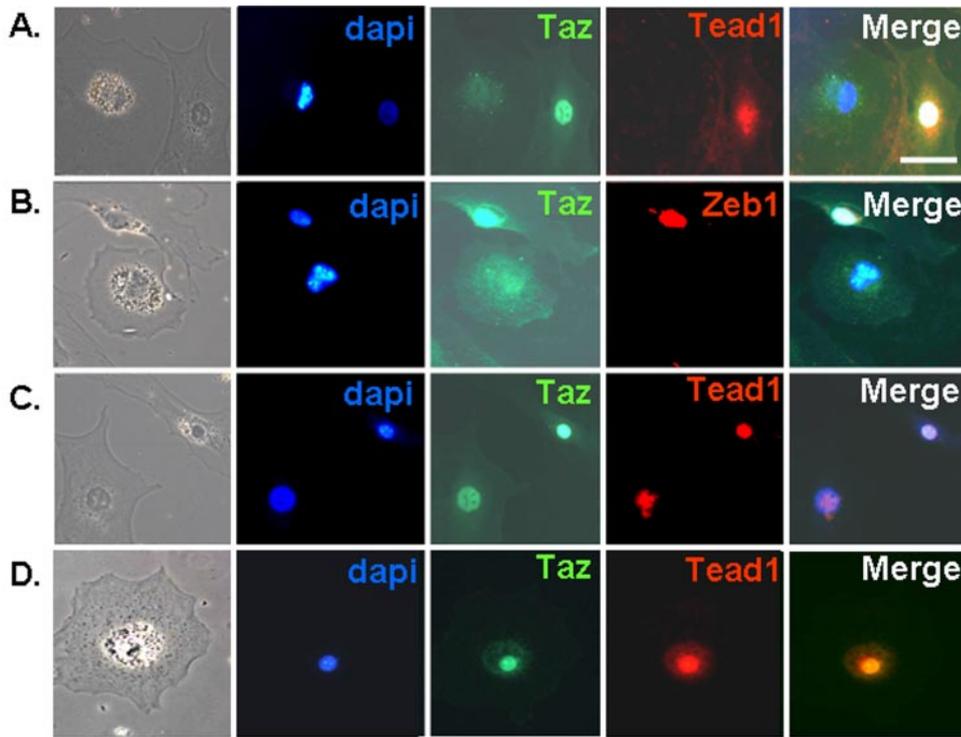


FIGURE 2. Nuclear translocation of Taz and Tead1 correlates with Zeb1 expression in cultured RPE cells. (A, B) Cells after 1 week in primary culture were immunostained as indicated. (C) Immunostaining of RPE cells after 2 weeks in culture. (D) Immunostaining of RPE cells lacking cell-cell contacts on day 2 in primary culture. Scale bar, 25 μ m. (E) Quantification of results in Figures 1 and 2 showing nuclear versus cytoplasmic localization in differentiated and dedifferentiating RPE cells in culture. A small number of cells showed both nuclear and cytoplasmic staining for Tead1.



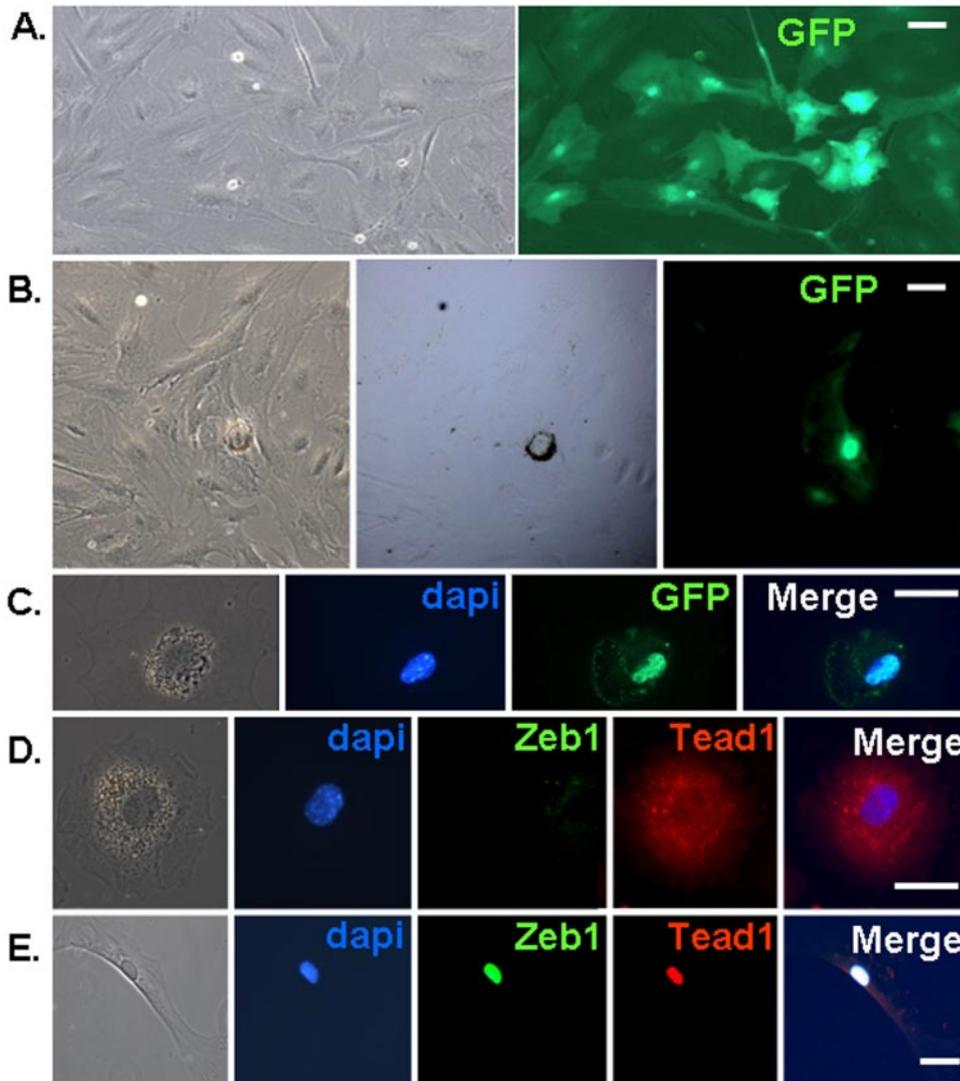


FIGURE 4. Knockdown of Taz prevents proliferation, EMT, expression of Zeb1, and Tead1 nuclear localization in cultured RPE cells. RPE cells were infected with either a control lentivirus (A) or a Taz shRNA lentivirus (B), and cells were allowed to grow out in culture for 2 weeks. Lentiviruses expressed GFP, and in the control both infected and uninfected cells proliferated, lost pigment, and underwent EMT. However, the Taz shRNA-infected cells failed to proliferate, lost pigment, and underwent EMT. (C-E) RPE cells were infected with Taz or control shRNA lentiviruses on day 2 in culture, and infected GFP-positive cells were enriched by cell sorting. (C) Live GFP+ Taz shRNA cell is shown to retain epithelial morphology and pigment. (D) Taz shRNA-sorted cells were fixed and immunostained for Zeb1 and Tead1. Fixation eliminated GFP fluorescence. (E) Control shRNA sorted cells were fixed and immunostained for Zeb1 and Tead1. Scale bar, 25 μ m.

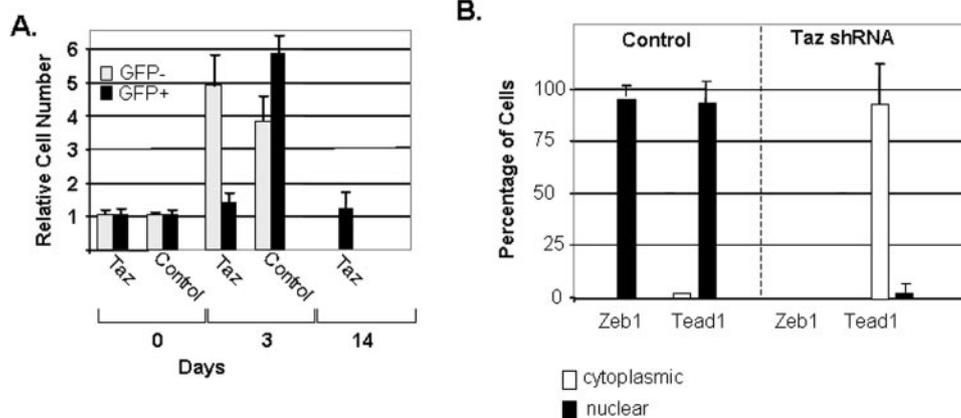


FIGURE 5. Taz shRNA blocks the proliferation of RPE cells in culture and nuclear expression of Tead1 and Zeb1. RPE cells were infected on day 2 in primary culture with lentiviruses expressing GFP and either Taz shRNA or a control scrambled shRNA sequence. (A) Two days after infection (day 0), GFP+ (infected) and GFP- (uninfected) RPE cells were initially counted. Three days later (day 3), the same areas were again counted. GFP+ cells were counted again after 14 days. GFP- cells were not counted because they had become too numerous. Results represent an average of five different areas. (B) After 2 weeks in culture, cells were immunostained, and nuclear localization was quantified in comparison with cytoplasmic localization of Zeb1 and Tead1.

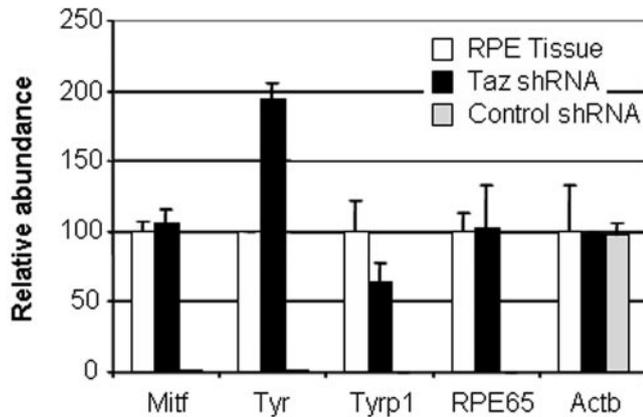


FIGURE 6. Real-time PCR comparing RNA levels in RPE tissue from neonatal mice at P3 (the time of RPE cells cell isolation) and in sorted GFP-positive Taz and control shRNA cells after 2 weeks in culture. Results were normalized to Actb mRNA. Similar results were seen on normalization GAPDH mRNA (results not shown).

synthesis, remained similar to RPE tissue in the Taz shRNA cells, whereas these mRNAs were greatly diminished in the control cells (Fig. 6). Zeb1 induction in cultured RPE cells is responsible for repression of Mitf and, in turn, for its target genes and for onset of proliferation and EMT.¹⁹ Results of Taz knockdown were similar, thus linking Taz to Zeb1 expression and RPE differentiation and proliferation. Taken together, our results suggest that cell-cell contacts regulate Zeb1 expression by Taz nuclear localization and that knockdown of Taz and Zeb1 lead to similar effects in the cultured RPE cells.

DISCUSSION

Our results suggest that cell-cell contacts regulate Zeb1 expression in RPE cells by way of Taz-Tead1 nuclear localization. Zeb1, in turn, links EMT to proliferation in the RPE cells. The Hippo signaling pathway, which senses cell-cell contacts and regulates nuclear translocation of Yap and Taz, is crucial for establishing organ size and tissue topology from flies to mammals.¹⁻³ Taken together, our results suggest that disruption of cell-cell contacts when RPE tissue is placed in culture deregulates the Hippo signaling pathway leading to Taz-Tead1 nuclear localization. We suggest that this event is important for both the onset of proliferation and the resultant EMT of the cultured cells. The potential corollary to these findings is that cell-cell contact and the resultant proliferation arrest initiated by the Hippo signaling pathway may serve to impose the topological monolayer of RPE in vivo.

The primary reason for failure in the surgical repair of retinal detachment is proliferative vitreoretinopathy (PVR), which is mediated at least in part by RPE cells that become detached into the vitreous at the time of rhegmatogenous retinal detachment.²⁷ In the vitreous, the RPE cells most frequently adhere to the inner surface of the detached retina, proliferate, undergo EMT, and contract the retina, thereby preventing retinal reattachment. Our results raise the possibility that deregulation of the Hippo signaling pathway when cell-cell contacts are disrupted as RPE cells are detached and shed into the vitreous may be responsible for the onset of proliferation and EMT in the cells and the development of PVR.

Tea1 is an essential coactivator for Taz and is required to target Taz to gene promoters. We show that Taz is required for Tea1 nuclear localization. Thus, Taz-Tea1 appears to be the functional transcriptional complex, in agreement with previous reports. How might the mutation of Tea1 cause Svein-

son's chorioretinal atrophy with diminished RPE viability if loss of the Taz-Tea1 complex simply leads to the maintenance of RPE differentiation and the inhibition of proliferation, as our results in culture suggest? One possibility is that that loss of Tea1 blocks the proliferation of RPE required to replace cells during normal turnover. In the rat, a recent study found a low level of proliferation in the adult RPE—approximately 20 cell divisions every 30 days—and this was elevated to 200 cell divisions in the albino during this same period.²⁸ As in the CNS, this low-level proliferation likely reflects normal turnover of the RPE. An inability of the RPE to proliferate in Tea1 mutants may prevent the replacement of cells during normal turnover. Such a defect conceivably would lead to a gradual, progressive loss of the RPE.

Taz is crucial for maintaining embryonic stem cells in an undifferentiated state,²⁹ and Tea1 is also pivotal in the earliest embryonic differentiation event—defining embryonic stem cells versus trophoblasts.³⁰ EMT and EMT transcription factors such as Zeb are crucial for the generation of cancer stem cells, which share many properties of stem cells, from differentiated somatic cells.^{17,18} Thus, nuclear localization of Taz-Tea1, overexpression of Zeb1, and EMT seen in RPE dedifferentiation suggest that this dedifferentiation may share some features of cell reprogramming seen in early embryonic development and cancer initiation.

Acknowledgments

The authors thank Diego Montoya-Durango for assistance with chromatin preparation and Ewa Zuba-Surma for cell sorting.

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