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Distinct intracellular sAC-cAMP domains regulate ER calcium signaling and OXPHOS function

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Summary statement: We demonstrate that soluble adenylyl cyclase (sAC) regulates ER calcium release. While ER and mitochondria sAC domains are distinct, ER calcium release provides a functional link between the two organelles.
Abstract
cAMP regulates a wide variety of physiological functions in mammals. This single second messenger can regulate multiple, seemingly disparate functions within independently regulated cell compartments. We previously identified one such compartment inside the matrix of the mitochondria, where soluble adenylyl cyclase (sAC) regulates oxidative phosphorylation (OXPHOS). We now show that sAC KO fibroblasts have a defect in OXPHOS activity and attempt to compensate for this defect by increasing OXPHOS proteins. Importantly, sAC KO cells also exhibit decreased probability of endoplasmic reticulum (ER) Ca\textsuperscript{2+} release associated with diminished phosphorylation of the inositol 3-phosphate receptor. Restoring sAC expression exclusively in the mitochondrial matrix rescues OXPHOS activity and reduces its biogenesis, indicating that these phenotypes are regulated by intramitochondrial sAC. In contrast, ER Ca\textsuperscript{2+} release is only rescued when sAC expression is restored throughout the cell. Thus, we show that functionally distinct, sAC-defined, intracellular cAMP signaling domains regulate metabolism and Ca\textsuperscript{2+} signaling.
Introduction

Cyclic AMP (cAMP) has been implicated in a wide variety of (often contradictory) physiological processes, including different aspects of cell proliferation, apoptosis, differentiation, migration, development, ion transport, pH regulation, and gene expression. A long-standing challenge in cAMP biology has been to understand how this single second messenger could simultaneously mediate so many disparate processes. This conundrum was especially puzzling when examining a single cell type. For example, Brunton and co-workers found that different hormones elicited distinct responses in a single cell type, yet both hormones worked via classically defined cAMP signaling cascades (Buxton and Brunton, 1983; Buxton and Brunton, 1986). They postulated that cAMP signals in physically separated compartments within a cell. Functional compartmentalization of cAMP signaling was ultimately demonstrated for membrane-anchored and membrane-proximal, hormone-initiated signaling cascades (Davare et al., 2001; Marx et al., 2002), and it is now widely accepted that cAMP acts locally within independently-regulated, spatially-restricted compartments comprised of anchoring proteins (e.g., AKAPs) as well as the enzymes which synthesize (i.e., adenylyl cyclases; ACs) and degrade (i.e., phosphodiesterases; PDEs) cAMP.

A number of distinct cAMP compartments affect mitochondria (Monterisi and Zaccolo, 2017). Inside the matrix, cAMP is synthesized by the bicarbonate and Ca\(^{2+}\) sensitive soluble adenylyl cyclase (sAC) in response to both metabolically generated CO\(_2\)/HCO\(_3^-\) (Acin-Perez et al., 2009b), as well as to the entry of Ca\(^{2+}\) through the mitochondrial Ca\(^{2+}\) uniporter (MCU) upon its release from the endoplasmic reticulum (ER) (Di Benedetto et al., 2014; Di Benedetto et al., 2013; Lefkimmiatis et al., 2013). sAC activation in mitochondria regulates OXPHOS and ATP production, (Acin-Perez et al., 2009b; Di Benedetto et al., 2013), and this pathway appears to be conserved in yeast (Hess et al., 2014).
Important additional roles of cAMP in mitochondria depend on signaling occurring outside the matrix, at the outer mitochondrial membrane, where a cAMP/PKA/PDE2A2 signaling domain regulates mitochondrial dynamics through phosphorylation of the fission protein Drp1 (Monterisi et al., 2017). Monterisi and co-workers also demonstrated that the outer mitochondrial membrane cAMP signaling depends on PDE sculpting of second messengers generated at the plasma membrane by hormone and G protein regulated transmembrane adenylyl cyclases (tmACs).

Here, we find that the intramitochondrial sAC-defined domain regulates OXPHOS biogenesis by initiating a signaling cascade to the nucleus and uncover a novel extramitochondrial sAC-defined domain that regulates inositol 3-phosphate receptor (IP3R) phosphorylation, ER metabotropic Ca\(^{2+}\) release, and the functional coupling between ER Ca\(^{2+}\) and mitochondrial OXPHOS.

**Results**

*OXPHOS is impaired in sAC KO MEFs*

We investigated mitochondrial bioenergetic properties in isolated mitochondria from sAC KO MEFs cell lines in which the enzyme was genetically ablated and isogenic wild type (wt) control MEFs (Bitterman et al., 2013; Rahman et al., 2016; Ramos-Espiritu et al., 2016a; Ramos-Espiritu et al., 2016b). Complex I (CI) activity (NADH:decylubiquinone oxidoreductase, DBQ) was significantly decreased in sAC KO cells (Fig. 1A). Furthermore, the NADH oxidoreductase activity of CI measured with an artificial electron acceptor (NADH:hexaammineruthenium oxidoreductase activity, HAR), which indicates the total amount of flavin-containing CI, was similar in sAC KO and wt mitochondria (Fig. 1B). As a result, the DBQ:HAR ratio was significantly decreased in sAC KO MEFs (Fig. 1C), indicating a decrease in activity, but not a loss of CI content. In addition, sAC KO mitochondria displayed a small decrease in complex IV (COX) activity compared to wt (Fig. 1D), supporting the notion that COX is regulated by sAC (Acin-Perez et al., 2009a).
To study intact cell energy metabolism, mitochondrial oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in a flux analyzer. In sAC KO MEFs, OCR was significantly decreased (Fig. 2A), while ECAR was increased (Fig. 2B), indicating lower cell respiration and greater lactate production. The OCR:ECAR ratio was decreased in sAC KO cells (Fig. 2C), suggesting a shift toward glycolytic metabolism. The maximal OCR achieved upon addition of the uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was similar in sAC KO and wt cells (Fig. 2D), indicating that sAC regulated respiration only in coupled (i.e., ADP-phosphorylating) mitochondria. Accordingly, mitochondrial ATP synthesis was significantly decreased in sAC KO cells (Fig. 2E). Since it was shown that PKA phosphorylates and inactivates mitochondrial IF1, a negative modulator of the F1F0 ATPase (Garcia-Bermudez et al., 2015), we investigated whether the absence of SAC affected IF1 phosphorylation. However, we did not detect IF1 phosphorylation in WT or sAC KO cells (data not shown), suggesting that IF1 phosphorylation is marginal in MEFs.

To exclude that the OXPHOS impairment associated with sAC KO was specific to one MEF line, ATP synthesis and COX defects were also confirmed in an independent sAC KO MEF line (sAC KO2 MEF, Fig. S1A,B).

In addition, mitochondrial membrane potential (ΔΨm) assessed as the accumulation of the potentiometric fluorescent probe tetramethylrhodamine methyl ester (TMRM), was lower in sAC KO than in wt cells (Fig. 2F,G), while in both lines FCCP completely dissipated ΔΨm, indicating equal loading of the probe (Fig. 2F).

We then measured the ability of cells to proliferate under regular glucose-containing medium or under obligatory oxidative conditions, induced by replacing glucose with galactose (Robinson, 1996). In glucose medium, sAC KO cells displayed better growth, both at 48h and 72h, but not at 24h, compared to wt MEFs (Fig. 2H). In contrast, in galactose medium, the growth of sAC KO cells
was significantly decreased in respect to wt (Fig. 2I), further demonstrating that sAC KO cells had defective mitochondrial energy production.

**sAC KO increases the content of OXPHOS components**

In light of the decrease in mitochondrial respiration and ATP synthesis in sAC KO MEFs, we investigated the steady state levels of OXPHOS proteins. Surprisingly, Western blots of cell homogenates revealed that sAC KO MEFs had increased levels of NDUFSB8 of CI, 30 KD subunit of CII, core2 of complex III, subunit 1 of COX, and α subunit of complex V, relative to β-actin (Fig. 3A,B). However, the increase in the levels of mitochondrial proteins was selective and did not involved all proteins, as the voltage dependent anion channel protein (VDAC), the inner membrane translocator protein TIM23, and the matrix chaperone HSP60 were unchanged (Fig. 3A,B). Furthermore, in isolated mitochondria from sAC KO cells, there was a significant increase subunit 1 of COX and subunit NDUFSB8 of complex I, relative to TIM23, but not in other OXPHOS subunits (Fig. S2A, B).

The levels of PKA were also unchanged suggesting that lack of sAC did not affect the expression of the cAMP effector. Since it was shown that transfection efficiency another cAMP effector, the exchange protein directly activated by cAMP 1 (EPAC1), modulates mitochondrial function in response to Ca\(^{2+}\) and bicarbonate stimulation (Wang et al., 2016), we investigated EPAC1 expression levels, which were unchanged in the absence of sAC (Fig. 3A,B). sAC KO cells also contained more mtDNA (COX1 gene) relative to nuclear DNA (18S rRNA gene) than wt (Fig. 3C). In addition, the average area occupied by mitochondria in each cell, visualized by microscopy with the non-potentiometric fluorescent dye MitoTracker Green FM, was increased in sAC KO MEFs (Fig. 3D). No significant differences were detected in mitochondrial morphology, aspect ratio, and form factor between wt and sAC KO MEFs (Fig. S3A,B,C). The increase in OXPHOS proteins, mtDNA, and mitochondrial density suggested that sAC KO cells had greater mitochondrial content than wt.
Taken together, these data suggest that sAC KO cells had increased mitochondrial content and a selective increase of OXPHOS proteins and mtDNA. However, other mitochondrial proteins did not increase proportionally, which can explain why sAC KO cells had impaired OXPHOS function, despite the increase in several OXPHOS components.

AMP-activated protein kinase (AMPK) is a key regulator of cellular energy homeostasis, involved in mitochondrial biogenesis, which is activated by phosphorylation (Hardie, 2007; Shaywitz and Greenberg, 1999). In sAC KO MEFs, the P-AMPK/AMPK ratio was increased (Fig. 3E,F), indicating that the AMPK signaling pathway was activated. Accordingly, the mRNA levels of two downstream effectors of AMPK involved in mitochondrial biogenesis, PGC-1α (Fig. 3G) and NRF1 (Fig. 3H), were also upregulated. Furthermore, to confirm the involvement of AMPK in the signaling pathway leading to PGC-1α activation, we treated cells with the AMPK inhibitor Dorsomorphin (compound C 10 µM overnight). Dorsomorphin did not alter PGC-1α in wt cells, whereas in sAC KO cells treated with Dorsomorphin, the activation of PGC-1α expression was essentially prevented (Fig. 3I), indicating that AMPK phosphorylation was indeed involved in the pathway.

Since PGC-1α could also be activated by reactive oxygen species (ROS) (Han et al., 2010; Zmijewski et al., 2010), we tested the effects of treatment with the antioxidant Trolox (500 µM) for 72 hours, which was shown to decrease ROS in OXPHOS deficient cells (Distelmaier et al., 2012). Trolox did not abolish the difference in PGC-1α between sAC KO and wt MEFs (Fig. S3D), suggesting that ROS did not play a major role in regulating mitochondrial biogenesis in sAC KO cells. Taken together, the data suggest that loss of sAC induced an AMPK-driven adaptive mitochondrial biogenesis response to OXPHOS deficiency.

mtsAC restores P-AMPK level and OXPHOS function in sAC KO MEFs

Because sAC can be found in multiple locations inside cells (Acin-Perez et al., 2009b; Zippin et al., 2003), we sought to determine if the bioenergetics and signaling effects of sAC KO were
specifically caused by the lack of sAC in mitochondria. To this end, we transfected cells with an enzymatically active recombinant sAC exclusively targeted to mitochondria (mtsAC) (Acin-Perez et al., 2009a). mtsAC localization by immunofluorescence coincided with that of the mitochondrial protein TOM20, while an untargeted version of the construct (sAC) showed diffused intracellular distribution (Fig. 4A). The transfection efficiency was 70-80% for both constructs. By western blot analyses, using antibodies against the HA tag, KO MEFs transfected with sAC contained the protein in the enriched mitochondrial fraction, although at lower levels than cells transfected with mtsAC (Fig. 4B). In the cytosolic fractions, HA immunoreactivity was detected in the KO+sAC samples, whereas KO+mtsAC did not display any detectable signal.

At 48 hours post-transfection, both sAC and mtsAC expressing sAC KO MEFs had significantly increased CI (DBQ/HAR) and COX activities (Fig. 4C,D), as compared to sAC KO cells transfected with empty vector, and these activities were no longer significantly lower than in wt cells. TMRM accumulation (Fig. 4E) and mitochondrial ATP synthesis (Fig. 4F) were also increased by sAC and mtsAC in sAC KO cells to levels comparable to wt. Furthermore, we investigated the cell growth of wt and sAC KO in glucose and galactose medium. In glucose, KO MEFs showed increased cell growth relative to wt, which was not altered by expression of sAC or mtsAC (Fig. 4G). Importantly, in galactose medium, KO MEFs transfected with sAC or mtsAC showed comparable cell growth to wt (Fig. 4H), confirming that sAC expression restored mitochondrial energy production in sAC KO cells.

In agreement with the hypothesis that increased mitochondrial biogenesis was the result of the lack of sAC in mitochondria, both sAC and mtsAC expression decreased P-AMPK/AMPK ratio in sAC KO MEFs to levels comparable to wt (Fig. 4I,J). As expected, the levels of other mitochondrial proteins, which were unchanged in sAC KO MEFs, such as HSP60, TIM23, appeared unaffected by sAC or mtsAC overexpression (Fig. 4I). To further investigate the effect of mtsAC on KO cells, we analyzed the expression of the OXPHOS complexes in KO cells and KO+mtsAC cells after 48h of
transfection. The OXPHOS protein expression was decreased in KO+mtsAC cells compared to KO (Fig. S4A,B), but not complex V. Similarly the PGC1α mRNA expression (Fig. S4C) and the mtDNA content (Fig. S4D) were decreased in KO+mtsAC cells in respect to KO, indicating that mtsAC overexpression partially restored mitochondrial biogenesis in KO cells.

These results demonstrated that sAC localized in mitochondria was capable of improving mitochondrial bioenergetics and extinguishing the adaptive P-AMPK signaling of mitochondrial biogenesis.

*ER-mitochondria Ca\(^{2+}\) transfer is altered in sAC KO MEFs*

Mitochondria are key players in intracellular Ca\(^{2+}\) homeostasis, as they are able to accumulate Ca\(^{2+}\) from the cytosol through the mitochondrial Ca\(^{2+}\) uniporter (MCU) in a ΔΨm-dependent manner (De Stefani et al., 2015; Kamer and Mootha, 2015). Ca\(^{2+}\) release from the ER through the inositol-3-phosphate receptor (IP3R) is an essential signaling process, which participates in the regulation of mitochondrial bioenergetics (Denton, 2009) and intramitochondrial sAC activity (Di Benedetto et al., 2014; Di Benedetto et al., 2013). To study the effect of sAC KO on ER-mitochondrial Ca\(^{2+}\) signaling, we utilized a fluorescent Ca\(^{2+}\) sensor GCaMP6 (Chen et al., 2013) targeted to the mitochondrial matrix (mitoGCaMP6). Mitochondrial localization of mitoGCaMP6 was assessed by co-transfection with a fluorescent reporter localized to mitochondria (mitoDsRed) (Fig. 5A). Upon Ca\(^{2+}\) release from the ER triggered by stimulation of purinergic receptors with ATP (1mM, for 30 sec, in perfusion buffer containing 1mM Ca\(^{2+}\)), mitoGCaMP6 fluorescence increased (Fig. 5B), indicating mitochondrial Ca\(^{2+}\) uptake. As expected, treatment with FCCP (1µM) to depolarize mitochondria prior to ATP stimulation resulted in a complete disappearance of the mitochondrial Ca\(^{2+}\) signal (Fig. 5C), demonstrating that the sensor detected mitochondrial Ca\(^{2+}\) specifically. After ATP stimulation, a smaller subset (33.1% ± 10.1) of sAC KO cells showed a mitochondrial Ca\(^{2+}\) response in comparison to wt (79.9% ± 4.7) (Fig. 5D), while the average mitoGCaMP6 fluorescence peak (F/F0) in
responding cells was similar in wt and sAC KO cells (Fig. 5E). These results suggested that the probability of mitochondria taking up Ca\(^{2+}\) released from the ER was decreased in sAC KO cells.

The contacts between ER and mitochondria can be involved in the regulation of Ca\(^{2+}\) entry in mitochondria (Area-Gomez et al., 2012; Hayashi et al., 2009; Szabadkai and Rizzuto, 2004). Therefore, we investigated ER-mitochondria co-localization by transfecting cells with mitoDsRed and the fluorescent ER reporter Sec61-GFP. We did not detect differences in the colocalization of the two reporters between sAC KO and wt cells (Fig. S5A,B).

To test mitochondrial Ca\(^{2+}\) uptake of ER Ca\(^{2+}\) released in an IP3R-independent manner, we blocked the sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase pump with thapsigargin (TG, 1\(\mu\)M). As expected, 100% of wt and sAC KO cells showed mitochondrial Ca\(^{2+}\) uptake upon TG addition (Fig. 5F). This result suggested that IP3R-dependent Ca\(^{2+}\) release was impaired in sAC KO cells, while ER Ca\(^{2+}\) content was unaffected. Interestingly, we also found that upon TG stimulation sAC KO cells had higher mitochondrial Ca\(^{2+}\) uptake than wt (Fig. 5G). Therefore, we investigated the levels of critical components of the mitochondrial Ca\(^{2+}\) uptake machinery, MCU, its regulator mitochondrial Ca\(^{2+}\) Uptake 1 (MICU1), and the Na\(^{+}/Ca^{2+}\) exchanger SLC24A6 (NCLX). Normalized to TIM23, MCU (Fig. 5H,I) and NCLX (Fig. 5J,K) were significantly increased in sAC KO MEFs, whereas MICU1 was unchanged (Fig. 5L,M). We then measured Ca\(^{2+}\) uptake capacity in isolated mitochondria from wt and sAC KO MEFs using Calcium Green fluorescence, and found that sAC KO mitochondria had higher Ca\(^{2+}\) capacity than wt (Fig. S5C).

Higher Ca\(^{2+}\) uptake in sAC KO MEFs could potentially be explained by lack of cAMP-dependent activation of EPAC and consequent decreased inhibition of MCU by EPAC1 (Wang et al., 2016). Therefore we tested mitochondrial Ca\(^{2+}\) uptake in wt and sAC KO cells treated with the EPAC1/2 inhibitor (ESI-09 10 \(\mu\)M, 30 minutes pretreatment and throughout the measurements). Ca\(^{2+}\) release from the ER was induced with TG, ESI-09 did not significantly alter the amount of Ca\(^{2+}\) taken up by
mitochondria, and the differences between wt and sAC KO persisted (Fig. S6A,B), suggesting that EPAC1 did not have a significant impact on mitochondrial Ca\(^{2+}\) uptake in MEFs.

Taken together, these results indicated that sAC KO cells had decreased sensitivity to metabotropic stimulation of ER Ca\(^{2+}\) release. However, when ER Ca\(^{2+}\) was released in an IP3-independent manner using TG, mitochondria of sAC KO cells took up Ca\(^{2+}\) more efficiently, likely due to increased levels of MCU, accompanied by higher Ca\(^{2+}\) capacity, possibly resulting from increased levels of NCLX that prevented Ca\(^{2+}\) overload.

**ER-mitochondria Ca\(^{2+}\) mediated functional coupling is altered in sAC KO MEFs**

Since mitochondrial Ca\(^{2+}\) uptake has well known regulatory functions on mitochondrial respiration (Denton, 2009), we measured OCR upon ATP stimulation of ER Ca\(^{2+}\) release. For these experiments, cells were treated with the uncoupler 3,5-di(tert-butyl)-4-hydroxybenzylidenemalononitril (SF6847), which allows for maximal electron transfer activity in the respiratory chain. ATP induced a small but significant increase in OCR in wt cells. In contrast, ATP did not affect sAC KO cells (Fig. 6A), consistent with their decreased probability of ER Ca\(^{2+}\) release. Furthermore, we monitored TMRM fluorescence upon ATP stimulation of ER Ca\(^{2+}\) release and found that a smaller proportion (21.1% ± 3) of sAC KO cells showed TMRM fluorescence increase in compared to wt cells (70% ± 4) (Fig. 6B). Lastly, we observed that the average intensity of the TMRM fluorescence peak in responding cells was lower in sAC KO cells as compared to wt (Fig. 6C). This suggested that even in the minority of responding cells, which took up Ca\(^{2+}\) in mitochondria, the underlying bioenergetic defect attenuated the ΔΨm increase. Taken together, these results indicated that the absence of sAC causes decreased probability of IP3R opening and defective coupling of metabotropic ER Ca\(^{2+}\) release with mitochondrial bioenergetics.
Metabotropic ER Ca\(^{2+}\) release is altered in sAC KO MEFs

We investigated if the decrease in the number of sAC KO cells that took up Ca\(^{2+}\) in mitochondria upon ATP stimulation was associated with alterations of ER Ca\(^{2+}\) release in the cytosol. Using the cytosolic Ca\(^{2+}\) reporter GCaMP6, we observed that ATP stimulation induced a cytosolic Ca\(^{2+}\) increase in the majority of wt cells (80.3% ± 3), while the proportion of responsive sAC KO cells was significantly lower (42.4% ± 8) (Fig. 7A). Furthermore, the cytosolic Ca\(^{2+}\) peak height (F/F0) in the smaller subset of responsive sAC KO cells was lower than wt (Fig. 7B). Similarly to ATP, bradykinin (1µM) stimulated fewer sAC KO cells, which also had lower cytosolic Ca\(^{2+}\) peaks (Fig. S7A,B), suggesting that ER Ca\(^{2+}\) signaling abnormalities were not due to changes in purinergic receptors or IP3 generation. Furthermore, upon TG (1µM) administration, 100% of wt and sAC KO MEFs released ER Ca\(^{2+}\) (Fig. 7C) and the fluo4 fluorescence peaks were similar in the two cell lines (Fig. 7D), indicating that the decreased metabotropic ER Ca\(^{2+}\) response in sAC KO cells was not due to lower ER Ca\(^{2+}\) content.

Although the presence of ryanodine receptor (RyR) has not been reported in fibroblasts, to ensure that in our system there was no contribution of the RyR to ER Ca\(^{2+}\) release (Reiken et al., 2003), we treated cells with the RyR inhibitor dantrolene (10µM for 1 hour), prior to ATP administration. Dantrolene did not affect the proportion of cells that responded to ATP nor the difference in Ca\(^{2+}\) peak amplitude between sAC KO and wt cells (Fig. S7C,D), confirming that the RyR did not play a role in the metabotropic Ca\(^{2+}\) response in these cells.

Together, these results pointed to abnormal IP3R mediated ER Ca\(^{2+}\) release in sAC KO cells.
Mechanisms of IP3R dysregulation in sAC KO MEFs

IP3R phosphorylation is involved in Ca\(^{2+}\) signaling (Vanderheyden et al., 2009). PKA phosphorylates mouse IP3R1, which is abundantly expressed in MEFs (Oakes et al., 2005), at two sites, S1588 and S1756 (Ferris et al., 1991), leading to an increase in the open probability of the receptor (Nakade et al., 1994; Tang et al., 2003; Wagner et al., 2004). Therefore, we hypothesized that sAC KO could impair IP3R regulation by PKA-mediated phosphorylation.

We determined by Western blot using phospho-IP3R1 antibodies that the levels of IP3R1 phospho-S1756 relative to total IP3R were decreased in sAC KO cells (Fig. 7E,F). Treatment of wt and sAC KO MEFs with calf intestinal alkaline phosphatase (CIP) caused a significant decrease of phospho-S1756 IP3R1 signal in both cell lines, confirming the specificity of the antibody (Fig. 7E). Furthermore, expression of recombinant sAC increased the levels of IP3R1 phospho-S1756 in sAC KO cells (Fig. S8A,B).

Pharmacological blockage of sAC in wt cells with the specific inhibitor KH7 (100 nM, for 30 min) resulted in a decrease in the proportion of cells that responded to ATP stimulation, similar to sAC KO cells (Fig. 7G). In addition, we assessed the effects of PKA inhibition on ER Ca\(^{2+}\) release probability with the inhibitor H89 (1µM), which resulted in decrease in the proportion of responding wt, but not sAC KO cells (Fig. 7H). The tmAC activator forskolin causes supraphysiological levels of cAMP which are able to simultaneously stimulate any cytoplasmic cAMP microdomain. Forskolin increased the probability of response in both wt and sAC KO MEFs, suggesting that tmAC-generated cAMP under pathophysiological conditions, is capable of reactivating the IP3R in sAC KO cells. Accordingly, IP3R phosphorylation was increased by forskolin in sAC KO cells, whereas KH7 decreased IP3R phosphorylation in wt, but not in sAC KO, cells (Fig. S8C,D).
Taken together, these data highlighted a novel role for sAC in modulating IP3R activity, and indicated that defective PKA-dependent IP3R phosphorylation resulted in decreased probability of IP3R opening in sAC KO cells.

**IP3R regulation is mediated by a sAC-defined cAMP functional domain separate from mitochondrial bioenergetics**

To discriminate whether defective ER Ca\(^{2+}\) signaling was due to lack of mitochondrial or cytosolic sAC we expressed mtsAC-HA (mtsAC) or sAC-HA (sAC) in sAC KO MEFs. Cells were co-transfected with mitoDsRed, which demonstrated that HA expression co-existed with mitoDsRed in approximately 90% of the transfected MEFs (Fig. S8E). mtsAC did not increase the number of mitoDsRed positive sAC KO cells, in which ER Ca\(^{2+}\) was released into the cytosol in response to ATP (wt=71.2\%\pm7.1, sAC KO=24.5\%\pm3, sAC KO+mtsAC=15.3\%\pm2.9, ) (Fig. 8A). The height of the cytosolic Ca\(^{2+}\) peak (F/F0) was also unchanged by the expression of mtsAC (Fig. 8B). On the contrary, sAC expression increased the proportion of responding sAC KO cells (57.3\%\pm3.6) (Fig 8A), although the Ca\(^{2+}\) peak was only modestly increased (by 16.4\%, Fig. 8B), suggesting that sAC expression partially rescued metabotropic ER Ca\(^{2+}\) signaling. These results indicated that impaired sensitivity to metabotropic stimulation was due to lack of sAC-mediated modulation of IP3R and not to mitochondrial effects of sAC KO. As expected, mitochondrial Ca\(^{2+}\) uptake measured with mitoGCaMP6 in sAC KO cells after ATP stimulation was not restored by mtsAC (Fig. 8C,D). Also, sAC expression failed to increase mitochondrial Ca\(^{2+}\) uptake in sAC KO cells (Fig. 8C,D), despite the modest increase observed in cytosolic Ca\(^{2+}\) peak (Fig. 8B), presumably because the Ca\(^{2+}\) concentration in the cytosol did not reach a sufficient threshold to activate the MCU. In order to confirm this hypothesis, mitochondrial Ca\(^{2+}\) was measured in KO+sAC cells and in KO+sAC cells treated with forskolin/IBMX and the number of cells responding to ATP stimulation was quantified. Addition of forskolin/IBMX in KO+sAC cells significantly increased the number of responding cells
compared to KO+sAC (Fig. 8E), indicating that Ca\(^{2+}\) concentration must reach a threshold to activate the MCU.

**Discussion**

The intramitochondrial sAC defined cAMP compartment regulates mitochondrial energy production. In sAC KO MEFs, where this regulation is chronically absent, the cells respond with a compensatory increase of OXPHOS biogenesis. sAC KO MEFs also exhibit impaired coupling of ER Ca\(^{2+}\) and OXPHOS. In sAC KO MEFs, Ca\(^{2+}\) entry into mitochondria after stimulating ER Ca\(^{2+}\) release is diminished, and this attenuation is caused by desensitization of the IP3R to metabotropic stimulation. In contrast to mitochondrial bioenergetics and OXPHOS biogenesis, the cAMP dependent regulation of IP3R Ca\(^{2+}\) release is controlled by extramitochondrial sAC. Our identification of an intramitochondrial domain regulating OXPHOS and an extramitochondrial domain, that modulates the coupling between ER Ca\(^{2+}\) and OXPHOS defines multiple distinct sAC-regulated cAMP signaling compartments affecting mitochondrial function.

The original studies by Brunton et al. suggesting that second messenger signaling had to be compartmentalized were performed in cardiac myocytes. Stimulation by β-adrenergic agonists increased contractility while prostaglandins altered metabolism, and both seemed to be mediated via cAMP and PKA (Buxton and Brunton, 1983; Buxton and Brunton, 1986). In these studies, the molecular details of the distinct compartments were not identified, but since both β-agonists and prostaglandins signal via GPCRs, functionally distinct plasma membrane-bound tmAC dependent compartments were postulated. Ultimately, theories of compartmentalization were confirmed by studies demonstrating that hormones could stimulate functionally separate tmAC-dependent cAMP compartments (Davare et al., 2001; Marx et al., 2002), and current models of cAMP signaling are dependent upon compartmentalization of the second messenger into independently regulated cAMP signaling domains (Lefkimmiatis and Zaccolo, 2014).
We previously demonstrated that bicarbonate and Ca\textsuperscript{2+} sensitive sAC is distributed throughout the cell (Zippin et al., 2003), which suggested that cAMP could be generated locally to fuel signaling at intracellular cAMP compartments (Bundey and Insel, 2004). One such sAC defined intracellular compartment is the mitochondrial matrix, where cAMP generated by sAC regulates OXPHOS (Valsecchi et al., 2013). For other intracellular cAMP compartments, the situation has been less clear. A variety of studies suggest sAC can generate cAMP locally; for example on trafficking endosomes (Inda et al., 2016). However, other results point to PDE activity regulating intracellular cAMP compartments originating from second messenger produced by tmACs (Monterisi et al., 2017). Data presented here demonstrate that cAMP regulates Ca\textsuperscript{2+} release from the ER via an extramitochondrial, sAC-defined signaling domain. Thus, cells contain at least two distinct sAC regulated cAMP compartments that control mitochondrial function, one inside the matrix and a second one outside, in addition to the outer membrane tmACs-mediated cAMP regulation of mitochondrial dynamics. There may be other sAC defined cAMP compartments revealed by additional phenotypes in sAC KO MEFs. We previously showed that sAC KO MEFs display a lysosomal acidification defect (Rahman et al., 2016) and are more susceptible to oncogenic transformation than wt cells (Ramos-Espiritu et al., 2016a). However, neither of these phenotypes has been ascribed to a specifically localized isoform of sAC, so it is unclear whether they define unique cAMP functional compartments.

The absence of sAC impairs mitochondrial energy metabolism causing a decline in CI activity, COX activity, cell respiration, $\Delta \Psi m$, ATP production, and impaired growth in galactose medium. Furthermore, sAC KO cells display a shift towards glycolytic metabolism, as indicated by a decreased OCR:ECAR ratio. Taken together, these results are consistent with data obtained with pharmacological modulators of the enzyme (Acin-Perez et al., 2009a; Acin-Perez et al., 2009b; De Rasmo et al., 2015; Di Benedetto et al., 2013). In addition, recent reports have shown that pharmacological inhibition of sAC causes CI activity defects in fibroblasts (De Rasmo et al., 2015) and silencing of sAC resulted in decreased TMRM accumulation potential in cardiomyocytes (Wang...
et al., 2016). Importantly, we demonstrate that bioenergetic defects in sAC KO cells are due to the loss of the matrix sAC-defined cAMP domain, since they can be fully rescued by expression of a recombinant sAC selectively targeted to the mitochondrial matrix. Importantly, we find that the effects of sAC ablation extend to broader intracellular signaling mechanisms, involving OXPHOS biogenesis, possibly as an adaptive response to energy depletion, which are also complemented by targeting sAC to the matrix.

Energy deprivation induces activation of AMPK, one of the key enzymes responsible for mitochondrial biogenesis regulation (Scarpulla, 2011). AMPK is activated by phosphorylation and maintains energy homeostasis by promoting catabolism and inhibiting ATP consuming processes (Hardie et al., 2012; Witczak et al., 2008). Since AMPK can be phosphorylated by PKA in a cAMP-dependent manner (Hawley et al., 2010), it could be expected that sAC KO MEFs have less P-AMPK. Instead, P-AMPK is upregulated in sAC KO MEFs. Therefore, a cAMP-independent mechanism may be involved. For example, AMPK can be phosphorylated by LKB1 (liver kinase B1), a serine threonine kinase activated in conditions of energy deprivation (Ruderman et al., 2010). Another mechanism of AMPK phosphorylation is through CaMKKβ (Ca²⁺/calmodulin-dependent protein kinase kinase beta). However, since this kinase is activated by increased cytosolic Ca²⁺ levels, and in sAC KO MEF ER Ca²⁺ release is decreased, it is unlikely that CaMKKβ represents a major effector of AMPK phosphorylation in these cells. Another activator of AMPK phosphorylation is ROS production (Han et al., 2010; Zmijewski et al., 2010). However, the downstream effector PGC-1α, was unaffected by antioxidants in sAC KO MEFs, suggesting that a reduction in intracellular ATP levels, rather than ROS, is likely responsible for AMPK phosphorylation. The activation of AMPK suggests that an energy deprivation response takes place in sAC KO cells, leading to a signaling cascade that increases OXPHOS biogenesis (Scarpulla, 2012). In support of this hypothesis, we observed that the expression of the downstream effectors of P-AMPK, PGC-1α and NRF1, is
upregulated in sAC KO MEFs. Furthermore, normalization of mitochondrial energy production by mtsAC decreases the levels of P-AMPK.

Mitochondrial Ca$^{2+}$ is a modulator of cellular energy homeostasis (Denton, 2009) and the release of ER Ca$^{2+}$ was shown to affect, not only mitochondrial biogenesis, but also signaling through AMPK (Cardenas et al., 2010) and mitochondrial substrate transporters (Satrustegui et al., 2007). Furthermore, it was reported that Ca$^{2+}$ regulates sAC activity in mitochondria (Di Benedetto et al., 2014; Di Benedetto et al., 2013), highlighting a pathway of metabolic regulation that involves Ca$^{2+}$, sAC, and energy production. sAC KO mitochondria take up more Ca$^{2+}$ when Ca$^{2+}$ release from the ER is caused by TG or when isolated mitochondria are exposed directly to Ca$^{2+}$. We interpret this observation in light of the increased complement of proteins involved in Ca$^{2+}$ handling in mitochondria, notably MCU. However, sAC KO cells have attenuated cytosolic and mitochondrial Ca$^{2+}$ responses upon metabotropic stimulation of ER Ca$^{2+}$ release. We determined that this was not due to lower ER Ca$^{2+}$ stores, but to decreased IP3R phosphorylation. IP3R1 phosphorylation increases the activity of the receptor (Ferris et al., 1991; Nakade et al., 1994; Tang et al., 2003; Wagner et al., 2004), and we find that sAC plays a role in this regulation. Lack of sAC-generated cAMP results in decreased PKA-mediated IP3R phosphorylation in the ER and desensitization of the receptor, leading to lower probability of the open state.

In conclusion, our data support the model (summarized in Figure 8F), whereby signaling pathways initiated by distinct sAC-defined intra and extramitochondrial domains converge in fine-tuning the regulation of cellular bioenergetics through ER-mitochondria Ca$^{2+}$ signaling and OXPHOS biogenesis.

Material and Methods

MEF cultures. Wt and sAC KO MEFs were cultured in DMEM supplemented with 25 mM glucose, 110 mg/L pyruvate, 1% Penicillin-Streptomycin, and 5% fetal bovine serum (FBS). Where indicated, MEFs were cultured in galactose medium, in which glucose was substituted with 5 mM
galactose, plus 5% dialyzed FBS. Cells were grown in galactose or glucose medium for 48-72 hours and then counted in a particle counter (Coulter Beckman). The cells genotype was assessed by PCR using the following primers: *wt sAC gene* forward TCTGGCCACACACTAAGG, reverse CTCCAGCTCCGATGAAGG; *KO sAC gene* forward CTGGGCTGTCTCCTCAAGCTC, reverse GCAGCGCATCGCCTTCTATC. Cells were regularly tested free for mycoplasma test.

**Mitochondria isolation.** Cells were grown to 70% confluence in P150 flasks, washed twice with phosphate buffer saline (PBS) and scraped off in 2 ml of cold isolation buffer (in mM: 225 mannitol, 20 HEPES, 75 sucrose, pH 7.4), containing a protease inhibitor cocktail (Roche Diagnostics) and phosphatase inhibitors (in mM: 1 NaF,1 Na₃VO₄, 1 pyrophosphate, 2 Imidazole). Cells were collected by centrifugation at 2000 x g for 5 min, at 4°C. The resulting pellet was resuspended in cold isolation buffer containing 1 mg/ml fatty acid free BSA and homogenized on ice in a glass-glass homogenizer. The homogenate was cleared at 800 x g for 5 min, at 4°C. The supernatant was centrifuged at 10,000 x g, for 10 min, at 4°C. The resulting pellet containing the mitochondrial fraction was washed twice in isolation buffer, resuspended in 50-80 μl of isolation buffer, snap-frozen in liquid nitrogen, and stored at −80°C.

**OXPHOS function measurements.** ATP synthesis was measured in cells (1.5 × 10⁶) permeabilized with digitonin 50μg/ml, using a kinetic luminescence assay, as described (Vives-Bauza et al., 2007). Pyruvate (5 mM) and malate (2 mM) were used as substrates. COX activity was measured spectrophotometrically in isolated mitochondria (5–10μg of protein), as described (Birch-Machin and Turnbull, 2001). NADH-dependent enzymatic activities of Complex I were assayed spectrophotometrically using a Lambda 35 spectrophotometer (Perkin Elmer) or a SpectraMax plate reader (Molecular Devices) and expressed as a decrease in absorption at 340nm (ε340nm = 6.22 mM⁻¹ cm⁻¹) with 150μM NADH in SET pH 7.5 (0.25M sucrose, 0.2mM EDTA, 20μg/ml alamethicin,
1mM MgCl₂, 50mMTris-HCl) buffer containing 5–30µg protein/ml of isolated mitochondria. For the measurement of NADH:DBQ (decylubiquinone) oxidoreductase activity, the concentrations of additions were 15-30 µg protein/ml, 45µM DBQ, 1 mg/ml BSA (fatty acids free) and 1 mM cyanide. The activity was sensitive to rotenone (1 µM). For the measurement of NADH:HAR (hexaammineruthenium) oxidoreductase activity, isolated mitochondria (5-10 µg of protein per ml) were assayed in the presence of 1mM cyanide with 1mM HAR. The activities were measured at 25°C and expressed in nmol NADH×min⁻¹×mg⁻¹. NADH:DBQ oxidoreductase activity was normalized to complex I content as estimated from NADH:HAR reductase activities (DBQ/HAR).

Quantification of mitochondrial membrane potential and morphology. MEFs were incubated with 10nM tetramethyl rhodamine methyl ester (TMRM) for 25 min at 37°C in DMEM medium. Cells were washed twice and then imaged in HEPES-Tris (HT) imaging buffer (in mM: 132 NaCl, 4.2 KCl, 1 MgCl₂, 5.5 D-glucose, 10 HEPES, 1 CaCl₂, pH 7.4 with Tris base) at 37°C. Images from ten random fields per coverslips were acquired using a Leica TCS SP5 Confocal Microscope (Leica Microsystems) with an oil-immersion 63 × [1.4 (NA)] lens. Mitochondrial morphology was measured using Image Pro software (Universal Imaging) in cells loaded with Mitotracker Green (50nM) for 25 min at 37°C as previously described (Koopman et al., 2006; Koopman et al., 2005). Briefly, cells were imaged using life-cell confocal laser scanning. Next, the acquired images were sequentially processed using an algorithm that yields quantitative descriptors for mitochondrial length and degree of branching (Aspect ratio AR, form factor F) and the number of mitochondria per cell. In essence, the image-processing algorithm converts a background-corrected image into a binary image displaying mitochondrial structures (white) on a black background. This conversion consists of a number of subsequent calculations involving: (i) background correction of the acquired raw image, (ii) linear contrast optimization, (iii) top-hat filtering, (iv) median filtering, and (v) thresholding.
**OCR and ECAR measurement.** Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were determined by Seahorse XF 96 flux analyzer (Seahorse Bioscience). MEFs were plated at 30,000/well. The next day the media was replaced with KHB XF Assay media supplemented with 1mM sodium pyruvate and 1mg/mL D-glucose (Sigma G5400) and incubated at 37°C in a non-CO₂ incubator for 1 hour, prior to the measurement. For normalization, protein determination of each well was done using DC™ Protein Assay kit (Biorad). To assess OCR after induction of ER Ca²⁺ release in wt and sAC KO cells, cells were treated with SF6847 to prevent matrix NADH accumulation and then stimulated with ATP (1mM).

**Western blot electrophoresis.** Cell homogenates or isolated mitochondria fractions were analyzed by Western blot, as described before (Valsecchi et al., 2012). The following antibodies were used for protein detection: AMPKα, P-AMPKα (Thr172) (Cell signaling, catalog number 2532 and 2531 respectively, 1:1000); IP3R and P-IP3R Ser 1756 (Cell Signaling, catalog number 8568 and 3760 respectively, 1:500); EPAC1 (Cell Signaling, catalog number 4155, 1:500); MCU (Sigma, catalog number 16480, 1:1000); MCU1 (Abcam, catalog number 102830, 1:1000); OXPHOS rodent cocktail (Abcam, catalog number 110413, 1:1000); SLC24A6 (Abcam, catalog number 83551, 1:1000); VDAC (Abcam, catalog number 34726, 1:1000); HSP60 (Enzo Life Sciences, catalog number 828, 1:1000); PKA (Upstate, catalog number 06-903, 1:1000); β-actin (Sigma, catalog number A5316, 1:1000). Appropriate fluorescently labeled secondary antibodies (Licor) were used for detection in the Odyssey CLx imaging system (Licor). For quantification of band intensities, the Image Studio Software package (Licor) was used. For the quantification of phospho-proteins, such as P-AMPK and P-IP3R1, relative to the total protein, replicates of the samples were run in parallel. The blot was then cut and the two halves probed with the antibodies against phospho-protein or the antibodies against the total proteins.
**IF-1 Immunoprecipitation.** Immunoprecipitation of IF-1 was performed using a co-immunoprecipitation kit (Thermo Scientific Pierce). Briefly, IF-1 antibody or normal mouse IgGs (ThermoFisher) were covalently coupled to IP resins. Mitochondria (0.5 mg) were resuspended in Co-IP lysis/wash buffer and incubated with the resins overnight. Proteins were eluted in elution buffer and eluates neutralized with Tris-HCl pH 8.5. Western blot was performed with anti IF-1 antibody to assess the presence of the target protein in the Co-IP eluates. Two different anti phospho Ser/Thr antibodies (Cell Signaling and BD Biosciences) were used to assess the phosphorylation of immunoprecipitated IF-1.

**mtDNA and RNA qPCR.** Total DNA was isolated from cell pellets using the Wizard genomic DNA Purification kit (Promega). The abundance of the mtDNA COX1 gene was assayed by qRT-PCR using SYBR Green (Roche) in a Light Cycler (Roche). 18S rRNA gene was used as an internal control. For mRNA analyses total mRNA was isolated from cells using the RNAqueous-4PCR kit (Life Technology). cDNA was generated using the ImProm-II Reverse Transcription System (Roche). The relative mRNA levels of PGC1α and NRF1 were determined by qRT-PCR and normalized by β-actin mRNA. Primers and PCR conditions were as follows.

Primers:

*COX1 gene*, forward CATCCCTTGACATCGTG, reverse CTGAGTAGC GTCGTGG.

*18S rRNA gene*, forward CCGACAGGATTGACAGA, reverse CCAGTCAGTGTAGCGC.

*PGC1α cDNA*, forward TGAAAAAGCTTGACTGGCGTC, reverse CGCTAGCAAGTGTTCCTCAT.

*NRF1 cDNA*, forward CTTCATGGAGGAGCACGGAG, reverse ATGAGGCCGTTTCCGTTTCT.

*β-actin cDNA*, forward CTTTGCAGCTCCTTGCGTGC, reverse CCTTCTGACCCATTCCACC.
Forward and reverse primers (10µM) were mixed with the master mix SYBR Green (Roche), 25mM Mg\(^{2+}\), and H\(_2\)O, in the presence of 2.5ng of DNA, according to the manufacturer’s protocol.

PCR conditions: initial denaturation: 95°C 10 min; denaturation: 95°C 15 sec, anneal: 50°C 10 sec, extend: 72°C 15 sec, 30 cycles; final extension: 72°C 10 min.

The reactions were optimized using a linear concentration range of DNA standards. In each experiment, samples were analyzed in triplicate.

Transgenic sAC and mtsAC expression. Mt-sAC and sAC expression constructs were previously described (Acin-Perez et al., 2009a). For imaging experiments, wt and sAC KO MEFs were transfected with sAC, mtsAC or empty vector using lipofectamine 2000 (Life Technology), according to the manufacturer’s instructions.

Cytosolic and mitochondrial Ca\(^{2+}\) imaging. For cytosolic Ca\(^{2+}\), cells were incubated with 4 µM fluo4 (Life Technology) in DMEM for 25 min at 37°C, washed three times with HT buffer and placed in a heated live imaging station (lens, stage, and perfusion lines were maintained at 37°C). Alternatively, cells were transfected with pGP-CMV-GCaMP6s (Addgene) plasmid to express the cytosolic Ca\(^{2+}\) reporter. Live imaging was performed on a Leica TCS SP5 confocal microscope using a water-immersion 20× [0.7 NA] lens. During the recordings, cells were perfused with agents that induce release of intracellular Ca\(^{2+}\) stores (1 mM ATP, 1 µM bradikynin or 1 µM thapsigargin) in HT imaging buffer. Images were taken every 2 seconds.

For mitochondrial Ca\(^{2+}\), MEFs were transfected with pTurbo-mitoGCaMP6. GCaMP6 coding region was amplified from pGP-CMV-GCaMP6s, a N-terminal mitochondrial-targeting signal from the F\(_{0}\) subunit C of ATPase was added, and the ligated product cloned into the AgeI and NotI sites of the pTurbo vector (Evrogen).
Changes in fluorescent intensities over time were measured in individual cells after thresholding of the images using Metamorph. Individual data points were plotted as F/F0, and graphs and statistical analyses performed using OriginPro software.

**Immunocytochemistry.** For immunocytochemistry, cells grown on glass coverslips were fixed with 4% paraformaldehyde (PFA; 10 min), followed by three washes in PBS at room temperature. The following primary antibodies were used: HA-Tag (6E2) Cell Signaling, 1:200), TOM20 (FL-145, Santa Cruz Biotechnology, 1:500). Secondary Cy2 anti-mouse and Cy3 anti-rabbit antibodies (Jackson ImmunoResearch, 1:250) were used for fluorescent immunodetection.

**Mitochondrial Ca\(^{2+}\) uptake.** Mitochondrial Ca\(^{2+}\) uptake was estimated fluorometrically with the Ca\(^{2+}\)-sensitive fluorescent dye Calcium green 5N (CaGr5N). Mitochondria (0.15-0.25 mg /ml) were added to 2 ml of incubation medium (125 mM KCl, 20 mM HEPES (pH 7.2), 10 mM NaCl, 4 mM Na\(_2\)PO\(_4\), 5 mM glutamate, 2.5 mM malate, 40 mM EGTA, 100 nM CaGr5N). Changes in fluorescence were recorded in a F-7000 spectrofluorometer (Hitachi) using 340/380 nm excitation and 510 nm emission wavelengths. Experiments were performed in a fluorimeter cuvette at 37°C. Bolus additions of Ca\(^{2+}\) (25 µM CaCl\(_2\)) were made to the mitochondrial suspension every 150 sec, until mitochondria were unable to take up Ca\(^{2+}\). To correlate the CaGr5N fluorescence response to Ca\(^{2+}\) concentration, consecutive Ca\(^{2+}\) additions by 40, 40, 20 nmol, and then by 10 nmol were done, until Ca\(^{2+}\) was no longer accumulated.

**ER-mitochondria co-localization.** MEFs were transfected using lipofectamine 2000 with Sec61-GFP (ER marker, green) and mito-Dsred (mitochondrial marker, red). After 48 hours, cells were fixed with 4% PFA for 10 min, followed by three washes in PBS. Z-stack images were obtained using a
Leica TCS SP5 spectral confocal microscope. Ten random fields per coverslip were imaged and the colocalization of the two fluorochromes was assessed by the colocalization function in MetaMorph.

Statistical analyses. Data were expressed as the mean ± SEM. Differences between two groups were evaluated by two-tailed, unpaired, Student’s t test, with significance set at P<0.05. Differences among multiple groups were evaluated by ANOVA with Tukey’s correction. In the figures, * indicates p<0.05; ** p<0.01; *** p<0.001.

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References


Fig. 1: OXPHOS complex activities in wt and sAC KO MEFs

A) NADH:DBQ oxidoreductase activity (DBQ) (Complex I activity) (n=8). B) NADH:HAR oxidoreductase activity (HAR) (n=8). C) DBQ:HAR ratio (n=8). D) COX activity (n=8). Data is expressed as mean±s.e.m. in 8 different biological replicates (**p<0.001, Student’s t-test).
**Fig. 2: Mitochondrial respiration, ATP synthesis and membrane potential in wt and sAC KO MEFs**

A) Oxygen consumption rate (OCR, n=3). B) Extracellular acidification rate (ECAR, n=3). C) OCR/ECAR ratio (n=3). D) Maximal OCR (n=3). E) Mitochondrial ATP synthesis (n=6). F) Trace of TMRM fluorescence in cells, before and after the addition of FCCP (1 μM). G) TMRM average intensity expressed as % of wt (n=3, >60 cells measured in each experiment). H) Cell number of wt and sAC KO MEFs cultured for 48 and 72h in glucose medium (n=3). I) Cell number of wt and sAC KO MEFs cultured for 48 and 72h in galactose medium (n=3). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (*p<0.05, **p<0.001, Student’s t-test).
Fig. 3: OXPHOS complexes, mtDNA, mitochondrial content and mitochondrial biogenesis signaling

A) Western blot of OXPHOS complexes subunits and structural component of the mitochondria machinery in cell homogenates (n=3-6). B) Quantification of the relative content of OXPHOS complexes normalized by β-actin (n=3-6). C) mtDNA content measured by qPCR of COX1 gene normalized by 18S rRNA gene (n=4). D) Mitochondrial density expressed as the average area of mitotracker Green labeled mitochondria in each cell (n=3, >20 cells per experiment). E) Western blot of AMPK and P-AMPK (Tyr 172). F) Quantification of the P-AMPK/AMPK intensity ratio (n=4). G) PGC-1α mRNA levels relative to β-actin (n=4). H) NRF1 mRNA levels relative to β-actin (n=4). All data are expressed as % of wt. Data is expressed as mean±s.e.m. in indicated number of different biological replicates (*p˂0.05, **p˂0.01, ***p˂0.001, Student’s t-test). I) PGC-1α mRNA levels relative to β-actin in cells treated with (+D) or without Dorsomorphin (n=3). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (***p˂0.001, ANOVA with Tukey’s correction).
**Fig. 4: Effect of mtsAC and sAC expression on mitochondrial bioenergetics in sAC KO cells**

A) Representative images of sAC KO MEFs transfected with mtsAC-HA or sAC-HA tag and immunostained for HA (green) and TOM20 (red) antibodies. B) Western blot of total cell homogenate.
and enriched mitochondrial fractions from KO MEFs transfected with sAC and mtsAC and immunoblotted for HA, TIM23 and β-actin. C) DBQ-HAR ratio of complex I activity in wt, KO, KO+sAC and KO+mtsAC (n=3). D) COX activity in wt, KO, KO+sAC and KO+mtsAC (n=4). E) TMRM average intensity (expressed as % of KO) in wt, KO, KO+sAC and KO+mtsAC (n=3). F) ATP synthesis in wt, KO, KO+sAC and KO+mtsAC (n=4). G) Cell number of wt, KO, KO+sAC and KO+mtsAC MEFs cultured in medium containing glucose at 24, 48 and 72h. H) Cell number of wt, KO, KO+sAC and KO+mtsAC cultured in medium containing galactose at 24, 48 and 72h (n=3). The lines for statistical comparisons encompass both bars representing average values at 48h and 72h. I) Western blot of AMPK, P-AMPK, HSP60, TIM23 in wt, KO, KO+sAC, KO+mtsAC and J) P-AMPK:AMPK ratio expressed as % of wt (n=4). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (*p<0.05, **p<0.01, ***p<0.001, ANOVA with Tukey’s correction).
Fig. 5: mitochondrial Ca\textsuperscript{2+} responses to ER Ca\textsuperscript{2+} release stimulation in wt and sAC KO MEFs

A) Representative image of wt MEFs co-transfected with mitoGCamp6 and mitoDsRed and merged images. B) Representative average intensity curve of mitoGCamp6 upon ATP stimulation in wt cells (n=6 cells). C) Average intensity of mitoGCamp6 upon FCCP (first) and ATP (after) addition in wt
cells (n=3 cells). D) Average percentage of responding cells in wt versus KO cells upon ATP stimulation. E) Top panel: curves of average mitoGCaMP6 fluorescence in MEFs stimulated with 1mM ATP (n=14-78 cells, in 3 independent experiments). Bottom panel: quantification of mitoGCaMP6 fluorescence peak values in responding cells. F) Percentage of responding cells upon TG stimulation. G) Top panel: average curve of mitoGCaMP6 fluorescence in wt and sAC KO MEFs stimulated with 1µM TG (n=26-37 cells, in 3 independent experiments). Bottom panel: quantification of mitoGCaMP6 fluorescence peak values in responding wt and sAC KO cells. H) Western blot of MCU in isolated mitochondria from wt and sAC KO MEFs (n=3) and I) quantification of the relative intensity normalized on TIM23 and expressed as % of wt. J) Western blot of NCLX in isolated mitochondria from wt and sAC KO MEFs and K) quantification of the relative intensity normalized on TIM23 and expressed as % of wt. L) Western blot of MICU1 in isolated mitochondria of wt and sAC KO cells (n=3) and M) quantification of the relative intensity normalized on TIM23 and expressed as % of wt. Data is expressed as mean±s.e.m. in indicated number of different biological replicates (*p<0.05, **p<0.01, ***p<0.001, Student’s t-test).
Fig. 6: ER-mitochondria bioenergetic coupling through Ca\(^{2+}\) signaling

A) OCR measured in uncoupled mitochondria (SF 1\(\mu\)M) of wt and sAC KO cells in the presence or absence of ATP (n=3). B) Percentage of responding wt and sAC KO cells loaded with fluo4 and stimulated with ATP 1mM. C) TMRM average intensity of wt and sAC KO MEFs upon ATP 1mM stimulation and loaded with fluo4. Insert: peak average intensity of fluo4 in wt and sAC KO MEFs upon ATP stimulation (n=30-55, in 3 independent experiments). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (**p<0.01, ***p<0.001, Student’s t-test).
Fig. 7: Expression of mitochondrial Ca$^{2+}$ handling proteins and IP3R phosphorylation in wt and sAC KO MEFs

A) Percentage of wt and sAC KO cells responding to ATP stimulation. B) Top panel: average GCaMP6 fluorescence in wt and sAC KO MEFs stimulated with 1mM ATP (n=18-32 cells, in 3
independent experiments). Bottom panel: quantification of GCaMP6 fluorescence peak values in responding wt and sAC KO cells. C) Percentage of responding cells upon TG stimulation in wt and sAC KO cells. D) Top panel: curves of average fluo4 fluorescence in MEFs stimulated with 1 µM TG (n=165-254 cells, in 3 independent experiments). Bottom panel: quantification of fluo4 fluorescence peak values in responding wt and sAC KO cells. E) Western blot of IP3R1 P-Ser1756 and IP3R1 in wt and sAC KO cells in the absence or presence of CIP. F) Quantification of the P-IP3R1 Ser1756:IP3R1 ratio expressed as a % of wt (n=6). G) Percentage of responding wt and sAC KO cells loaded with fluo4 and pretreated with KH7 100mM for 30 minutes. Data is expressed as mean±s.e.m. in indicated number of different biological replicates (*p<0.05, ***p<0.001, Student’s t-test). H) Percentage of responding wt and sAC KO cells loaded with fluo4 upon ATP stimulation and treated with the sAC inhibitor H89 1µM or the tmAC activator forskolin 50 µM (n=3). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (*p<0.05, ***p<0.001, ANOVA with Tukey’s correction).
**Fig. 8: Effects of sAC and mtsAC expression on ER Ca^{2+} release in sAC KO MEFs**

A) Percentage of cells responding to ATP stimulation in wt, KO, KO+mtsAC and KO+sAC MEFs and loaded with fluo4. B) Top panel: average fluo4 fluorescence in MEFs stimulated with 1mM ATP. Bottom panel: quantification of the peak values in responding cells. (n=29-52 cells, in 3 independent experiments). C) Percentage of cells responding to ATP stimulation in wt, KO, KO+mtsAC and KO+sAC MEFs and loaded with mitoGCaMP6. D) Top panel: average mitoGCaMP6 fluorescence in MEFs stimulated with 1mM ATP. Bottom panel: quantification of the peak values in responding cells. (n=35-42 cells, in 3 independent experiments). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (***p<0.001, ANOVA with Tukey’s correction). E) Percentage of cells responding to ATP stimulation in KO, KO+sAC and KO+sAC treated with forskolin/IBMX and loaded with mitoGCaMP6 (n=30-54 cells, in 3 independent experiments). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (***p<0.001, Student’s t-test).

F) Schematic representation of sAC-cAMP domains regulating ER calcium signaling and OXPHOS: cytosolic sAC produces cytosolic cAMP that leads to PKA-mediated phosphorylation and activation of the IP3R in the ER membrane and increased probability of the open state upon IP3 stimulation. Ca^{2+} from the ER is taken up by MCU and activates mitochondrial OXPHOS. Mitochondrial sAC regulates OXPHOS in a cAMP mediated manner, independently of cytosolic sAC. The involvement of PKA as the cAMP-activated kinase in the mitochondrial matrix remains controversial (Valsecchi et al., 2014). In turn, OXPHOS regulates the PGC1α/NRF1 signaling pathway through p-AMPK.
Figure S1: COX activity and ATP synthesis in wt2 and sAC KO2 MEFs

A) COX activity in independent lines of wt2 and sAC KO2 MEFs (n=3). B) ATP synthesis in permeabilized wt2 and sAC KO2 MEFs (n=3). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (*p<0.05, ***p<0.001, Student’s t-test).
**Figure S2: Quantification of OXPHOS proteins in isolated mitochondria from wt and sAC KO cells.**

A) Representative western blot of OXPHOS subunits and TIM23. B) Quantification of band intensity normalized by TIM23 (n=5). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (*p<0.05, Student’s t-test).
Figure S3: mitochondrial morphology and effect of antioxidants on PGC-1α expression.

A) Representative image of wt and sAC KO cells loaded with MitoTracker Green 50nM. B) Mitochondria aspect ratio in wt and sAC KO MEFs expressed as % of wt. C) Mitochondrial form factor in wt and sAC KO MEFs expressed as a % of wt. In B and C, n=48-83 in 3 experiments. D) Effects of the antioxidant Trolox on PGC-1α mRNA content (n=3) in wt and sAc KO cells. Data is expressed as mean±s.e.m. in indicated number of different biological replicates (*p<0.05, ANOVA with Tukey’s correction).
Figure S4: mtsAC expression decreases OXPHOS component content in KO cells. A) representative western blot of KO and KO+mtsAC cells probed with OXPHOS cocktails antibody. B) Quantification of the western blot. C) mRNA level of PGC1α expressed as % of KO. C) Mitochondrial DNA content expressed as % of KO (COXI gene normalized by 18S rRNA). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (***p<0.001, Student’s t-test).
**Figure S5: mitochondria Ca^{2+} uptake and ER-mitochondria colocalization**

A) Representative images of wt and sAC KO cells co-transfected with the ER protein Sec61-GFP (green) and mitochondrial mitoDsRed (red). B) Quantification of ER-Sec61-GFP and mitoDsRed colocalization assessed by the co-localization function of MetaMorph expresses as % of red over green. C) Mitochondrial Ca^{2+} uptake in isolated mitochondria from wt and sAC KO MEFs (n=3). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (***p<0.001, Student’s t-test).
Figure S6: Effect of EPAC inhibitor ESI-09 on mitochondrial Ca\textsuperscript{2+} uptake

A) Fluorescent traces of mitoGCaMP6 in wt and sAC KO (KO) MEFs treated with and without ESI-09 (ESI) and stimulated with TG.

B) Quantification of mitoGCaMP6 fluorescence peaks (n=40, 32, 28, and 44 cells, for wt, wt + ESI, sAC KO, and sAC KO + ESI, respectively). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (***p<0.001, ANOVA with Tukey’s correction).
Figure S7: cytosolic ER Ca\textsuperscript{2+} release induced by Bradykinin stimulation, effects of dantrolene on ER Ca\textsuperscript{2+} release,

A) Percentage of responding wt and sAC KO MEFs upon Bradykinin (BK) stimulation. B) Top panel: Average fluo4 fluorescence curve in wt and sAC KO cells stimulated with 1\,\mu M bradykinin; Bottom panel: quantification of Ca\textsuperscript{2+} peak in responding wt and sAC KO cells.
(n=33-66 cells, in 3 independent experiments). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (***p<0.001, ***p<0.001, Student’s t-test). C) Percentage of responding wt and sAC KO cells with and without dantrolene (10µM for 1 hour pre-incubation and in the perfusion buffer) and ATP (1mM). D) Top panel: fluo4 fluorescence in wt and sAC KO cells stimulated with ATP (1mM) in the presence or absence of dantrolene. Bottom Panel: quantification of the Fluo4 peak intensity in cells stimulated by ATP with and without dantrolene (n=15-27 cells, in 3 independent experiments). Data is expressed as mean±s.e.m. in indicated number of different biological replicates (***p<0.001, ANOVA with Tukey’s correction).
Figure S8: IP3R phosphorylation is regulated by sAC by forskolin or KH7.

A) Representative western blot of IP3R P-S1756 and total IP3R in wt, KO and KO+sAC cells.
B) Quantification of P-S1756 normalized by total IP3R, in KO and KO+sAC cells expressed as % of wt (n=3).
C) Representative western blot of IP3R P-S1756 and total IP3R in wt, and KO cells treated with forskolin and KH7.
D) Quantification of P-S1756 normalized by total IP3R, in KO and KO+sAC cells expressed as % of wt (n=3). Data is expressed as mean±s.e.m. in
indicated number of different biological replicates (**p<0.001, ANOVA with Tukey’s correction). E) Representative images of MEF cells co-transfected with mtsAC-HA or sAC-HA (green) and mitoDsRed (red) and merged images.