Identification of the stef Gene That Encodes a Novel Guanine Nucleotide Exchange Factor Specific for Rac1*

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The Rho family GTPases are involved in a variety of cellular events by changing the organization of actin cytoskeletal networks in response to extracellular signals. However, it is not clearly known how their activities are spatially and temporally regulated. Here we report the identification of a novel guanine nucleotide exchange factor for Rac1, STEF, which is related in overall amino acid sequence and modular structure to mouse Tiam1 and Drosophila SIF proteins. STEF protein contains two pleckstrin homology domains, a PDZ domain and a Dbl homology domain. The in vitro assay showed that STEF protein specifically enhanced the dissociation of GDP from Rac1 but not that from either RhoA or Cdc42. Expression of a truncated STEF protein in culture cells induced membrane ruffling with altered actin organization, which implies that this protein also activates Rac1 in vivo. The stef transcript was observed in restricted parts of mice, including cartilaginous tissues and the cortical plate of the central nervous system during embryogenesis. These findings suggested that STEF protein participates in the control of cellular events in several developing tissues, possibly changing the actin cytoskeletal network by activating Rac1.

Members of the Rho family GTPases, which include RhoA, Rac1, and Cdc42, act as molecular switches that control the organization of actin cytoskeleton in response to extracellular signals (1). Experiments using cultured cells showed that each member of the Rho family has a different influence on the cytoskeletal structure and eventually on cellular morphology. RhoA induces stress fibers associated with focal adhesions, Rac1 produces lamellipodia or membrane ruffling, and Cdc42 evokes filopodia on the plasma membrane of fibroblasts (2–5).

Evidence has accumulated that the Rho family proteins have a variety of roles in distinct types of cells and tissues. For instance, they are involved in extension and collapse of neurites (6, 7), chemotaxis of macrophages when stimulated by colony-stimulating factor-1 (8), and the generation of tissue polarity in Drosophila (9). However, it is not clear how the activities of Rho family proteins are spatially and temporally regulated to express their functions in vivo.

There are three kinds of proteins that regulate the activities of Rho family proteins by controlling the ratio of the GDP-bound active form to the GTP-bound inactive form. GEFs activate the G proteins by converting the GDP-bound inactive state to the GTP-bound active state, whereas GTPase-activating proteins and guanine nucleotide dissociation inhibitors inactivate Rho family proteins (10). Recently, more than 20 putative GEFs for Rho family GTPases have been identified and are characterized by the presence of the conserved amino acid sequence, Dbl homology (DH) domain, which catalyzes the guanine nucleotide exchange reaction. These proteins have been shown to regulate various cellular events including morphological changes of cells, oncogenesis, or activation of transcription factors (11). Genetic analyses also revealed that GEFs play important roles in development; DRhoGEF2 mediates cell shape changes in gastrulation of Drosophila embryos (12), and UNC-73A is required for cell and growth cone migrations in Caenorhabditis elegans (13). Although the Rho family GTPases appear to be widely distributed in a variety of tissues, several GEFs are preferentially expressed in a limited number of tissues at certain developmental stages. Therefore, it is hypothesized that each of the GEFs has a distinct role in the regulation of various cellular events by activating the GTPases during development.

One of the GEFs whose tissue and subcellular localization has been clearly shown is SIF protein, which is encoded by the still life (sif) gene of Drosophila, identified by our behavioral mutant screen (14). SIF protein is predominantly expressed in the nervous system and confined to the synaptic terminals. At the ultrastructural level, it is found in lateral regions of the active zones for neurotransmission. A loss-of-function sif mutation causes reduced motor activities, which can be rescued by expression of a sif minigene in the nervous system. Moreover, expression of a truncated SIF protein induced membrane ruffling with altered actin localization in human KB cells. These data suggested that SIF protein regulates the formation or

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AB022915.

1 The abbreviations used are: GEF, guanine nucleotide exchange factor; DH, Dbl homology; PH, pleckstrin homology; PCR, polymerase chain reaction; RT, reverse transcription; kb, kilobase pair(s); GTPase, guanosine 5’-3-O-(thio)triphosphate; GST, glutathione S-transferase; PBS, phosphate-buffered saline; HA, hemagglutinin; En, embryonic day n.
maintenance of synapses, possibly by organizing the actin cytoskeleton through the activation of Rho family GTPases. SIF protein contains a DH domain, two pleckstrin homology (PH) domains (15), and a PDZ domain (16, 17). The organization and amino acid sequences of these domains are highly related to those of mouse Tiam1.

The Tiam1 gene was originally identified by its ability to induce invasion of T-lymphoma cells (18). Tiam1 protein functions as a GEF that specifically reacts to Rac1 and Cdc42 in vitro and induces membrane ruffling in NIH 3T3 cells by activating Rac1 (19). Tiam1 affects the morphology of neuroblastoma cells, including neurite outgrowth (20), and E-cadherin-mediated cell-cell adhesion of epithelial cells (21). The transcripts are mainly expressed in the brain and testis, and the developmental distribution patterns in the brain suggest that Tiam1 contributes to cytoskeletal reorganization required during cell migration and neurite extension in a specified population of neurons (22).

To further understand the molecular mechanisms underlying the activation of Rho family GTPases, particularly Rac1, and the biological phenomena that the related signaling cascade may regulate, we have made efforts to identify a new GEF. Here we report a novel mouse protein, STEF (SIF and Tiam1-like exchange factor), which was isolated on the basis of sequence similarity to both SIF and Tiam1. Our biochemical and cell biological analyses showed that STEF protein functions as a GEF specific for Rac1 in vitro and in vivo. These data together with the similar domain organization of STEF and Tiam1 suggest that these two proteins execute their functions in similar molecular environments to activate Rac1 cascades in cells. However, in situ hybridization revealed that the stef transcript is preferentially localized in narrower regions when compared...
with the distribution pattern of the Tiam1 transcript, suggesting a distinct or more limited role of STEF in mouse development.

**EXPERIMENTAL PROCEDURES**

**Isolation of the STEF Gene**—To isolate a murine gene homologous to the Drosophila sif gene, we designed degenerated oligonucleotide PCR primers corresponding to the conserved amino acid residues between SIF and Tiam-1. The first set of primers (PF1, PR1, and PR3) was designed in the PHn-TSS domain. The second set (DF1, DR1, and DR2) was designed in the DH domain. The primer sequences are as follows: PF1, 5'-GG(A/T/C/G)C(A/C/G/T)(A/C/G/T)AA(A/G)AC-3' (amino acids G(A/T)VRKA); PR1, 5'-GC(A/C/G/T)GC(A/G)CA(A/C/G/T)G-3' (complementary to amino acids IHSACAA); PR3, 5'-CCA(A/G/T)CA(A/C/G/T)A(A/G)(C/T)TC-3' (complementary to amino acids Q(V/T)ELENW); DF1, 5'-AC(A/C/G/T)GA(A/G/C/G/T)AC(A/C/G/T)TA(C/T)AA3- (amino acids TERTYVK); DR1, 5'-AC(A/C/G/T)TA(C/T)AA3- (complementary to amino acids HINEMQ(R/K)); and DR2, 5'-TC(G/T)TC(A/G/T)GC(A/C/G/T)TG(A/G/T)GA(G/T)GC(T)GA(G/T)GC(T)G-3' (complementary to amino acids MQ(R/K)IHEE).

Poly(A)+ RNA was isolated from the brains of ICR mice with the QuickPrep mRNA Purification kit (Amersham Pharmacia Biotech). 4 μg of RNA was used as a template for first strand cDNA synthesis using the First-strand cDNA Synthesis kit (Amersham Pharmacia Biotech), and the entire product was subsequently used as a template for one PCR reaction. PCR was performed in the 100-μl scale with the primer set PF1 and PR1 or the primer set DF1 and DR2, employing the following protocol: 30 cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 2 min.
72 °C for 2 min. Then 1 μl of each of the products was used as a template for a second PCR reaction with the nested primer set PF1 and PR3 or nested primer set DF1 and DR1. The DNA fragments of predicted sizes were recovered from agarose gels, cloned into pBluescriptII SK (Stratagene), and sequenced. The FhN-TSS domain primer pair yielded mrf-23, and the primers derived from primer pair mrf-1-5. To test whether these fragments were derived from the same gene, RT-PCR was again executed using the LA-PCR kit version 2 (Takara) with a primer pair to amplify a fragment spanning the two fragments. The primers used were: mrfF1, 5′-GGAGAAGATCCAGACGACAGAGTGGCC-3′ and mrfR3, 5′-ACTTAAAATGATCCATGAGAAGGAAAG-3′. The PCR products were incubated for 40 cycles of 90 s at 94 °C, 1 min at 55 °C, and 2 min at 72 °C in a 100-μl reaction mixture. Oligonucleotide primers used in this study are shown in the Table. The PCR products were then subcloned into M13mp18 and M13mp19 (Stratagene) for sequencing analysis. The DNA fragment amplified in our procedure was derived from one novel gene, we again performed the RT-PCR experiment with a new set of primers, one in the sequence of mrf-23 and the other in the sequence of mrf-1-5. Two sets of degenerate oligonucleotide PCR primers were designed from conserved amino acid sequences found in SIF and Tiam1 to amplify two corresponding DNA fragments expected to contain the first PH (PHn) domain or the DH domain (Fig. 1B). The first primer set produced an unidentified DNA sequence that encodes a PH domain with high similarity to the PHn domains of SIF and Tiam-1 proteins. Using the second primer set, we obtained another new DNA sequence that encodes a DH domain, which is highly related to the DH domains of SIF and Tiam-1. To test whether these two DNA fragments were derived from one novel gene, we again performed the RT-PCR experiment with a new set of primers, one in the sequence of PHn domain and the other in DH domain. A 2-kb DNA fragment was successfully amplified, and the sequencing analysis revealed that this fragment contained nucleotide sequences encoding a PDZ domain as well as the PH and DH domains described above. The overall amino acid sequences and the organization of the domains were highly conserved among the novel protein, SIF and Tiam1. These data suggested that the DNA fragment amplified in our procedure was derived from a novel gene that may be a mouse homologue of the Drosophila sif gene. Screening a cDNA library prepared from the mouse newborn brain RNA using the 2.0-kb DNA fragment as a probe identified several cDNA clones. We further obtained the corresponding cDNA fragments expected to extend to the 5′ or 3′ ends of the full-length transcript by 5′ or 3′ rapid amplification of cDNA ends. Sequencing several overlapping cDNAs revealed the structure of the sif transcript, a stretch of 6157-base pair sequence that contains the 5′-untranslated region, a long open reading frame, the 3′-untranslated region, and poly(A) tail.

The sequence of the 6157-base pair cDNA contains a long open reading frame that predicts a protein of 1715 amino acids (Fig. 1A). As found in Tiam1 and SIF proteins, this protein has a potential myristoylation site at the N terminus, two PH domains, one PDZ domain, and one DH domain (Fig. 1B). Because these motifs are present in many intracellular signaling molecules and STEF protein does not contain a signal sequence or a transmembrane region, STEF is likely to be a cytoplasmic protein. Several regions of STEF protein are highly related in amino acid sequence to Tiam1 and SIF proteins (Fig. 1, B–E); the PHn domain together with its C-terminally flank-

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2 M. Sone and C. Hama, unpublished data.
The dissociation of $[^{3}H]$GDP from Rho family GTPases and the association of $[^{35}S]GTP \cdot S$ to Rac1 were assayed. In these experiments, the DH-PHc region of STEF conjugated with GST was used instead of the full-length STEF. Dbl protein showed GEF activity for all RhoA, Rac1, and Cdc42 and was therefore employed as a positive control. Time courses of the effects of STEF (1 μM) and Dbl (0.5 μM) proteins on the dissociation of $[^{3}H]$GDP from Rac1 (A) and on the association of $[^{35}S]GTP \cdot S$ to Rac1 (B). In each experiment, both proteins showed GEF activity for Rac1 in a time-dependent manner. C, substrate specificity of STEF protein. The dissociation of $[^{3}H]$GDP from Rac1, Cdc42, and RhoA was assayed in the presence of various doses of STEF or Dbl. STEF catalyzed the exchange reaction specifically for Rac1 in a dose-dependent manner.

Because it has been reported that Tiam1 is a GEF for Rac1 (18, 31, 32), we constructed the N-terminally truncated SIF homologous domain.

Several DH domain-containing proteins including Tiam1 have been shown to be GEFSs that specifically function for the Rho family proteins in vitro (11). We therefore examined whether STEF has any influence on the dissociation of GDP from and the association of GTP to Rho family proteins. Because the truncated GEFSs comprising only the DH domain and the adjacent PH domain have been shown to sufficiently exhibit the GDP/GTP exchange activity for the specific Rho family proteins (30, 31, 39), we employed a recombinant GST fusion protein carrying the DH-PHc domain of STEF to assay its exchange activity. In addition, the GST-Dbl protein was used as a positive control, which is known to exhibit GEF activity for all RhoA, Rac1, and Cdc42 (25).

Because it has been reported that Tiam1 is a GEF for Rac1 (19), we first analyzed whether GST-STEFS-enhanced the dissociation of GDP from Rac1. The results obtained in this assay indicated that GST-STEFS as well as GST-Dbl stimulated the dissociation of $[^{3}H]$GDP from Rac1 in a time-dependent manner (Fig. 2A). To confirm these findings, we investigated the influence of STEF on the exchange of preloaded GDP for $[^{35}S]GTP \cdot S$ on Rac1. GST-STEFS and GST-Dbl enhanced the incorporation of $[^{35}S]GTP \cdot S$ to Rac1 (Fig. 2B). We then examined the kinetic properties of GST-STEFS and GST-Dbl for Rac1. Both stimulated the dissociation of $[^{3}H]$GDP from Rac1 in a dose-dependent manner (Fig. 2C). However, measurement of the kinetic parameters (e.g. $k_{cat}$ and $K_m$) of GST-STEFS was unsuccessful because the reaction was not saturated under our experimental conditions (data not shown). We subsequently performed the dissociation assay for RhoA and Cdc42 under the same conditions. GST-STEFS did not show the dissociation activity for RhoA or Cdc42, while GST-Dbl clearly increased the release of $[^{3}H]GDP$ from RhoA and Cdc42 (Fig. 2C). Consistently, GST-STEFS did not prompt the exchange of preloaded GDP for $[^{35}S]GTP \cdot S$ on RhoA or Cdc42 (data not shown). From the data obtained in these experiments, we conclude that STEF is a GDP/GTP exchange factor that specifically reacts to Rac1.

We next examined whether STEF also activates Rac1 in cultured cells. When KB cells were transfected with the cDNA of constitutively activated Rac1 (V12Rac1), the cells exhibited membrane ruffling accompanied by the accumulation of actin filaments along the altered plasma membrane as previously reported (Fig. 3, g–i) (2), whereas constitutively activated Cdc42 (V12Cdc42) induced filopodia (Fig. 3, j–l). Because many DH domain-containing proteins express their oncogenic or invasive activities when their N-terminal portions are truncated (18, 31, 32), we constructed the N-terminally truncated SIF (SIFΔN) or STEFS (STEFSΔN) and made them overexpressed in KB cells to examine their effects on cytoskeleton and cellular morphology. SIFΔN, as previously reported (14), was found to induce membrane ruffling in KB cells (Fig. 3, d–f). When STEFSΔN was introduced into KB cells (Fig. 3, a–c) and NIH3T3 cells (data not shown), membrane ruffles were also evoked, where STEFSΔN colocalized with F-actin along the periphery. However, STEFSΔN induced neither filopodia nor stress fibers in KB cells or NIH3T3 cells. These data implied that STEF activated Rac1 but not Cdc42 or RhoA in vivo, which was consistent with the results of the GEF assay in vitro.
To determine when the stef gene is expressed during embryogenesis, we performed a Northern blot analysis for the RNA samples prepared from embryos at various stages (Fig. 4A). A weak signal of the stef RNA was detected at 6.3 kb in the E7 embryo, and the signal intensity increased as development proceeded up to E17 during embryogenesis.

To reveal the tissue distribution of the stef transcript, we performed further Northern blot analysis for the RNA samples extracted from various tissues of adult mice (Fig. 4B). A strong signal was detected in the brain, and much weaker signals were found in the heart, lung, skeletal muscle, kidney, and testis. This expression pattern was different from the Tiam1 profile, which showed strong signals in the brain and testis (18).

We also performed in situ hybridization for mouse embryos to reveal the spatial distribution of the stef transcript. During late embryonic stages, stef was mainly expressed in the cartilaginous tissues and in restricted parts of the central nervous system (Fig. 4C). In E14.5 embryos, signals were detected in cartilaginous tissues including Meckel’s, costal, vertebral, and tracheal cartilage. In the central nervous system at the same stage, stef was very strongly expressed in the cortical plate, moderately expressed in a part of the striatum, and very weakly expressed at the floor of the fourth ventricle. In E16.5 embryos, the distribution pattern of the stef transcript was similar to that in E14.5 embryos (data not shown).

**FIG. 3. Human KB cells expressing STEF, SIF, Rac1, and Cdc42 proteins.** The N-terminally truncated STEF (STEFN in A–C), SIF (SIFAN, in D–F), constitutively activated Rac1 (Rac1V12, in G–I), and Cdc42 (Cdc42val12, in J–L) were overexpressed in human KB cells. In each experiment, the same view was visualized with fluorescein isothiocyanate (green) for the FLAG or HA-tagged proteins (a, d, g, and j) and rhodamine (red)-phalloidin for F-actin (b, e, h, and k). Merged pictures are c, f, i, and l. White arrowheads in c, f, and i and arrows in l indicate ruffling membranes and filopodia, respectively, where the tagged proteins and F-actin are colocalized. In d and f, nuclei are labeled with GFP. The scale bar represents 20 μm.

**FIG. 4. Temporal and spatial distribution of the stef transcript.** A and B, Northern blot analyses of the stef transcript. Each lane contained poly(A)⁺ RNA extracted from a mouse embryo at the indicated developmental stage (A) and from eight different tissues of an adult mouse (B). The stef transcript was strongly detected in brain, weakly in heart, lung, skeletal muscle, and kidney, and faintly in testis. Each lane contained approximately 2 μg of poly(A)⁺ RNA. The same blots were reprobed with G3PDH DNA as a control (lower panels). C, a section of an E14.5 mouse embryo was hybridized with the stef antisense RNA probes. Signals were detected in several tissues including cartilaginous tissues and restricted parts of the central nervous system. D, a section of the same stage was hybridized with the sense probe of stef as a negative control. No significant signal was detected. cx, cortical plate; ffv, floor of the fourth ventricle; tc, tracheal cartilage; M, Meckel’s cartilage; c, cartilage primordium of vertebrae; cc, cartilage primordium of costae.

**DISCUSSION**

Utilizing RT-PCR, we cloned DNA fragments that represent a novel gene, stef, which is highly related to Drosophila sif and mouse Tiam1 genes. From a cDNA library prepared from the brain of a newborn mouse and by means of 5’ and 3’ rapid amplification of cDNA ends experiments, we succeeded in obtaining overlapping cDNA clones that nearly covered the full-length transcript of the stef gene. The longest open reading frame predicts an amino acid sequence of the STEF protein that consists of 1715 amino acid residues. This protein contains a potential myristoylation site at its N terminus, a PH (PHn) domain, a PDZ domain in the middle part, and a DH domain followed by a second PH (PHc) domain in the C-terminal part. All these motifs are also found in SIF and Tiam1 in the same order, and their sequences are remarkably conserved. In addition, in the C-terminal flanking region of PHn, the TSS domain was defined on the basis of sequence similarity among these
proteins. This conservation found in amino acid sequence and domain organization indicates that both stef and Tiam1 are orthologues of the Drosophila sif gene. Computer analyses revealed that the similarity between STEF and Tiam1 is greater than that between SIF and STEF or Tiam1. From an evolutionary point of view, these relationships suggest that at the time of the divergence of arthropods and chordates, a common ancestral gene of these three genes separated into sif and a second ancestral gene, and the latter further split into stef and Tiam1 afterward.

Rho-like GTPases become activated when bound GDP is exchanged for GTP, a process catalyzed by GEFs. More than 20 putative GEFs specific for Rho family GTPases have been identified, which contain a conserved catalytic domain, the DH domain (11). The substrate specificity for the exchange reaction differs among members of the DH family. For example, Lce, Lfc, and Lsc only catalyze the exchange for RhoA (33, 34), whereas Ost and Dbs react for Cdc42 and RhoA (11, 35). We found that the STEF protein is a GEF that specifically functions for Rac1 in vitro and in cultured cells.

We initially examined whether the DH-PHc domain of STEF conjugated with GST activated the dissociation of GDP from each member of the Rho family. Although the DH-PH domain of Dbl showed activity with Rac1, Cdc42, and RhoA (25), the DH-PHc of STEF only enhanced GDP release from Rac1, not from the other molecules. This substrate specificity of STEF revealed in vitro was further examined in cultured cells. It has been shown that each of the activated Rho family GTPases induces a distinct cellular morphology; Cdc42 induces filopodia; Rac1 causes lamellipodia or membrane ruffling, and RhoA exhibits stress fibers in fibroblasts or partly in KB cells (2–5). Our experiment showed that N-terminally truncated STEF (STEFΔN) induced membrane ruffling but did not induce either filopodia or stress fibers in KB or NIH3T3 cells. In addition, STEFΔN colocalized with F-actin at the edge of the ruffled membrane, suggesting local activation of Rac1 in the altered structure. Thus, STEF appears to specifically activate Rac1 in culture cells as well as in vitro.

Many Rho family proteins are believed to be widely distributed in various tissues, each having multiple functions during development. For example, Rac1 is involved in the fusion of myoblasts (6) and migration of neuronal cells (36). Furthermore, each member is potentially able to regulate several developmental processes even in the same type of cells; in Purkinje cells, the activated Rac1 appears to affect the formation of dendritic spines and also perturbs axonal extension (37). These facts imply that a temporally and spatially organized regulation of the Rho family proteins is required for each cell to properly differentiate, possibly through activation by GEFs localized to subcellular sites during a specific developmental time window. Indeed, SIF protein, a Drosophila putative GEF, is localized in synaptic terminals of mature synapses but is hardly detected during axonogenesis (14). Expression of each GEF may therefore correspond to the spatiotemporally programmed activation of Rho family members and thereby induce a subset of cellular events during development.

We reported that the stef transcript is preferentially distributed in a limited number of tissues during embryogenesis and at the adult stage. In E14.5 and E16.5 embryos, stef is expressed in several tissues including the developing cerebral cortex and cartilage. Notably, in the cortex during these stages, there are numerous neurons migrating through the intermediate zones toward the cortical plates and some extending neurites. It is therefore possible that STEF may regulate neuronal migration or neurite extension by locally activating Rac1. This possibility is supported by the previous findings that Rac1 is involved in cellular migration and neurite extension (6, 19, 36). At the same embryonic stage, Tiam1 expression is also observed in the brain and cartilage (22) and spatially overlaps stef expression. The similarities in the expression pattern as well as in protein structure suggest that the two proteins may essentially have overlapping functions in similar molecular environments and affect the same cellular events. Alternatively, these proteins may be localized at distinct subcellular sites to differentially activate Rac1 and regulate distinct processes of neuronal differentiation. To clarify these possibilities, the subcellular localization of both gene products should be investigated.

Although stef and Tiam1 transcripts are distributed in several overlapping tissues, we are aware that stef expression is confined to narrower areas compared with Tiam1 expression. At the adult stage, Tiam1 is strongly expressed in testis (18), where stef is detected at a much lower level. In E14.5 embryos, there are a number of tissues in which Tiam1 is expressed, but stef expression is hardly detected; among them are the roof of the midbrain and the olfactory epithelium (22). These distinct expression patterns suggest that stef and Tiam1 have differential functions, and stef may play a more limited role in the regulation of tissue development.

We have shown that STEF, Tiam1 and SIF share several conserved domains. PDZ is a domain for protein interaction and is found in many proteins associated with specialized junctions (16, 17), and the PH domain is implicated in the binding to membranes and is frequently present in many signaling molecules (15). The TSS domain also appears to mediate protein interaction (38). These conserved domain structures suggest that the three proteins interact with similar molecules to constitute a protein complex at the plasma membrane and activates Rac1 in a similar mode, possibly responding to extracellular cues. Through this possible signaling cascade, STEF may control neuronal migration or neurite extension and also synaptic events, as suggested for SIF. To understand what biological events STEF regulates during and after development, genetic analyses will be necessary after the gene has been disrupted.

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