Ndæl Expression and Regulation in Drosophila Embryos
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Abstract

The construction and prediction of cell fate maps at the whole embryo level require the establishment of an accurate atlas of gene expression patterns throughout development and the identification of the corresponding cis-regulatory sequences. However, while the expression and regulation of genes encoding upstream developmental regulators such as transcription factors or signaling pathway components have been analyzed in detail, the number of cis-regulatory sequences identified for downstream effector genes, like ion channels, pumps, and exchangers, is very low. The control and regulation of ion homeostasis in each cell, including at blastoderm stages, are essential for normal embryonic development. In this study, we analyzed in detail the embryonic expression pattern and cis-regulatory modules of the Drosophila Na⁺-driven anion exchanger 1 (Ndae1) gene, involved in the regulation of pH homeostasis. We show that Ndae1 is expressed in a tight and complex spatial-temporal pattern. In particular, we report that this downstream effector gene is under the control of the canonical dorsal-ventral patterning cascade through dorsal, Toll, twist and snail at early embryogenesis. Moreover, we identify several cis-regulatory modules, some of which control discrete and non-overlapping aspects of endogenous gene expression throughout development.

Introduction

Throughout embryogenesis, morphogen gradients and signaling molecules lead to a precise combination in the spatial distribution of gene expression. During this process, effector genes are switched on and off through their cis-regulatory sequences in a highly dynamic manner to grant cell fate and achieve a specific developmental program. For the construction, prediction and validation of cell fate maps at the whole embryo level, a main issue is to acquire an accurate atlas of gene expression patterns and their corresponding cis-regulatory sequences [1–3]. In addition, one of the major challenges of today embryology is to infer spatial-temporal cis-regulatory activity based on in vivo transcription factor binding and enhancer activity data. In Drosophila, numerous methods to computationally predict or validate cis-regulatory sequences at a genome wide scale have been developed [2,4–6]. Despite these many efforts, the number of cis-regulatory sequences of downstream effector genes is very low. Hence, in this era of genomics and high throughput analysis, traditional methods are still useful to complement and refine findings, adding accuracy to databases for the community.

Among the downstream effector genes whose regulatory network is yet to be unraveled are ion channels, pumps and exchangers. Normal embryonic development depends on the control of essential functions like ion homeostasis and the inability to maintain ion homeostasis leads to severe developmental defects [7] and diseases, called channelopathies [8,9]. However, there are still few studies on how morphogen gradients and master regulators affect ion homeostasis in Drosophila embryos.

One such study reveals how calcium gradients are required for specification of the dorsal embryonic region and how this gradient is inhibited in pipe, Toll (Tl), and dorsal (dTl) mutants, but is unchanged in decapentaplegic or punt mutants. At stage 5 of Drosophila embryonic development a calcium gradient is formed and is used for patterning the embryo through the suppression of ventral calcium concentrations [10,11]. Ion channels, pumps and exchangers are also useful markers of cell fate determination, as it has been observed during Drosophila neuronal specification. Indeed, different neurons are specified by different sets of TFs activating different sets of ion channels, which in turn confer distinct electrophysiological properties to each particular type of neuron [12,13].

Drosophila Ndae1 encodes a membrane-associated protein of 1,030 aminoacids, with homology to the vertebrate family of Na⁺/HCO₃⁻ exchangers [14]. When expressed in Xenopus oocytes, Ndae1 mediates the exchange of Na⁺, Cl⁻ and HCO₃⁻ independently of HCO₃⁻, in order to maintain intracellular hydrogen ion concentration (pH) [14–16]. The regulation of pH is fundamental for cell viability, metabolism and action potentials [15,17].

In Drosophila embryos, Ndae1 expression has been detected at blastoderm stages, and later in the alimentary tract, Malpighian tubules, salivary glands, mesectoderm, anal pads and nervous system [14,16]. Altogether these data reinforce the notion that Ndae1 may be a key pH and osmoregulatory protein during early development and may contribute to baso-lateral ion transport in Drosophila epithelia and in the nervous system. Hence, Ndae1...
expression must be under tight dynamic control throughout development and therefore constitutes an interesting candidate to study its regulation.

In vertebrates, Na⁺-dependent Cl⁻/HCO₃⁻ exchangers are a very extensive gene family constituted by a large number of proteins and isoforms [16,19,20]. Like Drosophila Ndae1, many members of this family are widely expressed. Among their functions, is the maintenance of intracellular pH. The importance of these channel functions is illustrated by studies in knock out models and the human diseases to which they have been linked (reviewed in [16,20]). For example, SLC4A4 has been demonstrated to be expressed in the colon and kidneys and knock out mice (NBCe1/−) show metabolic acidosis and intestinal blockage ([21] and references therein). Further, members of this family of transporters, like SLC4A10 has been found to be mutated in patients with epilepsy [21], and REV5-CCGGATATCGTTGATCGATATAGTTAAACCA-3’ (oMC018) and cloned into XhoI/EcoRI. pMC024: 2,628 bp corresponding to a fragment of the 5’UTR overlapping with pMC027 and pMC029 amplified with the primers FWD 5’-GGATCCGGTGTGCTAATCAAGTTTACGGTCG-3’ (oMC014) and cloned into BamHI/SacII. pMC026: 6,609 bp corresponding to introns II and III amplified with the primers FWD 5’-GGTACTGAGGTGTCGATCGATATAGTTAAACCA-3’ (oMC037) and REV 5’-GGGATATCGGTGCTAATCAAGTTTACGGTCG-3’ (oMC039) and cloned into XhoI/EcoRI.

NotI/BamHI. pMC013: a 4,000 bp BamHI-Xba I fragment corresponding to the most 5’ fragment of the 5’UTR was CRE excised from a lambda PS library, subcloned in BamHI/XbaI of pBluescript KS+ excised with BamHI and NotI and cloned into CHAB in BamHI/NotI. pMC020: 963 bp corresponding to the complete 3’UTR of Ndae1 amplified with the primers FWD 5’-CCGGATATCGTTGATCGATATAGTTAAACCA-3’ (oMC031) and REV 5’-GGGATATCGGTGCTAATCAAGTTTACGGTCG-3’ (oMC032) and cloned into XhoI/EcoRI. pMC024: 1,353 bp corresponding to introns VIII and IX in addition to exon IX amplified with the primers FWD 5’-TTGCTGAGCTGTAATTAAACATTGGGTACGGTTAATGCTG-3’ (oMC033) and REV 5’-CCGGATATCGTTGATCGATATAGTTAAACCA-3’ (oMC034) and cloned into XhoI/EcoRI. pMC025: 2,628 bp corresponding to a fragment of the 5’UTR overlapping with pMC027 and pMC029 amplified with the primers FWD 5’-GGTACTGAGGTGTCGATCGATATAGTTAAACCA-3’ (oMC037) and REV 5’-GGGATATCGGTGCTAATCAAGTTTACGGTCG-3’ (oMC014) and cloned into BamHI/SacII. pMC026: 6,609 bp corresponding to introns II and III amplified with the primers FWD 5’-GGTACTGAGGTGTCGATCGATATAGTTAAACCA-3’ (oMC037) and REV 5’-GGGATATCGGTGCTAATCAAGTTTACGGTCG-3’ (oMC039) and cloned into XhoI/EcoRI.

Materials and Methods

Transformation constructs

All DNA fragments have been amplified by PCR using the KOD or KOD XL (when greater than 3,000 bp) DNA Polymerase (Novagen, Tokyo). The following constructs (except for pMC035) have been cloned from a w¹¹¹⁰ genomic DNA template into the P{CaSpeR-hs43-AUG-betagal} (CHAB) vector (V. Pirrotta, unpublished data) in their endogenous orientation. In the oligonucleotides, the underlined is shown in bold and the restriction sites are underlined. pMC008: 2,400 bp corresponding to intron II amplified with the primers FWD 5’-GGGCGCGCGCCGCTGAAAGAGAGAGGTGCGGATT-3’ (oMC007) and REV 5’-GGGATATCGTTGATCGATATAGTTAAACCA-3’ (oMC008) and cloned into NotI/BamHI. pMC013: a 4,000 bp BamHI-Xba I fragment corresponding to the most 5’ fragment of the 5’UTR was CRE excised from a lambda PS library, subcloned in BamHI/XbaI of pBluescript KS+ excised with BamHI and NotI and cloned into CHAB in BamHI/NotI. pMC020: 963 bp corresponding to the complete 3’UTR of Ndae1 amplified with the primers FWD 5’-CCGGATATCGTTGATCGATATAGTTAAACCA-3’ (oMC031) and REV 5’-GGGATATCGGTGCTAATCAAGTTTACGGTCG-3’ (oMC032) and cloned into XhoI/EcoRI. pMC024: 1,353 bp corresponding to introns VIII and IX in addition to exon IX amplified with the primers FWD 5’-TTGCTGAGCTGTAATTAAACATTGGGTACGGTTAATGCTG-3’ (oMC033) and REV 5’-CCGGATATCGTTGATCGATATAGTTAAACCA-3’ (oMC034) and cloned into XhoI/EcoRI. pMC025: 2,628 bp corresponding to a fragment of the 5’UTR overlapping with pMC027 and pMC029 amplified with the primers FWD 5’-GGTACTGAGGTGTCGATCGATATAGTTAAACCA-3’ (oMC037) and REV 5’-GGGATATCGGTGCTAATCAAGTTTACGGTCG-3’ (oMC039) and cloned into XhoI/EcoRI. pMC026: 6,609 bp corresponding to introns II and III amplified with the primers FWD 5’-GGTACTGAGGTGTCGATCGATATAGTTAAACCA-3’ (oMC037) and REV 5’-GGGATATCGGTGCTAATCAAGTTTACGGTCG-3’ (oMC039) and cloned into XhoI/EcoRI.

Fly strains

Fly strains, d¹ (BL#3256), d² (BL#7096), T¹ (BL# 30914), d¹ (BL#3281), sn¹ (BL#2311), sn¹ (BL#3299) mutants and w¹¹¹⁰ (BL#5905) flies were acquired from the Bloomington Drosophila Stock Center (Bloomington, IN, USA). The d¹ and d² stocks resulted homozygous lethal, transheterozygote d¹/ d² females were generated and used to lay eggs after crossing to w¹¹¹⁰ males. All constructs were injected into w¹¹¹⁰ embryos by standard procedures to make transgenic flies bearing the same transgene as the transgene they carry.

In situ hybridization and immunohistochemistry

The Ndae1 sense probe was synthesized from a full length Ndae1 cDNA (AF047468) cloned into the pGEM-T easy plasmid [32] linearized with SacII and transcribed with the SP6 RNA polymerase using the Riboprobe Combination System kit (Promega, Madison, WI) and the DIG RNA Labeling Mix (Roche, Indianapolis, IN). This cDNA encompasses the full Ndae1 coding sequence and therefore allows revealing all characterized Ndae1 transcripts. The control sense probe was synthesized using the T7 RNA polymerase. The GP probe was made as previously [33]. Embryos were fixed in 4% formaldehyde for 25 minutes and stored in 100% ethanol. Endogenous peroxidase was inactivated by incubation in 3% H₂O₂ for 30 minutes. Then, embryos were post-fixed in PBS containing 8% Triton X-100 as in [34].
Embryos were stained using the Tyramide Signal Amplification (TSA) Plus Biotin System (Perkin Elmer n. NEL700A001KT) following instructions except that SA-HRP (Perkin Elmer n. NEL750001EA) and TSA were diluted at 1:500. Endogenous biotin was blocked using the Avidin/Biotin Blocking kit (Vectorlab n. SP2001) before adding the biotinylated anti-Digoxigenin antibody. Details of the protocol are available in Text SI. All antibodies were previously pre-absorbed on embryos at a 1:10 dilution. The anti-Dig antibody [Jackson ImmunoResearch] was used at a 1:250 dilution. For immunohistochemistry, rabbit anti-dl-Galactosidase (Life Technologies) and rabbit anti-dl-GFP (Molecular Probes, Invitrogen) were used at a 1:500 dilution. Histochomical detection was carried out using biotinylated goat anti-rabbit IgG or biotinylated goat anti-mouse IgG (1:500 dilution) and the avidin/biotin reagents of the Vectastain Elite Kit (Vector labs).

Color was revealed using 0.5 mg/ml 3,3’-Diaminobenzidine tetrahydrochloride hydrate and approximately 0.0015–0.003% H2O2. For immunofluorescence, chicken anti-GFP (Aves Labs, Inc), rabbit anti-Me2 (made by BioGenes) and Mab 22C10 (Hybridoma Bank) antibodies were used at a 1:1,000 dilution. Alexa 488 coupled goat anti-chicken and Alexa 555 coupled goat anti-rabbit secondary antibodies (Molecular Probes, Invitrogen) were used at 1:500. Slides were observed on a Zeiss LSM 510 confocal microscope.

Results and Discussion

Previously, Ndae1 transcripts have been detected by RT-PCR in embryos, larvae, pupae and adults [14]. In embryos, RNA was localized to the gut primordium, the mesoderm, the central nervous system (CNS) and the anal pads [14]. Subsequently, Ndae1 has been detected by tyramide-amplified FISH in the heart [32]. Using polyclonal antibodies, Ndae1 protein was found to be present at blastoderm stages, in the mesoderm, the alimentary tract (gut and Malpighian tubules), the central nervous system (CNS), the peripheral nervous system (PNS), the head sensilla and the anal pads [18]. In order to clear the discrepancies between Ndae1 transcript and protein localization, we performed in situ hybridization using a digoxigenin (DIG) labeled probe followed by tyramide amplification and revealing the staining by histochemistry (see Materials and Methods and Supporting Information). We found that Ndae1 transcripts clearly begin to be expressed at the cellular blastoderm stage (stage 5), in a ventral band that extends dorsally at the anterior and posterior ends of the embryo (Figure 1B). Ndae1 does not seem to be expressed in pole cells (Figures 1C and 1D, arrows) as instead previously reported [18]. At the beginning of gastrulation, Ndae1 transcripts are observed in the head and weakly in the tail (Figure 1E). At germ band extension, Ndae1 is detected in numerous cells that resemble yolk nuclei (vitellogaphes) (Figure 1F and see also http://insitu.fruitfly.org/cgi-bin/ex/report.pl?type=1&xftext=CG1675). At stage 13, expression in few amnioserosa cells is detected (Figure 1G, arrows) and Ndae1 begins to be expressed in the anal pads (Figures 1G and 1H, block arrows). At stage 14, lateral cells are labeled (Figure 1H, arrowhead). At stage 15, transcripts are detected in the CNS, in the brain and in lateral clusters of cells with a segmented pattern (Figures 2A-D, arrowheads). Expression in the anal pads (block arrows in figures 1 and 2) persists until the end of embryogenesis and could potentially contribute to the mechanisms of osmoregulation and salt stress tolerance. No expression is detected with the sense strand except for salivary glands (data not shown and see [33]). We did not detect RNA expression in the mesoderm, in the gut, in the Malpighian tubules and in the heart. This discrepancy could be due to the time window of expression of the transcripts in those tissues, which might be very narrow as Ndae1 expression changes very rapidly. However, in spite of the differences in the detection methods used (TSA-amplified in situ versus traditional in situ, our results comply with those found by BDGP (http://insitu.fruitfly.org/cgi-bin/ex/report.pl?type=1&xftext=CG1675), despite few differences. Thus, our data reinforce the confidence in high throughput analysis of gene expression patterns in Drosophila that are publicly available while providing important complementary information.

Establishment of DV asymmetry in the early Drosophila embryo is largely under maternal control [35–38]. In the oocyte, the dl morphogen is distributed in the cytoplasm. Upon fertilization, activation of the Tl pathway is triggered when the maternal ligand spatzle binds to the transmembrane receptor Tl on the ventral side of the embryo [39–41]. Ultimately, Tl signaling leads to the nuclear localization of the dl morphogen specifically on the ventral side [42–44]. This is the initial cue for DV axis formation in the embryo. We investigated whether this pathway regulates the early ventral expression of Ndae1. In the majority (approximately 80%) of embryos from dl/dl mothers, no Ndae1 expression is observed (Figure 3A compared with figures 1A and 1B). Consistently, in embryos from Tlmo mothers, where the Tl receptor is constitutively active, Ndae1 expression extends dorsally (Figure 3B) emphasizing the role of the DV pathway in the control of early Ndae1 expression. Noticeably, in few overstained dl/dl embryos some residual expression is observed anteriorly (Figure 3E), suggesting that Ndae1 is also under the control of the anterior and/or the terminal pathways.

Due to the activation of the Tl receptor, DI nuclear localization is highest in the ventral region. The activation of dl target genes occurs in a concentration dependent manner and has been subdivided in three categories [reviewed in 38,45,46]. tvi and sna are the first two zygotic genes expressed in the ventral regions of the embryo in a domain where the levels of nuclear DI are highest [47–49]. During gastrulation, dl, tvi and sna cooperate to pattern ventral cell invagination to form the mesoderm [50,51]. Mutants for either tvi or sna fail to undergo a ventral invagination and lack mesodermal tissue. As Ndae1 transcripts are expressed ventrally until gastrulation (Figures 1A and B), we explored Ndae1 expression in tvi and sna mutants. In tvi (Figure 3C) or sna (Figure 3D) mutant embryos, no Ndae1 expression is detected. Accordingly, we observed that in tvi, sna double mutants Ndae1 transcripts are also not present (data not shown). Altogether these data indicate that Ndae1 is under control of the classical DV patterning cascade, in agreement with high throughput studies attempting to establish clusters of co-expressed genes at blastoderm stages, where Ndae1 was predicted to be co-expressed with sna and tvi [3].

To fully understand animal transcription networks and predict cell fate, it is essential to measure the 3D spatial and temporal expression patterns of TFs and their downstream effectors and accurately map the cis-regulatory sequences of these TF targets [1,32]. To this purpose, Ndae1 is an appealing candidate because it is a downstream effector gene with a highly dynamic expression pattern during development. From a different point of view, the study of the cis-regulatory sequences of Ndae1 might provide insights on its expression pattern discrepancies. Thus, we decided to study Ndae1 enhancers through traditional methods. We first made lacZ reporter constructs covering the whole genomic region of Ndae1 from the previous gene (CG13786) to the next one (iconoclast) (Figure 4). Using immunohistochemistry, we observed that four out of nine reporter constructs do not drive any expression (Figure 4A, crossed constructs). We found that construct pMC024 (Figure 4B) drives lacZ expression in the
hemocytes, thus it probably belongs to a different gene. To better investigate the activity of this enhancer, we carried out in situ hybridization to detect lacZ mRNA in pMC024 embryos (Figure 4C). Protein and mRNA expression appear very similar. Construct pMC028, covering the fourth Ndae1 intron, drives lacZ expression in the CNS (pMC028, Figure 4D), a domain of expression of Ndae1 (Figure 2). Ndae1 is one of the few ion exchangers with known functions in the control of cellular pH that have been described in the nervous system of Drosophila and pMC028 is a rare ion exchanger enhancer. We hope that in the future it will be a useful tool for studies on transcription control, specification in cells of the nervous system and network excitability. It would be interesting to understand whether the concentration of Ndae1 is altered upon an action potential and whether the neuronal/glial selective loss of Ndae1 affects excitability.

Construct pMC028 drives expression also in a dorsal line of cells. In order to determine their belonging, we cloned the same intronic fragment into the pH-Stinger vector [31] (pMC035; Figure 4E) and observed that it drives GFP expression in the same domains as the lacZ construct, but with a higher signal. To determine the nature of these dorsal cells we performed double labeling with anti-Mef2 antibodies, which label all mesodermal cells including those of the heart where expression of Ndae1 has been detected. We observed that the dorsal GFP-positive cells are large, polygonal and internal to the cardiac cells labeled by Mef2 (Figure 5A). Hence, we conclude they are amnioserosa cells. The amnioserosa is an extraembryonic epithelium that plays a key role in dorsal closure [53], the process by which the two dorsal epidermal margins, one on each side of the embryo, meet and fuse at the dorsal midline of the embryo at the end of embryogenesis. Inside the amnioserosa sheet, the external line of cells making direct contact with the dorsal epidermis has been described to have distinctive characteristics and plays a major role in the integrin-dependent adhesion of the amnioserosa cells to the dorsal epidermis in response to Dpp signaling [54–56]. We propose that the cells labeled by the reporter construct pMC035 (Figure 5A) belong to the peripheral or marginal amnioserosa group of cells, playing a role in germ band retraction [57]. This expression pattern is in agreement with previous proposals that Ndae1 may

**Figure 1. Ndae1 expression in early embryogenesis.** Ndae1 expression patterns in embryos detected by in situ hybridization with TSA amplification followed by histochemistry (same as in figures 2 and 3). (A) Ventral expression in a ventral view of a stage 5 embryo. (B) Ventral expression in a lateral view of a stage 5 embryo. (C) Lateral view of the posterior end of a stage 5 embryo. Ndae1 is not expressed in pole cells (arrow). (D) Lateral view of the posterior end of a stage 6 embryo. No Ndae1 transcripts are observed in pole cells (arrow). (E) Lateral view of a gastrulating stage 6 embryo showing strong expression in the head and weak in the tail. (F) Lateral view of a stage 10 embryo. Ndae1 is likely expressed in yolk cells. (G) Stage 12 embryo with amnioserosa expression (arrows) and the beginning of anal pad expression (block arrow). (H) Lateral view of a stage 14 embryo showing expression in lateral cells (arrowhead) and in the anal pads (block arrow).
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contribute to baso-lateral ion transport in *Drosophila* epithelia. Expression of *Ndae1* in these cells might be too weak or transient to be detected even by enhanced *in situ* hybridization.

Both pMC028 and pMC035 constructs label also lateral cells (Figures 4D and 4E) that resemble a domain of expression of *Ndae1* (arrowheads in Figures 1H and 2). To learn more about the identity of these lateral cells we stained pMC035 embryos with anti-GFP and anti-22C10 antibodies (Figures 5B and 5C), which at the end of embryogenesis label several CNS neurons, PNS neurons and sensory neurons [58]. However, at dorsal closure, these lateral cells were not labeled with 22C10, but are interestingly in very close apposition, immediately below the...
We also found that these cells do not belong to the mesoderm, as they are not labeled by anti-Mef2 antibodies (Figures 5D and 5E).

Moreover, three overlapping constructs in the 5' region (pMC025, pMC027 and pMC029) drive expression in the anal pads (Figures 4F-H), a domain of expression of \textit{Ndae1} (Figure 2).

Because the anal pads are involved in osmoregulation [59–61], this enhancer may be very useful to study the contribution of \textit{Ndae1} to osmotic balance in \textit{Drosophila} and its role in the stress response due to changes in osmolarity of the medium.

Finally, none of the constructs reported expression at early blastoderm stages. We conclude that this blastoderm enhancer is probably lying in a genomic region far from the gene itself. We have not found any enhancer even for the pole cells, mesectoderm, Malpighian tubules, gut and heart where \textit{Ndae1} had been previously described to be present [18,32], nor did we detect \textit{Ndae1} expression in these tissues. This could be due to the different techniques used or to a high instability of the \textit{Ndae1} mRNA.

**Conclusions**

Altogether, the data presented here indicate that \textit{Ndae1} is under tight control during development. Some expression domains previously found have not been validated by our sensitive \textit{in situ} hybridization procedure. We have revealed that the ventral blastoderm expression of \textit{Ndae1} is regulated directly or indirectly by the classical DV pathway through \textit{dl}, \textit{Toll}, \textit{tux} and \textit{sha}, which had not been previously reported. Moreover, we have presented a sequential cloning of the whole genomic region of \textit{Ndae1} in order to reveal all the areas positive for enhancer activity. The \textit{Ndae1} enhancers found, especially the anal pads and CNS ones, provide new tools to validate future studies of \textit{cis}-regulatory sequence prediction at a genome wide scale and to study \textit{Ndae1} function. The different requirements of \textit{Ndae1} in different neuron and/or glia adult subtypes as well as its role in network excitability in embryos and adult flies should be further explored. In addition, it would be interesting to understand whether \textit{Ndae1} is altered in fly models for epilepsy or neurodegenerative diseases.

**Supporting Information**

**Text S1** Detailed protocol to carry out \textit{in situ} hybridization with tyramide amplification on \textit{Drosophila} embryos. (PDF)

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Author Contributions
Conceived and designed the experiments: MC LP. Performed the experiments: MFT CT MC DS. Analyzed the data: MFT LP MC. Contributed reagents/materials/analysis tools: MFT LP MC. Wrote the paper: MFT MC.

References

Figure 5. GFP expression driven by the fourth Ndae1 intron. All embryos carry the pMC035 reporter construct and are stained by immunofluorescence with anti-GFP antibodies (green). (A) Magnification of a dorsal view of a stage 14 embryo double-stained with anti-Mef2 antibodies (red), labeling muscle cells including the cardiac tube. GFP is expressed in amnioserosa cells. (B) Lateral view of a stage 16 embryo double labeled with anti Mef-2 antibodies (red). (C) Magnification of a boxed portion of embryo in (B). The GFP-positive cells do not overlap the Mef-2-positive cells. (D) Lateral view of a stage 16 embryo double labeled with 22C10 antibodies (red). (E) Magnification of a boxed portion of embryo in (D). The GFP-positive cells lay below the 22C10-positive neurons.
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Author Contributions
Conceived and designed the experiments: MC LP. Performed the experiments: MFT CT MC DS. Analyzed the data: MFT LP MC. Contributed reagents/materials/analysis tools: MFT LP MC. Wrote the paper: MFT MC.


