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Decoding Calcium Signaling Dynamics during *Drosophila* Wing Disc Development

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ABSTRACT The robust specification of organ development depends on coordinated cell-cell communication. This process requires signal integration among multiple pathways, relying on second messengers such as calcium ions. Calcium signaling encodes a significant portion of the cellular state by regulating transcription factors, enzymes, and cytoskeletal proteins. However, the relationships between the inputs specifying cell and organ development, calcium signaling dynamics, and final organ morphology are poorly understood. Here, we have designed a quantitative image-analysis pipeline for decoding organ-level calcium signaling. With this pipeline, we extracted spatiotemporal features of calcium signaling dynamics during the development of the *Drosophila* larval wing disc, a genetic model for organogenesis. We identified specific classes of wing phenotypes that resulted from calcium signaling pathway perturbations, including defects in gross morphology, vein differentiation, and overall size. We found four qualitative classes of calcium signaling activity. These classes can be ordered based on agonist stimulation strength Gαq-mediated signaling. In vivo calcium signaling dynamics depend on both receptor tyrosine kinase/phospholipase C γ and G protein-coupled receptor/phospholipase C β activities. We found that spatially patterned calcium dynamics correlate with known differential growth rates between anterior and posterior compartments. Integrated calcium signaling activity decreases with increasing tissue size, and it responds to morphogenetic perturbations that impact organ growth. Together, these findings define how calcium signaling dynamics integrate upstream inputs to mediate multiple response outputs in developing epithelial organs.

INTRODUCTION

Organ development requires the coordination of many cells to form a structurally integrated tissue. Important properties of the final organ architecture include its shape, size, and spatial distribution of cell types. Notably, the information processing network required for development resembles a "bow-tie" network structure with many input signals that are funneled through a limited number of second messengers (1–3) (Fig. 1 *A*). Signal integration and pathway crosstalk result in many possible downstream outputs that are determined by effector proteins that regulate cellular processes, including cell division, migration, mechanical properties, death, and cell differentiation state (1). However, how these diverse input signals regulate the dynamics of second messengers is poorly understood. Further, how organ-level properties, such as size and shape, emerge from the integration of second messenger signaling remains to be fully elucidated.

A key second messenger that serves as a central node in the bow-tie structure is the calcium ion (Ca^{2+}) (4). Ca^{2+} signaling is a ubiquitous transducer of cellular information and plays key roles in regulating cell behaviors, such as cell division, growth, and death (5). Ca^{2+} dynamics regulate cellular properties and behavior during animal development, and perturbations to Ca^{2+} signaling often lead to disease (3,6–16). Cells can encode complex signals into a Ca^{2+} signaling "signature," which includes amplitude, frequency, and integrated intensity of Ca^{2+} oscillations (13,17). Cells decode these signaling signatures by modulating the activities of downstream enzymes and transcription factors (Fig. 1 *A*).

Intercellular Ca^{2+} signaling is correlated with many developmental processes. For example, they have been found to regulate scale development in the butterfly (18). Ca^{2+} waves are indispensable to activate *Drosophila* egg development (19), and Ca^{2+} spikes are important for development of *Drosophila* and *Xenopus* embryos (8,11,16,20,21). Ca^{2+} signaling responds to Hedgehog (Hh) signaling in the frog

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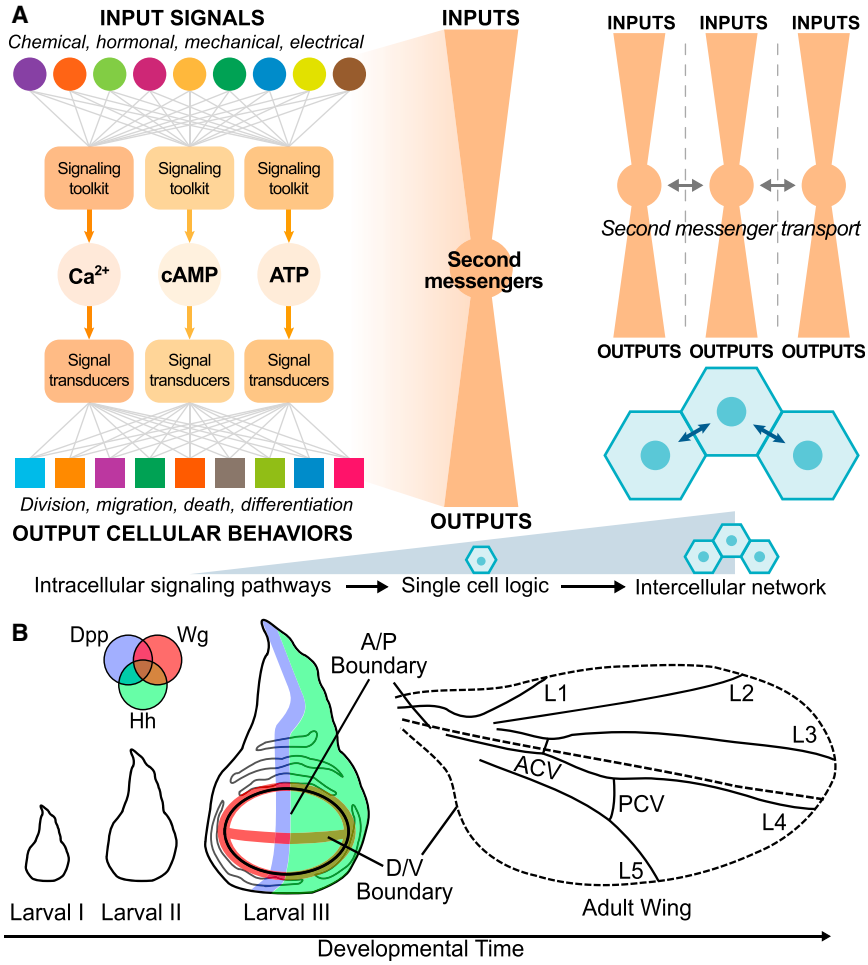

FIGURE 1 The wing disc as a model system of signal integration during organogenesis. (*A*) Second messengers are central nodes of a bow-tie structure that integrates various input signals to inform cellular behaviors. The information from a coupled intercellular network results in emergent tissue-scale phenomena. (*B*) Schematic describing the development of the *Drosophila* wing from the wing disc is shown. The wing disc grows rapidly during larval stages and develops into the adult wing. Spatial patterning of cell fates is determined by the combined actions of morphogenetic signals, including Hedgehog (Hh), BMP/Decapentaplegic (Dpp), and Wnt/Wingless (Wg). After larval growth, the wing disc undergoes morphogenesis to form the wing and the thorax. The larval pouch is approximately an oval. The pouch cells later form the wing blade. In the adult wing, there are L1–L5 veins, anterior crossvein (ACV), and posterior crossvein (PCV). This study focuses on Ca^{2+} signaling dynamics in the third instar wing disc pouch, after primary patterning of anterior/posterior and dorsal/ventral compartments has occurred. To see this figure in color, go online.

neural cord (22), correlates with Decapentaplegic (Dpp) secretion in *Drosophila* imaginal discs (23), and is indispensable for human neural rosette development (24). Ca^{2+} dynamics also are essential for cell migration and tissue contractility in zebrafish, Japanese newt, and chick embryos (9,20,25). Recently, intercellular Ca^{2+} transients (ICTs) have been observed in the *Drosophila* wing disc, both in vivo and ex vivo (15,26–28), and have been implicated as a first response to wounding (27) and robustness in regeneration (15), tissue homeostasis (26), and mechanotransduction (15,29). Inhibition of Ca^{2+} significantly also rescues cancerous overgrowth of wings, thus showing its regulatory role in tissue growth (30). However, a quantitative characterization of Ca^{2+} dynamics in organ development is lacking, in part because of a lack of image-processing methods and a suitable model system to analyze the stochastic nature of the signals. Consequently, there is a need for a systems-level description of Ca^{2+} signaling dynamics to decode the role of Ca^{2+} signaling in organ development.

The *Drosophila* wing imaginal disc pouch is a premier model system to study how epithelial cells undergo specific morphogenetic steps to form the intricate structure of an adult wing (31–36) (Fig. 1 *B*). The wing disc is a powerful model system because of the availability of tools to perturb gene expression in a specific region of a tissue (37). Multiple conserved regulatory modules for tissue development have been discovered in the wing disc. In the larval organ, morphogens divide the wing disc pouch into regions that define the differentiation state of cells and coordinate morphogenesis (Fig. 1 *B*). Morphogen signals that are important for wing disc development include Hh (reviewed in (38)) and Dpp (35,39), which define the anterior/posterior axis. Wg (reviewed in (40)) patterns the dorsal/ventral axis. Widely available genetic tools and simple geometry make the *Drosophila* wing disc a powerful platform to decode Ca^{2+} signaling at the systems level.

Here, we have developed an image-processing pipeline to quantitatively investigate the relationships between Ca^{2+} signaling and organ size. We first genetically inhibited key components of the core Ca^{2+} signaling pathway, termed elsewhere as the "Ca^{2+} signaling toolkit" (5), to define the range of adult wing phenotypes. Next, we performed a dose-response experiment of fly extract (FEX) to order the

specific classes of Ca^{2+} signaling based on the relative concentration of agonist-based stimulation. We use the term "Ca^{2+} signaling activity" to collectively refer to these four Ca^{2+} signaling classes. We investigated how these classes of Ca^{2+} signaling correlate with disc age and size, both in vivo and ex vivo. We established that FEX stimulates Ca^{2+} through Gαq/phospholipase C (PLC) β signaling through genetic perturbation experiments. Next, we developed advanced image-analysis tools to handle the large data sets to extract quantitative Ca^{2+} dynamics measurements. Using this image-analysis pipeline, we identified a negative power-law correlation between integrated Ca^{2+} signaling activity and wing disc pouch size. We examined how the genetic state of the tissue modulates Ca^{2+} signaling dynamics through genetic perturbation. Ca^{2+} signaling activity responds to perturbations that impact the morphogenic state of the tissue, resulting in deviations from the quantitative correlation curve between Ca^{2+} signaling activity and developmental progression. Together, these trends indicate that Ca^{2+} signaling provides a biochemical readout of organ size. The results suggest Ca^{2+} could be involved in modulating cell proliferation activity during larval growth. In sum, this study provides significant evidence that Ca^{2+} signaling contributes to intercellular consensus-building during organ development (41). This research paves the road of revealing the quantitative and mechanistic regulation of organ development by Ca^{2+} signaling in future studies.

MATERIALS AND METHODS

Fly strains and genetics

Phenotypic analysis in Fig. 2 was performed using the MS1096-Gal4 line (BL#8860). A *nub > GCaMP6f* reporter tester line was used to measure relative Ca^{2+} signals in the wing disc pouch (29,42). Gene perturbations were generated by crossing the tester line to either RNA interference

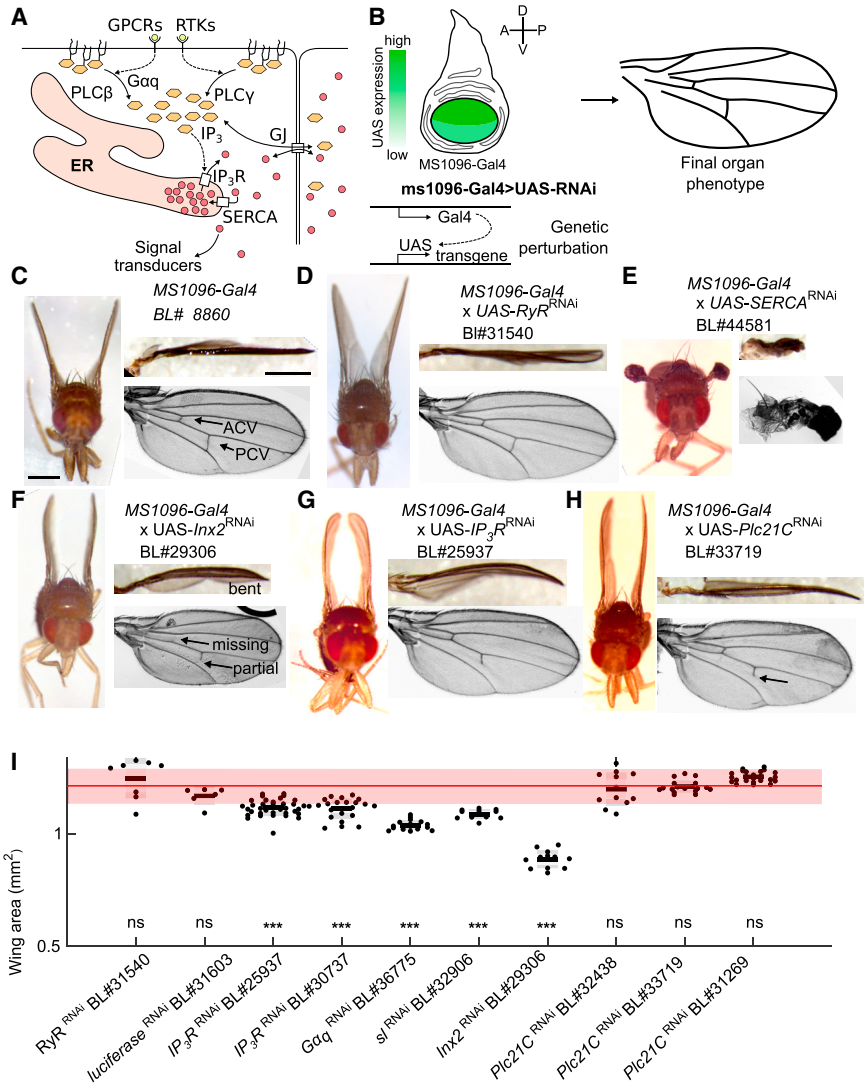

FIGURE 2 Core calcium signaling components modulate wing size, shape, and vein differentiation. (*A*) Schematic representing mechanism of the core IP_3R-mediated Ca^{2+} signaling pathway is shown. ER, endoplasmic reticulum; Gαq, G protein α q subunit; GJ, gap junction; GPCR, G protein-coupled receptor; IP_3, inositol trisphosphate; IP_3R, IP_3 receptor; PLC, phospholipase C, which includes multiple isoforms: PLCγ homolog (sl) and PLCβ homologs (Plc21C and norpA); RTK, receptor tyrosine kinase; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase. (*B*) Schematic of expression pattern of MS1096-Gal4, which is expressed more strongly in the dorsal compartment, is shown. The GAL4/UAS system was used to express RNAi constructs starting during the third instar larval stage. Adult wing phenotypes were used to provide a readout of final phenotype. (*C–H*) Micrographs of adult fly, orthogonal view of wing, and mounted view of wing for indicated crosses are shown. Scale bars represent 0.5 mm. (*I*) Total area of adult wings under various perturbations of Ca^{2+} signaling genes driven by MS1096-Gal4. The gray boxes indicate standard deviation. ns, not significant. $*p < 0.05$; $***p < 0.001$ by *t*-test after Bonferroni correction. The red band provides the average and SD of multiple control crosses. To see this figure in color, go online.

(RNAi)-based transgenic lines (UAS-*Gene X*^{RNAi}) or gene overexpression lines (UAS-*Gene X*). When possible, multiple independent RNAi lines were tested for each gene investigated. As a second round of controls, the tester line was also crossed to *UAS-mCherry* to obtain ratiometric measurements (Fig. S6). Validation of Ca^{2+}-based RNAi lines is reported in the Supporting Materials and Methods, Section 5 and in (29). Further details on genetic crosses and validation are detailed in the Supporting Materials and Methods.

In vivo imaging setup

Wandering third instar larvae were collected for imaging and rinsed in deionized water. Larvae were dried and then adhered to a coverslip for imaging with Scotch tape covering the larvae. The larvae were attached with their spiracles facing toward the coverslip to align the wing discs toward the microscope. The larvae were imaged at 20× magnification for 20 min on an EVOS FL Auto microscope (Thermo Fisher Scientific, Waltham, MA). Images were taken every 15 s.

Organ culture media

Organ culture studies were performed as detailed in (29,43) with modifications. We used 15% FEX for genetic perturbation studies performed ex vivo because it improved the signal/noise ratio for reliable quantification of Ca^{2+} dynamics, although there was more activity than in vivo measurements. ZB media +15% FEX contains 79.4% (v/v) ZB media (44), 0.6% (v/v) of 1 mg/mL insulin (Sigma-Aldrich, St. Louis, MO), 15% (v/v) ZB-based FEX, and 5% penicillin/streptomycin (Gibco, Carlsbad, CA). ZB media was developed as a chemically defined media to support *Drosophila* cell culture (44) and was used as the basal media for all experiments. The text of Supporting Materials and Methods provides details of FEX preparation.

Wing disc imaging setup

Wing discs were dissected from third instar larvae, cultured, and imaged as detailed in the Supporting Materials and Methods. All experiments were performed immediately after dissection to minimize the time in culture. Discs were imaged at three *z*-planes with a step size of 10 μm and 20× magnification and 10 s intervals for a total period of 1 h, with 200 ms exposure time and 50 mW 488 nm laser exposure at 44–70% laser intensity (Fig. S5). For some experiments, mCherry signal was imaged with a 560 nm laser (50% laser intensity).

Ex vivo data preprocessing

Microscopy resulted in four-dimensional time-lapse data (512 pixels by 512 pixels by three z-planes by 361 time points). The *z*-stack data was max-projected in FIJI (45) to yield *z*-projected time-lapse videos. Time points were selected so that discs were only analyzed during times in which disks did not move in the *z*-direction, with the shortest time lapse analyzed being 20 min.

Image segmentation and registration

For the image frame at each time point, a region of interest (ROI) defining the pouch was obtained using a novel deep-learning-based segmentation algorithm that we developed and is described in (46). Each frame was segmented with a fully convolutional network (FCN) module, and the ROI boundaries were refined by a graph-search algorithm. The FCN module was trained on 800 images of wing discs expressing *nub-GCaMP6f* with various stages of Ca^{2+} signaling activity. B-spline-based registration was used to transform image frames onto a shape defined by the first time point. The pipeline ensures that coordinates within the pouch are consistent from frame to frame, allowing spatial analysis of Ca^{2+} signatures.

The FCN was used to segment only the pouch region, excluding the *nub*-expressing hinge region dorsal to the pouch. Note that the traditional image segmentation methods explored could not distinguish well between these two types of regions. For data that had both a GCaMP6f and RFP channel, Rigid Registration was used in FIJI (45), as the RFP channel does not exhibit oscillatory dynamics.

Identification of the axes

The posterior and D compartments were identified manually based on the characteristic shapes of the pouch (Figs. 1 *B* and S1). Pouches were flipped so that the A compartment was on the left and the D compartment was on the top. A custom MATLAB (The MathWorks, Natick, MA) graphical user interface was used to reduce error in manual pouch orientation (Fig. S2). The graphical user interface also helped to reduce error in the manual pouch orientation. Details are found in Supporting Materials and Methods and Fig. S2.

Feature extraction

Images of each wing disc pouch were divided into square ROIs of 2.8 μm. We obtained time-averaged Ca^{2+} signatures for each ROI and represented them as a spatial map (see Fig. 6 *D*). Each set of spatial maps was averaged to obtain a composite spatial map for each feature. The Ca^{2+} signatures include amplitude, frequency, and integrated intensity. The Ca^{2+} signature was extracted by taking average intensity (F(t)) from 4 × 4 pixel (2.8 × 2.8 μm) spatial bins arrayed with square packing across the segmented disc pouch (Fig. S1, *A–C*). Video durations ranged from 20 to 60 min. Further details are included in the Supporting Materials and Methods Text and Figs. S1 and S2.

Qualitative analysis

The in vivo data were analyzed blindly and qualitatively by dividing each video into 135-s-clip segments and scoring them in random order. Ca^{2+} signaling activity in each segment was manually classified into one of the four previously mentioned categories. One sample may, in this way, be classified in multiple bins for the full video. Each clip was classified as having either no activity exceeding basal levels, cellular spikes, ICTs, intercellular Ca^{2+} waves (ICWs), fluttering, or being unanalyzable because of larval motion. If a time-lapse segment includes two or more categories, the most active category was annotated. This was done with a custom MATLAB script that divided each video into clips. One random clip was looped at a time until all the data had been classified. Afterwards, the fraction of time spent in each class was reported for each condition. Additional procedural details are included in the Supporting Materials and Methods.

Statistical analysis and visualization

The median value of each summary statistic for each ROI in each disc was compared across genetic conditions, pouch sizes, and between compartments (Fig. 6). ROIs within two spatial bins of the edge were excluded from the median to avoid edge effects. A two-tailed, unpaired, Student's *t*-test was performed in comparisons across conditions. The visualization of the manifold identified in Fig. 8 is a multivariate kernel density estimate with a cutoff value selected to display a three-dimensional region that encompasses a uniform density of measurements. As discussed in the Supporting Materials and Methods, this was done to clarify the manifold along which developing wing discs lie in the "morphological cell signaling" space.

RESULTS

The core calcium signaling pathway regulates final wing size, shape, and patterning robustness

The regulation of cytosolic Ca^{2+} levels occurs by regulating fluxes of Ca^{2+} between Ca^{2+} stores, such as the endoplasmic reticulum, and the cytosol (Fig. 2 *A*). We knocked down genes known to regulate Ca^{2+} signaling dynamics and characterized final wing morphologies as an initial step to systematically identify phenotypic outputs. We used *MS1096-Gal4* to express RNAi. *MS1096-Gal4* is expressed in the wing pouch, the precursor of the wing blade, and has stronger expression in the dorsal than the ventral compartment (Fig. 2 *B*; (47,48)). The parental *MS1096-Gal4* line was crossed to control lines such as UAS-RyR^{RNAi} (where RYR is not expected to have significant expression (49)) or other UAS-RNAi constructs that do not target endogenous messenger RNA (such as luc^{RNAi}). These did not exhibit significant morphological or size defects (Fig. 2, *C* and *D*).

Based on the wealth of literature on wing development, adult wing phenotypes provide important functional information into the downstream outputs of calcium signaling. We found that there are four major categories of wing defects in adult wings when perturbing Ca^{2+} signaling genes: 1) crumpling and blistering (Fig. 2 *E*), 2) wing bending (Fig. 2, *F–H*), 3) loss of crossveins and other vein defects (Fig. S11), and 4) reduction in wing size. *MS1096* > $SERCA^{RNAi}$ led to a severe shriveled wing (Fig. 2 *E*) (15,26). Knocking down gap junctions through *MS1096* > $Inx2^{RNAi}$ (RNAi phenotype for line is described in Pézier et al. (50); see Table S1) led to significantly smaller wings with defects, including partial loss, total loss, and deviation in the posterior crossvein (PCV) and anterior crossvein (Fig. 2, *F* and *I*). We observed that partial loss of the PCV was always the loss of the posterior side of the PCV and never the anterior side (n = 33). *MS1096* > IP_3R^{RNAi} exhibited similar wing defects (Fig. 2, *G* and *I*). The loss of crossveins is consistent with Ca^{2+} signaling playing a role in the secretion of growth and patterning molecules, such as Dpp (23). Earlier reports have also implicated Ca^{2+} signaling genes (including *Stim* and *Orai*) in vein patterning (26,51).

Notably, wing size was also significantly reduced for RNAi knockdowns to many of the core Ca^{2+} signaling components. *MS1096* > $Inx2^{RNAi}$ (Fig. 2 *I*), *MS1096* > IP_3R^{RNAi} (Fig. 2 *G*), and *MS1096* > $G\alpha q^{RNAi}$ (data not shown) wings are all bent and show a significant reduction of wing size. If a certain gene regulates growth, the asymmetric perturbation by *MS1096-Gal4* could lead to a differential reduction of dorsal and ventral wing area. This differential reduction of area could lead to bending of the final wing, which is formed by apposition of the dorsal and ventral compartments during pupal morphogenesis (Fig. 2, *C–G*). Together, these results demonstrate that Ca^{2+} signaling plays important roles in wing morphogenesis, vein patterning, and growth control.

Three homologs of the PLC family are present in *Drosophila*. *Plc21C* (PLCβ1) and *norpA* (PLCβ4) are stimulated by G protein-coupled receptors (GPCRs), and *sl* (PLCγ) is stimulated by receptor tyrosine kinases (RTKs), such as insulin receptor and epidermal growth factor receptor (52). We observed that *MS1096* > $Plc21C^{RNAi}$ wings had a similar bent wing phenotype and defects in crossvein patterning compared to the *MS1096* > IP_3R^{RNAi} and *MS1096* > $Inx2^{RNAi}$ wings, but did not have the same wing size phenotypes (Figs. 2, *H* and *I* and S9). *MS1096* > *norpA* also did not cause significant size phenotype (data not shown). An explanation for the weaker defect of *MS1096* > $Plc21C^{RNAi}$ compared to knockdown of IP_3R or $G\alpha q$ could be that it is caused by incomplete knockdown for the given RNAi lines tested or redundancy between PLCβ homologs. Another possibility is that the bent wing phenotype arises from defects in the mechanical regulation of cells during morphogenesis and is a separate phenotype from growth regulation. Previously, *sl* has been shown to modulate wing size (53), which is in agreement with our results (Fig. 2 *I*).

Taken together, these results show that IP_3R-mediated Ca^{2+} signaling contributes to multiple aspects of wing morphogenesis. Here, we have established that Ca^{2+} plays an important role in wing size control. These observations propelled us to investigate further the interplay and correlation between Ca^{2+} signaling dynamics and larval wing disc size, which is the stage of development governing overall organ growth as reviewed in (34,54).

Varying agonist concentration results in four ordinal classes of calcium signaling activity ex vivo

Previously, FEX has been reported to stimulate Ca^{2+} activity by our group and others (15,26,28,29). We first tested whether the extent of Ca^{2+} signaling exhibits a concentration dependence on FEX. To do so, we performed ex vivo imaging of Ca^{2+} signaling activity in the wing disc pouch using a genetically encoded Ca^{2+} sensor (*nub* > *GCaMP6f*). Wing discs were cultured with variable concentrations of FEX. We observed a consistent progression of four classes of Ca^{2+} signaling activity as the concentration of FEX increased (Fig. 3, *A–E*): 1) local Ca^{2+} spikes that occur in single cells (0% v/v FEX, Fig. 3 *A* and *A′*; Video S14); 2) stochastic, short-distance ICTs (2.5% v/v FEX, Fig. 3, *B* and *B′*; Video S15); 3) oscillatory long-range ICTs that we term ICWs (5–20% v/v FEX, Fig. 3, *C* and *C′*; Videos S16, S17, and S18); and 4) elevated cytoplasmic Ca^{2+} exhibiting rapid, low-amplitude oscillations that we term "fluttering" (40% v/v FEX, Fig. 3, *D*and *D′*; Video S19). Because the observed Ca^{2+} activity classes depend on the dose of FEX, we established the Ca^{2+} signaling activity to be ordinal, with spikes being the least active and fluttering being the most active. These results suggest that transitions

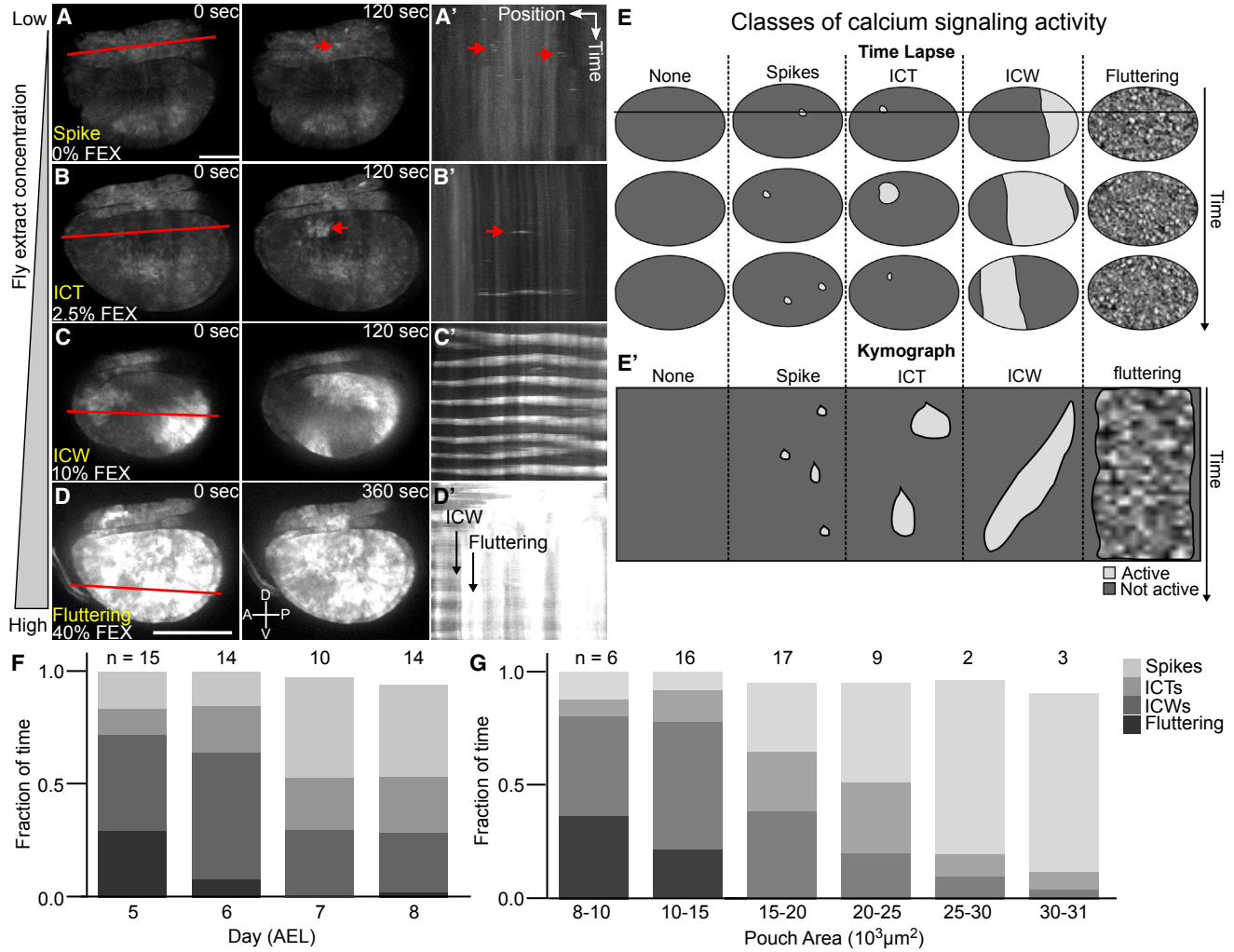

FIGURE 3 Increased stimulation of calcium signaling results in a progression of signaling classes. Wing discs were cultured in chemically defined ZB media with varying concentrations of fly extract (FEX): (*A*) 0%, (*B*) 2.5%, (*C*) 10%, and (*D*) 40% v/v FEX. These conditions stimulate four classes of Ca^{2+} signaling activity: spikes, ICTs, ICWs, and "fluttering" Ca^{2+} oscillations (Videos S14, S15, S16, S17, S18, and S19). (*A–D*) Montages from ex vivo time-lapse videos are shown. (*A′–D′*) Kymographs of the corresponding time-lapse videos along the indicated red lines are shown. Note: the kymograph slice in A was selected in the hinge region to clearly indicate examples of spikes. (*E*) Schematic of four classes of Ca^{2+} signaling activity is shown. The horizontal line represents the *x-y* position of the kymograph. (*E′*) Schematic representation of kymographs taken from (*E*) is shown. (*F* and *G*) Average fraction of time in each class of Ca^{2+} signaling activity for 15% FEX varies by (*F*) pouch age and (*G*) pouch size. Wing disks are oriented with anterior to the left and dorsal compartment to the top. AEL indicates days after egg laying. Position and timescale bars, 25 μm and 15 min. For (*F* and *G*), $p < 0.001$ for ordinal logistic regression model versus constant model. n indicates the sample size. To see this figure in color, go online.

in Ca^{2+} activity occur as the overall level of IP_3R-based signaling increases.

Next, we quantified the percentage of each class of Ca^{2+} signaling activity in wing discs of different ages and sizes throughout development while keeping the FEX concentration constant at 15%. The highest activity observed within each time segment established the annotated signaling class. For example, if ICTs and ICWs occur in the same time within the pouch, we characterized that fraction of time as ICW activity. We found that the occurrence of the highest Ca^{2+} signaling activities (ICWs and fluttering) decreased from 72% at 5 days after egg laying (AEL) to 29% at 8 days AEL (Fig. 3 *F*). Also, the occurrence of the highest Ca^{2+} signaling activity deceased from 80% in small pouches ($<8 \times 10^3$ μm^2) to 5% in large pouches ($>30 \times 10^3$ μm^2, Fig. 3 *G*). Importantly, our observations demonstrate that ex vivo Ca^{2+} signaling activity in 15% FEX correlates strongly with both the age of the larvae and the size of the wing disc pouch.

To investigate the mechanism of ICW formation, we systematically inhibited Ca^{2+} signaling genes using RNAi when stimulated with 15% v/v FEX. Importantly, we found significantly fewer ICWs when *Plc21C*, *IP_3R*, or *Gαq* were knocked down (Fig. 4, *A–D*; Videos S11, S12, and S13). Knockdown of *Plc21C* still resulted in localized cell-level spike activity but not ICWs. This suggests PLC is either

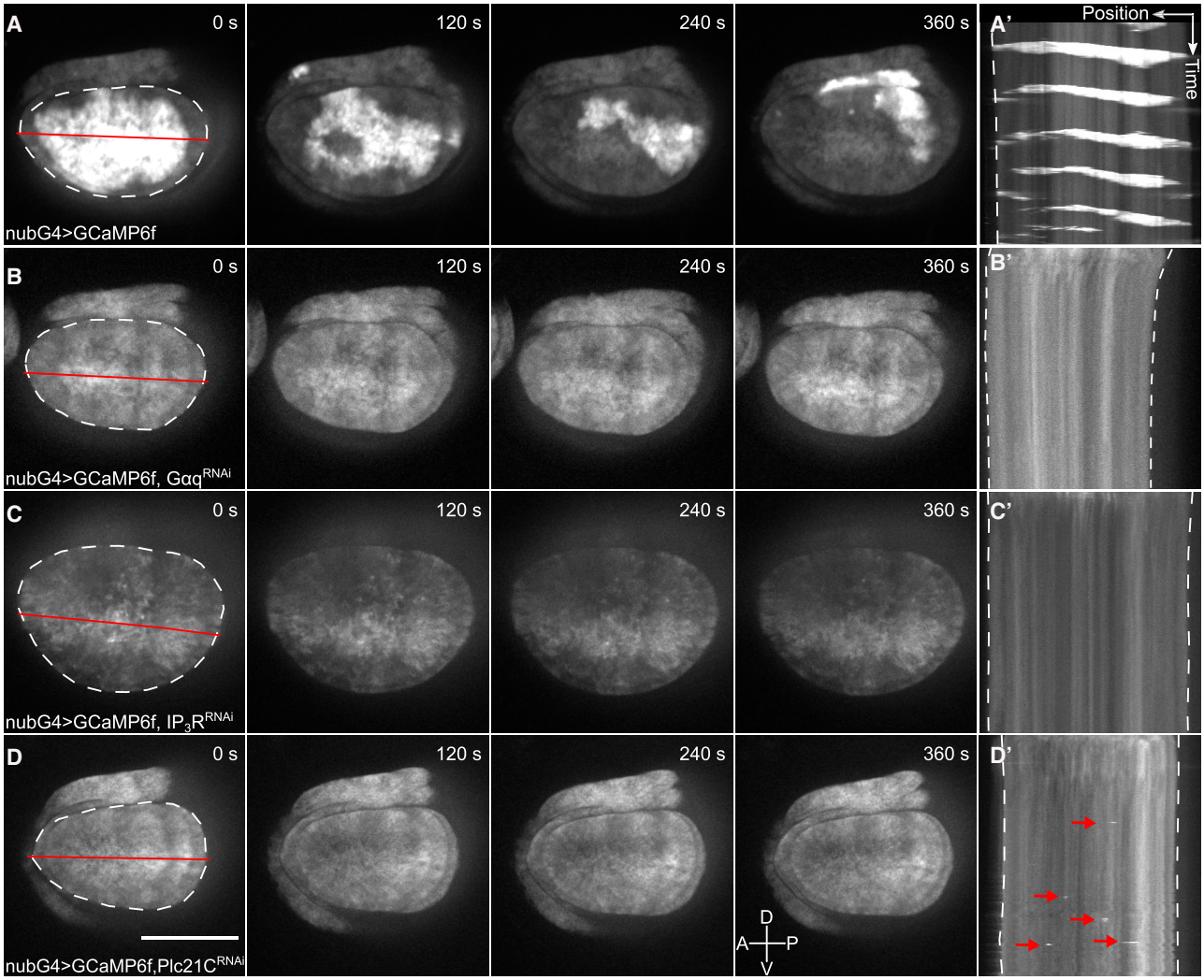

FIGURE 4 FEX-induced calcium waves require GPCR/Gαq signaling. (*A–D*) Montages from ex vivo time-lapse videos and (*A′–D′*) kymographs of the corresponding time-lapse videos are shown. The genotypes of the wing discs are indicated on the bottom left. We observed ICWs in (*A*) and no Ca^{2+} signaling activity in (*B–D*). The cross indicates the orientation of the wing disc (Videos S10, S11, S12, and S13). The red arrows indicate single-cell spikes. Position and timescale bars, 25 μm and 15 min. White dotted line represents the pouch boundary. Red line represents the *x-y* position of the kymograph. The RNAi lines are as follows: $G\alpha q^{RNAi}$, BL#36775; IP_3R^{RNAi}, BL#25937; and $Plc21C^{RNAi}$, BL#31719. To see this figure in color, go online.

partially knocked down, or there is redundancy between PLC isoforms (Fig. 4 *D*). Overall, these results demonstrate that FEX-induced ICWs require GPCR/Gαq-based signaling. Thus, our ex vivo results provide additional insights into the mechanism of intercellular Ca^{2+} signaling propagation in the wing disc.

Calcium signaling dynamics are regulated during wing disc development

In vivo Ca^{2+} transients were noted in previous reports. However, a systematic characterization has not been performed because of the technical challenges of in vivo imaging. To address this knowledge gap, we tested whether in vivo Ca^{2+} signaling activity is a regulated phenomenon. We gently immobilized the larvae with tape on a coverslip to enable in vivo imaging for durations of 20 min. We developed a semi-automated pipeline to blindly classify Ca^{2+} signaling activity in video segments, matching the qualitative ex vivo analysis. Interestingly, we identified and quantified the same four classes of Ca^{2+} signaling activity that are observed ex vivo (Fig. 3, *E* and *E′*): 1) local Ca^{2+} spikes (Fig. 5 *A*; Video S1), 2) ICTs (Fig. 5 *B*; Video S2), 3) ICWs (Fig. 5 *C*; Video S3), and 4) fluttering (Fig. 5 *D*; Video S4).

Next, we quantified the percentage of four classes in wing discs of different ages and sizes throughout development (n = 103 videos). We found that observable Ca^{2+} signaling activity decreases from 27% to 6% of the time from 5 to 8 days AEL (Fig. 5 *E*). Ca^{2+} signaling activity also shifts from fluttering and ICW classes at day 5 to spikes and

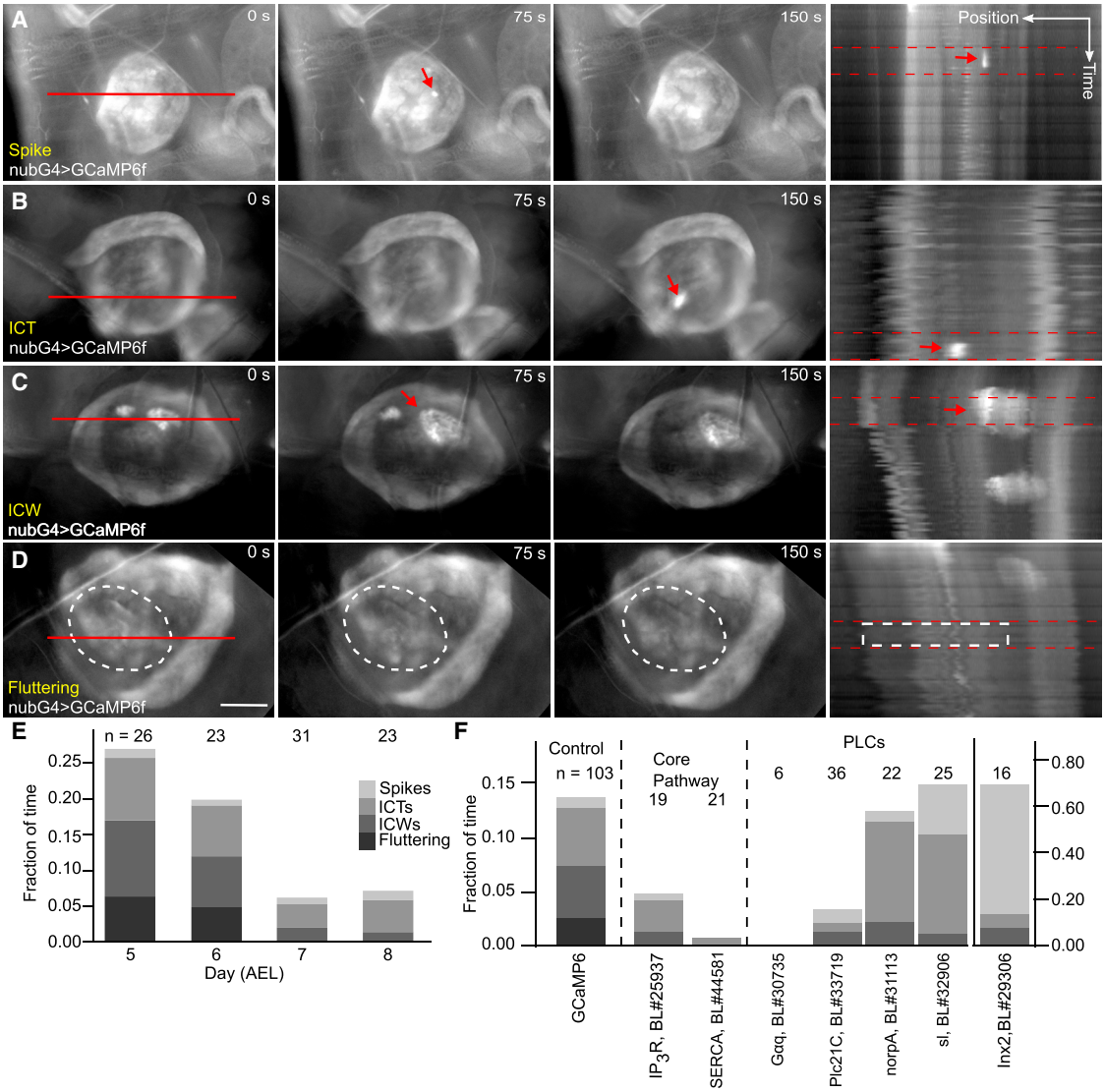

FIGURE 5 Overall calcium activity decreases throughout development in vivo and is regulated by multiple PLCs. (*A–D*) Montages from in vivo time-lapse videos of *nub > GCaMP6f* larval wing discs are shown. A diverse range of Ca^{2+} dynamics are observed. The red lines represent the *x-y* position of the kymograph and are 200 μm long. The red arrows indicate the Ca^{2+} events. The white circle indicates the area of fluttering Ca^{2+} oscillation. Because of its nature, the supporting videos demonstrate the fluttering oscillation much more clearly (Videos S1, S2, S3, and S4). (*A′–D′*) The kymograph of the corresponding time-lapse videos is shown. The kymographs between red dotted lines correspond to the montage on the left. Scale bars, 50 μm. Position and timescale bars, 50 μm and 5 min. (*E*) The duration of each category of Ca^{2+} oscillation as a fraction of total time is shown. The total Ca^{2+} activation time (the height of the column) decreases as wing disks grow older. $p < 10^{-15}$ by ordinal regression. (*F*) In vivo Ca^{2+} signaling transients are regulated by multiple PLC isoforms, either through Gαq/PLCβ (Plc21C), which greatly inhibits large waves, or RTK/PLCγ, which also modulates Ca^{2+} signaling levels. Videos S5, S6, S7, S8, S9, and S10 provide representative examples for each genetic perturbation. For Inx2, the *y*-axis is on the right, showing upregulation of small cellular spikes. Labels represent crosses of UAS-RNAi lines to parental *nub* > GCaMP6f line. AEL indicates days after egg laying. To see this figure in color, go online.

ICTs at day 8 (Fig. 5 *E*, $p < 10^{-15}$ by ordinal regression). Note, the GCaMP6f test line shows slower growth compared to nontransgenic flies, resulting in delayed larval development.

To verify the mechanisms regulating in vivo Ca^{2+} activity, we performed similar perturbations with *UAS-RNAi* lines (Fig. 5 *F*; Tables S6 and S7; Videos S5, S6, S7, S8, S9, and S10). We observed that genetically knocking down *SERCA* and *IP$_3$R* greatly reduced Ca^{2+} signaling activity. Perturbation of GPCR-stimulated signaling through $Plc21C^{RNAi}$ inhibited ICWs. Perturbation of RTK signaling through *sl/PLC*γ also reduced the observed proportion of higher classes of Ca^{2+} signaling activity but increased the proportion of time with observed cellular spikes (Fig. 5 *F*; Tables S6 and S7). The disruption of gap junctions through $Inx2^{RNAi}$ led to a significant increase in the number of spikes throughout the tissue, but Ca^{2+} signals did not travel from cell to cell across the tissue. This suggests that gap-junction

communication synchronizes Ca^{2+} activity across the tissue and leads to the reduced frequency of initial stimulatory events. The increase in cellular Ca^{2+} spike activity with gap junctions inhibited may suggest that it is easier to stimulate smaller systems. $Inx2^{RNAi}$-expressing wings are significantly smaller, suggesting the possibility that part of the growth phenotype is due to reduced coordination in Ca^{2+} signaling synchronization between cells.

To summarize, the multiple classes of observed Ca^{2+} signaling activity are a recurring phenomenon during in vivo development (29). Importantly, we found that Ca^{2+} signaling activity in wing discs correlate with the larval age. This correlation suggests that Ca^{2+} signaling activity decreases as wing discs approach their final size (Fig. 5 *E*).

Calcium signaling activity is spatiotemporally patterned by morphogen signaling

It is challenging to perform quantitative spatial analysis of Ca^{2+} signaling in vivo because of larval motion, optical interference from the cuticle, and limited imaging duration. To overcome this challenge, we developed a quantitative pipeline to analyze ex vivo-imaged wing discs, which enabled more detailed spatiotemporal analysis (Fig. 6; Fig. S1–S4). In these experiments, we stimulated Ca^{2+} signaling with 15% FEX and recorded and analyzed Ca^{2+} signaling responses under a range of genetic perturbations. We then inferred how the "organ state" influences the calcium responses.

As suspended micro-organs, wing discs often exhibit movement, even in ex vivo cultures. Image registration is a problem with dynamic systems because the most distinct landmarks in the image are constantly changing as the signal moves along the tissue. To solve this problem, we built a pipeline that detects the borders of the pouch using a deep neural network approach that transforms the underlying images onto the initial mask (Figs. 6, *A* and *B* and S4; (46)). Ca^{2+} signatures were extracted from signals that are taken from a square grid across the tissue (Fig. 6, *C* and *D*). The resulting spatial maps were transformed onto a canonical domain represented by the average positions of the pouch boundaries and axes using a previously described method (Fig. 6 *E*; (55)). Finally, we report the median of the spatial maps (Fig. 6 *F*).

The spatial analysis revealed that a time-averaged patterning of amplitude emerges as the wing disc grows bigger. Across all wing discs, the average amplitude is 33% higher in the posterior compartment than in the anterior compartment (Fig. 7 *A*). In smaller discs (area $< 1.6 \times 10^4$ μm^2), the difference of amplitude between the anterior compartment and the posterior compartment is not significant (Fig. 7 *B*). In larger discs (area $> 1.6 \times 10^4$ μm^2), the patterning of amplitude emerges between the anterior and the posterior compartments, in which the amplitude is lower in the anterior compartment and higher in the posterior compartment, and the difference is significant (Fig. 7 *B*).

The discovery of anterior-posterior patterning of amplitudes suggests that upstream morphogen pathways may

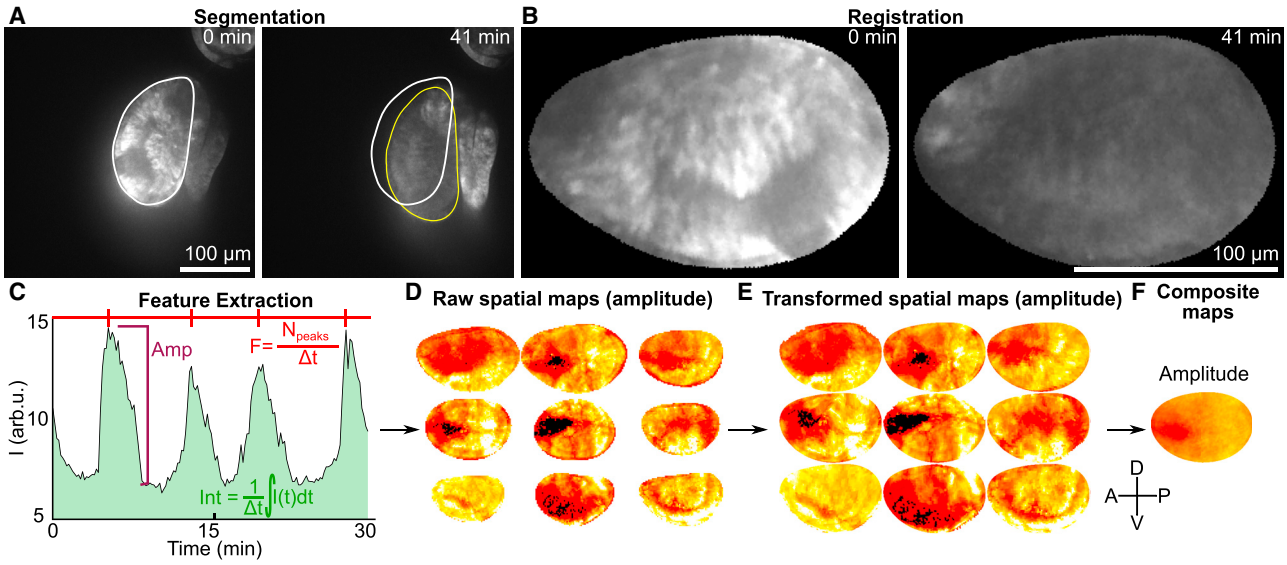

FIGURE 6 Pipeline for spatiotemporal quantification of calcium signatures. (*A*) Automatic segmentation of pouch region with deep-learning segmentation algorithm was developed for this study. Outlines represent the pouch at the initial time point (*white*) and the final time point (*yellow*). (*B*) Registration of raw images onto canonical pouch shapes is shown. (*C*) Extraction of amplitudes (Amp), frequency (F), and integrated intensity (Int) from Ca^{2+} traces for individual region of interest (ROI) was performed. (*D*) The Ca^{2+} signature is computed for each spatial position within each pouch to generate a spatial map. (*E*) Each spatial map is transformed onto a canonical coordinate system to align the anterior-posterior and dorsal-ventral axes. (*F*) Each set of transformed spatial maps is averaged at each position to generate a composite spatial map. The cross indicates the orientation of the wing disc. Scale bars, 100 μm. To see this figure in color, go online.

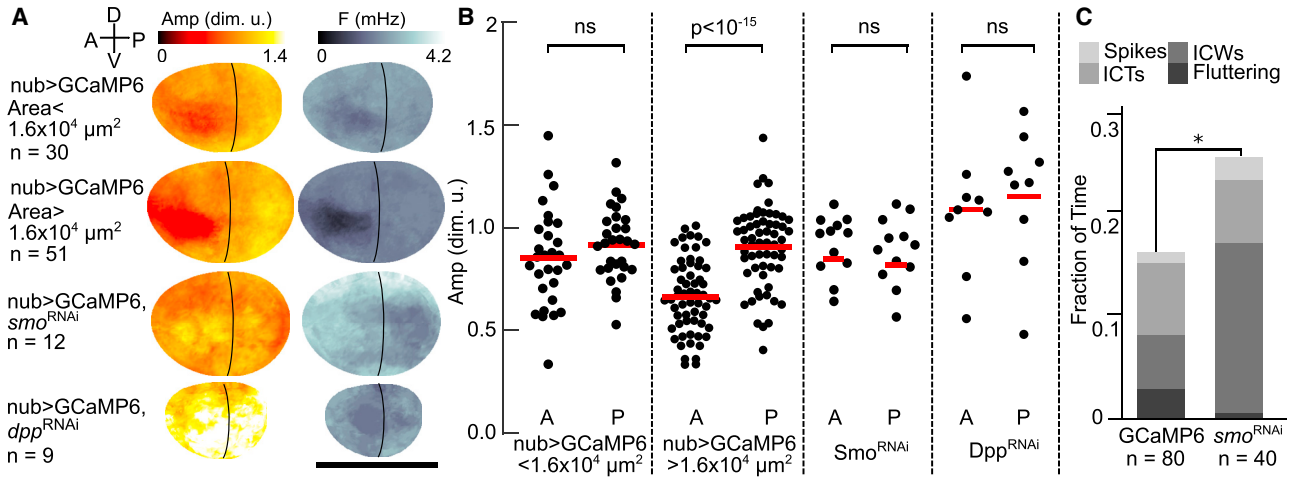

FIGURE 7 Calcium signaling is spatially patterned in the developing wing disc. (*A*) Composite spatial maps of median summary statistics of Ca^{2+} features are shown. Wing disc pouches expressing *nub > GCaMP6f* were grouped by pouch size. These wing disc pouches followed one of two patterns: small pouches ($<1.6 \times 10^4$ μm^2) with uniformly high amplitude in both compartments and larger pouches ($>1.6 \times 10^4$ μm^2) with a lower median amplitude in the anterior compartment. Inhibition of Hh and Dpp signaling led to an increase in frequency (F) and uniformly high amplitude. The black curve represents the approximate anterior-posterior boundary. Orientations of the wing discs are indicated in upper left corner. (*B*) The amplitude measurement of anterior and posterior compartments in control discs (*small* and *large*) and smo^{RNAi} and dpp^{RNAi} discs are shown. In *nub > GCaMP6f* discs, the difference of amplitude between the two compartments is significant in large disks. The difference is not significant in smo^{RNAi} (composite of three independent RNAi lines, Fig. S9) and dpp^{RNAi} discs, and the median amplitude appears uniform near the anterior dorsal-ventral boundary region. The *p* values were obtained by paired *t*-tests. (*C*) Inhibition of Hh signaling by smo^{RNAi} increases overall Ca^{2+} signaling activity in wing discs in vivo. Sample size represents the number of analyzed videos. Scale bars, 200 μm. Total Ca^{2+} activity by proportions test as described in Tables S6 and S7. To see this figure in color, go online.

affect Ca^{2+} dynamics. To test this, we perturbed the Hh signaling pathways, which direct the development of the anterior-posterior compartment boundary (56). Smoothened (Smo), a GPCR (57), is a primary transducer of Hh signaling (58). We also perturbed Dpp signaling, which is downstream of Hh signaling and also directs anterior-posterior patterning and growth (35,39,59). We knocked down *smo* and *dpp* using RNAi expression in the wing disc pouches of wandering larvae to test if the spatial asymmetry in average amplitudes is downstream of Hh signaling (*nub > GCaMP6f > smo*^{RNAi}; Video S21) or Dpp signaling (*nub > GCaMP6f > dpp*^{RNAi}; Video S22). We found that inhibition of either Hh or Dpp signaling was sufficient to abolish anterior-posterior spatial patterning of ICW amplitudes and leads to a higher frequency in the entire pouch (Fig. 7, *A* and *B*). These results were confirmed by evaluating multiple independent RNAi lines against *smo*, which are reported individually in Fig. S9, and combined together in Fig. 7, *A* and *B*. Further, the results of increased activity of *nub > GCaMP6f > smo*^{RNAi} were replicated in vivo (Fig. 7 *C*). Hh suppression resulted in roughly doubling the amount of time when fluttering or ICWs were observed in the pouch (Fig. 7 *C*). Additionally, Hh suppression led to a decrease in the average pouch size for wandering larvae, which are near the end of development. These results demonstrate that spatial patterning of the amplitude of Ca^{2+} oscillations is downstream of Hh and Dpp signaling, which are two morphogen pathways involved in regulating patterning and tissue growth. It provides support for a model in which agonist-stimulated Ca^{2+} signaling activity provides a readout of morphogenetic states of the organ during development.

Calcium signaling activity decreases with wing disc pouch size in a power-law relationship

To determine how stimulated Ca^{2+} signaling dynamics change as wing disc sizes increase, we created a high-dimensional summary map relating organ size to the features of Ca^{2+} signaling (Fig. 8 *A*; Videos S20, S21, S22, S23, and S24). We plotted the integrated intensity and frequency of Ca^{2+} signaling activity of all wing disc imaged against their pouch sizes (with wing disc pouch size as a proxy for overall organ size). The result demonstrates several interesting correlations between features of Ca^{2+} signaling activity and wing disc sizes, which form a manifold in high-dimensional morphological-cell signaling space (Fig. 8, *A–C*). Integrated intensity was found to decrease with pouch size (Fig. 8 *B*). Additionally, we found that frequency increases with integrated intensity and then decreases again (Fig. 8 *C*). Further, we analyzed the full data set for qualitative characteristics of calcium signaling, similar to the in vivo experiments. We found that there is a progression from fluttering disc to oscillatory waves to smaller-scale stochastic spiking activity (Fig. 8 *B*). Overall, these findings further confirm stimulated Ca^{2+} signaling responses decline with increasing organ size until the end of larval development.

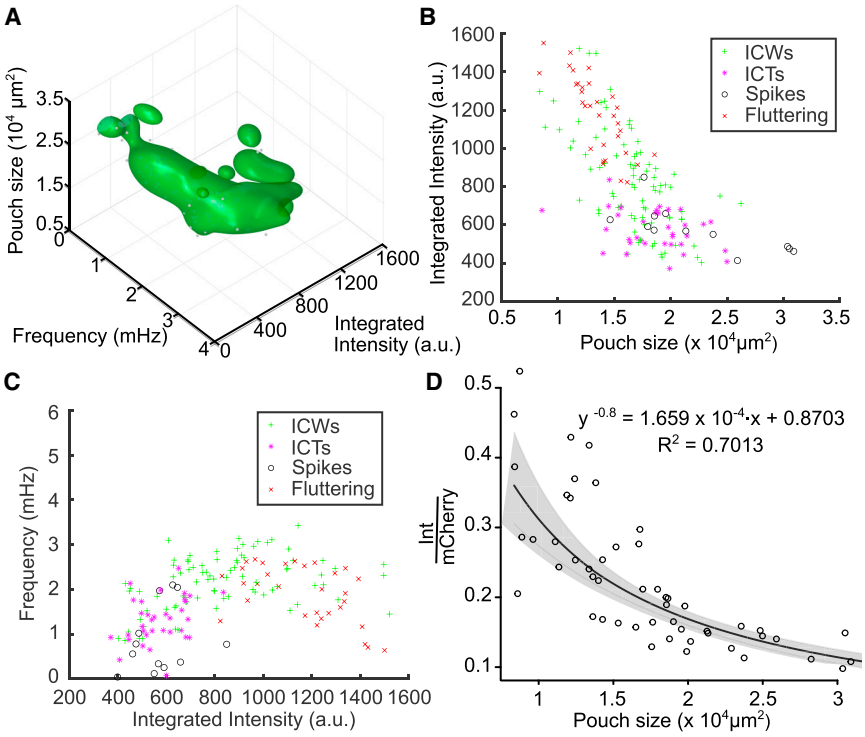

FIGURE 8 Transitions of agonist-stimulated calcium signaling as a function of pouch size. (*A*) Larval wing discs exhibit transitions in FEX (Gαq) agonist-stimulated Ca^{2+} signaling dynamics during development, forming a spiral in a three-dimensional plot of "morphological cell signaling space." (*B*) Integrated intensity decreases with increasing pouch size. Colors represent dominant Ca^{2+} dynamics observed in the particular sample. (*C*) A parametric plot of a data set from (*A*) shows frequencies of Ca^{2+} oscillations increasing with increasing integrated intensity and then decreasing at higher amplitudes. Note that low-amplitude fluttering does not contribute to the measured frequency signal. Each wing disc was imaged for 1 h and with a 10 s interval. Each video was divided into 31 time segments. As a second level of analysis, dominant Ca^{2+} activity was scored blindly in each segment, respectively (*colors*, see legend). Ca^{2+} dynamics that were observed for the largest total time for each separate wing disc sample are represented as color dots in (*B* and *C*). (*D*) A regression curve fit to ratiometric average integrated intensity of Ca^{2+} signaling in wing pouches as a function of pouch size is shown (n = 53). To see this figure in color, go online.

The full data set plotted in Fig. 8, *A–C* includes a subset of discs that also expressed a red fluorescent protein under the same Gal4 driver (expressing *nub > GCaMP6f/ > mCherry*; Video S24). Similar results between *nub > GCaMP6f* and *nub > GCaMP6f/ >mCherry* suggest Gal4 dilution is not a significant concern (Fig. S6 *E*). For discs coexpressing mCherry, we could normalize GCaMP6f fluorescence to mCherry fluorescence (Fig. S6 *C*). This allowed us to obtain ratiometric estimates of Ca^{2+} signaling that better account for sensor expression. For the data with mCherry expression, we observed a power-scaling relationship in which average ratiometric integrated intensity (normalized to mCherry expression) scales with pouch area ($R^2 = 0.70$, Fig. 8 *D*; Figs. S7 and S8). This relationship utilizes a Box-Cox power-scaling transformation (60) to maximize the log-likelihood estimation of the power exponent (Fig. S6 *A*). This power-scaling relationship served as a better fit compared to linear and exponential models because of a more normal distribution of residuals and the least variance between model residual values (Fig. S7).

Calcium signaling responds to perturbations to morphogen and growth factor signaling

Finally, we investigated the effects of multiple regulators of organ growth to further clarify the connections between organ growth and stimulated Ca^{2+} responses. We ectopically activated the Dpp pathway through the uniform expression of the constitutively active form of the Dpp receptor, tkv^{CA} (39,61,62). Additionally, we ectopically stimulated growth through the inhibition of the tumor suppressor phosphatase and tensin homolog (*Pten*), which results in high levels of insulin/AKT signaling without impacting wing patterning (63,64; Video S23). According to previous reports, both conditions lead to increases in cell division and growth (35,59,65). We found that these growth-inducing perturbations resulted in a decrease in Ca^{2+} signaling activity relative to control discs when exogenously stimulated (Fig. 9, *A* and *B*, *orange*). Inhibiting Hh and Dpp signaling with smo^{RNAi} and dpp^{RNAi} expression, respectively, lead to reduced growth (Fig. S8) and shows an increased integrated intensity, amplitude, and frequency of Ca^{2+} oscillations in wing disc pouches (Fig. 9, *A* and *B*, *blue solid points* of various shapes).

In sum, our results provide multiple lines of evidence that reveal general trends when wing disc growth is perturbed: morphogenetic perturbations that reduce growth lead to abnormally higher levels of integrated Ca^{2+} signaling activity above the manifold, relating size to integrated Ca^{2+} activity. In contrast, perturbations that promote growth lead to reduced levels of total (integrated) Ca^{2+} signaling.

DISCUSSION

Here, we have performed a systems-level analysis of Ca^{2+} signaling in a model system of organ growth and morphogenesis. To do so, we have established an innovative "Ca^{2+} decoding" image-processing pipeline. This work

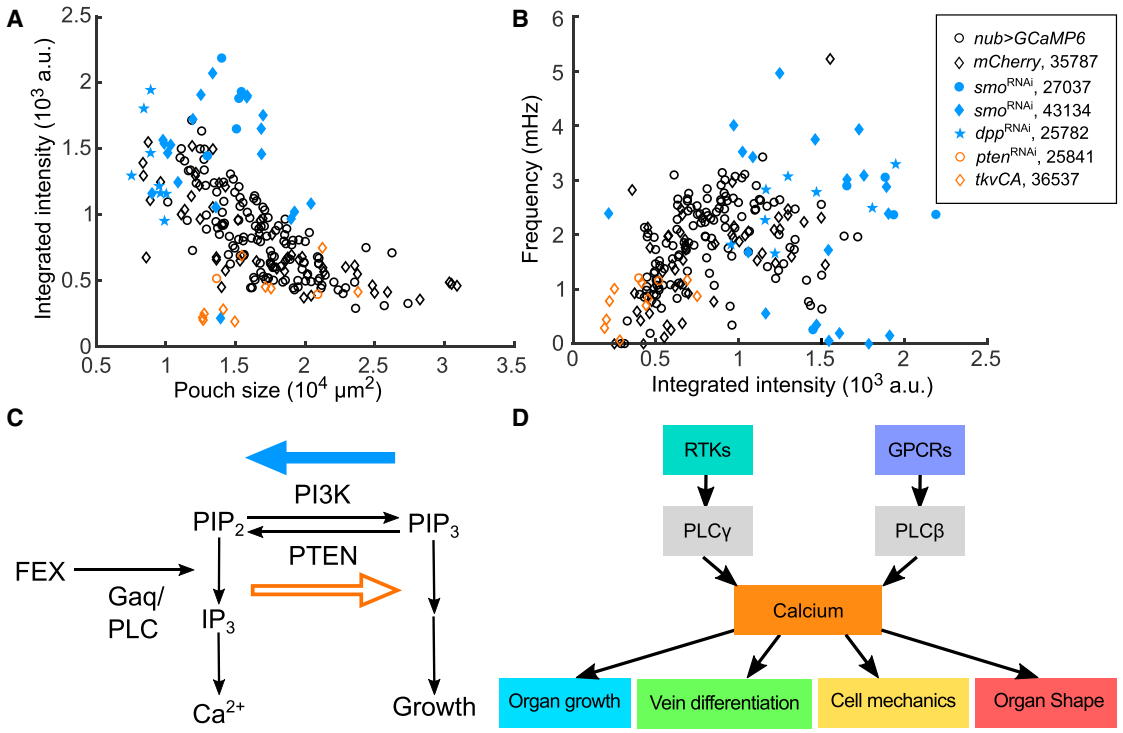

FIGURE 9 Integrated model of calcium signaling transduction in the *Drosophila* wing disc. (*A*) Genetic perturbations impacting morphogen and growth pathways result in deviations in agonist (FEX)-stimulated Ca^{2+} signaling responses. Data points of different colors and shapes represent wing discs with various genotypes that are indicated in the legend. Blue-colored, solid data points result in patterning defects and reduced wing growth. Higher Ca^{2+} signaling responses are produced for a given stimulus (15% FEX in all cases). Orange-colored, nonsolid data points result in increased growth. Reduced Ca^{2+} signaling responses are observed for the same stimulus. (*B*) The parametric plot of the same data set shows increased frequency in Ca^{2+} transients for the growth-suppression perturbations and a decreased frequency for growth-enhancement perturbations. Bloomington stock numbers for UAS-RNAi transgenic lines are included. All the perturbations shown here were driven by the *nub > GCaMP6f* tester line. (*C*) An inferred model is shown consistent with observations in (*A*) and (*B*). (*D*) Schematic summarizing key findings from this work and the literature is shown. Both RTK/PLC$\gamma-$ and GPCR/PLCβ-based signaling contributes to Ca^{2+} activity in vivo and ex vivo (inputs). Perturbations to core Ca^{2+} signaling pathway result in a range of developmental phenotypes (outputs), including wing size, vein differentiation, cell mechanics, and overall tissue shape. To see this figure in color, go online.

advances the analysis of Ca^{2+} signaling dynamics in organ systems. It demonstrates how a significant portion of cellular information processing occurs through the coupling of signaling dynamics during organ development. This pipeline also can be used to develop spatiotemporal maps of larval wing growth and patterning for other signal integrators such as cAMP (66) and for readouts of central growth pathways such as Hippo signaling (67).

This work has established multiple inputs and outputs for the calcium bow-tie network during wing development (Fig. 9 *D*). We also identified four classes of spontaneous Ca^{2+} signaling activity during in vivo development in the wing disc: 1) cellular Ca^{2+} spikes, 2) ICTs, 3) ICWs, and 4) elevated Ca^{2+} fluttering. We found that increasing Gαq-mediated signaling with increasing concentrations of FEX leads to a natural progression from low (class 1 and 2) to higher levels of Ca^{2+} signaling responses (classes 3 and 4). These four signaling classes occur both ex vivo and in vivo. Importantly, we found that multiple classes of Ca^{2+} activity occur and are a regulated phenomenon in vivo. These findings contradict previous suggestions that ICWs may be an ex vivo artifact (26,68). Future work is needed to specify the full set of specific RTKs, GPCRs, and morphogens that modulate Ca^{2+} dynamics in vivo.

We demonstrated a negative correlation between the stimulated Ca^{2+} signaling responses and the wing disc age and size for third instar larvae. Overall, these observations provide evidence for Ca^{2+} signaling as a readout for overall organ size in the developing wing and a regulator of cellular processes during larval wing development. Through linear regression analysis, we demonstrated a negative power-law correlation between larval age/pouch size and integrated Ca^{2+} signaling activity. These findings suggest that Ca^{2+} signaling decreases during the latter stages of larval wing disc growth. The maximal log-likelihood estimation of the power exponent occurred when the estimate had a value of -0.8 ± 0.5. This is consistent with many allometric scaling relationships observed in biological systems wherein quarter-power scaling frequently occurs (69,70). For example, quarter-power scaling has been observed in the organism metabolic rate, lifespan, growth rate, heart rate, and the concentrations of metabolic enzymes (69).

A −0.75-scaling relationship is consistent, near the maximal log-likelihood estimation, and within the 95% confidence interval of the optimal exponent power (Fig. S6 *A*). This, in turn, may indicate that the underlying metabolic trajectory of organ growth influences the level of agonist-stimulated calcium signaling activity.

Further, we observed anterior-posterior patterning of Ca^{2+} signaling activity amplitudes in the wing disc. The amplitude is higher in the posterior than in the anterior compartment. As these compartments have been shown to grow at different rates, this result is consistent with the correlation between Ca^{2+} signaling activity and the growth state of each compartment (71). There are several possible explanations for why there is an absence of amplitude patterning between anterior and posterior compartments for larger discs in Hh (smo^{RNAi}) or Dpp (dpp^{RNAi}) signaling-perturbed discs. First, Hh and Dpp signaling may be directly responsible for patterning the anterior-posterior amplitude difference, perhaps through regulation of cAMP levels (72,73). Second, this may be because the sizes of anterior and posterior compartments are similar under those conditions. Identifying the cause of this phenomenon may yield insight into additional patterning roles for Ca^{2+} signaling in wing development, including the pupal stages when vein differentiation occurs. Recently, Ca^{2+} signaling has been connected to proper Hh signaling in zebrafish embryo (74). Our work suggests that Ca^{2+} signaling may generally be involved in modulating morphogenesis mediated by Hh signaling and other morphogen pathways.

Future work is needed to identify specific mechanisms connecting signal transduction inputs to phenotypic outputs. In a recent article, cellular Ca^{2+} spikes were found to correlate with secretion of Dpp, a key regulator of wing disc size and tissue patterning (23). We speculate that local cellular spike activity might be connected to the positive regulation of organ growth. Smo^{RNAi} and dpp^{RNAi} leads to smaller wing discs and higher integrated Ca^{2+} intensity when Ca^{2+} signaling is stimulated by agonists. The data points from growth-reducing perturbation (smo^{RNAi} and dpp^{RNAi}, *blue solid data points*) lie above the negative correlation curve of the control wing discs (*black circle* or *diamond data points*, Fig. 9, *A* and *B*). In contrast, genetic perturbations leading to more growth (tkv^{CA} and $Pten^{RNAi}$, *orange data points*, Fig. 9, *A* and *B*) result in reduced Ca^{2+} signaling responses when stimulated.

These results imply a common underlying regulatory mechanism. As a launching point for future work, we speculate by proposing a simple model that explains the results reported here (Fig. 9 *C*). First, our experiments demonstrate that FEX stimulates $G\alpha q/PLC\beta$ activity, which results in IP_3 generation and IP_3-regulated Ca^{2+} release. Sufficient IP_3 production may lead to phosphatidylinositol bisphosphate (PIP_2) substrate depletion. In other systems, PIP_2 is often rate limiting for Ca^{2+} signaling (75,76). PIP_2 is also required for phosphatidylinositol trisphosphate generation, which then stimulates cell growth through PI_3K/AKT signaling (77). It follows that reduced PI_3K signaling resulting from decreased growth stimulation (indirectly through inhibition of Hh or Dpp signaling in our experiments) will lead to higher PIP_2 substrate availability and a stronger Ca^{2+} response. Conversely, decreased PIP_2 availability through the inhibition of PTEN (which converts phosphatidylinositol trisphosphate to PIP_2) (63,65) or through constitutively active Dpp signaling (78) would lead to attenuated Ca^{2+} signaling responses when stimulated by FEX (Fig. 9 *C*).

This interpretation of the data provides a generalizable and testable hypothesis for future work: if PIP_2 levels are more abundant (reduced PI_3K signaling and growth activity), more IP_3 can be generated, resulting in more Ca^{2+} signaling for a given agonist response. If PIP_2 substrate levels are limiting (as results when PTEN is inhibited or more growth is stimulated), less IP_3-stimulated Ca^{2+} signaling can occur. This hypothetical model would predict that sufficient overexpression of Gαq could lead to reduced organ growth by depleting PIP_2 substrate availability for growth stimulation. Future work may identify such relationships across biological systems because all of these molecular components are present in most eukaryotic cells. We term this hypothetical model the "Ca^{2+} shunt" hypothesis of growth control.

Ca^{2+} signaling likely modulates other aspects of growth control during larval development. Ca^{2+} may integrate signals about the availability of nutrients or about mechanical constraints on the tissue. Several known effectors of size control pathways, such as *kibra*, a regulator of Hippo signaling (79–81), have Ca^{2+} signaling binding domains as annotated by InterPro (82).

Additionally, this work motivates new questions regarding how gap-junction communication, and by extension, membrane voltage, influences the overall control of organ size (4). A decrease in cell-cell gap-junction permeability occurs over the course of wing development (83). As gap junctions become less permeable, Ca^{2+} and IP_3 diffuse a shorter distance before being reabsorbed into the endoplasmic reticulum or decaying, respectively. This would explain the transition from ICWs to ICTs and spikes as well as why amplitude is spatially patterned in large discs as development proceeds. Other studies have also implicated gap-junction communication in organ size control (84). For example, $Inx2^{RNAi}$ suppresses growth in the developing eye disc (85). *Connexin43* mutants that disrupt gap-junction communication lead to short fin in zebrafish (86). Gap-junction communication also regulates cell differentiation as Inx2-mediated Ca^{2+} flux is essential for border cell specification in *Drosophila* (87). Our results suggest that part of the role of gap-junction communication in regulating size and influencing tissue patterning is through the regulation of Ca^{2+} transients across the tissue. Taken together, it is

therefore likely that the role of Ca^{2+} signaling in wing growth is conserved in other organs.

Our phenotypic analysis provides additional evidence that the Ca^{2+} signaling module contributes to modulating wing morphogenesis during pupal development and vein cell differentiation. It should be noted that the crossvein defects suggest that these veins are particularly sensitive to levels of morphogen signaling, including Dpp (88). In particular, Dpp signaling has been linked to Ca^{2+} signaling in the developing wing (23). Perturbing Ca^{2+} signaling may also be enhancing the crossvein defects that can occur in the MS1096-Gal4 line, which impacts *Beadex* gene function (89). Future work will need to investigate the mechanisms leading to wing shape and vein differentiation defects, which are specified during pupal development.

Computational modeling is essential for future efforts to decode the regulation and function of Ca^{2+} signaling (3). Understanding the specific roles of Ca^{2+} signaling in organ development will require computational models that couple multiple signals of Ca^{2+} signaling across multiple spatio-temporal scales (90–93). For example, computational models are particularly useful at the systems level to understand mechanisms for the coupled transport of Ca^{2+} and wound healing (6). Regarding this study, our findings that the integrated Ca^{2+} intensity decreases with development is consistent with a model from the neocortex being applied to our wing disc, in which Ca^{2+} signaling dynamics are weakly coupled with cell-cycle progression and can influence cell-cycle synchrony with neighbors (94,95). In sum, this effort demonstrates key roles of Ca^{2+} signaling as a signal integrator in epithelial growth and morphogenesis.

SUPPORTING MATERIAL

Supporting Materials and Methods, 11 figures, 7 tables, and 24 videos are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(19)30023-2.

AUTHOR CONTRIBUTIONS

J.J.Z., Q.W., P.A.B., and C.N. designed and conceived the study. Q.W., D.K.S., F.J.H., C.N., M.K.L, and N.A.-W. performed experiments. P.A.B. developed methods for feature extraction, statistical analysis, and manual annotation. P.A.B. and F.J.H. implemented qualitative analysis pipeline, performed statistical analysis, and performed manual classification of in vivo data. J.C., P.L., and D.Z.C. developed automated methods for image segmentation and registration. Q.W., P.A.B., F.J.H., and J.J.Z. analyzed data and wrote the manuscript. J.J.Z supervised the study.

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