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.

References and Notes

1. A. Bachmair, D. Finley, A. Varshavsky, *Science* **234**, 179 (1986).
2. A. Varshavsky, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 12142 (1996).
3. Y. T. Kwon, Z.-X. Xia, I. V. Davydov, S. H. Lecker, A. Varshavsky, *Mol. Cell. Biol.* **21**, 8007 (2001).
4. A. Hershko, A. Ciechanover, A. Varshavsky, *Nature Med.* **10**, 1073 (2000).
5. Y. T. Kwon, A. S. Kashina, A. Varshavsky, *Mol. Cell. Biol.* **19**, 182 (1999).
6. G. C. Turner, F. Du, A. Varshavsky, *Nature* **405**, 579 (2000).
7. Y. T. Kwon *et al.*, *Mol. Cell. Biol.* **20**, 4135 (2000).
8. H. Rao, F. Uhlmann, K. Nasmyth, A. Varshavsky, *Nature* **410**, 955 (2001).
9. Y. T. Kwon *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7898 (1998).
10. D. K. Gonda *et al.*, *J. Biol. Chem.* **264**, 16700 (1989).
11. I. V. Davydov, A. Varshavsky, *J. Biol. Chem.* **275**, 22931 (2000).
12. Materials and methods are available as supplementary material on *Science* Online.
13. T. Tetaz *et al.*, *Biochem. Int.* **22**, 561 (1990).
14. T. Yagi, H. Kagamiyama, M. Nozaki, *Biochem. Biophys. Res. Commun.* **90**, 447 (1979).
15. J. Li, C. M. Pickart, *Biochemistry* **34**, 15829 (1995).
16. H. Kaiji, G. D. Novelli, A. Kaiji, *Biochim. Biophys. Acta* **76**, 474 (1963).
17. E. C. Griffith *et al.*, *Chem. Biol.* **4**, 461 (1997).
18. N. Sin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6099 (1997).
19. S. Hauf, I. C. Waizenegger, J.-M. Peters, *Science* **293**, 1320 (2001).
20. M. Ivan *et al.*, *Science* **242**, 464 (2001).
21. P. Jaakkola *et al.*, *Science* **242**, 468 (2001).
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.
23. G. C. Turner, A. Varshavsky, *Science* **289**, 2117 (2000).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/297/5578/96/DC1
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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F_1 fish by DNA resequencing. By nested PCR, we amplified two fragments that included exons 2 and 3 of the coding region (Fig. 2A). Subsequent sequence analysis with five different primers (Fig. 2A; Table 1) was done on a 96-capillary ABI3700 DNA analyzer (*12*). Heterozygosity detection was done with the programs Phred (*14*), Phrap (*15*), and PolyPhred (*16*), and was visualized with Consed (*17*).

Single nucleotide polymorphisms (SNPs) were encountered at a high frequency; they could be distinguished from ENU-induced mutations because they were detected in multiple fish. As shown in Fig. 2B, we detected two haplotypes that differ from the published sequence of *rag1* at about 1 in 116 nucleotides; five of these SNPs result in coding changes in the *rag1* gene. The two haplotypes indicate that the fish used for this screen, the so-called Tübingen strain, is not homogeneous at this locus. Other loci also have been found to show heterogeneity within this library of F_1 fish (*18*).

After we filtered the SNPs, we detected a total of 15 ENU-induced mutations (Table 1). Each of these mutations was verified by independent PCR and resequencing. Because most mutation spectra are based on the detection bias of the forward screen, we can use these data to get an initial unbiased picture of the mutation spectrum of ENU in the zebrafish. As shown in Fig. 2C, the distribution of the mutations found in *rag1* is similar to those found in previous forward screens. The mutation rate is 15 mutations in 7,268,581 base pairs among 2679 fish (Table 1), which corresponds to a frequency of 2.1×10^{-6} per base pair.

Of the 15 mutations in *rag1*, 3 are silent, 2 are in intronic sequences (some introns were partially covered), and 10 result in changes in the coding sequence, of which 9

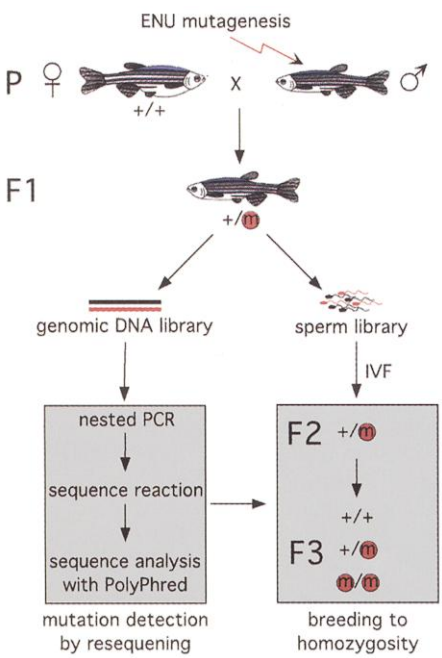

Fig. 1. Overview of target-selected mutagenesis in zebrafish. Ninety-nine adult male zebrafish were mutagenized by three to five consecutive treatments with 3 mM ENU, in accordance with (*32*). The mutagenized fish were crossed with wild-type females to give a nonmosaic F_1 generation of fish. Sperm was isolated and cryopreserved from 2679 fertile F_1 males. Genomic DNA was isolated, arrayed in PCR plates, and screened for mutations by nested PCR amplification of the target gene and subsequent DNA sequence analysis. After a particular mutation was identified, in vitro fertilizations (IVF) were performed to recover the F_2 line carrying the mutation (*12*). Finally, mutations can be bred to homozygosity and analyzed for phenotypes.

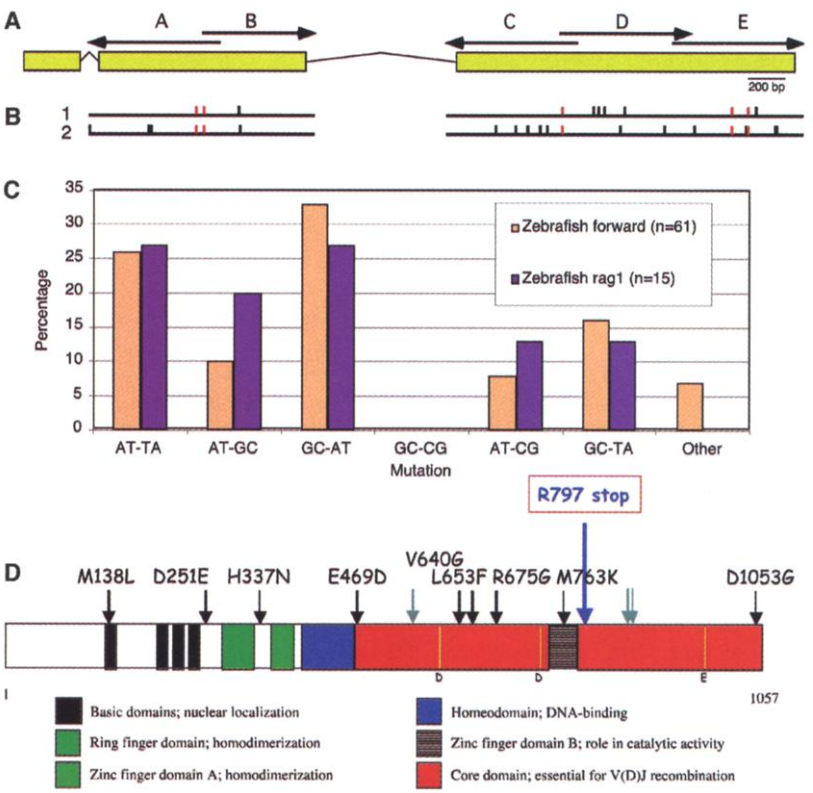

Fig. 2. Resequencing the *rag1* gene. (**A**) Genomic organization of the *rag1* gene. Coding regions are represented as yellow boxes. Regions A to E indicate segments screened by resequencing. (**B**) Distribution of SNPs in the two haplotypes of the *rag1* gene found in this study compared with the published sequence (*13*). Black bars indicate a SNP in one haplotype; red bars indicate a SNP in both haplotypes. (**C**) Spectrum of the ENU-induced mutations found in this screen compared with the mutations detected in previous forward screens. (**D**) Mutations changing the coding region of the *rag1* gene. RAG1 domain structure is adapted from (*33*). The DDE motif characteristic of recombinases (*34*) is indicated below the structure. Amino acid changes are indicated with black arrows above the structure: D, Asp; E, Glu; F, Phe; G, Gly; H, His; K, Lys; L, Leu; M, Met; N, Asn; R, Arg; V, Val. Purple arrow indicates stop codon. Gray arrows indicate silent mutations.

Table 1. *Rag1* resequencing statistics. Fragments correspond to the fragments indicated in Fig. 2A. All sequencing reactions were analyzed with PolyPhred (version 3.5 beta) with a ratio of 0.65, a background ratio of 0.15, and an average Phred score of 25. The total number of base pairs (bp) screened is corrected for fragment overlap and primer sequences. Failed sequences were excluded. Average lengths include standard errors. One mutation was found in fragment D, as well as in the overlapping part of fragment E.

Fragment	No. of reactions	Avg. length (bp)	No. of bp screened	No. of mutations
		Exon 2 (1118 bp)		
A	2,564	629 ± 2.2	1,613,420	4
B	2,615	602 ± 1.6	1,574,606	2
		Exon 3 (1748 bp)		
C	2,603	619 ± 1.5	1,611,149	4
D	2,053	581 ± 1.5	1,193,172	3
E	2,640	656 ± 1.2	1,730,797	3
Total	12,475	619 ± 0.8	7,723,144	16
Total corrected			7,268,581	15

are missense mutations and 1 is a premature stop (Fig. 2D). The latter is in the middle of the catalytic domain and, therefore, is expected to be a complete null allele. We genotyped the F_2 progeny of the fish that carry this allele (*rag1*^{t26683}) by isolating genomic DNA from fin clips, nested PCR, and sequencing. Crossing two F_2 heterozygotes gave Mendelian segregation of the mutant allele. The homozygous F_3 mutant embryos show *rag1* messenger RNA (mRNA) expression in the thymus (*19*) as seen by whole-mount in situ hybridizations (*18*). Adult mutant F_3 fish are viable and fertile in our standard nonsterile aquarium facilities.

A functional RAG1 protein is required for V(D)J recombination (*20*). Therefore, we examined functionality of the truncated RAG1 protein in mutant adults by a PCR assay (*21*) that checks for V(D)J rearrangements of the immunoglobulin heavy-chain genes (*22*) as outlined in Fig. 3A. Whereas heterozygous *rag1*^{t26683} mutant fish show joining of four different classes of heavy-chain genes, homozygous *rag1*^{t26683} mutant fish were found to be deficient in V(D)J joining (Fig. 3B). Apparently, as in other vertebrates, there is only one functional *rag1* gene; loss of function at this locus results in a complete block of immunoglobulin gene assembly and, presumably, in immunodeficiency.

Some of the missense mutations are at conserved amino acid positions; therefore, it is possible for these to affect gene function. Three other alleles have been tested for loss of function as heteroalleles over the null allele. These were not defective in V(D)J joining (Fig. 3B). Although we have assayed only for a loss-of-function phenotype, production of an allelic series is a major advantage of this method over knockout strategies that rely on homologous disruption or deletions. In some cases, complete loss of function may be lethal, and missense mutations may have informative viable phenotypes.

The clonal immune system was first acquired in evolution by teleost fish. Here we describe experimental inactivation of the immune system in a teleost fish. These presumably immunodeficient fish survive to adulthood without obvious signs of infectious disease in a nonsterile environment. Whether homozygous mutant lines can be bred without acquiring diseases at a higher rate than control cases remains to be seen. The *rag1*^{t26683} mutant is now available for detailed analysis of the role of the clonal immune system in resistance to infections and possibly in other processes.

We expect that the first-generation zebrafish mutant library described here will be followed by others, if only because the mutation efficiency can possibly be improved and the size of the library increased. Nevertheless, the current library has recently been used successfully to detect mutations in other genes (*18*), and new screens are ongoing. Once the DNA and the testes library were established, the time and reagent costs for the *rag1* screen were about 2 months and $12,500, respectively (*12*); these are currently being reduced by further automation and alternative mutation detection methods. With establishment of this technology, the zebrafish is now the second vertebrate model organism in which reverse genetics can be applied on a large scale.

Target-selected mutagenesis has also been successfully developed in the two major invertebrate model organisms, *Drosophila* (*23*–*25*) and *C. elegans* (*26*, *27*), as well as in *Arabidopsis* (*28*, *29*) and, recently, the mouse (*30*, *31*). The approach described here for zebrafish differs in that random mutagenesis is followed directly by straightforward, high-throughput resequencing of the target gene. This approach is insensitive to the presence of frequent SNPs in outbred organisms, because these are filtered out in the data analysis step. Therefore, this approach could be applicable for any organism on the condition that it can be bred and mutagenized efficiently. The advantage of multiple alleles, including partial loss-of-function mutants, may make it the preferred approach even for organisms for which gene knockout strategies already exist, such as *Drosophila* and *C. elegans*.

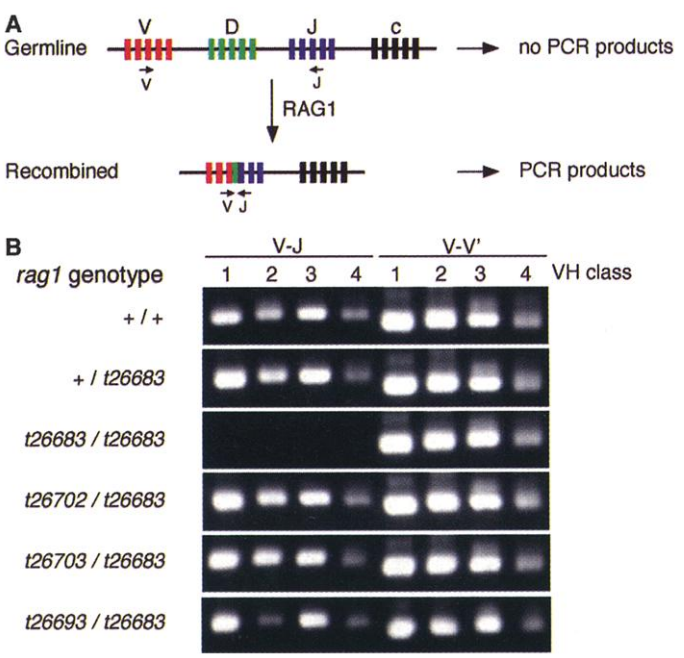

Fig. 3. Functionality of different RAG1 mutants. **(A)** Outline of the PCR assay used to test for V(D)J recombination of the immunoglobulin heavy-chain gene (*12*). PCR primers are represented as arrows. Semi-nested PCRs with an additional internal primer in the V segments were done to obtain a bright signal. C, constant domain. **(B)** V(D)J recombination in tail tissue from adult fish carrying different *rag1* alleles. The mutations tested are as follows: R797stop (*rag1*^{t26683}), D251E (*rag1*^{t26702}), L653F (*rag1*^{t26703}), and M138L (*rag1*^{t26693}). V-J, PCRs with primers in V segments and J segments. V-V', PCRs with primers in V segments only. VH, variable heavy chain.

References and Notes

1. P. Haffter *et al.*, *Development* **123**, 1 (1996).
2. W. Driever *et al.*, *Development* **123**, 37 (1996).
3. N. Hukriede *et al.*, *Genome Res.* **11**, 2127 (2001).
4. R. Geisler *et al.*, *Nature Genet.* **23**, 86 (1999).
5. I. G. Woods *et al.*, *Genome Res.* **10**, 1903 (2000).
6. A. Nasevicius, S. C. Ekker, *Nature Genet.* **26**, 216 (2000).
7. M. R. Capecchi, *Science* **244**, 1288 (1989).
8. S. Thompson, A. R. Clarke, A. M. Pow, M. L. Hooper, D. W. Melton, *Cell* **56**, 313 (1989).
9. C. Ma, L. Fan, R. Ganassin, N. Bols, P. Collodi, *Proc. Natl. Acad. Sci. U.S.A* **98**, 2461 (2001).
10. G. Jansen *et al.*, *Nature Genet.* **21**, 414 (1999).
11. L. X. Liu *et al.*, *Genome Res.* **9**, 859 (1999).
12. Materials and methods are available as supporting material on *Science* Online.
13. C. E. Willett, J. J. Cherry, L. A. Steiner, *Immunogenetics* **45**, 394 (1997).
14. B. Ewing, P. Green, *Genome Res.* **8**, 186 (1998).
15. D. Gordon, C. Desmarais, P. Green, *Genome Res.* **11**, 614 (2001).
16. D. A. Nickerson, V. O. Tobe, S. L. Taylor, *Nucleic Acids Res.* **25**, 2745 (1997).
17. D. Gordon, C. Abajian, P. Green, *Genome Res.* **8**, 195 (1998).
18. E. Wienholds, S. Schulte-Merker, B, Walderich, R. H. A. Plasterk, data not shown.
19. C. E. Willett, A. G. Zapata, N. Hopkins, L. A. Steiner, *Dev. Biol.* **182**, 331 (1997).
20. D. G. Schatz, M. A. Oettinger, D. Baltimore, *Cell* **59**, 1035 (1989).
21. N. Danilova and L. Steiner, personal communication.
22. N. Danilova, V. S. Hohman, E. H. Kim, L. A. Steiner, *Immunogenetics* **52**, 81 (2000).
23. D. G. Ballinger, S. Benzer, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9402 (1989).
24. K. Kaiser, S. F. Goodwin, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1686 (1990).
25. A. Bentley, B. MacLennan, J. Calvo, C. R. Dearolf, *Genetics* **156**, 1169 (2000).
26. R. R. Zwaal, A. Broeks, J. van Meurs, J. T. Groenen, R. H. Plasterk, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7431 (1993).
27. G. Jansen, E. Hazendonk, K. L. Thijssen, R. H. Plasterk, *Nature Genet.* **17**, 119 (1997).
28. S. Parinov, V. Sundaresan, *Curr. Opin. Biotechnol.* **11**, 157 (2000).
29. C. M. McCallum, L. Comai, E. A. Greene, S. Henikoff, *Nature Biotechnol.* **18**, 455 (2000).
30. D. R. Beier, *Mamm. Genome* **11**, 294 (2000).
31. E. L. Coghill *et al.*, *Nature Genet.* **19**, 19 (2002).
32. F. J. van Eeden, M. Granato, J. Odenthal, P. Haffter, *Methods Cell Biol.* **60**, 21 (1999).
33. L. D. Notarangelo, A. Villa, K. Schwarz, *Curr. Opin. Immunol.* **11**, 435 (1999).
34. J. Kulkosky, K. S. Jones, R. A. Katz, J. P. Mack, A. M. Skalka, *Mol. Cell Biol.* **12**, 2331 (1992).

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Materials and Methods

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Sustained Loss of a Neoplastic Phenotype by Brief Inactivation of *MYC*

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Pharmacological inactivation of oncogenes is being investigated as a possible therapeutic strategy for cancer. One potential drawback is that cessation of such therapy may allow reactivation of the oncogene and tumor regrowth. We used a conditional transgenic mouse model for *MYC*-induced tumorigenesis to demonstrate that brief inactivation of *MYC* results in the sustained regression of tumors and the differentiation of osteogenic sarcoma cells into mature osteocytes. Subsequent reactivation of *MYC* did not restore the cells' malignant properties but instead induced apoptosis. Thus, brief *MYC* inactivation appears to cause epigenetic changes in tumor cells that render them insensitive to *MYC*-induced tumorigenesis. These results raise the possibility that transient inactivation of *MYC* may be an effective therapy for certain cancers.

Activation of oncogenes plays an important role in tumorigenesis (*1*). Strategies that inactivate oncogenes for the treatment of cancer are in development; however, such approaches may be limited by the toxicity caused by the prolonged inactivation of the associated proto-oncogene. Moreover, cessation of the pharmacologic inactivation of an oncogene may result in tumor regrowth. To determine whether brief oncogene inactivation can produce sustained tumor regression, we used the tetracycline regulatory system to conditionally regulate *MYC* expression in transgenic mice. We previously described transgenic mice that conditionally express *MYC* in their lymphocytes (*2*). About 1% of these mice develop osteogenic sarcomas, and these tumors expressed abundant levels of MYC, presumably because the EμSRα enhancer causes *MYC* expression in immature osteoblasts. Consistent with this, *MYC* is commonly overexpressed in human and rodent osteogenic sarcomas (*3–11*).

The tumors in our transgenic model share some features with human osteogenic sarcoma (*12–14*). They present as invasive masses in the skeleton; they are associated with disorganized bone matrix; and they readily metastasize (fig. S1) (*15*). These properties were maintained as the tumors were adapted to in vitro growth and were inoculated into syngeneic hosts (*15*). To investigate the effects of *MYC* inactivation, we administered doxycycline (dox) treatment to mice with transplanted osteogenic sarcoma cells or primary transgenic tumors. After dox treatment in vivo, osteogenic sarcomas stopped expressing the *MYC* transgene, differentiated into mature bone, and exhibited sustained tumor regression (fig. S2). Similarly, primary transgenic tumors regressed and differentiated into bone (fig. S4). After dox treatment in vitro, the tumor cells exhibited a reduced growth rate, assumed a flattened morphology, lost alkaline phosphatase activity, and continued to express osteopontin (*15*) (Fig. 1). These phenotypic features are associated with the differentiation of immature osteoblasts into mature osteocytes (*7*, *16–18*). We conclude that *MYC* inactivation causes osteogenic sarcoma cells to differentiate into mature osteocytes.

To examine the effects of *MYC* inactivation and reactivation in individual tumor cells, we cultured osteogenic sarcoma cells

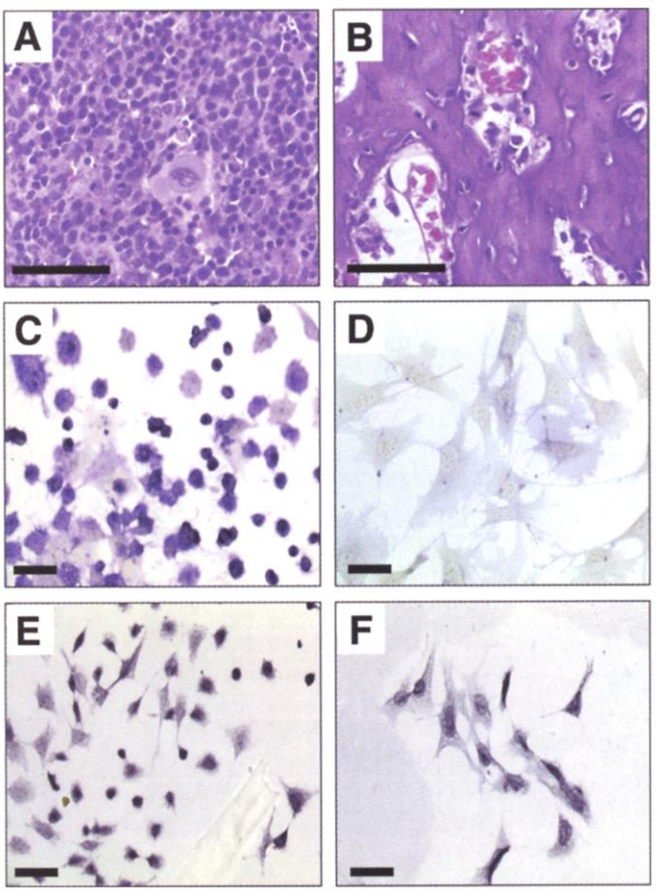

Fig. 1. Inactivation of *MYC* causes regression and differentiation of tumor cells. *MYC* inactivation resulted in the differentiation of **(A)** osteogenic sarcomas into **(B)** mature osteoid. Alkaline phosphatase activity **(C)** before and **(D)** after dox treatment. Osteopontin expression **(E)** before and **(F)** after dox treatment. Representative data from one of five experiments. At least five mice were injected per experiment. Similar results were seen for two other transplanted tumors and two independent primary transgenic tumors (*15*). Bars, 50 μm.

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