Figures and figure supplements

Assigning mitochondrial localization of dual localized proteins using a yeast Bi-Genomic Mitochondrial-Split-GFP

Gaétan Bader *et al*

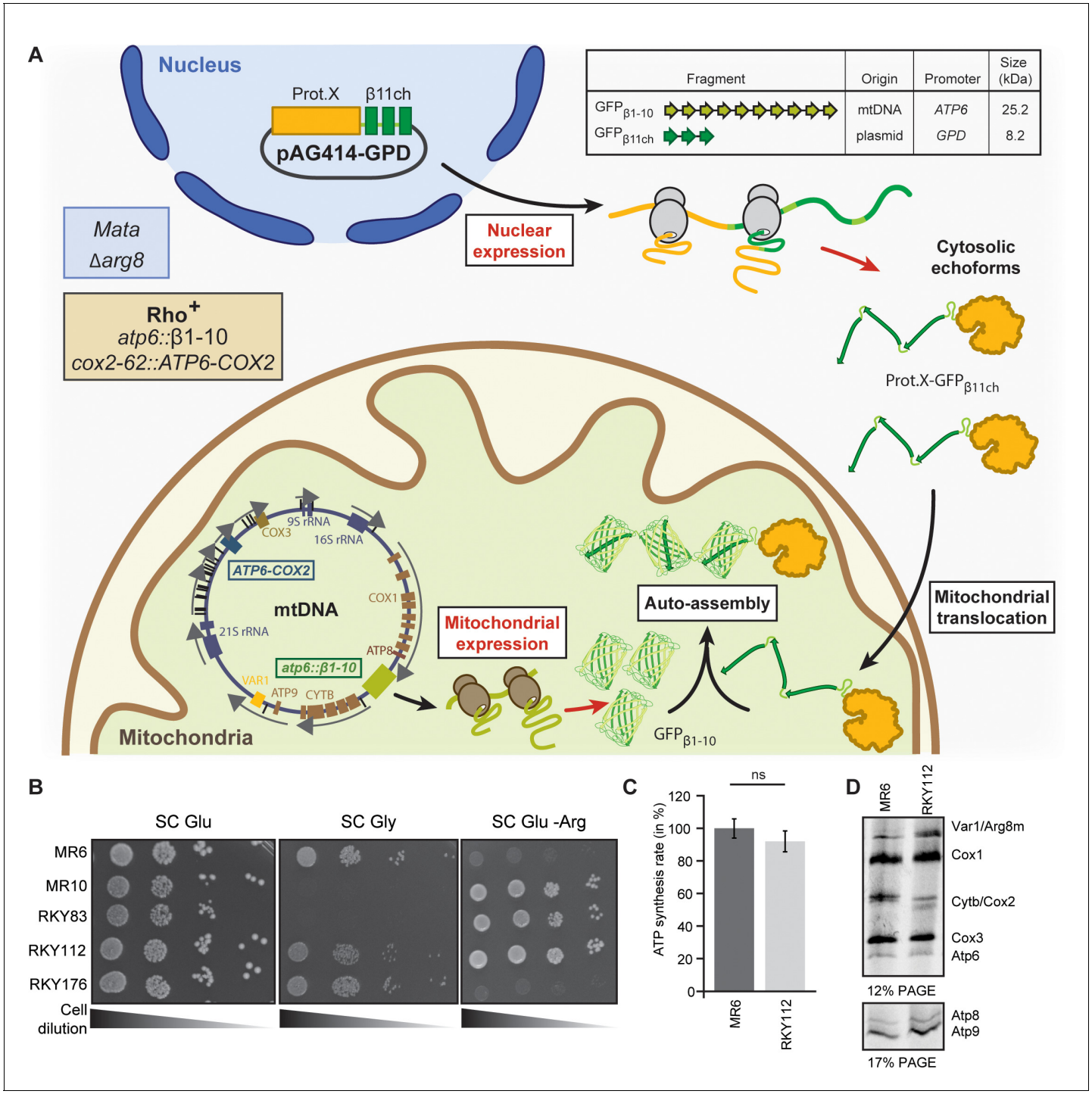

Figure 1. Engineering of the BiG Mito-Split-GFP system in *S. cerevisiae*. (**A**) Principle of the Split-GFP system. When present in the same subcellular compartment, two fragments of GFP namely $GFP_{\beta1\text{-}10}$ and $GFP_{\beta11ch}$ can auto-assemble to form a fluorescent BiG Mito-Split-GFP chaplet (three reconstituted GFPs). *$GFP_{\beta1\text{-}10}$* sequence encoding the first ten beta strands of GFP has been integrated into the mitochondrial genome under the control of the *ATP6* promoter. $GFP_{\beta11ch}$ consists of a tandemly fused form of the eleventh beta strand of GFP and is expressed from a plasmid under the control of a strong GPD promoter (pGPD). The molecular weight of the tag is indicated. (**B**) Growth assay on permissive SC Glu plates, respiratory plates (SC Gly), and restrictive media lacking arginine (SC Glu -Arg) of the different strains used in the study (N = 2). All generated strains are derivative from MR6. (**C**) ATP synthesis rates of the MR6 and RKY112 strains presented as the percent of the wild type control strain (N = 2). P-value was 0.7456 (not significant). 95% confidence interval was −273.4 to 229.9, R squared = 0.064 (**D**) Mitochondrial translation products in the MR6 and RKY112 strains (N = 2). Cells were grown in rich galactose medium. Pulse-chase of radiolabeled [^{35}S]methionine + [^{35}S]cysteine was performed by a 20 min incubation

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in the presence of cycloheximide. Total cellular extracts were separated by SDS PAGE in two different polyacrylamide gels prepared with a 30:0.8 ratio of acrylamide and bis-acrylamide. Upper gel: 12% polyacrylamide gel containing 4 M urea and 25% glycerol. Lower gel: 17.5% polyacrylamide gel. Gels were dried and exposed to X-ray film. The representative gels are shown.

Figure 1—figure supplement 1. Optimized sequence and secondary structure of the $GFP_{\beta1\text{-}10}$ and $GFP_{\beta11ch}$ that were used in this study (related to ***Figure 1***). (**A**) The amino acid sequence and numbering of the residues of wild type $GFP_{\beta1\text{-}10}$ are shown. The β-strands are schematized as blue arrows. The amino acid residues of wild type GFP that were mutated to generate the Folding Reporter GFP are in green. The six amino acids of Folding reporter GFP that were then mutated to build the Superfolder GFP are in red (***Pédelacq et al., 2006***) and the seven amino acid residues of Superfolder GFP that were mutated to generate $GFP_{\beta1\text{-}10}$ OPT are indicated in orange (***Cabantous et al., 2005a***). (**B**) The amino acid sequence of the $GFP_{\beta11ch}$ is shown and the numbering corresponds to the aa residues of the β11-strand of wild type GFP. The three consecutive β11 strands are schematized as green arrows and the three mutations that were introduced into each β11 strand (GFP11M3) are in purple (***Cabantous et al., 2005a***). The linker sequences are colored gray.

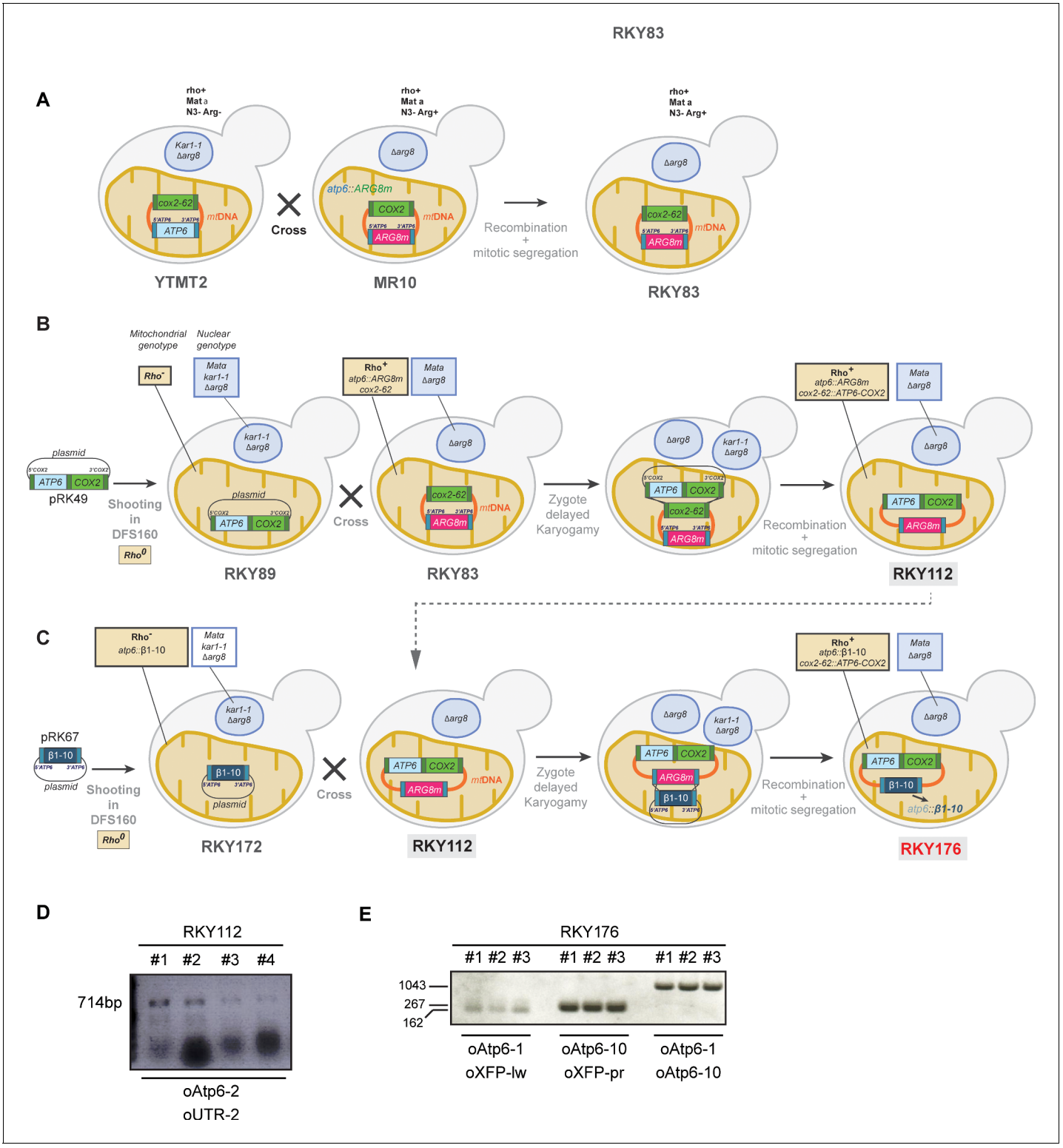

Figure 1—figure supplement 2. Engineering of the strains and verification of the correct integration of *ATP6* under the control of *COX2* gene UTRs or $GFP_{\beta 1\text{-}10}$ under the control of *ATP6* gene UTRs (related to ***Figure 1***). (**A**) Construction of the RKY83 strain. (**B–C**) Construction of RKY112 and RKY176, a strain that expresses $GFP_{\beta 1\text{-}10}$ from the mitochondrial genome. Detailed description can be found in the Materials and methods section. (**D**) Total DNA prepared from the RKY112 clones 1 to 4 was used as templates for a mplification of the 3'part of *ATP6* and the 3'UTR region of *COX2* (N = 4). (**E**) Total DNA prepared from or RKY176 clones 1 to 3 was used as templates for amplification of the 3' and the 5' *ATP6* gene UTRs/$GFP_{\beta 1\text{-}10}$ regions (N = 3). The oligonucleotides used for each reaction and products lengths are indicated (***Supplementary file 1***).

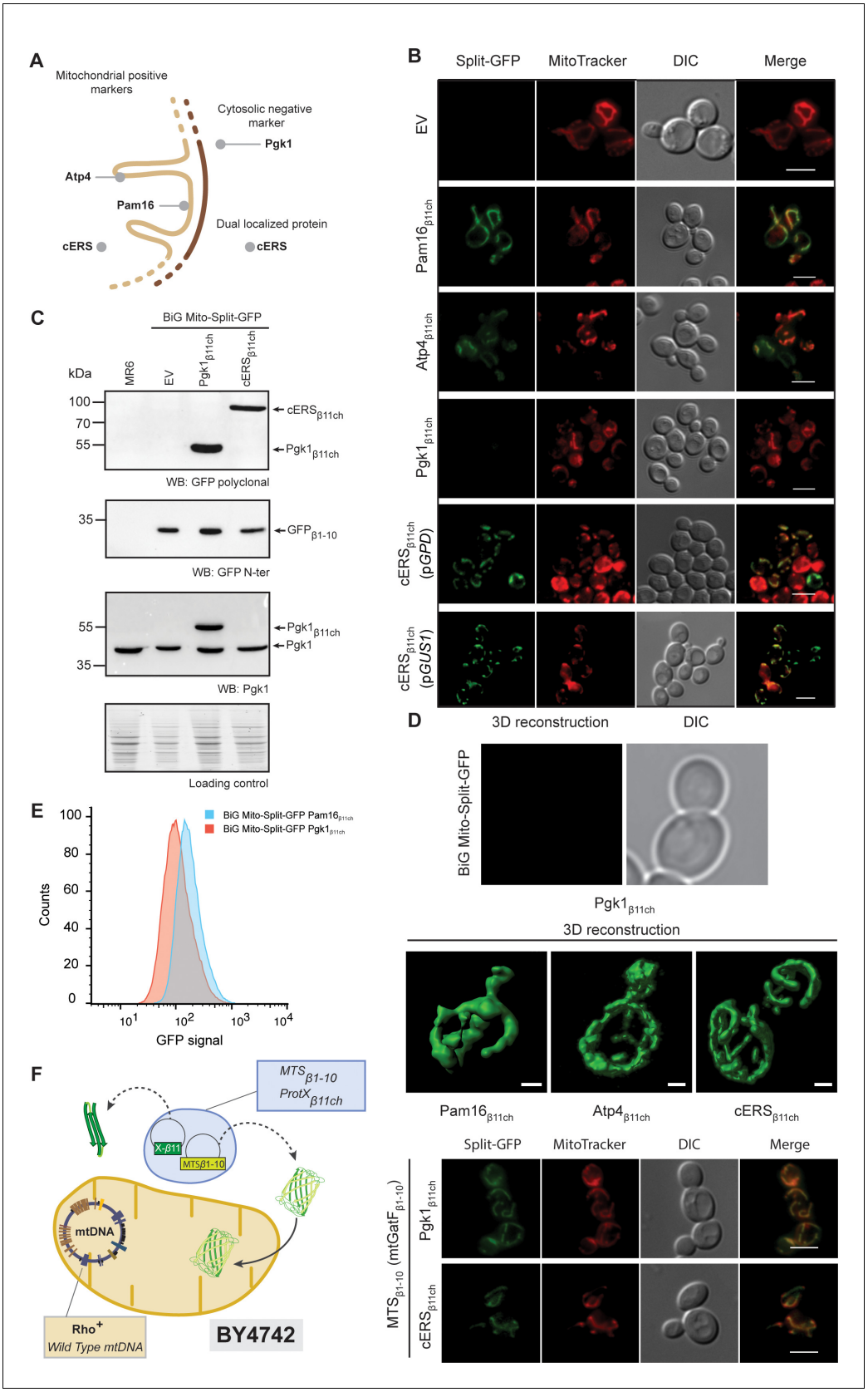

Figure 2. The reconstitution and fluorescence emission of the BiG Mito-Split-GFP is confined to mitochondria and exclusively generated by mitochondrial proteins. (**A**) Schematic of the spatial localization of proteins used as positive mitochondrial control proteins (Atp4, Pam16), negative

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Figure 2 continued

cytosolic control protein (Pgk1) and as dual localized protein (cERS) in *S. cerevisiae*. (**B**) Empty pAG414pGPD$_{\beta 11ch}$ vector (EV) or pAG414pGPD$_{\beta 11ch}$ vectors expressing each of the four GFP$_{\beta 11ch}$-tagged proteins used as markers in our study were transformed into the BiG Mito-Split-GFP strain. cERS$_{\beta 11ch}$ was either expressed under the dependence of the GPD (pGPD) or its own promoter (pGUS1) from a centromeric plasmid. GFP reconstitution upon mitochondrial import was followed by epifluorescence microscopy (N = 3). (**C**) Immunodetection of the GFP$_{\beta 1\text{-}10}$, cERS$_{\beta 11ch}$ and Pgk1$_{\beta 11ch}$ fusion protein in whole cell extract from the transformed BiG Mito-Split-GFP strain using anti-GFP and -Pgk1 antibodies, confirming expression of Pgk1$_{\beta 11ch}$. Loading control: stain-free. The representative gels are shown. (**D**) The strains described in the legend of panel (**B**) were used for three-dimensional reconstitution of yeast mitochondrial network (N = 1). Z-Stack images from Pam16$_{\beta 11ch}$, Atp4$_{\beta 11ch}$, cERS$_{\beta 11ch}$ and Pgk1$_{\beta 11ch}$ were taken using an Airyscan microscope. Scale bar: 1 µm. (**E**) Flow cytometry measurements of total GFP fluorescence of the BiG Mito-Split-GFP strain stably expressing Pgk1$_{\beta 11ch}$ or Pam16$_{\beta 11ch}$ (N = 3). (**F**) The mitochondrial GatF protein was fused to the GFP$_{\beta 1\text{-}10}$ fragment (mtGatF $_{\beta 1\text{-}10}$), thereby targeting the ten first GFP beta-strands to mitochondria after being transcribed in the nucleus and translated in the cytoplasm. This construct was co-expressed with either cERS$_{\beta 11ch}$ or Pgk1$_{\beta 11ch}$. The GFP reconstitution was monitored by epifluorescence microscopy. Mitochondria were stained with MitoTracker Red CMXRos. Scale bar: 5 µm. Representative fields are shown.

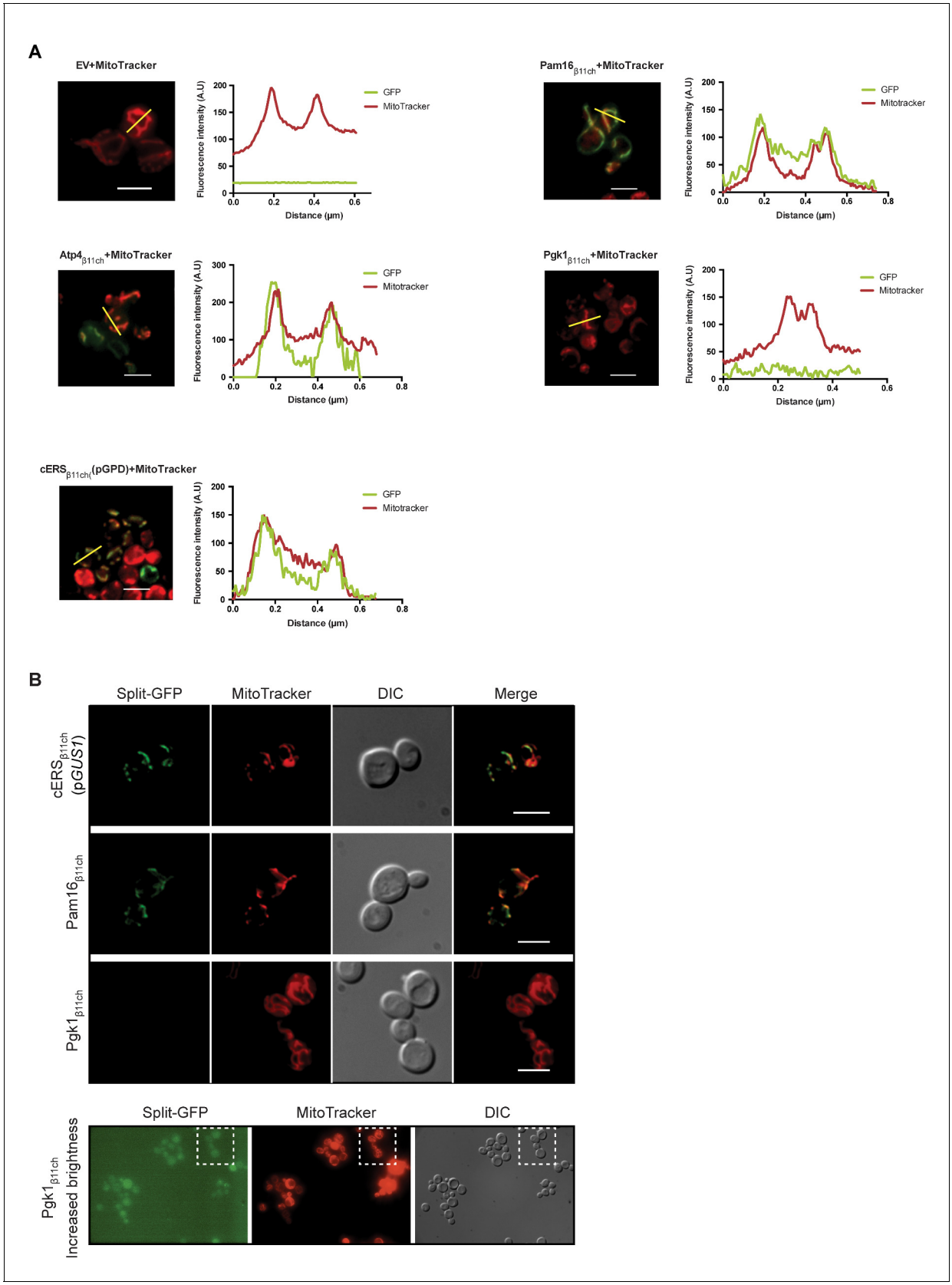

Figure 2—figure supplement 1. Mitochondrial relocation of mitochondrial proteins or echoforms tagged with $GFP_{\beta 11}$ (related to ***Figure 2***). (**A**) Colocalization measurement of the reconstituted GFP ($_{\beta 11+ \beta 1\text{-}10}$) with MitoTracker Red CMXRos-stained mitochondria on merged micrographs shown in

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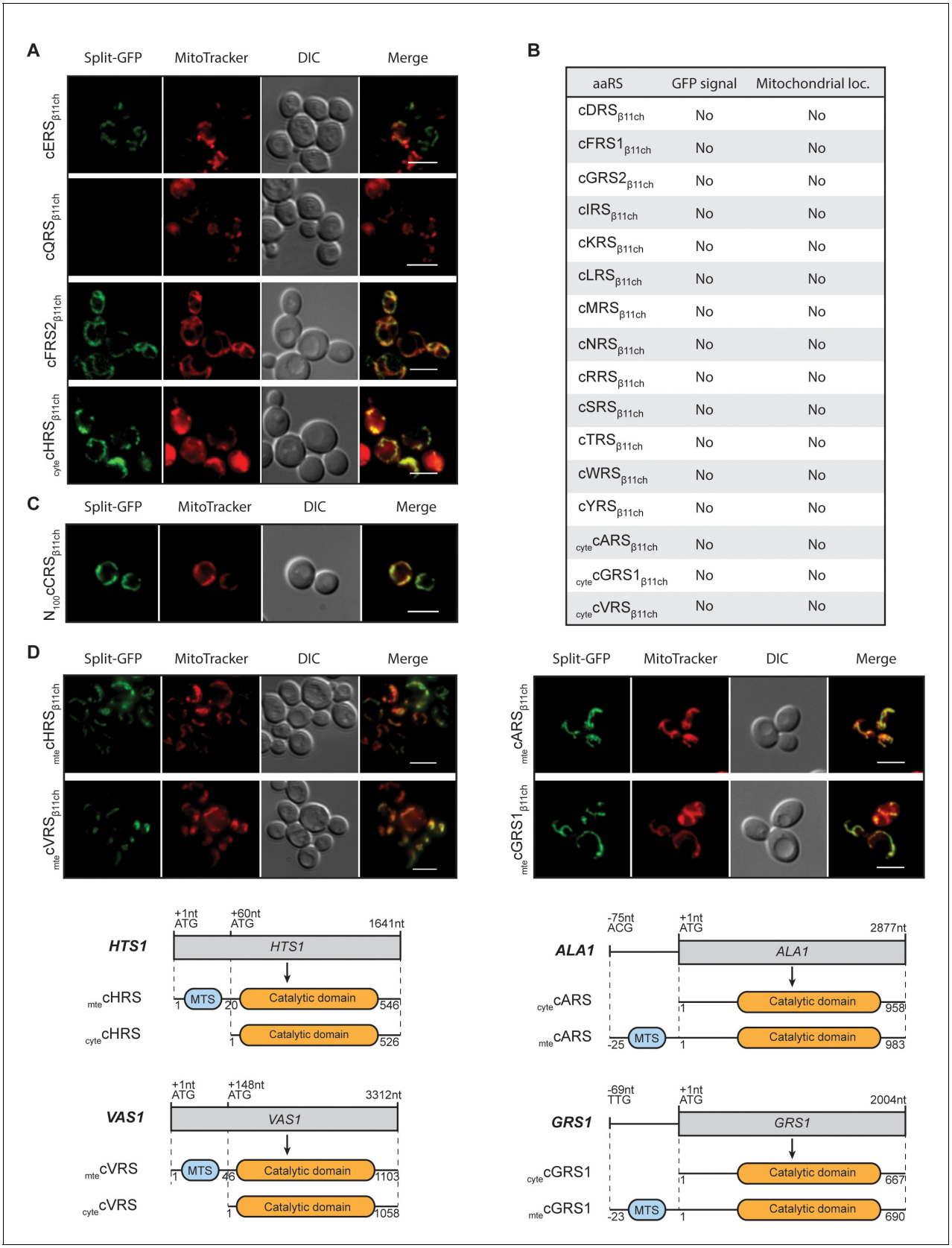
Figure 2B. Fluorescent signals were measured along the yellow line with the ImageJ software. (**B**) Fluorescence microscopy analysis of the BiG Mito-Split-GFP strains bearing integrated into the *TRP1* locus of *GUS1* (cERS), *PAM16* or *PGK1* genes fused to $GFP_{\beta 11ch}$. The $cERS_{\beta 11ch}$ is expressed from the own promoter (*GUS1*) while $Pam16_{\beta 11ch}$ and $Pgk1_{\beta 11ch}$ are expressed from GPD promoter. The last panel ($Pgk1_{\beta 11ch}$ Increased brightness) shows the full field from which the $Pgk1_{\beta 11ch}$ micrograph of the upper panel was taken from, with enhanced brightness, thereby illustrating the absence of any faint mitochondrial fluorescence. Mitochondria were stained with MitoTracker Red CMXRos. Scale bar: 5 μm.



aaRS	GFP signal	Mitochondrial loc.
cDRS β11ch	No	No
cFRS1 β11ch	No	No
cGRS2 β11ch	No	No
cIRS β11ch	No	No
cKRS β11ch	No	No
cLRS β11ch	No	No
cMRS β11ch	No	No
cNRS β11ch	No	No
cRRS β11ch	No	No
cSRS β11ch	No	No
cTRS β11ch	No	No
cWRS β11ch	No	No
cYRS β11ch	No	No
cytecARS β11ch	No	No
cytecGRS1 β11ch	No	No
cytecVRS β11ch	No	No

Figure 3. Identification and visualization of mitochondrial echoforms of yeast cytosolic aaRSs using the BiG Mito-Split-GFP strategy. Fluorescence microscopy analyses of BiG Mito-Split-GFP strain transformed with pAG414pGPD$_{\beta 11ch}$ expressing yeast caaRSs (also see Table S3). Genes encoding 18

Figure 3 continued on next page

Figure 3 continued

out of the 20 yeast caaRS, including those encoding the α- and β-subunits of the cytosolic $\alpha_2\beta_2$ FRS (cFRS2), and the cGRS2 pseudogene, as well as the four encoding the cytosolic echoforms of cGRS1 ($_{cyte}$cGRS1), cARS ($_{cyte}$cARS), cHRS ($_{cyte}$cHRS) and cVRS ($_{cyte}$cVRS) were cloned in the pAG414pGPD$_{\beta 11ch}$ and expressed in the BiG Mito-Split-GFP strain (N = 2). (**A**) From the set of caaRSs tested, only cERS, cQRS, cFRS2 and $_{cyte}$cHRS micrographs are shown. (**B**) Table summarizing the GFP emission and mitochondrial localization of the caaRSs not shown in **A**). The corresponding micrographs are shown in Fig. S4A. (**C**) Fluorescence microscopy analysis of the BiG Mito-Split-GFP strain expressing the first 100 amino acids of the N-ter region of the cCRS fused to GFP$_{\beta 11ch}$ (N = 2). (**D**) Fluorescence microscopy analyses of BiG Mito-Split-GFP strain transformed with pAG414pGPD$_{\beta 11ch}$ expressing the mitochondrial echoforms $_{mte}$cGRS1, $_{mte}$cARS, $_{mte}$cHRS and $_{mte}$cVRS. Schematics of cARS, cGRS1, cHRS and cVRS echoforms expression in yeast. Expression can be initiated upstream of the initiator ATG$_{+1}$ ($_{mte}$cARS at ACG$_{-75}$ and $_{mte}$cGRS1 at TTG$_{-69}$) but the synthesis of this echoform can also be initiated at the ATG$_{+1}$. In this case, the expression of the cytosolic echoform is initiated downstream ($_{cyte}$cHTS at ATG$_{+60}$ and $_{cyte}$cVRS at ATG$_{+148}$). Mitochondria were stained with MitoTracker Red CMXRos. Scale bar: 5 μm. Representative fields are shown.

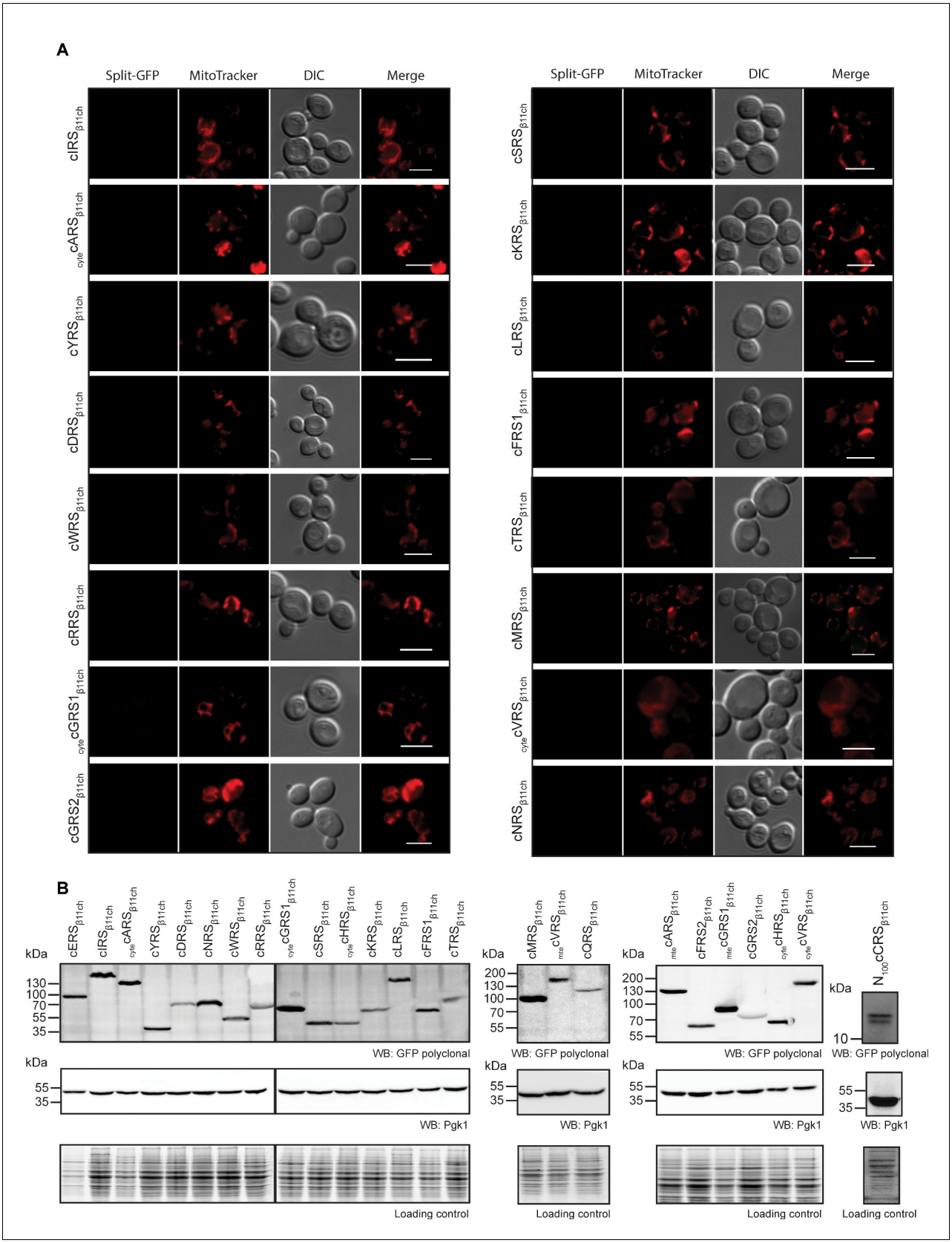


Figure 3—figure supplement 1. Screening of caaRSs and expression level of each GFP$_{\beta 11ch}$-tagged proteins (related to ***Figure 3***). (**A**) Micrographs of all the other caaRSs tested in ***Figure 3A***. Representative panels from two independent experiments are shown. Mitochondria were stained with

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MitoTracker Red CMXRos. Scale bar: 5 μm. Representative fields are shown. (**B**) Immunodetection of all the $GFP_{\beta 11ch}$-tagged aaRSs expressed in the BiG Mito-Split-GFP strain. $aaRS_{\beta 11ch}$ were detected by anti-GFP antibodies. Equal loading was verified by anti-Pgk1 antibodies and by stain-free technology (Loading control). caaRS: cytosolic aaRS, $_{cyte}$caaRS: cytosolic echoform of the caaRS, $_{mte}$caaRS: mitochondrial echoform of the caaRS.

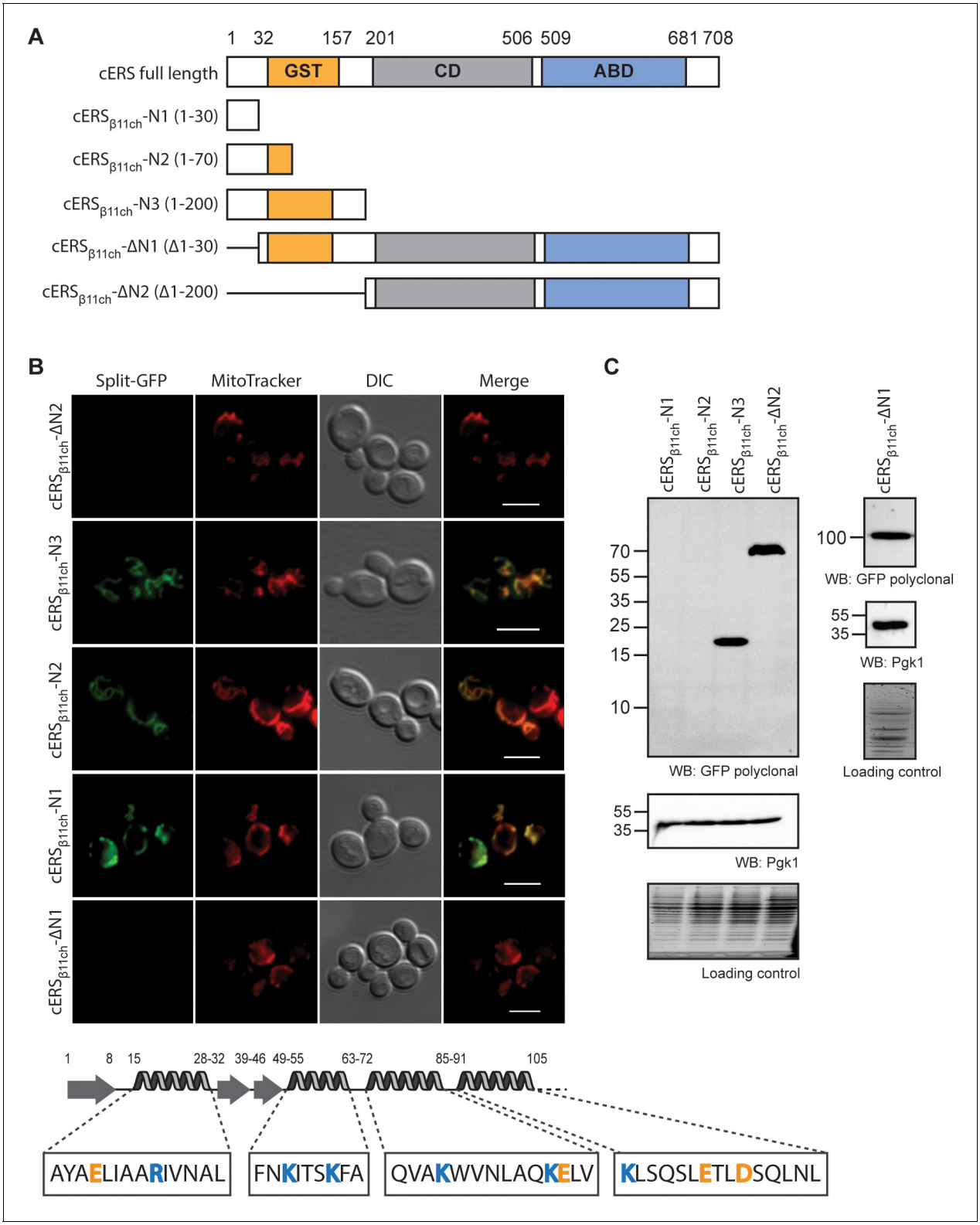

Figure 4. The BiG Mito-Split-GFP is a suitable tool to delimit regions containing non-canonical MTSs. (**A**) Schematic representation of the cERS fragments fused to GFP$_{β11ch}$. Orange boxes correspond to the GST-like domain necessary for Arc1 interaction (GST), the grey boxes represent the catalytic domain (CD), and the blue box, the tRNA-binding domain generally named anti-codon binding domain (ABD). Numbering above corresponds to cERS amino acids residues. (**B**) Fluorescence microscopy analyses of the BiG Mito-Split-GFP strain expressing the cERS variants shown on **A**. Mitochondria were stained with MitoTracker Red CMXRos; scale bar: 5 µm. The secondary structure (according to ***Simader et al., 2006***) of the smallest

Figure 4 continued on next page

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peptide that still contains the non-conventional MTS of cERS is described together with the amino acid sequence of each helices. Positively and negatively charged amino acids are shown in orange and blue respectively. (**C**) Immunodetection of the cERS variants in BiG Mito-Split-GFP whole cell extracts using anti-GFP antibodies. Quantity of proteins loaded in each lane was estimated using anti-Pgk1 antibodies or by the stain-free procedure. The bands corresponding to the mutants N1 and N2 could not be detected. The representative fields or gel are shown.

Figure 4—figure supplement 1. Analysis of N-terminal sequences of mitochondrial aaRSs and echoforms.

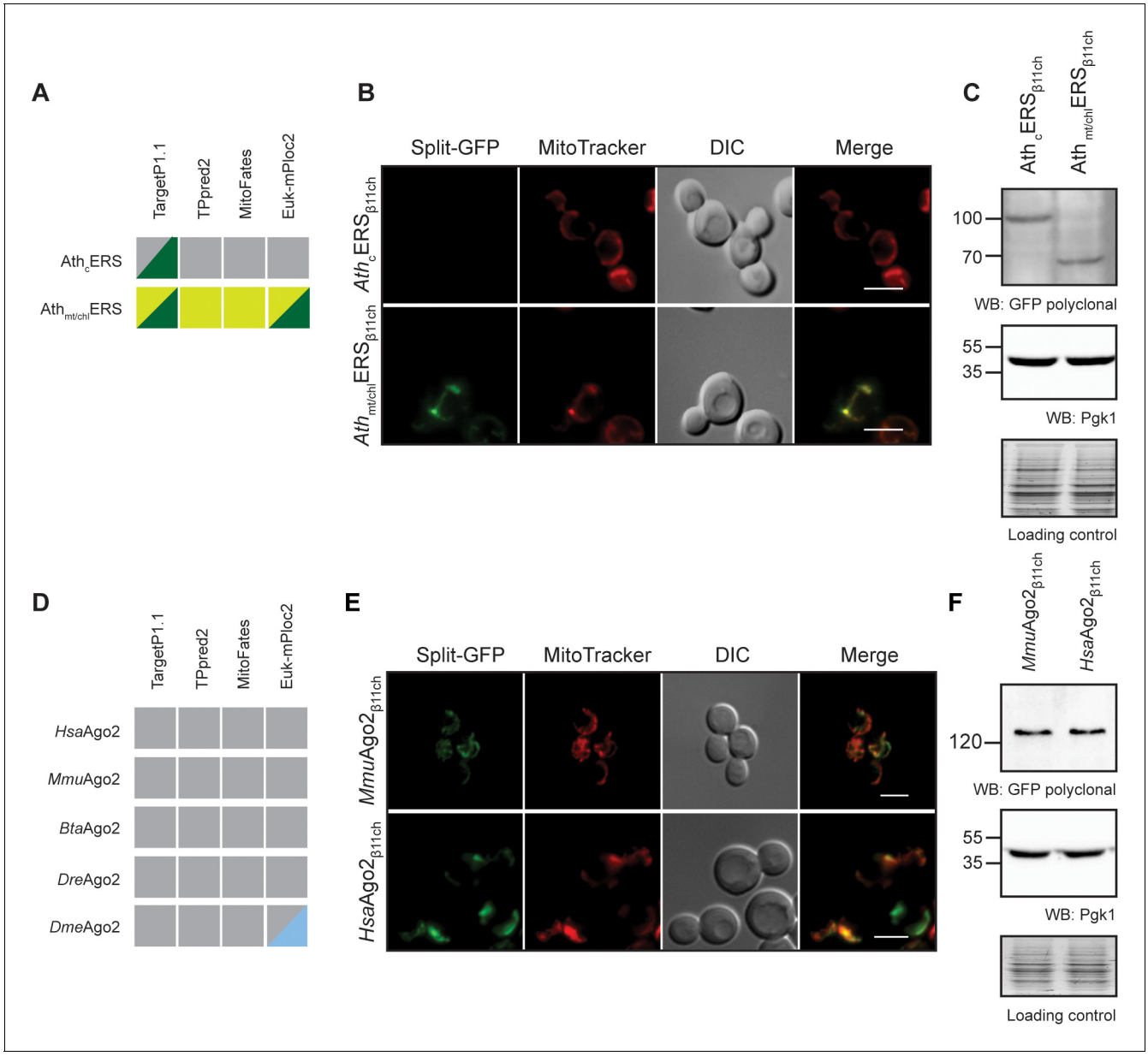

Figure 5. The BiG Mito-Split-GFP can be used to study mitochondrial importability of mammalian and plant proteins. (**A, D**) Prediction of MTS and mitochondrial localization of (**A**) two ERS from *Arabidopsis thaliana* (*Ath*cERS and *Ath*mt/chlERS) and (**D**) five eukaryotic Ago2 proteins [*Hsa*Ago2 (Protein argonaute-2 isoform X2 [Homo sapiens] NCBI sequence ID: XP_011515267.1), *Mmu*Ago2 (protein argonaute-2 *Mus musculus* NCBI sequence ID: NP_694818.3.), *Bta*Ago2 (*Bos Taurus*), *Dre*Ago2 (*Danio rerio*), *Dme*Ago2 (*Drosophila melanogaster*). MTS were predicted using TPpred2.0 (http://tppred2.biocomp.unibo.it/tppred2), TargetP1.1 (http://cbs.dtu.dk/services/TargetP/), MitoFates (http://mitf.cbrc.jp/MitoFates/cgibin/top.cgi) and the EukmPloc2 website (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/). Grey boxes indicate prediction of a cytosolic localization, light and dark green indicate prediction of mitochondrial or chloroplastic localization respectively. Blue boxes indicate prediction of nuclear localization. (**B, E**) Fluorescence microscopy analyses of the BiG Mito-Split-GFP strain expressing the GFP$_{β11ch}$-tagged $_{Ath}$cERS and $_{Ath}$mt/chlERS (N = 2) (**B**) and$_{Mmu}$Ago2, $_{Hsa}$Ago2 (N = 2) (**E**). Mitochondria were stained with MitoTracker Red CMXRos. Scale bar: 5 µm. Representative fields are shown. (**C, E**) Protein expression was checked by WB with anti-GFP antibodies and equal amount of loaded protein was controlled using anti-Pgk1 antibodies and by the stain-free technology (Loading control: stain-free). The representative gels are shown.

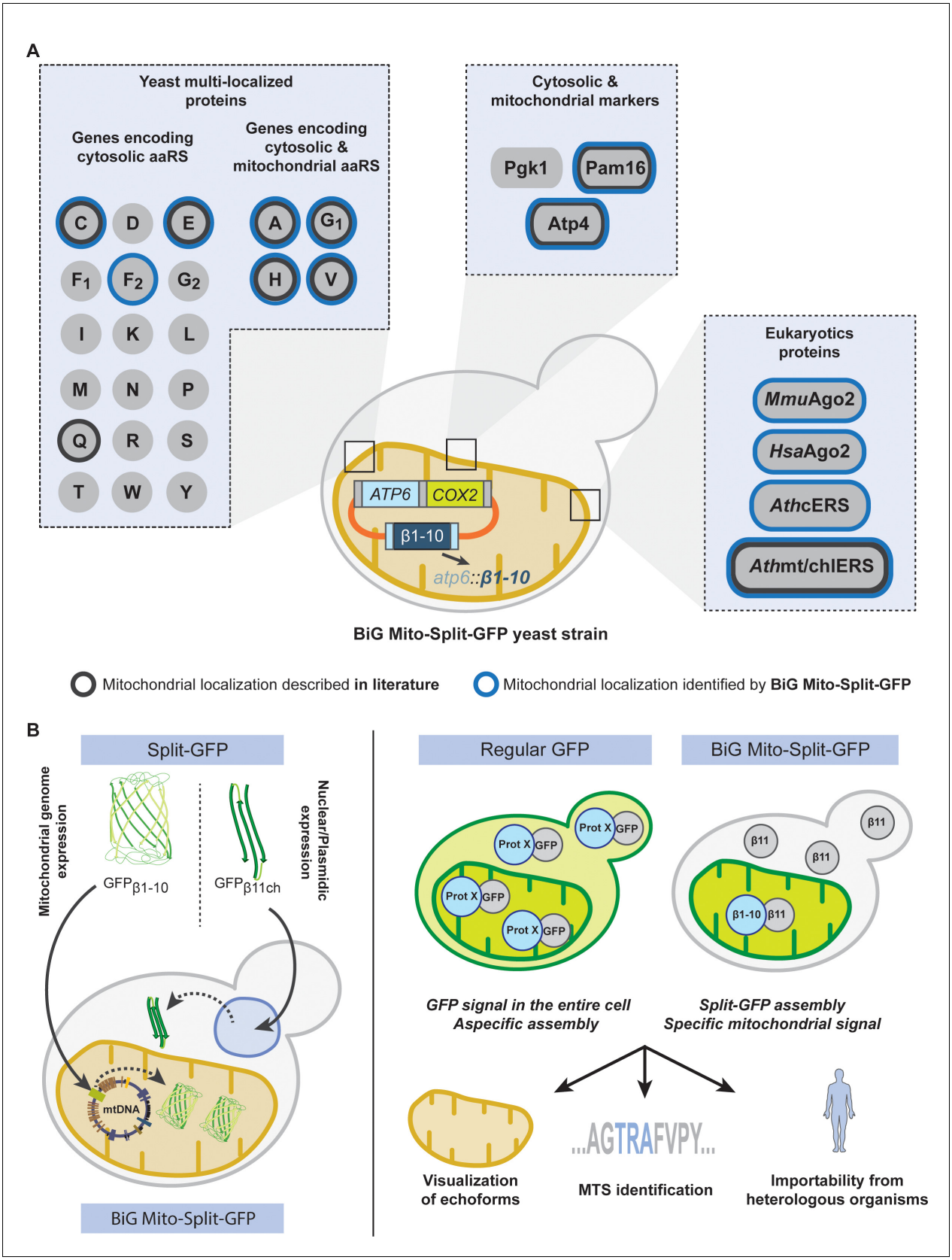

Figure 6. Schematic of the BiG Mito-Split-GFP system and its applications. (**A**) Using our engineered strain, we could show the dual localization of echoforms in the aaRS family of proteins and foster its power by studying localization of heterologous proteins originating from plants, mice and

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human. (**B**) The BiG Mito-Split-GFP strain was generated by integrating the sequence encoding the first 10 beta barrel segments into yeast mitochondrial DNA, and by either expressing any protein of interest fused to the 11th GFP segment from a plasmid or by integration in yeast nuclear DNA. As opposed to regular GFP-tagging where visualizing an echoform ultimately results in a GFP signal diffusing in the entire cell, our BiG Mito-Split-GFP system abolishes the fluorescence originating from cytosolic echoform to only display a specific mitochondrial signal. Further applications range from high-throughput experiments to identify relocating proteins involved in mitochondria homeostasis or metabolism, to identify non-conventional MTSs or seek for mitochondrial localization of heterologous proteins.