Deletion of a putative HDA-1 binding site in the *hlh-2* promoter eliminates expression in *C. elegans* dorsal uterine cells

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Abstract

The helix-loop-helix transcription factor *hlh-2* (E/Daughterless) has been shown to play an important role in regulating cell fate patterning, cell cycle, and basement membrane invasion in the context of the development of the *C. elegans* somatic gonad. Here, using CRISPR/Cas9 genome engineering, we generated a new *hlh-2* allele (*hlh-2(Δ-1303-702)*) in the endogenous, GFP-tagged *hlh-2* locus. This allele represents a deletion of a 601 bp region in the *hlh-2* promoter that contains a putative binding site of the histone deacetylase *hda-1* (HDAC) according to publicly available ChIP-sequencing data. Strikingly, we find that HLH-2 expression is virtually absent in the dorsal uterine cells of *hlh-2(Δ-1303-702)* animals compared to wild type controls. Levels of HLH-2 in the anchor cell and ventral uterine cells are only modestly reduced in the mutant; however, this does not seem to be functionally significant based on the lack of relevant phenotypes and expression levels of a downstream gene, NHR-67 (TLX/Tailless/NR2E1), in these cells. Taken together, these results support growing evidence that HDACs can potentially positively regulate transcription and provide a new reagent for studying *hlh-2* regulation.
**Figure 1. Characterization of new allele deleting putative HDA-1 binding site from the hlh-2 promoter:** (A) Schematic of the endogenous GFP::hlh-2 locus and promoter region, annotated with a previously characterized proximal regulatory element, putative HDA-1 binding site (based on published ChIP-sequencing data) and binding motif, and the genomic region deleted in the hlh-2(bmd231) mutant. (B) Nuclear expression of wild type (wt) GFP::hlh-2 in the anchor cell (AC), dorsal uterine (DU) cells, and ventral uterine (VU) cells. (C-D) Nuclear expression of GFP::hlh-2 in the AC (solid arrowhead), DU (arrows), and VU (unfilled arrowheads) cells of hlh-2(WT) and hlh-2(Δ-1303-702) animals. (D-E) Cytoplasmic expression of HLH-2 in the AC of hlh-2(WT) and hlh-2(Δ-1303-702) animals. (F-G) Expression of nhr-67::TagRFP-T in hlh-2(WT) and hlh-2(Δ-1303-702) animals. (H-I) Levels of GFP::hlh-2 following depletion of hda-1 via RNA interference compared to empty vector control in the DU (nuclei outlined hda-1(RNAi) micrograph). Statistical significance determined through using an unpaired two sample t-test, with Welch’s correction (*p ≤ 0.05; ***p ≤ 0.001; n.s., not significant). Scale bars: 5 µm.

**Description**

During *C. elegans* development, two cells, Z1 and Z4, give rise to the entire somatic gonad, including populations of dorsal uterine (DU) and ventral uterine (VU) cells, as well as the postmitotic anchor cell (AC), which invades the underlying basement membrane during uterine-vulval morphogenesis (Kimble & Hirsh, 1979; Sherwood & Sternberg, 2003). The helix-loop-helix transcription factor hlh-2 (E/ Daughterless) is expressed in these three cell types, among others, and is particularly enriched in the AC following post-transcriptional down-regulation of HLH-2 in the VU through dimerization-driven degradation (Karp & Greenwald, 2003; Sallee & Greenwald, 2015). At the L2 stage, HLH-2 regulates the Notch signaling event that specifies AC and VU fates, with its initial onset predicting the lineage ultimately giving rise to the presumptive VU (Karp & Greenwald, 2004; Attner et al., 2019). Following AC/VU specification, HLH-2 functions to regulate cell cycle arrest and invasion of the AC (Medwig-Kinney et al., 2020; Schindler & Sherwood, 2011). We and others have shown that HLH-2 does so by regulating expression of the nuclear hormone receptor and transcription factor NHR-67 (Tailless/TLX/NR2E1), and likely cell-cycle independent, pro-invasive targets as well (Bodofsky et al., 2018; Medwig-Kinney et al., 2020).

In addition to hlh-2 and nhr-67, the histone deacetylase (HDAC) hda-1 was also identified as a regulator of AC invasion through a reverse genetic screen (Matus et al., 2010). It was later shown that hda-1 function is necessary for AC invasive fate differentiation and plays a role in the pro-invasive pathway encompassing NHR-67 (Matus et al., 2015). In order to further elucidate the mechanism by which HDA-1 regulates AC invasion, we sought to test we sought to test whether HDA-1 regulated expression of hlh-2. Previous studies of the hlh-2 promoter identified a proximal regulatory element (hlh-2(prox)) that confers expression in the AC and VU cells specifically (Figure 1A) (Sallee & Greenwald, 2015). Approximately 3.3 kb downstream of this hlh-2(prox) element is an HDA-1 binding motif that lies within a putative HDA-1 binding site based on ChIP-sequencing data generated by the modENCODE Project (Figure 1A) (Shao et al., 2020; Celniker et al., 2009). Using CRISPR/Cas9 genome engineering, we edited the endogenous hlh-2 locus to introduce a deletion mutation (hlh-2(bmd231)) from 1303 to 702 base pairs (bp) upstream of the GFP start codon, hereafter referred to as hlh-2(Δ-1303-702) (Figure 1A).

Consistent with findings on immunostaining, we find that endogenous GFP::hlh-2 expression is normally enriched in the AC compared to the VU and DU (Karp & Greenwald, 2003) (Figure 1B). When compared to wild type, the hlh-2(Δ-1303-702) mutant showed a modest but statistically significant reduction in HLH-2 expression in the AC and VU cells (Figure 1C-D). The endogenous GFP::hlh-2 allele paired with high resolution microscopy allowed us to detect subtle regulation of HLH-2 expression by hda-1 in the AC and VU cells that was not previously reported using a transgenic reporter (Ranawade et al., 2013). DU expression, however, was virtually eliminated in the mutant when accounting for camera-derived background noise (Figure 1C-D). We also observed that cytoplasmic expression of HLH-2 in the AC was modestly reduced in the mutant as well (Figure D-E). However, the alterations in HLH-2 expression in the AC and VU do not seem to be functionally significant, as expression of a NHR-67, a downstream target of HLH-2 in the cell cycle dependent pro-invasive gene regulatory network (Medwig-Kinney et al., 2020), was not significantly reduced (Figure 1F-G). Furthermore, no defects in AC specification or invasion were observed in any of the strains containing the hlh-2(Δ-1303-702) allele (n > 50) based on both the presence of a single AC and an underlying gap in the basement membrane, in all animals examined (visualized by cdh-3p::mCherry::moeABD and laminin::GFP, respectively). RNAi-induced knockdown of hda-1 resulted in reduced levels of HLH-2 in the DU, providing further evidence of this regulatory interaction (Figure 1H-I).

In summary, here we generate and characterize a new mutant allele of hlh-2. Deletion of the genomic region containing a putative HDA-1 binding site from the hlh-2 promoter appears to primarily affect expression in the DU cells and is recapitulated through RNAi-induced knockdown of hda-1. Although HDA-1 is traditionally thought of as a repressor of transcription through chromatin deacetylation (Kadosh & Struhl, 1997; Kadosh & Struhl, 1998; Rundlett et al., 1998; Gui et al., 2003), this data adds to the growing evidence that HDACs can also function as activators of transcription (Vidal & Gaber, 1991; De Nadal et al., 2004; Wang et al., 2014). Whether there is a functional consequence of hlh-2 depletion in the DU cells...
is currently unknown. It is our hope that this reagent will be useful to the C. elegans community to further study the roles of hda-1 and hlh-2.

Methods
Request a detailed protocol

Strain maintenance:
Animals were reared under standard conditions and cultured at 25°C (Brenner, 1974). Animals were synchronized through alkaline hypochlorite treatment of gravid adults to isolate eggs (Porta-de-la-Riva et al., 2012). The RNAi clone targeting hda-1 was generated by cloning 923 bp of cDNA (available from wormbase.org; Harris et al., 2020) into the highly efficient T444T RNAi feeding vector (Sturm et al., 2018). RNAi experiments were performed by feeding synchronized L1s following hypochlorite treatment.

CRISPR/Cas9 injections:
In order to generate the deletion mutation in the hlh-2(Δ-1303-702) allele, Cas9 protein injections were performed as previously described (Paix et al., 2014; Ghanta et al., 2020) with minor modifications to the published protocols. In short, a 200 bp ssODN donor repair ultramer for hlh-2 (IDT) was constructed. To facilitate easy screening of our edit, a Pvul restriction site was engineered into the ssODN repair oligo as previously described (Paix et al., 2014), flanked on each side by 94 bp of sequence homologous to the region surrounding the putative HDA-1 binding site. Single guide RNAs for hlh-2 and dpy-10 (co-CRISPR marker) were purchased from CRISPRevolution by Synthego (Synthego Corporation). The Cas9/sgRNA ribonucleoprotein complex was formed by incubating 30 pmols of Cas9 NLS protein (California Institute for Quantitative Biosciences at UC Berkeley (QB3-Berkeley)) with 23.75 pmols of each hlh-2 gRNA, and 47.5 pmols of dpy-10 sgRNA for 15 minutes at 37°C. The hlh-2 and dpy-10 ssODN donor repair ultramers (IDT) were then added to the reaction at a final concentration of 2.2 µg.

TagRFP-T::AID was inserted into the C-terminus of the endogenous nhr-67 locus via CRISPR/Cas9 mediated genome engineering using the self-excising cassette method (Dickinson et al., 2013; Dickinson et al., 2015). The sgRNA targeting sequence in pDD122 was replaced with the sgRNA targeting the C-terminus of nhr-67 using Gibson cloning (Dickinson et al., 2013). The repair template for nhr-67 was generated by cloning homology arms, synthesized by Twist Biosciences (left homology arm) and by PCR using genomic DNA as a template (right homology arm), into pTNM063 (TagRFP-T::AID repair template) (Ashley et al., 2021).

Live cell imaging:
Micrographs were collected on a Hamamatsu Orca EM-CCD camera mounted on an upright Zeiss AxioImager A2 with a Borealis-modified CSU10 Yokagawa spinning disk scan head (Nobska Imaging) using 488 nm and 561 nm Vortran lasers in a VersaLase merge and a Plan-Apochromat 100×/1.4 (NA) Oil DIC objective. MetaMorph software (Molecular Devices) was used for microscopy automation. Animals were mounted into a drop of M9 on a 5% Noble agar pad containing approximately 10 mM sodium azide anesthetic and topped with a coverslip.

Image quantification:
Images were processed using Fiji/ImageJ (v.2.0.0) (Schindelin et al., 2012). Nuclear expression of HLH-2 and NHR-67 was quantified by measuring mean gray value as previously described (Medwig-Kinney et al., 2020). DU and VU cells were sampled by selecting the three most proximal to the AC and closest to the coverslip. Cytoplasmic expression of HLH-2 was measured using the freehand selection tool, tracing the outline of the cell but excluding the nucleus. For all quantification, background subtraction was performed by subtracting the mean gray value of a background region of an equal area to account for EM-CCD camera noise.

Data visualization and statistical analysis:
Representative micrographs were processed using Fiji/ImageJ and assembled into figures using Adobe Illustrator (v.23.0.6). RStudio (v.1.4.1717) was used to generate violin/sina plots and to perform statistical analyses.

Reagents
Strains:

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<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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DQM350: \(\text{hlh-2(bmd90[hlh-2p:\text{LoxP}\cdot\text{GFP}\cdot\text{HLH-2}]) I;}\)
\(\text{qyIs225[cdh-3p::mCherry\cdot\text{moeABD}] V;}\)
\(\text{qyIs7[laminin\cdot\text{GFP}]} \text{ X.}\)

**Medwig-Kinney et al., (2020)**

DQM704: \(\text{nhr-67(bmd212[nhr-67p:\text{NHR-67\cdot\text{TagRFP-T}\cdot\text{AID}}]) IV;}\)
\(\text{hlh-2(bmd90[hlh-2p:\text{LoxP}\cdot\text{GFP}\cdot\text{HLH-2}]) I.}\)

This study

DQM785: \(\text{hlh-2(bmd231[hlh-2p(\Delta-1303-702)\cdot\text{LoxP}\cdot\text{GFP}\cdot\text{HLH-2}]) I;}\)
\(\text{qyIs225[cdh-3p::mCherry\cdot\text{moeABD}]} \text{ V;}\)
\(\text{qyIs7[laminin\cdot\text{GFP}]} \text{ X.}\)

This study

DQM900: \(\text{hlh-2(bmd231[hlh-2p(\Delta-1303-702)\cdot\text{LoxP}\cdot\text{GFP}\cdot\text{HLH-2}]) I;}\)
\(\text{nhr-67(bmd212[nhr-67p::\text{NHR-67\cdot\text{TagRFP-T}\cdot\text{AID}}]) IV.}\)

This study

**Sequences:**

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<td>dpy-10 ssODN</td>
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<td>hda-1(RNAi) targeting sequence</td>
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**References**


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Writing - review and editing. David Q Matus: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review and editing.

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