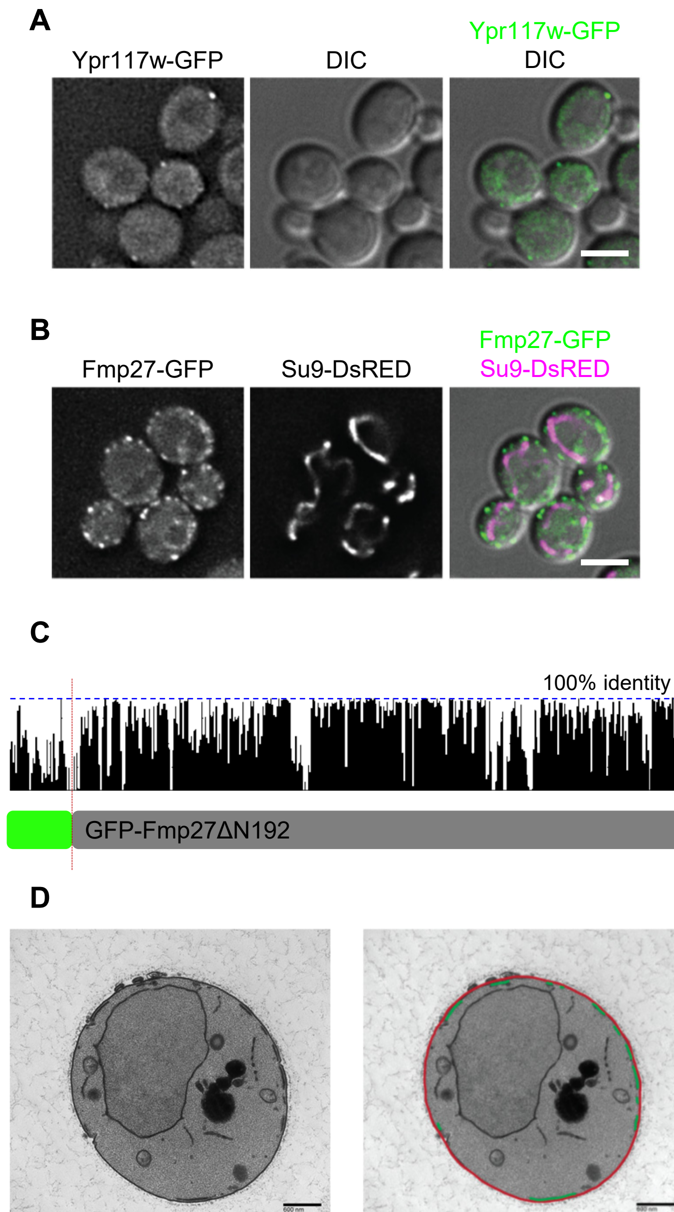

Fig. S1. Analysis of Ypr117w and Fmp27 localization and conservation. (A) Live-cell imaging of endogenously tagged Ypr117w-GFP (green) shows that Ypr117w is enriched in puncta at the cell cortex; however, this protein was expressed at very low levels. **(B)** Live-cell imaging of endogenously tagged Fmp27-GFP (green) with the mitochondrial marker Su9-DsRED (magenta) shows that Fmp27 does not localize to mitochondria. Su9-DsRED was expressed from a plasmid. Images shown are representative of three independent experiments with $n \geq 3$ images acquired per replicate. Scale bars: 2 µm. **(C)** Schematic depicting the conservation of *S. cerevisiae* Fmp27 and its orthologs in nine other fungal species (see methods section for list). Our goal was to identify "blocks" of conserved sequence separated by "gaps" with low conservation to inform our truncation analyses. GFP-Fmp27ΔN192 removes the first block of conserved sequence (192 amino acids) at the N-terminus. **(D)** Representative electron microscopy (EM) image acquired and used for quantification of cortical ER contacts in Fig. 3B. The image on the right illustrates how the quantification was done; cortical ER is marked in green, and the plasma membrane is marked in red. Cortical ER/plasma membrane ratios were calculated for each image.

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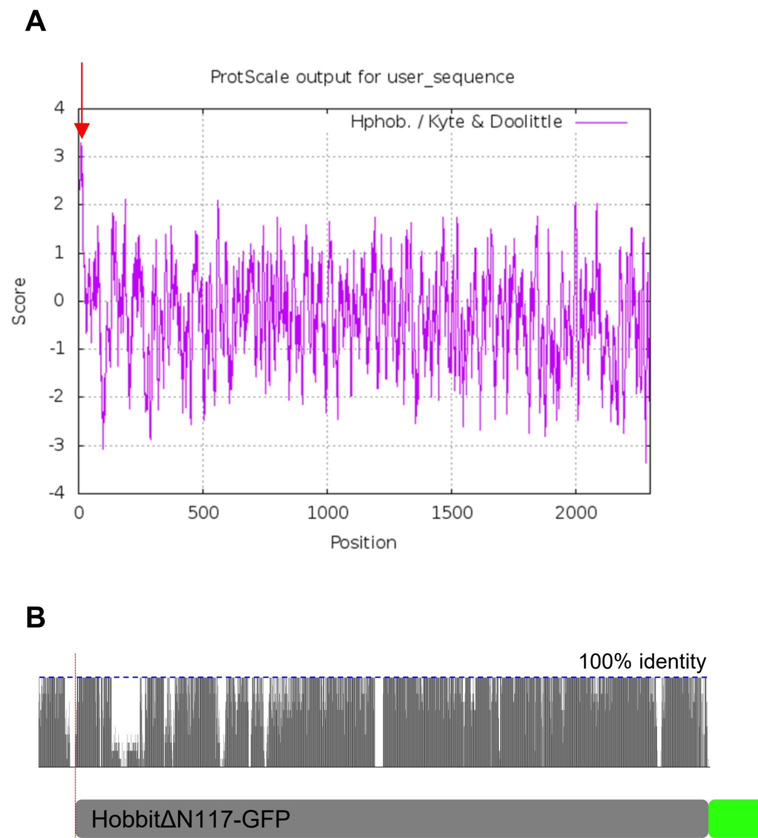

Fig. S2. Design of *D. melanogaster* N-terminal Hobbit truncation. (A) Kyte-Doolittle hydrophobicity plot of *D. melanogaster* Hobbit shows a short, highly hydrophobic region at the N-terminus of the protein (marked by red arrow), consistent with a possible transmembrane domain. Plot generated using Expasy ProtScale. **(B)** Schematic depicting the conservation of *D. melanogaster* Hobbit and its orthologs in nine other Drosophilid species (see methods section for list). Like Fmp27, our goal was to identify "blocks" of conserved sequence separated by "gaps" of low conservation. The HobbitΔN117-GFP transgene removes the first block of conserved sequence (117 amino acids) at the N-terminus.

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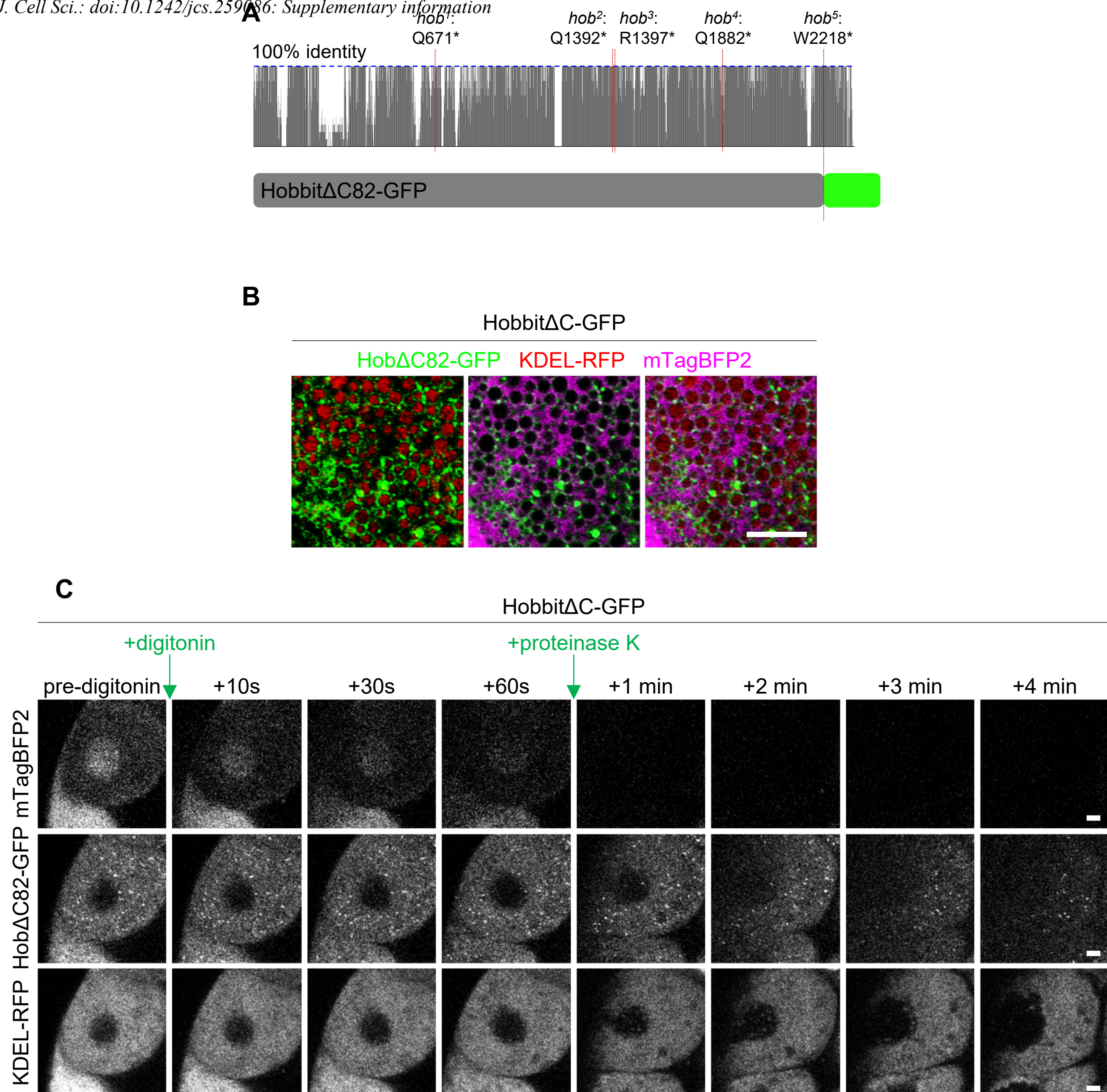

Fig. S3. C-terminal truncation of Hobbit does not affect ER membrane localization or topology. (A) Schematic depicting the conservation of *D. melanogaster* Hobbit and its orthologs in nine other Drosophilid species (see methods section for list) and the position of each of the identified *hobbit* mutant nonsense mutations. The HobbitΔC82-GFP transgene enables overexpression of a protein comparable to that in *hob*^{5}. **(B)** Live-cell imaging of HobbitΔC82-GFP (green), the ER lumen marker KDEL-RFP (red), and cytosolic mTagBFP2 (magenta) in salivary glands at the onset of metamorphosis (0 h after puparium formation) shows that C-terminally truncated Hobbit still localizes to the ER membrane. Full genotype: *UAS-KDEL-RFP/+; Sgs3>hobΔC82-GFP/UAS-mTagBFP2*. Images show a single slice from a z-stack comprising three optical sections at a 0.28 µm step size.**(C)** Imaging-based protease protection assay shows that the C-terminal truncation of Hobbit does not affect topology. Cytosolic mTagBFP2 (top) rapidly diffuses out of the cells after permeabilization with digitonin, while HobbitΔC82-GFP (middle) and KDEL-RFP (bottom) are unaffected. Like the full-length protein, HobbitΔC82-GFP (tagged at the C-terminus) is degraded after subsequent addition of proteinase K, while KDEL-RFP is unaffected. Note that the cells flatten after addition of proteinase K. Experiment was conducted using 0 h PF glands. Full genotype: *UAS-KDEL-RFP/+; Sgs3>hobΔC82-GFP/UAS-mTagBFP2*. Images shown are representative of three independent experiments with *n*≥10 salivary glands from independent animals analyzed per genotype. Scale b a r in (B): 5 µm; (C): 10 µm.

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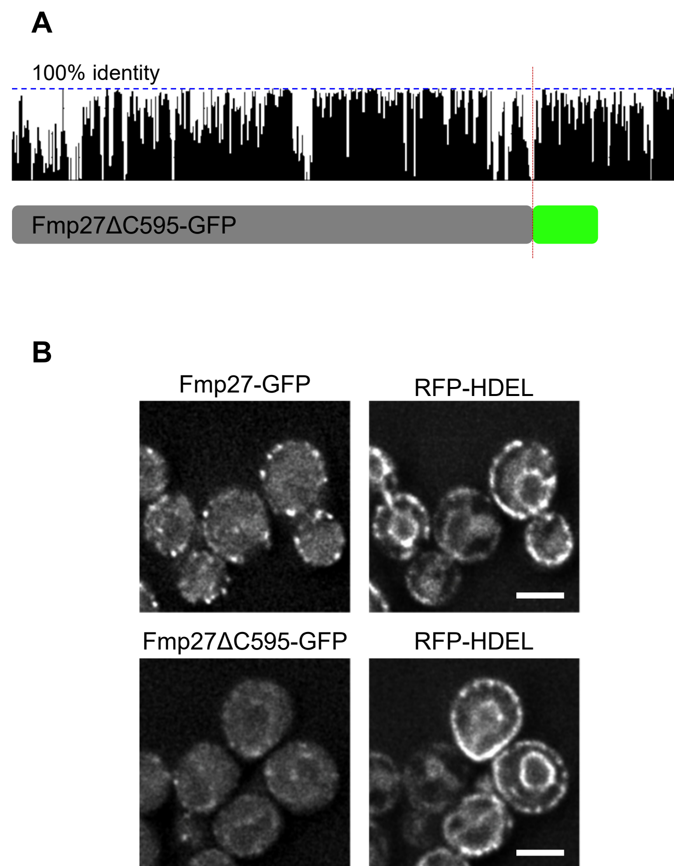

Fig. S4. C-terminal truncation of Fmp27 reduces cortical ER localization. (A) Schematic depicting the conservation of *S. cerevisiae* Fmp27 and its orthologs in nine other fungal species (see methods section for list; also displayed in Fig. S1C). Fmp27ΔC595-GFP removes the final block of conserved sequence at the C-terminus (595 amino acids) **(B)** Live-cell imaging of full-length Fmp27-GFP and Fmp27ΔC595-GFP with the ER marker RFP-HDEL shows that C-terminal truncation of Fmp27 reduces its localization to cortical ER. FMP27-GFP and C-terminal truncation were made at the endogenous *FMP27* locus and are expressed under the endogenous promoter; RFP-HDEL was expressed from a plasmid. Note that Fmp27ΔC595-GFP is expressed at lower levels than full-length Fmp27-GFP. Images shown are representative of three independent experiments with $n \geq 3$ images acquired per replicate. Scale bars: 2 µm.

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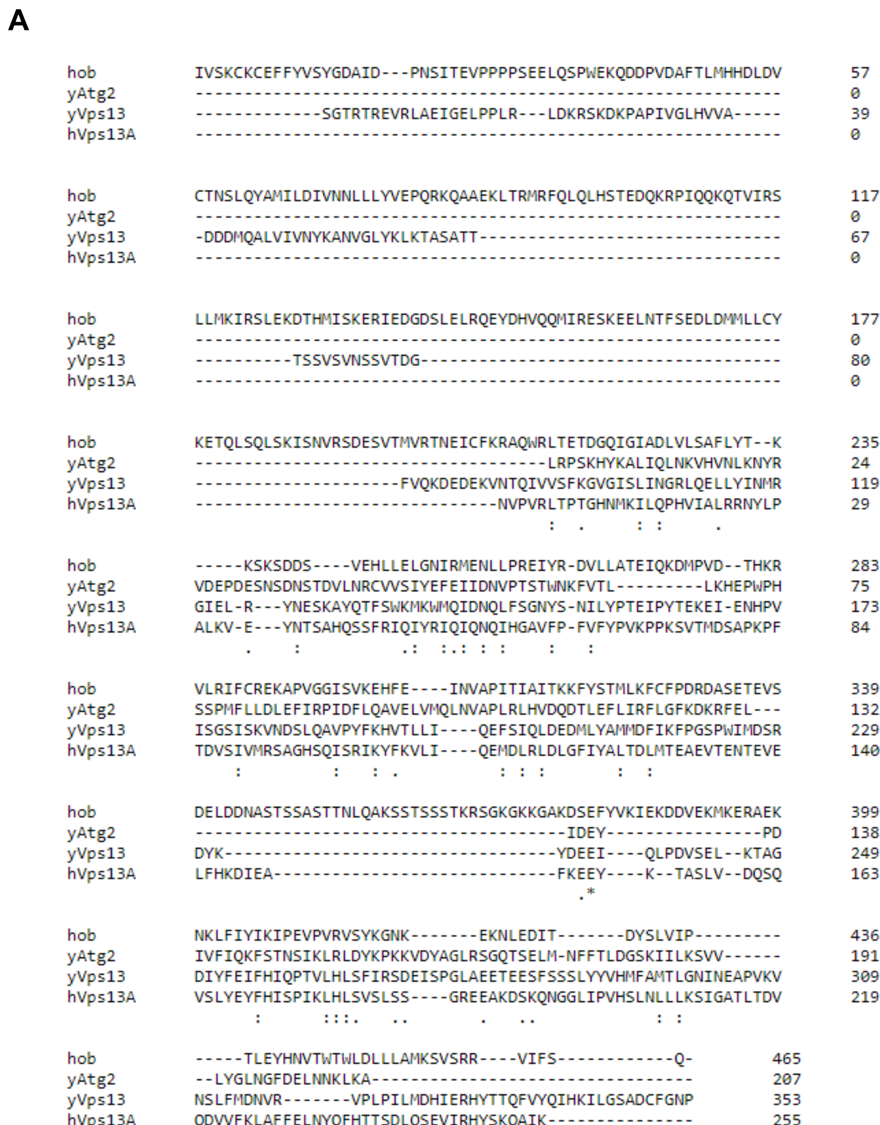
A



B

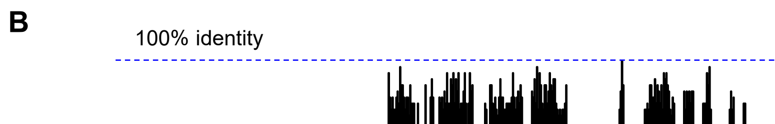
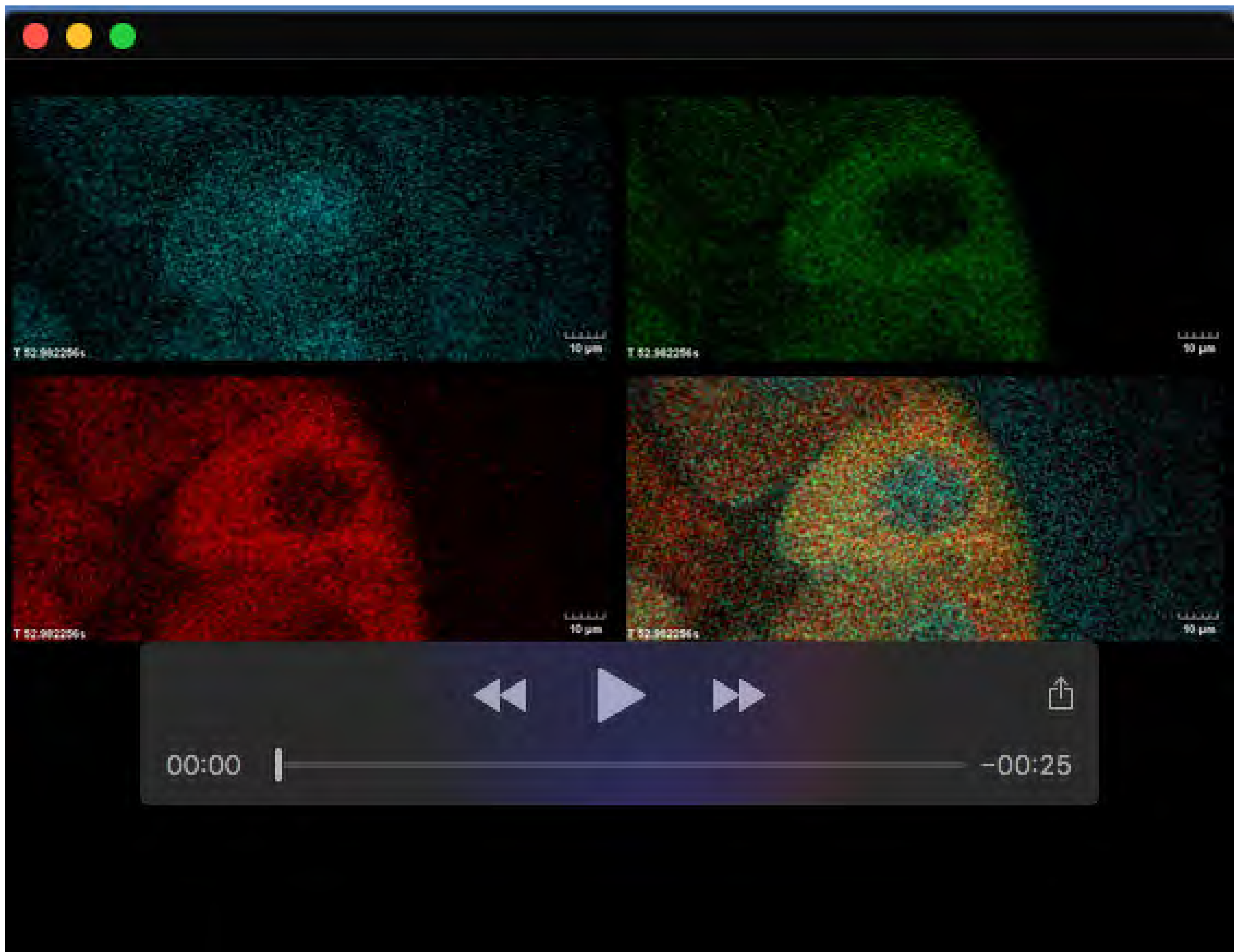


Fig. S5. Sequence alignment of Apt1 domains. (A) Alignment of the Apt1 domain sequence from *D. melanogaster* Hobbit (hob), *S. cerevisiae* Atg2 (yAtg2), *S. cerevisiae* Vps13 (yVps13) and *H. sapiens* VPS13A (hVps13A). The Hobbit Apt1 domain consists of the amino acid residues predicted by Pfam (1774-2239); the sequences of the other Apt1 domains were previously reported (Kaminska et al., 2016; Kolakowski et al., 2020; Rzepnikowska et al., 2017). Note that the Hobbit Apt1 domain is significantly longer than the other reported Apt1 domains. **(B)** Graphical identity plot depiction of the sequence alignment shown in (A).



Movie 1. Imaging-based protease protection assay with full-length Hobbit-GFP. Live-cell time-lapse imaging of cytosolic mTagBFP2 (cyan), full-length Hobbit-GFP (green), and KDEL-RFP (red) in an imaging-based protease protection assay. The selective detergent digitonin, which permeabilizes the plasma membrane but not intracellular membranes, was added at timestamp 28.049430 s, and proteinase K was added at timestamp 2 min 4.664132 s. Still images from this time-lapse are pictured in Fig. 4C.

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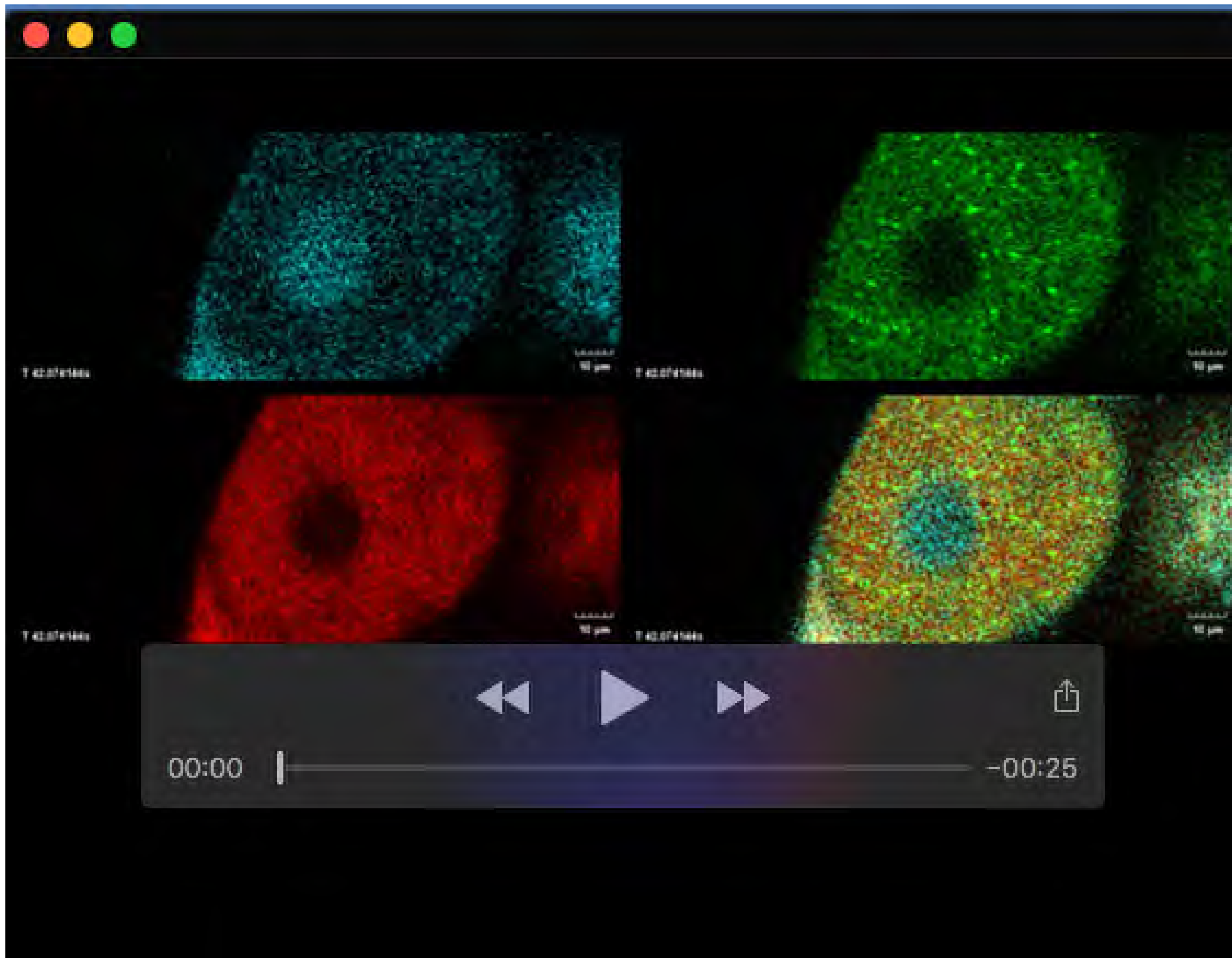

Movie 2. Imaging-based protease protection assay with HobbitΔC82-GFP. Live-cell time-lapse imaging of cytosolic mTagBFP2 (cyan), HobbitΔC82-GFP (green), and KDEL-RFP (red) in an imaging-based protease protection assay. The selective detergent digitonin, which permeabilizes the plasma membrane but not intracellular membranes, was added at timestamp 1 min 45.964512 s, and proteinase K was added at timestamp 3 min 47.512041 s. Still images from this time-lapse are pictured in Fig. S3C.