Structure-Function Analysis of Axonal Fragile X Mental Retardation Protein

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Abstract

Structure-Function Analysis of Axonal Fragile X Mental Retardation Protein
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Silencing of the Fragile X Mental Retardation Protein (FMRP)-encoding gene *Fmr1* causes Fragile X Syndrome, the leading known cause of autism. FMRP is an alternatively spliced, multidomain, RNA-binding protein most highly expressed in the brain that regulates mRNA transport and translation. Its function is well-characterized in dendrites but it is also found in the cell body, axons, and in the nucleus. Functioning throughout neurons suggests that some mechanism exists whereby it is appropriately transported and that this mechanism requires one or more of its domains. FMRP is alternatively spliced to yield at least 12 splice forms. These differ from each other in which domains and post-translational modification sites are present or absent. Our hypothesis is that one or more domains is required for axonal localization of FMRP. This is tested using EGFP-tagged FMRP splice form constructs to identify whether certain ones are preferentially localized to axons over others as well as mutation-containing constructs to identify one or more domains that are required for the mechanism of axonal localization of FMRP. These constructs are transfected into cultured rat cortical neurons with tdTomato and examined for differences in axonal localization, length, puncta distribution and density, and axonal arbor complexity, a process regulated by FMRP. All splice forms showed axonal localization at equivalent efficiencies and quantities suggesting that all are able to function in axons. Since the N-terminus is well-conserved between splice forms while the C-terminus is more variable due to the alternative splicing

events, it was concluded that the domain required for axonal localization is N-terminal to the first splice site. Using a model for FMRP regulation of axonal arbor complexity in which overexpression of splice forms that function in axon growth and branching would oversimplify axonal arbors, SF7 and SF9-transfected neurons had reduced complexity. The domain(s) required for this function may be C-terminal. Because there are several domains in this region affected by alternative splicing, individual mutant analysis was required to narrow down the possibilities. All FMRP-SF7 mutant constructs showed an ability to localize to axons, supporting the idea that the region required for axonal localization is in the N-terminus. Intriguingly, the NES34A mutation in which the nuclear export sequence (NES) is deleted showed an increased efficiency over WT to localize axonally. Furthermore, the S500A, S500D and the ΔRGG mutants showed decreased puncta densities in axons. Axonal arbor complexity was not affected in neurons transfected with NES deletion or S500 mutants suggesting these domains may be required for FMRP to function in axon growth and branching regulation. Our results suggest a mechanism of axonal localization of FMRP that requires a region in the N-terminus which allows for all splice forms to be localized and function axonally. A region in the C-terminus is required for FMRP to function in regulation of axonal arbor complexity and this region may be the either the NES or S500 phosphorylation site or both. This is the first known attempt at elucidating a mechanism for differential localization of FMRP splice forms and the role of FMRP domains in axonal localization.

Chapter I: Introduction

Autism, Fragile X Syndrome, and Synaptic Plasticity

There are an estimated 86.1 billion neurons in the human brain which form as many as 150 trillion synapses with each other (Azevedo et al., 2009; Pakkenberg et al., 2003). By strengthening or weakening these synapses, the brain is able to both learn and respond to the environment. This is known as synaptic plasticity and is required for cognitive function. But if this process is not properly maintained, learning and memory can become impaired (Bear et al., 2004). Intellectual disabilities and neurological disorders such as autism are considered the result of faulty synaptic plasticity. However, autism is difficult to study because it is a complex disease involving many factors. Monogenic diseases with similar manifestations offer a simpler disease mechanism to study brain circuitry and synapses in relation to autism itself.

Fragile X Syndrome (FXS), an X-linked cognitive disorder, is one such disease caused by a loss of the Fragile X Mental Retardation Protein (FMRP). FMRP is an RNA-binding protein important in regulating translation, generally by repressing mRNAs. Though present in a variety of tissues such as kidney, lung, and placenta, its expression levels are highest in the brain and testes. These are the two organs affected in FXS (Hinds et al., 1993). Affected individuals show large, prominent ears and forehead, intellectual disability, developmental delay, macroorchidism, and obsessive compulsive disorder (Bear et al., 2004). Furthermore, FXS is the leading known cause of autism; up to 5% of autistic individuals also have FXS. (Kelleher and Bear, 2008). Genetically, FXS usually occurs when the gene encoding FMRP, *Fmr1*, is silenced by hypermethylation due to an expanded CGG trinucleotide repeat in the 5’ untranslated region (UTR) (Pieretti et al.,

1991). As FMRP regulates the translation of an estimated 4% of neuronal mRNAs and is crucial in protein synthesis-dependent synaptic plasticity (Darnell et al., 2011), it follows that dysregulation as a result of its absence throughout development would lead to neurological defects.

Of Splice Forms and Domains

Though much of the work on FMRP has focused on its role in dendrites and postsynaptic protein synthesis-dependent synaptic plasticity, more recent studies have begun to show its importance in axons and presynapses as well (Antar et al., 2006; Christie et al., 2009). The functions discovered thus far have been found to be independent of translation, suggesting that this role of FMRP is dendritic. Though the presynaptic and postsynaptic functions of FMRP may be independent of each other, they are both linked to neuronal and circuit hyperexcitability (Myrick et al., 2015b). For instance, FMRP has been implicated in presynaptic modulation of various voltage-gated ion channels including Slack, $Ca_v2.2$, and BK channels (Brown et al., 2010; Zheng et al., 2012; Ferron et al., 2014; Deng et al., 2013), linking FMRP with control of the duration of action potential and release of neurotransmitters.

These findings increase our understanding of the complexity of FMRP's roles. Indeed, the protein itself is complex. The 614 amino acid protein possesses multiple functional domains and sites of post-translational modifications (Fig. 1.1a) as well as multiple alternative splice sites that give rise to at least 12 splice forms. The domains include three RNA binding domains as well as nuclear export and localization sequences (Siomi et al., 1993; Bardoni et al., 1997). The protein can be further modified via a conserved phosphorylation site and methylation site(s) (Siomi et al., 2002; Dolzhanskaya

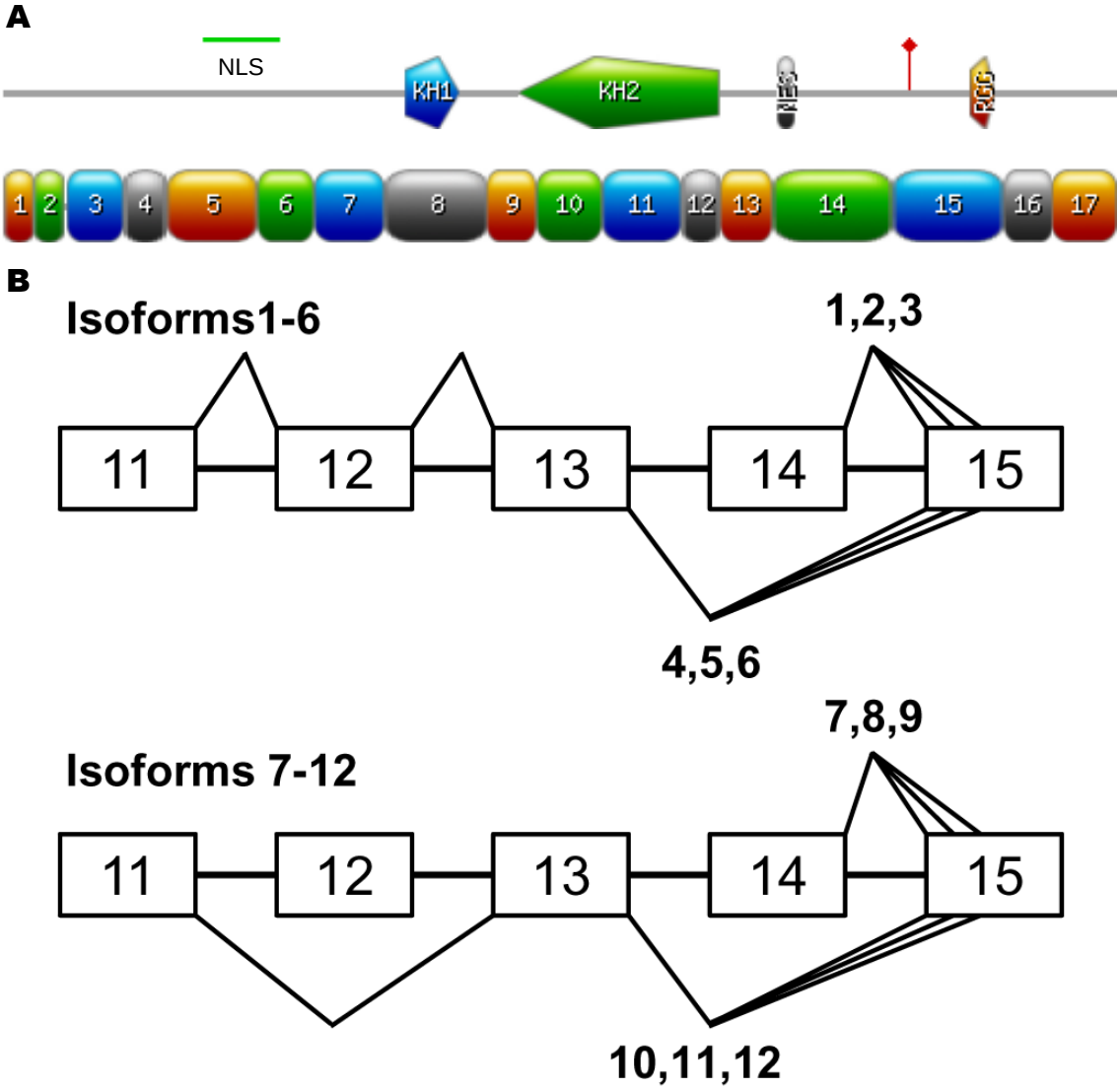

Figure 1.1 FMRP splice forms and domains. A) Locations of FMRP domains and S500 phosphorylation site within exons. B) Alternative splice site locations in *Fmr1* transcript and resulting splice form (Brackett, et al, 2013).

et al., 2006). The importance of more than one of the domains has been seen in patients with FXS whose *Fmr1* genes are not silenced but contain missense mutations. The first of these to be discovered was found in the K Homology 2 (KH2) domain of a patient with severe FXS. This is one of the RNA-binding domains and the altered amino acid at this position (I304N) leads to partial loss of function in the protein (Siomi et al., 1994). One other missense mutation is R138Q. This patient presented with milder symptoms and none of the morphological features typically seen. R138Q is located in the region of the nuclear localization sequence (NLS), an area notorious for being difficult to study (Collins et al., 2010). More recently, a novel KH motif was discovered at positions 126-202, suggesting that the R138Q mutant loses its RNA binding ability at this domain (Myrick et al., 2015a).

Though these domains are all present in the full-length protein, some cannot be found in protein products translated from alternatively spliced transcripts. The alternative splice sites in FMRP can give rise to up to 12 different mRNA transcripts (Verkerk et al., 1993; Brackett et al., 2013). The domains most likely to be missing are the Nuclear Export Sequence (NES) and one RNA-binding domain, the RGG box. One or more of the sites for post-translational modification can be lost as well leading to differences in biochemical properties. It can be inferred that such biochemical differences could lead to localizational and functional differences. These combinations of splice forms, domains, and post-translational modification sites confer versatility to FMRP.

FMRP Domains and Post-translational Modification Sites

The best characterized function of FMRP is translational control mediated by RNA-binding. To this purpose, FMRP has three long-known RNA-binding domains,

tandem KH domains (KH1 and KH2) and an RGG box. As mentioned previously, a third novel KH domain (KH0) was recently discovered. Of these, the KH2 domain is the best characterized due to its previously discussed relation to FXS. While it has been estimated that FMRP targets 4% of neuronal mRNAs, whether or not each RNA-binding domain specifically targets subsets of this population is unknown. In regards to this, little is known about what the KH1 domain binds, but its loss of function by mutation results in a protein less able to inhibit translation than WT proteins (Chen et al., 2014). The KH2 domain and RGG box each recognize different motifs. The KH2 domain targets mRNAs containing a "kissing complex" motif while the RGG box identifies with targets possessing G-quartet motifs (Darnell et al., 2005; Darnell et al., 2001). Intriguingly, the KH0 domain appears to not be involved in translational control by FMRP, but has a presynaptic role in BK channels, arbitrating action potential duration (Myrick et al., 2015b).

FMRP and RNA-binding in translational regulation are crucial regardless of target motif, but FMRP must also be regulated. The S500 phosphorylation site regulates FMRP's activity. Though the mechanism of repression by FMRP is unclear, it is able to bind polysomes and/or mRNA and stall translation when phosphorylated. Even when dephosphorylated, FMRP can remain bound to polysomes without stalling translation (Siomi et al., 2002; Ceman et al., 2003). This ability to alter FMRP's activity by phosphorylation places it in signaling pathways involved in processes that require quick translation of new protein such as translation-dependent synaptic plasticity. Methylation has also been found to occur at four positions within the RGG box. However, little is

known about its role beyond a possible modulation of FMRP-bound mRNA quantity or identity (Blackwell et al., 2010).

The role that post-translational modifications may have on the mechanism of FMRP binding to the ribosome remains undetermined except that a conformational change related to phosphorylation/dephosphorylation is likely involved. Chen et al. (2014) found that the KH1 and KH2 domains bind the ribosome while the RGG domain interacts with the mRNA. In this way, translational repression is mediated by the interactions of FMRP with both the ribosome and mRNA. The location of FMRP binding also blocks various translation factors and tRNA such that translation is paused during the elongation phase. Ribosome stalling by phosphorylated FMRP may allow mRNAs to be transported along processes, partially translated, so that they can be readily available for use in synapses following signal transmission (Darnell et al., 2011; Graber et al., 2013).

FMRP is characterized as a nucleocytoplasmic protein playing a role in shuttling mRNA (Feng et al., 1997); the two remaining domains illustrate this need for shuttling. The nuclear localization sequence (NLS) allows FMRP to be shuttled to the nucleus where there is evidence that it binds nuclear mRNAs while the nuclear export sequence (NES) keeps it in the cytoplasm or allows it to return (Sittler et al., 1996; Kim et al., 2009). The NLS is noncanonical. Located in exon 5, it has been placed closer to the N-terminus than the other domains and sites already discussed (Bardoni et al., 1997; Kim et al., 2009). The NES is in exon 14 and is of interest in relation to splice forms, discussed below. The role of FMRP in the nucleus was thought to be minor because only about 4% of it was localized there while the remainder was in the cytoplasm (Feng et al., 1997).

However, some findings have led to the discovery that FMRP is a chromatin-binding protein important in regulating replication stress through the DNA damage response pathway (Alpatov et al., 2014). This role is important in spermatogenesis and may explain, at least partly, defective sperm development in FXS individuals. Whether or not this chromatin-binding role is important in neurons is yet to be determined.

FMRP Splice Forms

Of the twelve possible versions of the FMRP protein, at least five have been identified in the mouse brain by Western blot (Verheij et al., 1995). Furthermore, all mRNA transcripts are able to associate with polysomes, suggesting that they are translated in mouse brain. There is evidence that their expression levels fluctuate spatially and developmentally (Bracket et al., 2013). Beyond this, more in-depth analysis has been impossible due to a lack of splice form-specific reagents. Work done on individual splice forms as well as within subsets has provided some information.

To start, simply comparing the amino acid sequences shows where these splice forms differ (Fig. 1.1b). The first splice site results in the presence or absence of exon 12. If removed, the KH2 domain is shortened, impacting but not eradicating its RNA-binding abilities (Xie et al., 2009). This differential binding of the KH2 domain occurs in half of the splice forms. The next splice site results in the presence or absence of exon 14 where the NES is located. Again, half of the splice forms lack the NES and have been seen to localize exclusively to the nucleus (Sittler et al., 1996). This site also leads to the most variation among the structures because the ending can shorten exon 15 in several ways. This results in the loss of the S500 phosphorylation site in all but splice forms 1 and 7 as well as the RGG box in some. For splice forms 4, 5, 6, 10, 11, and 12, removal of exon

14 also results in a +1 frame shift such that the last ~100 amino acids are completely different from the other six splice forms. To what extent this alteration affects activity and function is mostly unknown, but it is clear that the structures are highly variable.

Here we asked first whether FMRP splice forms localize axonally, whether the axons contained different densities of FMRP, and whether there were differences in the efficiency with which they localized based on how far into axons they travelled. Next we considered what role the axonally localizing splice forms might be playing in the axons and examined differences in axonal arbor complexity. All splice forms localized axonally with equal efficiencies and densities suggesting that all have a function in this neuronal compartment and the domain required for this localization is located in the N-terminus. A model for a regulatory role of FMRP in axonal arbor complexity was developed in which splice forms that function in this role would oversimplify axonal arbors. SF7 and SF9 led to reduced complexity when overexpressed. These results suggested that the shortened KH2 domain is required and that the C-terminus of FMRP contains a domain required for axon growth and branching. Because several domains are in the C-terminus, the next step used a battery of FMRP-SF7 mutants to ask the same questions of the splice forms with the goal of identifying domains required for axonal localization and axonal arbor complexity regulation. Surprisingly, no mutants lost the ability to localize axonally and did so with the same efficiency except NES34A-transfected neurons. Puncta density was decreased in neurons expressing ΔRGG, S500A, and S500D mutants while axonal arbor complexity did not decrease from endogenous FMRP neurons in neurons expressing S500A, S500D and NES34A mutants. Coupled with the splice form findings, these further suggest that the domain required for axonal localization is located in the N-

terminus while the domain required for regulating axon growth and branching is in the C-terminus and may be the NES and/or S500 phosphorylation site. Though more work is needed to verify these results, the findings here serve as a starting point for the elucidation of mechanisms involving axonal localization of FMRP and its regulatory role in axon growth and branching.

Chapter II: Alternatively Spliced Forms of Fragile X Mental Retardation Protein Localize to Axons

Abstract

The Fragile X Mental Retardation Protein (FMRP) is alternatively spliced to yield at least 12 mRNA transcripts, all of which are translated and can be found at highly variable levels in mouse brain. When absent due to hypermethylation in the *FMR1* gene, Fragile X Syndrome (FXS) occurs in individuals whose gene contains a CGG repeat that has been expanded to over 200 repeats. Intellectual disability, distinctive facial features, macroorchidism, and autistic symptoms are common characteristics of FXS. FMRP is an RNA binding protein that regulates translation at the synapse and can be found in the soma, dendrites, and axons. The mechanism behind trafficking FMRP to the appropriate subcellular domain within neurons is unknown. To begin to unravel this question, we examined the localizational patterns of all 12 splice forms in rat cortical axons. All localized axonally with equivalent efficiencies, suggesting that the domain for axonal localization of FMRP is in the N-terminus, a region common to all splice forms. FMRP functions in regulation of axon growth and branching. Overexpression of splice forms that function in this role was predicted to decrease complexity, a result found in SF7 and SF9-transfected neurons suggesting that the region involved in this role is in the C-terminus and may be the S500 phosphorylation site. More work is required to piece these results together, but this is a first known attempt at characterizing the axonal localization patterns of FMRP splice forms.

Introduction

Fragile X Syndrome (FXS) is an X-linked genetic disorder affecting males more severely than females. In addition to intellectual disability, it is characterized by developmental delay, macroorchidism, and distinctive facial features and shares many characteristics with Autism Spectrum Disorder (ASD) (Bear et al., 2004). Indeed, FXS is the leading known cause of ASD (Kelleher and Bear, 2008). The disorder is caused by silencing of the *FMR1* gene. This gene becomes hypermethylated and silenced following expansion of a CGG trinucleotide repeat in the 5' untranslated region (UTR) (Verkerk et al., 1991; Pieretti et al., 1991).

The *FMR1* gene encodes Fragile X Mental Retardation Protein (FMRP), an RNA binding protein which functions in translational repression of its mRNA targets. It is expressed most highly in the brain and is predicted to regulate the translation of 4% of neuronal mRNAs (Hinds et al., 1993; Darnell et al., 2011). As structure begets function, FMRP possesses a number of domains and post-translational modification sites. Among these are three RNA binding domains, tandem K homology (KH) domains and an RGG box, a serine phosphorylation site which regulates FMRP, as well as nuclear localization and export sequences (NLS, NES; Siomi et al., 1993; Bardoni et al., 1997; Siomi et al., 2002, Dolzhanskaya et al., 2006). Together, these domains coordinate the various functions of FMRP.

In addition to these functional domains, the *Fmr1* transcript has several alternative splice sites, giving rise to up to 12 mRNA transcripts. At least five have been identified in Western blot from mouse brain and all transcripts are spatially and temporally regulated during mouse development (Verheij et al., 1995; Bracket et al., 2013). The *Fmr1* splice

forms are of interest due to the potential effects of biochemical alterations as a result of the alternative splicing events. Splice Form 1 (SF1) is the full-length transcript. The first alternative splice site does not occur until exon 12 which encodes part of the KH2 domain. This splice site does not fully remove the domain but does shorten it. SF7-12 possess the shorter KH2 domain. The next splice site occurs at exon 14, either removing or retaining the NES domain. SF4-6 and SF10-12 lack this domain. Past research has shown that loss of this domain prevents FMRP from exiting the nucleus (Sittler et al., 1996). There are two possible acceptor sites for the last splice site, which occurs in exon 15, the location of the S500 phosphorylation site. Interestingly, only SF1 and SF7 retain this site, raising the question of what regulates other FMRP splice forms. This splice site also introduces a +1bp frameshift such that half of the splice forms (4-6, 10-12) have an entirely different C-terminus in addition to losing the RGG domain. It is speculated that unidentified domains could be present in these transcripts.

Though the exact effects of alternative splicing events in *Fmr1* are unknown, the localizational preferences of these can be evaluated. Here, we used splice form specific constructs of *Fmr1* fused to EGFP to examine the axon localization capabilities of each in cultured rat cortical neurons. All splice forms were able to traffic into axons and localized with equivalent levels and efficiencies, suggesting that the domain required for axonal localization is found in the N-terminus which is conserved among splice forms. Overexpression of FMRP was predicted to reduce axonal arbor complexity; SF7 and SF9-transfected neurons displayed this simplification suggesting a C-terminal domain or post-translational modification site is involved in this role for FMRP in axon growth and branching. As intriguing as these results are, more work, including mutation analysis

(Chapter 3) is required to further understand the role of splice forms in axonal localization and function of FMRP.

Materials and Methods

Constructs

Splice forms 1, 3, 7, 8, and 9 including the 3’ UTRs were provided by the laboratory of Dr. David Morris at the University of Washington. PCR primers were designed to subclone the coding sequence starting at amino acid 2 with 5’ extensions to introduce *XmaI* and *SacI* restriction sites. The forward primer was 5'-GAGCTCAAGAGGAGCTGGTGGTGAAGTGC-3' and the reverse primer was 5'-CCCGGGTTAGGGTACTCCATTCACCAGC-3'. Two As were added between the *XmaI* site and the beginning of the coding sequence so that the PCR product could be inserted in frame with an N-terminal EGFP. The PCR products were placed in pCAGES-EGFP vector with CAG as the promoter.

Splice forms 2, 4, 5, 6, 10, 11, and 12 were obtained as fragments from GeneArt Strings services (LifeTechnologies) with 5’ extensions to introduce *XmaI* and *SacI* restriction sites as well as two As to place them in frame with an N-terminal EGFP. The fragments were blunt-end ligated into ZeroBlunt vector using the ZeroBlunt® TOPO® PCR Cloning kit (LifeTechnologies), sequenced, and then cut out using the *XmaI* and *SacI* sites and placed in pCAGES-EGFP.

The laboratory of Dr. Justin Fallon at Brown University provided pCAGES-tdTomato.

Neuronal Cell Cultures

Coverslips for cortical neuron cultures were cleaned and sterilized with nitric acid and ethanol and prepared for cell culture with PDL followed by laminin coating. P0 rat cortical neurons were obtained from Neurons-R-Us at the University of Pennsylvania and plated at a density of 400,000 neurons per well in a 24-well plate. They were maintained in Neurobasal media supplemented with GlutaMax, penicillin-streptomycin, and B27. Cultures were grown at 37C, 5% CO_2 for 3 days prior to transfection.

Transfections

Transfections were performed by magnetofection using Neuromag (Oz Biosciences) at a ratio of 1ul Neuromag to 1ug DNA (0.5ug TdTomato; 0.5ug EGFP-FMRP-SF) in 1x OptiMem (Life Technologies). pCAGES-tdTomato and each splice form construct were cotransfected into 3 days *in vitro* cultured cortical neuron cultures from P0 rat pups. The cultures were then maintained at 37°C, 5% CO_2 for 1 week.

Fixation

Neurons were fixed after a total of 10 days *in vitro* using 4% PFA/4% sucrose in 1x PBS for 15 minutes. They were then washed 3x in 1x PBS and stained with DAPI (1:10000). The coverslips were mounted using 85% NPG (4% n-propylgallate (Sigma P3130), 85% glycerol, phosphate buffer (pH 7.4), water) and stored at 4°C.

Imaging, Analysis, and Statistics

Neurons were imaged with a Leica confocal microscope using tile-scan and z-stacks to capture the entire length of axons at 40X oil immersion. Stacks were collapsed in Fiji. Axons were traced and puncta counted using Neurolucida. Traces were analyzed

using “Branched Structure Analyses” in Neurolucida Explorer. Data types including number of puncta, distance from puncta, longest axon, size of axonal arbor, complexity (calculated by [Sum of terminal orders + Number of terminals] * [Total axonal length] (MBF Bioscience)), branch order number, node number, and terminal numbers were obtained from these and analyzed. One-way ANOVA followed by Dunnett Contrasts test for multiple comparisons of means was done in R. Plots were made in Prism; the Kruskal-Wallis test for non-parametric data followed by Dunn’s post-hoc for multiple comparisons was performed using GraphPad Prism (version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

Results

Splice form 7 (SF7) of FMRP exhibits axonal localization (Antar et al., 2006). Whether the other 11 splice forms would localize axonally was yet to be determined. In order to investigate this, cortical neurons isolated from P0 rat pups were grown for 10 days *in vitro*. They were transfected with EGFP-tagged FMRP splice forms to visualize their axonal localization and tdTomato to visualize whole neurons. In order to determine which splice forms localize axonally as well as identify functional roles for these splice forms, several questions were asked: 1) which splice forms exhibit axonal localization, 2) are there differences in the amount of FMRP localizing to axons among these splice forms, 3) are some splice forms more efficient at localizing throughout the axon, and 4) which splice forms function in regulation of axonal arbor complexity?

Prior to determining answers for these questions, we first examined the expression levels of the individual constructs. This was done to determine if there were any

construct-specific effects on the efficiency of protein synthesis. Mean pixel intensities of cell bodies were measured and compared between splice forms (Fig. 2.1). There were no differences in the expression levels between splice form constructs ($p = 0.5222$). The axonal localization of each splice from was determined by sight (Fig. 2.2-2.13). All splice forms exhibited axonal localization.

Since all splice forms exhibited axonal localization, we next looked for differences in the amount of FMRP puncta entering axons. Puncta densities were calculated by determining the number of puncta per 100um per neuron (Fig. 2.14a). This showed that all splice forms were expressed at equivalent levels. None were significantly different from SF7 (Dunn's post-hoc for multiple comparisons), but differences did exist among other splice forms ($p = 0.0125$; $p = 0.0315$ (SF3 vs SF9); $p = 0.0309$ (SF3 vs SF12)). An interesting but uncommon feature of some splice forms was the formation of elongated puncta (Fig. 2.14b). Termed elongated structures, these were distinct from typical, rounded puncta and appeared to fill the axonal segments through which they occurred. Among the neurons examined, SF3, SF7, and SF8-transfected neurons never formed these. They seemed to occur most frequently in SF11, appearing in about 1 in every 5 neurons. Furthermore, these structures typically formed only once or twice in a neuron and were never seen longer than 15um (a puncta is typically less than 1um in length).

In order to examine possible differences in the distribution of puncta throughout axonal arbors, cumulative distributions were generated using the distance of each puncta from the cell body (Fig. 2.14c). There were no differences between the splice forms; all were able to localize axonally at equivalent efficiencies. Together, these results suggest

that all splice forms localize axonally at equivalent quantities and efficiencies. Because the first alternative splice site does not occur until exon 12, this suggests that a region N-terminal to this exon is required for axonal localization of FMRP. Furthermore, these localizational abilities imply that all splice forms also function in axons.

FMRP has been implicated in a variety of axonal and presynaptic functions including axon growth and branching, ion channel regulation, and protein synthesis-dependent long term plasticity (Li et al., 2009; Brown et al., 2010; Zheng et al., 2012; Ferron et al., 2014; Deng et al., 2013; Till et al., 2010). Here, we chose to investigate its role in the regulation of axon growth and branching. We began by comparing the length of the longest axon as well as the size of the axonal arbor among the splice forms (Fig. 2.15a, b). When compared to neurons expressing only endogenous FMRP, there were no differences among the splice form-transfected neurons. However, comparing the overall complexities of the axonal arbors to that of arbors from neurons expressing only endogenous levels of FMRP showed that neurons overexpressing splice forms 1, 7, and 9 had significantly reduced axonal arbor complexities (Fig. 2.15c). This result was repeated for SF7 and SF9 and sometimes for SF1 and SF8 in analyses of highest branch order, number of nodes, and number of axon terminals, all readouts related to axonal arbor complexity (Fig. 2.15d-f). FMRP regulates axonal arbor complexity by mediating Semaphorin 3A signaling (Li et al., 2009). Axonal arbors are overelaborated in *Fmr1* KO neurons. Thus, overexpression of splice forms which function in axonal arbor localization was predicted to result in oversimplification of axonal arbors. Thus, SF7 and SF9 and possibly SF1 and SF8 may function in regulation of axonal arbor complexity while the

others do not. This may also implicate a requirement for the C-terminus that results from no frame shift in this function.

Discussion

Alternative splicing in *Fmr1* transcripts offers versatility and specificity in FMRP structure and function. Here, we attempted to identify whether all splice forms can localize axonally as well as whether they differ in localizational efficiency or the quantity of FMRP that enters axons. This was done by co-transfecting GFP-labeled FMRP and tdTomato into cultured rat cortical neurons and analyzing axons for localizational differences between mutants as well as puncta densities and axonal arbor complexities. These analyses showed that all splice forms seemed to be capable of localizing axonally at equal efficiencies. There were no differences between SF7 and the others in terms of axonal localization, efficiency, or puncta density. Axonal arbor complexity was significantly decreased in SF7 and SF9 compared to neurons expressing endogenous levels of FMRP. These results show that all splice forms have axonal functions and also implicate the N-terminus in axonal localization but the C-terminus in regulation of axon growth and branching.

Of the 12 FMRP splice forms, SF 1-3, 6-9, and 12 are the most highly expressed in mouse brain (Brackett et al., 2013), but SF 4, 5, 10, and 11 are still present. Because these are natural variants, it is fairly unsurprising that no differences between SF7 and the others were found in either axonal arbor length or longest axon length. SF7 is used as a baseline here because it is the most highly expressed transcript in four cell lines (Dury et al., 2013) as well as P0 mouse brain (Brackett et al., 2013). Cumulative frequency

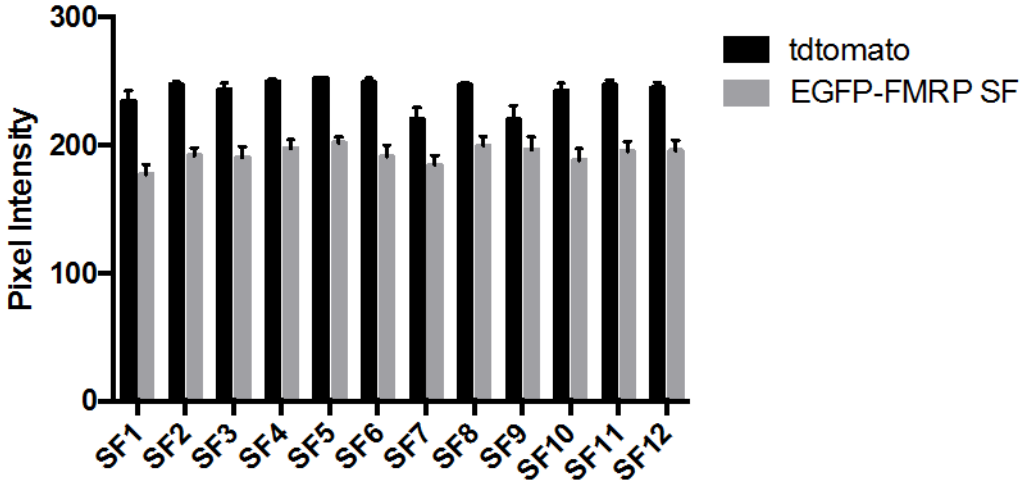

Figure 2.1 Construct expression levels do not differ between splice forms. Mean pixel intensity of cell bodies of each splice form for tdTomato and EGFP-FMRP SF (Kruskal-Wallis, p = 0.5222).

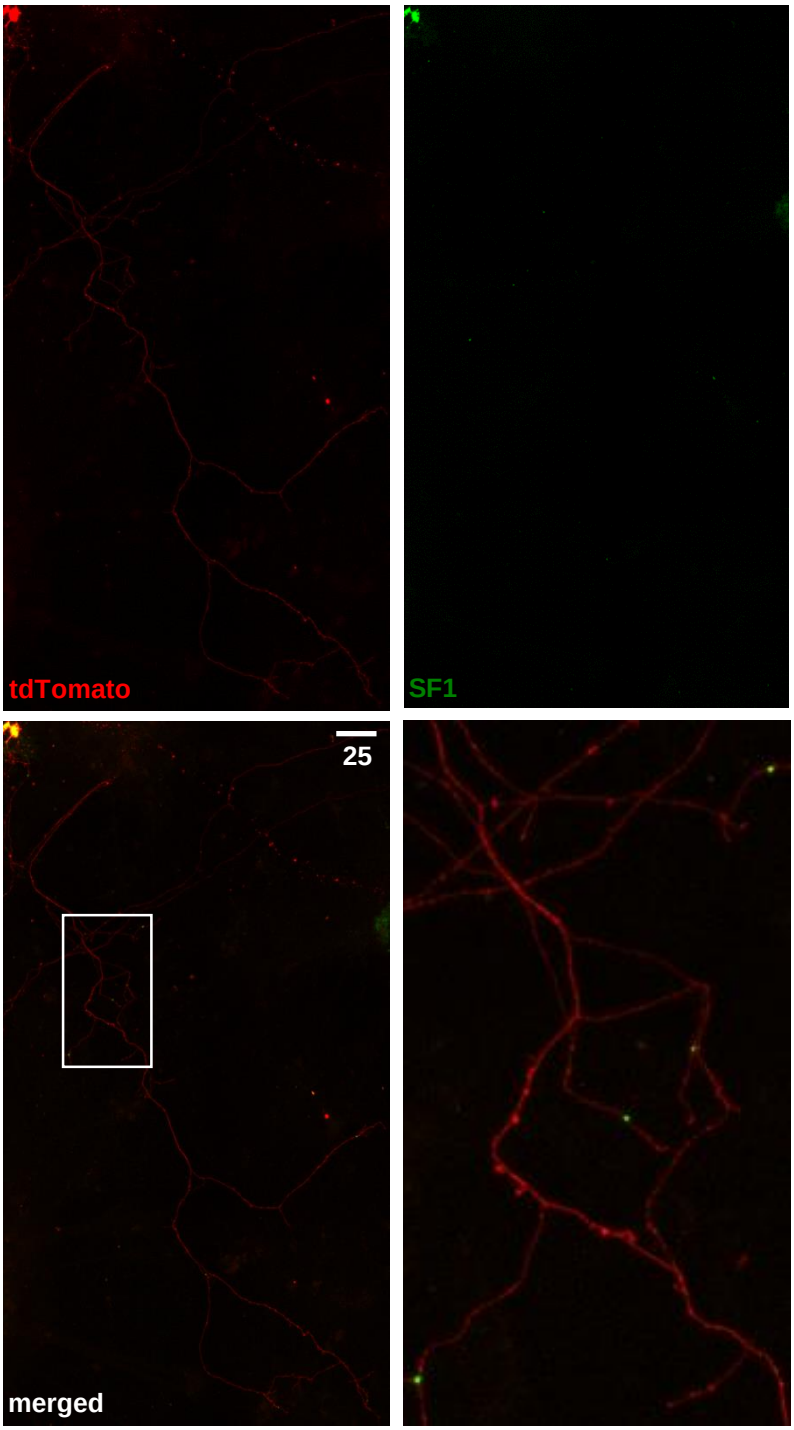

Figure 2.2: Axonal localization of EGFP-FMRP-SF1.

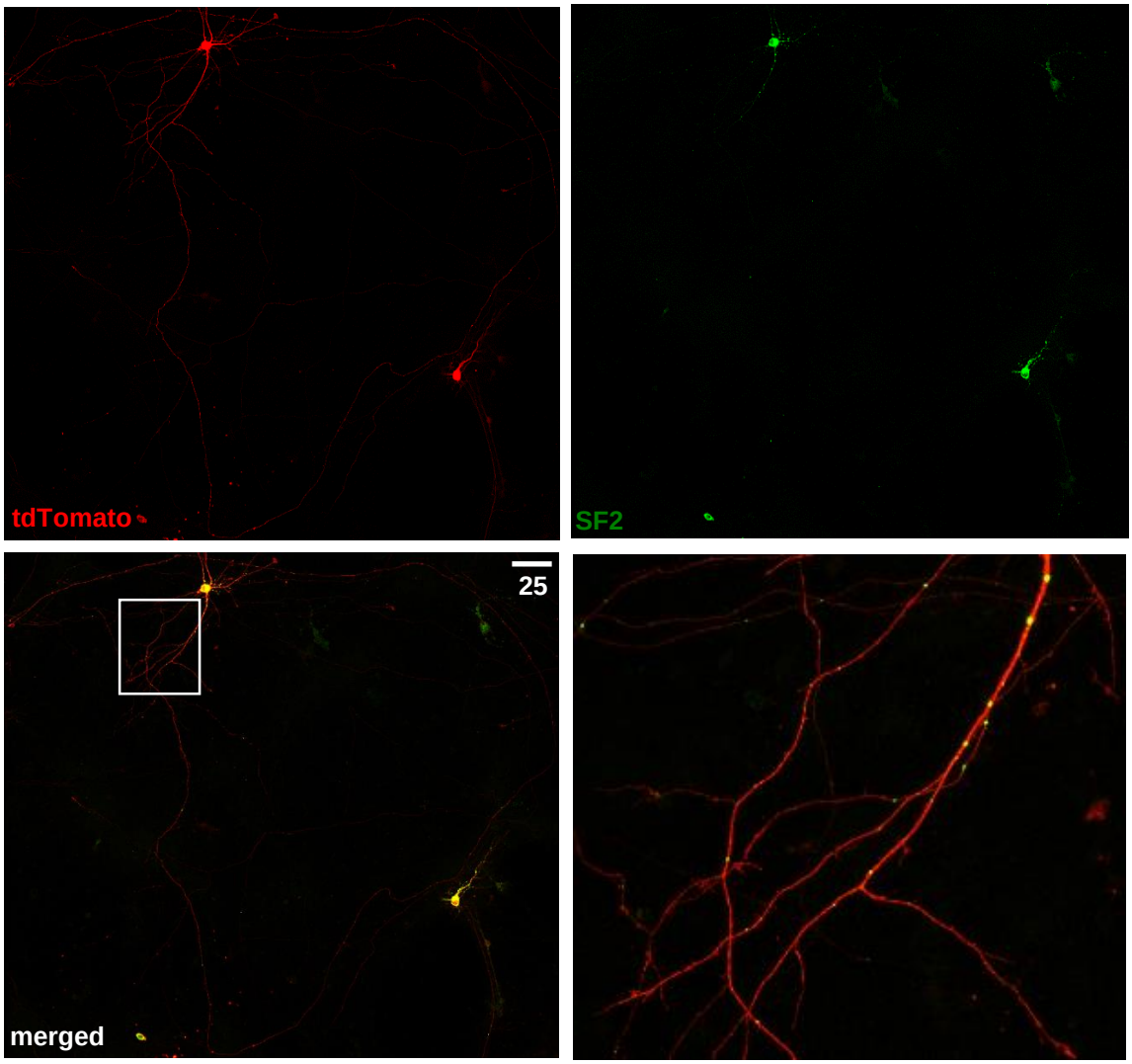

Figure 2.3: Axonal localization of EGFP-FMRP-SF2.

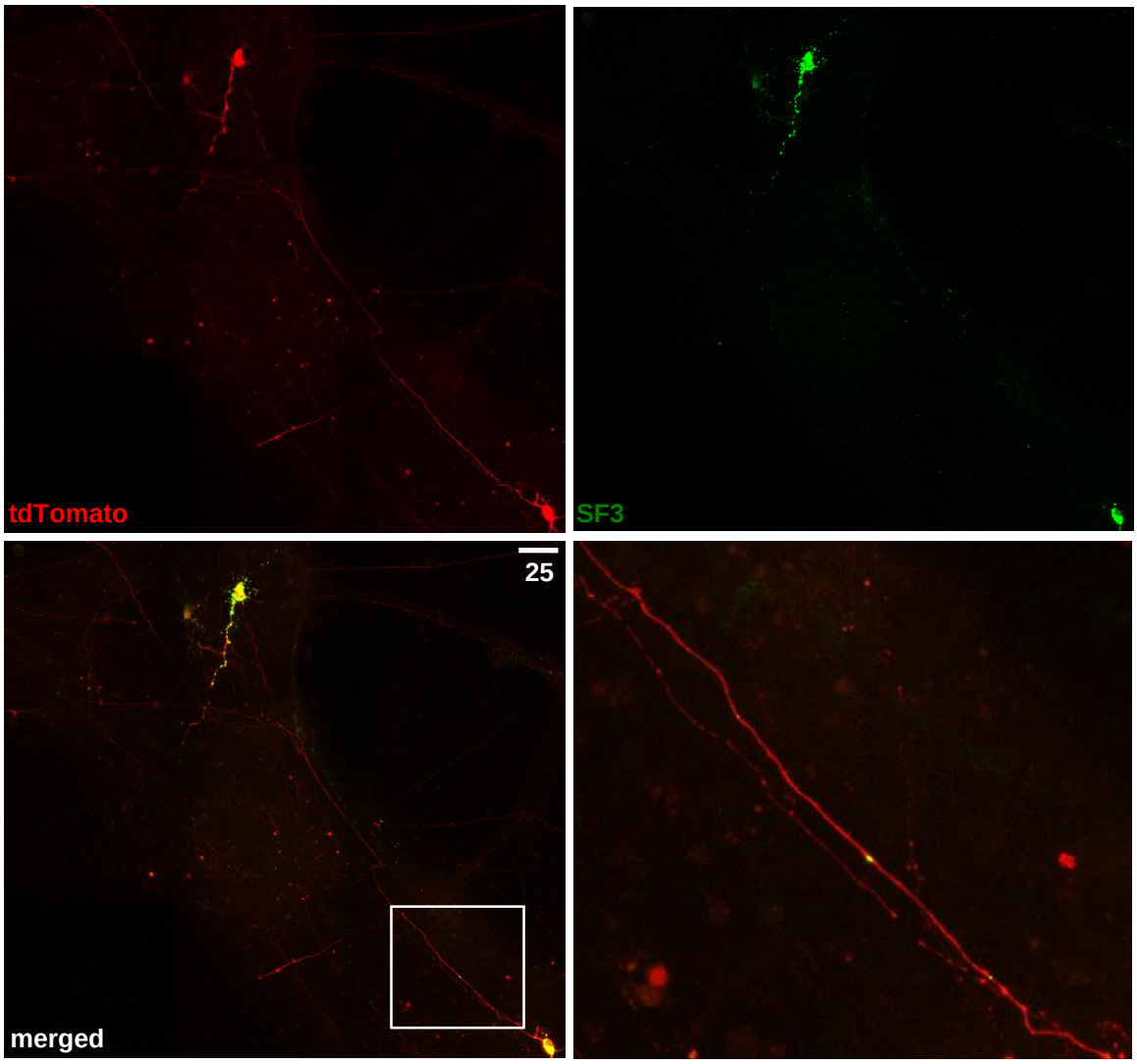

Figure 2.4: Axonal localization of EGFP-FMRP-SF3.

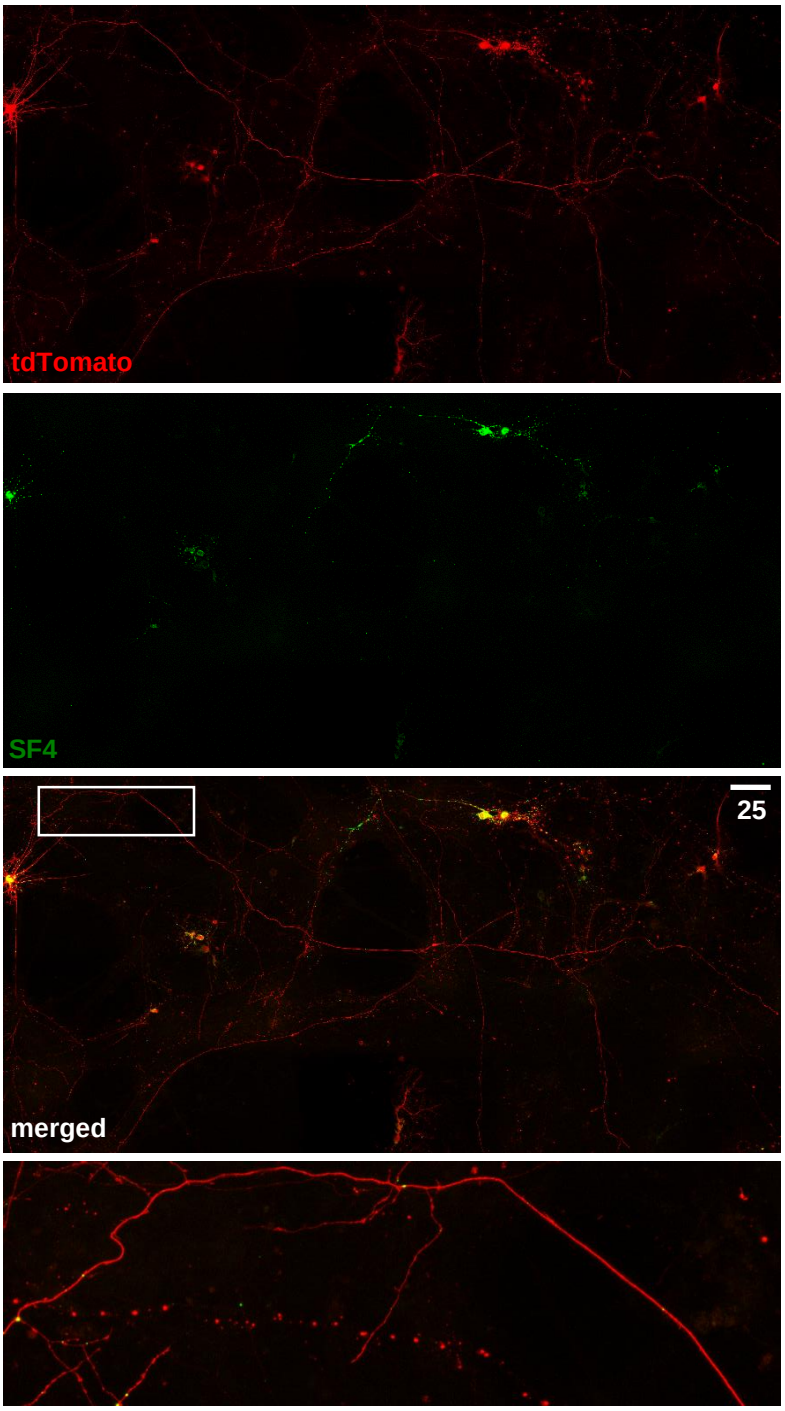

Figure 2.5: Axonal localization of EGFP-FMRP-SF4.

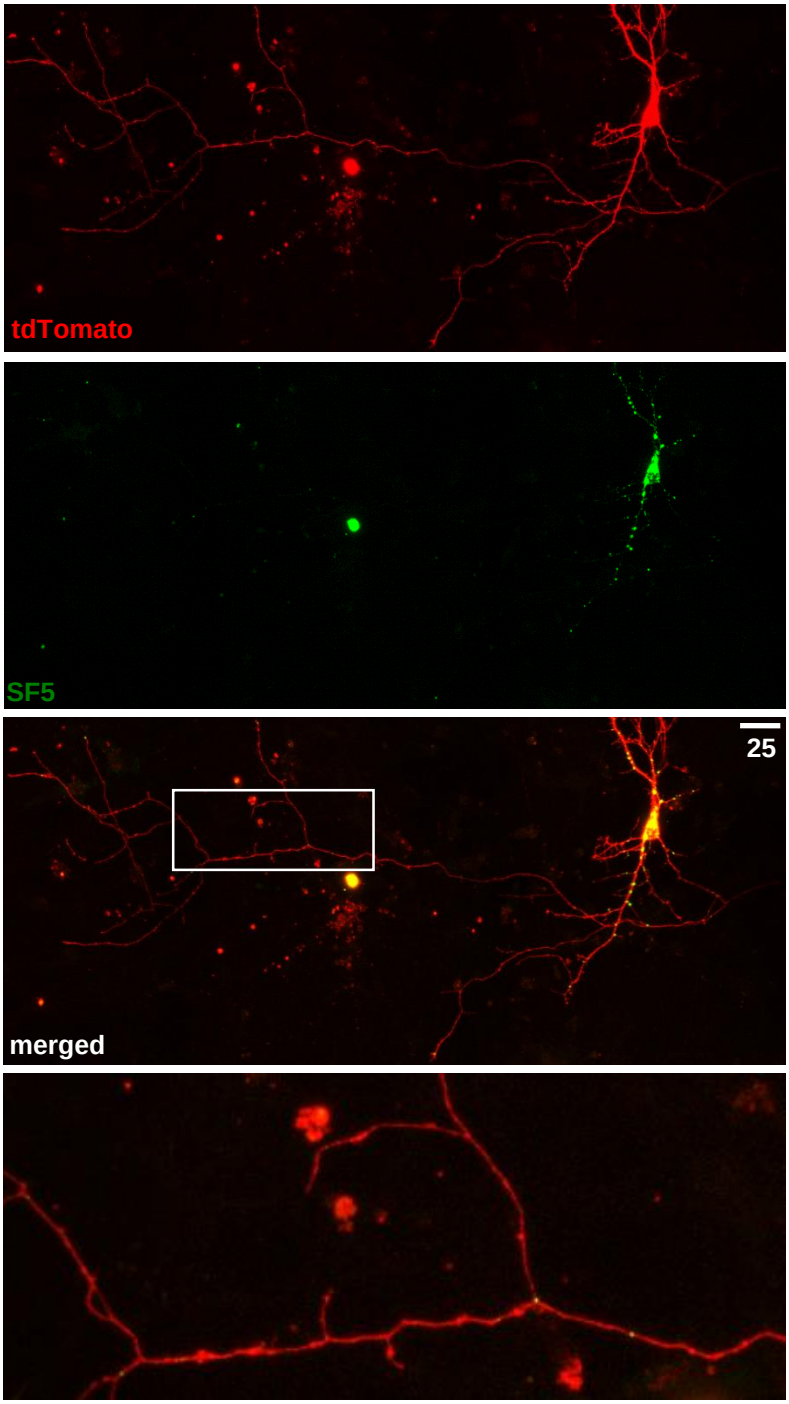

Figure 2.6: Axonal localization of EGFP-FMRP-SF5.

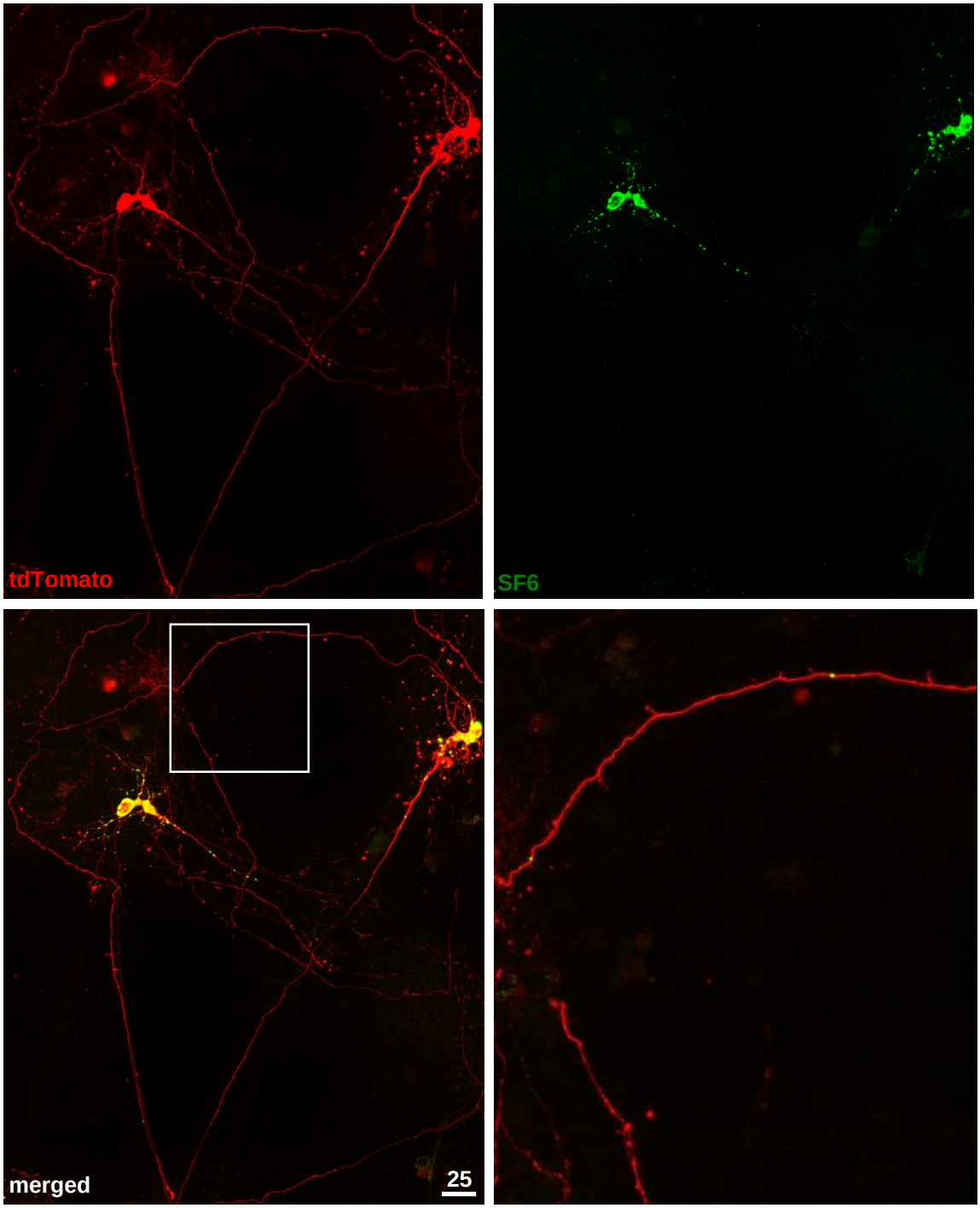

Figure 2.7 Axonal localization of EGFP-FMRP-SF6.

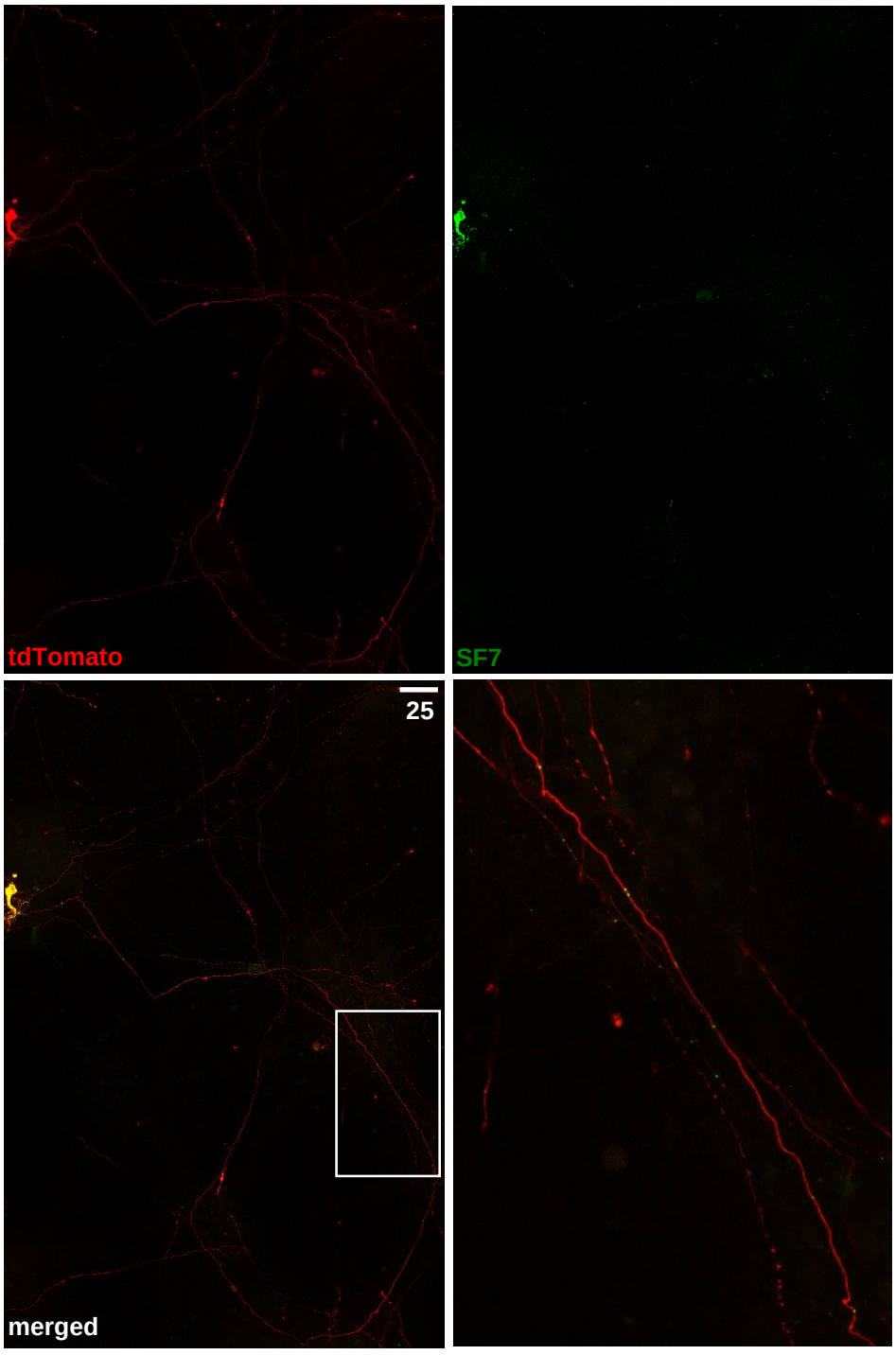

Figure 2.8 Axonal localization of EGFP-FMRP-SF7.

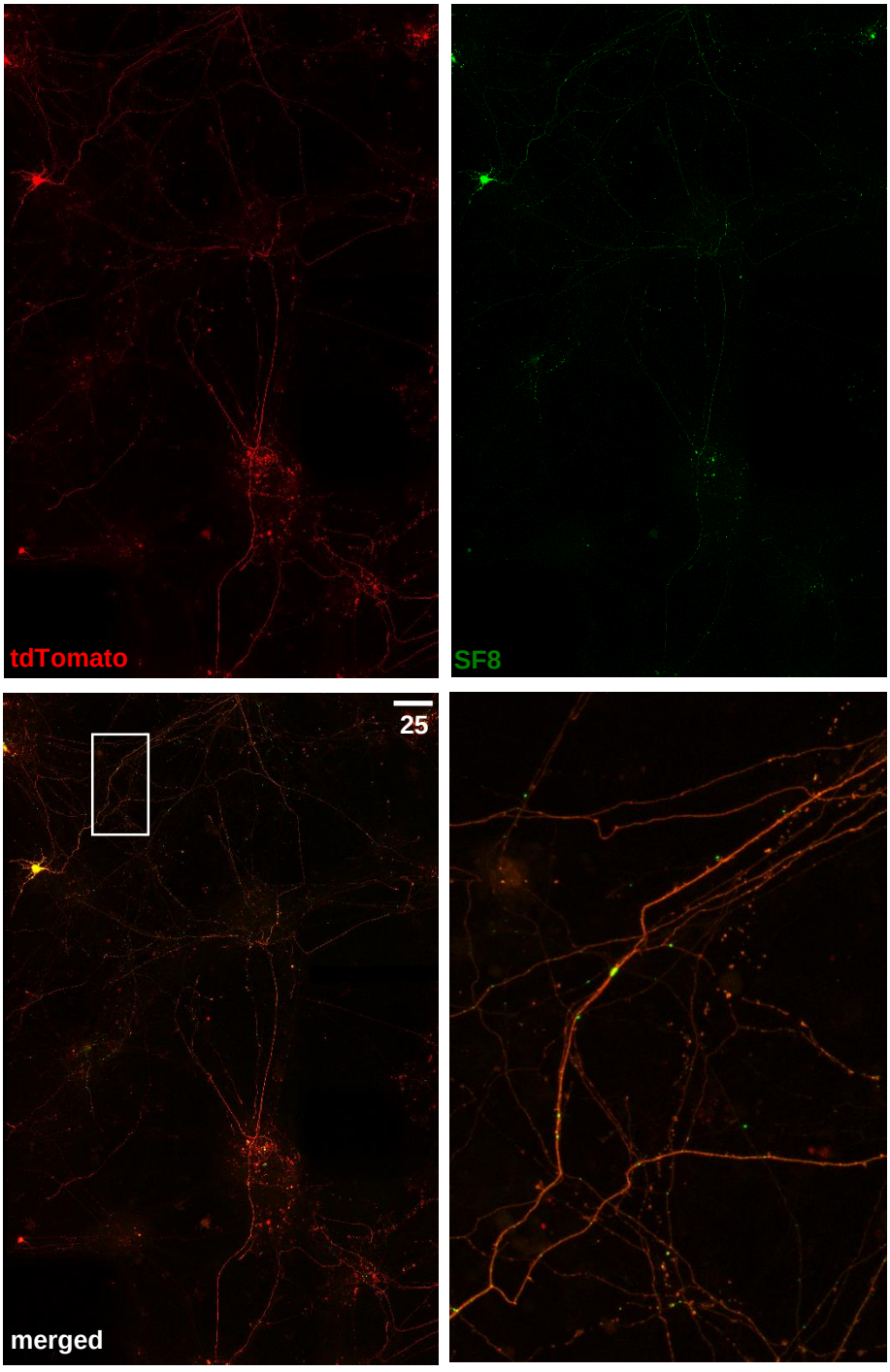

Figure 2.9: Axonal localization of EGFP-FMRP-SF8.

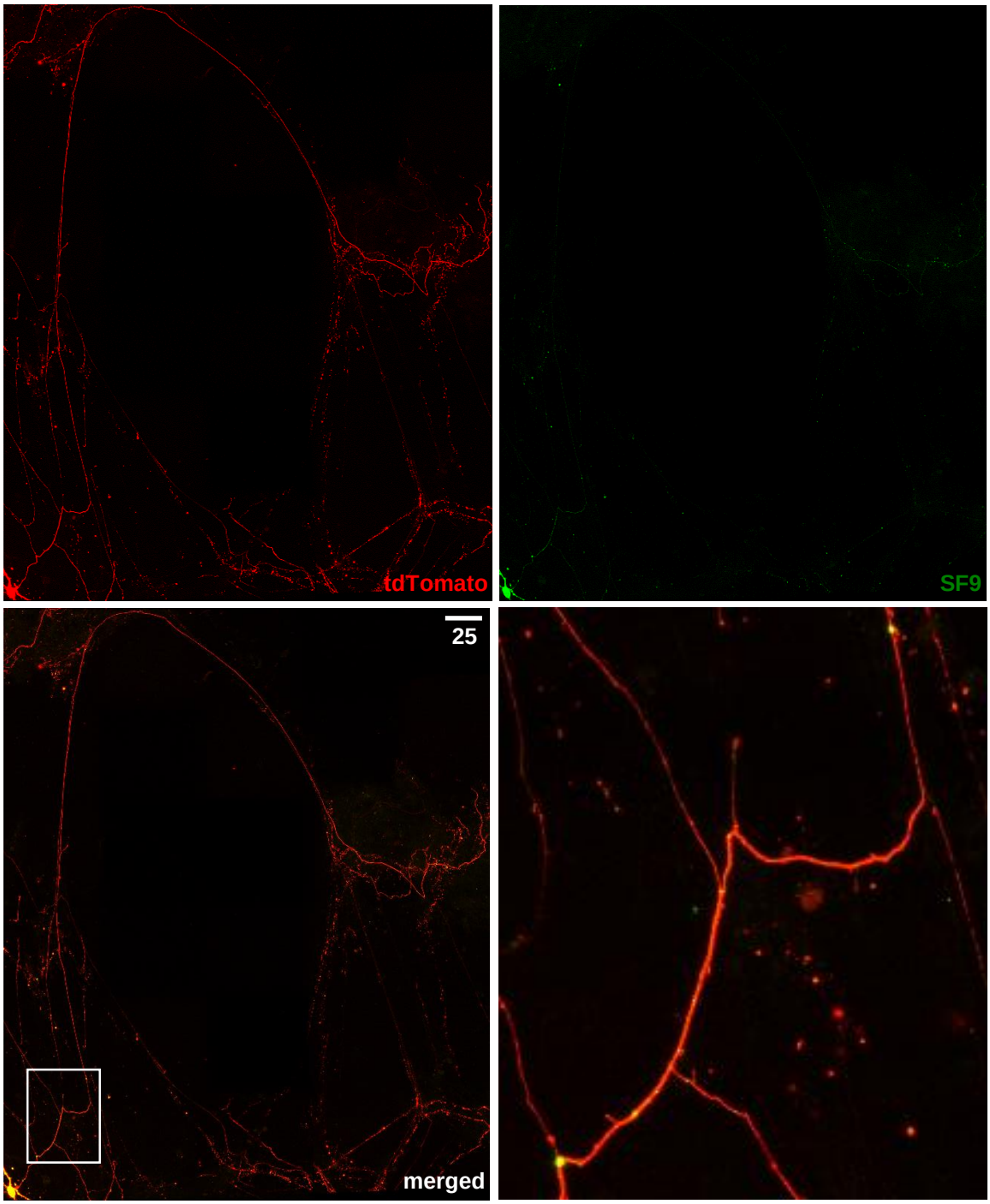

Figure 2.10: Axonal localization of EGFP-FMRP-SF9.

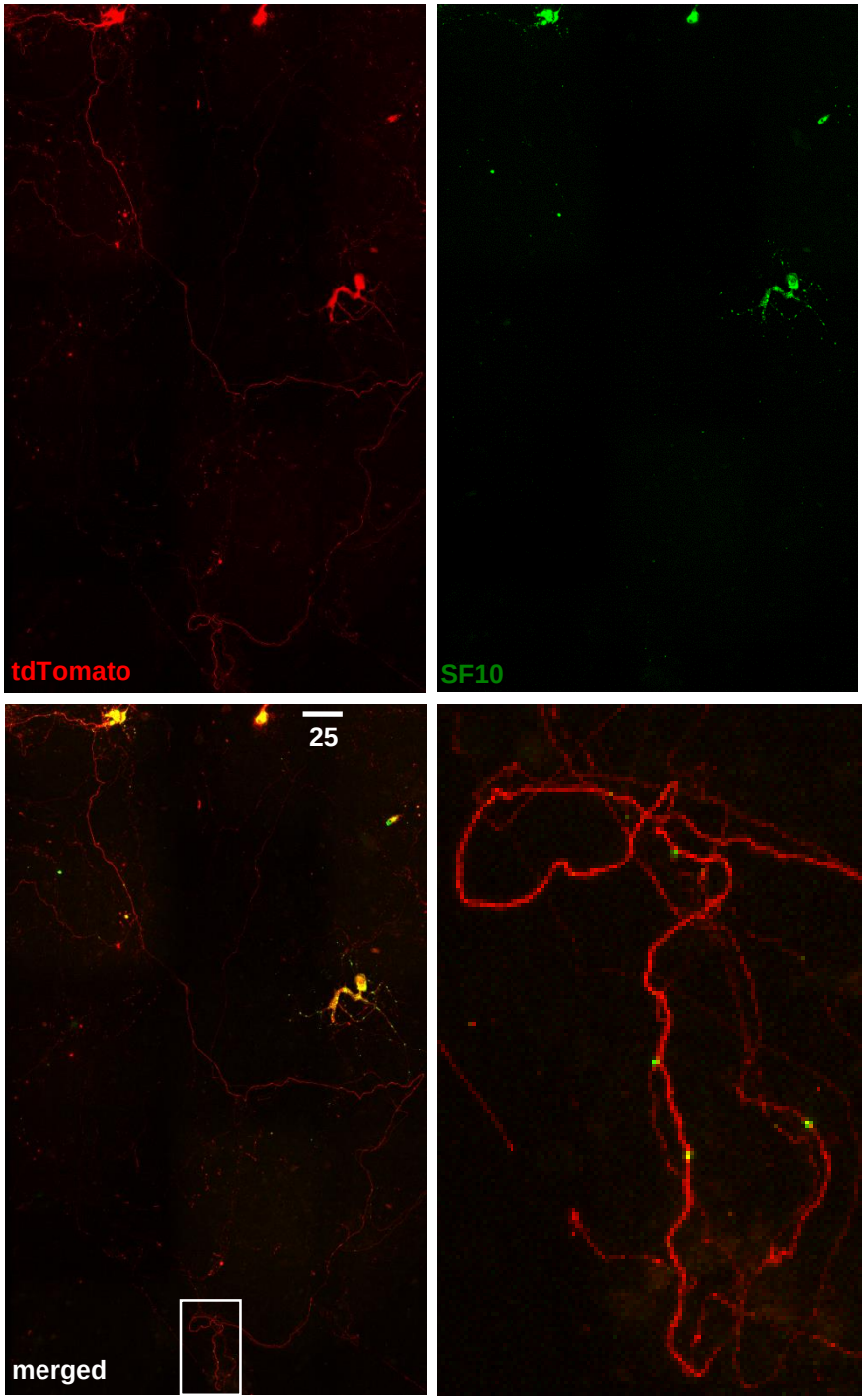

Figure 2.11: Axonal localization of EGFP-FMRP-SF10.

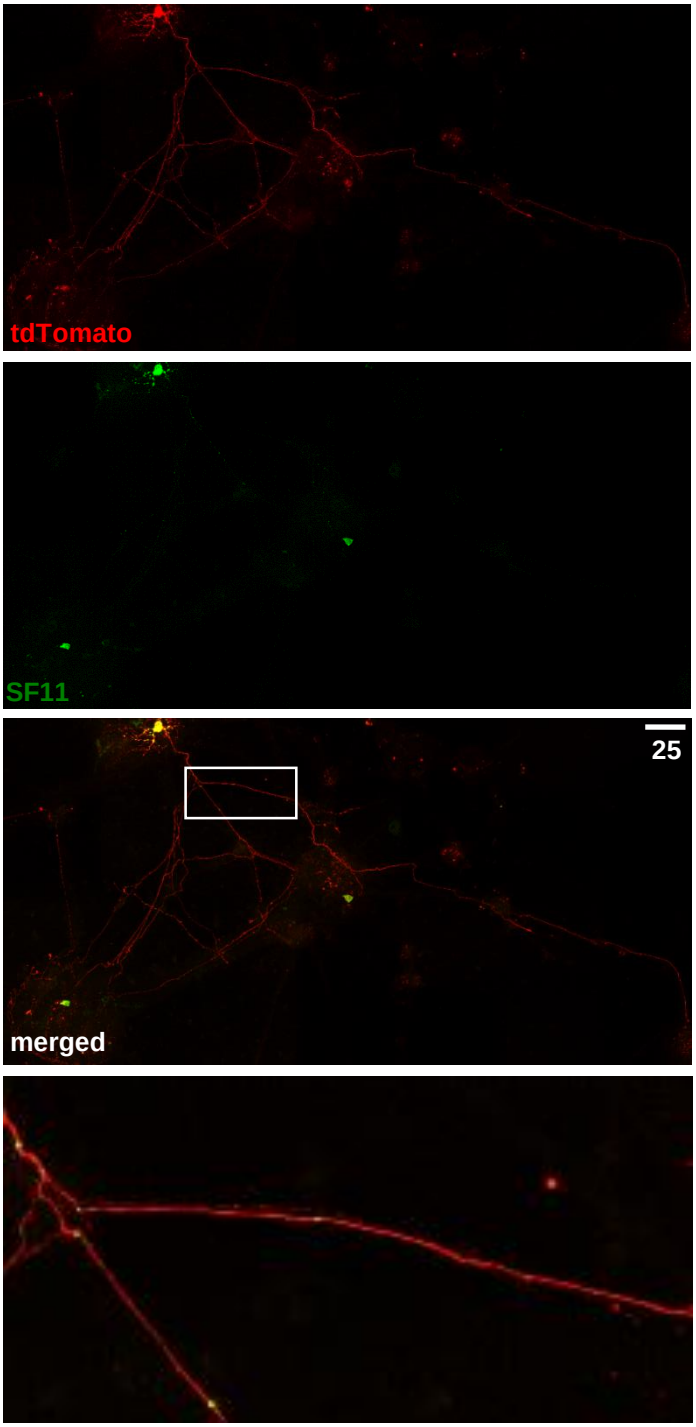

Figure 2.12: Axonal localization of EGFP-FMRP-SF11.

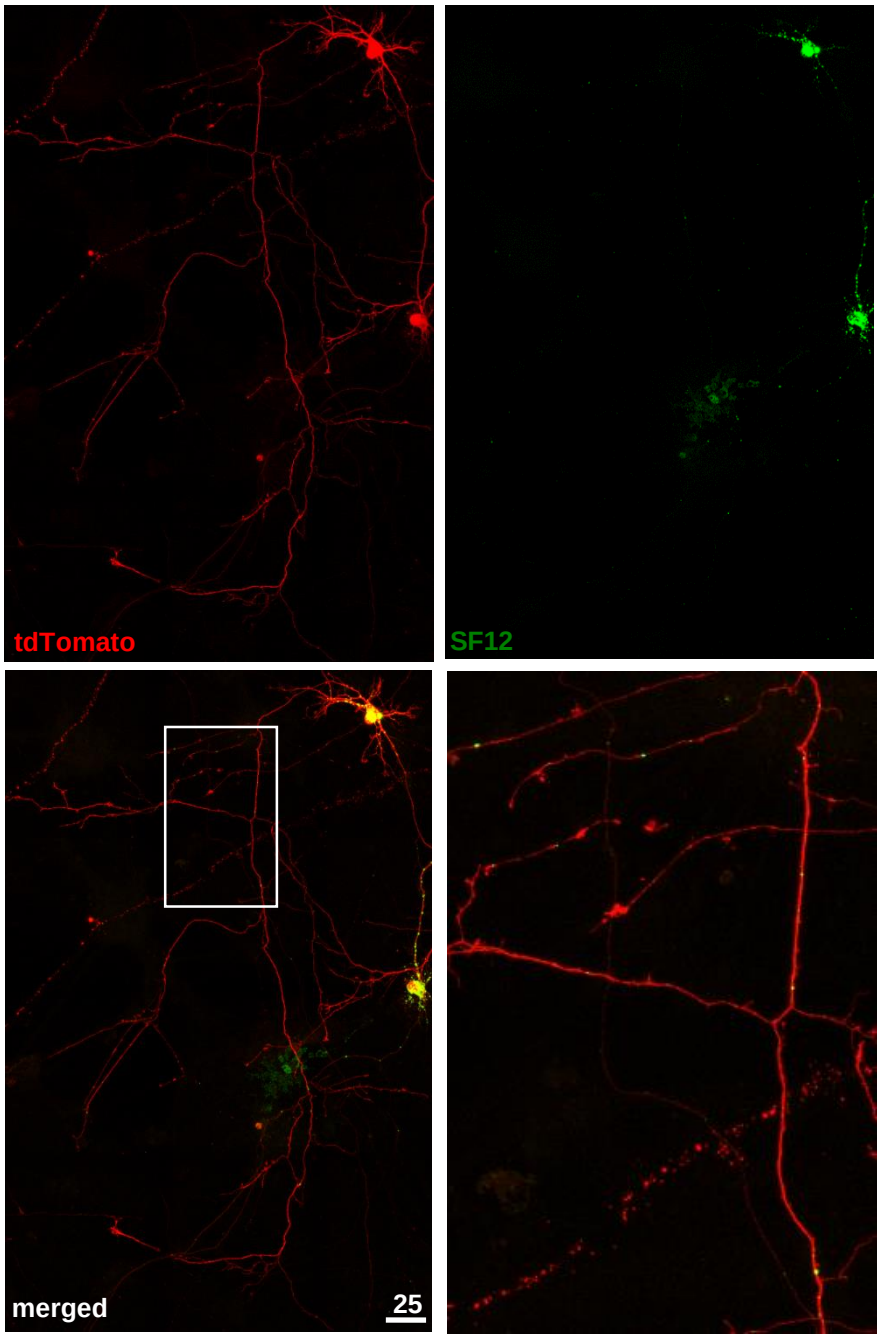

Figure 2.13: Axonal localization of EGFP-FMRP-SF12.

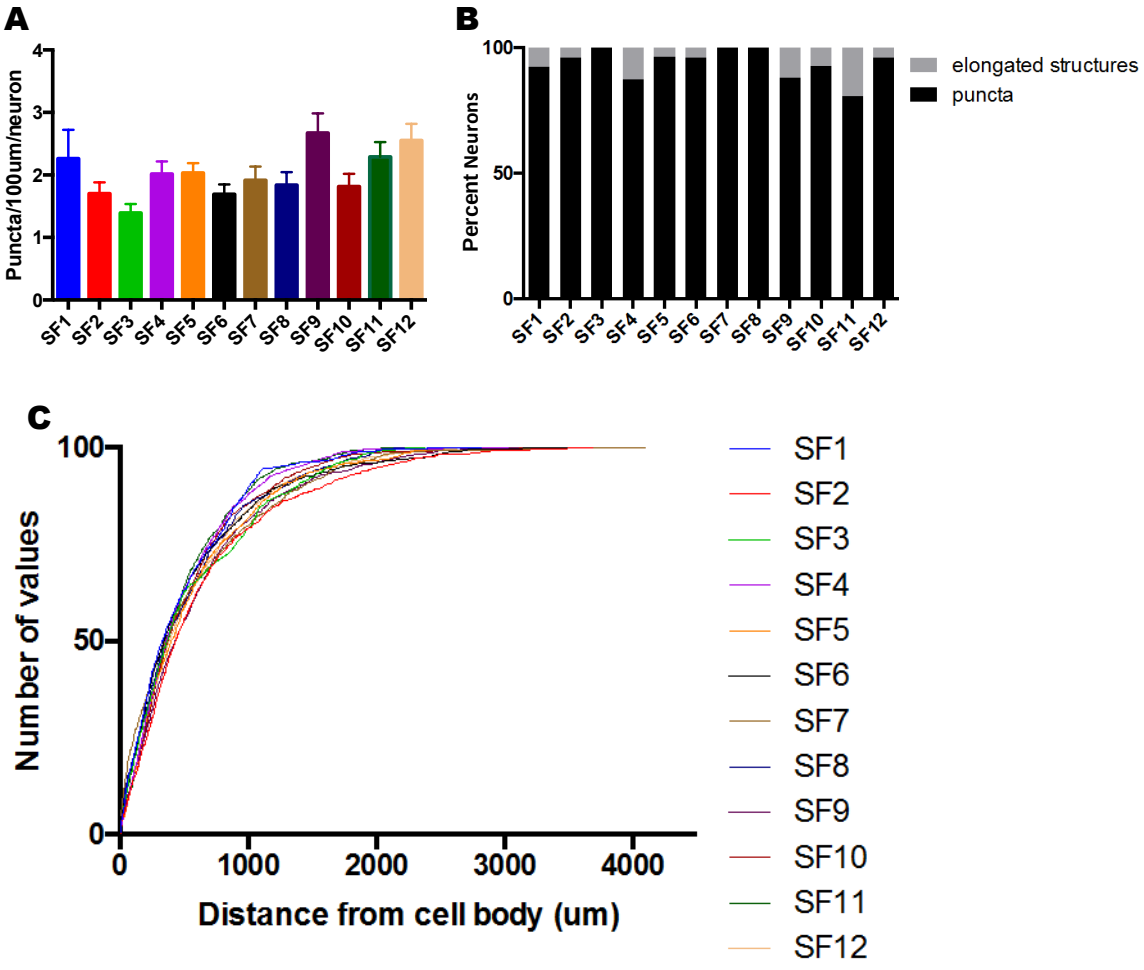

Figure 2.14 All FMRP splice forms localize to axons at equivalent levels and efficiencies. A) Mean density of puncta through axonal arbors. None were significantly different from SF7 (Kruskal-Wallis, p = 0.0125; Dunn's post-hoc for multiple comparisons, p = 0.0315 (SF3 vs SF9); p = 0.0309 (SF3 vs SF12) B) Percent neurons in which elongated structures form for each splice form C) Cumulative distributions of splice forms. None are significantly different from SF7 (Tukey Contrasts). Error bars represent SEM.

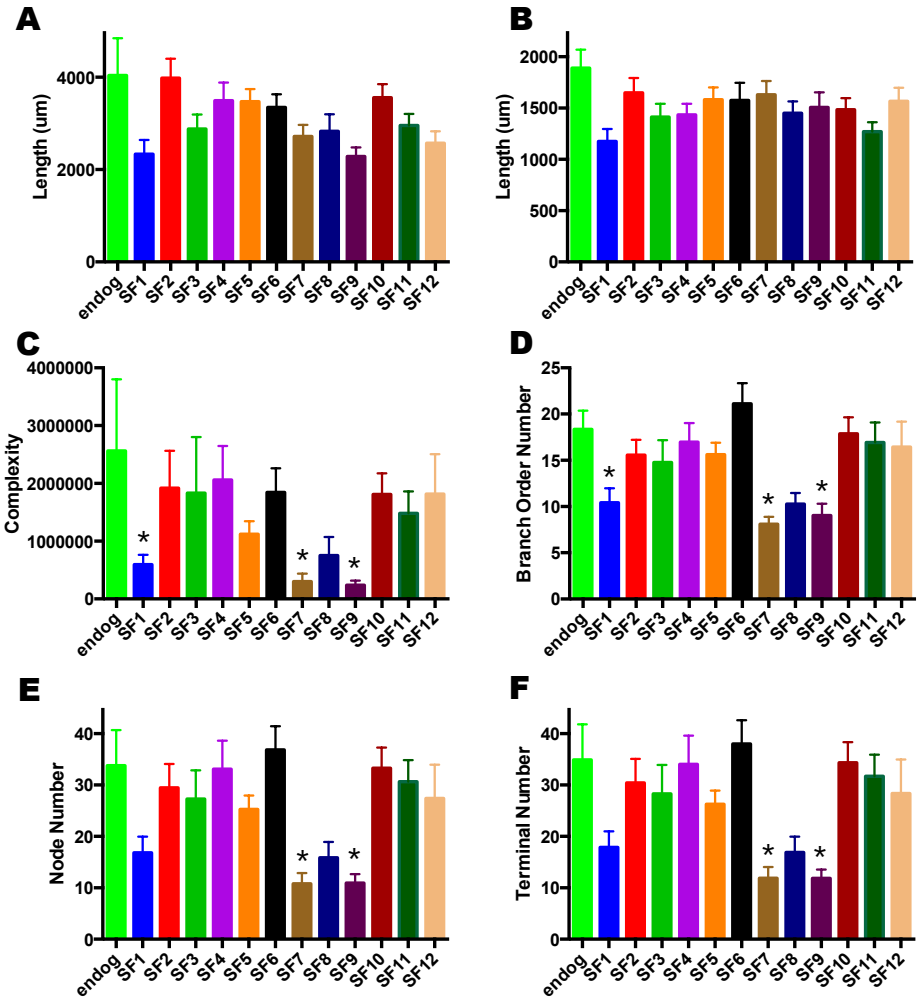

Figure 2.15 Axonal arbors are oversimplified when SF7 and SF9 are overexpressed compared to neurons expressing endogenous levels of FMRP A) Axonal arbor length means varied among splice forms but were not different from endogenous levels of FMRP ($p = 0.0004$). B) Longest axon means were not different ($p = 0.1211$). C) Complexity means [Sum of terminal orders + Number of terminals] * [Total axonal length]. SF1, SF7, and SF9 less elaborate than endogenous ($p < 0.0001$; $p = 0.0345$ (SF1), $p = 0.0034$ (SF7), $p = 0.0882$ (SF8), $p = 0.0042$ (SF9)) D) Highest branch order means. SF1, SF7, SF9 have fewer branches ($p < 0.0001$; $p = 0.0268$ (SF1), $p = 0.0019$ (SF7), $p = 0.0683$ (SF8), $p = 0.0074$ (SF9)) E) Number of nodes means. SF7, SF9 have fewer nodes ($p < 0.0001$; $p = 0.0864$ (SF1), $p = 0.0024$ (SF7), $p = 0.0798$ (SF8), $p = 0.0064$ (SF9)) F) Number of axon terminals means. SF7, SF9 have fewer terminals ($p < 0.0001$; $p = 0.0877$ (SF1), $p = 0.0025$ (SF7), $p = 0.0793$ (SF8), $p = 0.0059$ (SF9)). Statistics run using Kruskal-Wallis test followed by Dunn's post-hoc for multiple comparisons. Error bars represent SEM.

distributions of all puncta distances from the cell body for each splice form also showed no difference in efficiency of axonal localization. That all splice forms are able to localize axonally and at the same efficiency may suggest either that an N-terminal structure common to all splice forms is responsible for this transport or that the structure of FMRP is not behind the mechanism of axonal localization. Very few of the FMRP domains are common to all splice forms. The N-terminus contains a nuclear localization sequence (NLS), an Agenet (Tudor) domain, a recently identified KH domain named KH0, and the long-known KH1 domain (Ashley et al., 1993; Sittler et al., 1996; Alpatov et al., 2014; Myrick et al., 2015a). The NLS is involved in nucleocytoplasmic shuttling of FMRP while the Agenet domain has been implicated in a chromatin-binding function for FMRP. Neither are obvious candidates for axonal localization. The KH1 domain is one of the RNA binding domains. Little is known about it, but its mutation does affect translational inhibition by FMRP (Chen et al., 2014). The KH0 domain was discovered from an R138Q point mutation in a FXS patient and is thought to be important in a presynaptic-specific function of FMRP as it was unable to rescue neuromuscular junction synaptic overgrowth in *Drosophila* as well as cortical pyramidal and hippocampal neuron AP broadening, both presynaptic functions (Myrick et al., 2015b). Myrick and colleagues do not investigate why this mutation is not able to rescue these phenotypes, but we speculate that the ability of FMRP to localize axonally and therefore be present in the presynapses was inhibited. A function in axonal localization for this domain would explain why all splice forms were able to traffic to axons since they all possess the KH0 domain.

Intriguingly, a number of splice forms including SF1, 7, 8, and 9 appeared to have simplified axonal arbors compared to neurons expressing endogenous levels of FMRP, however only SF7 and SF9 were consistently significantly different when we also looked at highest branch number, number of nodes, and number of axon terminals. These observed differences suggest that there are one or more FMRP splice forms involved in regulation of axonal arbor branching though the other splice forms are expected to have some other axonal function not identified here. The role of FMRP in axonal complexity is regulatory and translation-dependent (Li et al., 2009). Semaphorin 3A (Sema3A) signaling results in increased levels of MAP1B as a result of derepression of these mRNAs by FMRP such that axon branching is reduced without affecting axon length. *Fmr1* KO neurons show reduced growth cone collapse and thus increased axonal arbor complexity. But what occurs in conditions of FMRP overexpression like we have here? Our prediction is that overexpression of FMRP would repress axon branching, thereby reducing elaboration. In this model, SF2-6, 8, and 10-12 are unchanged compared to baseline complexity while SF7 and SF9 show oversimplified axons suggesting that SF7, SF9, and possibly SF1 have a role in axonal arbor complexity.

Based on the model, most splice forms do not function in regulating axon growth and branching. The first alternative splice site gives rise to a shortened KH2 domain in SF7-12; SF1-6 have the full-length KH2 domain (Xie et al., 2009). This is an RNA-binding domain that is required for translation regulation (Darnell et al., 2005). The pattern of splice forms affected versus those that are not suggests that the shortened KH2 domain is probably required. The second alternative splice site leads to removal of the nuclear export sequence (NES) as well as the S500 phosphorylation site. This occurs in

SF4-6 and SF10-12. This splice event is particularly interesting because a +1 frameshift is also introduced such that these splice forms also lack the RGG box, another RNA-binding domain. These three domains are present in SF7 and SF9, so this C-terminal is required over the +1 frameshift C-terminal. An argument could be easily made for both the RGG box and the S500 phosphorylation site as being required for axonal arbor complexity regulation by FMRP. However, the RGG box may be ruled out here. The region removed in SF9 has been found to be necessary for regulation of the RGG box through methylation (Evans et al., 2012). One splice form in which this regulation is not lost and one splice form in which this regulation is lost suggests that the RGG box may not be important in this role. However, SF9 lacks the S500 phosphorylation site, so one could rule that out, too. Because SF7 has regulation from both S500 and the RGG box while SF9 does not have any known method of regulation, it may be the shortened KH2 domain that is crucial here. Since the process requires translation, this could very well be the case. However, it is not clear why SF1, 2, 3, and 8 would be excluded. Clearly, mutant analysis is necessary to determine which one might be involved and can be found in Chapter III. These comparisons still suggest that FMRP is able to regulate axonal arbor growth and branching through a domain located in its C-terminus.

The discovery that all splice forms are able to localize axonally at equivalent efficiencies was surprising. However, given the number of roles FMRP is being implicated in within axons, axons may need the variation and specificity that the collection of splice forms can provide. Therefore, perhaps it is unsurprising that the well-conserved N-terminus seems to contain the domain required for axonal localization. Since the C-terminus is highly variable between splice forms, the domains required for

specific functions within axons are more likely to be located here as we see with the regulation for axonal arbor complexity by FMRP. These findings are exciting and will ultimately lead to enhanced understanding regarding FMRP's many axonal roles and how it is able to coordinate specificity for each one.

Chapter III: Analysis of Fragile X Mental Retardation Protein Domains in Axonal Localization

Abstract

Fragile X Syndrome typically occurs when the gene encoding Fragile X Mental Retardation Protein (FMRP) becomes silenced following hypermethylation of a trinucleotide expansion. The disease presents as intellectual disability, macroorchidism, distinct facial features, and autistic symptoms. FMRP is a multidomain RNA binding protein which functions in translational regulation at synapses. It is expressed most abundantly in neurons where it is found in the soma, dendrites, and axons. In order to gain insight into a mechanism behind its localization to axons, EGFP-tagged FMRP mutants were generated by systematic mutation of each of its domains and posttranslational modification sites and transfected into P0 rat cortical neurons with tdTomato. No mutants were restricted from axons, but deletion of the nuclear export sequence (NES) seemed to confer increased localizational ability to the protein. Axonal puncta densities were decreased for phosphorylation site mutations S500A and S500D as well as the RGG box deletion. Furthermore, axonal arbor complexity and branching were increased for these as well as the NES34A mutant compared to overexpressed WT. Although no axonal localization mechanism was identified, the remaining domains that were not analyzed here are still possibilities. Also, these results may point to a regulatory role for the S500 phosphorylation site in axon growth and branching.

Introduction

Fragile X Syndrome (FXS) is an inherited form of intellectual disability. Affecting more males than females, FXS is caused by an expanded CGG trinucleotide repeat in the 5' UTR of the *FMR1* gene located on the X chromosome (Verkerk et al., 1991; Pieretti et al., 1991). Under normal conditions, CGG repeats fewer than 60 times. When these are increased to over 200 repeats, the region becomes hypermethylated, silencing *FMR1*. This results in the absence of the protein product of *FMR1*, Fragile X Mental Retardation Protein (FMRP). FXS manifests as developmental delay, characteristic facial features, and macroorchidism in addition to intellectual disability (Bear et al., 2004). A number of these symptoms overlap with Autism Spectrum Disorder symptoms. About 50% of FXS individuals are also diagnosed with autism, making FMRP the leading known cause of autism (Kelleher and Bear, 2008).

How might the absence of a single protein lead to FXS and its symptoms? FMRP is an RNA binding protein functioning in translational regulation, typically by repression. It is ubiquitously expressed but is highest in the brain where it has been estimated to regulate about 4% of neuronal mRNAs (Hinds et al., 1993; Darnell et al., 2011). For the regulation of FMRP targets as well as regulation of the protein, FMRP has an assortment of domains and post-translational modification sites. Consistent with its function for RNA binding, FMRP has three RNA binding domains, tandem K homology (KH) domains and an RGG box (Siomi et al., 1993). Recently, a third, novel KH domain was discovered, named KH0 (Myrick et al., 2015a). There is a phosphorylation site at S500 for FMRP's own regulation which acts as a switch (Siomi et al., 2002). Phosphorylation at this site activates FMRP, allowing it to bind ribosomes and repress translation. When

dephosphorylated, FMRP is still able to interact with the ribosome but is no longer able to repress translation. These sites are important in FMRP's association with ribosomes. The KH domains are thought to interact with L5 of the large ribosomal subunit while the RGG box interacts with the mRNA, inhibiting translation during the elongation stage (Chen et al., 2014). Lastly, for shuttling mRNAs between the nucleus and cytoplasm, FMRP possesses a nuclear localization sequence (NLS) as well as a nuclear export sequence (NES) (Bardoni et al., 1997).

FMRP is well-characterized in dendrites where it plays an important role in protein synthesis-dependent synaptic plasticity but more and more is coming to light about FMRP's role in axons. Thus far, these newly discovered functions are translation-independent but are still involved in regulation of presynaptic voltage-gated ion channels, suggesting possible functions in neurotransmission and action potential duration (Deng et al., 2013). In order to discover more about its role in axons as well as possible mechanisms behind its transport, we investigated the impact of mutating FMRP on axonal localization. Each mutant was generated either by point mutation or deletion so that one domain or post-translational modification site would be rendered non-functional. FMRP mutants lacking functional NLS or NES sites were created by deletion. A mutant lacking the RGG box was also created by deletion. All other mutants were created by point mutation. The I304N mutation in the KH2 domain was originally identified in an individual with severe FXS (Siomi et al., 1994). Its counterpart in KH1 is I241N. R138Q is a second missense mutation initially identified in a patient with developmental delay but few FXS characteristics (Collins et al., 2010). The KH2 domain will be further examined through the R290A mutation. The S500 phosphorylation site has also been

mutated both to be constitutively phosphorylated (S500D) and constitutively dephosphorylated (S500A).

By comparing P0 rat cortical neurons transfected with each mutant FMRP, we set out to identify which domains or post-translational modification sites might be required for axonal localization. It was found that no mutant was excluded from axons. Furthermore, the efficiency with which each mutant localized axonally was not inhibited compared to WT. Instead, the NES34A mutant showed an increased ability to infiltrate axons. Interestingly, axonal arbor complexity was increased in this mutant as well as in the S500 phosphorylation site mutants when compared to WT. These results suggest that the domain required for FMRP localization to axons is N-terminal to the tested domains while part of the mechanism behind axon growth and branching regulation requires the presence of a functional NES and/or S500 phosphorylation site. Though more work is required to further elucidate these findings, this FMRP mutant analysis serves as a jumping off point for further experimentation in axonal localization and function of FMRP.

Materials and Methods

Constructs

pCAGES-EGFP-FMRP WT, I241N, I304N, NES34A, S500A, S500D, and pCAGES-tdTomato came from the laboratory of Dr. Justin Fallon at Brown University. The construct containing the RGG box deletion also came from this lab but had to be transferred to the pCAGES-EGFP vector by restriction digest.

Neuronal Cell Culture

Coverslips for cortical neuron cultures were cleaned and sterilized with nitric acid and ethanol and prepared for cell culture with PDL followed by laminin coating. P0 rat cortical neurons were obtained from Neurons-R-Us at the University of Pennsylvania and plated at a density of 400,000 neurons per well in a 24-well plate. They were maintained in Neurobasal media supplemented with GlutaMax, penicillin-streptomycin, and B27. Cultures were grown at 37C, 5% CO_2 for 3 days prior to transfection.

Transfections

Cortical neurons were transfected after 3 days *in vitro* using magnetofection. Mixtures of pCAGES-tdTomato and the construct of interest were prepared at a ratio of 1ul Neuromag (Oz Biosciences):1ug DNA (0.5ug TdTomato; 0.5ug EGFP-FMRP mutant) in 1x OptiMem (Life Technologies) and incubated together for 15mins prior to addition to neurons. Magnetofection took place on a magnet at 37C for 15 minutes. Neurons were maintained for 1 additional week prior to fixation.

Fixation

10-day old transfected rat cortical neurons from P0 rat pups were fixed in 4% PFA/4% sucrose for 15 minutes followed by 3 washes in 1x PBS. DAPI was added at 1:10000 to stain nuclei. Coverslips were mounted on slides using 85% NPG (4% n-propylgallate (Sigma P3130), 85% glycerol, phosphate buffer (pH 7.4), water) and stored at 4C.

Imaging, Analysis, and Statistics

Fixed transfected P0 rat cortical neurons were imaged using a Leica confocal. Images were taken as a tiled z-stack at 40X oil immersion to capture the entire lengths of axons

and depths of neurons. Stacks were collapsed using Fiji. Axons were traced and FMRP puncta labeled in Neurolucida. Puncta had to be at least 3 pixels wide to be counted. Traces were analyzed in Neurolucida Explorer using Branched Structure Analysis. Data types collected from this analysis included puncta counts and distances along axons, longest axon, axonal arbor length, complexity (calculated by [Sum of terminal orders + Number of terminals] * [Total axonal length]), branch order number, node number, and terminal numbers. One-way ANOVA followed by Dunnett Contrasts test for multiple comparisons of means was done in R. Plots were made in Prism; the Kruskal-Wallis test for non-parametric data followed by Dunn's post-hoc for multiple comparisons was performed using GraphPad Prism (version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

Results

FMRP localizes axonally, but also has a presence in the soma and dendrites (Antar et al., 2006; Feng et al., 1997). Therefore, it follows that a mechanism exists whereby it is able to traffic to the appropriate subcellular compartment. We hypothesize that one or more of FMRP's domains and/or post-translational modification sites is important for this localization. In order to investigate this, FMRP mutants were constructed either by introducing point mutations or deletions to render each domain non-functional. These included the KH1 and KH2 domains, the NES, the S500 phosphorylation site, and the RGG box. Because splice form 7 (SF7) exhibits axonal localization (see Chapter 2), is the most abundant form in multiple cell lines (Dury et al., 2013), and functions in regulation of axonal arbor complexity (see Chapter 2; Li et al.,

2009), these mutants were made in the SF7 background. Thus, all mutants possess the shortened KH2 domain. The mutants were placed in frame with EGFP and co-transfected with tdTomato into cultured P0 rat cortical neurons. The hypothesis that one or more domains would be required for axonal localization of FMRP and function in axon growth and branching was addressed. It was broken down into several questions, 1) are FMRP mutants able to localize axonally, 2) are there differences in the amount of puncta localizing to axons among the mutants, 3) are some mutants more efficient at localizing than others, and 4) which mutants are able to function in regulation of axonal arbor complexity?

Prior to answering these questions, we examined the protein synthesis levels of the individual mutant constructs to look for any construct-specific differences in expression. This was done by comparing the mean pixel intensities of both tdTomato and EGFP-FMRP mutant expression in the cell bodies (Fig. 3.1a). All mutant constructs showed expression levels comparable to WT (SF7) FMRP although there was variation elsewhere (Kruskal-Wallis, $p = 0.0198$). It was determined that all FMRP mutant constructs localized axonally by sight (Fig. 3.2-3.9). However, for most mutants, except I241N, a small subset of neurons showed no axonal localization (Fig. 3.1b). The frequency with which this occurred in any mutant was not significant so these neurons were removed from the data sets for further analysis.

Although it was somewhat surprising that all mutants still localized axonally, we expected to find differences in the localization patterns. In order to investigate this, we began by looking at the amount of EGFP-FMRP puncta entering axons. This was done by determining the puncta density in axonal arbors by measuring the number of puncta per

100um per neuron (Fig. 3.10a). The FMRP-S500A, FMRP-S500D, and the FMRP-ΔRGG conditions had significantly fewer puncta per 100um per neuron than WT (ANOVA, Dunn's post-hoc for multiple comparisons; $p < 0.0001$ (S500A and S500D), $p = 0.0408$ (ΔRGG)).

In Chapter 2, we reported the formation of elongated structures among certain splice forms. These never exceeded 15um, nor were they observed in more than 20% of neurons for a single condition. For the I241N, I304N, and NES deletion mutants, these elongated structures were observed about 50% of the time (Fig. 3.10b). These structures were found at greater lengths compared to those observed in splice form-transfected neurons, extending up to 175um without breaking. Furthermore, elongated structures tended to form frequently in those neurons in which they appeared. The formation of elongated structures was also observed in a couple of R290A and ΔRGG-expressing neurons. However, these were more similar in length and frequency to those observed among the splice form-transfected neurons and were not analyzed further.

The formation of these structures was unexpected. In order to determine their cause, we first asked if they could be due to aggregation of the mutant proteins. If this were the case, it was reasoned that tdTomato would be excluded from the areas in which the elongated structures occurred. However, comparing unmerged images showed that tdTomato was not excluded from these regions. Another possibility for the development of these elongated structures was an overabundance of FMRP in these neurons compared to other conditions. Because *Fmr1* mRNA is a target of FMRP, this could suggest decreased regulation of *Fmr1* translation. However, we already saw that there are no differences in the expression levels of different constructs (see Fig. 1a). Furthermore, we

saw that none of these mutants showed increased formation of puncta compared to WT (see Fig. 3.10a). Visually, neurons in which elongated structures form do have more FMRP entering axons than axons from WT-transfected neurons. In order to keep the analyses comparable to WT, elongated structures were scored the same as puncta although it is highly unlikely that these contain the same amount of protein. Using this method, the abundance of I241N and I304N are not statistically significant. Another method of quantifying elongated structures that remains comparable to WT is required in order to evaluate whether there really is more FMRP in the axons of neurons transfected with these mutants compared to WT-transfected neurons. The formation of these elongated structures could also be an artifact of the overexpressed mutant protein. Until further tests can be devised, we may have to leave these at that.

In order to investigate whether the distribution of puncta throughout the axonal arbors varied among the mutant forms of FMRP, cumulative distributions were generated using the distance that each puncta traveled from the cell body (Fig. 3.10c). Rather than see that one or more mutants displayed decreased localizational efficiencies, the NES deletion mutant showed an increased efficiency for infiltrating the axonal arbor (Dunnett Contrasts, $p = 0.0147$). This result was unexpected as the NES deletion was expected to retain the protein in the nucleus. Together, these results are consistent with the findings from the FMRP splice form analysis in that the N-terminus is still implicated as containing the region required for axonal localization of FMRP.

In order to examine the functionality of these mutants in axon growth and branching, we compared the axonal arbor size and longest axons. No statistically significant differences were found when mutants were compared to the WT form ($p =$

0.1224 (longest axon); p = 0.0859 (axonal arbor)). The axonal arbor complexity of each set of neurons was examined next as well as highest branch order, number of branches, and number of terminals (Fig. 3.11). In Chapter 2, neurons overexpressing SF7 had axonal arbors about 8x less complex than those of neurons expressing only endogenous FMRP. The S500A, S500D, and NES deletion neurons had significantly more elaborate arbors than WT (~8x; ANOVA, Dunn's post-hoc for multiple comparisons, p = 0.0008 (NES), p = 0.0066 (S500A), p = 0.0151(S500D)). Because the WT-transfected neurons have reduced elaboration compared to axonal arbors from neurons expressing only endogenous FMRP, these actually exhibit no change from complexity under normal conditions. Based on this pattern, the NES and/or the S500 phosphorylation site may be required for FMRP's function in regulation of axon growth and branching. Consistent with the results in Chapter 2, this again implicates the C-terminus in this role.

Discussion

The intention of these experiments was to identify FMRP domains that regulate axonal localization in rat cortical neurons. In this way, it would be possible to begin pinpointing functional domain(s) necessary for this trafficking as well as begin to elucidate the mechanism behind axonal transport of FMRP. Surprisingly, only the NES34A mutant was found to be significantly different from WT-FMRP based on the extent to which each mutant infiltrated axons, but it was shown to localize more

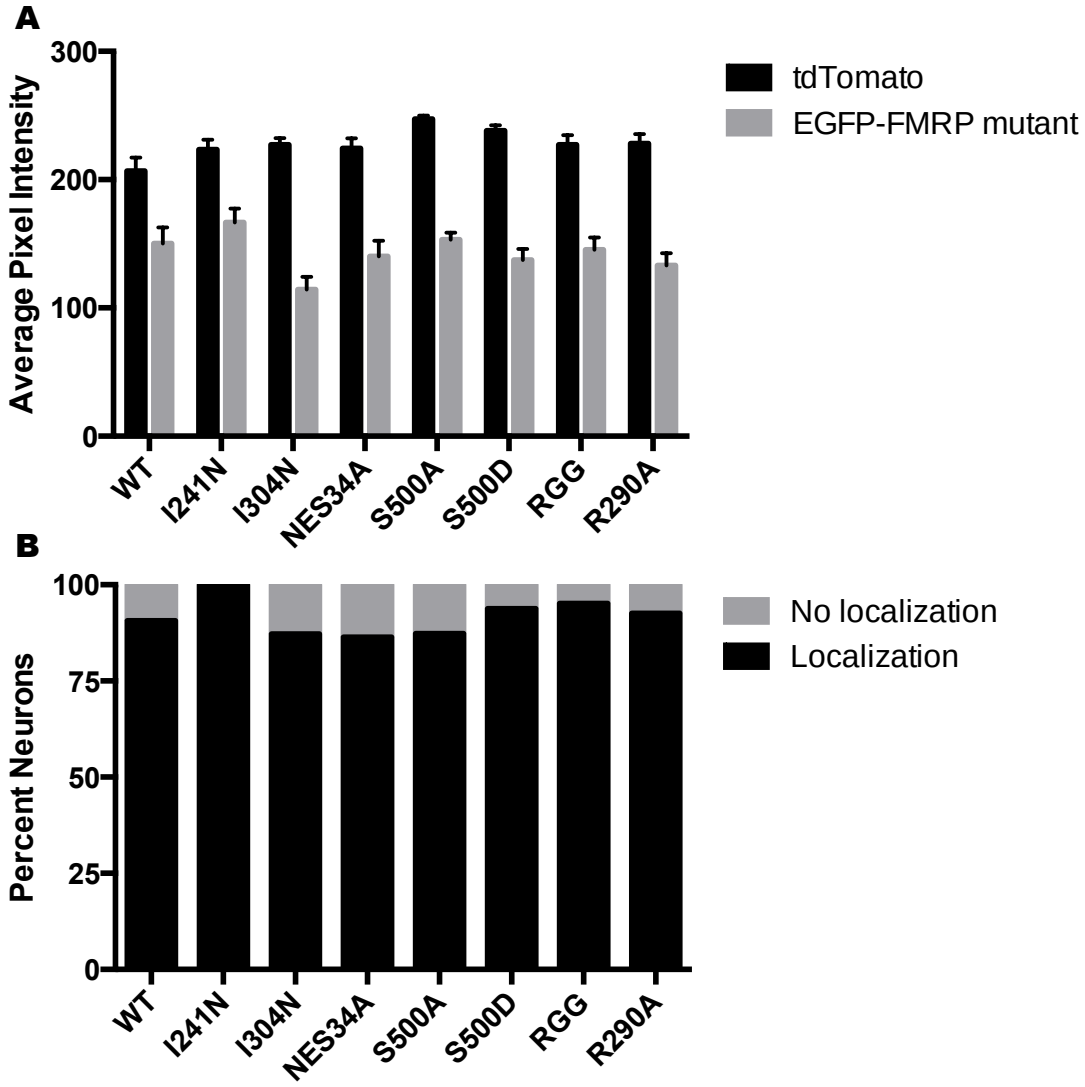

Figure 3.1 All FMRP mutant constructs are expressed at equivalent levels but do not always localize axonally. A) Mean pixel intensities of cell bodies were not significantly different between eGFP-FMRP mutants and WT although differences did exist elsewhere (Kruskal-Wallis, p = 0.0198) B) Most neurons show eGFP-FMRP mutant localization regardless of mutant form. Error bars represent SEM.

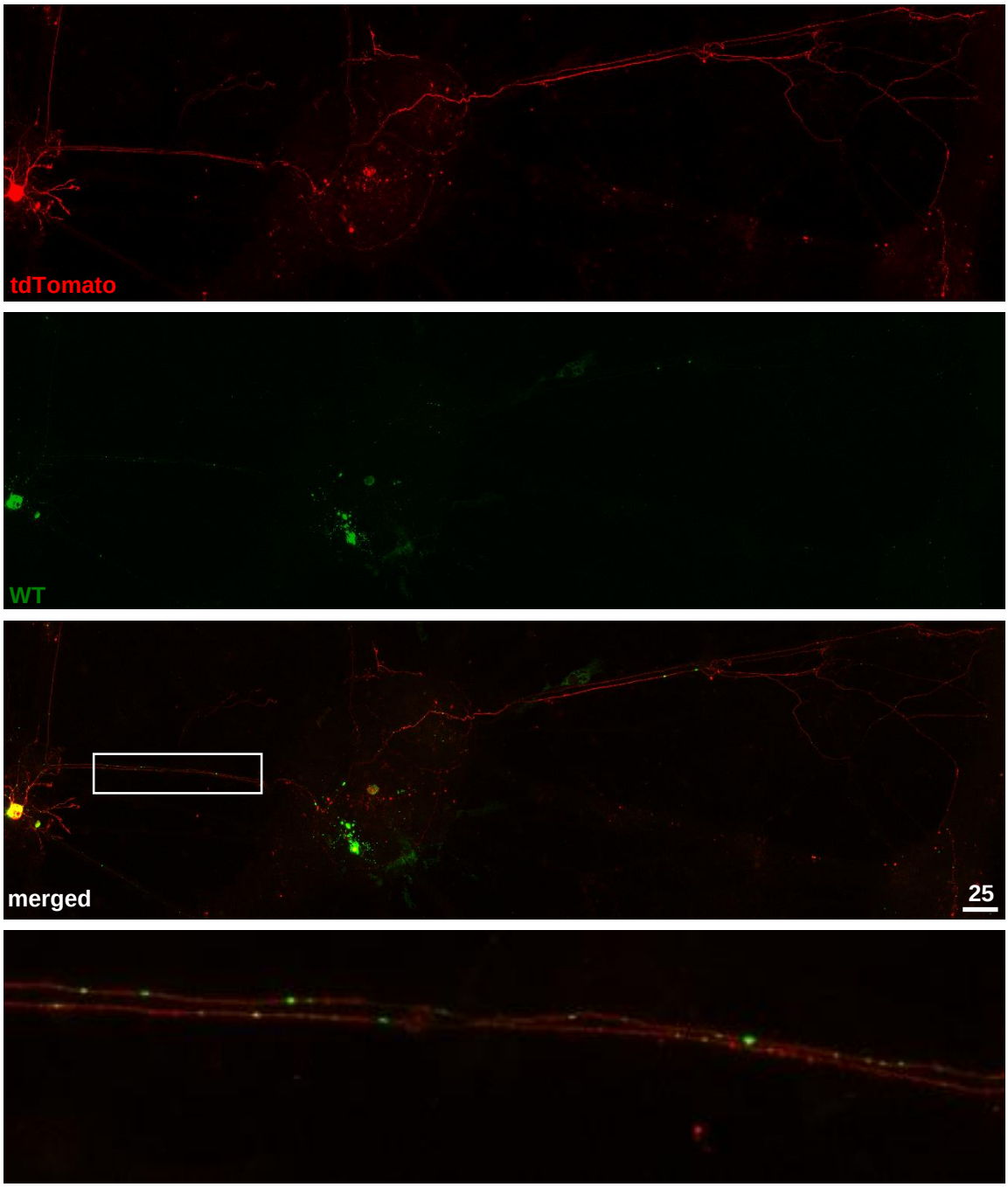

Figure 3.2: Axonal localization of EGFP-FMRP-WT.

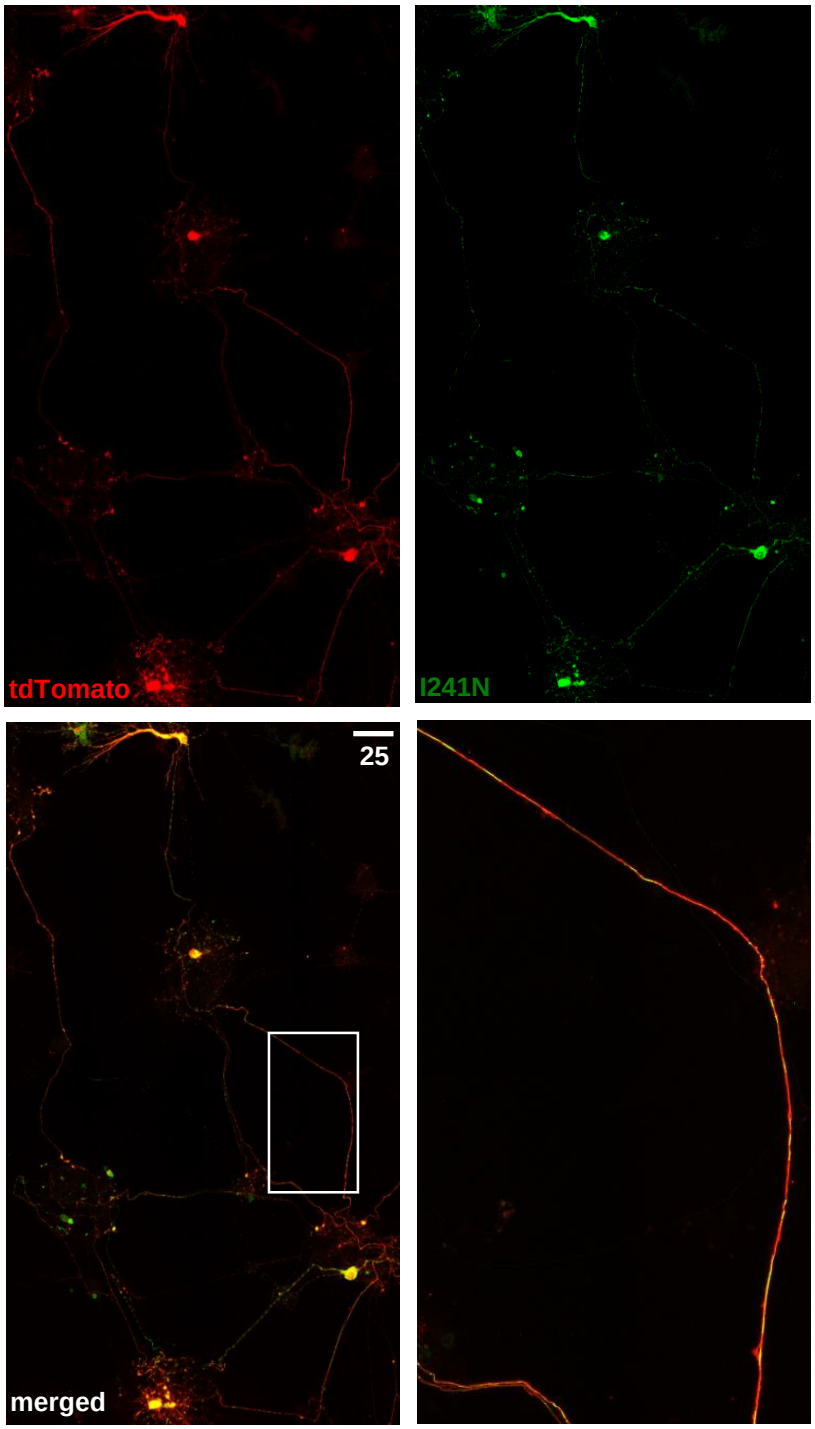

Figure 3.3: Axonal localization of EGFP-FMRP-I241N.

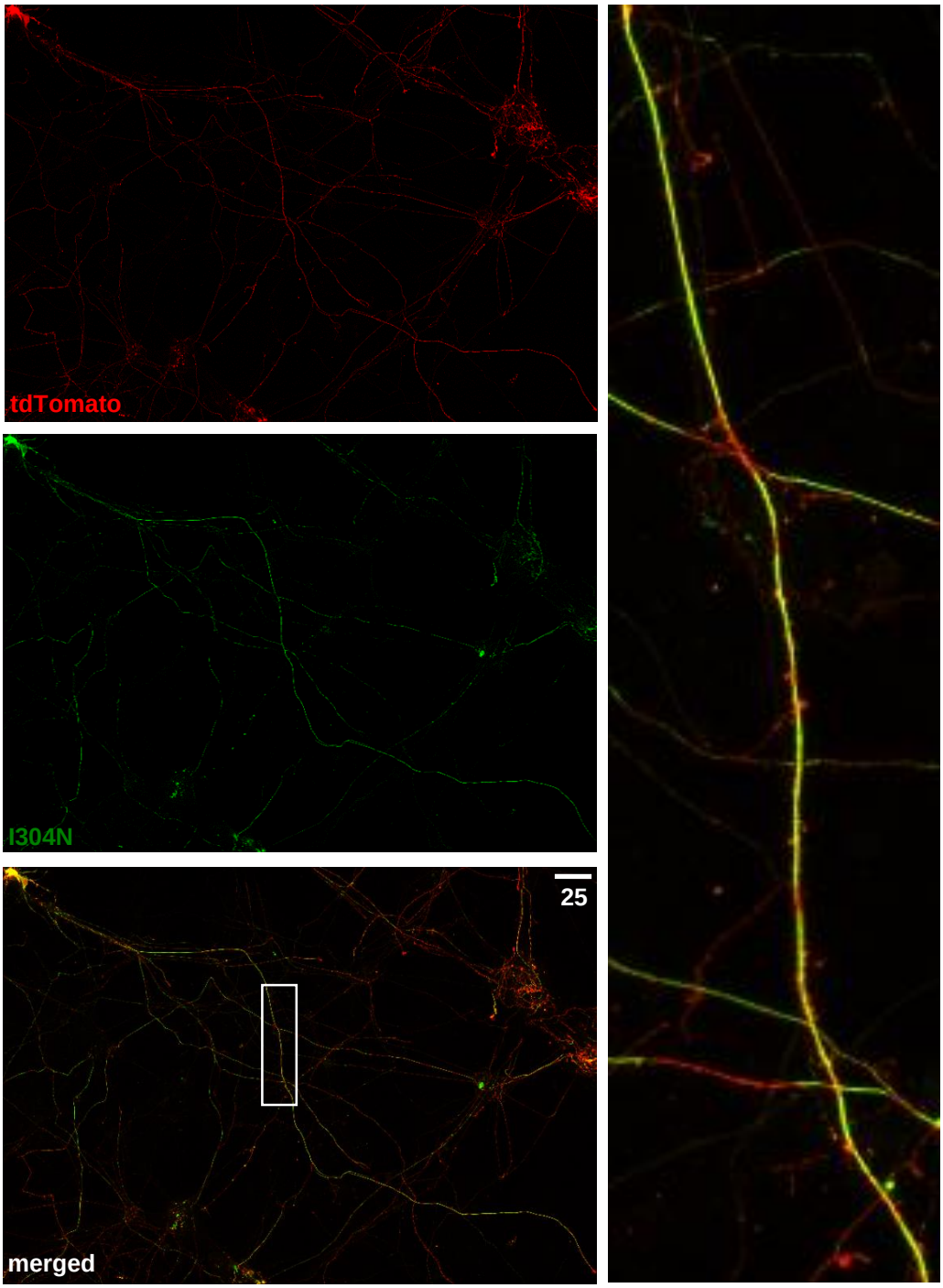

Figure 3.4: Axonal localization of EGFP-FMRP-I304N.

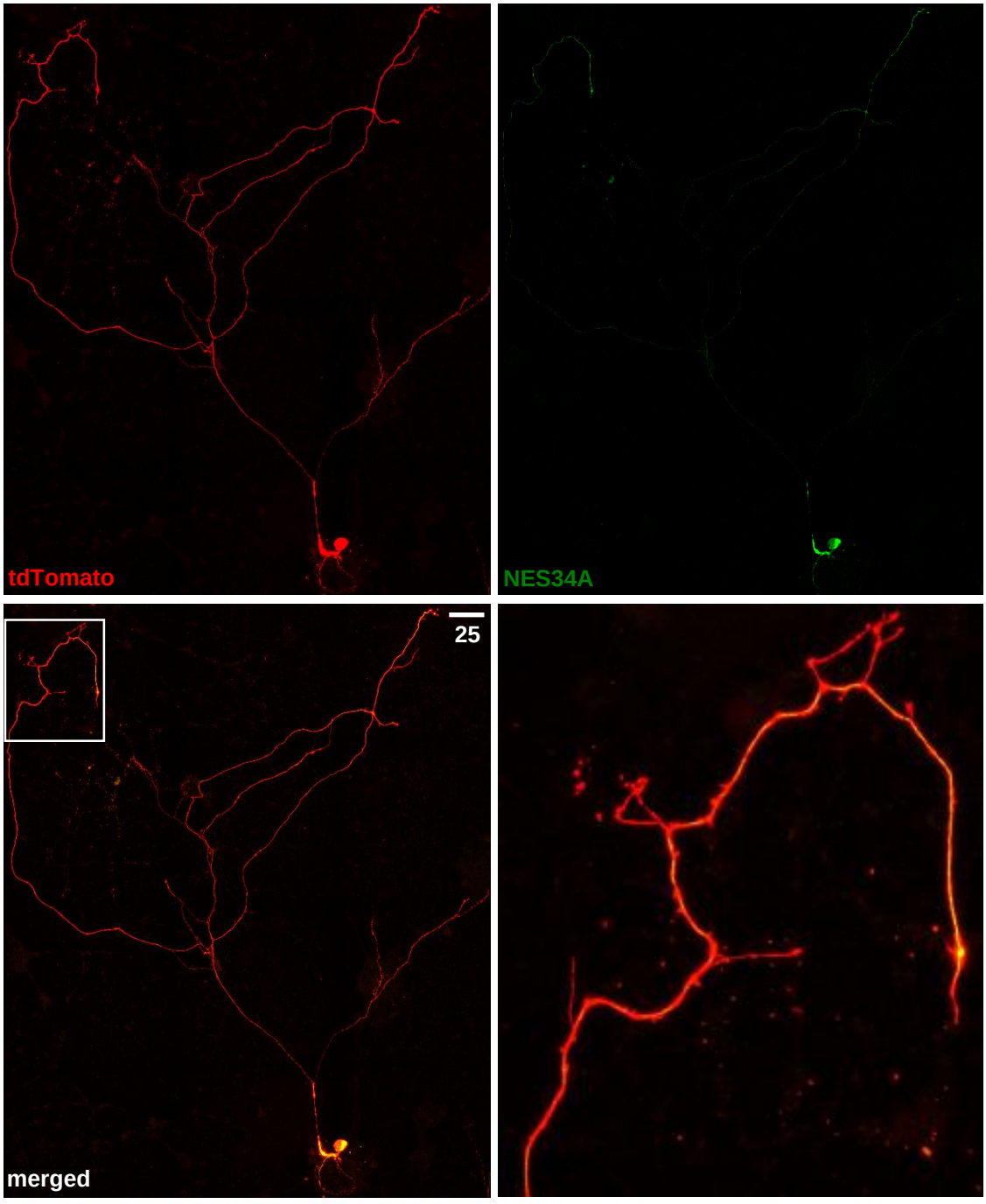

Figure 3.5: Axonal localization of EGFP-FMRP-NES34A.

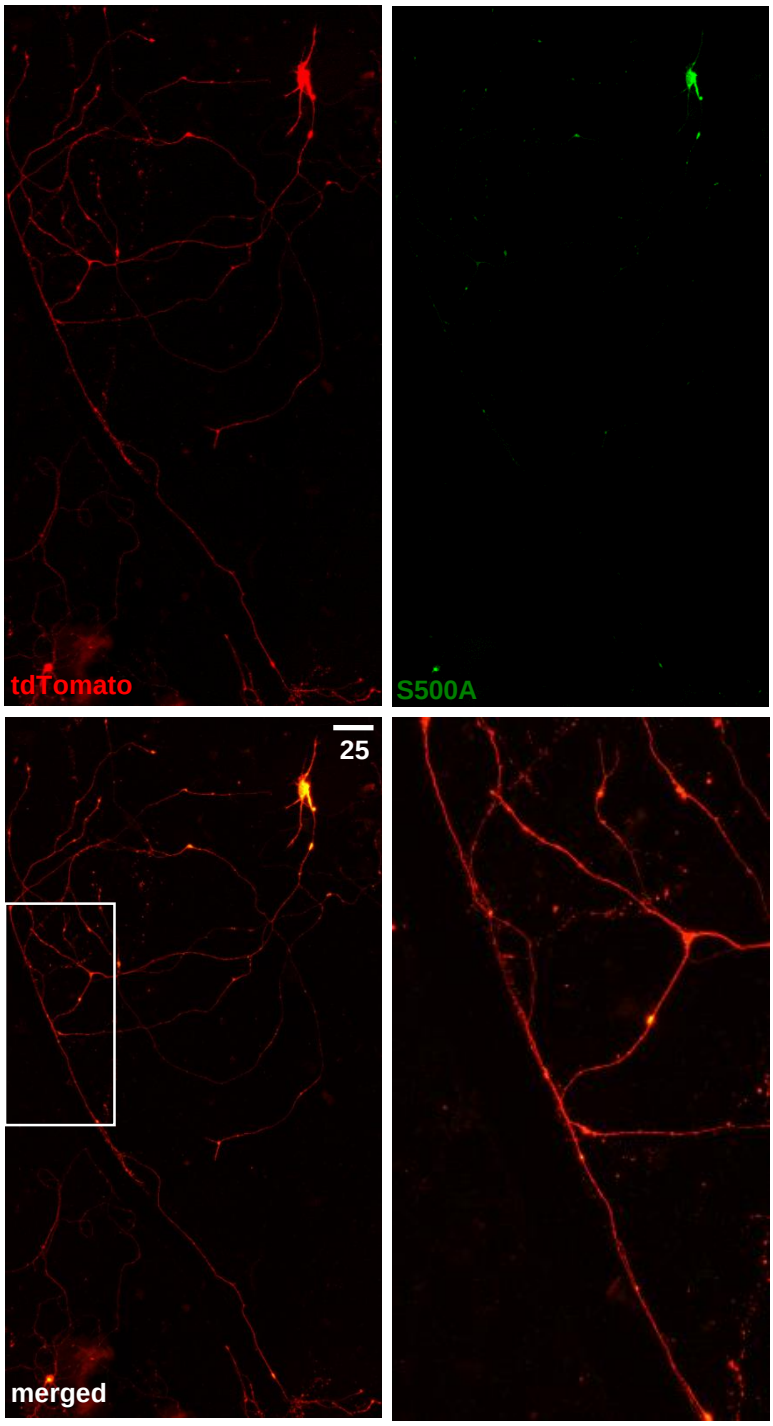

Figure 3.6: Axonal localization of EGFP-FMRP-S500A.

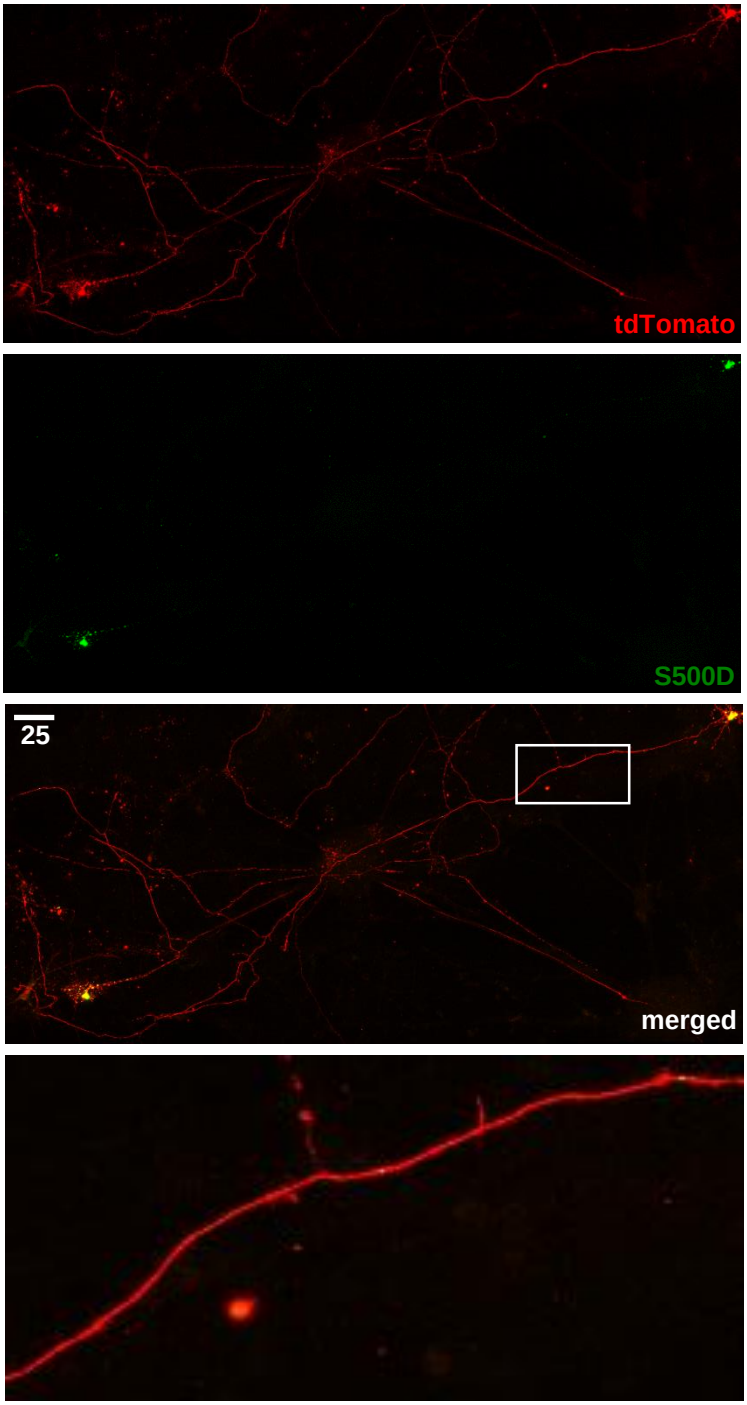

Figure 3.7: Axonal localization of EGFP-FMRP-S500D.

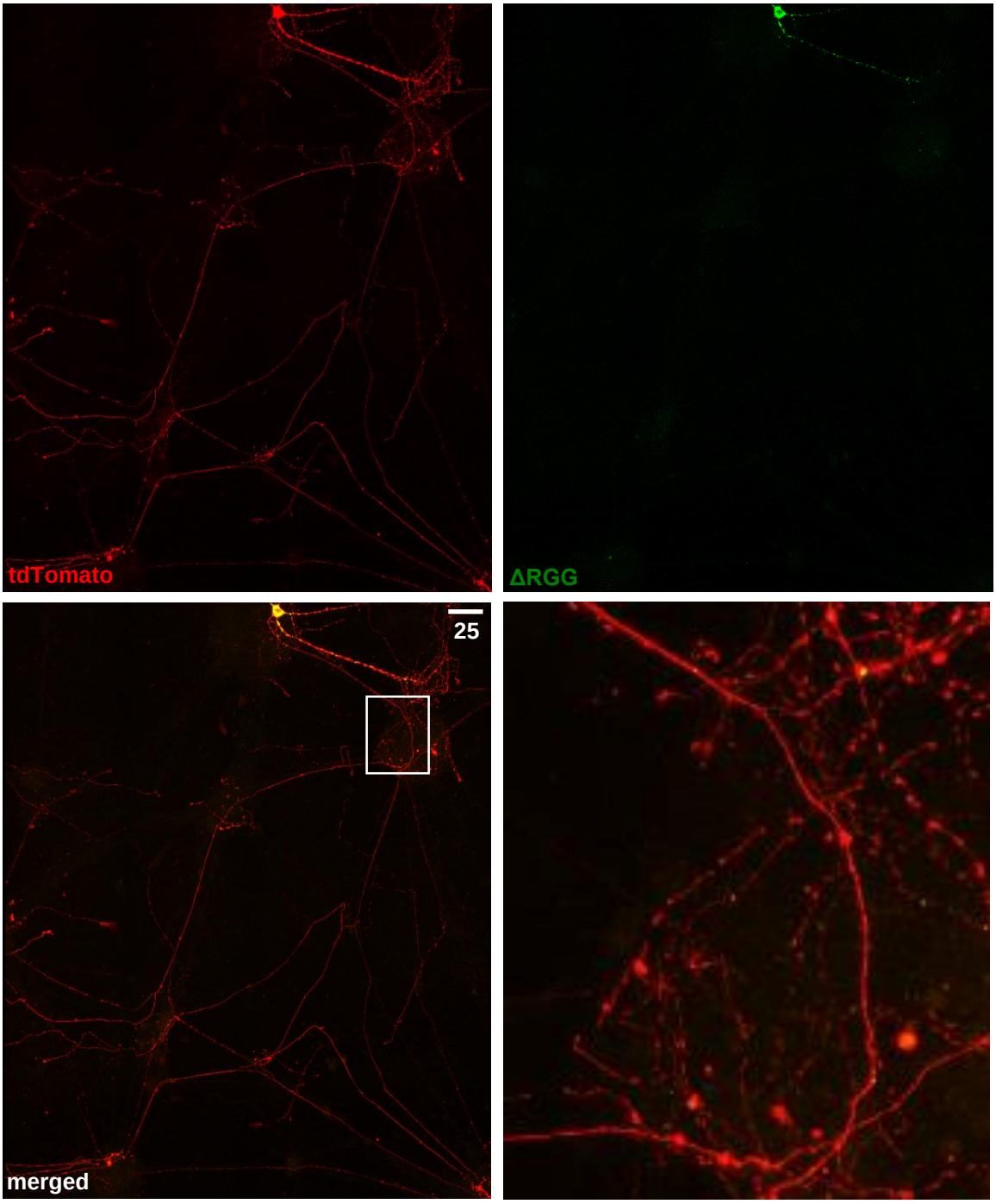

Figure 3.8: Axonal localization of EGFP-FMRP-ΔRGG.

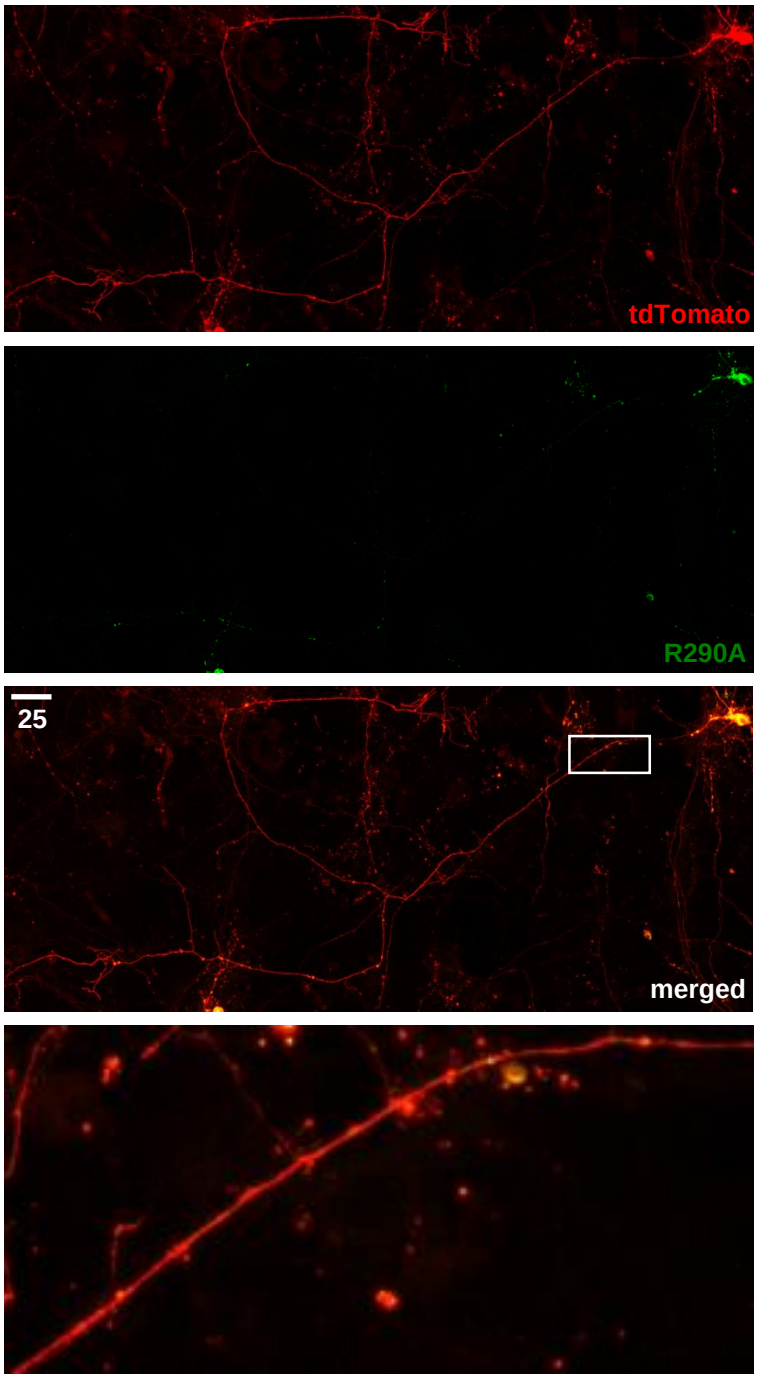

Figure 3.9: Axonal localization of EGFP-FMRP-R290A.

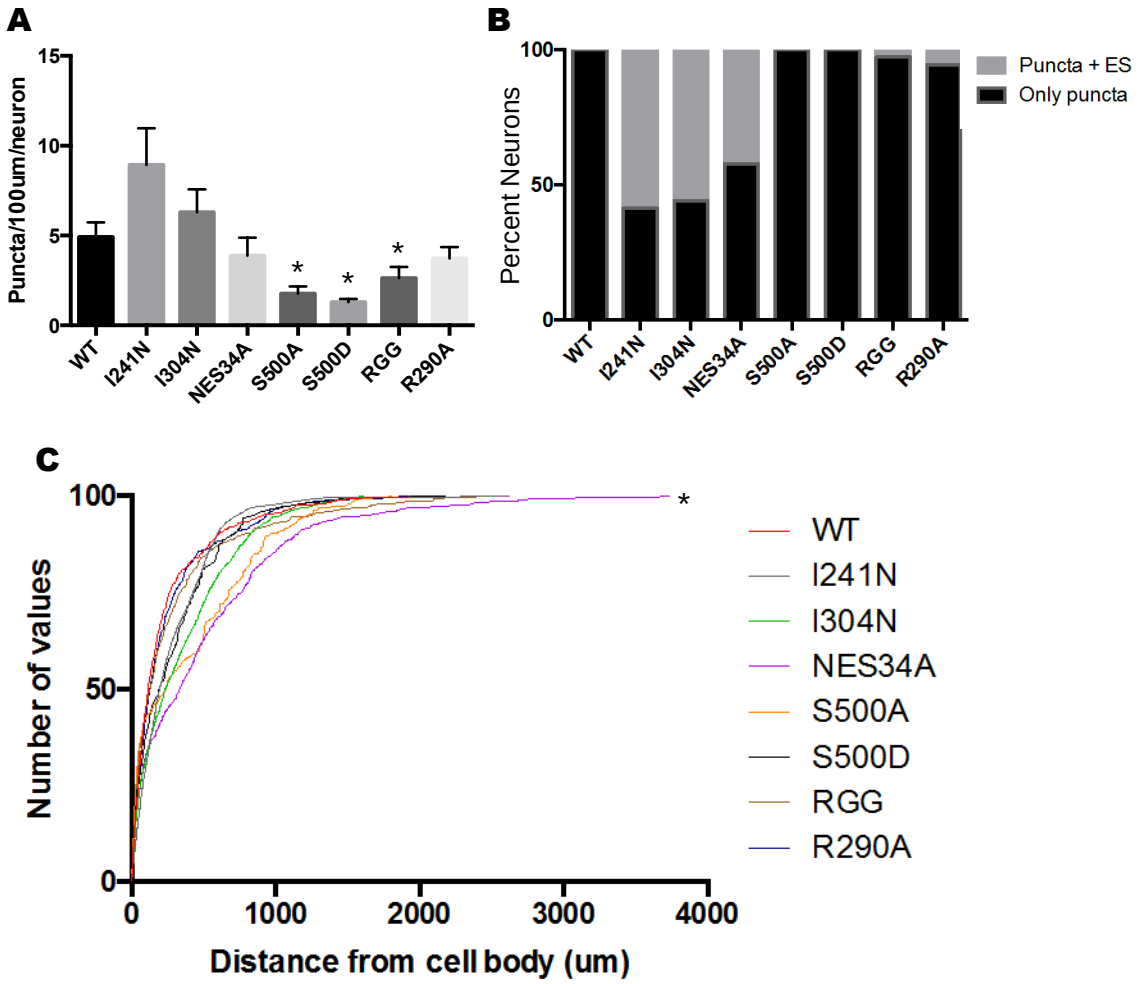

Figure 3.10 Not all FMRP mutants localize at equivalent levels and efficiencies A) Puncta density of axonal arbors among EGFP-FMRP mutant-transfected neurons varied. S500A, S500D, and ΔRGG mutants were significantly less dense than WT (Kruskal-Wallis, $p < 0.0001$; Dunn's post-hoc for multiple comparisons, $p < 0.0001$ (S500A, S500D), $p = 0.0408$ (ΔRGG)). B) Some EGFP-FMRP mutants (I241N, I304N, NES34A) form elongated structures (ES) frequently. RGG and R290A form ES rarely. ES were never observed in WT, S500A, or S500D-expressing neurons. C) Cumulative distributions of distance of each puncta from cell body for each mutant. NES34A infiltrates axonal arbors more than WT FMRP (Dunnett Contrasts, $p = 0.0147$). Error bars represent SEM.

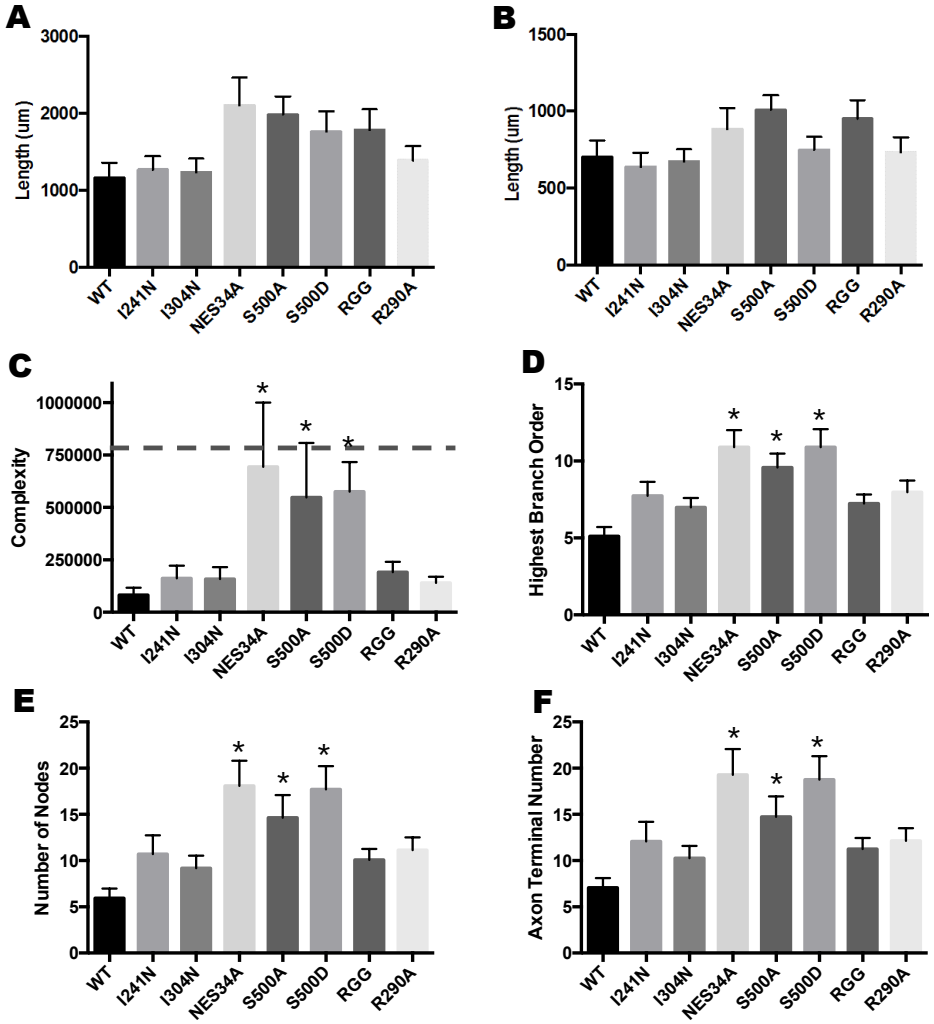

Figure 3.11 Axonal arbors are not oversimplified when EGFP-FMRP-S500A, S500D, or NES34A are overexpressed compared to neurons expressing endogenous levels of FMRP. A, B) Axonal arbor size and longest axon means not different from WT ($p = 0.0859$ (axonal arbor); $p = 0.1224$ (longest axon)). C) Complexity means [Sum of terminal orders + Number of terminals] * [Total axonal length]. Dashed line is expected complexity of neurons expressing endogenous levels of FMRP. S500A, S500D, NES34A more complex than WT ($p = 0.0019$; $p = 0.0008$ (NES34A), $p = 0.0066$ (S500A), $p = 0.0151$ (S500D)). D) Highest branch order. S500A, S500D, NES34A greater than WT ($p = 0.0002$; $p < 0.0001$ (NES34A), $p = 0.0025$ (S500A), $p = 0.0005$ (S500D)). E) Number of nodes. S500A, S500D, NES34A greater than WT ($p = 0.0006$; $p < 0.0001$ (NES34A), $p = 0.0114$ (S500A), $p = 0.002$ (S500D)). F) Number of axon terminals S500A, S500D, NES34A greater than WT ($p = 0.0012$; $p = 0.0001$ (NES34A), $p = 0.0408$ (S500A), $p = 0.0029$ 9S500D)). Statistics done using Kruskal-Wallis test followed by Dunn's post-hoc for multiple comparisons. Error bars represent SEM.

efficiently. Interestingly, the I241N, I304N, and NES34A mutants formed long axon-filling assemblies termed “elongated structures” that were measured to be as long as 175um. Though there were no apparent differences in trafficking ability, puncta densities were decreased in S500A, S500D, and ΔRGG. Examining differences in a functional role for FMRP affected by these mutations showed that axonal arbor complexities and numbers of nodes, branches, and terminals were not significantly decreased in NES34A, S500A, and S500D mutants. All together the results suggest that none of the domains analyzed here are required for axonal localization of FMRP but the NES and/or the S500 phosphorylation may be required for a regulatory role in axon growth and branching.

Initially, the NES34A mutant was expected to be confined to the nucleus and would not localize axonally as a result. This followed from the presence of NES and NLS domains and evidence that FMRP is a nucleocytoplasmic protein that shuttles mRNA from the nucleus to the cytoplasm (Feng et al., 1997; Sittler et al., 1996). Rather than be unable to traffick out of the nucleus, this construct was more efficient in localizing axonally. The apparent ability of this mutant to export out of the nucleus may support a model whereby it is mRNAs bound to FMRP that allow it to exit the nucleus by interacting with the bulk mRNA transporter NFX1 than the presence of an NES (Kim et al., 2009). Lai et al. (2006) showed that FMRP interacts with NFX2 but not NFX1 for transport out of the nucleus in hippocampal neurons from mice. Their findings may have been because the interaction with NFX1 occurs between mRNAs and the transporter. It is also possible that the nonnuclear NES34A mutant we see in axons never entered the nucleus in the first place. The overexpression conditions may have resulted in so much protein that the nuclear transporters could not traffic all of it, allowing much of it to never

enter the nucleus. There are two domains not examined here, the Agenet (Tudor) domain and the KH0 domain (see Chapter 2 for discussion of these), both located N-terminally. These results narrow down the number of possible domains that could be required for axonal localization of FMRP and suggest that this region is in the N-terminus.

While the NES is not entirely necessary for nuclear export of FMRP, the NLS is thought to be essential not only for nuclear localization but also for proper functioning. Kim, et al (2009) found that FMRP lacking the NLS did not fold properly and had reduced affinity for RNA and polyribosomes. The NLS mutant generated here showed a lack of specific localization in that it appeared diffuse throughout neurons, did not appear to form puncta, and could not be quantified due to this pattern (Appendix A).

The NES34A mutant was also among those that developed "elongated structures." These could be nearly 200um in length, filling the axon where they occurred. Because tdTomato was not excluded from these regions, it was concluded that they were likely due to large amounts of protein building up in areas but did not aggregate. Initially, this effect was thought to be due to a greater expression efficiency in these constructs over the others as a result of construct-specific effects rather than the FMRP mutant product. However, no differences were detected in cell body pixel intensities, suggesting that all constructs were expressed equally and that the excess was caused by the mutation. This phenomenon occurs in nearly half of NES34A mutant-transfected neurons. It occurs in over half of the neurons transfected with either the I241N and I304N mutant-transfected cells. While this effect may be an artifact of working *in vitro*, there are possible explanations specific to these mutations that could also cause it. In *Drosophila*, both the KH1 and KH2 mutants show decreased affinities for the ribosome and do not inhibit

translation as efficiently as fully functional FMRP (Chen et al., 2014). FMRP binds its own mRNA (Doyle and Kiebler, 2011), thus mutations causing reduced translational inhibition could result in increased FMRP in cells. Increased protein synthesis levels are observed in *Fmr1* KO neurons (Qin et al., 2005). The neuron is likely able to detect that translation of a subset of mRNAs is not properly regulated and sends more FMRP into axons to correct for this. Why is this behavior not seen in the ΔRGG mutant? The RGG box is thought to be important for binding mRNA targets; its deletion does not impact translation inhibition. Interestingly, the KH1 mutation was predicted to unfold FMRP (Musco et al., 1997). While this would explain its loss of translational repression, it does not shed light on trafficking as an unfolded protein would not be expected to be transported normally.

The ΔRGG, S500A, and S500D mutants both showed significantly fewer puncta per 100um per neuron when compared to WT. For ΔRGG, this result has been seen in fibroblasts in which fewer FMRP-containing granules formed compared to WT (Blackwell and Ceman, 2011). It also makes sense in the context of how FMRP binds to ribosomes and forms granules. mRNA synthesis is required for formation of FMRP-containing granules, and granules are more likely to contain FMRP in the presence of mRNA. Since the RGG box binds the mRNA while the KH domains bind the ribosome, it may be that FMRP must be bound to mRNA before it can be incorporated into granules. If this lack of granule formation in these neurons had any effect it was not related to either efficiency of axonal localization or arbor complexity.

The S500 phosphorylation site is necessary for regulation of FMRP's ability to repress translation. When phosphorylated, FMRP actively represses mRNA translation

but does not when dephosphorylated. Dephosphorylation by PP2A also marks FMRP for degradation by the ubiquitin proteasome system (UPS) (Nalavadi et al., 2012). This has been seen in dendrites; whether the same mechanism occurs in axons remains unknown. Loss of FMRP in dendrites due to its degradation could lead to more being sent into dendrites to replace the degraded proteins which may explain why the S500A mutant appears to be less abundant in axons. However, such an explanation does not work for the S500D mutant. FMRP represses translation by stalling ribosomes during the elongation step of translation. Thus, constitutively phosphorylated FMRP would be expected to keep ribosomes stalled. Unless there is another specific mechanism for degradation of FMRP, this protein would be stuck, allowing it to accumulate.

S500A and S500D were also significantly different from WT in regards to axonal arbor complexity, branch order number, terminal number, and number of nodes. The axonal arbor and longest axon lengths do not differ in these, so the increased elaboration may be at the expense of branch length. Whether this is related to decreased puncta density cannot be determined from the data. However, calcium/calmodulin-dependent protein kinase II (CAMKII), a known target of FMRP, and Semaphorin 3A (Sema3A), an upstream regulator of FMRP are both involved in cortical axon growth and branching. CAMKII responds to increased Ca^{2+} levels and has been shown to increase cortical axon branching when over expressed (Kalil et al., 2011). Sema3A signaling reduces axon branching as well as branch length but does not affect axon length which may explain why axon length is not significantly different in these mutant conditions compared to others. Sema3A acts through FMRP to regulate translation locally in distal axons (Li et al., 2009). In Chapter 2, we hypothesized that overexpression of FMRP would

oversimplify axonal arbors due to the observation that *Fmr1* KO neurons show increased complexity. In the case of the mutant forms, if the affected domain is not essential for FMRP to regulate axon growth and branching, then this oversimplification will be seen. However, if the affected domain is essential, then there will be no change from expected complexity.

Increased axonal arbor complexity also occurs in the NES34A mutant. It is not immediately clear how or why this is the case. However, the NES domain is in close proximity to the S500 phosphorylation site. It is possible that its removal altered the ability of this site to be phosphorylated or dephosphorylated such that it acted similarly to the phospho- and dephosphomimetic conditions. However, altering the S500 phosphorylation site does not affect axonal localization efficiency, so the increased efficiency seen from the NES34A mutant may be independent of the phosphorylation site.

As intriguing as some of these results are, it is important to remember that these experiments were done in wild type rat neurons in which endogenous FMRP was still present. As such, these results are confounded by the presence of the endogenous protein. Further experiments in either a KD or KO system to better determine the effect of mutations on axonal localization of FMRP are needed. It may be that the results seen here will be magnified. The use of double mutants to further unravel the effects of FMRP domain involvement in axonal localization, efficiency, puncta densities, and axonal arbor complexities are also expected to be useful in future experiments. These experiments serve as a jumping off point for determining the mechanism behind axonal localization of FMRP as well as its role in axonal growth and branching. This is especially true as they

represent a first attempt at identifying a mechanism for axonal localization of FMRP using all splice forms and systematic mutation of nearly all FMRP domains.

Chapter IV: Discussion

Analysis of axonal localization among different FMRP splice forms and mutants was expected to lead to the beginning of determining a mechanism behind the transport of FMRP along axons. Transfecting EGFP-tagged FMRP splice forms into cultured rat cortical neurons showed that all were able localize axonally with the same efficiency. Performing the same experiment with EGFP-tagged FMRP mutants also showed that all but one of those investigated were also able to localize with similar efficiencies. The mutant in which the NES domain had been deleted was found to be more efficient than WT. Differences were observed among puncta densities as well as axonal arbor complexity. The S500 phosphorylation site mutants showed reduced puncta density but increased complexity compared to WT FMRP. The RGG box deletion also showed decreased puncta density while the NES deletion showed increased complexity though puncta density remained the same. Some of the results in the splice form analysis were recapitulated in the mutants; many of the splice forms that lacked the NES showed increased complexity as the NES deletion mutant had. These same splice forms also lack the S500 phosphorylation site. Together, our findings show that all FMRP splice forms are able to localize axonally and suggest that the FMRP domain required for axonal localization is located in the N-terminal region, while a C-terminal domain, possibly the NES and/or the S500 phosphorylation site, is required for a regulatory role in axon growth and branching.

The N-terminus of FMRP is well-conserved between splice forms as the first alternative splice site does not occur until exon 12. All of the splice forms traffic to axons

with similar efficiencies; the six splice forms in which a +1 frameshift changes the last ~100 amino acids are no different in this aspect. This suggests that, if an FMRP domain is required for axonal localization, then that domain would be in the N-terminus. In further support of this, none of the mutants showed an inability to localize axonally or even a reduced efficiency compared to WT FMRP. The earliest that any of these domains occur is the KH1 domain which does not begin until over 200 amino acids into the sequence. In the region N-terminal to the KH1 domain are the nuclear localization sequence (NLS), an Agenet (Tudor) domain, and the KH0 domain (Sittler et al., 1996; Alpatov et al., 2014; Myrick et al., 2015a). As discussed in Chapter 2, the KH0 domain is the most likely candidate among these because the NLS functions in nucleocytoplasmic shuttling into the nucleus and the Agenet domain has been implicated in a chromatin-binding function for FMRP. The KH0 domain was discovered following identification of an FXS individual with an R138Q point mutation instead of the usual expanded trinucleotide repeat. This region has been shown to be involved in presynaptic functions whose mechanisms are unknown (Myrick et al., 2015b). Therefore, it is possible that the phenotypes are due to the absence of FMRP-R138Q at the presynapses due to an inhibition in axonal localization.

The observation that all splice forms and mutants localized axonally was unexpected and could call into question whether FMRP is specifically localized subcellularly or if something unrelated to its structure such as the mRNA target is responsible for appropriate localization. Reeve et al. (2008) concluded that the N-terminus is essential for neuronal functions following identification of numerous conserved N-terminal residues whose mutation impacted protein-protein interactions

critical for proper functioning of FMRP. FMRP is transported along dendrites and axons in granules; this transport is dependent on microtubules (Davidovic et al., 2007; Schrier et al., 2004), but the mechanism has only been studied in detail in dendrites. Kinesins KIF5, KIF1, and the neurospecific KIF3C have all been shown to associate with FMRP and transport it along dendrites (Kanai et al., 2004; Ling et al., 2004). Interestingly, Dictenberg et al. (2008) identified the C-terminus in binding between FMRP and kinesin light chain (KLC), but Ling et al. (2004) found no association between FMRP and KLC. FMRP interacts with dyneins, too (Ling et al., 2004). Assuming the mechanism of transport is similar in axons, this would suggest that FMRP is incorporated into granules with other proteins and mRNAs and these granules are transported along axons via motor proteins, thereby becoming localized.

The lack of identifying any splice forms or mutant forms that did not localize axonally could also suggest that the mechanism is independent of FMRP. "Zipcodes" have been identified in the 3' UTRs of mRNAs such as chicken *β-actin* and zebrafish *Tubb5* that traffic them to axons (Zhang et al., 2001; Baraban et al., 2013). ZBP1 binds the *β-actin* mRNA zipcode, promoting its axonal localization and later regulating its translation in growth cones (Doyle and Kiebler, 2011). While FMRP could play a similar role with its mRNA targets, its involvement in ribonucleoprotein granules suggests otherwise. FMRP was identified with more than 40 other proteins and mRNA for CaMKIIα and Arc in a KIF5-transported granule (Kanai et al., 2004). Further work suggested that FMRP served as a molecular adaptor between granules and motor proteins (Dictenberg et al., 2008; Yao et al., 2011). It seems most likely that different domains would be required for the different roles of FMRP in this transport. Both the N-terminus

and C-terminus have been implicated in protein-protein interactions (Reeves et al., 2008; Menon et al., 2004; Dictenberg et al., 2008). In order for FMRP to act as a molecular adaptor, it would need to bind at least two proteins simultaneously. Presumably, one would be the motor protein and the other a granule protein, possibly another FMRP, as FMRP can form homo-oligomers (Agulhon et al., 1999). However, the involvement of more than one domain would suggest that we should have seen localization impacted in one or more mutants. It may be that endogenous FMRP is sufficient to act in this role, o transport was still able to occur. Experiments using *Fmr1* KO neurons as well as double mutants may help elucidate this issue.

Splice forms and mutant forms displayed formation of elongated structures. However, the occurrence, frequency within neurons, and length varied. Nearly all splice forms developed these, except for SF3, SF7, and SF8. They occurred most frequently in SF11-transfected neurons in which 1 in every 5 neurons displayed these. Within neurons, they only appeared once or a few times, and their lengths never exceeded 15um. Among the mutants, the WT (SF7) form did not display elongated structures, consistent with the earlier observation. The S500 mutants did not develop these either. The R290A and ΔRGG mutants developed elongated structures rarely, on par with the lengths and frequencies of their appearance among most of the splice forms. Elongated structures developed in 50% of neurons expressing I241N, I304N or NES34A mutants. Furthermore, they often appeared many times within a single neuron and were measured at lengths up to 175um. Based on these observations, there seemed to be something different between the splice forms and some mutants versus these three mutants. In Chapter 3, we discussed various possibilities for the formation of elongated structures

(see Discussion). Others have reported that overexpressed mutated FMRP (I304N) forms artificial aggregates and that overexpressed mutated FMRP is capable of increasing granule formation (Schrier et al., 2004). This could explain why the elongated structures are more extensive in these three mutants than in other mutants or splice forms.

While the N-terminus may be important in axonal localization, the results suggest a role for FMRP in regulation of axonal arbor complexity and that the C-terminus is necessary for this. FMRP has been previously linked with the regulation of axon growth and branching as a mediator of Semaphorin 3A (Sema3A) signaling and MAP1B protein levels (Li et al., 2009). Sema3A is a secreted factor that functions as a guidance cue in axon growth and branching by reducing branch formation through growth cone collapse without affecting over all axon length (Tillo et al., 2012; Antar et al., 2006). MAP1B associates with FMRP in granules and shows increased levels in response to Sema3A signaling while eukaryotic initiation factor 4E shows increased phosphorylation, both of which are reduced in the absence of *Fmr1* (Antar et al., 2005). *Fmr1* KO neurons have overelaborate axonal arbors compared to WT neurons. Thus, axonal arbor complexity is regulated in a translation-dependent manner through Sema3A and FMRP. A model was developed to analyze the effects of overexpression of splice forms in the context of FMRP's role in axon growth and branching. In this model, splice forms that do not have a role in axonal arbor complexity remain unchanged compared to baseline complexity while those that do have a role reduce complexity when overexpressed. Thus, splice forms (SF) 1, 2, 3, 4, 5, 6, 8, 10, 11, and 12 do not have a role in complexity while SF7 and SF9 are involved. The involvement of these two splice forms suggests that the

shortened KH2 domain is used over the full-length version and the C-terminal domain that results when there is not a frameshift is required.

This model can also be used to determine whether each domain affected in the FMRP mutants might be necessary for regulation of axonal arbor complexity. In this model, a mutated domain required for the role of FMRP in axon growth and branching will not have an effect on the complexity of the axonal arbor while a mutated domain not involved will allow the protein to function as normal. Thus, transfection of the NES34A, S500A, and S500D constructs did not result in any change in axonal arbor elaboration, suggesting that these domains are required to be present and functional in order for FMRP to be able to regulate axonal arbor complexity. All three of these are C-terminal and are present in most of the splice forms shown to function in regulation of axon growth and branching while those that do not have a role tend to not have these domains.

Prior to beginning these experiments, the NES deletion mutant and splice forms which lacked the NES domain were expected to be negative controls since they should have been trapped in the nucleus. Not only were the NES-lacking splice forms equally able to localize axonally, but the NES deletion mutant showed increased efficiency in traveling down axons. FMRP is able to get out of the nucleus using nuclear transporters NFX1 or NFX2 (Kim et al., 2009; Lai et al., 2006). It interacts directly with NFX2 but only the mRNA interacts with NFX1, thus the NES would not necessarily be required in order to be exported. It may be that deletion of this site causes some conformational change that impacts how FMRP is transported along microtubules allowing for this gain of axonal localization efficiency. In order to test this, we can try another type of mutation. Bardoni et al. (1997) identified three leucines that could be mutated to serines which

would localize FMRP to the nucleus. Generating this mutant and analyzing it as the other mutants were analyzed could yield different results, suggesting that it was the biochemical changes caused by the deletion and not actually the loss of the NES that led to the increase in axonal localization efficiency.

There are other possibilities to be considered. First, the protein may have never localized to the nucleus in the first place. Since the FMRP constructs are overexpressed, there could be so much that the transporters cannot handle all of it, such that much of the FMRP is not localized to the nucleus. Furthermore, the potential effects of expressing FMRP beyond biological conditions should be considered. Overexpression of FMRP correlates with increased levels of cyclic AMP (cAMP) (Berry-Kravis and Ciurlionis, 1998). Since cAMP levels are important in many cellular processes, this increase could have far-reaching effects that impact certain mutants more than others. Indeed, Azhderian et al. (1994) found that increased cAMP levels increased axonal transport rates in bag cell neurons from *Aplysia*, organisms which possess a homolog of FMRP. Numerous neuronal signaling pathways are conserved between *Aplysia* and mammals and a presynaptic function for FMRP has been identified in *Aplysia* (Till et al., 2010). Thus, increased cAMP levels in response to overexpressed FMRP could impact the rate at which granules are transported along axons and mutations in FMRP could result in either loss- or gain-of-function in FMRP in relation to one of these mechanisms. Among the splice forms, we saw no change in localizational efficiency. This might suggest that these factors impacted the splice forms equivalently. Since half of these splice forms do not possess the NES, the question of why a mutant lacking this domain would localize more

efficiently is still up for debate and could still be due to conformational changes in the protein.

Both S500 mutants showed no change in axonal arbor complexity as well as decreased puncta densities. However, an S500-dependent effect would have been expected to only be seen from one mutant and not the other. How might this be? Phosphorylation of FMRP at the S500 site is reversible; it is phosphorylated by S6K as part of the mTOR pathway and dephosphorylated by PP2A (Narayanan et al., 2008; Bassell and Warren, 2008). When phosphorylated, FMRP binds ribosomes and actively represses translation of target mRNAs. When dephosphorylated, it remains bound to ribosomes but does not repress translation (Siomi et al., 2002; Ceman et al., 2003). The S500D mutant mimics phosphorylation; mRNAs are never derepressed due to the loss of dephosphorylation. The S500A mutant mimics dephosphorylation; mRNAs are not repressed due to a loss of activity in FMRP. FMRP regulates axon growth and branching in a translation-dependent mechanism through two known actors: MAP1B and CAMKII (Li et al., 2009; Kalil et al., 2011). Increases in MAP1B levels following derepression of translation of its mRNA results in growth cone collapse and simpler axonal arbors. Increases in CAMKII levels for the same reason results in increased arborization. Regulation of both of these factors by FMRP may suggest that some sort of negative feedback loop exists to prevent the axonal arbor from becoming either too elaborate or too simplified. With FMRP at the heart of this, axon growth and branching will become independent of Sema3A signaling if it is not functional. This pathway would start with signaling from Sema3A which ultimately will lead to dephosphorylation of FMRP. MAP1B will be translated, allowing reduced axonal growth and branching. FMRP

dephosphorylation also induces degradation by the ubiquitin proteasome system (Nalavadi et al., 2012), so renewed repression of MAP1B mRNAs would require a new protein. Another signal, possibly from a different Semaphorin or something else entirely, will then lead to phosphorylation of other FMRP to repress MAP1B again as well as separate dephosphorylation of yet another FMRP such that CAMKII is translated, allowing increased growth and branching. An S500D mutant would not allow derepression of either MAP1B or CAMKII such that growth and branching is neither decreased nor increased in response to semaphorin signaling. Neither MAP1B nor CAMKII will be repressed in FMRP-S500A-expressing neurons such that growth cone collapse due to MAP1B and growth due to CAMKII will occur without regulation, resulting in axonal arbors that appear unchanged compared to neurons expressing endogenous levels of FMRP. In order to determine whether any of this occurs, other semaphorins would need to be identified as promoting growth and branching in cortical neurons, as well as determine if this is through FMRP and CAMKII translation.

As the first known analysis of all FMRP splice forms and nearly all domains in axonal localization, this work serves as a starting point for future work in the mechanism for axonal localization of FMRP, as well as mechanisms behind the role of FMRP in axon growth and branching, and, perhaps eventually, other axonal functions of FMRP. Finding that all splice forms localize axonally but can function in different roles gives support to the idea that alternative splicing in FMRP alters the protein just enough to allow for specificity in a variety of roles. This also underscores the importance of FMRP in the development of the brain and why its absence in Fragile X Syndrome (FXS) individuals is so devastating. Furthermore, identifying the N-terminus as the possible

sight of the domain required for axonal localization suggests that this well-conserved region is involved in general characteristics of FMRP such as subcellular localization. Similarly, identifying the C-terminus as the location of the domain required for a regulatory role in axon growth and branching suggests that variation in this region allows for variable functions such as postsynaptic protein synthesis-dependent synaptic plasticity, presynaptic action potential broadening, axonal arbor complexity, and others. These findings further our understanding of FMRP and how it functions.

How might these findings relate back to FXS and autism? Increased synapses have been observed in both FXS and autistic individuals (Robichaux et al., 2014). This is typically thought to be due to reduced pruning, but the observation that axonal arbors are overelaborated in *Fmr1* KO neurons could suggest that it is also due to increased branching, allowing for more synapses to form. If this is so, it lends support to the idea that FMRP is involved in more than post-synaptic translation-dependent synaptic plasticity. However, it may also increase the complexity of the mechanisms behind FXS and autism. If increased synapses are due both to increased arborization and reduced pruning, then identifying therapeutics that can address FXS and autistic phenotypes may become more difficult as more pathways dysregulated by the loss of FMRP come to light. These processes are likely to become more complex before they are fully elucidated, but each step takes us closer to a treatment for FXS and eventually autism.

Chapter V: Future Directions

FMRP Mutants and Double Mutants in Fmr1 KO background

As discussed, the above mutant experiments were done in WT rat cortical neurons in which FMRP had not been knocked down. We will also compare the differences in expression levels of endogenous FMRP and transfected FMRP to determine how much more mutant FMRP is present. This will be done by staining for endogenous FMRP while the EGFP-tagged FMRP is also present in the cells.

Steps have been begun to validate these results in KD conditions. We have designed and tested several shRNAs against the 3' untranslated region (UTR) of FMRP (Fig. 5.1a, b). Our constructs do not use the endogenous 3' UTR so they will not be knocked down following transfection. In our current system, puncta seen in axons either contained RNA granules in which both endogenous and transfected FMRP co-existed or RNA granules that contained either endogenous FMRP or transfected FMRP. For the most part, we do not expect to see differences from our current data in axonal localization, efficiency, or quantity entering axons, although it may be possible that mutant constructs which had approached significancy above may be truly significant once endogenous FMRP is absent. Furthermore, there could be loss of axonal localization if the endogenous FMRP was sufficient to allow for appropriate axonal localization in granules also containing the mutant form. Redoing the splice form experiments in this context is not necessary because they are already naturally occurring.

Our results from both splice form and mutant analyses suggest that the N-terminus is required for axonal localization. Because of this, we will introduce the R138Q

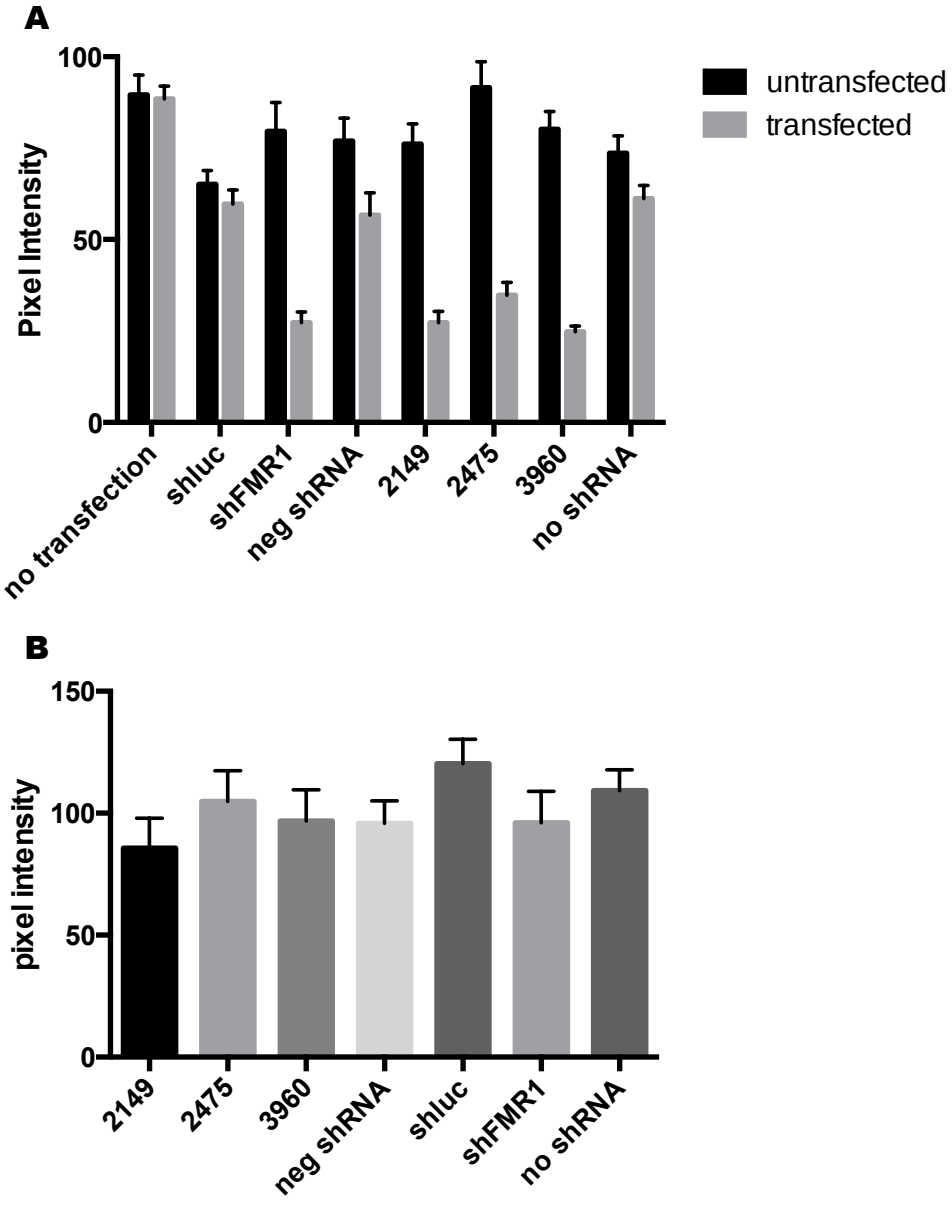

Figure 5.1 3' UTR-targeting shRNAs knockdown FMRP A) Neurons transfected with shRNA and stained for FMRP show knockdown under shRNA conditions. Mean cell body pixel intensities for FMRP were measured for transfected (indicated by EGFP) and untransfected cells within the same image B) Mean pixel intensities of EGFP in all transfected cells measured show no change. Error bars represent SEM.

mutation (see Chapter 2 discussion) into future experiments. In the event that this still does not reduce axonal localization, we may have to consider that specific mRNAs bound to FMRP determine whether it is transported axonally. Since there is evidence that FMRP binds mRNAs before being incorporated into granules (Blackwell and Ceman, 2011), this is a possible result. Whether it is FMRP or its target mRNA that localizes FMRP to axons was discussed in Chapter 4.

Our results implicate the N-terminus in axonal localization and the C-terminus in regulation of axon growth and branching. In order to directly test these, we would begin with fusing these regions only to EGFP and testing them as we have done previously. Because the C-terminus shows so much variation, we would test multiple splice versions and lengths. If the N-terminus is required for axonal localization, then we should see that the C-terminal constructs are not able to localize axonally, but it will have no effect on axon growth and branching. If the N-terminus is required, then we can further reduce the size of the fragment in order to identify which part is necessary. Should the C-terminus actually be required here, the same could be done in this region. Since the N-terminus is required for various protein-protein interactions (Reeves et al., 2008), we could also use FRET on the full-length protein as well as various fragments to identify which terminus interacts with motor proteins. By narrowing down the fragment size, we can ask whether various regions or domains are sufficient to localize the EGFP axonally.

Lack of loss of axonal localization due to mutations in FMRP domains could also mean that the coordination of more than one domain is required for axonal localization to occur (see Chapter 4). Based on our results, several pairs have been selected which might be interesting. These include the KH1 (I241N) or KH2 (I304N) mutations paired with

either the RGG box deletion, S500A or S500D mutations as well as pairing the nuclear export sequence (NES) deletion with either the S500A or S500D mutations. For the most part, the rationale for each of these pairs centers around either elongated structure formation in the KH and NES domains mutations versus the reduction in puncta density in the RGG box deletion and S500A/D mutants.

Growth Cone Collapse Assay

Raper et al. (1990) developed a growth cone collapse assay that could be used to study factors that inhibit growth cone motility, a process related to axon growth and branching dynamics. This assay has previously been used in conjunction with Sema3A and FMRP (Luo et al., 1993; Li et al., 2009) but never with splice forms or mutants. *Fmr1* KO neurons show increased elaboration and decreased growth cone collapse. So each splice form can be applied to these neurons in order to attempt to rescue ordinary levels of growth cone collapse. Ideally, only the splice forms we have identified as being able to regulate axonal arbor complexity (SF7, SF9, and possibly SF1 and SF8) will be able to rescue the phenotype (increase growth cone collapse compared to *Fmr1* KO neurons). Each mutant FMRP can also be applied to this assay with the expectation that most will be able to rescue growth cone collapse but S500A, S500D, and NES34A will not. In conjunction with this assay, we can also measure levels of MAP1B mRNA and protein to look for repression of MAP1B mRNA in rescued conditions. This assay will provide another line of evidence that the FMRP splice forms and domains already implicated in axon growth and branching are involved in this function. If we can provide this second line of evidence implicating the S500 mutants in regulation of axon growth

and branching, we could then proceed with the negative feedback loop model discussed in Chapter 4 and design various experiments to test this.

Synapse Formation and Mixed Culture Assay

Another role for presynaptic FMRP has been implicated in synapse formation based on observations that the probability of synapse formation decreases when FMRP is absent (Hanson and Madison, 2007). A number of proteins are affected by the loss of FMRP. One such protein is neurexin 1α which is decreased in *Fmr1* knockout (KO) mouse synaptosomes (Liao et al., 2008). Neurexin 1α is a presynaptic transmembrane protein that binds the postsynaptic neuroligin, thus regulating both formation and structure of the synapse, *in vitro* (Nguyen and Sudhof, 1997). Alternative splicing of neurexin transcripts is predicted to yield up to 2000 splice forms expressed in different neuronal subsets (Ullrich et al., 1995). Because of this variation potential and their enrichment at synaptic plasma membranes, the connection between neurexin and neuroligin is speculated to be essential for synaptogenesis (Irie et al., 1997). Preliminary data from mixed culture assays shows that neurons from *Fmr1* KO mice form fewer synapses than their wild type counterparts (Schieffele et al., 2000; Biederer and Schieffele, 2007; Akins and Berk-Rauch, preliminary).

Further future studies in the role of axonal FMRP splice forms and domains can involve the use of a mixed culture assay under *Fmr1* KO conditions to rescue the synapse formation phenotype. In this assay, mammalian cell line cells are transfected with HA-tagged neuroligin 1 (NL1) and seeded with neurons transfected with tdTomato and synaptophysin-EGFP, a protein found in synaptic vesicles. Synapse formation is counted based on the presence of synaptophysin-EGFP-containing puncta.

Prior to conducting these experiments, we need to verify that 1) NL1 induces synapse formation between neurons and HEK cells and that 2) this induction is reduced in *Fmr1* KO conditions. These have been started. Thus far, we have seen reduced synapse formation in FMRP KD conditions compared to WT (shluc) conditions (Fig. 5.2). In this system, synapses form with both the NL1-expressing HEK293T cells and crossing untransfected neuronal processes. Because of this, puncta were counted based on whether they occurred crossing a HEK293T cell or not and compared both within conditions and between conditions. Thus, we see induction in the WT condition compared to baseline synapse formation but little induction in the FMRP KD condition. At this point, we still need to conduct experiments showing that the NL1 transfected into HEK293T is causing synapse formation. These will be compared to HEK293T that have been transfected with HA-UPRT which should not be involved in synapse formation.

If verification of preliminary data is successful, we can test each splice form and FMRP domain for rescue of synapse formation in *Fmr1* KO conditions using the same 3' UTR-targeting shRNA discussed above. Whether different splice forms or domains are required for this function or the same as we have already seen in axon growth and branching will be interesting.

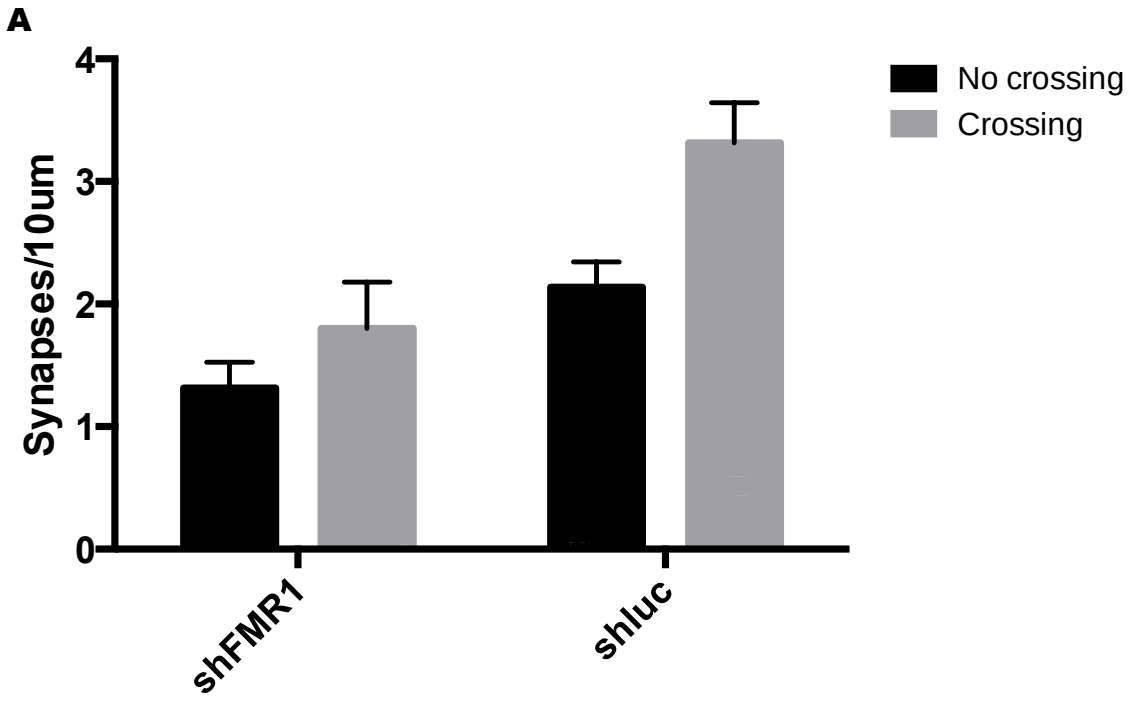

Figure 5.2 Synapse formation is reduced under FMRP KD conditions. The density of synapses was determined by counting synapses based on whether they occurred while an axon was crossing a NL1-transfected HEK293T cell or not. Synapse induction is increased when axons cross NL1-transfected HEK293T cells in shluc (control) conditions but is unaffected by NL1-trasnfected HEK293T cell crossing when FMRP is knocked down. Error bars represent SEM.

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Appendix A: Nuclear Localization Sequence

The nuclear localization sequence (NLS) of FMRP allows for nucleocytoplasmic shuttling in conjunction with the nuclear export sequence (NES). Though we had and worked with a construct in which this domain was deleted, we were unable to analyze axonal localization as other splice forms and mutants were analyzed due to a lack of puncta formation or specific localization (Fig. A). Kim et al. (2009) had similar troubles with an NLS deletion construct in which they found that protein products in which this domain has been deleted did not form functional protein and were unable to bind RNA. Based on this, our lack of puncta formation may be unsurprising since RNA binding is thought to be required as an initial step in the formation of FMRP-containing granules (Blackwell and Ceman, 2011). The FMRP NLS is a non-canonical NLS and the amino acids required for its function are unknown. If one or several point mutations are found that would be sufficient to lose NLS function without sacrificing other functions, then future studies could potentially involve this mutation.

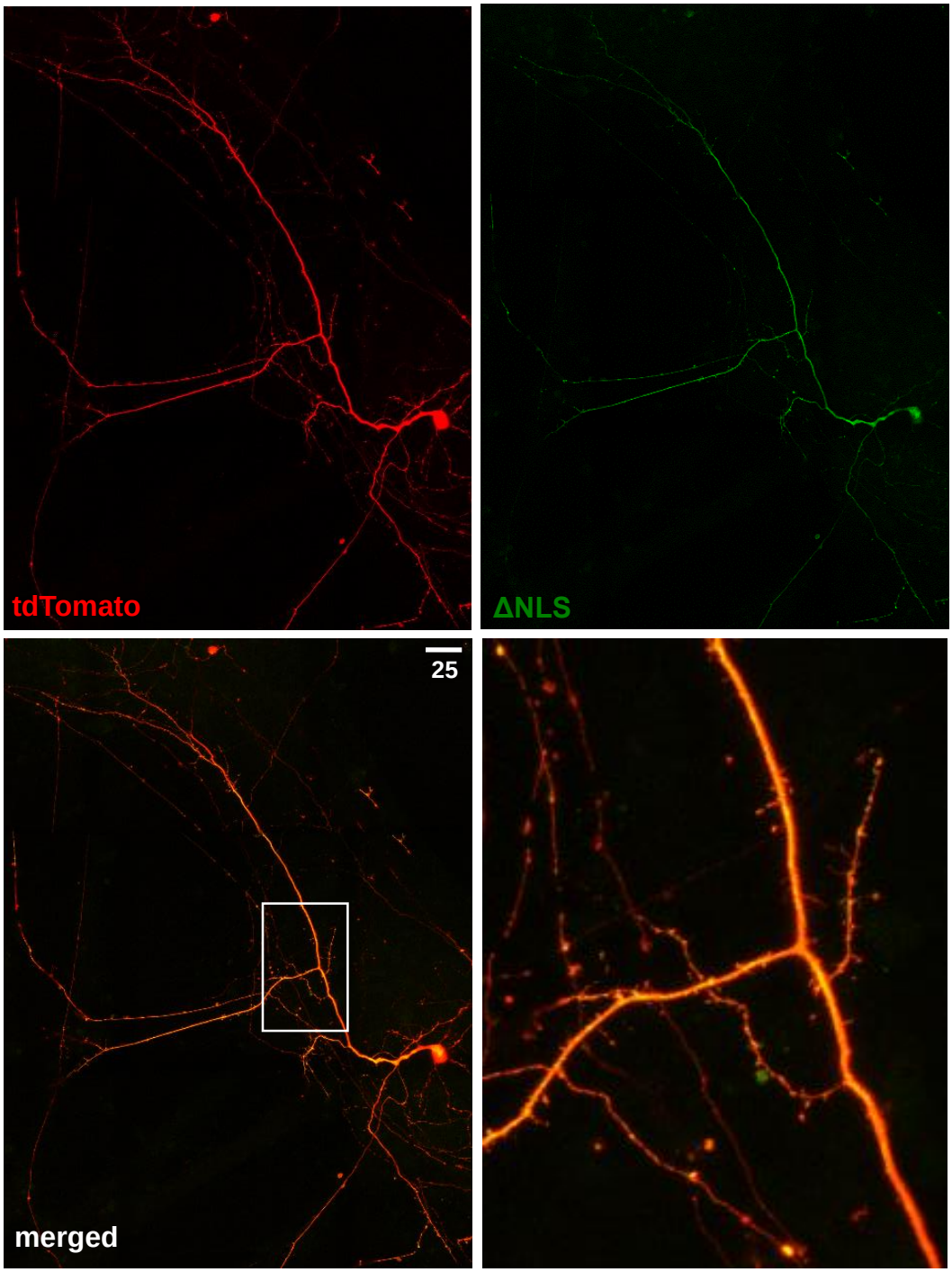

Figure A: Expression and localization of EGFP-FMRP-ΔNLS