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Genomes and developmental control

Regulation of UNC-130/FOXD-mediated mesodermal patterning in *C. elegans*

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ABSTRACT

Spatial polarity cues in animals are used repeatedly during development for many processes, including cell fate determination, cell migration, and axon guidance. In *Caenorhabditis elegans*, the body wall muscle extends the length of the animal in four distinct quadrants and generates an UNC-129/TGF-β-related signal that is much higher in the dorsal two muscle quadrants compared to their ventral counterparts. This pattern of *unc-129* expression requires the activity of the proposed transcriptional repressor UNC-130/FOXD whose body wall muscle activity is restricted to the ventral two body wall muscle quadrants. To understand how these dorsal-ventral differences in UNC-130 activity are established and maintained, we have analyzed the regulation of *unc-130* expression and the distribution of UNC-130 protein. We have identified widespread, *cis*-acting elements in the *unc-130* promoter that function to positively regulate ventral body wall muscle expression and negatively regulate dorsal body wall muscle expression. We have defined the temporal distribution of UNC-130 protein in body wall muscle cells during embryogenesis, demonstrated that this pattern is required to establish the dorsal-ventral polarity of UNC-129/TGF-β, and shown that UNC-130 is not required post-embryonically to maintain the asymmetry of body wall muscle *unc-129* expression. Finally, we have tested the impact of the depletion of a variety of transcription factors, repressors, and signaling molecules to identify additional regulators of body wall muscle UNC-130 polarity. Our results confirm and extend earlier studies to clarify the mechanisms by which UNC-130 is controlled and affects the pattern of *unc-129* expression in body wall muscle. These results further our understanding of the transcriptional logic behind the generation of polarity cues involving this poorly understood subclass of Forkhead factors.

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1. Introduction

During development, spatial cues are utilized to determine cell fates, direct cell migration and patterning, and organize tissues. The molecular mechanisms involved in establishing and maintaining these cues are quite complex and they are critical for proper development in metazoa. In *Caenorhabditis elegans,* a non-canonical UNC-129/TGF-β is preferentially generated by, and presumably secreted from, dorsal body wall muscle cells to establish a dorsal-ventral gradient. This asymmetric signaling cue is utilized throughout development in *C. elegans* by a variety of cell types as they migrate and extend cellular processes. Although the transcription factor UNC-130 was identified genetically as a key transcriptional regulator of *unc-129* (Nash et al., 2000), the molecular mechanisms that establish and maintain dorsal-ventral polarity of *unc-130* expression in body wall muscle cells are unknown.

UNC-130 is a member of a large group of evolutionarily conserved Forkhead Box (FOX) transcription factors that can act as transcriptional activators or repressors; 15 canonical FOX factors have been identified in *C. elegans* (Hope et al., 2003). Characterized by a conserved 110-amino acid 'Forkhead' DNA-binding domain, also referred to as the 'winged-helix' domain (Hansen et al., 2007), the FOX factors are classified into nineteen subclasses (FOXA to FOXS) (Tuteja and Kaestner, 2007). In Xenopus, the FOXD subclass factor (FOXD3) acts as a transcriptional repressor that, in a non-cell-autonomous manner, results in the induction and patterning of dorsal mesoderm through maintenance of *Nodal* (a TGF-β superfamily member) expression in the Spemann organizer (Steiner et al., 2006). In *C. elegans*, the FOXD subclass member, UNC-130, genetically negatively regulates UNC-129/TGF-β in mesodermal body wall muscle and other cell types, suggesting conserved aspects of developmental regulation (Nash et al., 2000).

To gain insight into the mechanisms that drive mesoderm formation, spatial gradients, and animal polarity, we investigated the developmental expression pattern for *unc-130* using a

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combination of transcriptional and translational reporter genes and antibody staining. Dissection of *cis*-acting *unc-130* DNA sequences revealed promoter elements that are necessary to enhance ventral, while restricting dorsal, body wall muscle expression that are distributed over more than 10 Kb upstream of the translational start site. Some of these *cis*-acting regions contain sequences that are highly conserved and include potential FOX and other transcription factor DNA binding sites. Reporter genes and antibody staining demonstrated that the pattern of ventral body wall muscle *unc-130* expression, which is required to repress *unc-129* expression, is established during the last half of embryogenesis and is cell autonomous; mis-expression of *unc-130* in dorsal body wall muscle is sufficient to silence *unc-129*::*gfp*. Interestingly, the wild type pattern of post-embryonic body wall muscle expression for *unc-129*::*gfp* appears to be UNC-130-independent, suggesting that other factors act to maintain the UNC-129/TGF-β asymmetric pattern that is established during embryogenesis. Our results more clearly define the function of this family of transcription factors that are generally poorly understood as a result of the complex and pleiotropic roles they play in metazoan development.

2. Materials and methods

2.1. C. elegans strains and alleles

Standard culture conditions (Brenner, 1974) were used unless otherwise stated, with N2 (variety Bristol) serving as the wild-type strain. Other strains utilized in the experiments included: KM499 ($P_{unc\text{-}130\ (5.9\ Kb)}$::*unc-130*:*gfp*) transgenic for a 5.9 Kb region upstream of *unc-130* driving expression of the coding region of (lacking codons for the last 12 amino acids) fused to GFP with the *unc-54* 3′ UTR provided in the plasmid pPD95.79 (Fire Lab Vector Kit), KM510 & KM511 ($P_{unc\text{-}129\ (4.2\ Kb)}$::*gfp*) transgenic for the 4.2 Kb region upstream of the translational start site of *unc-129* driving GFP expression, KM512 [$P_{unc\text{-}129\ (4.2\ Kb)}$::*gfp*; *unc-130(oy10)*], KM513 [$P_{unc\text{-}129\ (4.2\ Kb)}$::*gfp*; *unc-130(ev505)*], KM514 ($P_{unc\text{-}130\ (10.5\ Kb)}$::*unc-130*::*gfp*) was identical to KM499 with the exception that the promoter region of KM514 extends 10.5 Kb upstream of the *unc-130* translational start codon, KM515 [*zag-1(hd16)*; *unc-17 (e113) dpy-13(e184)IV*], KM516 [$P_{unc\text{-}129\ (4.2\ Kb)}$::*gfp*; *pag-3(ls20)X*], KM517 and KM518 ($P_{unc\text{-}130\ (10.5\ Kb)}$::*unc-130* cDNA::*unc-130* 3′*UTR*), KM520 ($P_{unc\text{-}129\ (4.2\ Kb)}$::*unc-130* cDNA::*unc-130* 3′ UTR), and KM521($P_{unc\text{-}130\ (10.5\ Kb)}$::*gfp*).

The following strains were obtained from the *Caenorhabditis* Genetics Center: PY1133 *unc-130 (oy10) II*, EA81 *pag-3(ls20) X*, CB1893 *unc-17(e113) dpy-13(e184) IV*, *mef-2(gv1)*. Strains kindly provided for these studies were VH661 *zag-1(hd16)* from Harold Hutter (Simon Fraser University, Vancouver, Canada), PY1438 *unc-130*(*ev505*) from Piali Sengupta (Brandeis University, MA), and *evIs120(unc-130::gfp; pRF4)* from Joe Culotti (Mount Sinai Hospital).

2.2. Generation of unc-130 reporter and mutant rescue genes

A PCR fusion-based approach (SOEing) (Hobert, 2002) was used to generate a series of reporter genes in order to map putative *cis*-acting DNA elements in the *unc-130* upstream promoter region. Fragments ranging from −10.5 Kb to −1 Kb relative to and including the translation initiation codon of *unc-130* (WormBase, WS243) were fused immediately upstream of a green fluorescent protein (GFP) reporter gene (constructs 3010–3017; see Fig.3). For each SOEing construct, at least two extra-chromosomal transgenic lines were established to observe the expression pattern of the reporter gene. Analysis of various *unc-130* promoter regions included clones in which the 1 Kb segment immediately upstream of the *unc-130* translational start site was fused downstream of additional 1 Kb segments of *unc-130* regions between −10.5 Kb and −4 Kb. These reporter genes used vector pPD95.67 (Fire Vector Kit) to generate constructs pKM3037 to pKM3042. Sequence-specific mutations in reporter gene constructs (pKM3030-pKM3036) were generated using the QuikChange® XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Mutant clones were verified by Sanger sequencing.

Additional GFP transcriptional reporter constructs were made by amplifying specific regions of the *unc-130* upstream promoter region by PCR and cloning them individually into the reporter gene vector pPD95.67. Assayed fragments included clones extending upstream from the *unc-130* ATG codon to positions −1000, −879, −574 and −308 bp (pKM3018 to pKM3021, respectively). Several *unc-130* promoter fragments generated by amplification of genomic DNA were also cloned upstream of the *unc-54* minimal promoter GFP reporter plasmid pPD94.81. This plasmid vector has 238 bp from the *unc-54* promoter that on its own drives strong, uniform GFP expression in all body wall muscle and the vulva muscles. Specifically, we tested the influence of the −1000, −879, and −574 bp fragments from the *unc-130* upstream promoter region on GFP expression from the *unc-54* promoter by generating plasmids pKM3022, pKM3024, and pKM3029, respectively. The *unc-130* promoter fragments, between −879 and −574 that were fused to *unc-54::gfp,* were subsequently deleted from the −1000 bp *unc-130* promoter region and the resulting fragment was cloned into pPD94.81 (pKM3023). Additional deletion constructs included: −879 to −752 (pKM3025), −777 to −630 (pKM3026), −751 to −630 (pKM3027) and −630 to −557 (pKM3028).

The *unc-130* reporter gene or mutant rescue constructs that contained *mCherry* (*mCh*) were derived from the *mCh::unc-54 3′ UTR* fragment in Erik Jorgenson's pCFJ90 construct (Addgene, plasmid #19327) that was transferred into the pVZ-1 vector to generate plasmid (*mCh::unc-54 3′ UTR*); this starting plasmid was used to clone multiple *unc-130* promoter and coding regions (genomic or cDNA) fragments upstream of mCherry. Either −5.9 Kb or −10.5 Kb *unc-130* promoter fragments were generated by PCR from N2 genomic DNA; those fragments were individually cloned into *mCh::unc-54* to generate pKM3044 ($P_{unc\text{-}130\ (5.9\ Kb)}$::*unc-130*$_{(genomic)}$::*mCh*::*unc-54 3′UTR)* and pKM3045($P_{unc\text{-}130\ (10.5\ Kb)}$::*unc-130*$_{(genomic)}$::*mCh*::*unc-54 3′UTR*) respectively.

To direct the expression of *unc-130* cDNA under control of either the *unc-129* or *unc-54* promoter, we followed the same procedures as that for pKM3048, with the exception that a new forward primer (mCherry Forward *NotI-1*) was used; this generated plasmid pKM3052 (*mCh*::*unc-54 3′UTR*). The *unc-129* and *unc-54* promoter fragments were generated by PCR from N2 DNA; those fragments were individually cloned into pKM3052 to generate pKM3053 ($P_{unc\text{-}129\ (4.2\ Kb)}$::*unc-130::mCh::unc-54 3′ UTR*), pKM3054 ($P_{unc\text{-}54\ (pPD30.35)}$::*unc-130::mCh::unc-54 3′ UTR*), and pKM3055 ($P_{unc\text{-}54\ (pPD94.81)}$::*unc-130::mCh::unc-54 3′ UTR*) respectively.

Transgenic animals harboring reporter genes were generated by standard DNA injection techniques (Mello et al., 1991). DNA was injected at various concentrations depending upon the plasmid. The plasmid pRF4 was often used as a co-injection marker at 60 ng/μl. For mutant rescue constructs and subsequent assays in *unc-129::gfp*, *rol-6* transgenic strains, the *myo-2::gfp* reporter plasmid (1 or 5 ng/μl) and/or *elt-2::mTomato* (pJM371; 100 ng/μl) were used as a transgenic marker with the rescuing gene plasmid (5–25 ng/μl). We noted difficulty obtaining viable F1 and stable lines with any construct designed to express the *unc-130* cDNA; a bit less toxicity was observed using the *unc-130* genomic coding region, but strongly expressing strains were difficult to maintain.