# SUPPLEMENTARY INFORMATION

## SUPPLEMENTARY TEXT

### Markers of glutamatergic identity

The enzyme glutaminase generates 70% of all neuronal glutamate in vertebrates (Hertz, 2004). However, the expression of glutaminase is not restricted to glutamatergic neurons within the vertebrate nervous system (e.g. (Kaneko et al., 1990; Kaneko and Mizuno, 1992)). Glutamate plasma membrane reuptake transporters are also not good markers of glutamatergic neurons since, unlike the reuptake transporters for other neurotransmitters, glutamate reuptake transporters are primarily expressed in cells surrounding glutamatergic neurons, rather than in the glutamatergic neurons themselves (Huang and Bergles, 2004; Mano et al., 2007).

Glutaminase also does not serve as a selective marker for *C. elegans* glutamatergic neurons. Sequence homolog searches identify three glutaminase-encoding genes in the *C. elegans* genome, *glna-1, glna-2* and *glna-3*. *glna-1* and *glna-2* reporter genes are expressed outside the nervous system and in very small (<5) number of neurons, while a *glna-3* reporter is expressed in many neuron types (**Suppl. Fig.1**). However, co-labeling transgenic *glna-3::gfp* worms with markers that label other neurotransmitter populations show that *glna-3* is not restricted to glutamatergic neurons (**Suppl. Fig.1**). The non-selectivity of glutaminase expression for glutamatergic neurons mirrors the situation in the vertebrate nervous system and argues that like in vertebrates, the VGLUT gene *eat-4* is the only currently available identity marker of glutamatergic neurons.

### Examination of the antagonism of glutamatergic and GABAergic identity

In several distinct regions of the vertebrates CNS, excitatory glutamatergic and inhibitory GABAergic identity of neuron types can be controlled in an antagonistic,

binary switch-type manner, such that loss of glutamatergic identity is accompanied by gain of GABAergic fate (for example in the dorsal spinal horn of Tlx mutant mice; (Ma and Cheng, 2006)). A similar excitatory/inhibitory antagonism is also observed in the cholinergic system of the basal ganglia, where loss of cholinergic identity of a neuron type can result in a switch to a GABAergic identity (Fragkouli et al., 2009). We examined whether such antagonism is built into the glutamatergic differentiation programs of *C. elegans* neurons as well. Aside from the 38 classes of glutamatergic neurons described above, the *C. elegans* nervous system contains 26 GABAergic neurons (defining six different classes)(McIntire et al., 1993). We examined potential switches of glutamatergic to GABAergic identity in eight mutant strains that lack distinct regulators of 18 distinct glutamatergic neuron classes (*ceh-36, ttx-1, ceh-37, vab-3, unc-42, ets-5, che-1, ceh-14*) using anti-GABA immunostaining. We observed no ectopic GABA staining (data not shown). Considering the number of cells and regulators we examined, we can conclude that GABAergic and glutamatergic identity are not commonly executed in an antagonistic, binary switch-type manner in *C.elegans*. This is consistent with previous studies of terminal selector transcription factors, whose disruption does not usually result in cell identity switches, but mere failures to differentiate into any specific state (Hobert, 2011).

## SUPPLEMENTARY EXPERIMENTAL PROCEDURES

### *eat-4* reporter genes

The *eat-4* fosmid reporter was generated by fosmid recombineering using fosmid WRM0623aF12 and an SL2-based, nuclear localized *yfp* reporter (Tursun et al., 2009). The reporter was injected at 15 ng/uL into *pha-1(e2123)* mutant animals with *pBX* as injection marker (2 ng/uL) [100 ng/uL OP50 gDNA as filling DNA]. One extrachromosomal array was integrated to yield *otIs388* III.

The *eat-4* locus reporter (shown in **Figure 2**) was generated by *in vivo* recombination (Boulin et al., 2006), using two overlapping fragments of the *eat-4* locus. Briefly, a *5'-eat-4prom-mChOpti*$^{[1\text{-}516]}$ fragment was generated by PCR fusing an upstream *eat-4* region from position -5.6 kb to the *eat-4* start codon (using the primer 5'-GGATTGAAGTAGCTCACTGATGGATCG-3') to the first two thirds of the codon-optimized *mCherry*. A second fragment, *mChOpti*$^{[116\text{-}861\Delta stop\ codon]}$ *–EAT-4::eat-4 3'-UTR,* was generated PCR fusing a C-terminus fragment (+116-861 bp) of codon-optimized *mCherry* to the 5' end of the *eat-4* locus plus 560 bp of *eat-4* 3'UTR (using the primer 5'-GAACATCCTTGATTTCCTTCTTGCTCA-3'). Both fragments contained a 400 bp overlap within the *mCherry* sequence, so only upon successful *in vivo* recombination an intact fluorescent protein can be generated. Both fragments were injected at equal molar ratios with *rol-6 (su1006)* (50 ng/ul) as a co-injection marker. One extrachromosomal array was integrated to yield *otIs292*.

We identified neurons that express a *eat-4* reporter constructs based on their cell position and, in many cases, by co-labeling individual neurons with mCherry-based reporters.

The *eat-4* reporter transgenes are as follows:

*otIs388* III: *eat-4*$^{FOS}$*::sl2::yfp::H2B*; injected in *pha-1(e2123)*; pBX

*otIs292: eat-4* in vivo recombineered reporter; *rol-6*

*adIs1230: eat-4* reporter construct from Lee at al/, 1999.

*otIs376*: *eat-4$^{prom2}$::gfp;rol-6*

*otIs392*: *eat-4$^{prom6}$::gfp;ttx-3::dsRed*

*otEx4478, otEx4479, otEx4480:* 3 lines for *eat-4$^{prom1}$::gfp; rol-6*

*otEx4492, otEx4432, otEx4494:* 3 lines for *eat-4$^{prom12}$::gfp; rol-6*

*otEx5292, otEx5293, otEx5294: :* 3 lines for *eat-4$^{prom5}$::gfp; ttx-3::dsRed*

*otEx5298, otEx5299, otEx5300:* 3 lines for *eat-4$^{prom6\Delta3}$::gfp; ttx-3::dsRed*

*otEx5311;otEx5312:* 2 lines for *eat-4$^{prom6\Delta4}$::gfp; rol-6*

*otEx5295, otEx5296, otEx5297:* 3 lines for *eat-4$^{prom7}$::gfp; ttx-3::dsRed*

*otEx4488, otEx4489, otEx4490:* 3 lines for *eat-4$^{prom8}$::tagRFP; rol-6*

*otEx5301, otEs5310:* 2 lines for *eat-4$^{prom10}$::gfp; rol-6*

*otEx5098, otEx5099, otEx5100:* 3 lines for *eat-4$^{prom2\Delta7}$::gfp;rol-6*

*otEx5313, otEx5314, otEx5315:* 3 lines for *eat-4$^{prom16}$; ttx-3::dsRed*

*otEx5316, otEx5316:* 2 lines for *eat-4$^{prom2\Delta11}$;rol-6*

*otEx5318, otEx5319, otEx5320:* 3 lines for *eat-4$^{prom2\Delta12}$;rol-6*

*otEx5345, otEx5346, otEx5347:* 3 lines for *eat-4$^{prom2\Delta6}$;rol-6*

*otEx5330, otEx5331, otEx5332:* 3 lines for *eat-4$^{prom10\Delta6}$;rol-6*

*otEx5333, otEx5334, otEx5335:* 3 lines *eat-4$^{prom10\Delta7}$;rol-6*

**Serotonin and GABA antibody staining**

Antibody staining was performed using a tube fixation protocol (adapted from (McIntire et al., 1992)). Briefly, young adult worms well fed were fixed with PFA 4% for 24 hours at 4°C; for GABA staining animals were fixed with PHA 4% - Glutaraldehyde 1%. The next day they were washed with 1% PBS – 0.5% Triton X-100 three times and incubated for 18 hours at 37°C in a nutator mixer with 5% □-mercapto-ethanol-1% Triton X-100 - 0.1M Tris (pH 7.5). The third day the worms were rinse three or four

times with 1% PBS -0.5% Triton X-100 and treated with collagenase type IV (Sigma Aldrich, C-5138) in collagenase buffer (1% Triton X-100 / 0.1M Tris, pH 7.5 / 1mM CaCl2) for 1 hour at 37°C/700 rpm. Worms were washed with 1% PBS – 0.5% Triton X-100 and proceed to stain. Blocking solution (PBS 1X - 0,2% Gelatin - 0.25% Triton X-100) was added to the worms for 30 minutes at room temperature and then they were incubated for 24 hours at 4°C in primary antibody [anti-5HT antibody 1/100 (Sigma Aldrich, S-5545); anti-GABA antibody 1/500 (AbCam, ab17413)] in PBS 1X - 0,1% Gelatin - 0.25% Triton X-100. The worms were washed three times and incubated with secondary antibody [anti-rabbit Alexa Fluor 1/1000 (BD Biosciences) for anti-5HT and anti-guinea pig Alexa Fluoro 1/100 (BD Biosciences) for anti-GABA] for 2 hours at room temperature. Finally worms were washed three times and mounted on Fluoro-Gel II with DAPI (EMS).

**Isolation, identification and characterization of *vab-3***

We utilized a transgenic strain that expresses a reporter gene for the tyramine receptor *ser-2* (*otIs138* transgenic animals) which is exclusively expressed in the OLL and PVD sensory neuron classes (Tsalik et al., 2003). After EMS mutagenesis, we identified with a Copas Biosort machine (Doitsidou et al. 2008) three non-allelic mutant strains in which expression of *ser-2::gfp* is lost specifically in the OLL neurons. The mutation *ot569* completely abolishes *ser-2::gfp* expression in the OLL but not the PVD neurons (**Figure 5A**). Whole genome sequencing was used to determine the molecular identity of *ot569*. DNA from *ot569* mutants was sequenced to an average depth of 8x in an Illumina GA2 sequencer as previously described (Doitsidou et al. 2010). Analysis of the WGS data with MAQGene (Bigelow et al. 2009) resulted in the identification of 280 variants on the X chromosome, to which the mutant had been previously mapped genetically. A list of background variants was compiled by combining variants present in the whole genome sequencing data of two other mutants isolated in the same screen and subtracted from the *ot569* dataset. Of the resulting 85 variants only 9 are predicted to be splice site or protein coding variants and only one of these affected a transcription factor, namely the *vab-3* locus, which codes for the *C. elegans* ortholog of the

vertebrate Pax6/Drosophila Eyeless gene (Chisholm and Horvitz, 1995). The mutation converts a highly conserved glycine residue in the linker region of the paired domain to a serine. The linker region makes extensive contacts with the minor groove of DNA suggesting this mutation affects the ability of VAB-3 to bind DNA (Cohen and Melton, 2011). A canonical allele of *vab-3*, *e648* showed a similar OLL differentiation defect as *ot569* and the *ot569* allele can be rescued with a fosmid that contains the *vab-3* locus (**Figure 5A,B**).

The *vab-3* fosmid reporter was generated by fosmid recombineering based in fosmid WRM0623aF12 (Tursun et al., 2009) using a *gfp* reporter fused to the N-terminus of the protein and replacing the stop codon. It was injected at a concentration of 15ng/ul, in combination with 2ng/ul of *elt-2::DsRed* and 100 ng/ul OP50 gDNA (as filling DNA) to generate 3 extrachromosmal arrays (*otEx5057, otEx5058, otEx5059*) none of which rescue the *vab-3(ot569)* phenotype. An untagged fosmid array does rescue the mutant phenotype (**Fig.5A**).

**Neuronal identity markers**

The following neuronal identity markers were used: *otIs92 [flp-10::gfp], inIs179 [ida-1::gfp], ynIs30 [flp-4::gfp], gmIs12 [srb-6::gfp], otIs33 [kal-1::gfp], otIs358 [ser-2$^{prom2}$::gfp], gmIs21 [nlp-1::gfp], ynIs80 [flp-21::gfp], ynIs2022 [flp-8::gfp], ynIs40 [flp-11::gfp], vsIs28 [dop-1::gfp], sEx12012 [srab-12::gfp], zdIs13 [tph-1::gfp], otEx5336 [gpa-11$^{prom2}$::gfp], pkIs589 (gpa-13::gfp), pkIs591(gpa-15::gfp), otEx5323 (dkf-2b::gfp), ynIs54 (flp-20::gfp)*, *otIs138* and *otEx449 [ser-2$^{prom3}$::gfp], otIs396 [ace-1$^{prom2}$::tagrfp], sEx15238 [grd-8::gfp]*, *myEx741[pdfr-1::NLS::rfp]*, *vdEx078 [tol-1::gfp], otEx2540 [gcy-23::gfp], ynIs37 [flp-13::gfp], rtEx247 [nlp-14::gfp], otEx5428[glna-1::NLS::gfp], sEx10131[glna-2::gfp], otEx5429[glna-3$^{prom1}$::NLS::gfp].*

The *ace-1* reporter was generated by PCR fusion of 697 bp promoter sequence to *NLS::tagRFP*. This construct was injected in *pha-1(e2123)* and in combination with wildtype *pha-1* (pBX plasmid). One extrachromosomal array line spontaneously integrated to generate *otIs396 [ace-1$^{prom2}$::NLS::tagRFP; pha-1(+)]*.

The *gpa-11* reporter was generated by amplifying 1603 bp of the promoter cloned into pPD95.77. The PCR was injected in N2 at 5 ng/ul with *rol-6* (*su1006)* and 100 ng/ul OP50 gDNA as filling DNA.

The *glna-1* reporter was generated by PCR fusion of 2103 bp promoter sequence to *NLS::gfp.* The PCR was injected in N2 at 15 ng/ul with *rol-6* (*su1006)* and 100 ng/ul OP50 gDNA as filling DNA.

The *glna-3*$^{prom1}$ reporter was generated by PCR fusion of 1300 bp of the promoter sequence, the first exon of the gene and the first intron to *NLS::gfp.* The PCR was injected in N2 at 15 ng/ul with *rol-6* (*su1006)* and 100 ng/ul OP50 gDNA as filling DNA.

**Staining of mouse brain sections**

Primary antibodies used in this study are anti-Brn3a (1:50, mouse monoclonal; Santa Cruz Biotechnology), anti-VGlut2 (1:100, guinea pig polyclonal)(Brumovsky et al., 2007), anti-glutaminase (1:600, rabbit polyclonal)(Kaneko and Mizuno, 1992) and Lhx1 (1:20,000, kindly provided by Jane Dodd). Secondary antibodies were donkey antisera coupled with Alexa dyes (Invitrogen). Sections were counter-stained with DAPI (1:1000) (Invitrogen).

Animals were perfused intracardially with 4% paraformaldehyde. Brains were cryoprotected in Optimal Cutting Temperature compound (Tissue-Tek) and sectioned in 12 μm sections.

In situ hybridization was performed as previously described (Wallen-Mackenzie et al., 2006). The Vglut2 probe was kindly provided by Dr. Kullander, Uppsala University, Sweden (Wallen-Mackenzie et al., 2006). Neurons in the inferior olive were visualized using the Nissl staining with cresyl violet (Sigma).

## SUPPLEMENTAL FIGURE LEGENDS

**Figure S1: Expression pattern of glutaminase genes.** Related to Figure 1 and 2. We examined whether glutaminase could perhaps serve as a marker for *C. elegans* glutamatergic neurons. Sequence homolog searches identify three glutaminase-encoding genes in the *C. elegans* genome, *glna-1, glna-2* and *glna-3*.
**(A)** Expression pattern of a *glna-1* reporter that contains 2.1 kb sequences upstream of the first exon of the gene. Expression is observed in a very small number of neurons in the head (white arrowheads).
**(B)** Expression pattern of a *glna-2* reporter that contains 3 kb sequences upstream of the first exon of the gene. Expression is observed in a very small number of neurons in the head (white arrowheads).
**(C)** Expression pattern of a *glna-3* reporter that contains 1.3 kb sequences upstream of the first exon of the gene and the first intron. Expression of the *gfp* reporter is seen in many neuron types in the head (as seen in the yellow overlap of the green *gfp* reporter and a red fluorescent marker that labels all neurons). In the middle and lower panel, the overlap of the *gfp* reporter with cholinergic neurons (*cho-1::rfp*) and GABAergic neurons (*unc-47::rfp*) is shown. Some examples of clear overlaps in expression are indicated with white arrowheads. The non-selectivity of glutaminase expression for glutamatergic neurons mirrors the situation in the vertebrate nervous system and argues that like in vertebrates, the VGLUT gene *eat-4* is the only currently available identity marker of glutamatergic neurons.

**Figure S2: The role of terminal selectors in maintaining and ectopically inducing neuronal identity and their effect on panneuronal identity features.** Related to Figure 3, 4 and 5.
**(A-C)** *mec-3* and *unc-42* are required to maintain the expression of *eat-4/VGLUT*.
**(A)** Temporally controlled *mec-3* expression was achieved through driving *mec-3* expression under the control of the heat shock promoter in a *mec-3(e1338)* mutant background. A brief pulse of *mec-3* expression, achieved through 30 minutes of heat shock, is able to restore the expression of *eat-4* (assayed with the *adIs1240* transgene)

in the touch receptor neurons and to ectopically express *eat-4* in BDU. Three days after the transient, 30 minute-pulse of *mec-3* expression (when *mec-3* expression has presumably faded away), *eat-4* expression is reduced compared with the expression at 24 hours after heat shock. Animals carrying the heat-shock *mec-3* array but that were not heat-shocked do not show a restoration of *eat-4* expression at any time point.
**(B)** Postembryonic removal of *unc-42* results in a decrease in *eat-4* expression (assayed with the *otIs376* array) in the ASH neurons. RNAi-sensitized *nre-1 lin-15; otIs376* animals were fed *unc-42(dsRNA)* from the L1 stage onward and assayed 4 days later. As an internal control, *gfp* expression in ASH was compared to *gfp* expression in the AUA neurons. RNAi against *unc-42* showed a reduction in the expression of *eat-4* in ASH compared to AUA fluorescence. In animals fed with control (empty vector) RNAi the GFP intensity in ASH is always higher or similar to that of AUA.
**(D-F)** Ectopic expression of *eat-4/VGLUT* regulators induces ectopic *eat-4/VGLUT* expression.
*ceh-36, ceh-37* and *che-1* were misexpressed with the pansensory promoters *osm-6* and *ift-20* and *mec-3* was misexpressed using the heat-shock promoter. Heat shock was induced at the L1 stage for 30 minutes and animals were scored 48 hours later at the L4 stage.
**(D)** Quantification of effects of misexpression of glutamatergic regulators. We ascribe the cellular context-dependency of ectopic *eat-4* expression to the limited availability of cofactors with which these factors act in their normal cellular context.
n.a.: not applicable because promoter is not expressed in these cells.
n.d.: not determined.
**(E,F)** Representative examples of the effects of misexpression of glutamatergic regulators.
**(G-I):** Expression of the panneuronal marker *rab-3* is unaffected in terminal selector mutants.
**(G)** *rab-3* expression (monitored with *otIs356 = rab-3::NLS::tagRFP)* is unaffected in phasmid sensory neurons in 3/3 *ceh-14* null mutant animals.
**(H)** *rab-3* expression (monitored with *otIs291= rab-3::NLS::yfp)* is unaffected in the AUA neurons of 6/6 *ceh-6* null mutant animals. Identification of AUA was facilitated by DiI

staining (red) which labels a closely neighboring neuron.

**(I)** *rab-3* expression (monitored with *otIs291(rab-3::NLS::yfp); otIs396(ace-1$^{prom2}$::NLS::tagRFP)* is unaffected. Since the position of neuronal cell bodies is somewhat variable in *vab-3* mutants, it is difficult to unambiguously identify OLL in these animals. We therefore counted overall *rab-3::yfp(+)* neuron number in the anterior ganglion. Adult wild-type animals (n=22) have an average of 37.8 neurons in the anterior ganglion and *vab-3* mutants have an average of 36.9 (n=51), which is not statistically significantly different.

**Figure S3: *unc-86* and neuroblast identity of glutamatergic lineages.** Related to Figure 3.

**(A)** Expression of *unc-86* (diagram taken from (Finney and Ruvkun, 1990)) and its overlap with *eat-4* expression. While *unc-86* affects the terminal differentiation programs of specific neuron types (e.g. ALM and PLM), in a small number of cases, it is expressed and acts earlier in the lineage to affect neuroblast identity (Chalfie et al., 1981; Finney and Ruvkun, 1990). Considering the expression pattern analysis of UNC-86, the common theme emerges that whenever UNC-86 is expressed through 3 cell generations, its loss results in a reiteration of the fate of the mother cell. To corroborate the loss of glutamatergic neuron identity in these lineage-defective mutants with a molecular marker, we examined *eat-4/VGLUT* expression in these lineages in *unc-86* mutants. We indeed observed a loss of *eat-4/VGLUT* expression in the Q neuroblast-derived AVM touch receptors neuron, in the T lineage-derived PHC tail sensory neurons and in the AIZ interneurons (**Figure 4**). In the Q neuroblast-derived AVM and PVM neurons, *unc-86* is known to not only affect neuroblast identity, but to also act later during terminal differentiation (Duggan et al., 1998). It is possible that in the PHC and AIZ lineages *unc-86* may also have late roles in controlling terminal differentiation via regulating *eat-4* expression in addition to defining neuroblast identity. Notable, *unc-86* appears to cooperate with distinct LIM homeobox genes in distinct neuron types. In the case of the Q lineage that produces the AVM/PVM neuron, *unc-86* has been found to cooperate with the LIM homeobox gene *mec-3* to control the terminal identity state of the neuron. In the lineage that produces PHC, a similar cooperation with *ceh-14* may

occur. In the lineage that produces AIZ, such a cooperation with the resident LIM homeobox gene *lin-11* appears, however, less likely since AIZ identity is not affected in *lin-11* mutants (data not shown)(Tsalik et al., 2003).
**(B)** *unc-86* does not affect expression of *eat-4* in I2 or URY. The effect of *unc-86* on *eat-4* expression in AQR, PQR and ADA was inconclusive.

**Figure S4: ModEncode data reveals CEH-14 binding sites in the *eat-4* locus.** Related to Figure 2. Wormbase genome browser representation of *ceh-14* binding sites (dark blue boxes in genome browser image) and their specific location in *eat-4*$^{prom5}$. The picture shows a representative projection of the expression of *eat-4*$^{prom5}$ in the tail in PHC, PHB and DVC. The expression of *eat-4 (otIs388)* was abolished in these neurons in a *ceh-14* null mutant. The light blue bars in the promoter represent TAAT homeodomain binding sequences corresponding to the region within the blue boxes.

**Figure S5: Summary of the effect of regulation of *eat-4* expression in distinct neuron types by distinct transcription factor combinations, each involving an Otx-type homeodomain transcription factor.** Related to Figure 2 and 3. Figure 2 shows the data for the involvement of the TAATCC and the ETS domain binding sites. We hypothesize that other, as yet unidentified TFs act in parallel. The cofactor of *ceh-36* in the AWC neurons is likely *sox-2* (unpubl. data).

**Figure S6: The LIM homeobox genes *lin-11/Lhx1* and *ttx-3/Lhx2* control the identity of the ADL and ASK sensory neurons.** Related to Figure 2 and 3.
**(A)** *ttx-3* affects expression of *eat-4 (*assayed with the *otIs376* transgene*)* and several other terminal features (*gcy-27::gfp* transgene *otEx2540*, *nlp-14::gfp* transgene *rtEx247* and *flp-13::gfp* transgene *ynIs37*) of the ASK neurons.
**(B)** *lin-11* affects *eat-4* expression (assayed with the *otIs392* transgene) in the ASK neurons.

**Figure S7: LHX1 is not expressed in all glutamatergic neurons and not all glutamatergic neurons are LHX1-positive.** Related to Figure 6.

Immunostaining analysis for LHX1 and BRN3A and VGLUT2 *in situ* hybridization in sequential coronal sections of different regions of the adult mouse brain.

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