

# A Role for $\beta$ A3/A1-Crystallin in Type 2 EMT of RPE Cells Occurring in Dry Age-Related Macular Degeneration

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**PURPOSE.** The RPE cells have a major role in the development of dry age-related macular degeneration (AMD). We present novel evidence that  $\beta$ A3/A1-crystallin, encoded by the *Cryba1* gene, a protein known to be important for lysosomal clearance in the RPE, also has a role in epithelial-to-mesenchymal transition (EMT) of RPE cells.

**METHODS.** RPE from dry AMD globes, genetically engineered mice lacking *Cryba1* globally or specifically in the RPE, spontaneous mutant rats (Nuc1) with a loss-of-function mutation in *Cryba1*, and the melanoma OCM3 cell line were used. Spatial localization of proteins was demonstrated with immunofluorescence, gene expression levels were determined by quantitative PCR (qPCR), and protein levels by Western blotting. Cell movement was evaluated using wound healing and cell migration assays. Co-immunoprecipitation was used to identify binding partners of  $\beta$ A3/A1-crystallin.

**RESULTS.**  $\beta$ A3/A1-crystallin is upregulated in polarized RPE cells compared to undifferentiated cells. Loss of  $\beta$ A3/A1-crystallin in murine and human RPE cells resulted in upregulation of Snail and vimentin, downregulation of E-cadherin, and increased cell migration.  $\beta$ A3/A1-crystallin binds to cortactin, and loss of  $\beta$ A3/A1-crystallin resulted in increased *P*-cortactin<sup>Y421</sup>. The RPE from AMD samples had increased Snail and vimentin, and decreased E-cadherin, compared to age-matched controls.

**CONCLUSIONS.** We introduced a novel concept of dry AMD initiation induced by lysosomal clearance defects in the RPE and subsequent attempts by RPE cells to avoid the resulting stress by undergoing EMT. We demonstrate that  $\beta$ A3/A1-crystallin is a potential therapeutic target for AMD through rejuvenation of lysosomal dysfunction and potentially, reversal of EMT.

**Keywords:** crystallin, lysosomes, EMT, dry AMD, autophagy

Crystallins, the highly abundant proteins of the lens, are essential determinants of the transparency and refractivity required for lens function.<sup>1-3</sup> Initially thought to be lens-specific and to have evolved as lens proteins, crystallins are now known to be recruited to the lens from proteins that existed before lenses evolved.<sup>3</sup> Crystallins are expressed outside of the lens and most have been shown to have cellular functions distinct from their roles as structural elements in the lens. For,  $\beta$ A3/A1-crystallin, one member of the  $\beta/\gamma$ -crystallin family, it appears that its “nonlens” function is mediated via signaling pathways.<sup>4,5</sup>

In the past several years, we explored possible functions for the polypeptides ( $\beta$ A3- and  $\beta$ A1-crystallins) encoded by *Cryba1*, one of the six  $\beta$ -crystallin genes. In a spontaneous rat mutant (Nuc1)<sup>6-10</sup> and genetically engineered mouse models (*Cryba1* knockout [KO]<sup>11-13</sup> and *Cryba1* conditional [RPE-specific] knockout [cKO]),<sup>14,15</sup> a striking age-related macular degeneration (AMD)-like phenotype with compro-

mised lysosome-mediated clearance<sup>12,13,15</sup> was observed. Our findings suggested that  $\beta$ A3/A1-crystallin localizes to the lysosomal lumen of RPE cells and binds with the V<sub>0</sub> subunit of V-ATPase, the proton pump responsible for endo-lysosomal acidification.<sup>8,14</sup>  $\beta$ A3/A1-crystallin also regulates amino acid transport via the proton-assisted amino acid transporter 4 (PAT4)/solute-linked carrier 36A4 (SLC36A4).<sup>13</sup> Therefore, it has an important role in the complex process of recruiting and assembling the mechanistic target of rapamycin, complex 1 (mTORC1) signaling platform to the lysosomal surface.<sup>13,14</sup> As a result,  $\beta$ A3/A1-crystallin influences the clearance functions of lysosomes, both phagocytosis and autophagy.

Lysosomal efficiency declines with age, and this decline has been implicated in age-related diseases, such as Parkinson’s and Huntington’s diseases, and recently, AMD.<sup>5,16-18</sup> In dry AMD, lysosomal dysfunction may drive RPE cells into epithelial-mesenchymal transition (EMT) to survive a stressful microenvironment. Different forms of EMT are associated with three

distinct biological settings, with varying functional consequences. While, type 1 EMT has a role during development and type 3 EMT occurs in most cancers, type 2 EMT is associated with wound healing and tissue regeneration.<sup>19</sup> It now is well documented that in AMD, some RPE cells appear to degenerate, losing normal cell shape, exhibiting migratory behavior, and losing their epithelial function.<sup>20,21</sup> This degeneration is especially evident in the transition zone of geographic atrophy (GA), the advanced dry form of AMD.<sup>22</sup> Previous studies have described these RPE cells as severely dysmorphic, often multilayered, with migration into the retina and sub-RPE space.<sup>23</sup> While described classically as “degeneration,” a closer examination of these “degenerating” cells suggests that some are not dying, but instead may have transformed into mesenchymal cells to survive the harsh microenvironment during disease progression.<sup>23–25</sup> While cells undergoing Type 2 EMT would lose critical epithelial function, they also become resistant to cell death.<sup>26</sup> Since EMT is reversible, these cells are logical targets for novel therapies aimed at reversing dry AMD. Such treatments would greatly benefit patients who currently have very limited prevention or treatment options.

We report that  $\beta$ A3/A1-crystallin is highly expressed in polarized, differentiated (RPE) cells, but is not detected in undifferentiated cells, and further, that the absence of  $\beta$ A3/A1-crystallin causes RPE cells to display molecular and functional features of type 2 EMT. Therefore,  $\beta$ A3/A1-crystallin, through its regulatory role on lysosomes, may influence EMT in the RPE, and may offer a novel approach to therapy for AMD.

## MATERIALS AND METHODS

### Human Samples

Fresh postmortem eyes obtained from the Portland, Oregon Eye Bank or the National Disease Research Interchange (Philadelphia, PA, USA) were processed within 14 hours after death. Donor information has been summarized previously.<sup>27</sup> The disease conditions were determined by medical record, and the globes were examined further by an experienced retinal physician with expertise in AMD (JTH). The retinas were defined as normal when there were no abnormalities observed using a dissecting microscope. Early-stage AMD was defined by the presence of any RPE pigmentary changes and/or large-size drusen (>125  $\mu$ m diameter). Late-stage AMD was defined by areas of geographic atrophy due to loss of the RPE. We only included dry AMD and excluded wet AMD. Under direct visualization with a dissecting microscope, the RPE was mechanically separated from the choroid and used for Western analysis.

All research including human samples followed the tenets of the Declaration of Helsinki, informed consent was obtained from the study subjects and the research was conducted under protocols approved by the respective institutional review boards.

### Generation of *Cryba1* cKO and *Cryba1* KO Animals

$\beta$ A3/A1-crystallin cKO (*Cryba1* cKO) and corresponding *Cryba1*<sup>fl/fl</sup> control mice were generated as described previously.<sup>14</sup> *Cryba1* complete KO mice were generated as explained previously and wild type (WT) mice were used as controls.<sup>11</sup> All studies including animals were performed in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and under approved Institutional Animal Care and Use Committee (IACUC) protocols.

### Human RPE Cell Culture

Human RPE cells were isolated from postmortem fetal eyes (gestational age 16–18 weeks) obtained from Advanced Bioscience Resources, Inc. (Alameda, CA, USA) and cultured according to published protocols.<sup>28,29</sup> Primary cultures of nonpolarized RPE cells were established as described previously.<sup>30,31</sup> Isolation of nonpolarized and polarized cells were performed according to the previously described protocol.<sup>28</sup>

### Culture of OCM3 Cell Line

OCM3 is a human primary uveal melanoma cell line that is fibroblastic in nature with adherent properties.<sup>32</sup> These cells were cultured following a previously reported method.<sup>33</sup> The cells were grown in RPMI medium with 10% fetal bovine serum (FBS), HEPES buffer, sodium pyruvate, penicillin/streptomycin, and L-glutamine to 90% confluency.

### RNA Isolation and Real-Time RT-PCR

Total RNA was isolated and cDNA was generated as described previously.<sup>13</sup> Gene expression levels were normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and reported as fold change over controls. The primer sequences used were as follows:  $\beta$ A3/A1-crystallin, 5'-CCCTGAAGGTGGAAAGTGG-3' and 5'-CATCCAGCGAGGGTATTC-3'; and GAPDH, 5'-CCACATCGCTCAGAACACC-3' and 5'-GCCCAATACGACCAAATCC-3'. The mRNA expression of mouse *Snai1* and *Snai2* genes was evaluated by using Taqman probes, for which the *Hprt* gene was used as an internal control.<sup>13</sup>

### Microarray Analysis

Microarray was performed on cultured nonpolarized and polarized RPE cells with a mean TER of  $385 \pm 13.1 \Omega \cdot \text{cm}^2$  grown to confluency in RtEGM (Retinal Pigment Epithelial Cell Growth Medium; Lonza, Walkersville, MD, USA) with 1% FBS. Total RNA was isolated using RNeasy kit (Qiagen, Germantown, MD, USA). Analysis was performed using a commercial array service (Agilent Expression Array; Agilent, Tokyo, Japan).

### Immunofluorescence Staining and Immunohistochemistry

Human RPE cells were cultured in 4-well multichamber slides. Cells were subjected to immunofluorescence staining using primary  $\beta$ A3/A1-crystallin goat polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Mouse RPE flat mounts (*Cryba1*<sup>fl/fl</sup>, *Cryba1* cKO, *Cryba1* KO, and WT) from freshly enucleated eyes were immunostained as described previously.<sup>13,14</sup> Images were acquired by a Zeiss LSM 710 (Carl Zeiss Meditec, Jena, Germany) confocal workstation.

### Transfection Studies

*Cryba1* siRNA or *Cryba1* pcDNA was transfected using a previously reported protocol.<sup>14</sup>

### Co-Immunoprecipitation

Co-immunoprecipitation was performed using the Pierce Co-Immunoprecipitation Kit (Thermo Fisher Scientific, Waltham, MA, USA).<sup>12,15</sup> RPE-choroid preparations from seven rats of each genotype (WT and Nuc1) were sonicated in IP Lysis/Wash Buffer (provided in the kit) with 1% protease inhibitors

**TABLE.** Gene Expression of Crystallins in Polarized RPE Cells Normalized to Nonpolarized RPE Cells Determined by Microarray Analysis

Crystallin Genes	Fold Changes	Crystallin Genes	Fold Changes
Crystallin, $\beta$ A1	23.75	Crystallin, $\gamma$ B	1.45
Crystallin, $\gamma$ N	7.13	Crystallin, $\beta$ A4	1.44
Crystallin, $\lambda$ 1	6.40	Crystallin, $\beta$ B1	1.43
Crystallin, $\gamma$ B	2.34	Crystallin, $\beta$ B3	1.30
Crystallin, $\beta$ B1	1.46	Crystallin, $\alpha$ B	1.06
Crystallin, $\beta$ A4	1.45	Crystallin, $\beta$ B2	1.02

Nonpolarized RPE cells = 1.0. Microarray was performed in confluent nonpolarized RPE and polarized RPE cells cultured by RtEGM with 1% FBS. RNA was collected 24 hours after changing the medium. Ratios of crystallins higher than 1.0 in polarized cells normalized to nonpolarized RPE cells are shown.

(Millipore Sigma, St. Louis, MO, USA) and the procedure was performed according to manufacturer's instructions.

### Western Blot Analysis

Lysates were prepared from RPE-choroid preparations from freshly dissected mice (*Cryba1*<sup>fl/fl</sup>, *Cryba1* cKO), rat (WT and Nuc1), normal, and AMD RPE cells from human tissues, fetal human RPE cell cultures, and OCM3 cell line. Total protein (50  $\mu$ g) was loaded for fetal human RPE cell culture and 10  $\mu$ g protein was loaded for the other samples. Western blots were performed as described previously.<sup>12,13</sup> The primary antibodies used in this study were  $\beta$ A3/A1-crystallin (Cat# sc-22398; Santa Cruz Biotechnology and Cat# ab151722; Abcam, Cambridge, MA, USA) as well as a polyclonal antibody developed in our laboratory and previously characterized,<sup>7</sup> E-cadherin (Cat# 610181, BD Biosciences, Franklin Lakes, NJ, USA), Vimentin (Cat# 550513; BD Biosciences and Cat# MA5-11883; Thermo Fisher Scientific, Waltham, MA, USA),  $\beta$ -Catenin (Cat# ab16051; Abcam), Cortactin (Cat# ab33333; Abcam), phospho(Y421)-cortactin (Cat# 4569S; Cell Signaling Technology, Danvers, MA, USA), Snail1 (Cat# 14-9859-82; Invitrogen, Carlsbad, CA, USA), and Actin (Cat# A2066; Sigma Aldrich Corp., St. Louis, MO, USA). Secondary antibodies were peroxidase labeled goat anti-rabbit (Cat# 074-1506; KPL, Gaithersburg, MD, USA) and peroxidase labeled goat anti-mouse (Cat# 074-1806; KPL).

### Wound Healing Assay

Wound healing assay to assess the migration of OCM3 cells was performed following previously reported methods.<sup>34</sup>

### Cell Migration Assay

Cell migration assay was performed by following the manufacturer's instructions for the Cell Migration Assay Kit (M1000; Biocolor Ltd, Carrickfergus, United Kingdom).

### Statistics

Statistical analysis was performed using Microsoft Excel and GraphPad 5.0 software. The *P* values were determined using the Mann-Whitney *U* test in at least four biological replicates representative of three independent experiments. Multiple comparisons were made using the Kruskal-Wallis test. Significance was defined as *P*  $\leq$  0.05. Results are presented as mean  $\pm$  SD. Each biological replicate has at least three technical replicates.

## RESULTS

### $\beta$ A3/A1-Crystallin Is Expressed in Polarized RPE Cells

A variety of RPE cell functions are dependent on its polarized state, including trafficking of ions, fluid, and metabolites across the RPE monolayer.<sup>35</sup> Recently it has been shown that polarized RPE cells exhibit distinct proteomes on the apical and basal plasma membranes.<sup>35</sup> We report the expression profile of  $\beta$ A3/A1-crystallin in polarized and nonpolarized human fetal RPE cells.<sup>36</sup> The differentiation of cultured fetal human RPE cells was assessed by the detection of RPE-65, a marker of terminal differentiation in the RPE, which was 6.8 times higher in polarized than nonpolarized fetal human RPE cells (data not shown). Using microarray analysis, the expression of 12 crystallin genes, from the  $\alpha$ - and  $\beta$ / $\gamma$ -crystallin families was compared in polarized and nonpolarized fetal human RPE cells (Table). While most crystallins showed little, if any, increased expression in polarized cells,  $\beta$ A3/A1-crystallin expression was increased 23.75-fold compared to nonpolarized cells (Table). RNA (Fig. 1A) and protein levels (Fig. 1B) were significantly increased in polarized fetal human RPE cells as validated by quantitative RT-PCR (qRT-PCR) and Western analysis, respectively. Our data also showed that  $\alpha$ B-crystallin, a small heat shock protein with known regulatory function in the RPE,<sup>37</sup> did not show significant expression change with polarization.

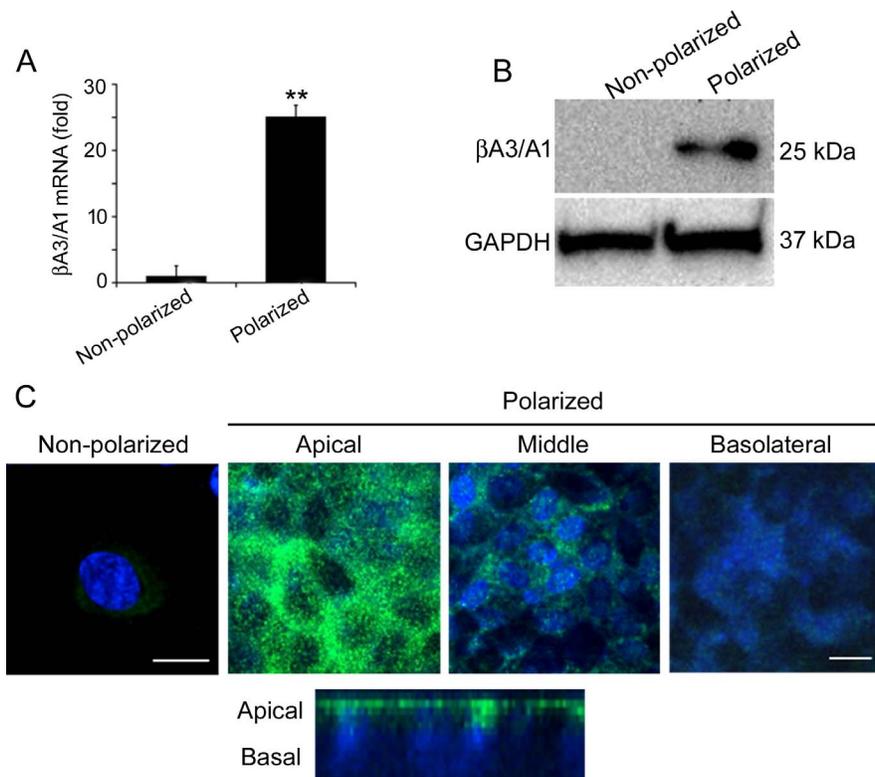
Immunofluorescence studies showed that  $\beta$ A3/A1-crystallin was localized predominantly to the apical side of polarized fetal human RPE cells in contrast with nonpolarized RPE cells, which have very weak expression of  $\beta$ A3/A1-crystallin that was distributed throughout the cytosol (Fig. 1C). Cultured RPE cells from WT mouse or rats only express detectable  $\beta$ A3/A1-crystallin when the culture is confluent and the cells are polarized (data not shown).

### Loss of *Cryba1* Leads to EMT in RPE Cells

EMT is a process that allows a polarized epithelial cell, which normally interacts with its basement membrane via its basal surface, to assume a mesenchymal cell phenotype, conferring increased migratory capacity, invasiveness, resistance to apoptosis, and production of ECM (extracellular matrix) components.<sup>38,39</sup> It is known that RPE cells undergoing EMT assume a mesenchymal morphology, lose cell-cell adhesion, deposit ECM proteins, and migrate to the retina or subduct into Bruch's membrane.<sup>39,40</sup> In EMT, the transcription factor Snail1 is associated with upregulation of vimentin, an intermediate filament protein that is expressed in mesenchymal cells, and downregulation of E-cadherin within tight junctions despite its potential for mosaic expression in certain epithelial cells, such as the RPE.<sup>41-45</sup> Therefore, we investigated whether  $\beta$ A3/A1-crystallin might have a role in the onset of EMT in RPE cells. RPE cells from *Cryba1* cKO mice have elevated mRNA expression of the EMT transcription factors *Snail1* and *Snai2* (Figs. 2A, 2B), elevated Vimentin protein expression, and loss of E-cadherin (Fig. 2C).

Dynamic reorganization of the actin cytoskeleton is a prerequisite for the migration and invasion of cells undergoing EMT.<sup>46</sup> This led us to evaluate whether the actin cytoskeleton in the RPE of *Cryba1* KO mice was reorganized compared to that in WT controls. We found that staining for the high-affinity filamentous actin probe Phalloidin<sup>13</sup> indicated significant change in the size and shape of RPE cells from *Cryba1* KO compared to WT mice (Fig. 3A).

In normal epithelial tissues with high E-cadherin expression,  $\beta$ -catenin is sequestered at the membrane, preventing it



**FIGURE 1.**  $\beta$ A3/A1-crystallin is differentially expressed in nonpolarized and polarized human RPE cells. (A, B) Polarization of RPE cells results in a highly significant increase in the mRNA and protein expression of  $\beta$ A3/A1-crystallin, compared to nonpolarized RPE cells. All values are represented as mean  $\pm$  S.D. \*\* $P < 0.01$  (Mann-Whitney  $U$  test),  $n = 4$ . (C) The distribution of  $\beta$ A3/A1-crystallin in polarized cultured human RPE cells as seen by confocal microscopy (upper) indicates that  $\beta$ A3/A1-crystallin is predominantly localized to the apical region of RPE cells. This was confirmed by a confocal cross-section image (lower). Scale bar: 20  $\mu$ m for nonpolarized cells, 60  $\mu$ m for polarized cells.

from being released into the cytoplasm and entering the nucleus to activate genes required for cell proliferation and survival.<sup>43</sup> It also is known that complexes of cadherin and catenins connect adjacent epithelial cells through adherens junctions, which then link to the actin cytoskeleton via  $\alpha$ -catenin and its interacting protein,  $\alpha$ -actinin.<sup>43</sup> However, in proliferating or migrating cells, due to decreased expression of cadherins (E-cadherin), the sequestration of  $\beta$ -catenin to the membrane is lost, enabling its translocation to the nucleus.<sup>43</sup> We observed that in floxed controls,  $\beta$ -catenin was predominantly localized to the cell membrane, especially in the central region of the RPE cell, while in the *Cryba1* cKO mice, it was distributed at the plasma membrane and in the cytoplasm (Fig. 3B).

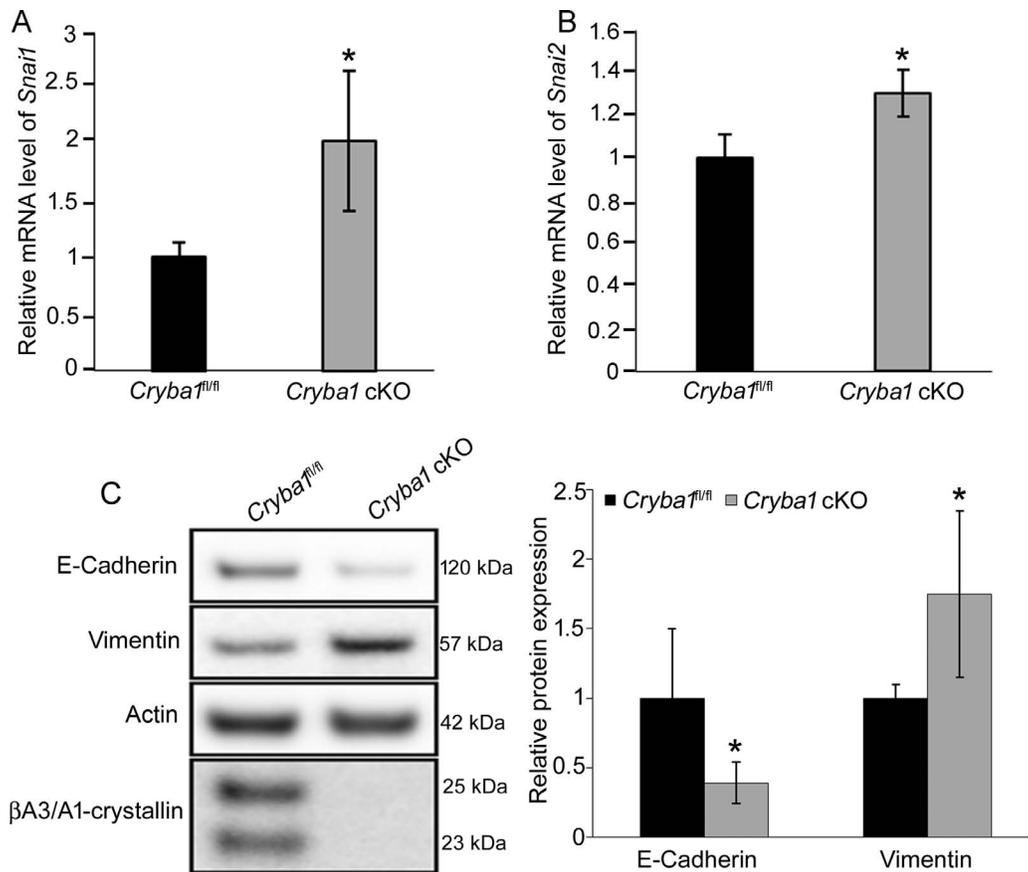
### Loss of $\beta$ A3/A1-Crystallin Induces EMT by Acquisition of Vimentin and Migratory Properties

It is well documented that cancer cells have intrinsic migratory properties and are susceptible to EMT.<sup>19</sup> Thus, as a model system for evaluating how  $\beta$ A3/A1-crystallin might modulate EMT in cells, we used uveal melanoma cells (OCM3),<sup>33</sup> which are susceptible to EMT and express  $\beta$ A3/A1-crystallin (Supplementary Fig. S1). When OCM3 cells were transfected with *Cryba1* short interfering RNA (siRNA) to knockdown the expression of  $\beta$ A3/A1-crystallin<sup>8</sup> (Supplementary Fig. S2), we observed loss of E-cadherin and acquisition of vimentin (Fig. 4A), two critical steps in EMT. EMT is a cellular differentiation regime and is associated with cellular migration.<sup>47</sup> We sought to evaluate whether loss of  $\beta$ A3/A1-crystallin could potentiate migration in the OCM3 cells undergoing EMT. We performed *Cryba1* siRNA silencing studies to downregulate  $\beta$ A3/A1-

crystallin and found that there was increased migration of the cells as evident from wound healing (Fig. 4B) and horizontal cellular migration experiments (Fig. 4C), further emphasizing the fact that loss of *Cryba1* is responsible for EMT-induced cell migration. As a control, we attempted the converse experiment to upregulate expression of  $\beta$ A3/A1-crystallin by transfecting with the expression vector *Cryba1* pcDNA. Since this did not generate any increase in  $\beta$ A3/A1-crystallin (not shown), probably because OCM3 cells already are producing it, this had no effect on EMT-related parameters (Figs. 4A, 4C).

### Association of Cortactin and $\beta$ A3/A1-Crystallin Is Necessary for EMT-Induced Migration

To further delineate how  $\beta$ A3/A1-crystallin controls EMT-mediated migration, we re-evaluated our previous proteome high-throughput microarray data to identify proteins associated with  $\beta$ A3/A1-crystallin.<sup>13</sup> We identified cortactin, a protein known to have a major role in cell migration.<sup>48</sup> Co-immunoprecipitation assay using OCM3 cells supported the microarray data and demonstrated that  $\beta$ A3/A1-crystallin was associated with cortactin (Fig. 5A). Further, we observed significant increase in p-Cortactin<sup>Y421</sup> expression in OCM3 cells transfected with *Cryba1* siRNA (Fig. 5B), with no change in the total cortactin level (Fig. 5B). Cortactin, a substrate of sarcoma (Src) kinases, is an actin-binding protein that is involved in cytoskeletal regulation.<sup>48</sup> The tyrosine phosphorylation (Y421) of cortactin increases its turnover at focal adhesions to promote cell motility.<sup>49</sup> Binding to the actin-related protein 2/3 (Arp2/3) complex stimulates cortactin activity, which promotes F-actin nucleation and assembly.<sup>48</sup>



**FIGURE 2.** *Cryba1* is involved in the onset of EMT in the mouse RPE cells. (A, B) The mRNA levels of *Snai1* and *Snai2* were increased in the RPE from 5-month-old *Cryba1* cKO mice compared to age-matched *Cryba1<sup>fl/fl</sup>* controls, as assessed by qRT-PCR. *Hprt* was used as internal reference gene. (C) Western blotting revealed reduced E-cadherin and increased vimentin with no noticeable expression of  $\beta$ A3/A1-crystallin in 5-month-old *Cryba1* cKO RPE cells compared to age-matched *Cryba1<sup>fl/fl</sup>* RPE cells. Actin was used as internal control. The graphs show mean  $\pm$  SD and  $*P < 0.05$  (Mann-Whitney *U* test),  $n = 4$ .

Cortactin promotes cancer cell migration and invasion, and has a pivotal role in invadopodia formation and ECM degradation.<sup>48</sup> The cortactin N-terminus associates with F-actin, while its C-terminus interacts with focal adhesions, dynamic protein complexes through which the cytoskeleton of a cell connects to the ECM.<sup>49</sup>

To demonstrate the involvement of  $\beta$ A3/A1-crystallin and the cortactin complex in RPE cell migration, we performed a co-immunoprecipitation assay and evaluated the expression of p-Cortactin<sup>Y421</sup> in RPE+Choroid lysates harvested from spontaneous mutant Nuc1 rats, which lack functional  $\beta$ A3/A1-crystallin. We found that there was decreased association of cortactin and  $\beta$ A3/A1-crystallin (Fig. 5C), along with increased phosphorylation of cortactin (Fig. 5D) in the RPE+Choroid of Nuc1 compared to WT rats. Taken together, our data suggested that functional  $\beta$ A3/A1-crystallin is required for regulating cortactin phosphorylation, which may be a critical regulatory mechanism in migration of RPE cells.

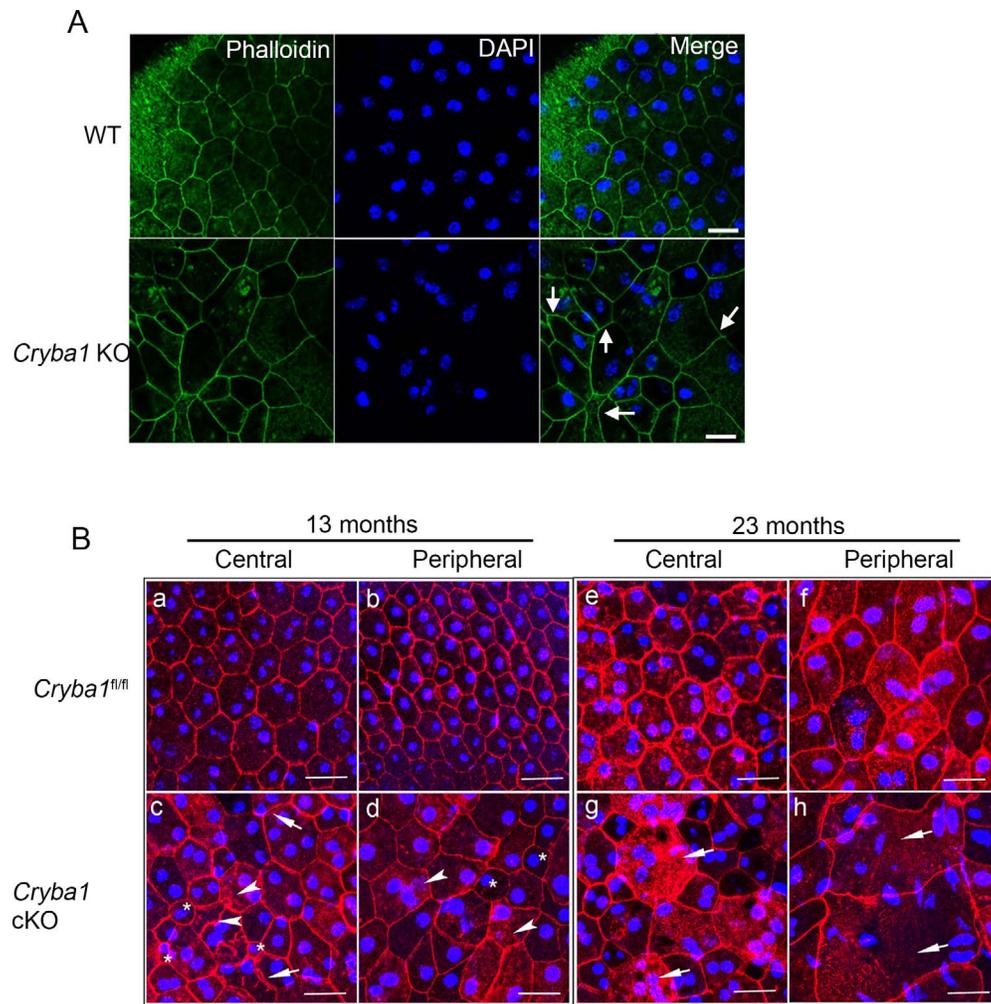
### The RPE of Nonneovascular AMD Expresses EMT Markers

Finally, to provide molecular evidence of EMT in human dry AMD, we found that the RPE from nonneovascular AMD eyes had increased Snai1 and vimentin and decreased E-cadherin, as detected by Western analysis compared to age-matched controls (Fig. 6), which is consistent with the onset of EMT.

### DISCUSSION

AMD is a major cause of vision loss in the elderly.<sup>50</sup> With the increasing human life span, the prevalence of aging-related diseases, including AMD, is increasing.<sup>51</sup> Despite this growing population of afflicted individuals, no definitive treatment other than the Age-Related Eye Disease Study (AREDS) II formulation for intermediate AMD, or prevention for dry AMD currently is available. Therefore, a new treatment that targets dry AMD before significant loss of vision occurs would have great benefit for patients.

The RPE, which is critically important to retinal homeostasis, is a central cell type involved in dry AMD pathobiology.<sup>27</sup> As AMD progresses, some RPE cells appear to degenerate while others appear to be normal. The morphologic changes observed are characteristic of type 2 EMT, a process that can be activated by oxidative stress-induced impairment of autophagy and lysosomal function.<sup>52-54</sup> EMT is a transcriptional program allowing cells to survive stresses, such as oxidative stress, but at the expense of losing their specialized function. We showed that the RPE obtained from AMD patients and genetically engineered mouse models with an AMD-like phenotype express key molecular markers of EMT. RPE dysfunction undeniably drives AMD pathophysiology.<sup>27,50</sup> Since EMT is reversible and cells in EMT are resistant to death, targeting RPE cells in EMT is a promising novel strategy to reverse AMD pathobiology and ultimately stabilize vision.

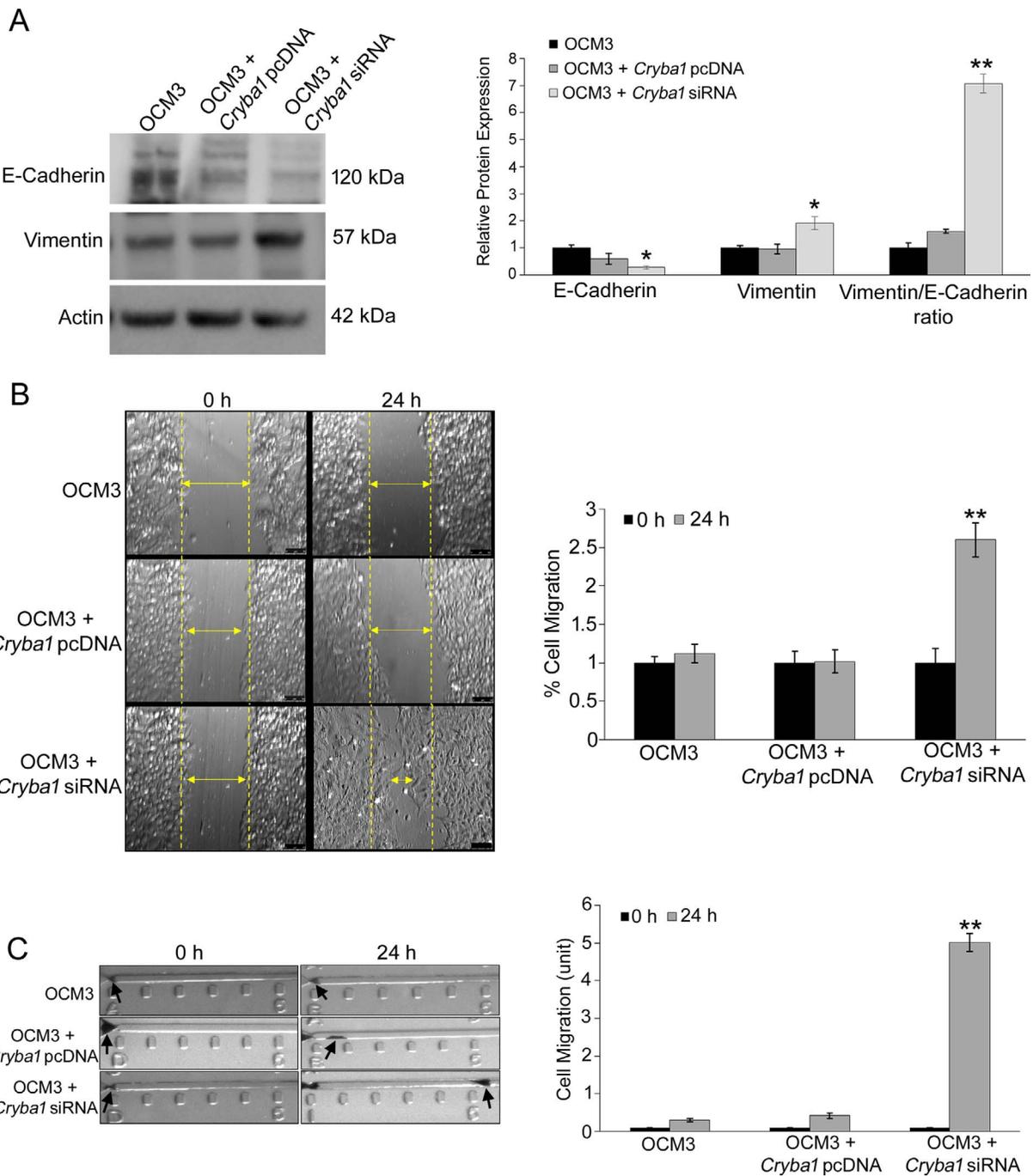


**FIGURE 3.** *Cryba1* regulates cytoskeletal organization during EMT. (A) RPE flat mounts prepared from the eyes of 20-month-old *Cryba1* KO and WT mice were stained with phalloidin (green) and DAPI (blue). Phalloidin staining revealed changes in the shape of *Cryba1* KO RPE cells compared to WT control mice (white arrows). Scale bar: 20  $\mu$ m. (B) RPE-choroid-sclera flat mounts prepared from *Cryba1* cKO and *Cryba1*<sup>fl/fl</sup> control mice were stained with anti- $\beta$ -catenin antibody (red) and 4',6-diamidino-2-phenylindole (DAPI; blue). At 13 months of age, *Cryba1* cKO RPE cellular architecture shows highly variable geometry, where some cells are enlarged while, others show significantly smaller size (asterisk in c and d) at central and peripheral regions of the RPE layer compared to *Cryba1*<sup>fl/fl</sup> control mice (a, b). In control RPE cells,  $\beta$ -catenin is located mainly at the cell membrane, especially at the central region of the RPE layer; while in cKO cells,  $\beta$ -catenin is not restricted to the plasma membrane, but also is located in the cytoplasm (arrowheads). Double-layer staining also is seen in some cKO cells (arrows in c). At 23 months of age, there is noticeable enlargement of cells especially at the peripheral region of RPE layer (arrows in g and h). Scale bar: 20  $\mu$ m.

EMT in the RPE of *Cryba1* deficient mice probably is due to impaired autophagy, as we have shown previously that autophagy flux and autophagy-mediated clearance is compromised in these mice.<sup>14</sup> In RPE cells,  $\beta$ A3/A1-crystallin is absolutely essential for normal lysosomal-mediated clearance by the processes of autophagy and phagocytosis.<sup>13,14</sup> The literature indicates that lysosomal dysfunction is involved in AMD since autophagosome clearance and autophagy flux are significantly impaired in tissue sections from human donor AMD eyes,<sup>55</sup> and circulating autoantibodies from AMD patients point to compromised autophagy.<sup>56</sup> In fact, autophagy can prevent EMT by clearing Snail and Twist.<sup>57</sup> In differentiated hepatocytes, autophagy degrades Snail while lysosomal inhibition impairs the autophagic clearance of Snail and Twist to cause EMT.<sup>57,58</sup> It is highly likely that in our genetically engineered mice, impaired autophagic clearance also leads to upregulation of Snail, as shown in this study. Moreover, our results suggest that  $\beta$ A3/A1-crystallin is required for regulating cortactin phosphorylation, which is critical for quality control

(QC) autophagy.<sup>59</sup> QC autophagy has been shown to selectively remove toxic protein aggregates and damaged organelles, the accumulation of which may lead to various forms of neurodegenerative disease.<sup>60</sup> Since our previous and recent data suggested that  $\beta$ A3/A1-crystallin is a key component of starvation-induced and QC autophagy, it is highly likely that loss of this protein in RPE cells is linked to EMT.

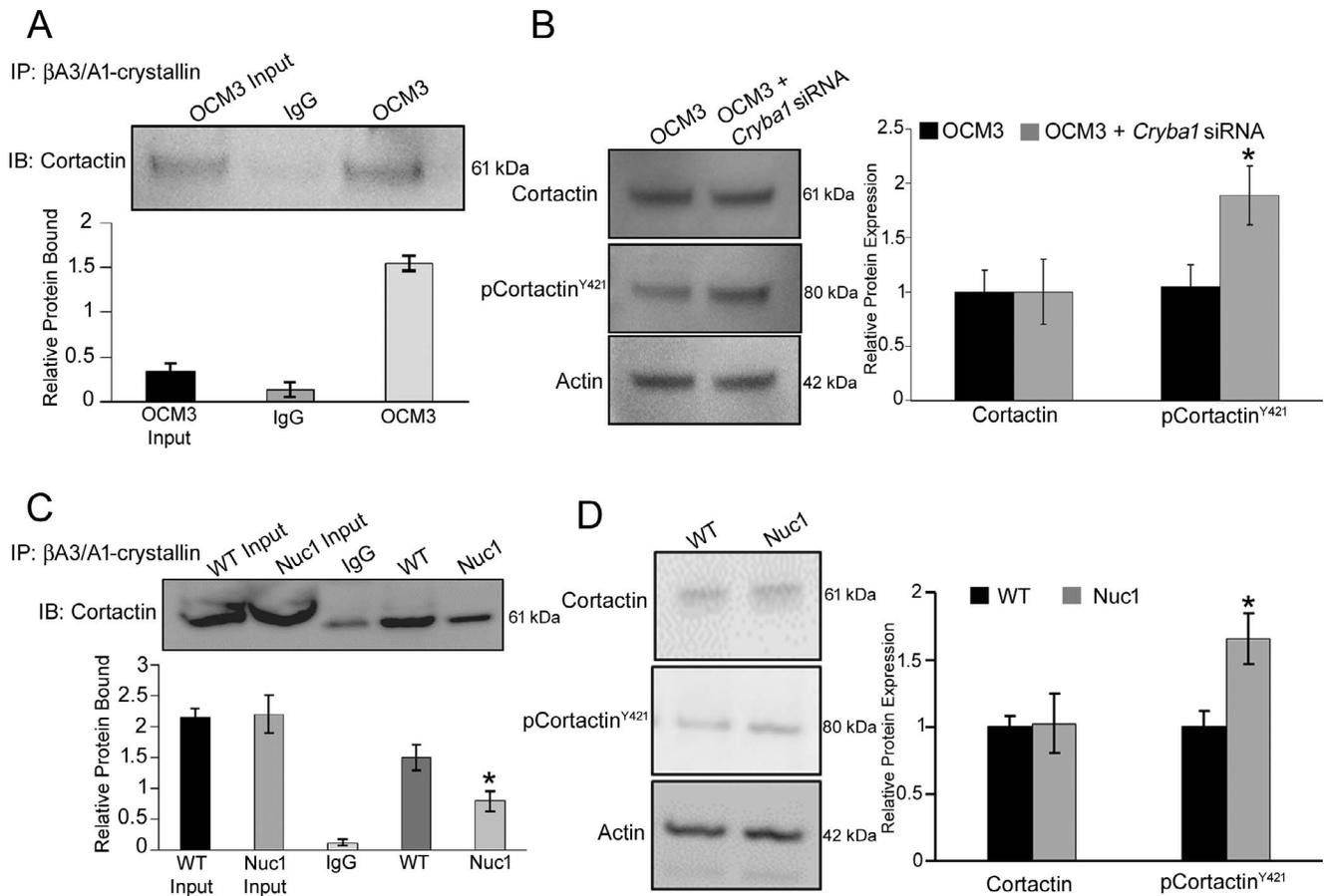
In summary, we introduced a new concept regarding one aspect of dry AMD pathology. We believe that RPE cells take refuge from the stressful microenvironment produced by oxidative insult via initiation of Type 2 EMT, a mechanism that the cells adopt to avoid cell death. However, in AMD patients,<sup>61,62</sup> as well as in our genetically engineered animal models,<sup>15</sup> the cells ultimately die due to the chronic nature of the insult as the disease progresses. The RPE may switch on EMT due to malfunctioning of various pathways/processes that are required to maintain homeostasis of the postmitotic RPE cells. One such process that is immensely important for RPE cell survival is lysosomal-mediated clearance by phagocytosis



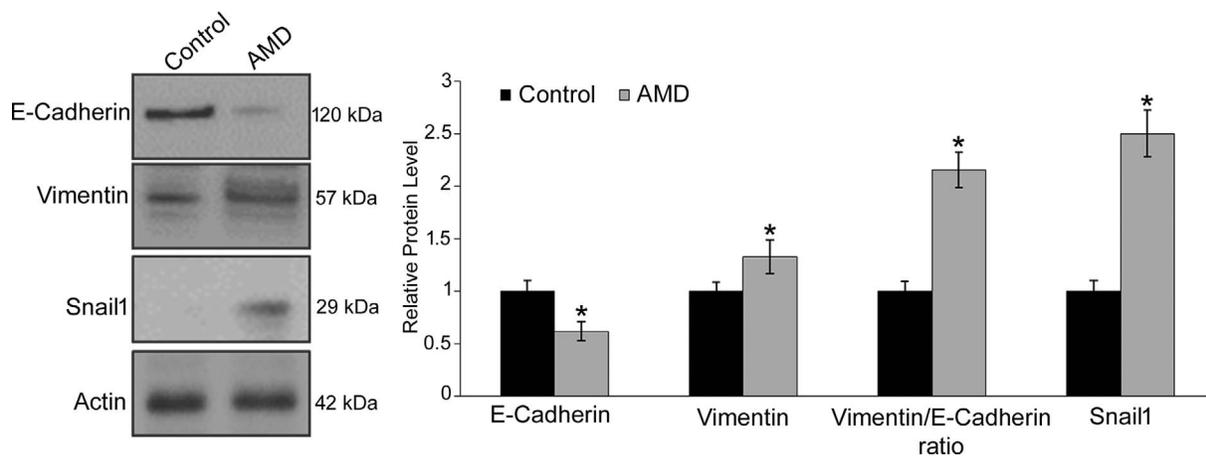
**FIGURE 4.** Knockdown of *Cryba1* triggers EMT-like phenotype in ocular melanoma cells. (A) Knockdown of *Cryba1* (OCM3 cells transfected with *Cryba1* siRNA) resulted in decrease in E-cadherin expression with an increase in vimentin expression, while overexpression of  $\beta$ A3/A1-crystallin (OCM3 cells transfected with *Cryba1* pcDNA) did not cause any changes in the expression of these proteins in OCM3 cells. Representative Western blot images are shown. The graphs show mean  $\pm$  S.D.; \* $P < 0.05$ , \*\* $P < 0.001$  (Mann-Whitney  $U$  test),  $n = 4$ . (B) Wound healing assay revealed that OCM3 cells transfected with *Cryba1* siRNA showed increased wound healing after 24 hours of exposure compared to OCM3 cells transfected with *Cryba1* pcDNA. The migration rate of control cells was taken as 1% and healing rate of other plates were compared with respect to control cells. (C) Increased cell migration is evident after 24 hours in *Cryba1* siRNA transfected cells compared to cells transfected with *Cryba1* pcDNA, suggesting the role of  $\beta$ A3/A1-crystallin in inhibiting ocular melanoma cell migration. The migration strips are divided into 5 units (each unit = 100  $\mu$ m), black arrows indicate the cluster of cells. The migration was quantified under microscope and replicated thrice for each group. The data are represented as 'units' migrated. All values are mean  $\pm$  SD and \*\* $P < 0.01$  indicates significant change compared to *Cryba1* siRNA transfected OCM3 cells at 0 hours.

and autophagy. It now is becoming increasingly clear that defects in these clearance processes can contribute to AMD. We suggest that there is a defined window of time during which patients suffering from dry AMD may be amenable to

treatment aimed at reversing the subpopulation of RPE that have entered EMT. While malfunction of a myriad of proteins involved in the cellular processes that maintain healthy RPE can lead to such dysfunction, our data indicated that  $\beta$ A3/A1-



**FIGURE 5.** Association between  $\beta$ A3/A1-crystallin and cortactin regulates cell migration. (A) Pull-down assay using antibody to  $\beta$ A3/A1-crystallin demonstrates that cortactin and  $\beta$ A3/A1-crystallin interact in OCM3 lysates. (B) Western blot analysis revealed that phosphorylated (Y421) cortactin is present at a higher level in cultured OCM3 cells transfected with *Cryba1* siRNA compared to untreated control. (C) Pull down assay followed by Western blot showed increased cortactin and  $\beta$ A3/A1-crystallin association in the RPE+Choroid from WT (control) compared to Nuc1 (mutation in *Cryba1* gene, forming nonfunctional  $\beta$ A3/A1-crystallin protein) rats. (D) Western blotting demonstrated increased expression of p-cortactin<sup>Y421</sup> in RPE+Choroid from Nuc1 rat compared to WT, with no change in the total cortactin level. These results suggested that mutated  $\beta$ A3/A1-crystallin loses its ability to bind cortactin and the loss of this association triggers phosphorylation of cortactin at Y421 resulting in actin disassembly and cell migration. All values are mean  $\pm$  S.D and are evaluated from three independent experiments. \*Denotes significant change ( $P < 0.05$ ) with respect to control OCM3 cells or RPE cell lysates from WT rats (Mann-Whitney *U* test),  $n = 4$ .



**FIGURE 6.** Prevalence of EMT-like phenotype in the RPE cells of human AMD tissues. RPE cell lysates from human AMD samples showed increased Snail1 and vimentin expression concomitant with decreased expression of E-cadherin compared to age-matched controls. Representative Western blot images are shown. The graphs show mean  $\pm$  SD and \* $P < 0.05$  (Mann-Whitney *U* test),  $n = 4$ .

crystallin is an attractive target for rejuvenation of lysosomal function, and potentially, for reversal of EMT. Thus, it may provide a novel and effective means of preventing or delaying the progression of the disease.

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