Crumbs Affects Protein Dynamics in Anterior Regions of the Developing *Drosophila* Embryo

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Abstract

Maintenance of apico-basal polarity is essential for epithelial integrity and requires particular reinforcement during tissue morphogenesis, when cells are reorganised, undergo shape changes and remodel their junctions. It is well established that epithelial integrity during morphogenetic processes depends on the dynamic exchange of adherens junction components, but our knowledge on the dynamics of other proteins and their dynamics during these processes is still limited. The early *Drosophila* embryo is an ideal system to study membrane dynamics during morphogenesis. Here, morphogenetic activities differ along the anterior-posterior axis, with the extending germ band showing a high degree of epithelial remodelling. We developed a Fluorescence Recovery After Photobleaching (FRAP) assay with a higher temporal resolution, which allowed the distinction between a fast and a slow component of recovery of membrane proteins during the germ band extension stage. We show for the first time that the recovery kinetics of a general membrane marker, SpiderGFP, differs in the anterior and posterior parts of the embryo, which correlates well with the different morphogenetic activities of the respective embryonic regions. Interestingly, absence of *crumbs*, a polarity regulator essential for epithelial integrity in the *Drosophila* embryo, decreases the fast component of SpiderGFP and of the apical marker Stranded at Second-Venus specifically in the anterior region. We suggest that the defects in kinetics observed in *crumbs* mutant embryos are the first signs of tissue instability in this region, explaining the earlier breakdown of the head epidermis in comparison to that of the trunk, and that diffusion in the plasma membrane is affected by the absence of Crumbs.

Introduction

Epithelia are characterised by a pronounced apico-basal polarity of their cells with the apical side facing the outside and the baso-lateral side facing neighbouring cells and/or a basal lamina. Their cells are closely connected to each other by different types of junction, such as adherens junctions or tight junctions, which guarantee integrity and tightness of these tissues. Epithelia are of crucial importance for shaping the embryo, for example during gastrulation, neurulation or tissue elongation during organogenesis. Several processes contribute to morphogenetic changes of epithelia, such as oriented cell division, changes in cell shape and cell size, remodelling of junctions, reorganisation of the actomyosin cytoskeleton, modification of apical and baso-lateral surface areas and cell intercalation (reviewed in: [1,2,3,4]).

Cell intercalation is the major driving force for tissue and organ elongation and largely depends on convergence and extension movements. It contributes to shaping of embryos and organs and is instrumental for vertebrate axis elongation, tube formation or germ band extension in the *Drosophila* embryo, to mention just a few [1,3,6,7]. Germ band extension in the fly embryo is an ideal model system to study the genetic and cell biological basis underlying tissue elongation. During elongation, the germ band, which develops into the segmented trunk of the larvae, doubles in length along the anterior-posterior axis and narrows along the dorso-ventral axis [8,9]. The process can be subdivided into the first, rapid phase, which takes about 25 minutes, during which most of elongation occurs and the second, slow phase, covering the following 70 minutes [10,11]. Several processes contribute to the elongation of the tissue, which differentially affect the anterior and the posterior region of the germ band. While tissue elongation in the anterior region mostly depends on cell intercalation [8,12,13], taking place as response to mechanical forces exerted by the invaginating mesoderm [14] and anisotropies in cortical tension [15,16,17], extension of the posterior region substantially relies on cell divisions oriented along the anterior-posterior axis [18].

During morphogenetic processes, including germ band extension, epithelial integrity and polarity are controlled by a number of mechanisms, which are closely interconnected. One of the key regulators of epithelial polarity in the *Drosophila* embryo is the Crumbs complex, which contains the transmembrane protein Crumbs (Crb) and the scaffolding proteins Stardust (Sdt), DLin-7 and DPATJ as core components. Other components, such as DPar-6, a member of the Par protein group or Yurt, a negative regulator of Crb, can be transiently recruited into the complex (reviewed in [19,20]). Embryos lacking *crb* function fail to maintain apico-basal polarity in many of their epithelia, which eventually leads to a complete breakdown of tissue integrity, followed by apoptosis [21]. In particular the developing epidermis is strongly


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Figure 1. Stranded at Second-Venus and a toolkit of plasma membrane proxies used in this study. A) Organisation of Stranded at Second (Sas)-Venus used here. Note that part of the native protein was replaced by the fluorophore (green) (see methods section). vWC: von Willebrand factor type C; FN3: Fibronectin type 3; blue bar: transmembrane domain. B) Localisation of the transgene-encoded Sas-Venus resembles the localisation of Sas in wild-type embryos. Left: Sas (top) and DE-Cadherin (middle) staining of the epidermis in wild-type embryos of stage 16 (scale bar: 10 μm). Right: Sas-Venus (top) and DE-Cadherin (middle) staining of the epidermis in Sas-Venus embryos (scale bar: 10 μm). C) Diagram showing the localisation of SpiderGFP, DEcadGFP, LacGFP, and Sas-Venus in the epidermis.
affected. Here, an intact Crb complex is essential to position and form the zonula adherens (ZA), a belt-like structure encircling the apex of the cell [22,23]. On the other hand, overexpression of Crb can lead to an expansion of the apical membrane domain, both in embryos [24] and photoreceptor cells [25,26,27]. These results point to a role of Crb in maintaining the apical membrane, but data demonstrating this role are still missing.

Fluorescence Recovery After Photobleaching (FRAP) is an ideal method for in vivo measurements of protein turnover. Using this method, it was recently shown that biosynthetic DE-Cadherin turnover was higher at early stages of Drosophila embryogenesis, when cells are polarising, compared to polarised epilayer at later stages [28]. Using the same technique, we were interested to find out whether the turnover of general and polarised plasma membrane markers was spatially regulated during germband extension – a stage where cells necessarily need to remodel their plasma membrane and junctions – and whether the polarity regulator Crb plays a role in this process.

Results

To better understand protein dynamics during germband extension in the Drosophila embryo, we developed a FRAP assay with a higher temporal resolution. Since an exclusively apical marker was lacking, Stranded at Second (Sas) was fluorescently tagged with Venus (Fig. 1A). Sas is a type I transmembrane protein composed of 1693 amino acids, with four predicted von Willebrand factor type C (vWC) and three fibronectin 3 (FN3)-domains [29]. Sas is expressed during germband retraction in ectodermally derived tissues, where it is restricted to the apical membrane [24]. A low complexity region of the protein (isof orm B; aa 1092–1244) was replaced with Venus (Fig. 1B; aa 1092–1244) was replaced with Venus, a YFP derived fluorophore with high brightness levels [30,31] (Fig. 1A) and put under the control of UAS-elements or a tubulin promoter. Flies expressing Sas-Venus from either transgene were viable and fertile under the control of UAS-elements or a tubulin promoter. Flies expressing Sas-Venus from either transgene were viable and fertile under the control of UAS-elements or a tubulin promoter. Flies expressing Sas-Venus from either transgene were viable and fertile under the control of UAS-elements or a tubulin promoter. Flies expressing Sas-Venus from either transgene were viable and fertile under the control of UAS-elements or a tubulin promoter. Flies expressing Sas-Venus from either transgene were viable and fertile under the control of UAS-elements or a tubulin promoter. Flies expressing Sas-Venus from either transgene were viable and fertile.

The experimental setup of our FRAP assay consisted in varying temporal imaging acquisition rates post-bleach, performed consecutively in the anterior and posterior region of the embryo (see Materials and Methods) (Fig. 2B). This allowed grasping at the same time the very brisk recovery immediately after bleach and the slower, more ample increase over longer times. A constant acquisition rate would miss either the fast response when using long time intervals, or the complete recovery in case of imaging short intervals only (Fig. 2C-D’). We observed that in all cases the recovery curves resembled the sum of two exponentials $y = A_1(1 - e^{-(t - t_1)/\tau_1}) + A_2(1 - e^{-(t - t_2)/\tau_2})$ (Fig. 3). We retained this function as an empirical fitting model. Other ad-hoc models with less parameters, e.g. the single exponential, could not fit as well the experimental curves (see the fit correlation values ($R^2$) in Fig. 3). The two-exponential model has the benefit of having a very limited number of free parameters and hence better describes the experimental recovery curves (Fig. 3). Moreover, the two exponential components each grasp separate time-scales: the early, quick recovery is described by the exponential with the smallest $\tau$ ($\tau_1$, here) and the later, slower recovery is described by the largest $\tau$ ($\tau_2$). This separation in the fitting process works, because the quick and slow responses have time-scales that differ by several orders of magnitude (see below). As suggested previously [35] we propose that the fast and slow recovery could be the result of diffusion of molecules from outside the region of interest (ROI) and of biosynthetic delivery of proteins via intracellular trafficking routes, respectively, though other alternatives, such as fast and slow membrane trafficking routes cannot be excluded.

Once all double exponential fitting curves were obtained from all FRAP experiments for all different markers and normalised (see Materials and Methods), we performed statistical analysis of all parameters. The time-scale parameters $\tau_1$ and $\tau_2$ gave us the recovery kinetics, whilst $A_1$ and $A_2$ measured the relative amount of fluorophore (mobile fraction) used in the quick and slow recovery, respectively. By following these four parameters, we could monitor changes in the recovery dynamics and changes in the pre-eminence of one versus another.

No differences in $\tau_1$ and $\tau_2$ of Sas-Venus, DE-CadherinGFP and LachesinGFP were observed between the anterior and the posterior region (Fig. 4.; blue symbols mark the anterior and red/green circles the posterior regions). However, a significant difference of SpiderGFP in $\tau_1$ recovery was observed between the two areas of the embryo, in that the fluorescence in the anterior recovered more slowly than in the posterior of the embryo (25.13s vs 14.17s) (Fig. 4, top, last two data sets). A similar behaviour was observed for $\tau_2$, as the recovery rate in the anterior was slower than that in the posterior of the embryo (583.8s vs 368.04s) (Fig. 4, bottom, last two data sets). To summarise, only the general membrane marker SpiderGFP showed a difference in recovery, in that the recovery was slower in the anterior region in comparison to the posterior region. These differences reflect the varying levels of morphogenetic activity in the embryo – cells where no intercalation ocurs (anterior) have longer recovery times after bleaching, whereas cells undergoing intercalation (posterior) are
Figure 2. The FRAP assay. A) Imaged areas of the embryo – the region localised anteriorly to the cephalic furrow shows lower levels of morphogenetic activity whilst the posterior region shows high levels of morphogenetic activity. The shown embryo is expressing DE-CadherinGFP. Scale bar - 50 μm. B) A FRAP experiment consists in 4 different phases of image acquisition characterised by different temporal resolution and duration: prebleach (1 frame/5 seconds; 25 seconds); fast postbleach (1 frame/1 second; 60 seconds); medium postbleach (1 frame/5 seconds; 600 seconds); slow postbleach (1 frame/30 seconds; 600 seconds). Still images of a SpiderGFP FRAP experiment movie are shown as an example to
much faster in recovering the levels of fluorescence of the general membrane marker SpiderGFP.

To reveal a role of crb for trafficking of membrane proteins, we performed FRAP assays of SpiderGFP and Sas-Venus in crb mutant embryos. Again, we measured $t_1$ and $t_2$ in the anterior and posterior region. As described above, $t_1$ and $t_2$ of SpiderGFP had higher values in the anterior compared to the posterior in wild-type embryos (25.13s vs. 14.17s and 583.8s vs. 368.04s) (Fig. 5, left; blue symbols mark the anterior and red/green circles the posterior regions). In the absence of crb, the difference of $t_1$ was completely abolished and the recovery rates were similar in the anterior and posterior crb mutant embryos (14.65s vs. 17.32s) (Fig. 5, top, left). The difference of $t_2$ values between anterior and posterior observed in wild-type embryos was maintained in crb mutant embryos. $t_2$ showed higher values in the anterior compared to those in the posterior region (wild-type: 583.8s anterior vs. 368.04s posterior, and crb: 504.19s anterior vs. 364.73 s posterior) (Fig. 5, bottom, left). We recognised that the kinetic values of $t_1$ in the anterior of crb mutant embryos (14.65s) were decreased in comparison to wild-type (25.13s), while the posterior values remained similar (14.17s vs. 17.32s) Fig. 5, top, left). A similar observation was obtained for $t_2$, which was reduced in crb mutant embryos in comparison to wild-type only in the anterior region (583.8s in wild-type vs. 504.19s in crb), but not in the posterior (368.04s in wild-type vs. 364.73s in crb). To summarise, absence of crb affected the kinetics of a general membrane marker, SpiderGFP, only in the anterior region of the embryo. SpiderGFP recovered faster than in wild-type, so that the values measured in the anterior were more similar to that in the posterior region, thus abolishing the difference between anterior and posterior observed in wild-type embryos.

We next analysed the consequences of the loss of crb on the behaviour of the apical marker Sas-Venus. The only significant difference we observed was a decrease in the mean value of $t_1$ in the anterior region of the embryos (21.58s in wild-type vs. 18.1s in crb) (Fig. 5, top, right, blue symbols). No effect on the behaviour of Sas-Venus was observed in the posterior (21.01s in wild-type vs. 20.5s in crb). Equally, no significant differences in the $t_2$ values of wild-type and crb mutant embryos were observed in the anterior (323.96s vs. 397.3s) and the posterior regions (344.17s vs. 430.74s).

Taken together, our results show that the absence of crb enhances the diffusion/fast delivery ($t_1$) of both SpiderGFP as well as Sas-Venus, but only in the plasma membrane of the anterior region.

**Discussion**

The FRAP experiments presented here aimed to analyse whether the differences observed in morphogenetic behaviour of epithelia in the head and the extending germband of the Drosophila embryo were reflected by the kinetics of membrane proteins. Strikingly, from the four proteins analysed, only SpiderGFP showed a significant difference between the anterior and the posterior. Cells in the anterior, where no intercalation occurs take longer to recover their fluorescence after the bleach, whereas cells undergoing intercalation (posterior) are much faster in recovering the levels of fluorescence. In wound-healing assays performed in Drosophila embryos, SpiderGFP showed the same behaviour as the
pleckstrin homology domain of PLC (phospholipase Cγ) and GAP43 (growth-associated protein 43), despite the fact that all three are differently attached to the membrane. This suggested that all of them reflect the behaviour of the membrane in general [36].

The unique behaviour of SpiderGFP observed could be explained by the fact that it is the only one of the four proteins analysed that is linked to the membrane via palmitoylation. Therefore, the anterior-posterior difference in its dynamics cannot easily be explained by differences in protein/vesicle trafficking. They may rather hint to differences in trafficking and/or mobility within the membrane.

Unexpectedly, however, the higher morphogenetic activity in the germband due to convergent extension movements is not reflected by a higher turnover of DE-Cadherin in this region compared to the anterior region, suggesting that the apico-basal boundary is maintained. This is different from the pupal wing epithelium, where the hexagonal packing of cells depends on...
polarised trafficking of DE-Cadherin during junction remodelling [37].

There is a prominent effect of loss of crb on \( \tau_1 \) of SpiderGFP and Sas-Venus, but only in the anterior region. Recovery is enhanced in the absence of crb and approximates its values to the ones measured in the germband. This suggests that crb plays an important role on membrane dynamics particularly in the procephalic region. Common to both proteins is their association with the apical membrane. This suggests that Crb affects specifically apical proteins, independent of the way they are associated with the membrane, a result that is in agreement with the apical localisation of Crb itself. So far, we can only speculate about the mechanism by which Crb influences the dynamics of SpiderGFP and Sas-Venus. Crb could stabilise the underlying membrane-associated cytoskeleton, and/or it may modify the characteristic features of the membrane. Both mechanisms could act on transmembrane (Sas) and palmitoylated proteins (Spider). Support of the former model comes from the observation that loss
of Crb results in loss of βH spectrin [23,38]. Loss of βH spectrin, in turn, could lead to a destabilisation of the membrane-associated cytoskeleton and enhanced protein turnover. In fact, a higher rate of endocytosis upon reduction of spectrin was described previously [39,40], but this is more likely to act on the slow phase of recovery. Alternatively, the effect of crb on the fast phase of recovery could be explained by a faster diffusion in the membrane, which could be due to the deterioration of a diffusion barrier within the membrane due to the loss of the Crb complex. It is well established that the highly complex organisation of the plasma membrane itself has an impact on the diffusion, stability and trafficking of proteins [41,42]. It is tempting to speculate that Crb may, directly or indirectly, modify membrane characteristics, which would result in a faster recovery of membrane proteins by lateral diffusion.

Unexpectedly, lack of crb affects the dynamics in the anterior region, rather than in the germband. In fact, the head epidermis falls apart earlier in crb mutant embryos than the epidermis in the trunk [43], although no major defects in epithelial integrity were observed in the anterior region at early stages of development (data not shown). Nevertheless, the anterior region is subject to morphogenetic changes due to postblastodermal divisions [44] and delamination of neuroblasts, the precursors of the nervous system [45,46,47]. Both processes occur earlier in the head than in the trunk and may require additional mechanisms ensuring tissue stability. Therefore we suggest, that the faster recovery of SpiderGFP and Sas-Venus observed in crb mutant embryos in the procephalic region are the first signs of tissue instability. The data presented here reveal a novel function of crb in epithelial morphogenesis by influencing the dynamics of membrane proteins.

Materials and Methods

Cloning of Sas-Venus and establishment of transgenic lines

Stranded at Second (Sas) CDS was obtained from the Drosophila Genomics Resource Center (LD44801). The low complexity region of Sas replaced by Venus consisted in 461bp as determined by the restriction sites SpeI and XhoI. Venus was placed between two linker sequences (GGSGGGGSGG) in order to optimise its solubility and folding within SAS. SAS-Venus was cloned into pCasper4 with a tubulin promoter (gift from Suzanne Eaton lab), tub-SAS-Venus, and into pUAST, giving rise to UAS-SAS-Venus. P-element transformation of the constructs was done according to the procedure described by [48]. We used w1118 as recipient strains. Several independent transgenic lines were established. Correct localisation of transgene-encoded Venus-tagged Sas protein in embryos was confirmed by comparing Venus fluorescence with antibody stainings against endogenous Sas protein, using anti-Sas (dilution 1:500; kindly provided by D. Cavener), and anti-DE-cadherin antibody to mark the zonula adherens (dilution 1:50 [49]) and standard fixation protocols of Drosophila embryos [50].

Drosophila stocks

Flies were raised on conventional cornmeal agar at 25°C. See fly list in Table 1.

Embryo Collections

Flies were placed in cages with apple juice agar plates containing yeast. After two hours, the plates were collected and left at 25°C for roughly 5h, giving rise to embryos at germband elongation (stage 8–10).

Imaging of live embryos by laser confocal microscopy

Embryos were dechorionated in bleach for 2m45s, rinsed with water and placed on slides containing HaloCarbon Oil 700 (Sigma-Aldrich) with two coverslips (thickness 1.5; 22mm×22mm, Corning) on each side, creating an artificial chamber when covered with a coverslip (thickness 1; 24mm×50mm, Menzel-Glaeser).

FRAP experiments were conducted in an inverted microscope with motorised stage (Zeiss LSM 510 DuoScan, Carl Zeiss MicroImaging, Inc.), using the 488nm line of an Argon laser with a 505–530 emission filter for GFP and Venus and a 405nm laser diode for the bleaching. All images were captured with a C-Apochromat 1.2 NA 40× water immersion objective (Carl Zeiss MicroImaging, Inc.) with a zoom of 3 for the FRAP experiments. All images consisted in 2 μm optical slices.
Photobleaching and analysis

FRAP experiments were performed by photobleaching a circular ROI (region of interest) encompassing the target cell and its surrounding neighbours and then monitoring fluorescence recovery. Since FRAP experiments were performed in both the anterior and posterior regions of the same embryo, there is a time delay of roughly 22 minutes between the start of both movies (the duration of a FRAP experiment).

To achieve the different temporal acquisition rates, a macro was created using VisualMacro Editor (Carl Zeiss MicroImaging, Inc.). Images were analysed with FIJI software [51]. To compensate for cell drift, the Linear Stack Alignment with SIFT plugin was used. Fluorescence value measurements were then exported to Microsoft Excel where they were normalised and scaled between 0–1 using:

\[ I_{\text{norm}} = \frac{(I_{\text{bleach}} - I_{\text{nonbleach}}) - \text{max}(I_{\text{bleach}} - I_{\text{nonbleach}})}{\text{max}(I_{\text{bleach}} - I_{\text{nonbleach}}) - \text{min}(I_{\text{bleach}} - I_{\text{nonbleach}})} \]

Subsequently, these Excel files containing the normalised values of fluorescence recovery were imported to a MATLAB script, which performed the curve fitting analysis, plotted these and then did a statistical analysis of the various parameters obtained from the double exponential equation used for the fit. Since the FRAP experiments were conducted in the same optical slice throughout the whole duration of the experiment, eventual shifts in the z-axis had to be manually curated and then compiled in a master file stating the timepoint at which an eventual z-axis shift had occurred. This was then used as a reference by the MATLAB script when using the curve fitting module.

Outliers in the fitted parameters were identified as follows – for each parameter distribution, q1 and q3, were calculated, which represent 25% and 75% percentile, respectively. Values larger than q3+1.5×(q3−q1) were deemed as outliers and not included in the mean, standard deviation and t-tests calculations.

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Author Contributions

Conceived and designed the experiments: EK, JF. Performed the experiments: JF. Analyzed the data: JF, JYT. Wrote the paper: JF, EK.

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