

organismal fitness (for example, metabolic efficiency, growth rate) (2, 17–21) and have concluded that allozyme polymorphisms themselves underlie physiological energetic differences by virtue of their influence on metabolite flux through central biochemical pathways (5, 22). Such balancing selection on allozyme polymorphisms could counter the influence of genetic drift, even in the face of population subdivision due to historical demographic events or contemporary restrictions on gene flow that are reflected in geographically divergent frequencies of neutral genetic markers. Hidden molecular variation that would also distinguish Atlantic and Gulf oysters may exist at the allozyme loci, but this possibility can in a sense be subsumed under the hypothesis of balancing selection, provided that the selection operates with respect to the observed electromorph classes instead of the level of the hidden variation. A slower rate of evolution for allozymes as compared to *scnRFLPs* can also be eliminated, because both cases in oysters involve the population level sorting of ancestral polymorphisms that affect both equally.

Regardless of the specific underlying causes, the heterogeneity in geographic pattern among allozyme, *scnDNA*, and *mtDNA* data sets cannot be accommodated under a single evolutionary model involving either neutrality or balancing selection (23). In this example where an allozyme survey had suggested high levels of gene flow, dramatic population genetic separation nonetheless was present in both the nuclear and the cytoplasmic genomes. Results of this study emphasize the need for caution in inferring population genetic structure and gene flow from any single class of genetic markers.

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12. S. A. Karl, B. W. Bowen, J. C. Avise, *Genetics*, in press.
13. Locations and sample sizes for American oysters in the present study are as follows: MA, Woods Hole, MA ($n = 35$); SC, Charleston, SC (23); GA, Cumberland Island, GA (33); FL1, New Smyrna Beach, FL (30); FL2, Stuart, FL (29); FL3, Port Charlotte, FL (29); FL4, Panacea, FL (39); FL5, Carabelle River, FL (18); and LA, Grand Isle, LA (41). Living oysters were collected and placed on wet ice for transportation to the laboratory. Total cell DNA was extracted from mantle-gonad tissue by homogenizing in 50 mM tris-HCl (pH 8.0), 100 mM EDTA, and 100 mM NaCl followed by one cycle of phenol, phenol-chloroform, and chloroform extraction. RNA was removed by ribonuclease A digestion for 3 hours followed by a repeat of the organic extractions described above. We precipitated DNA by adjusting the aqueous fraction to a final concentration of 300 mM sodium acetate and 70% ethanol; it was then vacuum-dried and resuspended in 1 × TE (10 mM tris-HCl, pH 8.0, 1 mM EDTA).
14. In general, 1 μ l of genomic DNA was amplified in a 100- μ l reaction volume with 25 pmol of each primer and 2.5 units of *Taq* polymerase, according to manufacturer's instructions (Promega), with the addition of $MgCl_2$ and bovine serum albumin to a final concentration of 2.5 mM and 0.1 μ g/ μ l, respectively. PCR cycling parameters consisted of an initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 94°C for 1 min with annealing at 55° to 62°C for 1 min and extension at 72°C for 2 min, and a final extension at 72°C for 7 min.
15. Approximately 10 μ l of the amplified DNA was digested directly with 5 units of each restriction enzyme in a 20- μ l reaction according to the manufacturer's directions. Electrophoresis was performed in 2.5% agarose gels stained with ethidium bromide (180 ng/ml). Restriction pat-

terns were visualized with shortwave ultraviolet light. Polymorphisms were indicated by the gain or loss of fragments in the restriction profiles.

16. To eliminate the possibility that our nuclear loci mistakenly may have represented *mtDNA* polymorphisms, we probed a Southern (DNA) blot of the amplified *scnDNA* products with purified oyster *mtDNA*. No bands appeared in the autoradiogram except in control lanes. The hypothesis of *mtDNA* contamination is further discounted by the nature of the *scnDNA* polymorphisms themselves, which involved diploid genotypes with general conformance to Hardy-Weinberg expected genotypic frequencies. This result further supports the idea that the loci are inherited in a Mendelian fashion. In addition, all pairwise comparisons of the four loci show insignificant deviations from gametic equilibrium [B. S. Weir and C. C. Cockerham, in *Mathematical Evolutionary Theory*, M. E. Feldman, Ed. (Princeton Univ. Press, Princeton, NJ, 1989), pp. 86–110].
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25. We thank M. Schexnayder and S. McAlpine for oyster specimens. Supported by an NIH predoctoral training grant to S.A.K. and by NSF grant BSR-9005940.

4 December 1991; accepted 14 February 1992

Identification of *ras* Oncogene Mutations in the Stool of Patients with Curable Colorectal Tumors

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Colorectal (CR) tumors are usually curable if detected before metastasis. Because genetic alterations are associated with the development of these tumors, mutant genes may be found in the stool of individuals with CR neoplasms. The stools of nine patients whose tumors contained mutations of *K-ras* were analyzed. In eight of the nine cases, the *ras* mutations were detectable in DNA purified from the stool. These patients included those with benign and malignant neoplasms from proximal and distal colonic epithelium. Thus, colorectal tumors can be detected by a noninvasive method based on the molecular pathogenesis of the disease.

Colorectal cancer is the third most common malignancy in the world, with 570,000 new cases expected in 1992. In the United States alone, over 60,000 people

will die from colorectal cancer this year (1). Whereas individuals with advanced disease have a poor prognosis, colorectal tumors diagnosed at an earlier stage can usually be cured by surgical or colonoscopic excision (2). Methods to detect surgically resectable tumors could therefore reduce deaths from this disease (3). The only noninvasive test for such a purpose involves testing stool for blood, but the appearance of hemoglobin in stool is not specific for neoplasia (4, 5).

Tumor-derived mutations in oncogenes and suppressor genes potentially provide more specific markers (6). Mutations in

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these genes appear to be responsible for the initiation and progression of most human tumors, including those of the colon and rectum (7). Theoretically, colorectal tumors should shed cells containing these mutations into the stool. However, stool is a complex mixture consisting of diverse microorganisms, undigested food residues, mucus, and soluble and insoluble products of the gastrointestinal

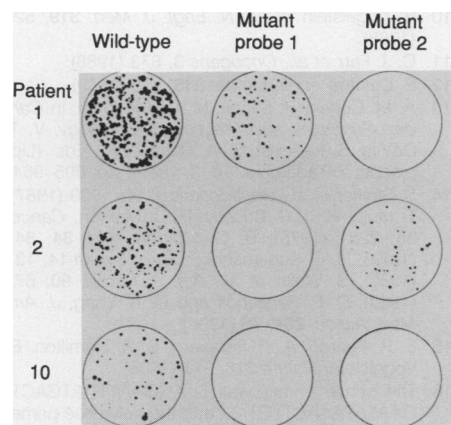


Fig. 1. Identification of *ras* mutations in stool by plaque hybridization. PCR products that contained the first coding exon of *K-ras* were generated from stools of patients 1, 2, and 10 (Table 1) and cloned into a bacteriophage vector (16, 17). The plaque lifts were hybridized to an oligonucleotide specific for wild-type *Ras*, an oligonucleotide specific for Val¹² (mutant probe 1), and to an oligonucleotide specific for Asp¹³ (mutant probe 2), as indicated (20). The number of plaques used for hybridization to the mutant specific oligonucleotides was fivefold higher than that used for the wild-type-specific oligonucleotide so that a statistically significant number of hybridizing plaques was obtained. The ratios of plaques hybridizing to the mutant-specific probe compared to the wild-type-specific probe were 0.08:1 and 0.04:1 in patients 1 and 2, respectively.

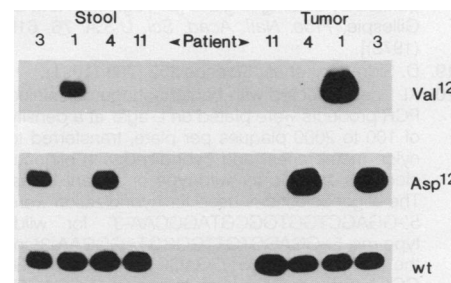


Fig. 2. Southern blot assay for *ras* mutations. PCR products that contained the first coding exon of *K-ras* were generated from stools and tumors of patients 1, 3, 4, and 11, as indicated (16). PCR products were subjected to electrophoresis through a 2% agarose gel and transferred to nylon filters (21). The blots were hybridized to oligonucleotide probes specific for wild-type *ras* (bottom), the Asp¹² mutation (middle), or the Val¹² mutation (top).

tract. Furthermore, it contains numerous degradative enzymes derived from cells, food, and bacteria. It was therefore unclear whether mutant genes from tumor cells could survive in this hostile environment and be detectable in clinical specimens.

To investigate this possibility, we examined stools from individuals with CR tumors for mutations of *K-ras* at codons 12 or 13, which occur commonly in these neoplasms (8–12). We first analyzed tumors from 24 patients for the presence of *K-ras* gene mutations. These cases comprised randomly chosen individuals from our clinics from whom we obtained stool samples before bowel preparation for colonoscopy or surgery and who were subsequently found to have either a malignant colorectal tumor (carcinoma) or a benign tumor (adenoma) greater than 1 cm in diameter. Adenomas of this size are clinically the most important, as they are much more likely to progress to malignancy than smaller tumors (13, 14).

The first exon of *K-ras* gene was amplified from DNA purified from cryostat sections of these tumors (15) by the polymerase chain reaction (PCR), (16). The PCR products were cloned, and pooled clones were sequenced to identify mutations (17). Nine of the 24 tumors (37%) were found to contain mutations of this exon. Three different mutations were identified (Gly¹² to Val¹² or Asp¹²; and Gly¹³ to Asp¹³). These data were consistent with previous studies that showed *ras* gene mutations in about 50% of such tumors, with 84% of the mutations confined to codons 12 or 13 of *K-ras* (10).

We next analyzed the stools from the first two of the nine patients. Several methods to purify DNA were evaluated, and the

most reproducible procedure was used (18). Approximately 100 mg of stool frozen at -80°C was diluted with 300 μl of lysis buffer [500 mM tris, 16 mM EDTA, 10 mM NaCl (pH 9.0)], and particulates and most bacteria were removed by centrifugation. Proteins were digested with proteinase K and extracted with phenol and chloroform. After ethanol precipitation, the DNA was further purified by binding to glass beads. From 0.5 to 5.0 μg of DNA was typically obtained. The first exon of *K-ras* was then PCR-amplified from this DNA as described above. Because we initially expected that mutant *ras* would represent only a small fraction of the total *ras* DNA in stool (if present at all), we used a sensitive technique for analysis. This technique allowed the identification of a small fraction of mutant p53 genes in the urine of patients with advanced bladder cancers and can reveal the existence of even one mutant gene among several thousand normal genes (19). The PCR products were cloned in a bacteriophage vector and the phage DNA transferred to nylon filters (20). These filters were then incubated with ^{32}P -labeled oligonucleotides that recognized either wild-type *K-ras*, the mutant *K-ras* found in the tumor, or another mutant *K-ras* gene as a negative control. With this assay, we found that both patients contained mutant *ras* in the DNA purified from their stool samples. The mutant genes detected in the stool were the same as those detected in the tumors (Val¹² in the stool and tumor of patient 1; Asp¹³ in the stool of patient 2, Fig. 1). A control stool sample from a patient without a *ras* mutation in his tumor contained no mutation at either of these positions (Fig. 1, patient 10).

Table 1. Patients studied for stool *ras* gene mutations.

Patient	Age/sex	Tumor location	Tumor type/stage*	Tumor size (cm ³)	Tumor mutation†	Mutant <i>ras</i> gene in stool‡
1	52/F	Rectum	Carcinoma/C	5.8 × 6.5 × 2.7	Val ¹²	+
2	63/M	Sigmoid colon	Adenoma	1.5 × 1.5 × 0.6	Asp ¹³	+
3	51/F	Rectum	Carcinoma/C	2.8 × 2.0 × 0.4	Asp ¹²	+
4	61/M	Rectum	Carcinoma/C	2.5 × 4.7 × 1.8	Asp ¹²	+
5	70/M	Rectum	Carcinoma/A	1.0 × 0.9 × 0.4	Asp ¹³	–
6	71/M	Rectum	Carcinoma/B	5.9 × 6.4 × 1.7	Asp ¹²	+
7	51/F	Ascend colon	Carcinoma/B	4.3 × 3.4 × 1.4	Asp ¹²	+
8	69/M	Sigmoid colon	Carcinoma/B	4.8 × 3.0 × 1.2	Asp ¹³	+
9	67/M	Cecum	Adenoma	6.0 × 4.0	Asp ¹²	+
10	61/M	Splenic flex	Carcinoma/C	12 × 6.5	None	–
11	34/M	Sigmoid colon	Carcinoma/B	7 × 3 × 2	None	–
12	63/M	Sigmoid colon	Carcinoma/C	2.1 × 3.1 × 0.4	None	–
13	42/F	NA§	NA		NA	–
14	53/F	NA§	NA		NA	–
15	63/F	NA§	NA		NA	–

*Carcinomas were classified according to Duke (13): A, confined to muscularis propria; B, extension through muscularis propria, but confined to colon; C, metastatic to regional lymph nodes. †Determined by sequence analysis of codons 12 or 13 of *K-ras*. ‡As assessed by the plaque hybridization or Southern blot (DNA) assay with three mutant specific oligomers (Val¹², Asp¹², and Asp¹³) as probes. §Patients 13, 14, and 15 had no colorectal neoplasms found at colonoscopy (NA = not applicable). ||Only measurements of the external surfaces of these tumors were available.

The fraction of phage plaques hybridizing to the mutant-specific oligonucleotide in patients 1 and 2 was high, representing 8 and 4%, respectively, of the phage hybridizing to the oligonucleotide specific for the wild-type K-ras. This suggested that a less sensitive, but simpler, assay could be used to identify mutant genes in stool samples. For this purpose, crude PCR products were simply subjected to electrophoresis through an agarose gel and transferred to nylon filters by the method of Southern (21). These blots were then incubated with ³²P-labeled oligonucleotides that recognized wild-type or mutant K-ras. The Val¹² mutation of patient 1 was easily observed in stool with the Southern blot (DNA) assay (Fig. 2, top). The oligonucleotide specific for the Asp¹² mutant provided a negative control (Fig. 2, middle). The wild-type-specific oligonucleotide hybridized to DNA from both tumor and stool, as expected (Fig. 2, bottom). Similarly, Southern blot analysis revealed that the tumors and stool of the DNA from patients 3 and 4 both contained the Asp¹² mutation, whereas neither hybridized to the Val¹²-specific oligonucleotide (Fig. 2). The ratio of mutant to wild-type hybridization in the stool samples was five- to tenfold lower than that in the tumors, consistent with the plaque hybridization assays.

Southern blot analysis detected mutations in stool from eight of the nine patients (Table 1). Mutations originating in benign tumors (patients 2 and 9) and malignant tumors were detected. Tumors as small as 1.3 cm³ gave rise to detectable mutant genes in the stool (patient 2). Moreover, proximal as well as distal tumors (patient 9, cecum; patient 7, ascending colon) yielded positive results.

As controls, we examined six stool samples, three from individuals with no colorectal neoplasia and three from individuals with colorectal tumors that did not contain K-ras mutations at codons 12 or 13. In all six cases, strong hybridization to the wild-type-specific oligonucleotides, but not to the oligonucleotides specific for Val¹², Asp¹², or Asp¹³ mutations, was observed (Table 1 and Figs. 1 and 2).

We were surprised at the ease with which K-ras mutations were identified in the stool. However, rough calculations indicate that colorectal tumors could constitute a significant fraction of the human DNA present in stool. The colon is more than a meter in length, but the epithelium is confined to a lining only a few millimeters thick. It is estimated that the normal adult colon contains 5 × 10¹⁰ epithelial cells (22). One-sixth to one-third of these are shed every 24 hours, giving rise to approximately 10¹⁰ normal cells per day (23). A tumor of 1 cm³ may contain more

than 10⁹ cells. These cells turn over at similar or elevated rates compared to normal cells (24). Thus, it is conceivable that more than 1% of the epithelial cells shed from colon into the stool could be derived from tumors of this size. It is also possible that tumor cells are more resistant to the degradative processes in feces or that apoptosis, which degrades DNA in normally differentiating cells, is not fully operative in tumor cells.

Mutations of *ras* are particularly amenable for these studies because they are present in both benign and malignant CR tumors and mutations occur at relatively few codons (8–12). However, *ras* mutations are found in only 50% of such tumors. Other mutant genes present in colorectal tumors (7) could probably be detected in the stool, which would increase the potential sensitivity of this strategy. With the help of *ras* and other gene probes, additional studies, in which the stools from a large number of patients with colorectal tumors of varying size, stage, and anatomical position are analyzed, will be needed to more accurately determine the sensitivity of such tests.

These results provide the conceptual and practical basis for a new approach for detecting the presence of colorectal tumors in a noninvasive fashion. The approach could have use in monitoring patient populations on different diets or treatments designed to minimize the incidence of colorectal neoplasia (25). It also could eventually find use in screening asymptomatic patients, especially those at risk by virtue of inherited or environmental factors (25, 26), for the presence of colorectal neoplasia. An analysis of data from only *ras* probes suggests that some early colorectal cancers and premalignant lesions might be identifiable through this strategy. Because colorectal tumors are so common, this approach has implications for public health.

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18. The stool-lysis buffer mix was vortexed for 30 s and cleared by centrifugation at 12,000g for 2 min. DNA in the supernatant was purified by SDS-proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation [as described in S. E. Goelz, S. R. Hamilton, B. Vogelstein, *Biochem. Biophys. Res. Commun.* **130**, 118 (1985)]. The DNA from stool was then further purified by binding to glass [B. Vogelstein and D. Gillespie, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 615 (1978)].
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17 January 1992; accepted 2 March 1992

CD19: Lowering the Threshold for Antigen Receptor Stimulation of B Lymphocytes

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Lymphocytes must proliferate and differentiate in response to low concentrations of a vast array of antigens. The requirements of broad specificity and sensitivity conflict because the former is met by low-affinity antigen receptors, which precludes achieving the latter with high-affinity receptors. Coligation of the membrane protein CD19 with the antigen receptor of B lymphocytes decreased the threshold for antigen receptor-dependent stimulation by two orders of magnitude. B lymphocytes proliferated when approximately 100 antigen receptors per cell, 0.03 percent of the total, were coligated with CD19. The B cell resolves its dilemma by having an accessory protein that enables activation when few antigen receptors are occupied.

The immune system must respond to low concentrations of antigen for the efficient elimination of infections. The growth and differentiation of lymphocytes are mediated by antigen receptors that have low affinity for their ligands because they are products of recombinatorial gene rearrangement that takes place in the absence of selection by antigen. Therefore, lymphocytes must have mechanisms that enable them to be stimulated when relatively few receptors have bound antigen. The T lymphocyte has the accessory membrane proteins CD4 and CD8, which, when coligated with the T cell antigen receptor (TCR) by the major histocompatibility complex (MHC)-peptide complex, decrease the number of TCRs that must be ligated (1). No membrane protein of the B lymphocyte that is analogous to CD4 and CD8 has been identified.

The CD19 membrane protein, a member of the immunoglobulin (Ig) superfamily, is B cell-specific and is expressed at each developmental stage except that of the terminally differentiated plasma cell (2). It is a component of a complex that contains at least two other membrane proteins, complement receptor type 2 (CR2, also called CD21) and TAPA-1 (3). The CR2 protein mediates the capacity of the complement system to enhance the production of antibody in response to low concentrations of

antigen in vivo (4). Both CD19 and CR2 augment activation of phospholipase C in B cells when coligated with membrane Ig (mIg), but independent ligation of CD19 suppresses B cell activation (5, 6).

To determine whether ligating CD19 alters the capacity of mIgM to induce DNA synthesis, replicate samples of human B cells and mitomycin-treated murine L cells that express human Fc γ RII (Fc γ RII-L cells) (7) were cultured for 2.5 days in the presence of incremental concentrations of a monoclonal antibody (MAb) to IgM, a saturating concentration of MAb to CD19, or an equal concentration of an irrelevant MAb, MOPC-21, and interleukin-4 (IL-4). In the absence of anti-CD19, the lowest concentration of anti-IgM that induced incorpo-

ration of [^{3} H]thymidine by B cells above the background incorporation was 6.7×10^{-11} M. In the presence of anti-CD19, this was reduced to 6.7×10^{-13} M (Fig. 1) (8, 9). At higher concentrations of anti-IgM, anti-CD19 also increased the magnitude of [^{3} H]thymidine incorporation to twice that induced by anti-IgM alone. Anti-CD19 alone did not induce proliferation. The ligation of CD19 both lowered the threshold for B cell activation by mIgM and increased the magnitude of the B cell response to optimal levels of mIgM stimulation.

In a parallel experiment to assess the binding characteristics of the monoclonal anti-IgM, replicate samples of B cells and Fc γ RII-L cells were incubated with incremental concentrations of [^{125} I]-labeled anti-IgM in the presence or absence of 2×10^{-7} M unlabeled anti-IgM. Cell-bound and free antibody were separated, and specific binding was calculated. The L cells alone did not specifically bind the radiolabeled antibody. Scatchard analysis showed that the anti-IgM bound to the mixture of B cells and L cells with a dissociation constant (K_d) of 1.8×10^{-9} M, and to 2.7×10^5 sites per cell at saturation. The presence of anti-CD19 did not alter the K_d of the anti-IgM for B cells in the presence of Fc γ RII-L cells (10). Therefore, in Fig. 1, the threshold concentration of anti-IgM that induced B cell proliferation in the presence of anti-CD19 bound to only 92 mIgM molecules per cell, or 0.03% of the total mIgM expressed per cell.

We measured the fraction of CD19 that must be ligated to augment B cell proliferation by incubating replicate samples of B cells and Fc γ RII-L cells in the presence of suboptimal anti-mIgM, IL-4, and incremental concentrations of anti-CD19 for 2.5 days, after which incorporation of [^{3} H]thymidine was assayed. The enhancing effect of anti-CD19 increased incrementally from 6.7×10^{-12} M, which bound <0.2% of CD19, up to a saturating concentration of 6.7×10^{-8} M (Fig. 2A). To determine

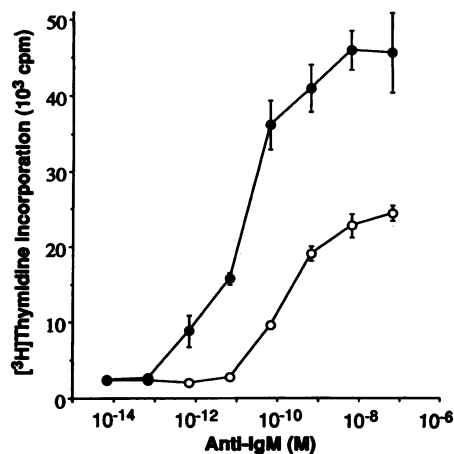


Fig. 1. Enhancement of DNA synthesis in B lymphocytes by the coaggregation of mIgM and CD19. Replicate samples of 5×10^4 peripheral blood B cells (8) were cultured in 0.2 ml of RPMI-1640 with 10% fetal calf serum in flat-bottom culture plates with 2×10^4 mitomycin-treated fibroblastic L cells expressing human CDw32/Fc γ RII and recombinant IL-4 (200 units/ml, Genzyme). The B cells were stimulated with a range of concentrations of MAb DA4.4 anti-IgM (9) together with either control antibody MOPC-21 (○) or HD37 anti-CD19 (●). Cells were pulsed with [^{3} H]thymidine for the last 16 hours of a 60-hour culture. Results are means $\pm$ SD of triplicates. Cells cultured with either control antibody or anti-CD19 alone incorporated 2726 ± 256 and 2475 ± 255 counts per minute (cpm), respectively. Representative of four experiments.

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Identification of *ras* Oncogene Mutations in the Stool of Patients with Curable Colorectal Tumors

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Science **256** (5053), . DOI: 10.1126/science.1566048

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