One gene - two proteins: The C-terminus of the prototypical M2 muscarinic receptor localizes to the mitochondria

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Abstract

Muscarinic acetylcholine receptors are G protein-coupled receptors (GPCRs) activated by the endogenous neurotransmitter acetylcholine. We show here that the carboxyl terminal fragment of the muscarinic M₂ receptor, containing the transmembrane regions 6 and 7 (M₂tail), is expressed by virtue of an internal ribosome entry site. Radioligand binding, biochemical and molecular biology experiments, combined to advanced fluorescence microscopy reveal that the M₂tail, whose expression is upregulated in cells undergoing integrated stress response, does not follow the normal route to the plasma membrane, but is almost exclusively sorted to the mitochondria inner membrane: here it controls oxygen consumption, cell proliferation and the formation of reactive oxygen species via reduction of oxidative phosphorylation. Genetic editing of the key methionine where cap-independent translation occurs, reveals the physiological role of this process in influencing cell proliferation and oxygen consumption. The expression of the carboxyl-terminal of a GPCR, capable of regulating mitochondrial function, constitutes a hitherto unknown mechanism that cells may use for controlling their metabolism under variable environmental conditions, notably unrelated to the canonical signalling function of the GPCR at the plasma membrane.

Muscarinic receptors include five structurally related receptors, belonging to class A of the G protein-coupled receptor (GPCR) family¹. They are comprised of seven trans-membrane domains (TM domains 1-7) connected by intra and extracellular loops. Of these structural elements, the third intracellular (i3) domain is the one conferring a coupling specificity for G proteins, β -arrestin recruitment and endocytic internalization². However, only a small portion of the i3 loop is required for receptor function, and for this reason large deletions of this segment have no effect on receptor activation and internalization³.

We could previously show that the M₂ receptor can be split at the level of the i3 loop into two segments, each behaving as an autonomous folding unit capable of targeting the plasma membrane and surprisingly reconstituting a physiologically active receptor when expressed together in transfected cells⁴. In the context of these studies, a mutant of the receptor carrying a stop codon in position 228 was surprisingly observed to retain (albeit reduced) ligand binding in radioligand binding assays¹¹. This finding cannot be explained by the

fact that the insertion of the stop codon results in the expression of an N-terminal receptor fragment, constituted by the TM regions 1-5, since this fragment alone has been shown to be not able to bind muscarinic ligands and to trigger signal transduction¹². Therefore, an alternative explanation for the behavior of stop-codon mutant 228 is needed. Since the N-terminal fragment can reconstitute a physiologically active receptor when co-transfected with the C-terminal fragment, a plausible possibility is that the C-terminal segment of the receptor can be independently produced and reconstitute an active receptor with the N-terminal portion on the cell membrane. This scenario opens the interesting question of how the C-terminal fragment can be translated endogenously as an independent polypeptide.

In eukaryotes, the most common mechanism of translation initiation is the cap-dependent process, which requires the interaction of several key proteins at the 5'-end (5' cap) of mRNA molecules. However, in recent years, it has been demonstrated that the presence of internal ribosome entry site (IRES) sequences, which are common features of virus replication⁵, can mediate cap independent translation of eukaryotic RNA(s)⁶. This presents the suggestive possibility that a cap-independent translation of the M₂ C-terminal domain indeed occurs in this mutant, opening a number of important questions about the molecular processes involved in the synthesis and subcellular behaviour of the C-terminal domain fragment.

GPCRs are normally distributed at the plasma membrane, where, after activation by an agonist and coupling to G proteins, regulate a number of intracellular effectors. Nevertheless, non-canonical localizations of GPCRs have also been described, such as in endosomes⁷, cell division compartments (centrosomes, spindle midzone and midbodies)⁸, and mitochondria^{9 10}, underpinning non-traditional roles for this protein family.

In this work, we therefore set out to explore whether the expression of the C-terminal domain of the M₂ muscarinic receptor, a region containing the TM regions 6 and 7, occurs naturally by a cap-independent translation mechanism. Combining radio-ligand binding, molecular biology approaches, advanced fluorescence imaging and genetic editing of human induced pluripotent stem cells (hiPSCs) we have observed that the C-terminal domain of this receptor is indeed expressed as an independent polypeptide. Surprisingly, using bimolecular fluorescence complementation, in-vitro mitochondrial import assays and cell metabolism

assays we observe that the C-terminal domain is strongly targeted to the cell mitochondria. Importantly, this mitochondrial targeting has functional ramifications since both cap-dependent mediated overexpression of the C-terminal domain alone, as well as cap-independent mediated translation starting from the mRNA of the full length M₂ receptor, resulted in a sizable reduction of oxidative phosphorylation.

In summary, our study reports the new observation that alternative reading of a GPCR receptor gene can translate into a fragment of the receptor that serves a cellular function entirely different from canonical GPCR signalling.

Results

Ligand binding and functional properties of M₂ receptors mutants.

To investigate and distinguish the functional roles of the two known N-terminal (TM1-5) and C-terminal (TM6-7) domains of the M₂ muscarinic receptor, we expressed C-terminally truncated variants of the receptor in COS-7 cells. We first observed that the M₂stop228 construct, a receptor mutant bearing a stop codon at the beginning of the i3 loop in position 228 (but containing the downstream sequence, i.e. leading to the transcription of an mRNA of the same length as that of the wt M₂R) (Fig. 1a and Supplementary Fig. 1), exhibited unexpected binding activity¹¹ albeit at a very low level of expression, i.e. 45 fmol/mg of protein. Both K_D value of [³H]NMS (N-methylscopolamine, an antagonist) calculated from direct saturation experiments, and the IC₅₀ values, calculated in competition experiments for the agonist carbachol, were in fact similar to those calculated for the wild type M₂ receptor (Table 1). Furthermore, the M₂stop228 receptor was found functionally active as it was capable to inhibit adenylyl cyclase¹¹ and increase ERK phosphorylation (Supplementary Fig. 2b), even though the extent of response was reduced if compared to the wild type M₂ receptor. On the other hand, if a stop codon is inserted upstream or downstream of position 228, e.g. at positions 196 or 400, no radioligand binding can be observed (Fig. 1a and Supplementary Table 1).

As we demonstrated in an earlier report¹², another receptor fragment whose sequence runs only until position 283 (i.e. comprising only TM1-5) of the corresponding wt receptor, called M₂trunk(1-283) (Supplementary Fig. 1), did not show any radio-ligand binding activity upon expression in COS-7 cells, despite comprising the first five trans-membrane regions of the wild type M₂ receptor, as the M₂stop228 construct (Table 1).

114 Consistently, a construct containing only the M₂ receptor sequence until amino acid 228 (M₂trunk(1-228)), did
115 not show any radio-ligand binding (**Table 1**).

116 On the other hand, we observed binding and downstream signaling ability in co-transfection experiments of
117 the M₂trunk(1-283) and M₂tail(281-466) (**Table 1**), the latter being a construct containing only the sequence
118 between aminoacid 368 and the end of the M₂ receptor, i.e. comprising TM6 and 7. Therefore, co-expression
119 of the M₂trunk(1-283) (TM1-5) and M₂tail(368-466) (TM6-7) reconstitutes a functional full length receptor at
120 the cell membrane (**Fig. 1b**).

121 These data suggest that, for the M₂stop228 receptor to display binding and functional activity, the receptor
122 sequence downstream of the stop codon in position 228 (containing TM6 and 7) has (i) to be translated and
123 (ii) to presumably interact with the first five TM regions of the M₂ receptor. This notion is graphically
124 summarized in **Fig. 1b**. For this hypothesis to be true, however, the C-terminal portion of the receptor needs
125 to be expressed despite the presence of stop codons in the i3 loop in our M₂stop228 mutant. Indeed, we
126 confirmed expression of the C-terminal fragment by fusing an enhanced Green Fluorescent Protein (EGFP) at
127 the C terminus of several receptor mutants (**Fig. 1c**).

128 Interestingly, though, while cells expressing the M₂-EGFP displayed the normal, predominant plasma
129 membrane expression (**Fig. 1c**), cells expressing the M₂stop228-EGFP displayed a visible intracellular green
130 fluorescence, which is in stark contrast to cells expressing M₂stop400-EGFP, which do not show any
131 fluorescence, in line with the non-transfected control.

132 To further analyze the atypical behavior of M₂stop228-EGFP, which by displaying green fluorescence
133 confirms that the sequence downstream of the stop in position 228 is indeed translated, we generated a frame
134 shift mutant: we inserted a single guanine nucleotide in the sequence of the M₂-EGFP, changing the methionine
135 in position 368 into an aspartate, which in turn generates an in-frame stop codon at position 370. We named
136 the construct, which notably still displays an EGFP signal in transfected cells, M₂fr.sh.-EGFP (**Fig. 1c**). The
137 observation that the mutant retains EGFP fluorescence is most remarkable, since the EGFP is now out of the
138 original reading frame and it can only be properly translated by a cap-independent translation mechanism,
139 starting from a methionine in the new reading frame.

Furthermore, the observation that both M₂stop228-EGFP and M₂fr.sh.-EGFP retain EGFP fluorescence, whereas M₂stop400-EGFP does not, indicates not only that a cap independent translation mechanism occurs, but also that the starting methionine for the process must be downstream of stop 228 and upstream of stop 400, leaving as a leading candidate the third in-frame methionine of the i3 loop. This is in line with the radio-ligand binding data from co-expression of receptor mutants carrying a double stop codon, the first in position 228 and the second in positions ranging from 248 to 400, including mutations of the first (M248) and second (M296) in-frame methionines in the third i3 loop (**Supplementary Table 1, Fig. 1a** and **Supplementary Fig. 2a**), further pointing to M368 as the most likely site for the cap-independent initiation of translation of the C-terminal domain.

Immunodetection of the M₂ carboxyl terminal fragment

To further characterize the expression of the C-terminal fragment and verify the hypothesis of cap-independent translation, we tagged several of the M₂ receptors mutants described above with a myc-tag for immune detection. The constructs were subcloned in a bicistronic vector backbone, allowing also the expression of the red fluorescent protein mRuby2 as a fluorescent reporter, in order to verify comparable expression levels. HEK293 cells were therefore transfected with the wild type M₂ receptor M₂-myc, the C-terminal fragment starting at the third in-frame methionine M₂tail(368-466)-myc, a mutant of the M₂ receptor with the third in-frame methionine mutated to an alanine, M₂M368A-myc that as discussed above should affect the cap-independent translation, and finally the M₂stop400-myc; the constructs were resolved by SDS-PAGE and assayed by Western blotting, as displayed in **Fig. 2a**. Based on the amino acid sequence, the expected molecular weights for the full length receptor and the C-terminal fragment (inclusive of the myc tag) are ~75 kDa and ~13 kDa respectively.

Interestingly, M₂tail(368-466) runs with two clear bands ~20 and ~15 kDa (**Fig. 2a**), indicating the presence of post-translational modifications and protein processing (see **Discussion**) and thus defining the qualitative pattern expected for the M₂ carboxyl terminal fragment starting at the third in-frame start codon (M368). The full-length receptor was observed, as expected, at about ~75 kDa together with commonly observed higher order oligomers¹³. Notably, the M₂ receptor clearly displays the lower MW band pair also seen for M₂tail(368-466). The strongest confirmation for a cap-independent translation process is found in the M₂M368A: here the

~20 kDa band disappears. In line with what we observed for its EGFP tagged version, the M₂stop400 does not exhibit any band, comparable to the negative control using untransfected cells. The absence of any band further excludes the possibility of a downstream cap-independent translation site yielding a fragment originating from the next in frame methionine (406) (Fig. 2a). Altogether these results support the notion that the band at ~20 kDa observed in the wild type M₂ receptor lane is the bona-fide tail fragment originated by a cap-independent translation mechanism, and that the origin of this process is at the third in frame methionine (M368) of the M₂ receptor.

We then tested whether a similar phenomenon, namely the translation of the C-terminal portion of the M₂ receptor, could be observed endogenously in physiological settings. We therefore prepared protein extracts from heart, brain and spleen tissues from a line of knock-in mice (see Methods), designed to have expression of a Chrm2/tdtomato fusion protein driven by the endogenous M₂ muscarinic acetylcholine receptor promoter/enhancer sequences. The heart and brain are known to express sizable levels of the M₂ receptor, whereas the spleen, where no expression of the M₂ receptor is reported in the proteomic databases, was used as a negative control.

The use of a transgene tag was necessary, since antibody against the M₂ receptor are notoriously ineffective, in particular when targeted at the C-terminal portion of the receptor. Instead, the tdtomato fluorescent protein can be targeted by a specific polyclonal antibody, as shown by the Western blot displayed in Fig. 2b. As expected, the full-length receptor-tdtomato fusion was observed at ~130 kDa (~75 kDa contributed by the receptor and ~55 kDa contributed by tdtomato).

Notably, both in the heart and brain, but not in the spleen, of these knock-in animals we observed a band doublet at ~75 kDa, which corresponds to the sum of the molecular weights of the tail fragment (~20 kDa, see above and Fig. 2a) and the molecular weight of tdtomato.

Testing bicistronic constructs for the presence of an IRES.

To explain the translation of the carboxyl terminal fragment of the M₂ receptor, a mechanism enabling a cap-independent initiation of protein synthesis should be hypothesized^{5,6}. Accordingly, an IRES should be found downstream of the artificial stop codon 228 and upstream of the third in-frame methionine (codon 368) of the i3 loop. Before testing this hypothesis, we first ruled out several alternative mechanisms that could lead to the

translation of M2tail, such as stop codon read-through, termination-reinitiation and alternative splicing in the translation of M2tail, as discussed in the **Supplementary Information** and in **Supplementary Figure 3**. Then, to test the hypothesis that an IRES is leading to the cap-independent translation of the C-terminal domain, we prepared a fluorescence reporter construct in which the i3 loop of M₂ (from nucleotide 685 (a.a. 228) to nucleotide 1101 (a.a. 368)) was inserted between the two fluorescent proteins Sirius and EGFP (**Fig. 3a**). This construct, named Sirius-M₂i3(417n)-EGFP, would translate Sirius according to the classical cap-dependent mechanism, and, at the same time, it would drive EGFP translation only if the M₂i3 loop contains an IRES sequence, effectively acting as a bicistronic expression cassette. We transfected several cell lines with this construct, all positive for the blue and green fluorescence (**Supplementary Fig. 4**). HEK293, used throughout our study, also appeared positive for both the blue and green fluorescence, as shown in the inset of **Fig. 3a**. To rule out the possibility that EGFP translation from the Sirius-M₂i3(417n)-EGFP construct could be due to read-through and termination-re-initiation mechanisms, a mRNA hairpin loop was inserted in the sequence after the stop codon of the Sirius protein. The resulting construct (named Sirius-H-M₂i3(417n)-EGFP) showed only a slight reduction of the EGFP/Sirius with respect to the value measured for the analogous reporter without hairpin (~90%) (**Fig. 3a**). In contrast the Sirius-H-EGFP plasmid, where the entire M₂i3 loop after the Sirius was removed, showed a negligible EGFP/Sirius ratio (below 5%), underlining the importance of the i3 loop for EGFP expression (**Fig. 3a**). Notably, when the hairpin was inserted within the i3 loop of the full length M₂-EGFP, upstream of the M368, it was still possible to observe GFP fluorescence (**Supplementary Fig. 5**). To narrow down the position of the IRES sequence we divided the M₂ i3 loop in three parts and the resulting nucleotide sequences, 685-699, 685-1071 and 685-699/1072-1101, were inserted downstream of the hairpin loop in three different constructs (**Fig. 3a** and **Supplementary Fig. 6**). EGFP fluorescence was not significantly rescued upon insertion of the first 15 nucleotides (685-699) of the i3 loop after the hairpin, nor by the addition of the subsequent 372 nucleotides (685-1071). However, when the last portion of the M₂ i3 loop was inserted in the construct after the Hairpin, leading to Sirius-H-M₂i3(15n/30n)-EGFP, ~110 % of the value measured for the Sirius-M₂i3(417n)-EGFP plasmid was recovered (**Fig. 3a**). These results strongly suggest that the IRES is located in a region of the i3 loop sequence immediately upstream of the third methionine (M368) (see **Fig. 1a**), as also confirmed from ribosome profiling data from human left ventricle tissue, displaying increased p-sites as well as overall ribosomal coverage in correspondence of this

region. This conclusion is further supported by the finding that when each of the three in-frame i3loop methionines (namely M248, M296 and M368) after the stop codon 228 of the M₂stop228 were mutated to an additional stop codon, only the mutation of M368 to a stop codon proved capable of completely abolishing [³H]NMS binding (**Supplementary results** and **Supplementary Fig. 1 and Supplementary Table 1**). These binding results can furthermore rule out read-through and termination re-initiation: if these mechanisms were responsible for translation of the C-terminal of the M₂stop228, suppression of the first methionine would have abolished the binding. Finally, the origin of the carboxyl terminal fragment of M₂ at the third in-frame methionine (M368) is also supported by the key observation that the mutant M₂-M368A does not display the ~20 kDa band for the M₂ C-terminal fragment as compared to the wild type M₂ receptor in the Western blot of **Fig. 2a**.

Based on this evidence, we mutated the conserved residues highlighted in yellow in **Supplementary Fig. 6a**, in order to check for their relevance in recruiting the ribosome on the M₂ i3 loop sequence, as shown in **Fig. 3b**. As discussed in detail in the **Supplementary Results**, we found that the four nucleotides AAGA1090-1093 next to the methionine 368 play a pivotal role in recruiting the ribosome on the M₂ mRNA and in translating the M₂ C-terminal fragment.

Generalization to other members of the GPCR superfamily

In order to determine if the observed behavior is specific to the M₂ receptor, or could highlight a feature common to other members of the GPCR superfamily, we repeated several of our experiments in another GPCR, the related muscarinic M₃ receptor. The M₃ displays an in-frame methionine at the end of the i3loop, in position 481 (**Supplementary Fig. 6a**). We then inserted a stop codon in position 273, generating the construct M₃stop273 that still displayed radio-ligand binding. Several other mutants, mimicking the single and double stop mutations performed for the M₂ displayed a behavior consistent with M481 being the start point of a cap-independent translation of the C-terminal also for the M₃ receptor (**Supplementary Tables 2 and 3**), thus indicating that IRES-mediated cap-independent translation of the C-terminal domain could be a phenomenon not restricted solely to the M₂ receptor, but may be shared at least by some other members of the GPCR superfamily. The presence of an IRES sequence upstream of M481 further appears to be supported by the

observation that 558 nucleotides from the i3 loop of the M₃ receptor (from nucleotide 880 to 1437) were inserted in the same Sirius-EGFP reporter construct as used for the M₂ (Supplementary Fig. 6b). This appears to be indeed supported by the sequence-alignment of the five muscarinic receptors. The alignment of the last portion of the M₂ i3 loop (from nucleotide 1072 to 1104) with regions adjacent to the second (M₁), fourth (M₃ and M₄) and first (M₅) in-frame Methionines, clearly shows that some nucleotide residues are conserved (Supplementary Fig. 6a). Overall these data underline that cap-independent translation could be a more general feature of GPCRs mRNAs.

Localization of the M₂ C-terminal fragment generated by cap-dependent expression under the control of a viral promoter

After having established that the expression of the M₂ C-terminal domain is regulated by an IRES and that its translation starts at M368, we went on to study the role it might play in the cell. For this purpose, M₂tail(368-466) fused to the fluorescent protein EGFP in pcDNA3, that is under the control of a strong CMV promoter, was expressed in HEK293 cells in order to study the subcellular localization of this domain. Confocal microscopy demonstrated a striking mitochondrial localization of the M₂tail(368-466)-EGFP construct (Fig. 4a). We confirmed this observation by co-staining HEK293 cells with a conventional mitochondrial stain (Mitotracker Deep Red) and by co-expressing M₂tail(368-466)-EGFP with Tom20-mCherry, a fluorescently labeled construct targeted to the outer mitochondrial membrane (Supplementary Fig. 7a). The observed mitochondrial localization of the M₂tail(368-466)-EGFP appeared in stark contrast to the canonical cellular localization of the M₂ receptor, as shown in Fig. 4b where the M₂ muscarinic receptor was C-terminally labeled with the red fluorescent protein mRuby2. Interestingly, very little expression of M₂tail(368-466)-EGFP was observed on the plasma membrane, as we confirmed by fluorescence correlation spectroscopy¹⁴ (Supplementary Fig. 7b-d). By contrast, when the M₂tail(368-466)-EGFP was co-expressed with M₂stop228, a modest localization could eventually be detected also on the cell membrane thanks to a reduction in diffusion coefficient of M₂tail(368-466)-EGFP, suggesting a chaperon activity of the M₂ N-terminal domain (M₂trunk) (Supplementary Fig. 7e-g) and hence explaining the [³H]NMS binding observed in cells transfected with either M₂stop228 or cotransfected with M₂trunk(1-228) and M₂tail(368-466) (Table 1). Likewise, constructs such as M₂tail(368-466)-mRuby2 (a red monomeric fluorescent protein) and M₂tail(368-466)-tdTomato (a

tandem-dimer red fluorescent protein) also displayed clear mitochondrial localization (**Supplementary Fig. 7h-i**), ruling out bias due to the fluorophore used.

Localization of the M₂ C-terminal fragment generated by cap-independent expression under the control of the i3loop IRES.

We then sought to express the C-terminal fragment of the M₂ receptor under the control of the i3loop IRES rather than a viral promoter, to further exclude the possibility that the observed localization of the M₂ carboxyl terminal fragment were due to overexpression of the construct. HEK293 cells were transfected with the construct M₂-mRuby2-STOP-M₂-i3-tail-EGFP which contains the full wild type M₂ receptor, mRuby2-labeled at the C-terminal, fused to the C-terminal part of the M₂ receptor starting at nucleotide 685 of the i3 loop, following the stop codon of mRuby2, and ending with a C-terminal EGFP sequence. Through this experimental strategy, the expression of M₂tail(368-466)-EGFP is regulated by the IRES, and the mechanism of C-terminal fragment production is therefore close to physiological. The expression of EGFP confirmed that the *in vivo* production of the M₂tail(368-466) is regulated by the IRES within the i3 loop. In addition, the processed receptor fragment was eventually observed to be predominantly confined to the cytosol and the mitochondrial network (**Fig. 4c**).

Notably, when the M₂-EGFP fusion protein was expressed and the cells were co-stained with Mitotracker, a faint EGFP signal was also observed in the mitochondrial regions (**Supplementary Fig. 8a**), although the expression of the full-length receptor masks this effect and makes quantification difficult. On the other hand, **Fig. 4d** shows that, when the M₂fr.sh-EGFP mutant was expressed in HEK293 cells, the IRES generated C-terminal fragment of the M₂ receptor still localized to the mitochondria. As mentioned above, in this construct the masking effect of the EGFP fused to the full-length receptor is removed (see also **Supplementary Fig. 1**). Similar mitochondrial localization was also observed when we transfected HEK293 cells with M₂fr.sh-mRuby2 (**Supplementary Fig. 8b**), ruling out possible effects due to the specific fluorophore used as a tag. We obtained comparable findings also in COS-7 cells (**Supplementary Fig. 8c**).

Mitochondrial import of the C-terminal fragment of the M₂ receptor was further verified by immunoblotting of protein extracts from purified mitochondria of HEK293 cells transfected with myc-tagged M₂ receptor, M₂tail(368-466) and M₂M368A. **Fig. 4e** shows that in purified mitochondria (unlike in the cytosolic and

308 microsomal supernatants originating from the purification process, **Supplementary Fig. 8d**), only the protein
309 bands associated to the M₂ C-terminal fragment were visible.

310 Notably, no mitochondrial import was visible for the M₂M368A, confirming that the third in frame methionine
311 (M368) in the i3 loop of the M₂ receptor is necessary for the C-terminal fragment production and its localization
312 in the mitochondria.

313 A consistent result was observed when mitochondria were purified from the heart and spleen of the
314 Chrm2/tdtomato mice **Fig. 4f** (the cytosolic fraction is shown in **Supplementary Fig. 8e**). A band at ~75 kDa,
315 matching the molecular weight of the M₂ C-terminal domain, was clearly visible. We conclusively confirmed
316 that this is the fingerprint associated to the M₂ C-terminal fragment, by purifying mitochondria from HEK293
317 cells expressing the construct M₂tail(368-466)-tdtomato. **Fig. 4g** (cytosolic fraction is shown in
318 **Supplementary Fig. 8e**) shows a ~75 kDa protein band matching closely the bands observed in mitochondria
319 from mice tissues, in addition to lower molecular weight bands most likely arising from degradation of the
320 tdtomato tag.

321 Mitochondrial localization of the M₂tail originating from the wild type M₂ receptor was finally confirmed by
322 relative abundance estimates of EGFP and M₂ receptor from parallel reaction monitoring (PRM)-based mass
323 spectrometry measurements **Supplementary Fig. 9**. Samples were prepared by generating mitochondrial and
324 cytosolic fractions (**Methods**) from HEK293 cells, expressing M₂-EGFP and a set of control constructs,
325 including M₂tail(368-466)-EGFP, pmito-EGFP (an EGFP fused to the mitochondrial leading sequence of the
326 cytochrome c oxidase subunit 8A (COX8A)), lifeact-EGFP (a cytosolic protein) and Epidermal Growth Factor
327 receptor fused to EGFP (EGF-EGFP). A 15-aminoacid long peptide originating from EGFP was screened. The
328 bands intensities of the cytosolic fraction, were normalized to consider both the overall loading variability as
329 well as the relative transfection efficiencies among the samples. Notably, we observed a mitochondrial
330 enrichment of the EGFP derived peptide as well the receptor-specific peptide originating from the M₂ receptor,
331 in agreement with the positive mitochondrial localization control.

332

333 **Mechanism of import of the M₂ carboxyl terminal fragment into the mitochondria and localization**
334 **within the organelle**

335 To gain further insight into the dynamics of the M₂ C-terminal fragment import within the mitochondria, we
336 first monitored the kinetics of colocalization between mitochondria and EGFP in cells transfected with
337 M₂tail(368-466)-EGFP. In cells observed for several hours, starting at 5h after transfection (**Fig. 4h**), the
338 construct was found initially expressed in the perinuclear region, likely the endoplasmic reticulum, indicating
339 that at least the initial step of subcellular trafficking of the M₂tail(368-466)-EGFP is the same as for the full
340 length receptor¹⁵. At this time (5h post-transfection), almost no colocalization with the Mitotracker-stained
341 mitochondria was visible. However, at 6h post-transfection, the colocalization level increased significantly,
342 and the M₂ C-terminal fragment was either found in the cytosol or in the mitochondria. At 9h post-transfection
343 the colocalization at the mitochondria was well visible. We quantified the colocalization level over time using
344 the Manders' coefficients (See **Methods**). The resulting chart, indicates that almost half of the mitochondrial
345 import occurred within 6h post-transfection.

346 This value is qualitatively in agreement with what we observed using an in-vitro assay to determine the import
347 of the M₂ carboxyl terminal into isolated mitochondria (see **Methods**). To this end, we used mitochondria from
348 *S. cerevisiae*, which is a robust and well-studied system for protein import assays. **Supplementary Fig. 10a**
349 shows the autoradiography experiment of isolated, intact, mitochondria incubated with 35S-labelled full length
350 muscarinic M₂ receptor or M₂tail(368-466), made using a reticulocyte in vitro translation system. The data
351 show that the full-length receptor was not imported into mitochondria. On the other hand, the M₂tail(368-466)
352 displays a clear mitochondrial import already after 5 minutes and increasing over time, up to 30 minutes.
353 Interestingly, this import (**Supplementary Fig. 10b**) appears to be independent upon the transporters of the
354 outer membrane Tom5, Tom6, Tom7 and Tom70, whereas the import of an inner membrane protein, the
355 ADP/ATP carrier (AAC), used here as a control, depends on Tom70. Upon extraction with carbonate most of
356 M₂tail(368-466) was found in the pellet, and therefore presumably integrated in a membrane. Furthermore Blue
357 native analysis suggests that the mitochondrially imported M₂tail(368-466) is not a monomer but rather it is
358 part of a larger complex of approximately 180 kDa **Supplementary Fig. 10c**.

359 All together these data indicate that the import of the M₂tail(368-466) leads to its localization in one of the
360 mitochondrial membranes, to form a complex with other native mitochondrial proteins.

361

362 To further discriminate if the construct is on the outer or inner mitochondrial membrane, we set up a
 363 fluorescence assay based upon GFP fluorescence complementation¹⁶. HEK293 cells were first transfected with
 364 two constructs: M₂tail(368-466)-GFP11, in which a short 16 amino acids peptide of GFP was fused at the C-
 365 terminus of M₂tail(368-466), and mito-GFP1-10, in which the COX8A was N-terminally fused to the other
 366 GFP fragment containing the remaining 10 beta strands (See **Methods**). **Supplementary Figure 10d** shows
 367 the successful recombination of the two within the mitochondrial matrix. We then applied this approach to the
 368 full length M₂ receptor, by generating the M₂-GFP11 construct and by transfecting it in HEK293 cells. **Fig. 4i**
 369 shows a green signal from the recombined GFP visible in the mitochondria. All together these data suggest
 370 that the M₂ C-terminal fragment is localized in the inner mitochondrial membrane, with its own C-terminal
 371 domain facing the matrix.

372 This interpretation appears to be confirmed in cells expressing the M₂stop228-myc receptor and examined by
 373 electron microscopy (**Supplementary Fig. 10e**) after immune-gold labelling. As shown in **Supplementary**
 374 **Fig. 10f-g**, gold particles were observed only in mitochondria, and at this stage they appeared localized either
 375 to the mitochondrial matrix or to the lumen of the cristae (**Supplementary Fig. 10h-k**).

376

377 **Functional role of the C-terminal fragment in the mitochondria**

378 The next step of our investigation concerned the obvious question about the functional effects, if any, that the
 379 observed localization of the M₂ C-terminal fragment might have on the mitochondrial activity, and -more
 380 generally- on the energy metabolism of the cell. To investigate this aspect, we measured the extent of oxygen
 381 consumption in COS-7 cells expressing M₂-EGFP, M₂tail(368-466)-EGFP or the sole EGFP as a control.
 382 Initially, we normalized the expression of the three proteins to the amount of EGFP expressed in the cells, then
 383 the metabolic functions were assayed on a Seahorse instrument using cells transfected with normalized
 384 amounts of plasmids (**Fig. 5a**).

385 Our data did not show a significant difference in total oxygen consumption capacity between cells transfected
 386 with cytosolic EGFP and the untransfected control. However, a significant decrease of oxygen consumption
 387 was found in cells transfected with M₂-EGFP and even more so with M₂tail(368-466)-EGFP (**Fig. 5a**). Notably,
 388 the basal oxygen consumption rate (OCR), in starvation-stress conditions, was profoundly decreased in
 389 M₂tail(368-466) cells and to a smaller extent in M₂ cells compared to EGFP and empty control cells (see also

the time-course in **Supplementary Fig. 11a**). These results are in line with what was observed using a Clarke-type oxygen electrode (**Supplementary results and Supplementary Fig. 11b and 11c**).

A cell apoptosis assay, based on the detection of activated caspase-3¹⁷, did not show substantial differences among the tested mutants (**Supplementary Fig. 11d**). The overexposed western blot showed a tiny amount of activated caspase-3 in most of the transfected COS-7 cells, indicating that the expression of M₂tail(368-466) in mitochondria, in agreement with the qualitative post-starvation data shown in **Supplementary Fig. 11e**, does not induce fragmentation of the mitochondrial network and cell apoptosis.

To test whether the C-terminal-M₂ fragment effects on mitochondrial oxygen consumption could alter the production of Reactive Oxygen Species (ROS), the measurements were combined in a single cell assay. Remarkably, ROS production (assayed using a fluorescence readout kit, where the fluorochrome becomes activated in contact with oxidized species) was clearly reduced within and in proximity of mitochondria of cells expressing a high level of the M₂tail(368-466) (**Fig. 5b**). By regrouping individual mitochondria in classes according to the level of the ROS marker expression, an actual inverse correlation between the amount of receptor fragment (Green Fluorescence) and the production of ROS (Red Fluorescence) was revealed.

Cell growth was also found to be affected depending on which of the constructs was transfected. Notably, transfection of HEK293 cells with the wild type M₂ receptor reduced significantly cell growth compared to the control, whereas cells transfected with the mutant M₂M368A, that do not express the M₂ C-terminal fragment (**Fig. 5c**), displayed a significantly higher growth. All cells were incubated with saturating concentration of the M₂ antagonist atropine throughout, in order to exclude effects on cell growth and proliferation due to the canonical receptor signaling.

Cell stress upregulates M₂ C-terminal expression and mitochondrial localization

Having established that the C-terminal fragment localizes in the mitochondria and affects the cell oxygen consumption rate, we looked at the effect of stress on the level of expression of the C-terminal fragment. Stressed cells attenuate their translational activity via phosphorylation of the initiation factor eIF2 α . However, certain mRNAs may be insensitive to eIF2 α phosphorylation level if their translation is regulated by IRES. Under these conditions their expression is bound to increase rather than to attenuate^{18,19}.

Therefore, we transfected COS-7 cells with the Sirius-M₂i3(417n)-EGFP reporter construct and stressed by starvation for three hours by replacing the culture medium with HBSS or PBS. During this time interval, the green fluorescence was seen to gradually increase up to 1.4 times the basal level observed in HBSS-starved cells and 3.5 times for PBS-starved cells (**Fig. 5d**), confirming that the C-terminal fragment expression is upregulated in response to stress conditions such as cell starvation. The same experiment was reproduced in single cells, displaying an increase of the average EGFP/Sirius ratio upon HBSS starvation in HEK293 cells (**Fig. 5e**).

We further used the GFP fluorescence complementation assay to emphasize the importance of stress in the pattern of expression of the M₂ gene. HEK293 cells were co-transfected with M₂-GFP11 and Mito-GFP1-10. HEK293 cells grown in culture medium predominantly expressed the full M₂ receptor protein (**Fig. 5f**). The green fluorescence detected in the plasma membrane is explained with the chaperoning effect of M₂-GFP11 on Mito-GFP1-10 before its sorting to the mitochondria. In sharp contrast, when HEK293 cells were first transfected with Mito-GFP1-10 and, 24h later, transfected with M₂-GFP11 followed by a 6 h starvation in HBSS, the pattern of expression changed drastically: IRES mediated translation of the M₂tail(368-466) fragment led to a predominantly mitochondrial green fluorescence signal of the complemented GFP (**Fig. 5f**). These results are in line with our observation that the IRES mediated C-terminal fragment localization to the mitochondria in cells expressing the construct *M₂-mRuby2-STOP-M₂-i3-tail-EGFP* was enhanced upon cell starvation, and drastically reduced upon mutation of the M368 (**Supplementary Figure 12** and **Supplementary Methods**).

This observation was also supported by the lack of complementation of M₂tail-GFP11 with GFP1-10 when this last fragment was N-terminally fused to the targeting sequence of SMAC, a protein expressed in the mitochondrial intermembrane space²⁰ (**Supplementary Fig. 13c**).

Mutation of the M368A via CRISPR/Cas9 affects cell growth and oxygen consumption rates in hiPSCs

We finally asked the question of what would be the effect of abolishing the translocation of the M₂ C-terminal fragment to the mitochondria, in a physiological and entirely label-free setting. To reach this goal we genetically engineered (see **Methods**) human induced pluripotent stem cell (hiPSC), to incorporate in both alleles of the M₂ gene the M368A mutation, that, based on our data, should be sufficient to significantly impair

the M₂ C-terminal production and mitochondrial translocation. Wild typ (wt)e and M368A hiPSCs were cultured in pluripotency maintaining medium, and subsequently undirectedly differentiated upon exposure for seven days to DMEM-F12 medium, as described in the **Methods**. **Fig. 6a** shows confocal micrographs for wild type and M368A hiPSCs cultured in E8 medium respectively, with the M368A hiPSCs displaying a mildly more spread-out phenotype. We shall note here that the M₂ receptor has been found to be expressed in PSCs lines, and it has been shown to play a role in stem cell differentiation^{21,22}. In this context, however, we were not interested in the canonical signaling of the receptor, and all experiments were performed with a saturating concentration of atropine, to block any activity of the receptor. We then evaluated cell proliferation, observing a significant increase in the proliferation of the M368A clone 72h after seeding **Fig. 6b**. The observation of an increased proliferation of the cells harboring the mutation that impairs the production and localization of the C-terminal fragment in the mitochondria, suggests that the same effect is at play here, as observed in the overexpression system (**Fig. 5a,b,c**). These results are supported by extracellular oxygen consumption assays performed, for both hiPSCs and differentiated hiPSCs (**Fig. 6c**). Our data suggest that the M368A cells indeed display an accelerated oxygen consumption rate, and that this effect increases as the cell progressively differentiates.

For comparison, the assay was also carried on HEK293 overexpressing the wild type M₂ receptor and the mutant M₂M368A (**Supplementary Fig. 11f**). We could observe with this assay a reduced oxygen consumption of the wild type M₂ receptor compared to untransfected cells, in line with what was observed in **Fig. 5a**, and an increased oxygen consumption of the M₂M368A expressing cells, in line with what observed for the hiPSCs.

Discussion

Over the last 10 years, growing evidence has accumulated indicating that GPCRs can be functional and signal also at cellular locations other than the plasma membrane. These locations can be associated to their canonical trafficking pathways (such as sorting vesicles and endosomes)²³, but also to non-canonical locations, such as the nucleus, the nuclear membrane, the Golgi apparatus and the mitochondria (reviewed in Jong et al. BPJ 2018²⁴). Some of these locations are surprising, since many ligands are non-membrane permeable, and it is still debated how they can reach their target receptors at such intracellular locations.

473

474 Interestingly, the specific localization of these receptors appears to go hand in hand with a specific function.
475 In these cases, activation/inhibition of mitochondrially localized GPCRs have been shown to specifically affect
476 the organelle function, ranging from oxygen consumption rates to Ca^{2+} handling and even to apoptosis. Notably
477 Benárd et al.²⁵ showed that the CB_1R on the outer mitochondrial membranes affects neuronal energy
478 metabolism; Suofu et al.¹⁰ demonstrated that activation of the MT_1R by a mitochondrial melatonin pool can
479 affect cytochrome C release. This is particularly interesting, since cytosolic cAMP does not appear to be able
480 to activate mitochondrial PKA, and hence a mitochondrially localized GPCR signalosome appears to be
481 required to enable such downstream signaling cascade²⁶.

482

483 However, other non-canonical mechanisms are also possible for the regulation of mitochondrial function. It is
484 well known that GPCRs are subject to interindividual variability, arising, for example, from mRNA alternative
485 splicing (i.e. the process which allows the recombination of exons) which leads to different isoforms which
486 may present distinct signaling and localization properties²⁷. Receptor variants with different functional and
487 localization properties may also occur due to other processes. For example, our comprehension of eukaryotic
488 translational regulation is facing a rapid expansion, as exemplified by the observation that small open reading
489 frames (smORFs), also within lncRNAs, are an important class of genes, oftentimes with mitochondrial
490 localization²⁸.

491

492 In this study, for the first time, we provide evidence that an IRES sequence drives expression of a membrane
493 receptor fragment and acts as a regulator of its expression levels and function. IRES sequences are distinct
494 regions of the mRNAs that are capable of recruiting ribosomes in the proximity of an internal initiation codon
495 and translate transcripts in a cap-independent manner^{5,6,29-31}. Here, we show that an IRES sequence is located
496 within the third intracellular loop of the M_2 muscarinic receptor, where it plays a crucial role in regulating the
497 expression of its carboxy-terminal domain, which we identified as M_2tail (368-466).

498

499 We assessed the IRES-driven expression of the C-terminal fragment through a broad set of constructs, meant
500 to exclude alternative synthesis mechanisms such as stop-codon read-through, and a diversified palette of

501 techniques. Radioligand binding data (**Table 1**) pointed to the necessity of the C-terminal fragment synthesis,
 502 in order to explain the observed downstream signaling in mutants carrying a stop codon in the initial portion
 503 of the i3loop of the M₂ receptor, upstream of M368. Fluorescence microscopy allowed us visualizing the
 504 production of the EGFP-tagged C-terminal fragment in these receptor mutants (**Fig. 1d**). We further detected
 505 its production by Western blotting on protein extracts from cells; here the C-terminal fragment gives rise to
 506 two bands: the lower of the two (~15 kDa) appears to be proportional to the ~75 kDa band, suggesting that this
 507 may result from subsequent processing or degradation. On the other hand the ~20 kDa band intensity appears
 508 independent of the M₂ full-length band (~75 kDa), as would be expected for a cap-independent translation
 509 mechanism, and this band disappears in the M368A mutant.

510 We further performed Western blotting from heart and brain tissues from knock-in mice carrying a C-
 511 terminally tagged M₂ receptor (tdtomato) and under the control of the endogenous promoter/enhancer
 512 sequences (**Fig. 2a-c**).

513
 514 Mutagenesis of key residues within the i3 loop of the M₂ receptor allowed us to pinpoint the 3rd in-frame
 515 methionine as the most likely candidate for the beginning of the cap-independent translation (**Fig. 3**). This
 516 observation appears to be supported by the observation of a peak in ribosome coverage around the M368 seen
 517 in ribosome profiling databases (as reviewed in Ingolia et al.³²), and particularly prominent in those from left
 518 ventricle cardiomyocytes, where M₂ receptor expression is known to be abundant²⁸.

519
 520 To our surprise, in contrast to the plasma membrane localization of the wild type, full length M₂ receptor, the
 521 C-terminal fragment was localized mostly in mitochondria. This localization occurred markedly when
 522 overexpressing the C-terminal fragment M₂tail(368-466), but could be also clearly detected when the C-
 523 terminal fragment was expressed under the control of the IRES sequence of the full length receptor (**Fig. 4**
 524 **c,d**).

525
 526 We devised several strategies to detect the localization of the IRES-driven fragment in the mitochondria by
 527 avoiding the cell-wide background signal from the full-length receptor. **Fig. 4c,d** show two prominent
 528 examples, namely relying on spectrally unmixing the two components, or by sending out of frame the EGFP

at the C-terminus of the full-length receptor. By studying mitochondria purified from HEK293 cells we could confirm that the mutation of the M368, which we identified as the starting point of the IRES-mediated translation, is sufficient to suppress any mitochondrial targeting of the M₂ C-terminal fragment, and most likely, its synthesis altogether (Fig. 4e).

We then addressed the mechanism of mitochondrial import of the C-terminal fragment and its localization within the organelle. Our data, originating both from live cell import kinetics (Fig. 4h) as well as from in-vitro import into isolated mitochondria (Supplementary Fig. 10), support the notion that the fragment becomes rapidly imported into mitochondria following translation in the cytosol. Once inside the mitochondria, it undergoes processing leading to the appearance of a lower molecular weight band, observed both in Western blots from whole cell and mice tissue protein extracts, as well as from purified mitochondria. The fragment is found predominantly in a carbonate-insoluble fraction, i.e. a membrane, and it appears to form a high molecular weight complex of ~180 kDa with other mitochondrial proteins (Supplementary Fig. 10c).

Fluorescence complementation assays (Fig. 4i) and electron microscopy (Supplementary Fig. 10d) suggest that the M₂ C-terminal fragment predominantly resides in the inner mitochondrial membrane, with its own C-terminal domain exposed towards the matrix. Future work should clarify the precise import pathway.

Most importantly, our data show that mitochondrial oxygen consumption was reduced by the M₂tail(368-466) (Fig. 5a,b), and also cell proliferation was affected (Fig. 5c). Under integrated stress response, namely aminoacid starvation, IRES-mediated production of the C-terminal fragment was increased (Fig. 5d-f). On these bases, the M₂tail might be needed in mitochondria as a means to reduce mitochondrial basal oxygen consumption and ATP-linked respiration. An important consequence of reduced respiration is the lower level of ROS produced in mitochondria, as we observed in agreement with this hypothesis.

This observation was confirmed in physiological, label-free conditions, by genetically editing human induced pluripotent stem cells in order to suppress the synthesis of the M₂ C-terminal fragment mutating the starting methionine M368. Increase of cell proliferation of this clone compared to its wild type counterpart, together with an increase of oxygen consumption rates (Fig. 6), corroborates our hypothesis that the M₂ C-terminal fragment reduces oxygen consumption upon reaching the mitochondria. The effect was further amplified when

557 the cells were undirectededly differentiated, and it was consistent with the shift of the cell phenotype towards
558 oxidative phosphorylation³³.

559

560 The mechanism by which the M₂tail(368-466) may reduce oxygen consumption in mitochondria and its
561 meaning needs to be further explored. Nonetheless, since ROS production is implicated in the pathogenesis of
562 several diseases³⁴, we would propose the hypothesis that increased localization of the M₂ C-terminal fragment
563 in mitochondria, as it occurs under integrated stress response, might have protective effects (**Fig. 7**).

564

565 In broader terms, protection of mitochondria by unconventional import mechanisms to allow localization
566 within the organelle of specific proteins has been previously observed for cells that undergo stress conditions
567 ³⁵. The mechanism of IRES-dependent modulation of translation and consequent mitochondrial targeting of
568 the translation product of a GPCR may provide another means, completely unrelated to the canonical signaling
569 function of this class of membrane receptors, of a generic cellular response to overcome different types of
570 stress by modulating the mitochondrial proteome and adjusting the mitochondrial bioenergetic state.

571 Tables, Figures and Supplementary Tables and Figures

572 **Table 1. Radioligand binding and activation properties of M₂ receptor mutants.** Binding properties and inhibition of
573 forskolin stimulated adenylyl cyclase by M₂, M₂stop228 and the co-transfected M₂trunk(1-283)/M₂tail(M-281-466) and
574 M₂trunk(1-228)/M₂tail(368-466). Untransfected COS-7 cells did not show any [³H]NMS specific binding. N.B. = no
575 specific [³H]NMS binding.

Binding data				Adenylyl cyclase inhibition assay	
Receptor	B _{max} (fmol/mg)	[³ H]NMS KD (pM)	Carbachol IC ₅₀ (μM)	Carbachol EC ₅₀ (μM)	Maximum inhibition of forskolin stimulated cAMP accumulation (%)
M ₂	980 ± 60	62 ± 7	2.1 ± 0.6	0.04 ± 0.01	53 ± 5
M ₂ trunk(1-283)	N.B.				
M ₂ trunk(1-228)	N.B.				
M ₂ stop228	45 ± 2	70 ± 10	1.8 ± 0.4	0.13 ± 0.02	27 ± 3
M ₂ trunk(1-283) +M ₂ tail(M-281- 466)	480 ± 20	79 ± 10	0.7 ± 0.2	0.15 ± 0.03	37 ± 4
M ₂ trunk(1-228) +M ₂ tail(368- 466)	70 ± 10	72 ± 8	0.6 ± 0.2	0.17 ± 0.03	29 ± 4

Fig. 1. Mutations of the M₂ receptor confer unexpected functional and localization properties. **a**, Snake-like plot of the human muscarinic M₂ receptor: in red are indicated the aminoacids substituted with stop codons in the mutants: M₂stop196, M₂stop228 and M₂stop400 (Supplementary Fig. 1). The three in-frame methionines of the i3 loop, 248 and 368, are highlighted in yellow; the translation of the C-terminal fragment starts from methionine 368. **b**, Schematic representation of the gene products that reach the plasma membrane of the wild type M₂, M₂trunk, M₂trunk and M₂tail and M₂stop228 (Supplementary Fig. 1). The M₂trunk and M₂tail genes generate the respective protein fragments that, only when co-transfected together, are able to form functional receptor complexes capable to bind ligands. The M₂stop228 gene mutant generates both M₂trunk (in green), when translation starts from the cap site, and M₂tail (in brown) when translation is cap-independent driven. This makes it likely that the truncated fragment of the M₂stop228 receptor may act as a chaperon driving part of the C-terminal-M₂ fragment to the plasma membrane, as supported by the binding and functional data shown by the co-transfection of M₂trunk(1-228) with M₂tail(368-466) (Table 1). **c**, Fluorescence micrographs of HEK293 cells respectively expressing M₂-EGFP, M₂stop228-EGFP, M₂fr.sh.-EGFP and M₂stop400-EGFP, together with an untransfected control. Imaging settings are the same for the five images, whereas contrast was adjusted to improve visualization. In the untransfected cells and stop400 panels, the contrast was set to 0. Insets display DIC image of the same field of view (scale bars 10 μm).

-24-

594 **Supp. Fig. 1. Schematic representation of wild type muscarinic M₂ (human) receptors and derived mutants.** Each
595 construct was obtained as described under **Methods**. The left column indicates the mRNA product from each construct,
596 the right column the expected protein product. For the last two constructs, above mRNA product, and below the expected
597 protein product.

598

599 **Supp. Fig. 2. M₂ receptor mutants with single and double stop codons.** **a**, Schematic representation of the gene
600 products M₂stop196 and M₂stop400, that are not functional by themselves but that are rescued by co-transfection with
601 M₂Trunk(1-283) and M₂Tail(M-281-466), respectively. **b**, Dose response curve of carbachol induced ERK
602 phosphorylation in HeLa cells transiently transfected with M₂ and M₂stop228 muscarinic receptors. Cells were stimulated
603 for 5 minutes with the indicated concentration of carbachol. Phosphorylated ERK (P-ERK) was normalized vs total ERK
604 (Tot ERK). Significance (*p < 0.05, **p < 0.01) was calculated against carbachol 0 μM.

605 **Supplementary Table 1. Ligand binding and functional properties of M₂ receptor mutants with single and double**
606 **stop codons.** Number of [³H]NMS binding sites for the indicated mutants. Untransfected COS-7 cells did not show any
607 [³H]NMS specific binding. N.B. = no specific [³H]NMS binding.

Receptor	Bmax (fmol/mg)	Receptor	Bmax (fmol/mg)
M ₂ stop228	45.3 ± 2.4	M ₂ stop228/stop368	N.B.
M ₂ stop196	N.B.	M ₂ stop228/stop368 + M ₂ tail(281-466)	370 ± 20
M ₂ stop400	N.B.	M ₂ stop228/stop400	N.B.
M ₂ stop196+ M ₂ trunk(1-283)	47 ± 3	M ₂ stop228/stop400+ M ₂ tail(281-466)	380 ± 20

M ₂ stop400+ M ₂ tail(281-466)	400 ± 30	M ₂ stop196/stop400+ M ₂ trunk(1-283)	N.B.
M ₂ stop196/stop400	N.B.	M ₂ stop196/stop400+ M ₂ tail(281-466)	N.B.
M ₂ stop228/stop248	49 ± 5	M ₂ stop228/fr.sh.	47 ± 3
M ₂ stop228/stop296	38 ± 3	M ₂ stop228/Hairpin	37 ± 2

Supp. Fig. 3. Lack of alternative splicing in M₂ wild type and M₂stop228 receptors. mRNAs were extracted by COS-7 cells transfected with M₂ wild type and M₂stop228, subjected to reverse transcriptase and the resulting cDNA amplified by PCR with two oligos directed to the 5' and 3' end of the receptors. The gel shows, in both M₂ wild type and M₂stop228, a single band of 1401 bp corresponding to the full-size receptor, running at the same level of the band amplified directly from the M₂ pcD plasmid.

Fig. 2. Western blot of tagged M₂ receptor mutants in HEK293 cells and in tdtomato knock-in mice. a, left Graphical guide of the fragments observed on the gel when using myc-tag. **right**, M₂-myc, M₂tail(368-466)-myc, M₂M368A-myc and M₂stop400-myc were transiently transfected in HEK293 cells and immunodetected via antimyc primary antibody, together with an untransfected control. Loading control normalization was performed vs β-actin **b, left** Graphical guide of the fragments observed on the gels when using the tdtomato tag. **right**, Western blot from tissue preparations originating from the indicated organs of M₂-tdtomato knock-in mice (HT) and wild-type animals (WT). Immunodetection was performed using an anti-tdtomato primary antibody. Loading control normalization was performed using GAPDH in the heart, and β-actin in the brain and spleen.

Supp. Fig. 4. Expression of the bicistronic plasmid Sirius-M₂i3(417n)-EGFP in four different cell lines

Expression of Sirius was driven by the canonical scanning mechanism of translation initiation, while EGFP expression was driven by an IRES dependent mechanism. All Sirius-M₂i3(417n)-EGFP transfected cells expressed both the Sirius

and EGFP proteins. On average, $25 \pm 4\%$ of the COS-7 cells transfected with Sirius-M₂i3(417n)-EGFP plasmid were blue fluorescence positive, while $17 \pm 2\%$ were green fluorescent positive, with only a few cells being only green.

Fig. 3. Fluorescence assay of cap-independent translation of EGFP driven by the i3 loop mutants of the muscarinic M₂ receptor. a, left Schematic representation of bicistronic plasmids bearing the M₂ i3 loop between the coding regions of the Sirius and EGFP fluorescent proteins. A guanine nucleotide was originally inserted before the EGFP in the plasmid Sirius-H-EGFP in order to maintain in frame the sequences of the two fluorescent proteins. In all the other plasmids the sequence of the two fluorescent proteins is not in frame. Fifteen nucleotides derived from the M₂ i3 loop were inserted between the end of Sirius and the hairpin loop, in order to allow the easy scanning of the ribosome across the stop codon. **right**, Relative fluorescence of EGFP compared to Sirius, highlighting the efficiency of cap-independent translation for the control Sirius-M₂i3(417n)-EGFP and the six mutants. The insert shows the difference in the green fluorescence between two of the mutants. **b, left** Mutations of the bicistronic Sirius-H-M₂i3(15n/30n)-EGFP plasmids in the region of the M₂ i3 loop from nucleotides 1972 to 2001, in yellow are the modified nucleotides. In Sirius-H-M₂i3(15n/30n)mut#6-EGFP the four nucleotides in green (AAGA) were reinserted in the stretch of 20 T of Sirius-H-M₂i3(15n/30n)mut#5-EGFP. **right** Relative fluorescence of EGFP compared to Sirius, highlighting the efficiency of cap-independent translation for the control Sirius-H-M₂i3(15n/30n)-EGFP and the six mutants illustrated. Significance ($p < 0.01$) was calculated in **a** against Sirius-M₂i3(417n)-EGFP and in **b** against Sirius-H-M₂i3(15n/30n)-EGFP. Error bars are s.e.m.

Supp. Fig. 5. Expression of the construct M₂-Hairpin-EGFP in HEK293 cells. (left) Confocal images of M₂-Hairpin-EGFP, (middle) Mitotracker Deep red staining, (right) DIC. Scale bar 5 μm.

Supp. Fig. 6. Alignment of the nucleotide sequence 1072-1104 of the M₂ i3 loop with analogous sequences of the other four muscarinic receptors, and expression of the bicistronic plasmid Sirius-M₃i3(558n)-EGFP. **a**, The criteria for the alignment are described in the supplementary data. In yellow are highlighted the conserved nucleotides. In bold are the in frame ATG codons. Next to each sequence is indicated the codon number of the in-frame ATG and its distance in nucleotides from the beginning of TMDVI. **b**, Schematic representation of the bicistronic plasmid bearing the i3 loop of the rat muscarinic M₃ receptor (558 nucleotides from nucleotide 880 to nucleotide 1437) between the coding regions of the Sirius and EGFP fluorescent proteins. **c**, EGFP expression in COS-7 cells transfected with the bicistronic plasmid Sirius-M₃i3(558n)-EGFP.

Supplementary Table 2. Radioligand binding and activation properties of M₃ receptor mutants. Binding characteristics and stimulation of phosphatidylinositol accumulation by M₃, M₃stop273 and the co-transfected M₃trunk(1-272) and M₃tail(M-388-586). Untransfected COS-7 cells did not show any [³H]NMS specific binding. N.B. = no specific [³H]NMS binding.

Binding data				Phosphatidylinositol accumulation assay	
Receptor	Bmax (fmol/mg)	[³ H]NMS KD (pM)	Carbachol IC50 (μM)	Carbachol EC50 (μM)	Maximum increase in IP ₁ level above baseline (%)
M ₃	1120 ± 71	29 ± 2	59 ± 4	4.2 ± 0.5	183 ± 15
M ₃ trunk(1-272)+ M ₃ tail(388-589)	114 ± 17	23 ± 2	48 ± 5	1.4 ± 0.1	126 ± 12
M ₃ stop 273	52.3 ± 0.5	31.3 ± 2.8	56 ± 2	1.1 ± 0.1	89 ± 13

Supp. Table 3. Ligand binding of M₃ receptor mutants with single and double stop codons. Number of [³H]NMS binding sites in COS-7 cells transiently transfected with the indicated M₃ receptor mutants with single and double stop codons.

Receptor	Bmax (fmol/mg)	Receptor	Bmax (fmol/mg)
M ₃ stop273	54 ± 1	M ₃ stop273/stop503	N.B.
M ₃ stop240	N.B.	M ₃ stop273/stop503 + M ₃ tail(388-589)	120 ± 15
M ₃ stop503	N.B.	M ₃ stop240/stop503+ M ₃ trunk(1-272)	N.B.
M ₃ stop240+ M ₃ trunk(1-272)	44 ± 3	M ₃ stop240/stop503+ M ₃ tail(388-589)	N.B.
M ₃ stop503+ M ₃ tail(388-589)	135 ± 12	M ₃ stop273/fr.sh.	120 ± 4
M ₃ stop240/stop503	N.B.	M ₃ stop273/Hairpin	30 ± 2

Fig. 4. Localization of fluorescently labeled M₂ mutant constructs: microscopy and western blots analysis. **a**, Colocalization of M₂tail(368-466)-EGFP (green) together with the mitochondrial targeted dye Mitotracker (magenta) in HEK293 cells. Separate spectral channels are displayed below. **b**, Co-expression of M₂tail(368-466)-EGFP (green) and wild type M₂-mRuby2 (red) in HEK293 cells. Separate spectral channels are displayed below. **c**, Cellular localization of the construct M₂-mRuby2-STOP-M₂-i3-tail-EGFP in HEK293 cells. Expression of the M₂tail(368-466)-EGFP (green) after the STOP codon is an indicator of the endogenous IRES activity. While M₂-mRuby2 localizes correctly and predominantly to the cell plasma membrane, M₂tail(368-466)-EGFP localizes in the cytosol as well as, strongly, to the cell mitochondria. Separate spectral channels are displayed below. **d**, M₂-fr.sh.-EGFP (green) expressed in conjunction with Mitotracker (magenta). The EGFP is expressed despite the creation of an in-frame stop after M368, confirming endogenous cap-independent translation of the M₂tail(368-466). Arrows indicate areas of colocalization with the mitochondria. Separate spectral channels are displayed below. **e**, Western blot (10% TRIS-Glycine PAA gel) of mitochondria purified from Hek293 cells transfected with the M₂-Myc, M₂tail(368-466)-Myc, M₂M368A-Myc, together with an untransfected control are blotted using an anti-myc antibody. Loading control was verified by immunoblotting COXIV. **f**, Western blot (8% TRIS-Glycine PAA gel) of mitochondria purified from tissue preparations originating from the indicated organs of M₂-tdtomato knock-in mice (HT) and wild-type animals (WT), detected using an anti-tdtomato antibody. Please note that the band at 80kDa is aspecific, and is more prominent in the WT lane due to higher loading. **g**, Western blot (8% TRIS-Glycine PAA gel) of mitochondria purified from HEK293 cells transfected with the M₂tail(368-466)-tdtomato, together with an untransfected control. Mitochondrial proteins were blotted using an anti-tdtomato antibody. Loading control was verified by detecting the 15 kDa mitochondrial matrix protein Cox-IV. **h** Time course of M₂tail(368-466)-EGFP import in the mitochondria of a HEK293 cell over a 4h interval, starting 5h after cell transfection, as indicated. Panels, from top to bottom show M₂tail(368-466)-EGFP (green), Mitotracker (magenta) and overlay displaying increasing colocalization over time. Chart at the bottom quantifies colocalization of the M₂tail(368-466)-EGFP

in the mitochondria by calculating the Manders Coefficients (n=7 cells, from 3 independent transfections, error bars are s.e.m.) (See **Methods**). **i**, Detail of a HEK293 cell expressing the M₂-GFP11 construct together with pmito-GFP1-10. The M₂-GFP11 was sequentially transfected 24 h after the pmito-GFP1-10 (see **Methods**) Magenta overlay indicates mitotracker. White signal in the overlay indicates M₂tail(368-466) trafficking to the organelle. All confocal micrographs were obtained by sequential imaging using 488 nm excitation and detection in the 520-600 nm (EGFP), 561 nm excitation and detection in the 580-650 nm (mRuby2) and at 633 nm and detected in the 650-700 nm range (Mitotracker) using HyD detectors in Photon Counting Mode. Scale bars are 10 μ m throughout.

Supp. Fig. 7. Subcellular localization of the M₂tail(368-466). **a**, Confocal image displaying the colocalization (top) of M₂tail(368-466)-EGFP (green, middle)) in HEK293 cells, co-expressed with the outer mitochondrial membrane marker Tom20-mCherry (red, bottom). **b** expression of M₂tail-EGFP displaying marked mitochondria and cytosolic localization. **c**, Glasbey colorscale representation of panel (**b**) highlighting the low-intensity pixels. **d**, autocorrelation function of the M₂tail(368-466)-EGFP diffusion taken at or in proximity of the basal membrane, yielding a mean diffusion coefficient of 0.11 μ m²/s, incompatible with membrane diffusion. **e**, co-expression of M₂stop228 and M₂tail(368-466)-EGFP. M₂stop228 is unlabeled. M₂tail(368-466)-EGFP maintains the localization to the mitochondria observed in cells expressing M₂tail(368-466)-EGFP alone, but it is also localized to the plasma membrane, visible in panel **f**, Glasbey colorscale representation of panel **e** highlighting the low-intensity pixels. **g**, autocorrelation function of the M₂tail(368-466)-EGFP diffusion taken at or in proximity of the basal membrane, yielding a mean diffusion coefficient of 0.013 μ m²/s, compatible with membrane diffusion. **h**, Cellular localization of M₂tail-mRuby2, together with Mitotracker deep red staining of the mitochondrial network and corresponding DIC image. **i**, Cellular localization of M₂tail-tdtomato and corresponding DIC image. All panels originate from Laser Scanning Confocal Microscope sequential acquisitions, with laser lines 488 nm (EGFP) 561 nm (mRuby2 and tdtomato) and 633 nm (Mitotracker deep red), and corresponding emission filters in the ranges 520-600 nm, 570-620 nm and 640-750 nm. Scale bars 10 μ m.

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Supp. Fig. 8. Mitochondrial localization of the M2tail fragment. **a**, Confocal image of M2-EGFP (green) expressed in conjunction with Mitotracker (magenta) in HEK293 cells. Separate panels below. **b**, Confocal image of HEK293 cell expressing *M2fr.sh-mRuby2*, together with DIC, below. **c**, Confocal sequential images displaying the localization of fluorescently labeled M2tail(368-466)-EGFP (green), together with the mitochondrial network (magenta), colocalization (white) and DIC image in COS-7 cells. Scale bars are 10 μ m. Confocal sequential acquisitions were performed with 488 nm excitation and 520-600 nm detection (EGFP), and with 561nm excitation and 600-750 nm detection (mCherry) and 633 nm excitation and 650-750 nm detection (Mitotracker) using HyD detectors in Photon Counting Mode. **d**, Western blot (10% TRIS-Glycine PAA gel) of the cytosolic and microsomal fractions resulting from the mitochondria purification of lysates of HEK293 transfected with myc-tagged constructs, as displayed in Fig. 4e. **e**, Western blot (8% TRIS-Glycine PAA gel) of the cytosolic fraction resulting from the mitochondria purification of lysates of HT and WT tissues of a tdtomato transgene mice, as displayed in Fig. 4f. **f**, Western blot (10% TRIS-Glycine PAA gel) of the cytosolic fraction resulting from the mitochondria purification of lysates of HEK293 transfected with M2tail(368-466)-tdtomato, as displayed in Fig. 4g.

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Supp. Fig. 9 Relative abundance estimates of EGFP and M₂ receptor from PRM based mass spectrometric measurements. Normalized fragment intensities were calculated for the peptide FSVSGEGEGDATY GK (specific for EGFP) for cytosolic and pellet fractions and for different constructs. Each preparation was normalized relative to the cytosolic fraction, if data was available. Median levels of cytosolic, plasma membrane and mitochondrial control markers are shown in the left panel. For each identified peptide the normalized spectrum contrast angle, indicating the similarity of the recorded spectrum to the reference spectrum, is indicated below each plot.

gold particles confined to the mitochondria. Bar: 500 nm. **h-k**, Gold particles appear associated with the mitochondrial cristae, highlighted by the dotted line. A total number of 236 gold particles in 40 mitochondria from 19 sections were detected.

Fig. 5. Effects of M₂ mutants on mitochondrial function and cell metabolism in transfected HEK293 cells. **a**, Basal oxygen consumption upon HEK293 cell stress -following starvation- measured by a Seahorse assay at a constant temperature of 37 °C. **b**, Localization of M₂tail(368-466)-EGFP in HEK293 (green) cells stained with a marker for reactive oxygen species (Cell Rox Deep Red, ThermoFisher). Relationship between green (M₂tail abundance) vs red (amount of ROS produced) fluorescence intensities measured from regions of the mitochondrial network. The data were binned in four groups as a function of the amplitude of Cell Rox Deep Red signal (0-2.5, 2.5-5, 5-7.5 and 7.5-10 intensity counts). **c**, Cell viability assay performed on HEK293 cells transfected with M₂, M₂tail(368-466) and M₂(M368A), compared to an untransfected control. Cells were counted 24h and 72h after transfection in T-25 flasks, exposed throughout to 100 nM atropine. The experiments were performed in two biological replicates, each with two technical replicates. Transfection efficiency (to the right of the chart) was monitored thanks to the mRuby2 fluorescent reporter within a bicistronic cassette within each vector (see **Methods**). **d**, 96-well plate reader measurement of the increase of EGFP fluorescence relative to Sirius in COS-7 cells transfected with the bicistronic Sirius-M₂i3(417n)-EGFP plasmid and starved for three hours respectively in PBS (left panel) (n= 3 transfections, 16 wells) and HBSS (right panel) (n=1 transfection, 16 wells). **e**, Representative single cell confocal and DIC micrographs of HEK293 cells expressing the Sirius-M₂i3(417n)-EGFP construct in basal conditions, together with scatter plot of the average EGFP and Sirius fluorescence intensity values measured from single cells in basal (red dots) and 3h HBSS starvation (blue dots) conditions. **f**, HEK293 cells were sequentially transfected with pMito-GFP1-10 (24h) and M₂-GFP11 (8h, of which 6h under starvation conditions). (left panels) HEK293 cells grown in full culture medium and (right panels) HEK293 cells starved for 6 h after transfection of M₂-GFP11. Shown are EGFP fluorescence (top, with DIC inset) and Mitotracker (bottom).

Supp. Fig. 11 Supplemental data on the effect of the M₂tail(368-466) fragment on mitochondrial function and cell metabolism. **a**, Representative experiment of Seahorse assay showing the time course of oxygen consumption inhibition by M₂tail(368-466)-EGFP and wild type M₂-EGFP, compared to the same experiments performed in control cells transfected with EGFP. The rate of ATP-linked respiration measured after oligomycin administration is decreased in M₂tail and slightly decreased in M₂ cells as well as compared to the controls. FCCP, an ionophore, was added to each cell type to investigate the cellular bioenergetic reserves that seem to be slightly reduced only in the M₂ tail cells. **b**, Oxygen consumption measured by a Clark type electrode-based-polarographic method at a constant temperature of 37 °C. After

15 minutes of basal respiration, the ATP synthase inhibitor oligomycin was added at a concentration of 17 nM. Oxygen consumption in the absence and presence of oligomycin is represented by black and red bars, respectively. The blue bars represent the percentage of mitochondrial oxygen consumption (total minus oligomycin treated) in cells transfected with each construct. **c**, is a representative experiment showing the time course of inhibition of oxygen consumption by M₂tail(368-466), compared to the same experiments performed in control cells and cells transfected with the M₂ wild type construct, upon addition of oligomycin. Results are given as ng-atom of oxygen/min/10⁶ cells. **d**, Western blot of caspase-3 cleavage in COS-7 cells transfected with the indicated M₂ receptor mutants 48h after transfection (top panel, normal contrast; bottom panel, increased contrast). **e**, Confocal sequential images of M₂tail(368-466)-EGFP (green), Mitotracker (magenta) and overlay upon 2h of serum starvation. Scale Bars are 10 μm. **f**, Extracellular oxygen consumption assay of HEK293 cells transfected with the indicated M₂ receptor mutants using a fluorogenic dye quenched by Oxygen. Blank indicates the dye lifetime measured in wells containing the dye, but no cells.

Supp. Fig. 12. Functional aspects of mitochondrial localization of the M₂tail(368-466) fragment. **a-e**, Mitochondrial localization of the IRES mediated C-terminal fragment is enhanced upon cellular stress. HEK293 cells transfected with the super construct M₂-mRuby2-STOP-M₂-i3-tail-EGFP display increased localization of the EGFP labelled portion to the mitochondria, upon serum starvation for 2 hours and incubation of the cells in HBSS buffer, in presence of Mitotracker: **a**, is IRES-driven M₂tail(368-466)-EGFP. **b**, Cap dependent M₂-mRuby2 + IRES-driven M₂tail(368-466)-mRuby2, both derived from the M₂-mRuby2 gene. **c**, Mitotracker Deep Red **d**, Overlay of Mitotracker Deep Red and EGFP channel. **e**, Overlay between GFP and mRuby2. **f-l**, Localization of the mutant construct M₂(M368A)-mRuby2-STOP-M₂-i3-tail-EGFP in HEK293 cells after 2 hours incubation in HBSS buffer. The same legend as in **a-e** applies. Bottom: Schematic structure of the mega constructs M₂(M368A)-mRuby2-STOP-M₂-i3-tail-EGFP. This construct is similar to the M₂-mRuby2-STOP-M₂-i3-tail-EGFP construct but the methionine 368 in the M₂ sequence has been replaced with alanine. The asterisks represent the stop codons at the end of mRuby2 and EGFP. EGFP was excited at 488 nm and fluorescence collected between 500-600 nm; mRuby2 was excited at 561 nm and fluorescence collected between 580-

814 620 nm. Mitotracker deep red was excited at 633 nm and fluorescence collected between 650-750 nm. Scale bars are 10
815 μ m.

817 **Supp. Fig. 13.** Cellular localization of split GFP constructs in HEK293 cells. Confocal micrographs of **a**, co-
818 expression of SMAC-GFP11 and SMAC-GFP1-10 (positive control) and corresponding Mitotracker deep red image. **b**,
819 mito-GFP11 and mito-GFP1-10 (positive control). **c**, M₂tail(368-466)-GFP11 and SMAC-GFP1-10. **d**, M₂tail(368-466)-
820 GFP11 and mito-GFP1-10. **e**, mito-GFP1-10 alone (negative control). EGFP (green) was excited at 488 nm and
821 fluorescence collected between 500-600 nm. Mitotracker deep red (magenta) was excited at 633 nm and fluorescence
822 collected between 650-750 nm. Scale bars are 10 μ m.

823 **Fig. 6.** Effects of M368A mutation on cell metabolism in hiPSCs **a**, Confocal micrographs of wt hiPSCs (left) and
824 those harboring the M368A mutation within the M₂ gene (right), stained with DAPI (cyan) and Mitotracker (red). **b**, Cell
825 proliferation assay performed for wt hiPSCs and M368A hiPSCs grown in T25 flasks. Cells were counted 24h and 72h
826 after seeding. **c**, Extracellular oxygen consumption assay, performed on wt and M368A hiPSCs, prior to and after 7 days

undirected differentiation. The lifetime of a commercial dye increases as oxygen in the medium is progressively consumed by the cells. The data displayed are collected after 60 minutes incubation.

830

Fig. 7. Schematic representation of the wild type M₂ receptor gene products, their destination in the cell and their function. The M₂ receptor gene give rise to two different transcripts: when translation starts from the canonical cap site the wild type M₂ receptor is generated (in blue). When translation is driven by the internal ribosome entry site (IRES) the C-terminal fragment M₂tail is generated (in green). The two proteins are then sorted in two different cell compartments where they play completely different roles: the M₂ receptor is transported to the plasma membrane where it functions as a G protein-coupled neurotransmitter receptor and inhibits the adenylyl cyclase and β-arrestin mediated signalling. The M₂tail is instead transported in the mitochondria where it inhibits mitochondrial respiration and reduces ROS formation. IRES driven M₂tail translation is probably stimulated in para-physiological and pathological conditions and might have a protective role in cells expressing muscarinic M₂ receptors.

840

841 **Methods**

842 **Statistical analysis.** Significance values reported in the graphs, in comparing tabulated values, were
 843 determined by a one-tailed Student t-test using Prism 9 (GraphPad Software), and significance values
 844 (p-values) are indicated according to the following legend: **** $p < 0.0001$; *** $0.0001 < p < 0.001$; **
 845 $0.001 < p < 0.01$; * $0.01 < p < 0.05$. p-values larger than 0.05 are deemed non-significant. Unless otherwise
 846 indicated, bar charts and scatter plots indicate average value with s.e.m.

847
 848 **Generation of mutant muscarinic M₂ receptors.** Multiple receptor mutants were constructed starting
 849 from the human M₂ muscarinic receptor expressed in the pcD vector ³⁶. The constructs are sketched in
 850 **Supplementary Fig. 1**. The two expression plasmids referred as M₂trunk(1-283) (containing
 851 transmembrane domains I-V and the N-terminal portion of the third cytoplasmic loop) and M₂tail(281-
 852 466) (containing transmembrane domains VI and VII, and the C-terminal portion of the third
 853 cytoplasmic loop) were described previously ¹².

854 *M₂stop228* – This plasmid was created by substituting codon 228 (CAA) with a stop codon (TAA) at
 855 the N-terminal of the i3 loop of the wild type M₂ receptor.

856 *M₂stop228/stop400* – This plasmid was created by inserting an additional stop codon – TGG was
 857 substituted with TAA – at the position codon 400 of the TM region VI of the M₂stop228
 858 mutant.

859 *M₂stop400* – This plasmid was created by substituting codon 400 (TGG) with a stop codon (TAA) in
 860 the TM region VI of the wild type M₂ receptor.

861 *M₂stop196* – This plasmid was created by substituting codon 196 (TAT) with a stop codon (TAA) in the
 862 TM region V of the wild type M₂ receptor.

863 *M₂stop196/stop400* – This plasmid was created by inserting an additional stop codon in the TM region
 864 VI of the M₂stop196 mutant, where TGG was substituted with TAA at position of the
 865 codon 400.

866 *M₂stop228/fr.sh.* – This plasmid was created by inserting four bases (AATT) fifteen nucleotides
 867 downstream of the stop codon to create a shift in the correct reading frame of the
 868 M₂stop228 mutant.

869 *M₂stop228/hairpin* – This construct was created by inserting a 42 bases long sequence
870 (AGGGGCGCGTGGTGGCGGCTGCAGCCGCCACCACGCGCCCC). fifteen
871 nucleotides downstream of the stop codon of the M₂stop228 mutant. Upon transcription,
872 this palindromic sequence has been shown to form a hairpin structure with a ΔG value
873 of –64 kcal/mol that blocks effectively ribosome scanning^{37,38}.

874 *M₂stop228/stop248*, *M₂stop228/stop296* and *M₂stop228/stop368* – These three constructs were obtained
875 by substituting the three in-frame ATG codons (1. ATG 248, 2. ATG 296 and 3. ATG
876 368) with the stop codon TAA downstream of the stop228 of the M₂stop228 mutant.

877 *M₂trunk(1-228)* – This plasmid was created by substituting codon 228 (CAA) with a stop codon (TAA)
878 at the N-terminal of the i3 loop of the wild type M₂ receptor followed by removal of the
879 downstream sequence.

880 *M₂tail(368-466)* – This construct was created by removing all bases of the M₂ receptor sequence up to
881 codon 368 (ATG) of the C-terminal of the i3 loop. The resulting plasmid encodes for a
882 polypeptide fragment that contains the trans-membrane domains VI and VII along with
883 the C-terminal portion of the third cytoplasmic loop.

884 *M₂(M368A)* – In this construct the third in-frame methionine of the i3-loop of the M₂ muscarinic
885 receptor (ATG) was mutated to alanine (GCG).

886 *M₂-Myc*, *M₂stop228-Myc*, *M₂stopM368A-Myc*, *M₂tail(368-466)-Myc* and *M₂stop40-Myc* – M₂-Myc was
887 purchased from OriGene. This plasmid encodes the human M₂ receptor with a Myc (
888 tag at the C-terminus of the protein. M₂stop228-Myc was obtained by replacing the 0.9
889 Kb BmtI-PspOMI fragment of the M₂stop228 mutant with the corresponding fragment
890 of M₂-Myc. M₂stop400-Myc was obtained by substituting the codon 400 (TGG) with a
891 stop codon (TAA) of the M₂-Myc. All the constructs have an additional DDK tag after
892 the C-Myc. Subsequently, all C-Myc constructs were extracted by PCR and subcloned
893 into a bicistronic pVitro2-MCS plasmid (Invivogen), allowing for the expression of a
894 reporter gene (in our case the red fluorescent protein mRuby2) after an IRES sequence,
895 in order to check for transfection efficiency.

896

897 **Generation of bicistronic constructs expressing Sirius and EGFP proteins (Fig. 3a).**

898 *Sirius[PacI-M₂i3(685-1101)]EGFP* – This bicistronic construct was created by inserting the i3 loop of
 899 the muscarinic M₂ receptor, from nucleotide 685 to nucleotide 1101, between the
 900 ultramarine fluorescent protein (Sirius) and the green fluorescent protein (EGFP). Eight
 901 nucleotides corresponding to the recognition site of the PacI enzyme were inserted
 902 between Sirius and the i3 loop of the wild type M₂ receptor to alter the reading frame
 903 downstream of the Sirius stop codon. Hereafter this plasmid is referred to as Sirius-
 904 M₂i3(417n)-EGFP.

905 *Sirius[PacI-M₂i3(685-699)-Hairpin-G]EGFP* – This bicistronic construct was created by inserting a 42
 906 nucleotides hairpin loop (see above) between Sirius and the EGFP fluorescent protein.
 907 The hairpin loop was spaced from the Sirius stop codon by inserting a PacI recognition
 908 sequence and 15 nucleotides of the M₂ i3 loop sequence comprised between nucleotide
 909 685 and nucleotide 699. A G nucleotide was also inserted following the 42 nucleotides
 910 of the hairpin loop and upstream of the initial ATG triplet of the EGFP to restore a
 911 correct reading frame. Throughout the text we referred to this plasmid as Sirius-H-
 912 EGFP.

913 *Sirius[PacI-M₂i3(685-699)-Hairpin-G-PacI-M₂i3(685-1101)]EGFP* – This bicistronic construct was
 914 created by adding 417 nucleotides of the i3 loop of M₂ from nucleotide 685 to nucleotide
 915 1101 to the plasmid *Sirius[PacI-M₂i3(685-699)-Hairpin-G]EGFP*. An additional PacI
 916 restriction site was also inserted upstream of this segment of the i3 loop. Throughout
 917 the text we referred to this plasmid as Sirius-H-M₂i3(417n)-EGFP.

918 *Sirius[PacI-M₂i3(685-699)-Hairpin-G-PacI-M₂i3(685-699/1072-1101)]EGFP* – This bicistronic
 919 construct was created by deleting from the plasmid *Sirius[PacI-M₂i3(685-699)-Hirpin-
 920 G-PacI-M₂i3(685-1101)]EGFP* 372 nucleotides of the M₂ i3 loop, from nucleotide 700
 921 to nucleotide 1071. Throughout the text we referred to this plasmid as Sirius-H-
 922 M₂i3(15n/30n)-EGFP.

923 *Sirius[PacI-M₂i3(685-699)-Hirpin-G-PacI-M₂i3(685-699)]EGFP* – This bicistronic construct was
 924 created by adding 15 nucleotides of the i3 loop of M₂ from nucleotide 685 to nucleotide

699 to the plasmid *Sirius[PacI-M₂i3(685-699)-Hirpin-G]EGFP*. An additional PacI restriction site was also inserted upstream of this short segment of the i3 loop. Throughout the text we will refer to this plasmid as Sirius-H-M₂i3(15n)-EGFP.

Sirius[PacI-M₂i3(685-699)-Hirpin-G-PacI-M₂i3(685-1071)]EGFP – This bicistronic construct was created by adding 387 nucleotides of the i3 loop of M₂ from nucleotide 685 to nucleotide 1071 to the plasmid *Sirius[PacI-M₂i3(685-699)-Hirpin-G]EGFP*. An additional PacI restriction site was also inserted upstream this long segment of the i3 loop. Throughout the text we will refer to this plasmid as Sirius-H-M₂i3(387n)-EGFP.

Mutants of the Sirius-H-M₂i3(15n/30n)-EGFP plasmid – Based on the sequence alignment of amine and adenosine GPCRs, several conserved nucleotides were mutated in order to define the putative IRES sequence. These mutants are reported in **Fig. 3b**.

The fluorescence intensities of the two spectral bands were quantified by the use of a fluorometer and the EGFP intensity values normalized to those of Sirius.

Constructs for fluorescence microscopy.

M₂-EGFP – The wild type M₂ muscarinic receptor was cloned into the pEGFP-N1 expression cassette, between the restriction sites HindIII and XbaI.

M₂-mRuby2 – The wild type M₂ muscarinic receptor was cloned into the mRuby2 vector (Addgene plasmid #40260).

M₂tail(368-466)-EGFP – The M₂tail(368-466)-EGFP was constructed so that the receptor protein would start from the third in-frame methionine of the M₂ i3 loop (M368). The EGFP gene was fused C-terminally following a short restriction site sequence (TCTAGA) of the XbaI enzyme.

M₂tail(368-466)-mRuby2 – The M₂tail(368-466)-mRuby2 was constructed so that the receptor protein would start from the third in-frame methionine of the M₂ i3 loop (M368). The mRuby2 gene was fused C-terminally following a short restriction site sequence (TCTAGA) of the XbaI enzyme.

952 *M₂-mRuby2-STOP-M₂-i3-tail-EGFP* – This mega construct was obtained by fusing two preceding
 953 constructs, M₂-mRuby2 and M₂tail(368-466)-EGFP, in a unique plasmid, but the M₂tail
 954 started at codon 229 (M₂tail(229-466)-EGFP). After the stop codon of mRuby2 and
 955 before the codon 229 of M₂tail was inserted a short restriction site sequence (TCCGGA)
 956 of the BspEI enzyme (Fig. 4).

957 *M₂(M368A)-mRuby2-STOP-M₂-i3-tail-EGFP* – This mega construct is analogous to *M₂-mRuby2-STOP-*
 958 *M₂-i3-tail-EGFP*, but the third in-frame methionine of the i3-loop of the M₂ muscarinic
 959 receptor was mutated to alanine (M368A) (Supplementary Fig. 1).

960 *M₂fr.sh-EGFP* – A single base insertion (G) upstream of nucleotide 1102 of the wild type M2 receptor,
 961 just before the third in-frame methionine of the i3-loop (M368), induces a frame shift
 962 that, following 2 aminoacids, generates a stop codon (TAA) in the amino acid position
 963 370 of the new reading frame. The construct is then fused to EGFP.

964 *M₂fr.sh-mRuby2* – As above, but the EGFP is replaced by a mRuby2.

965 *M₂-GFP11* – A short peptide 16 aminoacids long from the GFP protein, GFP11, was fused to the C-
 966 terminus of the muscarinic M₂ receptor. GFP11 was custom synthetised and inserted as
 967 a linker between the XbaI and XhoI restriction sites in the plasmid backbone of M₂-
 968 EGFP.

969 *Mito-GFP1-10* – The mitochondrial targeting sequence of the cytochrome c oxidase subunit 8A
 970 (COX8A) was fused to the N-terminal of GFP1-10 fragment of GFP. GFP1-10 was
 971 purchased from Addgene as Addgene plasmid 70219.

972 *Mito-GFP11* – The mitochondrial targeting sequence of COX8A was fused to GFP11 fragment. Mito
 973 sequence was obtained from Addgene plasmid 23348.

974 *SMAC-GFP1-10* – The mitochondrial targeting sequence of the SMAC protein was fused to the N-
 975 terminal of GFP1-10 fragment. SMAC was obtained from Addgene plasmid 40881.

976 *SMAC-GFP11* – The mitochondrial targeting sequence of the SMAC protein was fused to GFP11
 977 fragment.

978 *M₂tail(368-466)-GFP1-10* – The GFP1-10 fragment was fused to the C-terminus of the muscarinic
 979 M₂tail(368-466) receptor fragment.

980 *M₂tail(368-466)-GFP11* – The GFP11 fragment was fused to the C-terminus of the muscarinic
981 *M₂tail(368-466)* receptor fragment.

982

983 **Constructs for in-vitro mitochondrial import assays**

984 *PSP64-M₂tail(368-466)-5Met* - The vector backbone PSP64 (courtesy of Ruairidh Edwards). The

985 following primers: FWD: ATGATGTAActtagaggaggcgacgc, REV:

986 CATCATCATcctttagcgcctatgttcttataatg

987 were used to add five methionines to the C-terminal domain of the *M₂tail(368-466)-*

988 GFP11 template (see above) using Q5® Site-Directed Mutagenesis Kit (New England

989 Biolabs® Inc.). The construct was then extracted with appropriate restriction enzymes

990 (HindIII and XbaI) and ligated into the vector backbone PSP64 (courtesy of Ruairidh

991 Edwards).

992 *PSP64-M₂R-5Met* – The following primers: FWD: ATGATGTAActtagaggaggcgacgc REV:

993 CATCATCATcctttagcgcctatgttcttataatg were used to add five methionines to the C-

994 terminal domain of to the *M₂-GFP11* template (see above) using Q5® Site-Directed

995 Mutagenesis Kit (New England Biolabs® Inc.). The construct was then extracted with

996 appropriate restriction enzymes (HindIII and XbaI) and ligated into the vector backbone

997 PSP64.

998

999 All constructs were sequenced either with a Genetic Analyzer 3500 (Applied Biosystem®) or by a

1000 professional sequencing service (LGC Genomics, Berlin).

1001

1002 **Reverse transcription.** Reverse transcription was performed using a 24-nucleotide antisense oligo

1003 (TTACCTTGTAGCGCCTATGTTCTT) directed against the last 24 nucleotides at the 3' end of the *M₂*

1004 open reading frame. cDNA amplification was carried out with the same antisense oligo used for reverse

1005 transcription, together with a 24 nucleotides sense oligo (ATGAATAACTCAACAACTCCTCT)

1006 directed against the first 24 nucleotides at the 5' end of the *M₂* open reading frame.

1007 **Cell culture and transfection.** COS-7 (African green monkey kidney fibroblast-like SV40 transformed
1008 cell line) and CHO (Chinese Hamster Ovary) cells (from American Type Culture Collection) were
1009 grown at 5% CO₂ and 37 °C, in high glucose Dulbecco's modified Eagle's medium (DMEM)
1010 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 IU/ml penicillin, and 50
1011 mg/ml streptomycin, and non-essential amino acids. HeLa cells (from American Type Culture
1012 Collection) were cultured in the same medium, but with the double amount of L-glutamine. SHSY-5Y
1013 cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS,
1014 2 mM L-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin. Media and reagents used for cell
1015 cultures were from Euroclone, PAN Biotech, Biochrome and Sigma-Aldrich. Cell transfection was
1016 performed using FuGene[®] transfection reagents (Promega) as recommended by the manufacturer.
1017 Eighty percent confluent cells were washed twice with free-serum medium and the mix of FuGene and
1018 plasmid DNA was added. In every experimental condition 6-8 µg of plasmid DNA per Petri (10 cm²)
1019 were used. Cells were grown for 48 h post-transfection before use. In co-transfection experiments, half
1020 amount of each plasmid DNA was used. All microscopy experiments were performed with transiently
1021 transfected HEK293 (Human Embryonic Kidney, ECACC 96121229 from Sigma-Aldrich Chemie
1022 GmbH) and COS-7 cells. HEK293 cells were cultured in DMEM (Dulbecco's modified Eagle's medium
1023 PAN biotech, Aidenbach, Germany), supplemented with 4.5 g/L Glucose, 2 mM L-Glutamine, 10%
1024 FCS, 100 units/mL penicillin and 0.1 mg/mL streptomycin and maintained at 37 °C and 5% CO₂. Cells
1025 cultured in 15-cm dishes were split at a 1:36 ratio into 6-well plates containing poly-D-lysine (PDL)-
1026 coated 24 mm glass coverslips. Cells were transfected using Effectene transfection reagent (QIAGEN)
1027 according to the manufacturer's instructions. Cells seeded on PDL-coated coverslips in 6-well plates
1028 were transfected 16 hours after seeding with 0.6 µg plasmid/well.

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1030 **Fluorescence microscopy.** Fluorescence microscopy experiments were performed either on a Leica
1031 SP5 (or SP8) Confocal Microscopes, using HyD photon counting detectors and an Argon/Ion laser (or
1032 white light laser) source for 488 nm/633 nm excitation and a solid-state diode laser (or white light laser)
1033 for 561 nm excitation. A solid-state diode laser was used for 405 nm excitation. A 40x 1.3 NA objective
1034 was used, and the electronic zoom was set to achieve a pixel size of 50 nm. For visualization,

1035 mitochondria of transfected cells were labelled for 30 minutes at 37 °C with 50nM MitoTracker® Deep
1036 Red FM (Invitrogen™, Thermo Scientific).

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1038 **Ligand binding studies.** Cells were lysed 48 h post-transfection with ice-cold 2 mM EDTA, 1 mM Na-
1039 HEPES (pH 7.4), scraped with a rubber policeman in the presence of ~4 ml rinsing medium, and
1040 homogenized with a Polytron homogenizer (ULTRA-TURRAX) in binding assay buffer (50 mM Tris-
1041 HCl, pH 7.4, 155 mM NaCl, 0.01 mg/ml bovine serum albumin). Radioligand bindings were carried out
1042 at 37 °C for 3 h in a final volume of 1 ml. Non-specific binding was determined in presence of 1 µM
1043 atropine. For competition binding assays, increasing concentrations of carbachol (from 0.01 µM up to
1044 10 µM) were used in presence of 200 pM [³H]N-Methylscopolamine ([³H]NMS; 78.9 Ci/mmol;
1045 PerkinElmer).

1046 The bound ligand was separated from the unbound ligand using glass-fiber filters (Whatmann, GF/B)
1047 with a Brandel Cell Harvester, and filters were counted with a scintillation β-counter. K_D values of
1048 [³H]NMS were calculated in direct saturation experiments, whereas carbachol Inhibitory Concentration
1049 (IC)₅₀ values were calculated in competition curves, against 400 pM [³H]NMS, fitted to one binding
1050 models using the iterative, non-linear least-squares regression analysis of OriginPro 7.5 software
1051 (OriginLab Corporation).

1052 **Phosphatidylinositol Breakdown Assay.** Transfected COS-7 cells were incubated with myo-
1053 [³H]inositol (23 Ci/mmol; PerkinElmer) for 48 h. Immediately before the experiment the cells were
1054 washed twice with phosphate-buffered saline and incubated for 15 min in Eagle's medium containing
1055 10 mM LiCl and 20 mM HEPES. The medium was then replaced by 0.25 ml of the same medium
1056 containing the experimental agents. After a 1-hour incubation at 25 °C the reaction was arrested by the
1057 addition of 0.75 ml of 7.5% (w/v) ice-cold trichloroacetic acid followed by a 30-min incubation on ice.
1058 The trichloroacetic acid was extracted with water-saturated diethyl ether (3 × 4 ml), and levels of IP₁
1059 determined by anion exchange chromatography.

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1061 **Adenyl Cyclase Assay.** COS-7 cells were transfected with plasmid(s) containing the receptor of interest

plus adenylyl cyclase V. 24 h after transfection the cells were detached with trypsin and re-cultured in 24-well plates. Following an additional period of 24 h culture, they were assayed for adenylyl cyclase activity. The assay was performed in triplicate. In brief, cells were incubated in 24-well plates for 2 h with 0.25 ml/well of fresh growth medium containing 5 μ Ci/ml [3 H]adenine (PerkinElmer). The medium was then replaced with 0.5 ml/well of DMEM containing 20 mM HEPES, pH 7.4, 0.1 mg of bovine serum albumin, and the phosphodiesterase inhibitors 1-methyl-3-isobutylxanthine (0.5 mM) and Ro-20-1724 (0.5 mM). AC activity was stimulated by the addition of 1 μ M forskolin in the presence or absence of carbachol. After 10 min of incubation at +30 °C the medium was removed, and the reaction terminated by addition of perchloric acid containing 0.1 mM unlabeled cAMP followed by neutralization with KOH. The amount of [3 H]cAMP formed under these conditions was determined by a two-step column separation procedure (referenza?).

Immunoblot of muscarinic M₂ receptor mutants transfected in HEK293 cells. Cells were seeded in 10 cm plates and transfected after 24h according to manufacturer protocols (Effectene, Qiagen) using 2 μ g of plasmid DNA. 48h after transfection cells were washed (2x) in ice cold PBS. 200 μ L per plate of ice-cold lysis buffer were added. Lysis buffer was a RIPA buffer: 50 mM Tris-HCL pH 8, 150 mM NaCl, 1% TRITON, 0.5% sodium deoxycholate and 0.1% SDS. The following compounds were added to the buffer to a final 1x dilution: 100x Halt Protease Inhibitor Cocktail (Thermo Fisher), 100x 0.5 M EDTA and (10x) 10 mM PMSF. Cells were then scraped and the cell lysate transferred to pre-cooled 1.5 mL Eppendorf tubes. The tubes were placed in a Thermomixer at 4 °C, 300 rpm for 30 minutes. Then the Eppendorf tubes were centrifuged for 30 minutes at 14000 rpm at 4 °C. The supernatant was then transferred to new Eppendorf tubes. Protein concentration in the cell lysates was quantified by a BCA assay (Pierce BCA Protein Assay Kit, from Thermo Fisher) according to manufacturer instructions.

Cell lysates were loaded into 10% polyacrylamide gels. Cell lysates were loaded using Laemmli Buffer 2x (Sigma Aldrich) in equal mass. As a reference marker we loaded 5 μ L of PageRuler Prestained Protein Ladder (Thermo Fisher). Gels were mounted into a Mini Protean Tetra Cell kit (BioRad) using 1x SDS running buffer. Gels were ran using a constant voltage of 90V for approximately 15 minutes,

and afterwards at 130V for 45 minutes. Gels were transferred to PVDF membranes. Wet transfer was achieved by using the Mini Trans Blot Module (BioRad) in Wet Transfer Buffer (25 mM Tris pH 8.3, 192 mM Glycine and 20% MetOH) at 350 mA for 90 minutes. The immunoblotted membranes were then blocked in 5% Milk in TBS-T for 1 hour at room temperature. The membrane was then incubated overnight at 4 °C with primary antibody solution using the following concentrations depending on the antibody used: 1:1000 anti β -actin (13E5) rabbit primary antibody (Cell Signaling), 1:1000 anti myc-Tag (9B11) mouse primary antibody (Cell Signaling). After overnight incubation, the membranes were washed 3x 10 minutes in TBS-T and incubated at RT for 1 h with a secondary antibody (1:10000 anti-mouse IgG/(anti rabbit) HRP-linked antibody from Cell Signaling). After the incubation with the secondary antibody, the membrane was washed 3x 10 minutes in TBS-T. Detection was achieved by eliciting HRP luminescence by incubating the membrane with SuperSignal WestFemto solution (Thermo Fisher) according to the manufacturer's instructions, and imaging the membrane using a c600 Transilluminator from Azure Biosystems.

Animal Experiments. All animal experiments were carried out according to the German animal welfare act, considering the guidelines of the National Institute of Health and the 2010/63/EU Directive of the European Parliament on the protection of animals used for scientific purposes. The animals had free access to food and water and were kept in individually ventilated cages under a 12h:12h light/dark regime (light from 6:30 am to 6:30 pm), a constant 22±2°C temperature and 55±10% humidity.

The transgenic line B6.Cg-Chrm2tm1.1Hze/J, also known as Chrm2-tdT-D knock-in, was obtained from 'The Jackson Laboratory' (Stock No: 030330). They are heterozygous animals, designed to have expression of a Chrm2/tdTomato fusion protein directed by the endogenous M2 muscarinic acetylcholine receptor promoter/enhancer sequences.

Tissue preparation and immunoblot of endogenous M₂ receptor in Chrm2-tdT-D knock-in mice.

Mouse tissues were weighed out and ice-cold lysis buffer was added to approx. 0.2-0.5mg tissue/ml buffer (10mM K₂HPO₄, 150mM NaCl, 5mM EDTA, 5mM EGTA, 1% Triton X-100, 0.2% Na-deoxycholate. Fresh protease inhibitors PMSF 0.5mM, Na₃VO₄ 0.1mM, NaF 50mM, Jenny's mix

1118 (Soybean trypsin inhibitor 3.2µg/ml, Aprotinin 2µg/ml, Benzamidin 0.5mM) were added to the buffer.
 1119 Samples were first homogenized, and then pellet debris were centrifuged at 4°C for 30 minutes at max
 1120 speed (tabletop) and the supernatant (soluble proteins) was transferred into fresh tube (pellets = non-
 1121 soluble proteins)
 1122 Protein concentration were determined with BCA assay. Cell lysates were loaded using Laemmli Buffer
 1123 2x (Sigma Aldrich) in equal mass (see above for the immunoblot procedure) into 8% polyacrylamide
 1124 gels.
 1125 The membrane was then incubated overnight at 4 °C with primary antibody solution (1:1000 anti β -
 1126 actin (13E5) rabbit primary antibody #4970 from Cell Signaling) (1:1000 anti GAPDH) (1:1000 anti-
 1127 TdTomato rabbit primary antibody #632496 from Takara).
 1128
 1129 **Isolation of mitochondrial fractions and immunodetection.** Cells were seeded in 15 cm plates and
 1130 transfected after 24h according to manufacturer protocols (Effectene, Qiagen) using 5 µg of plasmid
 1131 DNA. 48h after transfection cells were harvested and centrifuged at 1000 rpm for 10 minutes,
 1132 resuspended in NaCl 0.9% (w/v) and centrifuged at 1000 rpm for 10 minutes.
 1133 Mouse tissue were freshly excised and placed in ice cold PBS (1x) . The samples were washed with
 1134 NaCl 0.9% (w/v). The homogenization steps were performed with Precellys Evolution Homogenizer, at
 1135 4500 rpm for 10 seconds.
 1136 The samples were processed according to manufacturer protocols (Qproteome Mitochondria Isolation
 1137 Kit, Qiagen). From the different steps were obtained three different fractions: the cytosolic, the
 1138 microsomal and the mitochondrial fraction.
 1139 The fresh mitochondrial pellet was resuspended in 150µL of ice-cold lysis buffer and the protein
 1140 concentration of the three fractions from each sample was quantified with a BCA assay (see above).
 1141 Cell lysates were loaded into 12% polyacrylamide gels using an SDS loading buffer (6x solution: 2.28g
 1142 Tris-HCl, 2.313 DTT, 6g SDS, 30% Glycerol and 0.03% bromophenol blue in a final volume of 50 mL)
 1143 in equal mass (see above for the immunoblot procedure).
 1144 The membrane was then incubated overnight at 4 °C with primary antibody solution using the following
 1145 concentrations: 1:1000 anti β -actin (13E5) rabbit primary antibody (#4970 from Cell Signaling), 1:1000

1146 anti-tdtomato rabbit primary antibody (#632496 from Takara), 1:1000 anti β -tubulin mouse primary
1147 antibody, 1:1000 anti GAPDH mouse primary antibody, 1:1000 anti COXIV mouse primary antibody
1148 (#ab33985 from abcam).

1149

1150 Quantification of M₂tail(368-466) mitochondrial import kinetics from confocal time sequences.

1151 Before imaging, 2.5×10^4 HEK-TSA cells were seeded in each PDL-coated well of the μ -Slide 8 well
1152 (Ibidi) with DMEM (ScienCell™) supplemented with 10% FBS and 1% P/S overnight, and transfected
1153 with a total of 2 μ g C-terminal EGFP tagged M₂ tail using Lipofectamine 2000 (Thermo Fisher
1154 Scientific) according to the manufacturer protocol after washing with 200 μ L PBS and refreshing with
1155 100 μ L DMEM (ScienCell™) supplemented with 10% FBS and 1% P/S. After transfection for five
1156 hours, mitochondria of transfected cells were rapidly labelled with 50nM MitoTracker® Deep Red FM
1157 (Invitrogen™, Thermo Scientific) in FluoroBrite DMEM medium (Thermo Scientific) supplemented
1158 with 1% P/S for 20 minutes after the removal of old culture medium. After mitochondria labeling, the
1159 μ -Slide were washed twice with FluoroBrite DMEM medium (Thermo Scientific) and subsequently
1160 mounted onto the TCS SP8 confocal microscope (Leica) (See imaging). Firstly, the stage of the
1161 microscope was equilibrated in temperature, and the acquisition mode was set to the tile scanning. After
1162 storing the position of each desired transfected cells, the imaging mode was set to XYT and a HC PL
1163 APO CS2 40 $\times$ /NA1.3 oil immersion objective was selected. Both DIC channel and HyD were utilized
1164 to obtain 512 $\times$ 512 pixels images. Laser wavelengths were set as described in the section Fluorescence
1165 Microscopy above. All settings remained the same throughout the whole measurement. Images were
1166 taken at every 30 minutes over a 5h interval. The images were afterwards analyzed by ImageJ software,
1167 using the Plugin ‘JACoP’. Briefly, the degree of colocalization between EGFP-tagged M₂tail(368-466)
1168 (CH1) and MitoTracker stained mitochondria (CH2) was quantified by Mander’s colocalization
1169 coefficients³⁹. The two colocalization coefficients M1 ($M1 = \frac{CHB}{CH1}$) and M2 ($M2 = \frac{CHB}{CH2}$), CHB indicates
1170 overlapping pixels in channel 1 and channel 2. A threshold was determined for individual channel in
1171 order to distinguish specific signals from nonspecific ones. Due to their sensitivities to thresholds, the
1172 Pearson correlation coefficient (PCC)⁴⁰, as well as the faster decaying attribute of nonspecific signals,
1173 were also taken into account while estimating thresholds for each channel. For the analysis of each cell,

the threshold for Mitotracker channel was kept constant. Whereas the threshold for EGFP channel was evaluated based on the PCC value.

Transfection and expression of split-GFP constructs in HEK293 cells. To perform the sequential split GFP complementation assay illustrated in **Figures 4h-i and 5f**, 3×10^5 HEK-TSA cells were seeded on a 10% poly-D-lysine (PDL)-coated 25mm coverslip in each well of a Falcon® 6-well flat-bottom plate (StemCell™) and cultured overnight. Cells were transfected using Effectene (see above) with a 0.4 µg Mito-GFP1-10 plasmid. The cells were then washed once with fresh 2 mL phosphate-buffered saline (PBS) first and supplemented with 1.5 mL fresh DMEM medium (ScienCell™) supplemented with 10% FBS and 1% P/S. After adding the transfection mixture, the cells were cultured overnight. After the first transfection, a second transfection of a 0.6 µg plasmid encoding either the C-terminal GFP11 tagged M₂tail(368-466) or M₂ receptor was performed using the Effectene Transfection Reagent (Qiagen). After washing each well with 2 mL PBS, the old medium was exchanged with 1.5 mL low glucose Minimum Essential Medium (MEM) (Gibco™, Thermo Scientific) supplemented with 1% P/S, in order to create a stress-like cell starvation condition. After a four hours starvation, the cells were fixed using 4% polyformaldehyde (PFA)/PBS at 37°C for 30 minutes and washed twice with PBS before imaging.

Oxygen consumption measured with the Seahorse assay. 5×10^4 COS7 cells were seeded in seahorse 24MW plates with Complete media (DMEM supplemented with 10%FBS and 1X PS) and then incubated for about 4h at 37C in a cell incubator. After this incubation, cells were washed twice with Seahorse media supplemented with glutamate (1 mM), pyruvate (1 mM) and glucose (20 mM) and then incubated for 1h at 37 °C in an incubator without CO₂ before being analyzed in the Seahorse XF Analyzers. In particular, the baseline cell oxygen consumption rate (OCR) was measured at the beginning of the assay, followed by measurements of ATP linked respiration and maximal respiratory capacity after the addition of respectively oligomycin (1 µM) and the FCCP protonophore (1 µM). Finally, rotenone (1 µM), an inhibitor of complex I, and antimycin (1 µM), inhibitor of complex III,

1201 were added to shut down completely the electron transport chain (ETC) function, revealing the non-
1202 mitochondrial respiration.

1203

1204 **Generation of M368A hiPSC clone.** The human induced pluripotent stem cell (hiPSC) line BIHi005-
1205 A (<https://hpscreg.eu/cell-line/BIHi005-A>) was used to generate an isogenic iPSC clone (BIHi005-A-
1206 39) with **M368A mutation** using a protocol previously described⁴¹. In brief, small guide RNA (sgRNA)
1207 targeting the M₂ gene close to the mutation site to be corrected were designed using the
1208 <http://crispor.tefor.net/> webpage and the sgRNA (5'- AGTCATCTTCACAATCTTGC-3') were
1209 synthesized by Integrated DNA technologies (<https://eu.idtdna.com>). The donor ssODN template was
1210 designed to convert the ATG to GCT and was synthesized as an Ultramar DNA oligo by IDT. (5'-
1211 AAAAGTGACTCATGTACCCCACTAATAACACCGTGGAGGTAGTGGGGTCTTCAGGTCAG
1212 AATGGAGATGAAAAGCAGAATATTGTAGCCCGCAAGATTGTGAAGGCTACTAAGCAGCC
1213 TGCAAAA-3'). The Ribonucleoprotein (RNP) complexes were prepared by incubating of 1.5µg Cas-
1214 9 protein and 360 ng gRNA for 10 minutes at room temperature. For delivery of RNPs and ssODN
1215 template, 10µl cell suspension containing Cas9 RNPs and ssODN and 1x10⁵ cells were electroporated
1216 using the Neon transfection System (ThermoFisher Scientific). The electroporated cells were plated in
1217 one well of 6 well plate with StemFlex media (ThermoFisher Scientific) supplemented CloneR™
1218 (Stemcell technologies). Three days after the transfection we analyzed the bulk population using the
1219 Sanger sequencing to estimate the editing efficiency. Thereafter, the automated single cell cloning of
1220 the genome edited cell pool was performed as described in protocol ⁴². The clones were screened by
1221 SANGER sequencing. The positive confirmed clones were banked and characterized for pluripotency
1222 and karyotype stability as described in ⁴³.

1223

1224 Briefly, for pluripotency assay, single cells were labelled with conjugated antibodies. Surface markers
1225 (SSEA1, SSEA4 and TRA1-60) staining was performed in unfixed cells by incubation with antibodies
1226 diluted in 0.5% BSA/PBS (10 min, 4 °C, dark). Intracellular markers (OCT3/4, NANOG) were stained
1227 by fixation/permeabilization solution (Miltenyi Biotec, 130-093-142) (30 min, 4 °C, dark) followed by

1228 antibody incubation in permeabilization buffer (Miltenyi Biotec) (30 min, 4 °C, dark). Analysis was
1229 done using the MACSQuant® AnalyzerVYB and FlowJo v10.4.

1230

1231 Briefly, for karyotype stability, genomic DNA was isolated using the DNeasy blood and tissue kit
1232 (Qiagen, Valencia, CA, United States) and samples were analyzed using the human Illumina OMNI-
1233 EXPRESS-8v1.6 BeadChip. First, the genotyping was analyzed using GenomeStudio 1 genotyping
1234 module (Illumina). Thereafter, KaryoStudio 1.3 (Illumina) was used to perform automatic normalization
1235 and to identify genomic aberrations utilizing settings to generate B-allele frequency and smoothened
1236 Log R ratio plots for detected regions. The stringency parameters used to detect copy number variations
1237 (CNVs) were set to 75 kb (loss), 100 kb (gain) and CN-LOH (loss of heterozygosity) regions larger than
1238 3 MB.

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1240 **Culture and handling of hiPSCs cells.** hiPSCs were cultured on Geltrex (Thermo Fisher) coated plates
1241 using: Essential 8 medium (Thermo Fisher). The culture was routinely monitored for mycoplasma
1242 contamination using a PCR assay. 10 µM ROCK Inhibitor Y-27632 2HCl (Selleck Chemicals, #SEL-
1243 S1049) was added after cell splitting to promote survival. hiPSC cultures were kept in a humidified
1244 atmosphere of 5% CO₂ at 37°C and 5% oxygen. hiPSCs were undirectedly differentiated to a mixture
1245 of three lineages (ectodermal, endodermal and mesodermal) by replacing Essential 8 medium with
1246 DMEM-F12 medium supplemented with 10% FBS for seven days. 10 µM ROCK Inhibitor Y-27632
1247 2HCl (Selleck Chemicals) was added after cell splitting to promote survival.

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1249 **Cell proliferation assay with HEK293 cells.** 4x10⁵ HEK293 cells 24h after transfection and 4x10⁵
1250 hPSCs were seeded respectively in T25 flask and pre-coated T25 flask, in culture medium with Atropine
1251 at final concentration of 100nM; the medium with Atropine was replaced every day. After 72h from
1252 transfection of the HEK293 and from seeding of the hiPSCs, the medium and the cells were collected
1253 and centrifuged, the pellet was resuspended in the same amount of medium for each pellet; an aliquots
1254 of the resuspension was mixed with the same amount of Trypan blue (ratio 1:1), 10 microliters were
1255 used for counting in a Neubauer chamber.

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Fluorescence plate reader assays. HEK293 cells were grown on 6 well plates without coverslips, and, upon reaching 60-70% confluence, the cells were transfected using Effectene (Quiagen, Venlo Netherlands) according to the manufacturer's instructions. After 24 h the cells were split and transferred to 96 well (black plate) at a seeding density of 50,000 cells/well. The 96 well plate was pre-coated with Poly-D-Lysine for 30 min at RT. The medium was removed and each well was washed 3x with 100µl HBSS. The cells were finally analyzed by a fluorescence plate reader (Neo2, BioTek, Bad Friedrichshall) in HBSS.

Extracellular oxygen consumption. HEK293 cells 24h after transfection and hPSCs were harvested and seeded respectively in 96 multiwell black plate and pre-coated (Geltrex A1413302, Thermo Fisher) 96 multiwell black plate, 5x10⁴ cells for well; after incubation O/N at 37 °C and in 5% CO₂, the cell culture medium was replaced by HBSS and the assay were performed according to the manufacturer protocols (abcam Extracellular Oxygen Consumption Assay).

Author Contributions

PA, FP, IF, ZW, MR, IP, RE, HZ, UZ, MET, JDG, SS, ART, SA conducted primary experiments; MG, CP, GA, FM, BP, AR contributed to the experimental data; NT and SD contributed tools and reagents. PA, IP, IF, MR, UZ, AT, KT, VF, FA, FG, MS contributed to experimental design and assisted in writing paper, PA, MJL and RM conceived and lead the study, and wrote the paper.

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Competing Interests

The authors declare that they have no conflicts of interest with the contents of this article.

1284

1285 **Data Availability Statement**

1286 Source data are provided with this manuscript.

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