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Engineering the ABA Plant Stress Pathway for Regulation of Induced Proximity

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Abstract

Chemically induced proximity (CIP) systems use small molecules and engineered proteins to control and study biological processes. However, small molecule-based systems for controlling protein abundance or activities have been limited by toxicity, instability, cost, and slow clearance of the small molecules in vivo. To address these problems, we modified proteins of the plant abscisic acid (ABA) stress response pathway to control the proximity of cellular proteins and showed that the system could be used to regulate transcription, signal transduction, and subcellular localization of proteins in response to exogenously applied ABA. We also showed that the ABA CIP system can be combined with other CIP systems to simultaneously control multiple processes. We found that, when given to mice, ABA was orally available and had a 4-hour half-life. These properties, along with its lack of toxicity and low cost, suggest that ABA may be well suited for therapeutic applications and as an experimental tool to control diverse cellular activities in vivo.

INTRODUCTION

The probability of an effective reaction between two molecules varies with the cube or third order of the distance between them. For this reason, many biological processes are controlled by regulation of the distance between interacting proteins or substrates and enzymes, among others. For example, protein interaction domains, such as Src homology 2 (SH2), plant homeodomain (PHD), and chromo- and bromodomains, mediate induced proximity in response to various modifications and thereby regulate signaling or transcriptional mechanisms. Thus, changes in molecule or domain proximity are naturally occurring and commonly used biological regulatory mechanisms (1). The ability to induce proximity and in turn modulate the production or activity of proteins in a tunable and temporal fashion is useful for both biological research and therapeutic applications.

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Author contributions: F.-S.L. designed the overall experiments; designed, made, and tested the DNA constructs in mammalian cells; performed luciferase transcription activation assays, protein localization studies, and immunofluorescent staining; designed and made the mutant and tested its phosphatase activity and dimerization capability; developed and conducted the ABA stability assay in serum and bioavailability assay in mice; and wrote the manuscript. W.Q.H. made the SOS-FKBP construct, performed SOS activation, and analyzed ERK phosphorylation; performed bioavailability assay in mice; and wrote the manuscript. G.R.C. conceived the strategy, suggested the approach, provided advice, supervised the project, and wrote the manuscript.

Competing interests: G.R.C., W.Q.H., and F.-S.L. report that there is a patent pending on the methods of inducing proximity of chimeric molecules in cells with alkenyl substituted cycloaliphatic (ASC) inducer compounds. Stanford University requires a material transfer agreement (MTA) for the DNA constructs of the ABA system.

Chemically induced proximity (CIP) is a tool for controlling and studying biological processes as diverse as receptor function, signaling, protein secretion, protein stability, transcription, chromosomal separation, apoptosis, protein splicing, and protein glycosylation (2–13). This technique has also been used for gene therapy in murine and primate models to induce the activation of therapeutically relevant genes (14, 15). CIP uses membrane-permeable, small-molecule inducers to control dimerization between proteins of interest that are fused to the inducer-binding proteins. The original CIP was FK1012, a symmetric molecule that binds two FKBP12 proteins and induces homodimerization of proteins fused to FKBP12. Additional methods use (i) rapamycin (Rap), which binds FKBP (FK506 binding protein) and Frb [FKBP-Rap binding domain of mammalian target of Rap (mTOR)], or (ii) FK506, which binds FKBP and calcineurin, or cyclosporin A, to regulate cellular processes in response to the addition of either Rap or FK506, respectively. However, these approaches have been limited by the instability, toxicity, or expense of these small molecules. In addition, all these methods involve FKBP fusion or cyclophilin fusion proteins, which complicate their use in vivo because of the existence of abundant endogenous mammalian FKBP and cyclophilin that compete for binding of the fusion proteins.

To develop a more broadly useful CIP system that may potentially circumvent currently encountered problems, we focused our attention on the plant phytohormone *S*-(+)-abscisic acid (ABA). The ABA signaling pathway mediates stress responses and developmental decisions in plants. ABA produces its effects by binding to the pyrabactin resistance (PYR)/PYR1-like (PYL)/regulatory component of ABA receptor (RCAR) family of intracellular receptors, and the resulting complexes inhibit the activity of protein phosphatase type 2Cs (PP2Cs), which leads to the activation or repression of downstream target genes (16). In its mechanism, ABA is similar to the small molecules cyclosporin A or FK506, which neutralize the phosphatase activity of calcineurin (17). ABA is inexpensive, nontoxic (18), and present at low amounts in our daily diets. Because the ABA signaling pathway does not exist in mammalian cells, there should be no competing endogenous binding proteins as in the Rap, FK506, and cyclosporin A CIP systems. The structures of ABA in complex with various members of the PYR/PYL/RCAR family and PP2Cs revealed that the interaction involves a gate-and-latch mechanism where the binding of ABA to PYR/PYL/RCAR induces a conformation change and creates an extensive binding surface for PP2C (19–21). We envisioned that this pathway could be engineered to chemically induce the proximity of any two proteins linked to fragments of members of the PYR/PYL/RCAR family and PP2C and, in turn, regulate various cellular processes. On the basis of the crystal structure of the PYL1–ABA–ABI1 (ABA insensitive 1) complex (fig. S1) (19), we hypothesized that the interacting complementary surfaces (CSs) of PYL1 (PYL_{CS}, amino acids 33 to 209) and ABI1 (ABI_{CS}, amino acids 126 to 423) (Fig. 1A) would confer ABA-induced proximity to proteins fused to these fragments.

We confirmed the induced interaction of such chimeric proteins and the ability of ABA to induce proximity-regulated gene transcription, protein subcellular localization, and signal transduction in cultured mammalian cells. We also examined the stability and bioavailability in mammalian cell culture and in mice.

RESULTS

Regulation of gene expression with the ABA-induced proximity system

To assay the ability of ABA to induce effective proximity of chimeric proteins, we tested the ability of ABA to reconstitute protein domains. Because most proteins are made of two or more domains that must function in proximity, a general strategy to regulate the activity of a protein is to separate its domains and then reconstitute the activity by inducing the proximity

of the separated domains. In this strategy, we used ABA to induce the proximity of the yeast Gal4 DNA binding domain (Gal4DBD) to the herpes simplex virus VP16 transactivation domain (VP16AD) (Fig. 1B). The Gal4DBD binds specifically to the upstream activation sequence (UAS), but does not activate transcription without a transactivation domain (22). The VP16AD, when tethered to DNA binding domains, strongly activates transcription but cannot act without proximity to a DNA binding domain. To test gene activation by ABA-induced proximity of Gal4DBD and VP16AD, we constructed an ABA-activator cassette by fusing Gal4DBD to ABI_{cs} and VP16AD to PYL_{cs} (Fig. 1B). A similar strategy has been used to identify the PP2C binding partners of PYR1 in a yeast two-hybrid screen (23). When the ABA-activator cassette was co-transfected with a UAS-luciferase reporter into murine embryonic stem (ES) cells (Fig. 1C) or NIH 3T3 and human embryonic kidney (HEK) 293T cells (fig. S2), ABA induced luciferase production by more than three orders of magnitude (Fig. 1C).

We also compared the ABA-induced domain reconstitution leading to induction of gene expression to a similar system in Chinese hamster ovary (CHO) cells controlled by Rap, which has wild-type FKBP12 and Frb replacing ABI_{cs} and PYL_{cs}, respectively, in the chimeric proteins. In contrast to the large linear range of ABA responsiveness (Fig. 1D), Rap-induced transactivation exhibited an essentially “off-on switch” over a factor of 10 concentration change (Fig. 1D). Such a linear dose response of ABA-induced transactivation has the potential to provide precise control of therapeutic protein abundance induced by ABA. The relative activation change induced by ABA was much higher than that by Rap in E14 cells (Fig. 1E) but was comparable in CHO cells (Fig. 1D and fig. S3). Activation of luciferase was first observed after 1.5 hours of ABA addition and reached a maximum within 24 hours, which was faster than that observed in the Rap system (Fig. 1F and fig. S3) and is most likely due to the lack of competition with endogenous FKBP. To evaluate the reversibility of the ABA system, we first induced cells to produce luciferase for 24 hours, and then withdrew ABA by washing cells five times with fresh medium without ABA and incubating cells at 37°C for 5 min between each wash. ABA-induced gene activation was reversed to background within 24 hours in CHO cells (Fig. 1G). On the contrary, the same washing conditions did not reverse Rap-dependent activation over the same time period. A more extensive washing or the addition of competitive binders of Rap against FKBP, such as FK506, is required to reverse Rap-dependent activation (fig. S4). The observed differences in the inducible gene activation between ABA and Rap may not simply be due to differences in fusion protein production (fig. S5) but may also result from the combination of differences in mechanisms and affinities of inducer–fusion protein recognition, as well as their association–dissociation rates. ABA-induced gene activation was observed in all cell types tested (fig. S6), although we noticed a large range of responsiveness with the Jurkat T cell line and mouse embryo fibroblasts (MEFs) exhibiting the smallest induction (factor of ~30 relative to uninduced cells) and the mouse ES cell lines (E14 and TC1) exhibiting the largest induction (factor of >1000 relative to un-induced cells). These variations may reflect the difference in transfection efficiency, therefore the amount of chimeric proteins produced, as well as the intrinsic difference between cell lines.

Controlling protein localization and signal transduction with the ABA-induced proximity system

The activities of proteins are often regulated by their subcellular localization. In another variation of domain reconstitution, we investigated whether ABA-induced proximity can be used to control the localization of a chosen protein. We constructed fusions of ABI_{cs} to specific subcellular localization domains and used ABA to dimerize these ABI_{cs} localization fusion proteins to green fluorescent protein (GFP)–fused PYL_{cs}. We induced the relocation of GFP-PYL_{cs} to four subcellular locations by fusing ABI_{cs} to (i) Numb to serve

as a cytoplasmic localization partner (24), (ii) to a nuclear localization sequence (NLS) or to Brg1 (Brm/SWI2-related gene 1) to serve as nuclear localization partners (25), (iii) to CD4 (cluster of differentiation 4) to serve as a plasma membrane localization partner (26), or (iv) to a myristoylation (myr) sequence to serve as a plasma membrane and an intracellular membrane localization partner (5).

GFP-PYL_{CS} alone showed a pan-cellular localization with or without ABA (fig. S7), whereas when cytoplasmic Numb-ABI_{CS} was also present, within 30 min ABA induced the cytoplasmic accumulation of GFP-PYL_{CS} at the expense of its nuclear localization in HEK 293T cells (Fig. 2A, left panel, and fig. S8). When Numb was replaced with either the nuclear protein Brg1 or the membrane protein CD4, GFP-PYL_{CS} relocated to the nucleus or to the membrane, respectively, upon ABA addition within 30 min (figs. S9 and S10). The nuclear or membrane localization was also achieved with the NLS-fused ABI_{CS} or the myr-fused ABI_{CS} (figs. S9 and S10).

We found that ABA-induced relocation competed with an endogenous localization mechanism. In the presence of NLS-ABI_{CS}, ABA triggered the redistribution of cytoplasmic Numb-GFP-PYL_{CS} fusion protein into the nucleus (Fig. 2A, right panel, and fig. S11). The degree of Numb-GFP-PYL_{CS} nuclear localization varied among cells, and even with a 450 μ M ABA we did not find complete relocation of all Numb-GFP-PYL_{CS} in most cells. However, many cells showed near-complete localization, as shown in fig. S11, indicating that complete localization should be achievable upon optimization.

In response to growth factor signaling, for example, the guanine nucleotide exchange factor SOS (son of sevenless) moves to the membrane where it activates the guanosine triphosphatase Ras, resulting in activation of the mitogen-activated protein kinase (MAPK) pathway and phosphorylation of extra-cellular signal-regulated kinases 1 and 2 (ERK1/2) (27). Localization of SOS depends on the binding of its SH2 domains to phosphotyrosines in the adaptor protein GRB2 (growth factor receptor-bound protein 2) or other membrane-localized proteins. We showed that ABA induced the phosphorylation of ERK in cells expressing SOS-PYL_{CS} chimeric proteins and myr-ABI_{CS} (Fig. 2B), which we assume reflected relocation of SOS-PYL_{CS} to the plasma membrane. The induction of ERK phosphorylation was not observed when SOS-FKBP fusion protein was used instead of SOS-PYL_{CS}. These studies indicate that ABA can be used to rapidly relocate proteins and thereby activate or inhibit their functions.

Combining ABA- and Rap-induced proximity systems to control independent biological processes

To assess the specificity and independence of ABA- and Rap-induced proximity and thereby their potential to construct genetic circuits with Boolean logic, we treated cells expressing VP16-PYL_{CS} and Gal4DBD-ABI_{CS}, VP16-Frb and Gal4DBD-ABI_{CS}, or VP16-Frb and Gal4DBD-FKBP transgenes, along with the UAS-luciferase reporter, with either ABA or Rap. Luciferase production was only induced by matching small-molecule and dimerizing protein pairs (Fig. 3A and fig. S12). This result illustrates the potential of using both ABA and Rap as chemical inducers to regulate the expression of two individual genes in the same cell or organism.

To explore whether ABA and Rap can be used independently to relocate different cellular proteins, we transfected 293T cells with either GFP-PYL_{CS} and myr-ABI_{CS} or GFP-Frb and myr-FKBP. The membrane localization of GFP proteins was observed only when the matching small molecule was added (fig. S13). One of the advantages of having orthogonal CIP systems is the ability to manipulate two proteins individually at the same time in the same cell. To demonstrate this application, we introduced mCherry-PYL_{CS} and Brg-ABI_{CS}

together with GFP-Frb and CD4-FKBP into 293T cells. When transfected cells were treated with ABA or Rap alone, mCherry-PYL_{CS} or GFP-Frb proteins were recruited to the nucleus or membrane, respectively (fig. S14). When both small molecules were added, each of these two pan-cellular distributed proteins was relocalized to the desired subcellular compartment within 30 min (Fig. 3B). Thus, the ABA system can be used together with the Rap system to regulate two cellular processes at the same time.

Engineering a phosphatase-free ABA-CIP system

ABA is present in many foods containing plant extracts and oils. Its lack of toxicity is supported by an extensive evaluation by the Environmental Protection Agency (EPA) (18). Although we found that the ABA-CIP system had no observable toxic effects in cultured mammalian cells, a potential problem is the phosphatase domain of ABI1, which is present in our constructs. To avoid any potential long-term toxic effects when used in animal models or in humans, we engineered an ABI_{CS} domain lacking the phosphatase activity that maintained the ABA-induced PYL_{CS} binding ability. Several amino acid residues are conserved in the PP2C domain and are reported to be involved in catalytic function, such as metal or phosphate binding (Fig. 4A) (28). Among these residues, Asp¹⁴³ does not make direct contact with PYL1 and is positioned away from the PYL-ABI binding surface (fig. S1) (19). Therefore, we predicted that a point mutation of Asp¹⁴³ to Ala (D143A) might cause the loss of the phosphatase activity with minimal disturbance to the overall ABI1 PP2C domain structure and its PYL1 binding ability. We made and purified the recombinant glutathione *S*-transferase (GST) fusion proteins of ABI_{CS} with the D143A point mutation. When tested for phosphatase activity, the mutant protein showed activity close to background (Fig. 4B and fig. S15). Using wild-type or mutant GST-ABI_{CS} to pull down GFP-PYL_{CS} from whole-cell lysates of GFP-PYL_{CS} transgene-expressing 293T cells, we found that the D143A mutant showed similar ABA-dependent PYL binding relative to the wild-type ABI (Fig. 4C and fig. S16). When we introduced the same point mutation into the ABA-activator cassette used previously and tested its ability to induce luciferase production upon ABA addition, the mutant showed induction comparable to that of the wild-type ABI_{CS} (Fig. 4D and fig. S17). These results indicate that the phosphatase activity of ABI_{CS} can be eliminated without sacrificing induction efficiency; therefore, unregulated phosphatase activity should not be an issue with the experimental or therapeutic use of this system.

Assessing ABA stability, oral availability, and toxicity

In plants, ABA had been reported to be unstable due to active enzymatic degradation (29). To examine its stability in mammalian cells, we incubated ABA with CHO cells for up to 48 hours and then assayed the functional ABA concentration by the ability of the ABA-incubated cell culture medium to induce luciferase production in cells expressing VP16-PYL_{CS} and wild-type Gal4DBD-ABI_{CS}. After 24 hours of incubation with cells, about 60% of ABA activity was retained, and after 48 hours, the activity was reduced to around 23% (Fig. 5A), indicating that ABA has a desirable half-life in mammalian cells. For potential therapeutic application in humans, we examined the stability of ABA in the serum and its bioavailability in mice (Fig. 5B). We measured the activity of residual ABA in the serum by its ability to induce the transcriptional activation of the luciferase reporter in cells expressing the ABA-activator cassette. We tested the stability of ABA in isolated serum by incubating ABA with fresh human serum or heat-inactivated fetal bovine serum (FBS) for up to 48 hours and then applying the serum as the ABA source to activate luciferase. We found that ABA was stable in serum for up to 48 hours, retaining about 64 to 77% activity (Fig. 5C and fig. S18). Using this assay, we evaluated the bio-availability in mice. When ABA was injected intraperitoneally, it entered circulation rapidly, and activity was detectable within 30 min (fig. S19). When ABA was administered orally, it had a half-life of about 4 hours (Fig. 5D).

Although ABA has been shown to be nontoxic by the EPA (18), we tested one potential toxicity, using a lymphocyte proliferation assay, and found no statistically significant effect upon proliferation induced by antigen receptor signaling (fig. S20). These studies suggest that ABA is orally bioavailable, its half-life is favorable, and it is likely to be nontoxic. Therefore, the ABA CIP system should be useful for controlling biological processes in vivo in the mouse. Human pharmacologic studies are required to determine whether these findings are generalizable.

DISCUSSION

Our studies indicate that components of the ABA signaling pathway from *Arabidopsis thaliana* can be engineered to induce proximity of intracellular proteins and thereby regulate many diverse cellular processes with exogenously applied ABA. The ABA-CIP system that we have developed seems well suited for these purposes because ABA is stable, inexpensive, and nontoxic to cultured cells and mice, and has favorable pharmacokinetics in mice.

ABA is present in our daily consumption of fruits and vegetables; for example, avocados contain 0.76 mg of ABA per kilogram of fresh weight (30, 31), and no toxic effects to humans have been associated with consumption of ABA to date. ABA has an acute oral median lethal dose (LD₅₀) of >5000 mg/kg in rat and a “no observable adverse effect level” (NOAEL) of 20,000 mg/kg per day in subchronic toxicity studies reported by the EPA (18). These amounts are much higher than the amounts of ABA used in our studies. Compared to the original dimerizers, FK1012, FK506, or Rap, ABA is harmless. FK506 is reported to be a powerful inhibitor of T lymphocyte activation (32). Rap has a number of favorable and unfavorable activities, including suppression of interleukin-2–driven lymphocyte proliferation and suppression of transplant rejection (33), and inhibition of proliferation of a number of specific tumors (34). ABA has been shown to be nontoxic by the EPA, and in agreement with that, we found that ABA did not have an adverse effect in a lymphocyte proliferation assay. The other components in our ABA-induced proximity system, the PYL1 and ABI1 fragments from the plant ABA signaling pathway, are also present in our daily diet; therefore, immune tolerance toward these protein fragments may already be established in humans. This may reduce the risks of eliciting unfavorable immune responses when introducing the ABA-inducible system into humans for therapeutic purposes. Studies with mice indicate that ABA has biologic activities that include enhancing PPAR γ (peroxisome proliferator–activated receptor γ) activity and suppressing macrophage inflammatory cytokine production. Its administration to mice has been associated with improved insulin sensitivity and glucose tolerance and amelioration of atherosclerosis in mouse models (35, 36). However, human studies are required to demonstrate its therapeutic potential and whether there are any adverse or side effects associated with its use.

Several Rap analogs (rapalogs) were developed in combination with engineered Frb (37, 38) to overcome the toxic effects of Rap due to its inhibition of mTOR and protein synthesis (39, 40). However, compared to Rap, these rapalogs did not show superior dimerization capability when assessed in transcription activation assays (7, 37). These rapalogs, although not toxic, were generally much more expensive and too unstable to be clinically useful or even useful for animal studies. For example, C20-methyl-Rap (MaRap) has a half-life of less than an hour in aqueous solution and is not bioavailable in mice (7), in contrast to ABA, which we found was stable in serum over 48 hours and was orally available in mice. Our studies suggest that in murine ES cells, the ABA system may be used to achieve a factor of >1000 change in protein activity relative to untreated cells. We also found that the ABA system was independent of the Rap-based CIP system, and thus, it may be possible to combine the ABA system with other inducible systems, such as those based on adenosine 5′-

triphosphate (ATP) analogs (41), tetracycline (42), or tamoxifen (43), to engineer genetic circuits with Boolean characteristics.

MATERIALS AND METHODS

Plasmid construction

All DNA fragments were amplified by polymerase chain reaction (PCR) from other intermediate constructs or genomic DNA or complementary DNA (cDNA) purchased from the *Arabidopsis* Biological Resource Center at Ohio State University or Open BioSystem. PCR was performed with Phusion DNA Polymerase (New England Biolabs), Expand Long Template PCR System (Roche), or In-Fusion PCR Cloning Kit (Clontech) with MJ Research Peltier Thermal Cycler (Bio-Rad). All constructs were made by inserting into or replacing parts of the actin-IRES (internal ribosomal entry site)-eGFP (enhanced GFP) vector (44), myr-FKBP (MF3E) (45), or pGEX vector (GE Healthcare) with the amplified DNA fragments. All the plasmids were sequenced to confirm the sequence and avoid cloning errors. The UAS-luciferase reporter construct was provided by A. Shalizi. Primers used for preparing constructs used in this study were as follows: ABI forward (F): 5'-CCGACAACGCGTGTGCCTTTGTATGGTTTTACTTC-3'; ABI FLAG reverse (R): 5'-CCGACAGCGGCCGCTCACTTATCGT-CATCGTCCTTGTAATCCTTCAAATCAACCACCACCACAC-3'; GFP K F: 5'-CCGACAGTCGACGCCACCATGGTGAGCAAGGGCGAGGAG-3'; GFP R: 5'-CCGACAGGCGCGCCCTTGTACAGCTCGTCCATGCC-3'; PYL F: 5'-CCGACAGGCGCGCCAACTCAAGACGAATTCACCCAAC-3'; PYL HA R: 5'-CCGACAGCGGCCGCTCAAGCGTAATCTGGAACATCG-TATGGGTAGTTCATAGCTTCAGTGATCGAAG-3'; m-Numb F: 5'-CCGA-CAGTCGACATGAACAACTACGGCAAAGCTTC-3'; m-Numb R: 5'-CCGACAACGCGTACCCCCACCAGAACCCCCACCA-GAAAGTTCTATTTCAAACGTTTTTC-3'; hCD4 F: 5'-CCGA-CAACGCGTAATGGGGCTACATGTCTTCTGA-3'; hCD4 R: 5'-CCGACAGCGGCCGCAATGGGGCTACATGTCTTCTGA-3'; SOS F: 5'-CCGACAGTCGACGCCACCATGCAGGCGCAGCAGCTG-CCCTAC-3'; SOS R: 5'-CCGACAGGCGCGCCGGAAGAATGGG-CATTCTCCAAC-3'; ABIc NLS R: 5'-CCGACAGCGGCCGCTCA-TACCTTTCTCTCTTTTTTTGGATCTACCTTTCTCTCTTTTTTG-GATCGTTCAAGGGTTTGCTCTTGAG-3'; ABI1 myr F: 5'-CCGACAGAATTCGCCACCATGGGTAGCAACAAGAGCAAGG-GAGGTGTGCCTTTGTATGGTTTTACTTC-3'; Frb F: 5'-CCGA-CAGGCGCGCTGGAATGTGGCATGAAGGCCTGGAA-3'; Frb R: 5'-CCGACAGCGGCCGCTCACTGCTTTGAGATTGTCGGAACAC-3'; VP16 F: 5'-CCGACAGAATTCGCCACCATGGGCCCTAAAAAGAAGCG-TAAAG-3'; VP16 R: 5'-CCGACAGGCGCGCCTCCACCGTACTCGTCAATT-CCAAG-3'; Gal4 F: 5'-CCGACAGAGCTCATGAAGCTACTGTCTTCTATCG-3'; Gal4 R: 5'-CCGACAACGCGTCGATACAGTCAACTGTCTTT-GAC-3'; ABI D143A F: 5'-CCGACAACGCGTATGGTGCCTTTG-TATGGTTTTACTTCGATTTGTGGAAGAAGACCTGAGATGGA-AGCTGCTGTTTCGACTATAC-3'; ABI1 G F: 5'-CCGACAGGATC-CGTGCCTTTGTATGGTTTTACTTC-3'; ABI1 G R: 5'-CCGACAG-GATCCTCACTTCAAATCAACCACCACCACAC-3'; simian virus 40 (SV40) F: 5'-CCGACAAGTAGTCTGTGGAATGTGTGTCAGTTAG-3'; SV40 R: 5'-CCGACAGAATTCGAAAATGGATATACAAGCTCC-3'; hemagglutinin (HA) R: 5'-CCGACAGGATCCTCAAGCGTAATCTGGAA-CATCGTATG-3'.

Cell culture and transfection

CHO, HEK 293T, NIH 3T3, B35, MEF, and COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% FBS (Omega Scientific), 1× glutamate (Gibco), and 1× penicillin/streptomycin (Pen/Strep; Gibco). Jurkat cells were cultured in RPMI 1640 (Gibco) with 10% FBS, 1× glutamate, and 1× Pen/Strep. E14 and TC1 were cultured in Knockout DMEM (Gibco) with 7.5% ES FBS (Applied Stem Cell), 7.5% knockout SR (Gibco), 1× Hepes (Gibco), 1× sodium pyruvate (Gibco), 1× MEM NEAA (nonessential amino acids; Gibco), 1× 2-mercaptoethanol (Gibco), 1× glutamate, 1× Pen/Strep, and leukemia inhibitory factor (LIF) on gelatin (Millipore)-coated plates. Cells (50,000 to 200,000) were plated in a well of a 24-well plate the day before transfection. An amount of 0.1 to 0.5 µg of DNA of each construct was mixed with 50× (v/w) Opti-MEM (Gibco), and then 3× (v/w) FuGENE HD (Roche) was added and thoroughly mixed. After incubation at room temperature for 20 min, the mixture was added to the cells and cultured for 24 to 48 hours before experiments were performed.

Luciferase assay

Cells in 24-well plates were lysed with 100 µl of 1× Passive Lysis Buffer (Promega) at room temperature for 10 min on a shaker (~80 rpm). Cell lysates were then spun down (16,000g) and 10 µl of supernatant was used for luciferase assay. Luciferase substrate solution [100 µl; 5 mg of D-luciferin (BD Biosciences) and 7 mg of coenzyme A (Sigma) in 33 ml of luciferase reading buffer: 20 mM tricine, 1.07 mM (MgCO₃)₄Mg(OH)₂·5H₂O, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM dithiothreitol (DTT), and 0.53 mM ATP in water] was added to the cell lysates, and signal was read with a 3-s delay and 1-s integration with a Turner BioSystem Modulus microplate reader. Obtained data were analyzed by Prism 5 (GraphPad Software).

Immunofluorescence staining

Cells grown on glass coverslips were washed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA, prepared in PBS) at room temperature for 20 min. After three 5-min washes in PBS, the cells were permeabilized with 5% normal goat serum and 0.1% Triton X-100 in PBS at room temperature for 30 min and incubated with primary antibodies (FLAG antibody M2, Agilent, cat. #200472-21) at 4°C overnight in the same buffer. The slides were subsequently washed three times with PBS (5 min each wash) and then incubated with Alexa fluorophore 594-conjugated secondary antibodies (Molecular Probes) for 1 hour at room temperature in the dark. Slides were washed three times in PBS for 5 min. Finally, the slides were mounted in Vectashield containing 4',6-diamidino-2-phenylindole (DAPI; H-1500, Vector Laboratories) and imaged (63×) with a Leica DM5000B microscope.

Fluorescence quantification

The area and integrated fluorescence intensity of the whole cell and the nucleus were measured with Fiji (an open-source image processing package based on ImageJ) for images from the cytoplasmic or nuclear localization experiments. The area and the integrated fluorescence intensity of the cytoplasm were calculated by subtracting the value of the nucleus from the whole cell. The mean fluorescence intensity of nucleus or cytoplasm was calculated by dividing the integrated fluorescence intensity with the area of each subcellular compartment. The ratio between nuclear or cytoplasmic distribution was calculated by comparing the corresponding mean fluorescence intensity. Twenty cells were measured in each experiment.

SOS activation

Six-well plates of 293T cells containing 0.5×10^6 cells per well were transfected with 3 μ g of total DNA using FuGENE HD (Roche) according to the manufacturer's recommendations. Cells were transfected with expression plasmids encoding myr-ABI, SOS-PYL, SOS-FKBP, myr-SOS, and an expression vector control, pUC19. ABA (250 μ M) was added at 3, 6, 12, and 24 hours before harvesting the cells. Cells were lysed in 1 $\times$ lysis buffer [50 mM tris-HCl (pH 7.5), 300 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 20 mM NaF, 1 mM Na_3VO_4 , 1 mM phenylmethyl-sulfonyl fluoride (PMSF), and 1 $\times$ protease inhibitor mixture (Calbiochem)] for 20 min at 4°C. After centrifugation at 14,000g for 15 min, proteins were separated on 4 to 12% bis-tris NuPAGE gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. After incubation with 10% bovine serum albumin (BSA) in PBST (PBS and 0.1% Tween 20), membranes were incubated with antibody against HA 6E2 (Cell Signaling Technologies, cat. #2367), antibody against Brg H88 (Santa Cruz Biotechnology, cat. #sc-10768), antibody against Hsp90 (BD Biosciences, cat. #610418), antibody that recognizes phosphorylated ERK1/2 (Cell Signaling Technology, cat. #4377), antibody that recognizes total ERK1/2 (Cell Signaling Technology, cat. #9107), and antibody against FLAG M2 (Agilent, cat. #200472-21) in 10% BSA-PBST overnight at 4°C. Infrared dye 800 and 680 anti-rabbit or anti-mouse secondary antibodies were incubated at room temperature for 1 hour in 10% BSA-PBST and detected with a LI-COR/Odyssey scanner.

Phosphatase activity assay

Wild-type and mutant GST-ABI_{CS} fusion proteins were produced from BL21(DE3)pLysS (Promega) and purified by Glutathione Superflow resin (Clontech) following standard procedures. Phosphatase activity of GST-ABI_{CS} was measured by ProFluor Ser/Thr PPase Assay Kit (Promega, V1260) following the manufacturer's protocol. Briefly, in a 96-well plate, GST-ABI_{CS} was supplied with or without 40 mM MgCl_2 and incubated with fluorophore-conjugated phosphorylated PP2C substrate peptides at room temperature for 30 min. Protease for dephosphorylated peptide was then added and incubated at room temperature for 90 min. Termination buffer was added and the reaction mixture was read at an excitation wavelength of 485 nm and an emission wavelength of 530 nm with a Molecular Device SpectraMax M2 plate reader.

ABA-dependent pull-down

Wild-type or mutant GST-ABI_{CS}-bound glutathione beads (50 μ l) were incubated with 400 μ g of GFP-PYL_{CS}-containing cell lysates in 200 μ l of PBS with or without 500 μ M ABA overnight at 4°C. Beads were then washed three times with 1 ml of PBS and then processed for Western blot analysis. GFP-PYL_{CS} and GST-ABI_{CS} were detected with antibodies against GFP (Invitrogen, cat. #A11122) and GST (Upstate, cat. #16-209), respectively.

Mouse gavage and serum collection

Mice were housed in the Stanford University Research Animal Facility in accordance with federal and institutional guidelines. Mice aged 6 to 8 weeks and weighing about 30 g were orally gavaged with 10 mg of ABA in EtOH/Tween 20/Cremophor (4:3:1; 100 μ l) or just vehicle with syringes. Blood was collected from the tails of mice at the indicated time after gavage and left on ice for 20 min. After blood was coagulated, the samples were spun down at 1500g for 20 min and serum was collected for immediate use or snap-frozen and stored at -80°C.

Lymphocyte proliferation assay

Naive T cells were isolated from spleens of C57BL/6J mice with MACS T cell isolation kit (Miltenyi Biotec). Isolated T cells were stimulated for 72 hours at 37°C with plate-bound monoclonal antibodies against CD3 (10 µg/ml) and CD28 (2 µg/ml) before cell count measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

The use of ABA-induced proximity for domain reconstitution, detected as induction of gene expression. **(A)** Regions of PYL_{CS} and ABI_{CS} used in our studies. **(B)** Scheme of ABA-induced luciferase activation and the design of the constructs. **(C)** Dose response of ABA-induced luciferase activation for 24 hours in TC1 ES cells. **(D)** Dose response of ABA- or Rap-induced luciferase activation for 24 hours in CHO cells. **(E)** Time course (0 to 24 hours) of luciferase activation by ABA or Rap in E14 cells. **(F)** Time course (0 to 3 hours) of luciferase activation by ABA or Rap in E14 cells. **(G)** Time course of luciferase activity upon drug withdrawal after induction for 24 hours in CHO cells. For **(C)** through **(G)**, the cells were transfected with the ABA- or Rap-activator cassette and the luciferase reporter for 24 hours before addition of ABA. For all experiments, the induction fold change was

calculated relative to the values of noninduced samples. Data are the means $\pm$ SEM ($n = 3$) from experiments of three independent transfections and induction from the same passage of cells. Independent experiments were repeated six or more times.

Fig. 2.

The use of ABA-induced proximity to control protein subcellular localization and signal transduction. **(A)** Left: cytoplasmic localization of GFP-PYL_{CS} to Numb-ABI_{CS} induced by ABA in 293T cells. Numb-ABI_{CS} was detected with an antibody that recognizes the FLAG tag. Right: nuclear localization of Numb-GFP-PYL_{CS} to ABI_{CS}-NLS induced by ABA in 293T cells. In both panels, nuclei are stained with DAPI. **(B)** ABA-induced ERK phosphorylation by SOS localization. Left panel: Cells were transfected with myr-ABI_{CS} and SOS-PYL_{CS} (lanes 1 to 5), myr-ABI_{CS} and SOS-FKBP (lanes 6 and 7), or SOS-PYL_{CS} only (lanes 8 and 9) for 24 hours, and then 250 μ M ABA was added for the indicated period. Right: quantification of ERK phosphorylation in the left panel. The fold change was calculated by a-phos ERK/a-HA (for SOS fusion proteins) and then normalized to lane 1. The results are representative of three independent experiments. a-phos ERK, an antibody that recognizes phosphorylated ERK1/2; a-HA, an antibody that recognizes the HA tag; a-FLAG, an antibody that recognizes the FLAG tag; a-Hsp90, an antibody that recognizes heat shock protein 90; a-ERK, an antibody that recognizes total ERK1/2.

Fig. 3.

Independent control of transcription and protein localization by the ABA and Rap systems. **(A)** Independent induction of luciferase by ABA and Rap for 24 hours in CHO cells. The cells were transfected with the ABA- or Rap-activator cassette and the luciferase reporter for 24 hours before addition of drug. Induction fold change was calculated relative to the values of noninduced samples. Data are the means $\pm$ SEM ($n = 3$) from experiments of three independent transfections and induction from the same passage of cells. Six independent repeats were performed. **(B)** Independent localization of mCherry-PYL_{CS} and GFP-Frb by ABA and Rap. 293T cells were transfected with all of the indicated constructs 24 hours before the addition of ABA, Rap, or both at the indicated concentrations for 2 hours.

Fig. 4. ABA-induced proximity can be engineered free of phosphatase activity. **(A)** Sequence alignment of the PP2C domain from different species. Abbreviations for the amino acids are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. **(B)** Phosphatase activity of wild-type and mutant GST-ABI_{CS}. Phosphatase activity was measured by the ProFluor Ser/Thr PPase Assay Kit (20). Data are the means $\pm$ SEM ($n = 3$) of three independent experiments. **(C)** Pull-down of GFP-PYL_{CS} from whole-cell lysates by wild-type (WT) or mutant GST-ABI_{CS}. GFP-PYL_{CS} and GST-ABI_{CS} were detected by antibodies against GFP (a-GFP) or GST (a-GST). Data shown are representative of three independent experiments. **(D)** Luciferase activation by ABA in CHO cells expressing VP16-PYL_{CS} and wild-type or mutant Gal4DBD-ABI_{CS} transgenes for 24 hours. Data are the means $\pm$ SEM from experiments of three independent transfections and induction from the same passage of cells.

Fig. 5.

ABA is stable and orally available. CHO cells were transfected with the ABA activator cassette and luciferase reporter for 24 hours before addition of ABA-containing serum. **(A)** ABA stability in cell culture. ABA (100 μ M) was incubated with CHO cells for the indicated times and then the medium was used for luciferase activation. **(B)** Bio-functional assay used to evaluate ABA concentration in serum. **(C)** ABA stability in human serum. ABA (1 mM) was incubated with fresh human serum for the indicated times and then used [10% (v/v) of culture medium] for luciferase activation. **(D)** Oral availability of ABA in mice. Serum was collected from mice at the indicated times after gavage of ABA in a mixture of ethanol, Tween 20, and Cremophor (4:3:1), and then 10% (v/v) of culture medium was added and applied to cells for luciferase activation. Induction fold change was calculated on the basis of the values of cells that were not exposed to serum. The data in (A) and (C) are the means $\pm$ SEM of triplicates from representative experiments; the data in (D) are the means $\pm$ SEM ($n = 6$ to 8).