Supplemental Materials

Molecular Biology of the Cell

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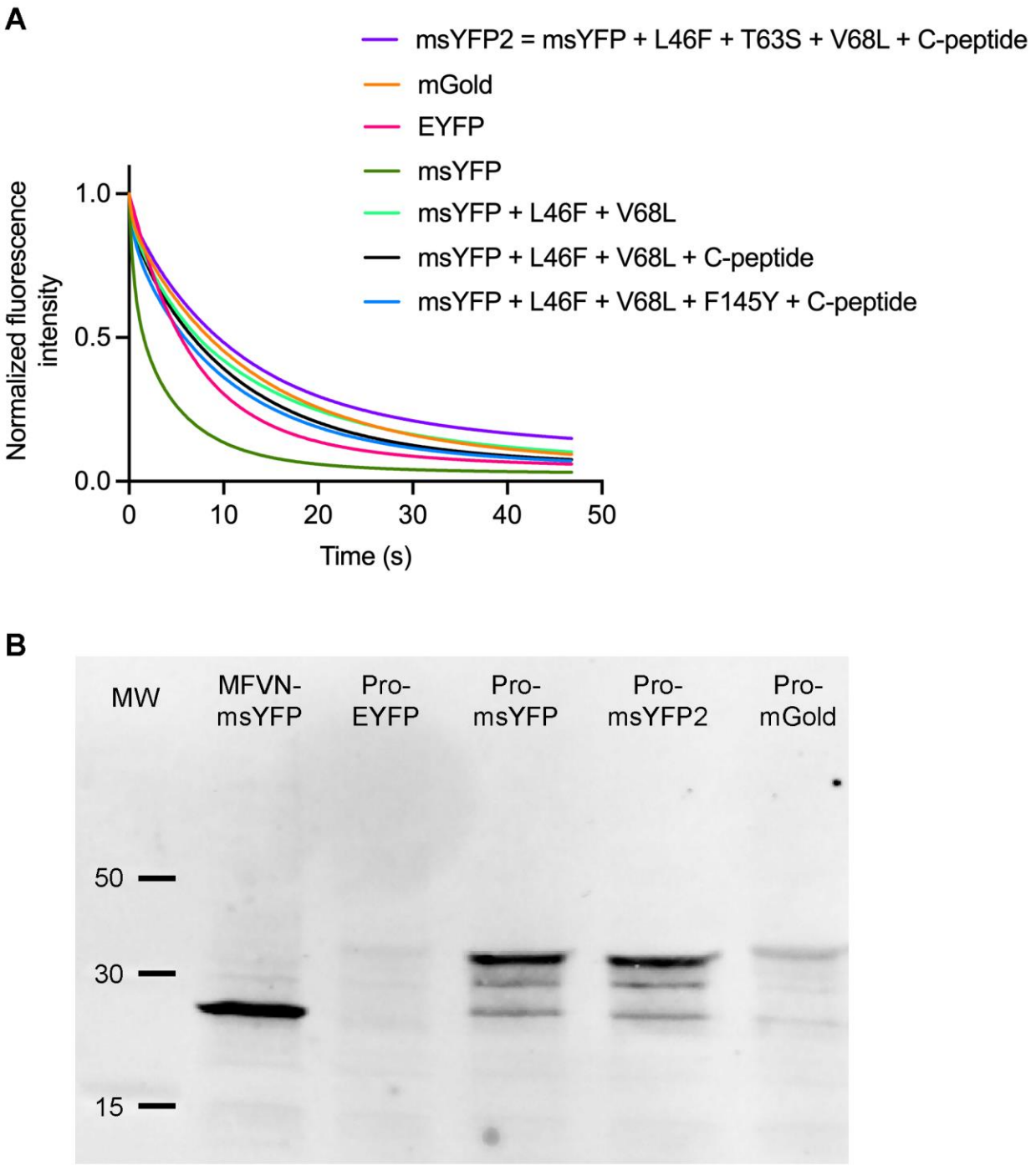

FIGURE S1: Supporting data for Figure 1. (A) Photobleaching of purified YFP variants. The indicated YFPs were imaged repeatedly by widefield microscopy as in Figure 1A. L46F, V68L, and F145Y are reversions of mutations originally introduced into msYFP, and "C-peptide" indicates replacement of the original msYFP C-terminal peptide with the C-terminal peptide from msGFP2. (B) Expression levels of YFP variants in *E. coli*. The indicated YFPs were expressed as fusions to proinsulin ("Pro"), and whole cell extracts were separated by SDS-PAGE followed by immunoblotting with an anti-GFP antibody. The sample labeled "MFVN-msYFP" was msYFP

preceded by the first four amino acids of proinsulin. “MW” is molecular weight markers, with the sizes indicated in kDa.

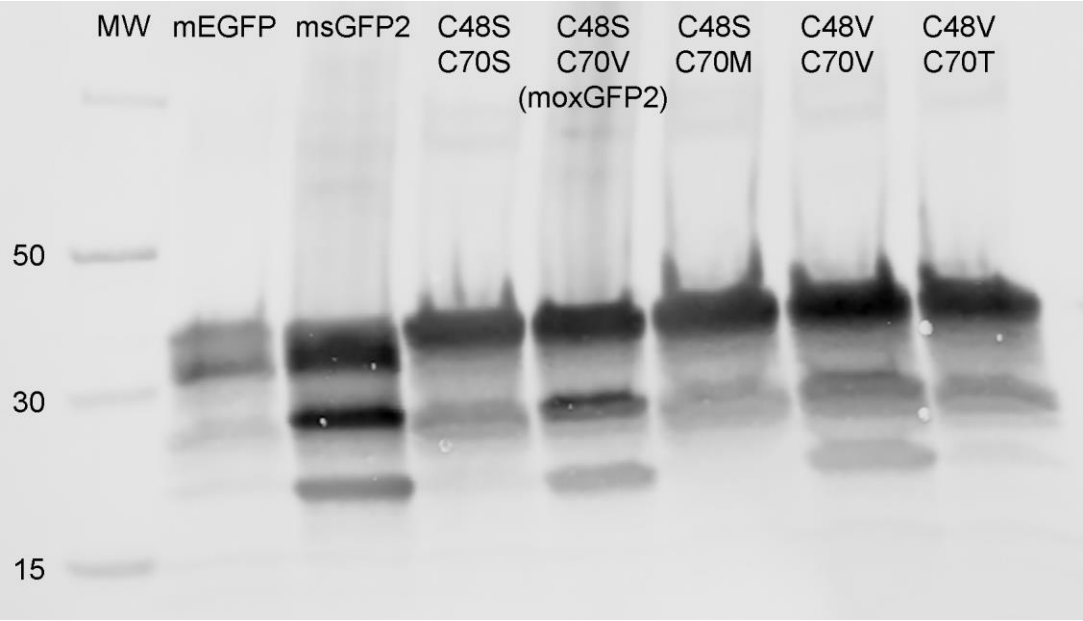

FIGURE S2: Expression levels of mEGFP and of msGFP2 derivatives in *E. coli* (supporting data for Figure 5A). The indicated GFPs were expressed as fusions to proinsulin, and whole cell extracts were separated by SDS-PAGE followed by immunoblotting with an anti-GFP antibody. Cysteine substitutions indicate mutant derivatives of msGFP2. “MW” is molecular weight markers, with the sizes indicated in kDa.

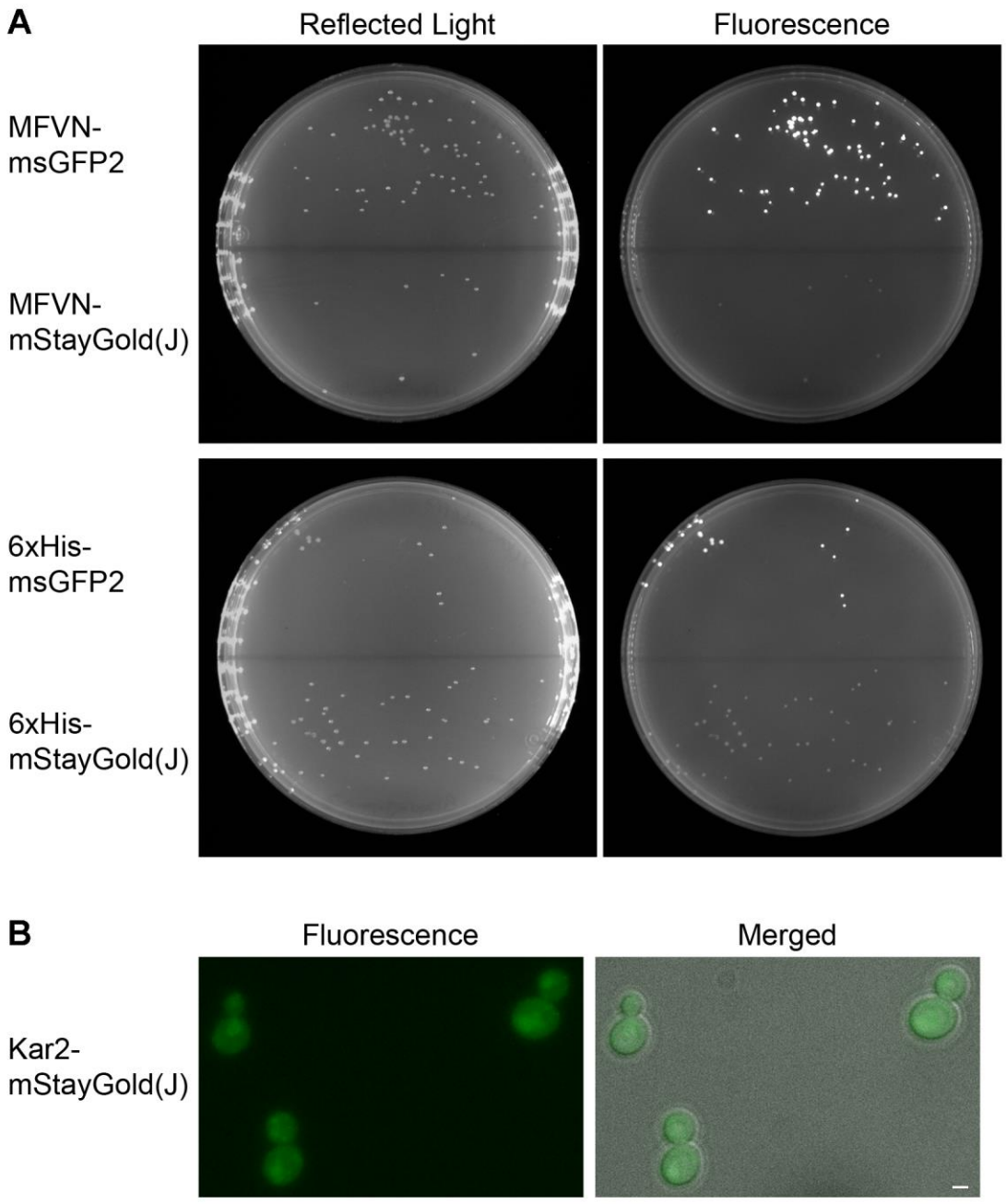

Figure S3: N-terminal fusions to mStayGold(J) are not consistently well-behaved *in vivo*. (A) mStayGold(J) yields weak fluorescence when expressed in bacteria. We routinely add the tetrapeptide MFVN to the N-terminus of an FP to create an expression control for superfolder assays, as illustrated in Figure S1B, and the resulting constructs tend to be brightly fluorescent when expressed in bacteria. For the experiment shown in the top panels, expression plasmids encoding MFVN-msGFP2 or MFVN-mStayGold(J) were transformed in parallel into bacteria, and colonies were generated by overnight growth at 37°C on an LB + ampicillin plate followed by incubation at 23°C for 24 h. The plate of colonies was then illuminated with white light

("Reflected Light") or blue light ("Fluorescence") in an Axygen GDBL-1000 gel documentation system. Whereas MFVN-msGFP2 was brightly fluorescent, MFVN-mStayGold(J) was not visibly fluorescent. For the experiment shown in the bottom panels, the same procedure was performed except that the expression plasmids encoded 6xHis-msGFP2 or 6xHis-mStayGold(J). Whereas 6xHis-msGFP2 was brightly fluorescent, 6xHis-mStayGold(J) was only weakly fluorescent. In all cases, the same plasmid samples used for bacterial transformation were also used to verify the constructs by whole-plasmid sequencing. (B) mStayGold(J) is not suitable as a fluorescent tag for yeast Kar2. Shown are fluorescence and merged fluorescence/brightfield images of a yeast strain in which the endogenous *KAR2* gene was replaced with a *KAR2-mStayGold(J)* fusion gene. Compare to the fluorescence pattern seen with *KAR2-moxGFP2* in Figure 6A. Scale bar, 2 µm.