**On Protein Recruitment Dynamics in Clathrin-Mediated Endocytosis**

**Dissertation**

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By

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**Fields of Study**

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# Introduction

## 1.1 The Plasma Membrane

The plasma membrane is a bilayer of lipids arranged in a way such that it is impermeable to large or electrically charged molecules, but permeable to other molecules, like water.

It plays a uniquely important role in cell biology: It defines the boundary of the cell. Anything large or electrically charged is partitioned into the cytoplasm or the space outside the cell. For large, necessary proteins to cross the plasma membrane into the cell, the plasma membrane must be circumvented by a process called endocytosis (Conner, 2003).

There are several distinct variants of endocytosis (Conner/Schmid fig). All of these use the same basic idea, akin to how an amoeba eats (fig?): The cargo to be internalized must be surrounded by an envelope of plasma membrane, at which point the envelope can close around the cargo forming a vesicle. This vesicle is then effectively inside the cell without a hole ever being made in the membrane.

Working against these processes, though, is the fact that the plasma membrane is resistant to being curved or stretched. Because of this, active cellular processes must work against the plasma membrane’s tendency to be as flat as possible.

(membrane tension?)

## 1.2 Clathrin and friends

The method of endocytosis of interest in this work is clathrin-mediated endocytosis (CME). In this process, the energy needed to curve the membrane into a vesicle is partially supplied by the bonds of a molecule called clathrin.

Clathrin is a triskelion shaped molecule (fig) that spontaneously self-assembles into round, closed cages if it is present at sufficient concentration and appropriate pH (Crowther, 1975). It does not, however, bind to the plasma membrane, so it cannot create vesicles alone.

Aside from clathrin, AP2 and CALM are the most abundant proteins in clathrin-coated structures (Borner, 2012). These proteins can bind clathrin and the membrane simultaneously. As such, they are called adaptor proteins. AP2, specifically, will be of particular interest in this work because it, unlike clathrin, only localizes to CME-associated structures. CALM is a less-studied adaptor that will be the subject of Chapter 5.

## 1.3 Fluorescence Microscopy (work on later)

(history stuff?)

Fluorescence microscopy is important to our work because it allows us to track the cellular location and relative numbers of different specific proteins in live cells. Because proteins are so small, light microscopes cannot resolve them inside a cell directly. To get around this problem, we give our cells DNA that allows them to make our protein of interest, attached to a fluorescent tag. This way, when we shine a laser with the correct wavelength at a cell, it will fluoresce only in the subcellular locations where our protein is (fig). For the specific case of AP2, we see discrete punta on the cell membrane corresponding to active CME events.

The single images in fig only contain limited information. From them, we can only calculate the locations of the various clathrin-coated structures (CCSs) and their intensities. If we wish to study dynamic quantities such as the time it takes for a clathrin-coated pit to internalize, we must track these puncta over time. To this end, software has been developed that uses advanced, optimized algorithms to track clathrin-coated structures on the plasma membrane (Aguet, 2013).

## 1.5 The Life of the Average Clathrin-Coated Pit

A typical intensity profile corresponding to the AP2 content of a clathrin-coated pit over time is illustrated in fig. The pit slowly and linearly accumulates clathrin and AP2 over 40 or so seconds, then reaches a plateau, in which dynamin and other proteins must be recruited to perform scission on the neck of the pit. Once scission is complete, the newly formed vesicle quickly diffuses away and uncoats, causing a steep decrease in intensity.

## 1.6 Thesis Overview

This thesis will cover the extent of my study of clathrin mediated endocytosis and how membrane tension has far-reaching effects on the machinery therein. Chapter 2 will describe methods to be used later in the measurement of CME dynamics. Chapter 3 will describe how elevated membrane tension affects CME. Chapter 4 will explore the link between CME and membrane tension in a more physiological context. Chapter 5 will show how the protein CALM is connected to membrane tension. And chapter 6 will summarize this work and describe where more work remains to be done.

# Deciphering Dynamics of Clathrin-Mediated Endocytosis in a Living Organism (Later)

Derived from: Ferguson, J.P., Willy, N.M., Heidotting, S.P., Huber, S.D., Webber, M.J., and Kural, C. (2016). Deciphering dynamics of clathrin-mediated endocytosis in a living organism. J. Cell Biol. *214*, 347–358.

*In this chapter my contributions are some experiments of cholesterol depleted cells; and development of the 2D trace rejection algorithm*

## 2.1 Abstract

Current understanding of clathrin-mediated endocytosis (CME) dynamics is based on detection and tracking of fluorescently tagged clathrin coat components within cultured cells. Because of technical limitations inherent to detection and tracking of single fluorescent particles, CME dynamics is not characterized in vivo, so the effects of mechanical cues generated during development of multicellular organisms on formation and dissolution of clathrin-coated structures (CCSs) have not been directly observed. Here, we use growth rates of fluorescence signals obtained from short CCS intensity trace fragments to assess CME dynamics. This methodology does not rely on determining the complete lifespan of individual endocytic assemblies. Therefore, it allows for real-time monitoring of spatiotemporal changes in CME dynamics and is less prone to errors associated with particle detection and tracking. We validate the applicability of this approach to in vivo systems by demonstrating the reduction of CME dynamics during dorsal closure of Drosophila melanogaster embryos.

## 2.2 Introduction

Clathrin-mediated endocytosis (CME) is the major pathway responsible for internalization of lipids and receptor-bound macromolecules from the plasma membrane of eukaryotic cells (Conner and Schmid, 2003). During internalization of a cargo molecule, clathrin triskelions assemble into submicron-sized polyhedral structures upon their recruitment to the plasma membrane by the endocytic clathrin adaptor protein AP2 (Ehrlich et al., 2004; Saffarian and Kirchhausen, 2008; Boucrot et al., 2010; Cocucci et al., 2012; Hong et al., 2015). Live-cell imaging studies designed for tracking of fluorescently tagged clathrin coat components have revealed the dynamics of formation, internalization, and dissolution of distinct classes of clathrin-coated structures (CCSs; Gaidarov et al., 1999; Merrifield et al., 2002; Ehrlich et al., 2004; Loerke et al., 2009; Mettlen et al., 2010; Taylor et al., 2011; Kural and Kirchhausen, 2012; Aguet et al., 2013). The best-characterized structures are highly curved, cage-like assemblies that deform the plasma membrane into pits and vesicles. In conventional fluorescence time-lapse acquisitions, clathrin-coated pits appear as diffraction-limited spots with mean lifetimes of ∼1 min (Ehrlich et al., 2004; Saffarian et al., 2009; Kural et al., 2012; Aguet et al., 2013). CCSs disappearing within the first ∼20 s of their initiation are abortive structures that fail to construct bona fide endocytic carriers (Hong et al., 2015). Flat arrays of clathrin, also known as plaques, are larger than coated pits and slower in their internalization dynamics (Saffarian et al., 2009; Grove et al., 2014). Physiological relevance of clathrin-coated plaques has been equivocal, because they only appear at the substrate contact sites of cultured cells and, because of their long lifetimes, they are not effective endocytic carriers.

Dynamics of endocytic pathways are inversely related to plasma membrane tension, because membrane internalization machinery are required to do work against the two major constituents of tension (i.e., in-plane tension and membrane-cytoskeleton adhesion) to create invaginations (Dai et al., 1997; Raucher and Sheetz, 1999; Sheetz, 2001; Apodaca, 2002; Gauthier et al., 2012; Diz-Muñoz et al., 2013). Tension regulates formation and curvature of clathrin coats reconstructed on giant unilamellar vesicles (Saleem et al., 2015). Studies in yeast and in polarized and mitotic mammalian cells show that CME is inhibited unless plasma membrane tension is counteracted by actin dynamics (Aghamohammadzadeh and Ayscough, 2009; Boulant et al., 2011; Kaur et al., 2014).

Regulation of endocytic rates by mechanical cues has important roles in development; during the early stages of Drosophila melanogaster embryogenesis, increased tension inhibits Fog receptor endocytosis, which is required for completion of ventral furrow formation (Pouille et al., 2009).

Our current understanding of CME dynamics is based on in vitro imaging studies that are limited in their potential to mimic physical properties of tissue microenvironments. In a majority of these studies, dynamics of CCSs were monitored at the plasma membrane–coverglass interface, which has no physiological correspondence. Plating conditions, membrane–substrate interactions, and cell spreading area can regulate clathrin dynamics in in vitro experiments (Batchelder and Yarar, 2010; Tan et al., 2015). A holistic understanding of CME requires elucidating clathrin coat dynamics in cells residing within tissues of multicellular organisms.

Determining lifetime distributions of CCSs is the prevalent technique for monitoring CME dynamics. This approach necessitates identifying complete traces of individual CCSs (from initiation to dissolution), which is error prone within high-density particle fields and regimes with low signal to noise (Aguet et al., 2013; Mettlen and Danuser, 2014). CME dynamics have not been reported for any in vivo systems, because determining lifetimes of individual CCSs is more challenging within complex, 3D geometries of living tissues. In this study, we show that the rate of incorporation or dispersion of clathrin coat components during formation of endocytic vesicles can be used as reporters for clathrin dynamics. Because hundreds of clathrin-coated endocytic carriers can be detected within a cell at a given instant, distributions spanning the entire range of formation and disassembly rates can be obtained within temporal windows shorter than the lifetime of clathrin coats. This advantage makes growth rate distributions a superior alternative to clathrin lifetime analyses, especially within cellular contexts where the fidelity of fluorescent particle tracking is low. Using this approach, we provide the first experimental evidence of CME mechanoregulation in tissues of live Drosophila embryos.

## 2.3 Results

### 2.3.1 CCS growth rates are robust reporters of CME dynamics

In fluorescence imaging assays, endocytic CCS formation is marked by appearance of a diffraction-limited spot that steadily increases in intensity because of accumulation of fluorescently tagged coat components. The internalization of the clathrin-coated vesicle is followed by a relatively fast dimming of the fluorescence caused by dissolution of the coat (Kural and Kirchhausen, 2012; Fig. 2.1, A and B). Lifetime (i.e., the time required for formation and internalization of CCSs from the plasma membrane) is an extensively used metric for characterizing CME dynamics. Factors that elongate CCS lifetimes reduce the efficacy of ligand endocytosis (Cureton et al., 2010; Boulant et al., 2011). Any factor that affects the rate of formation and/or dissolution of clathrin coats is a potential regulator of CCS lifetimes and, hence, CME dynamics. To establish whether we can use growth rates of CCS signal as metrics for endocytic dynamics, we quantified formation and dissolution (negative growth) rates of individual CCSs by determining the slope of the normalized fluorescence intensity within 12-s temporal windows (Fig. 2.1 C and online supplemental software). Because hundreds of CCS traces can be detected within a single cell at a given instant, we were able to assemble distributions of growth rates for each frame of time-lapse acquisitions (Video 1). When applied to cells that produce predominantly clathrin-coated pits, the majority of the growth rates obtained in this way were positive (i.e., corresponding to increasing fluorescence signal; Fig. 2.2 B). This is consistent with the characteristic intensity profile of pits, which has a steady formation phase followed by a relatively abrupt dissolution (Ehrlich et al., 2004; Massol et al., 2006; Fig. 2.1 B).

Figure 2.1 Determining CCS growth rate distributions.

Figure 2.1 Determining CCS growth rate distributions.

(A) Fluorescence intensity trace of a clathrin hotspot imaged at the ventral surface of a BSC-1 cell expressing AP2-GFP. Traces segments that are not used in growth rate calculation are shown in red, as they were considered the background signal. (B) Zoomed version of the clathrin-coated pit trace marked by the arrow in A. Shaded regions show 12-s-long fragments dwelling at formation (red), plateau (green), and dissolution (purple) phases of the pit. (C, left) For the intensity trace in A, slope values representing the growth rates are determined from 12-s-long fragments centered on each time point. Red, green, and purple arrowheads mark the slopes corresponding to the growth, plateau, and dissolution fragments highlighted in B, respectively. (C, right) Bar plot shows the distribution of the growth rates shown in the left panel. Positive and negative values correspond to formation and dissolution phases, respectively.

As a means to alter CME efficiency, we used micropipette aspiration to increase in-plane membrane tension in cells (Houk et al., 2012). We detected a significant increase in CCS lifetimes when micropipette aspiration was applied (45.5 ± 27.1 s versus 80.1 ± 86.6 s, Ncells = 7, Ntraces= 38,136; Fig. 2.2 A). Asymmetry of the growth rate distributions is abolished upon aspiration, and a greater fraction of intensity traces were associated with steady levels of fluorescence signal (i.e., the plateau phase). High-magnitude slopes, which represent fast formation and dissolution phases, are diminished with the increasing membrane tension (Fig. 2.2, B and C). These transformations in the growth rate distributions suggest that obstruction of both coat formation and dissolution is the prevalent factor behind elongation of the mean CCS lifetime under increased membrane tension. We used two independent visualization tools to validate these results. First, we created 2D histograms assembled by superposition of CCS intensity profiles that are synchronized at the beginning, middle, or end of traces (Fig. 2.2 D). Even though CCSs have a wide distribution of lifetimes, 2D histograms assembled from traces obtained before aspiration displayed the characteristic intensity profile of coated pits (Fig. 2.2 D, top row, middle column). However, the histograms assembled using aspirated cell traces were wider and displayed significant elongation in formation, plateau, and dissolution phases (Fig. 2.2 D, bottom row). In the second approach, we used a hierarchical clustering algorithm to create groups of CCS traces that have similar intensity profiles. As expected, clusters obtained from aspirated cells displayed longer plateau phases and slower formation and dissolution rates.

Figure 2.2 Using growth rate histograms as reporters of clathrin dynamics.

(A) Kymographs are generated from the same BSC-1 cell before and during microaspiration, respectively. Elongated AP2 traces demonstrate longer CCS lifetimes under increased membrane tension. (B) Growth rate distributions are shown for seven different cells before and during aspiration. Change in CCS dynamics induced by micropipette aspiration can be observed in growth rate distributions. In the control experiments (cells before aspiration), CCSs spend more time in the formation phase (i.e., the distribution is inclined toward positive slopes). The asymmetry was abolished upon aspiration and plateau phases got relatively longer (Ncells= 7 and Ntraces = 38136). (C) Growth rate distributions in B are assembled in five bins to better delineate different phases of clathrin-coated vesicle formation (FD, fast dissolution; FF, fast formation; P, plateau; SD, slow dissolution; SF, slow formation). The bars show mean + standard deviation to illustrate dispersion between cells. P-values were obtained using the two-tailed t test. (D) 2D histograms of normalized intensity traces aligned at different time points (beginning, trace maximum, and end) and superposed as represented by the cartoons. In each alignment, the aspirated cells show a significantly widened distribution, demonstrating a preference for slopes lower in magnitude. Bins corresponding to multiples of 12s are more populated, as they contain trace data from both 3- and 4-s frame rate acquisitions.

To elucidate the effects of membrane–substrate interactions on CCS growth rates, we used a 3D particle tracking algorithm to determine the relative axial positions and fluorescence intensities of CCSs at the ventral surface of cultured cells (Kural et al., 2012, 2015). Along with pits, we observed formation of clathrin-coated plaques at the ventral surfaces of BSC-1 cells that are plated for >72 h (Fig. 2.3 A). Based on the intensity and position information, we categorized the CCSs as plaques (bright and close to substrate), “low” pits (dim and close to substrate), and “high” pits (dim and far from the substrate; Fig. 2.3, B and C). In good agreement with the previous studies, we found that CCSs positioned closer to the substrate contact sites are significantly longer lived (plaques, 174.5 ± 160.5 s; low pits, 75.2 ± 78.3 s; high pits, 48.3 ± 27.8 s, Ncells = 6, Ntraces= 11,482; Fig. 2.3 C; Batchelder and Yarar, 2010). Growth rate analyses establish that impairment of clathrin dynamics in the proximity of adhesion regions is associated with extension of the plateau and diminishing of the high slope phases, consistent with the measurements performed in aspirated cells (Fig. 2.3 D). Our combined results demonstrate significant alterations in CCS growth rates caused by physical factors that hinder endocytic dynamics.

Figure 2.3 CCSs in proximity of substrate adhesion sites have reduced dynamics.

(A) Arrowheads mark clathrin-coated plaques at the ventral surface of a BSC-1 cell stably expressing AP2-GFP. Bar, 5 µm. Scatterplot shows maximum intensity versus the axial positions of CCSs detected at the ventral surface. The positions are relative to the lower bound of the confocal volume used for 3D tracking (Kural et al., 2012). CCS traces are divided into plaque (red) and pit (green and blue) populations based on their maximum intensities. Clathrin pits positioned closer to the substrate are in green (Ncells = 6 and Ntraces = 11,482). Scatterplot shows the lifetime versus axial positions of the CCSs shown in B. (D) Growth rate distributions of the pit and plaque populations. The inset shows the distributions as bar plots.

### 2.3.2 Accuracy of growth rate distributions does not depend on determining complete traces of CCSs

Errors associated with particle detection and tracking result in significant underestimation of CCS lifetimes (Aguet et al., 2013; Mettlen and Danuser, 2014). Slope values populating the growth rate histograms, however, are calculated using trace information within 12-s-long windows and thus do not rely on determining the complete CCS traces. We tested the reproducibility of growth rate distributions on 24-s-long integrated movie segments (IMSs) that are the quadrature sum of four different temporal sections of a movie (Fig. 2.4, A–C). Faithful assessment of CCS lifetimes from IMSs is impractical because (1) density of CCSs is on average four times greater than the original acquisition, (2) background noise level is increased due to error propagation, and (3) IMSs are significantly shorter than the mean coated pit lifetime (24 s versus 1 min). Despite these impediments, we found that CCS growth rate distributions could be reproduced very accurately from the IMSs (Fig. 2.4, B and C). We also found that distortion of the growth rate distributions caused by increased tension could be observed in the IMSs of microaspirated cells regardless of the algorithm used for tracking CCSs (Fig. 2.4 D).

Figure 2.4 Growth rate analysis does not depend on determining the complete traces of CCSs.

(A, left) Thumbnails represent 12 temporal sections (24 s each) of a 288-s-long fluorescence movie of a BSC-1 cell stably expressing AP2-GFP. (right) CCS growth rate distribution of the entire movie. (B, left) Integrated movie segments (IMSs) are the quadrature sum of four 24-s-long segments in which density of CCSs is approximately four times larger than the original movie. Temporal separation is maximized between the segments to minimize the number of self-overlapping clathrin spots. (right) Growth rate distributions obtained from each IMS is plotted in comparison with the distribution obtained from the entire movie (black). (C) Growth rate distributions of the IMSs and the entire movie are displayed as bar plots. (D) We tested the reproducibility of growth rate distributions using the CCS traces produced by cmeAnalysis (Aguet et al., 2013) and TraCKer (homemade particle tracking program; see online supplemental software). Changes in slope distributions upon micropipette aspiration can be observed in both datasets. Numbers of the analyzed IMSs are 25 and 64 for before and during aspiration conditions, respectively (Ncells = 7). Error bars show standard deviations. FD, fast dissolution; FF, fast formation; P, plateau; SD, slow dissolution; SF, slow formation.

### 2.3.3 Spatiotemporal variations in CME dynamics can be resolved in real time using CCS growth rates

When CCS lifetime distributions are used as metrics for CME efficiency, the mean lifetime of clathrin-coated pits (∼1 min) sets the limit of the temporal resolution for distinguishing the changes in clathrin coat dynamics. Consequently, the factors that influence endocytic processes at shorter time scales become indiscernible. Growth rate distributions, however, are constructed before the completion of traces and thus enable real-time monitoring of CCS dynamics in cells. We used growth rate analysis to monitor gradual changes in CCS dynamics upon acute cholesterol depletion. Structural studies show that cholesterol depletion reduces CME efficiency by replacing clathrin-coated pits with flat clathrin arrays (plaques) at the cell membrane (Subtil et al., 1999; Fig. S2 A). When cells are treated with methyl-β-cyclodextrin (MβCD), in line with our micropipette aspiration experiments, we found that impairment of CCS dynamics is coupled with disappearance of high-magnitude slopes in CCS growth rate distributions (Fig. 2.5, A and B; and Video 2). This result was anticipated, as cholesterol depletion increases the effective membrane tension through escalation of membrane–cytoskeleton adhesion energy (Khatibzadeh et al., 2012; Fig. S2 B). We used standard deviation of regional CCS growth rates to generate a visualization tool for monitoring spatiotemporal changes in clathrin dynamics in real time (Fig. 2.5 C). In regions with impeded CCS dynamics, standard deviations of the growth rates were reduced because of the disappearance of the high-magnitude slopes (Fig. 2.5 D). Using this approach, we found that MβCD treatment can affect CME dynamics at different rates in neighboring cells (Fig. 2.5 D, E). Cell-to-cell variation may stem from cells’ mitotic state or position with respect to cell islet edges, as described earlier (Snijder et al., 2009).

Figure 2.5 Real-time monitoring of CCS dynamics in cholesterol-depleted cells.

(A) Fluorescence images show snapshots of three BSC-1 cells stably expressing AP2-GFP. 10 mM MβCD was applied at the sixth minute (t = 360 s) to initiate cholesterol depletion (Video 2). Dashed lines demarcate the cell boundaries. Bar, 10 µm. (B) For the three cells shown in A, percentage frequency of the five growth phases are plotted as a function of time. The dashed lines denote the time point of MβCD addition. The fastest change in CCS dynamics is observed in cell 1. (C) Figures show standard deviation (of local clathrin growth rates for the snapshots shown in A. Each pixel in the image is given the value of standard deviation of the growth rates detected from CCSs found in a radius of 4.8 µm. This representation is used for illustrating temporal and spatial variations in CCS dynamics. Standard deviation is lower in regions where the growth rate distribution is narrow, a signature of impeded CCS dynamics. Cell 1 responds to cholesterol depletion the earliest, as observed at t = 816 s. (D) Growth rate histograms are shown for the three cells at the time points of the snapshots in A and C. Spatial and temporal heterogeneity of CCS dynamics can be detected using the standard deviation of growth rate histograms, which change at different rates for the three cells. (E) Plots show spatial variation in standard deviation of local CCS growth rates over the distances shown by the dashed arrows in C. Positions of the cell boundaries are marked by the dashed red lines.

Reduction in CCS formation rates (positive slopes) upon cholesterol depletion could be attributed to depletion of free clathrin coat components in the cytosol caused by increased CCS lifetime, as they are contained within existing coats. In that case, we would expect dissolution rates to disappear the earliest. However, we found that both positive and negative slopes (i.e., fast formation and dissolution phases) diminish simultaneously (Fig. 2.5 B), which indicates that flat clathrin arrays have slower formation rates than clathrin-coated pits (Fig. S2 A). Therefore, in contrast to earlier interpretations (Subtil et al., 1999), our results indicate that flat clathrin arrays found in cholesterol-depleted cells cannot be considered as direct precursors of clathrin-coated pits, as they have distinct formation dynamics.

### 2.3.4 CCS intensity profiles can be reproduced from growth rate distributions

Hierarchical clustering of CCS traces allows us to determine growth rate distributions corresponding to different intensity profiles (Fig. 2.6 A). An interesting question that arises is whether we can reverse the flow of information in this process (i.e., reproduce the mean intensity profiles using growth rate distributions). Our IMS analyses show that growth rate distributions can be accurately assembled even in the absence of complete CCS intensity profiles (Fig. 2.6 B). Therefore, whatever the design, such a methodology would be immensely useful for interpreting the CCS growth rates that are obtained from acquisitions in which monitoring complete CCS traces is infeasible. We used a strategy based on determining the analogous growth rate distributions within a library of CCS clusters (Fig. 2.6 C). We assembled a library of growth rate histograms obtained from 361 clusters containing a total count of 6,958 CCS traces. When we used test clusters obtained from various cellular contexts, we were able to make accurate predictions of their intensity profiles solely by determining the most analogous growth rate distribution within the cluster library (Fig. 2.7 A and online supplemental software). When applied to cells coexpressing fluorescently tagged AP2 and clathrin, we found that the AP2 growth rate distribution has higher frequencies at the low-magnitude slope region, and its predicted intensity profile has a longer plateau before dissolution (Fig. 2.7 B). This result is in accord with the previous studies, which show that AP2 fluorescence growth halts earlier than the clathrin signal during the completion of the coat (Saffarian and Kirchhausen, 2008). When cells are treated with MβCD, incremental changes in mean CCS intensity profiles taking place within short intervals could be resolved in real time (Fig. 7 C). Upon cholesterol depletion, intensity profiles predicted using growth rate distributions became longer lived and reached higher maximum intensities in time. This was expected, as clathrin-coated pits are gradually replaced by larger and less dynamic CCSs in these cells (Fig. S2 A). Although the extent of the cluster library is a critical determinant of the fidelity of predicted intensity profiles, the presented strategy is a powerful tool for analyzing the changes in CCS growth rates obtained from in vivo datasets.

Figure 2.6 A novel analytical toolbox for CME dynamics.

(A) CCS traces with similar intensity profiles can be grouped using a hierarchical clustering algorithm. This is applicable to acquisitions longer than the mean CCS lifetime. Growth rate distributions obtained from different clusters are used to develop a cluster library. (B) As validated by IMS analysis, growth rate distributions can be assembled using short fragments of CCS traces. (C) For a given growth rate distribution, an accurate estimation of the corresponding intensity profile is possible by determining analogous growth rate distributions in the cluster library.

(A) Trace clusters from test cells (excluded from the library) are matched with clusters from the CCS library by their growth rate histograms. Successful reproductions of the cluster traces occurred in multiple cell types and conditions. Two clusters and library matches are shown per condition. Intensity profiles of test clusters and corresponding growth rate distributions are shown in blue, and library matches are in red. Shaded regions in intensity profiles mark the standard deviation of the cluster. (B) In a U373 cell coexpressing fluorescently tagged AP2 and clathrin, the growth rate of AP2 is more peaked at low slope magnitudes (left). Corresponding library matches show continuing increase in clathrin signal after AP2 signal plateaued (right). (C) MβCD is added to BSC1 cells at t = 0. Growth rate distributions determined at different time points of the treatment are shown on the left. (right) Predicted intensity profiles for the given growth rate distributions. Gradual changes in growth rates result in increasing trace lifetime and peak intensity.

Figure 2.7 Determining CCS intensity profiles using growth rate distributions.

### 2.3.5 CME dynamics slow down during dorsal closure of Drosophila embryos

Taking advantage of the new set of analytical tools at hand, we investigated the dynamics of CME in amnioserosa tissue of developing Drosophila embryos. Amnioserosa consists of a single layer of polarized cells that covers the dorsal surface of the embryo after germ band retraction. The tissue is entirely curtained by lateral epidermal cells in ∼4 h by a process called dorsal closure (Jacinto et al., 2002). CCSs originating at the apical and basal surfaces of the amnioserosa can be detected in embryos expressing clathrin light chain (CLC) fused with GFP, using spinning disk confocal fluorescence imaging (Fig. 2.8 A). CCSs forming on intracellular organelles appear as bright blobs at the perinuclear regions. To determine individual CCS traces at the aminoserosa, we used a robust 2D particle tracking software (Aguet et al., 2013) and processed the output data using a trace combination algorithm to correct for particle disappearances caused by movements in the axial dimension (Fig. 2.8 B and Video 3). We used local distributions of the number of CCS traces to determine the axial positions of apical and basal surfaces (Fig. 2.8 C). We found that lifetime distributions for both of the surfaces are dominated by short CCS traces in comparison with the distributions obtained for clathrin-coated pits originating in cultured cells (Fig. 2.8 D). We believe the anomaly in lifetime distributions obtained from the amnioserosa has no biological basis but is a consequence of single-particle tracking errors caused by high CCS motility in this tissue and increased ratio of incomplete trace fragments (Fig. S3). When we used CCS growth rates as the alternative approach, we noted rapid changes in the distributions obtained from individual amnioserosa cells. However, we found no correlation between the temporal evolution of the distributions at the apical and basal surfaces (Fig. 2.9, A and B). We extended the duration of the imaging assays by acquiring CCS growth rates from 30-s-long acquisitions that are separated by temporal gaps of 3 or 10 min (Fig. 2.9 C). In this experimental scheme, monitoring CCS dynamics of individual cells throughout the entire assay was not achievable because the cellular organization of amnioserosa is not preserved over long durations, as the tissue is gradually replaced by lateral epidermis. When the growth rates are calculated for the entire tissue, we found that the intensity traces predicted using CCS growth rates were significantly longer lived than typical clathrin-coated pits (Fig. 2.9 E), in striking contrast with the results of the lifetime distributions (Fig. 2.8 D). In all of the four embryos we have analyzed in this way, we recorded significant change in growth rate distributions at the apical surface, which correspond to slower CCS dynamics and elongated lifetimes over time (Fig. 2.9, D–F).

Figure 2.8 3D tracking of CCSs in apical and basal surfaces of Drosophila amnioserosa.

(A) Three sections of a confocal z-stack show apical, perinuclear, and basal regions of Drosophila amnioserosa cells expressing clathrin light chain fused with GFP (CLC-GFP). CCSs originating on organelles are observed mostly at the perinuclear regions as nondiffraction-limited bright fluorescent puncta (blobs). Bar, 10 µm. (B, left) Maximum projection image of a z-stack acquired at the amnioserosa tissue of a live Drosophila embryo. The image is divided into windows of 10 × 10 µm for determination of basal and apical surfaces (expanded in C). (right) Snapshot of CCS traces that are detected within the left panel, color-coded according to their axial positions. The projection of the entire 3D time-lapse acquisition and the corresponding CCS traces are shown in Video 3. (C) In each frame of the 3D time-lapse acquisition, axial positions of the apical and basal surfaces are determined separately for the 10 × 10 µm windows. Cartoon represents an amnioserosa cell oriented in a way that its apical surface faces the detection optics. The adjacent histogram shows the distribution of traces detected within the orange square shown in B with respect to their axial positions. The distribution is bimodal because of increased CCS density at the apical and basal surfaces and can be fit with a sum of two Gaussians. CCSs falling within one standard deviation of the respective means (± σ) are considered apical or basal. (D) A comparison of CCS lifetime distributions obtained from apical and basal amnioserosa with clathrin-coated pits (CCPs) detected in cultured BSC-1 cells stably expressing AP2-GFP (Amnioserosa: Nembryos = 2, Ncells = 75, and Ntraces = 124,013; CCPs: Ncells = 3 and Ntraces = 12,002).

Continued

(A) Amnioserosa tissue of a late Drosophila embryo is imaged for 4 min using confocal z-stacks acquired every 3 s. Representative frame is an image section at the middle of a stack. Red lines represent the boundaries between amnioserosa cell centers, which are marked by numbers. Cell boundaries determined in each frame of a 3D time-lapse acquisition. Bar 10 µm. (B) Histograms show evolution of the growth rates corresponding to different cells selected from the amnioserosa tissue in A. Frequencies of the five phases are plotted as a function of time for the apical and basal surfaces. (C) Thumbnails represent 30-s-long 3D time-lapse acquisitions separated by intermissions.

Figure 2.9 CME dynamics in Drosophila amnioserosa.

Figure 2.9: Continued

(D) Transformation of CCS growth rates at the basal and apical surfaces of the amnioserosa during dorsal closure of a *Drosophila* embryo. Each bar in the histograms represents the frequency of growth rates obtained from CCS traces detected in individual 30-s-long acquisitions. A significant change in the growth rates is observed at the apical surface. (E) CCS intensity profiles predicted using the growth rates in (D). The change in the apical CCS dynamics is observed as gradually elongated lifetime (right). No major change is observed in the basal surface dynamics (left). (F) Histograms show the CCS growth rates at the apical amnioserosa of three embryos. Increasing frequency of the plateau phase is a hallmark of reduced CCS dynamics. FD, fast dissolution; FF, fast formation; P, plateau; SD, slow dissolution; SF, slow formation.

## 2.4 Discussion

We show that clathrin coat growth rates can be used as quantitative reporters of CME dynamics in cellular contexts where errors associated with single-particle tracking are significant. Growth rate distributions obtained from short fragments of CCS traces offer the ability to assess clathrin dynamics from acquisitions shorter than the mean CCS lifetime. Gradual changes in endocytic dynamics can be detected in real time. Mean intensity profiles of the CCS traces that are used to generate growth rate distributions can be reconstructed by finding analogous growth rates in a library of trace clusters. 0These advantages make growth rate analysis a strong alternative to existing methodologies, which rely on quantifying lifetimes of individual CCSs.

Growth rate analyses reveal that physical factors increasing the energy cost of membrane deformation (e.g., in-plane tension, membrane–cytoskeleton adhesion, and membrane–substrate interactions) slow down formation and dissolution of CCSs. It has been shown that the same energy cost also reduces the curvature of CCSs (Saleem et al., 2015). In-plane tension is assumed to be homogenous in a cell, but membrane–cytoskeleton adhesion and membrane–substrate interactions are not uniform and, therefore, may induce spatial heterogeneities in dynamics and geometries of CCSs. This heterogeneity may account for the coexistence of clathrin-coated pits and plaques at the ventral surface of certain cell types (Saffarian et al., 2009). Growth rate analyses suggest that flat clathrin arrays and clathrin-coated pits have distinct formation dynamics. Even though flat lattices may acquire curvature over time (Avinoam et al., 2015), our data suggest that this mechanism cannot account for the formation of clathrin-coated pits. Transition of flat clathrin lattices to curved pits requires fourfold reduction in the projected area of clathrin coats on the x–y plane (Fig. S4). Such abrupt shape transformations are not observed in recent superresolved fluorescence acquisitions displaying the formation of clathrin-coated pits (Li et al., 2015), which suggests that curvature of the clathrin coat is constant during pit formation.

Strategies described here lay the groundwork for other quantitative assays that aim to elucidate endocytic processes in various multicellular contexts. Experimental and analytical deficiencies that distort CCS lifetime distributions are less detrimental to growth rates, as their quantification does not rely on tracing the complete lifetime of CCSs. Our study shows that CME dynamics can be assessed within tissues of developing organisms through quantification of the fluorescence growth rates of individual endocytic assemblies. Lifetime distributions obtained from Drosophilaamnioserosa are overpopulated by traces that last <20 s, which would be regarded as abortive structures in in vitro assays. The intensity traces reproduced using growth rate distributions extended to durations longer than the mean clathrin-coated pit lifetime. Longer CCS lifetimes may be a result of the intrinsic tension of the amnioserosa tissue (Kiehart et al., 2000). Likewise, gradual increase in CCS lifetimes during dorsal closure may be induced by the changes in tension levels. This assumption is in accord with a recent study showing that reduction in volume of amnioserosa cells leads to increasing tension during dorsal closure (Saias et al., 2015). Drosophila amnioserosa cells undergo periodic but asynchronous shape changes at shorter time scales (Solon et al., 2009). Further research is needed to elucidate if rapid fluctuations of CCS growth rates observed in individual cells are correlated with temporal changes in amnioserosa mechanics. Such studies may also elucidate whether dynamics of CCSs can be used as reporters for mechanical states of cells and tissues.

## 2.5 Materials and Methods

### 2.5.1 Fluorescence imaging

The imaging system was an Eclipse TI-E microscope (Nikon) equipped with a temperature controlled chamber, a CSU-W1 spinning disk confocal unit (Yokogawa Electric Corporation), a 100× objective lens (Nikon CFI Plan-Apochromat Lambda, NA 1.45) and an EMCCD camera (iXon DU897 Ultra; Andor Technology). 2D and 3D time series were obtained using NIS Elements image acquisition software.

S. Boulant (University of Heidelberg, Heidelberg, Germany) provided BSC-1, MDCK, and U373 cells stably expressing σ2-EGFP. Imaging of cultured cells was performed 8–24 h after plating on glass bottom dishes at 37°C ambient temperature (Greiner Bio-One), unless stated otherwise. Cells were imaged at a rate of 0.25–0.5 Hz and laser exposure of 50–300 ms per frame. Imaging medium was phenol red–free L15 (Thermo Fisher Scientific) supplemented with 10% FBS. Serum-free L15 was used for cholesterol depletion experiments. Final concentration of MβCD (Sigma-Aldrich) was 10 mM in Fig. 2.5, Fig. 2.7 C, and Video 2 and 4 mM in Fig. S2 B. Latrunculin B (SigmaAldrich) was used at the final concentration of 2 µM.

Drosophila embryos were harvested from a cross between male flies homozygous for GAL4-Arm and female flies homozygous for CLC-GFP (Bloomington Drosophila Stock Center). Eggs laid by this cross were collected and allowed to develop for ∼11 h at 25°C (stage 14 of development). The embryos were then mounted on a slide with glue (ventral side up), immersed in halocarbon oil (to prevent drying out), and covered with a coverslip. Amnioserosa tissues were imaged at 22°C using 3D time-lapse acquisitions containing ∼20 planes (400 nm apart) exposed for 50–100 ms. Temporal gap between adjacent z-stacks was 3 s.

### 2.5.2 Micropipette Aspiration

Glass micropipettes (BF100-58-10) were pulled using a micropipette puller (P-97; Sutter Instrument). A custom stage was built to mount a Sutter Instrument BRM/E micromanipulator to the live-cell imaging platform. Suction pressure was controlled with a Sutter Instrument BRE110/E microinjection system. Cell aspiration was performed with a 5- to 10-µm microneedle at the dorsal surface of cells, and the clathrin activity at the ventral surface was recorded with spinning-disk fluorescence imaging. P. Selvin (University of Illinois at Urbana-Champaign, Urbana, IL) provided the micromanipulator and injection system.

### 2.5.3 Single-particle tracking

2D particle tracking was performed using the cmeAnalysis software (obtained from http://lccb.hms.harvard.edu/software.html) unless stated otherwise. TraCKer software is a less sophisticated but faster tracking algorithm that lacks the advanced forward-backwardforward rechecking and intelligent thresholding of cmeAnalysis. It also lacks the 2D Gaussian fitting of point-spread functions and uses integrated pixel intensity as the CCS signal. TraCKer uses a simple threshold determined over a Mexican hat filtered image for detection of fluorescent spots. CCS positions are located using the intensity- weighted center of the point-spread function. In this work, TraCKer was used for the sole purpose of testing growth rate analysis on IMSs.

cmeAnalysis software occasionally detects objects that last a single frame or persist consistently in the background without following an intensity path that could be considered as a CCS. We implemented a sorting scheme for these traces, requiring that they be at least three frames long and at some point meet statistical criteria for demonstrating a linear increase or decrease in intensity (corresponding to CCS growth and dissolution, respectively). We went over every three or four consecutive intensity points (three for traces that last for 0.75 were rejected. Rejected traces were not used in calculation of growth rate distributions. Traces with at least two separate high r2 values of both positive slope (growth) and negative slope (dissolution) were valued most highly. Fig. S5 shows the classification of CCS traces obtained from amnioserosa tissues.

### 2.5.4 3D tracking of CCSs within amnioserosa tissues

Each z-plane of a 3D time-lapse movie was analyzed using cmeAnalysis software. Detected 2D traces were then run through our trace rejection scheme. The resulting data were analyzed to combine traces that occur at the same lateral position in two adjacent z-planes. Coincident traces had to be within one pixel (160 nm) x–y distance for at least three consecutive frames. For each time point in the movie, the resulting trace contains the maximum intensity value of all traces considered for combination. Axial positions were assigned by calculating the intensity-weighted mean z-position of all traces considered. The algorithm for trace combination ran from the outermost z-planes to the innermost (alternating between the top and the bottom) to ensure that there was no directional bias and all possible trace combinations were considered. The resulting data structure will have the duplicate traces deleted and contain subplane z-position data where possible.

### 2.5.5 Classification of apical and basal CCSs and blobs

Because of the curvature of the dorsal surface, cells in the amnioserosa tissue are not coplanar. Therefore, the field of view was divided into 64 equal square regions, and apical and basal surfaces were determined for each region independently (Fig. 2.8 B). Axial positions of the traces detected inside each square region were put together within in a two-frame temporal radius. These values were binned into discrete z-plane positions, and the resulting count-value graph was fit with two Gaussian functions. The fit for the apical surface has a low standard deviation. The basal surface was found at a higher axial position and the corresponding Gaussian fit had a larger standard deviation in general. Traces found at the mean or within one standard deviation of their respective Gaussian fits were classified as apical or basal for each of the 64 squares. The brightest 2% of all traces were classified as blobs and were excluded from the apical and basal populations.

### 2.5.6 Determining amnioserosa cell boundaries

To determine the positions of amnioserosa nuclei, the middle imaging plane of each z-stack was filtered with a Gaussian filter. Each frame was then inverted and local maxima were determined using the FastPeakFind function (A. Natan, PULSE Institute, Stanford, CA). After multiple nuclei positions were determined for each frame, they were tracked using the TraCKer algorithm. Once the centers were known and connected for each frame, the boundaries were determined by creating a Voronoi diagram using the cell centers in each frame.

### 2.5.7 Growth rate distributions

Slopes were extracted from CCS traces that pass the trace rejection scheme. We found that CCS lifetime distributions of obtained from accepted (nonrejected) traces were very similar to those reproduced by others who used different parameters to distinguish genuine endocytic transporters (Fig. S5; Aguet et al., 2013). Each trace was normalized by subtracting a global background and dividing by the new maximum of the trace. From this normalized trace, every 12-s interval was used in a leastsquares fit to determine the slope of the trace at all frames (Fig. 2.1 C). A trace had to be at least 12 s long to be considered for slope extraction. 12 s was chosen because it evenly divides 1-, 2-, 3-, and 4-s frame rates, which are the most common frame rates used. An arbitrary bin width (0.03) was assigned for the histograms displaying the five distinct growth phases.

# Mechanoregulation of clathrin-mediated endocytosis (Later)

Derived from: Ferguson, J.P.\*, Huber, S.D.\*, Willy, N.M., Aygün, E., Goker, S., Atabey, T., and Kural, C. (2017). Mechanoregulation of clathrin-mediated endocytosis. J. Cell Sci. *130*, 3631–3636.

*In this chapter my contributions are some osmotic shock experiments and analysis; development of two-color master-slave analysis; two-color clathrin/actin experiments and analysis; and average z-position analysis.*

## 3.1 Abstract

We characterized the tension response of clathrin-mediated endocytosis by using various cell manipulation methodologies. Elevated tension in a cell hinders clathrin-mediated endocytosis through inhibition of de novo coat initiation, elongation of clathrin coat lifetimes and reduction of high-magnitude growth rates. Actin machinery supplies an inward pulling force necessary for internalization of clathrin coats under high tension. These findings suggest that the physical cues cells receive from their microenvironment are major determinants of clathrin-mediated endocytic activity.

## 3.2 Introduction

Clathrin-coated structures bear a major fraction of the endocytic load from the plasma membrane of eukaryotic cells. During formation of an endocytic vesicle, clathrin heterohexamers assemble into a multifaceted cage that is linked to the plasma membrane by clathrin adaptors. Tension on the membrane hinders this process as it increases the energy cost of curvature formation (Sheetz, 2001). Curvature-bearing clathrin-coated pits are replaced by less-dynamic shallow coats when tension is elevated (Saleem et al., 2015). In various cellular contexts, actin dynamics supplements the energy required for formation of clathrin-coated vesicles under high membrane tension (Aghamohammadzadeh and Ayscough, 2009; Boulant et al., 2011; Kaur et al., 2014). However, actin-dependent clathrin-mediated endocytic events have a longer duration than their counterparts taking place at lower tension levels (Boulant et al., 2011). Here, we characterized the regulation of clathrin coat dynamics by membrane tension by using cell manipulation techniques (i.e. microaspiration, cell squeezing and hypo-osmotic swelling) coupled with fluorescence live-cell imaging. Our results show that the density of endocytic clathrin-coated structures on the plasma membrane depends on tension, and actin machinery rescues internalization of clathrin coats under high tension by moving clathrin coats away from the membrane.

## 3.3 Results

We used three independent approaches to increase the tension on the plasma membrane while monitoring clathrin coat dynamics at the ventral (adherent) surface of cells. Applying negative pressure on the plasma membrane by micropipette aspiration is an effective way to increase tension (Herant et al., 2005; Houk et al., 2012). We detected a significant increase in average clathrin coat lifetime (time elapsed between the origination and conclusion of the coat; 44±23 s versus 88±73 s, mean±s.d., P< 0.001; ncells=9, ntraces=40,943; Fig. 3.1A,B) in BSC1 cells upon microaspiration of the membrane. The impeded clathrin coat dynamics is also observed as gradual disappearance of high-magnitude growth rates in clathrin traces (Movie 1) (Ferguson et al., 2016). As a result, the standard deviation of clathrin growth rate distributions reduced [0.038±0.003 (before aspiration) versus 0.027±0.004 (during aspiration), P< 0.001; Fig. 3.1C]. We also found that increased tension reduced the surface density of clathrin coat initiation and conclusion events (Fig. 3.1D).

Figure 3.1 Aspiration of the plasma membrane slows down clathrin coat dynamics.

(A) Kymograph showing the clathrin activity at the ventral surface of a BSC1 cell expressing AP2–eGFP. Clathrin coat traces elongate gradually upon microaspiration (dashed line; Movie 1). Blue and red arrowheads mark the initiation and conclusion of a clathrin-coated structure, respectively. Δt is its lifetime. (B) Clathrin coat lifetime distributions are shown for nine BSC1 cells imaged before and during microaspiration (ntraces=40,943). (C) For the same nine cells, the standard deviation of the clathrin growth rate distributions are shown in boxplots. Lines connect the standard deviation values obtained from the same cell before and during aspiration. The narrower growth rate distributions indicate slower clathrin coat dynamics. (D) Box plots are the initiation and conclusion densities of clathrin-coated structures before and during aspiration. In the boxplots, the box represents the 25–75th percentiles, and the median is indicated. The whiskers show the 10–90th percentiles. P-values were obtained with a two-tailed t-test.

To induce faster changes in plasma membrane tension, we increased the hydrostatic pressure in cells by squeezing them with a micromanipulator-controlled polymer cushion (Fig. 3.2A,B). We used growth rate distributions obtained from clathrin coat intensity profiles to temporally resolve the fast alterations in endocytic dynamics (Ferguson et al., 2016). In good agreement with the microaspiration experiments, fast dissolution and fast formation phases in the growth rate distributions diminished with the increasing tension, whereas the frequency of the plateau phase increased (Fig. 3.2C; Fig. S1, Movie 2). Discrete changes in the tension could be resolved as stepwise reduction in the standard deviation of clathrin growth rates (Fig. 3.2D). Furthermore, the average clathrin coat lifetimes increased while initiation and conclusion densities reduced (Fig. 3.2B,E). When we relieved the squeezing to verify the viability of cells, we found that the parameters determining clathrin coat dynamics reverted to normal (Fig. 3.2F; Fig. S2, Movie 3).

Hypotonic swelling is a straightforward and widely used approach to increase membrane tension (Boulant et al., 2011; Cocucci et al., 2014; Diz-Muñoz et al., 2016). Upon reducing the osmolarity of the imaging medium, we found that the hypotonic swelling takes effect within minutes but the volume of cells converges back to the original values within an hour (Fig. 3.3A). As expected, the temporary increase of the tension due to stretching of the membrane affected endocytic clathrin coat dynamics only temporarily (Fig. 3.3B). We found that the average lifetime of clathrin-coated structures increased significantly in SUM159 cells (Aguet et al., 2016) under hypoosmotic shock (87±86 s versus 161.2±208.1 s, mean±s.d., P< 0.001; ncells=12, ntraces=34,113; Fig. 3.3C). In accordance with the microaspiration and cell squeezing assays, increased tension resulted in reduction of the standard deviation of clathrin growth rates [0.018 ±0.003 (pre-osmoshock) versus 0.014±0.002 (post-osmoshock), P< 0.001; Fig. 3.3D], and initiation and conclusion densities (Fig. 3.3E).

Figure 3.2 Cell squeezing induces fast and reversible alterations in clathrin coat dynamics.

(A) Cartoon and representative frames of a BSC1 cell are shown at different stages of squeezing. (B) Kymograph showing the temporal evolution of the clathrin traces detected at the ventral surface of the cell shown in A. Dashed lines mark the squeezing steps. (C) For the cell in A, normalized distributions of clathrin growth rates are plotted for different squeezing levels. The standard deviation of the distribution reduces as the tension increases. (D) For the same cell, the time variation of the ventral surface area (upper) and the standard deviation of the clathrin growth rates (lower). The stepwise changes in these parameters are due to discrete levels of squeezing. (E) The response of the same cell to squeezing is shown as the mean clathrin lifetime (upper) and initiation and conclusion densities (lower). Dashed lines indicate change in squeezing (ntraces=8217). (F) Standard deviation of clathrin growth rates (upper), mean lifetime (middle), and initiation and conclusion densities (lower) from a cell that undergoes increased stepwise squeezing (orange dashed lines) and relaxation (blue dashed lines) (ntraces=8255).

(A) Change in the volume (normalized to the initial value) is plotted for three BSC1 cells during hypotonic swelling (i.e. osmoshock). (B) Mean clathrin coat lifetime (upper), and initiation and conclusion densities (lower) are plotted against time for a BSC1 cell treated with hypotonic shock (dashed line). (C) Clathrin lifetime distributions are assembled pre- and post-osmoshock for 12 gene-edited SUM159 cells expressing AP2–eGFP (ntraces=34,113). (D, E) For the same cells, the standard deviation of clathrin growth rates (D) and initiation and conclusion densities of clathrin-coated structures pre- and post-osmotic shock (E) are shown in boxplots. Lines connect the standard deviation values obtained from the same cell pre- and post-osmoshock. In the boxplots, the box represents the 25–75th percentiles, and the median is indicated. The whiskers show the 10–90th percentiles. P-values were obtained with a two-tailed t-test.

Figure 3.3 Hypotonic swelling inhibits clathrin coat dynamics temporarily.

As plasma membrane tension increases, actin polymerization energy becomes indispensable for narrowing of the neck between clathrin-coated pits and the plasma membrane. Therefore, inhibition of the actin machinery arrests clathrin coats prior to the scission phase (Boulant et al., 2011). There are two proposed models for actin-dependent formation of clathrin-coated vesicles under high tension (Hassinger et al., 2017). The first model predicts a vertical force generated by actin polymerization to move the clathrin coat away from the plasma membrane. The second model suggests formation of an actin collar that constricts the neck region directly (Collins et al., 2011). By tracing the three-dimensional (3D) displacement of clathrin-coated structures, we found that coats move ∼100 nm into the cell before uncoating, and the inward displacement is significantly higher when the membrane tension is increased by hypotonic swelling (Fig. 3.4A,B). We also found that the axial velocity of the inward movement is the highest during the fast dissolution phase of clathrin coats (Fig. 3.4C). We adapted a master–slave approach (Aguet et al., 2013) to monitor the intensity profiles of clathrin coats (AP2S1–eGFP as the master; hereafter denoted AP2–eGFP) and colocalizing actin filaments (LifeAct– mCherry as the slave), simultaneously. As expected, the LifeAct signal peaked during the later stages of clathrin-coated vesicle formation (Fig. 3.4D), and the axial velocity detected during the fast dissolution phase reduced significantly when the actin dynamics is inhibited upon jasplakinolide treatment (Fig. 3.4E). These results indicate that actin polymerization provides the inward force that is required for constriction of the neck under high membrane tension, a mechanism analogous to clathrin-mediated endocytosis in yeast (Aghamohammadzadeh and Ayscough, 2009).

(A) Mean±s.e.m. values for of normalized AP2 intensity traces (determined for a SUM159 cell before and after hypotonic swelling; ntraces=3728). The traces are aligned at the end point before averaging. (B) Mean±s.e.m. z displacements are shown for the two trace groups in A. (C) Top, growth rate distributions are assembled for eight SUM159 cells before and after hypotonic swelling (ntraces=30,409). Different growth phases (ff, fast formation; sf, slow formation; p, plateau; sd, slow dissolution; fd, fast dissolution) were determined by quantifying the change in the clathrin coat signal over 12-s-long time windows (Ferguson et al., 2016). Bottom, for the same cells, bar plots show the mean+s.e.m. of the z velocities of the trace fragments (12 s long) that are used to generate the growth rate distributions above. Trace fragments that have the highest z velocity are found in the fast dissolution (fd) phase. (D) Top, representative intensity traces of AP2 (green) and LifeAct (red) fluorescence during the formation of a clathrin-coated vesicle at the ventral surface of a BSC1 cell. Bottom, the relative LifeAct intensity (mean±s.e.m.) colocalizing with clathrin coats is shown for different growth phases (ncells=4, ntraces=28,795). Note that the growth phases are determined by using the master (AP2–eGFP) signal. (E) Bar plots show the z velocities (mean±s.e.m.) corresponding to different growth phases for AP2 traces obtained from BSC1 cells in the absence and presence of jasplakinolide (Jasp) (Control, ncells=7, ntraces=20,204; Jasp, ncells=6, ntraces=35,972). \*P<0.0001; \*\*P<0.001 (two-tailed t-test).

Figure 3.4 Actin dynamics mediate the inward movement of clathrin coats prior to disassembly.

## 3.4 Discussion

In this study, we used quantitative live-cell imaging in combination with diverse cell manipulation techniques to detect the changes in clathrin coat dynamics as cells undergo mechanical perturbations. This powerful approach allowed us to investigate the response of individual cells to mechanical stimuli in real time, rather than making a comparative analysis between different cells. Collectively, our assays reveal an inverse relationship between plasma membrane tension and endocytic clathrin coat dynamics. Increased tension manifests itself as reduced initiation and conclusion densities, elongated lifetime, and a reduced standard deviation of clathrin coat growth rates. These results suggest that the reduced density of clathrin-coated structures observed during mitosis (Aguet et al., 2016) and at the lamellae of migrating cells (Kural et al., 2015) can be a product of increased membrane tension (Fogelson and Mogilner, 2014; Kaur et al., 2014; Lieber et al., 2015; Raucher and Sheetz, 1999). Correspondingly, previously described feedback regulation between membrane tension and membranebending proteins in migrating cells (Tsujita et al., 2015) can explain the stark increase in clathrin coat density upon mechanical inhibition of cell protrusion (Movie 4).

Our results show that tension is an effective, fast-acting and reversible regulator of clathrin-mediated endocytosis. To induce hypotonic swelling, we reduced the osmolarity of the imaging medium to 63 mOsm. In a recent study, comparable changes in osmolarity are shown to increase the membrane tension ∼2-fold (Diz-Muñoz et al., 2016). This is within the boundaries of physiologically relevant variances in plasma membrane tension given that spreading of a cell results in an ∼3-fold reduction in membrane tension (Gauthier et al., 2009), and the tension at the apical surface of polarized cells is ∼2.5-fold higher than that at the basal surface (Dai and Sheetz, 1999). Compression of cells by surrounding mechanical cues has been proposed to control tissue morphogenesis at different stages of metazoan development (Desprat et al., 2008; Legoff et al., 2013; Rauskolb et al., 2014). In our cell-squeezing assays, we observed changes in clathrinmediated endocytic activity even when the relative fold change in the cell area is lower than the levels detected in developmental processes associated with cell compression (Aegerter-Wilmsen et al., 2012) (Fig. S3). Dynamics and organization of the actin cytoskeleton were unperturbed in these assays (Movie 5). These findings suggest that morphological alterations involving mechanical forces within physiological contexts can induce abrupt changes in clathrin coat dynamics. Consequently, mechanoregulation of clathrin-mediated endocytosis can influence related biological processes that are central for development and homeostasis of multicellular organisms, such as signal transduction and cell shape regulation.

## 3.5 Materials and Methods

### 3.5.1 Cell culture, reagents and fluorescence imaging

BSC1 cells stably expressing AP2–eGFP (gift of Steeve Boulant, Department of Infectious Diseases, Virology, Heidelberg University, Germany) were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, penicillin/streptomycin. SUM159 cell gene edited to express AP2–eGFP (Aguet et al., 2016) (gift of Tomas Kirchhausen, Departments of Cell Biology and Pediatrics, Harvard Medical School Boston, MA) were grown in F-12 medium containing 5% fetal bovine serum (FBS), penicillin-streptomycin and hydrocortisone. Transient expression of LifeAct–mCherry (gift of Patrick M. Reeves, Vaccine & Immunotherapy Center, Charlestown, MA) in BSC1 cells stably expressing AP2–eGFP was carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions, and imaging was performed 24–48 h after transfection. The final jasplakinolide (Enzo Life Sciences) concentration used to inhibit actin dynamics was 1 µm.

The fluorescence imaging system is composed of an Eclipse TI-E microscope (Nikon) equipped with a perfect focusing system (PFS), a temperature-controlled chamber, a CSU-W1 spinning disk confocal unit (Yokogawa Electric Corporation), a 100× objective lens (Nikon CFI PlanApochromat Lambda, NA 1.45) and an EMCCD camera (iXon DU897 Ultra; Andor Technology). All image acquisition was performed by using NIS Elements software.

Imaging of cultured cells was performed 30 min after plating on glass bottom dishes (Greiner Bio-One) in the case of squeezing cells. The plating time prior to aspiration and osmotic shock experiments was 24 h. All cells were maintained at an ambient temperature of 37°C during imaging. Images were acquired at rate of 0.25–0.5 Hz with a laser exposure of 50–300 ms per frame. Imaging medium was Phenol Red-free L15 (Thermo Fisher Scientific) supplemented with 10% FBS. The snapshots and movies of fluorescence acquisitions are inverted to increase visibility.

### 3.5.2 Squeezing, micropipette aspiration and osmotic shock

In squeezing experiments, a 15 μl suspension of cells and imaging medium is plated in the middle of the imaging dish, forming a small droplet. A polydimethylsiloxane (PDMS) brick of ∼10 mm×10 mm×2.5 mm is placed on top of the droplet. The dish is capped and the cells are allowed to spread for ∼30 min (at this point cells are not in contact with the PDMS). After spreading, a micromanipulator (Narishige MMO-202ND, Narishige MMN-1) fitted with a rounded glass pipette tip is slowly brought into contact with the PDMS brick from above. The micromanipulator is used to press down on the PDMS while observing the cells under brightfield illumination. Fluorescence acquisitions start after the PDMS brick is brought into contact with the cells. The maximum level of compression is signaled by complete halting of clathrin coat activity. Further imaging is performed at various stages while the compression is released. In the microaspiration experiments, a microinjection system (BRE110/E; Sutter Instrument) was used to control the negative pressure applied on the dorsal surface of cells via a 5–10-μm-thick microneedle.

For osmotic shock experiments, SUM159 cells are cultured on four-well glass bottom plates (Fisher Scientific) and imaged every 3 s. At 5 min after the start of the experiment, 800 µl of ddH2O is added to the 200 µl of imaging medium to induce hypo-osmotic shock. The measured osmolarity level after this dilution is 63 mOsm. The cells are then imaged for another 20 min to study the cellular response. To compare clathrin coat dynamics before and after osmotic shock, two time windows are analyzed: the preosmoshock time window consists of the 5 min immediately prior to addition of water, whereas the post-osmoshock time window starts 2.5 min after water addition (to allow time for osmotic shock effects fully take hold) and runs for 5 min. For lifetime analyses, only traces whose mean time-point lies within the window are considered.

For the calculation of the cell volume, we used the 3D time-lapse spinning-disk confocal microscopy acquisitions. A custom MATLAB program was written to allow the user to select the boundary of the cell for each plane in a z-stack (available from the corresponding author upon request). The number of pixels inside of these boundaries was multiplied by the size of the pixels to determine the area of the cell in that stack. The volume was calculated by multiplying this area by the difference in position between the stacks and adding all those values together.

### 3.5.3 Single-particle tracking

cmeAnalysis software was used for two dimensional (2D) particle tracking (obtained from http://lccb.hms.harvard.edu/software.html) (Aguet et al., 2013). We used exclusionary criteria for the traces that last a single frame or persist consistently in the background without following a characteristic clathrin intensity profile (Ferguson et al., 2016). Selected traces are at least three frames long and contain a sequence which meets statistical criteria for demonstrating a linear increase or decrease in intensity (corresponding to clathrin coat growth and dissolution, respectively). For each group of three or four consecutive intensity points (three for traces 0.5 were rejected. Rejected traces were excluded from the calculation of initiation and dissolution densities, growth rate distributions, lifetime distributions and lifetime dipoles.

We used the traces that passed the rejection scheme to determine the average clathrin coat lifetime per frame. In each frame, we added together the lifetime of each trace that exists in that frame, and divided by the number of traces considered. The beginning and end of each trace is considered as an initiation and conclusion event, respectively. For each frame, initiation and conclusion densities (number/µm2 /minute) were determined by finding the number of traces that begin and end in that frame, multiplying by the frame length (2–4 s), dividing by the visible cell area (in µm2 ) and by 60 s.

A custom MATLAB program was used for the master–slave analysis (available from the corresponding author upon request). The traces in the master channel were determined by using the cmeAnalysis software as described above. To quantify the intensity in the slave channel, we determined the average intensity in a 5×5 pixel region around the structure, and then subtracted the background intensity, which was calculated as the average intensity of the outside pixels of the 7×7 pixel region around the structure.

3D traces and growth rate distributions of clathrin coats were determined as described previously (Ferguson et al., 2016). z-velocities are calculated for each 12-s long trace fragments, which were used to determine the corresponding clathrin growth rates.

# Membrane mechanics govern spatiotemporal heterogeneity of endocytic clathrin coat dynamics (Later)

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*In this chapter, my contributions are deoxycholate experiments and some cholesterol depletion and osmotic shock experiments and analysis; some spreading analysis; and all optical tweezer experiments and analysis*

## 4.1 Abstract

Dynamics of endocytic clathrin-coated structures can be remarkably divergent across different cell types, cells within the same culture, or even distinct surfaces of the same cell. The origin of this astounding heterogeneity remains to be elucidated. Here we show that cellular processes associated with changes in effective plasma membrane tension induce significant spatiotemporal alterations in endocytic clathrin coat dynamics. Spatiotemporal heterogeneity of clathrin coat dynamics is also observed during morphological changes taking place within developing multicellular organisms. These findings suggest that tension gradients can lead to patterning and differentiation of tissues through mechanoregulation of clathrin-mediated endocytosis.

## 4.2 Introduction

Clathrin-mediated endocytosis (CME) is the most prominent internalization mechanism of membrane lipids and proteins from the cell surface. The major building blocks of endocytic clathrin coats, that is, clathrin triskelions, can assemble into (complete or partial) polyhedral cages in a seemingly infinite number of geometries (Heuser et al., 1987; Heuser, 1989). The dynamic properties of clathrincoated structures can be strikingly diverse as well. Lifetime, the time it takes for formation and internalization of endocytic clathrin coats, can be an order of magnitude disparate within the same cell (Ferguson et al., 2016). Here we show that cellular processes associated with membrane tension gradients, that is, spreading and migration, result in increased spatiotemporal heterogeneity of endocytic clathrin coat dynamics. The variations in clathrin coat dynamics coincide with the gradients in plasma membrane tension, which is a potent regulator of endocytic processes (Dai and Sheetz, 1995). We also show that spatiotemporal changes in clathrin coat dynamics take place during developmental processes shaping Drosophila melanogaster embryos.

## 4.3 Results

Physical factors that increase the energy cost of curvature generation on the plasma membrane slow down formation of clathrincoated vesicles. Using quantitative imaging of fluorescently tagged clathrin coat components (clathrin or AP2) within live cells, this phenomenon can be observed as elongated coat lifetime (Figure 1A) (Boulant et al., 2011). Alternatively, mechanoregulation of CME dynamics can be monitored at distinct surfaces of a cell through growth rate distributions which are assembled by quantifying the changes in the fluorescence signal of individual clathrin coats within short time windows (Ferguson et al., 2016). High magnitude growth rates, that is, rapid changes in the clathrin coat intensity corresponding to fast formation and fast dissolution of the coat, diminish with increasing plasma membrane tension. Therefore, the standard deviation (SD) of the growth rate distributions reduces when the effective membrane tension is increased by cholesterol depletion or hypotonic swelling (Figure 1, B–E) (Dai et al., 1998; Khatibzadeh et al., 2012; Diz-Muñoz et al., 2016; Sun et al., 2007). Conversely, SD of growth rate distributions increases when tension is reduced on deoxycholate treatment (Figure 1, D and E) (Raucher and Sheetz, 1999; Batchelder et al., 2011).

Continued

Figure 4.1 Monitoring mechanoregulation of clathrin coat dynamics in real time.

(A) Depletion of plasma membrane cholesterol by methyl-ß-cyclodextrin (MßCD) increases the adhesion energy between membrane and the cytoskeleton and, thereby, inhibits curvature formation by clathrin-coats (Subtil et al., 1999; Sun et al., 2007; Khatibzadeh et al., 2012). A 35-min-long kymograph shows clathrin coat dynamics at the ventral surface of a BSC1 cell stably expressing the σ2 subunit of AP2 fused with enhanced green fluorescent protein (EGFP). The dashed line marks the addition of MßCD. The traces corresponding to individual clathrin-coated structures elongate as clathrin pits are gradually replaced by less dynamic flat arrays (plaques).

Figure 4.1: Continued

(B) For the cell in A, growth rate distributions are assembled using the traces detected before cholesterol depletion (Control; the first 5 min of the acquisition) and after (MßCD; the last 5 min of the acquisition). As clathrin coat dynamics slow down, growth rate distribution gets narrower (top). The distribution is also displayed using five bins corresponding to five growth phases (bottom; fd: fast dissolution, sd: slow dissolution, p: plateau, sf: slow formation, ff: fast formation). High-magnitude slopes, corresponding to fd and ff phases, diminish due to inhibition of CME. (C) SD of clathrin growth rate distributions are shown for the ventral and dorsal surfaces of four BSC1 cells before and after MßCD treatment. (D) Growth rate distributions are assembled using AP2 traces detected in SUM159 cells under different conditions (top; Ncells = 4). With application of hypotonic shock (Dai et al., 1998; Diz-Muñoz et al., 2016), CME slows down due to increasing in-plane tension. Deoxycholate treatment reduces membrane tension (Raucher and Sheetz, 1999; Batchelder et al., 2011) and results in increased clathrin coat dynamics. The distributions are also displayed using five bins corresponding to distinct growth phases (bottom). (E) Temporal change in the SD of clathrin coat growth rates is plotted for two SUM159 cells. The dashed line marks the application of hypotonic shock and deoxycholate.

### 4.3.1 Clathrin coat dynamics in spreading and migrating cells

Membrane tension reduces gradually during cell spreading (Gauthier et al., 2009). Rounded-up cells have approximately threefold and Supplemental Movie 1). Changes in the SD of growth rates in both ventral and dorsal surfaces demonstrate that clathrin coat dynamics increase across the entire cell (Figure 2, D and G). We also found that initiation and dissolution densities of clathrin-coated structures increase significantly with the completion of spreading (Figure 2, E and F).

Continued

Figure 4.2 Clathrin coat dynamics reflect changing membrane tension throughout cell spreading.

(A) Optically trapped beads are used to measure membrane tether forces of BSC1 cells at early and late stages of spreading. Membrane tension values (mean + standard error) are shown for cells plated for 10 and 120 min (Ncells = 12). (B) Snapshots show spreading of a BSC1 cell expressing AP2-EGFP. Detected clathrin coat traces are colored according to the lifetime. (C) Time variations of the average clathrin coat lifetime (orange) and visible spreading area (black) of the cell in B. SD of growth rates (D), and initiation and dissolution densities of clathrin coats (E) are plotted for the same cell. Shortening of lifetimes, increasing SD of growth rates, and increased initiation and dissolution rates establish increased endocytosis rates with the completion of spreading. (F) Box plots show the cumulative comparison of the clathrin coat lifetime and initiation/dissolution densities obtained during and after spreading of BSC1 cells (Ncells = 24, Ntraces = 41,989).

Figure 4.2: Continued

(G) SD of growth rates are calculated for the ventral and dorsal surfaces of BSC1 cells at early and late stages of spreading (measurements are separated by 30–40 min; Ncells = 11). Boxes extend to the quartiles, with a line at the median. Whiskers extend from the 10th to 90th percentiles. (H) The plot shows the normalized clathrin coat lifetime (mean±SD) vs. normalized extension rate of the ventral surface (area/time) obtained from 24 spreading cells. (I) Spreading area (blue) and average clathrin coat lifetime (orange) in cells featuring periods of spreading (shaded green regions) as well as retraction or pause. p values were obtained using the two-tailed t test.

Extension of the cell surface area is associated with increasing membrane tension (Gauthier et al., 2011; Houk et al., 2012; Masters et al., 2013). In good agreement with this observation, we detected a strong correlation between the rate of area extension and average clathrin coat lifetime in spreading cells (Pearson’s r = 0.67; Figure 2H). This phenomenon is particularly conspicuous in cells that undergo multiple rounds of extension. When spreading is interrupted temporarily, clathrin coat lifetimes converge to the values observed during low tension phases. Lifetimes elongate back to the values observed under high tension as soon as the cells start to spread again (Figure 2I and Supplemental Movie 2). Together, our findings show that temporal variations in tension have direct effects on dynamics and distribution of endocytic clathrin coats in cells.

Polarization of cells induces spatial heterogeneity in effective membrane tension (Dai and Sheetz, 1999; Lieber et al., 2015). Theoretical studies predict a strong front-to-rear tension gradient at the ventral surface of protruding cells (Fogelson and Mogilner, 2014). We found that the spatial heterogeneity in clathrin coat dynamics outlines the predicted tension gradient at the ventral surface of asymmetrically spreading cells (Figure 3). Figure 3B shows clathrin lifetime maps of two spreading cells in which every data point is given the average value of the closest three clathrin coats’ lifetime. We found that long-lived clathrin-coated structures are predominantly located in the vicinity of the leading edge. To better quantify this trend, for each time point of spreading, we calculated the lifetime dipole moment, which is a vector pointing in the direction of increasing clathrin coat lifetime. We detected a significant correlation between the direction of the lifetime dipole and the cells’ center-of-mass displacement even when cells change directions (Pearson’s r = 0.53; Figure 3, B–D, and Supplemental Movie 3). As a control, we randomly exchanged the lifetime values between clathrin coats and recalculated the dipoles. The rose diagrams generated using the angular separation between the simulated lifetime dipoles and cells’ original displacement directions were omnidirectional, indicating that the control analyses had no preference for the correct direction (Figure 3D). We also found that clathrin coat distribution is significantly heterogeneous even when the net cellular displacement is due to slight asymmetry of the spreading. Initiation and dissolution densities are the lowest within cellular regions with the highest extension rate (Figure 3E).

Continued

(A) Snapshots show two asymmetrically spreading BSC1 cells expressing AP2-EGFP. (B) Lifetime maps of the cells in A. This representation allows analyzing the local lifetime and density information by the color and size of the domains, respectively, that is, the domain sizes are inversely related to the local density of clathrin coats. The clathrin coat lifetime dipole moments are shown by black vectors for each cell. The displacement directions of the cellular centers of mass for the given frames are shown by red vectors. ΘL and ΘD represent the angles of the lifetime dipoles and displacement vectors, respectively. (C) Rose plots are assembled using the angular separation between the lifetime dipole vectors and the displacement vectors (ΔΘ = ΘL – ΘD) for the two spreading cells in A and B.

Figure 4.3 Heterogeneous clathrin dynamics maps the tension gradient in protruding cells.

Figure 4.3: Continued

(D) The blue angular histogram shows ΔΘ values obtained from 15 spreading cells (total spreading time is 123 min). The red histogram shows the cumulative result of five simulations (using the same 15 cells) in which ΘL values are determined after clathrin coat lifetimes are randomly exchanged within a cell. (E) Asymmetrically spreading cells are sectioned into three regions (front: 30% of the cell area next to the leading edge; back: 30% of the cell area at the opposite side of the leading edge; middle: the remaining 40% of the cell area in between the front and back regions) in each frame of spreading movies. Both the front and back are extending regions. However, initiation and conclusion densities (shown as bar plots; mean + SD) are the lowest at the front region, which has the highest extension rate. p values were obtained using the two-tailed t test.

Tether force measurements revealed a significant front-to-rear tension gradient at the lamellipodial fragments of migrating keratocytes (Lieber et al., 2015). Such fragments cannot be isolated from migrating astrocytes for tension measurements. However, at the dorsal surface of these cells, we detected significant spatial heterogeneity in CME dynamics accompanying the expected tension gradient. Clathrin coats originating in the proximity of the leading edge have longer lifetimes (69 ± 51 s [leading edge] vs. 58 ± 39 s [lamella], p < 0.001; Figure 4D) and narrower growth rate distributions (0.035 ± 0.003 [leading edge] vs. 0.041 ± 0.005 [lamella], p < 0.02; Figure 4E). As a visualization tool for the spatial distribution of the clathrin dynamics, we generated growth rate maps in which each pixel is given the value of the SD of the growth rates detected in a circular neighborhood. In this representation, regions of the cell that have slower clathrin dynamics have smaller SD values (Figure 4C). A comparative analysis of clathrin coat initiation and dissolution densities at the two regions is infeasible due to the complex three-dimensional (3D) geometries of the membrane ruffles appearing at the leading edge (Kural et al., 2015). Collectively, our results demonstrate that the cellular processes associated with spatial divergences in plasma membrane tension increase the heterogeneity of CME in cells.

Figure 4.4 Heterogeneous CME during in vitro and in vivo cell migration.

(A) Maximum z-projection image shows clathrin-coated structures at the ventral and dorsal surfaces of an U373 astrocyte expressing AP2-GFP. The arrow points toward the direction of extension. (B) Clathrin coat traces obtained from the cell in A are color-coded according to their z-position relative to the substrate. (C) Growth rate map of the dorsal surface is created by calculating the SD of clathrin growth rates within a 4.8-µm radius. SD values are lower in the vicinity of the leading edge due to slower clathrin dynamics. Dashed lines represent the cell boundary. (D) Normalized distributions show lifetimes of dorsal clathrin coats positioned within an 8-µm neighborhood of the leading edge (indigo) and the dorsal coats positioned in the lamellar region between 8 and 16 µm from the leading edge (orange). (E) Box plots show the SD of growth rate distributions assembled using the leading edge and lamellar clathrin coat populations from eight astrocytes (Ntraces = 11,386). Connected lines indicate the values obtained from the same astrocyte. The narrower distribution obtained for the leading edge group (indigo) indicates slower clathrin dynamics at this region. Boxes extend to the quartiles, with a line at the median. Whiskers extend from the 10th to 90th percentiles. p value was obtained using the two-tailed t test. (F) Hemocytes expressing clathrin-GFP (green) and CD4-tdTomato (red) are imaged at the ventral surface of late Drosophila embryos. (G) CD4 signal is used to generate a 3D mask representing the surface of the hemocyte. Positions of the clathrin coats within 320-nm neighborhood of the surface are shown with green dots. (H, I) Grayscale represents the thickness of the hemocyte. Endocytic clathrin coats are color coded according to their z-positions in H and local density of clathrin spots calculated within a 5-µm cube in I. (J) Each pixel in the hemocyte image is color coded according to its average distance to the three closest clathrin coats. Scale bars in H–J are 8µm.

### 4.3.2 Spatiotemporal variations in clathrin dynamics of Drosophila embryos

We expect spatiotemporal heterogeneity in clathrin coat dynamics to be prominent during developmental processes associated with dynamic tissue mechanics. It was previously shown that tensionbased regulation of receptor endocytosis have important functions in development of Drosophila embryos (Pouille et al., 2009). Since quantification of clathrin lifetimes is error prone within tissue contexts, we used growth rates and spatial distribution of clathrincoated structures to probe CME in this system (Ferguson et al., 2016). During late stages of Drosophila embryogenesis, hemocytes migrate along the ventral nerve cord to populate the entire embryo. Unlike in vitro migration systems, embryonic hemocytes are physically constrained by the 3D environment and therefore do not form membrane ruffles at their lamellipodial extensions (Tucker et al., 2011). Figure 4F shows the maximum z-projection image of a hemocyte expressing fluorescently tagged clathrin and CD4 (membrane marker). We assessed the 3D positions of clathrin structures to distinguish the endocytic coats, which are in the vicinity of the cell surface (Figure 4, G and H) (Kural et al., 2012), and used alternative visualization tools to analyze the spatial distribution of endocytic clathrin coats. In Figure 4I, positions of endocytic clathrin coats are color coded according to the density of neighboring coats within the 5-µm neighborhood. In Figure 4J, the heat map shows each pixel’s average distance to the three closest clathrin coats.

During dorsal closure of the Drosophila embryo, tension on the amnioserosa (AS) tissue increases and the tissue volume reduces gradually (Ma et al., 2009; Saias et al., 2015). As expected, we detected significant reduction in clathrin dynamics at later stages of the dorsal closure (SD: 0.036 ± 0.003 [early] vs. 0.032 ± 0.002 [late], p < 0.01; Figure 5A). Using SD maps, we also discovered that CME dynamics is spatially heterogeneous at the dorsal surface of the embryo (Figure 5, B and D). The growth rate analysis revealed that clathrin dynamics are markedly slower at the AS compared with the two flanks of the lateral epidermis (LE) tissue (SD: 0.032 ± 0.002 [AS] vs. 0.035 ± 0.002 [LE], p < 0.02; Figure 5C). Such a divergence in endocytic dynamics was anticipated, considering the distinct physical properties of AS and LE cells and the mechanical roles they play during dorsal closure (Brodland et al., 2014; Ducuing and Vincent, 2016; Pasakarnis et al., 2016).

(A) Clathrin dynamics slow down with increasing tension during late stages of the dorsal closure. Box plots show the SD of clathrin growth rate distributions obtained from early and late stage AS tissues. Reduced SD is a hallmark of slowed-down endocytosis (Ferguson et al., 2016). (B) Left, clathrin-coated structures at the dorsal surface of a Drosophila embryo. AS appears as narrow opening between the two flanks of lateral epidermis (LE). Right, SD map of the clathrin growth rates obtained from the same area. The map is created by calculating the SD of apical clathrin growth rates within an 8-µm radius. Lower SD values in the AS region display slower clathrin dynamics with respect to the neighboring LE. (C) More examples demonstrating the heterogeneous clathrin dynamics at the dorsal surface of late Drosophila embryos. (D) Box plots show the SD of clathrin growth rate distributions obtained from LE and AS of eight embryos. Connected lines indicate the values obtained from the same embryo. Boxes extend to the quartiles, with a line at the median. Whiskers extend from the 10th to 90th percentiles. p values were obtained using the two tailed t test.

Figure 4.5 Spatiotemporal variations in clathrin dynamics can be detected within tissues of Drosophila embryo.

## 4.4 Discussion

In this study, we show that spatial and temporal variations in cell membrane tension dominate the dynamics of clathrin-coated structures. Endocytic machinery must overcome the major constituents of the effective membrane tension, that is, in-plane tension and membrane-cytoskeleton adhesion, to deform the plasma membrane (Sheetz, 2001). In-plane tension is assumed to be in equilibrium across the entire plasma membrane due to fast flow of membrane lipids. However, membrane-cytoskeleton adhesion can be heterogeneous and induce stark differences in the clathrin dynamics between distinct surfaces of a cell (Dai and Sheetz, 1999; Boulant et al., 2011). Similarly, adhesion to the substrate can inhibit curvature formation and slow down clathrin coat dynamics locally. Therefore, nonuniform adhesion of a cell to the substrate creates another layer of heterogeneity in clathrin dynamics (Batchelder and Yarar, 2010; Ferguson et al., 2016).

We believe that spatiotemporal heterogeneity in clathrin coat dynamics plays important roles in central cellular processes. Mechanoinhibition of endocytosis at early stages of cell spreading might elevate the rate of extension of the plasma membrane area (Gauthier et al., 2009, 2011). Inhibition of endocytosis at the leading edge of migrating cells may facilitate cell protrusion by allowing net membrane deposition to this region (Bretscher, 2014). Similarly, increased tension at the amnioserosa tissue of developing embryos may account for the gradual reduction of the cell volume through inhibition of endocytosis in the late stages of the dorsal closure. Future studies should be directed toward investigating the mechanoregulation of endocytosis in situ and elucidating the roles it plays at the organismal level.

## 4.5 Materials and Methods

### 4.5.1 Cell culture and fluorescence microscopy

BSC1 and U373 cells stably expressing σ2-EGFP were cultured in DMEM (Life Technologies) containing penicillin/streptomycin and 10% fetal bovine serum (FBS). Gene-edited SUM159 cells were grown in F-12 medium containing 5% FBS and 1 µm/ml hydrocortisone (Aguet et al., 2016). Live cells and embryos are imaged using a Nikon Eclipse (TI-E) microscope equipped with a 100× objective lens (Nikon CFI Plan-Apochromat Lambda, NA 1.45), a CSU-W1 spinning disk confocal head (Yokogawa Electric Corporation) and an electron-multiplying charge-coupled device (EMCCD) camera (iXon DU897 Ultra; Andor Technology). Sample temperature and z-position are stabilized using a temperature controlled chamber and perfect focusing system (PFS), respectively. NIS Elements software is used for image acquisition.

Spreading cells were imaged on glass bottom dishes (Greiner Bio-One) directly after plating. The plating time prior to astrocyte migration experiments was 8–24 h. Live cell imaging is performed at 37°C ambient temperature within L15 (Thermo Fisher Scientific) supplemented with 10% FBS. Images were acquired at 0.25–0.5 Hz, and laser exposure lasted for 50–300 ms per frame. The final concentrations of MβCD and deoxycholic acid (Sigma-Aldrich) were 10 and 0.4 mM in serum-free L15, respectively. Hypotonic shock was performed using 1:5 dilution of the imaging medium using deionized water. In the figures and movies fluorescence acquisitions are inverted to increase visibility.

### 4.5.2 Fly strains and in vivo imaging

We used the UAS/GAL4 system to monitor clathrin dynamics in Drosophila embryos. Arm-GAL4, CLC-GFP, and CD4-tdTom strains were provided by the Bloomington Drosophila Stock Center. srpHemo-GAL4 was a gift from Norbert Perrimon (Harvard Medical School). Embryos were collected and aged for 11–13 h at 25°C. After dechorionation, embryos were mounted on coverslips and immersed in halocarbon oil. Clathrin dynamics at the dorsal and ventral surfaces were imaged at 22°C using 3D time-lapse acquisitions. In amnioserosa, apical clathrin coats were determined by filtering out the bright puncta corresponding to organelle-bound clathrin-coated structures as described earlier (Ferguson et al., 2016). In Figure 5A, maximum amnioserosa openings for the early- and late-stage embryos were 81.5 ± 13.0 and 28.2 ± 10.6 µm, respectively.

To analyze hemocyte images, clathrin coats were found using a simple threshold of the clathrin channel and localized using the center of intensity of the fluorescence signal (Kural et al., 2012). Similarly, the membrane was identified using a threshold on the CD4 channel. Using the built-in MATLAB function, isosurface, a triangular mesh of the membrane surface was generated. Clathrin coats were defined to be endocytic if they were < 320 nm from the nearest surface voxel. The spot density was determined by counting all endocytic coats around a spot within a 5-µm cube and then dividing that by the sum of the area from the triangulated mesh found within the cube. The distance map was generated by determining the average distance of the three closest endocytic clathrin spots for each pixel on the surface.

### 4.5.3 Two-dimensional tracking of clathrin-coated structures

We used cmeAnalysis software for 2D single particle tracking (Aguet et al., 2013). We used a previously developed trace rejection scheme to filter traces that do not follow a characteristic clathrin coat intensity profile (Ferguson et al., 2016). We used the traces that pass the rejection scheme in the calculation of lifetime distributions, growth rate distributions, initiation/dissolution densities and lifetime dipoles.

To determine the temporal evolution of the average clathrin coat lifetime, for each frame of a movie, we added together the lifetime of each trace that exists in that frame and divided by the number of traces considered.

### 4.5.4 Three-dimensional tracking of clathrin-coated structures

We used the z-position information to distinguish the dorsal and ventral clathrin coats in cells (Figures 1C, 2G, and 4B). cmeAnalysis software was used to analyze each z-plane of 3D time-lapse movies (followed by the trace rejection scheme detailed above). The resulting data were combined to link traces which occur at the same lateral position in two adjacent z-planes. Coincident traces had to remain within one pixel (160 nm) x–y distance for at least three frames. The resulting trace was assigned the maximum intensity value among all traces considered for combination. Axial positions were calculated using the intensity-weighted mean z-position of all traces considered. The algorithm for trace combination ran from the outermost z-planes to the innermost, alternating between the top and the bottom to ensure that there was no directional bias and all possible trace combinations were considered.

### 4.5.5 Growth rate distributions

Clathrin coat intensity traces were normalized by subtracting a global minimum and dividing by the resulting maximum. From this normalized trace, each 12-s interval was used in a least-squares fit to determine the growth rate of each interval. A trace had to be at least 12 s long to be included in the distribution. An arbitrary bin width (0.03) was found to delineate five distinct growth phases (fast dissolution, slow dissolution, plateau, slow formation, and fast formation) (Ferguson et al., 2016).

To determine the SD of the growth rates per frame, we generated a list of the intensity slope of each trace within that frame and took the SD of that list. When the data are sparse, we used the walking average of three adjacent frames. We used PFS to eliminate sample defocusing triggered by the squeezing procedure. We found that the adjustment of the PFS resulted in a single frame of artificial growth rate values due to abrupt changes in the clathrin coat fluorescence intensities. Those frames are excluded from the analyses.

SD maps of clathrin coat growth rates in Figures 4 and 5 are made by, for each pixel within the cell, calculating the SD of all growth rates within 4.8 and 8 µm, respectively.

### 4.5.6 Lifetime maps and dipole vectors

Lifetime maps in Figure 3 and Supplemental Movie 3 are made of a given frame by calculating the average lifetime of the three closest clathrin-coated structures for each pixel within the cell. Patches of color are regions where the set of closest clathrin coats are the same, so color is an indication of local lifetime and the size of the patch is an indication of local clathrin coat density (larger patches indicates lower density).

Lifetime dipoles were calculated using the equation , where and are the lifetime and the position of the ith clathrin-coated structure and is the average lifetime of all clathrin-coated structures in the frame. Abortive clathrin coats with lifetimes less than 20 s, hotspots, and clathrin-coated plaques that do not disappear until the end of the acquisitions were excluded from the calculation of lifetime dipoles. In the randomization scheme used for the control analyses, the positions of clathrin-coated structures remained untouched to validate that the reciprocity between the lifetime dipole and cell displacement is due only to the spatial distribution of lifetimes within the cell.

### 4.5.7 Tether force measurements

We used optically trapped beads to quantify membrane tether forces. An optical tweezers system was built based on a previous design; however; only one of the two traps was used in this application (Bustamante et al., 2009). Polystyrene beads (1 µm; Spherotech) were coated with fibronectin (Sigma-Aldrich) before the experiments. Cells were incubated for 10 min or 2 h before the experiments for measuring membrane tension at different stages of spreading. Membrane tension values (*T*) are calculated using , where is the measured membrane tether forces and *B* is the bending modulus of the plasma membrane, and the value is assumed to be 0.27 pN µm (Hochmuth et al., 1996).

# In Vitro Characterization of CALM recruitment to clathrin coated structures

Derived from a manuscript in submission by: (later)

## 5.1 Abstract

## 5.2 Introduction

Clathrin-mediated endocytosis (CME) is an essential process in all mammalian cells that is responsible for the uptake of extracellular proteins and receptors (Conner, 2003).

The most prevalent protein in CME and namesake for the process, clathrin, can self-assemble into a round cage (Crowther, 1975). This provides some of the energy necessary to bend the initially flat plasma membrane into a round clathrin-coated pit (CCP), which can then pinch off from the surrounding membrane. However, clathrin by itself cannot bind to the membrane, so it could only form empty cages alone.

Aside from clathrin, the two most prevalent CME proteins, AP2 and CALM, can simultaneously bind to clathrin and membrane lipids, which allows them to act as clathrin-membrane adaptors (ref). Using overexpression of clathrin-GFP and AP2-GFP, the recruitment dynamics of clathrin and AP2 were studied *in vitro* as early as 1999 and 2004 (?), respectively (Gaidarov, 1999; Ehrlich, 2004). Unfortunately, overexpression of CALM inhibits CME by sequestration of clathrin, so CALM could not be studied in the same way (Tebar, 1999).

Only recently was CALM recruitment dynamics studied at all (Miller, 2015). In that study, it was found that CALM functions in curvature generation as a cuvature sensor and inducer. Along with their important biochemical characterization of CALM, Miller et. al. also briefly described the *in vitro* recruitment of CALM, and found that it followed AP2 recruitment very closely except during a short window shortly before scission.

(ref Hoppe)?

In this study, we seek to further test the *in vitro* recruitment dynamics of CALM to clathrin-coated structures using a cell line that we (?) gene-edited to express CALM-eGFP at endogenous levels.

## 5.3 Results

Stuff about making the cells (later)

### 5.3.1 Characterization of protein recruitment numbers

The first quantity we wished to characterize in these cells was the number of each protein recruited to pits by the time of scission. With knowledge of the substitution ratio of the proteins, we could calculate the numbers of each protein in a pit by dividing its intensity by the intensity of a single eGFP and diving by the substitution ratio. To calculate the intentity of a single eGFP, we imaged a slide of sparese, free eGFP molecules with a long exposure time, and calculated the intensity change during bleaching events as in Cocucci et. al. (Cocucci, 2012).

We first characterized the recruitment numbers of our Dynamin-eGFP cells so that we could compare to the results of Cocucci et. al. (Cocucci, 2014). We used cmeAnalysis package for automated detection of dynamin-containing structures (Aguet, 2013), along with our previously described selection code for recognizing full traces (ref Ferguson). We then plotted the distribution of dynamin peak intensities calibrated with our single eGFP intensity value as in Cocucci et. al. The resulting distribution was in very good agreement with the previous result (fig).

We then calculated the calibrated peak intensity distribution of our AP2-eGFP and CALM-eGFP cells (fig). We found that the variation of CALM intensities was much less than that of AP2. In order to compare AP2 and CALM numbers of similar pits, we grouped our traces into lifetime cohorts and compared the median AP2 and CALM numbers of corresponding cohorts. We saw that AP2 increases more drastically with lifetime (fig). This suggests that larger pits need proportionally fewer CALM molecules per AP2 to internalize.

### 5.3.2 Clathrin coated pit dynamics in CALM siRNA cells

Next, we quantified the effects of lowered CALM expression on clathrin dynamics. To do so, we transiently transfected our cells with CALM siRNA to partially block CALM expression. In order to avoid potential bias caused by tracking puncta labelled by CALM-eGFP, we simultaneously transfected our cells with CLCa-ruby to use as a marker for CCS tracking. We also transfected a control group of cells with the CLCa-ruby plasmid in the same way without CALM siRNA.

We compared the clathrin and CALM signal between the control and siRNA cells (fig). As expected, the clathrin signal in the control cells was on average the same as the clathrin signal in the siRNA cells. The CALM signal in the siRNA cells was roughly 40% of that of the control cells, confirming the efficacy of the siRNA. Dynamically, the control and siRNA cells were very similar; the medians of the lifetime distributions differed by only 2 seconds, despite the fact that the siRNA pits recruited far less CALM by the time of scission (fig). This suggests that, under ideal conditions, pits can function normally with 40% of their normal available CALM.

To probe the recruitment dynamics of the siRNA cells further, we used our previously described hierarchical trace-clustering algorithm to separate clathrin intensity traces of single clathrin-coated pits by shape (Ferguson, 2016). We found three highly populated clusters: cluster 1 had highly plateaued recruitment, cluster 2 had highly linear recruitment, and cluster 3 had recruitment in between 1 and 2 (fig). The average clathrin traces of these clusters looked very similar between control and siRNA cells; however, the average CALM traces of the siRNA cells showed CALM recruitment that lagged behind that of the control cells (fig). Additionally, we found that the siRNA traces had a slightly higher tendency towards cluster 2 (fig), suggesting that the scarcity of CALM causes an increased probability of linear pit growth. Lastly, we found that the average lifetime of each cluster was somewhat higher in the siRNA cells (fig).

### 5.3.3 Clathrin coated pit dynamics in CALM siRNA expressing cells subject to elevated membrane tension

CALM has been previously found to help in the generation of membrane curvature in clathrin-coated pits (Miller, 2015). We have previously found that an increase in membrane tension decreases the internalization rate of pits, likely due to the additional energy required to curve the membrane (Ferguson, 2017). Combining these two findings, we hypothesized that cells with lowered CALM expression would be more strongly affected by increased membrane tension. To test this, we subjected our control and CALM siRNA cells to osmotic shock, which we have previously shown transiently elevates membrane tension (Ferguson, 2017).

We first subjected our cells to an osmotic shock that lowered the osmolarity of the medium by 80%. Under this very strong shock, we found that the dynamics of all cells were almost completely stalled after 10 minutes (fig). In the time between the addition of water to the medium and 10 minutes, though, we saw a significantly higher internalization rate in our control cells compared to that of our CALM siRNA cells (fig). This suggests that while the cells were swelling, they were temporarily in a state where the membrane tension was too high for siRNA cells to internalize pits, but not so high as to completely halt the dynamics of the control cells.

We then tried to replicate this intermediate-tension state by subjecting our cells to the weaker osmotic shock of 66% water. In this condition, the internalization rate of the control cells was not affected, even after 10 minutes, while the internalization rate of the siRNA cells was reduced by roughly 2/3 (fig). This provided further evidence that cells with reduced CALM expression have reduced CME efficiency under elevated membrane tension.

Additionally of interest was the effect osmotic shock had on the CALM content of pits at scission. We hypothesized that the minimum number of CALM molecules needed to successfully complete a pit would increase when tension increased. In actuality, we saw that the distribution of peak CALM intensities was always very wide with no apparent change in minimum. There was a change in the medians of these distributions under osmotic shock, however: We found that the median peak CALM intensity of pits in our siRNA cells increased markedly after 10 minutes of either 66% or 80% shock (fig). Interestingly, these medians did not change in the first 10 minutes of the shock suggesting that some delayed mechanism causes pits to start accumulating more CALM to oppose tension.

### 5.3.4 Super-resolved localization of CALM in large, clathrin-coated structures

To further characterize these gene-edited CALM-eGFP cells, we imaged them with TIRF-SIM. TIRF-SIM is a super-resolution imaging technique that combines the low cytosolic background of Total Internal Flourescence Microscopy (TIRF) with the sub-diffraction resolution of Structured Illumination Microscopy (SIM) (Chung, 2006).

We ran 2-color TIRF-SIM on our CALM-eGFP cells overexpressing clathrin light chain A-ruby. Upon inspection of the images, it appeared that large structures seen the clathrin channel appeared as collections of closely spaced small structures in the CALM channel (fig). To assess the possibility of this being an artifact of the naturally better resolution of the shorter wavelength CALM channel, we repeated these experiments on similarly gene-edited SUM AP2-eGFP cells, also overexpressing clathrin light chain A-ruby. In these cells, large structures in the clathrin channel appeared as similarly large structures in the AP2 channel indicating that the localization of AP2 in large structures does indeed differ from that of CALM.

To quantify this effect, we used image autocorrelation analysis. We exploited the fact that at the center of the autocorrelation image of a series of objects is a global maximum that decays with a width that roughly corresponds to times the average width of the objects (Robertson, 2012). With that in mind, we calculated the radial average of the autocorrelation image of areas of interest with the large structures we wished to probe (fig). We consistently found that the full width at half max of CALM autocorrelation images was much smaller than that of their corresponding clathrin images, while the full width at half max of AP2 autocorrelation images roughly matched that of their clathrin images (fig). This however did not hold for areas of interest containing only small clathrin coated structures.

To quantify this final assertion, we manually selected 467 total clathrin structures among the two cell types and classified them as either “pits” (a single Gaussian-shaped object that internalizes in one step), or “plaques” (large, non-Gaussian-shaped objects). We then ran our autocorrelation width analysis on just the small regions containing each object. In pits, both AP2 and CALM are spread across similarly sized regions, as evidenced by their autocorrelation widths (fig). However, in plaques, AP2 is spread over a region that is roughly 40% wider than that of CALM (fig). Furthermore, the median CALM autocorrelation width in plaques is very similar to that of pits, suggesting that CALM may localize to small, pit-sized regions of plaques.

## 5.4 Discussion

(Later)

(comment on smaller spread in CALM calibrated numbers)

(comment on increasing calm 10 minutes after osmoshock)

Our finding that CALM may localize to small, pit-sized regions of clathrin-coated plaques may be related to the fact that CALM has been shown to be a sensor and driver of membrane curvature (Miller, 2015). It has been previously suggested that the edges of flat clathrin-coated plaques may be able to gain curvature and eventually pinch off vesicles (ref). Indeed, EM images often show large, clathrin-coated objects with heterogeneous curvature profiles (ref). Perhaps the pit-sized regions we see CALM in are curved sections of plaques, which attract more CALM than the adjacent flat sections. In support of this idea, we have documented instances of these regions quickly leaving the field of view, as if that section of the plaque were invaginated (sup fig).

## 5.5 Materials and Methods

### 5.5.1 Cell culture

Talk to Ema for gene-editing details (later) and transfection stuff (check all my red tags are accurate)

### 5.5.2 Fluorescence Imaging

Confocal stuff (later)

TIRF-SIM Janelia stuff (later)

### 5.5.3 Image Detection and Tracking

Tracking was done using the cmeAnalysis MATLAB package (Aguet, 2013). The output traces were then filtered using our previously described trace-rejection algorithm (Ferguson, 2016). All 2-channel tracking was done only using data from the 560 nm (clathrin) channel in order to not introduce bias to siRNA cells with lower 488 (CALM) signal. Intensity in both channels was then calculated using the pixel-sum scheme described above.

### 5.5.4 Single GFP Intensity Calibration

Free eGFP (source later) was plated onto coverslips using protocol (ref later). These slides were then imaged with 4 s exposure per frame for 2-5 minutes. The resulting movies were then tracked and instances of step-like bleaching events were manually selected. Intensity of puncta was calculated as the mean intensity of a 5x5 pixel window around the puncta minus a background value calculated as the median intensity of the 24 pixels immediately surrounding the signal window.

### 5.5.5 Object Dissolution Verification

We developed an algorithm for screening all clathrin fluorescent traces for true scission events. The goal thereof was to create a set of criteria that identified most events while strictly avoiding false positives, so that quantities of CALM at scission could be measured. Briefly, this algorithm scanned all traces for spans of fast, linear intensity decrease that ended with a sufficiently dim timepoint. A maximum displacement per frame was also imposed to preclude tracking errors. This algorithm was tuned and checked using the below described manual analysis software. In situations of especially low scission rates where a few false positives could be problematic, manual verification was used.

### 5.5.6 Autocorrelation Analysis

A MATLAB function was previously developed to facilitate manual pit and plaque selection (ask about ref to in prep work (later)). This was used to select suitable structures for 2-channel autocorrelation analysis from the clathrin channel of our movies. Some areas that were in-focus in the clathrin channel appeared out-of-focus in the adaptor channel, so a cross-correlation minimum of .5 was imposed to filter out these structures.

Manually selected and cropped 2-channel TIRF-SIM structures were aligned using by the maximum of the cross-correlation of the images. Autocorrelation images were then made using MATLAB built-in “xcorr2”.

# Conclusions and Future Work (Later)

Trace classification improvements

Optimized imaging-analysis pipeline

More TIRF-SIM

Further Actin-AP2 experiments

# Bibliography

Aegerter-Wilmsen, T., Heimlicher, M.B., Smith, A.C., de Reuille, P.B., Smith, R.S., Aegerter, C.M., and Basler, K. (2012). Integrating force-sensing and signaling pathways in a model for the regulation of wing imaginal disc size. Development 139, 3221–3231.

Aghamohammadzadeh, S., and Ayscough, K.R. (2009). Differential requirements for actin during yeast and mammalian endocytosis. Nat. Cell Biol. 11, 1039–1042.

20 Aguet, F., Antonescu, C.N., Mettlen, M., Schmid, S.L., and Danuser, G. (2013). Advances in analysis of low signal-to-noise images link dynamin and AP2 to the functions of an endocytic checkpoint. Dev. Cell 26, 279–291.

20 Aguet, F., Upadhyayula, S., Gaudin, R., Chou, Y., Cocucci, E., He, K., Chen, B.-C., Mosaliganti, K., Pasham, M., Skillern, W., et al. (2016). Membrane dynamics of dividing cells imaged by lattice light-sheet microscopy. Mol. Biol. Cell 27, 3418–3435.

Apodaca, G. (2002). Modulation of membrane traffic by mechanical stimuli. Am. J. Physiol. Renal Physiol. 282, F179–F190.

Avinoam, O., Schorb, M., Beese, C.J., Briggs, J.A.G., and Kaksonen, M. (2015). Endocytic sites mature by continuous bending and remodeling of the clathrin coat. Science (80-. ). 348, 1369–1372.

Batchelder, E.M., and Yarar, D. (2010). Differential Requirements for Clathrin-dependent Endocytosis at Sites of Cell–Substrate Adhesion. Mol. Biol. Cell 21, 3070–3079.

Batchelder, E.L., Hollopeter, G., Campillo, C., Mezanges, X., Jorgensen, E.M., Nassoy, P., Sens, P., and Plastino, J. (2011). Membrane tension regulates motility by controlling lamellipodium organization. Proc. Natl. Acad. Sci. 108, 11429–11434.

Borner, G. H. H., Antrobus, R., Hirst, J., Bhumbra, G. S., Kozik, P., Jackson, L. P., … Robinson, M. S. (2012). Multivariate proteomic profiling identifies novel accessory proteins of coated vesicles. The Journal of Cell Biology, 197(1), 141–160.

Boucrot, E., Saffarian, S., Zhang, R., and Kirchhausen, T. (2010). Roles of AP-2 in clathrin-mediated endocytosis. PLoS One 5.

13 Boulant, S., Kural, C., Zeeh, J.-C., Ubelmann, F., and Kirchhausen, T. (2011). Actin dynamics counteract membrane tension during clathrin-mediated endocytosis. Nat. Cell Biol. 13, 1124–1131.

Bretscher, M.S. (2014). Asymmetry of Single Cells and Where That Leads. Annu. Rev. Biochem. 83, 275–289.

Brodland, G.W., Veldhuis, J.H., Kim, S., Perrone, M., Mashburn, D., and Hutson, M.S. (2014). CellFIT: A cellular force-inference toolkit using curvilinear cell boundaries. PLoS One 9.

Bustamante, C., Chemla, Y.R., Moffitt, J.R., Bustamante, C., Chemla, Y.R., and Moffitt, J.R. (2014). High-Resolution Dual-Trap Optical Tweezers with Differential Detection : Instrument Design High-Resolution Dual-Trap Optical Tweezers with Differential Detection : Instrument Design. 4, 1–9.

Chung, E., Kim, D., and So P. T. (2006). Extended resolution wide-field optical imaging: objective-launched standing-wave total internal reflection fluorescence microscopy. Opt. Lett. 31, 945-947

Cocucci, E., Aguet, F., Boulant, S., and Kirchhausen, T. (2012). The first five seconds in the life of a clathrin-coated pit. Cell 150, 495–507.

12 Cocucci, E., Gaudin, R., and Kirchhausen, T. (2014). Dynamin recruitment and membrane scission at the neck of a clathrin-coated pit. Mol. Biol. Cell 25, 3595–3609.

Collins, A., Warrington, A., Taylor, K.A., and Svitkina, T. (2011). Structural organization of the actin cytoskeleton at sites of clathrin-mediated endocytosis. Curr. Biol. 21, 1167–1175.

Conner, S.D., and Schmid, S.L. (2003). Regulated portals of entry into the cell. Nature 422, 37–44.

Crowther, R.A., Pinch, J.T., Pearse B.M.F. (1976). On the structure of coated vesicles. Journal of Molecular Biology 103 (4), 785-798

Cureton, D.K., Massol, R.H., Whelan, S.P.J., and Kirchhausen, T. (2010). The length of vesicular stomatitis virus particles dictates a need for actin assembly during clathrin-dependent endocytosis. PLoS Pathog. 6.

Dai, J., and Sheetz, M.P. (1995). Regulation of endocytosis, exocytosis, and shape by membrane tension. Cold Spring Harb. Symp. Quant. Biol. 60, 567–571.

Dai, J., Sheetz, M.P., Wan, X., and Morris, C.E. (1998). Membrane tension in swelling and shrinking molluscan neurons. J. Neurosci. 18, 6681–6692.

Dai, J., Ting-Beall, H.P., and Sheetz, M.P. (1997). The Secretion-coupled Endocytosis Correlates with Membrane Tension Changes in RBL 2H3 Cells. J. Gen. Physiol.

Dai, J., and Sheetz, M.P. (1999). Membrane tether formation from blebbing cells. Biophys. J. 77, 3363–3370.

Ref for chap 5 to be added den Otter, W.K., and Briels, W.J. (2011). The Generation of Curved Clathrin Coats from Flat Plaques. Traffic 12, 1407–1416.

Desprat, N., Supatto, W., Pouille, P.A., Beaurepaire, E., and Farge, E. (2008). Tissue Deformation Modulates Twist Expression to Determine Anterior Midgut Differentiation in Drosophila Embryos. Dev. Cell 15, 470–477.

Diz-Muñoz, A., Fletcher, D.A., and Weiner, O.D. (2013). Use the force: membrane tension as an organizer of cell shape and motility.

Diz-Muñoz, A., Thurley, K., Chintamen, S., Altschuler, S.J., Wu, L.F., Fletcher, D.A., and Weiner, O.D. (2016). Membrane Tension Acts Through PLD2 and mTORC2 to Limit Actin Network Assembly During Neutrophil Migration. PLoS Biol. 14, 1–30.

Ducuing, A., and Vincent, S. (2016). The actin cable is dispensable in directing dorsal closure dynamics but neutralizes mechanical stress to prevent scarring in the Drosophila embryo. Nat. Cell Biol. 18, 1149–1160.

Ehrlich, M., Boll, W., van Oijen, A., Hariharan, R., Chandran, K., Nibert, M.L., and Kirchhausen, T. (2004). Endocytosis by Random Initiation and Stabilization of Clathrin-Coated Pits. Cell 118, 591–605.

Ferguson, J.P., Willy, N.M., Heidotting, S.P., Huber, S.D., Webber, M.J., and Kural, C. (2016). Deciphering dynamics of clathrin-mediated endocytosis in a living organism. J. Cell Biol. 214, 347–358.

Ferguson, J.P., Huber, S.D., Willy, N.M., Aygün, E., Goker, S., Atabey, T., and Kural, C. (2017). Mechanoregulation of clathrin-mediated endocytosis. J. Cell Sci. 130, 3631–3636.

Ref for chapter 5? Fiolka, R., Shao, L., Rego, E.H., Davidson, M.W., and Gustafsson, M.G.L. (2012). Time-lapse two-color 3D imaging of live cells with doubled resolution using structured illumination. Proc. Natl. Acad. Sci. 109, 5311–5315.

Fogelson, B., and Mogilner, A. (2014). Computational estimates of membrane flow and tension gradient in motile cells. PLoS One 9.

Gaidarov, I., Santini, F., Warren, R.A., and Keen, J.H. (1999). Spatial control of coated-pit dynamics in living cells. Nat Cell Biol 1, 1–7.

Gauthier, N.C., Rossier, O.M., Mathur, A., Hone, J.C., and Sheetz, M.P. (2009). Plasma Membrane Area Increases with Spread Area by Exocytosis of a GPI-anchored Protein Compartment. Mol. Biol. Cell 20, 3261–3272.

Gauthier, N.C., Fardin, M.A., Roca-Cusachs, P., and Sheetz, M.P. (2011). Temporary increase in plasma membrane tension coordinates the activation of exocytosis and contraction during cell spreading. Proc. Natl. Acad. Sci. 108, 14467–14472.

Gauthier, N.C., Masters, T.A., and Sheetz, M.P. (2012). Mechanical feedback between membrane tension and dynamics. Trends Cell Biol. 22, 527–535.

Hassinger, J.E., Oster, G., Drubin, D.G., and Rangamani, P. (2017). Design principles for robust vesiculation in clathrin-mediated endocytosis. Proc. Natl. Acad. Sci. U. S. A. 114, E1118–E1127.

Herant, M., Heinrich, V., and Dembo, M. (2005). Mechanics of neutrophil phagocytosis: behavior of the cortical tension. J. Cell Sci. 118, 1789–1797.

Heuser, J.E., Keen, J.H., Amende, L.M., Lippoldt, R.E., and Prasad, K. (1987). Deep-etch visualization of 27S clathrin: A tetrahedral tetramer. J. Cell Biol. 105, 1999–2009.

Heuser, J. (1989). Effects of cytoplasmic acidification on clathrin lattice morphology. J. Cell Biol. 108, 401–411.

Hochmuth, R.M., Shao, J.Y., Dai, J., and Sheetz, M.P. (1996). Deformation and flow of membrane into tethers extracted from neuronal growth cones. Biophys. J. 70, 358–369.

Hong, S.H., Cortesio, C.L., and Drubin, D.G. (2015). Machine-Learning-Based Analysis in Genome-Edited Cells Reveals the Efficiency of Clathrin-Mediated Endocytosis. Cell Rep. 12, 2121–2130.

Houk, A.R., Jilkine, A., Mejean, C.O., Boltyanskiy, R., Dufresne, E.R., Angenent, S.B., Altschuler, S.J., Wu, L.F., and Weiner, O.D. (2012). Membrane tension maintains cell polarity by confining signals to the leading edge during neutrophil migration. Cell 148, 175–188.

Jacinto, A., Woolner, S., and Martin, P. (2002). Dynamic analysis of dorsal closure in Drosophila: From genetics to cell biology. Dev. Cell 3, 9–19.

Kaur, S., Fielding, A.B., Gassner, G., Carter, N.J., and Royle, S.J. (2014). An unmet actin requirement explains the mitotic inhibition of clathrin-mediated endocytosis. Elife 2014, 1–17.

Khatibzadeh, N., Gupta, S., Farrell, B., Brownell, W.E., and Anvari, B. (2012). Effects of cholesterol on nano-mechanical properties of the living cell plasma membrane. Soft Matter 8, 8350.

Kiehart, D.P., Galbraith, C.G., Edwards, K.A., Rickoll, W.L., and Montague, R.A. (2000). Multiple forces contribute to cell sheet morphogenesis for dorsal closure in Drosophila. J. Cell Biol. 149, 471–490.

Kural, C., and Kirchhausen, T. (2012). Live-cell imaging of clathrin coats (Elsevier Inc.).

Kural, C., Tacheva-Grigorova, S.K., Boulant, S., Cocucci, E., Baust, T., Duarte, D., and Kirchhausen, T. (2012). Dynamics of Intracellular Clathrin/AP1- and Clathrin/AP3-Containing Carriers. Cell Rep. 2, 1111–1119.

Kural, C., Akatay, A.A., Gaudin, R., Chen, B.-C., Legant, W.R., Betzig, E., and Kirchhausen, T. (2015). Asymmetric formation of coated pits on dorsal and ventral surfaces at the leading edges of motile cells and on protrusions of immobile cells. Mol. Biol. Cell 26, 2044–2053.

Legoff, L., Rouault, H., and Lecuit, T. (2013). A global pattern of mechanical stress polarizes cell divisions and cell shape in the growing Drosophila wing disc. Development 140, 4051–4059.

Li, D., Shao, L., Chen, B.C., Zhang, X., Zhang, M., Moses, B., Milkie, D.E., Beach, J.R., Hammer, J.A., Pasham, M., et al. (2015). Extended-resolution structured illumination imaging of endocytic and cytoskeletal dynamics. Science (80-. ). 349.

Lieber, A.D., Schweitzer, Y., Kozlov, M.M., and Keren, K. (2015). Front-to-rear membrane tension gradient in rapidly moving cells. Biophys. J. 108, 1599–1603.

Ma, X., Lynch, H.E., Scully, P.C., and Hutson, M.S. (2009). Probing embryonic tissue mechanics with laser hole drilling. Phys. Biol. 6.

Masters, T.A., Pontes, B., Viasnoff, V., Li, Y., and Gauthier, N.C. (2013). Plasma membrane tension orchestrates membrane trafficking, cytoskeletal remodeling, and biochemical signaling during phagocytosis. Proc. Natl. Acad. Sci. 110, 11875–11880.

Merrifield, C.J., Feldman, M.E., Wan, L., and Almers, W. (2002). Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. Nat. Cell Biol. 4, 691–698.

Mettlen, M., Loerke, D., Yarar, D., Danuser, G., and Schmid, S.L. (2010). Cargo- and adaptor-specific mechanisms regulate clathrin-mediated endocytosis. J. Cell Biol. 188, 919–933.

Mettlen, M., and Danuser, G. (2014). Imaging and Modeling the Dynamics of Clathrin-Mediated Endocytosis. Cold Spring Harb. Perspect. Biol. 6, a017038–a017038.

Miller, S.E., Mathiasen, S., Bright, N.A., Pierre, F., Kelly, B.T., Kladt, N., Schauss, A., Merrifield, C.J., Stamou, D., Höning, S., et al. (2015). CALM Regulates Clathrin-Coated Vesicle Size and Maturation by Directly Sensing and Driving Membrane Curvature. Dev. Cell 33, 163–175.

Pasakarnis, L., Frei, E., Caussinus, E., Affolter, M., and Brunner, D. (2016). Amnioserosa cell constriction but not epidermal actin cable tension autonomously drives dorsal closure. Nat. Cell Biol. 18, 1161–1172.

Pouille, P.A., Ahmadi, P., Brunet, A.C., and Farge, E. (2009). Mechanical signals trigger myosin II redistribution and mesoderm invagination in drosophila embryos. Sci. Signal. 2, 1–9.

Raucher, D., and Sheetz, M.P. (1999). Membrane expansion increases endocytosis rate during mitosis. J. Cell Biol.

Rauskolb, C., Sun, S., Sun, G., Pan, Y., and Irvine, K.D. (2014). Cytoskeletal tension inhibits Hippo signaling through an Ajuba-Warts complex. Cell 158, 143–156.

Robertson, C., & George, S. C. (2012). Theory and practical recommendations for autocorrelation-based image correlation spectroscopy. *Journal of Biomedical Optics*, *17*(8), 080801.

Saffarian, S., and Kirchhausen, T. (2008). Differential evanescence nanometry: Live-cell fluorescence measurements with 10-nm axial resolution on the plasma membrane. Biophys. J. 94, 2333–2342.

Saffarian, S., Cocucci, E., and Kirchhausen, T. (2009). Distinct dynamics of endocytic clathrin-coated pits and coated plaques. PLoS Biol. 7.

Saias, L., Swoger, J., D’Angelo, A., Hayes, P., Colombelli, J., Sharpe, J., Salbreux, G., and Solon, J. (2015). Decrease in Cell Volume Generates Contractile Forces Driving Dorsal Closure. Dev. Cell 33, 611–621.

Saleem, M., Morlot, S., Hohendahl, A., Manzi, J., Lenz, M., and Roux, A. (2015). A balance between membrane elasticity and polymerization energy sets the shape of spherical clathrin coats. Nat. Commun. 6, 6249.

Sheetz, M.P. (2001). Cell control by membrane – cytoskeleton adhesion. Nature 2, 366–369.

Snijder, B., Sacher, R., Rämö, P., Damm, E.M., Liberali, P., and Pelkmans, L. (2009). Population context determines cell-to-cell variability in endocytosis and virus infection. Nature 461, 520–523.

Ref for chapter 5--Sochacki, K.A., Dickey, A.M., Strub, M.P., and Taraska, J.W. (2017). Endocytic proteins are partitioned at the edge of the clathrin lattice in mammalian cells. Nat. Cell Biol. 19, 352–361.

Solon, J., Kaya-Çopur, A., Colombelli, J., and Brunner, D. (2009). Pulsed Forces Timed by a Ratchet-like Mechanism Drive Directed Tissue Movement during Dorsal Closure. Cell 137, 1331–1342.

Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M.A., Keen, J.H., and McGraw, T.E. (1999). Acute cholesterol depletion inhibits clathrin-coated pit budding. Proc. Natl. Acad. Sci. 96, 6775–6780.

Sun, M., Northup, N., Marga, F., Huber, T., Byfield, F.J., Levitan, I., and Forgacs, G. (2007). The effect of cellular cholesterol on membrane-cytoskeleton adhesion. J. Cell Sci. 120, 2223–2231.

Tan, X., Heureaux, J., and Liu, A.P. (2015). Cell spreading area regulates clathrin-coated pit dynamics on micropatterned substrate. Integr. Biol. 7, 1033–1043.

Taylor, M.J., Perrais, D., and Merrifield, C.J. (2011). A high precision survey of the molecular dynamics of mammalian clathrin-mediated endocytosis. PLoS Biol. 9.

Tebar, F., Bohlander, S. K., & Sorkin, A. (1999). Clathrin Assembly Lymphoid Myeloid Leukemia (CALM) Protein: Localization in Endocytic-coated Pits, Interactions with Clathrin, and the Impact of Overexpression on Clathrin-mediated Traffic. Molecular Biology of the Cell, 10(8), 2687–2702.

Tsujita, K., Takenawa, T., and Itoh, T. (2015). Feedback regulation between plasma membrane tension and membrane-bending proteins organizes cell polarity during leading edge formation. Nat. Cell Biol. 17, 749–758.

Tucker, P.K.P., Evans, I.I.R., and Wood, W. (2011). Ena drives invasive macrophage migration in Drosophila embryos. Dis. Model. Mech. 4, 126–134.

Willy, N.M., Ferguson, J.P., Huber, S.D., Heidotting, S.P., Aygün, E., Wurm, S.A., Johnston-Halperin, E., Poirier, M.G., and Kural, C. (2017). Membrane mechanics govern spatiotemporal heterogeneity of endocytic clathrin coat dynamics. Mol. Biol. Cell 28, 3480–34

# TO DO:

To be done (Later):

Revise intro

list of abbreviations

fix figure numbers in e.g. (figure 4K)—should be (figure 4.4K)

fix figure wrapping

Code reference (so that future people can find/use code)

Simple-english section

Read most important citations—remove citation numbers from bibliography

Kirchhausen, T. (1993). Coated pits and coated vesicles - sorting it all out. Curr. Opin. Struct. Biol. 3, 182–188.

Kirchhausen, T. (2009). Imaging endocytic clathrin structures in living cells. Trends Cell Biol. 19, 596–605.

Robinson, M.S. (2015). Forty Years of Clathrin-coated Vesicles. Traffic 16, 1210–1238.

**Late: Make sure all references go somewhere in the bibliography**

**Table of figures**

Make sure all “later”s and “?” from editing are gone