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Amelia Burch April 15, 2015

Analysis of Dysbindin-BLOC-1 Complex Mechanisms in the Neuromuscular Synapse of the Fruit Fly *Drosophila melanogaster*

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2015

Abstract

Analysis of Dysbindin-BLOC-1 Complex Mechanisms in the Neuromuscular Synapse of the Fruit Fly *Drosophila melanogaster*

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The Biogenesis of lysosome-related organelles complex-1 (BLOC-1) is an evolutionarily conserved, endosomally-localized protein complex involved in the biogenesis of lysosome-related organelles, including synaptic vesicles. Polymorphisms in the human gene *DTNBP1,* which encodes the BLOC-1 subunit dysbindin, confer an increased risk of schizophrenia. Moreover, defects in neurotransmission and synapse morphology have been observed in mouse and Drosophila BLOC-1 loss-of-function mutants. This suggests that molecular pathways downstream of dysbindin deficiency may intersect with schizophrenia disease mechanisms to regulate neurotransmission. Here, we studied molecular phenotypes arising from loss-of-function defects in the dysbindin-BLOC-1 schizophrenia susceptibility network. Using quantitative mass spectrometry, we measured changes in protein expression associated to dysbindin-BLOC-1 down-regulation in mammalian cells. We identified components of the actin polymerization machinery that were down-regulated in neuronal cells following reduced dysbindin-BLOC-1 content, and confirmed that these actin polymerization regulators biochemically interacted with BLOC-1. For my thesis, I tested the hypothesis that these BLOC-1 sensitive factors identified by mass spectrometry participate in the same molecular pathway to regulate synaptic morphology in the *Drosophila* neuromuscular junction. Here, I aimed to assess synaptic phenotypes using transheterozygotic analysis between loss-of-function alleles of the actin nucleator Arp2/3 and BLOC-1 subunits; however, I instead unintentionally tested the *Arp1* null allele. Arp1 is a subunit of the dynactin complex, a protein complex critical for retrograde axonal transport. I found that *Arp1* null mutants generate an abnormal synaptic morphology, which is phenocopied in BLOC-1 deficient animals. This synaptic phenotype is reproduced in animals carrying null alleles for BLOC-1 expressed *in trans* with *Arp1* loss-of-function alleles, suggesting Arp1 acts in the same pathway as BLOC-1 to regulate synaptic architecture in *Drosophila.* These findings suggest an unforeseen connection between BLOC-1 complex and dynein-dynactin mechanisms.

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Introduction

Schizophrenia Susceptibility Genes

Schizophrenia is a debilitating mental disorder that affects approximately 1% of the American population. This affliction is characterized by positive symptoms such as hallucinations and delusions, as well as negative symptoms, which include lack emotion and motivation and cognitive deficits (Kay et al 1987). While the exact cause of onset is not understood recent research has strongly indicated that schizophrenia is a complex polygenic disorder where multiple genes interact with environmental factors and impinge on neuronal signaling pathways at specific developmental stages ultimately resulting in pathogenesis. For example genome-wide association studies (GWAS) have identified over 120 gene variants and 108 genetic loci associated with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014). Genes disrupted in schizophrenia affected individuals include those involved in neurotransmission, synaptic plasticity, calcium channel biology, and cellular immunity.

One gene of particular interest due to its strong association with schizophrenia onset is the dysbindin-encoding *DTNBP1* gene. Copy number variations in *DTNBP1* have been identified in several genetic association studies as a potential risk factor for schizophrenia in multiple human populations (Straub et al. 2002; van den Oord et al. 2003; Kirov et al. 2004; Allen et al. 2008; Zuo et al. 2009). Notably, postmortem studies of human schizophrenic brains have shown reduced levels of mRNA and protein expression of dysbindin in regions of the brain associated with schizophrenia pathogenesis (Harrison & Eastwood 2001; Dwork 2002; Talbot et al. 2004). Interestingly, these reductions occurred in ~80% of the 17 schizophrenic patients in the study, a

number far greater than the frequency of *DTNBP1* haplotypes (0-18%) which confer risk for the disorder (Talbot et al. 2004) (Figure 1A). Reduction of dysbindin mRNA levels has also been reported in patients with schizophrenia in the dorsolateral prefrontal cortex (Weickert et al. 2004), a region also implicated in the pathology of the disorder (Callicott et al. 2000).

The BLOC-1 Complex

DTNBP1 encodes the protein Dysbindin, which is a part of a larger, ubiquitous octameric biogenesis of lysosome related organelles-1 complex (BLOC-1 complex). BLOC-1 comprises of subunits dysbindin, pallidin, muted, cappuccino, BLOS1, BLOS2, BLOS3 and snapin (Di Pietro & Dell'Angelica 2005) (Figure 2). There is a high degree of primary sequence similarity among subunits of the BLOC-1 complex from *Drosophila* to humans (Cheli et al. 2010) (Table 1). All subunits demonstrate the same binary interactions conserved across *Drosophila* and mammals in yeast two hybrid assays and the whole protein complex has been identified in humans and *Drosophila*, suggesting conserved structure and functions of this complex throughout evolution (Starcevic & Dell-Angelica 2004; Mullin et al. 2015) (Table 1). Mutations in any of the subunits of the BLOC-1 complex result in systemic defects including reduced pigmentation, pulmonary fibrosis, impaired blood coagulation and abnormal neuronal function in mouse knock-out models(Figure 3). Of these phenotypes pigmentation and neuronal phenotypes are conserved from *Drosophila* to humans. These phenotypes are consistent with molecular studies, which have shown BLOC-1 to be involved in the biogenesis of lysosome-related organelles, such as melanosomes, platelet granules and lamellar bodies, as well as

synaptic vesicles (Falcon-Pérez et al. 2002; Newell-Litwa 2009; Newell-Litwa et al. 2010).

We are particularly interested in the neuronal function of BLOC-1. Notably, unpublished data from Konrad Talbot laboratory (Mount Sinai Hospital Los Angeles, CA) has demonstrated that in addition to dysbindin the other BLOC-1 complex subunits pallidin, and BLOS3 are reduced in the hippocampus of schizophrenia patients at post-mortem (Figure 1B), suggesting that this reduction in BLOC-1 levels may be a cause or effect of schizophrenia endophenotypes or behavioral phenotypes induced by BLOC-1 genetic defects (Talbot et al. 2004). This finding is reinforced in mutant mouse models of dysbindin and pallidin, which exhibit behavioral impairments similar to those observed in schizophrenia patients (Talbot 2009; Horowitz 2012). Therefore, studying dysbindin and BLOC-1 cellular mechanisms is critical for understanding the pathogenesis of schizophrenia.

Published data from the Faundez lab has shown that BLOC-1 is necessary for the biogenesis of synaptic vesicles (Newell-Litwa et al. 2009; Newell-Litwa et al. 2010). Recent studies from our laboratory have established a role for dysbindin and the BLOC-1 complex in synaptic morphology, plasticity, and synaptic vesicle trafficking mechanisms at the *Drosophila* neuromuscular junction (Mullin et al 2015). However, the precise molecular pathways by which dysbindin and the BLOC-1 complex participate in these presynaptic mechanisms both in invertebrates and vertebrates are mostly unknown (Mullin et al 2015).

My thesis focuses on determining the identity of these unknown pathways associated with dysbindin and BLOC-1 complex function. I take advantage of the

evolutionary conserved primary and quaternary structure of the BLOC-1 complex and the commonality of presynaptic phenotypes in *Drosophila* and mammals to identify and test functionally and genetically new mechanisms associated to dysbindin/BLOC-1 deficiency.

To identify pathways associated to dysbindin and BLOC-1 complex function, our laboratory conducted a quantitative proteomic analysis of BLOC-1 loss-of-function using shRNA down-regulation of BLOC-1 complex subunits to identify factors sensitive to BLOC-1 cellular levels. This approach is inspired by the observation that the loss of one BLOC-1 subunit results in the down-regulation of the other members of the complex. For example, *sandy* (*Bloc1s8*$^{syd/syd}$) mice deficient in the dysbindin subunit exhibit reduced levels of muted, snapin, and pallidin proteins (Starcevic & Dell'Angelica 2004; Feng et al. 2008). Similarly, knockout mice in other BLOC-1 subunits exhibit similar phenotypes in all BLOC-1 subunits (Larimore et al. 2014). Thus, we predicted that the expression of molecules interacting with BLOC-1 complex or participating in mechanisms downstream of BLOC-1 would be sensitive to BLOC-1 downregulation. Using quantitative mass spectrometry, we quantified the expression of ~4,000 proteins in neuronal cells lines down-regulated for the BLOC-1 complex. Among these proteins we identified actin polymerization regulatory factors whose expression was decreased after BLOC-1 perturbation. Of particular interest were the multiple subunits of the Arp2/3 complex and nucleation-promoting factors (NPFs) such as Annexin II and members of the WASH complex, factors involved in the recruitment of the Arp2/3 complex. This thesis focuses on one of these actin regulatory factors, the Arp2/3 complex. In the next sections I will

introduce the Arp2/3 complex and present this thesis hypothesis, which I will test utilizing *Drosophila melanogaster* as a model organism.

The Arp2/3 Complex

Actin-related proteins 2/3 (Arp2/3) is a cytosolic protein complex constituted by seven-subunits. Two of the subunits are actin-related proteins of the ARP2 and ARP3 subfamilies, giving the complex its name. The other five subunits are referred as ARPC1 (actin-related protein complex-1), ARPC2, ARPC3, ARPC4 and ARPC5 (Figure 4). ARPC1 has two isoforms in humans, ARPC1A and ARPC1B. The Arp2/3 complex induces the polymerization of branched actin filaments in multiple subcellular compartments at precise 70° angles from a mother filament (Amann & Pollard 2001). The Arp2/3 complex resides on endosomes and endocytic vesicles and it has been implicated in the formation of vesicles, participating in actin polymerization that occurs at the neck of a forming vesicle (Qualmann et al 2000). This is an attractive mechanism as our laboratory has found that the BLOC-1 complex resides on endosomal vesicles as well as endosomes (Salazar et al. 2006; Newell-Litwa et al. 2009). As mentioned above quantitative mass spectrometry of BLOC-1 down-regulated cells identified a decrease in the levels of ARPC1a, ARPC2, ARPC5, ACTR3.

The function of the Arp/3 complex at the synapse

Prior studies in mammals have reported that Arp2/3 plays an important role in post-synaptic morphology and synaptogenesis (Wegner et al. 2008; Kim et al 2013), processes which are known to be impaired in psychiatric disorders like schizophrenia (Fiala et al. 2002). Mouse knock-out Arp2/3 models in Kim *et al* demonstrate loss of

spine synapses and abnormal spine morphology, resulting in abnormal social behaviors. These results suggest the importance of Arp2/3 and the actin cytoskeleton in post-synaptic phenotypes. However they leave open the question of whether Arp2/3 mechanisms modulate the function and morphology of presynaptic terminals.

Central Hypothesis

In this thesis I postulate that the BLOC-1 complex and the Arp2/3 complex participate in the same molecular pathway. I will test this hypothesis using *Drosophila melanogaster* neuromuscular synapse. I focus on this synapse because our laboratory has demonstrated that loss-of-function alleles in BLOC-1 subunits alter the synapse morphology by increasing the complexity of branches and number of synapses (boutons). This is a reliable assay that will allow me to test the central hypothesis of this proposal that:

BLOC-1 and Arp2/3 act in the same molecular pathway to regulate synaptic morphology.

From this hypothesis I predict:

1) BLOC-1 synaptic morphological phenotypes should be similarly observed in Arp 2/3 subunit mutants.
2) Combined BLOC-1 and Arp2/3 subunit mutants should have phenotypes similar to single gene deficiencies if both complexes participate on the same molecular pathway.

Foundational Results

A post-doctoral fellow in our lab, Dr. Avanti Gokhale, conducted a proteomic analysis of BLOC-1 down-regulated neuroblastoma cells to identify factors sensitive to BLOC-1 cellular levels. Stable isotopic labeling by amino acids in cell culture (SILAC) was employed to detect changes in protein levels in response to BLOC-1 down-regulation (Figure 5). Human neuroblastoma cells were grown to equilibrium in isotopically-labeled media, infected with either lentiviruses carrying shRNA against the muted and pallidin subunits of the BLOC-1 complex or control shRNA. Protein content was quantified by tandem mass spectrometry and compared to cells infected with control shRNA (Figure 5). Using this method, Dr. Gokhale profiled over 4,000 proteins sensitive to BLOC-1 down-regulation. We summarized these results from the SILAC proteomic analysis with a gene ontology bioinformatics studies in Figure 6. These results revealed that BLOC-1 sensitive proteins participate in a wide array of cellular functions (Figure 6) but have in common actin cytoskeletal factors and synaptic proteins sensitive to BLOC-1 deficiency (Figure 7).

The SILAC mass spectrometry data identified a decrease in the levels of the Arp2/3 subunits Arpc1a, Arpc2, Arpc5, Actr3, as well as the actin nucleation-promoting factor (NPF) Annexin II. Dr. Gokhale confirmed the down-regulation of Arp2/3 by western blot analysis in human neuroectodermal cells, demonstrating that Arp2/3 shows a near 50% reduction in the absence of BLOC-1 (Figure 8). Down-regulation of Arp2/3 was reproduced in multiple BLOC-1 deficient cell types, suggesting a ubiquitous phenotype. Our laboratory previously identified the WASH complex an actin nucleation factor that associates with the BLOC-1 complex (Ryder et al 2013), which is involved in

the recruitment of the Arp2/3 complex to endosomes. My advisor, Dr. Victor Faundez, used bioinformatic tools to predict putative yeast two hybrid and genetic interactions from human gene databases (Genemania.org). As shown in Figure 8B the BLOC-1, WASH and Arp2/3 complexes are predicted to undergo biochemical association as well as to participate in similar genetic pathways. These data collectively support the hypothesis that the BLOC-1 complex and the Arp2/3 complex participate on a common pathway.

Since the BLOC-1 and Arp2/3 complex reside in endosomes, we hypothesized that reduced levels of Arp2/3 secondary to decreases in the BLOC-1 content should impair actin polymerization in endosomes. We tested this hypothesis in collaboration with Dr. Manoj Puthenveedu (Carnegie Mellon University) using fluorescence recovery after photobleaching (FRAP) of actin filaments using the probe LifeAct GFP. LifeAct GFP is a genetically encoded probe that selectively binds to polymeric actin (Riedl et al. 2008). To selectively identify actin filaments in endosomes with LifeAct GFP Dr. Puthenveedu's group marked endosomes with internalized beta-adrenergic receptor (Figure 9A). FRAP experiments demonstrated that LifeAct GFP fluorescence recovery after photobleaching was decreased in endosomes from BLOC-1 down-regulated cells (Figure 9B). We interpret these observations as to indicate that actin at endosomes is impaired in BLOC-1 deficient cells.

Methods and Results of this Honor Thesis

Methods

Drosophila Stocks

Fly stocks were bred and maintained at 25°C. Flies containing the *Arp1* null allele were obtained from Bloomington Stock Center (Stock ID number: 11424). Hypomorph $Dysb^{1}$ flies were a gift from collaborator Graeme Davis while $Blos1^{ex2}$ null animals were acquired from Esteban Dell'Angelica. Appropriate chromosome balancers were used.

Immunohistochemistry and confocal microscopy

Immunofluorescence confocal microscopy was used to visualize synaptic morphology of approximately 10-15 animals per genotype. The neuromuscular junction (NMJ) of third instar female larvae were dissected in HL3 and fixed in 4% paraformaldehyde for 45 minutes. After a 10 minute wash in 1x phosphate buffer saline + .01% Triton X-100 (PBS-T), NMJs were stained overnight at 5°C with HRP-FITC and Phalloidin on shaker. Dissections were washed on shaker three times for 1 minute, then washed three times for 10 minutes in PBS-T the following day. For the final washes 1x phosphate buffer saline was used three times for 1 minute. NMJs were mounted on uncharged slides with mounting medium and dried overnight. Motoneurons innervating muscles 6/7 of either segments A2 or A3 were imaged (Figure 10). Confocal images were obtained by an inverted 510 Zeiss LSM microscope.

Quantification

LSM images were stacked in ImageJ. Relative muscle area size was measured in pixels using the freehand line selection tool. Bouton number was quantified using the count tool in Adobe Photoshop.

Statistical Analysis

Experimental conditions were compared using Synergy Kaleida-Graph, version 4.1.3 (Reading, PA), or StatPlus Mac Built5.6.0pre/Universal (AnalystSoft, Vancouver, Canada)

Results

My hypothesis predicts that BLOC-1 synaptic morphological phenotypes should be similarly observed in Arp 2/3 subunit mutants. Additionally I predict that combined BLOC-1 and Arp2/3 subunit mutants should have phenotypes similar to single gene deficiencies if both complexes participate on the same molecular pathway. However, in the final stages of the preparation of this written thesis document I discovered that the mutant allele used in my studies is a null mutation in the Arp1 gene, a subunit of the dynactin complex, rather than a null allele of the Arpc1 gene, which is the subunit of the Arp2/3 complex. Therefore, instead of testing the hypothesis described in the introduction of this thesis I tested the hypothesis that the BLOC-1 complex participates in a similar pathway with the dynactin complex. The dynactin complex is required for the motor activity of most cytoplasmic dyneins and has been shown to interact with the BLOC-1 complex (Mead et al. 2010). In the following sections I will describe my findings and discuss the implications of my results with the dynactin subunit Arp1 for the biology of the BLOC-1 complex.

Thus, my revised thesis hypothesis predicts that BLOC-1 synaptic morphological phenotypes should be similarly observed in dynactin subunit mutants. Additionally, I predict that combined BLOC-1 and dynactin subunit mutants should have phenotypes similar to single gene deficiencies if both complexes participate on the same molecular pathway.

I tested the hypothesis that BLOC-1 and the dynactin complex participate in the same pathway staining *Drosophila* larval motoneurons located at muscle 6/7 of segments A2 or A3 with HRP- FITC, a neuronal specific marker, and quantified bouton number

(Figure 10). Boutons correspond to individual synapse units with the muscle and its number and morphology are correlated with alterations in neurotransmission (Mullin et al. 2015). In the case of the BLOC-1 mutant allele *Blos1*, we previously observed an increase in the number of boutons. I confirmed findings previously reported by us in Mullin *et al,* demonstrating that homozygous and heterozygous *Blos1* mutants display a significantly greater number of boutons compared to the w1118 wild-type animals. Additionally, heterozygous $Dysb^{1}$ hypomorphs also exhibit the same bouton count phenotype confirming that BLOC-1 regulates synaptic morphology. Animals with a single copy loss-of-function dynactin complex subunit Arp1 (*Arp1)* also demonstrated a significant increase in bouton number, which was of the same magnitude as BLOC-1 mutant animals (Figure 11H). I could not generate double *Arp1* null mutants as they are early embryonic lethal. My findings that BLOC-1 and dynactin complex deficiencies generate similar phenotypes support the hypothesis that these two complexes participate in the same molecular mechanism.

I then tested the second prediction creating mutants that carry mutations in both BLOC-1 subunits and dynactin complex subunits. Because single gene copy loss of BLOC-1 and dynactin complexes were enough to produce phenotypes, I generated double mutants carrying a single copy of the mutant *Arp1* in *trans* with either a single copy loss of the BLOC-1 mutant alleles $Dysb^{1}$ or $Blos1^{ex2}$. I observed an increased number of boutons in double mutants. This increase was of the same magnitude as the increase observed in single mutants (Figure 11H). Statistical analysis among different genotypes indicated that although they all have a significant increase as compared with wild type (w1118) there were no differences among any of the mutant animals (Figure

11J). These effects of BLOC-1 and Arp1 mutations on the presynaptic terminal are unlikely to result from changes in muscle area induced by these mutations. I infer this as the small changes in muscle size (reductions of less than 10%) did not follow the pattern of increase in the bouton number in these mutants (Figure 11I).

These results indicate that genetic defects in BLOC-1 and dynactin complex produce similar bouton phenotypes and that combination of these mutations in the same animal do not increase or attenuate this morphological phenotype. We conclude that *Drosophila* BLOC-1 and the dynactin subunit Arp1 act on the same pathway to regulate the morphology of the presynaptic terminal at the neuromuscular junction.

Discussion

Here, we sought to test whether BLOC-1, a complex identified as a schizophrenia risk factor, acts in the same molecular pathway as the actin cytoskeletal nucleator Arp2/3 to regulate synaptic morphology in *Drosophila*. However, the mutant allele used in these studies did not allow us to test the original hypothesis. We used mutants in the Arp1 gene rather than the Arpc1 gene. The former is a subunit of the dynactin complex, a protein complex identified as a cytosolic activity required for cytoplasmic dynein to drive long-range movements of membrane vesicles on microtubules in vitro (Schroer & Sheetz 1991) (Figure 12). Dynactin acts as a bridge or adaptor between cargoes such as membrane bound organelles and the minus directed motor dynein (Schroer & Sheetz 1991). Among the cargoes that dynactin binds are endosomes which is the organelle where the BLOC-1 complex primarily resides and where it participated in membrane protein sorting into vesicles.

Although we did not address the original hypothesis that BLOC-1 participates in the same molecular pathway as the actin cytoskeletal nucleator Arp2/3, my results offer novel insight into possible mechanisms whereby BLOC-1 and the dynactin complex reside in the same molecular pathway. These accidental findings are consistent with the identification of cytosolic dynein subunits and dynamitin by mass spectrometry in BLOC-1 and AP-3 coated vesicles reported by our laboratory previously (Salazar et al. 2005; Salazar et al. 2009). Although the identification of cytosolic dynein and dynamitin were not confirmed by methods independent of mass spectrometry, my present results offer insight into this potential molecular pathway. In particular it is exciting that dynamitin, also known as p50, is a subunit of the dynactin complex that directly interacts

with Arp1, which we previously found in a complex with BLOC-1 and AP-3 (Salazar et al 2009).

The genetic predictions of the original hypothesis that BLOC-1, belongs to the same molecular pathway as the actin cytoskeletal nucleator Arp2/3 are similarly valid for my findings with the dynactin complex subunit Arp1. My genetic findings argue in favor of a role of dynactin-dynein complex in the function of the BLOC-1 complex. First, a null allele of the dynactin complex subunit Arp1 generates an increase in the number of boutons at the neuromuscular junction. This synaptic phenotype is similar to the increased bouton count observed in two mutants affecting BLOC-1 subunits. Importantly, combined genetic defects in the dynactin subunit Arp1 and the BLOC-1 complex generate phenotypes similar to those generated by a double copy loss in the BLOC-1 complex subunit blos1.

Another interpretation of my results could be that BLOC-1 and the dynactin complex participate in parallel and independent pathways to regulate synaptic morphology. Further studies are necessary to provide further evidence for my main conclusion that these complexes act in the same molecular pathway to regulate synaptic architecture. In the following questions I outline future experiments, which would further support my conclusion and also expand upon the findings of this thesis.

1) Does the BLOC-1 complex interact with dynein-dynactin complexes in the human brain?

   Prior studies have demonstrated that Arp1 and Dynamitin co-immunoprecipitate with Dysbindin in clonal mouse striatal cells (Mead et al. 2010). We should,

therefore, address this question in human neuronal cells by co-immunoprecipitation experiments. An interaction between the BLOC-1 complex and the dynein-dynactin complex would be of great interest as it would suggest that BLOC-1 complexes bound to membranes could serve as an adaptor between the membrane with dynactin and dynein.

2) As previously mentioned down-regulation of BLOC-1 subunits affects the expression of proteins that participate in BLOC-1 dependent mechanisms or associate with the BLOC-1 complex. This raises the question of whether BLOC-1 defects associate with changes in the expression of dynein-dynactin complex subunits. Our results suggest that this may be the case. SILAC analysis of BLOC-1 down-regulated neuroblastoma cells identified upregulation of Cytoplasmic dynein 2 heavy chain 1 and Dynein light chain 2A. Although we have not confirmed these results by complementary approached to SILAC mass spectrometry, it is of interest to explore these changes as well as the content of dynactin complex subunits.
3) Does the BLOC-1 complex or organelles coated with BLOC-1 undergo minus end directed microtubule dependent transport via dynactin-dynein mechanisms? We have identified that the BLOC-1 complex is enriched in nerve terminals in brain synapses (Larimore et al. 2013) suggesting that BLOC-1 coated vesicles undergo anterograde plus directed transport. However, we have no evidence of retrograde transport mechanisms directing BLOC-1 or BLOC-1-positive organelles from the nerve terminal to cell bodies. Our laboratory has *Drosophila* strains that express GFP-tagged BLOC-1 complexes that could be used to test retrograde transport of

BLOC-1. A simple prediction of this hypothesis is that the Arp1 mutants used in this thesis should lead to the accumulation of BLOC-1 complexes in presynaptic terminals at the *Drosophila* neuromuscular junction.

4) By what mechanism do BLOC-1 and the dynactin mutations result in an increase in the number of synapses? From my results we can hypothesize how the endosomal complex BLOC-1 may act in conjunction with the retrograde transport motors (dynein-dynactin complex) to affect synaptic structure. BLOC-1 and dynactin-dynein complexes could interact and regulate the transport of endosomes from the axon terminal to the cell body. However, when BLOC-1 and dynactin function is perturbed, it may result in the accumulation of endosomes at the presynaptic compartment, leading to an impairment in neurotransmission and an increase in the number of synapses as a compensatory effect. We could test this hypothesis using electron microscopy and determine if flies carrying BLOC-1 and dynactin mutations have an accumulation of endosomes at the neuromuscular junction.

5) Finally, if biochemical, functional and genetic evidence were to confirm a role of dynactin-dynein complexes in BLOC-1 dependent mechanisms at the nerve terminal it will suggest a participation of minus end directed motor processes in the pathogenesis of schizophrenia. Point mutants in dynein light intermediate chain 1 alter cortex development in mice and generate behavioral phenotypes some of them observed in schizophrenia and mood disorders (Banks et al. 2011). However, the most interesting case is the gene DISC-1 that when mutated increases the risk of schizophrenia. DISC-1 is a component of the microtubule-

associated dynein motor complex whose genetic defect in mice alters the development of the cerebral cortex (Kamiya et al. 2005).

Summary

This thesis started with a defined question and solid foundational data presented in the introduction. However, accidentally I generated evidence of a previously unexplored connection between the BLOC-1 complex and dynein-dynactin mechanisms. A pathway where BLOC-1 complex and dynein-dynactin participating in a common mechanism need additional experimentation to solidly establish its existence. However, my findings have led us to revise our hypothesis where we have found significant circumstantial evidence that the BLOC-1 complex and dynein-dynactin participate in a common mechanism.

Human subunit	*Drosophila* gene Current name	Proposed name	*E*-value[a]	Amino acid identity[b] (%)
BLOS1	*CG30077*	*blos1*	6×10^{-31} (1)	55
BLOS2	*CG14145*	*blos2*	7×10^{-16} (1)	40
BLOS3	*CG34255*	*blos3*	9×10^{-6} (2)	22
Cappuccino	*CG14149*	*blos4*	2×10^{-4} (1)	25
Dysbindin	*CG6856*	*dysbindin*	6×10^{-15} (1)	38
Muted	*CG34131*	*muted*	2×10^{-7} (2)	18
Pallidin	*CG14133*	*pallidin*	1×10^{-3} (1)	24
Snapin	*snapin*	*snapin*	3×10^{-13} (1)	33

Table 1: Homology of *Drosophila* BLOC-1 genes to humans. Using, PSI-BLAST algorithm homologies between *Drosophila* and human BLOC-1 amino acid sequences were identified. All *Drosophila* BLOC-1 subunits were shown to be 18-55% identical to humans, making the fruit fly an effective model for studying BLOC-1 function. Numbers in the parentheses indicate iteration number. Taken from: Cheli *et al,* Hum Mol Genet, 19:861-78, 2010.

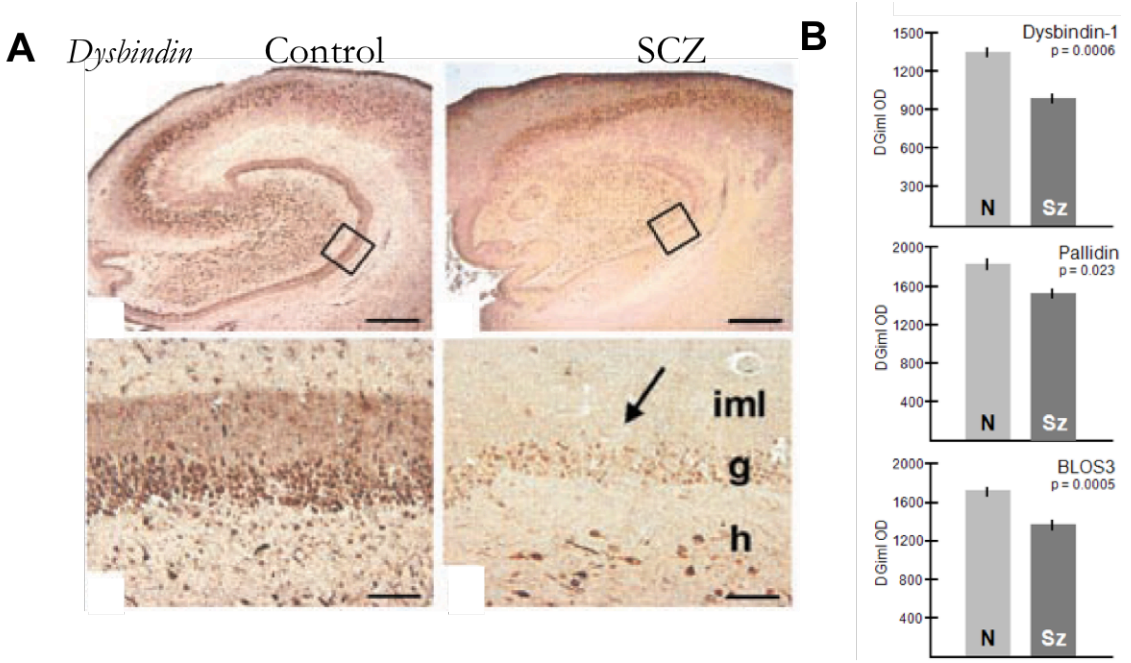


Figure 1: Dysbindin and other BLOC-1 subunits are reduced in the hippocampal formation in postmortem schizophrenia patients. *A* Immunohistochemical staining for dysbindin in the dentate gyrus indicated by the boxes in the top panels. The left panels show dysbindin distribution in control patients, while the right panels show a clear reduction in dysbindin expression. *B* These graphs show the immunoreactivity for three different BLOC-1 subunits. All three subunits are significantly reduced in the dentate gyrus of postmortem schizophrenia patients. Modified from: Talbot *et al*, Journal of Clinical Investigation, 113:1353-63, 2004.

Figure 2: The human BLOC-1 complex. Image illustrates all eight subunits, which comprise the BLOC-1 complex. Many of the human binary interactions between subunits are depicted by the dotted lines and are conserved in both invertebrates and vertebrates. Taken from: Mullin *et al,* Journal of Neuroscience, 35:325-338, 2015.

Figure 3: *Dysbindin* null mice exhibit a reduced pigment phenotype. Compared to the wild type mice, *dysbindin* deficient animals or *sandy* mice show less pigmentation. This is consistent with the fact that patients who carry a loss-of-function *dysbindin* allele are affected with Hermansky-Pudlak syndrome, a disorder characterized by albinism. This phenotype can also be attributed to dysbindin's function in the context of BLOC-1 as molecular studies have shown the complex is involved in the biogenesis of melanosomes.

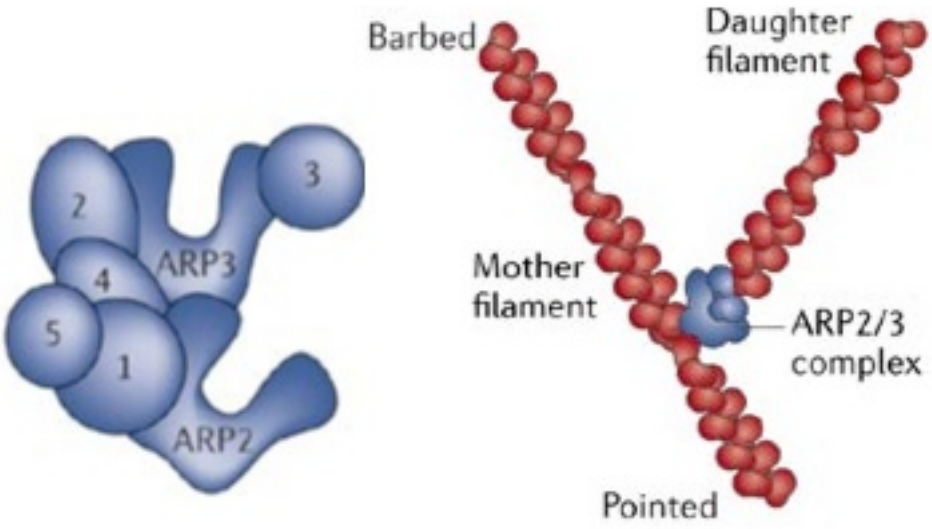


Figure 4: Schematic of the Arp2/3 complex. Shown here is the Arp2/3 complex with subunits: Arp2, Arp3, ARPC1, ARPC2, ARPC3, ARPC4 and ARPC5. Arp2/3 has been shown to play an important role in the polymerization of actin branches from a mother filament. Modified from: Goley & Welch, Nat Rev Mol Cell Biol, 7:713-726, 2006.

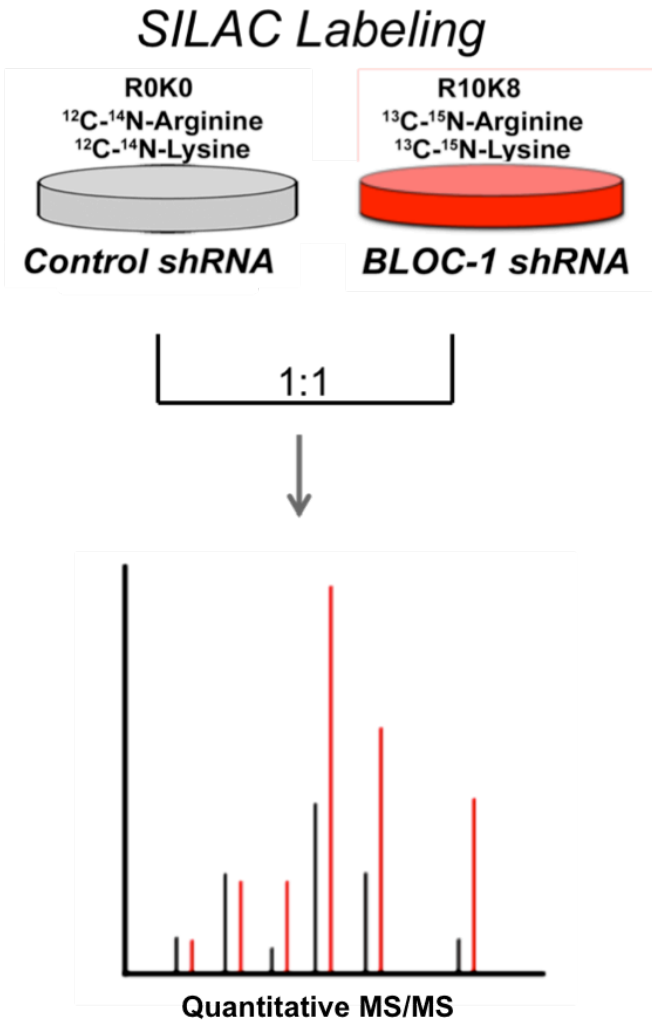


Figure 5: Schematic of a quantitative analysis of the cellular proteome by SILAC (Stable Isotope Labeling of Amino Acids in Cell Culture). SH-SY5Y neuroblastoma cells infected with either control shRNA or shRNA against either the pallidin or muted subunits of the BLOC-1 complex were isotopically labeled with either "light" (R0K0) or "heavy" (R10K8) media respectively followed by cell lysis. Cytoplasmic lysates were then combined at a 1:1 ratio and analyzed by nano LS MS/MS. Peptides that were labeled with the R10K8 amino acids and enriched or down-regulated two-fold or more were considered as preponderant BLOC-1 sensitive factors. Gokhale *et al*, 2015 (unpublished).

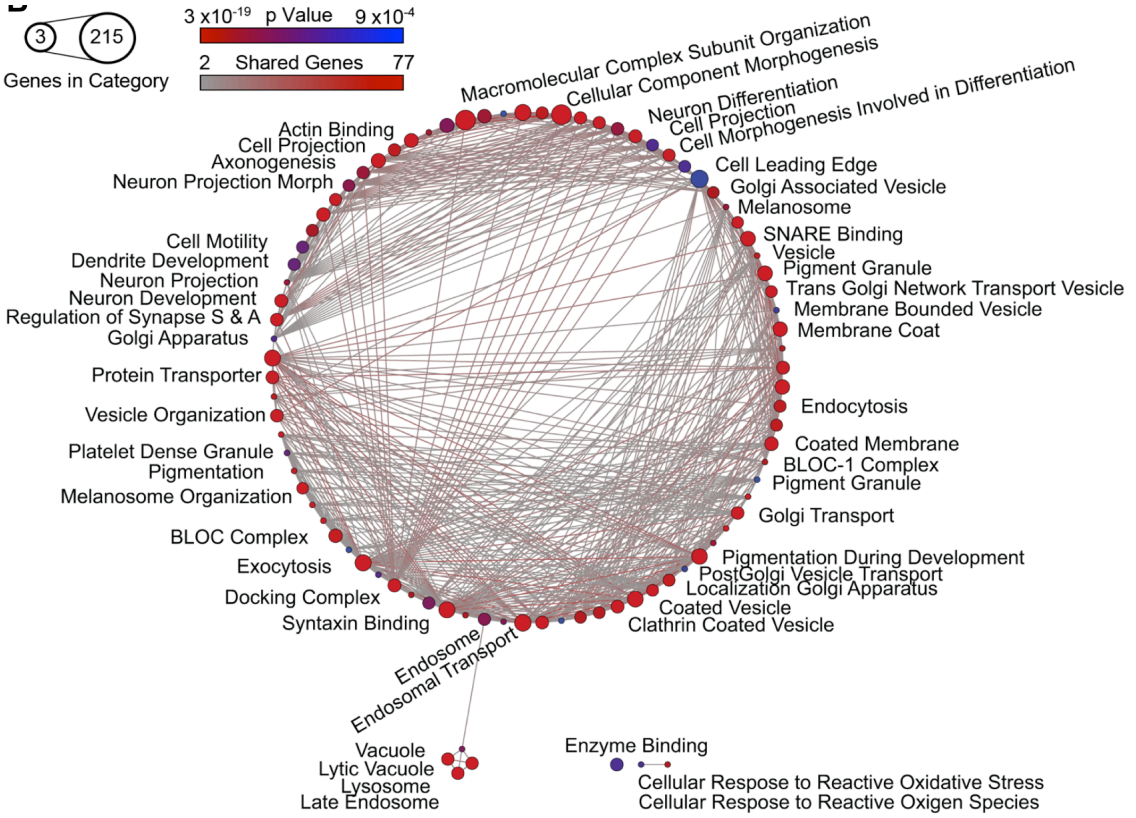


Figure 6: Proteins sensitive to BLOC-1 deficiency are involved in a wide array of cellular functions. The diagram depicts a gene ontology analysis of all proteins whose content is sensitive to BLOC-1 deficiency. Lines depict interactions between genes within each category. Ontology analysis was performed using the DAVID 6.7 engine (http://david.abcc.ncifcrf.gov/) and GO Term relationships were analyzed using Cytoscape. Gokhale *et al*, 2015 (unpublished).

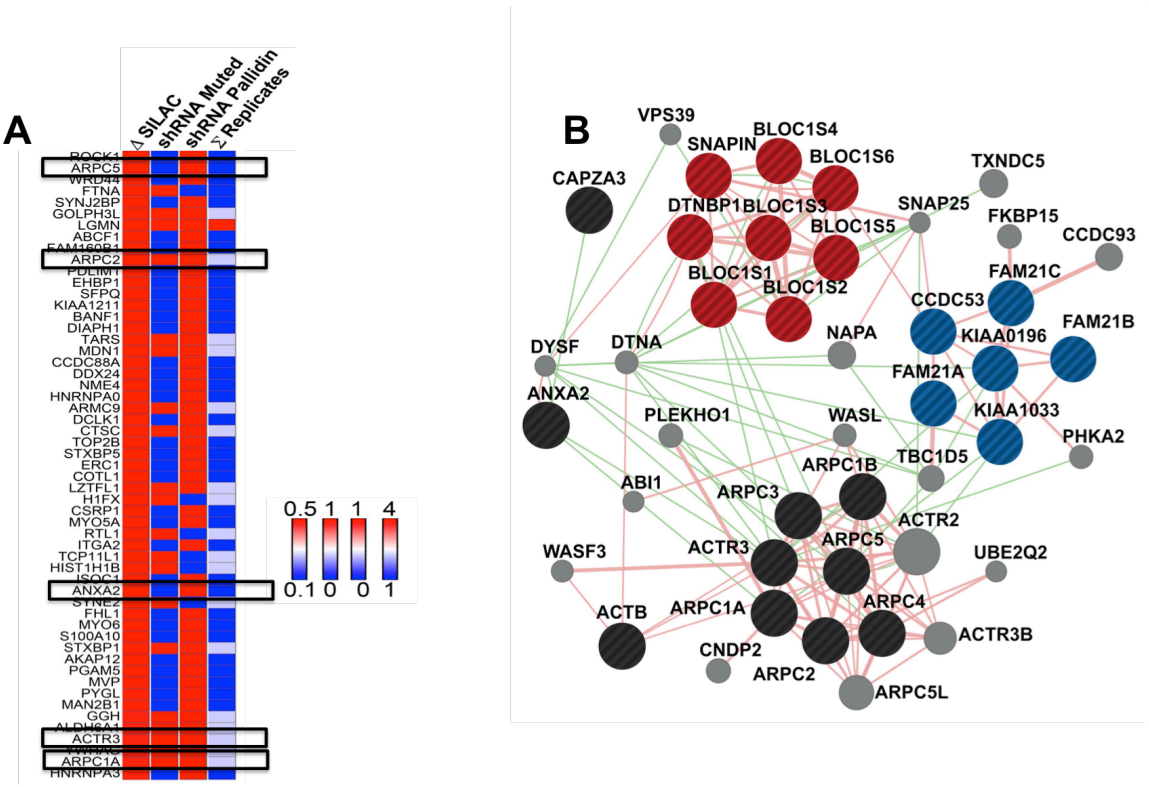


Figure 7: SILAC detects reduced protein levels in proteins related to actin cytoskeletal regulation in response to BLOC-1 perturbation. *A* SILAC detected changes in protein abundance in the absence of BLOC-1. Diagram depicts a heat map representation of fold of change, the shRNA treatment where the protein abundance change was detected and the sum of all independent SILAC experiments were a protein was modified. Highlighted by boxes are proteins that regulate actin dynamics. Subunits from the Arp2/3 complex and nucleation promoting factors such as Annexin II were identified (boxed). *B* In silico network analysis of the BLOC-1 and Arp2/3 interactome components built from Genemania software. We included the WASH complex, an Arp2/3 activator, that we previously demonstrated interacts with the BLOC-1 complex. BLOC-1 subunits (red), WASH subunits (blue) and Arp2/3 subunits (black). The lines represent putative physical protein interactions (pink) and genetic interactions (green) curated from mammalian databases by Genemania. Gokhale *et al*, 2015 (unpublished).

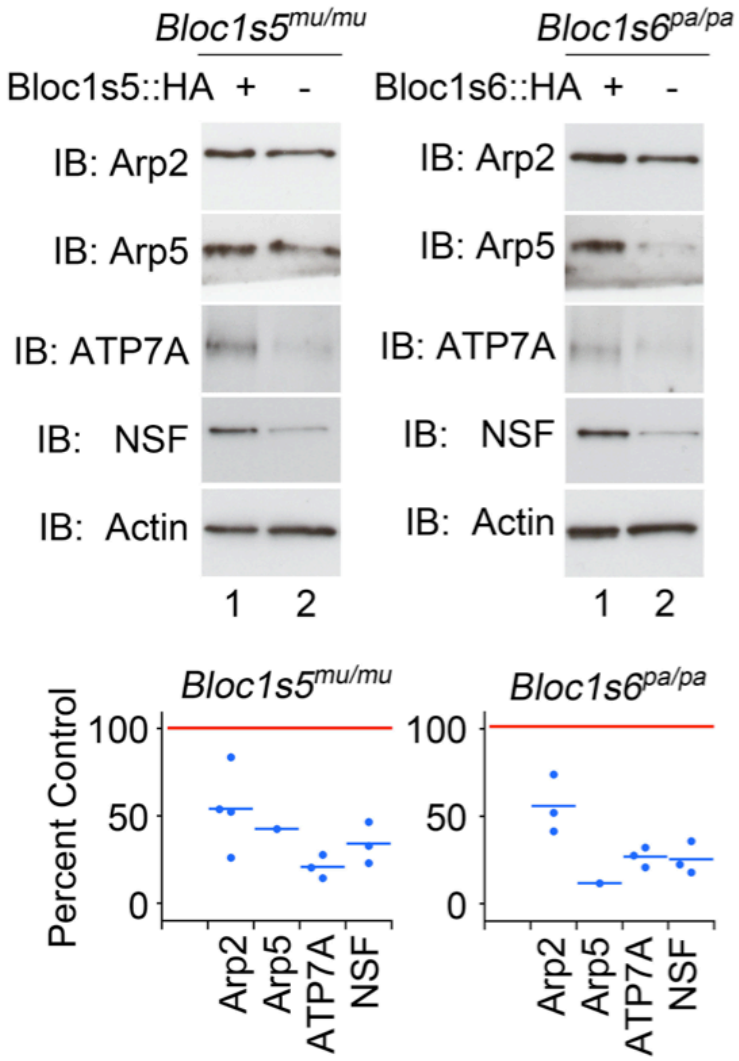


Figure 8: Western blot analysis shows Arp2/3 subunits are down-regulated in pallidin and muted deficient cells. *A* Western blot analysis confirms that Arp2/3 subunits are down-regulated in BLOC-1 deficient cells. Neuroectodermal pigmentary cells from either pallidin or muted mutant cells (lane 2) show a significant reduction in the amount of Arp 2 and 5 compared to the control lysates in lane 1. *B* The blots also confirmed the SILAC results showing the down-regulation of NSF, a presynaptic protein also shown to converge with BLOC-1 at the synapse. The plots depict relative protein content of transfected to wild type cells from the western blots. All plotted antigens are significantly reduced $p < 0.05$ ANOVA followed by Dunnett Comparisons. Western blot experiments were conducted by Avanti Gokhale. Gokhale *et al*, 2015 (unpublished).

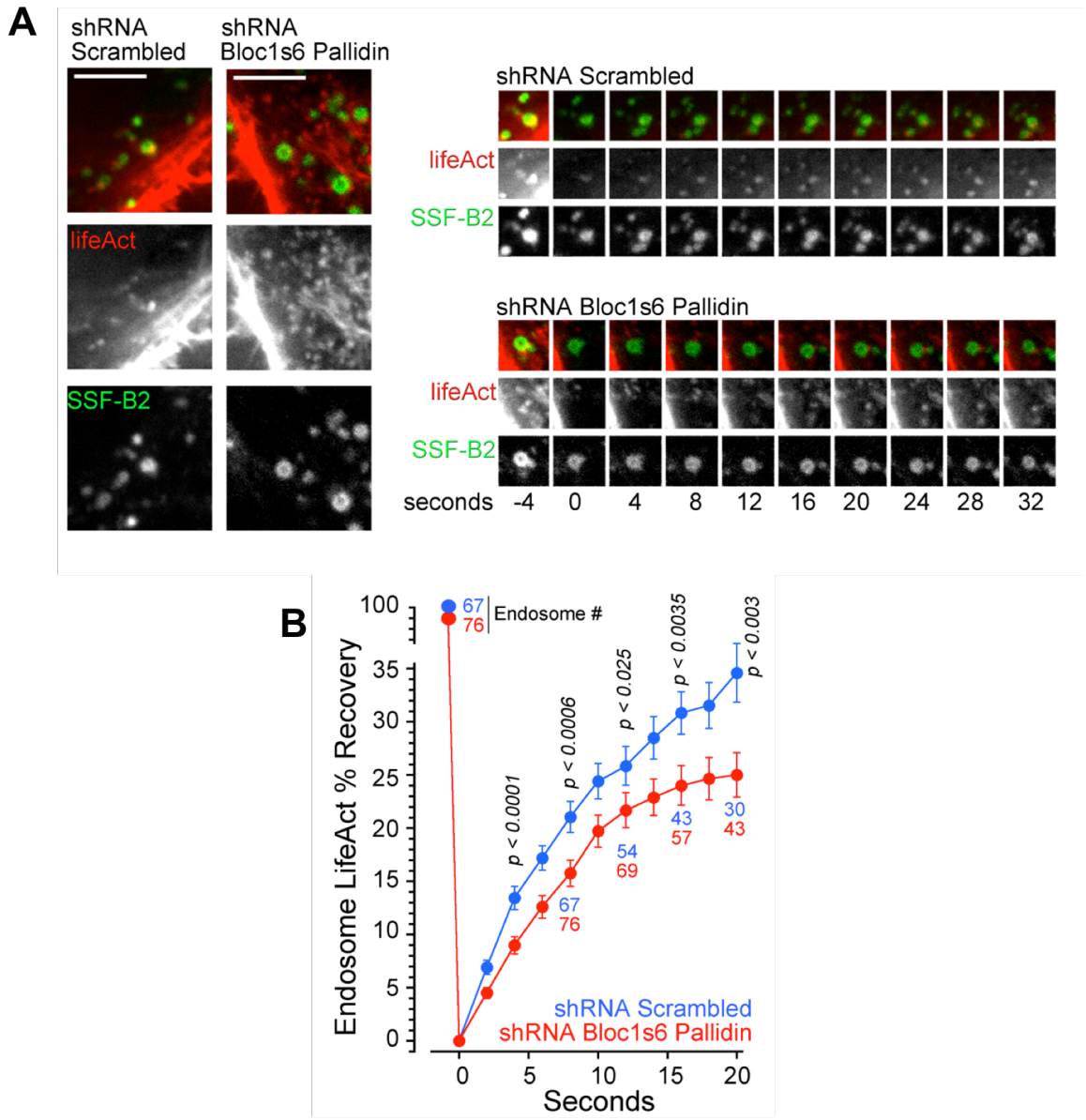


Figure 9: Actin polymerization at endosomes is impaired in the absence of BLOC-1. *A* Using fluorescence recovery after photobleaching of actin filaments, we measured the rate of newly polymerized actin at endosomes. The probe LIfeAct GFP allowed for the visualization of actin filaments present in endosomes detected with internalized beta adrenergic receptor (SSF-B2). Cells were transfected with the shRNA scrambled control or with shRNA pallidin, a BLOC-1 subunit. *B* The graph shows that BLOC-1 deficient cells have a significantly slower rate of fluorescence recovery compared to the scrambled controls. This rate of recovery is indicative of rate of actin polymerization at endosomes. Data collected in collaboration with Manoj Puthenveedu, Carnegie Mellon University. Gokhale *et al*, 2015 (unpublished).

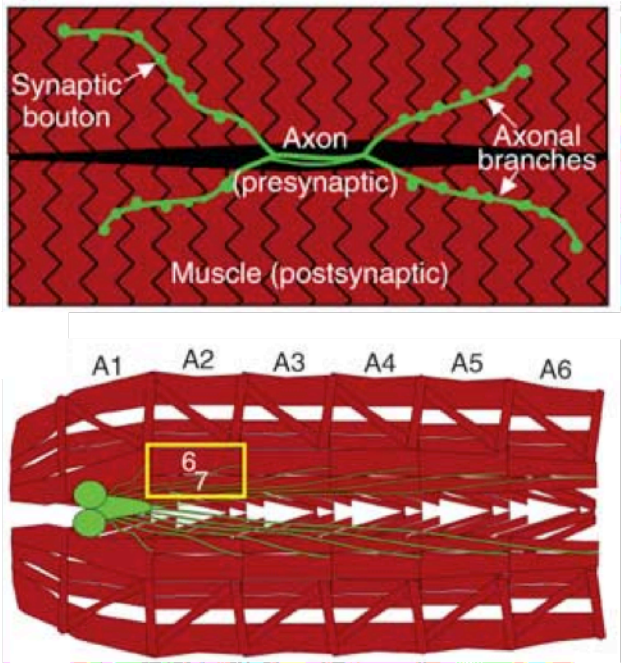
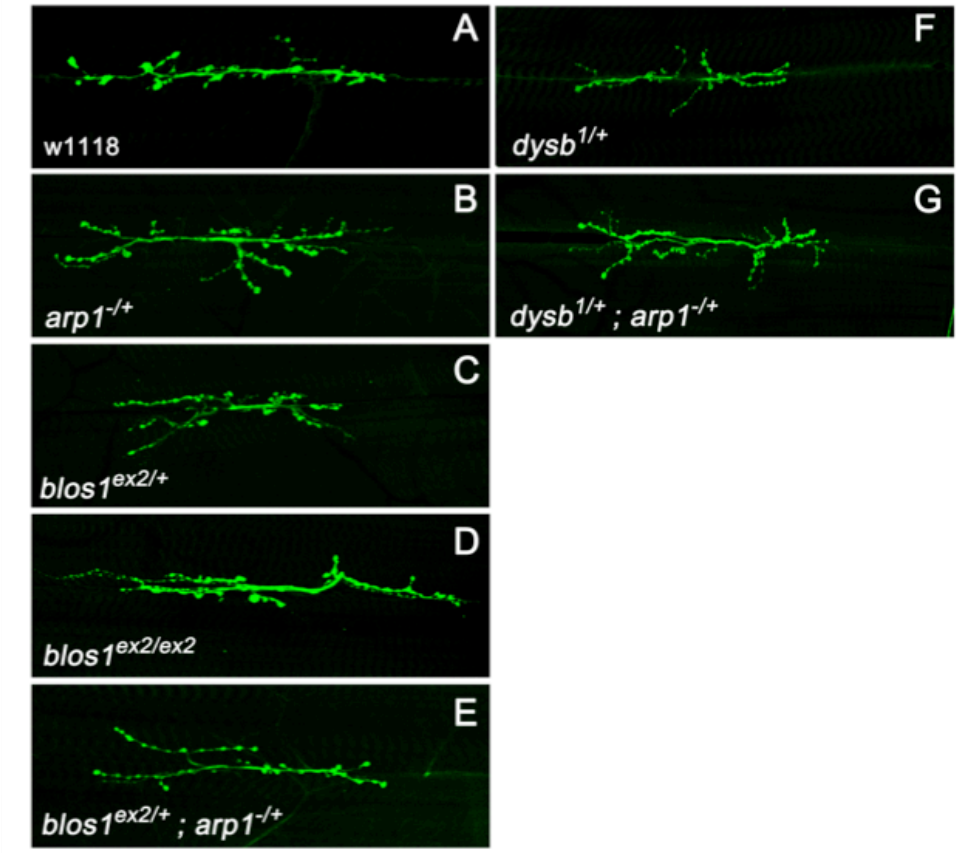
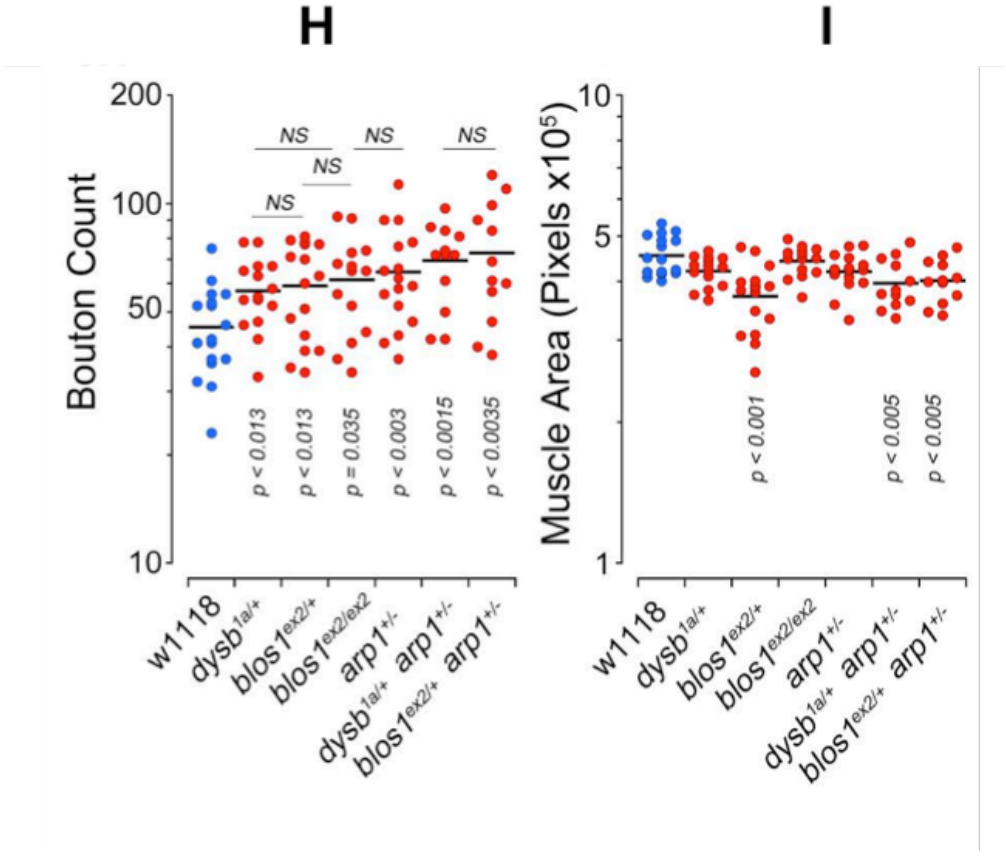


Figure 10: Preparation of the *Drosophila* neuromuscular junction. For this thesis, 3rd instar female larvae were dissected and immunohistochemical stains HRP-FITC (neuronal marker) and Phalloidin (actin) were used. Motoneurons from muscles 6/7 of segments A2 or A3 were imaged. Synaptic bouton number was measured in each animal and used as a proxy for synaptic morphology in our study. Modified from: Loya CM *et al,* Nature Methods, 6:897-903, 2009.


A
w1118
F
dysb1/+
B
arp1-/+
G
dysb1/+ ; arp1-/+
C
blos1ex2/+
D
blos1ex2/ex2
E
blos1ex2/+ ; arp1-/+


H
I
Bouton Count
200
100
50
10
NS
NS
NS
NS
NS
p < 0.013
p < 0.013
p = 0.035
p < 0.003
p < 0.0015
p < 0.0035
Muscle Area (Pixels x10^5)
10
5
1
p < 0.001
p < 0.005
p < 0.005
w1118
dysb1a/+
blos1ex2/+
blos1ex2/ex2
arp1+/-
dysb1a/+ arp1+/-
blos1ex2/+ arp1+/-

J

	w1118_ dysb	w1118 _blos1	blos1_ blos1	w1118_ arp1	arp1_ dysb	arp1_ blos1
w1118	0.01259	0.035	0.01799	0.00292	0.001406	0.003452
w1118_dysb		0.7516	0.5406	0.4403	0.06682	0.4162
w1118_blos1			0.9007	0.6107	0.1251	0.2272
blos1_blos1				0.368	0.2363	0.368
w1118_arp1					0.4162	0.4859
arp1_dysb						1
arp1_blos1						

Figure 11: BLOC-1 and the dynactin complex act in the same pathway to regulate synaptic morphology. *A-G* Images depicting motoneurons innervating muscle 6/7 stained with HRP for indicated genotypes. *H* Bouton count number was compared across all genotypes. Compared to w1118 wild-type controls there are significant increases in bouton number in animals with a single copy loss of *Dysbindin* ($Dysb^{1/+}$), the dynactin complex *Arp1* subunit ($Arp1^{-/+}$) and *Dysb-Arp1* transheterozygotes ($Arp1^{-/+}$ $Dysb^{1/+}$). The same bouton count phenotype is also observed in animals homozygous ($Blos1^{ex2/ex2}$) and heterozygous ($Blos1^{ex2/+}$), for *Blos1*, and *Blos1-Arp1* transheterozygotes ($Arp1^{-/+}$; $Blos1^{ex2/+}$). *H* Select genotypes demonstrate small (<10%) but statistically significant differences in muscle area. These differences were not consistent with the excess bouton phenotype shown across all genotypes. *J* Excessive bouton count phenotype is statistically significant in all genotypes compared to wild-type (w1118). The phenotype is neither significantly attenuated nor exacerbated across animals carrying different mutations. Kruskal-Wallis Test was performed followed by pairwise comparisons with Wilcoxon-Mann-Whitney Test.

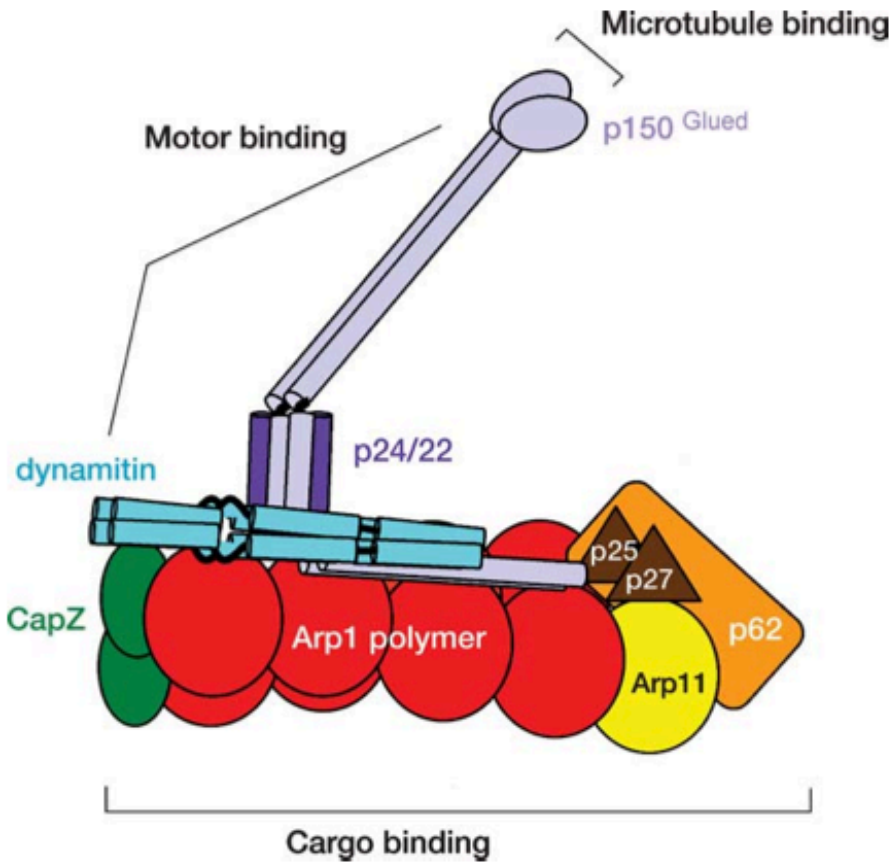


Figure 12: Schematic of the dynactin complex. Subunits of the dynactin complex include: $p150^{Glued}$, p62, Dynamitin (p50), Arp11, Arp1, B-Actin, CapZ, p24, p27 and p25. The dynactin complex acts as a linker between retrograde motor protein, dynein and its cargo. The Arp1 subunit (red) is an actin-like protein, which acts as the "backbone" of the dynactin complex, binding to cargo. Taken from: Schroer, Annu. Rev Cell Dev Biol, 20:759-79, 2004.

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