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Annie Shen March 10, 2023

Muscleblind Like Splicing Regulator 1 (MBNL1) Associates with Endosomes in Mouse Neuroblastoma Cells

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Abstract

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Myotonic Dystrophy Type 1 (DM1) is a genetic disorder that affects multiple organ systems, including the central nervous system, and causes symptoms such as cognitive difficulties and hypersomnia. Muscleblind-like (MBNL) proteins are a family of RNA-binding proteins (RBPs) that contain zinc-finger domains. In DM1, MBNL proteins are sequestered in the nucleus by CUG trinucleotide repeats. It is unclear how the sequestration of MBNLs contributes to DM1. RBPs participate in RNA localization, associating with motor proteins and/or endomembranes during transport. Recent findings have shown that RNA is able to be transported with motile endosomes, leading to the possibility that Muscleblind Like Splicing Regulator 1 (MBNL1) transports mRNA by interacting with the endomembrane system. This thesis investigates MBNL1 trafficking within the endomembrane system. I hypothesized that MBNL1 associates with varying degrees with different kinds of endosomes. Here, we show that MBNL1 colocalized with early, late, recycling endosomes, and exosomes/lysosomes. Using Rab5, Neep21, and EEA1 antibodies that correspond to early endosomes, I demonstrated that MBNL1 colocalized to a greater degree to early endosomes compared to late endosomes, recycling endosomes, and lysosomes. From these findings, I explored how Rab5 function affects MBNL1 mobility and trafficking in the endomembrane system. I showed that MBNL1 exhibits a trend towards more

colocalization with more in constitutively active Rab5 than wild-type Rab5. Live imaging showed a trend towards greater MBNL1 movement in both constitutively active Rab5 and dominant negative Rab5 as compared to wild-type Rab5. Results show endosomes, especially early endosomes, are involved in MBNL1 trafficking and anchoring. This thesis motivates further research using live cell imaging to study if the mobility of MBNL1 is disturbed when early endosome mobility is disturbed. More research is needed to understand the underlying mechanisms of MBNL1 transportation and anchoring to regulate mRNA localization and translation.

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I. Introduction

Myotonic Dystrophy

Myotonic dystrophy (DM) is the leading form of muscular dystrophy affecting adults and is an autosomal dominant neuromuscular disease (Brook et al., 1992). There are two types of Myotonic Dystrophy: Myotonic Dystrophy Type 1 (DM1) and Myotonic Dystrophy Type 2 (DM2). DM1 is a multisystemic disease with symptoms affecting skeletal muscle, cardiac muscle, the central nervous system, the gastrointestinal tract, and the endocrine system. Symptoms include skeletal muscle weakness, wasting, and pain, as well as myotonia. Cardiac arrhythmias, cataracts, insulin resistance, and neurological problems may also occur (Holt et al., 2008). In DM1, patients have an expanded CTG triplet repeat in the 3' non-coding region of the gene encoding the DM protein kinase (DMPK). The expanded repeats fold into stable RNA hairpin structures, toxic RNAs, that can sequester muscleblind-like (MBNL) proteins and other nuclear factors in the form of ribonuclear foci in the nucleus (Konieczny et al., 2014). In unaffected patients, the number of triplets varies between 5 and 37, but in DM1 patients, there can be up to several thousand repeat units (Yum et al., 2017).

Many molecular mechanisms are proposed to cause DM1 symptoms, including titration of muscleblind-like (MBNL) RNA-binding proteins (RBPs) by expanded CUG repeats (Todd and Paulson, 2010), microRNA dysregulation, and elevated levels of CUGBP/ETR-like factors due to hyperactivated protein kinase C (Kuyumcu-Martinex et al., 2007). The best-characterized pathway is the sequestration of MBNL proteins, containing zinc-fingers, by RNAs that have the CUG repeats. The depletion of MBNL causes changes in alternative splicing (Ravel-Chapuis et al., 2004), alternative polyadenylation (Batra et al., 2014), RNA localization (Wang et al., 2012),

and microRNA processing. MBNL splicing targets are affected differently in DM1, and patients show a broad range of alternative exon inclusion levels (Jog et al., 2012). Thus, the extent of MBNL target mis-splicing correlates with muscle strength and is the foundation of developing biomarkers of disease severity.

Role of Muscleblind-like protein (MBNL) in DM1

Muscleblind-like protein is an RNA-binding protein that regulates metabolism and is encoded by the MBNL gene. There are three paralogs: Muscleblind Like Splicing Regulator 1 (MBNL1), Muscleblind Like Splicing Regulator 2 (MBNL2), and Muscleblind Like Splicing Regulator 3 (MBNL3). All three paralogs have four zinc-finger domains that are essential for recognizing pre-mRNA and mRNA targets. MBNL proteins are highly conserved and contribute to muscle, cardiac, and neuron differentiation. Increased concentrations of MBNL1 and MBNL2 during differentiation promote differentiation of embryonic stem cells as well as induce a shift from fetal to adult splice pattern of mRNAs. Downregulated MBNL leads to adult-to-fetal splicing transitions (Du et al., 2010, Edge et al., 2008). MBNL plays an important role in tissue-specific alternative splicing regulation, and sequestering of MBNL to toxic RNAs can lead to mis-splicing of muscle chloride channels, insulin receptor, bridging integrator 1, and calcium channel voltage-dependent L type alpha 1S subunit. This can result in myotonia, insulin resistance, and muscle weakness (Konieczny et al., 2014).

The MBNL1 gene contains 12 exons, 10 of which encode protein. The zinc fingers are encoded by exons 2-6. The C-terminal of MBNL1 leads to multiple functionally distinct protein isoforms (Tran et al., 2011). There are multiple MBNL1 isoforms. For the purpose of this thesis, I have overexpressed MBNL1 isoform 41, which contains one nuclear signal (NLS) and shuttles

between the nucleus and the distal processes. MBNL proteins bind to YGCY motifs, especially UGCU (Du et al., 2010). MBNL typically binds to the introns upstream or downstream of an alternative exon to prompt exclusion or inclusion (Kalsotra et al., 2008). In the cytoplasm, MBNL binds to the 3' UTR of target mRNAs, affecting mRNA localization and stability (Llamusi et al., 2012). In DM1, the 3' CTG repeats form CUG RNA stem-loops that bind and sequester MBNL1. MBNL1 interacts with RNA through its four CHHC Zinc fingers. The zinc fingers fold into two compact domains and bind single-stranded RNA (Teplova et al., 2008).

Though the role of MBNLs in regulating alternative splicing has been well established, studies have also shown a role for MBNLs in regulating mRNA localization and translation. It has been shown that nuclear MBNLs bind introns or exons to activate or repress splicing. Cellular MBNLs bind 3' UTRs and facilitate transport from the cytoskeleton to the rough ER or other membranes (Wang et al., 2012). MBNL2 was shown to interact with the 3'-UTR of Integrin α3 and carry it to the plasma membrane for local translation (Adereth et al., 2005). Through genomics and biochemical approaches, previous studies showed that MBNL was a global regulator of RNA localization and membrane-associated translation. In previous conventional assays, the C-terminal region of MBNL1 was inactive but remained active in tethered assays where the MS2 coat protein recruited it to target RNAs (Edge et al., 2013). The C-terminal region is able to mediate dimerization, leading to the idea that the C-terminal may allow for it to interact with intact endogenous MBNL proteins, promoting their recruitment to RNA. Recent studies showed that the C-terminal tail of MBNL1 mediated membrane anchoring with specific motor proteins (Hildebrandt et al., in review). However, RNA localization regulation through MBNLs in DM1 has not been explored, and it is unclear whether regulation is achieved through diffusion/anchoring or active transport along cytoskeletal filaments.

RNA Binding Proteins

Local translation, a highly conserved process, allows for spatial and temporal control of the proteome at a subcellular level (Martin and Ephrussi, 2009). Several examples of mRNA localization involve transcripts whose proteins play specialized roles. In yeast, mRNA encoding for ASH1 to inhibit mating-type switching is transported to the budding tip (Paquin and Chartrand, 2008). In brain development, mRNA localization in axonal growth cones allows neurons to respond to their environmental cues at axonal processes (Lin and Holt, 2007). Hundreds of mRNAs are present in dendrites and axons, where translation occurs and they encode a plethora of functionalities (Martin and Zukin, 2006). Localized translation is important because it allows for gene expression to be restricted in the cytoplasm. Local stimuli can regulate translation. Localized translation is beneficial since there is no longer a need for a signal to be delivered to the nucleus to initiate transcription, followed by mRNA export and translation, and subsequent targeting of the protein at the site of stimulation. In addition, localized mRNAs can be translated repeatedly to produce multiple copies of a protein. This is more efficient than translating mRNA near the nucleus and transporting each protein to the distal parts of the cell (Martin and Ephirussi, 2009).

In highly polarized and differentiated cells, such as multinucleated skeletal muscle cells and neurons, mRNA localization and translation are essential to influence cell function (Donlin-Asp et al., 2017). RNA binding proteins (RBPs) bind to RNA in cells and form ribonucleoprotein complexes. Specifically, in neurons, RBPs deliver mRNA to distal parts of neurons, such as the dendrites and axons, to be translated locally. RBPs contain different structural motifs including RNA recognition motif, dsRNA binding domain, RGG box, and zinc

fingers (Lunde et al., 2007). Zinc fingers are classical DNA-binding proteins that are also able to bind RNA. They are typically classified based on the residues used to coordinate zinc: Cys2His2 (C2H2), CCCH, and CCHC are typically present in multiple repeats in a protein (Lunde et al., 2007). Most CCCH zinc finger proteins act as regulators of RNA metabolism, including mRNA splicing, polyadenylation, export, translation, and decay (Fu and Blackshear, 2017). MBNL homologs contain two distantly spaced pairs of CCCH zinc fingers that play a large role in RNA metabolism.

Endosomal Trafficking

Endocytosis is the process in which cells internalize macromolecules and surface proteins. After molecules enter the cell, different endocytic pathways traffic the molecules through endosomes. The endosomal network is dynamic and interconnected, allowing for vectorial trafficking and transfer of cargo between membrane-bound compartments. Endosomes can recycle the internalized macromolecules and surface proteins back to the plasma membrane, delivered to lysosomes for degradation, or transported to their targets (Houtari, 2011). Endosomes can go through maturation from early endosomes to late endosomes which are done through decreasing pH and differential recruitment of Rab-family GTPases (Elkin et al., 2016).

For proteins to be translated locally, RNA must be transported long distances to the distal parts of the cell. For organelles that are membrane-bound, such as mitochondria and endosomes, microtubule-based motors kinesin and dynein interact directly or indirectly with membrane proteins to enable long-range transport. However, RNAs typically do not exist in membrane-enclosed structures, but rather interact with RBPs to form RNA granules that traffic to neuronal axons and dendrites (Gopal et al., 2017). Recently, studies have shown that not all

cargos directly interact with motor proteins for transport. Some cargos are indirectly transported along microtubule networks by docking onto other membrane-bound organelles, such as endosomes in a process called “hitchhiking” (Guimaraes et al., 2015). Endosome hitchhiking is the most common mechanism by which peroxisomes, lipid droplets, and ER travel to distal parts of the cell. In filamentous fungi, RNA granules have also been shown to hitchhike on moving endosomes, leading to the hypothesis that this mechanism occurs in higher-order organisms as well (Pohlmann et al., 2015). Recently, research has shown that RNA granules can hitchhike on lysosomes for long-distance trafficking in mammalian cells using ANXA11 as a tether that couples RNA granules and lysosomes (Liao et al., 2019). Additionally, previous studies have demonstrated that ribonucleoprotein complexes are present on mobile Rab7 endosomes along axons, supporting a model in which Rab7 endosomes are sites for mRNA translation in axons (Cioni et al., 2019). These endosomes frequently attach to mitochondria where they act as hubs for new protein synthesis.

Recent findings show that MBNLs are involved in regulating RNA localization and translation. Cellular MBNLs bind 3’ UTRs and facilitate transport from the cytoskeleton to the rough ER or other membranes (Wang et al., 2012, Hildebrandt et al., in review). It is hypothesized that MBNL transports RNA or participates in targeting them to translation sites by interacting with endomembranes through its long unstructured domains. However, the molecular mechanism of MBNL involvement in RNA transport in neurons has not been studied.

Rab GTPases

The Rab family of GTPases regulates intracellular membrane trafficking through biogenesis, transport, tethering, and fusion of membrane-bound organelles and vesicles.

Typically, Rab genes encode for a GTPase of 200-250 amino acids and approximately 60 Rab genes have been identified in mammals (Klopper et al., 2012). When activated, Rabs localize to specific membranes of different compartments, such as the endoplasmic reticulum, Golgi apparatus, secretory vesicles, endosomes, or lysosomes. There, they recruit effector proteins that allow for membrane trafficking regulation (Homma et al., 2021).

Rab5 is a key member of the Rab family and regulates early endocytosis. Rab5 recruits effector proteins to early endosomes to regulate membrane trafficking. The effectors of Rab5 are early endosome antigen-1 (EEA1), ravaotub-5, rabenosyn-5, APPL1/2, and ZFYVE21 (Yuan and Song, 2020). Rab5 affects the internalization and intracellular transport of several receptors such as receptor tyrosine kinases and G protein-coupled receptors. When receptors are internalized, the vesicles fuse with early endosomes that are Rab5/EEA1-positive early endosomes. The receptors are then transported to late endosomes along with the early endosomes, fusing into a multivesicular body. Rab5 promotes the formation of early endosomes by regulating vesicle fusion (Pagano et al., 2004). Rab5-positive organelles are generally referred to as early endosomes and Rab5 is commonly used as an early endosome marker.

Rab7 is primarily associated with late endosomal structures. Research has shown that Rab7 plays a key role in regulating the transport of lysosome-destined enzymes and internalized surface proteins to lysosomes in the endocytic pathway (Zhang et al., 2009). Rab5 and Rab7 membrane domains on endosomes are not mutually exclusive, but rather work dynamically and cooperate in the endocytic pathway. At the early endosomes, cargo is sorted to different destinations: the recycling endosome, the late endosomes, the lysosome, or the Golgi apparatus. The Rab cascade determines the specific trafficking pathway (Markgraf et al., 2007). The pathway from early endosome to late endosome is determined by recruiting Rab5 to early

endosomes and then recruiting Rab7 endosomes while losing Rab5 in the late endosomes (Rink et al., 2005). In this research, Rab7-positive organelles will be referred to as late endosomes.

Rab11 associates primarily with recycling endosomes and regulates the recycling of endocytosed proteins (Stenmark, 2009). The recycling pathway involves the transport of cargo from early endosomes to endocytic recycling compartments to the plasma membrane. During early endosome maturation, the endosome extends tubules that become the endocytic recycling compartments and the main body of the endosome becomes multivesicular bodies. Live imaging has shown early endosomes lose Rab5 and acquire Rab11 (Sonnichsen et al., 2000). Rab11 then recruits motor proteins to the recycling endosomes to facilitate their transport back to the plasma membrane. Rab11 has also been shown to play a role in cell growth, cytokinesis, tumorigenesis, and exocytosis (O'Sullivan and Lindsay, 2020). Rab11-positive organelles will be referred to as recycling endosomes.

Other Endosome Markers

Early Endosome Antigen-1 (EEA1) is a tethering protein that is involved in homotypic fusions of early endosomes. EEA1 localizes exclusively to early endosomes and plays an important role in endosome trafficking. EEA1 is an effector protein that is recruited by Rab5 to early endosomes. EEA1 has a Rab5-binding domain at both the N-terminus and C-terminus, allowing it to anchor Rab5-positive structures at the first step of the fusion process. Due to EEA1 being a mediator for homotypic fusions of early endosomes, Rab5 and EEA1 are considered identical markers for early endosomes by default (Kamentseva et al., 2020).

Neurons have neuronal-specific endosomal regulators and machinery to address neuronal-specific demands. Neep21 is a transmembrane protein that is highly expressed in

neurons. Neep21 is a neuronal early endosome protein that is found in the early endosomal population, distinct from EEA1-positive endosomes (Steiner et al., 2002). Knockdown approaches in neurons have shown that Neep21 is involved in regulating the endosomal transport of several neuronal receptors (Steiner et al., 2005). Recent studies show that Neep21 are not resident proteins of dendritic endosomes, but proteins that transverse early endosomes after endocytosis and then enter degradative compartments (Yap and Winckler, 2012). In addition, studies show Neep21 was found transiently with early endosomes that contain EEA1 and Rab5. Functional interference with Rab5 led to an accumulation of Neep21 on the cell surface, which led to the conclusion that Rab5 plays a role in regulating the endocytosis of Neep21 (Yap et al., 2017). Live imaging has shown Neep21 moves bi-directionally in dendrites, making Neep21 an attractive candidate to visualize motile early endosomes (Yap et al., 2017). Neep21 will be used as an early endosome marker, specifically targeting motile endosomes.

Lysosome-associated membrane protein-1 (LAMP1) is a transmembrane protein that targets lysosomes. Lysosomes serve as the terminal degradation hubs for endocytic components. Cargo destined for degradation traffic through early endosomes to late endosomes and finally to lysosomes. LAMP1 leaves the trans-Golgi network after being synthesized and enters the plasma membrane and endolysosomal pathway (Cook et al., 2004). LAMP1-positive vesicles include both degradative lysosomes and non-acidic endolysosomes (Cheng et al., 2018). Studies have shown that RNA granules and lysosomes were tightly associated (Buchan et al., 2013) and RNA granules hitchhike on motile lysosomes (Liao et al., 2019). LAMP1 is a routinely used marker for neuronal lysosomes and LAMP1-positive organelles are referred to generally as lysosomes.

CD63 is a member of the tetraspanin family, a group of proteins involved in the regulation of cell adhesion, migration, and signaling (Pols and Klumperman, 2009). It is a

transmembrane protein expressed on the surface of various cells and is also present in intracellular vesicles, including endosomes, lysosomes, and exosomes. CD63 is also involved in the fusion of multivesicular bodies with lysosomes, which is important for the degradation of cargo that is not destined for secretion (Andreu and Yanez-Mo, 2014). CD63 is specially enriched in the membrane of exosomes and they are often used as exosome markers (Pols and Klumperman, 2009).

Transferrin Pathway

Transferrin, a monomeric glycoprotein, binds to specific receptors on cell surfaces and delivers iron via clathrin-mediated endocytosis (Pearse and Robinson, 1990). When transferrin enters the cell through clathrin-mediated endocytosis, endocytosed transferrin is sorted along the trafficking pathway into three main endosomes: early endosomes, late endosomes, and recycling endosomes (Gruenberg and Stenmark, 2004). Once inside the cell, transferrin is sorted for recycling or degradation. Transferrin can be trafficked to recycling endosomes before returning to the cell surface (Grant and Donaldson, 2009). Or, transferrin can remain in early endosomes as they mature into late endosomes, leading to degradation. Due to its pathway to recycling endosomes, transferrin can be used as a tool to elucidate trafficking pathways that go through the recycling pathways.

Research Aims

MBNL1 and MBNL2 are critical for regulating RNA splicing. Additionally, previous findings implicate their involvement in RNA transport in neurons. However, the underlying

molecular mechanisms are unknown. This project examined MBNL1 association with various endosomal markers to elucidate how MBNL1 transports to distal parts of the cell.

The first research aim of this thesis was to explore the degree of colocalization of MBNL1 with various endosomal markers in the endomembrane system. Neuro2a (n2a) mouse neuroblastoma cells were transfected with EGFP-MBNL1 and colocalization was assessed with early, late, and recycling endosomes. From these experiments, it was determined that MBNL1 primarily colocalizes with early endosomes.

The second research aim of this thesis was to use transferrin colocalization with early and recycling endosome markers, Rab5 and Rab11, at different time points to validate endosomal markers representing different stages of the endocytosis and recycling endosome pathway. Transferrin colocalization with anti-Rab5 and anti-Rab11 antibody at early and late time points of the endocytosis pathway were measured.

The third research aim of this thesis was to explore the degree of colocalization with other endomembrane markers including Neep21, EEA1, LAMP1, and CD63. Mouse n2a neuroblastoma cells were transfected with MBNL1 to measure colocalization with each endomembrane marker to assess whether MBNL1 associates with the specific endosomes or lysosomes. Through colocalization of endosome markers, MBNL1 showed the greatest colocalization with all the investigated markers of early endosomes, Rab5, Neep21 and EEA1.

The fourth research aim of this thesis focused on the function of Rab5 to determine if Rab5 GTPase activity influences MBNL1 mobility and activity. Fixed cell imaging and live cell imaging were used to assess how Rab5 influences MBNL1 colocalization with early endosomes and its mobility.

II. Methods

Cell Culture and Transfection

Mouse neuro-2A (n2a) neuroblastoma cells were cultured in Gibco Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS), 0.5% penicillin-streptomycin (Pen-Strep), and 1% HEPES. Cells were passed every 3 to 4 days once they reached about 90% confluency. Cells were washed with HBSS (Corning Hanks' Balanced Salt Solution), dissociated with 1mL 0.25% Trypsin, and split to 20% confluency.

Cells were transfected with a plasmid containing EGFP-MBNL1 isoform 41 fusion protein with Lipofectamine 2000 reagent (Invitrogen). 0.9ng DNA was added to each 15cm coverslip with 1.8uL of Lipofectamine 2000 reagent. Lipofectamine-DNA mixture was intubated at room temperature for 25 minutes before 100uL Lipofectamine-DNA mixture was added to each coverslip and incubated at 37°C overnight.

Immunofluorescence

For indirect immunofluorescence, n2a cells were plated at 10% confluence on 15mm coverslips, lipofectamine transfected the next day, and fixed after overnight incubation at 37 °C using 4% paraformaldehyde in PBS (phosphate buffered saline, ph 7.4) for 15 minutes at room temperature in the dark. Cells were then washed 3 times with PBS and permeabilized with a solution containing 0.1% saponin, 0.2% gelatin, 50 mg/mL BSA in PBS (Solution 1). Cells were incubated in 40uL of primary antibodies diluted in a solution containing 0.2% gelatin and 0.01% saponin in PBS (Solution 2) overnight at 4 degrees. Primaries antibodies used for

immunofluorescence include Rab5 rabbit mAb (1:100, Cell Signaling Technologies), Rab7 rabbit mAb (1:100, Cell Signaling Technologies), Rab11 rabbit mAb (1:100, Cell Signaling Technologies), rabbit mAb to LAMP1 (1:10,000, Abcam), rabbit pAb to EEA1 (1:1000, Abcam), rabbit polyclonal Neep21 (1:100, Bioss), mouse αnti-CD63 (1:100, Novus Biologicals). Cells were then washed two times with Solution 2 and incubated in 40 uL of secondary antibody in Solution 2 for 90 minutes at room temperature. Secondary antibodies include donkey Cy5 (1:600, anti-mouse, Jackson ImmunoResearch Labs), Alexa Fluor 555 donkey (1:600, anti-rabbit IgG, Invitrogen). Cells were washed three times with PBS, mounted using Prolong Gold with DAPI, and imaged.

Transferrin Treatment

After mouse n2a neuroblastoma cells were transfected with the protocol above and incubated overnight, transferrin (transferrin from human serum, Alexa Fluor 555 conjugate, Thermo Fisher, 1:100 in DMEM without supplements) was introduced to the coverslip. To verify anti-Rab5 antibody colocalization with internalized transferrin in early endosomes, there was no refeeding of cells after transferrin introduction. Briefly, cells were starved for 30 minutes in DMEM at 37°C, then each coverslip was placed on parafilm with a drop of 40 uL transferrin (1:100). Coverslips were put on ice and incubated in the dark for 30 minutes, then immediately moved for a 5-minute incubation at 37°C. Coverslips were then washed twice with DMEM and subsequently fixed with 4% paraformaldehyde using the protocol above. To verify anti-Rab5 antibody colocalization with internalized transferrin in recycling endosomes, cells were refed with DMEM supplemented with 10% fetal bovine serum (FBS), 0.5% penicillin-streptomycin (Pen-Strep), and 1% HEPES after transferrin treatment. Briefly, cells were starved for 30 minutes

in DMEM at 37 °C, then coverslips were placed on 40 uL transferrin solution drops as above. Subsequently, coverslips were put on ice and in the dark for 30 minutes and immediately moved for a 20-minute incubation at 37 °C. Coverslips were washed twice with pure DMEM and reintroduced to DMEM supplemented with 10% fetal bovine serum (FBS), 0.5% penicillin-streptomycin (Pen-Strep), and 1% HEPES for 25 minutes in 37 °C. Coverslips were then washed twice with DMEM and subsequently fixed with 4% paraformaldehyde using the protocol above.

Fixed Cell Imaging

Fixed cell imaging for MBNL1 and endosome/lysosome in n2a cells was performed using widefield illumination on a Nikon Eclipse TE300 inverted microscope with a Plan-Neofluar 1.4 NA x 40 oil objective.

For quantitative colocalization experiments, images were taken in a z-series at 0.2 um steps and deconvolved using a 3-D blind-constrained interactive algorithm (AutoQuant). Imaris imaging software (Bitplane), namely the ‘Coloc’ module, was used to analyze the deconvolved images to determine the degree of colocalization. Quantitative colocalization analysis involved creating a 3-D mask of the green channel from selected neurite-like processes of the n2a cells and excluding the background signal from outside this volume. Within the masked volume, two measures of colocalization, Pearson’s correlation coefficient, and Mander’s overlap coefficient were calculated between MBNL1 and respective endosomes or lysosomes.

Testing Rab5 Variants

For experiments testing Rab5 function, wild-type Rab5, constitutively active Rab5 (Q79L mutant), and dominant negative Rab5 (S34N mutant) were chosen. Cells were cotransfected with a plasmid containing EGFP-MBNL1 isoform 41 fusion protein and a plasmid containing mCherry-Rab5 with Lipofectamine 2000 reagent (Invitrogen). 0.9ng total DNA, with 1:1 ratio of MBNL1:Rab5, was added to each 18cm coverslip with 1.8uL of Lipofectamine 2000 reagent. Lipofectamine-DNA mixture was intubated at room temperature for 25 minutes before 100uL Lipofectamine-DNA mixture was added to each coverslip and incubated at 37 °C overnight. mCherry-Rab5a-7 was from Michael Davidson (Addgene plasmid # 55126 ; http://n2t.net/addgene:55126 ; RRID:Addgene_55126), mCherry-Rab5DN(S34N) and mCherry-Rab5CA(Q79L) were from Sergio Grinstein (Addgene plasmid # 35139 ; http://n2t.net/addgene:35139 ; RRID:Addgene_35139) (Bohdanowicz M et al., 2012)

Live Imaging

Mouse neuroblastoma n2a cells were plated on 18 mm coverslips and transfected with the above-mentioned protocol. Subsequently, they were transferred to a preheated to 37 °C Chamlide magnetic chamber (Live Cell Instruments) and immersed in 0.5mL Hibernate E CO2 independent medium (Gibco) supplemented with B-27 plus supplement (Gibco) and GlutaMAX (Gibco). Then, the magnetic chamber was transferred to a Chamlide live cell imaging chamber, preheated to 37 °C. Coverslips were imaged on a Nikon Eclipse TE2000-E microscope with a Plan-Apo 1.4 NA x 60 oil objective.

For live imaging experiments, 51 images in two color channels were taken every 1.17 seconds and deconvolved using a 2-D blind-constrained interactive algorithm (AutoQuant). MBNL1 total travel distance was analyzed using ImageJ plug-in Trackmate (Tinevez J-Y et al., 2017) by Aleksandra Janusz-Kaminska.

III. Results

To assess if MBNL1 associates with endosomes, MBNL1 colocalization was examined with early, late, and recycling endosomes through immunofluorescence (IF) in mouse neuroblastoma cells. Fixed cell imaging, as seen in Figure 1, demonstrated that MBNL1 colocalized with early, late, and recycling endosomes. MBNL1 colocalized to approximately the same degree with early endosomes (63.71% ± 4.39, number of replicates (N)=3, number of cells per variant (n)=5) and late endosomes (57.72% ± 4.07, N=3, n=5) as the difference was not statistically significant (p=0.0755) as determined by one-way ANOVA with Tukey's test. There was a greater degree of colocalization in early endosomes (63.71% ± 4.39, N=3, n=5) than in recycling endosomes (45.26% ± 4.87, N=3, n=5), which was statistically significant (p=0.0004). MBNL1 colocalized slightly less in recycling endosomes than in late endosomes, however, the difference in colocalization is not statistically significant (p=0.1302).

Transferrin was visualized in the transferrin endocytosis pathway to verify the endosome markers. Fixed cell imaging shows Rab5 antibodies and Rab11 antibodies used as endosome markers accurately represent their specific endosomes. Figure 2 shows partial colocalization of Rab5 and Rab11 with transferrin without refeeding with DMEM/FBS (early endosome protocol) and after refeeding with DMEM/FBS (recycling endosome protocol). Colocalization with

transferrin and the Rab5 antibody was observed in the early endosome protocol as well as colocalization between transferrin and Rab11 antibody in the recycling endosome protocol. Observations are consistent with previous findings of colocalization of transferrin and endosomes (Mayle et al., 2012), indicating the accuracy of using Rab markers as indicators of endosomes.

After the results showed that MBNL1 colocalizes with Rab5, Rab7, and Rab11, markers corresponding to other types of endosomes were tested. Figure 3 shows MBNL1 colocalization with neuronal-specific early endosomes, marked by Neep21. MBNL1 colocalized with early endosomes marked by Neep21 (67.15% ± 4.90, N=3, n=5) and Rab5 (62.67% ± 4.33, N=3, n=5) to relatively the same degree ($p=0.9950$). This further shows that MBNL1 is colocalizing with early endosomes. This set of experiments supports the difference in colocalization as shown in the previous experiment as colocalization between early and recycling endosomes was statistically significant ($p=0.0038$).

Other markers, including EEA1, CD63, LAMP1, were tested to assess colocalization. Figure 5 shows MBNL1 colocalization with early endosomes (EEA1), which is congruent with the earlier results with Rab5 and Neep21, exosomes (CD63), and less frequently lysosomes (LAMP1). MBNL1 strongly colocalized with early endosomes (66.86% ± 5.62, N=3, n=5). MBNL1 colocalized with exosomes (63.78% ± 5.32, N=3, n=5). MBNL1 colocalized with lysosomes to a lesser degree (25.38% ± 5.40, N=3, n=5). One-way ANOVA with Tukey's test demonstrated a significant difference in colocalization ($p<0.0001$) between early endosomes and lysosomes, and between CD63 and lysosomes ($p<0.0001$). There was no significant difference in colocalization between EEA1 and CD63 ($p=0.9705$).

MBNL1 showed the greatest degree of colocalization with early endosomes, so an additional assessment of the activity of Rab5 and its influences on MBNL mobility and activity was undertaken. Constitutively active and dominant negative variants of Rab5 were chosen and compared to wildtype to assess for differences in colocalization with MBNL1 and MBNL1 transport. Constitutively active Rab5 cannot detach from the endosome membrane, blocking the replacement of Rab5 by Rab7. This stops early endosomes from maturing into late endosomes. The dominant negative Rab5 variant binds transiently to the membrane, therefore it cannot participate in endocytosis. Thus, dominant negative Rab5 is highly cytoplasmic and only transiently associated with endosomes. Fixed cell imaging showed varying degrees of colocalization in constitutively active Rab5 cells, ranging from 2.97% to 95.67% with a median of 49.87%. MBNL1 colocalized slightly more in wild-type Rab5 (62.95% ± 4.33, N=3, n=5) than constitutively active Rab5 (51.46% ± 8.41, N=3, n=5) variants, though the difference was not significant (p=0.6238). Interestingly, there are enlarged endosomes in the constitutively active Rab5 cells. In the cells, MBNL1 colocalized mainly with smaller endosomes, with minimal to no colocalization with the enlarged endosomes. Figure 5 illustrates enlarged granules and colocalization of MBNL1 and endosomal granules. Live imaging analysis, shown in Figure 6, showed a trend towards more processive movement of MBNL1 particles in cells cotransfected with constitutively active Rab5 (2.435 um ± 0.311, n=4) and dominant negative Rab5 (2.455 um ± 0.260, n=4) when compared to wild-type Rab5 (2.136 um ± 0.2239, n=4). However, the results are preliminary and without statistical significance.

IV. Discussion

This thesis aimed to investigate MBNL1 association with endosomes through fixed and live cell imaging and colocalization studies to help understand how MBNL1 is transported to distal parts of the cell. The findings of this thesis provide preliminary evidence regarding the role of Rab GTPases in MBNL1 mobility and the role of endosomes in MBNL1 trafficking and mobility.

One main aim of this thesis was to examine if RNA-binding protein, MBNL1, associates with early, late, and recycling endosomes to suggest colocalization of MBNL1 and endomembranes. Findings indicate that MBNL1 colocalizes with early and late endosomes, and to a lesser degree, recycling endosomes. This suggests that MBNL1 utilizes endosomes, in particular early endosomes, for transportation and targeting its cargo mRNAs to distal parts of the cell. Recent studies have shown that RNA granules can be transported on moving endosomes through a process called “hitchhiking” (Liao et al., 2019). It is possible that MBNL1 uses a molecular tether to attach to endosomes and travel along the endosomal pathway for distal translation. Moreover, MBNL1 colocalized to the greatest degree with early endosomes, which suggests that MBNL1 selectively binds to specific endomembranes.

Rab5 and Rab11 were used as proxies for early and recycling endosomes. To validate that the Rab GTPases corresponded to their respective endosomes, Rab antibodies were colocalized with transferrin. Previous studies have shown transferrin to colocalize with Rab5 and Rab11, indicating that transferrin delivery to endocytic recycling compartments from early endosomes (Mayle et al., 2012). Experimental data from this project was consistent with previous work, validating the usage of the chosen antibodies for Rab5 and Rab11 proteins as indicators for early and recycling endosomes.

Another aim of this thesis was to determine if MBNL1 colocalized with other endomembrane markers. Results show colocalization with Neep21, specific to neuronal early endosomes, as well as EEA1, an early endosome-specific Rab5 tether. This again highlights that MBNL1 associates with early endosomes and is transported on early endosomes and utilizes a binding mechanism to travel to distal parts of the cell. Additionally, previous work has shown that ribosomes can perform translation on early endosomes and the colocalization results may also represent MBNL1 assisting in localized translation (Cioni et al., 2019), docking on endosomes rather than traveling on them. However, docking for localized translation and attaching for distal transportation are not mutually exclusive events, and it is possible that MBNL1 is colocalizing with different endosomes to facilitate separate tasks. Further research is needed to fully understand the functional implications of these colocalization patterns and to determine molecular mechanisms of MBNL1 association with endosomes within the endosomal system.

Furthermore, the results show that MBNL1 colocalizes with CD63, an exosome/lysosome marker, more than LAMP1, a lysosome-associated membrane protein. It is possible that MBNL1 associates with exosomes and lysosomes for transportation or localized translation. The difference in colocalization between CD63 and LAMP1 may suggest that MBNL1 is able to use CD63-positive exosomes for transportation, but detaches when it is delivered to a lysosome and the cargo is released. This, however, requires further investigation in the future. The varying levels of colocalization between MBNL1 and exosomes and lysosomes indicate that while MBNL1 is able to bind to different endomembranes, lysosomes are not the primary transport vehicle for MBNL1 transportation.

The last aim of this thesis was to determine how Rab5 function affects MBNL1 trafficking with endosomes. The results showed that the constitutively active Rab5 led to the formation of enlarged endosomes located in the cell body, and to a lesser degree, in the processes. However, MBNL1 did not co-localize significantly with these enlarged endosomes, which suggests that MBNL1 selectively travels on smaller endosomes and selectively chooses endosomes that are functional for transport. It is possible that due to the size of the enlarged endosomes, the enlarged endosomes are bottlenecked in the cell body and unable to mobilize towards the processes, thus not a suitable candidate for transportation. The range of colocalization in the constitutively active variant Rab5 may be due to the overexpression of Rab5 due to over-transfection, resulting in the formation of non-functional endosomes and impairing MBNL1 transport. This would result in a decrease in colocalization between Rab5 and MBNL1, increasing the variability of colocalization data. Additionally, it is important to consider the possibility that early endosome mobility may also impact MBNL1 mobility. Possible disruption of endosome mobility could have downstream effects on endosome-associated proteins and could also affect the degree of colocalization. Further work is needed to study if the mobility of MBNL1 is disturbed when early endosome mobility is disrupted.

Dominant negative variants of Rab5 colocalization with MBNL1 were qualitatively analyzed. Qualitative results showed anti-colocalization of MBNL1 and Rab5 DN, highlighting that MBNL1 is not associated with non-functional Rab5. The dominant negative Rab5 is largely cytoplasmic, which may suggest that MBNL1 requires Rab5 to be on the endomembrane surface to facilitate binding for travel or localized translation. Without the active Rab5 on the surface, MBNL1 may not be able to anchor to the endosome. Due to the dominant negative Rab5 variant being largely cytoplasmic, assessing the degree of colocalization proved to be challenging. The

Imaris software and AutoQuant deconvolution used in the experiment did not accurately cut off cytoplasmic fluorescence, making it difficult to accurately determine the degree of colocalization.

Interestingly, preliminary live imaging results show a trend of increased MBNL1 movement in both constitutively active and dominant negative variants of Rab5 compared to wild-type Rab5. This suggests that Rab5 GDP to GTP switch is necessary for MBNL1 anchoring to early endosomes. The results also indicate that MBNL1 is able to travel through a different mechanism than the endocytic pathway, or on other endomembranes. However, further work is needed to assess the colocalization between MBNL1 and Rab5 in live cells.

Colocalization studies would provide more definitive evidence of the relationship between these two proteins and would help to clarify the specific mechanism of MBNL1 trafficking. It is highly likely that Rab5 plays a role in MBNL1 transport with early endosomes, but additional studies are needed to explore this further. The impact of Rab5 function on MBNL1 transportation underscores the need for further research to fully understand the underlying mechanisms involved. Ultimately, gaining a better understanding of the role of the endosomal system in MBNL1 trafficking and localized translation could provide new insights into the pathogenesis of myotonic dystrophy.

V. Figures

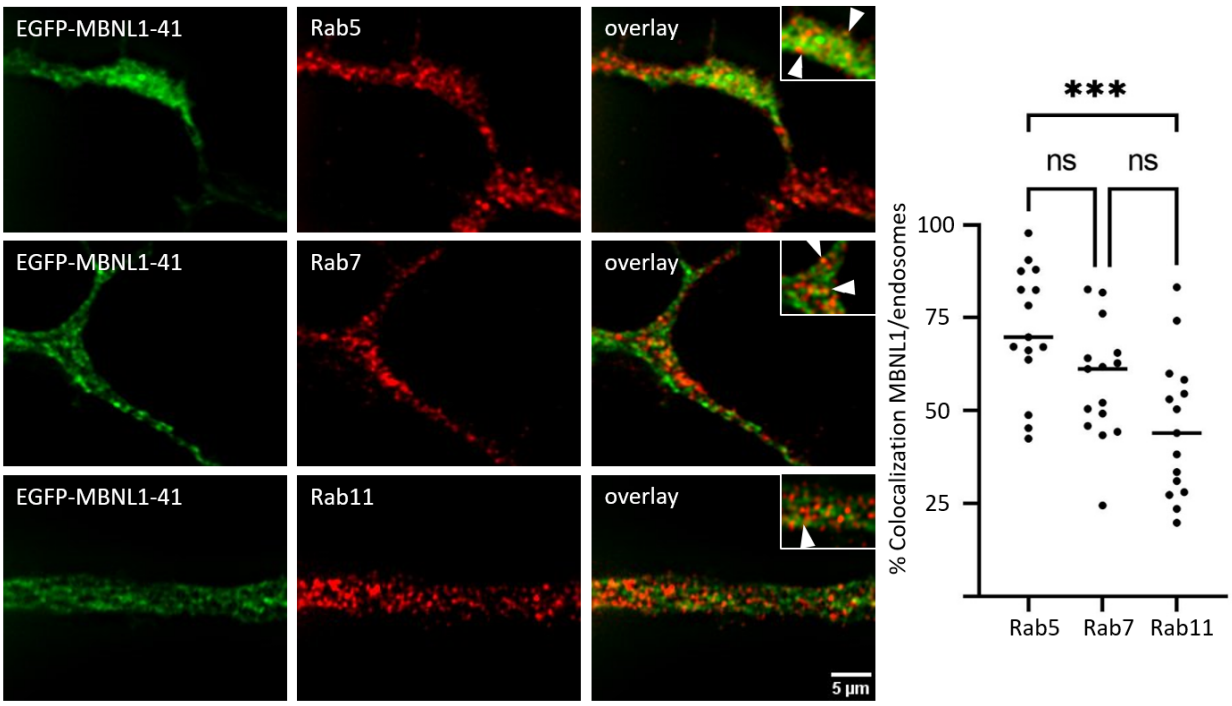

Figure 1. MBNL1 colocalizes with early, late, and recycling endosome markers in mouse neuroblastoma cells. EGFP-MBNL1-41 isoform overexpressed in mouse neuroblastoma cell (n2a) overnight (green). Images represent distal, neurite-like protrusions. Indirect immunofluorescence on endogenous endosome markers (red). 3D quantitative colocalization analysis done through Imaris software in distal processes shows colocalization with early endosomes (Rab5), late endosomes (Rab7), and to a lesser degree recycling endosomes (Rab11). Arrows demonstrate examples of colocalization spots (*** $p<0.001$, one-way ANOVA with Tukey's post hoc test). Number of cells per variant (n) =5, number of replicates (N)=3.

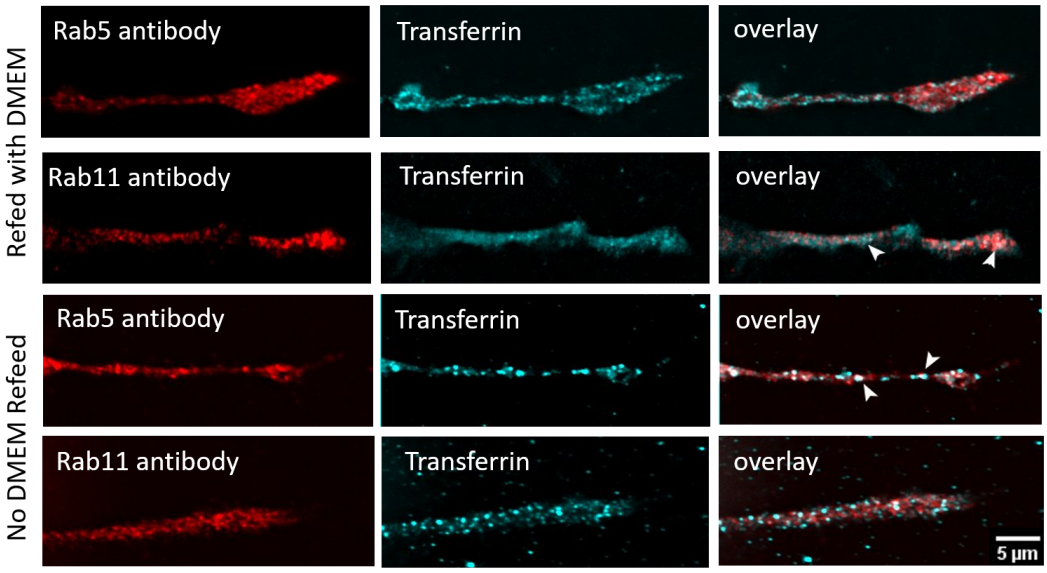

Figure 2. Transferrin partially colocalizes with early and recycling endosome markers in mouse neuroblastoma cells. Indirect immunofluorescence on endogenous endosome markers (red). Tagged fluorescent transferrin internalization (cyan). Images represent distal, neurite-like protrusions. Arrows demonstrate examples of colocalization spots. n=5, N=3.

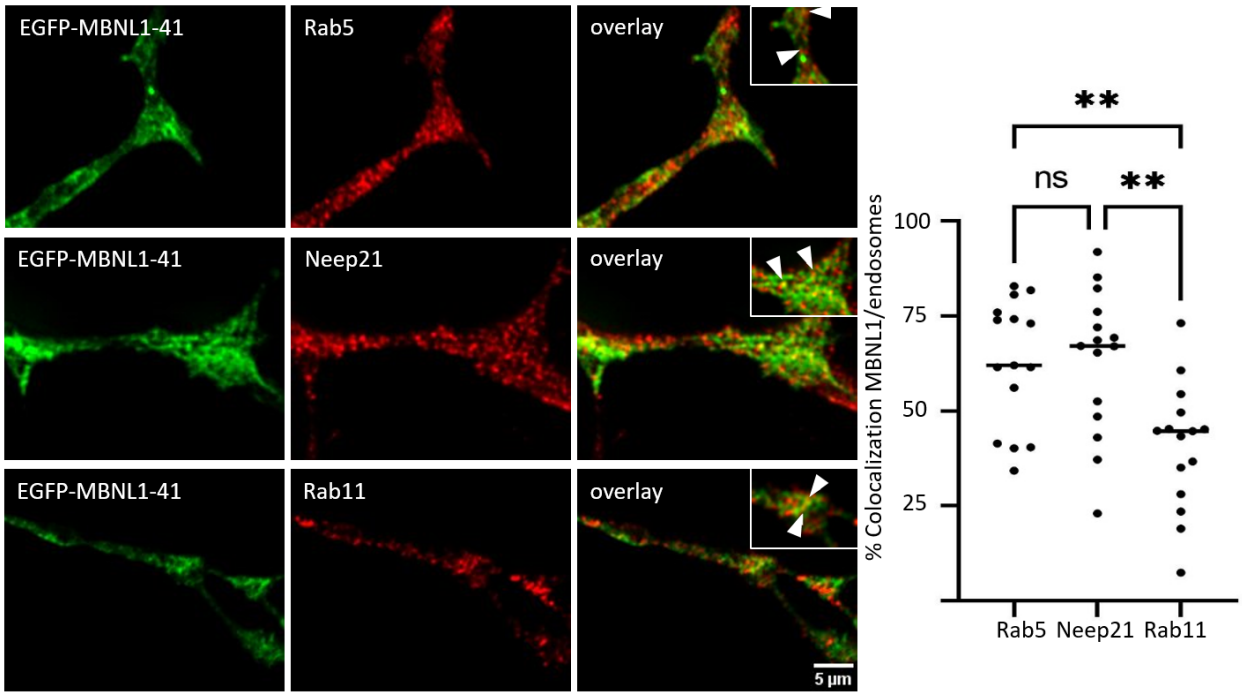

Figure 3. MBNL1 colocalizes with early, and recycling endosome markers in mouse neuroblastoma cells. EGFP-MBNL1-41 isoform overexpressed in mouse neuroblastoma cells (n2a) overnight (green). Indirect immunofluorescence on endogenous endosome markers (red). Images represent distal, neurite-like protrusions. 3D quantitative colocalization analysis done through Imaris software in distal processes shows colocalization with early endosomes (Rab5 and Neep21) and to a lesser degree recycling endosomes (Rab11). Arrows demonstrate examples of colocalization spots (** $p<0.01$, one-way ANOVA with Tukey's test). n=5, N=3.

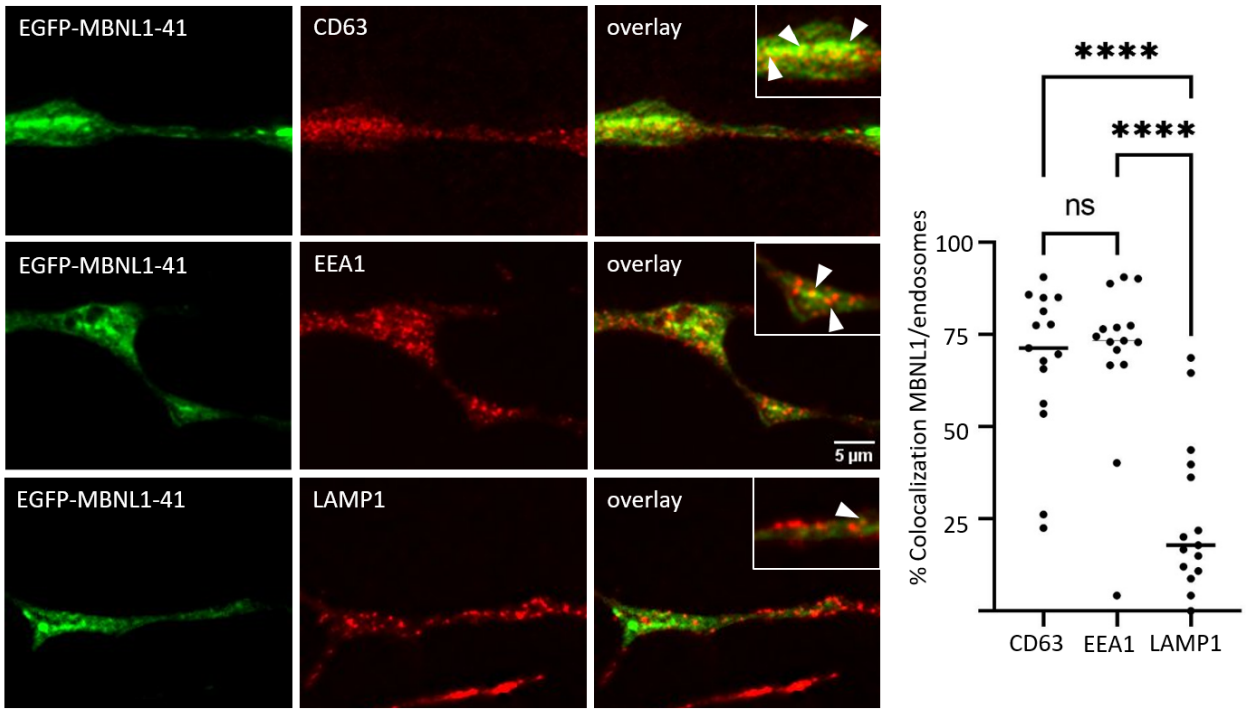

Figure 4. MBNL1 colocalizes with early endosome and exosome markers in mouse neuroblastoma cells. EGFP-MBNL1-41 isoform overexpressed in mouse neuroblastoma cell (n2a) overnight (green). Indirect immunofluorescence on endogenous endosome markers (red). Images represent distal, neurite-like protrusions. 3D quantitative colocalization analysis done through Imaris software in distal processes shows colocalization with early endosomes (EEA1), late exosomes (CD63), and to a lesser degree lysosomes (LAMP1). Arrows demonstrate examples of colocalization spots (**** $p < 0.0001$, one-way ANOVA with Tukey's test). n=5, N=3.

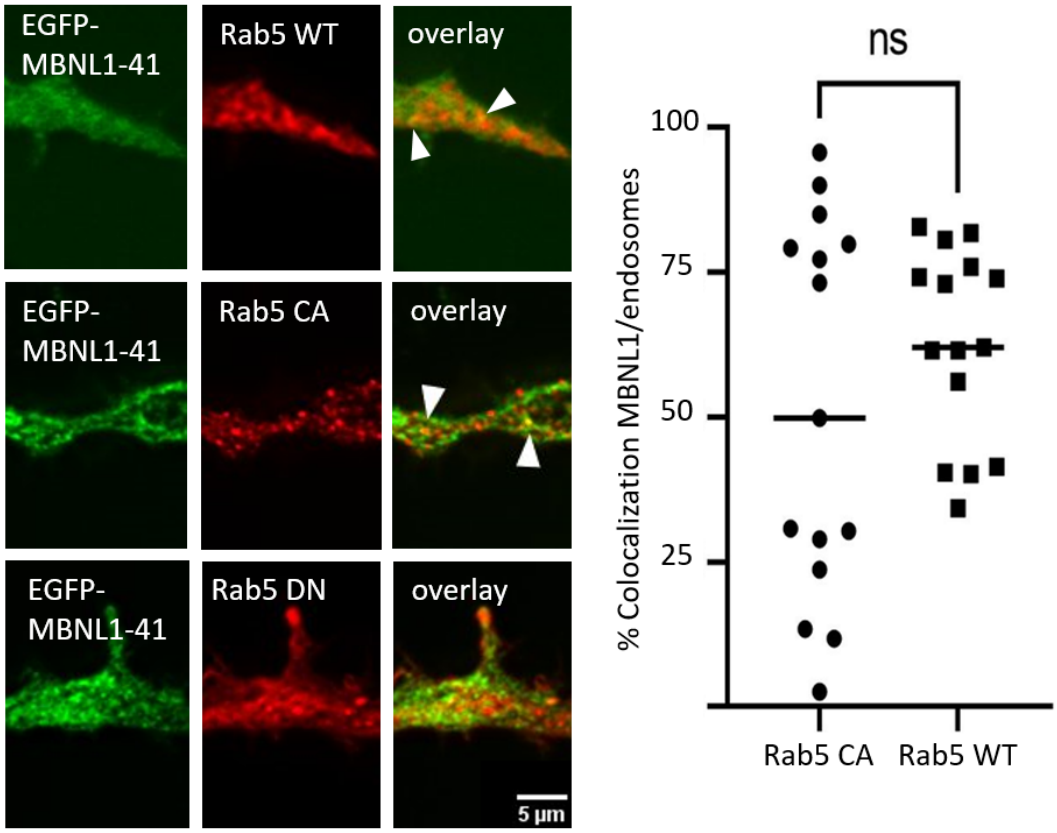

Figure 5. MBNL1 colocalizes with constitutively active (CA) Rab5 and wild-type (WT) Rab5 early endosome markers in mouse neuroblastoma cells. EGFP-MBNL1-41 isoform (green) co-transfetected with Rab5 variants (red) in mouse neuroblastoma cell (n2a) overnight. Images represent distal, neurite-like protrusions. 3D quantitative colocalization analysis done through Imaris software in distal processes shows colocalization with constitutively active Rab5 and wild-type Rab5 early endosomes. Arrows demonstrate examples of colocalization spots (* $p< 0.05$, one-way ANOVA with Tukey's test). n=5, N=3.

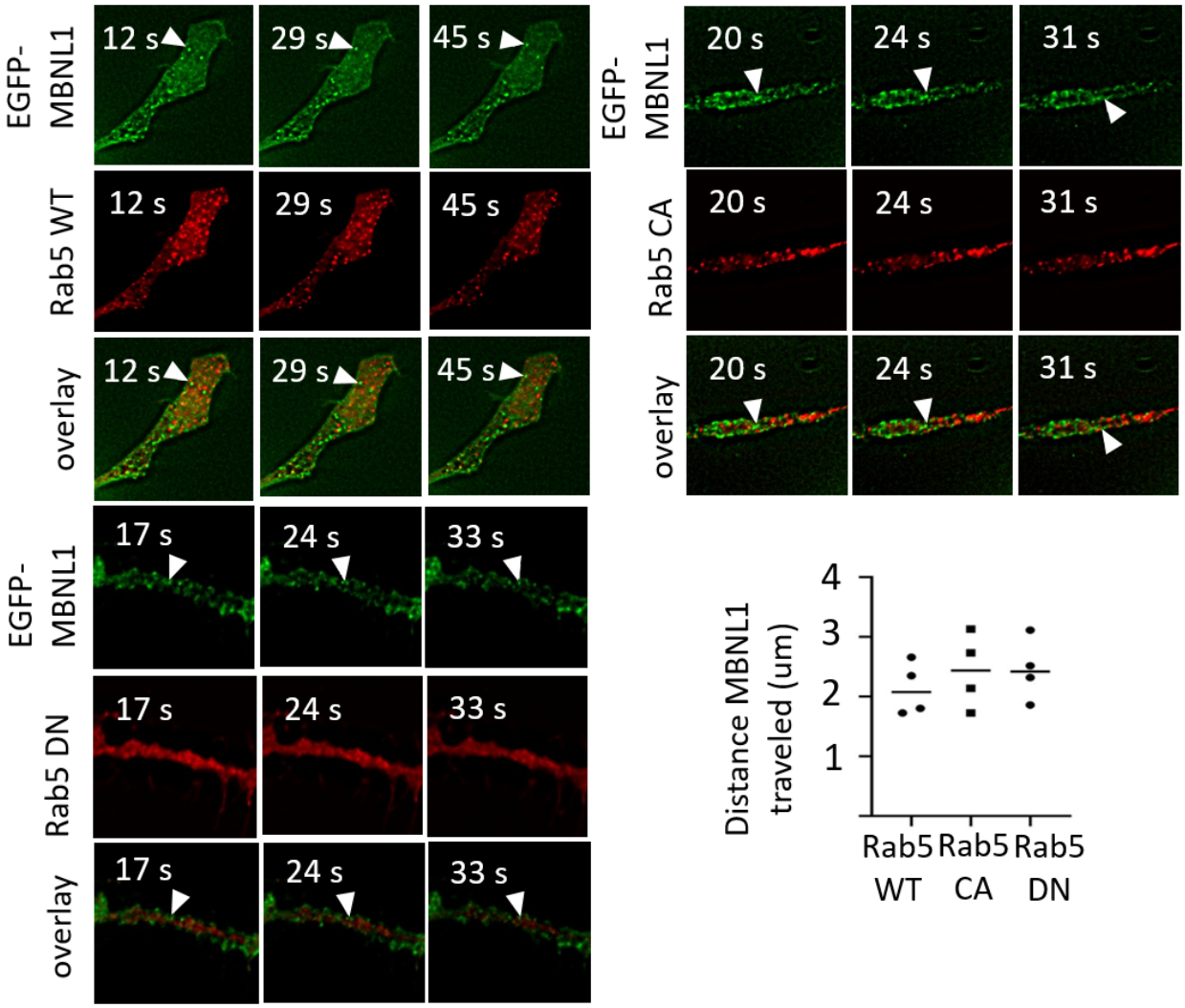

Figure 6. MBNL1 travels in constitutively active and dominant negative variants of Rab5 in mouse neuroblastoma cells. EGFP-MBNL1-41 isoform (green) co-transfetected with Rab5 variants (red) in mouse neuroblastoma cell (n2a) overnight. Images represent distal, neurite-like protrusions. MBNL1 Travel distance analysis done through Imaris software in distal processes shows more processive movement of MBNL1 particles in CA and DN Rab5 than WT Rab5. n=4.

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