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Supplementary Materials for

Mitochondrial metabolic regulation by GRP78

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SUPPLEMENTARY RESULTS

Results

We first determined the close localization of ER and mitochondria in the rat testis (fig. S1A and B). To confirm the subcellular localization of StAR and GRP78, we immunostained testicular tissue individually with StAR (S1C-E) and GRP78 (S1F-H) antibodies and analyzed their localization by EM. StAR staining was detected throughout the cell (fig. S1C), and enlargement of the image showed ER (fig. S1D) and mitochondria (fig. S1E) staining. However, staining for GRP78 (S1G-H) confirmed that it was primarily localized to the ER. Taken together, the above results support the view that StAR and GRP78 may interact directly to regulate mitochondrial metabolism.

GRP78 and StAR may transiently interact at the MAM prior to StAR reaching the mitochondrial membrane. To understand the rationality of the presence of StAR and GRP78 in the same complex, we fractionated organelles isolated from the MA-10 cells (fig. S2, left panels) or testes (fig. S2, right panels) and then probed them with ER and mitochondrial antibodies against phosphofurin acidic cluster sorting protein 2 (PACS2), calnexin, GRP78 and StAR, Tom40. PACS2 and calnexin were present in the ER and MAM fractions (fig. S2). As expected, the mitochondrial import channel protein, Tom40, was only detected in the mitochondrial fraction, confirming the accuracy of the organelle fractionation (fig. S2). In both cells (left panel) and tissues (right panel), a greater proportion of StAR was present in the MAM than in the mitochondria; however, the proportion of GRP78 was greater in the ER as compared to the MAM (fig. S2). Thus, organelle fractionation analysis suggests the colocalization of StAR and GRP78.
We next analyzed the organelle-specific localization of StAR and GRP78 in MA-10 cells following stimulation with cAMP for 30 min, 2 h and 6 h to confirm their colocalization at the MAM before StAR transits to the mitochondria. We hypothesized that if GRP78 has a role in StAR folding at the MAM, there will be a gradual increase of StAR at the OMM after leaving MAM. As shown in fig. S3A, EM analysis revealed that StAR was localized minimally to mitochondria in the absence of cAMP, and an enlarged view showed that it is specifically localized at the OMM and also within cristae of the IMS, but not in the free mitochondrial space (fig. S3A, mito). Analysis of StAR in MAM fractions obtained through Percoll density fractionation showed that a StAR was present in the MAM fraction in the absence of cAMP (fig. S3A, MAM).

On stimulation with cAMP for 30 min, the expression of StAR was increased as evidenced by the increased number of gold labels (fig. S3B, total). StAR was present around mitochondria specifically at the OMM as well as the matrix (fig. S3B, mito). Interestingly, there were more StAR present at the MAM

region (fig. S3B, enlarged MAM). At 2 h cAMP stimulation, even more StAR was observed both at the MAM and mitochondria (fig. S3C, mito and MAM). However, at 6 h, most of the StAR was present at the OMM and inside the mitochondria (fig. 3D, mito and MAM). As controls, the localization of the matrix resident, aldosterone synthase (AS), was determined, showing that indeed it is localized in the mitochondria (fig. S4A); Tom22 showed OMM-specific localization (fig. S4B, enlarged MAM lane). StAR was primarily present at the MAM on shorter cAMP stimulation, moving to the OMM on longer stimulation (fig. S3H and I). A similar analysis of GRP78 revealed that in the absence of cAMP stimulation, it was localized all over the cytoplasm and at the OMM and MAM (fig. S3E total and MAM). As the distribution and expression level of GRP78 did not change significantly in response to cAMP, the association between StAR and GRP78 is likely transient and facilitated by StAR expression (fig. S3F, G, J and K). The expression of calnexin was present only in the MAM fractions confirming an accuracy of our MAM preparation (fig. S4C).

The stimulation of cells with cAMP did not change the mitochondrial structure as determined by the localization of Aldosterone synthase (fig. S4A) and Tom22 (fig. S4B) antibodies. Similarly, the ER architecture remained unchanged as amplified MAM section was unchanged for calnexin (fig. S4C). To confirm their colocalization at the MAM, EM analysis of MA-10 cells after stimulation with cAMP was undertaken by probing the cells with GRP78 and StAR antibodies together. We analyzed the distributions of GRP78 and StAR at 30 min and 6 h following cAMP stimulation. As shown in fig. S5A, GRP78 was abundantly expressed (18 ± 1.8 per $81\mu m^2$ field of view), but StAR levels were minimal (8 ± 0.8 per grid per $81\mu m^2$ field of view in the MAM and 5 ± 0.5 per $81\mu m^2$ field of view in the mito). After 30 min of cAMP, StAR levels reached 12 ± 1.2 per $81\mu m^2$ field of view in the MAM and 7 ± 0.7 per grid per $81\mu m^2$ field of view in the mito. GRP78 levels remained similar to that observed with untreated cells (20 ± 2 per grid per $81\mu m^2$ field of view; fig. S5B). After 6 h of stimulation with cAMP, StAR levels were 10 ± 1.2 per grid per $81\mu m^2$ field of view in the MAM and 12 ± 1.2 per grid per $81\mu m^2$ field of view in the mito (fig. S5C). As expected, GRP78 expression remained almost unchanged (19 ± 1.9 per $81\mu m^2$ field of view) (fig S5 A–C). The quantitative analysis shown in fig. S5E and S5F confirms that StAR levels in the MAM increased after 30 min, returning to baseline levels at 6 h; however, GRP78 levels remained unchanged. To confirm the accuracy of the experiment, we also performed colocalization analysis of calnexin and the mitochondrial resident, AS (fig. S5D). Whereas AS was localized in the matrix, calnexin was present at the MAM (fig. S5D, enlarged MAM and mito panels). As the number of StAR increased after 30 min and becomes maximal at 3 h at the MAM without a significant change in GRP78 level, the interaction is not likely permanent, but StAR may be passing through GRP78 sites.

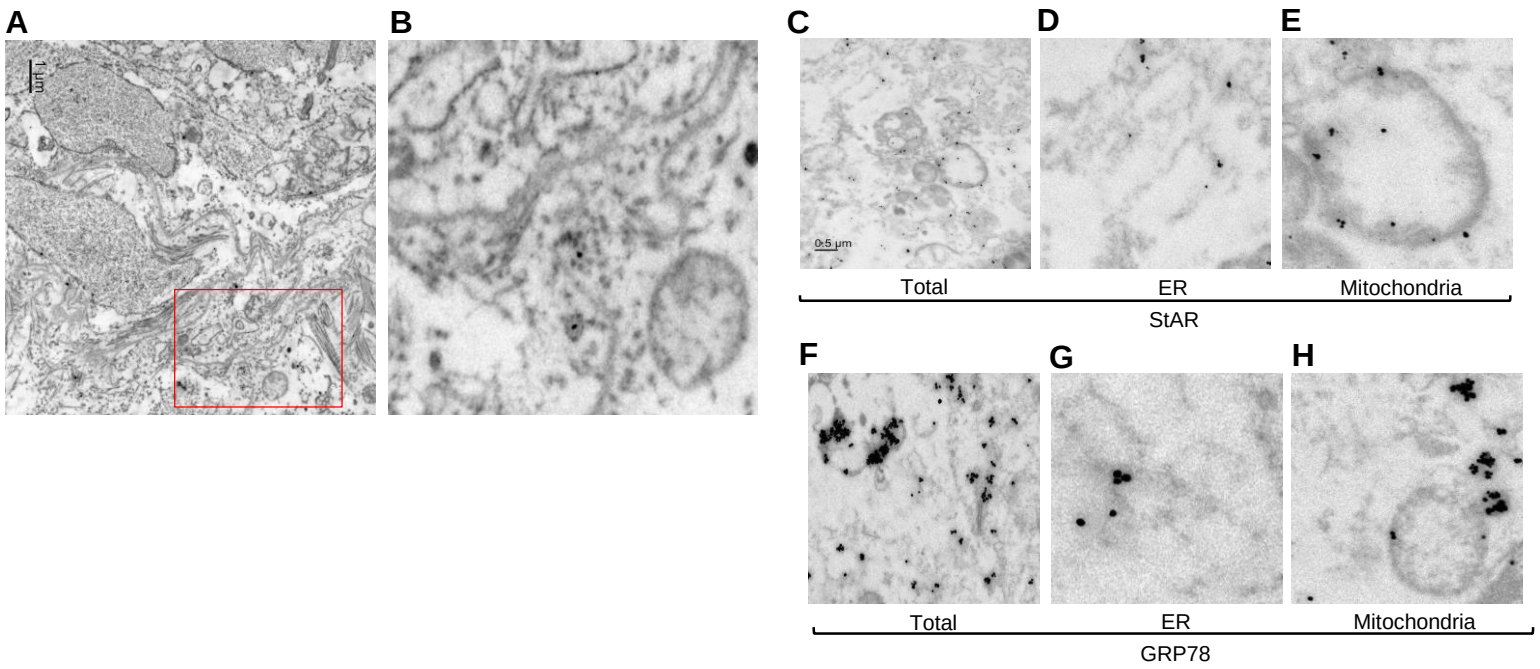

fig. S1. EM analysis of testicular tissues with a StAR and GRP78 antibody. (**A**) Electron microscopic analysis of ER and mitochondrial localization in murine testis. (**B**) An enlarged image of the red box in panel A. The right panel shows the regions of interest shown in red box schematically drawn from the original section. Em analysis of testicular tissues with a StAR (**C**–**E**) and GRP78 (**F**–**H**) antibodies showing its localization in the cytoplasm near a mitochondrion.

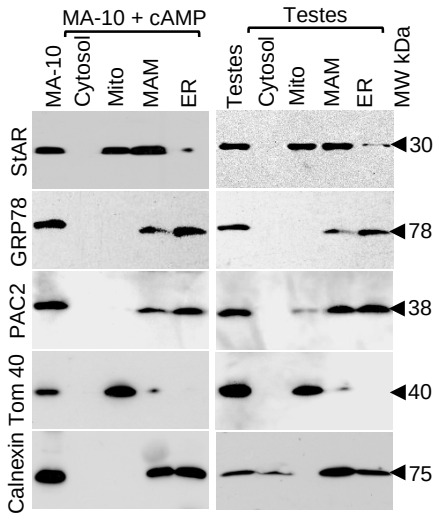

fig. S2. Identification of compartment-specific localization of StAR and GRP78 in rat testes (right) and cAMP-stimulated MA-10 cells (left). Organelle fractions were probed with the ER markers, PACS2 and calnexin, and the mitochondrial OMM-associated protein, Tom40. StAR was present in the mitochondria and MAM fractions, whereas the GRP78 was present in the MAM and ER fractions.

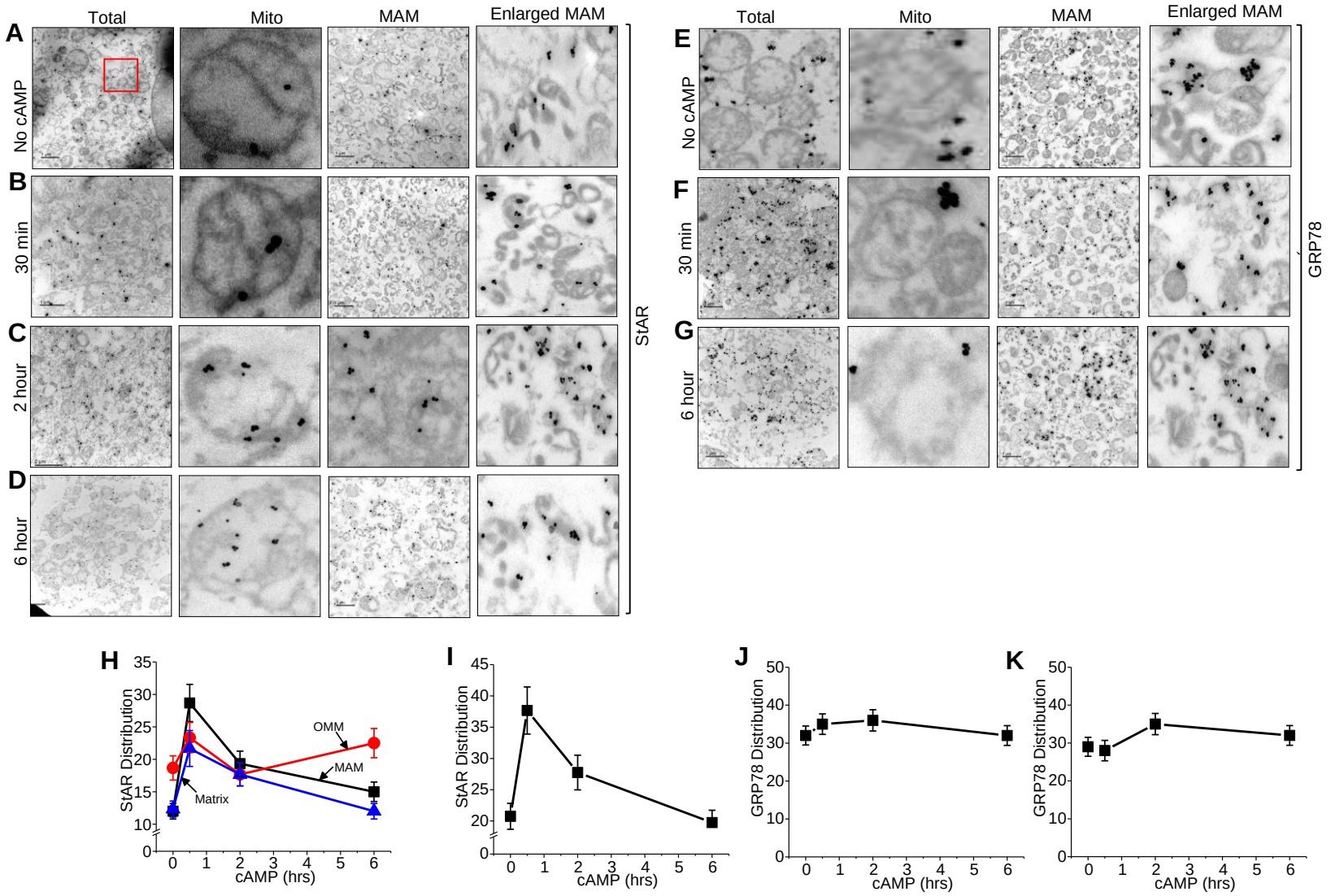

fig. S3. Kinetics of StAR folding by GRP78. (**A**–**D**) Identification of StAR localization in MA-10 cells by EM in the absence of cAMP (A) and after stimulation with cAMP for 30 min (B), 2 h (C) and 6 h (D). (**E**–**G**) Similar identification of GRP78 without stimulation (E) or following cAMP stimulation for 30 min (F) and 6 h (G). (**H**, **I**) Quantitative and kinetic analysis of the presence of StAR expression at different time points and in different cellular compartment with and without stimulation of MA-10 cells with cAMP (H), asa well as in MAM fractions (I). (**J**, **K**), Quantitative analysis after probing with whole MA-10 cells (J) or purified MAM fractions (K) with the GRP78 antibody. Data presented in panels H–K are the mean ± SEM of at least five different experiments performed at three different times.

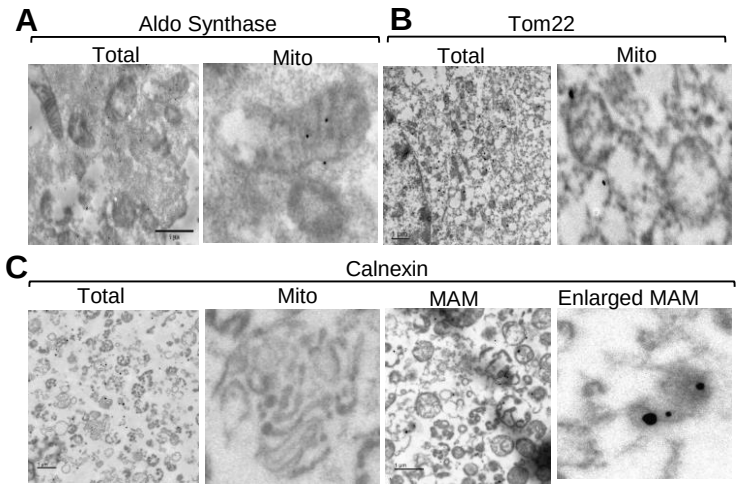

fig. S4. EM colocalization kinetics of aldosterone synthase (A), Tom22 (B), and Caln (C) in MA-10 cells in total and mito lane. Tom22 antibody staining in the MAM lane and one mitochondrion is shown in the Enlarged MAM lane.

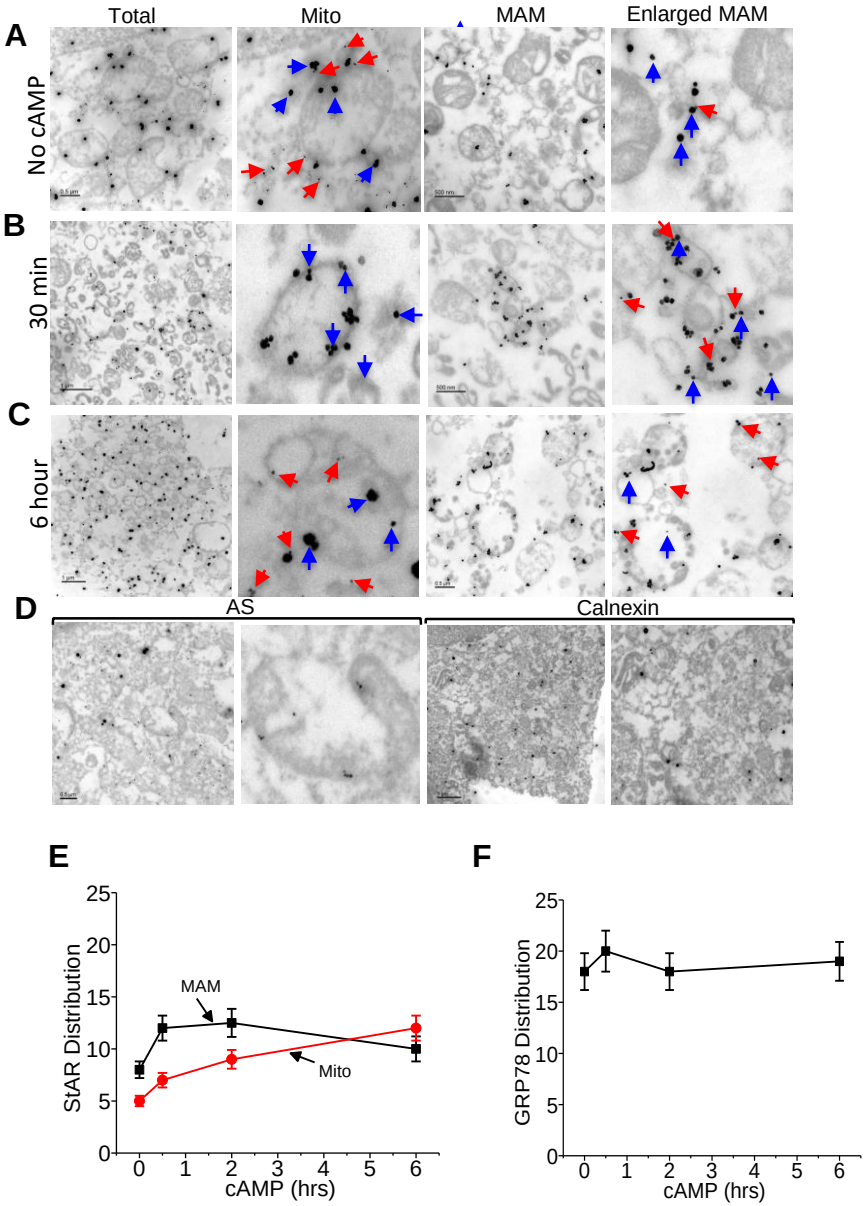

fig. S5. EM colocalization kinetics of StAR and GRP78 after cAMP stimulation after staining with StAR and GRP78 antibodies together. (**A**–**D**) Identification of the time at which StAR is transported from the MAM to mitochondria. MAM fractions isolated from control MA-10 cells (A) or those treated with cAMP for 30 min (B) or 6 h (C) and were probed with a StAR and GRP78 antibodies. (D) Colocalization of aldosterone synthase (AS) and GRP78 in whole cells, mitochondrion, MAM and Enlarged MAM sections. (**E**, **F**) Quantitative analysis of the expression pattern of StAR (E) and GRP78 (E) in the MAM and mitochondria (mito) fractions from panels A–C.

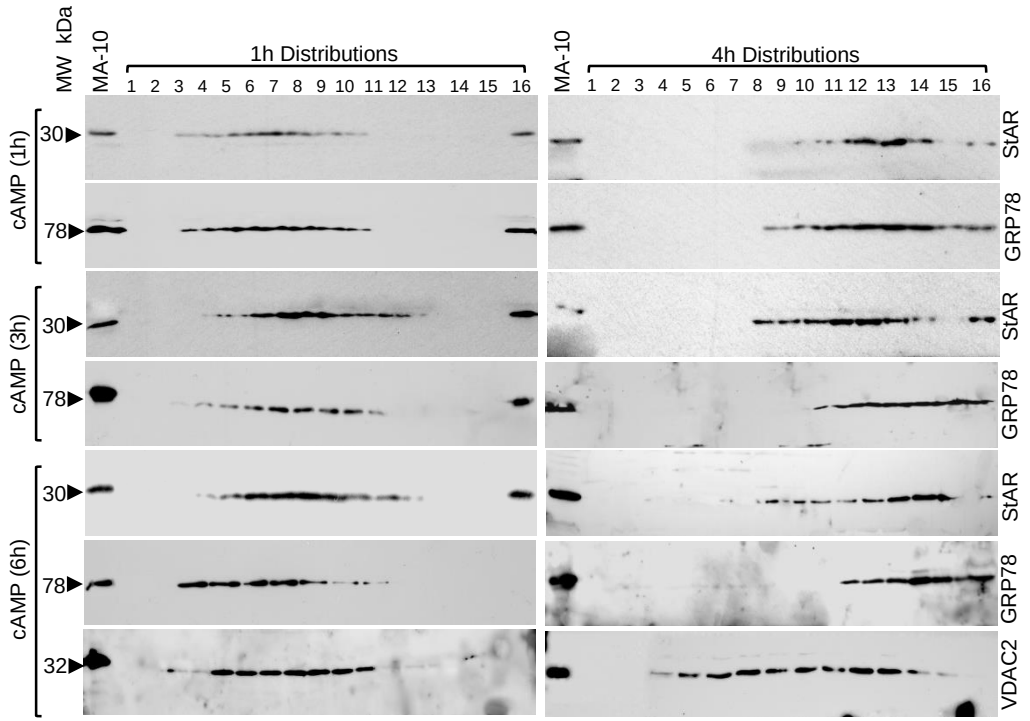

fig. S6. Colocalization kinetics of StAR and GRP78 after cAMP stimulation. Density gradient fractions of MA-10 cells or those treated with cAMP for 1, 3 and 6 h and were probed with a StAR and GRP78 antibodies after density gradient fractionation. The left panel is for 1 hour and the right panels is for 4 hours of separation. The bottom left (1h) and right (4h) panels indicate the distribution pattern of VDAC2 with the indicated time.

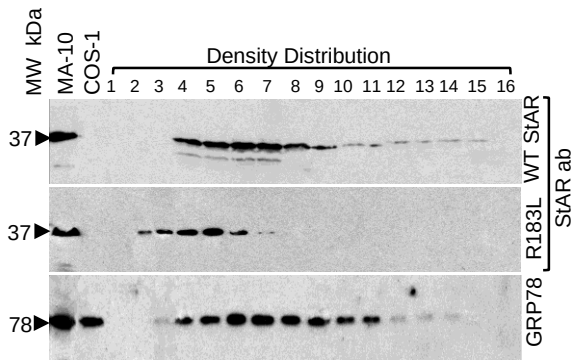

fig. S7. Colocalization kinetics of WT (top) and mutant R182L (middle) StAR after cAMP stimulation. Density gradient fractions of wild-type and mutant R182L StAR following transfection in COS-1 cells and stained with StAR antibody independently. Similar fractionation with GRP78 antibody is presented as control in the bottom panel.

table S1. One-dimensional native PAGE (3 to 16%) of mitochondrial-associated membrane native complex stained with StAR antibody (450-kDa MAM complex).

Accession No.	Protein	MS/MS Score	No. of unique peptides
50345982	ATP synthase subunit alpha c, mitochondrial	1050	15
763111	Desmolase (P450scc)	543	8
47522750	Voltage-dependent anion-selective channel protein 1	495	7
1304157	78 kDa glucose-regulated protein (GRP78)	381	5
10716563	Calnexin precursor	303	4
190200	Porin	285	6
6755965	Voltage-dependent anion-selective channel protein 2	285	6
558099117	ADP/ATP translocase 2	196	5
15928608	Solute carrier family 25	193	5
1710248	Protein disulfide isomerase-related protein 5	190	3
179468	3-beta-hydroxysteroid dehydrogenase	158	2
4557303	Fatty aldehyde dehydrogenase isoform 2	135	2
431902221	Voltage-dependent anion-selective channel protein 3	128	2
66547450	60 kDa heat shock protein (HSP60)	95	2
*52630888	Steroidogenic acute regulatory protein, mitochondrial	90	2
553254	NADH cytochrome b5 reductase	83	1
585110061	Transitional endoplasmic reticulum ATPase	82	1

*Observed 50% times

table S2. One-dimensional native PAGE (3 to 16%) of mitochondrial-associated membrane native complex stained with GRP78 antibody (450-kDa MAM complex).

Accession No.	Protein	MS/MS Score	No. of unique peptides
50345982	ATP synthase subunit alpha c, mitochondrial	823	14
1304157	78 kDa glucose-regulated protein (GRP78)	398	6
4507879	Voltage-dependent anion-selective channel protein 1	347	4
4503327	NADH-cytochrome b5 reductase 3 isoform 1	338	4
4504509	3 beta-hydroxysteroid dehydrogenase	337	6
10716563	Calnexin precursor	267	5
6755965	Voltage-dependent anion-selective channel protein	243	3
15928608	Solute carrier family 25	191	4
4557303	Fatty aldehyde dehydrogenase isoform 2	163	3
306890	60 kDa heat shock protein (HSP60)	160	2
126723203	Cholesterol side-chain cleavage enzyme, mitochondrial precursor	124	2
6755967	Voltage-dependent anion-selective channel protein 3	87	2
521026634	Protein disulfide-isomerase A6	75	2
*52630888	Steroidogenic acute regulatory protein, mitochondrial	75	2
159592	Heat shock protein 70	63	1

*Observed 50% times

table S3. Chemical cross-linked complex analysis (135-kDa complex).

Accession No.	Protein	MS/MS Score	Peptide Sequence
4105605	Voltage dependent anion channel	34	R.WTEYGLTFTEK.W
121570	78 kDa glucose-regulated protein	50	K.NQLTSNPENTVFDAK.R
52630888	StAR	4	K.VVPDVGK.V
			R.LYEELVER.M