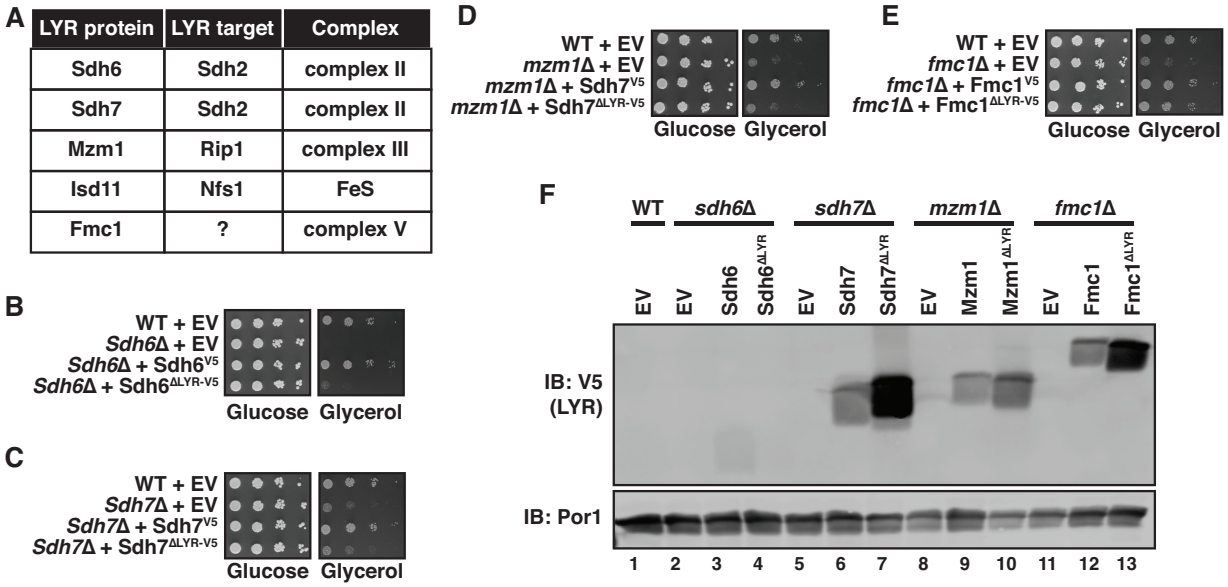
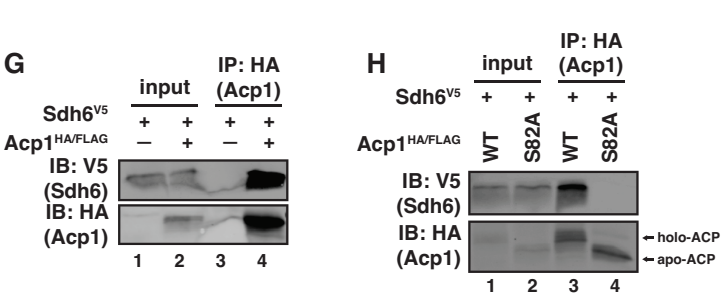
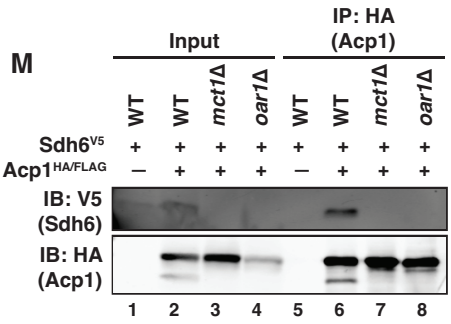
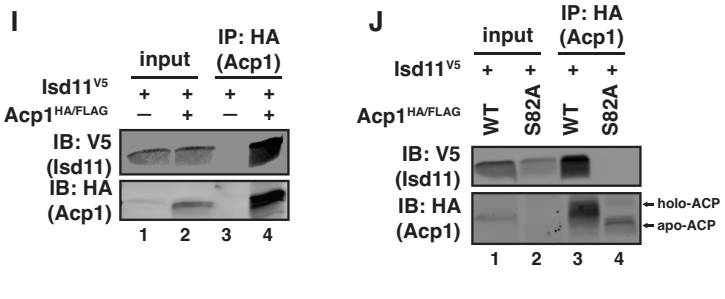
A

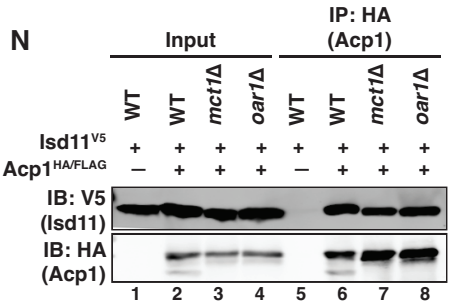
LYR protein	LYR target	Complex
Sdh6	Sdh2	complex II
Sdh7	Sdh2	complex II
Mzm1	Rip1	complex III
Isd11	Nfs1	FeS
Fmc1	?	complex V

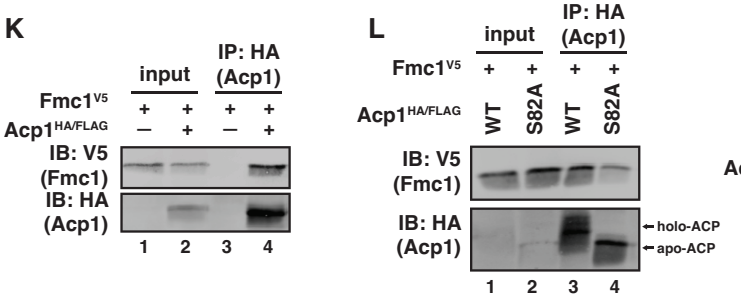


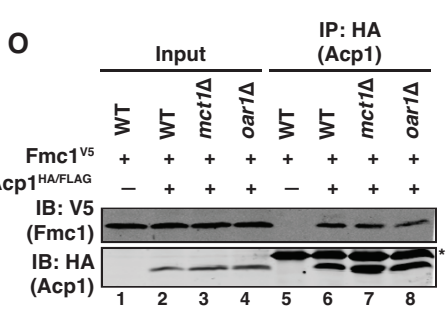






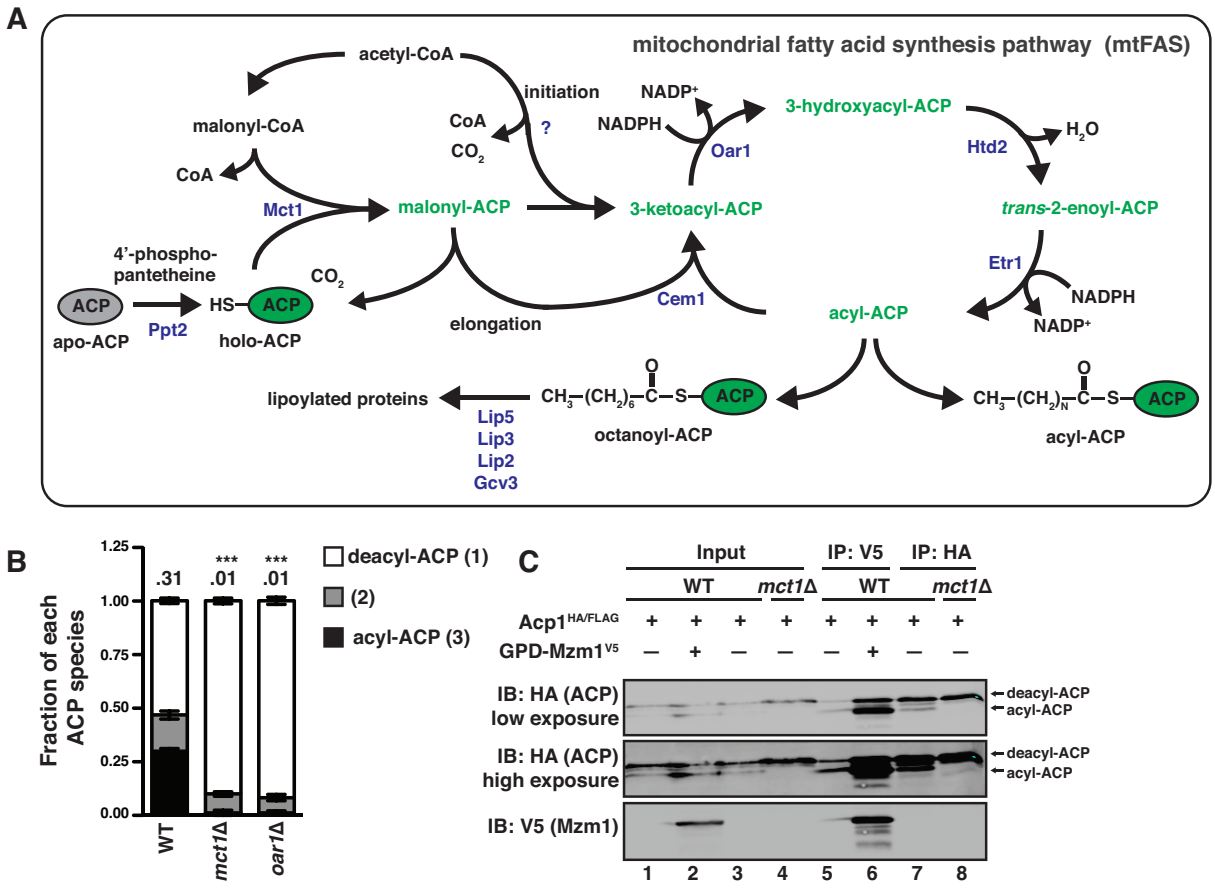






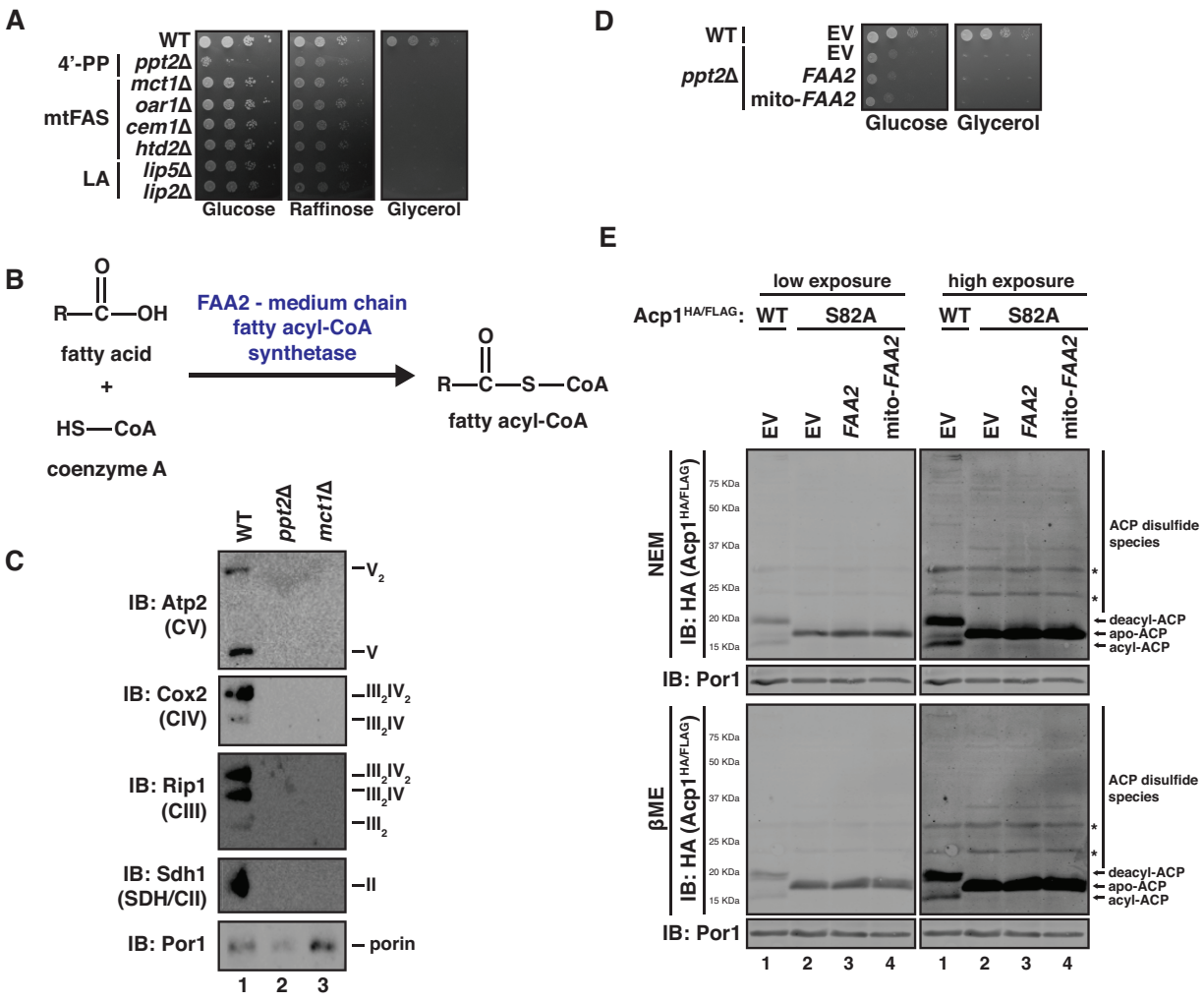
Supplemental Figure 1

Figure S1, Related to Figure 1. LYR proteins require ACP to execute their functions as complex-specific ETC assembly factors. A. Table summarizing the function of yeast LYR proteins. Each LYR protein physically binds a direct LYR target. This interaction facilitates the integration of the LYR target into its specific complex. B-E. Ten-fold serial dilutions of the indicated strains harboring the indicated plasmids were plated on synthetic medium with either glucose or glycerol and grown for 2-3 days. F. Mitochondrial lysates generated from the indicated strains harboring the indicated plasmids were separated by SDS-PAGE and immunoblotted with the indicated antibodies. G-L. $Acp1^{HA/FLAG}$ or $Acp1^{S82A\text{-}HA/FLAG}$ were immunoprecipitated from cells expressing $Sdh6^{V5}$ (G,H), $Isd11^{V5}$ (I,J), or $Fmc1^{V5}$ (K,L). The inputs and eluates were immunoblotted with the indicated antibodies. M-O. $Acp1^{HA/FLAG}$ was immunprecipitated from WT, *mct1*Δ, and *oar1*Δ cells expressing $Sdh6^{V5}$ (M), $Isd11^{V5}$ (N), or Fmc1V5 (O). The inputs and eluates were immunoblotted with the indicated antibodies. * indicates non-specific bands. Inputs represent 5% of the total lysate.



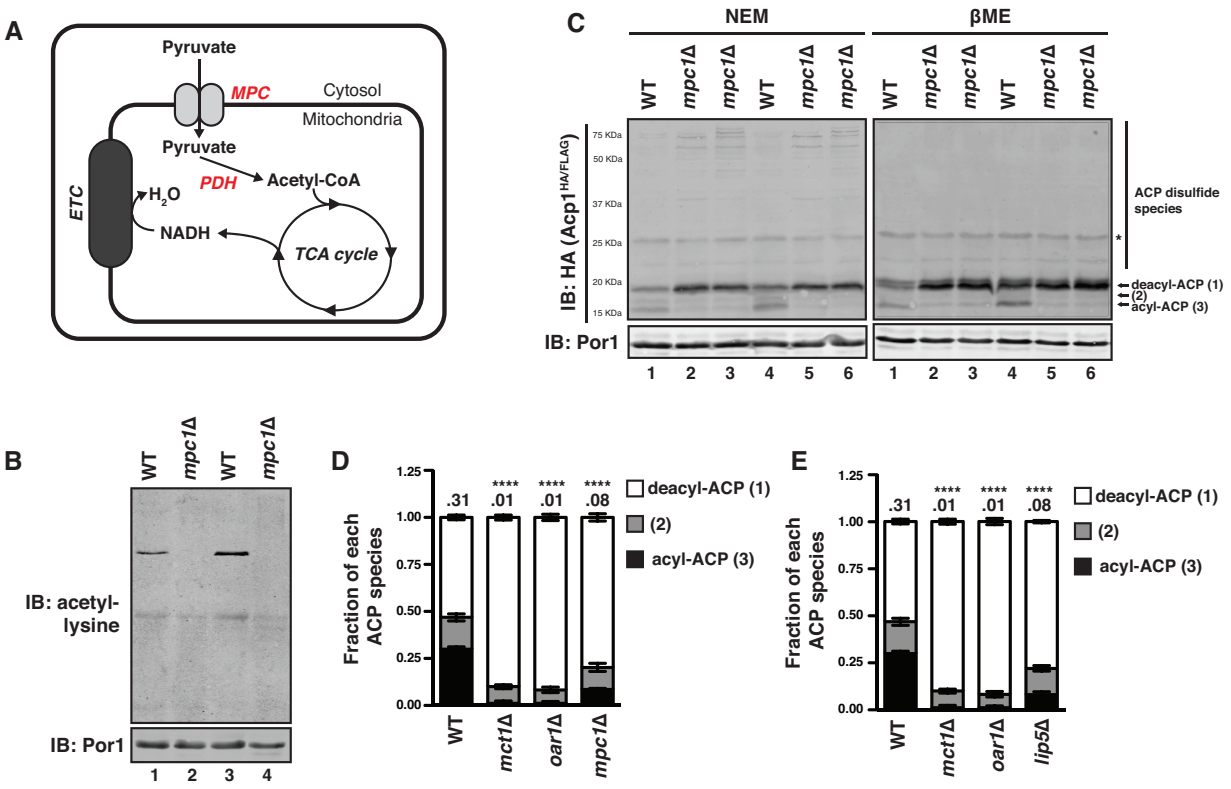
Supplemental Figure 2

Figure S2, Related to Figure 2. Defining the steady state abundance of modified ACP species. A. Model of mitochondrial fatty acid synthesis (mtFAS). B. The fraction of Acp1$^{HA/FLAG}$-containing species was quantified using densitometry for each indicated strain. The fraction was determined as the abundance of each species divided by total Acp1$^{HA/FLAG}$. N≥3. Error bars represent the standard deviation. ****p<0.0001. C. Acp1$^{HA/FLAG}$ or Mzm1^{V5} (expressed from the *GPD1* promoter) were immunoprecipitated from mitochondrial lysates expressing the indicated proteins, separated by SDS-PAGE in the presence of 10 mM NEM, and immunoblotted for HA (Acp1$^{HA/FLAG}$) and V5 (Mzm1^{V5}). Inputs represent 5% of the total lysate.



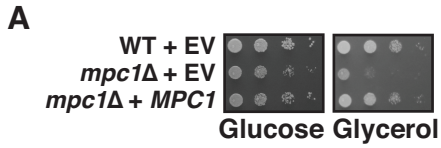
Supplemental Figure 3

Figure S3, Related to Figure 3. ACP acylation is required for ETC biogenesis. A. Ten-fold serial dilutions of the indicated strains were plated on synthetic medium with either glucose or glycerol and grown for 2-3 days. B. Faa2 catalyzes the ligation of CoA to acyl chains. C. Mitochondrial lysates generated from the indicated strains were separated by BN-PAGE and immunoblotted with the indicated antibodies. D. Ten-fold serial dilutions of the indicated strains harboring the indicated plasmids were plated on synthetic medium with either glucose or glycerol and grown for 2-3 days. E. Mitochondrial lysates generated from the indicated strains expressing $Acp1^{HA/FLAG}$ or $Acp1^{S82A\text{-}HA/FLAG}$ were separated by SDS-PAGE in the presence of 10mM NEM or 1% βME and immunoblotted for HA to visualize $Acp1^{HA/FLAG}$-containing species. * indicates non-specific bands.



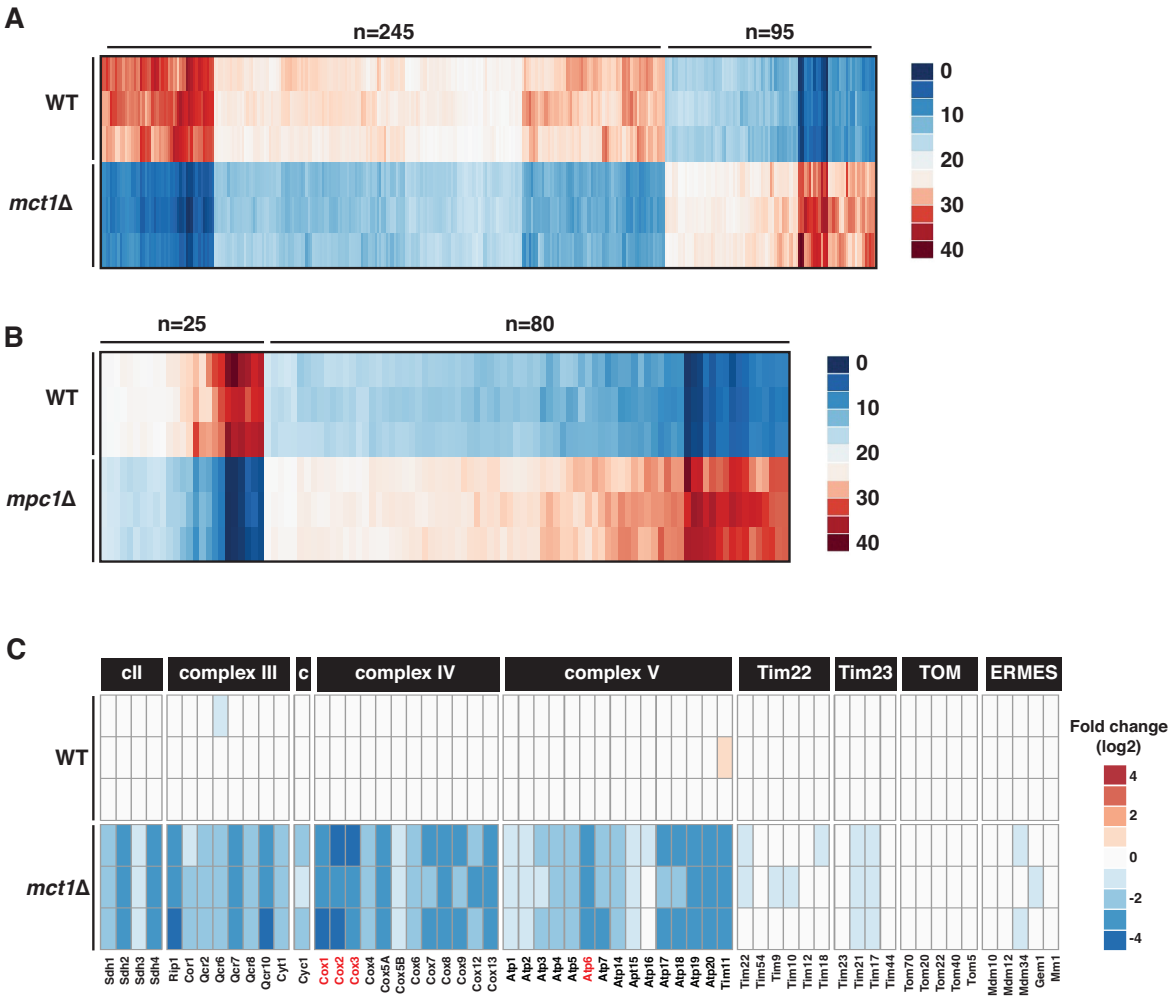
Supplemental Figure 4

Figure S4, Related to Figure 4. ACP acylation is sensitive to perturbation in mitochondrial acetyl-CoA. A. Model of yeast mitochondrial acetyl-CoA generation. In the absence of Mpc1, mitochondria will be prevented from importing pyruvate and therefore pyruvate dehydrogenase will not have the substrate needed to generate mitochondrial acetyl-CoA. In the absence of Lip5, defects in lipoic acid synthesis prevent PDH activity. These cells can import pyruvate into mitochondria normally but will be unable to use it to generate acetyl-CoA. B. Mitochondrial lysates generated from the indicated strains were separated by SDS-PAGE and immunoblotted with an acetyl-lysine antibody to identify acetylated proteins. C. Mitochondrial lysates generated from the indicated strains harboring $Acp1^{HA/FLAG}$ were separated by SDS-PAGE in the presence of 10mM NEM or 1% βME and immunoblotted for HA to identify $Acp1^{HA/FLAG}$-containing bands. D,E. The fraction of $Acp1^{HA/FLAG}$-containing species was quantified using densitometry for *mpc1*Δ (D) and *lip5*Δ (E) cells. The fraction was determined as the abundance of each species divided by total $Acp1^{HA/FLAG}$. N≥3. Error bars represent SEM. ****p<0.0001.



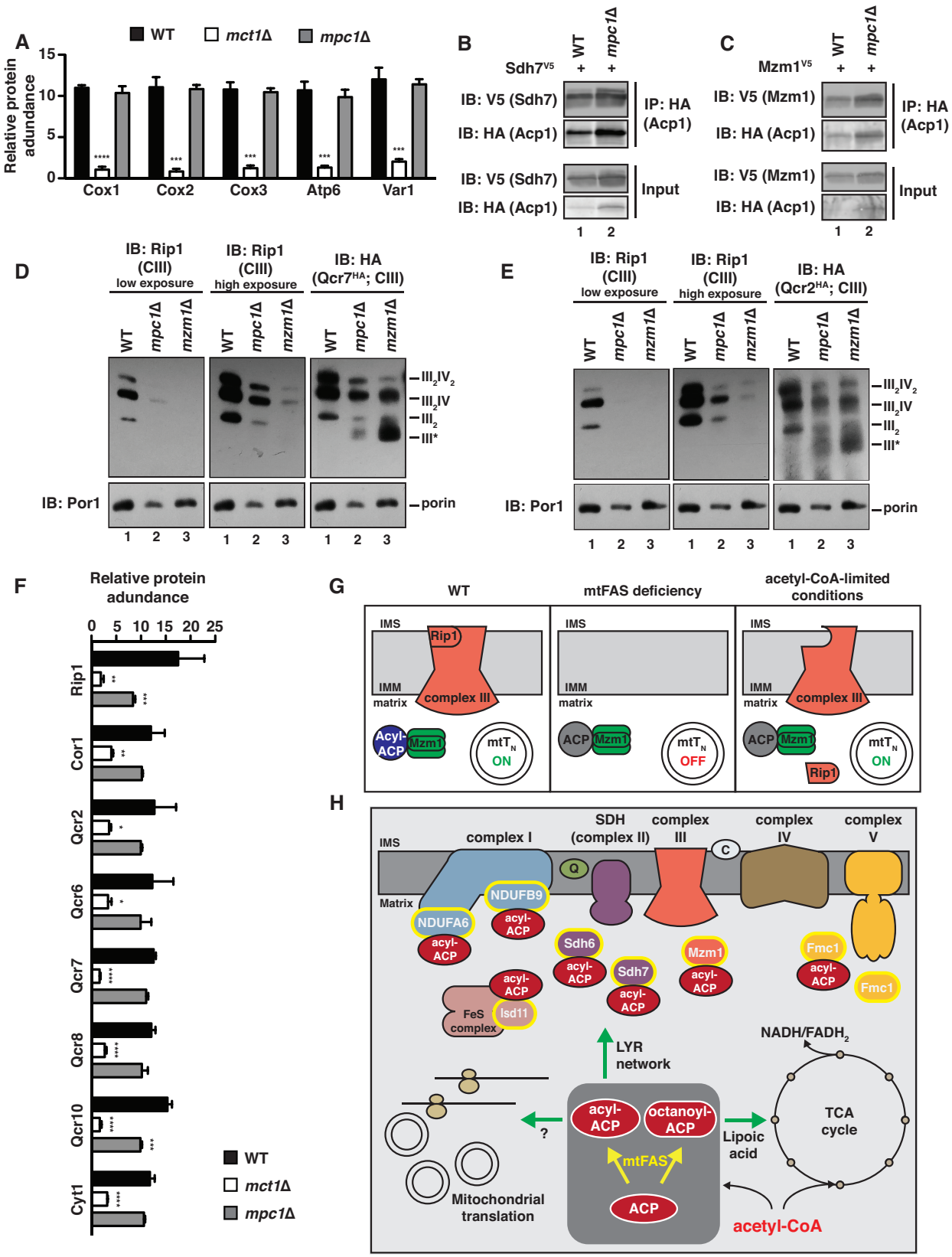
Supplemental Figure 5

Figure S5, Related to Figure 5. Expression of mito-FAA2 restores ACP acylation, OXPHOS assembly and growth in *mpc1*Δ cells. A. Ten-fold serial dilutions of the indicated strains harboring the indicated plasmids were plated on synthetic medium with either glucose or glycerol and grown for 2-3 days.



Supplemental Figure 6

Figure S6, Related to Figure 6. Assessing the phenotypic differences between *mct1*Δ and *mpc1*Δ cells. A,B. Heat map indicating significantly changing proteins comparing (A) WT and *mct1*Δ and (B) WT and *mpc1*Δ. From left to right: significantly down-regulated proteins (corrected p-value < 0.01 and fold-change < 0.75) and significantly up-regulated proteins (corrected p-value < 0.01 and fold-change < 1.5). Intensities represent % of TMT reporter ion signal for each of the six channels. C. Heat map showing the fold change (log2) of the indicated proteins.



Supplemental Figure 7

Figure S7, Related to Figure 7. Acetyl-CoA limitation prevents LYR-mediated ETC assembly and activation. A. Relative abundance of the indicated proteins in WT, *mct1*Δ, and *mpc1*Δ cells (N=3). B,C. $Acp1^{HA/FLAG}$ was immunprecipitated from mitochondria isolated from WT or *mpc1*Δ expressing $Sdh7^{V5}$ (B) or $Mzm1^{V5}$ (C). The inputs and eluates were immunoblotted with the indicated antibodies. D,E. Mitochondrial lysates isolated from cells expressing either $Qcr7^{HA}$ (D) or $Qcr2^{HA}$ (E) were separated by BN-PAGE immunoblotted with the indicated antibodies. These images are the same as in Figure 6D and E but have been oriented differently. F. Relative abundance of the indicated proteins in WT, *mct1*Δ, and *mpc1*Δ cells (N=3). G. In WT cells, acyl-ACP and Mzm1 work together to integrate Rip1 and Qcr10 into complex III to complete the assembly and activate the complex. In mtFASΔ cells, all complex III subunits fail to accumulate. In acetyl-CoA limited cells (*mpc1*Δ), a stable sub-assembly of complex III accumulates, which contains all subunits except for Rip1 and Qcr10. In these cells, complex III biogenesis stalls at the point in which Rip1 and Qcr10 are supposed to be integrated by acyl-ACP and Mzm1 due to a decrease in acyl-ACP abundance. H. mtFAS-dependent acylation of ACP is required for three critical mitochondrial processes—synthesis of lipoic acid, activation of mitochondrial translation, and assembly of the ETC. In the presence of mitochondrial acetyl-CoA, mtFAS converts ACP to octanoyl-ACP and acyl-ACP. Octanoyl-ACP is the substrate of lipoic acid synthesis, which is required for the TCA cycle by lipoylating the PDH and α-KGDH enzymes. In addition, acyl-ACP functions as an activator of the LYR protein family. Upon binding on acyl-ACP, LYR proteins become competent for supporting ETC complex assembly. In addition to direct LYR-mediated ETC assembly, acyl-ACP also supports ETC biogenesis through the activation of mitochondrial translation through an unknown mechanism. Interestingly, upon limitation of mitochondrial acetyl-CoA (e.g. in an *mpc1*Δ strain), mitochondrial translation functions normally, while LYR-mediated ETC assembly is inhibited. Error bars represent the standard deviation. *$p<0.05$, **$p<0.005$, ***$p<0.0005$, ****$p<0.00005$. Inputs represent 5% of the total lysate.