The Akt-mTOR Pathway Drives Myelin Sheath Growth by Regulating Cap-Dependent Translation

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In the vertebrate CNS, oligodendrocytes produce myelin, a specialized membrane, to insulate and support axons. Individual oligodendrocytes wrap multiple axons with myelin sheaths of variable lengths and thicknesses. Myelin grows at the distal ends of oligodendrocyte processes, and multiple lines of work have provided evidence that mRNAs and RNA binding proteins localize to myelin, together supporting a model where local translation controls myelin sheath growth. What signal transduction mechanisms could control this? One strong candidate is the Akt-mTOR pathway, a major cellular signaling hub that coordinates transcription, translation, metabolism, and cytoskeletal organization. Here, using zebrafish as a model system, we found that Akt-mTOR signaling promotes myelin sheath growth and stability during development. Through cell-specific manipulations to oligodendrocytes, we show that the Akt-mTOR pathway drives cap-dependent translation to promote myelination and that restoration of cap-dependent translation is sufficient to rescue myelin deficits in mTOR loss-of-function animals. Moreover, an mTOR-dependent translational regulator was phosphorylated and colocalized with mRNA encoding a canonically myelin-translated protein in vivo, and bioinformatic investigation revealed numerous putative translational targets in the myelin transcriptome. Together, these data raise the possibility that Akt-mTOR signaling in nascent myelin sheaths promotes sheath growth via translation of myelin-resident mRNAs during development.

Key words: mTOR; myelin; oligodendrocyte; zebrafish

Significance Statement

In the brain and spinal cord, oligodendrocytes extend processes that tightly wrap axons with myelin, a protein- and lipid-rich membrane that increases electrical impulses and provides trophic support. Myelin membrane grows dramatically following initial axon wrapping in a process that demands protein and lipid synthesis. How protein and lipid synthesis is coordinated with the need for myelin to be generated in certain locations remains unknown. Our study reveals that the Akt-mTOR signaling pathway promotes myelin sheath growth by regulating protein translation. Because we found translational regulators of the Akt-mTOR pathway in myelin, our data raise the possibility that Akt-mTOR activity regulates translation in myelin sheaths to deliver myelin on demand to the places it is needed.

Introduction

During development, oligodendrocytes extend numerous processes that survey the environment and ensheath target axons with myelin. Notably, not all myelin sheaths produced by an individual oligodendrocyte are uniform in length and thickness (Murrie et al., 2007; Almeida et al., 2011; Chong et al., 2012), and changes in neural activity can influence the amount of myelin made on an axon (Hines et al., 2015; Koudelka et al., 2016; Mitew et al., 2018). Together, this suggests a mechanism whereby oligodendrocytes use localized cell signaling events to coordinate myelin production at distal locations with extracellular cues.

One strong candidate to mediate distal myelin production is the PI3K-Akt-mTOR signaling pathway, whose precise regulation is critical for proper myelin formation. When pathway activity was increased through either oligodendrocyte specific deletion of the antagonist phosphatase PTEN (Goebbels et al., 2010; Harrington et al., 2010) or overexpression of constitutively active Akt (caAkt) (Flores et al., 2008) oligodendrocytes made thicker myelin. Chronic treatment of mice overexpressing caAkt in oligodendrocytes with rapamycin, an mTOR inhibitor, reduced hypermyelination to WT levels (Narayanan...
et al., 2009) and decreased pathway activity resulting from oligodendrocyte-specific KO of mTOR or Raptor, the defining protein of the mTORC1 complex, resulted in severe hypomyelination of the spinal cord (Bercury et al., 2014; Lebrun-Julien et al., 2014; Wahl et al., 2014). Additionally, animals harboring a mutation in the ubiquitin ligase Fbxw7, which targets mTOR for degradation (Mao et al., 2009), made longer myelin sheaths in an mTOR-dependent manner (Kearns et al., 2015).

Protein translation and myelin protein synthesis are among the numerous cell biological processes regulated by the Akt-mTOR pathway. One way that Akt-mTOR signaling regulates protein translation is through the eukaryotic translation initiation factor 4E binding protein (4E-BP) family of proteins. Unphosphorylated 4E-BPs inhibit cap-dependent translation initiation by binding to eIF4E on the 5' cap of mRNAs (Pause et al., 1994). Upon phosphorylation by the mTORC1 subcomplex, 4E-BP1 dissociates from its binding partner eIF4E, thereby allowing for recruitment of eIF4A and eIF4G to assemble the eIF4F translation initiation complex and promotion of translation (Pause et al., 1994). Dysregulation of Akt-mTOR signaling in oligodendrocytes results in aberrant levels of some myelin proteins (Flores et al., 2008; Narayanan et al., 2009; Bercury et al., 2014; Lebrun-Julien et al., 2014; Wahl et al., 2014; Zou et al., 2014). In vitro, myelin basic protein mRNA is packaged into granules and actively transported into some, but not all, myelin sheaths (Aigner et al., 1993, 1997), where it is presumably translated. In support of this possibility, free ribosomes have been observed by electron microscopy in the distal ends of cultured oligodendrocytes (Lunn et al., 1997), and recently hundreds of other myelin resident mRNAs have been identified (Thakurela et al., 2016) and a subset validated in vivo (Yergert et al., 2021).

Here, using zebrafish as a model system, we directly tested the hypothesis that protein translation downstream of Akt-mTOR signaling promotes myelin development. We found that modulation of the Akt-mTOR signaling pathway controlled myelin sheath length and stability. By performing cell-specific manipulations of protein translation, we found that Akt-mTOR signaling required cap-dependent translation to promote proper myelin development. Moreover, oligodendrocytes colocalized mRNA and translational regulators downstream of mTOR in myelin; and through bioinformatic analysis, we identified hundreds of putative mTOR translational targets in myelin. Together, these data raise a model wherein mTOR signaling in myelin sheaths regulate sheath growth by promoting translation of myelin-resident mRNAs.

### Materials and Methods

#### Contact for reagent and resource sharing
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, B.A. (bruce.appel@cuanschutz.edu).

| Construct | Template | Primers |
|-----------|----------|---------|
| pME-4EBP1 | Addgene Plasmid 64927 | Forward: 5'-GGGGACAAAGTTTGTAACAAAAAGCAGCCTAATGGCGGCGGAAGAGCAG-3' Reversive: 5'-GGGGACACCATTTGCTACAGAAGAGGCTATGTTAAATGCTTACCTCAAACTGCTAGTCT-3' |
| pME-4EBP1^97/964A | Addgene Plasmid 64928 | Forward: 5'-GGGGAAEAGTTTGTAACAAAAAGCAGCCTAATGGCGGCGGAAGAGCAG-3' Reversive: 5'-GGGGACACCATTTGCTACAGAAGAGGCTATGTTAAATGCTTACCTCAAACTGCTAGTCT-3' |
| pME-mScarletCAAX | p3E-P2A-mScarletCAAX | Forward: 5'-GGGGACAAAGTTTGTAACAAAAAGCAGCCTAATGGCGGCGGAAGAGCAG-3' Reversive: 5'-GGGGACACCATTTGCTACAGAAGAGGCTATGTTAAATGCTTACCTCAAACTGCTAGTCT-3' |
| pME-eIF4E | Addgene Plasmid 17343 | Forward: 5'-GGGGACAAAGTTTGTAACAAAAAGCAGCCTAATGGCGGCGGAAGAGCAG-3' Reversive: 5'-GGGGACACCATTTGCTACAGAAGAGGCTATGTTAAATGCTTACCTCAAACTGCTAGTCT-3' |
| P3E-P2A-myrAkt | pME-myrAkt | Forward: 5'-AAGAGAGCTCTCATTTTCGTTTGACAGCAG-3' Reversive: 5'-TAAGAGAGCTCTCATTTTCGTTTGACAGCAG-3' |

#### Experimental model and subject details

Zebrafish lines and husbandry. All procedures were approved by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee and performed to their standards. WT non-transgenic embryos were generated by crosses of males and females of the AB strain. Tg(sox10:mRFP) embryos were generated by outcrossing Tg(sox10:mRFP)^97/964 (Kucenas et al., 2008) carriers with WT AB fish. mtor mutant larvae were generated by incrosses of heterozygote mtor^+/+ (Ding et al., 2011) carriers. Tg(mbpaeGF-P-CAAX) larvae were produced by injecting heterozygous Tg(mbpaeGF-P-CAAX)^97/964 (Yergert et al., 2021) carriers. Embryos and larvae were raised at 28.5°C in E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4 at pH 7.4 with sodium bicarbonate) and staged according to hours and days after fertilization. Only larvae displaying good health and normal developmental patterns were used experimentally. Animals used were of unknown sex.

#### Method details

**Genotyping.** mtor mutant larvae were genotyped following imaging. Whole anesthetized larvae were lyzed by incubation in 50 mM NaOH at 95°C for 30 min followed by neutralization with 1/10th volume Tris, pH 8.0. PCR amplification was performed using a small volume of lysis and the primers: forward primer: 5'-ATAAGAAAAAAAGGCAACTTGCTG TACCC; reverse primer: 5'-CTTACACTCTGACAGAGACAAAGG-3'; 5'LTR primer: 5'--CCCTAAGTACTTGTACTTTCACTTG-3'.

**Plasmids and expression construct generation.** Expression constructs were generated for Multisite Gateway cloning using LR Clonase II Plus (Thermo Fisher Scientific) to recombine entry clones p5E, pME, and p3E into a destination vector, pDEST. All entry clones and expression constructs were screened by restriction digestion and sequence confirmed by Sanger sequencing. The following entry clones were used in this study: p5E: p5E-sox10; p5E-mbpA pME: pME-mNeonCAAX; pME-myrAkt; pME-4EBP1; pME-4EBP1^97/964A; pME-mScarletCAAX; pME-eIF4E; pME-mbpaCDS-24xMBS; pME-HA-NLS-tdMCP-tagREP p3E: p3E-P2A-mNeonCAAX; p3E-eGFP; p3E-P2A-myrAkt; p3E-poly A; p3E-mbpa-3'UTR pDEST: pDEST-Tol2-pA pcDNA3-TORCAR (Addgene Plasmid 64927) (Zhou et al., 2015) and pcDNA3-TORCAR(T/A) (Addgene Plasmid 64928) (Zhou et al., 2015) were gifts from Jin Zhang and used to create pME-4EBP1 and pME-4EBP1^97/964A, respectively. pHA-eIF4E (Addgene Plasmid 17343) (Okamura et al., 2007) used to generate pME-eIF4E was a gift from Dong-Er Zhang. pme-HA-NLS-tdMCP-tagREP (Addgene Plasmid 86244) (Campbell et al., 2015) was a gift from Florence Marlow (Table 1).

**Microinjections.** Microinjections were performed into newly fertilized eggs generated by incrosses of WT AB and heterozygote mtor^+/+ carriers. Injection solutions contained 5 μl 0.4 x sKCl, 250 ng Tol2 mRNA, a total of 250 ng plasmid DNA, and water to 10 μl. In instances where multiple plasmids were injected, equal concentrations of each plasmid were used. Larvae were raised to the developmental time point indicated in individual experiments and selected for good health before inclusion in experiments.

**Pharmacological treatment.** Tg(mbpaeGF-P-CAAX) were treated from 3 to 4 dpf with 10 μM rapamycin (LC Laboratories) or 0.1%
DMSO in embryo medium before being fixed and processed for immunohistochemistry.

**Immunohistochemistry.** Tg(sox10:mRFP) and Tg(mbpa:eGFP-CAAX) larvae were fixed at 4°C overnight in 4% PFA/1× PBS. Larvae were washed in PBS and embedded in 1:5% agar/30% sucrose blocks placed in 30% sucrose overnight at 4°C. Blocks were frozen in 2-methyl butane chilled by immersion in liquid nitrogen; 20 μm sections were cut and collected on microscope slides. Sections were rehydrated with PBS and then blocked with 5% goat serum/2% BSA in PBS for 1 h at room temperature. Rabbit anti-4E-BP1 (Cell Signaling Technology, catalog #53H11) or rabbit anti-P-4E-BP1 (Cell Signaling Technology, catalog #T37/46) was applied at 1:500 dilution in blocking solution overnight at 4°C. The sections were washed with PBS and then incubated with Alexa Fluor-488 goat anti-rabbit antibody (Invitrogen, catalog #A11008) or Alexa Fluor-568 goat anti-rabbit antibody (Invitrogen, catalog #A11011) at 1:200 dilution in blocking solution for 2 h at room temperature. Slides were washed again with PBS, covered with Vectashield, and coverslipped.

**Microscopy.** For static imaging experiments, larvae were anesthetized with 0.06% tricaine and immobilized in 0.6% low-melt agarose with 0.4% tricaine. For dynamic live imaging experiments, larvae were immobilized in 0.3 mg/ml pancuronium bromide aided by a small tail slit and embedded in 1.2% low-melt agarose. For analysis of myelin sheath length and dynamics, we acquired images using a C-Apochromat 63×/1.20 NA water immersion objective on a Carl Zeiss CellObserver Spinning Disk confocal microscope equipped with a Photometrics Prime 95B camera. For analysis of subcellular localization, we acquired images using a Plan-Apochromat 63×/1.4 NA oil immersion objective on a Carl Zeiss LSM 880. Images were collected with Zen (Carl Zeiss), blinded, and processed/analyzed in ImageJ/Fiji (Schindelin et al., 2012).

**Quantification and statistical analysis.**

**Quantification of myelin sheath length and number.** Three dimensional images of single spinal cord oligodendrocytes were collected in Zen. We then used the Fiji plugin Simple Neurite Tracer (Longair et al., 2011) to quantify myelin sheath length and number. Briefly, blinded 8-bit z-stack images of single oligodendrocytes were opened in Simple Neurite Tracer. We traced each sheath and saved the path length (sheath length), path number (sheath number), and the sum of the path lengths (cumulative sheath length) in Excel for further analysis in R and Prism 9.

**Quantification of myelin sheath dynamics.** Three dimensional images of the spinal cord were collected every 15 min for 4 h in Zen. Z-volume movies collected from individual larvae were opened in Fiji. We used the trace segmented line tool to trace individual myelin sheaths. Single myelin sheaths were measured in all frames and exported to Excel before continuing to the next. Myelin sheath dynamic data were then transferred to Prism 9 for visualization and analysis.

**Colocalization quantification.** Three-dimensional images of spinal cord oligodendrocytes coexpressing sox10:4E-BP1-eGFP and mbpa MS2 reporter constructs were collected in Zen. We imported these images to Fiji and used JACoP (Just Another Colocalization Plugin) (Belte and Cordelières, 2006) to quantify colocalization. Specifically, we isolated single optical planes of individual myelin sheaths and used JACoP to calculate Mander’s Colocalization Coefficients, M1 and M2. M1 and M2 describe the proportion of protein 1 that colocalizes with protein 2 and vice versa, independent of fluorescent intensity. These data were saved in Excel and exported to Prism 9 for visualization and analysis.

**Statistics.** Plots were generated in R (version 3.6.3) with RStudio using ggplot2 (Wickham, 2016) or biomaRt (Durinck et al., 2009) or Prism 9. All statistical tests were performed in Prism 9. We first tested normality of data. In no instances were all datasets normal. For unpaired comparisons, we used Mann–Whitney test to compare ranks. For multiple comparisons, we first assessed overall significance with the Kruskal–Wallis test. If the Kruskal–Wallis test showed significance, we then performed pairwise Mann–Whitney tests with Bonferroni–Holm correction for multiple comparisons.

**Bioinformatic analysis**

**Find Individual Motif Occurrences (FIMO) analysis.** We used FIMO (version 5.3.3), part of the MEME suite software, to identify transcripts containing terminal oligopyrimidine (TOP)-like or Cytosine Enriched Regulator of Translation (CERT) motifs in the myelin transcriptome. We downloaded the 5’ untranslated region (5’UTR) sequences from the myelin transcriptome and created position weight matrices corresponding to TOP-like or CERT motifs. We uploaded the 5’UTR sequences and position weight matrices to FIMO. We changed options to scan the given strand only and otherwise used default settings. We downloaded the resulting file and removed duplicates to generate the final list of unique genes containing TOP-like or CERT motifs.

**Gene ontology analysis.** We used DAVID software (version 6.8) to identify gene ontology terms associated with transcripts containing TOP-like or CERT motifs. We submitted Ensembl Gene IDs of transcripts identified as having these motifs and selected categories for biological process, cellular compartment, and up_keywords. We filtered for false discovery rate <0.05 and removed any duplicate terms. We sorted terms from lowest to highest false discovery rate and selected the top 20 for analysis.

**Results**

The Akt-mTOR pathway drives myelin sheath growth

We previously showed that homozygous mtor mutant zebrafish larvae express abnormally low levels of myelin genes (Kearsn et al., 2015), consistent with studies from mice showing that conditional loss of mTOR function in oligodendrocytes results in a decreased expression of some myelin genes and proteins and decreased myelin sheath thickness (Wahl et al., 2014). For this study, we assessed the number and length of myelin sheaths formed by oligodendrocytes as a sensitive measure of how mTOR activity promotes myelin development. To label individual oligodendrocytes, we used Tol2-mediated transgenesis (Kwan et al., 2007) and sox10 regulatory DNA to express the membrane-localized fluorophore mNeonCAAX in oligodendrocyte lineage cells. We injected sox10:mNeonCAAX into newly fertilized eggs generated from incrosses of WT or mtor heterozygous zebrafish to generate homozygous mtor mutant loss-of-function animals. At 5 d postfertilization (dpf), we imaged spinal cord oligodendrocytes (Fig. 1B) and analyzed oligodendrocyte morphology. On average, myelin sheaths in mtor mutant larvae were ~35% shorter than those in WT larvae (Fig. 1C). Additionally, oligodendrocytes in mtor mutant larvae produced fewer sheaths on average than those in WT larvae (Fig. 1D). Together, this reduction of both myelin sheath length and number resulted in a nearly 50% reduction in the cumulative length of myelin produced by individual oligodendrocytes of mtor mutants compared with WT controls (Fig. 1E). These data therefore show that mTOR function promotes the length and number of myelin sheaths formed by spinal cord oligodendrocytes.

In mice, oligodendrocytes programmed to express a constitutively active form of Akt expressed elevated levels of myelin mRNA and protein and produced significantly thicker myelin sheaths (Flores et al., 2008). To test whether elevated Akt signaling also increases myelin sheath length and number, we drove oligodendrocyte-specific expression of a constitutively active Akt1 allele. This allele, hereafter referred to as myrAkt, has an N-terminal myristoylation signal as well as threonine 308 and serine 473, which are the phosphorylation sites that result in full activation, mutated to aspartic acid (Aoki et al., 1998). To identify cells expressing this allele, we appended the viral P2A sequence followed by mNeonCAAX to generate the construct sox10:myrAkt-P2A-mNeonCAAX, which drives expression of both myrAkt and mNeonCAAX in oligodendrocyte lineage cells from the same construct. We injected sox10:myrAkt-P2A-mNeonCAAX into newly fertilized WT eggs and at 5 dpf imaged mNeonCAAX*
spinal cord oligodendrocytes for ensheathment analysis. On average, myelin sheaths generated by cells expressing myrAkt were ~30% longer than those made by WT controls (Fig. 1C). Additionally, cells expressing myrAkt had slightly more sheaths than cells expressing a control, but the difference did not reach statistical significance (Fig. 1D). Together, the increased individual myelin sheath length and number resulted in ~40% more myelin generated on constitutive activation of Akt (Fig. 1E). These data show that constitutive Akt activation in WT cells autonomously promotes myelin sheath elongation.

In mice, Akt and mTOR function in a common pathway to promote myelin gene and protein expression and myelin sheath thickness (Narayanan et al., 2009). Because Akt has numerous downstream targets, it was important to determine whether the myelin sheath length-promoting activity of myrAkt requires mTOR function. To test this, we elevated Akt signaling in oligodendrocytes of mtor mutant larvae by injecting sox10:myrAkt-P2A-mNeonCAAX into newly fertilized eggs generated from incrosses of mtor mutant heterozygotes. If Akt and mTOR function in a common pathway to promote myelin sheath growth, we predicted that driving constitutively active Akt in oligodendrocytes of mtor mutant larvae would result in a myelin phenotype indistinguishable from mtor mutants alone. By contrast, if Akt does not require mTOR function to promote myelin sheath growth, we predicted driving constitutive Akt activation in oligodendrocytes of mtor mutant larvae would increase myelin sheath length. Strikingly, oligodendrocytes in mtor mutant larvae that expressed myrAkt produced shorter (Fig. 1C) and fewer (Fig. 1D) myelin sheaths than WT controls. These cells produced an average of ~50% less cumulative myelin sheath lengths than cells of WT larvae (Fig. 1E). We then compared the myelin profiles of oligodendrocytes of mtor mutant larvae with those of mtor mutant larvae that also expressed constitutively active Akt and found the average length, number, and cumulative amount of myelin produced per cell were indistinguishable (Fig. 1C–E). From this, we conclude that Akt requires mTOR function to promote myelin sheath length, a result that is comparable to the requirement in myelin sheath thickness (Narayanan et al., 2009).

**Decreased myelin sheath length in mtor mutant larvae is not because of reduced body size**

The Akt-mTOR pathway is a key regulator of many processes, including cell size (Fingar et al., 2002). Because in these experiments mtor mutant larvae were global loss of function, it was conceivable that the observed decrease in myelin sheath length was the result of an overall decrease in body size. To rule out this possibility, we quantified the body size of WT and mtor mutant larvae generated from incrosses of heterozygous mtor mutant animals. At 5 dpf, we imaged WT and mtor-/- larvae laterally (Fig. 2A). To approximate the size of larvae, we traced the body of the entire animal and calculated the area contained within the

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**Figure 1.** Akt-mTOR pathway activity drives myelin sheath length. **A,** Schematic representation of the Akt-mTOR pathway. **B,** Representative images of single spinal cord oligodendrocytes of 5 dpf WT or mtor mutant larvae with or without expression of myrAkt. Dashed line indicates an individual myelin sheath. Scale bar, 10 μm. Quantification of average sheath length (C), sheath number per cell (D), and cumulative myelin sheath length (E). Violin plot solid lines indicate median. Dashed lines indicate 25th and 75th percentiles. Overall significance was assessed using the Kruskal–Wallis test: sheath length, \( p < 1 \times 10^{-5} \); cumulative sheath length, \( p = 2 \times 10^{-12} \), \( h = 57.40 \). Orange dots represent individual data points. \( p \) values are pairwise Mann–Whitney tests with Bonferroni correction for multiple comparisons. Sample sizes: WT, 16 fish, 16 cells, 161 sheaths; mtor-/-, 23 fish, 23 cells, 206 sheaths; myrAkt, 18 fish, 18 cells, 197 sheaths; myrAkt + mtor-/-, 22 fish, 22 cells, 184 sheaths.

**Figure 2.** mtor mutant larvae do not differ in size from WT larvae. **A,** Lateral image of WT (top) and mtor mutant (bottom) larvae at 5 dpf. Scale bar, 1 mm. **B,** Quantification of the lateral area of WT and mtor mutant larvae. Mann–Whitney, \( p = 0.1972 \), \( U = 78 \). WT, 14 animals; mtor-/-, 15 animals.
trace in a measure we called lateral area. In comparing the lateral area between WT and mtor mutant larvae, we found no difference in the mean size between the groups. From this, we conclude that the shorter myelin sheaths of mtor mutant larvae were not simply the result of an overall smaller body size.

**mTOR pathway activity promotes myelin sheath stabilization**

Changes in myelin sheath length could result from differences in growth rate, stability, or both. To understand which of these parameters was altered on mTOR pathway loss of function, we performed time-lapse imaging of actively myelinating oligodendrocytes beginning at 72 h after fertilization, when myelin sheath growth is highly dynamic. We collected three-dimensional images of WT transgenic sox10:mRFP and mtor mutant larvae transiently expressing sox10:mScarletCAAX every 15 min for 4 h (Fig. 3A). Myelin sheath length was quantified at each time point during the imaging period. To understand the net change in myelin sheath length during the imaging period, we subtracted the length of a given sheath in the first frame from its length in the last frame. We found that, on average, myelin sheaths in WT larvae grew longer during the imaging period, whereas sheaths of mtor mutant larvae, on average, became shorter (Fig. 3B). We calculated the mean change in sheath length at each time point by subtracting the length in the initial frame and analyzing those as a function of time. On average, sheaths in mtor mutant larvae displayed a steady net negative change in size during the imaging period compared with the net positive change observed in sheaths of WT larvae (Fig. 3C). Together, these data show that mTOR signaling is required to drive myelin sheath growth during development.

Why did myelin sheaths in mtor mutant larvae not show a net growth during development? Did these sheaths not grow at all or did they grow but were not stabilized? To distinguish between these possibilities, we calculated the total amount of space an individual myelin sheath occupied during the imaging period. This measure, which we referred to as dynamic range, is defined by the maximum length of a given sheath subtracted by the minimum length of that sheath. There was no difference in the average dynamic range of myelin sheaths in WT and mtor mutant larvae (Fig. 3D), which shows that, in the absence of mTOR signaling, myelin sheaths are capable of growth but are not stabilized.

**Cap-dependent translation regulated by Akt-mTOR signaling drives myelin sheath growth**

The Akt-mTOR pathway coordinates numerous cell biological pathways, including transcription, translation, metabolism, and cytoskeletal dynamics. Because myelination demands protein and lipid synthesis, we tested the role that protein translation downstream of mTOR signaling plays in myelin development. To assess this, we performed cell-specific manipulation of the downstream translational regulators 4E-BP1 and eIF4E. To first test whether 4E-BP1 function is required for myelin development, we overexpressed a human allele of 4E-BP1 where threonines 37 and 46, which are obligate mTOR phosphorylation sites, were mutated to alanine (4EBP1T37/46A). These mutations render the protein unable to be phosphorylated by mTORC1 and released from its translational repressor state (Gingras et al., 1999), thereby inhibiting 4E-BP1-mediated translation (Fig. 3A). Using the Tol2-transgenesis system, we transiently expressed mNeonCAAX, a WT allele of human 4E-BP1 (4EBP1WT) and mNeonCAAX, or 4EBP1T37/46A and mNeonCAAX in oligodendrocyte lineage cells of WT animals using sox10 regulatory DNA. Overexpression of 4EBP1WT did not change myelin sheath length, number, or cumulative length of myelin made compared with WT controls (Fig. 4D,E). However, when mTOR-4E-BP1 signaling was perturbed by overexpression of the 4EBP1T37/46A allele, oligodendrocytes generated myelin...
Figure 4. 4E-BP1-dependent translation downstream of Akt-mTOR signaling promotes myelination. A, Schematic illustrating the mechanism by which 4E-BPs promote translation initiation and the function disruption strategy. B, Representative images of single 5 dpf spinal cord oligodendrocytes expressing controls or 4E-BP1 manipulation with or without simultaneous mTOR pathway manipulations. Scale bar, 10 μm. Quantification of myelin sheet length (C), number (D), or cumulative myelin sheet length (E). Violin plot solid lines indicate median. Dashed lines indicate 25th and 75th percentiles. Overall significance was assessed using the Kruskal–Wallis test: sheath length, \( p < 1 \times 10^{-13}, h = 458.4 \); sheath number, \( p = 8.62 \times 10^{-6}, h = 33.44 \); cumulative sheath length, \( p < 1 \times 10^{-13}, h = 86.76 \). Orange dots represent individual data points. Sample sizes: WT, 19 fish, 19 cells, 190 sheaths; 4EBP1wt, 18 fish, 18 cells, 169 sheaths; Fedder-Semmes and Appel mTOR-Mediated Translation Drives Myelination J. Neurosci., October 13, 2021 – 41(41):8532–8544 – 8537.
sheaths that were ~35% shorter than those of WT larvae or those expressing 4EBP1WT (Fig. 4D). Additionally, cells expressing 4EBP1T37/46A produced fewer myelin sheaths than those of controls (Fig. 4E). Together, the reduction in myelin sheath length and number resulted in a 50% reduction in the total length of myelin produced by cells expressing 4EBP1T37/46A (Fig. 4F). From these data, we conclude myelin development requires 4EBP1-dependent translation.

As a direct test of whether the Akt-mTOR pathway requires 4E-BP1 function to drive myelination, we simultaneously manipulated Akt-mTOR signaling and 4E-BP1. To drive Akt-mTOR pathway activity in oligodendrocytes, we created the construct sox10:mscarletCAAX-P2A-myrAkt. Using the Tol2-transgenesis system, we expressed either sox10:mscarletCAAX-P2A-myrAkt alone or in combination with 4EBP1T37/46A and mNeonCAAX in WT larvae. Simultaneous expression of myrAkt and 4EBP1T37/46A resulted in an ~35% reduction in average myelin sheath length (Fig. 4D). This was accompanied by a decrease in myelin sheath number (Fig. 4E) and resulted in a nearly 50% reduction in the total length of myelin produced (Fig. 4E). Individual and cumulative myelin sheath length as well as number produced by cells expressing both myrAkt and 4EBP1T37/46A were not different from those of cells expressing 4EBP1T37/46A alone (Fig. 4D, E). To test whether mTOR and 4EBP1 function in a common pathway to promote myelin development, we performed ensheathment analyses in mtor mutant larvae expressing either mNeonCAAX alone or 4EBP1T37/46A and mNeonCAAX. Myelin sheath length, number, and cumulative length of myelin were not appreciably changed in WT larvae expressing 4EBP1T37/46A alone, mtor mutants, or mtor mutant larvae expressing 4EBP1T37/46A (Fig. 4D,E). From these data, we conclude that 4E-BP1-mediated translation downstream of Akt-mTOR signaling is required for proper myelin development.

Because our 4E-BP1T37/46A data showed that dampening cap-dependent translation inhibits myelin development, we tested whether increasing cap-dependent translation would drive myelin sheath elongation. Constitutive overexpression of eIF4E in the mouse brain exaggerates cap-dependent translation that results in alterations to synaptic morphology and physiology and animal behavior (Gligorjas et al., 2013; Santini et al., 2013). To test the role of eIF4E-mediated translation in our system, we expressed human eIF4E and mNeonCAAX in oligodendrocytes by Tol2-transgenesis. Oligodendrocytes expressing human eIF4E had, on average, a 10% increase in the average length of myelin sheaths (Fig. 4D,E). This was accompanied by no change in the number of myelin sheaths produced (Fig. 5C). Cumulatively, cells expressing human eIF4E produced a greater length of myelin sheaths (Fig. 5D), but this did not reach statistical significance. Together, these data indicate that exaggerated protein translation alone is capable of driving myelin sheath elongation.

Our results showing 4E-BP1-dependent translation promotes myelination led us to conclude that translation initiation downstream of Akt-mTOR is required for myelin sheath elongation. As a direct test of this possibility, we exaggerated translation in oligodendrocytes of mtor mutant larvae by expressing human eIF4E and mNeonCAAX using Tol2 transgenesis and sox10 regulatory elements. If translation downstream of mTOR signaling promotes myelination, we predicted mtor mutant myelin sheath length and number deficits would be rescued on exaggerated translation achieved by eIF4E overexpression. Indeed, eIF4E overexpression in oligodendrocytes of mtor mutant larvae restored average myelin sheath length (Fig. 5B), number (Fig. 5C), and cumulative myelin sheath length (Fig. 5D) to WT levels. These experiments provide strong evidence that protein translation regulated by mTOR signaling is required for oligodendrocytes to produce proper length and number of myelin sheaths.

Oligodendrocytes localize mTOR-regulated translational machinery to myelin sheaths

The localization of mRNAs (Aigner et al., 1993; Thakurela et al., 2016; Yergert et al., 2021), ribosomes (Lunn et al., 1997), and RNA binding proteins (Doll et al., 2020) in distal myelin sheaths supports a model where local protein translation promotes myelination. We therefore investigated the possibility that translation factors that are regulated by mTOR signaling are localized to nascent myelin sheaths. To test this possibility, we used immuno-histochemistry to detect endogenous 4E-BP1 in spinal cord myelin sheaths of 4 dpf Tg(mbpa:eGFP-CAAX) larvae. We found that endogenous 4E-BP1 localizes to discrete puncta in myelin sheaths of developing zebrafish (Fig. 6A). We then tested whether 4E-BP1 localized to myelin sheaths was phosphorylated by mTOR signaling. To test this possibility, we performed immuno-histochemistry using an antibody to detect 4E-BP1 phosphorylated at the obligate mTOR phosphorylation sites threonines 37 and 46. We found that phosphorylated 4E-BP1 was indeed localized to myelin sheaths of 4 dpf Tg(mbpa:eGFP-CAAX) larvae (Fig. 6B, left). Importantly, the phospho-4E-BP1 signal was abolished on inhibition of mTOR signaling with rapamycin (Fig. 6B, right). Together, these observations raise the possibility that translation regulated by 4E-BP1 is locally activated by Akt-mTOR signaling in myelin sheaths in vivo.

For Akt-mTOR signaling to promote localized translation, we predicted that Akt-mTOR-dependent translational regulators would colocalize with myelin localized mRNAs. To investigate this possibility, we used fluorescent reporters to test whether 4E-BP1 colocalized with mbpa mRNA, which encodes myelin basic protein, in vivo using the MS2-MCP RNA visualization system. The MS2-MCP system consists of an mRNA containing 24 MS2 binding sites (24xMBS), which form repetitive stem loops, and a fluorescently tagged MS2 coat protein (MCP), which specifically binds the 24xMBS stem loops to visualize mRNA via fluorescent reporter (Bertrand et al., 1998). Our laboratory has previously used this system to reveal localization of RNA encoded by mbpa, a zebrafish ortholog of Mbp, to myelin sheaths (Yergert et al., 2021). We coexpressed mbpa Tg MS2-mScarlet and a fluorescently tagged 4E-BP1 fusion protein (4E-BP1-eGFP) in oligodendrocytes using the Tol2-transgenesis system and imaged eGFP/mScarlet+ spinal cord oligodendrocytes at 4 dpf (Fig. 6C). Expression of 4E-BP1-eGFP and mbpa MS2 was highly colocalized in myelin sheaths (Fig. 6D, inset). We performed colocalization analysis using JACoP and calculated Mander’s coefficient to quantify the amount of 4E-BP1-eGFP colocalized with mbpa mRNA and vice versa. We found that ~85% of mbpa mRNA colocalized with 4E-BP1-eGFP in myelin sheaths (Fig. 6D), whereas 4E-BP1-eGFP colocalized with mbpa mRNA ~70% of the time (Fig. 6D). Additionally, the percent of 4E-BP1-eGFP that colocalized with mbpa mRNA was highly variable, raising the possibility that 4E-BP1 regulates the translation of other myelin-resident mRNAs.
Identification of putative translational targets in myelin

What myelin-resident mRNAs might be targets of mTOR-regulated translation? To address this question, we used bioinformatics to identify putative myelin-resident translational targets. Loss of 4E-BPs in cultured cells was sufficient to render translation of mRNAs containing TOP or TOP-like motifs in their 5'UTR resistant to inhibition by the mTOR inhibitor Torin 1 (Thoroen et al., 2012), indicating that mTOR signaling through 4E-BPs promotes the translation of 5' TOP or TOP-like motif containing transcripts. To identify putative 4E-BP1 translational
targets, we used the function FIMO in the package MEME Suite to scan the myelin transcriptome defined by Yergert et al. (2021) for TOP or TOP-like motifs (Fig. 7A). We found that 324 of the 1195 annotated 5′UTRs in the myelin transcriptome contained a TOP-like motif (Fig. 7B; Extended Data Fig. 7-1), indicating that nearly 25% of mRNAs in the myelin transcriptome could be subject to translational regulation by mTOR-4E-BP1 signaling. Canonical TOP-motif containing mRNAs encode proteins that are components of translational machinery (Yoshihama et al., 2002; Iadevaia et al., 2008). We identified such proteins in our dataset, including the 40S ribosomal proteins S2 and S18 (Rps2 and Rps18, respectively). Additionally, we identified other transcripts with known TOP motifs, such as vimentin (Meyuhas and Kahn, 2015), together indicating that our bioinformatic analysis accurately identified TOP and TOP-like motif containing mRNAs in the myelin transcriptome. To ask whether myelin-resident mRNAs with TOP or TOP-like motifs encode specific functions, we performed gene ontology analysis. From this, we identified some processes indicative of developmental myelination, such as cell junction, membrane, nervous system development, and cytoplasm (Fig. 7C). Similar to previous analysis of myelin-resident transcripts (Yergert et al., 2021), we also identified terms associated with synapses and cell signaling (Fig. 7C). These data support the possibility that 4E-BP1 regulated translation of specific myelin-resident mRNAs could promote myelination via synaptic-like mechanisms.

We next asked whether any myelin-resident mRNAs were putative translational targets of the 4E-BP1 binding partner eIF4E. Studies from eIF4E haploinsufficient mice identified the shared cis-regulatory element CERT present in ~70% of mRNAs that are translational targets of eIF4E (Truitt et al., 2015). To identify transcripts containing a CERT sequence in the myelin transcriptome, we used the FIMO function in MEME suite to search the 5′UTRs of myelin resident transcripts for CERT (Fig. 8A). We found that nearly 50% (570 of 1195) of the annotated 5′UTRs in the myelin transcriptome contained the CERT sequence (Fig. 8B; Extended Data Fig. 8-1). Similar to 4E-BP1 targets, CERT-containing transcripts were enriched for gene ontology terms associated with synapses and intracellular signaling, but also included different categories, such as lipoprotein and ion channel (Fig. 8C).

Because both 4E-BP1 and eIF4E regulate translation downstream of mTOR signaling, we defined the transcripts that contain both of these sequences. Of the 1195 annotated 5′UTRs in the myelin transcriptome, 245 possessed both a TOP-like motif and a CERT sequence (~20%) (Fig. 9A). Approximately 75% of TOP-like motif containing also contained a CERT sequence (245 of 324) (Fig. 9B; Extended Data Fig. 9-1), whereas only ~40% of CERT-containing transcripts also contain a TOP-like motif (245 of 570) (Fig. 9B). Myelin-resident transcripts containing both a TOP-like motif and CERT sequence represented gene ontology terms associated with myelin development, such as cell junction, membrane, and nervous system development, but also represented distinct categories, such as those associated with potassium and neurotransmitter transport (Fig. 9C). Together, the similarities and differences among putative 4E-BP1 and eIF4E translational targets support the possibility that translation regulated by these two proteins has both overlapping and distinct functions in myelin development.

**Discussion**

During development, oligodendrocytes ensheathe axons with myelin to support nervous system function. The Akt-mTOR pathway is a key regulator of radial myelin sheath growth, but our understanding of how this pathway promotes myelin sheath elongation and the downstream signaling events it regulates to promote myelination remains limited.

Using a series of manipulations to increase and decrease Akt-mTOR pathway activity, we found that Akt-mTOR signaling bidirectionally controls myelin sheath length. Oligodendrocytes of larval zebrafish homozygous for a loss-of-function mutation in mtor make myelin sheaths that are significantly shorter than WT controls (Fig. 1). Overexpressing a constitutively active allele of Akt specifically in oligodendrocytes makes myelin sheaths longer in an mTOR-dependent manner (Fig. 1). These data provide a detailed cellular study of how this pathway promotes myelin development and build on previous reports in the larval zebrafish implicating mTOR signaling as a key regulator of myelin sheath length (Kearns et al., 2015; Preston et al., 2019). In addition to shorter myelin sheaths, oligodendrocytes in mtor mutant larvae made significantly fewer myelin...
sheaths than controls (Fig. 1). Consistent with these observations, loss of function of mTOR and mTORC1 subcomplex activity in spinal cord oligodendrocytes of mice results in fewer myelinated axons during development (Bercury et al., 2014; Lebrun-Julien et al., 2014; Wahl et al., 2014). On the other hand, overexpression of constitutively active Akt in oligodendrocytes did not result in individual oligodendrocytes generating more myelin sheaths (Fig. 1). Studies in mice overexpressing caAkt in oligodendrocytes did not quantitate the number of myelinated axons, so it is difficult to assess whether these phenotypes are consistent. However, when mTORC1 activity was elevated through conditional deletion of TSC1, fewer myelinated axons were observed during development (Jiang et al., 2016). Inhibition of mTOR signaling can partially rescue this phenotype. In these genetic mouse studies, it is unclear whether the deficit in myelination is because of a decrease in the number of myelinating cells or fewer sheaths being generated by each oligodendrocyte. Future studies should focus on teasing these two mechanisms apart. Collectively, these data highlight the importance of precise regulation of Akt-mTOR signaling in myelin development.

The Akt-mTOR pathway controls numerous cell biological processes. By inhibiting 4E-BP1 function we found that protein translation initiated by Akt-mTOR signaling drives myelin sheath growth (Fig. 4). Previous studies have shown that Akt-mTOR signaling in oligodendrocytes promotes both transcription and translation of some myelin genes and proteins (Flores et al., 2008; Narayan et al., 2009; Tyler et al., 2009, 2011; Bercury et al., 2014; Lebrun-Julien et al., 2014; Wahl et al., 2014; Zou et al., 2014; Kearns et al., 2015) and translation only of other myelin proteins (Tyler et al., 2009, 2011; Bercury et al., 2014; Lebrun-Julien et al., 2014; Wahl et al., 2014). Consequently, the independent roles that transcription and mTOR mediated processes, such as cytoskeletal regulation (Musah et al., 2020; Brown et al., 2021), are critical of driving myelin development.

Consistent with the role of protein translation driving myelination downstream of Akt-mTOR signaling, we found that eIF4E overexpression cell-autonomously rescued myelin sheath length and number deficits observed in mTOR loss-of-function mutants (Fig. 5). eIF4E represents a point of convergence between Akt-mTOR signaling and the MAPK/ERK pathway. Phosphorylation of eIF4E at Ser201 by Mnk1 directly regulates eIF4E cap-binding activity downstream of ERK signaling (Waskiewicz et al., 1997). Pharmacological treatment of mice with the mTOR inhibitor rapamycin led to an upregulation of phospho-ERK1/2 in oligodendrocytes of the corpus callosum (Dai et al., 2014), indicating that mTOR signaling may negatively regulate ERK signaling during development. To date, crosstalk between mTOR and ERK signaling during myelination has proposed Insulin Receptor Substrate-1 (Dai et al., 2014) or p70S6K (Michel et al., 2015) as points of convergence. Our data raise the possibility that eIF4E represents yet another integration point of these pathways, and that upregulation ERK signaling during development could overcome myelin sheath elongation deficits seen on mTOR loss-of-function.

Do 4E-BP1 and eIF4E promote myelin sheath growth by targeting translation of the same transcripts? By mining the myelin transcriptome for cis-regulatory elements that confer translational control by 4E-BP1 or eIF4E, we identified populations of mRNAs whose translation may be regulated by either 4E-BP1 or eIF4E alone or the two in tandem (Figs. 7-9). In support of our identification of different subsets of genes, yeast genetic studies show that 4E-BP family members can regulate both eIF4E-

Figure 8. Identification of putative eIF4E myelin-resident translational targets. A, Schematic representation of CERT position weight matrix used to identify transcripts of interest. B, Percentage of myelin-resident mRNAs identified as having a CERT motif in the 5’UTR. C, Gene ontology of transcriptions containing identified CERT motif. Top 20 terms shown, ordered most to least significant by -log2 of false discovery rate. Counts correspond to number of genes associated with each term. For the list of candidate targets, see Extended Data Figure 8-1.
The myelin transcriptome is composed of hundreds of mRNAs with diverse functions (Thakurela et al., 2016). Gene ontology analysis revealed that the subsets of mRNAs we identified as potentially regulated by 4E-BP1 and/or eIF4E have distinct functions, raising the possibility that 4E-BP1 and eIF4E regulated transcripts have different functional consequences for myelin development. Another possibility is that 4E-BP1 and eIF4E function provides a level of regulation of selective translation based on extracellular signals. Additionally, interactions with RNA binding proteins, such as FMRP, which localizes to and promotes myelination (Doll et al., 2020), could provide complex translational regulatory networks in myelin sheaths.

Together, our data provide preliminary support for a model wherein mTOR signaling in myelin sheaths promotes selective translation of myelin-resident mRNAs. Such a model has been tested in other cell types of the nervous system. In cultured cortical neurons, BDNF stimulation promoted the phosphorylation of mTOR downstream targets 4E-BP and p70S6K in dendrites and drove neurite-localized translation of Arc and CaMKII in an mTOR-dependent manner (Takei et al., 2004). Our bioinformatic analyses revealed some CaMKII subunits as putative 4E-BP1 translational targets in myelin. Genetic studies in mice showed that both some CaMKII subunits (Waggener et al., 2013) and BDNF (Xiao et al., 2010; Lundgaard et al., 2013; Wong et al., 2013; Fletcher et al., 2018) promote CNS myelination, raising the possibility that BDNF coordinates new myelin synthesis by mTOR-dependent translation.

This model of mTOR signaling activating the translation of specific myelin-resident transcripts in a context-dependent manner is predicated on localized signaling and translation in myelin sheaths. In support of this possibility, we found a 4E-BP1 fusion
protein and \textit{mbpa} mRNA localized to myelin during development. Additionally, a reporter for \textit{PPIP5K1} formation, the phospholipid that activates Akt, and phosphorylated Akt localized to the membrane of cultured oligodendrocytes (Snaidero et al., 2014). Moreover, in oligodendrocyte-DRG cocultures, action potentials induced \textit{de novo} translation of an \textit{Mbp}-fluorescent reporter construct (Wake et al., 2011). In contrast to our proposed mechanism, these studies attributed \textit{Mbp} translational regulation to Fyn kinase (Wake et al., 2011); however, it is possible that differences in translational control could be dependent on context or developmental age or even specific transcripts being regulated by different signaling mechanisms. Future investigation into this topic should use biosensors of cell signaling activity to identify that spatial and temporal dynamics of cell signaling networks in myelin sheaths as well as imaging-based readouts of \textit{de novo} protein translation to validate the local translation of myelin-resident mRNAs \textit{in vivo}.

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