Hox genes are highly conserved transcription factors renowned for their roles in the segmental patterning of the embryonic anterior-posterior (A/P) axis. We report functions for Hox genes in A/P tissue segmentation and transverse fission behavior underlying asexual reproduction in adult planarian flatworms, *Schmidtea mediterranea*. Silencing of each of the Hox family members identifies 5 Hox genes required for asexual reproduction. Among these, silencing of *hox3* genes results in supernumerary fission segments, while silencing of *post2b* eliminates segmentation altogether. The opposing roles of *hox3* and *post2b* in segmentation are paralleled in their respective regulation of fission behavior. Silencing of *hox3* increases the frequency of fission behavior initiation while silencing of *post2b* eliminates fission behavior entirely. Furthermore, we identify a network of downstream effector genes mediating Hox gene functions, providing insight into their respective mechanisms of action. In particular, we resolve roles for *post2b* and effector genes in the functions of the marginal adhesive organ in fission behavior regulation. Collectively, our study establishes adult stage roles for Hox genes in the regulation of tissue segmentation and behavior associated with asexual reproduction.
We recently uncovered and characterized the size-dependent behaviors and adult tissue structures underlying asexual reproduction in the planarian, *Schmidtea mediterranea* (Fig. 1a). These highly regenerative flatworms exist in both sexual and asexual biotypes. The latter reproduces solely through transverse fission – an asexual reproductive behavior in which torn posterior tissue fragments regenerate and give rise to clonal progeny. Fission behavior is size-dependent, and its establishment and regulation coincide with growth-dependent patterning of the planarian central nervous system (CNS). Our study also revealed a cryptic form of segmentation that allocates tissue for fission progeny in coordination with the dynamic growth and de-growth of the adult planarian body plan. Presently, the molecular mechanisms mediating this segmentation and its coupling to asexual reproductive behavior are still unknown.

The ancestral roles of Hox genes, a family of transcription factors with evolutionarily conserved functions in embryonic head to tail patterning, are tightly linked to the emergence of segmented animal body plans. Additionally, Hox gene functions in neuronal development throughout taxa suggest potentially ancient roles in the evolution of the nervous system. As asexually reproducing animals, planarians preserve many embryonic developmental programs within their adult tissues, including the ability to establish and

![Diagram of patterning and behavioral aspects of planarian asexual reproduction.](image)

**Fig. 1 Planarian Hox genes are required for adult asexual reproduction.** 

**a** Diagram of the patterning and behavioral aspects of planarian asexual reproduction. 

**b** Fission activity following RNAi against Hox genes. Plot depicts fission progeny number on day 12 of primary RNAi screen (n = 54, 12, and 18 animals for Control#1, Control#2, and all other RNAi samples, respectively; p-value calculated by Welch’s two-tailed t-test versus corresponding control; Control#1 vs. *loxA*, *post2d*, *hox4b*, *post2c*, *hox4a*, *post2a*, *hox3b*, *hox1*, *loxA*, *post2b* p-values = 0.1733, 0.5958, >0.9999, 0.8582, 0.0455, 0.0348, <0.0001, <0.0001, <0.0001, <0.0001, and <0.0001, respectively, Control#2 vs. *post1* p-value = 0.0384). Box and whisker plot depicts individual data points, median (center), 25th/75th percentile (bounds of box), and minima/maxima (whiskers). Source data are provided as a Source Data file. 

**c, d** Heatmaps depicting fission activity following RNAi treatment for both the (c) 2-phase primary and (d) secondary Hox RNAi screens. Cumulative fissions over time are displayed for individual worms from each RNAi condition (n = 12 or 18 animals). Source data are provided as a Source Data file. 

**e** Representative images and phenotypic frequency of RNAi animals 15 days post-amputation after eight dsRNA feedings (n = 18–53 animals, three independent repeats). Scale, 1 mm.
maintain the polarization and patterning of the body plan along the A/P axis\textsuperscript{9,10}. We therefore hypothesized that Hox genes play a role in A/P directed tissue segmentation and/or transverse fission behavior underlying asexual reproduction in adult planarians.

There has been a great deal of interest in understanding Hox gene function in planaria. Planarian Hox genes were first cloned over 30 years ago\textsuperscript{1,12}. Since then, multiple studies have characterized the spatio-temporal regulation of planarian Hox genes during homeostasis and regeneration\textsuperscript{11,13–15}. With the introduction of RNA-mediated genetic interference (RNAi)\textsuperscript{16}, many investigators made extensive efforts to perturb planarian Hox gene function (both individually and combinatorially) with no discernible phenotypic defects reported\textsuperscript{14,17,18}. Only recently has a requirement for the planarian Hox gene post2d in proper tail regeneration been reported\textsuperscript{19}. Thus, to date, the functions of Hox genes in planaria remain largely a mystery.

In this work, we report functions for Hox genes in the asexual reproduction of the planarian flatworm, \textit{S. mediterranea}. We identify multiple planarian Hox genes required for adult tissue segmentation and behavior underlying transverse fission. Among these, RNAi-mediated silencing of hox3 genes increases both fission segmentation number and fission behavior initiation. In contrast, RNAi of post2b eliminates both fission segmentation and fission behavior. Using RNAseq expression analysis and RNAi functional screening, we identify downstream effector genes mediating Hox gene function in asexual reproduction. We find that post2b regulation of effector genes is required for the function of the planarian adhesive organ in the anchoring of the body form to a substrate to initiate transverse fission. Our data support a model in which the coordinate functions of Hox genes regulate asexual reproductive segmentation and behavior within adult planaria.

**Results**

**Planarian Hox genes are required for asexual reproduction.** The \textit{S. mediterranea} genome encodes 13 Hox genes: Hox1, Hox3a, Hox3b, Hox4a, Hox4b, Lox4, Lox5a, Lox5b, Post1, Post2a, Post2b, Post2c, Post2d\textsuperscript{15} (Supplementary Fig. 1a, b). In planaria, Hox genes are dispersed throughout the genome in an atomization of the ancestral cluster\textsuperscript{4} (Supplementary Fig. 1c). This genomic organization may have implications for Hox gene expression and function in planaria as Hox genes typically display spatial collinearity (correspondence between gene order along the chromosome with domains of expression along the head to tail axis) in other animals\textsuperscript{20}. RNAseq analysis of sexual and asexual planarian biotypes indicates that Hox gene expression increases from embryogenesis to adulthood (10/13 genes) with comparatively minor changes during whole animal regeneration/remodeling\textsuperscript{21,22} (Supplementary Figs. 2, 3). Despite an absence of genomic clustering, whole-mount in situ hybridization (WISH) reveals both axially restricted (hox3b, hox4b, lox5a, post2c, and post2d) and radially layered (post2a and post2b) domains of Hox gene expression within adult stage planarians\textsuperscript{15}. Notably, hox3b is enriched in a band of expression anterior to the pharynx in 4–5 mm animals\textsuperscript{15}. This region overlaps with the location of newly arisen fission planes as planarians grow from 3 to 5 mm in length\textsuperscript{15}. Additionally, hox1, hox3a, hox4b, post2d, and post2b exhibit overlapping expression with the cholinergic neuron marker, \textit{chat} in the planarian brain and nerve cord\textsuperscript{15}. The expression of planarian Hox genes within these axially restricted domains and neuronal cells are consistent with potential functions in the tissue segmentation and behavior underlying asexual reproduction.

We determined the role of each of the planarian Hox genes in asexual reproduction. Individual RNAi of 5/13 Hox genes (hox1, hox3a, hox3b, lox5b, and post2b) in adult stage planaria resulted in a >50% reduction in the generation of fission progeny (Fig. 1b, c, Supplementary Fig. 4a–c). Targeting hox1, hox3a, hox3b, lox5b, and post2b with an extended RNAi treatment in a secondary screen either entirely or largely eliminated fission activity (Fig. 1d, f, Supplementary Fig. 4d). Consistent with previous reports, knockdown of hox1, lox5b, post2b, hox3a, or hox3b did not result in evident morphological changes during growth, homeostasis, or regeneration\textsuperscript{15,17,18} (Fig. 1e–g). These results identify a set of planarian Hox genes with prominent roles in asexual reproduction.

**Role of Hox genes in adult tissue segmentation.** We hypothesized that planarian Hox genes regulate the adult tissue segmentation underlying asexual reproduction. We utilized our previously established physical compression assay to reveal the fission planes dividing tissue segments in RNAi animals and thereby determine the respective effects of Hox gene silencing\textsuperscript{3}. Knockdown of hox1 or lox5b had little to no effect on segment number (Fig. 2a, b). In contrast, knockdown of post2b eliminated fission segmentation, while knockdown of hox3a or hox3b resulted in supernumerary segments (Fig. 2a, b). Although simultaneous knockdown of hox3a + hox3b increased fission segment number, it also reduced fission progeny yield (Fig. 2a, b, Supplementary Fig. 4e–g). The increased fission segmentation following hox3 RNAi was slightly biased in the region anterior of the pharynx, coinciding with the expression domain of hox3b\textsuperscript{15} (Fig. 2c). We reasoned that increasing the number of fission planes dividing the animal should reduce the size of each segment and the resultant fission progeny. We quantified the size of rare fission progeny in \textit{hox3} RNAi animals, revealing a decrease in progeny size consistent with the increase in segmentation observed via physical compression (Fig. 2d, e). Altogether, these data indicate that the planarian Hox genes post2b and hox3 are required for A/P tissue segmentation associated with asexual reproduction.

**Role of Hox genes in asexual reproductive behavior.** We next determined the roles of planarian Hox genes in the frequency, duration, and success of asexual reproductive behavior. Using time-lapse imaging, we recorded fission events in Hox RNAi-treated animals\textsuperscript{3} (Fig. 3a, Supplementary Figs. 5, 6, Supplementary Movies 1–8). Individual knockdown of either hox1 or lox5b substantially reduced the success of fission behavior (Supplementary Fig. 6b–f, I, Supplementary Movies 2, 5). Mirroring their opposing roles in segmentation, post2b RNAi eliminated fission behavior (Supplementary Figs. 6j, 7b) while hox3a + hox3b RNAi increased fission behavior frequency and duration (Fig. 3b–d, Supplementary Figs. 6g–h, 7a, c, Supplementary Movies 6, 7). Individual RNAi of hox3b, but not hox3a, largely phenocopied hox3a + hox3b RNAI effects on fission behavior (Supplementary Figs. 6b–d, g, h, 7c, Supplementary Movies 3, 4). These results suggest unequal levels of functional redundancy for the hox3 genes in the regulation of fission behavior in contrast to their similar contributions to fission segmentation (Fig. 2). Importantly, these results indicate that hox1 and lox5b regulate fission behavior independent of segmentation while post2b and hox3 mediate opposing roles in fission behavior regulation that parallels their roles in segmentation.

Our analysis indicates that planarian hox3 genes have shared roles in the negative regulation of adult tissue segmentation and asexual reproductive behavior. Yet, while hox3 RNAi increases the frequency and duration of fission behavior, these attempts are ultimately unsuccessful (Fig. 3b–e). Therefore, we hypothesized that hox3 is also required at a later stage of the asexual reproduction process. Detailed analyses of fission behavior in control and hox3 RNAi-treated animals revealed two distinct
phases of behavior (I and II) common to individual fission attempts (Fig. 3f, g). During phase I, animals elongate their bodies anteriorly in a contorted manner. During phase II, animals further stretch out, thinning and rupturing the fission plane dividing segments to release a tissue fragment. Analysis of hox3 RNAi-treated animals reveals an inability to progress from phases I to II, thus preventing completion of asexual reproduction (Fig. 3h–j). Furthermore, high-resolution time-lapse imaging of hox3a + hox3b and hox3b RNAi-treated animals revealed that phase I contortions are coincident with A/P directed peristaltic
contractions (Fig. 3k, l, Supplementary Movies 9, 10). This peristalsis was previously observed in live imaging of fissioning S. mediterranea, and a similar process was observed in fissioning Dugesia japonica. To date, the significance of these peristaltic contractions in planarian asexual reproduction is still unclear. We conclude from these data that hox3 is required for both the initiation and successful progression of asexual reproductive behavior.

Resolving effector genes mediating Hox gene function. Our findings indicate that Hox genes regulate asexual reproduction via the emergence and modulation of A/P segmentation and behavior in adult animals. We previously identified requirements for the Wnt signaling genes β-catenin, apc, dshB, and wnt11-6 as well as the TGF-β signaling genes actR-1 and smad2/3 in the regulation of fission behavior. Their functions coincided with their roles in the size-dependent patterning of putative mechanosensory neurons expressing gabrg3L-2 and pdk1L-2, genes with roles in the inhibition of fission activity. The interplay between Hox and Wnt signaling is an ancient feature of axial patterning broadly conserved throughout phyla. Additionally, TGF-β and/or BMP signaling has been shown to regulate Hox gene expression and collaborate with HOX proteins for target gene regulation.

Fig. 3 post2b and hox3 are opposing regulators of asexual reproductive behavior. a Diagram of webcam time-lapse imaging and timeline visualization depicting successful (upward displacement) and unsuccessful (downward displacement) fission attempts. b Representative activity timelines of fission behavior from live imaging of control, post2b, and hox3a + hox3b RNAi animals. c–e Plots of (c) total number of fission attempts (Control vs. post2b and hox3a + hox3b p-values = 0.005 and <0.0001, respectively), (d) duration of each attempt (Control vs. hox3a + hox3b, p-value < 0.0001), and (e) percentage of successful attempts for RNAI-treated animals (Control vs. hox3a + hox3b p-value = 0.194). n = 8, 6, and 6 animals for Control, post2b, and hox3a + hox3b RNAI samples, two independent repeats. Source data are provided as a Source Data file. f–h Representative images (5 min per frame, A = anterior, P = pre-fission posterior, and P’ = post-fission posterior) of (f) successful and (g, h) unsuccessful fission attempts divided into Phase 1 (Contortion) and Phase 2 (Stretch) for (f, g) control and (h) hox3a + hox3b RNAI-treated animals (experiment independently repeated once). i, j Plots of the duration of (i) Phase 1 and (j) Phase 2 within each fission attempt for RNAI-treated animals (n = 18, 50, and 476 fission events for Ctl Successful, Ctl Unsuccessful, and hox3a + hox3b Unsuccessful, respectively; Ctl Successful vs. Ctl Unsuccessful p-value = 0.0005; Ctl Unsuccessful vs. hox3a + hox3b Unsuccessful p-value < 0.0001). Source data are provided as a Source Data file. k, l Representative images of the (k) entire body or the (l) pre-pharyngeal region of hox3a + hox3b RNAI-treated animal stalled in Phase 1 (n = 10/10 animals, arrows indicate tissues undergoing peristaltic contractions, two independent repeats). Animals were given 17 bacterial RNAI feedings. Scale, 1 mm. Box and whisker plot depicts individual data points, median (center), 25th/75th percentile (bounds of box), and minima/maxima (whiskers). p-values calculated by Welch’s two-tailed t-test. Scale, 1 mm.
This raises the question as to the extent to which hox1, hox3a, hox3b, hox5b, and/or post2b interact with Wnt/TGF-β signaling in the regulation of asexual reproduction.

We explored the potential interplay between Wnt/TGF-β genes and the identified fission regulatory Hox genes in planarian asexual reproduction. While knockdown of β-catenin alters expression of multiple Hox genes, including hox4b (aka hoxD), hox5a, post2c, and post2d (aka abdlBα), there is no significant effect on the expression of hox1, hox5b, hox3a, hox3b, or post2b. Additionally, while the expression domain of post2b along the lateral edge of the animal resembles that of gabreg3L-2 and pdkHL-2, single-cell RNAseq (scRNAseq) analysis indicates that they are expressed within non-overlapping cell clusters. Finally, the RNAi phenotypes of Wnt/TGF-β pathway genes are distinct from that of the identified fission-regulatory Hox genes. Animals treated with hox1, hox3a, hox3b, hox5b, or post2b RNAi lack detectable anterior–posterior regeneration phenotypes (Fig. 1e, g) and knockdown of hox3a, hox3b, or post2b alters fission segmentation (Fig. 2). In contrast, knockdown of fission regulatory Wnt/TGF-β genes yields anterior–posterior regeneration defects and has little to no effect on fission segmentation. The lack of an obvious functional integration of fission-regulatory Hox genes into the Wnt/TGF-β pathway motivated a de novo investigation of the mechanism by which Hox genes regulate asexual reproduction.

Hox genes mediate their functions via direct and indirect regulation of downstream effector genes. To elucidate Hox downstream effector genes regulating asexual reproduction, we devised a two-part strategy to (I) identify putative candidates via differential gene expression analysis and (II) functionally validate their roles in fission (Fig. 3a). RNAsseq analysis identified 1724 DEGs following perturbation of Hox gene function (Fig. 4b, Supplementary Data File 1). We selected and successfully cloned 423 putative effector genes into RNAi vectors for functional analysis. We determined the role of each gene in asexual reproduction by scoring resultant fission progenesis following RNAi-mediated gene perturbation (Supplementary Fig. 8a, b).

We focused on RNAi conditions that phenocopied Hox gene RNAi—i.e., robustly decreased fission progenesis (>4-fold reduction, p-value < 0.01) independent of evident alterations to body plan morphology or size (Fig. 1e–g, Supplementary Fig. 4d). Using these criteria, our functional screen identified 24 putative Hox downstream effector genes required for asexual reproduction: ganglioside GM2 activator (gma2), zing finger protein 816 (znf816), sterile alpha motif-containing protein (same), sex determining region Y-box 5 (sox5), carboxy anhydrase VII (ca7), thymidine DNA glycosylase (tdg), dd25334, G elongation determining region Y-box 5 (sox5), carbonic anhydrase VII (znf816), sterile alpha motif-containing protein (samc), sex determining region Y-box 6, 8, 10, 13, and epidermal 10 (Supplementary Fig. 9a–e). Consistent with this analysis, published WISH staining confirms post2b expression in a subset of smedwi+ cells, the CNS, and layers of parenchymal and epidermal cells along the lateral edge of the animal. Amongst the 11 effector genes co-enriched with post2b, rcn-1 was expressed in smedwi+ PP, and parenchymal-8/13 cell clusters (Fig. 4e, Supplementary Data File 2). The remaining 10 effectors were co-enriched in the parenchymal and epidermal cell clusters: rcn-1 and rcn-2 in parenchymal 8/13, eve in parenchymal 6/10, and plgl1, ifb, lmmAC, post2a, barH2, dd13286, and dd20390 in epidermal 10 (Supplementary Fig. 9a–e, Supplementary Data File 2).

Expression and function of post2b effector genes. To gain insight into the mechanism of action by which Hox genes regulate asexual reproduction, we set out to determine the spatial and cellular context of Hox genes and their effectors. In situ hybridization analysis of Hox gene transcripts poses a significant challenge due to their low expression in planarian tissues. We therefore utilized published scRNAseq expression data to resolve the cell populations where Hox genes were co-expressed with their cognate downstream effectors. This analysis identified overlapping expression for post2b and 11/15 of its downstream effectors (Supplementary Fig. 9a–e). Post2b is enriched in the single-cell clusters smedwi+ PP, non-ciliated neurons 0, parenchymal 6, 8, 10, 13, and epidermal 10 (Supplementary Fig. 9a–e). Consistent with this analysis, published WISH staining confirms post2b expression in a subset of smedwi+ cells, the CNS, and layers of parenchymal and epidermal cells along the lateral edge of the animals that paralleled published post2b expression patterns (Fig. 4e, Supplementary Fig. 9i). These scRNAseq and in situ analyses indicate that post2b and its respective downstream effector genes are co-expressed within specific planarian cell populations.

We next set out to determine whether post2b regulated the expression of these cognate downstream effectors within the identified cell populations. We used WISH to analyze gene expression in post2b RNAi-treated worms. Knockdown of post2b, but not hox3, eliminated lateral edge staining of rcn-1, syt1, lmmAC, and ifb (Fig. 4f). These results are consistent with previous reports that post2b regulates ifb expression in planarian lateral epidermal cells. Notably, RNAi targeting of post2b did not affect rcn-1 expression in parapharyngeal cells outside the domain of post2b expression. Collectively, this expression analysis indicates that post2b maintains the expression of genes within lateral parenchymal and epidermal cell populations that are required for asexual reproduction.

To gain insight into the mechanism of action of these post2b effectors, we determined whether they were required for segmentation similar to post2b. Surprisingly, RNAi of tested post2b effectors did not phenocopy the loss of segmentation observed in post2b RNAi animals (Fig. 4g). We reasoned that these effectors were instead required for fission behavior. Knockdown of rcn-1, plgl1, syt1, ifb, lmmAC, or post2a significantly reduced or eliminated fission attempts, largely phenocopying post2b RNAi (Fig. 4h, Supplementary Fig. 10, Fig. 3b–e, Supplementary Movies 11–17). RNAi phenotypes of most
effectors were not as strong as that of post2b, suggesting that multiple effectors work in concert to give rise to the post2b phenotype. These findings indicate that post2b-mediated regulation of effector gene expression within these lateral parenchymal and epidermal cell populations is required for fission behavior.

Role of post2b and effectors in the MAG. Our screening analysis identified multiple Hox gene effectors required for asexual reproduction (Fig. 4). ScRNAseq analysis and WISH staining indicate that the effectors required for fission are expressed within multiple cell populations in the adult animal29 (Fig. 4,
Discussion

Our study identifies roles for planarian Hox genes as mediators of adult tissue segmentation and fission behavior in addition to providing evidence that Hox genes function in asexual reproduction. In combination with prior work, our current findings indicate complementary roles for Hox and Wnt/TGF-β genes during planarian asexual reproduction. The coordinate roles of hox1, loc5b, hox3a, hox3b, and post2b regulate segmentation for progeny tissue.

Fig. 4 Elucidation of downstream effectors mediating fission: (I) use RNAseq analysis to identify putative effector genes from the differentially expressed genes in Hox RNAi-treated animals, then, (II) use RNAi to determine the effects of putative effector genes on fission. b Heatmap of 1724 DEGs from RNAseq that are significantly regulated following RNAi of Hox genes. c Heatmap of the top 24/423 genes from RNAi screen of putative downstream fission effectors (see Supplementary Fig. 8) 16–19 Bacterial RNAi feedings. Heatmap depicts normalized fission number on Day 12 across six RNAi-treated worms. Effector genes are color coded with respect to their putative upstream Hox gene: hox1 (red), loc3b (orange), hox3 (green), post2b (purple). Source data are provided as a Source Data file. d Representative images of post2b effector RNAi-treated animals 15 days post fission induction (n = 6 animals, 17 RNAi feedings, two independent repeats). Scale, 1 mm. e Double fluorescent whole-mount in situ staining of post2b effectors rcn-1 and syt1, ifb, or lmnAC (n = 6–7 animals, two independent repeats). Whole animal image (top) and zoom in of posterior lateral section (bottom). Scale, 500 and 100 μm, respectively. f Expression of post2b effector genes detected by whole-mount in situ hybridization in control, hox3a + hox3b, and post2b RNAi-treated animals (n = 4–13 animals, 1 independent repeat). Scale, 500 μm. g Plot of the number of fission planes/animal length following post2b effector RNAi treatment (n = 4, 10, 11, 10, 7, 4, and 17 animals for Ctl, rcn-1, plg, syt1, ifb, lmnAC, post2a, and post2b, respectively; Ctl vs. rcn-1, plg, syt1, ifb, lmnAC, post2a, and post2b p-values = 0.9581, 0.6815, 0.1552, 0.1634, 0.5967, 0.4605, and 0.0001, respectively; replicates pooled from 1 to 4 independent repeats, 21 bacterial RNAi feedings). p-Values were calculated by Welch’s two-tailed t-test. Source data are provided as a Source Data file. h Plot of the total number of fission attempts for RNAi-treated animals (n = 14, 5, 5, and 6 animals for Ctl, syt1, post2a, and all other RNAi samples, respectively; Ctl vs. rcn-1, plg, syt1, ifb, lmnAC, post2a, and post2b p-values = 0.0145, <0.0001, <0.0001, 0.0006, 0.0157, and 0.0218, respectively; 21 bacterial RNAi feedings). Box and whisker plot depicts individual data points, median (center), 25th/75th percentile (bound of box), and minima/maxima (whiskers).

Supplementary Fig. 9). These observations suggest that gene function within multiple tissues and cell types, in addition to the CNS, are required for asexual reproduction and even different aspects of fission behavior. But, in the case of the identified post2b effectors, how are gene functions within lateral parenchymal and epithelial cells required for fission behavior initiation? Cross-referencing scRNAseq data, in situ hybridization data, and published literature revealed that the lateral parenchymal and epithelial cell populations are mag-1+ viscid gland cells and ifb+ epithelial anchor cells of the marginal adhesive gland (MAG). Within this duo gland organ, conserved across platyhelminthes, the coordinate functions of adhesive gland cells (viscid gland cells), releasing cells, and anchor cells mediate transient adhesion to substrates. Viscid gland cells within the parenchyma expel their adhesive secretions onto the animal’s surface via long cellular processes that cross the basal lamina and travel through epithelial anchor cells to outer pores surrounded by adhesive papilla. In Macrostomum lignano, RNAi targeting of the anchor cell-specific intermediate filament gene macfil results in severe morphological alterations in anchor cells and inability to adhere to substrates. Assuming conservation of gene functions, RNAi targeting of the anchor cell-specific planarian intermediate filament gene ifb likely compromises the structure and function of the epithelial secretory pore. Furthermore, the post2b effector syt1 is expressed in viscid gland cells and is homologous to synaptotagmin, a calcium sensor for regulated exocytosis. Additionally, the post2b effect rcn-1, also expressed in viscid gland cells, is homologous to reticulocalbin, a calcium-binding protein of the secretory pathway. Based on the functions of these homologous genes, we predict that RNAi targeting of rcn-1 and syt1 likely compromises calcium-regulated exocytosis of adhesive secretions from the viscid gland. Notably, RNAi targeting post2b or downstream effectors expressed within the marginal adhesive organ (rcn-1, plg1, syt1, ifb, lmnAC, and post2a) results in animals constantly drifting across substrates while adopting a resting position. This phenotype is consistent with a defect in substrate adherence and inability to stop cilia-driven locomotion and remain stationary. Given that fission behavior begins with the anchoring of the worm’s posterior end to a substrate and requires functional effectors specifically expressed within the MAG, we conclude that control of MAG constitutes a key facet of planarian asexual reproduction.

To investigate the function of post2b in the MAG, we used electron microscopy to characterize the cellular structure of the viscid gland and adhesive cells in post2b RNAi animals. Scanning electron micrographs (SEM) of an amputated planarian tail region revealed a distinct mucus stripe along the lateral edge epithelial surface, corresponding to the adhesive secretions of the MAG (Fig. 4i, Supplementary Fig. 11a). Knockdown of post2b eliminated or vastly reduced this stripe of mucosal secretions. Notably, the exposed lateral edge epithelial surface of post2b RNAi animals had expected complements of cilia and microvilli but lacked detectable adhesive papillae. Analysis of transverse sections in control animals resolved parenchymal viscid gland cells extending processes that crossed the basal lamina to connect to adhesive papillae-covered pores on the epithelial anchor cell surface (Fig. 4j, Supplementary Fig. 11b). Consistent with our exterior SEM analysis, post2b RNAi animals lacked a distinct anchor cell region in the lateral margin epithelium and displayed a reduced number of viscid gland cell extensions crossing the basal lamina and terminating at the epithelial surface. Of the few viscid gland cells detected, all lacked adhesive papillae around the pore termini. Altogether, our findings indicate that post2b functions in the MAG are required for initiation of planarian fission behavior.
allocation, adhesion for posterior anchoring to substrates, and the execution of transverse fission behavior to ultimately separate tissue fragments that will regenerate into clonal progeny (Fig. 4k). In parallel, Wnt/TGF-β activity coordinates growth with size-dependent patterning of fission-modulatory mechanosensory neurons. Given the preponderance of evidence for the interplay of Hox genes with the Wnt and TGF-β pathways,19,24–27, it would be of interest to further investigate the extent of their interdependence in the regulation of asexual reproduction. Our study also elucidates Hox gene-mediated segmentation within historically designated unsegmented flatworms.28 In combination with the discovery of Hox-mediated segmentation in the basal metazoan NematoMuta vectensis, these findings support ancient roles for Hox genes in the regulation of body segmentation in addition to their well-established roles in segmental patterning.43 Finally, we identify roles for Hox genes in multiple aspects of asexual reproductive behavior, building upon the known roles for Hox genes in developing nervous systems and functions linked with specific animal behavior.6–8,44,45 From cnidarians to deuterostomes, Hox genes play conserved roles in the regulation of germ-layer patterning.43,44 Notably, asexual reproductive organ budding or fission, is also distributed throughout these groups and phyla.45 We speculate that the regulation of asexual reproduction and/or key genes underlying the neural circuitry or specific cell types utilized in this process represent ancestral and potentially conserved functions for Hox genes.

Methods

Animal husbandry. Clonal CIW4 strain S. mediterranea were maintained in 1x Montjuïc salts according to standard husbandry protocols.46 CIW4 animals were sourced from a large recirculation culture and placed directly into a unidirectional flow system culture for RNAi feeding experiments.1–4,36 The planaria recirculation culture system is composed of three culture trays (96 vessels) stacked vertically with water bunching over a sump. Planarian water flows from the sump pump through a chiller, canister filter, and a UV sterilizer into the top tray. Water subsequently flows down drains at the opposite ends of the source through the series of three trays. Water then re-enters the sump where it is filtered through two vertically stacked 400 and 200 μm sieves. Beyond the sieves, water is gravity fed and mechanically filtered through a set of filter/loss pads. Finally, water passes through Water Garden Oasis Pond Matrix and Kaldness media to remove nitrogenous waste. Water is then able to flow back through the chiller, canister filter, and UV sterilizer back into the top tray. For the unidirectional flow system, planaria were cultured in disposable plastic cups lined with a pock of 150 μm nylon mesh (Saatites Ltd.). Cups were perforated with holes at the desired planaria water level for outflow to complete the flow vessel. These flow vessels were placed in an enclosed container on a perforated tray suspended above a collection basin and centralized waste drain. Planarian water was pumped through spigots directly above the flow vessels. The system actively pumped 2 vessel exchanges of water every 12 h. After RNAi feedings uneaten food was removed.

Gene cloning and RNAi feeding protocol. Gene cloning was performed as previously described but with the following distinctions1. Primer3 version 4.1.0 (https://primer3.ucl.ceb) was used to design primer pairs targeting genes of interest, using the Sánchez Alvarado lab transcriptome as a reference. 5′ overhangs were added to ends of both forward and reverse primers to enable downstream Gibson Assembly. Amplified products were designed to be 400–600 base pairs in length. PCR was performed using cDNA as a template. Products were inserted into a modified version of the pMR-T4P vector and transformed directly into E. coli strain HT115. Final cloning products were verified by sequencing. A list of all accession numbers and primer sequences for all the Hox genes and effector genes in this study are available in Supplementary Data File 3. Preparation of dsRNA and RNAi feedings were performed as previously described with the following distinctions4. Purified dsRNA food was prepared by mixing 1 volume of dsRNA at 400 ng/ml with 1 volume of beef liver paste. Bacterial RNAi food was prepared by mixing 8 ml of pelleted bacteria with 120 μl of beef liver. Food was administered every 7 days, fed directly from a starting size of ~4 mm to a final size of 10–15 mm. To achieve this, worms were either fed 7–9 times with purified dsRNA food or 16–19 times with bacterial RNAi food as indicated. Control animals were fed RNAi food targeting unc-22.

Fission assay. For fission induction, animals were removed from recirculation or unidirectional flow system, rinsed 10 times with planarian water, and individually placed into wells of a six-well tissue culture plate. The number of fission fragments was scored every 1–2 days. Data for analysis, the number of daily cumulative fissions was directly plotted or normalized to the average of the control RNAi fissions where indicated. Fission number was converted to a heat color code for visualization.

Fission segmentation compression assay. Fission segmentation was revealed by compression of worms between a glass coverslip and a fusion dish. Animals were inverted with their ventral side up, compressed using four fingertips, then imaged. To ensure that all compression/fission planes were revealed for every animal, images were acquired sequentially using a Leica M205 microscope as each fission plane was revealed by mechanical compression.

Microscopy. Images of live worms and fixed colorimetric in situ samples were acquired using a Leica M205 microscope. Fission segmentation assay was calculated using ImageJ (NIH). Multicolor fluorescent in situ hybridization (FISH) single confocal images were acquired using a Nikon CSU-W1 inverted spinning disk confocal microscope. Fiji plugins were used to stitch tiles for whole planarian images. Following image acquisition, brightness and contrast were adjusted, and the images were rotated and cropped for data presentation.

Live imaging of fission behavior. For all data sets, worms were plated in six-well plates and imaged in one of two ways. For time-lapse A (Supplementary Figs. 5, 6, Supplementary Movies 1–5), worms were imaged from above with a single 4x webcam (Logitech Brio). Four inverted LED ring lights (AmScope) mounted above the camera were used for illumination. Images were acquired using an in-house written Python script described previously4. In time-lapse B and C (Figs. 3 and 4, Supplementary Movies 7, 10, Supp. Movies 6–17), imaging was acquired with a configuration wherein the camera was mounted below a large (2.8 x 2.6) plexiglass panel that was topped with large white plexiglass panels. The same four LED lights were placed at the bottom of the setup next to the camera. In this format, a webcam (Logitech c920) was used to acquire images. The time-lapse was acquired using micro-manager version 1.4.22 (https://micro-manager.org/) and the open-source framework. In both formats, camera gain, exposure, and autofocus were controlled using the Logitech Webcam Controller software (https://download01. logi.com/web/fbp/video/lws/lws280.exe).

Videos of individual animals were analyzed, manually annotated, and example frames were extracted using ImageJ. Fission attempt initiation was scored at the frame in which a rapid stationary elongation occurred following a period of contraction and immobility. Termination of fission attempt was scored at the frame in which the animal ruptured and released a fragment (successful), or the animal returned to its original pre-elongation state (unsuccessful). Fission events were divided into phase I (initial elongation accompanied by body contractions) and phase II (second elongation accompanied by thinning and/or rupture). Data were visualized with timelines plotted using Graphpad Prism version 9.2.0. Worms that crawled out the well and desiccated during the experiment were not considered for further analysis.

Planarian activity of hox3b and hoxa3b + hox3b RNAi-treated worms was captured using a Leica M205 microscope (acquired at 10 frames per second, Supplementary Movies 9,10).

RNAseq analysis. Control or Hox gene RNAi animals (7× purified dsRNA feedings) were starved for 5 days and snap-frozen for RNA collection (4 replicate samples with three animals each). RNA was extracted using Trizol Reagent, purified and DNase treated (Qiagen RNeasy Mini Kit Cat. No. 74104). Libraries were prepared according to the manufacturer’s instructions using the TruSeq Stranded mRNA Prep Kit (Illumina). The resulting libraries were purified using the Agencourt AMPure XP system (Beckman Coulter) then quantified using a Bioanalyzer (Agilent Technologies) and a Qubit fluorometer (Life Technologies). Libraries were re-quantified, normalised, pooled and sequenced on an Illumina NextSeq 500 instrument as High Output 75 bp single read runs using NextSeq Control Software version 2.2.0.4. Following sequencing, Illumina NextSeq Real-Time Analysis version 2.4.11 and bcl2fastq2 version 2.2.0 were run to demultiplex reads and generate FASTQ files. These FASTQ files were then aligned to the smed_dd_gt4 genome using STAR and parameters:--outFilterMultimapNmax 2 --alignIntronMax 60. Mode GeneCounts, along with the Sm_ad_v3 gene models from the Max Plank Institute, obtained via Planmine, to generate read counts to genes. Differentially expressed genes were determined using the edgeR library in R48, p-Values were adjusted for multiple hypothesis testing by the method of Benjamini and Hochberg49. Cutoff for significant differential gene expression for hox1a, hoxa3b, and hox3b RNAi versus control RNAi was a log2 fold change of 2 and an adjusted p-value < 0.5. Cutoff for significant differential gene expression for hox3b RNAi versus control RNAi was a fold change of 2 and an adjusted p-value < 0.1.

Planarian Hox gene, transcript, and protein sequence analysis. Planarian Hox gene mRNA and amino acid sequences were obtained from Planosphere (https://planosphere.stowers.org/find/gene). The genomic contig location of planarian Hox genes was determined using PlanMine version 3.0 (http://planmine.mpi-cbg.de). Homology domain signatures were annotated using Pfam 33.1 (https://pfam.xfam.org/150). Homology domain sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

1. Sánchez Alvarado, M. & Keshishian, H. Nematostella vectensis. NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-021-26986-2 | www.nature.com/naturecommunications 9
Planarian Hox gene expression. Expression of Hox genes during regeneration and development was obtained from Zeng et al. (2018) and Davies et al. (2017), respectively. Single-cell data and t-SNE plots were obtained from a planarian cell transcriptome atlas (https://digiworm.wim.mit.edu/)29.

Data visualization and statistical tests. Heatmaps were generated using Microsoft Excel (version 16.49) and Multiple Experiment Viewer (MeV version 4.81). Graphs were generated using GraphPad Prism (version 9.2.0). Illustrations were made using Adobe Illustrator 2020 (version 24.1.3). Images were prepared for figures using Image (version 2.1.0), and Adobe Photoshop 2020 (version 21.1.3). For pairwise comparisons, significance was calculated using an unpaired t-test with Welch’s correction using GraphPad Prism (version 9.1.0).

Whole-mount in situ hybridization. Colorimetric WISH hybridization was performed as follows46. Mucus was removed with 7.5% NAC in PBS for 8 min. Worms were fixed in 4% PFA 0.3% Triton X for 30 min and washed twice with 1× PBS 0.3% Triton-X (PBSTx). Worms were washed twice with PBSTx 0.3%, and then dehydrated with a 50% MeOH:50% PBSTx 0.3% solution, and washed and stored in 100% MeOH (O/N) at −20 °C. In preparation for sample bleaching, worms were rehydrated with a 50% MeOH:50% PBSTx 0.3% wash, washed twice with PBSTx 0.3%, and washed once in 1× SSC. For bleaching, worms were incubated in a 1.2% H2O2, 5% Formamide, 0.5× SSC solution for 3 h over a box. Worms were washed once with 1× SSC and twice with 1× PBSTx 0.2% (PBSTx 0.3%). Worms were incubated with 4% paraformaldehyde (PFA, Sigma) in 1× PBSTx for 20 min with a 1× PBS 0.1% SDS solution containing 1−2 μg/ml Proteinase K (in accordance with worm size). Worms were post-fixed in 4% PFA 0.3% Tw for 10 min and washed twice with PBSTx 0.3%. Worms were prepared for hybridization with a 10 min 50% solution wash and subsequent 2 h 56 °C incubation with a prehybridization solution containing 50% Formamide, 1× Denhardts solution, 100 μg/ml Heparin, 1% Tween-20, 50 mM DTT, and 1 mg/ml Sigma Torula Yeast RNA in 5× SSC. DIG-labeled probes were denatured 5 min at 70 °C, and incubated O/N at 56 °C in a hybrid solution identical to prehybridise solution but with Deionized Formamide in place of Formamide, Calbiochem Yeast RNA 0.25 mg/ml in place of Sigma Torula Yeast RNA, and an addition of 5% Dextran Sulfate.

The next day non-specific probe binding was washed at 56 °C. The samples were washed twice for 30 min in a Wash Hybe solution containing 50% Formamide, 0.5% Tween-20, and 1% Denhardt’s in 5× SSC. Worms were then washed twice in 50% Wash Hybe:50× SSC 0.1% Tw for 30 min, thrice with 2× SSC 0.1% Tw for 10 min, and twice with 0.2× SSC 0.1% Tw for 15 min. The samples were then returned to room temperature and washed twice with 1× MAB Tw 0.1−0.3% Tw (MABTw) pH 7.5. The samples were blocked for 2 h in a solution of 1× MABTw containing 10% Horse Serum and 0.5% Roche Western Blocking Reagent (RBWR) and stained with anti-DIG and/or anti-DNP Fab fragment conjugated to alkaline phosphatase at a 1:1000 dilution O/N at 4 °C. Non-specific binding was removed with washing six times with MABTw for 2–3 h and then processed for colorimetric development. Worms were incubated for 15 min in solution of 0.1 M Tris pH 9.5, 0.1 M NaCl, 0.05 M MgCl2, 0.1% Tween containing 0%, 5%, and 10% O/N. The final solution of 80% PVA was supplemented with 1× MABTw containing 0.25% Tween-20 and worms monitored for color development. The reaction was stopped by rinsing 1–2 times in PBS, fixed in 4% PFA 0.3% Tw for 45 min, and washed three times with PBSTx 0.3%. The samples were then washed with 100% EtOH for 20 min and then with 50% EtOH:50% PBS for 5 min. The samples were then rehydrated with 1× PBS washes and mounted in 80% glycerol.

Downregulation of FISH with a Hox effector genes in whole axialplanar s. mediterranea52,55. To kill and remove mucus, animals were placed in 5% N-Acetyl Cysteine (NAC, Sigma) in 1× PBS solutions at room temperature with gentle shaking by hand for 5 min. To fix, animals were placed in 4% paraformaldehyde (PFA, Sigma) in 1× PBSTx 0.5% Triton-X at room temperature for 30 min. The reduction solutions step was skipped to preserve the fragile epidermis of the animals. Animals were bleached with 5% non-deionized formamide, 1.2% H2O2 in 0.5× SSC for 2 h under direct light. Proteinase K treatment (2 μg/ml Proteinase-K, Invitrogen, 0.1% SDS in 1× PBSTx) was performed for 10 min at room temperature, followed by a post-fixation (4% PFA) point-fixation overnight. Samples were subsequently incubated in two riboprobes overnight at 56 °C. Following hybridization, DIG-probes were detected with antibodies (Roche anti-DIG-POD 1:1000) in MABTw containing 5% horse serum and 0.5% RBWR. DIG-probes were developed with rhodamine tyramide (1:500) in Borate Buffer for 45 min. For multicolor FISH, peroxidase activity was stained with Sato’s lead and 0.5% uranyl acetate and images with a BSD at 7 kV, 1.2 nm. Both gross brightness and contrast and local contrast were adjusted using CLAHE (blocksize=127, bins: 256, slope=1.5) in Fiji55.

Summary of data presentation and statistics. All data points derived from distinct single measurements of individual biological replicates except for plots of the size of fission fragments or duration of fission attempts wherein multiple distinct data points (not technical replicates) are derived from each biological replicate. p-Values calculated with Welch’s t-test were unpaired, assumed a Gaussian distribution, and did not assume equal standard deviations. Data points were omitted under the following conditions: animal compressions failed due to technical error, animals crawled out of wells and desiccated on lids during the fissioning experiment, and animals recently fissioned prior to recording of animal body length. Details of all statistical analyses performed in Supplementary Data File 4.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The original data generated in this study have been deposited in the Stowers Original Data Repository under accession code LJBPB-1567. The Hox RNAi RNAseq data generated from this study has been deposited in NCBi GEO with accession number: GSE159876. The fission progeny scoring data, compression and fission segment scoring data, fission progeny area calculations, fission behavior scoring data, and animal length measurements generated in this study are provided in the Source Data File. The RNAseq data for Supplementary Figs. 2 and 3 used in this study are available in the NCBi GEO database under accession codes GSE82280 and GSE107874. The scRNAseq data used to generate t-SNE plots used in this study is available at https://digiworm.wim.mit.edu/ and NCBi GEO: GSE111764. Source data are provided with this paper.

Code availability

The fission behavior timelapses were acquired using micro-manager version 1.4.22 (https://micro-manager.org/) and the openpyramidal framegraber. In both cases, the camera gain, exposure, and autofocus were controlled using the Logitech Webcam Controller software (https://download01.logi.com/web/fp/pub/video/lws/lws280.exe). Source data are provided with this paper.

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