Regulatory cocktail for dopaminergic neurons in a protovertebrate identified by whole-embryo single-cell transcriptomics

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The CNS of the protovertebrate *Ciona intestinalis* contains a single cluster of dopaminergic (DA) neurons, the coronet cells, which have been likened to the hypothalamus of vertebrates. Whole-embryo single-cell RNA sequencing (RNA-seq) assays identified *Ptf1a* as the most strongly expressed cell-specific transcription factor (TF) in DA/coronet cells. Knockdown of *Ptf1a* activity results in their loss, while misexpression results in the appearance of supernumerary DA/coronet cells. Photoreceptor cells and ependymal cells are the most susceptible to transformation, and both cell types express high levels of *Meis*. Coexpression of both *Ptf1a* and *Meis* caused the wholesale transformation of the entire CNS into DA/coronet cells. We therefore suggest that the reiterative use of functional manipulations and single-cell RNA-seq assays is an effective means for the identification of regulatory cocktails underlying the specification of specific cell identities.

Dopaminergic (DA) neurons mediate a variety of "reward" behaviors in vertebrates, such as feeding, mating, and response to external stimuli (Iversen and Iversen 2007). There are several classes of DA neurons in the vertebrate CNS, including those mediating motor functions in the midbrain and others controlling secretion of neuropeptides in the hypothalamus (Flames and Hobert 2011; Grattan 2015). There is considerable information about the specification of midbrain DA neurons due to their importance in Parkinson's disease (Flames and Hobert 2011; Kee et al. 2017; Kirkeby et al. 2017; Parmar 2018). Characterization of the molecular programs controlling differentiation of DA neurons is crucial for understanding this important neuronal cell type and developing stem cell-based therapies for DA deficiencies such as Parkinson's disease (Parmar 2018).

Previous studies identified the *Ets* transcription factor (TF) *AST-1* as a key determinant of DA neurons in the nematode worm *Caenorhabditis elegans* (Flames and Hobert 2009; Doitsidou et al. 2013). This mechanism is conserved in olfactory bulb DA neurons in vertebrates (Flames and Hobert 2009; Doitsidou et al. 2013). However, vertebrates possess additional classes of DA neurons, and additional studies are required to identity corresponding mechanisms. Several TFs have been implicated in the differentiation of midbrain DA neurons in vertebrates, including *Nurr*, *Lmx*, and *Pitx3* (Flames and Hobert 2011). Considerably less is known about other classes of DA neurons, such as those associated with the hypothalamus. For this purpose, we sought to identify selector genes for DA neurons in the protovertebrate *Ciona intestinalis*.

The CNS of *C. intestinalis* consists of 177 neurons that share a number of similarities with the vertebrate CNS (Ryan et al. 2016). The tadpole contains a single cluster of DA neurons, the coronet cells, which are located in ventral regions of the sensory vesicle in close proximity to group III photoreceptor cells (Fig. 1A; Moret et al. 2005a, b; Horie et al. 2008; Razy-Krajka et al. 2012; Ryan et al. 2016; Sharma et al. 2018). This region of the ascidian CNS shares a number of similarities with the hypothalamus (Moret et al. 2005a,b; Razy-Krajka et al. 2012) and is evocative of the deep brain light sensory system of lower fish (Nakane et al. 2013). Here we used whole-embryo single-cell RNA sequencing (RNA-seq) assays to elucidate the regulatory networks underlying the specification of DA neurons/coronet cells in the *Ciona* tadpole.

Results and Discussion

Mid-tail bud embryos (~1500 cells) were dissociated and barcoded using the 10x Genomics Chromium system (Fig. 1B). RNA was extracted from individual cells and reverse-transcribed, and the resulting cDNAs were sequenced. A total of ~5000 cells was sequenced to ensure effective coverage of the entire embryo. The single-cell transcriptome profiles identified all of the major tissues,

A

B

*Ciona intestinalis
mid tailbud stage*

Dissociate cells

Single cell sequencing
(10XGenomics Chromium)
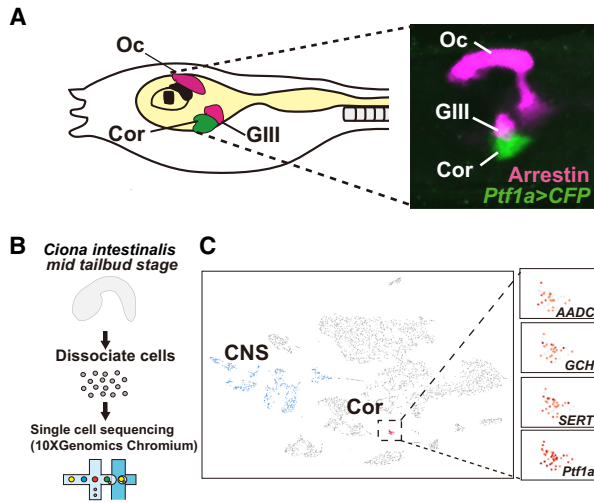
C

CNS

Cor

AADC

GCH

SERT

Ptf1a

Figure 1. Whole-embryo single-cell RNA-seq analysis of coronet cells. (*A, left*) Diagram of a *Ciona* tadpole showing the position of coronet cells (DA neurons [green]) and photoreceptor cells, including the ocellus and group III cells (magenta). (*Right*) Coronet cells visualized by a *Ptf1a>CFP* reporter gene (green) containing 5′ flanking regulatory sequences from *Ptf1a* and photoreceptor cells (magenta) visualized by immunostaining with an Arrestin antibody. (Oc) Ocellus; (Cor) coronet cells; (GIII) group III photoreceptor cells. (*B*) Schematic illustrating the workflow for single-cell RNA-seq analysis of *Ciona* embryos using the 10x Genomics Chromium system. (*C*) A t-distributed stochastic neighbor embedding (tSNE) projection map of mid-tail bud stage embryos highlighting the distribution of DA neuron marker genes (*AADC*, *GCH*, and *SERT*) and *Ptf1a*. Each dot corresponds to the transcriptome of a single cell. Red dots indicate DA neurons/coronet cells clusters, blue dots indicate CNS, and gray dots indicate other tissues.

including notochord, endoderm, mesenchyme, tail muscles, and CNS (Fig. 1C; Horie et al. 2018). DA neurons/coronet cells were identified on the basis of their expression of a variety of dopaminergic markers genes, including those encoding the dopamine biosynthetic pathway such as *TH*, *GCH*, and *AADC* (Fig. 1C; Supplemental Figs. S1, S2; Supplemental Table 1). These cells form a discrete cluster that is distinct from all other neuronal cell types in the CNS (Fig. 1C, red dots).

The 10x analysis also identified a number of transcripts in DA neurons/coronet cells that encode secreted neuropeptides (Supplemental Fig. S2; Hamada et al. 2011; Kawada et al. 2011). This observation supports and extends previous proposals that coronet cells are a component of an ancient protohypothalamic–retinal territory (Fig. 1A; Razy-Krajka et al. 2012). In vertebrates, there are separate DA neurons and neurosecretory neurons (Grattan 2015). In contrast, both activities are contained within individual coronet cells, raising the possibility that cellular subfunctionalization contributed to the evolution of the hypothalamus (Arendt 2008).

The transcriptome profiles of DA neurons/coronet cells identified a number of regulatory genes, including *Ptf1a*, a basic helix–loop–helix (bHLH) gene implicated in the development of the pancreas and GABAergic/glutamatergic neurons in the cerebellum of vertebrates (Hoshino et al. 2005; Fujitani et al. 2006; Dullin et al. 2007; Nakhai et al. 2007). It is specifically expressed in DA neurons/coronet cells and absent in all other major neuronal cell types in mid-tail bud embryos (Fig. 1C; Supplemental Figs. S1, S2; Razy-Krajka et al. 2012). To determine the

role of *Ptf1a* in the specification of DA neurons/coronet cells, we inhibited gene activity by injecting a *Ptf1a* morpholino (MO) that targets the 5′ untranslated region (Supplemental Fig. S3). The resulting morphants appear normal, although DA marker gene expression is lost in ventral regions of the sensory vesicle (Fig. 2B, cf, with A; Supplemental Fig. S4). There is a corresponding expansion in the expression of *arrestin*, raising the possibility that DA neurons/coronet cells are transformed into photoreceptor cells in *Ptf1a* morphants (Supplemental Fig. S5). These results suggest that *Ptf1a* is important for the development of DA neurons/coronet cells in the *Ciona* CNS.

To determine whether *Ptf1a* functions as a "master control gene" for DA neuron/coronet cell identity, we misexpressed it throughout the nervous system (CNS and peripheral nervous system [PNS]) using 5′ regulatory sequences from the *β2tubulin* gene. Injection of the *β2tubulin>Ptf1a* transgene resulted in expanded expression of the *TH>Kaede* marker gene throughout the sensory vesicle and portions of the nerve cord (Fig. 2C, cf. with A). This result strengthens the evidence that *Ptf1a* functions as a determinant of DA neurons/coronet cells. It would appear that misexpression of *Ptf1a* is sufficient to transform some regions of the CNS into DA neurons/coronet cells but not others.

To explore the nature of the transformations, we performed single-cell RNA-seq assays with transgenic embryos expressing the *β2tubulin>Ptf1a* transgene along with a *β2tubulin>CFP* reporter gene that identifies all of the cells expressing *Ptf1a* (Fig. 2D; Supplemental Fig. S6). Most tissues do not show any changes in gene activity (e.g., notochord, endoderm, and tail muscle) (Supplemental Fig. S6). In contrast, the CNS and PNS display strong expression of *Ptf1a* (Fig. 2D, top panel, Supplemental Fig. S7A). *Ptf1a*-expressing cells were clustered based on their transcriptome profiles (Fig. 2D, bottom panel; Supplemental Fig. S7A; Satija et al. 2015; Butler et al. 2018). Cluster 1 displays the most complete transformation, since it expresses nearly the complete suite of DA neurons/coronet identity genes (Fig. 2E, heat map; Supplemental Figs. S6, S7B). It is possible that these cells derive from posterior regions of the sensory vesicle and nerve cord (Fig. 2C). In contrast, clusters 2–4 display progressively fewer DA neurons/coronet marker genes and continue to express a variety of marker genes reflecting their origins from anterior regions of the sensory vesicle and PNS.

Misexpression of *Ptf1a* suppresses the development of photoreceptor cells (Horie et al. 2008) and ependymal cells (Supplemental Fig. S5; Horie et al. 2011) but does not significantly alter glutamatergic, GABAergic/glycinergic, and cholinergic neurons (Supplemental Fig. S8). The loss of photoreceptor cells and ependymal cells suggests that they are transformed into supernumerary DA neurons/coronet cells upon misexpression of *Ptf1a*. It seems likely that *Ptf1a* works with additional sequence-specific TFs to specify DA neurons/coronet cells. These factors may be present in photoreceptor cells and ependymal cells but absent or only weakly expressed in other neurons. We found that the homeobox gene *Meis* fulfills these criteria (Fig. 2E; Supplemental Fig. S9). Previous studies have shown that *Meis* functions cooperatively with a number of Hox TFs (Moens and Selleri 2006; Agoston et al. 2014). To determine whether it might also work in concert with *Ptf1a* to specify DA neurons/coronet cells, we simultaneously misexpressed both genes (Fig. 3).
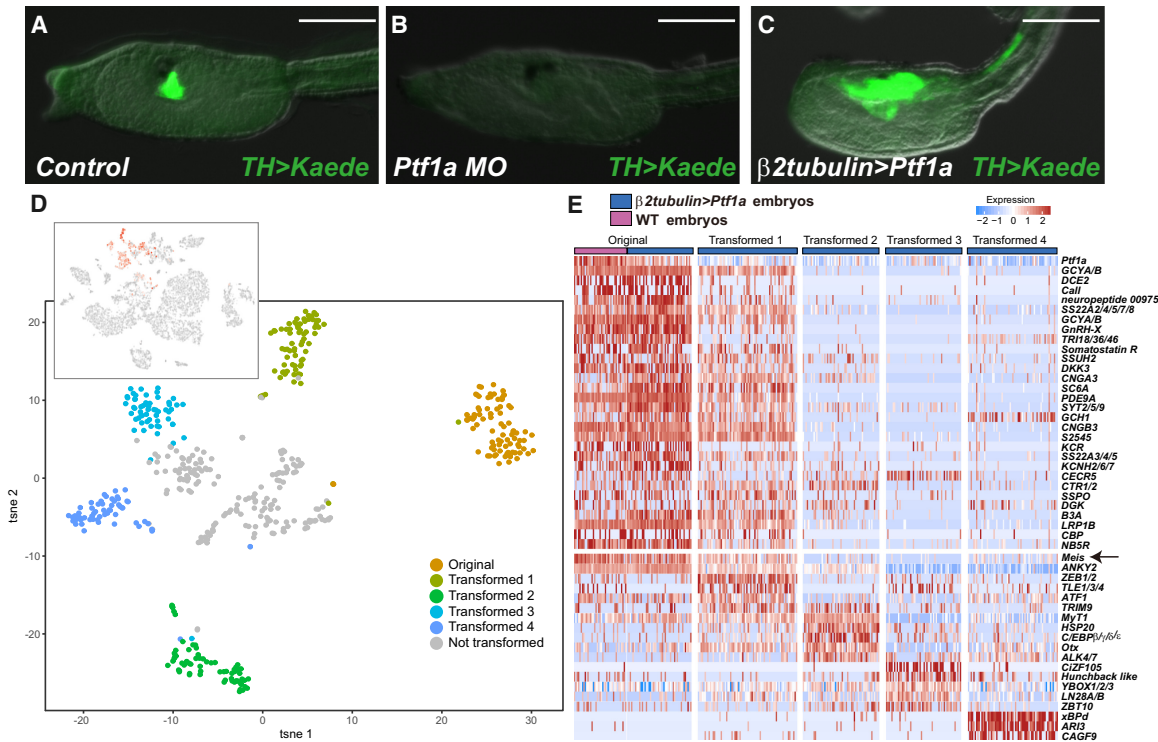
Figure 2. *Ptf1a* is required for the differentiation of DA/coronet cells. (*A–C*) Head regions of *TH>Kaede* transgenic larvae. (*A*) *Kaede* expression in the coronet cells of control larvae (51 of 103 larvae displayed this expression pattern). (Note that the transgenic line is a heterozygote for the *TH>Kaede* transgene.) (*B*) Same as *A* except that it was injected with a *Ptf1a* MO (111 of 121 larvae displayed this expression pattern) (see Supplemental Fig. S3 for more details). (*C*) Same as *A* except that *Ptf1a* was misexpressed throughout the CNS by *β2tubulin* 5′ regulatory sequences (29 of 60 larvae displayed this expression pattern). Bar, 100 µm. (*D, top*) The tSNE projection map of mid-tail bud embryos expressing the *β2tubulin>Ptf1a* transgene. Red dots identify cells expressing a *β2tubulin>CFP* reporter gene. (*Bottom*) tSNE "subclustering" of cells expressing *Ptf1a* and the *CFP* marker gene. (*E*) Heat map of native DA neurons/coronet cells and four different groups of transformed cells (shown in *D*) showing the relative expression of a select group of genes encoding cellular effectors and TFs. Gene expression profiles of transformed cells in cluster 1 are similar to those of native DA neurons/coronet cells. The arrow identifies *Meis*, which is expressed in native DA neurons/coronet cells and transformed cluster 1 but not the partially transformed cells in clusters 2, 3, or 4.

β2tubulin>Ptf1a and *β2tubulin>Meis* transgenes were coinjected in unfertilized eggs and grown to the late tail bud I (LTB I) stage. The resulting embryos exhibit a dramatic transformation of the entire CNS into DA neurons/coronet cells (Fig. 3C, cf. A and B). To determine the nature of this transformation, we performed single-cell RNA-seq assays on transformed embryos (Fig. 3D,E; Supplemental Figs. S10, S11). Most of the transformed cells express the complete suite of DA neuron/coronet cell marker genes. They lack expression of marker genes identifying their developmental origins and original neuronal identities, such as *VACHT/ChAT* (cholinergic neurons) and *GAD* (GABAergic neurons) (Fig. 3E; Supplemental Fig. S12). In contrast, the other major site of expression mediated by *β2tubulin* regulatory sequences—epidermal sensory neurons—displays little or no transformation toward a DA neuron/coronet cell identity.

It is likely that *Ptf1a* and *Meis* work directly to regulate target genes that are specifically expressed in DA neurons/coronet cells. The 5′ flanking regions of many such genes contain tightly linked *Ptf1a*-binding (E box) and *Meis*-binding sites (Agoston et al. 2014), and DNA fragments containing these motifs mediate restricted expression in DA neurons/coronet cells when attached to reporter genes and expressed in transgenic embryos (Fig. 4; Supplemental Figs. S13–S15). Given the parallels between DA neurons/

coronet cells and the hypothalamus, it seems reasonable to suggest that the regulatory "cocktail" of *Ptf1a* and *Meis* might also control the development of DA neuronal cell types in vertebrates. Indeed, *Ptf1a* has been suggested to play a role in the specification of DA neurons in the hypothalamus in mice (Fujiyama et al. 2018), while *Meis* TFs have been implicated in the specification of DA neurons in the olfactory bulb (Agoston et al. 2014) and hypothalamus (Hook et al. 2018) in mice.

Single-cell technologies are pervasively used to provide descriptive cell atlases of gene expression (Karaiskos et al. 2017; Briggs et al. 2018; Farrell et al. 2018; Fincher et al. 2018; Plass et al. 2018; Wagner et al. 2018). Here, we attempted to extend the promise of these technologies by combining them with classical approaches in experimental embryology. We used whole-embryo single-cell transcriptome assays to identify the determinants of DA neurons/coronet cells in the *Ciona* larval CNS. The identification of *Meis* as a critical cofactor of *Ptf1a* in the specification of DA neurons/coronet cells depended on single-cell analysis. It is broadly distributed in the CNS, PNS, and other tissues but preferentially expressed in those regions of the CNS most susceptible to transformation by *Ptf1a* (e.g., photoreceptor cells and ependymal cells). Previous studies have focused on individual determinants, although these are not always sufficient to
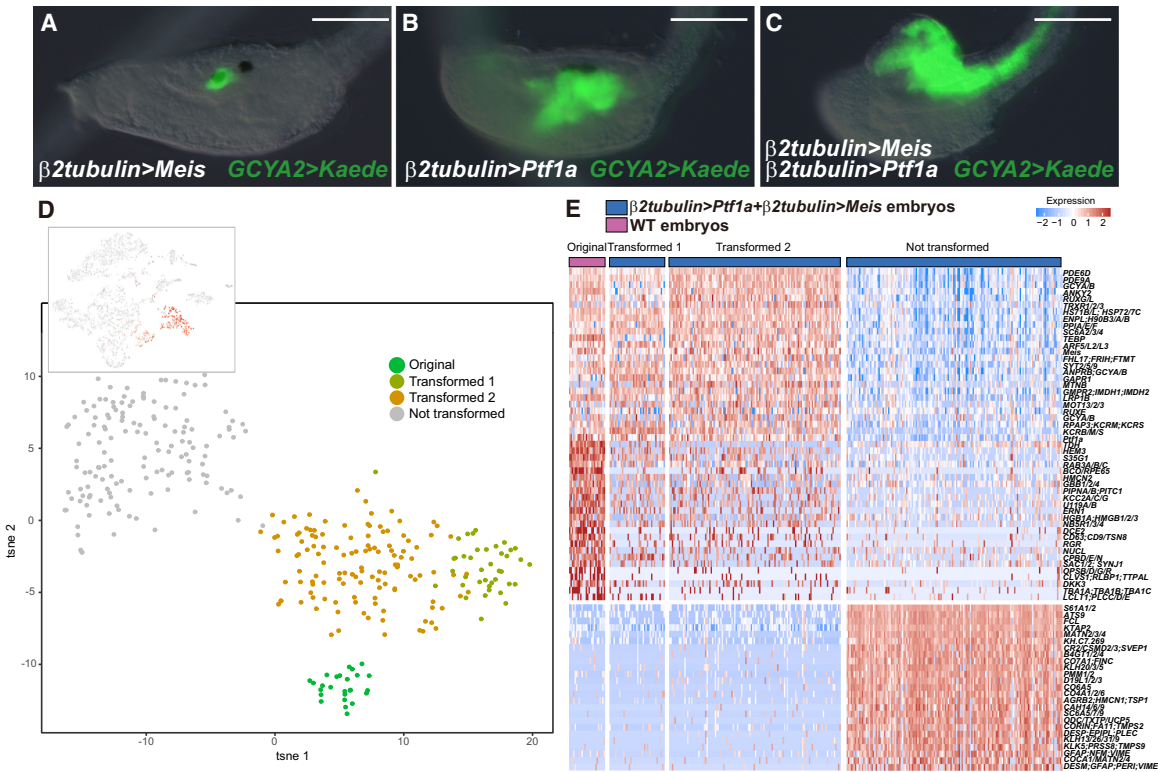
Figure 3. A *Ptf1a* + *Meis* cocktail promotes the differentiation of DA neurons/coronet cells. (*A–C*) Head regions of larvae that were injected with a *GCYA2>Kaede* reporter gene that is specifically expressed in DA neurons/coronet cells (see Supplemental Fig. S2). (*A*) The embryo was coinjected with a *β2tubulin>Meis* transgene. Misexpression of *Meis* does not alter the normal expression of the reporter gene within DA neurons/coronet cells (100 of 100 larvae displayed this expression pattern). (*B*) The embryo was coinjected with the *β2tubulin>Ptf1a* transgene. *Kaede* expression is expanded into posterior regions of the sensory vesicle and anterior neural tube (104 of 104 larvae displayed this expression pattern) (see Fig. 2C). (*C*) The embryo was coinjected with both *β2tubulin>Ptf1a* and *β2tubulin>Meis* transgenes. The *GCYA2* reporter gene is now expressed throughout the entire CNS (89 of 89 larvae displayed this expression pattern). Bar, 100 μm. (*D, top*) A tSNE projection map of late tail bud stage embryos expressing both the *Ptf1a* and *Meis* transgenes. Red dots correspond to cells expressing the *β2tubulin>CFP* reporter gene, which identifies cells that misexpress *Ptf1a* and *Meis*. (*Bottom*) tSNE subclustering of coexpressing cells. (*E*) Heat map of native DA neurons/coronet cells, transformed cluster 1, transformed cluster 2, and untransformed cells. A select group of genes encoding cellular effectors and TFs is shown. Clusters 1 and 2 display transcriptome profiles that are very similar to those seen for native coronet cells. The untransformed cells are likely to correspond to epidermis based on their transcriptome profiles.

specify specific cell types (Flames and Hobert 2009; Doitsidou et al. 2013). The reiterative use of experimental manipulations (e.g., misexpression of *Ptf1a*) and single-cell analysis provides a potent one–two punch in the identification of the complex gene networks underlying development.

Materials and methods

Biological materials

Wild-type *C. intestinalis* type A (also called *Ciona robusta*) adults were obtained from M-Rep and the National Bio-Resource Project for *Ciona* in Japan. Sperm and eggs were collected by dissecting the sperm and gonadal ducts. Transgenic lines were cultured and maintained in an island system.

Isolation and characterization of Ci-Ptf1a cDNA

A partial cDNA fragment of *Ci-Ptf1a* was found in the Ghost database (http://ghost.zool.kyoto-u.ac.jp/cgi-bin/gb2/gbrowse/kh). To obtain the full-length coding sequence, we performed 5′ RACE using Generacer kit (Invitrogen). The nucleotide sequences of oligonucleotide primers used for 5′ RACE were 5′-CACCACCCCTTCTTCGGTAAATTGGAAG-3′

(for the primary PCR) and 5′-TCGGGAGGCTAGTACCTCACGAAG CAACG-3′ (for the nested PCR). The cDNA fragments were cloned into a pGemT vector (Promega). The cDNA clone was sequenced on both strands with automatic DNA sequencer (Applied Biosystems).

Whole-mount in situ hybridization

A cDNA clone of *Ci-Ptf1a* that contained the full ORF was used as the template to synthesize a digoxigenin-labeled antisense RNA probe using a DIG-RNA labeling kit (Roche). In situ hybridization of the whole-mount specimens was carried out as described previously (Kusakabe et al. 2002).

Constructs

Reporter genes were designed using previously published enhancer sequences *TH*, *AADC*, *GCH*, *SERT*, and *Ptf1a* (Razy-Krajka et al. 2012). To generate pSPCiPtf1a ΔMO target sequence C, 5′ upstream regions of *Ci-Ptf1a* were amplified by PCR using a thermostable DNA polymerase (PrimeSTAR HS DNA polymerase, Takara) and oligonucleotide primers (Supplemental Table 2). The PCR products were digested with BamHI and inserted into the BamHI pSPeCFP. To generate pSPCiGCYA2K, pSPCiGCYBK, pSPCiGnRHXK, pSPCiNtlBK, pSPCiPDE9aK, pSPPDEdK, pSPLectinK, and pSPSS23A3K, 5′ upstream regions of *Ci-GCYA2* *Ci-GCYB*, *Ci-GnRHX*, *Ci-NtlB*, *Ci-PDE9a*, *Ci-PDEd*, *Ci-Lectin*, and *Ci-SS23A3* were amplified by PCR using a thermostable DNA polymerase
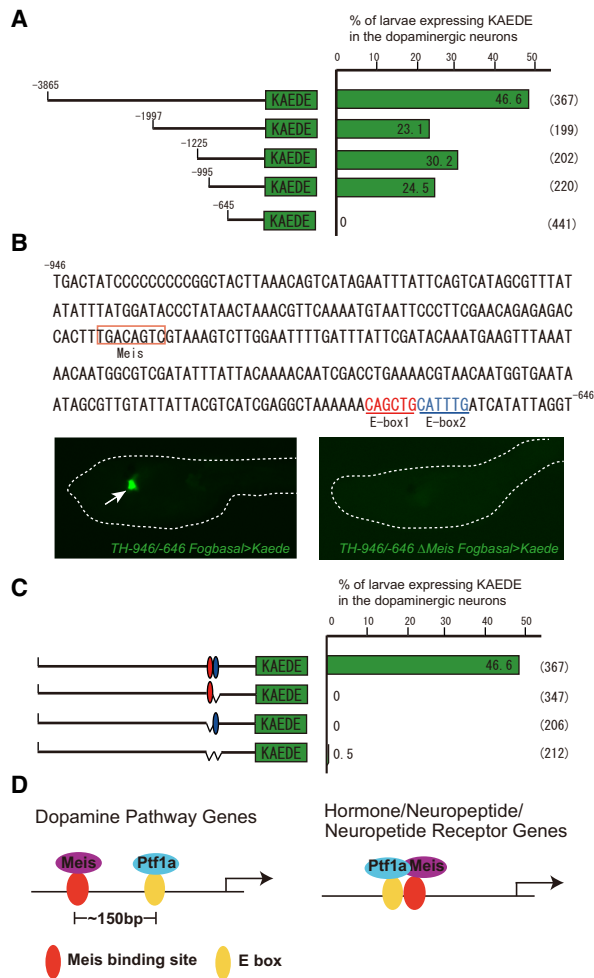
Figure 4. Gene network for the specification of DA neurons/coronet cells. (*A*) Deletion analyses of the *cis* regulatory region of *Ci-TH*. An ~350-base-pair (bp) DNA fragment (−995 to −646 bp upstream of the translation start site) from the 5′ flanking region of *Ci-TH* is sufficient to mediate Kaede expression in DA neurons/coronet cells. (*B*) This 5′ regulatory DNA contains two E-box sequences (E-box1 and E-box2) and one *Meis*-binding site. Deletion in the *Meis*-binding site eliminates expression in DA neurons/coronet cells (116 of 119 larvae displayed this pattern). (*C*) Deletion and mutation analysis of the E-box sequences. Deletion and mutation analysis of E-box1 and E-box2 showed that binding sites are necessary for *Ci-TH* expression in DA neurons/coronet cells. (*D*) Schematic diagrams of enhancer regions mediating localized expression in DA neurons/coronet cells. (*Left*) Three of three DA neuron marker genes contain enhancers with distant linkage of *Meis*- and *Ptf1a*-binding motifs (~150 bp). (*Right*) In contrast, two of two neuroendocrine genes contain enhancers with tightly linked *Meis*- and *Ptf1a*-binding motifs.

and oligonucleotide primers (Supplemental Table 2). The PCR products were digested with BamHI/NotI and inserted into the BamHI/NotI site of pSPKaede (Hozumi et al. 2010).

To generate pMiCiTHK, a promoter-Kaede cassette was amplified by PCR using a thermostable DNA polymerase and vector-specific oligonucleotide primers (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT GAACTCGAGCAGCTGAAGCTTG-3′ and 5′-GGGGACCACTTTGT ACAAGAAAGCTGGGTGCAGATCTGATGGCCGCTTTGAC-3′). The PCR product was subcloned into pMiDestF (Sasakura et al. 2008) with a gateway system (Invitrogen). To generate pSPCiPtf1acDNA, the coding sequence of *Ci-Ptf1a* was amplified by PCR with oligonucleotide primers (Supplemental Table 2). The PCR product was digested with NotI and inserted into the NotI and blunted EcoRI sites of pSPeGFP. To generate

pSPCiMeiscDNA, the coding sequence of *Ci-Meis* was amplified by PCR with oligonucleotide primers (Supplemental Table 2). The PCR product was digested with BamHI/EcoRV and inserted into the BamHI and blunted EcoRI sites of pSPeGFP.

To generate the pSPCiβ2tubulinCiPtf1acDNA and pSPCiPtf1a ΔMO target sequence Ptf1acDNA, 5′ upstream regions of *Ci-β2tubulin* and *Ci-Ptf1a* ΔMO target sequences were inserted into the BamHI site of pSPCiPtf1acDNA. To generate pSPCiβ2tubulinCiMeisacDNA, 5′ upstream regions of *Ci-β2tubulin* were inserted into the XhoI and BamHI sites of pSPCiMeiscDNA.

Generation of tranagenic lines

TH>Kaede transgenic lines were created by coelectroporation of in vitro synthesized transposase mRNA and pMiCiTHK (Sasakura 2007). Electroporated animals were cultured and maintained in an island system. Screening of transgenic lines was performed as described previously (Sasakura et al. 2007).

Microinjection of antisense MO oligonucleotides

MO oligonucleotides were obtained from Gene Tools, LLC. The antisense oligonucleotide sequence of the MO against *Ci-Ptf1a* was 5′-CGTTGA TAACTCACAAACACATAGG-3′. MOs were dissolved in DEPC-treated water containing 1 mg/mL tetramethylrhodamine dextran (Invitrogen, D1817). The concentrations of MO and plasmid DNA in the injection medium were 0.5 mM and 2.5–10 ng/μL, respectively. Microinjections of MOs and reporter constructs were performed as described previously (Horie et al. 2018). All experiments were repeated at least twice with different batches of embryos.

Single-cell RNA-seq assays

Eggs injected with 2.5 ng/μL *β2tubulin*>*Ptf1a* or 2.5 ng/μL *β2tubulin*>*Ptf1a* + 2.5 ng/μL *β2tubulin*>*Meis* and control eggs were fertilized side by side and allowed to develop to the middle or LTB stage (11 h or 13.5 h after fertilization at 18°C). For each sample, 120 morphologically normal embryos were used for single-cell RNA-seq assays. Dissociation of the embryos and single-cell RNA-seq assays by the 10x Genomics Chromium system were done as described previously (Horie et al. 2018) and are detailed in the Supplemental Material.

Image acquisition

Images of transgenic larvae were obtained with a Zeiss AxioPlan, Zeiss AX 10 epifluorescence microscope, and Olympus Fluoview FV10i confocal microscope.

Accession number

10x single-cell RNA-seq data for Ptf1a, Ptf1a and Meis overexpression, and control are available from Gene Expression Omnibus (GEO) under the accession number GSE120035.

Acknowledgments

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