The microRNA *bantam* regulates a developmental transition in epithelial cells that restricts sensory dendrite growth

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**ABSTRACT**

As animals grow, many early born structures grow by cell expansion rather than cell addition; thus growth of distinct structures must be coordinated to maintain proportionality. This phenomenon is particularly widespread in the nervous system, with dendrite arbors of many neurons expanding in concert with their substrate to sustain connectivity and maintain receptive field coverage as animals grow. After rapidly growing to establish body wall coverage, dendrites of *Drosophila* class IV dendrite arborization (C4da) neurons grow synchronously with their substrate, the body wall epithelium, providing a system to study how proportionality is maintained during animal growth. Here, we show that the microRNA *bantam* (*ban*) ensures coordinated growth of C4da dendrites and the epithelium through regulation of epithelial endoreplication, a modified cell cycle that entails genome amplification without cell division. In *Drosophila* larvae, epithelial endoreplication leads to progressive changes in dendrite-extracellular matrix (ECM) and dendrite-epithelium contacts, coupling dendrite-substrate expansion and restricting dendrite growth beyond established boundaries. Moreover, changes in epithelial expression of cell adhesion molecules, including the beta-integrin *mysopheroid* (*mys*), accompany this developmental transition. Finally, endoreplication and the accompanying changes in epithelial *mys* expression are required to constrain late-stage dendrite growth and structural plasticity. Hence, modulating epithelium-ECM attachment probably influences substrate permissivity for dendrite growth and contributes to the dendrite-substrate coupling that ensures proportional expansion of the two cell types.

**KEY WORDS:** Dendrite, Endoreplication, Extracellular matrix, Growth control, Plasticity, *Drosophila*

**INTRODUCTION**

A central question in growth control of multicellular organisms is how growing organisms maintain proportionality. This problem is particularly complex when different types of interacting cells must grow in a coordinated fashion, a scenario that is widespread in the nervous system. For example, as animals grow, dendrite arbors of many neurons expand proportionally to sustain proper connectivity and maintain receptive field coverage (Bentley and Tororian-Raymond, 1981; Bloomfield and Hitchcock, 1991; Hitchcock, 1987; Parrish et al., 2009; Truman and Reiss, 1988). This scalar expansion of dendrite arbors to accommodate growth is widely documented in sensory systems, including many types of invertebrate sensory neurons and vertebrate retinal ganglion cells (RGCs). In many cases, dendrites outpace substrate growth to establish appropriate coverage, and subsequently scale with substrate expansion to maintain coverage. Thus, neurons and their substrates differentially respond to common growth cues, receive distinct growth cues, or some combination of both.

Several observations suggest that neuron non-autonomous growth-inhibitory signals contribute to the fidelity of dendrite arbor expansion by restricting dendrite arbors to target fields. Following ablation of RGCs or starburst amacrine cells, the surviving cells developed regularly spaced dendrite arbors that exhibited a limited ability to expand into unoccupied territory (Farajian et al., 2004; Lin et al., 2004). Therefore, interactions between neighboring dendrites are largely dispensable for maintenance of coverage in these neurons and unknown constraints limit their growth potential. Supporting the argument in favor of an extrinsic component, these dendrite arbors expand in sync with retinal growth, whereas exuberant growth is limited.

*Drosophila* peripheral nervous system (PNS) class IV dendrite arborization (C4da) neurons completely and non-redundantly cover (‘tile’) the larval epidermis early in development and maintain this tiling by growing in precise synchrony with their substrate, the body wall epithelium (Emoto et al., 2006; Grueber et al., 2002; Parrish et al., 2007, 2009). Before establishment of tiling, ablating C4da neurons leads to dendrite growth into vacated territory by adjacent neurons (Grueber et al., 2003; Parrish et al., 2009; Sugimura et al., 2003). However, after tiling is established the invasive growth potential is lost; growth occurs only to maintain proportional receptive field coverage, showing that, as with RGCs, signals constrain late-stage growth of these dendrites. Notably, this signaling does not involve the homotypic repulsion required to establish tiling. Instead, epithelium-derived signals restrict exuberant arbor expansion; the miRNA *ban* acts in epithelial cells to regulate substrate-derived growth-inhibitory signals that constrain dendrite growth (Parrish et al., 2009).

Here, we report our characterization of the *ban*-regulated epithelial signaling that regulates dendrite growth. We found that *ban* regulates epithelial endoreplication, which is required for postembryonic body wall epithelial growth, and that manipulating epithelial endoreplication using *ban*-independent approaches recapitulates *ban*-mediated effects on dendrite growth. Endoreplication influences epithelium-dendrite and epithelium-extracellular matrix (ECM) interactions, providing the cellular basis for the reduced dendrite growth potential that accompanies larval development. At a molecular level, endoreplication alters epithelial expression of cell adhesion molecules, including the integrin Mys, which is required for proper coupling of dendrite and substrate expansion and to restrict dendritic structural plasticity. Thus, a developmental transition in epithelial growth constrains dendrite growth to ensure synchronous expansion of dendrites and their substrate.
RESULTS
Identification of ban-regulated pathways
The microRNA ban functions as a regulatory switch for substrate-derived signaling that restricts PNS dendrite growth/plasticity and ensures proportional expansion of dendrite and substrate (Fig. 1A) (Parrish et al., 2009). To identify the substrate-derived factors required for proportional dendrite/substrate growth, we conducted microarray-based expression profiling of epithelial cells from wild-type and ban mutant larvae (Fig. 1B). We identified ~100 transcripts that were significantly deregulated in ban mutant epithelial cells (Fig. 1C; supplementary material Table S1). Lexical analysis (Kim and Falkow, 2003) indicated that genes associated with the cell cycle were enriched in this dataset, suggesting that ban regulates the cell cycle in epithelial cells; we also identified a large number of transcripts associated with cell growth and adhesion.

Drosophila increase their mass ~200-fold during larval development, and this growth is accomplished by cell expansion rather than cell addition (Britton and Edgar, 1998; Church and Robertson, 1966). Indeed, we found that body wall epithelial cell number is constant from late embryogenesis to late larval stages, and that epithelial cells infrequently turn over, as epithelial cell clones persist through larval development >98% of the time (n=200; Fig. 1D,E). Body wall epithelial cell number is unresponsive to ban activity (Fig. 1D); thus ban regulation of epithelial growth must involve growth of existing cells rather than proliferation. Although ban is known to regulate proliferation, and hence growth, in mitotically active tissues (Brennecke et al., 2003; Hipfner et al., 2002), how ban regulates growth of differentiated cells is unknown.

To facilitate growth, many larval cell types undergo endoreplication, a modified cell cycle that entails DNA replication without cell division (Britton and Edgar, 1998; Smith and Orr-Weaver, 1991). Consistent with a change in endoreplication, ban mutant epithelial cells exhibited dysregulation of cell-cycle-associated genes, including reduced expression of two regulators of endoreplication, double parked (dup) and retina aberrant in pattern (rap) (Park and Asano, 2008; Pimentel and Venkatesh, 2005; Sigrist and Lehner, 1997; Zielke et al., 2008), which encode orthologs of the DNA replication factor CDT1 and the APC/C activator CDH1/FZR1, respectively (Fig. 1C). We therefore hypothesized that ban regulates growth of mitotic (e.g. imaginal discs) and postmitotic body wall epithelial cells by regulating different forms of the cell cycle.

To monitor larval endoreplication during the period of ban activity required for dendrite growth, we fed first instar larvae BrdU for 1 day and monitored BrdU incorporation in third instar larvae. As a positive control we monitored BrdU incorporation in the ventral ganglion, which contains mitotically active neuroblasts and endoreplicating glia (Truman and Bate, 1988; Unhavaithaya and Orr-Weaver, 2012), and observed extensive labeling (Fig. 2A,B). Likewise, we observed extensive labeling of epithelia and muscle, but no labeling of sensory neurons, even when BrdU was constantly administered (Fig. 2C). We therefore conclude that larval body wall epithelia and muscle, but not sensory neurons, endoreplicate.

To monitor the timing and extent of endoreplication in the larval epidermis we measured DNA content in epithelial cells over development. During embryogenesis, epithelial cells and PNS neurons had comparable levels of DAPI staining, and hence DNA content (Fig. 2D,E). Similar to other larval tissues (Britton and Edgar, 1998), body wall epithelia exhibited low levels of endoreplication in first instar larvae; DNA content in epithelial cells was 2.8-fold higher than in PNS neurons. Epithelial ploidy increased throughout larval development, and the rate of endoreplication increased dramatically at 48 h after egg laying (AEL), leading to a ~25-fold increase in genome content by 96 h AEL. Epithelial cell size and ploidy increased at
comparable rates, with the pace of epithelial growth dramatically increasing after 48 h AEL (Fig. 2F). Indeed, we observed a strong linear relationship between epithelial DNA content and cell size, suggesting that the two are tightly coupled (Fig. 2G). By contrast, C4da dendrites expanded most rapidly before 48 h AEL, when dendrites establish coverage of a fixed portion of the body wall (Fig. 2H) (Parrish et al., 2009).

Ban is required in epithelial cells to dampen dendrite growth beginning at ∼48 h AEL (Parrish et al., 2009), after dendrites tile the body wall and coincident with the rapid increase in epithelial endoreplication. We therefore assayed for ban function in endoreplication by monitoring BrdU incorporation in ban mutant larvae and found that BrdU incorporation was reduced by an average of 56% in ban mutant body wall epithelial cells compared with controls (Fig. 2I). Likewise, epithelial ploidy was significantly reduced in ban mutants, as in dup mutants and larvae with epithelium-specific expression of dup(RNAi) (Fig. 2J; supplementary material Figs S2 and S3); rap mutants exhibited reduced ploidy, but the change was not significant, perhaps owing to protein perdurance. By contrast, epithelial ploidy was significantly increased by overexpression of ban or diminutive (dm; encodes Drosophila Myc), which promotes endoreplication in a variety of Drosophila cell types (Pierce et al., 2004). Thus, ban is necessary and sufficient to promote endoreplication in larval body wall epithelial cells, and the major wave of endoreplication is initiated in second instar larvae, corresponding to the time when ban functions in epithelial cells to coordinate dendrite/epithelial growth (Parrish et al., 2009).

We next investigated effects of ban activity and endoreplication on epithelial growth. Previously, we found that ban mutant body wall lysates had decreased levels of phosphorylated Akt (p-Akt) (Parrish et al., 2009), which is often associated with cell growth. Likewise,
epithelial p-Akt levels were significantly decreased in \textit{ban} mutant and \textit{dup} mutant larvae, whereas p-Akt levels were significantly increased in epithelial cells overexpressing \textit{ban} or \textit{dm} (supplementary material Fig. S1), consistent with \textit{ban} and endoreplication promoting epithelial growth. Indeed, \textit{ban} mutant larvae exhibited significant decreases in epithelial cell size, as did \textit{dup} or \textit{rap} mutants, whereas \textit{ban} or \textit{dm} overexpression significantly increased cell size (Fig. 2K). Thus, \textit{ban} regulates larval endoreplication, which promotes growth of larval epithelial cells.

\textbf{Epithelial endoreplication influences dendrite growth}

\textit{C4da} dendrite growth outpaces substrate growth to establish tiling, but growth is altered coincident with onset of epithelial endoreplication, such that dendrite arbors expand synchronously with the epidermis to maintain tiling of the growing body wall (Parrish et al., 2009). \textit{C4da} dendrites in \textit{ban} mutants tile the body wall properly, but dendrite and substrate growth are not synchronized at the first/second instar transition, hence dendrite growth outpaces substrate growth, causing dendrites to occupy larger territories and more densely populate the body wall. As shown in Fig. 3, \textit{C4da} dendrites in \textit{ban} mutant larvae exhibit a ∼30% increase in the territory they cover (coverage index) and a ∼140% increase in dorsal midline occupancy as a result of unchecked late-stage growth.

We hypothesized that local signals, independent of systemic cues that promote larval growth and dendrite expansion, coordinate dendrite/substrate growth and that epithelial endoreplication may be a crucial component of this local substrate-derived control of dendrite growth. We therefore assayed effects of epithelial endoreplication on dendrite growth. First, we monitored effects of reducing endoreplication on dendrite growth. In larvae homozygous for mutations in \textit{dup} or \textit{rap}, which attenuate epithelial endoreplication (Fig. 2J), we observed exuberant late-stage dendrite growth similar to \textit{ban} mutants (Fig. 3C,G). Expression of \textit{dup} (RNAi) with an epithelium-specific driver (supplementary material Fig. S2) similarly reduced...
epithelial endoreplication and caused exuberant dendrite growth, whereas resupplying UAS-dup to epithelial cells rescued the dup mutant dendrite growth defects (supplementary material Fig. S3), demonstrating that dup is required in epithelial cells to regulate endoreplication and dendrite growth. Finally, sustained epithelial expression of Cyclin E, which inhibits progress through endoreplication cycles (Weiss et al., 1998), caused dendrite defects similar to ban mutants (Fig. 3D,G), demonstrating that epithelial endoreplication is necessary for modulation of C4da dendrite growth. By contrast, ban or dm overexpression, which increases epithelial endoreplication, dampened late-stage dendrite growth, leading to decreased dendrite coverage and midline occupancy (Fig. 3E-G), consistent with a role for epithelial endoreplication in constraining dendrite growth. As with epithelial ban overexpression (Parrish et al., 2009), epithelial dm overexpression caused dendritic ‘wrapping’ of epithelial cells, possibly reflecting tighter dendrite-epithelium coupling (Fig. 3F,G). Whereas wild-type C4da dendrites were confined to a thin cross-sectional area along the basal surface of epithelial cells (Fig. 3F’), Han et al., 2012; Kim et al., 2012, epithelial dm or ban overexpression caused dendrites to occupy a larger three-dimensional space, particularly in regions exhibiting the ‘wrapping’ behavior.

To test whether ban control of dendrite growth involves epithelial endoreplication, we examined the epistatic relationship between ban and endoreplication regulators (Fig. 3H-N). First, we assayed for genetic interactions between ban and dup. On its own, heterozygosity for mutations in either gene had no significant effect on dendrite coverage or epithelial ploidy, but larvae doubly heterozygous for ban and dup mutations exhibited modest but significant increases in dendrite coverage and reductions in epithelial ploidy (Fig. 3H,L-N), suggesting that ban and the endoreplication effector dup function in a genetic pathway to regulate epithelial endoreplication and hence dendrite growth. Second, we overexpressed ban in dup mutant larvae and found that reducing dup function blocked the effects of epithelial ban overexpression on dendrite growth and epithelial ploidy (Fig. 3I), demonstrating the requirement for endoreplication in ban-mediated dendrite growth control. Third, we overexpressed dm in epithelial cells of ban mutant larvae, taking advantage of the temperature-sensitive nature of the Gal4-UAS system to drive dm expression at different levels. Consistent with dm functioning downstream of ban to promote endoreplication and constrain dendrite growth, dm expression suppressed the dendrite overgrowth and epithelial endoreplication defects of ban mutants in a dose-dependent fashion (Fig. 3J,K). Altogether, these results demonstrate that ban regulates epithelial endoreplication to modulate dendrite growth.

**Developmental control of dendrite-epithelium interactions**

Increased epithelial endoreplication alters the relative position of dendrites and epithelial cells (Fig. 3F). We hypothesized that epithelial endoreplication affects dendrite-substrate interactions by promoting epithelium-dendrite adhesion, modulating ECM permissivity to dendrite growth, or some combination of the two. Using a genetically encoded proximity sensor, high-resolution confocal imaging of dendrite/ECM markers, and transmission electron microscopy (TEM) of the dendrite/epithelium interface we examined whether dendrite/epithelium interactions change over developmental time in response to ban and endoreplication.

We used GFP reconstitution as a proximity detector (GFP-PD) to monitor dendrite-epithelium apposition with the underlying hypothesis that increased dendrite-epithelium adhesion would be manifest as increased dendrite-epithelium proximity. Based on physical dimensions of the components (Becker et al., 1989; Morell et al., 2008), GFP reconstitution indicates dendrite-substrate proximity of <30 nm, a distance spanned by known adhesion molecules (Fig. 4A). GFP reassembly occurs slowly (Pédelaître et al., 2006); thus dendrite-epithelium apposition must be stable to generate GFP-PD signal. Expressing either half of the proximity sensor produced no GFP-PD signal in vivo, whereas neuronal co-expression of both split-GFP fragments resulted in GFP-PD signal throughout the dendritic arbor of first instar larvae (supplementary material Fig. S4), demonstrating that GFP reconstitution occurs on a timescale amenable to analysis of larval dendrite-epithelium proximity.

To monitor dendrite-epithelium juxtaposition we expressed one-half of the proximity sensor in C4da neurons and the other half in epithelial cells. In first instar larvae, before the surge in epithelial endoreplication, GFP-PD signal was almost undetectable (Fig. 4B). Epithelial ploidy rapidly increases in second instar larvae, and we likewise observed dendritic accumulation of GFP-PD in second instar larvae (Fig. 4C). Epithelial overexpression of ban or dm led to a significant increase in GFP-PD in second instar larvae (Fig. 4D,F,G), suggesting that endoreplication promotes dendrite-epithelium juxtaposition. Conversely, dup mutants exhibited reduced GFP-PD signal that was most pronounced in terminal dendrites (Fig. 4E-G). We conclude that epithelial endoreplication is necessary and sufficient to trigger developmental changes in dendrite-epithelium juxtaposition.

GFP-PD signal was apparent throughout the majority of the dendritic arbor in third instar larvae (Fig. 4H), suggesting that dendrite-epithelium apposition, and probably adhesion, progressively increases throughout larval development. However, GFP-PD signal was unevenly distributed and markedly reduced/absent from many terminal dendrites, suggesting that dendrite-epithelium apposition varies across the dendritic arbor, with the most dynamic portions (terminal dendrites) coupled to epithelial cells to a lesser degree (Fig. 4H). Epithelial ban or dm overexpression, by contrast, led to increased levels of GFP-PD signal throughout the dendrite arbor, including terminal dendrites (Fig. 4I), consistent with a role for epithelial endoreplication in promoting dendrite-epithelium adhesion.

C4da dendrites grow along the basal surface of epithelial cells, attached to the ECM by virtue of dendritic integrins, with a small proportion of dendrites embedded in epithelial cells (Han et al., 2012; Kim et al., 2012). Based on our observation that dendritic and epithelial membranes become more closely juxtaposed as larval development progresses, we hypothesized that dendrite-ECM interactions might be developmentally regulated as well. Using high-resolution confocal imaging (Han et al., 2012), we monitored colocalization of dendrites and ECM components labeled by GFP exon traps, including Collagen IV (vkg-gfp), and Perlecan (trol-gfp) (Fig. 5; supplementary material Figs S5 and S6) (Morin et al., 2001). In first instar larvae, >98% of dendrites co-localize with ECM markers (Fig. 5A,G), but 12% of dendrites in third instar larvae were detached from the ECM and apically shifted (Fig. 5B,G; supplementary material Fig. S5); these detached dendrites are likely embedded in epithelial cells (Han et al., 2012). Significantly fewer dendrites were detached from the ECM in ban or dup mutant third instar larvae, whereas epithelial ban or dm overexpression significantly increased apical ECM detachment of dendrites (>25% of dendrites; Fig. 5C-G; supplementary material Fig. S6). Taken together, these results demonstrate that dendrite-epithelium proximity and dendrite-epithelium interactions (embedding) are developmentally regulated by epithelial endoreplication. Thus, after dendritic coverage of the body wall is established, dendrites become increasingly coupled to epithelial cells.

To corroborate our finding that dendrite-epithelium interactions are developmentally regulated by endoreplication, we examined...
dendrite-epithelium interactions using TEM. In thin sections of abdominal segments cut along the apicobasal body wall axis, we monitored distribution of dendrites (identified as processes near the basal epithelial surface containing arrays of parallel microtubules) and the frequency of plasma membrane invaginations, as internalized dendrites are frequently found in membrane invaginations (Han et al., 2012; Kim et al., 2012). In first instar larvae, most dendrites were positioned at the surface of epithelial cells in direct contact with the ECM; only 3/50 dendrites were enclosed in epithelial cells (Fig. 5H; supplementary material Fig. S7). We observed a substantial increase in epithelium-embedded dendrites in third instar larvae (27/91 dendrites; Fig. 5H). Thus, dendrite-epithelium interactions change substantially from early larval development when dendrites establish body wall coverage to late larval development when dendrites expand proportionally with their substrate. The frequency of membrane invaginations significantly increased in third instar larvae (supplementary material Fig. S7), and this may facilitate dendrite enclosure by epithelial cells. Mutations that inhibited endoreplication blocked the developmental increase in epithelial plasma membrane invagination and dendrite enclosure, whereas treatments that increased epithelial endoreplication had the opposite effects (Fig. 5H; supplementary material Fig. S7). These findings confirm our results using a GFP-based proximity sensor and our in vivo imaging of dendrite-ECM colocalization; altogether, these studies indicate that dendrite-epithelium interactions are developmentally regulated and that endoreplication in epithelial cells is a crucial component of this control.

Next, we set out to identify molecular mediators of this developmentally regulated change in dendrite-epithelium interaction. Most ban-responsive adhesion-related transcripts were differentially expressed in first and third instar epithelial cells, consistent with ban playing a role in developmental control of epithelial adhesion (Fig. 1C and Fig. 6A). We therefore examined whether expression of these adhesion-related genes was responsive to endoreplication. Notable among these transcripts were mys, which encodes the lone somatically expressed Drosophila β-integrin, and hep, which encodes a Drosophila JNK kinase; integrins are key mediators of cell-ECM interactions including epidermis-basement membrane attachment (DiPersio et al., 1997), and JNK regulates adhesive properties of Drosophila epithelial cells (Jacinto et al., 2000; Jasper et al., 2001; Martin-Blanco et al., 2000). Epithelial Mys levels, in particular on the basal surface of epithelial cells outside of the basolateral junctional domain (Fig. 6B; arrows, adherens junctions), significantly increased...
during larval development, concomitant with the rapid increase in larval endoreplication, suggesting that Mys expression is coupled to endoreplication. Indeed, Mys expression was dampened by mutations that reduced endoreplication, and epithelial ban or dm overexpression, which increase endoreplication, further enhanced Mys levels (Fig. 6B). Likewise, phosphorylated JNK (P-Jnk) levels significantly increased during larval development and this increase was dependent on ban as well as endoreplication (supplementary material Fig. S8). Thus, developmental changes in epithelial expression of adhesion-related genes are triggered by endoreplication. We therefore hypothesized that endoreplication affects dendrite growth via changes in epithelial adhesion.

Epithelium-ECM interactions influence dendrite growth and plasticity

To examine whether epithelial integrins influence dendrite growth, we expressed UAS-mys (RNAi) in epithelial cells, which attenuated epithelial Mys protein levels (supplementary material Fig. S9), and monitored effects on dendrite patterning in third instar larvae. Epithelial mys (RNAi) caused exuberant late-stage dendrite growth similar to ban mutants or other endoreplication-defective mutants, albeit to a lesser extent (Fig. 6C,F). Thus, increased expression of Mys, which mediates ECM attachment, contributes to coordination of dendrite/epithelial growth by constraining late-stage dendrite growth. One model to account for this finding is that increased epithelium-ECM attachment makes the ECM less permissive to dendrite growth. Alternatively, increased epithelium-ECM attachment may potentiate epithelial-dendrite contacts that constrain dendrite growth.

To ascertain whether developmental control of Mys expression is a functionally relevant output of ban in regulating dendrite growth, we examined the epistatic relationship between epithelial ban and mys in control of C4da dendrite development. First, we simultaneously overexpressed ban and knocked down mys in epithelial cells and found that mys (RNAi) attenuated the dendrite growth defect of ban overexpression, resulting in dendrite overextension beyond normal boundaries (Fig. 6D,F). Second, we assayed effects of epithelial overexpression of integrins (UAS-mys + UAS-inflated) on dendrite growth and found that integrin overexpression caused a dendrite undergrowth phenotype similar to that of ban or dm overexpression (Fig. 6E,F). Third, we overexpressed integrins in ban mutant larvae and found that epithelial integrin overexpression only partially suppressed the dendrite overgrowth of ban mutants (Fig. 6F). We conclude that increased epithelial Mys expression is necessary but not sufficient for ban-mediated control of dendrite growth. Taken together with our observation that modulating integrin expression causes less severe dendrite defects than modulation of ban or endoreplication, these results suggest that additional epithelium-derived factors contribute to ban-mediated control of dendrite growth. Ablating C4da neurons before establishment of tiling leads to exuberant dendrite growth into unoccupied territory by spared neurons and this invasive growth potential is lost concomitant with the onset of epithelial endoreplication (Grueber et al., 2003; Parrish et al., 2009; Sugimura et al., 2003). We therefore hypothesized that developmental restriction in C4da dendrite plasticity is the result of alterations in dendrite-substrate and dendrite-epithelium interactions triggered by epithelial endoreplication, including increased proximity dendrite-epithelium proximity. Indeed, following ablation of a C4da neuron, invading dendrites of spared neurons were less closely associated with epithelial cells than neighboring non-invading dendrites, as assessed by relative levels of GFP-PD intensity (Fig. 7A-C).

ECM modification plays important roles in regulating dendrite structural plasticity in several contexts (Mataga et al., 2002; Onay et al., 2004; Yasunaga et al., 2010), thus we reasoned that ban-regulated changes in epithelium-ECM attachment might alter substrate permissivity for dendrite growth and hence C4da dendrite plasticity. To test this possibility, we ablated second instar C4da neurons and monitored dendrite invasion of the unoccupied territory by spared
neurons. In this paradigm, wild-type dendrites exhibit little invasive activity, covering ∼18% of vacated territory (Fig. 7D,G). By contrast, when we ablated C4da neurons in dup mutant larvae, which are defective in endoreplication and the associated developmental changes in dendrite-epithelium interactions, we observed robust invasive activity comparable to ban mutants (Fig. 7E,G). Likewise, when we reduced epithelial Mys levels via epithelia-specific mys (RNAi), we observed a significant potentiation of dendrite invasion (Fig. 7F,G). Notably, mutations in ban or dup potentiate dendrite invasion to a greater degree than mys (RNAi) (Fig. 7G; Parrish et al., 2009), suggesting that factors other than mys function downstream of endoreplication to control dendrite expansion. We conclude that the developmentally programmed growth transition to endoreplication in epithelial cells regulates substrate permissivity for dendrite growth, in part by regulating epithelium-ECM interactions.

DISCUSSION

Local and systemic control of dendrite growth

During embryonic and early larval development, C4da dendrites expand faster than their substrate to achieve complete body wall coverage. C4da neurons respond to nociceptive stimuli (Tracey et al., 2003), and the rapid early dendrite growth ensures that the larval body wall is completely covered by these sensory dendrites shortly after hatching, when larvae must interact with their surroundings. Subsequently, C4da dendrites expand synchronously with the body wall epithelium as larvae grow while maintaining proportionality (Fig. 8A). Thus, multiple growth signals are likely to be at work in this system. In response to systemic growth cues, dendrites and epithelial cells continuously expand during larval development. Blocking these growth cues, for example by ablating dILP neurons or compromising insulin signaling, similarly affects both neuron and substrate, resulting in growth-arrested larvae with properly scaled dendrite arbors (Parrish et al., 2009). Beginning in second instar larvae, sensory neurons and epithelial cells respond differently to growth cues: epithelial growth relies on endoreplication; sensory neuron growth does not (Fig. 8B). During this latter period of larval growth, epithelium-derived signals constrain dendrite expansion to ensure synchronous dendrite/substrate growth. Larval activation of ban in epithelial cells is an essential component of this signaling cascade (Parrish et al., 2009), and here we demonstrate that ban functions in epithelial cells to regulate endoreplication. By altering adhesive properties of epithelial cells, hence epithelium-ECM and epithelium-dendrite interactions, endoreplication curtails C4da dendrite growth and plasticity (Fig. 8C). Many types of neuron expand their arbors synchronously with their substrate to maintain proportional coverage of their receptive field; developmental control of substrate adhesion may be similarly regulated in other contexts to couple dendrite/substrate growth, and to regulate dendrite structural plasticity.

Endoreplication in nervous system development

Growth control is particularly complex in the nervous system, where different cell types are continuously incorporated and cells must grow while maintaining connections. In some contexts, programmed polyploidy facilitates neuronal growth. For example, in the terrestrial slug Limax valentianus endoreplication occurs...
throughout the nervous system in proportion to animal growth, presumably to facilitate neuron expansion (Yamagishi et al., 2011). Neuronal polyploidy has been documented in vertebrates as well; tetraploid neurons exist in the retina and cortex (López-Sánchez and Frade, 2013; Morillo et al., 2010), and Purkinje neurons may be polyploid (Lapham, 1968; Mann et al., 1978).

In addition to supporting neuronal growth, developmentally regulated polyploidy (endoreplication) is suited for tissue growth in circumstances where division might disrupt patterning or connectivity. For example, endoreplication allows glial cells that wrap the Drosophila ventral ganglion to maintain blood-brain barrier integrity even as glia grow to accommodate brain expansion (Unhavaithaya and Orr-Weaver, 2012). Likewise, we found that epithelial endoreplication allows for substrate growth without cell divisions that might disrupt body wall innervation. In addition, epithelial endoreplication drives a differentiation program that regulates epithelium-dendrite interactions to influence sensory dendrite growth and patterning. Notable among these interactions is an increase in epithelial dendrite embedding, which may serve to tether dendrites to epithelial cells, increases as the capacity for structural plasticity decreases, and manipulations that increase plasticity decrease the prevalence of epithelium-embedded dendrites. Third, epithelial adhesion changes as dendritic plasticity is restricted and epithelial integrin expression contributes to restriction of plasticity. Large-scale plasticity would seem to be incompatible with synchronous growth of neurons and their substrate, so it will be intriguing to see whether dendritic plasticity is broadly constrained during periods of growth that maintain proportionality.

**Materials and Methods**

**Fly Stocks**

See supplementary material Table S2 for alleles used in this study.

**Live Imaging**

Embryos were collected on yeasted grape juice agar plates, aged at 25°C in a moist chamber, mounted in 90% glycerol under coverslips sealed with
To analyze dendrite phenotypes, image stacks of dendrites in segments A2-A4 were captured from 8-10 larvae. For high-resolution imaging of the dendrite-ECM interface, larvae were anesthetized with ether before mounting and stacks with a 0.2 µm z-step were acquired.

GFP reconstitution
GFP proximity detection (GFP-PD) is based on GRASP constructs (Feinberg et al., 2008) using extracellular GFP fragments tethered to the transmembrane carrier CD4. Briefly, CD4-spGFP1-10 was PCR amplified and cloned into pUAST containing a C-terminal mCerulean tag. Transgenic lines were obtained from BestGene (Chino Hills, CA). UAS-spGFP1-10-CD4-mCer was recombined with ppk-spGFP11-CD4-tdTomato (Han et al., 2012) and epithelium-dendrite GFP reconstitution was monitored using the epithelium-specific A58-Gal4 driver to express UAS-spGFP1-10-CD4-mCer with the C4da neuron-specific ppk-spGFP11-CD4-tdTomato. GFP-PD signal was imaged under identical conditions for all samples of a given time point, taking care to avoid pixel saturation. Dendrite arbors were used to generate a mask, and mean GFP-PD pixel intensity within the mask was measured using ImageJ (NIH).

Laser ablation
A single larva was mounted, as for live imaging, and the nucleus of a C4da neuron was targeted under a 100×-1.4NA objective using a 337 nm pulsed nitrogen laser (Andor Micropoint; 12 Hz, 15 s) mounted on a Leica DM550 microscope. Following ablation, animals were recovered to cornmeal agar and imaged 48 h later.

Immunohistochemistry
Larval immunohistochemistry was as described (Grueber et al., 2002) using the following: HRP-Cy2 or Cy3 (1:200; Jackson ImmunoResearch), mCD8 (1:100; Life Technologies), phospho-D-Akt Ser505 (1:500; Cell Signaling), Myospheroid-CF.6G11 (1:20; Developmental Studies Hybridoma Bank), BrdU (1:250; Abcam), phospho-JNK (1:500; Cell Signaling), DAPI (50 ng/ml; Life Technologies) and secondary antibodies from Jackson ImmunoResearch. For BrdU labeling, larvae were fed cornmeal-molasses food containing BrdU (10 µg/ml; Sigma), dissected in PBS, fixed in 4% formaldehyde/PBS, permeabilized in PBS-Tx, acid treated with 2.5 N HCl for 30 min, neutralized with 100 mM sodium tetraborate, and processed for immunostaining.

TEM
Larvae were perforated with insect pins, fixed in 2.5% glutaraldehyde/0.1 M sodium cacodylate buffer with centrifugation [15,000 rpm (21,330 g), 1 h], washed 4×5 min in PBS and post-fixed at 4°C in buffered 2% osmium tetroxide overnight. Samples were spun [12,500 rpm (14,674 g), 1 h], washed with distilled water in 20 ml scintillation vials (3×20 min), and dehydrated in a graded series of ethanol, followed by two changes of propylene oxide. This was followed by infiltration in a 1:1 mixture of propylene oxide:epon-araldite overnight, two changes in epon-araldite (2 h each), and overnight polymerization (60°C). 70 nm sections were stained with Reynolds’ lead citrate and viewed on a JEOL-1230 microscope with an AMT XR80 camera.

Microarray analysis
Fluorescence-activated cell sorting (FACS) cell purification and microarray analysis were conducted as described (Parrish et al., 2014). Details are available in in the supplementary materials and methods.

Measurements
Dendrite metrics: 2D projections of z-stacks were used for computer-assisted dendrite tracing (Neurolucida); arbor features were measured using the traces. Details are provided in the supplementary materials and methods.

DNA content: DAPI quantitation was as described (Unhavaithaya and Orr-Weaver, 2012). Briefly, we measured pixel intensity of DAPI stain in each optical section of z-stacks (500 nm step) and normalized epithelial DAPI intensity to mean DAPI intensity of ten diploid PNS neurons from the same fillet imaged using identical settings. PNS neuron DAPI intensity varied less than 10%.

Cell size: We traced the outline of Nrx-IV-GFP or anti-Mys immunoreactivity for ≥50 epithelial cells of each genotype using ImageJ (NIH). We obtained similar results with volume measurements of epithelial cells.
Statistical analysis  
Differences between group means were analyzed via ANOVA with a post-hoc Dunnett’s test. Significance of microarray expression was calculated using Significance analysis of microarrays (SAM) (Tusher et al., 2001) using a 5% false discovery rate and fold-change threshold of 1.5-fold. Lexical analysis was as described (Kim and Falkow, 2003).

Accession number  
Microarray data are available in the NCBI Gene Expression Omnibus (GSE50540).

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Competing interests  
The authors declare no competing financial interests.

Author contributions  
N.J. and J.P. conceived and designed the experiments. N.J. and J.P. conducted the experiments with assistance from P.S. on GFP reconstitution assay, C.K. on microarray analysis, and E.P. on TEM. N.J., C.K. and J.P. analyzed the data. J.P. wrote the manuscript.

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Supplementary material  
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References  


