Designed Endocytosis-Triggering Proteins mediate Targeted Degradation

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Abstract

Endocytosis and lysosomal trafficking of cell surface receptors can be triggered by interaction with endogenous ligands. Therapeutic approaches such as LYTAC[1,2] and KineTAC[3], have taken advantage of this to target specific proteins for degradation by fusing modified native ligands to target binding proteins. While powerful, these approaches can be limited by possible competition with the endogenous ligand(s), the requirement in some cases for chemical modification that limits genetic encodability and can complicate manufacturing, and more generally, there may not be natural ligands which stimulate endocytosis through a given receptor. Here we describe general protein design approaches for designing endocytosis triggering binding proteins (EndoTags) that overcome these challenges. We present EndoTags for the IGF-2R, ASGPR, and Transferrin receptors, and show that fusing these tags to proteins which bind to soluble or transmembrane protein leads to lysosomal trafficking and target degradation; as these receptors have different tissue distributions, the different EndoTags could enable targeting of degradation to different tissues. The modularity and genetic encodability of EndoTags enables AND gate control for higher specificity targeted degradation, and the localized secretion of degraders from engineered cells. The tunability and modularity of our genetically encodable EndoTags should contribute to deciphering the relationship between receptor engagement and cellular trafficking, and they have considerable therapeutic potential as targeted degradation inducers, signaling activators for endocytosis-dependent pathways, and cellular uptake inducers for targeted antibody drug and RNA conjugates.

Main Text

The endocytosis of many cell surface receptors is triggered by binding of their endogenous ligands which can shift the conformational or oligomerization state of the receptor[4] and induce receptor clustering and adaptor protein recruitment[5,6]. Native endocytosis inducing ligands have been utilized to enhance intracellular delivery and prompting extracellular protein degradation[1–3]. While powerful, these approaches have the limitations that native ligands can trigger off-targeting signaling[3,7], their binding sites may be occupied by existing ligands, and instability and in some cases the need for modification can complicate manufacturing[8]. Bio-orthogonal inducers of endocytosis could have therapeutic utility not only for targeted degradation but also for initiating signaling through pathways involving endocytosis[4], and provide powerful tools for investigating the association between cellular trafficking and receptor structures. Antibodies have been identified that stimulate endocytosis, but this can require considerable empirical screening for any target receptor[9,10]. To date there does not exist a completely synthetic and systematic approach to design such orthogonal and synthetic activators of cellular trafficking.

We reasoned that de novo protein design could enable the creation of bio-orthogonal endocytosis inducing proteins that avoid the above limitations by using strategies customized for the target receptor. For receptors such as transferrin receptor (TfR), that constitutively traffic between the cell surface and the endosome/ lysosome, binding to a site on the receptor non-overlapping with the native ligands could be sufficient (**Fig. 1a**).

For receptors such as the IGF-2R receptor, for which conformational change triggers endocytosis, binding must induce rearrangement of receptor extracellular domains, while for others, such as ASGPR, where endocytosis is stimulated by clustering, binding should induce oligomerization. Fusion of designed proteins with these properties to a second target binding protein could promote endocytosis and lysosomal trafficking of the target. We set out to design such endocytosis targeting proteins, which we call EndoTags, for all four receptor systems, and to explore their utility for modulating protein degradation and cellular signaling.

To enable tissue-specific control over endocytosis for downstream applications, we selected target receptors with distinct tissue expression profiles. IGF-2R is expressed in most tissues, ASGPR is expressed primarily in the liver, and TfR is abundant in the brain[11,12]. We developed customized design approaches to optimize endocytosis through each of these receptor systems, as described in the following sections.

Design of EndoTags orthogonal to endogenous ligands

TfR constitutively cycle between the cell surface and intracellular compartments. Thus, for these receptors, the challenge is not to actively induce endocytosis, but to bind to the receptor at a site that does not compete for the natural ligand, which could have undesired side effects and reduced efficiency. De novo protein design has the advantage of being able to target binders to specific sites of interest on a target[13–15], and

thus is well suited to designing protein binders that target receptor sites that do not overlap with those of native ligands.

We applied orthogonal binding strategy with TfR (**Fig. 1b**) whose native function is to transport iron-bound transferrin into cells and across the blood brain barrier[12,16]. We previously reported the design of TfR binders with high binding affinity at a binding site distant from the transferrin binding site[17]; here we explore the use of this binder as an EndoTag (TfR_EndoTag). We found that the TfR_EndoTag directed endocytosis in U-251MG glioblastoma cells, with a fifty fold increase in cellular uptake over control after two hour incubation (**Fig. 1c**). Confocal imaging again indicated lysosome targeting of the TfR_EndoTag after 24 hours incubation in U-251MG cells (**Extended Data Fig. 5b**).

Taken together, these results with TfR indicate that binding to bio-orthogonal sites with single high-affinity binding domains is an effective strategy for hijacking constitutively endocytosing receptors. Our bio-orthogonal binder design approach could be readily generalized to a wide range of other tissue specific targets.

Design of EndoTags for triggering conformational change

IGF-2R is a large cell surface receptor that rapidly transports its natural ligands IGF-2 and M6P to the lysosome for degradation[1]. IGF-2R has high abundance across tissues, and considerable overall lysosomal trafficking capacity which has made it an attractive target for protein degradation approaches. Structure investigation suggests that IGF-2 binding induces a conformational change in IGF-2R which brings together domain 6 (D6) and domain 11 (D11), and promotes dimerization of the receptor[18]. With the ability

to design de novo binders at arbitrary interfaces[13], we hypothesized that a designed binding protein that brings together domain 6 and 11 could similarly trigger IGF-2R endocytosis and lysosomal targeting without triggering off-target signaling activation like IGF-2[7].

We sought to use the Rosetta RIFdock method[13,14] to design small proteins that bind to D11 and D6 of IGF-2R independently. Inverse rotamer fields were generated around residues interacting with IGF-2R in both domains (**Extended Data Fig. 1d-g**), and scaffolds were docked into these fields with RIFdock[19]. After sequence optimization and refinement with RosettaFastdesign, the best scoring binding motifs were identified and grafted back on to the scaffold library with RosettaMotifgraft[15], followed by another round of RosettaFastdesign. Genes encoding the top-ranked 15,000 designs based on Rosetta interface metrics (ddg, contact_molecular_surface)[13], were synthesized on an oligonucleotide array, transformed into a yeast display vector, and fluorescence-activated cell sorting (FACS) was carried out using biotinylated human IGF-2R domain 6 and domain 11. After next-generation sequencing, we identified one high-affinity candidate binder for domain 6 and two candidate binders for domain 11; these were expressed and purified in E coli. The IGF-2R domain 6 binder (D6mb) affinity of 41nM KD was measured using biolayer interferometry (BLI) (**Extended Data Fig. 2a**), and the best domain 11 minibinder (D11mb) bound IGF-2R domain 11 at an affinity of 190nM KD (**Extended Data Fig. 2b**). After affinity optimization with mutation library screening, the highest affinity variant for D11 (D11mb2) bound domain 11 at 6.5nM KD in BLI (**Extended Data Fig. 2c**).

We next sought to develop IGF-2R EndoTags (IGF_EndoTags) utilizing the distinct IGF-2R D6 and D11 minibinders (**Fig. 1e**). Initially, we explored the use of flexible fusions between D11mb and D6mb, with different loop lengths and domain orders. We then expressed these fusions in E. coli and incorporated an avi-tag for biotinylation. Finally, we evaluated their cellular uptake in Jurkat cells by utilizing Alexa-647 (AF-647) and flow cytometry. A construct connecting D11mb and D6mb with a GGS linker (D11mb-GGS-D6mb, IGF_EndoTag1) was the most readily uptaken, with increased cell associated fluorescence signal over native IGF-2 or D6mb/D11mb alone in Jurkat cells (**Extended Data Fig. 4c**). Co-localization imaging indicated that IGF_EndoTag1 is targeted to lysosomes (**Fig. 1g**): this synthetic ligand thus recapitulates the trafficking of the endogenous IGF-2 ligands. Longer linkers decreased the uptake level, while a shorter GS linker abolished all uptake (**Extended Data Fig. 4b**), suggesting the orientation and distance of the two binding domains modulates the IGF-2R endocytosis process. Constructs with two copies of one mini-binder (D6mb-linker-D6mb, D11mb-linker-D11mb did not display any cellular uptake (**Extended Data Fig. 4a**); engagement of both domains (likely driving their reorientation within the receptor structure) appears to be necessary to trigger efficient cellular uptake. Substitution of D11mb with the higher affinity variant D11mb2 in IGF_EndoTag1 (generating IGF_EndoTag2) increased internalization to 2 fold compared to native IGF-2 in Jurkat cells (**Fig 1f**); IGF_EndoTag2 was clearly detectable in lysosomes after a 30 minute incubation, unlike IGF-2 or IGF_EndoTag1 (**Extended Data Fig. 4d-e**).

We reasoned that still more potent stimulation of endocytosis could be achieved by two domain constructs in which the individual domains are rigidly fused to each other to

drive specific receptor conformational changes. We generated such fusions by using RFInpainting[20] to generate three-helix bundles combining the major interface helix from D11mb and two interface helices from D6mb (**Fig. 1e**, **Extended Data Fig. 3**). Designed residues within 3 angstroms (A) of the receptor were kept fixed, the D11mb helix was randomly perturbed by rigid body translations (up to 5 angstroms) and rotations (up to 10 degrees), the two chains were connected by inpainting, and the sequence of the fusion designed in the context of IGF-2R using ProteinMPNN[21] keeping residues interacting with the receptor constant. Models with RosettaFold LDDT metrics (>0.5) for the inpainted region and Alphafold2[22] (AF2) structure predictions for the fusions matching the design models (RMSD < 2.0) were experimentally screened for binding with yeast display against both IGF-2R domains. Out of 170 screened designs, the 8 most enriched candidates were selected for protein expression and BLI affinity evaluation. Different designs had distinct affinities for domains 6 and 11; for example, IGF_EndoTag3 possessed strong binding affinity to both domains (6nM for domain 6 and 190nM for domain 11, **Extended Data Fig. 2d**), while EndoTag4 bound more tightly to domain 6 (15nM for domain 6 and 4.3μM for domain 11, **Extended Data Fig. 2e**). In cellular uptake assays, IGF_EndoTag3 had internalization activity equivalent to IGF-2, and IGF_EndoTag4 showed 2 fold higher internalization than IGF-2 (**Extended Data Fig. 4c**) and co-localized with lysosomes within 30 minutes as we observed in co-localization imaging (**Extended Data Fig. 4f**).

Design of EndoTags for inducing receptor clustering

The endocytosis of receptors such as ASGPR and EGFR is stimulated through dimerization or oligomerization[23]. ASGPR is a liver-specific ligand shuttling receptor to transport GalNAc labeled proteins into lysosomes for clearance[24]. Multivalent GalNAc ligands have been used for multiple liver-specific degradation applications[2,25] and RNA delivery platforms[26]. However, these require chemical modification and hence are not genetically encodable, and must compete with native ligands which raises challenges.

We designed binders to ASGPR that do not overlap with the glycan binding sites (**Extended Data Fig. 1h&i**) using an augmented version of the Rosetta design approach described above that uses ProteinMPNN[21] for sequence design and Alphafold2[18] for design evaluation. 2689 designs passed Alphafold2 pae_interaction cutoff and were selected for experimental screening. After yeast display enrichment and NGS, we identified 4 candidates that bound to ASGPR on the yeast surface. Following expression and purification from E coli, biolayer interferometry showed that the highest affinity of these, (ASmb1), binds to ASGPR with an affinity of 2.7 μM (**Extended Data Fig. 2f**).

To stimulate ASGPR endocytosis through clustering, we connected two or three ASmb1 domains with GS linkers to generate ASGPR EndoTags, which we refer to as AS_EndoTags-2C and AS_EndoTags-3C (**Fig. 1h**). Cellular uptake assays in Hep3B cells indicated that multivalent AS_EndoTags-2C and AS_EndoTags-3C were endocytosed more efficiently than monomeric ASmb1 (following a two hour incubation and extensive washing, 2.5-fold and 4.5-fold more fluorescence was associated with

cells, respectively (**Fig. 1i**)). Confocal imaging showed that AS_EndoTag-3C strongly co-localized with lysosomes after 24h (**Fig. 1j**, **Extended Data Fig. 5c**).

Cell surface receptor degradation

Targeted degradation of receptors and extracellular soluble proteins is a promising therapeutic strategy for cancer, autoimmune diseases and neurodegenerative diseases. Lysosome-targeting Chimeras (LYTACs) utilize mannose-6-phosphonate (M6Pn) ligands which triggers lysosomal delivery and degradation of the targeted proteins through the IGF-2R[1,27], or N-acetylgalactosamine (GalNAc) to trigger the ASGPR lysosomal trafficking pathway[2]. While very promising, the LYTAC approach is hindered by the reliance on existing native ligands and by the sophisticated chemistry required to generate multivalent modifications that increase endocytosis potency, complicating their manufacturing. Given their potent and rapid endocytosis and lysosomal targeting ability, we hypothesized that the fusion of EndoTags with POI-specific binders to generate protein-LYTACs (pLYTAC) (**Fig. 2a**), could provide an orthogonal and genetically-encoded approach for efficient extracellular protein degradation, and the different tissue distributions of the different receptors could allow targeting of degradation to different tissues.

To explore the potential of pLYTACs in promoting protein degradation, we investigated their ability to target and degrade cell surface receptors. We started by focusing on the

Epidermal Growth Factor Receptor (EGFR), which is often overexpressed in various cancer types and plays an important role in regulating cell proliferation[28]. We first assessed the degradation efficiency of the liver specific AS_EndoTags. Consistent with the cellular uptake results (**Fig. 1k**), introduction of EGFRn fusions to AS_EndoTags-2C and AS_EndoTags-3C resulted in a 40% decrease in total EGFR levels, while fusions to the monomeric ASGPR binder had little effect (**Fig. 2b**). Thus, designed protein mediated clustering of ASGPR can drive lysosomal targeting and cargo degradation, and the ASGPR-EndoTags could serve as liver-specific targeted degraders. To generate EGFR-pLYTACs targeting in the brain, we fused TfR_EndoTag with a minibinder targeting EGFR N-terminal (EGFRn)[13]. We observed efficient clearance of EGFR in HeLa cancer cells after 48h incubation, with 55% reduction of EGFR by EGFRn-TfR_EndoTag (**Fig. 2c**). Given the abundant expression of the corresponding receptors in the brain, TfR_EndoTag could function as pLYTACs for neurodegenerative disease applications.

We next sought to make systemically active pLYTACs that act through the ubiquitously expressed IGF-2 receptor. To this end, we fused IGF_EndoTags to EGFRn, and found that in H1975 and HeLa cells, EGFR was effectively cleared from the whole cell lysate, as evidenced by western blot analysis (**Fig. 2e&f, Extended Data Fig. 6b**). EGFRn-IGF_EndoTag2 was the most effective, leading to more than 80% clearance of EGFR, while EGFRn-IGF_EndoTag1 resulted in 70% clearance. The flexible EGFRn-IGF_EndoTag1&2 had higher degradation capacity than the rigid constructs (IGF_EndoTag3&4) (**Fig. 2f**), perhaps due to better interactions with EGFR. Mass spectrometry-based proteomic analyses confirmed that EGFRn-IGF_EndoTag2 and

EGFRn-IGF_EndoTag1 significantly reduced EGFR levels in both Hela and H1975 cells without affecting IGF-2R levels (**Fig. 2g, Extended Data Fig. 6e**); EGFRn without EndoTag had no effect on EGFR levels (**Fig. 2g, Extended Data Fig. 6d**).

To compare with the original M6P-based LYTACs, we generated genetic fusions of EndoTag1 with Cetuximab (CTX), a clinically-approved therapeutic antibody that targets EGFR with high affinity[29]. In H1975 cells, CTX-IGF_Endotag1 led to more effective degradation of EGFR than the M6P-based LYTAC (**Fig. 2e**)[1]. Proteomic analyses demonstrated that CTX-IGF_Endotag1 elicited a significantly greater reduction in EGFR levels compared to CTX alone (**Extended Data Fig. 6f-g)**, with little effect on IGF-2R levels. CTX has a higher affinity for EGFR, and hence required a lower dose for effective degradation: CTX-IGF_Endotag1 achieved 85% degradation at a concentration of 10nM (**Extended Data Fig. 6a**).

We next investigated targeted degradation of Programmed Death-Ligand 1 (PD-L1), an immune checkpoint in cancer immunotherapy, and therefore an attractive target for this approach[30]. We genetically fused pLYTACs at the C-term of Atezolizumab (Atz), a potent PD-L1 inhibitor[31], then tested PD-L1 clearance ability. All of the Atz-EndoTags effectively degraded PD-L1 on the cell surface of MDA-MB-231 cells within 4 hours, with EndoTag2 achieving the highest clearance percentage of 90% after 24 hours of incubation (**Extended Data Fig. 6h**). Western blot analysis of the whole cell lysis indicated that Atz-EndoTag2 molecules were able to deplete 77% of PD-L1 in the whole cell after 48h incubation (**Fig. 2h**). Like PD-L1, cytotoxic T lymphocyte antigen 4 (CTLA4) is an immune checkpoint component for which inhibitors have shown promising antitumor effects[18,19]. To target CTLA4, we generated a genetic fusion

between EndoTag1 and a minibinder against CTLA4 (CTLA4mb) which resulted in a 45% decrease of CTLA4 in Jurkat-CTLA4 cells after only 3 hours (**Extended Data Fig. 6c**).

Clearance of soluble proteins

We next investigated the ability of EndoTags to degrade targeted soluble proteins as soluble pLYTACs (**Fig. 3a**). As a first proof of concept we employed the nanomolar affinity de novo designed protein heterodimer LHDA and LHDB, which has been used as the basis for synthetic signaling systems[34]). We fused LHDA to IGF_EndoTags, and LHDB to AF647, and found that the EndoTags significantly enhanced the uptake of LHDB-AF647 in both Jurkat and K562 cells (**Fig. 3b, Extended Data Fig. 7a&b**), with IGF_EndoTag3 producing a remarkable 40-fold increase in mean fluorescence intensity (MFI) compared to the control. Incubation of Jurkat cells with 100nM LHDA-IGF_EndoTag3 resulted in a 50% clearance of 100nM LHDB from the solution after 48h incubation in Jurkat cells (**Fig. 3c;** clearance may be limited by the number of available receptors).

We next investigated the potential of the pLYTAC system to eliminate disease-relevant soluble protein targets such as autoantibodies that recognize self-antigens which have been linked to multiple autoimmune diseases[35]. We first tested whether the fusion of EndoTags with IgG binding protein G[36] could clear IgG in solution. proteinG-IGF_EndoTags were generated by genetically fusing proteinG with various EndoTags and purified after expression in E coli. As a benchmark comparison to

glycan-based LYTAC, ProteinG-M6Pn was generated by conjugating proteinG with azido-NHS ester followed by M6Pn-BCN peptide. All fusion proteins including EndoTag constructs together with proteinG-M6Pn were then incubated with IgG-AF647 and Jurkat cells. All candidates triggered considerable uptake of IgG in both Jurkat and K562 cells (**Extended Data Fig. 7c&e**). ProteinG-IGF_EndoTag3 elicited 2 fold higher cellular uptake of IgG than proteinG-M6Pn, leading to an overall 80 fold increase in IgG in K562 cells and 360 fold increase in Jurkat cells (**Extended Data Fig. 7e**). To quantify the clearance of IgG in the solution, we measured the fluorescence intensity in the cell culture supernatant normalized by that in control cells treated with IgG-AF647 alone. Incubation of 100nM proteinG-IGF_EndoTag3 with 133nM IgG resulted in depletion of 70% of the IgG after 48 hours in Jurkat cells (**Fig. 3e**). IGF_EndoTag3 elicited the higher clearance of IgG in Jurkat cells compared to the flexible IGF_EndoTag1&2, and a proteinG-M6Pn control (**Extended Data Fig. 7d&f**). IGF_EndoTag4 was less potent (**Extended Data Fig. 7h**). Utilizing confocal microscopy in HeLa cells, we observed enhanced co-localization of IgG with lysosomes following treatment with proteinG-IGF_EndoTag3 for 24 hours, indicating that the EndoTags actively facilitated the transportation of IgG to the lysosomes for degradation (**Fig. 3f**); this co-localization was not observed in HeLa cells not expressing IGF-2R (**Fig. 3g**).

Logic gated targeted degradation and locally secretable degraders

Many targets of interest are expressed not only in diseased tissues but also on healthy cells, and the ability to target protein degradation to specific cell subpopulations could reduce toxicity. For example, in the case of cancer, target degradation conditioned on the presence of a specific marker in the tumor microenvironment could help avoid undesired effects on healthy cells. Such logic gated targeted degradation has not been achieved with current extracellular protein degradation systems. To address this limitation, we utilized the colocalization-dependent protein switches (Co-LOCKR) system, which functions as an AND logic gate only exposing a recruitment motif when two target cell markers are present on the same cell (**Fig. 4a**). We utilized Co-LOCKR to selectively degrade EGFR when HER2 was also present on the surface of cancer cells[37]. We fused EndoTag with Bcl2, which binds the Bim peptide exposed upon coincident binding in this version of Co-LOCKR, and evaluated EGFR degradation level in both HER2+ and HER2- cells. In K562 cells overexpressing both EGFR and HER2, Bcl2-IGF_EndoTag2 resulted in 80% degradation of EGFR, while in K562 cells expressing only EGFR without HER2, the EGFR level remained unchanged (**Fig. 4b**). Thus the EndoTag system can be precisely targeted to specific cells based on combinations of surface markers.

Another major challenge is the degradation of the degrader itself upon binding cells that express the target endocytosis receptor, which could increase the dose of drug required. In the context of adoptive cell therapies or mRNA delivery, the secretion of a degrader locally in response to stimuli could potentially overcome this challenge. Our system offers a solution as it is fully protein based and consequently can be locally secreted with high specificity and efficiency[38,39]. To investigate this possibility, we

transiently transfected IGF-2R KO HeLa cells with plasmids encoding EGFRn-IGF_EndoTag, collected the cell supernatants, and incubated them with EGFR+ K562 cells while monitoring EGFR expression levels (**Fig. 4c**). Secreted EGFRn-IGF_EndoTag1 led to similar EGFR clearance as the exogenously added EGFRn-IGF_EndoTag1 (**Fig. 4d**), while secreted EGFRn-IGF_EndoTag2 was more potent, consistent with the results obtained with the purified proteins. Thus EndoTags remain functional when secreted from cells, providing a potential avenue for their use in adoptive cell therapy control systems.

Conclusions

Targeted degradation has shown considerable promise as a therapeutic approach[1–3] by using native ligands or chemical modifications to engage with rapidly trafficking cell surface receptors. Our designed EndoTag approach extends the power of this therapeutic modality in several ways. First, while native ligands can trigger off-target signaling and competition with endogenous proteins can reduce potency , our designed EndoTags, as illustrated by TfR and ASGPR cases (**Fig. 1b,e,h**), can be targeted to sites on endocytosing receptors that are not bound by native ligands. Second, high valency chemical modification[1,2,41] has been used to enhance endocytosis, but this complicates manufacturing[1,23,14]; as illustrated by our multi domain ASGPR EndoTags, small synthetic domains can be readily combined to create all protein receptor clustering and endocytosis stimulating proteins. The all-protein nature of our pLYTACs not only simplifies manufacturing, but also enables employment of targeted degradation

approaches in adoptive cell therapies through secretion from engineered cells (**Fig. 4c**). The small, stable and readily producible synthetic ligands that could be useful not only for therapeutic applications but as molecular tools for probing how receptor conformations modulate cellular trafficking.

Moving forward, there are many avenues to pursue using our computational design approach to generating EndoTag stimulated enhancers of cell surface receptor endocytosis and trafficking. First, there are likely many more receptor targets which can undergo rapid endocytosis upon suitable triggering at the cell surface; our ability to design endocytosis stimulators without requiring native ligands or identification of chemical modifications should enable utilization of the full range of these receptors to achieve more tissue restricted targeting and modulatable intracellular trafficking (different receptors likely will have different intracellular compartment residence times and transition dynamics). In addition to the targeted degradation application pursued here, such designed endocytosis stimulators could be of great utility for enhancing uptake of nucleic acid (siRNA for example) and small molecule drug conjugates. Second, there are likely natural signaling pathways which, can be more potently activated by promotion of receptor endocytosis. Third, as illustrated by the use of the LOCKR system to make logic gated protein degradation systems and the secretion of EndoTag-degrader constructs from cells, the robustness and modularity of de novo designed protein and capability for logic gated activation and cell based expression open the door to a wide range of more precise and controllable strategies for protein and cell based therapies.

Conflict of interest

B.H., M.A., I.S., L.S., L.C., and D.B. are co-inventors on a provisional patent application that incorporates discoveries described in this manuscript.

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Figures

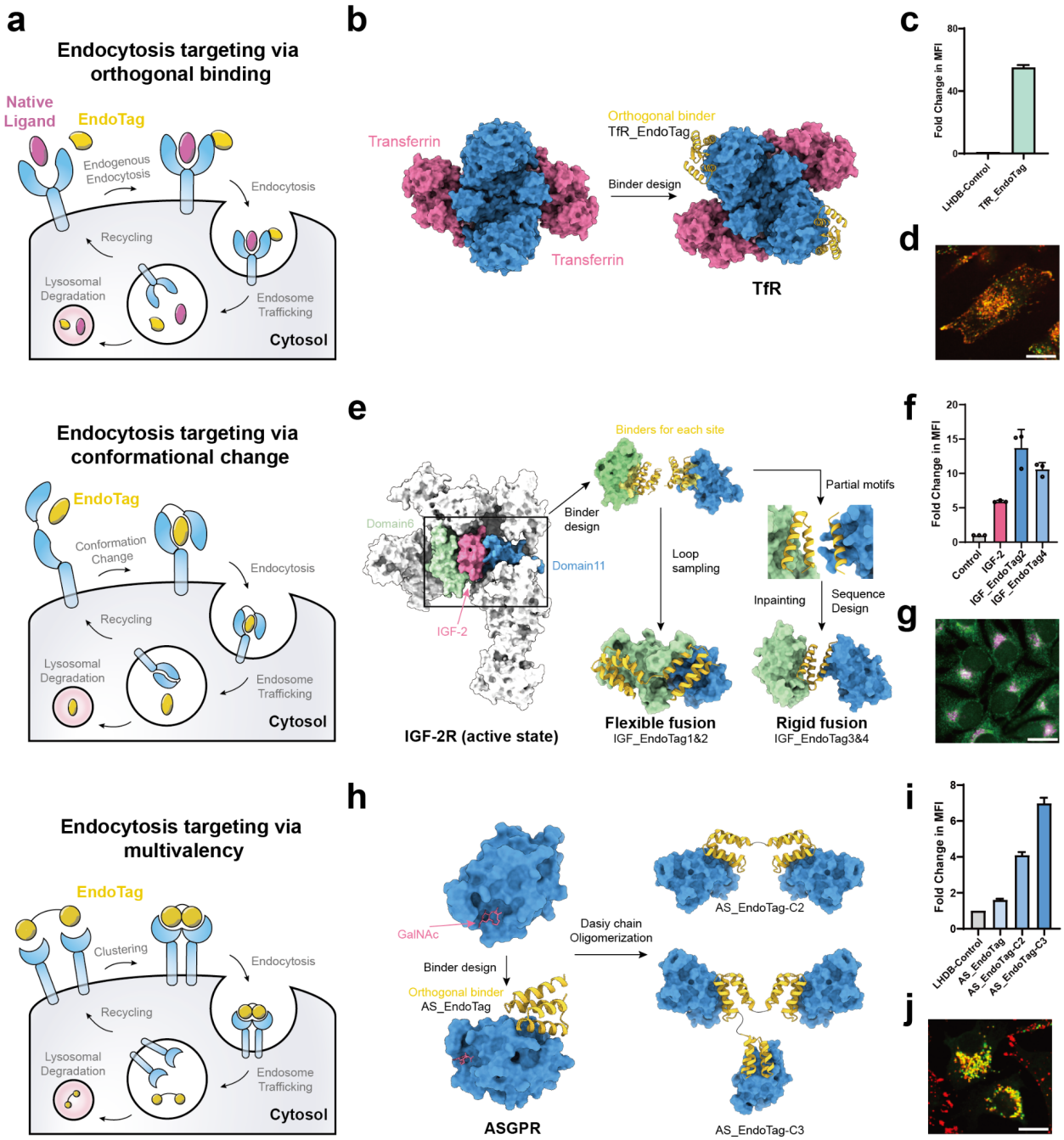

Figure 1: Design strategies for endocytosis-triggering EndoTags. a, Schema of designed endocytosis mechanisms. (top) Design of binding to constitutively cycling receptors at sites that do not overlap with those of natural ligands to avoid competition. (middle) Design of binders that trigger endocytosis by eliciting a conformational change in the receptor. The EndoTag binds at two distinct epitopes on the target and actively triggers the conformational change. (bottom) Designed endocytosis via receptor

clustering. The multivalent EndoTag clusters multiple copies of the target receptor and induces endocytosis. **b,** Design strategy for TfR EndoTags. A designed binder to TfR binds at a site distant from that of transferrin[42]. **c,** Cellular uptake of TfR EndoTags. U-251MG cells were treated with 100nM AF647-labelled TfR-EndoTags for 2h. After washing 3 times, cellular uptake was measured by flow cytometry. The data were normalized with respect to a control group treated with 100nM AF647-labelled LHDB (supposed no endocytosis). **d,** Confocal imaging of EndoTag (red) and a lysosomal marker (green). 500nM AF647 labelled EndoTag was incubated with U-251MG cells for 24h. The lysosomes were stained with AF488-labelled Lysotracker. **e,** Design strategy for IGF_EndoTags. Starting from the structure of IGF-2 in complex with IGF-2R, de novo minibinders were generated and screened against the IGF-2 binding sites at IGF-2R domain 6 and domain 11, separately. Individual binders for each domain (D6mb and D11mb) were fused with flexible linkers or a rigid fusion interdomain connection. For flexible fusion, multiple linker lengths and fusion directions were sampled. For rigid fusion, the two major binding helices from D6mb and one major binding helix from D11mb were extracted as starting motifs. With protein Inpainting, geometries and fusion orders were sampled, and ranked based on Rosetta and alphafold2 metrics. **f,** Cellular uptake of IGF_EndoTags. Jurkat cells were treated with biotinylated 100 nM IGF_EndoTags or IGF-2, and 33 nM Strapavidin-AF647 for 24h. After washing 3 times, cellular uptake was measured by flow cytometry. The data were normalized with the control group treated with 33 nM Strapavidin-AF647 alone. **g,** Microscopy imaging of IGF_EndoTag1 (pink) co-localization with lysosomes (green). Hela cells were incubated with 100nM biotinylated EndoTag1 and 33nM Strapavidin-AF647 for 2h. After washing and fixing the cells, the cells were stained with anti-LAMP2A antibody followed by fluorophore-labeled secondary antibody and DAPI. **h,** Design strategy for ASGPR EndoTags. Based on the crystal structure of ASGPR[43], binders were designed and then connected with flexible linkers. **i,** Cellular uptake of ASGPR EndoTags. Hep3B cells were treated with 100 nM AF-647 conjugated ASGPR EndoTags for 24h. After washing 3 times, the cellular uptake was measured by flow cytometry. The data were normalized with respect to a control group treated with 100nM AF647-labelled LHDB (supposed no endocytosis). **j,** Confocal imaging of EndoTag (red) with lysosome (green). 500nM AF647 labelled EndoTag was incubated with Hep3B cells for 24h. The lysosomes were stained with AF488-labelled Lysotracker. For **d**,**g**,**j**, the scale bar indicates 20um. For **c,f,i**, the mean values were calculated from triplicates, and error bars represent standard deviations.

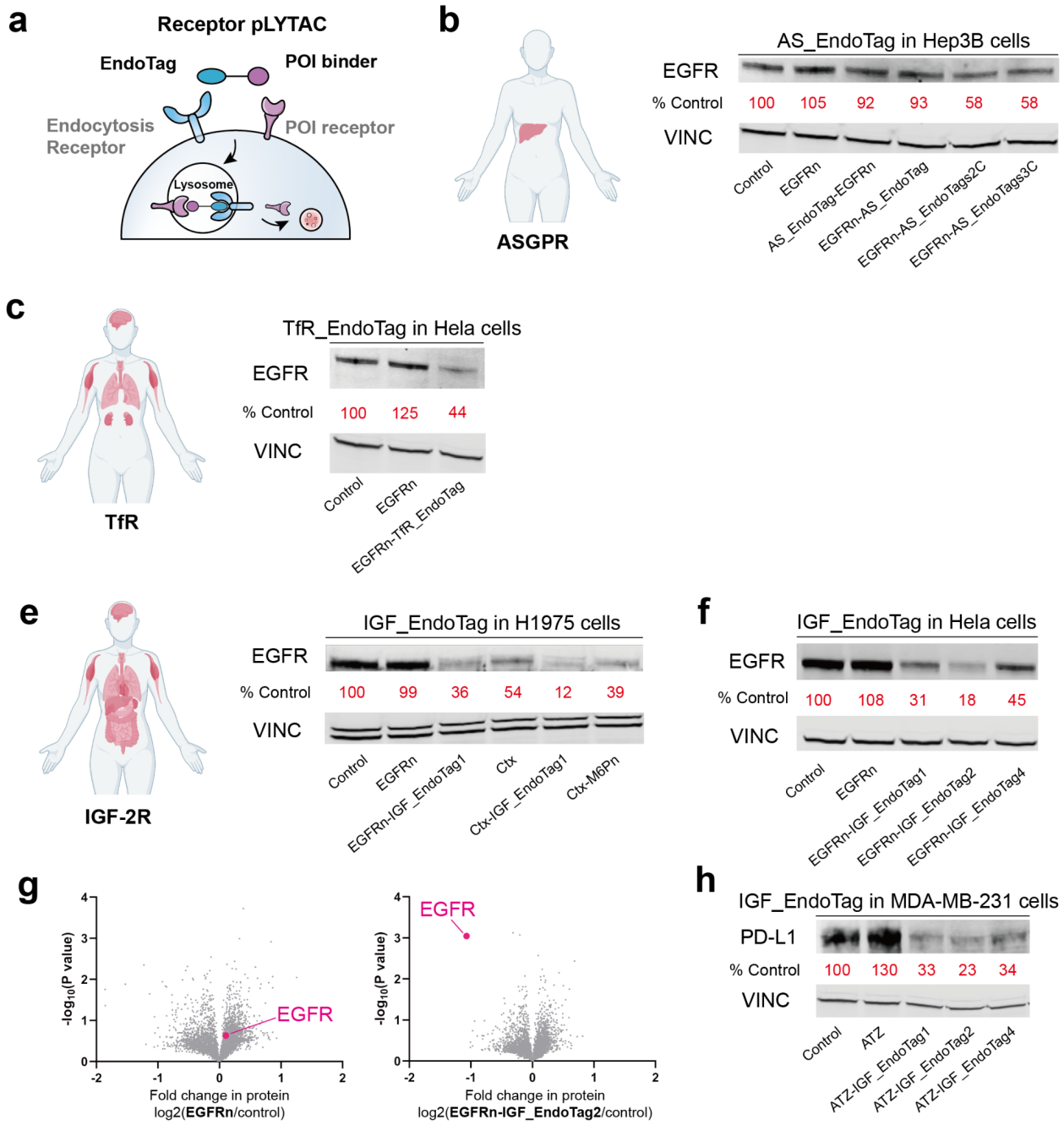

Figure 2. Surface receptor degradation with tissue-specific pLYTACs.
a, Schema of tissue-specific pLYTACs for receptor degradation. **b,** ASGPR. Left: Tissue expression of ASGPR. Right: Western blot analysis of total EGFR levels in Hep3B cells after treatment with 200 nM EGFRn or EGFRn-AS_EndoTag for 48 h. **c,** TfR. Left: Tissue expression of TfR. Right: Western blot analysis of total EGFR levels in Hela cells after treatment with 200 nM EGFRn or EGFRn-TfR_EndoTag for 48 h. **e,** IGF-2R. Left: Tissue expression of IGF-2R. Right: Western blot analysis of total EGFR levels in H1975 cells after treatment with 200 nM of EGFRn/CTX with/without the fusion with EndoTag1 or M6Pn for 48 h. **f,** Western blot analysis of total EGFR levels in Hela cells

after treatment with 200 nM EGFRn or EGFRn-IGF_EndoTags for 48 h. **g,** Quantitative proteomics analysis of protein abundance in H1975 cells after treatment with EGFRn or EGFRn-IGF_EndoTag2 for 48h relative to untreated cells. Data are the mean of three biological replicates. **h,** Western blot analysis of total PD-L1 levels in MDA-MB-231 cells after treatment with 200 nM of ATZ or ATZ-pLYTACs for 48 h**.**

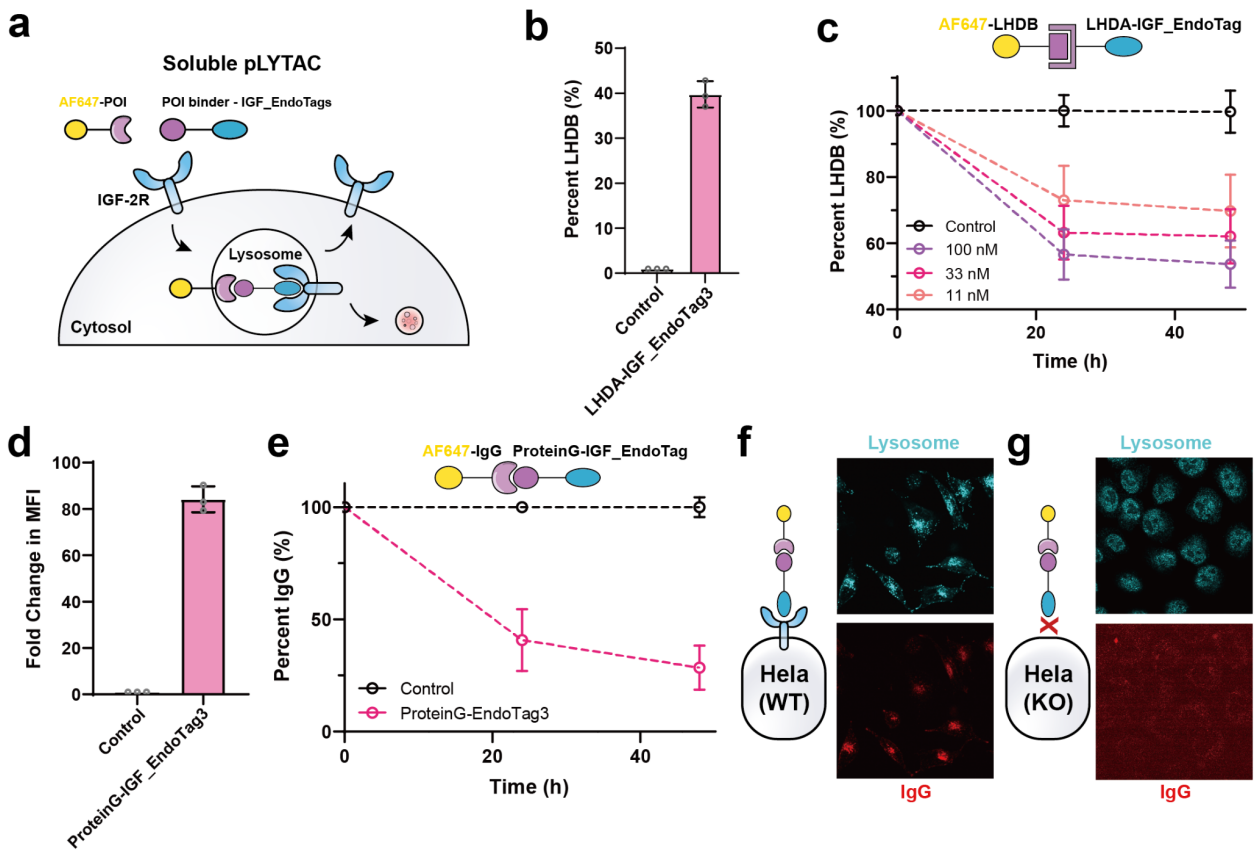

Figure 3: Clearance of soluble proteins by IGF-2R pLYTACs. a, Schema of soluble pLYTACs with IGF_EndoTags. **b**, Cellular uptake of LHDB-AF647 via LHDA-IGF_EndoTags in Jurkat cells. Cells were incubated with 33nM LHDB-AF647 with/wihtout 1uM LHDA-IGF_EndoTags for 24h, washed twice with cold PBS and analyzed by flow cytometry. **c**, Remaining supernatant LHDB-AF647 levels in Jurkat cells. Jurkat cells were incubated with 100nM LHDB-AF647 with/wihtout 500nM LHDA-IGF_EndoTags. At timepoints 24h, 48h, the cells were pelleted down, and supernatant IgG levels were quantified by Neo2 plate reader. The percentage of IgG level was normalized with the IgG alone control group. **d**, Cellular uptake of IgG-AF647 via proteinG-IGF_EndoTags in K562 cells. Cells were incubated with 33nM IgG-AF647 with/without 1uM proteinG-IGF_EndoTag3 for 24h, washed twice with cold PBS and analyzed by flow cytometry. The fold change in MFI (mean fluorescence intensity) was calculated by normalizing the IgG-AF647 alone group. **e**, Remaining supernatant IgG-AF647 levels in Jurkat cells. Jurkat cells were incubated with 133nM IgG-AF647 with/wihtout 100nM proteinG-IGF_EndoTag3. At timepoints 24h, 48h, the cells were pelleted down, and supernatant IgG-AF647 levels were quantified by Neo2 plate reader. The percentage of IgG-AF647 level was normalized with the IgG-AF647 alone control group at each time point. **f**, Confocal imaging of lysosome co-localization of IgG-AF647 with lysosome in Hela cells. **g**, Confocal imaging of lysosome co-localization of IgG-AF647 with lysosome in Hela (IGF-2R KO) cells. For **f**,**g**, the cells were incubated

with 200nM IgG-AF647 and 1uM of proteinG-IGF_EndoTag3 for 24h, washed and stained with Lysotracker.

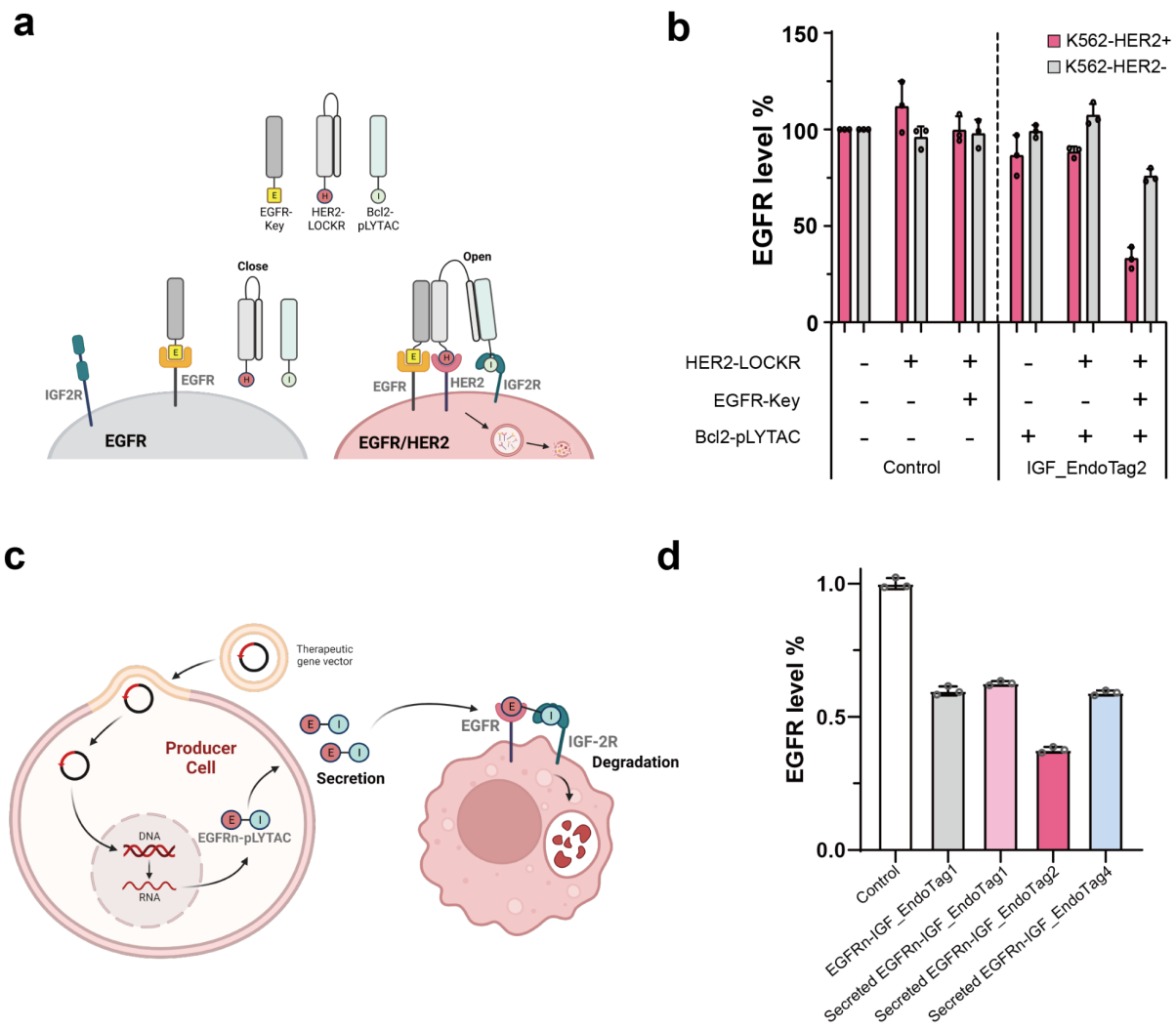

Figure 4. Logic gated targeted degradation and locally secretable degraders. a, Schema of AND-gate logic for EGFR degradation in presence of HER2**. b,** Flow cytometry quantification of EGFR level in cell surface. The K562-EGFR or K562-EGFR/HER2 cells were incubated with combinations of 100nM of EGFRn-Key, HER2-LOCKR, and Bcl2-EndoTag2 for 24h. **c,** Schema of secreted EGFR-pLYTAC. **d,** Flow cytometry quantification of EGFR level in cell surface treated with cell supernatant or exogenous EGFRn-IGF_EndoTag1 for 24h. For the secretion groups, Hela (IGF-2R KO) cells were transfected with viral vectors encoding the EGFRn-IGF_EndoTag1 or LHDA-pLYTACs. The supernatant of the cells were collected and incubated with K562-EGFR cells.