

Dynamic Delta-like1 expression in presomitic mesoderm cells during somite segmentation

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Abstract

During somite segmentation, the expression of clock genes such as *Hes7* oscillates synchronously in the presomitic mesoderm (PSM). This synchronous oscillation slows down in the anterior PSM, leading to wave-like propagating patterns from the posterior to anterior PSM. Such dynamic expression depends on Notch signaling and is critical for somite formation. However, it remains to be determined how slowing oscillations in the anterior PSM are controlled, and whether the expression of the Notch ligand Delta-like1 (*Dll1*) oscillates on the surface of individual PSM cells, as postulated to be responsible for synchronous oscillation. Here, by using *Dll1* fluorescent reporter mice, we performed live-imaging of *Dll1* expression in PSM cells and found the oscillatory expression of *Dll1* protein on the cell surface regions. Furthermore, a comparison of live-imaging of *Dll1* and *Hes7* oscillations revealed that the delay from *Dll1* peaks to *Hes7* peaks increased in the anterior PSM, suggesting that the *Hes7* response to *Dll1* becomes slower in the anterior PSM. These results raise the possibility that *Dll1* protein oscillations on the cell surface regulate synchronous *Hes7* oscillations, and that the slower response of *Hes7* to *Dll1* leads to slower oscillations in the anterior PSM.

1. Introduction

Cell-cell interactions play an important role in coordinating gene expression during tissue morphogenesis. One such example is the somite segmentation process, in which a bilateral pair of somites is generated periodically by segmentation of the anterior regions of the presomitic mesoderm (PSM). This periodic event is controlled by the segmentation clock genes, such as the transcriptional repressors *Hes1* and *Hes7*, whose expression oscillates synchronously between neighboring cells (Hubaud and Pourquié, 2014; Kageyama et al., 2012; Oates et al., 2012). This synchronous oscillatory gene expression slows in the anterior PSM, thereby leading to wave-like propagating patterns from the posterior to anterior PSM. This dynamic expression is critical for somite formation, as dampening and/or desynchronization of the oscillations leads to segmentation defects, causing the fusion of somite-derived tissues such as the vertebrae and ribs (Takashima et al., 2011; Niwa et al., 2011). When PSM cells are dissociated, oscillatory gene expression becomes unstable and desynchronized, suggesting that cell-cell interactions play an important role in synchronized oscillations (Maroto et al., 2005; Masamizu et al., 2006).

Notch signaling regulates such cell-cell interactions; the Notch ligand Delta-like1 (*Dll1*) activates Notch signaling in neighboring cells, in which *Hes1* and *Hes7* are induced (Artavanis-Tsakonas et al., 1999; Jiang et al., 2000; Horikawa et al.,

2006; Riedel-Kruse et al., 2007; Mara et al., 2007; Özbudak et al., 2008; Kageyama et al., 2012). Hes1 and Hes7 expression oscillates with a periodicity of ~2 hours by negative feedback in mouse PSM cells (Hirata et al., 2002; Bessho et al., 2003), and these oscillations lead to the oscillatory expression of Dll1 (Shimojo et al., 2016). Optogenetic induction of Dll1 oscillations can entrain Hes1 oscillations in neighboring cells (Isomura et al., 2017), suggesting that Dll1 oscillations regulate synchronous oscillations in the PSM. Indeed, when dissociated PSM cells are re-aggregated, they exhibit synchronous oscillations in a Notch signaling-dependent manner (Tsiairis and Aulehla, 2016). Furthermore, when Dll1 oscillations are dampened, Hes7 oscillations are also dampened, resulting in severe somite fusion (Shimojo et al., 2016). All of these data suggest that Dll1 oscillation-dependent cell-cell interactions are essential for coordinated gene expression during somite segmentation.

Despite the above findings, the dynamics of Dll1 expression in the PSM are still obscure. Particularly, it has been difficult to analyze Dll1 protein dynamics, because Dll1 protein oscillations exhibit amplitudes that are too small to be detected by immunostaining (Okubo et al., 2012), but they have been reliably detected by time-lapse imaging (Shimojo et al., 2016). Furthermore, Dll1 protein, as a ligand, should function on the cell surface, but the oscillatory expression of Dll1 protein in the PSM has only been shown at the tissue level (Bone et al., 2014; Shimojo et al., 2016). Dll1 protein is present not only on the cell surface but also in cytoplasmic vesicles including the Golgi apparatus (Geffers et al., 2007); therefore, the total expression levels of Dll1 protein in the PSM do not reflect the functional Dll1 expression levels. We previously examined Dll1 expression dynamics by using Dll1 reporter mice, in which luciferase cDNA was inserted into the Dll1 gene so that Dll1-luciferase fusion protein was expressed from the endogenous locus. Although we confirmed Dll1 protein oscillations in the PSM (Shimojo et al., 2016), it was technically difficult to examine its expression at the cellular level, because luciferase-dependent luminescence imaging exhibits only a low spatial resolution.

Here, to overcome this problem, we used a new Dll1 reporter mouse line (Dll1-Venus-T2A-mCherry mice), in which Venus fluorescent protein cDNA was inserted into the Dll1 gene so that a Dll1-Venus fusion protein was expressed from the endogenous Dll1 locus (Tateya et al., 2019). We performed time-lapse imaging using these mice to examine Dll1 protein expression dynamics at the cellular level and the mechanism of how oscillatory gene expression slows in the anterior PSM.

2. Results

2.1. Dll1 oscillations in the PSM

To examine Dll1 expression in the PSM, we used Dll1-Venus-T2A-mCherry mice, in which the cDNA for Venus-T2A-mCherry was inserted at the end of the coding sequence of the Dll1 gene (Fig. 1A). In these mice, in addition to the Dll1-Venus fusion protein, mCherry protein was also expressed from the Dll1 locus, but because it is cleaved from the Dll1-Venus fusion protein, mCherry expression is stable and not oscillatory. We first examined the somite segmentation phenotypes of these mice. Heterozygous Dll1-Venus-T2A-mCherry mice were apparently normal, but homozygous mice showed some mild segmentation defects only in the tail region, indicating that the Dll1-Venus fusion protein is slightly hypomorphic (Fig. S1). However, the vertebrae and ribs in the trunk region were segmented normally in the homozygous mice (Fig. S1), suggesting that the Dll1-Venus fusion protein functions normally in most regions. Therefore, we decided to use this line to analyze Dll1 protein dynamics.

To determine Dll1 protein expression dynamics during somitogenesis, caudal parts containing the PSM of Dll1-Venus-T2A-mCherry embryos at embryonic day (E) 10.5 were cultured with luciferin, and fluorescence and bioluminescence images were acquired. Time-lapse imaging analysis showed that Dll1-Venus expression clearly oscillated in the PSM, propagating from the posterior to the anterior PSM, while mCherry expression occurred broadly throughout the PSM and somites (Fig. 1B and Movie 1). Dll1-Venus protein was mainly expressed in the membrane, whereas mCherry expression occurred diffusely throughout the cytoplasm (Fig. 1C). Dll1-Venus-T2A-mCherry mice were next crossed with pMesp2-UbELuc mice, which expressed ubiquitinated ELuc periodically under the control of the Mesp2 promoter in S-1 of the anterior PSM, a region just caudal to the next forming somite (Niwa et al., 2011). Time-lapse imaging analysis of PSM explant cultures of these embryos showed that Dll1-Venus oscillations propagated, reaching S-1, and that this dynamic expression pattern was repeated with the same period as the segmentation clock (Fig. 1D,E and Movie 2). This expression pattern agreed well with the previous observations using Dll1-Fluc knock-in mice (Shimojo et al., 2016), suggesting that Dll1-Venus expression in this reporter mouse line represents normal Dll1 expression.

2.2. Comparison of Dll1 and *Hes7* oscillations

To compare the expression patterns of Dll1 and *Hes7*, Dll1-Venus-T2A-mCherry mice were crossed with *Hes7* reporter mice (pHes7-UbLuc), which expressed a destabilized luciferase reporter under the control of the *Hes7* promoter (Takashima et al., 2011;

Niwa et al., 2011). The expression of this *Hes7* reporter has been shown to represent *Hes7* mRNA production (Takashima et al., 2011; Niwa et al., 2011). *Dll1*-Venus and *Hes7* expression was monitored by live imaging of PSM explant cultures (Fig. 2A). *Dll1* protein and *Hes7* reporter oscillations were mostly anti-phase in the posterior PSM but overlapped with each other in the anterior PSM (Fig. 2B,C and Movie 3). Signal intensity kymographs of E10.5 *Dll1*-Venus-T2A-mCherry;pHes7-UbLuc PSM showed that the speed of wave propagation changed in the middle of the PSM (indicated by a broken line in the second panel from the left of Fig. 2B). The anterior and posterior PSM was defined as the region anterior and posterior, respectively, to this broken line, and quantification of the delay time was done for the anterior and posterior PSM separately. This analysis indicated that the delay from the *Hes7* peaks to *Dll1* peaks was not significantly changed between the anterior and posterior PSM (Fig. 2D, pHes7 to *Dll1*), whereas the delay from the *Dll1* peaks to *Hes7* peaks increased from 114.8 ± 4.3 min in the posterior PSM to 152.0 ± 3.9 min in the anterior PSM (Fig. 2D, *Dll1* to pHes7). These results indicated that the *Hes7* response to *Dll1* became slower in the anterior PSM compared to the posterior PSM. Accordingly, the peak intervals (= periodicity) of both *Dll1*-Venus and pHes7-Luc oscillations increased in the anterior PSM compared to the posterior PSM (Fig. 2D, *Dll1* and pHes7), indicating that *Dll1* and *Hes7* oscillations slow down in the anterior PSM.

2.3. *Dll1* oscillations in tail bud cultures

We next sought to examine *Dll1*-Venus expression on the surface of PSM cells. However, although we were able to monitor *Dll1* protein expression in PSM explant cultures of *Dll1*-Venus-T2A-mCherry embryos, it was technically difficult to monitor its expression in the membrane. To overcome this difficulty, we next performed tail bud cultures of these reporter mice. In these cultures, tail bud cells expanded outward (peripherally), and segmentation occurred at the peripheral regions, indicating that this culture system mimicked the intact PSM undergoing somite segmentation (Tsiaris and Aulehla, 2016). Time-lapse imaging analysis showed that *Dll1* expression oscillated in the tail bud cultures (Fig. 3A,B and Movie 4), and *Dll1* protein oscillations propagated peripherally (downward in Fig. 3C,D and Movie 5). By using this system, we examined *Dll1* protein expression dynamics at the cellular level. We found that *Dll1* protein was present in cytoplasmic vesicles as well as on the cell surface (Fig. 3E). Thus, we next removed the cytoplasmic signals and quantified only the cell surface signals (Fig. 3F-I). Quantification analyses showed that *Dll1* protein expression on the cell surface oscillated synchronously with neighboring PSM cells (Fig. 3G,I and Fig. S2). These

data raised the possibility that Dll1 protein oscillations on the cell surface are responsible for the synchronized oscillations in the PSM.

3. Discussion

How cell-to-cell communication is controlled is a major question to understand the mechanism of synchronized oscillations during somitogenesis. Dll1 protein oscillations can entrain oscillatory expression in neighboring cells, and dampening Dll1 oscillations lead to severe somite fusion, demonstrating the important roles of Dll1 oscillations in the somite segmentation (Shimojo et al., 2016; Isomura et al., 2017). However, although it has been shown that Dll1 protein expression oscillates at the tissue level in the PSM, there was no experimental evidence for Dll1 protein oscillations on the cell surface. Here, by using time-lapse imaging of tail bud cultures, we demonstrated that Dll1 protein expression oscillates on the surface of PSM cells. This oscillation is synchronous with neighboring PSM cells. This result supports the hypothesis that Dll1 oscillations may be responsible for the synchronization of clock genes between PSM cells via cell-cell interactions.

Another issue is that the delay from the peak of Dll1 protein oscillations to that of *Hes7* oscillations (coupling delay) increases in the anterior PSM compared to the posterior PSM, leading to longer periodicity (slower oscillations). It has been shown that oscillatory expression slows down as the waves propagate from the posterior PSM to the anterior PSM, but the mechanism is not clear (Giudicelli et al., 2007). Our data showed that the slower oscillations are due to the increased delay from Dll1 expression to *Hes7* expression, which includes the time required for Dll1 expression on the cell surface and that for inducing *Hes7* expression in neighboring cells in response to Dll1. Further analyses will be required to elucidate the mechanism by which the coupling delay is controlled.

It has been proposed that the coupling delay affects oscillation dynamics (Momiji and Monk, 2009; Shimojo et al., 2016; Yoshioka-Kobayashi et al., 2020). According to mathematical modeling, neighboring cells exhibit in-phase or out-of-phase oscillations depending on the delay required for Dll1-Notch signaling transmission (Momiji and Monk, 2009; Shimojo et al., 2016). While PSM cells show in-phase oscillations, neural stem cells show out-of-phase oscillations, and it will be important to compare the delays between PSM cells and neural stem cells. Our live imaging method described here will be useful for such analyses.

4. Experimental procedures

4.1. Mice

All animals were handled in accordance with the Kyoto University Guide for the Care and Use of Laboratory Animals. Dll1-Venus-T2A-mCherry, pHes7-UbLuc, and pMesp2-UbEluc transgenic mice were developed previously (Takashima et al., 2011; Niwa et al., 2011; Tateya et al., 2019).

4.2. Bone and cartilage staining

Skeleton of neonates were stained with alcian blue and alizarin red, respectively, as described previously (Bessho, et al., 2001).

4.3. PSM and tail-bud explant cultures

Embryos were collected at E9.5 or E10.5. Tails were dissected in pre-warmed PBS, incubated in culture medium, and placed under 5%CO₂ and 80%O₂ at 37°C. The culture medium was prepared as 2:1 mixture of DMEM/F12 (without glucose, pyruvate, or phenol red, Cell Culture Technologies) and DMEM/F12 (with HEPES, 17.5mM D-glucose, no phenol red, Gibco), supplemented with 1% BSA (A3059, Sigma-Aldrich) and penicillin-streptomycin. For tail-bud cultures, tissues were dissected in culture medium and transferred to glass bottom dish (φ3.5cm, Matsunami) coated with fibronectin (F1141-1MG, Sigma) and incubated for an hour to allow attachment to the dish prior to imaging in 5%CO₂ at 37°C.

4.4. Real-time imaging of bioluminescent and fluorescent reporters

For bioluminescence imaging, PSM tissues were placed on glass-bottom dish and imaged using an inverted microscope (Olympus IX83) equipped with a cooled CCD camera (iKon-M 934, Andor) as described previously (Masamizu et al., 2006; Niwa et al., 2011). 0.1mM luciferin (Nacalai Tesque) was added to the culture medium. This concentration allowed the reduction of background noise in YFP channel compared to 1mM luciferin condition. Both Luminescence and YFP/Venus signals were captured using 4x4 binning. For YFP/Venus excitation, 535 nm LED light source was used. Time lapse images were collected at 6- or 10-min intervals for pHes7-UbLuc, and at 20-min intervals for pMesp2-UbELuc. Exposure time for each channel was assigned as follows: 5 min 30 sec in 6-min intervals, 9 min 30 sec in 10-min intervals, and 19 min 30 sec in 20-min intervals for chemiluminescence; 300 ms for Venus; 100 ms for bright field.

For fluorescence imaging, Zeiss LSM 780/880 microscopes equipped with QUASAR GaAsP spectral detector were used. For the imaging of the PSM explants, upright confocal microscope (LSM780, Zeiss) with 20x dipping objective (W-PlanApo

20x, N.A. 1.0, Zeiss) was used. To examine the cellular Dll1-Venus expression, inverted confocal microscope (LSM880, Zeiss) with 40x objective (Plan-Apochromat, N.A. 1.4, oil, Zeiss) was used. Venus and mCherry fluorescent proteins were activated by 514-nm Argon laser and 633-nm DPSS laser, respectively. Images were acquired with photon counting mode. Data were collected in 12 bit, 512x512 pixels. Voxel size was 1.1861x1.1861x8.690 μm^3 for Fig. 1B, 1.1861 x1.1861 x11.429 μm^3 for Fig. 3A, and 0.461 x0.461 x0.708 μm^3 for Fig. 3C.

4.5. Image analysis and quantification

ImageJ software was used for image analysis. Custom written “SpikeNoise Filter” was applied to remove cosmic rays from bioluminescence images, as previously described (Isomura et al. 2017). Venus images were subjected to moving average detrending with 27-frame temporal window. Savitzky-Golay filter was applied to smoothen the Venus signals. Kymographs were created as described previously (Niwa, 2011). To estimate the spatial offset from combined reporters, ROIs parallel to newly formed somite boundaries were drawn on kymographs, and the signal intensity along the ROIs was plotted. To estimate the peak-to-peak intervals, peaks were selected manually as the highest point or the midpoint between the troughs. To quantify the time intervals in the anterior and posterior regions of the PSM, each peak or trough was classified based on their location in respect to the point where the velocity of the pHes7 travelling wave changed (i.e., where the steepness of the wave in the kymograph changed, which is approximately around the middle of the PSM). At least 6 waves from 3 independent samples were analyzed at least at 4 time points. To define the region for plasma membrane to quantify the membrane signal levels, the membrane was manually segmented, and ROI was created by applying Dilate/Erode plugins once and by image subtraction (Dilate minus Erode). Statistical analyses were performed with R software. Welch’s t test was performed for Fig. 2D.

More information is listed in the Key Resources Table.

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Figure legends

Figure 1. Real-time imaging of Dll1 protein oscillations during mouse somitogenesis. (A) The structures of the Dll1 locus of wild-type (upper) and Dll1-Venus-T2A-mCherry mice (lower). In the latter, cDNA encoding Venus and mCherry fluorescent proteins tethered by a T2A sequence was inserted in-frame to the 3' end of the Dll1 coding region. (B) Real-time imaging of a PSM explant of an E9.5 Dll1-Venus-T2A-mCherry embryo (see Movie 1). A bright field (BF) image merged with Venus signals is shown on the left. The expression patterns of Venus (green) and mCherry (magenta) signals are shown on the right. Scale bar, 100µm. Asterisk indicates the most recently formed somite boundary. (C) Snapshots of the anterior, posterior and tail-bud regions of the PSM of a Dll1-Venus-T2A-mCherry embryo. Merged images of BF, Venus (green), and mCherry (magenta) signals are shown. Venus signals are mostly located on the membrane whereas the mCherry signals are diffuse throughout the cytosol. The signals were collected from an optical section with a thickness of 2.5 µm. Scale bar, 100 µm. (D) Real-time imaging of a PSM explant culture of an E10.5 Dll1-Venus-T2A-mCherry;pMesp2-ELuc embryo (see Movie 2). Dorsal view of the PSM. I, II, and III indicate different phases of Dll1 protein expression patterns in the PSM. pMesp2-ELuc signals are detected in the S-1 region, where the Dll1-Venus expression intensity peaks. Scale bar, 200µm. (E) Intensity kymographs from real-time imaging of Dll1-Venus and Mesp2-ELuc reporter expression in an E10.5 PSM explant culture. Signals along the anterior-posterior axis of the PSM from the region of interest in (D, left panel) were acquired. The right panel shows de-trended Venus signals. Time-series were captured every 20 min, with 19.5 min of exposure to detect the luminescent signal of pMesp2-ELuc.

Figure 2. Estimation of the collective time delay between Dll1 protein and *Hes7* reporter expression via Notch signaling in the mouse PSM. (A) Real-time imaging of a PSM explant culture of an E10.5 Dll1-Venus-T2A-mCherry;pHes7-UbLuc embryo. Dll1-Venus (magenta) and *Hes7* expression (green) were examined. Dorsal view of the PSM. A bright field (BF) image is shown in the left panel. I, II, and III indicate different phases of Dll1 protein expression patterns in the PSM. Scale bar 200 µm. (B) Signal intensity kymographs of E10.5 Dll1-Venus-T2A-mCherry;pHes7-UbLuc PSM (see Movie 3). The time-series was captured every 6 min, with a 5.5 min exposure to detect luminescence signals from pHes7-Luc. Signal intensity kymographs of Dll1-Venus and *Hes7* reporter expression from Movie 3. Signals obtained along the anterior-posterior axis indicated by the region of interest shown in (A) were plotted over time. The 4th

panel shows de-trended Venus signals. The speed of wave propagation changed in the middle of the PSM (indicated by a broken line in the second panel from the left), and the anterior and posterior PSM in (D) was defined as the region anterior and posterior, respectively, to this broken line. (C) Signals in the region indicated by the horizontal lines #1-4 in (B) are plotted over time. The distance of the peaks from the two reporters indicates the delay between Dll1 protein and *Hes7* reporter expression via Notch signaling. (D) Quantification of the time differences between the Dll1-Venus and pHes7-Luc peaks and the periods of Dll1-Venus and pHes7-Luc oscillations in the anterior- and posterior halves of the PSM. The oscillation periods of pHes7-Luc and Dll1-Venus were estimated from the peak-to-peak intervals represented in (C). Time intervals between the peaks of corresponding waves of Dll1-Venus/pHes7-Luc were also quantified in each area. The peaks of at least 6 travelling waves for each of 3 PSM samples were measured. Please note that the periodicity of Dll1 and pHes7 here was longer than the reported value due to a slightly lower temperature during imaging. Sample mean $\pm$ standard error of the mean is shown. * $p < 0.05$, unpaired t test.

Figure 3. Dll1-Venus protein expression dynamics in the membrane regions of PSM cells. (A) Real-time imaging of a tail bud culture of an E9.5 Dll1-Venus-T2A-mCherry embryo. Scale bar, 100 μ m. (B) Quantification of the raw and de-trended Dll1-Venus signal intensities from the regions of interest (ROIs #1-3) shown in (A) (see Movie 4). In the right panel, Savitzky-Golay filtering was applied to smoothen the raw data. (C) Real-time imaging of a tail bud culture of an E9.5 Dll1-Venus-T2A-mCherry embryo. Scale bar, 40 μ m. (D) Quantification of the raw and de-trended Dll1-Venus signal intensities from the ROIs shown in (C) (#1-2) (see Movie 5). In the right panel, Savitzky-Golay filtering was applied to smoothen the raw data. (E) Representative snapshots of a tail bud culture of an E9.5 Dll1-Venus-T2A-mCherry embryo. Dll1-Venus signals were observed in vesicles and on the cell membrane. Time is shown in minutes. (F) Representative snapshots of the z section ROI indicated in (C) (ROIa). The lower column shows an example of a manually segmented ROI (yellow) of the plasma membrane of a single cell. Time is shown in minutes. (G) Plots of signal intensity from a ROI (z5-ROIa) and single cells (z5_30-1 and z5_30-2) compared to the whole field (z5). To create the plot, the total signal intensity inside the ROI (a.u.) was divided by the area of each ROI (pixels) at each time point. (H) Representative snapshots of the peak and trough phases of cell ROI6, which undergoes cell division twice. ROIs are shown in red in the lower panels. Time is shown in minutes. Scale bar, 20 μ m. (I) Plot of the signal intensities of single cells (ROI6-1,2,3) shown in (H). The

gray line shows the signal intensity of the whole field (z_4).

Fig.1

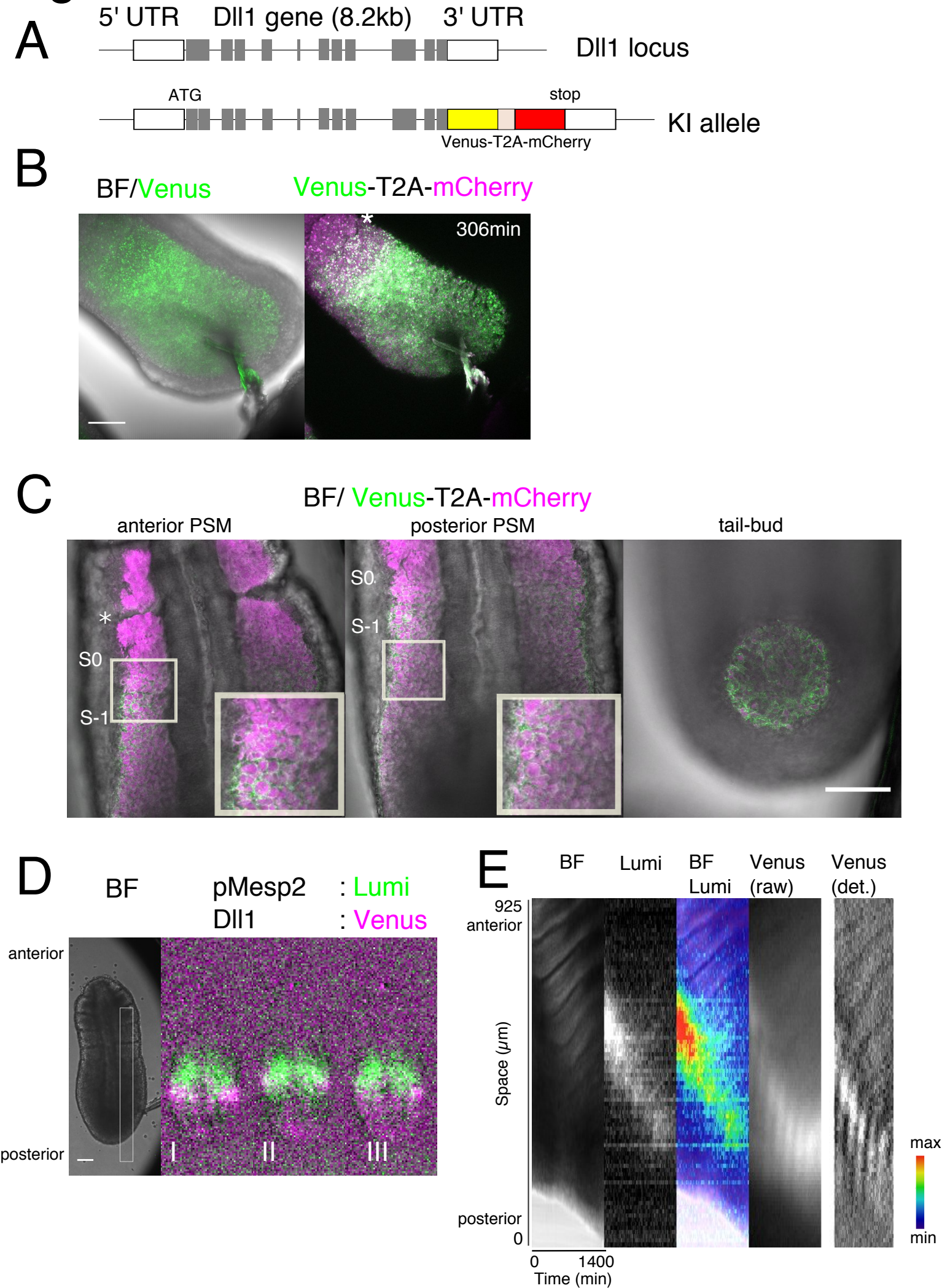


Fig.2

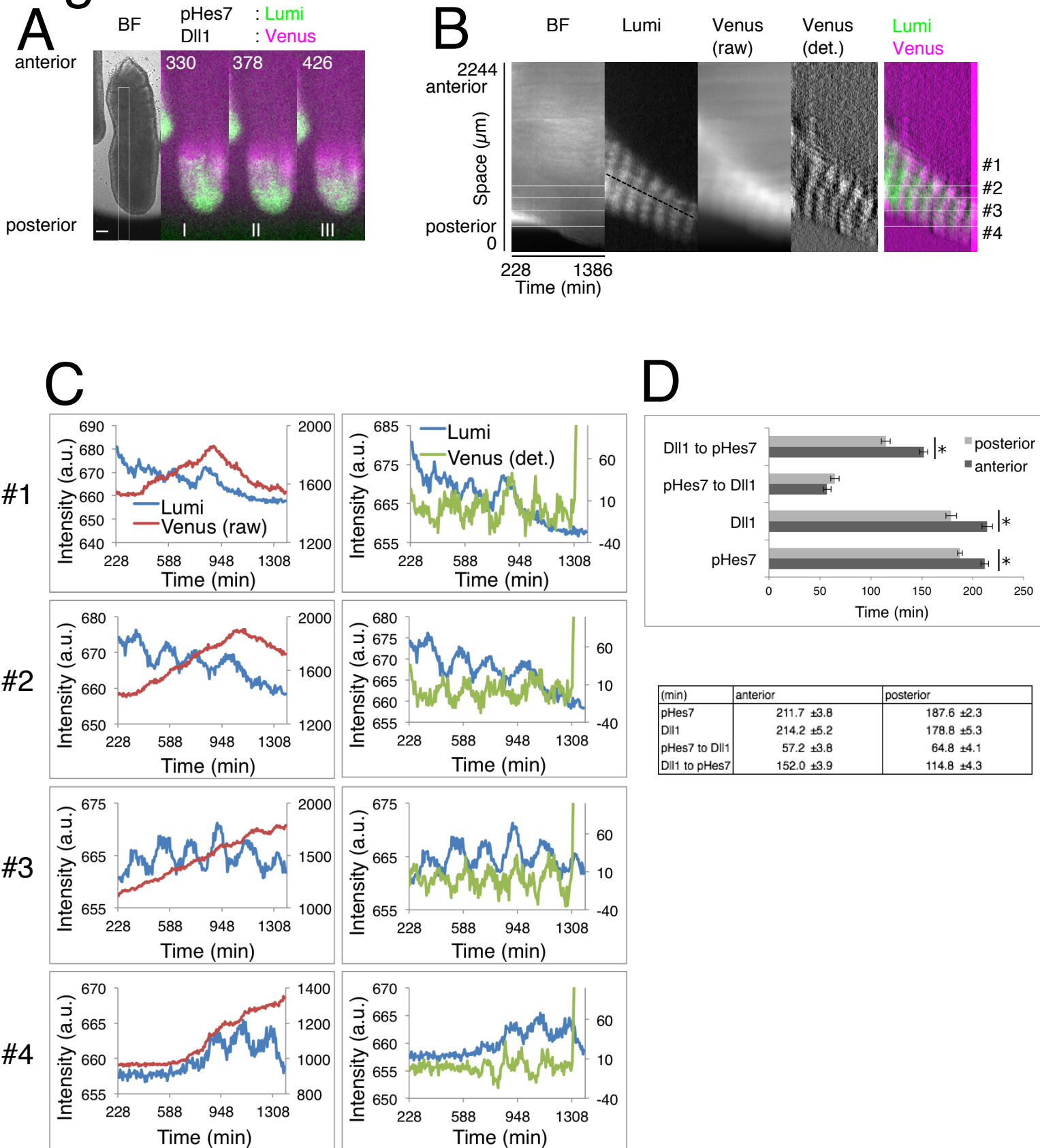
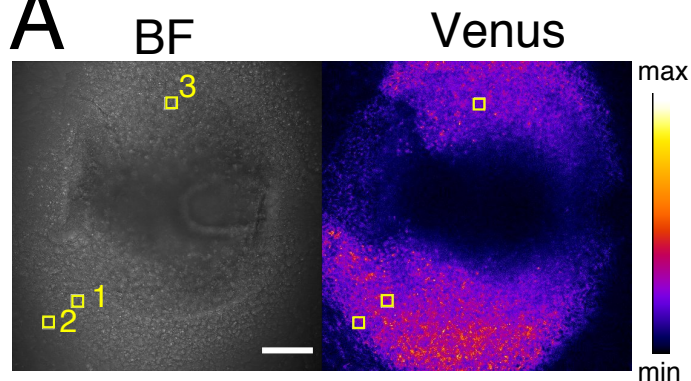
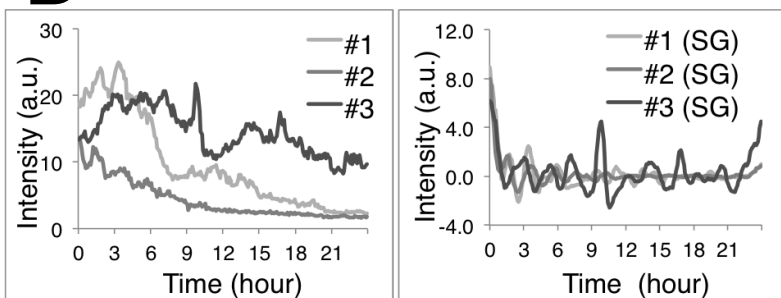
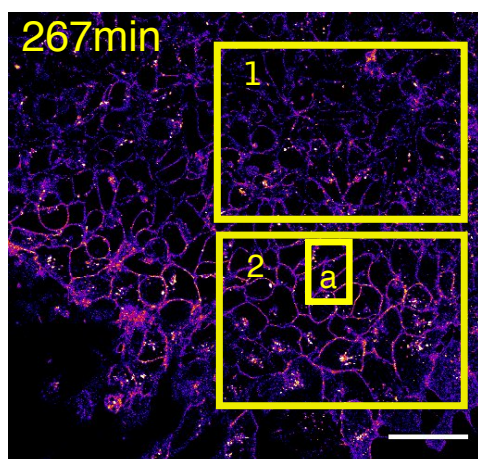


Fig.3**A****B**

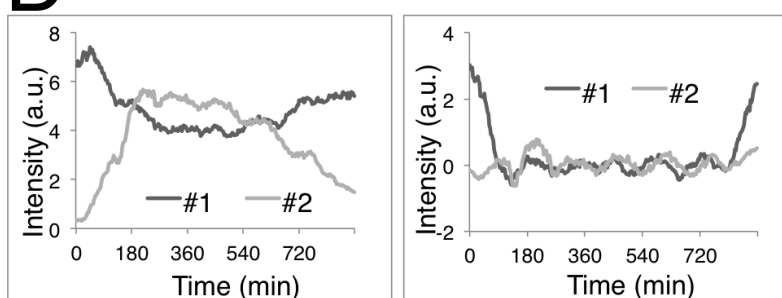
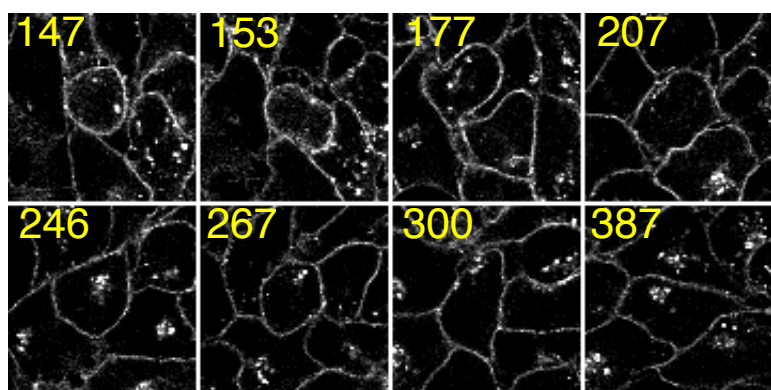
raw

detrended

**C**posterior
(center)anterior
(periphery)**D**

raw

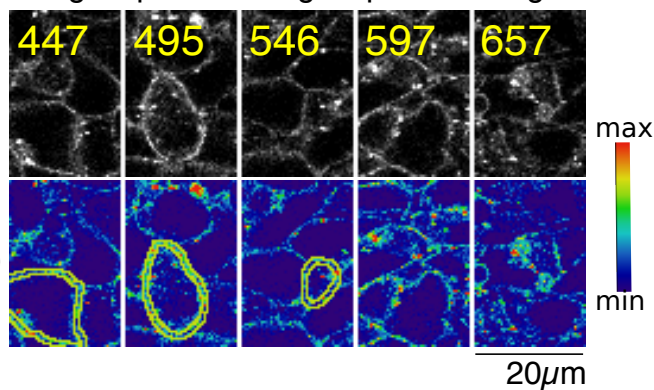
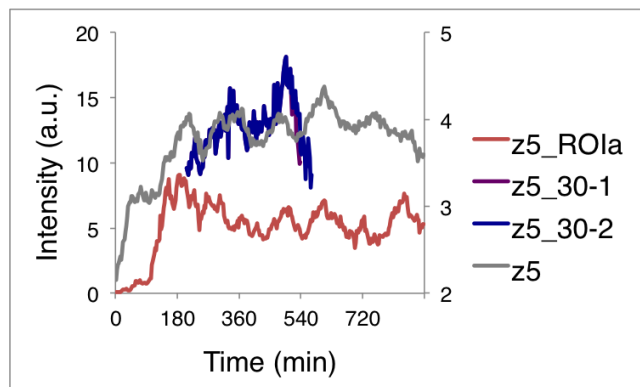
detrended

**E**40 μ m**F**

trough peak trough peak trough

z5_ROIa

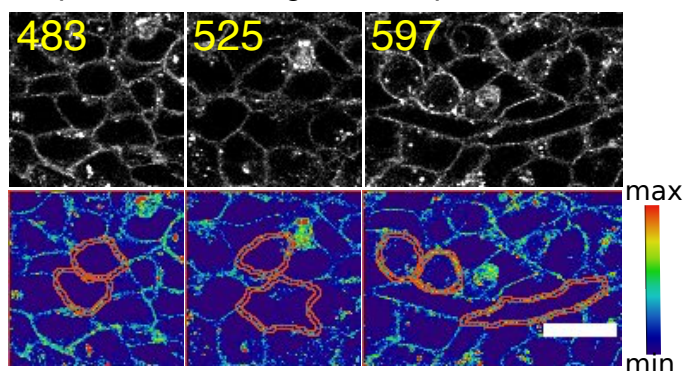
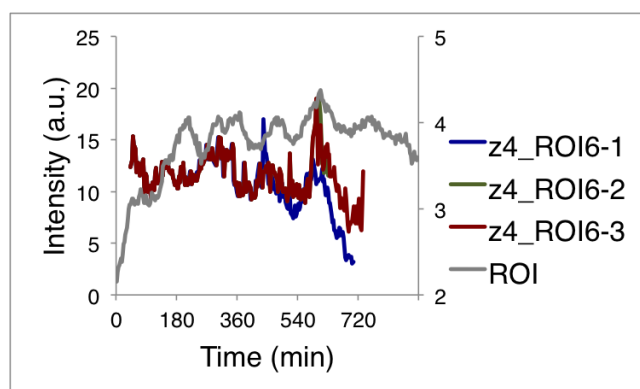
z5_ROI30

**G****H**

peak

trough

peak

ROI6-1
6-2
6-3**I**

Supplemental Figures

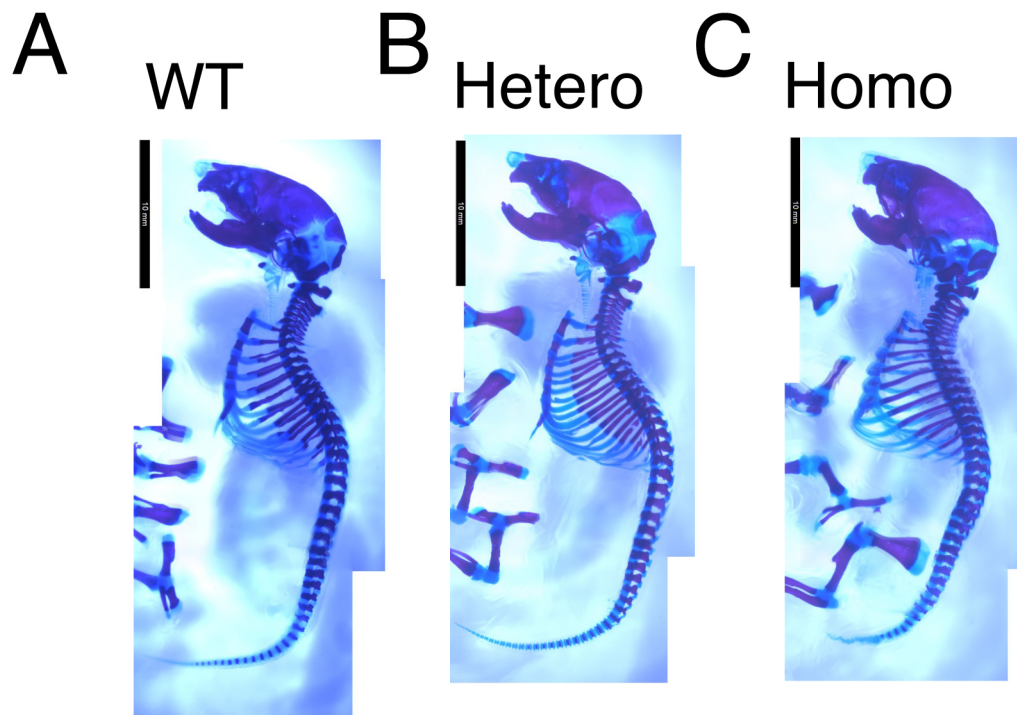


Figure S1. Bone and cartilage staining of wild-type and heterozygous and homozygous *Dll1*-Venus-T2A-mCherry mice. Mendelian proportion of homozygotes with 100% penetrance was observed, confirmed by PCR genotyping. Heterozygous mice show no apparent defects, but homozygous mice have mild kinked tails. Mouse neonatal skeletons were stained with Alcian blue and Alizarin red to differentiate cartilage (blue) and bone (red). Scale bar, 10mm.

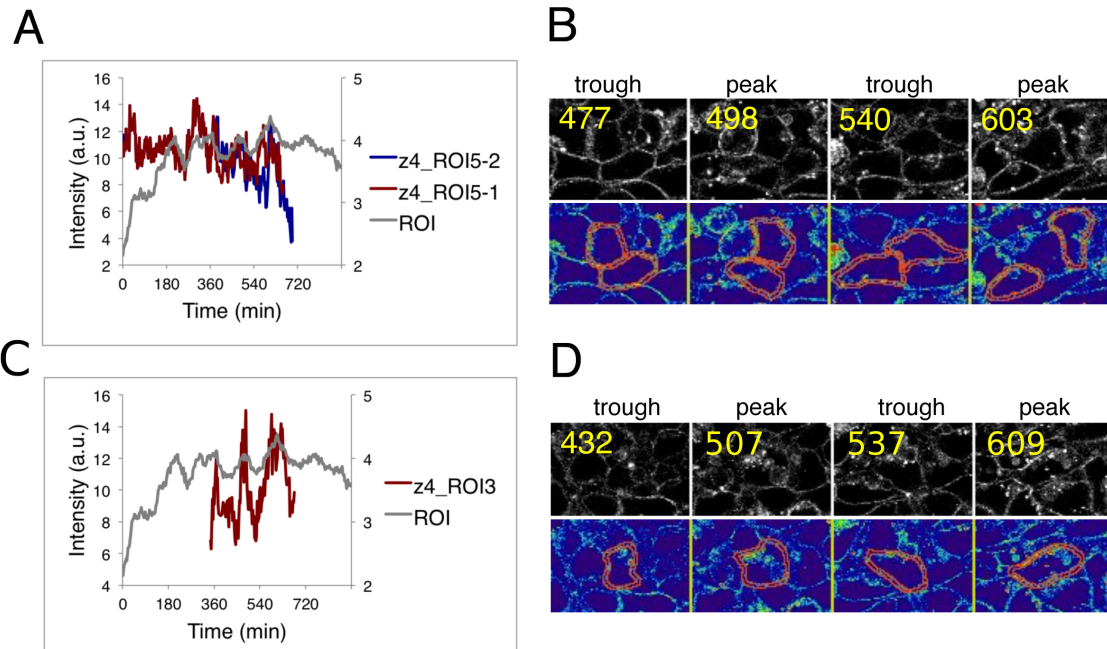


Figure S2. Dll1-Venus protein expression dynamics in the membrane regions of PSM cells in tail bud culture of an E9.5 Dll1-Venus-T2A-mCherry embryo. Representative snapshots and plots from manual segmentation and tracking of the membrane of single cells.