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**A fluorogenic chemically induced dimerization technology for controlling, imaging and sensing protein proximity**

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## **ABSTRACT**

Proximity between proteins plays an essential and ubiquitous role in many biological processes. Molecular tools enabling to control and observe the proximity of proteins are essential for studying the functional role of physical distance between two proteins. Here we present CATCHFIRE (Chemically Assisted Tethering of CHimera by Fluorogenic Induced REcognition), a chemically induced proximity technology with intrinsic fluorescence imaging and sensing capabilities. CATCHFIRE relies on genetic fusion to small dimerizing domains that interact upon addition of fluorogenic inducers of proximity that fluoresce upon formation of the ternary assembly, allowing real-time monitoring of the chemically induced proximity. CATCHFIRE is rapid and fully reversible, and allows the control and tracking of protein localization, protein trafficking, organelle transport and cellular processes, opening new avenues for studying or controlling biological processes with high spatiotemporal resolution. Its fluorogenic nature allowed furthermore the design of a new class of biosensors for the study of various processes, such as signal transduction and apoptosis.

## **INTRODUCTION**

The many distinct activities of a cell are coordinated through the spatial and temporal organization of functionally interacting proteins via sequestration/translocation into specific compartments (e.g. organelles) or at the plasma membrane, or through the physical assembly of protein complexes via scaffold proteins. Technologies to chemically induce protein dimerization/proximity have been transformative for studying the role of spatiotemporal protein organization in various processes such as gene regulation, protein transport, signal transduction, metabolism, immune response, cell-cell communication<sup>1</sup>. Based on fusion to dimerization domains that interact in presence of chemical inducers of proximity, chemically induced proximity (CIP) enables to modulate protein interactions and thus cellular processes with high temporal control. Ideally, CIP should (i) rely on small dimerizing domains to avoid dysfunctional fusions, (ii) use non-toxic, easy to synthesize inducers of proximity, (iii) be rapid and reversible for dynamic studies, and (iv) have detection capabilities for real-time monitoring. Currently no technology meets all these criteria. CIP based on the rapamycin-inducible interaction of the FK506 binding protein (FKBP) and the FKBP-rapamycin binding domain (FRB) of the mammalian target of rapamycin (mTOR), is efficient and rapid, but is not reversible because of the high stability of the ternary assembly, and is limited

by the biological activity of rapamycin<sup>2,3</sup>. These limits can be mitigated using (i) non-toxic rapamycin analogs<sup>4</sup>, (ii) alternative technologies based on dimerization domains involved in the sensing of plant hormones<sup>5-8</sup>, although these latter can suffer from rather bulky dimerizing domains, or (iii) light-cleavable inducers of proximity that enable to terminate a chemically induced interaction between two proteins by photolysis<sup>9</sup>, enabling to control processes with high spatial and temporal resolution.

In this study, we present a technology that addresses the limitations of CIP technologies while incorporating unprecedented fluorescence imaging and sensing capabilities in its design. Currently, the monitoring of CIP requires fusion to fluorescent proteins (for colocalization studies or measurements of proximity by Förster resonance energy transfer<sup>10</sup>), or split reporters<sup>11-13</sup>. These additions inherently increase the risk of dysfunctional fusions and complicates the tagging of endogenous proteins by genome editing. In addition, colocalization or FRET studies require controlled stoichiometry. Split fluorescent reporters can circumvent this issue, however systems such as split fluorescent proteins<sup>11</sup> suffer from slow and irreversible complementation, and, because of their irreversibility, can stabilize the assembly, preventing dynamic studies.

Our technology, dubbed CATCHFIRE (Chemically Assisted Tethering of CHimera by Fluorogenic Induced REcognition), relies on a small peptide of 11 amino acid residues dubbed <sup>FIRE</sup>tag and a small protein domain of 114 amino acid residues dubbed <sup>FIRE</sup>mate, whose interaction is reversibly mediated by fluorogenic inducers of proximity (called *match*) that fluoresce upon formation of the ternary assembly (**Fig. 1a,b**). The fluorogenic nature of CATCHFIRE allows real-time monitoring of proximity without the need for additional reporters. We showed that CATCHFIRE is rapid and reversible, and enables the control and tracking of cellular protein localization, intracellular protein trafficking, organelle positioning/transport and cellular processes, opening new avenues for studying and controlling biological processes with high spatiotemporal resolution. Moreover, we showed that the fluorogenic nature of CATCHFIRE allows the design of innovative biosensors for the detection of cellular activities.

## RESULTS

### CATCHFIRE design

$\text{FIRE}_{\text{tag}}$  and  $\text{FIRE}_{\text{mate}}$  were generated by bisection of the fluorescent chemogenetic reporter pFAST<sup>14</sup>. pFAST is a promiscuous and improved version of FAST (Fluorescence-Activating and absorption-Shifting Tag), a 14 kDa protein that binds and stabilizes the fluorescent state of fluorogenic chromophores. Non-fluorescent when free, these so-called fluorogens enable to label fusion proteins with high contrast without the need for fluorogen washout<sup>15</sup>. FAST was previously used to design a split fluorescent reporter for detecting protein-protein interactions through bisection at position 114-115. Because the two complementary fragments of FAST exhibit low complementation in presence of fluorogen but can fully complement when in close proximity, they allow the detection of interacting proteins<sup>12</sup>. When applying the same splitting strategy on pFAST, we discovered that pFAST<sub>1-114</sub> (dubbed  $\text{FIRE}_{\text{mate}}$ ) and pFAST<sub>115-125</sub> (dubbed  $\text{FIRE}_{\text{tag}}$ ) interact in presence of fluorogens, allowing the development of a fluorogenic recruitment system that we called CATCHFIRE.

We showed that fluorogens can act as molecular glue and force the assembly of  $\text{FIRE}_{\text{mate}}$  and  $\text{FIRE}_{\text{tag}}$  by fusing the two fragments to the C-termini of FKBP and FRB. Evaluation by flow cytometry of the complementation efficacy in presence of fluorogens in HEK293T cells treated or not with rapamycin (to induce or not FKBP and FRB interaction), revealed that the ternary fluorescent assembly forms efficiently regardless of the presence of rapamycin (**Extended Data Fig. 1**). We showed that some fluorogens (hereafter called '*matches*') can induce the interaction between  $\text{FIRE}_{\text{tag}}$  and  $\text{FIRE}_{\text{mate}}$  with half maximum effective concentrations between about 100 and 500 nM (**Extended Data Fig. 1**). In vitro analysis showed that these *matches* undergo >100-fold fluorescence increase upon formation of the ternary complex, reaching about 80 % of the fluorescence observed within full-length pFAST (**Extended Data Fig. 2**). Such high fluorogenicity enabled to obtain high contrast imaging in cells, even in excess of *match* (*vide infra*).

To evaluate whether  $\text{FIRE}_{\text{mate}}$  and  $\text{FIRE}_{\text{tag}}$  self-complement in absence of *match*, we spatially separated the two fragments. We fused  $\text{FIRE}_{\text{tag}}$  to the C-terminus of the red fluorescent protein mCherry to produce a diffuse cytosolic expression, and  $\text{FIRE}_{\text{mate}}$  to the C-terminus of the N-terminal domain of the mitochondrial outer membrane protein TOM20 (TOM20<sub>1-34</sub>), so that  $\text{FIRE}_{\text{mate}}$  is immobilized, facing the cytosol. An enhanced cyan fluorescent protein (ECFP) was additionally inserted to monitor the localization of the  $\text{FIRE}_{\text{mate}}$  fusion. In untreated cells, mCherry- $\text{FIRE}_{\text{tag}}$  showed diffused cytosolic localization while co-expressed TOM20-ECFP- $\text{FIRE}_{\text{mate}}$

localized at mitochondria, suggesting that the two fragments do not spontaneously complement at the expression levels explored (**Fig. 1c,d**). Treatment with match<sub>550</sub> triggered rapid translocation of the red fluorescent mCherry fusion from the cytosol to the mitochondria surface and led to the simultaneous appearance of a strong green-yellow fluorescent signal at the mitochondria (**Fig. 1c,d** and **Supplementary Video 1**). Match<sub>550</sub>-induced proximity was further evidenced by a decrease of the mitochondrial ECFP signal, in agreement with Förster resonance energy transfer (FRET) between ECFP and the ternary fluorescent assembly (**Supplementary Fig. 1**). These experiments showed that match<sub>550</sub> could induce the proximity of proteins fused to FIRE<sub>mate</sub> and FIRE<sub>tag</sub> respectively, while lighting up the recruitment process. Fast time-lapse confocal microscopy showed that the half-time of recruitment was about 20 seconds, suggesting that CATCHFIRE is rapid and mainly limited by cellular uptake and diffusion of the *match* (**Extended Data Fig. 3a,b** and **Supplementary Video 2**, see also **Fig. 2h**). Long-term time-lapse microscopy showed that the fluorogenic recruitment was stable over long period of time (**Fig. S4c,d** and **Supplementary Video 3**). Fusion of FIRE<sub>tag</sub> at the N-terminus of mCherry, or insertion of FIRE<sub>tag</sub> in between two mCherry further demonstrated the efficiency of the approach regardless of the position of FIRE<sub>tag</sub> (**Extended Data Fig. 4** and **Supplementary Videos 4-5**).

### **CATCHFIRE enables imaging with various colors**

Match<sub>550</sub> induces the formation of a green-yellow fluorescent ternary assembly. As chromophores with various spectral properties can bind pFAST<sup>14</sup>, we next explored their use as (fluorogenic) chemical inducer of proximity to extend the ‘colors’ available for detecting CATCHFIRE. We showed that the green fluorescent match<sub>540</sub> and the red fluorescent match<sub>600</sub> enabled efficient fluorogenic induction of proximity (**Figure 1e-h** and **Supplementary Video S6-S7**). As match<sub>600</sub> provides red fluorescence, we used EGFP-FIRE<sub>tag</sub> instead of mCherry-FIRE<sub>tag</sub> to monitor the recruitment at the mitochondria. In addition, the non-fluorogenic match<sub>dark</sub> was also shown to induce efficient recruitment (**Figure 1i,j** and **Supplementary Video 8**), which can be interesting when rapid recruitment, but no fluorescence induction, is needed. The ability to induce proximity without generating fluorescence could find various applications in biological and medical research as demonstrated by conventional CIP tools.

## Control and tracking of protein localization

To demonstrate the versatility of CATCHFIRE, we next anchored <sup>FIRE</sup>mate at the membrane of various organelles or cellular structures so that it faced the cytosol. We fused the transmembrane domain (3131-3259 amino acid residues) of Giantin<sup>16</sup>, or the transmembrane domain (100-134 amino acid residues) of the cytochrome b5 (Cb5) to the C-terminus of <sup>FIRE</sup>mate to target it to the Golgi apparatus and the endoplasmic reticulum respectively. We also fused <sup>FIRE</sup>mate at the C-terminus of Lyn11, the inner membrane targeting sequence (composed of the 11 amino acid residues GCIKSKGKDSA) of the Lyn protein. For each fusion, an ECFP was inserted to monitor the localization of the fusion. Each fusion was co-expressed together with cytosolic mCherry-<sup>FIRE</sup>tag. Addition of match<sub>550</sub> or match<sub>540</sub> resulted in rapid and specific rerouting of the red fluorescent mCherry-<sup>FIRE</sup>tag to the organelle expressing the <sup>FIRE</sup>mate and the simultaneous appearance of green-yellow fluorescence on the respective organelle (**Fig. 2a-f, Extended Data Fig. 5, Supplementary Videos 9-14**), demonstrating efficient fluorogenic induced recruitment. The rerouting of mCherry-<sup>FIRE</sup>tag occurs with comparable kinetics regardless of the organelle. CATCHFIRE can thus be used to induce fast fluorogenic recruitment of proteins to sub-cellular compartments.

## CATCHFIRE is reversible upon *match* washout

Next, we tested if CATCHFIRE was reversible through simple washout of the fluorogenic inducer of proximity. The *match* molecules used in this study can bind full-length pFAST non-covalently with dissociation constants ranging from 10 to 100 nM, allowing to reverse the labeling of pFAST through washout<sup>14</sup>. As the bisection of pFAST reduces the binding affinity (see **Extended Data Fig. 1**), we hypothesized that CATCHFIRE should be reversible upon *match* washout. To demonstrate CATCHFIRE reversibility, we expressed mCherry-<sup>FIRE</sup>tag and <sup>FIRE</sup>mate-IRFP-Giantin, and induced rapid fluorogenic recruitment by adding match<sub>550</sub> to the cells. Washout of match<sub>550</sub> led to simultaneous release of mCherry fusion into the cytosol and disappearance of the green fluorescence, in agreement with the rapid dissociation of the fluorescent ternary assembly, demonstrating the full reversibility of the recruitment (**Fig. 2g,h, Supplementary Video 15**). Catch and release cycle could be repeated by sequential addition and washout of match<sub>540</sub>, showing the possibility to repeat the process (**Fig. 2g,h, Supplementary Video 15**). Fast time-lapse microscopy allowed us to estimate

that the half-times of association (upon addition of *match*) and of dissociation (upon washout) were both about 25 s (**Fig. 2h**), allowing full dimerization and full dissociation within about 100 s. The ability to reversibly control protein proximity on-demand opens great prospects to study cellular processes with high temporal resolution.

### **CATCHFIRE combination with other CIP technologies**

We next asked whether CATCHFIRE could be combined with the FRB-FKBP-*rapamycin* CIP system in order to control the dimerization of two pairs of proteins. We expressed the chimeric fusion mCherry-FKBP-<sup>FIRE</sup>tag together with both FRB-ECFP-Giantin and TOM20-<sup>FIRE</sup>mate, so that mCherry-FKBP-<sup>FIRE</sup>tag was in excess. Sequential addition of, first, *match*<sub>550</sub> and, then *rapamycin*, allowed the rerouting of a fraction of the total pool of mCherry-FKBP-<sup>FIRE</sup>tag to mitochondria (through <sup>FIRE</sup>tag-<sup>FIRE</sup>mate interaction) and of a second fraction to the Golgi apparatus (through FKBP-FRB interaction) (**Extended Data Fig. 6**), demonstrating that the two systems are orthogonal and can be used for controlling the dimerization of two pairs of proteins in the same experiment.

### **CATCHFIRE set-up without basal activity**

Our results showed that <sup>FIRE</sup>tag and <sup>FIRE</sup>mate have weak affinity for each other in absence of the fluorogenic inducer of proximity in the range of protein concentrations that we explored, allowing CATCHFIRE with high dynamic range. However, we anticipated some residual self-association in cells or compartments with high protein expression levels, because of the residual affinity between the two complementary fragments. We observed for instance a small degree of self-association in some of the experiments where cytosolic mCherry-<sup>FIRE</sup>tag was recruited at the surface of the Golgi apparatus via *match*-induced interaction with Giantin-ECFP-<sup>FIRE</sup>mate (see **Fig. 2a** and **Extended Data Fig. 5a**). Here, we assume that the compactness of the Golgi apparatus leads to high local concentrations of Giantin and thus to a certain degree of self-association. Although this residual self-association is always much lower than the fluorogenic induced association and should have no impact for most applications, we were concerned that, for some applications, even a very small degree of self-induction might lead to an undesirable basal activity. Therefore, we developed a caged version of <sup>FIRE</sup>tag to prevent any interaction with <sup>FIRE</sup>mate in case one needs to ensure very low *match*-independent interaction. To do so, we took advantage of the photoactive yellow



protein (PYP), pFAST's ancestral protein<sup>14,15</sup>, which cannot bind any pFAST chromophores. Fusion of <sup>FIRE</sup>tag to the N-terminal domain of the apo PYP (PYP<sub>2-114</sub>) resulted in a protein (caged<sup>FIRE</sup>tag) that folds like a circular permutation of PYP because of the intramolecular interaction between <sup>FIRE</sup>tag and PYP<sub>1-114</sub> (**Extended Data Figs. 7-8a**), masking thus <sup>FIRE</sup>mate. Analysis of the complementation efficacy of FKBP-caged<sup>FIRE</sup>tag and FRB-<sup>FIRE</sup>mate in presence and absence of rapamycin at various match<sub>550</sub> concentrations by flow cytometry allowed us to show that caged<sup>FIRE</sup>tag could still complement with <sup>FIRE</sup>mate in presence of match<sub>550</sub>, regardless of the proximity of the two domains (**Extended Data Fig. 8b**). We observed that, in agreement with the caging of <sup>FIRE</sup>tag, higher concentrations of match<sub>550</sub> were however needed to reach full complementation in absence of rapamycin. We propose that, in presence of match<sub>550</sub>, the formation of a more stable ternary assembly provides the driving force to uncage <sup>FIRE</sup>tag for efficient association with <sup>FIRE</sup>mate via a mutually exclusive folding mechanism. Next, we expressed mCherry-caged<sup>FIRE</sup>tag together with <sup>FIRE</sup>mate-ECFP-Giantin in HeLa cells. In absence of match<sub>550</sub>, we did not observe any colocalization of the two proteins, confirming that caged<sup>FIRE</sup>tag and <sup>FIRE</sup>mate exhibit no significant binding affinity (**Extended Data Fig. 8c,d**). Treatment with match<sub>550</sub> resulted in efficient, albeit slower, recruitment of mCherry-caged<sup>FIRE</sup>tag to the Golgi, demonstrating efficient complementation of the two domains (**Extended Data Fig. 8c,d and Supplementary Video 16**). This set of experiments suggested that caging <sup>FIRE</sup>tag can eliminate residual self-association. However, as the caging increases the size of the tag and slows down the kinetics of the dimerization process, we recommend the use of caged<sup>FIRE</sup>tag only for experiments in which residual self-association might cause undesirable basal activity

### **Control and tracking of nucleocytoplasmic protein trafficking**

Next, we showed that CATCHFIRE could allow the development of systems to induce protein motility such as nucleocytoplasmic protein trafficking. Nucleocytoplasmic trafficking is tightly regulated through mechanisms typically involving nuclear localization signals (NLS) and nuclear export signal (NES). CATCHFIRE-mediated nuclear protein export was demonstrated using NLS-mCherry-<sup>FIRE</sup>mate as a NLS-containing cargo and NES-ECFP-<sup>FIRE</sup>mate as an export NES-containing partner. NES-ECFP-<sup>FIRE</sup>mate is mainly localized in the cytoplasm at steady state, but continuously shuttles between the cytoplasm and the nucleus (**Fig. 3a**). Treatment with match<sub>550</sub>

resulted in rapid nuclear export of NLS-mCherry-<sup>FIRE</sup>tag through interaction with NES-ECFP-<sup>FIRE</sup>mate, as judged by a nuclear-to-cytoplasmic shift of mCherry fluorescence and the appearance of cytoplasmic green fluorescence (**Fig. 3a-c** and **Supplementary Video 17**). This result confirmed that in protein assemblies containing both NLS and NES, the export activity prevails over the import activity<sup>17</sup>.

Conversely, CATCHFIRE enabled to mediate nuclear accumulation of a cytosolic cargo containing a NES and NLS (NLS-mCherry-<sup>FIRE</sup>tag-NES). H2B-ECFP-<sup>FIRE</sup>mate was used here as a stable nuclear anchor to trap NES-mCherry-<sup>FIRE</sup>tag-NLS transiently shuttling between the cytoplasm and the nucleus. Treatment with match<sub>550</sub> allowed to sustainably trap shuttling NES-mCherry-<sup>FIRE</sup>tag-NLS in the nucleus, inducing overall a cytosol-to-nucleus localization shift through a sink effect (**Fig. 3d-f** and **Supplementary Video 18**).

Overall this set of experiments suggests that the CATCHFIRE assay could be suitable to control and track protein trafficking between different cell compartments.

### **Control and tracking of secretory protein traffic**

As CATCHFIRE allows reversible interaction, we tested its use for the Retention Using Selective Hooks (RUSH) assay<sup>18</sup> to control and track the traffic of secretory proteins. The RUSH system allows the synchronization of secretory protein traffic through the expression of two proteins: the hook, which is stably expressed in a donor compartment, and the reporter, which can reversibly interact with the hook. Upon reversion of the interaction, the reporter is released and traffics to its final compartment. We thus developed a Retention Using CATCHFIRE Hook (RUCH) system by creating an endoplasmic reticulum (ER) hook by fusing <sup>FIRE</sup>mate with a C-terminal ER retention signal (Lys-Asp-Glu-Leu; KDEL), and three reporter proteins by fusing either the tumor necrosis factor- $\alpha$  (TNF) or the  $\alpha$ -mannosidase II (ManII) to the N-terminus of a red fluorescent protein (mCherry or mApple)-<sup>FIRE</sup>tag, or a glycosylphosphatidylinositol (GPI) anchor to the C-terminus of mApple-<sup>FIRE</sup>tag (**Fig. 4** and **Extended Data Fig. 9**). To ensure efficient retention of the reporter by the hook, cells were treated after transfection with match<sub>550</sub> for 24 h. At steady-state, TNF-mCherry-<sup>FIRE</sup>tag, mApple-<sup>FIRE</sup>tag-GPI and ManII-mApple-<sup>FIRE</sup>tag were localized in the ER (**Fig. 4** and **Extended Data Fig. 9**). To release the reporters, match<sub>550</sub> was washed out, leading to a rapid loss of the green fluorescence and fast ER export. After 10 min, the three reporters were detected in the Golgi (**Fig. 4**, **Extended Data Fig. 9** and **Supplementary Videos**

**19-21**). For TNF-mCherry-<sup>FIRE</sup>tag, granule-like post-Golgi carriers appeared after 15 min (**Fig. 4b, Supplementary Video 19**), while plasma membrane expression of mApple-<sup>FIRE</sup>tag-GPI was visible after 60 min (**Extended Data Fig. 9, Supplementary Video 20**). Interestingly, in the case of ManII-mApple-<sup>FIRE</sup>tag, KDEL-mediated retrograde traffic from the Golgi apparatus to the ER could be induced by re-addition of match<sub>550</sub> (**Fig. 4d-f, Supplementary Video 21**). This set of experiments demonstrated the ability of CATCHFIRE to be used to synchronize and control the trafficking of secretory proteins. Compared to the conventional RUSH system that uses tetrameric streptavidin as a hook for the retention of reporters fused to streptavidin-binding peptides, and addition of biotin for the release, our RUCH system, in addition to bringing intrinsic fluorescence to monitor cargo release, benefits from the use of a monomeric hook, <sup>FIRE</sup>mate, and from being reversible, opening new prospects for studying the mechanisms of trafficking.

### **Control and tracking of organelle positioning**

CATCHFIRE can efficiently control the movement of proteins between compartments, and we wondered whether it could be used to alter and track the positioning of organelles through recruitment of specific molecular motors<sup>19</sup>. We focused on lysosomes, whose motility and positioning are essential in various cellular processes, such as destruction of pathogens, plasma membrane repair, cell adhesion and migration, tumor invasion and metastasis, apoptosis, metabolism and gene regulation<sup>20,21</sup>. <sup>FIRE</sup>tag was targeted to lysosomes using a lysosomal-associated membrane protein (LAMP)1-mCherry-<sup>FIRE</sup>tag fusion, and <sup>FIRE</sup>mate was fused to the N-terminus of the motor domain of kinesin-like KIF17<sup>22</sup>, a member of the kinesin-2 family of plus-end directed microtubule-based motor proteins. Addition of match<sub>550</sub> induced movement of the lysosomes from the perinuclear region to the cell periphery, in agreement with an induced anterograde transport. Lysosomes were repositioned within 30-45 minutes (**Fig. 5, Supplementary Video 22**). Washout of match<sub>550</sub> led to repositioning of the lysosome back to the perinuclear region. This experiment demonstrated that CATCHFIRE could efficiently control organelle positioning in a reversible fashion opening exciting prospects for getting insights into the functional effects of organelle displacement or to alter intracellular organization.

### **Control of mitophagy**

As spatiotemporal control of protein localization can allow to tune cell behavior, we explored the use of CATCHFIRE to control key cellular mechanisms. We focused on mitophagy, the selective degradation of mitochondria by autophagy. One of the main pathways involves the protein kinase PINK1<sup>23</sup>. In unhealthy cells, depolarization of the mitochondrial membrane impairs PINK1 internalization and leads to its accumulation at the mitochondrial outer membrane, initiating mitophagy. We investigated CATCHFIRE-mediated mitophagy through translocation of PINK1 at the mitochondrial outer membrane using PINK1-mCherry-FIRE<sub>tag</sub>. A fusion FIRE<sub>mate</sub>-ECFP-(mitochondrial fission 1 protein)Fis1 was generated as mitochondrial outer membrane anchor. Addition of match<sub>550</sub> resulted in rapid translocation of PINK1-mCherry-FIRE<sub>tag</sub> to the mitochondrial outer membrane (**Supplementary Fig. 2** and **Supplementary Video 23**). Observations after 2h of treatment revealed strong morphology change of mitochondria in agreement with the triggering of mitophagy (**Extended Data Fig. 10** and **Supplementary Fig. 3**), showing that recruitment of PINK1 at the mitochondrial outer membrane was sufficient to initiate mitophagy. This experiment showed how CATCHFIRE could be used to control cellular processes through recruitment of specific effectors.

### **Fluorescent sensors for monitoring cellular activity**

The fluorogenic nature of CATCHFIRE provides unprecedented opportunities for the design of sensors that switch on upon changes in cellular activity. Activation of signaling pathways or cellular processes often results in change of localization of one or several proteins. As CATCHFIRE requires FIRE<sub>tag</sub> and FIRE<sub>mate</sub> to be in the same cellular compartment to occur, it is possible to design sensors that switch on upon activation of a specific pathways by stably anchoring FIRE<sub>mate</sub> within a specific organelle and tracking a reporter fused to FIRE<sub>tag</sub> through change in CATCHFIRE efficiency. To demonstrate the ability to design such fluorescent sensors, we first designed a sensor of signaling cascade activation. We used p65, a transcription factor of the NF- $\kappa$ B family involved in cell proliferation, apoptosis, cytokine production, and oncogenesis<sup>24</sup>. In most unstimulated cells, p65 is sequestered in the cytoplasm by I $\kappa$ B inhibitory proteins. Stimulation of cells leads to degradation of I $\kappa$ B, and allows the translocation of p65 to the nucleus, resulting in expression of target genes<sup>24</sup>. A sensor of p65 nuclear import was designed using p65-mCherry-FIRE<sub>tag</sub> as reporter and H2B-

ECFP-<sup>FIRE</sup>mate as a stable nuclear anchor. In unstimulated cells, no nuclear green fluorescence was observed upon treatment with match<sub>550</sub> in agreement with p65-mCherry-<sup>FIRE</sup>tag being fully sequestered in the cytoplasm and unable to translocate in the nucleus, preventing thus efficient fluorogenic induced interaction with nuclear H2B-ECFP-<sup>FIRE</sup>mate (**Fig. 6a-c**). Cell stimulation with TNF $\alpha$  led to a strong increase in nuclear CATCHFIRE signal, in agreement with nuclear translocation of p65-mCherry-<sup>FIRE</sup>tag (**Fig. 6d-f**), demonstrating that CATCHFIRE can be used for sensing signal transduction.

To explore the generality of this approach, we designed a sensor of apoptosis by relying on apoptosis-mediated nuclear translocation. We fused mCherry-<sup>FIRE</sup>tag at the N-terminus of the mitochondrial monoamine oxidase (MoA) protein using a DEVDG linker and expressed it together with H2B-ECFP-<sup>FIRE</sup>mate as nuclear anchor. DEVDG sequence can be cleaved by caspase-3 during cell apoptosis, thus releasing mCherry-<sup>FIRE</sup>tag. Prolonged treatment of cells with staurosporine resulted in the release of mCherry-<sup>FIRE</sup>tag by action of the caspase-3 and its diffusion into the nucleus where it interacted with H2B-ECFP-<sup>FIRE</sup>mate inducing a nuclear green fluorescence (**Fig. 6g-i**). The principle of such CATCHFIRE-based fluorescent sensors is general and could be applied to design optical sensors for various other cellular activities. Importantly, in both cases, a global analysis of the green fluorescence signal is sufficient to quantify signaling without the need for sub-cellular image analysis. As CATCHFIRE-based sensing does not strictly require the use of microscopes, techniques such as flow cytometry or other fluorometric techniques may also be used for the detection.

## DISCUSSION

CATCHFIRE uses fluorogenic inducers of proximity to conditionally induce and visualize the proximity of two proteins fused to a small peptide tag (<sup>FIRE</sup>tag) and a small protein domain (<sup>FIRE</sup>mate). We show here that CATCHFIRE enabled the development of both original inducers and sensors for use in living cells. Compared to traditional CIP assays, CATCHFIRE has several advantages. First, it allows both to induce and visualize the recruitment process without the need for additional reporters. Fluorogenic inducers of proximity with various spectral properties exist allowing to adjust the color to a given experimental set-up. Second, CATCHFIRE is the CIP tool with the smallest dimerizing domains (11 amino acids for <sup>FIRE</sup>tag and 114 amino acids for <sup>FIRE</sup>mate), which should reduce the risk of dysfunctional fusions. The small size of <sup>FIRE</sup>tag may

also enable fast genome editing for endogenous tagging. Third, CATCHFIRE allows one to rapidly initiate or terminate an interaction between two proteins through addition and washout of *match*, opening very exciting prospects for the acute control and regulation of dynamic biological processes. We observed full dimerization within about 1 min upon addition of *match*, and full dissociation of the ternary complex within the same time scale upon *match* washout. Compared to reversible CIP techniques based on photocleavable inducers of proximity, such as the zapalog<sup>9</sup>, CATCHFIRE shows similar temporal resolution for the diffusion-limited dimerization step (about 100 s), but slower dissociation (100 s versus < 1s). The dissociation in the case of zapalog is achieved using photolysis rather than a simple washout, which allows to terminate the interaction on a much shorter time scale. However, rapid photolysis step requires illuminating cells with 405 nm light, which necessitates additional controls to exclude any light-induced cellular effects.

In this study, we demonstrated the suitability of CATCHFIRE to control and track with high temporal resolution protein rerouting, nucleocytoplasmic protein trafficking, secretory protein trafficking, organelle transport and key cellular mechanisms such as mitophagy. Among these examples, the reversibility of CATCHFIRE was essential for the development of a new assay enabling to synchronize protein secretion. In the Retention Using CATCHFIRE hook (RUCH) assay, *match* washout releases target proteins from their hook, enabling their synchronous trafficking. The ability to perform release and catch cycle (by *match* washout and re-addition) allowed us moreover to study anterograde and retrograde traffic between the ER and the Golgi apparatus with high temporal resolution. The ability to induce and terminate an interaction between two proteins by *match* addition and washout allowed us moreover to control organelle positioning with very high temporal resolution, opening great prospect to study the dynamic and kinetics of such processes. The reversibility of CATCHFIRE could allow in the future the reversible rerouting of target proteins to a given organelle or cell compartment for the rapid tuning of cellular functions through reversible protein knock-sideway<sup>25</sup>.

The fluorogenic nature of CATCHFIRE opens also unprecedented possibilities for the design of sensors with built-in multi-color fluorescence activation. As the efficiency of CATCHFIRE depends on the ability of <sup>FIRE</sup>tag and <sup>FIRE</sup>mate to meet each other, we could design sensors that switch on upon activation of signaling pathways or cellular mechanisms involving protein localization changes. We anticipate that

CATCHFIRE will enable the development of novel sensors with built-in fluorescence activation that will allow to study a large diversity of cellular processes. Such set-up will be particularly well-suited for cell imaging but because of the fluorogenic nature of CATCHFIRE, assays independent from microscopy analysis (e.g. fluorometry, cytometry) can also be developed, opening great prospect for larger scale screenings.

Future efforts will focus on improving, expanding and exploring the limits of the technology. We reported a tendency of  $\text{FIRE}^{\text{tag}}$  and  $\text{FIRE}^{\text{mate}}$  to interact in the absence of *match* molecules when overexpressed. Although the caged  $\text{FIRE}^{\text{tag}}$  is an efficient answer to this limitation, the engineering of improved  $\text{FIRE}^{\text{tag}}:\text{FIRE}^{\text{mate}}$  pairs with minimal residual affinity may alleviate this limitation. Additionally, we also aim to identify additional *match* molecules to cover more extensively the visible spectrum (for example in the far-red or near-infrared region), and develop orthogonal systems for the control and visualization of multiple interactions. Last, when developing the FAST system<sup>15</sup>, we observed that chromophore binding could be pH sensitive and we thus still need to explore this aspect in the context of CATCHFIRE.

Finally, the validation of CACHFIRE in vivo will be the next challenge for both fundamental studies in Biology and applications in Biomedicine. The *match* molecules presented in this study belong to a family of chromophores that are highly cell-permeant and that have been used successfully in the past to label proteins in vivo in zebrafish embryo and larvae<sup>15,26,27</sup>, as well as ex vivo in chicken embryo tissues<sup>14,26</sup>, allowing to expect efficient CATCHFIRE in these systems and in other multicellular organisms.

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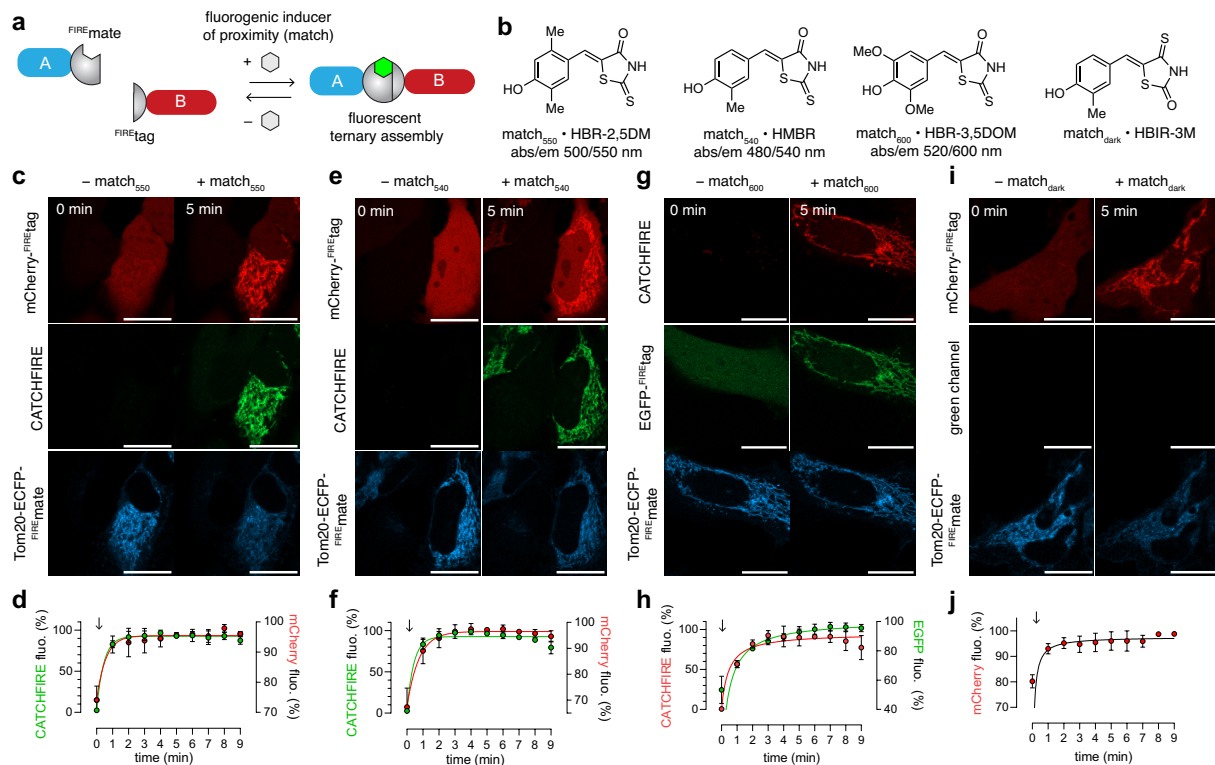
## **AUTHOR CONTRIBUTIONS**

S. B., O.J., Z.V.C., L.E.H., L.M.R., G.B., F.P. and A.G. designed the experiments. S.B., O.J., Z.V.C., L.E.H., L.M.R., and G.B. performed the experiments. S. B., O.J., Z.V.C., L.E.H., L.M.R., G.B., F. P. and A.G. analyzed the experiments. S. B., F. P. and A.G. wrote the paper with the help of all the authors.

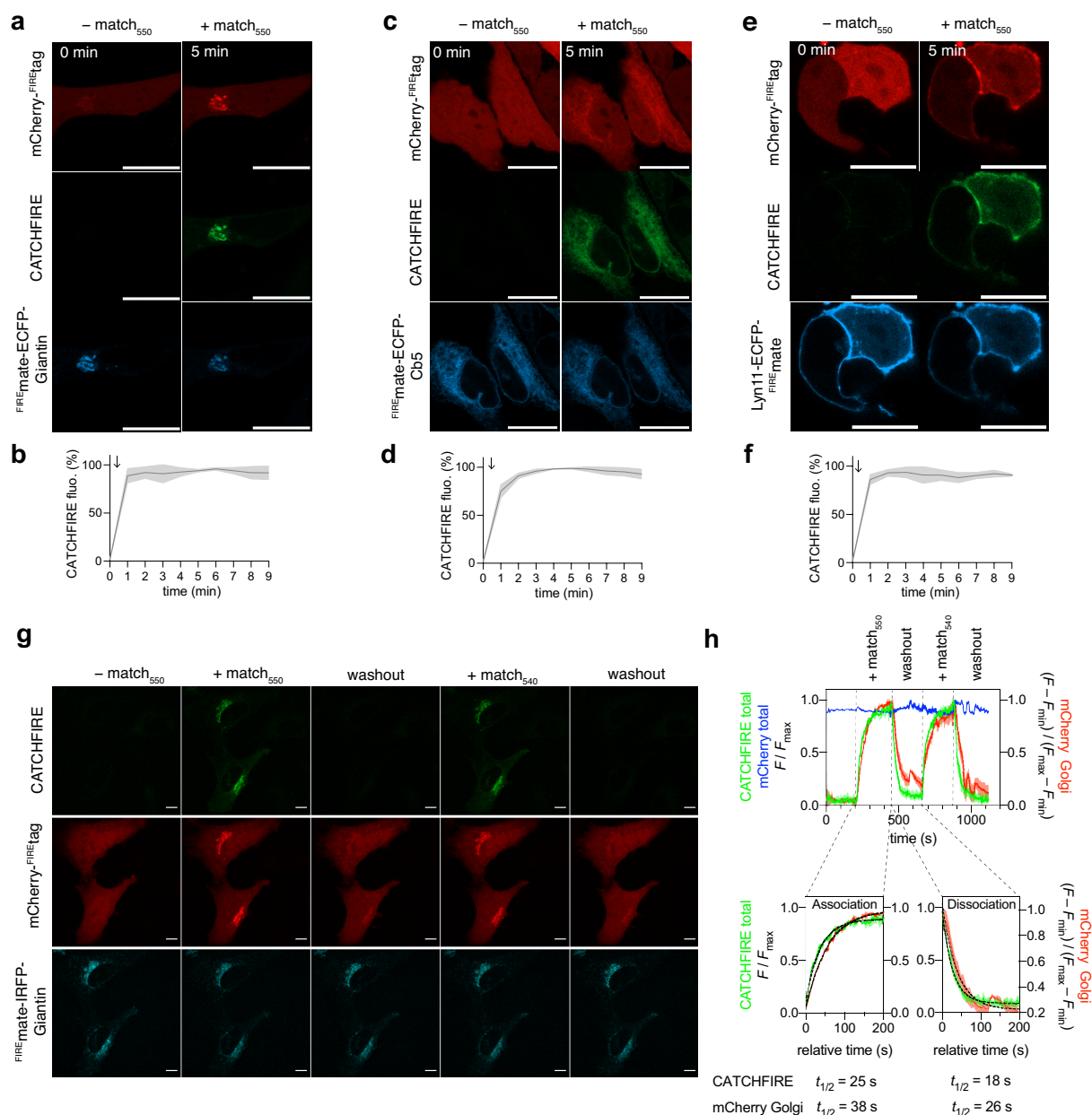
## **COMPETING INTERESTS**

The authors declare the following competing financial interest: A.G., F.P., S.B., Z.V.C. and O.J. are listed as inventors on a patent application related to the present work and filed by Sorbonne University, the École Normale Supérieure – PSL University, the CNRS and the Institut Curie. A.G. and F.P. are also co-founder of The Twinkle Factory, a start-up which will commercially distribute the *match* molecules for research use. The remaining authors declare no competing interest.



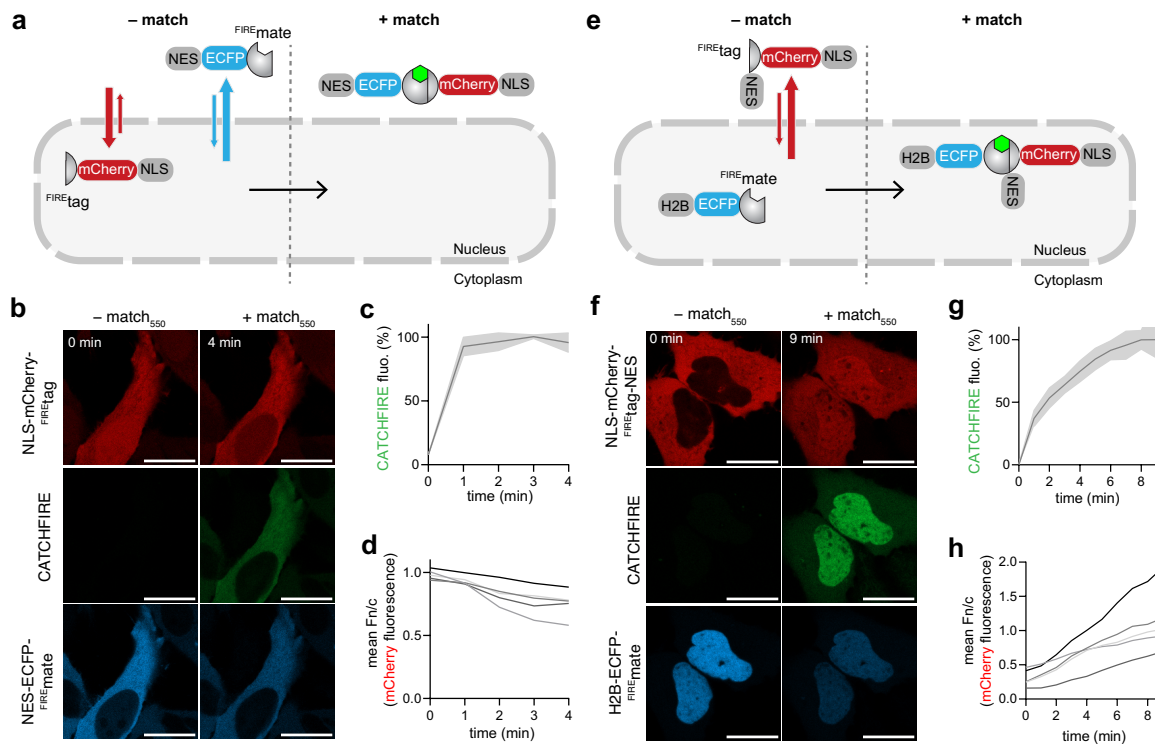


**Fig. 1. Chemically Activated Tethering of Chimera by Fluorogenic Induced REcognition (CATCHFIRE).** **a** CATCHFIRE relies on the genetic fusion of two proteins A and B to  $\text{FIRE}_{\text{tag}}$  and  $\text{FIRE}_{\text{mate}}$ , two domains that can interact in a specific manner in presence of a fluorogenic inducer of proximity (called the *match*) that becomes fluorescent upon formation of the ternary assembly, enabling to induce the proximity of the proteins and visualize the recruitment process by fluorescence imaging. **b** Structures of the different inducers of proximity used in this study. **c-j** HeLa cells co-expressing  $\text{mCherry-FIRE}_{\text{tag}}$  (**c-f,i,j**) or  $\text{EGFP-FIRE}_{\text{tag}}$  (**g,h**) together with  $\text{Tom20-ECFP-FIRE}_{\text{mate}}$  were treated with  $10 \mu\text{M}$  of either  $\text{match}_{550}$  (**c,d**) or  $\text{match}_{540}$  (**e,f**) or  $\text{match}_{600}$  (**g,h**), or  $\text{match}_{\text{dark}}$  (**i,j**), and imaged by time-lapse confocal microscopy. **c,e,g,i** Representative confocal micrographs of cells before (0 min) and after (5 min) addition of the *match* (see **Supplementary Videos 1, 6-S8**). Experiments were repeated three times with similar results. **d,f,h,j** Temporal evolution of the recruitment. Data represents the mean values  $\pm$  SD of three independent experiments ( $n=17$  cells (**d**), 16 cells (**f**), 10 cells (**h**), 10 cells (**j**)). Microscopy settings: Red: ex/em 561/606-675 nm; Green: ex/em 488/508-570 nm; Cyan: ex/em 445/455-499 nm; Scale bars are 20  $\mu\text{m}$ .

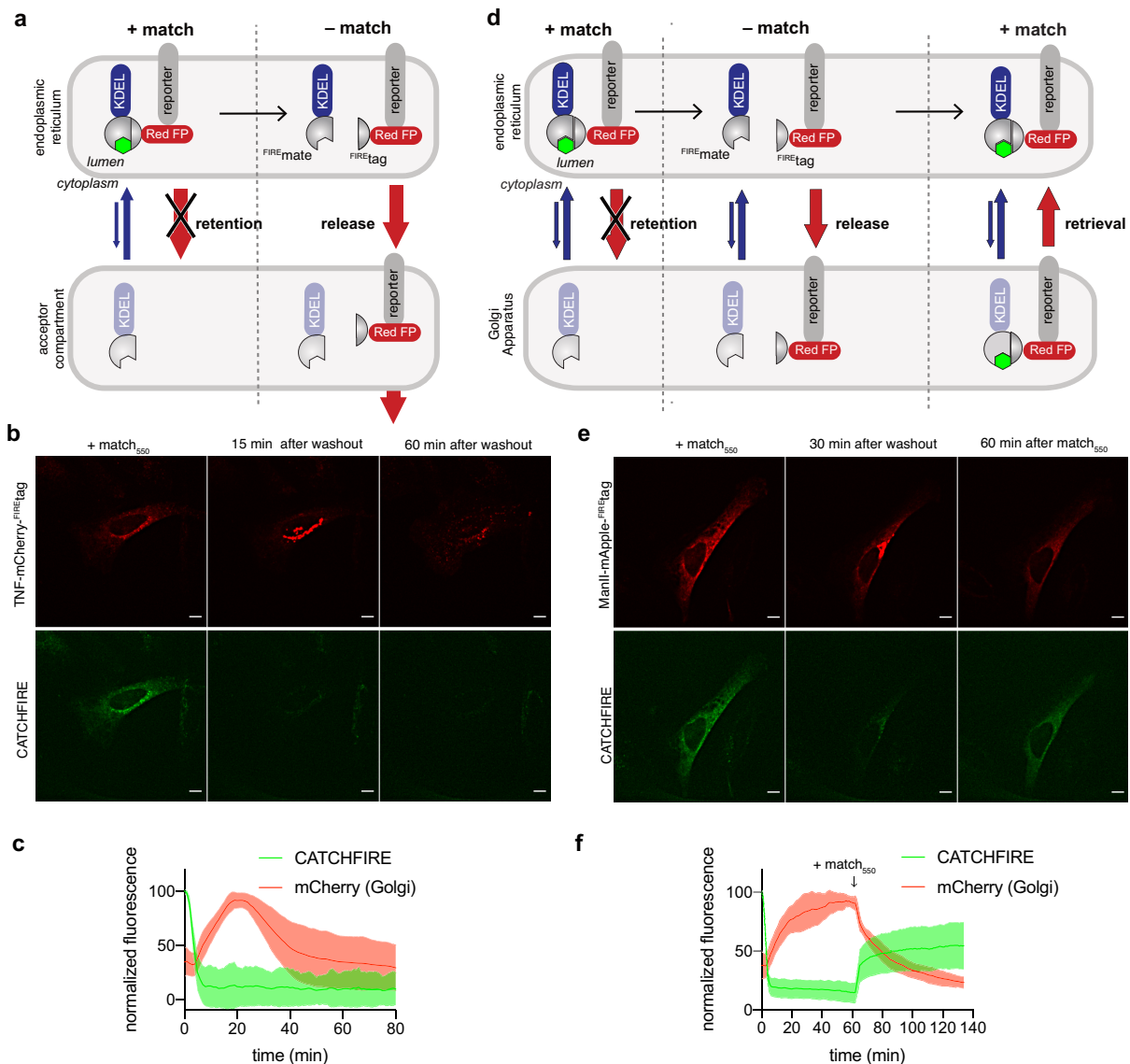


**Fig. 2. CATCHFIRE enables the control of protein dimerization with high temporal control.** **a-f** HeLa cells co-expressing mCherry-FIREtag and FIREmate-ECFP-Giantin (**a,b**), FIREmate-ECFP-Cb5 (**c,d**), Lyn11-FIREmate-ECFP (**e,f**) were treated with 10  $\mu$ M match<sub>550</sub>, and imaged by time-lapse confocal microscopy. **a,c,e** Representative confocal micrographs of cells before (0 min) and after (5 min) addition of match<sub>550</sub> (see **Supplementary Videos 9-11**). Experiments were repeated three times with similar results. **b,d,f** Temporal evolution of the CATCHFIRE signal. Data represents the mean values  $\pm$  SD of three independent experiments ( $n = 11$  cells (**b**), 15 cells (**d**), 15 cells (**f**)). **g,h** HeLa cells co-expressing mCherry-FIREtag and Giantin-IRFP-FIREmate were treated with 10  $\mu$ M match<sub>550</sub> for 240 s, washed for 212 s and then treated with 10  $\mu$ M match<sub>540</sub> for 212 s and washed again. Cells were imaged by time-lapse spinning-disk microscopy. **g** Representative micrographs of the different steps (see also **Supplementary Video 15**). Experiments were repeated five times with similar results. **h** Temporal evolution of the total CATCHFIRE (green) and mCherry (blue) signals, and of the mCherry signal at the Golgi apparatus (red). Data represents the mean value  $\pm$  SD of the two cells shown in panel **g**. The inserts show the kinetic analysis of the

association and dissociation steps using either the CATCHFIRE signal or the Golgi mCherry signal. Microscopy settings: **a-f** Red: ex/em 561/606-675 nm; Green: ex/em 488/508-570 nm; Cyan: ex/em 445/455-499 nm ; Scale bars are 20  $\mu\text{m}$ ; **g,h** Green (CATCHFIRE): ex/em 488/545-575 nm. red (mCherry): ex/em 561/605-664 nm; cyan (IRFP): ex/em 633/700-775 nm. Scale bars are 10  $\mu\text{m}$ .

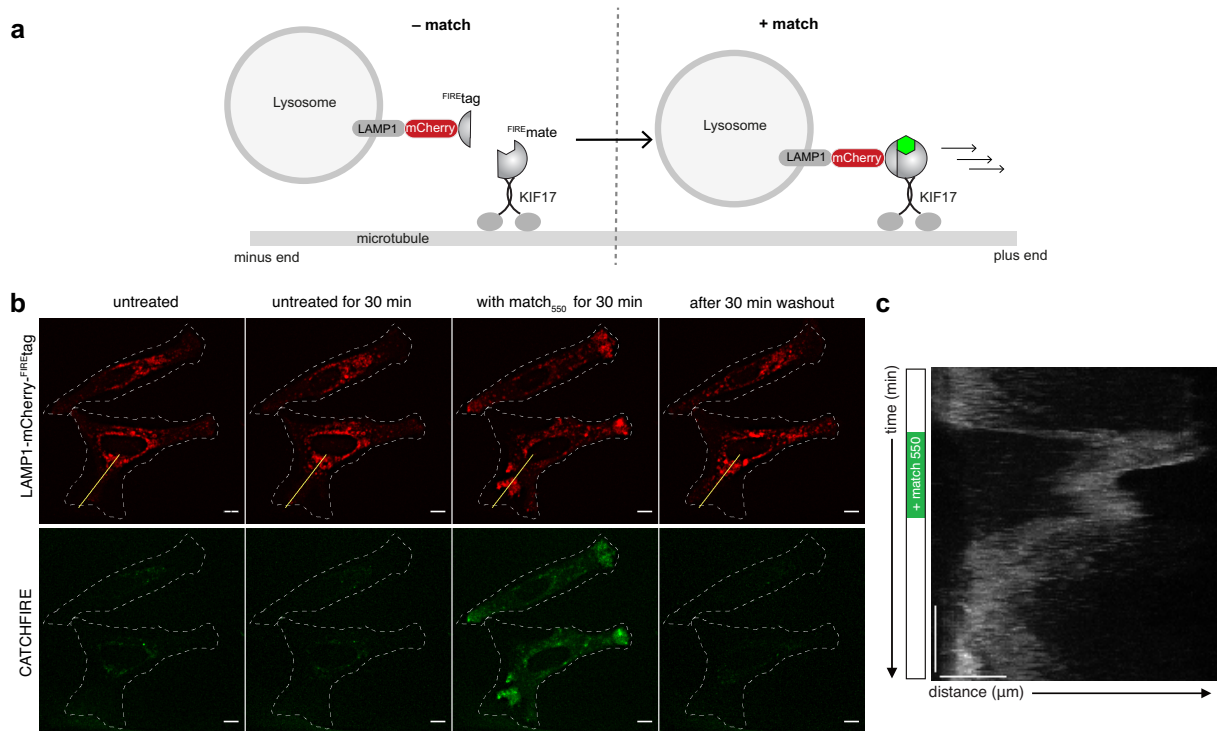


**Fig. 3. Control and tracking of nucleocytoplasmic shuttling.** **a-d** HeLa cells co-expressing NLS-mCherry-<sup>FIREtag</sup> and NES-ECFP-<sup>FIREmate</sup> were treated with 10  $\mu$ M match<sub>550</sub> and imaged by time-lapse confocal microscopy. **b** Representative confocal micrographs before and after treatment with match<sub>550</sub> (see also **Supplementary Video 17**). Experiments were repeated three times with similar results. **c** Temporal evolution of the CATCHFIRE signal. Data represent the mean  $\pm$  SD of  $n = 5$  cells from two independent experiments. **d** Temporal evolution of the ratio nucleus-to-cytoplasm mCherry fluorescence of  $n = 5$  cells from 2 independent experiments. **e-h** HeLa cells co-expressing NLS-mCherry-<sup>FIREtag</sup>-NES and H2B-ECFP-<sup>FIREmate</sup> were treated with 10  $\mu$ M match<sub>550</sub> and imaged by time-lapse confocal microscopy. **f** Representative confocal micrographs before and after treatment with match<sub>550</sub> (see also **Supplementary Video 18**). Experiments were repeated three times with similar results. **g** Temporal evolution of the CATCHFIRE signal. Data represent the mean  $\pm$  SD of  $n = 5$  cells from two independent experiments. **h** Temporal evolution of the ratio nucleus-to-cytoplasm mCherry fluorescence of  $n = 5$  cells from 2 independent experiments. **b,f** Red: ex/em 561/606-675 nm; green: ex/em 488/508-570 nm; cyan: ex/em 445/455-499 nm. Scale bars are 20  $\mu$ m.

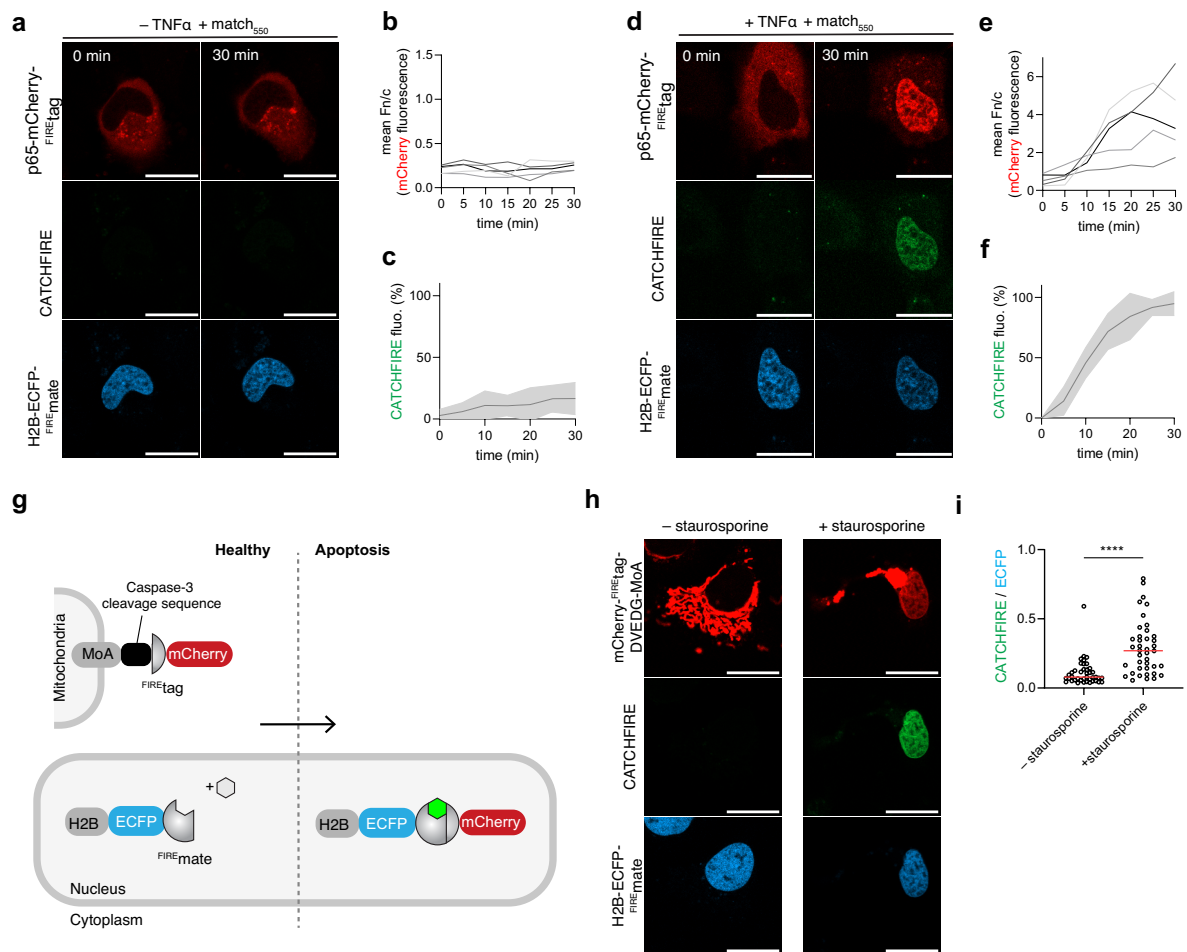


**Fig. 4. Control and tracking of secretory protein trafficking through Retention Using CATCHFIRE Hook (RUCH).** **a** Schematic illustrating how  $\text{FIRE}_{\text{mate}}$ -KDEL acts as a hook to retain  $\text{FIRE}_{\text{tag}}$ -reporter in the endoplasmic reticulum in presence of the *match*. Release is induced by washing of the *match*, allowing trafficking of the reporter to its acceptor compartment. **b,c** HeLa cells co-expressing  $\text{FIRE}_{\text{mate}}$ -KDEL and  $\text{TNF-mCherry-FIRE}_{\text{tag}}$  were treated with  $\text{match}_{550}$  for 24 h and imaged by spinning-disk microscopy after washout of  $\text{match}_{550}$ . **b** Representative micrographs before washout, 15 min and 60 min after washout (see also **Supplementary Video 19**). Experiments were repeated three times with similar results. **c** Temporal evolution of the global CATCHFIRE signal (observed in the ER, green) and the mCherry signal quantified in the Golgi apparatus (red). Data represents the mean  $\pm$  SD of 8 cells. **d** Schematic illustrating how the retention using CATCHFIRE hooks (RUCH) approach can be used to study the anterograde and retrograde traffic between the ER and the Golgi apparatus. **d,e** HeLa cells co-expressing  $\text{FIRE}_{\text{mate}}$ -KDEL and  $\text{ManII-mApple-FIRE}_{\text{tag}}$  were treated with  $\text{match}_{550}$  for 24 h and imaged by spinning-disk microscopy after washout of  $\text{match}_{550}$ .  $\text{Match}_{550}$  was re-added after 60 min. Experiments were done in 25  $\mu\text{g}/\text{mL}$  cycloheximide. **e** Representative micrographs before washout, 30 min after washout and 60 min after re-addition of  $\text{match}_{550}$  (see also **Supplementary Video 21**). Experiments were repeated five times with similar results. **f** Temporal evolution of the

global CATCHFIRE signal (observed in the ER, green) and the mCherry signal quantified in the Golgi apparatus (red). Data represents mean  $\pm$  SD of 11 cells. Red: ex/em 561/604-664 nm; green: ex/em 488/545-575 nm. Scale bars are 10  $\mu$ m. All images were enhanced by 2D-deconvolution on NIS-Elements Ver.5.42.



**Fig. 5. Control and tracking of organelle positioning.** **a** Schematic illustrating how *match*-induced interaction between LAMP1-mCherry-FIREtag and FIREmate-KIF17 allows the chemically induced anterograde transport of lysosomes. **b** HeLa cells co-expressing LAMP1-mCherry-FIREtag and FIREmate-KIF17 were imaged by spinning-disk microscopy for 35 min, then match<sub>550</sub> was added. After 50 min of treatment, match<sub>550</sub> was washed out and cells were imaged for 75 min. Representative micrographs of each step (see also **Supplementary Video 22**). Experiments were repeated five times with similar results. Red: ex/em 561/604-664 nm; green: ex/em 488/545-575 nm. Scale bars are 10 μm. Images were enhanced by 2D-deconvolution on NIS-Elements Ver.5.42. **c** Kymograph showing the LAMP1-mCherry-FIREtag along the yellow line shown in **b** over time.



**Fig. 6. CATCHFIRE-based biosensors for studying cell signaling.** **a-f** Signal transduction sensor. HeLa cells expressing p65-mCherry-FIREtag and H2B-ECFP-FIREmate pretreated with 10  $\mu$ M match<sub>550</sub> were treated without (**a-c**) or with 10 ng/ml TNF $\alpha$  (**d-f**). **a,d** Representative micrographs of cells after 0 min and 30 min. Experiments were repeated three times with similar results. **b,e** Temporal evolution of the ratio nucleus-to-cytoplasm mCherry fluorescence of  $n = 5$  cells (**a-c**) and  $n = 5$  cells (**d-f**) from three independent experiments. **c,f** Temporal evolution of the CATCHFIRE signal. Data represent the mean  $\pm$  SD of  $n = 5$  cells (**a-c**) and  $n = 5$  cells (**d-f**) from three independent experiments. **g** Schematic of the apoptosis sensor. **h,i** HeLa cells expressing mCherry-FIREtag-DVEDG-MoA and H2B-ECFP-FIREmate pretreated with match<sub>550</sub> were treated without or with 2  $\mu$ M staurosporine for 3.5 h. **h** Representative micrographs. Experiments were repeated three times with similar results. **i** Quantification of the apoptosis-induced CATCHFIRE. Data represent  $n = 41$  cells (- staurosporine) and  $n = 40$  cells (+ staurosporine) from two independent experiments. The median of CATCHFIRE/ECFP ratio is reported as a red line. Two-tailed Student's *t*-test assuming equal variance was used to compare the two distribution. \*\*\*\*  $p$  value = 0.000000693. **a,d,h** Red: ex/em 561/606-675 nm; green: ex/em 488/508-570 nm; cyan: ex/em 445/455-499 nm. Scale bars are 20  $\mu$ m.



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## METHODS

The presented research complies with all relevant ethical regulations.

**General** Synthetic oligonucleotides used for cloning were purchased from Integrated DNA technology. PCR reactions were performed with Q5 polymerase (New England Biolabs) in the buffer provided. PCR products were purified using QIAquick PCR purification kit (Qiagen). DNase I, T4 ligase, Fusion polymerase, Taq ligase and Taq exonuclease were purchased from New England Biolabs and used with accompanying buffers and according to manufacturer protocols. Isothermal assemblies (Gibson assembly) were performed using homemade mix prepared according to previously described protocols<sup>28</sup>. Small-scale isolation of plasmid DNA was done using QIAprep miniprep kit (Qiagen) from 2 mL overnight bacterial culture supplemented with appropriate antibiotics. Large-scale isolation of plasmid DNA was done using the QIAprep maxiprep kit (Qiagen) from 150 mL of overnight bacterial culture supplemented with appropriate antibiotics. All plasmid sequences were confirmed by Sanger sequencing with appropriate sequencing primers (GATC Biotech). All the plasmids used in this study are listed in **Supplementary Table 1**, as well as DNA sequences. The synthesis of match<sub>540</sub> (a.k.a. HMBR), match<sub>550</sub> (a.k.a. HBR-2,5DM), match<sub>600</sub> (a.k.a. HBR-3,5DOM), match<sub>dark</sub> (a.k.a. HBIR-3M) were previously reported<sup>14,15,29</sup>. These molecules will be available from The Twinkle Factory (<https://www.the-twinkle-factory.com>)

**Cloning.** The plasmids used in this study have been generated using isothermal Gibson assembly or restriction enzymes and allow mammalian expression of relative insert under the control of the CMV promoter. The plasmid pAG1141 allowing the expression of FKBP- caged<sup>FIRE</sup>tag-IRES-IRFP670 was constructed by replacing the sequence coding for CFAST10 by the <sup>FIRE</sup>tag sequence and the sequence of mCherry by the sequence of IRF670 in the vector pAG496<sup>12</sup> coding for FKBP- CFAST10-IRES-mCherry. The plasmid pAG1151 allowing the expression of FKBP- <sup>FIRE</sup>tag -IRES-IRFP670 was constructed by removing the sequence coding for PYP<sub>2-114</sub> from the vector pAG1141. The plasmid pAG1142 allowing the expression of FRB-<sup>FIRE</sup>mate-IRES-mTurquoise2 was constructed by replacing the sequence coding for FAST<sub>1-114</sub> by <sup>FIRE</sup>mate sequence, in the previously described vector pAG641<sup>12</sup>. The plasmid

pAG1159 allowing the expression of TOM20-ECFP-<sup>FIRE</sup>mate was constructed by replacing the sequence coding for FRB by <sup>FIRE</sup>mate sequence in the Addgene vector #171461 Tom20-CR coding for TOM20-ECFP-FRB<sup>30</sup>. Tom20-CR was a gift from Takafumi Miyamoto (Addgene plasmid # 171461 ; <http://n2t.net/addgene:171461> ; RRID:Addgene\_171461). The plasmid pAG1160 allowing the expression of mCherry-<sup>FIRE</sup>tag was constructed by replacing the sequence coding for PYP by <sup>FIRE</sup>tag sequence in the previously described vector pAG97<sup>15</sup> coding for mCherry-PYP. The plasmid pAG1209 allowing the expression of <sup>FIRE</sup>tag-mCherry was constructed by adding the sequence coding for <sup>FIRE</sup>tag in the vector pAG1189 (Gautier et al. unpublished) coding for mCherry. The plasmid pAG1241 allowing the expression of <sup>FIRE</sup>tag-mCherry-<sup>FIRE</sup>tag was constructed by adding the sequence coding for <sup>FIRE</sup>tag in the vector pAG1209 coding for <sup>FIRE</sup>tag-mCherry. The plasmid pAG1188 allowing the expression of mCherry-caged<sup>FIRE</sup>tag was constructed by adding the sequence coding for PYP<sub>2-114</sub> in the vector pAG1160. The plasmid pAG1344 allowing the expression of EGFP-<sup>FIRE</sup>tag was constructed by replacing the sequence coding for mCherry by EGFP sequence in the vector pAG1160. The plasmid pAG1169 for the expression of <sup>FIRE</sup>mate-ECFP-Cb5 was constructed by replacing the sequence coding for FKBP1C by <sup>FIRE</sup>mate sequence in the Addgene vector (#162438) coding for FKBP1C-ECFP-Cb5<sup>31</sup>. FKBP1C-CFP-Cb5 was a gift from Takanari Inoue (Addgene plasmid # 162438 ; <http://n2t.net/addgene:162438> ; RRID:Addgene\_162438). The plasmid pAG1171 for the expression of <sup>FIRE</sup>mate-ECFP-Giantin was constructed by replacing the sequence coding for FRB-ECFP(W66A)-Giantin by <sup>FIRE</sup>mate-ECFP sequence in the Addgene vector (#67903) coding for FRB-ECFP(W66A)-Giantin<sup>16</sup>. FRB-ECFP(W66A)-Giantin was a gift from Dorus Gadella (Addgene plasmid # 67903 ; <http://n2t.net/addgene:67903> ; RRID:Addgene\_67903). The plasmid pFP5364 for the expression of <sup>FIRE</sup>mate-IRFP-Giantin was constructed by replacing the sequence coding for a FRAP domain in a plasmid for the expression of FRAP-Giantin with the sequence of coding for <sup>FIRE</sup>mate-IRFP (IRFP = infrared fluorescent protein IRFP670). The plasmid pAG1186 for the expression of Lyn11-ECFP-<sup>FIRE</sup>mate was constructed by replacing the sequence coding for TOM20 by Lyn11 sequence in the vector pAG1159. The plasmid pAG1211 for the expression of FRB-ECFP-Giantin was constructed by replacing the sequence coding for ECFP(W66A) by ECFP sequence in the Addgene vector (#67903) coding for FRB-ECFP(W66A)-Giantin<sup>16</sup>. The plasmid pAG1212 for the expression of mCherry-FKBP-<sup>FIRE</sup>tag was constructed by inserting the sequence

coding for <sup>FIRE</sup>tag in the vector pAG1190 coding for mCherry-FKBP. The plasmid pAG1242 for the expression of NLS-mCherry-<sup>FIRE</sup>tag was constructed by inserting the sequence coding for NLS in the vector pAG1160. The plasmid pAG1243 for the expression of NES-ECFP-<sup>FIRE</sup>mate was constructed by replacing the sequence coding for TOM20 by NES sequence in the vector pAG1159. The plasmid pAG1280 for the expression of NLS-mCherry-<sup>FIRE</sup>tag-NES was constructed by inserting the sequence coding for NES in the vector pAG1242. The plasmid pAG1247 for the expression of H2B-ECFP-<sup>FIRE</sup>mate was constructed by replacing the sequence coding for TOM20 by H2B sequence in the vector pAG1159. The plasmid pAG1244 for the expression of p65-mCherry-<sup>FIRE</sup>tag was constructed by inserting the sequence coding for p65, amplified from the Addgene vector GFP- RelA (#23255)<sup>32</sup>, in the vector pAG1160. GFP-RelA was a gift from Warner Greene (Addgene plasmid # 23255; <http://n2t.net/addgene:23255>; RRID:Addgene\_23255). The plasmid pAG1237 for the expression of <sup>FIRE</sup>mate-ECFP-Fis1 was constructed by inserting the sequence coding for Fis1, amplified from the Addgene vector pHAGE-mt-mKeima-P2A-FRB-Fis1 (#135295)<sup>33</sup>, in the vector pAG1165. pHAGE-mt-mKeima-P2A-FRB-Fis1 was a gift from Richard Youle (Addgene plasmid # 135295; <http://n2t.net/addgene:135295>; RRID:Addgene\_135295). The plasmid pAG1238 for the expression of PINK1-mCherry-<sup>FIRE</sup>tag was constructed by inserting the sequence coding for PINK1, amplified from the Addgene vector pEYFP-N1-Pink1 (#101874)<sup>34</sup>, in the vector pAG1160. pEYFP-N1-Pink1 was a gift from Richard Youle (Addgene plasmid # 101874; <http://n2t.net/addgene:101874>; RRID:Addgene\_101874). The plasmid pAG1345 for the expression of mCherry-<sup>FIRE</sup>tag-DEVDG-MoA was constructed by inserting the sequence coding for DEVDG-MoA, amplified from the Addgene vector ECFP(W66A)-FRB-MoA (#67904)<sup>16</sup>, in the vector pAG1160. ECFP(W66A)-FRB-MoA was a gift from Dorus Gadella (Addgene plasmid # 67904; <http://n2t.net/addgene:67904>; RRID:Addgene\_67904). The plasmid pFP5290 for the expression of <sup>FIRE</sup>mate-KDEL-IRES-mApple-<sup>FIRE</sup>tag-GPI was constructed by insertion of <sup>FIRE</sup>tag-GPI obtained from gene synthesis (Integrated DNA technology) into a vector for the expression of Streptavidin-KDEL-IRES-SBP-mApple-GPI (built from the plasmid pFP1774<sup>18</sup>, Addgene plasmid # 65294; <http://n2t.net/addgene:65294>; RRID:Addgene\_65294; see also Addgene plasmid #166904, <http://n2t.net/addgene:166904>), and then insertion of <sup>FIRE</sup>mate obtained from gene synthesis (Integrated DNA technology) instead of Streptavidin. The plasmid pFP5299

for the expression of <sup>FIRE</sup>mate-KDEL-IRES-ManII-mApple-<sup>FIRE</sup>tag was constructed by addition of <sup>FIRE</sup>tag into a vector for the expression of Streptavidin-KDEL-IRES-ManII-SBP-mApple (constructed from pFP1708<sup>18</sup>, Addgene plasmid # 65252; <http://n2t.net/addgene:65252>; RRID:Addgene\_65252) and then insertion of ManII-SBP-mApple-<sup>FIRE</sup>tag into pFP5290 instead of mApple-<sup>FIRE</sup>tag-GPI. The plasmid pFP5370 for the expression of <sup>FIRE</sup>mate-KDEL-IRES-TNF-mCherry-<sup>FIRE</sup>tag was constructed by insertion of <sup>FIRE</sup>tag in pFP1752<sup>18</sup>, (Addgene plasmid # 65279; <http://n2t.net/addgene:65279>; RRID:Addgene\_65279) and then insertion of TNF-mCherry-<sup>FIRE</sup>tag into pFP5290 instead of mApple-<sup>FIRE</sup>tag-GPI. The plasmid pFP5369 for the expression of KIF17MD-flag-<sup>FIRE</sup>mate was constructed by insertion of <sup>FIRE</sup>mate obtained from gene synthesis (Integrated DNA technology) into a vector for the expression of KIF17MD-flag-FRB (constructed from a vector given by Lukas Kapitein<sup>19,22</sup>) instead of FRB. The plasmid pFP5380 for the expression of LAMP1-mCherry-<sup>FIRE</sup>tag was constructed by insertion of mCherry-<sup>FIRE</sup>tag from pFP5370 in a plasmid for the expression of LAMP1-SBP-EGFP. The plasmid pAG1281 for the bacterial expression of 6xHIS-TEVcs-<sup>FIRE</sup>mate was constructed by removing the sequence encoding C-pFAST(115-125) from the plasmid pAG641<sup>14</sup> coding for 6xHIS-TEVcs-pFAST by Gibson assembly. The plasmid pAG1282 for the expression of 6xHIS-TEVcs-mCherry-<sup>FIRE</sup>tag was constructed by inserting the sequence coding for <sup>FIRE</sup>tag in the vector pAG224 (Gautier, unpublished) coding for 6xHIS-TEVcs-mCherry by Gibson assembly.

**Cell culture** HeLa cells (ATCC CRM-CCL2) were cultured in Minimal Essential Media (MEM) supplemented with phenol red, Glutamax I, 1 mM of sodium pyruvate, 1% (vol/vol) of non-essential amino-acids and 10% (vol/vol) fetal calf serum (FCS), at 37 °C in a 5% CO<sub>2</sub> atmosphere. HEK 293T (ATCC CRL-3216) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with phenol red and 10% (vol/vol) fetal calf serum at 37 °C in a 5% CO<sub>2</sub> atmosphere. U2OS cells (ATCC HTB-96) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with phenol red and 10% (vol/vol) fetal calf serum and 1% (vol/vol) penicillin–streptomycin at 37°C in a 5% CO<sub>2</sub> atmosphere. For imaging, cells were seeded in  $\mu$ Dish IBIDI (Biovalley) coated with poly-L-lysine. The ratios of plasmids used for each experiment are given in **Supplementary Table 2**. Cells were transiently transfected using Genejuice (Merck) according to the manufacturer's protocols for 24 prior to imaging.

Cells were washed with DPBS (Dulbecco's Phosphate-Buffered Saline), and treated with DMEM media (without serum and phenol red) supplemented with the compounds at the indicated concentration. For the p65 nuclear translocation experiments, cells were serum-starved for 4 hours before activation by  $\text{TNF}\alpha$ . For experiments on reversibility, secretory protein trafficking and organelle positioning, HeLa cells were cultured in DMEM supplemented with 10% FCS, 1 mM sodium pyruvate and 100  $\mu\text{g}/\text{mL}$  penicillin and streptomycin at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  atmosphere. HeLa cells were transfected using calcium phosphate as previously described<sup>35</sup>. For imaging, cells were seeded onto 25 mm-diameter glass coverslips. The day after transfection, coverslips were transferred into L-shape tubing Chamlide (Live Cell Instrument) filled with carbonate independent Leibovitz's medium supplemented with 10% FCS, and the compounds at the indicated concentration. Washouts were done by addition into the Chamlide of pre-warmed PBS (Phosphate-Buffered Saline) 2 mL at a time for a total volume of 15 mL, and then addition of Leibovitz's medium.

### **Protein expression in bacteria and purification**

Plasmids were transformed in BL21 (DE3) Competent *E. coli* (New England Biolabs). Cells were grown at  $37^\circ\text{C}$  in lysogen Broth (LB) medium supplemented with 50  $\mu\text{g}/\text{ml}$  kanamycin to  $\text{OD}_{600\text{nm}}$  0.6. Expression was induced overnight at  $16^\circ\text{C}$  by adding isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were harvested by centrifugation ( $4300 \times g$  for 20 min at  $4^\circ\text{C}$ ) and frozen. For purification, the cell pellet was resuspended in lysis buffer (PBS supplemented with 2.5 mM  $\text{MgCl}_2$ , 1 mM of protease inhibitor PhenylMethaneSulfonyl Fluoride PMSF, 0.025 mg/mL of DNase, pH 7.4) and sonicated (5 min, 20% of amplitude) on ice. The lysate was incubated for 2 h on ice to allow DNA digestion by DNase. Cellular fragments were removed by centrifugation ( $9000 \times g$  for 1 h at  $4^\circ\text{C}$ ). The supernatant was incubated overnight at  $4^\circ\text{C}$  by gentle agitation with pre-washed Ni-NTA agarose beads in PBS buffer complemented with 20 mM of imidazole. Beads were washed with 10 volumes of PBS complemented with 20 mM of imidazole and with 5 volumes of PBS complemented with 40 mM of imidazole. His-tagged proteins were eluted with 5 volumes of PBS complemented with 0.5 M of imidazole. The buffer was exchanged to PBS (0.05 M phosphate buffer, 0.150 M NaCl) using PD-10 desalting columns (GE

Healthcare). Purity of the proteins were evaluated using SDS-PAGE electrophoresis stained with Coomassie blue.

**Physico-chemical measurements** Steady state UV-Vis and fluorescence spectra were recorded at 25°C on a Spark 10M (Tecan). Data were processed using GraphPad Prism v9.3.0.

**Flow cytometry analysis** Flow cytometry in HEK 293T cells was performed on a MACSQuant® analyzer equipped with 405 nm, 488 nm and 635 nm lasers and seven channels. To prepare samples, cells were first grown in cell culture flasks, then transiently co-transfected 24 hours after seeding using Genejuice (Merck) according to the manufacturer's protocol for 24 hours. After 24 hours, cells were centrifuged in PBS with BSA (1 mg/ml) and resuspend in PBS-BSA supplemented with the appropriate amounts of compounds. For each experiment, 20,000 cells positively expressing mTurquoise2 (Ex 434 / Em 450 ± 25) and iRFP670 (Ex 638 nm / Em 660 ± 10 nm) were analyzed with the following parameters: Ex 488nm, Em 525 ± 20 nm. Data were analyzed using FlowJo v10.7.1. A figure presenting the gating strategy is presented in supporting information on **Supplementary Fig. 4**.

**Fluorescence microscopy** The confocal micrographs were acquired on a Zeiss LSM 980 Laser Scanning Microscope equipped with a plan apochromat 63× /1.4 NA oil immersion objective. ZEN software was used to collect the data. Confocal spinning disk images were acquired on a Nikon Inverted Eclipse Ti-E (Nikon) microscope equipped with a Spinning disk CSU-X1 (Yokogawa), an iXon EMCCD camera (Andor) integrated in Metamorph software by Gataca Systems, using an 60× CFI plan apochromat VC 1.4 NA oil immersion objective (Nikon). The images were analyzed using Icy (2.4.0.0), Fiji (Image J) and NIS-Elements 5.42 (Nikon). Images from **Fig. 4**, **Fig. 5** and **Extended Data Fig. 9** were enhanced by 2D-deconvolution on NIS-Elements Ver.5.42. To track fluorescence signal in a specific organelle, the organelle contour was determined by masking the signal in the ECFP channel with the plug-in *HK-means* with the following parameters: intensity class equals 100, min object size (px) equals 500, max object size (px) equals 1500-3000. The signal intensity of the ROI was tracked over the time for each channel by using plug-in *Active Contours*. The



background signal was subtracted. Data were processed using GraphPad Prism v9.3.0. The change in mitochondria morphology in the mitophagy experiments was performed measuring the area of mitochondria in each cell. The cells were segmented with CellPose 2.0 [www.cellpose.org](http://www.cellpose.org) using the mCherry channel. The area occupied by the mitochondria was quantified with the ECFP channel.

## DATA AVAILABILITY

The data supporting the findings of this study are available within the article and supplementary information, and are available from the corresponding authors upon reasonable request. Source data are provided with this paper. The plasmids developed in this study will be available from Addgene.

## METHODS-ONLY REFERENCES

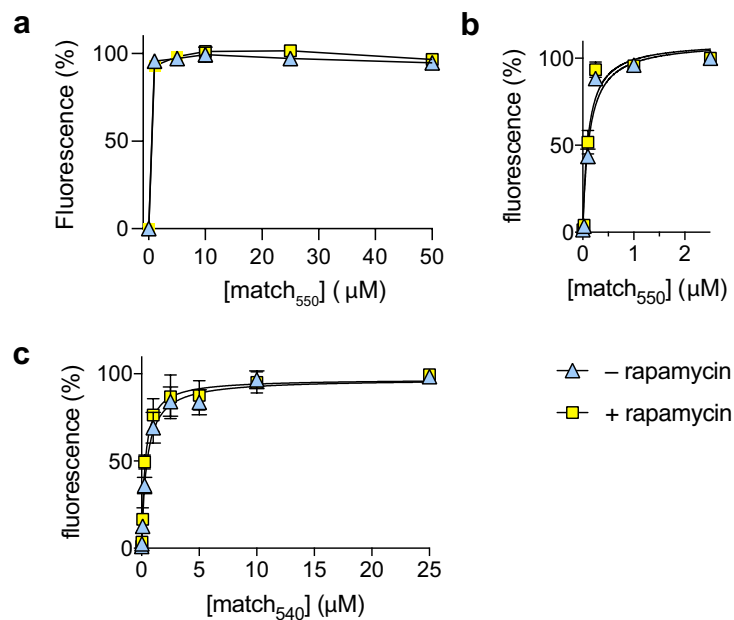
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## **Extended Data**

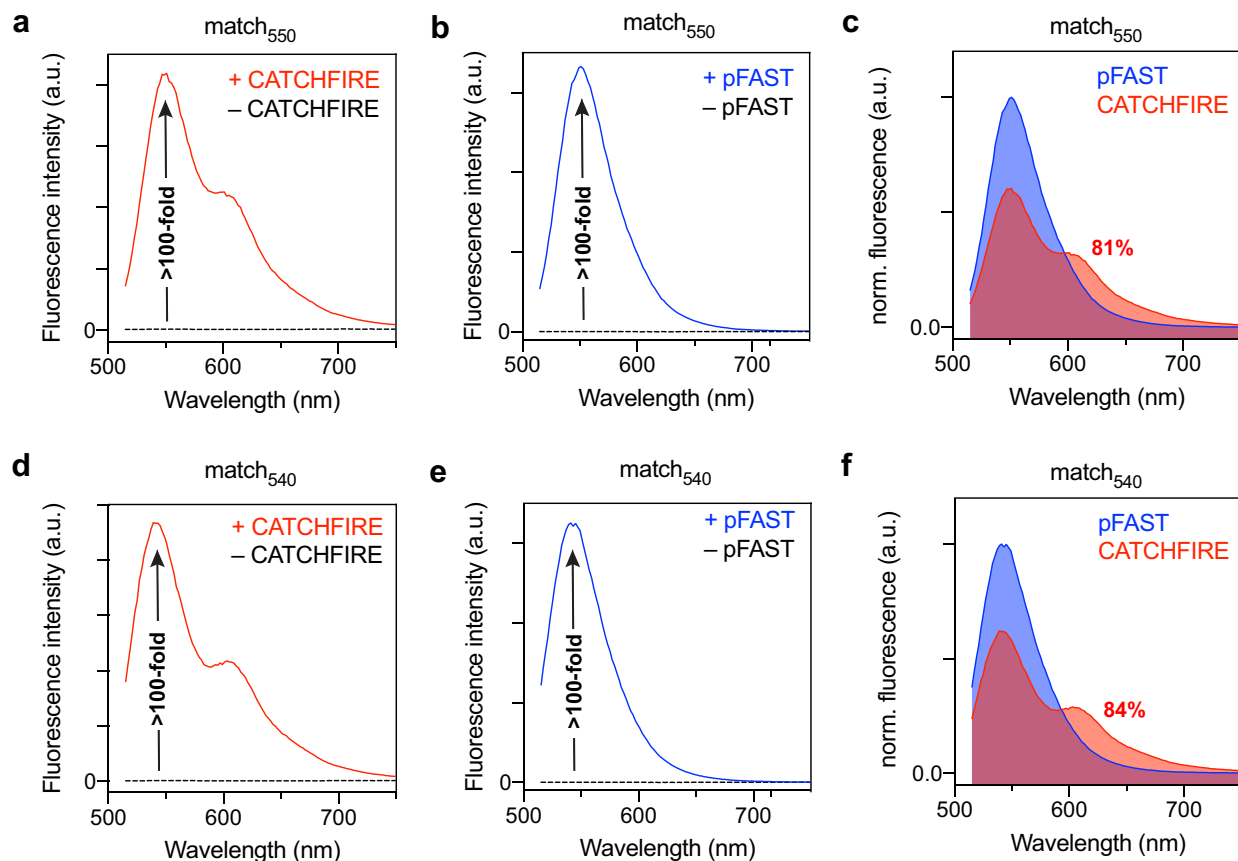
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Extended Data Figs. 1-10

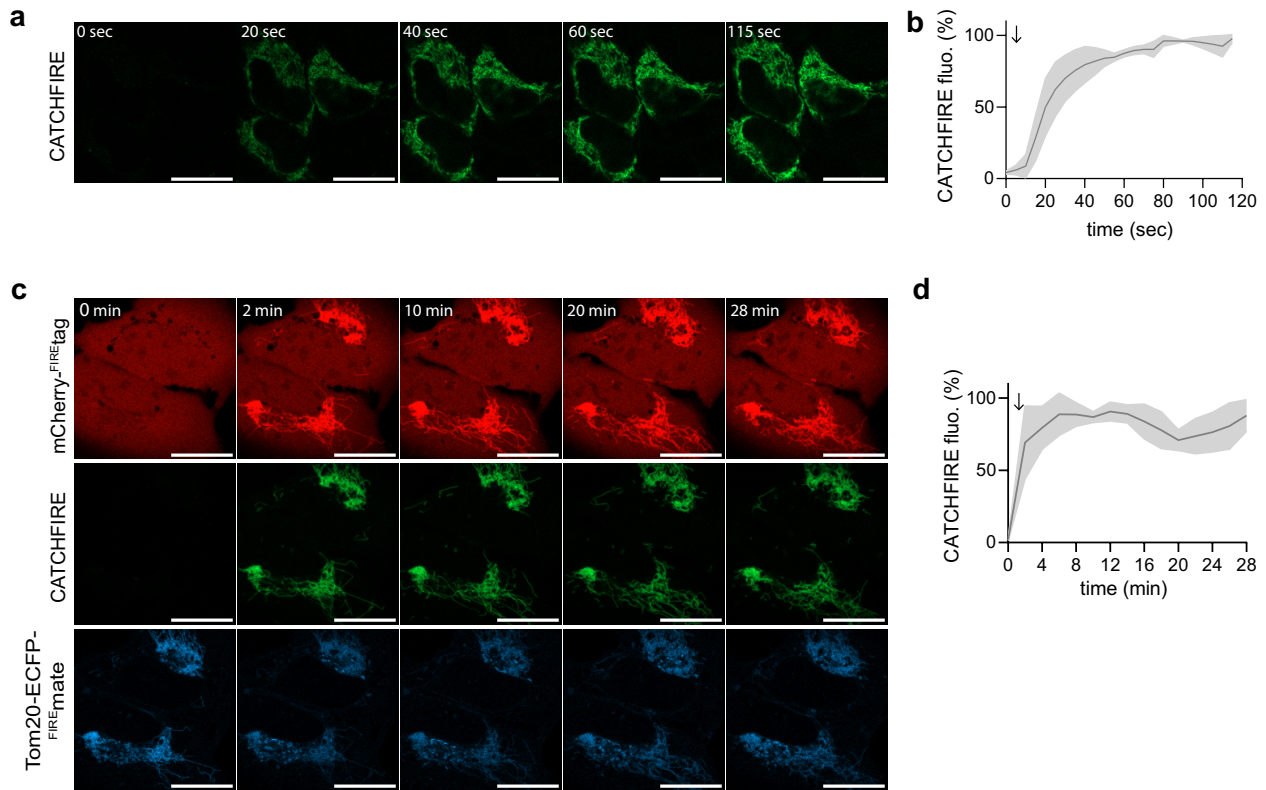
Extended Data References



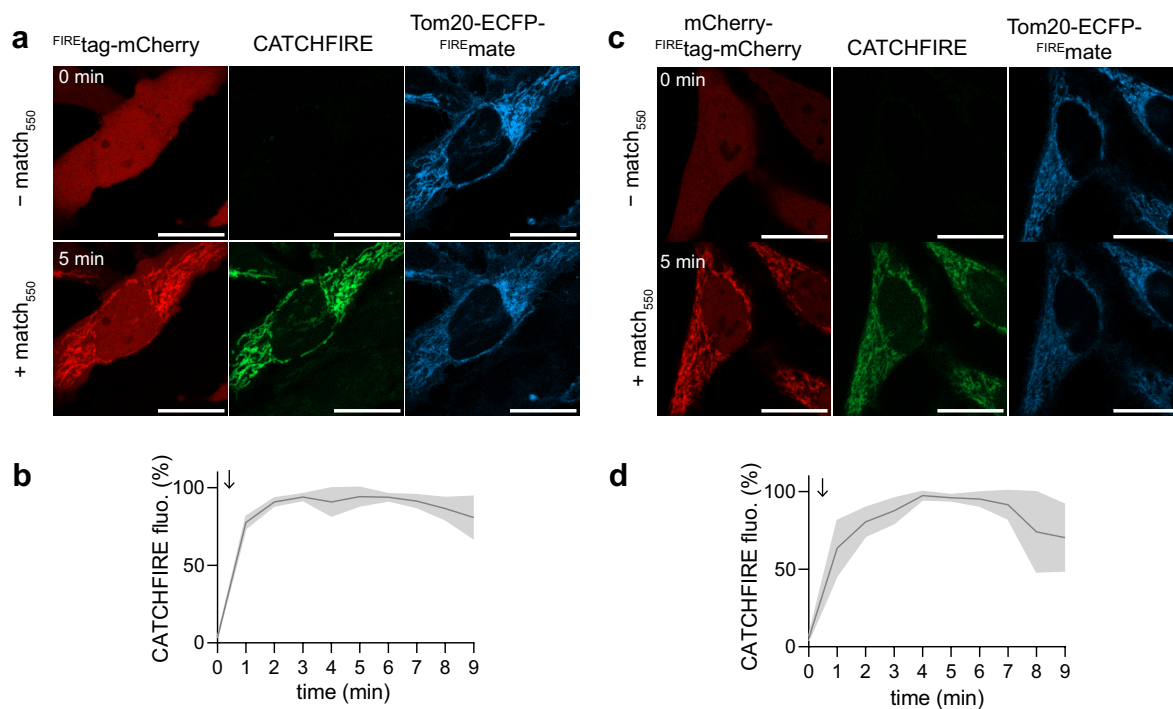
**Extended Data Fig. 1. Match-induced complementation of <sup>FIRE</sup>mate (a.k.a. pFAST<sub>1-114</sub>) and <sup>FIRE</sup>tag (a.k.a. pFAST<sub>115-125</sub>).** Normalized average fluorescence of about 20,000 HEK293T cells co-expressing the FK506-binding protein (FKBP) fused to <sup>FIRE</sup>tag and the FKBP-rapamycin-binding domain of mammalian target of rapamycin (FRB) fused to <sup>FIRE</sup>mate treated without or with 500 nM of rapamycin, and with **a** 1, 5, 10, 25 or 50 μM of match<sub>550</sub> (a.k.a. HBR-2,5DM), **b** 0.1, 0.25, 1 or 2.5 μM of match<sub>550</sub> (a.k.a. HBR-2,5DM), and **c** 0.1, 0.25, 1, 2.5, 5, 10 or 25 μM of match<sub>540</sub> (a.k.a. HMBR). Individual cell fluorescence was analyzed by flow cytometry. Data represent the mean values ± SD of three independent experiments. A figure presenting the gating strategy is presented on **Supplementary Fig. 4**.



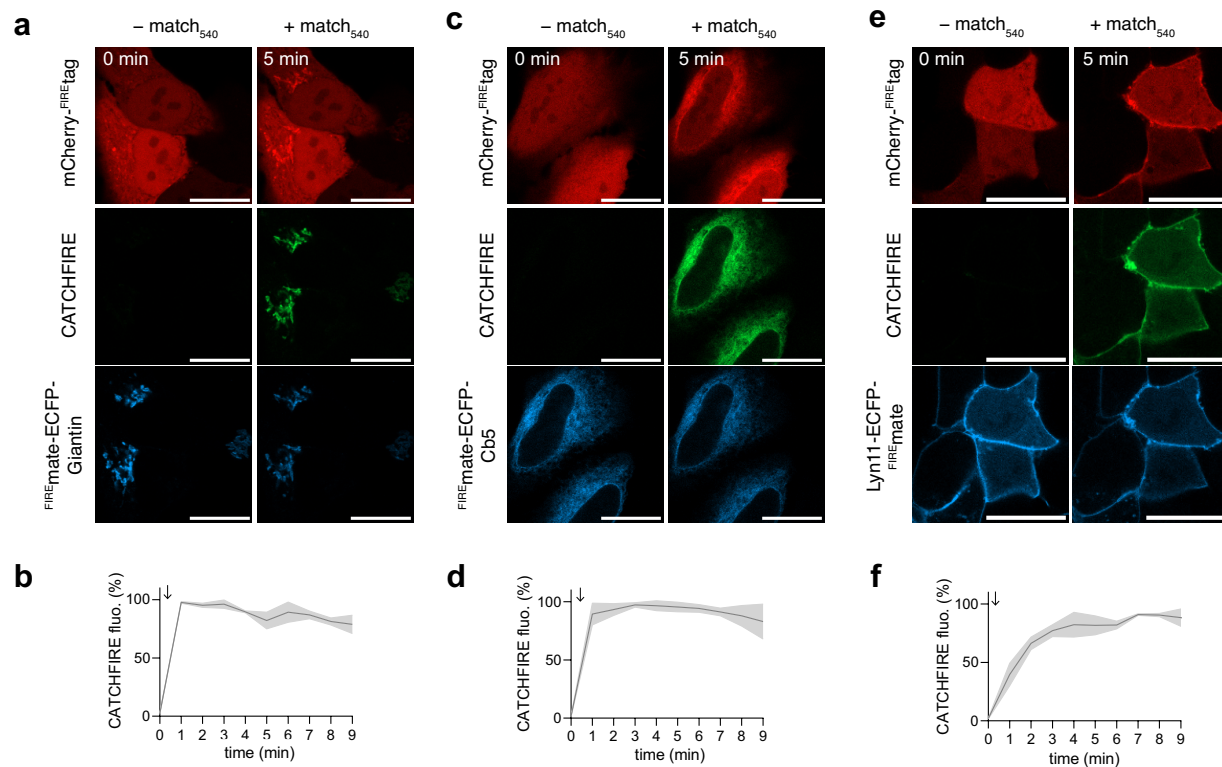
**Extended Data Fig. 2. Fluorescence activation of match<sub>550</sub> and match<sub>540</sub> upon complementation of <sup>FIRE</sup>mate and <sup>FIRE</sup>tag.** **a** Fluorescence spectra of match<sub>550</sub> when free (black dotted line) or in presence of recombinant <sup>FIRE</sup>mate and mCherry-<sup>FIRE</sup>tag (red solid line). [match<sub>550</sub>] = 6 μM, [<sup>FIRE</sup>mate] = 12 μM, [mCherry-<sup>FIRE</sup>mate] = 12 μM, λ<sub>exc</sub> = 470 nm. Note the observed shoulder at 615 nm corresponds to the signal resulting from the FRET between bound match<sub>550</sub> and mCherry. **b** Fluorescence spectra of match<sub>550</sub> when free (black dotted line) or in presence of recombinant pFAST (blue solid line). [match<sub>550</sub>] = 6 μM, [pFAST] = 6 μM, λ<sub>exc</sub> = 470 nm. **c** Fluorescence spectra of match<sub>550</sub> within CATCHFIRE vs pFAST. Spectra were normalized by the absorbance of bound match<sub>550</sub> at 470 nm for allowing direct comparison. **d** Fluorescence spectra of match<sub>540</sub> when free (black dotted line) or in presence of recombinant <sup>FIRE</sup>mate and mCherry-<sup>FIRE</sup>tag (red solid line). [match<sub>540</sub>] = 6 μM, [<sup>FIRE</sup>mate] = 12 μM, [mCherry-<sup>FIRE</sup>mate] = 12 μM, λ<sub>exc</sub> = 470 nm. Note the observed shoulder at 615 nm corresponds to the signal resulting from the FRET between bound match<sub>540</sub> and mCherry. **e** Fluorescence spectra of match<sub>540</sub> when free (black dotted line) or in presence of recombinant pFAST (blue solid line). [match<sub>540</sub>] = 6 μM, [pFAST] = 6 μM, λ<sub>exc</sub> = 470 nm. **f** Fluorescence spectra of match<sub>540</sub> within CATCHFIRE vs pFAST. Spectra were normalized by the absorbance of bound match<sub>540</sub> at 470 nm for allowing direct comparison.



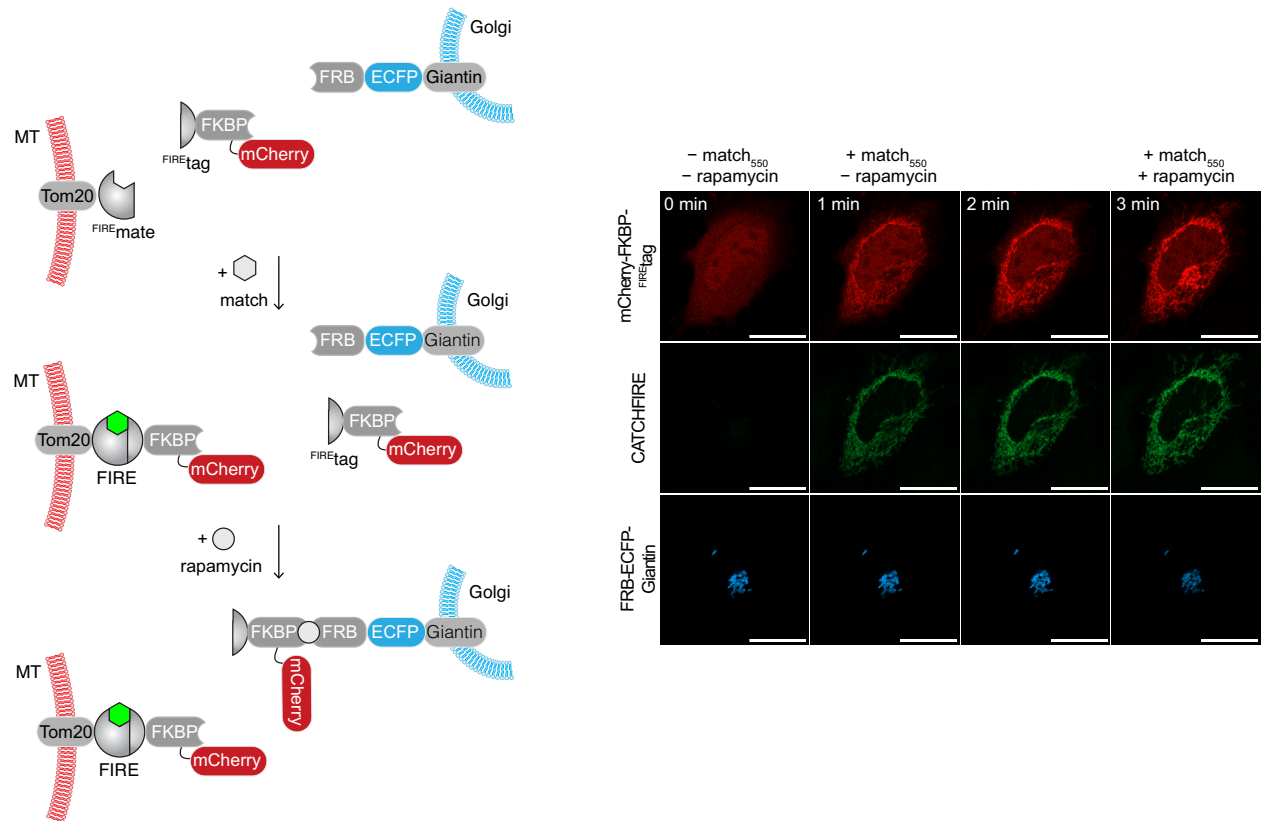
**Extended Data Fig. 3. Fluorogenic induced recruitment of cytoplasmic proteins to mitochondria.** **a,b** HeLa cells co-expressing mCherry-FIREtag and Tom20-ECFP-FIREmate were treated with 10  $\mu$ M match<sub>550</sub> and imaged by time-lapse confocal microscopy at 1 image per 5 seconds. **a** Representative time-lapse of FIRE (ex/em 488/508-570 nm) (see also **Supplementary Video 2**). Experiment was repeated three times with similar results. Scale bars are 20  $\mu$ m. **b** Temporal evolution of the FIRE signal. Data represents the mean value  $\pm$  SD of three independent experiments (n = 17 cells). **c,d** U2OS cells co-expressing mCherry-FIREtag and Tom20-ECFP-FIREmate were treated with match<sub>550</sub> and image by time-lapse confocal microscopy at 1 image every 2 minutes. **c** Representative time-lapse (mCherry: ex/em 561/606-675 nm; FIRE: ex/em 488/508-570 nm; ECFP: ex/em 445/455-499 nm) (see also **Supplementary Video 3**). Experiment was repeated three times with similar results. Scale bars are 20  $\mu$ m. **d** Temporal evolution of the CATCHFIRE signal. Data represents the mean value  $\pm$  SD of three independent experiments (n = 13 cells).



**Extended Data Fig. 4.  $\text{FIRE}^{\text{tag}}$  position does not influence CATCHFIRE efficiency.** HeLa cells co-expressing  $\text{FIRE}^{\text{tag}}$ -mCherry (a,b) or mCherry- $\text{FIRE}^{\text{tag}}$ -mCherry (c,d) and Tom20-ECFP- $\text{FIRE}^{\text{mate}}$  were treated with 10  $\mu\text{M}$  match<sub>550</sub> and imaged by time-lapse confocal microscopy. **a,c** Representative confocal micrographs of cells before (0 min) and after (5 min) addition of match<sub>550</sub> (mCherry: ex/em 561/606-675 nm; CATCHFIRE: ex/em 488/508-570 nm; ECFP: ex/em 445/455-499 nm) (see also **Supplementary Videos 4-5**). Experiments were repeated three times with similar results. Scale bars are 20  $\mu\text{m}$ . **b,d** Temporal evolution of the CATCHFIRE signal. Data represents the mean values  $\pm$  SD of three independent experiments (n = 19 cells (**b**) and 12 cells (**d**)).

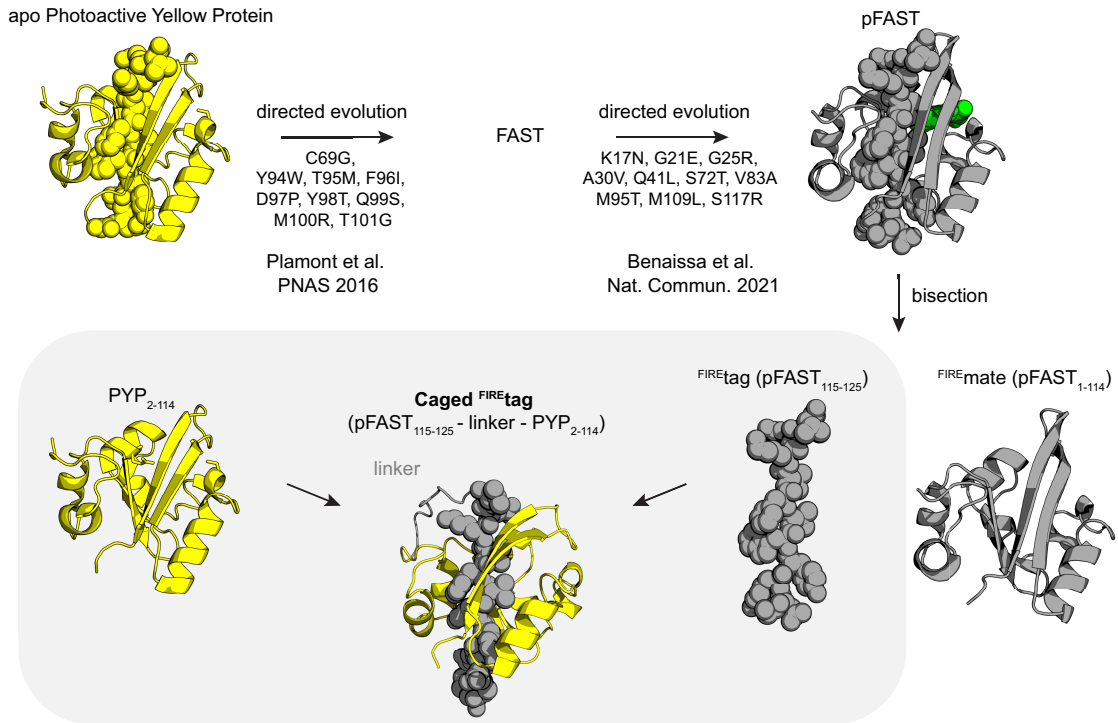


**Extended Data Fig. 5. CATCHFIRE to various organelles.** HeLa cells co-expressing mCherry-FIREtag and FIREmate-ECFP-Giantin (**a,b**), FIREmate-ECFP-Cb5 (**c,d**), Lyn11-FIREmate-ECFP (**e,f**) were treated with 10  $\mu$ M match<sub>540</sub>, and imaged by time-lapse confocal microscopy. **a,c,e** Representative confocal micrographs of cells before (0 min) and after (5 min) addition of match<sub>540</sub> (see **Supplementary Videos 12-14**). Experiments were repeated three times with similar results. **b,d,f** Temporal evolution of the CATCHFIRE signal. Data represents the mean values  $\pm$  SD of three independent experiments (n = 12 cells (**b**), 16 cells (**d**), 13 cells (**f**)).

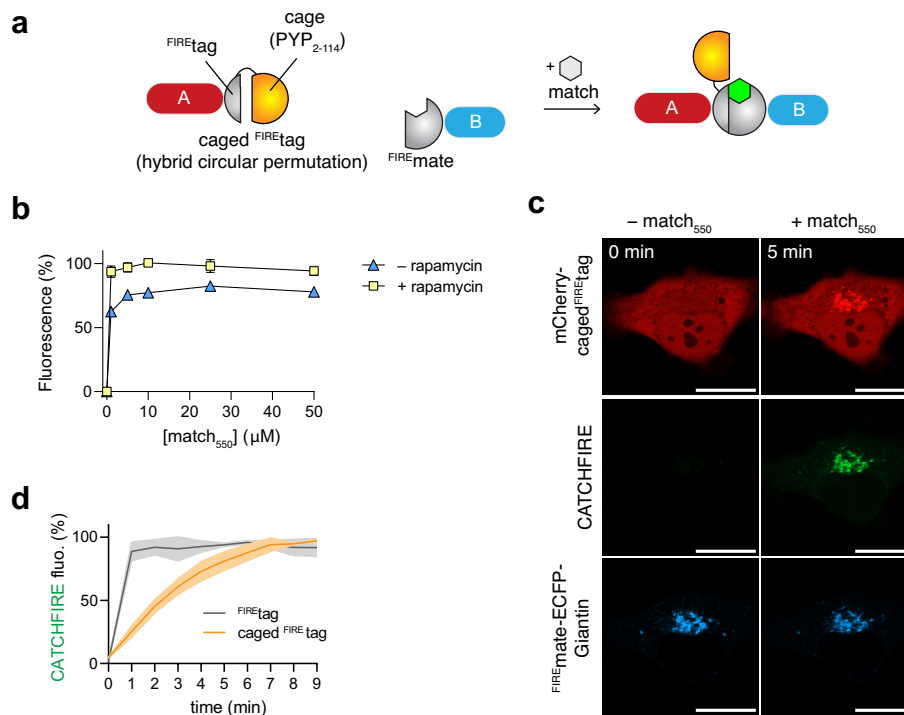


**Extended Data Fig. 6. Dual control with CATCHFIRE and FRB-rapamycin-FKBP.** HeLa cells co-expressing mCherry-FKBP<sup>-FIREtag</sup>, TOM20<sup>-FIREmate</sup> and FRB-ECFP-Giantin were treated with 10  $\mu$ M match<sub>550</sub> and then, after 2 min, with 500 nM rapamycin, and imaged by time-lapse confocal microscopy. Representative confocal micrographs of cells before, after addition of match<sub>550</sub>, and after addition of rapamycin. Red: ex/em 561/606-675 nm; green: ex/em 488/508-570 nm; cyan: ex/em 445/455-499 nm. Scale bars are 20  $\mu$ m.

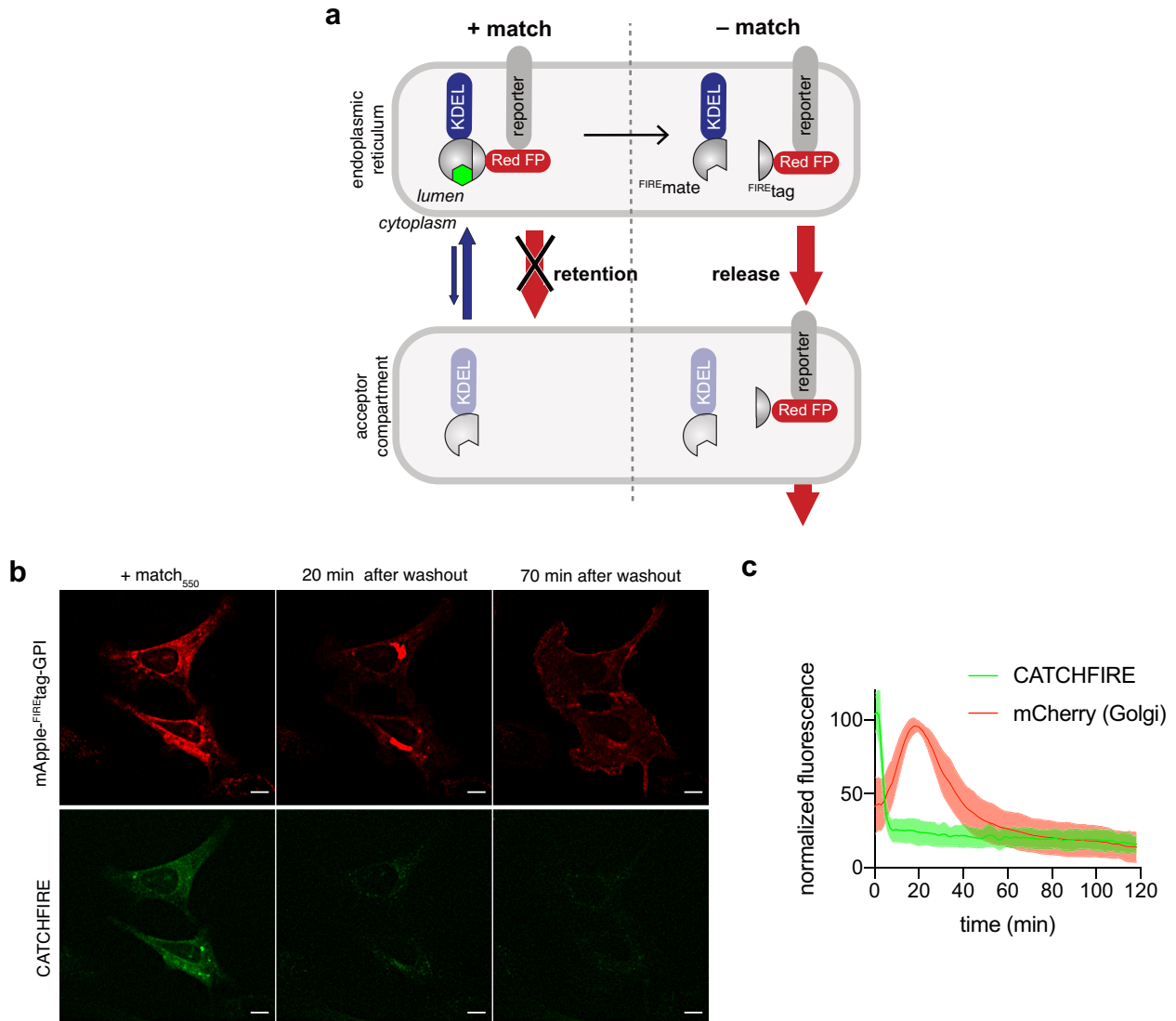




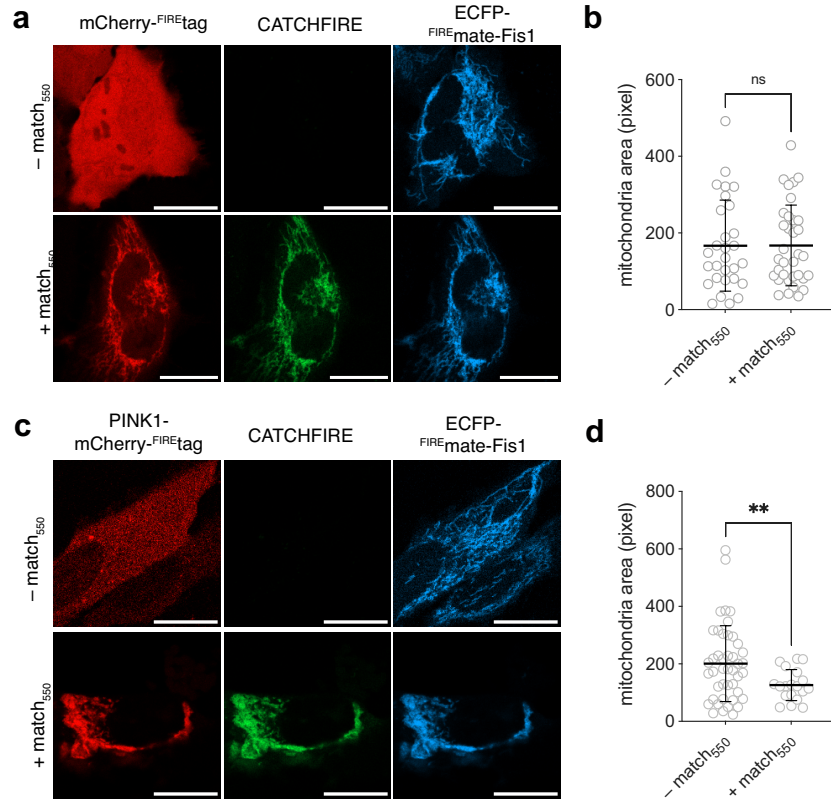
**Extended Data Fig. 7. Design of caged<sup>FIRE</sup>tag.** Fusion of <sup>FIRE</sup>tag at the N-terminal domain of the apo photoactive yellow protein (PYP<sub>2-114</sub>) resulted in a protein (caged<sup>FIRE</sup>tag) that folds like a circular permutation of PYP because of the intramolecular interaction between <sup>FIRE</sup>tag and PYP<sub>1-114</sub>, masking thus <sup>FIRE</sup>mate. The structure of apo PYP was generated from the crystal structure (PDB: 1NWZ). The model of fluorogen-bound pFAST was generated by homology modeling and molecular dynamics in ref.<sup>1</sup>. The model of caged<sup>FIRE</sup>tag was predicted using AlphaFold<sup>2</sup>.



**Extended Data Fig. 8. Caging  $\text{FIRE}^{\text{tag}}$  avoids residual self-association.** **a** Design of caged $\text{FIRE}^{\text{tag}}$ . **b** Normalized average fluorescence of about 50,000 HEK293T cells co-expressing the FK506-binding protein (FKBP) fused to caged $\text{FIRE}^{\text{tag}}$  and the FKBP-rapamycin-binding domain of mammalian target of rapamycin (FRB) fused to  $\text{FIRE}^{\text{mate}}$  treated without or with 500 nM of rapamycin, and with 1, 5, 10, 25 or 50  $\mu\text{M}$  of match $_{550}$ . Individual cell fluorescence was analyzed by flow cytometry. Data represent the mean values  $\pm$  SD of three independent experiments. **c,d** HeLa cells co-expressing mCherry-caged $\text{FIRE}^{\text{tag}}$  and  $\text{FIRE}^{\text{mate}}$ -ECFP-Giantin were treated with 10  $\mu\text{M}$  match $_{550}$ , and imaged by time-lapse confocal microscopy. **c** Representative confocal micrographs of cells before (0 min) and after (9 min) addition of match $_{550}$  (see also **Supplementary Video 16**). Experiments were repeated three times with similar results. Red: ex/em 561/606-675 nm; green: ex/em 488/508-570 nm; cyan: ex/em 445/455-499 nm. Scale bars are 20  $\mu\text{m}$ . **d** Temporal evolution of the CATCHFIRE signal. Data represents the mean values  $\pm$  SD of three independent experiments ( $n = 7$  cells).



**Extended Data Fig. 9. Control and tracking of secretory protein trafficking. a** Schematic illustrating how  $\text{FIRE}^{\text{mate}}$ -KDEL acts as a hook to retain  $\text{FIRE}^{\text{tag}}$ -ged reporter in the endoplasmic reticulum in presence of the match. Release is induced by washing of the match, allowing trafficking of the reporter to its acceptor compartment. **b,c** HeLa cells co-expressing  $\text{FIRE}^{\text{mate}}$ -KDEL and mApple- $\text{FIRE}^{\text{tag}}$ -GPI were treated with match<sub>550</sub> for 24 h and image by spinning-disk microscopy after washout of match<sub>550</sub>. **b** Representative micrographs before washout, 20 min and 70 min after washout (see also **Supplementary Video 20**). Experiments were repeated five times with similar results. **c** Temporal evolution of the CATCHFIRE signal in the ER (green) and the mCherry signal in the Golgi apparatus (red). Data represents the mean  $\pm$  SD of 14 cells. Red: ex/em 561/604-664 nm; green: ex/em 488/545-575 nm. Scale bars are 10  $\mu\text{m}$ . All images were enhanced by 2D-deconvolution on NIS-Elements Ver.5.42.



**Extended Data Fig. 10. Fluorogenic induced recruitment of PINK1 to mitochondria induces mitophagy.** **a-d** HeLa cells co-expressing ECFP-FIREmate-Fis1 and either **a,b** mCherry-FIREtag (negative control) or **c,d** PINK1-mCherry-FIREtag were treated without or with 10  $\mu$ M match<sub>550</sub> for 2 h. **a,c** Representative micrographs (see also **Supplementary Fig. 3** for additional examples). Experiments were repeated three times with similar results. Red: ex/em 561/606-675 nm; green: ex/em 488/508-570 nm; cyan: ex/em 445/455-499 nm. Scale bars are 20  $\mu$ m. **b,d** Quantification of the mitochondria area in individual cells. The mean  $\pm$  SD is reported (**b** n = 29 (- match<sub>550</sub>), n = 34 (+ match<sub>550</sub>); **d** n = 44 (- match<sub>550</sub>), n = 19 (+ match<sub>550</sub>)). A two-tailed Welch's *t*-test was used to compare the two distributions. \*\* p value = 0.0022, ns: non significant p value = 0.9859.

## Extended Data References

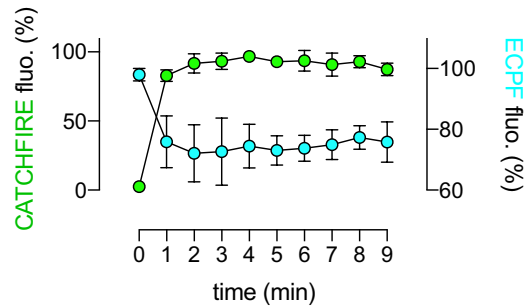
1. Benaissa, H. *et al.* Engineering of a fluorescent chemogenetic reporter with tunable color for advanced live-cell imaging. *Nat. Commun.* **12**, 6989 (2021).
2. Jumper, J. *et al.* Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583–589 (2021).

## **Supplementary Information**

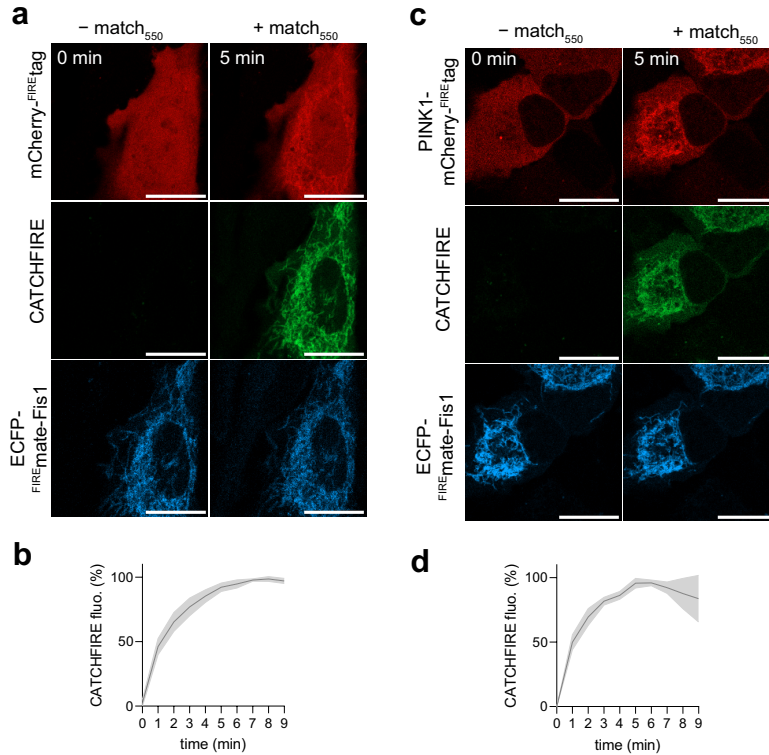
### **This PDF file includes:**

Supplementary Figs. 1-4

Supplementary Tables 1-2

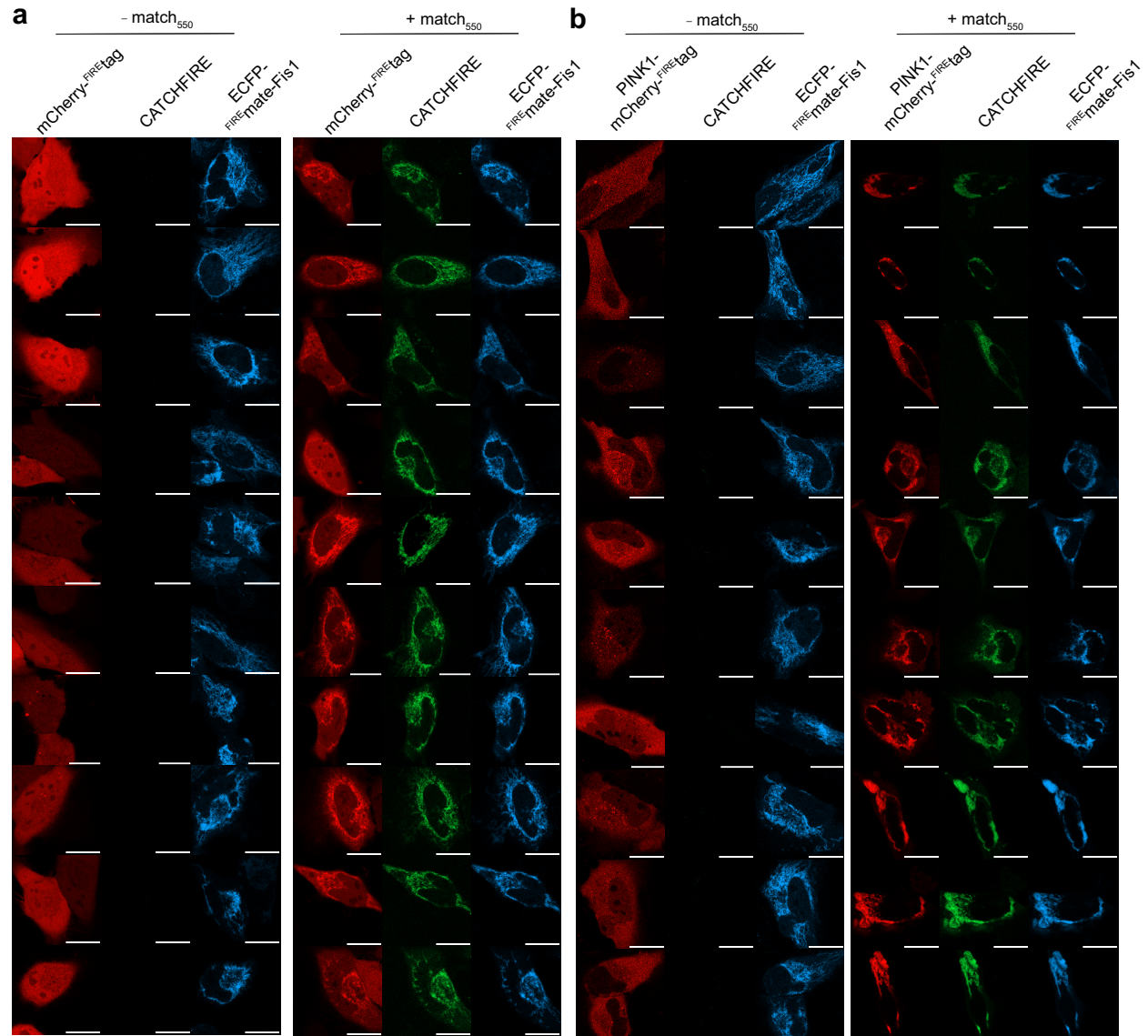


**Supplementary Fig. 1. FRET between ECFP and CATCHFIRE leads to a loss of ECFP fluorescence.** When using ECFP as an additional reporter of localization, we observed a decrease of the ECFP signal upon induction of CATCHFIRE, which we attributed to a loss of fluorescence by Förster resonance energy transfer between ECFP and the CATCHFIRE-induced ternary fluorescent assembly. As an example, the graph shows the temporal evolution of the ECFP fluorescence signal (right axis) upon CATCHFIRE induction (left axis) in HeLa cells co-expressing mCherry-<sup>FIRE</sup>tag and Tom20-ECFP-<sup>FIRE</sup>mate treated with 10  $\mu$ M match<sub>550</sub> (data shown on **Fig. 1c,d**). Data represents the mean  $\pm$  SD of three independent experiments (n = 17 cells).

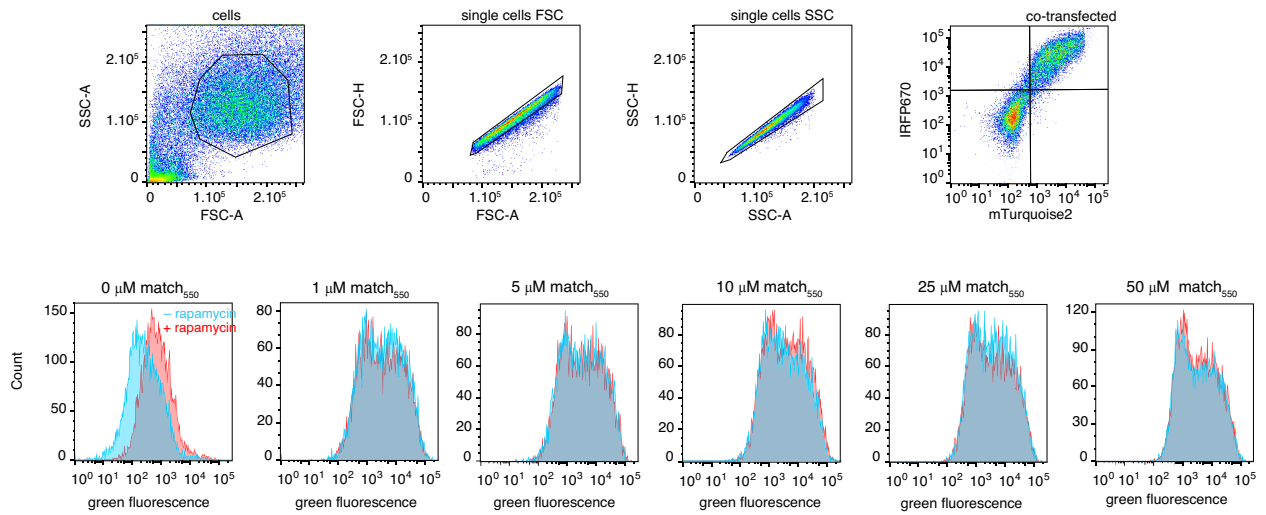


**Supplementary Fig. 2. Fluorogenic induced recruitment of PINK1 to mitochondria.** HeLa cells co-expressing ECFP-FIREmate-Fis1 and either **a,b** mCherry-FIREtag or **c,d** PINK1-mCherry-FIREtag were treated without or with match<sub>550</sub>, and imaged by time-lapse confocal microscopy. **a,c** Representative confocal micrographs of cells before (0 min) and after (5 min) addition of the fluorogenic inducer of proximity. Experiments were repeated three times with similar results. **b,d** Temporal evolution of the recruitment. Data represents the mean values  $\pm$  SD of three independent experiments (n = 8 cells (**b**) and 16 cells (**d**)). Red: ex/em 561/606-675 nm; green: ex/em 488/508-570 nm; cyan: ex/em 445/455-499 nm). Scale bars are 20  $\mu$ m





**Supplementary Fig. 3. Fluorogenic induced recruitment of PINK1 to mitochondria induces mitophagy.** HeLa cells co-expressing ECFP-FIREmate-Fis1 and either **a** mCherry-FIREtag (negative control) or **b** PINK1-mCherry-FIREtag were treated without or with match<sub>550</sub> for 2 h. Experiments were repeated three times with similar results. Representative micrographs are shown. Red: ex/em 561/606-675 nm; green: ex/em 488/508-570 nm; cyan: ex/em 445/455-499 nm). Scale bars are 20 μm



**Supplementary Fig. 4. Gating strategy for the cytometry experiments.** The figure presents the gating strategy for the flow cytometry experiments. The plasmids allowing the expression of FRB-FIRE<sub>mate</sub> and FKBP-FIRE<sub>tag</sub> also allowed the expression of mTurquoise2 and IRFP670, respectively, for transfection reporting. Measurement of the green fluorescence of doubly transfected cells enabled to quantify the complementation efficiency of FRB-FIRE<sub>mate</sub> and FKBP-FIRE<sub>tag</sub> in presence of various concentrations of match (see also **Extended Data Fig. 1**).



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pFP5299	ss-FIRE <sup>mate</sup> -HA-KDEL-IVS-IRES-ManII-SBP-mApple-FIRE <sup>tag</sup>	<p>ATGGATGTATGCGTCCGCTTTGCCCTTGCGCTCCTCTGGGACTCCTCTGCACCAGGGCCAGAGCCTCAGCCATAGTCA  CAGTGAGAAGGCGACAGAACAGCTCGGGCCGCGgtttaaacgagcagtgtgctttggcagtgaggacatcgagaaca  ctctggccaatatggacgacgaacaactggataggttggcctttggcgtaattcagctcgatggtagcgggaatctctg  ctgtacaatgctgctgaaggggacatcactggcagagatcccaaacaggtgattgggaagaactcttccaagatgttgc  acctggaacgggatactcccgagttttacggcaaatcaaggaagggcagcgtcagggaaatctgaacacatgttcgaat  ggcgcgataccgacaagcggggccaacaaggtcaaggtgcactgaagaagcccttccggaggtggcgggaagt<b>TAT</b>  <b>CCTTAAGCAGTACCAGACTACGCA</b> tccggaccg<b>AAGATGAACGTGA</b>ggggccGATAGATAACTGATCCAGTGTGCTG  GAATTAATTCGCTGTCTGCGAGGGCCAGCTGTGGGGTGAAGTACTCCCTCTCAAAGCGGGCATGACTCTGCGCTAAGA  TTGTCAGTTTCCAAAACAGGAGGATTTGATATTACCTGGCCCGCGGTGATGCCTTTGAGGTTGGCCCGCTCATCTG  GTCAGAAAAGCAACTCTTTTGTCTCAAGCTTGAGCTGTGGCAGGCTTGAGATCTGCCATACACTTGAGTCAAAATGA  CATCCACTTTGCCTTTCTCTCCACAGGTGCCACTCCCAGGTCCAACTGCAGGTCGAGCATGCATCTAGGGCGGCCAAT  CCGCCCCCTCCCTCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGAATAAGCCGGTGTGCGTGTGTCTATATGT  TATTTTCCACCATATGGCGCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACAGGACTTCTTAGG  GGTCTTTCCCTCTCGCCAAAGGAATGCAAGGTCTGTGAATGCTGGAAGGAAGCAGTCTCTGGAAGCTTTTGAAG  ACAAACAACGCTCTGAGCAGCCCTTTGACAGGACGGAAACCCCACTGGCAGCAGGTCCTCTGCGCCAAAAGCCAC  GTGTATAAGTACACTTGCAAAAGGGCCACAACCCAGTCCACAGTGGCCCTGCAGGCTGACCCAGAGACTTTGGATCCAC  CTCTCTCAAGCTATTCAACAGGGCTGAAGGATGCCAGAAGTACCOCATTTGATGGGATCTGATCTGGGGCCCTG  GTGCACATGCTTTACATGTGTTTGTAGTCAGGTTAAAAAACGCTTAGGCCCCCGAACCCAGGGGAGCGTGTTCCTTT  GAAAAACAGATGATAAGCTTGCACAAACcggggaGGcgcc<b>ATGAAGTTAAGTCGCAGTTCACCGTTCGCGAGG</b>  CGATCTTCTCGCTCGTAATCTTCTCACTTCACTTCACTTCACTTCACTTCACTTCACTTCACTTCACTTCACTTCACTT  GAGGCTCTCTTCCGCAAGGCGACGTTTCAATATTGCAAGAAAAGATTGACCATTTGGAGCGTTCCTCGCTGAGAACAA  CGAGATTATCTCAAAATCAGAGACTCAGTCACTCACTGAGCAGTCTGTGGAGGACGGCCCGGGGGTCCACCGAAC  AGCGCCAGCAAGGCTCCATCCACTCCACTCGCCACAGTGGCCCTGCAGGCTGACCCAGAGACTTTGGATCCACCC  GGTCGCCACCGG<b>aattcc</b> <b>GACGAGAAGACCCTGGTTGGCGAGGTGGACACTGTTTGAAGGACTGGCTGGGAACTTGA</b>  <b>ACAACCTCTGCAGCTGGAGCATCCACCACAAGCTCAACGTGAACCA</b> ctggcaggt<b>GTGAGCAAGGGCCAGGAGTA</b>  ACATGGCCATCATCAAGGAGTTCATGGCTTCAAGGTGCACATGGAGGGCTCCGTGAAGGCCACGAGTTCGAGATCGAG  GGCGAGGGGGAGGGCCCGCCCTACGAGGGCTTTCAGACCGCTAAGCTGAAGGTGACCAAGGGTGGCCCGCTGCCCTTCG  CTGGGACATCCTGTCCCTTCACTGACGGCTCCAAGGTCTACATTAAAGACCACCGGCACATCCCGCCACTTCCG  AGCTGTCTTCCCGAGGGCTTCAAGTGGGAGCGCTGATGAATCTGAGGACGGCGGCATTTACAGTTAAACAGGAC  TCCTCCCTGAGGACGGCGTGTATCTACAAGGTGAAGTGGCGGCCACCACTTCCCTCCGAGCGAGTTCAGATGA  GAAGAAGCCATGGGTGGGAGGCTCCGAGGAGCGGATGTACCCGAGGACGGCCCTGAAGAGCGAGATCAAGAAGA  GGCTGAAGCTGAAGGACGGCGCCACTACGCCCGGAGGTCAAGACCCTACAAGGCCAAGAAGCCCTGCAGCTGCC  GGCGCTACATCTGCACATCAAGTTGGACATCTGTGCCACAACGAGGACTACACCCTCGTGAACAGTACGACAGCCG  CGAGGGCCCGCACTCCACCGGGCATGGACGAGCTGT<b>ACAAG</b>ggcgccCaggcggggctccggtgacagatattggg  tctttgtgaaacgggtgggcctcgagGGCaaTTAatTAActtagATAA</p>
pFP5370	ss-FIRE <sup>mate</sup> -HA-KDEL-IVS-IRES-TNF-SBP-mCherry-FIRE <sup>tag</sup>	<p>ATGGATGTATGCGTCCGCTTTGCCCTTGCGCTCCTCTGGGACTCCTCTGCACCAGGGCCAGAGCCTCAGCCATAGTCA  CAGTGAGAAGGCGACAGAACAGCTCGGGCCGCGgtttaaacgagcagtgtgctttggcagtgaggacatcgagaaca  ctctggccaatatggacgacgaacaactggataggttggcctttggcgtaattcagctcgatggtagcgggaatctctg  ctgtacaatgctgctgaaggggacatcactggcagagatcccaaacaggtgattgggaagaactcttccaagatgttgc  acctggaacgggatactcccgagttttacggcaaatcaaggaagggcagcgtcagggaaatctgaacacatgttcgaat  ggcgcgataccgacaagcggggccaacaaggtcaaggtgcactgaagaagcccttccggaggtggcgggaagt<b>TAT</b>  <b>CCTTAAGCAGTACCAGACTACGCA</b> tccggaccg<b>AAGATGAACGTGA</b>ggggccGATAGATAACTGATCCAGTGTGCTG  GAATTAATTCGCTGTCTGCGAGGGCCAGCTGTGGGGTGAAGTACTCCCTCTCAAAGCGGGCATGACTCTGCGCTAAGA  TTGTCAGTTTCCAAAACAGGAGGATTTGATATTACCTGGCCCGCGGTGATGCCTTTGAGGTTGGCCCGCTCATCTG  GTCAGAAAAGCAACTCTTTTGTCTCAAGCTTGAGCTGTGGCAGGCTTGAGATCTGCCATACACTTGAGTCAAAATGA  CATCCACTTTGCCTTTCTCTCCACAGGTGCCACTCCCAGGTCCAACTGCAGGTCGAGCATGCATCTAGGGCGGCCAAT  CCGCCCCCTCCCTCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGAATAAGCCGGTGTGCGTGTGTCTATATGT  TATTTTCCACCATATGGCGCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACAGGACTTCTTAGG  GGTCTTTCCCTCTCGCCAAAGGAATGCAAGGTCTGTGAATGCTGGAAGGAAGCAGTCTCTGGAAGCTTTTGAAG  ACAAACAACGCTCTGAGCAGCCCTTTGACAGGACGGAAACCCCACTGGCAGCAGGTCCTCTGCGCCAAAAGCCAC  GTGTATAAGTACACTTGCAAAAGGGCCACAACCCAGTCCACAGTGGCCCTGCAGGCTGAGATTTGGAAGGATCAAATG  CTCTCTCAAGCTATTCAACAGGGCTGAAGGATGCCAGAAGTACCOCATTTGATGGGATCTGATCTGGGGCCCTG  GTGCACATGCTTTACATGTGTTTGTAGTCAGGTTAAAAAACGCTTAGGCCCCCGAACCCAGGGGACCTGGTTCCTTT  GAAAAACAGATGATAAGCTTGCACAAACcggggaGGcgcc<b>atgtctaccgagctctatgattaggagctggaactgg</b>  ctgaggaggaactgccccaaaaaacggcggaaccaagggctc  taggagatgtctgtttctgtctctgttctctttct  gcgtgattggaccacagcagcaggaatcttcccgagctctgctcaactcttccaccagtggaacagcagctctccagatct  tctcgactccatccgacaacactgtcgcactgtcgtcgcaaccccaagggcagggccagctccagtggtccaatag  gagggcaaacgctctgctcgccaatggcgtggaactccgggataaccagctctgctgctgctatgtaggactccactca  tctactccaggtgctgttaaggggcagggatgctctctacacatgtgctgctcaacacacaacttcaaggtatgccc  gtgtcttaacagactaaagtcaactctgctctctgccaataatccccatgtcagcgggaaacaactgagggcgctgagggc  taaaccttggtagcaaacctctactcggagggctgttccagctgggaaagggcagatagactgagtgatgagataact  gaccogactacctcgacttgcgaacttggccaggtctactttggcactattgctctggccctcaggggocagctcagct  ggcgacactaggcctttgaatt<b>cc</b> <b>ATGACGAGAAGACCCTGGTTGGCGAGGTGGACACTGTTTGAAGACTGGCTCG</b>  <b>GGAACTTGAACAACCTTCGTGCAGACTGGAGCATCCACCACAAGGTCAACGTGAACCA</b>  cctgaggtatggtgagcaagggcagggagataaacatggccatcaacagggagtcaactgagcttcaaggtgcaacatgga  gggctccgtgaaacggccacagctcagatcgagggcagggcagggccgcccctacagggccaccocagcccaagc  tgaagtgacaaggggtggccccctgctctgctgggaaactctgctccccctcagttcaatgagctcaagggctcaag  gtgaaaccccccgacacccccactacttgaactgctctccccagggcttcaagtgaggagcggctgtagtaact  cgaggacggcggtggtgacogtgaccaggaactctccctacaggaacggcaggttcaactcaaggtgaagctgcgagc  gcaccaacttccccccagcggccccgtaatgcagaagaagaccatgggctgggagggcctcccgagcggatgacccc  gaggacggcgccctgaagggcgagatcaagcagaggtggaagtgaggacggcgccactacagcgtgagctcaagac  caoctacaagccaaagcccctgcaagctccccggccctacaacgtcaacatcaagttggacatcaactccccacaagc  aggactacacatcgtgaaacagtcagaacggccgagggccgcccactccaccggcgcatggacagctgtacaagggc  cggccatctagagggcgggctcc<b>ggtgacagatattgggtctttgtgaaacgggtgctcgagtga</b></p>
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## Supplementary Table 2. Experimental conditions

Figure	plasmids	Ratio
Fig. 1c,e,i	pAG1159 + pAG1160	5:1
Fig. 1g	pAG1159 + pAG1344	5:1
Fig. 2a	pAG1171 + pAG1160	5:1
Fig. 2c	pAG1169 + pAG1160	5:1
Fig. 2e	pAG1186 + pAG1160	5:1
Fig. 2g	pFP5364 + pAG1160	5:1
Fig. 3b	pAG1243 + pAG1242	5:1
Fig. 3f	pAG1247 + pAG1280	5:1
Fig. 4b	pFP5370	
Fig. 4e	pFP5299	
Fig. 5b	pFP5380 + pFP5369	5:1
Fig. 6a	pAG1237 + pAG1160	5:1
Fig. 6c	pAG1237 + pAG1238	5:1
Fig. 7a,d	pAG1247 + pAG1244	5:1
Fig. 7h	pAG1247 + pAG1345	5:1
Fig. S1	pAG1142 + pAG1151	1:1
Fig. S4a,c	pAG1159 + pAG1160	5:1
Fig. S5a	pAG1159 + pAG1209	5:1
Fig. S5c	pAG1159 + pAG1241	5:1
Fig. S6a	pAG1171 + pAG1160	5:1
Fig. S6c	pAG1169 + pAG1160	5:1
Fig. S6e	pAG1186 + pAG1160	5:1
Fig. S7	pAG1192 + pAG1211 + pAG1212	5:5:1
Fig. S9b	pAG1141 + pAG1142	1:1
Fig. S9c	pAG1171 + pAG1188	5:1
Fig. S10	pFP5290	
Fig. S11a	pAG1237 + pAG1160	5:1
Fig. S11c	pAG1237 + pAG1238	5:1

