Published in final edited form as:

Nature. 2009 April 16; 458(7240): 885–889. doi:10.1038/nature07929.

Gene regulatory logic of dopaminergic neuron differentiation

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Abstract

Dopamine signaling regulates a variety of complex behaviors and defects in dopaminergic neuron function or survival result in severe human pathologies, such as Parkinson's disease 1. The common denominator of all dopaminergic neurons is the expression of dopamine pathway genes, which code for a set of phylogenetically conserved proteins involved in dopamine synthesis and transport. Gene regulatory mechanisms that result in the activation of dopamine pathway genes and thereby ultimately determine the identity of dopaminergic neurons are poorly understood in any system studied to date 2. We show here that a simple *cis*-regulatory element, the DA motif, controls the expression of all dopamine pathway genes in all dopaminergic cell types in *C. elegans*. The DA motif is activated by the ETS transcription factor, AST-1. Loss of *ast-1* results in the failure of all distinct dopaminergic neuronal subtypes to terminally differentiate. Ectopic expression of *ast-1* is sufficient to activate the dopamine production pathway in some cellular contexts. Vertebrate dopaminergic pathway genes also contain phylogenetically conserved DA motifs that can be activated by the mouse ETS transcription factor Etv1/ER81 and a specific class of dopaminergic neurons fails to differentiate in mice lacking Etv1/ER81. Moreover, ectopic Etv1/ER81 expression induces dopaminergic fate marker expression in neuronal primary cultures. Mouse Etv1/ER81 can also functionally substitute for *ast-1* in *C.elegans.* Our studies reveal an astoundingly simple and apparently conserved regulatory logic of dopaminergic neuron terminal differentiation and may provide new entry points into the diagnosis or therapy of conditions in which dopamine neurons are defective.

Nervous systems generally harbor distinct populations of dopaminergic (DA) neurons that derive from different precursor cells. Despite their diverse origin, all DA neurons share the expression of a core set of 5 genes that code for enzymes and transporters which synthesize, package and re-uptake dopamine (“dopamine pathway genes”; Fig.1a). The regulatory logic of the terminal differentiation of DA neurons, manifested by the induction of the DA pathway genes, can, in theory, be described by two distinct models. In model #1, each dopamine pathway gene is independently activated by a distinct set of regulatory factors and, as a reflection of their distinct developmental history, each DA neuron subtype utilizes a distinct set of regulatory molecules (Fig. 1b). In model #2, each dopamine pathway gene is regulated by the same regulatory factor(s) and those factor(s) are the same in each DA neuron subtype (Fig. 1b). These two models make specific predictions about the *cis*-

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regulatory architecture of dopamine pathway genes. In model #1, each dopamine pathway gene is controlled by distinct *cis*-regulatory motifs and different motifs are active in individual DA neuron subtypes. In model #2, there is a single motif for all pathway gene that is utilized in all different DA neuron subtype (Fig. 1b). To test these models, we made use of the dopaminergic system of the nematode *C. elegans*, which contains 4 distinct, lineally unrelated classes of dopaminergic neurons that express the same set of highly conserved dopamine pathway genes (Fig. 1c) 3. We systematically dissected the *cis*-regulatory regions of all DA pathways genes in the context of *gfp* reporters expressed in transgenic worms (Fig. 1c-h; Suppl. Fig. S1). The *cis*-regulatory analysis of two genes exclusively expressed in the DA neurons, the dopamine transporter gene *dat-1*/DAT and the tyrosine hydroxylase gene *cat-2*/TH, reveals the existence of a small *cis*-regulatory module (CRM) in each promoter that is required and sufficient to drive expression in all DA neurons (Fig. 1d and e). Dopamine pathway genes expressed in both DA and serotonergic (5-HT) neurons (*cat-1*/VMAT, *cat-4*/GTPCH, *bas-1*/AAA) contain separable CRMs for expression in DA and 5-HT neurons (Fig. 1f-h).

The DA-specific CRM of the *dat-1*/DAT locus contains a small sequence motif that is conserved in three other *Caenorhabditis* species (Fig. 1d; Suppl. Fig. S5) and through mutation was found to be required for *dat-1* expression in all DA neurons (Fig. 1d). This motif is also sufficient to drive expression in all DA neurons, either when tested in isolation or when appended to the CRM of another neuron-specific gene (Suppl. Fig. S2a). Bioinformatics analysis predicts the binding of six different types of transcription factors to this conserved motif (Suppl. Fig. S2b). Point mutations that specifically abolish the predicted binding of some factors while keeping others intact reveal that the only predicted motif that can be made responsible for *cis*-regulatory motif activity in the DA neurons is a predicted ETS transcription factor binding site (EBS) defined by an invariant GGAW core sequence (Suppl. Fig. S2b). The DA-expressed CRM of all other dopamine pathway genes also contain predicted EBSs and mutational analysis corroborates their requirement for the correct expression in all DA neurons of *C. elegans* hermaphrodites (Fig.1f-h; Suppl. Fig.S3) and in the three additional DA pairs present in the male (Suppl. Fig. S4). All the functionally characterized EBSs are conserved in other *Caenorhabditis* species, they can occur in either orientation and at different distances from the transcriptional start (Fig. S5). The weight matrix generated with all these sequences defines a consensus EBS sequence motif that we term the “DA motif” (Fig.1i; Suppl. Fig.S5).

Analyzing the expression of the DA marker *dat-1::gfp* in mutants that lack each of the ten *C. elegans* ETS family members (Fig. S6), we find that loss that all *ets* mutants showed wild-type *dat-1::gfp* expression except for animals lacking the *Axon STeering defect-1 (ast-1)* gene, previously identified as a gene controlling axon outgrowth in the ventral nerve cord 4. Moreover, we found that a mutant allele, *ot417*, that we retrieved from an unbiased forward genetic screen for mutants in which DA fate is inappropriately executed 5, is an allele of *ast-1* (Fig.2a). The expression of all five dopamine pathway genes is strongly affected if not completely lost in *ast-1* mutants (Fig.2b,c; Suppl. Table S1; Suppl. Fig.S7). Two other DA terminal differentiation markers, the ion channels *asic-1* 6 and *trp-4* 7, also fail to be expressed in the DA neurons of *ast-1* mutants (Suppl. Fig. S8). Both genes contain

phylogenetically conserved DA motifs in their regulatory regions. *ast-1* therefore appears to affect DA fate broadly, which is further corroborated by axon pathfinding defects we observe in *ast-1* mutants (Suppl. Fig. S9). Loss of DA marker gene expression is not a reflection of early lineage specification defects and/or absence of the neurons, as assessed by analysis of additional fate marker (Suppl. Fig. S8).

ast-1 is expressed in several neurons 4, including all DA neurons (Fig. 2d) and acts cell-autonomously in DA neurons, as the *ast-1* mutant phenotype can be rescued by expression of *ast-1* specifically in the DA neurons (Fig. 2e). *ast-1* expression persists in DA neurons throughout postembryonic stages, suggesting that *ast-1* is not only required to initiate DA terminal cell fate, but to also maintain DA neuron identity, a notion we confirmed through temporally controlled addition and removal of *ast-1* gene activity (Fig. 2f; Suppl. Fig.S10).

To address whether *ast-1* function is not only necessary for proper DA neuron differentiation, but also sufficient, we ectopically induced *ast-1* expression throughout all cell and tissue types at different stages of development (Fig.3). Ectopic induction during embryogenesis leads to a substantial ectopic expression of both *dat-1::gfp* (Fig.3a-d) and *cat-2::gfp* (data not shown). The morphology, location and pan-neuronal fate marker expression of these cells suggests that the effects of *ast-1* are confined to the nervous system, in which some (20 cells; ∼10% of the embryonic nervous system) but clearly not all cells can be induced to ectopically express both *dat-1*/DAT and *cat-2*/TH. Ectopic *ast-1* is maximally effective when expressed around the time of neurogenesis (Suppl. Fig. S11). Moreover, ectopic *ast-1* expression under control of the DA- and 5-HT-specific *bas-1* promoter induces *dat-1*/DAT expression in 5-HT neurons (Fig. 3e) demonstrating that *ast-1* acts autonomously to control DA neuron specification and that 5-HT neurons provide the appropriate cellular context to allow *ast-1* to induce DA neuron specification. The related ETS domain transcription factor LIN-1 is not able to induce ectopic DA neuron production when expressed under similar conditions, demonstrating the specificity of AST-1 function.

To assess whether ETS transcription factor(s) have a similar function in vertebrate DA neuron specification, we analyzed their expression in the DA areas of the brain (Suppl. Table S2). Distinct ETS factors appear to be expressed in distinct types of DA neurons and we focus here on the ETS factor Etv1/ER81, which is expressed in the DA neurons of the olfactory bulb (Suppl. Fig. S12)8,9. Mice lacking Etv1 10 display a dramatic reduction in the number of TH positive cells in their olfactory bulb compared to wild-type siblings whereas other periglomerular interneuron subtypes were not affected or less severely reduced (Fig. 4a-c; Suppl. Fig. S13). This phenotype is not paralleled by increased cell death, nor by a reduction in the overall density of cells in the glomerular layer, nor by a reduction in overall neuron number, nor by proliferation defects (Fig. 4d-f; Suppl. Fig. S14). Moreover, the identity of DA progenitor cells in the lateral ganglionic eminence (LGE), which already express Etv1 11, appears unaffected in Etv1 mutants (Suppl. Fig. S15). Therefore, Etv1 may affect a late stage in olfactory DA neuron differentiation.

Like *ast-1*, Etv1 appears not only required for DA neuron differentiation but also sufficient, as ectopic expression of Etv1 in olfactory bulb primary cell culture increases the number of cells expressing the DA marker TH (Fig.4g). Etv1 is also able to directly activate the *cis*-

regulatory region of the mouse TH locus in a heterologous context (Fig. 4h). This activation depends on the presence of two phylogenetically conserved DA motifs (Fig. 4h). Like in *C. elegans* , phylogenetically conserved DA motifs can also be found in the 5' upstream regulatory region of all four other mouse dopamine pathway genes (Fig. S16). Another indicator for a conserved function of mouse Etv1 and worm AST-1 is that mouse Etv1 is able to rescue the *ast-1* mutant phenotype when expressed in transgenic worms (Fig.2e).

In conclusion, we have described here a surprisingly simple regulatory logic for DA specification. Our *cis*-regulatory analysis in worms reveals that all dopamine pathway genes are co-regulated through a similar *cis*-regulatory motif and *trans*-acting factor and this regulatory logic applies to dopaminergic neurons of distinct lineage origin. Our analysis demonstrates that the ETS factor *ast-1* is a terminal selector gene for dopaminergic cell fate, akin to other terminal selector genes that control the terminal identity of other neuron types 12-15. Terminal selector genes are transcription factors that directly regulate the "nut-and-bolts" differentiation gene batteries that determine the specific properties of a neuron by binding to simple *cis*-regulatory motifs shared by members of the terminal gene batteries, termed "terminal selector motifs" (in the case of the DA neurons, the DA motif) 15. As exemplified by AST-1, terminal selector genes are continuously expressed throughout the life of a neuron to ensure that the terminal differentiation state is properly maintained.

The regulatory logic of DA neuron specification appears to be phylogenetically conserved. Vertebrate dopamine pathway genes also contain DA motifs that are required for the activation by a *trans*-acting factor that is homologous to the *C. elegans trans*-acting factor. Loss of the *trans*-acting factor either in worms or mice leads to a loss of the dopaminergic phenotype. Both Etv1 and *ast-1* are continuously expressed throughout the postmitotic life of DA neurons, and our analysis in worms indicate that these factors also maintain the terminal identity of DA neurons. The function of vertebrate ETS proteins in DA specification may have been distributed over several different ETS domain transcription factors, as Etv1 is not expressed in other DA neuron population in the brain and as it does not affect the generation of these other types of DA neurons (data not shown). Those other areas express a related ETS factor, Etv5, which may fulfill a similar role as Etv1 in olfactory DA neurons; in support of this notion, Etv5 can also transactivate the TH promoter in a heterologous assay system (Supp.Fig. S17). The logic of distributing an ancestral gene function, observed in an invertebrate species, over several vertebrates paralogs of the ancestral invertebrate ortholog has been noted for other transcription factors as well 16 and appears an important component of driving neuronal diversification processes in more complex brains.

While AST-1 and Etv1 both act as selector genes for DA terminal differentiation, their presence is not strictly sufficient to activate DA genes as both AST-1 and Etv1 are expressed in other neurons apart from DA neurons 4,17. Our ectopic expression experiments also show that AST-1's ability to induce ectopic DA fate is restricted to some cellular and temporal contexts. Classic "master regulators", like *eyeless* or MyoD also show similar context-dependencies in their mode of action 18,19. AST-1 and Etv1 function may be actively inhibited in cells "refractory" to AST-1/Etv1 activity. Alternatively, AST-1/Etv1 function may require additional, cell type-specific factors for appropriate function in DA neurons.

Such “combinatorial coding” mechanisms are a common theme in neuron type specification 20 and our identification of a conserved role of ETS factors as a central component of such a code is the first important step in decoding the regulatory logic of DA neuron specification. It will be interesting to see whether the additional specificity determinants of ETS factors are also conserved from worms to vertebrates.

METHOD SUMMARY

Transgenic and mutant *C.elegans* strains

Reporter gene constructs were generated by subcloning into the pPD95.75 backbone vector, which contains the *gfp* coding sequence and the *unc-54 3’ UTR*. Mutagenesis and deletions were performed using the Quickchange II XL Site-Directed Mutagenesis Kit (Stratagene). Reporter constructs were injected into *otIs181*(*Is[dat-1::mCherry;ttx-3::mCherry]*) to facilitate the identification of the DA cells. DNA was injected at 50ng/µl using *rol-6* as injection marker. For every construct, 30 or more animals were scored from at least 2 different transgenic lines. Ets mutant strains were obtained from the CGC. For the heat shock experiments, a transgenic strain of the following genotype was generated: OH7546 *[otIs198*(*hsp16−2::ast-1;hsp16−2::NLS-mCherry*;*ttx-3::ds-red*),*vtIs1(dat-1::gfp;rol6)].* Heat-shock induction conditions, as well as a list of transgenic and mutants strains, are provided in the Suppl. Methods.

Analysis of vertebrate *Etv1*

Standard histological protocols were used to analyze wild-type and *Etv1* mutant mouse samples. For ectopic *Etv1* expression, olfactory bulbs were dissected from P0-P1 wild-type mice, dissociated and electroporated using the Amaxa Nucleofector System and the mouse neuron kit, following the manufacturer's protocol. For the TH promoter analysis, COS cells were transfected with Lipofectamine (Invitrogen) and luciferase activity was measured using the Luciferase Assay kit (Stratagene) and the ß-galactosidase Enzyme Assay System (Promega). See supplementary methods for full description on histology, cell culture and quantification methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Q. Chen for expert injection assistance, M. Doitsidou for kindly providing the *ot417* allele, E. Savner for initiating some of the promoter analysis, A. Kruyer for technical help, the CGC for strains, the *C. elegans* Gene Knockout Consortia, led by Shohei Mitani at Tokyo Women's Medical University School of Medicine and by Don Moerman at the University of British Columbia for knockout alleles, H. Hutter, N. Tavernarakis and T. Jessell for strains and reagents, several members of Columbia University community for generously sharing their equipment, Hobert lab members, especially Maria Doitsidou, for helpful discussion and A. Abeliovich, R.J.Johnston and I. Greenwald for comments on the manuscript. This work was funded by the National Institutes of Health (R01NS039996−05; R01NS050266−03), the Howard Hughes Medical Institute and a EMBO long term fellowship and Marie Curie Outgoing International fellowship to N.F.

METHODS

Strains and transgenes

For the ETS family analysis the following mutants were crossed into *vtIs1* (*dat-1::gfp*) strain to check for a DA phenotype: *tag-97*(*ok284*); C50A2.4(*tm440*); C52B9.2(*tm413*); *ets-4*(*ok165*); C24A1.2(*tm801*); *ets-5*(*tm866, tm1734*); *lin-1*(*e1777*) *ast-1*(*hd1, rh300, hd92, gk463*). F19F10.1(*tm456*); F19F10.5(*tm436*) were crossed into *otIs181*. For further *ast-1* analysis the following strains were crossed into *ast-1*(*gk463* or *hd92*): *nIs118* (*cat-2::gfp*), *zdIs13* (*tph-1::gfp*), *oyIs59* (*osm-6::gfp*), AX43 [*lin15*(*n765*);Ex(*gcy-36::gfp*], TQ886 [*trp-4::YFP+pha-1*(+);*pha-1*(ts);*him-5*]. The *cat-1*^{prom1}*::mCherry*, *bas-1* ^{prom1}*::mCherry*, *cat-4* ^{prom1}*::mCherry* and *asic-1::gfp* reporters were injected into the *ast-1* balanced strain *ast-1*(*gk463*) *bli-2*(*e768*) *unc-4*(*e120*)/*mIn1*[*mIs14 dpy-10*(*e128*)] II at 50ng/µl. AST-1 expression was analyzed injecting *ast-1::yfp* 4 at 50 ng/µl into *otIs181*.

In regard to our reporter gene analysis we note that we have previously reported that, in some cases, PCR products are expressed in more cells than the equivalent cloned product 21. Some of the reporter gene constructs that gave no expression in the DA cells were injected both as subcloned plasmids or as PCR products (using vector primers). No difference in expression was found in any of them except for the single copy 31bp EBS motif from *dat-1* (Fig. 3A) that gave no expression injected as a clone product and PDE expression when injected as PCR fragment.

Heat shock experiments

Strain OH7546 *[otIs198*(*hsp16−2::ast-1;hsp16−2::NLS-mCherry*;*ttx-3::ds-red*),*vtIs1(dat-1::gfp;rol6)]* was used for the heat shock experiments. For the rescue experiment shown in Fig.2f (postembryonic heat shock), first larval stage worms were heat shocked at 37°C three times for 30 minutes with one hour incubation at 20°C between each heat shock to let worms recover. After heat shocks worms were kept at 15°C and scored at the indicated times.

For the ectopic DA fate induction experiments (Fig.3), two cell embryos were released from hermaphrodite mothers by sectioning them in half, mounted in slides, incubated at 20°C, heat shocked at 37°C for 20 minutes at different stages of development and analyzed the following morning (approximately 24 hours after first cleavage).

hsp16−2 promoter activity was monitored by *mCherry* expression from *hsp16−2::NLS-mCherry* construct present on the same array. Even though red fluorescence was first detected two hours after the heat shock, immunodetection of protein production from the same promoter has been reported as early as 30 minutes after heat shock 22.

Histology

P0 mice were anesthetized in ice and transcardially perfused with 4% paraformaldehyde (PFA). P10 mice were anesthetized with an overdose of ketamine HCL (Ketaset) and xylazine HCL (AmTech) and transcardially perfused with 4% PFA. Brains were removed,

postfixed for 2 h at 4°C, cryoprotected in 30% sucrose in PBS, embedded in Tissue-Tek® OCT compound and stored frozen at −80°C. 20 μm coronal sections were cut on a cryostat.

The following antibodies were used: sheep anti-Tyrosine hydroxylase (1/1000, Peel-Freeze), rabbit anti-Er81 (1/32.000, provided by Tom Jessell), rabbit anti-calbindin (1/5000, Swant), rabbit anti-Calbindin (1/2000, Chemicon), rabbit anti-caspase3 cleaved (1/200, Cell Signaling), mouse anti-ßtubulin (TUJ1) (1/500 Covance), rabbit anti-phospho-histone H3 (1/500 Upstate).

Etv1 mutant analysis was performed using a confocal microscope (Leica TCSSP5). For P0 tyrosine hydroxylase, P10 tyrosine hydroxylase, P10 calretinin and P10 calbindin olfactory bulb quantification eight different fields corresponding to two different levels were scored in each animal. For P0 cleaved caspase 3 and phosphohistone 3 quantification 2 whole sections of olfactory bulb section were scored for each animal. For cell density quantification DAPI nuclei were scored in a field of the glomerular layer for each animal. Olfactory bulb volume was estimated by the Cavalieri method 23. Briefly, the whole extent of the olfactory bulb was cut in 20 μm coronal sections, one every twenty sections were analyzed using a grid of $0.04mm^2$. Data were statistically analyzed using ANOVA tests.

In situ hybridization was performed as described before 11. CD-1 mouse embryos were analyzed using the following probes: *dlx2* (probe kindly provided by Dr. Rubenstein); Ehf (image clone 5008262 and 3991773); *Elf3* (MTF387 from Gray et al. library 24); *Elf5* (image clone 5252409); *Elk1* (image clone 423774); *Elk3* (image clone 3471706); *Elk4* (image clone 3589378); *Etv1/Er81* (probe kindly provided by Dr. Tom Jessell); *Erg* (image clone 7105647 and 7442975); Ets1 (image clone 5720204); Ets2 (image clone 3385111 and 3511332); *Etv3* (image clone 5290278); *Etv4* (antibody kindly probided by Dr. Tom Jessell); *Etv5* (image clone 3673190 and 3674281); Etv6 (probe kindly provided by Dr. Stuart H.Orkin); *Fli1* (image clone 717781 and 808955); *ngn2* (probe kindly provided by Dr. Guillemot), *spedf* (image clone 6529808 and 5003573); *Spib* (probe kindly provided by Dr. M.Celeste Simon); *Spi-c* (MTF416 from Gray et al. library 24).

Olfactory bulb primary cultures

Olfactory bulbs were dissected from P0-P1 mice, dissociated and electroporated using the Amaxa Nucleofector System and the mouse neuron kit, following the manufacturer's protocol. Approximately 3×10^6 cells were electroporated in each reaction with 0.7 μg of pmaxGFP and 2μg of PCDNA3.1 and 0.7 μg of pmaxGFP and 2μg of Etv1/PCDNA3.1, respectively. Cells were inmediately plated in 8 well glass chamber slides and cultured for 4 days as described before 25. Cells were then fixed with 4%PFA and immuno-stained against with GFP and TH antisera. Two independent sets of experiments were performed and in each, 2 wells were scored for the GFP expressing cells and two for the GFP+Etv1 expressing cells. In un-transfected cultures, the percentage of TH positive cells was 1/3000 plated cells. For each well we scored the total number of TH positive cells and the number of co-labeled TH/GFP positive cells (ranking from 100 to 200 TH cells and 20 to 50 TH/GFP cells per well). The percentage of TH transfected cells versus TH not transfected cells was calculated.

Luciferase assays

COS cells were plated in M6 plates and incubated overnight, at approximately 70% confluence cells were transfected with lipofectamine (Invitrogen). Each well was transfected with 2.7 μg of total DNA (200ng of ß-galactosidase, 500ng of PGL3 luciferase, 2 μg of Etv1/PCDNA3.1(−) or PCDNA3.1(−). 24 hours after transfection luciferase and ß-galactosidase activity was measured using Luciferase Assay kit (Stratagene), ß-galactosidase Enzyme Assay System (Promega) and 20/20^{n} Luminometer (Turner BioSystems) and Biorad Model 680 Microplate reader. For each experiment each value represents the mean luciferase activity in three different wells and each construct was analyzed in three independent experiments. Statistical analysis was performed applying the t-Test.

DA motif mutagenesis were performed using the Quickchange II XL Site-Directed Mutagenesis Kit (Stratagene). In both cases the core GGAT of the DA motif was mutated to AGAT.

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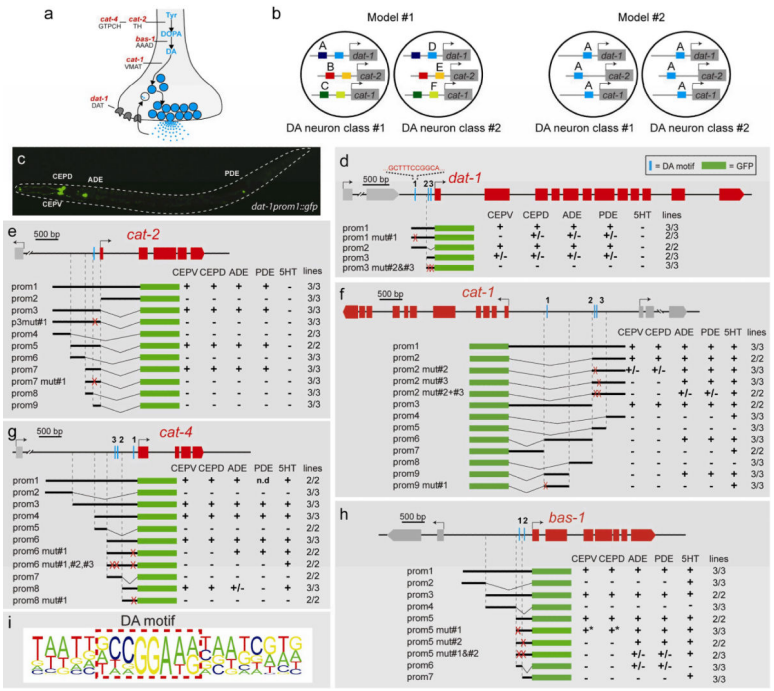

Fig. 1. Characterization of the DA motif in *C. elegans*

(a) Schematic representation of a DA neuron synapse. AAAD: aromatic L-amino acid decarboxylase; *bas-1*: *Biogenic Amine Synthesis related* 1; *cat: abnormal CATecholamine distribution;* DA: dopamine*; dat: DopAmine Transporter;* GTPCH: GTP cyclohydrolase; TH: Tyrosine hydroxylase; Tyr: tyrosine; VMAT: vesicular monoamine transporter.

(b) Schematic representation of two different models for DA terminal differentiation. See text for explanations.

(c) Picture of an adult worm expressing GFP under the control of the full length *dat-1/*DAT promoter, labeling all *C. elegans* DA neurons. Similarly, *cat-2/*TH is also exclusively expressed in DA neurons (not shown), as *C.elegans* contains no adrenergic or noradrenergic neurons.

(d) *dat-1/*DAT promoter analysis. Schematic representation of the *dat-1/*DAT locus with its upstream region. Exons are represented as red blocks, the upstream gene is shown in grey. Below: representation of cloned and injected constructs, and expression pattern in the DA and serotonergic (5-HT) neurons. Thick black lines symbolize the promoter piece placed in front of GFP (green box). Red cross represents a mutated EBS. “+” indicates >10% penetrant expression in more than half of the transgenic lines examined; “+/−“ also means >10% penetrant expression, but the penetrance is lower than in the corresponding full-length construct, “-” indicates <10% penetrant expression in more than half of the transgenic lines examined, “n.d” means not determined.

(e-h) Analysis of the regulatory regions of all other dopamine pathway genes. “+*” means dimmer gfp expression than corresponding wild type construct. See Fig. S1 and S3 for all primary data and nature of the mutations.

(i) The sequence alignment of all functional EBSs defines a position weight matrix (PWM) that is represented by a sequence logo. The conserved core in all sequences constitutes the DA motif. See Fig. S5 for sequences used to define the DA motif.

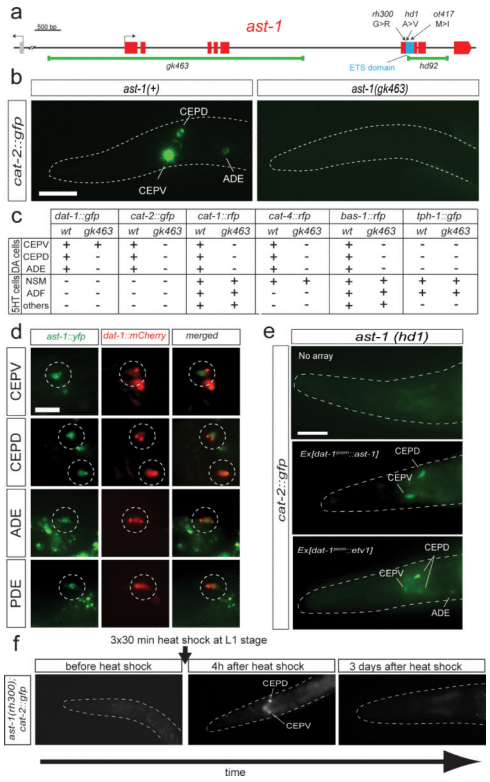

Fig. 2. *ast-1* is required to induce and maintain DA neuron differentiation

(a) Schematic representation of *ast-1* locus and the mutants available for this gene.

(b) Representative example of loss of DA fate marker in *ast-1* mutants. See Fig. S7 and S8 for other examples and Table S1 for quantification of data.

(c) Summary of *ast-1* null mutant phenotype. +: fate marker expressed; -: fate marker not expressed. Due to early larval lethality, only the embryonically generated DA head neurons, but not the postembryonically generated PDE neurons could be scored for developmental defects in *ast-1* null mutants. Markers that were expressed in both DA and 5-HT neurons were assayed with an *rfp* reporter in a transgenic background in which 5-HT neurons were labeled with *gfp (Is[tph-1::gfp*) so as to allow for loss of expression specifically in the DA neurons. See Fig. S7 for primary data.

(d) Expression of an *ast-1::yfp* reporter gene 4 in DA neurons. DA neurons are labeled with *dat-1::mCherry*. Scale bar: 10 μm.

(e) Rescue of the *ast-1* mutant phenotype. We used a hypomorphic allele, *hd-1*, in which *dat-1* expression is unaffected (Table S1), to drive *ast-1* or a mouse homolog, *etv-1*, under control of the DA-specific *dat-1* promoter and assayed expression of *cat-2::gfp* (*otIs199)*.

(f) Developmentally staged *ast-1(rh300)* animals, containing the heat shock-inducible *ast-1* array *otIs198* and the DA fate marker *cat-2::gfp (otIs199)*, were grown under non-inducible condition to the first larval stage (resulting in an absence of *cat-2::gfp* expression in 100% of animals); *ast-1* was then induced by heat shock at the L1 stage and animals scored 4 hours and 3 days after heat shock. Of the 40 animals found to turn on expression of *cat-2::gfp* 4 hours after heat shock, all lost expression after 3 days. Data with a temperature-sensitive allele of *ast-1* corroborate sustained *ast-1* activity (Supp.Fig.10).

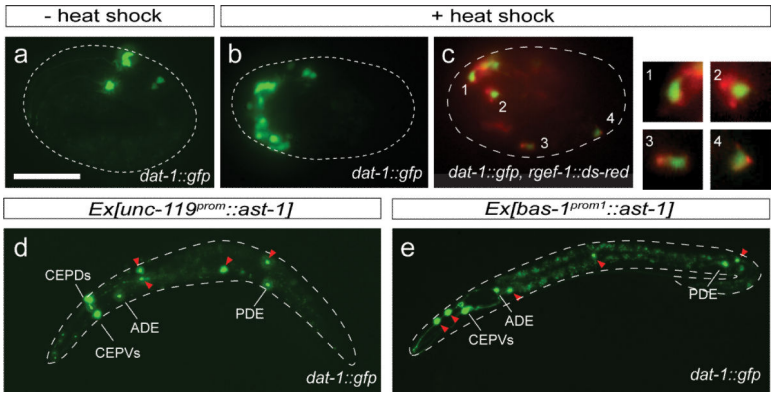

Fig. 3. Ectopic *ast-1* expression can induce DA cell fate

(a) Representative picture of a control embryo after the 3-fold stage. *dat-1::gfp* expression starts at late three fold stage and can be detected in the six embryonically generated DA neurons.

(b,c) Representative picture of an embryo heat shocked four hours after the two cells stage and analyzed ten hours after the heat shock. *dat-1::gfp* is ectopically expressed in many cells of the embryo. Scale bar: 20 μm. In the presence of a pan-neuronal marker (*rgef-1::rfp)* (panel c), the ectopic DA-fate expressing cells can be identified as neurons.

(d,e) Ectopic expression of *ast-1* under the control of the ectodermal promoter *unc-119* and the DA /5HT-neuron specific promoter *bas-1* leads to ectopic expression of *dat-1::gfp* in additional neurons compared to wild-type worms (red arrowheads). Similar effects were observed in multiple lines (2/2 lines for *bas-1* driver; 2/3 lines for *unc-119* driver). Scale bar: 100 μm.

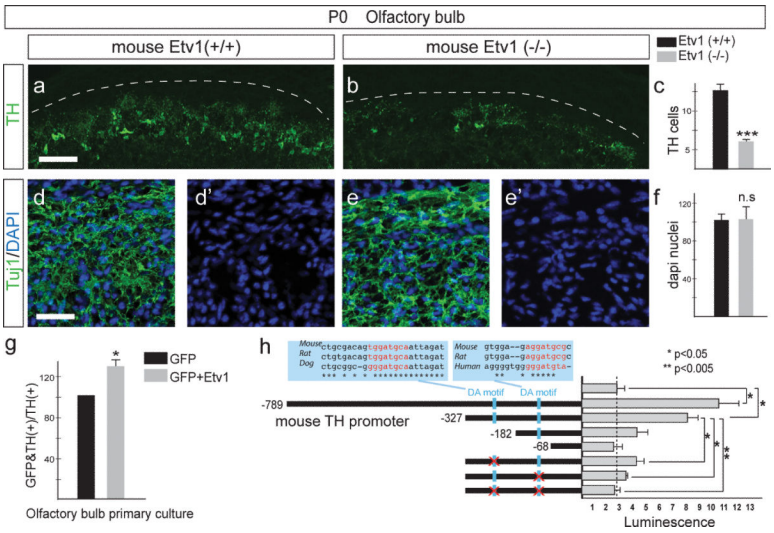

Fig. 4. Mouse Etv1/Er81 is necessary for the olfactory bulb dopaminergic neuron specification

(a,b) Coronal section TH immunostaining of a wild type (A) and Etv1 mutant (B) newborn pup (P0) olfactory bulb. Scale bar: 150 μm.

(c) Quantification of TH positive cells in wild type and Etv1 mutants at P0. Etv1 mutants show a significant reduction of the TH positive cells already at this stage (n=3, p-value=0.00009).

(d, e) Coronal section tuj1 immunostaining and DAPI staining of a wild type (d, d') and Etv1 mutant (e, e') P0 glomerular layer to label neurons and cell nuclei. Scale bar: 40 μm.

(f) Quantification of DAPI nuclei in wild type and Etv1 mutants at P0. Glomerular layer cell density is similar between wild type and Etv1 mutants (n=3, p-value=0.93).

(g) Overexpression of Etv1 can induce DA differentiation. Dissociated P0 olfactory bulbs were transfected with GFP and PCDNA3.1 (control) or GFP and Etv1 cloned into the PCDNA expression vector, plated and cultured for 4 days. Etv1 overexpression leads to increased number of tyrosine hydroxylase positive cells.

(h) Analysis of the activation of TH promoter by Etv1 in COS cells. The dotted line indicates the level of luciferase activation of the empty luciferase vector observed upon Etv1 transfection. "DA motif" = phylogenetically conserved match to the a VGGAWRNV consensus. n=3 independent experiments for each construct.