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Exchange Dynamics of Dynamin Measured in Living Cells During Endocytic Vesicle Formation

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During clathrin mediated endocytosis (CME) at the plasma membrane, the GTPase dynamin is recruited to the neck of nascent clathrin-coated vesicles (CCVs) where it oligomerizes into helical filaments. Conformational changes induced by the hydrolysis of GTP catalyze the scission of the vesicle neck. This process has been studied in great detail with *in vitro* reconstitution on membrane tubules [1,2] but it needs to be established in living cells, where dynamin interactions with SH3 domain containing proteins such as amphiphysin are critical [3]. Live cell TIRF imaging with the ppH assay [4] allows the detection of CCV formation with high spatial (~100 nm) and temporal (2 s) resolutions. It has revealed that dynamin is recruited to maturing clathrin coated pits (CCPs) in two phases with a peak at the time of scission [5] but the parameters of its recruitment in living cells remain unclear.

To determine these parameters, we have performed live cell imaging of dynamin recruitment at collective and single molecule levels during acute perturbations of its function. First, we showed that Dyngo4a, a cell permeable blocker of dynamin GTPase activity, or GTPγS dialyzed through a patch-clamp pipette, quickly blocked CME and led to the accumulation of dynamin-mCherry at CCPs. Partial block decreased the rate of dynamin recruitment before CCV formation, suggesting that GTPase activity regulates its recruitment at early stages of CCV formation. To avoid any effect of diffusion, we have performed photo-activation of DMNPE-GTPγS: this compound is inactive before UV illumination, but blocks endocytosis within 4 s while provoking dynamin accumulation on a longer timescale. We next investigated the parameters of dynamin recruitment to forming CCVs. FRAP analysis showed that dynamin exchange is fast and complete and was only moderately impaired by saturating concentrations of Dyngo4a, suggesting that dynamin exchanges with an extra-CCP pool at all times, including association in the oligomeric helix around the tubular neck.

To get better insight into the modes of dynamin recruitment at all stages of CCP maturation we conducted dual ppH/single protein tracking photo-activation localization microscopy (sptPALM) imaging in cells expressing dynamin-mEos3.2 (Figure 1). Dynamin is recruited to the plasma membrane, diffuses outside of CCPs and is trapped at CCPs, as revealed by the existence of a large number of trajectories outside of CCPs (Figure 1A) but its diffusion is slower within CCPs (Figure 1B). The number of detected molecules increases as scission approaches (Figure 1C) but single molecules are equally immobilized at all stages of CCP maturation. We conclude that dynamin exchanges with an extra-CCP pool at all times (Figure 1D): this would allow for its further recruitment by addition of new binding sites and its ability to narrow the vesicle neck after GTP hydrolysis [6].

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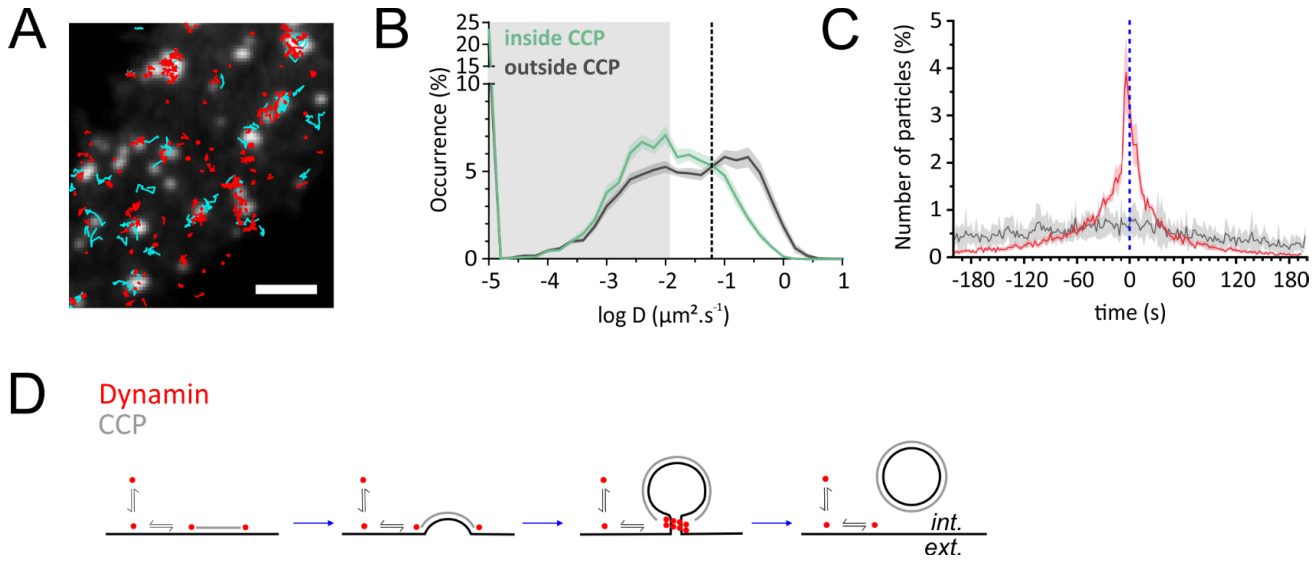

Figure 1: Imaging of single dynamin proteins at the surface of living cells. A. Portion of an NIH 3T3 cell transfected with TfR-SEP and dynamin1-mEOS3.2 and imaged with TIRF microscopy with time lapse at 0.25 Hz (for TfR-SEP) and sptPALM at 50 Hz (for dyn1-mEOS3.2). The image of CCPs labelled with TfR-SEP is overlaid with trajectories of single dynamin1-mEOS3.2 molecules. *Red lines* show trajectories of single molecules classified as immobile ($D < 10^{-2}$ µm².s^{-1}) and *cyan lines*, single molecules classified as diffusive ($D > 10^{-2}$ µm².s^{-1}). Scale bar 2 µm. **B**. Distribution of diffusion coefficients measured inside CCPs (*green curve*) and outside CCPs (*black curve*) for 31 cells (4 independent experiments). **C**. Normalized number of particles in CCPs relative to the time of scission measured with the ppH protocol. *Red*, real data (10 cells); *grey*, randomized data. **D.** Model of dynamin recruitment to CCPs at all stages of vesicle formation.

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