Fibroblasts as a Window into the Cell Biology of Ankyrin –B and –G.

by

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Thesis submitted in partial fulfillment of
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ABSTRACT

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Abstract

The ankyrin family of proteins form specialized membrane domains in various cell types, including neurons and cardiomyocytes but little is known about how this process occurs. The majority of ankyrins localize to the plasma membrane in these cells while ankyrins in fibroblasts are largely intracellular. This property of fibroblasts allows us to study intracellular ankyrin and potentially how ankyrin forms membrane domains in other cell types. In this thesis, we use western blots, immunofluorescence, RT-PCR to characterize the expression pattern of ankyrin in fibroblasts and find that both ankyrin-B and -G localize to both nuclear and intracellular compartments. The size of the compartments of both ankyrin-B and -G is affected by the genetic deletion of the large isoforms of ankyrin-B and -G. The ankyrin-B compartment has a weak association with recycling endosomes suggesting that ankyrin-B may be involved in membrane protein trafficking.

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1. Introduction

1.1 The discovery of ankyrins

There are three vertebrate ankyrins: erythrocyte ankyrin (ankyrin-R), brain ankyrin (ankyrin-B), and giant ankyrin (ankyrin-G). Each ankyrin contains, from N-terminus to C-terminus: an ankyrin repeat domain, two ZO-1 and Unc5-like netrin receptor (Zu5) domains, a domain present in Unc5, PIDD, and ankyrins (UPA) domain, a death domain, and an unstructured C-terminal regulatory domain. Ankyins share thef the c-terminal Zu5-UPA-DD cassette with Unc5 (axonal guidance receptor) and PIDD (programmed cell death) (Wang et al, 2009). To date, these are the only proteins known to possess this structural cassette. The ankyrin repeat domain is largely responsible for the interaction of ankyrin with membrane proteins while the first Zu5 domain is the site for spectrin binding.

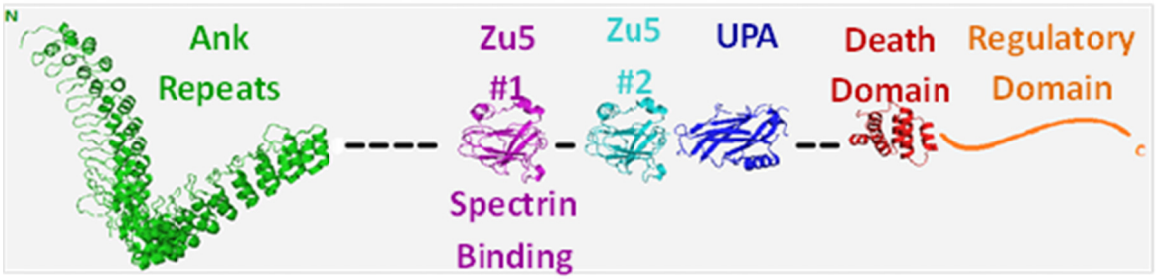

Figure 1. Ankyrin domain model. The domains of ankyrin that have been structurally solved are shown in order from N-terminus to C-terminus.

In 1978, a 72 kDa proteolytic fragment was discovered and found to serve as the membrane attachment site for erythrocyte spectrin (Bennett, 1978). Soon after the discovery of this 72 kDa fragment the full length ankyrin molecule was purified and its properties identified (Bennett and Stenbuck, 1979a). The protein was coined red cell ankyrin (ankyrin-R) since it was discovered in eyrthrocytes. Ankyrin-R is an asymmetrical monomeric protein with hydrophobic domains and associates with both spectrin and the erythrocyte anion channel, band 3 (Bennett and Stenbuck, 1980 and Bennett and Stenbuck, 1979b). These findings classify ankyrin as an adaptor protein that links membrane-associated proteins to the spectrin based-cytoskeleton. The coordination of ankyrin-R, spectrin, and membrane proteins is essential to the integrity of erythrocytic membranes as disruption of this complex results in hereditary erythrocyte spherocytosis and anemia (Bodine et al,1984 and White et al, 1990).

1.2 Ankyrin B

Since immunoreactive forms of ankyrin-R have been found in non-erythrocyte tissues, including brain, isoforms of ankyrin were sought and identified in brain (Bennett, 1979 and Davis and Bennett, 1984). Brain ankyrin (ankyrin-B) was identified as two polypeptides from pig brain that reacted with an anti-ankyrin-R antibody (Davis and Bennett, 1984). The site of the Ankyrin-B gene was mapped to human chromosome 4 whereas the gene for ankyrin-R maps to human chromosome 8; therefore these

proteins are differen gene products (Otto et al, 1991). Ankyrin-B has a pattern of expression distinct from that of Ankyrin-R and is spliced into various transcripts with some containing large inserts and others lacking ankyrin repeats almost completely (Kunimoto et al, 1991).

Ankyrin B has been demonstrated to play an important role in the function of the heart. In cardiomyocytes, ankyrin B coordinates the formation of a complex at the T-tubule containing the Na/K ATPase, Na/Ca cation exchanger, and inositol triphosphate ($InsP_3$) receptor (Tuvia et al, 1999 and Mohler et al, 2005). A point mutation (E1425) in ankyrin-B found in patients with an inherited cardiac arrhythmia syndrome abrogates the formation of this complex and causes the mislocalization of its components (Mohler et al, 2003). Patients with this mutation have type 4 long QT syndrome, exhibiting prolonged QT intervals in EKG and sudden death (Mohler et al, 2003). Several other human ankyrin-B mutations result in a similar condition, displaying long QT syndrome to varying degrees and/or various other cardiac phenotypes (Mohler et al, 2004). Murine cardiomyoctes expressing ankyrin-B loss of function mutations exhibit abnormal contraction rates and altered Ca^{2+} dynamics (Mohler et al, 2004). Some of these ankyrin-B mutation variants also result in human sinoatrial node dysfunction (Le Scouarnec et al, 2008). The inherited conditions that arise from ankyrin-B loss of function vairiants have been collectively termed “ankyrin-B syndrome”.

The importance of ankyrin-B in human physiology is not limited to the heart as the targeting of dystrophin to costameres and neuromuscular junctions in skeletal muscle also requires ankyrin-B (Ayalon et al, 2008). Reduced ankyrin-B expression in skeletal muscle results in catastrophic misorganization of the costamere (Ayalon et al, 2008). Specifically, abbrogation of the interaction of ankyrin-B with dystrophin either by dystrophin mutation or ankyrin-B depletion results in dystrophin and β-distroglycan mislocalization. The dystrophin mutations involved in ankyrin-B binding cause either Becker or Duchenne's muscular dystrophy. Reduction in ankyrin-B expression also disrupts the organization and localization of costamere associated mictrotubules and dynactin-4 (Ayalon et al, 2008). This catatrophic disorganization results in the failure of muscle fibers to withstand exercise induced injury and provides a mouse model for muscular dystrophy (Ayalon et al, 2008 and Ayalon et al, 2011).

With ankyrin-B playing important roles in the function of both the heart and skeletal muscle, it would not be surprising to discover that it has roles in the function of other organs and cell types. However, the fundamental cellular function of ankyrin-B remains elusive.

1.3 Ankyrin G

The ankyrin family of proteins has long been known to associate with the voltage gated sodium channel *in vitro* and to colocalize with the sodium channel at nodes of

Ranvier and the axon initial segment (AIS) (Srinivasan et al, 1988 and Kordeli, et al, 1990). An ankyrin isoform that cross reacted with an ankyrin-R antibody but was still expressed in ankyrin-R knockout mice was discovered (Kordeli, et al, 1990 and Kordeli, et al, 1991). In the mid 1990's this isoform was identified as, ankyrin-G, using a human frontal cortex library (Kordeli et al, 1995). Like ankyrin-R and ankyrin-B, ankyrin-G has multiple splice variants with some variants lacking the ankyrin repeats domain (Peters et al, 1995). The biggest splice variant of ankyrin-G is 480 kDa and is where the name "giant ankyrin" originated (Kordeli et al, 1995). In additon, ankyrin-G has a 190 kDa form that is is expressed ubiquitously. Expression of the 480 kDa isoform of ankyrin G is restricted to nodes of Ranvier and axon initial segments of neurons. (Kordeli et al, 1995).

Like ankyrin-R and ankyrin-B, ankyrin-G binds ion channels and transporters. The most well studied of these are voltage gated sodium and potassium channels expressed in the brain and heart (Pan et al, 2006 and Zhou et al, 1998). Ankyrin-G targets voltage gated sodium channels to the AIS and nodes of Ranvier in neurons (Zhou et al, 1998). Sodium channel mislocalization in ankyrin-G cerebellar knockout mice results in abnormal axonal firing and ataxia (Zhou et al, 1998). The localization of sodium channels in the heart is just as dependent on ankyrin-G as those expressed in the brain (Mohler et al, 2004b). A mutation (E1053K) in ankyrin-G in patients with Brugada

syndrome abbrogates the interaction of Nav1.5 with ankyrinG resulting in Nav1.5 mislocalization (Mohler et al, 2004b). Patients with the ankyrin-G mutation causing Brugada syndrome die suddenly usually during the night from ventricular fibrillation (Brugada and Brugada, 1992). These data show that ankyrin-G expression is critical for the correct electrophysiological function of both the brain and the heart.

Recently, new data has been produced showing that ankyrin-G is important for maintaining the structural organization of the intercalated disc of cardiomyocytes (Sato et al, 2011). Ankyrin-G binds to and affects the localization of the demosomal proten, plakophilin-2 and the gap juntion protein, connexon 43. When ankyrin-G is knocked down via siRNA plakopilin-2, N-cadherin, and connexin 43 lose their association with the intercalated disc but are still expressed on the cell membrane (Sato et al, 2011). This implies that ankyrin-G expression is important for the structural organization and integrity of the intercalated disc in cardiomyocytes.

Expression of ankyrin-G is important for the proper functioning of neurons as well as the proper functioning of cardiomyocytes. Ankyrin-G expression is required for the formation and maintenance of the axon initial segment (AIS) in developing neurons (Jenkins and Bennett, 2001 and Sobotzik et al, 2009). Silencing of ankyrin-G by shRNA results in the disassembly of the AIS and mislocalization of Nav channels, βIV spectrin, and neurofascin-186 (Zhou et al, 1998). The clustering of these proteins at the AIS is

important for the firing of action potentials (Zhou et al, 1998). Ankyrin-G is required for the clustering of ion channels at the axon initial segment as the diffusion coefficient of a mutant (EA4SA) version of the ion channel chimera, Kv-Nav, that lacks ankyrin binding is significantly greater than that of wild-type Kv-Nav (Brachet et al, 2010). Neurons infected with shRNA against neurofascin-186 retain the AIS segment suggesting that ankyrin-G, and not neurofascin-186, is required for short term maintenance of the AIS (Hedstrom et al, 2007). However, recent data suggests that neurofascin 186 is required for long term maintenance of the AIS (Zonta et al, 2011). Without the AIS neurons are non functional, demonstrating an important role for ankyrin-G in the formation, development, and proper functioning of the nervous system.

Both ankyrin-B and ankyrin-G are expressed in rod photoreceptors but ankyrin-G is the only one that localizes to the rod photoreceptor outer segement where CNG channels are located (Kizhatil et al, 2009). Ankyrin-G silencing via shRNA in the photoreceptors of mice shows that ankyrin-G is required for the correct localization of the CNG channel in rod photoreceptors. The length of the outer segments of the rod photoreceptors is also significantly shortedned in shRNA transfected receptors (Kizhatil et al, 2009).

In epithelial cells, ankyrin-G binds to and localizes E-cadherin to the basolateral membrane. When ankyrin-G expression is abbrogated in epithelial cells, the cells

become shorter and E-cadherin gets sequestered in the trans-golgi network (TGN) (Kizhatil et al, 2007). Similar results are seen when treating cells with the microtubule depolymerizing agent nocadazole and silencing of β2-spectrin with siRNA. These data suggest that transport of E-cadherin from the TGN to the lateral membrane requires both tubulin and β2-spectrin presumably via endosomal transport (Kizhatil et al, 2007). This finding sheds light on the fundamental cellular function of ankyrin-G and establishes an *in vitro* model for studing the basic cellular function of ankyrin-G that may not be possible to study in other cell types such as neurons and cardiomyocytes.

1.4 Fibroblasts as a model

Until now, ankyrin research has focused on studying polarized, terminally differentiated cells such as cardiomyocytes, neurons, and epithelial cells. With the exception of one paper published in 1996, the expression and function of ankyrin-B and -G in fibroblasts has not been studied. The paper shows that ankyrin is expressed in fibroblasts but does not distinguish between the three canonical ankyrins or attempt to identify what functions the three ankyrins perform in fibroblasts (Kato et al, 1996). As ankyrin-B and -G are believed to establish specialized membrane domains in cells, it would be interesting to know what the function of these proteins is in a cell type that largely lacks stable specialized membrane domains. In this thesis, we attempt to answer

that question by characterizing the expression of ankyrin-B and -G in murine embryonic fibroblasts (MEFs).

2. Results

2.1 Ankyrin-B

2.1.1 Genotyping of Ankyrin-B Mutant Mice

Ankyrin-B mutant mice were produced in the laboratory by Paula Scotland using a neomycin cassette to disrupt the reading frame of large ankyrin-B isoforms. The neomycin cassette was inserted into an exon in the spectrin binding domain of ankyrin-B resulting in loss of expression of ankyrin-B 220 (Scotland et al, 1998). Small ankyirn-B isoforms lacking an ankyrin repeat domain escape knockout making this mouse useful for the study of both large and small ankyrin-B isoforms. Hereafter, homozygous mutant mice will be referred to as AnkB 200 KO mice. AnkB 220 KO mice die around postnatal day one (P1) and therefore, have to be genotyped before any experiments can be done. P1 pups were sacrificed by decapitation and tail samples were taken for DNA isolation and genotyping (Figure 2). Genotyping was done by Janelle Hostettler and Kayla Dowless. AnkB 22 KO mice were generated at Mendelian frequencies. Pups that were determined to be AnkB 220 KO mice were used in all ankyrin-B experiments with wild-type littermates used as controls.

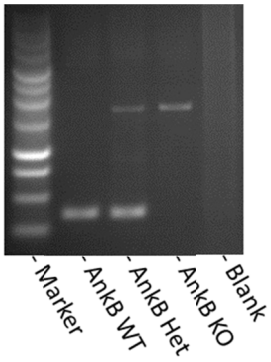

Figure 2. Genotyping of ankyrin-B mice. PCR showing genotyping results from a wild-type (400bp), heterozygous (400 and 1700bp), and AnkG 220 KO (1700bp) ankyrin-B mice.

2.1.2 Ankyrin-B Protein Expression in Fibroblasts

The expression of ankyrin B isoforms has never been characterized in fibroblasts though several isoforms have been identified in other tissues. All isoforms identified to date contain the C terminal regulatory domain therefore; we used an antibody raised against this domain of ankyrin-B. As ankyrin-B and ankyrin-G have high homology, the antibody was cross absorbed against ankyrin-G to reduce the possibility of cross reactivity by Lydia Davis.

MEFs isolated from wild-type pups express two ankyrin-B isoforms, one at 220 kDa and one at 150 kDa while MEFs from AnkB 220 KO littermates only express the 150 kDa isoform. In addition, AnkB 22 KO MEFs express the 150 kDa isoform at higher levels than wild-type MEFs (Figure 3b). Presumably, the increased expression of the 150 kDa isoform in the AnkB 220 KO MEFs is an attempt to compensate for the loss of the

220 kDa isoform.

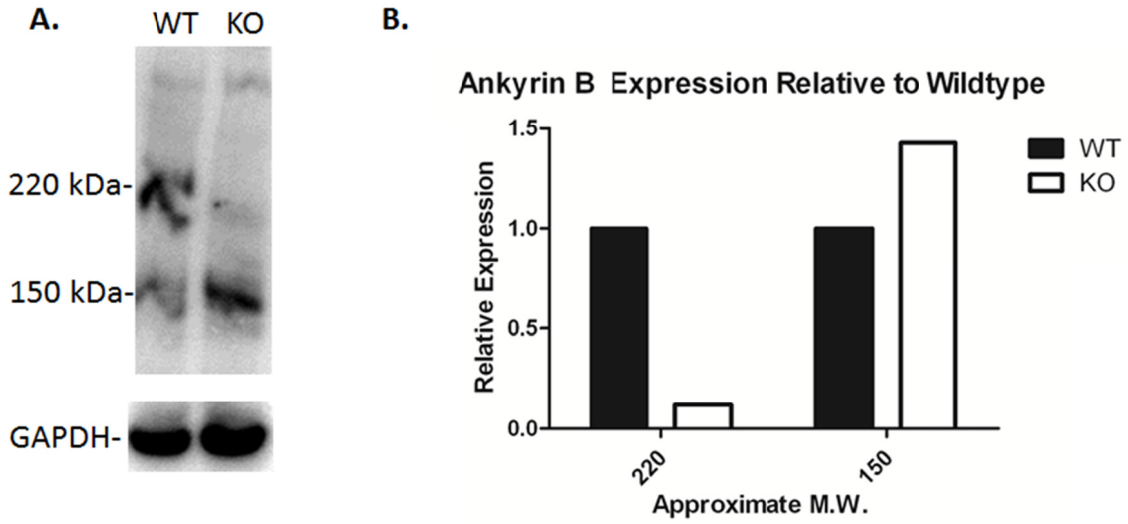

Figure 3. Ankyrin B Isoforms Expressed in Fibroblasts. (A) Western Blot of AnkB showing expression of the 220 and 150 kDa isoforms. A GAPDH loading control is below the AnkB blot. (B) Expression levels relative to wild-type for each isoform normalized to GAPDH. n=1

2.1.3 Differences in expression between WT and AnkB 220 KO cells

We attempted to perform northern blots on wild-type and AnkB 220 KO MEFs but the ankyrin-B transcripts were present in too low abundance to be visualized using a fibroblast cell culture system. Therefore, we decided to use reverse transcriptase PCR (RT-PCR) to identify which domains of ankyrin-B were present in wild-type and AnkB 220 KO cells. We found that AnkB 220 KO cells retain the spectrin binding domain (Zu5) and death domain but lack the ankyrin repeats domain (Figure 4). AnkB 220 KO cells produce a smear in the ankyrin repeat PCR which we believe is due to errant

binding of the ankyrin repeat primers to ankyrin-G transripts since ankyrin-B and -G have high homology even at the DNA level.

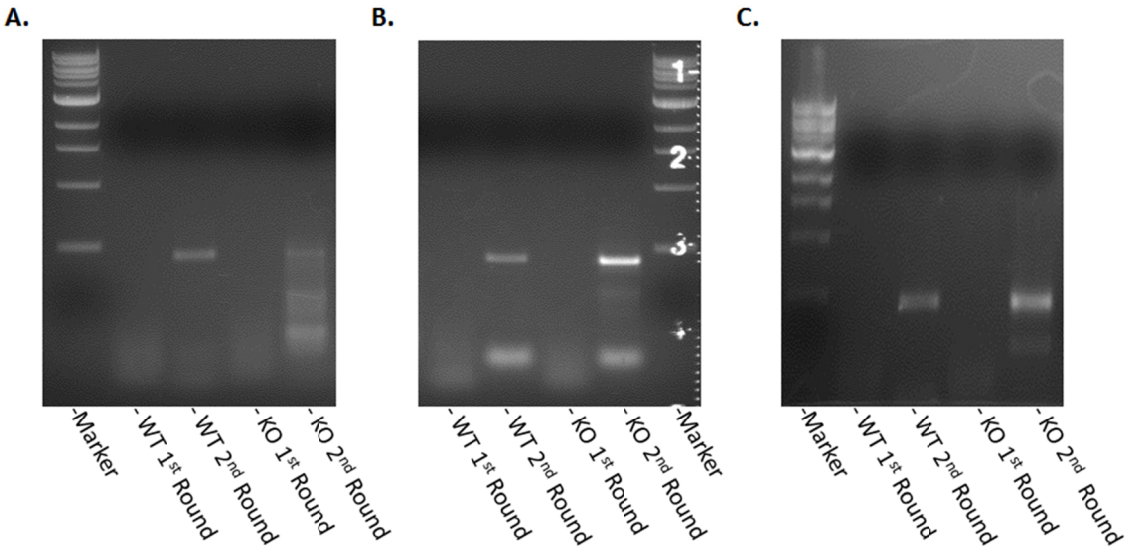

Figure 4. RT-PCR of Wild-type and AnkB 220 KOMEFs. (A) RT-PCR of the ankyrin repeats domain of ankyrin-B. (B) RT-PCR of the spectrin binding domain (Zu5) of ankyrin-B. (C) RT-PCR of the death domain of ankyrin-B.

2.1.4 Ankyrin-B localization in Fibroblasts

Ankyrin-B wild-type and AnkB 220 KO MEFs were fixed in 4% paraformaldehyde (PFA), permeabilized in 0.1% saponin, and stained for ankyrin-B with our rabbit antibody generated against the DD and C-terminal regulatory domain of ankyrin-B. AnkB 220 KO MEFs display a quantitative difference in average fluorescence intensity when compared with wild-type MEFs. The average fluorescence intensity of the AnkB 220 KO MEFs is only about ~30% lower than that of wild-type MEFs adding

support to the hypothesis that there is increased expression of the 150 kDa isoform to compensate for loss of the 220 kDa isoform (Figure 5c).

In addition, we measured the size of the ankyrin-B positive objects in wild-type and AnkB 220 KO cells in an attempt to determine the phenotype of AnkB 220 KO MEFs. We found that the ankyrin-B compartment is smaller in AnkB 220 KO cells suggesting that ankyrin-B 220 might play a role in maintaining the size and stability of the compartment (Figure 5d).

Both wild-type and AnkB 220 KO MEFs have ankyrin-B staining in the nucleus. This is an unexpected result as nuclear ankyrin-B has never been seen in cells up to this point. It is not likely that the ankyrin-B staining in the nucleus is due to an antibody artifact as this staining is present with several antibodies that have been produced in the laboratory and is affected by shRNA knockdown of ankyrin-B (data not shown). What isoforms of ankyrin-B are responsible for this localization remains to be discovered; however, the working hypothesis is that small isoforms of ankyrin-B that lack the ankyrin repeat domain are responsible for the nuclear signal as cells transfected with ankyrin-B 220 and 150 GFP do not display nuclear localization (data not shown).

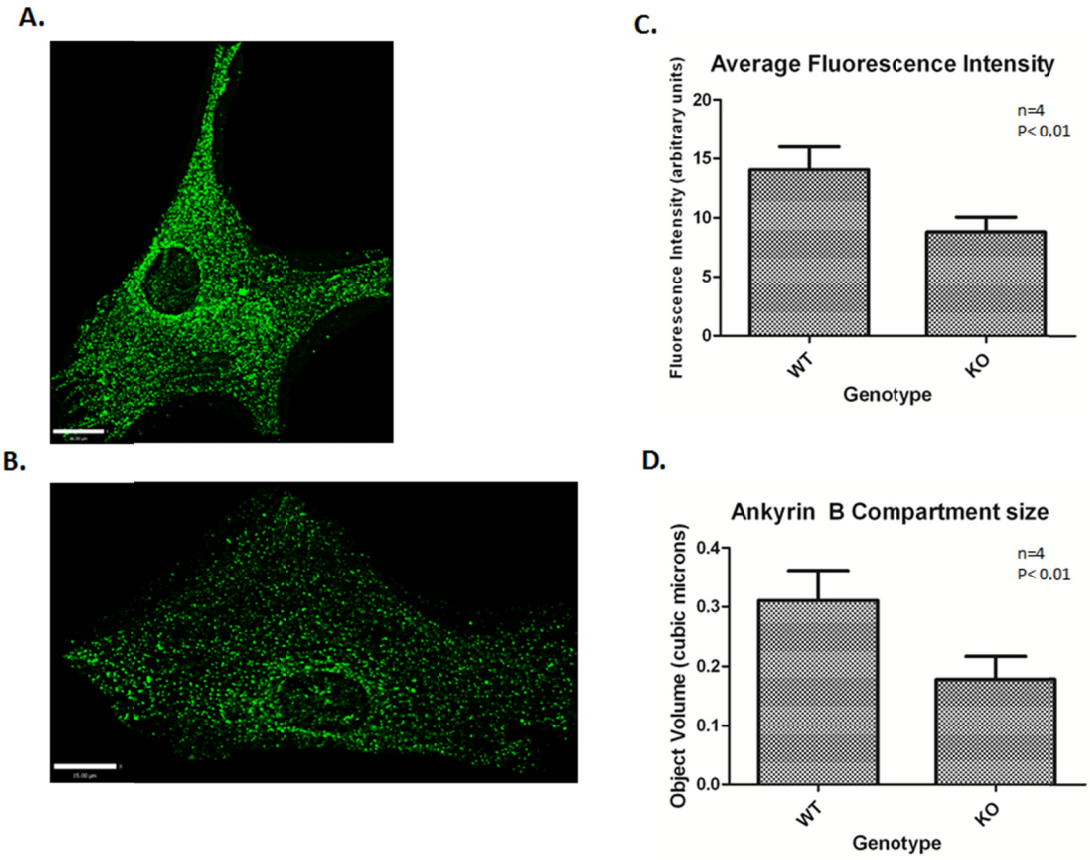

Figure 5. Ankyrin B localization in fibroblasts. (A) A representative image of a wild-type cell. Scale bars: 20 μm. (B) A representative image of a AnkB 220 KO cell. Scale bars: 20 μm (C) Quantification of average fluorescence in wild-type and AnkB 220 KO cells.N=4. (D) Quantification of the ankyrin B compartment size in wild-type and AnkB 220 KO cells. n=4. Student's t-tests were used to calculate statistical significance.

To try to identify the cytoplasmic ankyrin-B compartment, we did colocalization studies with various organelle markers and our sheep anti-ankyrin-B antibody generated against the C-terminal regulatory domain by Lydia Davis. We used antibodies against Rab11 (recycling endosomes), Rab 7 (late endosomes), Giantin (Golgi), Grp78 (endoplasmic reticulum), and LAMP2 (lysosomes). In addition, an antibody against β2spectrin was used as a positive control because β2 spectrin is a

known binding partner of ankyrin-B. We found that ankyrin-B and β2 spectrin have a strong association, but do not completely colocalize. Lack of complete colocalization between β2-spectrin and ankyrin-B is explained by the fact that β2-spectrin is also a binding partner of ankyrin-G. Ankyrin-B has a weak association with Rab11 but ankyrin-B does not display an association with any of the other markers suggesting that the ankyrin-B compartment may either be a novel compartment or a compartment containing more than one endosomal organelle (Figure 6).

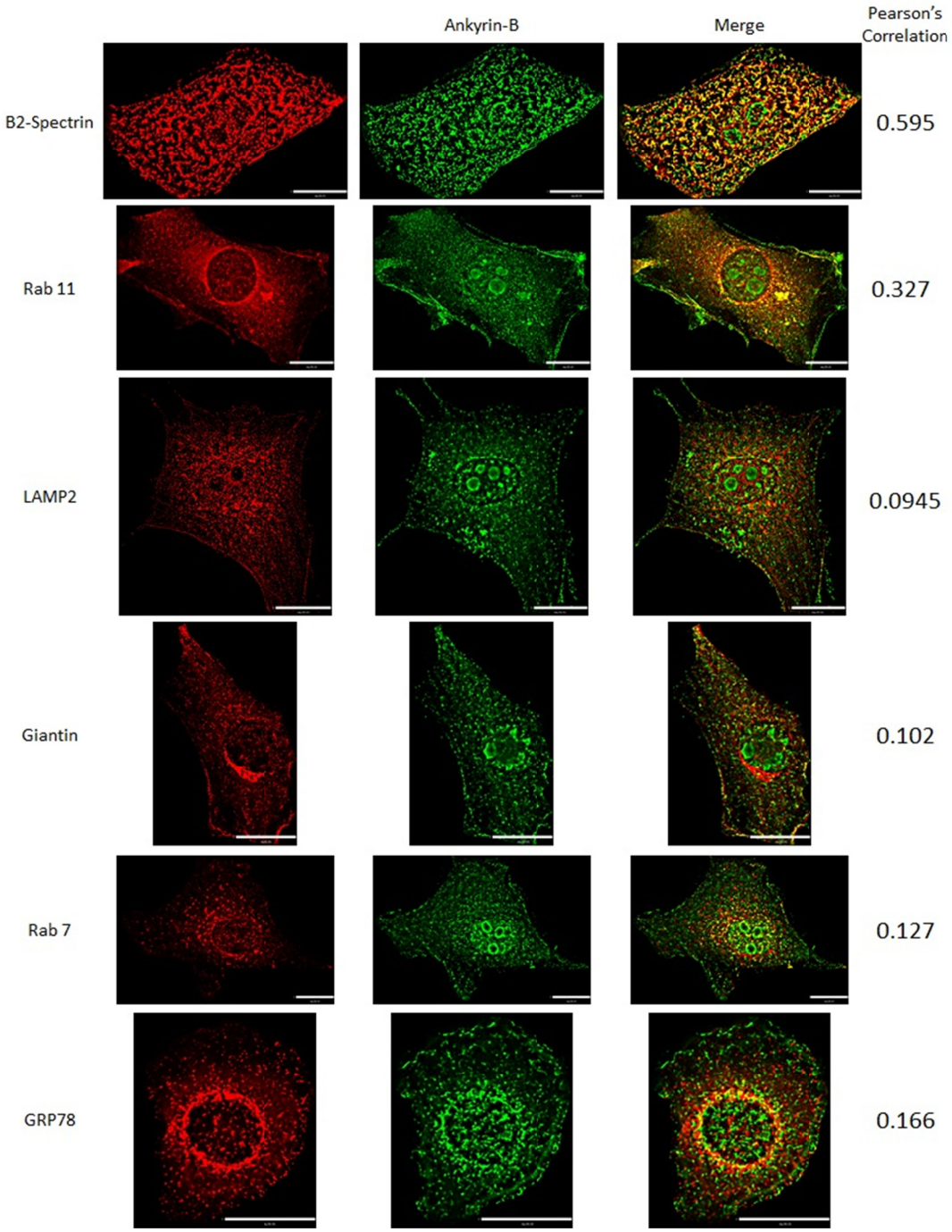

Figure 6. Ankyrin B Colocalization. MEFs were stained for ankyrin-B using a sheep anti-anykrin-B antibody generated against the c-terminus and one of the following: β2-spectrin, rab11, LAMP2, rab7, giantin, and GRP78. Image deconvolution and colocalization calculations were done in Volocity. Scale bars: 20 μm.

2.2 Ankyrin-G

2.1.1 Genotyping of Ankyrin-G Mutant Mice

Ankyrin G 190 KO pups were generated by Krishnakumar Kizhatil and the Duke Transgenic Mouse Facility by flanking exons 22 and 23 with flox sites. The floxed ankyrin-G 190 mice were then crossed against mice expressing CRE under control of the β-actin promoter by Kadar Abdi. When the β-actrin CRE removes exons 22 and 23, expression of the large ankyrin-B isoforms is abolished but expression of the smaller isoforms remains intact. Therefore, ankyrin-G null mice will be here after referred to as AnkG 190 KO mice. AnkG 190 KO mice die on postnatal day one (P1) and therefore, have to be genotyped before any experiments can be done. P1 pups were sacrificed by decapitation and tail samples were taken for DNA isolation and genotyping (Figure 7). Genotyping was done by Janelle Hostettler. AnkG 190 KO pups were generated at Mendelian frequencies. Pups determined to be AnkG 190 KO were used in all ankyrin-G experiments with wild-type littermates used as controls.

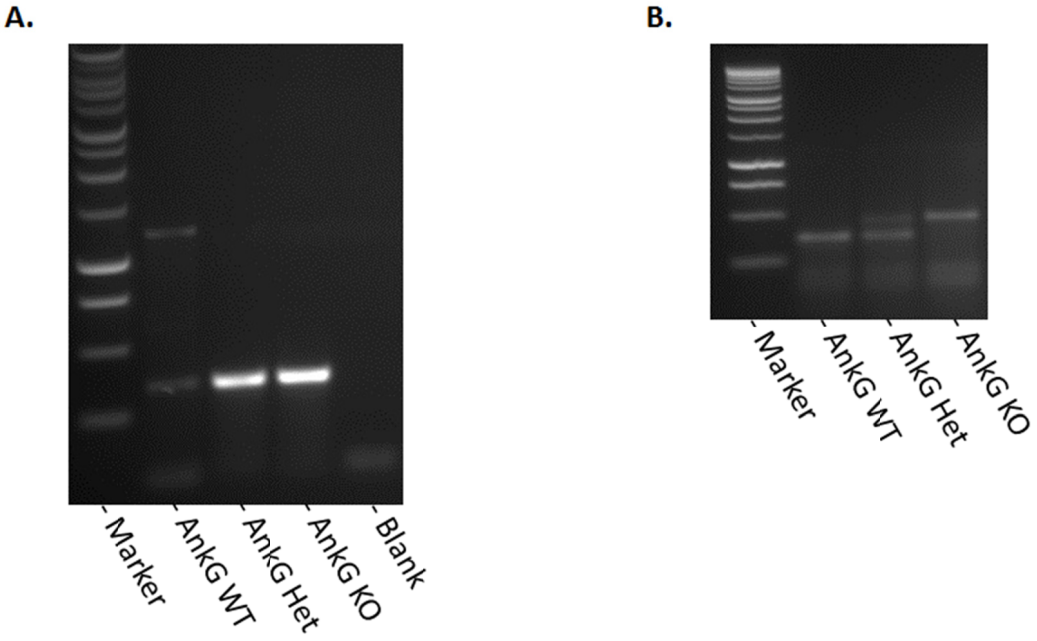

Figure 7. Ankyrin G Genotyping. (A) Ankyrin-G flox C/J genotyping PCR showing bands at 356 bp in heterozygous and AnkG 190 KO pups and no band in the wild-type pups. (B) Ankyrin-G flox G/J genotyping PCR showing a band at 288bp in wild-type pups, bands at 288 and 356 bp in heterozygous pups, and a band at 356 bp in AnkG 190 KO pups.

2.1.2Ankyrin-G Protein Expression in Fibroblasts

The expression of ankyrin-G isoforms has never been characterized in fibroblasts though, like ankyrin-B, several isoforms identified in other cell and tissue types. All isoforms identified to date contain the C terminal regulatory domain therefore; we used an antibody raised against the c-terminal regulatory domain of ankyrin-G. As ankyrin-B and ankyrin-G have high homology, the antibody was cross absorbed against ankyrin-B to reduce the possibility of cross reactivity by Lydia Davis.

Wild-type MEFs express four ankyrin-G isoforms, one at 210 kDa, one at 190 kDa, one at 120 kDa, and one at 100 kDa while AnkG 190 KO littermates only express the 120 and 100 kDa isoforms. In addition, AnkG 190 KO MEFs appear to express the 120 and 100 kDa isoforms at higher levels than wild-type MEFs (Figure 8). Presumably, the increased expression of the 120 and 100 kDa isoforms in the AnkG 190 KO MEFs is an attempt to compensate for the loss of the 210 and 190 kDa isoforms.

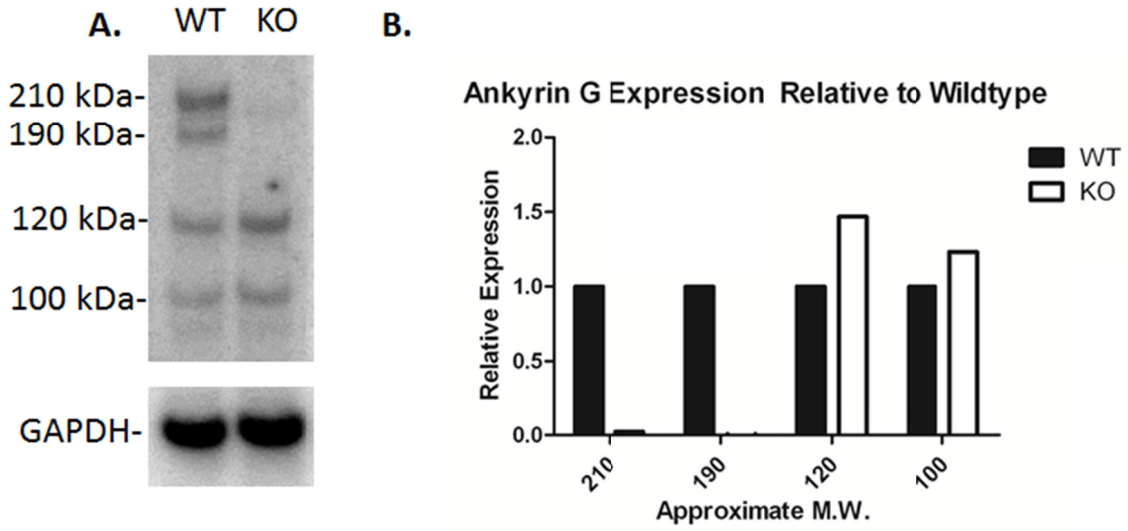

Figure 8. Ankyrin G Isoforms Expressed in Fibroblasts. (A) Western Blot of AnkG showing expression of the 210, 190, 120, and 100 kDa isoforms. A GAPDH loading control is below the AnkG blot. (B) Expression levels relative to wild-type for each isoform normalized to GAPDH. n=1

2.1.4Ankyrin-G localization in Fibroblasts

Ankyrin-G wild-type and AnkG 190 KO MEFs were fixed in 4% PFA, permeabilized with 0.1% saponin, and stained for ankyrin-G with our goat antibody raised against ankyrin-G's C-terminal regulatory domain by Lydia Davis. AnkG 190 KO MEFs display a quantitative difference in average fluorescence intensity when compared with wild-type MEFs. The average fluorescence intensity of the AnkG 190 KO MEFs is about ~50% lower than that of wild-type MEFs adding support to the hypothesis that there is increased expression of the 120 and 100 kDa isoforms to compensate for loss of the 210 and 190 kDa isoforms (Figure 9c).

Like ankyrin-B, the ankyrin-G antibody recognizes an intracellular compartment. We tested whether the size of this compartment is reduced in AnkG 190 KO cells and found similar, though less dramatic, results to those seen in AnkB 220 KO MEFs. The compartment in AnkG 190 KO cells was somewhat smaller than the compartment in wild-type cells (Figure 9d).

Ankyrin-G staining in fibroblast results in a small percentage of nuclear staining and like ankyrin-B this staining is seen with more than one antibody. However, the hypothesis is that small isoforms of ankyrin-G that lack the ankyrin repeat domain are responsible as there is no signal in the nucleus when cells are transfected with ankyrin-G 190 GFP (data not shown).

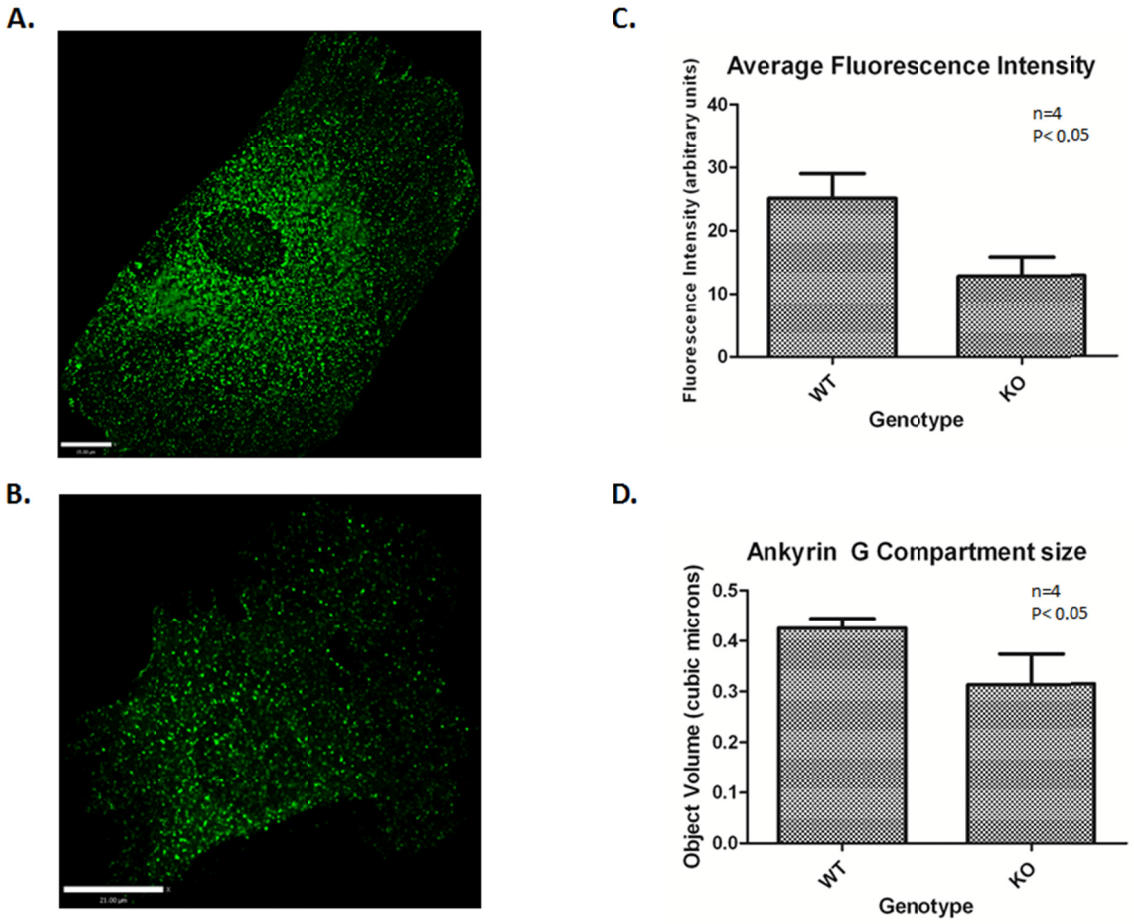

Figure 9. Ankyrin G localization in fibroblasts. (A) A representative image of a wilt-type cell. Scale bars: 20 μm. (B) A representative image of a AnkG 190 KO cell. Scale bars: 20 μm. (C) Quantification of average fluorescence in wild-type and AnkG 190 KO cells. n= 4. (D) Quantification of the ankyrin-G compartment size. n=4. Student's t-tests were used to calculate statistical significance.

2.3 Ankyrin-B and -G and the Fas Receptor

2.3.1 Ankyrin-B and -G Do not bind Fas or FADD

In 2004, a paper was published claiming that the death domain of ankyrin-G 190 binds the pro-apoptotic receptor, Fas (Del Rio et al, 2004). Fibroblasts provide the perfect model for studying apoptosis and the role of ankyrin-B and -G in this process. We attempted to reproduce the results from the paper that identified fas receptor as being an ankyrin-G binding partner. Since, ankyrin –B and –G share such high sequence homology, we tested ankyrin-B for binding as well.

We performed a yeast two hybrid cotransformation assay using the death domain of either ankyrin-B or –G as bait and the full length fas receptor or fas associated death domain containing protein (FADD) as prey. We found that neither ankyrin-B or -G bind to either protein but were able to show that the fas receptor and FADD bind each other. This suggests that the data published in the 2004 paper claiming that ankyrin-G 190 and fas receptor are binding partners may be flawed and needs to be reviewed.

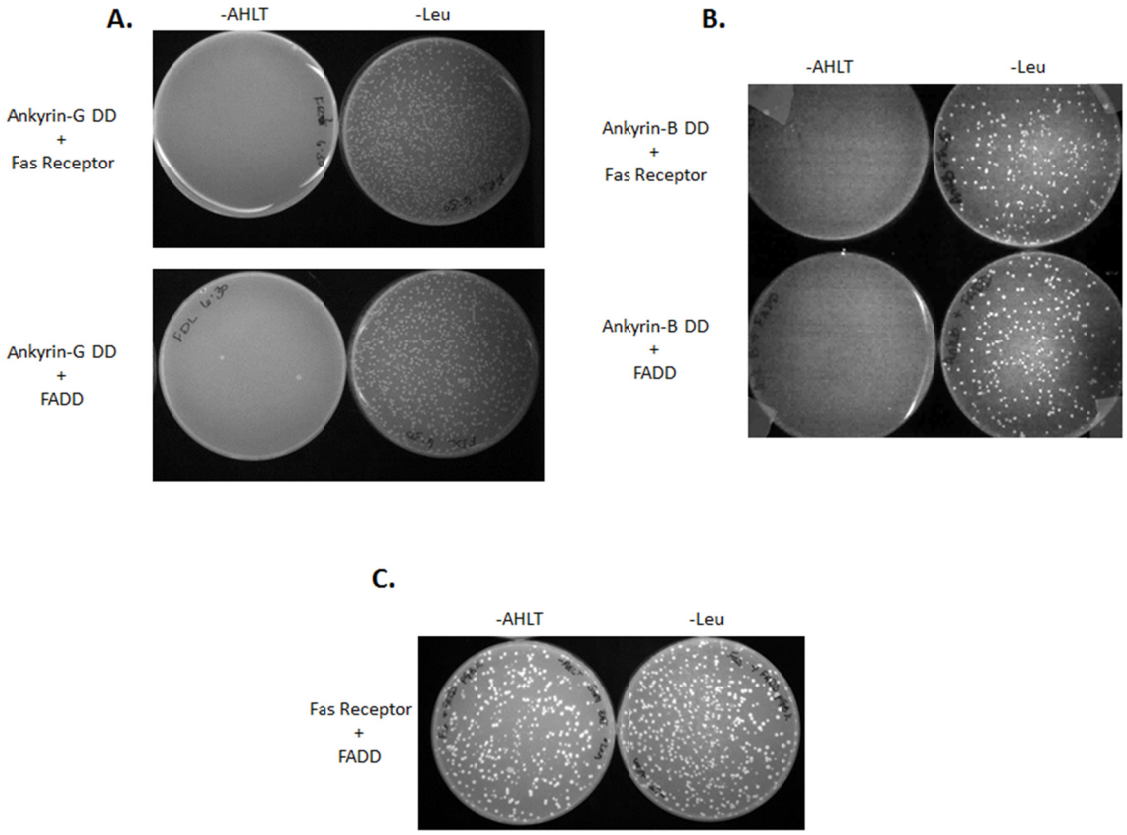

Figure 10. Ankyrin-B and –G death domains do not bind the fas receptor or FADD. (A) Y2H cotransformation assay with the death domain of ankyrin-G as bait and either fas receptor or FADD as prey showing no colonies on reporter plates. (B) Y2H cotransformation assay with the death domain of ankyrin-B as bait and either fas receptor or FADD as prey showing no colonies on reporter plates. (C) Y2H cotransformation assay with the FADD as bait and fas receptor or FADD as prey showing numerous colonies on reporter plates.

3. Discussion

Our laboratory has observed that even cells with stable ankyrin-based membrane domains retain a population of both ankyrin-B and -G in the cytoplasm. Until now, Ankyrin-B and –G have been studied as proteins linking plasma membrane proteins to the cytoskeleton. Since the vast majority of ankyrin is located on the plasma membrane in the cell types normally used in ankyrin research, a cell with a sizeable intracellular ankyrin population would be useful for studying intracellular ankyrin. To this end, we chose to study ankyrin-B and –G in MEFs as they are a fundamental cell type with a sizeable intracellular pool of ankyrin-B and -G and little polarity. We have now used MEFs to characterize ankyrin expression in both wild-type, AnkB 220 KO, and AnkG 190 KO mice.

Fibroblasts express multiple isoforms of both ankyrin -B and -G with the small isoforms being upregulated in AnkB 220 KO and AnkG 190 KO cells. The upregulation of the small isoforms in the MEFs could be a mechanism by which AnkB 220 and AnkG 190 mice attempt to compensate for the loss of the large isoforms of ankyrin –B and –G. However, more studied need to be done to confirm these observations. This small isoform upregulation could explain why AnkB 220 KO and AnkG 190 KO pups survive gestation but die soon after birth, as the expression of the large isoforms of ankyrin-B

and -G is required for the proper development and functioning of neurons and cardiomyocytes.

Both ankyrin -B and -G are expressed in an intracellular compartment in fibroblasts and the size of both compartments is affected by knockout of the large isoform. To identify the ankyrin-B compartment, colocalization studies were done with markers for recycling endosomes, late, endosomes, lysosomes, Golgi, and endoplasmic reticulum. Ankyrin-B displayed a weak association with recycling endosome but none of the other organelles was found to have an association with the ankyrin-B compartment. The ankyrin-B compartment strongly associated with β2-spectrin, its known binding partner. The weak association of ankyrin-B with rab11, a marker for recycling endosomes, suggests that ankyrin-B may somehow be involved in the endocytic recycling pathway. Ankyrin-B, but not ankyrin-G, has recently been shown to bind Eps15 homology domain containing (EHD) proteins important for endosomal transport and recycling (Gudmundsson, 2010). Ankyrin could potentially be involved in the formation of protein trafficking compartments via its association with EHD proteins 1-4. EHD proteins are expressed on many different endocytic compartments (Naslavsky, 2011). EHD domain positive compartments have similar structures to the ankyrin-B compartment seen in these MEFs (Blume et al, 2007). Thus, rather than being expressed on one specific organelle, the ankyrin-B compartment might include several

different endosomal organelles. It would be interesting to perform colocalization studies to see if ankyrin-B colocalizes or partially colocalizes with EHD positive compartments.

There is less information available on ankyrin-G intracellular compartment than there is on the ankyrin-B compartment but there is ongoing work suggesting that this ankyrin-G compartment may be an endosomal sorting compartment similar to that proposed for ankyrin-B.

Ankyrin-B and -G display nuclear as well as cytoplasmic localization. At first, it was thought that the nuclear staining may be an antibody artifact however, we have seen nuclear staining with many of the antibodies against ankyrin-B and -G that we use in the laboratory suggesting that the nuclear signal may be real. In addition, the nuclear staining is decreased in ankyrin-B cells infected with shRNA targeted against the regulatory domain (data not shown). Elucidation of ankyrin-B function in the nucleus could unveil a surprising role for ankyrin family proteins in the nucleus.

There is little known about the function of the Zu5-UPA-DD cassette found at the c-terminus of ankyrin protein family members. A paper published in 2004 claimed that the death domain of ankyrin-G 190 bound fas receptor and that through this interaction ankyrin-G 190 had pro-apoptotic activity. Unfortunately, we could not reproduce the yeast two hybrid results found in the paper using the same yeast two hybrid system.

These and ongoing studies give us valuable insight into the fundamental cell biology of ankyrin-B and –G. Originally identified as static structural proteins in the spectrin cytoskeleton, the ankyrin family of proteins are being revealed as dynamic players in membrane biology and protein trafficking.

4. Methods

4.1 Cell Culture

Ankyrin B 220 KO mouse embryonic fibroblasts (MEFs) were harvested from neonatal mice on post natal day 1. Wild-type MEFs were harvested from wild-type littermates. Internal organs and limbs were removed from the torso and then torso was then used for the production of MEFs. Harvested pups were stored at 4^0C in Hibernate (GIBCO, Carlsbad, CA) overnight until genotyping results were available. Pups were then minced in 5 mL 0.25% Trypsin EDTA. The following was then added to the tissue suspension: 3 mL 2.5% Trypin and 1 mL 1mg/ml DNAse. The tissue suspension was then incubated at 37^0C for 1 hour on a rotator. After incubation, the suspension was filtered using a 100 micron cell filter. Complete DMEM (10% FBS, 100 units penicillin, 100 μg streptomycin, and 100 μM non-essential amino acids) was added to the cells and spun at 1500xg for 5 minutes. Supernatant was aspirated and cells were resuspended in 10 mL complete DMEM and plated on a 10 cm plate. Cells were allowed to adhere to the plate for 24 hours before being washed twice with 1XPBS to remove any non-adherent cells 10 mL of fresh complete DMEM was then added to the plate. MEFs were allowed to grow to confluence at 37^0C and 5% CO_2 before passaging.

4.2 Genotyping

AnkB 220 KO mice were generated by Paula Scotland (Scotland et al, 1998). All pups were sacrificed by decapitation and the tailed removed. DNA was extracted from cells using the REDExtract-N-Amp Tissue PCR kit (Sigma, St. Louis, MO). Genotyping primers are located in Table 1. The ankyrin-B genotyping PCR results in a 400 bp band in wild-type pups, a 1700 bp band in AnkB 220 KO pups, and a band of each size, in heterozygous pups.

AnkG 190 KO pups were generated by Krishnakumar Kizhatil and the Duke Transgenic Mouse Facility by flanking exons 22 and 23 with flox sites. The floxed ankyrin-G 190 mice were then crossed against mice expressing CRE under control of the β-actin promoter by Kadar Abdi. Genotyping the floxed ankyrin-G pups requires two PCRs. The ankyrin-G flox C/J PCR results in no bands in wild-type pups and one 356 bp band in both AnkG 190 KO and heterozygous pups. The ankyrin-G flox G/J PCR results in a 356 bp band in AnkG 190 KO pups, bands at 288 and 356 bp in herterozygous pups, and one 288 bp band in wild-type pups. Therefore, genotyping results for the floxed ankyrin-G 190 pups are as follows (C/J PCR/G/J PCR), wild-type: no band/288bp band, heterozygous: 356 bp band/288 bp band, and AnkG 190 KO: 356 bp band/no band.

Table 1. List of primers used in genotyping PCR for identification of ankyrin B 220 and ankyrin G 190 wild-type, heterozygous, and knockout pups.

	Forward Primer	Reverse Primer
Ankyrin-B 220 KO PCR	5′-AACAACATAGCAGCAATCACAAAAGACC-3′	5′-GCCTAACAGTAATGAATCTCTTCTCTTGTA-3′
Ankyrin-G Flox C/J PCR	5′-CTGTGCTTGAGGAAGATTTTTGGG-3′	5′-TTGGGATGCTTTGATTCAGGG-3′
Ankyrin-G Flox G/J PCR	5′-TTAATTTGGGGAGGGGGGAGTC-3′	5′ TTGGGATGCTTTGATTCAGGG-3′-

4.3 Antibodies

Antibodies against ankyrin B, ankyrin G, and β2 spectrin were generated in the lab. The following antibodies were purchased: rabbit anti-giantin (Cat. No. ab24586, Abcam, Cambridge MA), rabbit anti-rab7 (Cat. No. 9367S, Cell Signaling Technologies, Danvers, MA), rabbit anti-rab11 (Cat. No. 71-5300, Invitrogen, Carlsbad, CA), rabbit anti-LAMP2(Cat No. L0668, Sigma-Aldrich, St. Louis, MO), and rabbit anti-GRP78 (Cat No. ab21685, Abcam, Cambridge, MA). Secondary antibodies used were donkey anti-rabbit Alexa 488, donkey anti-sheep Alexa 568 (Invitrogen, Carlsbad, CA).

4.4 Secondary Antibody Preabsorbtion

All secondary antibodies were preabsorbed against mouse tissues. Heart, kidney, and brain tissue were harvested from wild-type C57BL/6 mice and then fixed in

4% PFA overnight at 4ºC. Tissues were then washed three times in 1XPBS, flash frozen in liquid nitrogen, and then ground to a powder. The resulting homogenate was then resuspended in 1XPBS to a final concentration of 100mg/mL. 5% (w/v) of each tissue was then added to 1XPBS to a total volume of 500 μL for each antibody to be preabsorbed. 5 μL of secondary antibody was added to the homogenate and incubated on a rotator overnight at 4°C and then centrifuged at 100,000xg for 20 minutes at 4ºC. The supernatant was collected and used at a dilution of 1:10.

4.5 Immunofluorescence

Cells were plated on collagen coated 1.5 cm Mat-Tek plates (Cat. No. P35GCOL-1.5-14-C, Mat-Tek Corporation, Ashland, MA) and allowed to adhere overnight. Cells were washed once with PBS before being fixed in 4% PFA in 1XPBS for 15 minutes at room temperature. Cells were permeabilized with 0.1% saponin in 1XPBS for 10 minutes and washed three times with 1XPBS, 5 minutes each. Antibodies used for immunofluorescence were diluted in 2% BSA in 1XPBS and used at the indicated dilutions. Antibodies were used at the following dilutions: sheep anti-ankyrin B C-terminus (1:1500), rabbit anti-ankyrin B death domain (1:50), rabbit anti-ankyrin G death domain (1:500), rabbit anti-β2 spectrin (1:500), rabbit anti-giantin (1:1000), rabbit anti-rab7 (1:100), rabbit anti-rab11 (1:10), and rabbit anti-GRP78 (1:300). Plates were incubated with primary antibody for 1 hour at room temperature and then washed three

times with 1XPBS. Preabsorbed secondary antibody was then added to the plates and incubated for two hours at room temperature. After secondary incubation, plates were washed three times in 1XPBS, 5 minutes each and mounted in Vectashield (Vector Labs, Burlingame, CA) for imaging. Images were gathered using a Zeiss 510 confocal microscope with a 100X objective. All image quantification was done using Volocity software (Perkin Elmer, Waltham, MA).

4.6 Western Blots

Cells from a 10 cm plate were trypsinized, and centrifuged at 1500xg for 5 minutes. The supernatant was aspirated and the cells were washed twice with PBS. After the last wash, 200 μL of 5x SDS-PAGE loading buffer was added to the cells. The sample was then sonicated in 15 one second pulses using a sonicator equipped with a microtip. Samples were then heated to 95^0C for 5 minutes. After heating, the samples were loaded on to a bis-tris acrylamide gel with a 3.5 to 17% acrylamide gradient. The gel was run in 1X Fairbanks Running buffer pH 7.4 (40 mM tris-HCL, 20 mM sodium acetate, 2 mM EDTA pH 8.0, 10% SDS) at 200 volts until the dye front entered the gel. The voltage was then lowered to 100 volts and the dye front was allowed to run to the bottom of the gel.

The gel was transferred onto a nitrocellulose membrane in 1X transfer buffer (20 mM tris-HCL pH 7.4, 0.01 mM sodium acetate, 1 mM EDTA ph 8.0, 10% SDS) at 0.3

miliamperes overnight. The membrane was then blocked with 2% BSA in Blot Buffer 1 (BB1; 10 mM phosphate buffer pH 7.4, 1 mM EDTA pH 8.0, 1 mM sodium azide, 150 mM sodium chloride, 0.2% triton X-100) for 30minutes. Primary antibody was then added to the membrane in 2% BSA in BB1 and incubated overnight at 4^0C. The membrane was washed three times in BB1 and ^{125}I-protein A/G was added to the blot at a 1:200 dilution. The blot was then incubated at room temperature for 2 hours before being washed three times in BB1. The blot was dried and then exposed to film for 24 hours before being developed. All image quantification was done in ImageQuant2 (GE Healthcare, Pittsburgh, PA).

4.7 RNA Purification

RNA from either wild-type or AnkB 220 KO MEFs was purified using TRIzol (Invitrogen, Carlsbad, CA) with modifications to the manufacturer's protocol. The RNA was precipitated in isopropanol twice. The initial precipitation was done 1 ml isopropanol per milliliter of TRIzol used and stored at -20^0C for 1 hour. The second precipitation was done by combining the RNA pellets in 1 mL of isopropanol. The RNA was then allowed to precipitate at -20^0C for an hour. After all precipitations and washes, the RNA was resuspended in TE and the concentration determined using a NanoDrop spectrophotometer. All other aspects of the manufacturer's protocol were adhered to.

4.8 Reverse Transcriptase – Polymerase Chain Reaction

RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) with poly dT oligos. Nested PCR was then carried out on the cDNA from the reverse transcription reaction. Primer sets were used to amplify 500 basepair sections of ankyrin corresponding to the ankyrin repeats, spectrin binding (first Zu5), and death domains (Table 2). Five microliters of DNA was used in the first PCR while two microliters of DNA from the first PCR was used in the second PCR. Both PCR reactions were loaded and run on a 1% agarose gel then visualized under ultraviolet light.

Table 2. List of nested DNA primers used for RT-PCR of ankyrin B 220 KO and wild-type MEFs.

Probe Target	Outer Forward Primer	Outer Reverse Prmier	Inner Forward Primer	Inner Reverse Primer
Ankyrin Repeats	5′ CTTCACATGG CTGCGCAGGG GGAC 3′	5′ GTGTAAAGGT GTCTGCTCCTC CCTGGCTCTG G 3′	5′ CACGTGGAAT GTGTGAAGCA CCTGCTC 3′	5′ CATCCACAAG AGCACCATTC CTCAGAAGAC ATCG 3′
Spectrin Binding Domain	5′ GTGATCGTGG AGATCCCTCA CTTC 3′	5′ GAAGCTTTGG GGACAGGAAT AGTCATAG 3′	5′ GCCGCTCTTC GAGGAAAGG AGAGG 3′	5′ GTAATTGGCT TGTGGAATTTT CTTCTCCTGGG TTCC 3′
Death Domain	5′ GGGATGGGAA GCATGCCACA GATAC 3′	5′ GTCCTCAGGA GTCTTACAGA GAGAGTCAGG 3′	5′ CATCCTCATC GAATGCCTCA CCAAGATCAA CAGG 3′	5′ GGCTGTGGCC TTCTGAGGGT CTTC 3′

4.9 Y2H Cotransformation Assay

The Y2H co-transformation assay was performed using the Matchmaker yeast two hybrid system (Clontech, Mountain View, CA). Wild-type AH109 yeast were co-transformed with either the death domain of ankyrin-B or -G in the bait vector, pGBK-T7 and either full length fas receptor or FADD in the prey vector, pGAD-T7. Yeast cultures were inoculated with one colony of AH109 and allowed to grow overnight in 1X YPDA media (20g/L peptone, 10g/L yeast extract, 1% dextrose, 0.0015% adenine hemisulfate) at 30^0C. The optical density (OD) of the cells was determined at OD 600 and the cells were diluted to 0.3 OD and allowed to grow to an OD of 0.6 before being used for transformation. After an OD of 0.6 was reached, cells were centrifuged at 1,500xg for 5 minutes and the media removed. Cells were washed once with distilled water and then twice with 50 mL of 1X TE LiAc (10 mM tris pH 7.4, 1 mM EDTA pH 8.0, 100 mM lithium acetate (LiAc)). The cell pellet was resuspended in 1X TE LiAc to an OD of 5. 50 μL of cells was used for each transformation. 500 ng of both the bait and prey vectors was added to each 50 μL aliquot of cells along with 5 μL of a 10 mg/mL solution of salmon sperm DNA. 250 μL of fresh PEG/LiAc (40% PEG, 10 mM tris pH 7.4, 1mM EDTA pH 8.0, 100 mM LiAc) was added to each aliquot, mixed thoroughly, and incubated at 30^0C for 30 minutes. Cells were then heat shocked at 42^0C for 15 minutes and allowed to cool 30 minutes on ice. Cells were spun down at 7,000xg for 5 minutes

and the supernatant drawn off. Transformed cells were then resuspended in 1 mL of 0.5X YPDA then plated on selective media. 75 μL of cells was plated on –Leu dropout plates (6.7g/L yeast nitrogen base, 20g/L agar, 0.69 g/L –Leu amino acid supplement, 2% dextrose) and 150 μL plated on –AHLT reporter dropout plates (6.7g/L yeast nitrogen base, 20g/L agar, 0.6 g/L –AHLT amino acid supplement, 2% dextrose). Plates were placed in a 30^0C incubator and allowed to grow 3-4 days before checking for colonies.

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