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

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Abstract

The helix-loop-helix transcription factor \textit{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 \textit{C. elegans} somatic gonad. Here, using CRISPR/Cas9 genome engineering, we generated a new \textit{hlh-2} allele (\textit{hlh-2(Δ-1303-702)}) in the endogenous, GFP-tagged \textit{hlh-2} locus. This allele represents a deletion of a 601 bp region in the \textit{hlh-2} promoter that contains a putative binding site of the histone deacetylase \textit{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 \textit{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 \textit{hlh-2} regulation.
In summary, here we generate and characterize a new mutant allele of $hlh-2$ by using CRISPR/Cas9 genome engineering, we edited the endogenous $hlh-2$ locus to introduce a deletion mutation ($hlh-2(\Delta-1303-702)$) from 1303 to 702 base pairs (bp) upstream of the GFP start codon, hereafter referred to as $hlh-2(\Delta-1303-702)$ (Figure 1A). Consistent with findings from immunostaining, we find that endogenous GFP::$hlh-2$ expression is normally enriched in the AC compared to the DU and VU cells. (Figure 1B). When compared to wild type, the $hlh-2(\Delta-1303-702)$ 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 $hlh-2(\Delta-1303-702)$ 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).
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 ulramer for *hlh-2* (IDT) was constructed. To facilitate easy screening of our edit, a PvuI 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 pmoles of each *hlh-2* gRNA, and 47.5 pmoles of *dpy-10* sgRNA for 15 minutes at 37°C. The *hlh-2* and *dpy-10* ssODN donor repair ulramers (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:**

| Strain | Genotype | Source |
|--------|----------|--------|

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*9/2/2021 - Open Access*
| Reagent | Sequence |
|---------|----------|
| dpy-10 sgRNA | GCUACCAUAGGCACCACGAG + Synthego modified EZ Scaffold |
| dpy-10 ssODN | CACCTGGAACCTTCAATACGCGAACATGAAATGACTGGAACACCTGGCATGCAGTGCCCTATGGTAGCGGAGCTTCACATGGCTTCAGACCATAGT |
| hda-1(RNAi) targeting sequence | TTGATGGAGACCCGTAAGGCGCCTGTCGCTACTTACTATGACTCCAAACATTGAAATTACTATAATGCTCAAGGACTCAGACATGACATCTGAAACAGCCTATGAGGCAATCCAGAATTTTCATGAAATACGAGTTGAAATTATGGATGGAGTATGAGGAGGCGTTCTATACGACTGATCGAGTAATGACAGTGTCATTCCATAAATATGGAGATTTCTTCCAGGAGACCTGAAAGATATAGGAGCTGGAAAAGGAAAGCTCTATTCAGTCAATGTTCCACTTCGCGATGGGAATACCGACGTCTCTTACCAGAGTATTTTTAAACCAATCATGACAAAGGTTATGGAGAGATTTGATCCCTGTGCTGTTGTTCTTCAATGTGGAGCTGATTCTCTCTAACGGAGATAGACTTGGACCATTCAATCTGACCTTGAAAGGCCACGGAGAATGTGCTCGTTTCTTCCGAAAGCTACAACGTTCCACTTATGATGGTCGGTGGAGGTGGATACACTCCAAGAAATGTGGACAG |
| hlh-2 (-1303 bp) gRNA | AUGAAUGUACUCCCUACAGU + Synthego modified EZ Scaffold |
| hlh-2 (-702 bp) gRNA | UAAGGAAUUCGUAACACAUGU+ Synthego modified EZ Scaffold |
| Gene  | Description | Sequence |
|-------|-------------|----------|
| **hhl-2(Δ-1303-702)** ssODN | | CTCTCACTCTTACCATTACATTCTGAAAGAGATCTCCCTACAAAAACTCTAATAACATGGCTTCAAAAATGAAATGACTCCCTACAGTGATAAGCAAAATGTTTACGAATCCCTTACCAATTTTGAAATTTAACAAGAAACGCAGATGTATTGAGGCAGGCTTTTTTCTCTATTTAGATTTAGAGAAAAATTGAAAAGCAGAATATTTTTTCAAAGACTTTCAAAATTATAGGCGTAGGCTTTATTTTTGACTGTCAGTAAAATATTTATTCAAAAAAAATTTTAAAAGTTTTACCATAATATTTGGGCATGGGCATTTTACTTTTAAAAACGATTTCTAAAGAAACCATTTTTATATGTAAAACAGTTTTGCTCAATTTTACCAGTTATCAA |
| **nhr-67 C-terminus sgRNA** | | AGAGAGGTGTTAATGGTGAAG |
| **nhr-67 C-terminus left homology arm** | | GGAATAATGTGAGACTTCCACTATAAAGGTTAACAGCCTGTATTCTGTAGGTTGCAACGATCAAAGGTTAACAGCCTGCTATTTCAGCATGTTATTTAATAAGTTAATTTAACAAATGAAATGACGTCTTTCCTCAGAAATTTAAGGAAGAGAATGATGAACGTTTTTCTGAGTGGGTTGCAACGATCAGCTCTAGTACCATCTTTCCTTATGAAAACCTCAGTGAACCTAAATTTGTAGGGCAGTTTTTTTTTCAATTATTGAGTTTATCAAAA |
| **nhr-67 C-terminus right homology arm** | | TAAATAGTTAATTCATGTITTTCCATATACAGTAACCTCAATTATTTCTAAGAICTCTTTTTCATCTGCTTTTCATCCGTTTCTGCTCCGCGGCAATTTTCTGAGTTTTGCTAAATTTCAGAAAAATTGAAAAGCAGAATATTTTTTCAAAGACTTTCAAAATTATAGGCGTAGGCTTTATTTTTGACTGTCAGTAAAATATTTATTCAAAAAAAATTTTAAAAGTTTTACCATAATATTTGGGCATGGGCATTTTACTTTTAAAAACGATTTCTAAAGAAACCATTTTTATATGTAAAACAGTTTTGCTCAATTTTACCAGTTATCAA |
### Forward primer to amplify nhr-67 C-terminus right homology arm from gDNA

CGGCAGCGTTGCGAATAAATAGTAATTTGTTGCTATAC

### Reverse primer to amplify nhr-67 C-terminus right homology arm from gDNA

GCTATGACCATTCTGATTCTCCTGATTGATAACTGGTAAAATTGAGCA

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