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Cooperation of axial and sex specific information controls Drosophila female genitalia growth by regulating the Decapentaplegic pathway

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A B S T R A C T

The specification and morphogenesis of an organ requires the coordinate deployment and integration of regulatory information, including sex specific information when the organ is sex specific. Only a few gene networks controlling size and pattern development have been deciphered, which limits the emergence of principles, general or not, underlying the organ-specifying gene networks. Here we elucidate the genetic and molecular network determining the control of size in the *Drosophila* abdominal A9 primordium, contributing to the female genitalia. This network requires axial regulatory information provided by the Hox protein Abdominal-BR (Abd-BR), the Hox cofactors Extradenticle (Exd) and Homothorax (Hth), and the sex specific transcription factor Doublesex Female (DsxF). These factors synergize to control size in the female A9 by the coordinate regulation of the Decapentaplegic (Dpp) growth pathway. Molecular dissection of the *dpp* regulatory region and in vivo protein interaction experiments suggest that Abd-BR, Exd, Hth and DsxF coordinately regulate a short *dpp* enhancer to repress *dpp* expression and restrict female A9 size. The same regulators can also suppress *dpp* expression in the A8, but this requires the absence of the Abd-BM isoform, which specifies A8. These results delineate the network controlling female A9 growth in Drosophila.

1. Introduction

The understanding of the mechanisms that control the size and pattern of different organs is a major goal in developmental biology. Significant size differences are observed, for instance, between homologous structures of an animal. These differences depend on the activity of Hox genes (Crickmore and Mann, 2008), genes conserved in evolution that determine structures along the A/P axis of bilaterians (Rezsohazy et al., 2015). Hox proteins regulate hundreds of downstream targets, frequently in association with cofactors and collaborators, thus providing unique features to particular organs. The main Hox cofactors described in Drosophila, belonging to the TALE (Three Aminoacid Loop Extension) family of homeodomain proteins, are Extradenticle (Exd; Pbx1-4 in vertebrates) and Homothorax (Hth; Meis1-3 in vertebrates) genes (Mann et al., 2009).

Another source of size variation is sex. Sexual dimorphism is common to many animals, and frequently results in significant size differences between homologous organs in males and females. Both Hox genes and sex-determination genes likely act together to regulate size and pattern in different structures. A clear example of such collaboration is found in the development of the posterior abdomen of *Drosophila melanogaster*: only males have pigmentation in the whole fifth and sixth abdominal segments, and only females have a seventh abdominal segment (A7). Both characteristics depend on sex determination and Hox input (Kopp et al., 2000; Williams et al., 2008; Wang et al., 2011; Foronda et al., 2012). However, the more striking sexual difference observed in the adult of *Drosophila* and other species is the genitalia.

The genitalia of *Drosophila* derive from the genital disc, located at the back of the larva. This disc is made of three different primordia, corresponding to the eighth, ninth and tenth embryonic abdominal segments (A8, A9 and A10). The A8 gives rise to most of the structures of the genitalia (except for the parovaria and part of the uterus) in females while it gives rise to a tiny A8 segment in males. By contrast, the A9 forms only a reduced region of the uterus and the parovaria in females, while it develops most of the genital structures in the male. The A10 will form anal structures of the adult in both sexes (Nöthiger et al., 1977; Schüpbach et al., 1978; Keisman et al., 2001). Thus, the size of two of the three primordia in the third instar genital disc significantly diverges in males

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and females. The A9 primordium does not grow in the female disc as much as the A8 (or the male A9), whereas the male A8 is much smaller than the female one (or the male A9) (Fig. 1A). The A10 is of similar size in male and female genital discs (reviewed in Sánchez and Guerrero, 2001; Christiansen et al., 2002; Estrada et al., 2003).

In the genitalia, as in the posterior abdomen, development is dictated by the combined activity of sex determining genes and the Hox gene *Abdominal-B* (*Abd-B*). This gene codes for two different proteins, Abd-BM and Abd-BR. Both proteins are identical in the C-terminal region, including the DNA-binding domain, the homeodomain, but they differ in the N-terminal part: the Abd-BM protein has 223 aminoacids in the N-terminal end that are absent in the Abd-BR form (Zavortink and Sakonju, 1989; Celniker et al., 1989). In the female genital disc, the transcript encoding Abd-BM is present in the A8 and RNAs encoding Abd-BR can be detected in the A9 (Casares et al., 1997; Foronda et al., 2006) (Fig. 1A).

The different size of the A8 and A9 primordia between the male and female genital discs depends on the sex determination pathway. The gene at the end of the sex-determination genetic cascade is *doublesex* (*dsx*). This gene is differently spliced in males and females, giving rise to the DsxM (in males) and DsxF (in females) products. The alternative splicing is regulated by the presence of the protein encoded by the gene *transformer* (*tra*), only present in females, which together with the Transformer2 (Tra2) protein (present in both sexes), directs the splicing machinery to make the DsxF product in females (reviewed in Sánchez, 2008). The absence of *tra* results in the production of the DsxM protein and male development.

Several pathways have been involved in the growth of imaginal discs, such as the Decapentaplegic (Dpp) pathway (Schwank and Basler, 2010). A role for the Dpp pathway in genitalia growth control has been suggested. If a clone of cells mutant for *tra2* is induced in the female A9 (and therefore, the splicing of *dsx* directed to make the DsxM protein), there is ectopic expression of *dpp* and overgrowth (Sánchez et al., 2001). *dpp* encodes a factor that spreads and regulates growth and pattern (reviewed in Schwank and Basler, 2010), suggesting this ectopic expression could be the reason for the increased size of the primordium (Sánchez et al., 2001).

The regulatory information specifying the size and identity of the organ likely needs to be integrated during genitalia development. To understand the underlying molecular mechanisms we studied the respective contribution of the Hox Abd-BR, Exd and Hth Hox cofactors, and DsxF proteins in genitalia size control. Our results show that axial and sex specific information cooperate in the control of growth of the female A9 by converging in the regulation of the Dpp signaling pathway.

2. Material and methods

2.1. Genetics

We have used the following mutations, P-Gal4 and P-UAS lines: hth^{P2}, a strong hypomorph (Kurant et al., 1998), $Abd\text{-}B^{M1}$, a strong hypomorph or a null allele of *Abd-B* (Casanova et al., 1986), $Abd\text{-}B^{D18}$, a small deficiency for *Abd-B* (Hopmann et al., 1995), $tra\text{-}2^{ts1}$ (Belote and Baker, 1982), dsx^{1} (Bloomington #1679), *Df(3R)f01649-d09625* (Chatterjee et al., 2011) *dpp-lacZ* (BS3.0) (Blackman et al., 1991), *dpp-lacZ* (G132) (Theisen et al., 2007), *Abd-B*-$Gal4^{LDN}$ (de Navas et al., 2006b), *dsx*-Gal4 (Rideout et al., 2010), *btd*-Gal4 (Estella et al., 2003), *AC69*-Gal4, UAS-*traRNAi* (*Bloomington, BL-28512*), UAS-*tra2IR* (Fortier and Belote, 2000), referred to in the text as UAS-*tra-2RNAi*, UAS-*dpp-shmiR* (Haley et al., 2008), referred to in the text as UAS-*dppRNAi*, UAS-*Abd-BRNAi* (VDRC, line 12,024), UAS-*hthRNAi* (VDRC, lines 12,763 and 12,764),

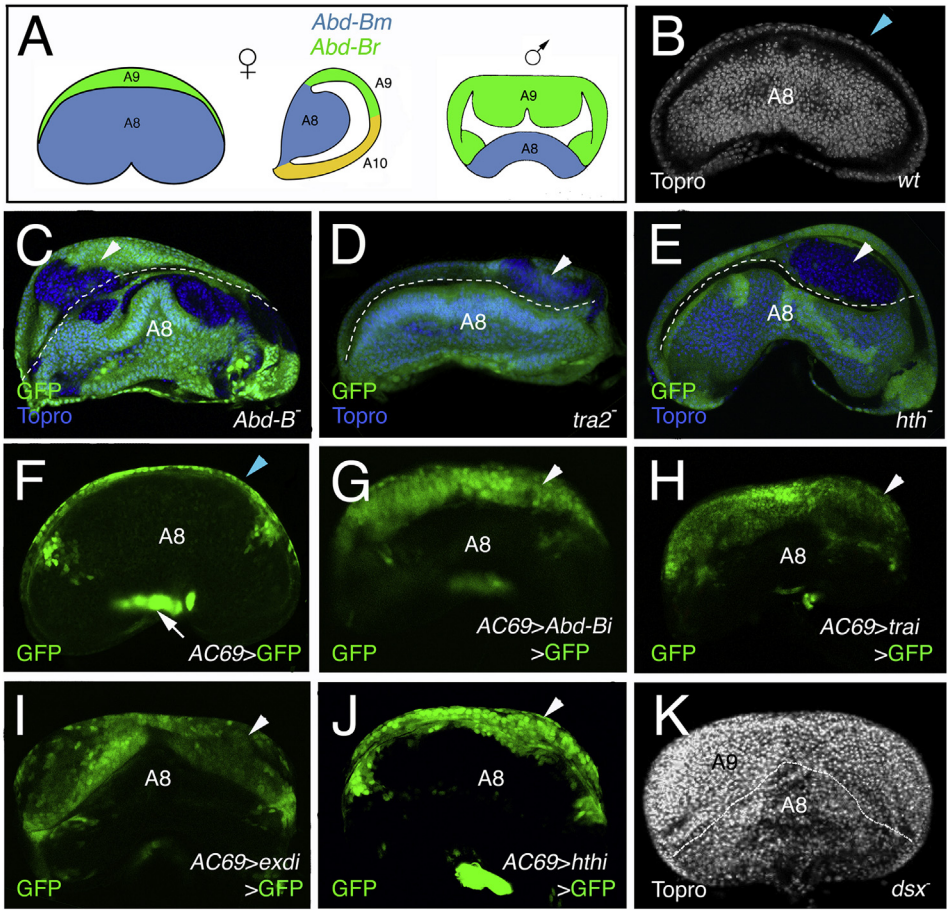

Fig. 1. *Abd-B*, *tra*, *hth* and *exd* repress growth in the A9 primordium of the female genital disc. (A) Drawings of the female (left, ventral and lateral views), and male (right, ventral view) discs, showing the A8, A9 and A10 primordia and the expression of *Abd-Bm* and *Abd-Br* transcripts. (B) Female genital disc showing the A8 and the A9 segment (arrowhead). In this and subsequent Figures, ventral views of the third instar female genital disc are shown. (C-E) $Abd\text{-}B^{M1}$ (C), $tra\text{-}2^{ts1}$ (D) or hth^{P2} (E) clones induced in the A9 cause overgrowths (arrowheads). F) Pattern of expression driven by the *AC69*-Gal4 line, showing GFP signal in the A9 (blue arrowhead) and in the stalk (arrow). (G-J) If the levels of expression of *Abd-B* (*AC69*-Gal4 UAS-*Abd-BRNAi*; G), *tra* (*AC69*-Gal4 UAS-*traRNAi*; H), *exd* (*AC69*-Gal4 UAS-*exdRNAi*; I) or *hth* (*AC69*-Gal4 UAS-*hthRNAi*; J) are reduced, the A9 increases its size (arrowheads). (K) In dsx^{1}/*Df(3R)f01649-d09625* genital discs the A9 segment overgrows (both A8 and A9 grow as the wildtype segments of female and male, respectively). In this and subsequent Figures the dashed lines indicate separation between the A8 and A9 segments.

UAS-*exdRNAi* (VDRC, lines 7802 and 7803), UAS-*hth* (Pai et al., 1998), UAS-*exd.nls* (gift of N. Azpiazu, CBMSO, Madrid), UAS-tkv^{QD} (Nellen et al., 1996), UAS-*brk* (Jaźwińska et al., 1999), UAS-Abd-BM (Castelli--Gair et al., 1994), UAS-Abd-BR (Rivas et al., 2013), UAS-DsxF (Sánchez et al., 2001), and UAS-GFP-*RNAi* (BDSC 9331). *tub*-$Gal80^{ts}$ was used for the temporal control of Gal4 expression (McGuire et al., 2003). Larvae were normally shifted from 17ºC to 29ºC at the beginning or the middle of the third larval instar.

2.2. Clonal analysis

Clones were induced at 48-72h or 72-96h after egg laying (AEL) by a 1 h heat shock at 37ºC. For *tra-*2^{ts1} clones larvae were transferred to 29ºC after clone induction. Clones were induced in larvae of the following genotypes:

y w hs-flp122/+; FRT82B hth^{P2}/ FRT82B *Ubi*-GFP

y w hs-flp122/+; *dpp-lacZ* (BS3.0)/+; FRT82B hth^{P2}/ FRT82B *M(3)RpS3 Ubi*-GFP

y w hs-flp122/+; FRT82B *Abd-*B^{M1}/ FRT82B *Ubi*-GFP or FRT82B *M(3)RpS3 Ubi*-GFP

y w hs-flp122/+; *dpp-lacZ* (BS3.0)/+; FRT82B *Abd-*B^{D18}/ FRT82B *M(3)RpS3 Ubi*-GFP

y w hs-flp122/+; FRT42D *tra-*2^{ts1} / FRT42D *Ubi*-GFP

2.2.1. MARCM clones (Lee and Luo, 2001)

Abd-B$^-$ UAS-*Abd-Br* clones: *tub*-Gal4 UAS-GFP *hs*-flp/+; *dpp-lacZ* (G132)/+; FRT82B *tub*-Gal80/ FRT82B *Abd-*B^{M1} UAS- *Abd-Br*

Abd-B$^-$ UAS-*Abd-Br* UAS-*hth* clones: *tub*-Gal4 UAS-GFP *hs*-flp/+; UAS-*hth* *dpp-lacZ* (G132)/+; FRT82B *tub*-Gal80 / FRT82B *Abd-*B^{M1} UAS- *Abd-Br*

***In situ* hybridization:** It was done according to standard protocols. The *dsx* RNA probe was synthesized from the GH08308 clone (DGRC, Bloomington) by in vitro transcription with the SP6 RNA polymerase and DIG-RNA labeling mix (Roche) The mRNA localization was revealed using Fast Red (Roche).

Immunohistochemistry. Dissection, fixation and staining of imaginal discs were done according to standard protocols (Sullivan et al., 2000) with the following antibodies: mouse anti-Abd-B (Developmental Studies Hybridoma Bank) at 1:50, rat anti-Exd (González-Crespo and Morata, 1995) at 1:200, rabbit anti-Hth (Azpiazu and Morata, 2002) at 1:500, guinea-pig anti-Hth (gift of N. Azpiazu, CBMSO, Madrid) at 1:300 and rat anti-Tropomyosin (Babraham Tech.) at 1:100. Secondary antibodies were conjugated anti-mouse or anti-rabbit Fluor 488, 555 or 647 (Alexa) used at a 1:200 dilution.

2.3. Constructs

2.3.1. Constructs of the dpp-enhancer

The G132 *dpp* enhancer is a 2,8 Kb fragment located at chr2L: 2,480,609-2,483,498. G136 and G137 are fragments of the G132 enhancer. The three DNA fragments were previously described (Theisen et al., 2007); transgenic flies containing these fragments upstream of the *lacZ* reporter gene were kindly provided by Dr. J. L. Marsh. The rest of the *dpp* enhancer fragments were generated in this work and cloned into the pH-pelican vector for the assay of their expression patterns using the *lacZ* reporter gene (Barolo et al., 2000). The different genomic sequences were obtained from the BAC R34M11 (BDGP) by standard PCR amplification with the corresponding primers (see Table 1). The obtained products were subcloned into pGEMteasy vector (Promega) and then transferred to pH-pelican digested with the same restriction enzymes present in the primers (underlined sequences) except for pHp-GE307, pHp-GE308b, pHp-GE396, pHp-GE496 and pHp-GE217, which were cloned in pH-pelican digested with BglII/EcoRI. pHp-GE308a construct is a 308bp SacII/NheI fragment of GE610, cloned into pH-pelican digested with SacII/NheI.

2.4. Bimolecular Fluorescence complementation assays

Bimolecular Fluorescence complementation assays in imaginal discs were carried out basically as previously described for Drosophila embryos (Hudry et al., 2011; Duffraisse et al., 2014).

2.4.1. BiFC constructs

The DsxF cDNA was obtained by PCR amplification from GH08308 clone (DGRC) by using the following primers: Fw = gaa<u>gcatgc</u>atcatggtttc (Sph-I) Rv = agta<u>gtcgac</u>gttactctagttaatg (Sal-I). The product was cloned into pGEMTeasy vector (Promega). The insert coding for Abd-BR protein was generated by PCR from full length complementary DNA (cDNA). pGEMT-DsxF and the previously obtained pGEMT-Abd-BR constructs were used for PCR amplification and fusion with the DNA fragments coding for the N-terminal (VN: 1–173) and C-terminal (VC: 155–238) moieties of Venus, which were generated by polymerase chain reaction (PCR) and cloned into EcoRI-XhoI restriction sites of the pUASTattB vector (Duffraisse et al., 2014). The Dsx^F insert to obtain fusions downstream the Venus fragment was cloned into KpnI-XbaI sites of the $pUASTVN_{173}$attB vector while AbdBr was cloned into XhoI-XbaI sites of the $pUASTVC_{155}$ attB vector. The transgenic flies UAS-^{VN}Exd, UAS-^{VC}Exd and UAS-Hth^{VN} used in this study were previously generated and described (Hudry et al., 2011).

2.5. In silico analysis

We searched for putative binding sites of the different TFs within the GE610 *dpp* enhancer region. Putative binding sites for Hth, Exd and Abd-B were identified by using Jaspar database (http://jaspar.genereg.net), setting the core identity to ≥80%. Consensus Dsx binding site described

Table 1
Oligonucleotides used for the dpp-enhancer constructs.

Construct in pHp vector	Fw-primer	Rv-primer	Length (bp)	Position relative to G132
GE546	caagaattcaggatcgaag(EcoRI)	aagggatcctataaaataag(BamHI)	546	1-546
GE856	caagaattcaggatcgaag(EcoRI)	ccggatccacacacacctgc(BamHI)	856	1-856
GE610	aggatccgatggatctgaag(BamHI)	ctcgagctagccaattccgg(XhoI)	610	2262-2876
GE307	aggatccgatggatctgaag(BamHI)	ggagaattccgcggagccc(EcoRI)	307	2262-2568
GE308b	ggatccctgtttgccg(BamHI)	gaattcgaaaagcgctctaaac(EcoRI)	308	2451-2752
GE396	aggatccgatggatctgaag(BamHI)	gaattcgcctggtggtc(EcoRI)	396	2262-2657
GE496	aggatccgatggatctgaag(BamHI)	gaattcgaaaagcgctctaaac(EcoRI)	496	2262-2752
GE217	ggatccctgtttgccg(BamHI)	gaattcgcctggtggtc(EcoRI)	217	2451-2657
GE427	ggatccctgtttgccg(BamHI)	ctcgagctagccaattccgg(XhoI)	427	2451-2876

in (Luo et al., 2011) was screened by using rVista (http://rvista.dcode.org/).

2.6. Chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) assay

A cDNA of *Abd-Br* was transferred from pGEMT-easy to pAC5.1 by using KpnI/NotI restriction enzymes. The resulting construct, pAC5.1-Abd-BR, contains a V5 epitope fused to the C-terminal of Abd-BR. Around 20 x10^6 Dmd8 cells transfected with pAC5.1-Abd-BR-V5 or non-transfected cells (as negative control) were used for Chromatin IP experiments using 2ug of anti-V5 antibody (Abcam). Similarly, the same number of non-transfected DmD8 cells was used for ChIP experiments with rabbit anti-Hth or a non-related IgG (Sigma). ChIP assay was performed by using ChIP-IT High Sensitivity kit (Active Motif) according to the supplier instructions. 50 ng of precipitated DNA of each sample was analyzed by qPCR (Roche). For the Abd-BR-V5 experiments, fold enrichment was expressed as the ratio of V5 immunoprecipitated signal in cells transfected with Abd-BR-V5 against the signal in non-transfected control cells. For the Hth experiment, fold enrichment was calculated as the ratio of the signal obtained with anti-Hth to the non-related IgG signal. As a negative control, an exonic region of the *RpII* gene was used. The following primers were used for qPCR: *RpII* gene: Fw; tggcaagaccattacccttc and Rv; gaatcttgcagaacttgtagcc. GE-610 Fw; 5′-ctttttggccgtcgttatgt-3’; GE-610 Rv; 5′-cgctctaaactggccatca-3’; *ct-340* Fw; 5′- acagcagtagcagcccaaaa-3′ and Rv; 5′-cagagcggggaatcatcata-3’ (Zhai et al., 2011); *Bn-A* Fw; 5′ aatccaaacgtgcagacggc-3′ and Rv; 5′-agcggtgtctaagcacagcg -3′ sequence kindly provided by R. Mann (Peng et al., 2009).

3. Results

3.1. Abd-B, homothorax, extradenticle and the sex determination genes repress growth in the female A9

Mutations (like *tra* or *tra-2* mutations) that transform females into males increase the size of the female A9 to reach the bigger size of the male A9 (Belote and Baker, 1982; Wieschaus and Nöthiger, 1982; Epper and Nöthiger, 1982; Keisman et al., 2001). Clones that disrupt *tra* or *tra-2* function induced in the female A9 also cause overgrowth (Epper and Nöthiger, 1982; Sánchez et al., 2001; Keisman and Baker, 2001). We wondered if other genes that specify genitalia development, like *Abd-B* (Estrada and Sánchez-Herrero, 2001) or the Hox cofactors Extradenticle (Exd) and Homothorax (Hth), which are highly expressed in this primordium (Estrada and Sánchez-Herrero, 2001), might also be needed to determine the size of this segment. To test this, we decided to study the requirement of DsxF, Abd-BR, Exd and Hth in the development of the female A9. We selected ventral views of female genital discs to better observe the size of the A9 in relation to the A8.

Mutant clones for *Abd-B* (20/23 clones), *hth* (8/13), or *tra-2* (12/15) induced in the female A9 during larval development cause overgrowths (Fig. 1C-E, compare with the wildtype in Fig. 1B). Overgrowing clones are normally located in a medial-lateral side of the segment. A similar increase in size was observed when we reduced the expression of *Abd-B*, *tra*, *exd* or *hth* with UAS-*RNAi* lines and a Gal4 insertion, *AC69*-Gal4, isolated from a large screen for Gal4 lines (Calleja et al., 1996), and which drives expression in the female A9 (Fig. 1G-J, compare with 1F). Overgrowths are more variable in size and observed frequently only in one side of the segment when reducing *Abd-B* expression. We note that the absence of *tra* involves two changes in protein expression: the elimination of DsxF and the expression of DsxM. However, in *dsx* mutants, in which both DsxF and DsxM proteins are absent or their expression strongly reduced, there is also A9 overgrowth (Sánchez et al., 2001; Keisman and Baker, 2001; Fig. 1K), thus suggesting that DsxF, present in the wildtype female A9, represses growth. A control cross in which a UAS-GFP-*RNAi* construct is driven in the A9 does not give any effect (not shown).

3.2. Mutiple independent contribution of Abd-B, DsxF, hth and exd for size control in the female A9

One possible interpretation of the preceding results is that there are cross regulations between the genes needed to reduce A9 size, so that growth repression is not independently achieved by each of them. There is, for instance, a tight link between Hth and Exd: absence of Hth prevents the nuclear translocation of Exd (Kurant et al., 1998; Pai et al., 1998; Rieckhof et al., 1997) and the absence of Exd reduces Hth stability (Abu-Shaar and Mann, 1998). Exd and Hth are highly expressed in the female A9 (Fig. 2A-A″) and we have also observed a mutual requirement between them (Fig. 2B-C’). To check if the effect of *hth* on growth was mediated by *exd*, we reduced *hth* expression and simultaneously forced the nuclear localization of Exd with a construct in which a nuclear localization signal, in addition to those already present in the protein (Abu-Shaar et al., 1999; Berthelsen et al., 1999; Saleh et al., 2000), is attached to Exd; we name it UAS-Exd.nls. This resembles previous experiments that directed Exd to the nucleus in a region of the leg disc without *hth* expression (Stevens and Mann, 2007), or that increased Exd expression in *hth* deficient embryos (Corsetti and Azpiazu, 2013). We observed nuclear (but also cytoplasmic) Exd protein in the female A9 of these mutant larvae, but there is still A9 segment overgrowth (Fig. 2E-E”). The sole expression of Exd.nls, however, does not change A9 size (Fig. 2D). These results suggest that, similarly to what has been shown before (Stevens and Mann, 2007), *hth* can function (in this case, it can regulate growth in the female A9) independently of its role in translocating Exd to the nucleus, although *hth* may be needed for Exd activity (Stevens and Mann, 2007).

We checked then regulatory interactions among *Abd-BR*, DsxF and *hth* and their impact on growth control. Given the link between *hth* and *exd* expression, we took the former as representative of the two. As shown in Fig. 3A, A′ and 3B, B′, *tra-2^{ts1}* mutant clones do not reduce the expression of *Abd-B* or *hth*, respectively. Similar results were obtained when using the *AC69*-Gal4 line: reducing *tra* with a UAS-tra*RNAi* construct does not modify Abd-B (Fig. S1A, A′) or Hth (Fig. S1B, B′) expression. Downregulation of *dsx* does not significantly change Hth or Abd-B levels, either (Figs. S1C and D). *hth* mutant clones do not change Abd-B expression (Fig. 3C, C′) and reducing *hth* in *AC69*-Gal4 UAS-hth*RNAi* discs does not, or just barely, change Abd-B protein levels (Fig. S1E, E′). However, in 4/5 discs of the genotype *AC69*-Gal4 UAS-Abd-B*RNAi* there is a slight downregulation of Hth (Fig. 3D, D′). It has also been recently reported that *Abd-B* regulates the expression of *doublesex* (*dsx*), the gene at the end of the sexual determination genetic cascade (reviewed in Sánchez, 2008), in the posterior abdomen (Foronda et al., 2012; Wang and Yoder, 2012). We also observed that downregulation of *Abd-B* in the A9 reduces *dsx* expression (Fig. 3F, compare with *dsx* wildtype expression in 3E). However, if in discs with reduced *Abd-B* expression we increase both *hth* and DsxF (Fig. 3G), there is still overgrowth in the female A9, indicating that *Abd-B* prevents growth independently of its role in maintaining *dsx* and *hth*. The control discs expressing just *hth* (Fig. S1F, F’) or *hth* and DsxF (Fig. 3H) have a normal sized A9. Taking together, although *Abd-B* influences Hth and DsxF expression, the control of size in the female A9 also relies on action independent of these regulatory effects.

3.3. Abd-B, hth, exd and the sex determination pathway regulate growth in the female A9 through the dpp pathway

It was previously described that *tra-2^{ts1}* mutant clones in the female A9 show ectopic *dpp* expression and overgrowth (Sánchez et al., 2001). We checked therefore if the absence of *Abd-B*, *hth* or *exd* also results in ectopic *dpp* expression in this segment. To this end, we used the *dpp*-lacZ BS3.0 reporter line, which reproduces *dpp* transcription in the genital discs (Blackman et al., 1991). In females, *dpp-lacZ* is expressed in two bands in the A8 and A10 but not in the A9 (Casares et al., 1997, Blackman et al., 1991; Freeland and Kuhn, 1996; Chen and Baker, 1997) (Fig. 4A). Clones mutant for *Abd-B* (Fig. 4B) or *hth* (Fig. 4C) overgrow and activate

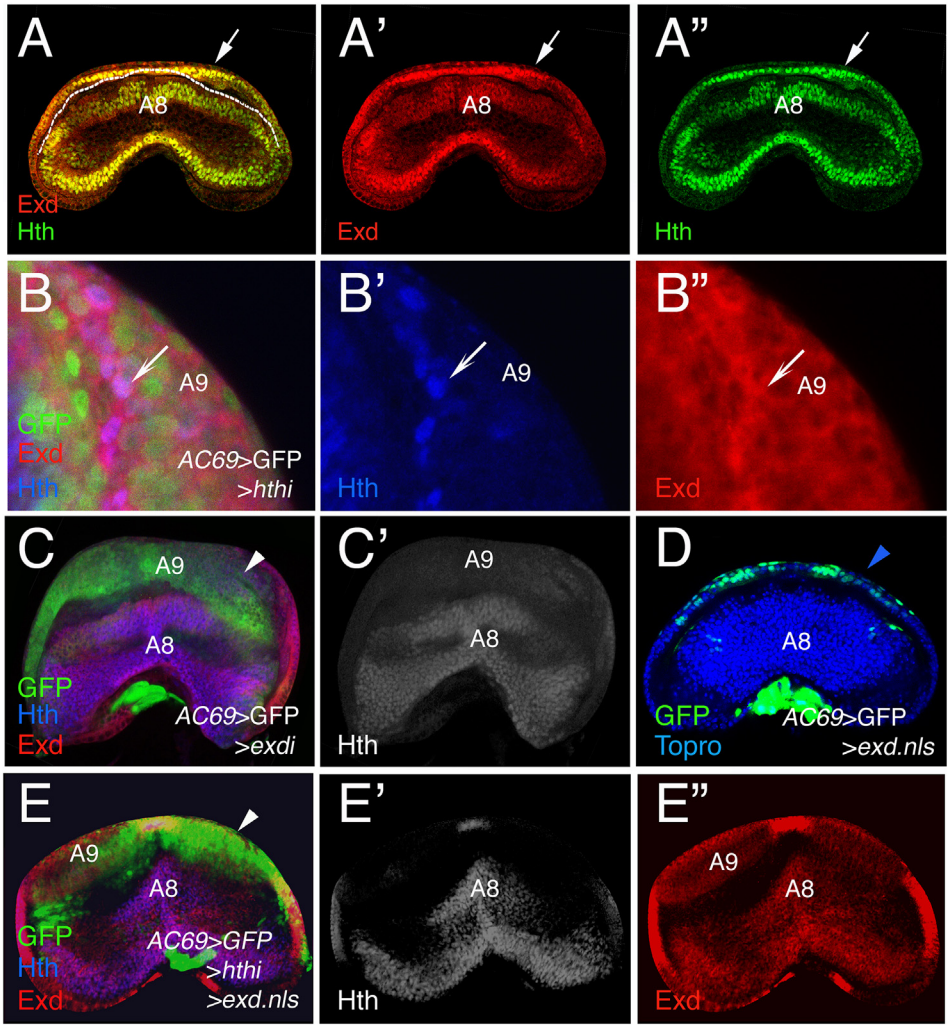

Fig. 2. Expression of Hth and Exd in the female genital disc and cross-regulatory interactions between them. (A-A") Female genital disc stained with anti-Exd and anti-Hth antibodies (arrows mark the A9). (B-B″) Detail of part of the A9 of a female genital disc where a UAS-*hth*RNAi construct was expressed under the control of the *AC69*-Gal4 line (GFP marks expression driven by the Gal4 line in green). *hth* expression (in blue) is reduced in the segment and the Exd protein (in red) is cytoplasmic. A line of cells where Hth protein remains high (arrows in B and B′) shows nuclear localization of Exd (arrow in B″). (C, C′) In *AC69*-Gal4 UAS-*exdRNAi* female genital discs the expression of Exd (in red) is reduced in the A9 (marked with GFP) and so is that of *hth* (in blue). The A9 segment shows size increase. D) The expression of just *exd.nls* does not change the size of the A9 (blue arrowhead). (E-E″) If *hth* expression is reduced and *exd* simultaneously expressed, in *AC69*-Gal4 UAS-*exd.nls* UAS-*hthRNAi* larvae, there is also overgrowth of the A9. The A9 Exd protein is both in the nucleus and the cytoplasm, as has been observed with other Exd mutant proteins in which there is a balance between the Nuclear Export Signals and the Nuclear localization Signals (Stevens and Mann, 2007).

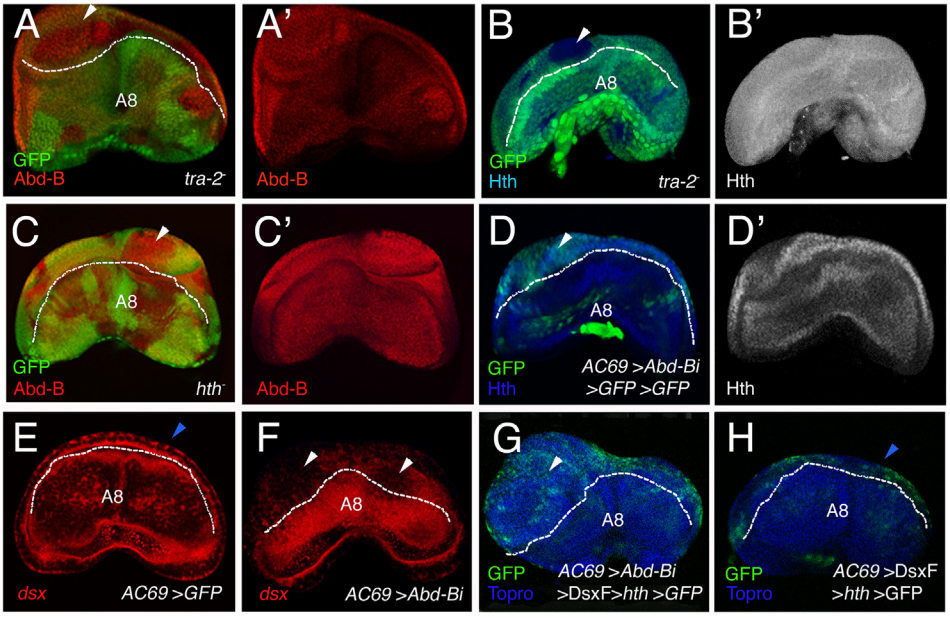

Fig. 3. Regulatory interactions between *Abd-B*, *dsx* and *hth*. (A-D′) Clones mutant for *tra-2*^{ts1} (A-B′) or *hth*^{P2} (C, C′), marked by absence of GFP (arrowheads in A, B and C), do not change the expression of *Abd-B* (A, A′), *hth* (B, B′) or *Abd-B* (C, C′), respectively. (D, D′) There is a slight downregulation of *hth* in the A9 of 4/5 discs in which *Abd-B* expression is reduced (*AC69*-Gal4 UAS-*GFP* UAS-*GFP* UAS-*Abd-BRNAi* female discs; arrowhead) (E) *dsx* RNA ubiquitous expression in the female genital disc (the arrowhead marks the A9). (F) Reduction of expression of *Abd-B* (*AC69*-Gal4 UAS-GFP UAS-*Abd-BRNAi*) down-regulates *dsx* expression, detected as in E, in the overgrown A9 (arrowheads). G) Female genital disc in which *Abd-B* expression is reduced and Hth and DsxF protein levels increased in the A9 (*AC69*-Gal4 UAS-GFP UAS-*Abd-BRNAi* UAS-*hth* UAS-DsxF). The A9 shows increased growth (arrowhead). H) Overexpression of both *hth* and DsxF does not modify A9 size.

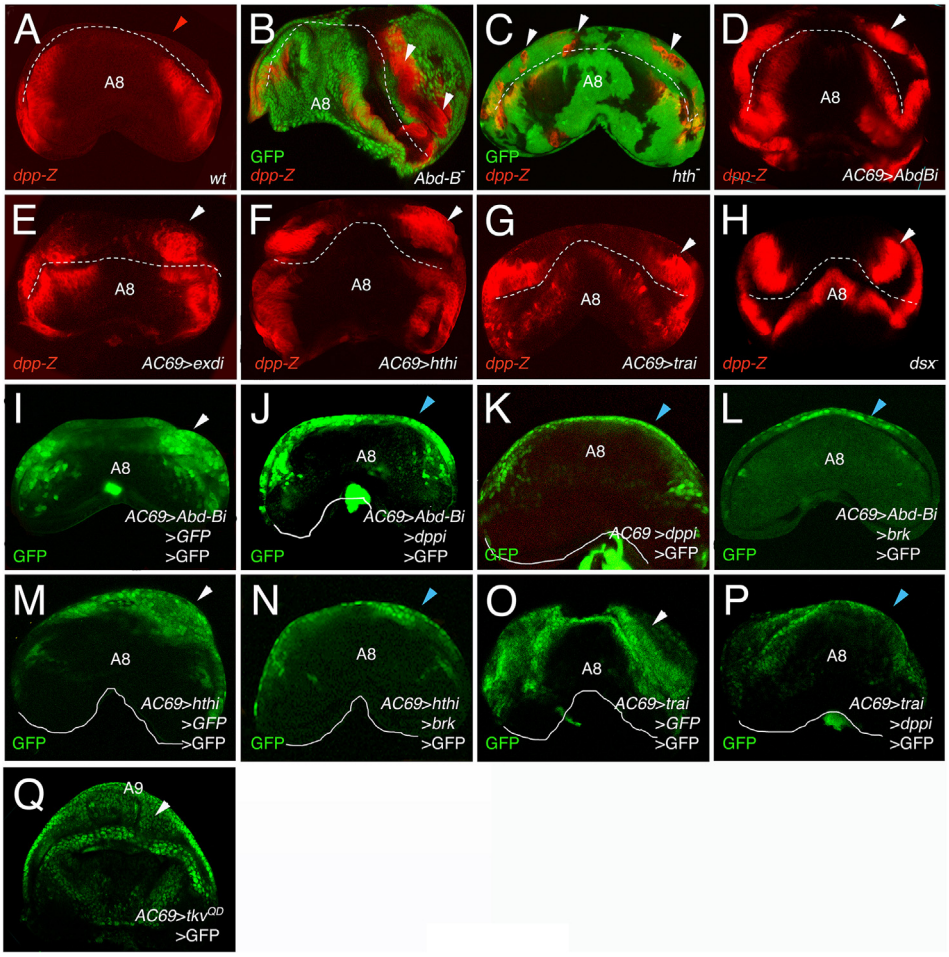

Fig. 4. Ectopic activation of *dpp* when *Abd-B*, *tra*, *exd* or *hth* are reduced causes increased growth of the female A9. (A) The BS3.0 *dpp-lacZ* reporter is expressed in the female A8 but not in the A9 (red arrowhead). (B, C) Clones mutant for *Abd-B* (*Abd-B*^{D18}; B) or *hth* (*hth*^{P2}; C) induce ectopic expression of *dpp*-lacZ in the A9 (arrowheads). (D-G) If *Abd-B* (*AC69*-Gal4 UAS-*Abd-BRNAi*; D), *exd* (*AC69*-Gal4 UAS-*exdRNAi*; E), *hth* (*AC69*-Gal4 UAS-*hthRNAi*; F) or *tra* (*AC69*-Gal4 UAS-*traRNAi*; G), expression is reduced, the A9 increases its size and shows ectopic *dpp-lacZ* expression (white arrowheads). (H) When *dsx* is strongly down-regulated in *dsx*^{1}*/Df(3R)f01649-d09625* genital discs, there is also ectopic *dpp*-lacZ signal in the A9 (arrowhead). (I, J) The strong overgrowth in the female A9 (in 14/36 discs) when *Abd-BRNAi* is expressed in this segment (*AC69*-Gal4 UAS-GFP UAS-GFP UAS-*Abd-BRNAi*, I, white arrowhead) is substantially reduced (3/27 discs show overgrowth) if *dpp* is concomitantly downregulated (*AC69*-Gal4 UAS-GFP UAS-*Abd-BRNAi UAS-dppRNAi*) (J, blue arrowhead). K) Inhibition of *dpp* expression alone does not affect A9 growth. L) A strong reduction of the phenotype is also observed if *brk* is expressed in the *AC69*-Gal4 UAS-GFP UAS-*Abd-BRNAi* background. (M-P) In *AC69*-Gal4 *tub*-Gal80^{ts} UAS-GFP UAS-GFP UAS-*hthRNAi* (M) (larvae shifted from 17ºC to 29ºC at the end or larva II), or *AC69*-Gal4 UAS-GFP UAS-GFP UAS-*traRNAi* (O) female genital discs there is overgrowth of the A9 (white arrows). Such overgrowths are reduced when there is simultaneous expression of *brk* in *AC69*-Gal4 *tub*-Gal80^{ts} UAS-GFP UAS-*brk* UAS-*hthRNAi* larvae (N, larvae shifted from 17ºC to 29ºC at the end or larva II), or reduction of *dpp* in *AC69*-Gal4 UAS-GFP UAS-*dppRNAi* UAS-*traRNAi* larvae (P) (blue arrows). Q) The constitutive activation of the *dpp* pathway (*AC69*-Gal4 UAS-*tkv*^{QD}) also augments A9 size.

dpp-lacZ ectopically in the A9. Similarly, the down-regulation of *tra*, *Abd-B*, *exd* or *hth* in this segment with the *AC69*-Gal4 line induces ectopic *dpp-lacZ* expression and overgrowth (Fig. 4D-G, compare with 4A). However, it was unclear in this and previous experiments (Sánchez et al., 2001) if the ectopic *dpp* expression observed when *tra* was down-regulated was due to the elimination of DsxF, the ectopic expression of DsxM or both. To check if DsxF was indeed repressing *dpp*-lacZ we reduced *dsx* expression in the A9: we observed in these discs ectopic *dpp-lacZ* (Fig. 4H), arguing that DsxF represess *dpp* expression (which does not exclude a function for DsxM in *dpp* regulation as well). The ectopic expression in all these mutant combinations is limited to a small domain of central-lateral regions of the A9, possibly due to regulation by *hedgehog* input from the A8 and repression by *wingless* (Sánchez et al., 1997).

We then analyzed whether the ectopic expression of *dpp* could indeed account for the increased growth of the A9 in the absence of *Abd-B, hth or tra*. Compared to the single loss of *Abd-B*, which induces strong overgrowths in the A9 region in 39% of the cases (n = 36) (Fig. 4I), the double *Abd-B* and *dpp* knockdown led to smaller outgrowths and in only 11% of the genital discs (n = 27) (Fig. 4J). The expression of the dppRNAi construct alone does not change A9 size (Fig. 4K). The A9 overgrowth was also reduced in the absence of *Abd-B* when co-expressing *brinker* (*brk*), an antagonist of Dpp activity (Jaźwińska et al., 1999; Minami et al., 1999) (Fig. 4L). A similar reduction in A9 size was also observed when *hth* and *brk* (Fig. 4N), or *tra* and *dpp* (Fig. 4P), were simultaneously downregulated, in comparison with discs diminishing just *hth* (Fig. 4M) or *tra* (Fig. 4O), respectively. Further, ectopic activity of *dpp* is sufficient to induce such overgrowths in this primordium (Fig. 4Q). Collectively, our data indicate that change of female to male sex, or a reduction of *Abd-B*, *exd* or *hth* activity, induces growth of the female A9, at least in part, through the ectopic expression of *dpp*.

3.4. A specific Abd-B isoform and high levels of hth are sufficient to down-regulate dpp expression in the female A8

Next, we aimed at identifying the genetic requirements sufficient for *dpp* repression. To this end, we focused our study in the female A8, in which DsxF is also present but that, contrary to the A9, presents *dpp*-lacZ signal (Fig. 4A). A difference between the two segments is the specific expression of *Abd-B* RNAs: *Abd-Br* transcripts are expressed in the A9 and the *Abd-Bm* RNA in the A8 (Casares et al., 1997; Foronda et al., 2006). We therefore manipulated the distribution of *Abd-B* transcripts and analyze *dpp* expression using the G132-*lacZ dpp* reporter construct, which is part of the BS3.0 fragment (Theisen et al., 2007) and mimics its expression (see below). Forcing Abd-BR in the A8 is not sufficient to repress *dpp* (Fig. 5A, A′). Reciprocally, the ectopic expression of the Abd-BM protein in the A9 induces just weak *dpp*-lacZ expression, and in less than half of the discs (Fig. 5B, B'). This suggests that the sole presence of one or another Abd-B isoform in A8 or A9 is not sufficient to determine, in a fully reproducible manner, whether *dpp* is transcribed or not.

We then considered the possibility that the presence of the Abd-BM isoform in the A8, although not needed for *dpp* expression (Estrada and Sánchez-Herrero, 2001), could counteract Abd-BR activity. To probe this, the expression of the Abd-BR protein was induced in clones that, at the same time, eliminate the activity of the two endogenous Abd-B proteins. Most of these clones (7/10) do not reduce *dpp* expression (Fig. 5C, C′)

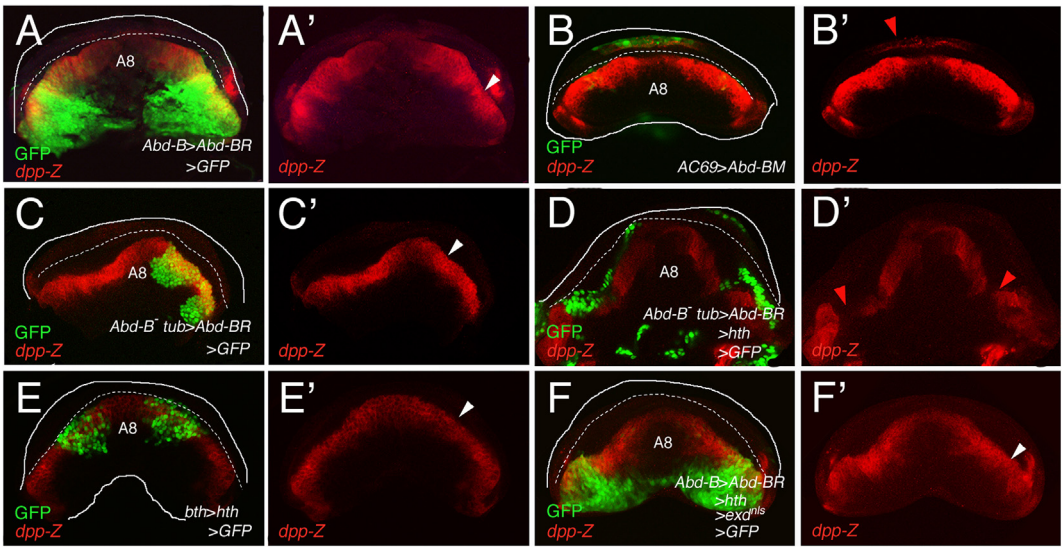

Fig. 5. Hth and Abd-Br down-regulate *dpp* expression in the female A8 in the absence of endogenous Abd-B. (A, A′) Ectopic expression of Abd-BR in the more ventral region of the A8 (*Abd-B*-Gal4^{LDN} UAS-*Abd-BR*), marked by GFP, does not repress *dpp-lacZ* expression (arrowhead in A'; line G132; in this and following panels, in red). (B, B′) The ectopic expression of *Abd-Bm* in the A9 induces weak expression of *dpp*-lacZ (arrowhead in B′). (C, C′) MARCM clones expressing Abd-BR (marked by GFP) in the absence of endogenous *Abd-B* (*Abd-B*^{M1} UAS-*Abd-BR* UAS-GFP) do not repress *dpp-lacZ* expression (arrowhead in C′). (D, D′) Most MARCM clones (marked by GFP) that express Abd-BR and *hth* in the absence of endogenous *Abd-B* (*Abd-B*^{M1} UAS-*Abd-BR* UAS-*hth* UAS-GFP) downregulate *dpp-lacZ* expression (red arrowheads in D′). (E-F′) The increased expression of *hth* under the control of the *btd*-Gal4 line (E, E′), or the simultaneous expression of Abd-BR, Hth and Exd.nls in the more ventral region of the A8 (F, F′) (*Abd-B*-Gal4^{LDN} UAS-*Abd-BR* UAS-*hth* UAS-*exd.nls*) (both Gal4 lines driving GFP expression), do not repress *dpp-lacZ* expression (arrowheads in E′ and F′).

whereas in 3 of them there is a slight downregulation. This suggests that the absence of Abd-BM is not enough in all the clones for full *dpp* repression by Abd-BR in the female A8. To discover which other regulators may cooperate to repress *dpp* in the A8 we noticed that Hth and Exd are highly expressed in the A9 (Estrada and Sánchez-Herrero, 2001, Fig. 2A-A″), which might influence *dpp* expression. Hence, we decided to make clones that express both Abd-BR and Hth in the absence of endogenous Abd-B proteins, and check for *dpp* signal. Some of the clones (something also observed in *Abd-B*$^{-}$ UAS-Abd-BR clones) seem to "push" the *dpp-lacZ* bands of expression. However, most clones (6/9) substantially reduce *dpp* expression (Fig, 5D, D′). Just increasing *hth* expression in the A8 or the combined expression of Abd-BR, Hth and nuclear Exd (Exd.nls) is not enough to repress *dpp* (Fig. 5E-F'). These results suggest that, although *hedgehog* would activate *dpp* in both female A8 and A9, the Abd-BR isoform and high *hth* levels, along with the absence of Abd-BM, can repress *dpp* in the A8. Our experiments, however, also suggest that the expression of Abd-BM in the A9 is not sufficient to fully prevent this repression.

3.5. DsxF, exd, Hth and Abd-BR form an interaction network in vivo

We have shown that Abd-BR, DsxF, Exd and Hth repress female A9 *dpp* expression and growth. The similar effect and independent contribution of these proteins suggest that they may act together in the context of a regulatory complex. We explored this possibility by using Bimolecular Fluorescence Complementation assays (BiFC), which have been used before in *Drosophila* to demonstrate protein-protein interactions in vivo (Hudry et al., 2011; Baëza et al., 2015). We fused the N-terminal or C-terminal parts of the Venus fluorescent protein to the Abd-BR, DsxF, Hth or Exd proteins and expressed them under UAS control. The *AC69*-Gal4 line, used to direct expression in the female A9, is also active in the wing disc, allowing us to assess protein-protein interactions in another developmental context, different from the genital disc.

With the exception of Abd-BR and Hth, we detected interactions between Abd-BR, DsxF, Hth and Exd proteins in the female A9 and wing pouch (Fig. 6B-E, G, I-L, N, the Gal4 patterns for genital and wing disc are shown in 6A and 6H, respectively). The fluorescence signal in the female A9 is consistently observed in all these protein combinations, although at low levels. This may be due to two reasons: the tagged proteins may compete with the endogenous ones for positive interactions, or the level of expression of the Gal4 protein may be low. Two observations give some support to these explanations. First one is that we noticed that the GFP signal is weaker for all the combined genotypes in the genital disc than in the wing pouch (Fig. 6I-L, N), a territory with no *Abd-B*, *dsx* or *hth* expression (Celniker et al., 1989; Casares and Mann, 2000; Azpiazu and Morata, 2000; Robinett et al., 2010). Second, the GFP signal for the Hth/Exd pair, whose direct interaction is well established (Abu-Shaar et al., 1999), is similar to the other positive protein pairs, suggesting that the low signal in the genital disc may be due to low levels of protein expression. Further supporting this idea we noticed that we see a much robust signal in the wing disc, and also that when we used a stronger Gal4 driver in the genital disc (*dsx*-Gal4), we obtained higher and more widespread GFP signal in the A9 (Fig. S2). The most parsimonius explanation for these observations is that AbdB, DsxF, Hth and Exd are part of the same protein complex, although this is not demonstrated. However, we could not observe BiFC between AbdBR and Hth (Fig. 6F, M), which either suggests that the above described interactions results from multiple protein complexes (AbdB/Exd/DsxF; Hth/DsxF and Exd/Hth), or that something prevents vizualising the AbdB/Hth interaction by BiFC. In any case, the absence of GFP signal in the Abd-BR/Hth pair (while each of these two tagged proteins interacts with other proteins, see Fig. 6B, C, E, I, J and L), argues against a non-specific GFP signal in the different combinations.

3.6. Abd-BR, DsxF, Exd and Hth act at the level of a common enhancer to repress dpp in the female genital disc

The control of female A9 size relies mainly on repressing *dpp*. Therefore, we resolved to study *dpp* regulation in more detail. Since Abd-BR, DsxF, Exd and Hth regulate *dpp* and may be part of the same regulatory complex, they may act on a common *dpp* enhancer. A 10kb fragment (BS3.0), located 3′ to the *dpp* transcription unit (Fig. 7A), when fused to the *lacZ* reporter gene, reproduces *dpp* expression in the genital discs (Blackman et al., 1991). A 2.8kb fragment (G132) within BS3.0

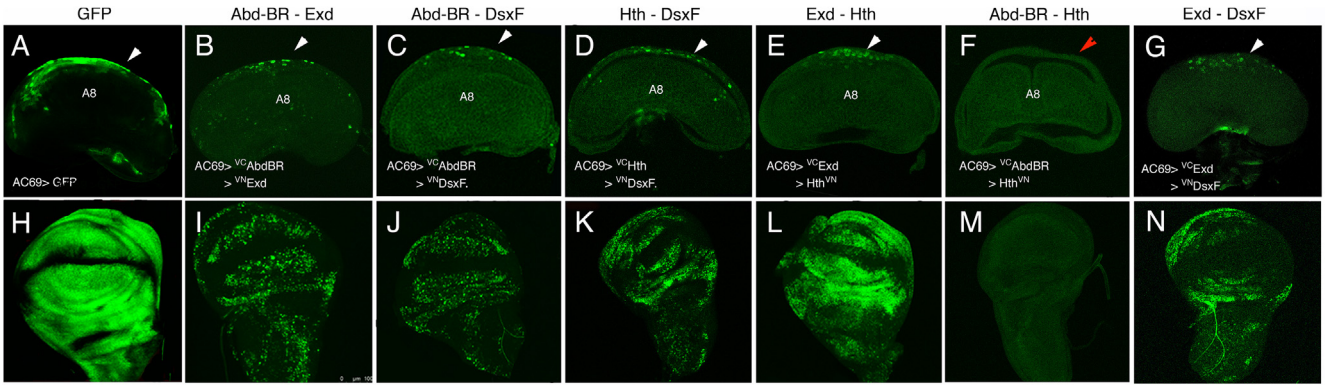

Fig. 6. Protein interactions between *Abd-B*, Exd, Hth and DsxF. (A, H) *AC69*-Gal4 UAS-GFP female genital (A) and wing (H) discs, showing GFP expression in the A9 (arrowhead) and in most of the wing disc except for the dorsal-ventral boundary region. (B-G, I-N) Bifluorescence complementation in the A9 of female genital discs (B-G) and in the wing pouch (I-N) of different proteins bearing N-terminal (VN) or C-terminal (VC) parts of Venus in the N-terminal or C-terminal regions of either Abd-BR, Exd, Hth or DsxF. The different combinations of the chimeric proteins are: Abd-BR-Exd (B, I), Abd-BR-DsxF (C, J), Hth–DsxF (D, K) Exd-Hth (E, L), Abd-BR-Hth (F, M) and Exd-DsxF (G, N). See signal in the female A9 (white arrowheads in B-E, G) and a much better expression in the wing disc than in the genital disc in the different combinations except Abd-BR-Hth (F, M, red arrowhead in F).

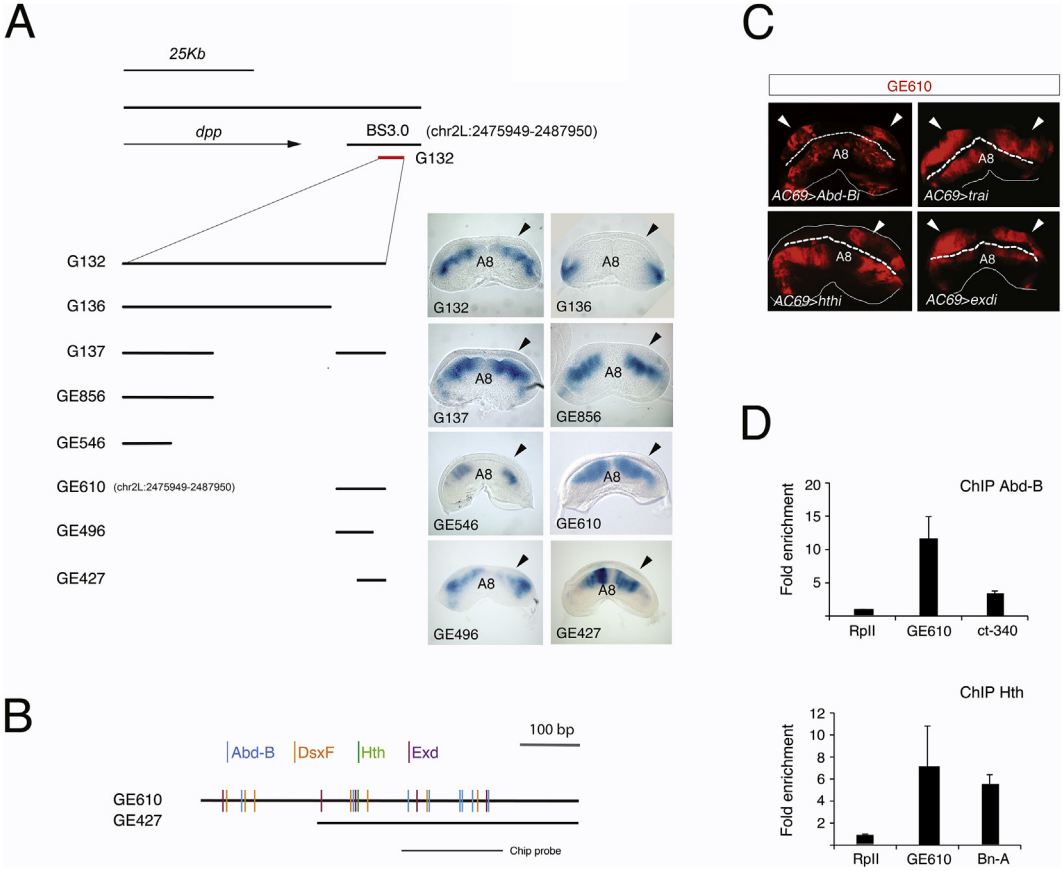

Fig. 7. Dissection of the *dpp* regulatory region. (A) Scheme of the different constructs in which the BS3.0 fragment of the regulatory region of *dpp* was subdivided and the *lacZ* expression driven by each of them in the female genital disc (to the right). The G132 2.8Kb fragment (Theisen et al., 2007) reproduces the pattern of expression of *dpp* in the A8, and the absence of signal in the A9 (arrowhead). Two fragments that subdivide G132, GE856 and GE610, of 856 and 610bp, respectively, show a similar pattern. Two fragments in which GE610 was subdivided, GE496 and GE427, of 496 and 427bp, respectively, show also no expression in the A9 (arrowheads) but the pattern of GE427 reproduces more precisely than that of GE496 the A8 *dpp* expression. (B) Putative binding sites for DsxF, Abd-B, Hth and Exd in the GE610 fragment, indicated by vertical bars of different colors. Below, the ChIP probe used. (C) The GE610 fragment responds like the G132 one to the reduction of expression of either *Abd-B* (*AC69*-Gal4 UAS-*Abd-BRNAi*), *tra* (*AC69*-Gal4 UAS-*traRNAi*), *hth* (*AC69*-Gal4 UAS-*hthRNAi*), or *exd* (*AC69*-Gal4 UAS-*exdRNAi*) (the arrowheads mark the ectopic expression of the *lacZ* gene.) (D) Chromatin Immunoprecipitation (ChIP) in the disc-derived cell-line DmD-8 with anti-Abd-B and anti-Hth antibodies. There is an enrichment of Abd-B and Hth binding as compared to the RpII controls. The previously described bindings of Abd-B to a region of the *cut* gene (Zhai et al., 2011) and of Hth to the Bn-A region (Peng et al., 2009) were used as positive controls.

directs *lacZ* expression in leg discs in a pattern similar to that of *dpp* (Theisen et al., 2007). We decided then to concentrate on this fragment. The G132 construct reproduces the *dpp* expression pattern in the female A8 and the absence of *dpp* in the A9 (Fig. 7A). Two fragments that subdivide G132, GE856 and GE610, of 856bp and 610bp, respectively, also reproduce the *dpp* expression pattern in the female genital disc (Fig. 7A). The GE856 fragment is not robustly derepressed when we downregulate *Abd-B*, *tra* or *hth* (Figs. S3A–C). By contrast, the GE610 fragment is consistently expressed in the A9 in the same mutant backgrounds (Fig. 7C), indicating it contains the regulatory sequences required for both activation and repression in A9. By using the Jaspar database we have identified in GE610 eight putative binding sites for Abd-B, five for Exd and one for Hth (Fig. 7B; Fig. S4). Regarding Dsx, a 13bp consensus sequence has been described (Luo et al., 2011). Although the 13bp optimal DSX-binding sequence is frequently associated with direct DSX targets, mismatches are allowed in the sequences through which DSX functions in vivo (Luo et al., 2011; Clough et al., 2014), and functional enhancers that match up to only 7 of the 13bp have been described (Burtis et al., 1991; Luo et al., 2011). We have found within GE610 seven putative DSX-binding sites that match 9 to 11 of the 13bp (Fig. 7B; Fig. S4).

To gain further insight into *dpp* regulation we asked whether the AbdB-R, Hth or DsxF proteins bind in vivo to the GE610 fragment. Clough et al. (2014) studied Dsx genomic occupancy to identify Dsx targets and inspection of their data reveals the Dsx protein binds to two close sites within the GE610 fragment. We decided then to know if Abd-BR or Hth, for which appropriate controls have been published (Peng et al., 2009; Zhai et al., 2011), are also able to bind the GE610 region by performing Chromatin Immunoprecipitation (ChIP). Given the small size and difficult isolation of the female A9, we decided to use the disc-derived cell line DmD-8 because this line expresses the highest levels of Hth (modENCODE data; www.modencode.org). ChIP experiments using anti-Hth antibody on this cell line show an enrichment of Hth binding to the *dpp*-GE610 region (see probe in Fig. 7B) as well as to the previously described Bn-A region, used as positive control (Peng et al., 2009), but no enrichment for an exonic region of the *RpII* gene (Fig. 7D). We also asked if Abd-BR could also bind to this region. Since *Abd-B* is not expressed in this cell line we transfected the Abd-BR protein fused to V5 epitope (Abd-BR-V5) in the DmD8 cell line and used an anti-V5 antibody to make the ChIP. We found that Abd-B also binds to the *dpp*-GE610 region. An enrichment of a region of the *cut* gene (*ct*-340), previously described (Zhai et al., 2011), was confirmed and used as positive control, whereas no enrichment was found for the *RpII* region (negative control) (Fig. 7D). These results indicate that Hth and Abd-BR, as well as DsxF (Clough et al., 2014), are able to bind the GE610 region.

We further subdivided GE610 into different fragments, fused them to *lacZ* and checked their expression in the genital disc. The constructs do not give signal or do not reproduce the endogenous *dpp* pattern with the exception of the 427bp GE427 fragment and, less precisely, GE496 (Fig. 7A; Fig. S5). Comparison to GE610 shows that GE427 loses 1 Exd, 1 Abd-B and 3 Dsx putative binding sites, but conserves the Hth site and several ones for the three other proteins (Fig. 7B). We found that GE427 is expressed in the A9 in the absence of Abd-B, Hth or Tra (Figs. S3D–F), indicating it accommodates sequences used by these proteins for repression.

Overall, the derepression of *dpp*-GE610 and *dpp*-GE427 when *AbdB*, *tra* or *hth* are downregulated, the pairwise BiFC data, the identification of putative binding sites for AbdB-R, Exd, Hth or DsxF in GE610, and the chromatin immunoprecipitation data suggest that the activity of these proteins occurs by integration at the level of *dpp* cis-regulatory region. However, although the lack of any of these proteins similarly increases size, the absence of each of them results in particular tissue specification: the reduction of *Abd-B* in the female A9 transforms it into leg tissue (Fig. 8A, A'), the down-regulation of *tra* (or *tra-2*) into male genitalia (Epper and Nöthiger, 1982; Wieschaus and Nöthiger, 1982; Belote and Baker, 1982, Fig. 8B), and that of *hth* duplicates an oviduct (Fig. 8C). This suggests that Dsx, Abd-B and Hth coordinately regulate growth, but each of them suppresses particular developmental pathways.

4. Discussion

We have centered our analysis of growth control on the A9 segment of the Drosophila female genital disc. This primordium has peculiar characteristics that make its study interesting compared with the wing disc (the main organ to study growth): it is sexually dimorphic (much smaller in females than males) and it does not express *dpp*, a main regulator of growth in the wing pouch. The main results obtained is that there is a coordinate action of sex determination, Hox and TALE proteins on size control and *dpp* repression, possibly occurring though the assembly of one or multiple protein complexes whose transcription repressive activity converges on a short *dpp* enhancer. In what follows, we highlight the main characteristics of size control in the female genitalia and discuss how they compare with that of other organs.

4.1. A combination of exd, hth and isoforms of Abd-B and DsxF regulates the activity of the dpp pathway to control growth in the female A9

We have shown that the absence of *Abd-B*, DsxF, *hth* or *exd* results in overgrowth in the female A9. These factors provide multiple cues that collectively limit growth. BiFC experiments suggest that axial and sex

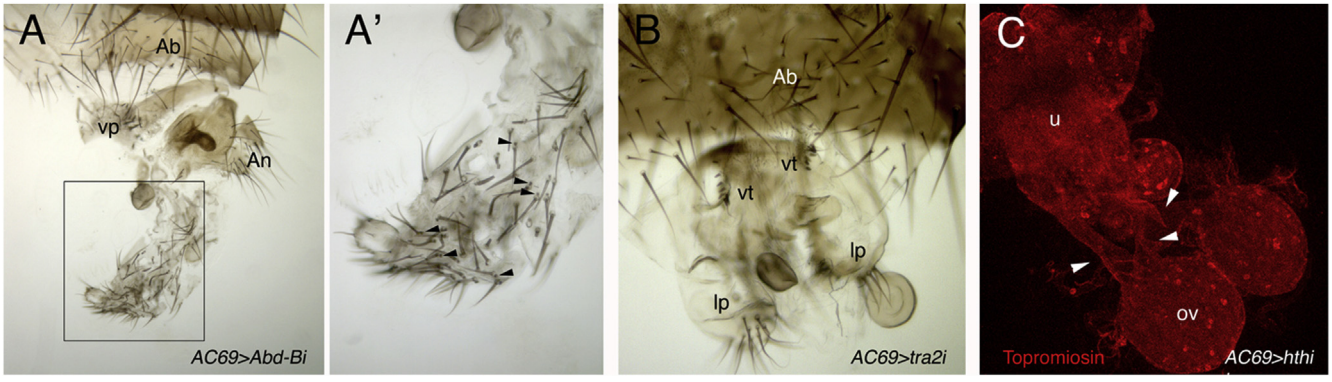

Fig. 8. Reduction of the expression of *Abd-B*, *tra-2*, or *hth* in the female A9 results in different transformations. (A, A′) Posterior part of an *AC69*-Gal4 UAS-*Abd-BRNAi* female, showing transformation of part of the genitalia into a leg. A′ is a magnification of the square in A. The arrows in A′ mark the bracteae adjacent to the bristles, revealing transformation into leg tissue. (B) If *tra-2* expression is reduced, the segment is transformed into male genitalia. Since the female A8 segment is not transformed, and this gives rise to the external female genitalia, elements of both male (lp, lateral plate, or epandrial ventral lobe) and female (vt, vaginal teeth) genitalia are observed. (C) If there is down-regulation of *hth* in the same segment, the oviducts seem duplicated (arrows); u, uterus; ov, ovaries.

determination information, provided by Abd-BR and DsxF, respectively, together with Hth/Exd activity, may cooperate in this growth restriction. A crucial element in this control is the repression of *dpp* expression. We have identified a 610bp fragment within the *dpp* regulatory region that reproduces *dpp* expression in the female A8, its absence in the A9, and is ectopically expressed in this segment in the mutant conditions. Abd-B and Hth bind to this region and DsxF has also been reported to do so (Clough et al., 2014). Our results, therefore, indicate that the sex determination pathway and the Hox gene *Abd-B* and its cofactors regulate the same enhancer, and since the absence of one of these elements is sufficient to cause ectopic *dpp* expression and overgrowth, this suggests that they do not repress *dpp* independent- and redundantly. We conclude then that the architecture of the *dpp* cis-regulatory region for genitalia expression is compatible with an AbdB-R-Exd-Hth-Dsx complex acting as a repressor of *dpp* in the female A9. The confirmation of the existence of such a complex, however, demands further experiments.

4.2. Similarities with size control in other organs

Size control by regulating expression of *dpp* has been reported for another Hox gene, *Ultrabithorax* (*Ubx*). *Ubx* reduces haltere size, as compared to the homologous structure, the wing, by down-regulating *dpp* expression and spread (Crickmore and Mann, 2006, 2007; de Navas et al., 2006a; Makhijani et al., 2007). The regulation of *dpp* expression and activity seems, therefore, to be a common feature of the regulation of size by the Ubx and Abd-B proteins in particular regions of the haltere and female genital disc.

Previous work demonstrated that a combination of sex determination and Hox proteins is required for the development of Drosophila sexually dimorphic characteristics such as pigmentation in the posterior abdomen (Kopp et al., 2000; Williams et al., 2008), suppression of the male A7 segment (Wang et al., 2011; Foronda et al., 2012, 2015; Wang and Yoder, 2012) or development of the sex comb (Tanaka et al., 2011). In the genital disc it was suggested that the expression of *dpp* and *wingless* in the A8 and A9 primordia of male and female genital discs, and subsequent growth, depends on a combination of Dsx and Abd-B isoforms (Sánchez and Guerrero, 2001; Sánchez et al., 2001). A combination of axial and sex specific information thus appears as a common theme in the control of pattern and size. However, contrary to what happens in sex comb or abdomen, the coordinate activity of Hox and sex-determination genes in the female A9 regulates size and not only differentiation.

4.3. Features specific to size control in the female genitalia

Our results also show differences with respect to a previous model that suggested that Abd-BR and DsxF would repress *dpp* in the A9 (and therefore growth) whereas Abd-BM and DsxF would activate *dpp* in the A8 (Sánchez and Guerrero, 2001; Sánchez et al., 2001). We have previously shown that *Abd-B* is not needed to maintain *dpp* expression in the A8 (Estrada and Sánchez-Herrero, 2001). We further show here that the forced expression of Abd-BR in the A8, where *dpp* is normally expressed, is not sufficient to repress it. Rather, absence of the Abd-BM isoform and high levels of Hth seem to be also required for such repression in most of the mutant clones. However, this is different in the A9, where the presence of Abd-BM does not fully prevent *dpp* repression. In addition, we have shown that DsxF represses *dpp* expression, but only in the A9, suggesting this Dsx isoform requires a specific combination with Abd-B and Hth proteins for repression of *dpp*.

It has recently been reported that differences in size between males and females depends on the expression of *Sex lethal* (*Sxl*), the gene at the top of the sex determination cascade (reviewed in Sánchez, 2008), in the central nervous system. If *Sxl* is active on specific neurons, as it occurs in females, the size of the adult structures is bigger in females. The size of the corresponding imaginal discs, however, seems to depend also in local cues (Sawala and Gould, 2017). We have shown in the genital disc that differences in size between male and female A9 primordia depend on the segment-autonomous *dpp* expression. The size of the male and female adult A8 and A9 derivatives seems also to reflect size differences observed in the imaginal disc. Thus, the male genitalia (A9 derivative) are clearly bigger than the parovaria and part of the uterus, which come from the female A9 primordium (Keisman et al., 2001). Therefore, local, *dpp*-dependent size regulation is probably superimposed on the general central nervous system control to specify the relative sizes of the adult male and female A9 genitalia.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.06.014.

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