EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF RD3, THE PROTEIN
ASSOCIATED WITH LEBER’S CONGENITAL AMAUROSIS TYPE 12

by

THOMAS JEFFERIES

B.Sc., Juniata College, 2009

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

Master of Science

in

The Faculty of Graduate Studies

(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

May 2013

© Thomas Jefferies, 2013
Abstract

RD3 is a highly conserved 23 kDa soluble protein expressed in the retinal and testicular tissues required for the trafficking of GC1 (Guanylate Cyclase 1). A lack of expression of this protein, or of GC1, results in a rapid loss of photoreceptor cells after retinal development, indicating their importance in photoreceptor function and survival. Previous work has demonstrated that RD3 shows disperse and membrane-associated distribution in HEK293T cells. When expressed in *E. coli*, RD3 forms inclusion bodies exclusively. RD3 is non-amenable to vigorous centrifugation, resistant to purification by way of size exclusion, and binds to and inhibits GC1.

The current investigation demonstrates that RD3 is highly conserved throughout sight capable vertebrates, forms large proteolipid macro-molecular structures of 10nm size or greater and that *E. coli* expressed protein conforms to the predicted secondary structure of RD3. Additionally, methods of isolating RD3 used in previous studies are carried out here and found to result in the formation of the expected structures. Multiple alignment and secondary structure prediction software, circular dichroism, dynamic light scattering, electron microscopy, and standard protein purification and visualization techniques are utilized in these endeavors.
Preface

Work first began on RD3 before my time within Dr. Molday’s lab. As it was linked to both Leber’s congenital amaurosis and GC1, RD3 was of great interest. Initial studies focused on the localization of the protein within the retina and cultured mammalian cells. Given my background in protein chemistry as opposed to microscopy, I embarked on a structural study of RD3 and while it lead to very interesting findings we deviated from our original goal of obtaining a crystal structure for reasons which will become obvious within.
Table of Contents

Abstract ................................................................................................................................. ii
Preface ................................................................................................................................... iii
Table of Contents .................................................................................................................. iv
List of Tables ........................................................................................................................ vii
List of Figures ....................................................................................................................... viii
List of Abbreviations .......................................................................................................... ix
Acknowledgements .............................................................................................................. xi

1.0 INTRODUCTION .............................................................................................................. 1

1.1 The Eye ............................................................................................................................. 1
1.2 The Retina ......................................................................................................................... 1
1.3 The Outer Segment .......................................................................................................... 4
1.4 Phototransduction .......................................................................................................... 4
1.5 GC1 .................................................................................................................................. 6
1.6 RD3 .................................................................................................................................. 8
1.7 Leber’s Congenital Amaurosis ....................................................................................... 12
1.8 Thesis Investigation ....................................................................................................... 12

2.0 METHODS ...................................................................................................................... 14

2.1 Bioinformatics ............................................................................................................... 14
2.2 Agarose Gel Electrophoresis ......................................................................................... 14
2.3 SDS-PAGE ...................................................................................................................... 15
List of Tables

Table 1: DLS analysis of LCA12 behavior in varying buffers .................................................. 32
List of Figures

Figure 1: The Eye ................................................................................................................. 2
Figure 2: Phototransduction ................................................................................................. 5
Figure 3: The relationship between RD3 and RetGC (GC1) .................................................... 7
Figure 4: RD3 expression in mouse retina ............................................................................. 9
Figure 5: RD3/GC1 coexpression in HEK293T cells ................................................................. 11
Figure 6: Secondary structure and topology predictions ......................................................... 25
Figure 7: Phylotree and secondary structure analysis of RD3 orthologs ................................. 28
Figure 8: Expression and purification of RD3 from E. coli ....................................................... 29
Figure 9: Size Exclusion Purification of RD3 ......................................................................... 30
Figure 10: Purification of RD3 under Denaturing Conditions ................................................. 31
Figure 11: Analysis of secondary structure by circular dichroism .......................................... 33
Figure 12: Analysis of folding and unfolding by pH differential ............................................. 34
Figure 13: Electron microscopy analysis of RD3 .................................................................... 35
Figure 14: Analysis of protein obtained using the Dizhoor method ........................................ 37
Figure 15: Purification and analysis of RD3-MBP .................................................................. 38
Figure 16: Purification and analysis of RD3 murine ortholog .................................................. 39
Figure 17: Purification and analysis of protein from mammalian cell lines ............................. 40
Figure 18: Analysis of lipid extraction from purified RD3 ...................................................... 41
List of Abbreviations

BBS – Borate Buffered Saline

\( \beta \)-ME – \( \beta \)-Mercaptoethanol

CD – Circular Dichroism

cGMP – cyclic Guanidine Monophosphate

CNG – Cyclic Nucleotide Gated

DLS – Dynamic Light Scattering

DMEM – Dulbecco’s Modified Eagle Media

DOPC - 1,2-Dioleoyl-sn-glycero-3-phosphocholine

DOPE - 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine

DOPS - 1,2-Dioleoyl-sn-glycero-3-phosphoserine

EDTA – Ethylenediaminetetraacetic acid

EM – Electron Microscopy

GC1 – Guanylate Cyclase 1

GCAP – Guanylate Cyclase Activating Protein

GDP – Guanidine Diphosphate

GMP – Guanidine Monophosphate

GPCR – G Protein Coupled Receptor

GTP – Guanidine Triphosphate

IPTG - Isopropylthio-\( \beta \)-Galactoside

LCA – Leber’s Congenital Amaurosis
MBP – Maltose Binding Protein
PBS – Phosphate Buffered Saline

PDE - Phosphodiesterase

PML – Premyelocytic Leukemia (bodies)

PVDF - Polyvinylidene Fluoride

RD3 – Retinal Degeneration 3

RPE – Retinal Pigment Epithelial

SDS-PAGE - Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
Acknowledgements

This thesis could not have been completed without the help and assistance of a great many people over a great span of time but none more than Dr. Robert Molday. In admitting me to his research group Dr. Molday began this project and through access to his experience, knowledge, criticisms, and advice this thesis progressed over the course of three years to where it is today. Additionally, the help of his wife and lab manager Laurie Molday was invaluable in both her knowledge of the thesis subject and related works plus the lessons given on unfamiliar laboratory methods.

Within the Molday lab group, Dr. Seifollah Azadi was working on LCA12 at the same time and was most helpful during his time in the lab. Jonathan Coleman, Theresa Hii, Karen Chang, or Aleeza Tam, and Faraz Quazi also helped on many occasions in less direct, but still greatly appreciated, ways. While outside of our direct lab group, Dr. Calvin Yip deserves a great deal of thanks for his willingness to perform much of the work in imaging LCA12 preparations with Dr. Molday’s electron microscope assembly.

Dr. Frank Duong and Dr. Natalie Strynadka deserve thanks also. Both for serving on the committee to oversee this thesis and additionally Dr. Strynadka made available the DLS within her lab while Dr. Duong kindly made a strain of Rhodococcus available to us which we would not ordinarily have had on hand.

Having been a wonderful advisor during my time at Juniata College, for her patronage, and for her willingness to let an eager first year student loose in her lab I would like to thank Dr. Ruth Reed. I would also like to acknowledge Dr. Fisher, Dr. Matter, Dr. Glazier, and the many great professors of the department of biology and chemistry.
Last but definitely not least, my supportive family and parents south of the border deserve a great deal more than I can give them for suffering through my lapses in regular communication and for their emotional and financial support. Simply put, thanks for being Mom and Dad, there were a few tight times I would not have gotten through without you.
1.0 INTRODUCTION

1.1 The Eye

The eye is an organ solely responsible for the detection of light and the subsequent signaling of the resulting image to the brain. While the eye consists of over a dozen layers of epithelial, sensory, muscle, and capillary tissues, only two are necessary, and alone sufficient, for the detection of light. The others are required for focused, clear vision and the seemingly random movements associated with complex sight (Rodieck 1998)(Figure 1A).

Light first enters the eye through cornea, aqueous humour, and pupil, passing through these tissues into the lens. The lens is a fluid filled tissue shaped to focus light onto the retinal tissue located at the back of the eye. To reach the retinal tissue the light must first pass through the vitreous fluid, filling the center of the eye, consisting of mostly water with very small concentrations of cells, proteins, salts, and sugars. Upon reaching the retina, light passes through the optic nerve, the connecting neuronal layers of the retina, and the nuclei of the photoreceptors, before reaching the sensory organelles (outer segment) of the photoreceptors (Rodieck 1998). (Figure 1C)

1.2 The Retina

Dedicated to the detection of light, the retina is a complex tissue consisting of eight distinct layers (Figure 1B). From the perspective of light traveling inwards through the lens, the layers are arranged as the axon, ganglion cell, inner plexiform, inner nuclear, outer plexiform, outer nuclear, the inner segment, the outer segment, and the retinal pigment epithelial layers.
Figure 1: The Eye

A) The principle tissues of the eye necessary for eye movement, the detection of light, signal transduction to the brain, and general tissue upkeep. Reproduced with permission (Kolb, Fernandez, et al. 2008)

B) The cell layers of the retina organized with respect to the direction of light, reproduced with permission (Forschungszentrum Jülich GmbH).

C) A cartoon of the rod and cone photoreceptor cells demonstrating the similarities and differences. (Forschungszentrum Jülich GmbH)
The detection of light begins within the outer segment layer, and the signal generated travels down through to the axon layer, and finally to the brain. The remaining tissue layer, the retinal pigment epithelial cell layer, is responsible for housekeeping, pigment recycling, and phagocytosis of the exhausted ends of the photoreceptor cells. (Rodieck 1998)

While each individual photoreceptor cell (rod or cone) detects light, it is the intricate cross-connectivity of these cells and the gated bipolar, amacrine, and horizontal cells that generate the rich signals necessary for complex vision (Figure 1B). Each of the cells between the inner plexiform layer and the outer plexiform layer form a particular kind of logic gate, activating under only the correct arrangement of cell signaling events; the inputs coming from the photoreceptors or the other plexiform cells. Amacrine cells are mainly inhibitory neurons that interconnect rod and cone bipolar cells, and feed into the ganglion cells. Bipolar cells connect to horizontal cells, rods, and cones and pass the resulting signals onward through amacrine cells to ganglion cells or directly to ganglion cells. Finally, horizontal cells make connections with multiple cone cells, acting as a buffer to allow better perception of light intensity shifts. (Rodieck 1998)

After filtering through the cells of the inner plexiform and outer plexiform layers, the signal then passes through the axon layer to the optic nerve and onward to the brain (Figure 1B). Prior to reaching the visual cortex of the brain, the optic nerve, crosses itself, delivering the vision of the right eye to the left hemisphere of the brain and vice versa (Rodieck 1998).
1.3 The Outer Segment

Both rod and cone photoreceptor cells contain a unique organelle referred to as the outer segment (Figure 1C). This body of cytoplasm is cut off from the rest of the cell (inner segment) by a ciliary body. The outer segment consists of many hundred stacked membranous disks, each containing the necessary components for the sensing of light. The difference between rod and cone cells, aside from the wavelengths of light detected and the general shape of the cell, is the presence of a cellular membrane around the disks of the outer segment in rod photoreceptor cells and the absence of this membrane in cone cells. (Rodieck 1998)

As the disks mature they move upwards toward the apical tip of the outer segment, drawn by both the continuous generation of disks at the basal end, and the phagocytosis of old disks at the apical tip. Typically, 10% of the disks in a given rod cell are phagocytosed each day by the retinal pigment epithelial (RPE) cells. The phagocytic action of the RPE cells helps to recycle the used cellular components, such as lipids and protein. In addition, the RPE functions in the conversion of all-trans retinal to 11-cis retinal for the regeneration of rhodopsin. Thus the RPE cells play an important role in the long term survival and function of the photoreceptor cells, despite being removed from the primary events of phototransduction. (Yau 2008)

1.4 Phototransduction

The G-Protein Coupled Receptor (GPCR) rhodopsin consisting of the protein opsin and cofactor 11-cis retinal is at the heart of the process of phototransduction through the initial absorption of a photon of light (Figure 2). Specifically, a photon is absorbed by the 11 cis-retinal causing it to isomerize to
Figure 2: Phototransduction

A cartoon depicting the varying stages of phototransduction. First, light is absorbed by the chromophore 11-cis-retinal which isomerizes to all-trans-retinal, resulting in a conformational change in opsin. This perturbation releases α-transducin, activating PDE, hydrolyzing cGMP in solution and closing the CNG channels generating the necessary membrane potential. GC1 re-synthesizes cGMP after phototransduction to re-open the CNG channels and reset outer segment for further phototransduction. Reproduced with permission (Leskov, Handy, et al. 2000)
all-\textit{trans} retinal, resulting in a conformation change in rhodopsin to meta-rhodopsin II. Meta-rhodopsin II then activates the α-subunit of transducin via the displacement of GDP with GTP in the transducin nucleotide binding site. This activated transducin subunit then activates phosphodiesterase (PDE), which cleaves the 3’ bond of cGMP to produce GMP. The drop in cGMP levels closes the cyclic nucleotide gated (CNG) channels, allowing for the hyperpolarization necessary for a signaling event. To recover from this process, the GTP bound to the α-subunit of transducin is hydrolyzed to GDP, allowing for the dissociation of α-transducin from PDE. While this halts the hydrolysis of cGMP in the outer segment, the synthesis of additional cGMP falls to guanylate cyclase I (GC1). (Berg 2007)

The decreased calcium levels resulting from the closing of the CNG channels activate GC1 by way of the calcium-modulated guanylate cyclase activating protein (GCAP). GCAP in an inactivated state binds Ca\textsuperscript{2+} and upon the closing of the CNG channels and the drop in Ca\textsuperscript{2+} concentration will bind Mg\textsuperscript{2+} (Figure 2). The increased level of cGMP opens the CNG channels, resetting the membrane potential and subsequently the signal. Thus GC1 can be seen as the ‘reset’ that helps in priming the photoreceptor for to a photoreceptive state. This reset function is necessary for the long term survival of the photoreceptor, as seen by the plethora of diseases, such as Leber’s Congenital Amaurosis (LCA), that arise from a loss of GC1 function. (Yau 2008)

\textbf{1.5 GC1}

GC1 itself is a 120kDa enzyme with a single span transmembrane domain, a disk lumenal domain with a signal sequence, and a cytoplasmic domain consisting of the kinase,
Figure 3: The relationship between RD3 (RD3 murine ortholog) and RetGC (GC1)

A) GC1 activity depicted in the basal, activated (GCAP bound), and inhibited (RD3 bound) states. Lacking GCAP, GC1 exists in a basal state of low cGMP synthesis activity. Bound Mg$^{2+}$ activated GCAP stimulates the full catalytic potential of GC1. Bound RD3 (themurine RD3 ortholog) inhibits GCAP binding and reduces GC1 activity to below the basal state. Reproduced with permission (Peschenko, Azadi, et al. 2011).

B) A comparison of predicted secondary structure and notable domains within the proteins RD3 and GC1. GC1 is comprised of a disk luminal, a luminal domain, transmembrane domain, kinase domain, dimerization domain, and a catalytic domain. RD3 is predicted to consist of two alpha helical segments each of which is thought to be a coiled coil motif. Reproduced with permission (Den Hollander, Koenekoop, et al. 2008)
dimerization, GCAP1 binding, and catalytic sub-domains (Figure 3)(Lange 1999). Responsible for the recovery of the dark state of the photoreceptor prior to the detection of light, GC1 catalyzes the formation of the 5’-3’ phosphodiester bond needed to synthesize cyclic GMP from GTP. Given that cGMP is the sole secondary messenger in the outer segment, a lack of GC1 leads to sustained signaling events as despite the eventual dissociation of α-transducin the cyclic gated channels remain closed until more cGMP is synthesized. These sustained signaling events are inherently stressful to the photoreceptor cell and usually lead to cell death a short time after birth. (Pugh 1997)

Functional studies of LCA1 patients have found a number of missense mutations both in the catalytic domain and in the lumenal domain, both of which greatly diminish the catalytic activity of GC1 (Perrault 2000). To be functionally active, GC1 must dimerize in a head to head fashion and be associated with GCAP in its activated state (Mg$^{2+}$ bound), thus presenting many different sites at which a missense mutation could disrupt either the dimerization, GCAP binding, modulation of basal activity, or the catalytic domain itself (Perrault 2000). Regardless of whether or not GC1 is capable in a given system, of catalyzing the formation of cGMP, it must first be present in the outer segment in order to fulfill its purpose and for this, RD3 is required.

1.6 RD3

RD3 is a 23 kDa retina specific protein required for GC1 trafficking to the outer segment, and thus photoreceptor recovery from the ‘dark state’ (Azadi 2010). First identified as an open reading frame, C1Orf36 (Chromosome 1 Open reading frame 36), associated with retinal degeneration, the protein
Figure 4: RD3 expression in mouse retina

A) Protein immunoprecipitated from solubilized WT and rd3 mouse retina using beads conjugated with the monoclonal α-RD3 antibody, 9D12 and subsequently analyzed by western blot using 9D12 and LI-COR α-mouse secondary antibody. Reproduced with permission (Azadi 2010).  B) WT and rd3 mouse retina fixed and labeled with 9D12 α-RD3 antibody. Reproduced with permission (Azadi, Molday, et al. 2010).
product was named RD3 and the defective form of RD3 responsible for rapid photoreceptor loss prior to birth was identified to be a truncation mutant (Friedman 2006). The downstream effectors of cell death are likely similar to those in other cases of oxidative stress following prolonged Ca\(^{2+}\) mediated signaling events, as is the case in LCA1 (GC1) cases. With similar symptoms to many Leber’s Congenital Amaurosis, this gene product joined GC1 in the list of 13 genes each of which, individually, cause LCA in human patients (Den Hollander 2008).

While the complete nature of the relationship between RD3 and GC1 is still not known, published data show that the two proteins bind directly when co-expressed in HEK293T cells, that RD3 inhibits GC1 function, as well as inhibiting the binding of GCAP to GC1 (Peschenko 2011). Complicating these results is the preparation of RD3 used in these assays, a preparation lacking in schemes to purify the protein post solubilization. Some published data have suggested that RD3 demonstrates a sub-nuclear localization in mammalian cell lines whilst other cell lines have shown RD3 to localize throughout the cytoplasm, cellular membranes, and vesicles (Friedman 2006, Azadi 2010) (Figure 5). In the latter case RD3 co-localizes with GC1 to a large extent. The tendency of RD3 to precipitate out of solution, or to be excluded from solution post expression in bacteria, have made attempts to study the function and structure of RD3 quite difficult (Peschenko 2011).

Preliminary bioinformatic analysis concluded that RD3 was likely to be soluble and was predicted to consist of several \(\alpha\)-helical domains, of which up to two were possibly coiled coil domains. No localization sequences or common cleavage sites were detected, though protein kinase sites were found. (Lavorgna 2003)
**Figure 5: RD3/GC1 co-expression in HEK293T cells**

As labeled above, each row corresponds to a culture of COS-7 cells transiently transfected with the constructs listed on the left with each image having been visualized with an antibody against the protein labeled in the top left. Reproduced and modified with permission (Azadi, Molday, et al. 2010).
1.7 Leber’s congenital amaurosis

Leber’s congenital amaurosis is a congenital retinal degeneration discovered by Dr. Leber in the 1800’s. The most severe form of retinal degeneration, the 13 sub-types of LCA account for up to 18% of cases of blindness amongst children (Den Hollander 2008). It is characterized by swift photoreceptor loss after birth and symptoms include nystagmus (sporadic eye movement), a lessened or absent pupillary response, a lack of lesions (or at least, a lack of lesions contributing to the rapid loss of photoreceptors), and a lack of electroretinogram signal. Regardless of the originating gene, all identified forms LCA are autosomal recessive. (Den Hollander 2008)

1.8 Thesis Investigation

This work attempts to expand our knowledge of RD3 through analysis of the sequence of RD3 and its homologs, characterization of the secondary structural features, and characterization of the macro-structure of RD3. Utilizing publicly available software, electron microscopy (EM), circular dichroism (CD), dynamic light scattering (DLS), and lipid analysis, the macro-molecular behavior of RD3 will be placed in context with previous work demonstrating a lack of solubility and yet significantly potent inhibition of its binding partner GC1.

Firstly, the sequences of known RD3 orthologs will be compared with each other and a general pattern of conservation will be established. Secondly, the bacterially-expressed RD3 will be characterized. By DLS and EM analysis, it will be shown that RD3 forms large 10nm macro-molecular structures with a secondary structure obtained by CD similar to that already predicted. Thirdly, alternate preparations will demonstrate that the macro-molecular structure is present despite changes in the purification method or the sequence expressed. Fourthly,
protein expressed in mammalian cells will be analyzed by DLS to corroborate the data collected in the bacterial system.
2.0 METHODS

2.1 Bioinformatics

The primary sequence of RD3 was uploaded into the PredictProtein server (Rost 1994) to generate a set of predictions for secondary structure, solvent accessibility, transmembrane helices, globular regions, coiled-coil regions, structural switch regions, β-sheet values, disordered regions, intra-residue contacts, protein-protein and protein-DNA binding sites, subcellular localization, domain boundaries, beta-barrels, cysteine bonds, metal binding sites and disulphide bridges using the default settings for each of the server tools. The membrane topology predictions of the PredictProtein server were double checked by loading the RD3 sequence into the TOPPRED servers (GVH 1992) and running the algorithm with the standard settings.

The sequence for human RD3 was uploaded into the PubMed pBlast server software (Altschul 1997) and run against all known sequences. Matches of greater than 50% were then taken and arranged in FASTA (Appendix A) format. The primary sequences for these homologs or orthologs of RD3 were taken and aligned using the ClustalW server software (Altschul 1997). The alignment file was uploaded to the Phylotree server (Dereeper 2008) for phylogenetic distance mapping using only the default settings for the server. Selected sequences were then loaded into the PHD server (Rost 1994) and analyzed with a PHD secondary structure prediction using the default settings of the server.

2.2 Agarose Gel Electrophoresis

Gels were cast from 1% (w/v) agar in 1x TAE with 0.5% (v/v) SyberSafe (New England Biolabs; Ipswich, MA) and poured to a depth of no less than one centimeter. Loading buffer
(30% glycerol, 0.25% bromophenol blue) was added in a 1:5 ratio to all samples and standards (New England Biolabs; Ipswich, Mass) before loading. Gels were subsequently run for 20 minutes at 100V and 100A, or until the dye front had migrated 80% of the length of the gel. Gels were then visualized in an EpiChem3 Darkroom (UVP Bioimaging Systems; Upland, CA).

2.3 SDS-PAGE

Samples and standards (Biorad) were mixed with loading buffer in a 2:1 ratio and loaded onto a 10% Tris-acrylamide SDS-PAGE gel (Shapiro, 1967). Both the upper and lower reservoirs were filled with 1x SDS-PAGE running buffer and the gel was allowed to run for 50 minutes at 180V, 25mA, or until the dye front was less than half a centimeter from the bottom of the gel. Gels were then removed, stained with Coomassie blue (Thermo Fisher Scientific, Waltham, Mass), or used immediately for transfer to PVDF membrane used for western blotting.

2.4 Western Blotting

Six filter papers (GE Healthcare, Little Chalfont, UK) and 1 of PVDF blotting paper (Millipore; Billerica, Mass) were cut and soaked in Towbin transfer buffer with 10% methanol (Towbin 1979) for 20 minutes prior to blotting. After incubation with the transfer buffer, three filter papers were placed on top of each other and pressed free of air bubbles. The PVDF was then layered on top of these filters with the gel above the PVDF. The last three filter papers were layered on and the stack inspected for air bubbles. The transfer was run for 22 minutes at 20V, 0.35mA after which the PVDF membrane was dried overnight.

The following day, the membrane was reactivated with methanol and washed for 40 minutes in blocking buffer, 10% condensed milk power in PBS. After three washes in PBS for 20 minutes each the membrane was incubated with PBS diluted primary antibody for 40 minutes.
After this incubation, the membrane was washed three times for 20 minutes in PBS-Tween before incubation with the PBS diluted secondary antibody for 40 minutes. This incubation was followed by another three 20 minute washes with PBS-Tween. After the final wash, the membrane was imaged on an Odyssey scanner (LICOR; Lincoln, NE) using the appropriate emission filters for the secondary antibody.

2.5 Cloning and Transformation

Sequences were amplified from cDNA using PCR with a primer to each of the 5’ ends of each template cDNA strand (Appendix B, Integrated DNA Technologies; San Diego, CA). Primers and restriction sites were designed for use within the pET or pMal vector systems (New England Biolabs; Ipswich, Mass). 5% T4 DNA polymerase, 10% T4 DNA polymerase buffer, 2.5% of each nucleotide, 10% of each primer, and <1% template DNA were mixed and subject to a standard 30 cycle PCR regime with a 1 minute elongation phase. The PCR product was purified using a Qia-quick PCR Purification Kit (Qiagen; Valencia, CA). Purified PCR product and vector, prepared by GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific; Waltam, Massachusetts) were cut with the restriction enzymes simultaneously, where possible, for 30 min at 37°C. Digests were run on a 1% agarose gel, excised and purified using the Qia-quick Gel Extraction Kit (Qiagen; Valencia, CA).

Extracted digests were mixed in a 2:1 vector to insert ratio by mass and ligated for 10 minutes at room temperature with 0.5 µl T4 DNA ligase (New England Biolabs; Ipswich, Mass), 1 µl T4 DNA ligase buffer (New England Biolabs; Ipswich, Mass), and de-ionized water added to a volume of 10 µl. Three µl of ligation mixture was then added to 50 µl of competent *E. coli* DH5α cells (Invitrogen; Carlsbad, CA) and incubated for 20 minutes on ice. After a 3 minute
temperature shock, 30 seconds incubation at 41°C followed by a 2 minute incubation at 0°C, the transformed DH5α cells were streaked onto LB plates with the appropriate selection antibiotic (antibiotics obtained from Goldbio; St. Louis, MO) and incubated overnight at 37°C. Resulting colonies were screened with the original primers using the ‘ColIPCR’ thermocycler program. Positive colonies were picked and grown overnight in 1ml of LB media with the appropriate selection antibiotic for miniprep generation.

Purified construct DNA was digested and analyzed for the presence of insert DNA on a 1% agarose gel as above. Three µl of construct DNA was then used to transform competent E. coli BL21 (Invitrogen; Carlsbad, CA) cells, incubating for 20 minutes on ice. After a 3 minute temperature shock, 1:2 41°C to 0°C, the transformed BL21 cells were streaked onto LB plates with the appropriate selection antibiotic and left overnight at 37°C. Colonies were screened using the original two primers before being picked and left overnight at 37°C in 10ml LB media containing the appropriate selection antibiotic.

2.6 Protein Expression, BL21

For expression in E. coli cells, overnight cultures picked from plates of BL21 cells were added to 500ml fresh LB media and incubated at 37°C until the cells reached an OD₆₀₀ of 0.6. One µg/ml ITPG (Goldbio; St. Louis, MO) was then introduced to induce protein expression in the BL21 cells for four hours. Cells were subsequently harvested by centrifugation for 20 minutes at 3,000 rpm and then frozen at -30°C until needed.

2.7 Protein expression, HEK293T

Expression of protein in HEK293T cells was achieved via transient transfection. HEK293T cells were grown to confluence, split, and grown again to 60% confluence in DMEM (Invitrogen;
Carlsbad, CA). Thirty-six µg of pcDNA3.1, with RD3 cloned into a site containing a c-terminal TETSQVAPA tag, was subsequently mixed with a CaCl$_2$/BBS transfection buffer and added to the cells. Media was changed the following day. On the third day, cells were collected and washed twice in the desired buffer by centrifugation. Washed cells were then used on the same day for purification of protein and analysis.

2.8 Solubilization from Inclusion Bodies

*E. coli* cell pellets containing the desired expressed protein were thawed on ice in 10ml Tris-EDTA buffer (pH 8.0) (Sigma Aldrich; Carlsbad, CA) before being sonicated, 12W for 60s, three times on ice. Lysate was spun at 15,000 rpm for 20 minutes to pellet the insoluble material. The supernatant and pellet were then tested for protein expression by SDS-PAGE analysis, before continuing to the purification.

The lysate pellet was washed three times in Tris-EDTA buffer before being resuspended in 20mM Tris (pH 8), 6M de-ionized urea and homogenized for 20 minutes on ice. The homogenate was subsequently spun at 15,000 rpm for 20 minutes. The supernatant was dialyzed overnight in 20mMTris, 1mM EDTA, 12µM β-ME or used immediately in the case of experiments requiring denatured or homogenized protein samples.

2.9 Nickel affinity purification

Prior to use, a 5ml HiTrap nickel affinity column (GE Healthcare; Little Chalfont, UK) was equilibrated at 1ml/min with 10 column volumes of 20% ethanol (Thermo Fisher Scientific; Waltham, Mass), followed by 10 column volumes of water, followed by 10 column volumes of 20mM Tris, (pH 8.0). The dialyzed or homogenized protein solution was spun at 15k rpm for 20 minutes again before being diluted 1:10 in Tris buffer. Ten column volumes of Tris buffer was
run prior to 2 column volumes of 20mM Tris (pH 8.0) 250mM imidazole. Elution fractions were analyzed by their absorbance at 280nm prior to being pooled and dialyzed overnight prior to analysis.

2.10 Amylose affinity purification

Amylose conjugated beads (New England Biolabs; Ipswich, Mass) were loaded onto a spin filter column (Millipore; Billerica, Mass) and equilibrated with 5 column volumes of 20mM Tris twice. *E. coli* lysate supernatant was then incubated with the beads for 1 hour at 4°C. Unbound fraction was eluted and collected and the beads washed with buffer twice for 20 minutes at 4°C. Tris buffer with 12mM maltose (Thermo Fisher Scientific; Waltham, Mass) was then incubated with the beads for 20 minutes at 18°C. The elution fraction was collected for analysis by SDS-PAGE and western blot for the presence of protein and dialyzed overnight for analysis.

2.11 Size exclusion purification

Prior to purification, a size standard calibrated Sephacryl 100 column (GE Healthcare; Little Chalfont, United Kingdom) was connected to a Pharmacia Akta Purifier FPLC (GE Healthcare; Little Chalfont, United Kingdom) and washed at 1ml/min with a 20% ethanol solution for 5 column volumes then equilibrated with water for 5 column volumes before being equilibrated with 5 column volumes Tris-EDTA buffer (pH 8.0). Samples of protein (100 µg/ml to 5 mg/ml) were loaded into a 1ml injection syringe. After vigorously removing air from the syringe, the sample was loaded into the injection loop of the FPLC and the collection system adjusted to reflect the desired number of fractions. Run settings were as follows: 100% buffer A, pump head A; 0.5ml/min; max pressure 0.5mPa. Samples were collected starting with the
void volume, as determined by a blue dextran standard (GE Healthcare; Little Chalfont, United Kingdom), and ceased with the elution of a final sharp conductance peak. Samples were drawn from the fractions based on overlaid absorbance values at 280nm, 255nm, and 215nm. Drawn fractions were analyzed by SDS-PAGE and western blotting to confirm the presence and purity of protein, before being stored at 4°C for further analysis.

2.12 Immunopurification

An aliquot of 1d4 monoclonal antibody coupled Sepharose 2B beads (GE Healthcare; Little Chalfont, United Kingdom) was loaded onto a filter column and equilibrated twice with 5 column volumes 20mM Tris (pH 8.0). Solubilized protein samples were spun at 20,000 rpm for 10 minutes and the supernatants incubated with Tris buffer-equilibrated-Rho 1D4 beads for one hour. Unbound fractions were collected and the beads washed twice for 30 minutes in buffer. Beads were subsequently incubated with buffer and 250 µg/µl peptide (TETSQVAPA). The elution was analyzed by SDS-PAGE and western blot for the presence of protein.

2.13 Dynamic Light Scattering

Prior to analysis, the DynaPro NanoStar dynamic light spectrophotometer (Wyatt Technology Corporation; Santa Barbara, CA) was set to ‘laser on’ on the control board and ‘DLS = true’ and ‘temp = 25’ within the control program Astra Dynamic 7 (Wyatt Technology Corporation; Santa Barbara, CA). Ten µl sample was loaded into the cuvette with care to avoid air bubbles or particulate contaminants. The cuvette was aligned within the sample cell and left to equilibrate to 25°C for sample analysis.

Once equilibrated to 25°C, data collection was initiated and preceded automatically through 10 sets of 10 acquisitions to build an average distribution of particle sizes for each
sample run. The output was then adjusted to reflect percentage intensity under an isotropic sphere model and saved for interpretation. Determination of particle size in differing buffers was performed as above with purified protein, either dialyzed overnight in the desired buffer conditions, or simply diluted 1:100 into the desired buffer conditions (see appendix). These samples were then analyzed as above.

2.14 Electron Microscopy

Purified RD3 samples diluted to 3 µg/ml and Dr. Calvin Yip (Assistant professor, Dept. of Biochemistry and Molecular Biology, UBC) kindly performed the experiment, given the experience required to operate the EM. Purified samples were adsorbed to carbon coated copper grids, glow discharged, and subsequently stained with 0.75% uranyl formate prior to drying. Specimens were imaged with a Tecnai Spirit transmission electron microscope equipped with a LaB₆ filament and operated at an accelerating voltage of 120kV. Images were taken with an FEI Eagle 4K charged couple device camera.

2.15 Circular Dichroism

Prior to analysis, the Jasco 810 spectropolarimeter (Jasco Inc; Easton, MD) was purged with nitrogen and put through the standard start up procedure. The sample was diluted to 3 µM in water and loaded into the sample cell. The scanning set was adjusted to between 180nm and 290nm, the temperature brought and held at 23°C, and the voltage of the spectropolarimeter checked before starting the sample run. Data was collected and stored as an excel spreadsheet with a spectra of the molar ellipticity automatically generated. These data points were then uploaded along with the protein sequence to the K2D2 server (Rost 1994) for the generation of the percentage secondary structure composition, as well as
closeness of fit to a predicted CD spectrum generated from the amino acid sequence. Analysis was repeated with differing buffer and temperature conditions as described above (see appendix).

2.16 Bligh-Dyer Lipid Extraction

This method was adapted from that of Jensen (Jensen 2008). Protein samples were mixed 1:2:1 sample:methanol:chloroform (Thermo Fisher Scientific; Waltam, Massachusetts) and vortexed thoroughly. Another volume of chloroform and water was added to the sample prior to vortexing thoroughly once again. Samples were centrifuged at 1,000 rpm for 5 minutes before the bottom layer (chloroform) was extracted and dried under a nitrogen atmosphere. All samples were then resuspended in 20 µl chloroform and spotted onto a TLC plate (Sigma Aldrich; Carlsbad, CA) alongside 100 µg standards of DOPE, DOPS, and DOPC (Avanti; Alabaster, AL). The running buffer was comprised of 64:25:4 chloroform:methanol:ammonia and was allowed to run for approximately 2 hours, or until the running front was within two inches from the top of the plate. Samples were then labeled with iodine and visualized in an EpiChem3 Darkroom (UVP Bioimaging Systems; Upland, CA)
3.0 RESULTS

3.1 Bioinformatics

PSIPRED servers generated several hits for both secondary structural features, membrane topography, and post-translational modifications (Figure 6A, 6B). With regards to secondary structures, it also predicted the presence of alpha helical domains and a coiled coil region as seen previously (Lavorgna 2003) (Figure 6A). No membrane spanning regions were detected throughout the sequence. Six protein kinase sites were detected, with three sites located on either terminus. The TOPPRED servers did not plot the hydropathy of the RD3 sequence within frame of the lower bound for potential transmembrane domains, confirming the result from the PSIPRED servers regarding membrane spanning regions (Figure 6B).

The pBlast servers determined that RD3 had no known homologs but did have orthologs throughout the sight capable species of phyla chordata (Appendix A). Organization of these orthologs by species and distance of relation according to a phylogenetic tree resulted in three distinctive groupings, a lower order consisting of non-mammalian vertebrates, a middle order consisting of non-primate mammals, and a higher order consisting of primates (Figure 7A). Comparison of the secondary structure predictions for two species within each of the different groupings demonstrated a conservation of the size and position of the α-helical secondary structures characteristics throughout the selected species (Figure 7B).

3.2 RD3 Expression

Analysis of bacterially expressed RD3 confirms previous work (Peschenko, 2011) demonstrating that hexahistidine tagged RD3 is not detectable in the supernatant of lysed cells using SDS-PAGE and western blotting analysis though it is present in sizeable quantities.
(>10mg/100ml cultured cells) in the pellet (Figure 8). Solubilization of RD3 from the pellet can be achieved. Nevertheless, despite thorough washing of the inclusion bodies, a great deal of protein contaminant is still present. Upon renaturing, less than half of the solubilized product remains within the supernatant of a centrifuged sample, again confirming literature reports (Peschenko, 2011) of RD3 being non-amenable to vigorous centrifugation post-renaturation. RD3 expressed with an MBP tag via the pMal vector system was present in both the supernatant and the pellet of lysed cells, and could be used without the need for solubilization.

3.3 Recombinant RD3 Purification (native)

Purification of 20mg of RD3 from a 500ml starting culture can be achieved by HiTrap nickel affinity chromatography, using protein solubilized from inclusion bodies and renatured overnight. Protein contaminant is perceptible by SDS-PAGE analysis, though the RD3 band identified with the 9d12 monoclonal antibody by western blotting, is the major band (Figure 8). The use of an S100 size exclusion column does not resolve the RD3 from the majority of the contaminants, as the RD3 in addition to contaminating proteins of size from size 10 kDa to greater than 200 kDa are restricted to the void volume of the column, with only a few exceptions that test negative for the presence of RD3 by western blotting (Figure 9A, 9B).

3.4 Recombinant RD3 Purification (Non-Native)

Purification of RD3 using HiTrap nickel affinity chromatography under denaturing conditions from solubilized inclusion bodies leads to a purified RD3 product with little contaminating protein present (Figure 10A). RD3 can be purified successfully up to 20mg from a 500ml starter culture. Despite the purity of the sample, this preparation still cannot be vigorously centrifuged nor is it amenable to further purification by way of the S100 size exclusion column.
Figure 6: Secondary Structure and Topology Predictions

A) Secondary structure prediction obtained by uploading the RD3 sequence into the PSIPRED servers (McGuffin, Jones, et al. 2000). The symbols for the secondary structure predictions are as follows: H, alpha helix; E, extended; B, beta sheet; D, disordered; C, coiled-coil.

B) Membrane topology predictions obtained by uploading the RD3 sequence into the TOPPRED servers (GVH 1992). The blue line corresponds to the predicted hydropathy. The lower cutoff for membrane spanning regions is off scale, above y=0.
3.5 DLS Analysis

Analysis of purified samples of RD3 shows 10nm to 100nm sized particles in solution, with no peak of monomeric, monodisperse protein of the expected size of approximately 2nm (Figure 10B). Several different buffer components, additives, and pH conditions were tested using the DLS, all of which yielded particle sizes greater than 8nm (Table 1). Size standards; tobacco etch mosaic virus protease, maltose binding protein, and soy bean trypsin inhibitor were analyzed using the DLS and found to be within the 2nm to 4nm range expected of soluble, monomeric proteins. RD3 purified simply solubilized from ‘cleaned’ inclusion bodies without a purification step exhibited similar particle sizes in solution with no detectable signal of less than 10nm (Figure 14B).

3.6 CD Analysis

Analysis of purified RD3 samples with a Jasco 810 yielded reproducible spectra between 190nm and 230nm. Analysis of the spectra with K2D2 showed secondary structure percentages to be 19% alpha helix and 22% beta sheet (Figure 11A). Analysis of the purified samples at a fixed wavelength over variable heat and pH conditions was inconclusive, with little change in the 208nm, 215nm, and 222nm peaks detected (Figure 11B, 12A-C) in comparison to previous work on coiled coil protein folding studies (Figure 11C, 12D) (Steinmetz 1998).

3.7 Electron Microscopy

EM imaging of the purified protein sample fixed to charged carbon-coated grids demonstrated the existence of spherical structures roughly 10nm in diameter and larger, amorphous structures of up to or exceeding 100nm in a single dimension (Figure 13). Images of control protein, maltose binding protein, solutions yielded only punctuate monomer bound to
the charged carbon plates, while a negative control demonstrated no observable structures that could be differentiated from the carbon background.

### 3.8 Alternate Methods of Protein Preparation

Protein samples prepared by simple solubilization (Figure 14A) without a purification step show a particle size in solution similar to that of fully purified RD3, with 10nm bodies and a collection of polydisperse peaks at and above 100nm (Figure 14B). Whilst the percentage mass in each of the preparation was variable, this was attributed to the larger, amorphous particles and their differing hydrodynamic profiles, as these non-spheroids tumbled through solution. Attempting to purify this preparation on a Sephacryl 100 size exclusion column led to a concentration of the RD3 within the void volume as previously seen (Figure 14C, 14D).

Protein expressed with an n-terminal MBP tag can be purified from the supernatant of lysed cells (Figure 15A). The resulting pure fractions have between 30% and 50% of the mass in solution as particles of 3.0nm (Figure 15B), with the remainder of the protein forming the 10nm structures seen in other preparations when analyzed by DLS, with the large peaks greatly diminished or absent entirely.

### 3.9 Purification and analysis (RD3 murine ortholog) (E. coli expressed)

pET28b vector containing the mouse Rd3, insert was obtained from Dr. Seifollah Azadi and expressed in E.Coli. Under non-native purification conditions, RD3 was purified to a single band (Figure 16A). Analysis by DLS demonstrated formation of the 10nm and 100nm bodies (Figure 16B).
Figure 7: Phylotree and secondary structure analysis of RD3 orthologs

A) RD3 ortholog sequences obtained by BLAST search (see Appendix X) were arranged in FASTA format with human opsin sequence added for scale purposes and input into the Phylotree server (Dereeper, Blanc, et al. 2008) using the default settings to obtain a phylogenetic grouping tree of the orthologs.

B) Select orthologs were each loaded into the PredictProtein secondary structure prediction server with a simple visual output. These were arranged above with the red bars corresponding to α-helical domains and the yellow boxes corresponding to β-sheet domains.
Figure 8: Expression and purification of RD3 from *E. coli*

A coomassie blue stained 10% SDS-PAGE gel of the expression and purification of RD3 under native conditions on a 5ml HiTrap nickel affinity column. Elutions were concentrated using an Amicon spin filter column for 1 hour at 4°C and 3750 rpm.
Figure 9: Size exclusion purification of RD3

A) Chromatograph of RD3 purified under native conditions on a Sephacryl 100 size exclusion column. The blue line represents the 280nm absorbance profile while the brown line indicates the conductivity. The black bar has been added to indicate the void volume as determined by blue dextran.

B) A 10% SDS-PAGE gel and western blot, labeled with the anti-RD3 antibody 9D12, of the fractions eluted above replete with input diluted 1:10. The black bar has been added to indicate the void volume fractions as labeled above.
Figure 10: Purification of RD3 under denaturing conditions

A) Protein solubilized from the inclusion bodies of *E. coli* expressing full length RD3 with a 6x His tag is purified by way of HiTrap nickel affinity chromatography under denaturing conditions. Column wash consisted of 20mM Tris buffer (pH 8.0), 50mM imidazole, and 6M de-ionized urea. Column elution buffer consisted of 20mM Tris buffer (pH 8.0), 250mM imidazole, and 6M urea. B) A sample of the protein purified above analyzed by DLS to determine the distribution of sizes in solution.
Table 1: DLS analysis of RD3 behavior in varying buffers

RD3 purified under denaturing conditions was dialyzed overnight or diluted 1:100 in the buffer conditions above and analyzed by DLS to determine the average size(s) of particles and structures in solution. Several conditions, such as alcohols and SDS, could not be analyzed as the buffer additives interfered with data collection and are not listed above.
Figure 11 Analysis of secondary structure by Circular Dichroism

A) RD3 was analyzed using a spectropolarimeter for ellipticity changes from a baseline obtained using double distilled water. The green line refers to the observed spectra and the red line refers to the predicted values as determined by K2D2 software analysis (Gell, Eaton, et al. 2002), indicating that the sequence is comprised of 24.99% α-helix and 19.75% β-sheet. B) A graph of the change in the secondary structure (as determined by change in ellipticity) at 222nm of an RD3 sample from 0°C to 85°C as measured by spectropolarimeter. C) A graph of temperature change on secondary structure (at 222nm) of cortexillin-1, a coiled-coil containing protein, reproduced with permission (Steinmetz, Schulthess, et al. 1998) for comparison with figure 11B.
Figure 12: Analysis of folding and unfolding by pH differential

A) RD3 equilibrated to a range of pH values was analyzed for folding changes by spectropolarimeter set to record ellipticity at 208nm and 55°C.  B) RD3 equilibrated to a range of pH values was analyzed for folding changes by spectropolarimeter set to record ellipticity at 215nm and 55°C.  C) RD3 equilibrated to a range of pH values was analyzed for folding changes by spectropolarimeter set to record ellipticity at 222nm and 55°C.  D) The changes in observed ellipticity of cortexillin-1 equilibrated to a range of pH values at 23°C (solids) and 55°C (blanks), reproduced with permission (Steinmetz, Schulthess, et al. 1998).
Figure 13: Electron microscopy analysis of RD3

An electron micrograph depicting the 10nm and 100nm structures of fully purified RD3 observed by DLS in solution. Imaging and staining performed by Dr. Calvin Yip (Department of Biochemistry and Molecular Biology, UBC). Inset micrograph is of a control preparation, 20mM Tris (pH 8) spotted onto charged plates and imaged.
3.10 Purification and Analysis (Mammalian Cell Expression)

RD3 expressed in transfected HEK293T cells can be detected in both the soluble and membrane fractions of lysed cells, when run on a 5% to 60% sucrose gradient. Protein isolated from the soluble fraction by 1D4 peptide purification (Figure 17A) demonstrates similar behavior to that purified by His$_6$ tag from bacterial lysate, with 10nm, 100nm, and larger structures detectable (Figure 18B).

3.11 Lipid Extraction and Analysis

Iodine-labeled TLC plates of lipid extracted from RD3 protein samples, control protein, and standards indicated that there was lipid present in the RD3 preparations in quantities greater than 0.1mg/ml from a starting concentration of 0.5mg/ml sample. Lipid bands, extracted from RD3, aligned with the DOPE standards, whilst control protein preparations displayed no bands aligning with any lipid standards (Figure 18).
Figure 14: Analysis of protein obtained using the Dizhoor method

A) A coomassie blue stained SDS-PAGE gel of bacterially expressed RD3 prepared with the method referenced by Peschenko (Peschenko, Azadi, et al. 2011). B) DLS spectra of the protein prepared above. C) The 10% SDS-PAGE (bottom) and 9d12 labeled western blot (top) of the fractions collected from D. The black bar above the fraction number was added for convenience to identify the fractions associated with the void volume. D) Chromatograph resulting from the injection of RD3 prepared in A and loaded onto an Sephacryl 100 column. The black bar above the fraction numbers was added for convenience to identify the void volume fractions.
Figure 15: Purification and analysis of RD3-MBP

A) A coomassie blue stained 10% SDS-PAGE gel of RD3 cloned into the pMal vector, expressed in *E. coli*, and purified by amylose affinity chromatography  

B) DLS analysis of bacterially expressed RD3-MBP purified as above indicating the presence of monomeric RD3-MBP in addition to the 10nm structures observed previously with the presence of 100nm structures greatly diminished.
**Figure 16: Purification and analysis of RD3 ortholog RD3**

a) A coomassie blue stained 10% SDS-PAGE gel of *E. coli* expressed RD3 homogenized from cell lysates and purified under denaturing conditions using a HiTrap nickel affinity column.

b) RD3 purified as above was analyzed by DLS to determine the average particle sizes in solution demonstrating the presence of the 10nm and 100nm structures observed in RD3 preparations.
Figure 17: Purification and analysis of protein from mammalian cell lines

A) A 9d12 labeled western blot of RD3 purified by 1D4α-TETSQVAPA antibody bound to Sephacryl 2B beads from lysed HEK293T cells. B) DLS analysis of 1d4 tagged RD3 isolated from HEK293T cells as above indicating the presence of 10nm+ structures and a lack of detectable monomer.
Figure 18: Analysis of lipid extracted from purified RD3

RD3 purified under non-native conditions was subjected to a standard Bligh Dyer lipid extraction (Jensen 2008) prior to TLC plate analysis for lipid content. Plates were visualized with iodated silica. Common lipid size standards are indicated on the right with purified maltose binding protein and glutathione-s-transferase also subjected to lipid extraction serving as controls.
4.0 DISCUSSION

4.1 Bioinformatics

The PredictProtein and TOPPRED servers corroborated early evidence that RD3 should be soluble in solution, and would consist of several alpha helical or coiled coil regions in addition to some beta sheet. In addition, the PredictProtein server specifically indicated some homology in the N-terminal region with a nuclear localization signal, and six protein kinase sites, three in each of the protein’s termini. As shown above, the prediction of solubility was greatly exaggerated and the nuclear localization is questionable given the role of RD3 in the binding of GC1, a protein with no observed tendency for nuclear, or even sub-nuclear, localization. This apparent error of predicted hydrophilicity is even more profound when it is seen that the prediction software could not bring the lower cut off for a probable membrane spanning region into the visible frame.

A standard pBlast search revealed no true homologs of RD3 in any genome, but did show over a dozen orthologs across phyla chordata. With a lower end alignment score of 53% by residue, and a higher end of over 99%, these orthologs were either known RD3 orthologs (RCD2, RD3, etc.) or predicted proteins from genomic studies automatically given the label ‘RD3-like’.

After alignment and organization, three distinctive groupings appear within the orthologs of RD3. Non-mammalian vertebrates, non-primate mammals, and primates all form distinctive distance groupings, the orthologs in each group share specific amino acid residue identity not seen in cross group alignments. After alignment and multiple sequence secondary
structure prediction it is apparent that only the specific amino acid content and the slight variations within the C terminus distinguish the orthologs.

Each sequence consists of three alpha helical domains, two of which were predicted to be coiled coil domains. From this, the general structure of RD3 appears to be conserved across Phyla Chordata, presumably as a fundamental component for recovery from phototransduction as is known to be the case in human, murine, and canine models. As little is known about the retinas of species such as the elephant or the horse, it is hard to say what functional or structural changes exist between the varying groupings. Given the evidence that a lack of RD3 and RD3 result in similar diseases in both species the differences could be trivial and have less of an impact on the function of the gene than the lower percentage conservation between species would imply. Given the important nature of GC1 in vision, it is certainly possible that the RD3/GC1 dependency developed early in evolutionary history and remained, functionally if not specifically, unchanged.

Two main segments of alpha helical domains, each consisting of one possible coiled coil domain, appear throughout all species found. As the truncation mutant responsible for the retinal degeneration occurs in mice and human sequences in between the two segments of predicted alpha helical content, the presence of the latter alpha helical domain would appear to be necessary for the function of RD3. Whether it is responsible for the formation of macrostructure, the trafficking of GC1, the inhibition of GC1, or all three is as yet unknown.

4.2 RD3 Expression

As has been reported previously, RD3 forms inclusion bodies exclusively, when expressed with small peptide tags of less than 9AA. The protein can be solubilized from these
inclusion bodies, liberating less than half of the total expressed protein content. This solubilized protein can then be purified by way of affinity chromatography, but not by size exclusion chromatography. Expression as a construct with a larger MBP tag is possible and can generate a population of RD3 fusion protein in the soluble fraction of lysed cells without the need for prior solubilization. Expression of murine RD3 mirrored that of RD3, with the expressed protein restricted to the pellet of homogenized cells and workable only after homogenization with 6M urea solutions. Despite a 30% difference in sequence, the expression was still confined to the insoluble fraction or to inclusion bodies, suggesting that despite the large distance between the species and the alignment on the phylogenetic tree to difference groups, the two orthologs were behaving in identical ways.

Transfection of HEK293T cells showed approximately half the expressed RD3 protein in the soluble fraction and half within the cell pellet. When lysed cells are run on a 5%:60% sucrose gradient instead of simple centrifugation, the protein can be detected in both the membrane fraction and the soluble fraction, and is readily purified from either fraction.

4.3 Analysis of Purified Protein

RD3 can be easily purified from *E. coli* inclusion bodies by way of nickel affinity chromatography under denaturing conditions, with near total purity achieved using 6M urea. When run through a size exclusion column, the protein elutes within the void volume and not in the fractions that similarly sized protein standards elute in. DLS analysis points to the cause of the size exclusion behavior, the RD3 in solution forming 10nm diameter structures or larger structures greater than 100nm diameter.
Electron microscopic analysis shows that the structures are spheroid in nature when 10nm in diameter, or fairly amorphous and elongated when 100nm in diameter or larger. The spheroid structures themselves are not amorphous but are lacking in observable, repeating order. Each spheroid structure consists of an outer lattice of protein surrounding an inner, heavily stained mass. Even the larger, amorphous structures exhibit this lattice behavior with regular and heavily stained gaps existing periodically in the outer layer.

Solutions of protein expressed from HEK293T cells, the RD3 ortholog, and RD3 expressed with an MBP tag all demonstrated the same propensity to form 10nm structures when analyzed by DLS, with one exception. RD3-MBP fusion protein, as suggested by its ability to be handled without a homogenization step, would exist as a monomer in solution, with no more than 40% of the mass as detected by DLS falling into this state. The 10nm structures were also detected, suggesting that the placement or existence of the MBP tag (N-terminal) disrupted, but did not completely inhibit, the formation of the 10nm structures.

For the protein expressed from HEK293T cells and the RD3 expressed from BL21 cells, there was no difference in particle size as determined by DLS. This could be due to a lack of some unknown retina specific protein associated with GC1 and RD3. Given the inhibitory effect of even bacterially expressed protein on GC1 though, this point seems moot in terms of RD3 function. The ability of RD3 to prosecute its putative function is unchanged regardless of expression system as is formation of large, spheroid structures.

Given the proposed model for GC1 inhibition, it is brought into question whether the spheroids are densely packed excluding the internal protein monomers from the solvent or whether the form simply a protein shell around the solvent, lipid, protein, or other component.
Lipids were investigated first using a standard Bligh-Dyer lipid extraction on protein samples purified under denaturing conditions. While the initial work demonstrates the presence of lipid in solution, the identity of these lipids, phosphatidylethanolamine (DOPE), should not be thought to extend to a more heterogeneous lipid population given that the extraction was done in the DOPE rich background of *E. coli*.

The presence of these structures helps us to rationalize both the previously reported aversion of RD3 to vigorous centrifugation to the presence of RD3 solely within the void volume of size exclusion columns. RD3 alone, as a monomer, should have a diameter in solution of no more than 2nm, if it were a spherical or globular protein. As no protein was detected, even by western blot, in the expected elution fractions, it would appear that RD3 does not exist in solution as a monomer in sufficient quantities to be detected by specific antibodies.

Whether the mechanism of the inhibition observed by Peschenko (Peschenko 2011) lies within an encapsulation of the GC1 dimer by a cage of RD3 bound lipids, the direct inhibition of GC1, or by some other mechanism entirely remains to be demonstrated. Nevertheless, the macro-molecular, lipo-protein structures of RD3 should be investigated for their potential role in the inhibition of GC1.

### 4.4 Structural Durability Analysis

Due to the fact that the 10nm structures of RD3 and lipid formed even after purification by nickel affinity chromatography under denaturing conditions, the question arose whether the protein actually purified in a denatured state or whether the structure was somehow being stabilized, perhaps by way of the bound lipids. Purified protein was diluted into various buffers to induce the denatured state and the resulting solution was analyzed by DLS. Salts, buffers
with varying effective pH, chaotrophic agents, and alcohols were all tested with little success. No salt or buffer condition lacking high concentrations of chaotrophics led to the reduction of the 10nm or 100nm diameter structures the formation of a monomer, let alone a monomer.

Of the chaotrophics, urea (at any concentration) failed to induce the formation of monomer from the macro-molecular structures. Guanidine hydrochloride could induce formation of the monomer but only at concentrations greater than 3M. SDS, at concentrations present in an SDS-PAGE gel, can obviously induce the monomeric state by way of denaturation, given the presence of a monomeric band on the gel. The previously observed result of small amounts of 50kDa and 100kDa bands, staining with coomassie blue or with 9d12, now fit with a larger pattern of resistance to denaturation.

Even a concentration of 1% SDS fails to fully break down the larger structures of RD3 on an SDS PAGE gel. Often, bands of 50kDa, 100kDa, and 200kDa could be observed by coomassie blue staining or by western blotting using the 9d12 αRD3 antibody. These structures exist despite the use of reducing agents (casting doubt on the immediate assumption that these bands were the result of disulphide bonds) and could be evidence that RD3 stability is rooted in the formation of an initial dimer pairing, required for the propagation of a larger structure. This also brings into question the reliability of using SDS PAGE gels; how much of the protein could be retained in the interface between the stacking and resolving layers? Anecdotal accounts of ‘disappearing’ protein could well be attributed to insufficiently solubilized macro-structures failing to migrate through a PAGE stacking/resolving interface.

Temperature was also utilized as a tool to examine the denaturation of RD3, although for these measurements the secondary structure was analyzed by circular dichroism.
spectroscopy as opposed to the particle size analyzed by dynamic light scattering given the adverse effects of increased heat on the signals from the DLS. Single wavelengths were monitored during pH and temperature trials. Previous work on another coiled-coil containing protein, cortexillin-1, demonstrates a profound (75%) reduction in secondary structure at the wavelengths denoting alpha helical structure, 208nm and 222nm. A repetition of this work with RD3 demonstrated only a 33% loss in structure during any condition, from 4C to 85C and from pH 2 to pH 12.

This stability is puzzling given the strong inhibition of GC1 and the blocking of GCAP binding. If RD3 were required for trafficking and inhibition of GC1, then the two proteins would eventually need to separate. Given the resistance to denaturation and degradation, the as-of-yet uninvestigated role of the six phosphorylation sites could shed some light on this problem.
5.0 CONCLUSIONS

RD3 is a retina specific, coiled-coil protein with orthologs throughout the sight capable species of phyla chordata. These orthologs all display similar alpha helical and coiled coil patterning in their sequence predictions with slight differences recurring throughout some species and falling into three groupings as demonstrated by a phylogenetic tree. The presence of RD3 in a relatively unchanged form in sight capable, non-mammal chordates demonstrates the importance of RD3 in the workings of the retina, integral as it seems to be for so many species.

While RD3 does not express in the soluble fraction of bacterial cells, it can be homogenized and purified from lysate pellets under denaturing conditions. With or without a purification step, RD3 will form 10nm diameter spherical structures and larger, amorphous structures of 100nm diameter or larger. These structures are incredibly difficult to break down into the constitutive monomer, requiring SDS or high concentrations of guanidine hydrochloride. Temperatures nearing the boiling point, extreme pH changes, salt conditions, and even high concentrations of urea fail to dissociate monomers of RD3 from the larger structures. These structures are observed in the murine ortholog, RD3, in protein expressed in mammalian cells, and to a lesser extent to protein expressed with large tags such as maltose binding protein. Application of the Bligh-Dyer lipid extraction on bacterially expressed RD3 shows there to be phosphatidylethanolamine present in the purified samples, possibly making up the core of the 10nm structures.
6.0 FUTURE DIRECTIONS

While the discovery of the RD3 structures dug to the root of the problems relating to expression, purification, and analysis, there is still much to be done. Ignoring the wetwork entirely, the function of the known RD3 orthologs still needs to be confirmed in less developed species such as *Danio rerio* and *Xenopus laevis*. Indeed, the morphology of *Xenopus laevis* rod photoreceptors makes that species a prime target for the visualization and, more importantly, the specific localization of RD3 within the photoreceptor.

On the subject of the structures themselves, the lipid content in protein purified from retinal samples and mammalian cell lines should be determined. The lipid content of bacterial expression systems is mainly PE and so the effects of a more retina-applicable lipid background should be investigated. Additionally, the relation of these structures to the inhibition of GC1 and the requirement of RD3 for the trafficking of GC1 needs to be determined. While it has not been shown nor claimed that RD3 is alone sufficient for the trafficking of GC1, the structures present in solution suggest the role of RD3 may not be solely one of targeting or chaperoning to the rod outer segment.

Additionally, questions as to the composition and formation of the structures in the retina must be raised. Do these structures exist in rod cells as observed in bacterially expressed protein? If so, is the protein content homogenous or are there components missing from the mammalian and bacterial systems that are present in the rod cell? Do these structures form spontaneously after expression in the rod cell or are they kept apart and in reserve until needed for GC1 trafficking? Hopefully future work will make quick progress now that the monomeric assumptions of the past have been dealt with.
REFERENCES


Yau, KW; Hardie, R. “Phototransduction Motifs and Variations.” *Cell* **139**: 246-264
APPENDIX A: cDNA Primers

LCA12 Reverse (HindIII) – AAG CTT CTA GTC GGC TTT GGG CGC CCG GAA
LCA12 Forward (Ncol) – CCA TGG GGT CTC TCA TCT CAT GGC TTC GG
LCA12 Reverse (XbaI) – GCG TCT AGA ATG TCT CTC ATC TCA TGG CTT CGG
LCA12 Forward (BamHI) – GCG GGA TCC TTA GTC GGC TTT GGG CGC CCG GAA
RD3 reverse, 1D4 (XbaI) – GCG TCT AGA TCA GGC AGG CGC CAC TTG GCT GGT CTC TGT GTC
GCG CTG GGG GCG CCC TGA A
RD3 Forward (BamHI) – GCG GGA TCC CTC ATG TCC CTC ATC CCG TGG CTC CGG
APPENDIX B: Buffers used during pH trials

pH 3 – 100mM citrate, pH 3  
pH 4 – 100mM citrate, pH 4  
pH 5 – 100mM citrate, pH 5  
pH 6 – 50mM MOPS, pH 6.5  
pH 7 – 50mM MOPS, pH 7  
pH 8 – 50mM MOPS, pH 8  
pH 9 – 50mM Glycine, pH 9  
pH 10 – 50mM Glycine, pH 10  
pH 11 – 50mM Glycine pH 11
APPENDIX C: Solutions used during buffer optimization trials

20mM Tris (pH 8), 50mM NaCl
20mM Tris (pH 8), 50mM arginine
20mM Tris (pH 8), 50mM glucose
20mM Tris (pH 8), 50mM sucrose
20mM Tris (pH 8), 10%-50% ethanol
20mM Tris (pH 8), 10%-50% propanol
20mM Tris (pH 8), 3M urea
20mM Tris (pH 8), 6M urea
20mM Tris (pH 8), 8M urea
20mM Tris (pH 8), 3M guanidine HCl
20mM Tris (pH 8), 6M guanidine HCl
50mM HEPES (pH 7)
PBS (pH 7)
MOPS (pH 7)
APPENDIX D: RD3 Orthologs (% homology)

protein RD3 - 100%
PREDICTED: protein RD3 [Pan troglodytes] - 99%
PREDICTED: protein RD3-like [Nomascus leucogenys] - 98%
hypothetical protein EGK_01683 [Macaca mulatta] - 96%
PREDICTED: protein RD3-like [Pongo abelii] - 96%
PREDICTED: protein RD3-like [Callithrix jacchus] - 96%
PREDICTED: LOW QUALITY PROTEIN: protein RD3-like [Equus caballus] - 90%
PREDICTED: protein RD3-like [Ailuropoda melanoleuca] - 90%
PREDICTED: RIKEN cDNA 3322402L07-like [Oryctolagus cuniculus] - 88%
PREDICTED: RIKEN cDNA 3322402L07-like [Rattus norvegicus] - 86%
protein RD3 isoform 1 [Mus musculus] - 86%
PREDICTED: protein RD3-like [Sus scrofa] - 85%
PREDICTED: protein RD3-like [Loxodonta africana] - 84%
Protein RD3 [Cricetulus griseus] - 84%
PREDICTED: protein RD3-like [Cavia porcellus] - 77%
PREDICTED: similar to retinal degeneration 3 [Taeniopygia guttata] - 68%
protein RD3 [Danio rerio] - 53%