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p75 is required for the establishment of postnatal sensory neuron diversity by potentiating Ret signaling

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Summary

Producing the neuronal diversity required to adequately discriminate all elements of somatosensation is a complex task during organogenesis. The mechanisms guiding this process during dorsal root ganglion (DRG) sensory neuron specification remain poorly understood. Here we show that the p75 neurotrophin receptor interacts with Ret and its GFR α co-receptor upon stimulation with glial cell line-derived neurotrophic factor (GDNF). Furthermore, we demonstrate that p75 is required for GDNF-mediated Ret activation, survival, and cell surface localization of Ret in DRG neurons. In mice in which p75 is deleted specifically within sensory neurons beginning at E12.5, we observe that approximately 20% of neurons are lost between P14 and adulthood, and these losses selectively occur within a subpopulation of Ret⁺ nonpeptidergic nociceptors, with neurons expressing low levels of Ret impacted most heavily. These results suggest that p75 is required for the development of the nonpeptidergic nociceptor lineage by fine-tuning Ret-mediated trophic support.

Graphical abstract

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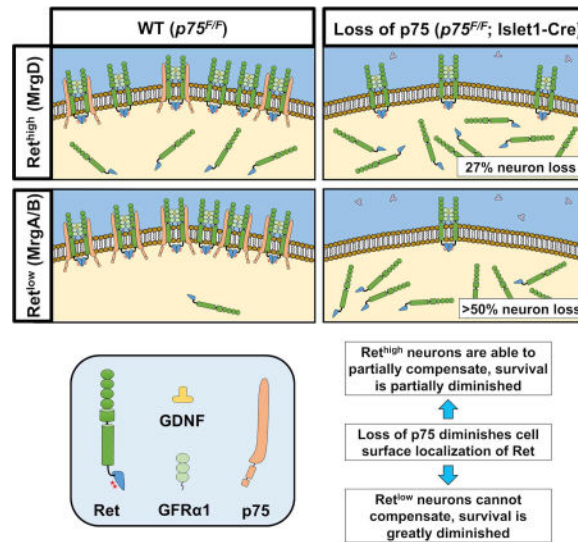
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Additional methods are included in the Supplementary Materials.

Author contribution

Z.C., C.R.D., B.A.P., and K.F.L. designed experiments, interpreted the data, and wrote the manuscript. Z.C., C.R.D., A.S.H. and B.D. performed the experiments. Y. H. and W.L. generated p75 floxed mice. B.A.P. and K.F.L. were responsible for the overall direction and communication of the experiments.



Introduction

The generation of the diverse array of sensory neurons necessary for discriminating all aspects of somatosensation is critical for animals to interact and respond to their environment. Sensory neurons in the dorsal root ganglia (DRG) innervate the peripheral tissues of the body below the neck and communicate sensory information to higher order neurons within the central nervous system. DRG neurons are greatly diversified with respect to their sensory functions, which is mirrored by their unique morphological, physiological, and molecular characteristics. Large, medium, and small diameter neurons carry proprioceptive stimuli (proprioceptors), tactile stimuli (mechanoreceptors) and nociceptive stimuli (nociceptors), respectively. Each of these morphologically distinct groups can be further subdivided based on their expression of different neurotrophic factor receptors, G protein coupled receptors, ion channels and transcription factors. Using these morphological and molecular properties, combined together with electrophysiological properties as well as distinctive central and peripheral innervation patterns, sensory neurons have been categorized into multiple subpopulations (Liu and Ma, 2011).

In adult mice, nociceptors can be divided into two major populations. One population, the peptidergic nociceptors, expresses neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P (SP), and the nerve growth factor (NGF) receptor, TrkA. CGRP- or SP-negative nonpeptidergic nociceptors, in contrast, express the tyrosine kinase Ret and the GFR α co-receptors for the GDNF family ligands. Interestingly, the Ret^+ nociceptive population of neurons emerges from TrkA-expressing neurons in early postnatal development, and the deletion of TrkA results in the complete loss of Ret-expressing unmyelinated neurons (Molliver et al., 1997; Silos-Santiago et al., 1995). This Ret-expressing population of nociceptors further differentiates into three subclasses of neurons that express specific Mas-related G protein-coupled receptors (Mrgpr), namely, neurons that express MrgprA3, MrgprB4 or MrgprD (Dong et al., 2001; Liu et al., 2008). Recent physiologic studies suggest that these three classes of neurons are responsive to pruritogens,

the gentle stroking of hair, and noxious mechanical stimulation, respectively (Han et al., 2013; Liu et al., 2012; Liu et al., 2007; Zylka et al., 2005). One central question in developmental biology is how such neuronal diversity is developed and maintained throughout life.

The development of sensory neurons in the DRG is regulated by the interplay between neurotrophic factors and various transcription factors (Marmigere and Ernfors, 2007). Recent studies indicate that the expression of transcription factors such as Runx1, Runx3, Er81, Shox2, and MafA play significant roles in the diversification of DRG neurons (Bourane et al., 2009; Chen et al., 2006; Inoue et al., 2007; Kramer et al., 2006; Scott et al., 2011). Neurotrophin family members have been shown to support the survival of various types of DRG neurons during development. Previous loss-of-function studies of neurotrophin family members Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 3 (NT3) and their respective high-affinity receptors TrkA, TrkB and TrkC, demonstrated that different sensory neuron subtypes require different neurotrophins for their survival. For example, in *Ngf*^{-/-} and *TrkA*^{-/-} mice, nociceptors are lost (Crowley et al., 1994; Smeyne et al., 1994), while *Nt3*^{-/-} and *TrkC*^{-/-} mice lose proprioceptors (Ernfors et al., 1994; Tessarollo et al., 1994), and *TrkB*^{-/-} and *Bdnf*^{-/-} mice lose mechanoreceptors (Jones et al., 1994; Klein et al., 1993).

The GDNF family ligands (GFLs) consist of glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN) and persephin (PSPN). They bind to one of four GDNF family co-receptors (GFR α s) to initiate their signaling, GFR α 1-GFR α 4. GDNF preferentially binds to GFR α 1, neurturin to GFR α 2, artemin to GFR α 3 and persephin to GFR α 4. GFR α s are glycerophosphatidylinositol (GPI) linked proteins that do not have an intracellular domain and, after binding to GFLs, they bind to and activate the tyrosine kinase Ret and initiate downstream signaling. Ret has two unique C-terminal isoforms, Ret9 and Ret51, each with unique signaling properties and functions (Airaksinen and Saarma, 2002). *In vitro* studies have demonstrated that GFLs promote the survival and axonal growth of various DRG neurons including nociceptive neurons (Molliver et al., 1997). *In vivo* loss-of-function studies on GFLs and GFR α s have shown that they are necessary for the survival of DRG neurons, their proper peripheral projections and cell body size (Ernsberger, 2008; Lindfors et al., 2006). The analysis of three different conditional Ret mutant mice demonstrated that Ret signaling is important for several aspects of DRG neuron development such as cell survival, central and peripheral neuronal projection and expression of phenotypical markers (Franck et al., 2011; Golden et al., 2010; Luo et al., 2007).

The p75 neurotrophin receptor (p75) is a member of the tumor necrosis receptor super family. p75 binds members of the Trk receptor family and modulates the cell survival functions of neurotrophins (Ceni et al., 2014). p75 is highly expressed in migrating neural crest cells and most sensory neurons during development. p75 null mice lose approximately 50% of DRG neurons across different sensory neuron subtypes, but the mechanism of these deficits is unclear in part due to expression of p75 in multiple cell types in the peripheral sensory system including neurons, Schwann cells and targets (Lee et al., 1992). One hypothesis is that neuronal loss is due to impairment in neurotrophin signaling. Indeed, *in vitro* experiments have shown that sensory neurons isolated from p75 null mutant mice are

2–3 fold less sensitive to the survival promoting effect of NGF treatment (Davies et al., 1993; Lee et al., 1994). In addition, *in vitro* experiments suggest that p75 prolongs the activation of TrkA receptors by preventing poly-ubiquitination and degradation of TrkA (Makkerh et al., 2005). These results argue that p75 can serve as a modulator of the neuronal response to neurotrophins. Although the role of p75 for modulating neurotrophin signaling is clearly established, whether the DRG neuron loss in p75 null mutant mice is due to a lack of neurotrophin signaling still remains to be determined.

Here we show that p75 is co-localized with GFR α 1, GFR α 2 and Ret in a subset of DRG neurons. p75 forms a complex with GFR α 1 and Ret *in vitro* and *in vivo*. Removal of p75 from neurons reduced the survival effect of GDNF *in vitro* and impacted a subset of nonpeptidergic nociceptors *in vivo*, especially those that express low levels of Ret. Surprisingly, p75 deletion did not affect TrkA⁺ or TrkB⁺ populations of DRG neurons. p75, therefore, serves to augment GFL signaling and maintains the balance of cell specification and diversity during DRG neuron development.

Results

p75 is expressed in a subset of Ret expressing nociceptive neurons

Ret expressing cells in the DRG encompass several neuronal populations mediating different sensory modalities. Previous studies have shown that small diameter nonpeptidergic nociceptors which mediate pain sensation up-regulate Ret expression and down-regulate TrkA expression postnatally. This process is not complete until 2–3 weeks after birth (Molliver et al., 1997). p75 is expressed in a variety of DRG neurons subclasses, and germline deletion of p75 results in a 50% loss of DRG neurons across different neuronal types (Murray et al., 1999). To determine whether the Ret-expressing nonpeptidergic nociceptive neurons express p75, immunostaining was performed on postnatal day 14 (P14) L4 DRG sections with antibodies against p75, Ret, GFR α 1 and GFR α 2. As shown in Figure 1B, 1E and 1H, small diameter nonpeptidergic nociceptors are Ret⁺ and most of them are also immunopositive for GFR α 1 or GFR α 2. p75 expression overlapped with some small diameter neurons that are immunopositive for Ret, GFR α 1 or GFR α 2 (Figures 1C, 1F, and 1I). When we counted neurons that were immunopositive for both p75 and Ret, we found that ~23.8% of small diameter Ret⁺ cells are also p75⁺ (Figure 1J). Previous studies have also shown that Ret⁺ nociceptive neurons have different levels of Ret expression and can be divided into groups that express a high level of Ret (Ret^{high} neurons) and a low level of Ret (Ret^{low} neurons), each group with their own characteristic markers (Zylka et al., 2003). When we investigated the co-localization of p75 with Ret in small diameter Ret^{high} (as indicated by arrows in Figure 1A–C) and Ret^{low} neurons (as indicated by arrowheads in Figure 1A–C), we found that among Ret^{high} neurons, only 22.1% of them co-express p75, while 67.6% of Ret^{low} neurons express p75 (Figure 1J). The specificity of Ret staining was confirmed by staining sensory neurons taken from tamoxifen (TMX)-treated *Rosa26^{L-SL-tdTomato/+}; Ret-Cre/ERT2* mice (Figure S1). These results suggest that p75 is in position to regulate the development of a subset of Ret⁺ nociceptive neurons, and its role may be more significant in Ret^{low} sensory neurons.

p75 is a component of the GFR α /Ret receptor complex

To investigate potential interactions between p75 and the components of the GDNF receptor complex, namely GFR α 1 and Ret, we stained primary cultures of dissociated DRG neurons with antibodies against p75 and GFR α 1. As shown in Figure 2A, punctate staining of p75 is often co-localized with GFR α 1, although non-overlapping staining exists. Based on these findings, we next explored whether a functional interaction exists between p75 and GFR α 1 and Ret. p75 was co-expressed with GFR α 1 or Ret in HEK-293 cells and a co-immunoprecipitation assay was performed. As shown in Figures 2B and 2C, p75 co-immunoprecipitates with GFR α 1 and Ret. GDNF treatment enhanced the association of p75 with GFR α 1 (Figure 2B). On the other hand, GDNF had no effect when myc-tagged p75 were co-expressed with Ret alone in HEK293 cells. Quantification of the western blot signals from 3 independent experiments showed that no statistically significant changes in the amount of normalized Ret in the immunoprecipitate pulled down by an anti-myc antibody (fold change +GDNF/-GDNF=1.17, p=0.19) or an anti-p75 antibody (fold change +GDNF/-GDNF=1.08, p=0.58). The amount of normalized p75 in the immunoprecipitate pulled down by an anti-Ret antibody is also unchanged with GDNF treatment (fold change +GDNF/-GDNF = 0.86, p=0.43). This is likely due to the lack of GFR α 1 expression. To determine whether p75 forms a complex with GFR α 1 under physiological conditions *in vivo*, co-immunoprecipitation assays were performed using lysates from superior cervical ganglia (SCG). We found that p75 and GFR α 1 were able to be co-immunoprecipitated regardless of which protein was isolated by IP (Figure 2D). Furthermore, to determine the time course of the interaction between p75 and Ret, primary DRG neuron cultures were generated from P0 rats and maintained in culture for 7–10 days. Importantly, DRG neurons cultured under similar conditions were previously shown to develop in a manner comparable to their *in vivo* development regarding the expression of ion channels and neurotrophic factor receptors (Molliver et al., 1997). 7–10 DIV DRG neurons were stimulated with GDNF for the indicated times, followed by immunoprecipitation of Ret and immunoblotting for p75 and Ret. Interestingly, we observed that GDNF led to a statistically significant increase in p75-Ret association at 15 minutes, 1 hour, and 6 hours (Figure 2E–F) after stimulation. These results suggest that p75 forms a complex with GFL receptor components that mediate GFL signaling.

p75 augments GDNF-mediated Ret activation by increasing cell surface localization of Ret

To determine whether p75 modulates GDNF-induced Ret autophosphorylation, primary DRG neuron cultures were generated from p75 conditional knockout mice (*p75^{F/F}*), followed by infection with HSV-expressing Cre to knockdown their expression of p75, or with HSV-expressing GFP alone as a control. The cultures were then treated with GDNF, followed by immunoprecipitation to assess the level of phosphorylated Ret using an anti-phosphotyrosine antibody. As shown in Figure 3A, GDNF induces a rapid phosphorylation of Ret, which is sustained above baseline for at least 60 minutes. Transduction of primary DRG neurons with HSV-GFP-Cre resulted in successful knockdown of approximately 80% of p75 compared to HSV-GFP-transduced neurons (Figure 3A–B). Following knockdown of p75, GDNF-induced Ret phosphorylation is dramatically reduced at both 15 minutes and 60 minutes following GDNF treatment (Figure 3A, 3C). These results suggest that p75 augments GDNF-mediated Ret activation.

Given the striking loss of GDNF-mediated Ret activation, we hypothesized that loss of p75 may result in changes of cell surface localization of Ret. To explore this possibility, we first transfected NIH/3T3 cells with Ret9 or Ret51, with or without p75 co-transfection. Cell surface proteins were biotinylated using NHS-LC-Biotin and precipitated using neutravidin. Interestingly, we observed that co-transfection of p75 with Ret9 and Ret51 significantly enhanced cell surface levels of both isoforms (Figure 3D). To test whether p75 plays a similar role in DRG neurons, we performed cell surface biotinylation experiments in HSV-treated *p75^{F/F}* DRG neurons. We observed a striking reduction in the amount of total cell surface Ret using an antibody that detects both isoforms, as well as a reduction of both individual Ret9 and Ret51 isoforms in HSV-Cre treated neurons compared to HSV-GFP controls (Figure 3E, quantifications in 3F, 3G).

p75 is required for GFL-mediated survival of Ret⁺ nonpeptidergic nociceptors *in vitro*

Given that p75 is required for GDNF-mediated Ret activation, we sought to determine whether loss of p75 impacted GFL-mediated DRG neuron survival. Further, because TrkA⁺ peptidergic nociceptors selectively depend on NGF, while Ret⁺ nonpeptidergic nociceptors depend on GDNF and NRTN (Molliver et al., 1997; Snider and Silos-Santiago, 1996) for their survival, we hypothesized that p75 deletion would selectively impair GFL-mediated survival within the nonpeptidergic nociceptor population. To this end, we crossed *p75^{F/F}* mice with *UBC-Cre/ERT²* mice, in which a tamoxifen (TMX)-inducible Cre is driven by the ubiquitin C promoter expressed in all cells (generating p75-WT and p75-cKO neurons) (Ruzankina et al., 2007), or *Ret-Cre/ERT²* mice, in which TMX induces recombination selectively within Ret⁺ neurons (generating p75-WT and p75-RC neurons) (Luo et al., 2009). DRG neurons were cultured in the presence of NGF and 4-hydroxy-TMX to induce recombination. Importantly, we observed a substantial knockdown of p75 in p75-cKO neurons compared to p75-WT ($p < 0.0001$; greater than 98% reduction; Figures 4A–B). No changes were observed in the total amount of TrkA (Figure 4A, 4C) or Ret (Figure 4A, 4D) protein levels, further validating this system. Due to the difficulty of detecting GFR α 1 and GFR α 2 protein levels by western blotting in cultured DRG neurons, we performed immunostaining to determine whether loss of p75 alters the proportion of GFR α 1 or GFR α 2 neurons. While we observed no difference in the proportion of GFR α 1⁺ neurons ($p = 0.2723$), a small but statistically significant reduction in the proportion of GFR α 2⁺ neurons was observed ($p = 0.0103$; p75-WT: 80.12 ± 1.32 and p75-cKO: $66.76 \pm 4.34\%$; Figure S2C–D).

To fully characterize how p75 deletion alters Ret and TrkA expression, we next treated 7–10 DIV, TMX-maintained p75-WT and p75-cKO DRG neurons with NGF, a NGF blocking antibody (α NGF), α NGF and GDNF, or α NGF and NRTN. 48 hours later, p75-WT and p75-cKO DRG neurons were fixed and immunostained for DAPI, TuJ1 (a pan-neuronal marker), Ret, and TrkA. The number of TrkA⁺/Ret⁻ and Ret⁺/TrkA⁻ neurons were quantified in each condition. We observed no genotype-dependent differences in the number of TrkA⁺ neurons regardless of treatment condition (Figure S2A), although we did observe a significant reduction of TrkA⁺ neurons following NGF deprivation, as expected (Snider and Silos-Santiago, 1996). In addition, we observed an increase in the percentage of Ret⁺ neurons following NGF-deprivation ($p < 0.0001$ for all genotypes) (Figure S2B), as expected

due to the death of the TrkA⁺ population of neurons. Interestingly, the proportion of Ret⁺ neurons was increased in p75-cKO compared to p75-WT neurons treated with αNGF, αNGF and GDNF, and αNGF and NRTN, but not in NGF-maintained neurons. Collectively, these data indicate that removal of p75 does not substantially alter levels of TrkA, but enhances upregulation of Ret following NGF deprivation.

To determine whether loss of p75 altered GFL-mediated survival of TrkA⁺ peptidergic or Ret⁺ nonpeptidergic neurons, p75-WT and p75-cKO neurons were treated as described above and the number of apoptotic Ret⁺ (Figure 4E) and TrkA⁺ (Figure 4F) neurons was quantified by counting pyknotic nuclei. As expected, NGF deprivation led to an increase in the number of apoptotic Ret⁺ and TrkA⁺ neurons (Figures 4E–F) regardless of genotype. GDNF and NRTN treatments were able to partially rescue Ret⁺ neurons from apoptosis (Figure 4E; $p < 0.001$ for each), and this rescue effect was substantially diminished in p75-cKO ($p < 0.0001$ for each). As expected, GDNF and NRTN were not able to rescue TrkA⁺ neurons and loss of p75 had no effect (Figure 4F). To further substantiate these findings, these experiments were repeated using DRG neurons cultured from p75-RC, or p75-WT littermate mice. Interestingly, we observed that GDNF and NRTN were each able to rescue Ret⁺ neurons from apoptosis in p75-WT ($p < 0.0001$), but not p75-RC neurons, with a corresponding increase in apoptosis observed in p75-RC neurons treated with αNGF and GDNF ($p < 0.01$), and αNGF and NRTN ($p < 0.001$) compared to p75-WT neurons (Figure 4G). Additionally, as expected, GFL treatment did not rescue TrkA⁺ neurons from apoptosis, and no genotype-dependent effects were observed in TrkA⁺ neurons (Figure 4H). Collectively, these results indicate that p75 functions specifically to augment the GFL-mediated survival of Ret⁺ DRG neurons.

Removal of p75 expression in DRG neurons induces a loss of 20% of adult DRG neurons

Based on our findings that p75 is necessary for GFL-mediated survival of DRG neurons *in vitro*, we asked whether it plays similar roles *in vivo*. Previous studies analyzing p75 germline knockout mice have demonstrated that 50% of DRG neurons are lost, and these losses occur across sensory modalities. It is unclear, however, whether this drastic phenotype reflects a cell-autonomous requirement for p75 within sensory neurons themselves. Thus, we generated $p75^{F/F}$ mice (described in Supplemental Experimental Procedures and Figure S3) and crossed them with an *Islet1-Cre* driver that expresses Cre in spinal motor and sensory neurons at later stages (Srinivas et al., 2001). Grossly, *Islet1-Cre^{+/-}; p75^{F/F}* mice are indistinguishable from their littermates. To determine at what stage p75 is deleted in these animals, p75 immunostaining was performed on E10.5, E12.5, and adult *Islet1-Cre^{+/-}; p75^{F/F}* mice (compared to $p75^{F/F}$ controls). We observed no reduction of p75 immunolabeling at E10.5 (Figure 5A, 5D), but a drastic loss of p75 by E12.5 (Figure 5B, 5E). At both E12.5 and adulthood (Figure 5C, 5F), the vast majority of neurons have lost p75 immunolabeling, with primarily non-neuronal cells expressing the residual p75. Therefore, *Islet1-Cre^{+/-}; p75^{F/F}* mice lose neuronal p75 expression between E10.5 and E12.5. To determine whether neuronal survival is altered in the *Islet1-Cre^{+/-}; p75^{F/F}* mice, we performed Nissl staining and counted total cell numbers in L4 DRGs from *Islet1-Cre^{+/-}; p75^{F/F}* (and $p75^{F/F}$ control) mice at P1, P14 or adulthood. As a control, we also analyzed L4 DRG counts in p75 germline knockout mice. As expected, we observed a 50% reduction in P1 $p75^{-/-}$ DRGs compared to

p75^{+/+} controls (Figure 5G). Strikingly, given the drastic results observed in P1 *p75^{-/-}* mice, we observed no difference in total L4 DRG neuron numbers at P1 or P14 in *Isl1-Cre^{+/-}*; *p75^{F/F}* mice (Figure 5G). We did, however, observe that approximately 20% of DRG neurons were lost in adult *Isl1-Cre^{+/-}*; *p75^{F/F}* mice compared to *p75^{F/F}* controls (Figure 5G). Collectively, these results indicate that neuron-specific deletion of *p75* by E12.5 is insufficient to impact neuronal survival during embryogenesis, and suggest neuronal *p75* functions postnatally in sensory neuron diversification.

Removal of *p75* does not alter the generation of Ret⁺ or Runx1⁺ neurons

Loss of NGF-TrkA signaling in a *Bax^{-/-}* background (thereby preventing apoptosis) results in significantly reduced levels of Ret, as well as reduced levels of the transcription factor Runx1 (Luo et al., 2007). Given that *p75* has also been shown to enhance NGF signaling through TrkA, we tested whether the generation of Ret⁺ or Runx1⁺ neurons is affected in *Isl1-Cre^{+/-}*; *p75^{F/F}* mice. Upon immunostaining P14 DRG neurons for Ret or Runx1, we observed no differences in the intensity of immunostaining (Figure 5H–I, 5K–L) or total number of Runx1⁺ and Ret⁺ neurons in *Isl1-Cre^{+/-}*; *p75^{F/F}* compared to *p75^{F/F}* control mice (Figure 5J, 5M), suggesting that the removal of *p75* does not impact the NGF-TrkA-Runx1 signaling axis, thus unlikely to alter subsequent Ret expression. We further characterized the expression level of TrkA, TrkB, GFR α 1 and GFR α 2 at P0, P14 and adult DRGs. There is no obvious difference in any of these markers between *Isl1-Cre^{+/-}*; *p75^{F/F}* mice and controls at P0 and P14 (Figure S4, Figure S5). These results suggest that loss of *p75* in the *Isl1-Cre^{+/-}*; *p75^{F/F}* mice does not impact the expression level of TrkA, TrkB, or the relevant GFL co-receptors while Ret⁺ nonpeptidergic nociceptors are being generated.

p75 is required for survival of a subset of nonpeptidergic nociceptors

Given that we observed losses of approximately 20% of DRG neurons, and our *in vitro* findings linking *p75* to GFL-Ret signaling, we hypothesized that nonpeptidergic nociceptors were selectively lost in *Isl1-Cre^{+/-}*; *p75^{F/F}* mice. To answer this question, adult *p75^{F/F}* and *Isl1-Cre^{+/-}*; *p75^{F/F}* L4 DRG neurons were immunostained for NF200 (mechanoreceptors and proprioceptors), CGRP (peptidergic nociceptors), parvalbumin (proprioceptors), and IB4 (nonpeptidergic nociceptors). Interestingly, we observed no difference in the number of CGRP⁺ (Figure 6A, 6E), parvalbumin⁺ (Figure 6B, 6F), or NF200⁺ (Figure 6D, 6H) neurons between *Isl1-Cre^{+/-}*; *p75^{F/F}* and *p75^{F/F}* mice (Figure 6O). In contrast, we observed a 35% decrease in IB4⁺ neurons (Figure 6C, 6G, 6O), indicating that *p75* is selectively required for the survival of a subset of nonpeptidergic nociceptors. Given that the IB4⁺ immunolabeling may not sufficiently label all nonpeptidergic nociceptors, we sought to corroborate these results by performing co-immunostaining for peripherin and Ret to count the number of small diameter Ret⁺ nociceptors (Figure 6I–N). As shown in Figure 6O, we observed a 25% decrease in the total number of peripherin⁺/Ret⁺ neurons. We also observed ~30% decrease in the GFR α 2⁺ population (Figure S5G) and ~50% decrease in small diameter GFR α 1⁺ population (Figure S4N). Collectively, these results indicate that *p75* is selectively required to promote the survival of nonpeptidergic Ret⁺ neurons, although we cannot rule out the possibility that *p75* has additional functions in other unexplored subpopulations.

To characterize the neuron loss within the IB4⁺ population, we performed *in situ* hybridization using probes against MrgA, MrgB, and MrgD on adult (6 months old) L4 DRG sections (Dong et al., 2001). Importantly, MrgA⁺ and MrgB⁺ neurons are known to express Ret weakly (Ret^{low}), while MrgD⁺ neurons express Ret more strongly (Ret^{high}) (Zylka et al., 2003). Interestingly, compared to *p75^{F/F}* mice, we observed that *Isl1-Cre^{+/-}; p75^{F/F}* DRGs had a greater than 50% reduction in MrgA⁺ (Figure 7A–B, 7G) and MrgB⁺ neurons (Figure 7C–D, 7H), while only 27% of MrgD⁺ neurons were lost (Figure 7E–F, 7I). We cannot rule out the possibility that the loss of p75 in DRG neurons causes down regulation of Mrg family gene expression, as previous studies have shown a dependence on Ret signaling (Luo et al., 2007). Nonetheless, these data are consistent with the model that the p75-dependence of Ret⁺ neurons is correlated with the level of p75 expression in the Ret^{low} and Ret^{high} neurons.

Discussion

In this study, we demonstrate that p75 forms a GDNF-activated receptor complex with GFR α 1 and Ret. Furthermore, knockdown of p75 levels within DRG neurons resulted in a drastic impairment in GDNF-mediated Ret activation, and a subsequent loss of cell surface localization of Ret, suggesting that p75 potentiates GDNF-Ret signal transduction through enhancing receptor availability to ligand, or by enhancing recycling. Correspondingly, p75 deletion results in a substantial reduction in GDNF and NRTN-mediated survival of Ret⁺ nonpeptidergic neurons, but not TrkA⁺ peptidergic neurons. Strikingly, especially when considering the 50% loss of DRG neurons during embryonic development in p75 germline null mutants, neuron-specific deletion of p75 by E12.5 resulted in no embryonic deficits. In these animals, 20% of neurons were lost postnatally, with losses occurring selectively within the IB4⁺/peripherin⁺/Ret⁺ class of nonpeptidergic nociceptors during the transition from P14 to adulthood. MrgA⁺ and MrgB⁺ neurons expressing the lowest levels of Ret had the highest degree of p75 expression, and were more significantly affected than Ret^{low} MrgD⁺ neurons. In light of these findings, we propose the following model to explain the role of p75 modulation of Ret signaling in the survival of adult DRG neurons (Figure 7J). The expression of p75 increases the level of Ret on the cell surface in *p75^{F/F}* mice, increasing sensitivity of these neurons to GDNF, thereby potentiating GFL-induced Ret activation and downstream survival. Upon loss of p75 expression in *Isl1-Cre^{+/-}; p75^{F/F}* mice, the level of Ret on the cell surface is reduced. Ret^{high} MrgD⁺ neurons may be better able to compensate for the loss of p75, resulting in lower neuron losses. However, in Ret^{low} MrgA⁺ or MrgB⁺ neurons, the loss of cell surface Ret cannot be adequately buffered, leading to more substantial losses within the MrgA⁺ and MrgB⁺ neuron population. This model highlights the ability of p75 to fine-tune Ret signaling and maintain the balance of different types of DRG neurons in postnatal development. Our findings further expand the repertoire of receptor complexes that p75 modulates and the developmental processes that p75 impacts.

The developmental diversification of DRG neurons is an intricate process regulated by transcription factors and growth factors. Different types of sensory neurons rely on specific neurotrophic factors for their survival as they differentiate and mature. The p75 receptor can modulate both neurotrophin and GFL signaling pathways, playing a significant role in the development and maintenance of DRG neuron diversity. In the p75 knockout mice, 50% of

lumbar DRG neurons are lost by the first week of postnatal life (Figure 5G) (Murray et al., 1999). This loss is across all DRG neuronal types and occurs early during embryonic DRG development. In *Isl1-Cre^{+/-}; p75^{F/F}* mice, p75 expression is removed at E12.5 specifically within nearly all sensory neurons, yet no neuronal deficits are observed before P14, and only 20% of the total neurons are lost in adult *Isl1-Cre^{+/-}; p75^{F/F}* mice. The striking disparity between neuronal losses in p75 knockout mice compared to *Isl1-Cre^{+/-}; p75^{F/F}* mice suggests that the early deficits in p75 knockout mice may be due to its function in non-neuronal cells. For example, previous studies have indicated that p75 is expressed in neural crest stem cells, which give rise to DRG neurons, satellite cells in the DRG, and Schwann cells. Given that germline p75 knockout animals lack p75 expression in all cell types from the onset of development, the previously observed loss of 50% of neurons across DRG subtypes may be due to defects in neural crest progenitors. Another possibility is that removing p75 from satellite cells and Schwann cells may create a less supportive environment for the survival of DRG neurons. While we cannot rule out the possibility that the small amount of p75 remaining following *Isl1-Cre* mediated deletion is responsible for the differences observed between germline p75 knockout mice and *Isl1-Cre^{+/-}; p75^{F/F}* mice, previous studies have also suggested a non-neuronal role for p75 early in sensory neuron development. For example, migration of p75-deficient Schwann cells is impaired and may thereby contribute to a reduced ability of developing sensory neurons to reach their proper target cells (Bentley and Lee, 2000). Nevertheless, the results reported here indicate that p75 has a postnatal function specifically serving to augment Ret signaling in nonpeptidergic nociceptors.

Another unexpected finding of this study is that p75 expression is required for the survival of nonpeptidergic nociceptors, but not other neuronal populations. In our analysis of *Isl1-Cre^{+/-}; p75^{F/F}* mice, we observed no deficits in the number of CGRP⁺ peptidergic nociceptive neurons, nor is their *in vitro* survival impaired, despite the fact that p75 is expressed in the majority of CGRP⁺ neurons and p75 expression is removed in most CGRP⁺ neurons in *Isl1-Cre^{+/-}; p75^{F/F}* mice at a developmental period during which these neurons are highly dependent on NGF/TrkA signaling (Figure S6). In addition, we observed no deficits in numbers of TrkA⁺, Runx1⁺, Ret⁺, GFR α 1⁺, or GFR α 2⁺ neurons at P14. These results are surprising because p75 has been shown in many studies to enhance NGF signaling via TrkA through increased NGF binding affinity (Esposito et al., 2001; Hempstead et al., 1991), as well as through reduced ubiquitination and subsequent degradation of TrkA (Makkerh et al., 2005). The pro-survival function of p75 in sensory neurons has been proposed by many (Davies et al., 1993; Lee et al., 1994) to be through modulation of NGF/TrkA signaling. Based on these results, we conclude that the intrinsic level of NGF signaling is sufficient to maintain their survival and, therefore, p75 is dispensable for NGF/TrkA-dependent survival of peptidergic neurons, although the trophic status of these neurons may still be impacted.

In conclusion, we have found that p75 physically associates with receptors in the GDNF family and plays a novel role in mediating the survival-promoting effects of the GFLs by enhancing cell surface localization of Ret, thereby augmenting GFL/GFR α /Ret signaling. Surprisingly, *in vivo*, deletion of p75 specifically within neurons resulted in no deficits until after P14, suggesting a potential early, non-neuronal role for p75 followed by a postnatal,

neuron-specific function in nonpeptidergic nociceptors. Within the nonpeptidergic nociceptor population, neurons of the MrgA⁺ and MrgB⁺ subclasses demonstrate a greater dependence on p75 to potentiate Ret signaling compared to MrgD⁺ neurons. Thus, our data clarify the function of this enigmatic receptor in sensory neurons and identify a unique mechanism by which neurotrophic factor support can be fine-tuned to allow the selective survival of discrete subpopulations, ultimately expanding sensory neuron diversity.

Experimental Procedures

Statistics

Statistical tests were carried out by using GraphPad Prism (GraphPad Software); statistical tests used are indicated throughout the results section. A student's t-test was used to assessing statistical significance between two conditions, while one-way ANOVA was utilized when multiple variables were compared. All data are reported as the mean \pm s.e.m. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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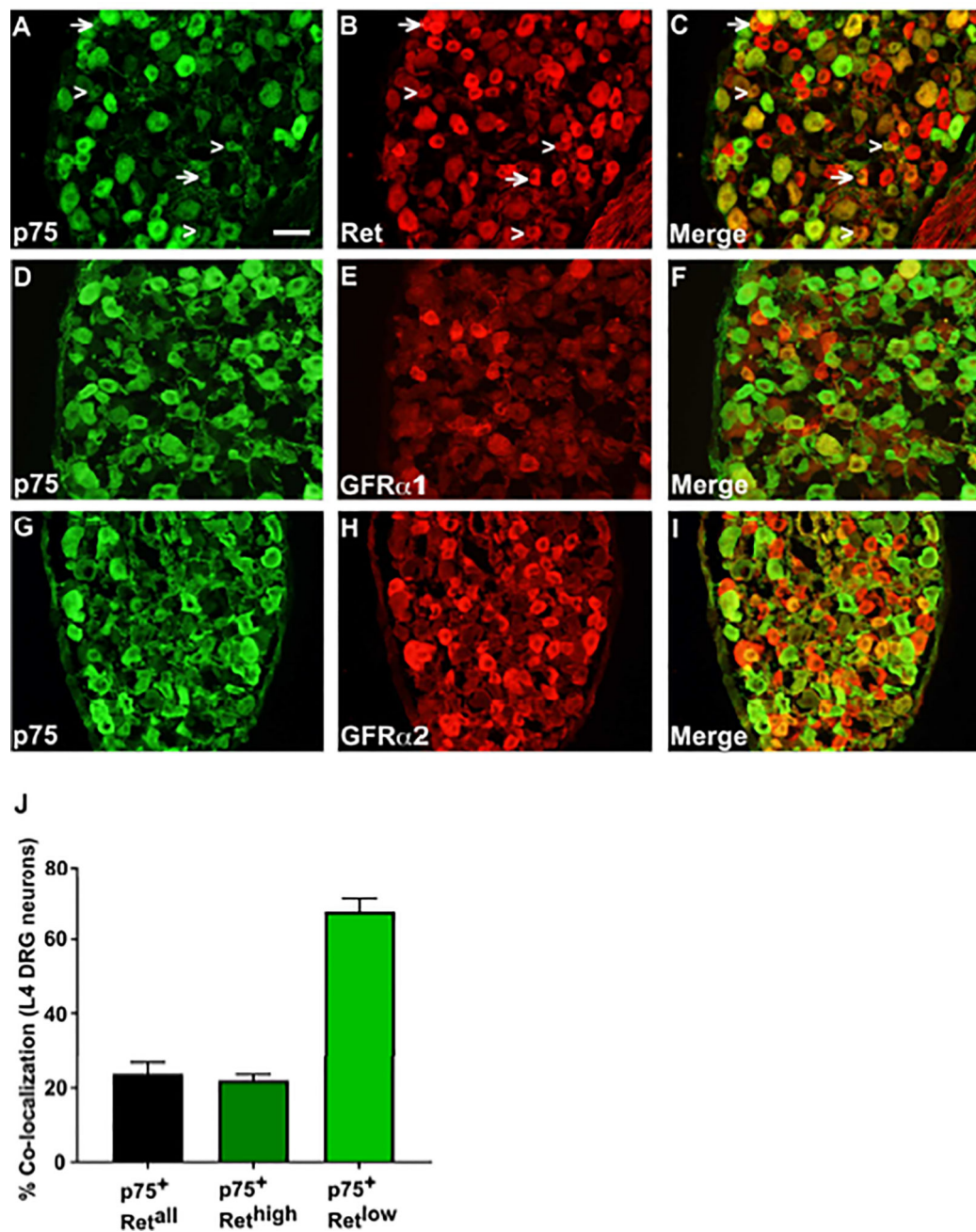


Figure 1. p75 is co-expressed with GFR α 1, GFR α 2 and Ret in DRG neurons

Immunofluorescence staining of p14 DRG sections with anti-p75 (panel A, D, G), anti-Ret (panel B), anti-GFR α 1 (panel E), or anti-GFR α 2 (panel H) antibodies. The quantitation of co-localization is presented in panel J. Arrows in panel A–C indicate examples of Ret^{high} cells and arrowheads indicate examples of Ret^{low} cells. Scale bar, 100 μ m

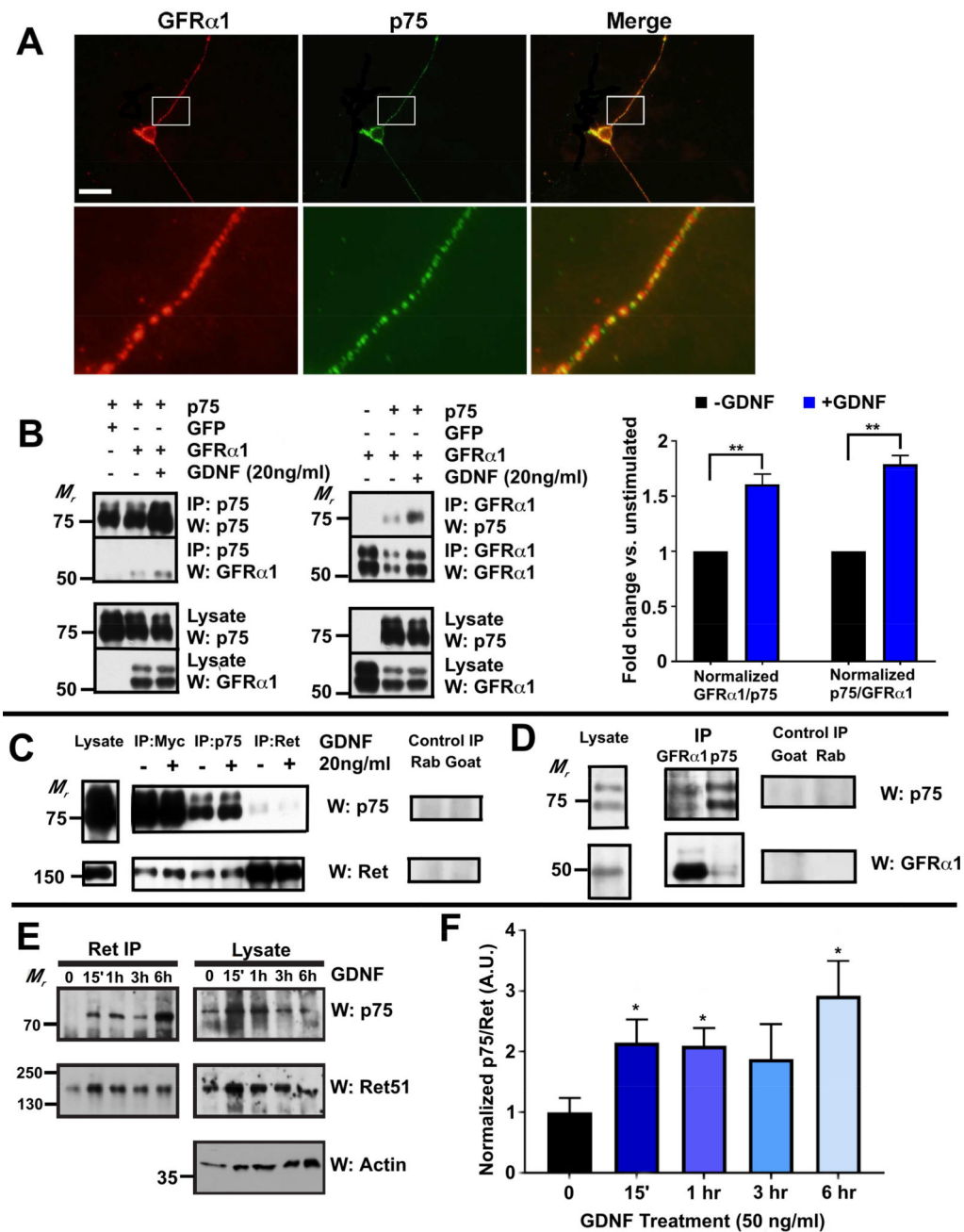


Figure 2. p75 forms a receptor complex with GFR α 1 and Ret

(A) Primary DRG neurons were cultured from P14 mice and immunostained with anti-GFR α 1 and anti-p75 antibodies without fixation. Images in the second row are magnified regions from the rectangles from images in the first row. Scale bar, 100 μ m (only for the top panels). (B) Immunoprecipitation assay of HEK293 cells co-transfected with GFR α 1 and p75. When GFR α 1 and p75 are co-expressed in HEK293 cells, they can be co-immunoprecipitated with an anti-GFR α 1 antibody or an anti-p75 antibody. Quantification of the GFR α 1 and p75 signals in co-IP experiments indicates GFR α 1 and p75 association increased with GDNF treatments. (C) Immunoprecipitation assay of HEK-293 cells co-

transfected with Ret and a myc-tagged p75. Ret and p75 can be co-immunoprecipitated with an anti-myc antibody, a rabbit anti-p75 antibody or a goat anti-Ret antibody. Treating the cells with GDNF did not increase the association between p75 and Ret. Normal rabbit (rab) or goat IgG was used as a negative control. (D) Immunoprecipitation assay of P1-2 SCG. GFR α 1 can be co-immunoprecipitated with p75 using a rabbit anti-p75 antibody or a goat anti-GFR α 1 antibody. Normal rabbit (rab) or goat IgG was used as control. (E) Primary DRG neurons were cultured from E18-P0 rats in the presence of 50 ng/ml NGF. After 7–10 DIV, the neurons were stimulated with medium alone or with GDNF for the indicated times. Ret receptor complexes were immunoprecipitated and immunoblotted for p75 (top panel; left), followed by Ret51 (middle; left). Lysates were immunoblotted for p75, Ret, and for actin (as a loading control). (F) Quantification of p75 co-immunoprecipitating with Ret at each timepoint.

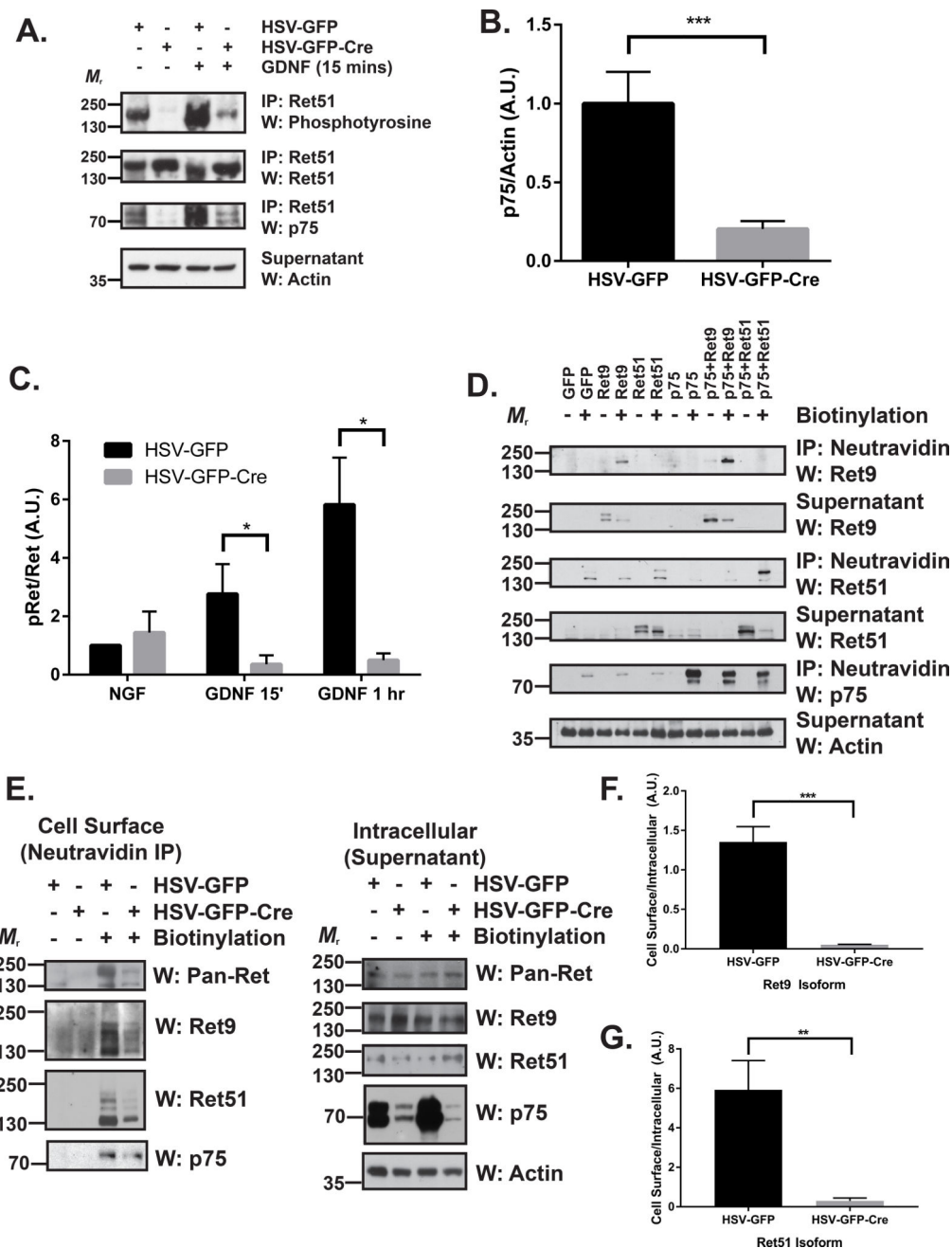


Figure 3. p75 augments GDNF-Ret signaling by increasing the cell surface localization of Ret (A) DRG neurons were cultured from P0 $p75^{F/F}$ mice in the presence of 50 ng/ml NGF. After two days, neurons were exposed to HSV-GFP or HSV-GFP-Cre viruses overnight (indicated above the blots). Neurons were maintained in culture until 7 DIV, followed by stimulation with medium alone or with 50 ng/ml GDNF for 15 minutes or 1 hour. Ret was immunoprecipitated and its level of activation was determined by phosphotyrosine immunoblotting. p75 immunoblotting confirmed that p75 protein levels were substantially reduced upon Cre expression. Actin immunoblotting of the supernatants served as a loading control. (B) Quantifications of p75 levels in 10 separate experiments demonstrate that

approximately 80% of p75 is removed following treatment with HSV-GFP-Cre compared to HSV-GFP alone (** $p < 0.001$). (C) Quantification of the mean proportion of phospho-Ret (pRet) over total Ret \pm the standard error (5 experiments were quantified; * $p < 0.05$). (D) NIH/3T3 cells were transfected with GFP, Ret9, Ret51, p75, p75 and Ret9, or p75 and Ret51. After 24–36 hours, the cells were surface biotinylated using NHS-LC-Biotin in PBS (or treated with PBS alone as a control). The cells were then subjected to neutravidin precipitation. Precipitated cell surface proteins and supernatants (containing intracellular proteins) were then immunoblotted for Ret9, Ret51, and p75. Actin immunoblotting served as a loading control. (E) DRG neurons were cultured from P0 $p75^{F/F}$ mice and treated as described in A. Neurons were subsequently surface biotinylated as in D followed by neutravidin immunoprecipitation. Precipitated cell surface proteins (left) and supernatants (right; containing intracellular proteins) were analyzed by immunoblotting for pan-Ret (upper), Ret9 (2nd panel), and Ret51 (3rd panel). Actin immunoblotting served as a loading control. p75 immunoblotting confirmed efficient knockdown of greater than 70% of p75. (F) Quantifications of the cell surface levels of Ret9 (** $p < 0.001$) and (G) Ret51 (** $p < 0.01$) indicate a highly significant difference in HSV-GFP-Cre treated neurons compared to HSV-GFP controls. *IP*, immunoprecipitation; *W*, western blot.

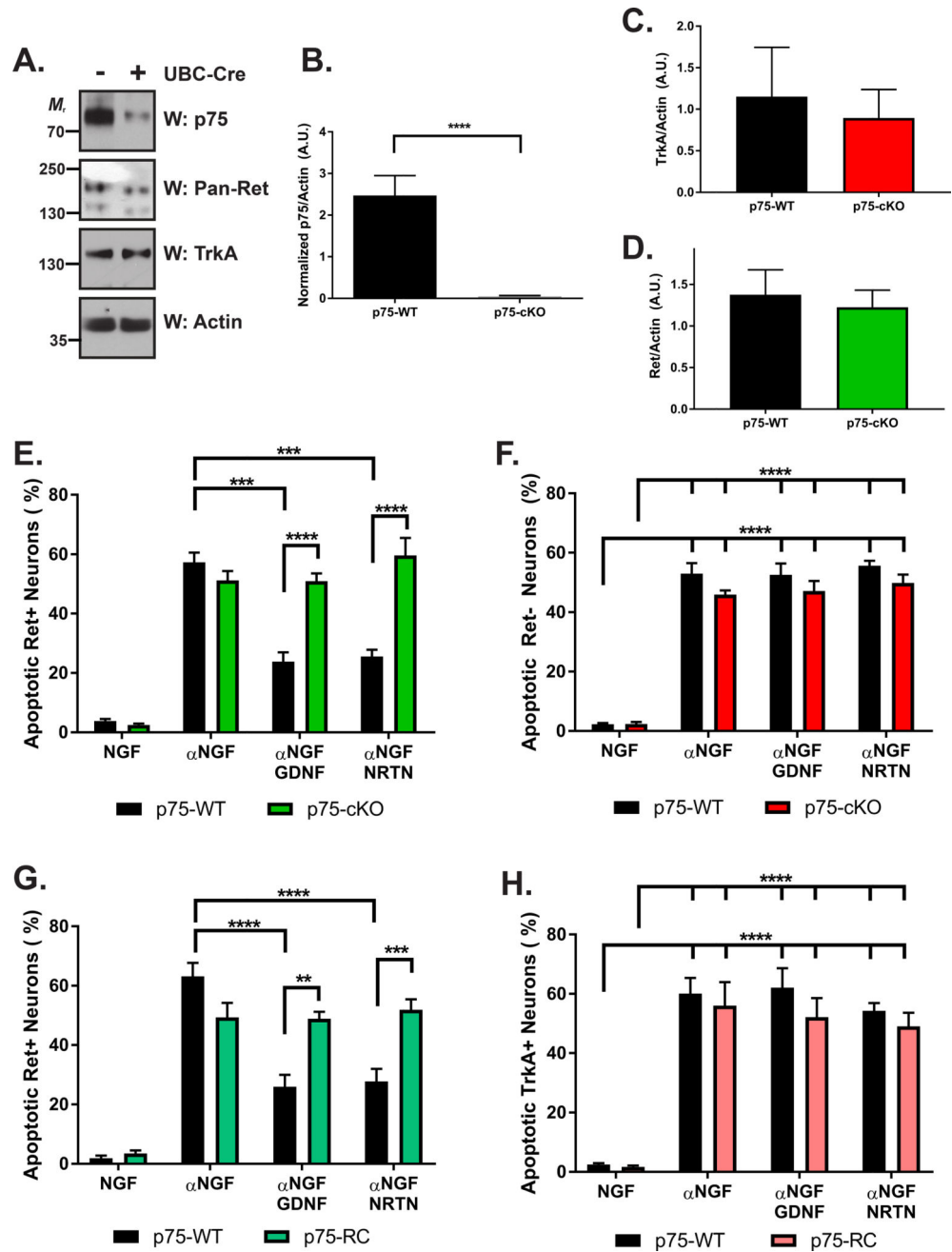


Figure 4. p75 mediated Ret-mediated survival of nonpeptidergic nociceptors *in vitro*
 (A) Primary DRG neuron cultures were generated from P0 $p75^{F/F}$; UBC-Cre/ER^{T2} (p75-cKO) or $p75^{F/F}$ control mice (p75-WT) and maintained in the presence of 50 ng/ml NGF and 4-OH-TMX. 7 DIV neurons were lysed and immunoblotted for p75 (top panel) to confirm efficacy of deletion, pan-Ret, TrkA, and actin (as a loading control). (B) Quantification of normalized p75 levels indicated a highly significant reduction of p75 levels in p75-cKO neurons (n=10) compared to p75-WT neurons (n=8; **** p < 0.0001). (C) No difference was observed in total levels of TrkA (n=5 p75-WT and n=7 p75-cKO; p = 0.6982) or (D) Ret protein levels (p=0.6737). (E, F) 7 DIV neurons were treated as described in A

and subsequently treated with NGF, an anti-NGF blocking antibody (α NGF), α NGF with GDNF, or α NGF with NRTN (as indicated on the horizontal axis). 48 hours post-treatment, neurons were fixed and stained with Ret, TrkA, and DAPI (to determine nuclear pyknosis, indicating apoptosis) The number of apoptotic Ret⁺/TrkA(E) or Ret/TrkA⁺ neurons (F) was quantified for each treatment group (n=5 for p75-WT and n=3 for p75-cKO for each condition), with all statistical differences noted. GDNF and NRTN treatment was sufficient to rescue Ret⁺, but not TrkA⁺ neurons from apoptosis in p75-WT, but not p75-cKO neurons. (G, H) DRG neuron cultures were generated from P0 p75^{F/F} (p75-WT) and p75^{F/F}; *Ret-Cre/ERT2* (p75-RC) mice and treated, immunostained, and quantified as in E and F (treatments indicated on the horizontal axis). The number of apoptotic Ret⁺/TrkA (G) or Ret/TrkA⁺ neurons (H) was quantified for each treatment group (n=6 for p75-WT and n=5 for p75-RC for each condition). Similar results were noted as in E and F, with GFL treatment able to selectively rescue Ret⁺ neurons from apoptosis in p75-WT, but not p75-RC neurons.

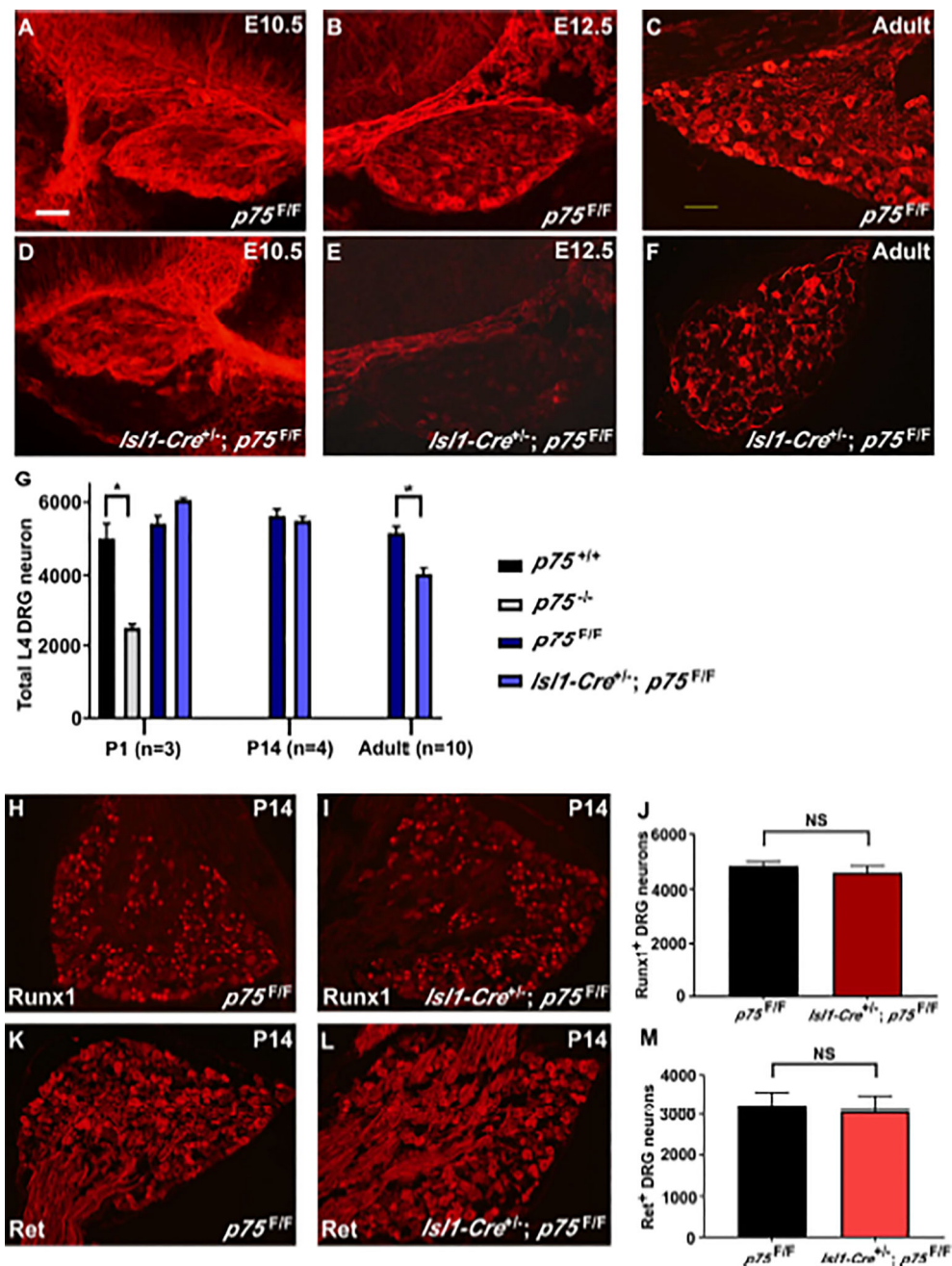


Figure 5. $Is11-Cre^{+/-}; p75^{F/F}$ mice lose 20% of DRG neurons
 $p75$ immunofluorescence staining of DRG sections from E10.5, E12.5 and adult $p75^{F/F}$ and $Is11-Cre^{+/-}; p75^{F/F}$ mice is shown in A-F. As shown in panel D, $p75$ immunoreactivity in DRG sensory neurons is not reduced at E10.5 in $Is11-Cre^{+/-}; p75^{F/F}$ mice. However, the number of $p75$ -immunoreactive neurons is markedly reduced in E12.5 (panel E) and adult animals (panel F). The immunoreactivity observed in adult DRG is likely coming predominantly from satellite cells, which normally express $p75$. Scale bar, 100 μ m. (G) Total neuron numbers in L4 DRGs from $p75^{F/F}$ mice were compared to $Is11-Cre^{+/-}; p75^{F/F}$ mice at different postnatal ages. Adult $Is11-Cre^{+/-}; p75^{F/F}$ mice lose approximately 20% of DRG

neurons. In contrast, $p75^{-/-}$ mice lose 50% of DRG neurons at P1 (* $p < 0.05$). At p14, Runx1 expression in $p75^{F/F}$ mice (H) is similar to *Isl1-Cre*^{+/-}; $p75^{F/F}$ mice (I). The Ret expression level is also not changed between $p75^{F/F}$ (K) and *Isl1-Cre*^{+/-}; $p75^{F/F}$ (L) mice. There is no change in the number of Runx1⁺ neurons (J) or Ret⁺ neurons (M) at p14 in *Isl1-cre*^{+/-}; $p75^{F/F}$ mice compared to $p75^{F/F}$ mice (n=3).

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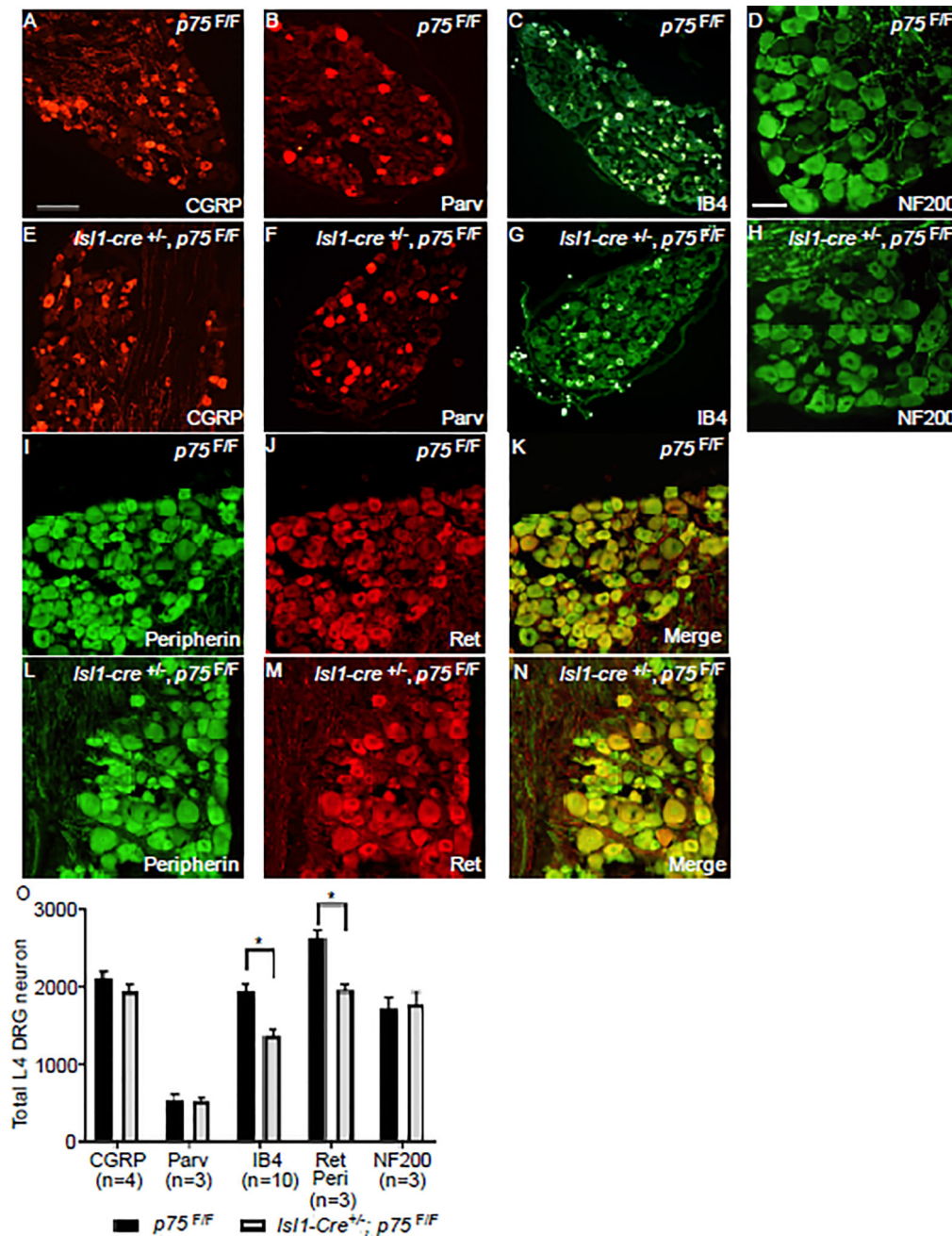


Figure 6. IB4⁺ nonpeptidergic nociceptors are selectively lost in *Isl1-Cre^{+/-}; p75^{F/F}* mice
 Immunofluorescence staining of adult L4 DRG with anti-CGRP (panel A and E), anti-Parvalbumin (panel B and F), anti-NF200 (panel D and H) antibody, IB4 (panel C and G) or a combination of anti-peripherin (I, L) and anti-Ret (J, M) antibodies from *p75^{F/F}* (panel A–D, I–K) and *Isl1-Cre^{+/-}; p75^{F/F}* (panel E–H, L–N) mice. Scale bar for A–C, E–G, 200 μ m (located in panel A), Scale bar for D, H–N 100 μ m (located in panel D). (O) The quantification of CGRP⁺, Parvalbumin⁺, IB4⁺, Peripherin⁺/Ret⁺ and NF200⁺ neurons in adult L4 DRGs from *p75^{F/F}* and *Isl1-Cre^{+/-}; p75^{F/F}* mice. Only the IB4⁺ and Peripherin⁺/Ret⁺ neurons were significantly reduced in the *Isl1-Cre^{+/-}; p75^{F/F}* mice. Values

are expressed as mean \pm SEM, * $p < 0.05$. N indicates the number of animals being counted (each animal provides two DRGs).

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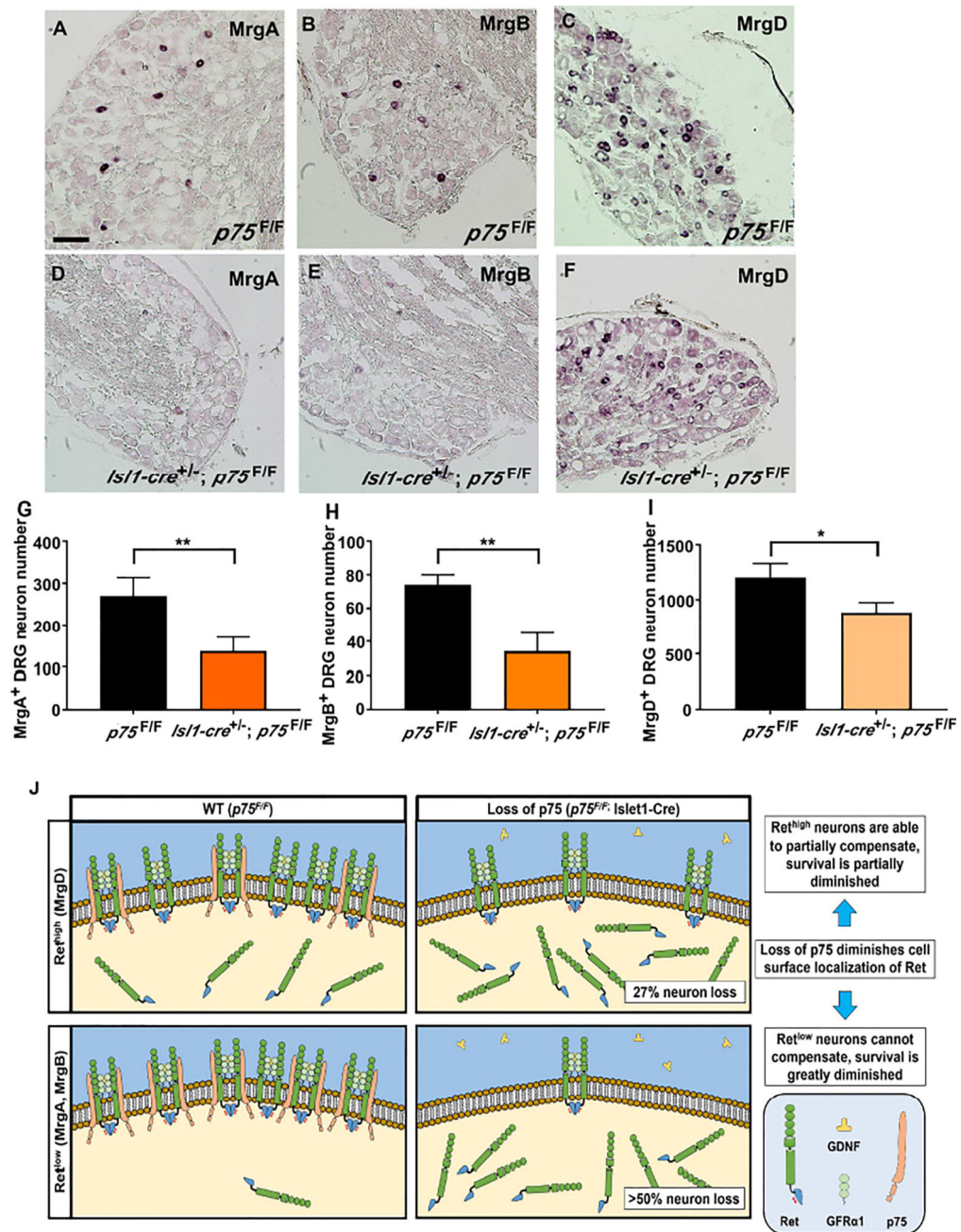


Figure 7. Nonpeptidergic neuron deficits in *Islet1-Cre^{+/-}; p75^{F/F}* mice correlates with level of p75-Ret co-expression

In situ hybridization of adult (6 months old) L4 DRG with MrgA (panel A and D), MrgB (panel B and E) and MrgD (panel C and F) *in situ* probes from *p75^{F/F}* mice (panel A–C) and *Islet1-Cre^{+/-}; p75^{F/F}* (panel D–F). Scale bar, 100 μ m. Quantification of MrgA⁺ (panel G), MrgB⁺ (panel H) and MrgD⁺ (panel I) neurons showed that in *Islet1-Cre^{+/-}; p75^{F/F}* mice, over 50% of MrgA⁺ and MrgB⁺ neurons were lost, while approximately 20% of MrgD⁺ neurons were lost. Importantly, MrgA⁺ and MrgB⁺ neurons were shown to express low level of Ret while MrgD⁺ neurons express high level of Ret. The value is expressed as mean \pm SEM (n=3 for each data point, * p<0.05, ** p<0.01). (J) The expression of p75 increases the level

of Ret on the cell surface in $p75^{F/F}$ mice and potentiates GFL-induced Ret phosphorylation and its downstream survival effects. When p75 expression is lost in $Isl1-Cre^{+/-}; p75^{F/F}$ mice, the level of Ret on the cell surface is reduced. For neurons that express high levels of Ret (MrgD⁺), the high level of Ret expression may compensate for the loss of p75 and results in more modest deficits. In neurons that express low levels of Ret (MrgA⁺ and MrgB⁺), however, the loss of Ret on the cell surface cannot be compensated for, leading to substantial cell loss.

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