PROTEIN TARGETING

# An ER surface retrieval pathway safeguards the import of mitochondrial membrane proteins in yeast

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**The majority of organellar proteins are translated on cytosolic ribosomes and must be sorted correctly to function. Targeting routes have been identified for organelles such as peroxisomes and the endoplasmic reticulum (ER). However, little is known about the initial steps of targeting of mitochondrial proteins. In this study, we used a genome-wide screen in yeast and identified factors critical for the intracellular sorting of the mitochondrial inner membrane protein Oxa1. The screen uncovered an unexpected path, termed ER-SURF, for targeting of mitochondrial membrane proteins. This pathway retrieves mitochondrial proteins from the ER surface and reroutes them to mitochondria with the aid of the ER-localized chaperone Djp1. Hence, cells use the expanse of the ER surfaces as a fail-safe to maximize productive mitochondrial protein targeting.**

Despite our detailed understanding of the translocation routes into mitochondria, little is known about cytosolic targeting of mitochondrial precursors (*1*, *2*). To identify factors that take part in early targeting steps of mitochondrial membrane proteins, we designed a genetic screen in yeast, monitoring the cytosolic accumulation of nonimported mitochondrial precursors. To this end, we integrated the coding sequence of orotidine-phosphate decarboxylase (Ura3) into the C terminus of the nuclear encoded inner membrane protein Oxa1 while maintaining the endogenous flanking regions of the *OXA1* gene (Fig. 1A). The corresponding Oxa1-Ura3 protein, expressed in the absence or presence of endogenous Oxa1, was efficiently targeted to mitochondria, integrated into the inner membrane, and fully functional (Fig. 1B and fig. S1, A to D). Owing to efficient targeting of Oxa1-Ura3 to mitochondria, Ura3 was sequestered from the cytosol, causing a severe growth defect on media lacking uracil. This effect was reverted when the presequence of Oxa1-Ura3 was deleted (ΔN-Oxa1-Ura3), causing its cytosolic accumulation and subsequent uracil-independent growth (Fig. 1C). Hence, a defect in mitochondrial targeting could be monitored by growth on medium lacking uracil.

Using automated mating approaches, the Oxa1-Ura3 construct was introduced into yeast libraries covering 4916 deletion mutants of nonessential genes as well as 1102 DAmP (decreased abundance by mRNA perturbation) mutants of essential genes (*3*) (Fig. 1D). Twelve mutants displayed particularly strong growth on uracil-deficient media, suggesting critical roles of corresponding proteins in preventing cytosolic accumulation of the Oxa1-Ura3 precursor (Fig. 1E). Whereas some of the identified factors were expected (e.g., Tim50, an essential subunit of the TIM23 translocase), several of the hits were nonmitochondrial proteins for which a role in mitochondrial protein import or precursor quality control was unknown. These include the uncharacterized proteins Yil029c, Ylr050c, and Ycr100c, which we named Ema17, Ema19, and Ema35, respectively (for efficient mitochondria targeting–associated proteins). These three components are predicted to be membrane proteins but were not previously found in mitochondria (*4*). Ema19 is embedded in the endoplasmic reticulum (ER) membrane and conserved among eukaryotes. Deletion mutants lacking Ema19 or Ema35 showed respiration problems at elevated temperatures (fig. S2, A to E).

One of the identified components was Djp1, an abundant yet poorly characterized member of the J-protein/Hsp40 cochaperone family (*5*). Oxa1-Ura3 growth assays with strains lacking

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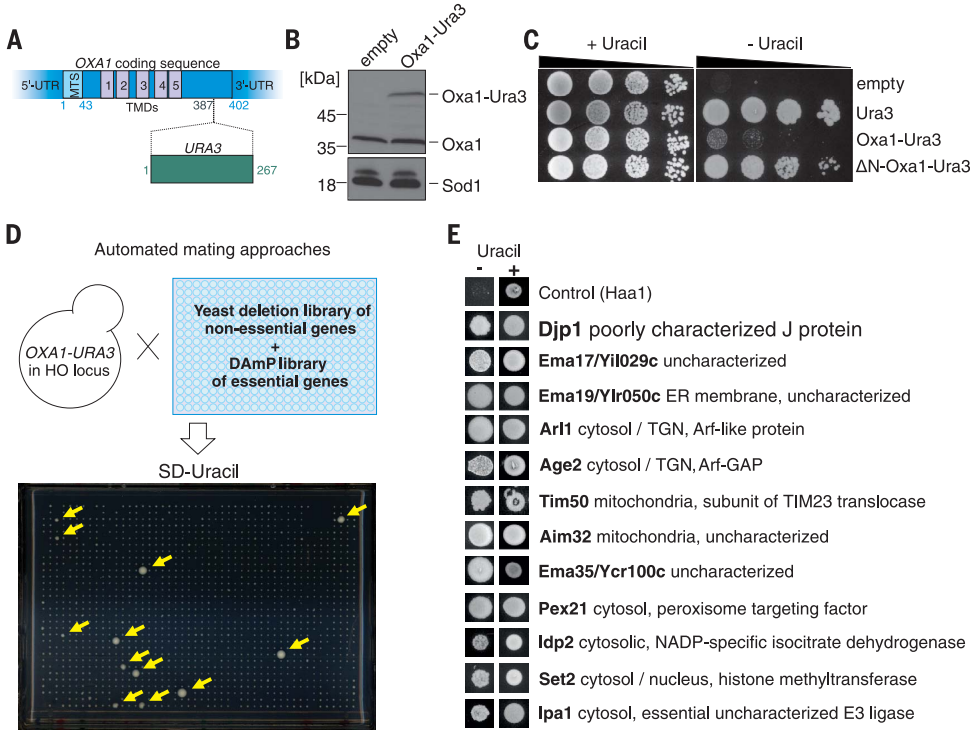


**Fig. 1. Genetic screen for mutants that accumulate Oxa1-Ura3 in the cytosol.** (**A**) Structure of the Oxa1-Ura3 construct. TMD, transmembrane domain; MTS, mitochondrial targeting sequence; UTR, untranslated region. (**B**) Immune blot of Oxa1-Ura3. (**C**) Growth of Δ*ura3* mutants expressing the indicated constructs. (**D**) Representative array plate on uracil-deficient medium. Yellow arrows indicate mutants accumulating Oxa1-Ura3 in the cytosol. SD, synthetic dextrose medium. (**E**) Proteins critical for the prevention of cytosolic accumulation of Oxa1-Ura3. TGN, trans-Golgi network; GAP, guanosine triphosphatase activating protein; NADP, nicotinamide adenine dinucleotide phosphate.

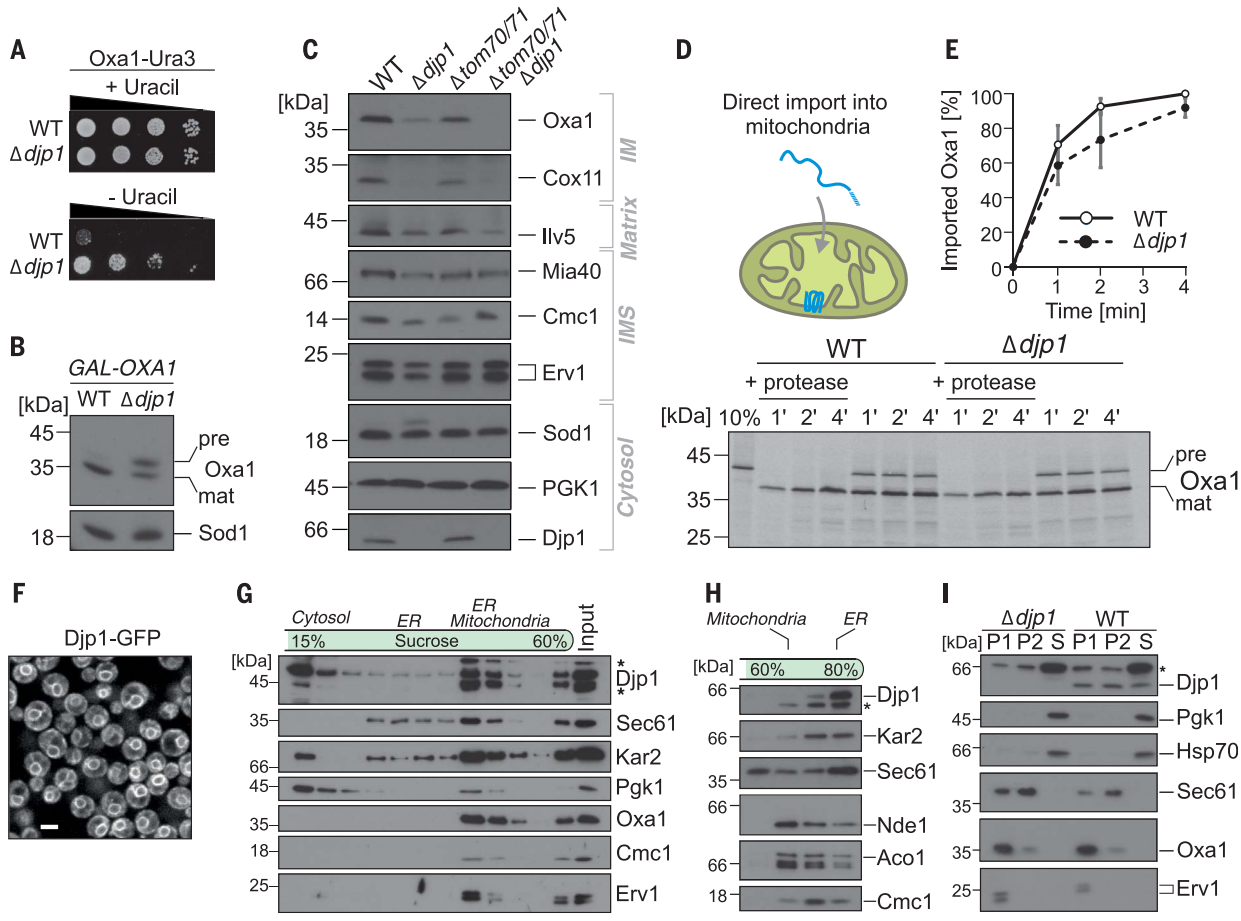


**Fig. 2. Djp1 is critical for mitochondrial biogenesis.** (**A**) Growth on synthetic medium with or without uracil. (**B**) Oxa1 was overexpressed in wild-type (WT) and Δ*djp1* using the GAL promoter. Cell extracts were analyzed by immune blot; pre, precursor; mat, mature. (**C**) Cellular extracts were analyzed by immune blotting. IMS, intermembrane space. (**D** and **E**) Radiolabeled Oxa1 precursor (pre) was incubated with WT or Δ*djp1* mitochondria. Data shown are means ± SD; *n* = 4 replicates. (**F**) Djp1-GFP shows a perinuclear staining typical for ER proteins. Scale bar, 5 μm. (**G** to **I**) Cell extracts were separated on sucrose gradients or by differential centrifugation. A large fraction of Djp1 cofractionates with ER membranes. 12,000*g* and 30,000*g* pellets are labeled as P1 and P2, respectively. S, supernatant. Asterisks indicate cross-reactions of antibodies.

other J-proteins confirmed a specific role for Djp1 (fig. S3, A to E). Djp1 is involved in peroxisomal import and the biogenesis of the mitochondrial outer membrane protein Mim1; however, the mechanism of its function was not elucidated (*6*, *7*). The robust growth of the Oxa1-Ura3–expressing Δ*djp1* mutant in the absence of uracil suggests that Djp1 plays a role in targeting or import of Oxa1 (Fig. 2A). Indeed, in Δ*djp1* cells we observed reduced levels of endogenous Oxa1 and a strong accumulation of the precursor when Oxa1 was overexpressed (Fig. 2, B and C, and fig. S4A). The relevance of Djp1 was not restricted to Oxa1. The steady-state levels of multiple mitochondrial proteins were considerably reduced in Δ*djp1* cells. Djp1 was particularly important in mutants lacking the mitochondrial preprotein receptors Tom70/71 (*6*) (Fig. 2C and fig. S4, B and C). However, in vitro, we did not observe a considerably reduced Oxa1 import efficiency of isolated Δ*djp1* mitochondria (Fig. 2, D and E). This suggests that Djp1 plays a role in Oxa1 targeting that is upstream of the translocation reaction.

Systematic localization studies previously identified Djp1 as an ER-associated protein (*8*), which we confirmed by fluorescence microscopy (Fig. 2F) and subcellular fractionation (Fig. 2, G to I). In addition, a fraction of Djp1 was present in the cytosol where it did not appear to interact with ribosomes (fig. S5A). We could not exclude that a small fraction of Djp1 may be bound to mitochondria. The ER-binding of Djp1 was very tight, nucleotide independent, and also observed in mutants lacking Ema19 or Ema35, although it appeared to be reduced in these mutants (fig. S5, B to E).

Why would an ER protein affect mitochondrial targeting? A fraction of Oxa1–green fluorescent protein (GFP) was ER-localized in Δ*djp1* but not in wild-type cells (Fig. 3A). This fraction considerably increased upon depletion of Cdc48, a component crucial for the proteasomal degradation of aberrant ER-associated proteins. Accordingly, Oxa1 lacking its mitochondrial presequence (ΔN-Oxa1) was partially glycosylated (fig. S6A). Glycosylated Oxa1 was also observed upon overexpression of Oxa1, particularly in Δ*djp1* cells. This suggests that in Δ*djp1* cells, a fraction of Oxa1 that accumulates on the ER surface gets integrated into the membrane, glycosylated, and recognized as mislocalized.

Because Djp1 is present at different cellular locations, we tested whether the ER-bound Djp1 is critical for Oxa1 biogenesis: We expressed Djp1-GFP in the presence of GFP-binding chromobody traps that restricted Djp1 either to the ER (Erg11-binder) or to the vacuole (Vph1-binder) (Fig. 3B and fig. S6, B to D). The ER-tethered Djp1 version, but not that on the vacuolar membrane, fully promoted Oxa1 import into mitochondria (Fig. 3, C to E). In the absence of Djp1, Oxa1 may be inserted into the ER membrane, glycosylated, and recognized as aberrant and degraded (fig. S6E).

In vitro binding experiments showed that the ER surface binds Oxa1 precursor in a Djp1-mediated manner (Fig. 3F and fig. S7, A to D). It has previously been assumed that any targeting of mitochondrial proteins to the ER would be a dead end, resulting in recognition of mistargeting and degradation. However, our results suggest that the association with the ER surface could

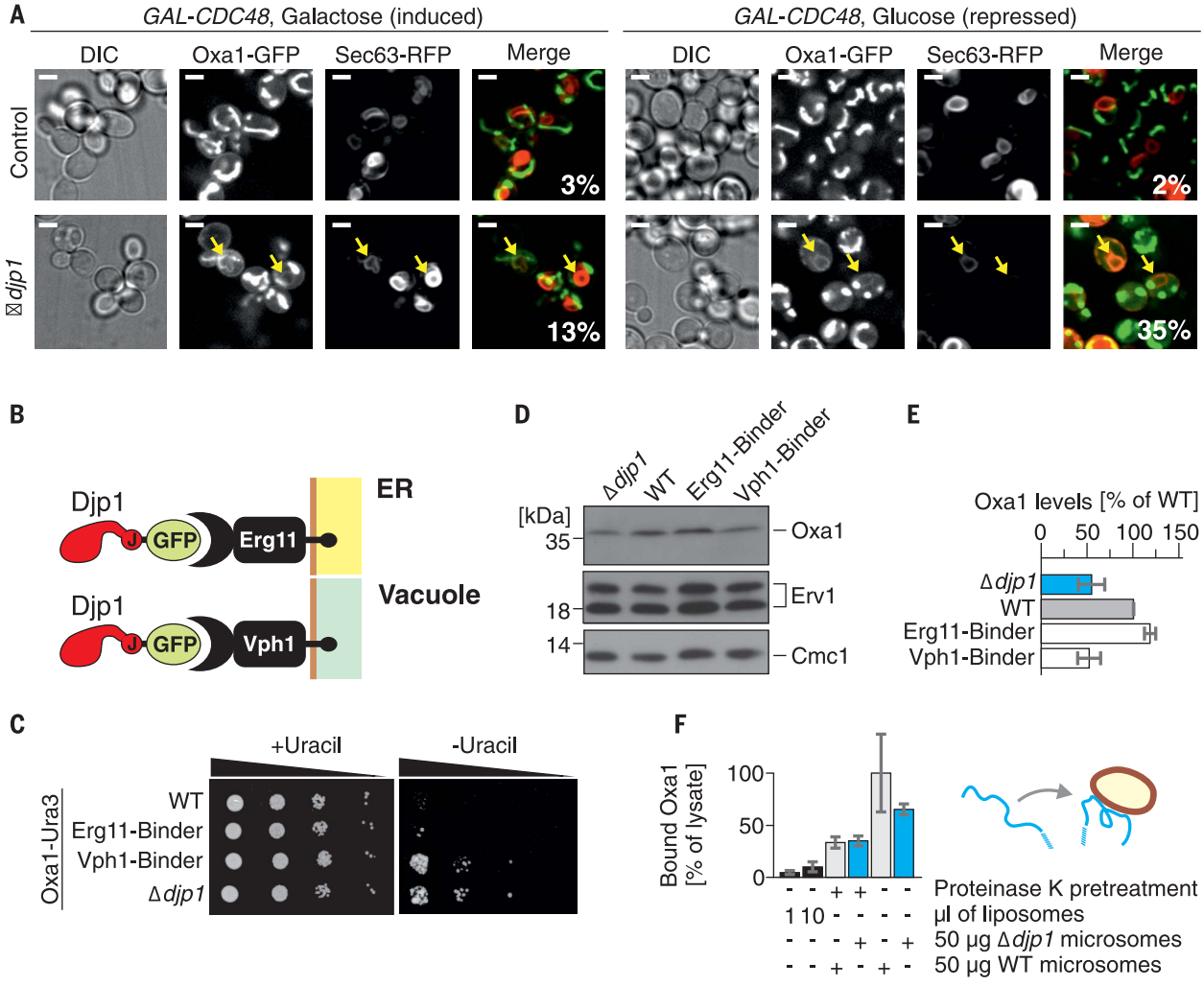


**Fig. 3. Djp1 prevents the accumulation of Oxa1 on the ER.** (**A**) In the absence of Djp1, Oxa1 partially accumulated on the ER, especially upon depletion of Cdc48. Sec63-RFP served as an ER marker. The percentage of cells showing ER-localized Oxa1 is depicted. Scale bars, 5 µm. RFP, red fluorescent protein; DIC, differential interference contrast. (**B**) Chromobody-based binders to trap Djp1 on the ER or the vacuole. (**C** to **E**) ER-localized Djp1 promotes efficient import of Oxa1-Ura3 into mitochondria and leads to WT levels of Oxa1. Data shown are means ± SD; $n = 3$. (**F**) Oxa1 precursor was incubated with liposomes or protease-treated and -untreated microsomes. Membranes were reisolated, and the associated Oxa1 was quantified. The bound Oxa1 was normalized to the WT microsome sample. Means ± SE are shown; $n = 3$.

instead be an intermediate in productive protein targeting to mitochondria. To test whether the ER-localized Oxa1 is imported into mitochondria, we tethered Oxa1 mRNA to the ER surface such that all Oxa1 was translated on ER membranes (*9*). Under these conditions, Djp1 became critical for respiration competence, indicating a crucial role in the productive Oxa1 transfer from the ER to mitochondria (fig. S7, E and F).

To investigate mitochondrial import of Oxa1 in a more physiological environment, we employed semi-intact cells with permeabilized plasma membranes (*10*) (Fig. 4A). The in vitro import into semi-intact cells was similar to the import into isolated mitochondria requiring mitochondrial membrane potential and mitochondrial translocases (fig. S8, A to C). However, in semi-intact cells, the mitochondrial import of Oxa1 and other mitochondrial membrane proteins was considerably less efficient in the absence of Djp1 (Fig. 4, B and C, and fig. S8, D to F). Preloading of Δ*djp1* semi-intact cells with purified Djp1 restored their competence to import Oxa1 (fig. S8J). Oxa1 import was almost fully blocked in semi-intact Δ*tom70/71*/Δ*djp1* cells, indicating that Djp1 and Tom70 cooperate (fig. S8, A and B).

In vitro Djp1 considerably stimulated the import of ER-bound mitochondrial precursors, including that of Oxa1. Hence, Djp1 and other ER proteins maintained import-competent precursors (Fig. 4, D to G, and fig. S8G). Djp1 was particularly critical for the microsome-to-mitochondria transfer of the very hydrophobic inner membrane protein Coq2 (fig. S8H), which showed a profound ER association in Δ*djp1* cells (fig. S8I). When we co-incubated wild-type and Δ*djp1* microsomes with low, rate-limiting amounts of mitochondria, a strong Djp1-dependent stimulation of the Oxa1 import was observed (Fig. 4, H and I, arrows). Thus, the ER supports import into mitochondria rather than competes with it. In this reaction, we observed a direct binding of Oxa1 precursors to Djp1, particularly when the mitochondrial membrane potential was depleted (Fig. 4J).

Taken together, Oxa1 precursor was found to absorb onto but not translocate into microsomes from where it was transferred to mitochondria in a Djp1-stimulated reaction (fig. S9, A to C). Soluble translocation intermediates of Oxa1 were not observed in this process, nor did mutation of the HPD motif in the J domain of Djp1 compromise its function (fig. S9, D to G).

The early stages of mitochondrial preprotein targeting are poorly understood. Cytosolic chaperones (*11*, *12*) and stabilizing factors called ubiquilins (*13*) associate with mitochondrial preproteins to prevent precursor-mediated proteotoxic

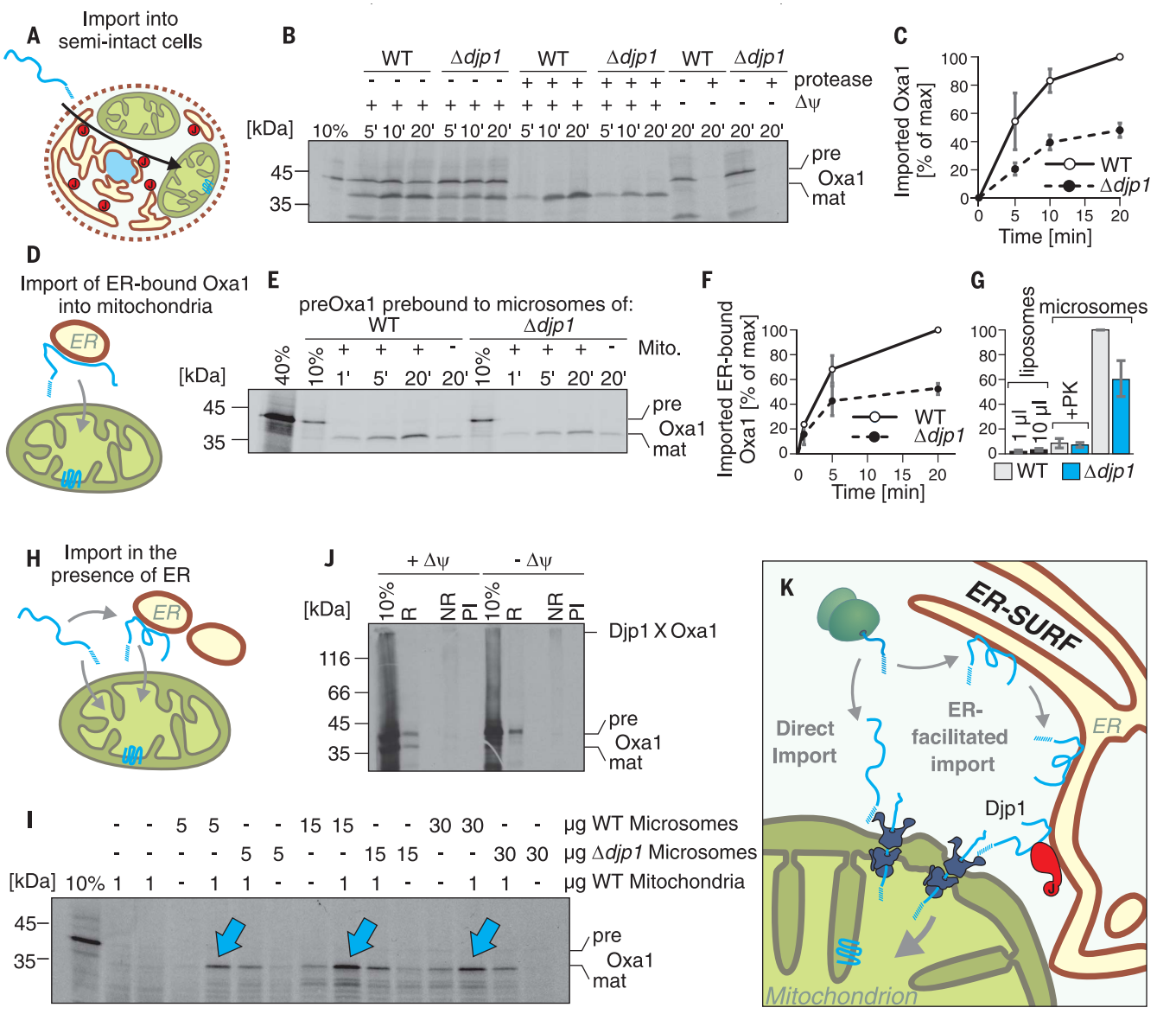


**Fig. 4. The ER facilitates Oxa1 import in a Djp1-dependent process.** (**A** to **C**) Import of radiolabeled Oxa1 precursor into semi-intact cells. Amounts of protease-resistant mature Oxa1 were quantified. Data shown are means ± SD; *n* = 4. (**D** to **G**) Djp1 promotes the import of ER-bound Oxa1. Quantification shows means ± SD; *n* = 3. (G) Microsomes can transfer Oxa1 to mitochondria. Graph shows means ± SE; *n* = 3. (**H** and **I**) Radiolabeled Oxa1 was incubated with WT and Δ*djp1* microsomes to which low (rate-limiting) amounts of WT mitochondria were added. Nonimported Oxa1 was removed by protease. Arrows indicate Oxa1 import. (**J**) Radiolabeled Oxa1 precursor was incubated with semi-intact cells. After cross-linking with 400 µM DSP, Djp1 was immune-precipitated. NR, nonreducing; R, reducing (i.e., cross-links were broken); PI, preimmune serum. (**K**) Mitochondria can import Oxa1 precursor directly. However, in vivo, a fraction of Oxa1 associates with the ER surface. The ER-SURF pathway maintains the Oxa1 precursor in an import-competent state and facilitates its rerouting to mitochondria in a Djp1-dependent reaction.

stress (*14*, *15*). Here we found that the ER surface can function as a capture net to salvage and redirect mitochondrial precursors and thus facilitate early targeting reactions by an import route that we termed the ER-SURF pathway (for ER surface–mediated protein targeting) (Fig. 4K). Our findings are consistent with a previous study that used a comprehensive proximity-based ribosome profiling approach showing that many mitochondrial membrane proteins are preferentially synthesized on the ER surface (*2*). The conservation and importance of Ema19 in this pathway (fig. S8, K and L) suggests that this rerouting mechanism may be conserved among eukaryotes, including humans.

Hydrophobic mitochondrial proteins were previously observed on the ER, which was interpreted as mislocalization (*16*–*19*). However, our observations suggest that the ER surface can serve as a safeguard in targeting of mitochondrial precursor proteins, from where they are retrieved in a Djp1-mediated reaction. This ER-SURF targeting pathway could explain the difficulty in identifying targeting factors for mitochondrial proteins.

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**SUPPLEMENTARY MATERIALS**

www.sciencemag.org/content/361/6407/1118/suppl/DC1
Materials and Methods
Figs. S1 to S9
References (*20–28*)

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**ER-SURF protein import into mitochondria**

Eukaryotic cells contain membrane-bound organelles, defined by distinct protein compositions. Almost all cellular proteins are synthesized in the cytosol, and thus, organelle-resident proteins must be directed to their appropriate location after synthesis. Working in yeast, Hansen *et al.* identified a protein-targeting paradigm termed ER-SURF, in which the membrane expanse of the endoplasmic reticulum (ER) serves as a "capture net" for mitochondrial proteins. This process productively redirected mitochondrial precursor proteins for efficient mitochondrial import. Thus, two distinct organelles, once thought to be mutually exclusive protein destinations, can cooperate during protein targeting.

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