UNC-16/JIP3 inhibits the function of the regeneration promoting isoform of DLK-1

Running title: UNC-16 hinders axon regeneration

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Summary statement

Our work demonstrates that the MAPKinase cascade scaffolding protein, UNC-16/JIP3, is a negative regulator of the regeneration promoting MAPKKK DLK isoform.

Abstract

The extent of neuronal regeneration after injury depends on the genetic control of the intrinsic growth potential of neurons and their extracellular environment. We show that UNC-16, a *C. elegans* JIP3 (JNK Interacting Protein 3) homologue, plays an inhibitory role after axotomy in the early stages of neuronal regeneration. UNC-16 inhibits the regeneration promoting long isoform of DLK-1, an essential MAPKKK for neuronal regeneration, while acting independently of the inhibitory DLK-1 short isoform. UNC-16 sequesters DLK-1L along the axon in a concentration dependent manner and prevents its accumulation at the cut site immediately after axotomy thereby preventing the early steps in regrowth. The inhibitory control of UNC-16 may provide a general means to control DLK in regenerating neurons.

Introduction

The extent of neuronal regeneration in adult neurons, post-injury, depends on the balance between extrinsic and intrinsic factors that inhibit and promote neuronal regeneration. MAP Kinases such as Erk (Hanz, Perlson et al. 2003), JNK (Lindwall and Kanje 2005) and Dual Leucine zipper Kinase (DLK) (Hammarlund, Nix et al. 2009, Kim and Jin 2015) have been reported to regulate the intrinsic growth potential of neuronal regeneration. JIP3, a member of the JNK interacting protein (JIP) group of scaffolding proteins, regulates JNK signalling by scaffolding specific kinases viz., JNK (MAPK), MKK7 (MAPKK) and the most upstream kinase, MLK3 (MAPKKK). The MAPK cascade is known to modulate neuronal growth (Ito, Yoshioka et al. 1999, Kelkar, Gupta et al. 2000, Byrd, Kawasaki et al. 2001).

JIPs are also known to regulate the activity of DLK. DLK is an essential MAPKKK required for neuronal regeneration in multiple species (Nakata, Abrams et al. 2005, Hammarlund, Nix et al. 2009, Rishal and Fainzilber 2014). JIP3 is in a complex with DLK and DLK homodimerization is important for its activation (Nihalani, Merritt et al. 2000, Holland, Collura et al. 2016). JIP1, maintains DLK in an inactive state by preventing its dimerization (Nihalani, Meyer et al. 2001). Thus, the interaction of scaffolding molecules and kinases they scaffold can regulate activity of the kinases involved in neuronal regeneration.

We investigated the role of *C. elegans* JIP3/UNC-16, a well-established member of the JIP family, in regeneration. We show that UNC-16 inhibits regeneration of *C. elegans* touch receptor neurons after axotomy. The faster regeneration in *unc-16* mutant animals is a result of early initiation of neuronal regrowth and is dependent on DLK-1. The *dlk-1* locus encodes two isoforms in *C. elegans*: regeneration promoting DLK-1L and DLK-1S that inhibits DLK-1L (Yan and Jin 2012). Our data suggest that UNC-16, in a concentration dependent manner, inhibits regeneration by acting on DLK-1L and is independent of the inhibition through DLK-1S. Our data suggest that UNC-16's inhibitory activity likely occurs by sequestering DLK-1L into puncta and preventing DLK-1L accumulation at the cut site after injury. The dual inhibitory control by both UNC-16 and DLK-1S can calibrate the intrinsic growth promoting function of DLK-1L *in vivo*.

Results and Discussion

Loss of UNC-16 leads to an increase in neuronal regeneration

To study UNC-16's role in regeneration, we compared the post-axotomy regrowth in the Posterior Lateral Mechanosensory (PLM) neuron of *unc-16* and wild type animals. We observed regeneration in four different alleles of *unc-16,* namely, *ju146, e109, tb109* and *n730* with mutations at different locations (Fig. 1A) (Byrd, Kawasaki et al. 2001, Edwards, Yu et al. 2013). *tb109*, isolated in an unrelated genetic screen in our lab, has a stop codon at amino acid 423 and is a partial loss-of-function allele. All *unc-16* alleles examined showed greater number of animals whose neurons regenerate as compared to wild type. Our experiments were carried out 9 hours post-axotomy as they gave us clear differences between genotypes and data is represented as percentage regeneration that refers to the number of animals that show neuronal regeneration. The inhibitory effect of *unc-16* on neuronal regeneration is not allele specific varying from 46% to 88% in different loss of function (lf) alleles. A similar inhibitory role for *unc-16* in motor neuron regeneration was identified in a *C. elegans* modifier RNAi screen (Nix, Hammarlund et al. 2014).

We found that expression of the transgene encoded UNC-16::GFP (Byrd, Kawasaki et al. 2001) in *unc-16(tb109)* animals reduces regeneration from 81% to 27% (Fig. 1B). Further, expression of UNC-16::GFP in wild type animals resulted in 19% of the severed processes regenerating compared to 36% in wild type. This suggests that levels of UNC-16 may control regeneration following injury.

Loss of UNC-16 results in faster initiation of regeneration

To examine the altered regeneration in *unc-16,* we monitored the neuronal processes of *unc-16(tb109)* [*unc-16(lf)*], at different time points after axotomy. Measurements started 6 hours post-axotomy since that is the earliest time point where we first observe regeneration in wild type animals. At 6 hours 57% of *unc-16(lf)* animals initiated neuronal regrowth compared to only 16% in wild type (Fig. 1C, D). This three-fold increase suggests that loss of *unc-16* promotes initiation of regrowth. This increased incidence of regrowth persists even 24 hours post-axotomy (Fig. 1C).

6-9 hours post-axotomy, neuronal processes in the *unc-16(lf)* animals grew at the rate of 2.1 µm/hour, significantly higher than the wild type neuronal growth rate of 0.6 µm/hour (Fig. 1E). This led to a six-fold increase in the regenerated process

length in *unc-16* compared to wild type 9 hours post-axotomy (Fig. 1F). The difference in growth rate between wild type (1.7 µm/hour) and *unc-16(lf)* (6.4 µm/hour) animals was greatest in the 9-12-hour time window. This faster growth rate led to a five-fold increase in neuronal length 12 hours post-axotomy in *unc-16* compared to wild type. But at 18 hours post-axotomy the neuronal regrowth in *unc-16(lf)* reduced and became comparable to wild type (Fig. 1E). 24 hours post-axotomy, more than 50% of the processes of *unc-16(lf)* reconnect to their distal ends, thus an accurate assessment of true growth rates is not readily measureable. Taken together our data suggest that the loss of *unc-16* results in a quicker initiation of regrowth and a faster rate of process regrowth up to 12 hours post-axotomy.

Our findings show that UNC-16 acts as an inhibitory factor in early steps of axon regeneration and prevents the initiation and early axon extension after injury.

Increased regeneration in *unc-16* depends on DLK-1

UNC-16 is a scaffolding protein that plays multiple roles in intracellular transport and MAPK signalling. It interacts with the Kinesin-1 motor (Sun, Zhu et al. 2011), the Dynein motor (Arimoto, Koushika et al. 2011), a RUN domain protein UNC-14 (Sakamoto, Byrd et al. 2005), a leucine-rich repeat kinase 2 (LRRK2) (Hsu, Chan et al. 2010) and MAP kinases such as JNK-1 (Kelkar, Gupta et al. 2000) and DLK (Ghosh, Wang et al. 2011). We thus examined if UNC-16 dependent regeneration depended on the above proteins.

We assessed neuronal regeneration in lf mutants of the Kinesin heavy chain-1 (*unc-116*), the JNK MAP kinase (*jnk-1*), RUN domain protein (*unc-14*), leucine-rich repeat kinase 2 (*lrk-1*), Dynein heavy chain (*dhc-1*) and the Dual-leucine zipper kinase (*dlk-1*). The single mutants either regenerate similar to wild type as seen in the *unc-116* mutant and the *jnk-1* mutant or regenerate significantly lower as seen in mutants of *unc-14*, *lrk-1*, *dhc-1* and *dlk-1* (Fig. 2A, B). In addition, double mutants between *unc-16(lf)* and the above mentioned genes did not influence UNC-16 dependent regeneration with the exception of *dlk-1* (Fig. 2B). Since *dlk-1(ju476)*; *unc-16(tb109)* animals do not regenerate, we infer that the inhibitory role of UNC-16 in neuronal regeneration is dependent on DLK-1.

UNC-16 inhibits the regeneration promoting function of DLK-1L

DLK is an essential MAPKKK that functions in the initiation of regrowth of neuronal processes after injury and in growth cone formation in many model systems (Hammarlund, Nix et al. 2009, Yan, Wu et al. 2009, Xiong, Wang et al. 2010, Wang and Jin 2011, Shin, Cho et al. 2012). *dlk-1* in *C. elegans* encodes two isoforms, DLK-1L (long) and DLK-1S (short), that have antagonistic functions. DLK-1S forms a heterodimer with DLK-1L and inactivates the regeneration promoting role of DLK-1L (Yan and Jin 2012). JIP3, the mammalian orthologue of UNC-16, is known to form a complex with DLK (Kelkar, Gupta et al. 2000, Holland, Collura et al. 2016). Thus we wished to determine whether UNC-16 influences neuronal regeneration through a specific isoform of DLK-1.

We assessed the effect of loss of UNC-16 on neuronal regeneration in animals expressing a single copy of DLK-1S or DLK-1L. Consistent with previous studies, expression of single copy of DLK-1S or DLK-1L in wild type respectively suppresses and enhances neuronal regeneration (Fig. 3A) (Yan and Jin 2012). Similarly, results consistent with past studies were observed when either *dlk-1(ju476)* (does not express DLK-1L but expresses endogenous levels of intact DLK-1S) referred to as *dlk-1(onlyS)* or *dlk-1(tm4024)* (a DLK-1 null) referred to as *dlk-1(null)*, expressed either a single copy of DLK-1S or DLK-1L (Yan and Jin 2012).

To determine if DLK-1S and UNC-16 act independently to inhibit DLK-1L mediated regeneration we assessed regeneration in the following genotypes: Single copy DLK-1L with *dlk-1(onlyS);unc-16(lf)* and *dlk-1(null); unc-16(lf).* Single copy DLK-1L is introduced so that some regeneration occurs allowing us to assess the inhibitory effects of DLK-1S and UNC-16. We see that *unc-16(lf)* mutants lacking DLK-1S show significantly higher regeneration compared to animals that contain DLK-1S suggesting that both DLK-1S and UNC-16 can both inhibit DLK-1L (Fig. 3A).

Loss of *unc-16* is unable to promote regeneration in animals expressing DLK-1S in wild type, *dlk-1(onlyS)* or *dlk-1(null)* backgrounds (Fig. 3A). However, animals expressing DLK-1L in *dlk-1(onlyS);unc-16(lf)* had enhanced regeneration compared with animals expressing DLK-1L in *dlk-1(onlyS)* alone (Fig. 3A). Additionally, animals expressing DLK-1L in *dlk-1(null);unc-16(lf)* further enhanced regeneration compared with animals expressing DLK-1L in *dlk-1(null)* greatest at 6 hours post-axotomy consistent with UNC-16's role early in regeneration (Fig. 3B). Moreover, the loss of UNC-16 leads to a two-fold increase in regeneration in *dlk-1(onlyS)*

animals and a further 1.5-fold increase in regeneration in *dlk-1(null)* (Fig. 3B). However, in *dlk-1(null)*, 9 hours post-axotomy, these differences were not very significant (Fig. 3A) and this may be due to the larger amounts of DLK-1L available in the single copy DLK-1L line, soon after axotomy, resulting in faster initiation of regeneration. Our data shows that UNC-16 inhibits the growth promoting activity of DLK-1L.

The levels of UNC-16 modulate the function of DLK-1L

JIPs are known to either activate or inhibit signalling depending on their cellular levels (Whitmarsh 2006). Hence we evaluated the role of elevated UNC-16 on regeneration. Elevated levels of UNC-16 decreased the regeneration in animals with single copy DLK-1L in both wild type and *dlk-1(null)* (Fig. 4A). These data show that levels of UNC-16, rather than the mere loss of UNC-16 function, regulates regeneration through DLK-1L. In addition, regeneration observed in overexpression of UNC-16 is not altered by the loss of DLK-1S. Our genetic data suggests that UNC-16 may act additively with DLK-1S to inhibit regeneration.

UNC-16 changes the localization of DLK-1L

Vertebrate DLK, *C. elegans* GFP-DLK-1L, GFP-DLK-1S and endogenous UNC-16 are all shown to localize in punctae along the neuron (Yan and Jin 2012, Edwards, Yu et al. 2013, Holland, Collura et al. 2016). The punctate localization of *C. elegans* GFP-DLK-1S depends on its association with DLK-1L (Yan and Jin 2012). Therefore, we examined the localization of DLK-1L in both *unc-16(lf)* and in neurons with elevated levels of UNC-16. The punctate localization of GFP-DLK-1L is lost in all *unc-16(lf)* animals examined (Fig. 4B, C). Further, upon UNC-16 over-expression, GFP-DLK-1L showed a significant increase in the intensity of puncta in wild type and *dlk-1(null)* animals although the density of puncta remains at ~2.5±0.4/10µm similar to wild type (Fig. 4B, C). Our observations show that the punctate DLK-1L localization is dependent on the presence and levels of UNC-16 and the UNC-16 dependent DLK-1L localisation is independent of the presence of DLK-1S.

Upon axotomy DLK-1L is known to increase locally at the injury site while the DLK-1S does not (Yan and Jin 2012). We thus also investigated whether the post-axotomy localization of DLK-1L is dependent on UNC-16. Similar to the earlier report we observe that 4 minutes after axotomy, the levels of GFP-DLK-1L at the

anterior end of the cut site increased (Fig. 4D, E). However, in *unc-16(lf)* there was a greater accumulation of GFP-DLK-1L at the proximal cut site (Fig. 4D, E). Further, overexpression of UNC-16 led to a great reduction of GFP-DLK-1L accumulation at the proximal cut site with the GFP signal comparable to the GFP alone control (Fig. 4D, E). Thus the presence and levels of UNC-16 controls the post-axotomy levels of DLK-1L at the cut site.

Taken together, our data suggest that UNC-16 may contribute to making DLK-1L unavailable at the cut site, perhaps by sequestering it in punctae along the neuronal process. We think UNC-16 acts independently and in addition to DLK-1S because [i] both *unc-16(lf)* and lack of DLK-1S enhance each other (Fig. 3A, B), [ii] the overexpression of DLK-1S is not suppressed by the *unc-16(lf)* mutation (Fig. 3A) and [iii] the overexpression of UNC-16 is not suppressed by DLK-1S deletion (Fig. 4A).

The early effects we see with UNC-16/JIP3 suggests an inhibitory role to enable tight temporal and spatial control of DLK-1 function. Cytoskeletal damage is thought to trigger DLK activity that subsequently initiates regeneration post injury (Valakh, Frey et al. 2015). Consistent with this hypothesis we observe that lack of UNC-16 leading to elevated DLK at the cut site controls early steps in regeneration which include axonal growth initiation and extension (Fig 1. C, F); both microtubule dependent processes known to be controlled by DLK-1 (Hammarlund, Nix et al. 2009, Yan, Wu et al. 2009). The sequestration of DLK depends on palmitoylation (Holland, Collura et al. 2016) and thus DLK bound UNC-16 is likely to be palmitoylated. JIP3 has also been shown to control axon elongation in cultured neurons although whether this depends on DLK is unclear (Sato, Ishikawa et al. 2015). Independently, JIP3-DLK complex is retrogradely transported and may regulate regeneration (Cavalli, Kujala et al. 2005, Arimoto, Koushika et al. 2011, Holland, Collura et al. 2016), but UNC-16's prominent role in touch neurons appears to be in the early post-axotomy steps.

Since JIP3 is also known to form a complex with DLK (Kelkar, Gupta et al. 2000, Holland, Collura et al. 2016) we propose that the effects of UNC-16 on DLK-1L might be direct. UNC-16 and its vertebrate homologue JIP3 may act as a general inhibitor of DLK mediated signalling in multiple species by altering its localisation. Other members of the JIP family may also share a similar role since JIP1 is also known to both directly bind and inhibit DLK activity (Nihalani, Meyer et al. 2001).

We think that after injury DLK-1 plays a dual role: an immediate effector through its role in microtubule dynamics and a long-term effector through its role in retrograde transport dependent signalling.

Materials and Methods

Strains

C. elegans strains were grown on NGM agar plates seeded with *E. coli* OP50 bacteria (Table S1). $_{p}$jkk-1::T7::UNC-16 plasmid was made by inserting T7-UNC-16 DNA excised from $_{p}$CMVT7-UNC-16 plasmid (Byrd, Kawasaki et al. 2001) into $_{p}$NHjkk-1p (Kawasaki, Hisamoto et al. 1999).

Laser Axotomy

Nanosecond laser (Spitlight 600, Innolas) pulses (λ = 355 nm) of energy 0.8 μJ in single shot mode was used to generate neuronal cuts (Rao, Kulkarni et al. 2008). One PLM neuron was cut approximately 30-50 μm away from the cell body. All experiments were done using L4 animals grown at 16°C.

Imaging and analysis

Images were acquired using a 100X 1.4 NA objective and were processed using ImageJ. Regeneration was represented as percent neuronal regeneration i.e. number of neuronal processes showing regrowth out of total number of axotomised neurons (Hammarlund, Nix et al. 2009). The length of the regrowing process was measured from the cut site (Wu, Ghosh-Roy et al. 2007). Neurons showing >4 μm regrowth were considered as regenerating.

Statistical Analysis

The data has been represented as mean ± s.e.m. The data when represented as % regeneration is an average of at least two separate trials. One-way ANOVA was used as a test of significance.

Localization of GFP tagged DLK isoforms.

PLM neurons of 1 day adult animals were imaged using a Perkin Elmer spinning-disc confocal microscope (100X, 1.4 NA) with a EMCCD C9100-13 camera.

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Competing Interests

The authors declare no competing or financial interests.

Author contributions

S.S.K., S.S. and S.P.K. designed experiments and wrote the manuscript. S.S.K., S.S. performed experiments, K.M., N.H. provided reagents.

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Legends

Figure 1. Loss of UNC-16/JIP3 promotes neuronal regeneration. ***A***, Percentage neurons regenerating post-axotomy in *unc-16* alleles. ***B***, Percentage neurons regenerating after expression of UNC-16::GFP in wild type(*zdIs5*) and *unc-16(tb109)*. ***C***, Percentage neurons regenerating at specified time-points post-axotomy in wild type(*zdIs5*) and *unc-16(tb109)*. ***D***, Representative images of the PLM process at specified time-points post-axotomy. Arrow indicates position of axotomy. Scale bar 10 µm. ***E***, Growth rate of the neuronal process in each three-hour interval after axotomy. ***F***, Length of the regenerated neuronal process at different time points after axotomy. p-value *<0.05, **<0.005, ***<0.001. Number of animals inside bars.

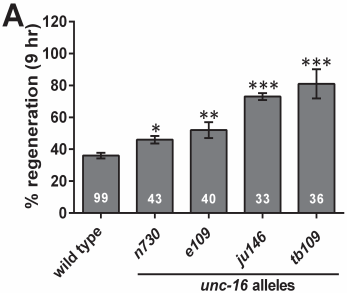
Figure 2. *unc-16*'s effect on neuronal regeneration is *dlk-1* dependent. ***A***, Neuronal regeneration in *unc-116(e2310)* and *jnk-1(gk7)* and their doubles with *unc-16(tb109)*. ***B***, Neuronal regeneration in *unc-14(e57)*, *dhc-1(js319)* and *dlk-1(ju476)* and their doubles with *unc-16(tb109)*. p-value *<0.05, ***<0.001. Number of animals inside bars.

Figure 3. UNC-16 inhibits DLK-1L. ***A***, Genetic analysis of effect of *unc-16* in single copy DLK-1L and DLK-1S expressing strains (*MosSci*) in wild type and *dlk-1* background 9 hours post-axotomy. ns Not significant. ***B***, Genetic analysis of effect of *unc-16(tb109)* in single copy DLK-1L in wild type and *dlk-1* background 6 hours post-axotomy. p-value *<0.05, **<0.005, ***<0.001. Number of animals inside bars.

Figure 4. UNC-16 regulates DLK-1L in a dose-dependent fashion. ***A***, Regeneration in wild type, T7::UNC-16, *unc-16(tb109)* and *dlk-1(tm4024)* all expressing single copy of DLK-1L (*MosSciL)* 6 hours after axotomy. ***B***, Normalised intensity quantification of effect of *unc-16(tb109)* and T7::UNC-16 on GFP-DLK-1L localization. Intensity of $_{p}$*mec-4::mCherry* was used as a negative control. ***C***, Representative picture of GFP::DLK-1L localisation. White arrows show examples of puncta quantified. Scale bar 10 µm. ***D***, Normalised intensity quantification of GFP-DLK-1L in the first 3 µm near the proximal cut end of an axotomised PLM neuron. ***E***, Representative picture of axotomised PLM neurons expressing GFP-DLK-1L, 4

minutes post-axotomy. Arrow points to the proximal cut end with respect to cell body. Scale bar 5 µm. p-value *<0.05, ***<0.001. Number of animals inside bars.

Fig. 1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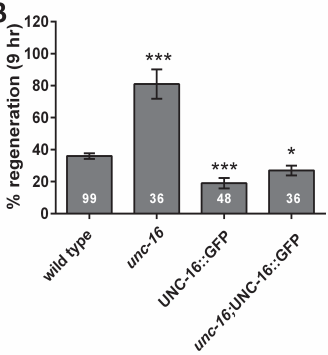
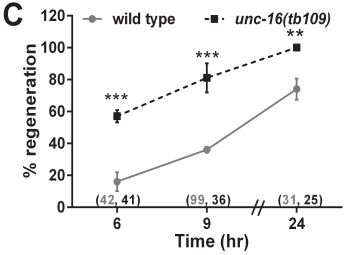
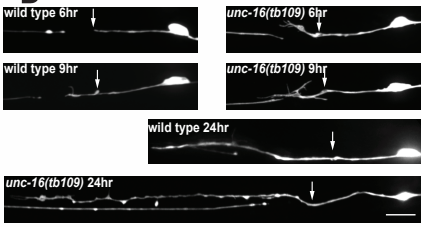
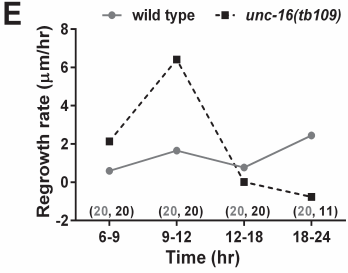
***
***
wild type
n730
e109
ju146
tb109
unc-16 alleses


B
% regeneration (9 hr)
120
100
80
60
40
20
0
99
36
48
36
***
***
*
wild type
unc-16
UNC-16::GFP
unc-16;UNC-16::GFP


C
wild type
unc-16(tb109)
% regeneration
120
100
80
60
40
20
0
***
***
**
(42, 41)
(99, 36)
(31, 25)
6
9
24
Time (hr)


D
wild type 6hr
unc-16(tb109) 6hr
wild type 9hr
unc-16(tb109) 9hr
wild type 24hr
unc-16(tb109) 24hr


E
wild type
unc-16(tb109)
Regrowth rate (μm/hr)
8
6
4
2
0
-2
(20, 20)
(20, 20)
(20, 20)
(20, 11)
6-9
9-12
12-18
18-24
Time (hr)

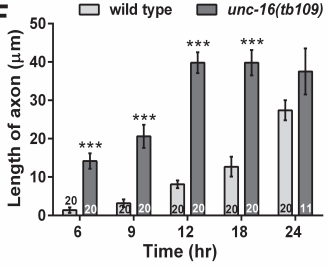
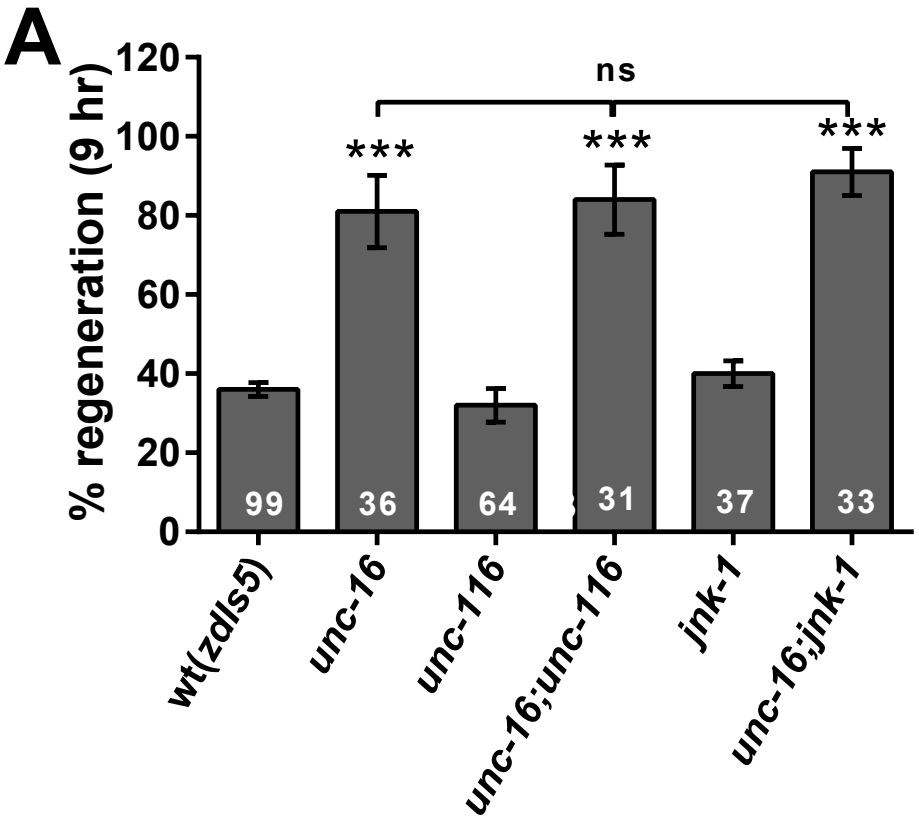

F
wild type
unc-16(tb109)
Length of axon (μm)
50
40
30
20
10
0
***
***
***
***
20
20
20
20
20
20
20
20
20
11
6
9
12
18
24
Time (hr)

Fig. 2


A
% regeneration (9 hr)
120
100
80
60
40
20
0
ns
***
***
***
99
36
64
31
37
33
wt(zdls5)
unc-16
unc-116
unc-16;unc-116
jnk-1
unc-16;jnk-1

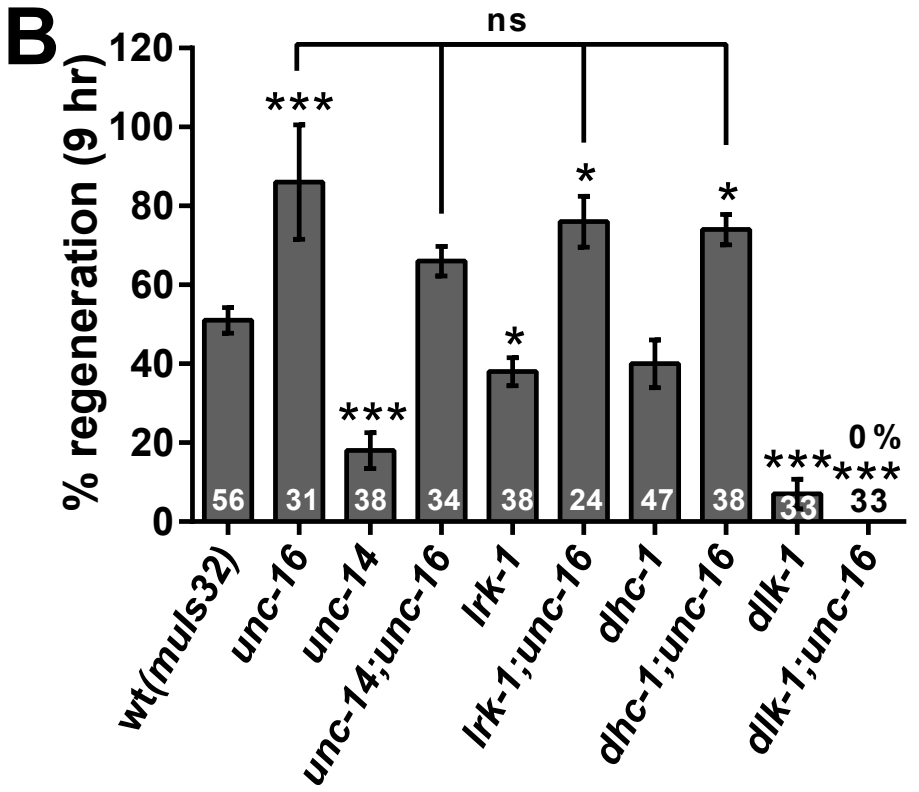

B
% regeneration (9 hr)
120
100
80
60
40
20
0
ns
***
***
*
*
*
***
0 %
***
56
31
38
34
38
24
47
38
33
33
wt(muls32)
unc-16
unc-14
unc-14;unc-16
lrk-1
lrk-1;unc-16
dhc-1
dhc-1;unc-16
dlk-1
dlk-1;unc-16

Fig. 3

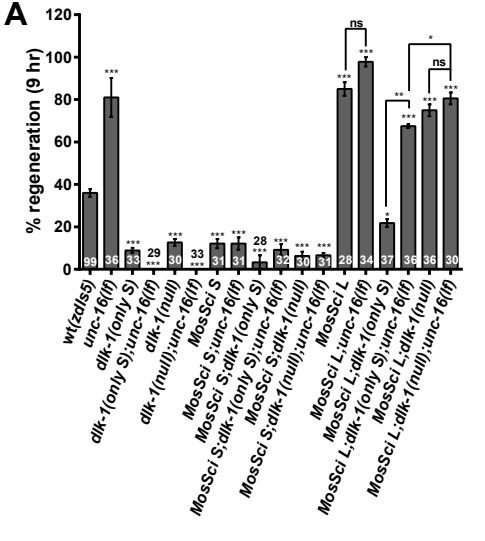
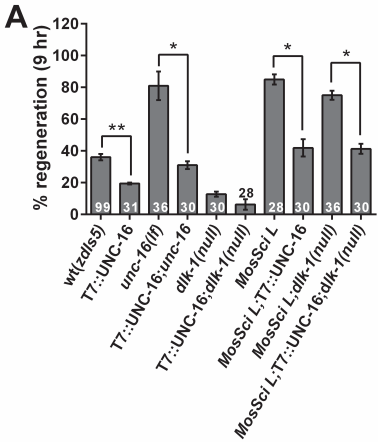
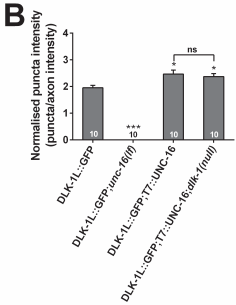
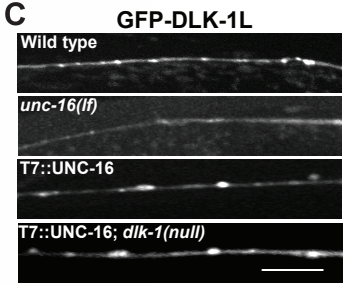
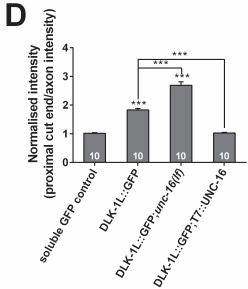

A
% regeneration (9 hr)
120
100
80
60
40
20
0
39
36
33
29
30
33
31
31
28
32
30
31
28
34
37
36
36
30
***
***
***

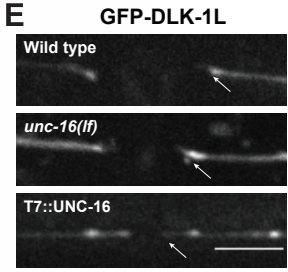
Fig. 4


A
% regeneration (9 hr)
120
100
80
60
40
20
0
**
*
*
*
99
31
36
30
30
28
28
30
36
30
wt(zdls5)
T7::UNC-16
unc-16(lf)
T7::UNC-16;unc-16
dlk-1(null)
T7::UNC-16;dlk-1(null)
MosSci L
MosSci L;T7::UNC-16
MosSci L;dlk-1(null)
MosSci L;T7::UNC-16;dlk-1(null)


B
Normalised puncta intensity
(puncta/axon intensity)
4
3
2
1
0
ns
*
*
***
10
10
10
10
DLK-1L::GFP
DLK-1L::GFP;unc-16(lf)
DLK-1L::GFP;T7::UNC-16
DLK-1L::GFP;T7::UNC-16;dlk-1(null)


C
GFP-DLK-1L
Wild type
unc-16(lf)
T7::UNC-16
T7::UNC-16; dlk-1(null)


D
Normalised intensity
(proximal cut end/axon intensity)
4
3
2
1
0
***
***
***
***
10
10
10
10
soluble GFP control
DLK-1L::GFP
DLK-1L::GFP;unc-16(lf)
DLK-1L::GFP;T7::UNC-16


E
GFP-DLK-1L
Wild type
unc-16(lf)
T7::UNC-16