

**α B-CRYSTALLIN-MEDIATED RESCUE OF CENTRIOLIN
DEPLETION-INDUCED CELL DEATH IN RETINAL
PIGMENT EPITHELIAL CELLS**

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Catherine Campbell

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APPROVED:

Stephen Doxsey, Ph.D.
Program in Molecular Medicine
UMass Medical Center
Major Advisor

David Adams, Ph.D.
Biology and Biotechnology
WPI Project Advisor

ABSTRACT

Centrosomes are the polar anchors of the mitotic spindle, the apparatus which separates the chromosomes during cell division, and are required for cell cycle progression. Centriolin is one of the core centrosomal proteins, and its depletion is known to result in cytokinesis defects and cell cycle arrest, which may ultimately lead to cell death. Therefore, one of the major goals for this project was to investigate the effect of centriolin depletion in retinal pigment epithelial (RPE) cells. Preliminary experiments had suggested centriolin depletion induces cell death. A second major aim was to determine if the centriolin depletion phenotype could be rescued by overexpression of α B-crystallin (CRYAB). CRYAB is a small heat shock protein whose phospho-form localizes to centrosomes, and which is known to have an antiapoptotic effect. This study shows an inverse correlation between the expression of Ki-67, a cell proliferation marker, and the expression of cleaved caspase-3, an apoptosis marker, in centriolin depleted RPE cells. In contrast, centriolin depleted RPE cells overexpressing CRYAB exhibited increased Ki-67 expression, while cleaved caspase-3 expression decreased. Based on these observations, CRYAB appears to rescue RPE cells from centriolin depletion-induced cell death, however, the precise mechanism of rescue remains to be determined.

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BACKGROUND

The Centrosome and the Cell Cycle

The cell cycle is an omnipresent process responsible for cell growth and proliferation and organismal development. The cell cycle is divided into 4 major stages, including G1, S, G2, and M phase, where G1 and G2 represent the “gap” phases, S represents DNA synthesis, and M phase signifies mitosis. G1, S, and G2 are the subdivisions of interphase. Cells in G1, the first gap phase, are preparing for S phase, while cells in G2, the second gap phase, are preparing for mitosis. M phase consists of prometaphase, metaphase, anaphase, and telophase (reviewed in Schafer, 1998). Cytokinesis is characterized by the separation of the nascent dividing daughter cells, and occurs just after telophase. In mammalian cells, the contractile ring is assembled and contracts to form the cleavage furrow during early cytokinesis. The contractile ring is composed of actin and myosin filaments, and its constriction is powered by myosin motor activity, which recruits actin filaments and ultimately results in its contraction (reviewed in Glotzer, 2005). As the contractile ring continues to furrow, the plasma membrane constricts to form a cytoplasmic intercellular bridge between the two nascent daughter cells. Bundled microtubules and the midbody are located within the intercellular bridge (reviewed in Glotzer, 2001, 2005; Guertin et al., 2002). During abscission, the final event in cytokinesis, the daughter cells separate once the intercellular bridge is cleaved (reviewed in Barr and Gruneberg, 2007).

The centrosome is a juxta-nuclear organelle composed of two perpendicular centrioles in close proximity to each other surrounded by pericentriolar material (reviewed in Doxsey, 2001; Doxsey et al., 2005). The pericentriolar material (PCM) is

composed of a network of fibers and protein aggregates, and is the site of microtubule nucleation. The centrioles are symmetrical, barrel-shaped structures that each consist of nine sets of triplet microtubules. During S phase, the centrioles split and are duplicated, giving rise to two centrosomes. Each of these centrosomes has a younger daughter centriole and an older mother centriole, characterized by its supplementary appendages (reviewed in Doxsey, 2001). The mother centriole is the docking site for cytoplasmic microtubules and may anchor the centrioles to the cell membrane (reviewed in Bettencourt-Dias and Glover, 2007).

As the primary microtubule organizing center in mammalian cells, the centrosome directs the nucleation of polarized microtubules, which organize cytoplasmic organelles, arrange the mitotic spindle, synchronize cytokinesis, and arrange primary cilia in interphase (reviewed in Doxsey, 2001; Doxsey et al., 2005). The centrosome anchors regulatory molecules, and may receive, integrate, and propagate signals which control basic cellular functions (reviewed in Doxsey et al., 2005). The mitotic centrosome plays a role in the mitotic spindle's function and orientation. Conversely, interphase centrosomes organize the microtubules which serve as intercellular tracks for protein, organelle, and chromosome transport (reviewed in Nigg and Raff, 2009). Although the centrosome is well known for its role in microtubule nucleation, it has also been shown to function in cytokinesis and cell cycle progression (reviewed in Doxsey et al., 2001, 2005). For example, Piel et al. (2001) reported that the maternal centriole relocates to the intercellular bridge, a microtubule filled connection between the nascent daughter cells, during cytokinesis. When the intercellular bridge narrows and the microtubules depolymerize, the centriole begins to move away from this bridge. Cell cleavage, or

abscission, correlates with this event. This observation led to the prediction that the maternal centriole may regulate the pathway which controls cytokinesis and abscission in vertebrate cells (Piel et al., 2001).

Centriolin and the Cell Cycle

Centriolin, one of the major components of the maternal centriole in vertebrate cells, may be required to associate with the maternal centriole for the initiation and completion of cytokinesis (Piel et al., 2001; Gromley et al., 2003). In addition, centriolin colocalizes with the exocyst-complex to the midbody-ring during cytokinesis, and is suggested to be required for abscission. The exocyst-complex plays a role in membrane-vesicle tethering during abscission in mammalian cells, and is composed of many proteins which target secretory vesicles to specific regions on the plasma membrane. Upon impairment of the exocyst, vesicles accumulate at this complex and cell cleavage is disrupted (Dobbelaere and Barral, 2004; Verplank and Li, 2005). When cells are depleted of centriolin, the ability of the exocyst-complex to localize to the midbody is disrupted, demonstrating that centriolin is required for exocyst midbody localization (Gromley et al., 2005). Altogether, such results suggest centriolin may be required for the completion of cell cleavage during abscission in mammalian cells.

Centriolin depletion is known to induce several cell cycle phenotypes, including G1 arrest and cytokinesis failure. This cytokinesis failure is characterized by the presence of persistent, long intercellular bridges between dividing nascent daughter cells which fail to cleave. Intercellular bridges may remain even after several mitotic cycles, resulting in syncytia formation, binucleated cell formation, or cell death. Collectively,

these data suggest that centrosomes may have a role in cell cycle progression in vertebrate cells (Gromley et al., 2003).

Heat Shock Proteins and the Heat Shock Response

Following translation or denaturation, most proteins cannot spontaneously achieve their native conformation, especially when exposed to stressful conditions (reviewed in Broadley and Hartl, 2009; Hartl and Hayer-Hartl, 2009). This inability to fold into their functional conformation is a result of high intracellular protein concentrations that favor the misfolding and aggregation of cellular proteins. During such potentially catastrophic events, certain molecular chaperone proteins assist these misfolded and aggregated proteins in refolding into their native conformation via chaperone activity (commentary in Liang and MacRae, 1997). Heat shock proteins (HSPs) are present in nearly all organisms, are some of the most highly conserved proteins identified, and are ubiquitously expressed in a majority of tissues (Schlesinger, 1990). HSPs are categorized into 5 major families based on their size, structure, and function, and include HSP100, 90, 70, 60 and the small HSP, or α -crystallin proteins (Craig et al., 1994; Kampinga et al., 2009). Once activated by stressful conditions, molecular chaperone proteins bind to and stabilize misfolded proteins to prevent protein aggregation, assist in protein refolding, and if necessary, mediate protein targeting for proteasomal degradation before misfolded proteins form aggregates (Ellis and van der Vies, 1991; McClellan and Frydman, 2001; Sherman and Goldberg, 2001; Slavotinek and Biesecker, 2001; Meriin and Sherman, 2005). Aside from their production in stressed cells, HSPs are also present in unstressed cells (Hendrick and Hartl, 1993, 1995). A

majority of HSPs exercise strong cytoprotective effects which are essential to cellular well being. In general, most molecular chaperone HSPs protect against protein damage and promote cell survival (Garrido et al., 2006).

Small HSPs: α B-crystallin (CRYAB)

Small HSPs/ α -crystallin proteins are characterized by their low molecular weights (15 to 30 kDa), have molecular chaperone activity, and form oligomeric complexes to protect various proteins from destabilization and denaturation (Bennardini et al., 1992; Horwitz, 1992; de Jong et al., 1993; Nicholl and Quinlan, 1994). In 1982, it was observed that certain crystallin proteins were also small HSPs, and expressed shared sequence homology (Ingolia and Craig, 1982). Small HSPs, including α B-crystallin (CRYAB), exhibit molecular chaperone activity and thermoprotection *in vitro* and *in vivo* (Landry et al., 1989; Horwitz, 1992; Lavoie et al., 1993; Arrigo and Landry, 1994; Parsell and Lindquist, 1993; Muchowski et al., 1997). This information makes CRYAB of interest to researchers studying molecular chaperone proteins.

CRYAB is a member of the Class I subdivision of the small HSP/ α -crystallin family and is ubiquitously expressed in several tissues, particularly lens and muscle tissue (Iwaki et al., 1989; Iwaki et al., 1990; Piatigorsky, 1990; Kato et al., 1991; Klemenz et al., 1991; Srinivasan et al., 1992; Fluegel et al., 1993; Sax and Piatigorsky, 1994; Horwitz, 2000; reviewed in Taylor and Benjamin, 2005). Small HSPs lack intrinsic ATPase activity, indicating that they do not depend of ATP as a source of energy to drive chemical reactions (reviewed in Taylor and Benjamin, 2005). In addition, CRYAB phosphorylated at Ser-59 is localized to the centrosome and midbodies during

microtubule assembly (Inaguma et al., 2001). These observations show a potential function for CRYAB in various cellular processes involving microtubule organization and assembly, particularly during mitotic spindle assembly and mitosis.

aB-Crystallin and Apoptotic Cell Death

Oxidative stress and heat shock are just two of the many stress stimuli which can trigger apoptosis and induce synthesis of cytoprotective HSPs, which influence various apoptosis cascades (Garrido et al., 2001; Alge et al., 2002). The two main apoptosis pathways are the mitochondrial (intrinsic) and death receptor (extrinsic) pathways (Earnshaw et al., 1999; Miller, 1999; Wolf and Green, 1999; Stennicke and Salvesen, 2000). Both the mitochondrial- and death receptor-mediated apoptosis pathways activate apical caspases, caspase-9 and caspase-8, respectively. Caspase-8 and -9 are two of the four apoptotic initiators, and both proteolytically activate caspase-3, an apoptotic executioner. Caspase-3 is a member of the cysteine protease family of caspases, which are apoptosis effectors that selectively cleave target proteins at aspartate residues (reviewed in Wolf and Green, 1999; Alge et al., 2002). Caspase-3 activation eventually results in cellular protein cleavage and nuclease activation, which ultimately result in cell death via apoptosis (reviewed in Wolf and Green, 1999; reviewed in Riedl and Salvesen., 2007).

Recently, it was demonstrated that small HSP family proteins may interact directly with the cell's apoptotic machinery and act as pro-survival factors (Mehlen et al., 1996; Bruey et al., 2000; Dimberg et al., 2008). Similar to other HSPs, CRYAB expression is elevated several fold after HS and other stresses in numerous cell lines, and

may function to prevent stress induced protein aggregation and denaturation (Klemenz et al., 1991; Kamradt et al., 2001). Increased CRYAB levels have also been reported in diverse conditions, such as neurodegenerative disease (van Rijk and Bloemendal, 2000), autoimmune disease (van Noort et al., 1995; Bajramovic et al., 1997), cardiomyopathy (Rajasekaran et al., 2007), and certain cancers (Strik et al., 2000; Hermisson et al., 2000). Apoptosis induced by staurosporine (Mehlen et al., 1996), UVA radiation (Andley et al., 2000), and okadaic acid (Li et al., 2001) can be prevented by CRYAB expression in cultured cells. CRYAB has been shown to enhance cell survival by inhibiting the autoproteolytic maturation of caspase-3, which may be the mechanism for prevention of cell death (Kamradt et al., 2001).

CRYAB is constitutively expressed in RPE cells and may have an antiapoptotic effect, in which CRYAB overexpression reduces oxidative stress-induced caspase-3 activity and global cell death. Of particular interest, heat shock and oxidative stress significantly increased caspase-3 activity and CRYAB expression simultaneously. Conversely, cells stably transfected with CRYAB, then exposed to oxidative stress, exhibited relatively low caspase-3 activity levels. This observation indicates CRYAB may be a stress-inducible protein with antiapoptotic effects in RPE cells. Overall, overexpression of CRYAB and other stress proteins may provide supplementary techniques to postpone cell death in age-related degenerative diseases (Alge et al., 2002).

CRYAB is a caspase-3 inhibitor that is up-regulated by Bcl2L12 (Bcl2-Like-12) in normal and transformed glial cells. Bcl2L12 is a proapoptotic molecule that is overexpressed in a majority of human primary glioblastoma multiforme (GBM) samples. Both Bcl2L12 and CRYAB are gliomagenic oncoproteins which work together to

encourage resistance to apoptosis in GBM. Collectively, these results demonstrate CRYAB's oncogenic role as a Bcl2L12-downstream effector in cell signaling in GBM specimens (Stegh *et al.*, 2008). In addition, CRYAB expression is upregulated and the CRYAB protein phosphorylated during endothelial cell tubular morphogenesis, which results in vascularization of tumor masses. This information suggests CRYAB promotes survival of tumor cells by encouraging tumor mass vascularization, which enhances tumor progression (Dimberg *et al.*, 2008). These observations provide further evidence of CRYAB's antiapoptotic and prosurvival properties, which may make CRYAB a potential target for chemotherapeutic agents in cancer biology.

PROJECT PURPOSE

The centrosome, the major microtubule organizer in mammalian cells, is comprised of a pair of centrioles embedded in a matrix of proteins, known as the pericentriolar material. Abnormalities in the centrosome may lead to a number of pathologies, including chromosomal instabilities, thus linking centrosomal proteins to cancer. The centrosome has a complex and dynamic protein composition, and depletion of any of a variety of the centrosomal proteins results in G1 cell cycle arrest. Centriolin is one of the core centrosomal proteins and is localized to the maternal centriole and the mitotic midbody ring with its best-described function in cytokinesis. Depletion of centriolin has been shown to result in G1 arrest and cytokinesis failure, which is characterized by the presence of long, persistent intercellular bridges between dividing nascent daughter cells which fail to cleave. Intercellular bridges may remain even after several mitotic cycles, resulting in syncytis formation, binucleated cell formation, or cell death. α B-crystallin (CRYAB) is a small heat shock protein/ α -crystallin family member, and is present at the centrosome and midbody in its phospho-form. CRYAB has cytoprotective properties, and may interact with caspase proteins directly to protect cells from apoptotic cell death. The focus of this study was to examine the effect of centriolin depletion on cellular survival, and potential rescue by CRYAB via its chaperone activity, which may occur at the centrosome.

METHODS

Maintenance and Transfection of RPE Cells

All experimental studies in this project used pseudo-diploid, telomerase-immortalized retinal pigment epithelial cells (RPE-1) (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium/F12 supplemented with 1% HEPES, 1% glutamate, 1% penicillin/streptomycin, and 10% fetal bovine serum. Cells were plated onto glass coverslips 24 hours before transfection. Cells were transfected with siRNAs using Lipofectamine2000 (Invitrogen) according to manufacturer's directions (Dharmacon, Lafayette, CO). siRNA sequences targeting centriolin and GFP were used as described previously (Gromley et al, 2003). Cells were transfected using Lipofectamine2000 with plasmid DNA according to the manufacturer's instructions. Myc-CRYAB construct was a kind gift from I. Benjamin (University Of Utah). Cells were collected at the indicated times after transfection.

Antibodies

Anti-centriolin 883 antibody was generated by our lab as previously described (Gromley et al, 2003). Commercially available primary antibodies against GFP, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), myc (Santa Cruz Biotechnology, Santa Cruz, CA), Hsc70 (Abcam, Cambridge, MA) were used for immunoblotting. Secondary antibodies labeled with anti-rabbit, anti-mouse, and anti-rat HRP were purchased from Jackson Laboratories (Bar Harbor, ME) and were used for immunoblotting. For immunofluorescent staining, commercially available primary antibodies against cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), Ki-67 (Abcam, Cambridge, MA),

gamma-tubulin (Doxsey Lab), myc (Abcam, Cambridge, MA), and GFP (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Secondary antibodies labeled with anti-rabbit-568, anti-mouse-488, and anti-mouse-Cy3 were used for immunofluorescent staining (Jackson Laboratories, Bar Harbor, ME).

Immunofluorescent Staining and Microscopy of Cultured Cells

Cultured cells were fixed in ice cold methanol and stored at -20°C. Following a PBS wash and blocking with PBSAT, coverslips were incubated with primary antibody for 90 minutes. Secondary antibody incubations were performed for 45 minutes, and coverslips were counterstained with DAPI. Coverslips were mounted in Prolong Gold, then analyzed and imaged with a Zeiss Axiophot microscope equipped with a 100X plan Fluor lens. For time lapse imaging, a Zeiss Axiovert 200 with a 20X plan Fluor lens was used. All imaging used MetaMorph for image analysis and labeling (Molecular Dev., Downington, PA).

Immunoblot Assay

Cell lysates were prepared and analyzed for protein depletion and expression. Following siRNA and plasmid DNA treatment for 48-72 hours, cells were harvested and lysed on ice for 30 minutes in NP-40 budder (1% PMSF, 1% sodium orthovanadate, and supplemented with Complete Mini protease inhibitor (Roche Applied Science, Indianapolis, IN). Cell lysates were cleared by centrifugation. The protein concentration of each cell lysate was determined using Bio-Rad protein Assay, and gel loadings were

adjusted accordingly. Proteins were resolved by SDS-PAGE and analyzed by Western Blot.

RESULTS

Verification of Centriolin Knockdown and CRAYB Overexpression

The initial phase of this project involved using siRNA to knockdown the expression of centriolin in RPE cells, and to overexpress myc-CRAYB as a potential rescue. RPE cells were transfected with siRNA targeting centriolin (siR2), and control GFP (siGFP) as a control, at 0 hours. Samples designated “siMock” indicated no siRNA was used, rather, cells were treated only with transfection reagent. Cells were then transfected with plasmid encoding myc-CRYAB or EGFP-N1 24 hours after siRNA transfection. Cells were harvested and lysed at 48 and 72 hours. Verification of the depletion of centriolin, GFP knockdowns, and overexpression of myc-CRYAB and EGFP-N1 were assayed by Western Blots (**Figure 1, A and B**). GAPDH and Hsc70 were used as loading controls, and showed equal signals. As shown in Figure 1 A, the endogenous level of centriolin decreased at 48h in siCentriolin-transfected cells compared to siGFP-transfected cells, and remained low at 72h post-transfection (Fig 1, B). The expression of myc-CRYAB (detected using anti-myc antibodies) was consistently high in all samples transfected with plasmid myc-CRYAB at either 48 or 72h post-transfection. Expression of the EGFP construct was decreased in cells double-transfected with siGFP, demonstrating the efficiency of the siGFP treatment. The figure is representative of 6 experiments showing similar results.

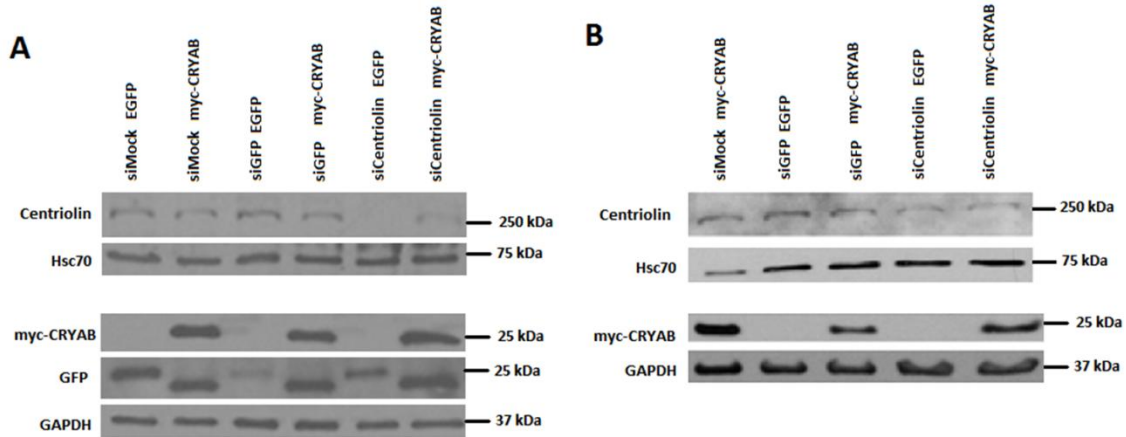


Figure 1. Verification of Centriolin Knockdown and Overexpression of myc-CRYAB in RPE Cells at 48 and 72 Hours. (A) Western Blot analysis of RPE cell lysates at 48 hours. (B) Western Blot analysis of RPE cell lysates at 72 hours.

Effect of Centriolin and CRYAB on Cell Proliferation and Morphology

The next step in the project was to examine the possible differences in cell proliferation between controls and centriolin depleted cells. As discussed in the Background section, cell cycle arrest at the G1 stage is known to occur in RPE-1 cells following centriolin depletion. It was of particular interest to determine whether overexpression of myc-CRYAB would rescue a centriolin depletion phenotype. RPE cells were transfected with siRNA targeting centriolin (siCentriolin) and GFP (siGFP) (as a control) at 0 hours (siMock indicates no siRNA was used, only transfection reagent) (**Figure 2**). Cells were then transfected with plasmid DNA for myc-CRYAB or EGFP-N1 24 hours after siRNA transfection. Each image, at 10X, is representative of 6 experiments with similar results. The data indicate that cells transfected with siCentriolin and control EGFP plasmid (5th column) displayed a decrease in cell number and numerous rounded up cells, while RPE cells transfected with siCentriolin but also overexpressing myc-CRYAB (6th column) appeared comparable to control cells (columns 1-4) (**Figure 2**). White arrows indicate dead cells seen in the centriolin-depleted cells.

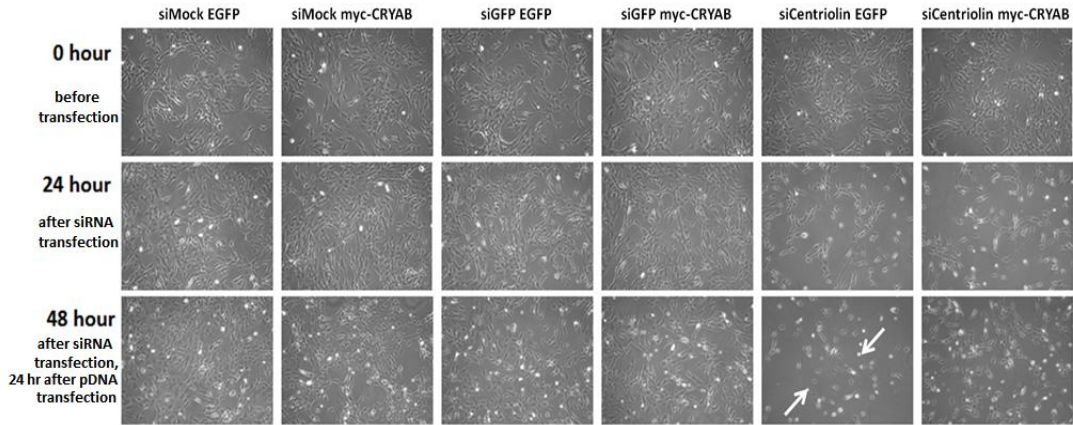


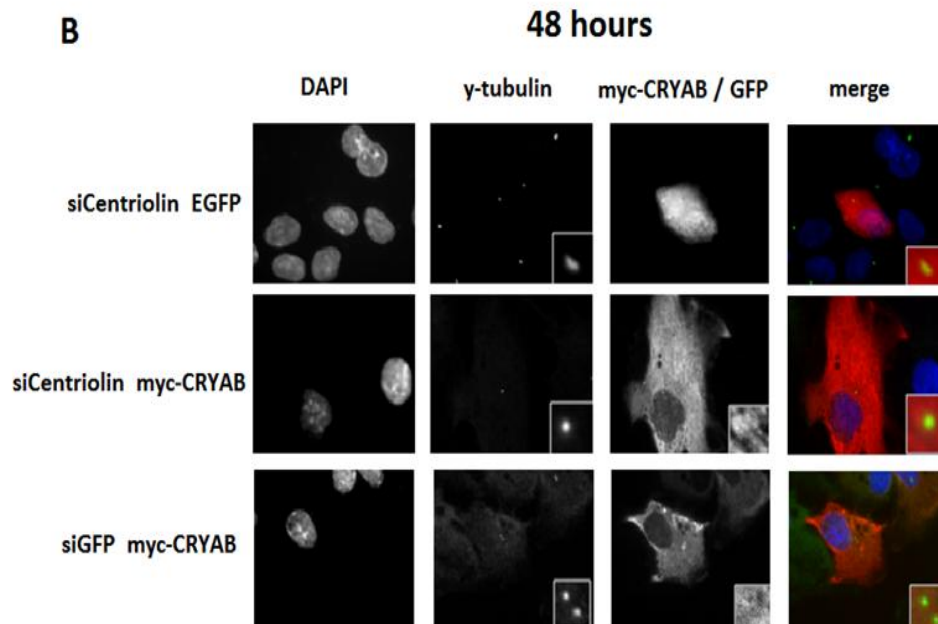
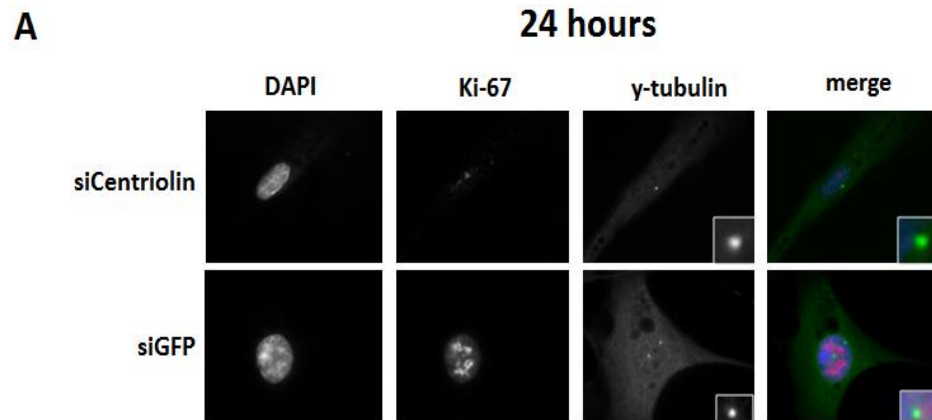
Figure 2. Assay of Cell Numbers and Morphology in Transfected Cells. myc-CRYAB overexpression rescues RPE cells from centriolin depletion-induced cell death by 48 hours. White arrows indicate dead cells in the centriolin-depleted cells. Cell culture images were taken at 0, 24, and 48 hours post siRNA transfection at 10X magnification.

To assess whether the observed decreased cell number seen in centriolin-depleted cells was due to decreased cell proliferation, staining was performed with cell proliferation marker, Ki-67, for 24 hour samples (**Figure 3A**). Control GFP-depleted cells (lower row) robustly expressed Ki-67 (red), while centriolin-depleted cells (upper row) expressed relatively low levels of Ki-67 at 24 hours. Thus, the low cell numbers observed for centriolin-depleted cells appears to result from decreased cell proliferation.

Cellular Location of myc-CRYAB

One of the possible mechanisms for myc-CRYAB rescuing the decreased cell proliferation phenotype was that CRYAB may translocate to the centrosome and prevent changes induced by the centriolin depletion. Coverslips were stained for γ -tubulin (green), and either GFP (red) or myc (CRYAB, red), and counterstained with DAPI (blue) (**Figure 3B and C**). The intracellular localization of myc-CRYAB in methanol-

fixed cells (3rd column) showed cytoplasmic distribution, often with granular structures. No enrichment or exclusion of myc-CRYAB was observed in centrosomal regions, where γ -tubulin was used as a marker.



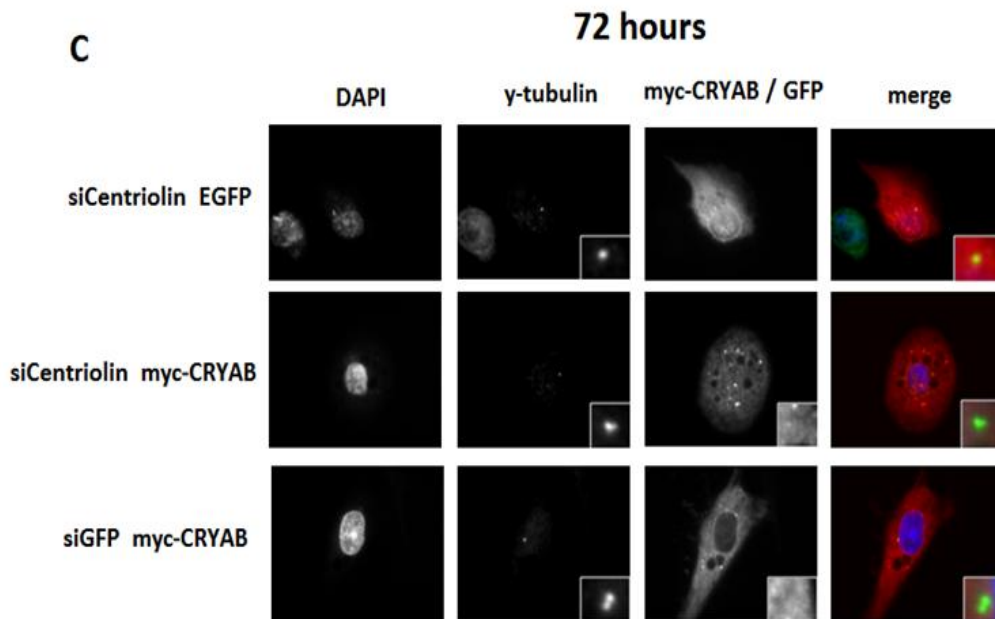


Figure 3. Assay of myc-CRYAB Localization. Immunofluorescent staining of cultured RPE cells at 24, 48, and 72 hours at 100X. (A) Staining of cultured RPE cells 24 hours post siRNA transfection. Cells were stained with gamma-tubulin (AATR), Ki-67, and counterstained with DAPI. Inserts represent enlarged images of centrosomal staining. (B) Staining of cultured RPE cells 48 hours post siRNA transfection. Cells were also transfected with plasmid DNA at 24 hours. Cells were stained with either myc (CRYAB) or GFP, gamma-tubulin (AATR), and counterstained with DAPI. Inserts represent enlarged images of centrosomal staining. (C) Staining of cultured RPE cells 72 hours post siRNA transfection. Cells were also transfected with plasmid DNA at 24 hours. Cells were stained with either myc (CRYAB) or GFP, gamma-tubulin (AATR), and counterstained with DAPI. Inserts represent enlarged images of centrosomal staining.

Effect of Centriolin and CRYAB on Centriolin Depletion-Induced Apoptosis

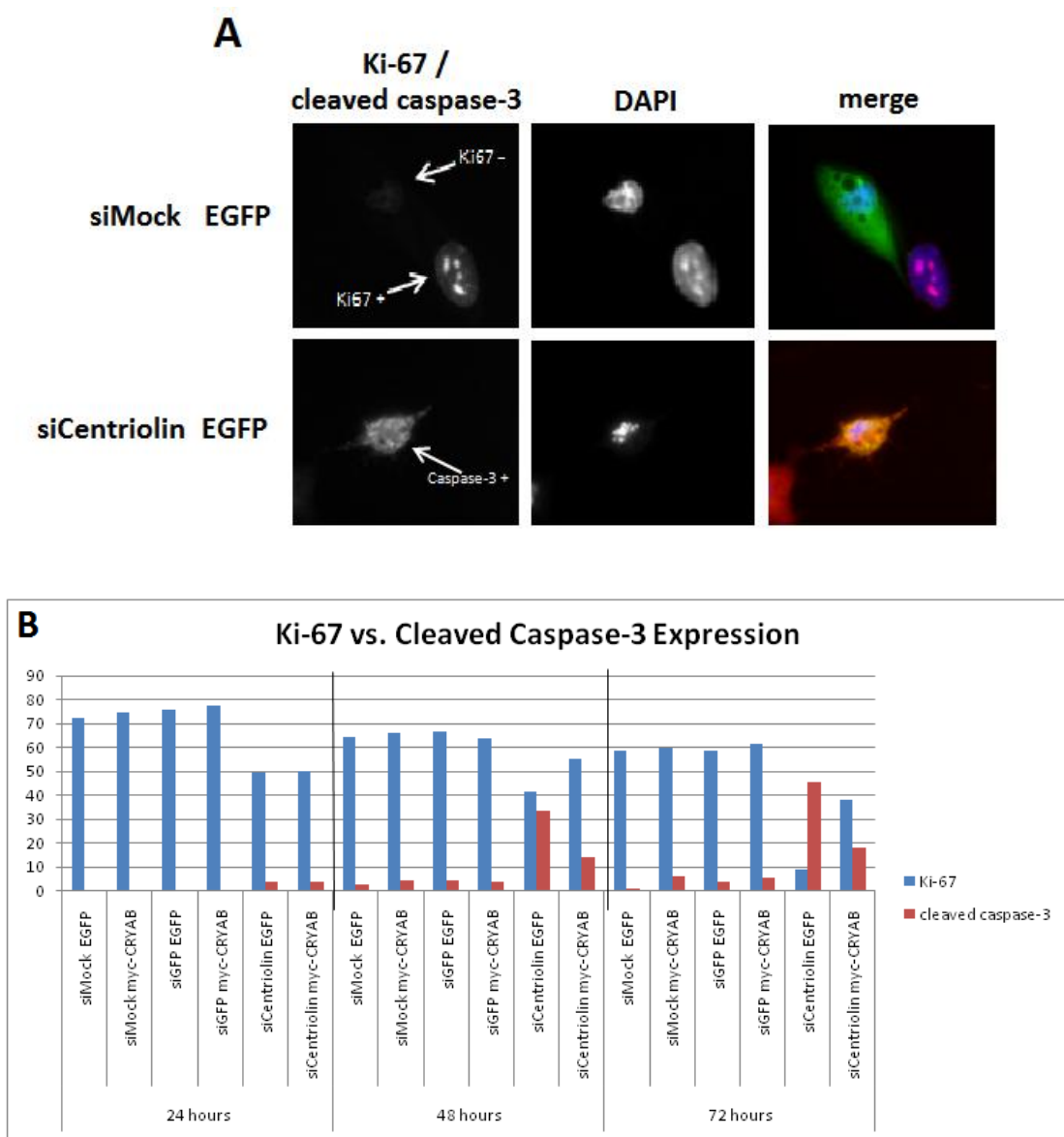
Further experiments focused on determining the relationship between cell proliferation and cell death in centriolin-depleted cells expressing either the control EGFP plasmid or the myc-CRYAB construct. As discussed previously, G1 cell cycle arrest can result in apoptosis in some cell lines. Cells were co-stained for cell proliferation marker Ki-67 (red), and an apoptosis marker, cleaved caspase-3 (red), and counterstained for DAPI (blue) (**Figure 4**). Cells shown were also stained for GFP

(green). Panels 4B and D represent cell counts viewed on a widefield microscope (n = 500). Results are representative of 3 experiments with similar results.

Since a proper centriolin antibody for immunofluorescence assays was unavailable, all cells were considered to be depleted of centriolin, since immunoblot analysis already confirmed centriolin depletion in the siCentriolin-treated population (Figure 1, A and B). Cells expressing Ki-67 were considered to be proliferating, and cells expressing cleaved caspases-3 were considered to be apoptotic (**Figure 4A, B**). In centriolin-depleted cells expressing GFP, at 72 hrs the percentage of Ki-67-positive cells decreased to 9% compared to approximately 60% for control cells, while cleaved caspases-3 expression increased from roughly 4% to 46%. In contrast, Ki-67 positive centriolin-depleted cells expressing myc-CRYAB remained at 38%, while cleaved caspases-3 expression lingered at 18% at 72 hours. These results demonstrate an inverse correlation between cell proliferation and cell death, and suggest centriolin-depleted cells are undergoing apoptosis (**Figure 4B**). Thus, the results show that centriolin-depleted cells overexpressing the myc-CRYAB construct might be rescued from cell death, based on the 3-fold increase in Ki-67 expression and 2-fold decrease in cleaved caspases-3 expression relative to centriolin depleted cells expressing EGFP plasmid (**Figure 4B**).

To further investigate whether myc-CRYAB overexpression rescues centriolin depleted cells from cell death, additional cell counts were conducted. In contrast to the previous cell count in which all cells were included, in this case only cells expressing either GFP or myc-CRYAB were included in the cell count (centriolin depletion was previously confirmed via immunoblot assay (Figure 1A and B) (**Figure 4C**). Cell proliferation for centriolin-depleted cells expressing GFP remained at 25% and 20%, at

48 and 72 hours, respectively. In contrast, cell proliferation for centriolin depleted cells expressing myc-CRYAB was 55% and 58%, at 48 and 72 hours, respectively (**Figure 4D**). Based on the 2-fold difference in cell proliferation at 48 and 72 hours, this result provided further evidence that overexpressing myc-CRYAB in centriolin-depleted RPE-1 cells rescues them from apoptotic cell death.



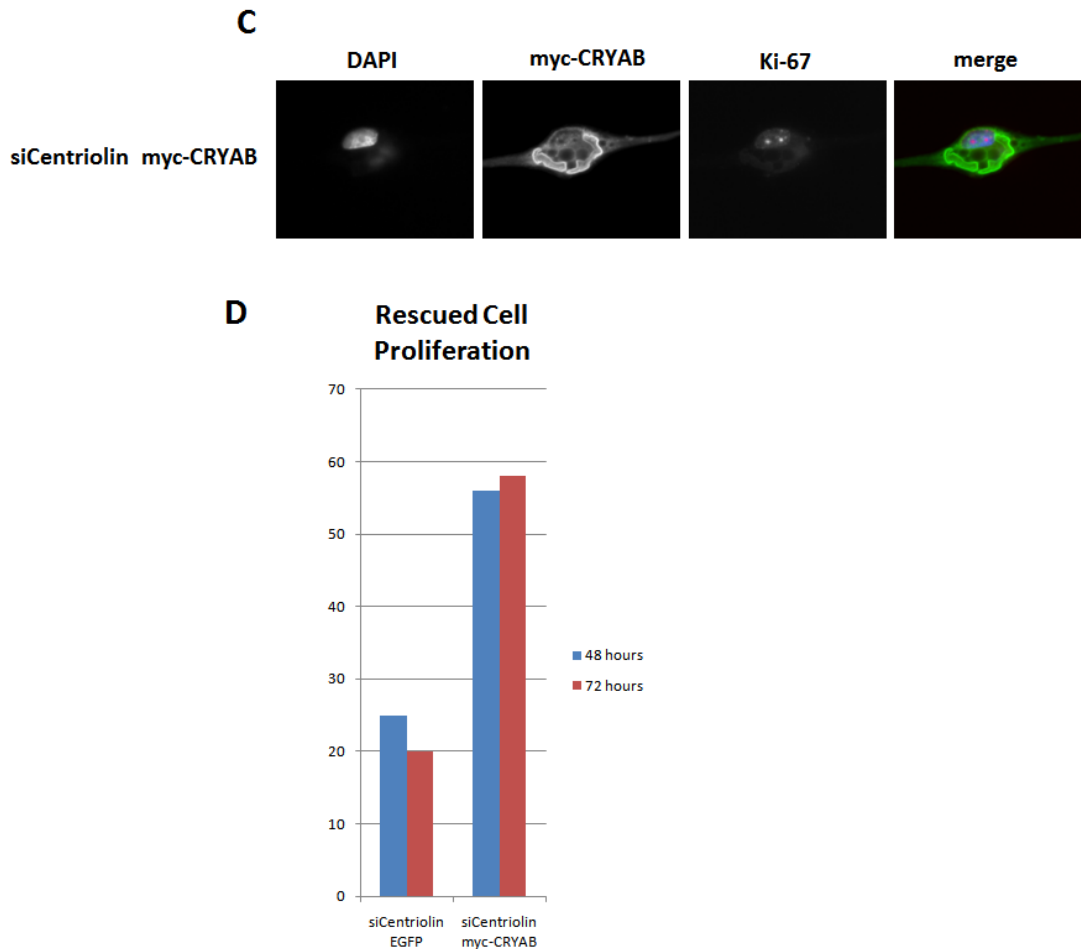
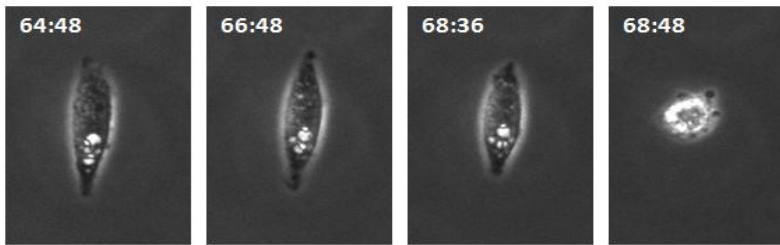


Figure 4. Effect of Centriolin and CRAYB on Centriolin Depletion-Induced Apoptosis. Ki-67 Positive and Cleaved Caspase-3-Positive Cell Counts in RPE at 24, 48, and 72 hours. (A) RPE cells stained for Ki-67 and counterstained with DAPI. Representations of both Ki-67 positive and negative cells are shown. RPE cells stained with cleaved caspase-3 and counterstained with DAPI. Representation of cleaved caspase-3 positive cell is also shown. (B) Cell counts at 24,48, and 72 hours for Ki67- or cleaved caspase-3-positive RPE cells. (C) Representation of a rescued RPE cell positively expressing myc-CRYAB and Ki-67. RPE cells were stained with myc, Ki-67, and counterstained for DAPI. (D) Rescued cell proliferation count at 48 and 72 hours.

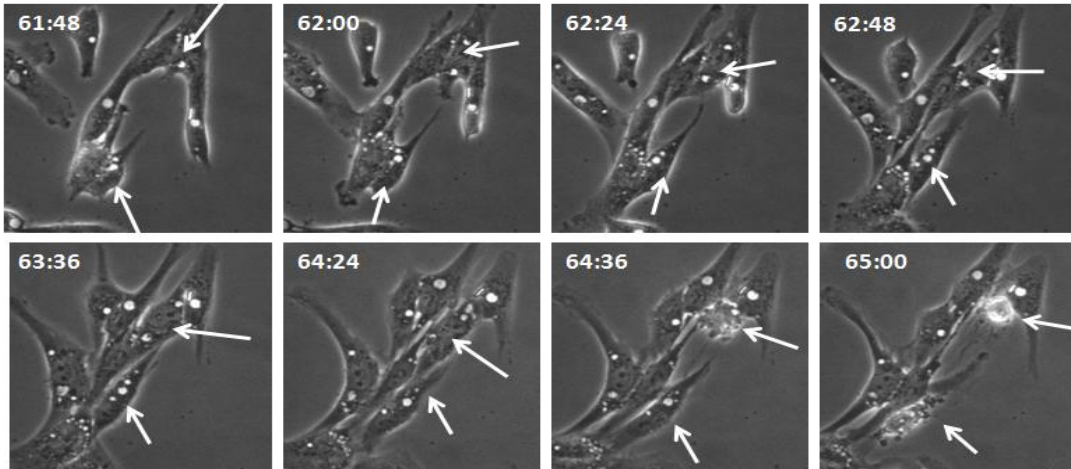
In an effort to determine at what stage of the cell cycle centriolin-depleted cells were dying, time lapse imaging was employed (**Figure 5**). At 0 hours, cells were transfected with siRNA targeting centriolin. Time lapse imaging (20X) began 48 hours after transfection, and continued for 24 hours. In **Panel A**, a single cell dies after remaining in interphase for an extended period of time. In **Panel B**, two cells (marked by

white arrows) extend toward each other then retract their projections before dying. The upper row in **Panel C** shows a cell which appears to round up, and possibly arrests in mitosis for about 15 hours. The future viability of this particular cell remained undetermined, since time lapse imaging was terminated at this final time point. The bottom row in Panel C shows a cell which appears to round and possibly arrests in mitosis for about 9 hours before dying. All data shown in Figure 4 is preliminary, and a proper control is still required before analyzing the results.

A



B



C

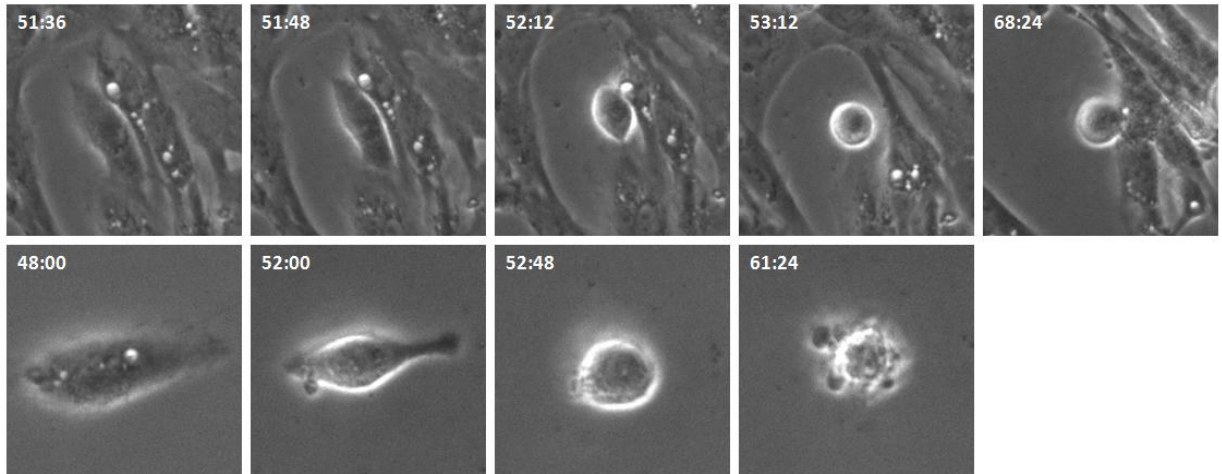


Figure 5. Time Lapse Imaging of siCentriolin-Transfected Cells. RPE cells were transfected with siRNA targeting centriolin at 0 hours. Time lapse imaging continued from 48 hours to 72 hours after siRNA transfection. (A) Cell death in centriolin depleted RPE cell in interphase. (B) Cell death in centriolin depleted RPE cells. White arrows denote two cells whose projections extend towards each other before apoptosis. (C) Possible mitotic arrest and cell death in centriolin depleted RPE cells. Top row shows cell possibly arrested in mitosis, while bottom row displays cell potentially arrested in mitosis and resulting in cell death.

DISCUSSION

Based on the data obtained during this project, it was concluded that CRYAB may have an anti-apoptotic effect on centriolin-depleted hTERT-immortalized retinal pigment epithelial (RPE-1) cells. Western Blot analysis showed centriolin protein levels were depleted in siCentriolin-treated cells, and myc-CRYAB was strongly expressed at levels near to the endogenous housekeeping protein GAPDH and the hsc70 loading controls, at 48 and 72 hours (Figure 1, A and B). Cells treated with siCentriolin and EGFP plasmid showed a reduction in global cell number and an increase in dead cells at 48 and 72 hours (Figure 2). However, cells treated with siCentriolin and myc-CRYAB plasmid were more abundant in cell number with a lower percentage of dead cells by 72 hours (Figure 2). This result was consistent with previous data reported by Alge et al (2002), in which CRYAB overexpression reduced global cell death. These data indicate that centriolin depleted cells expressing myc-CRYAB can be rescued from cell death, and may have increased cell proliferation rates compared to centriolin-depleted cells expressing only EGFP.

Based on immunofluorescence microscopy, it was determined that the plasmid expressed myc-CRYAB protein was distributed uniformly throughout the cytoplasm with some expression in the nucleus (Figure 3b, c). Although myc-CRYAB was not excluded at the centrosome, the protein was not observed to be specifically accumulating at the centrosome (Figure 3b, c). Therefore, a conclusion could not be made as to whether myc-CRYAB served its rescue function at the centrosome itself, or whether the protein was performing its presumed rescue function elsewhere, such as in the cytoplasm.

However, it was previously reported that CRYAB localizes to the centrosome in its phospho-form (Inaguma et al., 2001). Since only the localization of the myc-CRYAB construct was investigated in these experiments, it is difficult to determine whether CRYAB localizes to the centrosome during rescue, since endogenous CRYAB protein localization was not examined. Therefore, as a next step, careful examination of endogenous CRYAB protein in control and centriolin-depleted cells would be useful. At this stage, it appeared that myc-CRYAB may be rescuing centriolin-depleted cells from cell death, possibly by inhibiting the autoproteolytic maturation of caspase-3 as shown by Kamradt et al (2001). However, it was unclear whether its rescue function was due to myc-CRYAB's interaction with a caspase intermediate or its unknown centrosomal function.

In order to determine whether myc-CRYAB rescued centriolin-depleted cells from cell death, cell proliferation rates and cleaved caspase-3 expression were compared. The results showed that centriolin-depleted cells overexpressing myc-CRYAB might be rescued from cell death, based on the 3-fold increase in Ki-67 expression, and the 2-fold decrease in cleaved caspases-3 expression relative to centriolin-depleted cells expressing EGFP plasmid (Figure 4, B). Similarly, cells stably transfected with CRYAB have been previously shown to exhibit relatively low caspase-3 activity levels following oxidative stress (Alge et al., 2002). These results demonstrate an inverse correlation between cell proliferation and cell death, and suggest centriolin-depleted cells are undergoing apoptosis (Figure 4, B). In addition, the rescued cell count data showed a 2-fold increase in cell proliferation between centriolin-depleted cells expressing myc-CRYAB and control GFP (Figure 4, D). This study shows for the first time that overexpression of

myc-CRYAB can rescue RPE-1 cells from centriolin depletion-induced apoptotic cell death.

Preliminary time lapse imaging analysis of centriolin-depleted cells was conducted to determine which cell cycle stage cells were dying (Figure 5). Results showed that centriolin-depleted cells may undergo cell death while in interphase or mitosis (Figure 5A, B, and C). Many cells appeared to undergo apoptosis after remaining in interphase for extended periods of time (Figure 5A). However, some cells appeared to make contact with each other via cellular processes, then undergo cell death as they separated (Figure 5B). More interestingly, many cells appeared to arrest in mitosis for extended periods of time or eventually underwent cell death (Figure 5C). Numerous centriolin-depleted cells also seemed to arrest in mitosis, perhaps in prometaphase or metaphase, for about 10 hours and subsequently undergo cell death (Figure 5C, bottom row). Although Gromley et al (2003) observed long, persistent intercellular bridges between dividing nascent daughter cells as a result of cytokinesis failure, none of these were observed in this study. Since these studies used RPE-1 cells and the previous experiments used HeLa, these inconsistencies are not surprising given the different origins of the two cell types. Although these results are particularly intriguing and exciting, the data are preliminary and further analysis will require more controls, especially siRNAs targeting GFP and centrin, a centrosomal protein similar to centriolin, will be used as controls in future experiments.

For the first time, this study shows that centriolin depletion induces apoptotic cell death in RPE-1 cells. In addition, this study is the first to show that overexpression of myc-CRYAB in centriolin-depleted RPE-1 cells prevents apoptotic cell death. Further

experiments will be aimed at determining the mechanism for cell death and rescue. In particular, activity should be induced at the centrosome using other heat shock proteins which lack antiapoptotic activity, such as HSP90. Such an experiment would help us determine whether it is the chaperone activity of heat shock proteins which results in rescue after centriolin depletion. Alternatively, CRYAB can be mutated to inhibit its apoptotic activity, which may provide further evidence of CRYAB's antiapoptotic activity following centriolin depletion. In addition, a second siRNA sequence targeting centriolin will be used, in order to exclude off-target effects, and cells should be rescued from centriolin-induced cell death by centriolin overexpression.

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