Chemically responsive protein switches for the precise control of biological activities

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ABSTRACT

Chemically induced proximity/dimerization (CIP/D) is a powerful tool for basic biological research, biotechnology and cell therapy applications. In this approach, small molecules inducing the proximity or interaction of proteins enable the chemical regulation of cellular functions. Here, we employ CATCHFIRE (chemically assisted tethering of chimera by fluorogenic induced recognition) for creating chemically responsive proteins switches for the precise and reversible control of various biological activities. We demonstrated that CATCHFIRE could be used to chemically induce the assembly and thus function of various split enzymes such as luciferases, proteases or DNA recombinases. We extended this approach to the control of transcription activators and developed CATCH-ON for chemically inducible gene expression. Relying on the chemically induced dimerization of the DNA-binding domain GAL4 and the truncated transcription factor p65Δ, CATCH-ON allowed us to precisely regulate the expression of cellular enzymes such as proteases, DNA recombinases, or suicide switches, as well as to control the secretion of therapeutically relevant proteins such as insulin. We showed that the CATCH-ON system is fast-acting, reversible, titratable, non-toxic and

compatible with other CIP/D systems, opening exciting possibilities for its application in basic research, biotechnology and cell therapy.

INTRODUCTION

Protein-protein interactions are key molecular recognition events controlling biological processes in living systems. Chemically induced proximity/dimerization (CIP/D) tools that rely on small molecules to induce the interaction of two proteins enable to study the functional role of protein proximity in specific biological processes and to create chemically responsive systems for precise control of biological activities. The use of non-toxic small-molecules as specific triggers present various advantages. Their ease of administration, good biodistribution and cell permeability allow simple and rapid control of biological processes in a dose-dependent and in some cases in a reversible manner. This approach has been widely used in the last two decades to regulate various cellular processes, including gene transcription[1–4], signal transduction[5–7], proteasome-[8–10] and lysosome-based protein degradation[11], protein post-translation modifications[12,13] or protein folding and localization[14–16]. Beyond their use in basic research, chemically responsive protein switches are increasingly used to tune the function of therapeutic cells, such as CAR-T cells[6,7,17–19], and mitigate potentially life-threatening side effects[20–23].

One of the most popular CID approaches is based on rapamycin and its rapalogs that promote the interaction of two dimerization domains derived from FK506-binding protein (FKBP) and FKBP-binding protein (FRB)[21]. We recently expanded the CID toolbox with CATCHFIRE (chemically assisted tethering of chimera by fluorogenic-induced recognition)[15], a unique CID system with fluorescence read-out. CATCHFIRE was designed by splitting pFAST[24], a chemogenetic fluorescent reporter made of a 14 kDa protein domain that binds and stabilizes the fluorescent state of fluorogenic ligands. The two complementary fragments of pFAST, dubbed ^{FIRE}mate (114 aa, 12 kDa) and ^{FIRE}tag (11 aa, 1.4 kDa), only assemble in the presence of the fluorogenic ligands. These small ligands, called match, act thus as fluorogenic molecular glues that light up in the process, enabling to monitor the dimerization process in real-time by fluorescence microscopy. Because match molecules display high cell permeability, CATCHFIRE enables rapid and reversible control of protein dimerization. This approach

allowed for the precise temporal control of protein localization, organelle positioning and protein secretion. Furthermore, its intrinsic fluorogenic nature enabled the design of sensors of cellular processes[15].

In this study, we explored the potential of CATCHFIRE for the design of chemically responsive protein switches for the precise control of protein activities and cellular processes. We successfully utilized CATCHFIRE to chemically modulate the activity of split versions of enzymes such as luciferases, proteases and DNA recombinases, and to manipulate the assembly of chimeric transcription regulators for the accurate regulation of gene expression. These chemically responsive systems enabled us to precisely regulate various cellular processes relevant for cell biology, synthetic biology or for more applied applications in biotechnology and cell therapy.

RESULTS

Chemically responsive luciferase

Chemically responsive enzymes have important applications in synthetic biology. To demonstrate the suitability of CATCHFIRE to control protein function, we first engineered chemically responsive bioluminescent enzymes. Split luciferases are ideal systems as their activity can be followed by measuring their bioluminescence. We fused ^{FIRE}mate and ^{FIRE}tag to the SmBiT and LgBiT fragments of the NanoBiT technology derived from the blue-emitting luciferase NanoLuc[25,26] (**Figure 1a-b** and **Supplementary Figure 1**). The chemical responsiveness of the different possible topologies was studied by measuring bioluminescence in the absence or the presence of $match_{550}$ (a.k.a. HBR-2,5DM). The different systems were compared to a rapamycin-inducible NanoBiT based on FRB and FKBP (**Figure 1** and **Supplementary Figure 1**). A preliminary screening using a two-plasmid transfection strategy allowed us to identify LgBiT-^{FIRE}tag and ^{FIRE}mate-SmBiT as the best topology for recapitulating the NanoBiT technology (**Figure S1**). To alleviate the mosaic effect and better

control the ratios of the two protein parts, we co-expressed the two proteins using a polycistronic expression cassette containing a viral P2A sequence for ribosomal skipping during translation. We observed high bioluminescence efficiency, achieving about 7% of that of the full-length NanoLuc (**Figure 1**), similar to the rapamycin-responsive system, demonstrating the efficiency of CATCHFIRE for recapitulating the function of NanoBiT. In an attempt to reduce self-complementation in the absence of match and increase the dynamic range of the system, we replaced ^{FIRE}tag with a previously described caged version[15] incorporating a masking domain (NPYP), derived from the N-terminal fragment of the *Halorhodospira halophila* Photoactive Yellow Protein PYP used to design the FAST technology[27]. By masking ^{FIRE}tag, NPYP prevents residual complementation with ^{FIRE}mate, reducing thus self-complementation. The use of caged ^{FIRE}tag allowed to reduce self-complementation by three-fold but with the trade-off of reducing the dynamic range, and decreasing the total signal in the presence of match to only 1% of that of full-length NanoLuc.

Using our best chemically responsive NanoBiT, we could furthermore (i) determine the optimal match concentration for efficient complementation, and (ii) expand the palette of fluorogenic ligands suitable for CATCHFIRE. The molecules $match_{550}$ (a.k.a. HBR-2,5DM) and $match_{600}$ (a.k.a. HBR-3,5DOM) were previously used for inducing CATCHFIRE with green and red fluorescence (λ_{em} = 550 and 600 nm respectively)[15]. Since pFAST can bind and activate the fluorescence of a palette of fluorogenic ligands with different wavelengths[24], we tested some of them in the context of CATCHFIRE (**Supplementary Figure 2**). These experiments showed that several ligands could efficiently induce NanoBiT complementation with half-maximum effective concentration (EC_{50}) values ranging from 0.1 to 1 μM.

Chemically responsive protease

Next, we demonstrated that CATCHFIRE could be used to design chemically responsive proteases leveraging known split proteases. Split proteases are modular enzymes used to

control various cellular functions[2,5,28–34]. Chemically responsive proteases can enable precise regulation of proteins containing their specific cleavage sequence. The irreversible cleavage can change the structure and/or the localization of the protein, leading to a gain or loss of function and downstream cellular effects[5,28,29]. One of the most popular split protease systems is derived from the tobacco etch virus protease (TEVp)[5]. Its activity was previously controlled with small molecules[5,30–33] or light[28]. Upon activation, TEVp cleaves a specific sequence composed of seven amino acids (ENLYFQ/S), allowing, for example, the control of gene expression[5,30,31], protein secretion[2,32], cellular viability[34] or the creation of logic gates for cellular control[33,34].

To design split TEVp controllable with CATCHFIRE, we fused ^{FIRE}tag and ^{FIRE}mate at the N-terminus of the two split fragments of TEVp, generating two chemically responsive proteases. We analyzed their match-responsiveness using a proteolysis assay based on a circularized inactive Luc2 that opens and activates upon cleavage by TEVp. The two systems were compared to a pre-existing version of abscisic acid (ABA)-responsive split TEVp based on the ABA-dependent interaction of PYL1 and ABI[33] (**Figure 2a-c**). After 1 h of treatment with match, we observed a 5-fold increase of Luc2 bioluminescence, reaching 18% (for ^{FIRE}tag-NTEVp/^{FIRE}mate-CTEVp) and 6% (for ^{FIRE}mate-NTEVp/^{FIRE}tag-CTEVp) of the level reached with full-length TEVp. By comparison, treatment of ABA-responsive TEVp with ABA led only to a two-fold bioluminescence increase. The lower dynamic range of the ABA-responsive TEVp is due in part to a higher self-complementation.

An interesting feature of proteases is their composability with other modular systems to create complex networks thanks to their unique cleavage sequences[33,34].To test this hypothesis, we challenged our match-responsive split TEVp (hereafter called CATCH-TEVp) with other chemically responsive split proteases of the polyviral family derived from the Sunflower Mild Mosaic Virus protease (SUMMVp) and the Plum Pox Virus protease (PPVp) (**Figure 2d**). Our results showed that CATCH-TEVp is orthogonal to the rapamycin- and ABA-responsive split proteases previously reported[33,34], opening great prospects to create chemically-controlled

cascades of events, in which the output of one protease system can serve as the input of the following one to build logic gates that could control cell functions in new ways.

To demonstrate the utility of CATCH-TEVp for the control of cellular processes, we designed a TEVp activatable transcription factor. We anchored the transcriptional co-activator GAL4-VP16 at the plasma membrane by fusing it to the dopamine receptor D1 (DRD1) with a linker composed of ^{FIRE}tag-NTEVp followed by a TEVp cleavage site (**Figure 2e,f**). Match-induced interaction with cytosolically expressed ^{FIRE}mate-CTEVp leads to GAL4-VP16 release and translocation to the nucleus, where it activates the transcription of a UAS-controlled gene. We compared our CATCH-TEVp-based system with a previously reported dopamine-sensitive system in which split TEVp is similarly reconstituted upon dopamine-induced interaction with β-arrestin-2[31]. After 24 h of activation, we measured the UAS-controlled expression of Luc2P (a Luc2 variant bearing a degron sequence). We observed a four-fold increase in Luc2P expression upon activation of CATCH-TEVp-based system, while only a two-fold increase was observed with the previously reported dopamine-activated system (**Figure 2g**).

The modest fold increase observed in these experiments can be explained by self-complementation of the split TEVp in the absence of inducer, increasing the system's leakiness and limiting thus the dynamic range of the assay[31]. Western blot analysis showed that indeed the CATCH-TEVp-based system suffers from leakiness in the absence of the inducer, in agreement with self-complementation of the split TEVp (**Figure 2h**). To confirm this, we conducted control experiments in which we anchored the transcriptional co-activator GAL4-VP16 at the plasma membrane through fusion to DRD1 using a linker composed of only NTEVp followed by a TEVp cleavage site, and co-expressed either full-length TEVp or ^{FIRE}tag-CTEVp (**Supplementary Figure 3**). Quantification of the UAS-controlled expression of Luc2P showed only a five-fold higher expression in the case of full-length TEVp compared to ^{FIRE}tag-CTEVp, in agreement with a rather high self-complementation of the split TEVp itself. This result suggests moreover that, with a four-fold dynamic range, the CATCH-TEVp-based system reaches 80% of the maximal possible dynamic range.

To demonstrate that our CATCH-TEVp-based expression system could be used to control the expression of other proteins, we replaced the gene coding for Luc2 by the gene coding for d2EGFP (a EGFP variant containing a degron)[35]. Confocal microscopy analysis allowed us to show that d2EGFP expression increase by three-fold upon match addition, in agreement with the dynamic range observed in the bioluminescence assay (**Figure 2i-j**).

Chemically responsive DNA recombinase

Next, we developed chemically responsive DNA recombinases leveraging previously developed split CRE recombinase. The CRE recombinase enables gene editing for the study of gene functions. It catalyzes site-specific recombination between two DNA sequences called *loxP* sites, enabling gain or loss of function by sequence removal. Split versions controllable by the addition of rapamycin[36] or the use of light[37,38] have been previously described. These activatable CRE allowed precise control of gene recombination in cells and organisms[36–38]. In this part, we developed a split CRE controllable by CATCHFIRE, hereafter called CATCH-CRE, by fusing ^{FIRE}tag at the N-terminus of the C-terminal domain of CRE and ^{FIRE}mate at the C-terminus of its N-terminal domain. We constructed a bicistronic plasmid containing a P2A sequence for co-expression of the two parts of CATCH-CRE. To study the responsiveness of CATCH-CRE, we used a target plasmid containing a *loxP*-STOP-*loxP* cassette upstream of Luc2 gene[37]. CRE-induced removal of the STOP codon enables permanent Luc2 expression (**Figure 3**). Preliminary experiments showed us that the ratio of the CATCH-CRE plasmid and the *loxP*-STOP-*loxP*-Luc2 target plasmid was critical to obtain efficient chemically induced gene recombination. Indeed, we observed that a 10:1 target:CATCH-CRE plasmid ratio led to gene recombination in absence and in presence of match (**Supplementary Figure 4**). We assume that the leakiness observed in that case is very likely due to the high recombination efficiency of the CRE recombinase. Even weak self-complementation is likely sufficient to generate small concentration of CRE able to induce a recombination event. To circumvent this problem and obtain efficient chemically induced gene recombination, we ultimately opted for a

200:1 target:CATCH-CRE plasmid ratio (**Figure 3c**). In these conditions, low leakiness was observed in absence of match. Addition of match led to a 13-fold increase in Luc2 expression, reaching 80% of the expression obtained with full length CRE in the same conditions. These results were confirmed by Western blot analysis (**Figure 3d**).

To further demonstrate the use of CATCH-CRE for the control of gene recombination, we used CATCH-CRE to chemically control the expression of the red fluorescent protein mCherry using a *loxP*-STOP-*loxP*-mCherry target plasmid. Analysis by confocal fluorescence microscopy showed efficient match-induced gene recombination when using a 200:1 target:CATCH-CRE plasmid ratio, reaching efficiency of recombination corresponding to 27% of that observed with full length CRE (**Figure 3e-f**).

Chemically responsive transcription activators

To further demonstrate the usefulness of CATCHFIRE for the design of chemically responsive cellular control systems, we next engineer chemically responsive transcription activators. To achieve this, we leveraged the mammalian two-hybrid system based on the DNA-binding domain GAL4 and the VP16 transcription activation domain[39]. Interaction-induced assembly of GAL4 and VP16 enables to induce the expression of genes under the control of a UAS sequence[39]. This approach has been used (i) to detect protein-protein interactions such as the MyoD/Id interaction[39], or leucine zippers[40], (ii) to create chemically responsive transcription activators using ABA-induced PYL1/ABI interaction[3,41] or rapalog-induced FRB/FKBP interaction[3,42] or (iii) to design light-responsive transcription activators using the light-induced Cry2-CIB1 interaction[43]. This approach was demonstrated in various system from cultured cells to mice and *Drosophila*.

To design CATCHFIRE-controlled transcription activators, we fused ^{FIRE}tag (or caged ^{FIRE}tag) and ^{FIRE}mate to GAL4 and VP16. We designed different combinations to identify the optimal topology (**Supplementary Figure 5**). We used the MyoD/Id pair as a positive control.

Formation of the GAL4-VP16 assembly was assessed by monitoring the expression of a Luc2P gene under the control of a UAS sequence 24 h after addition of match (**Figure 4a-c** and **Supplementary Figure 5**). The best system was composed of GAL4-^{FIRE}tag and VP16-^{FIRE}mate (hereafter called CATCH-ON v1): we observed a 24-fold increase in expression of Luc2P after match treatment, reaching expression level comparable to the MyoD/Id positive control. Low leakiness was observed in absence of match: we detected a minimal basal expression only 2-fold higher than the negative control with no GAL4 and VP16 (**Figure 4c**). The efficiency and low leakiness of CATCH-ON v1 was further confirmed by Western blot analysis (**Figure 4e**). Kinetics analysis showed that expression of Luc2P peaked 7 h after match addition (**Figure 4d**), similarly to what was previously observed with similar expression systems[39]. We confirmed the ability of CATCH-ON v1 to chemically control gene expression by quantifying the match-induced expression of a d2EGFP gene under the control of a UAS sequence. Fluorescence microscopy analysis confirmed the high responsiveness of CATCH-ON v1 upon match addition and its very low leakiness in absence of match (**Figure 4f-g**).

Encouraged by the good performance of CATCH-ON v1, we optimized it to further increase the expression level and dynamic range. To achieve this, we created a new set of pairs using two new types of modification. First, we replaced GAL4 by a truncated version, named GAL4Δ, containing only the 65 first residues ($GAL4_{1-65}$) where a fraction of the dimerization domain is removed[44]. Second, we replaced VP16 by a truncated version of the transcription activator p65, named p65Δ ($p65_{285-550}$), lacking the Rel Homology Domain (RHD) responsible for DNA binding and homo and heterodimerization (**Supplementary Figure 6** and **Figure 5**)[43,45]. The combination of GAL4Δ and p65Δ was previously shown to increase expression efficiency in Cry2-CIB1 optogenetics-based systems[43]. Evaluating the different possible combinations, we observed that replacing VP16 by p65Δ significantly improved the system: when using GAL4-^{FIRE}tag and p65Δ-^{FIRE}mate (hereafter called CATCH-ON v2), we observed a 160-fold increase of Luc2P expression upon match addition, reaching, in addition, expression levels one order of magnitude higher than CATCH-ON v1. This improvement came however at the cost of a

slight increase of the system's leakiness. This study also identified the NLS-p65Δ-^{FIRE}mate/GAL4Δ-^{FIRE}tag pair as an interesting system showing similar dynamic range than CATCH-ON v1 but having a five-fold reduced leakiness (**Supplementary Figure 6**).

Using various concentrations of match to activate CATCH-ON v2, we showed that gene expression could be finely regulated in a dose-dependent manner (**Figure 5d**). In addition, monitoring expression of Luc2P over 24 h allowed us to characterize the kinetics of expression upon match addition (**Figure 5e**). We showed that CATCH-ON v2 could be efficiently switched off by washing out the match molecule: expression of Luc2P reached basal level with a halftime of about 4 h upon match washout, demonstrating the high tunability and the reversibility of the system (**Figure 5e**)[15]. The reversibility of CATCH-ON v2 was further confirmed by Western blot analysis (**Figure 5f**). We further confirmed the superior performances of CATCH-ON v2 by chemically controlling the expression of d2EGFP (**Figure 5g-h**). In accordance with the bioluminescence assay, we observed that when using CATCH-ON v2 the expression level of d2EGFP increased by 200-fold upon match addition (versus 20-fold when using CATCH-ON v1), and that overall CATCH-ON v2 allowed to reach expression levels 10-fold higher than with CATCH-ON v1.

We next demonstrated the usability of CATCH-ON for chemically controlling the expression of proteins involved in the regulation of cellular functions (**Figure 5i-l**). First, we showed that the CATCH-ON v2 system could be used to conditionally control the expression of full-length TEVp and full-length CRE. The expression of full-length TEVp was evaluated by monitoring the activation of the TEVp bioluminescent sensor described above, while the expression of full-length CRE was evaluated by monitoring the expression of Luc2 upon excision of an upstream *loxP*-STOP-*loxP* cassette. We observed a 12-fold increased activity of full-length TEVp upon match addition (**Figure 5j**), reaching activity comparable to that obtained expressing constitutively TEVp under the control of a CMV promoter. This approach allowed us to chemically control TEVp activity with a higher dynamic range than the CATCH-TEV system described above. Similarly, we observed a 10-fold increased activity of full-length CRE upon

match addition, reaching activity comparable to that obtained expressing constitutively CRE under the control of a CMV promoter (**Figure 5k**).

One exciting application of CATCH-ON could be to control the function of therapeutic cells on demand. To explore this idea, we used CATCH-ON v2 to chemically control the expression of the suicide switch DmrB-iCasp9 previously used to conditionally control CAR-T cell activity[17,19,46,47]. This suicide switch consists in the fusion of the truncated caspase iCasp9 and DmrB, an FKBP variant that can homodimerize in the presence of the rapalog AP1903. AP1903-induced dimerization of iCasp9 triggers the apoptotic cascade, leading ultimately to cell death. We generated a construct with the gene coding for DmrB-iCasp9 downstream a UAS sequence (**Figure 5i,j**). Control of its expression with CATCH-ON v2 allowed us to create an AND logic gate enabling to tightly control cell death. We experimentally showed that the presence of both match and AP1903 was required to significantly reduce cell viability (**Figure 5k**). The use of such an AND logic gate enables to get rid of the intrinsic leakiness of DmrB-iCasp9 in absence of AP1903. This experiment opens exciting prospects for applying CATCH-ON in cell therapy. It additionally confirmed the compatibility of CATCHFIRE with other described CID systems, opening the possibility for the creation of various Boolean systems.

To further explore the potential of CATCH-ON for cell-based therapies, we used CATCH-ON v2 to chemically control the secretion of a therapeutically relevant protein. As a model, we focused on insulin used for regulating blood glucose level in diabetes treatment. To monitor insulin secretion, we used a chimeric protein incorporating NanoLuc as a secretion reporter[48]. In this design, NanoLuc was flanked by the peptides A and B of insulin using Furin cleavage sites as linkers. Once synthesized and marked for secretion, this chimera travels from the endoplasmic reticulum to the trans-Golgi network where the Furin enzyme cleaves its cleavage site, releasing NanoLuc and functional insulin (**Figure 6a**)[2,32]. The NanoLuc secreted is used as a proxi for insulin secretion. CATCH-ON v2 allowed us to chemically control insulin secretion in a match-dependent manner. Treatment of cells with match increased insulin secretion by 55-fold (**Figure 6b,c**). This experiment indicates that CATCH-ON v2 could be used in the future

for controlling the secretion of various therapeutically relevant proteins such as chimeric antigen receptors (CAR), cytokines or hormones.

DISCUSSION

Engineering chemically responsive protein switches that enable the control of cellular processes is a very powerful and general approach with a broad range of applications in basic research, biotechnology and biotherapy. An ideal chemically responsive protein switch should be versatile, fast-acting, reversible, non-toxic, and compatible with existing technologies. In this article, we show that, thanks to its versatility, speed and reversibility, CATCHFIRE enabled the successful design of chemically responsive protein-based switches through precise control of split enzymes such as Luciferases, the TEV protease and the CRE recombinase. These systems open exciting prospects for modulating various cellular processes on demand. Our approach has the potential to be applied to other enzymes, although designing such systems presents inherent challenges as observed in this study. Split enzymes typically exhibit lower activity compared to their full-length counterparts, leading to reduced efficiency in chemically responsive split systems. Furthermore, split proteins often display some level of intrinsic self-complementation, resulting in inherent leakage in the absence of an inducer of proximity, thereby limiting the dynamic range. Even split proteins engineered for minimal self-complementation still retain residual self-complementation due to the inherent affinity between the two split fragments. This residual self-complementation is particularly limiting for highly efficient enzymes such as the split CRE, for which a single recombination event is then amplified by the gene expression machinery. Our study demonstrates that designing chemically responsive split systems with a high dynamic range is however feasible but necessitates a comprehensive investigation of all components and conditions within the system. Optimization should involve a deep structural analysis of the split sites and the fragment interactions. This should be followed by a careful analysis on how the dimerization domains are connected to the split fragments. Despite the possibility to place ^{FIRE}tag or ^{FIRE}mate

either at the N- or C-terminus of proteins, our study showed that the design of efficient chemically responsive systems requires systematic testing of more than one topology to find the optimal one, as no general rule or “magic bullet” exists. Similarly, a careful examination of the linker length and composition is crucial. Short linkers can mask active sites and hinder their function, while long flexible linkers can prevent the split fragments to get into proximity. Rigid linkers, on the other hand, can potentially prevent the interaction and reconstitution of the desired function. All these considerations must be followed by an optimal selection of promoter and expression conditions to ensure sufficient expression of the inducible system's components for a measurable effect upon inducer addition. This must be achieved without exceeding a concentration threshold where self-complementation can occur. The numerous conditions that must be considered render the quest of chemically or light-responsive protein-based switches a challenging yet achievable task compared to using full-length proteins[5,25,30,36–38,49]. The CATCH-TEVp and CATCH-CRE systems developed in this study can still be optimized. The dynamic range of their responsiveness could be optimized by reducing the self-complementation of the split TEVp, split CRE, and CATCHFIRE itself. This optimization could be achieved by leveraging recent developments in de novo protein design[50], by using directed protein evolution or by exploring the use of protein homologs[51] as we recently did for optimizing the dynamic range and complementation efficacy of splitFAST, a split fluorescent reporter of PPI related to CATCHFIRE[51].

Splitting multidomain proteins rather than monodomain proteins simplifies the design of highly efficient chemically responsive protein-based switches, as shown in this study with the design of the CACTH-ON transcriptional regulation system. CATCH-ON displays minimal self-activation and high dynamic range (about 200-fold for CATCH-ON v2) allowing precise control of gene expression in a dose-dependent and reversible manner. CATCH-ON allowed us to chemically control the expression of various proteins such as luciferases, fluorescent proteins, proteases, and DNA recombinases, and to create Boolean systems with other CID technologies, revealing exciting potential for applications in synthetic biology and

biotechnology. Beyond fundamental research, the ability to regulate the expression of suicide switches or therapeutic proteins like insulin offers also promising avenues for developing controllable therapeutic cells with enhanced therapeutic control and increased safety. The road towards such applications is of course long and will require to demonstrate the functionality of CATCH-ON in vivo and in therapeutic cells.

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AUTHOR CONTRIBUTIONS

J.F.P and A.G. designed the overall project and wrote the paper with the help of the other authors. J.F.P., N.D., S.D., F.P. and A.G. designed the experiments. J.F.P., N.D., S.D. performed the experiments. J.F.P., N.D., F.P. and A.G. analyzed the experiments.

COMPETING INTERESTS

The authors declare the following competing financial interest: A.G. and F.P. are co-founders and hold equity in Twinkle Bioscience/The Twinkle Factory, a company commercializing the FAST, split-FAST and CATCHFIRE technologies. J.F.P. is occasionally consultant for Twinkle Bioscience/The Twinkle Factory. The other authors declare no competing interests.

MATERIALS AND METHODS

Plasmid construction

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 or QIAquick® Gel Extraction Kit (QIAGEN). Isothermal assemblies (Gibson Assembly) were performed using a

homemade mix prepared according to previously described protocols.[52] GAL4-Id VP16-MyoD plasmid were bought from PROMEGA as the CheckMate™ Mammalian Two-Hybrid System. Small-scale isolation of plasmid DNA was conducted using a QIAprep miniprep kit (QIAGEN) from 2-5 mL overnight DH10β in LB culture supplemented with appropriate antibiotic. Large-scale isolation of plasmid DNA was conducted using the QIAprep maxiprep kit (QIAGEN) from 150-180 mL overnight bacterial culture supplemented with the corresponding selection antibiotics. All plasmid sequences were confirmed by Sanger sequencing with appropriate sequencing primers (Eurofins). See **Supplementary Information** and **Supplementary Table 1** for ORFs and primers used to synthesize them, and **Supplementary Table 2** for primers sequence.

Cell culture experiments

General considerations. HeLa cells (ATCC, CCL2 Lot 700756809) were grown in Minimal Essential Medium media (MEM, Gibco™) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Gibco™), 1% (v/v) of Sodium Pyruvate (Gibco™) and 1% (v/v) of Penicillin-Streptomycin (Sigma-Aldrich) at 37 °C in a 5% CO_2 atmosphere.

Ligands. Match ligands (in-house synthesized)[24,27,53], rapamycin (TCI), abscisic acid (Sigma-Aldrich), dopamine (Sigma-Aldrich), doxorubicin (Sigma-Aldrich), AP1903 (TOCRIS) were dissolved in DMSO (Sigma-Aldrich) at 3 to 20 mM stock solutions and kept at –20 °C. Intermediate solutions were prepared at 200-400× final concentration in DMSO.

Chemically responsive Luciferase study

Chemically responsive NanoBiT. 130 µL of a 2×10^4 cells/mL suspension were added in a 96-white well plate (Corning®) and incubated for 24 h in the abovementioned conditions. Each sample to be added to the wells for transfection contains half volume of GeneJuice® (Sigma-Aldrich): in OptiMEM™ (Gibco™, 8% v/v) and half volume of DNA: Opti-MEM™ that was incubated for 15 to 20 minutes at RT before addition to the wells according to GeneJuice® protocol. For each well, cells were transfected with 6.3 µL of the mixture to obtain a final

amount of 20 ng of DNA per well. Cells were incubated for 24 h, media was removed and replaced with 100 µL of OptiMEM™ containing a final concentration of DMSO 0.5% (v/v), $match_{550}$ 5 µM, or rapamycin 0.5 µM, and then incubated for 2 h. Cells were treated with Nano-Glo® Live Cell Assay System (PROMEGA) as described in the protocol. Luminescence was recorded with a POLARstar OPTIMA plate reader (BMG Biotech) at 37 °C for 90 minutes with a 2-minute scan interval and 1-second integration time. Results show the luminescence values 40 minutes after reagent addition. Values are shown as mean ± SD of n ≥ 3 independent experiments (each composed of two technical replicates).

Determination of the effective concentration of match for half maximal complementation (EC_{50}) using chemically induced NanoBiT system. Cells were seeded, transfected as above for 24 h after which media was removed and replaced with OptiMEM™ containing a final ligand concentration between 8 to 0 µM, maintaining DMSO at 0.5%. From each independent experiment, values were normalized attributing 0 at the lowest signal value and 1 at the highest. Curves were fitted using GraphPad Prism (V7.00) using a non-linear fitting, one site-specific binding equation: $Y = Y_{max}*X/(EC_{50}+ X)$ where X is the concentration of match. Values are shown as mean ± SD of n ≥ 3 independent experiments (each composed of two technical replicates).

CATCH-TEVp and orthogonality with other inducible proteases

130 µL of a 4×10^4 cells/mL suspension was added in a 96-white well plate and incubated for 24 h in the abovementioned conditions. For each well, cells were transfected as reported above with 30 ng of a circularly permuted Firefly Luciferase (Luc2) encoding plasmid (target plasmid), 15 ng of each plasmid encoding the inducible split protease system, and 2.5 ng of a Renilla encoding plasmid used as a transfection control. After for 24 h, media was removed and replaced with 200 µL of fresh media containing DMSO 0.5%, Absicic Acid (ABA) 100 µM, rapamycin 3 µM or $match_{550}$ 5 µM, and cells were incubated for 1 h at 37 °C. Luciferase activity was measured using the Dual-Luciferase® Reporter System (PROMEGA). Briefly, media was removed, and wells were washed with 150 µL of DPBS. The supernatant was removed, and

cells were lysed with 20 µL per well of Passive Lysis Buffer (PROMEGA) while gently rocking at room temperature for 20 minutes. Luciferase activity was measured as indicated by the supplier. Luminescence was recorded 2-3 minutes after reagent addition, using a 1-second integration time at 25 °C. Values represent the mean of the Luc2 signal divided by the signal of the Renilla luciferase signal, constitutively expressed and used as a transfection reporter, ± SD of n ≥ 3 independent experiments (each composed of two technical replicates). Fold increase represents the mean of the normalized signal of cells treated with match, rapamycin or ABA *vs.* cells treated with DMSO.

Gene expression induced by CATCH-TEVp

130 µL of a 4×10^4 cells/mL suspension was added in a 96-white well plate and incubated for 24 h in the abovementioned conditions. For each well, cells were transfected as reported above with, in each well, 30 ng of target plasmid (UAS-Luc2P plasmid), 15 ng of each plasmid encoding the inducible splitTEVp system and 2.5 ng of Renilla encoding plasmid as transfection control. DMSO 0.5%, dopamine 5 µM or $match_{550}$ 5 µM were added and cells were incubated for 24 h. Luc2P expression was studied, gene expression was measured by using the Dual-Luciferase® Reporter System as indicated by the supplier and as described before. Fold increase represents the mean of the Luc2P signal divided by the signal of a Renilla protein used as reporter, of dopamine or match treated *vs.* DMSO treated cells, ± SD of n = 3 independent experiments (each composed of two technical replicates). One-Way ANOVA analysis followed by a Tukey's multiple comparison was performed.

Control of luciferase expression using CATCH-CRE

130 µL of a 4×10^4 cells/mL suspension was added in a 96-white well plate and incubated for 24 h in the abovementioned conditions. For each well, cells were transfected as reported above using 50 ng of *loxP*_STOP_*loxP*_Luc2 target plasmid in each well, 0.25 ng of CATCH-CRE encoding plasmid, CMV_CRE or empty plasmid and 2.5 ng of Renilla encoding plasmid as transfection control. After 24 h, media was removed and replaced with 150 µL of fresh media

containing DMSO 0.5% or $match_{550}$ 5 μM and incubated for 24 h. As indicated before, Luc2 expression was measured using the Dual-Luciferase® Reporter System. Values are shown as mean ± SD of 3 independent experiments (each composed of two technical replicates).

Control of gene expression with CATCH-ON

Control of luciferase expression with CATCH-ON. 130 μL of a 4×10^4 cells/mL suspension was added in a 96-white well plate and incubated for 24 h in the abovementioned conditions. For each well, cells were transfected with 30 ng of reporter (UAS-Luc2P plasmid) and 15 ng of each plasmid encoding CATCH-ON parts or the MyoD/Id parts were added. DMSO 0.5% or $match_{550}$ 5 μM final concentration were added and co-incubated for 2–26 h with the transfection mixture. For degradation experiments, media was removed, wells were washed with 150 μL of DPBS and replaced by 150 μL of fresh media. Luciferase expression was measured by using the Dual-Luciferase® Reporter System as indicated above. Values represent the mean of the Luc2P signal divided by the signal of the Renilla protein used as a transfection reporter, ± S. D of n = 3 independent experiments (composed of two technical replicates).

Control of a suicide gene with CATCH-ON. 130 μL of a suspension of 6×10^4 cells/mL was added in a 96-white well plate and incubated for 24 h in the abovementioned conditions. For each well, cells were transfected with 20 ng of target (UAS-DmrB-iCasp9 plasmid) and 10 ng of each plasmid encoding CATCH-ON parts for 8h, media was removed and replaced by 200 μL of fresh media containing DMSO 0.25%, $match_{550}$ 5 μM, AP1903 5 μM or the combination of both as final concentration. To control molecules' cytotoxicity, non-transfected cells were treated equally, using doxorubicin at 5 μM as the positive control. Cells were treated for 16 h and viability was measured using Cell-Titer Glo® (PROMEGA) where 100 μL were removed from the well and the remaining supernatant was treated with 100 μL of reagent, the mixture was incubated at 30 minutes at room temperature. Luminescence was recorded at 25 °C using a 1-second integration time. Values are shown as mean ± SD of 3 independent experiments (each composed of two technical replicates). Viability (%) is expressed as $V = 100 \times$

(Luminescence treatment – Luminescence Blank) / (Luminescence DMSO control – Luminescence Blank).

Control of TEVp expression with CATCH-ON. 130 µL of a suspension of 4×10^4 cells/mL was added in a 96-white well plate and incubated for 24 h in the abovementioned conditions. For each well, cells were transfected as reported above where in each well with 30 ng of a plasmid containing a circularly permuted Luc2 used as a target and 10 ng of GAL4-^{FIRE}tag plasmid that contains also a Renilla cassette used as a transfection control. For the treatments were added for positive control 10 ng of CMV_TEVp encoding plasmid and 10 ng of an EGFP encoding plasmid (as mock plasmid), for negative control 10 ng of empty plasmid and 10 ng of EGFP encoding plasmid and for the test 10 ng of UAS-TEVp encoding plasmid and 10 ng of p65Δ-^{FIRE}mate encoding plasmid. The wells were co-incubated with DMSO 0.5% or $match_{550}$ at 5 µM for 24 h. TEVp activity was assessed by measuring Luc2 expression using the Dual-Luciferase® Reporter System (PROMEGA). Values are shown as mean ± SD of 3 independent experiments (each composed of two technical replicates).

Control of CRE expression with CATCH-ON. 130 µL of a suspension of 4×10^4 cells/mL was added in a 96-white well plate and incubated for 24 h in the abovementioned conditions. For each well, cells were transfected as reported above with 50 ng of target plasmid and 5 ng of GAL4-^{FIRE}tag plasmid. For the treatments were added for positive control 0.05 ng of CMV_CRE plasmid and 5 ng of EGFP encoding plasmid (as mock plasmid), for negative control 0.05 ng of empty plasmid and 5 ng of EGFP encoding plasmid (as mock plasmid) and for the test 0.05 ng of UAS-CRE plasmid and 5 ng of p65Δ-^{FIRE}mate plasmid. The cells were cultured for 24 h, media was removed and replaced with fresh media containing DMSO 0.5% or $match_{550}$ at 5 µM for 24 h as above. CRE activity was assessed by measuring Firefly signal as described above using the Dual-Luciferase® Reporter System. Values are shown as mean ± SD of 3 independent experiments (each composed of two technical replicates).

Control of insulin secretion with CATCH-ON (NanoLuc measurement). 1 mL of a 2.5 × 10^5 cells/mL suspension was added to a 12-well plate and incubated for 24 h under the aforementioned conditions. The media was removed and replaced with 930 µL of fresh MEM, then completed with 70 µL of a transfection mixture containing 680 ng of DNA. For all conditions, 280 ng of Furin encoding DNA were added; for the negative control, 400 ng of EGFP encoding plasmid (as mock plasmid) were used. For the positive control, 100 ng of CMV_Insulin-NanoLuc encoding plasmid and 300 ng of EGFP encoding plasmid (as mock plasmid) were used. For the test, 100 ng of UAS-insulin-NanoLuc encoding plasmid, 100 ng of GAL4-^{FIRE}tag encoding plasmid, 100 ng of p65Δ-^{FIRE}mate encoding plasmid, and 100 ng of EGFP encoding plasmid (as mock plasmid) were used. Additionally, DMSO at 0.5% or $match_{550}$ at a final concentration of 5 µM was added and co-incubated for 24 h with the transfection mixture. The media was collected and centrifuged for 5 min at 500 g rpm at 4°C. NanoLuc secretion was measured at room temperature from 100 µL of the supernatant using the Nano-Glo® Luciferase system with a 1-second integration time. Values are shown as mean ± SD of 3 independent experiments.

Western Blot analysis

2 mL of a HeLa cells suspension (1-2.5 × 10^5 cells/mL) were seeded in a 6-well plate and incubated for 24 h in the described conditions. Media was removed and replaced with 1.9 mL of fresh media and 100 µL of transfection solution containing 1 µg of DNA per well. See **Supplementary Table 3** for transfection mixture. For CATCH-TEVp and CATCH-ON experiments, cells were co-incubated with DMSO 0.5% or $match_{550}$ at 5 µM. For CATCH-CRE experiments, cells were transfected for 24 h, media was removed and replaced by 2 mL of fresh one containing DMSO 0.5% or $match_{550}$ at 5 µM. After 24 hours, the media was removed, and the cells were washed with DPBS (2 × 1 mL). Lysates were obtained by adding 200 µL of a mixture 1:100 of a protease cocktail (Halt™ Protease Inhibitor Cocktail, 100X, Thermo Scientific™) and RIPA lysis and extraction buffer (Thermo Scientific™) on ice for 5 minutes. The supernatant containing the proteins was collected after centrifugation (15000 g at 4°C for

30 min). In the case of CATCHFIRE reversibility, media was removed and washed as stated and replaced by 2 mL of media for 8 h. Samples were treated as before. Protein concentrations were evaluated thanks to a calibration curve following Pierce™ BCA Protein Assay Kit (Thermo Scientific™) instructions. The calibration samples (0-2000 µg/mL) were prepared from a Serum Bovine Albumin solution following the instructions provided in the kit. The absorbance was read after 30 min incubation at 37°C at 562 nm on a TECAN Spark® 10 plate reader. Solutions of 10–40 µg of the protein mixture and Laemmli Buffer (5×) were boiled for 5 min at 95°C and loaded onto a gel (Premade NuPAGE™ Bis-Tris, 10% MES buffer, Thermo Fisher Scientific). After 45 min migration at 150 V using a PowerPac™ Basic device (BioRad), the proteins were wet-transferred to a nitrocellulose membrane (Nitrocellulose Blotting membrane 0.2 µm, General Electric) applying current for 90 minutes at 100 V in using Transfer Buffer (Towbin 1×, MeOH 20% (v/v) and milliQ H_2O). The membranes were blocked in PBS (PB 50 mM, NaCl 150 mM, pH = 7.4) and skimmed milk 5% (w/v) for one hour at RT. After blocking, the membrane was incubated with the primary antibody diluted in PBS + skimmed milk 0.5% (w/v) overnight at 4°C with gentle rocking. The membranes were washed 3 times in PBS + skimmed milk 0.5% (w/v). The membrane was then probed with the secondary antibody diluted in PBS + skimmed milk 5% (w/v) for 45 min at RT. The membranes were washed 1 time in PBS + skimmed milk 0.5% and then 2 times in PBS. The proteins were visualized using the following primary antibodies: monoclonal mouse anti-LgBit (N7100, PROMEGA, 1:500), rabbit anti-FAST (Covalab, 1:1000), and polyclonal rabbit anti-β-actin (4967S, Cell Signaling, 1:1000). Secondary antibodies were used as follow: goat anti-rabbit IgG HRP-linked antibody (7074S, Cell Signalling, 1:2500), goat anti-mouse IgG (H+L) antibody (W4021, PROMEGA, 1:2500). The membranes were revealed by Chemiluminescent detection with Chemi-Doc™ imager (Biorad) after being incubated for one minute in 3-4 mL of a 1:1 solution of SuperSignal West Pico PLUS (Horse Radish Peroxidase) and luminol (Thermo Fisher Scientific). The membranes were stripped by washing them with PBS (2 × 5 mL) and then using Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific) for 15-20 minutes while shaking at

room temperature, then washed again with PBS (2 × 5 mL) and re-probed with the corresponding antibodies.

Confocal microscopy experiments

A suspension of 4.2 to 6 × 10^4 HeLa cells in a total volume of 2 mL was seeded in a 35 mm µDish (Ibidi®) and incubated for 24 h in the conditions described before. Medium was removed, replaced with 1.9 mL of fresh media and 100 µL of transfection solution containing 1 µg of DNA per dish. See **Supplementary Table 4** for transfection mixture. For CATCH-TEVp and CATCH-ON experiments, cells were incubated for 8 h and media was removed and replaced by fresh media containing DMSO 0.5% or $match_{550}$ at 5 µM for 16 h. For CATCH-CRE, media was removed after 24 h and replaced by fresh media containing DMSO 0.5% or $match_{550}$ 5 µM for 24 h. After incubation, the media was removed, and the cells were washed with DPBS (2 × 1 mL). 1 mL of fresh Dubelcco's Modified Eagle Medium (DMEM, Gibco) without phenol red was added and samples were analysed on a Zeiss LSM 980 Laser Scanning Microscope equipped with a 20× or a 63× objective. ZEN software was used to collect the data and then images were analysed using Icy software (v 2.5.2.0) or Fiji software. To track fluorescence signal in the cytoplasm in the CATCH-TEVp and CATCH-ON experiments, cell contours were determined by masking the signal in the iRFP713 channel with the plug-in HK-means with the following parameters: intensity class equals 100, min object size (px) equals 20000, max object size (px) equals 50000. For the CATCH-CRE experiments, cell contours were determined by masking the signal in the EGFP channel with the plug-in HK-means with the following parameters: intensity class equals 75, min object size (px) equals 10000, max object size (px) equals 80000. The ROI's signal intensity was measured for each channel using plug-in Active Contours.

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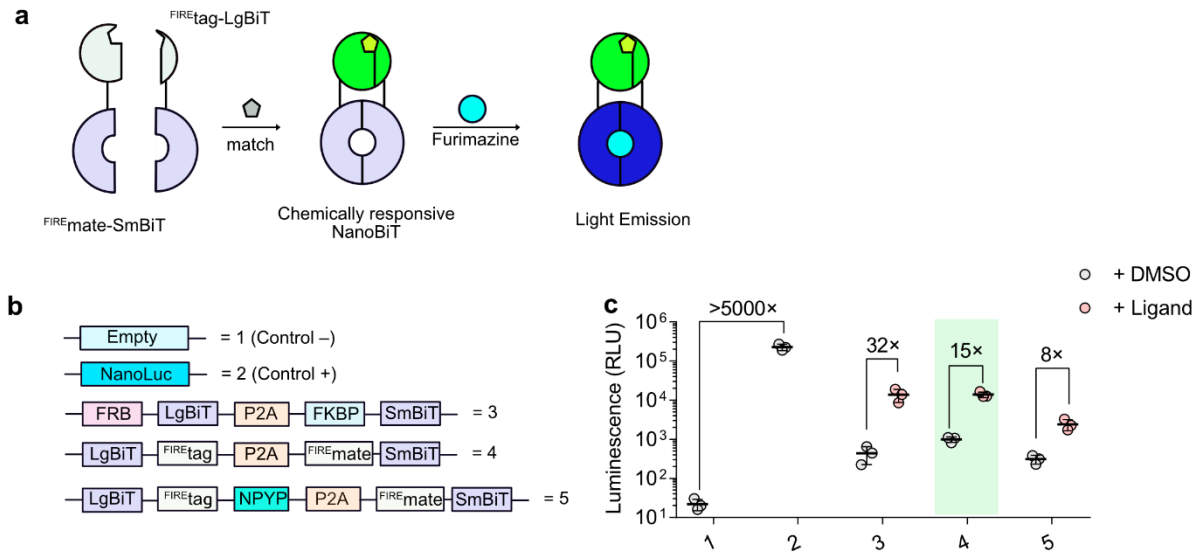

Figure 1. Chemically responsive luciferase. **a** Principle. **b** Constructs used in this study. **c** Luminescence of HeLa cells expressing the chemically responsive luciferase candidates incubated for 2 h either with DMSO 0.5% or their cognate inducer (0.5 μM of rapamycin or 5 μM of $match_{550}$). Luminescence values are shown as mean ± SD of n = 3 independent experiments (each composed of two technical replicates).

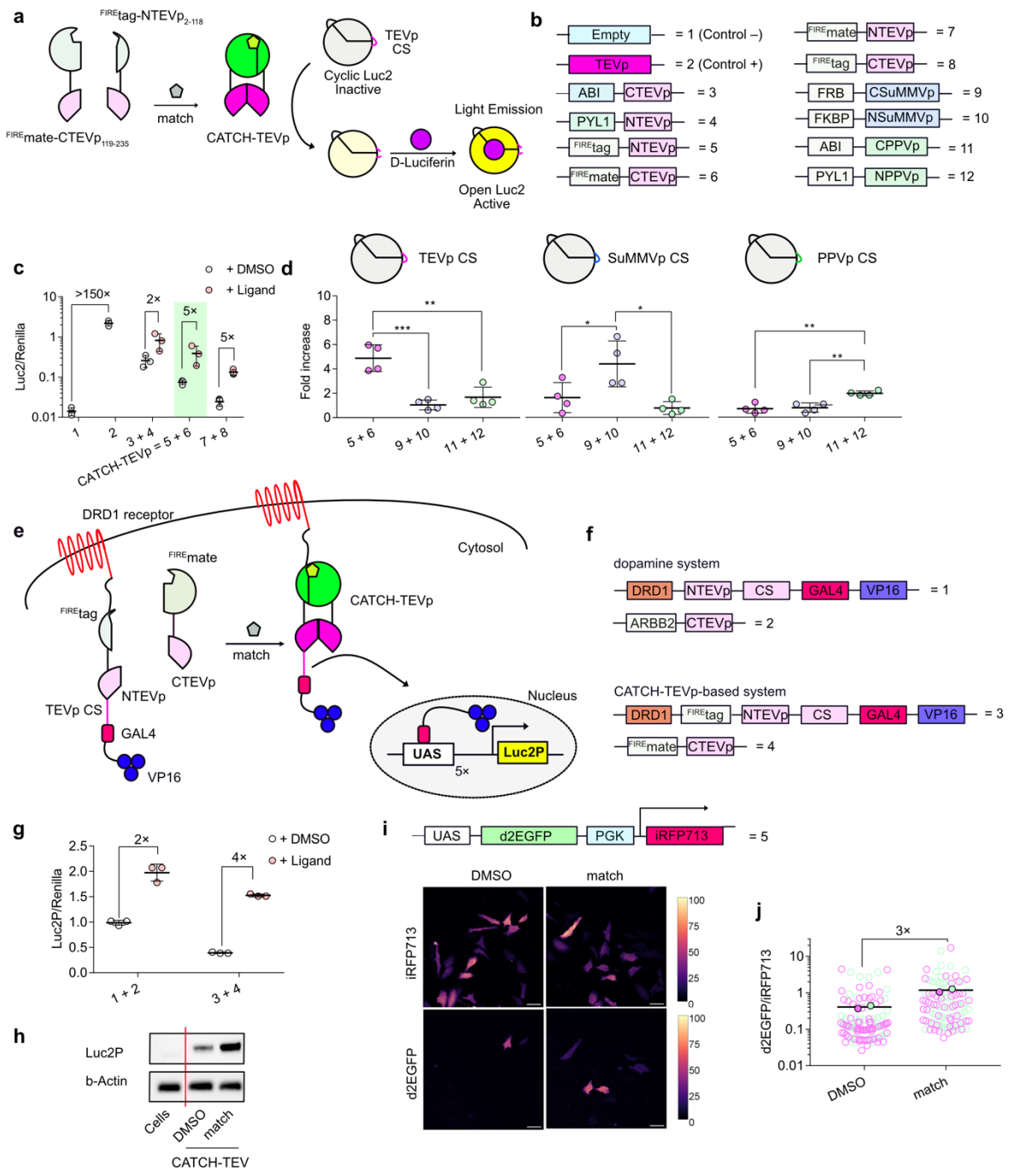

Figure 2. Chemically responsive protease. a Principle of CATCH-TEVp and the bioluminescent assay based on circularized Luc2. **b** Constructs used in this study (CS, cleavage site). **c** Bioluminescence intensity of HeLa cells co-expressing the CATCH-TEVp candidates together with the bioluminescent TEVp sensor and incubated for 1 h with 0.5% DMSO or 5 μM of $match_{550}$. Luc2 signals were normalized by the signal of a co-expressed Renilla luciferase to overcome differences in expression levels. Control experiments using full-

length TEVp or the ABA-inducible TEVp are shown (100 μM of abscisic acid (ABA) were used). Values are shown as mean ± SD of n = 3 independent experiments (each composed of two technical replicates). **d** Orthogonality of CATCH-TEVp, ABA-inducible PPVp and Rapamycin-inducible SuMMVp. The different chemically responsive proteases were expressed either with a bioluminescent TEVp sensor (left), a bioluminescent SUMMVp sensor (middle) or a bioluminescent PPVp sensor (right). Bioluminescent fold increases upon addition of the inducer are shown. ONE-way ANOVA analysis followed by a Tukey's multiple comparisons test was performed where *p*-values are: 0.0003 and 0.0010 for TEVp, 0.0103 and 0.0406 for SUMMVp, 0.0015 and 0.0010 for PPVp. **e** Principle of CATCH-TEVp-based gene expression control. **f** Constructs used in this study (CS, cleavage site). **g** Expression fold increase upon activation of the CATCH-TEVp-based system or the dopamine-sensitive system. HeLa cells expressing the different components were treated for 24 h either with 0.5% DMSO or with the cognate inducer (5 μM of dopamine or 5 μM of $match_{550}$). Values are shown as mean ± SD of n = 3 independent experiments (each comprised of two technical replicates). **h** Western blotting showing Luc2P expression after 24 h of incubation with DMSO 0.5% or 5 μM of $match_{550}$. The experiment shows one representative blot out of two experiments. **i,j** Evaluation of the induced expression of d2EGFP upon activation of CATCH-TEVp by confocal microscopy experiments after 16 h of incubation with DMSO 0.5% or 5 μM of $match_{550}$. **i** Representative confocal micrographs showing d2EGFP expression. iRFP713 was used as transfection control. Images were taken with identical microscope settings. Scale bars represent 50 μm. **j** d2EGFP fluorescence intensity (normalized by iRFP713 signal) of single cells. Each cell is color-coded according to the biological replicate it came from. The solid circles correspond to the mean of each biological replicate. The black line represents the mean values of the two biological replicates (each with n ≥ 45 cells).

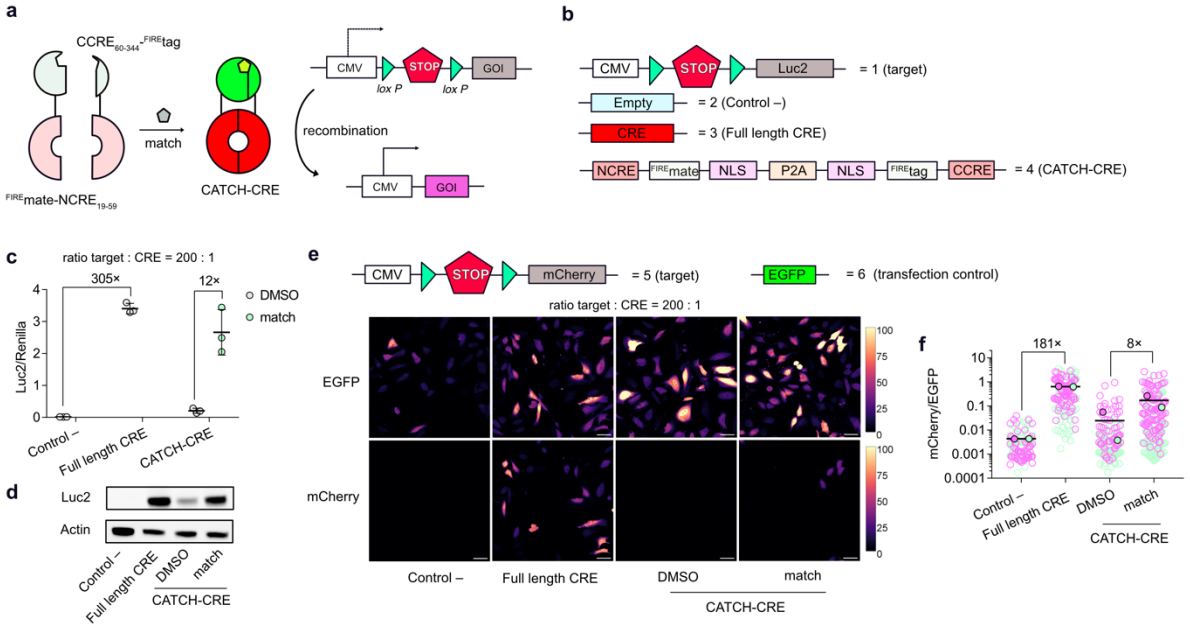

Figure 3. Chemically responsive DNA recombinase. a Principle of CATCH-CRE-based DNA editing. **b** Constructs used in this study. **c** HeLa cells transfected with the *loxP*-STOP-*loxP*-Luc2 plasmid and the CATCH-CRE plasmid (200:1 ratio) were incubated for 24 h with 0.5% DMSO or 5 μM of match$_{550}$. Values are shown as mean ± SD of n = 3 independent experiments (each composed of two technical replicates). Control experiments with no CRE (control –) or full-length CRE are also shown. Luc2 signals were normalized by the signal of a co-expressed Renilla luciferase to overcome differences in transfection efficiency. **d** Western blot showing Luc2 expression after 24 h of incubation with DMSO 0.5% or 5 μM of match$_{550}$. The experiment shows one representative blot out of two independent experiments. **e,f** Evaluation of mCherry expression upon activation of CATCH-CRE by confocal microscopy after 24h of incubation with DMSO 0.5% or 5 μM of match$_{550}$. **e** Representative confocal micrographs showing mCherry expression. EGFP was used as a transfection control. Images were taken with identical microscope settings. Scale bars represent 50 μm. **f** mCherry fluorescence intensity (normalized by EGFP signal) of single cells. Each cell is color-coded according to the biological replicate it came from. The solid circles correspond to the mean of

each biological replicate. The black line represents the mean values of the two biological replicates (each with n ≥ 50 cells).

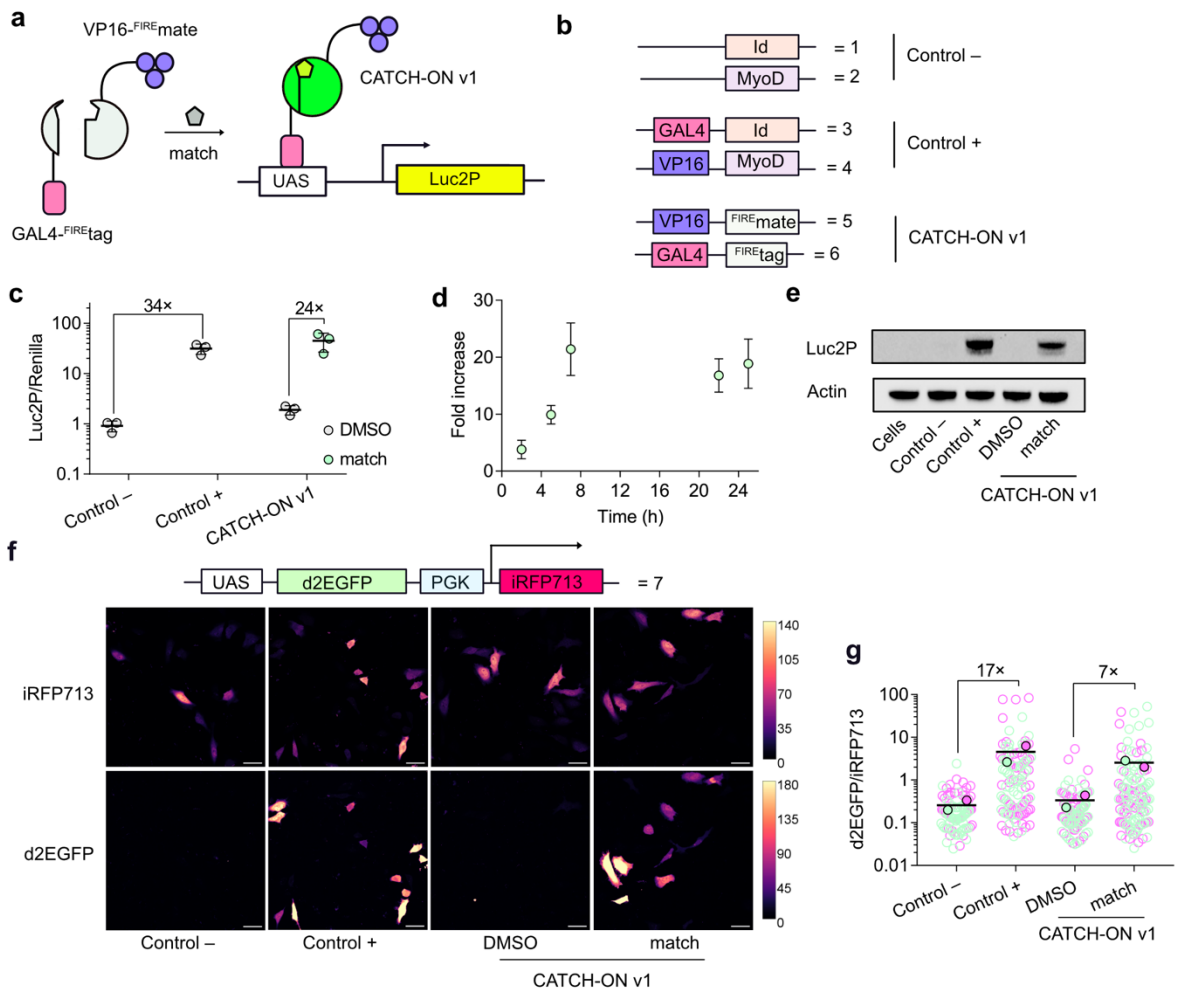

Figure 4. Chemical control of gene expression. a Principle of CATCH-ON v1. **b** Constructs used in this study. **c** HeLa cells transfected with UAS-Luc2P plasmid and the CATCH-ON v1 plasmid were incubated with DMSO 0.5% or 5 μM of $match_{550}$ for 24 h. Results are depicted as a normalized signal of Luc2P *vs.* Renilla signal used as a transfection control. **d** Time-lapse of Luc2P gene expression upon activation of CATCH-ON v1. Expression is shown as a fold increase in the normalized Luc2P signal of $match_{550}$-treated *vs*. DMSO-treated cells. Values are shown as mean ± SD of n = 3 independent experiments (each composed of two technical replicates). **e** Western blot showing Luc2P expression controlled with CATCH-ON v1. Transfected cells were incubated with DMSO 0.5% or 5 μM of $match_{550}$ for 24 h. The experiment shows one representative Western blot out of two independent experiments. **f,g** Evaluation of CATCH-ON v1-controlled d2EGFP expression by confocal microscopy after 16 h of incubation with DMSO 0.5% or 5 μM of $match_{550}$. **f** Representative confocal micrographs

showing d2EGFP expression. iRFP713 was used as transfection control. Images were taken with identical microscope settings. Scale bars represent 50 µm. **g** d2EGFP fluorescence intensity (normalized by iRFP713 signal) of single cells. Each cell is color-coded according to the biological replicate it came from. The solid circles correspond to the mean of each biological replicate. The black line represents the mean values of the two biological replicates (each with $n \geq 35$ cells).

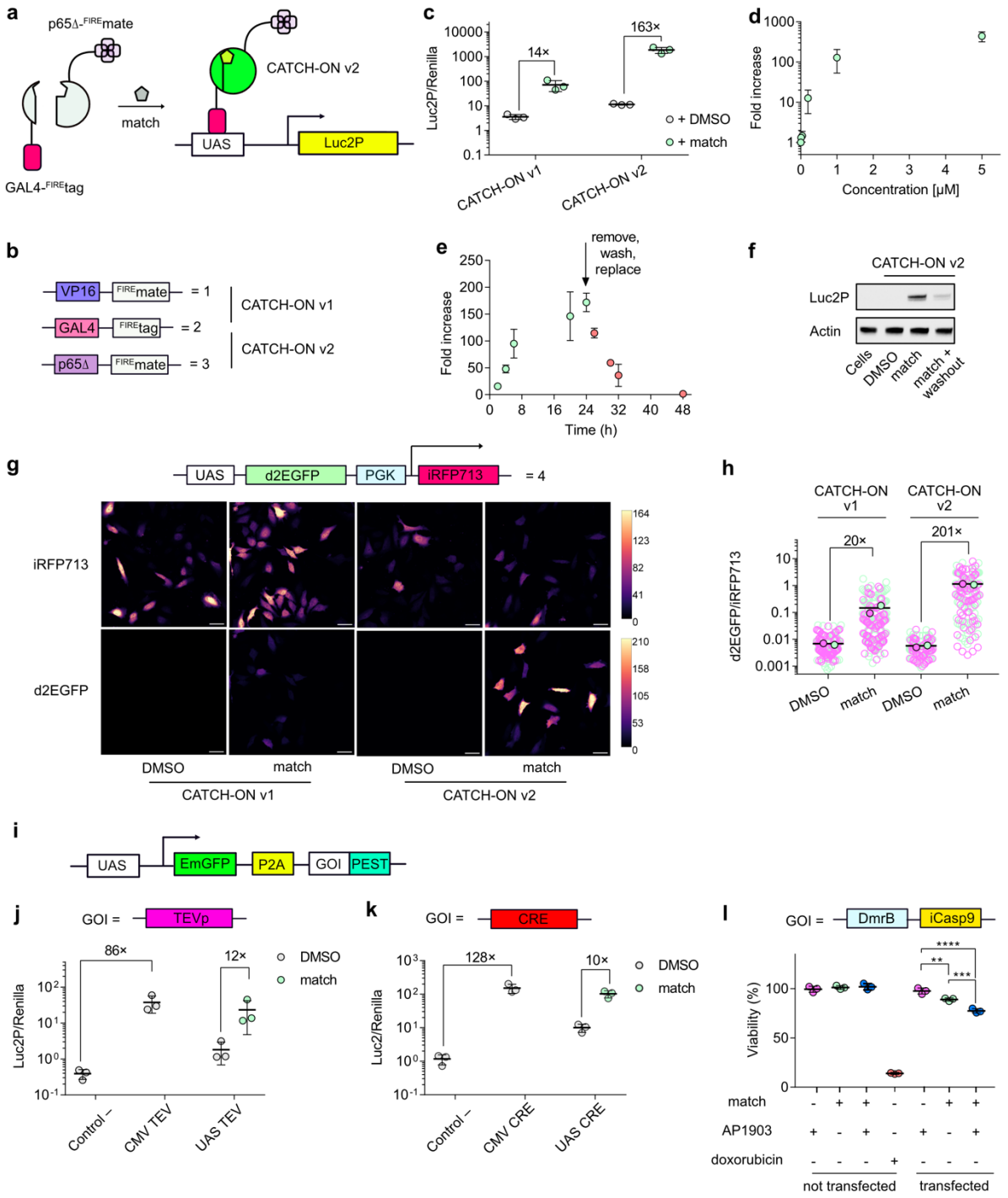

Figure 5. Chemical control of gene expression with improved CATCH-ON. **a** Principle of CATCH-ON v2. **b** Constructs used in this study. **c** HeLa cells transfected with UAS-Luc2 plasmid and CATCH-ON v1 or v2 plasmids were incubated with DMSO 0.5% or match$_{550}$ 5 μM for 24 h. Results are depicted as a normalized signal of Luc2P *vs.* Renilla signal used as a transfection control. **d** Dose response of Luc2P gene expression after 26 h of incubation with

DMSO 0.5% or 0-5 μM of $match_{550}$. **e** Kinetics of Luc2P gene expression shown as a fold increase in the normalized Luc2P signal of $match_{550}$(5 μM)-treated *vs*. DMSO-treated cells. Values are shown as mean ± SD of n = 3 independent experiments (each composed of two technical replicates). **f** Characterization of CATCH-ON v2 by Western blot. Cells transfected with UAS-Luc2 plasmid and CATCH-ON v2 plasmids were incubated with DMSO 0.5% or $match_{550}$ 5 μM for 24 h. For the reversibility experiment, the media was removed and replaced with fresh media and incubated for 8 h. The experiment shows one representative blot out of two independent experiments. **g,h** Evaluation of CATCH-ON-controlled d2EGFP expression by confocal microscopy after 16 h of incubation with DMSO 0.5% or 5 μM of $match_{550}$. **g** Representative confocal micrographs showing d2EGFP expression. iRFP713 was used as transfection control. Images were taken with identical microscope settings. Scale bars represent 50 μm. **h** d2EGFP fluorescence intensity (normalized by iRFP713 signal) of single cells. Each cell is color-coded according to the biological replicate it came from. The solid circles correspond to the mean of each biological replicate. The black line represents the mean values of the two biological replicates (each with n ≥ 50 cells). **i-l** Control of the expression of biologically relevant proteins using the CATCH-ON v2 system**. i** Generic plasmid for inducible UAS-based gene expression. **j** TEVp protease and **k** CRE recombinase activity in HeLa cells upon activation of CATCH-ON v2 with DMSO 0.5% or 5 μM of $match_{550}$. Their activity was measured after 24 h. **l** Viability of HeLa cells transfected with UAS-DmrB-iCasp9 plasmid and CATCH-ON v2 plasmids after treatment with 5 μM of $match_{550}$ and 5 μM of AP1903 for 16 h. Induction of cell death with 5 μM of doxorubicin was used as a positive control (For normalization details, see Materials and Methods section). Values are shown as mean ± SD of n = 3 independent experiments (each composed of two technical replicates). ONE-way ANOVA analysis followed by a Sidak's multiple comparisons test was performed where *p*-values are 0.018, 0.001 and <0.001 for **, *** and **** respectively.

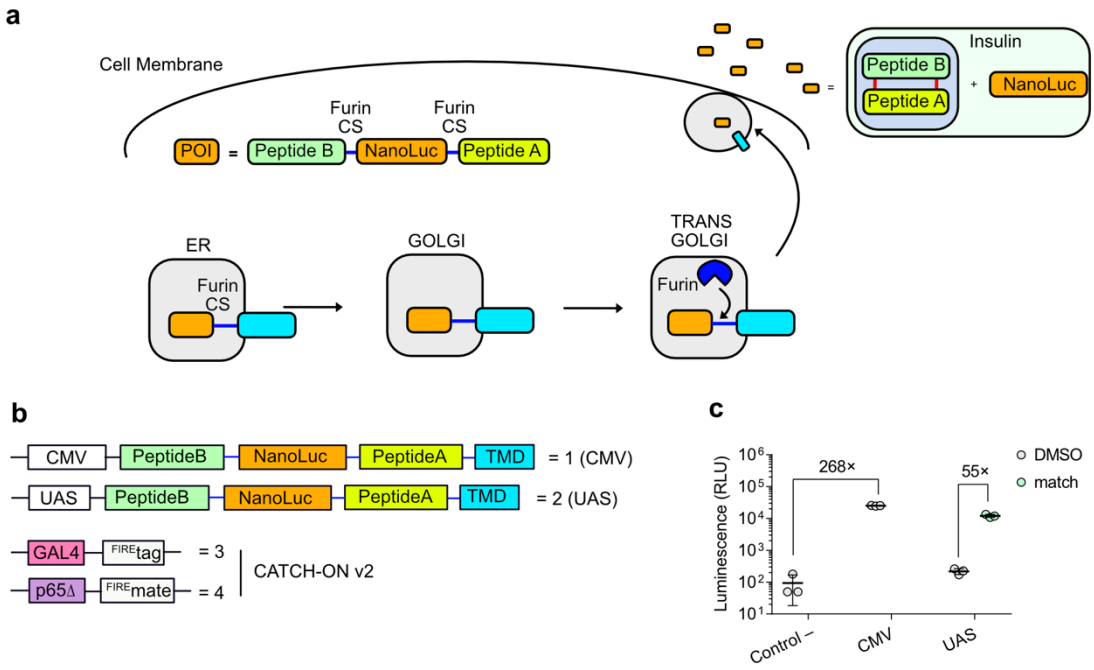

Figure 6. Control of insulin secretion with CATCH-ON. a Insulin/NanoLuc secretion scheme and **b** constructs used in this study. **c** HeLa cells were transfected and incubated with DMSO 0.5% or 5 μM of $match_{550}$ for 24 h. The supernatant was collected and NanoLuc activity was measured. Values are shown as mean ± SD of n = 3 independent experiment.