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Org-1 is required for the diversification of circular visceral muscle founder cells and normal midgut morphogenesis

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Abstract

The T-Box family of transcription factors plays fundamental roles in the generation of appropriate spatial and temporal gene expression profiles during cellular differentiation and organogenesis in animals. In this study we report that the *Drosophila Tbx1* orthologue *optomotor-blind-related-gene-1* (*org-1*) exerts a pivotal function in the diversification of circular visceral muscle founder cell identities in *Drosophila*. In embryos mutant for *org-1*, the specification of the midgut musculature *per se* is not affected, but the differentiating midgut fails to form the anterior and central midgut constrictions and lacks the gastric caeca. We demonstrate that this phenotype results from the nearly complete loss of the founder cell specific expression domains of several genes known to regulate midgut morphogenesis, including *odd-paired* (*opa*), *teashirt* (*tsh*), *Ultrabithorax* (*Ubx*), *decapentaplegic* (*dpp*) and *wingless* (*wg*). To address the mechanisms that mediate the regulatory inputs from *org-1* towards *Ubx*, *dpp,* and *wg* in these founder cells we genetically dissected known visceral mesoderm specific cis-regulatory-modules (CRMs) of these genes. The analyses revealed that the activities of the *dpp* and *wg* CRMs depend on *org-1*, the CRMs are bound by Org-1 in vivo and their T-Box binding sites are essential for their activation in the visceral muscle founder cells. We conclude that Org-1 acts within a well-defined signaling and transcriptional network of the trunk visceral mesoderm as a crucial founder cell-specific competence factor, in concert with the general visceral mesodermal factor Biniou. As such, it directly regulates several key genes involved in the establishment of morphogenetic centers along the anteroposterior axis of the visceral mesoderm, which subsequently organize the formation of midgut constrictions and gastric caeca and thereby determine the morphology of the midgut.

Keywords

visceral mesoderm patterning; midgut morphogenesis; T-box transcription factor; combinatorial enhancer binding

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Competing interests statement
The authors declare no competing financial interests.

Material and Methods

Drosophila strains

In this study the following strains were used: *org-1*^{OJ487}, *org-1*^{OJ423} and *UAS-org-1* (Schaub et al., 2012), *tin*^{346}, *bap*^{Df} (a *Df(3R)e*^{D7} chromosome carrying the *tin* genomic rescue construct *tin* Re28–58) (Azpiazu and Frasch, 1993), *bin*^{R22}, *bap3-lacZ* and *bap3-GAL4* (Zaffran et al., 2001), *UAS-tkv*$^{Q-D}$ (Nellen et al., 1996), *UAS-wg-HA* (Bloomington Stock Center, Indiana), *2xPE-GAL4; how*^{24B}*-GAL4* (Wang et al., 2005). GAL4/UAS induced overexpression was carried out at 28°C.

Construction of the reporter constructs

For the creation of the *dpp-GFP* and *wg-GFP* reporter lines the genomic regions *chr2L: 2446030…2446286* (*dppPB*, Manak et al., 1994) and *chr2L:7300474…7301020* (*wgXC*, Grienenberger et al., 2003) according to R5.41 were amplified using *yw* genomic DNA as template. All fragments were cloned into *Eco*RI/*Kpn*I of a modified *pH-Stinger* vector, which has *AttB* sequences inserted into its *Avr*II site (H. Jin and M. Frasch, unpublished). For the analogous creation of *dppPB-OrgImut-GFP* and *wgXC-OrgImut-GFP* reporters the Org-1 binding sites within the *dppPB* and *wgXC* sequences were predicted using Target Explorer (Sosinsky et al., 2003) searches with a positional weight matrix generated by SELEX with Org-1-GST (Schaub et al., 2012) and mutated via site directed mutagenesis as follows: *dppPB* TCACACCC → *dppPB-OrgImut* AAGCTTCC (Fig. 7E) and *dppPB-OrgImut2* TCAATACC (with same result as with *dppPB-OrgImut*; CS and MF, unpublished data); *wgXC* TCGCACTT → *WgXC-OrgImut*-GFP TCAAGCTT (Fig. 7H). For transformation, the landing sites *ZH-35B* (Bischof et al., 2007) or *AttP2* (Groth et al., 2004) were used.

Immunohistochemistry

Antibody staining and in situ hybridization were performed as described in (Azpiazu and Frasch, 1993; Knirr et al., 1999). The following primary antibodies and probes were used: rat anti-Org-1 (1:100, Schaub et al., 2012) mouse anti-Ubx monoclonal (1:50, White and Wilcox, 1985), rabbit anti-Teashirt (1:4000, Gallet et al., 1998), rat anti-Abd-A (1:500, Macias et al., 1990), mouse anti-Antp, mouse anti-FasciclinIII, mouse anti-Scr, mouse anti-Wg (all monoclonal, 1:20, Developmental Studies Hybridoma Bank, Univ. of Iowa), rabbit anti-Homothorax (1:1000, Kurant et al., 1998), rabbit anti-β3-Tubulin (1:3000, a gift from R. Renkawitz-Pohl, University of Marburg), rat anti-Tropomyosin (1:400, Brabraham Monoclonal Antibody Facility), rabbit anti-GFP (1:3000, Molecular Probes), mouse anti-GFP (1:200, Molecular Probes), rabbit anti-βGal (1:2000, Promega), mouse anti-βGal (40–1a, 1:20, Developmental Studies Hybridoma Bank, Univ. of Iowa) and digoxigenin labeled *opa* (Cimbora and Sakonju, 1995), *dpp* (St Johnston and Gelbart, 1987), *GFP* as well as *pntP1* (Klambt, 1993) antisense RNA probes.

All mutant lines were balanced with lacZ or GFP balancers and mutant embryos were distinguished from wild type embryos by staining with β-galactosidase or GFP antibodies. Confocal pictures were taken with a Leica SP5 II (20×/1.3 PL APO Glycerol, 63×/1.3 PL APO Glycerol). Projections were done with Leica LAS AF.

Chromatin immunoprecipitation assays (ChIP)

Chromatin preparation using ChIP were performed using stage 11–14 *yw* embryo collections and Org-1 antibodies as described (Schaub et al., 2012). The precipitated DNA was quantified using FastStart Universal SYBR Green Master (Rox) (Roche Applied Science) on an Eco™ Real-Time PCR System (Illumina). In each experiment Real-Time

PCR was performed in triplicate. Three independent precipitations per amplicon were analyzed.

Introduction

The musculatures of higher organisms can be subdivided into three major types: the skeletal, cardiac, and visceral muscles. These muscle types have distinct developmental origins and differ in terms of their morphological, physiological and molecular features including their contractile properties.

The visceral musculature of the *Drosophila* midgut, which is in the focus of this study, consists primarily of the musculature of the digestive tract and is composed of an inner layer of circular muscles and an outer layer of longitudinal muscles. Whereas the circular musculature derives from segmental portions of the dorsal mesoderm in the trunk under the inductive influence of *decapentaplegic* (*dpp*, a TGFβ signals (Frasch, 1995), the anlagen of the longitudinal muscles are located in the caudal visceral mesoderm (Nguyen and Xu, 1998; Kusch and Reuter, 1999; Ismat et al., 2010). The transcriptional response of the prospective trunk visceral mesoderm cells to the inductive cues of Dpp includes the induction of the NK homeobox genes *tinman* (*tin*), *bagpipe* (*bap*) and the FoxF family member *biniou* (*bin*) (Staehling-Hampton et al., 1994; Frasch, 1995; Zaffran et al., 2001). The segmental blocking of these inductive activities of Tin and Dpp in the dorsal mesoderm by the Wingless-induced repressor Sloppy-paired (Lee and Frasch, 2000; Lee and Frasch, 2005) leads to the expression and function of *bap* and *bin* exclusively in the metameric primordia of the circular visceral muscles of the midgut. Whereas *tin* and Dpp are required for the formation of all dorsal mesodermal derivatives (heart, dorsal body wall muscles, visceral muscles) (Azpiazu and Frasch, 1993; Bodmer, 1993; Zaffran et al., 2001; Lee and Frasch, 2005), *bap* and *bin* are both essential for the specification of the trunk visceral mesoderm and the formation of midgut muscles from it (Azpiazu and Frasch, 1993; Zaffran et al., 2001). Moreover, the sac-like appearance of the remnant of the midgut in *bap* and *bin* mutant embryos highlights the importance of the visceral mesoderm in shaping the proper morphogenesis of the midgut. In particular, it triggers the formation of three constrictions at defined positions along the developing midgut that evolve into gut loops and induces the formation of four blind-ending pouches termed gastric caeca at its anterior end (Tepass and Hartenstein, 1994; Campos-Ortega and Hartenstein, 1997).

The circular visceral muscles (cVM) are built from the trunk visceral mesoderm by the fusion of circular visceral muscle founder cells (cFC) and visceral fusion competent myoblasts (FCM) into binucleated muscle fibers (Martin et al., 2001; Klapper et al., 2002). The founder cells are specified by spatially restricted *Jelly belly* (*Jeb*) signals acting via the *Anaplastic lymphoma kinase* (*Alk*) receptor tyrosine kinase along the ventral margin of the trunk visceral mesoderm, whereas the dorsally adjacent cells become fusion-competent myoblasts by default (Englund et al., 2003; Lee et al., 2003).

The morphogenetic events along the developing midgut are the readout of regulatory patterning networks that act within specific anteroposterior areas of the cVM. The networks integrate the spatial activities of homeotic selector genes and secreted growth factors, leading to the establishment of morphogenetic centers at defined positions. In the most anterior part of the midgut the gene products of S*ex combs reduced* (*Scr*) and *decapentaplegic* (*dpp)* are required for proper formation of the gastric caeca (Panganiban et al., 1990; Reuter and Scott, 1990). The formation of the anterior midgut constriction is initiated by the activity of *Antennapedia* (*Antp*) and its downstream targets, the zinc finger protein encoding genes *odd-paired* (*opa*) and *teashirt* (*tsh*) (Reuter and Scott, 1990; Mathies et al., 1994; Cimbora and Sakonju, 1995). The formation and placement of the central

midgut constriction depends crucially on *Ultrabithorax* (*Ubx*), *dpp* and *wingless* (*wg*, a *Wnt* member), which act together in a regulatory feedback loop that has been subject to detailed dissection (Muller et al., 1989; Panganiban et al., 1990; Reuter et al., 1990; Hursh et al., 1993; Capovilla et al., 1994; Sun et al., 1995). *teashirt* (*tsh*), another Wg downstream gene, is additionally required for the formation of the central constriction and, independently of Wg, also for the anterior constriction (Mathies et al., 1994). The identification and analysis of cis-regulatory modules (CRMs) of *Ubx, dpp* and *wg* expression in the visceral mesoderm has further shown that all three genes are direct regulators of each other (Thuringer et al., 1993; Capovilla et al., 1994; Manak et al., 1994; Sun et al., 1995; Yang et al., 2000; Grienenberger et al., 2003) The formation of the posterior constriction is dependent on *abdominal-A* (*abd-A*) expression (Tremml and Bienz, 1989). For this event, Wg needs to activate the expression of the ETS transcription factor encoding gene *pointed* (*pnt*) in the cells of the anterior *abd-A* domain. In the cells of the same region, Dpp signals are essential to prevent Abd-A from activating the transcription of the zinc finger factor-encoding gene *odd-paired* (*opa*) (Cimbora and Sakonju, 1995; Bilder et al., 1998).

Because the above-described signals, Hox factors, and other transcriptional regulators are also present in tissues outside of the visceral mesoderm, the visceral mesoderm-specific establishment and maintenance of the various morphogenetic centers they participate in must additionally require tissue-specific cofactors. In this context, it was found that *bin* functions as a direct upstream regulator of *dpp* and is also required for *wg* expression at the prospective central constriction (Zaffran et al., 2001). However, the expression of *Ubx* in the visceral mesoderm was independent of *bin* (and *bap*), thus indicating a requirement for additional visceral mesodermal competence factors. Furthermore, it was found that the absence of cFCs in *Alk* mutant backgrounds prevents *Ubx*, *dpp,* and *wg* expression in the visceral mesoderm even though *bin* is not affected. This observation implied that the initial activation of these genes is either directly dependent on Alk signals or dependent on factor(s) induced transcriptionally by Jeb/Alk in circular muscle founder cells (Shirinian et al., 2007).

In the present study, we identify *org-1,* a Jeb/Alk-induced gene encoding the *Drosophila Tbx1* ortholog (Lee et al., 2003), as a crucial factor required for the full activation and maintenance of *opa*, *tsh*, *Ubx, dpp* and *wg* expression in the founder cells of the circular visceral musculature. Consequently, the morphogenesis of midgut structures depending on these activities, namely the gastric caeca and the anterior as well as the central midgut constrictions, is disrupted in *org-1* mutant embryos. We demonstrate that the activation of the respective visceral mesoderm specific enhancer elements of *dpp* and *wg* by *org-1* requires T-box binding motifs and show in vivo occupancy of these elements by Org-1. Thus, by directly regulating *dpp* and *wg*, *org-1* represents a component of the intrinsic founder cell program of the cFCs that helps implementing their anteroposterior diversification and organizing subsequent midgut morphogenesis.

Results

org-1 is expressed in the circular visceral muscles and their progenitors

As described previously, *org-1*, the *Drosophila* orthologue of *Tbx1,* shows a highly restricted expression pattern in the cells of the developing somatic and visceral mesoderm (Lee et al., 2003; Schaub et al., 2012). In the dorsal mesoderm Org-1 expression is induced at stage 10 in all cells of the 11 bilateral patches of cells of the prospective trunk visceral mesoderm (TVM) (Fig. 1A,A′), showing an analogous expression pattern to *bagpipe* (*bap*) and *biniou (bin)* at this stage. During stages 11–12, the visceral Org-1 expression is refined and restricted to the progenitor cells, and after their division, the founder cells of the circular visceral musculature (cVM) (Fig. 1B,B′). By contrast, *bap* and *bin* expression is maintained

in all TVM cells although *bap* expression ceases after stage 12 (Azpiazu and Frasch, 1993; Zaffran et al., 2001). Upon fusion of the circular visceral muscle founder cells (cFC) with visceral fusion competent myoblasts (FCM) at stage 13 (Fig. 1C,C′) until late embryogenesis (Fig 1D,D′,E,E′), Org-1 protein is found, like Biniou, in all nuclei of the cVM.

org-1 expression in the trunk visceral mesoderm is regulated by *tinman* and *dpp* but largely independent of *bap* and *bin*

Previous work has shown that the expression domains of *bap* and *bin* in the 11 bilateral patches of the TVM are defined by the intersecting dorsal activities of *dpp*/*tin*, which act positively, and the segmental activities of *wg*/*slp*, which have repressing effects. (Azpiazu and Frasch, 1993; Staehling-Hampton et al., 1994; Frasch, 1995; Azpiazu et al., 1996; Riechmann et al., 1997; Lee and Frasch, 2000; Zaffran et al., 2001; Lee and Frasch, 2005). As shown in Fig. 2C,D (compare with A, B), like *bap* and *bin*, also *org-1* requires *tin* activity for its full activation in the TVM. In a *tin* mutant background, only partial activation of *org*-1 expression in the visceral mesoderm primordia occurs (Fig. 2C; note that most of the residual striped expression corresponds to somatic mesodermal (sm) expression) and there is no refinement of *org-1* expression to the two rows of cFCs (Fig. 2D). Apart from being downstream genes of Tin, *bap* and *bin* also regulate each other through a cross-regulatory feedback loop (Zaffran et al., 2001). To elucidate if additional inputs from Bap and Bin are necessary for *org-1* regulation, we analyzed Org-1 expression in bap^{Df} and bin^{R22} genetic backgrounds. At stage 10, neither the loss of *bap* (Fig. 2E) nor of *bin* function (Fig. 2G) have any severe effects on the expression pattern of *org-1*, thus indicating that activation of *org-1* in the trunk visceral mesoderm patches is largely independent of *bap* and *bin*. During stages 11–12, loss of *bap* results in perturbed restriction of Org-1 to the visceral muscle founder cells (if any are present; Fig. 2F), indicating some influence of *bap* on or*g-1* expression during later stages. Unlike *bap* mutants, bin^{R22} mutant embryos show only slight aberrations with regard to Org-1 expression at this stage (Fig. 2H).

To test if Dpp signaling from the overlying ectoderm is involved in the induction of *org-1* expression (which may explain the residual visceral mesodermal *org-1* expression in *tin* mutant embryos), we overexpressed a constitutively-activated version of the Dpp receptor Thickveins (tkv^{Q-D}) (Nellen et al., 1996) panmesodermally. As shown in Fig. 2J (compare with Fig. 2I), this leads to an expansion of Org-1 expression into the ventral somatic mesoderm, thus demonstrating direct or indirect activation of *org-1* by Dpp signaling. Taken together, our data suggest a model in which *org-1* expression is activated by *tin* and *dpp* in the visceral mesoderm primordia in parallel to and largely independently of *bap* and *bin*.

Org-1 is required for proper midgut morphogenesis, but not for the specification of the visceral musculature itself

To analyze the function of *org-1* during visceral mesoderm development, we examined the expression patterns of various markers in hemizygous embryos carrying the lethal *org-1* null alleles $org\text{-}1^{OJ487}$ or $org\text{-}1^{OJ423}$ (Schaub et al., 2012) at different developmental time points. It is well documented that proper specification of the endodermal as well as the mesodermal cell layers of the embryonic midgut is required for normal morphogenesis and differentiation of the midgut (Azpiazu and Frasch, 1993; Reuter et al., 1993; Tepass and Hartenstein, 1994; Zaffran et al., 2001; Wolfstetter et al., 2009). To examine whether potential abnormalities in the visceral mesoderm of *org-1* mutants affect the embryonic midgut we stained embryos with antibodies against Tropomyosin (Fig. 3A), which is expressed in all visceral muscles and provides a clear picture of midgut morphology. The analysis of stage 16 $org\text{-}1^{OJ487}$ and $org\text{-}1^{OJ423}$ mutant embryos revealed pronounced abnormalities in midgut morphology. In particular, the midgut fails to form the anterior and

the central midgut constrictions whereas the remaining posterior constriction is displaced (Fig. 3B). In order to investigate gastric caeca differentiation, we visualized them by reporter staining in stage 17 *dppPB-GFP* embryos. Whereas in wild type embryos the two pairs of gastric caeca are clearly visible and show strong reporter signals (Fig. 3C), midguts of *org-1* mutant late stage 17 embryos show an arrest of gastric caeca formation and only few cells with reporter signals (Fig. 3D, asterisks). These midgut phenotypes of the *org-1* mutant alleles can be rescued by the expression of *org-1* under the control of a *bap3-GAL4* driver in the trunk visceral mesoderm of *org-1* mutant embryos (data not shown), clearly demonstrating that the *org-1* gene is required within the visceral mesoderm for proper midgut organogenesis.

To test whether the observed midgut phenotypes are due to the failure of TVM specification in the absence of functional *org-1*, the IgG-domain adhesion molecule Fasciclin III (FasIII) was used as an early marker for specified TVM and developing cVM (Patel et al., 1987). However, in stage 11 *org-1* mutant embryos, no changes were observed in the expression pattern of FasIII and all other tested trunk visceral mesoderm markers (data not shown). Likewise, the formation of circular gut muscle founder cells (cFC) was not disrupted because, as shown with a *bap-lacZ* marker in the *org-1* mutant background, the trunk visceral mesoderm precursors (including fusion-competent cells) are still strictly fated to contribute to midgut muscles (data not shown). By contrast, in genetic situations that prevent the formation of cFCs, fusion of the FCMs occurs with somatic founder cells, which causes their subsequent integration into the somatic musculature (Lee et al., 2003).

Org-1 is required for proper activation of distinct patterning genes in the founder cells of the developing circular midgut musculature

Previous work has shown that the formation and the placement of the gastric caeca and the three midgut constrictions depend on regulatory networks in the visceral muscle lineage that integrate the spatial and temporal inputs of specific transcriptional regulators and signaling activities. These include the homeotic selector genes *Sex combs reduced* (*Scr*), *Antennapedia* (*Antp*), *Ultrabithorax* (*Ubx*) and *abdominal A* (*abd-A*), as well as the secreted growth factors *decapentaplegic (dpp)* and *wingless* (*wg*), and lead to the establishment of distinct expression domains of the downstream effectors *teashirt (tsh), pointed* (*pnt*) and *odd-paired* (*opa*) in the midgut musculature (Tremml and Bienz, 1989; Immergluck et al., 1990; Panganiban et al., 1990; Reuter et al., 1990; Reuter and Scott, 1990; Andrew et al., 1994; Bienz, 1994; Mathies et al., 1994; Staehling-Hampton and Hoffmann, 1994; Cimbora and Sakonju, 1995; Bilder et al., 1998).

As the loss of *org-1* function disrupts gastric caeca formation and causes the loss and misplacement of midgut constrictions (Fig. 3B,D), it was conceivable that *org-1* represents a new and founder cell specific regulator within this network. If this were the case, then at least some members of the network would be expected to show founder cell specific expression domains. For *Ubx*, *dpp*, *wg* and *Abd-A* this has already been suggested (Martin et al., 2001; Shirinian et al., 2007).

This notion was further underpinned by our colocalization analyses of the respective expression patterns in stage 11 embryos with Org-1. Although it has been described that the visceral expression of *tsh* and *Antp* starts in defined parasegments of stage 13 embryos (Reuter and Scott, 1990; Mathies et al., 1994), our analyses revealed that the expression of these genes in fact already initiates in the founder cells at stage 11 in the respective visceral mesodermal parasegments (Fig. 4A,B). Tsh expression is detected in the founder cells of PS4-5 as well as in the anterior part of PS6, with the expression in PS5-6 being very robust as compared to that in PS4 (Fig. 4A, bracket, asterisk). Early Tsh expression is still very weak in PS8 (Fig. 4A, arrowhead) where it is upregulated later as a response to Wg and Dpp

signals (Mathies et al., 1994) (see also Fig. 6E, asterisk). Likewise, we demonstrate that that the visceral mesoderm specific expression domains of *Ubx* (Fig. 4C, in PS7, red bracket), *dpp* (Fig. 4C,D in PS3, white asterisk and PS6-7, white bracket) and *wg* (Fig. 4D, in PS8, red asterisk) as well as *Abd-A* (data not shown) in stage 11–12 embryos are founder cell specific. Interestingly, the *dpp* expression domain in the founder cells is broader than described for the visceral musculature (Panganiban et al., 1990). Instead of being expressed exclusively in PS7, it extends into the posterior part of PS6, whereas Ubx is expressed solely in PS7 (Fig. 4C, white bracket, compare to red bracket). Wg is expressed in the founder cells of PS8 posteriorly adjacent to the *dpp* domain (Fig 4D, white bracket and red asterisk). Whereas the data for the visceral musculature had indicated that *wg* is expressed in the entire PS8 (van den Heuvel et al., 1989), the smaller Wg domain in the founder cells indicates that, at least at stage 11, Wg expression is limited to the anterior part of PS8. Expression of the homeotic target gene *opa* has been documented in PS5 and PS9-12 of the visceral musculature (Cimbora and Sakonju, 1995) and we find that it is initiated already at stage 11 in the founder cells of visceral mesodermal PS3-5 (only weakly in PS3) and PS9-12 (Fig. 4E).

To investigate potential regulatory inputs from Org-1 towards these genes that are expressed in localized domains within the visceral muscle founder cells we tested their expression patterns in *org-1* loss and gain of function backgrounds. As shown in Fig. 4F,H,J,L,N loss of *org-1* function causes near absence of the expression of *opa*, *tsh*, *Ubx*, *dpp* and *wg* within the founder cells of the visceral musculature (compare to Fig. 4E,G,I,K,M). By contrast, *Antp* and *abd-A* expression in the founder cells is not affected by the loss of *org-1* function (data not shown). In light of this result the loss of founder cell specific *opa* and Tsh expression was surprising, because it has been shown that *Antp* regulates both, *tsh* in PS4-6 and *opa* expression in PS4-5, and that *abd-A* is required for *opa* activation in PS9-12 of the visceral musculature (Mathies et al., 1994; Cimbora and Sakonju, 1995). Therefore we conclude that *org-1* is an essential regulator of *opa* and *tsh* expression in these visceral founder cells in parallel with *Antp* and *abd-A* respectively.

To test whether the founder cell-specific expression of *org-1* is limiting the induction of the patterning genes to the founder cells in the TVM we expressed *org-1* ectopically. Forced o*rg-1* expression in the whole trunk visceral mesoderm, including the fusion-competent cells of the TVM, causes an expansion of the *opa*, *tsh*, *Ubx*, *dpp* and *wg* expression domains into these FCMs (Fig. 5F,G,H,I,J compare to Fig. 5A,B,C,D,E). Hence, *org-1* expression is required and in the proper tissue context sufficient for visceral lineage specific *opa*, *tsh*, *Ubx*, *dpp* and *wg* expression.

The phenotypes in the developing visceral musculature of *org-1* mutant embryos with regard to the expression of various patterning genes resembles those seen in stage 11–12 embryos. The expression domains of *opa*, *tsh*, *Ubx*, *dpp* and *wg* are nearly abolished (Fig. 6D,F,H,J,L compare to Fig. 6C,E,G,I,K). In addition, the visceral mesoderm expression domain of *Scr,* which is normally induced at stage 13 in PS4 of the developing midgut musculature, is barely detectable in *org-1* mutant embryos (Fig. 6B compare to A). This effect could explain the observed defects in the formation of gastric caeca in *org-1* mutant embryos because Scr is known to be indispensible for the development of these sacs (Reuter and Scott, 1990). Other defects in the developing midgut musculature of stage 13–14 *org-1* mutant embryos include the lack of expression of the zinc finger factor encoding gene *tsh* in PS8 (Fig. 6F, asterisk) and the ETS domain factor encoding gene *pointed* in PS8-9 (Fig. 6N). It has been reported that, in the normal situation, both *pnt* and *tsh* (in PS8) require Wg for their induction. To clarify if the absence of *tsh* and *pointed* expression at the future central midgut constriction are solely due to the loss of sufficient *wg* expression in *org-1* mutants or if there are additional regulatory inputs from Org-1 we forced *wg* expression in the whole visceral

mesoderm in the *org-1* mutant background. We found that, in this situation, *pnt* expression is re-initiated (data not shown), whereas *tsh* (Fig. 6P) is only slightly activated, demonstrating that even in the presence of sufficient doses of activating factors and signals the full activation of *tsh* transcription in PS8 of the visceral musculature is dependent on Org-1 function.

Taken together, our analysis provides strong evidence that *org-1* is required for the lineage specific activation of *opa*, *tsh*, *Ubx*, *dpp* and *wg* in the visceral muscle founder cells and of *Scr* in the developing visceral musculature. These findings implicate *org-1* as an additional tissue-specific regulator in the regulatory network controlling midgut morphogenesis.

Org-1 is a direct upstream regulator of *dpp* and *wg* expression in the visceral mesoderm

Previous work has shown that the expression patterns of *dpp*, *Ubx* and *wg* in PS7/8 of the visceral mesoderm are established through a regulatory feedback loop which integrates the direct inputs of all three genes together with Biniou as a tissue specific activator (Muller et al., 1989; Panganiban et al., 1990; Reuter et al., 1990; Hursh et al., 1993; Manak et al., 1994; Sun et al., 1995; Yu et al., 1996; Zaffran et al., 2001; Grienenberger et al., 2003). Our current data raise the question of whether Org-1 is an additional direct upstream regulator of *Ubx*, *dpp* and *wg* that acts together with Biniou but in this case exclusively in the founder cells of the circular visceral muscles. To test this possibility we dissected visceral mesoderm specific cis-regulatory modules (CRMs) of *dpp* (*dppPB*, Fig 7A) and *wingless* (*wgXC*, Fig. 7F) which are known to mediate the signal integration of these two genes in PS7 and PS8, respectively (Manak et al., 1994; Grienenberger et al., 2003). The activity of both enhancers at stage 11–12 is indeed restricted to founder cells in the TVM, with the *dppPB-GFP* reporter being initiated in the cFCs of PS3 and PS7 of the visceral mesoderm (Fig. 7B) and *wgXC-GFP* in cFCs of PS8 (*wgXC-GFP*, Fig. 7G). By contrast, in *org-1* loss of function genetic backgrounds the *dppPB-GFP* (Fig. 7C) and *wgXC-GFP* (Fig. 7H) enhancer constructs show a loss of the reporter signals, which mimics the effects on *dpp* and *wg* expression upon loss of functional Org-1.

To address if Org-1 contributes directly to the activation of these enhancers, we searched their sequences for putative Org-1 binding motifs (Schaub et al., 2012) and identified one good match in *dppPB* and one within *wgXC*. The sequences spanning the predicted T-Box motifs were tested for in vivo binding of Org-1 by anti-Org-1 ChIP experiments, which demonstrated significant binding of Org-1 to the tested sites in the enhancer fragments in stage 11–14 embryos (Fig. 7K). To address their functionality in vivo we introduced 2–3 base pair changes in the T-Box motifs at positions known to be important for T-box protein binding (see Materials & Methods) (Muller and Herrmann, 1997) These mutations had dramatic effects as neither the *dppPB-OrgImut-GFP* reporter (Fig. 7E) nor the *wgXC-OrgImut-GFP* reporter (Fig. 7J) allowed any *GFP* expression in the cFCs of transgenic embryos during stages 11–12. Both mutant constructs did become active in the cVM of stage 13 embryos, albeit with strongly reduced intensities (data not shown). Altogether, these data strongly suggest that the normal activation of *dpp* and *wg* in the visceral mesoderm requires Org-1 binding to the T-box binding motifs within the *dppPB* and *wgXC* CRM sequences.

Discussion

The analysis of *org-1* expression and function during visceral mesoderm development defined this gene as a new and essential lineage specific regulator of circular visceral muscle founder cell identities and midgut patterning in *Drosophila*. Our data add new insights into the developmental regulatory mechanisms responsible for the diversification of the circular visceral muscle founder cell lineage and midgut morphogenesis.

Regulation and functions of *org-1* in trunk visceral mesoderm primordia versus circular visceral muscle founder cells

The initial expression of *org-1* occurs in the segmented TVM, where it is coexpressed with *tin*, *bap, bin* and *Alk* (Azpiazu and Frasch, 1993; Zaffran et al., 2001; Loren et al., 2003). It has been documented that the induction of *tin* and *bap* in the dorsal mesoderm involves the combined binding of Smad proteins (Medea and Mad) and Tin to Dpp-responsive enhancers of the *tin* and *bap* genes (Xu et al., 1998; Lee and Frasch, 2005), whereas the segmental repression of *bap* is mediated by binding of the *sloppy paired* (*slp*) gene product (Lee and Frasch, 2005). Our genetic analysis of *org-1* has shown that *org-1* is activated downstream of *tin* but independently of *bap* and *bin*, and that *dpp* provides the key signals for its induction. This suggests a regulatory mechanism analogous to that of *bap*, in which the combined binding of Smads and Tin activates a Dpp-responsive *org-1* enhancer, whereas Wg activated Slp is required for its mutual segmental repression (Fig. 8A).

The similarities in the early expression patterns of *bap*, *bin*, *Alk* and *org-1* in the trunk visceral mesoderm primordia raise the question of the contribution of *org-1* to the early development of the TVM as such. Whereas *bap* and *bin* are crucially required for the specification of the trunk visceral mesoderm and visceral musculature (Azpiazu and Frasch, 1993; Zaffran et al., 2001), loss of *org-1* function, like the loss of *Alk* (Englund et al., 2003), has no obvious impact on the specification of the early TVM. Therefore, it is notable that during the subdivision of the visceral mesoderm primordia into founder and fusion-competent myoblasts (cFCs and FCMs), *org-1* expression is extinguished in the FCMs and only sustained in the cFC lineage of the circular visceral musculature. This lineage-specific restriction and maintenance of *org-1* expression crucially depends on Jeb mediated Alk/Ras/MAPK signaling (Lee et al., 2003) and points towards a possible cFC lineage specific function of *org-1*. Our genetic analysis demonstrates that *org-1* is not required for cFC specification, but plays a decisive role in the induction of the visceral mesoderm specific expression of patterning genes in the founder cells of the circular musculature. Thus, *org-1* is critical for the processes of cell fate diversification that provide individual fields of cells along the anteroposterior axis of the visceral mesoderm with their specific identities (Fig. 8B).

org-1 as a founder cell-specific regulator of visceral mesodermal patterning genes

Proper anteroposterior patterning of the trunk visceral mesoderm and the formation of localized organizer fields are prerequisites for eliciting the morphogenetic events that shape the midgut. The formation of these organizer fields depends on the appropriate spatial expression domains of the homeotic selectors *Scr*, *Antp*, *Ubx* and *abd-A*, the secreted factors *dpp* and *wg*, as well as the zinc finger proteins *opa* and *tsh*, which are required for the formation of the midgut constrictions as well as the gastric caeca (Bienz and Tremml, 1988; Tremml and Bienz, 1989; Immergluck et al., 1990; Panganiban et al., 1990; Reuter and Scott, 1990; Masucci and Hoffmann, 1993; Mathies et al., 1994; Cimbora and Sakonju, 1995). The regulatory mechanisms responsible for the establishment of the spatial, temporal and tissue-specific expression patterns of these genes in the TVM are only partially understood. Genetic and molecular analyses with the FoxF gene *bin*, which is expressed in all trunk visceral mesoderm precursors and their descendents, have demonstrated that *bin* is a direct upstream regulator of *dpp* in PS7 and is also required for the expression of *wg* in PS8 of the TVM (Zaffran et al., 2001). Thus, Bin serves as an essential TVM-specific competence factor in conjunction with the *dpp*/*wg* signaling feedback loop. Our current findings have defined Org-1 as an additional tissue-specific regulator with an even broader range of downstream patterning genes in the TVM, but with a narrower spatial range of action. We have shown that *org-1* acts specifically within the visceral muscle founder cell lineage as a positive regulator upstream of *opa*, *tsh, Ubx, dpp* as well as *wg*.

Our combination of genetic data and functional enhancer analyses provides convincing evidence that both *dpp* and *wg* are direct transcriptional targets of Org-1 in the cFCs. Prior dissections of the *dpp* visceral mesoderm (VM) enhancer had shown that it is also regulated by the direct binding of Ubx, Exd, dTCF (a Wg effector) and Bin, and that minimal synthetic variants that contain only the binding motifs for Ubx, Exd, Bin, and dTCF within conserved sequence contexts (which happen to include the Org-1 motif) are active as VM enhancers (Manak et al., 1994; Yang et al., 2000; Zaffran et al., 2001; Johnson et al., 2008). Likewise, the *wgXC* enhancer fragment integrates Org-1 with the direct regulatory inputs of Abd-A as well as CREB and Smad (Mad/Medea) proteins mediating Dpp signaling (Grienenberger et al., 2003).

Org-1 is the first transcription factor known to be required for *Ubx* expression in PS7 of the visceral musculature. Extensive work on an *Ubx* visceral mesoderm CRM (*UbxRP*) indicated that *dpp* and *wg* regulate *Ubx* through indirect autoregulation (Thuringer and Bienz, 1993; Thuringer et al., 1993). Of note, in *bin* embryos, which also lack visceral mesodermal *dpp* and *wg* expression, *Ubx* is still expressed (Zaffran et al., 2001). Our genetic data show that the *UbxRP* element, while requiring *org-1,* is not directly regulated by Org-1 as mutation of its four predicted T-Box binding sites did not have any effects (CS and MF, unpublished data). Taking into account that we were not able to detect *UbxRP* reporter activity in the cFCs at pre-fusion stages, we suggest that *UbxRP* represents a late enhancer element and responds to *dpp* and *wg* only after they are activated by Org-1 in the founder cells. To clarify whether the regulation of *Ubx* by Org-1 is direct or indirect, the identification and dissection of a founder cell specific CRM will be required.

tsh and *opa* were described as homeotic target genes of *Antp* in PS4-6 (*tsh*) and PS4-5 (*opa*) as well as of *abd-A* in PS8 (*tsh*) and PS9-12 (*opa*) of the visceral musculature (Mathies et al., 1994; Cimbora and Sakonju, 1995). Our data show that *tsh* and *opa* expression is already activated in the respective cFCs of the visceral parasegments where it requires *org-1*. The later activation of *tsh* in PS8 during muscle fusion follows the *org-1* dependent founder cell specific initiation of *wg* in PS8, which acts upstream of *tsh* (Mathies et al., 1994). Thus it was conceivable that the regulation of *tsh* by *org-1* is indirect. However, ectopic activation of *wg* in an *org-1* loss of function background is not able to rescue *tsh* expression and *Antp* and *abd-A* expression is not altered upon loss of *org-1*. These observations suggest that Org-1 acts directly on *tsh* and *opa*, e.g., via functional cooperation with Antp and Abd-A, respectively, during the early activation of *tsh* and *opa* in the founder cells.

Functional relationships between *org-1* and of Jeb/Alk signals during visceral mesoderm patterning

It was reported that the absence of Jeb/Alk signaling causes loss of *dpp* expression in the founder cells in PS7 of the visceral mesoderm (Shirinian et al., 2007). In light of our current findings that *org-1* loss-of-function produces a similar phenotype, and of our previous demonstration that *org-1* expression is downstream of Jeb/Alk, this observation could simply be explained by the action of a linear regulatory cascade from Jeb/Alk via *org-1* towards *dpp*. Alternatively, Jeb/Alk may provide additional inputs towards *dpp* (and other patterning genes) in parallel to *org-1*, which could explain the slightly stronger phenotype of *Alk* as compared to *org-1* mutations with respect to *dpp*. A possible candidate for an additional effector of Jeb/Alk signals in this pathway is *extradenticle* (*exd*), which is known to be required for normal *dpp* expression in PS7 of the visceral mesoderm, presumably through direct binding of Exd in a complex with Hox proteins and Homothorax (Hth) to a PS7-specific enhancer element (a derivative of which was used herein) (Rauskolb and Wieschaus, 1994; Ryoo et al., 1999; Stultz et al., 2006). Like *org-1, exd* is also needed for the expression of *tsh* and *wg* in the visceral mesoderm (Additionally, it represses *dpp* in PS4-6 through sequences not contained in the minimal PS7 enhancer). It is thought that Exd

complexed with Hox proteins and Hth increases the binding preference of these Hox complexes for specific binding sites within visceral mesodermal enhancers of their target genes (Rauskolb and Wieschaus, 1994; Mann and Chan, 1996; Grieder et al., 1997; Slattery et al., 2011).

As *exd* is expressed in both founder and fusion-competent cells in the visceral mesoderm, it is unlikely that it fulfils its roles in the regulation of *dpp*, *wg*, and *tsh* in the founder cells as a downstream gene of *org-1*. However, it is known that Exd requires nucleocytoplasmic translocation for it to be functional (Mann and Abu-Shaar, 1996; Aspland and White, 1997) and, interestingly, Shirinan et al. (2007) showed that Jeb/Alk signals trigger nuclear localization of Exd specifically in the cFCs of the visceral mesoderm. Because nuclear Exd appears to be hyperphosphorylated as compared to cytoplasmic Exd (Stultz et al., 2006), nuclear translocation of Exd may be triggered by Alk-mediated phosphorylation, as proposed by Shirinan et al. (Shirinian et al., 2007). Alternatively, Jeb/Alk signals may induce the expression of *hth* in the cFCs and Hth could then translocate Exd to the nuclei, as it has been shown in other contexts (Rieckhof et al., 1997; Pai et al., 1998; Abu-Shaar et al., 1999; Berthelsen et al., 1999). This would be compatible with our observation that Hth is upregulated in the founder cells in an *org-1*-independent manner (CS and MF, unpublished data).

The combined data show that Jeb/Alk signals exert at least two parallel inputs towards patterning genes in the cFCs, which are the induction of *org-1* and the nuclear translocation of Exd. Taken altogether, we suggest a model in which combinatorial binding of Org-1, nuclear Exd/Hth and the homeotic selector proteins to the corresponding visceral mesoderm specific CRMs is required for the initiation of lineage specific expression of *opa*, *tsh*, *dpp, Ubx* and *wg* in the founder cells of the respective parasegments. As shown in the examples of *dpp* (PS7) and *wg* (PS8), accessory Bin is required for the activation as a general visceral mesodermal competence factor, whereas Dpp and Wg effectors mediate autoregulatory stabilization of their expression (Fig. 8B, C).

Muscle identity factors in somatic versus visceral muscle founder cells

Extensive work has shown that during somatic muscle development individual founder myoblasts acquire distinct identities, which are adopted by the newly incorporated nuclei upon myoblast fusion, thus leading to the morphological and physiological diversification of the differentiating muscles (Abmayr et al., 1995; de Joussineau et al., 2012). We propose that the same principle is active during visceral muscle development. In this view, Org-1 acts as a muscle identity factor in both the somatic and visceral mesoderm. In the visceral mesoderm, Org-1 helps diversifying founder cell identities and, after myoblast fusion, their differential identities are transmitted to the respective differentiating circular gut muscles. The activation of downstream targets of this identity factor in the developing muscles leads to the observed morphogenetic differentiation events of the midgut and the establishment of the signaling center in PS7/8 that is also required for Dpp and Wg mediated induction of *labial* in the endodermal germ layer (Fig. 8C). As is the case for identity factors in the somatic muscle founders, Org-1 in the visceral mesoderm acts in concert with other, spatially restricted activities such as Hox factors and signaling effectors to achieve region-specific outputs. The main difference is that, in the trunk visceral mesoderm, Org-1 is present in all founder cells whereas in the somatic mesoderm this identity factor (like others) is expressed in a particular subset of founder myoblasts. Thus, in contrast to the somatic mesoderm, the spatial expression of Org-1 does not contribute to its function in visceral muscle diversification and instead, it solely relies on spatially-restricted co-regulators during this process.

The pool of trunk visceral mesodermal fusion-competent cells contributes to the formation of both circular and longitudinal midgut muscles, depending on whether they fuse with resident founder cells of the trunk visceral mesoderm or with founders that migrated in from the caudal visceral mesoderm. The restricted expression of the identity factor Org-1 in the founder myoblasts in the trunk visceral mesoderm and its exclusion from the FCMs represents an elegant mechanism to ensure that the respective patterning events only occur in the developing circular musculature but not in the longitudinal muscle fibers, which extend as multinucleate syncytia throughout the length of the midgut.

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Highlights

- Circuit of Hox, *dpp*, and *wg* regulation is established in visceral founder myoblasts
- Org-1 is a tissue-specific co-factor during *Drosophila* visceral mesoderm patterning
- Org-1 is needed for activation of majority of known patterning genes in founder cells
- It is required for visceral muscle founder diversification and midgut morphogenesis
- Org-1 functions via direct activation of *dpp* and *wg* enhancers in vm founder cells

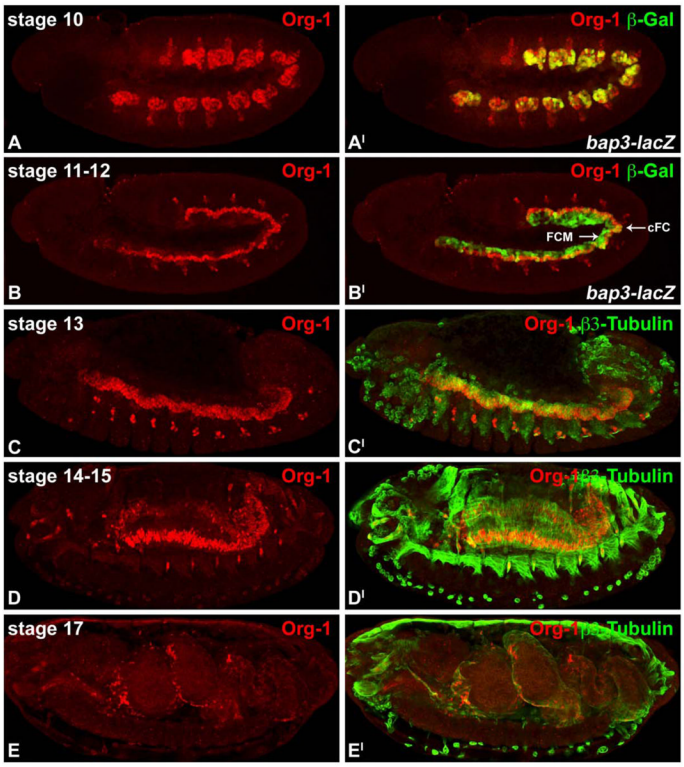

Figure 1. *org-1* shows a highly dynamic expression pattern during visceral mesoderm development

(A,A^{I}) Org-1 protein (Org-1) is found during stage 10 in all cells of the trunk visceral mesoderm primordia, visualized by *bap3-lacZ* expression (β-Gal). **(B,B^{I})** At stage 11 the visceral mesodermal expression domains of Org-1 are narrowed down to the founder cells of the circular visceral musculature (cFC), whereas the visceral fusion competent myoblasts (FCM) have become negative for Org-1 protein. **(C,C^{I})** Visceral muscle fusion takes place in stage 13, leading to the activation of Org-1 expression in the newly incorporated nuclei in the binucleated muscle syncytia. **(D,D^{I})** From stage 14 until the end of embryogenesis **(E,E^{I})** all nuclei of the circular visceral musculature continue to express Org-1.

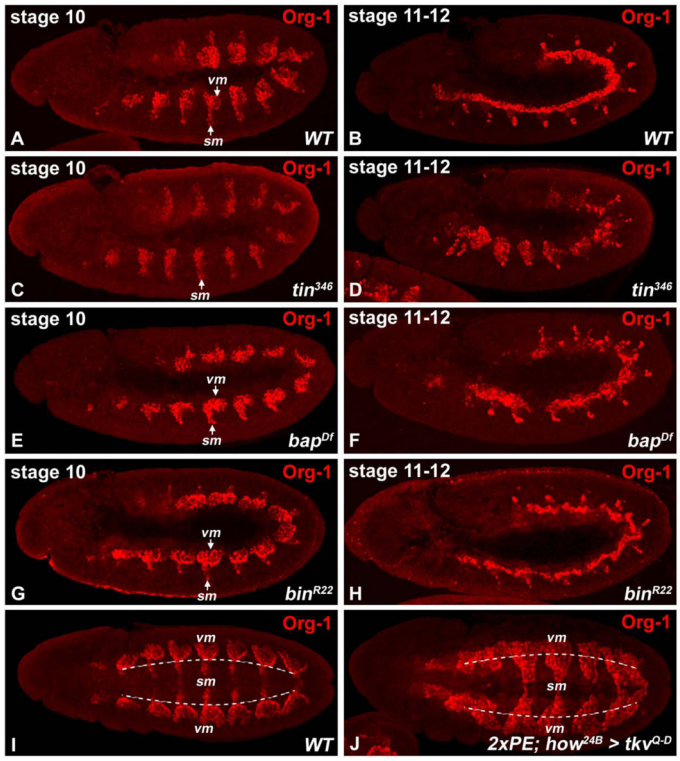

Figure 2. Regulation of *org-1* expression in the trunk visceral mesoderm

(A,B) Stage 10 and stage 11–12 wildtype embryos stained for Org-1 protein. **(C,D)** Stage 10 and stage 11–12 tin^{346} embryos stained for Org-1 showing strongly reduced expression in the trunk visceral mesoderm and missing founder cell restriction. **(E)** Stage 10 bap^{Df} mutant embryos display only slight reduction of Org-1 expression domains during early development. **(F)** bap^{Df} embryos of stage 12 display missing founder cell restriction of Org-1. **(G,H)** Loss of Bin function has no influence on Org-1 expression at early stages as well as during stage 12. **(I)** Ventral view of a stage 12 wildtype embryo stained for Org-1. **(J)** Ventral view of a stage 12 embryo with forced panmesodermal expression of a constitutively activated version of the Dpp-receptor Thickveins (tkv^{Q-D}). The Org-1 expression domains have been extended into the ventral somatic mesoderm. Abbreviations: (vm) visceral mesoderm, (sm) somatic mesoderm.

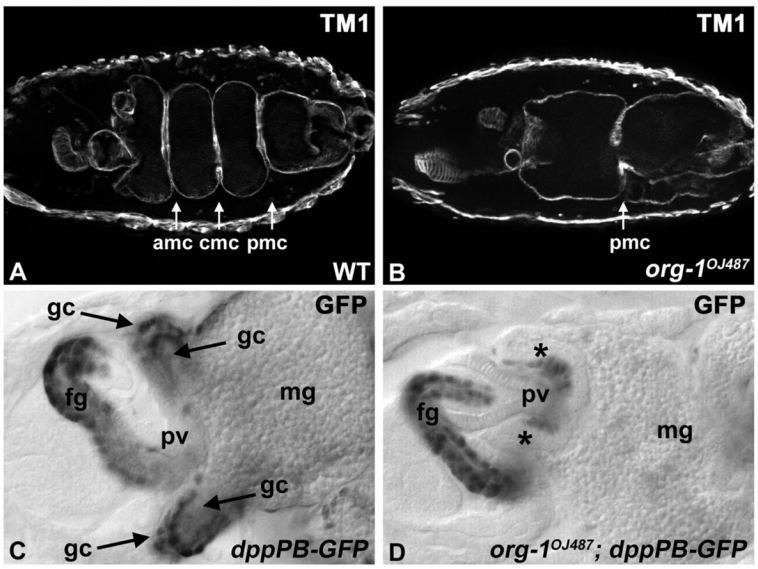

Figure 3. *org-1* is required for proper midgut morphogenesis

(A,B) Comparison of midgut morphology of stage 16 *WT* and *org-1*^{OJ487} mutant embryos (stained with antibodies against Tropomyosin (TM1)) reveals the loss of the anterior (amc) and central midgut (cmg) constrictions as well as a mislocated posterior (pmc) constriction in the mutant. **(C,D)** Anterior midguts from late stage 17 *dppPB-GFP* embryos, stained against GFP. In wildtype background the reporter is active in the visceral musculature around the 2 pairs of gastric caeca (gc, arrows). In the *org-1*^{OJ487} genetic background gastric caeca morphogenesis is arrested (asterisks). Abbreviations: (fg) foregut, (pv) proventriculus, (mg) midgut.

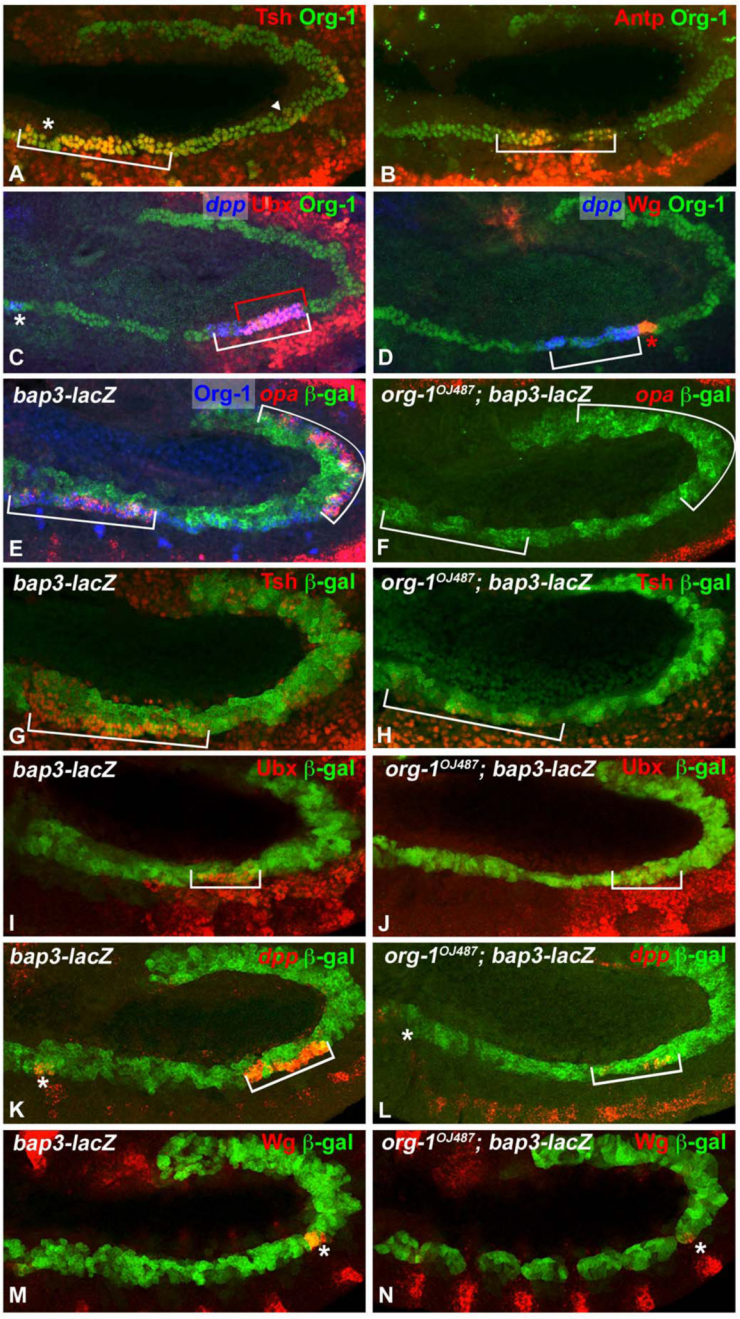

Figure 4. Lineage specific *odd paired, teashirt, Ultrabithorax, decapentaplegic* and *wingless* expression in the founder cells of the circular visceral musculature depends on Org-1

(A) At stage 11 Teashirt (Tsh) shows expression within the circular visceral muscle founder cells of parasegments (PS) 4–6 (bracket), albeit being weaker in PS4 (asterisk) than in PS5-6, and traces of expression in PS8 (arrowhead). **(B)** In an embryo of the same stage Antennapedia (Antp) is detected in the founder cells of PS5-6 (bracket). **(C)** In the visceral mesoderm, Ultrabithorax (Ubx) is solely expressed in the founders of PS7 (red bracket), whereas *decapentaplegic* (*dpp*) transcript can be found in the anterior founders of PS3 (asterisk), posterior PS6 and PS7 (bracket). **(D)** Some founder cells of PS8 are positive for Wingless (Wg) (red asterisk), whereas the adjacent founders of posterior PS6 and PS7 are

positive for *dpp* transcript (bracket). **(E)** *odd-paired* (*opa*) transcript can be found in the founders of PS3-5 (weak in PS3) and PS9-12 (brackets). **(F)** In *org-1* loss of function background *opa* expression in the visceral mesoderm is absent. **(G)** Tsh shows a strong expression domain in the founder cells of PS4-6 (bracket). **(H)** Org-1 loss of function leads to the nearly complete abolishment of Tsh in these founder cells. **(I)** In a stage 11–12 wildtype embryo Ubx is detected in the visceral founder cells of PS 7 (bracket). **(J)** In *org-1* mutant background Ubx expression in the nuclei of the founder cells of PS7 is absent. **(K)** *Dpp* is expressed in wildtype embryos in the founders of PS3 (asterisk) and PS6-7 (bracket) whereas **(L)** the loss of Org-1 leads to heavy reduction of visceral mesodermal *dpp* transcription in PS3 and PS6-7. **(M)** The visceral expression domain of Wg is seen in founder cells of PS8 (asterisk). **(N)** The removal of functional Org-1 leads to the nearly complete loss of Wg expression in the founders of PS8. The founder cells are visualized by stainings with Org-1 antibodies. The visceral mesoderm and musculature are visualized by *bap3-lacZ* reporter stainings (β-Gal).

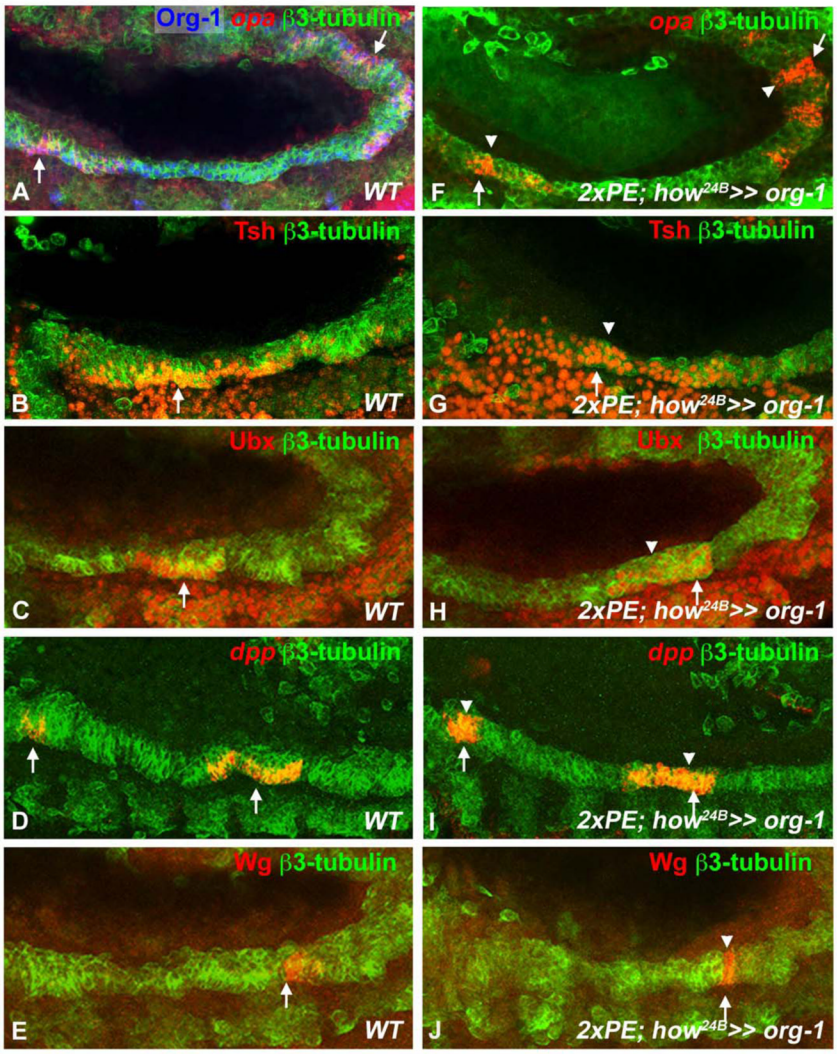

Figure 5. Org-1 is sufficient for visceral *opa*, *tsh*, *Ubx*, *dpp* and *wg* expression in the proper visceral mesodermal context

(A,B,C,D,E) Wildtype stage 12 embryos showing founder cell specific expression of (A) *opa* in PS3-5 and PS9-12 PS, (B) Tsh in PS4-6 (C) Ubx in parasegment (PS) 7, (D) *dpp* in PS3 and PS7 and (E) Wg in PS8 in the visceral founder cells (arrows). **(F,G,H,I,J)** Forced expression of Org-1 in all cells of the visceral mesoderm leads to the expansion of (F) *opa*, (G) Tsh, (H) Ubx, (I) *dpp* and (J) Wg expression domains from the visceral founder cells (arrows) into the visceral fusion competent myoblasts (arrowheads). The visceral mesoderm is visualized by 3-Tubulin stainings.

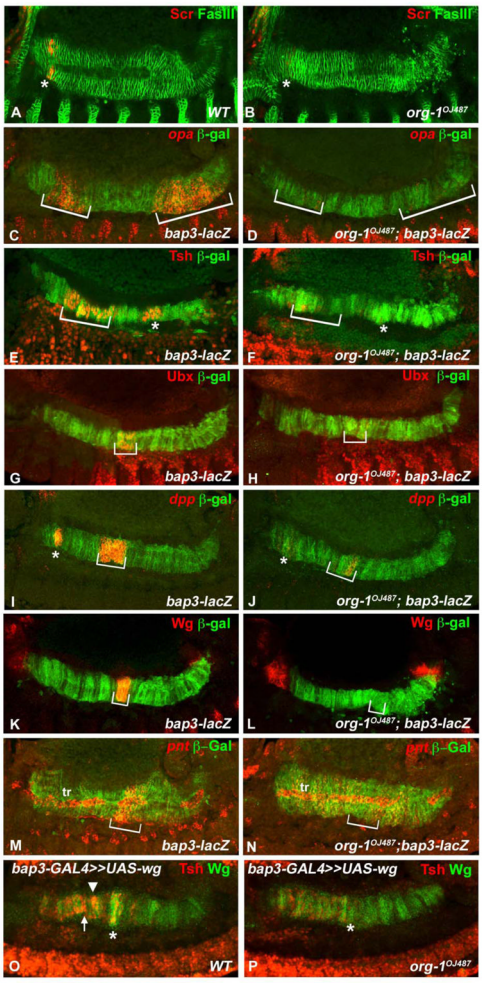

Figure 6. Org-1 is required for proper anteroposterior patterning of the developing visceral musculature

(A) Sex combs reduced (Scr) expression is initiated in the visceral musculature (visualized by Fasciclin III (FasIII) staining) of stage 13 embryos in parasegment (PS) 4 (asterisk). **(B)** In *org-1* mutant background visceral mesodermal Scr expression is nearly completely abolished. **(C)** Visceral muscle specific *odd-paired* (*opa*) expression can be detected in PS5 (asterisk) and PS10-12 (bracket). **(D)** In an *org-1* mutant embryo both expression domains are absent. **(E)** Teashirt (Tsh) shows two expression domains in the visceral musculature: one in PS4-6 (bracket) and another one in PS8 (asterisk). **(F)** Loss of Org-1 expression leads to abolishment of both expression domains of visceral mesodermal Tsh. **(G)** Stage 13 wildtype embryo showing Ubx expression in PS7 of the developing visceral musculature (bracket). **(H)** In the *org-1*^{OJ487} genetic background Ubx expression in PS7 is completely abolished. **(I)** *Dpp* shows strong expression domains in PS3 (asterisk) and PS6-7 (bracket) of the developing visceral musculature. **(J)** Only weak *dpp* expression can be detected in PS3 and PS7 in the *org-1* mutant background**. (K)** In a stage 13 wildtype embryo, Wg can be detected in PS8 (bracket) of the developing visceral musculature. **(L)** In an *org-1* mutant embryo Wg expression can no longer be detected in PS8. **(M)** In a stage 13 embryo *pointed* (*pnt*) transcript is detectable in PS8-9 of the developing visceral musculature (bracket) and the trachea adjacent to it (tr). **(N)** In an *org-1* mutant embryo the visceral musculature specific expression of *pnt* is not present. **(O)** Overexpression of Wg in the whole visceral mesoderm of wildtype embryos via *bap-Gal4* induces between PS4-6 (bracket) and PS8

(asterisk) a strong ectopic Tsh expression domain (arrowhead). **(P)** In an *org-1*^{OJ487} embryo, forced induction of Wg expression in the visceral mesoderm causes only faint Tsh expression anterior to PS8 (asterisk). The visceral musculature is visualized by *bap3-lacZ* reporter stainings (β-Gal).

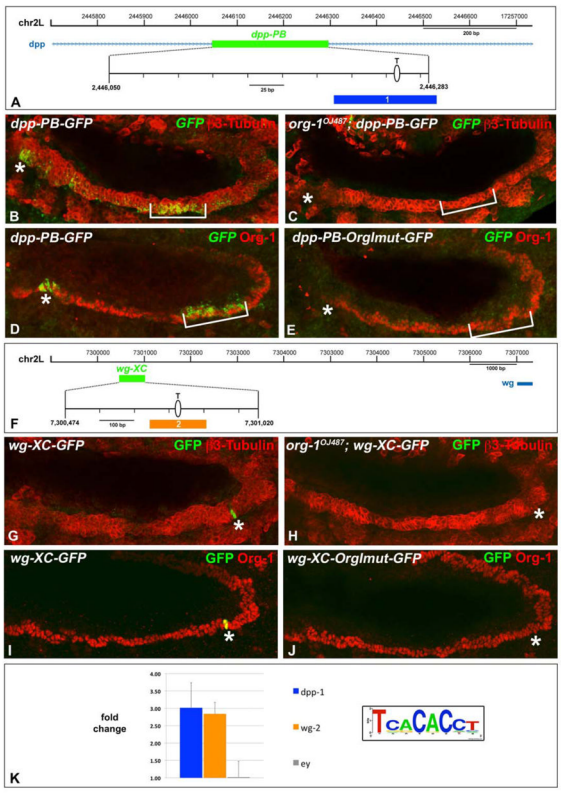

Figure 7. Org-1 is a direct regulator of *dpp* and *wg* expression in the circular visceral muscle founder cell lineage

A) Schematic diagram of the genomic region used for the generation of the *dppPB* reporter. The predicted T-Box binding site is indicated in the blow-up view. **(B)** *dppPB* driven *GFP* expression in parasegment (PS) 3 (asterisk) and PS6-7 (bracket) of a stage 11–12 embryo. **(C)** Stage 11–12 *org-1* mutant embryo carrying *dppPB-GFP* showing complete absence of visceral *GFP* transcript. **(D)** The *dppPB* reporter (*GFP*) is coexpressed with Org-1 in visceral mesodermal PS3 (asterisk) and PS6-7 (bracket). **(E)** The *dppPB-OrgImut-GFP* enhancer construct displays nearly complete loss of reporter activity in PS3 and PS7. **(F)** Schematic diagram of the genomic region used for the generation of the *wgXC* reporter. The predicted T-Box binding site is indicated in the blow-up view. **(G)**The *wgXC* enhancer fragment drives GFP expression in the visceral founder cells of parasegment (PS) 8 (asterisk) of a stage 11–12 embryo. **(H)** Complete absence of visceral *GFP* expression in a stage 11–12 *org-1* mutant embryo carrying *wgXC*. **(I)** *wgXC* reporter signal (GFP) is colocalized with Org-1 in visceral mesodermal PS8 (asterisk). **(J)** The *wgXC-OrgImut-GFP* reporter shows nearly complete loss of reporter activity in PS8. **(K)** Amplicons covering the T-Box binding sites (indicated in A and F) and an *ey* exonic amplicon as negative control were assayed by qPCR for enrichment in Anti-Org-1 ChIP. Three independent biological replicates were used to generate the average bar normalized to negative controls from the *C15* gene. The Org-1 consensus motif identified by SELEX (Schaub et al., 2012) is visualized by its sequence logo.

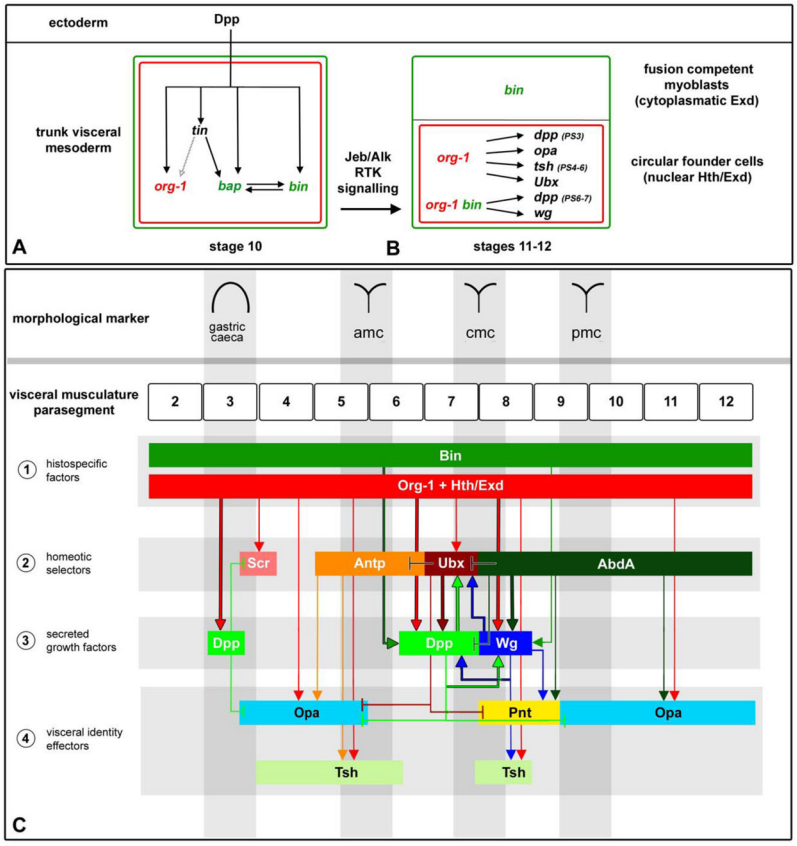

Figure 8. Regulatory interactions during trunk visceral mesoderm specification, founder cell diversification and midgut differentiation

(A) At stage 10 all cells of the trunk visceral mesoderm primordia initiate the expression of *tinman* (*tin*), *bagpipe* (*bap*), *binou* (*bin*) and *org-1* due to inductive *decapentaplegic* (*dpp*) cues from the ectoderm (only positive inputs are shown). **(B)** Whereas *bap* expression diminishes during stage 11, *bin* expression persists in all cells of the visceral mesoderm and *org-1* expression is maintained under the influence of receptor tyrosine kinase signaling (RTK) from the Anaplastic lymphoma kinase (Alk) only in the founder cells of the visceral musculature. Alk signaling induces nuclear translocation of Homothorax/Extradenticle (Hth/Exd) that activate together with Org-1 in the visceral founder cells of PS3 *dpp*, of PS3-5 and PS9-12 *opa*, of PS4-6 *tsh*, and of PS7 *Ubx*, and together with Org-1 and Bin the founder cell specific expression of *dpp* in PS6-7 and of *wg* in PS8. This process results in the diversification of visceral muscle founder cell fates along the anteroposterior axis. **(C)** After muscle fusion the spatial expression patterns of *opa*, *tsh*, *Ubx*, *dpp* and *wg* in the founder cells expand to the respective muscle fibers. Additionally Org-1 initiates the expression of *Sex combs reduced* (*Scr*) in the visceral musculature and is required in addition to Wg for the activation of *tsh* expression in PS8. *abd-A*, *wg* and *dpp* initiate the expression domains of Pointed (*pnt*) in PS8-9. Demonstrated direct regulation is indicated by arrows outlined in black.