N-Glycosylation Regulates Fibroblast Growth Factor Receptor/EGL-15 Activity in Caenorhabditis elegans in Vivo

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The regulation of cell function by fibroblast growth factors (FGFs) classically occurs through a dual receptor system of a tyrosine kinase receptor (FGFR) and a heparan sulfate proteoglycan co-receptor. Mutations in some consensus N-glycosylation sites in human FGFR result in skeletal disorders and craniosynostosis syndromes, and biophysical studies in vitro suggest that N-glycosylation of FGFR alters ligand and heparan sulfate binding properties. The evolutionarily conserved FGFR signaling system of Caenorhabditis elegans has been used to assess the role of N-glycosylation in the regulation of FGF signaling in vivo. The C. elegans FGF receptor, EGL-15, is N-glycosylated in vivo, and genetic substitution of specific consensus N-glycosylation sites leads to defects in the maintenance of fluid homeostasis and differentiation of sex muscles, both of which are phenotypes previously associated with hyperactive EGL-15 signaling. These phenotypes are suppressed by hypoactive mutations in EGL-15 downstream signaling components or activating mutations in the phosphatidylinositol 3-kinase pathway, respectively. The results show that N-glycans negatively regulate FGFR activity in vivo supporting the notion that mutation of N-glycosylation sites in human FGFR may lead to inappropriate activation of the receptor.

Additional tissue specificity is generated by alternative splicing of the FGF receptor genes. For example, splicing of the third immunoglobulin domain (D3) to B and C isoforms is tissue-dependent and determines a degree of the ligand binding specificity. Some of the FGF ligands, such as FGF1, are promiscuous and bind to all FGFRs, whereas other ligands bind only specific FGFR isoforms and/or splice variants (5, 6). In canonical FGF signaling, the growth factor is thought to assemble in a ternary complex with the FGFR and a glycosaminoglycan co-receptor, usually heparan sulfate. Specific heparan sulfate structures can either activate or inactivate FGFR signaling (7).

Mutations in genes encoding FGF receptors give rise to a variety of human disorders and diseases (for review see Ref. 8). The activating mutations lead to excessive receptor signaling (9, 10), increased ligand binding affinity (11, 12), or altered ligand specificity (13), whereas inactivating mutations such as in Kallmann syndrome result in decreased signaling (14). Some of the activating mutations of human FGF receptors 1–3 abolish asparagine residues in consensus N-glycosylation sites and result in a diverse group of skeletal disorders. These mutations include asparagine 330 to isoleucine (N330I) in FGFR1 (15), asparagine 331 to isoleucine (N331I) in FGFR2 (16), and asparagine 328 to isoleucine (N328I) in FGFR3 (17), which lead to osteoglophonic dysplasia, Crouzon syndrome, and hypochondroplasia, respectively.

Studies using soluble and membrane-bound recombinant receptors produced in mammalian cells corresponding to the extracellular ligand binding domain of FGFR1 IIIc have demonstrated extensive and highly heterogeneous N-glycosylation of FGF1 (18). Furthermore, N-glycosylation of FGFR1 IIIc regulates the association of FGFR2 and heparin to the receptor by decreasing the association rate of the respective binding reactions, suggesting that N-glycosylation of the receptor may negatively regulate signaling through FGFR (18).

Glycosylation is one of the most common post-translational modifications, thought to occur in more than 50% of all proteins in eukaryotes (19). Although a large number of membrane proteins, including growth factor receptors such as epidermal growth factor receptor (20) and ion channels, are heavily glycosylated, the role of the glycans for the regulation of the protein function is still poorly understood. N-Glycans and sialic acid modulate the gating of Shaker potassium channels (21). N-Glycosylation of BMP6 (bone morphogenetic protein 6) ligand, a member of the transforming growth factor-β superfamily, is suggested to provide receptor specificity and a recognition site for the activin receptor type I (22). Changes to N-glycan processing by expression of N-acetylgalactosaminyltransferase V (Mgat5), which promotes poly N-acetyllactosamine substitu-
tion, modifies mouse mammary cell responses to growth factors, including epidermal growth factor, FGF, and transforming growth factor-β and the endocytosis of their receptors (23). A large number of human congenital disorders of glycosylation caused by deficiency in N-glycosylation have been identified (for a review see Ref. 24). The congenital disorders of glycosylation result from mutations in enzymes involved in N-glycan synthesis and processing and are likely to be caused by changes in a large repertoire of N-glycan-modified proteins.

The Caenorhabditis elegans genome contains a single homologue of vertebrate FGFR, egl-15 (for egg laying-defective), and two homologues of FGF ligands, egl-17 and let-756 (for lethal; see Refs. 25–27). EGL-15/FGFR has two isoforms, EGL-15 (5A) and EGL-15 (5B), as a result of alternative splicing of the fifth exon (28). EGL-15 (5B) and LET-756 have essential function in C. elegans, and null mutations in the corresponding genes cause larval lethality. Null mutations in egl-15 (5A) and egl-17, however, lead to very specific cell migration and axon maintenance defects (25, 29). The intracellular signaling cascades activated by EGL-15 are relatively well characterized and share a high degree of conservation with mammalian FGFR (reviewed in Refs. 30–32). Hypoactive mutations in egl-15 lead to various degrees of phenotypic effects from scranny to egg laying-defective, whereas hyperactive mutants of egl-15 accumulate fluid in the body cavity and appear clear (Clr). Mutations in a phosphatase clr-1 also lead to the Clr phenotype and are suppressed by hypoactive mutations in egl-15 (33), suggesting that the Clr phenotype is caused by excess FGFR signaling.

The characteristic phenotypes of hypo- and hyperactive egl-15 mutations provide an excellent model in which to assess the role of N-glycosylation for FGFR regulation in vivo. Three of the consensus N-glycosylation sites of immunoglobulin domain 3 (D3) are conserved between human FGFRs and C. elegans EGL-15. Mutations in two of these conserved sites lead to skeletal disorders in humans. We have introduced alanine substitutions to the consensus sites leading to skeletal disorders in humans. We have introduced alanine substitutions to the consensus sites leading to skeletal disorders in humans. We have introduced alanine substitutions to the consensus sites leading to skeletal disorders in humans. We have introduced alanine substitutions to the consensus sites leading to skeletal disorders in humans.

**Experimental Procedures**

**Strains**—C. elegans strains were maintained at 20 °C essentially as described (34) unless otherwise stated. Wild type strain used in this study is N2 var. Bristol. The following previously described mutations were used: LGIV, daf-18 (ok480), soc-2 (n1774); LGV, akt-1 (mg144); LGX, egl-15 (n1456).

**DNA Constructs and Transgenic Lines**—All egl-15 constructs were derived from the plasmid NH112 (a gift from M. Stern), which contains a full-length wild type genomic hybrid (egl-15(5A+B++) of egl-15 DNA capable of rescuing egl-15 (lf) lethality (26). Asparagine to alanine mutations were introduced using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. A 1-kb Rsrl/BamHI fragment of NH112, which encodes for seven consensus N-glycosylation sites, was subjected to site-directed mutagenesis. Primer sequences for each construct can be obtained upon request. After mutagenesis, the 1-kb Rsrl/BamHI fragment was cloned into the original NH112 vector to replace the wild type Rsrl/BamHI fragment. All DNA constructs generated by PCR were confirmed by sequencing. Transgenic arrays were generated using standard germ line transformation techniques (35). All mutant DNA constructs were injected to heterozygous egl-15 (n1456;lf)/+ hermaphrodites at 2.5 ng/µl with either Pymo-3::gfp, Ptxx-3::rfp, or Psur-5::gfp at 50 ng/µl as injection markers and pBluescript. Wild type egl-15 construct (NH112) was injected at concentrations from 0.5 to 25 ng/µl as indicated in the figures and the text. All transgenic constructs were analyzed as extrachromosomal arrays. At least three independent transgenic lines were analyzed for each DNA construct.

**Analysis of Egg Laying**—Egg laying was measured essentially as described (36). Briefly, single hermaphrodites were placed on nematode growth medium agar plates seeded with Escherichia coli OP50 as a food source in the presence or absence of 7.5 mM serotonin (SHT; Sigma). The number of eggs laid was counted after 1 h. Significance of results was tested using z test.

**Purification of EGL-15 Protein**—C. elegans were ground in liquid nitrogen, and the frozen worm powder was solubilized in a buffer containing 1% Igepal (Nonidet P-40; Sigma) in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl. 1 µM phenylmethylsulfonfluoride, 2 mg/ml aprotinin, and 1 µg protease inhibitor mixture (Roche Diagnostics) were added to prevent proteolysis. Protein lysates were cleared by centrifugation at 10,000 × g for 30 min at 4 °C. In some experiments, the supernatants were applied to heparin-agarose beads (Sigma). Heparin-agarose was washed with 20-bead volumes of the binding buffer, followed by low salt washing with the solubilization buffer containing 0.20 M NaCl. Proteins were eluted either in a single step using 1.5 M NaCl or in a gradient of 0.20–1.5 M NaCl in the solubilization buffer. The eluted proteins were treated with N-glycosidase F (PNGaseF) or endoglycosidase H (Roche Diagnostics) for 2 h. Proteins were separated on 7.5% SDS-PAGE and transferred to Immobilon P membranes (Millipore, MA). Proteins were detected in Western blot using polyclonal anti-EGL-15 antibody Crackle (a gift from M. Stern) and anti-actin C4 (MP Biochemicals) and anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) and ECL chemiluminescence detection kit (Biological Industries, Beit Haemek, Israel).

**Microscopy**—Vulval muscles were visualized using rhodamine-conjugate phalloidin (Sigma) as described previously (37). For whole mount immunostaining, worms were fixed using a protocol modified from Finney-Ruvkun (38). Polyclonal anti-EGL-15 antibody, Pop (a gift from M. Stern), was used at a 1:10 dilution, as described previously (37), followed by fluorescently conjugated anti-rabbit secondary antibodies (Jackson Immunoresearch, West Grove, PA) at 1:100. Fluorescent and differential interference contrast images were acquired using a Zeiss AxioCam MRm camera mounted on a Zeiss Axioskop2 micro-
scope equipped with \( \times 10, \times 20, \times 40, \) and \( \times 63 \) optics. Images were captured using Axiovision and further cropped and scaled using Adobe Photoshop CS3.

RESULTS

Putative N-Glycosylation Sites of FGFR Immunoglobulin Domain 3 Are Conserved in Evolution—ClustalW alignment of the immunoglobulin (Ig)-like D3 of \( C. \) elegans, \( C. \) briggsae, \( D. \) melanogaster Breathless (Btl), and human FGFRs shows that three of the four putative \( N \)-glycosylation sites (Asn-Xaa-(Ser/Thr)) in the human FGFRs are conserved across the species (Fig. 1A). The only exception is \( C. \) briggsae, where one of the conserved asparagines (Asn-464) does not constitute a consensus \( N \)-glycosylation site, as it is followed by histidine rather than serine/threonine. \( C. \) elegans has a consensus \( N \)-glycosylation site (Asn-449; depicted as N5 in Fig. 1A) in the proximity of the fourth conserved consensus site of human FGFRs (317 in human FGFR1) and a site, designated N4, in proximity to the conserved N3 site. However, because sequence changes more rapidly than structure, it was important to identify if these putative \( N \)-glycosylation sites were conserved between worms and mammals at the level of their spatial position in the three-dimensional structure of the receptor.

Molecular modeling of the EGL-15 D2 and D3 shows that the three asparagines that are conserved across species, Asn-401, Asn-433, and Asn-474, are at the surface of the protein (supplemental Fig. S1) and occupy a similar spatial position to the orthologous sites in human FGFRs (18, 39, 40). Asparagine 449 of EGL-15 occupies a similar spatial position to asparagine 317 of human FGFR1, suggesting conserved functions for these residues, although they do not align in the two-dimensional sequence comparison. In the spatial comparison, asparagine 407 of EGL-15, which does not have an obvious orthologue in the human FGFRs, is in close proximity to asparagine 474. Mutations in two of the three sites conserved between human and \( C. \) elegans FGFRs have been associated with human skeletal syndromes. These include Asn-262 of human FGFR3 associated with hypochondroplasia (41), which is orthologous to Asn-401 in the \( C. \) elegans EGL-15 and Asn-330 of human FGFR1 associated with osteoglophonic dysplasia (15), which is orthologous to Asn-474 in \( C. \) elegans EGL-15. Mutations in orthologous sites (Asn-331) of human FGFR2 and of FGFR3 (Asn-328; see Ref. 17) lead to Crouzon syndrome and hypochondroplasia, respectively.

\( C. \) elegans EGL-15 has three additional consensus \( N \)-glycosylation sites in D3, all of which are conserved in the \( C. \) briggsae EGL-15 (Fig. 1A).
EGL-15 Is N-Glycosylated in Vivo—EGL-15 was purified from protein extracts of mixed stage wild type C. elegans. Polyclonal anti-EGL-15 antibody (Crackle) detects EGL-15 from mixed stage C. elegans as four different polypeptides migrating in SDS-PAGE with molecular masses of ~105, 95, 85, and 75 kDa (Fig. 2A). Two of the separate polypeptides are likely to represent the different isoforms of EGL15, 5A and 5B (42). It is well established that vertebrate FGFR receptors form ternary complexes with the FGF ligands and with heparan sulfate, a tissue form of heparin. EGL-15/FGFR could be enriched from complexes with the FGF ligands and with heparan sulfate, a well established that vertebrate FGF receptors form ternary complexes with heparin. EGL-15/FGFR could be enriched from fractions eluted from heparin-agarose with 1.5 M NaCl. The 105-kDa isoform of EGL-15 binds predominantly to heparin.

TABLE 1
Summary of phenotypes observed for EGL-15 Asn → Ala mutants

| Construct | Phenotype |
|-----------|-----------|
| WT (egl-15 (+)) | + | + | + | + | 0 |
| N401A | NA | NA | NA | NA | NA |
| N407A | + | + | + | + | 0 |
| N433A | + | + | + | 0 | 0 |
| N440A | + | + | + | 0 | 0 |
| N449A | + | + | + | 0 | 0 |
| N474A | + | + | + | 0 | 0 |
| N497A | + | + | + | 0 | 0 |
| N401A,N407A | + | + | + | 0 | 0 |
| N440A,N449A | + | + | + | 0 | 0 |
| N401A,N407A,N433A | + | + | + | 0 | 0 |
| N401A,N407A,N433A,N440A | + | + | + | 0 | 0 |
| N401A,N407A,N433A,N440A,N449A | + | + | + | 0 | 0 |
| N401A,N407A,N433A,N440A,N449A,N474A | + | + | + | 0 | 0 |

The abbreviations used are as follows: Egl, egg laying-defective; Clr, clear; Dpy, dumpy; Unc, uncoordinated movement; Pvl, protruding vulva. +/+ indicates weak phenotype; the number of + indicates severity of the phenotype; −/−, not detected; NA, not applicable.

During purification from protein extracts of mixed stage wild type C. elegans, even if it would not normally contain sugar modifications. This could either restore the original function or alter the function of the mutant EGL-15 protein. Hence, where two consensus N-glycosylation sites reside in close proximity, the sites were abolished individually and in combination (Fig. 1B). The wild type and mutant egl-15 (Asn → Ala) DNA constructs were introduced into an egl-15 (n1456) background. The n1456 allele is a loss-of-function (lf) mutation, and the parental strain was maintained as heterozygous. Transgenic expression of wild type egl-15 rescued larval lethality of the egl-15 (lf) almost completely (83% of all laid eggs survived through larval stages; n >1000) but did not fully rescue the egg laying phenotype (Egl). Phenotypes observed for single or multiple EGL-15 Asn → Ala mutants are summarized in Table 1. Substituting asparagine 433 of EGL-15 with alanine (EGL-15 N433A) led to subtle phenotypes, most notably the Egl phenotype (Fig. 3C; Table 1). Asparagine 433 is conserved in human FGFRs, and based on molecular modeling, it occupies a similar spatial position to the human counterpart. Mutations in the orthologous sites of the human FGFRs have not been associated with human disorders to date. EGL-15 N474A mutation led to more severe phenotypes, and most notably these mutants displayed the Clr phenotype (Fig. 3D; Table 1), which is characteristic of EGL-15 overactivation (33). The N474A is the only single mutant that showed the Clr phenotype. EGL-15 N474A mutants were also Egl and had uncoordinated movement. The EGL-15 N407A mutation did not lead to severe phenotypes, although these animals were slightly dumpy (Dpy; Table 1). Asn-407 does not have an orthologous site in the human FGFRs, but it is in close sequence proximity to Asn-401, which is orthologous to Asn-262 in human FGFR3. Asn-407 is also in close spatial proximity to Asn-474 (supplemental Fig. S1). Despite several independent attempts, we failed to obtain a transgenic strain.
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FIGURE 3. Phenotypic consequences of EGL-15 N→A mutations. A, wild type (N2 WT); B, transgenic expression of wild type egl-15 (+); C, egl-15 N433A animals are egg laying-defective; D, egl-15 N474A mutants display low penetrance of the Clr phenotype; E, almost 90% of egl-15 N401A,N407A,N433A,N440A,N449A,N474A mutants are Clr; F, Clr phenotype is suppressed in double mutants of soc-2 (n1774);egl-15 N401A,N407A,N433A,N440A,N449A,N474A; G, egl-15 N401A,N449A are also Clr, although the phenotype is slightly less severe than in N401A,N407A,N433A,N440A,N449A,N474A mutants; H, egl-15 N401A,N407A,N433A,N440A,N449A,N474A,N440A,N449A,N474A,N497A mutant. Scale bars 100 μm.

with the egl-15 N401A mutant DNA construct. The fact that several independent attempts failed suggests that the nature of the N401A mutation leads to the inability of the mutant EGL-15 to rescue the larval lethality of the egl-15 (l(2f)) background. Using the same conditions, we have successfully obtained at least three independent transgenic lines for 12 of the other egl-15 Asn→Ala mutant DNA constructs. Single amino acid substitutions of Asn-440, Asn-449, and Asn-497 led to weak phenotypes; most notably the animals were weak Egl (Table 1).

The observed phenotypes are likely to be associated with the specific mutation in EGL-15 rather than too much EGL-15 protein, as introducing the wild type copy of egl-15 DNA at the same DNA concentration does not lead to a clear phenotype, but although it rescues larval lethality, it also leads to the animals being Egl. EGL-15 protein levels were also very similar in wild type and transgenic C. elegans expressing either wild type or mutant egl-15 DNA constructs (Fig. 2A). We also tested the wild type egl-15 DNA at higher DNA concentrations, and although high DNA concentrations lead to increased embryonic lethality (not shown) presumably caused by over-expression of EGL-15 protein, we could not detect any Clr phenotype, suggesting multiple levels of regulation of EGL-15 signaling over and above that dictated by expression of the protein.

Multiple asparagine to alanine mutations lead to a marked increase of the Clr phenotype characteristic of excessive EGL-15 signaling (Fig. 3; Table 1). EGL-15 N401A,N407A,N433A triple mutant displayed intermediate penetrance of the Clr phenotype, and the severity of this phenotype increased as more mutations were introduced. Almost 90% of EGL-15 N401A,N407A,N433A,N440A,N449A,N474A mutants were Clr (Fig. 4). The clear phenotype was suppressed to 30% by inactivating mutation in soc-2 (soc = suppressor of clear; Fig. 4), a homologue of a mammalian leucine-rich repeat protein and a component of FGFR downstream signaling (44). These results strongly suggest that asparagine substitutions of consensus N-glycosylation sites lead to overactivation of EGL-15 signaling in C. elegans.

Single mutants N407A and N474A, as well as double mutants of N401A,N407A and N440A,N449A are uncoordinated. The uncoordinated movement phenotype may result from dysfunctional EGL-15 signaling affecting either neurons or the body wall muscles, and the exact nature of this defect warrants further study. Mutations in egl-15 have been shown to lead to axon guidance and maintenance defects in ventral nerve cord interneurons (29).

Genetic Removal of EGL-15 N-Glycosylation Sites Results in Defective Sex Muscle Function.—The finding that most of the EGL-15 Asn→Ala mutants accumulate eggs within the hermaphrodite uterus suggests defects either in the egg laying muscles or in the neurons that innervate the vulval muscles. Egg laying in C. elegans is initiated when serotonergic hermaphrodite-specific neurons stimulate the vulval muscles. If the neurons that innervate the egg laying muscles are dysfunctional, but the muscle function is intact, exposing the hermaphrodites to exogenous 5HT stimulates the vulval muscles and induces egg laying. To assess the functionality of the sex muscles, single wild type and EGL-15 Asn→Ala mutant hermaphrodites were allowed to lay eggs for 1 h with or without serotonin, after which the number of eggs laid were counted. Because the availability of food influences serotonin levels, all assays were performed on...
Egg laying of EGL-15 Asn → Ala mutants display significantly reduced egg laying as compared with wild type. **Table 2** shows the egg laying at concentration of 2.5 ng/μl DNA, which was also used for all mutant DNA constructs, wild type egl-15 rescued egg laying almost to the control levels (5.4 eggs/h ± 3.4 without 5HT, n = 48; 6.0 eggs/h ± 3.4 with SHT, n = 67). However, at higher concentrations of the wild type DNA, the animals laid fewer eggs and displayed stronger Egl phenotype (Table 2). C. elegans with the wild type construct introduced at 25 ng/μl laid 2.4 eggs/h ± 2.0 (n = 61) without serotonin, and addition of serotonin increased egg laying to 3.5 eggs/h ± 2.3 (n = 45). All EGL-15 Asn → Ala mutants laid a significantly lower number of eggs than wild type animals or mutants rescued with equal DNA concentrations (2.5 ng) of the wild type egl-15. EGL-15 N474A mutants lay 2.2 eggs/h (+2.5; n = 39; p < 0.01) without 5HT. Addition of exogenous serotonin has no significant effect on EGL-15 Asn → Ala mutants; N474A mutants lay 2.8 eggs/h (+2.5; n = 60) in the presence of serotonin (p > 0.05). Taken together these results suggest that sex muscle function is defective in EGL-15 Asn → Ala mutants.

**Abolishing N-Glycosylation Sites of EGL-15 Leads to Defects in Sex Muscle Differentiation**—The finding that serotonin does not stimulate egg laying in the Egl-15 Asn → Ala mutants suggested that sex muscles have not developed or are not functional. This could be caused by a failure of the sex myoblast to migrate during development, a process that requires the EGF-17/EGF-15 (5A) signaling pathway (25, 26) and EGF-15 (5B) (42) or by defects in the differentiation of the sex myoblasts to vulval and uterine muscles, a process that is negatively regulated by EGF-15 (37). To assess if the vulval and uterine muscles were present in the mutants and to analyze their morphology, fluorescently labeled phalloidin was used to visualize filamentous muscle actin. Wild type C. elegans have eight vulval and eight uterine muscles assembled in four muscle quadrants (Fig. 5A, panel i). In all of the EGL-15 Asn → Ala mutants, some or all of the four vulval muscle quadrants are missing (Fig. 5A, panels ii–iv and vi). Absence of one or more vulval muscle quadrants was evident in 27–90% of all mutants with the severity of the defect depending on the position of the mutation and the number of asparagine to alanine substitutions (Fig. 5B). Almost half of all the EGL-15 N474 mutants analyzed (47%; number of animals scored = 178) lacked at least one of the four vulval muscle quadrants, with 15% of all animals (27 of 178) having no differentiated vulval muscles. Similar results were observed for the EGL-15 N433A mutant, which does not display other phenotypes than Egl. 46% (207 of 450 animals scored) of EGL-15 N433A mutants lacked one or more of the vulval muscle quadrants, and 10% had no differentiated vulval muscles. Because 85% of the EGL-15 N474A and 90% of the EGL-15 N433A mutant animals had at least one vulval muscle quadrants present at the correct location in relation to the length of the animal, we suggest that sex myoblast migration per se is unaffected in the mutants and the defects observed result from failure of the myoblasts to differentiate. This is supported by the findings that anti-EGL-15 antibodies detect undifferentiated descendants of the sex myoblasts in adult animals that lack differentiated vulval muscles (see below and Fig. 5C). These descendants have migrated to the approximate position of the hermaphrodite vulva but failed to differentiate to muscles that contain filamentous actin.

Constitutive activation of EGL-15 using a transgenic construct in which the transmembrane domain of EGL-15 has been replaced with that of the neu oncogene has been shown to inhibit sex myoblast differentiation (37). The findings that EGL-15 Asn → Ala mutants lack some or all of the differentiated vulval muscles, and thus phenocopy in this respect the C. elegans carrying constitutively active EGL-15 (neu+), suggest that removing N-glycans from EGL-15 leads to increased receptor activity, which would result in muscle differentiation defects and the Egl phenotype. This is supported by the findings that transgenic expression of the wild type copy of egl-15 leads to a dose-dependent lack of differentiated vulval muscles. All four vulval muscles are present in 79% (number of animals scored = 152) of animals obtained with wild type egl-15 at DNA concentrations of 0.5 ng/μl and in 84% (126 of 150) of animals obtained with wild type egl-15 at DNA concentrations of 2.5 ng/μl, which is the concentration used for all Asn → Ala mutant DNA constructs (Fig. 5B). However, at higher DNA concentrations of 25 ng/μl, 57% (128 of 224) of all animals show absence of one or more vulval muscles, and 10% of these animals lack all four vulval muscle quadrants. Most of the single Asn → Ala mutants display more severe vulval muscle phenotypes than animals with high dosage of the wild type egl-15 DNA (Fig. 5B). The most severe sex muscle differentiation phenotype was observed in animals with six Asn → Ala mutations (N401A,N407A,N433A,N440A,N449A,N474A), where 98% of animals analyzed (117 of 119) lack one or more vulval muscle quadrants and 42% (50 of 119 animals) have no differentiated vulval muscles. Mutations in soc-2 suppress the Clr phenotype.
of the six asparagine substitution mutants (see above and Fig. 4); however, soc-2 does not suppress the muscle differentiation defect (Fig. 5B). This is consistent with the findings of Sasson and Stern (37) where mutations in soc-2 failed to suppress muscle phenotype of the constitutively active egl-15 (neu*) mutant.

PI 3-kinase signaling positively regulates myoblast differentiation and antagonizes FGF function in vertebrates (45) and in C. elegans where the EGL-15 (neu*)-dependent muscle differentiation defect can be suppressed by overactivating PI 3-kinase signaling (37). If the vulval muscle phenotype observed in the EGL-15 Asn → Ala N-glycosylation mutants is due to too much EGL-15 signaling, overactivation of PI 3-kinase signaling in these mutants should suppress the myoblast differentiation defect and restore wild type vulval muscle phenotype. Consistent with this hypothesis, mutations in daf-18/pten partially suppress the sex muscle differentiation defect in EGL-15 N401A, N407A,N433A,N440A mutants. The lipid phosphatase DAF-18/PTEN dephosphorylates phosphatidylinositol 3,4,5-trisphosphate, thus antagonizing PI 3-kinase signaling. In 61% of EGL-15 N401A,N440A mutants (132 of 221), one or more sex muscles fail to develop normally. This phenotype is suppressed to 34% (115 of 337) by reducing DAF-18/PTEN and hence activating PI 3-kinase signaling in daf-18 (ok480);egl-15 N401A, N407A,N433A,N440A double mutants (Fig. 5B). All differentiated vulval muscles are absent in 16% (35 of 221) of egl-15 N401A,N407A, N433A,N440A mutants; daf-18 (ok480) suppresses this phenotype to 2% (7 of 337). Similarly, gain-of-function mutation in akt-1 (mg144), which acts downstream of PI 3-kinase, suppresses the sex muscle differentiation phenotype to 44% (49 of 112) in akt-1 (mg144);egl-15 N401A,N407A,N433A,N440A double mutants (Fig. 5B). These results further support the hypothesis that removal of consensus N-glycosylation sites from EGL-15 leads to overactivation of the receptor.

Mutant EGL-15 Is Expressed in Descendants of Sex Myoblasts—Polyclonal anti-EGL-15 antibody (Pop) was used to detect the EGL-15 Asn → Ala proteins in the mutant C. elegans. EGL-15 could be detected at vulval muscles of adult hermaphrodites expressing wild type egl-15 (Fig. 5C, panel i) and in mutants that had differentiated vulval muscles (data not shown). Furthermore, EGL-15 was detected in undifferentiated descendants of sex
myoblasts that have migrated from their origin and have undergone several rounds of cell divisions but remain undifferentiated in adults (Fig. 5C, panel ii). In some of the mutants EGL-15 can be detected in the muscle precursors, which are apparently correctly positioned relative to the vulval opening, but which have failed to differentiate to fully functional vulval muscles (Fig. 5C, panels iii–iv).

**DISCUSSION**

We have shown that the *C. elegans* FGF receptor, EGL-15, is *N*-glycosylated in vivo and that genetic removal of consensus sites for *N*-glycosylation in the D3 of EGL-15 leads to phenotypic consequences associated with receptor overactivation. These phenotypes can be suppressed by hypomorphic mutations in components of the EGL-15 downstream signaling pathway or by mutations that overactivate signaling pathways antagonistic to EGL-15/FGFR. Some of the mutations introduced are orthologous to FGFR mutations found in human patients of Crouzon syndrome (17) and in hypochondroplasia (15), which have been suggested to result in receptor activation. Our results thus confirm the hypothesis of receptor overactivation in vivo upon loss of *N*-glycosylation.

Intriguingly, despite numerous attempts we were not able to obtain a *C. elegans* strain in which the EGL-15 Asn-401 is substituted with alanine. The fact that several independent attempts to introduce the eggl-15 *N401A* DNA construct have failed suggests that the nature of the N401A mutation renders the mutant EGL-15 protein incapable of rescuing larval lethality in the eggl-15 (lf) background. However, we were able to obtain *c. elegans* strains with EGL-15 mutant constructs that contain the N401A mutation in combination with other asparagine to alanine substitutions, most notably whenever a consensus *N*-glycosylation site in the close proximity, Asn-407, is also alanine-substituted. One explanation could be that Asn-401, which is conserved in human FGFRs, is normally *N*-glycosylated and that removal of Asn-401 *N*-glycosylation shifts glycosylation to the neighboring Asn-407, which is normally not glycosylated or glycosylated with a small *N*-glycan structure. This would result in more severe functional consequences than simply removing *N*-glycans from Asn-401, for example the inappropriate processing of the mutant EGL-15 protein by either inducing transient intracellular retention in the endoplasmic reticulum or in the Golgi organelle and the subsequent degradation or accumulation of the inappropriately processed protein. Eliminating both asparagine residues, Asn-401 and Asn-407, abolishes this possibility and leads to a mutant EGL-15 protein that is capable of rescuing viability in eggl-15 null background albeit with phenotypic consequences. It is noteworthy that none of the human FGFRs contain a consensus *N*-glycosylation site that is orthologous to the EGL-15 Asn-407, although molecular modeling suggests that this residue is spatially located in close proximity to Asn-474, which occupies a similar position to Asn-330 of FGFR1.

Protein *N*-glycosylation affects protein translocation from the endoplasmic reticulum and the Golgi to the plasma membrane and acts as a means to quality control newly synthesized proteins. The fact that all other EGL-15 Asn → Ala mutants that were analyzed were able to rescue the larval lethality caused by eggl-15 (lf) mutations suggests that a sufficient fraction of the mutant protein is correctly localized to the site where EGL-15 normally functions. Antibodies against EGL-15 also detect the mutant EGL-15 Asn → Ala proteins at the cell surfaces of vulval muscles and descendants of sex myoblasts. The essential function of EGL-15 is required in the hypodermis (43). Using antibodies, we could not detect EGL-15 protein in the hypodermis, but because the mutant EGL-15 proteins are capable of rescuing larval lethality, we can only conclude that the expression levels are too low to enable protein detection in the hypodermis, which is consistent with findings by others (42).

EGL-15 *N433A* mutation leads to very weak phenotypes, and predominantly the mutants are Egls. Similarly, animals rescued with a wild type copy of eggl-15 are Egls. Asparagine 433 is fully conserved across the species, but thus far mutations affecting this consensus *N*-glycosylation site have not been associated with human disorders. Our results suggest that Asn-433 is either not *N*-glycosylated or its *N*-glycans are not critical for EGL-15 function. The most severe phenotype of the mutant strains with a single Asn → Ala amino acid substitution is the N474A mutation, which leads to low frequency of CIR animals. Asparagine 474 is orthologous to Asn-330 of human FGFR1, Asn-331 of human FGFR2, and Asn-328 of human FGFR3, mutations in which lead to skeletal disorders. Substituting asparagine 474 with alanine in the multiple Asn → Ala mutants also causes a marked increase in the severity of the phenotype. EGL-15 N401A,N407A,N433A,N440A,N449A,N474A mutants show only a moderate CIR phenotype, whereas almost 90% of EGL-15 N401A,N407A,N433A,N440A,N449A,N474A animals are Clrs. These results thus suggest an evolutionarily conserved role for *N*-glycosylation of this asparagine residue in negatively regulating signaling by the FGF receptor.

![FIGURE 5. A, absence of differentiated vulval muscles in EGL-15 Asn → Ala mutants. Muscles are visualized by rhodamine-conjugated phalloidin. Body wall muscles can be seen as broad bands below the focal plane. Vulval muscles are labeled in panel i which shows the wild type control. The position of the vulval opening is marked with dashed line. Panels ii and iii, differentiated vulval muscle quadrants are either absent or display abnormal morphology in eggl-15 N433A mutants. Panel iv, eggl-15 N401A,N440A, with sex muscle differentiation partially defective. Panel vi, increasing the activity of PI 3-kinase signaling in dau-18 (ok480)eggl-15 N401A,N440A double mutants partially suppresses the vulval muscle differentiation phenotype. Panel vii, 43% of all eggl-15 N401A,N474A mutants have no differentiated vulval muscles. Scale bars, 10 μm. All animals are positioned in a ventrolateral view with heads to the left. B, sex muscle differentiation defect in EGL-15 Asn → Ala mutants. Black bars indicate percentage of animals with all four vulval muscle quadrants present; white, light gray, gray, and dark gray bars represent animals with three, two, one, or none of the four vulval muscle quadrants present, respectively. n = animals scored >75 for all strains. C, immunostaining with anti-EGF-15 antibodies shows that EGL-15 is localized to the vulval muscles. Panel i, transgenic expression of eggl-15(+). The position of vulval opening is marked with a dashed line. EGL-15 is detected in vulval muscles. Panel ii, strain expressing eggl-15 N433A. In 45% of the animals, one or all of the vulval muscles are missing, and undifferentiated descendants of sex myoblasts that express EGL-15 at their cell surface can be detected. Panel iii, eggl-15 N401A,N440A, which shows EGL-15 in descendants of sex myoblasts at their correct location. These descendants have failed to differentiate to muscles. Panel iv, undifferentiated descendants of sex myoblasts in eggl-15 N401A,N474A. Scale bars, 10 μm.](https://www.jbc.org/content/284/48/33037/F5)
**N-Glycosylation Regulates FGFR Activity**

The *egl-15* DNA we have used in our mutagenesis experiments is a genomic hybrid, encoding for both EGL-15 (5A) and EGL-15 (5B) isoforms. We can assume that the mutations affect both isoforms. The EGL-15 (5B) isoform is required for viability, and excessive EGL-15 (5B) activity confers Clr phenotype, whereas the EGL-15 (5A) isoform is necessary for the gonad-derived attraction of sex myoblasts (28). Recent findings have also revealed a role for the EGL-15 (5B) isoform in gonad-dependent repulsion of sex myoblasts and suggested that structural differences in the two isoforms contribute to their functional differences (42). Expression of constitutively active 5A isoform results in animals that completely lack differentiated sex muscles (37). The EGL-15 Asn → Ala mutants display both the Clr phenotype and the sex muscle differentiation phenotype suggesting that signaling pathways of both EGL-15 isoforms 5A and 5B are overactivated in these mutants.

Wild type EGL-15 from mixed stage animals was biochemically detected as four polypeptides of ~105, 95, 85, and 75 kDa. Although we do not currently have EGL-15 isoform-specific antibodies, we can only speculate that two of these four polypeptides represent the two different EGL-15 isoforms 5A and 5B. The two other isoforms detected may represent alternatively glycosylated forms of EGL-15. Following partial purification on heparin, N-glycosidase F digestion clearly caused a band shift for the 105-kDa isoform, indicating that it is N-glycosylated. Endoglycosidase H, which cleaves high mannose type N-glycans, also resulted in a band shift of EGL-15. In mammals, high mannose-type glycans are often present as immature unprocessed form in the endoplasmic reticulum/Golgi. However, biochemical studies of mixed stage *C. elegans* have shown that high mannose-type N-glycans are the most abundant types of N-glycans (46–48). The findings that the EGL-15 N-glycosylation mutant containing seven asparagine to alanine mutations in D3 changes its mobility on SDS-PAGE upon N-glycosidase F treatment suggest that there are additional N-glycans present at other extracellular domains of EGL-15. Because the potential N-glycosylation sites of the D3 are primarily associated with human osteo- and chondrodysplasia syndromes and evolutionarily conserved, they were the focus of our study and were only targeted in the mutagenesis. There are three additional consensus N-glycosylation sites in EGL-15, which are not conserved in human FGFRs. Residual sugar modification of any or all of these sites may explain the change in gel mobility of the N-glycosidase F-treated EGL-15 proteins with D3 Asn → Ala mutations.

Although the *C. elegans* genome contains homologues of the vertebrate enzymes required for the biosynthesis of N-linked oligosaccharides (49) and biochemical analysis have revealed that *C. elegans* has a rich repertoire of N-linked oligosaccharide structures (for reviews see Refs. 50, 51), not much is known about the physiological roles of the *C. elegans* N-glycoproteins in vivo. N-glycosylation of calnexin/CNX-1, a Ca^{2+}-binding protein chaperone of the endoplasmic reticulum membrane, is important for its function, as a glycosylation-defective construct of cnx-1 fails to rescue *cnx-1* loss-of-function phenotype (52). Several enzymes involved in N-glycan biosynthesis and processing have been cloned and biochemically characterized, and null mutant phenotypes of some of them have been reported (53). However, approaches aiming at characterizing mutants in enzymes involved in N-glycan biosynthesis may be difficult to interpret at the molecular level, as the effects will affect a large number of proteins and be pleiotrophic. Our results show that abolishing specific consensus N-glycosylation sites of EGL-15/FGFR alters receptor activity leading to dramatic phenotypic consequences characteristic of excess receptor activity. Alanine substitution of a single asparagine 401 or 474 of EGL-15/FGFR leads to inability to rescue loss-of-function phenotype or to excess receptor signaling, respectively. Combinations of multiple asparagine substitutions lead to increased severity of the overactivation phenotype, suggesting decreased control of receptor signaling. The phenotypes seen in the EGL-15 N-glycosylation mutants are not simply a result of overexpression of EGL-15/FGFR as introducing higher concentrations of wild type egl-15 DNA into the egl-15 (lf) background does not confer the Clr phenotype. Abolishing specific N-glycosylation sites thus leads to dramatic phenotypic consequences supporting a model in which N-glycans add a level of control to FGFR signaling in vivo.

The findings that some of the single asparagine to alanine mutants and most of the double and multiple mutants display phenotypes that are characteristic of EGL-15 overactivation support the hypothesis that N-glycans negatively regulate FGFR receptor signaling. FGFR signaling is thus regulated not only at the levels of expression of specific receptor isoforms, the expression and availability of specific ligands, and the structure of the heparan sulfate co-receptor but also by N-glycosylation. N-Glycans reduce ligand and co-receptor affinity for FGFR (18) and may prevent self-dimerization or oligomerization, which might lead to ligand-independent receptor activation.

In conclusion, we have shown that the *C. elegans* EGL-15/FGFR is N-glycosylated, and removal of specific consensus N-glycosylation sites of EGL-15 immunoglobulin domain 3 (D3) leads to phenotypes associated with EGL-15 overactivation. This is the first demonstration of N-glycosylation carrying a specific biological function in *C. elegans*. Orthologous mutations in human FGFRs have been suggested to be receptor-activating. Our results confirm this hypothesis. Finally, our results demonstrate that the *C. elegans* model can be used to study human FGFR mutations and assess the mechanism used to affect FGFR function in vivo.

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