Compact Programmable Control of Protein Secretion in Mammalian Cells

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Abstract:
Synthetic biology currently holds immense potential to engineer the spatiotemporal control of intercellular signals for biomedicine. Programming behaviors using protein-based circuits has advantages over traditional gene circuits such as compact delivery and direct interactions with signaling proteins. Previously, we described a generalizable platform called RELEASE to enable the control of intercellular signaling through the proteolytic removal of ER-retention motifs compatible with pre-existing protease-based circuits. However, these tools lacked the ability to reliably program complex expression profiles and required numerous proteases, limiting delivery options. Here, we harness the recruitment and antagonistic behavior of endogenous 14-3-3 proteins to create RELEASE-NOT to turn off protein secretion in response to protease activity. By combining RELEASE and RELEASE-NOT, we establish a suite of protein-level processing and output modules called Compact RELEASE (compRELEASE). This innovation enables functions such as logic processing and analog signal filtering using a single input protease. Furthermore, we demonstrate the compactness of the post-translational design by using polycistronic single transcripts to engineer cells to control protein secretion via lentiviral integration and leverage mRNA delivery to selectively express cell surface proteins only in engineered cells harboring inducible proteases. CompRELEASE enables complex control of protein secretion and enhances the potential of synthetic protein circuits for therapeutic applications, while minimizing the overall genetic payload.

Keywords: intercellular communication, synthetic biology, proteases, 14-3-3 proteins, protein circuits, mRNA delivery

Introduction:

The field of synthetic biology is uniquely suited to develop biomolecular circuits to engineer cells[1–4] for various biomedical applications, such as improving cancer immunotherapies[5,6] and cell therapies[7]. These engineered circuits can enable cells to respond to combinatorial environmental inputs[8,9], interrogate natural systems[10], and produce controlled therapeutic responses[11]. In particular, an emerging strategy for programming cellular function in mammalian cells is to use orthogonal proteases to create synthetic protein circuits[12,13]. Protein-based circuits can implement synthetic signal-processing, featuring advantages such as fast operation, compact design, and robust context-independent performance compared to traditional transcriptional circuits[12–14]. Previously, we[15] and others[16] described generalizable platforms ("RELEASE" and "membER/lumER", respectively), which interfaced with pre-existing protease-based circuits[12,13] and enabled the control of intercellular signals through proteolytic removal of ER-retention motifs (**Fig. 1a-c**). However, to program complex expression patterns such as logic operations or quantitative processing, multiple orthogonal proteases were required[12,13], which may hinder their use in viral vectors with limited packaging capacities[17]. Therefore, to fully unlock the potential of these synthetic protein circuits for biomedicine, there is a need to harness additional post-translational modifications to develop synthetic protein components that condense genetic payloads while retaining or even enhancing circuit functionality.

Post-translational modifications of proteins represent a cornerstone in cellular regulation, orchestrating a diverse array of functions crucial for cellular homeostasis and responsiveness[18–20]. Among these, the interplay between post-translational modifications and protein-protein interactions, particularly with 14-3-3 scaffolding proteins, stands as a pivotal nexus of cellular control[21,22]. The 14-3-3 protein family is expressed in all eukaryotes and are adept at recognizing phosphorylated motifs on target proteins[23]. When 14-3-3 proteins bind to phosphorylated motifs, they can mediate diverse cellular outcomes, including sequestration from specific cellular compartments, stabilization of protein complexes, or modulation of enzymatic activity[21,24]. Of particular interest is their ability to block interactions between the COPI retrograde transport machinery and ER-retention motifs to control the surface expression of membrane proteins[25–30] (**Fig. 1d**). Although surface expression is mediated by phosphorylation-dependent recruitment of 14-3-3 proteins, this specific protein-protein interaction may be exploitable when using pre-existing synthetic protease-based circuits[12,13,31].

Given the size limitation of viral vectors for genetic delivery[17], we sought to expand the RELEASE platform and enable compact programmable control of protein secretion. Specifically, we used a small 14-3-3 recruiting peptide to manipulate protein-protein interactions with endogenous 14-3-3 scaffolding proteins to create RELEASE-NOT, an approach to turn off protein secretion in response to protease activity. With the ability to control the activation or repression of protein secretion in response to protease activity, we created a suite of RELEASE variants known as Compact RELEASE (compRELEASE). The compRELEASE suite achieved functional completeness of all Boolean logic gates and enabled analog signal filtering without requiring additional processing proteases[12,13]. We also focused on the compact delivery of synthetic protein circuits, and optimized polycistronic constructs under the control of a single promoter to create engineered cells with inducible control of protein secretion. Finally, to highlight the utility of post-translational circuits, we leveraged the mRNA delivery of RELEASE to selectively express cell surface proteins only in engineered cells harboring inducible proteases. This study builds on the RELEASE platform to unlock its potential as a protein-level processing and output module to enable circuit-level control of protein secretion, while minimizing the overall genetic payload.

Materials and Methods:

Plasmid generation:

All plasmids were constructed using general practices. Backbones were linearized via restriction digestion, and inserts were generated using PCR, or purchased from Twist Biosciences. A complete list of plasmids and their respective amounts used for each experiment can be found in **Supplementary Table 1**. Furthermore, all new plasmids used in this study will be deposited with annotations to Addgene (https://www.addgene.org/Xiaojing_Gao/).

Tissue culture: Human Embryonic Kidney (HEK) 293 cells (ATCC, CRL-1573) and HEK293T-LentiX (Takara Biosciences) were cultured in standard culture conditions (37ºC and 5% CO2) in Dulbecco's Modified Eagle Media (DMEM), supplemented with 10% Fetal Bovine Serum (FBS, ThermoFisher; catalog# FB12999102), 1X Pen/Strep (Genesee; catalog# 25-512), 1X non-essential amino acids (Genesee; catalog# 25-536), and 1X sodium pyruvate (Santa Cruz Biotechnology; catalog# sc-286966). APRE-19 cells (ATCC, CRL-2302) were cultured in standard culture conditions in DMEM/F-12 (1:1) GlutaMAX media (ThermoFisher; catalog# 10565018), supplemented with 10% FBS, and 1X PenStrep. K562 cells (ATCC, CCL-243) were cultured in RPMI-1640 (Sigma-Aldrich; catalog# R8758) media supplemented with 10% FBS, and 1X PenStrep. HEK293 cells were used for all transient transfection experiments and K562 cells were used for mRNA transfection experiments. HEK293T cells were used to produce lentivirus, described below. Cells tested negative for mycoplasma.

Transient transfection: HEK293 cells were cultured in 96-well tissue-culture treated plates under standard culture conditions. At 70-90% confluency, cells were transiently transfected with plasmids using the jetOPTIMUS® DNA transfection Reagent (Polyplus transfection; catalog# 117-15), as per manufacturer's instructions.

Lentiviral transduction: To generate lentivirus, HEK293T-LentiX cells (Takara Biosciences) were transfected with 750 ng of an equimolar mixture of the three third-generation packaging plasmids (pMD2.G, pRSV-Rev, pMDLg/pRRE) and 750 ng of donor plasmids using 200 uL of jetOPTIMUS® DNA transfection Reagent (Polyplus transfection, catalog# 117-15). pMD2.G (Addgene plasmid #12259, https://www.addgene.org/12259/), pRSV-Rev (Addgene plasmid #12253, https://www.addgene.org/12253/), and pMDLg/pRRE (Addgene plasmid # 12251, https://www.addgene.org/12251/) were gifts from Didier Trono. After 2 - 3 days of incubation, the lentivirus was harvested and filtered through a 0.45 micron filter (Millipore) to remove any cellular debris. The harvested virus was precipitated using the Lentivirus Precipitation Solution (Alstem; catalog# VC100) and centrifuged at 1,500 x g for 30 minutes to remove any residual media. The concentrated virus was then resuspended in supplemented DMEM, aliquoted and frozen at -80C for later use.

Lentivirus was titrated on HEK293 or APRE-19 cells by serial dilution in supplemented DMEM or DMEM/F-12, respectively. 72 hours after incubation, the percentage of mCherry-positive cells were quantified using flow cytometry. The cells were selected using 1000 ng/mL of puromycin (ThermoFisher Scientific; catalog# J61278-MB) for one week until >90% of cells were mCherry-positive before being used for *in vitro* or *in vivo* experiments.

K562 inducible reporter cell line: To generate the reporter cell line, K562 were electroporated in Amaxa solution (Lonza Nucleofector 2b, setting T0-16) with 1µg of reporter donor (pEF1a-p450-rapalog-split-TEVP-T2A-mCherry) and 1 µg of SuperPiggyBac transposase. After 7 days, the cells were treated with 1000 ng/mL of puromycin for two weeks to ensure that the construct was stably integrated. A monoclonal population was generated through serial dilution. The expression of the fluorescent reporter was measured by microscopy and flow cytometry. The SuperPiggyBac transposase plasmid was a gift from the Bassik lab.

mRNA Synthesis and Transfection: The IgG-RELEASE construct was cloned into a backbone containing optimized 5' and 3' UTRs and a T7 promoter. The plasmid backbone was a gift from the Elowitz lab. Using a reverse primer containing a 200 bp polyA sequence, we added a Poly-A tail to the DNA template via PCR. In vitro transcribed mRNA was produced and purified by GeneScript Inc. based on the DNA template, with Cap1 and modified bases (100% N1-methyl-pseudouridine).

Briefly, $2.5x10^5$ wildtype K562 cells and $2.5x10^5$ reporter K562 cells were mixed for a total of $5.0x10^5$ cells per well and transfected with various amounts of the purified mRNA using the TransIT®-mRNA Transfection Kit (Mirus Bio, catalog# MIR2250). To induce the reporter cell lines, Rapalog AP21967 (also known as A/C heterodimerizer, purchased from Takara Biosciences; catalog# 635056) was administered at the time of transfection. All experiments were induced with 100 nM of rapalog, unless otherwise stated.

Flow cytometry and data analysis: Following 24 hours after mRNA transfection, K562 cells were pelleted at 300 x g for 7 minutes and reconstituted in PBS supplemented with 5% Bovine Serum Albumin (BSA, company name). Following two wash steps, the cells were incubated with an anti-human IgG antibody conjugated to Alexa Fluor-488 (Southern Biotech Catalog# 2015-30; 1/750 dilution in PBS + 2% BSA) for 1 hour. Following incubation, the cells were washed twice in PBS + 2% BSA and then filtered through a 40 µm sieve. Cells were analyzed by flow cytometry (BioRad ZE5 Cell Analyzer) and Everest Software (version 3.1). FlowJo (version 10.9.0) was used to process the flow cytometry data.

For analysis, cells were gated for cells that were expressing the IgG-RELEASE-T2A-BFP construct (BFP^+). The median fluorescence intensity (MFI) of AF488 was calculated for wildtype ($mCherry^-$) and reporter ($mCherry^+$) cells, and then compared between uninduced and induced conditions. For each condition, an IgG isotype control (Southern Biotech Catalog# 0109-30) to correct the MFI of AF488 for non-specific staining.

Measuring protein secretion: Protein secretion was measured by a Secreted Embryonic Alkaline Phosphatase (SEAP) assay, as previously described[15]. Briefly, two days after transient transfection or induction with rapalog, the supernatant was collected and heat-inactivated at 70C for 45 minutes. After heat-inactivation, 10 - 40 uL of supernatant was mixed with dH_2O to a final volume of 80 uL. This mixture was then mixed with with 100 uL of 2X SEAP Buffer (20 mM homoarginine (ThermoFisher catalog# H27387), 1 mM MgCl2, and 21% (v/v) dioethanolamine (ThermoFisher, catalog# A13389)) and 20 µL of the p-nitrophenyl phosphate (PNPP, Acros Organics catalog# MFCD00066288) substrate (120 mM). Samples were measured via kinetic measurements (1 reading/minute) for a total of 30 minutes at 405 nm using a SpectraMax iD3 spectrophotometer (Molecular Devices) with the Softmax pro software (version 7.0.2)

Secreted NanoLuc® (secNLuc) was measured by taking the supernatant from samples that were previously transiently transfected or induced with rapalog. Briefly, 10 uL of supernatant was mixed with 45 µL of dH2O and then mixed with 50 uL of the Nano-Glo® Luciferase Assay system (Promega; catalog# N1110), per the manufacturer's instructions. Samples were measured using a SpectraMax iD3 spectrophotometer (Molecular Devices) with the Softmax pro software (version 7.0.2).

Statistical Analysis:
Values are reported as the average of four biological replicates, and representative of at least two independent biological experiments. For experiments comparing two groups, a Student's T-test was used to determine statistical significance, following confirmation that equal variance could be assumed (F-test). If equal variance could not be assumed, then a Welch's correction was used. For experiments with more than two groups, a one-way ANOVA with a post hoc Tukey's test was used for multiple comparisons among

the different means. Data was considered statistically significant when the p-value was less than 0.05. Data presented are average +/- SEM, unless otherwise stated. All statistical analysis was performed using Prism 9.0 (GraphPad).

Results:

Using additional protein motifs to modulate secretion with RELEASE:

The original RELEASE platform used the dilysine ER-retention motif (**Fig. 1b**) to retain tagged proteins of interest via retrograde transport, mediated through interactions with the COPI complex[32–35] (**Fig. 1a**). Only after proteolytic removal of the retention motif would the protein of interest be transported through the conventional secretory pathway, processed into its secretory form via furin (**Fig. 1c**), and secreted. However, the dilysine motif (**Fig. 1b**, -KKXX-COOH) only functioned when placed at the C-terminus[34,36]and limited the potential of RELEASE to be used in polycistronic constructs separated by self-cleaving 2A peptides[14,37]. Indeed, since 2A peptides leave a small peptide scar at the C-terminus, when transiently transfecting a polyprotein encoding Secreted Alkaline Embryonic Phosphatase (SEAP) fused to RELEASE and the mNeon reporter protein (**Supplementary Fig. 1a**), we observed protein secretion with and without co-expression of TEVP (**Supplementary Figure 1b**). Therefore, we identified additional ER-retention protein motifs such as the diarginine motif (-RXR-, **Fig. 1b**)[38] that were not limited to the C-terminus of membrane proteins and created updated RELEASE constructs (**Fig. 1e – left panel**). Using these new RELEASE constructs, we transiently transfected HEK293 cells (**Fig. 1e – right panel**) and observed a significant increase in SEAP secretion when co-expressing the cognate protease relative to when the protease was absent (**Fig. 1e – left panel**). As expected, these new RELEASE variants retained their ER-retention capabilities when encoded as the first protein in a polypeptide construct separated via a T2A peptide (**Supplementary Fig. 1c**). For the remainder of this study, we used RELEASE variants with the RXR retention motif unless otherwise stated. Furthermore, this new design unlocked the ability to generate fusion proteins with RELEASE to potentially impart new behaviors.

To test the inhibitory effect of 14-3-3 proteins on the ER-retention activity of RELEASE to facilitate protein secretion (**Fig.1d**)[27,39], we fused the 14-3-3ζ protein to the C-terminus of RELEASE (**Supplementary Fig. 2a**). As expected, the fusion of 14-3-3ζ resulted in a significant increase in protein secretion, compared to RELEASE alone (**Supplementary Fig. 2b**). This fusion highlighted the potential of using 14-3-3 proteins to block the ER-retention capabilities of RELEASE, however since our focus was to keep constructs as small as possible, we sought an alternative strategy focused on the recruitment of endogenous 14-3-3 proteins to drive protein secretion.

Previously, the R18 peptide has been described to bind the conserved amphipathic ligand groove of 14-3-3 proteins[40] via the WLDLE motif[41], and we hypothesized that its fusion to the C-terminus of RELEASE would be sufficient to recruit 14-3-3 proteins and drive protein secretion (**Fig. 1f – left panel**). To evaluate this, we created two RELEASE constructs: one with the R18 peptide and the other containing a mutant variant lacking the two key acidic residues (referred to as R18 mutant) critical for mediating binding with 14-3-3 proteins[40]. The addition of R18 was sufficient in blocking the retention capabilities of RELEASE and resulted in a significant increase in SEAP secretion relative to the RELEASE construct with the R18 mutant peptide (**Fig. 1f**). This result shows that the ER-retention capabilities of RELEASE can be inhibited through the addition of the R18 peptide at the C-terminus to drive protein secretion.

Negative regulation of protein secretion and functional completeness:

An important capability of synthetic protein circuits is the ability to integrate multiple inputs[12–14,31]. The ability of negative regulation (i.e., NOT logic) is essential for implementing all possible Boolean logic operations, in addition to the previous RELEASE-enabled compact implementation of AND/OR[15]. We attempted to use CHOMP to create a two-protease circuit to turn off secretion in response to the expression of the first protease (**Supplementary Fig.** 3a, TEVP, orange pac-man), however, protein secretion did not completely return to baseline even at high levels of the repressing protease (**Supplementary Fig. 3b**). Instead of relying on two proteases to impart negative regulation with CHOMP, we asked if we could program NOT logic directly into the RELEASE platform.

By placing a protease cut site (such as TEVP) between the ER-retention motif and R18 peptide we created RELEASE-NOT (**Fig. 2a**). RELEASE-NOT relies on the recruitment of 14-3-3 proteins to enable constitutive secretion of a tagged protein of interest, until proteolytic removal of the R18 peptide results in restoration of the ER-retention activity (**Fig. 2a**). Co-expression of TEVP with a TEVP-inducible RELEASE-NOT significantly reduced protein secretion relative to when protease was not expressed (**Fig. 2b**). Like RELEASE, we validated the modularity of RELEASE-NOT by switching the cytosolic protease cut site to respond to other proteases such as the hepatitis C virus protease (HCVP) (**Supplementary Fig. 4**). Since protein secretion with RELEASE-NOT is dependent on recruitment of 14-3-3 proteins, we overexpressed exogenous 14-3-3ζ protein and observed a significant increase in SEAP secretion (**Supplementary Fig. 5b**). However, exogenous expression of 14-3-3ζ proteins reduced the dynamic range by increasing baseline secretion (**Supplementary Fig. 5c**).

In addition to R18, we wanted to assess the compatibility of RELEASE-NOT with phosphorylation-dependent binding motifs that recruit 14-3-3 proteins, such as the SWTY motif[42]. The SWTY motif is phosphorylated by kinases within the PI3K/Akt pathway, which can be activated by the presence of FBS[29]. As expected, RELEASE-NOT using the SWTY motif, resulted in SEAP secretion in the absence of protease and a significant decrease when co-expressing the cognate protease (**Supplementary Fig. 6a**). To validate that protein secretion was due to phosphorylation-dependent recruitment of endogenous 14-3-3 proteins, we created a variant where the phospho-responsive threonine residue was mutated to alanine (i.e., SWTY → SWAY). The RELEASE-NOT using SWAY resulted in a complete abrogation of SEAP secretion (**Supplementary Fig.** 6b), suggesting phosphorylation was important for recruiting 14-3-3 proteins to inhibit ER-retention and drive SEAP secretion. Although the SWTY motif was suitable for imparting NOT logic, similar to the dilysine motif (**Supplementary Fig.** 1a), SWTY functions only at the C-terminus[42], therefore making it incompatible when encoded by polycistronic constructs separated by self-cleaving 2A peptides. In comparison, RELEASE-NOT with the R18 peptides preserved negative regulation of protein secretion when encoded in polycistronic constructs (**Supplementary Fig. 7**).

Now that we could implement OR, AND, and NOT logic, we created a suite of RELEASE variants known as Compact RELEASE (compRELEASE) for the different Boolean Logic gates and validated their behavior through co-transfection with and without different combinations of input proteases (**Fig. 2c**). An important characteristic of these variants is that the R18 peptide cannot inhibit the ER-retention capabilities of the N-terminal p450 motif since it confers ER retention by directly inserting into the ER membrane[43,44]. For example, to secrete SEAP under the control of RELEASE-NIMPLY, only the N-terminal p450 motif must be removed since the C-terminal retention motif is being inhibited by the R18 peptide (**Supplementary Fig. 8a**). Any other combination of binary inputs resulted in either the p450 motif remaining intact, or the removal of the R18 peptide and restoration of the C-terminal ER-retention motif activity (**Supplementary Fig. 8b**). Most of the Boolean gates (5 of 8) were implemented using single RELEASE constructs, but some gates, such as XNOR were created by co-expressing RELEASE variants with NOR and AND logic (**Fig. 2c**). The compRELEASE suite highlights the modularity of the RELEASE design to create variants with predictable logic operation functions without requiring additional proteases[12,13,45].

Quantitative processing using compRELEASE:

In addition to implementing Boolean logic, it would be advantageous to use the compRELEASE suite to enable more core circuit functions, such as analog signal filtering[46]. Considering some intercellular signals such as cytokines and growth factors have pleiotropic activity based on their concentrations[47–49], the ability to selectively respond to specific concentrations ranges of inputs would be critical for enabling future biomedical therapies. Proteolytic activation and repression of protein secretion within the compRELEASE suite are dependent on cleavage efficiencies of the protease cut sites, which can be manipulated by changing the canonical amino acid sequence (**Fig. 3a**). For example, the canonical TEVP cut site has a serine amino acid (S) at the P1' position (**Fig. 3a – bolded residue**), and mutations to this residue to alternative amino acids such tyrosine (Y) or leucine (L) have been reported to reduce cleavage efficiency[50]. Indeed, when using modified protease cut sites harboring these mutations, we observed a shift in the activation thresholds for both RELEASE (**Fig. 3b**) and RELEASE-NOT (**Fig. 3c**).

Now that we could reliably manipulate the cleavage efficiencies of our constructs, we sought to achieve analog signal processing by modifying the RELEASE-NIMPLY and RELEASE-IMPLY variants so that both of their cut sites were cleaved by the same protease, but with different cleavage efficiencies. In addition, these two variants were selected because they were single constructs that contained both activating and repression arms for protein secretion (**Fig. 2c**). To implement band-pass filtering of protein secretion (**Fig. 3d**), RELEASE-NIMPLY was modified to contain two TEVP cut sites with the activating arm of protein secretion to be more efficiently cleaved compared to the repression arm (**Fig. 3e**). This ensured that protein secretion would first increase and then decrease, to increasing amounts of TEVP (**Fig. 3f**). We implemented a similar strategy using the RELEASE-IMPLY variant to enable band-stop filtering (**Fig. 3g**), except that the repressing arm of protein secretion was more efficiently cleaved than the activating arm. In this design, protein secretion would first decrease and then increase, to increasing amounts of TEVP (**Fig. 3i**). These two variants enabled quantitative processing using the compRELEASE suite to improve the compactness of the circuit and while reducing the number of required proteases previously shown to be required to achieve the same behavior[12].

Delivering RELEASE:

Unlike traditional gene regulation circuits that often require multiple integration events to create functional circuits[51,52], synthetic protein circuits function at the post-translational level and can be encoded within a single polycistronic construct. However, manipulating the relative concentrations of individual protein components in single transcript designs is not trivial and may require additional optimization. To demonstrate the utility of using synthetic protein circuits to engineer new behaviors into cells, we designed a polycistronic construct encoding a puromycin-resistant cassette, a mCherry reporter protein, a rapalog-inducible split HCVP and a secNLuc-RELEASE all under the control of the EF1α promoter (**Fig. 4a**). Using lentiviral transduction, we created a stable HEK293 cell line with rapalog-inducible control of secNluc secretion (**Fig, 4b**).

The initial single-transcript design encoded a split HCVP protease into the cytoplasm to activate secNluc secretion in response to rapalog (**Fig. 4c – left panel**), however the dynamic range was poor (2.26-fold increase). We have previously shown that sub-cellular localization of intermediate protease components can modulate the activation of RELEASE[15], so we localized one half of the split protease to the ER using the signal anchor sequence of cytochrome p450[43,44]. This fusion was expected to improve RELEASE activation (**Fig. 4c – middle panel**), and we observed an increase in secNluc secretion following induction with rapalog. However, this topology resulted in an increased baseline and did not significantly improve the

dynamic range (1.96-fold increase). Therefore, our next approach was designed to manipulate the cleavage efficiency of the HCVP cut site, reducing the baseline of secNluc secretion, and improving the dynamic range of the circuit. We have previously shown that modifying the flanking residues of the HCVP cut site from their native sequences to GS-linkers significantly reduced the cleavage efficiency[15]. Indeed, using a single transcript design with an ER-localized split HCVP and a less efficient HCVP-inducible RELEASE construct resulted in the highest dynamic range (6.14-fold increase) due decreasing baseline secretion, with comparable activation levels as the original design (**Fig. 4c – right panel**).

In addition to stable integration, synthetic protein circuits have the potential to be delivered as mRNA transcripts without any risk of insertional mutagenesis[53]. Since RELEASE requires expression of the cognate protease to control protein secretion, we hypothesized that we could leverage this property to selectively express protein payloads in engineered cells, without activating RELEASE in wildtype cells (**Fig. 4d**). To validate this selectivity, we created an mRNA transcript encoding a human IgG-RELEASE and the BFP reporter separated by a T2A peptide (**Fig. 4d**). Previously, human IgG has been used as a synthetic surface marker[54], and we transfected this mRNA into a 50/50 mixture of wildtype (mCherry$^-$) and engineered (mCherry$^+$) K562 cells. The engineered K562 cells contained a rapalog-inducible TEVP, and 24 hours after transfection we assessed the surface expression of IgG using flow cytometry (**Fig. 4e**). From the mixture of cells, only engineered K562 cells (mCherry$^+$) showed a significant increase in the surface expression of IgG when induced with rapalog (**Fig. 4f**). In comparison wildtype cells (mCherry$^-$) did not show any significant induction and comparable surface expression with the uninduced population of engineered cells (**Fig. 4f**). Since mRNA expression is transient, the IgG retained in the ER of wildtype cells should eventually degrade via the proteasomal degradation pathway. This work highlights the potential of using mRNA to deliver RELEASE-tagged protein payloads that were selectivity expressed only when delivered into engineered cells.

Discussion:

Here, we introduced the compRELEASE suite, a collection of engineered proteins with expanded input-processing capabilities, enabling the precise control over intercellular signals in mammalian cells. The compRELEASE suite was engineered by harnessing the complementary functions of RELEASE and RELEASE-NOT to combine them in various configurations introduce novel behaviors. By functioning as both the processing and output module, the compRELEASE suite allowed for the integration of multiple inputs (**Fig. 2c**) and the implementation of analog-signal filtering (**Fig. 3**) to control protein secretion. Notably, this functionality was achieved without the need of additional processing proteases[12,13]. The reduction in the number of proteases is important as it streamlines the entire circuit, reduces the genetic payload, and frees up proteases for use in designing synthetic receptors[55,56]. However, it should be noted that the compactness enabled by compRELEASE is specifically applicable for protein secretion (**Fig. 2 and 3**) and the surface expression of proteins. Furthermore, given that the programmable features of compRELEASE are independent of specific tagged protein of interest, we hypothesized that these variants would uphold the plug-and-play adaptability of outputs demonstrated with the original RELEASE platform[15].

Since 14-3-3 proteins are ubiquitously expressed in all eukaryotic cells[23], we expect compRELEASE to maintain its functionality across various cell types. Nonetheless, it is important to acknowledge that differential expression of 14-3-3 proteins[23] in specific cells and tissues may influence the extent of protein secretion, particularly when employing logic gates (**Fig. 2c**) or quantitative processing (**Fig. 3i**) dependent on RELEASE-NOT (**Fig. 2a**). Notably, the reversible process of 14-3-3 recruitment and binding to the R18 peptide could be enhanced by overexpressing the 14-3-3ζ isoform to increase protein secretion using RELEASE-NOT (**Supplementary Fig. 5c**). While this study primarily emphasized the use of the R18 peptide to modulate protein secretion (**Fig. 1f and 2a**), it is important to note that RELEASE-NOT also

demonstrated compatibility with phosphorylation-dependent binding motifs for recruiting 14-3-3 proteins (**Supplementary Fig. 6)**. Through harnessing the interactions between phosphorylated peptides and 14-3-3 proteins[57], there exists an opportunity to develop a class of phospho-responsive components to modulate protein secretion and further expand the programming capabilities of compRELEASE. With over 200 distinct human proteins documented to interact with 14-3-3 proteins[58], the potential to monitor shifts in vital cellular processes such as cell cycle progression, glucose metabolism and apoptosis[21,59] may hold significant implications for advancing therapeutics applications, such as cell transplantation and cancer immunotherapies in the future.

The obstacle of delivery remains a significant challenge in the integration of synthetic protein circuits. While these circuits can be encoded within a single polycistronic construct, it's imperative to fine-tune individual components to ensure proper circuit functionality. Instead of relying on large and cumbersome Internal Ribosomal Entry Sites (IRES) to regulate expression levels[60], we capitalized on the inherent modularity and composability of engineered proteases and RELEASE to optimize our circuit's dynamic range at the post-translational level (**Fig. 4c**). Although not explicitly tested here, fine-tuning the relative expression levels of individual proteins has been found to be contingent on the positioning of individual genetic elements and the selection of specific 2A peptides[61]. The ability to optimize the relative expression levels at the genetic levels, in parallel with their respective activities at the protein level, holds the potential to enhance the tuneability of synthetic protein circuits.

The examples presented thus far have employed a single compRELEASE construct to govern a single output. However, the potential for more complex circuit functions is substantial, as multiple variants introducing different logic (**Fig. 2c**) or quantitative processing (**Fig. 3e, f**) can be integrated. For instance, by stacking RELEASE and RELEASE-NOT inducible variants to the same protease, one can establish single-input multi-output behavior driven by the presence or absence of a given protease input. Introducing these behaviors within engineered cells and co-delivering them with therapeutic cells to modulate the dynamic immune response may be a suitable approach for improving the long-term engraftment without the need of systemic immunosuppression. Moreover, the ability to exert on-demand control of outputs via mRNA (**Fig. 4f**), ensures that only engineered cells equipped with the synthetic protease circuit can activate and regulate their expression. The inherent compactness, delivery potential, and composability of synthetic protein circuits enhanced by compRELEASE will be an invaluable tool for implementing programmable cell therapies that control intercellular signals for biomedicine.

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Figures:

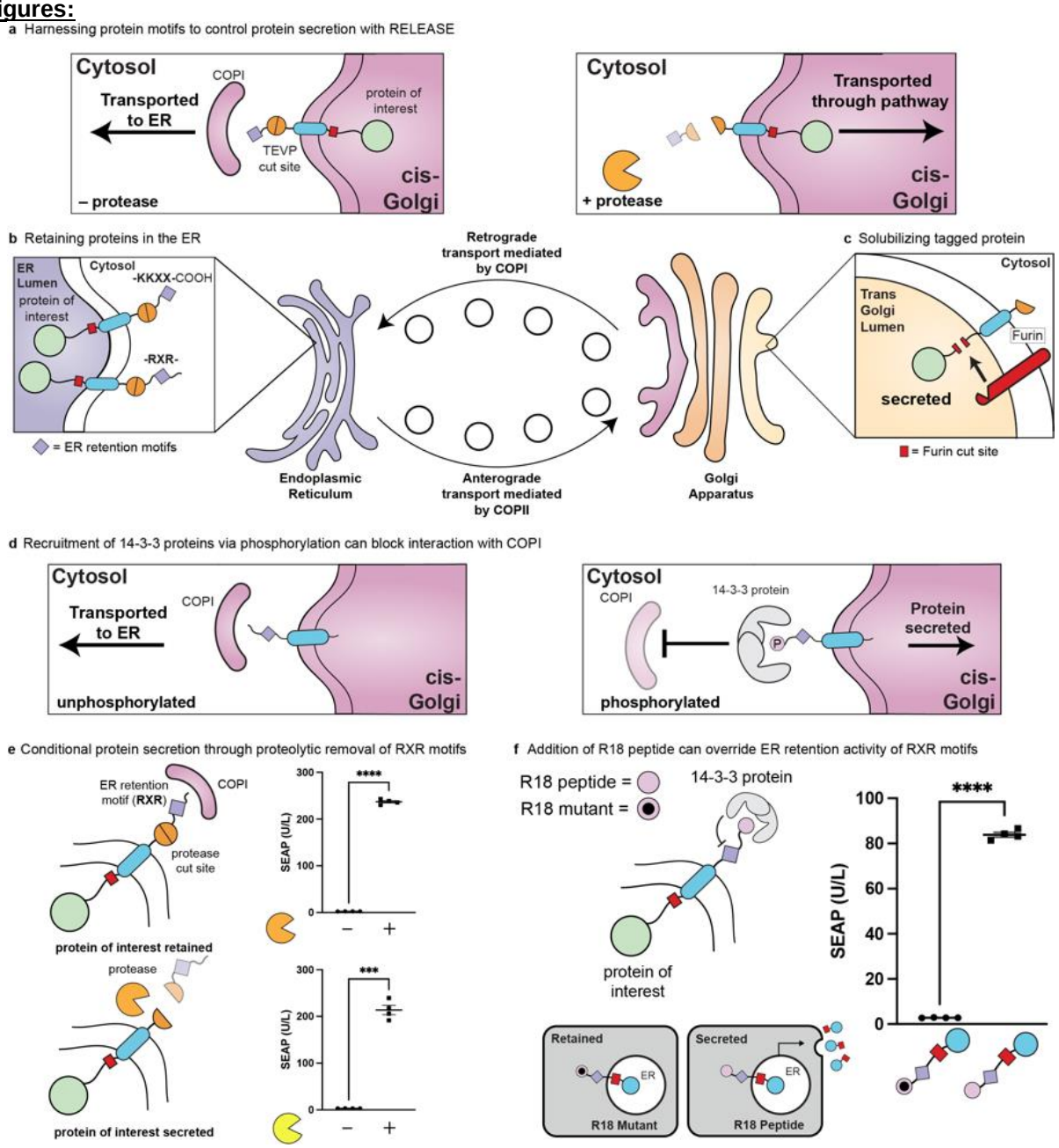


Figure 1 – Harnessing protein motifs to control protein secretion with RELEASE: **a)** Co-translated secreted proteins are normally transported through the conventional secretory pathway and secreted into the extracellular space. The Retained Endoplasmic Cleavable Secretion (**RELEASE**) platform harnesses **b)** native ER retention motifs (purple diamond) to retain tagged proteins of interest in the ER. Only through the activation or expression of a protease such as TEVP (orange partial circle), the ER-retention motif will be removed, and the protein of interest will be transported through the conventional secretory pathway. Upon reaching the trans-golgi apparatus, the tagged protein of interest will be solubilized through cleavage from the **c)** furin endoprotease and then secreted. **d)** Phosphorylation-dependent recruitment of endogenous 14-3-3 scaffolding proteins to control the surface expression of membrane proteins. **e**) Additional ER-retention motifs such as the diarginine (-RXR-) motif are compatible with RELEASE. Co-expression of proteases such as TEVP (orange partial circle), or HCVP (yellow partial circle) with the respective RELEASE constructs significantly increased SEAP secretion. **f**) The fusion of the 14-3-3 antagonist (R18 peptide) to the C-terminus of RELEASE inhibited ER retention activity and resulted in the

secretion of SEAP. In comparison, a fused R18 mutant which had two acidic residues required for 14-3-3 recruitment mutated into lysines (D12K and E14K) could not overcome the retention capabilities of RELEASE, and SEAP was not secreted. Each dot represents a biological replicate. Mean values were calculated from four replications (**d** and **f**). **The** error bars represent ± SEM. The results are representative of at least two independent experiments; significance was tested using an unpaired two-tailed Student's *t*-test between the two indicated conditions for each experiment. ****$p < 0.0001$.

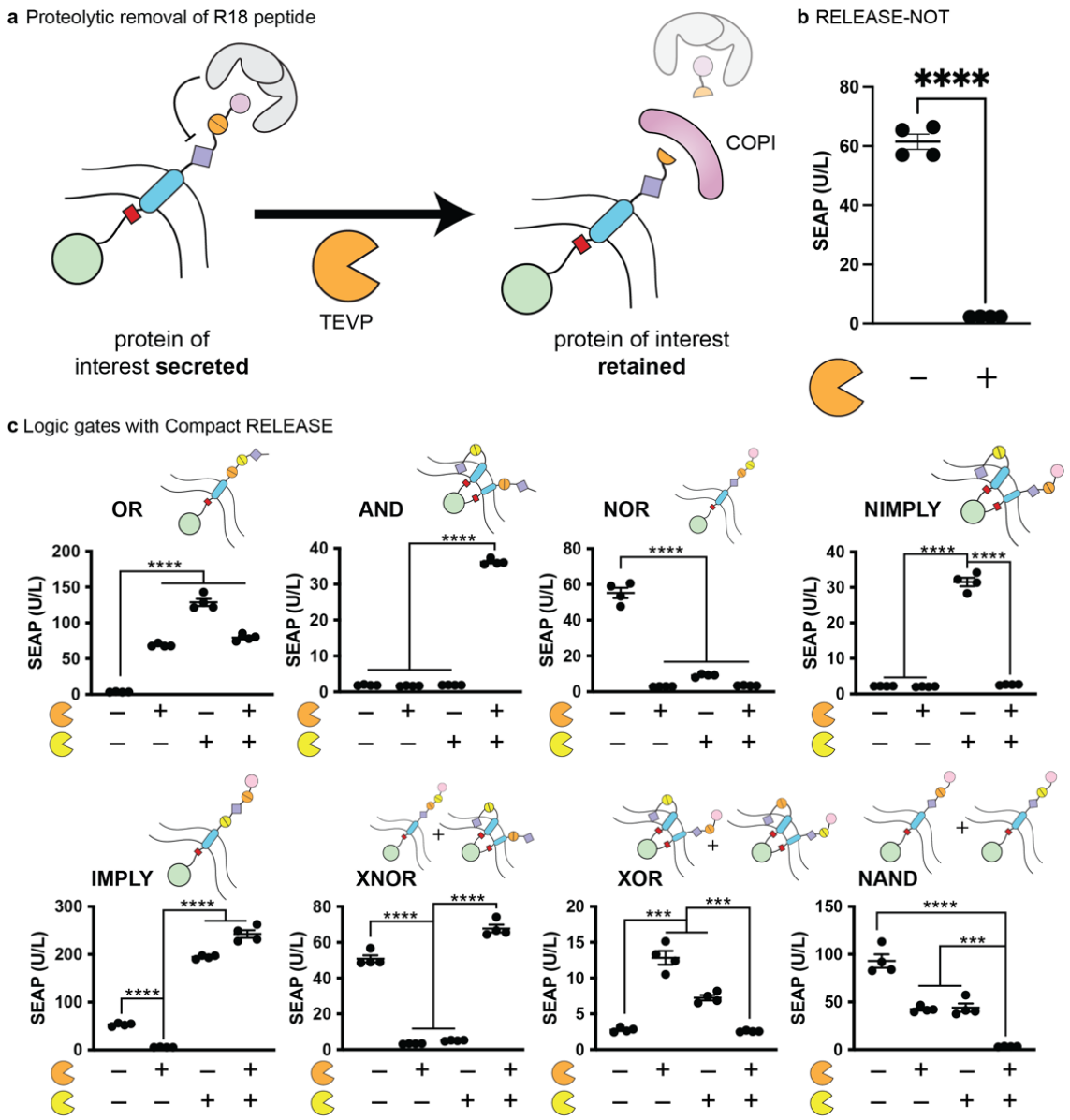


Figure 2 – Functional completeness using Compact RELEASE: a) The RELEASE platform was modified to enable negative regulation of protein secretion (RELEASE-NOT) in response to protease activity. Without protease activity, the tagged protein of interest in secreted due to the antagonistic activity of the R18 peptide on the ER-retention motif. Proteolytic removal of the R18 peptide restores the ER-retention activity, resulting in the tagged protein of interest to be retained (**NOT** logic). **b)** With RELEASE-NOT, the co-expression of proteases such as TEVP resulted in a significant decrease in SEAP secretion. **c)** Due to the modularity of engineered proteins, RELEASE variants were engineered to enable binary logic gates. This collection of RELEASE variants is referred to as the Compact RELEASE (compRELEASE) suite. For each indicated gate, TEVP and HCVP served as binary inputs, which are either included or excluded in transfections. SEAP secretion served as an output. The design of each compRELEASE variant is shown above each respective graph. Each dot represents a biological replicate. Mean values were calculated from four replications (**c-d**). **The** error bars represent ± SEM. The results are representative of at least two independent experiments; significance was tested using an unpaired two-tailed Student's *t*-test between the two indicated conditions for each experiment. For experiments with multiple conditions, a one-way ANOVA with a Tukey's post-hoc comparison test was used to assess significance. ***$p < 0.001$. ****$p < 0.0001$.

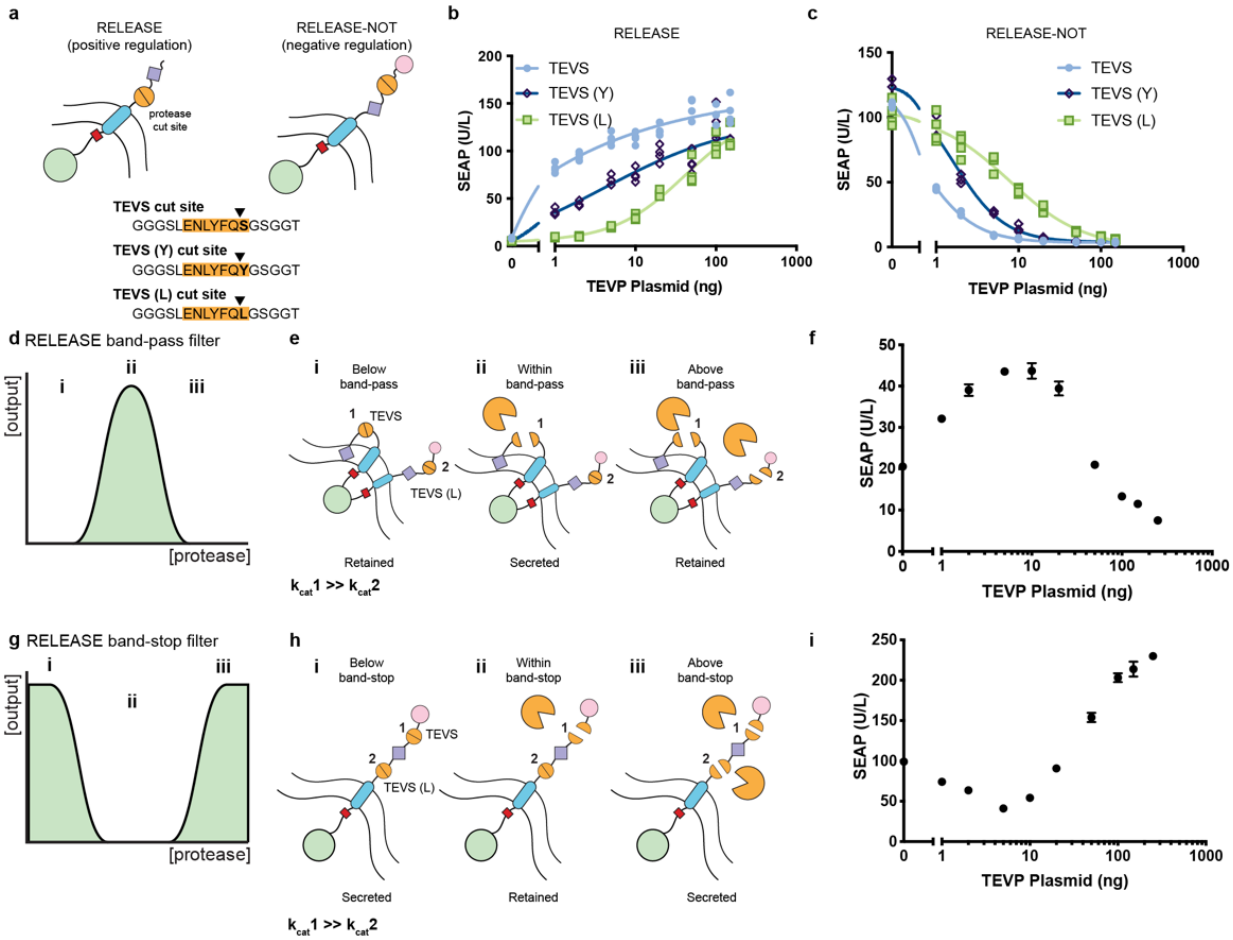


Figure 3 – Quantitative processing using the Compact RELEASE suite: a) RELEASE and RELEASE-NOT control the activation or repression of protein secretion in response to protease activity, respectively. The cleavage efficiencies of RELEASE and RELEASE-NOT to TEVP were modified by creating constructs using protease cut sites with different residues at the P1 position (bolded letter). The inverted triangle indicates the position of proteolytic cleavage. **b)** Input-output curve of three RELEASE constructs (positive regulation) with modified protease cut sites titrated with TEVP plasmid. Changing the TEVP cut site from the canonical sequence reduced the cleavage efficiency and increased the activation threshold required for protein secretion. **c**) The same changes in TEVP cut site sequences affected the repression thresholds of proteins tagged with RELEASE-NOT. The data is fitted to a Hill equation with a variable slope represented by the respective lines. **d)** Expected input-output curve of band-pass activation. **e)** To achieve band-pass activation of protein secretion, a NIMPLY compRELEASE variant was modified to contain two TEVP cut sites with different cutting efficiencies (K_{cat}). Band-pass filter activation requires the first TEVP cut site (indicated as cut site 1) to have a higher K_{cat} than the second TEVP cut site (indicated as cut site 2). This ensures that at low concentrations of TEVP, the first cut site will be cleaved, and the tagged protein of interest will be secreted. As the concentration of TEVP increases, the second cut site will be cleaved, restoring the ER-retention activity and the protein of interest will be retained. **f**) Input-output curve of the RELEASE band-pass construct titrated with TEVP plasmid. **g)** Expected input-output curve of band-stop filter activation. **h)** To achieve band-stop activation of protein secretion, an IMPLY compRELEASE variant designed with two TEVP cut sites with different cutting efficiencies was created. Under basal conditions without TEVP, the tagged protein of interest will be secreted since the ER-retention motif is inhibited by the R18 peptide. As the concentration of TEVP increases, the distal cut site will be preferentially cleaved (indicated as cut site 1), restoring ER-retention activity and reducing SEAP secretion. If the concentrations of TEVP continue to increase, then the proximal cut site will be cleaved (indicated as cut site 2) and the tagged protein of interest will be secreted. **i)** Input-output curve of the RELEASE band-stop construct titrated with TEVP plasmid. Each dot represents a biological replicate. Mean values were calculated from four

replications (**b-c, f and i**). The error bars represent ± SEM. The results are representative of at least two independent experiments; significance was tested using an unpaired two-tailed Student's *t*-test between the two indicated conditions for each experiment. For experiments with multiple conditions, a one-way ANOVA with a Tukey's post-hoc comparison test was used to assess significance. ***$p < 0.001$. ****$p < 0.0001$.

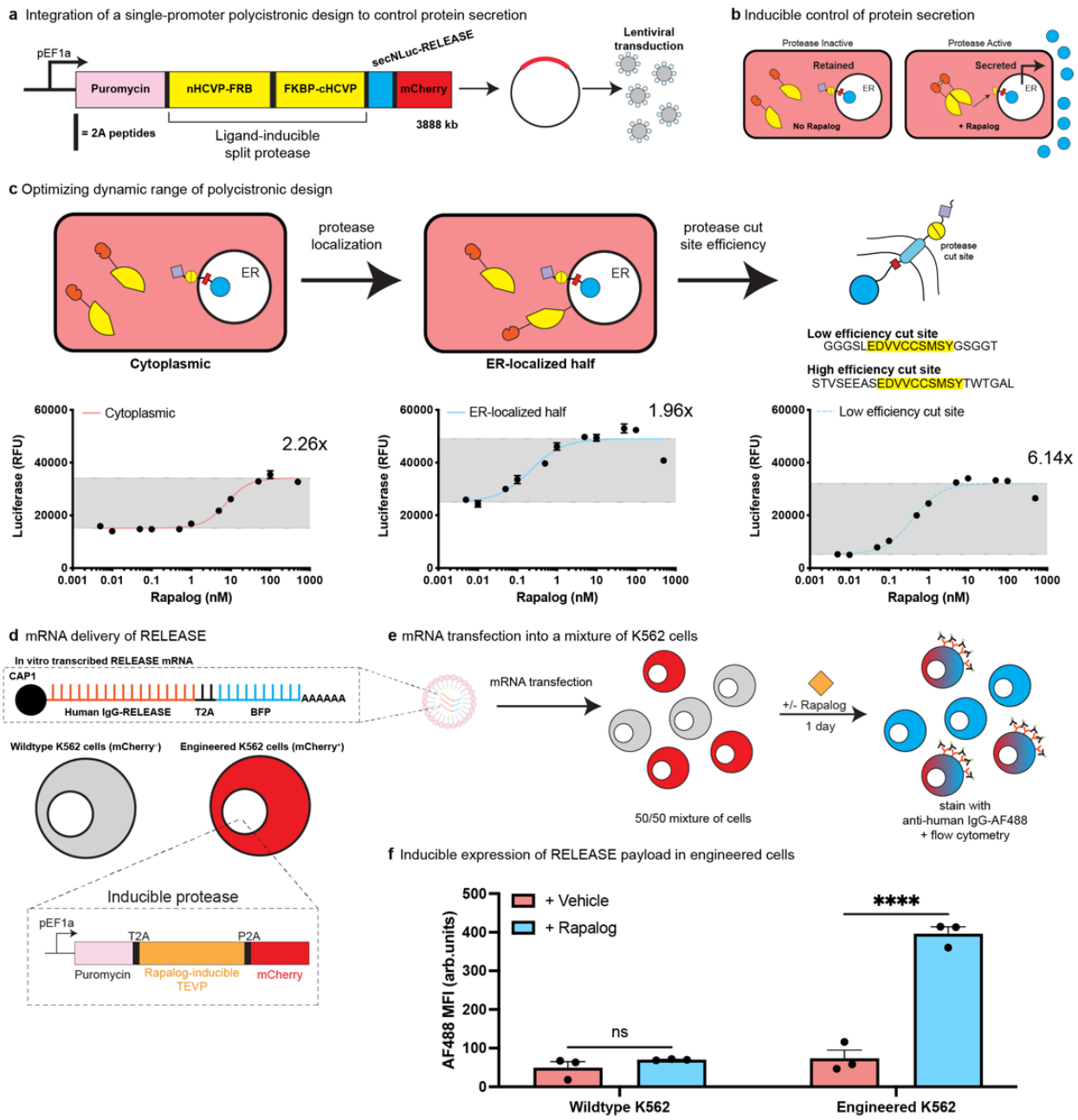
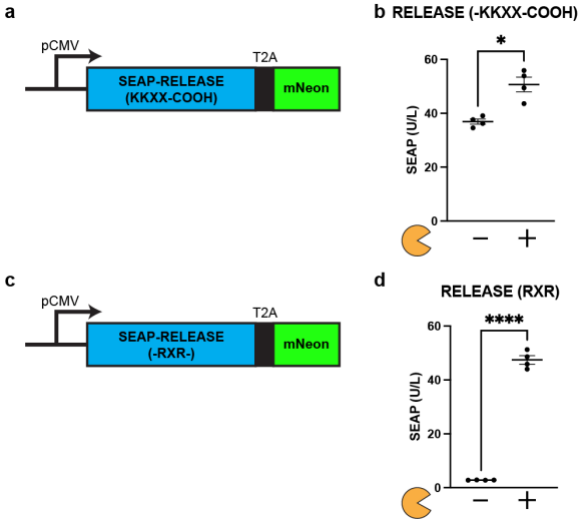


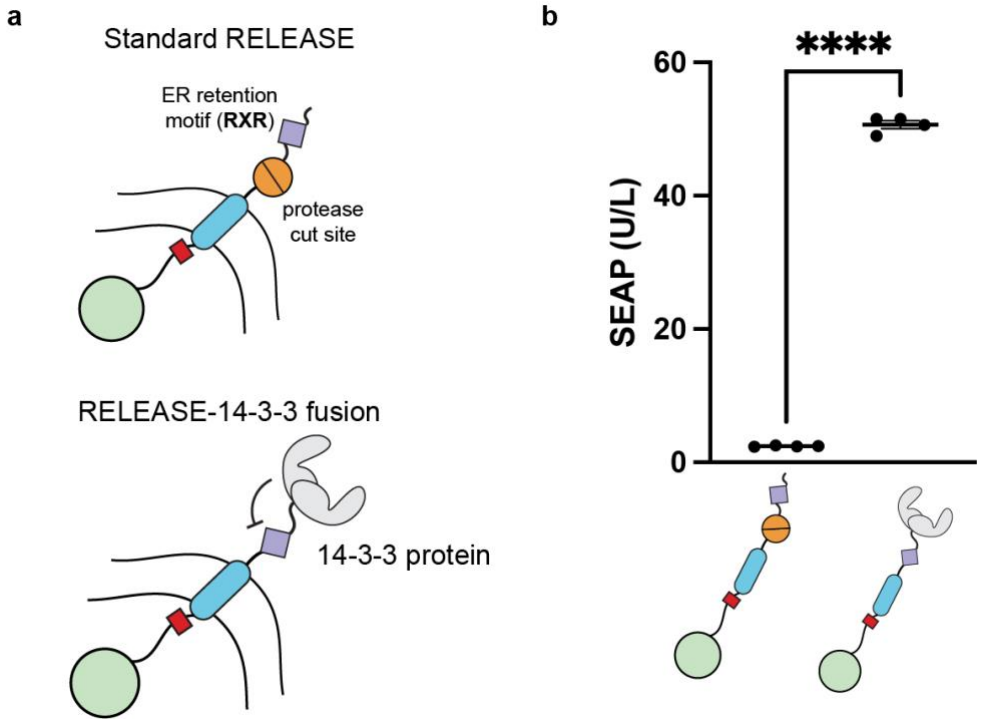
Figure 4 – Delivery of RELEASE: a) Schematic of the stable integration of a polycistronic gene encoding all the protein components required for inducible protein secretion via lentiviral transduction. **b)** Engineered cells contain a split HCVP protease expressed in the cytoplasm to control the secretion of Nluc following induction with rapalog. **c)** Input-output curves for engineered cells where secreted Nluc functions as the output and rapalog concentration serves as the input. The curve represents a Hill equation with a variable slope, and the grey box represents the top and bottom plateaus of the fit. To increase Nluc secretion, one half of the split HCVP was localized to the ER via the p450-motif, however this design resulted in an increase in background secretion and a reduction in the dynamic range. To reduce the background and increase the dynamic range, the cleavage efficiency of the HCVP cut site of RELEASE was reduced by modifying the flanking residues, as previously described[15]. **d)** mRNA delivery of a synthetic surface marker (IgG) tagged with RELEASE and BFP into wildtype and engineered K562 cells. K562 cells were engineered to stably express a rapalog-inducible TEVP protease and the mCherry reporter protein. **e**) Experimental setup of RNA-delivered IgG-RELEASE into a mixture of wildtype and engineered K562 cells with and without rapalog. Following 24 hours, the cells were harvested, stained for the surface expression of IgG

and processed through flow cytometry. **f)** Only engineered K562 cells containing the inducible split TEVP showed a significant increase in the surface expression of IgG. Wildtype K562 cells did not show any significant differences. Each dot represents a biological replicate. Mean values were calculated from four replicates (**c** and **f**). The error bars represent ± SEM. The results are representative of at least two independent experiments; significance was tested using a one-way ANOVA with a Tukey's post-hoc comparison test was used to assess significance. ****$p < 0.0001$.

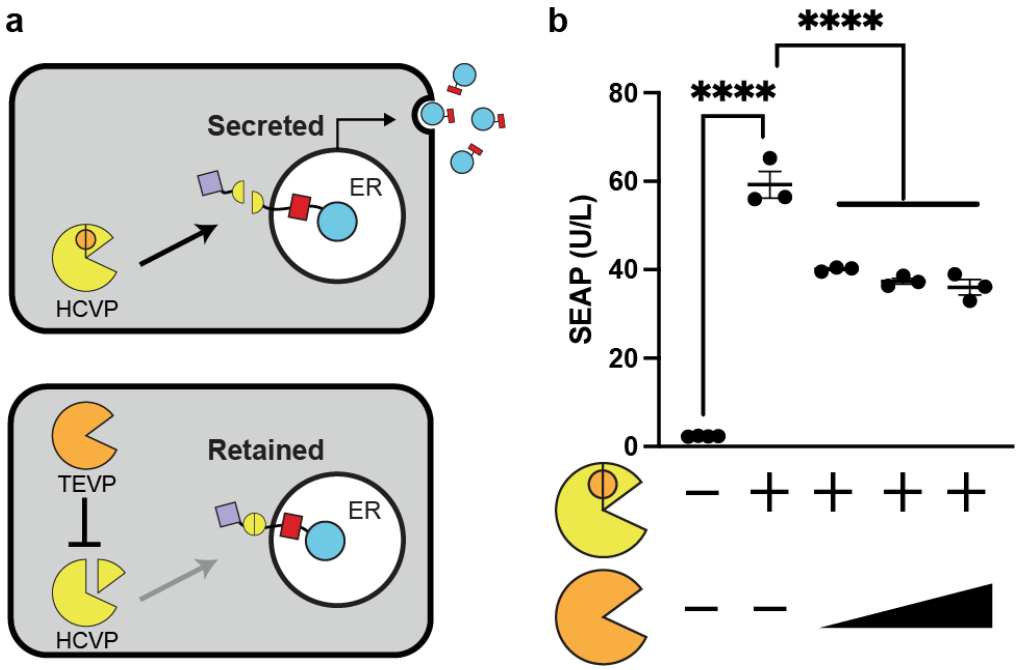
Supplementary Figures:



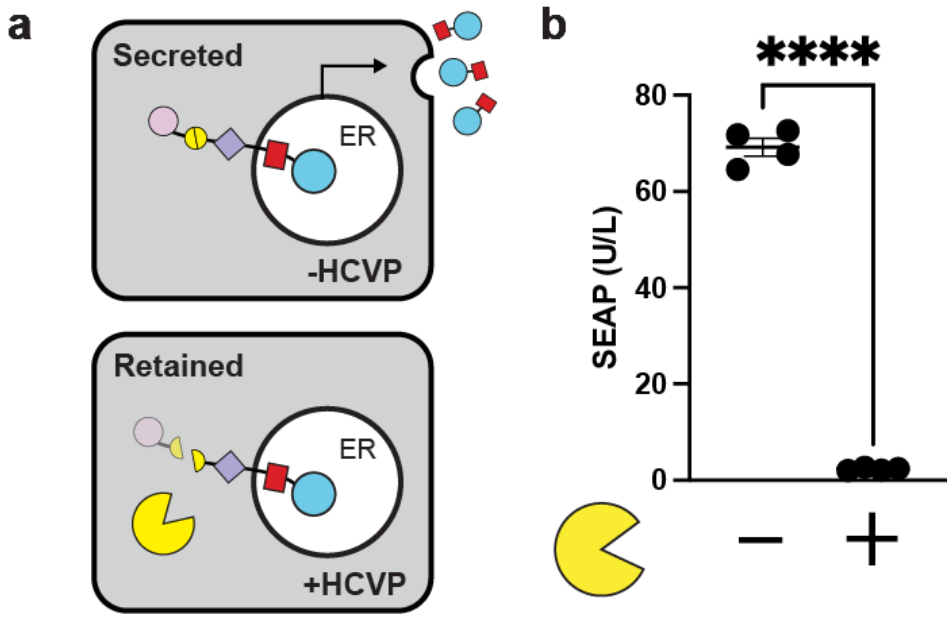
Supplementary Figure 1 – ER retention motifs in polyprotein constructs: a) Schematic of polyprotein construct containing SEAP-RELEASE under the control of the dilysine retention motif (KKXX-COOH) and the mNeon reporter protein separated via a T2A self-cleaving peptide. **b)** The retention capabilities of RELEASE were negatively affected, and SEAP secretion was measured even in the absence of TEVP. There was a significant increase in SEAP secretion when expressing TEVP, however the dynamic range was lower than previously reported[15]. **c)** Schematic of polyprotein construct containing SEAP under the control of the diarginine retention motif (-RXR-) and the mNeon reporter protein separated via a T2A self-cleaving peptide. **d)** The retention capabilities of these RELEASE variant using the diarginine retention motif were intact and we observed a significant increase in SEAP secretion when co-expressing TEVP. Each dot represents a biological replicate. Mean values were calculated from four biological replicates (**b**, **d**). The error bars represent ± SEM. The results are representative of at least two independent experiments; significance was tested using an unpaired two-tailed Student's *t*-test between the two indicated conditions. $^{**}p < 0.01$, $^{****}p < 0.0001$.



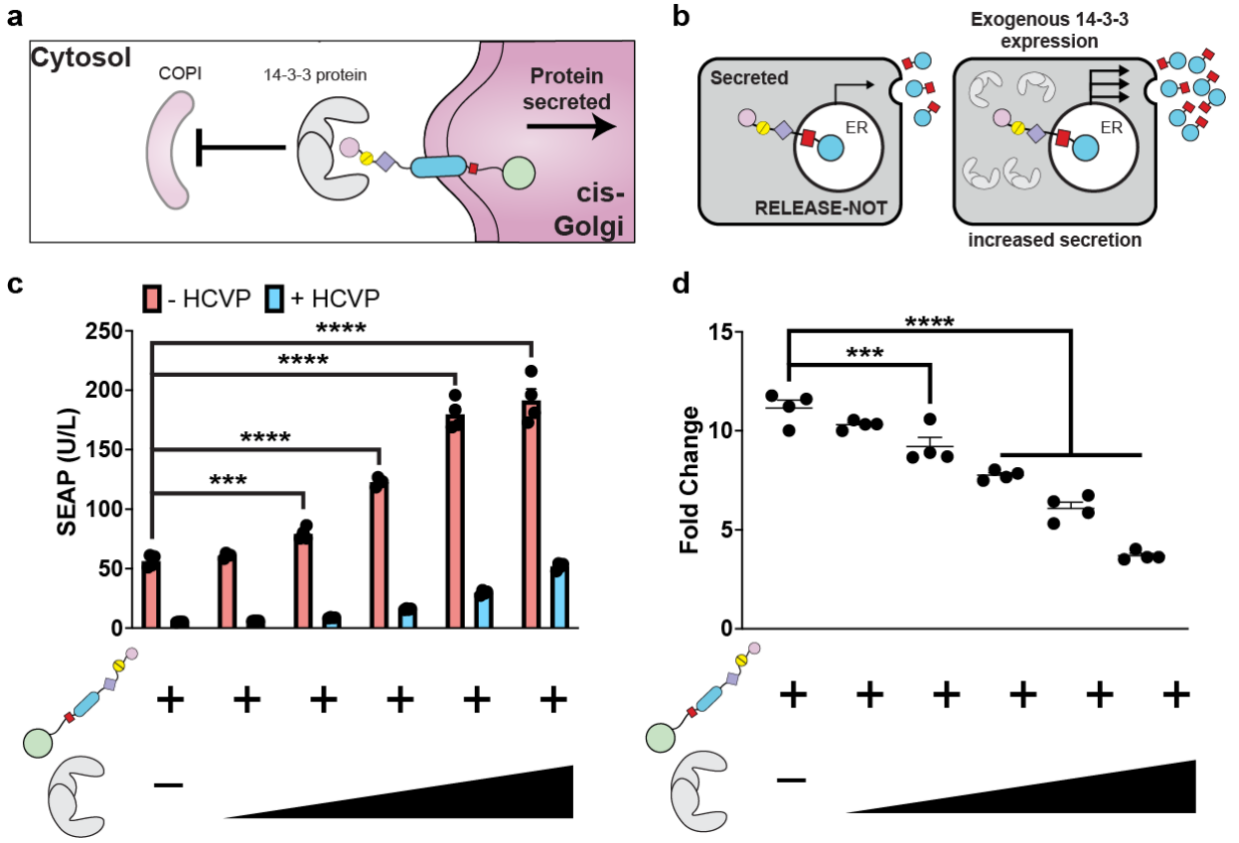
<u>Supplementary Figure 2 – Fusing 14-3-3ζ to block ER-retention activity of RELEASE:</u> a) Schematic of the standard RELEASE construct and a RELEASE-construct fused to the 14-3-3ζ protein. **b)** In the absence of protease, there was a significant increase in SEAP secretion when transiently transfecting the RELEASE-14-3-3 fusion compared to the standard RELEASE alone. Each dot represents a biological replicate. Mean values were calculated from four biological replicates (**b**, **d**). The error bars represent ± SEM. The results are representative of at least two independent experiments; significance was tested using an unpaired two-tailed Student's *t*-test between the two indicated conditions. $^{**}p < 0.01$, $^{****}p < 0.0001$.



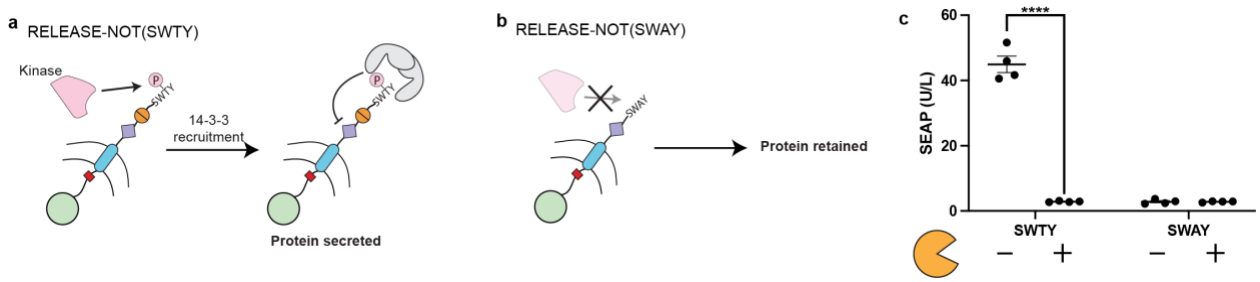
Supplementary Figure 3 – Negative regulation of protein secretion using CHOMP: a) Schematic of CHOMP circuit for negative regulation of protein secretion. A HCVP-inducible RELEASE construct controls the secretion of SEAP. The HCVP protease can be repressed by TEVP, by cleaving the co-peptide of HCVP, which will significantly reduce its activity. **b)** SEAP secretion was dependent on the co-expression of HCVP. Increasing the amount of TEVP significantly reduced SEAP secretion, however negative regulation of SEAP secretion plateaued and could not reach basal levels. Each dot represents a biological replicate. Mean values were calculated from four replicates (**b**). The error bars represent ± SEM. The results are representative of at least two independent experiments; significance was tested using a one-way ANOVA with a Tukey's post-hoc comparison test was used to assess significance. ****$p < 0.0001$.



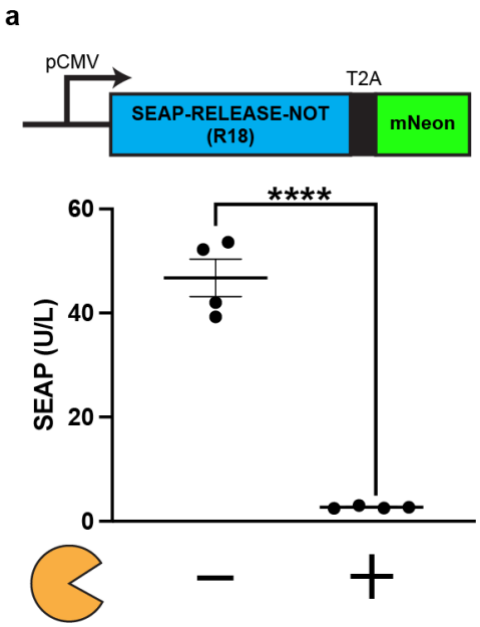
<u>Supplementary Figure 4 – HCVP-inducible RELEASE-NOT:</u> a) Schematic of HCVP-inducible RELEASE-NOT. When co-expressed with HCVP, SEAP secretion decreased relative to when HCVP was absent. Each dot represents a biological replicate. Mean values were calculated from four biological replicates +/- SEM. The results are representative of at least two independent experiments; significance was tested using an unpaired two-tailed Student's *t*-test between the two indicated conditions for each experiment. **** = $p < 0.0001$.



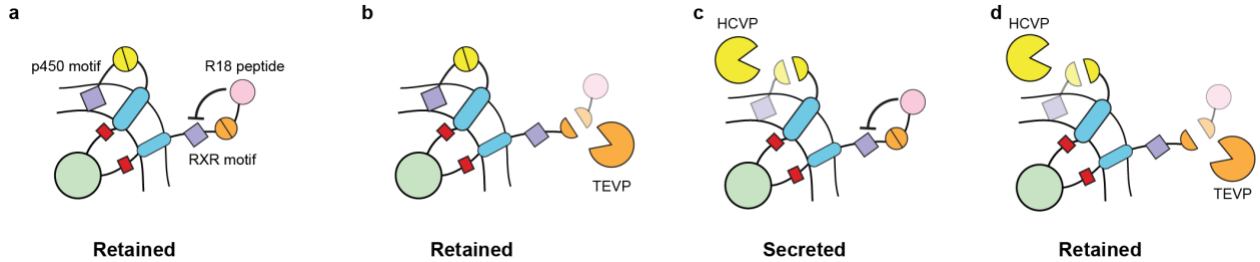
Supplementary Figure 5 – Exogenous expression of 14-3-3ζ to boost activity of RELEASE NOT: **a)** Mechanism of action for RELEASE-NOT to control protein secretion in the absence of protease. **b)** Schematic of adding exogenous 14-3-3 scaffolding proteins to boost the secretion of RELEASE-NOT in the absence of protease. **c)** Increasing the amount of exogenous 14-3-3ζ resulted in a significant increase in SEAP secretion with and without the co-expression of HCVP. **d)** Although we observed an increase in SEAP secretion as increasing the amount of exogenous 14-3-3ζ, the fold change between the ON and OFF state decreased. 14-3-3ζ also increased SEAP secretion in the presence of HCVP, resulting in a reduction in the overall fold change as 14-3-3ζ increased. Each dot represents a biological replicate. Mean values were calculated from four replicates (**b-c**). The error bars represent ± SEM. The results are representative of at least two independent experiments; significance was tested using a one-way ANOVA with a Tukey's post-hoc comparison test was used to assess significance. ***$p < 0.001$, ****$p < 0.0001$.



Supplementary Figure 6 – Phosphorylation-dependent recruitment of 14-3-3 proteins to program protein secretion: **a)** Schematic of RELEASE-NOT using the SWTY motif to enable phosphorylation-dependent recruitment of 14-3-3 proteins to block the retention activity of RXR. **b)** Schematic of RELEASE-NOT where the phospho-responsive threonine residue is mutated to alanine (SWTY-COOH → SWAY-COOH). **c)** When co-expressing TEVP we observed a significant decrease in SEAP secretion only when using the RELEASE-NOT construct with the SWTY motif. RELEASE-NOT with the SWAY motif did not result in any secretion of SEAP. Each dot represents a biological replicate. Mean values were calculated from four replicates. The error bars represent ± SEM. The results are representative of at least two independent experiments; significance was tested using a one-way ANOVA with a Tukey's post-hoc comparison test was used to assess significance. ****$p < 0.0001$.



Supplementary Figure 7 – R18 function is compatible when encoded by polycistronic constructs: **a)** RELEASE-NOT encoded by a polycistronic construct separated by self-cleaving T2A peptides is compatible with negative regulation of protein secretion. Each dot represents a biological replicate. Mean values were calculated from four biological replicates +/- SEM. The results are representative of at least two independent experiments; significance was tested using an unpaired two-tailed Student's *t*-test between the two indicated conditions for each experiment. **** = $p < 0.0001$.



<u>Supplementary Figure 8 – Schematic of RELEASE-NIMPLY:</u> a) In the absence of any protease, the tagged protein of interest is retained due to the ER-retention capabilities of the N-terminal signal anchor of cytochrome p450. **b)** When TEVP is co-expressed, the R18 peptide is removed restoring the retention capabilities of the RXR ER-retention motif and the protein of interest is retained. **c)** When HCVP is co-expressed, the N-terminal ER-retention motif is removed, and the C-terminal retention motif is being inhibited by the recruitment of 14-3-3 proteins via the intact R18 peptide. This results in the tagged protein of interest being secreted. **d**) Co-expression of HCVP and TEVP resulted in the tagged protein of interest being retained. Although the N-terminal ER-retention motif is removed by HCVP, the activity of the C-terminal ER-retention motif is restored by removal of the R18 peptide.

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