

4(P23L) mutant protein was diffusely cytoplasmic, suggests that CED-4 is recruited to nuclear membranes, possibly by interacting with another protein or protein complex. The identification of such a CED-4 receptor should help us understand the mechanism of action of CED-4 in the execution of programmed cell death.

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11. A partial *ced-9* cDNA was cloned into vector pET19b to generate a His-10-CED-9 fusion protein lacking the COOH-terminal 29 amino acids of CED-9. The fusion protein was purified on Ni²⁺-nitrilotriacetic acid-agarose (Qiagen) and injected with RIBI adjuvant into rabbits. The antiserum was affinity-purified against His-10-CED-9 immobilized on nitrocellulose. For the generation of anti-CED-4, full-length *ced-4S* cDNA was cloned into pXHA [S. J. Elledge et al., *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2907 (1992)]. The hemagglutinin-CED-4 fusion was purified from inclusion bodies of *Escherichia coli* and injected into rabbits and rats. The rabbit antiserum was used without further purification, and rat antiserum was affinity-purified against glutathione-S-transferase-CED-4 immobilized on nitrocellulose. Embryos were collected by bleaching mixed-stage worms in a 0.8 N NaOH, 8% hypochlorite solution. Embryos were fixed and permeabilized essentially as described [C. Guenther and G. Garriga, *Development* **122**, 3509 (1996)]. Embryos were incubated in a 1:100 dilution of primary antibody, washed four times, incubated for 2 hours in a 1:25 dilution of secondary antibody, washed four times, washed once in phosphate-buffered saline plus 4',6'-diamidino-2-phenylindole (DAPI, 1 µg/ml), and resuspended in an equal volume of VECTASHIELD mounting medium (Vector Labs). Embryos for mitochondrial colocalization experiments were collected from worms grown in the dark on NGM agar plates containing MitoTracker Red CMXRos (2 µg/ml, Molecular Probes).
12. Supplementary data can be found in Web figure 1 at www.sciencemag.org/feature/data/1046764.shl.
13. Mutant strains carrying the following alleles of cell-death genes were used in this study: *ced-1(e1735)*, engulfment-defective; *ced-3(n717)*, splice acceptor mutation, exon 7; *ced-9(n2812)*, Q46amber; *ced-9(n1950)*, G169E; *ced-9(n1950 n2161)*, *ced-9(n1950 n2077)*, loss-of-function mutations; *ced-9(n1653)*, Y149N; *ced-4(n1162)*, Q79ochre; *ced-4(n2860)*, E263K; *ced-4(n2879)*, E276K; *ced-4(n3040)*, P23L; *ced-4(n3043)*, D20N; *ced-4(n3100)*, S339P; *ced-4(n3141)*, R53K; *egl-1(n1084)*, G-to-A nucleotide transition at nucleotide +5631; *egl-1(n1084 n3082)*, n1084 lesion plus 5-base pair (bp) deletion in *egl-1* coding region.
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18. Embryos for fractionation were collected as described (17). Embryos were resuspended in five volumes of cold hypotonic buffer (10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 250 mM sucrose) and homogenized in a 1-ml Dounce tissue grinder. The homogenates were centrifuged at 40g briefly to remove worm debris. The supernatant was centrifuged twice at 750g for 10 min, and the resulting pellets were pooled as the nuclear fraction. The supernatant was further centrifuged at 100,000g for 1 hour. The pellet was designated the organelle and membrane fraction and the supernatant the soluble cytosolic S100 fraction. The pooled nuclear fraction was washed once with homogenization buffer. One-fifth of each fraction was used for immunoblotting analysis. Rabbit polyclonal antibody directed against human acetylated histone H4 (Upstate Biotechnology) was used as a marker for the nuclear fraction. Monoclonal anti-Ce HSP90 was used as a marker for the cytosolic fraction.
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20. P_{hsp} *egl-1* (5) was injected at a concentration of 2 ng/µl, along with p76-16B [L. Bloom and H. R. Horvitz, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3414 (1997)] at 50 ng/µl, into a *ced-1(e1735)*; *egl-1(n1084 n3082) unc-76(e911)* strain as described [C. Mello and A. Fire, *Methods Cell Biol.* **48**, 451 (1995)]. For immunofluorescence, mixed-stage transgenic worms were incubated at 33°C for 1 hour followed by a 2-hour recovery at 20°C. Embryos were then collected as in (17). For analysis of cell corpses, transgenic adults were allowed to lay eggs at 20°C for 2 hours, subjected to a 1-hour heat shock at 33°C, allowed to lay eggs for an additional 2 hours at 20°C, and then removed from the plates. Hatched L1 transgenic animals were examined by Nomarski microscopy for corpses in the head.
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32. We thank members of the Horvitz laboratory for helpful comments and discussions concerning this manuscript. We thank Y. Yamaguchi and J. Miwa for anti-Ce HSP90 monoclonal antibody; D. Xue for generating pET19b-CED-9ΔC; B. Davies, A. Madi, S. Shaham, E. Spiliotes, and G. Stanfield for *ced-4* missense mutations; B. Castor for DNA sequence determinations; and M. Boxem and S. van den Heuvel for assistance with microscopy. B.M.H. was supported by a Howard Hughes Predoctoral Fellowship. B.C. was supported by a postdoctoral fellowship from the Jane Coffin Childs Memorial Fund for Medical Research and a Leukemia Society Special Fellowship. Z.Z. was supported by a Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Fellowship (DRG 1343). H.R.H. is an Investigator of the Howard Hughes Medical Institute.

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Regulation of Cell Fate Decision of Undifferentiated Spermatogonia by GDNF

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The molecular control of self-renewal and differentiation of stem cells has remained enigmatic. Transgenic loss-of-function and overexpression models now show that the dosage of glial cell line-derived neurotrophic factor (GDNF), produced by Sertoli cells, regulates cell fate decisions of undifferentiated spermatogonial cells that include the stem cells for spermatogenesis. Gene-targeted mice with one *GDNF*-null allele show depletion of stem cell reserves, whereas mice overexpressing GDNF show accumulation of undifferentiated spermatogonia. They are unable to respond properly to differentiation signals and undergo apoptosis upon retinoic acid treatment. Nonmetastatic testicular tumors are regularly formed in older GDNF-overexpressing mice. Thus, GDNF contributes to paracrine regulation of spermatogonial self-renewal and differentiation.

The stem cells for spermatogenesis are single cells in the periphery of seminiferous tubules. The stem cells either self-renew by forming single stem cells or they become interconnected pairs of cells destined to differentiate. Such cells divide further into syncytial chains of usually not more than 16 cells that enter mitosis and apoptosis synchronously (1, 2).

Stem cells, pairs, and chains are collectively called undifferentiated spermatogonia, which subsequently become differentiating spermatogonia, spermatocytes, spermatids, and sperm cells. All types of undifferentiated spermatogonia are morphologically and molecularly alike, but they can be distinguished by the absence or presence of synchronized mitotic

and apoptotic figures (2) and by their spatial relation to differentiating sperm cells. Sertoli cells, the somatic cells of the seminiferous tubules, are paracrine regulators of spermat-

ogenesis (3). These cells probably regulate the maintenance of the stem cell pool and sperm differentiation, but the molecular mechanisms by which this occurs have remained unresolved. Here we show that the dosage of GDNF, normally secreted by Sertoli cells (4), regulates the fate and lineage determination of undifferentiated spermatogonia.

GDNF, a distant member of the transforming growth factor- β family, promotes survival and differentiation of several types of neurons in the nervous system (5, 6) and regulates ureteric branching in the embryonic kidney (7, 8). The signaling receptor complex of GDNF includes Ret receptor tyrosine kinase and GDNF family receptor- $\alpha 1$ (GFR- $\alpha 1$) (9). GDNF-, GFR- $\alpha 1$ - and Ret-null mice exhibit similar phenotypes and die during the first postnatal day (7, 10). Their testicular morphology at this stage is normal. GDNF

promotes Sertoli cell proliferation in vitro (11). To approach the in vivo function of GDNF in spermatogenesis, we analyzed gene-targeted mice with either decreased or increased GDNF expression.

Although most *GDNF*^{+/-} mice survived to adulthood and were fertile, histological analysis of their testes showed that spermatogenesis was disturbed (12) (Fig. 1, A and B). However, sperm cells were observed in the epididymal ducts and in well-preserved segments (13). In the degenerating tubules, spermatids were in an abnormal position and Sertoli cells engulfed some of them (Fig. 1C). In older *GDNF*^{+/-} mice, the depletion of the germ cells often resulted in Sertoli cell-only seminiferous tubules without spermatogonia (13) and the cell proliferation rate was reduced (Fig. 1, D and E), reflecting the depletion of spermatogonia.

We then overexpressed GDNF in transgenic mice under the testis-specific (13) human translation elongation factor-1 α (EF-1 α) promoter (14–16). Four independent transgenic founders were analyzed: two males, C10 and C12, and two females, S6 and E19, with transgene copy numbers of 10, 3, 20, and 4, respectively. The male founders were infertile, and further analysis of the transgenic phenotype was done with offspring of the female founders. Their litters developed normally to adulthood and did not show defects in other organs than the testis. The testicular weights were 41 and 66% less than in controls at 4 and 8 weeks of age, respectively ($n = 40$ animals in both groups). All transgenic males were infertile. In 5 months of continuous breeding (3 males) and a short-term breeding test of 2 weeks (20 males) with FVB females, the transgenic males produced more than 200 vaginal plugs, but sired no pups. Strain dependence of the infertile phenotype was excluded by crossbreeding the transgenic FVB females to NMRI mouse strain.

Testicular morphology of GDNF-overexpressing mice was normal at birth. After 2 to 3 weeks, a chimeric histological pattern was observed. The tubular cross sections not only showed normal spermatogenesis but also displayed large cell clusters (Fig. 2, A and B). Because these cells in the clusters did not show much nuclear heterochromatin and did express a spermatogonial marker, EE2 (17), they could be morphologically classified as type A spermatogonia (13, 18). The clusters gradually degenerated after puberty, resulting in tubular atrophy, and Sertoli cells phagocytosed the dead cells (Fig. 2C). At 10 weeks of age, only remnants of clusters were seen, but a rim of spermatogonia at the periphery of atrophic seminiferous tubules remained (Fig. 2, D and E). No sperm was observed in seminiferous tubules or the epididymis (13).

The levels of *GDNF* and its receptors

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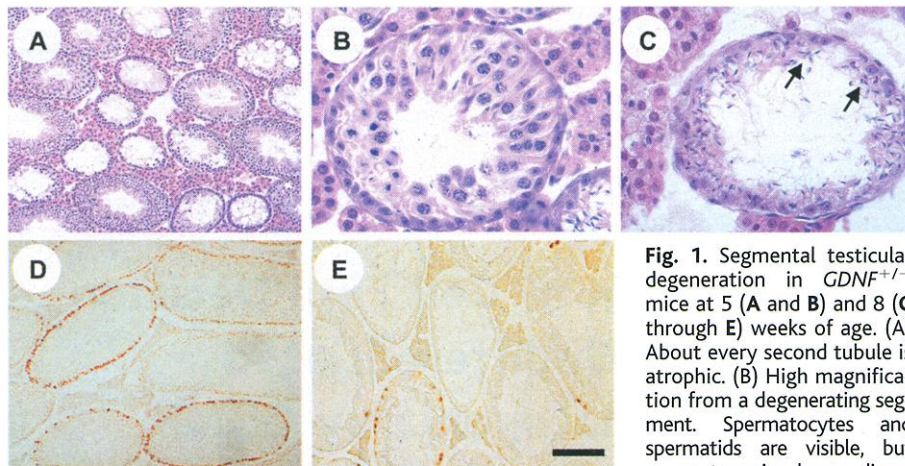


Fig. 1. Segmental testicular degeneration in *GDNF*^{+/-} mice at 5 (A and B) and 8 (C through E) weeks of age. (A) About every second tubule is atrophic. (B) High magnification from a degenerating segment. Spermatocytes and spermatids are visible, but spermatogonia have disappeared. The peripheral rim

consists mostly of Sertoli cells. (C) Mislocation of spermatids (arrows) in an atrophic tubule. The reduction of spermatid number is not statistically significant (18%) as compared to that in wild-type testes. (D) BrdU incorporation in a wild-type control and (E) reduced incorporation in a *GDNF*^{+/-} testis. Scale bar in (E), 40 μ m; bar indicates 200 μ m in (A), 100 μ m in (B), 60 μ m in (C), and 40 μ m in (D).

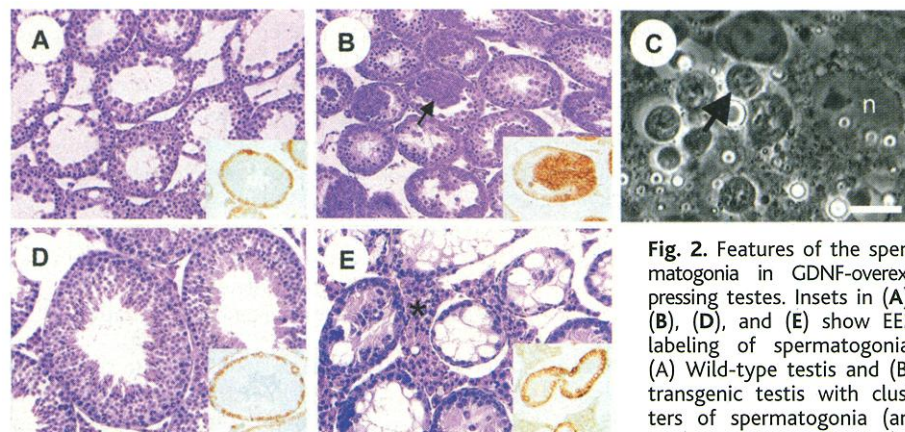


Fig. 2. Features of the spermatogonia in GDNF-overexpressing testes. Insets in (A), (B), (D), and (E) show EE2 labeling of spermatogonia. (A) Wild-type testis and (B) transgenic testis with clusters of spermatogonia (arrow) at 3 weeks of age. (C) High magnification of a live

cell preparation (18) from a transgenic seminiferous tubule with dying cells that resemble type A spermatogonia (arrow) and are engulfed by Sertoli cells. n, Sertoli cell nucleus. Histology of (D) normal 10-week-old testis and (E) advanced atrophy with Leydig cell hyperplasia (asterisk) in transgenic testis. Scale bar in (C), 10 μ m; bar indicates 100 μ m in (A), (B), (D), and (E).

were determined by Northern blotting in normal and GDNF-overexpressing testes (Fig. 3, A through F) (16). Although *GDNF*, *Ret*, and *GFR- α 1* were down-regulated in wild-type testes after the first postnatal weeks, their expression stayed high into adulthood in transgenic testes. Also, the endogenous *GDNF* mRNA levels (16) were elevated (Fig. 3G), reflecting altered cell type ratios. Immunoprecipitation with Western blotting (Fig. 3H) confirmed the high transgene expression. In situ hybridization for *GDNF* and its receptors is shown in Fig. 4 (19). *Ret* and *GFR- α 1* expression by a spermatogonial subset indicates that this group represents the GDNF-responsive cells. To further specify the target cell type of GDNF, GDNF-overexpressing testes were analyzed with markers for differentiating spermatogonia and Sertoli cells. The clusters did not express c-Kit, a marker for differentiating spermatogonia (12, 20). The distribution of GATA-1-positive nuclei (a DNA binding protein in Sertoli cells) (21) was unaltered, suggesting that Sertoli cell proliferation is not regulated in vivo by GDNF (13).

The cell cycle kinetics were analyzed by bromodeoxyuridine (BrdU) incorporation and apoptosis labeling (22). After the first postnatal week, the normal segmental nature of cell proliferation was disrupted in transgenic mice, but the overall proliferation rate of spermatogonia was not enhanced (Fig. 5, A through C). Thus, a differentiation block rather than hyperproliferation probably causes the accumulation of spermatogonia. Apoptosis was increased from the second postnatal week with a peak at 4 weeks of age (Fig. 5, D and E).

The interconnected spermatogonia enter apoptosis and undergo mitoses synchronously (2). The survey of 366 mitotic figures in spermatogonial clusters of 3-week-old transgenic mice ($n = 3$ mice) revealed predominantly single figures: 252 singles, 41 pairs, six groups of four, and one group of eight. Accordingly, only single or small groups of apoptotic cells were seen. The predominance of single mitotic figures and the morphological and molecular characteristics of the cells suggest that the clusters were mainly composed of stem cells. However, the changed microenvironment could have caused a premature disruption of the syncytial spermatogonial colonies, and the cluster cells could therefore also represent abnormal undifferentiated spermatogonia at other stages.

A lack of proper cell contacts between the clusters of spermatogonia and Sertoli cells could explain the disturbed spermatogonial differentiation. However, cytoplasmic extensions of Sertoli cells (Fig. 5H) often protruded into the clusters (23). The expression of *Ret* and *EE2* by the clusters further suggests that the differentiation defect is not caused by

unspecific distortion of the translational machinery by the transgene expression. This was also excluded by expressing neurturin, another GDNF family member expressed by Sertoli cells (24) in the presence of the EF-1 α promoter. These mice showed a different phenotype. They formed no clusters, had normal fertility, and had only a transient delay of spermatogenesis (25).

When rats or mice are fed a vitamin A-deficient diet or when the retinoic acid receptor- α gene is disrupted, differentiation of spermatogonia is blocked (26). We challenged the transgenic spermatogonia with daily injections of all-*trans* retinol (27). Instead of

undergoing differentiation, the clusters underwent apoptosis (Fig. 5, F and G). Thus, the overactivation of *Ret* or the abnormal localization of transgenic spermatogonia renders them incapable of proper response to a differentiation signal.

Testicular tumors developed regularly in older GDNF-overexpressing mice. The transgenic spermatogonia remained dormant after the clustering period, but invariably started to spread into the interstitium and formed non-metastatic tumors after a year of age. Of 12 old mice, 10 had bilateral and 2 had unilateral tumors.

The atrophy in adult GDNF-overexpress-

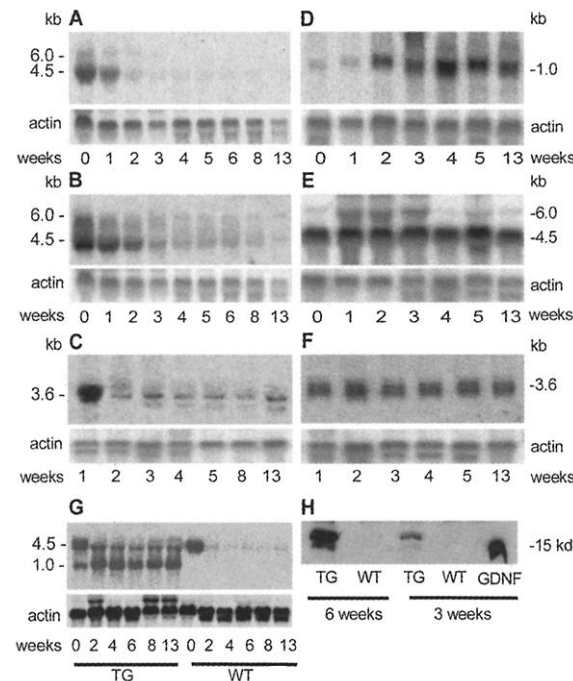


Fig. 3. Northern blotting of *GDNF* and its receptors in wild-type and transgenic testes. *GDNF* (A), *Ret* (B), and *GFR- α 1* (C) mRNA levels had been strongly down-regulated since the second postnatal week. In the transgenic testis, the levels of *GDNF* (D), *Ret* (E), and *GFR- α 1* (F) remain high to adulthood. (G) Also, the endogenous *GDNF* transcripts in transgenic (TG) versus wild-type (WT) testes are elevated (1- and 4.5-kb transcripts, respectively). (H) Western blotting of immunoprecipitated GDNF at 3 and 6 weeks of age. In WT testes, the GDNF protein is not detectable at these stages. Twenty-five nanograms of human GDNF protein serve as a control (right lane).

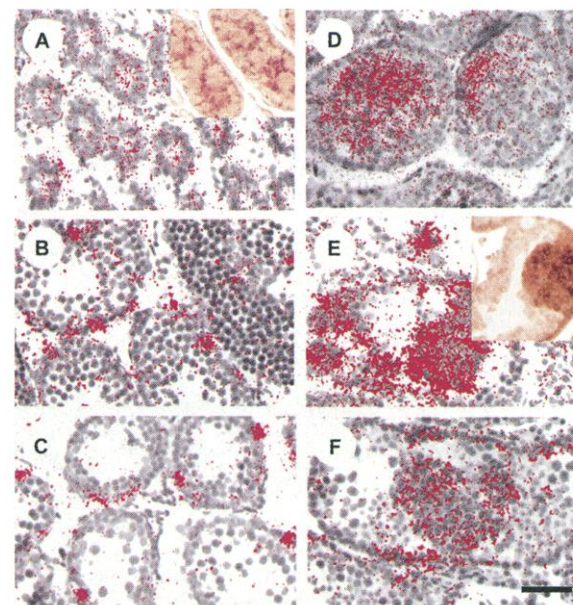


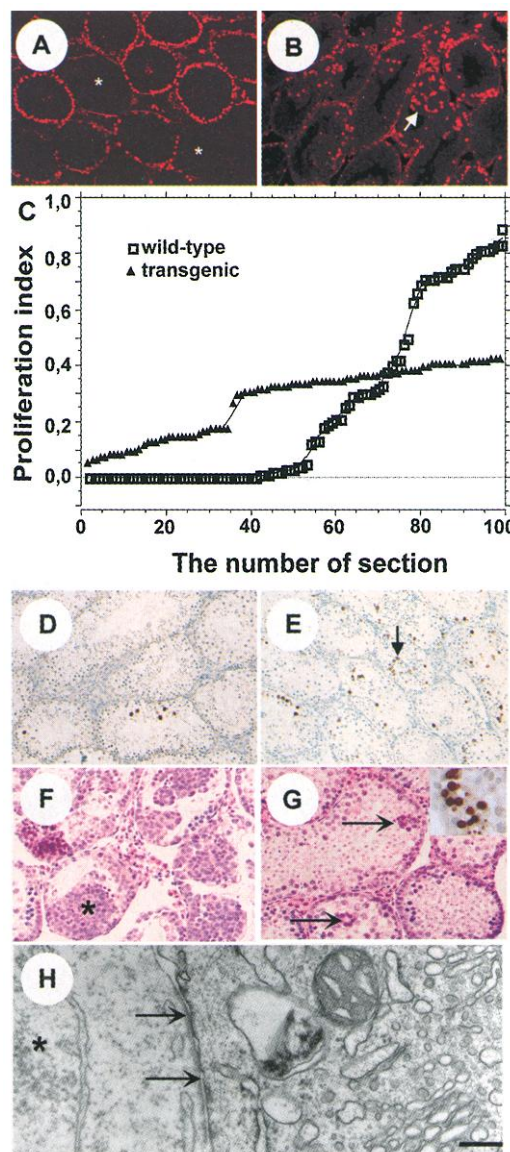
Fig. 4. In situ hybridization for *GDNF* and its receptors in wild-type (left) and transgenic (right) testes. (A) At 1 week of age, *GDNF* is highly expressed (red). The inset in (A) depicts GDNF protein expression by Sertoli cells in a newborn mouse. At 2 weeks of age, *Ret* (B) and *GFR- α 1* (C) are expressed in a subset of spermatogonia. They have a "starry sky" distribution. The spermatogonial clusters in an adult transgenic mouse continuously express the *GDNF* transgene (D), *Ret* (E), and *GFR- α 1* (F). There are peripheral rows of *Ret*- and *GFR- α 1*-positive cells outside the clusters. The inset in (E) shows Ret protein. Scale bar, 33 μ m.

ing testes might be caused by several mechanisms. First, the clusters block fluid flow in seminiferous tubules, which triggers testicular degeneration (28). Thus, obstruction might be the most important reason for the atrophy because spermatogenesis was partially restored after dissolution of the clusters by retinol treatment. Second, the blood-testis barrier, developing around three weeks of age (29), might prevent the protrusion of undifferentiated spermatogonia into the lumen. This would force them to spread horizontally under Sertoli cells. Indeed, sheets of Ret-positive spermatogonia were found in atrophic testes, suggesting that this subpopulation had overgrown and replaced Ret-negative spermatogonia.

Thus, GDNF dosage regulates the differentiation of undifferentiated spermatogonia. At a low GDNF level, spermatogonia

favor differentiation, and at a high level, they favor self-renewal (13). The disturbed spermatogenesis in the GDNF^{+/−} mice closely mimics the morphology of many human cases of impaired spermatogenesis and can be used as a new model for male infertility (30). Molecules activating the Ret signaling cascade may provide us with means to restore reduced spermatogonia pools in infertile men. Although the tumors in older GDNF-overexpressing mice remain uncharacterized, their regular development in GDNF-overexpressing mice suggests that GDNF is involved in the pathogenesis of germ line tumors. Furthermore, the effects on spermatogenesis should be taken into consideration when lead molecules activating the GDNF signaling cascade are designed for use in therapy for neurodegenerative disorders.

Fig. 5. Cell cycle kinetics of the spermatogonia in GDNF-overexpressing mice, their contacts with Sertoli cells, and their response to all-*trans* retinol. (A) In a 3-week-old wild-type testis, S-phase cells are only seen in the periphery of seminiferous tubules. Absent labeling of some tubules (stars) shows the segmental distribution of cell proliferation during normal spermatogenesis. (B) In a 3-week-old transgenic testis, numerous BrdU-labeled cells (arrow) are abnormally distributed in the luminal area of the tubules. (C) The average proliferation indices of spermatogonia in 100 trans-sections of wild-type (squares) and transgenic (triangles) tubules (BrdU-labeled nuclei per 100 spermatogonia) are not significantly different (0.28 ± 0.12 versus 0.25 ± 0.31 , respectively; $n = 19645$ cells), but the peak proliferation index is higher in wild-type than in transgenic mice (Student's *t* test, $P < 0.001$). (D and E) In situ labeling for apoptosis in a 4-week-old wild-type (D) and transgenic (E) testis. Apoptotic cells inside (arrow) and outside the clusters are nine times more frequent in the transgenic than in the wild-type testes (the average $\pm$ SD of apoptotic cells in tubular transsection is 0.47 ± 0.16 in wild-type and 4.22 ± 0.23 in transgenic testes). (F) Transgenic mouse testis without retinol treatment at the age of 4 weeks. (G) In its retinol-treated littermate, the clusters have almost disappeared after 8 days of all-*trans* retinol injections, and spermatogenesis has been partially recovered. The stem cells would have needed at least 22 days to reach the most advanced stages seen here (37). Thus, the differentiating cells are obviously not derived from the spermatogonial clusters but from the disturbed spermatogenesis outside the clusters (see Fig. 1B). Inset, in situ labeled apoptotic spermatogonia in a transgenic mouse injected for 3 days with retinol. (H) Electron micrograph depicts cell contacts (arrows) between a spermatogonium (asterisk) and a Sertoli cell within a cluster of spermatogonia in a 3-week-old transgenic mouse. Scale bar in (H), 0.4 μ m; bar indicates 100 μ m in (A) through (E), and 60 μ m in (F) and (G).



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- Testes were fixed in Bouin or 4% paraformaldehyde, processed in paraffin, sectioned, and stained with hematoxylin/eosin solution. Monoclonal antibodies to EE2 (1:200) and GATA-1 (1:50) (Santa Cruz, Santa Cruz, CA) were incubated on deparaffinized sections overnight and detected by indirect biotin-streptavidin-peroxidase (BSP) labeling (Vector, Burlingame, CA). Species-crossreactive rabbit polyclonal antibodies to GDNF (1:500; Santa Cruz), c-Kit (1:200; Santa Cruz), and c-Ret (1:500; IBL, Fujioka, Japan) were incubated for 48 hours at 4°C and detected with BSP labeling.
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General Acid-Base Catalysis in the Mechanism of a Hepatitis Delta Virus Ribozyme

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Many protein enzymes use general acid-base catalysis as a way to increase reaction rates. The amino acid histidine is optimized for this function because it has a pK_a (where K_a is the acid dissociation constant) near physiological pH. The RNA enzyme (ribozyme) from hepatitis delta virus catalyzes self-cleavage of a phosphodiester bond. Reactivity-pH profiles in monovalent or divalent cations, as well as distance to the leaving-group oxygen, implicate cytosine 75 (C75) of the ribozyme as the general acid and ribozyme-bound hydrated metal hydroxide as the general base in the self-cleavage reaction. Moreover, C75 has a pK_a perturbed to neutrality, making it "histidine-like." Anticooperative interaction is observed between protonated C75 and a metal ion, which serves to modulate the pK_a of C75. General acid-base catalysis expands the catalytic repertoire of RNA and may provide improved rate acceleration.

Eight different catalytic RNAs (ribozymes) occur in nature, and all catalyze phosphoryl transfer reactions (1, 2). The rate of phosphoryl transfer can be accelerated by numerous factors, including stabilization of unfavorable charge development in the transition state, positioning of atoms, and ground-state destabilization (3). Developing negative charges in the transition state of the *Tetrahymena* ribozyme are stabilized by direct interaction with metal ions (4). Because the nucleophile must be deprotonated and the leaving group protonated, proton transfer must occur during phosphoryl transfer. Thus, developing negative and positive charges could, in principle, be stabilized by partial proton transfer in the transition state by general acid-base catalysis (2, 5, 6). Optimal proton transfer in enzymes occurs with an atom having a pK_a near neutrality (5, 6). Thus, histidine often plays an important role in

proton transfer in protein enzymes (5, 6).

In RNA, adenine and cytosine have the potential for protonation of their ring nitrogens N1 and N3, respectively, but the pK_a s for the free nucleosides are relatively low at 3.5 and 4.2 (7). Perturbation of adenine and cytosine pK_a s to near neutrality has been observed in several different RNAs (8-10), which suggests that effective acid-base catalysis may be possible in RNA. Imidazole rescue experiments have shown that proton transfer is possible in RNA catalysis and occurs in the hepatitis delta virus (HDV) ribozyme cleavage mechanism (10). The work described herein involves further characterization of the mechanism for this ribozyme.

HDV is a human pathogen that uses a ribozyme in its replication cycle (11). The ~85-nucleotide HDV ribozyme is found as closely related genomic and antigenomic versions (11, 12), and it belongs to a class of small ribozymes that produce cleavage products with 5'-hydroxyl and 2',3'-cyclic phosphate termini (1) (Figs. 1 and 2).

To probe the catalytic mechanism of the

HDV ribozyme, we examined the pH dependence for self-cleavage of the precursor genomic ribozyme, with a wild-type cytosine at position 75 (C75). The logarithm of the observed rate constant increases with pH between 4.5 and 6 with a slope of ~1 (Fig. 3A). In the pH range 7 to 9, the observed rate constant is pH insensitive, providing an observed pK_a of 6.1 in 10 mM Mg^{2+} (Fig. 3A). The slope of 1 at low pH is consistent with an increase in the concentration of the functional unprotonated form of one general base with pH and a constant amount of the functional protonated form of a general acid. The slope of zero from pH 7 to pH 9 indicates that either the concentrations of the functional species do not change with pH, or the concentration of one species increases while the other decreases by the same amount. To test the nature of the rate-limiting step, we conducted a solvent deuterium isotope experiment (Fig. 3A). A substantial D_2O solvent isotope effect [$= k_{max}(H_2O)/k_{max}(D_2O)$] was observed throughout the pK_a range 5 to 8, which suggests that the observed pK_a of 6.1 reflects a real ionization rather than a change in the rate-limiting step.

The crystal structure of the self-cleaved form of the genomic HDV ribozyme has been solved (13) and reveals that N3 of cytosine 75 (C75) is located only 2.7 Å from the 5'-oxygen of G1 (Figs. 2 and 3B). Moreover, biochemical data suggest that the precursor, transition state, and self-cleaved forms of the ribozyme have similar structures (13). Because the 5'-oxygen of G1 is the leaving-group oxygen in the self-cleavage reaction, C75 could serve as the general acid during self-cleavage (Fig. 2). To test this hypothesis, we replaced C75 with adenine (C75A) or uracil (C75U). The C75U mutant did not result in detectable self-cleavage (14). In contrast, C75A did react, albeit more slowly (by a factor of 270), resulting in an observed pK_a of 5.7 and a ΔpK_a of -0.4 compared with C75 (Fig. 3A). A pK_a shift of -0.4 is consistent with the unperturbed ΔpK_a for N1 of adenosine and N3 of cytidine of -0.65

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