Safeguards for Cell Cooperation in Mouse Embryogenesis Shown by Genome-Wide Cheater Screen

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Safeguards for Cell Cooperation in Mouse Embryogenesis Shown by Genome-Wide Cheater Screen

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Ensuring cooperation among formerly autonomous cells has been a central challenge in the evolution of multicellular organisms. One solution is monoclonality, but this option still leaves room for exploitative behavior, as it does not eliminate genetic and epigenetic variability. We therefore hypothesized that embryonic development must be protected by robust regulatory mechanisms that prevent aberrant clones from superseding wild-type cells. Using a genome-wide screen in murine induced pluripotent stem cells, we identified a network of genes (centered on *p53*, *topoisomerase 1*, and olfactory receptors) whose down-regulation caused the cells to replace wild-type cells in vitro and in the mouse embryo—without perturbing normal development. These genes thus appear to fulfill an unexpected role in fostering cell cooperation.

Multicellularity has evolved independently a number of times. Under certain conditions, it offers selective advantages that outweigh the costs of constraining competition (*1*). It can afford protection against predation, increase metabolic efficiency through division of labor, promote more efficient resource consumption, and even create a defense against noncooperative individuals (*1–3*). Cooperation, then, can be both a means and a motivation for the rise of multicellular organisms.

Once multicellularity is achieved, however, a conflict can emerge between organismal and cellular fitness (*4–6*). For example, among the facultative multicellular organisms myxobacteria and *Dictyostelium discoideum*, cells with "defector" or "cheater" mutations (which produce more than their fair share of spores) can destroy the entire community of cells unless it is able to re-equilibrate toward cooperation (*7–9*). In the obligatory multicellular organism *Drosophila melanogaster*, cells with two different genotypes within a developmental niche can vie for occupancy; in most cases, the wild-type cells remove mutant cells that would reduce fitness at the level of the whole organism (*10–13*). Although it is reasonable to assume that normal mammalian development relies on coordinated responses within the organism to curtail the autonomous behavior of mutant cells, this prediction has not been tested by systematic screening for cheater or competitor mutations.

Cheater mutants have been identified in *D. discoideum* by genetic screening in which a mutagenized cell population was forced to pass through the three canonical stages of the amoeba life cycle: growth, development, and germination (*7*). We transposed this approach to an in vitro

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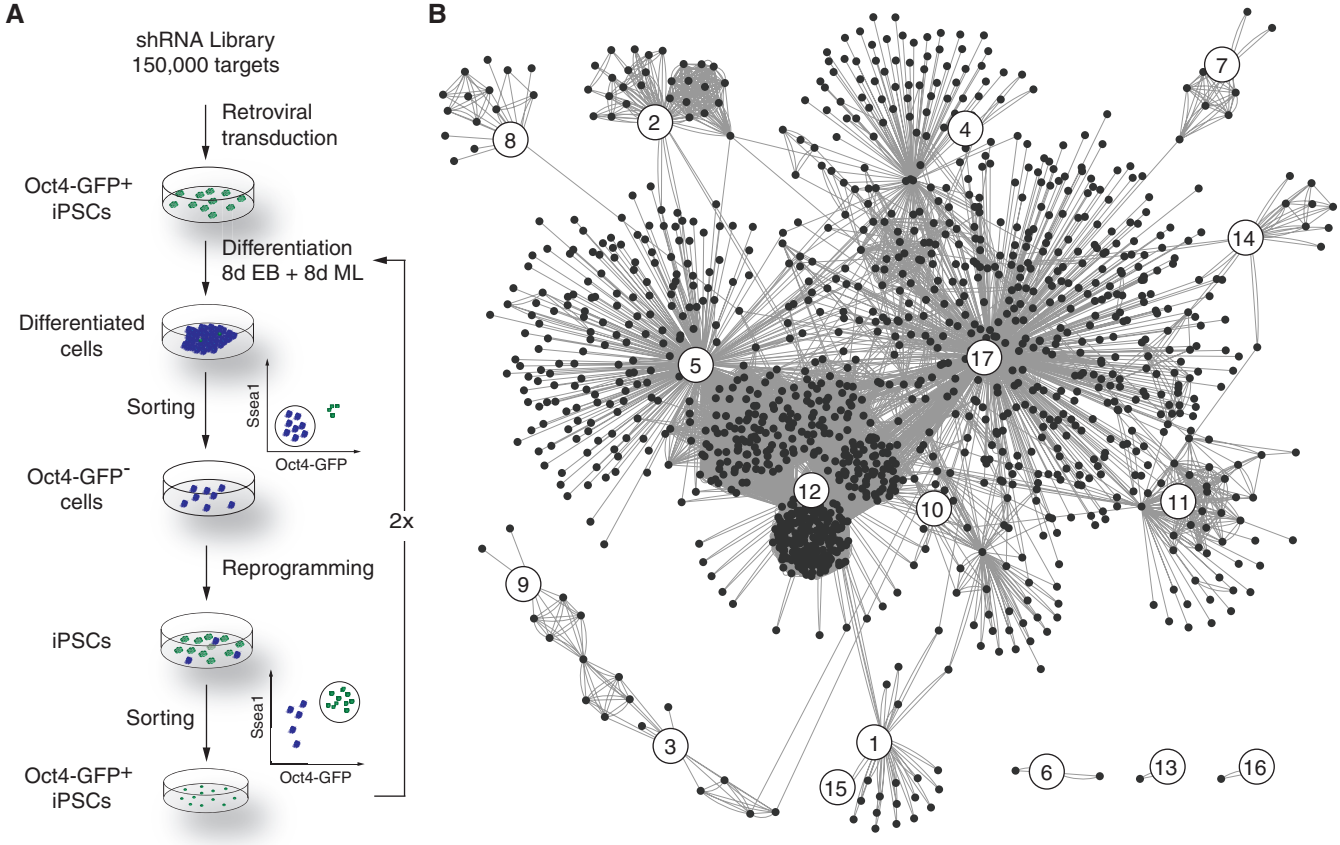

Fig. 1. Cell cheater screen. (**A**) Schematic of the screen used to identify candidate genes involved in cell cooperation, i.e., those whose knockdown confers a competitive advantage on stem cells. ML, monolayer. SSEA-1 and Oct4 are markers of pluripotency and self-renewal. (**B**) A cytoscape analysis of all candidate genes identified at least twice and for which network information was available shows that many of the candidate genes are tightly linked to each other. 1, *Mpp3*; 2, *Ptafr*; 3, *Galk1*; 4, *Dbc1*; 5, *Csnk2a1*; 6, *Ltb4r1*; 7, *Bet1*; 8, *Cat*; 9, *Gcat*; 10, *Hspa5*; 11, *Casp8*; 12, *Top1*; 13, *Lmtk3*; 14, *Bmf*; 15, *Crabp1*; 16, *Dnajc2*; 17, *p53*.

model of murine embryogenesis by introducing a library of small inhibitory (hairpin) RNAs into induced pluripotent stem cells [iPSCs, cell line PF159 (*14*)] and reasoning, by analogy to the *D. discoideum* screen, that egoistical phenotypes would be more likely to emerge among mutagenized cells under the considerable selective pressure of repeated rounds of growth [embryonic stem cell (ESC) expansion], development [embryoid body (EB) differentiation], and reacquisition of pluripotency (iPSCs) (*15*–*19*) (Fig. 1A; figs. S1 to S3; and see Methods for more detail).

Using this screen to select for cells that supersede wild-type cells simultaneously in all three categories (growth, differentiation, and reprogramming) in mixed populations of cells in a somatic context, we identified 131 putative cheater clones, a subset of which showed marked enrichment (fig. S4 and table S1). Gene ontology (GO) analyses revealed that a significant proportion of the candidate genes represent a relatively narrow set of functional categories, including cell communication, calcium channel activity, and kinase activity (fig. S4C and table S2). Inspection of table S1 reveals a number of notable genes, such as *p53*, *Top1* (*topoisomerase 1*), *Ptafr (platelet-activating factor receptor)*, *Olfr1387* (*olfactory receptor 1387*), *Olfr305* (*olfactory receptor 205*), *Dbc1* (*deleted in bladder cancer 1*), *Casp8* (*caspase 8*), *Mpp3* (*membrane protein, palmitoylated 3*), *Bmf* (*Bcl2-modifying factor*), *Calr* (*calreticulin*), and *I06Rik* (*E030030I06Rik*). A gene network analysis showed that the genes cluster closely together, and most link to *p53* and *Top1* (Fig. 1B).

To demonstrate that the isolated clones were authentic cheaters, we tested their performance in a developmental context in vivo using ESCs that expressed green fluorescent protein (GFP)–tagged knockdown versions of the top cheater mutants (fig. S4A and fig. S5A) (*shPtafr*, *shp53*, *shTop1*, *shOlfr1387*, *shOlfr305*, *shDbc1*, *shCasp8*, *shMpp3*, *shBmf*, *shCalr*, and *shI06Rik*) and a nontarget control (NTC). We also created a mock cell line containing the empty vector. In each

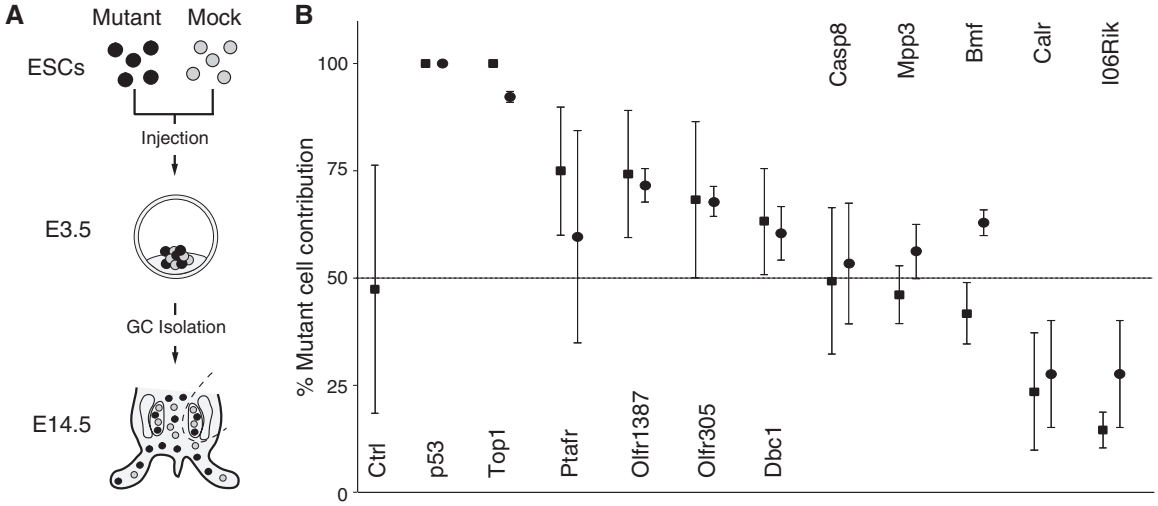

Fig. 2. In vivo analysis of cheater candidates. (**A**) Schematic illustration of the in vivo validation strategy (see text). (**B**) The contribution of mutant cells to embryonic tissue (somatic cells, squares) and germ cells (GC's, circles) at E14.5 relative to mock cells as determined by PCR. The data represent mean ± standard error. Ctrl, control; E, embryonic; ES, embryonic stem cells.

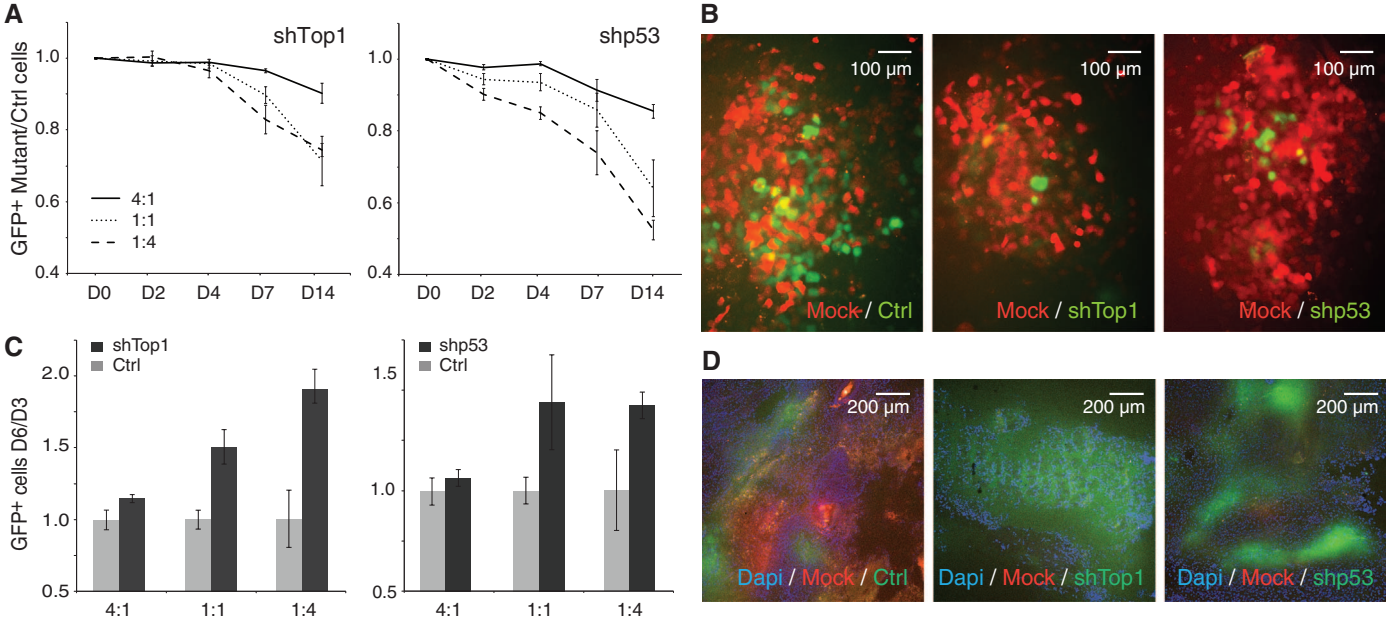

Fig. 3. In vitro behavior of pluripotent and differentiating cheater mutants. (**A**) Proliferation assay: Coculture of undifferentiated GFP-labeled mutant and RFP-labeled mock cells over the course of 14 days with different starting ratios of mutant:mock, as indicated (means ± SD). (**B**) Immunofluorescence images showing mutant and mock ESCs after 10 days of coculture as shown in (A) with an initial ratio of 1:4. (**C**) Differentiation assay: Mutant and mock cells were mixed as in (A), and differentiation was induced by EB formation in the absence of Lif (means ± SD). (**D**) Teratoma assay: Immunofluorescence analysis of sections from a teratoma grown from a 1:1 mixture injected into hindlegs of severe combined immunodeficiency mice indicates that knockdown cells contributed substantially more to the tumor tissue.

experiment, we injected five tester (provisional cheater mutant) cells and five mock cells into individual embryonic day (E) 3.5 (E3.5) blastocysts, implanted the embryos into pseudopregnant mice, harvested somatic embryonic tissue and germ cells at E14.5, and determined the relative contributions of mutant versus mock cells (Fig. 2A).

The effectiveness of cheating behavior in vivo can be grouped into four classes (Fig. 2B). *p53* or *Top1* knockdown cells (group 1) completely (100%) superseded control cells. It was remarkable that the E14.5 embryos and those that went to term were viable and appeared normal. A certain percentage of *p53* knockout embryos are known to develop defects in neural tube closure (*20*, *21*), whereas *Top1* knockout embryos are not viable. (*22*). Because our cells regained some Top1 function at E14.5, it is possible that the competitive phenotype arises from strong knockdown early on (so that later silencing of the retroviral construct is not relevant) or that only mild knockdown is sufficient for the phenotype (fig. S5B). The contribution of group 2 cheater clones (*Ptafr*, *Olfr1387*, *Olfr305*, and *Dbc1*) to the embryo was less striking but still significant (60 to 75%). Group 3 (*Casp8*, *Mpp3*, and *Bmf*) clones showed no evidence of competitive behavior (~50%), and group 4 (*Calr* and *106Rik*) clones contributed little (20%) to either the embryo or germ cells. It thus appears likely that mechanisms ensuring cooperation during development are more varied and stringent in vivo than in vitro.

To gain insight into the mechanistic basis for these outcomes, we recapitulated each step of the initial screen using the group 1 genes, beginning with proliferation. Thus, GFP-labeled *p53* or *Top1* knockdown or control cells were mixed with red fluorescent protein (RFP)–labeled mock cells at three different ratios (1:4, 1:1, and 4:1) and allowed to proliferate for a given time, and their relative contributions were determined (Fig. 3, A and B). This approach not only enabled us to capture the "cheating" cells for subsequent profiling studies (see below), but also allowed us to determine concentration-dependent effects, because homogeneous populations of p53 and Top1 mutants exhibited normal growth (fig. S6). This lack of a growth advantage indicates that rare escape mutants were not a major factor in our screening results. To our surprise, both *p53* and *Top1* mutants underperformed when grown in undifferentiated conditions (Fig. 3, A and B), which may suggest that the loss of *p53* or *Top1* activity by stem cells is the signal triggering removal of such defective cells from culture, reminiscent of the "cell competition" described in *D. melanogaster* (*10*).

Next, we tested the performance of *p53* or *Top1* knockdown cells under differentiation conditions, first in the context of EB development. Here, the outcome was in agreement with the results of the embryological studies presented earlier: The mutant cells expanded disproportionally when mixed with control cells (Fig. 3C). The magnitude of the effect was concentration-dependent, as in the proliferation assay, but in the opposite direction: here, the fewer the cheater cells, the greater their growth advantage. To corroborate our finding using a second differentiation system, we subjected a combination of mutant and control cells to teratoma differentiation conditions. A histologic examination of the resultant tumors revealed that *p53* and *Top1* knockdown cells contributed substantially more than wild-type cells to all three germ layers (Fig. 3D and fig. S7).

To elucidate the genetic framework that enabled the knockdown cells to predominate, we performed microarray expression analysis (fig. S8A). In these experiments, we mixed knockdown and mock ESCs and collected mRNA on day 4 of coculture, while cells were in the growth phase. These conditions allowed direct comparisons within a single cell type (ESCs) rather than a mixture of different cell types, as seen under differentiation conditions. To extract information specific to cell cooperation as opposed to canonical p53 and Top1 functions, we compared results obtained from our mixed (1:4) culture with those obtained after mixing mRNA (also 1:4) from *p53* or *Top1* knockdown cells and mock cells that were grown homotypically. These experiments showed that the cheating phenotype was firmly coupled to the gene network identified by our initial screen and further revealed that the gene signature consisted of a very similar set of genes or gene groups that included *Myc*, *Cdk6*, *Brachyury* (T), and *Jun* (Fig. 4 and fig. S8B). These signatures are closely linked to apoptosis and differentiation and resemble a signature previously linked to cell competition among hematopoietic stem cells (HSCs) (*23*) (fig. S9 and tables S3 and S4). Although the cells retained many defining characteristics of ESCs, including pluripotency and the ability to participate in three-germ-layer differentiation (as shown in our in vivo studies), it appears that the knockdown of *p53* or *Top1* prompted cells to co-opt a different gene program that facilitated

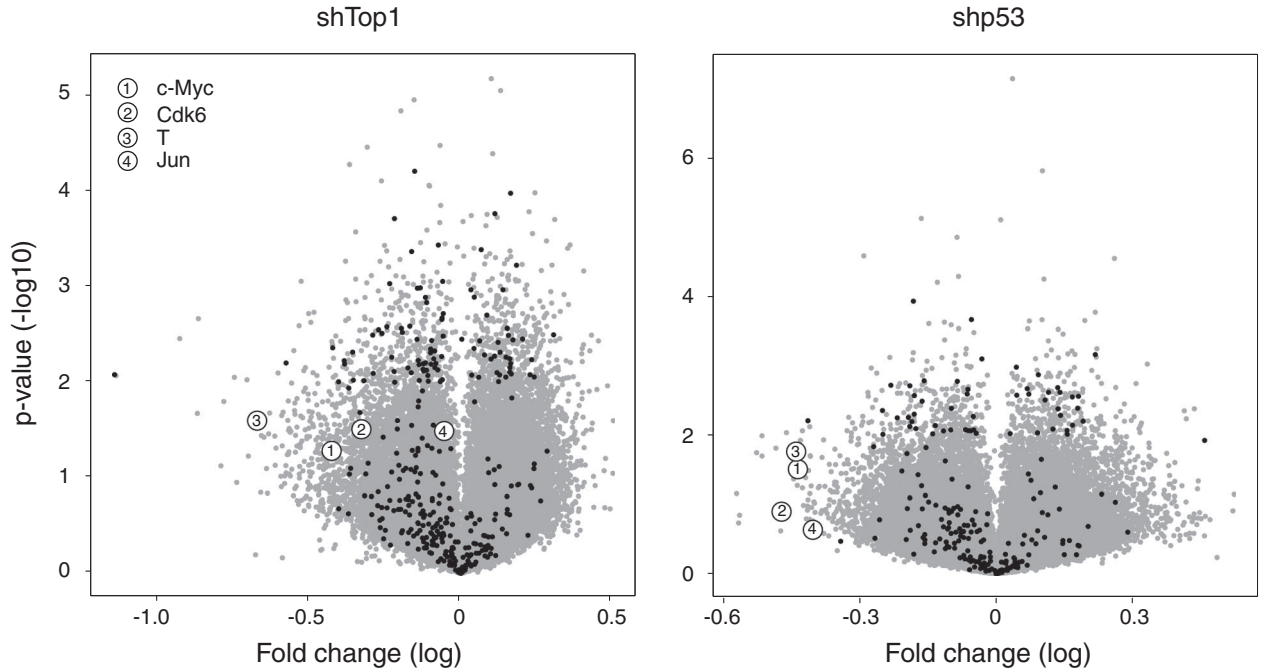

Fig. 4. Volcano plot analyses of microarray results. Black dots represent genes that belong to the Gene Set "GRAESSMANN APOPTOSIS BY DOXORUBICIN D" (tables S3 and S4) which is one of the top gene sets that are significantly enriched when mock cells are cocultured with *shTop1* ($P < 1 \times 10^{-16}$) or *shp53* ($P = 1.26 \times 10^{-9}$) knockdown cells.

more effective colonization of the embryonic niches available during early development.

The behavior of these cheater mutants points to the existence of genetic mechanisms that protect the ability of embryonic cells to cooperate. It is worth mentioning that several of the genes that responded to *p53* or *Top1* knockdown in our studies (*Myc*, *Jun*, and *Dbc1*) have been associated with cell competition in *D. melanogaster* (*11*–*13*). It is particularly noteworthy that in the context of DNA damage, murine HSCs with lower levels of p53 outcompete cells with higher levels of the protein (*23*, *24*). We found the opposite in ESCs, and only under differentiation conditions did cells with low levels of p53 (or Top1) outcompete wild-type cells. This could indicate a fundamental difference between ESCs and HSCs, or perhaps the influence of genotoxic stress in the earlier studies; it is also possible that in our experiments, survival of wild-type ESCs under undifferentiated conditions could have been promoted by p53-induced autophagy (*25*). Although one of the oldest known functions of p53, evident even in the sea anemone (*26*), is its role in the DNA damage-response pathway, our findings suggest that p53 serves a still more ancient function in enabling cells to respond to signals from other cells to coordinate their activities. Thus, the identification of olfactory receptors by our screen is not surprising, as the ability to cooperate with other cells must entail the ability to sense subtle changes in the microenvironment (indeed, chemotaxis proved to be an important component of the cooperative gene network in our analysis); this result is also in line with the so-called area-code hypothesis (*27*, *28*).

Biological competition has been defined explicitly in terms of a contest between "winners" and "losers," but the *Drosophila* studies established that cells communicate their growth or metabolic status, guiding each other's proliferation rate and determining survival (*10*). Cooperation need not be anthropomorphized to mean anything beyond coordinated activity; such functional interactions can be deemed selfish or altruistic, positive or negative, only in reference to some specific goal. What is surprising in the present study is that mutant cells were not only able to supersede wild-type cells but did so without affecting normal development.

More broadly, our studies place mechanisms of cell coordination during mammalian development in the larger matrix of evolutionary cooperation by identifying genes encoding several of the most ancient, yet still incompletely understood, proteins: p53, Top1, and olfactory receptors. Understanding how cells coordinate their functions might enable us to devise novel strategies for manipulating pluripotent stem cells in vitro (including reprogramming and transdifferentiation to alternative fates) and should illuminate other processes related to tissue homeostasis, including stem cell turnover (as in the skin and immune system) and oncogenesis.

References and Notes

1. A. Rokas, *Annu. Rev. Genet.* **42**, 235–251 (2008).
2. R. E. Michod, *Proc. Natl. Acad. Sci. U.S.A.* **104** (suppl. 1), 8613–8618 (2007).
3. T. Pfeiffer, S. Bonhoeffer, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 1095–1098 (2003).
4. A. Khare, G. Shaulsky, *Nat. Rev. Genet.* **7**, 577–584 (2006).
5. A. Sánchez Alvarado, H. Kang, *Exp. Cell Res.* **306**, 299–308 (2005).
6. K. R. Foster, G. Shaulsky, J. E. Strassmann, D. C. Queller, C. R. L. Thompson, *Nature* **431**, 693–696 (2004).
7. L. A. Santorelli *et al*., *Nature* **451**, 1107–1110 (2008).
8. F. Fiegna, Y.-T. N. Yu, S. V. Kadam, G. J. Velicer, *Nature* **441**, 310–314 (2006).
9. D. C. Hagen, A. P. Bretscher, D. Kaiser, *Dev. Biol.* **64**, 284–296 (1978).
10. L. A. Johnston, *Science* **324**, 1679–1682 (2009).
11. G. Morata, P. Ripoll, *Dev. Biol.* **42**, 211–221 (1975).
12. E. Moreno, K. Basler, *Cell* **117**, 117–129 (2004).
13. C. de la Cova, M. Abril, P. Bellosta, P. Gallant, L. A. Johnston, *Cell* **117**, 107–116 (2004).
14. M. Stadtfeld *et al*., *Nature* **465**, 175–181 (2010).
15. J. H. Hanna, K. Saha, R. Jaenisch, *Cell* **143**, 508–525 (2010).
16. M. J. Evans, M. H. Kaufman, *Nature* **292**, 154–156 (1981).
17. G. R. Martin, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7634–7638 (1981).
18. G. M. Keller, *Curr. Opin. Cell Biol.* **7**, 862–869 (1995).
19. K. Takahashi, S. Yamanaka, *Cell* **126**, 663–676 (2006).
20. J. F. Armstrong, M. H. Kaufman, D. J. Harrison, A. R. Clarke, *Curr. Biol.* **5**, 931–936 (1995).
21. V. P. Sah *et al*., *Nat. Genet.* **10**, 175–180 (1995).
22. S. G. Morham, K. D. Kluckman, N. Voulomanos, O. Smithies, *Mol. Cell. Biol.* **16**, 6804–6809 (1996).
23. T. Bondar, R. Medzhitov, *Cell Stem Cell* **6**, 309–322 (2010).
24. A. Marusyk, C. C. Porter, V. Zaberezhnyy, J. DeGregori, *PLoS Biol.* **8**, e1000324 (2010).
25. D. Crighton *et al*., *Cell* **126**, 121–134 (2006).
26. S. Pankow, C. Bamberger, *PLoS ONE* **2**, e782 (2007).
27. W. J. Dreyer, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9072–9077 (1998).
28. C. D. Buckley *et al*., *Cell. Immunol.* **236**, 29–41 (2005).

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Supplementary Materials

www.sciencemag.org/content/341/6153/1511/suppl/DC1
Materials and Methods
Figs. S1 to S9
Tables S1 to S4
References (*29*–*31*)

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Distinguishable Epidemics of Multidrug-Resistant *Salmonella* Typhimurium DT104 in Different Hosts

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The global epidemic of multidrug-resistant *Salmonella* Typhimurium DT104 provides an important example, both in terms of the agent and its resistance, of a widely disseminated zoonotic pathogen. Here, with an unprecedented national collection of isolates collected contemporaneously from humans and animals and including a sample of internationally derived isolates, we have used whole-genome sequencing to dissect the phylogenetic associations of the bacterium and its antimicrobial resistance genes through the course of an epidemic. Contrary to current tenets supporting a single homogeneous epidemic, we demonstrate that the bacterium and its resistance genes were largely maintained within animal and human populations separately and that there was limited transmission, in either direction. We also show considerable variation in the resistance profiles, in contrast to the largely stable bacterial core genome, which emphasizes the critical importance of integrated genotypic data sets in understanding the ecology of bacterial zoonoses and antimicrobial resistance.

Salmonella enterica subspecies *enterica* is one of the most common bacterial pathogens of humans and other animals (*1*, *2*). The global burden of disease caused by *Salmonella* infections is substantial, with more than 90 million human cases of gastroenteritis alone occurring each year (*3*). The annual cost of these infections is estimated to be about €3 billion in the European Union (*4*) and about $2.7 billion in the United States (*5*). The public health impact is exacerbated by antimicrobial resistance (AMR), which leads to increased morbidity, mortality, and treatment costs (*6*, *7*). In our study, the interrelated epidemiologies of *Salmonella* and AMR were examined at the level of the genome. Our results challenge the established view that the human and animal epidemics are synonymous (*8*–*11*) and show that the phylogenetic associations both within and between the resistance determinants and the host bacteria are different.

Throughout the 1990s, there was a global epidemic of multidrug-resistant *S.* Typhimurium definitive type 104 (DT104) in animals and humans (*12*, *13*). The DT104 epidemic was important because of its widespread prevalence and perceived zoonotic nature, as well as the high frequency of resistance to a wide range of commonly used antimicrobials, particularly ampicillin,