results demonstrate that our orthogonal **ET** and L/A mutant pairs achieve tailored high selectivity at the level of individual bromodomains.

To further demonstrate the feasibility of targeting the L/A mutation selectively, we characterized binding affinities and stoichiometries of **ET** within the context of a tandem bromodomain construct of Brd2 using ITC (Fig. 4A). In contrast to I-BET (K_d = 360 nM and expected stoichiometry of 2:1), no binding of **ET** to wild type was observed (K_d > 10 μM). The inactivity of **ET** against WT was further evidenced by its inability to induce upregulation of p21 mRNA levels, as reporter of downstream c-Myc activity (*14*), when compared with I-BET treatment in U2OS cells (fig. S10). However, **ET** exhibited K_d of 140 to 150 nM for the two single L/A mutants and 24 nM for the double mutant, with the expected 1:1 and 2:1 stoichiometries, respectively, confirming potent and selective targeting of mutant versus WT bromodomain (Fig. 4A).

To assess probe selectivity inside cells, we developed fluorescence recovery after photobleaching (FRAP) assays in U2OS cells transfected with full-length human Brd4. Control treatment with 1 μM I-BET accelerated the fluorescence recovery of the photobleached nuclear region of cells transfected with wild type (Fig. 4B, black, and fig. S11) relative to vehicle (Fig. 4B, white), indicating displacement of Brd4 from chromatin, as reported with JQ1 (*3*). As expected, exposure with 1 μM **ET** against wild type showed no significant reduction of recovery times relative to vehicle-treated cells (Fig. 4B, purple). Crucially, exposure of 1 μM **ET** against a double L(94,387)/A mutant showed recovery times comparable with the I-BET control in FRAP assays (Fig. 4B, red), and similarly fast recoveries were seen when the first domain only was mutated (Fig. 4B, blue) but not the second (Fig. 4B, green). Together, our data show that **ET** retains selectivity in cells and suggest that blockade of the first domain alone is sufficient to displace Brd4 from chromatin.

We describe a bump-and-hole approach to engineer controlled selectivity onto small-molecule modulation of BET bromodomains. We demonstrate that mutation of a conserved leucine residue within the bromodomain can be targeted by an ethyl derivative of I-BET with high potency and BET-subfamily selectivity in vitro and in cells. We also show proof of concept of applying orthogonal bromodomain:ligand pairs to dissect the role of individual bromodomains of Brd4 in chromatin binding. Future application of this approach could help identify which BET bromodomain target would be the most relevant therapeutic target in a given disease condition. To this end, recent advances in site-specific nuclease technologies for targeted genome engineering by use of clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9-based RNA-guided DNA endonucleases, among others (*15*, *16*), have opened up the possibility of systematically generating knock-in mutants in cells and living rodents (*17*). If a desired selectivity cannot be achieved at the KAc-binding site of WT bromodomains, it could be achieved instead by targeting allosteric sites or by modulating other specific protein-protein interactions of BET multiprotein complexes. Last, our approach could be extended to engineer selective chemical control within other subfamilies of the human bromodomain phylogenetic tree.

REFERENCES AND NOTES

1. A. C. Belkina, G. V. Denis, *Nat. Rev. Cancer* **12**, 465–477 (2012).
2. E. Nicodeme *et al.*, *Nature* **468**, 1119–1123 (2010).
3. P. Filippakopoulos *et al.*, *Nature* **468**, 1067–1073 (2010).
4. C. W. Chung *et al.*, *J. Med. Chem.* **54**, 3827–3838 (2011).
5. R. K. Prinjha, J. Witherington, K. Lee, *Trends Pharmacol. Sci.* **33**, 146–153 (2012).
6. GlaxoSmithKline, "A study to investigate the safety, pharmacokinetics, pharmacodynamics, and clinical activity of GSK525762 in subjects with NUT midline carcinoma (NMC)," ClinicalTrials.gov identifier NCT01587703; available at www.clinicaltrials.gov/show/NCT01587703.
7. J. E. Delmore *et al.*, *Cell* **146**, 904–917 (2011).
8. M. A. Dawson *et al.*, *Nature* **478**, 529–533 (2011).
9. J. Zuber *et al.*, *Nature* **478**, 524–528 (2011).
10. J. A. Mertz *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **108**, 16669–16674 (2011).
11. P. Filippakopoulos, S. Knapp, *Nat. Rev. Drug Discov.* **13**, 337–356 (2014).
12. K. Shah, Y. Liu, C. Deirmengian, K. M. Shokat, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3565–3570 (1997).
13. A. C. Bishop *et al.*, *Nature* **407**, 395–401 (2000).
14. F. Lamoureux *et al.*, *Nat. Commun.* **5**, 3511 (2014).
15. L. Cong *et al.*, *Science* **339**, 819–823 (2013).
16. P. Mali *et al.*, *Science* **339**, 823–826 (2013).
17. T. Gaj, C. A. Gersbach, C. F. Barbas 3rd, *Trends Biotechnol.* **31**, 397–405 (2013).

ACKNOWLEDGMENTS

We thank S. Knapp, O. Fedorov, and their team for constructs, assistance with BLI, and discussions; S. Swift for assistance with the Light Microscopy Facility; C. Conte, E. Griffis, V. Cowling, and M. Peggie for materials and discussions; and D. Chirgadze for assistance with the Crystallographic X-ray Facility. This work was supported by awards to A.C. from the UK Biotechnology and Biological Sciences Research Council (BBSRC, grant BB/J001201/1 and David Phillips Fellowship BB/G023123/1). E.L.S. and A.P. were supported by European Commission Erasmus work placement grants. Microscopy and biophysics were supported by Wellcome Trust strategic awards to the University of Dundee (097945/Z/11/Z and 100476/Z/12/Z, respectively). The University of Dundee and the authors have filed patent applications (GB1320994.5 and GB1401001.1) related to the use of the bump-and-hole BET bromodomain chemical probes and mutant pairs for examining the biological function of BET bromodomain proteins. Coordinates and structure factors have been deposited with the Protein Data Bank (PDB) under accession code 4QEU [Brd2(2)$_{L383A}$ apo], 4QEV (in complex with **ME**), and 4QEW (in complex with **ET**).

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/346/6209/638/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S11
Tables S1 to S6
References (*18*–*36*)

17 December 2013; accepted 1 October 2014
10.1126/science.1249830

INNATE IMMUNITY

A Spaetzle-like role for nerve growth factor *b* in vertebrate immunity to *Staphylococcus aureus*

Lucy Hepburn,[1,2]* Tomasz K. Prajsnar,[3,4,5]* Catherine Klapholz,[1,2] Pablo Moreno,[1] Catherine A. Loynes,[5,6] Nikolay V. Ogryzko,[5] Karen Brown,[1,2,7] Mark Schiebler,[1,2] Krisztina Hegyi,[1,2]† Robin Antrobus,[1] Katherine L. Hammond,[5,6] John Connolly,[3,4] Bernardo Ochoa,[8] Clare Bryant,[9] Michael Otto,[10] Bas Surewaard,[11] Suranjith L. Seneviratne,[12] Dorothy M. Grogono,[2,7] Julien Cachat,[13] Tor Ny,[14] Arthur Kaser,[2] M. Estée Török,[2] Sharon J. Peacock,[2,15] Matthew Holden,[15,16] Tom Blundell,[8] Lihui Wang,[17] Petros Ligoxygakis,[17] Liliana Minichiello,[18] C. Geoff Woods,[1,19] Simon J. Foster,[3,4] Stephen A. Renshaw,[3,5,6]‡ R. Andres Floto[1,2,7]‡

Many key components of innate immunity to infection are shared between *Drosophila* and humans. However, the fly Toll ligand Spaetzle is not thought to have a vertebrate equivalent. We have found that the structurally related cystine-knot protein, nerve growth factor β (NGFβ), plays an unexpected Spaetzle-like role in immunity to *Staphylococcus aureus* infection in chordates. Deleterious mutations of either human NGFβ or its high-affinity receptor tropomyosin-related kinase receptor A (TRKA) were associated with severe *S. aureus* infections. NGFβ was released by macrophages in response to *S. aureus* exoproteins through activation of the NOD-like receptors NLRP3 and NLRC4 and enhanced phagocytosis and superoxide-dependent killing, stimulated proinflammatory cytokine production, and promoted calcium-dependent neutrophil recruitment. *TrkA* knockdown in zebrafish increased susceptibility to *S. aureus* infection, confirming an evolutionarily conserved role for NGFβ-TRKA signaling in pathogen-specific host immunity.

Staphylococcus aureus causes a range of serious infections, including skin ulceration, osteomyelitis, pneumonia, and septicaemia (*1*, *2*). Several evolutionarily conserved components of antistaphylococcal immunity have been identified using *Drosophila* as a model organism (*3*, *4*). One of the key mediators of immunity to Gram-positive bacteria in *Drosophila* is the soluble protein Spaetzle which, when activated by Spaetzle processing enzyme (SPE) upon

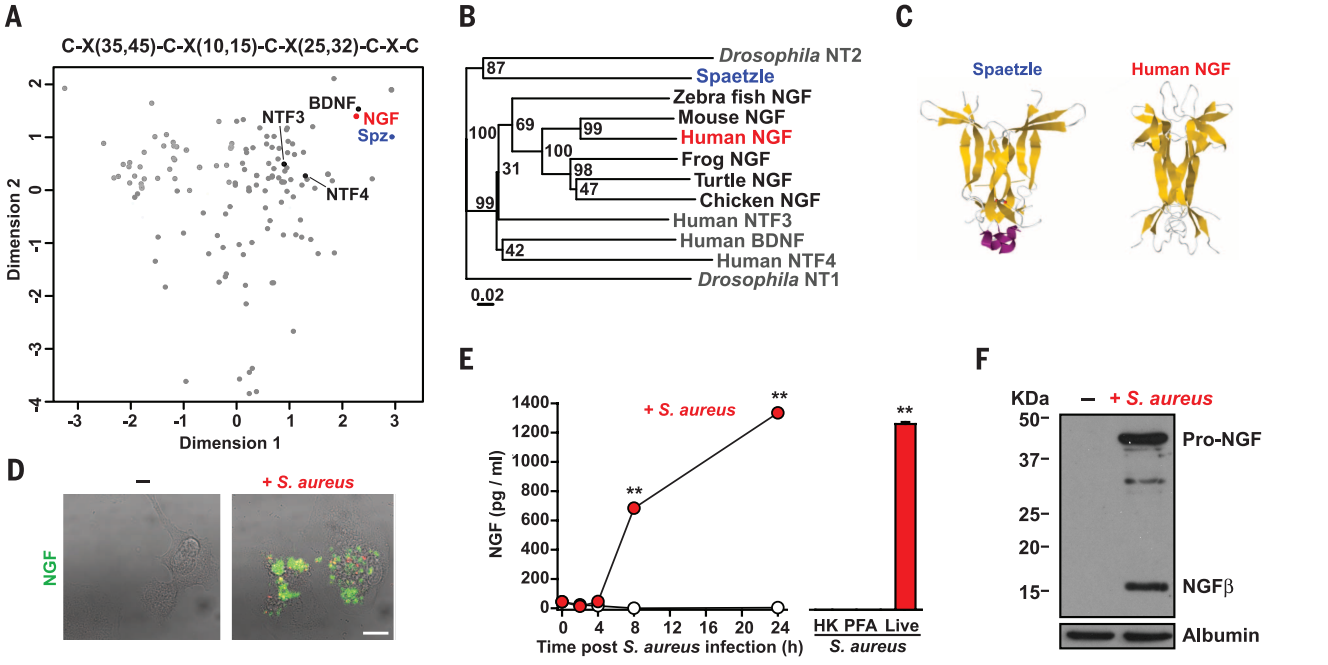

Fig. 1. NGFβ is implicated in antistaphylococcal immunity and is released from macrophages after *S. aureus* infection. (**A**) Bioinformatic identification of potential human orthologs of *Spaetzle*. The human proteome was searched using a PROSITE pattern to find soluble proteins potentially containing a >10-membered cystine-knot domain, which were then subjected to multifactorial analysis, incorporating structural prediction of disulphide bond formation with other structural and sequence parameters (see supplementary materials for details) to identify NGF (red) as the closest human ortholog to Spaetzle (Spz, blue). The other human neurotrophins [BDNF, neurotrophic factor 3 (NTF3), and NTF4 (black)] are also highlighted. (**B**) Phylogenetic alignment of vertebrate neurotrophic factors (NGF, BDNF, and NTF 3 and 4), *Drosophila* neurotrophin (NT) 1 and 2, and the *Drosophila* immune regulator Spaetzle, with bootstrap values. (**C**) Dimeric protein structures (from the Protein Data Bank) of Spaetzle and human NGFβ. (**D**) Intracellular staining of NGFβ (green) in primary human macrophages uninfected (left) or infected (right) with *S. aureus* (SH1000) (red) and then treated with monensin for 14 hours to prevent secretion. Scale bar, 5 μm. (**E**) Time course of NGFβ release from primary human macrophages after infection with *S. aureus*. NGFβ secretion requires live bacteria because heat-killed (HK) or paraformaldehyde (PFA)–killed *S. aureus* do not trigger NGFβ release. (**F**) Release of pro-NGF and NGFβ from differentiated THP-1 cells upon infection with *S. aureus* for 12 hours. *$P \leq 0.05$; **$P \leq 0.005$. All experiments were carried out in at least triplicate and are representative of at least three independent repeats.

infection, triggers effector immunity in an autocrine and paracrine manner through Toll receptor activation (*3*, *5*–*8*). To detect potential vertebrate equivalents of Spaetzle, we searched the human proteome using a relatively tolerant PROSITE pattern [C-X(35,45)-C-X(10,15)-C-X(25,32)-C-X-C; modified from (*9*)] to identify 166 soluble proteins potentially containing a >10-membered cystine knot domain (see the supplementary materials). We identified the neurotrophin nerve growth factor β (NGFβ) as a possible vertebrate ortholog of Spaetzle (Fig. 1, A and B). NGFβ regulates the survival, differentiation, and function of central and peripheral neurons (*10*, *11*), predominantly through activation of its high-affinity receptor, tropomyosin-related kinase receptor A (TRKA). Like Spaetzle, NGFβ is generated by enzymatic cleavage of a precursor proprotein to form a biologically active cystine-knot dimer (*11*) (Fig. 1C). Because NGFβ is implicated in the modulation of inflammation in non-neuronal cells (*12*–*14*), we asked whether NGFβ could play a Spaetzle-like role in coordinating vertebrate immunity to *S. aureus*.

Deleterious biallelic mutations in the genes encoding NGFβ (*NGF*) (*15*, *16*) or TRKA (*NTRK1*) (*17*) lead to a profound congenital sensory and autonomic neuropathy [termed hereditary sensory and autonomic neuropathy (HSAN) 4 and 5]. We found that these individuals also had frequent severe *S. aureus* infections of skin, teeth, joints, and bone (fig. S1), suggesting a pathogen-specific immune defect. To further explore the role of NGFβ in staphylococcal immunity, we measured its release from primary human macrophages obtained from healthy individuals. Infection of cells with live, but not killed, *S. aureus* stimulated de novo synthesis and secretion of both pro-NGF and mature NGFβ (Fig. 1, D to F). We found considerable variation in NGFβ stimulation by clinical isolates of *S. aureus*. Clones triggering lower levels of NGFβ were associated with increased all-cause patient mortality (fig. S1), again suggesting a protective role for NGFβ during *S. aureus* infection. The exact mechanisms generating mature NGFβ remain unclear, but it is likely that endogenous and exogenous host proteases [such as furins (*18*), matrix metalloproteinase (MMP) 7, and plasmin (*19*)], as well as bacterial proteases (fig. S1), combine to cleave pro-NGF during *S. aureus* infection, suggesting similarities with the regulation of Spaetzle processing (*20*).

We next examined whether other bacterial species were also able to stimulate NGFβ release from macrophages. Although a low-level response was seen with some other bacteria (such as

[1]Cambridge Institute for Medical Research, University of Cambridge, UK. [2]Department of Medicine, University of Cambridge, UK. [3]Krebs Institute, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK. [4]Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK. [5]Bateson Centre, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK. [6]Department of Infection and Immunity, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK. [7]Cambridge Centre for Lung Infection, Papworth Hospital, Cambridge, UK. [8]Department of Biochemistry, University of Cambridge, UK. [9]Department of Veterinary Medicine, University of Cambridge, UK. [10]Laboratory of Human Bacterial Pathogenesis, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, USA. [11]Department of Medical Microbiology, University Medical Centre, Utrecht, Netherlands. [12]Department of Clinical Immunology, Royal Free Hospital London, UK. [13]Department of Pathology and Immunology, Geneva University, Switzerland. [14]Department of Medical Biochemistry and Biophysics, Umea University, Sweden. [15]Wellcome Trust Sanger Institute, Hinxton, UK. [16]School of Medicine, University of St. Andrews, UK. [17]Biochemistry Department, Oxford University, UK. [18]Pharmacology Department, Oxford University, UK. [19]Department of Medical Genetics, University of Cambridge, UK.
*These authors contributed equally to this work. †Deceased.
‡Corresponding author. E-mail: arf27@cam.ac.uk (R.A.F.); s.a.renshaw@sheffield.ac.uk (S.A.R.)

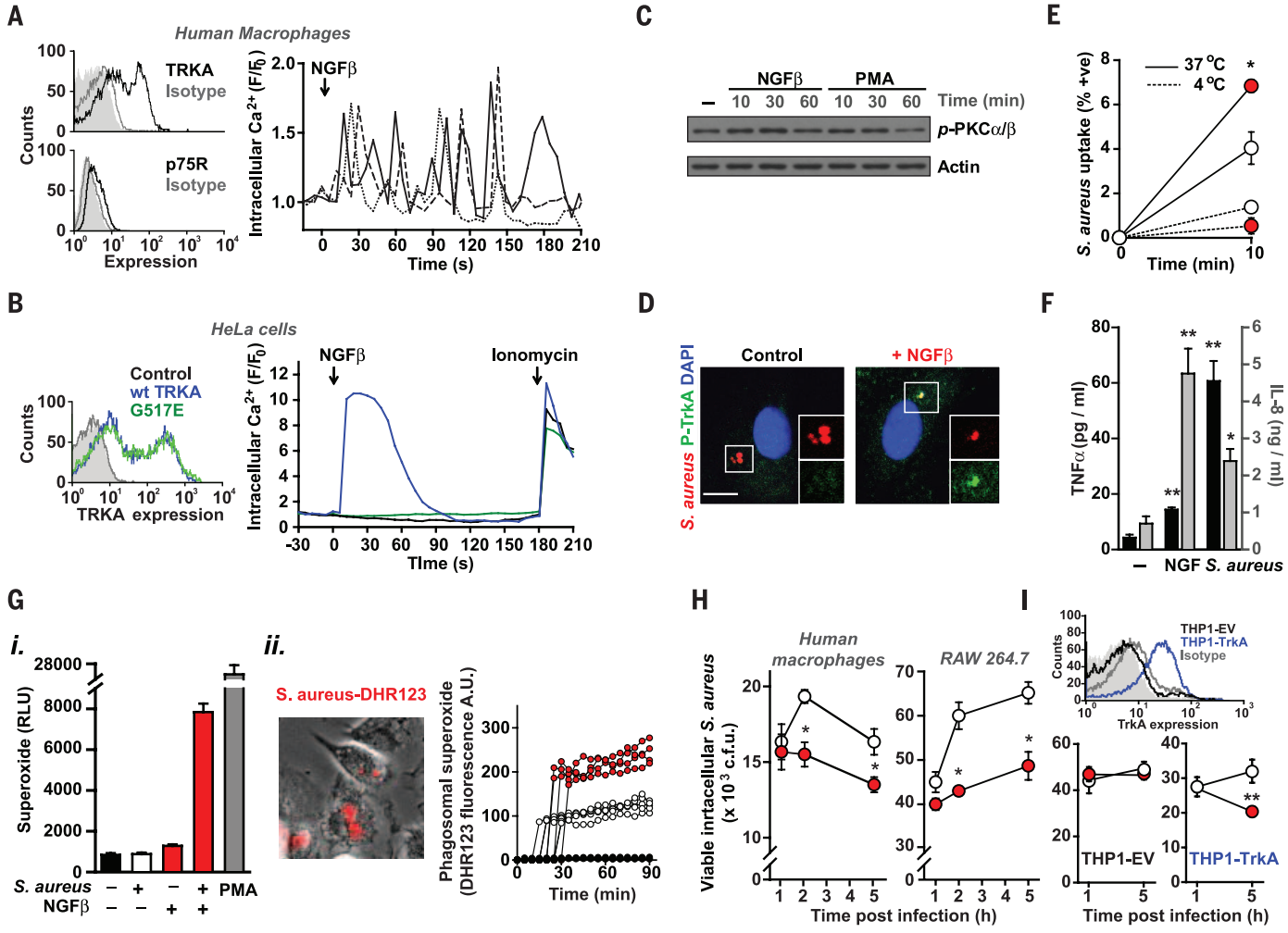

Fig. 2. Effects of NGFβ-TRKA signaling in human macrophages. (**A**) Addition of NGFβ (250 ng/ml; 9.25 μM) triggers sustained calcium oscillations in Fluo3-loaded primary human macrophages (detected by single-cell confocal imaging). Three representative recordings normalized for starting fluorescence (F/F_0) are shown. (Inset) Surface expression of TRKA and p75R (black) compared with isotype control (dark gray) or unstained cells (gray fill) on primary human macrophages. (**B**) Single-cell calcium signaling in GCamp3-expressing HeLa cells transfected with wild-type TRKA (blue), HSAN4-associated TRKA mutation (G517E; green) or empty vector (black) in response to NGFβ (250 ng/ml; 9.25 μM). (Inset) Surface expression of TRKA in transfected HeLa cells. (**C**) TRKA signaling in macrophages triggered rapid activation of calcium-dependent PKC isoforms. (**D**) Colocalization of intracellular phospho-TRKA (green) with red fluorescent protein (RFP)–labeled *S. aureus* (SH1000; red) in primary human macrophages treated for 30 min with 100 ng/ml (3.7 μM) NGFβ. (**E** and **F**) Addition of NGFβ to primary human macrophages increased (E) phagocytosis of RFP-labeled *S. aureus* and (F) release of TNFα and IL-8 (measured after 24 hours). (**G**) (i) Luminol-based detection of superoxide in response to *S. aureus*, NGFβ, or phorbol 12-myristate 13-acetate (PMA). (ii) The generation of phagosomal superoxide, monitored by DHR123-labeled heat-killed *S. aureus*, is increased in cells treated with the TRKA-specific agonist gambogic amide (250 nM; red) compared with vehicle (white; $P < 0.005$) or bacteria without cells (black). Four representative fluorescence traces from individual cells are shown for each group. (**H** and **I**) TRKA activation (by gambogic amide; 250 nM) enhanced intracellular killing of *S. aureus* in (H) primary human macrophages (left) and the mouse macrophage cell line RAW 264.7 (right) and (I) TRKA-transfected (blue), but not control (black), THP-1 cells. (Inset) Surface TRKA expression in THP-1 cells transfected with TRKA (blue) or empty vector (black) compared with isotype control (gray) and unstained cells (gray fill). All experiments were carried out in at least triplicate and are representative of at least three independent repeats.

Enterococcus faecalis), only *S. aureus* effectively triggered NGFβ release (fig. S1). Indeed, the closely related skin commensal *Staphylococcus epidermidis* was unable to stimulate NGFβ production effectively, suggesting that macrophages can discriminate between pathogenic and nonpathogenic staphylococcal species. Furthermore, macrophages only secreted NGFβ and not other neurotrophins [brain-derived growth factor (BDNF), NT3, and NT4] in response to infection (fig. S1). Thus, NGFβ may act as a specific and sensitive signal for *S. aureus* infection in man, potentially explaining the clinical phenotype of patients with HSAN 4 and 5 and suggesting a nonredundant and pathogen-specific role for NGFβ in innate immunity.

We then explored the cellular pathways triggering NGFβ generation. Rather than involving conventional surface pattern recognition receptors, *S. aureus* elicits NGFβ production through activation of nucleotide–binding and oligomerization domain (NOD)–like receptors (NLRs) (fig. S2), a well-recognized consequence of infection with this bacteria (*21*), and suggests an additional potential role for NGFβ during tissue damage.

To define the bacterial components responsible for NGFβ release from macrophages, we screened the Nebraska library of *S. aureus* transposon mutants (*22*) for their ability to stimulate NGFβ release from THP-1 cells. This identified a number of genes involved in bacterial cell wall synthesis, macromolecular transport, metabolism, and cellular regulation (fig. S3 and table S1), including the *saeR/saeS* 2 component gene system and autolysin, which regulate exoprotein

and peptidoglycan release, respectively (*23*, *24*). As expected, a number of purified *S. aureus*–derived exoproducts (protein A, peptidoglycan, and α-haemolysin) were able to stimulate NGFβ release in a proteinase K–dependent manner (fig. S3). Because most single exoprotein deletion mutants were still capable of stimulating NGFβ release, suggesting redundancy (fig. S4), we turned to comparative mass spectroscopy of conditioned media from wild-type and *saeS*-mutant *S. aureus* to define further bacterial components mediating NGFβ release (fig. S4) and identified alpha phenol–soluble modulins (α-PSMs), a recently described family of secreted peptides capable of membrane rupture (*25*), as putative factors (fig. S4). Thus, multiple *S. aureus* exoproteins can stimulate NGFβ release from macrophages. We asked whether this regulatory mechanism might be evolutionarily conserved to control Spaetzle production in *Drosophila*. Intriguingly, although the regulation of Spaetzle activity has focused on its SPE-mediated activation (*26*), pro-Spaetzle levels in *Drosophila* phagocytes (S2 cells) were stimulated by wild-type but not *saeR*-mutant *S. aureus*, by conditioned media, and by peptidoglycan (fig. S4), mirroring our results with NGFβ.

We then evaluated the effects of NGFβ on macrophage function. Primary human macrophages, which have constitutively high surface expression of TRKA but not the low-affinity NGF receptor p75, responded to NGFβ with sustained calcium signaling (Fig. 2A), which could be reconstituted in HeLa cells expressing wild-type TRKA but not the HSAN5-associated mutation G517E (Fig. 2B). TRKA signaling in macrophages also triggered rapid activation of calcium-dependent protein kinase C (PKC) isoforms (Fig. 2C), as well as other recognized components of TRKA signaling observed in neuronal cells (table S2). Because TRKA is thought to continue signaling after internalization, thereby permitting signal transmission along axons (*27*), we examined whether phagosomal TRKA activation might occur and found persistent tyrosine phosphorylation of TRKA within *S. aureus*–containing phagosomes (Fig. 2D). Functionally, TRKA activation led to enhanced phagocytosis (Fig. 2E), proinflammatory cytokine release from uninfected cells (Fig. 2F), and increased *S. aureus*–induced phagosomal superoxide generation (Fig. 2G). TRKA activation also enhanced intracellular killing of *S. aureus* in human and mouse macrophages (Fig. 2H) and in TRKA-transfected, but not control, THP-1 cells (Fig. 2I). This increased killing was dependent on intact receptor signaling (because it was not observed in cells from HSAN4 patients) and was principally mediated through enhanced superoxide generation (fig. S5) and autophagy (fig. S6). TRKA-dependent effector responses also depended on intact TLR signaling, because intracellular killing in *S. aureus*–infected cells and cytokine production in uninfected cells were abrogated in *Myd88*$^{-/-}$ and *Trif*$^{-/-}$ macrophages (fig. S7), suggesting an evolutionarily conserved interaction between cystine knot proteins and Toll family receptors.

We next determined the role of NGFβ-TRKA in human neutrophils, which are critical components of the host response to *S. aureus* infection (*28*). Neutrophils constitutively expressed TRKA (Fig. 3A) and released NGFβ in response to live *S. aureus* and peptidoglycan (Fig. 3B). As seen in macrophages, NGFβ stimulated neutrophils to generate superoxide (Fig. 3C) and secrete

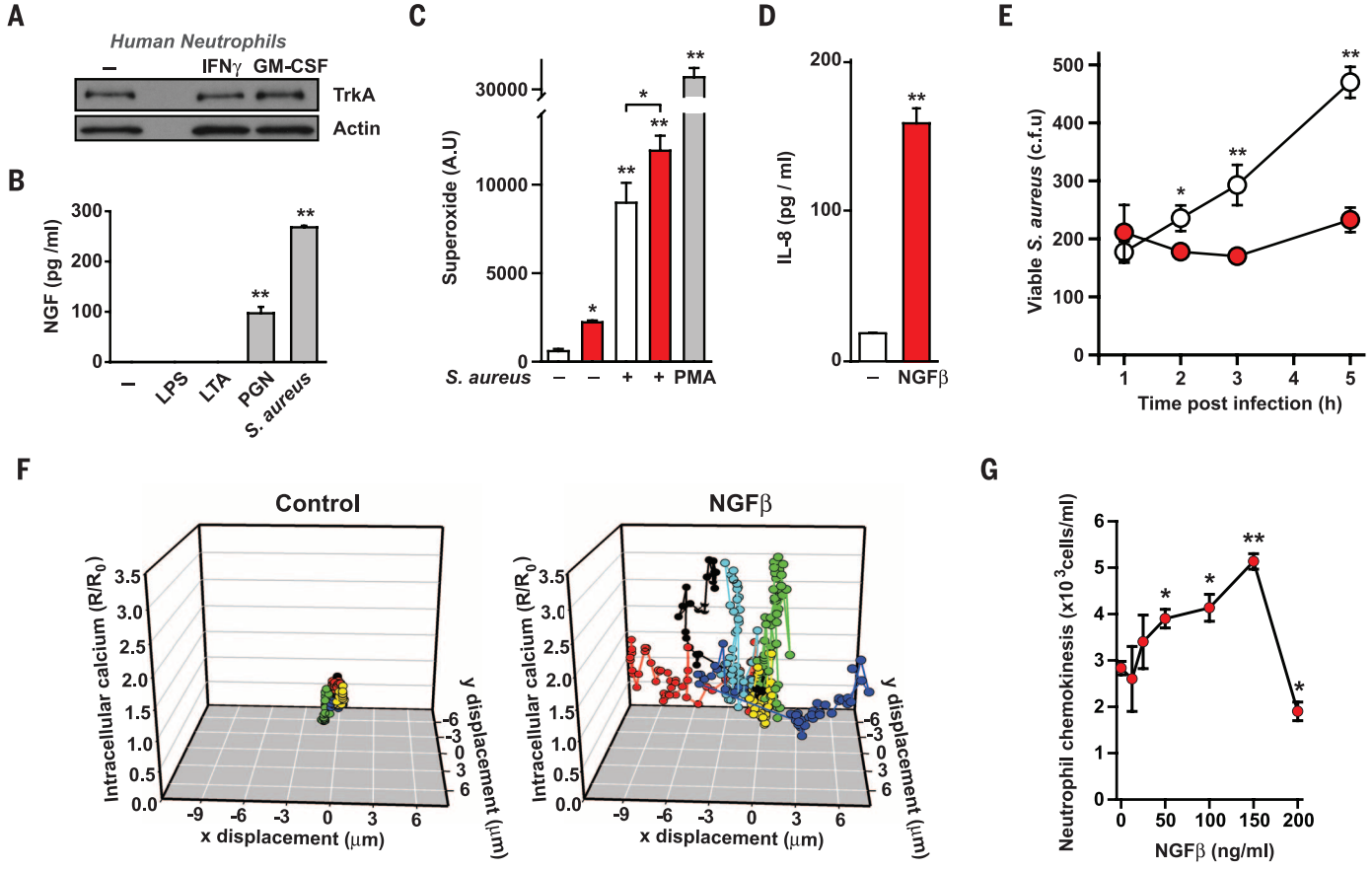

Fig. 3. NGFβ-TRKA signaling stimulates functional activation of neutrophils. (**A**) TRKA expression on untreated, IFN-γ (10 ng/ml) or granulocyte-macrophage colony-stimulating factor (100 ng/ml)–primed primary human neutrophils. (**B**) Neutrophils secrete NGFβ in response to live *S. aureus*, peptidoglycan (PGN) but not lipopolysaccharide (LPS; 100 ng/ml) or lipoteichoic acid (LTA; 5 μg/ml). (**C** and **D**) Neutrophils generate superoxide (C) and release interleukin-8 (IL-8) (D) in response to *S. aureus*, PMA, and/or NGFβ (red). (**E**) Killing of *S. aureus* by human neutrophils is enhanced by treatment with NGFβ (100 ng/ml; red) compared with control (white). (**F**) Representative plots of *x*-*y* displacement and calcium levels in individual neutrophils after addition of vehicle (control) or NGFβ. (**G**) Chemokinesis of human neutrophils assessed using a transwell assay in response to increasing concentrations of NGFβ. $*P \le 0.05$; $**P \le 0.005$. All experiments were carried out in at least triplicate and are representative of at least three independent repeats.

proinflammatory cytokines (Fig. 3D) and enhanced intracellular killing of *S. aureus* (Fig. 3E). NGFβ also stimulated chemokinesis and chemotaxis in a TRKA- and calcium-dependent manner (Fig. 3, F and G, movie S1, and fig. S8), suggesting that NGFβ may be an important chemotactic signal for neutrophil recruitment to sites of *S. aureus* infection.

To establish whether NGFβ-TRKA signaling represents a critical, evolutionarily conserved component of vertebrate immunity to *S. aureus* infection, we examined its role during in vivo infection of zebrafish. Effective morpholino knockdown of *trkA* was confirmed by immunohistochemistry, where we observed the expected loss of trkA protein in the forebrain and nose of zebrafish larvae (Fig. 4A). Knockdown of *trkA* had a major effect on the host response to *S. aureus*: *trkA* morphants were more susceptible to *S. aureus* infection than controls, a phenotype that could be rescued by concomitant injection of morpholino-resistant *trkA* RNA (Fig. 4B) and was only partially rescued in a transgenic line expressing trkA specifically in macrophages (fig. S9), suggesting the critical importance of trkA signaling in other cells (such as neutrophils). Bacterial counts in *trkA*-deficient fish rose faster and remained significantly higher than in controls (Fig. 4C). We then explored the relationship between the ability of bacteria to stimulate NGFβ release from macrophages and the in vivo effect of silencing trkA expression during infection (Fig. 4D). We observed a greater effect of *trkA* knockdown in fish infected with

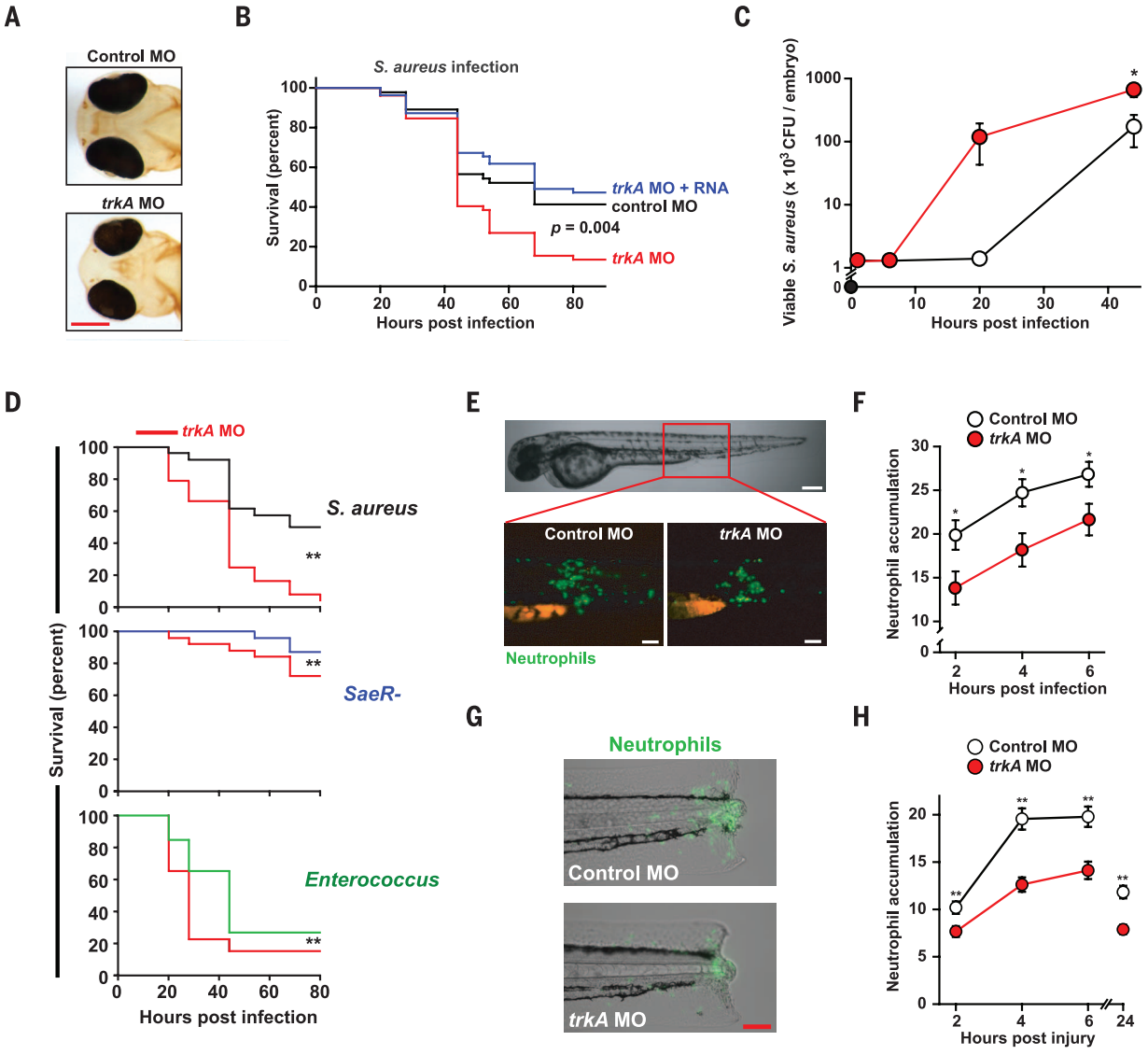

Fig. 4. Disruption of NGFβ-TrkA signaling compromises *S. aureus* immunity in vivo. (**A**) Reduced TrkA protein expression (assessed by immunohistochemistry) in the forebrain and nose of 72 hours post-infection zebrafish larvae injected with *trkA*-targeted (bottom) but not control (top) morpholinos. (**B**) Kaplan-Meier survival curves of fish infected with *S. aureus*. *TrkA* morphants (red) were more susceptible to *S. aureus* infection than controls (black) and could be rescued by concomitant injection of morpholino-resistant *trkA* RNA (blue). *N* of at least 45 fish per group performed as three independent experiments. (**C**) Numbers of viable *S. aureus* were significantly greater in *trkA* morphant (red) than control (white) fish, assessed as colony-forming units (CFU) per embryo. (**D**) Morpholino *trkA* knockdown (red) caused a greater effect on mortality in fish infected with wild-type (SH1000) *S. aureus* (black) compared to animals infected with bacteria less able to trigger NGFβ release from macrophages: the *saeR*–*S. aureus* mutant (causing a mild infection; blue) and *Enterococcus faecalis* (causing a severe infection; green). (**E** to **H**) Reduced migration of green fluorescent protein–tagged neutrophils to sites of *S. aureus* infection [(E) and (F)] or sterile inflammation [(G) and (H)] in *trkA* morphants (red) compared with controls (white). Representative images at 4 hours after infection (E) (scale bar: brightfield, 200 μm; fluorescence, 100 μm) or tail injury (G) (scale bar, 100 μm). *N* of at least 32 fish per group performed as three independent experiments. $^{*}P \le 0.05$; $^{**}P \le 0.005$; $^{***}P \le 0.0005$. Unless otherwise stated, data shown are representative of at least three independent experiments.

wild-type (SH1000) *S. aureus* compared to animals infected with bacteria less able to trigger NGFβ release from macrophages: the *saeR–S. aureus* mutant (causing a mild infection) and *Enterococcus* (causing a severe infection). Furthermore, *trkA* knockdown compromised neutrophil migration to sites of *S. aureus* infection (Fig. 4, E and F) as well as sterile inflammation (Fig. 4, G and H), supporting a role for NGFβ as an "alarmin" for both *S. aureus* infection and nonspecific tissue damage.

In summary, our results indicate a critical role for NGFβ-TRKA signaling in controlling vertebrate innate immunity during *S. aureus* infection. It is also conceivable that other vertebrate cystine-knot proteins might play similar roles to NGFβ for other bacterial pathogens. The recent finding that Spaetzle also functions as a neurotrophin in *Drosophila* (*29*) suggests an evolutionarily conserved dual function for cystine-knot proteins in both nerve development and antistaphylococcal immunity and may explain stimulation of aberrant nerve growth by soft-tissue infection by *S. aureus* (*30*). Our findings reveal pleotropic effects of the NGFβ-TRKA pathway that may particularly influence innate immunity to *S. aureus* infection, suggesting that, potentially, person-to-person variability in phagocyte secretion of, or response to, NGFβ may influence vulnerability to *S. aureus* infection and may provide opportunities for therapeutic intervention, particularly in multidrug-resistant disease.

REFERENCES AND NOTES

1. F. D. Lowy, *N. Engl. J. Med.* **339**, 520–532 (1998).
2. G. E. Thwaites *et al.*, *Lancet Infect. Dis.* **11**, 208–222 (2011).
3. B. Lemaitre, J. Hoffmann, *Annu. Rev. Immunol.* **25**, 697–743 (2007).
4. L. M. Stuart, R. A. Ezekowitz, *Nat. Rev. Immunol.* **8**, 131–141 (2008).
5. J. A. Hoffmann, *Nature* **426**, 33–38 (2003).
6. S. Valanne, J.-H. Wang, M. Rämet, *J. Immunol.* **186**, 649–656 (2011).
7. M. P. Belvin, K. V. Anderson, *Annu. Rev. Cell Dev. Biol.* **12**, 393–416 (1996).
8. A. N. Weber *et al.*, *Nat. Immunol.* **4**, 794–800 (2003).
9. U. A. Vitt, S. Y. Hsu, A. J. Hsueh, *Mol. Endocrinol.* **15**, 681–694 (2001).
10. S. Cohen, R. Levi-Montalcini, *Proc. Natl. Acad. Sci. U.S.A.* **42**, 571–574 (1956).
11. M. V. Sofroniew, C. L. Howe, W. C. Mobley, *Annu. Rev. Neurosci.* **24**, 1217–1281 (2001).
12. L. Aloe, R. Levi-Montalcini, *Brain Res.* **133**, 358–366 (1977).
13. U. Otten, P. Ehrhard, R. Peck, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 10059–10063 (1989).
14. S. C. Bischoff, C. A. Dahinden, *Blood* **79**, 2662–2669 (1992).
15. E. Einarsdottir *et al.*, *Hum. Mol. Genet.* **13**, 799–805 (2004).
16. O. P. Carvalho *et al.*, *J. Med. Genet.* **48**, 131–135 (2011).
17. A. Rotthier, J. Baets, V. Timmerman, K. Janssens, *Nat. Rev. Neurol.* **8**, 73–85 (2012).
18. N. G. Seidah, S. Benjannet, S. Pareek, M. Chrétien, R. A. Murphy, *FEBS Lett.* **379**, 247–250 (1996).
19. M. A. Bruno, A. C. Cuello, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 6735–6740 (2006).
20. L. El Chamy, V. Leclerc, I. Caldelari, J. M. Reichhart, *Nat. Immunol.* **9**, 1165–1170 (2008).
21. R. Muñoz-Planillo, L. Franchi, L. S. Miller, G. Núñez, *J. Immunol.* **183**, 3942–3948 (2009).
22. P. D. Fey *et al.*, *MBio* **4**, e00537-12 (2013).
23. M. A. Benson, S. Lilo, T. Nygaard, J. M. Voyich, V. J. Torres, *J. Bacteriol.* **194**, 4355–4365 (2012).
24. W. Vollmer, B. Joris, P. Charlier, S. Foster, *FEMS Microbiol. Rev.* **32**, 259–286 (2008).
25. A. Peschel, M. Otto, *Nat. Rev. Microbiol.* **11**, 667–673 (2013).
26. I. H. Jang *et al.*, *Dev. Cell* **10**, 45–55 (2006).
27. C. L. Howe, J. S. Valletta, A. S. Rusnak, W. C. Mobley, *Neuron* **32**, 801–814 (2001).
28. K. M. Rigby, F. R. DeLeo, *Semin. Immunopathol.* **34**, 237–259 (2012).
29. B. Zhu *et al.*, *PLOS Biol.* **6**, e284 (2008).
30. I. M. Chiu *et al.*, *Nature* **501**, 52–57 (2013).

ACKNOWLEDGMENTS

We thank S. Clegg and E. Henderson for help with patient samples, R. Mifsud and D. Cusens for initial phylogenetic and functional analysis, A. Segal for provision of *Nod2*$^{-/-}$ mouse bone marrow, and the aquarium staff of the Bateson Centre, University of Sheffield for zebrafish husbandry. This work was supported by The Wellcome Trust [Senior Clinical Research Fellowship to R.A.F. (084953), project grant to S.J.F./S.A.R. (089981), The Medical Research Council, UK (Research center grant (G0700091), Senior Clinical Fellowship to S.A.R. (G0701932)], Papworth Hospital and the National Institute for Health Research Cambridge Biomedical Research Centre, and the Intramural Research Program of NIAID, NIH.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/346/6209/641/suppl/DC1
Figs. S1 to S9
Movie S1
Databases S1 and S2
References (*31*–*64*)

14 July 2014; accepted 2 October 2014
10.1126/science.1258705

PLANT GENETICS

A Y-chromosome–encoded small RNA acts as a sex determinant in persimmons

Takashi Akagi,[1,2] Isabelle M. Henry,[1] Ryutaro Tao,[2]* Luca Comai[1]*

In plants, multiple lineages have evolved sex chromosomes independently, providing a powerful comparative framework, but few specific determinants controlling the expression of a specific sex have been identified. We investigated sex determinants in the Caucasian persimmon, *Diospyros lotus,* a dioecious plant with heterogametic males (XY). Male-specific short nucleotide sequences were used to define a male-determining region. A combination of transcriptomics and evolutionary approaches detected a Y-specific sex-determinant candidate, *OGI*, that displays male-specific conservation among *Diospyros* species. *OGI* encodes a small RNA targeting the autosomal *MeGI* gene, a homeodomain transcription factor regulating anther fertility in a dosage-dependent fashion. This identification of a feminizing gene suppressed by a Y-chromosome–encoded small RNA contributes to our understanding of the evolution of sex chromosome systems in higher plants.

Sexuality promotes and maintains genetic diversity in eukaryotic organisms. The characterization of sex chromosomes revealed evolutionary mechanisms governing sexuality in animals (*1*–*3*). However, most plant sex chromosomes, which could be present in up to 5% of species (*4*, *5*), remain poorly characterized (*5*–*8*). Dioecy, the separation of sex organs among male and female individuals, can be controlled by a heterogametic male system comparable to that of mammals and based on X and Y chromosomes, or on the X-to-autosome ratio (*5*–*8*). Species with heterogametic females, such as those of birds (ZW system), are less common (*8*). Studies of Y-chromosome structure and evolution in *Silene latifolia* (*9*–*11*), papaya (*Carica papaya*) (*12*–*15*), and date palm (*16*) have revealed a heterochromatic nonrecombining region controlling sex determination, a feature shared by loci controlling other sexual characters such as asexual reproduction via apomixis and the inability to self-fertilize via self-incompatibility systems (*7*). For Y-linked sex determination, as for apomixis, it has been challenging to identify genetic determinants in this heterochromatic context. A theoretical model postulates that two changes must occur during the transition from hermaphroditism to dioecy: a recessive mutation resulting in male sterility and a dominant female-suppressing mutation (*8*, *17*).

The *Diospyros* genus, within the Ebanaceae (Ericales), contains mostly tree species, including the economically important persimmons (*D. kaki*, *D. virginiana*, and *D. lotus*) and ebony (*D. ebenum*). Dioecy may predate the divergence of the *Diospyros* genus (*18*) and possibly even the origin of the Ebenaceae (35 to 65 million years ago) (*18*–*20*). Male flowers have fertile stamens but rudimentary, arrested carpels and are organized in a three-flower cyme. Female flowers display developed but defective anthers that normally do not produce pollen grains (fig S1, A to P). Although a single female flower is formed per inflorescence, lateral aborted flower primordia are often visible on the flower pedicel (fig. S1, Q and R).

We used de novo whole-genome sequencing and transcriptome approaches to characterize the sex determination system in the diploid *D. lotus*, located to a single sex determination (SD) locus on the Y chromosome (*21*).

[1]Department of Plant Biology and Genome Center, University of California Davis, Davis, CA, USA. [2]Laboratory of Pomology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan.
*Corresponding author. E-mail: rtao@kais.kyoto-u.ac.jp (R.T.); lcomai@ucdavis.edu (L.C.)

Science

A Spaetzle-like role for nerve growth factor β in vertebrate immunity to *Staphylococcus aureus*

Lucy Hepburn, Tomasz K. Prajsnar, Catherine Klapholz, Pablo Moreno, Catherine A. Loynes, Nikolay V. Ogryzko, Karen Brown, Mark Schiebler, Krisztina Hegyi, Robin Antrobus, Katherine L. Hammond, John Connolly, Bernardo Ochoa, Clare Bryant, Michael Otto, Bas Surewaard, Suranjith L. Seneviratne, Dorothy M. Grogono, Julien Cachat, Tor Ny, Arthur Kaser, M. Estée Török, Sharon J. Peacock, Matthew Holden, Tom Blundell, Lihui Wang, Petros Ligoxygakis, Liliana Minichiello, C. Geoff Woods, Simon J. Foster, Stephen A. Renshaw and R. Andres Floto

Science **346** (6209), 641-646.
DOI: 10.1126/science.1258705

Overcoming staph infections is hardwired

Several evolutionarily conserved components of antistaphylococcal immunity have been identified, using *Drosophila* as a model organism. However, no vertebrate ortholog has been identified for the Toll ligand Spaetzle, which plays a key role in controlling gram-positive infection in flies. Hepburn *et al.* have now identified NGF-β as a functional equivalent to Spaetzle in vertebrates. NGF- β acts as a paracrine "alarmin" orchestrating macrophage and neutrophil responses to *S. aureus* infection. People with deleterious mutations in genes encoding NGF-β or its high-affinity receptor TRKA are predisposed to recurrent and severe staph infections. *S. aureus* proteins selectively trigger macrophage production of NGF- β, which enhances uptake and superoxide-dependent killing of *S. aureus*, stimulates proinflammatory cytokine production, and promotes neutrophil recruitment. Moreover, TrkA silencing in vivo increases susceptibility to *S. aureus* . Thus, the NGF-β–TRKA pathway is a critical, evolutionarily conserved component of vertebrate immunity to *S. aureus* infection.

Science, this issue p. 641