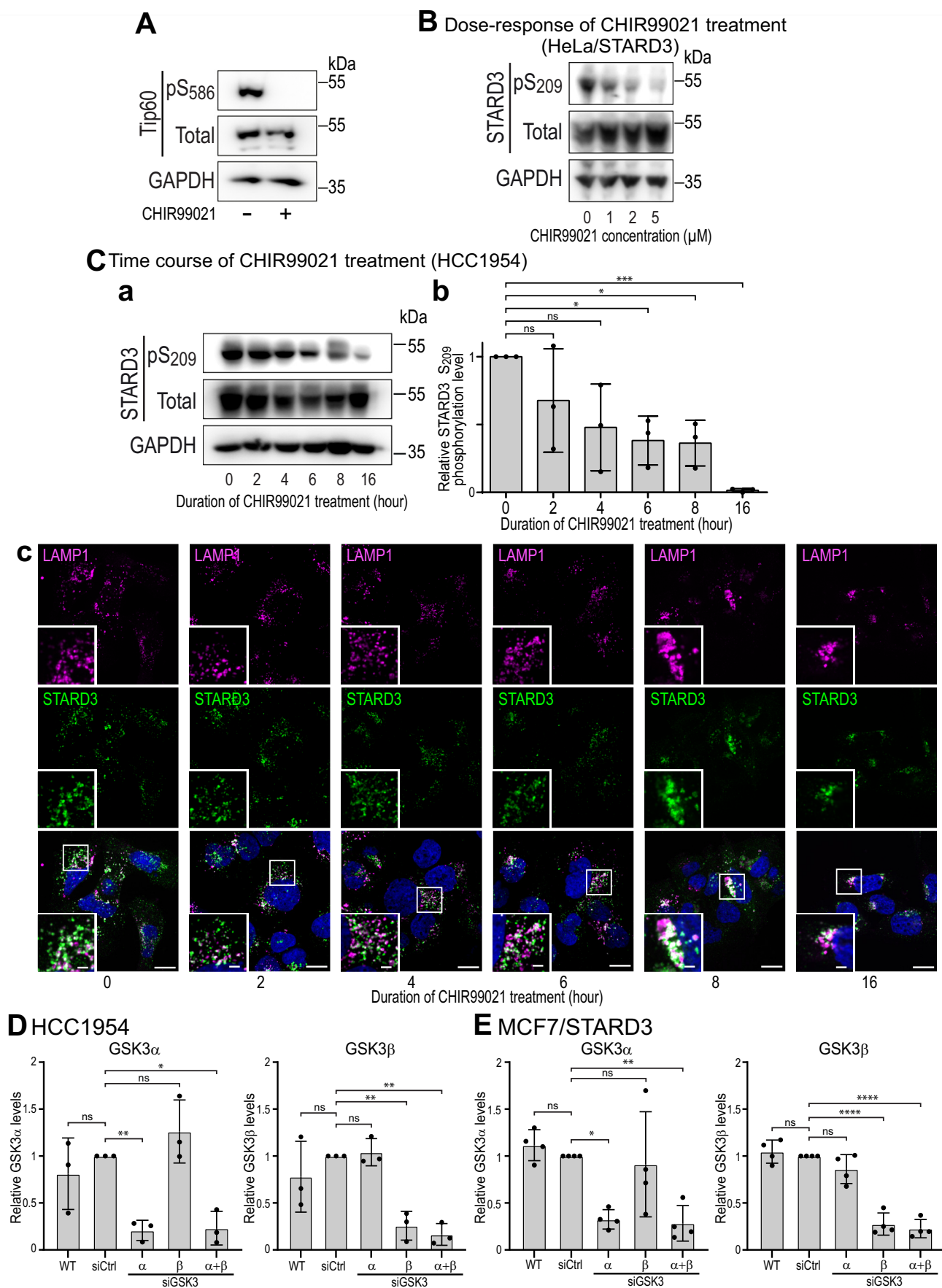


Expanded View Figures

Figure EV1. Dose and time-dependent effects of GSK3 inhibition on STARD3 phosphorylation.

(A) Western blot analysis of MCF7 cells overexpressing TIP60 treated or not with the GSK3 inhibitor CHIR99021 (5 μ M; overnight). TIP60 protein levels (Total) and S₅₈₆ phosphorylation (pS₅₈₆) were analyzed. (B) Western blot analysis of HeLa/STARD3 cells treated overnight with different concentration of CHIR99021 (1, 2 and 5 μ M) or left untreated. STARD3 protein levels (Total) and S₂₀₉ phosphorylation (pS₂₀₉) were analyzed. (C) (a) Western blot analysis of HCC1954 cells treated with 5 μ M of CHIR99021 for 2, 4, 6, 8 and 16 h, or left untreated. STARD3 protein levels (Total) and S₂₀₉ phosphorylation (pS₂₀₉) were analyzed. (b) Quantification of relative S₂₀₉ phosphorylation levels. Means $\pm$ SD. One-way ANOVA with Dunnett's multiple comparison test (*, $P < 0.05$; ***, $P < 0.001$; $n = 3$ independent experiments; 0 h vs 2 h, $P = 0.33$; 0 h vs 4 h, $P = 0.06$; 0 h vs 6 h, $P = 2.3 \times 10^{-2}$; 0 h vs 8 h, $P = 1.9 \times 10^{-2}$; 0 h vs 16 h, $P = 8 \times 10^{-4}$). (c) Representative images of HCC1954 cells expressing WT STARD3 and treated with the GSK3 inhibitor CHIR99021 for 2, 4, 6, 8 and 16 h, or left untreated. Cells were labeled with an anti-LAMP1 antibody (magenta), an anti-STARD3 antibody (green) and Hoechst for nuclei (blue). (D, E) Quantification of relative GSK3 α and GSK3 β in HCC1954 (D) and MCF7/STARD3 (E) cells transfected with control siRNAs (siCtrl) or siRNAs targeting GSK3 α (siGSK3 α), GSK3 β (siGSK3 β), or both (siGSK3 α + siGSK3 β) (see Fig. 1E,F). Means $\pm$ SD. One-way ANOVA with Dunnett's multiple comparison test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, $n = 3$ –4 independent experiments; D, GSK3 α : siCtrl vs WT, $P = 0.76$; GSK3 α , $P = 9 \times 10^{-3}$; siCtrl vs siGSK3 β , $P = 0.52$; siCtrl vs siGSK3 α + siGSK3 β , $P = 1.1 \times 10^{-2}$; (D), GSK3 β : siCtrl vs WT, $P = 0.5$; GSK3 α , $P = 0.99$; siCtrl vs siGSK3 β , $P = 3.6 \times 10^{-3}$; siCtrl vs siGSK3 α + siGSK3 β , $P = 1.6 \times 10^{-3}$; (E), GSK3 α : siCtrl vs WT, $P = 0.94$; GSK3 α , $P = 1.3 \times 10^{-2}$; siCtrl vs siGSK3 β , $P = 0.97$; siCtrl vs siGSK3 α + siGSK3 β , $P = 8 \times 10^{-3}$; (E), GSK3 β : siCtrl vs WT, $P = 0.93$; GSK3 α , $P = 0.28$; siCtrl vs siGSK3 β , $P < 10^{-4}$; siCtrl vs siGSK3 α + siGSK3 β , $P < 10^{-4}$). Source data are available online for this figure.



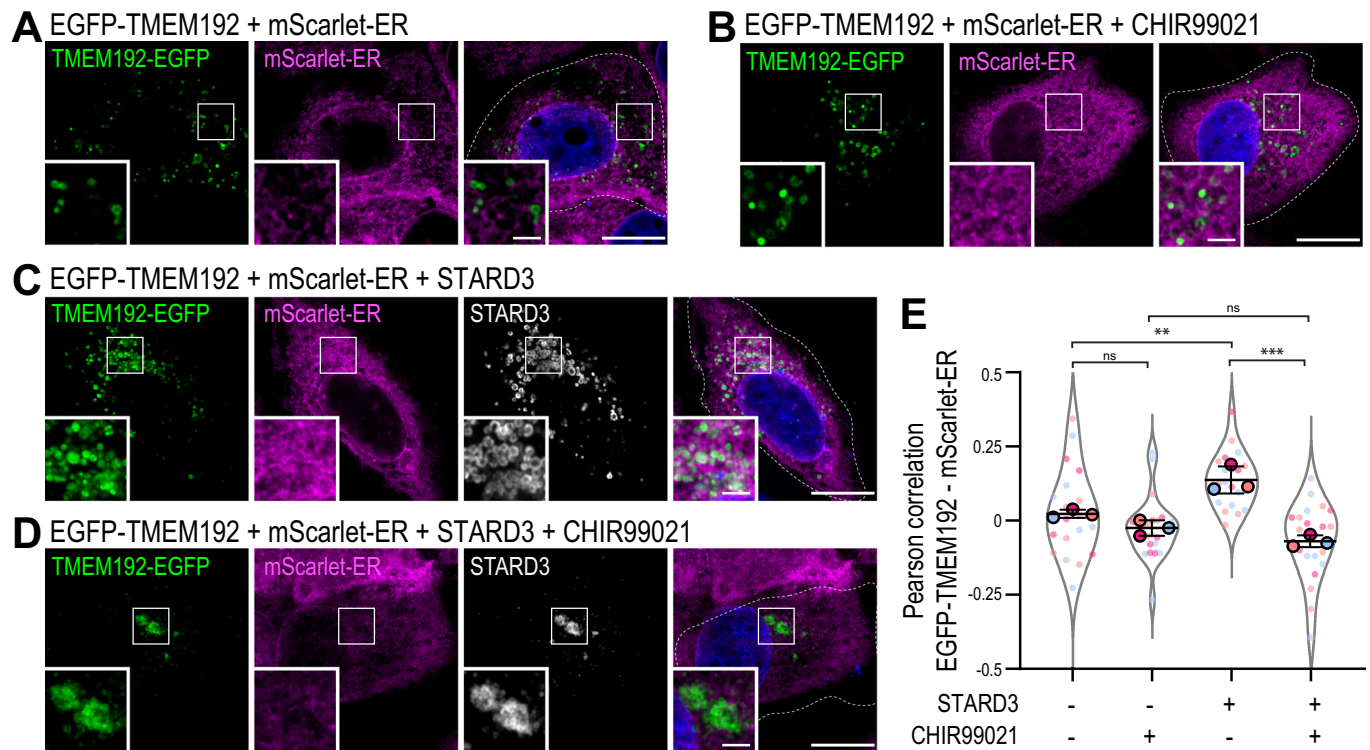


Figure EV2. In vivo, GSK3 activity governs the establishment of STARD3-mediated ER-endosome contacts.

(A–D) MCF7 cells expressing mScarlet-ER (magenta) were transfected with EGFP-TMEM192 (green) (A–D) and STARD3 WT (C, D). Cells were left untreated (A, C) or treated with CHIR99021 (B, D). STARD3 was labeled using anti-STARD3 antibodies (gray), and nuclei were stained with Hoechst (blue). Insets show higher magnification images of the areas outlined in white. Scale bars: 10 μ m. Inset scale bars: 2 μ m. Overlay panels show merged green, magenta and blue channels. In (A, B), endogenous STARD3 levels were too low to be detected with anti-STARD3 antibodies. (E) Pearson's correlation coefficients between EGFP-TMEM192 and mScarlet-ER in cells with or without STARD3 expression and with or without CHIR99021 treatment. Data are displayed as Superplots showing the clustering index per cell (small dots) and its mean per independent experiment (large dots). Number of cells: mScarlet-ER / EGFP-TMEM192: 21; mScarlet-ER / EGFP-TMEM192 treated with CHIR99021: 22; mScarlet-ER / EGFP-TMEM192 / STARD3: 22; mScarlet-ER / EGFP-TMEM192 / STARD3 treated with CHIR99021: 22, from three independent experiments. Means and error bars (SD) are shown. ANOVA with Tukey's multiple comparison test (**, $P < 0.01$; ***, $P < 0.001$; mScarlet-ER / EGFP-TMEM192 vs mScarlet-ER / EGFP-TMEM192-CHIR99021: $P = 0.26$; mScarlet-ER / EGFP-TMEM192 vs mScarlet-ER / EGFP-TMEM192 / STARD3: $P = 6 \times 10^{-3}$; mScarlet-ER / EGFP-TMEM192-CHIR99021 vs mScarlet-ER / EGFP-TMEM192 / STARD3-CHIR99021: $P = 0.31$; mScarlet-ER / EGFP-TMEM192 / STARD3 vs mScarlet-ER / EGFP-TMEM192 / STARD3-CHIR99021: $P = 1.9 \times 10^{-4}$). Source data are available online for this figure.

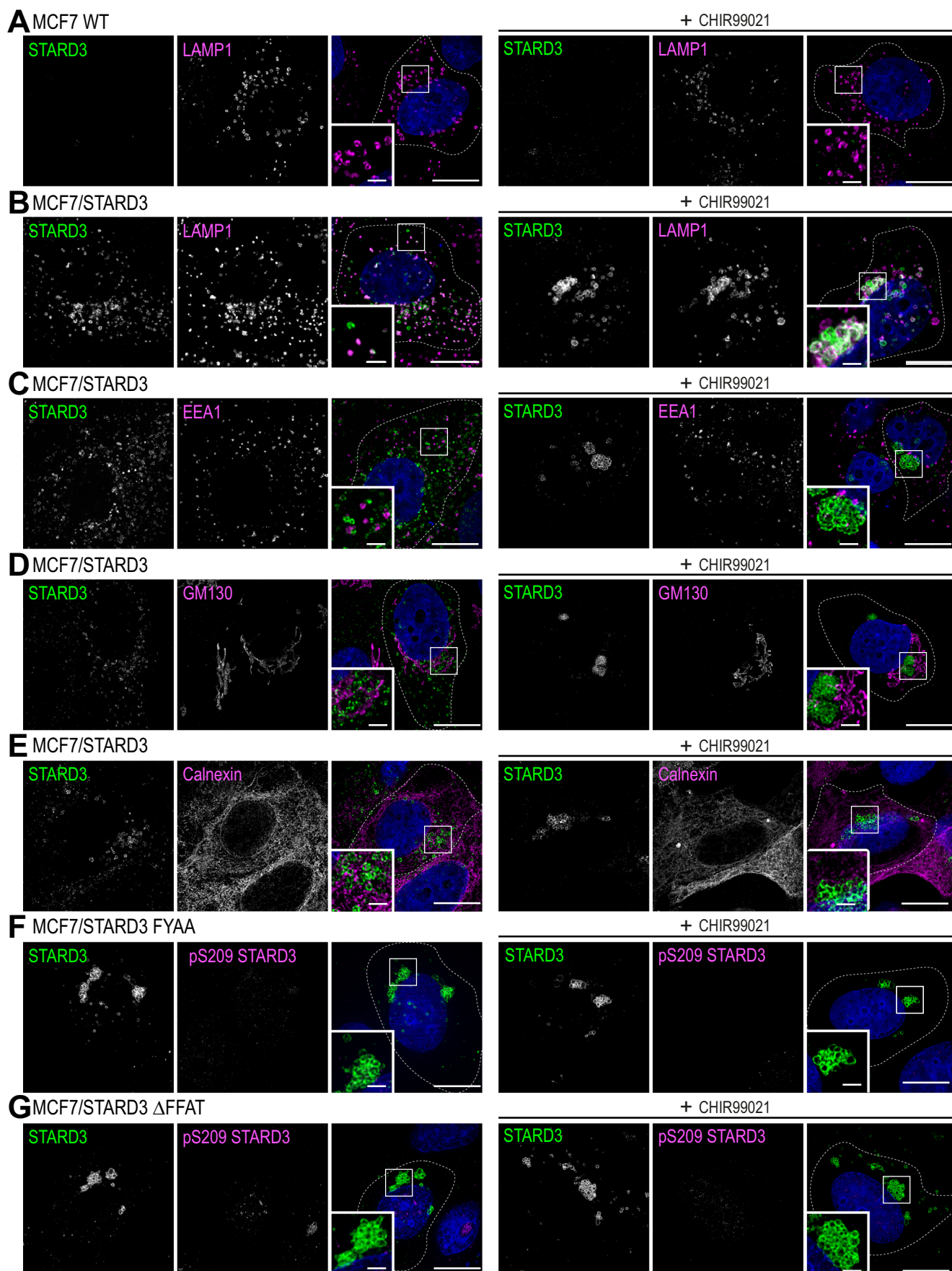


Figure EV3. STARD3 mediates the formation of LE/Lys clusters but does not affect other organelles.

WT MCF7 cells (A), MCF7 cells expressing STARD3 WT (B–F) or FFAT-motif deficient mutants (STARD3 FYAA (F), STARD3 Δ FFAT (G)) were left untreated (left) or treated with CHIR99021 (5 μ M, overnight; right). Cells were labeled with anti-STARD3 antibodies (green) (A–G) and in magenta with: anti-LAMP1 antibodies (A, B) to label LE/Lys, anti-EEA1 antibodies (C) to label early endosomes, anti-GM130 antibodies (D) to label the Golgi apparatus, anti-calnexin antibodies (E) to label the ER or phospho-specific antibodies (pS₂₀₉ STARD3, F, G). Nuclei were stained with Hoechst (blue). Subpanels show higher magnification images of the area outlined in white. Scale bars: 10 μ m. Inset scale bars: 2 μ m. Source data are available online for this figure.

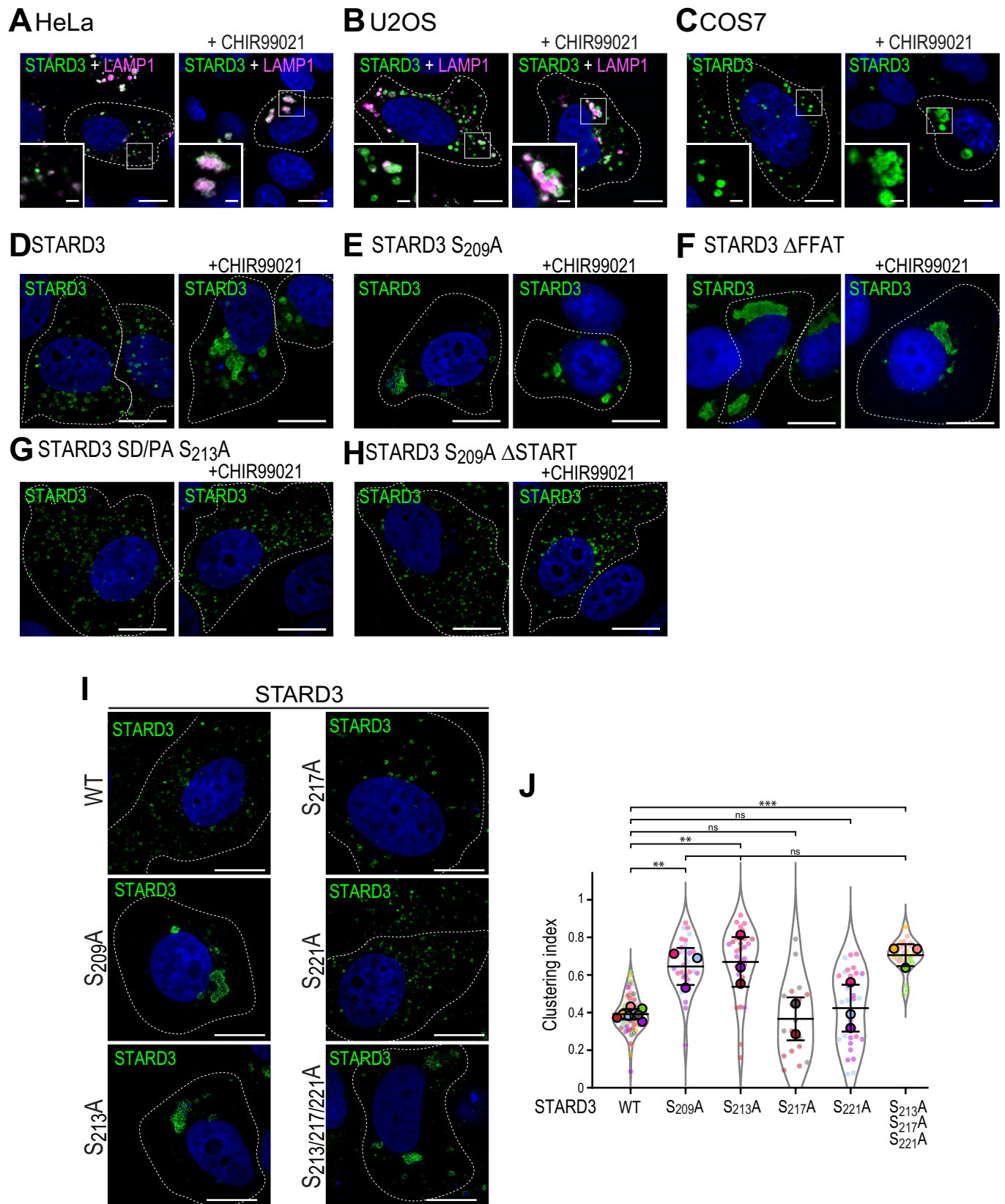



Figure EV4. Characterization of STARD3-induced LE/Lys clusters.

(A–C) HeLa cells (A), U2OS cells (B) and COS-7 cells (C) expressing STARD3 WT were left untreated (left) or treated with CHIR99021 (5 μ M, overnight) (right). Cells were labeled with anti-STARD3 antibodies (green) and anti-LAMP1 antibody (magenta). Nuclei were stained with Hoechst (blue). Subpanels show higher magnification images of the area outlined in white. Images were acquired with a SP8-UV confocal microscope. Scale bars: 10 μ m. Inset scale bars: 2 μ m. (D–H) MCF7 (A) expressing STARD3 WT (D), STARD3 S₂₀₉A (E), STARD3 Δ FFAT (F), STARD3 SD/PA S₂₁₃A (G) or STARD3 S₂₀₉A Δ START (H) were left untreated (left) or treated with CHIR99021 (5 μ M, overnight; right). Cells were labeled with anti-STARD3 antibodies (green). Nuclei were stained with Hoechst (blue). Scale bars: 10 μ m. Inset scale bars: 2 μ m. (I, J) Representative images of MCF7 cells expressing STARD3 WT, STARD3 S₂₀₉A, STARD3 S₂₁₃A, STARD3 S₂₁₇A, STARD3 S₂₂₁A, or STARD3 S₂₁₃A-S₂₁₇A-S₂₂₁A. Cells were labeled with anti-STARD3 antibodies (green) and Hoechst (blue). Scale bars: 10 μ M. (E) Quantification of LE/Lys clustering in cells shown in (D). Data are displayed as Superplots showing the clustering index per cell (small dots) and its mean per independent experiment (large dots). Number of cells: MCF7-STARD3: 70, MCF7-STARD3 S₂₀₉A: 31, MCF7-STARD3 S₂₁₃A: 31, MCF7-STARD3 S₂₁₇A: 18, MCF7-STARD3 S₂₂₁A: 32, MCF7-STARD3 S₂₁₃A-S₂₁₇A-S₂₂₁A: 31, from seven independent experiments). Independent experiments are color-coded. Means and error bars (SD) are shown as black bars. One-way ANOVA with Tukey's multiple comparison test (**, $P < 0.01$; ***, $P < 0.001$; $n = 3$ –7 independent experiments; WT vs S₂₀₉A, $P = 6.8 \times 10^{-3}$; WT vs S₂₁₃A, $P = 3 \times 10^{-3}$; WT vs S₂₁₇A, $P = 0.99$; WT vs S₂₂₁A, $P = 0.99$; WT vs S₂₁₃A-S₂₁₇A-S₂₂₁A, $P = 10^{-3}$; S₂₀₉A vs S₂₁₃A, $P = 0.99$; S₂₀₉A vs S₂₁₃A-S₂₁₇A-S₂₂₁A, $P = 0.95$; S₂₁₃A vs S₂₁₃A-S₂₁₇A-S₂₂₁A, $P = 0.99$). Source data are available online for this figure.

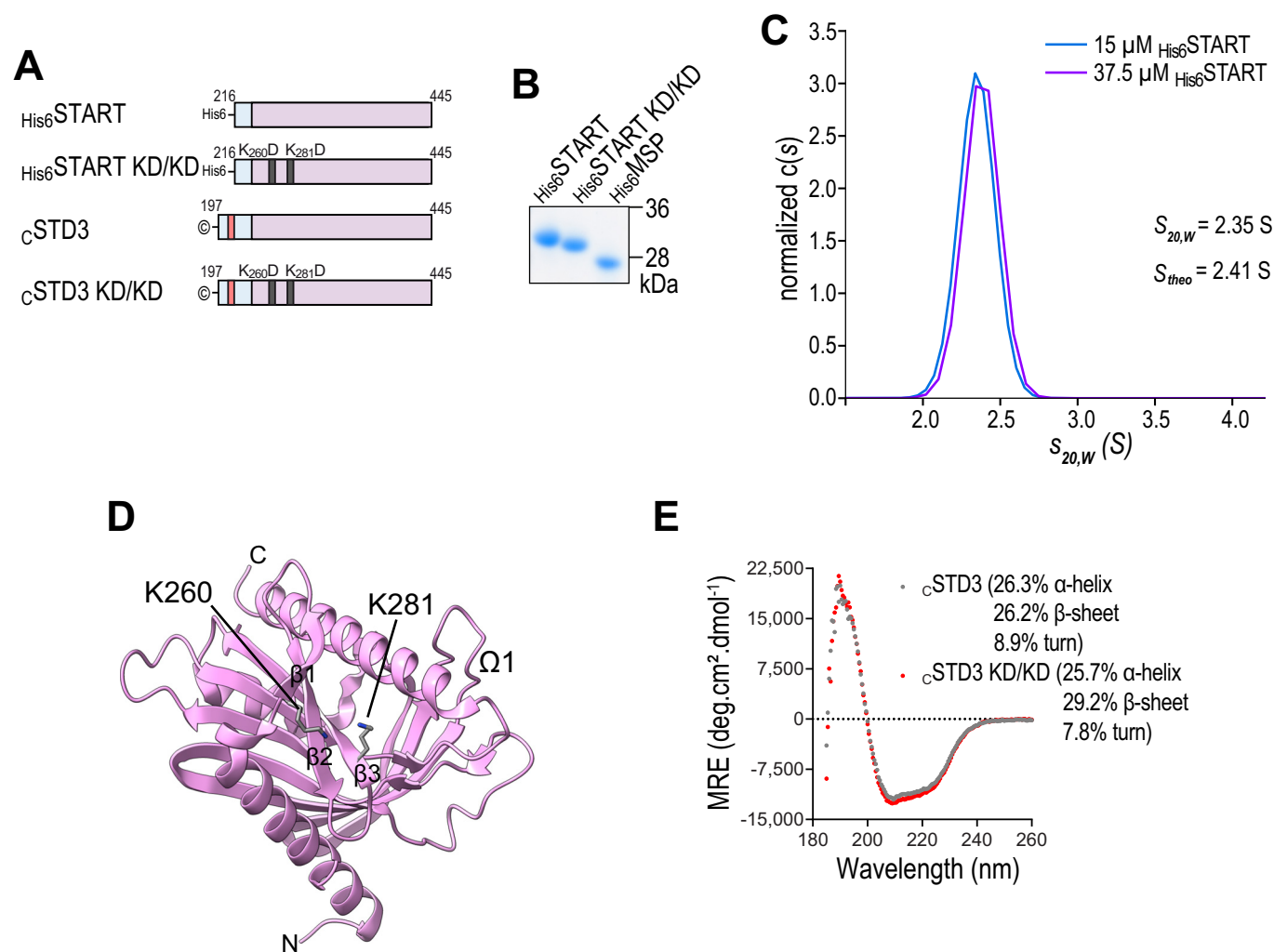


Figure EV5. The START domain is a monomer in solution and contains positively charged surface patches.

(A) Schematic representation of the different recombinant proteins used. (B) Coomassie blue staining of the recombinant START domain of STARD3 (WT and KD/KD mutant) and the MSP domain of VAP-B proteins after SDS-PAGE. (C) Superimposition of sedimentation coefficient distributions $c(s)$ obtained from sedimentation velocity (SV) experiments with either 15 μM (blue) or 37.5 μM (purple) of the recombinant START domain of STARD3. The experimental average sedimentation coefficient ($S_{20,W}$) of 2.35 S is close to the theoretical value (S_{theo}) of 2.41 S, indicating that the protein remains in a stable monomeric form at both concentrations. (D) Ribbon diagram of the START domain of STARD3. The positions of the two mutated residues, K260 and K281, are highlighted. Key structural features, including omega loop 1 ($\Omega 1$) and three beta strands ($\beta 1$, $\beta 2$, $\beta 3$), are also indicated (PDB ID: 1EM2) (Tsujishita and Hurley, 2000). (E) Far-UV CD spectrum of purified cSTD3 and cSTD3 KD/KD constructs in 20 mM Tris pH 7.4, 120 mM NaF buffer at room temperature. Source data are available online for this figure.