

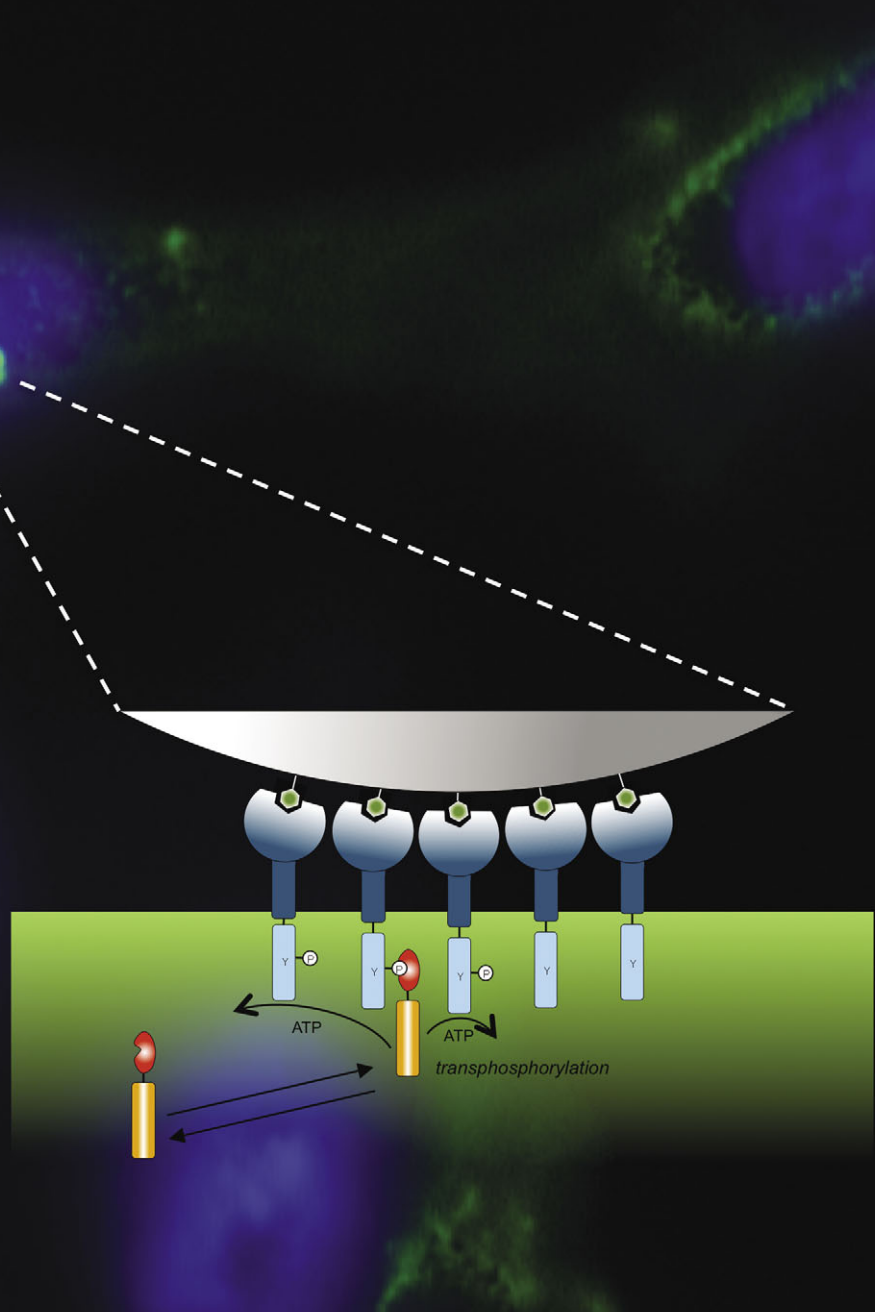
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PAPER

## *De novo* motif for kinase mediated signaling across the cell membrane†

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The binding of a ligand to a cell-surface receptor represents a first step in transmitting extracellular information to the cell in order to activate downstream intracellular signaling events. This report describes the *de novo* engineering of cells with a transmembrane signal transduction motif that is developed from first principles. The signaling event is initiated with a polyvalent ligand that clusters an engineered receptor in the membrane. The receptor presents a cytosolic kinase substrate that undergoes autocatalytic phosphorylation when organized in a cluster, but not in the unclustered arrangement. The example described here may represent a motif that can be used in synthetic biology for the engineering of cells with novel circuits.

### Introduction

Extracellular ligands initiate signaling pathways by associating with cell-surface receptors.<sup>1,2</sup> Receptor binding is often followed by phosphorylation of the intracellular domain of the receptor by protein tyrosine kinases, which in turn regulates a wide range of pathways and cellular activities.<sup>3</sup> Three motifs are commonly used to transduce receptor binding at the membrane to intracellular protein phosphorylation: dimerization of receptor tyrosine kinases,<sup>4</sup> conformational changes in the receptor,<sup>5</sup> and the assembly of multi-receptor complexes.<sup>6</sup> Designing cellular signaling pathways for the purpose of installing new functionality into cells is challenging and represents a significant goal for the synthetic biology community.<sup>7</sup> In this report, we describe an engineered receptor that is designed from first

principles and responds to a synthetic extracellular cue that induces clustering, upon which it is phosphorylated by an endogenous kinase.

Our engineered receptor takes advantage of an autocatalytic phosphorylation reaction that depends on the density of the receptor within the membrane.<sup>8</sup> At low densities there is no autocatalysis and phosphorylation of the receptor is rapidly reversed by local phosphatases. At densities above a threshold value, the autocatalytic motif can operate—where the SH2 domain of a kinase localizes the kinase to the membrane, where it can reach and rapidly phosphorylate a neighboring receptor, leading to efficient phosphorylation. Cells were engineered with an artificial transmembrane receptor that consists of an extracellular ligand binding domain and an intracellular substrate for Abelson tyrosine kinase (Abl).<sup>9</sup> Binding of a polyvalent bead to the cell surface results in clustering of the receptor, which can then support the autocatalytic phosphorylation of the receptor. Our results demonstrate an engineered signaling motif that can be utilized by synthetic biologists when designing new cellular signaling pathways.<sup>10</sup> By designing the signaling mechanism from first principles, we ensure that it is insulated from cross-talk with endogenous cellular processes.

The approach is based on our recent report of an autocatalytic reaction observed in the phosphorylation of immobilized peptides

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† Electronic supplementary information (ESI) available: Full sequences of the artificial receptor (Fig. S1) and SH2<sub>Abl</sub>-GFP reporter protein (Fig. S2); receptor expression analyzed by flow cytometry (Fig. S3); dose dependence of phosphorylation inhibition in response to imatinib and soluble benzenesulfonamide (Fig. S4). See DOI: 10.1039/c1ib00009h

### Insight, innovation, integration

Engineering cells to respond to new extracellular cues and to perform functions that are not otherwise endogenous is an important goal in advancing our basic understanding of cell function and in systems biology. This paper demonstrates a signaling motif that was engineered from first principles, and that relies on the autocatalytic phosphorylation of a clustered receptor. The chimeric receptor undergoes efficient

phosphorylation only when it is clustered by a bead on the extracellular face. Unlike natural transmembrane signaling motifs, which rely on receptor dimerization or conformational changes, this motif owes to an adaptor protein-mediated autocatalytic reaction by Abl kinase. This work provides an early example of the *de novo* design of signal transduction motifs in the cell.

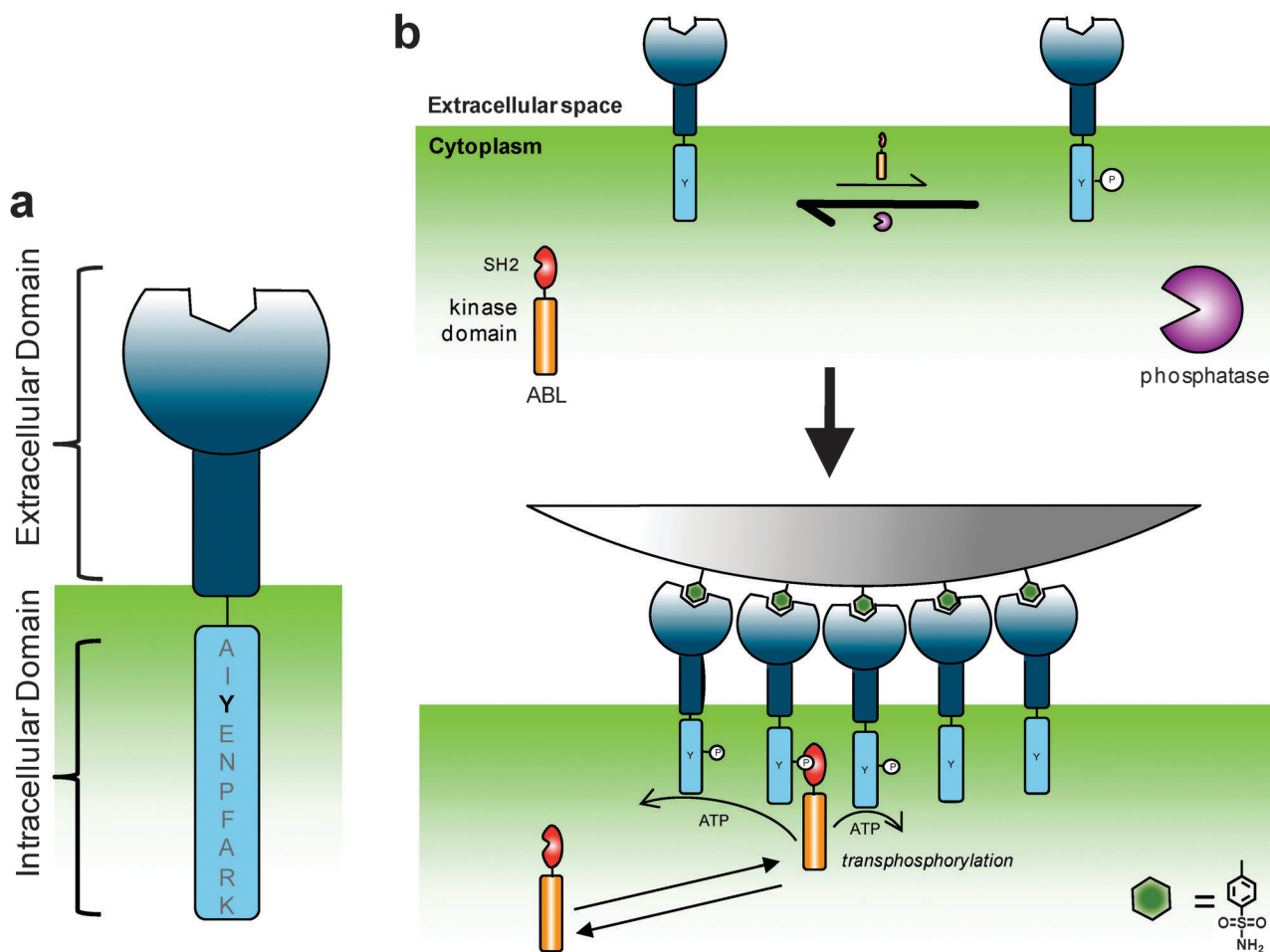
by the Abl kinase. This reaction requires that the phosphopeptide product of the reaction is also a ligand for the SH2 domain of the kinase.<sup>11,12</sup> In that case, the initial phosphorylation reaction is slow, but generation of the phosphopeptide serves to recruit the kinase by binding the SH2 adaptor domain. The kinase is then positioned to rapidly phosphorylate those peptides that are within its reach. At the beginning of the reaction, there is a marked lag phase in the phosphorylation reaction, followed by a rapid increase in rate. The phosphorylation that occurs during the lag phase reflects an 'intermolecular' reaction that requires the kinase in solution to associate with its substrate on the surface. However, the phosphorylated peptide is a ligand that can recruit kinase molecules, where they can more rapidly phosphorylate adjacent peptides on the surface through an 'intramolecular' reaction, where one end of the kinase is tethered to the surface through association of its SH2 domain with a phosphopeptide. It follows that this autocatalytic mechanism only operates when the density of peptide is sufficiently high such that a tethered kinase can

reach a neighboring peptide, as required for the intramolecular reaction. This density-dependence of the phosphorylation rate can be used to initiate a signaling event at the cell membrane and is the basis for our demonstration of signaling at the cell membrane.

## Results

### Designing the autocatalytic system

We use an engineered chimeric protein receptor that includes an extracellular 'receptor' domain, a single transmembrane helix, and a cytoplasmic domain that includes a substrate for Abl kinase (Fig. 1a). The extracellular receptor domain is carbonic anhydrase IV (CAIV), which binds to the small molecule benzenesulfonamide (BzS).<sup>13,14</sup> We engineered a stalk domain into the receptor to space the CAIV domain away from the membrane, and we used the integrin  $\beta 1$  transmembrane helix as the transmembrane domain. Finally, the Abl kinase substrate



**Fig. 1 Clustering of a membrane receptor into regions of high density triggers its autocatalytic phosphorylation by an endogenous kinase containing an adaptor domain that binds the phosphopeptide product.** (A) The receptor contains an extracellular ligand-binding domain (dark blue) with an intracellular phosphorylation site that is a substrate for Abl kinase (light blue). The extracellular domain consists of a BzS binding domain (carbonic anhydrase IV catalytic domain) that is spaced from the membrane by a fractalkine stalk (not drawn to scale). (B) When the receptor is diffusely spread throughout the membrane, endogenous phosphatase activity dominates and the receptor predominantly remains unphosphorylated (upper panel). Upon treatment with BzS coated beads (grey), the receptor becomes clustered into high density patches on the cell membrane. The resulting high density of substrate allows Abl to autocatalytically phosphorylate the receptor (lower panel).

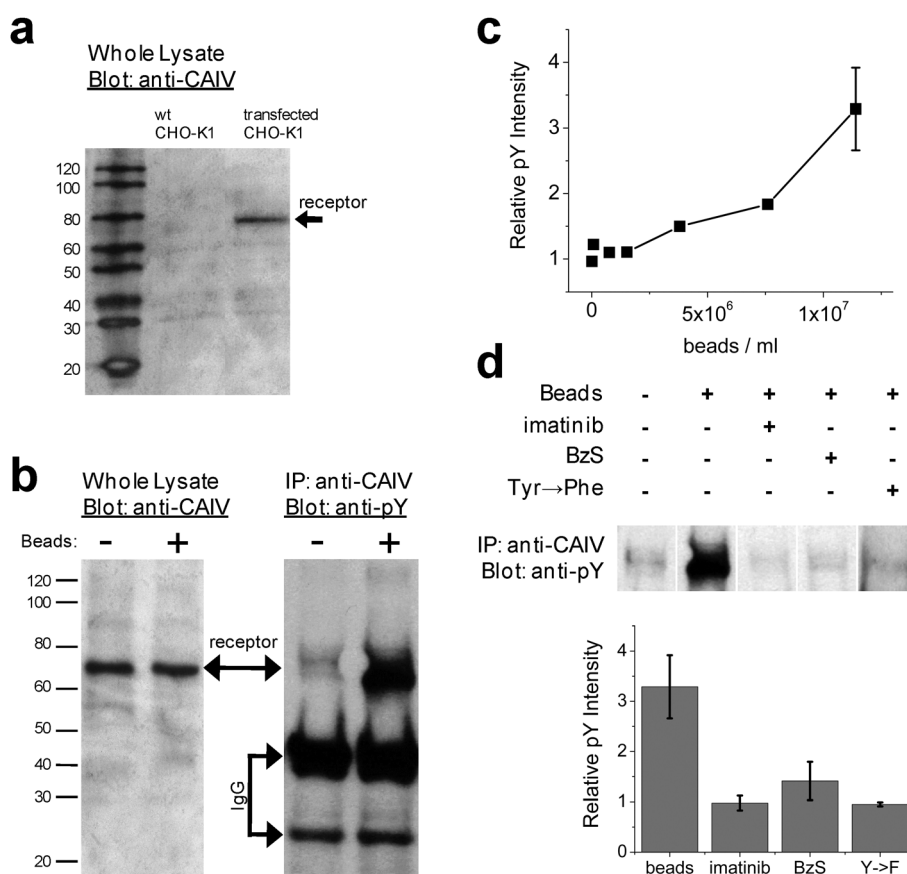
sequence AIYENPFARK was engineered into the cytoplasmic region of the receptor (see Supplementary Fig. 1 online for entire receptor sequence). This substrate sequence was chosen because it is a suboptimal catalytic substrate, but once phosphorylated, it has high affinity for the Abl SH2 domain.<sup>11,12</sup> *In vivo*, the phosphorylation state of the receptor is based on the competition between Abl kinase and native phosphatases in the cell.

In unstimulated cells, we reasoned that the receptor would be distributed across the membrane at densities that do not support the intramolecular reaction. Therefore, the endogenous phosphatase activity overwhelms the Abl kinase and keeps the receptor in a non-phosphorylated state. However, when the receptors are recruited into clusters, we expected the local density of kinase substrate to be sufficient to support the autocatalytic phosphorylation by Abl, and therefore the phosphorylated state of the receptor would dominate (Fig. 1b). To trigger clustering of the receptor, we use a polyvalent ligand that presents the BzS ligands on micron-sized beads and that can therefore be used to cluster the receptor and give high local densities of the receptor that support the autocatalytic branch of the reaction.

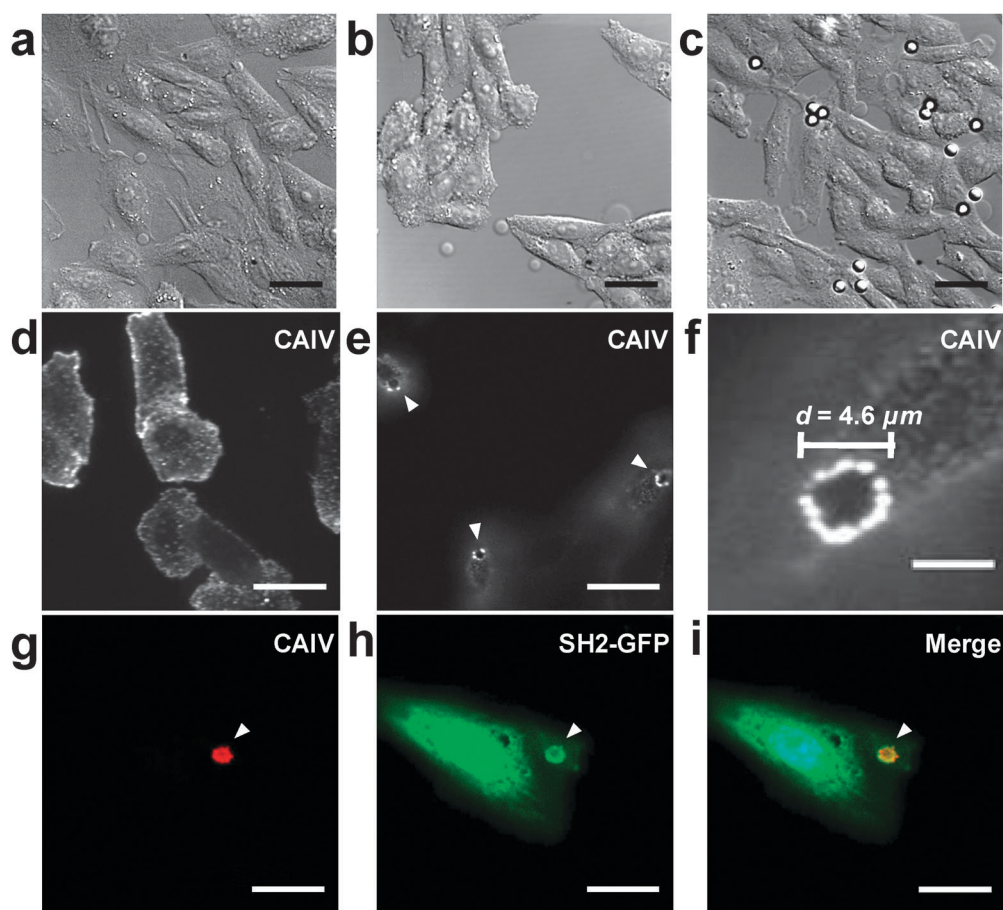
### Clustering of the receptor leads to phosphorylation

We first transfected cells with the receptor (Fig. 2a), and found that the receptor is trafficked to the membrane in the correct orientation as determined by flow cytometry analysis (see Supplementary Fig. 2 online). Receptor-expressing cells were treated with beads for one hour and then lysed. The receptor was immunoprecipitated from the whole cell lysate using anti-CAIV antibodies, and the immunoprecipitated receptor was then immunoblotted with an anti-phosphotyrosine antibody. When receptor-expressing cells are treated with BzS beads, we observe on average a 3.2-fold increase in phosphorylation relative to receptor-expressing cells that have not been treated with beads (Fig. 2b). As the cells are treated with higher quantities of beads, a greater proportion of the total receptor is clustered and higher levels of phosphorylation are observed (Fig. 2c). This result demonstrates that receptor clustering mediates the amplification in receptor phosphorylation. It is the local change in receptor density that triggers the kinetic nonlinearity and causes the kinase reaction to dominate over that of the phosphatase.

Several control experiments support the model of clustering-induced phosphorylation (Fig. 2d). Transfected cells with a



**Fig. 2 Receptor phosphorylation in response to clustering.** (A) CHO-K1 cells were transfected with DNA encoding the receptor. After sorting and selection, a stable cell line expressing the receptor was generated. An immunoblot against CAIV demonstrates that the receptor is only expressed in transfected cells, and it has the expected molecular weight of ~70 kDa. (B) Treatment of cells with beads leads to a large increase in the levels of phosphorylated receptor. Data are presented as ratios of phosphotyrosine staining between bead-treated and untreated cells. (C) Increased levels of phosphorylation are proportional to the number of beads added to the cells. Error bars represent the standard error of the mean ( $n = 5$ ). (D) Autocatalytic phosphorylation is susceptible to inhibition of Abl (imatinib treatment, 10  $\mu$ M), prevention of receptor/bead binding (BzS treatment, 1 mM), and mutation of tyrosine to phenylalanine (Tyr  $\rightarrow$  Phe). Error bars represent the standard error of the mean ( $n \geq 3$ ).



**Fig. 3 Visualizing receptor clustering and phosphorylation.** (A) Differential interference contrast (DIC) image of wild-type CHO-K1 cells. (B) DIC image of receptor expressing cells reveals that they are morphologically similar to wild-type. (C) Receptor expressing cells treated with beads. (D) Receptor expressing cells immunostained with anti-CAIV antibody. (E, F) Receptor expressing cells treated with beads and stained for CAIV. Upon treatment, visible clusters of receptor form with diameters that correspond well with the diameter of beads, 5  $\mu\text{m}$ . **g–i**, Fluorescent images of cells expressing both the receptor and SH2<sub>Abl</sub>-GFP treated with beads: CAIV (G), SH2<sub>Abl</sub>-GFP (H), merged (I). Scale bars: 20  $\mu\text{m}$  (A–E, G–I) and 5  $\mu\text{m}$  (F).

receptor having the active tyrosine-566 residue mutated to phenylalanine lacked the clustering-induced phosphorylation (Fig. 2d), even though this mutant receptor expressed well and was able to bind BzS beads identically to the tyrosine variant. Further, predictive software suggests that the Abl substrate sequence chosen for this study is not a viable substrate for other cellular kinases.<sup>15</sup> When cells were treated with imatinib—which is an approved drug for inhibition of Abl/Arg kinases<sup>16</sup>—the drug prevented phosphorylation of the receptor in the presence of beads (Fig. 2d). Finally, we performed experiments in the presence of saturating concentrations of soluble BzS and found that the BzS prevented engagement of the beads with receptor. In this case, again, we observe no increase in phosphorylation upon bead treatment (Fig. 2d). Susceptibility to imatinib and soluble BzS are both dose dependent (see Supplementary Fig. 3 online). These modifications in the system establish that the receptor phosphorylation is specific to the proposed auto-catalytic mechanism.

We also obtained direct visual evidence for clustering of the receptor by the beads. Receptor expressing cells (Fig. 3b–f) were treated with BzS beads (5  $\mu\text{m}$  diameter), fixed with formaldehyde, and then immunostained with an antibody directed against

CAIV (Fig. 3d–f). Only upon treatment with beads do cells exhibit discrete clusters of receptor, whereas cells treated with amine-terminated beads do not form clusters. The clusters form in a ring pattern whose diameter matches the size of the beads (Fig. 3e–f). We hypothesize that the ring pattern is an artifact of fixation and staining, where the antibody binding sites on the CAIV domain are physically occluded by the bound bead, and as such, remain unstained. Another possibility for this is that there is an energetic difference between areas of high and low membrane curvature, and the receptor prefers areas of high curvature. Receptor clustering is only possible if the protein can diffuse in the membrane. As such, this experiment clearly demonstrates that cluster formation is occurring upon bead treatment.

#### Receptor phosphorylation occurs locally in response to clustering

One of the assumptions attached to this signaling mechanism is that receptor phosphorylation occurs locally at the sites of clustered receptor. To determine if this is the case, we transfected receptor-expressing cells with a fusion protein that consists of

two domains: the SH2 domain of Abl fused to Green Fluorescent Protein (SH2<sub>Abl</sub>-GFP). The SH2<sub>Abl</sub>-GFP should only bind to the receptor after it has been phosphorylated, and therefore serves as an intracellular marker for the phosphorylation state of the receptor. Consistent with the expectation, the SH2<sub>Abl</sub>-GFP colocalized well with the clustered receptor (Fig. 3g–i), demonstrating that phosphorylation occurs locally at sites of clustered receptor directly as a result of the autocatalytic enzymatic activity of Abl.

## Discussion

This report demonstrates the use of an engineered receptor to transduce an extracellular cue across the membrane through the clustering of the receptor. Treatment of adherent cells with a polyvalent extracellular ligand—a bead having the small molecule BzS immobilized to its surface—results in a local clustering of the receptor that then supports the autocatalytic phosphorylation of the cytosolic domain. We showed that clustering markedly increases the phosphorylation levels of the receptor and can act as a mechanism for switching the receptor from the unphosphorylated to the phosphorylated state. This motif for tyrosine kinase signaling at the cell membrane takes advantage of a kinetic nonlinearity that occurs when a kinase can autocatalytically phosphorylate its substrates, but doesn't otherwise require protein-protein interactions or conformational changes of the transmembrane proteins.

The field of synthetic biology has made significant progress towards the broad goal of logically engineering biological systems for practical applications.<sup>17,18</sup> An ongoing effort is directed towards expanding the toolkit of synthetic biological parts and signaling motifs. Synthetic gene expression circuits have yielded many interesting behaviors, including switches,<sup>19</sup> oscillators,<sup>20</sup> memory, and counting.<sup>21</sup> These early successes with gene networks were facilitated by the strong understanding of gene expression control and regulation, and the components that regulate such systems—transcription factors, promoters, and operators—are generally independent of one another. Manipulating biological systems through protein signaling networks offers a new opportunity to install functions in cells. Many of the advances in synthetically designed signaling pathways have occurred by recognizing the inherent modularity of signaling proteins, including adaptor domains (e.g. SH3 and PDZ domains) and their cognate binding partners, as well as the modularity of certain catalytic domains. These modular pieces can then be rearranged and tied together into fusion proteins with new activities. Usually the fusion is between a catalytic domain and an adaptor domain, with the adaptor domain serving to link the catalyst to an activator, its substrate, or a different subcellular location, resulting in new behavior. Another method of regulation has been to fuse an adaptor domain, binding ligand, and catalytic domain together, where the enzyme is autoinhibited until a post-translational modification relieves the autoinhibition. These four motifs have constituted the bulk of design principles guiding the development of synthetic signaling pathways.

As mentioned in detail above, the common method of rewiring cellular circuitry is through the shuffling or redesign of endogenous protein domains. Such design principles have led to important progress in engineering cells and a greater

understanding of the organizational principles of biological systems.<sup>22</sup> In one study, a chimeric protein was created by fusing the SH2 domain of Grb2 to the death effector domain (DED) of Fadd.<sup>23</sup> Cells expressing this DED-SH2 chimera were 'rewired' to respond a stimulus for proliferation in a nonphysiological manner. Instead of proliferating, the cells underwent apoptosis as a result of the DED. In another example of signal transduction rewiring, Bashor *et al.* designed a synthetic protein scaffold in order to modulate the quantitative response of the MAP kinase pathway in response to  $\alpha$ -factor.<sup>24</sup> Through the physical recruitment of interacting proteins, the authors were able to tune the pathway to respond in either an accelerated, delayed, or ultrasensitive manner. For the purposes of biosensing, a complementary strategy has been utilized, where instead of rewiring the signaling circuitry, the receptor protein itself is manipulated to respond to unnatural ligands. Such methods have been used to alter the binding specificity of the periplasmic binding protein superfamily,<sup>25</sup> the histidine kinases,<sup>26</sup> and the FKBP, <sup>27</sup> and the modified proteins have the capability to respond to an extremely diverse array of small molecules.

Another approach to the problem is through the *de novo* design of new component proteins, which can operate in league with endogenous signaling pathways. This was the approach we took to designing the signaling motif used in this study. The development of new motifs to add to the toolkit of protein-based synthetic biology represents an important goal for chemical biologists, where the knowledge of chemical systems informs the design and engineering of new motifs based on known chemical mechanisms. In this report, we took advantage of a kinetic nonlinearity that occurs in an autocatalytic phosphorylation reaction to design a motif based on induced oligomerization. There are examples in nature of signaling pathways that operate through induced oligomerization, the most notable of which is B-cell signaling through Fc receptors, where the binding of multivalent immune complexes triggers oligomerization of the receptor.<sup>28</sup> Oligomerization leads to transphosphorylation by receptor-associated Src-family kinases,<sup>29,30</sup> and the subsequent recruitment of various effectors—including Syk and Lyn—which activate the immune response.<sup>31–33</sup> In another natural example, it has been shown that the oligomerization of Nck SH3 domains at the cell membrane induces the localized polymerization of actin through the recruitment of N-WASp.<sup>34</sup> While these examples take advantage of induced oligomerization, in both cases the motif operates through a multi-component system, where effective signaling is often dependent on three or more proteins, including receptors, scaffolding proteins, substrates, and kinases, making these challenging systems to repurpose for a non-natural application. Importantly, the example demonstrated in this report is a two-component system that relies on only two cellular proteins, the receptor and the kinase. Since the kinase is an endogenous protein, when designing a synthetic cellular circuit, only the receptor needs to be artificially installed in cells. The limited number of essential components increases the versatility of the motif.

Applications of induced oligomerization have been demonstrated on natural signaling systems, such as bacterial chemotaxis.<sup>35–37</sup> Even so, the idea of induced oligomerization, to our knowledge, has not been applied as a synthetic

biology motif. We believe that this motif is applicable to multiple systems, and can be modified to apply to diverse signaling pathways. Here, we employed induced oligomerization to regulate an autocatalytic phosphorylation reaction. The modularity of the receptor, however, allows the cytoplasmic tails to be modified to respond to other enzyme activities. Similarly, the receptor can be modified to respond to other extracellular stimuli by changing the ligand-binding domain. Additionally, the motif is not reliant only on endogenous enzymes, as the receptor can be modified to interact with non-natural enzymes. We believe that the versatility of this motif will allow for its use in a wide range of systems, and the focus of current work is to integrate this signaling motif with downstream activities that ultimately modulate gene expression activities in the cell.

Towards this end, one possibility is to engineer the receptor to recruit enzyme effectors to the intracellular face of the membrane. Membrane recruitment is a robust, and well studied mechanism of signal transduction.<sup>38–40</sup> There are many examples of enzymes that rely on membrane recruitment for activity, commonly by binding a membrane bound partner such as phosphatidylinositol (3,4,5)-trisphosphate (PIP3) or a modified protein such as scaffolding protein or a receptor tyrosine kinase. A well characterized example is the activation of Ras by Son of Sevenless (SOS). In this pathway, SOS is recruited to the membrane in response to receptor activation through the recruitment of adaptor proteins such as growth receptor-bound protein 2 (Grb2). Once localized, SOS activates Ras which then instructs downstream gene regulatory events.<sup>41</sup> The critical step in this gene regulation is the membrane recruitment of Ras.

Our signaling motif should enable approaches wherein signaling pathways are rewired by fusing the SH2<sub>Abl</sub> domain to an endogenous enzyme that relies on membrane recruitment. In the example discussed above, the SH2<sub>Abl</sub> domain could be fused to SOS to make Ras activation respond to the extracellular bead. Other possibilities include fusing the SH2<sub>Abl</sub> domain to phosphoinositide-3 kinase (PI3K), where membrane recruitment leads to increased levels of PIP3 in the membrane and subsequent activation of Akt, or fusing the adaptor domain to the WASP/WAVE family of proteins to locally stimulate actin polymerization.<sup>42</sup>

Our current strategy uses micron sized beads to trigger receptor clustering. Other inducers could be used provided that they are present in a polyvalent context and that a receptor is available that can be engineered into the extracellular domain of the transmembrane receptor. Plausible candidates would include engineering receptors to respond to viral or bacterial particles, or to rafts or other micro-domains present on the surface of a neighboring cell.

As the field of synthetic biology moves from bacterial systems into eukaryotic systems, the need to develop new motifs that are specific to eukaryotic systems is growing.<sup>18,43–45</sup> Currently, few motifs exist to translate an extracellular stimulus into an intracellular response, an essential step for engineering an artificial signaling cascade in eukaryotic cells. The intracellular phosphorylation event can then be connected to downstream events, allowing one to construct artificial signal transduction cascades, where the extracellular stimulus leads to intracellular changes, such as turning on the expression of a desired gene.

## Conclusion

The results of this study demonstrate that an artificial transmembrane receptor has the capacity to change its phosphorylation state in response to induced clustering by a polyvalent ligand. Upon receptor clustering, the cytoplasmic portion of the receptor is organized into high-density patches, which then support autocatalytic phosphorylation by Abl kinase. We have demonstrated that receptor phosphorylation is specific to this mechanism and that phosphorylation occurs locally at sites of clustering. Most importantly, the mechanism of induced oligomerization has important applications for synthetic biology, as the field moves to modifying eukaryotic systems and the resultant signaling pathways that need to be designed to operate in eukaryotes.

## Experimental

The antibodies used in this study were anti-CAIV (Abcam) and 4G10, HRP conjugated anti-phosphotyrosine (Millipore).

### Plasmid construction

Generation of the CAIV, fractalkine stalk, and transmembrane domains has been described previously.<sup>13</sup> Modification of this plasmid DNA to generate the sequence encoding the receptor (Supplementary Fig. 1a online) was generated using standard molecular biology techniques. This DNA was subcloned into the pCIneo (Promega) mammalian expression vector for transfection. For the phenylalanine mutant, the mutation was introduced using QuikChange Mutagenesis (Stratagene). For the assembly of SH2<sub>Abl</sub>-GFP, the SH2<sub>Abl</sub> gene was purchased from GeneArt and consisted of nucleotides 1873–2156 of the v-Abl oncogene (Genbank: V01541.1). This was fused N-terminal to Monster Green Fluorescent Protein (Promega), and subcloned into the pCI (Promega) mammalian expression vector for transient transfection. Please see Supplementary Fig. 1b online for the entire SH2<sub>Abl</sub>-GFP sequence.

### Cell culture

CHO-K1 cells were maintained in F-12 K medium supplemented with fetal bovine serum (10%), penicillin (100 U ml<sup>-1</sup>), and streptomycin (100 µg ml<sup>-1</sup>). CHO-K1 cells were transfected with the receptor DNA using FuGENE 6 transfection reagent (Roche), using a 6:1 FuGENE:DNA ratio. Cells positive for CAIV were sorted with FACS. For selection, cells were grown in growth medium supplemented with geneticin (0.5 mg ml<sup>-1</sup>). Successive rounds of geneticin selection and fluorescence-activated cell sorting yielded the stably transfected cell line, which was used in all the subsequent experiments.

### Bead labelling

5.24 µm diameter amine functionalized polystyrene beads (Bangs Laboratories) were treated with 100 mM 4-carboxybenzenesulfonamide, 110 mM PyBOP, and 110 mM N-methylmorpholine in dimethylformamide (DMF) for 1 h, with rotation, at room temperature. Beads were washed four times with DMF, and then washed four times with a solution of sterile phosphate buffered saline containing 0.01% Triton-X100.

## Bead treatment of cells

Receptor expressing cells were grown to confluency in 12-well plates. In all experiments, BzS coated beads were added to the cells for 1 h, followed by lysis or fixation. When cells were treated with beads in the presence of inhibitors, growth medium was exchanged for medium that contained the inhibitor at the appropriate concentration (imatinib, 10  $\mu$ M; 4-carboxybenzenesulfonamide, 1 mM); cells were kept in inhibitor containing medium for thirty minutes before the addition of beads at a concentration of 10<sup>7</sup> beads/ml.

## Immunoprecipitation and blotting

After treatment, cells were washed once with PBS containing 5 mM sodium vanadate, and then lysed in ice cold RIPA lysis buffer (25 mM Tris, 150 mM NaCl, 0.1% w/v sodium dodecyl sulfate, 1% w/v Igepal CA-630, 1% w/v sodium deoxycholate, and 2 mM EDTA) supplemented with 5 mM sodium vanadate, 2 mM phenylmethanesulfonyl fluoride, 1 mM  $\beta$ -glycerol phosphate, 1 mM sodium fluoride, and Complete Protease Inhibitor tablets (Roche). The whole cell lysate was then boiled for 5 min, before being centrifuged at 14k rpm in a tabletop centrifuge. The receptor was immunoprecipitated from lysate using protein G/Dynal beads cross-linked with CAIV specific antibodies. The beads were incubated with the lysate overnight at 4 °C, with rotation. The beads were then washed three times with PBS before the addition of Laemmli buffer, after which the purified receptor was eluted from beads by heating to 70 °C for 10 min, and the supernatant was collected for blotting and analysis. Blotting was done according to standard protocols and developed using chemiluminescent developing reagents (GE).

## Immunostaining and microscopy

Cells were fixed in a solution of PBS containing 4% formaldehyde for 4 min. They were stained with a primary antibody directed against CAIV (Abcam; 1 : 100 dilution in PBS containing 1% BSA) for 1 h. They were then treated with AlexaFluor-488 or AlexaFluor-647 conjugated anti-rabbit (Invitrogen; 1 : 200 dilution in PBS containing 1% BSA). Slides were imaged with a Hamamatsu back-thinned EM-CCD camera on an Olympus IX81 spinning disc confocal microscope using a 100X oil immersion objective. Cells were imaged using Slidebook (Intelligent Imaging Innovations, Inc.). Images were exported as 16 bit tagged image files for analysis in ImageJ (National Institute of Health).

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