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A new temperature-dependent strategy to modulate the epidermal growth factor receptor.

Zhe Li¹, David R. Tyrpak¹, Mincheol Park¹, Curtis T. Okamoto¹, and J. Andrew MacKay^{1,2,3,*}

¹Department of Pharmacology and Pharmaceutical Sciences, University of Southern California, USA

²Department of Biomedical Engineering, University of Southern California, USA

³Department of Ophthalmology, University of Southern California, USA

Abstract

The dynamic manipulation of kinases remains a major obstacle to unraveling cell-signaling networks responsible for the activation of biological systems. For example, epidermal growth factor (EGF) stimulates the epidermal growth factor receptor (EGFR/ErbB1); however, EGF also recruits other kinases (HER2/ErbB2) involved with various signaling pathways. To better study EGFR we report a new strategy to selectively activate receptor tyrosine kinases fused to elastin-like polypeptides (ELPs), which can be visualized inside mammalian cells using fixed and live-cell fluorescence microscopy. ELPs are high molecular weight polypeptides that phase separate abruptly upon heating. When an EGFR-ELP fusion is heated, it clusters, initiates receptor internalization, phosphorylates, initiates downstream kinase signaling, and undergoes retrograde transport towards the cell body. Unlike other strategies to block EGFR (small molecule inhibitors, RNAi, or transcriptional regulators) EGFR-ELP clustering can be specifically switched on or off within minutes. Live-cell imaging suggests that EGFR-ELPs assemble in most cells with only a 3 °C increase in temperature. This strategy was found reversible and able to dynamically control the downstream phosphorylation/activation of the ERK1/2 kinase pathway. For the first time, this strategy enables the rational engineering of specific temperature-sensitive receptors that may have broad applications in the study and manipulation of biological processes.

*Corresponding author: J. Andrew MacKay (jamackay@usc.edu), Telephone: 1-323-442-4118, Address: 1985 Zonal Avenue, PSC 306A, School of Pharmacy, University of Southern California, Los Angeles, CA 90089, USA.

Conflict of interest

The authors declare no conflict of interest

Data availability

Selected raw videos obtained from live-cell imaging have been uploaded as supplementary information; furthermore, other raw/processed data required to reproduce these findings are available on Mendeley Data.

Appendix A

Supplementary data and videos are available for this paper.

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Keywords

elastin-like polypeptides; thermo-responsive protein-polymer; ERK activity dynamics; green-fluorescent protein; synthetic biology; HEK293T

1. Introduction

Decisions on cell fate and function mainly rely on the coordinated intracellular signaling pathways that are activated by plasma membrane receptors, such as G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) [1, 2]. In particular, the dynamic behaviors of signaling networks have been regarded as the determinant for their specificity, resulting in different gene expression patterns and diverse physiological responses [3]. A range of tools has been developed to manipulate cell signals for understanding biological systems. Conventional approaches using chemical-based strategies [4, 5] or genetic perturbation [6] allow for activation and inhibition of selected receptors; however, they also suffer from poor temporal resolution and low reversibility. This makes it challenging to examine the dynamic properties of signaling networks in a highly specific manner. Existing methods to address these problems use various stimuli, such as light [7–9], magnetics [10–12] and electrics [13]. For instance, chimeric receptors fused with light-sensing domains can be activated in response to light in the absence of endogenous ligands. Although these systems have been proven to control various biological events, they have drawbacks including the introduction of exogenous materials (ligands, nanostructures, crosslinkers, etc.) and/or the need for a specialized stimulation system (optical, electromagnetic, etc.). Therefore, simple and convenient solutions remain highly desirable.

Recently our research group discovered that thermally responsive protein-polymers assemble functional proteins when expressed in live cells [14]. A small increase ($\sim 1^\circ\text{C}$) in temperature stimulates elastin-like polypeptides (ELPs) to phase separate, and this process can be fully reversed by decreasing the temperature. ELPs have been explored for drug delivery [15, 16] and tissue engineering [17]; however, they have untapped potential as biological tools with applications in synthetic biology. When expressed within cells and fused to key effector or fluorescent proteins, ELPs assemble cytosolic microdomains in a temperature dependent manner that can be tuned to target intracellular processes such as clathrin-mediated endocytosis [14, 18, 19]. Here, we extend this technology by developing a protein switch for manipulating intracellular signaling pathways utilizing ELPs as a regulatory component. In contrast to conventional ligand-based approaches, the ELP-based activation is fully reversible and enables non-invasive application and withdrawal of stimuli without further disturbing the cellular environment.

To demonstrate this strategy, we utilize epidermal growth factor receptor (EGFR) and its downstream signaling pathways as a model system. EGFR is an essential receptor tyrosine kinase (RTK) that regulates cell proliferation, differentiation, metabolism and is implicated in numerous human cancers [20–22]. Upon binding with extracellular ligands (such as EGF), EGFRs homo- and hetero- dimerize with other RTKs. This leads to trans-phosphorylation of the cytosolic arms of the RTKs. The phosphorylated tyrosine residues

then recruit other downstream signaling components that activate diverse intracellular pathways, including mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt and phospholipase C γ 1 (PLC γ 1)/Ca²⁺ pathways. Since receptor clustering plays an important role in both signal transduction and receptor internalization, we hypothesized that ELP-mediated assembly could functionally substitute for ligands. When induced to phase separate, expressed EGFR-ELPs might reasonably be expected to cluster and activate. To validate this strategy, this manuscript presents a comprehensive library of fusion proteins between EGFR and ELP and demonstrates their tunable modulation of intracellular signaling pathways through receptor clustering (Fig. 1). Prior to thermal stimulation, EGFR-ELPs remain freely distributed in the cytoplasmic membrane at the cell surface, remaining 'OFF' due to their lack of self-association. Once induced to phase separate, the EGFR-ELPs bring their catalytic domains into proximity, thereby switching downstream signaling 'ON'. The reversibility of ELP-mediated phase separation enables precisely controlled repetitive activation, providing a simple means for manipulating signaling dynamics.

2. Materials and Methods

2.1 Plasmid constructs

ELP expression vectors were synthesized using recursive directional ligation in pET25b(+) plasmid (MilliporeSigma, Burlington, MA), as previously reported [23]. The pCDNA6A-EGFR WT [Mien-Chie Hung, The University of Texas] was obtained from Addgene (#42665, Cambridge, MA) and modified by inserting DNA oligonucleotides with EcoRV/NotI digestion sites (Top strand: 5'-ccggTGCGATATCatgatGCGGCCGC-3'; Bottom strand: 5'-GCGGCCGCcatcatGATATCGCA-3') into AgeI/PmeI. Then DNA sequences encoding ELPs were cloned into the downstream of EGFR-myc after digesting the ELP plasmid with MsiI/NotI and the modified EGFR plasmid with EcoRV/NotI. To further engineer the EGFR-GFP-ELP fusions, GFP gene was amplified using PCR and inserted into the EGFR-ELP plasmids using the XbaI/AgeI digestion sites. The final opening frames of the fusion proteins were verified by DNA sequencing (Retrogen, San Diego, CA).

2.2 Cell culture and transfection

293T cells (#CRL-3216, ATCC, Manassas, VA) were maintained in DMEM (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (#35-011-CV, Corning, NY) in a humidified incubator with 5% CO₂ at 37 °C. CHO K1 cells were grown in DMEM/F12 1:1 (GE Healthcare Life Sciences, Logan, UT) containing 10% FBS at 37 °C with 5% CO₂. For the transient expression, cells were transfected with Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. After transfection, cells were cultured at 32 °C for 36-48 hours prior to assay.

2.3 Immunofluorescence and confocal microscopy

To assess the assembly of cell surface EGFR, 293T cells were plated on glass coverslips in 12-well plates and transfected with EGFR-ELPs. 24 hours after transfection, medium was replaced with serum free DMEM. After additional 16 hours, cells were cooled at 4 °C for 30 min with an Alexa Fluor 488-conjugated EGFR antibody (#sc-120 AF488, Santa Cruz

Biotechnology, Dallas, Texas) that specifically stains EGFR at the plasma membrane. After washing, cells were incubated at specified temperatures (22 °C, 27 °C, 32 °C, 37 °C) for 30 min with serum free DMEM prior to fixation. Coverslips were mounted on microscope slides in fluoromount (Diagnostic Biosystems, Pleasanton, CA) and images were captured on a LSM510 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY) with a Plan-Apochromat 63x oil objective. For detection of phosphorylated EGFR or EEA-1, first surface EGFR was labeled at 4 °C and then cells were assayed at different temperatures as described above. After 30 min incubation, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with a rabbit anti-pEGFR antibody (#2234, Cell Signaling Technology, Danvers, MA) or a rabbit anti-EEA1 antibody (#3288, Cell Signaling Technology, Danvers, MA) for 1 hour at 37 °C followed by another hour with Alexa Fluor 568-linked donkey antirabbit antibody (#A10042, Thermo Fisher Scientific, Waltham, MA). Cells were then washed and stained with DAPI before mounting.

2.4 Colocalization analysis

Colocalization analysis was performed in this study to quantify the receptor activation and internalization using ZEN2009 software (Carl Zeiss Microscopy, Thornwood, NY). Since the surface receptor was stained with a green fluorescent dye in both studies, Mander's colocalization coefficient (MCC) for the green channel was calculated to estimate the fraction of the surface receptor activated or internalized using the following Equation 1.

$$M_{green} = \frac{\sum_i G_{i,colocal}}{\sum_i G_i} \quad \text{Equation 1}$$

where $G_{i,colocal} = G_i$ if $R_i > 0$ and $G_{i,colocal} = 0$ if $R_i = 0$. R_i and G_i are the intensity values of the red and green fluorophore in individual pixels, respectively.

2.5 Flow cytometry

Internalization of cell surface receptors was assayed by flow cytometry. Cells transfected with EGFR-ELPs were serum starved for overnight and surface stained with Alexa Fluor 488-labelled EGFR antibody at 4 °C for 30 min. After washing, cells were ramped at either 22 °C or 37 °C for indicated durations to allow microdomain assembly and receptor internalization. The cells were then stripped with glycine buffer (0.2 M, pH 2.5, 1mg/ml BSA) to remove surface-bound antibodies and trypsinized into single-cell suspensions for further analysis with LSRII Flow Cytometer (BD Biosciences, San Jose, CA).

2.6 Immunoblot analysis

293T cells were seeded in 35mm dishes and transfected the following day with EGFR-ELPs. Cells were starved 24 hours before temperature stimulation. Whole cell lysates were prepared at different time points post temperature stimulations using RIPA buffer containing protease/phosphatase inhibitor (#5872, Cell Signaling Technology, Danvers, MA). 100µg of total protein was separated on a PAGEr EX 4–12% gradient gel (#59722, Lonza, Morristown, NJ) and then transferred onto a nitrocellulose membrane using iBlot2 dry blotting system (ThermoFisher Scientific, Waltham, MA). The membrane was first

immunoblotted with primary antibodies, including rabbit anti-phospho-ERK1/2 (#9101, Cell Signaling Technology, Danvers, MA), rabbit anti-ERK1/2 (#9102, Cell Signaling Technology, Danvers, MA), rabbit anti-phospho-STAT3 (#9145, Cell Signaling Technology, Danvers, MA), rabbit anti-GAPDH (#5174, Cell Signaling Technology, Danvers, MA), then incubated with secondary antibody anti-rabbit IgG (HRP-linked) (#7074, Cell Signaling Technology, Danvers, MA). Protein bands were visualized using ProSignal Dura Chemiluminescent Substrate (#20–301, Genesee Scientific, San Diego, CA) and imaged on a ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Hercules, CA).

2.7 Live cell imaging

Prepared cells were grown on 35mm glass bottom dishes (MatTek Corporation, Ashland, MA) and serum starved overnight before imaging. A DIAPHOT epifluorescent microscope equipped with a DS digital camera (Nikon Instruments, Melville, NY) was used to examine GFP-V60 self-assembly vs. GFP-V60 coassembly with EGFR-V96 while the temperature was increased from 4 °C to 60 °C at a rate of 4 °C/min on a temperature controlled microscope stage (Linkam Scientific). The actual medium temperature was monitored using a thermometer coupled with a probe from Sper Scientific (Scottsdale, AZ). To calculate the mean particle size and particle number, each single cell in the last image of the time-lapse video was analyzed using ImageJ (US NIH, Bethesda, MD). Briefly, 12-bit images were thresholded and converted to a binary black and white 8-bit image. The “Analyze Particles” function was used to count the particle number and measure the total area, the mean particle size was then calculated by dividing the total area by the number of particles. In the retrograde transport study, live cells were imaged on a LSM880 laser scanning confocal microscope with Airyscan (Carl Zeiss Microscopy, Thornwood, NY) and definite focus was used to compensate for thermally induced drift of focus. The decrease of fluorescence in the area encompassing filopodia/lamellipodia was quantified as a measurement of the retrograde transport of receptors towards the cell body. Two ROIs were defined by manually identifying the outlines of the protrusions and cell body, and the fluorescent intensity within each ROI at individual time point was measured using ImageJ (US NIH, Bethesda, MD). The percentage of fluorescent receptors remaining in the filopodia/lamellipodia was calculated using the following Equation 2.

$$\% \text{ receptor in filopodia} = \frac{I_{total} - I_{cell \text{ body}}}{I_{total}} \times 100 \quad \text{Equation 2}$$

where I_{total} and $I_{cell \text{ body}}$ represents the integrated density of the whole cell and cell body, respectively.

2.8 Statistics

All experiments were replicated at least three times and data were presented as Mean $\pm$ SD. The significance of changes in receptor internalization and signal activation were determined using t-tests. The upregulation of pSTAT3 after a repetitive heat stimulation was analyzed using a one-way ANOVA followed by Tukey’s post-hoc test using Prism (GraphPad Software, La Jolla, CA). A Wilcoxon rank sum test was performed with R to examine the

effect of EGFRV96 coassembly on GFP-V60 microdomain formation. A p value less than 0.05 was considered statistically significant.

3. Results

3.1 Generation of thermo-responsive EGFR-ELP fusion proteins.

A comprehensive library of chimeric receptors containing full-length EGFR and ELP was designed (Fig. 1). An ELP is a repetitive polypeptide with a sequence of (Val-Pro-Gly-Xaa-Gly)_n, where Xaa and n represents a guest residue and the number of repeats, respectively. ELPs phase separate above a transition temperature that can be easily tuned by changing its chain length or guest residue. Based on prior characterization [14], a panel of ELPs was genetically fused to EGFR, including V96, V72, 2VA96 as well as A96 as a negative control (Fig. 1b). These fusions vary in their intracellular transition temperature and composition (Table 1); therefore, it was expected that they could be used to tune microdomain assembly to relevant physiological temperatures. Expression of fusion proteins in mammalian cells was confirmed to demonstrate the correct molecular weight and expected glycosylation when assessed using a western blot (Fig. 1c).

3.2 Temperature-triggered clustering of cell-surface EGFR.

To characterize the behavior of EGFR-ELPs upon temperature stimulation, 293T cells expressing EGFR-ELPs were incubated at various temperatures (22, 27, 32, or 37 °C) for 30 minutes (Fig. 2a). Prior to assay, the extracellular domain of EGFR was pre-stained at 4 °C cells with an AlexaFluor-488 conjugated antibody that specifically assesses cell surface receptor (Fig. S1). Even in the absence of ligand EGF, EGFR-V96 assembled small clusters with strong green fluorescence after incubation at 32 °C and nearly all of the transfected cells showed evidence of receptor internalization at 37 °C (Fig. 2b). In contrast, EGFR-V96 remained evenly distributed on the cell surface when incubated at or below 27 °C. EGFR-V72 and EGFR-2VA96 also phase separate near physiological temperatures; however, they required a slightly higher temperature to reach their maximal assembly. In contrast, for nonswitchable EGFR-myc and thermo-insensitive EGFR-A96, no receptor internalization was detected at any temperature. (Fig. 2a, b). As our lead construct, the kinetics of EGFR-V96 internalization were followed at 37 °C, which show that within 15 minutes more than half of transfected cells show clear evidence of receptor internalization (Fig. 2c). This temperature-dependent clustering of EGFR-ELPs was reconfirmed using live cell imaging in 293T cells (Fig. S2, Video S1), and similar results were observed in CHO cells (Fig. S3). Among the temperature-sensitive EGFR fusion receptors, EGFR-V96 was selected for all follow-up studies since it reaches maximal microdomain assembly at 37 °C.

3.3 Temperature-triggered internalization of cell surface EGFR.

Having shown that cell-surface EGFR-ELPs can be clustered in response to heating (Fig. 2), EGFR internalization was probed using flow cytometry on cells that were stripped of surface antibody (Fig. 3). As shown in Fig. 3a, at 22 °C, cell-surface EGFR-V96 pre-labeled with an AlexaFluor-488 conjugated EGFR antibody was slowly endocytosed over time in a similar pattern to the control EGFR-A96, which could potentially result from non-specific fluid phase endocytosis. However, at 37 °C, internalization of EGFR-V96 increased significantly,

which suggested that ELP-mediated receptor clustering induced a rapid and effective internalization of EGFR. Moreover, the subcellular localization of internalized EGFRs was confirmed using confocal microscopy (Fig. S4). To further determine whether the endocytosed fusion proteins sort through early endosomes as the wild type EGFR, EEA-1, a protein involved in vesicular transport through early endosomes [24], was assessed for colocalization with microdomains. When soluble at room temperature, most of the EGFR-V96 remained on the plasma membrane and their colocalization with EEA-1 was low, whereas significantly more EGFR clustered and colocalized with EEA-1 after microdomain formation (Fig. 3b, c). EGFR-A96, which does not assemble microdomains at physiological temperatures, did not colocalize with EEA-1, suggesting the important role of receptor clustering in internalization.

3.4 Temperature-triggered activation of EGFR signaling pathways.

Having characterized the phase behavior and fate of EGFR-ELPs, their ability to activate known signaling pathways was explored. Using indirect immunofluorescence and colocalization analysis, the phosphorylation state of cell-surface EGFR was monitored. This method allows us to specifically quantify the percentage of cell surface receptors phosphorylated. At 22 °C, some of cells transfected with EGFR-V96 showed a strong intracellular red fluorescence signal arising from the auto-phosphorylation of receptors in the cytoplasm, which may be due to their overexpression through transient transfection. After 20 min incubation at 37 °C, in addition to pre-existing larger red fluorescent puncta, a pattern of smaller phosphorylation-positive puncta appeared to colocalize with the clustered cell-surface receptors (Fig. 4a). Colocalization analysis revealed that the activation of cell-surface EGFRV96 increased significantly post-stimulation (Fig. 4b). Subsequent activation of ERK1/2, indicated by a phosphorylation signal, was also observed in cells transfected with EGFR-V96 (Fig. 4c). While a certain level of auto-activation was detected at 22 °C, there remained a remarkable upregulation of phospho-ERK1/2 after heating up the cells to 37 °C for 20 min. Importantly, this response was completely blocked with the treatment of AG1478, a selective inhibitor of EGFR tyrosine kinase activity and PD0325901, a MEK inhibitor. These results indicate that temperature-induced ERK phosphorylation is mediated through the Ras/Raf/MEK signaling cascade following EGFR activation. As expected, similar activation was not observed when EGFR-A96 heated (Fig. S5). ERK1/2, known to be involved in the activation of many transcription factors, is strongly amplified by translocation to the nucleus [25]. The occurrence of nuclear translocation was confirmed by immunofluorescent staining of phosphorylated ERK1/2 (Fig. S6).

To compare the microdomain-mediated signaling with the ligand-triggered responses, the phosphorylation state of ERK1/2 was monitored over time (0, 10, 20, 60, 120, and 240 min) after applying heat or EGF. Given that EGF induces a dose-dependent activation, 1 ng/mL of EGF was used for comparison with the ELP fusions as it represents the physiological levels of EGFR ligands in human body [26]. Both stimuli resulted in rapid, but transient activation of ERK1/2 (Fig. 5a), consistent with previously published results [27]. Interestingly, the phosphorylation state of ERK1/2 in the presence of EGF is slightly stronger relative to the temperature-triggered activation, which may result from promiscuous binding of EGF to endogenous receptor tyrosine kinases. Taken together, these observations demonstrate that

EGFR-V96 enables transient activation of canonical ERK signaling in a temperature-dependent manner.

3.5 Dynamic control of microdomain-mediated signaling activation.

In comparison to small molecule inhibitors and inducible promoters, the potential to reversibly assemble microdomains in a biological environment is a strong advantage of ELP systems. To assess the reversibility of ELP-mediated EGFR signaling, 293T cells expressing EGFR-V96 were exposed to three rounds of heating (20 min) and cooling (40 min) and the activity of endogenous ERK1/2 was monitored. Western blots revealed that exposure of cells to 37 °C yielded approximately a four-fold increase in pERK1/2 and this activation was fully reversed upon cooling cells down to room temperature. Similar changes were observed after repetitively applying a second and third stimulation, demonstrating that this process can be reversibly controlled without losing activity (Fig. 5b).

It has been shown that dynamic changes of signaling in response to distinct stimuli can confer specificity to biological outcomes, especially for the ERK pathway [27]. Taking advantage of their reversible phase behavior, we hypothesized that ELP fusions can serve as a powerful platform for the dynamic control of signals by manipulating the temporal patterns of cell stimulation. To test this, various heating/cooling patterns were applied to 293T cells expressing EGFR-V96, and the changes in signaling outputs were assessed using pERK1/2 as a simple readout (Fig. 6). Low-frequency stimulation, such as one round of heating and cooling per hour, yielded transient and pulsatile patterns of ERK1/2 activation, which corresponded with its pattern of stimulation. By increasing the frequency of stimulation, it was possible to generate robust and sustained elevations in pERK1/2 over the entire study (Fig. 6a). To examine the effect of high frequency stimulation of EGFR-ELP, a downstream module of ERK signaling was assessed. STAT3 has been reported to selectively respond to sustained-- rather than transient-- ERK1/2 activation [28]. Using western blot, the phosphorylation of STAT3 was observed to increase after 2 hours of repetitive stimulation whereas no significant change was detected following a single round of stimulation (Fig. 6b). In addition, the effect of stimulation duration was also characterized by heating cells at 37 °C for different periods of time (5, 10, 15, and 20 min). The magnitude of ERK1/2 phosphorylation showed a proportional response to the heat duration (Fig. 6c), suggesting the quantitative control of ERK1/2 activity by EGFRV96. Collectively, these results suggest that EGFR-ELPs can be used to study distinct temporal patterns of dynamic and quantitative stimulation through the ERK pathway, which will be essential for studying and manipulating other physiological responses.

3.6 Directed transport of microdomain-mediated receptor clusters.

In addition to validating its functionality in signal activation, we also examined the functional regulation of cellular morphology by EGFR-V96 in 293T cells. To track the dynamic movements of EGFR-ELPs in real time, GFP was inserted between EGFR and ELP (Table S1, Fig. S7) and transfected into 293T cells for live cell imaging. The formation of filopodia and lamellipodia, and their dynamic activities, such as filopodial motility or retrograde transport of ligand-activated EGFR clusters, are characteristic of EGF-stimulated cells [29, 30]. Thus, the transport of fluorescent EGFR-GFP-V96 vs. EGFR-GFP-A96 was

monitored following a temperature ramp over 20 min (Fig. 7c). Interestingly, filopodia and lamellipodia-like structures (white arrowheads) formed at the plasma membrane of the cells with either EGFRGFP-V96 or EGFR-GFP-A96 (Fig. 7a). As temperature increased, EGFR-GFP-V96 apparently assembled into microdomains, and surprisingly these fluorescent receptor clusters were actively transported in a retrograde direction toward the cell body (Video S2, Fig. 7a, b). Although membrane structures, such as filopodia and lamellipodia, containing fluorescent EGFR-GFP-A96, were also observed in transfected cells, no significant changes were detected in their dynamic behavior or distribution (Video S3, Fig. 7a, b). These findings suggest that EGFR-GFP-V96, but not EGFR-GFP-A96, may mimic functional changes in a cell, such as with the cytoskeleton or dynamic behavior of the plasma membrane, that are also observed with EGF-stimulated cells [31, 32].

3.7 Indirect single-cell characterization of EGFR microdomain assembly in live cells.

Within individual cells the uptake of cell-surface EGFR-V96 occurs with a small change in temperature (Fig. S2c) [14]; furthermore, abrupt assembly within individual cells was confirmed by direct modification of these fusions with GFP (Fig. 7). Since direct modification by GFP significantly increases the molecular weight of EGFR-GFP-V96 fusion (Table S1), an indirect live cell imaging approach for following ELP-mediated assembly of EGFR-V96 was developed. To examine the variability between individual cells as a function of temperature, the exact transition temperature of each cell was monitored. Our prior studies have shown that co-expressed ELPs co-assemble different proteins within the cytosol [18]. Given this and the fact that the GFP-ELPs can be visualized in real time, a cytosolic GFP-ELP was selected as an indirect surrogate to follow the phase separation of EGFR-V96 (Fig. 8a). While the GFP fusion (EGFR-GFP-V96) also offers direct visualization under microscope, the addition of GFP may affect its expression level and transition temperature. To validate co-assembly of EGFR and GFP, 293T cells expressing both EGFR-V96 and GFP-V60 were incubated at 37 °C to induce intracellular microdomains, and their colocalization was confirmed in fixed cells using indirect immunofluorescence (Fig. S8a). This approach has significant applications during time-lapse imaging over temperature gradients, which can refine estimates of the exact phase transition temperature for individual cells without directly fusing a fluorescent protein to the EGFR-V96 fusion. As shown in Video S4 and S5, individual cells transitioned at slightly different temperatures/time points. However, each cell consistently formed puncta above its own transition temperature (Fig. 8b). In general, 293T cells dually transfected with GFP-V60 and EGFR-V96 assemble microdomains at a lower temperature (Fig. 8b, S9), which are larger and fewer in number than microdomains from cells transfected with the GFP-V60 reporter alone (Fig. 8c). Differences in the size and number of structures even enable the identification of both singly and double transfected subsets of cells in the same imaging field. Notably, the median transition temperature of EGFR-V96 is 32.8 °C, which is consistent with our previous observation using fixed cells (Fig. 2b) and that observed for EGFR-GFP-V96 (Fig. 7b). Moreover, co-assembly with GFP-V60 was confirmed to have no effects on the temperature-dependent activation of ERK by EGFR-V96 (Fig. S8b). These results reinforce the hypothesis that a GFP-ELP can serve as an indirect proxy for live-cell assembly of ELP fusion proteins.

4. Discussion

Controlled cell-signaling plays an undeniable role in diverse biological processes, and better tools to manipulate these signaling pathways will provide insight into how the temporal dynamics of signaling components regulate cellular function. Towards this end, this manuscript demonstrates the development of a protein switch that provides ON-OFF regulation of a specific cell-surface receptor, which enables dynamic control of downstream signaling pathways. The switch is designed using a thermally responsive ELP protein-polymer to drive reversible-assembly of a receptor tyrosine kinase expressed at the cell surface. A temperature sensitive ELP, V96, was genetically fused to EGFR, which is inactive in its default monomeric state and active in its clustered state. When heated above 32 °C, EGFR-V96 forms microdomains and activates the ERK signaling cascade, which demonstrates that ELPs confer their phase behavior without altering signaling targets of the native receptors. In comparison, a temperature-insensitive EGFR-A96 failed to show these effects at the same temperatures, which supports the hypothesis that ELP-mediated EGFR clustering is responsible for the receptor activation and signal transduction. While EGFR is known to activate Akt and PLC pathways in many cell lines, in the 293T cell line we only observed significant upregulation in pERK1/2 stimulated by either heating of EGFR-V96 or treatment with its endogenous ligand EGF. This suggests that signal sensitivity of different pathways is highly cell type-dependent and may vary across different cell lines.

As a genetically encodable protein, an ELP fusion only requires a single transgene to introduce temperature-dependent activation of signaling. In comparison to a stable expression system, transient transfections are widely used to rapidly express recombinant proteins for evaluation of activity. Many genetically encodable tools (optogenetic or chemical dimerizer systems) commonly rely on transient transfection [7, 33–36]. Based on these observations, fusion receptors in this study were delivered through transient transfection and visualized using confocal and epifluorescent microscopy. Immunofluorescent staining of myc revealed significant clusters of EGFR-V96 forming inside cells (Fig. S1). This likely resulted from culturing transfected cells at 37 °C prior to assay, above their phase transition temperature. Multiple experiments suggest that EGFR-V96 phase separates in cells above 32 °C by either fixed cell imaging (Fig. 2b) or by live cell imaging (Fig. 8b, S2). To visualize only the behavior of cell-surface EGFR-V96, an antibody was used to specifically label external EGFR-V96, which shows the clearest temperature-dependent internalization and phosphorylation (Figs. 3, 4, S2). However, future studies are required to determine if the EGFR-ELPs above their transition temperature become entrapped during recycling in the cytosol or are prevented from export from the endoplasmic reticulum or Golgi apparatus [37]. Despite the appearance of internalized clusters of EGFR-V96, when these cells are incubated at 37 °C they appear to proliferate normally. One challenge with transient expression is that receptor overexpression can elevate signaling above the physiologically basal state. Even though a certain level of phosphorylation of both EGFR and ERK was detected below the transition temperature, we still observed a remarkable increase in their activity at 37 °C, which far exceeded that of a temperature-insensitive EGFR-A96 control (Fig. 4c).

Temperature modulation is a common and powerful research strategy to block temperature-dependent processes, such as endocytosis [38–42]. While cold-incubation is not specific for a particular mechanism of endocytosis, it is used widely in research community to nonspecifically block all endocytosis. In contrast, this temperature-dependent approach induces cell-signaling and receptor internalization consequences that are highly specific to EGFR with a much smaller change in temperature (Figs. 2–4, S5). Live-cell characterization with GFP-ELP and EGFR-V96 (Fig. 7, 8, S2) suggests that ELP-mediated assembly within individual cells occurs abruptly over a sharp temperature shift ($\sim 1^\circ\text{C}$) [14]. Moreover, when the transition temperature for a population of transfected cells is characterized using live-cell imaging (Fig. 8b, S9), 70% of the EGFR-V96 positive cells phase separates between 31.0 and 34.0°C , suggesting only a 3°C increase is necessary to induce the majority of cells to phase separate. However, for fixed cell assays a wider range was used to clearly indicate differences in receptor status (Figs 2–6).

While ELP mediates similar responses as EGF at 22°C (Fig. 5a), it showed slower activation kinetics compared to that at 37°C , which was likely due to slow transfer of convective heat from air within the incubator. In contrast, signaling from EGF occurred instantaneously once mixed with the cell-culture media. To quantify this possibility, the temperature was monitored by a probe immersed in media above cells cultivated in a 37°C incubator. As in Fig. S10a, the temperature increase took 15 mins to reach the median T_f for EGFR-V96 (Figs. 2b, 8), which corresponds to the observed delay in signal activation (Fig. S10b). However, changing the heat source from air to conductive heating using a hot plate easily increased the rate of heating. The activation kinetics of pERK1/2 accelerated as the heat transfer rate increased, and the magnitude of activation remained the same. With the optimized system, we detected ERK phosphorylation within 2 minutes, which reached its maximum at 5 minutes. The rapid change of signaling clearly demonstrates the fast responsiveness of EGFR-V96, which was now even faster than for exogenous EGF (Fig. S10b). Beyond this, the temperature-triggered activation is reversible (Fig. 5), which allows controlled repetitive activation (Fig. 6); and specific to the receptors labeled with ELP, whereby co-transfected wild-type HER2/ErbB2 was unaffected by the assembly of EGFR-V96 (Fig. S11). HER2 is in the same family of receptor tyrosine kinases as EGFR, with which it is known to hetero-dimerize.

In addition, ELP-mediated assembly offers benefits compared with alternative magnetogenetic or optogenetic strategies. Molecular switches using magnetic nanoparticles that were previously developed for manipulation of cell signals, however, unless single-cell microinjection is used these methods can only be used with transmembrane proteins. In contrast, ELPs can be easily adapted to a broad range of signaling molecules including intracellular proteins. An advantage of ELPs over optogenetics is that it allows the use of green or blue light emitting fluorescent proteins for time-lapse imaging of receptor clustering in living cells. For example, GFP-V60, an ELP with a higher phase transition temperature than EGFR-V96, was explored as a live cell marker for microdomain formation. Co-assembly with EGFR-V96 not only lowered the apparent transition temperature of GFP-V60, but also changed the morphology of microdomains (Fig. 8). GFP-V60 thus enables real-time characterization of EGFR-V96 at a single-cell level without the need to directly link GFP to the EGFR gene. Furthermore, ELP phase separation results in receptor

oligomerization, which may differ significantly from ligand-induced dimerization. Although the dimerization of receptor tyrosine kinases has been reported for decades [43], there is increasing evidence that higher-order oligomers may also form in response to EGF binding [44, 45]. In these regards, the EGFR-ELP system offers multiple potential advantages to explore receptor clustering/oligomerization and its potential mechanisms in cell signaling and human disease.

While this ELP-based strategy may be useful to activate/inhibit signaling pathways in the short term, it does have some limitations. A stable cell line would be useful to study ELP-mediated proteins over longer durations; however, protein expression levels may be variable and interfere with the endogenous protein, making it difficult to control the activity of signaling molecules. Recent advancements in genomic editing using CRISPR/Cas9 may enable engineering of genomic proteins at endogenous levels, and could benefit from ELP-mediated control. Moreover, it should be noted that changes in temperature have been reported to induce hot or cold shock responses involving the modulation of transcription, translation, cell cytoskeleton, cell cycle and metabolic processes [46, 47]. To control for these effects, it is very important to include temperature-insensitive controls, such as EGFR-myc or EGFR-A96. In addition, it is important to minimize the shift in temperature necessary to drive an ELP-fused receptor to cluster, which live-cell imaging suggests can be accomplished in most cells using only a 3 °C increase (Figs. 7, 8, S2, S9). Although these changes did not show any effects on the EGFR signaling (Fig. S5), this strategy should be designed carefully for new pathways.

5. Conclusions

This report demonstrates that clustering of a receptor tyrosine kinase, EGFR, through thermoresponsive microdomain assembly can modulate signaling activities in a temperature-dependent manner, offering an approach to investigate the complex dynamic properties of signal transduction. Despite the limitations discussed above, we provide the first *in vitro* proof-of-concept for the use of ELPs to manipulate intracellular signaling pathways. Although at an early stage of development, the ELP-mediated switch has potential as a tool for studying biological processes and other biomedical applications. For example, since RTKs are foremost amongst cancer research targets [48], these engineered receptors may enable screening of drug candidates without the need for ligand-induced activation. From a synthetic biology perspective, we provide a new strategy for versatile control of protein clustering which can be extended to numerous signaling molecules and pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1. Design and function of EGFR-ELPs.

a, A schematic illustration of ligand-induced vs. ELP-mediated EGFR signaling. **b**, Genes encoding four ELPs (V72, V96, 2VA96, A96) were fused to the carboxyl terminus of EGFR with a myc tag for indirect immuno-detection of expressed proteins (Table 1). **c**, EGFR-ELPs were expressed in 293T cells and detected using an anti-myc antibody (red). GAPDH (green) was also included as a protein loading control, suggesting different expression levels of recombinant proteins. Lane: 1) No transfection; 2) EGFR-myc; 3) EGFR-V72; 4) EGFR-V96; 5) EGFR-2VA96; 6) EGFR-A96.

Fig. 2. Temperature-dependent clustering of surface EGFR-ELP is fast and tunable.

a, 293T cells expressing EGFR-ELPs were pre-stained with an anti-EGFR antibody at 4 °C prior to incubation (30 min) at indicated temperatures. After fixation, images were acquired using confocal laser scanning microscopy. Green: AlexaFluor-488 conjugated anti-EGFR antibody; Blue: DAPI; Scale bar: 10 μ m. **b**, The temperature of microdomain assembly can be tuned by changing ELP hydrophobicity and length. The majority of cells transfected with EGFR-V96, EGFR-V72, and EGFR-2VA96 clustered the label above 32, 37, and 42 °C respectively. A temperature-insensitive control, EGFR-A96 did not assemble microdomains at the temperatures evaluated. **c**, The kinetics of EGFR-V96 microdomain assembly were characterized at 37 °C. Phase separation occurs rapidly with more than 50% of cells showing microdomains within 15 min. Mean $\pm$ SD (n=3).

Fig. 3. EGFR-ELP assembly initiates receptor internalization to early endosomes.

293T cells were transiently transfected with EGFR fusions and the surface receptors were labeled by an anti-EGFR antibody (green) at 4 °C prior to incubation above (37 °C) or below (22 °C) the transition temperature. **a**, EGFR internalization was quantified using flow cytometry. After incubation, the cell surface was stripped with acidified glycine buffer (0.2 M, pH 2.5, 1 mg/ml BSA). By 30 min at 37 °C, there was a significant increase in receptor internalization for cells expressing EGFR-V96 compared to those with EGFR-A96, which does not phase separate at 37 °C (**** $p < 0.0001$, ** $p < 0.01$). Mean $\pm$ SD ($n = 3$). **b** and **c**, Alternatively, to follow the internalization pathway of surface-stained EGFR, 293T cells transfected with EGFR-V96 or EGFR-A96 were surface-labeled (green), incubated for 30 min, fixed, and stained for early endosomes (red). Immunofluorescence was observed using confocal laser scanning microscopy. Scale bar: 10 μ m. At room temperature, EGFRV96 remained on the cell surface. Once induced to phase separate at 37 °C, EGFR-V96 internalized to puncta that colocalize significantly with EEA1, a marker of early endosomes. *** $p = 0.0001$, Mean $\pm$ SD ($n = 11-23$).

Fig. 4. EGFR-V96 assembly specifically activates EGFR and the ERK1/2 pathway.

a, 293T cells transfected with EGFR-V96 were pre-chilled at 4 °C and surface EGFR was labeled by immunofluorescence (green). Cells were then incubated either below (22 °C) or above (37 °C) the transition temperature for 30 min, fixed, permeabilized, and the activation of EGFR was detected using an anti-pEGFR antibody (red). Scale bar: 20 μm. **b**, Colocalization coefficient of individual cells representing the percentage of surface EGFR phosphorylated was determined from the pixels in the overlapped region to the total green pixel intensity using Equation 1. Temperature-dependent activation of surface EGFR was detected in cells expressing EGFR-V96 but not EGFR-A96. * $p = 1e-16$. Mean $\pm$ SD (n=26–39) **c**, Using a western blot, temperature-dependent phosphorylation of ERK1/2 was observed in cells transfected with EGFR-V96 and this ELP-mediated activation was specifically blocked using an EGFR kinase inhibitor AG1478 (0.5 μM). Furthermore, a MEK inhibitor PD0325901 (0.1 μM) completely abolished the phosphorylation of ERK1/2.

Fig. 5. ELP-mediated ERK signaling is transient and reversible.

a, To verify that the construct retains activity similar to that obtained upon the addition of free ligand (EGF), 293T cells expressing EGFR-V96 were either heated to 37 °C or treated with 1 ng/mL EGF for the indicated time periods and monitored by western blot. A transient elevation in pERK1/2 was consistently observed in cells with different treatments. **b**, In EGFR-V96 transfected 293T cells, phosphorylation of ERK1/2 was monitored and quantified after three cycles (C1, C2, C3) of heating (20 min) and cooling (40 min), thus demonstrating reversibility of switching. **** $p=0.00004$, *** $p=0.0003$, ** $p=0.005$. Mean $\pm$ SD (n=3).

Fig. 6. EGFR-V96 mediated ERK signaling can be temporally modulated.

The change of ERK1/2 activity was monitored by western blot according to frequency and duration of heating (37 °C, red boxes) and cooling (22 °C, baseline) in 293T cells expressing EGFR-V96. **a**, Cells were heated for 10 min repetitively at 5, 10, 20, and 50 min intervals. High-frequency heating led to sustained ERK1/2 activation, whereas low-frequency heating showed pulsatile ERK1/2 activation. **b**, Cells were exposed either to constant heating (37 °C) or repetitive heating (10 min 37 °C, 5 min 22 °C). STAT3 is a transcription factor downstream of EGFR/ERK; furthermore, phosphorylation of STAT3 responds to repetitive heating and not to continuous heating after 2-hr treatment. GAPDH was included as a protein loading control. * $p = 0.04$. Mean $\pm$ SD ($n=4$). **c**, Cells were heated at 37 °C for 5, 10, 15, or 20 min over a total period of 30 min. pERK1/2 intensity increased as the duration increased to 20 min.

Fig. 7. EGFR-V96 assembly triggers directed transport of receptors.

293T cells were transfected with EGFR-GFP-V96/A96, and receptor transport in response to temperature was monitored by capturing time-lapse images every 30 s for 20 min. **a**, Representative images showing the dynamic behavior of receptors in transfected cells. The white arrowheads indicate filopodia and lamellipodiallike structures. When EGFR-GFP-V96 is induced to assemble into microdomains, dynamic cellular activities, such as retrograde transport of EGFR-GFP-V96 clusters are stimulated. These activities are not observed in cells expressing EGFR-GFP-A96. Scale bar: 10 μ m. **b**, Receptor transport toward the cell body was quantified by measuring the percentage of fluorescent receptors in filopodia (Equation 2), and the resulting numbers were plotted against time. The decrease observed in % receptor in filopodia with EGFR-GFP-V96 is consistent with retrograde transport of these receptor clusters. Mean $\pm$ SD (n=4). **c**, The real-time temperature was measured using a probe immersed above cells when cells were heated on a temperature controlled stage.

Fig. 8. GFP-ELP microdomains reveal intracellular phase separation of EGFR-V96 using live cell imaging.

a, Co-transfection with EGFR-V96 and GFP-V60 enables live cell imaging of microdomain assembly in individual 293T cells. **b**, Epifluorescence imaging was conducted over a temperature ramp of 10 to 40 °C at a rate of 4 °C per min. Co-transfection with EGFR-V96 and GFP-V60 significantly ($p = 0.002$) decreased intracellular T_i 32.8 °C [31.0, 34.0, $n=19$] compared to cells transfected with GFP-V60 alone 35.9 °C [33.0, 37.0, $n=19$]. Values are reported as Median [15th percentile, 85th percentile, sample size]. Scale bar: 10 μm . **c**, In addition to lowering the T_i , cotransfection alters the morphology of GFP-V60 microdomains. When compared to GFP-V60 alone, co-transfection significantly increased the puncta size ($p = 7\text{e-}06$) and reduced the puncta number ($p = 2\text{e-}04$) of GFP-V60.

Table 1.

ELP protein polymers fused with EGFR.

ELP Nomenclature	*Amino acid sequence	**Intracellular T_t (°C)	***Molecular weight (kD)
EGFR-A96	<u>EGFR-myc</u> -G(VPGAG) ₉₆ Y	>42	173.5
EGFR-V96	<u>EGFR-myc</u> -G(VPGVG) ₉₆ Y	32	176.2
EGFR-V72	<u>EGFR-myc</u> -G(VPGVG) ₇₂ Y	37	166.4
EGFR-2VA96	<u>EGFR-myc</u> -G(VPGVGVPVGVPAG) ₃₂ Y	42	175.3

* EGFR-myc sequence available in the Supplementary Table S2.

** Observed transition temperature yielding EGFR-ELP microdomains in more than 50% of 293T cells using fixed cell imaging.

*** Estimated molecular weight from amino acid sequence.