A Genome-Wide Screen for Dendritically Localized RNAs Identifies Genes Required for Dendrite Morphogenesis

Mala Misra,*, Hendia Edmund,*, Darragh Ennis,† Marissa A. Schlueter,*, Jessica E. Marot,* Janet Tambasco,* Ida Barlow,* Sara Sigurbjornsdottir,† Renjith Mathew,‡ Ana Maria Vallés,† Waldemar Wojciech,‡ Siegfried Roth,** Ilan Davis,† Maria Leptin,‡§ Elizabeth R. Gavis*,**

*Department of Molecular Biology, Princeton University, NJ 08544, †Department of Biochemistry, The University of Oxford, OX1 3QU, United Kingdom, ‡European Molecular Biology Laboratory, 69117 Heidelberg, Germany, and §Institute of Genetics, University of Cologne, 50674 Germany, **Biocenter, Institute of Developmental Biology, University of Cologne, 50674 Cologne, Germany

ABSTRACT Localizing messenger RNAs at specific subcellular sites is a conserved mechanism for targeting the synthesis of cytoplasmic proteins to distinct subcellular domains, thereby generating the asymmetric protein distributions necessary for cellular and developmental polarity. However, the full range of transcripts that are asymmetrically distributed in specialized cell types, and the significance of their localization, especially in the nervous system, are not known. We used the EP-MS2 method, which combines EP transposon insertion with the MS2/MCP in vivo fluorescent labeling system, to screen for novel localized transcripts in polarized cells, focusing on the highly branched Drosophila class IV dendritic arborization neurons. Of a total of 541 lines screened, we identified 55 EP-MS2 insertions producing transcripts that were enriched in neuronal processes, particularly in dendrites. The 47 genes identified by these insertions encode molecularly diverse proteins, and are enriched for genes that function in neuronal development and physiology. RNAi-mediated knockdown confirmed roles for many of the candidate genes in dendrite morphogenesis. We propose that the transport of mRNAs encoded by these genes into the dendrites allows their expression to be regulated on a local scale during the dynamic developmental processes of dendrite outgrowth, branching, and/or remodeling.

KEYWORDS mRNA localization local translation dendritic arborization neuron multidendritic neuron Drosophila peripheral nervous system

Copyright © 2016 Misra et al. doi: 10.1534/g3.116.030353
Manuscript received April 18, 2016; accepted for publication May 27, 2016; published Early Online June 1, 2016.
This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Supplemental material is available online at www.g3journal.org/lookup/suppl/doi:10.1534/g3.116.030353/-/DC1

1 Present address: Department of Biology, Colgate University, Hamilton, NY 13346.
2 Present address: School of Biological Sciences, National Institute of Science Education and Research, Bhubaneswar, India
3 Corresponding author: Princeton University, Princeton, NJ 08544. E-mail: gavis@princeton.edu
uncovered requirements for mRNA localization, and for mRNA regulatory proteins, in dendrite patterning of Drosophila sensory neurons (Brechbühl and Gavis 2008; Xu et al. 2013; Olesnicky et al. 2014). Despite the prevalence of mRNAs in neuronal processes, relatively few of these localized mRNAs have been studied in detail. As a result, our understanding of the molecular mechanisms that govern mRNA targeting to dendrites and axons and the functional implications of localization in neurons is still nascent. A long-standing obstacle to this goal has been the difficulty in visualizing localized mRNAs in the fine processes of neurons in vivo outside of dissociated culture systems. Detection by in situ hybridization has been hampered by the challenge of discriminating low endogenous neuronal transcript levels from expression in surrounding tissues. To overcome this difficulty, several studies have utilized the MS2/MCP system for the visualization of mRNA distributions in Drosophila class IV dendritic arboration (da) neurons—a subset of morphologically complex larval sensory neurons (Brechbühl and Gavis 2008; Xu et al. 2013). This system requires that targeted genes of interest be tagged with sequences encoding MS2 RNA stem-loops. Concurrent expression of MS2-tagged transcripts and fluorescent MS2 Coat Protein (MCP), a bacteriophage-derived protein that binds the MS2 stem loops, results in the formation of RNP particles detectable by fluorescence microscopy (Bertrand et al. 1998).

Here, we adapted a previously described methodology combining the MS2/MCP system with EP element transposition and GAL4/UAS-driven transgene expression to characterize novel localized transcripts in the processes of Drosophila class IV da neurons (JayaNandanan et al. 2011). This method allows the unbiased identification of candidate transcripts with the capacity to localize to neuronal processes. In addition, it enables the simultaneous in vivo visualization of these candidates to characterize their subcellular distributions. We have identified 55 candidate transcripts capable of localizing to neuronal processes. Quantitative mRNA analysis showed that the screen detected transcripts with a wide range of expression levels. Furthermore, many of these transcripts exhibit biased localization profiles, accumulating specifically in dendrites rather than axons. Post hoc genomic mapping revealed that 42 of the 55 transcripts are very likely to include a portion, or all, of a known, previously annotated RNA. Subsequent gene ontology (GO) analysis suggests that, although the corresponding genes encode molecularly diverse proteins, this candidate subset is significantly enriched for genes that function in neuronal development.

A secondary RNAi screen confirmed that the expression of many candidate genes is relevant to dendrite morphogenesis. RNAi-mediated knockdown of 18 candidate genes resulted in varied defects in dendritic arborization, which we have classified as “overbranching”, “reduced branching”, and “altered branch distribution”. We suggest that the transport of mRNAs encoded by these candidates into the dendrites may be an important method of regulating gene expression on a local scale during the dynamic developmental processes of dendrite outgrowth, branching, and/or remodeling.

**MATERIALS AND METHODS**

**Fly strains and genetics**

EP-MS2 insertion lines were generated as described in JayaNandanan et al. (2011). EP-MS2 lines were crossed to GAL4^{777}, UAS-mCD8:GFP/CyO, actin-GFP, UAS-MCP-RFP/TM6B (Brechbühl and Gavis 2008; Xu et al. 2013) at 25°C. The RNAi screen was conducted by crossing UAS-RNAi lines listed in Supplemental Material, Table S1 to ppk-GAL4, UAS-CD8:GFP (Han et al. 2011) at 29°C. Drosophila strains are available upon request.

**EP-MS2 screen for localized transcripts**

Screening for localized transcripts was performed using a semi-intact larval preparation. An individual wandering third instar larva was immersed in a droplet of 90% glycerol on a glass slide. A small incision was made near the posterior end to extrude the gut and associated tissue. A coverslip was then pressed over the larva and the sample was imaged immediately using a Leica SPE confocal microscope with a 63×/1.4 NA oil objective. ddaC neurons from abdominal segments 3 and 4 were imaged in extended z-stacks with a 500-nm step size.

![Image](image-url)
**Table 1 Positive candidates from EP-MS2 screen**

| Nearest Gene | FlyBase ID | Line ID | Position Relative to Gene | Neurons Analyzed (n) | # Particles (Mean ± SEM)* | P Value (t-Test)b | Dendrite Enrichmentc | RNAi Phenotype† |
|--------------|------------|---------|---------------------------|---------------------|--------------------------|-----------------|---------------------|----------------|
| antennal protein 10 (a10) | FBgn0011293 | EM-802 | Coding | 6 | 23 ± 4 | ** | – | – |
| apontic (apd) | FBgn0015903 | EM-842 | Coding | 6 | 21 ± 3 | ** | – | – |
| bruno-3 (bru-3) | FBgn0264001 | EM-402 | Intron | 6 | 19 ± 5 | ** | – | – |
| Calnexin 99A (Chx99A) | FBgn0015622 | EM-573 | Coding | 7 | 19 ± 3 | * | + | – |
| Calreticulin (Calr) | FBgn0005585 | EM-447 | 52 bp | 6 | 22 ± 5 | * | + | Not tested |
| CGS122 | FBgn0032471 | OX-061 | Coding | 7 | 44 ± 9 | ** | – | Not tested |
| CGS261 | FBgn0031912 | OX-015 | 60 bp | 6 | 20 ± 4 | * | + | – |
| CG8177 | FBgn0036043 | CO-044 | 1000 bp | 6 | 33 ± 6 | *** | – | Decreased branching |
| CG8420 | FBgn0037664 | OX-064 | 200 bp | 6 | 36 ± 8 | ** | – | – |
| CG9922 | FBgn0038196 | OX-012 | 22,000 bp | 8 | 37 ± 8 | ** | – | Increased branching |
| CG12535 | FBgn0029657 | EM-781 | Coding | 6 | 31 ± 6 | * | – | – |
| CG14805 | FBgn0023514 | CO-042 | Intron | 6 | 20 ± 5 | * | + | – |
| CG42524 | FBgn0260429 | OX-118 | 400 bp | 6 | 29 ± 6 | ** | + | Not available |
| CG42855 | FBgn0262102 | EM-532 | Intron | 9 | 23 ± 5 | * | – | Not available |
| CG43392 | FBgn00263249 | OX-116 | Intron | 6 | 44 ± 9 | *** | – | Not available |
| Chemosensory protein B 38c (CheB38C) | FBgn0032888 | OX-097 | 4700 bp | 6 | 34 ± 9 | * | – | – |
| ChHk1 | FBgn0045761 | CO-016 | Coding | 6 | 36 ± 8 | ** | – | Abnormal pattern |
| coracle (cora) | FBgn0010434 | OX-080 | 5′-UTR | 6 | 22 ± 5 | * | – | Increased branching |
| CR45669 | FBgn00267229 | OX-063 | 10 bp | 6 | 35 ± 8 | ** | – | Not available |
| escargot (esg) | FBgn0001981 | OX-031 | 300 bp | 4 | 33 ± 7 | ** | – | – |
| fatty acid binding protein (fapbp), scheggia (sea) | FBgn0037913, FBgn0037912 | OX-049 | 2500 bp | 8 | 44 ± 7 | *** | – | Increased branching |
| foraging (for) | FBgn0000721 | EM-066 | 3000 bp | 6 | 25 ± 3 | *** | + | Decreased branching |
| frayed (fray), CG7694 | FBgn0023083, FBgn0038627 | CO-033 | Intron | 8 | 29 ± 5 | ** | – | Increased branching; – |
| frizzled 2 (fz2) | FBgn0016797 | EM-019 | Coding | 6 | 18 ± 3 | * | + | – |
| High mobility group protein D (HmgD) | FBgn0004362 | CO-011 | Intron | 7 | 36 ± 7 | ** | – | Increased branching |
| Hormone receptor-like in 39 (Hr39) | FBgn00261239 | CO-060 | 2000 bp | 6 | 18 ± 4 | * | + | – |
| IGF-II mRNA binding protein (imp) | FBgn00262735 | EM-574 | 1000 bp | 8 | 20 ± 4 | * | + | – |
| Inositol 1,4,5-triphosphate kinase 1 (IIP3K1) | FBgn0032147 | EM-042 | Intron | 6 | 21 ± 3 | ** | + | Decreased branching |
| Ionotropic receptor 68a (Ir68a) | FBgn0036150 | OX-078 | 4000 bp | 6 | 20 ± 4 | * | – | Increased branching |
| jing interacting gene regulatory 1 (jigr1) | FBgn0039350 | EM-562 | Intron | 6 | 27 ± 6 | ** | + | – |
| Meltrin | FBgn00265140 | CO-051 | Intron | 6 | 35 ± 12 | * | + | – |
| Mi-2 | FBgn00262519 | EM-024 | Coding | 6 | 33 ± 12 | * | + | Decreased branching |
| mini spindles (msps) | FBgn0027948 | EM-404 | 80 bp | 4 | 23 ± 7 | * | + | Decreased branching |
| mir-315 stem loop (mir-315) | FBgn00262461 | EM-704 | 700 bp | 6 | 21 ± 3 | * | + | Not available |
| Multidrug resistance protein 4 ortholog (Mdr4) | FBgn00263316 | EM-043 | 500 bp | 6 | 19 ± 3 | * | + | Not tested |
| Phosphoinositide-dependent kinase 1 (Pdk1) | FBgn00020386 | PU-007 | 2400 bp | 6 | 27 ± 6 | ** | + | Decreased branching |
| schnurri (shn) | FBgn0003396 | EM-503 | 4000 bp | 6 | 22 ± 4 | ** | – | Increased branching |

(continued)
**Table 1, continued**

| Nearest Gene                  | FlyBase ID         | Line ID | Position Relative to Gene | Neurons Analyzed (n) | # Particles (Mean ± SEM) | P Value (t-Test) | Dendrite Enrichment | RNAi Phenotype |
|-------------------------------|--------------------|---------|---------------------------|----------------------|--------------------------|------------------|----------------------|-----------------|
| shibire (shi)                 | FBgn0003392        | EM-550  | Coding                    | 6                    | 19 ± 5                   |                 |                      |                 |
| spitz (spi)                   | FBgn0005672        | PU-003  | 3300 bp                   | 6                    | 22 ± 3                   | *                |                      |                 |
| Star (S)                      | FBgn0003310        | OX-043  | Intron                    | 6                    | 20 ± 3                   | **               |                      |                 |
| taranis (tara)                | FBgn0040071        | OX-032  | Intron                    | 6                    | 22 ± 4                   | **               |                      |                 |
| Thiolase                      | FBgn0025352        | CO-029  | Intron                    | 6                    | 36 ± 9                   | **               |                      |                 |
| three rows (thr)              | FBgn0003701        | CO-072  | Intron                    | 6                    | 33 ± 2                   | **               |                      |                 |
| u-shaped (ush)                | FBgn0003963        | EM-629  | Coding                    | 6                    | 25 ± 4                   | **               |                      | Decreased branching |
| unable to map (1)             | EM-047             |         |                           | 8                    | 20 ± 4                   | *                |                      |                 |
| unable to map (2)             | EM-063             | Intron  |                           | 6                    | 20 ± 4                   | *                |                      |                 |
| Vacular H+ ATPase 16kD subunit (Vhα16-1) | FBgn0262736         | EM-637  | Coding                    | 6                    | 24 ± 5                   | *                |                      |                 |

EP-MS2 insertion lines are identified according to their origin (EM, EMBL, Heidelberg; OX, Oxford University; PU, Princeton University; CO, University of Cologne). For each line, the gene disrupted by the insertion (and position within the gene) or the most proximal downstream gene (and the distance from the insertion to the transcription start site) are listed. In the case of CO-033 and OX-103, each line, the gene disrupted by the insertion (and position within the gene) or the most proximal downstream gene (and the distance from the insertion to the gene) gives rise to the localized transcript. Only RNAi targeting the nearest gene produced a phenotype. The two genes proximal to the OX-049 insertion share the same 5’-end and introns so it is not possible to distinguish which one is required in da neurons.

At least six neurons from three or more larvae were imaged and analyzed for each EP-MS2 line. Because particles are largely detected within the proximal dendrites and axon, nearly all particles could be captured by positioning the cell body near the center of the 174.6 × 174.6 μm image field. RNA particles were quantified from maximum z-series projections using fixed parameters in NIH ImageJ v.1.48. The red channel (for detection of MCP-RFP fluorescence) was thresholded to a fixed minimum value. The built-in particle detection function in ImageJ was then utilized to distinguish RFP-positive particles from background fluorescence levels. Particle identification parameters were set as follows: particle size, 0–20 pixels; particle circularity, 0.00–1.00. Distinct particles located within the neuronal processes (as demarcated by membrane-bound mCD8:GFP) were manually counted. Statistical significance was determined by Student’s t-test. Note that images shown in Figure 1 were cropped and adjusted identically in Adobe Photoshop.

**Mapping of insertions**

Genomic DNA was isolated from third-instar larvae according to Huang et al. (2009). TAIL-PCR was performed according to the method of Liu et al. (1995), using three consecutive rounds of PCR with a set of degenerative primers, and a set of primers complementary to sequences within the EP-MS2 element (Table S2). In each successive round of PCR, the EP-MS2 primer used is downstream of the primer used in the previous round. The PCR product of the third round was sequenced and mapped to the D. melanogaster genome using BLAST (NCBI).

**Quantitative RT-PCR analysis of EP-MS2 RNA expression**

For each EP-MS2 line analyzed, total RNA was purified from third-instar larvae using the illustra RNAspin Mini kit (GE Healthcare Life Sciences) and stored at –80°C. cDNA was synthesized from 100 ng of each RNA sample with RevertAid reverse transcriptase (ThermoFisher). Control reactions without reverse transcriptase were performed in parallel. Real-time PCR (qPCR) was carried out using a Bio-Rad CFX96. Each EP-MS2 line was analyzed in triplicate reactions for each of two primer sets: primers complementary to the EP-MS2 element, and primers complementary to rp49—a ribosomal protein gene used as a reference to normalize for differences in initial cDNA concentrations (Table S2). Three independent qPCR experiments were performed in all cases.

To quantify relative expression levels, ΔCt was calculated for each EP-MS2 line by subtracting the mean Ct-value obtained for reactions with EP-MS2 primers from the mean Ct-value of the corresponding rp49 reactions. Because there is no independent EP-MS2 control sample for this experiment, we compared expression of each line to that of the mean of all 24 lines. Statistical analysis was carried out using Prism 5 software (Graphpad); statistical significance was determined using Student’s t-test.

**RNAi screen for dendrite morphology defects**

Twelve ddaC neurons from six larvae were analyzed for each UAS-RNAi line (Table S1). To visualize dendrite morphology, wandering third instar larvae were mounted on slides in a 1:5 mixture of chloroform: halocarbon oil (2:1 ratio of halocarbon 95 and 200). Two 22 mm × 22 mm coverslips were placed, one on either side of the larva and a 22 mm × 60 mm coverslip was gently pressed on top. The ddaC neurons of abdominal segments 3 and 4 were imaged in z-stacks with 1 μm steps, using a Leica SPE confocal microscope with a 20× air objective. The number of terminal branches and total branch length were quantified from z-series maximum projections. Neuronal tracings used to determine branch length were made using...
Neuron] (Mrijiering et al. 2004). Statistical significance was determined using Student’s t-test.

**Data availability**

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

**RESULTS**

**A screen for localized mRNAs in Drosophila larval sensory neurons**

To identify novel localized transcripts in the processes of Drosophila larval class IV da neurons, we took advantage of the EP-MS2 method to generate MS2-tagged transcripts throughout the genome. In this method, a modified EP transposon containing GAL4 responsive UAS sequences and a minimal promoter followed by six MS2 stem-loops is integrated into the genome by P element-mediated transposition. Because P elements tend to insert near the 5'-ends of genes, activation by a GAL4 driver should frequently lead to transcription of a “trapped” gene, generating an extended 5’-UTR containing the MS2 tag. MS2-tagged transcripts can be visualized by simultaneous expression of MCP-RFP.

We generated a collection of EP-MS2 insertion lines that were then crossed to the cell type-specific driver GAL477 (Grueber et al. 2003) for expression in class IV da neurons. Neuronal coexpression of MCP-RFP generated fluorescently labeled mRNA that was visualized in semintact larval preparations by confocal microscopy. Consistent with previous results, MS2-tagged transcripts labeled with MCP-RFP formed bright particles that could be detected in the soma and/or processes of the neurons (Figure 1). Because MCP-RFP can form nonspecific particles in da neurons in the absence of MS2-tagged RNA, probably due to self-aggregation (Brechbiel and Gavis 2008; Xu et al. 2013), we compared the number and distribution of particles observed with EP-MS2 expression to those of control neurons expressing MCP-RFP alone (Figure 1, A and A'). “MCP-RFP-only” neurons. Particles were identified and quantified in micrographs using thresholding and particle-resolving functions in NIH Image (see Materials and Methods; Figure 1, A–C and A’–C).

Of 541 independent EP-MS2 lines screened, 10% (55 lines) yielded transcripts that were significantly enriched in the processes of class IV da neurons ($P < 0.05$ when compared to MCP-RFP-only neurons; Table 1). Qualitative visual analysis of RNA particles for these lines suggested that some transcripts preferentially localize to dendrites vs. axons. To quantify the likelihood of polarized localization among the positive lines, we compared the number of particles detected within each neuronal compartment to the corresponding number of particles in MCP-RFP-only neurons. Twenty-seven (49%) of the positives showed significant accumulation in dendrites but not the axon (Figure 1, B–B'). These particles were detected primarily in larger proximal processes rather than in thinner distal processes. The remaining positive lines showed patterns of accumulation that also included the axon (Figure 1, C–C').

**Identification of genes targeted by EP-MS2 insertions**

Genomic mapping of the positive EP-MS2 lines by thermal asymmetric interlaced (TAIL) PCR revealed that 27 (49%) of the lines had insertions within the transcription unit of a previously annotated gene

| Nearest Gene | FlyBase ID | Line ID | Position Relative to Gene (bp) | qRT-PCR |
|--------------|------------|---------|-------------------------------|---------|
| bruchpilot (brp) | FBgn0259246 | EM-405 | Intron | √ |
| CG1358 | FBgn0033196 | OX-112 | Coding | √ |
| CG3927 | FBgn0034739 | OX-017 | 24,000 | √ |
| CG3151 | FBgn00366576 | EM-836 | 100 | √ |
| CG3818 | FBgn0032218 | PU-017 | Intron | √ |
| CG819 | FBgn0019999 | EM-786 | 1100 | √ |
| CG8240 CR45196 | FBgn0264439 | OX-014 | 1100 | √ |
| CG9384 | FBgn0036446 | EM-648 | 2500 | √ |
| CG15358 | FBgn0031373 | PU-028 | 1900 | √ |
| CG42555 | FBgn0262102 | EM-951 | 100 | √ |
| chameau | FBgn0028387 | OX-107 | Intron | √ |
| circadian trip (ctrip) | FBgn0260794 | EM-758 | Intron | √ |
| CR43174 | FBgn0267794 | OX-087 | 300 | √ |
| escargot (esg) | FBgn001981 | EM-628 | 100 | √ |
| fatty acid binding protein (fabp), sheggia (sea) | FBgn0037913 | CO 066 | 2100 | √ |

* qRT-PCR results are shown in Figure 2.

Note: The table above lists the negative lines mapped and/or analyzed for expression levels.
(Table 1). An additional 15 lines had insertions ≤ 1 kb upstream of a transcriptional start site, likely generating a transcript with an extended 5’UTR. These sets included multiple independent insertions for several genes—escargot, jing interacting gene regulatory 1, and u-shaped—suggesting that these loci may be insertional hot-spots. Indeed, escargot was previously identified as a hot-spot locus (Spradling et al. 1999). For 11 lines, the nearest downstream transcription unit was ≥ 1 kb away, although whether the MS2-tagged transcripts include sequences from these genes or derive only from the intergenic region is not clear. In the latter case, we presume that these intergenic regions contain sequences that, if transcribed, can function as cryptic localization elements. Finally, we were unable to map two insertions. In total, 47 different genes were identified; in two cases two overlapping genes were targeted by the same EP-MS2 insertion.

We also mapped 23 negative lines to determine whether they represented insertions within genes or primarily intergenic insertions. Sixteen (69%) had insertions within or ≤ 1 kb upstream of an annotated transcription unit (Table 2). Thus, the majority of negative lines likely represent productive insertions that could generate MS2-tagged transcripts but that did not show specific localization patterns of the type we describe above. This analysis also revealed three cases in which the same genes were identified among both positive and negative lines. For Multidrug resistance protein 4 ortholog and escargot, both positive and negative lines contained insertions near the transcription start site and the negative lines produced transcripts (see below), suggesting that those insertions may lack MS2 stem-loops or be otherwise defective. The overlapping genes fatty acid binding protein (fadp) and scheggia (sea) were identified by insertions residing 2.4 kb (positive) and 2.1 kb (negative) upstream. The finding that neuronal knockdown of fadp/sea affects dendrite arbor morphology (see below; Table 1) suggests that the negative line similarly contains a nonproductive insertion, although we cannot rule out the possibility that the more upstream insertion is a false positive.

EP-MS2 expression levels do not correlate with RNA localization

The difference between positive and negative lines could reflect differences in transcript level and thus ease of detection rather than true differences in localization. To determine whether positive lines are generally associated with high transcript levels, we compared RNA expression levels among a set of 12 positive and 12 negative EP-MS2 lines using quantitative RT-PCR (qRT-PCR). Expression of MS2-tagged transcripts was activated in larval class IV da neurons using GAL4[27], and qRT-PCR was performed on RNA isolated from whole larvae with primers specific for expressed regions of the EP-MS2 transgene. To facilitate comparison among the different lines, expression levels were displayed relative to the average expression level of the 24 lines (Figure 2). Statistical analysis showed that there is no significant difference in the mean expression level of lines determined to be positive vs. lines determined to be negative ($P = 0.61$; Figure 2). We therefore conclude that there is no correlation between the level of expression and the categorization of a transcript as localized, and that the screen has the ability to detect localized transcripts whether highly expressed, or expressed at a low level.

Enrichment of developmental genes among the positive candidate group

Functional sorting of the genes tagged in positive lines using the Database for Annotation, Visualization and Integrated Discovery (DAVID v. 6.7; Huang da et al. 2009a, 2009b) revealed enrichment for genes encoding proteins involved in neuronal development and physiology as well as several other developmental processes (Table 3). The category of nervous system development includes a diverse group of genes encoding transcription factors and DNA binding proteins (escargot, High mobility group protein D, apontic, schnurti, and Mi-2), a serine-threonine kinase (frayed), an apico-basal polarity protein (coracle), RNA binding proteins (apontic and IGF II mRNA binding protein), a transferase (Star), a nucleotide exchange factor (RhoGEF64C), and membrane receptors (spitz and frizzled2). In sum, localized transcripts trapped by EP-MS2 transposons encoded functionally diverse developmental proteins.

Secondary screen of gene function in neuronal morphogenesis

To determine whether genes identified in the EP-MS2 screen function in class IV da neuron development, we knocked down expression of the majority (38) of these genes in the neurons by transgenic RNAi. UAS-RNAi was expressed using the class IV da neuron-specific ppp-GAL4 driver, and coexpression of the CD4:GFP membrane marker (Han et al. 2011) allowed visualization of neuronal morphology. Two independent UAS-RNAi lines were tested for each gene in order to minimize false positive and negative results. In all cases, both lines produced similar phenotypes.

Two parameters that reflect dendritic arbor branching, total dendrite length and the number of dendritic terminal branches, were quantified from confocal z-series projections of RNAi-expressing neurons. In total, knockdown of 18 genes (47% of those tested) produced a dendrite...
Table 3 Functional annotation of candidate genes

| GO Term                                      | Number of Genes | Fold Enrichment | Genes                                      |
|----------------------------------------------|-----------------|-----------------|--------------------------------------------|
| Peripheral nervous system development        | 7               | 19.1            | Calr, S, esg, fray, shn, spi, thr          |
| Neuron development (GO:0048666)              | 6               | 4.4             | Mi-2, HmgD, S, fray, fz2, spi              |
| Neuron differentiation (GO:0030182)          | 8               | 3.5             | Calr, S, a10, apt, for, fray, shn, shi     |
| Neurological system process (GO:0050877)     | 7               | 3.7             | CG14709, cora, msp5, shn, spi, tara, ush  |
| Regionalization (GO:0003002)                | 5               | 8.3             | Pdk1, S, shn, spi, ush                     |
| Enzyme linked receptor protein signaling pathway (GO:0007167) | 7               | 11.0            | S, apt, cora, esg, shi, spi, thr           |
| Open tracheal system development             | 7               | 8.2             | apt, cora, esg, fray, shn, shi, thr, ush  |
| Respiratory system development (GO:0007422)  | 7               | 19.1            |                                             |
| Epithelial development (GO:00600429)         | 7               | 8.2             |                                             |

Analysis of genes in Table 1 was performed using DAVID. The most highly represented functional annotation categories are listed.

DISCUSSION

By adapting a previously described EP transposon-MS2 aptamer tagging and visualization method (JayaNandanan et al. 2011), we have identified 47 genes that encode mRNAs with the capacity to localize to the axonal and/or dendritic processes of Drosophila class IV da neurons. Although confirming the endogenous localization of these candidates via in situ hybridization has not yet been possible due to technical limitations, the enrichment of genes with known roles in neuron development and the functional requirement for many of them in dendritic arborization demonstrated by RNAi support the validity of our initial results.

Utility of the EP-MS2 method

We took advantage of the tendency of MCP-labeled RNAs to form discrete RNP particles in class IV da neurons (Brechtieh and Gavis 2008; Xu et al. 2013) to quantitatively identify transcripts with statistically significant localization patterns. It is likely, however, that some truly localized transcripts were missed due to a high degree of variability in particle numbers among neurons from the same line. Such variability was particularly evident in some lines reimaged after months or years, suggesting that EP-MS2 insertions at some genomic loci may be susceptible to silencing over time. Screens performed in parallel for RNAs localized in the ovary, terminal cells of the trachea and in neuromuscular junction (NMJ) encountered difficulties due in part to the lack of discrete, quantifiable particles and confounding background fluorescence from the surrounding tissue layers. The class IV da neuron screen was indeed advantaged by the ease with which these very superficial neurons could be imaged. Despite the limitations, this method proved effective at identifying a collection of genes whose function may be locally required for neuronal development and/or function.

Localized mRNAs encode functionally diverse proteins

Consistent with transcriptome studies in neuronal processes (for examples, see Cajigas et al. 2012; Minis et al. 2014), the set of positive candidates identified in our screen comprise genes encoding proteins with diverse functions, including structural proteins, cell-surface receptors, intracellular signaling pathway components, and even transcription factors. Although the identification of transcription factors and DNA binding proteins within this candidate pool may seem surprising, mRNAs encoding several transcription factors including cAMP response element binding protein (CREB) have previously been shown to be axonally and/or dendritically localized. CREB synthesized in the processes is then transported retrogradely to the nucleus, linking events in the periphery to transcriptional responses (Eberwine et al. 2001; Jung et al. 2012). Interestingly, the chromosomal protein High mobility group protein D and the transcription factor Escargot, both identified in the screen, have previously been implicated in neuronal morphogenesis: High mobility group protein D was found to regulate branching of class I da neurons (Parrish et al. 2006), while Escargot has been implicated in axonal development (Ramat and Gho 2013). Functional sorting of our positive candidates revealed that at least 10 had previously been shown to play a role in nervous system development, with many contributing directly to the development of the peripheral nervous system. Our secondary RNAi screen confirmed functions for seven of these genes in class IV da neuron dendrite morphogenesis, and further identified 12 new genes that also regulate this process. These new candidates included a polarity protein, a microtubule-associated protein, several kinases, and an additional transcription factor, hinting at the possibility that the local translation of many functionally diverse proteins may be regulated coordinately to orchestrate dendrite morphogenesis.

mRNA localization in dendrite morphogenesis and beyond

As described above, results from the secondary RNAi screen indicate that a large number of the identified genes influence dendrite morphogenesis in class IV da neurons. While many studies have investigated requirements for the localization of mRNAs and/or RNA binding proteins and translation factors during the morphogenesis and remodeling of postsynaptic dendritic spines (Mikl et al. 2010; Thomas et al. 2014), the role of mRNA localization and local translation in the gross morphogenesis phenotype. A decrease in branching relative to control neurons was most frequently observed (nine genes; Table 1 and Figure 3, G and H). For example, knockdown of two kinases, Pdk1 and IP3K1, resulted in loss of higher order branches, and consequent reduction in overall coverage of the receptive field (Figure 3, C and D). Interestingly, Pdk1 protein has previously been shown to localize to the Drosophila neuromuscular junction, where it positively regulates synaptic bouton size (Cheng et al. 2011). Our data suggest that Pdk1 may also act locally in da neuron dendrites. In contrast, knockdown of seven genes resulted in increased branching relative to control neurons (Table 1 and Figure 3, F and G). Examples of genes displaying this phenotype include the ionotropic receptor-encoding gene Ir68A and the poorly characterized gene CG9922 (Figure 3, E and F). Notably, knockdown of CG9922 also caused defects in dendritic self-avoidance (Figure 3F), suggesting a broad regulatory function in dendrite morphogenesis. In several cases, we observed alterations in branch length that were not explained by changes in branch number (Figure 3, F and G). Finally, we observed one instance of patchy defects in patterning of the arbor and spacing of branches that was not reflected by quantification of total dendritic length or terminal branch number (Figure 3, B, G, and H).
Figure 3 RNAi screen for dendritic arborization defects. (A–F) Confocal z-series projections of representative ddaC neurons with ppk-GAL4 driving expression of UAS-CD4-gfp alone (control neuron; A) or together with the indicated UAS-RNAi transgene. (B) Representative neuron exhibiting abnormal patterning with patchy overbranching (arrowheads) not reflected by the quantitative measures used. (C, D) Representative images of neurons exhibiting underbranched phenotypes. Class III da neurons are occasionally labeled by ppk-GAL4 (asterisk). (E, F) Representative images of neurons with overbranched phenotypes. Arrowheads indicate dendrite crossing events, signifying a failure of self-avoidance. (G–H) Quantification of branch length and number of terminal branches in neurons of each genotype. Two neurons from each of six larvae (12 neurons in total) were analyzed for each RNAi line. Two neurons from each of 10 larvae (20 in total) were analyzed for the control. Values shown are mean ± SEM; * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. Scale bar = 100 μm.
Acknowledgments

We thank I. Aprill, A. Mathe, K. Wray, J. Halstead, O. Davis, R. Parton, U. Abdu, and L. Vogelsang for assistance at various stages of the work. This support was provided by National Institutes of Health (NIH) grants R01GM067758 (E.R.G.), DFG LE 54617-1 (M.L.), and DFG RO 890/5-1 (M.L. and S.R.), a Wellcome Trust Senior Research Fellowship (grant 096144 to I.D., supporting D.E. and A.M.V.), and Wellcome Trust Strategic Awards (grant 091911 and 107457 to I.D.) at Micron Oxford (http://micronoxford.com).

Literature Cited

Bertrand, E., P. Chartrand, M. Schaefer, S. M. Shenoy, R. H. Singer et al., 1998 Localization of ASH1 mRNA particles in living yeast. Mol. Cell 2: 437–445.

Brechbiel, J. L., and E. R. Gavis, 2008 Spatial regulation of nanos is required for its function in dendrite morphogenesis. Curr. Biol. 18: 745–750.

Cajigas, I. J., G. Tushev, T. J. Will, S. tom Dieck, N. Fuerst et al., 2011 Spatial regulation of Chong, L., C. Locke, and G. W. Davis, 2011 S6 kinase localizes to the ER to control dendrite morphogenesis in class IV da neurons (Brechbiel and Gavis 2008). The current results build on those initial findings to suggest that mRNA localization may be utilized widely for the regulation of dendrite growth and branching.

Genes involved in other dynamic processes such as sensory processing and adaptation may also be represented among the positive EP-MS2 lines but would not have been detected in our RNAi screen, which focused specifically on dendrite morphology. Furthermore, for 26 lines, we observed RNA particles in axons as frequently as in dendrites; the genes tagged in these lines may play important roles in axon development or function. Because class IV da neuron axons fasciculate with each other and with other peripheral neurons, visualization of axonal morphology requires analysis of single neuron mutant clones. Future mutant studies using mosaic analysis with a repressible cell marker (MARC) may reveal roles for these localized RNAs in axonal development and function.

Acknowledgments

We thank I. Aprill, A. Mathe, K. Wray, J. Halstead, O. Davis, R. Parton, U. Abdu, and L. Vogelsang for assistance at various stages of the work. This support was provided by National Institutes of Health (NIH) grants R01GM067758 (E.R.G.), DFG LE 54617-1 (M.L.), and DFG RO 890/5-1 (M.L. and S.R.), a Wellcome Trust Senior Research Fellowship (grant 096144 to I.D., supporting D.E. and A.M.V.), and Wellcome Trust Strategic Awards (grant 091911 and 107457 to I.D.) at Micron Oxford (http://micronoxford.com).

Literature Cited

Bertrand, E., P. Chartrand, M. Schaefer, S. M. Shenoy, R. H. Singer et al., 1998 Localization of ASH1 mRNA particles in living yeast. Mol. Cell 2: 437–445.

Brechbiel, J. L., and E. R. Gavis, 2008 Spatial regulation of nanos is required for its function in dendrite morphogenesis. Curr. Biol. 18: 745–750.

Cajigas, I. J., G. Tushev, T. J. Will, S. tom Dieck, N. Fuerst et al., 2011 Spatial regulation of Chong, L., C. Locke, and G. W. Davis, 2011 S6 kinase localizes to the ER to control dendrite morphogenesis in class IV da neurons (Brechbiel and Gavis 2008). The current results build on those initial findings to suggest that mRNA localization may be utilized widely for the regulation of dendrite growth and branching.

Genes involved in other dynamic processes such as sensory processing and adaptation may also be represented among the positive EP-MS2 lines but would not have been detected in our RNAi screen, which focused specifically on dendrite morphology. Furthermore, for 26 lines, we observed RNA particles in axons as frequently as in dendrites; the genes tagged in these lines may play important roles in axon development or function. Because class IV da neuron axons fasciculate with each other and with other peripheral neurons, visualization of axonal morphology requires analysis of single neuron mutant clones. Future mutant studies using mosaic analysis with a repressible cell marker (MARC) may reveal roles for these localized RNAs in axonal development and function.