A Receptor Tyrosine Kinase Network Regulates Neuromuscular Function in Response to Oxidative Stress in *Caenorhabditis elegans*

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ABSTRACT The transcription factor Nrf2 plays a critical role in the organism-wide regulation of the antioxidant stress response. The Nrf2 homolog *SKN-1* functions in the intestinal cells nonautonomously to negatively regulate neuromuscular junction (NMJ) function in *Caenorhabditis elegans*. To identify additional molecules that mediate *SKN-1* signaling to the NMJ, we performed a candidate screen for suppressors of aldicarb resistance caused by acute treatment with the *SKN-1* activator arsenite. We identified two receptor tyrosine kinases, *EGL-15* (fibroblast growth factor receptor, FGFR) and *DAF-2* (insulin-like peptide receptor), that are required for NMJ regulation in response to stress. Through double-mutant analysis, we found that *EGL-15* functions downstream of, or parallel to, *SKN-1* and *SPHK-1* (sphingosine kinase), and that the *EGL-15* ligand *EGL-17* FGF and canonical *EGL-15* effectors are required for oxidative stress-mediated regulation of NMJ function. *DAF-2* also functions downstream of or parallel to *SKN-1* to regulate NMJ function. Through tissue-specific rescue experiments, we found that FGFR signaling functions primarily in the hypodermis, whereas insulin-like peptide receptor signaling is required in multiple tissues. Our results support the idea that the regulation of NMJ function by *SKN-1* occurs via a complex organism-wide signaling network involving receptor tyrosine kinase signaling in multiple tissues.

KEYWORDS *SKN-1*; neuromuscular junction; oxidative stress; sphingosine kinase

THE transcription factor Nrf2 plays a crucial role in the maintenance of cellular redox homeostasis by directing the expression of a cascade of antioxidant, antiinflammatory, and detoxification enzymes in response to oxidative stress (Blackwell *et al.* 2015). In multicellular organisms, Nrf2 activation can confer organism-wide protection from oxidative stress by regulating stress responses in distal tissues through intertissue signaling. In *Caenorhabditis elegans*, activation of the Nrf2 homolog *SKN-1* in the intestine regulates longevity, survival against xenobiotics and pathogens, and neurotransmission (Kim and Jin 2015). Although the cell-autonomous effects of Nrf2/*SKN-1* in promoting cellular survival are well understood, less is known about how Nrf2/*SKN-1* activation leads to changes in oxidative stress responses in distal tissues.

Oxidative stress activates Nrf2/*SKN-1* by relieving it from proteolytic degradation in the cytoplasm and allowing entry into the nucleus, where it regulates gene expression. Nrf2/*SKN-1* activity is tightly regulated by phosphorylation and degradation (Inoue *et al.* 2005; Leung *et al.* 2014). Studies in *C. elegans* have shown that in combination with *CUL-4/DDB-1*, the ubiquitin ligase WD40 repeat protein *WDR-23* negatively regulates the function of *SKN-1* (Choe *et al.* 2009; Leung *et al.* 2014). *WDR-23* likely dissociates from *SKN-1* under conditions in which oxidative stress is increased, allowing *SKN-1* to accumulate in the nucleus (Choe *et al.* 2009). *SKN-1* activation is regulated by a conserved MAPK cascade composed of *NSY-1/MKKK*, *SEK-1/MKK*, and *PMK-1/MAPK*, which functions in the intestine to phosphorylate *SKN-1* leading to its stabilization and nuclear translocation (Inoue *et al.* 2005). Once activated, *SKN-1* directs the expression of hundreds of genes, including genes that comprise antioxidant responses such as *GST-4*, glutathione-S-transferase (Inoue *et al.* 2005).

We previously found that *SKN-1* activation in the intestine negatively regulates neuromuscular junction (NMJ) function

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via a mechanism that involves reducing neuropeptide secretion from motor neurons. Selective activation of *SKN-1* by *PMK-1* overexpression or by deletion of *WDR-23* in the intestine downregulates sphingosine-1-phosphate production by intestinal *SPHK-1*/sphingosine kinase, leading to the regulation of NMJ function by an intertissue signaling mechanism (Staab *et al.* 2013; Kim and Sieburth 2018b). Here, we identify two pathways that function downstream of or parallel to *SKN-1* to mediate the stress-induced regulation of NMJ function: the fibroblast growth factor (FGF) receptor (FGFR) pathway and the *DAF-2* insulin receptor pathway.

FGF signaling has well-defined functions in animal development, tissue repair, and remodeling. In neurons, FGF signaling regulates synapse formation and refinement during development. In *C. elegans*, FGF signaling functions in several developmental processes including cell migration, muscle differentiation, and axon guidance, and it also has a postdevelopmental role in regulating fluid homeostasis (Szewczyk and Jacobson 2003; Bülow *et al.* 2004; Huang and Stern 2004; Lo *et al.* 2008; Díaz-Balzac *et al.* 2015). The FGF receptor *EGL-15* is activated by one of two FGFs, *EGL-17* and *LET-756*. *EGL-15* activates a downstream signaling cascade composed of the *SEM-5*/GRB2 adaptor protein, the *SOS-1*/guanine nucleotide exchange factor, the *SOC-2*/SHOC2 adaptor protein, and *SOC-1*, a putative adaptor with a conserved plant homeodomain that interacts with *SEM-5* (Schutzman *et al.* 2001). *DAF-2* is the ortholog of insulin/*IGF-1* transmembrane receptor and is a key regulator of life span, stress resistance, metabolism, and development (Gottlieb and Ruvkun 1994; Libina *et al.* 2003; Hung *et al.* 2014). Neuronal functions of *DAF-2* include regulating motor activity, isothermal tracking, development of cholinergic axons and touch receptor neurons, and long- and short-term learning memory (Duhon and Johnson 1995; Murakami *et al.* 2005; Hsu *et al.* 2009; Kauffman *et al.* 2010; Li *et al.* 2016).

In this study, we found that the FGF pathway—consisting of *EGL-17*, *EGL-15*, *SOS-1*, *SOC-1*, and *SOC-2*—functions downstream of or parallel to *SKN-1* and *SPHK-1* to negatively regulate NMJ function in response to oxidative stress. We found that *EGL-15* functions primarily in the hypodermis to regulate NMJ function. In addition, we found that *DAF-2* functions downstream of or parallel to *SKN-1* in multiple distinct tissues to regulate NMJ function. These results suggest that the receptor tyrosine kinase (RTK) signaling network regulates NMJ function in response to oxidative stress.

Materials and Methods

C. elegans strains

All strains used in this study were maintained at 22° following standard methods. For temperature-shift experiments, we transferred L4-stage worms, which were grown at 22°, to 25° for 24 hr prior to assay. Young adult hermaphrodites were used for all experiments. The following mutant strains were

used, some of which were provided by the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health Office of Research Infrastructure Programs (P40 OD-010440): *egl-15(n1477ts)*, *egl-15(n484ts)*, *egl-17(ay6)*, *let-756(s2163)*, *sos-1(cs41ts)*, *soc-1(n1789)*, *soc-2(n1774)*, *sem-5(n1779)*, *daf-2(e1370)*, *daf-2(m596)*, *daf-16(mu86)*, *ZM8561[daf-2(m596);hpEx2906(Pmyo-2::RFP + Prgef-1::daf-2(mini-gene))]*, *ZM8562[daf-2(m596);hpEx2369(Pmyo-2::RFP + Pges-1::daf-2(mini-gene))]*, *ZM8988[daf-2(m596);hpEx2908(Pmyo-2::RFP + Pdp-30::daf-2(mini-gene))]*, *ZM9028[daf-2(m596);hpEx2905(Pmyo-2::RFP + Pmyo-3::daf-2(mini-gene))]*, and *NH2447[ayIs2(egl-15p::GFP + dpy-20(+))]*. The *wdr-23(tm1718)* strain was provided by the National BioResource Project (Japan). The wild-type reference strain was N2 Bristol. The genes and mutant strains tested in our screen are listed in the Supplemental Material, File S1.

Molecular biology

All genes were cloned from *C. elegans* genomic DNA (gDNA) from wild-type worms and inserted into *pPD49.26* using standard molecular biology techniques. Promoter DNA fragments were amplified from mixed-stage gDNA. The following plasmids were generated: *pSK46[Pges-1::egl-15(genomic)]*, *pSK47[Prab-3::egl-15(genomic)]*, *pSK48[Pcol-12::egl-15(genomic)]*, *pSK57[Pcol-12::daf-2(mini-gene)]*, and *pSK80[Pegl-15::gfp]*.

Transgenic lines and rescue experiments

Transgenic strains were generated by injecting expression constructs (10–25 ng/μl), and the co-injection marker *KP#708 (Pttx-3::rfp, 40 ng/μl)* or *KP#1106 (Pmyo-2::gfp, 10 ng/μl)*, into N2 or corresponding mutants. Microinjection was performed following standard techniques as previously described (Mello *et al.* 1991). At least three lines for each transgene were tested and a representative transgene was used for further experiments. The following transgenic arrays were made: *vjEx1309[Pcol-12::daf-2(mini-gene)]*, *vjEx1239[Prab-3::egl-15(genomic)]*, *vjEx1241[Pges-1::egl-15(genomic)]*, *vjEx1243[Pcol-12::egl-15(genomic)]*, and *vjEx1593[Pegl-15::gfp]*.

The *egl-15* genomic fragments used for rescue experiments span the entire *egl-15* locus and include coding sequences of all 16 *egl-15* isoforms, which arise by alternative splicing, as well as introns, which can potentially contain enhancer elements that may broaden gene expression beyond that dictated by the heterologous promoters. Since we observed promoter-specific rescue of *egl-15* mutants, we conclude that intronic sequences in these fragments do not influence the expression pattern of the transgenes in a meaningful way.

Microscopy and analysis

Fluorescence microscopy experiments were performed following previous methods (Kim and Sieburth 2018a). Briefly, L4-stage or young adult worms were immobilized by using 2,3-butanedione monoxime (30 mg/ml; Sigma [Sigma

Chemical], St. Louis, MO) in M9 buffer then mounted on 2% agarose pads for imaging. To image and quantify the intestinal fluorescence intensity of *Pgst-4::GFP* and *Pegl-15::GFP*, posterior intestinal cells were selected as a representative region. Images were captured with the Nikon eclipse 90i microscope equipped with a Nikon (Garden City, NY) PlanApo 40×, 60×, or 100× objective (NA = 1.4), and a PhotometricsCoolsnap ES2 or a Hamamatsu Orca Flash LT+ CMOS camera. Metamorph 7.0 software (Universal Imaging/Molecular Devices) was used to capture serial image stacks and the maximum intensity was measured (Kim and Sieburth 2018a). Intensity quantification analysis was performed on the same day to equalize the absolute fluorescence levels between samples within the same experimental set.

RNA interference

A feeding RNA interference (RNAi) knockdown assay was performed following the established protocol (Kamath and Ahringer 2003). Briefly, gravid adult animals were placed on RNAi plates seeded with HT115(DE3) bacteria transformed with L4440 vector, containing a genomic fragment of the gene to be knocked down (or empty L4440 vector as a control), to collect eggs then removed after 4 hr to obtain an age-matched synchronized worm population. Young adult animals were used for RNAi assays.

Pharmacology

For aldicarb assays, the percentage of paralyzed young adult animals was counted every 10–15 min starting ~1 hr after placing worms on 1 mM aldicarb (Bayer) plates. NGM plates containing *OP50* and aldicarb were freshly made 1 day before each assay. Wild-type animals were included in each set of assays to control for assay-to-assay variability arising from slightly different aldicarb concentrations in each batch of assay plates. Two-to-three replicates of at least 20 worms were performed per strain analyzed. For arsenite exposure, at least 50 young adult animals were transferred to NGM plates supplemented with 5 mM sodium arsenite (RICCA Chemicals) for 4 hr prior to aldicarb assay or *Pegl-15::gfp* imaging. For arsenite-induced *Pgst-4::gfp* expression, at least 50 young adult animals were incubated with 5 mM arsenite in M9 buffer for 1 hr, then images were taken after 4 hr of recovery.

Statistical analysis

For Figure 1, D and E, the Student's *t*-test (two-tailed) was used to determine the statistical significance. "ns" above the bars denotes *P*-values > 0.05. Error bars in the figures indicate ± SEM. The numbers of animals tested are indicated in each figure.

Data availability

Strains and plasmids used in this study are available upon request. The authors state that all data necessary for confirming the conclusions of the findings are present within the article, figures, and supplemental file. Supplemental

material available at Figshare: <https://doi.org/10.25386/genetics.7637873>.

Results

A screen for genes that promote aldicarb resistance in response to oxidative stress

We previously showed that acute (4 hr) exposure to the oxidative stressor arsenite causes resistance to the paralytic effects of aldicarb (Staab *et al.* 2013; Kim and Sieburth 2018b). Aldicarb is an acetylcholine esterase inhibitor, and aldicarb treatment leads to acetylcholine accumulation in synaptic clefts at NMJs and subsequent paralysis due to muscle hypercontraction. Animals defective in acetylcholine secretion exhibit delayed paralysis (referred to here as aldicarb resistance) because of a delay in acetylcholine accumulation. Arsenite causes aldicarb resistance by activating the SKN-1 pathway in the intestine (Staab *et al.* 2013; Kim and Sieburth 2018b). To identify additional genes that mediate the effects of arsenite on neuromuscular function, we performed a large-scale candidate screen for mutants that blocked or attenuated aldicarb resistance caused by arsenite treatment. We selected 90 candidate genes based on their known roles in presynaptic function, signaling transduction, protein secretion, or the stress response (File S1). Nearly all of the mutants corresponding to these genes had wild-type responses to arsenite, with the exception of *sphk-1*, *pmk-1*, *sek-1*, and *nsy-1* mutants, which were included as positive controls (Kim and Sieburth 2018b), as well as two additional mutants: *egl-15*/FGFR and *daf-2*/IR. Both *egl-15* and *daf-2* mutants significantly attenuated the ability of arsenite treatment to cause aldicarb resistance (File S1), revealing a potential role for FGF and insulin-like peptide receptor signaling in regulating stress-induced aldicarb response.

EGL-15 promotes arsenite-induced aldicarb resistance

egl-15 encodes the sole *C. elegans* ortholog of the FGFR. *egl-15* null mutants are lethal due to an essential role of EGL-15 in regulating fluid homeostasis (Schutzman *et al.* 2001; Huang and Stern 2004). The *egl-15*(n1477ts) allele encodes a W930Stop nonsense mutation that truncates EGL-15 at the C-terminal intracellular domain (DeVore *et al.* 1995). Temperature-sensitive *egl-15*(n1477ts) mutants are viable and display a scrawny phenotype due to destabilization of EGL-15 when grown at 25° (Mohammadi *et al.* 1996; Dixon *et al.* 2006). We found that arsenite-induced aldicarb resistance was significantly attenuated in *egl-15*(n1477ts) mutants grown at 20° and was completely blocked in *egl-15*(n1477ts) adults that had been shifted to 25° at the L4 stage (24-hr earlier, Figure 1, A and B). Because development is largely completed at the L4 stage, these results suggest that EGL-15 has a post-developmental role in regulating aldicarb responsiveness.

The *egl-15* gene encodes two receptor isoforms, EGL-15(A) and EGL-15(B), generated by alternative splicing, which differ in their extracellular domains and are proposed

Figure 1 EGL-15 functions downstream of SKN-1 and SPHK-1 to promote As-induced aldicarb resistance. (A and B) Time course of aldicarb-induced paralysis of wt or *egl-15* (*n1477ts*) adults shifted to the indicated temperature for 24 hr (denoted with →) with or without As treatment. (C) Time course of aldicarb-induced paralysis of wt or *egl-15* (*n484ts*) mutant worms cultured at 25° for 24 hr following As treatment. (D) Representative images of posterior intestines of indicated strains expressing GFP driven under the *gst-4* promoter (left) in the absence or presence of As. Quantification of the average GFP intensity (right). (E) Representative images and quantification of GFP driven under the *egl-15* promoter in hypodermis (left), intestine (middle), and vulva muscle (right) following control or As treatment. (F) Time course of aldicarb-induced paralysis of wild-type control, *skn-1*(*lax188gf*), *egl-15*(*n1477ts*), or *skn-1*(*lax188gf*);*egl-15*(*n1477ts*) double mutants. (G) Time course of aldicarb-induced paralysis of wt and *egl-15* mutant worms treated with e.v. control or *sphk-1* RNAi. Arrow (→) denotes that worms were shifted from 22° to the indicated temperature for 24 hr prior to the assay. “ns” above the bars denotes *P*-values > 0.05. Number of animals tested is indicated. Bar, 10 μm. Error bars indicate ± SEM. As, arsenite; e.v., empty vector; ns, not significant; RNAi, RNA interference; wt, wild-type.

to have different ligand-binding specificity. EGL-17 binds to EGL-15(A) while LET-756 binds to EGL-15(B) (Birnbaum *et al.* 2005). The *egl-15(n484ts)* allele encodes a W167Stop nonsense mutation in exon 5A that is predicted to specifically abolish EGL-15(A) expression without affecting EGL-15(B) expression (Goodman *et al.* 2003). Like *egl-15(n1477ts)* mutants, *egl-15(n484ts)* mutants shifted to 25° became significantly less aldicarb-resistant than wild-type controls following arsenite (Figure 1C). Taken together, we conclude that EGL-15(A) has a specific role in promoting arsenite-induced aldicarb resistance. Interestingly, in the absence of arsenite, *egl-15(n1477ts)* mutants exhibited significant hypersensitivity to aldicarb (Figure 1B), whereas *egl-15(n484ts)* mutants exhibited similar aldicarb sensitivity as wild-type controls (Figure 1C). Thus, the EGL-15(B) isoform may specifically regulate aldicarb responsiveness in the absence of stress.

EGL-15 functions downstream of, or parallel to, SKN-1 and SPHK-1 to promote arsenite-induced aldicarb resistance

To determine whether EGL-15 is a SKN-1 activator, we examined the requirement of *egl-15* in the stress-induced expression of *gst-4*/glutathione-S-transferase, which is a direct transcriptional target of SKN-1 (Choe *et al.* 2009; Wu *et al.* 2016). A transcriptional reporter in which GFP is expressed under the *gst-4* promoter (*Pgst-4::GFP*) is expressed at low levels in the absence of stress whereas arsenite treatment significantly increases *Pgst-4::GFP* expression in the intestine in a *skn-1*-dependent manner (Choe *et al.* 2009; Wu *et al.* 2016). We found that the expression of *Pgst-4::GFP* in the intestine was similar in *egl-15* mutants and wild-type controls, in both the absence and presence of arsenite (Figure 1D). Thus, both baseline SKN-1 activity and arsenite-induced activation of SKN-1 are normal in *egl-15* mutants, suggesting that EGL-15 does not regulate SKN-1 activity.

To determine whether EGL-15 is a transcriptional target of SKN-1, we examined the effects of arsenite treatment on the fluorescence intensity of *Pegl-15::GFP* reporters, in which the 2-kb promoter fragment of *egl-15* drives the expression of GFP. We found that transgenic animals expressing *Pegl-15::GFP* exhibited fluorescence in the intestine, vulval muscle, and hypodermis, in agreement with prior studies (Mounsey *et al.* 2002; Bülow *et al.* 2004). Arsenite treatment did not significantly alter the fluorescence intensity of *Pegl-15::GFP* in any of these tissues (Figure 1E). These results suggest that EGL-15 is not transcriptionally regulated by SKN-1 activation.

To determine whether EGL-15 functions in the SKN-1 pathway to regulate aldicarb resistance, we examined *skn-1* mutants that are constitutively active. The *skn-1(lax188gf)* mutation alters an amino acid in a protein-interaction domain that renders SKN-1 constitutively active (Paek *et al.* 2012). *skn-1(gf)* mutants are aldicarb-resistant (Staab *et al.* 2013). We found that *egl-15* mutations significantly reduced the aldicarb resistance of *skn-1(gf)* mutants (Figure 1F).

SKN-1 activation leads to aldicarb resistance in part by negatively regulating SPHK-1 signaling in the intestine (Kim and Sieburth 2018b). To determine whether EGL-15 mediates the effects of SPHK-1 in this pathway, we examined aldicarb responses of *egl-15* mutants in which intestinal *sphk-1* activity was knocked down by RNAi. Animals treated with *sphk-1* RNAi are strongly resistant to aldicarb (Chan *et al.* 2012; Kim and Sieburth 2018b). Strikingly, *egl-15* mutations nearly completely suppressed the aldicarb resistance caused by *sphk-1* RNAi (Figure 1G). These results indicate that EGL-15 and SPHK-1 function antagonistically to regulate aldicarb responsiveness, and that EGL-15 functions downstream of or parallel to SPHK-1. Together, these results reveal a role for EGL-15 in regulating neuromuscular function in response to SKN-1 activation.

The FGF ligand EGL-17 mediates arsenite-induced aldicarb resistance

The two FGF-related ligands signal through EGL-15 to control distinct functions of EGL-15. EGL-17/FGF promotes sex myoblast migration while LET-756/FGF promotes axon guidance and fluid homeostasis (DeVore *et al.* 1995; Sundaram *et al.* 1996; Burdine *et al.* 1997; Popovici *et al.* 1999; Birnbaum *et al.* 2005; Lo *et al.* 2008; Díaz-Balzac *et al.* 2015) (Figure 2A). *egl-17(ay6)* is a deletion allele that is predicted to eliminate *egl-17* activity (Burdine *et al.* 1997). *egl-17(ay6)* mutants displayed wild-type aldicarb responsiveness in the absence of arsenite, but exhibited a shift toward aldicarb resistance that was significantly smaller than that of wild-type controls following arsenite treatment (Figure 2B). This partial response suggests that EGL-17 contributes to arsenite-induced aldicarb resistance, but is not likely to be solely responsible for EGL-15 activation in this process.

To test the role of LET-765, we examined *let-756(s2613)* hypomorphic mutants, which are viable as adults, unlike null mutants, which die as larvae (Popovici *et al.* 2004). In the absence of arsenite, *let-756(s2613)* mutants were significantly more sensitive to aldicarb-induced paralysis than wild-type controls (Figure 2C), suggesting a role for EGL-15B activation by LET-765 in aldicarb responsiveness in the absence of stress. Arsenite treatment caused a large shift toward aldicarb resistance in *let-756* mutants that was similar to that of wild-type controls (Figure 2C). The partial defect in arsenite-induced aldicarb resistance of *egl-17* mutants was not enhanced by *let-756* mutations (Figure 2D). Thus, we conclude that EGL-17 contributes to arsenite-induced aldicarb resistance, whereas LET-756 does not.

The SOS-1/SOC-1/SOC-2 signaling cascade mediates arsenite-induced aldicarb resistance

We next examined mutants corresponding to each of the known components that make up the signaling cascade downstream of EGL-15 (Figure 2A). *sos-1(cs41ts)* mutants are temperature-sensitive (Abdus-Saboor *et al.* 2011) and, when shifted to 25° for 24 hr prior to assay, exhibited wild-type aldicarb responsiveness in the absence of stress.

Figure 2 EGL-17 FGF and the SOS-1/SOC-1/SOC-2 signaling cascade are required for As-induced aldicarb resistance. (A) Schematic diagram of the EGL-15 signaling pathway. (B–H) Time course of aldicarb-induced paralysis of indicated strains in the absence or presence of As. Temperatures at which aldicarb assays were performed are designated in each figure. Arrows (→) denote that worms were shifted from 22° to the indicated temperature for 24 hr prior to the assay. Number of animals tested is indicated. Error bars indicate $\pm$ SEM. As, arsenite; FGF, fibroblast growth factor; wt, wild-type.

sos-1(cs41ts) mutants became significantly less aldicarb-resistant following arsenite treatment than wild-type controls (Figure 2E). *soc-1(n1789)* and *soc-2(n1774)* are null and

hypomorph alleles, respectively (Schutzman *et al.* 2001). We found that *soc-1(n1789)* mutants were slightly aldicarb-resistant in the absence of arsenite whereas *soc-2*

(*n1774*) mutants were hypersensitive to aldicarb (Figure 2, F and G). However, both mutants became significantly less aldicarb-resistant than wild-type controls following arsenite treatment (Figure 2, F and G). Finally, *sem-5(n1779)* hypomorphic mutants were extremely resistant to aldicarb in the absence of arsenite, revealing a role of **SEM-5** in promoting NMJ function. Arsenite treatment elicited a further increase in aldicarb resistance in *sem-5(n1779)* mutants, but this shift was much smaller than the shift elicited in wild-type controls (Figure 2H). Thus, **SOS-1**, **SOC-1**, and **SOC-2** contribute to stress-induced aldicarb resistance. The contribution of **SEM-5** is more difficult to ascertain given that the mutants were so resistance to aldicarb in the absence of stress. Because **EGL-15** signaling appears to be left partially intact in each of the signaling mutants tested, we conclude that these components may function parallel to each other or may function with other unidentified components activated by **EGL-15** to contribute to stress-induced aldicarb responsiveness.

EGL-15 functions in the hypodermis to promote arsenite-induced aldicarb resistance

EGL-15 has been reported to be expressed in multiple tissues, including the hypodermis, vulval muscles, intestine, and neurons (Bülow *et al.* 2004; Huang and Stern 2004). To determine the site of action of **EGL-15** with respect to stress-induced aldicarb resistance, we performed tissue-specific rescue experiments by generating transgenic *egl-15(n1477ts)* mutants expressing *egl-15* gDNA selectively in the hypodermis [using the *col-12* promoter (Olofsson 2014)], intestine [using the *ges-1* promoter (Kim and Sieburth 2018a)], or nervous system [using the *rab-3* promoter (Kim and Sieburth 2018b)]. Expression of *egl-15* gDNA in each of these tissues did not rescue the weak aldicarb hypersensitivity of *egl-15(n1477ts)* mutants in the absence of stress, suggesting that the basal response of *egl-15(n1477ts)* mutants to aldicarb may require **EGL-15** signaling in multiple tissues or in other tissue(s) not tested here (Figure 3, A–C). However, **EGL-15** expression in the hypodermis nearly completely restored arsenite-induced aldicarb resistance to *egl-15(n1477ts)* mutants (Figure 3A). In contrast, expression of *egl-15* transgenes in the nervous system failed to rescue the aldicarb sensitivity of *egl-15(n1477ts)* mutants (Figure 3B), and expression of *egl-15* in the intestine partially restored stress-induced aldicarb resistance to *egl-15(n1477ts)* mutants (Figure 3C). These results suggest that **EGL-15** signaling in the hypodermis is critical for arsenite-induced aldicarb resistance. We propose a model whereby, under conditions of low stress, normal neuromuscular function is maintained by the inhibition of **EGL-15** signaling in the hypodermis (and possibly also the intestine). During stress, **SKN-1** activation negatively regulates neuromuscular function by promoting **EGL-15** signaling (possibly through intestinal **SPHK-1**). Alternatively, **EGL-15** may define a pathway parallel to **SKN-1** that regulates NMJ function during oxidative stress (Figure 3D).

DAF-2 is required for aldicarb resistance induced by oxidative stress

The second gene identified in our screen was *daf-2*. We examined two *daf-2* hypomorphic alleles: *daf-2(e1370)*, which encodes a P1547S missense mutation, and *daf-2(m596)*, which encodes a G471S missense mutation (Bulger *et al.* 2017). Mutants carrying these alleles exhibit a temperature-sensitive dauer constitutive phenotype when cultured at 25° (Gems *et al.* 1998). To test whether the aldicarb response of *daf-2* mutants is temperature-dependent, we cultured *daf-2* mutants at 20° until the L4 stage, and then shifted them to either 15, 20, 22, or 25° for 24 hr prior to assay. In the absence of arsenite, *daf-2(e1370)* mutants exhibited nearly normal aldicarb responsiveness following temperature-shift, with slight aldicarb resistance seen at 15, 20, and 22°, and slight aldicarb hypersensitivity seen at 25° (Figure 4, A–D). Following arsenite treatment, *daf-2(e1370)* mutants shifted to 15° exhibited aldicarb resistance that was similar to that of wild-type controls. However, arsenite-induced aldicarb resistance became progressively more attenuated in *daf-2(e1370)* mutants that had been shifted to higher temperatures. Following shift to 25°, arsenite-induced aldicarb resistance was nearly abolished in *daf-2(e1370)* mutants but not in wild-type controls (Figure 4D). These results reveal a critical requirement for **DAF-2** in promoting arsenite-induced aldicarb resistance.

DAF-2 functions downstream of or parallel to SKN-1

To determine whether **DAF-2** functions in the **SKN-1** pathway to regulate aldicarb responsiveness, we examined the aldicarb responsiveness of *daf-2* mutants in which **SKN-1** is constitutively active. **WDR-23** promotes neuromuscular function by negatively regulating **SKN-1** in the intestine. *wdr-23* null mutants show delayed aldicarb paralysis in the absence of stress that is completely dependent upon *skn-1* and is not enhanced by arsenite treatment (Staab *et al.* 2013; Kim and Sieburth 2018b). As expected, *wdr-23* knockdown by RNAi led to strong aldicarb resistance. However, knockdown of *wdr-23* was unable to cause aldicarb resistance in either *daf-2(e1370)* or *daf-2(m596)* mutants at 25° (Figure 4, E and F), but not at 15° (Figure 4G), indicating that the aldicarb-resistance phenotype caused by **SKN-1** activation is dependent upon *daf-2* signaling. **PMK-1** positively regulates **SKN-1** activity in the intestine by phosphorylating **SKN-1**, leading to its stabilization and its accumulation in the nucleus (Inoue *et al.* 2005). Animals overexpressing **PMK-1** cDNA specifically in intestine (under the *ges-1* promoter) exhibited enhanced aldicarb resistance compared to nontransgenic controls [Figure 4H and Kim and Sieburth (2018b)]. However, **PMK-1** overexpression was unable to make *daf-2(m596)* mutants more aldicarb-resistant than nontransgenic controls (Figure 4H). Together, these results are consistent with a function of **DAF-2** either downstream of or parallel to **SKN-1** in regulating aldicarb resistance in response to stress.

Figure 3 EGL-15 is required in the hypodermis to regulate As-induced aldicarb resistance. (A–C) Time course of aldicarb-induced paralysis of the indicated strains in the absence or presence of As. “hypodermis rescue, neuron rescue, intestine rescue,” denotes *egl-15* transgene expression using the tissue-specific promoters *col-12*, *rab-3*, or *ges-1*, respectively. (D) Schematic working model whereby EGL-15 signaling in the hypodermis (and possibly the intestine) is low during low-stress conditions. Following SKN-1 activation by oxidative stress, SPHK-1 activity is inhibited, which in turn causes aldicarb resistance possibly via increasing EGL-15 activity. Arrows (→) denote that worms were shifted from 22° to the indicated temperature for 24 hr prior to assay. Number of animals tested is indicated. Error bars indicate $\pm$ SEM. As, arsenite; wt, wild-type.

DAF-2 functions in multiple tissues to promote arsenite-induced aldicarb resistance

DAF-2 is expressed in multiple tissues including the intestine, nervous system, hypodermis, and muscle (McKay *et al.* 2003; Hunt-Newbury *et al.* 2007). To identify the tissue in which DAF-2 functions in SKN-1-mediated aldicarb resistance, we performed a series of tissue-specific rescue experiments. A prior study generated a panel of extrachromosomal arrays in which a *daf-2* mini-gene was expressed in different tissues (Hung *et al.* 2014). We examined strains bearing these extrachromosomal arrays for their ability to restore normal stress-induced aldicarb resistance to *daf-2(m596)* mutants. To activate the stress response, we knocked down *wdr-23* by RNAi in *daf-2(m596)* mutants. As expected, expression of *daf-2* in all tissues (using the *dpy-30* promoter) fully reverted aldicarb resistance to *daf-2* mutants treated with *wdr-23* RNAi (Figure 5A). Next, we tested whether *daf-2* expression selectively in the intestine (using the *ges-1* promoter), the nervous system (using the *rab-3* promoter), the hypodermis (using the *col-12* promoter), or in body wall muscle (using the *myo-3* promoter) could restore normal aldicarb responsiveness to *daf-2(m596)* mutants in which *wdr-23* was knocked down. Interestingly, we found that expression of *daf-2* in any single tissue failed to rescue *daf-2(m596)* mutants (Figure 5, B–E). These results suggest that DAF-2 may function in more than one tissue to regulate SKN-1-mediated

aldicarb resistance. Alternatively, DAF-2 may function in a tissue not tested here, such as the germline, to regulate aldicarb responsiveness.

Discussion

Multicellular organisms are exposed to a variety of stressors in the form of endogenous stress or environmental stress, induced by changes in their surroundings. Therefore, they have developed complex organism-wide defense mechanisms to prevent cellular damage, and maintain proper cellular and organism-wide homeostasis. Our results support the idea that the regulation of NMJ function by oxidative stress is mediated by complex signaling networks that act across multiple tissues and involves at least two RTK signaling cascades: FGFR and insulin-like peptide receptor. Our genetic analysis revealed that both of these RTKs likely function downstream of SKN-1 to promote aldicarb resistance caused by acute oxidative stress. Temperature-shift experiments show that these RTK pathways regulate NMJ physiology rather than development. We found that EGL-15 functions primarily in the hypodermis to regulate NMJ function, whereas DAF-2 signaling may be required in multiple tissues. Thus, EGL-15 and DAF-2 signaling may mediate intertissue communication between the intestine, where SKN-1 is activated at the NMJ during the oxidative stress response.

Figure 4 DAF-2 functions downstream of SKN-1 to regulate neuromuscular junction function. (A–D) Time course of aldicarb-induced paralysis of wt and *daf-2(e1370)* mutant worms grown at 15 (A), 20 (B), 22 (C), or 25° (D) for 24 hr prior to aldicarb assays following control or As treatment. (E–G) Time course of aldicarb-induced paralysis of indicated strains treated with e.v. control or *wdr-23* RNAi, at 25 (E and F) or 15° (G). (H) Time course of aldicarb-induced paralysis of indicated strains. PMK-1(OX) denotes animals overexpressing *pmk-1* transgenes in the intestine. Temperatures at which aldicarb assay were performed are designated in each figure. Arrows (→) denote that worms were shifted from 20° to the indicated temperature for 24 hr prior to assay. Number of animals tested is indicated. Error bars indicate $\pm$ SEM. As, arsenite; e.v., empty vector; RNAi, RNA interference; wt, wild-type.

Aldicarb resistance can arise from defects in acetylcholine release from NMJs, neuropeptide secretion, or muscle excitability. We previously found that the aldicarb resistance caused by *SKN-1* activation is not due to enhanced detoxification of aldicarb or alterations in muscle excitability, but instead is due to a reduction in neurotransmitter release from motor neurons (Staab *et al.* 2013). We subsequently found that inhibition of intestinal *SPHK-1* by *SKN-1* results in a reduction of neuropeptide secretion from motor neurons (Kim and Sieburth 2018b). Since *EGL-15* is required for the effects

of *SPHK-1* on NMJ function, we speculate that FGF signaling may also regulate neuropeptide secretion, although further analysis will be needed to determine the detailed mechanism by which FGFR signaling regulates NMJ function.

FGF signaling in regulating NMJ function

Our results are consistent with the idea that *EGL-17* activates *EGL-15* to regulate the aldicarb response during the oxidative stress response. First, the *egl-15(n484ts)* mutation, which specifically disrupts *EGL-15(A)*, nearly eliminates

Figure 5 DAF-2 functions in multiple tissues to regulate SKN-1-dependent neuromuscular junction function. (A–E) Time course of aldicarb-induced paralysis of wt animals or *daf-2(m596)* transgenic mutants, following control e.v. or *wdr-23* RNAi treatment. “all rescue, intestine rescue, neuron rescue, hypodermis rescue, muscle rescue” denotes DAF-2 transgenes expressing under the tissue-specific promoters *dyp-30*, *ges-1*, *rab-3*, *col-12*, or *myo-3*, respectively. Arrows (→) denote that worms were shifted from 20° to the indicated temperature for 24 hr prior to assay. Number of animals tested is indicated. Error bars indicate $\pm$ SEM. e.v., empty vector; RNAi, RNA interference; wt, wild-type.

arsenite-induced aldicarb resistance. Second, *egl-17* mutants attenuate arsenite-induced aldicarb resistance more effectively than *let-756* mutants. Finally, *let-756* mutations did not enhance the arsenite-induced aldicarb-resistance defects of *egl-17* mutants. Because *egl-17* null mutants did not fully block arsenite-induced aldicarb resistance, it is possible that EGL-17 and another unidentified EGL-15 ligand may function redundantly to regulate aldicarb responsiveness. Redundancy in the signaling cascade downstream of EGL-15 may also occur, since mutations in any single known downstream component of the EGL-15 pathway attenuate but do not block

the arsenite-induced aldicarb resistance. In mammals, the FGFR activates multiple different cytosolic signaling factors in a signal- and context-dependent manner. Additional downstream targets of FGFR include the adapter proteins FRS2a and CRKL, and well as STAT family members (Ornitz and Itoh 2015). It will be interesting to determine whether these genes function with SOC-1 and/or SOS-1 in this pathway. Interestingly, we found that in the absence of arsenite, *egl-15(a/b)*, *let-756*, or *soc-2* mutants were significantly hypersensitive to aldicarb-induced paralysis, whereas *soc-1* and *sem-5* mutants were resistant to aldicarb, revealing previously

unreported roles for these signaling components in negatively and positively regulating NMJ function, respectively. The aldicarb resistance of *sem-5* mutants complicates the interpretation of the relatively small shift to aldicarb resistance in *sem-5* mutants upon arsenite treatment; it may reflect a requirement of SEM-5 in promoting arsenite responsiveness or it could represent a ceiling effect given the extreme aldicarb resistance of the mutant under baseline conditions. Further experiments will be needed to determine whether SEM-5 is involved in this pathway.

How does EGL-15 signaling regulate aldicarb responsiveness in response to stress? EGL-15 in the hypodermis has a postdevelopmental function in regulating fluid homeostasis (Huang and Stern 2004). However, defects in fluid homeostasis are not likely to account for the aldicarb phenotypes of *egl-15* mutants, since *let-756* mutants, which are also defective in fluid homeostasis, did not block arsenite-induced aldicarb resistance, whereas *egl-17* mutants, which are not defective in fluid homeostasis, behaved similarly to *egl-15* mutants. Thus, the aldicarb phenotypes of the FGF mutants do not correlate with defects in fluid homeostasis. EGL-17 release from the hypodermis is required for sex myoblast migration during development. In adults, EGL-17 is expressed in several cells of the ventral hypodermis, as well as in a pharyngeal neuron (Burdine *et al.* 1998; Dixon *et al.* 2006; Hunt-Newbury *et al.* 2007). Thus, we speculate that EGL-17 secretion from either of these sites may activate EGL-15 signaling in the hypodermis in response to stress by an autocrine or paracrine mechanism.

Insulin-like receptor signaling in regulating NMJ function

Our results suggest that DAF-2 signaling is required for oxidative stress to negatively regulate NMJ function. We find that the blocking of arsenite-induced aldicarb resistance in *daf-2* mutants is temperature-dependent. Similarly, the dauer phenotype of most *daf-2* mutants is strictly temperature-dependent whereas other *daf-2*-associated phenotypes, such as increased longevity or thermotolerance, are not temperature-dependent (Gems *et al.* 1998). Therefore, the resistance to the effects of arsenite seen here may be a result of dauer-like characteristics in these mutants rather than a dependence on *daf-2* function *per se*. Indeed, even when cultured at “permissive” temperatures (as low as 20°), *daf-2* mutants can exhibit dauer-like characteristics (Gems *et al.* 1998) that may account for the partial effects in aldicarb responsiveness that we observe at semipermissive temperatures. Thus, the inability of oxidative stress to downregulate NMJ function may be a general feature of the dauer-like state, and this phenotype may contribute to the characteristic stress resistance associated with dauers (Androwski *et al.* 2017).

We speculate that DAF-2-regulated NMJ function in response to stress may involve intertissue signaling between multiple tissues, and could be mediated by one or more of the 40 insulins in *C. elegans* (Zheng *et al.* 2018). Prior studies have found that the biological function of DAF-2 is highly

tissue-specific; *daf-2* functions in both neurons and muscle to regulate resistance to hypoxia (Scott *et al.* 2002). DAF-2 has been reported to function in neurons, but not in muscles, to regulate life span extension, fat metabolism, and fertility (Apfeld and Kenyon 1998; Wolkow *et al.* 2000). A study using *daf-2* mini-genes expressed under tissue-specific promoters found an intestinal site-of-action (but not neuron or muscle) for *daf-2* in inhibiting dauer entry (Hung *et al.* 2014). Using the same set of transgenes, we found that intestinal *daf-2* expression was not sufficient to restore arsenite responsiveness to *daf-2* mutants. These results raise the possibility that the dauer-like state can be uncoupled from the stress-induced aldicarb-resistance defects of *daf-2* mutants. Notably, we found that selective expression of *daf-2* in the hypodermis led to aldicarb resistance (Figure 5D), a phenotype that was not observed when expressing *daf-2* in any other tissue tested, suggesting that enhanced DAF-2 signaling in the hypodermis may regulate NMJ function in the absence of stress. Indeed, DAF-2 signaling has been implicated in decreasing motor function in aged animals, but its site of action was not determined (Liu *et al.* 2013).

Our results suggest that DAF-2 signaling functions downstream of SKN-1 to regulate NMJ function. Consistent with this, tyrosine phosphorylation of the insulin receptor substrates 1 and 2 (IRS-1 and -2) by the insulin receptor is strongly reduced in the Nrf2-deficient mice (Beyer and Werner 2008). Furthermore, insulin receptor tyrosine kinase is activated by the oxidative stressor hydrogen peroxide (Dröge 2005). Interestingly, DAF-2 signaling has been implicated in the activation of SKN-1 during aging through the regulation of the Akt kinases AKT-1/2 (Tullet *et al.* 2008). The function of DAF-2 downstream of SKN-1 that we report here is likely to be distinct from the function identified for DAF-2 during ageing, further underscoring the complex role of insulin signaling in the SKN-1 pathway.

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