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Dual specificity phosphatase 15 regulates Erk activation in Schwann cells

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Abstract

Schwann cells and oligodendrocytes are the myelinating cells of the peripheral and central nervous system, respectively. Despite having different myelin components and different transcription factors driving their terminal differentiation there are shared molecular mechanisms between the two. Sox10 is one common transcription factor required for several steps in development of myelinating glia. However, other factors are divergent as Schwann cells need the transcription factor Egr2/Krox20 and oligodendrocytes require Myrf. Likewise, some signaling pathways, like the Erk1/2 kinases, are necessary in both cell types for proper myelination. Nonetheless, the molecular mechanisms that control this shared signaling pathway in myelinating cells remain only partially characterized. The hypothesis of this study is that signaling pathways that are similarly regulated in both Schwann cells and oligodendrocytes play central roles in coordinating the differentiation of myelinating glia. To address this hypothesis, we have used genome-wide binding data to identify a relatively small set of genes that are similarly regulated by Sox10 in myelinating glia. We chose one such gene encoding Dual specificity phosphatase 15 (Dusp15) for further analysis in Schwann cell signaling. RNA interference and gene deletion by genome editing in cultured RT4 and primary Schwann cells showed Dusp15 is necessary for full activation of Erk1/2 phosphorylation. In addition, we show that Dusp15 represses expression of several myelin genes, including myelin basic protein. The data shown here support a mechanism by which Egr2 activates myelin genes, but also induces a negative feedback loop through Dusp15 in order to limit overexpression of myelin genes.

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CONFLICT OF INTEREST DISCLOSURE

The authors have no conflict of interest to declare.

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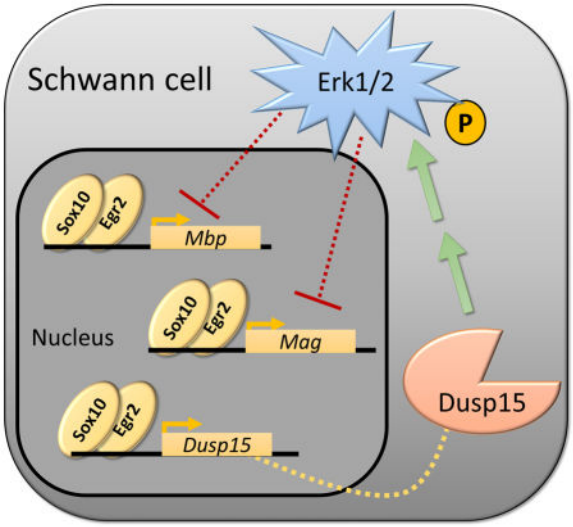
Graphical Abstract

Erk signaling in Schwann cells is essential, but its regulation is still poorly understood. We describe Dual Specificity Phosphatase 15 (Dusp15) as a novel positive regulator of Erk1/2 phosphorylation which leads to the transcriptional repression of some myelin genes. In addition, Dusp15 is a target of the transcription factors Sox10 and Egr2. These findings point to another layer of regulation over myelin genes that Sox10 and Egr2 activate but also repress through the activation of Dusp15.



Keywords

Schwann; Dusp15; Sox10; MEK-Erk; myelin

INTRODUCTION

Myelin is formed by myelinating glia called Schwann cells and oligodendrocytes in the peripheral and central nervous systems, respectively. Myelin is required for proper nerve transmission, and myelin formation is dependent on stage-specific actions of signaling pathways, many of which impinge on transcription factors regulating genes required for myelination of axons (Pereira *et al.* 2012, Salzer 2012, Grigoryan & Birchmeier 2015, Meijer & Svaren 2013, Mitew *et al.* 2013). Given the similar physiological roles of Schwann cells and oligodendrocytes, it is nonetheless clear that myelin constituents and gene regulatory networks diverge significantly between the two cell types. For example, principal myelin components include Myelin protein zero (Mpz) in Schwann cells of the peripheral nervous system, whereas Proteolipid protein 1 (Plp1) predominates in oligodendrocytes of the central nervous system. Indeed, even the developmental origins of these two cell types are distinct, as Schwann cells and oligodendrocytes arise from neural crest and neural tube, respectively (Stolt & Wegner 2015). Although some signaling pathways appear to be conserved in both cell types, there are significant differences in the physiological roles of neuregulin and PI3 kinase signaling (Noseda *et al.* 2016, Brinkmann *et al.* 2008).

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The transcription factors that drive myelination are also quite divergent in Schwann cells versus oligodendrocytes. Although a number of transcription factors have been characterized in myelinating glia, only Sox10, YY1, and Zeb2 are required for myelination in both cell types (Britsch *et al.* 2001, Stolt *et al.* 2002, He *et al.* 2007, He *et al.* 2010, Weng *et al.* 2012, Quintes *et al.* 2016, Wu *et al.* 2016). However, we recently reported a comparative analysis of Sox10 binding patterns in peripheral nerve and spinal cord, where we found that only a minority of binding sites are conserved between the tissues (Lopez-Anido *et al.* 2015). Sites unique to each tissue are co-localized with binding sites of transcription factors that are important for development of each cell type, indicating that Sox10 binding specificity is strongly influenced by cell type-specific factors (Emery 2013, Weider *et al.* 2013, Lopez-Anido et al. 2015).

Despite major differences between Schwann cells and oligodendrocytes, there is a core of myelin genes that are expressed in both cell types (e.g. *Mbp, Mag, Plp1, Gjb1/Cx32, Cnp*), even though Sox10 and cell type-specific regulators may utilize distinct binding sites in each cell type for these shared genes (Lopez-Anido et al. 2015). Recent studies have identified a key role for Myelin regulatory factor (Myrf), a transcription factor that is induced in myelinating oligodendrocytes and is required for terminal differentiation of oligodendrocytes (Hornig *et al.* 2013, Bujalka *et al.* 2013, Emery *et al.* 2009, Koenning *et al.* 2012). It has been suggested that Myrf plays an analogous role in oligodendrocytes to that of the Early growth response 2 (Egr2/Krox20) transcription factor (Emery 2013), which is induced in myelinating Schwann cells and is required for myelination (Topilko *et al.* 1994, Le *et al.* 2005a). Interestingly, both Egr2 and Myrf are regulated by Sox10 in Schwann cells and oligodendrocytes, respectively (Reiprich *et al.* 2010, Hornig et al. 2013, Ghislain & Charnay 2006).

Analogous to the core myelin genes expressed between oligodendrocytes and Schwann cells, the MEK-Erk signaling pathway promotes myelination in both myelinating cell types. For example, in vivo studies have shown hypermyelination of axons in both the central and peripheral nervous system when the MEK-Erk pathway is constitutively activated (Ishii *et al.* 2013, Ishii *et al.* 2016, Jeffries *et al.* 2016). We propose that identifying shared target genes in both Schwann cells and oligodendrocytes will shed light on potentially shared regulators of signaling mechanisms in myelinating glia. To examine the role of one factor that is coordinately regulated in both Schwann cells and oligodendrocytes, we identified Dusp15, a member of the Dual specificity phosphatase (DUSP) family that appeared to be strongly regulated by Sox10 in both cell types. Interestingly, Dusp15 is also targeted by Egr2 and Myrf in Schwann cells and oligodendrocytes, respectively. The following experiments test the role of Dusp15 in regulation of Schwann cell signaling and gene expression.

METHODS

Bioinformatics Analysis

Global binding profiles and enrichment at select loci were obtained from previously published ChIP-Seq data (Lopez-Anido et al. 2015, Srinivasan *et al.* 2012, Bujalka et al. 2013, Yu *et al.* 2013). To focus on high confidence gene targets of Egr2 and Myrf, binding sites were assigned to the nearest TSS within 50kb using the annotatePeaks program in

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Homer (Heinz *et al.* 2010). Comparison of gene targets identified targets that contain either Egr2 or Myrf near the TSS versus those that contain both Egr2 and Myrf nearby. 2653 and 707 genes contain either Egr2 or Myrf binding sites, respectively, and 304 genes contain both Egr2 and Myrf sites nearby. To perform gene ontology (GO) analyses we used David (Database for Annotation, Visualization, and Integrated Discovery) (Huang *et al.* 2009). We performed GO terms clustering using the highest stringency and also identified Kegg pathways with a p-value < 0.05.

Out of 304 genes with Egr2/Myrf nearby, 102 genes (147 gene probe sets) are expressed in sciatic nerve as determined from a previous microarray study (Le et al. 2005a, Le *et al.* 2005b). Using a stringent cut off, expressed genes in sciatic nerve were defined as those with detection values greater than 2000. Egr2-dependent genes were identified by comparing expression in control nerves versus Egr2-deficient nerves from mice with a hypomorphic allele of Egr2 (Le et al. 2005a, Le et al. 2005b).

Cell culture and transfection

S16 (Toda *et al.* 1994) and RT4-D6P2T (Hai *et al.* 2002) (referred to as RT4, obtained from ATCC) rat Schwann cells were grown in supplemented DMEM (Corning cellgro, 10-017-CV) with penicillin, streptomycin and 5% bovine growth serum (Hyclone, SH30541.03HI). Primary rat Schwann cells were grown in DMEM media, supplemented with 0.2% of bovine pituitary extract (Sigma) and 2uM forskolin (Sigma) (Fambrough *et al.* 1999).

Cells were transfected with Sox10 siRNA (Ambion, 4390771) Dusp15 siRNA (IDT, RNC.RNAI.N001108598.12.2) or control siRNA (IDT or Ambion-NC1) using Lipofectamine 3000 (L3000015). Luciferase assays were performed with the RT4 Schwann cell line, constructs were also transiently transfected with Lipofectamine 3000 and harvested for analysis 48hrs post-transfection. The reporter construct contained the following coordinates from the rat chromosome 13 (rn5), Pro_Dusp15 chr3:154,831,512-154,831,734, cloned upstream of the pGL4 luciferase reporter containing the minimal E1B TATA promoter. To test if Sox10 is required for activity, reporters were transfected together with Sox10 and control siRNA. The Dusp15-KO cell line was transfected with 500ng of pcDNA3-MmDusp15-T7c (Muth *et al.* 2016).

Genome editing was performed by cloning guide RNA's to remove the following sequence: (Rn5 chr3:154,823,256-154,831,564). Sequences for guide RNA were: CCGCCACGGCCCACGGGGGA and TGTGATTCCCGCGGCGATCG. The pX330 plasmid (Cong *et al.* 2013), purchased from Addgene (Fahmy & Khachigian 2004), was used to transfect the Cas9 system and clone each guide RNA.

Experimental animals and Nerve injury surgery

All animal experiments were performed according to protocols approved by the University of Wisconsin Graduate School. C57BL/6J mice were ordered from Jackson Laboratories and mice were selected at random. Sciatic nerves of 2-month old mice, 4 males and 3 females for 1 day experiment and 3 female mice for 4 day experiment, were transected following the surgery protocol described previously (Hung *et al.* 2015). As a control, the contralateral limb also received a sham operation consisting of only a skin incision. The nerve tissue distal to

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the transection and contralateral (sham) nerves were harvested and RNA was isolated, using the RNeasy Lipid Tissue Mini Kit (Qiagen Cat. 74804). Samples were coded prior to analysis and sham/injury samples were grouped to calculate changes in gene expression. The number of mice for these experiments was calculated in order to observe >2-fold changes in gene expression. Injury-induced gene expression changes were consistent with published gene expression profiles (Arthur-Farraj *et al.* 2012, Kim *et al.* 2012, Barrette *et al.* 2010).

Western Blot

RT4 Schwann cells were lysed in lysis buffer (150mM NaCl, 10% glycerol, 50nM Tris pH 8.0, 1% SDS 1% Triton and 1:100 protease inhibitor Sigma-Aldrich P8340) and heated for 5 minutes at 95°C before electrophoresis in an 10% SDS-PAGE (MIDSCI BCG01012). Total and phosphorylated Erk1/2 was measured using Erk1/2 (Cell Signal, #4695P) and P-Erk1/2 (Cell Signal, #4370P). Membranes were scanned and quantitated with the Odyssey Infrared Imager (LI-COR Biosciences).

RT-qPCR

RNA was isolated from cultured Schwann cells 48hrs after transfection using Tri Reagent (Ambion). RNA was converted to cDNA using the MMLV reverse transcriptase (Invitrogen). All cDNAs were analyzed from three independent experiments by RT-qPCR using Power SYBR Green Master Mix (Thermo Fischer Scientific) on the ViiA7 system (Applied Biosystems). Relative expression was calculated using Comparative Ct method (Livak & Schmittgen 2001). Primers used are listed in Table 1.

Statistics

P-values were obtained from the Student's two-tailed t-test.

RESULTS

Egr2 and Myrf have common target genes in Schwann cells and oligodendrocytes

While Egr2 and Myrf are required for terminal differentiation in Schwann cells and oligodendrocytes, respectively, it remains unclear if they fulfill similar roles by targeting unique or shared gene targets. Therefore, we annotated Egr2 and Myrf binding sites to nearby genes and performed a comparative analysis to identify unique and shared gene targets (Figure 1A). To focus on high confidence gene targets of Egr2 (7179 total) and Myrf (2049) (Lopez-Anido et al. 2015, Srinivasan et al. 2012, Bujalka et al. 2013), binding sites were assigned to the nearest transcription start site (TSS) within 50kb. A comparison of gene targets revealed 2653 and 707 genes that contain either Egr2 or Myrf binding sites, respectively, and 304 genes that contain both Egr2 and Myrf sites nearby (Figure 1B and Supplementary Table 1). It is intriguing that a relatively small group of shared genes have nearby Egr2/Myrf sites because it suggests that select genes exhibit converged Egr2/Myrf regulation. To better understand the function of these shared genes we performed gene ontology (GO) and Kegg pathway analysis using David (Database for Annotation, Visualization, and Integrated Discovery) (Huang et al. 2009). Our analysis revealed enrichment in genes associated with axon guidance (e.g. *Gas7),* regulation of cell death (e.g. *Ptprf, Foxo3*), cell projection (e.g. *Clic4, Ptprf, Gsn, Mbp*), and intracellular signaling

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cascades (e.g. *Gsn, Rab5b, Rtkn, Rhog*) (Figure 1C and Supplementary Table 2). Interestingly, two genes producing proteins involved in axoglial contacts in CNS and PNS node formation, contactin 2/Tag1 (Traka *et al.* 2002) and neurofascin, were in this group. Neurofascin is present in both axons and Schwann cells/oligodendrocytes, although the NF155 isoform is specific to myelinating glia (Roche *et al.* 2014).

To test if genes with nearby Egr2/Myrf sites are regulated by Egr2 in Schwann cells, we analyzed a previously published microarray on Egr2-deficient nerve from mice with a hypomorphic allele of Egr2 (Le et al. 2005a, Le et al. 2005b). 44 genes (e.g. *Clic4, Abca2, Mag, Mbp, Fa2h, Syt11, Thra, Psmf1, Dusp15, Ptprf, Klf9, Nfix, Gsn*) were downregulated <0.8-fold and 24 genes (e.g. *Map4k4, Sox10, P2rx4, Fem1b, Stard13,* Ninj1, Foxo3) were upregulated >1.25-fold in Egr2-deficient nerves (Figure 1D and Supplementary Table 3). One gene identified in the analysis was *Mag* (Myelin-associated glycoprotein), a critical myelin gene (Carenini *et al.* 1997, Yin *et al.* 1998) and a known Egr2 target (Jang *et al.* 2006). ChIP-Seq binding profiles indicate that while Mag is a shared target gene of Egr2/Myrf, Egr2 binds an intronic element and Myrf binds just upstream of the transcription start site (Figure 1E). Accordingly, our previous analysis on Sox10 binding events in Schwann cells and oligodendrocytes indicated that most sites are unique to one cell type or the other, and even shared genes have CNS- and PNS-specific enhancers (Lopez-Anido et al. 2015). Most of these genes have not yet been characterized in either oligodendrocyte or Schwann cell function, but the apparent parallel regulation by Sox10 and Egr2/Myrf may help identify critical genes required for myelination in both types of myelinating glia.

Sox10 and Egr2 control Dusp15 transcription

To determine the role of a gene that has not been characterized in Schwann cells, we turned to the Egr2-dependent gene Dual specificity phosphatase 15 (*Dusp15*), a gene with nearby Egr2/Myrf sites that is also reduced ~70% in Egr2-deficient nerve relative to wild type (Le et al. 2005a, Le et al. 2005b) (Figure 1D). Previous studies found that Dusp15 expression is induced upon differentiation of oligodendrocytes in culture (Schmidt *et al.* 2012) and PNS development (Patzig *et al.* 2011). A more recent study, in primary oligodendrocytes, showed how Sox10 and Myrf control Dusp15 transcriptional levels by binding to its promoter region and also observed an increase in Dusp15 mRNA in spinal cord after birth (Muth et al. 2016). Also, our analysis using Sox10 siRNA treatment in the S16 Schwann cell line identified Dusp15 as a potential Sox10 target gene (Srinivasan et al. 2012). To test if Dusp15 is a direct target of Sox10, we assayed ChIP-Seq datasets on Sox10 binding in P15 rat sciatic nerve. Sox10 ChIP-Seq in sciatic nerve (Srinivasan et al. 2012, Lopez-Anido et al. 2015) revealed a Sox10 binding site in the proximal promoter region (Figure 2A). ChIP-Seq in spinal cord (not shown), where Sox10 is only expressed in the oligodendrocyte lineage, also revealed Sox10 binding in the promoter region of Dusp15 (Lopez-Anido et al. 2015). Sox10 binding coincided with local enrichment with histone acetylation (histone H3K27 acetylation), which marks actively engaged enhancers (Figure 2B).

To confirm direct Sox10 binding at the promoter, we performed sequence analysis to identify the Sox10 binding motif and also tested if enhancer activity was Sox10-dependent. We found that the promoter region is GC-rich, but contains two potential binding motifs for

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Sox10 (GACAAAGcccTAGTGTG, GACAACGgcaGCTTGTT) that conform to the previously published consensus motif. The two motifs appear to be inverted dimeric sites that have been shown to bind Sox10 cooperatively (Peirano & Wegner 2000) and are conserved in mammalian species. To examine enhancer activity, the promoter region surrounding the Sox10 binding sites were cloned into a luciferase reporter vector (termed Pro_Dusp15 reporter), and transfected into the RT4 Schwann cell line. We found that level of luciferase activity from the Pro_Dusp15 reporter was significantly reduced when Sox10 siRNA was cotransfected (Figure 2C). Mutation of the first Sox10 motif listed above was shown to reduce Sox10 responsiveness of the Dusp15 promoter in N2A transfection assays (Muth et al. 2016).

In addition, we tested if Dusp15 expression was Sox10-dependent. We measured Dusp15 relative expression levels by qRT-PCR after siRNA-mediated depletion of Sox10 in RT4 cells (Figure 2D) and found a 5-fold reduction in Dusp15 levels by Sox10 siRNA. Finally, we tested if Dusp15 levels were also downregulated in mouse sciatic nerves lacking Sox10. We employed P16 sciatic nerve from a conditional knockout of Sox10 in which the floxed allele was mated to the Dhh::cre line to delete Sox10 selectively from Schwann cells (Finzsch *et al.* 2010). Previous analysis of this mouse revealed that Sox10 plays an important role in mature stages of Schwann cell development, and also showed strong reduction in Sox10 target genes (*Mbp, Mpz, Dhh, S100B, Erbb3*, and *Egr2*). Quantitative RT-PCR of these nerve samples (n=2) confirmed that Sox10 is necessary for Dusp15 expression in vivo (data not shown).

Our in vivo ChIP-Seq data also showed a pronounced Egr2 binding site in P15 rat sciatic nerve (Srinivasan et al. 2012) (Figure 2A). The region contained known, consensus Egr2 binding motifs (Swirnoff & Milbrandt 1995), which are also conserved in mammalian species (e.g. ACGGGGGAG, GCGTGGGAC, GTGGGGGCG, CTGGGGGCA). As noted above (Le et al. 2005a), Egr2-deficient nerve exhibits lower Dusp15 expression levels than wild type. We also found that siRNA-mediated depletion of Egr2 in S16 cells reduced Dusp15 levels ~50% (Figure 2F), while Sox10 expression levels remained unchanged. Our findings indicate that Dusp15 is regulated by two of the major regulators of Schwann cell development, Sox10 and Egr2, as has been shown for many other myelin-associated genes (Jones *et al.* 2007, Jones *et al.* 2011, Svaren & Meijer 2008, Jang & Svaren 2009, Srinivasan et al. 2012, Bondurand *et al.* 2001, Jones *et al.* 2012).

Dusp15 is regulated by nerve injury

Given the dependence of *Dusp15* expression on Egr2, we hypothesized that *Dusp15* expression would decline after peripheral nerve injury, since Egr2 and many of its target genes are downregulated in the demyelination phase after nerve injury (Le et al. 2005a, Nagarajan *et al.* 2002, Topilko *et al.* 1997, Zorick *et al.* 1996). To test this we performed a nerve transection injury, where we observed a decline in *Dusp15* expression 1 and 4 days after injury (Figure 2G). In addition, ChIP-Seq data for H3K27ac (Hung et al. 2015) around the Sox10 and Egr2 binding region upstream of *Dusp15* revealed a dramatic reduction in the enhancer mark in injury samples (3d post-transection) when compared with control (Figure 1B). Our results are consistent with microarray analyses of gene expression changes after

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nerve injury, which also show a similar decrease in *Dusp15* expression after injury (Arthur-Farraj *et al.* 2012, Kim *et al.* 2012, Barrette *et al.* 2010). Thus, in addition to being induced over myelination, *Dusp15* is also downregulated along with myelin genes after nerve injury.

Dusp15 increases Erk1/2 phosphorylation

Dusp15 is a member of the dual specificity phosphatase family and peptide array experiments identified potential substrates with important signaling molecules, such as ErbB3 and p38 (Schmidt et al. 2012). This suggested the hypothesis that Dusp15 is an important regulator of signaling molecules in Schwann cells. Dusp15 mRNA levels were repressed in the RT4 Schwann cell line using siRNA and phosphorylated levels of ErbB3 and p38 were measured by Western blot. While Dusp15 was repressed 4-fold (Figure 3A), phosphorylated levels of ErbB3 and p38 remained unchanged (data not shown). Because some DUSP family proteins are also known to interact with Erk and Akt signaling pathways, we hypothesized that Dusp15 may regulate other signaling molecules important for Schwann cell myelination. Instead of an expected increase in Erk1/2 phosphorylation levels, the opposite was observed as phosphorylation was reduced in Dusp15-repressed samples (Figure 3B and 3C). These results show that Dusp15 positively regulates the Erk1/2 signaling pathway, probably through intermediary factors.

Creation of a *Dusp15* Knockout in the RT4 Schwann cell line

One limitation of siRNA studies is that only partial depletion of the target gene is normally achieved. Therefore, to further investigate the function of *Dusp15*, the gene was deleted in RT4 Schwann cells using the new genome engineering technique, CRISPR-Cas9. The RT4 Schwann cell line was chosen due to the high levels of Dusp15 that it expresses when compared to other known Schwann cell lines like S16; we found that RT4 cells express 15-fold more Dusp15 than the S16 cell line (Figure 3D). Guide RNAs (gRNA) were designed to flank the coding sequence of Dusp15, spanning from the transcription start site to the 7^{th} exon and leaving the 3′UTR intact (Figure 3E). Two consecutive rounds of transfection yielded a clone that lacked the Dusp15 coding region. To assay the absence of *Dusp15* in the identified clonal population, genomic PCR was performed using primers that spanned the 3′UTR still present in the parent line (wildtype) and KO line and another primer set that flanked the 8 kb region between the two guide RNA targets. The wildtype line did not produce a band while the KO line amplified a 750bp band showing the absence of the *Dusp15* gene (Figure 3F). Furthermore, mRNA levels of Dusp15 were not detected by quantitative RT-PCR (Figure 3G). Our CRISPR-Cas9 deletion line provided another tool to test the role of Dusp15. We measured Erk1/2 phosphorylation in the deletion line, and observed a downregulation in the deletion line relative to the parental RT4 cell line, as was seen with Dusp15 siRNA (Figure 3H and 3I). Although Dusp15 does not seem to regulate ErbB3 phosphorylation (data not shown), it does appear to be required for the full activation and phosphorylation of Erk1/2.

Regulation of Erk1/2 target genes in Schwann cells

Erk1/2 signaling plays an important role in promoting Schwann cell development, particularly during embryogenesis, but has also been implicated in the injury response of Schwann cells (Harrisingh *et al.* 2004, Napoli *et al.* 2012, Newbern & Birchmeier 2010, Ishii

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et al. 2013, Sheean *et al.* 2014, Ishii et al. 2016). Previous studies identified several Erk1/2 target genes during Schwann cell injury (Napoli et al. 2012). Therefore, we hypothesized that reduced levels of Erk1/2 phosphorylation were correlated with targets of the MEK-Erk pathway in Schwann cells: *Ccl2/MCP*-1, Vegfc (Napoli et al. 2012, Fischer *et al.* 2008) and Pmp2 (Sheean et al. 2014). Quantitative RT-PCR revealed that *Ccl2* and *Vegfc* were significantly reduced by Dusp15 siRNA, while *Pmp2* was unchanged (Figure 4A). In addition, the same mRNA levels were also measured in the Dusp15 knockout line, which showed a more striking decrease of the Erk1/2 regulated genes (Figure 4B). Finally, we tested if changes in *Ccl2* mRNA levels could be rescued by ectopic expression of Dusp15, and found that transient transfection with a *Dusp15* expression plasmid in the knockout line led to partial restoration of the expression levels (Figure 4C). Our results indicate that Dusp15 functions upstream of Erk1/2 activity.

Dusp15 represses expression of myelin genes

As we observed that *Dusp15* loss leads to reduction of Erk1/2 phosphorylation, we also examined some Erk1/2-dependent myelin genes that have been characterized previously (Sheean et al. 2014, Napoli et al. 2012). Different myelin genes display different levels of Erk-dependence in various contexts, but we initially examined *Mbp* and *Mag* because these myelin genes were induced in Erk1/2-deficient Schwann cells (Newbern *et al.* 2011). In RT4 Schwann cells treated with siRNA against *Dusp15* we measured mRNA levels of *Mbp* and other myelin genes (*Mag*, *Mpz*, Gjb1/*Cx32*, *Pmp22)* and also an important regulator of lipid synthesis that is developmentally regulated in Schwann cells, *SREBP-1c* (LeBlanc et al. 2005, de Preux et al. 2007, Verheijen et al. 2003, Verheijen et al. 2009, Norrmén et al. 2014). Consistent with previous results (Newbern et al. 2011), some of the myelin genes were induced, mainly, *Mag*, *Mbp*, and *SREBP-1c*, while others remained unchanged (Figure 5A). We decided to further test the Dusp15 effects in primary Schwann cells (Primary SC). When Dusp15 was repressed using siRNA we measured an increase in *Mag* mRNA mimicking the results found in RT4 Schwann cells (Figure 5B). Somewhat surprisingly, *Mbp* expression was dramatically induced in the *Dusp15* knockout line compared with parental RT4 cells (Figure 5C). As it turns out, RT4 cells express relatively high levels of some myelin genes, but *Mbp* is virtually undetectable (Hai et al. 2002). Therefore, induction from the extremely low level of *Mbp* expression is quite dramatic.

The knockout line was also used to evaluate expression of the other myelin-associated genes by quantitative RT-PCR. In contrast to the siRNA experiments, *Mag*, was slightly reduced, but *Cx32/Gjb1*, *Pmp22* and *SREBP-1c* were induced (Figure 5D). Finally, it was important to show if the induction seen in the myelin genes was due to the lack of *Dusp15* rather than an off-target effect. Restoring *Dusp15* expression in the knockout line by transfection of an overexpression plasmid brought *Mbp* and *Pmp22* back to levels approximately equal to the expression in the parental RT4 line (designated WT in Figure 5E and 5F).

The Erk pathway represses myelin genes

Since Dusp15 reduction leads to decreased Erk1/2 phosphorylation, we tested if myelin gene expression changes would also be observed with more direct manipulation of Erk1/2 phosphorylation. The parental RT4 Schwann cell line was treated with Trametinib, a MEK

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inhibitor, and quantitative RT-PCR indicated that *Mbp* and *Mag* were induced 80-fold each (Figure 6A) while other myelin genes (*Pmp22*, *Mp11* and *Mpz*) were modestly induced (Figure 6B). Repression of Erk signaling was correlated with decreased expression of Erk-regulated target genes *Vegfc* and *Ccl2*, and the myelin gene *Pmp2* (Figure 6B). We next performed complementary experiments to activate Erk signaling by the phorbol ester PMA, which targets PKC upstream of Erk. Quantitative RT-PCR showed that Erk1/2 activation repressed most of the genes that were activated by Dusp15 depletion. *Mbp* and *Mag* were reduced 80% (Figure 6C) and other myelin gene mRNA levels (*Pmp22*, *Cx32* and *Mpz*) were modestly reduced (Figure 6D). Erk activated genes were also induced with PMA (*Vegfc* and *Ccl2),* while *Pmp2* was not induced (Figure 6D and 6E). Overall, our results indicate that Erk activity downstream of Dusp15 represses myelin gene expression.

Erk signaling represses myelin gene expression in primary Schwann cells

The experiments above were performed in the RT4 Schwann cell line, but we wanted to know if the effects of Erk signaling on myelin gene expression were present in primary Schwann cells. Primary rat Schwann cells were treated with Trametinib and several myelin genes, (*Mag, Mpz, Mbp and Pmp22*) were all induced as we had seen before (Figure 7A). The activation of Erk, through PKC using PMA, resulted in an almost mirror image effect on some of the genes, specifically *Mag, Pmp22 and Ccl2* (Figure 7B). The gain-of-function experiment using PMA resulted in the expected results as we observed *Mag* and *Pmp22* induced, while confirming an induction in Erk activity through increased *Ccl2* mRNA levels. An interesting effect observed with Trametinib was the reduction of Egr2 mRNA levels and the induction of the myelin genes. This was also observed in E12.5 DRG that lacked Erk1/2 expression (Newbern et al. 2011).

DISCUSSION

Although oligodendrocytes and Schwann cells perform similar functions in the central and peripheral nervous system, respectively, the molecular pathways of these two cell types are significantly divergent. To elucidate potentially common pathways we looked at genes associated with binding of Sox10, a transcription factor expressed in both cells. ChIP-Seq analysis from Sox10 genomic occupancy in sciatic nerve and spinal cord tissue (Schwann cells and oligodendrocytes, respectively) identified a core of common genes (Lopez-Anido et al. 2015). Here, we found that a relatively small subset of these genes are associated with Egr2 binding in peripheral nerve and Myrf in oligodendrocytes (Srinivasan et al. 2012, Bujalka et al. 2013). Of these ~300 genes, relatively few have been characterized previously.

To begin to identity novel regulators of myelination, we focused on Dusp15, a member of the dual specificity phosphatase family, since it had potential to be an important regulator of intracellular signaling pathways. ChIP-Seq revealed that the Dusp15 promoter is bound by Sox10 in both sciatic nerve and spinal cord tissue (Lopez-Anido et al. 2015). Luciferase activity of the promoter showed that Sox10 directly activates Dusp15 and Sox10 cKO nerve further confirmed Dusp15 as a target of Sox10 in vivo. Egr2 also appears to control Dusp15 expression levels based on our ChIP-Seq analysis (Srinivasan et al. 2012), siRNA effects on Dusp15 mRNA expression levels, and microarray analysis from Egr2-deficient mice (Le et

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al. 2005a). Finally, developmental gene expression analysis (Patzig et al. 2011, Verdier *et al.* 2012) revealed that Dusp15 is induced throughout Schwann cell differentiation and exhibits peak expression at P15. Dusp15 induction through differentiation can be explained by parallel activation of Egr2 during myelination (Topilko et al. 1994, Murphy *et al.* 1996). Interestingly, the promoter region is also a binding site for Myrf and Sox10 in oligodendrocytes and *lacZ* reporter controlled by the *Dusp15* promoter showed X-gal staining colocalizing with Sox10 positive cells in spinal cord and with Sox10/Egr2 expressing cells in peripheral nerve (Muth et al. 2016). The fact that Dusp15 is a common target of Egr2 and Myrf transcription factors suggested that it may have an important common role in both Schwann cells and oligodendrocytes.

While the direct targets of Dusp15 phosphatase activity are not resolved in this work, we show evidence for its regulation of the Erk1/2 pathway in Schwann cells. Positive regulation over the Erk pathway was unexpected since Dusp15 has been shown to have phosphatase activity (Schmidt et al. 2012, Alonso *et al.* 2004). Therefore, it is likely that Dusp15 targets an inhibitor of Erk1/2. We observed that Dusp15 is necessary for the full phosphorylation of Erk1/2, and depletion of Dusp15 led to reduction in previously described Erk1/2 target genes in Schwann cells, *Ccl2* and *Vegfc* (Napoli et al. 2012, Fischer et al. 2008). Interestingly, loss of Dusp15 not only reduced Erk1/2 signaling but also activated several myelin genes, mainly *Mbp*, *Mag* (in siRNA experiments) and *Pmp22*. We confirmed our results by using genome editing to remove the *Dusp15* open reading frame in the RT4 Schwann cell line and rescuing the effects described above by ectopic expression of Dusp15. While both siRNA and CRISPR technologies have limitations in terms of either a) partial depletion of target and b) potential compensatory/off-target changes in the selection of clones with the desired modification, the use of both technologies provide complementary benefits in analysis of Dusp15 function.

Dusp15-mediated repression of myelin genes and activation of the Erk pathway prompted us to test if the reduction of Erk1/2 phosphorylation leads to increased myelin gene expression. Indeed, we observed that inhibiting the Mek/Erk pathway also induced some myelin genes. Conversely, modulating the Erk pathway through PKC activation led to repression of *Mbp*, *Mag*, and *Pmp22*. Performing similar experiments in primary Schwann cells revealed the same trends in myelin gene expression as observed in RT4 Schwann cells. These effects are consistent with past findings of a Schwann cell-specific knockout of Erk1/2 (Newbern et al. 2011) which showed increased *Mbp* and *Mag* levels in E12.5 DRG samples even when *Egr2* levels were repressed.

The role of Erk1/2 signaling in Schwann cell differentiation has been the subject of several studies (reviewed in Grigoryan & Birchmeier 2015, Newbern 2015). Erk1/2 signaling is activated after nerve injury and plays a role in demyelinating processes triggered by injury and other pathological conditions (Harrisingh et al. 2004, Napoli et al. 2012). Indeed, increased Erk1/2 signaling was also demonstrated in rodent models of the CMT1A neuropathy (Fledrich *et al.* 2014). On the other hand, embryonic Erk1/2 signaling is required for myelination and for activity of the pro-myelinating YY1 transcription factor (Newbern et al. 2011, He et al. 2010). Enhanced expression of either Erk or Mek1 signaling in Schwann cells has also been shown to increase myelin thickness (Ishii et al. 2013, Sheean et al. 2014,

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Ishii et al. 2016). Moreover, expression of a constitutively active Mek1 allele in Schwann cells can rescue defects observed with knockout of either the Shp2 or Erbb3 gene (Sheean et al. 2014). It should be noted that Erk1/2 activation is high in the early postnatal myelination period in mouse up to at least P15, but then subsides by P30 until it is re-activated upon nerve injury (Sheean et al. 2014, Harrisingh et al. 2004).

Given the many potential targets of Erk1/2 activity, it is possible that the effect of Erk activation is dependent upon the degree of activation and the stage of Schwann cell development (Ishii et al. 2016). Therefore, it is important to identify potential regulators of Erk1/2 activation that may give rise to context-specific aspects of Schwann cell differentiation. An interesting parallel of Dusp15 is the Shp2 tyrosine phosphatase, which is required for full Erk1/2 activation (Grossmann *et al.* 2009), and it seems possible that Dusp15 may play a partially overlapping role with this protein. However, it is notable that Shp2 deficiency results in decreased Egr2 activation, which is not observed upon depletion of Dusp15 in our studies. Interestingly, Erk1/2 activation has been studied extensively in oligodendrocyte development, where it promotes differentiation and increased myelin thickness (reviewed in Gaesser & Fyffe-Maricich 2016). Therefore, Erk1/2 activation is important in both the development of oligodendrocytes and Schwann cells leading us to suggest that Dusp15 may have similar roles in both myelinating cells.

Based on our analysis and the developmental regulation of Dusp15, it is notable that the increase in Dusp15 up to P15 parallels a similar increase in Erk1/2 phosphorylation (Sheean et al. 2014). However, Dusp15 mRNA continues to be expressed in mature nerve even when Erk1/2 signaling decreases. It is possible that Dusp15 may limit full activation of certain myelin genes, such as *Mbp* and *Mag*, during the myelination stage in Schwann cell development through the activation of Erk signaling. It is noted that a preliminary analysis of Dusp15 in oligodendrocytes, showed a similar repression of some myelin genes (Schmidt et al. 2012). Ultimately we predict that myelin gene repression is not the only function by Dusp15 in Schwann cells and a full analysis of the Dusp15 molecule in vivo will require loss-of-function studies in vivo.

The actual targets of Dusp15 are yet to be determined, but since it is myristoylated (Alonso et al. 2004), a posttranslational modification that directs the protein to the cytoplasmic membrane, it is likely that its possible targets may rest in the cytoplasmic side of the cell membrane. However, a reliable antibody has not been identified for Dusp15 to confirm the predicted localization. Although we were unable to validate some putative targets of Dusp15 activity (Schmidt et al. 2012), some potential targets are Fyn and other Src family kinases (Wu *et al.* 2007), Erbin (Tao *et al.* 2009, Liang *et al.* 2012), which have been shown to be necessary for Erk phosphorylation and Schwann cell myelination (Hossain *et al.* 2010).

Supplementary Material

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Abbreviations

Dusp15	Dual specificity phosphatase 15
Egr2	Early growth response 2
Mbp	Myelin basic protein
Myrf	Myelin regulatory factor
Plp1	Proteolipid protein 1
YY1	Ying-Yang 1
ChIP-seq	Chromatin Immunoprecipitation-Sequencing
TSS	Transcription start site
Mag	Myelin associated glycoprotein
PNS	Peripheral nervous system
siRNA	short interfering RNA
Dhh	Dessert hedgehog
Mpz	Myelin protein zero
Erk	Extracellular signal-regulated kinases
CRISPR	clustered regularly interspaced short palindromic repeats
CCL2	chemokine C-C motif ligand 2
Pmp22	Peripheral myelin protein 22
Cx32	Connexin 32
MEK	MAPK/ERK kinase
PMA	phorbol-12-myristate-13-acetate
UTR	untranslated region
Kegg	Kyoto Encyclopedia of Genes and Genomes

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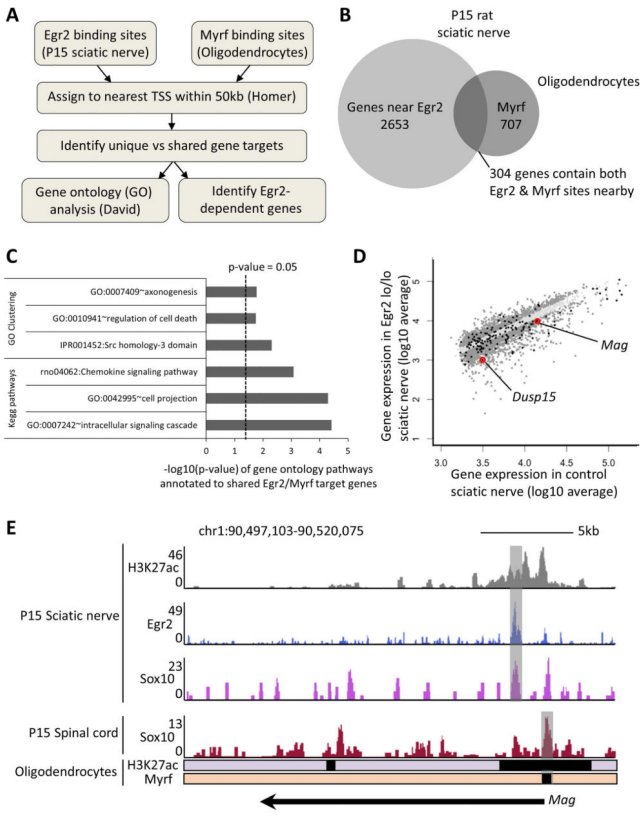

Figure 1. Egr2 and Myrf have common target genes in myelinating glia

(A) Schematic depicts bioinformatics approach to compare Egr2 and Myrf binding sites in Schwann cells versus oligodendrocytes, respectively. Egr2 binding sites in Schwann cell-rich P15 rat sciatic nerve and Myrf sites in cultured oligodendrocytes were recently published (Lopez-Anido et al. 2015, Srinivasan et al. 2012, Bujalka et al. 2013). (B) Overlap analysis of genes with Egr2 and Myrf binding sites nearby identifies 304 genes that may be shared targets in Schwann cells and oligodendrocytes. (C) Gene ontology (GO) terms clustering and Kegg pathway analysis on Egr2/Myrf shared target genes reveals enrichment in signaling pathways and other processes. (D) Scatter plot represents gene expression for a given gene (represented by dots) in control versus Egr2-deficient sciatic nerve. Gene expression values were obtained from a previous microarray study on mice with a hypomorphic allele of Egr2 (Le et al. 2005a, Le et al. 2005b). Dark grey dots are either upregulated in Egr2-deficient nerve by >1.25-fold or downregulated by <0.8-fold. The black dots represent genes that are expressed in control nerve and also have an Egr2/Myrf binding event nearby, and these include (highlighted in red) the well-studied myelin gene Myelin-associated glycoprotein (*Mag*) as well as Dual specificity phosphatase 15 (*Dusp15*), a gene with an unknown role in peripheral nerve myelination. (E) ChIP-Seq analysis profiles depict genomic regions enriched with transcriptional regulators and enhancers in P15 rat sciatic nerve and spinal cord. Included are binding profiles of Sox10 and Egr2 (Lopez-Anido et al. 2015, Srinivasan et al. 2012). Profiles of H3K27ac and the oligodendrocyte-specific regulator Myrf in cultured oligodendrocytes were also obtained from published datasets (Yu

et al. 2013, Bujalka et al. 2013, Lopez-Anido et al. 2015). Egr2- versus Myrf-enriched enhancers specific to Schwann cells versus oligodendrocytes are indicated by grey boxes.

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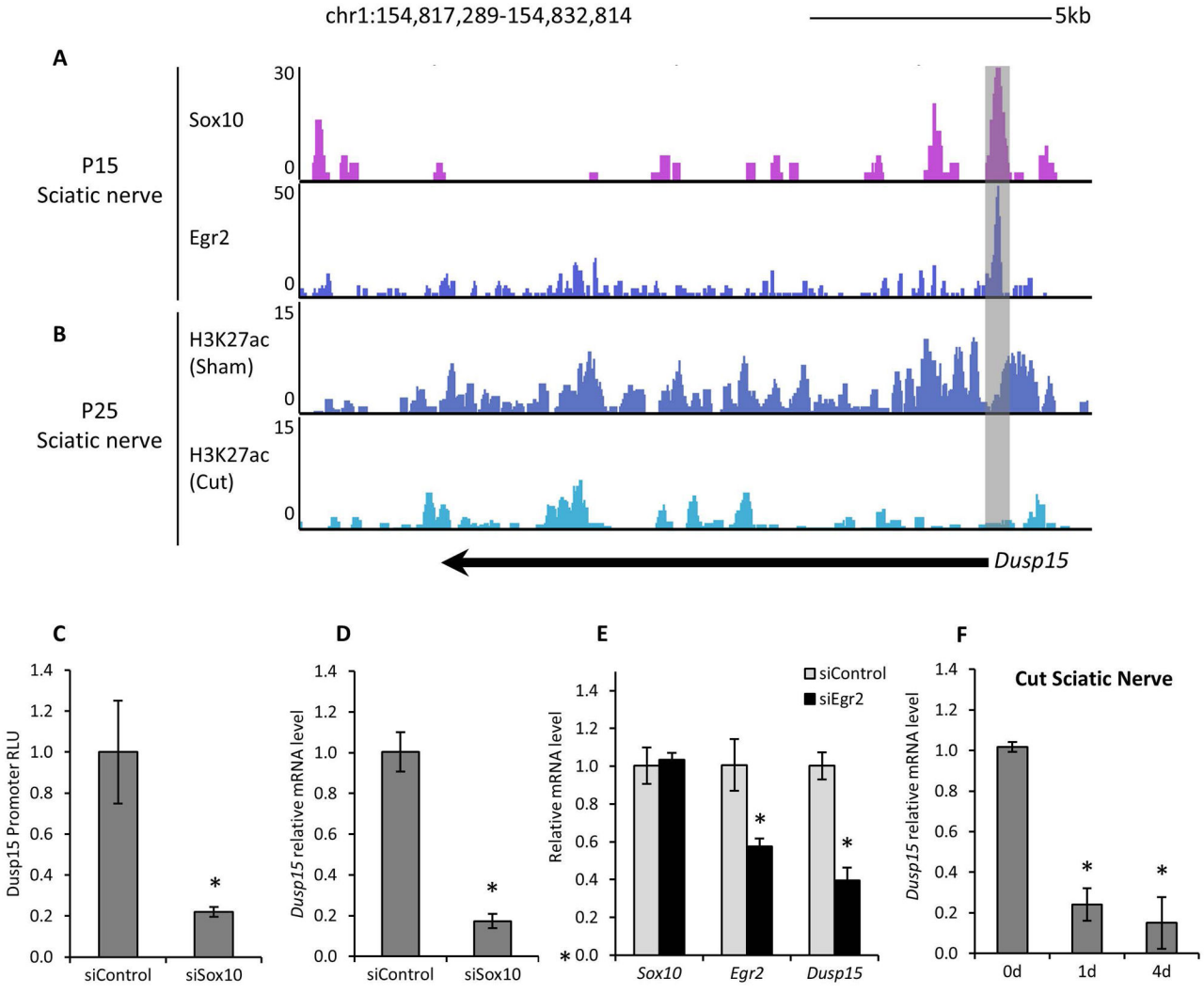

Figure 2. Sox10 and Egr2 regulate *Dusp15* transcription

(A) ChIP-Seq analysis of P15 sciatic nerve reveals Sox10 and Egr2 enrichment at the *Dusp15* gene, with a grey box highlighting the promoter region. (B) ChIP-Seq analysis on P25 rat sham sciatic nerve show the H3K27ac-marked enhancer (grey box), which is diminished in cut nerve (3 d post-injury). (C) Luciferase assays were performed in S16 Schwann cells with constructs containing the promoter region of *Dusp15* (Pro_Dusp15) and treated with Sox10 siRNA. Relative light units (RLU) are shown relative to siControl treatment after normalizing to β-galactosidase activity from a co-transfected CMV-lacZ plasmid. Quantitative RT-PCR was used to determine relative mRNA expression levels in (D) S16 rat Schwann cells treated with siRNA against Sox10. (E) S16 Schwann cells were treated with siRNA against Egr2 and quantitative RT-PCR was used to measure mRNA levels of Sox10, Egr2 and Dusp15. (F) Dusp15 levels were measured in uncut (sham) or cut sciatic nerve (1 and 4 d post-injury) using quantitative RT-PCR. 18S housekeeping gene was used to normalize all quantitative RT-PCR assays and error bars indicate the standard

deviation of three biological replicates (n=7 for 1 d post-injury and 3 for 4 d post-injury). (*P<0.05)

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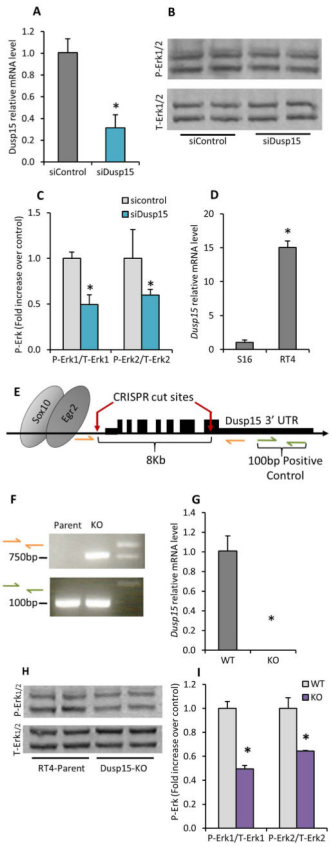

Figure 3. Dusp15 controls Erk1/2 phosphorylation levels

(A) Dusp15 levels were measured using quantitative RT-PCR in RT4 Schwann cells treated with siRNA against Dusp15. (B) Western Blot analysis of Erk1/2 phosphorylation was performed and (C) quantified in RT4 Schwann cells treated with siRNA against Dusp15. (D) Dusp15 mRNA levels were compared between S16 and RT4 Schwann cells using quantitative RT-PCR. (E) Diagram depicting guide RNA (red) target sites for CRISPR/Cas9 deletion of the *Dusp15* gene. (F) Genotyping of Cas9 treated cells was determined by PCR using primers (orange pair) flanking the deleted genomic and the uncut 3′UTR region in both lines (green). (G) Dusp15 mRNA levels in WT and KO cell lines were determined by quantitative RT-PCR. (H) Erk1/2 phosphorylation was measured in WT versus KO lines and compared to total levels of Erk1/2 by western blot analysis. (I) Band signals obtained from western blot analysis were normalized to Total-Erk (T-Erk) levels. All experiments represent average values of biological triplicates. (*P<0.05)

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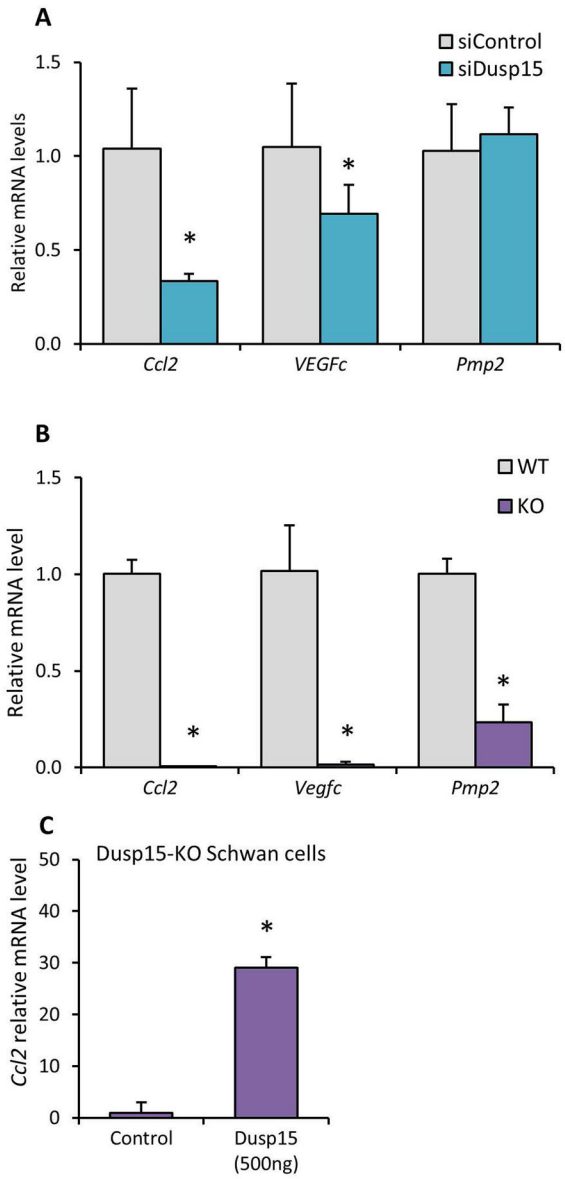

Figure 4. Dusp15 is necessary for Erk activity

Quantitative RT-PCR was used to determine Ccl2, Vegfc and Pmp2 mRNA levels in (A) RT4 Schwann cells treated with siRNA against Dusp15 and (B) Dusp15-KO-RT4 Schwann cell line. (C) Ccl2 mRNA levels were measured in Dusp15-KO Schwann cells transfected with an expression plasmid for Dusp15 using quantitative RT-PCR. (*P<0.05)

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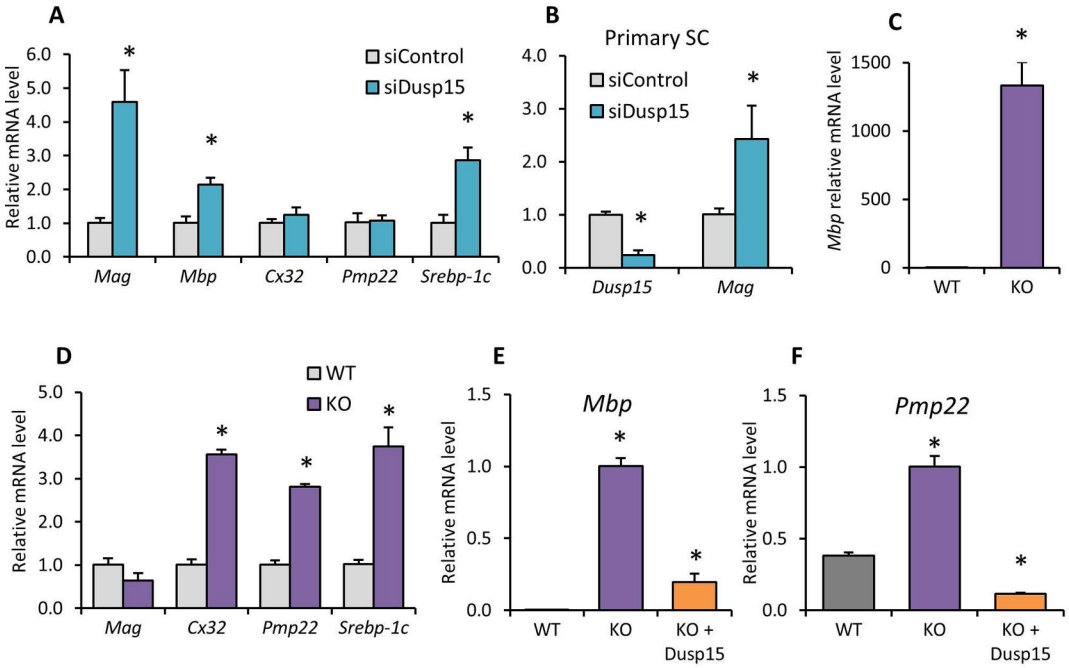

Figure 5. Dusp15 represses myelin gene expression

Mag, Mbp, Cx32, Pmp22 and Srebp-1c mRNA levels were measured by quantitative RT-PCR in (A) RT4 Schwann cells, Dusp15 and Mag in (B) Primary Schwann cells treated with siRNA against Dusp15 and in (C and D) Dusp15-KO Schwann cell line compared to parent RT4 Schwann cells. (E) Mbp and (F) Pmp22 mRNA levels were measured in Dusp15-KO Schwann cells treated with ectopic expression levels of Dusp15. All experiments represent biological triplicates, and expression levels were normalized to 18S rRNA. (*P<0.05)

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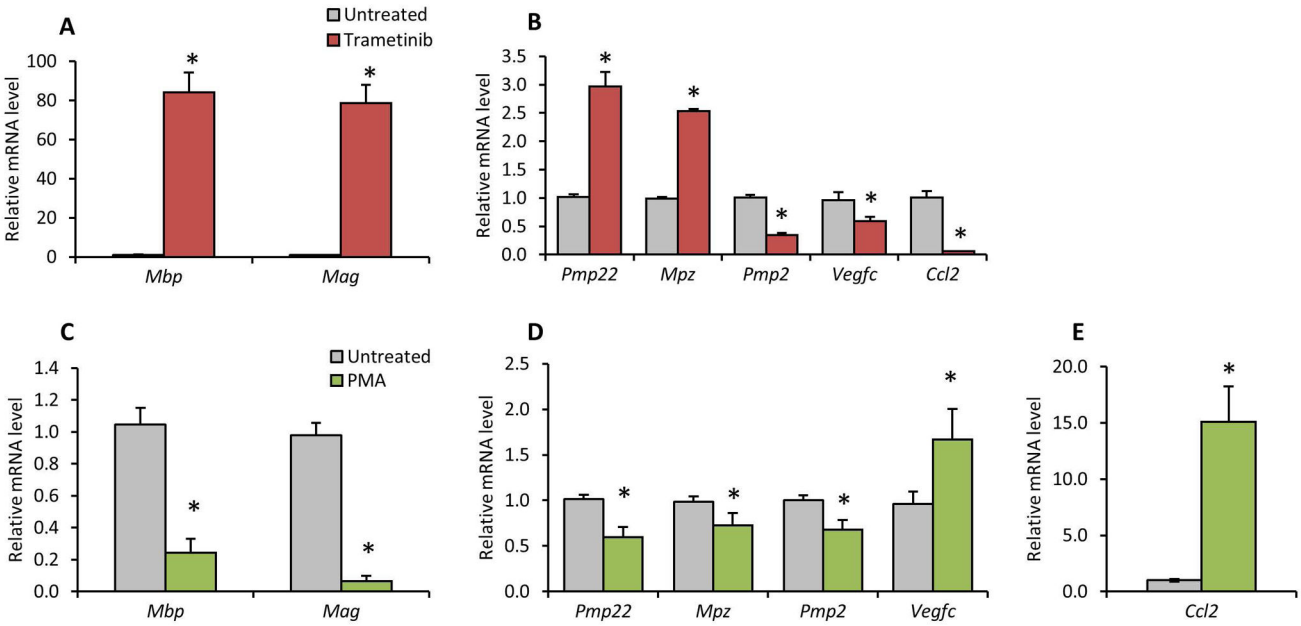

Figure 6. Erk activity represses myelin gene expression

Mbp, Mag, Pmp22, Mpz, Pmp2, Vegfc and Ccl2 mRNA levels were measured in RT4 Schwann cells treated with (A and B) Trametinib 18nM, or with (C, D and F) PMA using quantitative RT-PCR. Samples were normalized to the level of 18S rRNA. Error bars represent the standard deviation from biological triplicates.

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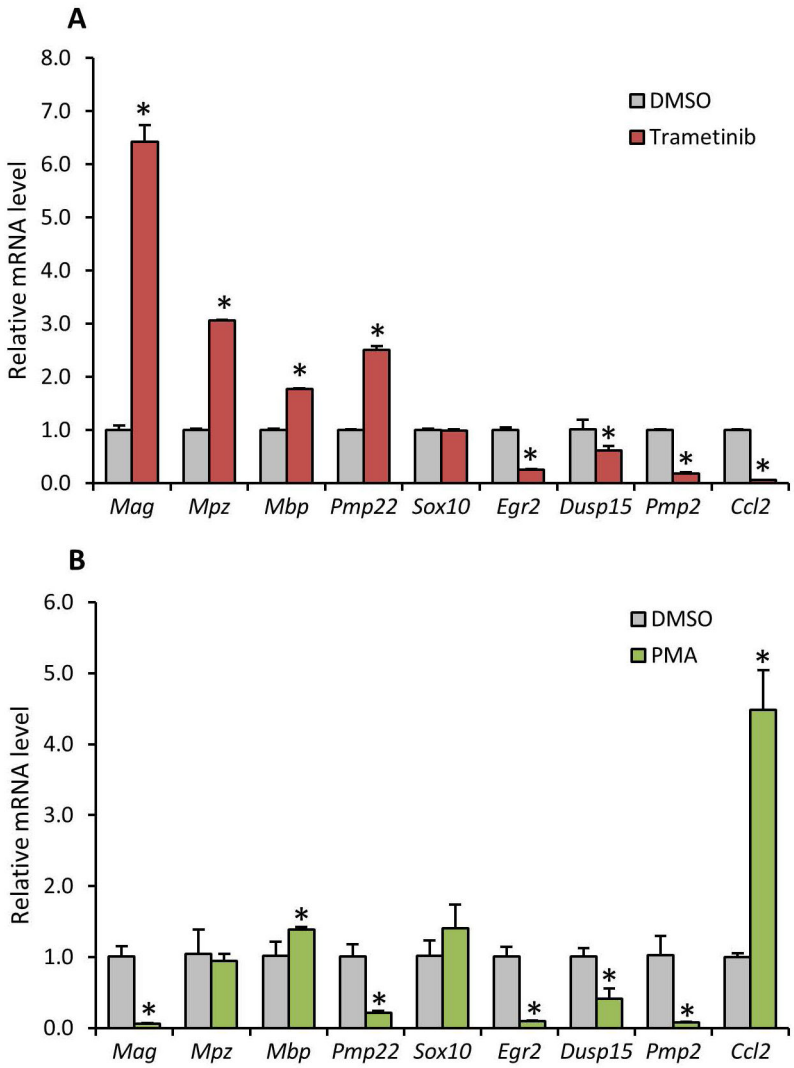

Figure 7. Downstream Erk effects is conserved in Primary Schwann cells

Mag, Mpz, Mbp, Pmp22, Sox10, Egr2, Dusp15, Pmp2 and Ccl2 were measured in Primary Schwann cells when these were treated with Trametinib (A) and PMA (B) as before. Genes were measured using quantitative RT-PCR and normalized to 18S rRNA. Error bars represent the standard deviation from biological triplicates. (*P<0.05)

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Table 1

Gene Name	Forward	Reverse
Dusp15	AGCCACTTAACGAGCCCCTTT	TCGGCTGAGCTGCAGTGTTTA
Sox10	CGAATTGGGCAAGGTCAAGA	CACCGGGAACTTGTCATCGT
Egr2	GCACTCTGTGGCCCTAGAACA	GGCTGAGATGGCTCGAGAAA
Ccl2	TGCATCCACTCTCTTTTCCA	CATTGAAAGTGTTGAACCAGGA
Vegfc	CGCTGTGTCCCATCATATTG	CTGTCTGGTCACTGGCAGAA
Mag	GGCTGAGTACGCAGAAATCC	AGGGGAGAGGGAGCTGTAAC
Mbp	CAGGATTCGGGAAGGCTGAG	GAGGAAGAGACAGCCGCTCTG
Gjb1	CTCTGCACTGTGGATGGAGA	TTCAGAGAGAGAGGCCTTGG
Pmp22	CAGATCCCTCCCTCCCATTC	TGTCCCCGCACTTTGGTTAT
Srebp-1c	GGAGCCATGGATTGCACATT	TCAAATAGGCCAGGGAAGTCA
Mpz	CCTGGAGGTGACGGTCACTT	CTGCAGTCAAATCCCCCAGTA