

LIN-39 and the EGFR/RAS/MAPK pathway regulate *C. elegans* vulval morphogenesis via the VAB-23 zinc finger protein

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SUMMARY

Morphogenesis represents a phase of development during which cell fates are executed. The conserved hox genes are key cell fate determinants during metazoan development, but their role in controlling organ morphogenesis is less understood. Here, we show that the *C. elegans* hox gene *lin-39* regulates epidermal morphogenesis via its novel target, the essential zinc finger protein VAB-23. During the development of the vulva, the egg-laying organ of the hermaphrodite, the EGFR/RAS/MAPK signaling pathway activates, together with LIN-39 HOX, the expression of VAB-23 in the primary cell lineage to control the formation of the seven vulval toroids. VAB-23 regulates the formation of homotypic contacts between contralateral pairs of cells with the same sub-fates at the vulval midline by inducing *smp-1* (*semaphorin*) transcription. In addition, VAB-23 prevents ectopic vulval cell fusions by negatively regulating expression of the fusogen *eff-1*. Thus, LIN-39 and the EGFR/RAS/MAPK signaling pathway, which specify cell fates earlier during vulval induction, continue to act during the subsequent phase of cell fate execution by regulating various aspects of epidermal morphogenesis. Vulval cell fate specification and execution are, therefore, tightly coupled processes.

KEY WORDS: Morphogenesis, *hox*, *ras*, *C. elegans*, Vulva

INTRODUCTION

Morphogenesis is the phase in development that occurs after cells have adopted their cell fates. A pivotal condition for proper morphogenesis is the proper specification of cell fates by the hox genes, which encode homeodomain-containing transcription factors that typically control anterior-posterior cell fate decisions during embryogenesis and the axial patterning of the limbs (Hombria and Lovegrove, 2003; McGinnis and Krumlauf, 1992).

The nematode *Caenorhabditis elegans*, which contains a single non-redundant hox gene cluster, has been used extensively to characterize hox gene functions. Six *C. elegans* hox genes have been identified: the *labial*-like gene *ceh-13* (Brunschwig et al., 1999); the *Antp* class genes *lin-39* (Clark et al., 1993) and *mab-5* (Kenyon, 1986); and the posterior *Abd-b*-like genes *egl-5*, *nob-1* and *php-3* (Chisholm, 1991; Van Auken et al., 2000). A central role for *lin-39* and its co-factors has been observed during vulval development in the hermaphrodite larva (Clark et al., 1993; Yang et al., 2005). The vulva forms a connection between the uterus and the outside, through which the fertilized eggs are laid. Composed of only 22 cells generated in a relatively simple and invariant cell lineage, the vulva serves as an excellent model to study cell fate determination (Sternberg, 2005). During the second larval stage, three out of six equipotent vulval precursor cells (VPCs) are induced by an epidermal growth factor (EGF) signal from the

uterine anchor cell (AC) to adopt a vulval cell fate (Hill and Sternberg, 1992). The EGF receptor, encoded by *let-23*, transduces the inductive signal in the VPCs via the RAS/MAPK pathway (Aroian and Sternberg, 1991; Beitel et al., 1990; Lackner and Kim, 1998). In response to the inductive signal, a lateral signal from P6.p activates LIN-12 NOTCH in P5.p and P7.p, which represses EGFR/RAS/MAPK signaling in these cells (Berset et al., 2001; Greenwald, 2005). Thus, high MAPK activity in P6.p results in the primary (1°) fate, whereas strong LIN-12 signaling in P5.p and P7.p specifies the secondary (2°) fate (Greenwald, 2005). Once the cell fates have been determined, P5.p–P7.p undergo three rounds of divisions to generate 22 vulval cells that are further divided into seven sub-fates. VulA, VulB1, VulB2, VulC and VulD are generated by the seven 2° descendants each of P5.p and P7.p, and VulE and VulF are formed by the eight 1° descendants of P6.p (Fig. 1A) (Shemer et al., 2000). During the subsequent phase of morphogenesis, cells migrate and form extensions towards the vulval midline, where they make homotypic contacts with their contralateral partner cells of the same sub-fates, thereby forming seven symmetric rings called toroids. With the exception of VulB1 and VulB2, cells within a toroid fuse, thus generating a stack of syncytial toroids arranged in a pyramidal manner.

LIN-39, which is activated through the combined actions of the EGFR/RAS/MAPK and WNT signaling pathways, performs at least two distinct functions during vulval development (Eisenmann et al., 1998; Maloof and Kenyon, 1998). First, LIN-39 prevents the fusion of the VPCs with *hyp7* at the early L2 stage (Clark et al., 1993). Second, during cell fate execution and morphogenesis, LIN-39 is required for cell proliferation and toroid formation (Shemer and Podbilewicz, 2002). The HOX co-factors CEH-20 PBX and UNC-62 MEIS play similar roles during vulval morphogenesis by regulating cell fusion and migration (Yang et al., 2005). Previous work has indicated that *let-60 ras* is also involved in controlling vulval morphogenesis (Shemer et al., 2000), though the exact role of EGFR/RAS/MAPK signaling during vulval cell fate execution is not well understood.

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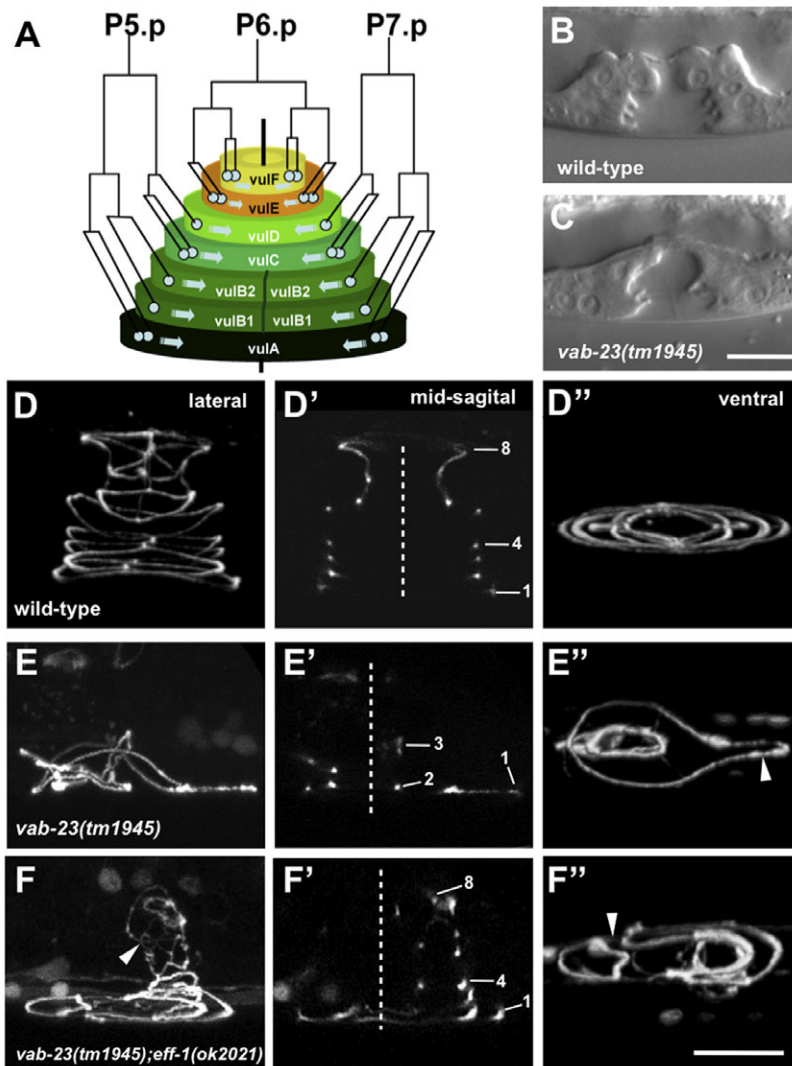


Fig. 1. VAB-23 regulates toroid formation and blocks cell fusion during morphogenesis in *C. elegans*. (A) A stack of seven concentric toroids forms the vulva. The cell lineages are indicated on the sides. The thick black line in the center marks the vulval midline. Arrows represent the cell movement towards the midline, where cells of the same sub-fates attach and fuse to each other (except for VulB1 and VulB2) to form toroids. (B,C) Nomarski images of the vulva in wild-type (B) and *vab-23(tm1945)* (C) L4 larva. (D-F'') Toroids visualized by AJM-1::GFP in wild-type (D-D''), *vab-23(tm1945)* (E-E'') and *vab-23(tm1945); eff-1(ok2021)* (F-F'') L4 larvae. D-F show lateral views of 3D confocal reconstructions, D'-F'' are single mid-sagittal optical sections showing the junctions between toroids, and D''-F'' are ventral views of the 3D reconstructions. Dashed lines indicate the vulval midline. The arrowhead in F points to a site where cells formed aberrant contacts on one side of the midline, and the arrowheads in E'' and F'' indicate defective migration of VulA to the midline. Anterior is left. Scale bars: 10 μ m.

We have previously identified the *vab-23* gene as an essential regulator of morphogenesis that controls the movements of ventral epidermal cells in the embryo (Pellegrino et al., 2009). VAB-23 is the first characterized member of a family of nuclear proteins containing a strongly conserved C4H2-type zinc finger domain at the C-terminus. All metazoans genomes encode a single VAB-23 homolog, and the human protein HCA127 (ZC4H2 – Human Gene Nomenclature Database) was found to be an auto-antigen in hepatocellular carcinomas (Wang et al., 2002). Here, we describe the functions of VAB-23 during vulval morphogenesis. The LIN-39 and RAS/MAPK target VAB-23 guides cell interactions during toroid formation. VAB-23 positively regulates, among others, the transcription of *smg-1* to allow proper interactions to occur between cells of the same sub-fates. In addition, VAB-23 inhibits EFF-1-dependent cell fusion during toroid formation. Thus, *vab-23* is an essential factor linking LIN-39 and the EGFR/RAS/MAPK pathway, which are first used during vulval fate specification, to the subsequent phase of morphogenesis.

MATERIALS AND METHODS

Strains

C. elegans strains were maintained at 20°C as described (Brenner, 1974) unless noted otherwise. Wild type refers to the Bristol N2 strain. Transgenic lines were generated by microinjection of the indicated

constructs at 10–20 ng/ μ l together with transformation markers at 50–75 ng/ μ l and bluescript carrier to a total DNA concentration of 150 ng/ μ l. Strains used are as follows: LGII: *vab-23(tm1945)/mIn1[mIs14 dpy-10(e128)]* (this study), *lin-7(e1413)*, *eff-1(ok1021)*. LGIII: *lin-39(n1760)*. LGIV: *lin-3(e1417)*, *let-60(n1046)*. LGV: *sos-1(cs41)*. Integrated arrays: LGII: *kuls36[P_{egl-26}::gfp,unc-119(+)]* (Hanna-Rose and Han, 2002). LGIII: *syIs90[P_{egl-17}::yfp,unc-119]*, *syIs107[unc-119(+),P_{lin-3}:: Δ pes-10::gfp]* (Chang et al., 1999). LGIV: *jcls1[ajm-1::gfp]* (Mohler et al., 1998), *zhIs1[lin-39::gfp]* (Szabo et al., 2009), *swIs79[ajm-1::gfp,unc-119]*, *mcls46[dlg-1::dsred;unc-119(+)]*. LGV: *syIs51[P_{cdh-3}::gfp,unc-119]* (Inoue et al., 2002), *gals36[hs-mpk-1(+),D-mek(gf)]* (Lackner and Kim, 1998), *zIs16[P_{eff-1}::gfp,rol-6(gf)]* (Fernandes and Sternberg, 2007). Extrachromosomal arrays: *zhEx229[vab-23::gfp,lin-48::gfp]* (Pellegrino et al., 2009), *zhEx271[P_{unc-119-vab-23a}::gfp,lin-48::gfp]* (Pellegrino et al., 2009), *zhEx275[vab-23::gfp,rol-6(d)]*, *zhEx276[vab-23::gfp,P_{sur-5}::dsred]*, *zhEx277[vab-23a::gfp,lin-48::gfp]*, *zhEx286[vab-23Hox(+),lin-48::gfp]*, *zhEx287[vab-23 Hox Δ 1,lin-48::gfp]*, *zhEx288[vab-23Hox Δ 2,lin-48::gfp]*, *zhEx294[vab-23Hox-mut,lin-48::gfp]*, *zhEx295[vab-23,P_{sur-5}::dsred]*, *zhEx317[P_{unc-119-vab-23a}::gfp,smg-1::gfp,plin-48::gfp]*, *zhEx372[P_{smg-1-2256-2987}::nls::gfp::lacZ,myo-2::mcherry]*, *zhEx373[P_{smg-1-2256-1::nls::gfp::lacZ,myo-2::mcherry]}*, *zhEx371[P_{smg-1-2256-2987 Δ 486-2497}::nls::gfp::lacZ,myo-2::mcherry]*, *zhEx375[P_{smg-1-2494-2987}::nls::gfp::lacZ,myo-2::mcherry]*, *zhEx391[P_{smg-1-2604-2987}::nls::gfp::lacZ,myo-2::mcherry]*, *zhEx390[P_{smg-1-2701-2987}::nls::gfp::lacZ,myo-2::mcherry]*, *zhEx388[P_{smg-1-2803-2987}::nls::gfp::lacZ,myo-2::mcherry]*, *zhEx389[P_{smg-1-2903-2987}::nls::gfp::lacZ,myo-2::mcherry]*, *zhEx400[P_{smg-1-2604-2987 Δ 2790-2809}::nls::gfp::lacZ,myo-2::mcherry]*.

RNA interference

RNA interference (RNAi) was performed using the feeding method (Kamath et al., 2001). Worms were synchronized at the L1 stage, transferred to nematode growth media plates containing 3 mM IPTG, 50 µg/ml ampicillin and RNAi bacteria, and allowed to grow for 3-5 days at 25°C. The F1 progeny were analyzed.

Microscopy and 3D reconstructions of vulval toroids

Images were recorded of anesthetized larvae using a Leica DMRA wide-field microscope equipped with a cooled CCD camera (Hamamatsu ORCA-ER) as described (Pellegrino et al., 2009) and analyzed using the Openlab 5.0 software package (Improvision/Perkin Elmer). To quantify signal intensities (Figs 5, 6), images were recorded under identical conditions and the mean fluorescence intensity subtracted by the background fluorescence was determined using the Openlab measurement tool. To create 3D reconstructions of vulval toroids (Figs 1, 7), confocal sections through the larvae were recorded with an Olympus FV1000 confocal microscope and processed with the Volocity 2.0 software package (Improvision/Perkin Elmer).

vab-23 and smp-1 promoter and chromatin immunoprecipitation (ChIP) analysis

vab-23 promoter deletion analysis was performed by PCR using the *vab-23::gfp* plasmid as template. The upstream region of the *vab-23* promoter for each PCR product is as follows: 3372 bp for *vab-23Hox(+)*, 1363 bp for *vab-23 HoxΔ1*, 1203 bp for *vab-23 HoxΔ2*. Point mutations in both conserved PBX sites (*vab-23 Hox mut*) were introduced using a combination of the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) and overlap PCR (Hobert, 2002). *smp-1* promoter analysis was performed by PCR amplification of the fragments indicated in Fig. 4E subcloned into the *PstI* and *SalI* sites of the Fire vector pPD96.04.

For ChIP analysis, chromatin prepared from animals carrying a functional LIN-39::GFP reporter was precipitated with GFP antibodies (Roche) as described by Mukhopadhyay et al. (Mukhopadhyay et al., 2008). As negative control, a mock precipitation omitting the primary antibody was performed in parallel. Binding was quantified by Q-PCR with the probes shown in Fig. 6B. For each sample, the signal was first normalized to the input DNA (Δct) and then to the signal obtained with a probe in the 3'UTR of *vab-23* ($\Delta\Delta\text{ct}$) as internal reference to calculate relative binding. Primers used for Q-PCR analysis were: CTCAAAGTGGTCCCAAAA and AGAGAAGAAAGAAGGAGG for region I; TACTTCCCCTCTGCACT and CGGACCACTATCAAAACAC for region II; CCCATTGACATTCTCTAC and GACAAGATTGATTCCGGCG for region III; and CAATTACTTTGTGATCTCCC and TCCCATATACTCCGCA for the 3' region. For ChIPseq analysis, 20 ng of immunoprecipitated DNA was used as template for the SOLiD 4 whole-genome sequencing platform yielding around 4×10^7 reads of 50 bp each. DNAs isolated from the input chromatin fraction and from a ChIP experiment using an unrelated GFP fusion protein were subjected to the same sequence analysis and used as background controls. Sequence alignment to the *C. elegans* reference sequence (WS192) was performed with the CLC genomics workbench software using default parameters. Peak detection was performed with a custom R script described in the legend to Table S1 in the supplementary material.

RESULTS

VAB-23 is essential for toroid formation during vulval morphogenesis

The function of *vab-23* was analyzed using the *vab-23(tm1945)* deletion allele (kindly provided by S. Mitani, Tokyo Women's University School of Medicine, Japan) that is likely to represent a null mutation (Pellegrino et al., 2009). The *vab-23(tm1945)* deletion results in a completely penetrant embryonic or early larval lethal phenotype due to defects in epidermal morphogenesis. The lethality could be rescued by expression of *vab-23a* cDNA with *gfp* fused at the C-terminus under control of the pan-neuronal *unc-119* promoter (*P_{unc-119::vab-23::gfp}*) (Pellegrino et al., 2009). This

neuron-specific rescue allowed us to study the function of *vab-23* during post-embryonic development. Homozygous *vab-23(tm1945)* mutants carrying the *P_{unc-119::vab-23::gfp}* transgene developed into fertile adults with a completely penetrant protruding vulva (Pv1) phenotype. At low penetrance (<15%), *P_{unc-119::vab-23::gfp}* was weakly expressed in the 1° vulval lineage, which we attribute to the leaky nature of the *unc-119* promoter. However, the low level of epidermal *vab-23* expression was insufficient to rescue the Pv1 phenotype. For the remainder of this study, we refer to *vab-23(tm1945)* larvae rescued by *P_{unc-119::vab-23::gfp}* as *vab-23(tm1945)* mutants.

We first investigated whether the vulval defects of *vab-23(tm1945)* were caused by an abnormal pattern of cell division. For this purpose, we directly followed the vulval lineage of *vab-23(tm1945)* animals, but observed no change in the cell division pattern or the orientations of the cleavage axes ($n=4$). In addition, we counted the number of vulval cells at the early L4 stage and found the normal number of 22 cells in all cases ($n=20$; data not shown). However, all *vab-23(tm1945)* L4 larvae displayed penetrant defects in vulval morphogenesis. The wild-type vulva is made of a stack of seven concentric toroid rings that are formed by the circumferential migration and subsequent fusion of contralateral pairs of cells of the same sub-fates (Fig. 1A,B). By contrast, the vulvae of *vab-23(tm1945)* larvae were asymmetric in configuration and abnormal in shape (Fig. 1C). These defects could be phenocopied by *vab-23* RNAi (see Fig. S1 in the supplementary material) and rescued by introducing the entire genomic *vab-23* locus into *vab-23(tm1945)* mutants (Fig. 8A, in this particular experiment in the absence of *P_{unc-119::vab-23::gfp}*). Thus, the observed vulval morphogenesis defects are caused by a loss of *vab-23* function.

Next, we used the AJM-1::GFP adherens junction marker to examine the shape of the vulval toroids in L4 larvae (Mohler et al., 1998). In the wild type, AJM-1::GFP appeared as a stack of eight concentric rings delineating the seven toroids (Fig. 1D). A mid-sagittal cross-section revealed eight dots representing the cell junctions of the toroids (Fig. 1D'). *vab-23(tm1945)* animals displayed two characteristic defects. First, fewer abnormally shaped toroids were formed with an average of 4.3 ± 0.2 vulval toroid junctions per animal ($n=43$; Fig. 1E,E'). The absent toroids were predominantly those normally formed by the 2° VulC, VulD and the 1° VulE and VulF cells. Second, cells failed to migrate towards the vulval midline in 45% ($n=64$) of *vab-23(tm1945)* L4 larvae (arrowhead in Fig. 1E''). Thus, *vab-23* is not necessary for the execution of the vulval cell lineage, but rather controls toroid formation during morphogenesis.

vab-23 represses eff-1-mediated vulval cell fusions

Next, we examined whether the reduced toroid number in *vab-23(tm1945)* mutants might be due to ectopic cell fusions resulting from inter-toroid fusion between cells of different sub-fates or from fusion with the surrounding hyp7. The fusogen EFF-1 is necessary for most somatic cell fusions, including those between vulval cells and hyp7 and between vulval cells within toroids (Mohler et al., 2002). We used a transcriptional *P_{eff-1::gfp}* reporter to examine whether ectopic *eff-1* expression might be responsible for the loss of toroids. In control RNAi animals, *P_{eff-1::gfp}* was expressed at the L4 stage in VulA, VulC and less frequently in VulF (21%, $n=43$) (Fig. 2A). Reduction of *vab-23* function by RNAi caused an increased expression of *P_{eff-1::gfp}* in VulF (65%, $n=34$) and additional expression in VulE (38%, $n=34$), where no expression was observed in control animals ($n=43$) (Fig. 2B). We then investigated whether loss of *eff-1* function suppresses the reduced toroid number in *vab-*

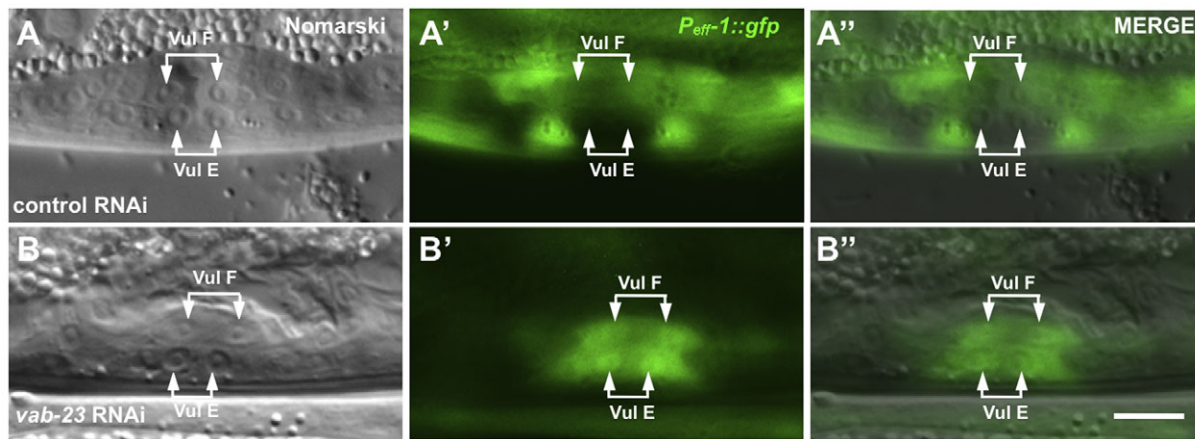


Fig. 2. VAB-23 represses the fusogen *eff-1* in VulE and VulF. (A–B'') Nomarski (A,B), GFP (A',B') and merged (A''–B'') images of control (A–A'') and *vab-23* RNAi-treated (B–B'') larvae carrying an *eff-1::gfp* reporter. The positions of the VulE and VulF nuclei are indicated by arrows. Anterior is left. Scale bar: 10 μ m.

23(tm1945) mutants. Vulval morphogenesis was still abnormal and toroids were malformed in *eff-1(ok1021); vab-23(tm1945)* double mutants, although the number of cell junctions was increased from 4.3 ± 0.2 to an average of 6.4 ± 0.2 per animal ($n=24$; Fig. 1F). Taken together, loss of VAB-23 function results in the ectopic expression of EFF-1, which is partially responsible for the reduced toroid number. However, as the toroids that formed in *eff-1(ok1021); vab-23(tm1945)* double mutants were still abnormally shaped, VAB-23 is not only required to repress EFF-1-mediated vulval cell fusions but also to mediate proper toroid formation.

VAB-23 represses the formation of ectopic cell contacts

VAB-23 regulates the formation of contacts between contralateral pairs of epidermal cells during morphogenesis of the embryo (Pellegrino et al., 2009). To assess whether VAB-23 plays a similar function during vulval morphogenesis, we followed cell junctions formed between adjacent vulval cells during larval development (Fig. 3). Cell contacts in *vab-23(tm1945)* worms appeared normal at the early L3 stage (Pn.p to Pn.px stage, $n=33$). However, beginning at the Pn.pxx stage, abnormal cell extensions between adjacent vulval cells formed in 59% of *vab-23(tm1945)* animals ($n=41$; Fig. 3D), and at the onset of vulval invagination (Pn.pxxx stage) ectopic contacts between 1° and 2° vulval cells were observed in 88% of the cases ($n=33$; Fig. 3F). Finally, in *eff-1(ok1021); vab-23(tm1945)* double mutants at the L4 stage, 1° and 2° cells failed to reach their contralateral partner cells across the midline (Fig. 1F), suggesting that the vulval morphogenesis defects are similar to the defects observed during ventral epidermal enclosure of the embryo (Pellegrino et al., 2009). Thus, besides repressing cell fusions, VAB-23 is also required for the proper interaction between vulval cells of the same sub-fates.

VAB-23 regulates *smg-1* transcription during vulval morphogenesis

The abnormal vulval cell migrations and cell contacts in *vab-23(tm1945)* mutants are reminiscent of mutations in *smg-1*, which encodes a transmembrane Semaphorin 1a homolog, or in *plx-1*, which encodes the putative SMP-1 receptor Plexin-A4 (Dalpe et al., 2005). We therefore examined whether *smg-1* and/or *plx-1* expression might be regulated by VAB-23, as we previously found

that the zinc finger domain of VAB-23 is essential for its nuclear function (Pellegrino et al., 2009). Using a translational SMP-1::GFP reporter (kind gift of Joseph Culotti, The Samuel Lunenfeld Research Institute, Ontario, Canada), for which expression and localization at the vulval midline has been previously described (Dalpe et al., 2005), we found that 59% of *vab-23(tm1945)* larvae ($n=29$) lacked SMP-1::GFP expression at the onset of vulval invagination, whereas SMP-1::GFP expression was absent in only 9% of wild-type animals at this stage ($n=34$) (Fig. 4A',B'). We verified that the loss of SMP-1 expression was not due to excess cell fusions by analyzing the adherens junction marker DLG-1::dsRED (Fig. 4A'',B'') (Bossinger et al., 2001). Despite the abnormal shape of the vulval invagination, no reduction in DLG-1::dsRED expression was detected in *vab-23(tm1945)* mutants at this stage, indicating that the ectopic cell fusions occur at a later stage and are therefore unlikely to be the cause of the lost SMP-1::GFP expression.

To determine whether VAB-23 controls *smg-1* expression at the transcriptional or post-transcriptional level and to identify the cis-regulatory elements, we generated a series of transcriptional *smg-1* reporters (Fig. 4E). A construct in which an *nls::gfp::lacZ* reporter cassette was fused in frame within exon II to include 2256 bp of the 5' *smg-1* promoter/enhancer region and the entire intron I yielded specific expression in the VulF, VulE and VulD cells ($P_{smg-1-2256-2987}$ in Fig. 4E and data not shown). However, a construct consisting only of the 2256 bp of the 5' promoter/enhancer region upstream of exon I did not show any detectable expression in the vulval cells but showed strong ectopic expression in body wall muscles and hypodermal cells ($P_{smg-1-2256-1}$ in Fig. 4E and data not shown). Thus, intron I must contain the regulatory elements required for vulva-specific transcription of *smg-1* mRNA. We further narrowed down the region that is sufficient for vulval expression to a 493 bp fragment at the end of intron I (Fig. 4C,C' and $P_{smg-1-2494-2987}$ in 4E). When the $P_{smg-1-2494-2987}$ reporter was introduced into the *vab-23(tm1945)* mutant background, expression was strongly reduced but not eliminated (Fig. 4D). Specifically, 30% ($n=30$) of *vab-23(tm1945)* L4 larvae showed detectable NLS::GFP expression in the vulval cells, whereas strong expression was observed in 85% ($n=20$) of wild-type larvae. Further deletion analysis identified a 102 bp region (between positions 2701 and

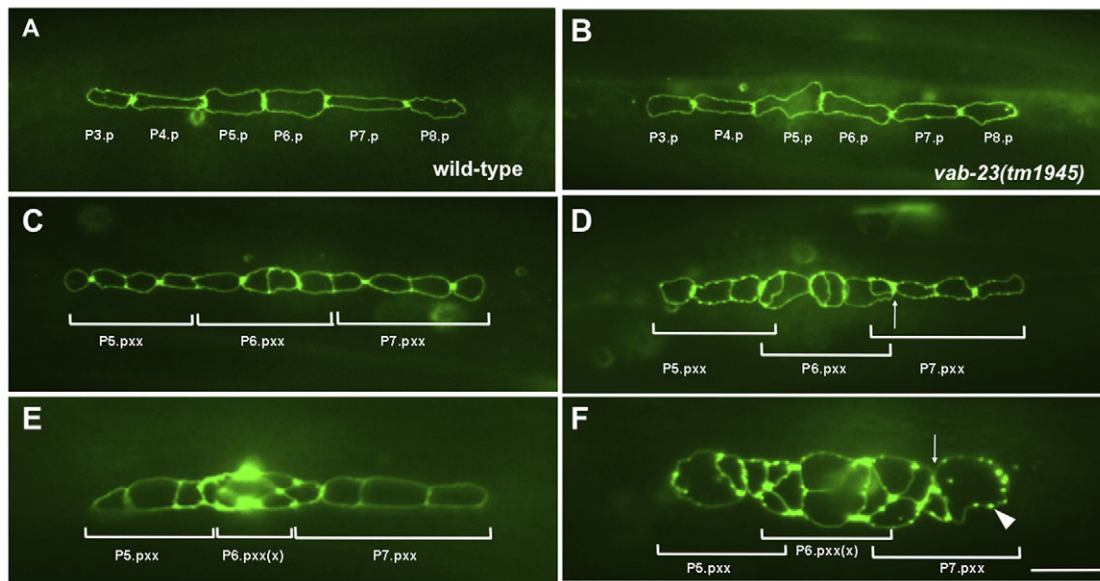


Fig. 3. VAB-23 regulates the formation of proper vulval cell contacts. (A-F) Ventral views of AJM-1::GFP in wild-type (A,C,E) and *vab-23(tm1945)* (B,D,F) larvae at the Pn.p, Pn.pxx to Pn.pxxx stages. Arrows in D and F indicate abnormal cell contacts, and the arrowhead in F shows punctate AJM-1::GFP expression. Scale bar: 10 μ m.

2803) that is absolutely required for expression of *smg-1* as no NLS::GFP expression was detectable in animals carrying the *P_{smg-1-2803-2987}* reporter. Possibly, this minimal region in intron I defines an alternative, vulva-specific promoter generating an *smg-1* mRNA that uses an alternative translational start codon present at the beginning of exon II (position 2884) or a further downstream start codon within exon II. This essential 102 bp region contains a stretch of 20 bp (positions 2790 to 2809) that are strongly conserved between the four closely related nematode species *C. elegans*, *C. briggsae*, *C. remanei* and *C. brenneri* (Fig. 4F). When this conserved 20 bp element was deleted in the minimal reporter (*P_{smg-1-2604-2987} Δ 2790-2809* in Fig. 4E), vulval expression was strongly reduced but not eliminated, similar to the reduction observed in *vab-23(tm1945)* mutants. Specifically, 22% of *P_{smg-1-2604-2987} Δ 2790-2809* L4 larvae showed NLS::GFP expression, whereas no expression was detected in 52% and asymmetric expression on one side of the vulval invagination in the remaining 25% of the cases ($n=36$).

To test whether the VAB-23 protein interacts directly with the *smg-1* locus, we performed chromatin immunoprecipitation of VAB-23 followed by deep sequencing of the bound DNA fragments (ChIPseq). For this purpose, we used the translational *vab-23::gfp* fusion construct described previously (Pellegrino et al., 2009) and isolated the VAB-23::GFP protein-chromatin complexes with anti-GFP antibodies. Importantly, the *vab-23::gfp* transgene efficiently rescued the embryonic and vulval morphogenesis defects of *vab-23(tm1945)* mutants (Fig. 8A). The ChIPseq analysis detected strong binding of VAB-23::GFP to the *smg-1* locus over a relatively large region with a peak (i.e. region of the highest read coverage) in the same region that had been identified by *smg-1* promoter analysis (left-right arrow in Fig. 4E; see Fig. S2A in the supplementary material).

Taken together, our promoter and ChIPseq analysis indicated that VAB-23 directly activates transcription of *smg-1* in the VulF, VulE and VulD cells. In addition to *smg-1*, we identified another

455 specific binding VAB-23 binding sites throughout the *C. elegans* genome, suggesting that VAB-23 controls the transcription of a large number of target genes (see Table S1 in the supplementary material and Materials and methods).

VAB-23 regulates the expression of late VulE and VulF sub-fate-specific genes

Next, we used various markers to determine whether early or late steps during cell fate specification are affected in *vab-23(tm1945)* mutants (Fig. 5). We examined the 1^o fate specification using a *P_{egl-17::yfp}* reporter, which is predominantly expressed in the 1^o cell lineage until the 4-cell stage (Pn.pxx). This early, 1^o-specific expression of *P_{egl-17::yfp}* was unchanged or even slightly elevated in *vab-23(tm1945)* larvae, indicating proper 1^o fate specification (Fig. 5A,B). To examine the VulE and VulF sub-fates, we used transcriptional *P_{egl-26::gfp}* and *P_{lin-3::gfp}* reporters, respectively (Chang et al., 1999; Hanna-Rose and Han, 2002). In *vab-23(tm1945)* mutants, 75% of L4 larvae ($n=61$) lacked *P_{lin-3::gfp}* expression in VulF cells, compared with 9% of wild-type larvae showing no expression at this stage ($n=23$; Fig. 5C,D). Similarly, a reduction of *vab-23* function by RNAi caused a loss of *P_{egl-26::gfp}* expression in VulE in 61% of the animals ($n=54$; Fig. 5E,F). Moreover, VAB-23::GFP ChIPseq analysis detected a small but significant peak in the *egl-26* 5' regulatory region contained within the *P_{egl-26::gfp}* reporter (see Fig. S2B in the supplementary material), whereas no binding to the *lin-3* locus was detected (data not shown).

LIN-3 EGF expression in VulF cells is necessary to specify the uv1 cell fate in the ventral uterine cells (Chang et al., 1999). Accordingly, *vab-23(tm1945)* mutants or RNAi-treated animals often had thick tissue blocking the connection between the vulval lumen and the uterus, suggesting a defect in uterine development (see Fig. S1B in the supplementary material). Finally, we examined the *P_{egl-17::yfp}* and *P_{cdh-3::gfp}* reporters in L4 larvae to assay the specification of the 2^o VulC and VulD sub-fates (Burdine et al., 1998; Inoue et al., 2002), but observed no obvious changes in their expression patterns (Fig. 5G-J). Thus, VAB-23 is not necessary for

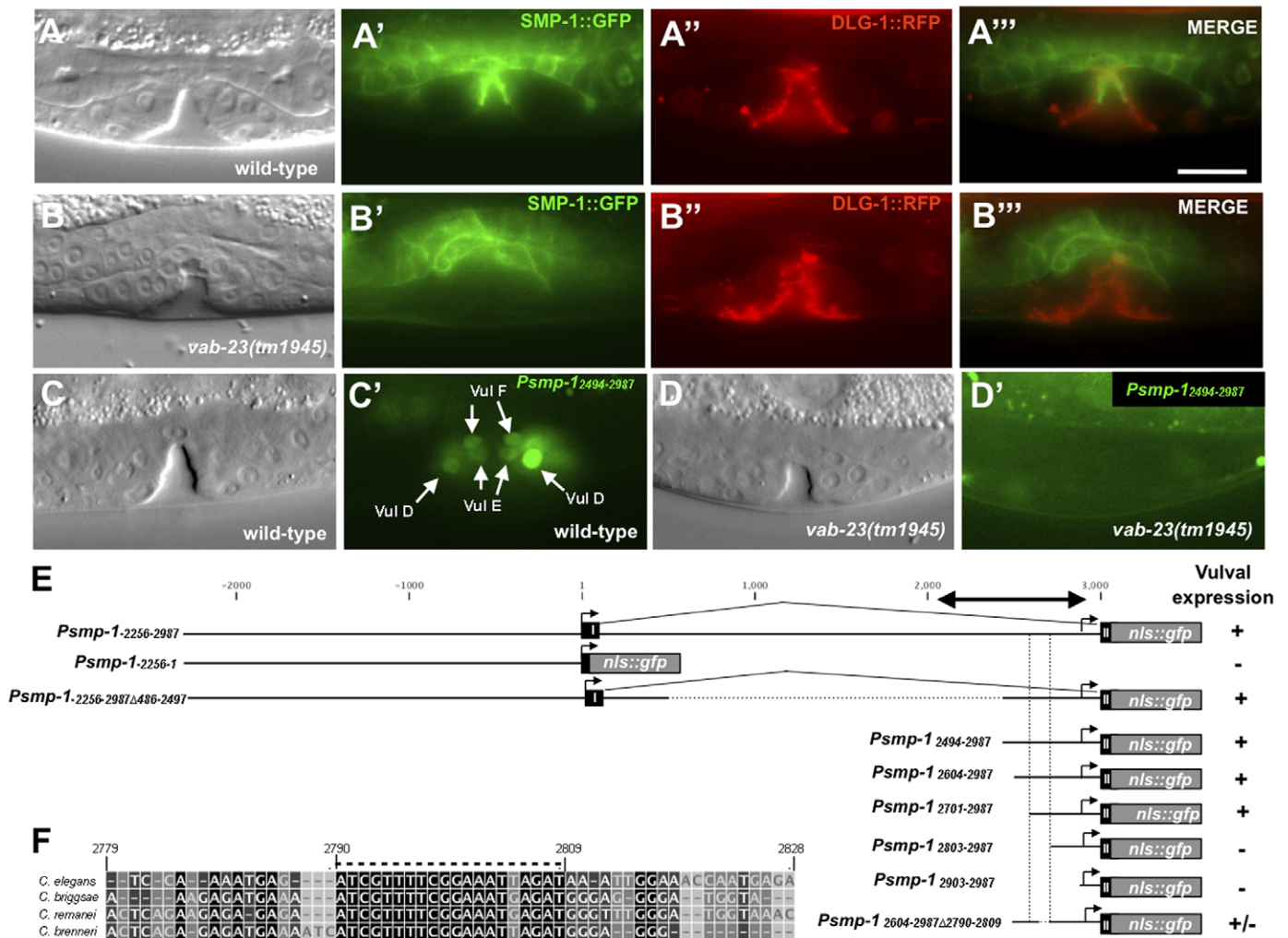


Fig. 4. VAB-23 induces *smp-1* transcription in VulF, VulE and VulD. (A-B'') Nomarski images (A,B), expression of the translational SMP-1::GFP reporter (A',B'), expression of the DLG-1::dsRED reporter (A'',B'') and merged images (A''',B''') in wild-type (A-A''') and *vab-23(tm1945)* (B-B''') L4 larvae at the onset of toroid formation. (C-D') Nomarski images (C,D) and expression of the transcriptional *Psm-1*²⁴⁹⁴⁻²⁹⁸⁷ reporter (C',D') in wild-type (C,C') and *vab-23(tm1945)* (D,D') L4 larvae. Anterior is left. Scale bar: 10 μ m. (E) Structures of the *smp-1* transcriptional reporters used for promoter analysis. The numbers indicate the positions relative to the translational start site. For each construct, three independent lines were analyzed except for *Psm-1*⁻²⁸⁰³⁻²⁹⁸⁷ which yielded only two lines. +, strong vulval expression; -, no detectable expression; +/-, reduced expression (see text). (F) Sequence alignment of the conserved element in the vulva-specific element in intron I. The dashed line indicates the 20 nucleotides deleted in reporter *Psm-1*^{-2604-2987Δ2790-2809}.

the specification of the 1° or 2° cell fates during vulval induction, but rather controls the patterning of the 1° VulF and VulE cells. Regulation of *egl-26* transcription by VAB-23 might be direct, whereas regulation of *lin-3* expression appears to be indirect.

VAB-23 expression is regulated by EGFR/RAS/MAPK signaling during vulval induction and morphogenesis

We analyzed the post-embryonic VAB-23 expression pattern using the translational VAB-23::GFP reporter described previously (Pellegrino et al., 2009). VAB-23::GFP was expressed in the AC, the vulval cells, in the ventral and dorsal uterine cells, the seam cells, the vulval muscle cells, a small cluster of unidentified tail cells, and some ventral cord neurons (Fig. 6 and data not shown). Vulval expression of VAB-23::GFP was observed predominantly in the 1° lineage beginning at the time of induction and persisting until adulthood (Fig. 6A-D and data not shown). Even though

VAB-23::GFP was initially expressed at low levels in all VPCs, expression was downregulated in the 2° lineage during induction and persisted at low levels in the tertiary (3°) cells. VAB-23::GFP continued to be strongly expressed in the VulE and VulF cells of L4 larvae during vulval morphogenesis, although relatively weaker expression was also observed in VulC and VulD at this later stage (Fig. 6D, VulC and VulD nuclei are out of focus).

The upregulation of VAB-23 in the 1° cell and simultaneous downregulation in the 2° lineage suggested that *vab-23* might be a target of the EGFR/RAS/MAPK signaling pathway. We therefore analyzed VAB-23::GFP expression in various mutants in which the activity of the EGFR/RAS/MAPK signaling pathway is changed. Diminished activation of LET-23 EGFR by a mutation in *lin-3* (Hill and Sternberg, 1992) or through mislocalization via a *lin-7* mutation (Simske et al., 1996) had two effects on VAB-23::GFP expression. First, expression in P6.p and its descendants was strongly reduced, and second, equally weak expression was observed in P5.p to P7.p

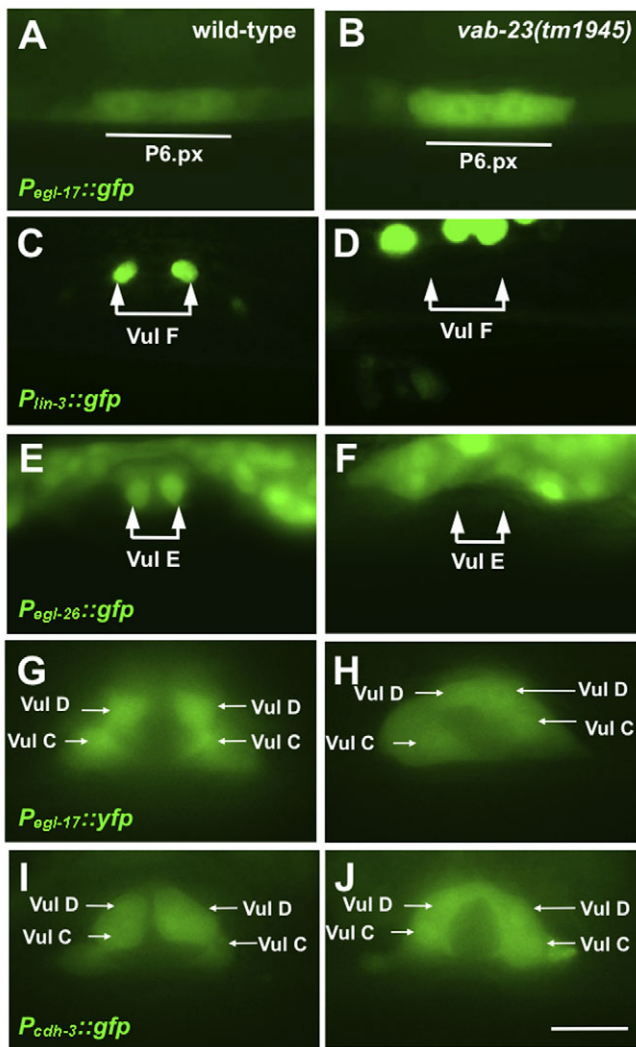


Fig. 5. VAB-23 controls the expression of late 1° cell fate markers. (A,B) Expression of EGL-17::YFP in wild-type (A) and *vab-23(tm1945)* (B) L3 larvae at the Pn.px stage, (C–J) LIN-3::GFP (C,D), EGL-26::GFP (E,F), EGL-17::YFP (G,H) and CDH-3::GFP (I,J) expression in wild-type (C,E,G,I) and *vab-23(tm1945)* (D,F,H,J) L4 larvae. Arrows indicate the nuclei of the indicated sub-fates on one side. Anterior is to left. Scale bar: 10 μ m.

and their descendants, probably owing to the loss of lateral inhibition from P6.p (Fig. 6E,F). By contrast, ectopic expression of VAB-23::GFP was induced in the pseudovulvae formed by P3.p, P4.p or P8.p descendants in animals carrying a constitutively active *let-60* allele or by overexpressing the MAP kinase MPK-1 along with MEK-2 (Fig. 6G,H) (Beitel et al., 1990; Lackner and Kim, 1998). Lastly, we examined whether EGFR/RAS/MAPK signaling continues to regulate VAB-23::GFP expression after vulval induction. For this purpose, we used a temperature-sensitive allele of *sos-1*, which encodes the major guanine nucleotide exchange factor for LET-60 (Chang et al., 2000). We observed a reduced or absent expression of VAB-23::GFP in ~80% of *sos-1(ts)* worms that had been shifted to the restrictive temperature after vulval induction had occurred (Fig. 6I,J; see Fig. S2 in the supplementary material). We conclude that EGFR/RAS/MAPK signaling is first required to upregulate *vab-23* expression during induction and later to maintain expression in the 1° lineage during morphogenesis.

***vab-23* is a direct target of LIN-39 and CEH-20**

LIN-39 performs at least two distinct functions during vulval development (Clark et al., 1993; Maloof and Kenyon, 1998; Shemer and Podbilewicz, 2002). First, LIN-39 represses the expression of the fusogen *eff-1* to prevent the fusion of VPCs with the surrounding hypodermis. Second, *lin-39* is required for the execution of the vulval cell lineage after the fates have been specified, because in *eff-1(0); lin-39(0)* double mutants the vulval cells fail to divide (Shemer and Podbilewicz, 2002). Because *lin-39* expression is positively regulated by EGFR/RAS/MAPK signaling and *vab-23* prevents *eff-1*-mediated cell fusions, we investigated whether *vab-23* might function downstream of LIN-39 during vulval morphogenesis (Guerry et al., 2007; Maloof and Kenyon, 1998). For this purpose, the loss-of-function allele *lin-39(n1760)* was examined together with the *eff-1(ok1021)* mutation, which suppresses the early VPC fusions. VAB-23::GFP expression was strongly reduced in the *eff-1(ok1021); lin-39(n1760)* double mutants, but unchanged in *eff-1(ok1021)* single mutants (Fig. 6K,L and data not shown). In early L3 larvae at the Pn.px cell stage, VAB-23::GFP was absent or barely detectable in 89% of *eff-1(ok1021); lin-39(n1760)* double mutants and strongly reduced in the remaining cases ($n=26$; Fig. 6K). Also, at the time of toroid formation, *eff-1(ok1021); lin-39(n1760)* animals showed, on average, a fourfold decrease in the intensity of VAB-23::GFP expression with 63% of the animals showing no or strongly reduced expression ($n=27$; Fig. 6L). In the remaining 37% of cases, VAB-23::GFP expression persisted albeit at a reduced level, indicating that late VAB-23 expression is partially regulated by a LIN-39-independent pathway. Similarly, a reduction-of-function mutation in *ceh-20*, which encodes a LIN-39 co-factor of the Extradenticle/PBX family (Yang et al., 2005), resulted in reduced VAB-23::GFP expression during the early (Pn.p to Pn.pxx) stages of vulval development, whereas the late (Pn.pxxx stage) expression was not affected by *ceh-20* (Fig. 6M,N).

Next, we determined whether *vab-23* is a transcriptional target of LIN-39. We aligned the *vab-23* 5' regulatory regions from *C. elegans* and the closely related nematodes *C. briggsae* and *C. remanei* and searched for conserved HOX/PBX consensus binding motifs, such as TGATNNAT (Cui and Han, 2003; Koh et al., 2002). Two conserved putative HOX/PBX binding sites were present in a 72 bp region located 1.3 kb upstream of the *vab-23* translation initiation site (Fig. 7A, region II in 7B). By performing a promoter deletion and mutation analysis, we found that the early (Pn.px stage) vulval expression of VAB-23::GFP depends on the 72 bp region, whereas a smaller deletion leaving the 72 bp region intact (Hox Δ 1) had no significant effect (Fig. 7B,C). By contrast, deletion of the two HOX/PBX sites had only moderate effects on the late VAB-23::GFP expression in L4 larvae (Hox Δ 2 in Fig. 7D). Moreover, point mutations in both conserved HOX/PBX sites (Hox-mut) eliminated the early but not the late VAB-23::GFP expression, suggesting that early on *vab-23* is a direct LIN-39 and CEH-20 target (Fig. 7C,D). Thus, LIN-39 regulates VAB-23 expression until the Pn.px stage together with CEH-20 and from the Pn.pxx stage onwards, in conjunction with another, unidentified co-factor.

Finally, we observed a direct interaction between LIN-39 and the region containing the HOX/PBX sites in ChIP experiments. Using a functional LIN-39::GFP reporter (Szabo et al., 2009), we detected strongest binding to region II, which contains the HOX/PBX sites, whereas regions I and III showed weaker binding relative a probe in the 3'UTR used as internal reference (Fig. 7B,E) (see Materials and methods). It should be noted that the ChIPseq experiments performed by the modENCODE consortium (Celniker et al., 2009)

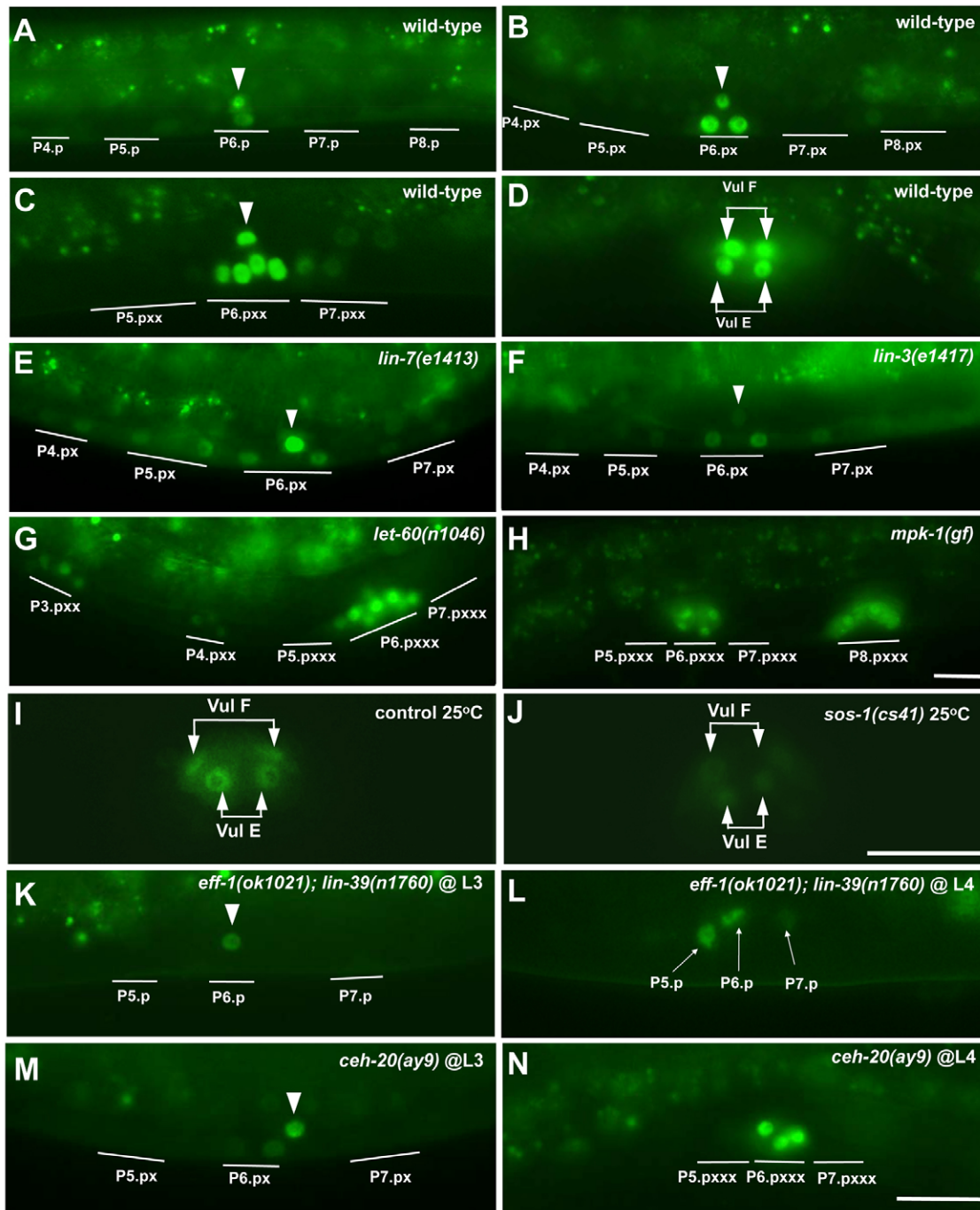


Fig. 6. VAB-23::GFP expression pattern and its regulation by EGFR/RAS/MAPK and LIN-39. (A-D) VAB-23::GFP expression in wild-type larvae from the Pn.p to the Pn.pxxx stages. (E-H) VAB-23::GFP expression in *lin-7(e1413)* (E) and *lin-3(e1417)* (F) L3 larvae at the Pn.px stage, and in *let-60(n1046)* (G) and *gals36[hs::mpk-1, dmek(gf)]* (H) L4 larvae. (I,J) VAB-23::GFP expression in wild-type control (I) and *sos-1(cs41ts)* (J) larvae, which had been up-shifted to 25°C in the L3 stage after induction and developed at 25°C until the L4 (Pn.pxxx) stage (see also Fig. S3 in the supplementary material). (K,L) VAB-23::GFP expression in the undivided VPCs of *lin-39(n1760); eff-1(ok1021)* mid-L3 (K) and L4 (L) larvae. (M,N) VAB-23::GFP expression in *ceh-20(ay9)* larvae at the L3 (M) and L4 (N) stages. Arrowheads indicate the position of the AC, which also expressed VAB-23::GFP. Anterior is left. Scale bars: 10 μ m.

detected binding of LIN-39::GFP to the same region in the *vab-23* gene that we identified in our analysis. Taken together, our data indicate that LIN-39 directly regulates *vab-23* expression by interacting with the conserved HOX/PBX sites in the 5' regulatory region.

LIN-39-induced expression of VAB-23 is required for toroid formation

Finally, we examined whether the early *vab-23* expression induced by LIN-39 and CEH-20 is necessary for toroid formation during the subsequent morphogenesis. For this purpose, we investigated

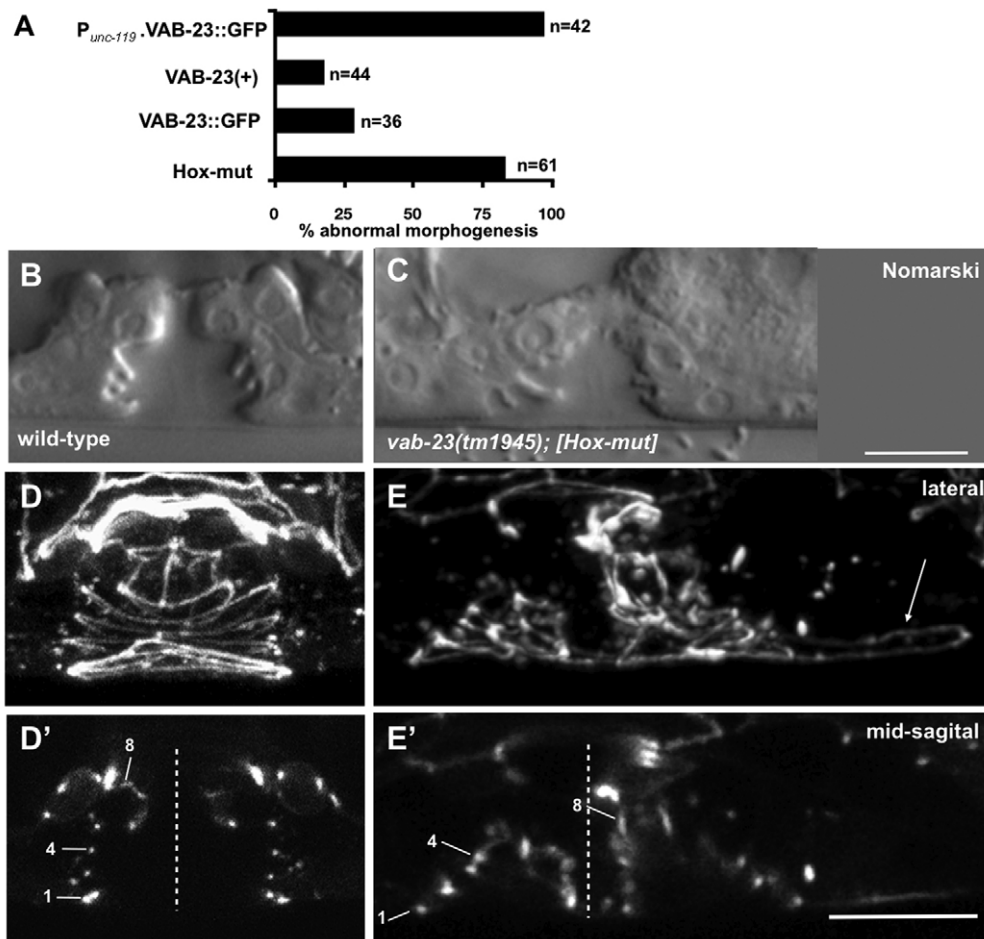


Fig. 8. The early, LIN-39-induced expression of VAB-23 is required for proper morphogenesis. (A) Rescue of the *vab-23(tm1945)* morphogenesis defects. VAB-23(+) represents a transgene covering the entire genomic locus (Pellegrino et al., 2009). Transgenic L4 larvae were inspected by Nomarski optics and the percentage of animals with abnormal vulval morphology was calculated. 'n' refers to the number of animals inspected for each transgene. (B, C) Nomarski images of wild-type (B) and *vab-23(tm1945); [Hox-mut]* (C) L4 larvae. (D-E') Toroid structures of wild-type (D) and *vab-23(tm1945); [Hox-mut]* (E) L4 larvae visualized with the DLG-1::dsRED reporter as described in Fig. 1. D' and E' are mid-sagittal cross sections showing the junctions between toroids (numbered). The arrow in E indicates abnormal migration of VulA, and the dotted lines mark the vulval midline. Scale bars: 10 μ m.

Similar functions of VAB-23 during embryo and vulval morphogenesis

Loss of VAB-23 function in the embryo causes the formation of ectopic cell contacts between ipsilateral ventral epidermal cells, resulting in defective ventral closure (Pellegrino et al., 2009). Interestingly, VAB-23 plays an analogous role during toroid formation by guiding cells towards the vulval midline, which forms an axis of symmetry for the developing organ (Fig. 1A). Similar to the embryonic phenotype, cells of the same sub-fate cannot form contacts across the vulval midline, resulting in the malformation or absence of toroids. The *vab-23* phenotype is in part due to reduced SMP-1 expression. To our knowledge, VAB-23 is the first example of a Semaphorin regulator, which links vulval morphogenesis via LIN-39 to the EGFR/RAS/MAPK pathway. However, both the vulval and embryonic morphogenesis defects of *smg-1* mutants are less penetrant and generally milder than the defects observed in *vab-23* mutants, suggesting that VAB-23 controls the expression of additional effectors of morphogenesis acting in parallel with SMP-1 (Dalpe et al., 2005). For example, the Rac GTPase MIG-2 and the guanine-nucleotide exchange factor UNC-73 are both required for vulval cell migration in parallel with the SMP-1/PLX-1 pathway (Dalpe et al., 2005; Kishore and Sundaram, 2002; Lundquist et al., 2001; Zipkin et al., 1997). Thus, VAB-23 might regulate multiple target genes performing diverse functions during vulval morphogenesis.

We have previously shown that during embryogenesis VAB-23 acts from the underlying neuroblasts in a cell non-autonomous manner to guide the ventral epidermal cells

(Pellegrino et al., 2009). Although VAB-23 expression was detected predominantly in the 1^o cell lineage (except for the weaker, later expression in VulC and VulD) and VAB-23 only controls the patterning of VulE and VulF cells, *vab-23* mutants display defects in the formation of all toroids. Even the distal-most VulA cells often failed to migrate towards the midline and form a toroid. It thus appears that during vulval morphogenesis, VAB-23 guides vulval cells in a cell non-autonomous manner, possibly by regulating the production of multiple secreted cues.

VAB-23 regulates various aspects of vulval and uterine morphogenesis

In addition to controlling the formation of homotypic cell contacts, VAB-23 inhibits cell fusions at a later stage of vulval morphogenesis. Ectopic expression of the fusogen EFF-1 in *vab-23* mutants is likely to be responsible for these abnormal fusions, as loss of *eff-1* function almost completely restored the normal number of cell junctions. LIN-39 acts upstream of *eff-1* to negatively regulate VPC cell fusions prior to vulval induction by inducing the GATA factors *egl-18* and *elt-6* (Koh et al., 2002; Shemer and Podbilewicz, 2002). It is possible that LIN-39 represses late vulval cell fusions indirectly by inducing *vab-23* expression. Interestingly, the cell lineage is unchanged in *vab-23* mutants, whereas the VPCs fail to proliferate in *lin-39(0)* single or *egl-18(0); elt-6(0)* double mutants. Thus, cell proliferation is controlled by LIN-39 through a VAB-23-independent branch of the cell fate execution pathway (Fig. 9).

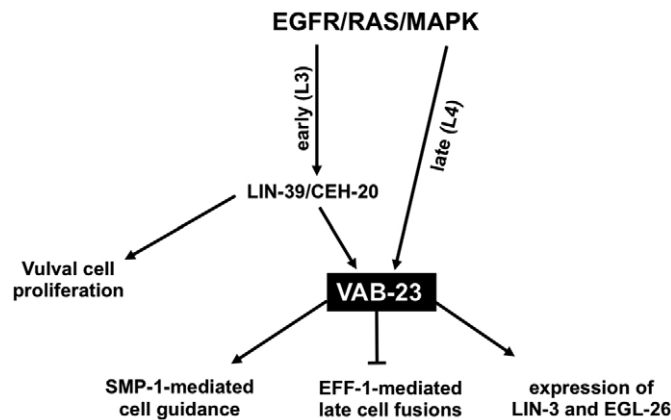


Fig. 9. Model for the regulation and function of *vab-23* during vulval morphogenesis. During early vulval development (Pn.p to Pn.pxx stage), EGFR/RAS/MAPK signaling induces *vab-23* transcription via LIN-39 together with its co-factor CEH-20. Early VAB-23 expression is required for cell guidance by regulating SMP-1 expression. Late VAB-23 expression (Pn.pxxx stage), which is maintained by EGFR/RAS/MAPK signaling acting in parallel with LIN-39 and CEH-20, is necessary to prevent cell fusions through inhibition of EFF-1. Furthermore, VAB-23 activates the expression of EGL-26 and LIN-3, which are necessary for the execution of the 1° VulE and VulF sub-fates. LIN-39 additionally promotes vulval cell proliferation independently of VAB-23.

Besides *eff-1* and *smp-1*, *vab-23* also regulates the later expression of *egl-26* and *lin-3* in VulE and VulF cells, respectively. Proper patterning of the VulE and VulF cells is necessary for vulval morphogenesis and uterine development. *egl-26* is required to maintain the shape of the VulF toroid after the AC has retracted (Estes and Hanna-Rose, 2009; Estes et al., 2007; Hanna-Rose and Han, 2002), and LIN-3 is required for the specification of the uv1 fate by activating LET-23 signaling in the ventral uterine cells. Therefore, the reduced LIN-3 expression in VulF cells probably accounts for the uterine-seam cell defects in *vab-23* mutants (Chang et al., 1999).

Based on our previous data it was not possible to distinguish whether VAB-23 acts as a transcriptional or post-transcriptional regulator of gene expression (Pellegrino et al., 2009). Here, we show that VAB-23 binds to regulatory regions in two of its target genes: to the first intron of *smp-1* and, more weakly, to the 5' regulatory region of *egl-26*. Finally, we have detected specific binding of VAB-23 to a relatively large number of sites throughout the genome. Although we could not extract an obvious consensus sequence from the VAB-23 binding sites identified by ChIPseq, the palindromic TTCNGAA motif in the center of the conserved regulatory element in *smp-1* is around sevenfold over-represented in the VAB-23 binding sites. (The TTCNGAA motif was found 286 times versus the 40 times that would be expected in a random distribution.) These results indicate that VAB-23 acts as a transcription factor that controls the expression of multiple target genes regulating various aspects of epidermal morphogenesis.

Vulval cell fate specification and morphogenesis are linked processes

During the specification of segment-specific structures and individual organs, the hox genes are thought to regulate a large number of target genes in a context-specific manner (Hueber and

Lohmann, 2008). However, only a relatively small number of hox targets that are directly involved in morphogenesis, the so-called 'realisators', have been identified. Furthermore, the conserved EGFR/RAS/MAPK, Notch and WNT signaling pathways control various aspects of epithelial morphogenesis in *Drosophila* and *C. elegans*, but their transcriptional targets during morphogenesis are poorly characterized (Galindo et al., 2005; O'Keefe et al., 2007; Rasmussen et al., 2008).

vab-23 reveals how EGFR/RAS/MAPK signaling coordinates via LIN-39 different aspects of organogenesis after the cell fate decisions have been made. *let-60* has previously been reported to regulate cell migration and fusions (Shemer et al., 2000), and *smp-1* is expressed in the pseudovulvae of *let-60(gf)* worms (Dalpe et al., 2005), suggesting that SMP-1 expression is indeed linked to EGFR/RAS/MAPK signaling. Moreover, conditional inactivation of *sos-1* indicates that continuous EGFR/RAS/MAPK signaling after induction is necessary to maintain VAB-23 expression in the 1° cell lineage. VAB-23 thus acts as a link between the cell fate specification and execution pathways. Although LIN-39 mediates most of the regulation of VAB-23 by the EGFR/RAS/MAPK pathway, LIN-39 clearly performs additional, VAB-23-independent functions. LIN-39 prevents VPC fusion before and promotes cell proliferation after vulval induction (Shemer and Podbilewicz, 2002). Owing to the lack of cell proliferation, it is difficult to determine whether any vulval sub-fates are specified and, thus, whether proper cell contacts are formed in *lin-39(0)* mutants. However, the few (usually one to four) toroids that are formed in *lin-39(0); eff-1(0)* double mutants appear disorganized (Shemer and Podbilewicz, 2002).

In summary, this study shows that vulval fate specification and morphogenesis are more tightly linked than previously thought, as there exists a considerable temporal overlap between these two phases of organogenesis. Continuous signaling by the EGFR/RAS/MAPK pathway after vulval fate specification might be necessary to induce and maintain the expression of key regulators of epidermal morphogenesis. The identification of additional late EGFR/RAS/MAPK targets might shed light on the regulatory network controlling vulval morphogenesis.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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