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RESEARCH ARTICLE

Autocrine and paracrine Wingless signalling in the *Drosophila* midgut by both continuous gradient and asynchronous bursts of *wingless* expression [version 1; referees: 3 approved with reservations]

Hsiao Yu Fang^{1,2}, Alfonso Martinez-Arias¹, Joaquín de Navascués³

¹Department of Genetics, University of Cambridge, Cambridge, UK

²National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, USA

³European Cancer Stem Cell Research Institute, School of Biosciences, Cardiff University, Cardiff, UK

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Abstract

Wingless (Wg)/ Wnt signalling is a major regulator of homeostasis in both the mammalian and *Drosophila* intestine. In *Drosophila* the organisation and function of Wingless signalling in the adult intestine remain poorly understood. Here we characterise the pattern of expression of *wg*, the stabilisation of its effector Armadillo in the adult *Drosophila* midgut, and correlate them with the response of the cells to Wg signalling activation. We show that in normal homeostasis there is a gradient of Wingless signalling in the intestinal stem cell (ISC) and the undifferentiated progenitor cell (enteroblast, EB) populations along the posterior midgut, with a high point at the midgut-hindgut boundary (pylorus). This gradient results from a combination of two sources of Wingless: a distant source outside the epithelium (the pylorus) and a local one from the ISCs and EBs themselves. Altogether, our studies show that Wingless expression and signalling in the epithelium is not continuous, but operates through bursts that occur randomly in space and time.

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Referee Status: **???**

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	1	2	3
version 1	?	?	?
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Corresponding authors: Alfonso Martinez-Arias (ama11@hermes.cam.ac.uk), Joaquín de Navascués (deNavascuesJ@cardiff.ac.uk)

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Abbreviations

Apc	Adenomatous polyposis coli
Arm	Armadillo
BBS	Borate buffered solution
EB	Enteroblast
EC	Enterocyte
EE	Enteroendocrine
Esg	Escargot
FLP	Flippase
FRT	Flippase recognition target
Fz	Frizzled
GFP	Green fluorescent protein
HRP	Horseshoe peroxidase
ISC	Intestinal stem cell
MARCM	Mosaic analysis with a repressible cell marker
Pros	Prospero
RFP	Red fluorescent protein
Su(H)	Suppressor of hairless
TA	Transit amplifying
TARGET	Temporal and regional gene expression targeting
TS	Temperature sensitive
Tub	Tubulin
Wg	Wingless

Introduction

The discovery of intestinal stem cells (ISCs) in the adult *Drosophila* midgut established an attractive model for the study of tissue homeostasis (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006). The *Drosophila* midgut displays similarities with the mammalian intestine in various aspects, such as cell composition and regulatory mechanisms (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006). Like the mammalian intestine, the *Drosophila* adult midgut consists of a tubular, monolayered epithelium lining the length of the midgut, surrounded by the basement membrane and two layers of visceral muscles (Micchelli & Perrimon, 2006). The ISCs are dispersed among the differentiated cells throughout the enteric epithelium (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006).

Two fully differentiated cells types populate the *Drosophila* midgut: large, polyploid enterocytes (ECs), the main absorptive cells in the epithelium, and small, diploid enteroendocrine (EEs) cells, the secretory cell type. ECs/EEs come from the differentiation of an intermediate cell type, called enteroblasts (EBs). EBs are conceptually similar to the Transit Amplifying (TA) cells in mammals, though unlike the TA cells, EBs do not divide before terminal differentiation. Lineage analysis has shown that ISCs undergo asymmetric divisions as well as symmetric self-renewal and symmetric differentiation (de Navascués *et al.*, 2012; O'Brien *et al.*, 2011), resulting in homeostasis by population asymmetry (de Navascués *et al.*, 2012; Klein & Simons, 2011).

Wnt signalling plays an indispensable role in the regulation of mammalian ISCs. In the mammalian intestine, Wnt signalling is crucial in the maintenance of stem cell crypts (Barker *et al.*, 2007; Barker *et al.*, 2009; Van der Flier & Clevers, 2009; Van der Flier *et al.*, 2007). Wingless (Wg), the *Drosophila* homologue of Wnt1, is expressed in the adult midgut, and Wg signalling has been shown to play a crucial role in tissue regeneration (Cordero *et al.*, 2012). Studies have shown that stress-induced epithelial Wg production from EBs is essential for ISC proliferation during tissue renewal, but not required for midgut maintenance under homeostatic conditions (Cordero *et al.*, 2012; Micchelli & Perrimon, 2006). However, other works reported that Wg signalling is required for ISC self-renewal: reduced proliferation and premature differentiation occur as a consequence of inhibiting downstream Wnt signalling (Lin *et al.*, 2008). By contrast, a separate study proposed that the loss of *Drosophila* adenomatous polyposis coli, Apc, does not affect ISC self-renewal nor EB cell fate specification (de Navascués *et al.*, 2012; Lee *et al.*, 2009; Lin *et al.*, 2008; O'Brien *et al.*, 2011). Lee *et al.* (2009) showed that Apc is required for midgut homeostasis and regulates ISC proliferation, and its absence leads to midgut hyperplasia and multilayering. Moreover, a recent work indicated that Wg signalling in ECs act non-autonomously to prevent ISC proliferation (Tian *et al.*, 2016). Thus the exact function of Wg signalling on ISC proliferation and EB differentiation remains controversial.

Reports have shown *wg* expression in the epithelium of the foregut-midgut and midgut-hindgut boundaries (pylorus) (Lee *et al.*, 2009; Singh *et al.*, 2011; Takashima *et al.*, 2008; Tian *et al.*, 2016), and also in the visceral muscle (Cordero *et al.*, 2012; Lin *et al.*, 2008; Tian *et al.*, 2016). The expression of *wg* at the midgut boundaries may take part in regulating foregut and hindgut development (Lee *et al.*, 2009; Singh *et al.*, 2011; Takashima *et al.*, 2008), though its function during adulthood is unclear. Moreover, the function of Wg emanating from the muscle has not been examined.

Using a Wg-responsive reporter transgene, *frizzled3-RFP* (*fz3-RFP*) (Olson *et al.*, 2011), it was observed that *fz3-RFP* is expressed in gradients in the midgut epithelium, comprising both ISCs/ECs, and coinciding with regional boundaries (Buchon *et al.*, 2013; Tian *et al.*, 2016). At the pylorus, the gradient of *fz3-RFP* correlates with other gene expression gradients and the morphology of enterocytes, suggesting that Wg signalling activity affects gene expression and enterocyte architecture (Buchon *et al.*, 2013; Lin *et al.*, 2008; Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006).

Here, we investigated the expression and localisation of Wg, several components of the signalling pathway, and the signalling reporter, *fz3-RFP*. Our results showed a Wg concentration gradient at the adult *Drosophila* posterior midgut, in agreement with previous reports (Tian *et al.*, 2016). The Wg protein is produced from two sources, one at the pylorus, and the other from the epithelial cells themselves. We show that in unchallenged conditions *wg* is sporadically expressed within the midgut epithelium, without any apparent spatial or temporal pattern. Using the stability of Arm as an instantaneous readout of Wg signalling, we further showed

that the epithelial cells respond to Wg signalling in a similarly stochastic manner. In this study, we describe in detail the expression and activity patterns of Wg signalling in the *Drosophila* midgut, as well as depicting the response of the midgut tissue to Wg signalling.

Results

Dataset 1. Raw microscopy images

<http://dx.doi.org/10.5256/f1000research.8170.d115432>

README.txt contains a description of the files.

Dataset 2. Raw data for the intensity values of wg-Gal4, UAS-GFP:wg (as plotted in Supplementary Figure S2)

<http://dx.doi.org/10.5256/f1000research.8170.d115434>

Wg establishes a gradient in the adult *Drosophila* posterior midgut

To observe the pattern of *wg* expression in the adult *Drosophila* posterior midgut (regions R4 and R5, (Buchon *et al.*, 2013)), we used the Wg antibody and several *wg* transcriptional reporters. An enhancer trap insertion in the *wg* locus (*wg-Gal4* > *UAS-GFP*) (Pfeiffer *et al.*, 2000) coincided with the anti-Wg antibody, and revealed that *wg* is highly expressed at the pylorus (Figure 1A–B),

in agreement with previous reports (Singh *et al.*, 2011; Takashima *et al.*, 2008). We did not observe Wg expression in the midgut cells adjacent to the pylorus, as recently reported (Tian *et al.*, 2016). Our observation was further confirmed with a *wg-lacZ* enhancer trap insertion (*wg⁰²⁶⁵⁷*), which showed a strong pyloric expression (Figure S1). To evaluate whether this Wg protein could be emanating from the pylorus, or instead came from areas of *wg* expression in the midgut that were not recapitulated by the enhancer trap insertions, we used *wg-Gal4* to drive the expression of a GFP-tagged Wg (*wg-Gal4* > *UAS-GFP:wg*) (Packard *et al.*, 2002). We observed GFP:Wg accumulation at the pylorus, and a decreasing gradient from the pylorus towards the anterior end of the midgut (Figure 1C–D and Figure S2). Notably, GFP:Wg diffused from the pylorus much further into the posterior midgut than into the hindgut epithelium. Interestingly, Wg travels in the midgut tissue to a distance longer than the width of the third larval instar imaginal wing primordium, where a Wg gradient is also established (Neumann & Cohen, 1997).

We also looked at flies expressing a membrane-tethered form of Wg (NRT-Wg) from the endogenous *wg* locus (Alexandre *et al.*, 2015). Anti-Wg again showed high Wg signals at the pylorus, with no obvious signalling gradient (Figure 1E,E'). These results suggest that the pylorus is the main source of Wg signal for the adult *Drosophila* posterior midgut, and that the Wg ligand can diffuse from this region, forming a gradient.

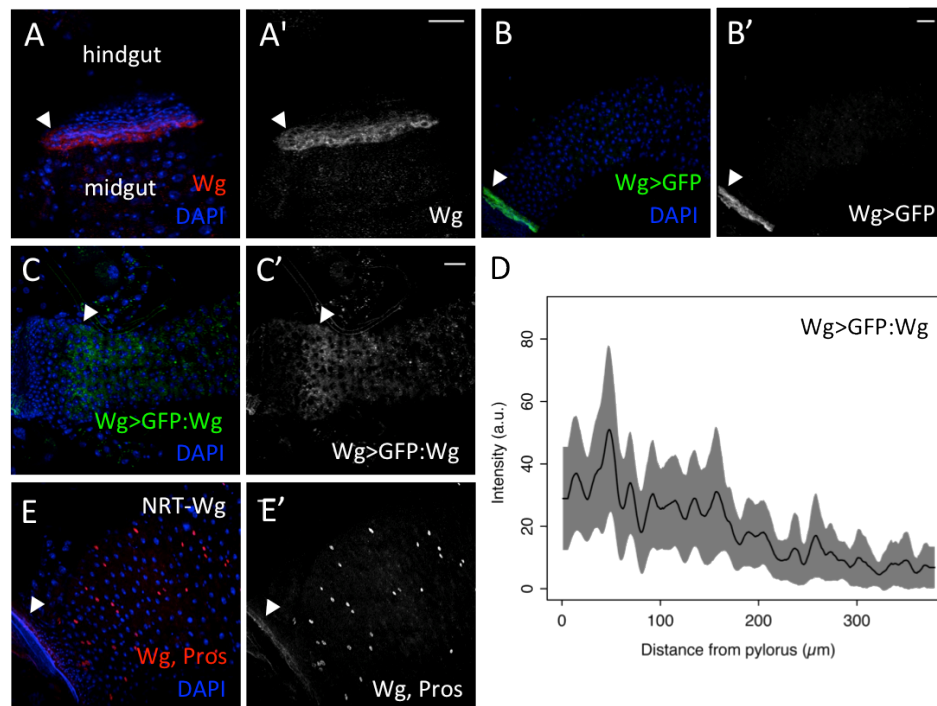


Figure 1. *wg* expression shows graded pattern along the posterior midgut. (A–A') Anti-Wg (red, A; grey, A') (B–B') *wg-Gal4*, UAS-GFP (green, B; grey, B') show high levels at the pylorus (arrowheads). (C–C') *wg-Gal4*, UAS-GFP:wg (green, C; grey, C') shows signalling gradient with high levels at the pylorus (arrowheads). (D) Intensity values of *wg-Gal4*, UAS-GFP:wg along thirteen parallel lines (as in the arrow in C'), averaged and smoothened with a Gaussian filter (5 μm wide) (black line). The corresponding raw data is represented in Figure S2. The limits of the grey area mark one standard deviation from the average value. (E–E') Flies expressing only NRT-Wg showed high Wg signals (anti-Wg) (red, E; grey, E') at the pylorus (arrowheads). EEs are marked by nuclear anti-Prospero staining (red, E; grey, E'). Scale bars: 25 μm.

Wg is expressed in asynchronous bursts within the adult *Drosophila* midgut epithelium

Our results so far indicate that most of the *Wg* protein in the posterior midgut comes from the pylorus. However, we still detected faint *Wg* antibody staining in midgut areas anterior to the lowest point of the *Wg* gradient, and we also observed low activity of *wg* transcriptional reporters (Figure 1). To ascertain whether there was *wg* expression in the homeostatic midgut epithelium, as reported during regeneration (Cordero *et al.*, 2012), we made use of the *wg*{*KO*; *Gal4*} line. In this line, the *wg* locus was edited to express *Gal4* instead of *Wg* (Alexandre *et al.*, 2015), and therefore is expected to accurately reproduce the expression of wild-type *wg*.

To amplify the signal of expression, *wg*{*KO*; *Gal4*} was crossed to *UAS-Flp*, *act<stop>lacZ*; *tub-Gal80^{ts}* (*wg*{*KO*}^{*ts*}*>Flp*, *act<stop>lacZ*). After switching to the restrictive temperature, *Gal4* activates and

over time all *Wg*-producing cells and their offspring will be strongly labelled with *LacZ*, even at minimal levels of *wg* expression. When the *wg*{*KO*}^{*ts*}*>Flp*, *act<stop>lacZ* flies were cultured at 18°C until 13–20 days of adulthood, some background activation was detected, as scattered, individual *LacZ*⁺ cells (Figure 2A,A') plus a field of cells in the posterior R5 region, abutting the pylorus (Figure S3A). However, after 2 days of incubation at 29°C (6–10 days old flies at dissection), the pylorus was marked with *LacZ* expression (Figure S3B–C), as expected, and the midgut epithelium showed more, sparse *LacZ*⁺ cells, either isolated or in pairs (Figure 2B,B'). When cultured for 8 days at 29°C (11–18 days old flies at dissection), more cells expressed *lacZ* in a salt and pepper pattern, with bigger groups that included polyploid ECs as well as diploid cells (Figure 2C,C'). These observations are likely the result of additional clonal induction accompanied by clonal expansion of previously labelled ISC, rather than of *wg* being expressed coordinately

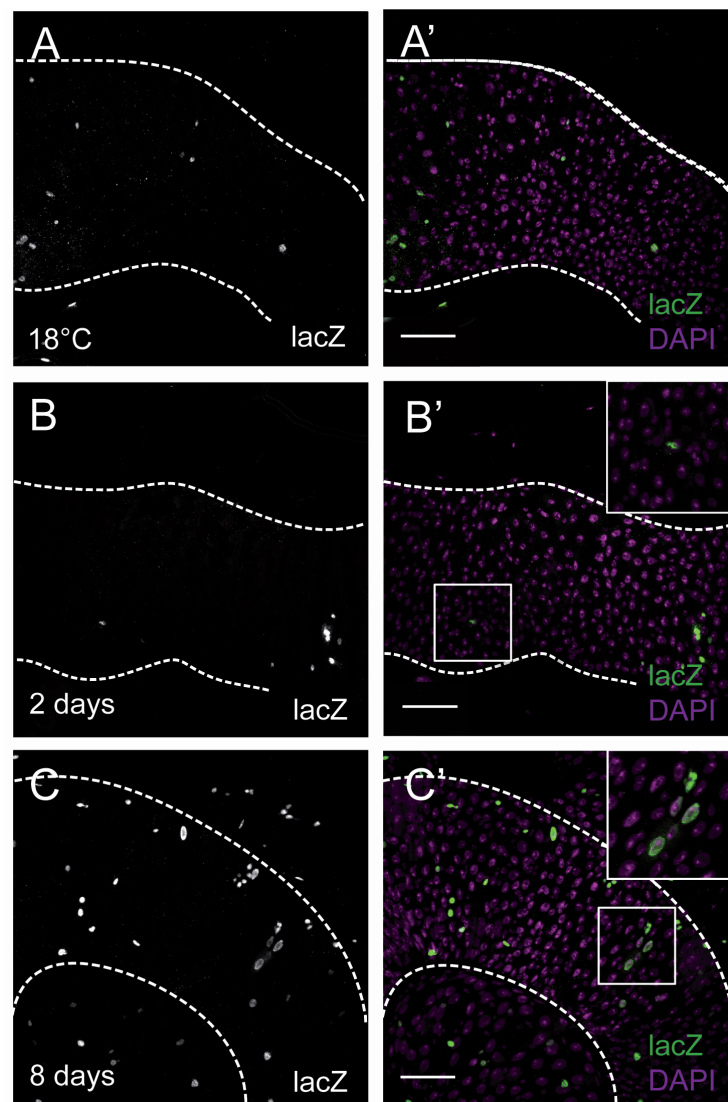


Figure 2. *Wg* expression is demonstrated by lineage tracing in the midgut epithelium. (A,A') *wg*{*KO*}-*Gal4*, *UAS-Flp*, *tub-Gal80^{ts}*, *Act<stop>lacZ* guts at 18°C (13–20 day old flies). (B–C') *wg*{*KO*}-*Gal4*, *UAS-Flp*, *tub-Gal80^{ts}*, *Act<stop>lacZ* guts after 2 days (6–10 day-old flies) or 8 days (11–18 day-old flies) of incubation at 29°C for *Gal4* activation. (B,B') Small *LacZ*⁺ (anti-βGal) (grey, B; green, B') clusters of 1–2 cells, mostly diploid, after 2 days at 29°C. (C,C') Larger *LacZ*⁺ (anti-βGal) (grey, C; green, C') clusters of both diploid and polyploid cells after 8 days at 29°C. DAPI nuclear staining is shown in magenta. Inset boxes show selected regions at higher magnification. Scale bars: 50 μm. Fields of view correspond to the anterior R5 region.

by patches of cells of multiple differentiated cell types. For the flies cultured for 8 days at 29°C, we also inspected the muscle layer of the entire posterior midgut, which showed no *wg* expression from the muscle cells (Figure 3). Taken together, our results indicate that under homeostatic conditions, *wg* is expressed intermittently and asynchronously in the *Drosophila* midgut epithelium, possibly in diploid cells including the ISCs.

Expression of other components of the Wg signalling pathway

In order to further characterise the spatial organisation of the Wg signalling pathway in the *Drosophila* midgut, we observed the expression patterns of the receptor *frizzled2* and the component of the destruction complex *shaggy* (*sgg*). Using a *frizzled-2* enhancer trap insertion (*fz2-GFP*), we found that *fz2-GFP* was highly expressed at the pylorus (Figure 4A,A'). *fz2-GFP* could also be

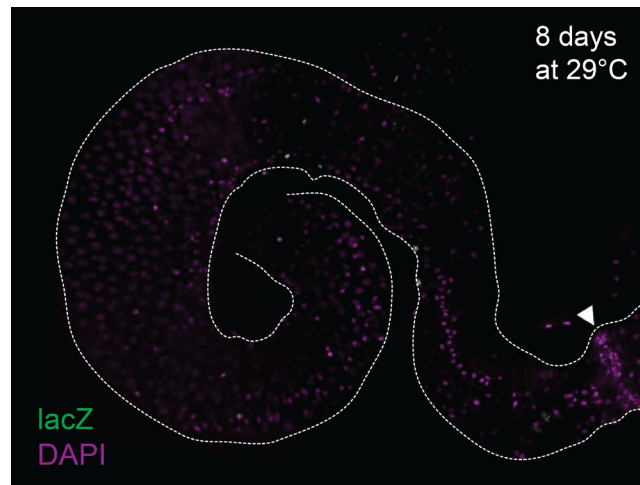


Figure 3. *Wg* is not expressed in the muscle layer of the posterior midgut. The muscle layer of the *wg*[*KO*]-*Gal4*, *UAS-Flp*, *tub-Gal80^{ts}*, *Act<stop>lacZ* gut after 8 days of incubation at 29°C for signal induction. No LacZ signals (anti-βGal, green) are detected in the muscle cells. DAPI nuclear staining is shown in magenta. Arrowhead points to the pylorus. Scale bar: 50 μm.

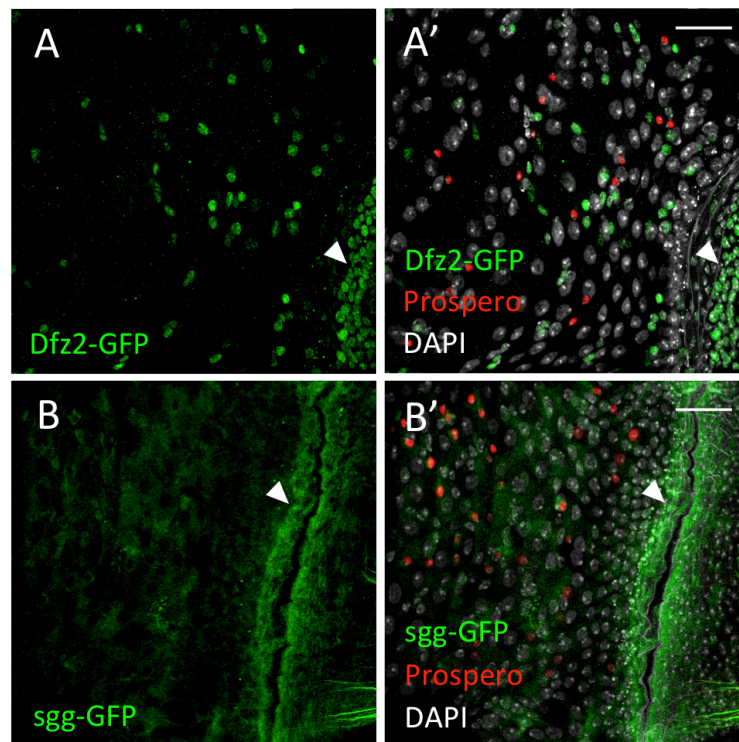


Figure 4. Characterisation of *fz2* and *sgg* expression patterns in the adult *Drosophila* posterior midgut. (A,A') *fz2-GFP* (green) is expressed in the small cells and the pylorus (arrowheads). (B,B') *sgg-GFP* (green) is expressed in all cell types, with strong signals at the pylorus (arrowheads). Prospero (red) marks the EEs. DAPI nuclear staining is shown in grey. Scale bars: 25 μm.

observed in diploid cells (presumably ISCs and EBs) and EEs away from the pylorus (Figure 4A'). There was no *fz2-GFP* expression in ECs.

To examine the expression pattern of *sgg*, we used a *Sgg:GFP* protein trap. *Sgg:GFP* could be detected throughout the posterior midgut, in all cell types, with strong expression at the pylorus (Figure 4B,B').

The adult *Drosophila* midgut displays asynchronous stabilisation of Armadillo

Next we wanted to examine the activation of Wg signalling in the adult *Drosophila* midgut. We used a *frizzled-3* (*fz3*) reporter to

monitor Wg signalling activity, *fz3-RFP*, since *fz3* is a direct target of the Wg pathway (Olson *et al.*, 2011). We inspected flies expressing *fz3-RFP* and *wg-Gal4 > UAS-GFP*, which both showed high expression at the pylorus (Figure 5A,C-C'), with *fz3-RFP* exhibiting a signalling gradient, culminating at the pylorus (Figure 5C). *fz3-RFP* also displayed strong epithelial expression in the region abutting the gastric zone (Figure 5A,C'). To identify the cell types expressing *fz3-RFP*, we used *esg-lacZ* to label ISCs/EBs, and GBE-Su(H)-GFP to distinguish EBs. We observed that in the regions of the gut away from the pylorus, *fz3-RFP* is only expressed in ISCs and EBs (Figure 6). However, the stability of RFP in this tissue is unknown, presumably long, and therefore *fz3-RFP* expression could be indicative of long-past Wg pathway activation.

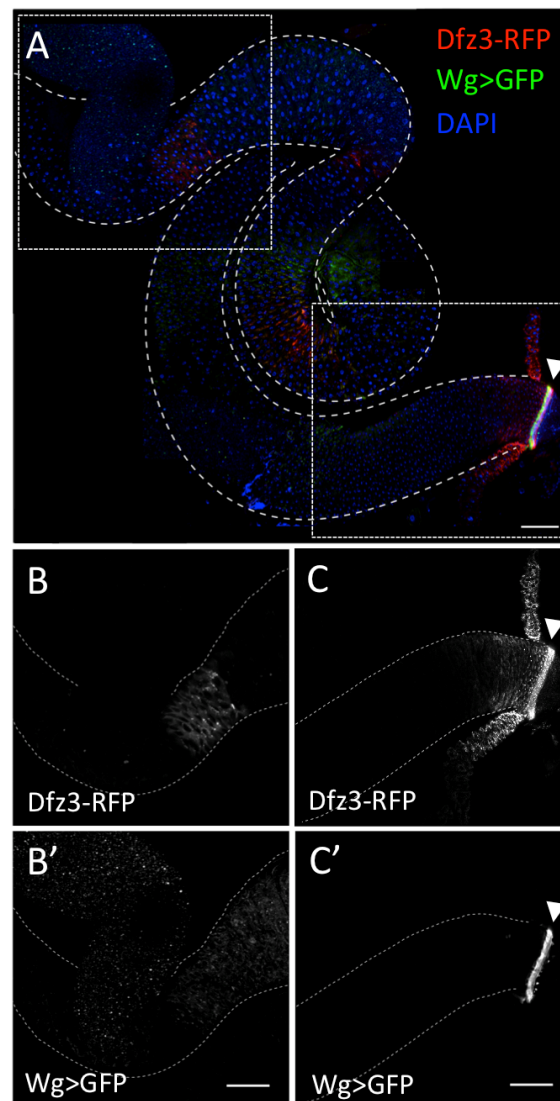


Figure 5. *fz3-RFP* and *wg-Gal4 > UAS-GFP* together show the patterns of Wg activity and expression in the adult *Drosophila* posterior midgut. *fz3-RFP* (red, A; grey, C) and *wg-Gal4 > UAS-GFP* (green, A; grey, C') are both highly expressed at the pylorus (arrowheads). *fz3-RFP* also displays epithelial expression in the region abutting the gastric zone (red, A; grey, B). (B,B') and (C,C') each show the region of the midgut within the dashed box above. Scale bars: 100 μ m.

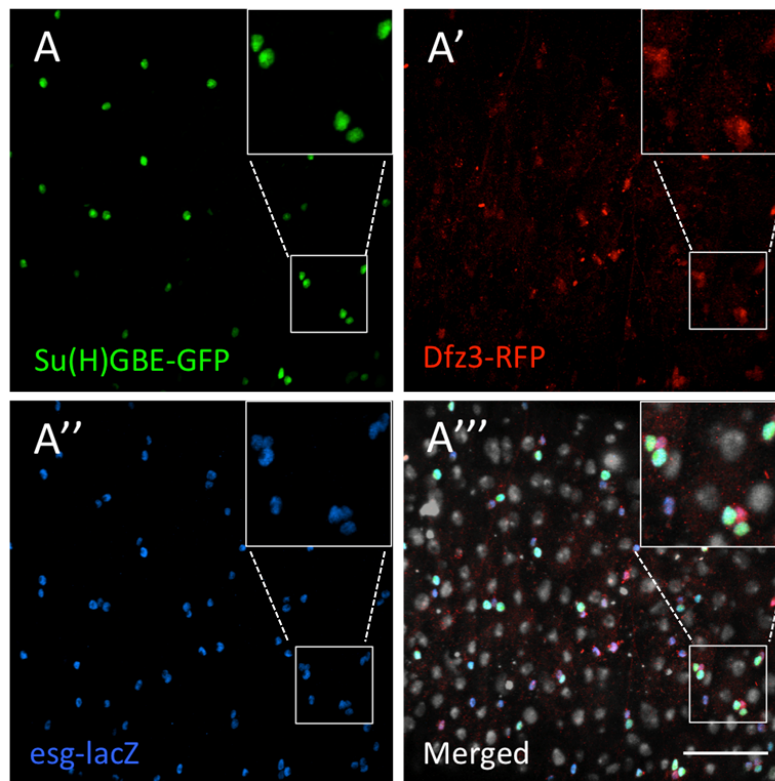


Figure 6. Fz3-RFP shows that ISCs/EBs away from the pylorus are responsive to Wg signalling. (A) Su(H)GBE-GFP (green) labels EBs. (A') Fz3-RFP (red) marks the cells responding to Wg signalling. (A'') Esg-lacZ (anti-βGal, blue) labels ISCs and EBs. (A''') Merged. Fz3-RFP (red) is detected only in the ISCs and EBs, and all the ISCs and EBs showed *fz3-RFP* expression. DAPI nuclear staining is shown in grey. Inset boxes show selected regions at higher magnification. Scale bar: 50 μm.

A more instantaneous reporter for Wnt signalling is the cytosolic levels of the overexpressed nuclear effector of the pathway, Armadillo/β-catenin (Alexandre *et al.*, 2015; Hayward *et al.*, 2005; Lin *et al.*, 2008). When full-length *armadillo* (*arm^{FL}*) is overexpressed in the epithelium of the imaginal wing disc, Armadillo protein is only accumulated close to the domains of *wg* expression (Hayward *et al.*, 2005). Arm is so rapidly degraded that its overexpression is only detected in cells where Wg signalling stabilises the protein. Therefore, the accumulation of UAS-Arm^{FL} correlates with presently active Wg signalling. We tested whether ISCs/EBs showed stabilisation of UAS-*arm^{FL}* using *esg-Gal4*, UAS-GFP. Surprisingly, only a proportion of the GFP-expressing ISCs and EBs showed elevated levels of Arm (Arm⁺GFP⁺/GFP⁺ per field of view: average% = 29%, highest% = 52%, lowest% = 19%) (Figure 7 and Table S1). This suggests that Wg signalling is activated in selected ISCs and EBs only in short periods of time, without any obvious spatial pattern. This is in good agreement with our observations of

wg expression, especially as reported with the “tracer amplifier”, *wg{KO}^{ts}>Flp*, *act<<lacZ*. The results indicate that Wg production from the ISCs and EBs occurs randomly, eliciting a paracrine/autocrine response that is similarly unpatterned.

We also overexpressed *arm^{FL}* in all cells with the *tub<stop<GAL4* driver, which was induced at adulthood by *hs-Flp*. Only a fraction of the cells, which included both differentiated and undifferentiated cells (ISCs/EBs, marked by anti-HRP), showed high levels of both cytoplasmic and nuclear Arm (Figure 8B–B''). Arm antibody detected irregular Arm distribution at the cell membranes (Figure 8B–B''). By contrast, uninduced tissue showed regular wild-type Arm staining at the cell membranes, with higher levels in ISCs and EBs (Figure 8A–A''). Global *arm* overexpression suggests that cells in the midgut tissue respond to Wg signalling asynchronously. Also, this forced global *Arm* induction appeared to perturb regular Arm distribution in the midgut, and possibly disrupting cell packaging,

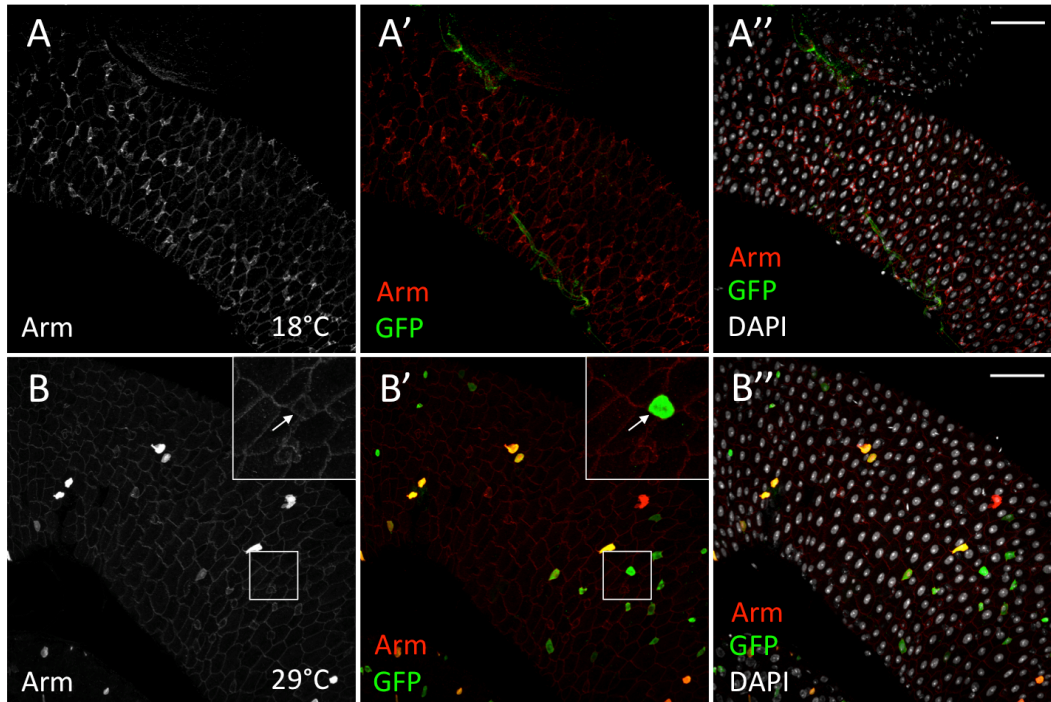


Figure 7. Only a fraction of ISCs/EBs shows stabilisation of Arm^{FL} . (A–A'') *esg-Gal4[TS], UAS-arm, UAS-GFP* intestines at 18°C show no GFP nor Arm^{FL} induction. (B–B'') GFP (anti-GFP, green) is detected in *esg*⁺ cells without Arm^{FL} accumulation (anti-Arm) (grey, B; red, B') (arrows). DAPI nuclear staining is shown in grey. Inset boxes show selected regions at higher magnification. Scale bars: 50 µm.

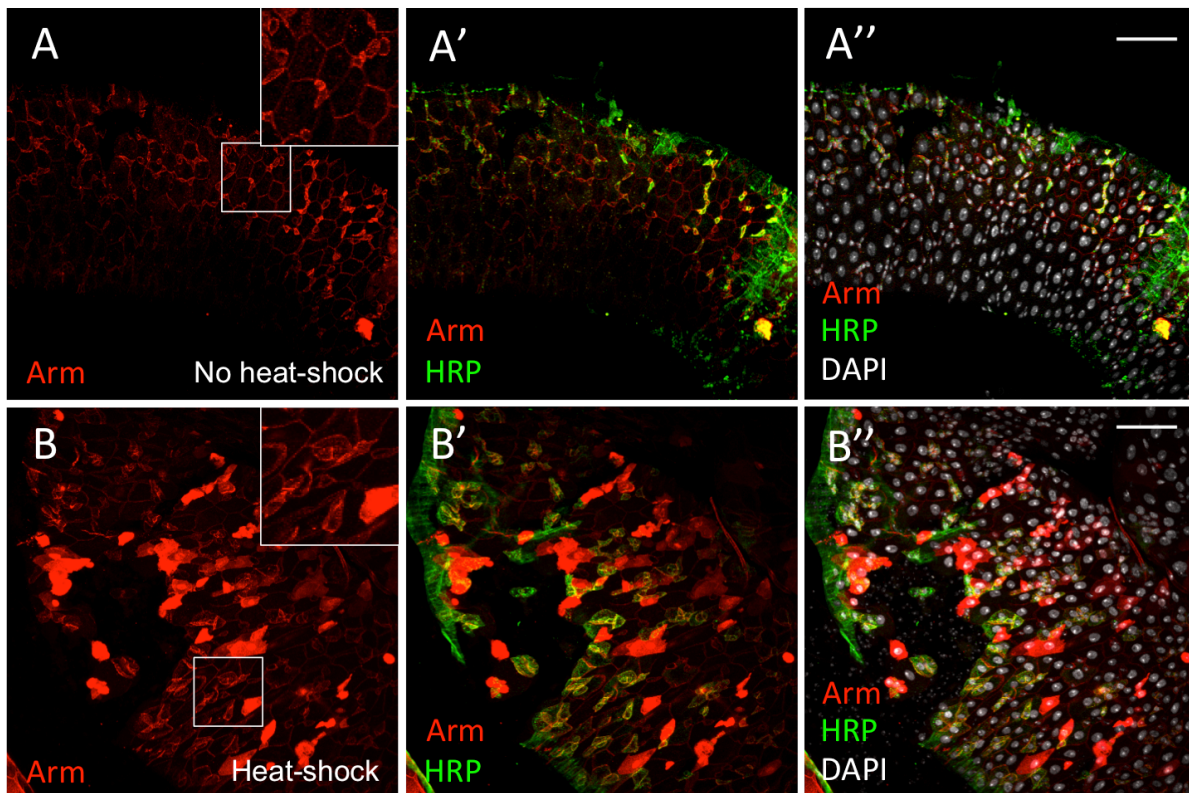


Figure 8. Midgut tissue responds to Wg signalling in a non-uniform way. (A–A'') *heat-shock FLP, tub<STOP>Gal4, UAS-arm* without heat-shock induction of *UAS-Arm^{FL}* overexpression. (B–B'') With the *tub<stop>GAL4* driver, *UAS-Arm^{FL}* is induced in all cells, but only a proportion of the cells shows elevated Arm (anti-Arm, red) signals in the cytoplasm and nucleus. HRP (anti-HRP, green) labels ISCs and EBs. DAPI nuclear staining is shown in grey. Inset boxes show selected regions at higher magnification. Scale bars: 50 µm.

leading to tissue dysplasia. Taken together, the adult *Drosophila* midgut tissue appears to respond to Wg signalling and stabilise Arm in an unpatterned way.

Discussion

Wnt signalling is the primary driving force in intestinal homeostasis and tumorigenesis in mammals. The adult *Drosophila* midgut is a powerful system to study intestinal homeostasis, but the role and organisation of Wg signalling in this tissue is still not well understood. We studied the expression, localisation and activity of Wg, and found that in homeostasis, Wg forms a gradient from the pylorus into the posterior midgut, and is also expressed in the diploid cells of the midgut. Moreover, midgut expression appears to be discontinuous and asynchronous, eliciting Wg immediate response in a seemingly random pattern, in turn possibly maintaining long-term expression of Wg signalling reporters.

Previous studies have suggested the visceral muscle as the main production site of Wg in the adult *Drosophila* midgut, which acts as a stem cell niche (Lin *et al.*, 2008). However, a source of Wg that could comprehensively regulate the widely distributed ISC lineages throughout the midgut is unclear. Using a variety of reagents, we observed a gradient of Wg signalling across the midgut with a source of high wg expression in the epithelium of the pylorus, which agrees with previous reports (Takashima *et al.*, 2008). However, in contrast to Lin *et al.* (2008) and Tian *et al.* (2016), we observed minimal levels of wg expression in the muscle cells using different lines and methods. It is possible that the wg expression detected in the muscle is confined to a specific region of the midgut, and that Wg is not being secreted from the entire muscle layer of the midgut, though we have not observed any area of wg expression from the visceral muscles. There might be discrepancies between the distribution of the Wg protein and the wg transcriptional reporters, which might differ in the readouts of the endogenous wg expression. This highlights the significance of the wg{KO}>Flp, act<<lacZ experiment we conducted, in which LacZ could precisely and sensitively reflect the spatial and temporal distribution of Wg. In these experiments, we were able to confirm Wg production from the midgut epithelial cells. Surprisingly, we observed sporadic LacZ⁺ clones composed of mainly diploid cells after a short gene induction time, while LacZ⁺ polyploid cells could be detected after longer gene induction. This suggests that wg is only expressed in the diploid cells including ISCs, which later give rise to polyploid ECs.

Using Wg signalling reporters, our work further showed that ISCs and EBs are the cell types responsive to Wg signalling, and that they respond in a stochastic manner. The fz3-RFP reporter seems to reveal a 'memory' of past Wg signalling, possibly explaining the results of Tian *et al.* (2016), where they saw that ISCs and EBs maintain fz3-RFP expression in the absence of Wg signalling. However, Arm stabilisation acts as a readout of the instantaneous response to Wg signalling, and this indicates that ISCs/EBs are indeed responding to Wg signalling. Depending on the stability of the fz3-RFP reporter, it is possible that the midgut epithelium only needs occasional bursts of Wg production to maintain signalling

levels. Together with our findings on the sources of Wg production, these results suggest that Wg might act as both paracrine and autocrine signals in the *Drosophila* midgut, and the two types of signals act in a complementary manner. The paracrine Wg signals would elicit a stronger cellular response in the vicinity of the pylorus, where Wg production is high, and contribute to the spatial patterning of ECs (Buchon *et al.*, 2013; Tian *et al.*, 2016). On the other hand, at the regions further away from the pylorus, where paracrine Wg signalling is weak, ISCs and EBs themselves produce, in asynchronous bursts, the required Wg signals.

Materials and methods

Fly strains maintenance

Flies were raised and maintained at 18, 25 or 29°C with 65–75% humidity and a 12 hour light/12 hour dark cycle on standard cornmeal/yeast medium (consisting of 1.25% agar, 10.5% dextrose, 10.5% maize, 2.1% killed yeast, and 3.5% nipagin. Supplier: Brian Drewitt, Cambridge, UK) seeded with live yeast. Stocks were obtained from the Bloomington *Drosophila* Resource Center unless otherwise stated. The NRT-Wg and UAS-GFP:Wg lines were provided by J. Vincent. The fz3-RFP line is from R. DasGupta. Experiments were conducted in well-fed, mated females, 3–20 days old of the following genotypes:

Figure 1:

Oregon R.

+; wg-Gal4/ CyO; UAS-GFP/TM3, Ser

+/- w; wg-Gal4/ UAS-GFP:Wg; +/- MKRS or TM6B

+; NRT-wg; +

Figure 2,3:

w; wg{KO; Gal4// UAS-Flp; tub-Gal80^{ts}/ Act<stop<lacZ

Figure 4:

w; +; fz2^{CB02997}

w, sgg^{CPT1000023}; +; +

Figure 5:

w; fz3-RFP/wg-Gal4; UAS-GFP/TM6B, Sb, Tb

Figure 6:

y, w; esg-lacZ/fz3-RFP; Su(H)GBE-GFP/TM6B

Figure 7:

y, w; esg^{NP7397}/ +; UAS-arm/ tub-Gal80^{ts}, UAS-GFP

Figure 8:

y, w, hs-FLP_{1,22}; tub<GFP, stop<Gal4/ +; UAS-arm/ +

Figure S1:

+; wg⁰²⁶⁵⁷ cn¹/ CyO; ry⁵⁰⁶

Histology and tissue analysis

The following fixation methods were used: (1) Adult intestines were dissected and collected in BBS for up to 30 minutes, then fixed for 2 hours at room temperature in 4% PFA diluted in BBS. This method was used for most of the experiments. (2) Adult intestines were dissected in ice-cold “wash solution” (ddH_2O + 0.7% NaCl + 0.05% Triton) for up to 15 minutes and collected within a mesh basket. The basket was submerged in a “double beaker” with wash solution at 90°C for 5 seconds, and then immediately placed in ice-cold wash solution for 2 minutes. The double beaker was prepared placing a 250 ml beaker inside a 600 ml beaker, both containing wash solution and set on a hotplate until the temperature in the 250 ml beaker reached 90°C (about 30–45 minutes). This method was used for stainings of Armadillo.

After fixation, the tissue was rinsed three times in PBT (PBS containing 0.1% Triton X-100), then washed three times with blocking buffer (PBT containing 2% BSA and 2% FCS), each time 15 minutes on the rotator at room temperature. Primary antibody incubations were overnight at 4°C. After washing with PBT (3 × 15 minutes), secondary antibodies were incubated for 2–4 hours rotating in the dark at room temperature. DAPI (1 µg/ml) was added after the final wash.

Primary antibodies were mouse monoclonal anti-Wg (1:100, gift from J. Vincent) (Alexandre *et al.*, 2015), goat polyclonal anti-HRP (1:500, Jackson, code number 123-001-021) (Hönigsmann *et al.*, 1975), rabbit polyclonal anti-βGal (1:10,000, Cappel) (de Navascués *et al.*, 2012), mouse monoclonal anti-Pros (1:200, Developmental Studies Hybridoma Bank) (de Navascués *et al.*, 2012), mouse monoclonal anti-N27 Arm (1:20, made in the Martínez-Arias lab), chicken polyclonal anti-GFP (1:200, Abcam, ab13970) (de Navascués *et al.*, 2012). Alexa fluorophor-conjugated secondary antibodies (1:500) were from Invitrogen: anti-mouse 568 (Catalog #A-11004, A10037), anti-rabbit 488 (Catalog #A-11034, R37118), anti-rabbit 633 (Catalog #A-21071), anti-goat A488 (Catalog #A-11055), anti-goat A633 (Catalog #A-21082), anti-chicken A488 (Catalog #A-11039). DNA dye was DAPI (Invitrogen).

Tissues were mounted in Vectashield and imaged on Zeiss LSM 700 confocal system using 40× objective and numerical aperture of 1.2.

Transgene activation by temperature-sensitive TARGET system and heat-shock flip out system

To induce gene expression, the temporal and regional gene expression targeting (TARGET) method was used with the GAL4, UAS and GAL80^{ts} elements (McGuire *et al.*, 2004). The flies were crossed at the restrictive temperature (18°C), and then the progeny of the desired genotype was allowed to age at 18°C for 3 to 20 days

post-eclosion to reach homeostatic condition. The flies were then incubated at 29°C to allow GAL4 activity, inducing the transcription of the UAS transgenes.

The heat-shock flip out system was also used to induce transgene expression (Gordon & Scott, 2009). Adult flies were raised at 25°C until they were 3–20 days old, then they were treated with heat-shock for 30 minutes in a 37°C water-bath. The hs-FLP recombinase was activated upon heat treatment, eliminating the GFP gene and the stop cassette. This activates *tub-GAL4*, leading to the stimulation of UAS-regulated genes.

Data analysis

Images and figures were assembled using ImageJ (1.47v). Images are maximum intensity projections or selected, representative layers of confocal stacks. The intensity values of *wg-Gal4*, *UAS-GFP:wg* were obtained by ImageJ (1.47v) (Figure 1C'), then plotted using RStudio (0.99.491) and Adobe Illustrator CS6 (Figure 1D and Figure S2). Cell counts were conducted using the PointPicker plugin in ImageJ (1.47v) (Table S1). The cellular percentages were calculated using Microsoft Excel 2011 (Table S1).

Data availability

F1000Research: Dataset 1. Raw microscopy images, 10.5256/f1000research.8170.d115432 (Fang *et al.*, 2016a).

F1000Research: Dataset 2. Raw data for the intensity values of *wg-Gal4*, *UAS-GFP:wg* (as plotted in Supplementary Figure S2), 10.5256/f1000research.8170.d115434 (Fang *et al.*, 2016b).

Author contributions

AMA and JdN conceived the project. HYF performed the experiments and collected the data. HYF and JdN analysed the results and wrote the manuscript. AMA discussed the results and commented on the manuscript.

Competing interests

The authors declare no competing or financial interests.

Grant information

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Supplementary material

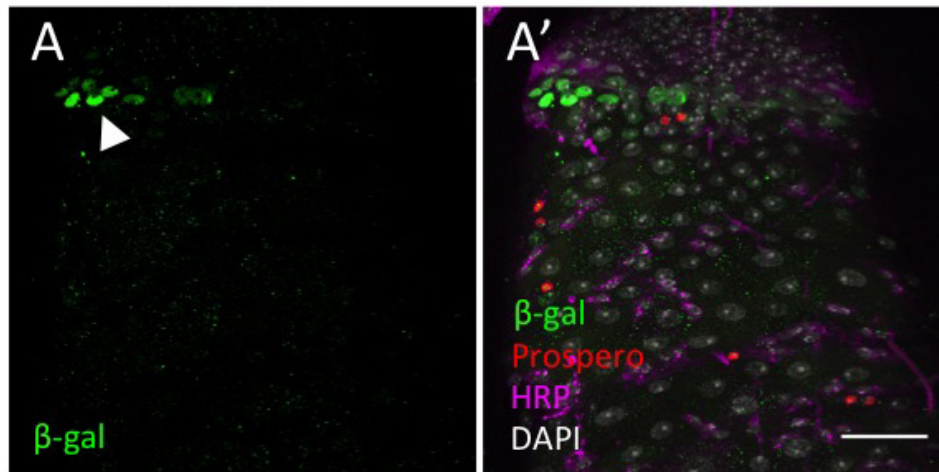


Figure S1. Expression of *wg-lacZ* enhancer trap in the adult *Drosophila* posterior midgut. (A) Nuclear β-galactosidase (green) is detected at the pylorus (arrowhead). (B) Prospero (red) identifies the EEs. HRP (magenta) marks ISCs and EBs. DAPI nuclear staining is shown in grey. Scale bar: 30 μm.

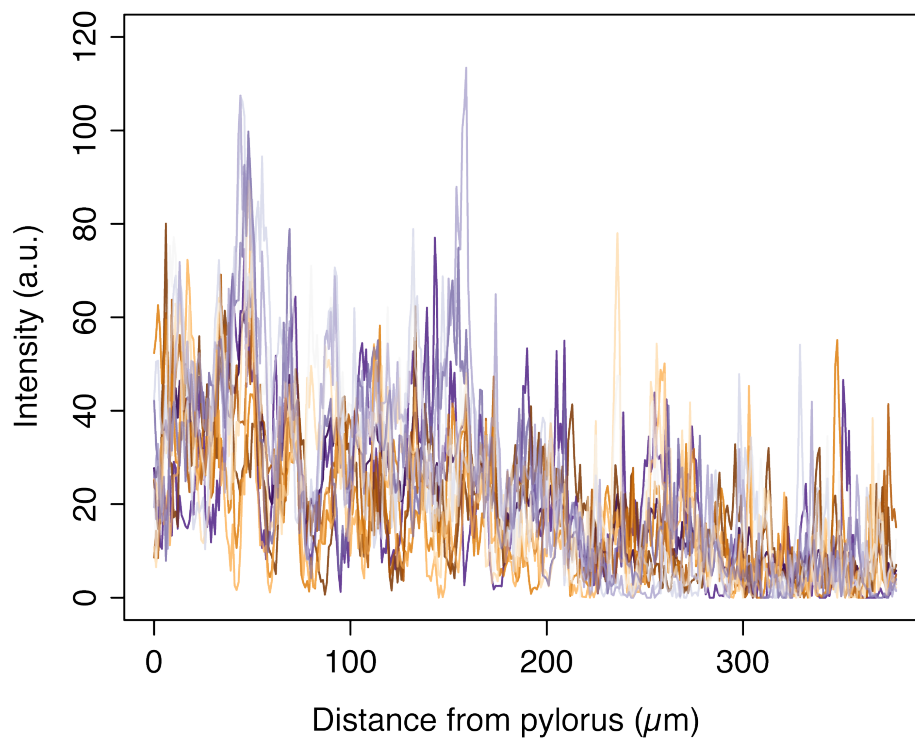


Figure S2. GFP:Wg graded localisation along the posterior midgut. Intensity values of *wg-Gal4*, *UAS-GFP:wg* (Figure 1C–C') along thirteen parallel lines from the pylorus into the posterior midgut (example shown as arrow in Figure 1C'), of which the smoothed average is shown in Figure 1D.

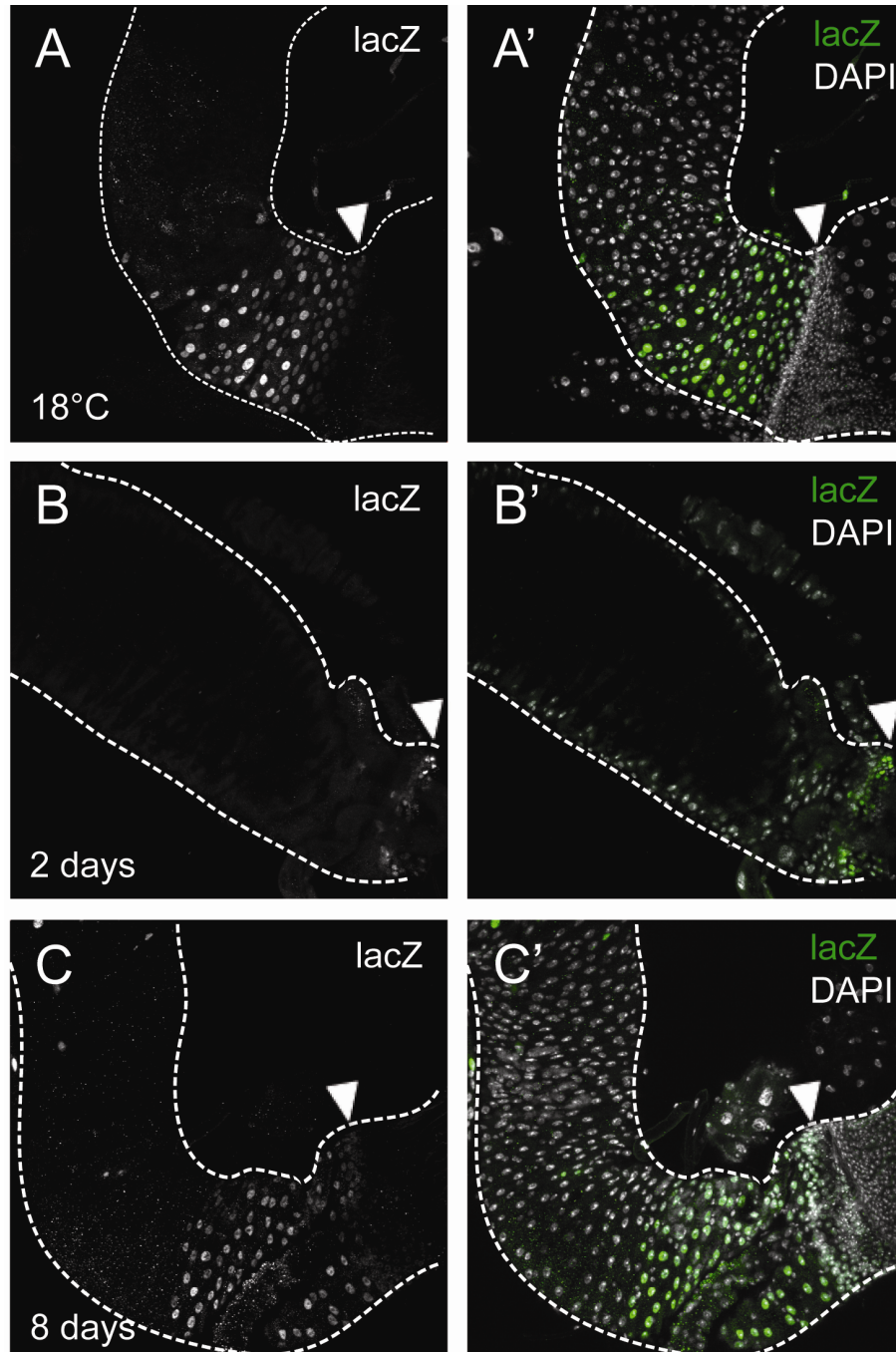


Figure S3. *Wg* is expressed in the pylorus after signal induction at 29°C. (A,A') *wg*[*KO*]-*Gal4*, *UAS-Flp*, *tub-Gal80^{ts}*, *Act<stop>lacZ* guts at 18°C (13–20 day-old flies). Note the non-specific induction at the midgut region connecting with the pylorus. (B–C') *wg*[*KO*]-*Gal4*, *UAS-Flp*, *tub-Gal80^{ts}*, *Act<stop>lacZ* clones after 2 (6–10 day-old flies; B,B') or 8 days (11–18 day-old flies; C,C') of incubation at 29°C for clonal induction. LacZ⁺ cells (anti-βGal, green) are detected in the pylorus (arrowheads) after induction at 29°C (B–C'), but not at 18°C (A,A'). Scale bars: 50 μm.

Table S1. Percentage of GFP-expressing ISCs/EBs overexpressing *arm^{FL}*. The percentage of *esg*⁺ cells overexpressing *arm^{FL}* is calculated for 5 guts (G1–G5), each with 3 fields of view (F1–F3), from the pylorus into the posterior midgut.

	Arm ⁺ GFP ⁺	GFP ⁺	Arm ⁺ GFP ⁺ /GFP ⁺
G1F1	17	58	29%
G1F2	8	25	32%
G1F3	11	30	37%
G2F1	9	18	50%
G2F2	6	26	23%
G2F3	10	54	19%
G3F1	15	74	20%
G3F2	16	64	25%
G3F3	36	105	34%
G4F1	31	70	44%
G4F2	24	123	20%
G4F3	25	114	22%
G5F1	25	48	52%
G5F2	26	76	34%
G5F3	34	91	37%
Average			29%

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Referee Report 13 September 2016

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Bruce A. Edgar

German Cancer Research Center (DKFZ)-Center for Molecular Biology (ZMBH) Alliance, University of Utah, Heidelberg, USA

This article addresses a topic of interest (Wnt signalling in the *Drosophila* gut), and brings new, relevant data to an issue that has remained controversial despite numerous publications. The article is well written, includes a complete up-to-date introduction, and the data that are shown are good quality and believable. Indeed the various methods employed to assess the spatio-temporal patterns of Wnt signalling in this organ are ingenious. Interest from the field is documented by the large number of internet hits already sustained (307). Notwithstanding these strengths, the paper has some notable weaknesses that should really be addressed before it is finalized for indexing.

First of all, it needs to be said that there is no data on Wnt function in this paper, and this is a limitation. Secondly, the two assays used to assess Wg activity, namely *fz3-RFP* expression and Arm stabilization, are not validated with the necessary controls. Although references are cited that support the accuracy of these markers, these references looked in different cell types and so controls in the gut still need to be done. The obvious controls would involve increasing or decreasing Wg expression and seeing increases or decreases in the activity reporters. Finally, the authors need to note that Wg is not the only Wnt expressed in the fly intestine, and so some of the activity patterns might be due to other ligands.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Referee Report 08 August 2016

doi:10.5256/f1000research.8787.r15309



Zhouhua Li

College of Life Sciences, Capital Normal University, Beijing, China

Summary:

It was previously shown that Wingless (Wg)/Wnt signaling is required for the proliferation and

differentiation of intestinal stem cells (ISCs) during development and tissue homeostasis. In this study, the authors examine the expression pattern of Wg ligand and the activation of Wg signaling in the posterior midgut of adult *Drosophila*. Consistent with previous studies, they find that Wg is expressed in the pylorus, but also sporadically in the diploid cells in the midgut epithelium. For unknown reason, they do not observe Wg expression in the visceral muscles (VMs), which is different from previous reports (Lin *et al.*, 2008; Takashima *et al.*, 2008; Tian *et al.*, 2016). Their observation complicates the expression pattern of Wg in adult posterior midgut. They should carefully examine Wg expression in the visceral muscles. Nevertheless, their examination of Wg expression and signaling activation in adult posterior midgut will contribute to the overall effort of understanding the function of Wnt signaling in stem cell biology and tissue homeostasis control.

Comments:

1. The panels in Figure 1 are randomly arranged. The panels should be uniformly arranged with the pylorus to the right side. Same rule should be applied for other Figure.
2. Higher magnification of Fig. 1B is required to better compare Wg expression with Fig.1A. It is very difficult to compare between the Wg antibody staining and *wg>GFP* results.
3. The authors examine flies expressing a membrane-tethered form of Wg (NRT-Wg) using anti-Wg antibody staining, and claim that “Anti-Wg again showed high Wg signals at the pylorus, with no obvious signaling gradient (Fig. 1E)”. They do not detect the signaling activation of Wg in this experiment, thereby it should be more proper to state “with no obvious Wg protein gradient”. Furthermore, the immunofluorescent intensity of Wg staining in Fig.1E is weaker than that in Fig.1A, could the authors explain the difference?
4. Did the authors use the *wgKO-Gal4* line to drive the expression of *UAS-GFP* to compare whether the results they obtain is same as that of *wg-Gal4>UAS-GFP*? Results obtained from *wgKO-Gal4* line should be more convincing as this KO line faithfully recapitulates endogenous Wg expression.
5. The authors do not find Wg expression in the VMs even though they use the same lines as previous studies. LacZ signal can be easily detected the VMs when *wg-lacZ* lines are examined. Can the authors explain why they fail to observe Wg expression in the VMs? Carefully examination of the lines they used, including *wgKO-Gal4* (by *UAS-GFP*), is required.
6. The authors claim that “However, we still detected faint Wg antibody staining in midgut areas anterior to the lowest point of the Wg gradient, and we also observed low activity of *wg* transcriptional reporters (Figure 1)”. However, where in the posterior midgut is the so-called low activity of *wg* transcriptional reporters the authors are referred to in Figure 1?
7. Flp-out system is used to detect Wg expressing cells. However, the experiment is not carried out in a strict manner. When the flies are put into the 18 incubator? Immediately after cross setup or at some stage after cross setup? The authors should specify it, as leaky lacZ expression is observed in flies raised in 18 for 13-20 days. It maybe due to the strong activity of *wgKO-Gal4*, or just handling problem. If it is due to the strong activity of *wgKO-Gal4*, the authors should observe the lacZ expression in the VMs. Moreover, it is critical to examine control flies from 18 at the time points of 2 days and 8 days in order to compare side-by-side to those of flies in 29. To further confirm that the identity of lacZ+ cells at 2 days in 29, it is better to perform DI and Pros staining.

8. In Figure 4, it is not clear to observe a gradient of Wg signaling activation using Dfz2-GFP and sgg-GFP. Lower magnification panels should be included to show whether a gradient exists, like Figure 5.
9. Green signal (likely GFP) can be observed in the left side of the lower dashed box in Fig. 5A. What are those cells? Midgut epithelium or VMs or other tissue? Is it true signal? Interestingly, this signal is not observed in Fig. 5C'.
10. In Figure 7, why do some cell shows strong Arm staining, but no GFP signal?
11. In Page 7, the authors state that "Global arm overexpression suggests that cells in the midgut tissue respond to Wg signalling asynchronously. Also, this forced global Arm induction appeared to perturb regular Arm distribution in the midgut...". This statement is inaccurate, as the authors use flip-out system to induce clones expressing *uas-ArmFL* in adult flies, which could only randomly result in a subset, not all, of cells expressing *uas-ArmFL*. Same phenomena as in Figure 7 are seen in Fig 8B, why some GFP negative cells show very high Arm signal? Closer examinations or explanations are needed.
12. If the authors indeed can not observe the expression of Wg in the VMs after further trials as suggested, they should explain the discrepancy in the discussion part.

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I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Referee Report 31 March 2016

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Todd Nystul¹, Rebecca Kim²

¹ Department of Anatomy, University of California, San Francisco, San Francisco, CA, 94143, USA

² School of Dentistry, University of California San Francisco, San Francisco, CA, USA

Summary:

In this study, the authors investigate the expression of Wg and the activation of Wg signaling in the *Drosophila* intestine. They find that wingless is expressed in the pylorus, consistent with previous studies, and also sporadically by diploid cells throughout the midgut epithelium. However, in contrast with other studies, they do not observe Wg expression in the visceral muscle. The source of wingless in the intestine has been investigated in multiple studies now and appears to be a vexing problem. The reason for the inconsistency between the studies remains unclear, but this study will contribute to the overall effort in the field to find the answer. In addition, we find their observation that Wingless signaling is activated sporadically to be an interesting and understudied aspect of wingless signaling in this tissue.

Comments:

1. Figures 1A and B show results from two methods of detecting Wg expression in the pylorus (antibody stain and Wg-Gal4 > UAS-GFP) but the differences in the presentation of the tissue in the two experiments make it difficult to compare the results. The Wg-Gal4 image is at a lower magnification and the relevant section of the tissue is not well centered in the panel. Comparable images for these two approaches should be provided.
2. Referring to these same panels, the authors contrast their findings to those reported in Tian, *et al* 2016, but we cannot see any apparent contradiction between these two studies. From the images presented, it would seem that approximately 3-4 rows of cells anterior to the pylorus express wingless as detected by antibody staining (Fig 1A' of this study) or Wg-LacZ expression (Fig 1I of Tian *et al*, 2016). The Wg-LacZ expression in Fig S1 of this study shows slightly more restricted expression, confined to maybe only 2 rows, but the row of lacZ+ cells in this image does not even extend across the width of the intestine (as one would expect based on the antibody stain presented in Fig 1A'), suggesting that this image is not representative of all of the wg expressing cells in this region of the tissue. More convincing images should be provided and the precise differences between the studies (if there are any) should be described in more detail.
3. In figure 2, the observation that the frequency of clones increases between 2 and 8 days after temperature shift is interpreted as an indication that Wg is expressed intermittently, but this could also be due to the inefficiency of flippase. Even in cell populations in which flippase is expressed constitutively, all cells do not undergo FRT recombination immediately. Instead, FRT recombination seems to depend at least in part on the phase of the cell cycle as well as the stochastic nature of the reaction, resulting in an inconsistent rate of clone induction. Although other data in this study support the notion that wingless signaling is intermittent, it is important to acknowledge the caveats of this clonal induction approach.
4. The authors state in the discussion "in contrast to Lin *et al*. (2008) and Tian *et al*. (2016), we observed minimal levels of wg expression in the muscle cells using different lines and methods." But the only method presented was the use of the wg-Gal4, UAS-flp system for inducing clones, which is not the same method used by these other studies. In addition, muscle cell nuclei are not visible (even by DAPI staining) in the low magnification image shown in figure 3. Moreover, there appear to be no LacZ positive clones in this tissue at all whereas the segment of intestine shown in figure 2c (also 8 days after temperature shift) has several LacZ positive clones. A much more careful analysis is required, especially because these observations appear to contradict published work. To validate the clonal labeling approach, it would be essential to (1) provide images in which the muscle cell nuclei are clearly visible and preferably stained with a muscle cell marker; (2) quantify the number of intestines analyzed and provide some description of number of muscle cells observed and where they were located in the intestine; (3) ensure that the intestines analyzed contained the expected frequency of LacZ+ clones in the epithelium, as a positive control. In

addition, if the images of wg-LacZ+ muscle cell nuclei in the published studies are wrong for some reason, the authors should replicate this simple experiment (i.e. stain intestines from wg-LacZ+ flies for LacZ and obtain high magnification images of the muscle cells in the same regions of the intestine that were analyzed in these studies) and incorporate the result with their other results into a discussion of whether muscle cells do or do not express wg.

5. What part of the gut is imaged in Figure 6? Is this still part of the posterior midgut?
6. In the assay using overexpression of full length arm to identify cells with active wg signaling, why do some cells express arm but not GFP, such as the cell just below the inset in Fig 7B-B" and the pair of cells in the lower right corner in these same panels?
7. In figure 8, the assumption that "UAS-armFL is induced in all cells", as stated in the figure legend, is inaccurate. In these experiments UAS-armFL is expressed within flipout clones generated by heat-shock induction of flippase. This would result in the random labeling of a subset of cells in the tissue and a clonal marker (such as GFP) would be necessary to determine which cells were part of a clone and which were not. The authors conclude from these experiments that the midgut tissue is responding to Wg signaling in a non-uniform way, but a much more likely possibility is that the cells with elevated arm signal are part of a clone and thus expressing UAS-armFL whereas those with lower arm signal are not part of a clone. A comparison of multiple cells that are all clearly part of a UAS-armFL-expressing clone (identified by the expression of a clonal marker such as GFP) is essential for this approach.
8. Please provide a reference or justification for the use of anti-HRP to detect ISCs and EBs

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We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.
