When examined at the single-cell level, NF-κB-negative feedback of inhibitor of NF-κB cullations due to transcriptionally inducible neg- also called CARD11). This modification causes a con- tinent with the prevalence of phage-encoded sulfur oxidation beyond hydrothermal plumes and in the wider pelagic oceans.

To date, deep-sea SUP05 has evaded growth in laboratory cultures; thus, direct host-phage manipulations and validation of the underlying mecha- nisms of phage-influenced sulfur oxidation remain a challenge. Yet, this study demonstrates the sequence- based elucidation of microbial community dynamics through the discovery of phages that infect a widespread deep-sea bacterium. The existence of rskr genes in viral genomes reveals a mechanism for horizontal transfer of genes associated with sulfur cycling (29) and implicates viruses in the evolutionary dynamics of a central step in the plan- etary cycling of sulfur.

References and Notes
22. D. Lindell, J. D. Jaffe, Z. I. Johnson, G. M. Church, S. W. Chisholm, Science 3300001676; Guaymas, 3300001683) and the National Center for Biotechnology Information (BioProject: PRJNA234377). We declare no competing financial interests.

Positive Feedback Within a Kinase Signaling Complex Functions as a Switch Mechanism for NF-κB Activation

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A switchlike response in nuclear factor–κB (NF-κB) activity implies the existence of a threshold in the NF-κB signaling module. We show that the CARD-containing MAGUK protein 1 (CARMA1, also called CARD11)—TAK1 (MAP3K7)—inhibitor of NF-κB (IkB) kinase-β (IKKβ) module is a switch mechanism for NF-κB activation in B cell receptor (BCR) signaling. Experimental and mathematical modeling analyses showed that IKK activity is regulated by positive feedback from IKKβ to TAK1, generating a steep dose response to BCR stimulation. Mutation of the scaffolding protein CARMA1 at serine-578, an IKKβ target, abrogated not only late TAK1 activity, but also the switchlike activation of NF-κB in single cells, suggesting that phosphorylation of this residue accounts for the feedback.

The transcription factor nuclear factor-κB (NF-κB) has a central role in determining cellular outcomes (1–3). Stimulus-driven NF-κB activity is highly dynamic and shows os- cillations due to transcriptionally inducible neg-ative feedback of inhibitor of NF-κB (IκB) (3–6). When examined at the single-cell level, NF-κB activity is triggered in a switchlike manner, and the number of fully activated cells underlies a shallow population dose response (6). The switch- like response in NF-κB activity implies the exist- ence of a threshold in the receptor-proximal signaling module, but this mechanism has not been elucidated.

In B cell receptor (BCR) signaling, NF-κB activity determines multiple B cell functions (7) (Fig. 1A). BCR stimulation by cognate antigen first induces activation of protein kinase C (PKCβ), which phosphorylates serine-668 (S668) of CARD- containing MAGUK protein 1 (CARMA1, also called CARD11). This modification causes a con- formational change in CARMA1, allowing recruit- ment and activation of both the protein

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kinase TAK1 (also called MAP3K7) and the adapter protein Bcl10, the caspase-like protein MALTI (CBM complex), and the associated IKK kinase (IKK) complex (7, 8). These interactions cause IKKβ activation, allowing for IkB phosphorylation and degradation, resulting in nuclear translocation of NF-κB and increased transcription of target genes. The assembly of the CARMA1 complex is regulated via multiple mechanisms (9–12). Notably, the activated IKKβ phosphorylates CARMA1 at S578, thereby serving as a positive modifier to enhance the assembly of the CARMA1 complex, which increases IKKβ activity (13). Regulatory motifs involving positive feedback may enhance and prolong stimulus-induced activities and, in some cases, provide the basis for switchlike behavior in signaling pathways (14, 15). We used iterative quantitative experiments and mathematical modeling to show that TAK1-IKKβ engages in positive feedback through CARMA1 and that this positive feedback loop can produce switchlike activation of NF-κB.

TAK1 may be critical for induction of NF-κB activity through the regulation of IKK activity (7). Indeed, we found that upon BCR stimulation, splenic B cells obtained from mice after conditional deletion of TAK1 (TAK1-cKO) showed very little nuclear translocation of RelA and NF-κB1 (fig. S1A), IKKβ activation, phosphorylation of IkB (fig. S1, B and C), and expression of NF-κB target genes (e.g., Fas, A20, and Il10) (Fig. 1, B and C). Steep induction of cellular proliferation in response to stronger stimuli observed in wild-type cells [Hill coefficient, 1.88; median effective concentration (EC50), 1.46 × 10^-6 g/ml] was abolished in the TAK1-cKO cells (Fig. 1D). Thus, TAK1 appears to be indispensable for BCR-mediated activation of NF-κB and cell proliferation.

To further investigate the regulation of NF-κB activity in response to BCR-stimulation, we assessed TAK1 and IKKβ activities in chicken DT40 B cells. We used an ultrafine, 90-s-interval time-course analysis of TAK1 activity to resolve two maxima at 1.5 and 6 min after BCR stimulation (Fig. 2A and fig. S2A). By contrast, IKKβ activity showed an initial plateau followed by a peak at 6 min (Fig. 2B and fig. S2B). When knock-in
cells expressing catalytically inactive IKKβ (IKKβ<sup>SSAA</sup>, S176A and S181A mutated) B cells (J3) were examined, TAK1 activity was significantly reduced and the second peak was completely absent (Fig. 2A and fig. S2A). Mutation of IKKβ also decreased the interaction of TAK1 with CARMA1 (fig. S3, A and C). The lack of correspondence between time-course patterns of TAK1 and IKKβ activities, as well as the reduced TAK1 activity in IKKβ<sup>SSAA</sup> cells, hinted at a positive-feedback regulation from IKKβ to TAK1.

To rigorously analyze the observed signaling dynamics, we constructed a mathematical model depicting the activity states of TAK1 and IKKβ with phosphorylation of CARMA1 S668 (P668) as an input (Fig. 2C and fig. S4). The model includes two IKKβ activation states, IKK2 and IKK3 (potentially reflecting oligomerization or different phosphorylation states), whose formation is mediated by trans-autophosphorylation (I6), and an inactivated form (IKK4) likely mediated by further phosphorylation and subsequent breakdown of the complex containing NF-κB essential modifier (IKKγ, also called NEMO) (I7) (Fig. 2C and table S1). In the absence of IKKβ self-activation and phosphorylation processes, the model failed to show late TAK1 and IKKβ activities and termination of the signals, respectively (fig. S5). After parameterization, the model simulations showed an excellent correspondence with the experimental time course (Fig. 2, D and E, and fig. S2) and dose-response profiles (Fig. 2, F and G, and figs. S6 and S7) of TAK1 and IKKβ activity. The high Hill coefficient of IKKβ at 6 min but not at 1.5 min (1.92 and 0.34, respectively; EC<sub>50</sub>, 1.83 × 10<sup>−7</sup> g/ml) in dose-response profiles is characteristic of a positive-feedback regulation.

Analysis of the model trajectory in the phase space showed that TAK1 operates close to quasi-equilibrium and that its initial activity is mainly driven by the input signal (but still helped by IKK
activity) but becomes driven by IKKβ as the event progresses (Fig. 3A). This explains loss of the second peak of TAK1 activity and reduced initial peak in IKKβSSAA cells (Fig. 2A). The model predicted that late TAK1 activity is only possible in the presence of persistent input signal (Fig. 3A, right), which is consistent with the loss of the second peak in the presence of a PKC inhibitor after 2 min of BCR stimulation (Fig. S8). Likewise, IKK2 activation mediated by the TAK1 activity (k1ta) is the most sensitive parameter for IKKβ activation (Fig. 3B). A positive-feedback loop from IKK3 to TAK1 (k5tb3) controls the amplitude of IKKβ and late TAK1 activities (Fig. 3C). We confirmed in mouse splenic B cells that the second wave of TAK1 activity was abolished by addition of an IKKβ inhibitor (BAY11-7985) after 2 min of BCR stimulation (Fig. 3D, fig. S9, and table S2). These results suggest that the IKKβ-mediated sustained activation of TAK1 is a common mechanism for TAK1 activation. Thus, IKKβ activity is essential for the steep dose response of TAK1 activity (Fig. 3E and fig. S6).

Deletion of TAK1 severely reduced phosphorylation of S578 in CARMA1 (fig. S3, B and F), without having any effect on the PKCβ-dependent phosphorylation of S668 (fig. S10), suggesting that an upstream feedback target is unlikely. By contrast, the second peak of TAK1 activity was completely abrogated in this CARMA1S578A mutant (Fig. 3F and fig. S11). Suppressing the IKKβ-driven feedback in the mathematical model resulted in a TAK1 activity profile that recapitulated the experimental data (Fig. 3F and table S3).

We investigated the role of the Bcl10 adaptor protein in the CARMA1 complex for IKK-dependent TAK1 activation. Bcl10 positively regulates NF-κB activation in antigen receptor signaling (18); however, negative regulation of Bcl10 by IKKβ is also implicated after T cell receptor (TCR) engagement (10–12). The time course profile of TAK1 activity in Bcl10−/− cells (fig. S12) was similar to that of IKKβSSAA cells (Figs. 2A and 3G and fig. S2), suggesting that Bcl10 has a positive role in IKKβ feedback regulation in this time frame. However, the overall reduction of TAK1 activity in these mutants raised the additional possibility that both Bcl10 and IKKβ may facilitate the initial activation of TAK1. Simulations with attenuated inputs of both CARMA1-P668 and IKKβ feedback (table S4) recapitulated dose response from experimental data in WT and IKKβSSAA cells at 6 min after ligand stimulation (n = 8 to 10). (F) Time-course experiment of TAK1 activation in WT and CARMA1S578A DT40 B cells (n = 3) (left). Simulation of TAK1 time-course activities under feedback attenuated conditions (right). (G) Time-course experiment of TAK1 activation in WT and Bcl10−/− DT40 B cells (n = 8) (left). Simulation of TAK1 time-course activities under feedback and input attenuated conditions (right). (H) Experimental data for TAK1 activity in an M4 dose-response study in Bcl10−/− cells (n = 8) (left). Simulation of the dose response of TAK1 activities under feedback and input attenuated conditions (right).
the time-course dynamics of TAK1 activity in Bcl10−/− cells (Fig. 3G and fig. S2A). The model’s dose response of TAK1 activity to BCR stimulation in this hypothetical condition accurately captured the experimental observations in the Bcl10−/− (Fig. 3H and fig. S13) and IKKβS32A cells (Fig. 3E and fig. S6). In support of the simulation, phosphorylation of CARMA1 at S668 (input signal) was reduced in cells deficient in IKKβ or Bcl10 (fig. S14). Bcl10 and MALT1 binding in the CARMA1 complex was also reduced in the IKKβ-deficient cells (fig. S3, B to E). Taken together, these data indicate that Bcl10 and IKKβ mediate feedback regulation of TAK1, but may also function in initial activation of TAK1 in the CARMA1 complex, as shown in T cells (10).

To determine whether the CARMA1-TAK1-IKKβ positive-feedback loop that functions to amplify IKKβ activity in BCR signaling also functions to induce switchlike activation of NF-κB, we examined nuclear translocation of RelA in cell populations (fig. S15A). In the steep dose-dependent RelA translocation profile at 45 min, peak maxima exhibited positive cooperativity (Hill coefficient, 2.46; EC50, 1.48 × 10−8 g/ml) for wild type, whereas CARMA1S578A mutant cells exhibited a more graded response even at high antibody concentrations.

Our study provides evidence that the TAK1-IKKβ positive-feedback loop mediated by phosphorylation of CARMA1 at S578 serves as a basis for switchlike activation of NF-κB, thereby determining an activation threshold in BCR signaling. A switchlike response is not always produced by simple positive feedback. Additional conditions, such as low activity before stimulation, should also be met to produce a large difference in the kinetic rates (19). The stoichiometry of the CARMA1 scaffolding protein and the binding kinetics must also be optimal to present a switchlike effect (20, 21). By this means, various modifications of CARMA1 may allow different thresholds of NF-κB to be set and thereby shape NF-κB signaling dynamics.

References and Notes

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Supplementary Materials
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Materials and Methods
Figs. S1 to S5
Tables S1 to S4
References (22–34)
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Fig. 4. Switchlike activation of NF-κB at population and single-cell levels. (A) Dose response of IKKβ activation. IKKβ activities were measured in WT (blue) and CARMA1S578A (green) DT40 B cells (n = 5) by kinase assay at 6 min after stimulation with the indicated concentration of mAb to chicken IgM (M4). (B) Dose response of NF-κB activation. NF-κB activity was examined in WT (blue) and CARMA1S578A (green) DT40 B cells (n = 5) by RelA translocation into the nucleus at 45 min after stimulation with the indicated concentration of M4. (C) and (D) Single-cell imaging analysis of GFP-tagged RelA nuclear translocation. (C) A representative time-course response in WT (top) and CARMA1S578A (bottom) cells. Live-cell snapshots up to 60 min after M4 (10−8 g/ml) stimulation. (D) Dose response analysis of NF-κB (green) cells. Nuclear-to-cytoplasmic ratio (N/C) of fluorescence at 45 min after stimulation was calculated (50 cells per experiment).