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- Using CRISPR/Cas9 to identify genes required for mechanosensory neuron development

and function

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16 Abstract:
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22 **Abstract:**
17 Tunicates
18 vertebrate:
19 and a sess
20 ability to se
21 tractable la
22 the anterio
23 initiate the
24 *Ciona* by u Tunicates are marine, non-vertebrate chordates that comprise the sister group to the
vertebrates. Most tunicates have a biphasic lifecycle that alternates between a swimm
and a sessile adult. Recent advances have shed ligh vertebrates. Most tunicates have a biphasic lifecycle that alternates between a swimming larva

and a sessile adult. Recent advances have shed light on the neural basis for the tunicate larva'

ability to sense a proper su and a sessile adult. Recent advances have shed light on the neural basis for the tunicate larva's
ability to sense a proper substrate for settlement and initiate metamorphosis. Work in the highly
tractable laboratory model ability to sense a proper substrate for settlement and initiate metamorphosis. Work in the highly

21 tractable laboratory model tunicate *Ciona robusta* suggests that sensory neurons embedded in

22 the anterior papillae tractable laboratory model tunicate *Ciona robusta* suggests that sensory neurons embedded in

the anterior papillae of transduce mechanosensory stimuli to trigger larval tail retraction and

initiate the process of metamo the anterior papillae of transduce mechanosensory stimuli to trigger larval tail retraction and

22 initiate the process of metamorphosis. Here, we take advantage of the low-cost and simplicit

23 *Ciona* by using tissue-s initiate the process of metamorphosis. Here, we take advantage of the low-cost and simplicity of

26 *Ciona* by using tissue-specific CRISPR/Cas9-mediated mutagenesis to screen for genes

25 potentially involved in mechano *Ciona* by using tissue-specific CRISPR/Cas9-mediated mutagenesis to screen for genes
potentially involved in mechanosensation and metamorphosis, in the context of an
undergraduate "capstone" research course. This small sc potentially involved in mechanosensation and metamorphosis, in the context of an

undergraduate "capstone" research course. This small screen revealed at least one

Vamp1/2/3, that appears crucial for the ability of the pa 26 undergraduate "capstone" research course. This small screen revealed at least one gene,

27 Vamp1/2/3, that appears crucial for the ability of the papillae to trigger metamorphosis. We

28 provide step-by-step protocols 27 *Vamp1/2/3,* that appears crucial for the ability of the papillae to trigger metamorphosis. We also
28 provide step-by-step protocols and tutorials associated with this course, in the hope that it might
29 be replicated 28 provide step-by-step protocols and tutorials associated with this course, in the hope that it might

29 be replicated in similar CRISPR-based laboratory courses wherever *Ciona* are available.

29 be replicated in simil 29 be replicated in similar CRISPR-based laboratory courses wherever *Ciona* are available.

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- 30 **Introduction**
31 Solitary tunica
32 developmenta
33 CRISPR/Cas9
4 employed to to
35 Sasakura and
36 animals an ide
37 CRISPR/Cas9
38 become more 31 Solitary tunicates (*Ciona spp.*) have emerged as highly tractable model organisms for
32 developmental, cell, and molecular biology (Cota 2018; Lemaire 2011). Tissue-specific
33 CRISPR/Cas9-mediated mutagenesis has bee
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developmental, cell, and molecular biology (Cota 2018; Lemaire 2011). Tissue-specific

CRISPR/Cas9-mediated mutagenesis has been adapted to *Ciona robusta* and is now re

employed to test the functions of genes in *Ciona* CRISPR/Cas9-mediated mutagenesis has been adapted to *Ciona robusta* and is now routinely

employed to test the functions of genes in *Ciona* embryos and larvae (Gandhi et al. 2018;

Sasakura and Horie 2023). The low-cost employed to test the functions of genes in *Ciona* embryos and larvae (Gandhi et al. 2018;

Sasakura and Horie 2023). The low-cost and ease of CRISPR/Cas9 in *Ciona* makes these

animals an ideal organisms for laboratory c 35 Sasakura and Horie 2023). The low-cost and ease of CRISPR/Cas9 in *Ciona* makes these
37 animals an ideal organisms for laboratory courses in higher education. Hands-on experienc
37 CRISPR/Cas9 might prepare students fo animals an ideal organisms for laboratory courses in higher education. Hands-on experience in

37 CRISPR/Cas9 might prepare students for a world in which CRISPR/Cas9-based technologies

38 become more prevalent (Thurtle-Sc CRISPR/Cas9 might prepare students for a world in which CRISPR/Cas9-based technologies
become more prevalent (Thurtle-Schmidt and Lo 2018).
Here we used *Ciona robusta* in the context of an undergraduate "capstone" researc become more prevalent (Thurtle-Schmidt and Lo 2018).

Here we used *Ciona robusta* in the context of an underg

the use of CRISPR/Cas9 in neurobiology, taught at the C

course, students selected 4 target genes from a list 39 Here we used *Ciona robusta* in the context of an undergraduate "capstone" research course on

40 the use of CRISPR/Cas9 in neurobiology, taught at the Georgia Institute of Technology. In this

41 course, students selec the use of CRISPR/Cas9 in neurobiology, taught at the Georgia Institute of Technology. In this

course, students selected 4 target genes from a list of genes putatively expressed in the

mechanosensory neurons of the anter course, students selected 4 target genes from a list of genes putatively expressed in the
mechanosensory neurons of the anterior papillae of the *Ciona* larvae. The papillae are a
of three small clusters of cells organized mechanosensory neurons of the anterior papillae of the *Ciona* larvae. The papillae are a group
of three small clusters of cells organized in a triangle at the anterior end of the larval head
(**Figure 1**). Basic characteri 43 of three small clusters of cells organized in a triangle at the anterior end of the larval head
44 (Figure 1). Basic characterization of the cell types contained in these papillae suggest mul
45 adhesive, contractile, a (**Figure 1**). Basic characterization of the cell types contained in these papillae suggest multiple adhesive, contractile, and sensory functions supporting the attachment of the larvae to the substrate and triggering the o adhesive, contractile, and sensory functions supporting the attachment of the larvae to the
substrate and triggering the onset of metamorphosis (Nakayama-Ishimura et al. 2009; Zen
al. 2019a; Zeng et al. 2019b). Recently, m substrate and triggering the onset of metamorphosis (Nakayama-Ishimura et al. 2009; Zeng et al. 2019a; Zeng et al. 2019b). Recently, mechanical stimulus of the papillae was shown to be sufficient for triggering tail retrac

al. 2019a; Zeng et al. 2019b). Recently, mechanical stimulus of the papillae was shown to be
sufficient for triggering tail retraction, the first stage of metamorphosis (Wakai et al. 2021). Thi
ability was shown to depend sufficient for triggering tail retraction, the first stage of metamorphosis (Wakai et al. 2021). This

49 ability was shown to depend on PKD2-expressing papilla neurons specified by the transcription

50 factor Pou4 (Sakam ability was shown to depend on PKD2-expressing papilla neurons specified by the transcription
factor Pou4 (Sakamoto et al. 2022).
With this in mind, students in the course hypothesized that one or more genes expressed in t factor Pou4 (Sakamoto et al. 2022).

51 With this in mind, students in the cou

papillae neurons might be required for

and validated single-chain guide RN.
 *hydroxylase (TH), Vamp1/2/3, Neuro

Vamp1/2/3 was the only gene* With this in mind, students in the course hypothesized that one or more genes expressed in the
papillae neurons might be required for tail retraction and metamorphosis. Students designed
and validated single-chain guide RN papillae neurons might be required for tail retraction and metamorphosis. Students designed
and validated single-chain guide RNAs (sgRNAs) targeting four selected genes: Tyrosine
bydroxylase (TH), Vamp1/2/3, Neuronal calci 53 and validated single-chain guide RNAs (sgRNAs) targeting four selected genes: *Tyrosine*
54 *hydroxylase (TH), Vamp1/2/3, Neuronal calcium sensor 1 (NCS1),* and *NARS1*. Of these,
55 *Vamp1/2/3* was the only gene that, *hydroxylase (TH), Vamp1/2/3, Neuronal calcium sensor 1 (NCS1), and NARS1.* Of these,
 Vamp1/2/3 was the only gene that, when knocked out, resulted in a metamorphosis defect

However, NARS1 knockout in the developing cen Vamp1/2/3 was the only gene that, when knocked out, resulted in a metamorphosis defect.
However, NARS1 knockout in the developing central nervous system resulted in major
morphological defects, indicating that our validate However, *NARS1* knockout in the developing central nervous system resulted in major
morphological defects, indicating that our validated sgRNAs might still be instrumental in
revealing the roles of these genes in other co 57 morphological defects, indicating that our validated sgRNAs might still be instrumental in
58 revealing the roles of these genes in other contexts. Here we describe our findings, in add
59 to providing detailed sequence 58 revealing the roles of these genes in other contexts. Here we describe our findings, in addition
to providing detailed sequence information and protocols. We hope that this study will help other
instructors who wish to 59 to providing detailed sequence information and protocols. We hope that this study will help other

instructors who wish to implement a similar lab course based on CRISPR and/or *Ciona*, or

researchers who wish to knock 60 instructors who wish to implement a similar lab course based on CRISPR and/or *Ciona,* or 61 researchers who wish to knock out these same *Ciona* genes out in other cell types.

62 **Methods**
63 **Ciona hai
64** *Ciona rob***
65 REP). Egg
66 (Christiae
67 juveniles v
68 1 mM EG⁻
69 NH4Cl for**

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- **Ciona handling, fixing, staining, and imaging**

Ciona robusta (intestinalis Type A) were collected

REP). Eggs were fertilized, dechorionated, and e

(Christiaen et al. 2009a; 2009b). Embryos were r

juveniles were fixed *Ciona robusta (intestinalis Type A)* were collected by and shipped from San Diego, CA (M-
REP). Eggs were fertilized, dechorionated, and electroporated according to published proto
66 (Christiaen et al. 2009a; 2009b). Emb
-
- REP). Eggs were fertilized, dechorionated, and electroporated according to published protocols

(Christiaen et al. 2009a; 2009b). Embryos were raised at 20°C. Embryos, larvae, and/or

inveniles were fixed in MEM-FA solutio
- 66 (Christiaen et al. 2009a; 2009b). Embryos were raised at 20°C. Embryos, larvae, and/or
67 juveniles were fixed in MEM-FA solution (3.7% formaldehyde, 0.1 M MOPS pH 7.4, 0.5 M
68 1 mM EGTA, 2 mM MgSO4, 0.1% Triton-X100),
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- iuveniles were fixed in MEM-FA solution (3.7% formaldehyde, 0.1 M MOPS pH 7.4, 0.5 M NaCl,
1 mM EGTA, 2 mM MgSO4, 0.1% Triton-X100), rinsed in 1X PBS, 0.4% Triton-X100, 50 mM
169 NH4Cl for autofluorescence quenching, and a
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- 68 1 mM EGTA, 2 mM MgSO4, 0.1% Triton-X100), rinsed in 1X PBS, 0.4% Triton-X100, 50 mM

69 NH4Cl for autofluorescence quenching, and a final 1X PBS, 0.1% Triton-X100 wash.

59 Specimens were imaged on a Leica DMI8 or Niko 69 NH4Cl for autofluorescence quenching, and a final 1X PBS, 0.1% Triton-X100 wash.

5 Specimens were imaged on a Leica DMI8 or Nikon Ti2-U inverted epifluorescence mi

71 Phylogenetic trees

Protein sequences were aligne
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- Specimens were imaged on a Leica DMI8 or Nikon Ti2-U inverted epifluorescence microscope.

71
 Phylogenetic trees

Protein sequences were aligned using online MAFFT version 7 (Katoh et al. 2019).

Phylogenetic trees wer 72 73 74 75 76 77 78 **Phylogenetic trees**

73 Protein sequences w

74 Phylogenetic trees w

75 sites), JTT substitution

76 bootstrapping. Trees

77 (https://github.com/cl

78 SMART (http://smart Protein sequences were aligned using online MAFFT version 7 (Katoh et al. 2019).

74 Phylogenetic trees were assembled in MAFFT also, using default parameters: NJ (c

57 sites), JTT substitution model, with heterogeneity Phylogenetic trees were assembled in MAFFT also, using default parameters: NJ (conserved
sites), JTT substitution model, with heterogeneity among sites ignored (α = infinite) and no
bootstrapping. Trees were visualized sites), JTT substitution model, with heterogeneity among sites ignored (α = infinite) and no

bootstrapping. Trees were visualized in MAFFT using Archaeopteryx.js

(https://github.com/cmzmasek/archaeopteryx-js). Protein d bootstrapping. Trees were visualized in MAFFT using Archaeopteryx.js

(https://github.com/cmzmasek/archaeopteryx-js). Protein domain analys

SMART (http://smart.embl-heidelberg.de/)(Letunic et al. 2021).

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 CRISPR/Cas9
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- (https://github.com/cmzmasek/archaeopteryx-js). Protein domain analysis was performed using

SMART (http://smart.embl-heidelberg.de/)(Letunic et al. 2021).

20 CRISPR/Cas9 sgRNA design and validation

2016)(crispor.tefor.n
-
- SMART (http://smart.embl-heidelberg.de/)(Letunic et al. 2021).

79
 CRISPR/Cas9 sgRNA design and validation

Single-chain guide RNA (sgRNA) templates were designed usir

2016)(crispor.tefor.net) and synthesized custom-cl 80 81 82 83 84 85 86 7 **CRISPR/Cas9 sgRNA design and validation**
81 Single-chain guide RNA (sgRNA) templates we
2016)(crispor.tefor.net) and synthesized custon
83 al. 2014) by Twist Bioscience (South San Franc
84 specificity scores were priorit
- Single-chain guide RNA (sgRNA) templates were designed using CRISPOR (Haeussler et al.

82 2016)(crispor.tefor.net) and synthesized custom-cloned into the U6>sgRNA-F+E vector (Stolfi

81 al. 2014) by Twist Bioscience (Sou 2016)(crispor.tefor.net) and synthesized custom-cloned into the U6>sgRNA-F+E vector (Stolfi et

al. 2014) by Twist Bioscience (South San Francisco, CA). High Doench '16 score, high MIT

specificity scores were prioritized al. 2014) by Twist Bioscience (South San Francisco, CA). High Doench '16 score, high MIT
specificity scores were prioritized, and targets containing known single-nucleotide
polymorphisms were avoided. Validation of sgRNAs 84 specificity scores were prioritized, and targets containing known single-nucleotide

85 polymorphisms were avoided. Validation of sgRNAs was performed by co-electrop

86 of *Eef1a>Cas9* (Stolfi et al. 2014) and and 75
-
- 86 of *Eef1a>Cas9* (Stolfi et al. 2014) and and 75 μg of the sgRNA plasmid, per 700 μl of total
87 electroporation volume. Genomic DNA was extracted from larvae electroporated with a give
88 sgRNA using a QIAamp DNA micro
- electroporation volume. Genomic DNA was extracted from larvae electroporated with a given
sgRNA using a QIAamp DNA micro kit (Qiagen). PCR products spanning each target site wer
amplified from the genomic DNA, with each am
-
- 85 polymorphisms were avoided. Validation of sgRNAs was performed by co-electroporating 25 μg
86 of *Eef1a>Cas9* (Stolfi et al. 2014) and and 75 μg of the sgRNA plasmid, per 700 μl of total
87 electroporation volume. Geno sgRNA using a QIAamp DNA micro kit (Qiagen). PCR products spanning each target site were
amplified from the genomic DNA, with each amplicon 150-450 bp in size. Amplicons were
purified using a QIAquick PCR purification kit 89 amplified from the genomic DNA, with each amplicon 150-450 bp in size. Amplicons were
90 purified using a QIAquick PCR purification kit (Qiagen) and Illumina-sequenced using Amp
91 EZ service from Azenta/Genewiz (New Je
- 90 purified using a QIAquick PCR purification kit (Qiagen) and Illumina-sequenced using Amplicon-
91 EZ service from Azenta/Genewiz (New Jersey, USA). Papilla-specific CRISPR knockouts were
92 performed using *Foxc>Cas9,* 91 EZ service from Azenta/Genewiz (New Jersey, USA). Papilla-specific CRISPR knockouts were
92 performed using *Foxc>Cas9,* as previously described (Johnson et al. 2023).
⁹
- 92 performed using *Foxc>Cas9,* as previously described (Johnson et al. 2023).

93 *Sox1/2/3>Cas9::GemininN* was constructed using the *Sox1/2/3* promoter (Stolfi et al. 2014) and
94 the *Cas9::GemininN* as previously published (Johnson et al. 2023; Song et al. 2022). All sgRNA
95 and primer sequences

- the *Cas9::GemininN* as previously published (Johnson et al. 2023; Song et al. 2022). All sgRNA
and primer sequences can be found in the **Supplemental Sequences File**. Detailed tutorials
and protocols used for classroom ac
- and primer sequences can be found in the **Supplemental Sequences File**. Detailed tutorials

and protocols used for classroom activities can be found at the OSF link: <u>https://osf.io/3fh89/</u>

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96 and protocols used for classroom activities can be found at the OSF link: https://osf.io/3fh89/
97 Please contact the corresponding author to inquire about more detailed modifications to
98 commercial kit manufacturers' Please contact the corresponding author to inquire about more detailed modifications to

98 commercial kit manufacturers' protocols.

99 **Results

91 Selecting genes and designing sgRNAs**

92 Genes to be targeted by CRISPR commercial kit manufacturers' protocols.
99
Results
Selecting genes and designing sgRNA
02 Genes to be targeted by CRISPR/Cas9 w
103 transcripts enriched in a cell cluster poter
104 neurons, identified from whole-larva 00
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06 100 **Results

101 Selectin**

102 Genes to

103 transcrip

104 neurons,

105 (Cao et a

106 tentative

107 *(KH.C6.1* Selecting genes and designing sgRNAs

102 Genes to be targeted by CRISPR/Cas9 were

103 transcripts enriched in a cell cluster potentia

104 neurons, identified from whole-larva singe-

105 (Cao et al. 2019) were reanalyze Genes to be targeted by CRISPR/Cas9 were chosen based on student preference, from a list of

transcripts enriched in a cell cluster potentially representing the papilla mechanosensory

neurons, identified from whole-larva transcripts enriched in a cell cluster potentially representing the papilla mechanosensory
neurons, identified from whole-larva singe-cell RNA sequencing data. Briefly, published c
(Cao et al. 2019) were reanalyzed (Johnso neurons, identified from whole-larva singe-cell RNA sequencing data. Briefly, published data

105 (Cao et al. 2019) were reanalyzed (Johnson et al. 2023) and papilla neuron identity was

106 tentatively confirmed by enrich (Cao et al. 2019) were reanalyzed (Johnson et al. 2023) and papilla neuron identity was

tentatively confirmed by enrichment with *Thymosin beta-related (KH.C2.140), Celf3/4/5*

(*KH.C6.128), Foxg (KH.C8.774), Synaptotagmi* tentatively confirmed by enrichment with *Thymosin beta-related (KH.C2.140), Celf3/4/5*

(*KH.C6.128), Foxg (KH.C8.774), Synaptotagmin (KH.C2.101), Pou4 (KH.C2.42), Pkd2

(<i>KH.C9.319), and TGFB (KH.C3.724)* based on previo (KH.C6.128), *Foxg (KH.C8.774), Synaptotagmin (KH.C2.101), Pou4 (KH.C2.42), Pkd2*

(KH.C9.319), and TGFB (KH.C3.724) based on previous reports (Horie et al. 2018; Kate

et al. 2002; Razy-Krajka et al. 2014; Sakamoto et al. 108 *(KH.C9.319), and TGFB (KH.C3.724)* based on previous reports (Horie et al. 2018; Katsuyama et al. 2002; Razy-Krajka et al. 2014; Sakamoto et al. 2022; Sharma et al. 2019; Zeng et al. 2019b) (**Supplemental Table 1**). T et al. 2002; Razy-Krajka et al. 2014; Sakamoto et al. 2022; Sharma et al. 2019; Zeng et al.

2019b)(**Supplemental Table 1**).To be clear, these are distinct from what we previously call

"palp neurons" (Sharma et al. 2019), 2019b)(**Supplemental Table 1**).To be clear, these are distinct from what we previously called

111 "palp neurons" (Sharma et al. 2019), which were later identified conclusively as a non-neurona

112 cell type, the Axial Co "palp neurons" (Sharma et al. 2019), which were later identified conclusively as a non-neuronal

112 cell type, the Axial Columnar Cells of the papillae (Johnson et al. 2020; Zeng et al. 2019b). The

113 genes and sgRNAs s cell type, the Axial Columnar Cells of the papillae (Johnson et al. 2020; Zeng et al. 2019b). The
113 genes and sgRNAs selected for this study are detailed below.
114 **Tyrosine hydroxylase (KH.C2.252)**
116 **Tyrosine hydrox**

genes and sgRNAs selected for this study are detailed below.

114
 Tyrosine hydroxylase (KH.C2.252)

116 The gene selected by the first group of students was Tyrosine

117 gene model ID: KH.C2.252), encoding the C. robus --
115
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121 Tyrosine hydroxylase (KH.C2.252)
116 The gene selected by the first group of
117 gene model ID: KH.C2.252), encoding
118 dopamine biosynthesis (Moret et al. 2
119 putative dopamine-releasing coronet
120 Razy-Krajka et al. The gene selected by the first group of students was *Tyrosine hydroxylase (TH;* KyotoHoya gene model ID: *KH.C2.252)*, encoding the *C. robusta* ortholog of the rate-limiting enzyme of dopamine biosynthesis (Moret et al. quare model ID: *KH.C2.252),* encoding the *C. robusta* ortholog of the rate-limiting enzyme of
118 dopamine biosynthesis (Moret et al. 2005). Previously, *TH* was reported to be a marker of
119 putative dopamine-releasing 118 dopamine biosynthesis (Moret et al. 2005). Previously, *TH* was reported to be a marker of putative dopamine-releasing coronet cells of the ventral larval brain vesicle (Moret et al. 20
120 Razy-Krajka et al. 2012; Tak

putative dopamine-releasing coronet cells of the ventral larval brain vesicle (Moret et al. 2005;

Razy-Krajka et al. 2012; Takamura et al. 2010). Dopamine immunoreactivity was also observe

in the papilla region of anothe

- 121 in the papilla region of another species, *Phallusia mammilata* (Zega et al. 2005).
122 Pharmacological treatments suggested roles for dopamine in neuromodulation of
123 swimming behavior in *Ciona* (Razy-Krajka et al.
- 120 Razy-Krajka et al. 2012; Takamura et al. 2010). Dopamine immunoreactivity was also observed

121 in the papilla region of another species, *Phallusia mammilata* (Zega et al. 2005).

122 Pharmacological treatments sugge 122 Pharmacological treatments suggested roles for dopamine in neuromodulation of larval
123 swimming behavior in *Ciona* (Razy-Krajka et al. 2012), and suppression of metamorpho
123 swimming behavior in *Ciona* (Razy-Kraj 123 swimming behavior in *Ciona* (Razy-Krajka et al. 2012), and suppression of metamorphosis in *P.*

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- mammillata (Zega et al. 2005)Three sgRNAs were selected from those predicted by the web-
hased CRISPOR prediction tool (crispor.tefor.net)(Haeussler et al. 2016), as described in det
in the methods section and online proto based CRISPOR prediction tool (crispor.tefor.net)(Haeussler et al. 2016), as described in detail

126 in the methods section and online protocols. Two were predicted to cut in exon 4 (named

"TH.4.114" and "TH.4.140") and
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- in the methods section and online protocols. Two were predicted to cut in exon 4 (named

127 imaged in the major catalytic domain of TH, these sgRNAs were predicted

128 encodes the beginning of the major catalytic domain
- TH.4.114" and "TH.4.140") and one in exon 5 ("TH.5.44")(**Figure 2A**). Because exon 5

encodes the beginning of the major catalytic domain of TH, these sgRNAs were predicte

generate frameshift mutations resulting in trunca encodes the beginning of the major catalytic domain of TH, these sgRNAs were predicted to
129 generate frameshift mutations resulting in truncated proteins lacking the catalytic domain.
130
131 Vamp1/2/3 (KH.C1.165)
132 generate frameshift mutations resulting in truncated proteins lacking the catalytic domain.

130
 Vamp1/2/3 (KH.C1.165)

132 The second student group picked Vamp1/2/3 (KH.C1.165), which encodes a member of the synaptobre 131
132
133
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137 **Vamp1/2/3 (KH.C1.165)**
132 The second student grou
133 synaptobrevin family of S
134 (Rizo 2022). Based on ph
135 appears to be orthologou
136 potentially evolutionarily of
137 system suggested an imp
138 including in the The second student group picked *Vamp1/2/3 (KH.C1.165),* which encodes a member of the
synaptobrevin family of SNARE complex proteins that carry out neurotransmitter vesicle rele
(Rizo 2022). Based on phylogenetic analysis synaptobrevin family of SNARE complex proteins that carry out neurotransmitter vesicle release

(Rizo 2022). Based on phylogenetic analysis in MAFFT (see methods), *Vamp1/2/3 (KH.C1.165)*

appears to be orthologous to *VAM*
-
-
- appears to be orthologous to *VAMP1, VAMP2,* and *VAMP3* in humans (**Figure S1**). Its
potentially evolutionarily conserved function and broad expression in the *Ciona* larval ne
system suggested an important role for *Vamp*
-
- (Rizo 2022). Based on phylogenetic analysis in MAFFT (see methods), *Vamp1/2/3 (KH.C1.165)*
appears to be orthologous to *VAMP1*, *VAMP2*, and *VAMP3* in humans (Figure S1). Its
potentially evolutionarily conserved functio potentially evolutionarily conserved function and broad expression in the *Ciona* larval nervous
system suggested an important role for *Vamp1/2/3* in neurotransmitter release in *Ciona*,
including in the papilla neurons d system suggested an important role for *Vamp1/2/3* in neurotransmitter release in *Ciona*,
including in the papilla neurons during settlement. The *Vamp1/2/3* gene in *Ciona* appears
give rise to a few different alternativ including in the papilla neurons during settlement. The *Vamp1/2/3* gene in *Ciona* appears to
give rise to a few different alternatively spliced isoforms. The sgRNAs selected from CRISPC
included one sgRNA targeting exon
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-
- give rise to a few different alternatively spliced isoforms. The sgRNAs selected from CRISPOR

included one sgRNA targeting exon 3 ("Vamp.3.49") and two sgRNAs targeting exon 4

("Vamp.4.26" and "Vamp.4.93") in the "v3" an included one sgRNA targeting exon 3 ("Vamp.3.49") and two sgRNAs targeting exon 4

141 ("Vamp.4.26" and "Vamp.4.93") in the "v3" and "v4" transcript variants (**Figure 2B**). The

142 exons become exons 2 and 3, respectively ("Vamp.4.26" and "Vamp.4.93") in the "v3" and "v4" transcript variants (**Figure 2B**). These

exons become exons 2 and 3, respectively, in all other transcript variants.

143
 Neuronal calcium sensor 1 (KH.C1.1067)

Group
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- exons become exons 2 and 3, respectively, in all other transcript variants.

143
 Neuronal calcium sensor 1 (KH.C1.1067)

Group number 3 selected the gene *Neuronal calcium sensor 1 (NCS1*, gen

145 Group number 3 select 144
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- **Neuronal calcium sensor 1 (KH.C1.1067)**

145 Group number 3 selected the gene Neurona

146 KH.C1.1067). According to our phylogenetic

147 to human NCS1 and its *Drosophila melanog*

148 the NCS family of proteins (Figure 145 Group number 3 selected the gene *Neuronal calcium sensor 1 (NCS1,* gene model

146 KH.C1.1067). According to our phylogenetic analysis, KH.C1.1067 appeared to be 1

147 to human NCS1 and its *Drosophila melanogaster* to human NCS1 and its *Drosophila melanogaster* orthologs, Frequenin1 and Frequenin2 within

the NCS family of proteins (**Figure S2A**). NCS1/Frq proteins regulate neurotransmission

through both pre- and post-synaptic mech the NCS family of proteins (**Figure S2A**). NCS1/Frq proteins regulate neurotransmission
through both pre- and post-synaptic mechanisms (Dason et al. 2012), likely on account o
ability to bind Ca2+ ions through their multip through both pre- and post-synaptic mechanisms (Dason et al. 2012), likely on account of their
150 ability to bind Ca2+ ions through their multiple EF hand domains. In *Ciona, NCS1* had been
151 previously identified as a ability to bind Ca2+ ions through their multiple EF hand domains. In *Ciona, NCS1* had been
previously identified as a transcriptional target of Neurogenin in the Bipolar Tail Neurons of t
larva, suggesting a broader role
-
-
- *KH.C1.1067).* According to our phylogenetic analysis, KH.C1.1067 appeared to be most similar
to human NCS1 and its *Drosophila melanogaster* orthologs, Frequenin1 and Frequenin2 within
the NCS family of proteins (**Figure** 151 previously identified as a transcriptional target of Neurogenin in the Bipolar Tail Neurons of the
152 larva, suggesting a broader role in neuronal function (Kim et al. 2020). However, no function ha
153 yet been shown 152 larva, suggesting a broader role in neuronal function (Kim et al. 2020). However, no function has
153 yet been shown for this gene in *Ciona*. Three sgRNAs targeting *NCS1* were selected for testing:
154 one sgRNA targ 153 yet been shown for this gene in *Ciona*. Three sgRNAs targeting *NCS1* were selected for testing:
154 one sgRNA targeting exon 1 ("NCS1.1.32") and two sgRNAs targeting exon 2 ("NCS1.2.43" and
one sgRNA targeting exon 1 154 one sgRNA targeting exon 1 ("NCS1.1.32") and two sgRNAs targeting exon 2 ("NCS1.2.43" and

"NCS1.2.56")(**Figure 2C**). As these sgRNAs are predicted to cut 5' to the exons encoding the

EF hand domains (exons 3-7, **Figure S2B**), the resulting frameshift mutations are predicted to

result in a truncated, non-funct EF hand domains (exons 3-7, **Figure S2B**), the resulting frameshift mutations are predicted to
157 result in a truncated, non-functional polypeptide.
158 **NARS1 (KH.C12.45)**
160 The fourth student group picked NARS1 (KH.C1 result in a truncated, non-functional polypeptide.

158
 NARS1 (KH.C12.45)

160 The fourth student group picked NARS1 (KH.C1

161 Aparaginyl tRNA synthetase 1 (cytoplasmic), wh

162 (Asn/N) to its cognate tRNAs (Shiba et 159
159
160
161
162
163
164
165 **NARS1 (KH.C12.45)**
160 The fourth student group
161 Aparaginyl tRNA synt
162 (Asn/N) to its cognate
163 shown that loss of NA
164 (Wang et al. 2020). M
165 syndromes such as m
166 regulation of protein s
167 Phylogenetic The fourth student group picked *NARS1 (KH.C12.45),* which encodes the *C. robusta* ortholog of
161 Aparaginyl tRNA synthetase 1 (cytoplasmic), which catalyzes the attachment of asparagine
162 (Asn/N) to its cognate tRNAs Aparaginyl tRNA synthetase 1 (cytoplasmic), which catalyzes the attachment of asparagine

162 (Asn/N) to its cognate tRNAs (Shiba et al. 1998). In a neurodevelopmental context, it has be

163 shown that loss of *NARS1* in (Asn/N) to its cognate tRNAs (Shiba et al. 1998). In a neurodevelopmental context, it has been

shown that loss of *NARS1* in human brain organoids impairs neural progenitor proliferation

(Wang et al. 2020). Mutations in shown that loss of *NARS1* in human brain organoids impairs neural progenitor proliferation

(Wang et al. 2020). Mutations in *NARS1* is associated with various neurodevelopmental

syndromes such as microcephaly and cognit (Wang et al. 2020). Mutations in *NARS1* is associated with various neurodevelopmental
165 syndromes such as microcephaly and cognitive delays (Wang et al. 2020), suggesting th
166 regulation of protein synthesis rates is syndromes such as microcephaly and cognitive delays (Wang et al. 2020), suggesting that
166 regulation of protein synthesis rates is indispensable for development of the nervous system
167 Phylogenetic analysis shows that regulation of protein synthesis rates is indispensable for development of the nervous system.

167 Phylogenetic analysis shows that these aminoacyl-tRNA synthetases are highly conserved in

168 their specificity, with simp Phylogenetic analysis shows that these aminoacyl-tRNA synthetases are highly conserved in

their specificity, with simple 1-to-1 orthology between *Ciona* and human genes of various type

and classes within this gene famil their specificity, with simple 1-to-1 orthology between *Ciona* and human genes of various types

and classes within this gene family (**Figure S3A**). For this gene, one sgRNA targeting exon 4

("NARS1.4.25") and two sgRNAs and classes within this gene family (**Figure S3A**). For this gene, one sgRNA targeting exon 4
170 ("NARS1.4.25") and two sgRNAs targeting exon 7 ("NARS1.7.86" and "NARS1.7.135") were
171 designed (**Figure 2D**). While the t ("NARS1.4.25") and two sgRNAs targeting exon 7 ("NARS1.7.86" and "NARS1.7.135") were
171 designed (Figure 2D). While the tRNA anti-codon domain is encoded by exons 5-7, and the
172 tRNA synthetase domain is encoded by exon designed (**Figure 2D**). While the tRNA anti-codon domain is encoded by exons 5-7, and the
172 tRNA synthetase domain is encoded by exons 7-13, these sgRNAs are predicted to result in
173 truncated NARS1 polypeptides lackin tRNA synthetase domain is encoded by exons 7-13, these sgRNAs are predicted to result in
173 truncated NARS1 polypeptides lacking both major functional domains (**Figure S3B**).
174 **Validation of sgRNA efficacy by Illumina** truncated NARS1 polypeptides lacking both major functional domains (**Figure S3B**).
174
Validation of sgRNA efficacy by Illumina amplicon sequencing
175 Validation of sgRNA efficacies was performed by sequencing amplicons

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181 **Validation of sgRNA efficacy by Illumina amplicon sequencing**

Validation of sgRNA efficacies was performed by sequencing amplic

177 target site, from larvae electroporated with a given sgRNA vector to

2178 expressed *E*

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- Validation of sgRNA efficacies was performed by sequencing amplicons surrounding each

target site, from larvae electroporated with a given sgRNA vector together with the ubiquito

expressed *Eef1a*>*Cas9* (Stolfi et al. target site, from larvae electroporated with a given sgRNA vector together with the ubiquitously-
expressed *Eef1a>Cas9* (Stolfi et al. 2014)(**Figure 3A**). Although we had previously reported a
Sanger sequencing-based meth expressed *Eef1a>Cas9* (Stolfi et al. 2014)(**Figure 3A**). Although we had previously reported a
179 Sanger sequencing-based method for estimating mutagenesis efficacy (Gandhi et al. 2018), th
180 strategy is frequently ha Sanger sequencing-based method for estimating mutagenesis efficacy (Gandhi et al. 2018), that

strategy is frequently hampered by naturally-occurring indels and poor sequencing quality. We

decided instead to quantify mut trategy is frequently hampered by naturally-occurring indels and poor sequencing quality. We
decided instead to quantify mutagenesis by sequencing amplicons using a commercially
available Illumina-sequencing based service decided instead to quantify mutagenesis by sequencing amplicons using a commercially
available Illumina-sequencing based service, as recently described (Johnson et al. 2023)
Briefly, 75 μ g/700 μ I total electroporati available Illumina-sequencing based service, as recently described (Johnson et al. 2023).

183 Briefly, 75 µg/700 µI total electroporation volume of each sgRNA plasmid was co-electropo

184 with 25 µg/700 µI of *Eef1a>Cas9*
- 183 Briefly, 75 μg/700 μl total electroporation volume of each sgRNA plasmid was co-electroporated
184 with 25 μg/700 μl of *Eef1a>Cas9* into zygotes, which were collected at larval stage (~17 hours
185 post-fertilization
- 184 with 25 µg/700 µl of *Eef1a>Cas9* into zygotes, which were collected at larval stage (~17 hours
185 post-fertilization, reared at 20°C). Larvae electroporated with the same sgRNA vector were
¹⁸⁵ post-fertilization, r 185 post-fertilization, reared at 20°C). Larvae electroporated with the same sgRNA vector were

pooled, and genomic DNA extracted from them. DNA fragments spanning each target site,

187 ranging from 150-450 bp as required by the sequencing service, were amplified by PCR fro

188 each genomic DNA pool. Negative contr ranging from 150-450 bp as required by the sequencing service, were amplified by PCR from
each genomic DNA pool. Negative controls for each amplicon were derived by repeating the
PCR on samples from larvae electroporated w each genomic DNA pool. Negative controls for each amplicon were derived by repeating the
189 PCR on samples from larvae electroporated with sgRNA vector targeting a different sequence
190 (e.g. targeting exon 2 instead of PCR on samples from larvae electroporated with sgRNA vector targeting a different sequence

(e.g. targeting exon 2 instead of exon 3). Amplicons were then submitted for library preparation

sequencing, and analysis by Gene (e.g. targeting exon 2 instead of exon 3). Amplicons were then submitted for library preparation,
191 sequencing, and analysis by Genewiz/Azenta. The sgRNAs chosen for further experiments
192 were those that resulted in a sequencing, and analysis by Genewiz/Azenta. The sgRNAs chosen for further experiments

192 were those that resulted in a larger portion of on-target indels based on visual examination of

193 the indel plot automatically g were those that resulted in a larger portion of on-target indels based on visual examination of

193 the indel plot automatically generated by the amplicon sequencing service. This was better the

194 relying on raw mutage the indel plot automatically generated by the amplicon sequencing service. This was better than

194 relying on raw mutagenesis rates provided by the service, as the plots revealed a high

195 frequency of naturally occurr 194 relying on raw mutagenesis rates provided by the service, as the plots revealed a high
195 frequency of naturally occurring indels that prevented the automatic quantification of the
196 efficacies of some sgRNAs. This frequency of naturally occurring indels that prevented the automatic quantification of the true

efficacies of some sgRNAs. This new approach is described in greater detail in the methods

online protocols.

Amplicon seque

- online protocols.

198 Amplicon sequen

199 was not evaluate

200 found that all thre

201 TH.4.140 and TH

202 For Vamp1/2/3, tl

203 Vamp.3.49 and V

204 wished to use a p

205 Vamp.3.49 for full was not evaluated due to failure to amplify its target site by PCR (**Figure 2, S4**). For *TH,* we
found that all three sgRNAs were effective at generating indels at the target sequences, tho
201 TH.4.140 and TH.5.44 were s
-
- efficacies of some sgRNAs. This new approach is described in greater detail in the methods and

online protocols.

Amplicon sequencing revealed the indels induced by all sgRNAs except for NARS1.4.25, which

was not evaluat 198 Amplicon sequencing revealed the indels induced by all sgRNAs except for NARS1.4.25, which

199 was not evaluated due to failure to amplify its target site by PCR (**Figure 2, S4**). For *TH*, we

1000 found that all th found that all three sgRNAs were effective at generating indels at the target sequences, though

201 TH.4.140 and TH.5.44 were selected, as having barely edged out TH.4.114 (Figure 2A, S4A).

202 For *Vamp1/2/3*, the most TH.4.140 and TH.5.44 were selected, as having barely edged out TH.4.114 (**Figure 2A, S4A**).

202 For *Vamp1/2/3*, the most efficient sgRNAs was Vamp.4.93 (>40% efficacy, **Figure 2B**), while

203 Vamp.3.49 and Vamp.4.26 wer For *Vamp1/2/3,* the most efficient sgRNAs was Vamp.4.93 (>40% efficacy, **Figure 2B**), while

203 Vamp.3.49 and Vamp.4.26 were less efficacious at ~20-30% indels (**Figure S4B**). Because w

204 wished to use a pair of sgRNA

-
-
- wished to use a pair of sgRNAs targeting different exons, we selected Vamp.4.93 and

205 Vamp.3.49 for further use. All three sgRNAs targeting *NCS1* generated indels, though

206 NCS1.1.32 efficacy was only 12% indels (**F** Vamp.3.49 for further use. All three sgRNAs targeting *NCS1* generated indels, though
206 NCS1.1.32 efficacy was only 12% indels (**Figure 2C, S4C**). Because NCS1.2.43 and
207 NCS1.2.56 targets overlapped, we paired the mos NCS1.1.32 efficacy was only 12% indels (**Figure 2C, S4C**). Because NCS1.2.43 and

NCS1.2.56 targets overlapped, we paired the most efficacious sgRNA (NCS1.2.56) w

NCS1.1.32. Finally, NARS1.7.86 and NARS1.7.135 resulted in
-
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- Vamp.3.49 and Vamp.4.26 were less efficacious at ~20-30% indels (**Figure S4B**). Because we
wished to use a pair of sgRNAs targeting different exons, we selected Vamp.4.93 and
Vamp.3.49 for further use. All three sgRNAs tar
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NCS1.2.56 targets overlapped, we paired the most efficacious sgRNA (NCS1.2.56) with
208 NCS1.1.32. Finally, NARS1.7.86 and NARS1.7.135 resulted in mutagenesis efficacy rate
209 >15% (Figure 2D). As these were the only two NCS1.1.32. Finally, NARS1.7.86 and NARS1.7.135 resulted in mutagenesis efficacy rates

209 > 15% (Figure 2D). As these were the only two *NARS1*-targeting sgRNAs for which amplice

210 were successfully amplified by PCR (F >15% (**Figure 2D**). As these were the only two *NARS1*-targeting sgRNAs for which amplicons

were successfully amplified by PCR (**Figure S4D**), we proceeded with both and did not further

use the untested sgRNA NARS1.4.25. were successfully amplified by PCR (**Figure S4D**), we proceeded with both and did not further
211 use the untested sgRNA NARS1.4.25.
212 **Papilla lineage-specific knockout of target genes by CRISPR/Cas9**
214 It was recentl use the untested sgRNA NARS1.4.25.
212
Papilla lineage-specific knockout of
214 It was recently shown that knockdown of
eliminates papilla neuron formation and
216 retraction and metamorphosis (Sakamor
217 shown to resul 213
214
215
216
217 **Papilla lineage-specific knockout of target genes by CRISPR/Cas9**
214 It was recently shown that knockdown or knockout of the neuronal trans
215 eliminates papilla neuron formation and subsequently, papilla neuron-in
216 214 It was recently shown that knockdown or knockout of the neuronal transcription factor *Pou4*
215 eliminates papilla neuron formation and subsequently, papilla neuron-induced signals for tail
216 retraction and metamorp eliminates papilla neuron formation and subsequently, papilla neuron-induced signals for tail
216 retraction and metamorphosis (Sakamoto et al. 2022). Other CRISPR gene knockouts were
217 shown to result in a mixture of ta 216 retraction and metamorphosis (Sakamoto et al. 2022). Other CRISPR gene knockouts were
217 shown to result in a mixture of tail retraction and body rotation defects during settlement and
317 shown to result in a mixture

217 shown to result in a mixture of tail retraction and body rotation defects during settlement and

1217 Shown to result in a mixture of tail retraction and body rotation defects during settlement and

1217

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- metamorphosis (Johnson et al. 2023). We therefore used papilla-specific CRISPR/Cas9-
mediated knockout in F0 embryos to test the requirement of our candidate genes in a sim
retraction assay. We used the *Foxc* promoter (W mediated knockout in F0 embryos to test the requirement of our candidate genes in a similar tail

retraction assay. We used the *Foxc* promoter (Wagner and Levine 2012) to drive expression of

Cas9 in the anteriormost cel retraction assay. We used the *Foxc* promoter (Wagner and Levine 2012) to drive expression of

221 Cas9 in the anteriormost cells of the neural plate, which gives rise to the entire papilla territory

222 and part of the o
- Cas9 in the anteriormost cells of the neural plate, which gives rise to the entire papilla territory

222 and part of the oral siphon primordium (**Figure 3**). Embryos were electroporated with 40 µg/700

223 µ *Foxc>Cas9*,
- and part of the oral siphon primordium (**Figure 3**). Embryos were electroporated with 40 μg/700 μl *Foxc>Cas9*, 10 μg/700 μl *Foxc>H2B::mCherry*, and gene-specific pairs of sgRNA vectors (40 μg/700 μl each sgRNA vector).
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- pd *Foxc>Cas9,* 10 μg/700 μl *Foxc>H2B::mCherry,* and gene-specific pairs of sgRNA vectors (40

1224 µg/700 μl each sgRNA vector). "Positive control" embryos were electroporated as above, using

225 a previously published 224 μ g/700 μl each sgRNA vector). "Positive control" embryos were electroporated as above, using

225 a previously published pair of sgRNA vectors targeting *Pou4* (Johnson et al. 2023), and

²²⁶ "negative control" a previously published pair of sgRNA vectors targeting *Pou4* (Johnson et al. 2023), and

²²⁶ "negative control" embryos were electroporated with 10 µg/700 µl *Foxc>H2B::mCherry a*

227 All embryos were raised through la ²²⁶ "negative control" embryos were electroporated with 10 µg/700 µl *Foxc>H2B::mCherry* alone.

227 All embryos were raised through larval hatching and settlement, and fixed at 45 hours post-

228 fertilization, upon All embryos were raised through larval hatching and settlement, and fixed at 45 hours post-
fertilization, upon which tail retraction and body rotation were scored (**Figure 4A**).
As previously reported, *Pou4* knockout in fertilization, upon which tail retraction and body rotation were scored (**Figure 4A**).

As previously reported, *Pou4* knockout in the papilla territory resulted in frequent b

resorption and body rotation compared to the
-
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- 229 As previously reported, *Pou4* knockout in the papilla territory resulted in frequent block of tail

230 resorption and body rotation compared to the negative control (**Figure 4B**). Of the gene-spec

231 CRISPR samples resorption and body rotation compared to the negative control (**Figure 4B**). Of the gene-specific

231 CRISPR samples, only *Vamp1/2/3* CRISPR showed a substantial effect on metamorphosis, with

232 only 56% of H2B::mCherr CRISPR samples, only *Vamp1/2/3* CRISPR showed a substantial effect on metamorphosis, with

only 56% of H2B::mCherry+ individuals having retracted their tails. This was closer to the *Pou4*

CRISPR (20% tail retraction) th only 56% of H2B::mCherry+ individuals having retracted their tails. This was closer to the *Pou4*

CRISPR (20% tail retraction) than to the negative control (97% tail retraction). The effect of

Vamp1/2/3 CRISPR on body ro CRISPR (20% tail retraction) than to the negative control (97% tail retraction). The effect of

234 Vamp1/2/3 CRISPR on body rotation was very similar (**Figure 4B**). An independent replicat

235 Vamp1/2/3 CRISPR confirmed
-
-

Vamp1/2/3 CRISPR on body rotation was very similar (**Figure 4B**). An independent replicate of
235 *Vamp1/2/3* CRISPR confirmed this result (**Figure S5**). Taken together, these data suggest that
236 knocking out *Vamp1/2/3* Vamp1/2/3 CRISPR confirmed this result (**Figure S5**). Taken together, these data suggest that

236 knocking out Vamp1/2/3 in the papilla territory impairs the ability of the larva to trigger the onset

237 of metamorphosis knocking out *Vamp1/2/3* in the papilla territory impairs the ability of the larva to trigger the onset
237 of metamorphosis.
Neural tube-specific knockout of *NARS1* **causes neurulation defects**
240 Because *NARS1* is ass 237 of metamorphosis.

238 **Neural tube-speci**f

240 Because *NARS1* is

241 al. 2020), NARS1 s

242 was targeted in the

243 reporter was used t

244 electroporated with ---
239
240
241
242
243
245 **Neural tube-specific knockout of** *NARS1* **causes neurulation defects

Because** *NARS1* **is associated with various neurodevelopmental defects in

al. 2020), NARS1 students also tested the requirement of** *NARS1* **in** *Ciona***
** Because *NARS1* is associated with various neurodevelopmental defects in mammals (Wang et al. 2020), NARS1 students also tested the requirement of *NARS1* in *Ciona* neurulation. *NARS1* was targeted in the neurectoderm us al. 2020), NARS1 students also tested the requirement of *NARS1* in *Ciona* neurulation. *NARS1*

242 was targeted in the neurectoderm using *Sox1/2/3>Cas9::GemininN*, and the *Nut>Unc-76::GFP*

243 reporter was used to vi was targeted in the neurectoderm using *Sox1/2/3>Cas9::GemininN*, and the *Nut>Unc-76::GFP*
reporter was used to visualize the central nervous system (Shimai et al. 2010). Embryos were
electroporated with 40 μg/700 μl *So* reporter was used to visualize the central nervous system (Shimai et al. 2010). Embryos were

electroporated with 40 µg/700 µl Sox1/2/3>Cas9::GemininN, 40 µg/700 µl Nut>Unc-76::GFP,

and 40 µg/700 µl each of both NARS1 sg electroporated with 40 μg/700 μl *Sox1/2/3>Cas9::GemininN,* 40 μg/700 μl *Nut>Unc-76::GFP,* and 40 μg/700 μl each of both *NARS1* sgRNA vectors. As a result of *NARS1* CRISPR in the neurectoderm, a high frequency of curle 245 and 40 μg/700 μl each of both *NARS1* sgRNA vectors. As a result of *NARS1* CRISPR in the neurectoderm, a high frequency of curled/twisting tails specifically in the CRISPR larvae, but in the negative control (**Figure** neurectoderm, a high frequency of curled/twisting tails specifically in the CRISPR larvae, but not
in the negative control (**Figure S6**). This was scored as well, and curved tails were observed in
248 249 out of 50 *NARS1* 247 in the negative control (**Figure S6**). This was scored as well, and curved tails were observed in
248 24 out of 50 *NARS1* CRISPR larvae (48%), compared to 0 out of 50 negative control larvae
249 (0%). Upwards curvatur 248 24 out of 50 *NARS1* CRISPR larvae (48%), compared to 0 out of 50 negative control larvae

249 (0%). Upwards curvature of the tail is a hallmark of impaired neural tube closure in *Ciona* (M 249 (0%). Upwards curvature of the tail is a hallmark of impaired neural tube closure in *Ciona* (Mita

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and Fujiwara 2007), suggesting *NARS1* may be required in the neural tube for proper

251 neurulation.

252

252

253 **Conclusion**

254 We have described the design and validation of sgRNAs targeting four different genes

251 neurulation.
252
253 **Conclusion**
254 We have des
255 in the contex
256 shown to be
257 Although the
258 validated sgl -- 253
253
255
256
257
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259 253 **Conclusion**
254 We have des
255 in the contex
256 shown to be
257 Although the
258 validated sgF
259 other context
260 Our results d We have described the design and validation of sgRNAs targeting four different genes in *Ciona*, in the context of a university-level laboratory course. Of these, only one gene (*Vamp1/2/3*) was shown to be required for ta in the context of a university-level laboratory course. Of these, only one gene (*Vamp1/2/3*) was

255 shown to be required for tail retraction and body rotation at the onset of metamorphosis.

257 Although the other CRISP shown to be required for tail retraction and body rotation at the onset of metamorphosis.

257 Although the other CRISPR knockouts did not result in a noticeable metamorphosis defe

258 validated sgRNAs may be of great int Although the other CRISPR knockouts did not result in a noticeable metamorphosis defect, our validated sgRNAs may be of great interest to other *Ciona* researchers studying these genes in other contexts.
259 other contexts validated sgRNAs may be of great interest to other *Ciona* researchers studying these genes in
259 other contexts.
260 Our results do not entirely rule out a role for the other three genes tested. For instance, there
261 m

259 other contexts.

260 Our results do r

261 may be similar

262 them. In fact, ar

263 putative papilla

264 that the gene m

265 substrates in th

266 long as the pap Our results do not entirely rule out a role for the other three genes tested. For instance, there

261 may be similar genes with overlapping functions that can compensate for the loss of one of

262 them. In fact, another may be similar genes with overlapping functions that can compensate for the loss of one of

them. In fact, another NCS family gene, *KH.C9.113*, was also found to be enriched in the

putative papilla neuron cell cluster by

putative papilla neuron cell cluster by scRNAseq (**Supplemental Table 1**). Another possibility is

that the gene may be required for fine-tuned mechanosensory discernment of settlement

substrates in the wild, while in our substrates in the wild, while in our laboratory assays most larvae eventually retract their tails as

266 long as the papilla neurons retain most of their functions.

267 The requirement of *Vamp1/2/3* for papilla neuron-m

them. In fact, another NCS family gene, *KH.C9.113*, was also found to be enriched in the

putative papilla neuron cell cluster by scRNAseq (**Supplemental Table 1**). Another possit

that the gene may be required for fine-t that the gene may be required for fine-tuned mechanosensory discernment of settlement
265 substrates in the wild, while in our laboratory assays most larvae eventually retract their ta
266 long as the papilla neurons retai The requirement of *Vamp1/2/3* for papilla neuron-mediated tail retraction is not surprising, given

268 its central role in synaptic transmission. However, our results and methods described here

269 establish a proof-of-

long as the papilla neurons retain most of their functions.

267 The requirement of *Vamp1/2/3* for papilla neuron-mediate

268 its central role in synaptic transmission. However, our res

269 establish a proof-of-principl

its central role in synaptic transmission. However, our results and methods described here
269 establish a proof-of-principle for future screens for genes potentially important for
270 mechanosensory papilla neuron develop establish a proof-of-principle for future screens for genes potentially important for

270 mechanosensory papilla neuron development and function in *Ciona* larvae.

271

272 **Acknowledgments**

273 We thank Dexter Dean and mechanosensory papilla neuron development and function in *Ciona* larvae.

271

272 **Acknowledgments**

273 We thank Dexter Dean and Alison Onstine for managing the Neuroscience i

274 teaching lab and granting access to eq --
272
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277
278 272 **Acknowledgments**
273 We thank Dexter Dea
274 teaching lab and gra
275 students in the previous
276 improvements to the
277 work was supported
278 to CJJ, NIH award K
279 R01GM143326 to As We thank Dexter Dean and Alison Onstine for managing the Neuroscience undergraduate

teaching lab and granting access to equipment and materials for the course. We thank the

students in the previous iteration of this cour teaching lab and granting access to equipment and materials for the course. We thank the

students in the previous iteration of this course for their constructive feedback and suggest

improvements to the teaching material

275 students in the previous iteration of this course for their constructive feedback and suggested
276 improvements to the teaching material. We thank Lindsey Cohen for technical assistance. Thi
277 work was supported by

276 improvements to the teaching material. We thank Lindsey Cohen for technical assistance. This
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278 to CJJ, NIH award K99NS126

277 work was supported by Georgia Tech student fees and institutional funds, an NSF GRFP award
1278 to CJJ, NIH award K99NS126576 to TMR, NSF IOS award 1940743 to AS, and NIH award
R01GM143326 to AS.
The Same of the Road o 278 to CJJ, NIH award K99NS126576 to TMR, NSF IOS award 1940743 to AS, and NIH award
R01GM143326 to AS.
R01GM143326 to AS.

279 R01GM143326 to AS.

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287
288 Figure 1. The sensory/adhesive papillae of the *Ciona* larva.

283 Brightfield image of a *Ciona robusta (intestinalis* Type "A") la

284 (hpf) raised at 20°C, showing the three protruding papillae o

285 number 3, the med 283 Brightfield image of a *Ciona robusta (intestinalis* Type "A") larva at 17 hours post-fertilization (hpf) raised at 20°C, showing the three protruding papillae of the head (numbered 1-3). Papi number 3, the medial/vent
- 284 (hpf) raised at 20°C, showing the three protruding papillae of the head (numbered 1-3). Papilla

285 number 3, the medial/ventral papilla, is out of focus. B) Image of electroporated *Ciona* larva at

286 17 hpf/20°C, number 3, the medial/ventral papilla, is out of focus. B) Image of electroporated *Ciona* larva at 17 hpf/20°C, papilla neurons (PNs) labeled by the reporter plasmid *KH.C4.78>Unc-76::GFP* (green, from Johnson et al. 2023) 286 17 hpf/20°C, papilla neurons (PNs) labeled by the reporter plasmid *KH.C4.78>Unc-76::GFP* (green, from Johnson et al. 2023). Nuclei counterstained by DAPI (blue). C) Summary diagra
of the arrangement and cell type dive 287 (green, from Johnson et al. 2023). Nuclei counterstained by DAPI (blue). C) Summary diagram
288 of the arrangement and cell type diversity of the papillae (from Johnson et al. 2023).
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- 288 of the arrangement and cell type diversity of the papillae (from Johnson et al. 2023).
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Based of the arrangement and cell type diversity of the papillae (from Johnson et al. 2023).
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298 292 Figure 2. Design and validation of sgRNAs for CRISPR/Cas9-mediated mutagenesis.

293 A-D) Diagrams of selected candidate gene loci and indel analysis plot for each selecte

294 based on next-generation sequencing of am

293 A-D) Diagrams of selected candidate gene loci and indel analysis plot for each selected sgRNA, based on next-generation sequencing of amplicons. Blue arrows indicate CRISPR/Cas9-induced indel peak, red asterisks indica based on next-generation sequencing of amplicons. Blue arrows indicate CRISPR/Cas9-

induced indel peak, red asterisks indicate naturally-occurring indels. Blue sgRNA identifie

indicate top sgRNAs selected for phenotypic

295 induced indel peak, red asterisks indicate naturally-occurring indels. Blue sgRNA identifiers
296 indicate top sgRNAs selected for phenotypic assay. Grey identifiers indicate sgRNA designe
297 tested, but not selected 296 indicate top sgRNAs selected for phenotypic assay. Grey identifiers indicate sgRNA designed, tested, but not selected for further use.
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330 Figure 4. Scoring metamorphosis defects in CRISPR larvae.

324 A) Example of a juvenile at 45 hours post-fertilization (hpf) in

325 showing the retracted tail and body rotation that occurs during

326 *Foxc>H2B::mCherry* 324 A) Example of a juvenile at 45 hours post-fertilization (hpf) in the "negative control" population,
325 showing the retracted tail and body rotation that occurs during metamorphosis.
326 $Foxc>H2B::mCherry$ (red) labels th showing the retracted tail and body rotation that occurs during metamorphosis.

Foxc>H2B::mCherry (red) labels the cells of the oral siphon and the papillae, th

are transformed into the stolon of the juvenile. Nuclei coun *Foxc>H2B::mCherry* (red) labels the cells of the oral siphon and the papillae, the latter of which are transformed into the stolon of the juvenile. Nuclei counterstained by DAPI (blue). B) Scoring of *Foxc>H2B::mCherry*+ are transformed into the stolon of the juvenile. Nuclei counterstained by DAPI (blue). B) Scoring
of *Foxc>H2B::mCherry*+ individuals upon papilla-specific CRISPR/Cas9-mediated mutagenesis
of the selected candidate genes. 328 of *Foxc>H2B::mCherry+* individuals upon papilla-specific CRISPR/Cas9-mediated mutagenesis
329 of the selected candidate genes. "Tailed juveniles" are individuals that have undergone body
330 rotation but not tail retr 329 of the selected candidate genes. "Tailed juveniles" are individuals that have undergone body
330 rotation but not tail retraction. $Pou4$ CRISPR served as the "positive control", eliminating the
331 papilla neurons tha

330 rotation but not tail retraction. *Pou4* CRISPR served as the "positive control", eliminating the

331 papilla neurons that trigger metamorphosis (see text for citations). Of the four genes tested,

332 Vamp1/2/3 CRISP

331 papilla neurons that trigger metamorphosis (see text for citations). Of the four genes tested, only Vamp1/2/3 CRISPR appeared to result in substantial loss of tail retraction and body rotation, though not as penetrant Vamp1/2/3 CRISPR appeared to result in substantial loss of tail retraction and body rotation,
though not as penetrant as the *Pou4* CRISPR.
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- 1333 though not as penetrant as the *Pou4* CRISPR.
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Figure S1. Phylogenetic tree of Vamp proteins.
344 Tree showing phylogenetic analysis of predicted
human (Hs) and *Ciona robusta* (KH gene mode
346 sequences file for protein sequences used.
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348 344 Tree showing phylogenetic analysis of predicted proteins encoded by *VAMP* family genes from
human (Hs) and *Ciona robusta* (KH gene models). See methods for details and supplemental
sequences file for protein sequence 345 human (Hs) and *Ciona robusta* (KH gene models). See methods for details and supplemental
346 sequences file for protein sequences used.
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Figure S2. Phylogenetic tree of NCS proteins and diagram of *Ciona robusta* NCS1 domains.

A) Tree showing phylogenetic analysis of proteins encoded by *Neuronal Calcium Sensor (NC*

family genes from human (Hs), *Drosophi*

356 family genes from human (Hs), *Drosophila melanogaster* (Dm) and *Ciona robusta* (KH gene
357 models). Efcab11 from *C. robusta* (Cr) was used to root the tree. See methods for details an
358 supplemental sequences fil

355 A) Tree showing phylogenetic analysis of proteins encoded by *Neuronal Calcium Sensor (NCS)*
356 family genes from human (Hs), *Drosophila melanogaster* (Dm) and *Ciona robusta* (KH gene
357 models). Efcab11 from *C. r* models). Efcab11 from *C. robusta* (Cr) was used to root the tree. See methods for details and
supplemental sequences file for protein sequences used. B) Protein domain analysis diagram
Ciona robusta NCS1 from SMART (Let

358 supplemental sequences file for protein sequences used. B) Protein domain analysis diagram of
359 *Ciona robusta* NCS1 from SMART (Letunic et al. 2021) showing its predicted three EF-hand
360 (Efh) domains. Dashed line 359 *Ciona robusta* NCS1 from SMART (Letunic et al. 2021) showing its predicted three EF-hand (Efh) domains. Dashed lines indicate exon-exon junctions.
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360 360 (Efh) domains. Dashed lines indicate exon-exon junctions.
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details and supplemental sequences file for protein sequences used. B) Protein domain analysis
376 diagram of *Ciona robusta* NARS1 from SMART (Letunic et al. 2021) showing its predicted
377 domains. Dashed lines indicate 376 diagram of *Ciona robusta* NARS1 from SMART (Letunic et al. 2021) showing its predicted
377 domains. Dashed lines indicate exon-exon junctions.
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396 Figure S4. Indel plots for all sgRNAs tested.

392 A-D) NGS indel validation plots (including ne

393 study. No amplicon was obtained for the third

394 Blue arrows indicate CRISPR-generated inde

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397 392 A-D) NGS indel validation plots (including negative controls) for all the sgRNAs tested in this
303 study. No amplicon was obtained for the third *NARS1* sgRNA nor the *NARS1* negative contro
394 Blue arrows indicate C 393 study. No amplicon was obtained for the third *NARS1* sgRNA nor the *NARS1* negative control.
394 Blue arrows indicate CRISPR-generated indels, red asterisks indicate naturally occurring indels
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413 Figure S5. Replicate of *Vamp1/2/3* CRISPR.

108 Independent replicate of papilla-specific *Vam*

109 electroporated with 40 µg/700 µl *Foxc>Cas9*

110 µg/700 µl each sgRNA vector). Negative con

111 *Foxc>Cas9* alone. Tai

Independent replicate of papilla-specific *Vamp1/2/3* CRISPR in *Ciona* larvae. Embryos were
electroporated with 40 μg/700 μl *Foxc>Cas9* and gene-specific pairs of sgRNA vectors (40
μg/700 μl each sgRNA vector). Negative electroporated with 40 μg/700 μl *Foxc>Cas9* and gene-specific pairs of sgRNA vectors (40 μg/700 μl each sgRNA vector). Negative control embryos were electroporated with 40 μg/704 μl *Foxc>Cas9* alone. Tail retraction was

410 µg/700 µl each sgRNA vector). Negative control embryos were electroporated with 40 µg/700 µl
411 Foxc>Cas9 alone. Tail retraction was scored at 48 hours post-fertilization without screening for
412 mCherry+ individual 411 *Foxc>Cas9* alone. Tail retraction was scored at 48 hours post-fertilization without screening for mCherry+ individuals.
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