Response to Comment on "Dynamics of Dpp Signaling and Proliferation Control"

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Schwank *et al*. challenge the temporal growth model with an experiment in which Dpp signal transduction is abrogated. They claim that in this condition, cellular Dpp signaling levels do not increase over time but that cells do grow normally. We repeated this experiment and observed an increase of signaling over time. The results are consistent with a temporal model of growth control.

In a recent study (*1*) we found that, in the *Drosophila* wing disc, the cell proliferation rate correlates with the relative temporal increase in signaling levels of the morphogen Dpp and that, therefore, relative temporal changes in signaling could control proliferation [among other possibilities discussed in the supporting online material (SOM) of (*1*) (text beginning on p. 79 and figure S46)]. A new experiment by Schwank *et al*. challenges this temporal growth model. These authors claim that clones of cells lacking the Dpp signal transducers Mad and Brk do not experience temporal increases in signaling but grow at the same rate as wild-type cells (*2*). They conclude that Dpp signaling is "permissive" for growth control.

In our recent paper, we did study a similar experimental condition: a brk^{XA} $dpp^{d12/d14}$ mutant (*1*). We found that the output of the Dpp signaling pathway, as measured by the transcriptional reporter dad-RFP, was strongly position-dependent and did increase over time in this condition [SOM of (*1*) (text beginning on p. 86 and figure S50)]. This difference from the recent findings by Schwank *et al*. in brk^{M68} mad^{12} clones could in principle be explained by either

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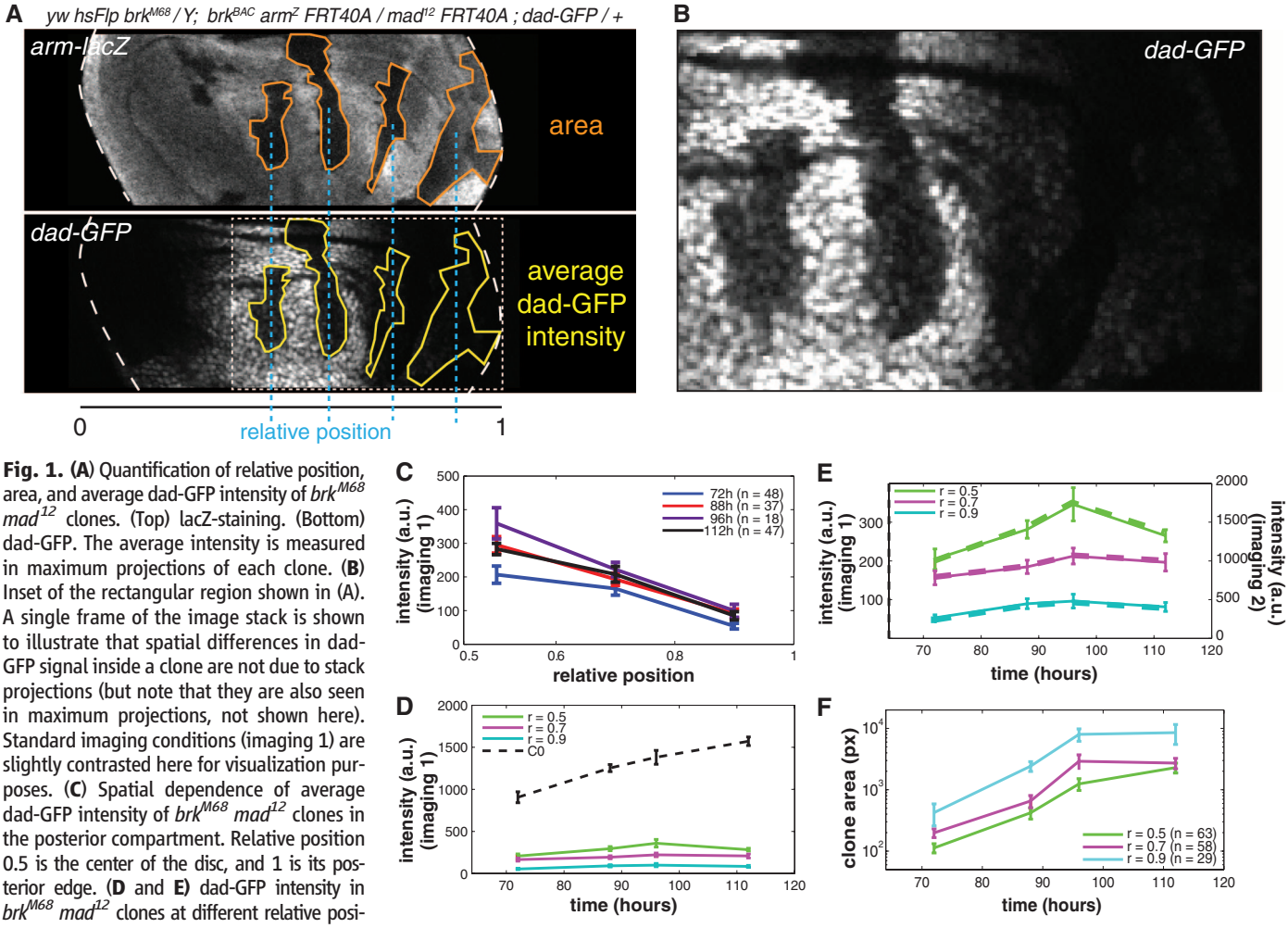

Fig. 1. (**A**) Quantification of relative position, area, and average dad-GFP intensity of brk^{M68} mad^{12} clones. (Top) lacZ-staining. (Bottom) dad-GFP. The average intensity is measured in maximum projections of each clone. (**B**) Inset of the rectangular region shown in (A). A single frame of the image stack is shown to illustrate that spatial differences in dad-GFP signal inside a clone are not due to stack projections (but note that they are also seen in maximum projections, not shown here). Standard imaging conditions (imaging 1) are slightly contrasted here for visualization purposes. (**C**) Spatial dependence of average dad-GFP intensity of brk^{M68} mad^{12} clones in the posterior compartment. Relative position 0.5 is the center of the disc, and 1 is its posterior edge. (**D** and **E**) dad-GFP intensity in brk^{M68} mad^{12} clones at different relative positions (r) over time, as compared to the amplitude of the endogenous gradient (C_0) (measured at the anterior-posterior boundary, not in wildtype clones), when imaged with standard imaging conditions (imaging 1) [(D) and dashed lines in (E)] and imaging conditions adjusted to low signaling levels in clones (imaging 2) [solid lines in (E)]. Independently of the imaging conditions, the signal intensity increases in clones (E). (**F**) Area over time for clones at different relative positions (r).

(i) the genotype—the brk^{M68} allele is amorphic, whereas brk^{XA} is hypomorphic; (ii) the imaging conditions—signaling levels are around an order of magnitude lower in the mutant cells than in wild-type tissue. The temporal model is based on relative temporal changes: ΔC/C rather than ΔC. Even small absolute increases in the signal (ΔC) lead to large relative temporal changes (ΔC/C) when the concentration (C) is small. When imaged with the standard imaging conditions used to quantify the wild-type dad-GFP profile, the dynamic range of the signal in brk^{M68} mad^{12} mutant clones might be too small to quantify temporal increases above noise levels. (We discussed this previously for the brk^{XA} $dpp^{d12/d14}$ mutant in the SOM of (*1*) (text beginning on p. 86 and figure S50); or (iii) the quantification method: Because the signal has a strong spatial dependence [see SOM of (*1*), figure S50, and Fig. 1, A to C], whether the minimum, maximum, or average signal intensity in the clone is measured will influence the result.

To address the genotype issue, we repeated the brk^{M68} mad^{12} mutant clone experiment with the fly lines kindly provided by Schwank *et al*. Like in our previous work (*1*), we quantified the average dad-GFP signal intensity and area of clones as a function of time and relative position. We found that, independently of imaging conditions, signaling levels in brk^{M68} mad^{12} clones (i) are position-dependent (Fig. 1, A to C) and (ii) increase over time (Fig. 1, C to E). Signal increases are accompanied by an increase in clone area (Fig. 1F). Consistent with a temporal growth model, the signal increase is statistically significant in medial clones where the signal (C) is higher and therefore its absolute increase (ΔC) is bigger [$P < 0.05$, two-way analysis of variance (ANOVA) (Fig. 1E). Laterally, where the average dad-GFP signal in clones is lower, the absolute increase is also smaller. Because we used the fly lines of Schwank *et al*. and the results were independent of imaging conditions, the differences between our results and those of Schwank *et al*. are most likely due to the quantification method (Fig. 1A).

The hypothesis that Dpp signaling is only permissive for growth was based on the observation that *tkv brk* or *mad brk* double-mutant clones grow, whereas *tkv* or *mad* mutant clones do not (*3*–*5*). This observation and the assumption that, when brk is mutated, Dpp targets are maximally and uniformly activated led to the hypothesis that growth is implemented by Brk and that spatial differences or temporal changes in Dpp are irrelevant for growth (*6*). However, as Schwank *et al*., we, and others have shown, the output of the Dpp signaling pathway is neither maximal nor uniform in space nor constant in time in brk^{XA} and brk^{XA} $dpp^{d12/d14}$ discs, or indeed in brk^{M68} mad^{12} mutant clones (*1*, *3*–*7*) (Fig. 1). Therefore, these conditions cannot be used as evidence for a permissive Dpp role.

The fact that Dpp signaling levels are not maximal but are very low in brk^{M68} mad^{12} and brk^{XA} $dpp^{d12/d14}$ mutant cells compared with wild type (Fig. 1, A and B) [see also SOM of (*1*), figure S50, and figure 1B in (*2*)] shows that removal of Brk is not sufficient to achieve full activation of Dpp target genes. This is consistent with the observation that both P-Mad and Brk regulate the enhancer of the Dpp target Dad (*8*). Furthermore, in *brk* mutants, dad-RFP expression levels are still graded in space and the repressive activity of Brk apparently has only a minor effect on the level of known output signals (Spalt and Dad) in lateral positions of the disc where extra proliferation effects are seen [see figure S1, H and J in (*6*) and figures S11F and S50A in the SOM of (*1*); discussed in (*9*)]. This is also difficult to reconcile with the permissive model by Schwank *et al*. (*6*), where Dpp signaling and its role in growth critically depend on absolute Brk activity levels.

The brk^{M68} mad^{12} clone experiments highlighted two key features of the Dpp system: (i) the Dpp output (dad-GFP levels) in the complete absence of Dpp input (Mad and Brk) is neither maximal nor zero but is very low and neither constant in space nor time, and (ii) the Dpp input is not essential for proliferation, because brk^{M68} mad^{12} clones still grow. It is worth noting, however, that growth is no longer homogeneous: lateral clones are larger than medial clones (Fig. 1F) [see also, e.g., (*1*, *6*)]. The increase in Dpp target gene expression over time in brk^{M68} mad^{12} clones shows that this experiment is consistent with a model in which growth is regulated by (normally Dpp-dependent) relative temporal increase in the expression of target genes implicated in proliferation. However, the fact that growth can occur in the absence of Dpp input uncovers another interesting aspect of growth control. We speculate that, in brk^{M68} mad^{12} clones, Dpp target genes respond to other inputs that dominate in this particular situation and can lead to growth in the absence of Dpp input. What would temporal changes in Dpp input achieve then in the wild-type situation? Because of scaling of the Dpp gradient, it would ensure position-independent growth and determine the decay time of the proliferation rate, the timing of growth arrest, and therefore the correct final size of the tissue.

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