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Supplemental Data

Local N-Cadherin Interactions Mediate Distinct Steps in the Targeting of Lamina Neurons

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Supplemental Experimental Procedures

Genetics

Specificity of *dacFLP* mosaics was assessed by MARCM with widely expressed markers (for example, *elav^{c155}GAL4* and *tubulin GAL4*, see genotypes [a] and [f] below). Labeled cells with processes in the outer medulla other than L1-L5 were only rarely observed. Several stocks (see below) contained *hsFLP* in addition to *dacFLP* transgenes but no heat-shocks were applied. *dacFLP* clones included all of L1-L5 regardless of whether *hsFLP* transgenes were present. We would wish to point out that in a previous study using *dacFLP* and MARCM on chromosome 3L with *actinGAL4 UAS mCD8GFP* as marker labeled cells other than L1 and L2 were only rarely observed (Millard et al., 2007). The 9-9 *GAL4*, 6-60 *GAL4* and 11-64 *GAL4* markers were identified by screening a collection of *GAL4* enhancer trap lines (generously provided by Ulrike Heberlein). Most *GAL4* drivers used are also expressed outside the lamina, but clones were almost completely restricted to lamina neurons (marker specificity in lamina neurons is as indicated below). Transgenes shown in parentheses (included to increase the GFP signal) were not used in all experiments with the respective genotype. *CadN* indicates either *CadN^{M19} or CadN$^{\Delta 14}$*. *FRT40* served as wild-type control.

Experimental genotypes :

(a) L1 and L2 MARCM (some other lamina neurons are also labeled occasionally): *tubulin GAL80 GMR-RFP FRT40* / *CadN FRT40; tubulin GAL4 UAS mCD8GFP* / *dacFLP*

(b) L3 MARCM (labeling is L3-specific throughout development): (*UAS mCD8GFP hsFLP)* / +; *tubulin GAL80 FRT40* / *CadN FRT40*; *9-9 GAL4 UAS mCD8 GFP* / *dacFLP*

(c) L4 and L3 MARCM (some other lamina neurons are also labeled occasionally): *UAS mCD8GFP hsFLP*/+; *tubulin GAL80 FRT40* / *CadN FRT40*; *11-64 GAL4 (UAS N-sybGFP*) / *dacFLP*

(d) L5 MARCM (strong labeling is L5-specific throughout development, weakly labeled L4s were also occasionally observed): *UAS mCD8GFP hsFLP*/+; *tubulin GAL80 FRT40* / *CadN FRT40*; *6-60 GAL4 UAS N-syb GFP* / *dacFLP*

(e) L5 reverse MARCM (labeling specificity as in [d]): *UAS mCD8GFP hsFLP*/+ ; *FRT40* / *CadN^{M19} tubulin GAL80 FRT40*; *6-60 GAL4 UAS N-syb GFP* / *dacFLP*

(f) L1, L2 and L4 MARCM for developmental analyses (all of L1-L5 are labelled): *elav^{C155} GAL4 hsFLP UAS mCD8GFP* / (*UAS mCD8GFP hsFLP)*; *tubulin GAL80 FRT40*/ *CadN FRT40*; *dacFLP* / +

(g) L4 MARCM for developmental analyses (labeling is L4 specific): *tubulin GAL80 hsFLP FRT19* / *FRT19; apterous GAL4 UAS mCD8GFP; dacFLP* / +

(h) R7 MARCM: *FRT40* / *CadN*^{M19} *tubulin GAL80 FRT40 GMR-FLP*; *tubulin GAL4 UAS N-sybGFP* / +
(i) R8 targeting was scored in *ey3.5FLP* (Bazigou et al., 2007)/ +; *cycE FRT40* / *CadN FRT40*; *Rh5-lacZ* / + animals.

L1-L5 subtype identification and phenotypic analyses
R7 and R8 (mAb 24B10) and anti-CadN (mAb DN-Ex#8) staining were used as references to estimate layer positions and column boundaries. The overall patterns of processes of *CadN* mutant lamina neurons in the lamina were similar to wild-type controls (see Figure S2). By such morphological criteria, the *GAL4* drivers used in the mosaic experiments (see genotypes listed above) are expressed in the same cell-type(s) in wild-type and *CadN* mosaic flies. To identify mutant L1-L5 in samples with labeling of different lamina neurons, axons were traced from medulla to the lamina, and cell types designated based on lamina dendrite patterns. In the case of L1 and L2, which have essentially identical dendrites, axons with arbors in M1 and M5 (and sometimes projections beyond M5) were classified as L1 and those with terminals in M2 were designated as L2 neurons. In the developmental analyses, specific markers were used for L3 and L5 and some L4s (see above). L1, L2 and the remaining L4s were identified by cell morphology.

Histology
Fly brains were dissected in PBS and fixed with PLP (2% paraformaldehyde, 75 mM lysine, 37 mM sodium phosphate buffer pH 7.4) for 1 hr (adult brains), or 40 min (pupal brains). Primary and secondary antibody incubations were in PBS with 0.5% Triton X-100 and 5 % normal goat serum, typically overnight at 4 °C. Brains were mounted for microscopy in vectashield (Vector Laboratories). Primary antibodies (used at the indicated dilutions) were rabbit anti lacZ (Cappel, 1: 1000), mAb 24B10 (1:20) (Zipursky et al., 1984), rat mAb DN-Ex #8 (1:20) (Iwai et al., 1997), anti-GFP rabbit serum (Molecular probes, 1:1000). Secondary antibodies were Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Molecular Probes, 1:1000), Cy3 F(ab')$_2$ Fragment Goat Anti-Mouse IgG (H+L) (Jackson Immuno Research, 1:300), Cy3 Goat Anti-Rat IgG (H+L) (minimal cross-reaction) (Jackson Immuno Research, 1:300), Cy5 F(ab')$_2$ Fragment Donkey Anti-Mouse IgG (H+L) (minimal cross-reaction) (Jackson Immuno Research, 1:200). The secondary antibodies with minimal cross-reaction were used for stainings that included both mAb 24B10 and mAb DN-Ex #8. Specificity of DN-Ex #8 mAb for CadN was confirmed by staining of mutant lamina neuron growth cones (see Figure S1) and has been previously demonstrated for labeling of embryos (Iwai et al., 1997) and pupal retinas (Nern et al., 2005).

Microscopy and image analysis
Zeiss LSM image files were imported into Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA,http://rsb.info.nih.gov/ij/, 1997-2007.) and converted to 8-bit TIF files. CadN and GFP signals across layer(s) or column(s) were measured by selecting a rectangular region of interest and using the "plot profile" command in Image J. The measurements were graphed using Microsoft Excel. Images were analyzed with Image J and Zeiss LSM software and figures were assembled

using Adobe CS2 programs. Image processing involved some linear adjustments to brightness and contrast, selection of smaller fields of view from larger images, adjustments of image orientation and LUT (look up table) changes (e.g. ImageJ 16-color LUT for pseudocolor images).
CadN staining patterns were reproducible between samples but overall fluorescence signal and noise unavoidably shows some variation between sections and samples. Some adjustments of laser power, gain and black level settings were therefore made during image acquisition to obtain similar overall fluorescence signals.To assess potential changes in anti-CadN staining in columns with mutant cells, staining of other columns in the same section (see Figure S1B) was used as an internal control for overall staining intensity and quality.

Supplemental References

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Iwai, Y., Usui, T., Hirano, S., Steward, R., Takeichi, M., and Uemura, T. (1997). Axon patterning requires DN-cadherin, a novel neuronal adhesion receptor, in the Drosophila embryonic CNS. Neuron *19*, 77-89.
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Nern, A., Nguyen, L. V., Herman, T., Prakash, S., Clandinin, T. R., and Zipursky, S. L. (2005). An isoform-specific allele of Drosophila N-cadherin disrupts a late step of R7 targeting. Proc Natl Acad Sci U S A *102*, 12944-12949.
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Supplemental Figures and Legends

Figure S1 CadN is expressed in lamina neurons

(A) CadN is strongly expressed in lamina neurons during development. Confocal images show CadN protein (mAb DN-Ex#8, purple [left] or pseudocolor [right]; see [C] for pseudocolor scale) and R cells (mAb 24B10, green [left]) in an optic lobe heterozygous for $CadN^{M12}$ at 35 hrs APF. $CadN^{M12}$ /+ pupae were used to facilitate CadN detection in cell bodies (see text). No defects in the overall organization of the optic lobes were observed in this genotype. Boxed region in top right panel is shown in higher magnification in lower panels. Letters in top left panel indicate the location of lamina (La) and medulla (Me) cell bodies (CB) and neuropils (NP). Lamina neuron cell bodies are located above the lamina plexus (brackets in bottom left panel). The strong CadN staining in the medulla is in the neuropil. Scale bar, 10 μm. (B, C) CadN is strongly expressed on growth cones of lamina neurons. Confocal sections with individual labeled wild-type or *CadN* null mutant ($cadN^{\Delta 14}$) lamina neurons at 48 hrs APF are shown. Lamina neuron clones were generated by MARCM with *dacFLP* (see text and Figure S2). Images show lamina neurons (anti-GFP staining, green in top and grey in middle panels) and CadN protein (purple in top and pseudocolor in bottom panels). Lamina neurons are marked with dashed outlines in the two lower panels in (C). The repetitive columnar structure of the medulla makes it possible to distinguish overall changes in staining intensity from specific changes in a column with a mutant cell (illustrated for a mutant L2 [circled] in [B]) Note that many areas of strong CadN staining overlap with growth cones of specific wild-type lamina neurons and are absent or reduced in columns with mutant growth cones ($n \geq 10$ growth cones examined for each cell type). Arrow points to the position of a hypothetical wild-type L3 growth cone in a column with a mistargeted mutant L3. We were unable to associate CadN protein with L4 growths cones this way (not shown). CadN is expressed in L4 as L4 requires CadN in a cell autonomous fashion (See text). Scale bars, 5 μm.

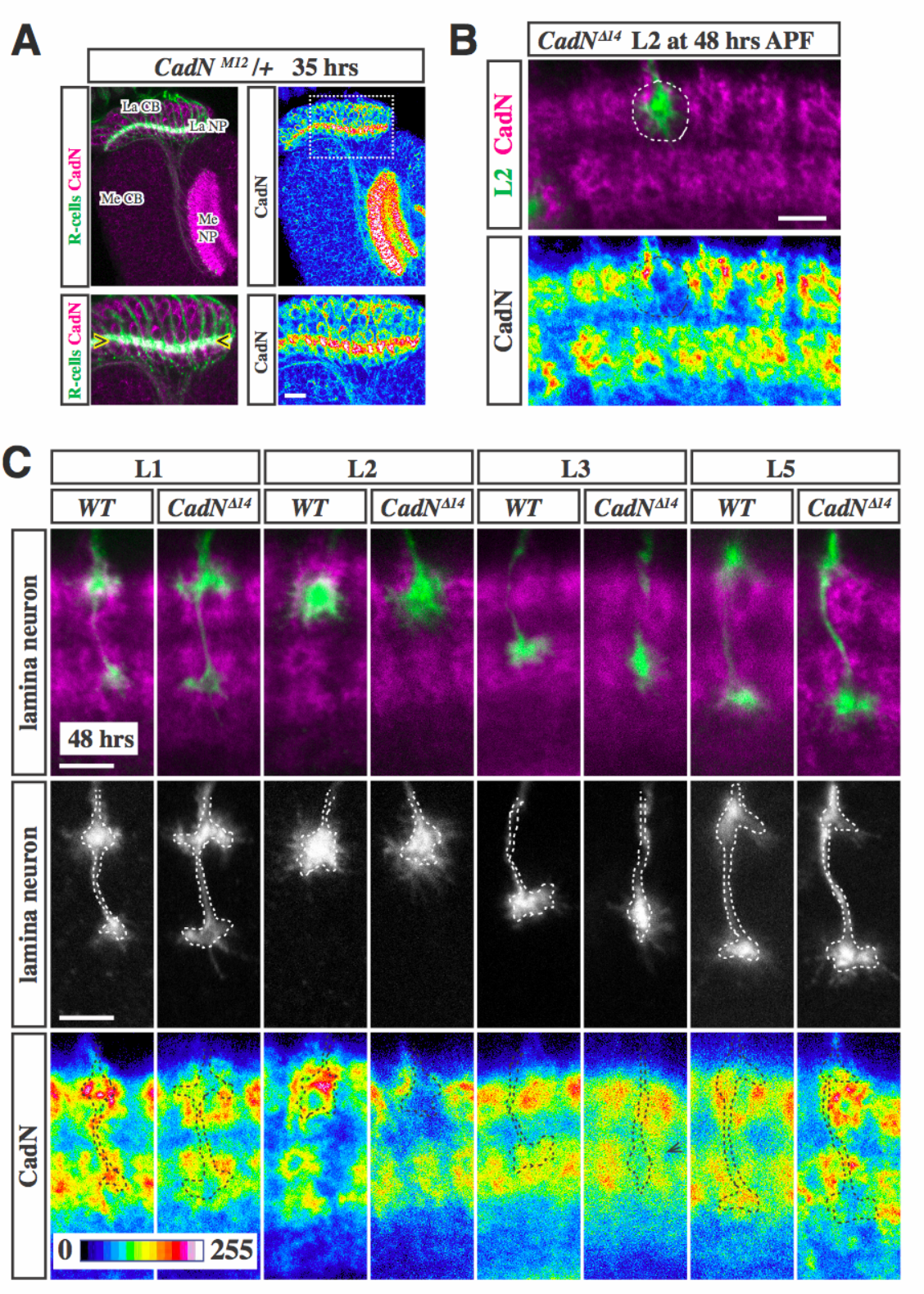
A
CadN M12 /+ 35 hrs
La CB
La NP
Me CB
Me NP
R-cells CadN
CadN
B
CadN Δ14 L2 at 48 hrs APF
L2 CadN
CadN
C
L1
L2
L3
L5
WT
CadN Δ14
lamina neuron
48 hrs
CadN
0
255

Figure S1

Figure S2

Single cell analyses of lamina neuron targeting. (A,B) Genetic mosaic techniques used in this study. *dacFLP* was used to induce FLP-recombinase mediated mitotic recombination between FRT sites (black rectangles) in lamina neuron precursor cells. Homozygous wild-type or mutant clones were visualized by MARCM (A) or reverse MARCM (B). In both cases, the presence of the Gal80 repressor prevents Gal4-mediated expression of UAS-GFP reporter constructs. As a result, only cells without Gal80 are labeled by GFP expression. (A) MARCM. Gal80 and the mutation of interest (pink asterisk) are located on the same arm of different chromosomes. Therefore only cells homozygous for the mutation (after mitotic recombination) are labeled with GFP. Unlabelled mutant cells may also be present depending on the GAL4 driver used. (B) Reverse MARCM. Because the mutation and Gal80 are on the same chromosome, only cells homozygous for the wild type allele are labeled. Unlabelled mutant lamina neurons are present and may affect the phenotype of the labeled wild-type cells. (C) Example of *dacFLP* MARCM clones. A late pupal (> 80 hrs APF) optic lobe with $CadN^{M19}$ *dacFLP* clones stained with antibodies specific for GFP (green), CadN (blue) and R-cells (red) is shown. With markers that label subsets of lamina neurons (e.g. mainly L1 and L2 with the *tubulin GAL4 UAS mCD8GFP* combination used here) clones were typically sparse enough for projections to be traced from lamina to medulla. (D) *CadN* mutant lamina neuron subtypes retain distinct branching patterns in the lamina. Cartoons illustrate wild-type morphology. Confocal images show MARCM clones of wild-type and $CadN^{M19}$ mutant L1-L5 in the lamina. With the exception of L1 and L2, which have very similar dendrites in both wild-type and mutant, wild-type and *CadN* L1-L5 classes can be distinguished by dendritic morphologies in the lamina. The distinction between L1 and L2 was based on medulla terminal morphology. Some abnormalities in the fine structure of dendritic processes in the lamina were observed in *CadN* mutants (see examples shown for L1/L2). Images are single confocal sections except L4 $CadN^{M19}$, which is a projection of a few sections. Scale bar, 5 μm.

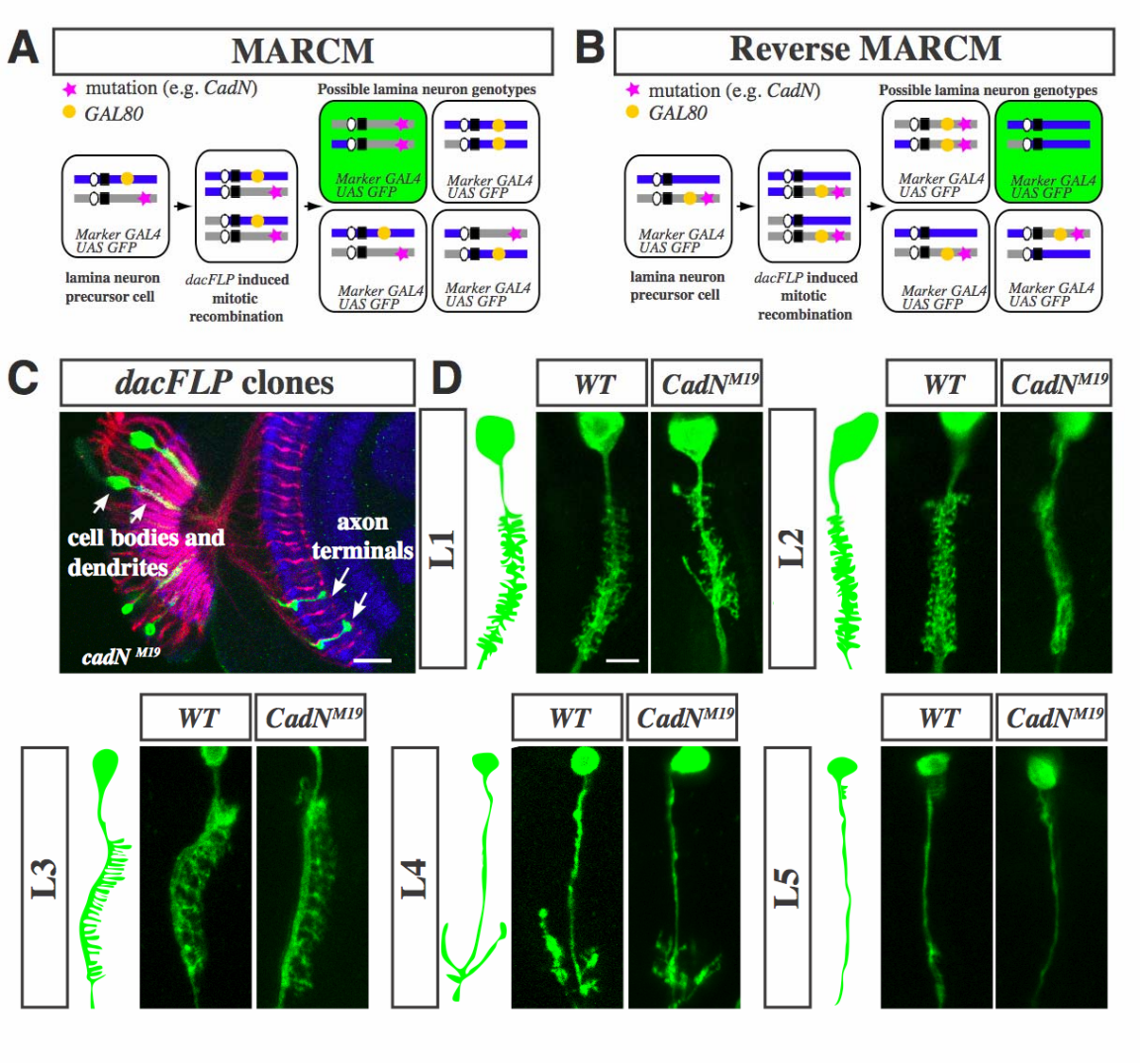
A
MARCM
mutation (e.g. CadN)
GAL80
Possible lamina neuron genotypes
Marker GAL4 UAS GFP
lamina neuron precursor cell
dacFLP induced mitotic recombination
B
Reverse MARCM
C
dacFLP clones
cell bodies and dendrites
axon terminals
cadN M19
D
WT
CadN M19
L1
L2
L3
L4
L5

Figure S2

Figure S3

Development of L1, L2 and L4 projections. (A) Time course of wild-type L1, L2 and L4 development. Single confocal sections of MARCM clones at indicated times APF are shown. To highlight the unusual intercolumnar position of L4 (see B), approximate column boundaries (based on R-cell and CadN labeling) are indicated by dotted lines in some panels. Images on the left show R-cells (mAb 24B10 staining, red), lamina neurons (anti-GFP, green), and CadN protein (mAb DN-Ex#8, blue); images on the right show only the GFP channel. Growth cones were labeled with *elav*^{C155} *GAL4* and *UAS mCD8 GFP*. Scale bar, 5 μm. Lower black rectangle in 33 hrs APF images is not part of actual confocal picture. (B) Specific patterns of CadN expression correlate with targeting of L4 collateral and terminal branches in the developing M2 and M4 layers. L4 axons turn anteriorly after entering the medulla neuropil (cartoon at top, also see A) and extend proximally between the two anterior neighbors of their column of origin. Confocal images show column cross-sections at the level of the collateral ("M2") and terminal L4 ("M4") branches and at an intermediate position in the developing M3 layer ("M3"). Quotation marks are used to distinguish layers at 48 hrs APF from adult layers. CadN protein (purple [top] or pseudocolor [bottom]) shows a distinct columnar pattern that overlaps with L4 processes. CadN (blue), R7 axons (red) and and collaterals of one L4 (green) in the developing M2 layer are also shown in cartoon form (Bottom Left). The dotted green line indicates the columnar origin of the illustrated L4. In *CadN* mutants, L4s often do not terminate in M4 and frequently have no or disorganized collaterals in M2 (see Figure 1C and not shown).

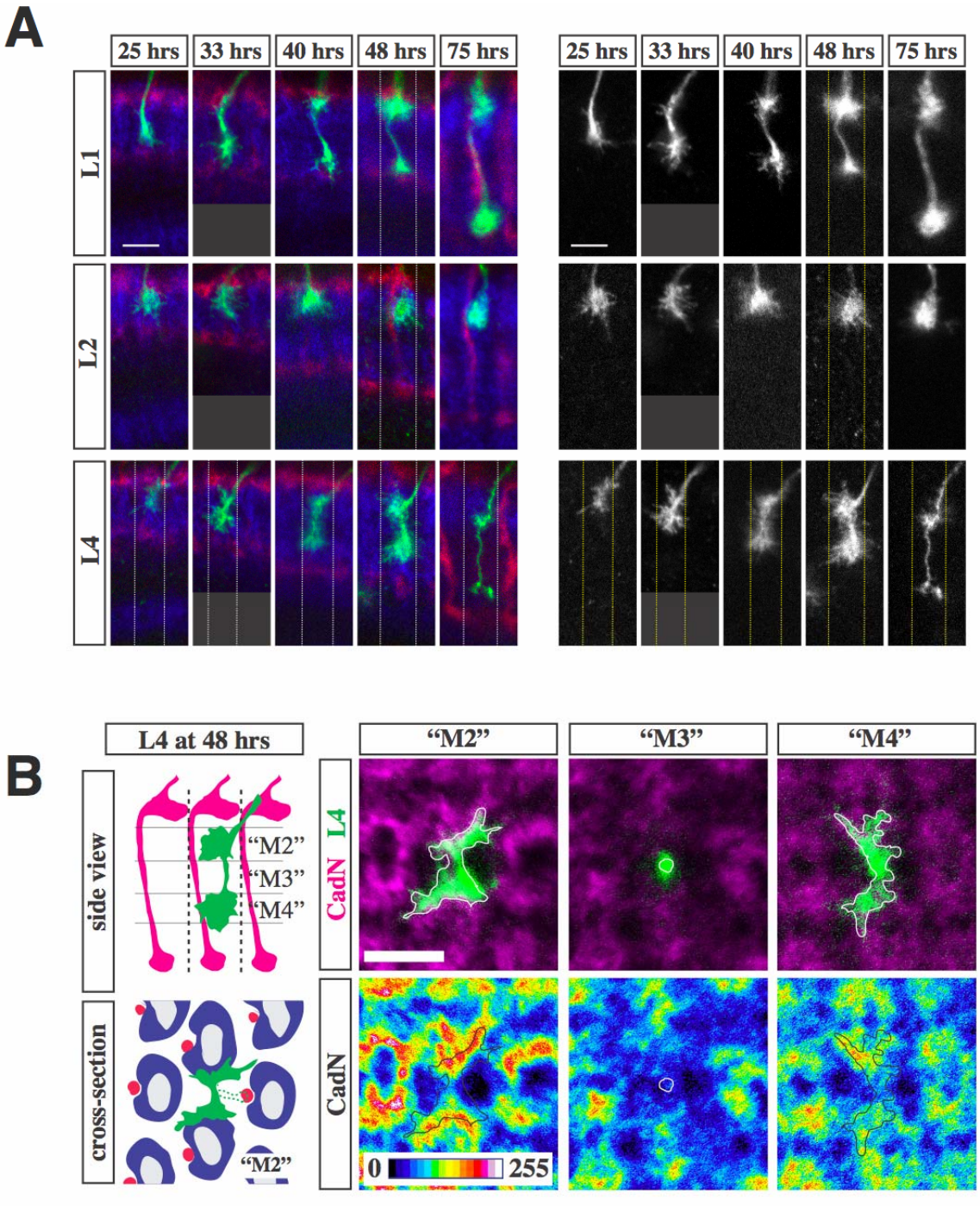
A
25 hrs
33 hrs
40 hrs
48 hrs
75 hrs
L1
L2
L4
B
L4 at 48 hrs
side view
cross-section
"M2"
"M3"
"M4"
CadN L4
CadN
0
255

Figure S3