The malectin-like receptor-like kinase LETUM1 modulates NLR protein SUMM2 activation via MEKK2 scaffolding

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The innate immune system detects pathogen-derived molecules via specialized immune receptors to prevent infections. Plant immune receptors include cell surface-resident pattern recognition receptors (PRRs), intracellular nucleotide-binding domain leucine-rich repeat proteins (NLRs). It remains enigmatic how PRR- and NLR-mediated signalling are connected. Disruption of an immune-activated MEKK1–MKK1/2–MPK4 MAPK cascade activates the NLR SUMM2 via the MAPK kinase kinase MEKK2, leading to autoimmunity. To gain insights into the mechanisms underlying SUMM2 activation, we used an RNA interference-based genetic screen for mkk1 mkk2 mpk4 null mutants in Arabidopsis (Arabidopsis thaliana) to identify a characterised malectin-like RLK named LETUM1 (LET1), as a specific regulator of mkk1 mkk2 mpk4 autoimmunity via complexing with both SUMM2 and MEKK2. MEKK2 scaffolds LET1 and SUMM2 for protein stability and association, and counter-regulates the F-box protein CPR1-mediated SUMM2 ubiquitination and degradation, thereby regulating SUMM2 accumulation and activation. Our study indicates that malectin-like RLK LET1 senses the perturbance of cellular homeostasis caused by the deficiency in immune-activated signalling and activates the SUMM2-mediated autoimmunity via MEKK2 scaffolding. The innate immune system detects pathogen-derived molecules via specialized immune receptors to prevent infections. Plant immune receptors include cell surface-resident pattern recognition receptors (PRRs) and intracellular nucleotide-binding domain leucine-rich repeat proteins (NLRs). It remains enigmatic how PRR- and NLR-mediated signalling are connected. Disruption of an immune-activated MEKK1–MKK1/2–MPK4 MAPK cascade activates the NLR SUMM2 via the MAPK kinase kinase MEKK2, leading to autoimmunity. To gain insights into the mechanisms underlying SUMM2 activation, we used an RNA interference-based genetic screen for mkk1 mkk2 mpk4 null mutants in Arabidopsis (Arabidopsis thaliana) to identify a characterised malectin-like RLK named LETUM1 (LET1), as a specific regulator of mkk1 mkk2 mpk4 autoimmunity via complexing with both SUMM2 and MEKK2. MEKK2 scaffolds LET1 and SUMM2 for protein stability and association, and counter-regulates the F-box protein CPR1-mediated SUMM2 ubiquitination and degradation, thereby regulating SUMM2 accumulation and activation. Our study indicates that malectin-like RLK LET1 senses the perturbance of cellular homeostasis caused by the deficiency in immune-activated signalling and activates the SUMM2-mediated autoimmunity via MEKK2 scaffolding.

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the robustness of the VIGS screen to uncover the components regulating plant cell death.

Here, we report the characterization of let1, which substantially reduced growth defects (Fig. 1a), cell death detected by trypan blue staining (Fig. 1b), elevated H₂O₂ accumulation by 3,3′-diaminobenzidine (DAB) staining (Fig. 1b) and constitutive expression of pathogenesis-related genes PR1 and PR2 (Fig. 1c), caused by silencing MEKK1. The let1-1 mutant did not affect cell death caused by silencing BAK1/SERK4 or BAK1-interacting RIK BIR1 (Supplementary Fig. 1a), suggesting the specific involvement of LET1 in mekk1 cell death. The let1-1 mutant is SALK_018793C, which bears a T-DNA insertion annotated in the promoter of PIRL1 (ATSG05850), encoding for a member of the plant intracellular ras-group-related leucine-rich repeat proteins (PIRLs) (Supplementary Fig. 1b,c). However, the transcripts of PIRL1 were not reduced in let1-1, probably due to the insertion in the promoter region (Supplementary Fig. 1d). In addition, another allele of T-DNA insertion mutant of PIRL1, SALK_020561C, did not suppress RNAi-MEKK1-triggered cell death (Supplementary Fig. 1e), implicating that pirl1 might not be the causal mutation in let1-1 suppressing mekk1 cell death. To identify the causal mutation in let1-1, we crossed the let1-1/mekk1 double mutant in the Col-0 background with the Ler-0 ecotype and genotyped plants with growth defects from an F₂ segregating population. All the plants with growth defects are mekk1 homozygous. Using 157 plants resembling let1-1/mekk1, which partially suppressed mekk1 cell death when grown on soil (Supplementary Fig. 2a), the mutation was mapped in a region around 1 million base pairs (bp) between markers FSH14 and T20D16 (Supplementary Fig. 2b). The whole-genome rescuencing of let1-1 revealed a 17-bp deletion in AT2G23200 in the mapped
LET1 functions genetically downstream of MEKK2 and upstream of SUMM2 in cell death control. a. The let1-1 mutant suppresses growth defects of mekk1, mkk1/2 and mpk4 mutants. Three-week-old plants grown on ½ MS medium plates with indicated genotypes are shown. Scale bars, 0.5 cm. b, c. The let1-1 mutant suppresses cell death (b) and H2O2 accumulation (c) in mekk1, mkk1/2 and mpk4. Cell death and H2O2 were stained by trypan blue and DAB respectively, with cotyledons of 2-week-old plants grown on ½ MS medium plates. Scale bars, 0.1 cm.

d. The schematic diagram of LET1 in the SUMM2-mediated cell death pathway. All the above experiments were repeated at least three times with similar results obtained.

e. The let1/mpk4mpk4 mutant cannot suppress the growth defects triggered by overexpressing the active SUMM2ac variant. The let1-1 mutant cannot suppress the growth defects triggered by overexpressing the active SUMM2ac variant. The let1-1 mutant suppresses the expression levels of PR1 and PR2 in mekk1, mkk1/2 and mpk4. The expression of PR1 and PR2 was normalized by the expression of UBQ10. P = 3.37 × 10⁻⁶ (PR1, left, columns 3 and 4), P = 3.81 × 10⁻⁶ (PR1, right, columns 1 and 2), P = 8.32 × 10⁻⁶ (PR1, right, columns 3 and 4), P = 4.21 × 10⁻⁵ (PR2, columns 3 and 4), P = 1.21 × 10⁻⁵ (PR2, columns 5 and 6) and P = 2.22 × 10⁻⁷ (PR2, columns 7 and 8). Data are shown as mean ± s.e.m. from four independent repeats. The asterisks indicate statistical significance by using two-sided two-tailed Student’s t-test (**P < 0.01). e. The let1-1 mutant suppresses growth defects triggered by overexpressing MEKK2. The 35S::MEKK2-HA transgenic plants in the WT background were grouped into four categories with representative plants showing dwarfism. The numbers are the percentages of plants displaying the indicated phenotype among a total of 251 transgenic plants analysed for 35S::MEKK2-HA/WT (n = 251). The 35S::MEKK2-HA transgenic plants in the let1-1 mutant background do not show severe growth defects and cell death (n = 56). Scale bars, 1 cm. f, MEKK2 protein expression and genotyping of LET1 in transgenic plants from e. Total proteins were subjected to immunoblotting using an anti-HA antibody (top panel), and CBB staining serves as a loading control (middle panel). PCR genotyping of LET1 with primers spanning the deletion site in let1-1 is shown (bottom panel). g. The let1-1 mutant cannot suppress the growth defects triggered by overexpressing the active SUMM2 (SUMM2ac), D478V variant. The 35S::SUMM2ac-HA transgenic plants show growth defects with a similar ratio in WT (53.3%, n = 75) and let1-1 (52.7%, n = 55) backgrounds. Scale bars, 1 cm. h. SUMM2 protein expression and genotyping of LET1 in transgenic plants from g. Assays were done similarly as in e, f. i. The schematic diagram of LET1 in the SUMM2-mediated cell death pathway. All the above experiments were repeated at least three times with similar results obtained.

Fig. 2 | LET1 functions genetically downstream of MEKK2 and upstream of SUMM2 in cell death control. a. The let1-1 mutant suppresses growth defects of mekk1, mkk1/2 and mpk4 mutants. Three-week-old plants grown on ½ MS medium plates with indicated genotypes are shown. Scale bars, 0.5 cm. b, c. The let1-1 mutant suppresses cell death (b) and H2O2 accumulation (c) in mekk1, mkk1/2 and mpk4. Cell death and H2O2 were stained by trypan blue and DAB respectively, with cotyledons of 2-week-old plants grown on ½ MS medium plates. Scale bars, 0.1 cm. d. The let1-1 mutant suppresses the elevated expression levels of PR1 and PR2 in mekk1, mkk1/2 and mpk4. The expression of PR1 and PR2 was normalized by the expression of UBQ10. P = 3.37 × 10⁻⁶ (PR1, left, columns 3 and 4), P = 3.81 × 10⁻⁶ (PR1, right, columns 1 and 2), P = 8.32 × 10⁻⁶ (PR1, right, columns 3 and 4), P = 4.21 × 10⁻⁵ (PR2, columns 3 and 4), P = 1.21 × 10⁻⁵ (PR2, columns 5 and 6) and P = 2.22 × 10⁻⁷ (PR2, columns 7 and 8). Data are shown as mean ± s.e.m. from four independent repeats. The asterisks indicate statistical significance by using two-sided two-tailed Student’s t-test (**P < 0.01). e. The let1-1 mutant suppresses growth defects triggered by overexpressing MEKK2. The 35S::MEKK2-HA transgenic plants in the WT background were grouped into four categories with representative plants showing dwarfism. The numbers are the percentages of plants displaying the indicated phenotype among a total of 251 transgenic plants analysed for 35S::MEKK2-HA/WT (n = 251). The 35S::MEKK2-HA transgenic plants in the let1-1 mutant background do not show severe growth defects and cell death (n = 56). Scale bars, 1 cm. f, MEKK2 protein expression and genotyping of LET1 in transgenic plants from e. Total proteins were subjected to immunoblotting using an anti-HA antibody (top panel), and CBB staining serves as a loading control (middle panel). PCR genotyping of LET1 with primers spanning the deletion site in let1-1 is shown (bottom panel). g. The let1-1 mutant cannot suppress the growth defects triggered by overexpressing the active SUMM2 (SUMM2ac), D478V variant. The 35S::SUMM2ac-HA transgenic plants show growth defects with a similar ratio in WT (53.3%, n = 75) and let1-1 (52.7%, n = 55) backgrounds. Scale bars, 1 cm. h. SUMM2 protein expression and genotyping of LET1 in transgenic plants from g. Assays were done similarly as in e, f. i. The schematic diagram of LET1 in the SUMM2-mediated cell death pathway. All the above experiments were repeated at least three times with similar results obtained.
is the personification of death in Roman mythology, which led us to name our mutants as lethality suppressor of mekk1 (letum or let). Two additional T-DNA insertion alleles, let1-2 and let1-3, also suppressed growth defects triggered by RNAi-MEK1 (Fig. 1e). In addition, expressing LET1, but not its kinase mutant variant bearing a mutation in a conserved ATP binding site (LET1K516E, LET1K516D), restored cell death in let1-1 (Fig. 1f,g). Thus, mutations in LET1 suppressed RNAi-MEK1 cell death and the kinase activity of LET1 is probably required for this function. Consistently, when purified from insect cells, the LET1 cytosolic domain (LET1CD) consisting of the juxtamembrane and kinase domains fused with His-tagged glutathione S-transferase (His-GST-LET1CD) displayed strong autoimmunity activity in an in vitro kinase assay (Fig. 1h).

MAPKKMKK1/2 and MAPKKPK4 function downstream of MEKK1 in SUMM2-mediated cell death control. Similar to mekk1, the mkk1/2 and mpk4 mutants are seedling lethal.5,6 To delineate the genetic position of let1 in suppressing mekk1–mkk1/2–mpk4 cell death, we generated the let1-1/mekk1, let1-1/mkk1/2 and let1-1/mpk4 mutants by genetic crosses. The let1-1 mutant suppressed the seedling lethality (Fig. 2a), cell death (Fig. 2b), elevated H2O2 accumulation (Fig. 2c) and defence gene PR1 expression (Fig. 2d) in the let1-1/mekk1, let1-1/mkk1/2 and let1-1/mpk4 mutants, suggesting that LET1 functions genetically downstream of the MEKK1–MKK1/2–MPK4 cascade. It is known that the MEKK1–MKK1/2–MPK4 cascade negatively regulates the transcript level of MEKK2/SUMM1, encoding another MAPKKK, in SUMM2-mediated autoimmunity.25,26 Overexpression of MEKK2 induced stunted growth, cell death, elevated H2O2 accumulation and PR gene expression in WT plants25,26 (Fig. 2e and Supplementary Fig. 3a,b). However, the elevated defence responses triggered by MEKK2 overexpression was largely reduced in the let1-1 mutant (Fig. 2e,f and Supplementary Fig. 3a,b), indicating that LET1 functions genetically downstream of MEKK2. Overexpression of an active form of SUMM2 (SUMM2ac), which bears an asparagine-to-valine mutation at the 478 amino acid residue in the MHD motif, triggers cell death in Nicotiana benthamiana.4 Overexpression of SUMM2ac also triggered growth defects and autoimmunity in Arabidopsis WT plants (Fig. 2g,h and Supplementary Fig. 4a,b). However, the let1-1 mutant did not interfere with cell death, H2O2 accumulation, stunted growth and defence gene activation triggered by overexpression of SUMM2ac (Fig. 2g,h and Supplementary Fig. 4a,b), indicating that LET1 functions genetically upstream or independent of SUMM2. The protein level of MEKK2 and SUMM2ac was similar in WT and let1-1 (Fig. 2f,h), indicating that LET1 might not affect MEKK2 and SUMM2 protein stability. Taken together, the above epistasis analysis demonstrates that LET1 functions genetically downstream of MEKK2 and upstream of SUMM2 in the mekk1 cell death pathway (Fig. 2i).

Since LET1 genetically functions between MEKK2 and SUMM2, we tested whether LET1 complexes with MEKK2 and/or SUMM2. A co-immunoprecipitation (Co-IP) assay with influenza hemagglutinin (HA) epitope-tagged LET1 and FLAG epitope-tagged MEKK2, SUMM2 or MPK4 indicated that LET1 associated with MEKK2 and SUMM2 but not with MPK4 in Arabidopsis protoplasts (Fig. 3a). The associations of LET1 with MEKK2 and SUMM2 were confirmed using reciprocally switched epitope tags in Arabidopsis protoplasts (Supplementary Fig. 5a,b) and in stable transgenic plants expressing LET1 under its native promoter (pLET1::LET1-FLAG) (Supplementary Fig. 5c,d). We also tested the association of LET1 with SUMM2 and MEKK2 with split-luciferase assays, in which LET1 was fused with the C-terminal luciferase (LET1-Cluc) and SUMM2 or MEKK2 was fused with the N-terminal luciferase (SUMM2-Nluc or MEKK2-Nluc). Co-expression of LET1-Cluc with SUMM2-Nluc (P = 9.00 × 10−9) or MEKK2-Nluc (P = 5.90 × 10−9) significantly increased luciferase signals compared to GFP controls (Fig. 3b and Supplementary Fig. 5e). Furthermore, Förster resonance energy transfer (FRET)–fluorescence lifetime imaging (FLIM) measurements revealed that LET1-GFP proteins were in the close proximity...
to MEKK2-mCherry but not BIR2-mCherry when co-expressed in Arabidopsis protoplasts (Fig. 3c,d). The FRET efficiency calculated on the basis of GFP fluorescence lifetime of LET1-GFP and MEKK2-mCherry (13.05 ± 1.76%) was similar to that of BAK1-GFP and BIR2-mCherry (13.46 ± 0.17%) (Supplementary Fig. 5g). MEKK2 is a modular protein with an N-terminal regulatory domain (MEKK2N) and a C-terminal kinase domain. MEKK2N directly interacted with the LET1CD in an in vitro pull-down assay with purified proteins (Fig. 3e) or in a Co-IP assay when expressed in protoplasts (Supplementary Fig. 5a,b). The interaction between MEKK2 and SUMM2 was further confirmed with split-luciferase (Supplementary Fig. 5g) and FRET–FLIM (Supplementary Fig. 6a,b) assays. The FRET efficiency of MEKK2-mCherry and SUMM2-GFP was 7.99 ± 0.19%. Together, the data reveal that LET1, MEKK2 and SUMM2 exist in a protein complex in plant cells.

Interestingly, we consistently observed that the protein levels of LET1-HA and SUMM2-HA were increased whenever co-expressed with MEKK2-GFP in N. benthamiana (Fig. 3f). Ectopic expression of MEKK2-GFP in protoplasts of N. benthamiana with MEKK2-GFP in protoplasts of transgenic plants also increased the protein levels of SUMM2-HA (Supplementary Fig. 7a). When 35S::MEKK2-HA was transformed into pSUMM2::SUMM2-FLAG or pLET1::LET1-FLAG transgenic plants, the protein levels of SUMM2 or LET1 were considerably increased in the double transgenic lines compared to the parental transgenic lines (Fig. 3g and Supplementary Fig. 7b). In addition, the SUMM2-GFP signal intensity was enhanced in the presence of MEKK2-FLAG when transiently expressed in N. benthamiana (Supplementary Fig. 7c). The data suggest that SUMM2 and LET1 may undergo protein degradation and MEKK2 probably stabilizes SUMM2 and LET1. Treatment of MG132, a proteasome-dependent protein degradation inhibitor, substantially stabilized SUMM2 and LET1 (Fig. 3h and Supplementary Fig. 7d). Notably, in the presence of MEKK2-GFP, MG132 showed a less pronounced effect on stabilizing LET1-HA (Supplementary Fig. 7d). Collectively, the data suggest that MEKK2 might regulate the protein homeostasis of SUMM2 and LET1, by protecting them from protein degradation. In line with this observation, MEKK2 promoted the association between LET1 and SUMM2 in N. benthamiana (Fig. 3i). In addition, expression of MEKK2 further aggravated cell death triggered by SUMM2ac in N. benthamiana, probably due to the MEKK2-mediated stabilization of SUMM2ac (Fig. 3j). Interestingly, the MEKK2 kinase
Fig. 4 | MEKK2 counter-regulates CPR1-mediated SUMM2 ubiquitination and degradation. a, CPR1 promotes SUMM2 protein degradation. SUMM2-HA was co-expressed with a vector or CPR1-FLAG in N. benthamiana. GFP was included as a control. Total proteins were immunoblotted by an anti-HA, anti-FLAG or anti-GFP antibody (top three panels). CBB is a loading control. b, SUMM2 associates with CPR1. SUMM2-HA was co-expressed with a vector (Ctrl) or CPR1-FLAG in WT protoplasts. Total proteins were immunoprecipitated with anti-FLAG affinity beads and then immunoblotted by an anti-HA or anti-FLAG antibody (top two panels). Immunoblots using total proteins before immunoprecipitation are shown as protein inputs (bottom two panels). c, CPR1 enhances SUMM2 ubiquitination. SUMM2-FLAG was co-expressed with HA-UBQ with or without CPR1-GFP in WT protoplasts. Total proteins were immunoprecipitated with anti-FLAG affinity beads and then immunoblotted by an anti-HA or anti-FLAG antibody (top two panels). Immunoblots using total protein before immunoprecipitation are shown as protein inputs (bottom three panels). The input for SUMM2 was adjusted to the similar level for Co-IP and immunoblot. The quantification of SUMM2 ubiquitination with and without CPR1-GFP is shown on the bottom; $P = 2.41 \times 10^{-2}$. Data are shown as mean ± s.e.m. from three independent repeats. The asterisk indicates statistical significance by using two-sided two-tailed Student’s $t$-test (* $P < 0.05$). d, The $cpr1-2$ mutant reduces SUMM2 ubiquitination. Ubiquitination assay was performed as in c with protoplasts from WT and $cpr1-2$. The input for SUMM2 in WT and $cpr1-2$ was adjusted to a similar level for Co-IP and immunoblot. The quantification of SUMM2 ubiquitination is shown on the bottom; $P = 3.36 \times 10^{-3}$. Data are shown as mean ± s.e.m. from three independent repeats. The asterisks indicate statistical significance by using two-sided two-tailed Student’s $t$-test (** $P < 0.001$). e, Overexpressing SUMM2 aggravates growth defects in $cpr1-2$. The 35S::SUMM2-HA construct was transformed into WT and $cpr1-2$. Plants were grown at 26 °C to reduce the growth defects of $cpr1-2$. All transgenic plants of 35S::SUMM2-HA/WT were phenotypically similar to WT ($n > 200$). A total 120 transgenic plants of 35S::SUMM2-HA/$cpr1-2$ were grouped into three categories (the ratio of each category is indicated) on the basis of the severity of growth defects. Pictures were taken 4 weeks after germination. Immunoblots with an anti-HA antibody show SUMM2-HA protein expression and CBB was used as a loading control (bottom). Scale bar, 1cm. f, CPR1 attenuates SUMM2–triggered cell death. SUMM2–HA was co-expressed with or without CPR1-FLAG in N. benthamiana. The GFP construct was used as a Ctrl. The images were taken 2 d after infiltration under the UV light. g, MEKK2 antagonizes CPR1-mediated SUMM2 protein degradation. MEKK2-FLAG was co-expressed with or without CPR1-FLAG in protoplasts of 35S::SUMM2-HA transgenic plants. h, Elevated SUMM2 ubiquitination in mekk2 compared to WT plants. SUMM2-FLAG was co-expressed with or without HA-UBQ in protoplasts of WT and mekk2. IP and immunoblot were performed similarly as in c. The input for SUMM2 in WT and mekk2 was adjusted to a similar level for Co-IP and immunoblot. i, A model for MEKK2 scaffolding LET1-SUMM2 complex and protecting SUMM2 from CPR1-mediated degradation in cell death control. MAMP-activated MEKK1–MKK1/2–MPK4 cascade regulates PRR-mediated immune signalling and suppresses SUMM2-mediated autoimmunity via modulating MEKK2 protein. MEKK2 scaffolds and stabilizes LET1-SUMM2 complex, which blocks SUMM2 ubiquitination and degradation mediated by SCF$^{cpr1}$ complex. All the experiments were repeated at least three times with similar results.
mutant (MEKK2\(^{26,43}\)) could also stabilize SUMM2 and LET1 (Fig. 3k) and enhance SUMM2\(^{-}\)-triggered cell death (Fig. 3l). This echoes the observations that the kinase activity of MEKK2 is not required for triggering cell death and MEKK2 may act as a structural protein, rather than a functional kinase, in the activation of SUMM2 (refs. 24,44). Taken together, the data indicate that MEKK2 probably scaffolds and stabilizes the SUMM2 and LET1 protein complex to activate autoimmunity.

The stabilization of SUMM2 by MG132 treatment prompted us to test whether any E3 ubiquitin ligase could mediate SUMM2 ubiquitination and degradation. The stability of several NLR proteins, including RPS2 and SNC1, is regulated by the SKP1–CULLIN1–F-box (SCF) complex-mediated proteasome degradation pathway20,22. The F-box protein CPR1 interacts with RPS2 and SNC1 in vivo11,24,25. We tested whether CPR1 could affect SUMM2 protein stability. When co-expressing with CPR1 in N. benthamiana, the protein level of SUMM2, but not a GFP control, was considerably reduced (Fig. 4a). Similarly, expression of CPR1 reduced the SUMM2 protein level in 35S::SUMM2-HA transgenic plants, which could be suppressed by MG132 treatment (Supplementary Fig. 8a), suggesting that CPR1 may mediate proteasome degradation of SUMM2. Importantly, CPR1 did not affect the protein level of LET1 nor MEKK2, indicating a specific regulation of CPR1 towards NLRs (Supplementary Fig. 8b). Further, CPR1 associates with SUMM2 in both Arabidopsis protoplasts (Fig. 4b) and N. benthamiana (Supplementary Fig. 8c), similar to its association with RPS2 (Supplementary Fig. 8d), in Co-IP assays. We next determined whether SUMM2 is ubiquitinated and the effect of CPR1 on its ubiquitination. We have previously established an in vivo ubiquitination assay in which HA-tagged ubiquitin (HA-UBQ) is co-expressed with FLAG-tagged target proteins in planta to detect the ubiquitinated proteins with an anti-HA immunoblot upon extensive washing to remove associated proteins and immunoprecipitation with an anti-FLAG antibody for target proteins44. As shown in Fig. 4c, in the presence of HA-UBQ, immunoprecipitated SUMM2-FLAG proteins were detected as a ladder-like smear migrating above its predicted molecular weight (~105kDa with 2×FLAG epitopes), suggesting that SUMM2 was polyubiquitinated in planta. The smear bands of the ubiquitinated SUMM2 were enhanced when co-expressing CPR1 (Fig. 4c, compare lanes 2 and 3) and reduced in the cpr1-2 mutant (Fig. 4d, compare lanes 2 and 4). The data suggest that SCF\(^{\text{CPR1}}\) ubiquitinates SUMM2, contributing to its degradation.

To determine the biological significance of CPR1-mediated ubiquitination and degradation of SUMM2 in cell death regulation, we generated 35S::SUMM2-HA transgenic plants in WT and cpr1-2 backgrounds. Notably, 35S::SUMM2-HA caused severe dwarfism and growth defects in cpr1-2 but not in WT plants (Fig. 4e). The severity of transgenic plant dwarfism was positively correlated with the SUMM2-HA protein accumulation and SUMM2 proteins in cpr1-2 were much higher than those in WT (Fig. 4e). Similarly, SUMM2\(^{-}\)-HA accumulated more and caused much more pronounced growth defects in cpr1-2 than in WT (Supplementary Fig. 9a,b). Furthermore, expression of CPR1 ameliorated SUMM2\(^{-}\)-triggered cell death (Fig. 4f) and reduced protein accumulation of SUMM2\(^{-}\) in N. benthamiana (Supplementary Fig. 9c). Taken together, our data indicate that CPR1 interacts with and ubiquitinates SUMM2 and negatively regulates the homoeostasis of SUMM2. The observation that MEKK2 stabilized SUMM2 (Fig. 3f,g) prompted us to test whether MEKK2 counter-regulates SCF\(^{\text{CPR1}}\)-mediated SUMM2 ubiquitination and turnover. Indeed, co-expression of MEKK2 blocked the CPR1-mediated SUMM2 degradation (Fig. 4g). Furthermore, the ubiquitinated SUMM2 appeared to be increased in the mekk2 mutant compared to WT (Fig. 4h). Conversely, SUMM2 ubiquitination was decreased when co-expressing MEKK2 (Supplementary Fig. 9d). Thus, the data implicate that MEKK2 suppresses SUMM2 ubiquitination, which is probably mediated by SCF\(^{\text{CPR1}}\), thereby stabilizing SUMM2.

It has been reported that Pseudomonas syringae effector HopAI1 associated with MPK4 and inhibited MAMP-induced MPK4 activation in vivo1. HopAI1 functions as a phosphothreonine lyase that targets multiple MAPKs, including MPK3 and MPK6 (ref. 45). It has been hypothesized that HopAI1-mediated inactivation of MPK4 results in the activation of SUMM2-mediated defence1. We tested whether HopAI1 affected the integrity of MEKK2–LET1–SUMM2 complex. Co-expression of HopAI1 with MEKK2, LET1 and SUMM2 in Arabidopsis protoplasts indicated that HopAI1 did not affect the association of LET1 with MEKK2 or SUMM2 (Supplementary Fig. 10a,b) but reduced the association of MEKK2 and SUMM2 (Supplementary Fig. 10c). The data suggest that HopAI1 might induce SUMM2 dissociation from MEKK2. HopAI1 also reduced the association of CPR1 and SUMM2 (Supplementary Fig. 10d), consistent with the reduced ubiquitination of SUMM2 in the presence of HopAI1 (Supplementary Fig. 10e). The data suggest that HopAI1 might induce SUMM2 dissociation from CPR1 for its abundance and activation.

Plant RLKs not only sense MAMPs but also recognize plant-derived DAMPs to regulate immunity46. Deficiency in the MAMP-activated MEKK1–MKK1/2–MPK4 cascade triggers the NLR SUMM2-mediated autoimmunity47,48, suggesting that NLRs also sense plant-derived molecules due to the disturbance of host cellular homeostasis. Here we show that a malectin-like RLK LET1 complexes with SUMM2 and is required for the NLR SUMM2 activation, linking RLKs directly to plant NLR-mediated signalling (Fig. 4i). In addition, the related malectin-like RLK, ANX1, complexes with NLR RPS2 and negatively modulates RPS2 immunity44. Furthermore, FER and ANX1/2 regulate plant MAMP-triggered immunity through association with PRRs38–40. Apparently, malectin-like RLKs regulate two-tiered plant immunity through modulation of both PRR and NLR immune receptors. Emerging evidence indicates the extracellular peptides of the RALF family act as the ligands of CrRLK1Ls\(^{48,49,50}\). RALFs or another type of ligands could be the potential ligands of LET1 in regulating SUMM2 activation.

Although the exact mechanism is not clear, the abundance of MEKK2 transcripts and proteins is positively correlated with the autoimmunity observed in the mekk1, mkk1/2 and mpk4 mutants24,48. Interestingly, MEKK2 has no detectable kinase activity and overexpression of WT MEKK2 and its kinase catalytic site mutant triggered a similar level of autoimmunity46. Thus, MEKK2 may play a structural role, rather than function as a kinase, in regulating SUMM2 accumulation. A recent study shows that MEKK2 might inhibit MPK4 activation by upstream MKKs41. We found here that MEKK2 interacts with both LET1 and SUMM2, and stabilizes LET1 and SUMM2 in a kinase activity-independent manner. The data hint that MEKK2 acts as a scaffold that stabilizes RLK LET1 and NLR SUMM2 for immune activation. The stabilization may be achieved by preventing proteasome-mediated SUMM2 degradation by SCF\(^{\text{CPR1}}\) complex. In WT plants, MEKK2 proteins remain at a basal level, resulting in a low abundance of LET1–SUMM2 complex. In the mekk1, mkk1/2 and mpk4 mutants, MEKK2 protein abundance is increased and sufficient to stabilize LET1–SUMM2 complex for autoimmune activation.

**Methods**

**Plant materials and growth conditions.** The A. thaliana ecotype Col-0 was used as WT in this study. The individual T-DNA insertion mutant lines (SALK\(_{025557}\), mekk1-1; SALK\(_{018793}\), let1-1; SALK\(_{020561}\), let1-2; SALK\(_{112949}\), let1-3; and SALK\(_{023522}\), ptrl1) and confirmed T-DNA insertion libraries (CS27941, 6,866 lines; CS27942, 3,980 lines; CS27943, 3,739 lines; CS27944, 3,263 lines; CS27945, 3,795 lines; and CS27946, 2,372 lines) were obtained from ABRC. The mutants of mekk1/2 from P. Krysan, mpk4 and sum2 (sum2-2) from Y. Zhang and cpr1-2 from J. Hua were reported previously24,47. The genotypes of the mutants

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were confirmed by PCR using the primers listed in Supplementary Table 1. The Arabidopsis and N. benthamiana plants used in this study were grown in soil (Metro Mix 366 for Arabidopsis and LPS for N. benthamiana) in a growth room at 23 °C (except where indicated), 45% relative humidity, and a photoperiod of 12-h light/12-h dark. Seedlings were grown on plates containing half-strength Murashige and Skoog medium (MS) with 0.5% sucrose, 0.8% agar and 2.5 mM MES at pH 5.7, in a growth room with the same condition as the above.

Quantification and statistical analysis. Data for quantification analyses are presented as mean ± s.e.m. or s.d. The statistical analyses were performed by using two-sided two-tailed Student’s t-test or one-way analysis of variance (ANOVA) test (*P < 0.05, **P < 0.01 and ***P < 0.001). Number of replicates is shown in the figure captions. The Microsoft Excel 2016 and Graphpad Prism 8 (Graphpad Prism software, v8.0.1) were used for statistics and bar graphs overlaid with dot plots.

Plasmid construction and generation of transgenic plants. The VIGS constructs of BAKI2ER4K, BIR1 and CLAI were reported previously.22 The fragments of MEKK1 (261 bp) were amplified from Col-0 complementary DNA by PCR using the primers containing restriction enzymes EcoRI at the 5’ end and Kpn1 at the 3’ end (except where indicated), 15% relative humidity, with a photoperiod of 12-h light/12-h dark. Seedlings were grown on plates containing half-strength Murashige and Skoog medium (MS) with 0.5% sucrose, 0.8% agar and 2.5 mM MES at pH 5.7, in a growth room with the same condition as the above.

To generate pHBT-LET1-HA, pHBT-LET1-KM was introduced into the binary vector pSB1A2::HA by BamHI and StuI digestion. The pHBT-LET1-KM mutant was crossed to the ecotype Landsberg ( Ler-0). Two pools of plants, including 20 WT-looking plants and 20 let1-1-mekk1-looking plants from the F2 population, were selected for genomic DNA isolation and mapping–PCR analysis. The genetic mapping, next-generation sequencing (NGS) and data analyses followed a previous report.44 Briefly, 22 pairs of simple sequence length polymorphism (SSLP) markers evenly distributed across five chromosomes of Arabidopsis were used to screen the length polymorphisms between two mixed DNA pools consisting of an equal amount of DNAs from 20 individual plants in each pool. The initial mapping placed LET1 on chromosome 2 and MEKK1 (AT4G08500) on chromosome 4 on the basis of the bulked segregant analysis. Additional SSLP markers from chromosome 2 with 157 let1-1-mekk1-looking plants from F2, population further mapped LET1 in a ~1 million-bp region between markers of F5H14 and T20D16. We then performed NGS of let1-1 with 100-nucleotide paired-end sequencing on an Illumina HiSeq 2000 platform at Texas AgriLife Genomics and Bioinformatics Service (TAGS). Forty-four genome coverage was obtained. Illumina reads were mapped to the TAIR10 release of the Arabidopsis genome using CLC Genomics Workbench 6.0.1 software (http://www.clcbio.com). The candidate variants between F5H14 and T20D16 were selected and a 17-bp deletion in T20D16 was identified (AT4G223200). The mutation was further confirmed with Sanger sequencing using genomic DNA.

Transient expression assay in Arabidopsis protoplasts and N. benthamiana. The indicated pHBT constructs were used for protoplast transformation following the protocol. Briefly, for Co-IP assay, 100 µl of plasmid DNA (2 µg µl⁻¹) was mixed with 1 µl of protoplasts (2 x 10⁶ cells µl⁻¹) and, for split-luciferase assays, 50 µl of plasmid DNA (2 µg µl⁻¹) was mixed with 500 µl of cells, for the PEG-mediated transfection.

For transient assays in N. benthamiana, the indicated constructs were transformed into A. tumefaciens strain GV3101 by electroporation. A single transformant was transferred into 2 ml of LB liquid medium containing 50 µg ml⁻¹ of kanamycin and 25 µg ml⁻¹ of gentamycin to incubate at 28 °C for 36 h. An Agrobacterium-mediated VIGS assay. The binary TRV vector pTRV-RNA1 and pTRV-RNA2 derivatives, pTRV-MEKK1, pTRV-CLAI and pTRV-GFP (the vector control), were transferred into A. tumefaciens strain GV3101 by electroporation. Positive transformants were selected on LB plates containing 50 µg ml⁻¹ of kanamycin and 25 µg ml⁻¹ of gentamycin by incubating at 28 °C for 36 h. An individual transformant was transferred into 2 ml of LB liquid medium containing 50 µg ml⁻¹ of kanamycin and 25 µg ml⁻¹ of gentamycin overnight at 28 °C with 100 rpm shaking. Cells were pelleted by 1,300 g centrifugation, resuspended in buffer containing 10 mM of MgCl₂, 10 mM of MES and 200 µM of acetylserotonin, adjusted to optimal density OD₆₀₀ ≈ 1.5 and incubated at 25 °C for at least 3 h. Bacterial cultures containing pTRV-RNA1 and pTRV-RNA2 derivatives were mixed at a 1:1 ratio and inoculated into the first pair of true leaves of 2-week-old soil-grown plants using a needleless syringe.

Trypan blue and DAB staining. Detached leaves were soaked in trypan blue staining solution (2.5 mg ml⁻¹ of trypan blue dissolved in lactophenol containing an equal volume of lactic acid, glycero, liquid phenol and double-distilled H₂O (ddH₂O) or DAB solution (1 mg ml⁻¹ of DAB dissolved in ddH₂O, pH 3.8) for overnight incubation. Samples were transferred into trypan blue staining solution containing lactophenol and ethanol in a ratio of 1:2 or DAB staining solution, gently agitated by the fluid–solid interface ratio of 1:1.33 and incubated at room temperature with gentle shaking until entirely destained. Samples were observed and recorded under a dissecting microscope.
lysed by vortexing and leaves were ground in the extraction buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris- HCl, pH 7.5, 2 mM NaF, 2 mM Na3VO4, 1 mM dithiobis(2-nitrobenzyl)disulfide (DTT), 0.5% Triton X-100, 10% glycerol and 1 μg protease inhibitor). After centrifugation at 12,500 g at 4°C for 15 min, 250 μl of extraction buffer were added to dissolve pellets and 20 μl of supernatant were collected for input controls and the remaining was incubated with anti-FLAG affinity beads (Sigma) at 4°C for 2 h with gentle shaking. Beads were collected and washed three times with washing buffer (20 mM Tris- HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100) and once with 50 mM Tris- HCl, pH 7.5. Proteins were eluted by 2× SDS-PAGE loading buffer and boiled at 94°C for 5 min. Immunoprecipitated and input proteins were analysed by immunoblot with indicated antibodies. For Co-IP assay with samples from transgenic plants, total proteins were extracted from 2 g of leaves of 4-week-old plants with 2 ml of IP buffer containing 1% Triton X-100. The supernatant was collected after centrifugation at 12,500 g for 10 min at 4°C. Beads were washed by 50 μl of IP buffer at 4°C for 15 min. The luciferase activity of the supernatant was measured with 0.2 mM luciferin with a luminometer (Perkin Elmer).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Original data that support the findings of this study are available from the corresponding author upon request. Source data are provided with this paper.

Received: 15 December 2019; Accepted: 21 July 2020;
Published online: 24 August 2020

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Acknowledgements
We thank the ABRC for *Arabidopsis* T-DNA insertion library and various mutant seeds.
We thank P. Krysan (University of Wisconsin, United States), Y. Zhang (University of
British Columbia, Canada) and J. Hua (Cornell University, United States) for *Arabidopsis*
seeds. We thank C. Franck and C. Zipfel for the critical reading of the manuscript and
members of the laboratories of L.S. and P.H. for discussions and comments on the
experiments. The work was supported by National Institutes of Health (NIH) grant no.
R01GM092893 and National Science Foundation (NSF) grant no. MCB-1906060 to P.H.
and NIH grant no. R01GM097247 and the Robert A. Welch Foundation grant no. A-1795
to L.S. Y.H. and D.G. were partially supported by China Scholarship Council (CSC) and
G.C.M was partially supported by INCT/CNPq Fellowship, Brazil.

Author contributions
Y.H., J.L. and L.S. conceived the project, designed experiments and analysed data.
J.L., Y.H., L.K., X.Y., B.F., D.L., B.Z., G.C.M., P.Y. and D.G. performed experiments and
analysed data. W.M.W, E.P.B.F. and E.L. analysed data and provided critical feedback.
L.S. and P.H. wrote the manuscript with inputs from all authors.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41477-020-0748-6.

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Software and code

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Data collection
Leica TCS SP8 confocal laser scanning microscope (Leica, Germany) was used for confocal imaging and data collection. Bio-Rad CFX Manager software (version 3.1) was used to collect qPCR raw data.

Data analysis
CLC Genomics Workbench 6.0.1 software (version 6.0.01) was used to map let-1-1 mutation site (http://www.clcbio.com)...
LAS X software (version 3.4.2.18368) was used to analyze confocal microscope Images and FLIM/FRET (https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/)
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | We chose at least two individual transgenic lines and more than three individual plants of each lines to observe the phenotype, gene transcription and the protein expression. For protoplast protein expression, about 2×10^5 cells were used for Co-IP and 2×10^4 cells for protein detection. Twenty protoplasts were used to determine the average value of t for each pair of proteins analyzed. Three individual N. benthamiana plants and more than 6 leaves were used for protein expression and cell death assay. There were more than 3 replications showing similar results for RT-PCR and qRT-PCR experiments. For quantification of Western band intensity, 3 replications were used in the statistical analysis showing similar pattern. Statistics were indicated in the figure legends. |
| Data exclusions | No data were excluded from analyses in the experiments. |
| Replication | All experiments were repeated at least three times and reliably produced. |
| Randomization | For all Arabidopsis transgenic plants, protoplast cells, N. benthamiana and bacterial strains, all samples were randomly selected throughout this study. |
| Blinding | The purpose of research is to observe a phenotype of gene knockout or over-expression, thus, the blinding design is not applicable to this study. |

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| Data collection | Provide details about the data collection procedure, including the instruments or devices used to record the data (e.g. pen and paper, computer, eye tracker, video or audio equipment) whether anyone was present besides the participant(s) and the researcher, and whether the researcher was blind to experimental condition and/or the study hypothesis during data collection. |
| Timing | Indicate the start and stop dates of data collection. If there is a gap between collection periods, state the dates for each sample cohort. |
| Data exclusions | If no data were excluded from the analyses, state so OR if data were excluded, provide the exact number of exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established. |
| Non-participation | State how many participants dropped out/declined participation and the reason(s) given OR provide response rate OR state that no participants dropped out/declined participation. |
| Randomization | If participants were not allocated into experimental groups, state so OR describe how participants were allocated to groups, and if allocation was not random, describe how covariates were controlled. |

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Study description | Briefly describe the study. For quantitative data include treatment factors and interactions, design structure (e.g. factorial, nested, |
Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

**n/a** Involved in the study

- [ ] Antibodies
- [xx] Eukaryotic cell lines
- [ ] Palaeontology
- [ ] Animals and other organisms
- [ ] Human research participants
- [xx] Clinical data

### Methods

**n/a** Involved in the study

- [xx] ChIP-seq
- [ ] Flow cytometry
- [ ] MRI-based neuroimaging

### Antibodies

**Antibodies used**

- Anti-HA-Peroxidase, Roche, Cat # 12013819001, 1:2000 dilution
- Anti-FLAG-Peroxidase, Sigma-Aldrich, Cat # A8592, 1:2000 dilution
- Anti-GFP, Roche, Cat # 11814460001, 1:2000 dilution
- Anti-Rabbit IgG, HRP-linked antibody, Cell Signaling, Cat # 7074S, 1:5000 dilution
- Anti-Mouse IgG, HRP-linked antibody, Cell Signaling, Cat # 7076, 1:5000 dilution
- Anti-GST-Peroxidase, Millipore Sigma, Cat # 16209, 1:2000 dilution
- Anti-MBP, Biolegend, Cat # 906901, 1:2000 dilution
- Anti-FLAG-Peroxidase, Sigma-Aldrich, Cat # A8592, 1:2000 dilution
- Anti-Mouse IgG, HRP-linked antibody, Cell Signaling, Cat # 7076, 1:5000 dilution
- Anti-GFP, Roche, Cat # 11814460001, 1:2000 dilution
## Anti-FLAG M2 Affinity gel, Sigma-Aldrich, Cat # 2220

Antibodies were validated by the manufacturers and further evaluated using the proper negative controls.

### Eukaryotic cell lines

| Policy information about | cell lines |
|--------------------------|------------|
| Cell line source(s)      | Insect sf9 cells were stocked in Pingwei Li’s lab at Texas A&M university. |
| Authentication           | Insect sf9 cells used were authenticated by the provider. |
| Mycoplasma contamination | Insect sf9 cells were tested negative for mycoplasma contamination. |
| Commonly misidentified lines (See ICLAC register) | none |

### Palaeontology

- **Specimen provenance**: Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).
- **Specimen deposition**: Indicate where the specimens have been deposited to permit free access by other researchers.
- **Dating methods**: If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.

[ ] Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

### Animals and other organisms

| Policy information about | studies involving animals, ARRIVE guidelines, recommended for reporting animal research |
|--------------------------|---------------------------------------------------------------------------------------|
| Laboratory animals      | For laboratory animals, report species, strain, sex and age OR state that the study did not involve laboratory animals. |
| Wild animals            | Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals. |
| Field-collected samples | For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field. |
| Ethics oversight        | Identify the organization(s) that approved or provided guidance on the study protocol, OR state that no ethical approval or guidance was required and explain why not. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

### Human research participants

| Policy information about | studies involving human research participants |
|--------------------------|---------------------------------------------------------------------------------------------|
| Population characteristics| Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write “See above.” |
| Recruitment              | Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results. |
| Ethics oversight         | Identify the organization(s) that approved the study protocol. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

### Clinical data

| Policy information about | clinical studies |
|--------------------------|--------------------------------------------------|
| All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions. |
| Clinical trial registration | Provide the trial registration number from ClinicalTrials.gov or an equivalent agency. |
| Study protocol            | Note where the full trial protocol can be accessed OR if not available, explain why. |
Data collection
Describe the settings and locales of data collection, noting the time periods of recruitment and data collection.

Outcomes
Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.

ChIP-seq

Data deposition
- Confirm that both raw and final processed data have been deposited in a public database such as GEO.
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links
For “Initial submission” or “Revised version” documents, provide reviewer access links. For your “Final submission” document, provide a link to the deposited data.

Files in database submission
Provide a list of all files available in the database submission.

Genome browser session
Provide a link to an anonymized genome browser session for “Initial submission” and “Revised version” documents only, to enable peer review. Write “no longer applicable” for “Final submission” documents.

Methodology

Replicates
Describe the experimental replicates, specifying number, type and replicate agreement.

Sequencing depth
Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

Antibodies
Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

Peak calling parameters
Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

Data quality
Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

Software
Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

Flow Cytometry

Plots
Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used.

Instrument
Identify the instrument used for data collection, specifying make and model number.

Software
Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details.

Cell population abundance
Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.

Gating strategy
Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between “positive” and “negative” staining cell populations are defined.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.
# Magnetic resonance imaging

## Experimental design

| Design type | Indicate task or resting state; event-related or block design. |
|-------------|---------------------------------------------------------------|
| Design specifications | Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials. |
| Behavioral performance measures | State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects). |

## Acquisition

| Imaging type(s) | Specify: functional, structural, diffusion, perfusion. |
| Field strength | Specify in Tesla |
| Sequence & imaging parameters | Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle. |
| Area of acquisition | State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined. |
| Diffusion MRI | Not used |

## Preprocessing

| Preprocessing software | Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.). |
| Normalization | If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization. |
| Normalization template | Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized. |
| Noise and artifact removal | Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration). |
| Volume censoring | Define your software and/or method and criteria for volume censoring, and state the extent of such censoring. |

## Statistical modeling & inference

| Model type and settings | Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation). |
| Effect(s) tested | Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used. |
| Specify type of analysis: | Whole brain, ROI-based, or Both |
| Statistic type for inference | Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods. |
| Correction | Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo). |

## Models & analysis

| n/a | Involved in the study |
| Functional and/or effective connectivity | Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information). |
| Graph analysis | Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.). |
| Multivariate modeling or predictive analysis | Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information). |
Multivariate modeling and predictive analysis

Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.