ing may repeat the lessons learned in attempts to control pests and pathogens (*1*), albeit over a somewhat longer time scale. Moreover, the genetic changes caused by selective harvest may be irreversible; cessation of harvest does not guarantee reverse selection back to the original state (*22*). Ignoring evolutionary consequences of selective harvest contradicts the precautionary approach to resource conservation.

What forms of management might help to reduce or incorporate evolutionary changes due to selective fishing? First, the establishment of no-take reserves or marine protected areas may, if properly designed, provide for the maintenance of natural genetic variation by allowing a portion of the stock to express an unconstrained range of sizes and growth rates (*26*, *27*). Second, reliance on minimum size restriction (all fish below a given size are protected) as a basis for management needs rethinking. Where feasible, maximum size limits (all fish above a given size are protected) may offer some important advantages: (i) fast-growing genotypes that pass more quickly through the period of vulnerability would be favored by selection; (ii) the age structure would broaden, thereby increasing spawning stock biomass; and (iii) the ecosystem services provided by large animals would be restored (*2*). Harvest regimes that account for the Darwinian effects of fishing need serious consideration if yields are to be truly sustainable.

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Materials and Methods

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An Essential Role of N-Terminal Arginylation in Cardiovascular Development

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The enzymatic conjugation of arginine to the N-termini of proteins is a part of the ubiquitin-dependent N-end rule pathway of protein degradation. In mammals, three N-terminal residues—aspartate, glutamate, and cysteine—are substrates for arginylation. The mouse *ATE1* gene encodes a family of Arg-tRNA-protein transferases (R-transferases) that mediate N-terminal arginylation. We constructed ATE1-lacking mouse strains and found that *ATE1*$^{-/-}$ embryos die with defects in heart development and in angiogenic remodeling of the early vascular plexus. Through biochemical analyses, we show that N-terminal cysteine, in contrast to N-terminal aspartate and glutamate, is oxidized before its arginylation by R-transferase, suggesting that the arginylation branch of the N-end rule pathway functions as an oxygen sensor.

Substrates of the ubiquitin (Ub)–dependent N-end rule pathway include proteins with destabilizing N-terminal residues (*1*–*4*). A set of amino acids that are destabilizing in a given cell yields a rule, called the N-end rule, that relates the in vivo half-life of a protein to the identity of its N-terminal residue (*1*–*3*, *5*–*8*). The N-end rule has a hierarchic structure. Specifically, N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their deamidation, by N-terminal amidohydrolases (*7*), to yield the secondary destabilizing residues Asp and Glu, whose activity requires their conjugation, by *ATE1*-encoded Arg-tRNA-protein transferases (R-transferases) (*5*), to Arg, one of the primary destabilizing residues. The latter are recognized by the Ub ligases (E3 enzymes) of the N-end rule pathway (Fig. 1A) (*3*, *4*, *9*).

In mammals, the set of destabilizing residues that function through their arginylation includes not only Asp and Glu but also Cys, which is a stabilizing (nonarginylated) residue in the yeast *Saccharomyces cerevisiae* (*5*, *10*, *11*). ATE1-1 and ATE1-2, the isoforms of mammalian R-transferase, are produced through alternative splicing of *ATE1* pre-mRNA and have the same specificity as the yeast R-transferase: They arginylate N-terminal Asp or Glu but not Cys (*5*). This raises the question of how N-terminal Cys is arginylated in mammalian cells. To address this issue and the physiological functions of arginylation, we constructed *ATE1*$^{-/-}$ mouse strains (*12*).

Whereas *ATE1*$^{+/-}$ mice were apparently normal, the *ATE1*$^{-/-}$ genotype conferred embryonic lethality (*12*). The *ATE1*$^{-}$ allele was marked with NLS-β-galactosidase (βgal) (*12*). During embryonic day (E) 9.5 to 12.5, the expression of βgal was high in the neural tube and other specific (often sharply delineated) regions of developing embryo (*12*). *ATE1*$^{-/-}$ embryos were pale and had thinner blood vessels and frequent edemas of the skin (Fig. 1, B and C; Fig. 2, A and B) (*12*). Hemorrhages were a consistent feature of *ATE1*$^{-/-}$ embryos and were the likely proximal cause of their death (Fig. 1, D and E). Of 22 *ATE1*$^{-/-}$ hearts (E13.5 to E15.5) examined, ~85% had a ventricular septal defect (VSD) (Fig. 1, I and J). The atria of *ATE1*$^{-/-}$ hearts were thin walled, with sparse trabeculae and a large atrial septal defect (ASD) (Fig.

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1J). About 90% of examined *ATE1*$^{-/-}$ hearts exhibited hypoplasia of both right and left ventricular myocardium (Fig. 1, G, I, and J). The compact zone of left ventricular myocardium was two or three cells thick, in contrast to 7 to 10 cells in +/+ embryos (Fig. 1, F and G). Whereas the aorta and pulmonary artery were completely separated in E13.5 +/+ hearts (Fig. 1K), ~70% of the examined *ATE1*$^{-/-}$ hearts (E13.5 to E15.5) had persistent truncus arteriosus, with the common root of aorta and pulmonary artery straddling a large VSD (Fig. 1L).

Vasculogenesis, the de novo formation of uniformly sized blood vessels that yields the primary capillary plexus, was apparently normal in E9.5 *ATE1*$^{-/-}$ embryos (Fig. 2, G and H) (*12*). In contrast, the process of angiogenic remodeling—such as, for example, the formation of vessels that normally sprout from intersegmental artery and cross the dorsal midline—was suppressed in E9.5 *ATE1*$^{-/-}$ embryos (Fig. 2, C and D) (*12*). The vessels in E13.5 *ATE1*$^{-/-}$ sacs often terminated prematurely (Fig. 2F, arrow), and many small vessels remained as a honeycomb-like meshwork of the primary-plexus capillaries (Fig. 2F, arrowhead), indicating a perturbation of angiogenic remodeling in *ATE1*$^{-/-}$ embryos.

To examine the N-end rule pathway in *ATE1*$^{-/-}$ cells, we transfected +/+ and *ATE1*$^{-/-}$ embryonic fibroblast (EF) cell lines with plasmids encoding X-nsP4^{f}, a set of 69-kD Flag-tagged Sindbis virus RNA polymerase proteins bearing different N-terminal residues (*3, 12*) (Fig. 1A). The R-transferase substrates Asp-nsP4^{f} and Glu-nsP4^{f} were short-lived in +/+ cells but were completely stabilized in *ATE1*$^{-/-}$ cells (Fig. 3, A and B).

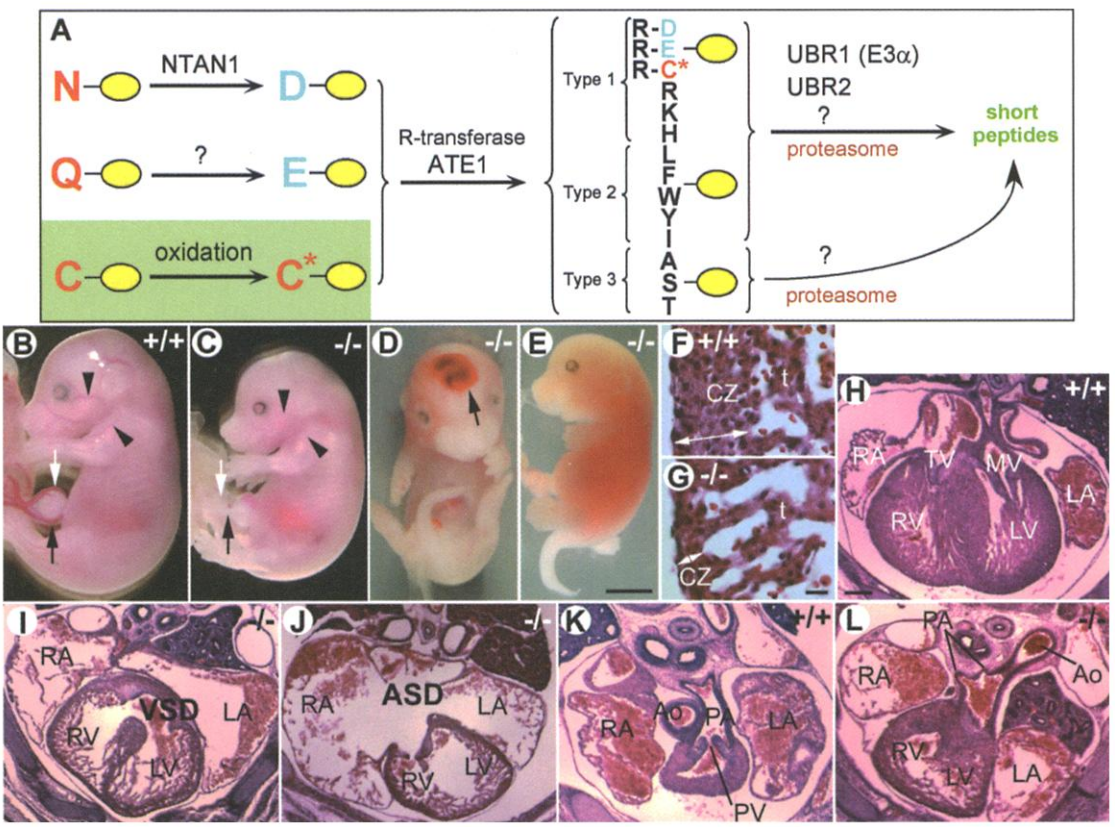

Fig. 1. The N-end rule pathway and phenotypes of *ATE1*$^{-/-}$ embryos. (**A**) N-terminal residues are indicated by single-letter abbreviations for amino acids (*22*). Yellow ovals denote the rest of a protein substrate. The area highlighted in green describes the understanding gained in the present work (see text). C* denotes an oxidized Cys residue. Type 1 and 2 primary destabilizing residues are recognized by functionally overlapping E3s that include UBR1 (E3α) and UBR2 (*3, 9*). N-terminal Ala, Ser, and Thr are recognized by an unidentified E3. (**B** to **E**) Whole mounts of +/+ (B) and *ATE1*$^{-/-}$ (C to E) E15.5 embryos. (**F** and **G**) Left ventricular wall in E14.5 +/+ (F) and *ATE1*$^{-/-}$ (G) embryos. (**H** to **L**) Transverse sections of hematoxylin and eosin–stained +/+ (H and K) and *ATE1*$^{-/-}$ (I, J, and L) E14.5 hearts. TV, tricuspid valve; MV, mitral valve; RV, right ventricle; LV, left ventricle; Ao, aorta; PA, pulmonary artery; RA, right atrium; LA, left atrium; CZ, compact zone; t, trabeculae. Scale bars, 2 mm (B to E), 10 μm (F and G), 200 μm (H to L).

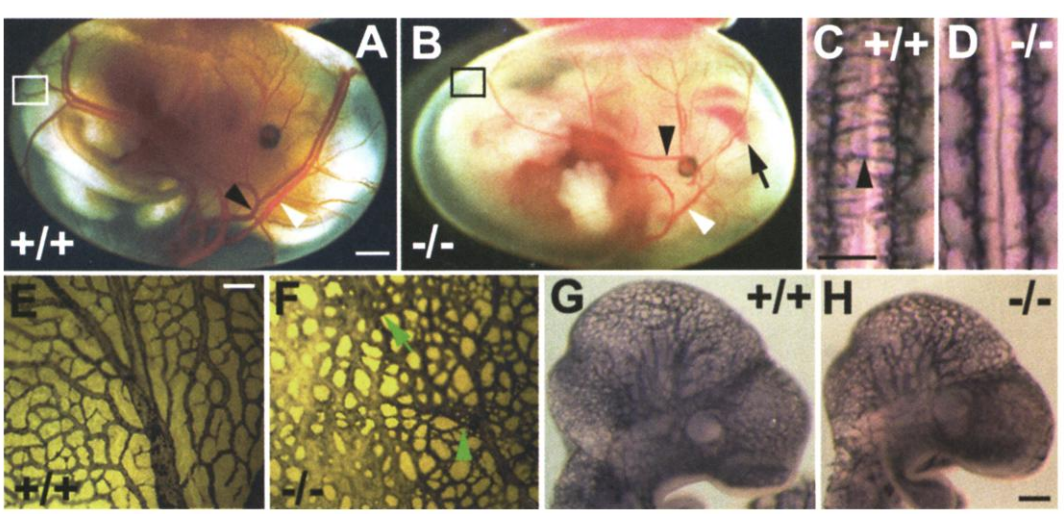

Fig. 2. Perturbation of angiogenic remodeling but not of vasculogenesis in *ATE1*$^{-/-}$ embryos. (**A**) +/+ and (**B**) *ATE1*$^{-/-}$ E13.5 embryos with yolk sacs. Black arrowheads, major artery; white arrowheads, major vein; arrow, a local hemorrhage. (**C** and **D**) Dorsal views of the trunks of PECAM-1–stained E9.5 +/+ (C) and *ATE1*$^{-/-}$ (D) embryos. (**E** and **F**) Similarly located areas (rectangles in A and B) of +/+ (E) and *ATE1*$^{-/-}$ (F) PECAM-1–stained E13.5 yolk sacs. (**G** and **H**) E9.5 +/+ (G) and *ATE1*$^{-/-}$ (H) embryos stained with antibody to PECAM-1. Scale bars, 1 mm (A), 250 μm (C and H), 100 μm (E).

Strikingly, although R-transferases cannot arginylate the N-terminal Cys (*5*), the normally short-lived Cys-nsP4^f also became long-lived in *ATE1*$^{-/-}$ cells (Fig. 3, A and B), indicating that N-terminal Cys, perhaps as a result of a preceding chemical modification, was also a substrate of *ATE1*-encoded R-transferases. To measure arginylation directly, we added purified Ub-X-βgal proteins (X = Met, Arg, Glu, or Cys) or human α-lactalbumin (bearing N-terminal Glu) to +/+ and *ATE1*$^{-/-}$ EF extracts supplemented with ATP, total tRNA, and aminoacyl-tRNA synthetases (Fig. 3C) (*10, 12*). Ub-X-βgals are rapidly deubiquitylated in vivo and in cell-free extracts, yielding X-βgals (*10*). Asp-βgal, Glu-βgal, and α-lactalbumin were arginylated in +/+ EF extracts, in contrast to Arg-βgal (bearing a primary destabilizing residue) and Met-βgal (bearing a stabilizing residue) (Fig. 3C). No arginylation of Asp-βgal, Glu-βgal, and α-lactalbumin was detected in *ATE1*$^{-/-}$ EF extracts (Fig. 3C) or in extracts from *ATE1*$^{-/-}$ embryos (fig. S5A), consistent with the in vivo results (Fig. 3, A and B). Surprisingly, the N-terminal Cys of Cys-βgal was not arginylated in +/+ or *ATE1*$^{-/-}$ extracts (Fig. 3C), suggesting that the demonstrated *ATE1* dependence of the in vivo degradation of Cys-bearing N-end rule substrates (Fig. 3, A and B) may involve a modification of N-terminal Cys before its arginylation. A protease called Asp-N cleaves peptide bonds N-terminal to the Asp and $CysO_3$ residues (*13*), and aspartate aminotransferases use both Asp and oxidized Cys as substrates (*14*). Thus, either the Cys sulfinic acid residue ($CysO_2$) or the cysteic acid residue ($CysO_3$) may be sufficiently close in structure to Asp (Fig. 3E) to serve as a substrate of R-transferases.

One prediction of the Cys-oxidation hypothesis was that the arginylated Cys residue should exist as $CysO_2$ or $CysO_3$. This was verified and confirmed with mouse RGS4, a guanosine triphosphatase–activating protein that bears N-terminal Cys and is arginylated and degraded by the N-end rule pathway in rabbit reticulocyte extract (*11*). RGS4-His_6 was expressed in mouse L cells, purified, treated with iodoacetamide to alkylate Cys residues (thereby making them identifiable by the sequencing procedure used), and N-terminally sequenced by Edman degradation. Both arginylated and unarginylated RGS4s were detected, the former being a major species (Fig. 3D). Remarkably, whereas the expected Cys at position 12 of arginylated RGS4 could be identified as alkylated Cys, the expected (alkylated) Cys at position 2 (position 1 in the unarginylated RGS4) could not be identified by the Edman procedure (Fig. 3D), indicating that N-terminal Cys of RGS4 had been modified before alkylation. Alkylated RGS4 was cleaved with cyanogen bromide (CNBr); HPLC fractionation and online mass spectrometric sequencing of CNBr-produced peptides identified residue 2 of RGS4 as cysteic acid ($CysO_3$) (*12*).

Another prediction of the Cys-oxidation hypothesis was that the yeast R-transferase should be able to rescue the destabilizing activity of Cys in mouse *ATE1*$^{-/-}$ cells, owing to the presence of Cys-oxidation activity in these cells. This was verified using pulse-chase assays with *ATE1*$^{-/-}$ EFs expressing X-nsP4^f and either *S. cerevisiae* ATE1 or enzymatically impaired ATE1^{C23A} mutant (*12, 15*). Both Asp-nsP4^f and Cys-nsP4^f, which were long-lived in mouse *ATE1*$^{-/-}$ EFs (Fig. 3, A and B), became short-lived in the presence of yeast ATE1 (*12*). The rescue by yeast R-transferase required its enzymatic activity, because ATE1^{C23A}, a catalytically impaired mutant (*12, 15*), had a significantly weaker effect (*12*). These findings, in conjunction with stoichiometric oxidation of N-terminal Cys in mouse cells (Fig. 3D) (*12*), indicate that the oxidation is an enzymatic (rather than uncatalyzed) reaction, because the intracellular solvent conditions, including redox potential, are likely to be similar in mammalian and yeast cells.

We have identified a physiological function—cardiovascular development—for the posttranslational conjugation of Arg to N-termini of proteins, a reaction first described 40 years ago (*16*). We have also shown that the N-terminal Cys undergoes two (rather than one) covalent modifications—oxidation and arginylation—by the N-end rule pathway (Fig. 1A). Met-aminopeptidases cleave off the N-terminal Met of a newly formed protein if a second residue is small enough. Among the arginylatable residues (Fig. 1A), only Cys satisfies this condition (*2, 3, 10*). Fumagillin and related suppressors of angiogenesis have

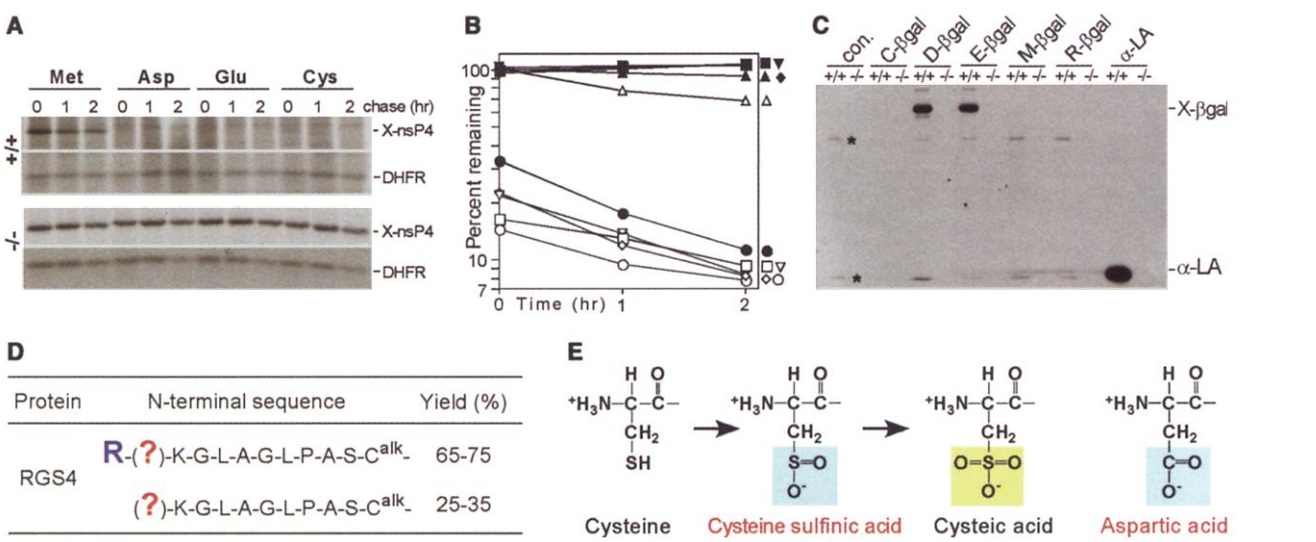

Fig. 3. Stabilization of N-end rule substrates in *ATE1*$^{-/-}$ cells, and oxidation-arginylation of N-terminal cysteine. (**A**) +/+ and *ATE1*$^{-/-}$ EF cells were transfected with plasmids expressing ^{f}DHFR^h-Ub^{R48}-X-nsP4^f, whose cleavage yielded the ^{f}DHFR^h-Ub^{R48} reference (DHFR) and X-nsP4^f reporter (X = Met, Arg, Asp, Glu, or Cys) (*3, 12*). Cells were pulse-labeled for 10 min with [^{35}S]methionine and chased for 1 and 2 hours (*12*). (**B**) Quantitation of results in (A). For each time point, the ratio of ^{35}S in X-nsP4^f (X = Arg, Asp, Glu, or Cys) to ^{35}S in the ^{f}DHFR^h-Ub^{R48} reference, at the same time point, was plotted as the percentage of this ratio relative to that for Met-nsP4^f at time 0 (*3, 23*). Open and closed symbols: +/+ and *ATE1*$^{-/-}$ cells, respectively. Triangles, Met-nsP4^f; circles, Arg-nsP4^f; squares, Asp-nsP4^f; inverted triangles, Glu-nsP4^f; diamonds, Cys-nsP4^f. (**C**) Cell-free assay for R-transferase using [^{3}H]Arg, S105 extracts from +/+ and *ATE1*$^{-/-}$ EFs, and unlabeled X-βgals (X = Cys, Asp, Glu, Met, or Arg) or α-lactalbumin (*12*). Asterisks indicate arginylated endogenous proteins in the extracts. (**D**) Determination, through Edman degradation, of N-terminal sequences (*22*) of RGS4-Flag-His_6 isolated from mouse L cells. C^{alk}, alkylated Cys residue. (**E**) Chemical formulas of N-terminal Cys, Cys sulfinic acid, cysteic acid, and Asp residues.

been shown to inhibit the Met-aminopeptidase MetAP2 (*17, 18*). The effect of these antiangiogenic reagents may stem from inhibition of the N-terminal Met-Cys cleavage in a normally short-lived regulator of angiogenesis that is targeted by the N-end rule pathway through its N-terminal Cys residue.

The oxidation (and subsequent arginylation) of N-terminal Cys may compete with its other known modifications, including acetylation and palmitoylation. N-end rule substrates with the arginylation-dependent destabilizing N-terminal residues (Asn, Gln, Asp, Glu, and Cys) (Fig. 1A) can also be produced through cleavages anywhere in a protein's polypeptide chain. For example, the conditional cleavage of a subunit of the mammalian cohesin complex at the metaphase-anaphase transition is predicted to produce a putative N-end rule substrate whose degradation would require N-terminal arginylation (*8, 19*).

HIF1α, a subunit of hypoxia-inducible factor 1 (HIF1) that functions as a key regulator of angiogenesis, is a conditionally short-lived protein. The degron of HIF1α, recognized by a distinct Ub-dependent proteolytic pathway, is activated through the oxygen-dependent hydroxylation of a specific Pro residue (*20, 21*). By analogy to prolyl-4-hydroxylases that regulate the degron of HIF1α, the currently unknown enzyme that oxidizes N-terminal Cys may also function as an oxygen sensor. If so, the formation and maintenance of the cardiovascular system may involve a battery of distinct, conditionally short-lived regulators such as HIF1 and the currently unknown substrate of the N-end rule pathway that bears N-terminal Cys.

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22. Single-letter abbreviations for amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Supporting Online Material

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Materials and Methods
Figs. S1 to S5
References

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Target-Selected Inactivation of the Zebrafish *rag1* Gene

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The zebrafish has become a favorite organism for genetic analysis of vertebrate development, but methods for generating mutants by reverse genetic approaches have been lacking. We report a method to obtain stable mutants of a gene based on knowledge of the gene sequence only. Parental fish were mutagenized with *N*-ethyl-*N*-nitrosourea; in 2679 F_1 fish, the *rag1* gene was analyzed for heterozygous mutations by resequencing. In total, we found 15 mutations: 9 resulted in amino acid substitutions and 1 resulted in a premature stop codon. This truncation mutant was found to be homozygous viable and defective in V(D)J joining. Although presumably immune deficient, these homozygous *rag1* mutant fish are able to reach adulthood and are fertile. As sperm samples from all 2679 F_1 fish were collected and cryopreserved, we have in principle generated a mutant library from which mutants of most zebrafish genes can be isolated.

Forward genetic screens in the zebrafish have identified thousands of mutants defective in many kinds of biological processes (*1, 2*). Cloning of the affected genes is accelerated by both an extensive and rapidly growing genetic linkage map (*3–5*) and the zebrafish genome sequencing project. However, until now a major drawback in zebrafish genetic analysis has been the inability to specifically study gene function through reverse genetics. It has been demonstrated that protein levels in embryos can be temporally reduced by antisense morpholino oligonucleotides (*6*), but this effect is transient and is not applicable to later stages of development.

In the mouse, reverse genetics was made possible by homologous recombination in embryonic stem cells, eventually giving rise to germ line transmission of the mutant alleles (*7, 8*). Recently, it has been shown that in zebrafish short-term embryonic stem cell cultures are able to produce germ line chimeras (*9*), but this has not yet been extended to targeted gene inactivation. Therefore, we have taken another approach: target-selected mutagenesis. This involves random mutagenesis, followed by targeted screening for induced mutations at the genomic DNA level. For example, in *Caenorhabditis elegans*, we and others (*10, 11*) have successfully performed ethylmethane sulfonate mutagenesis and have screened animals with a polymerase chain reaction (PCR) strategy for deletions in hundreds of target genes. In the case of zebrafish, we have now combined *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis with high-throughput resequencing of the target gene.

Random ENU mutagenesis in zebrafish is widely used in forward screens. Nonmosaic mutants are typically found at tester loci at an average frequency of 1 in 650 mutagenized genomes. Recessive embryonic lethal phenotypes occur at a similarly high rate, namely, an average loss-of-function frequency per locus of 1 in 1600 mutagenized genomes (*1, 2*). Therefore, we constructed a library of 2679 randomly ENU mutagenized F_1 males as outlined in Fig. 1, making use of the Tübingen 2000 screen fish. Genomic DNA was isolated, and testis samples were cryopreserved (*12*), generating a permanent library that could be screened for heterozygous mutations. In principle, we have isolated sufficient DNA samples to screen for mutations in most zebrafish genes, and the library is comprehensive enough that most genes should be represented by at least one null allele.

To test this approach, we screened for mutations in the *rag1* gene (*13*) of each individual

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