Supplemental Data

Dynasore, a Cell-Permeable Inhibitor of Dynamin

Eric Macia, Marcelo Ehrlich, Ramiro Massol, Emmanuel Boucrot, Christian Brunner, and Tomas Kirchhausen

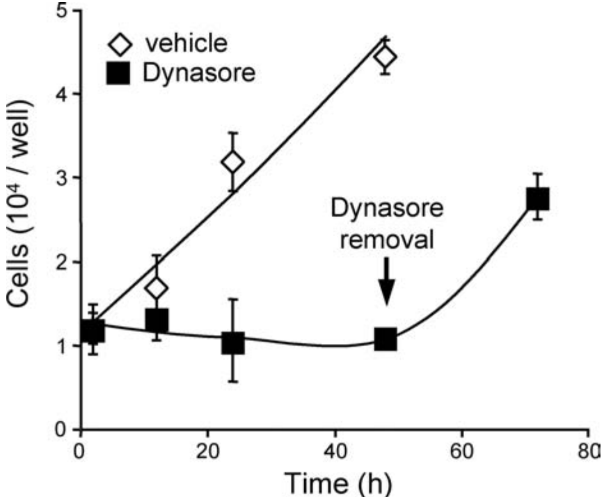

Figure S1. Extended Incubation of Cells with Dynasore Is Not Toxic and Prevents Cell Growth in a Reversible Manner

BSC1 cells were grown in a 6-well format at 37 °C in DMEM supplemented with 10% BD NuSerum (BD Biosciences) in the presence of 0.8% DMSO or 80 µM dynasore for the indicated times. Two days after continuous exposure to dynasore, a subset of the cells were transferred to fresh medium containing DMSO but not dynasore and incubated for another day. Results represent average of two independent experiments.

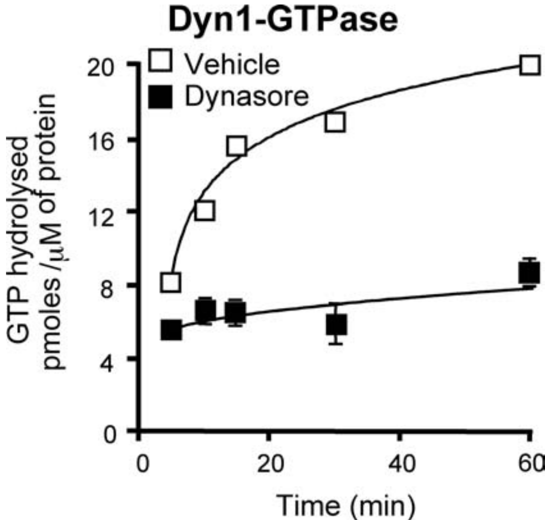

Figure S2. Effect of Dynasore on the GTPase Activity of Dynamin1 Determined at 37°C Using 2 μM GST-dyn2-GTPase Domain Purified from *E. coli*

The data points represent average +/- standard deviation from experiments performed in triplicate.

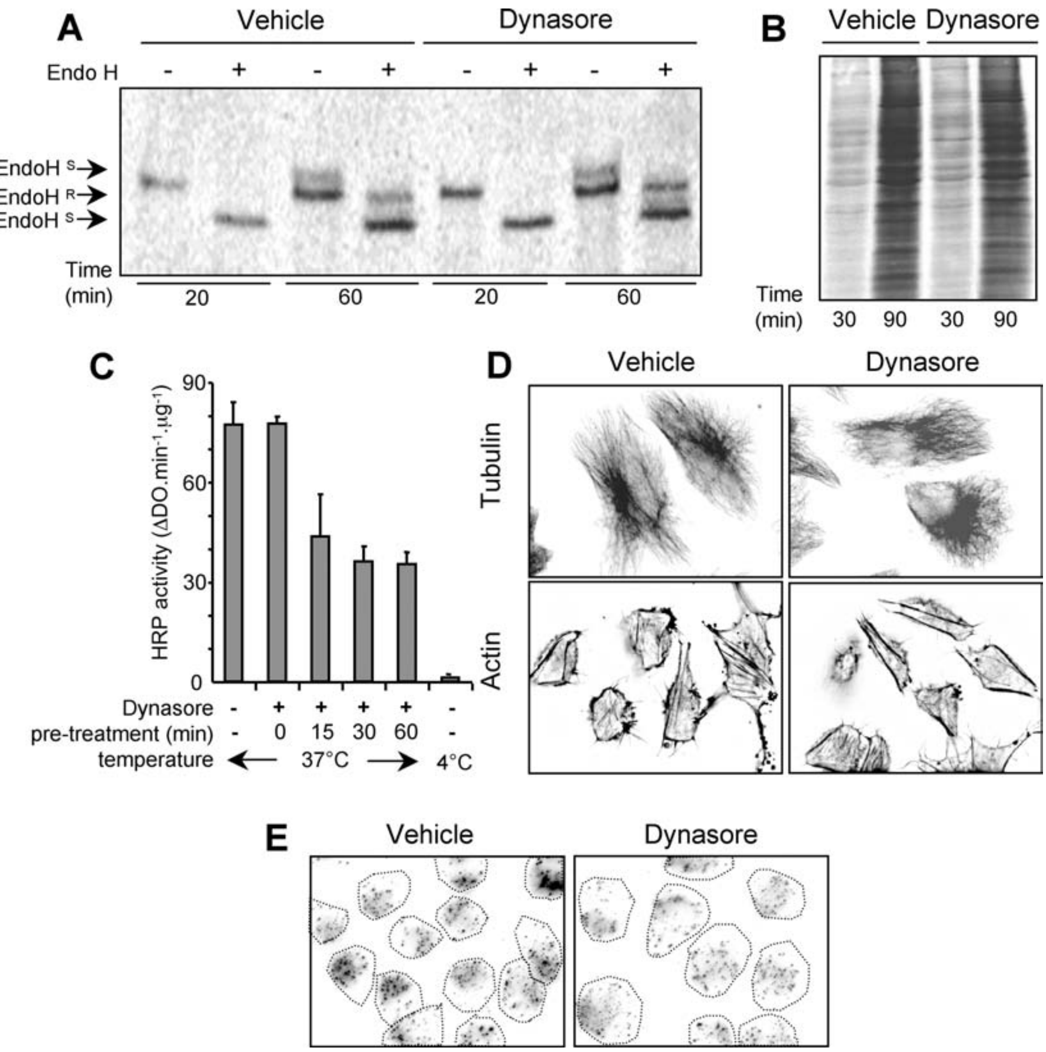

Figure S3. Dynasore Does Not Block Dynamin-Independent Processes

(A) Effect of dynasore in the biosynthesis and glycosylation of transferrin receptor. The extent of transferrin receptor synthesis was monitored at the indicated times in the presence or absence of 80 μM dynasore after brief exposure of BSC1 cells with S^{35} methionine followed by a chase period, immunoprecipitation and SDS-PAGE analysis (see Methods). TfR represents newly synthesized EndoH-sensitive transferrin receptor; the TfR* band represents receptor that reached the Golgi and undergo a further round of glycosylation. The cells were pre-treated for 30 min with vehicle or 80 μM dynasore. The experiment was done in duplicate.

(B) Effect of dynasore on overall protein synthesis. BSC1 cells were incubated in the presence or absence of 80 μM dynasore dissolved in media containing S^{35} methionine. Aliquots were obtained at the indicated times from total cell lysates, fractionated by SDS-PAGE and radioactive protein bands revealed by autoradiography. This gel is representative of two experiments.

(C) Effect of dynasore on the fluid phase uptake of horseradish peroxidase (HRP). BSC1 cells were pre-incubated at 37°C and for the indicated times with 80 μM dynasore or for 60 min with DMSO only (- dynasore) followed by the addition of HRP for 10 min (also at 37 °C). A cell sample was used as a negative control by keeping it 4°C during the incubation with HRP (complete block of fluid phase uptake). The data represents the average +/- standard deviation from experiments performed in triplicate.

(D) Effect of dynasore on the large-scale organization on the tubulin and actin cytoskeleton. BSC1 cells were incubated for 40 min with DMSO (vehicle) or 80 μM dynasore, followed by fixation and fluorescence visualization of tubulin with an α-tubulin antibody or of actin with phalloidin-rhodamin. The images represent experiments performed in triplicate.

(E) Effect of dynasore on the uptake of EGFR. Hela cells were starved in DMEM (with no serum) for 2 hr and then incubated with DMSO or 80 mM dynasore for 30 min. At this point EGF was added (100 ng/ml final) and incubated for another 10 min. Cells were transferred to 4 °C, fixed, permeabilized and stained with the monoclonal antibody 13A9 specific for EGFR. In the absence of EGF, most of the EGFR is located at the cell surface (not shown). Qunatitation of the fluorescence intensity in the (intracellular) spots indicates a reduction of about 50% in the uptake of EGFR.

The effect of dynasore on the biosynthesis of proteins was determined as follows: BSC1 cells were plated in 6 wells plates and kept overnight to achieve 50% confluency. Just before the experiment, the cells were washed three times with serum-free DMEM and incubated for 30 min at 37°C with DMEM containing 0.8% DMSO with or without 80 μM dynasore. A radioactive pulse was initiated by addition of 250 μCi of labeling mix containing L-[^{35}S]Methionine and L-[^{35}S]Cysteine (Redivue PRO-MIX; Amersham) for 30 min at 37°C. After a chase of 20 or 60 min, the wells were washed in PBS, and cells detached by incubation with with trypsin/EDTA for 5 min at 37°C. Released cells were concentrated at 4°C by centrifugation in a microfuge at 3000 rpm for 5 min and solubilized in PBS containing 0.1% Triton. Transferrin receptors were immunoprecipitated at 4 °C after incubation with a monoclonal antibody specific for the ectodomain of the transferrin receptor (gift of Dr. J. Cerny) and protein A and concentrated by centrifugation at 1500 rpm for 5 min. Samples were fractionated by SDS- 8% PAGE and radioactive bands revealed by 1 min exposure using a luminescent image analyzer (LAS-3000, Fuji Co). Total protein synthesis was followed in lysates of BSC1 cells continuously incubated for various time points with the same mixture of radioactive amino acids. The cells were washed at 4 °C for 3 times with 500 μl PBS, lysed with a solution containing PBS and 0.1% Triton and debris removed by centrifugation at 3000 rpm for 5 min and also at 4°C. Radioactive bands contained in 20 μl supernatant and corresponding to newly synthesized proteins were revealed by SDS-12% PAGE fractionation and imaging.

Fluid phase uptake of horse radish peroxidase (HRP) was determined using BSC1 monkey cells plated in a 6 well format for 16-18 hr to reach 50% confluency. The cells

were washed three times with serum-free DMEM followed by incubation with DMEM containing 0.8% DMSO in the presence or absence of 80 μM dynasore for 60 min at 37°C. Uptake was initiated by replacing with the same media containing 4 mg/ml HRP (Sigma Co.) for 10 min at 37°C. The uptake ended by removal of the media followed by 6 washes with PBS+ (PBS, 1mM $MgCl_2$, 1mM $CaCl_2$ pH 7.4) at 4°C. Cells were released from the plates by incubation with 0.25% trypsin/EDTA dissolved in PBS for 5 min at 37°C. The released cells were centrifuged at 3000 rpm for 5 min in a clinical centrifuge at 4°C and washed two more times with PBS+. The internalized HRP was released from the cells by incubation with 40 μl PBS containing 1% Triton and the enzymatic activity was determined using 10 μl of sample mixed with 90 μl of o-dianisidine as a substrate (0.34 mM o-dianisidine dissolved in $NaHPO_4$ pH 5.7, 0.003% H_2O_2, 0.3% Triton). Activity is expressed as the change in Absorbance (at 455 nm) per min and per μg protein. Protein concentration was determined using the BCA assay (Pierce Co).

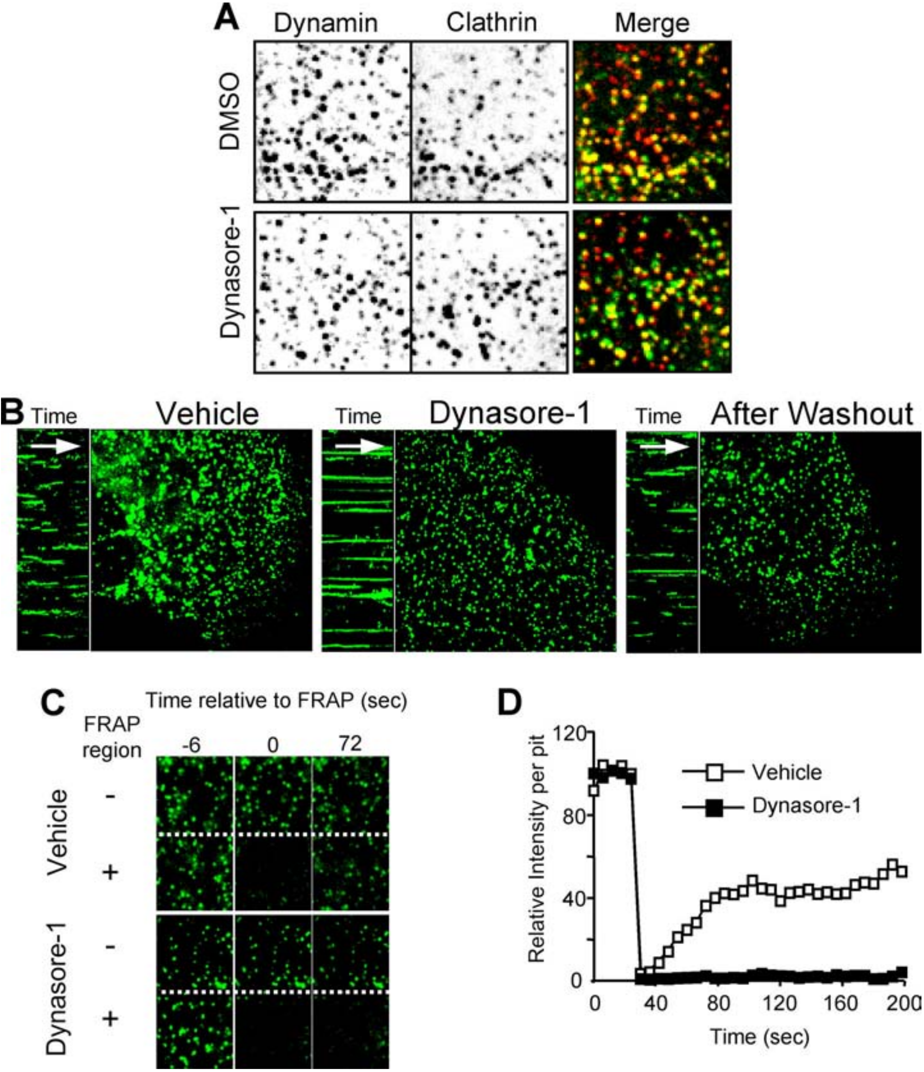

Figure S4. Dynasore Blocks the Assembly of Clathrin-Coated Pits

(A) Colocalization of Dynamin2 and clathrin containing pits in the presence and absence of dynasore. BSC1 cells stably expressing EGFP fused to LCa were used to label the clathrin coated structures; thee cells were treated with 0.8% DMSO (vehicle) or 80 μM dynasore (dynasore) at 37 °C for 30 min, fixed and stained with the antibody Hudy-2 (Upstate) specific for dynamin2 and then imaged by fluorescence microscopy. Many clathrin spots contain dynamin; a similar subset of dynamin spots colocalize with clathrin under both conditions.

(B) Live cell fluorescence imaging acquired from the bottom surface of BSC1 cells stably expressing EGFP-LCa. Time-series were collected for 6 min and at 37 °C in the presence or absence of dynasore from cells pre-incubated for 40 min with either DMSO (vehicle) or 80 μM dynasore (dynasore), or in the absence of dynasore from cells first incubated for 40 min with 80 μM dynasore and then for 20 min with DMSO (after washout). Each

still image corresponds to a frame acquired after 3 min of data collection while the kymographs represents the complete time-series obtained using the spinning disk confocal configuration (1 s exposures, acquired every 4 s). The data are representative of the experiment done in triplicate.

(C) Effect of dynasore on the fluorescence recovery after photobleaching (FRAP) of LCa-containing spots. BSC1 cells stably expressing EGFP-LCa were subjected to a FRAP protocol followed by immediate imaging of their bottom surface with the spinning disk confocal configuration. FRAP was done in cells exposed for 40 min to either DMSO (vehicle) or 80 μM dynasore (dynasore). The panels represent images obtained from areas of cells not subjected to bleaching (- FRAP region, negative controls) or from the photobleached area (+ FRAP region) acquired before (-6 s) or after (0.72 s) photobleaching.

(D) Plot of the integrated fluorescence intensity for all pits shown in C as a function of time. The data shows recovery of fluorescence intensity specific for the pits (e.g. corrected by the recovery from the surrounding background) and is representative of an experiment done in triplicate.

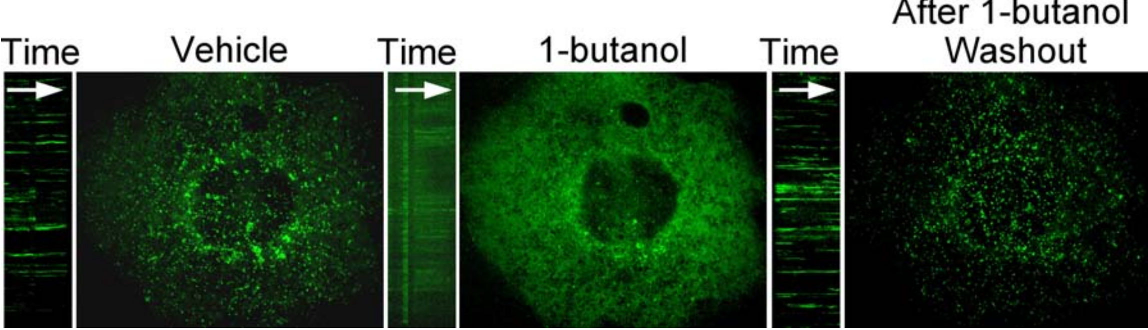

Figure S5. Effect of 1-Butanol in the Formation of Clathrin-Coated Structures

Live cell fluorescence imaging acquired from the bottom surface of BSC1 cells stably expressing EGFP-LCA. A control time-series was collected for 2min and at 37 °C in the absence of 1-butanol (medium only). A similar time series was then collected 1 min after addition of 1.2% 1-butanol (1-butanol). Finally, a third time series was collected 2min after removal of the 1-butanol (after 1-butanol washout). Each still image corresponds to a frame acquired after 3 min of data collection while the kymographs represents the complete time-series obtained using the spinning disk confocal configuration (1 s exposures, acquired every 4 s). The data are representative of the experiment done in triplicate.

		Coated pits %				Coated vesicles %
			U-shape	O-shape	O-shape	
Vehicle	(n=123)	10	7	2	0	82
Dynasore	(n=154)	0	13	14	12	60
Butanol/Dynasore	(n=178)	1	30	6	1	56

Figure S6. Quantification of Types of Coated Pits and Vesicles Observed in Fig. 6 (Vehicle, Dynasore) and Fig. 7E (Butanol/Dynasore)

Coated vesicles correspond to circular profiles removed 5 nm or more from the plasma membrane. O-shaped pits include circular profiles adjacent to the plasma membrane; presumably these structures represent a tangential section that missed the connecting neck between the pit and the plasma membrane. The number of pits (n) is indicated for each of the conditions analyzed.