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lacked endogenous synapsin I-immunopositive presynaptic terminals (fig. S7). Further, HA-Cbln1-coated beads induced clustering of GluD2 and GluD2^{ext}-GluK2, but not GluD2^{ΔNTD}, GluK2, or GluK2^{ext}-GluD2, in HEK293 cells (fig. S8).

The C terminus of GluD2 interacts directly with several intracellular molecules, such as shank-2 (15) and PSD-93/95 (16); many of these serve as scaffolds for other postsynaptic molecules, including homer-3, transmembrane AMPA receptor regulatory protein (TARP), and AMPA glutamate receptors (GluAs). Thus, we examined if the clustering of GluD2 induced by the Cbln1-coated beads might accumulate with other postsynaptic molecules in Purkinje cells. With GluD2, shank-2, homer-3, and GluA2 clustered in Purkinje cells around the beads coated with HA-Cbln1 (Fig. 4B). In contrast, HA-Cbln1-coated beads did not accumulate gephyrin [an anchoring protein for the γ -aminobutyric acid (GABA) receptor] (Fig. 4B) or excitatory amino acid transporter 4 (EAAT4, a neuronal glutamate transporter) (fig. S9) in Purkinje cells. HA-Cbln1-coated beads did not induce clustering of shank-2 or GluA2 in *cbln1/GluD2*-null Purkinje cells (Fig. 4C). Shank-2 and PSD-95 accumulated around HA-Cbln1-coated beads only when the responsible C-terminal domains of GluD2 were intact (fig. S10).

To further identify a role for Cbln1 as a postsynaptic organizer in vivo, we examined if the distribution of GluD2 was affected in *cbln1*-null Purkinje cells using the SDS-digested freeze-fracture replica labeling (SDS-FRL) method, which has a nearly one-to-one detection sensitivity for each iGluR on the surface of the postsynaptic membrane specialization (17). To exclude a possible effect of the presence of non-innervated spines in the *cbln1*-null cerebellum (Fig. 2C), we counted the number of immunoparticles detected by GluD2-specific antibody (fig. S11) in intact synapses, which were accompanied by the presynaptic protoplasmic face. The number of GluD2 immunoparticles located on postsynaptic membranes was significantly reduced in *cbln1*-null Purkinje cells (Fig. 4D, $P < 0.001$), which indicated that Cbln1 serves as a postsynaptic organizer in vivo and contributes to the clustering of postsynaptic GluD2.

We have demonstrated that Cbln1 is a ligand for the orphan receptor GluD2. Among known synapse-organizing molecules, such as neuroigin-neurexin (18), SynCAM-SynCAM (19), EphrinB-EphB (20), fibroblast growth factor (FGF) 22-FGF receptor 2b (21), Narp-GluAs (22), and netrin-G ligand-3 and leukocyte common antigen-related (NGL-3-LAR) (23), Cbln1-GluD2 signaling is unique in that without each component, synapse formation was severely abrogated in the cerebellum in vivo as well as in heterologous cells in vitro. Its bidirectional mode of action is also unique; at synaptic junctions, presynaptically derived Cbln1 accumulates and directly induces presynaptic differentiation, possibly by interacting with unidentified proteins on the pre-

synaptic membrane (Fig. 4E). Because beads coated with HA-Cbln1 induced accumulation of functional presynaptic terminals (Fig. 3), GluD2 may simply serve as a scaffold to accumulate and stabilize Cbln1 at synaptic junctions. Conversely, Cbln1 probably serves as a postsynaptic organizer by clustering GluD2, which may regulate synaptic plasticity via its interacting intracellular proteins (24).

Cbln1 is also expressed in various brain regions where GluD2 is not expressed, such as the olfactory bulb, the entorhinal cortex, and certain thalamic nuclei (25), which indicates that Cbln1 may bind to other receptors in these regions. An alternative candidate receptor is GluD1, which is highly expressed in these brain regions, especially during development (26). Indeed, HA-Cbln1 could bind to HEK293 cells that expressed GluD1 (fig. S12A) or beads coated with GluD1^{NTD}-Fc (fig. S12B). Furthermore, other Cbln family proteins (Cbln2 and Cbln4) are expressed in various brain regions (25). Therefore, further studies are warranted to elucidate the synaptic roles of Cbln and GluD family proteins in normal and pathological conditions in the CNS.

References and Notes

1. N. Kashiwabuchi *et al.*, *Cell* **81**, 245 (1995).
2. W. Kakegawa *et al.*, *J. Neurosci.* **29**, 5738 (2009).
3. T. Takeuchi *et al.*, *J. Neurosci.* **25**, 2146 (2005).
4. M. Yuzaki, *Cell. Mol. Life Sci.* **65**, 1698 (2008).
5. H. Hirai *et al.*, *Nat. Neurosci.* **8**, 1534 (2005).
6. M. Yuzaki, *Neuroscience* **162**, 633 (2009).
7. P. Wei, Y. Rong, L. Li, D. Bao, J. I. Morgan, *Mol. Cell. Neurosci.* **41**, 258 (2009).
8. Materials and methods are available as supporting material on Science Online.

9. D. Bao, Z. Pang, J. I. Morgan, *J. Neurochem.* **95**, 618 (2005).
10. A. Ito-Ishida *et al.*, *J. Neurosci.* **28**, 5920 (2008).
11. E. Miura, K. Matsuda, J. I. Morgan, M. Yuzaki, M. Watanabe, *Eur. J. Neurosci.* **29**, 693 (2009).
12. T. Uemura, M. Mishina, *Biochem. Biophys. Res. Commun.* **377**, 1315 (2008).
13. T. Kuroyanagi, M. Yokoyama, T. Hirano, *Proc. Natl. Acad. Sci. U.S.A.* **106**, 4912 (2009).
14. T. Iijima, K. Emi, M. Yuzaki, *J. Neurosci.* **29**, 5425 (2009).
15. T. Uemura, H. Mori, M. Mishina, *Mol. Cell. Neurosci.* **26**, 330 (2004).
16. K. W. Roche *et al.*, *J. Neurosci.* **19**, 3926 (1999).
17. M. Masugi-Tokita *et al.*, *J. Neurosci.* **27**, 2135 (2007).
18. B. Cih, H. Engelman, P. Scheiffele, *Science* **307**, 1324 (2005).
19. T. Biederer *et al.*, *Science* **297**, 1525 (2002).
20. M. S. Kayser, A. C. McClelland, E. G. Hughes, M. B. Dalva, *J. Neurosci.* **26**, 12152 (2006).
21. H. Umemori, M. W. Linhoff, D. M. Ornitz, J. R. Sanes, *Cell* **118**, 257 (2004).
22. R. O'Brien *et al.*, *J. Neurosci.* **22**, 4487 (2002).
23. J. Woo *et al.*, *Nat. Neurosci.* **12**, 428 (2009).
24. W. Kakegawa *et al.*, *J. Neurosci.* **28**, 1460 (2008).
25. E. Miura, T. Iijima, M. Yuzaki, M. Watanabe, *Eur. J. Neurosci.* **24**, 750 (2006).
26. H. Lomeli *et al.*, *FEBS Lett.* **315**, 318 (1993).
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Figs. S1 to S12
References

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Rapid Diversification of Cell Signaling Phenotypes by Modular Domain Recombination

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Cell signaling proteins are often modular, containing distinct catalytic and regulatory domains. Recombination of such biological modules has been proposed to be a major source of evolutionary innovation. We systematically analyzed the phenotypic diversity of a signaling response that results from domain recombination by using 11 proteins in the yeast mating pathway to construct a library of 66 chimeric domain recombinants. Domain recombination resulted in greater diversity in pathway response dynamics than did duplication of genes, of single domains, or of two unlinked domains. Domain recombination also led to changes in mating phenotype, including recombinants with increased mating efficiency over the wild type. Thus, novel linkages between preexisting domains may have a major role in the evolution of protein networks and novel phenotypic behaviors.

Domains are the basic functional and structural modules in proteins (1). In signaling networks, domains generally encode one of two major functions: (i) regulation or localization and (ii) catalysis. Catalytic domains directly transmit signaling information (e.g., through phosphorylation), whereas regu-

latory domains mediate interactions that either target or regulate this catalytic activity. The vast number of domain combinations found in the proteome suggests that domain shuffling could be a major source of evolutionary innovation in signaling behaviors (2–4). Three principal lines of evidence support this view. First, specific

changes in protein functions have been associated with domain recombination (5). Second, mutations leading to the fusion of protein-coding genes may lead to the improper activation of signaling networks that result in oncogenic transformations (6, 7). Third, fusions of diverse regulatory and catalytic domains can yield synthetic proteins with non-natural input/output relationships, both in vitro (8) and in vivo (9–11).

To investigate whether recombination of signaling protein domains provides a route for evolutionary innovation, analogous to the swapping of cis-regulatory elements and coding sequences in transcriptional circuits (12–14), we have systematically determined the effects of domain recombination on the behavior of a well-understood signaling network, the yeast mating pathway (Fig. 1A), and compared it to the effects brought about by gene or domain duplication. We used

the domains of 11 proteins belonging to the mating pathway to construct a library of 66 recombinant proteins (Fig. 1B). Specifically, all native proteins composed of at least two domains were split in a manner that separated regulatory and catalytic domains. The split points were chosen to ensure that domains were left intact and therefore are located within interdomain connecting regions. We then created a library of chimeric proteins that includes all possible recombinations of N-terminal and C-terminal blocks to systematically map the resulting phenotypic effects (Fig. 1C and fig. S1). Each protein was transformed into a yeast strain that retained the endogenous copies of the 11 mating pathway genes, such that an additional protein (with altered domain combination) was added to the existing network. To distinguish the effects of domain recombination from those of gene or domain duplication, we created three additional sets of strains (Fig. 1D): In the first one, each of the 11 genes analyzed was duplicated; in the second one, each of the N- or C-terminal blocks was duplicated; and in the third one, each possible pair of N- and C-terminal blocks were duplicated and coexpressed (all 66 combinations lacking domain recombination). To prevent any

bias that might be related to differential transcriptional control, we expressed all constructs at low abundance using a 250 base pair segment of the constitutive *cycl* promoter.

As a metric for how each additional protein altered signaling behavior, we measured the dynamics of mating pathway activation by flow cytometry. A green fluorescent protein (GFP) reporter was controlled by a mating-responsive promoter from the *fus1* gene (15) in an *a*-type, Δ *far1* strain [to prevent cell cycle arrest and the formation of mating projections that could affect flow cytometry measurements (16)]. We measured the intensity of GFP fluorescence before and after activation of the mating pathway with α -factor and used those values to calculate the baseline and slope of activation (Fig. 2A). The normalized baseline and slope values for each variant in our libraries (relative to wild type) were plotted on a “morphospace” diagram. Gene and domain duplications had little effect on the dynamics of pathway activation (Fig. 2, B and C). Only three domain duplication variants showed changes, slightly inhibiting pathway activation (variants with lower slopes in Fig. 2C), perhaps by acting as dominant negative fragments. In contrast, recombination of domains resulted in

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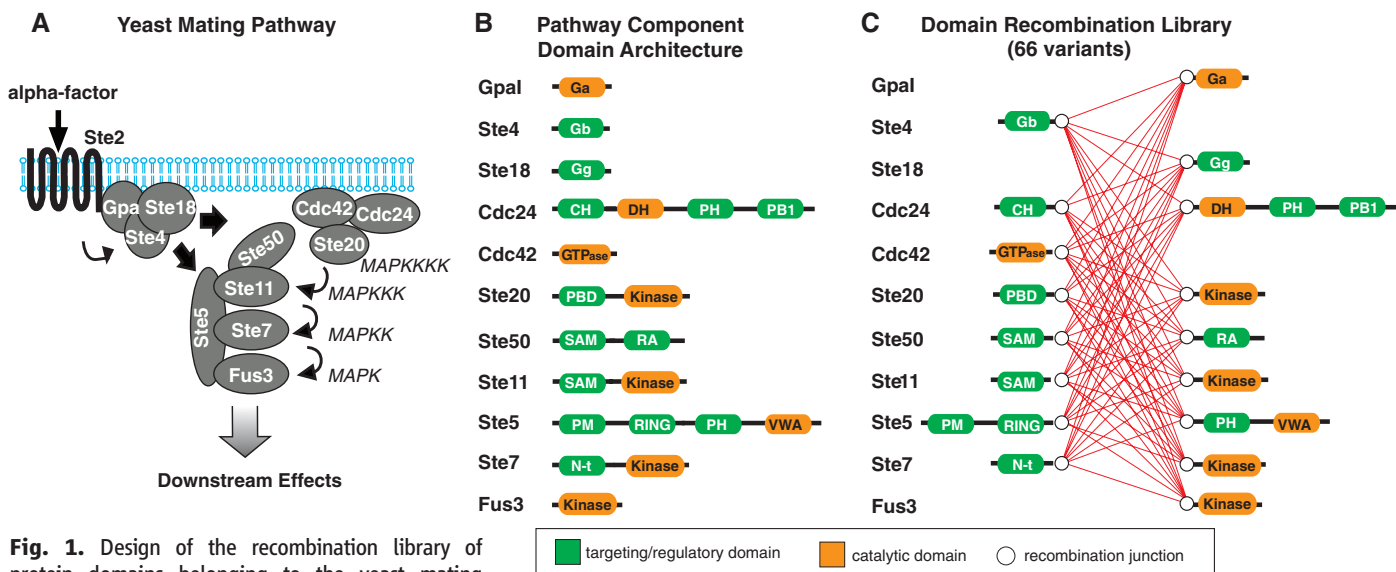


Fig. 1. Design of the recombination library of protein domains belonging to the yeast mating pathway. (A) The yeast mating pathway is activated by binding of the mating pheromone (α -factor) to the membrane receptor Ste2 in “a” cells (or α -factor to Ste3 in “ α ” cells), which causes the dissociation of the G protein alpha subunit (Gpal) from the G beta (Ste4) and gamma (Ste18) complex (20, 25). The scaffold protein Ste5 is then recruited to the membrane-localized Ste4, bringing along the MAPKKK Ste11, MAPKK Ste7, and MAPK Fus3. In addition, Ste11 interacts with the bridging protein Ste50, which by binding to the small guanosine triphosphatase (GTPase) Cdc42, positions Ste11 near its upstream activator, the PAK kinase Ste20 (26). Activated Ste11 phosphorylates Ste7, which in turn phosphorylates Fus3. The activated MAPK translocates to the nucleus, where it phosphorylates a number of transcription factors, leading to changes in gene transcription, cell cycle progression, and cell morphology and culminating in the fusion between “a” and “ α ” cells. (B) Domain

architecture of the yeast mating signaling pathway components. Regulatory domains are shown in green; catalytic domains are shown in orange. Fully annotated domain maps are given in fig. S8. (C) Domain recombination library. Recombination junctions are depicted as white circles; all possible recombinations are shown as red connecting lines. (D) Possible evolutionary events analyzed in this work. Gene duplication, domain duplication, domain recombination, and coexpression of two duplicated domains.

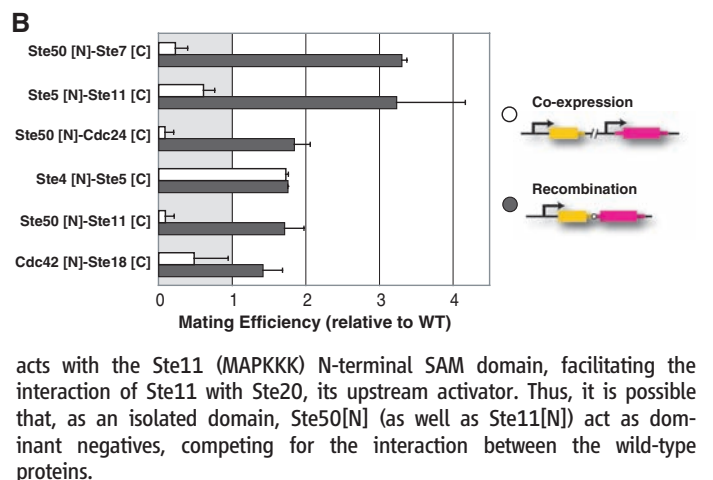
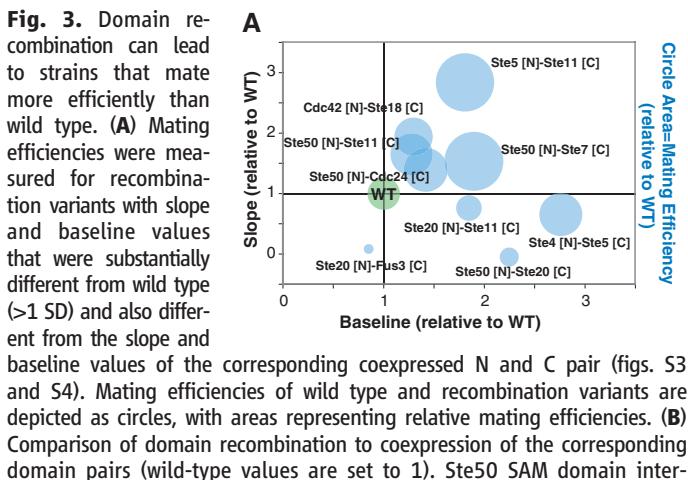
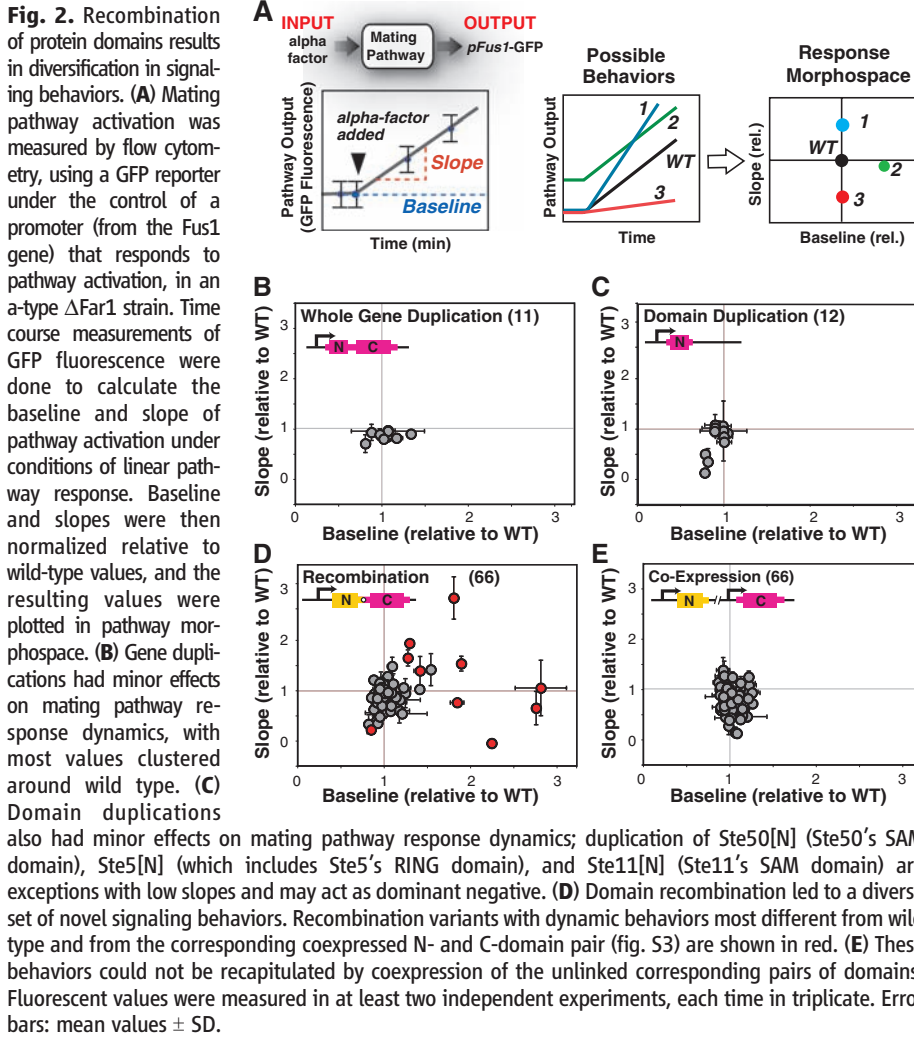
a wide range of altered dynamic behaviors, with variants that either prevented pathway activation or led to stronger activation of the mating pathway (Fig. 2D). These altered signaling behaviors appear to depend on domain recombination, because coexpression of all analogous pairs of unlinked N- and C-terminal domain blocks had limited effects on pathway activation (Fig. 2E). At least for the genes and signaling pathway

analyzed here, gene or domain duplication alone may contribute little to the immediate diversification of signaling phenotypes; changes in pathway behaviors probably require sequence divergence of the duplicates [e.g., by neofunctionalization or by differential transcriptional regulation of subfunctionalized duplicates (17, 18); see fig. S2]. In contrast, shuffling of domains provides a more direct path to functional divergence

(19), resulting in readily available alterations in signaling behaviors.

Beyond changes in gene expression, activation of the mating pathway leads to a coordinated response that arrests cell cycle, alters cell morphology, and ultimately results in the fusion of mating partners (20). To determine whether changes in reporter gene expression dynamics caused by domain recombination were mirrored by changes in overall pathway outcome, we measured the efficiency with which “a” strains, expressing domain recombination variants, mated with wild-type “α” cells. We focused on the 10 recombination variants with dynamic behaviors most different from wild type and from the corresponding coexpressed N- and C-domain pair (figs. S3 and S4) and measured the percentage of “a” cells that successfully mated when cocultured with “α” cells (21). Yeast strains expressing domain recombination variants with slopes of pathway activation greater than that of wild type mated more efficiently than did wild-type yeast (Fig. 3A and table S1). The same was true for one variant with high baseline of pathway activation but slightly lower slope (Ste4[N]-Ste5[C]). In contrast, yeast strains expressing variants with activation slopes lower than that of wild type mated more poorly. The observed changes in mating efficiency also appeared to depend on domain recombination, because there were marked differences between the mating efficiencies of corresponding recombination and coexpression variants (Fig. 3B). Thus, domain recombination can alter complex pathway outputs, such as the biochemical and morphological changes needed for mating. At least under laboratory conditions, recombination of protein domains can lead to strains that mate more efficiently than wild type, although further work is needed to determine whether the changes in mating efficiency we observed could confer a selective advantage.

Activation of the mating pathway response alters the regulation of the cell cycle (16). In addition, the mating pathway shares several proteins with other signaling pathways, such as the high osmolarity pathway. Thus, domain



recombination variants that alter the mating pathway response could also have pleiotropic effects on other cellular processes. To investigate this possibility, we measured growth rate, as well as the response to high osmolarity stimulus, for the recombination variants that most substantially affected mating response. We found that variants with growth rate deficiencies of only 2 to 3% compared to wild type (fig. S5A) mate up to ~3 times better than wild type. This suggests that, in some cases and under laboratory conditions, the cost in asexual growth likely imposed by recombination-induced network remodeling could be compensated in part by the benefit in mating efficiency it confers (fig. S5B). In addition, we observed that the response to high osmolarity is only marginally affected (fig. S5C). We cannot rule out the possibility that some of the variants analyzed might have detrimental effects on other signaling pathways or cellular processes.

Signaling responses are often characterized by their dynamics of temporal activation, as well as by the specific dose-response profile: Whereas some pathways follow a graded dose response, others have switch-like activation profiles (22). To explore whether domain recombi-

nation could also alter the dose-response profile, we measured pathway response at different concentrations of pheromone for two of the domain recombination variants that most markedly affected the mating pathway temporal response. We found that cells expressing the domain recombination variants Ste50[N]-Ste7[C] and Ste5[N]-Ste11[C] have dose-response profiles similar to that of wild-type cells, with only a small shift toward lower concentrations of pheromone for cells expressing Ste5[N]-Ste11[N] (fig. S6A). In addition, we observed a wider cell-to-cell variation in pathway response for the domain recombination variant Ste5[N]-Ste11[C] (fig. S6B). These results suggest that domain recombination might slightly alter the sensitivity of the mating pathway to pheromone levels.

We investigated the mechanisms by which recombination variants might alter the dynamics of the response. We first measured protein abundance for some duplication or recombination variants and found that there is no clear correlation between changes in mating response and protein abundance (fig. S7). Mating pathway signaling requires interactions between three major functional complexes: the membrane-bound G protein complex, the mitogen-activated protein

kinase (MAPK) complex, and the membrane-bound polarity complex (Fig. 1). Recruitment of the MAPK complex to the membrane, by its interaction with the G protein complex, positions the MAPKKK, Ste11, close to its p21-activated protein kinase (PAK) kinase activator, Ste20 (also referred to as the MAPKKKK), a member of the polarity complex (20, 23).

Close examination of the 10 recombination variants that most markedly changed signaling behavior revealed that 7 of the 10 created novel links between the different signaling complexes, whereas only three created linkages within an individual functional complex (Fig. 4A). Thus, new behaviors may arise when key components change in their localization or complex formation. To explore this hypothesis, we examined three recombinant variants in greater detail. The Ste20[N]-Ste11[C] fusion [which tethers the Cdc42 binding domain of Ste20 to the kinase domain of Ste11 (fig. S8)] resulted in higher baseline output (Fig. 4B). This fusion protein may result in the recruitment of Ste11 kinase domain to the polarity complex, even in the absence of α -factor stimulation, where it can be constitutively activated by Ste20 [the MAPKKKK (fig. S9A)]. This relocalization to sites of polarity was confirmed by microscopy experiments with the GFP-labeled fusion protein (Fig. 4C). The Ste5[N]-Ste11[C] fusion [which tethers the Ste4 binding domain of Ste5 to the kinase domain of Ste11 (fig. S8)] resulted in a large increase in the slope of output (Fig. 4D). This fusion may result in an additional population of Ste11 kinase domain that, because it is covalently fused to Ste5[N], is more efficiently recruited to the membrane upon α -factor stimulation, which may increase signaling (fig. S9B). Microscopy studies confirmed that this fusion protein is inducibly localized to membrane sites of polarization (Fig. 4E). The Ste50[N]-Ste20[C] fusion [which tethers Ste11 binding sterile alpha motif (SAM) domain of Ste50 to the kinase domain of Ste20 (Fig. S8)] resulted in high constitutive activation (Fig. 4F). This fusion protein may bring the Ste20 kinase domain to the MAPK complex, where it will constitutively activate Ste11 and trigger the MAPK cascade, without the need for membrane recruitment of the MAPK complex (fig. S9C). Microscopy studies confirmed that this fusion localizes to the cytoplasm both with and without α -factor stimulation (Fig. 4G). Overall, these more detailed observations are consistent with a model in which shuffling of a catalytic domain with different regulatory domains results in novel regulation or localization of the catalytic domain, leading to distinct changes in signaling behavior and cellular phenotype.

The high frequency with which the limited diversity encoded in our recombination library led to novel signaling behaviors suggests that domain recombination might have an important role in the generation of phenotypic novelty from simple genotypic changes and could likely complement the role of cis-regulatory elements

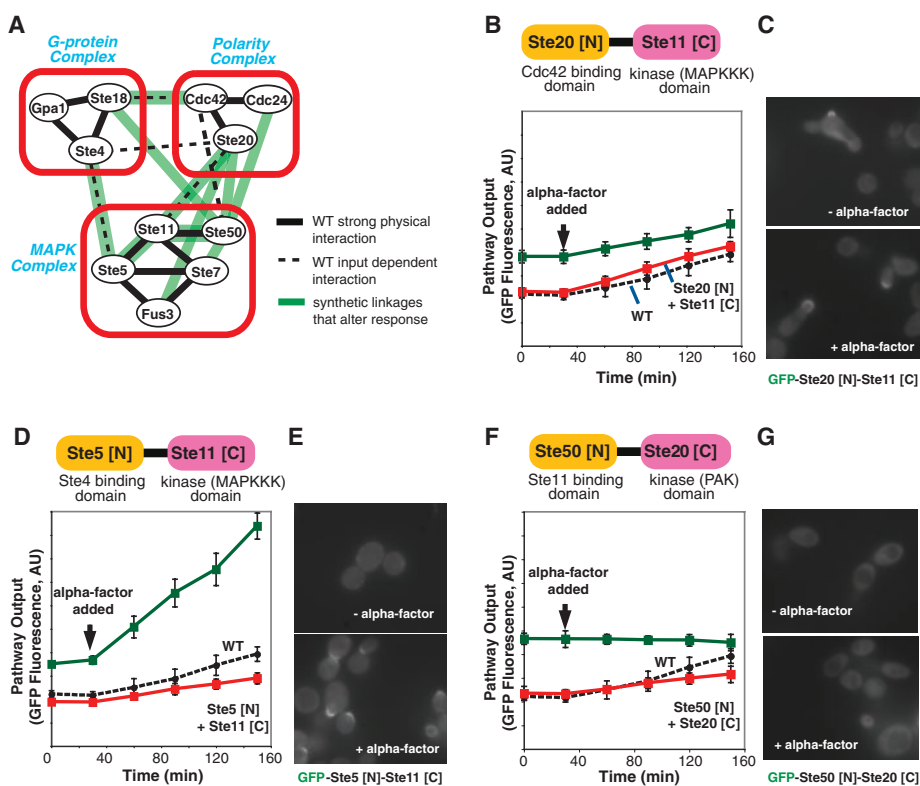


Fig. 4. Mechanisms of recombination-derived changes in signaling behavior. (A) Activation of the mating pathway requires interactions between three multiprotein complexes: the membrane-bound G protein complex, the membrane-bound polarity complex, and the MAPK complex. Seven novel connections between the three multiprotein complexes and three novel connections within an individual complex formed by the recombination variants were analyzed. (B, D, and F) Flow cytometry time course of pFus1-GFP for strains expressing Ste20[N]-Ste11[C], Ste5[N]-Ste11[C], and Ste50[N]-Ste20[C], respectively. (C, E, and G) Fluorescence microscopy of strains expressing GFP-labeled Ste20[N]-Ste11[C], Ste5[N]-Ste11[C], and Ste50[N]-Ste20[C], respectively.

in the evolution of global cellular regulatory networks composed of both transcriptional and signaling elements (24). Further work will be needed to compare in quantitative terms the contributions of gene duplication and recombination to the evolutionary process.

The strategy used here of targeted domain recombination between proteins that belong to a specific signaling network could facilitate the engineering of other protein networks of interest, a fundamental goal of synthetic biology. Genes known to belong to a target pathway could be deconstructed into domains and used to build small libraries of domain recombinations that are subsequently screened for the desired function.

References and Notes

1. C. Chothia, J. Gough, C. Vogel, S. A. Teichmann, *Science* **300**, 1701 (2003).
2. C. Vogel, M. Bashton, N. D. Kerrison, C. Chothia, S. A. Teichmann, *Curr. Opin. Struct. Biol.* **14**, 208 (2004).
3. T. Pawson, P. Nash, *Science* **300**, 445 (2003).
4. C. A. Voigt, C. Martinez, Z. G. Wang, S. L. Mayo, F. H. Arnold, *Nat. Struct. Biol.* **9**, 553 (2002).

5. M. Bashton, C. Chothia, *Structure* **15**, 85 (2007).
6. T. Pawson, N. Warner, *Oncogene* **26**, 1268 (2007).
7. C. A. Maher *et al.*, *Nature* **458**, 97 (2009).
8. J. E. Dueber, B. J. Yeh, K. Chak, W. A. Lim, *Science* **301**, 1904 (2003).
9. K. Harris *et al.*, *Curr. Biol.* **11**, 1815 (2001).
10. P. L. Howard, M. C. Chia, S. Del Rizzo, F. F. Liu, T. Pawson, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 11267 (2003).
11. B. J. Yeh, R. J. Rutigliano, A. Deb, D. Bar-Sagi, W. A. Lim, *Nature* **447**, 596 (2007).
12. M. C. King, A. C. Wilson, *Science* **188**, 107 (1975).
13. S. B. Carroll, *PLoS Biol.* **3**, e245 (2005).
14. B. Prud'homme, N. Gompel, S. B. Carroll, *Proc. Natl. Acad. Sci. U.S.A.* **104** (suppl. 1), 8605 (2007).
15. C. J. Bashor, N. C. Helman, S. Yan, W. A. Lim, *Science* **319**, 1539 (2008).
16. M. Peter, A. Gartner, J. Horecka, G. Ammerer, I. Herskowitz, *Cell* **73**, 747 (1993).
17. M. Lynch, A. Force, *Genetics* **154**, 459 (2000).
18. F. A. Kondrashov, I. B. Rogozin, Y. I. Wolf, E. V. Koonin, *Genome Biol.* **3**, RESEARCH0008 (2002).
19. R. L. Rogers, T. Bedford, D. L. Hartl, *Genetics* **181**, 313 (2009).
20. H. G. Dohlman, J. W. Thorner, *Annu. Rev. Biochem.* **70**, 703 (2001).
21. G. F. Sprague Jr., *Methods Enzymol.* **194**, 77 (1991).
22. S. Takahashi, P. M. Pryciak, *Curr. Biol.* **18**, 1184 (2008).

23. P. M. Pryciak, F. A. Huntress, *Genes Dev.* **12**, 2684 (1998).
24. J. Gerhart, M. Kirschner, *Proc. Natl. Acad. Sci. U.S.A.* **104** (suppl. 1), 8582 (2007).
25. L. Marsh, A. M. Neiman, I. Herskowitz, *Annu. Rev. Cell Biol.* **7**, 699 (1991).
26. L. Bardwell, *Peptides* **26**, 339 (2005).
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Supporting Online Material

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Materials and Methods

Figs. S1 to S9

Table S1

References

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Protein Kinase C- θ Mediates Negative Feedback on Regulatory T Cell Function

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T cell receptor (TCR)-dependent regulatory T cell (T_{reg}) activity controls effector T cell (T_{eff}) function and is inhibited by the inflammatory cytokine tumor necrosis factor- α (TNF- α). Protein kinase C- θ (PKC- θ) recruitment to the immunological synapse is required for full T_{eff} activation. In contrast, PKC- θ was sequestered away from the T_{reg} immunological synapse. Furthermore, PKC- θ blockade enhanced T_{reg} function, demonstrating PKC- θ inhibits T_{reg} -mediated suppression. Inhibition of PKC- θ protected T_{reg} from inactivation by TNF- α , restored activity of defective T_{reg} from rheumatoid arthritis patients, and enhanced protection of mice from inflammatory colitis. T_{reg} freed of PKC- θ -mediated inhibition can function in the presence of inflammatory cytokines and thus have therapeutic potential in control of inflammatory diseases.

CD4⁺ CD25⁺ regulatory T cells (T_{reg}) suppress the function of CD4⁺ and CD8⁺ effector T cells (T_{eff}) through a T cell receptor (TCR) engagement and cell contact-dependent mechanism (1–3). Inflammatory signals delivered by cytokines like tumor necrosis factor- α

(TNF- α) decrease T_{reg} activity (4, 5), perhaps as a mechanism to reduce interference by T_{reg} cells in immune responses to pathogens. In rheumatoid arthritis, T_{reg} cells circulate in normal numbers, but they have decreased activity *ex vivo* (5, 6). Besides the negative signals initiated by TNF- α , T_{reg} also receive inhibitory signals via the TCR. Akt activation by the TCR can reduce T_{reg} function and thus appears to be tightly regulated (7). This suggests TCR signaling in T_{reg} can negatively feed back to inhibit T_{reg} -mediated suppression. Moreover, TCR signaling leads to formation of the immunological synapse within seconds of T cell activation. Thus, the differences in TCR signaling in T_{reg} may emerge at the level of the immunological synapse (IS), a structured interface between T cells and antigen-presenting cells

(APCs) where TCR signalosomes are assembled (8). Whereas T_{reg} can form stable contacts with APCs with functional consequences both *in vitro* and *in vivo* (9–11), signaling events in the T_{reg} IS have not been investigated.

To study signaling in the human T_{reg} IS, we developed a model system on supported planar bilayers containing the mobile fluorescently labeled intercellular adhesion molecule-1 (ICAM-1) and antigen surrogate anti-CD3 (the signaling subunit of the TCR) antibodies and CD4⁺ CD25⁺ T_{eff} or CD4⁺ CD25⁺ T_{reg} freshly isolated from peripheral blood (fig. S1, A to C). T_{eff} and T_{reg} both formed IS, defined by a symmetric pattern consisting of a central cluster of anti-CD3 surrounded by a ring of ICAM-1 (12, 13) (Fig. 1A). T_{reg} ISs were more stable than T_{eff} ISs (fig. S2, A and B), which displayed symmetry breaking within 20 min as previously described (14). *Ex vivo* expanded, human umbilical cord blood T_{reg} (15) displayed similar behavior to adult peripheral blood T_{reg} (fig. S2, C to E). We measured recruitment of TCR proximal signaling molecules to IS by staining with phospho-Src kinase activation loop and Zap70 kinase interdomain A tyrosine 319 antibodies and imaging with total internal reflection fluorescence microscopy (TIRFM) (16). Signals were quantified on the basis of unbiased measurement of IS proximal fluorescence intensity. T_{eff} IS displayed significantly higher amounts of phospho-Src than T_{reg} (fig. S3A); however, we observed a similar intensity of phosphorylation of the downstream kinase Zap70 (fig. S3B). We next explored the protein kinase C- θ (PKC- θ) pathway, which is downstream of Src family kinases (17) and mediates IS breaking (14), because Zap70 phosphorylation appeared normal in T_{reg} .

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