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Supplementary Information for

CALM supports clathrin-coated vesicle completion upon membrane tension increase.

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Figures S1 to S7

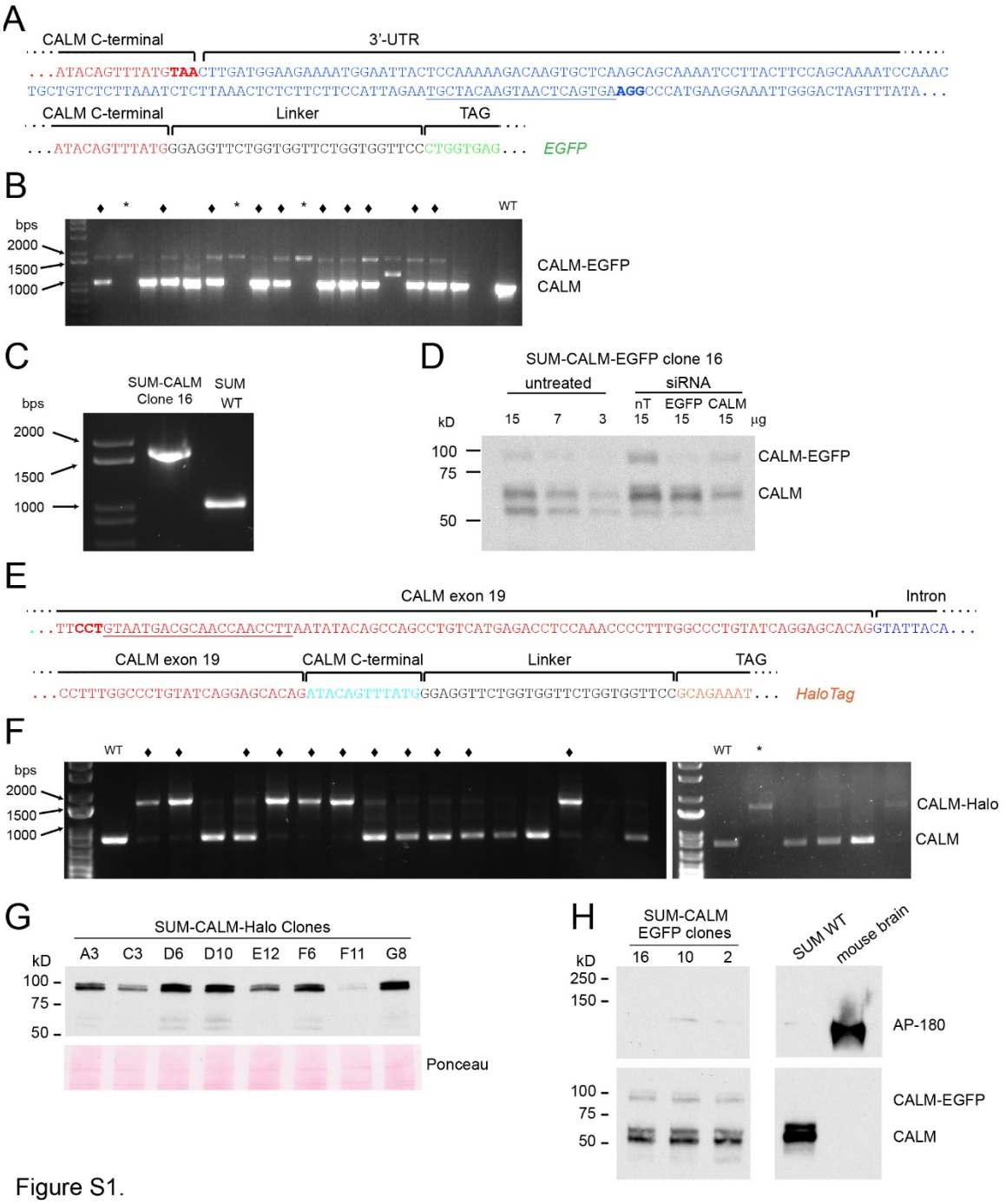


Figure S1.

Fig. S1. Gene editing strategies to express CALM-EGFP or CALM-Halo in SUM cells (Related to Figure 1).

(A) Scheme showing the CRISPR/Cas9 approach used to insert EGFP at the C-terminus of CALM. Top: original C-terminal region of CALM. The last exon of CALM is in red. In the 3' UTR region (blue), the underlined and the bold letters correspond to the Cas9 target sequence and the PAM

site, respectively. Bottom: modification obtained upon homologous recombination. EGFP (green) preceded by a linker (black) replaced the stop codon (top, bold red).

(B) Genomic PCR screening reveals EGFP integration into the CALM loci. Single (diamonds) and double edited clones (asterisks) were detected (exemple gel).

(C) Genomic PCR reveals the apparent integration in all CALM loci of EGFP in the SUM-CALM-EGFP clone 16. WT designates the parental cells, CALM the SUM-CALM clone.

(D) Western blot analysis of cell lysates from SUM-CALM-EGFP clone 16 untreated or transfected with non-targeting (nT), EGFP targeting (EGFP) or CALM targeting siRNAs. EGFP siRNA depleted CALM-EGFP protein without affecting the wild type isoform, while CALM siRNA reduces the expression of both protein species, suggesting that SUM-CALM-EGFP clone 16 cells are not completely edited despite SUM-CALM-EGFP appearing completely edited by genomic PCR in B.

(E) Scheme showing the CRISPR/Cas9 approach used to insert HaloTag at the second-last CALM exon (exon 19). The advantage of this approach, when compared to the one described in (A), was that we were able to design efficient Cas9 guides to induce double strand breaks less than 75 bps from the insertion site, which is known to increase the efficiency of homologous recombination [Ran FA, et al. 2013 Nat Protoc 8(11):2281–308]. Top: C-terminal region of CALM exon 19 (red), and initial portion of the subsequent intron (blue). The underlined and the bold letters correspond to the Cas9 target sequence and the PAM site, respectively. Bottom: modification obtained upon homologous recombination. Exon 19 (red) is followed by the coding sequence of the last CALM exon without the stop codon (cyan), a linker (black), and HaloTag (orange).

(F) Genomic PCR reveals HaloTag integration into the CALM loci. Single edited clones (diamonds) and completely edited clones (asterisks) were detected (example gels).

(G) Western blot analysis of cell lysates from SUM-CALM-Halo clones shows the complete substitution of CALM for CALM-Halo. The same nitrocellulose membrane stained with Ponceau is shown as a loading control.

(H) Western blot analysis of cell lysates from SUM cells and mouse brain shows that traces of AP180, the homologous neuronal form of CALM, are present in SUM cells.

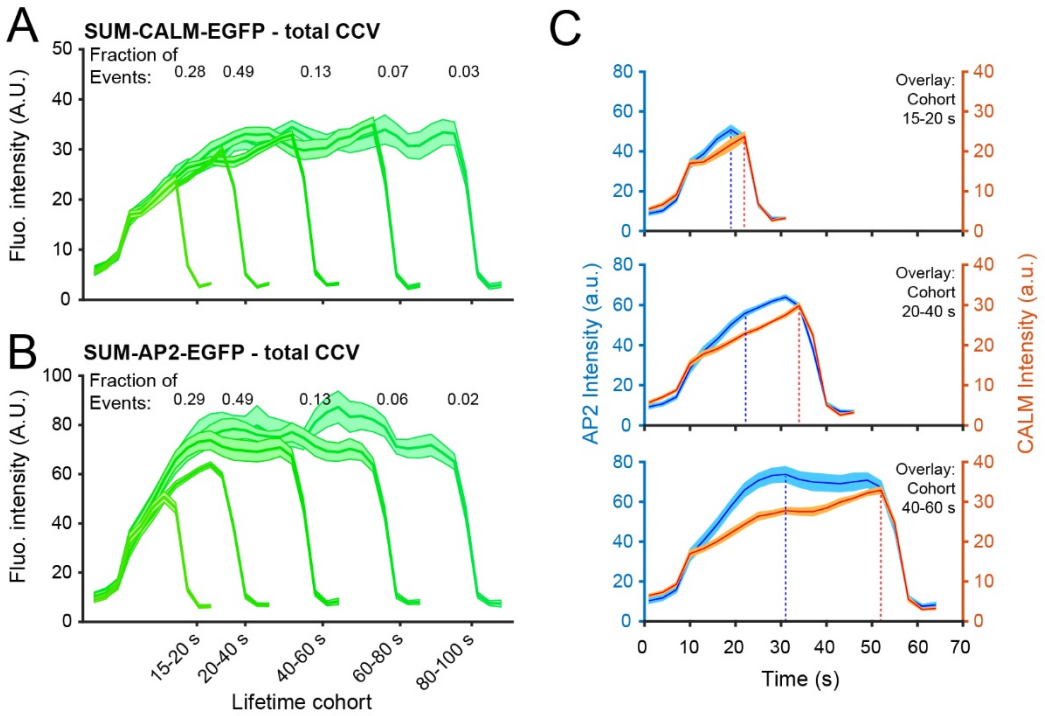


Figure S2.

Fig. S2. The recruitment of CALM and AP2 follows different dynamics (Related to Figure 2).

(A-B) Fluorescence intensity traces of CCVs organized in lifetime cohorts. Intensities were calculated averaging data obtained from seven SUM-CALM cells (2089 CCVs, A) and nine SUM-AP2-EGFP cells (4456 CCVs, B).

(C) The first three CCV cohorts, which correspond to 90% of the total events, obtained from SUM-AP2-EGFP cells (blue) were overlaid with the respective traces obtained from SUM-CALM cells (orange). Notice that AP2 reached the maximum intensity before CALM and tended to plateau. In contrast CALM recruitment was steadier until the end of the CCV.

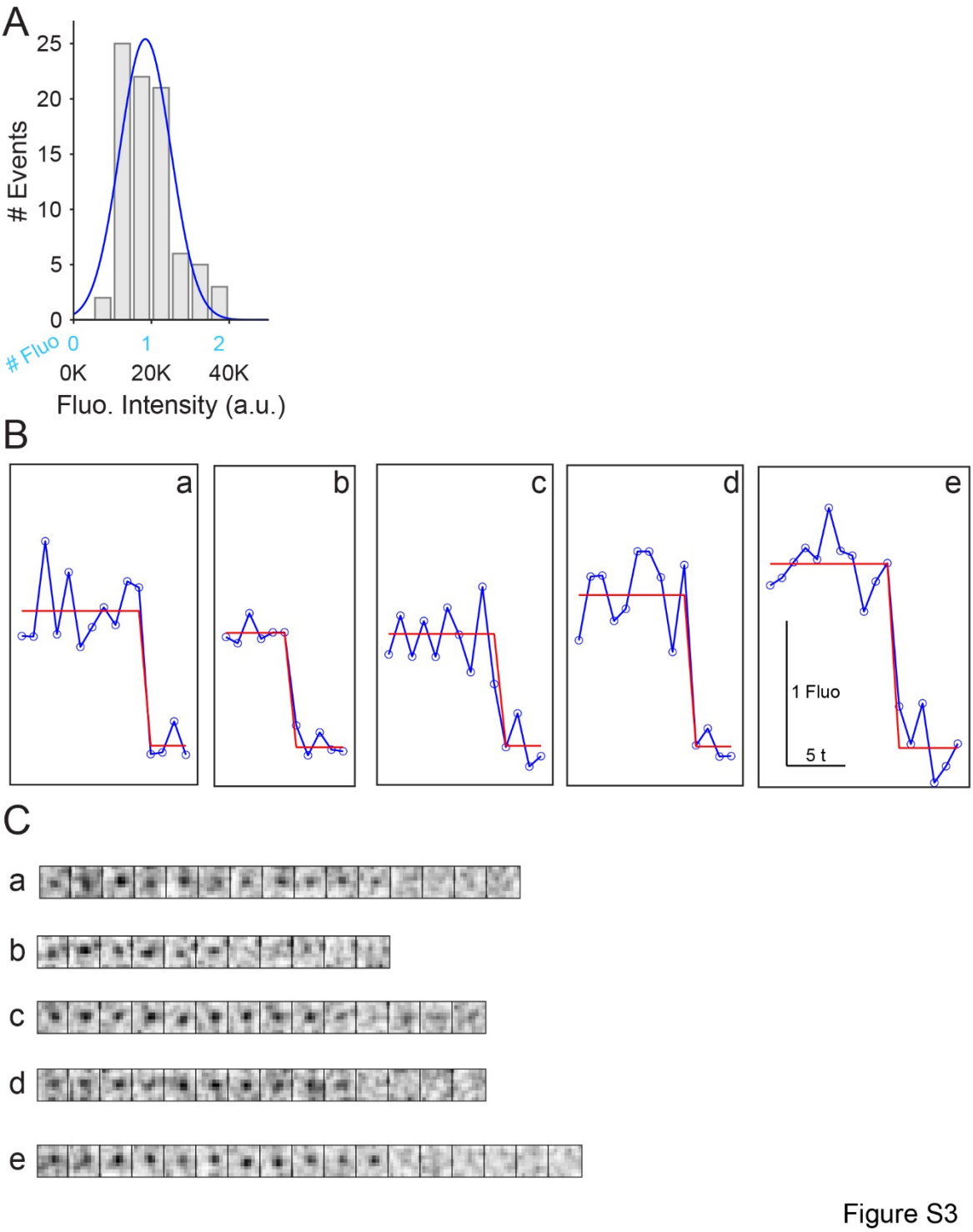


Figure S3

Fig. S3. Single molecule intensity calibration of EGFP (related to Figure 3).

EGFP molecules were adsorbed on functionalized coverslips and imaged. An exposure time of 4 s was sufficient to detect single molecules.

(A) Distribution of intensity changes of single bleaching steps, from 84 diffraction limited spots. The fit is a normal distribution centered at 18427±6572 (mean±SD).

(B) Single molecule photobleaching traces of EGFPs. The background corrected fluorescence intensities are plotted in blue, while the corresponding fitted step are shown in red. The vertical bar in **e** indicate the estimated intensity of a single molecule (1 Fluo.) obtained from A and the horizontal bar five consecutive time points (5 t).

(C) Sequential images corresponding to the traces in B. Images of 9x9 pixels containing the respective single detected molecule were extrapolated from the time series. A Gaussian filter (0.7 pixel) was applied to decrease the noise. The images were subsequently tiled in a filmstrip. Time flows from left to right.

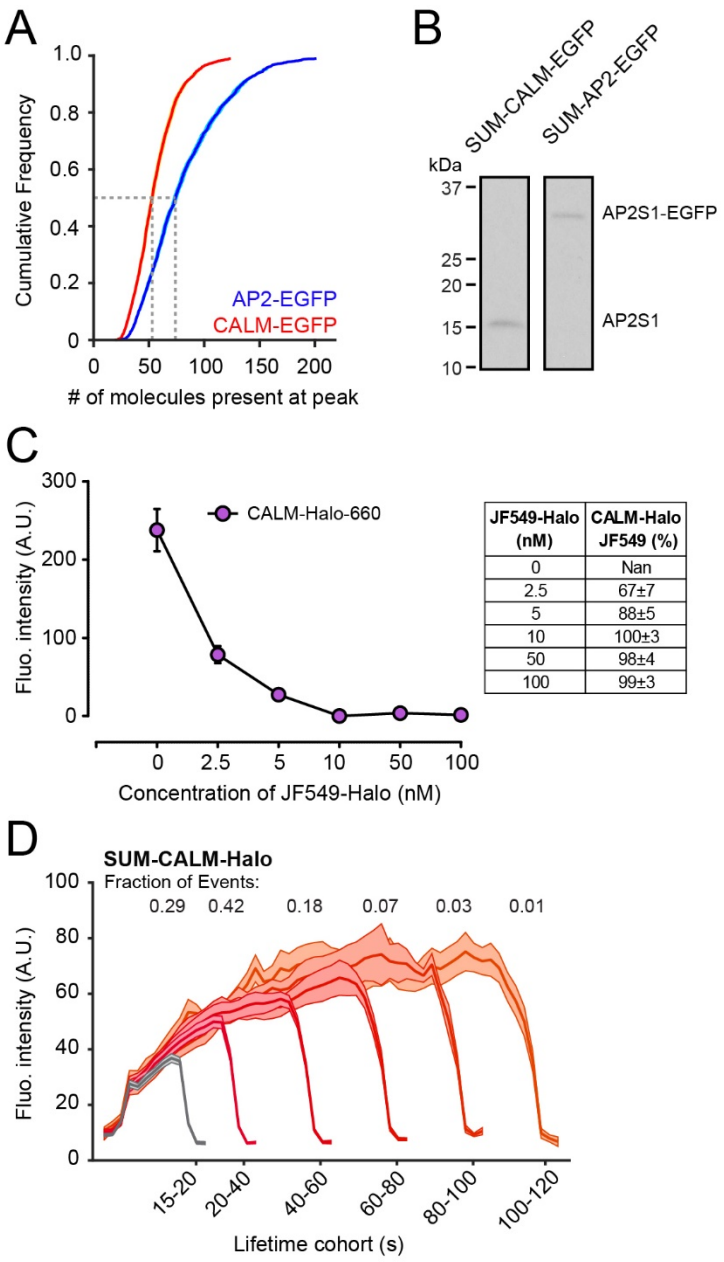


Figure S4.

Fig. S4. The maximum number of AP2 or CALM molecules recruited during the formation of CCVs (related to Figure 3).

(A) Frequency distribution of the maximum number of AP2 (blue) or CALM (red) fluorescent molecules identified per CCV in SUM-AP2-EGFP and SUM-CALM-EGFP, respectively. The dotted lines mark the median number of fluorescent AP2 (n = 74) or CALM (n = 53) adaptors recruited per CCV.

(B) Western blot analysis shows the complete substitution of AP2S1 by the AP2S1-EGFP chimera in SUM-AP2-EGFP cells.

(C) Competition assay to estimate the binding efficiency of JF549-Halo to CALM-Halo in living cells. The plot shows the fluorescence intensity of SUM-CALM-Halo cells labelled by AlexaFluor660-Halo (mean ± SEM) after exposure to increasing concentrations of JF549-Halo. The table on the right indicates the extrapolated fraction of CALM-Halo labelled with JF549-Halo expressed in percentage (mean ± SEM).

(D) Fluorescence intensity traces of CCVs detected in SUM-CALM-Halo cells organized in lifetime cohorts. The traces were calculated averaging data obtained from ten cells (2603 CCVs), and are shown as mean ± SEM; the relative frequency of each lifetime cohort is reported on the top of the chart (Fraction of Events). The 15-20 s cohort is color coded in gray. The datasets were composed of 150 z stacks, composed of 61 consecutive images acquired every 300 nm resulting in a total imaged volume of 50x50x18.3 μm acquired every 2.2 s. Each single plane was imaged for 35 ms using 500mW laser power and AOTF 1.

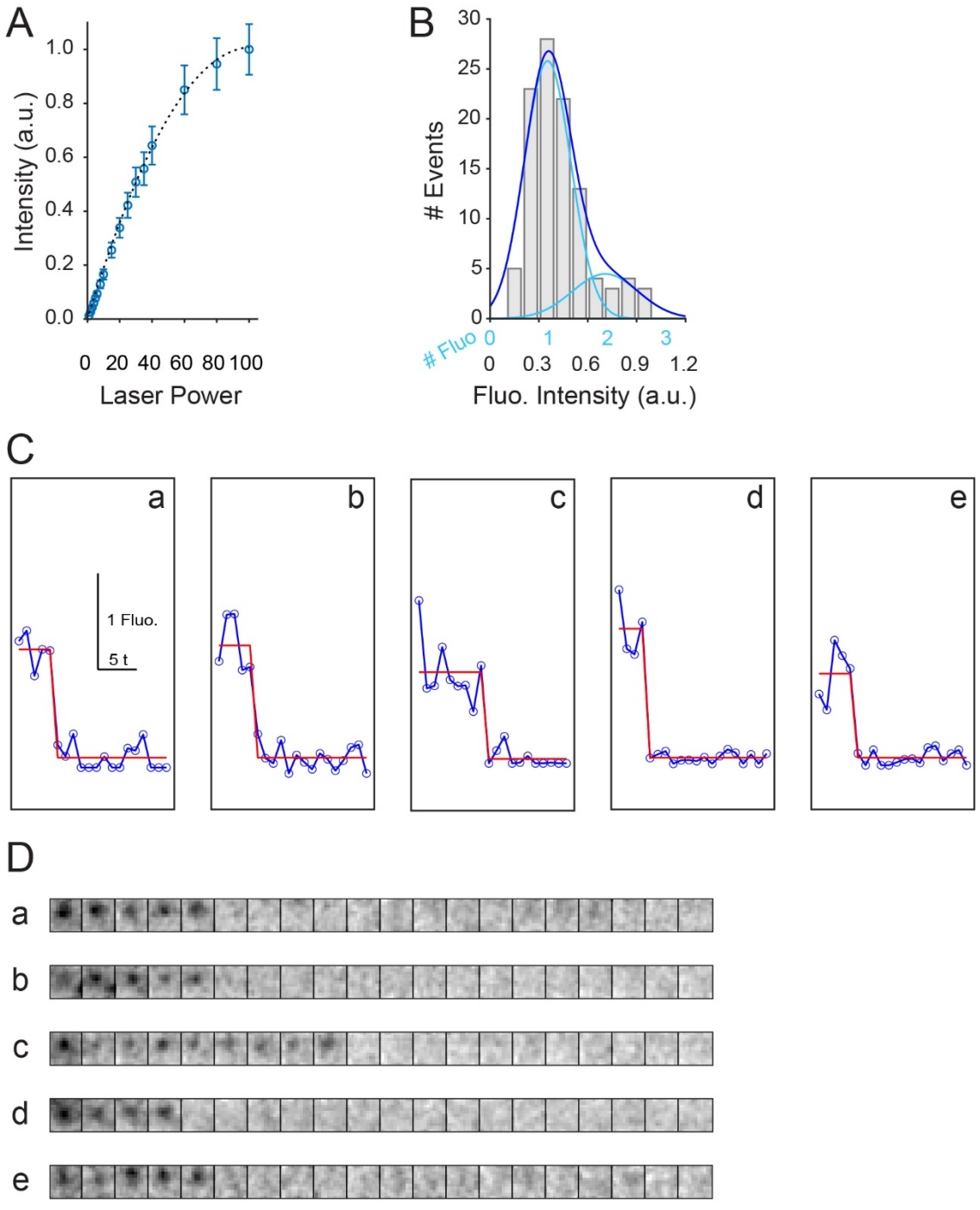


Figure S5

Fig. S5. Single molecule intensity calibration of CALM-Halo labelled with JF549 (related to Figure 3).

SUM-CALM-Halo cells were labelled with 5 nM of JF549 for 1 hr, and subsequently washed and lysed. The lysate was spread on 5 mm coverslips functionalized to promote protein absorption. 20

z-stacks of 61 sequential optical sections were acquired with 35 ms exposure and spaced 300 nm apart along the detection axis (resulting in a total volume of 50x50x18.3μm).

(A) Acousto-optic tunable filter calibration. Empirical determination of laser intensity at the sample in function of acousto-optic tunable filter (AOTF) excitation. Single molecules were imaged with AOTF=100 while cells were imaged with AOTF=1. Blue circles indicate the measured values (mean±SD), the black dotted line the fitting curve.

(B) Distribution of intensity changes of single bleaching steps, from 105 diffraction limited spots. The fit is a combination of two normal distributions. The most prominent (80% of the data) corresponds to the contribution of a single CALM-Halo and is centered at 0.35±0.14 (mean±SD). The intensities are normalized and plotted for an AOTF=1.

(C) Single molecule photobleaching traces of CALM-Halo labelled with JF549. The background corrected fluorescence intensities are plotted in blue while the corresponding fitted steps are shown in red. The vertical bar in **a** indicate the estimated intensity of a single molecule (1 Fluo.) obtained from A and the horizontal bar five consecutive time points (5 t).

(D) Sequential images corresponding to the traces in B. Volumes of 5x5x3 pixels containing the single detected molecule were extrapolated from the respective time series, and projected along the z axis. A Gaussian filter (0.7 pixel) was applied to decrease the noise. The images were subsequently tiled in a filmstrip. Time flows from left to right.

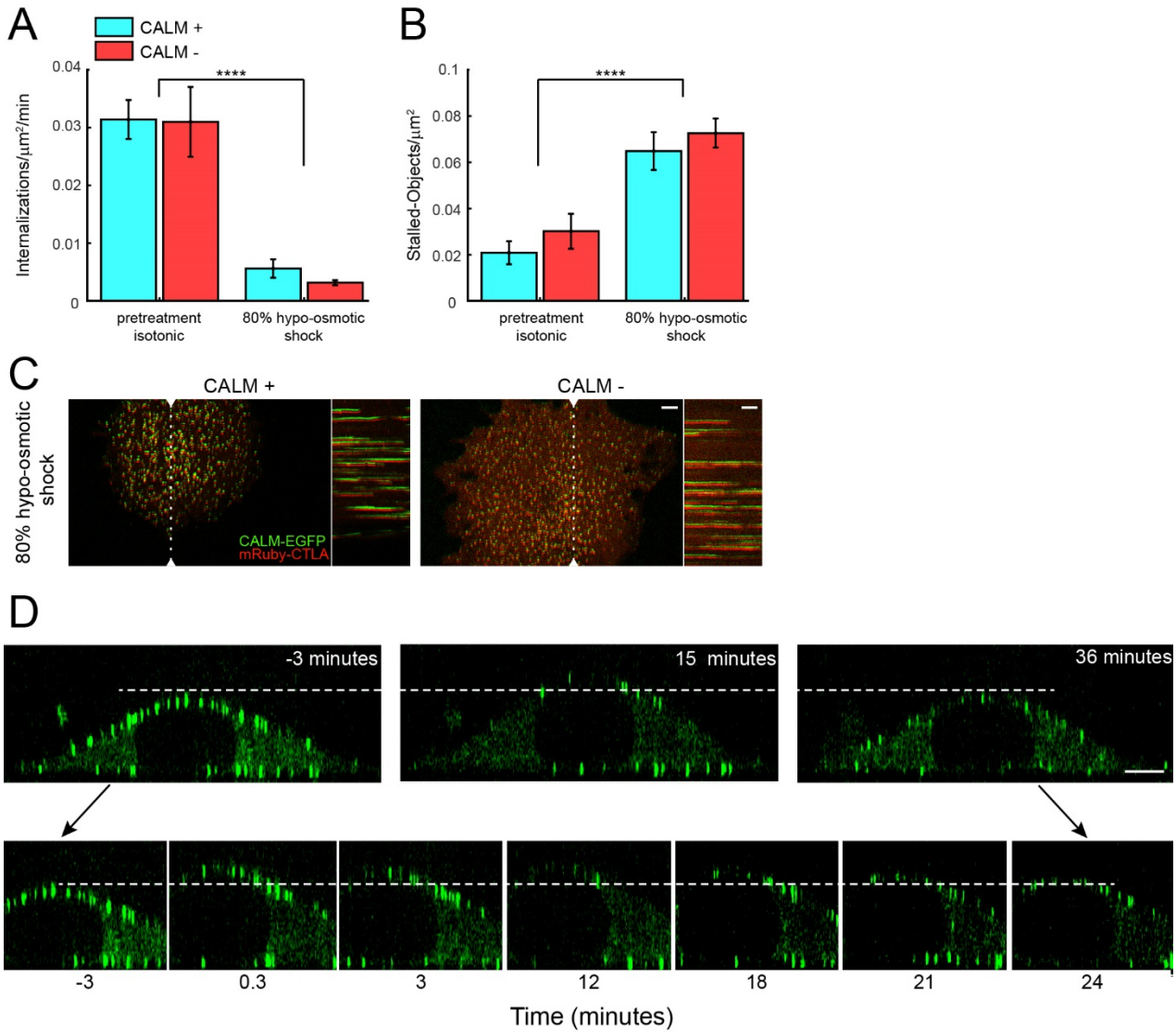


Figure S6

Fig. S6. Effect of hypo osmotic shock on SUM cells (related to Figure 4).

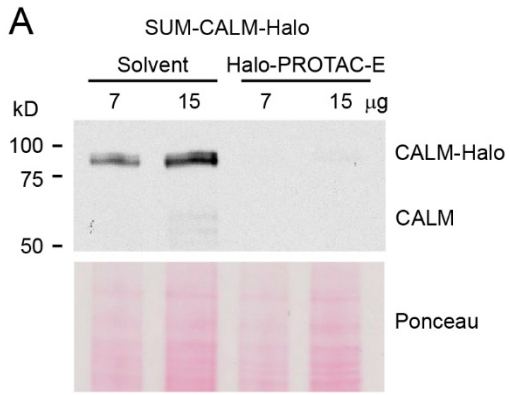
(A-C) Analysis of CCV formation at the ventral cell surface (median ± SEM) of SUM-CALM cells depleted (CALM-, red) or not (CALM+, cyan) for CALM and transiently transfected to express mRuby-CLTB in isotonic conditions or after exposure to 80% hypo osmotic shock for 3 min or 10 min. The data was obtained by analyzing 15 CALM+ and 13 CALM- cells in isotonic conditions; 25 CALM+ and 17 CALM- cells were analyzed in the 80% hypotonic condition. P-values were calculated by Student's t-test.

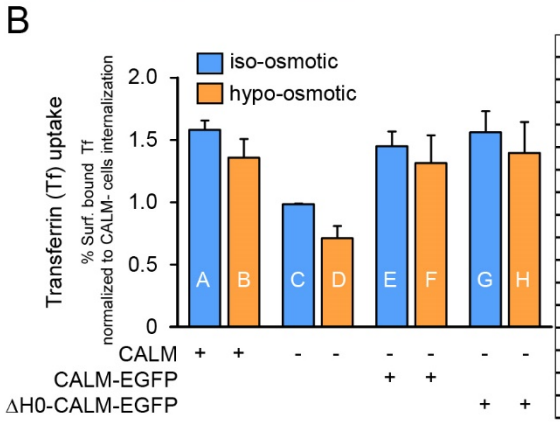
(A) Differences in the number of internalizations per micron meter squared per min were statistically significant when CALM+ or CALM- cells exposed to 80%-hypo were compared with their respective control (CALM+: $p = 9.2*10^{-8}$; CALM-: $p = 4.6*10^{-5}$). No significant differences were measured when the comparison was made in the same treatment group, suggesting that CCV dynamics were similarly affected upon exposure to 80% of hypo-osmotic shock, regardless of the different expression of CALM. Legend in A is also valid for B.

(B) Frequency of stalled objects (median ± SEM) in gene edited SUM-CALM cells depleted (CALM-, red) or not (CALM+, cyan) for CALM and transiently transfected for mRuby-CLTB detected in isotonic condition or upon exposure to 80%-hypo. Differences in the number of stalled objects were statistically significant when CALM+ or CALM- cells exposed to 80%-hypo were compared with their respective control (CALM+: pval = $4.7*10^{-4}$; CALM-: pval = $1.6*10^{-5}$). No significant differences were measured when the comparison was made in the same treatment group, suggesting that CCV dynamics were similarly affected upon exposure to 80% of hypo-osmotic shock, regardless of the different expression of CALM.

(C) Representative single frames and kymographs acquired by confocal spinning disk at the ventral surface of CALM+ and CALM- cells exposed to 80% osmotic shock. Notice the increased number of stalled objects in control (CALM+) and CALM depleted (CALM-) cells. The bar corresponds to 5μm, the arrow to 1 min and indicates the time flow of the kymograph. The dotted line indicates the plane at which we generated the kymographs.

(D) To verify the time needed for the cells to recover after swelling, we imaged SUM-AP2 cells every 3 minutes using spinning disc confocal microscopy using 0.3 μm step size in the axial dimension. Cells bathed in isotonic medium (-3 min) were exposed to 66%-Hypotonic solution at time 0. Cells were imaged every 3 min using spinning disc confocal microscopy using 0.3 μm step size in the axial dimension for 36 min. The top panel shows three entire projections while the lower panels cropped images to compare the time course. The dashed line represents the height of the cell prior to exposure to hypotonic shock. Notice that the recovery that is achieved ~24 min after exposure to hypotonic shock. The bar represents 5 μm on the x dimension.





Tested samples		p value	sig.	Diff (%)
A	B	0.23	ns	13
A	C	0.0002	***	38
A	E	0.38	ns	8
A	G	0.92	ns	1
B	D	0.011	*	48
B	F	0.87	ns	3
B	H	0.9	ns	3
C	D	0.033	*	28
C	E	0.0081	**	48
C	G	0.0148	*	60
D	F	0.0488	*	86
D	H	0.0424	*	97
E	F	0.61	ns	10
G	H	0.6	ns	10
E	G	0.6	ns	8
F	H	0.81	ns	6

Figure S7

Fig. S7. Complete CALM depletion affects transferrin endocytosis in isotonic and hypotonic condition (Related to Fig. 4).

(A) Immunoblotting of cell lysates with a specific anti-CALM antibody shows the complete depletion of CALM in SUM-CALM-Halo cells treated with Halo-PROTAC-E (500 nM) for 24 hr. Solvent indicates cells treated for 24 hours with DMSO. The same nitrocellulose membrane stained with Ponceau is shown as a loading control.

(B) A flow cytometry-based assay was performed to assess the role of CALM on transferrin endocytosis. SUM-CALM-Halo cells (CALM+) were transfected with plasmids to express CALM-EGFP or ΔH0–CALM-EGFP, and incubated for 24 hours with the solvent (DMSO) or Halo-PROTAC-E (500 nM) to completely deplete CALM (CALM-).

Cells were then exposed to fluorescent transferrin (Tf) dissolved in either iso- or hypo-osmotic solution for 10 min at 37ºC, then washed with an acidic solution to remove the surface bound Tf. Another set of samples were incubated with transferrin for 10 min at 4ºC to estimate the amount of transferrin receptor present on the cell surface.

The data presented in the plot (four independent experiments, mean ± SEM) are normalized over the respective amount of surface bound Tf and to the amount of internalized Tf by SUM-CALM-Halo cells completely depleted of CALM in isotonic solution. Transferrin internalization significantly decreased in CALM depleted cell in isotonic condition (compare A with C). Exposure of CALM depleted cells to hypo osmotic shock further and significantly reduced Tf uptake in comparison to CALM depleted cells exposed to the iso-osmotic condition (compare C with D). The re-expression of CALM (CALM-EGFP+) restored the uptake in iso-osmotic (compare A with E and C with E) and

hypo-osmotic conditions (compare B with F and D with F). Expression of ΔH0-CALM-EGFP+, recovered the phenotype to the same extend as the wild type form of CALM since no difference was detectable when compared to CALM-EGFP+ cells (both E-F and G-H tests resulted non-significant).

The conditions were compared using an unpaired Student's t-test. A table with the obtained p values, relative level of significance (sig.), and the absolute difference between the paired samples expressed as percentage (Diff. %) is plotted on the right.