

Alternative splicing affecting a novel domain in the *C. elegans* EGL-15 FGF receptor confers functional specificity

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SUMMARY

Fibroblast growth factor (FGF) receptors trigger a wide variety of cellular responses as diverse as cell migration, cell proliferation and cell differentiation. However, the molecular basis of the specificity of these responses is not well understood. The *C. elegans* FGF receptor EGL-15 similarly mediates a number of different responses, including transducing a chemoattractive signal and mediating an essential function. Analysis of the migration-specific alleles of *egl-15* has identified a novel EGL-15 isoform that provides a molecular explanation for the different phenotypic effects of lesions at this locus. Alternative splicing yields two EGL-15 proteins containing different forms of a domain located within the extracellular region of the receptors immediately after the first IG domain. Neither of these two domain forms is found in any

other FGF receptor. We have tested the roles of these EGL-15 receptor isoforms and their two FGF ligands for their signaling specificity. Our analyses demonstrate different physiological functions for the two receptor variants. EGL-15(5A) is required for the response to the FGF chemoattractant that guides the migrating sex myoblasts to their final positions. By contrast, EGL-15(5B) is both necessary and sufficient to elicit the essential function mediated by this receptor.

Supplemental data available online

Key words: FGF receptor, EGL-15, Alternative splicing, Chemoattraction, Achondroplasia, Sex myoblast

INTRODUCTION

FGF receptors are a class of receptor tyrosine kinases that play crucial roles in normal human health and development (Green et al., 1996; McKeehan et al., 1998; Ornitz, 2000). Mutations that perturb the normal function of FGF receptors have been implicated in dramatic developmental abnormalities such as those observed in a number of syndromes characterized by skeletal and craniofacial malformations (Webster and Donoghue, 1997).

The domain structure of FGF receptors comprises three extracellular immunoglobulin (IG) domains, a small extracellular region of predominantly acidic amino acids (the acid box), a single transmembrane domain and an intracellular tyrosine kinase domain (Dionne et al., 1991). Binding of an FGF ligand to IG domains 2 and 3 (IG2 and IG3) induces receptor dimerization and upregulates the intracellular tyrosine kinase activity, which in turn modulates the activity of downstream signaling pathways (Boilly et al., 2000; Green et al., 1996; Klint and Claesson-Welsh, 1999).

Understanding the factors that underlie the specific processes mediated by FGF signaling in vertebrates is

complicated by the tremendous diversity among the signaling complexes. At least 22 FGF ligands and five receptors have been identified in humans (Kim et al., 2001; Ornitz and Itoh, 2001). Receptor activity can also be modified through extensive alternate splicing that generates a diverse range of FGF receptor structures (McKeehan et al., 1998). The functional consequences and physiological relevance of only a few of these are known. One important alternate splicing event lies within the second half of IG3, which can be encoded by one of two alternate exons (IIIb or IIIc) (reviewed by Green et al., 1996). In vertebrate FGFR2, the use of exon IIIc confers the ability to bind FGF1 and FGF2 with high affinity but dramatically reduces the interaction of this receptor with FGF7. By contrast, use of exon IIIb enables robust interaction with both FGF7 and FGF1, but generates a receptor with very low affinity for FGF2 (Ornitz et al., 1996). The differential ligand responsiveness this alternate splicing creates confers mesenchymal/epithelial specificity to different forms of the same receptor. Finally, various heparan sulfate proteoglycans (HSPGs) participate in FGF signaling as part of the ligand/receptor/HSPG ternary complex, adding to the

molecular complexity of FGF signaling (Chang et al., 2000; McKeehan et al., 1998; Perrimon and Bernfield, 2000).

The analysis of FGF signaling in model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* has served as a paradigm for understanding many aspects of FGF signal transduction, in part due to the reduced molecular complexity of these systems. The *C. elegans* genome contains a single FGF receptor gene and two genes that encode FGF ligands, representing a relatively simple system to dissect the molecular mechanisms of FGF signaling (Borland et al., 2001). Similar to its vertebrate counterparts, EGL-15 plays a crucial role in multiple types of biological processes. Two major events mediated by EGL-15 control are the migrations of the hermaphrodite sex myoblasts (SMs) and an early essential function (DeVore et al., 1995).

Proper migration of the hermaphrodite SMs plays an important role in ensuring egg-laying proficiency. The SMs are a pair of muscle precursor cells that migrate anteriorly to functional positions flanking the precise center of the developing gonad. After migrating, each SM divides three times to generate a set of 16 cells that differentiate to generate the muscles required for egg laying (Sulston and Horvitz, 1977). Improper SM migration can result in the generation of egg-laying muscles in non-functional positions and, consequently, an inability to lay eggs (the *Egl* phenotype).

Several mechanisms cooperate to guide SM migration. Multiple central gonadal cells express EGL-17/FGF, which serves to attract the SMs to their precise final positions (Branda and Stern, 2000; Burdine et al., 1998; Thomas et al., 1990). This guidance mechanism has been termed the gonad-dependent attraction. In the absence of the EGL-17 chemoattractant, the SMs remain significantly posterior of normal due to a gonad-dependent repulsion (Stern and Horvitz, 1991). This dramatic posterior displacement of the SMs is also observed in a special class of mutations in *egl-15* termed the *egl-15(Egl)* alleles (DeVore et al., 1995; Stern and Horvitz, 1991).

A large number of *egl-15* alleles affect its essential function to varying degrees; these alleles can be ordered in an allelic series based on the degree of their defect in this function (Borland et al., 2001; DeVore et al., 1995). Complete loss of EGL-15 activity results in an early developmental arrest and larval lethality (*Let*) (DeVore et al., 1995). Severe hypomorphic alleles can confer a scrawny (*Scr*) body morphology. Certain EGL-15 mutations that cause a milder decrease in the activity of this receptor display only a *Soc* phenotype, named for their suppression of the *Clear* phenotype caused by mutations in *clr-1*. *clr-1* encodes a receptor tyrosine phosphatase that acts as a negative regulator of EGL-15 function (Kokel et al., 1998). Compromised function of CLR-1 results in the hyperactivation of the EGL-15 signaling pathway. *egl-15(Soc)* mutations compromise the level of EGL-15 activity sufficiently to bring signaling levels back to within the normal range even in the absence of the CLR-1 negative regulator.

The two *C. elegans* FGFs appear to be responsible for the two, distinct EGL-15-mediated processes. Loss of EGL-17/FGF results in migration defects very similar to those seen in the *egl-15(Egl)* mutants (Stern and Horvitz, 1991). Conversely, complete loss of LET-756/FGF function confers a larval arrest lethality similar to that observed in *egl-15* null animals (Roubin et al., 1999). This separation of ligand

function is similar to that observed for ligands of the *Drosophila* EGF receptor, where the *spitz*, *gurken* and *vein* ligands are each responsible for eliciting a subset of the functions of the receptor (Freeman, 1998; Schweitzer and Shilo, 1997). Functional differences of the *Drosophila* EGF receptor arise in part from temporal and spatial regulation of ligand expression. We report the identification of a novel EGL-15 receptor isoform that is generated by alternate splicing, and present evidence that the two different forms of EGL-15 are responsible for its two distinct functions.

MATERIALS AND METHODS

Genetic manipulations

All *C. elegans* strains were maintained according to standard protocols (Brenner, 1974) and constructed using standard genetic techniques (Herman, 1988).

Determination and representation of sex myoblast position

The final positions of sex myoblasts were determined with respect to the underlying hypodermal Pn.p cells as previously described (Thomas et al., 1990). SM distributions for each strain are depicted using box-and-whisker plots (Moore and McCabe, 1993) aligned to a schematic representation of the Pn.p cell metric. In brief, each set of SMs is ordered according to anteroposterior position and divided into quartiles. The 'box' includes the positions of SMs within the two central quartiles. An additional vertical line within the box indicates the median SM position at the boundary between the second and third quartiles. Overlap of the line representing median position with a right or left border of the box is depicted as a thickening of that line. A quartile length (1Q) is determined based on the range of positions covered by the 2Q box length. Bars ('whiskers') of up to 1.5 Q length extend from the edges of the box to additional data points. As whisker length does not extend beyond the range of data points, these bars may be shorter than a 1.5 Q length, or even absent. Data points beyond the edge of the bars ('outliers') are indicated by individual hash marks. This representation of SMs therefore depicts the overall range of SMs as well as their general distribution and median position. Sex myoblasts positioned dorsally are represented by asterisks and were treated outside of this data set (Fig. 5).

Sequence analysis

Lesions associated with *egl-15* mutations were identified by sequencing one strand for all the known exons and splice junctions for each mutant allele. PCR pools were derived from genomic DNA; lesions were confirmed by sequencing independently derived PCR pools. Sequence alterations for the 26 *egl-15* mutants can be found in Table S1 at <http://dev.biologists.org/supplemental/>. Six *egl-15* mutations confer the most severe phenotypic consequences [larval arrest lethality (*Let*)], and behave as null alleles by genetic criteria (DeVore et al., 1995). Four of these (*n1454*, *n1456*, *n1475*, *n1478*) are nonsense mutations, *n1476* (D815N) alters a residue conserved in all kinases, and *n1455* (R892I) changes a residue conserved in many tyrosine kinases, including all FGF receptors (Fig. 1) (Hanks and Quinn, 1991). As the nonsense mutation *egl-15(n1456)* is predicted to truncate EGL-15 extracellularly within the second IG domain, we have used this mutation as the canonical *egl-15* null allele.

Sequences classified the Y. Kohara EST cDNAs into the following categories based on C-terminal splicing patterns: yk36b5 (extends into exon 5B), yk234b8, yk251b4, yk288a9.5, yk322g6, yk348f12, yk34g9 and yk582c4 (Type 1); yk238a4 and yk129f10 (Type 2); and yk322c4 (Type 3, extends into exon 5B).

Sequences of newly discovered *egl-15* cDNAs are available with

the following GenBank Accession Numbers: the complete EGL-15(5A)/type I CTD cDNA (AY288941); type 2 CTD (AY268435); type 3 CTD (AY288942); type 4 CTD (AY268436); type 5 CTD (AY292532). Amino acids within the 5A domain are numbered D128(5A)-G193(5A), linking directly to T246, at which point the common sequences continue. 5B domain amino acids will continue to be numbered as previously, from D128-R245.

PCR analysis establishing exon 5A

Exon 5A was originally detected by nested PCR using a pool of single-stranded cDNA. The product of a first round PCR reaction using a primer within the putative beginning of exon 5A (5'-CCA-CTTAAACTGTTTCGATTGGC-3') and exon 7 (5'-GAATTCGTAT-CCGCCGGACCGAGCAC-3') was used as the template for a second round of PCR using nested primers (5'-AGATCTCGGAAATGAG-GAGAGTGAAGC-3' and 5'-GGTA-CCTTTCACACACCAT-AAACAAAATTCC-3') that generated a product of ~450 bp. The sequence of this product revealed the existence of a transcript containing 112 bp of exon 5A spliced to the beginning of exon 6.

Analysis of cDNA splicing patterns

RT-PCR of *egl-15* was performed on RNA isolated from L2-stage and mixed-stage populations of wild-type *C. elegans*. Reactions were carried out in duplicate using a common downstream primer located in exon 21 (5'-CTGGTCAAAAATGACTAGATC-3') together with either a non-specific primer located in exon 1 (5'-TGGTCAAGA-ATGACTAGATC-3') or an exon 5A-specific primer (5'-CCTTTA-CTCCTCTCACTTTTCGG-3'). These products were subsequently PCR amplified, in combination with the above primers, using either a nested exon 5A-specific primer (5'-GCTCTCGAGCCACTTAA-ACTGTTTCGATTGGC-3') or a nested non-specific primer (5'-CAGTAGATCTGATGAGTTATTCCTT-GCATCCTGCC-3'). This step yielded separate pools of non-specifically amplified L2-stage or mixed-stage cDNAs, as well as exon 5A-specific L2-stage or mixed stage cDNAs. Diagnostic PCR was used to determine whether transcripts encoding the exon 5A and exon 5B forms were represented in the cDNA pools.

5A-specific cDNAs from both the L2-derived cDNA pool as well as the mixed stage-derived cDNA pool were cloned using the TA Cloning Kit (Invitrogen) and characterized for their splicing pattern. In addition, all of the 5A-containing cDNAs from the L2-derived non-specific cDNA pool, a matching number of 5B-containing cDNAs from the same pool, and nine additional 5B-containing cDNAs from the mixed stage-derived non-specific cDNA pool were similarly cloned.

cDNA clones were subjected to a *Bam*HI/*Nde*I restriction digest to determine their 3' splicing patterns; PCR amplification of the 3' ends was also used to aid in the characterization of splicing patterns. Transcripts were categorized based on restriction patterns and the size of the PCR products; a single transcript from each class was then sequenced to determine the precise sites of the splice junctions. Several cDNAs were clearly splicing intermediates since the open reading frame did not extend much beyond the novel splicing pattern. These are not included in the list of alternatively spliced forms in Fig. 3.

Plasmid construction

All *egl-15* constructs were derived from the plasmid NH#112, which contains a rescuing genomic DNA fragment (DeVore et al., 1995). A nonsense mutation corresponding to that found in the *n1458* allele was generated by four-primer mutagenic PCR to yield EGL-15(5A⁺B⁻). The EGL-15(5A⁺B⁻) construct was similarly generated to create a nonsense mutation in the first codon of exon 5B. All plasmids constructed using PCR were confirmed by sequencing.

The *egl-17* genomic rescuing fragment was previously described (Burdine et al., 1997). A *let-756* genomic rescuing fragment was cloned from the cosmid CO5D11. To make the *P_{let-756::egl-17}*

chimera, a 0.8 kb *Pst*I-*Nco*I fragment was PCR generated and used to replace the full 4.1 kb *egl-17* promoter fragment of NH#354. Subsequent insertion of a 1.2 kb *Nco*I PCR product completed the downstream region of the *let-756* promoter. To make the *P_{egl-17::let-756}* chimera a *Bam*HI-*Nco*I *egl-17* promoter fragment wherein the start methionine was altered to contain a *Nco*I restriction site was ligated with a *Nco*I-*Xba*I *let-756* coding sequence fragment (*Nco*I restriction site also at the start methionine) into Bluescript KSII vector (Stratagene) using *Bam*HI and *Xba*I.

Transgenic rescue assays

Transgenic arrays were generated using standard germline transformation techniques (Mello et al., 1991).

egl-15 essential function

egl-15 tester DNA at 20 ng/μl was introduced into hermaphrodites of genotype *+/szTl*; *dpy-20(e1282ts)*; *unc-115(e2225)* *egl-15(n1456)/szTl* along with the *dpy-20(+)*-containing co-transformation marker plasmid pMH86 (50 ng/μl). Stable transgenic lines were established in the balanced heterozygous strain. Rescue of the *egl-15* Let defect was assessed by the segregation of viable Unc lines of genotype *dpy-20(e1282ts)*; *unc-115(e2225)* *egl-15(n1456)*; *ayEx[dpy-20(+); egl-15(tester)]*.

Egl rescue assay

To quantitate the Egl penetrance in transgenic lines, a minimum of 30 L4 transformants were placed on a seeded plate at 20°C. Animals were scored 36 hours and 48 hours later and categorized as non-Egl, semi-Egl, Egl or Bag-of-worms (Bag). The percent non-Egl animals was calculated from the proportion of animals that failed to become Egl or Bag. A line was considered to rescue if at least 60% of the transgenic animals were non-Egl. Data from the 48 hour time-point are represented throughout this manuscript. Transgenic rescue of the SM migration defect of *egl-15(Egl)* animals was assayed in a *dpy-20(e1282ts)*; *egl-15(n1458)* background.

let-756 essential function

Plasmids encoding either *let-756* or *egl-17* were introduced into *dpy-17(e164)* *let-756(s2887)* *unc-32(e189)*; *sDp3* hermaphrodites. The *sDp3* duplication covers both the *dpy-17* and *let-756* genes. The *P_{myo-2::GFP}* co-transformation marker plasmid was included at 5 ng/μl. Stable transgenic lines were established in the duplication-bearing strain. Rescue of the *let-756* Let defect was assessed by the segregation of viable Dpy animals of genotype *dpy-17(e164)* *let-756(s2887)* *unc-32(e189)*; *ayEx[let-756 tester]*.

Brood size assay

The average brood size of all *let-756(null)* rescued lines was determined. Ten L4-stage rescued animals per line were picked to an individual plate. Adults were transferred to new plates every 24 hours. The number of viable progeny (L4 stage or older) was determined for each plate at 48 and 72 hours post-egg lay. The average brood size per transgenic line was determined. Data from the rescued lines for each transgene were then averaged together, generating the data portrayed in Table 1.

RESULTS

The carboxy-terminal domain of EGL-15 is specifically required for SM chemoattraction

To understand the molecular basis of how EGL-15 carries out its various roles, we determined the lesions associated with 26 *egl-15* mutations. Lesions corresponding to all 22 mutations that affect the essential function of EGL-15 were found in the known coding regions or flanking splice sites (Fig. 1). By

contrast, a similar analysis of the four *egl-15(Egl)* alleles identified a lesion in only one of these mutations, *egl-15(n1457)*. No alteration corresponding to the three other *egl-15(Egl)* alleles was found in these regions.

The C-terminal domain (CTD) of EGL-15 appears to be required specifically for SM chemoattraction. The *egl-15(Egl)* allele *n1457* is a nonsense mutation (Q948ochre) predicted to remove the CTD; its only apparent defect is posteriorly displaced SMs and the resulting *Egl* phenotype (Fig. 2A). Animals bearing this mutation fail to exhibit either a scrawny body morphology or the Soc phenotype, even though minor perturbations of general EGL-15 function can confer a Soc phenotype with no effect on egg-laying competence [for example, the Soc allele *egl-15(n1783)*]. The behavior of the temperature-sensitive allele *n1477ts* is also consistent with the CTD being required for SM chemoattraction. This mutation, like *n1457*, corresponds to a nonsense mutation at the 3' end of the *egl-15*-coding region, but truncates the very C terminus of the kinase domain as well as the CTD (Fig. 1). *n1477ts* animals are *Egl* and have SM positioning defects at all temperatures, but display an increasingly severe scrawny body morphology only at higher temperatures. The truncation of the amino acids at the very end of the final α -helix of the kinase domain has been postulated to destabilize EGL-15, accounting for the temperature-sensitive effect on body morphology (Mohammadi et al., 1996). However, the elimination of the

CTD can account for the SM migration defects that are observed at all temperatures. The phenotypic characteristics of these two mutants suggest a specific requirement of the CTD for SM chemoattraction.

egl-15(Egl) lesions reveal a second EGL-15 isoform

EGL-15 is structurally and functionally similar to other FGF receptors (DeVore et al., 1995; Schutzman et al., 2001), the most dramatic difference being a large peptide insert found between IG1 and the acid box (Fig. 1). This domain is unique to EGL-15 and entirely encoded by exon 5. Interestingly, within the intron between exons 5 and 6, a G-to-A alteration was observed in the *egl-15(Egl)* mutant *n1458*. Immediately upstream of the lesion, we observed a perfect match to the consensus splice acceptor sequence. These observations suggested that the *n1458* lesion might lie within an alternate exon.

We tested for the presence of an *egl-15* transcript containing this sequence using RT-PCR. This yielded a product whose sequence corresponded to a portion of the 'intronic' region between exons 5 and 6 spliced to the known splice acceptor site of exon 6. The presence of a splice junction confirmed that we had identified a spliced transcript rather than a genomic DNA contaminant. Similar analysis of the splicing pattern upstream of this new exon revealed a junction between the normal exon 4 splice donor site and the predicted splice

acceptor site of the new exon. This new exon is therefore spliced between exons 4 and 6 as an alternative exon 5, known as exon 5A (Fig. 2B). The originally identified exon 5 is now termed exon 5B. Use of the alternative exon 5A maintains a continuous open reading frame, resulting in a putative new isoform of EGL-15, which we refer to as EGL-15(5A) (Fig. 2C).

Besides *egl-15(n1457)*, the other *egl-15(Egl)* alleles all have lesions affecting this newly identified exon 5A (Fig. 2B). The base change associated with *n1458* results in a nonsense mutation (Fig. 2B). A distinct lesion, also resulting in a nonsense mutation, was found in *n484* animals (Fig. 2B). The remaining *egl-15(Egl)* allele, *ay1*, is a base change that alters the splice acceptor site at the beginning of this alternate exon (Fig. 2B). These mutations are predicted to truncate or

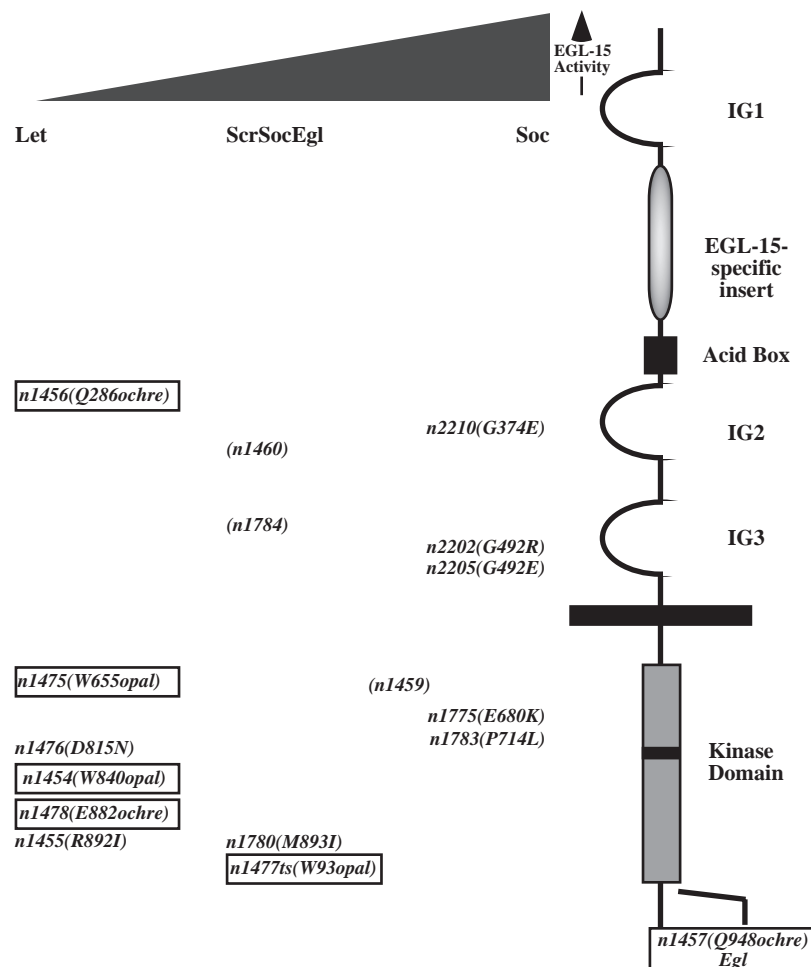


Fig. 1. Mutation analysis of *egl-15* alleles. Alleles are graphically positioned based on the severity of their phenotype (horizontal axis) and the location of their lesion (vertical axis). The axis representing the level of the essential function of EGL-15 signaling activity is portrayed above by the triangle, with minimal activity corresponding to the Let phenotype and near normal activity corresponding to the Soc phenotype. Nonsense mutations are boxed, splice site mutations are represented in parentheses. *n1457* and the other *egl-15(Egl)* alleles break this allelic series. An additional group of mutations isolated as suppressors of *clr-1* (Soc) were characterized only in a *clr-1(e1745ts)* background: *n2217(W633Opal)*, *n2182(A650V)*, *n2189(P876L)*, *n2184(G890E)*, *n2203(G871S)* and *n2206(W930Amber)*. The full-length EGL-15(5B) type 1 isoform is 1040 amino acids in length.

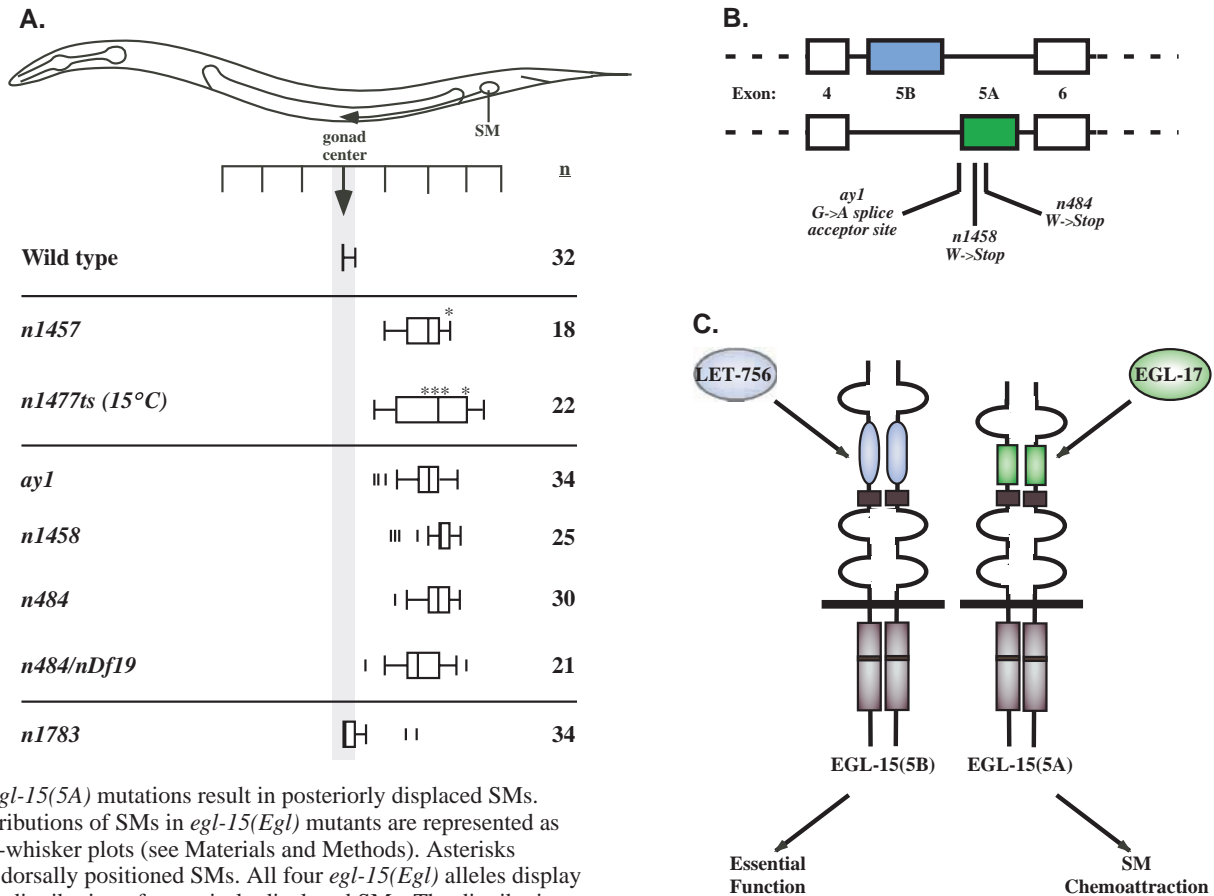


Fig. 2. *egl-15(5A)* mutations result in posteriorly displaced SMs.

(A) Distributions of SMs in *egl-15(Egl)* mutants are represented as box-and-whisker plots (see Materials and Methods). Asterisks indicate dorsally positioned SMs. All four *egl-15(Egl)* alleles display a similar distribution of posteriorly displaced SMs. The distribution of SMs in the *egl-15(Soc)* mutant, *n1783*, is shown for comparison. (B) An extracellular alternate splicing event gives rise to two distinct receptor isoforms. Three out of four *egl-15(Egl)* alleles contain point mutations that specifically affect the 5A exon. (C) Alternate splicing of exon 5 encoding the EGL-15-specific insert results in two distinct isoforms that specifically differ in this domain. A simple, modular hypothesis for normal signaling specificity is suggested by the phenotypic similarities of mutations affecting the ligands and receptor isoforms. Mis-expression of the ligands can allow them to stimulate the heterologous function via the heterologous receptor isoform.

severely reduce the product of *egl-15* transcripts containing this exon. As the truncation would occur in the extracellular region of EGL-15, these mutations are likely to destroy the function of the EGL-15(5A) isoform. Consistent with these mutations being null alleles for EGL-15(5A), the SM distribution in *n484/nDf19* animals resembles that of *n484* homozygotes (Fig. 2A). Because these *egl-15(Egl)* mutants only have SM migration defects, these data demonstrate that EGL-15(5A) is specifically required for SM chemoattraction (5A, for attraction). The original EGL-15 isoform is now referred to as EGL-15(5B).

EGL-15 isoform structure

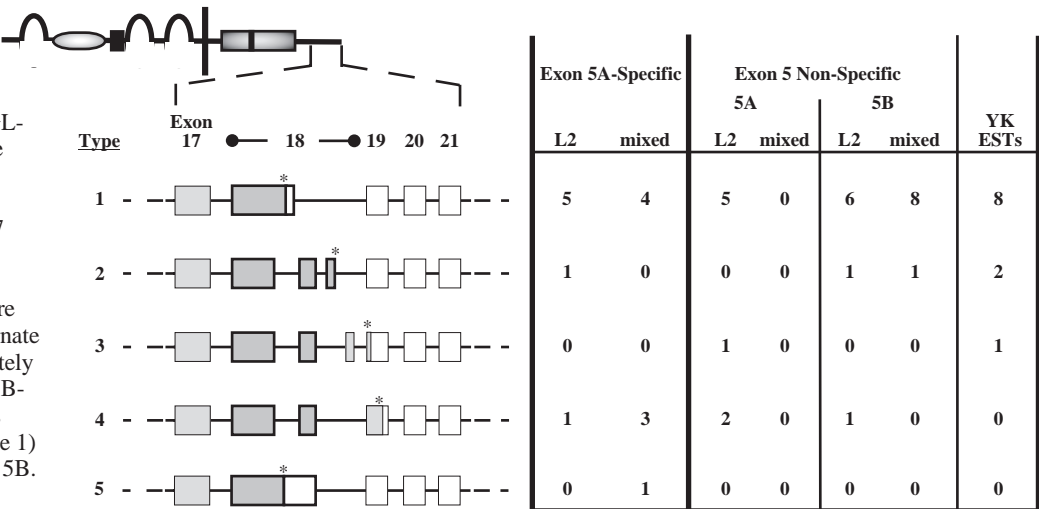
To understand the structural differences between the various isoforms of EGL-15, we characterized a large number of individual *egl-15* cDNAs. These cDNAs were characterized by restriction enzyme digestion, diagnostic PCR and/or sequence determination. Eleven independent *egl-15* cDNAs were available from an EST database (http://www.ddbj.nig.ac.jp/c-elegans/html/CE_INDEX.html). Analysis of these cDNAs revealed that two of these eleven cDNAs extend to exon 5 and contain exon 5B; the remaining cDNAs end prior to this exon. To characterize transcripts containing exon 5A, four sets of RT-

PCR pools were used to generate cDNAs containing exon 5 sequences. These were generated from RNA preparations either from mixed-stage populations or from L2 hermaphrodites, the stage during which the SMs migrate (Sulston and Horvitz, 1977). From each of these RNA preparations, both '5A-specific' and exon 5 'non-specific' RT-PCR cDNA pools were generated.

Analysis of the 'non-specific' exon 5-containing cDNAs revealed that *egl-15(5A)* is enriched at the L2 stage (Fig. 3). None of the cDNA clones (0/25) generated from the mixed-stage population were found to contain exon 5A. By contrast, 8/40 cDNA clones generated nonspecifically from a staged L2 hermaphrodite population contained exon 5A; the remaining clones all used exon 5B. Thus, exons 5A and 5B are used in a mutually exclusive manner, and the EGL-15 isoform required specifically for SM migration is enriched at the stage when SM migration is occurring. Interestingly, although removal of all exon 5 sequences entirely would result in normal FGF receptor architecture, no such transcripts were isolated.

Besides the alternative splicing of exon 5, the only additional alternative splicing detected was in the region between exons 17 and 19. Exon 18 encodes the extreme C terminus of the

Fig. 3. Splice variation of the EGL-15 C terminus. Analysis of *egl-15* transcripts revealed multiple splice variants affecting the very C terminus of EGL-15. The C-terminal *egl-15* exons are depicted on the left. Thickly edged rectangles represent alternate exons used between the common exons 17 and 19. Shading indicates coding regions. The numbers of cDNAs of each type isolated from each pool are shown on the right. C-terminal alternate forms are represented in approximately equal proportions in both 5A- and 5B-containing isoforms. Two YK ESTs extend to exon 5; both yk36b5 (Type 1) and yk322c4 (Type 3) contain exon 5B. Asterisks indicate stop codons.



CTD. A characterization of the 3' splicing pattern for 23 5A-containing, 17 5B-containing and the Kohara EST cDNA clones (YK ESTs) showed that there is one major variant in this region (type 1) and four minor variants (types 2-5) (Fig. 3). These variant transcripts encode a total of four different peptides at the extreme C terminus of EGL-15. Nine of the ESTs and one cDNA of each type were fully sequenced; no splice variation in other regions of *egl-15* was observed. Although five types of C termini were found, their distribution is roughly equivalent in the 5A- and 5B-containing cDNAs (Fig. 3).

EGL-15(5A) and EGL-15(5B) are required for distinct EGL-15 functions

To assess the importance of the 5A and 5B isoforms for the two distinct EGL-15 activities, we created constructs that lacked the ability to produce either EGL-15(5A) or EGL-15(5B). Nonsense mutations in either the 5A exon [*egl-15(5A⁻B⁺)*] or the 5B exon [*egl-15(5A⁺B⁻)*] were introduced into a genomic rescuing fragment. These were tested using transgenic rescue assays.

To test for the ability to carry out the essential function, these plasmids were assayed for rescue of the viability defect of an *egl-15(null)* mutant. If the construct rescued and generated homozygous viable transgenic lines, the lines were also tested for rescue of the SM migration defect. As shown in Fig. 4A, the Let defect of *egl-15(n1456)* was rescued in transgenic lines expressing EGL-15(5B) alone (2/2 lines) but not those expressing only EGL-15(5A) (0/7 lines). Combined with the fact that *egl-15* mutants that lack EGL-15(5A) are viable, these data indicate that EGL-15(5B) is both necessary and sufficient for the essential function of EGL-15.

Final SM positions were determined in the viable transgenic lines (Fig. 4B). Many SMs were precisely positioned in *egl-15(5A⁺B⁺)* transgenic lines, indicating that the genomic rescuing fragment rescues SM migration defects in addition to the essential function of EGL-15. By contrast, *egl-15(5A⁻B⁺)* failed to rescue SM migration in both transgenic lines (Fig. 4B). These distributions are similar to the SM distributions observed in *egl-15(Egl)* mutants, confirming that EGL-15(5A) is required for normal SM chemoattraction. *egl-15(5A⁻B⁺)* also fails to restore precise positioning in an *egl-15(Egl)*

background (Fig. 4A). Failure of *egl-15(5A⁺B⁻)* to rescue the lethality of *egl-15(n1456)* prevents us from determining whether EGL-15(5A) is sufficient for SM chemoattraction. EGL-15(5A⁺B⁻) was sufficient, however, to restore SM responsiveness to the EGL-17/FGF chemoattractant that is otherwise abrogated in the putative EGL-15(5A) null allele *n1458* (Fig. 4A,B).

Specificity of ligand function

The endogenous functions of the two *C. elegans* FGFs precisely match the normal specificity of the two EGL-15 isoforms: EGL-17 and EGL-15(5A) are required for SM chemoattraction, and LET-756 and EGL-15(5B) are required for the essential function. This ligand-receptor match suggests a simple model in which EGL-17 interacts specifically with EGL-15(5A) to trigger SM chemoattraction, while LET-756 interacts specifically with EGL-15(5B) to trigger the essential function (Fig. 2C). The response elicited by each ligand could be specified by the ligand itself, tissue-specific patterns of expression or something inherent in the different receptor isoforms.

To begin to test the molecular basis of this signaling specificity, we assessed whether the roles of the two ligands could be switched by altering their sites of expression. For each ligand, we used genomic constructs containing either the endogenous 5' flanking region or the heterologous 5' flanking region from the other gene; these constructs were tested in germline transformation rescue assays (Table 1 and Fig. 5). We assumed, but did not rigorously establish, that this exchange of the 5' flanking regions for the two *C. elegans* FGF genes would switch their sites of expression. To test for rescue of *egl-17*, transgenic lines were scored for rescue of both the Egl phenotype and the SM migration defect. To test for rescue of *let-756*, transgenic lines were qualitatively scored for rescue of the larval lethality, and the brood size of rescued animals was determined to assess the extent of rescue.

When expressed under the control of the heterologous promoter, each of the ligands could rescue the defect of the heterologous gene (Table 1). Thus, LET-756 can function as an SM chemoattractant and EGL-17 can trigger the essential function of EGL-15. However, the extent of rescue was not as robust as that observed with the ligand that normally mediates these activities (Table 1 and Fig. 5). The decreased rescue was

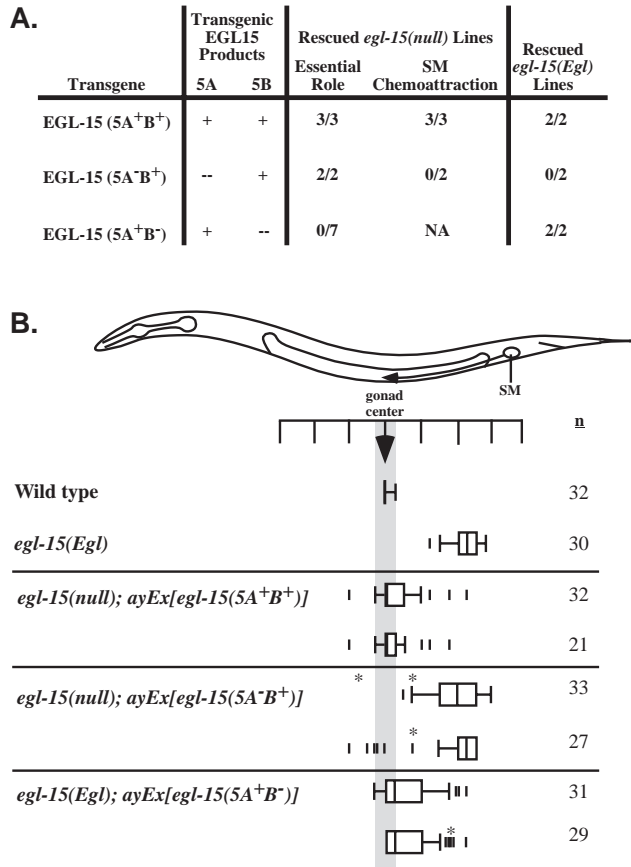


Fig. 4. EGL-15 splice variants mediate different responses. (A) The wild-type *egl-15* genomic rescuing fragment [EGL-15(5A⁺B⁺)] rescues defects associated with the *egl-15* (null) allele, including defects in both the essential role and SM chemoattraction. The essential role is also restored by expression of EGL-15(5A⁺B⁻); however, these lines exhibit aberrant SM migration. The EGL-15(5A⁺B⁻) transgene failed to restore the essential function in this null mutant, but rescued the chemoattraction defect of the *egl-15*(*Egl*) mutant, *egl-15*(*n1458*), based on final SM positions. (B) SM distributions for two transgenic lines for each of the constructs shown in A. As these transgenic animals bear extrachromosomal arrays that are mitotically lost at a significant frequency, some of these SMs may have lost the transgene. Complete loss of EGL-15 in the SMs results in centrally dispersed SMs (C.S.B., S.J.G. and M.J.S., unpublished), probably accounting for the outlying SMs.

manifested in any of a number of ways: rescue could require higher concentrations of the transgenic construct, the proportion of rescued lines could be significantly lower, or the extent of rescue could be less complete. For example, *let-756*, when expressed under the control of the *egl-17* promoter, could carry out the chemoattractive function of *egl-17* (Fig. 5). Despite its ability to carry out the heterologous function, a higher concentration of rescuing DNA was necessary and the proportion of rescued lines was reduced (Fig. 5). Similarly, although *egl-17* could rescue the lethality of *let-756* mutants

when expressed under the control of the *let-756* promoter, the brood size of the viable transgenic animals was significantly decreased (Table 1). Nevertheless, despite differences in efficiency, these two FGFs can stimulate either process.

When carrying out a heterologous function, the ligand might use either the receptor isoform normally associated with that function or the isoform normally associated with that ligand. To determine whether LET-756 functions as an SM chemoattractant via EGL-15(5A) or EGL-15(5B), we introduced the rescuing *P_{egl-17}::let-756* transgenes into a background lacking EGL-15(5A) (Fig. 6). For both lines tested, rescuing activity of the *let-756* chimeric transgene was completely lost in the absence of EGL-15(5A) (Fig. 6). Thus, LET-756 can function as an SM chemoattractant, but requires EGL-15(5A), the EGL-15 isoform that normally functions in chemoattraction.

Conversely, to test whether EGL-17 function as the essential FGF was dependent on the activity of EGL-15(5A), we introduced the *P_{let-756}::egl-17* transgenes into a background lacking EGL-15(5A) (Table 1). We found that rescue of the *let-756* Let defect by either LET-756 or EGL-17 was independent of EGL-15(5A) (Table 1). Thus, the two worm FGFs each are able to stimulate both of the normal functions of EGL-15, but they require the receptor isoform normally associated with the particular function. Therefore, some feature of the individual isoforms is critical in establishing signaling specificity.

Table 1. Summary of ligand replacement assays

Transgene	Dose (ng/μl)	Essential function			SM positioning		
		Rescued lines	Brood size	Lines rescued without EGL-15(5A)	Rescued lines	% non-Egl animals [§]	Lines rescued without EGL-15(5A)
None*	0	0/3	—*	—	0/4	13.1	—
<i>P_{let-756}::let-756</i>	5	10/10	75.0±7.9	1/1	—	—	—
	50	14/14	88.9±14.8	1/1	0/4	22.3	—
<i>P_{egl-17}::egl-17</i>	5	—	—	—	4/4	84.8	—
	50	0/4 [†]	—	—	5/6	72.9	0/1
<i>P_{let-756}::egl-17</i>	5	4/4 [‡]	2.0±1.0 [‡]	4/4	—	—	—
	50	3/3 [‡]	12.3±15.0 [‡]	—	0/5	6.8	—
<i>P_{egl-17}::let-756</i>	5	—	—	—	0/3	14.1	—
	50	3/3	7.7±5.2	—	2/8	36.3	0/2

*Co-transformation marker only; *let-756* homozygotes all die in these lines.

[†]Although this transgene fails to rescue the lethality of *let-756*, some of the inviable zygotes can develop beyond the normal stage of arrest.

[‡]Although all transgenic lines rescue the lethality of *let-756*, these animals were less healthy, developed at a slower rate and had a much reduced brood size.

[§]Average for all transgenic lines.

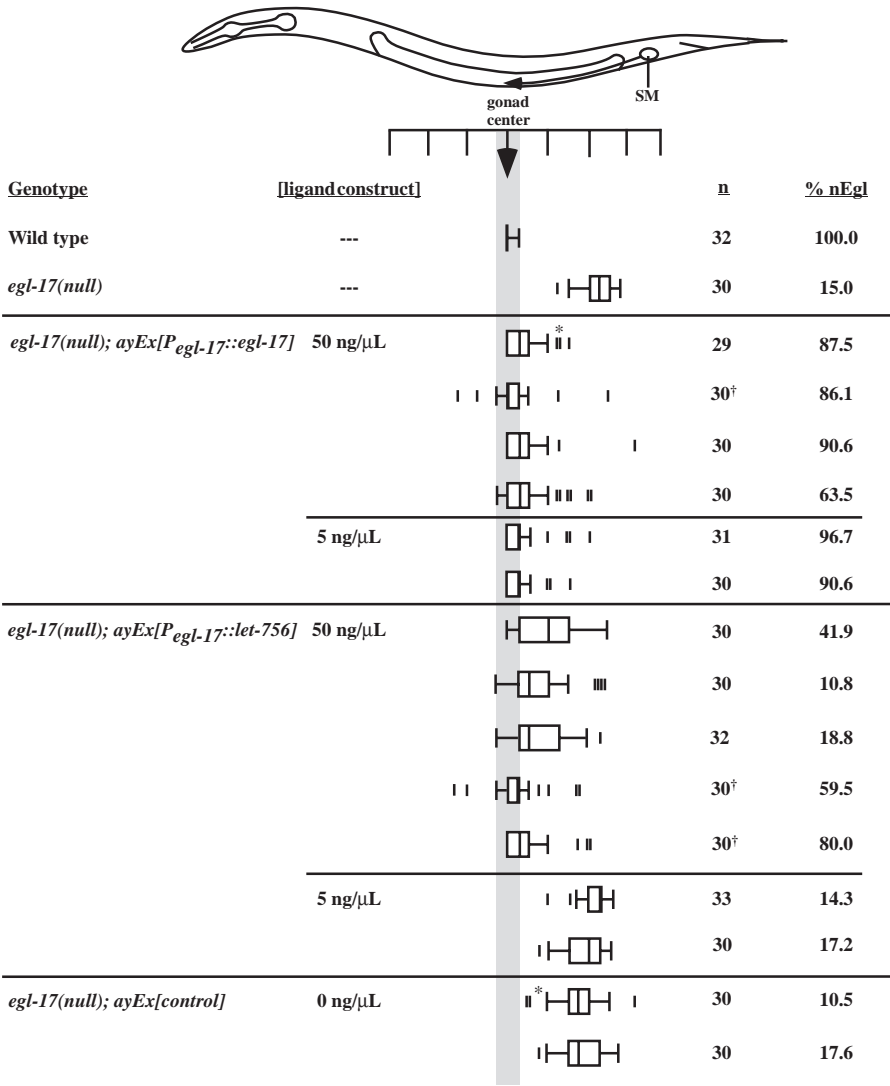


Fig. 5. LET-756 can mediate SM chemoattraction. SM distributions in an *egl-17(null)* background. EGL-17 expressed from its native promoter consistently rescues *egl-17(null)* at both high and low concentrations. Expression of LET-756 from the *egl-17* promoter can rescue the *egl-17(null)* chemoattraction defect in a subset of lines at a high concentration, but fails to rescue at the lower concentration. The *egl-17(null)* allele used was *n1377*. The control array contained only the transformation marker plasmid. The data for lines marked [†] are also represented for comparison in Fig. 6. Asterisks indicate dorsally positioned SMs. % nEgl, percent non-Egl transgenic animals.

et al., 2001) (P. Huang and M.J.S., unpublished). The second module, which is responsible for mediating SM chemoattraction, uses the EGL-17 ligand and the EGL-15(5A) receptor isoform. It is unclear whether the 5B isoform is also necessary for chemoattraction, as *egl-15(5A⁺5B⁻)* animals die prior to the stage at which the SMs migrate. The division of function for the two EGL-15 receptor isoforms explains the phenotypic specificity of the *egl-15(Egl)* alleles. Consistent with our model, reducing the dosage of EGL-15 by placing *egl-15(5A⁻5B⁺)* alleles in *trans* to an *egl-15(null)* allele results in hermaphrodites that display only an Egl phenotype and are otherwise viable and healthy (DeVore et al., 1995) (data not shown). Similar results obtained using the CTD truncation allele *egl-15(n1457)* support the specific role of the CTD in SM chemoattraction.

DISCUSSION

Receptor tyrosine kinases (RTKs) share a common catalytic function yet elicit many distinct biological responses. Multiple factors enable the specificity of signaling outputs achieved by RTKs in general and FGF receptors in particular. Our analysis of the Egl-specific alleles of *egl-15* has laid the groundwork for establishing the mechanisms used to confer EGL-15 FGF receptor signaling specificity and helped to clarify the role of EGL-15 in SM migration. Mutations in three out of four *egl-15(Egl)* alleles revealed the existence of an alternative exon 5 and led to the discovery of two isoforms of the EGL-15 FGF receptor. Our analysis of the respective functions of these isoforms has shown that FGF signaling in *C. elegans* is normally mediated by two parallel modules (see Fig. 2C). One module carries out the essential function of EGL-15, using the LET-756 ligand and the EGL-15(5B) receptor isoform. This module also appears to mediate the Soc function of EGL-15, as mutations affecting the 5B isoform confer a Soc phenotype, whereas mutations that eliminate the 5A isoform do not. The Soc function of this module extends to the ligand as well, because mutations in *let-756*, but not *egl-17*, also confer a Soc phenotype (Borland

Exon 5 of the *egl-15* gene encodes an additional FGF receptor domain that is inserted between the first and second IG domains within its extracellular region. A theoretical transcript in which exon 4 is spliced directly to exon 6 would encode a full-length FGF receptor that is structurally identical to vertebrate FGF receptors. Nevertheless, a comprehensive *egl-15* cDNA analysis did not reveal the existence of any *egl-15* transcripts encoding an FGF receptor architecture without one of the two EGL-15-specific inserts. The conservation of both exons 5A and 5B in the related nematode *Caenorhabditis briggsae* (http://www.sanger.ac.uk/projects/c_briggsae/BLAST_server.shtml) suggests an important biological function for this novel domain and its alternative splicing. Interestingly, the 5A and 5B domains are unique to EGL-15, with no significant homology to regions of any other protein in public databases or even within the genomic sequences of human FGFRs.

The C-terminal domain (CTD) is the only other site at which alternate splicing in *egl-15* was observed. This splicing variation generates four different C-terminal protein variants, an abundant Type 1 variant and three less abundant minor variants. In all cases, the first 91 amino acids of the CTD are common,

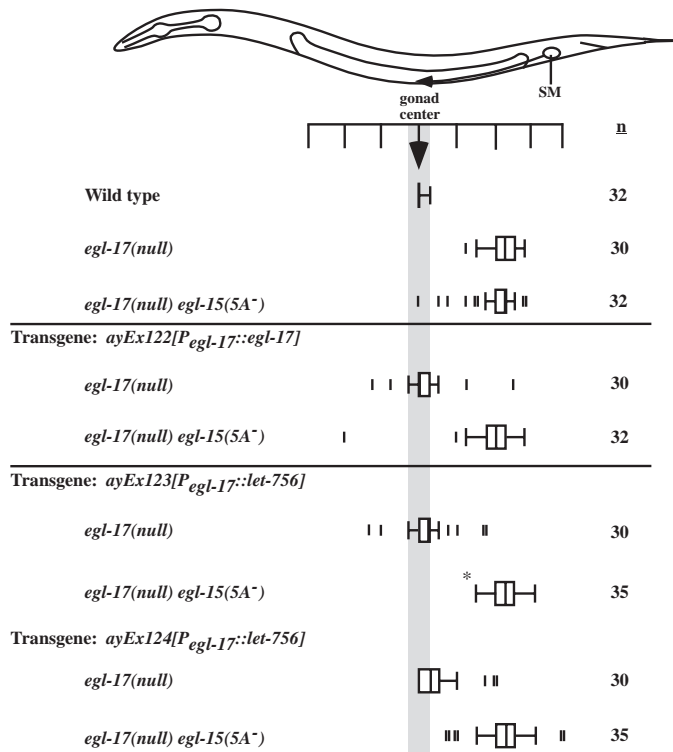


Fig. 6. LET-756-mediated SM chemoattraction requires EGL-15(5A) activity. The *egl-15(5A-5B⁺)* allele *n484* was introduced into the background of *egl-17(null)* rescued transgenic lines from Fig. 5. Removal of EGL-15(5A) in this manner results in the posterior displacement of SMs in all lines tested, demonstrating that this receptor isoform is crucial for SM chemoattraction independent of the ligand used as the chemoattractant.

each variant differing only at the extreme C terminus of the CTD. For comparison, the *n1457* truncation occurs after the first 15 amino acids of the CTD. Each CTD splice variant appears to be equivalently represented in both 5A- and 5B-containing cDNAs. The lack of a requirement for this domain in the essential function suggests that alternate splicing of this region cannot account for the functional differences observed among receptor isoforms. Nevertheless, our data reveal the crucial role the C-terminal domain serves during SM chemoattraction, a function that has precedent with other FGF receptors. Previous work has identified a 14 amino acid segment of the hFGFR1 CTD necessary for the chemotaxis function of this receptor (Landgren et al., 1998). This motif is not conserved in the EGL-15 CTD, although the function of this domain may be conserved. Analysis of components that interact with this portion of EGL-15 might provide important insights into the links between FGF receptors and the downstream signaling pathways that drive chemotactic movement.

The distinct functions of the two EGL-15 receptor isoforms raises the question of the roles of the two EGL-15-specific inserts in generating the specific in vivo responses to EGL-15 activation. The observed signaling specificity of the two modules of ligand/receptor pairs can derive from many sources, including the specific ligand used, the nature of the receptor mediating the response, and tissue-specific expression of either of these components or other factors that participate

in signal transduction. The discovery of two EGL-15 isoforms and the signaling modules they are associated with has allowed us to test signaling specificity in this system more rigorously.

We began this investigation by testing the interchangeability of the two FGF ligands and their dependence on the specific receptor isoforms. Our data demonstrate that the ligands exhibit a significant degree of functional equivalence; either ligand can be used to stimulate either EGL-15-mediated process. By contrast, we observed a specific functional requirement for each receptor isoform. EGL-15(5A) is required to mediate chemoattraction of the sex myoblasts, and EGL-15(5B) is required to elicit the essential function of this receptor. These data demonstrate that signaling specificity is determined in significant part by the specific receptor isoform, independent of the stimulating ligand.

It is likely that multiple mechanisms contribute to generate the specific cellular responses to EGL-15 activation. The sites of ligand expression are important for defining which of the two ligands triggers the two major EGL-15 activities, as swapping their promoters reveals a large degree of functional interchangeability. However, in addition to this mechanism, differences in their affinities for the two EGL-15 isoforms might also contribute to the observed signaling specificity. Although the major ligand-binding determinant for FGF receptors comprises the IG2 and IG3 domains, the alternative EGL-15-specific inserts are situated in a region that could easily affect ligand-binding affinity. In fact, other regions of the extracellular domain are known to modify ligand-binding affinity (Shimizu et al., 2001; Wang et al., 1995). Considering the decreased efficiency of heterologous ligand rescue, it remains possible that differences in ligand-receptor affinities contribute to the mechanism by which the different ligands stimulate different biological events. A detailed biochemical analysis of ligand binding affinities will be necessary to resolve this issue.

In addition to a region of signaling specificity arising from tissue-specific ligand expression, some aspect of the isoforms helps determine their functional specificity. At the moment, the mechanistic basis of this specificity is not clear, but several possibilities are likely. One possibility is that the normal specificity observed for the isoforms is due to tissue-specific alternative splicing. Modifying the expression of these isoforms could test this hypothesis. Alternatively, the structure of these domains might confer functional specificity. This could be effected by providing an alternative conformation that might alter intracellular signaling potentials, by allowing alternative modifications, or by permitting interactions with different co-receptors.

The set of missense mutations in the extracellular domain of EGL-15 bears an interesting relation to a specific achondroplasia (dwarfism) mutation in human FGFR3. Within the collection of *egl-15* alleles characterized, only three missense mutations were identified in the extracellular domain. *n2205* (*G492E*) is the identical alteration detected in a case of achondroplasia, affecting the analogous residue G346 at the C terminus of IG3 of human FGFR3 (Prinos et al., 1995). Interestingly, the other extracellular missense mutations in EGL-15 are related to this mutant allele. *n2202* (*G492R*) changes the same residue to a different, and oppositely charged, amino acid, while *n2210* (*G374E*) is the identical mutational alteration at the corresponding position within IG2 rather than IG3. These mutations were isolated as *soc* alleles

of *egl-15*, indicating that they have a net effect of reduced EGL-15 signaling. By contrast, achondroplasia mutations are thought to result in elevated FGF receptor activity (Webster and Donoghue, 1997). As these mutations affect the core of the ligand-binding region of FGF receptors, it is possible that their effects on activity are extremely context dependent. The net effect these mutations have on receptor function may reflect a balance between the impairment of ligand binding and an ability of the mutation to constitutively activate the receptor.

The discovery of the EGL-15(5A) isoform has provided a clear model for how EGL-15 contributes to the gonad-dependent attraction, and thereby clarified the contribution of FGF signaling to the mechanisms involved in SM migration guidance. The simplicity of this migration makes it ideal to probe the complex series of events that propel migrating cells: a bilaterally symmetric pair of cells move in a single anterior direction, at a fairly uniform rate from well-defined initial positions to precise final positions. Nonetheless, a complex series of guidance mechanisms is used to accomplish this apparently simple migration (Branda and Stern, 2000; Chen and Stern, 1998). The results of our current study present an important foundation from which to explore fully the mechanisms by which EGL-15 assists in the integration of these multiple migratory cues, as well as the mechanism by which FGF receptors achieve signaling specificity.

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