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Ubx dynamically regulates Dpp signaling by repressing Dad expression during copper cell regeneration in the adult *Drosophila* midgut

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Abstract

The gastrointestinal (GI) tract of metazoans is lined by a series of regionally distinct epithelia. To maintain structure and function of the GI tract, regionally diversified differentiation of somatic stem cell (SC) lineages is critical. The adult *Drosophila* midgut provides an accessible model to study SC regulation and specification in a regionally defined manner. SCs of the posterior midgut (PM) have been studied extensively, but the control of SCs in the middle midgut (MM) is less well understood. The MM contains a stomach-like copper cell region (CCR) that is regenerated by gastric stem cells (GSSCs) and contains acid-secreting copper cells (CCs). Bmp-like Decapentaplegic (Dpp) signaling determines the identity of GSSCs, and is required for CC regeneration, yet the precise control of Dpp signaling activity in this lineage remains to be fully established. Here, we show that *Dad*, a negative feedback regulator of Dpp signaling, is dynamically regulated in the GSSC lineage to allow CC differentiation. *Dad* is highly expressed in GSSCs and their first daughter cells, the gastroblasts (GBs), but has to be repressed in differentiating CCs to allow Dpp-mediated differentiation into CCs. We find that the Hox gene *ultrabithorax* (*Ubx*) is required for this regulation. Loss of Ubx prevents *Dad* repression in the CCR, resulting in defective CC regeneration. Our study highlights the need for dynamic control of Dpp signaling activity in the differentiation of the GSSC lineage and identifies Ubx as a critical regulator of this process.

Keywords

Drosophila; Intestinal stem cell; Regeneration; Ubx

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Author contributions

H.L. and H.J. designed and conceived the study, H.L. and Y. Q. performed all experiments, H.J. and H.L. analyzed data and wrote the manuscript.

Author information

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1. Introduction

Stem cell (SC) proliferation, differentiation, and maintenance have to be precisely controlled to maintain long-term tissue homeostasis. This is particularly relevant in barrier epithelia, including the intestine, stomach, and skin, that are continuously exposed to environmental challenges (Barker et al., 2010). In the gastrointestinal (GI) tract, intestinal stem cell (ISC) populations not only have to ensure accurate regenerative responses to tissue damage, but have to also maintain the diversity of the regionally defined epithelia with distinct function and morphology (such as the esophagus, stomach, and intestine, Barker et al., 2010; Buchon et al., 2013b; Li et al., 2016; Marianes and Spradling, 2013; Tasnim et al., 2016).

The adult *Drosophila* midgut has emerged as an important model to study somatic stem cell biology (Biteau et al., 2011; Buchon et al., 2013a; Buchon and Osman, 2015; Jiang and Edgar, 2011; Lemaitre and Miguel-Aliaga, 2013; Xu et al., 2016). ISCs can be found in all three regions of the midgut: anterior midgut (AM), middle midgut (MM), and posterior midgut (PM), and the SC lineages of the PM and MM regions have been characterized in detail (Biteau et al., 2011; Hou, 2010; Strand and Micchelli, 2011). Detailed molecular characterization of stem cells in 10–14 subdivided regions of the gut has further highlighted the diverse nature of the GI stem cell population, although mechanisms that maintain this diversity remain largely unexplored (Buchon et al., 2013b; Dutta et al., 2015; Marianes and Spradling, 2013).

ISCs in the PM are characterized by the expression of *escargot, esg,* and *Delta, Dl*. During regenerative episodes, these cells undergo asymmetric divisions to give rise to a new ISC and a precursor cell, an enteroblast (EB, esg+/Dl−), which can further differentiate into either an enterocyte (EC, pdm1+) or an enteroendocrine cell (EE, prospero+) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007). The MM contains a stomach-like copper cell region (CCR, (Dubreuil, 2004)), which is regenerated by gastric stem cells (GSSC). GSSCs, which also express esg, generate three differentiated cell types: acid-producing copper cells (CCs, Cut+/Labial+), interstitial cells (ISs, Cut−/weak Labial+), and enteroendocrine cells (EEs, prospero+) (Fig. 1A, Strand and Micchelli, 2011). GSSCs are mostly quiescent under homeostatic conditions, but can be stimulated to proliferate by stress (such as heat-shock). This activation of GSSCs seems to be mediated primarily by epidermal growth factor (EGF) signaling (Strand and Micchelli, 2011, 2013). Recent studies have refined our understanding of ISC lineage and suggest that two types of differentiated cells (ECs and EEs) are generated from pre-committed ISCs, and not from a common enteroblasts (EBs) (Beehler-Evans and Micchelli, 2015; Biteau and Jasper, 2014; Guo and Ohlstein, 2015; Wang et al., 2015; Zeng and Hou, 2015). These studies have focused on the stem cell lineage in the PM, and there is no published evidence for or against this model in the middle midgut yet. Based on the similarities of these lineages, it can be speculated that the same model applies in this region (Fig. 1A, Li and Jasper, 2016).

To date, numerous signaling pathways have been reported to regulate ISC function in the PM, and recent studies have begun to explore in detail how the integration of these pathways influences proliferation and differentiation of ISCs (Biteau et al., 2011; Buchon et al., 2013a; Buchon and Osman, 2015; Deng et al., 2015; Guo and Ohlstein, 2015; Jiang and

Edgar, 2011; Lemaitre and Miguel-Aliaga, 2013; Meng and Biteau, 2015). The regulation of GSSC proliferation and differentiation in the CCR, in turn, is still relatively poorly understood. Studies from others and us have recently shown that signaling by Decapentaplegic (Dpp) is required for CC regeneration in the adult CCR (Guo et al., 2013; Li et al., 2013a), while Dl/Notch signaling between GSSCs and gastroblasts (GBs) helps determine specification of GSSC daughter cells (Wang et al., 2014), similar to the regulation of ISC differentiation in the PM (Ohlstein and Spradling, 2007).

Dpp is a homologue of bone morphogenetic protein (BMP), and controls a number of vital events during development (Peterson and O'Connor, 2014). Canonically, Dpp signals through the BMP Type I receptor Thickveins (Tkv), the Type II receptor Punt, and the Smad transcription factors Mothers against dpp (Mad) and Medea, activating a wide range of target genes in a context and concentration dependent manner (Wartlick et al., 2011). One general transcriptional target is *Daughters against dpp* (*Dad*), which encodes an inhibitory Smad and creates a negative-feedback loop for Dpp signaling by preventing phosphorylation of Mad (Inoue et al., 1998; Tsuneizumi et al., 1997).

Several recent studies have revealed important roles of Dpp signaling in regulating ISC function in the PM (Ayyaz et al., 2015; Guo et al., 2013; Li et al., 2013b; Tian and Jiang, 2014; Zhou et al., 2015). In this study, we have characterized the regulation of Dpp signaling during CC differentiation in the MM in more detail. We find that *Dad* is highly expressed in GSSCs/GBs, but repressed in differentiated CCs, suggesting that Dpp signaling activity is dynamically regulated during CC regeneration. Accordingly, we find that the level of Mad phosphorylation (pMad) is significantly higher in CCs than in GSSCs/GBs, and that inhibition of *Dad* in CCs is required to maintain Dpp/Mad signaling activity during CC differentiation. Using a candidate RNAi screen, we identify the homeobox (hox) gene *Ultrabithorax* (*Ubx)* as a critical inhibitor of *Dad* expression in this context. Ubx is expressed in CCs, and is required to repress *Dad* expression to allow CC regeneration. Our study thus defines a new role for Ubx in regulating Dpp/Mad/Dad signaling during regeneration of the gastric region of the *Drosophila* midgut.

2. Results

2.1. Dad expression and Dpp signaling activity in the CCR

We have previously characterized the role of Dpp signaling in regeneration of the *Drosophila* CCR (Li et al., 2013a). In the course of this study, we also observed that the Dpp activity reporter *Dad:: nlsGFP* (Hamaratoglu et al., 2011) is differentially expressed in different cell types of the CCR, suggesting dynamic regulation of Dpp activity in this region. To characterize Dpp activity in more detail, we compared the expression of *Dad* using *Dad::nlsGFP* (Hamaratoglu et al., 2011) and the levels of Mad phosphorylation (using immunohistochemistry against pMad), in different cell types of the CCR. We found that *Dad::nlsGFP* was expressed in small diploid cells in the CCR, but not in polyploid Cut+ CCs (Fig. 1B). These small diploid cells also express esg (as determined using esg::Gal4, UAS:mcherry; Fig. 1C), which is a marker for gastric stem cells (GSSCs) and progenitor gastroblasts (GBs).

Using lineage tracing in the CCR, Strand and Micchelli (Strand and Micchelli, 2011) have proposed that GBs can generate three differentiated cell types: CCs, interstitial cells, and enteroendocrine cells (Fig. 1A). A recent study has further reported that GSSCs express Delta and activate Notch signaling in GBs (Wang et al., 2014). Consistent with these observations, we found that one of the two neighbor cells expressing *Dad::nlsGFP*+ in the CCR also expresses *Su(H)-GBE::lacZ* (Fig. 1D), a Notch signaling reporter and marker of EBs (the GB counterpart) in the PM (Ohlstein and Spradling, 2007). These data suggest that *Dad::nlsGFP*-expressing diploid cells are GSSCs and GBs (Fig. 1D). All cell types exhibited pMad immunoreactivity, but quantification indicated that pMad levels are significantly higher in the polyploid cells (including CCs) than that in GSSCs/GBs (Figs. 1D and 1E).

Taken together, our data suggest a dynamic regulation of *Dad* expression and, consequently, of Dpp signaling activity (pMad) in the GSSC lineage, with low signaling activity in progenitor cells and activation of Dpp signaling which correlates with reduced *Dad* expression in differentiating cells.

2.2. Dynamic regulation of Dad is required for CC differentiation

To test whether this dynamic regulation of Dpp activity is required for CC regeneration, we used UAS::Dad to constitutively maintain *Dad* expression in all cells of the GSSC lineage. We used the esg^{ts}F/O system, in which GFP-marked clones are generated from single esg+ ISCs when flies are transferred to the restrictive temperature (29 °C). Lineage tracing is achieved by expressing act::Gal4 after Flp-mediated excision of a transcriptional STOP cassette (Jiang et al., 2009). Because of the intrinsic quiescence of the GSSC, double heat-shock at 37 °C was performed to induce enough clones for analysis (Strand and Micchelli, 2011, Fig. 2A). We confirmed that continuous expression of *Dad* inhibits Dpp signaling activity (pMad staining) in clones observed in the PM (Fig. S1). Phosphorylation of Mad was also prevented in GSSC lineages with *Dad* overexpression, which, consistent with our hypothesis, resulted in defective CC regeneration (Fig. 2B). Clone sizes of UAS::Dad over-expressing GSSC clones did not differ from wild-type clones, supporting the notion that Dpp signaling does not influence GSSC proliferation (as shown before, Li et al., 2013a). Consistent with our previous finding that Labial is induced in differentiating CCs downstream of Dpp signaling (Li et al., 2013a, 2016), GSSC clones expressing UAS::Dad were also devoid of Labial-expressing cells (Fig. 2C).

While sustained inhibition of Dpp signaling thus impairs CC differentiation, our data also suggest that Dpp is maintained low in normal progenitor cells by high expression of *Dad*. To test the significance of this repression, we asked whether sustained activation of Dpp signaling also affects CC regeneration. We generated esg^{ts}F/O clones expressing a constitutively active form of the Dpp Type I receptor Tkv (*Tkv^{QD}*), and made clones from stem cells homozygous for the *Dad* loss of function allele *Dad^{J12}* (Ogiso et al., 2011). Dad mutant clones were generated using Mosaic Analysis with a Repressible Cell Marker (MARCM), a lineage tracing method that uses somatic recombination to generate GFP-marked cell clones derived from homozygous mutant cells (Lee and Luo, 2001). Both conditions resulted in higher pMad staining in the clone, confirming high Dpp signaling activity (Figs. 3A, B, and S2A). Clones from both conditions showed a defect in CC

regeneration (Figs. 3C, D and S2B), further supporting the notion that dynamic regulation of Dpp signaling activity in GSSC lineages is required for CC differentiation.

2.3. Ubx represses Dad expression in the CCR

Our data indicated that during differentiation from GBs to CCs, Dad expression is repressed, allowing for activation of Dpp signaling activity. To identify factor(s) involved in repressing *Dad* expression in differentiated CCs, we performed a limited RNAi screen, knocking down a selected set of genes in ECs and CCs using NP1::Gal4^{ts}, and monitoring *Dad::nlsGFP* expression. Tested candidates include the hox genes *scr*, *Antp*, and *Ultrabithorax* (*Ubx)*, and other genes (such as *wg*, *dve*, and *tsh*) that have reported roles in midgut development (Nakagoshi, 2005). Knockdown of Ubx (using two independent Ubx^{RNAi} lines) resulted in ectopic expression of *Dad::nlsGFP* in most cells of the CCR (Fig. 4A), suggesting that Ubx plays a critical role in repressing *Dad* in CCs. The repression of *Dad* expression by Ubx seems to be specific for the CCR, as loss of Ubx in the PM does not alter the pattern of *Dad:: nlsGFP* expression (Figs. 4A and 4B; note that *Dad::nlsGFP* expression is inducible in all cells of the midgut, as overexpression of Dpp can strongly induce *Dad::nlsGFP* both in CCs of the CCR and in ECs of the PM). Ubx thus plays a regionally restricted role in the repression of *Dad* expression in the CC lineage.

Ubx is a member of the *Drosophila* Hox gene family, which encodes transcription factors determining segment identity along the anterior-posterior (A/P) axis. In *Drosophila*, the two types of flight appendages, wings and halteres, develop from the second (T2) and third (T3) thoracic segments, respectively. Ubx is expressed in the haltere disc but not in the wing disc, and determines haltere identity. Accordingly, loss of Ubx results in transformation of the halteres into wings, while ectopic expression of Ubx transforms wings into halteres (Lewis, 1978; White and Wilcox, 1985). During *Drosophila* midgut development, the hox genes *Ubx* and *abd-A* regulate Dpp and Wg in Parasegment (PS) 7 and PS8, respectively, to specify the subdivision of the middle midgut (Nakagoshi, 2005). Whether Ubx continues to play a role in the adult gut remains unknown.

To assess the regulation of Ubx in the GSSC lineage, we examined the expression of Ubx in the GI tract using immunohistochemistry. Along the GI tract, we found that Ubx is expressed with regional specificity in different areas, including in tracheal cells surrounding the AM, CCR and PM, in visceral muscle cells predominantly in the AM, and in most Labial + epithelial cells in the CCR (Fig. 4C, S3B). We found that the expression level of Ubx in CCR epithelial cells is lower than in tracheal or muscle cells, yet that it can be clearly distinguished from AM and PM epithelial cells, where no expression was seen (data not shown). The efficiency of Ubx^{RNAi} to knock down Ubx expression was confirmed by antibody staining against Ubx (Fig. S3C), and the Ubx antibody used was validated by over-expressing Ubx (UAS::Ubx, Castelli-Gair et al., 1994) in the PM using the temperature sensitive ISC/EB-specific driver esg: Gal4^{ts} (Fig. S3A).

2.4. Ubx is required for CC differentiation by repressing Dad

Consistent with its regulation of *Dad*, and with the effects of *Dad* over-expression, knockdown of Ubx resulted in loss of Cut+ and Labial+ CCs (Fig. 5A). CCR MARCM

clones carrying the *Ubx*^{1} loss of function allele (Bender et al., 1983) also lack Cut+ CCs (Fig. 5B), further supporting the notion that Ubx is required for CC differentiation. To confirm that Ubx regulates CC regeneration by inhibiting *Dad* expression, we assessed whether knockdown of *Dad* can rescue CC differentiation in Ubx loss of function conditions, and found that double knockdown of *Ubx* and *Dad* (Ubx^{RNAi}, Dad^{RNAi}) resulted in normal Cut+ and Labial+ CCs (Fig. 5C).

We have previously shown that Labial is induced downstream of Dpp and is required for CC regeneration (Li et al., 2013a). To test whether Ubx may inhibit *Dad* expression by regulating Labial, we knocked down Labial in CCs using $NP1^{ts}$, and found that loss of Labial did prevent the formation of Cut+ CCs as expected, but did not affect *Dad::nlsGFP* expression (Fig. 5D). Taken together, our data suggest a model where Ubx-mediated repression of *Dad*, and thus activation of Dpp signaling (pMad) in differentiating CCs, is essential for the formation of Cut+ and Labial+ CCs, with Labial acting downstream of Mad activity to promote CC differentiation (Fig. 6).

3. Discussion

The regeneration of high-turnover epithelia needs to be precisely controlled to maintain regional identity and prevent metaplasias, diseases in which epithelial identity is perturbed. In the airway epithelium, it has been suggested that squamous metaplasia is caused by the mis-differentiation of basal stem cells (Hogan et al., 2014). In Barrett's metaplasia, the normally squamous epithelium of the esophagus is replaced by a columnar epithelium that resembles epithelia lining the stomach or intestine. Although the cellular origin of these metaplasias has not been conclusively determined, one proposed model is that metaplasia is due to the reprogramming of progenitors or stem cells (Lefort and Dotto, 2011; Li et al., 2016). Our study highlights the role of regionally expressed Hox transcription factors in maintaining regional identity during regenerative episodes.

Our previous work shows that differentiation in the GSSC lineage is controlled by Dpp signaling (Li et al., 2013a). Ectopic over-expression of Dpp in the adult GI tract leads to mis-differentiation of stem cells in the AM, resulting in Barrett's metaplasia-like phenotypes (Li et al., 2013a). Interestingly, this metaplasia is only observed when Dpp is over-expressed using NP1::Gal4, a strong EC driver (Li et al., 2013a), and not when it is expressed using a visceral muscle driver (Driver and Ohlstein, 2014), indicating that the source and/or strength of the Dpp signal determines the response of AM cells. Our findings here highlight the need for dynamic regulation of Dpp/Mad/Dad signaling in the differentiation of GSSC daughter cells, contributing to our understanding of stem cell differentiation in the maintenance of tissue homeostasis. In aging flies, chronic activation of JAK/Stat signaling in the CCR results in repression of Dpp signaling activity and of Dve/Labial expression, causing gastric metaplasia, characterized by transdifferentiation of Cut+ CCs into Pdm1+ ECs and loss of acid secretion into the lumen. This in turn results in microbiota dysbiosis and shorter lifespan (Li et al., 2016). Whether changes in Ubx function play a role in this age-related mis-regulation of the CCR remains unknown, and will be an interesting question to pursue in future studies.

Ubx has been shown, in the developing haltere disc, to regulate Dpp signaling at different levels, including the Dpp ligand, receptor, and target genes (Crickmore and Mann, 2006; de Navas et al., 2006; Weatherbee et al., 1998). One study in the haltere disc shows that Ubx collaborates with Smads to inhibit the Dpp target, *Spalt* (Walsh and Carroll, 2007), and genome-wide studies in the haltere disc, wing disc and/or whole embryo, reveal that Ubx, with its cofactor homothorax (Hth), regulates different groups of genes in a tissue-and stage-specific manner (Agrawal et al., 2011; Choo et al., 2011; Pavlopoulos and Akam, 2011; Slattery et al., 2011). However, the interaction between Ubx and Dad was unknown, and it was unclear whether Ubx continues to play a critical role in maintaining identity of cells in adult tissues. Our results provide genetic evidence that Ubx inhibits *Dad* expression to control Dpp/Mad signaling activity, and that this regulation is required for CC regeneration in the adult GI tract. How Ubx regulates *Dad* expression, and whether this process involves other cofactors remains unclear, and is a question that will be of interest for further study.

The role of Hox genes in controlling epithelial compartment identity in the adult GI tract may be conserved in mammals. During embryonic development, expression of the Hox genes Cdx1 and Cdx2 is restricted to prospective intestinal regions, but excluded from prospective stomach regions (Correa, 1992). This expression pattern is maintained in the adult GI tract and required for the maintenance of GI compartmentalization: forced expression of Cdx2 using a stomach-specific promoter in mice is sufficient to generate intestinal tissues in the stomach (Beck et al., 1999; Mutoh et al., 2002; Silberg et al., 2002). An interaction between Bmp signaling and CDX2 has been implicated in Helicobacter pylori – induced gastric metaplasia (Camilo et al., 2012). We thus anticipate that further characterization of the role of these interactions in maintaining regenerative fidelity of gastric epithelia, as well as of the potential role of other Hox transcription factors in maintaining epithelial compartmentalization and identity in other regions of the GI tract, will be of interest to explore causes and consequences of clinically relevant metaplasias.

4. Materials and methods

4.1. Fly lines and husbandry

Fly lines w^{1118}, FRT82, $Labial^{RNAi}$ (BL26753), UAS-mcherry (BL38245), UAS-Tkv^{QD} (BL36536), UAS-Dpp (BL1486), UAS-Ubx (BL911), Ubx^{1} (BL529), Ubx^{RNAi} line 1 (BL31913), Ubx^{RNAi} line 2 (BL34993), Dad^{RNAi} (BL33759) were obtained from Bloomington *Drosophila* Stock Center. esg-Gal4, UAS-GFP was a gift from Shigeo Hayashi; FRT82B, Dad^{212} from Hannele Ruohola-Baker; Btl-$Gal4^{ts}$, UAS-GFP from Dirk Bohmann; Dad::nlsGFP from Georgios Pyrowolakis; Su(H)-GBE-lacZ from Sarah Bray; esg^{ts}F/O (esgGal4, tubG80ts, UAS-GFP; UAS-flp, act > STOP > Gal4) from Huaqi Jiang; UAS::Dad from Thomas Kornberg; NP1::Gal4 from Dominique Ferrandon; MARCM82 (hsFlp; tub-Gal4, UAS-GFP; FRT82, tubGal80) from Norbert Perrimon.

Flies were cultured on yeast/molasses-based standard fly food (Recipe: 10 L H2O, 138 g agar, 220 g molasses, 750 g malt extract, 180 dry yeast, 800 g corn flour, 100 g soy flour, 62.5 ml propionic acid, 20 g Methyl 4-Hydroxybenzoate, and 72 ml ethanol) at 25 °C with a 12 h light/dark cycle. For TARGET (tubGal80ts) experiments, flies were raised at 18 °C to

allow Gal80 to inhibit Gal4, and 3–4 days after eclosion shifted to 29 °C to inhibit Gal80 and to allow Gal4 to drive UAS-linked transgene expression.

4.2. Immunostaining and microscopy

Female guts were dissected in phosphate-buffered saline (1 × PBS), fixed for 45 min at room temperature in fixative (100 mM glutamic acid, 25 mM KCl, 20 mM MgSO4, 4 mM sodium phosphate, 1 mM MgCl2, and 4% formaldehyde), washed for 1 h at 4 °C in washing buffer (1 × PBS, 0.5% bovine serum albumin and 0.1% Triton X-100), and then incubated in primary antibodies at 4 °C overnight and secondary antibodies at 4 °C for 4 h (primary and secondary antibodies were diluted in washing buffer). Staining with pMad antibody was performed using a phosphatase inhibitor (Roche PhosSTOP) during fixation and primary antibody incubation, following steps described above.

Primary antibodies and dilution: rabbit anti-pMad (pSMAD3, abcam, ab52903), 1:300; rabbit anti-β-galactosidase (Cappel), 1:5000; mouse anti-cut, anti-Ubx (Developmental Studies Hybridoma Bank), 1:100, 1:50, respectively; rabbit anti-labial (gift from Thom Kaufman), 1:200. Fluorescent secondary antibodies were from Jackson Immunoresearch. DAPI was used to stain DNA. All images were taken on a Zeiss LSM 710 confocal microscope and processed using Adobe Photoshop, Illustrator and Image J.

4.3. esg^{ts}F/O and MARCM Clone induction

Because of the intrinsic quiescence of gastric stem cells, the frequency of clone formation in the copper cell region (CCR) is very low for both MARCM system and esg^{ts}F/O system. And double heat-shock seems to increase the frequency of clone formation in the CCR (Strand and Micchelli, 2011). For MARCM system, 3 days old mated female flies were heat-shocked at 37 °C for 45 min, recovered for 2 h and then heat-shocked at 37 °C for 45 min again. Then flies were kept at 25 °C for 7 days before dissection. For the esg^{ts}F/O clone induction, 3 days old mated female flies (raised at 18 °C) were shifted to 29 °C for 2 days, double heat-shocked, and then kept at 29 °C for the time indicated before being dissected.

4.4. Generation of FRT82B, Ubx^{1}

To generate *FRT82, Ubx^{1}* fly line for MARCM clone analysis, the neomycin-resistant allele FRT82 (neo+) and loss-of-function allele U*bx^{1}* (BL529) were recombined using standard recombination protocol. In brief, the virgin female flies, FRT82(neo+)/*Ubx^{1}*, were crossed with male W^{1118} in neomycin food (1 mg/ml), and among the offspring only the ones with FRT82 (neo+) allele could survive. Then the flies with right haltere phenotype of ubx^{1} were chosen to get the stock FRT82, *Ubx^{1}*/TM3.

4.5. Statistical analysis

Statistical Analysis was performed using GraphPad Prism5 and Microsoft Excel. Statistical methods used and sample sizes are listed in Figure Legends. Sample sizes were chosen empirically based on observed effect sizes. For quantifications, averages and standard error are shown, and P values are from student *t*-test and Fisher's exact test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.08.027.

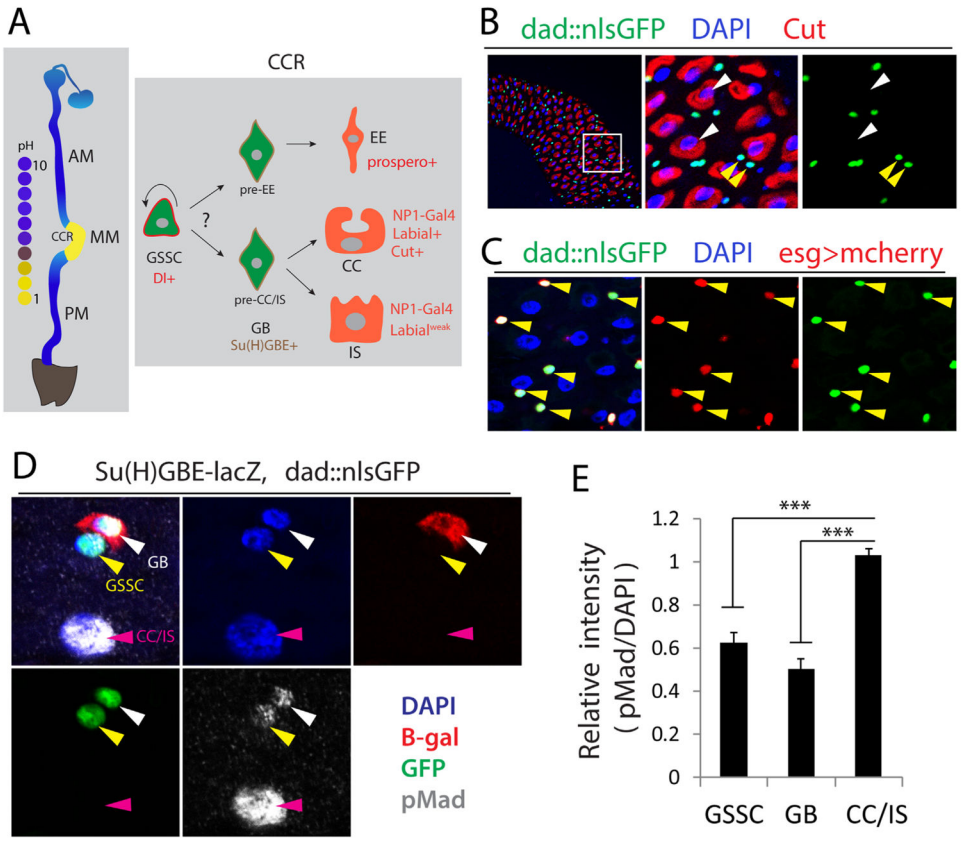

Fig. 1.

Dad expression and pMad staining in the CCR. (A) Left: Schematic of Drosophila midgut compartments with pH indicator. AM, anterior midgut; MM, middle midgut; CCR, copper cell region; PM, posterior midgut. Right: cell lineage in the CCR with markers and drivers indicated. The questionmark indicates that there is no experimental evidence about the existence of pre-committed GBs, and we speculate this model based on recent studies on stem cell lineage in the posterior midgut. GSSC, gastric stem cell; GB, gastroblast; EE, enteroendocrine cell; CC, copper cell; IS, interstitial cell. (B) Dad::nlsGFP (green) is expressed in small diploid cells (yellow arrowheads), not in Cut+ (red) CCs (white arrowheads). (C) Dad::nlsGFP (green) expressing cells are positive for esg > mcherry (red, esgGal4, uas-mcherry; esg is a marker for GSSC and GB). (D) One of the Dad::nlsGFP+ (green) doublet cells is Su(H)GBE-lacZ (red) positive in the CCR. pMad (white) antibody staining is positive for all cell types, but shows a higher level in the polyploidy CC/IS. (E) Quantification of pMad intensity relative to DAPI intensity from (D). Note that pMad intensity is significantly higher in CCs/ISs compared to GSSCs or GBs. N = 11 guts from three biological replicates (36 GSSCs, 33 GBs, 80 CCs/ISs). Averages and SEM are shown. One-way ANOVA with post-hoc *t*-test was performed,*** $p < 0.001$.

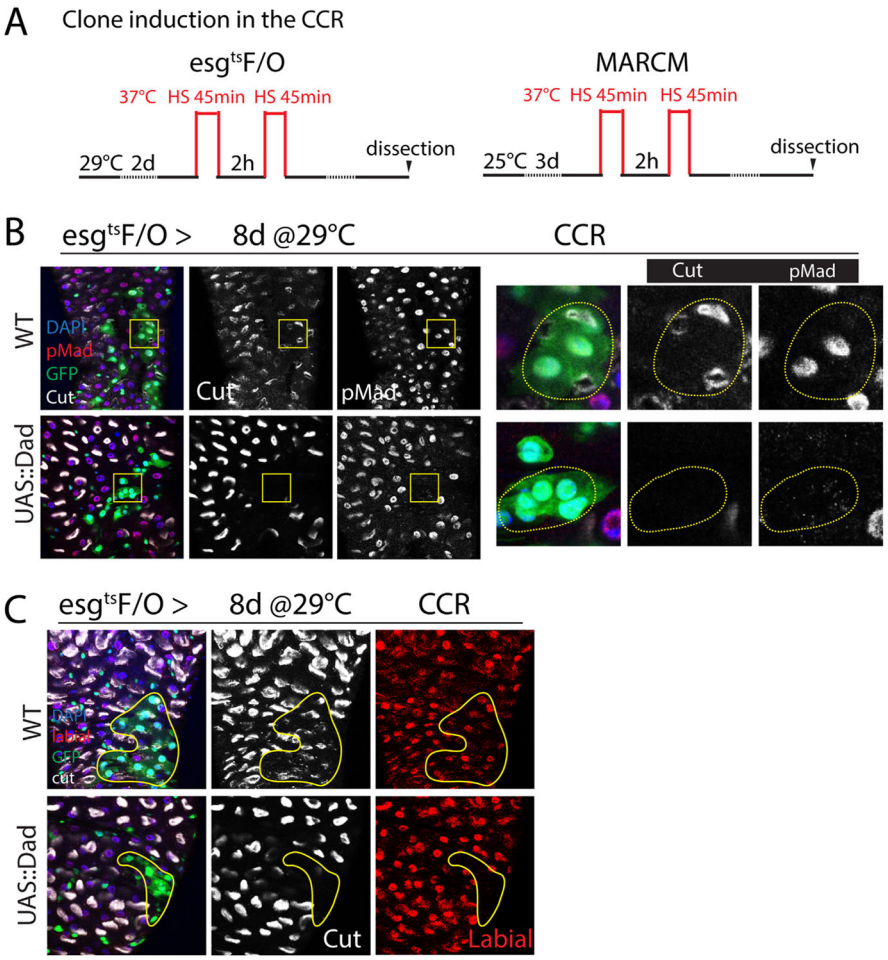

Fig. 2.

Ectopic Dad expression causes defective CC regeneration. (A) Strategies used to induce esg^{ts}F/O (flies were reared at 18 °C before eclosion) and MARCM (flies were reared at 25 °C before eclosion) clones in the CCR. HS, heat shock. (B) Overexpression of *Dad* (UAS-Dad) in esg^{ts}F/O clones blocks the phosphorylation of Mad (pMad), and causes defect of Cut+ CC formation. The right panel shows representative clones (GFP+, outlined). (C) Overexpression of *Dad* (UAS-Dad) in esg^{ts}F/O system generates clones (GFP+, outlined) devoid of Labial+ cells.

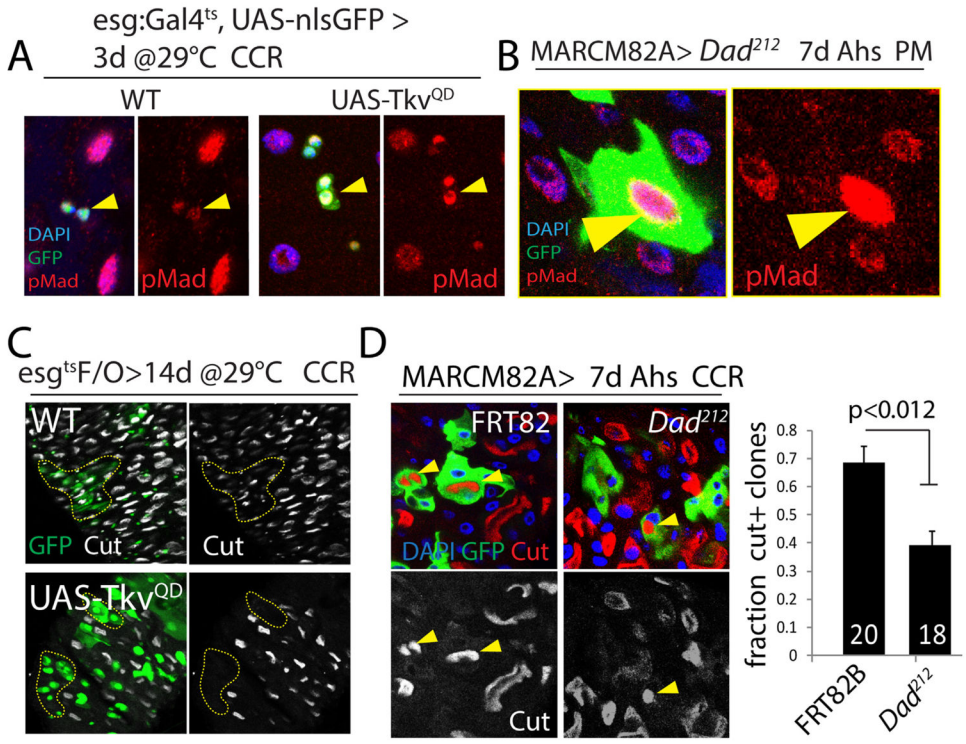

Fig. 3.

Ectopic Dpp activation causes defective CC regeneration. (A) Overexpression of *Tkv*^{QD} (UAS-Tkv^{QD}) by esg::Gal4^{ts} in the CCR induces high Dpp signaling activity, shown by pMad antibody staining, compared to the control. Arrowheads point to esg+ cells. (B) Cells in *Dad*^{212} mutant MARCM clones (arrowhead) have higher Dpp signaling activity, shown by pMad antibody staining, than surrounding non-GFP control cells. (C) Overexpression of the active form of Dpp signaling receptor *Tkv* (UAS::Tkv^{QD}) in esg^{ts}F/O clones (outlined) leads to defect of Cut+ CC formation. (D) *Dad*^{212} mutant has a significantly lower fraction of Cut+ (yellow arrowheads) clones compared to the control in the MARCM system. The right graph is the quantification. WT N = 83 clones from 20 guts, *Dad*^{212} N = 75 clones from 18 guts. Ahs: after heat shock. Averages and SEM are shown. P Value from Fisher's exact test.

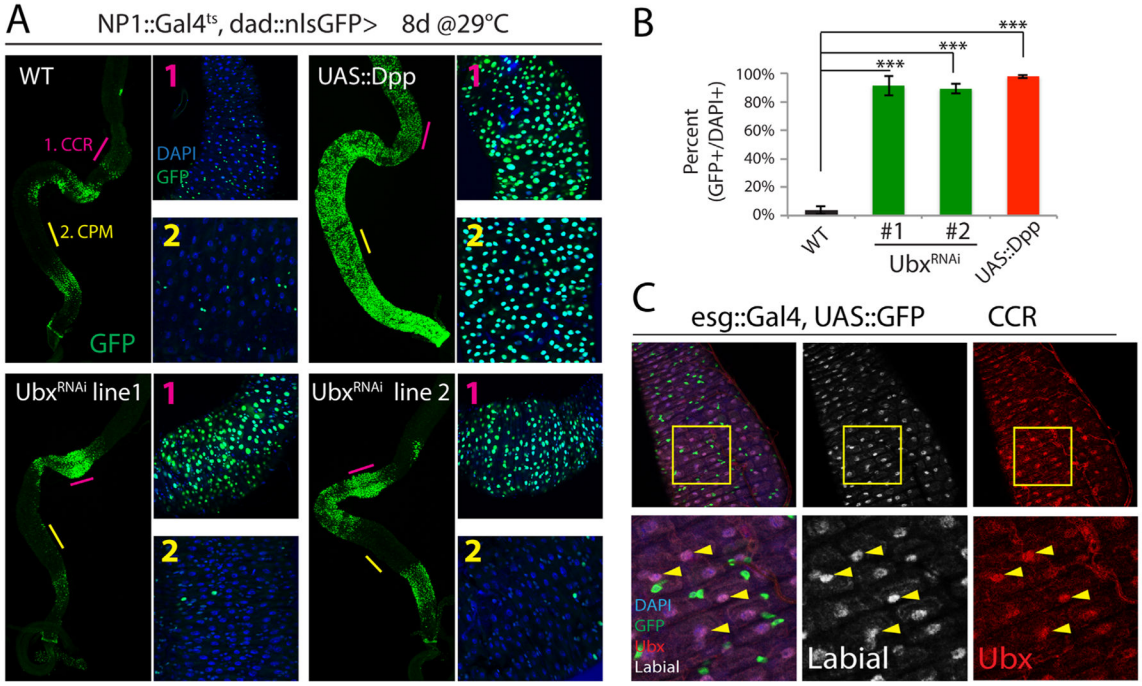

Fig. 4.

Ubx inhibits Dad expression specifically in the CCR. (A) Knockdown of *Ubx* (two Ubx^{RNAi} lines) by $NP1::Gal4^{ts}$ leads to ectopic expression of Dad::nlsGFP specifically in the CCR (region 1), but not in the central posterior midgut (CPM, region 2) compared to WT control, suggesting Ubx has a regionally specific role for repression of Dad. Note that over-expression of Dpp (UAS-Dpp) leads to Dad::nlsGFP overexpression in all regions, including the CCR and the CPM. Representative images are from 3 biological replicates of 13 guts for each genotype. (B) Percentage quantification of dad::nlsGFP+ polyploid cells over DAPI+ polyploid cells in the CCR from Figure (A). N = 13 guts from 3 replicates each genotype. Averages and SEM are shown. One-way ANOVA with post-hoc *t*-test was performed,*** $p < 0.001$. (C) In the CCR, Ubx (red, antibody against Ubx) is expressed in most of the Labial + CCs (white, arrowheads). Since Ubx expression is not very high in these epithelial cells, we have increased the intensity of the red (Ubx) channel, leading to high background noise. See also Fig. S2B for details of Ubx expression along the GI tract.

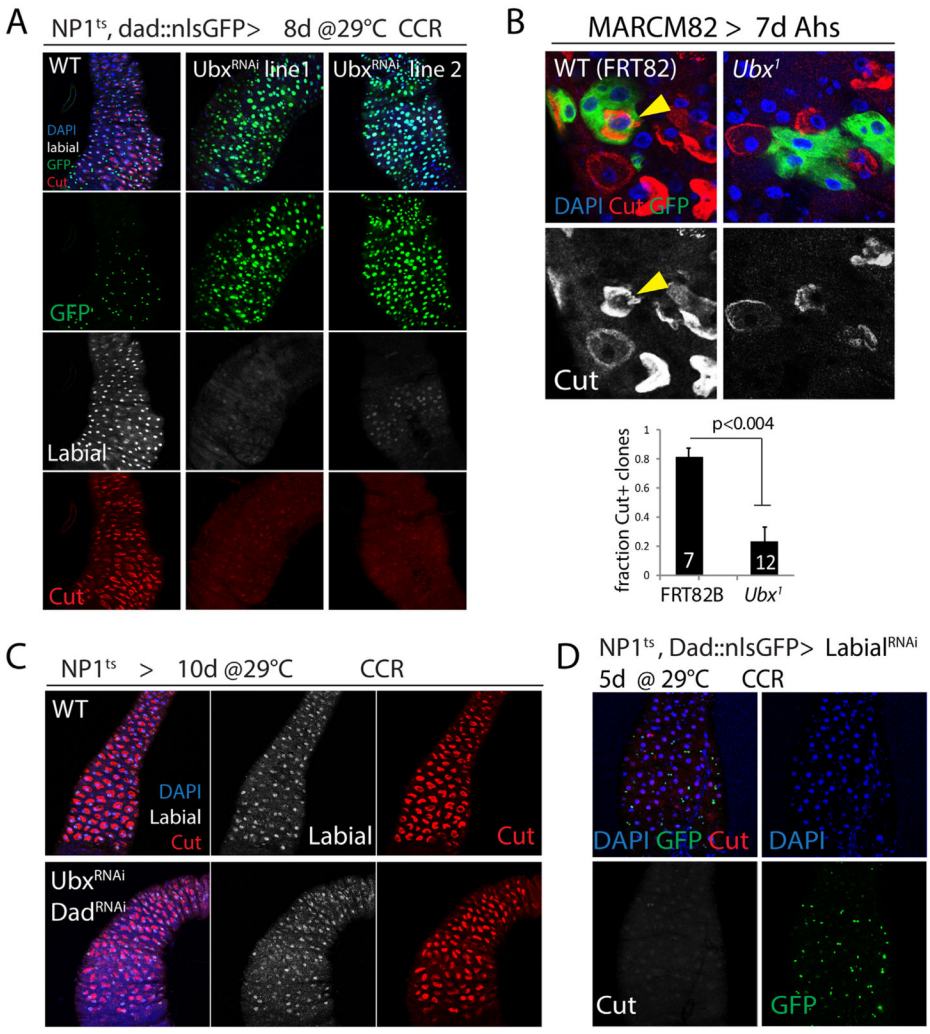

Fig. 5.

Inhibition of Dad expression by Ubx is required for CC differentiation. (A) Knockdown of Ubx (two Ubx^{RNAi} lines) by NP1::$Gal4^{ts}$ ($NP1^{ts}$) leads to *Dad::nlsGFP* (green) over expression in the CCR, and loss of Cut+ (red) and Labial+ (white) CCs. (B) Ubx^{1} mutant has a significantly lower fraction of Cut+ clones (arrowheads) compared to the control in the MARCM system. The lower graph shows the quantification. WT N = 35 clones from 7 guts, Ubx^{1} N = 51 clones from 12 guts. Ahs: after heat shock. Averages and SEM are shown. P Value from Fisher's exact test. (C) Double knockdown of *Ubx* and *Dad* (Ubx^{RNAi}, Dad^{RNAi}) by NP1::$Gal4^{ts}$ ($NP1^{ts}$) results in normal Cut+ (red) and Labial+ (white) CCs. (D) Knockdown of Labial ($Labial^{RNAi}$) by NP1::$Gal4^{ts}$ ($NP1^{ts}$) leads to loss of Cut+ (white) CCs, but does not induce Dad::nlsGFP (green) over-expression, suggesting that Labial does not regulate Dad.

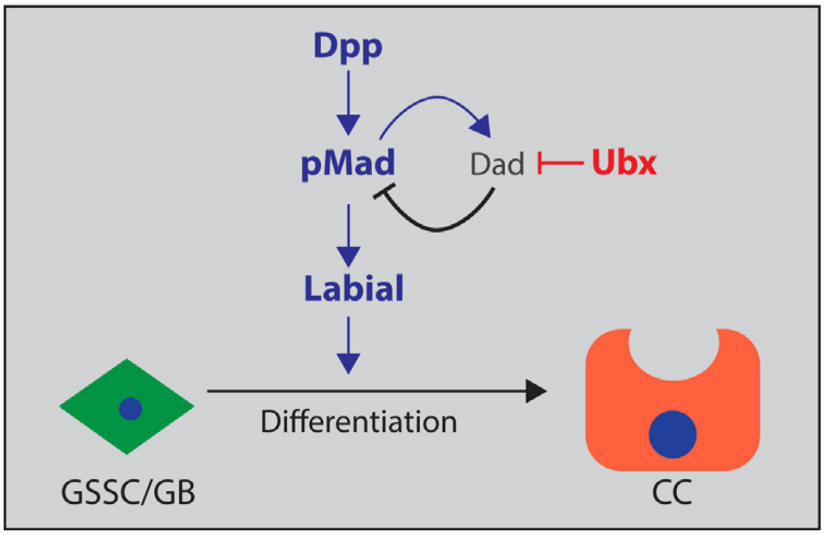

Fig. 6.

Ubx regulates Dpp signaling by inhibiting Dad for CC regeneration. Model: in the CCR, Ubx is required to inhibit Dad expression to allow the activation of Dpp signaling (high pMad levels) in differentiating CCs, and such regulation is essential for the CC regeneration.