NeuCode Labeling in Nematodes: Proteomic and Phosphoproteomic Impact of Ascaroside Treatment in Caenorhabditis elegans*†‡§

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The nematode Caenorhabditis elegans is an important model organism for biomedical research. We previously described NeuCode stable isotope labeling by amino acids in cell culture (SILAC), a method for accurate proteome quantification with potential for multiplexing beyond the limits of traditional stable isotope labeling by amino acids in cell culture. Here we apply NeuCode SILAC to profile the proteomic and phosphoproteomic response of C. elegans to two potent members of the ascaroside family of nematode pheromones. By consuming labeled E. coli as part of their diet, C. elegans nematodes quickly and easily incorporate the NeuCode heavy lysine isotopologues by the young adult stage. Using this approach, we report, at high confidence, one of the largest proteomic and phosphoproteomic data sets to date in C. elegans: 6596 proteins at a false discovery rate ≤ 1% and 6620 phosphorylation isoforms with localization probability ≥75%. Our data reveal a post-translational signature of pheromone sensing that includes many conserved proteins implicated in longevity and response to stress. Molecular & Cellular Proteomics 14: 10.1074/mcp.M115.049684, 2922–2935, 2015.

The field of proteomics has rapidly advanced in recent years, to the point that whole proteome sequencing within a few hours has become feasible (1). That said, simply identifying the components of the proteome is insufficient for addressing many complex biological phenomena, propelling a shift toward quantitative protein measurements (2). Isotope labeling techniques, such as stable isotope labeling by amino acids in cell culture (SILAC) and isobaric tagging, e.g. isobaric tags for relative and absolute quantification (iTRAQ) and tandem mass tags (TMT), have grown in popularity since their introduction in the early 2000s (3–5). We recently described a quantitative proteomic technique called NeuCode SILAC that provides comparable quantitative accuracy and higher degrees of multiplexing relative to traditional SILAC (6).

NeuCode SILAC utilizes several different isotopically heavy forms of lysine that all carry a nominal mass shift of +8 Da but differ in exact isotopic composition, i.e. the number of heavy isotopes of carbon, hydrogen, and nitrogen. Variations in the mass defect of different nuclei (7) result in subtle differences in exact mass between the heavy lysine isotopologues, which are then exploited for quantification using high resolution mass spectrometry. Unlike traditional SILAC, NeuCode SILAC permits additional multiplexing without a concomitant increase in spectral complexity because the multiple forms of each peptide can be masked or exposed by varying resolving power. In yeast, for example, we achieved quantitative accuracy identical to traditional SILAC, but quantified 26% more peptides with NeuCode SILAC, allowing for greater overall proteome coverage (6). We therefore sought to extend these methodological improvements to C. elegans for global protein and post-translational modification (PTM) analysis. Recent evidence in many organisms reveals only modest correlation between transcript and protein abundance, emphasizing the need for new tools to study the proteome and its post-translational regulation (8, 9).

1 The abbreviations used are: SILAC, stable isotope labeling of amino acids in cell culture; iTRAQ, isobaric tag for relative and absolute quantitation; TMT, tandem mass tags; PTM, post-translational modification; RCF, relative centrifugal force; RPM, revolutions per minute; CAD, collisionally activated dissociation; NCE, normalized collision energy; OMSSA, open mass spectrometry search algorithm; FDR, false discovery rate; CK2, casein kinase 2; CDK, cyclin-dependent kinase; MAPK, mitogen activated protein kinase; mTOR, mammalian target-of-rapamycin; CAMK2, Ca2+/calmodulin-dependent kinase 2; G-CK, Golgi-casein kinase; ER, endoplasmic reticulum; AMP, adenosine mono-phosphate; ATP, adenosine tri-phosphate.
The nematode *Caenorhabditis elegans*, a widely used model organism for genetic and biochemical investigation, is an excellent platform for developing emergent proteomic technologies (10). *C. elegans* is easy to grow in large quantities under standardized conditions, either on Petri dishes or in liquid culture. Moreover, *C. elegans* has a short generation time (~3 days at 20 °C) and yet is a multicellular, fully differentiated organism that exhibits many phenomena observed in higher organisms (e.g. response to stress or starvation, extended lifespan) (11–14). Approximately 38% of *C. elegans* genes have a human ortholog, providing direct insight on protein function and signaling pathways relevant for human biology and medicine (15, 16). Furthermore, the use of labeled *E. coli* as a food source makes *C. elegans* particularly amenable to metabolic labeling via SILAC (17–19).

The ascarosides comprise a large family of small, glycolipid signaling molecules synthesized and secreted into the environment by nematodes throughout their life cycle (20); in general, they are glycosides of the dideoxysugar ascarylose with fatty acid-like side chains, which may be linked to additional moieties originating from diverse primary metabolic pathways (21). Ascarosides are prototypic for excreted signaling small molecules in many organisms. They function as chemo- and/or pheromone signals that can act, in part, via conserved signaling pathways, and food and population density sensation (22–27). They also act, in part, via conserved signaling pathways (21). Ascarosides are prototypic for excreted signaling small molecules in many organisms. They function as chemosensory cues, binding to G-protein coupled receptors in specific chemosensory head neurons and controlling fundamental processes related to growth, development, and reproduction such as induction of the long-lived dauer state in larvae, and food and population density sensation (22–27). Ascarosides act, in part, via conserved signaling pathways, including the insulin and TGFβ pathways (28–30). Despite their structural similarity, individual ascarosides exert distinct phenotypic effects and can also act synergistically with other environmental stimuli, underscoring the complexity of the underlying signaling circuitry (22, 31–33). Moreover, exposure of adult *C. elegans* to certain ascarosides extends lifespan and increases stress resistance, including thermotolerance (20). Yet remarkably little is known surrounding the molecular mechanism and downstream effects of ascaroside exposure and endogenous signaling small molecules in general. We hypothesized that an investigation of the proteomic and phosphoproteomic remodeling following ascaroside treatment might reveal molecular signatures of key biological consequences in *C. elegans*.

Here we characterize the proteome dynamics of NeuCode-labeled *C. elegans* in response to two distinct but structurally similar ascarosides. To date, no study has analyzed organism-wide gene or protein expression changes upon ascaroside exposure at any life stage. We present, with high confidence, one of the largest proteomic and phosphoproteomic data sets in *C. elegans* to date (6,596 proteins at a false discovery rate ≤ 1%). We also identify 6,475 sites of phosphorylation (localization probability ≥ 75%). Treatment of young adult *C. elegans* with two ascarosides, ascr#2 and ascr#5, did not reveal any significant changes in protein abundance. Nevertheless, analysis of the phosphoproteomic data implicated several regulators of stress response and lifespan as potential targets. Our results propagate the notion that small structural discrepancies in related signaling pheromones can lead to remarkably different molecular profiles.

**EXPERIMENTAL PROCEDURES**

**NeuCode SILAC Bacterial Culture—** *E. coli* strain ET505, previously described for nematode SILAC (17, 18), was obtained from the Coli Genetic Stock Center (CGSC number 7088, New Haven, CT) and maintained on LB agar plates supplemented with tetracycline (50 μg/ml). To generate NeuCode SILAC food for the animals, a 10 ml preculture of ET505, picked from a single colony, was grown for 18 h, at 37 °C, shaking at 220 RPM in lysine drop-out EZ rich defined medium (Teknova Catalog No. M2105, Hollister, CA) supplemented with tetracycline (10 μg/ml) and 0.4 mM lysine K602, K341, or K080 (Cambridge Isotope Laboratories, Tewksbury, MA). The three labeled lysines are henceforth known as isotopologues with their isotopic composition indicated by the following nomenclature: K13C2H15N (K602) and K15N are both commercially available, whereas K341 was custom synthesized (34). The single-residue reference is not for an amino acid position, but for the designation of the labeling of heavy isotopes in the amino acids. In using the single-letter ‘K’ designation, we are following the convention established by a previous publication, in MCP, on NeuCode: Merrill AE, et al. “NeuCode labels for relative protein quantification.” Mol. Cell Proteomics. 2014 Sep; 13(9): 2503–2512. The labeled bacteria were used to seed a 1 L culture, containing media with lysine isotopologues which was grown for 18 h. We confirmed complete labeling of the bacteria after 18 h of growth. Bacteria were pelleted at 1,704 RCF and 16 °C for 15 min and washed twice with M9 buffer (per 1 L of buffer: 6g NaHPO4, 3g KH2PO4, 1g NH4Cl, 5g NaCl, 1m of 1m MgSO4) and then resuspended in M9 to produce a 30x culture.

**NeuCode SILAC Nematode Culture—** *Caenorhabditis elegans* wild-type N2 Bristol strain, cultivated at 20 °C, was used for all experiments (35). Developmental stage was evaluated by examining animals with a Zeiss Axiol Imager D1 compound microscope (Zeiss, Thornwood, NY) at 10× and 40× magnification for body size and stage-specific markers (e.g. vulva formation). For growth without labeling, animals were kept on standard NGM plates and fed *E. coli* OP50 as previously described (11). For growth with NeuCode SILAC labeling, age-synchronized first stage larvae (L1) were obtained by standard methods. Briefly, gravid unlabeled adults were treated with 2:1 bleach/5N NaOH to isolate embryos (36). The embryos were incubated in M9 buffer without food in a ventilated Erlenmeyer flask at 20 °C for 18 h, shaking at 170 RPM to obtain synchronized L1 larvae. The unlabeled L1s were pelleted at 2500 RCF for 2 min, washed twice with 15 ml of M9, resuspended in 10 ml of M9, and then distributed to 10 cm peptone-free NGM plates pre-equilibrated to 20 °C. Peptone was omitted because it contains unlabeled lysine. We confirmed complete labeling of the bacteria after 18 h of growth. Bacteria were pelleted at 1,704 RCF and 16 °C for 15 min and washed twice with M9 buffer (per 1 L of buffer: 6g NaHPO4, 3g KH2PO4, 1g NH4Cl, 5g NaCl, 1m of 1m MgSO4) and then resuspended in M9 to produce a 30x culture.
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labels were switched for each replicate to ensure that incorporation of the label itself did not cause a biological effect (Replicate 1: K602-control, K602-ascr#5; Replicate 2: K602-ascr#2, K602-Control; K602-ascr#5; Replicate 3: K602-ascr#5, K602-ascr#2, K602-Control). Each replicate consisted of 24,000 animals (4 plates, 6000 animals per plate), which yielded 3 to 6 mg of protein. Immediately after the final L4 molt (~50 h after release from L1 arrest), animals were rinsed off plates with 3 ml per plate of 20 °C M9 into a 15 ml falcon tube, pelleted at 200 RCF for 1 min, washed twice with 15 ml of 20 °C M9, and then transferred by glass Pasteur pipette to 25 ml Erlenmeyer flasks that contained: 10 ml of 20 °C M9 with 100 µl of the appropriate 30x ET505, and either ethanol vehicle, ascr#2, or ascr#5. Specifically, >99% pure, lyophilized ascr#2 and ascr#5 (synthesized as described elsewhere (22)) were dissolved in LC-MS grade ethanol and added to the treatment flasks to a final concentration of 1 µM. Control flasks received ethanol at the same concentration. Animals were treated for 6 h in a shaking incubator at 20 °C and 220 RPM and then rapidly transferred to 15 ml falcon tubes, pelleted at 200 RCF in a centrifuge at 4 °C, washed twice with 15 ml of 4 °C M9, transferred by glass Pasteur pipette to a 2 ml round-bottom tube, and the tubes snap frozen by liquid nitrogen in the smallest amount of M9 achievable without disturbing the pellet. C. elegans pellets were stored at ~80 °C until further processing.

Lysis and Protein Isolation—C. elegans pellets were thawed by adding 800 µl of ice cold lysis buffer (50 mM HEPES pH 7.5, 10 mM KCl, 100 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 10% glycerol, 0.1% Tween-20, 1× Roche Complete Mini EDTA-Free protease inhibitor tablets, 1× Roche PhosSTOP phosphatase inhibitor tablets, 10 mM nicotinamide and 10 mM sodium butyrate deacetylase inhibitors, 1× benzamidine to digest nucleic acids) and rotating on a Nutator in the cold room. Deacetylase inhibitors were included to enable analysis of lysine acetylation, however we ultimately did not perform that experiment. The thawed pellets were centrifuged at 1000 RCF and 4 °C for 1 min and washed 3 times with 800 µl of ice cold lysis buffer. After the final wash, 1 ml of lysis buffer was added to the pellet along with a 5 mm stainless steel ball (Retsch, Haan, Germany). Lysis was performed in the cold room using a Retsch 400 MM mill mixer. Lysis was completed after three 10 min cycles at a setting of 30 Hz, with 4 min freeze-thaws after the first and second cycles. Freeze-thaws were performed by immersion in liquid nitrogen for 1 min, then returning to liquid state by immersion in room temperature water for 3 min. This method yielded complete tissue lysis as confirmed by observing a small aliquot of final lysate at 40× magnification. Lysate was then cleared by centrifugation for 15 min at 16,100 RCF and 4 °C. Protein concentration was determined by Bradford assay. Cleared lysate was then transferred to a 15 ml falcon tube for chloroform/methanol protein precipitation as previously described (37).

Mass Spectrometry—Protein pellets were resuspended in 8 M urea, 50 mM Tris pH 8, 100 mM NaCl, 1 mM CaCl2, 1× Roche Complete Mini EDTA-Free protease inhibitor tablet, and 1× Roche PhosSTOP phosphatase inhibitor tablet. Proteins were reduced with dithiothreitol and alkylated with iodoacetamide. The urea concentration was diluted to 4 M with 50 mM Tris, 100 mM NaCl, and 1 mM CaCl2. The endoprotease LysC (Wako, Richmond, VA) was then added (1:50 w/w) and the digestion incubated at room temperature overnight. A subsequent addition of LysC (1:100 w/w) was performed for 1 h to ensure complete digestion. Peptides were desalted using a C18 solid phase extraction cartridge (Waters Sep-pak, Milford, MA). Peptides from each condition were then mixed prior to offline separation into 10 fractions via strong cation exchange chromatography. Five percent of each fraction was reserved for peptide abundance measurements, whereas the remainder was combined into four fractions which were subjected to phosphopeptide enrichment via immobilized metal affinity chromatography (38).

Samples were resuspended in 0.2% formic acid and subjected to online reversed-phase chromatography with an easyLC 1000 (Thermo Scientific, San Jose, CA). One technical replicate injection was performed for each protein fraction, whereas two were performed for each phosphopeptide fraction. Peptides were loaded onto an in-house packed analytical column (75 µm ID, packed with 1.7 µm BEH C18 particles, Waters) for 13.3 min at a flow rate of 300 nL/min. Samples were eluted using an 80 min gradient (0 min: 5%, 18 min: 12%, 65 min: 23%, 75 min: 30%, 80 min: 70% acetonitrile with 0.2% formic acid) at a flow rate of 350 nL/min. The LC was coupled to an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Medium-resolution MS scans in the Orbitrap (30,000 resolving power) guided data-dependent sampling (top 20 most intense peptides) and CAD fragmentation (NCE = 35) in the ion trap. A high-resolution MS scan (480,000 resolving power) in the Orbitrap followed the initial MS scan to reveal the NeuCode peaks and enable quantification (6, 39). Preview mode and predictive AGC were enabled, and peptides with unassigned or +1 charge states were excluded from MS/MS analysis. A maximum of 500 precursors were dynamically excluded for 45 s (-25 and +15 ppm surrounding the precursor and its isotopes). Target ion accumulation values were set to 1 × 106 (MS) and 5 × 105 (MS/MS).

Data Analysis—Spectra were searched with OMMSSA using COMPASS (v. 1.4) against the Uniprot/TREMBL C. elegans canonical and isoform database using a target-decoy approach (downloaded June 2014, 27,014 entries) (40–43). Enzyme specificity was set to LysC, with up to 3 missed cleavages. Oxidation of methionine was set to a variable modification, whereas carbamidomethylation of cysteine was used for a fixed modification. To account for the NeuCode mass shift, a single fixed modification representing the average mass increase of the K602 and K080 lysine isotopologues (+8.0322 Da), as compared with unmodified lysine, was included. Precursor and fragment mass tolerances were 150 ppm and 0.35 Da, respectively. The relatively wide precursor mass tolerance was used to account for the mass difference observed between isotopologues (6, 34, 44). Phosphorylation on serine, threonine, and tyrosine was included as a variable modification where appropriate. Peptides were filtered to a 1% FDR and combined to protein groups based on the rules of parsimony, with at least two peptides per protein (45). Phosphosite localization was performed with PhosphoRS (v. 3.0) (46).

Quantification was performed with in-house developed software that utilizes previously described algorithms (6) (Merrill, A. E., Bailey, D. J., Kwiecien, N. W., Hebert, A. S., Rose, C. M., Westphall, M. S., Coon, J. J., NeuQuant: open-source software for neutron-encoded protein quantification technologies. Manuscript in Preparation). Peptides will only be quantified if they meet the following criteria. First, the peptide must be theoretically resolvable at FTWAT with the given isotopologue spacing and resolving power. Second, the extracted isotopologue features must exhibit the proper spacing. Third, any features whose abundances do not match the predicted isotopic distribution of the peptide (to within ~20% error) will be discarded. Fourth, any remaining features must fall within the elution profile of the peptide. Finally, each individual feature must satisfy signal-to-noise and mass accuracy thresholds calculated from the entire population of extracted features. If a peptide had three or more quantitative measurements remaining, it will be quantified by summing the channel intensities across all measurements. Quantitative values were log2-transformed and mean-normalized. Ratios were calculated by subtracting the average mean-normalized, log2-transformed intensity of the control from the treatment and then performing the inverse-log operation to look at raw fold change. For protein quantification, statistical significance was assessed via t test (p value ≤ 0.05) using
the Benjamini-Hochberg correction for multiple tests (47). For phosphoprotein quantification, only phosphoisoforms with a localization probability greater than or equal to 75% were considered. Differential expression was assessed by considering only phosphoisoforms with a mean fold-change cutoff of 1.5 from at least two replicates, with a coefficient of variation $\leq$ 50% (48–50).

Fig. 1. NeuCode experimental design. Unlabeled larvae were grown on E. coli labeled with $K_{602}$, $K_{341}$, or $K_{080}$ to young adult, then transferred to liquid culture for 6 h for treatment with ascarosides (or ethanol control). Experiments were performed in biological triplicate, with the labels for each condition switched for each replicate.

Fig. 2. NeuCode scan sequence. A, MS scan from an LC-MS/MS analysis of a C. elegans fraction. Inset, the first MS is collected at a resolving power of 30,000, whereas the second, quantitative MS is collected at a resolving power of 480,000. B, MS/MS spectrum following collision-activated dissociation and ion trap analysis.
Motif Analysis and Phosphoprotein Interaction Network—Motifs were calculated for all ascr#2 and ascr#5 significant, localized phosphopeptides using Motif-X v. 1.2 (51), using a motif width of 13, a minimum occurrence threshold of 20, a significance threshold of 0.000001, and with the SwissProt C. elegans Proteome set as the background option. The central residue was “s” for serine. Predicted functional protein-association networks among the differential phosphoproteins were determined using STRING version 9.1 with default parameters at high confidence (52). The organism was set as C. elegans.

RESULTS

Quantitative Proteome Analysis—Three NeuCode lysine isotopologues (K<sub>602</sub>, K<sub>341</sub>, K<sub>080</sub>) were incorporated into overnight cultures of E. coli cells until full heavy lysine incorporation was achieved. Then the NeuCode E. coli cells were plated for consumption by C. elegans for ~55 h. Next, adult worms were washed from the plates into liquid cultures where they were treated with ascarosides, in physiologically relevant amounts, for six hours (21). Following treatment, C. elegans were harvested, lysed, and the extracted protein precipitated (Fig. 1). Each condition was enzymatically digested with endo LysC. The peptide mixtures were combined into triplicate sets and fractionated by strong cation exchange chromatography (SCX). Phosphorylated peptides were enriched from each fraction for nanoLC-MS/MS analysis. Note a small amount of each fraction was analyzed without enrichment for analysis of protein abundance. To facilitate peptide identification and NeuCode quantitative analysis we used a medium resolution (30,000 @ m/z 400) MS survey scan (Orbitrap) to inform a data-dependent top 20 MS/MS method (ion trap, Fig. 2). Concurrent with ion trap MS/MS, a high resolution (480,000 @ m/z 400) MS scan was performed in the Orbitrap, revealing quantitative information for all three NeuCode channels (Fig. 2, inset). This three-plex experiment (two ascarosides and one control) was performed in biological triplicate where the NeuCode label was rotated among the conditions to control for potential label effects (Fig. 1).

Overall, we identified 6,596 proteins with high inter-replicate overlap: 5,252 proteins with quantitative information across all replicates (Fig. 3A). This enables robust statistical analysis and high-confidence protein identification. Data pertaining to the 6,596 identified proteins are presented for reference in supplemental Table S1. Not surprisingly, these data did not reveal any statistically significant perturbations in protein abundance because of ascaroside treatment, a result possibly stemming from subtle differences in developmental stage of animals, which is very difficult to control within and across replicates. Alternatively, the largely unchanged proteome may suggest that early (~6 h) pheromone responses to ascarosides rely largely on protein post-translational modification. Of note, our analyses detected one of the two proteins previously reported to be down-regulated in C. elegans larvae in response to ascr#2 and ascr#5, the TGFβ signaling peptide DAF-7; however, DAF-7 levels did not significantly differ between ascaroside-treated and untreated adult worms. Given that the previous work reporting ascaroside-dependent changes in DAF-7 expression used worms at a different developmental stage and employed much higher ascaroside concentrations than the current study, the results may not be directly comparable (25).

Quantitative Phosphorylation Analysis—Given that protein phosphorylation is essential for many signal transduction pathways, we next measured ascaroside-mediated changes in the phosphoproteome, identifying 6,475 sites of phosphorylation (localization probability ≥ 75%). Note some of the detected phosphopeptides contain more than one site of phosphorylation. Consider a peptide having the sequence ArgAlaValAspAspSerSerAspGluSerAlaArgLeuValLys. Even though we observe only two sites of phosphorylation, we
detect three unique phosphopeptide sequences: ArgAlaValAspAsp-pSer-pSerAspGluSerAlaArgLeuValLys, ArgAlaValAspAspSer-pSerAspGluSerAlaArgLeuValLys, ArgAlaValAspAsp-pSer-pSerAspGluSerAlaArgLeuValLys. To simplify quantification we refer to each of these permutations as a phosphorylation isoform, of which we detect and quantify 6,620. The overlap between the three replicates is lower than observed for protein abundance measurements, as is expected because of separate PTM enrichments performed for each replicate (Fig. 3B). We focused our analyses on the subset of phosphoisoforms with both a fold change above 1.5 and a coefficient of variation below 50% in at least two biological replicates (highlighted in Fig. 4). Our data indicate the ascarosides induce changes in post-translational regulation as part of their signaling mechanism. We observed that only 11% of these phosphoisoforms shared fold change direction and magnitude between ascr#2 and ascr#5. This modest overlap suggests small structural differences in related pheromone signaling molecules can elicit distinct molecular responses. Interestingly, although the two ascarosides often appear to induce the same effect on a given site, they may also induce opposite effects (Fig. 4). We present all of the phosphorylated peptides with their modification sites in supplemental Table S2.

The assortment of altered phosphoisoforms includes many regulators of stress response and longevity. Interestingly, there is significant cross-talk between these two pathways in C. elegans and other organisms (53, 54). The set of ascr#2-regulated phosphoproteins contains 22 phosphorylated proteins with established or emerging roles in stress response (55–69) and 24 phosphorylated proteins with roles in aging and longevity (59, 63, 64, 66, 67, 70–81), some of which overlap (Table I). Several of these proteins harbor multiple differentially regulated phosphorylation sites. Similarly, the set of ascr#5-regulated phosphoproteins contains 17 proteins with roles in stress response (57, 61–66, 69, 70, 75, 82–90) and 31 with roles in aging and longevity (61, 63, 65, 66, 74, 75, 77–79, 81, 85, 91–98). From both the ascr#2 and ascr#5 data sets, the protein with the greatest number of altered phosphoisoforms was LEA-1 (Table II), which belongs to the conserved family of LEA proteins that are hypothesized to act as chaperone-like “molecular shields,” especially during desiccation and other stresses. LEA-1 transcripts and proteins are more expressed in long-lived daf-2 insulin/IGF-1 signaling knockout mutants (66, 91). Dauer larvae synthesize LEA-1, perhaps to survive water loss in their long-lived state (99). The five most phosphorylated proteins from each ascaroside data set include other proteins with emerging roles in stress response and/or lifespan determination, including UNC-44, HSR-9, and T07C4.3. UNC-44 is an ankyrin-like protein that is also dysregulated in daf-2 mutants, suggesting that its regulation plays a role in stress response and longevity (66). HSR-9 is a DNA damage response regulator (100). T07C4.3 is an uncharacterized protein that prevents aggregation of misfolded proteins in neurons (65).

To investigate which kinases are associated with phosphoproteomic changes following ascaroside treatment, we used the Motif-X tool to search for enriched amino acids around ascr#2- and ascr#5-dependent phosphorylation sites (51). We focused on serine phosphorylation sites for this analysis given their much higher abundance in our data than threonine and tyrosine sites. Both proline-directed (ser-Pro) and acidophilic
### TABLE I

| Gene Name | Phosphorylation Site | Implicated in: | References |
|-----------|----------------------|----------------|------------|
| **ascr#2** |                      |                |            |
| AAK-2     | Thr\(^{597}\); Ser\(^{601}\) | Stress response and lifespan | 55, 71 |
| ACBP-1    | Ser\(^{20}\)         | Lifespan       | 72         |
| BRE-1     | Ser\(^{17}\)         | Stress response | 56         |
| BRP-1     | Ser\(^{176}\)        | Stress response and lifespan | 61 |
| C25H3.6/MDT-26 | Thr\(^{211}\) | Lifespan       | 70         |
| C26B2.2   | Ser\(^{34}\)         | Lifespan       | 73         |
| CDC-48.1  | Ser\(^{767};\) Ser\(^{203}\) | Stress response | 57 |
| DAO-5     | Ser\(^{21}\)         | Stress response and lifespan | 61, 74 |
| DNJ-11    | Ser\(^{43}\)         | Stress response | 58         |
| F13G3.10  | Ser\(^{41}\)         | Lifespan       | 77         |
| FRM-1     | Ser\(^{402};\) Ser\(^{476};\) Ser\(^{407}\) | Stress response and lifespan | 61 |
| FZY-1     | Ser\(^{457}\); Ser\(^{458}\) | Lifespan       | 75         |
| HCF-1     | Ser\(^{551}\)        | Stress response and lifespan | 59 |
| IRE-1     | Ser\(^{372}\)        | Stress response and lifespan | 60,76 |
| K01G5.5   | Thr\(^{118}\)        | Lifespan       | 61         |
| K08E3.5   | Ser\(^{93}\)         | Lifespan       | 73, 77     |
| LEA-1     | Ser\(^{128};\) Ser\(^{258};\) Ser\(^{305};\) Ser\(^{80}\); Ser\(^{16};\) Ser\(^{976};\) Ser\(^{1068};\) Ser\(^{1074};\) Ser\(^{1382};\) Ser\(^{1385}\) | Stress response and lifespan | 66, 99 |
| LIG-1     | Ser\(^{745};\) Ser\(^{748}\) | Stress response | 61 |
| MAK-2     | Ser\(^{343}\)        | Lifespan       | 78         |
| NOL-1     | Ser\(^{580}\)        | Lifespan       | 79         |
| OSM-10    | Ser\(^{158}\)        | Stress response | 62         |
| PPTR-1    | Ser\(^{531};\) Ser\(^{634}\) | Stress response and lifespan | 63 |
| PRDX-2    | Ser\(^{92}\)         | Stress response and lifespan | 64 |
| SIP-1     | Ser\(^{151}\)        | Lifespan       | 66         |
| T03F1.1/UBA-5 | Ser\(^{43}\) | Stress response and lifespan | 67 |
| T07C4.3   | Ser\(^{116};\) Ser\(^{219};\) Ser\(^{220};\) Ser\(^{554}\) | Stress response and lifespan | 65 |
| TDP-1     | Ser\(^{18}\)         | Lifespan       | 80         |
| TOP-1     | Ser\(^{65}\)         | Stress response and lifespan | 61, 70 |
| TRAP-1    | Thr\(^{24};\) Ser\(^{246}\) | Stress response | 61 |
| UNC-16    | Ser\(^{789};\) Ser\(^{797}\) | Stress response and lifespan | 66 |
| UNC-44    | Ser\(^{3017};\) Ser\(^{3022};\) Ser\(^{3026};\) Ser\(^{3947};\) Ser\(^{4196};\) Ser\(^{5156};\) Ser\(^{6073};\) Ser\(^{6575}\) | Lifespan | 66 |
| UNC-54    | Ser\(^{146};\) Ser\(^{1291}\) | Stress response | 61 |
| UNC-89    | Ser\(^{1552};\) Ser\(^{1559}\) | Stress response | 69 |
| VIT-2     | Ser\(^{1499};\) Tyr\(^{1502};\) Thr\(^{1507}\) | Lifespan       | 66, 81, 91 |
| Y47G6A.18 | Ser\(^{311};\) Ser\(^{353}\) | Lifespan       | 70         |
| **ascr#5** |                      |                |            |
| ABL-1     | Tyr\(^{462}\)        | Stress response | 82         |
| BRP-1     | Ser\(^{176}\)        | Lifespan       | 61         |
| C03H5.3   | Ser\(^{48}\)         | Lifespan       | 93         |
| C36H8.1   | Ser\(^{12};\) Ser\(^{53};\) Ser\(^{275}\) | Lifespan | 73 |
| CDC-48.1  | Ser\(^{248}\)        | Stress response | 57         |
| CEY-2     | Ser\(^{163}\)        | Lifespan       | 81         |
| CST-1     | Ser\(^{380}\)        | Lifespan       | 95         |
| DAO-5     | Ser\(^{253};\) Ser\(^{652}\) | Stress response and lifespan | 61, 74 |
| EGL-45    | Ser\(^{978}\)        | Lifespan       | 79         |
| F23F1.5   | Ser\(^{74}\)         | Lifespan       | 70         |
| F53F4.11  | Ser\(^{158}\)        | Lifespan       | 79         |
| FZY-1     | Ser\(^{27}\)         | Lifespan       | 79         |
| HDA-3     | Ser\(^{444}\)        | Stress response | 83         |
| HTP-3     | Ser\(^{520}\)        | Lifespan       | 79         |
| IFG-1     | Ser\(^{311};\) Thr\(^{315};\) Ser\(^{328};\) Thr\(^{329}\) | Stress response and lifespan | 85 |
| IMMT-1    | Ser\(^{253}\)        | Stress response | 84         |
| K08E3.5   | Ser\(^{93}\)         | Lifespan       | 73, 77     |
| LEA-1     | Thr\(^{477};\) Ser\(^{589};\) Ser\(^{706};\) Ser\(^{971};\) Ser\(^{1056};\) Ser\(^{1074}\) | Stress response and lifespan | 66, 99 |
| LIG-1     | Ser\(^{406};\) Ser\(^{34}\) | Lifespan | 78 |
| MAK-2     | Ser\(^{433}\)        | Lifespan       | 78         |
| MET-1     | Ser\(^{1499}\)       | Lifespan       | 96         |
sequences (ser-x-Glu, ser-Asp-x-Glu, ser-x-x-Asp, ser-Glu, and ser-Asp-x-Asp) were enriched, whereas fewer basophilic sequences (ser-Pro-x-Lys, Arg-x-x-ser, Lys-x-x-ser) were enriched (Fig. 5). The primary motif was ser-Pro, which may represent phosphorylation by mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR), or cyclin dependent kinase (CDK) family members. Specifically, among phosphosites up-regulated by ascr#2, the Ca^{2+}/calmodulin-dependent kinase 2 (CAMK2) motif Arg-x-x-ser was enriched, whereas down-regulated phosphosites demonstrated a preference for the casein kinase 2 (CK2) motifs ser-Asp-x-Glu and ser-x-x-Asp, the Golgi-casein kinase (G-CK) motif ser-x-Glu, and a novel motif ser-Glu (51). In the ascr#5 data, up-regulated phosphosites were enriched for the following motifs: proline-directed and basophilic CDK (ser-Pro-x-Lys), CK2 (ser-x-x-Asp, ser-Asp-x-Asp, ser-Asp-x-Glu), and CAMK2 (Arg-x-x-ser, Lys-x-x-ser) (101–103). Among the down-regulated phosphosites, the single most enriched motif was ser-Pro. Together, these data suggest that MAPK, mTOR, CDK, CK2, CAMK2, and G-CK are among the kinases

| Gene Name | Phosphorylation Site | Implicated in: | References |
|-----------|---------------------|----------------|------------|
| NOL-1     | Ser^{580}           | Lifespan       | 79         |
| NPA-1     | Ser^{1029}          | Lifespan       | 66         |
| OSM-10    | Ser^{158};Ser^{161} | Stress response| 62         |
| PEPT-1    | Ser^{4}             | Lifespan       | 98         |
| PPTR-1    | Ser^{531}           | Stress response and lifespan | 63 |
| RICT-1    | Ser^{605};Ser^{607};Ser^{1209};Ser^{1246} | Lifespan | 97 |
| RPN-8     | Ser^{385}           | Lifespan       | 92         |
| SGP-1     | Ser^{268}           | Stress response and lifespan | 89, 90 |
| SMN-1     | Ser^{133}           | Lifespan       | 94         |
| T07C4.3   | Ser^{219},Ser^{220},Ser^{247},Ser^{254} | Stress response and lifespan | 65 |
| TOP-1     | Ser^{255},Ser^{256} | Stress response and lifespan | 61, 70 |
| TPA-1     | Ser^{345}           | Lifespan       | 79         |
| TRAP-1    | Thr^{244},Ser^{246} | Stress response | 61 |
| UNC-89    | Ser^{1489};Ser^{1529};Ser^{1559};Ser^{1700} | Stress response and lifespan | 69 |
| VHP-1     | Ser^{105}           | Stress response | 88         |
| VIT-2     | Ser^{1038};Ser^{1504};Ser^{1507} | Lifespan | 66, 81, 91 |
| VIT-3     | Ser^{1482};Ser^{1504} | Lifespan | 66, 81 |
| VIT-4     | Ser^{1502};Ser^{1504} | Lifespan | 66, 81 |
| Y47D3A.29| Ser^{163};Ser^{164} | Stress response and lifespan | 75 |

| Table I—continued |
|-------------------|

| Gene Name | Phosphorylation Site | Implicated in: | References |
|-----------|---------------------|----------------|------------|
| LEA-1     | Ser^{126}           | Lifespan       | 79         |
| LEA-1     | Ser^{298}           | Lifespan       | 66         |
| LEA-1     | Ser^{109}           | Lifespan       | 98         |
| LEA-1     | Ser^{584}           | Stress response and lifespan | 63 |
| LEA-1     | Ser^{816}           | Lifespan       | 97         |
| LEA-1     | Ser^{976}           | Lifespan       | 92         |
| LEA-1     | Ser^{1068}          | Stress response and lifespan | 89, 90 |
| LEA-1     | Ser^{1074}          | Lifespan       | 79         |
| UNC-44    | Ser^{382};Ser^{385} | Stress response | 61 |
| UNC-44    | Ser^{3002};Ser^{3006} | Stress response and lifespan | 69 |
| UNC-44    | Ser^{3947}          | Stress response | 88         |
| UNC-44    | Ser^{196}           | Lifespan       | 94         |
| UNC-44    | Ser^{1556}          | Stress response and lifespan | 65 |
| UNC-44    | Ser^{6573},Ser^{6575} | Lifespan | 66, 81, 91 |
| ATT-2     | Ser^{258}           | Lifespan       | 79         |
| ATT-2     | Ser^{209}           | Lifespan       | 66         |
| ATT-2     | Ser^{292}           | Lifespan       | 98         |
| ATT-2     | Ser^{3022},Ser^{3026} | Stress response and lifespan | 63 |
| ATT-2     | Ser^{1196}          | Lifespan       | 97         |
| ATT-2     | Ser^{164}           | Lifespan       | 92         |
| ATT-2     | Ser^{164}           | Lifespan       | 88         |
| CPNA-2    | Ser^{420},Ser^{427} | Lifespan       | 79         |
| CPNA-2    | Ser^{4649}          | Lifespan       | 81         |
| CPNA-2    | Ser^{777}           | Lifespan       | 79         |
| T07C4.3   | Ser^{216},Ser^{219},Ser^{220} | Lifespan | 66, 81 |
| T07C4.3   | Ser^{254}           | Lifespan       | 75         |

| Table II |
|----------|

Top five most phosphorylated proteins from each treatment by number of phosphosites

| Five Most Phosphorylated Proteins |
|-----------------------------------|
| Gene # sites | Isoforms | Fold Δ |
| LEA-1         | Ser^{126} | 1.54   |
| UNC-44        | Ser^{3022},Ser^{3026} | 1.72   |
| ATT-2         | Ser^{196} | 1.64   |
| CPNA-2        | Ser^{420},Ser^{427} | 2.21   |
| T07C4.3       | Ser^{216},Ser^{219},Ser^{220} | 1.91   |

| Gene # sites | Isoforms | Fold Δ |
| LEA-1         | Thr^{244},Ser^{246} | 1.91   |
| UNC-44        | Ser^{127} | 1.54   |
| ATT-2         | Ser^{196} | 1.64   |
| CPNA-2        | Ser^{420},Ser^{427} | 2.21   |
| T07C4.3       | Ser^{216},Ser^{219},Ser^{220} | 1.91   |
specifically activated or suppressed in response to ascaroside sensing.

**Interaction Network Analysis**—To generate a large-scale view of relationships between potential ascaroside-regulated phosphoproteins, we derived an interaction network for all phosphoproteins in each treatment group using experimentally known or computationally predicted interactions from the STRING database. This analysis yielded distinct networks for ascr#2 and ascr#5, yet a common signature of stress- and longevity-related proteins emerged (Fig. 6). The ascr#2 network included 99 of 338 significant ascr#2 phosphoproteins with 162 predicted interactions (Fig. 6A). The ascr#5 network included 122 of 318 significant ascr#5 phosphoproteins with 265 predicted interactions (Fig. 6B). Both networks were significantly enriched (p < 0.01) for protein-protein interactions. The phosphorylation profiles encapsulated in Figs. 5 and 6 may represent marks of post-translational modification that explain, in part, ascaroside-mediated lifespan extension and increased stress tolerance in adult animals.

![Motif Analysis](image)

**Fig. 5. Motif Analysis.** Significantly enriched motifs around phosphosites determined using Motif-X (p < 0.01) presented with likely associated kinases. The *C. elegans* Swissprot protein database was used as background to normalize the score against random peptide sequences.
DISCUSSION

NeuCode is a versatile strategy well-suited for robust and accurate protein quantification in a variety of paradigms (34, 39, 44, 104–108). By using mass resolving power to reveal or conceal quantitative information when desired, NeuCode SILAC affords greater sampling depth than traditional SILAC, while retaining comparable accuracy. Here we have extended this approach to nematode labeling, quantifying changes in both the proteome and phosphoproteome of adult animals in response to small molecule chemical signals. We quantified

**Fig. 6.** *Protein-protein interaction network.* Generated using predictions from STRING version 9.1 with default parameters at high confidence for *A*, ascr #2. *B*, ascr#5.
3,053 localized phosphoisoforms in at least two biological replicates, 741 of which were up- or down-regulated in response to at least one ascaroside treatment. This demonstrates the broad applicability of NeuCode SILAC for the quantitative analysis of proteins and PTMs.

Our data from two ascaroside family members did not reveal any significant ascaroside-induced changes in overall protein expression, suggesting that, at the time scale of this experiment, ascarosides may act primarily via translation-independent mechanisms. In contrast, we observed a vast array of changes in protein phosphorylation, suggesting an important role for PTMs in pheromone activity. The lack of significant changes in protein expression may also be because of technical limitations in the reproducibility of experimental conditions (e.g., ascaroside exposure time, temperature, or variation in synchronizing a large population of adults). In addition, small but nonetheless biologically relevant fold changes in protein levels may escape detection simply because of the relatively small number of replicates in this study (109).

Our global phosphoproteomic data reflect post-translational changes in stress response and longevity proteins from multiple pathways. The most phosphorylated protein from each data set is LEA-1, a protein with an emerging role in these two processes. Furthermore, LEA-1 is dysregulated at the transcript and protein level in long-lived daf-2 mutants (66, 99). Consistent with global phosphoproteomic studies in other organisms (110), we found that individual phosphorylation sites on the same protein were often regulated differently. Proteins with multiple regulated phosphorylation sites may represent key signaling hubs, integrating crucial inputs to affect increases in longevity and stress tolerance. The regulated phosphoproteins also include RNA-binding proteins, ribosomal proteins, and transcriptional regulators, suggesting that ascaroside signaling impacts many levels of gene regulation. In addition, phosphorylation likely works in synergy with other PTMs. Recent work implicates a sirtuin (NAD-dependent deacetylase) in ascaroside-mediated longevity and stress resistance in adult animals (111). Our data indicate a role for post-translational modification by select kinases.

Systems-level analyses expanded our view to consider possible relationships between regulated phosphoproteins. Some of the proteins suggested by our data set to exhibit ascaroside-dependent changes in phosphorylation patterns already have established roles in stress responses and/or aging (Table I). For example, IRE-1 is a master regulator of the endoplasmic reticulum (ER) stress response pathway and plays a key role in the unfolded protein response (UPR). IRE-1 is also necessary for the increase in lifespan that is induced by dietary restriction or by hypoxia inducible factor-1 (hypoxia, hypoxic, ischemic, or thermal), or other conditions that perturb the normal AMP:ATP ratio. Activated AAK-2 can increase lifespan and broadly increase stress resistance (55, 71). PPTR-1 is a phosphatase that regulates longevity and stress response in part by modulating DAF-16/FOXO (63). NOL-1 is a conserved nucleolar tRNA and rRNA cysteine-C5-methylase and knockdown of NOL-1 significantly increases lifespan in adult animals (79). TRAP-1 and FRM-1 show increased expression in stressed wild-type animals (61). NPA-1 (increased abundance in daf-2 animals) is critical for dauer formation and thus plays a role in longevity (66). These marks of post-translational modification on stress- and longevity-related proteins may increase stress tolerance and lifespan. Further, known modulators may interact with a broad net of other diverse proteins, including those with emerging significance in stress response and longevity or with as yet undiscovered roles.

Our phosphorylation data align with recent phenotypic and molecular studies of ascaroside exposure in adult animals (111). We found differential phosphorylation of many proteins associated with stress and longevity, some of which were identified by previous work on the daf-2 and daf-16 pathways and others that were not, although our experiments did not specifically target for the daf-2 and/or daf-16 pathways or known phosphorylation events in these two pathways. Our data help elucidate the molecular details of how ascaroside sensing is manifested phenotypically. However, our general understanding of stress response and lifespan at the level of protein phosphorylation and protein-protein interactions is still in its infancy. Phosphorylation can have many consequences in the cell (e.g., activate or deactivate a protein, modulate its stability, target it for degradation, or alter its localization) and it is unclear how the phosphorylation events in our data relate to these outcomes. Further in vivo studies on specific phosphorylation sites detected here will shed light on this and increase our understanding of pheromone responses in C. elegans and other animals, which remains largely unexplored.

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[§§] This article contains supplemental Tables S1 and S2.

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