A SPLICS reporter reveals α-synuclein regulation of lysosome-mitochondria contacts which affects TFEB nuclear translocation

Mitochondrial and lysosomal activities are crucial to maintain cellular homeostasis: optimal coordination is achieved at their membrane contact sites where distinct protein machineries regulate organelle network dynamics, ions and metabolites exchange. Here we describe a genetically encoded SPLICS reporter for short- and long-juxtapositions between mitochondria and lysosomes. We report the existence of narrow and wide lysosome-mitochondria contacts differently modulated by mitophagy, autophagy and genetic manipulation of tethering factors. The overexpression of α-synuclein (α-syn) reduces the apposition of mitochondria/lysosomes membranes and affects their privileged Ca\(^{2+}\) transfer, impinging on TFEB nuclear translocation. We observe enhanced TFEB nuclear translocation in α-syn-overexpressing cells. We propose that α-syn, by interfering with mitochondria/lysosomes tethering impacts on local Ca\(^{2+}\) regulated pathways, among which TFEB mediated signaling, and in turn mitochondrial and lysosomal function. Defects in mitochondria and lysosome represent a common hallmark of neurodegenerative diseases: targeting their communication could open therapeutic avenues.

Organelle contact sites are increasingly recognized as the bottleneck where nutrients, metabolites, ions, and lipids are fine-tuned to maintain cell homeostasis\(^1\). Indeed, even subtle variations in the bidirectional communication between different organelles can have a big impact on the biophysical and functional characteristics of the organelles themselves, and overall on the cell physiology, resulting in early sensitization to cell dyshomeostasis. Organelles tethering is hijacked in many pathological conditions and its molecular characterization has recently attracted major interest since it may represent a target for therapeutic intervention\(^1\)–\(^3\). In particular, mitochondria and lysosomes are functionally and physically\(^4\)–\(^11\) strictly interconnected being their dysfunction shared by many neurological disorders\(^12\),\(^13\). Despite the possibility that their crosstalk could be an important hub for neuronal homeostasis is recently emerging\(^2\),\(^9\),\(^14\)–\(^17\), the study of their interaction...
at specific membrane contact sites is still largely unexplored. Multiple studies using different imaging techniques have demonstrated that interorganelle contact sites between mitochondria and lysosomes occur at an average distance between membranes of ~10 nm and can dynamically form under healthy conditions. These contacts are clearly distinct from the contacts occurring under lysosomal degradation pathways such as mitophagy or involving mitochondria-derived vesicles. In this respect, molecular details at the basis of their dynamics have revealed the existence of a tight regulation by multiple proteins among which the small GTPase Rab7 is one of the major players. In its GTP-bound state it localizes to lysosomes and, by binding possible effector proteins on mitochondria, acts as a master regulator promoting contact formation. Rab7 GTP hydrolysis to a GDP-bound state mediates lysosome-mitochondria contact untethering and is driven by the Rab7 GTPase activating protein (GAP) TBC1D15, bound to dynamin that under healthy conditions binds to the outer mitochondrial membrane protein Fis1. The inability to undergo GTP hydrolysis, as observed upon the expression of the constitutively active Rab7 (Q67L)-GTP mutant, increased the number of contacts and prolonged the duration of the tethering. Accordingly, the inhibition of Rab7 GTP hydrolysis by the TBC1D15 (D397A) GAP-domain mutant led to inefficient untethering. Functionally, lysosome-mitochondria contacts play key roles in regulating the dynamics of both mitochondria and lysosomes as well as in metabolite homeostasis through bidirectional exchange of Ca²⁺, cholesterol, and iron. Impaired, formation and function of lysosome-mitochondria contact sites has been associated with the onset of different neurodegenerative diseases i.e., Charcot-Marie-Tooth (CMT) disease, Mucoepidermoid type IV (MLV), Niemann-Pick type C (NPC) and Parkinson’s disease (PD), suggesting that their modulation might represent a potential mechanistic pathway contributing to neurodegeneration, and thus become a target of possible therapeutic intervention. However, the molecular details of the consequences of impaired lysosome-mitochondria contact in PD pathogenesis are currently unknown. The gap is further hampered by the lack of reporters to easily image lysosome-mitochondria proximity over a range of distances in living cells and in vivo. We had previously designed split-GFP based contact site sensors of organelle proximity and tested them in vitro and in vivo.

Here we report on the generation of a reporter for lysosome-mitochondria proximity (SPLICSSMT) and on its validation in conditions well known to impact on lysosome-mitochondria tethering. We describe the existence of at least two types of lysosome-mitochondria contact sites in human cells, narrow and wide, which differently respond to mitophagy or autophagy stimuli and to changes in the levels of specific tethering/untethering factors. Here we also report on the application of the SPLICSSMT reporter to explore lysosome-mitochondria contact sites in model cells overexpressing the PD-related protein α-synuclein (α-syn). Mitochondria and lysosome dysfunction have been widely reported to contribute to the onset of many neurodegenerative conditions, including PD, and α-syn gene (SNCA) multiplication or point mutations are causally linked to dominant familial early-onset parkinsonism. Since α-syn interferes with both mitochondria and lysosome activities, we have directly tested whether α-syn accumulation, i.e., one of the most pathological hallmark of PD, could modulate the lysosome-mitochondria interface. Furthermore, we have searched for possible consequences of α-syn overexpression on the pathway of transcription factor EB (TFEB), the master regulator of autophagy, lysosomal biogenesis, and in intracellular clearance pathways which have been widely proposed as therapeutic target in cellular and mouse models for lysosomal storage disorders and neurodegenerative diseases. We show here that the overexpression of WT or the PD-related A30P and A53T α-syn mutants strongly impaired lysosome-mitochondria tethering, impinging on the efficient mitochondrial Ca²⁺ uptake upon its release from the lysosomes, thus reducing mitochondrial Ca²⁺ buffering and consistent with increasing local cytosolic Ca²⁺ concentration. Intriguingly, the cells overexpressing α-syn WT also display augmented TFEB nuclear localization, possibly by Ca²⁺-calcineurin activation and TFEB dephosphorylation. In summary, we show here the development and characterization of a genetically encoded SPLICSS reporter for in vitro and in vivo monitoring lysosome-mitochondria contact sites and propose that their modulation could open avenues to tune the TFEB pathway and the downstream protective cell responses, i.e., autophagy and lysosomal biogenesis, to counteract neurodegenerative processes.

### Results

**Generation and characterization of SPLICSSMT reporter for lysosomes mitochondria contact sites**

By exploiting the plasticity of split-GFP and of the second SPLICSS generation characterized by P2A peptide sequence ensuring the equimolar expression of organelle-targeted GFP fragments, we have generated a SPLICSS sensor (SPLICSSMT) to detect tethering between lysosomes and mitochondria. To achieve this goal, we have fused the β1 strand of the GFP to the C-terminus of the full-length human TMEM192 protein to target it to the lysosomal membrane. TMEM192 exposed both N- and C- termini on the cytosolic face and it is widely employed in the generation of chimeric proteins for its high specificity, ability to retain lysosomal localization upon overexpression, lack of glycosylation and perturbations of the lysosomal and cell’s physiology. Two glycine-repeat linkers of different length were introduced between the TMEM192 and the β1 strand fused moiety: the short linker was designed to detect interactions occurring over a range of ~4 nm (SPLICS), while the longer one to detect interactions at ~10 nm (SPLICSS). The non-fluorescent GFPβ1 moiety was targeted to the cytosolic face of the outer mitochondrial membrane as previously described (OMM-GFPβ1). To validate our probe HeLa cells were transfected with SPLICSSMT expressing vectors and observed under confocal microscope. An evident green fluorescence puncta pattern is appreciable from images in both short- and long-range contacts, due to the reconstitution of the two split SPLICSSMT-GFP moieties at lysosome-mitochondria (Lys-Mt) contact sites. Importantly the reconstituted GFP fluorescence colocalized with both the outer mitochondrial membrane marker (Tom20) and lysosome-associated membrane marker (LAMPI), as revealed by the immunocytochemistry analysis (Fig. 1c). The signal at the interface between lysosomes and mitochondria has been quantified as previously described and, interestingly, the number of Lys-Mt long-range contacts (Mean ± SEM: SPLICSSMT 94.7 ± 4.4 n = 35) was significantly higher respected to short-range ones (Mean ± SEM: SPLICSSMT 72 ± 4.4 n = 37; ***p < 0.001) (Fig. 1d).

The correct mitochondria targeting of the OMM-GFPβ1 moieties generated by these two probes was further verified by immunocytochemistry analysis on SPLICSSMT-GFPβ1 transfected cells using an anti-GFP antibody which detects the GFPβ1 portion and whose signal perfectly colocalized with the signal detected by an anti-Tom20 antibody (Fig. 1e).

The correct targeting of the β1 portion driven by TMEM192 was verified by the generation of a Ly-GFPβ1 construct, where the GFPβ1 portion was fused at the C-terminus of TMEM192. When the Ly-GFPβ1 construct was co-transfected with a Kateβ1 expressing vector, a typical lysosomal pattern was revealed after GFP reconstitution, which perfectly matched LAMPI signal (Fig. 1f). Similarly, when the Ly-GFPβ1 construct was transfected alone, an anti-GFP antibody stained the GFPβ1 portion whose signal perfectly colocalized with the signal detected by an anti-LAMPI antibody (Fig. 1f).
antibody, respectively, excluded the possibility that the differences in the number of contacts sites detected by the two SPLICS probes (short and long) could be dependent on differences in the protein amount of the two reporters.

Short- and long-range lysosomes–mitochondria interactions are differentially modulated by Rab7A and TBC1D15

As a proof-of-concept experiment, the flexibility of the SPLICS\textsubscript{SVT}-P2A\textsubscript{LY-MT}, in terms of ability to detect subtle changes in the number of contact sites, has been tested by altering the expression of proteins involved in the formation of Ly-Mt contacts. The data available from literature\textsuperscript{7} identified the GTP-binding protein Rab7A, enrolled on late endosomes/lysosomes, and the GTPase activating protein TBC1D15, recruited on the mitochondrial surface as the main proteins participating to Ly-Mt tethering and untethering processes (Fig. 2a)\textsuperscript{8}. In this respect, we co-transfected HeLa cells with SPLICS\textsubscript{SVT}-P2A\textsubscript{LY-MT} in presence of WT or mutated Rab7A or TBC1D15. Obtained data showed that, while the WT Rab7A affected in a significant manner the number of

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that blocks the Ly-Mt contact sites formation (Fig. 2a). Notably, also used a negative mutant, the constitutively GDP-binding Rab7A contact sites formation or their untethering, respectively (Fig. 2a). We TBC1D15 (D397A), which have been previously reported to induce (Q67L), and the constitutively inactive GTPase activating protein n.

**Mock 97.4 ± 3.9
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Rab7A (Q67L) and TBC1D15 (D397A) overexpression strongly compared to mock cells while both Rab7A (Q67L) and TBC1D15 d). It was of interest that the expression of Rab7A (T22N) was the only

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108.5 ± 7.2

of short Ly-Mt contacts, the WT TBC1D15 did not alter this interaction (Mean ± SEM; SPLICS− P2A LMVT + Mock 76.8 ± 3.5 n = 60 vs SPLICS− P2A LMVT + Rab7A 95.9 ± 9.6 n = 43 *p < 0.05; SPLICS− P2A LMVT + TBC1D15 63.5 ± 7.2 n = 33) (Fig. 2b, d). Interestingly, the number of long Ly-Mt contact sites was not affected by the overexpression of both Rab7A WT and TBC1D15 WT (Mean ± SEM; SPLICS− P2A LMVT + Mock 97.4 ± 3.9 n = 52; SPLICS− P2A LMVT + Rab7A WT 105.8 ± 6.5 n = 30 *p < 0.05; SPLICS− P2A LMVT + TBC1D5 WT 81.9 ± 4.6 n = 33) (Fig. 2b, e). To better validate the SPLICS sensitivity to lysosome-mitochondria interplay alteration, the same analysis has been performed using two mutants as positive controls, the constitutively GTP-bound Rab7A (Q67L), and the constitutively inactive GTPase activating protein TBC1D15 (D397A), which have been previously reported to induce contact sites formation or their untethering, respectively (Fig. 2a). We also used a negative mutant, the constitutively GDP-binding Rab7A (T22N) that blocks the Ly-Mt contact sites formation (Fig. 2a). Notably, the quantification of SPLICS− P2A LMVT signal has revealed that both Rab7A (Q67L) and TBC1D15 (D397A) overexpression strongly increased the mean of short contacts while, surprisingly, the negative mutant Rab7A (T22N) did not significantly change their number (Mean ± SEM; SPLICS− P2A LMVT + Rab7A (Q67L) 238.5 ± 9.8 n = 39 *p < 0.01; SPLICS− P2A LMVT + TBC1D15 (D397A) 103.2 ± 7.1 n = 38 *p < 0.01; SPLICS− P2A LMVT + Rab7A (T22N) 71.9 ± 7.7 n = 37) (Fig. 2b, d). It was of interest that the expression of Rab7A (T22N) was the only condition able to significantly decrease the Ly-Mt long contacts compared to mock cells while both Rab7A (Q67L) and TBC1D15 (D397A) did not affect them (Mean ± SEM; SPLICS− P2A LMVT + Rab7A (Q67L) 106.4 ± 6.8 n = 31; SPLICS− P2A LMVT + TBC1D15 (D397A) 108.5 ± 7.2 n = 37; SPLICS− P2A LMVT + Rab7A (T22N) 75.5 ± 5.8 n = 34 *p < 0.05) (Fig. 2b, e). No gross changes in the number and distribution of lysosomes (and mitochondria) were observed upon the overexpression of Rab7A and TBC1D15 variants as reported by LAMP1 or TOM20 immunofluorescence performed in cells co-expressing the SPLICS construct (Supplementary Fig. 1a, b) or expressing the Rab7A and TBC1D15 variants alone (Supplementary Fig. 1c, d). Fluorescence at 568 or 594 nm documented the presence of overexpressed Rab7A and TBC1D15 variants and Supplementary Fig. 1d confirmed that Rab7A and TBC1D15 variants were properly expressed. These data strengthened the reliability of SPLICS probes to detect changes in the number of Ly-Mt interactions occurring at different distances: short Ly-Mt contacts were finely sensitive to the overexpression of WT Rab7A, constitutively active Rab7A (Rab7A (Q67L)), and inactive TBC1D15 (TBC1D15 (D397A)) while the long ones were more susceptible to the presence of inactive Rab7A (Rab7A (T22N)). Radial analysis was also performed to exclude that changes in the contacts number could be due to non-specific perinuclear clustering of lysosomes and/or membrane crowding induced by Rab7 (Supplementary Fig. 2 panels a and b).

**Effects of Rab7A and TBC1D15 silencing on short- and long-range lysosomes–mitochondria contact sites**

To better evaluate the sensitivity of SPLICS− P2A LMVT to detect changes in Ly-Mt interaction, we silenced Rab7A and TBC1D15 by using two different siRNAs for each protein. Supplementary Fig. 1e, f documented that the silencing was effective for all the selected siRNAs. Interestingly, the silencing of Rab7A (Fig. 2c) did not affect short contact sites (Mean ± SEM; SPLICS− P2A LMVT + SCR 79.5 ± 5.8 n = 40; SPLICS− P2A LMVT + siRNA Rab7A #1 83.9 ± 8.1 n = 33; SPLICS− P2A LMVT + siRNA Rab7A #2 79.4 ± 6.2 n = 32; Fig. 2c, b) but instead reduced the long ones, i.e., the siRNA Rab7A #1 significantly reduced the number of long Ly-Mt contacts while a downward trend appeared evident with the siRNA Rab7A #2 (Mean ± SEM; SPLICS− P2A LMVT + SCR 107.5 ± 5.8 n = 47; SPLICS− P2A LMVT + siRNA Rab7A #1 80 ± 5.2 n = 39 *p < 0.05; SPLICS− P2A LMVT + siRNA Rab7A #2 91 ± 7.7 n = 30; Fig. 2g). Differently, the silencing of TBC1D15 significantly increased the number of short Ly-Mt interactions using both TBC1D15 siRNAs (Mean ± SEM; SPLICS− P2A LMVT + siRNA TBC1D15 #1 110.2 ± 7.1 n = 34; SPLICS− P2A LMVT + siRNA TBC1D15 #2 97.9 ± 6.3 n = 40 *p < 0.05; SPLICS− P2A LMVT + siRNA TBC1D15 #1 110.2 ± 7.1 n = 34) (Fig. 2f, g). Overall, these results suggest that our SPLICS− P2A LMVT probe is well suited to reveal differences in the formation of short and long Ly-Mt contact sites. An increase in Ly-Mt short contact sites was observed upon activation of Rab7A (by overexpressing it or by inhibiting TBC1D15), and a reduction of Ly-Mt long contact sites was detected by reducing Rab7A activity (by silencing it or by expressing a dominant negative form) suggesting the existence of a tunable regulation dependent on the degree/entity of Rab7A activity.

**Modulation of lysosome–mitochondria contact sites during autophagy and mitophagy**

To challenge whether our SPLICS− P2A LMVT probe was able to rule out some biological issues related to mitochondria and lysosome crosstalk under autophagy/mitophagy conditions, we carried out Ly-Mt contact sites quantification upon lysosomal and mitochondrial stressing conditions. HBSS and rapamycin application were used to induce cellular starvation condition (Supplementary Fig. 3) and Carbonyl Cyanide Chlorophenylhydrazone (CCCP) and oligomycin/antimycin A (OA/AA) treatment to evoke mitophagy. We found that only short Ly-Mt interactions were affected during autophagy and mitophagy activation, being their number increased significantly upon rapamycin or CCCP or OA/AA treatment (Fig. 3a, b) (Mean ± SEM; SPLICS− P2A LMVT CTRL 59.0 ± 6.7 n = 30; SPLICS− P2A LMVT + Rapamycin 118.7 ± 8.2 n = 38 *p < 0.05; SPLICS− P2A LMVT + CCCP 143.1 ± 6.7 n = 33 **p < 0.01; SPLICS− P2A LMVT + OA/AA 130.9 ± 10.4 n = 27 *p < 0.05). No effect was
observed on long Ly-Mt interactions in both autophagy and mitophagy conditions (Fig. 3c–d) (SPLICSL-P2 ALY-MT CTRL 125.1 ± 9.6 $n = 3$; SPLICSL-P2 ALY-MT + Rapamycin 128.3 ± 10.6 $n = 36$; SPLICSL-P2 ALY-MT + CCCP 150.9 ± 9.6 $n = 36$; SPLICSL-P2 ALY-MT + OA/AA 135.7 ± 10.4 $n = 31$). Curiously, the HBSS treatment did not alter either short or long Ly-Mt interactions (Mean ± SEM: SPLICSL-P2 ALY-MT + HBSS 90.2 ± 9.1 $n = 30$; SPLICSL-P2 ALY-MT + HBSS 143.5 ± 11.4 $n = 31$) (Fig. 3a–d). These data indicated that only Ly-Mt contact sites occurring at ≈4 nm are engaged during the autophagy and mitophagy activation and that our SPLICSL-P2 ALY-MT probe can discriminate the two types of contacts under these conditions.

Lysosome–mitochondria contact sites mediated Ca$^{2+}$ transfer between the two organelles

Mitochondria and lysosomes are both important and recognized players in intracellular Ca$^{2+}$ dynamics and their dysfunction has been...
reported in many disease conditions in which dysregulation of Ca²⁺ homeostasis is an observed phenotype. Pioneering studies from the Kranic group have established that Ly-Mt contacts facilitated the direct transfer of Ca²⁺ from lysosomes to mitochondria through the munc13-1 (TRPML1) lysosomal channel. Here, we take advantage of this finding to verify whether the expression of our SPLICSS-P2ALY-MT probe could impact on the formation of Ly-Mt contact sites. First, we checked whether the SPLICSS-P2ALY-MT expression could impact on mitochondrial (Fig. 3a, b) and cytosolic (Fig. 3c, d) Ca²⁺ transients induced by cell stimulation with the inositol 1,4,5 trisphosphate (InsP³)-linked agonist histamine by co-expressing the recombinant Ca²⁺ sensitive protein 1 (NPC1) inhibitor, as previously reported. Filipin III staining which an increase of lysosome-mitochondria interaction has been expected and previously reported. This cholesterol accumulation at lysosomes due to NPC1 inhibition, as previously reported, increases cholesterol accumulation in the mitochondrial matrix upon ML-SA1 stimulation with the SPLICSL-P2ALY-MT reporter and counterstained with Hoechst (488 nm), mCherry -Rab7A WT or -Rab7A Q67L or -Rab7A T22N (568 nm) and c-myc -TBC1D15 WT or -TBC1D15 D397A (594 nm) (red signal) are shown, mitochondrial or lysosomes were detected by anti-Tom20 or anti-LAMPI (405 nm) (blue and cyan fluorescence in pseudocolor, respectively). Representative Z projection images of HeLa cells transfected with SPLICSL-P2ALY-MT plus scramble or siRNA Rab7A #1 or siRNA Rab7A #2 or siRNA TBC1D15 #1 or siRNA TBC1D15 #2. Mitochondria morphology was detected by MitoTracker ® Red CMXRos upon excitation at 568 nm. a-e Quantification of short (Mean ± SEM: SPLICSL-P2ALY-MT + scramble 1.283 ± 0.055 μM: Mock 0.995 ± 0.043 M; SPLICSL-P2ALY-MT + siRNA Rab7A #1 8.38 ± 8.1; SPLICSL-P2ALY-MT + siRNA Rab7A #2 79.4 ± 6.2; SPLICSL-P2ALY-MT + siRNA TBC1D15 #1 110.2 ± 7.1; SPLICSL-P2ALY-MT + siRNA TBC1D15 #2 91 ± 7.7; SPLICSL-P2ALY-MT + siRNA TBC1D15 #3 95.3 ± 5.6 M: Mock 0.968 ± 0.047 M; Rab7A (Q67L) 1.240 ± 0.042 M; Rab7A (T22N) 1.095 ± 0.057 M: Mock 0.995 ± 0.043 M; SPLICSL-P2ALY-MT + siRNA TBC1D15 #1 1.192 ± 0.059 M; SPLICSL-P2ALY-MT + siRNA TBC1D15 #2 1.095 ± 0.057 M; SPLICSL-P2ALY-MT + siRNA TBC1D15 #3 1.192 ± 0.059 M. n= cells over 3 independent transfections. (*p < 0.05, **p < 0.01 one-way ANOVA). Source data are provided as a Source Data file.

Detection of lysosome-mitochondria contact sites in vivo
The experiments shown above, clearly demonstrated the ability of the SPLICSL-P2ALY-MT reporter to detect lysosome-mitochondria contacts and their changes in cultured cell lines. We therefore decided to test it on more polarized cells as well as in vivo. To this aim we have chosen three different approaches: (i) mouse primary cortical neurons, (ii) Rohon–Beard (RB) sensory neurons of living Zebrafish animals and (iii) wing sensory neurons of living Drosophila flies. As shown in Fig. 5a primary cortical neurons from wt mice were isolated and transfected with the SPLICSL-P2ALY-MT reporter and counterstained with Hoechst (405 nm) to detect nuclei and β-tubulin III (647 nm) as neuronal marker. Ly-Mt contact sites were clearly evident in the soma (Fig. 5a, red arrowheads) and in the axon (Fig. 5a, white arrows) of the transfected neuron. Interestingly, quantification of the number of Ly-Mt contacts revealed that most of them are present in the soma (Fig. 5b, Mean ± SEM: SPLICSL-P2ALY-MT 20.19 ± 2.17 M = 34, soma; 0.23 ± 0.035 M = 34, 0.589 ± 0.025 M = 16) (Fig. 4k, l); intriguingly, under the same conditions, we detected a significant increase in mitochondrial Ca²⁺ transients upon stimulation with ML-SA1 (Mean ± SEM, μM: CTRL 0.968 ± 0.047 M = 32; U8666A 1.240 ± 0.042 M = 32 **p < 0.0001) (Fig. 4m, n). These results support the existence of localized Ca²⁺ microdomains generated upon ML-SA1 induced Ca²⁺ release from lysosomes that are sensed by mitochondria and depend on lysosome-mitochondria tethering. This observation is very challenging since it is well established that lysosomes and mitochondria are in contact, but little is known on the functionality of these contacts. Considering this and the finding that Rab7A and TBC1D15 manipulation modulated the number of Ly-Mt contact sites (see above and Fig. 2), we explored their possible effect on lysosomal-mitochondrial Ca²⁺ transfer. Notably, the overexpression of Rab7A (Q67L) and TBC1D15 (D397A) mutants induced a strong increase in mitochondrial Ca²⁺ transients, while the wt forms and Rab7A (T22N) did not induce any changes (Mean ± SEM, μM: Mock 0.995 ± 0.043 M = 38; Rab7A WT 1.060 ± 0.045 M = 54; Rab7A (Q67L) 1.211 ± 0.042 M = 60 **p < 0.01; Rab7A (T22N) 1.125 ± 0.039 M = 60; TBC1D15 WT 1.012 ± 0.041 M = 60; TBC1D15 (D397A) 1.234 ± 0.057 M = 54 **p < 0.01) (Fig. 4o, p). Similarly, the silencing of TBC1D15 with two different siRNAs caused a significant Ca²⁺ transient rise, while the silencing of Rab7A protein did not alter them (Mean ± SEM, SCR 0.955 ± 0.044 M = 38; siRNA Rab7A #1 1.059 ± 0.057 M = 36; siRNA Rab7A #2 1.072 ± 0.045 M = 38; siRNA TBC1D15 #1 1.192 ± 0.059 M = 42 p < 0.01; siRNA TBC1D15 #2 1.193 ± 0.075 M = 39 p < 0.05) (Fig. 4q, r). Taken together, these data indicate that Ca²⁺ transfer from lysosomes to mitochondria is strictly dependent on their tethering and on the contact sites occurring in the short 4 nm range.
axon). Live imaging of Ly-Mt contact sites in zebrafish was performed by injecting the pT2-DsRed-UAS-SPLICSS-P2ALY-MT construct into fertilized eggs of s1022t:GAL4 fish, selectively expressing GAL4 in the RB neurons. At 24 hpf high-resolution images of anesthetized embryos were acquired in a confocal microscope. As shown in Fig. 5c, Ly-Mt contacts appeared either in the soma (red arrowheads) or distributed in the axons of RB neurons (white arrows) marked in red thanks to the expression of a cytosolic DsRed. We were not able to reliably quantify contacts in the soma since their high density makes the quantification potentially underestimated, axonal contacts in 50 μm of axonal tracts were instead quantified (Fig. 5d, Mean ± SEM: SPLICSS-P2A\textsubscript{LY-MT} 24.53 ± 2.607 n = 15). Finally, we also generated transgenic flies expressing the SPLICSS-P2A\textsubscript{LY-MT} under control of an UAS enhancer sequence. Figure 5e shows the pattern of probe expression under...
control of the pan neuronal driver Appl-Gal4. Ly-Mt contact sites were clearly evident in the soma (red arrowheads) and in the axon (white arrows). Also in this case those in 50 μm of axon could be reliably quantified (Fig. 5g) (Mean ± SEM: SPLICSS-P2A1VMT 18.81 ± 0.6471 n = 36). Altogether these experiments demonstrate that the SPLICSS-P2A1VMT can efficiently detect Ly-Mt contact sites in vivo.

**α-Synuclein decreases the number of short lysosome-mitochondria contact sites**

Once established that our tool was reliable and powerful in monitoring changes in lysosome-mitochondria contact sites we decided to investigate an important biological issue. Considering that defects in lysosome and mitochondria function and communication have been reported to occur in neurodegenerative processes we decided to explore whether the manipulation of the PD-related protein α-syn could impact on Ly-Mt tethering and Ly-Mt Ca2+ transfer. We had previously demonstrated that α-syn plays a role in ER-mitochondria tethering and thus impacts on Ca2+ transfer and cell bioenergetics. Here we investigated the role of α-syn WT and its two mutants, A30P and A53T, on Ly-Mt interactions by co-expressing them with the SPLICSS-P2A1VMT. We focused on contact sites occurring in the short-range since, based on our data, they are those involved in the process of Ca2+ transfer from lysosome to mitochondria. Intriguingly, we found that α-syn WT, A30P and A53T drastically decreased the number of short contact sites compared to Mock cells (Mean ± SEM: SPLICSS-P2A1VMT + Mock 87.84 ± 4.46 n = 64; SPLICSS-P2A1VMT + WT 30.67 ± 2.77 n = 50; SPLICSS-P2A1VMT + A30P 44.60 ± 3.39 n = 70; SPLICSS-P2A1VMT + A53T 56.35 ± 2.69 n = 69; ***p ≤ 0.0001) (Fig. 6a, b). To exclude that α-syn overexpression could modify the total lysosomal content, and eventually impact on Ly-Mt short contact sites for this reason, we quantified lysosomes abundance by immunocytochemistry and Western blotting analysis upon their labeling with an anti-LAMPI antibody (Fig. 6c–f). The results of this analysis revealed no differences in the lysosomal pool under the different conditions (Mean ± SEM: Mock 92.04 ± 2.77 n = 37; WT 94.41 ± 4.66 n = 27; A30P 89.92 ± 6.63 n = 26; A53T 115.7 ± 11.29 n = 16). Then, to evaluate whether the decrease in Ly-Mt interactions observed in α-syn overexpressing cells could impact on mitochondrial Ca2+ transients upon TRPML1-mediated lysosomal Ca2+ release, we co-transfected HeLa cells with wt mitochondrial aequorin and monitored Ca2+ concentration in the mitochondrial matrix upon ML-SAI stimulation. The results suggest that the overexpression of α-syn WT (documented in Supplementary Fig. 4) slightly but not significantly decreased the mitochondrial Ca2+ uptake in these conditions (Mean ± SEM, μM: Mock 1.230 ± 0.069 n = 37; α-syn WT 1.130 ± 0.059 n = 39) (Fig. 6g, h). Since ML-SAI has been reported to induce Ca2+ entry from the extracellular ambient via SOCE activation and α-syn as well has been reported to impact on Ca2+ entry to better appreciate the contribution of Ca2+ release from lysosomes we performed the same experiment in the absence of extracellular Ca2+ (Fig. 6i). Notably, the mitochondrial Ca2+ transients generated by ML-SAI in KRB medium containing 100 μM EGTA were reduced in respect to those generated in the presence of extracellular Ca2+, i.e., they are in the range of 0.5 μM. Under these conditions it was possible to detect that in cells overexpressing α-syn WT mitochondrial Ca2+ levels were significantly reduced compared to control cells (Mean ± SEM μM: Mock 0.46 ± 0.03 n = 10; α-syn WT 0.34 ± 0.03 n = 10; *p ≤ 0.01) (Fig. 6j, l). To exclude the possibility that the observed differences were due to differences in Ca2+ release from InsP3-sensitive intracellular compartments, after ML-SAI stimulation, the cells were stimulated with histamine. As expected, upon histamine addition mitochondrial Ca2+ uptake was observed, but no differences were detected between control and α-syn WT overexpressing cells (Mean ± SEM, μM: Mock 5.13 ± 0.83 n = 9; α-syn WT 5.35 ± 0.28 n = 9) (Fig. 6k). Altogether these results suggested that α-syn WT affects mitochondrial Ca2+ release, thus possibly interfering with the dissociation of cytosolic microdomains of local Ca2+ concentration generated by the opening of TRPML1 channel.

**α-Synuclein overexpression counteracts the increase of Ly-Mt short interactions induced by lysosomal cholesterol accumulation**

Considering that both dysregulation in cholesterol flux and α-syn clearance play key roles in PD pathogenesis and that, based on our findings, α-syn reduces, but cholesterol accumulation increases, the interplay between lysosomes and mitochondria, we analyzed the Ly-Mt interactions by combining the two conditions. HeLa cells were co-transfected with α-syn WT or A30P/A53T mutants and SPLICSS-P2A1VMT and then, incubated with UI8666A. According to the results reported above (see Fig. 4h, l), UI8666A treatment drastically increased the short contacts in control cells (Mean ± SEM: Untreated SPLICSS-P2A1VMT + Mock 60.37 ± 4.73 n = 43; UI8666A treated SPLICSS-P2A1VMT + Mock 83.49 ± 4.09 n = 39; ***p ≤ 0.0001, Fig. 7a, b), however, its action was completely blunted in presence of overexpressed α-syn WT, A30P and A53T (Mean ± SEM: Untreated: SPLICSS-P2A1VMT + WT 35.15 ± 3.83 n = 31; SPLICSS-P2A1VMT + A30P 38.07 ± 3.78 n = 36; SPLICSS-P2A1VMT + A53T 31.83 ± 3.39 n = 38; UI8666A treated: SPLICSS-P2A1VMT + WT 41.06 ± 4.09 n = 36; SPLICSS-P2A1VMT + A30P 43.38 ± 3.30 n = 31; SPLICSS-P2A1VMT + A53T 38.08 ± 3.37 n = 38) (Fig. 7a, b). Filipin III staining revealed that UI8666A treatment induced a strong cholesterol accumulation at lysosomes due to NPC1 inhibition regardless of the presence of α-syn WT (Supplementary Fig. 5). Notably, no relevant differences were reported comparing the wild-type form of α-syn with the two mutants. To elucidate functional consequences, we decided to evaluate the mitochondrial Ca2+ transients upon TRPML1-mediated lysosomal Ca2+ release, the overexpression of α-syn WT decreases mitochondrial Ca2+ transients even in the cells incubated with UI8666A (Mean ± SEM, μM: Mock 1.954 ± 0.116 n = 25; α-syn WT 1.567 ± 0.082 n = 39; **p ≤ 0.01) (Fig. 7c, d). These data demonstrated that α-syn WT abolished the upregulation of Ly-Mt interactions induced by lysosomal cholesterol accumulation and this was also reflected in a reduced mitochondrial Ca2+ uptake. Notably, α-syn WT impinged on Ly-Mt tethering also under HBSS induced starvation, which per se does not impact on Ly-Mt contact sites.
contact sites and α-syn overexpressing cells, upon HBSS treatment displayed an impaired autophagic capacity that was not observed under basal condition (Supplementary Fig. 6).

**α-Synuclein is involved in TFEB cellular localization**

In many forms of PD, accumulation of α-syn, which in turn leads to its improper aggregation, is a common hallmark for the disease progression. In this respect, lysosomal activity carries out a crucial role in controlling the clearance of α-syn meaning that lysosomal biogenesis could be stimulated to sustain this demand. The Transcription Factor EB (TFEB) is a master gene for lysosomal biogenesis sensitive to cellular homeostasis alterations such as amino acids deprivation and TFEB-mediated autophagy has been reported to rescue midbrain dopamine neurons from α-syn toxicity. Under
physiological conditions the mammalian target of rapamycin complex 1 (mTORC1) phosphorylates the cytosolic TFEB on the lysosomal membrane, inhibiting its transscriptional activity. mTORC1 inactivation reduces the phosphorylated TFEB pool, and meanwhile, the activation of the Ca2+-sensitive phosphatase calcineurin contributes to dephosphorylate TFEB inducing its nuclear translocation and activation. Calcineurin activation is directly dependent on lysosomal Ca2+ release, which can locally mediate TFEB dephosphorylation and activate it. Furthermore, it has been recently shown that lysosomal Ca2+ release also promotes specific inactivation of Rag/C/D GTPases, thus leading to TFEB nuclear translocation via impairment of mTORC1-mediated phosphorylation and calcineurin activation. Considering that in the presence of α-syn the amount of Ca2+ taken up by the lysosomal membrane is essentially unaffected, the same phenotype could be observed upon α-syn WT induced previous findings and further supporting the reliability of the SPLICS reporters, leaving the long-range interactions essentially unaffected. The same phenotype could be observed upon downregulation of the endogenous un tethered TBC1D15, while decreased levels of endogenous Rab7 showed no significant effect on either short- and long-range contact sites, suggesting that the remaining pool of Rab7 protein could be sufficient to support the tethering. Short-range lysosome-mitochondria contact sites were increased upon mitophagy induction with CCCP or oligomycin/antimycin treatment while rapamycin, but not HBSS, treatment only slightly increased them suggesting the intriguing possibility that different starvation conditions could well result in different engagement of this contact interface, which is also different from the one engaged under basal conditions. Long-range lysosome-mitochondria contact sites remained unaltered under the same conditions, suggesting that these two types of contact sites may respond differently to the same stimuli, implying their involvement in different functional pathways. Functional assessment of local lysosome-to-mitochondria Ca2+ transfer revealed no artificial tethering caused by the SPLICS reporters, rather confirming the action of the above-mentioned tethers/un tetheres, which exquisitely mirrored the foreseen action on the lysosome-
mitochondria contact sites. Accordingly, global and local Ca\textsuperscript{2+} handling was not affected by SPLICS reporters expression (Fig. 4a–f) per se, while the promotion of the lysosome-mitochondria interface by U18666A or by the overexpression of the constitutively active Rab7 (Q67L)-GTP mutant or the TBCID15 (D397A) GAP-domain mutant, enhanced the amount of Ca\textsuperscript{2+} sensed by mitochondria upon its release from lysosomes (Fig. 4m–p). According to the data above, down-regulation of Rab7 didn’t show any effect on lysosome-to-mitochondria Ca\textsuperscript{2+}-transfer while knockdown of the untether protein TBCID15 resulted in increased Ca\textsuperscript{2+} transient (Fig. 4q, r). Of note, U18666A treatment, selectively impinged on short-range lysosome-mitochondria contacts leaving unaffected the long-range interactions probably indicative of a sterol-transfer mediated by very close contact at the basis of the observed phenotype and further strengthening the ability of the SPLICS reporters in specifically labeling short and long contact sites. Our in vivo data also demonstrated that our SPLICS...
reporter can be used in living Zebrafish and Drosophila models to study the role and the function of the lysosome-mitochondria interface in both physiological and pathological relevant context. Lastly, the availability of a reporter to monitor mitochondrial-lysosomes tethering allowed us to identify the PD-related protein α-synuclein as a player in the regulation of this interface and to show that the reorganization of lysosome-mitochondria interface represents an early response to increased α-synuclein expression which in turn results in the calcium-dependent activation of TFEB. In agreement with our findings, TFEB nuclear translocation via calcineurin- and Rag GTPase-dependent pathways by TRPML1-mediated lysosomal Ca2+ release and early TFEB nuclear translocation upon α-syn overexpression in

Fig. 6 | Effects of α-synuclein overexpression on lysosomes–mitochondria contacts. a Representative Z-projection images of HeLa cells expressing SPLICS-P2A'Lys-MT alone (Mock) or co-expressing SPLICS-P2A'Lys-MT and α-Syn WT or α-Syn A30P or α-Syn A53T. SPLICS-P2A'Lys (488 nm), α-synuclein detected by anti-α-synuclein (633 nm) is visible in the merge panels. b Quantification of short LY-MT contacts (Mean ± SEM: SPLICS-P2A'Lys-MT + Mock 87.84 ± 4.46 n = 64; SPLICS-P2A'Lys-MT + WT 50.67 ± 2.77 n = 50; SPLICS-P2A'Lys-MT + A30P 54.60 ± 3.39 n = 70; SPLICS-P2A'Lys-MT + A53T 56.35 ± 2.69 n = 69), representative Z-projection images of HeLa cells transfected with pcDNA3 (Mock) or α-Syn WT or α-Syn A30P or α-Syn A53T. Lysosomes were stained by anti-LAMP1 (488 nm) and α-synuclein was detected by anti-α-synuclein (633 nm) and visible in the merge panels. d Quantification of LAMP1 dots per cell. The SPLICS dots and LAMP1 signal were quantified from the 3D rendering of a complete Z-stack, mean ± SEM (Mock 92.04 ± 7.27 n = 37; WT 94.41 ± 4.66 n = 27; A30P 89.92 ± 6.63 n = 26; A53T 115.7 ± 11.29 n = 16). e Western blotting analysis and quantification of LAMP1 expression in Mock, or α-Syn WT or α-Syn A30P or α-Syn A53T transfected cells. Equal amount of total loaded lysate was verified by incubation with anti-β actin antibody (Mock 1 ± 0.51, WT 1.66 ± 0.73, A30P 1.05 ± 0.26, A53T 1.31 ± 0.50). Data were obtained from 3 independent experiments. g, h Mitochondrial Ca2+ transients generated upon ML-SA1 stimulation in HeLa cells expressing mtAeqwt alone (Mock) or co-expressing mtAeqwt and α-Syn WT and average peak values, mean ± SEM: [Ca2+]mt, μM Mock 1.230 ± 0.069 n = 37; α-syn WT 1.130 ± 0.059 n = 39, I Mitochondrial Ca2+ transients in HeLa cells expressing mtAeqwt alone (Mock) or co-expressing mtAeqwt and α-Syn WT. The cells were first stimulated with ML-SA1 in a Ca2+ free KRB supplemented with 100 μM EGTA to release Ca2+ from lysosomes and then with histamine to release Ca2+ from the ER. The inset shows the Ca2+ transients induced by ML-SA1 application at a higher magnitude. j Average peak values upon ML-SA1 stimulation were graphed as mean ± SEM: [Ca2+]mt, μM Mock 0.46 ± 0.03 n = 10; α-syn WT 0.34 ± 0.03 n = 10) or (k) histamine stimulation were graphed as mean ± SEM: [Ca2+]mt, μM Mock 5.13 ± 0.83 n = 9; α-syn WT 5.55 ± 0.28 n = 9. Scale bar 10 μm. n = cells (b, d) or coverslips (h, j, k) examined over 3 independent experiments. **p ≤ 0.01 unpaired two-tailed t test, ****p ≤ 0.0001 one-way ANOVA. Source data are provided as a Source Data file.

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dopaminergic neurons have been previously reported, but no explanation for the signal "used" by α-syn to induce this response. We are aware that our data were obtained in HeLa cells, which do not represent a very relevant model to study α-syn-induced pathology, but the strong inhibitory effect of α-syn WT or α-syn A30P or α-syn A53T mutants on lysosome-mitochondria tethering, and on Ca\(^{2+}\) transfer into mitochondria is very interesting. All in all, our data support a model which proposes a role for α-syn as modulator of the lysosome-mitochondria interface and lysosomes-to-mitochondria Ca\(^{2+}\) transfer. Reduced mitochondrial Ca\(^{2+}\) uptake due the removal of lysosome-mitochondria tethering will result in perturbations of Ca\(^{2+}\)-microdomains near lysosomes with consequent calcineurin activation.

**Fig. 7 | Effects of α-synuclein overexpression on LY-MT contacts under cholesterol accumulation.** a Representative Z-projection images of HeLa cells transfected with SPLICS\(_{\alpha}\) P2A\(^{\alpha\text{MT}}\) (Mock) or co-transfected with SPLICS\(_{\alpha}\) P2A\(^{\alpha\text{MT}}\) and α-Syn WT or α-Syn A30P or α-Syn A53T in untreated condition or under U18666A treatment. SPLICS\(_{\alpha}\) P2A\(^{\alpha\text{MT}}\) was represented by fluorescent "dots" upon excitation at 488 nm (green) and α-synuclein was detected by anti-α-synuclein upon excitation at 633 nm (red) and shown in the merge panels. b Quantification of short LY-MT contacts. The SPLICS\(_{\alpha}\) P2A\(^{\alpha\text{MT}}\) MT contacts were quantified from the 3D rendering of a complete Z-stack, mean ± SEM Untreated: SPLICS\(_{\alpha}\) P2A\(^{\alpha\text{MT}}\) + Mock 60.57 ± 4.73 n = 43, SPLICS\(_{\alpha}\) P2A\(^{\alpha\text{MT}}\) + WT 35.15 ± 3.83 n = 31; SPLICS\(_{\alpha}\) P2A\(^{\alpha\text{MT}}\) + A30P 38.07 ± 3.78 n = 36; SPLICS\(_{\alpha}\) P2A\(^{\alpha\text{MT}}\) + A53T 31.83 ± 3.39 n = 38. U18666A treated: SPLICS\(_{\alpha}\) P2A\(^{\alpha\text{MT}}\) + Mock 83.49 ± 4.09 n = 39, SPLICS\(_{\alpha}\) P2A\(^{\alpha\text{MT}}\) + WT 41.06 ± 4.09 n = 36; SPLICS\(_{\alpha}\) P2A\(^{\alpha\text{MT}}\) + A30P 43.58 ± 3.30 n = 31; SPLICS\(_{\alpha}\) P2A\(^{\alpha\text{MT}}\) + A53T 38.08 ± 3.37 n = 38. c, d Mitochondrial Ca\(^{2+}\) transients generated upon ML-SA1 stimulation in HeLa cells treated with U18666A and cotransfected with mtAeqwt and pcDNA3 (Mock) or α-Syn WT (mean ± SEM: [Ca\(^{2+}\)]\(_{\text{mt}}\), μM Mock 1.954 ± 0.116 n = 25; α-syn WT 1.567 ± 0.082 n = 39). Scale bar 10 μm. n = cells (b) or coverslips (d) examined over 3 independent experiments. (****p ≤ 0.0001 two-way ANOVA) (**p ≤ 0.01, unpaired two-tailed t test). Source data are provided as a Source Data file.
and TFEB translocation according to the model we propose in Fig. 8g). This action could be an early signal used by the cells to activate the response of protective autophagic pathway and counteract further accumulation of α-syn, that however, when excessive turns out in cytosolic TFEB sequestration and impinges on autophagic response, as proposed by Decressac et al. Overall, our study reports the generation and experimental validation of a genetically encoded SPLICS probe to be used for in vitro and in vivo applications. It unravels the existence of at least two types of contact sites between mitochondria and lysosomes that are functionally distinct. Now, the challenge is to understand the mechanisms by which they are regulated and the physiopathological implications of their modulation. It will also be important to explore their role in vivo as well as to set up high throughput screening of compounds and/or players at the interface in
order to open avenues to prevent or delay debilitating diseases by targeting this local pathway.

Methods

Ethical statement

The research complies with all relevant ethical regulations. All experiments on mice were conducted according to the Italian Ministry of Health and the approval by the Ethical Committee of the University of Padova (Protocol Permit #690/2020-PR). No ethical committee is required for D. rerio experiments at 24 hpf (University of Padova and Italian Ministry of Education and Research) and for D. melanogaster experiments at Kings College (not classified as protected species under the UK ‘Animals Scientific Procedures Act (1986)’).

Cloning and fusion plasmid construction

To generate SPLICSS-L-P2A14,15-MT, simultaneously expressing (through a 2A peptide) lysosomal β1 short (Ly1; β1L) and outer mitochondrial membrane GTPase-dependent pathways and consequent TFEB nuclear translocation. Scale bar 10 μm. The data were obtained from at least 2 independent transfections. **p ≤ 0.01, unpaired two-tailed t test. Source data are provided as a Source Data file.

Cell Culture

HeLa ATCC cells and HeLa cells stably expressing TFEF-GFP, were grown at 37 °C in a 5% CO2 atmosphere in Dulbecco’s modified Eagle medium (DMEM) high glucose (Gibco; 41966-029), supplemented with 10% Fetal Bovine Serum (FBS) (Gibco; 10270-106), 100 μ/mL penicillin and 100 mg/ml streptomycin (Penicillin–Streptomycin solution 100X) (EuroClone; ECB3001D). All treatments were performed after transfection; particularly, U18666A (Sigma-Aldrich; 66215) treatment was performed for eighteen hours with 2 μg/ml vehicle (water) was added in untreated cells as control. Starvation experiments were performed incubating HeLa cells for three hours in Hanks’ Balanced Salt Solution (HBSS) (Gibco; 14025-050) adding in the grown medium 1 μM rapamycin (Sigma-Aldrich; 37094) for five hours. CCCP (Sigma-Aldrich; C2739) treatment was performed for three hours with 10 μM while oligomycin/antimycin A (Sigma-Aldrich; A6754) were both used at 10 μM for one-half-hour. Vehicle (DMSO) was added in untreated cells as control. Primary mouse cortical neurons were isolated and transfected as previously reported71. Primary mouse cortical neuronal cultures were obtained from post-natal day 0 pups by papain dissociation of the brain tissue and maintained as previously described70. At days-in-vitro 4, neurons plated in 24-well plates were transfected with lipofectamine (Lipofectamine 2000, Invitrogen) in a 1:2 ratio with DNA (w/w). The transfection was carried in Opti-MEM ( Gibco Life Technologies) for 45 min. Transfected neurons were then maintained in culture for additional 3–5 days prior to fixation. Four independent cultures and transfection were performed.

Transfection

Twenty hours before transfection, HeLa cells were seeded at 40–50% confluence onto 13 mm diameter glass coverslips (for immunofluorescence analysis) or 60–70% in 6-well plate (for Western blotting analysis). HeLa cells were transfected by standard Ca2+ phosphate protocol that was previously reported71. Briefly, for one 13 mm coverslip, 5 μl of 2.5 M CaCl2 (Sigma-Aldrich; Cat# C-5080) was added to 2 μg of total SPLICSS-L-P2A14,15-MT DNA dissolved in Milli-Q H2O to reach a final volume of 50 μl. CaCl2-DNA solution was added drop by drop to 50 μl HEPES Buffered Solution 2X (HBSS 2X; 280 mM NaCl, 50 mM HEPES, 1.5 mM Na2HPO4·7H2O (Sigma-Aldrich; Cat# S9390), pH 7.12) and incubated 30 min at room temperature. Before transfection, the growth medium was replaced with fresh medium. Eight hours after transfection cells were washed three times with Dulbecco’s Phosphate Buffered Saline (D-PBS) (EuroClone; Cat# EB40040L) to remove excess of Ca2+ phosphate precipitates and fresh medium was replaced, incubating cells for additional thirty-six hours. In co-transfection experiments, SPLICSS-L-P2A14,15-MT were co-transfected in a 1:1 ratio for mCh-Rab7AWT, mCh-Rab7A(Q67L) mCh-Rab7A(T22N), pEF6-myc-TBC1D15(D397A) using QuikChange® XL mutagenesis kit (For. CGATCTG ACTGGAACGGTTCCAGT for mCh-Rab7A(Q67L), (For. CTGGTTCATGAAACCAGGCAACGGCAGCGGCGGCA CGGCGCGGACCACTAGTGGTCAAGCTGACAGAGTACGTGAACGCCGCTGG CATCACAGGCCCCGGG). The PCR product was purified using the GenElute Gel Extraction Kit (Sigma), digested with BamHI and Smal and then ligated into the pcDNA3-SPLICSS-L-P2A14,15-MT vector digested with the same restriction enzymes34,36. Lysosomal β1L short (Ly1; β1L) were designed and obtained by custom gene synthesis (Vectorbuilder). Ly1; β1L was designed into the pcDNA3-SPLICSS-L-P2A14,15-MT vector digested with BamH and Smal enzymes and then ligated. mCh-Rab7A (mCherry- Rab7A) (Plasmid #61804), pEF6-myc-TBC1D15 (c-myc- TBC1D15) (Plasmid #79148) were purchased from Addgene. mCh-Rab7A plasmid was mutagenized to obtain mCh-Rab7A(WT) and empty vector in the same respectively. 

Fig. 8 | TFEB subcellular localization under α-synuclein overexpression.

a Representative single-plane images of HeLa cells expressing TFEF-GFP or co-expressing TFEF-GFP and α-Syn WT. b Quantification of nuclear TFEF-GFP intensity signal, mean ± SEM: Ctrl 0 ± 0 n = 7, TFEF-GFP 79.76 ± 8.07 n = 8 cells from 2 independent experiments. c Representative single-plane images of HeLa cells stably expressing TFEF-GFP and transiently transfected with pcDNA3 (Mock) or α-Syn WT. d Quantification of nuclear TFEF-GFP intensity signal. TFEF was represented by GFP fluorescence upon excitation at 488 nm (green) and α-synuclein was detected by anti-α-synuclein upon excitation at 633 nm (red); mean ± SEM: Ctrl 0 ± 0 n = 7, TFEF-GFP 69.44 ± 9.32 n = 9 cells from 2 independent experiments. e Representative confocal images of HeLa cells stably expressing TFEF-GFP, transfected with α-Syn WT and left untreated or treated with BAPTA-AM (20μM, 6 h), quantification of the nuclear TFEF signal is shown in (f) mean ± SEM: Untreated 88.33 ± 7.86 n = 10, BAPTA AM 0 ± 0 n = 11 cells from 3 independent experiments. g Proposed model: under basal conditions Ca2+ released from lysosomes is taken up by mitochondria in close proximity, thus reducing calcineurin activation and TFEF nuclear translocation. The unthethering of the Ly-Mt contacts by α-synuclein potentially increases the local Ca2+ concentration due to reduced buffering action by mitochondria, thus leading to increased local activation of calcineurin- and Rag GTPase-dependent pathways and consequent TFEF nuclear translocation. Scale bar 10 μm. The data were obtained from at least 2 independent transfections. **p ≤ 0.01, unpaired two-tailed t test. Source data are provided as a Source Data file.
Briefly, HeLa cells were seeded in a 6-well plate at 70–90% of confluence and transfected with Lipofectamine 3000 Transfection Reagent plus 1.5 μg SPLIC5a–P2A1–MT and 60 pmol of different siRNAs. A scramble siRNA was used as a control. Thirty-six hours after transfection cells were washed and re-plated at 40–50% confluence onto 13 mm diameter glass coverslips twenty hours before transfection. For one 13 mm coverslip, 25 ng of EGFP-TFEB plasmid and 300 ng of the empty vector were transfected in the same conditions. Before transfection, HeLa cells were seeded at 50,000 cells/mL. They were then processed using the Quantification 1 plugin (https://github.com/titocali1/Quantification-Plugins). A 3D reconstruction of the resulting image was obtained using the VolumeJ plugin (https://github.com/titocali1/Quantification-Plugins). A selected face of the 3D rendering was then thresholded and used to count short and long contact sites through the “Quantification 2” plugin (https://github.com/titocali1/Quantification-Plugins). Radial analysis was performed as described in§ by using the Radial Profile class plugin of ImageJ software (https://imagej.net/plugins/radial-profile.html).

Western blotting
Transfected cells were recovered from 6-well plate and cellular extracts were western blotting to evaluate GFP, TMEM192, LAMP1, Rab7A or TBC1D15 proteins expression. Cells were lysed in RIPA Buffer (50 mM Tris-HCl pH 7.4, NaCl 150 mM, 1% Triton X-100, 0.5% sodium deoxycholate, 10 mM EDTA, 0.1% SDS, 1 mM DTT, 2X Protease Inhibitor Cocktail (Sigma, Cat. # P8340)) for 20 min. Supernatants were collected after 20 min centrifugation at 16,000 x g at 4 °C. Extracted proteins were quantified by Bradford assay (Bio-Rad, Cat# 500-0025), resolved on SDS-PAGE in 12% SDS-PAGE Tris-HCl polyacrylamide gel, and then transferred to PVDF membranes (BioRad) using Trans-Blot™ Turbo™ Transfer System (BioRad). Membranes were blocked with 5% w/v non-fat dried milk (NFDM) in TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) and incubated overnight with the specific primary antibody at 4 °C. Signal was detected by incubation with secondary horseradish peroxidase-conjugated anti-rabbit (Santa Cruz, sc-2004) or anti-mouse (Santa Cruz, sc-2005) IgG antibodies for 1 h. At room temperature followed by incubation with the chemiluminescent reagent Luminata Classic HRP substrate (Merck Millipore, Cat# WBLU050). The following primary antibody were used: monoclonal anti-GFP 1L000 (Santa Cruz, Cat. Sc-9996), monoclonal anti-TMEM192 1L000 (Abcam, Cat. ab156737), monoclonal anti-tubulin 1L000 (Cell Signalling, Cat. #2146), monoclonal anti-LAMP1 (Santa Cruz, Cat. Sc-0011), monoclonal anti-beta actin (Sigma-Aldrich, Cat. A353), monoclonal anti-Rab7A 1L000 (Sigma-Aldrich, Cat. HA0078), monoclonal anti-TBC1D15 1L000 (Sigma-Aldrich, Cat. HPA01338), monoclonal anti-GAPDH 1L000 (Cell Signalling, Cat. 2128), monoclonal anti-vinculin 1L000 (Sigma-Aldrich, Cat. V9264).

Aequorin measurements
For Ca2+ measurements, cells were co-transfected with low-affinity mitochondrial aequorin (mTaqemt) or high-affinity mitochondrial aequorin (mtAeqwt) or cytosolic aequorin (cytAEQ) and SPLIC5a–P2A1–MT or α-synuclein WT plasmid following standard Ca2+ phosphate protocol. Briefly, cells were seeded at 40–50% confluence onto 13 mm diameter glass coverslips and twenty hours later cells were transfected adding 5 μl of 2.5 M CaCl2 (Sigma-Aldrich; Cat# C-5080) to 2.5 μg of total DNA (2:1 ratio favoring pcDNA3.1(+) empty vector or SPLIC5–P2A1–MT/α-synuclein WT protein expressing plasmids) dissolved in Milli-Q H2O to reach a final volume of 50 μl of HBS 2X. Eight hours after transfection cells were washed three times with Dulbecco’s Phosphate Buffered Saline (D-PBS) (EuroClone; Cat# ECOB4004L) to remove excess of Ca2+ phosphate precipitates and fresh medium was replaced for additional thirty-six hours. Forty-eight hours post-transfection, mitochondrial high affinity aequorin (mTaqemt) was reconstituted by incubating cells for 1 h with 5 μM WT coelenterazine (Invitrogen; C2944) in KR medium supplemented with 0.1% glucose and 1 mM CaCl2 (Sigma-Aldrich; 2111S) at 37 °C in a 5% CO2 atmosphere. After reconstitution, cells were transferred to the chamber of luminometer, and Ca2+ transient were measured by perfusion in KR medium added with 1 mM CaCl2 and 50 mM ML-SA1 (Sigma-Aldrich; SML0627-25MG) for SPLIC5–P2A1–MT transfected cells or 100 μM EGTA (Sigma-Aldrich; E-4378), 1 mM CaCl2, 100 μM Histine, 50 mM ML-SA1 (Sigma-Aldrich; SML0627-25MG) for α-synuclein WT transfected cells. Finally, cells were lysed with 100 μM digitonin in a hypotonic CaCl2 rich solution (10 mM CaCl2 in H2O) to displace the remaining 

Immunocytochemistry
Forty-eight hours post transfection cells, plated on 13-mm glass coverslips, were fixed for 20 min in a 3.7% (vol/vol) formaldehyde solution (Sigma-Aldrich; Cat# F5620). Cells were then washed three times with D-PBS (Euroclone). Cell permeabilization was performed by 10 min incubation in 0.3% Triton X-100 Bio-Chemica (PanReac AppliChem; A1388) in D-PBS, followed by three times washes in 1% gelatin/D-PBS (Type B from bovine skin) (Sigma-Aldrich; G9382) for 15 min at room temperature (RT). The coverslips were then incubated for 90 min at RT with the specific primary antibody diluted in D-PBS (50 ng anti-GFP; Santa Cruz, Cat# sc-9996, 150 ng α-synuclein WT plasmid and pcDNA3(+) empty vector in the same respectively conditions. Before transfection, the growth medium was replaced with fresh medium.

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reconstituted active aequorin pool. For cytosolic and mitochondria Ca\(^{2+}\) measurements under U18666A treatment or in presence of mCh-Rab7AWT or mCh-Rab7A(Q67L) or mCh-Rab7A(T22N) or pEF6-myc-TBC1D15WT or pEF6-myc-TBC1D15 (D397A) or α-synuclein WT, cells were co-transfected with cytosolic aequorin (cytAEQ) or wt mitochondrial aequorin (mtAEQwt) and pcDNA3.1(+) empty vector or protein expressing plasmids in a 1:2 ratio using Lipofectamine 2000 Transfection Reagent (Invitrogen, Cat. 11660-019) in accordance with the manufacturer’s instructions. Briefly, HeLa cells were seeded in a 6-well plate at 70–90% of confluence and transfected with Lipofectamine 2000 Transfection Reagent plus 1 μg mtAEQwt or cytAEQ and 2 μg of different DNA plasmids except for α-synuclein WT where 1 μg was used, pcDNA3.1(+) empty vector was used as a control. Six hours after transfection cells were washed and re-plated into a 96-well plate (Corning®; Cat# 3610) (for Ca\(^{2+}\) analysis) or in 12-well plates (for Western blotting analysis). For Ca\(^{2+}\) measurements in presence of Rab7A or TBC1D15 siRNAs, cells were transfected using Lipofectamine 3000 Transfection Reagent (Invitrogen, Cat. L3000000) in accordance with the manufacturer’s instructions. Briefly, HeLa cells were seeded in a 6-well plate at 70–90% of confluence and transfected with Lipofectamine 3000 Transfection Reagent plus 1 μg mtAEQwt and 60 pmol of different siRNAs. A scramble siRNA was used as a control. Thirty-six hours after transfection, cells were washed and re-plated into a 96-well plate (Corning®; Cat# 3610) (for Ca\(^{2+}\) analysis) or in 12-well plates (for Western blotting analysis). Thirty-six hours post re-plated, aequorins were reconstituted by incubating cells for 90 min with 5 μM coelenterazine (Santa Cruz Biotech.; Cat# sc-209504) in KRB solution supplemented with 0.1% glucose and 1 mM CaCl\(_2\) at 37 °C. Luminescence measurements were carried out using a PerkinElmer EnVision plate reader equipped with two injector units. For mitochondrial and cytosolic Ca\(^{2+}\) measurements, after reconstitution, cells were placed in 40 μl of KRB solution with 0.1% glucose and 1 mM CaCl\(_2\) and luminescence from each well was measured for 30–40 s. During the experiment, 50 nM ML-SA1 at the final concentration was first injected to activate Ca\(^{2+}\) transients, and then a hypotonic, Ca\(^{2+}\)-rich, digitonin (Sigma–Aldrich; Cat# D5628) containing solution was added to discharge the remaining aequorin pool. Output data were analyzed and calibrated with a custom-made macro-enabled Excel workbook.

**Zebrafish husbandry and imaging**

All animal experiments were conducted on wild-type fish. Adult fish were maintained and raised in 51 tanks with freshwater at 28 °C with a 12 h light/12 h dark cycle. Embryos were obtained from spontaneous spawnings and raised at 28 °C in Petri dishes containing fish water\(^{19}\). To perform experiments, both wt and s1102t:GAL4 fish were used. All experiments were performed on 24 h post fertilization (hpf) embryos. The pT2-DsRed-UAS-SPLICSS-P2A vector has been already described\(^ {35}\). Experiments were conducted on 24 h post fertilization (hpf) embryos. To perform experiments, both wt and s1102t:GAL4 fish were used. All experiments were performed using GraphPad Prism version 8.00 for Mac OS X (La Jolla, California, USA). Statistical significance threshold was set at \(p < 0.05\). The values of \(n\) is indicated in the figure legends. *\(p \leq 0.05\), **\(p \leq 0.01\), ***\(p \leq 0.001\), ****\(p \leq 0.0001\).

**Reporting summary**

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

**Data availability**

The data that support this study are present in the manuscript and supplementary information, and are available from the corresponding authors upon request. Requests will be fulfilled within 4 weeks. Source data are provided with this paper.

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Competing interests
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