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The t complex–encoded GTPase-activating protein Tagap1 acts as a transmission ratio distorter in mice

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Transmission ratio distortion in the mouse is caused by several t-complex distorters (Tcds) acting in *trans* on the t-complex responder (Tcr)[1–4]. Tcds additively affect the flagellar movement of all spermatozoa derived from t/+ males; sperm carrying Tcr are rescued, resulting in an advantage for t sperm in fertilization. Here we show that Tagap1, a GTPase-activating protein, can act as a distorter. *Tagap1* maps to the *Tcd1* interval and has four t loci, which encode altered proteins including a C-terminally truncated form. Overexpression of wild-type *Tagap1* in sperm cells phenocopied Tcd function, whereas a loss-of-function *Tagap1* allele reduced the transmission rate of the t^6 haplotype. The combined data strongly suggest that the t loci of *Tagap1* produce *Tcd1a*. Our results unravel the molecular nature of a Tcd and demonstrate the importance of small G proteins in transmission ratio distortion in the mouse.

Males heterozygous with respect to a complete t haplotype, a variant form of the t complex on mouse chromosome 17, transmit the t chromosome to up to 99% of their offspring. This transmission ratio distortion (TRD) is caused by the interaction of several mutant loci, *Smok1*^{Tcr} (encoding Tcr) and several Tcds[1–4]. Tcds are thought to have a harmful effect on all sperm produced by a t/+ heterozygous male, impairing their flagellar functions[5,6]. Tcr seems to counterbalance this harmful effect of the Tcds. In contrast to the Tcds, which are thought to be shared among all sperm cells, Tcr seems to be restricted to spermatozoa carrying the t chromosome. Thus, only t sperm are rescued by Tcr; wild-type sperm remain impaired, giving t sperm an advantage in fertilization.

So far, only *Smok1*^{Tcr} has been isolated molecularly by positional cloning. It encodes a mutant serine-threonine kinase that probably acts in a dominant negative manner[7]. The genomic intervals to which the Tcd loci have been mapped are several megabases in size each. This coarse localization of Tcds is mainly due to four inversions that suppress recombination between the t chromosome and the wild type-chromosome, preventing fine mapping of these loci.

We used a positional cloning approach to identify a candidate for *Tcd1* on the basis of four criteria: (i) the gene must be located in the genomic interval comprising *Tcd1*; (ii) it must be expressed in testis; (iii) it must show alterations in the t haplotype relative to the wild type; and (iv) it must encode a protein involved in signaling, in agreement with the idea that Tcds are components of signaling pathways that act upstream of Tcr (ref. 7). Candidates fulfilling all these criteria must also prove their ability to enhance (or reduce) t-haplotype transmission in transgenic mice.

Because chromosomal rearrangements have a high potential to affect gene function, we started our search for *Tcd1* candidates in the region *D17Leh119I*, which marks the end of a large inverted duplication in the wild-type chromosome[8]. We hybridized genomic fragments spanning the duplication breakpoint to a cDNA library and identified a gene with similarity to FGF receptor oncogene partner (*Fgfr1op*, also called *Fop*), called *Tagaps* (**Fig. 1a**). Northern-blot analysis showed that *Tagaps* was highly expressed in wild-type testes, whereas no transcripts were detectable in testes from males carrying t alleles in the *Tcd1* region (*e.g.*, haplotypes t^{h51}or t^{h49} or complete t haplotypes; data not shown). A detailed analysis of the gene structure showed that the 5′ region of *Tagaps* was derived from *Fgfr1op* whereas most of the coding region and the 3′ untranslated sequence came from an unrelated gene, indicating that the *Tagaps* mRNA is a fusion transcript (data not shown).

The part of *Tagaps* not derived from *Fgfr1op* occurs in a second locus on the wild-type chromosome, located close to *Tagaps*. Both loci are deleted from the T^{OR} chromosome (**Fig. 1b**). The partial t haplotype t^6 allows a more accurate assignment of both genes to the *Tcd1* region.

Gene targeting and mutant analysis excluded *Tagaps* as a candidate for *Tcd1* (data not shown). Therefore, we extended the analysis to the gene from which the 3′ end of *Tagaps* was derived. Cloning of a full-length cDNA and sequence analysis showed that it encodes a protein of 714 amino acid residues involving a domain with high similarity to GTPase-activating proteins (GAPs) for Rho small G proteins (**Fig. 1d**). This gene is called T-cell activation Rho GTPase-activating protein (*Tagap1*) in public databases. Genomic Southern-blot analysis using a *Tagap1*-specific 5′ fragment of the cDNA as probe showed a single band in wild-type strains, which was also present in t^{h2}/t^{h2} DNA, and two, more intense, polymorphic bands in t haplotypes carrying t alleles in

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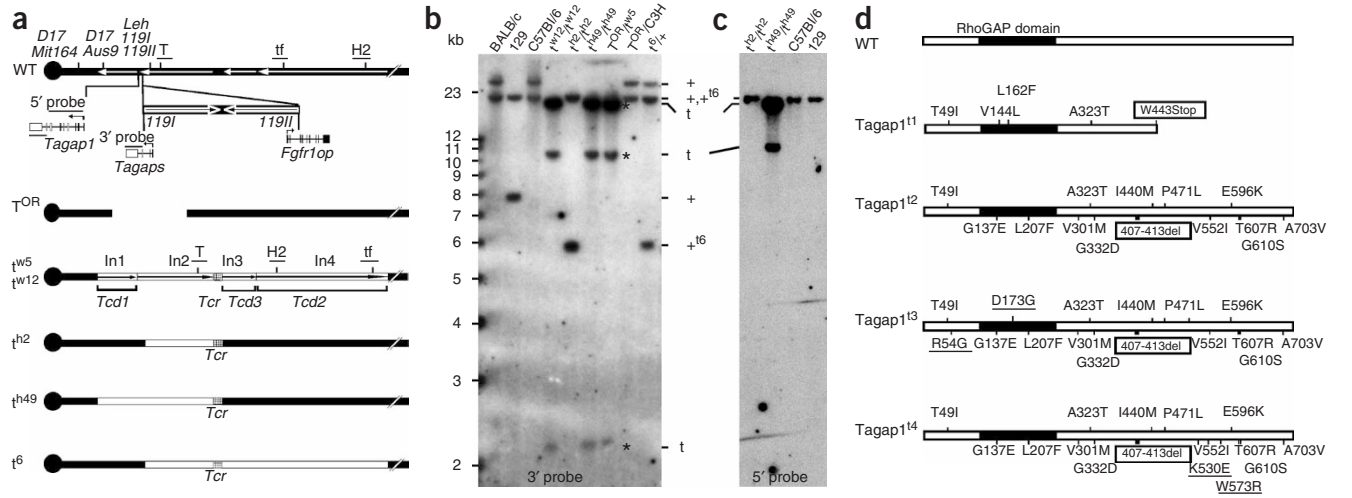

Figure 1 *Tagap1* is a candidate for *Tcd1*. (**a**) Schematic map of the t complex on chromosome 17. The approximate positions of *Tagap1*, *Fgfr1op* and the fusion gene *Tagaps* are indicated; gene maps are expanded. The centromere is indicated by a filled circle; wild-type chromatin, by filled bars; t chromatin, by open bars; and inversions (In1–In4), by arrows. The molecular and genetic markers and the structure of partial t haplotypes were described previously[3]. t^6 and t^{h2} contain wild-type chromatin in the *Tcd1* interval. The approximate extent of the deletion in T^{OR} is indicated by a gap; positions of Tcds, by brackets; and Tcr, by a hatched box. Maps are not to scale. WT, wild-type. (**b**) Genomic mapping of *Tagaps* and *Tagap1* to the *Tcd1* region in t haplotypes. A probe containing the 3′ end of *Tagaps* detects two wild-type bands, which are polymorphic in different strains and in t^6, and three t-specific bands (asterisks), which are absent from T^{OR} and not observed in t^6, localizing both genes to the *Tcd1* interval. (**c**) *Tagap1* is amplified in t haplotypes. A *Tagap1*-specific 5′ probe detects two polymorphic fragments in t^{h49}/t^{h49} DNA equal to four times the signal intensity of the wild-type band. (**d**) t haplotypes encode four different classes of *Tagap1* transcript. Schematic drawings of representative cDNA clones isolated from testes of t^{h49}/t^{h49} mutant mice, in comparison with the wild-type (WT) gene. Mutations resulting in deletion of amino acid residues are boxed; mutations distinguishing products derived from different *Tagap1* loci are underlined. None of these alterations were found in transcripts derived from wild-type strains or in t^6 or t^{h2} (data not shown).

the *Tcd1* region (**Fig. 1c**). Quantification of the wild-type and t-specific signals showed that the t haplotype in the *Tcd1* region contained four copies of *Tagap1*, whereas the wild-type sequence had only one.

Accordingly, sequencing of *Tagap1* cDNAs derived from the testis of a t^{h49}/t^{h49} male identified several classes of transcripts with multiple nonsilent nucleotide changes distinguishing them from the wild-type sequence (**Fig. 1d**). The main cDNA class (51 of 74 clones analyzed), derived from *Tagap1*^{t1}, contained a G→A transition at nucleotide position 1,471, changing the tryptophan at position 443 into a premature stop codon. This mutation produces a truncated protein with an intact N-terminal RhoGAP domain.

The remaining three t-specific *Tagap1* cDNA classes did not contain the transition resulting in the W443X mutation but had a number of nonsilent point mutations and a 21-bp deletion 3′ to the RhoGAP domain (**Fig. 1d**). Aside from common nucleotide changes, these cDNAs also had differences from each other, suggesting that they were derived from three distinct *Tagap1* genes, which arose by triplication of a single locus.

RNA expression analysis by RT-PCR and RNase protection assays showed that *Tagap1* was transcribed in the testis at the earliest stage analyzed, 7 d post partum (**Fig. 2a**,**b**). Therefore, *Tagap1* is expressed in diploid spermatocytes. This may be conducive to

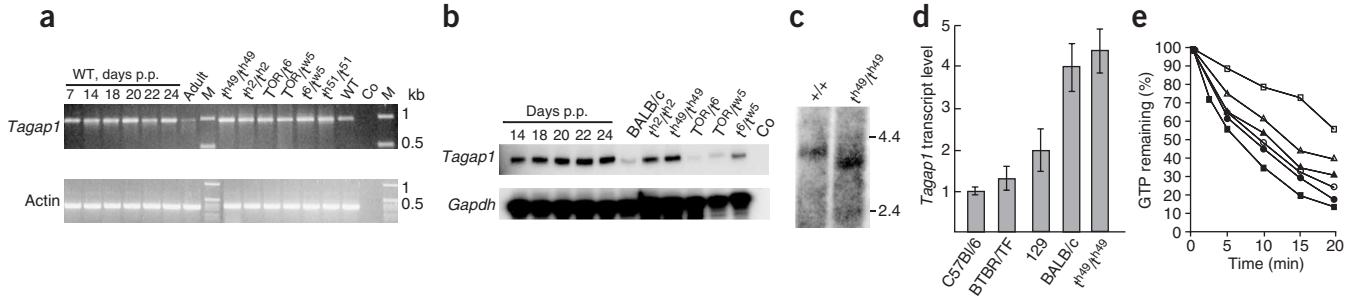

Figure 2 Expression analysis and GAP activity assays of *Tagap1*. (**a**) *Tagap1* is expressed at early stages of spermatogenesis. RT-PCR analysis of RNA isolated from testes of several post partum (p.p.) stages, adult wild-type mice or males carrying various t haplotypes. Actin served as control. Co, control; M, marker; WT, wild type. (**b**) RNase protection assay confirming the RT-PCR data. Co, control. (**c**) Northern-blot analysis of 8 μg of testis poly(A)$^+$ RNA hybridized with the *Tagap1*-specific 5′ probe. The t-specific mRNA migrates faster than wild-type mRNA. (**d**) Analysis of *Tagap1* transcripts by quantitative PCR shows higher levels of *Tagap1* transcript in t^{h49} than in wild-type strains, which differ considerably in *Tagap1* expression. (**e**) Tagap1 enhances the GTPase activity of RhoA. Squares, RhoA; triangles, cdc42; circles, Rac1; open symbols, reaction carried out without Tagap1; filled symbols, with Tagap1.

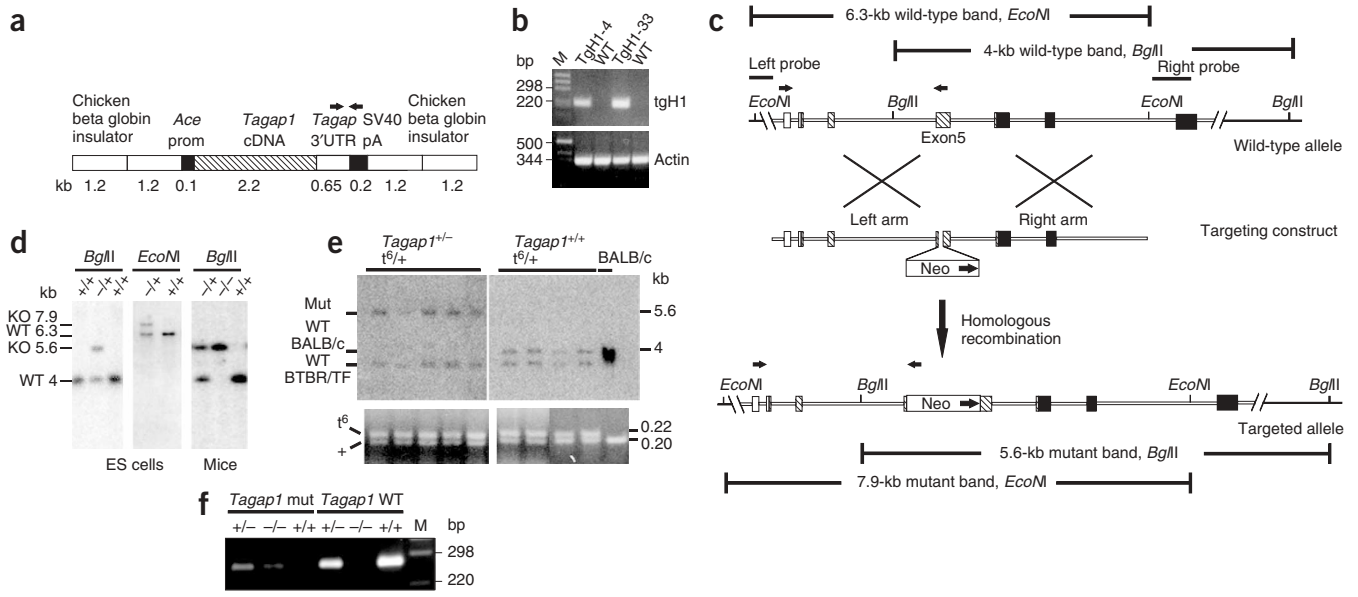

Figure 3 Construction of gain- and loss-of-function alleles of *Tagap1*. (**a**) Transgenic construct used for overexpression of wild-type *Tagap1*. Black arrows indicate primers for genotyping. (**b**) RT-PCR analysis of testis RNA verifying expression of the transgenic constructs. Actin served as positive control. M, marker; TgH1-4, *Tg(Tagap1)H1-4Bgh*; TgH1-33, *Tg(Tagap1)H1-33Bgh*, WT, wild-type. (**c**) Targeting of *Tagap1*. Introns are depicted as double lines; exons, as boxes. Coding regions are hatched; GAP domain–encoding regions, filled. A PGKneo selection cassette was integrated into exon 5. Primers used for expression analysis in **f** are indicated by black arrows. (**d**) Identification of the targeted allele *Tagap1*^{tm3Bgh} in clone A10. Genomic Southern-blot analysis identifies *BglII* and *EcoNI* fragments of the predicted sizes with the right and left probes, respectively, in the ES-cell clone A10. Right panel, genotyping of a heterozygous and a homozygous mutant male, confirming germline transmission of the mutated allele. KO, knockout; WT, wild-type. (**e**) Genotyping of males used for testing the effect of the *Tagap1* knockout allele on the transmission ratio of the t^6 haplotype. Mut, mutant; WT, wild-type. (**f**) RT-PCR analysis of testis RNA from wild type (+/+), heterozygous (+/–) and homozygous (–/–) mutant mice with primers specific for the mutated (mut; left) and wild-type (WT; right) alleles, showing loss of the wild-type *Tagap1* transcript in *Tagap1*$^{-/-}$ mice. M, marker.

distribution of the gene products to all sperm cells, because spermatids develop in a syncytium. Northern-blot analysis using testis poly(A)$^+$ RNA showed a low level of transcription in this organ (**Fig. 2c**).

The transcript detected in the t haplotype migrated slightly more quickly than the wild-type mRNA (**Fig. 2c**). The reason for this is not known, as we observed no large size differences (besides the 21-bp deletion) in any of the t-specific cDNA clones analyzed (data not shown). The smaller transcript size may be due to differences in polyadenylation. Shortening of the poly-A tail has been shown to accompany translational activation of some mRNAs during spermiogenesis[9]. Whether the observed difference is of functional relevance, however, remains to be determined.

We detected *Tagap1* transcripts in all organs examined by RT-PCR analysis (data not shown), and *Tagap1* expressed-sequence tags have been reported in public databases from a large variety of tissues and organs, suggesting that the gene is ubiquitously expressed.

Quantitative RT-PCR showed that t haplotypes expressed higher levels of *Tagap1* transcripts than did wild-type strains, which differed considerably in *Tagap1* expression (**Fig. 2d**). These differences in expression may contribute to the large differences in the transmission rates of t haplotypes maintained on various genetic backgrounds[10].

Finally we examined the specificity of the GAP domain of wild-type Tagap1 towards three 'classical' small GTPases (RhoA, cdc42 and Rac1), which, among several others, are expressed in testis[11]. GAPs enhance the intrinsic GTPase activity of small G proteins, promoting their inactive state. Our data show that the GTPase activity of RhoA was strongly enhanced by the GAP domain of Tagap1, whereas the other family members were only mildly (Cdc42) or hardly (Rac1) stimulated (**Fig. 2e**). Whether RhoA is a target of Tagap1 *in vivo*, however, remains to be determined.

The genetic and molecular data identified *Tagap1* as a *Tcd1* candidate and suggested that the t loci of *Tagap1* might result in a gain of function. Therefore, we tested whether overexpression of wild-type *Tagap1* from a transgene construct in elongating spermatids would enhance the transmission ratio of t^6, which lacks *Tcd1* (**Figs. 1a** and **3a**)[12]. We generated two independent transgenic lines expressing the transcript, crossed them to males carrying the t^6 haplotype and analyzed the transmission rate (**Fig. 3b** and **Table 1**). Both transgenic lines had significantly higher rates of transmission of t^6 than did

Table 1 Transmission rate of t^6 from males lacking or overexpressing *Tagap1*

		Offspring					
Genotype	*n*	t^6	Wild-type	Total	Percent t^6	χ^2	*P*
TgH1-33/0 t^6/+	5	218	39	257	85	7.08	0.01
t^6/+	5	125	43	168	74		
TgH1-4/0 t^6/+	7	190	19	209	91	5.17	0.025
t^6/+	5	200	39	239	84		
Combined data							
Tg/0 t^6/+	12	408	58	466	88	9.57	0.01
t^6/+	10	325	82	407	80		
Tagap1$^{tm3Bgh/+}$ t^6/+	9	245	109	354	69	21.09	0.001
Tagap1$^{+/+}$ t^6/+	9	292	56	348	84		

TgH1-33, *Tg(Tagap1)H1-33Bgh*; TgH1-4, *Tg(Tagap1)H1-4Bgh*.

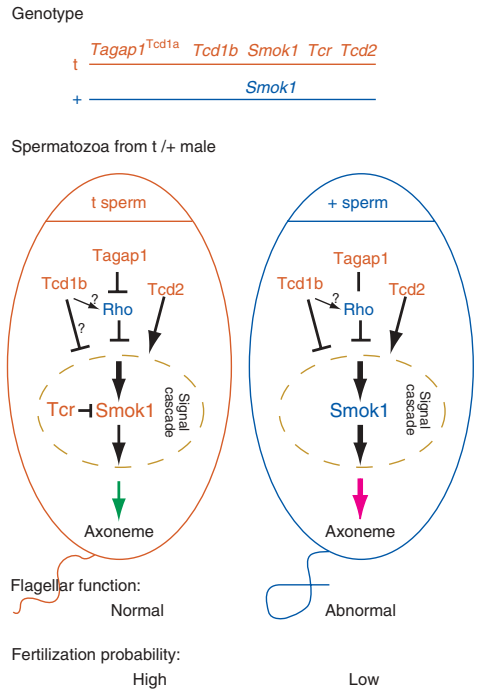

Figure 4 Model of the role of Tcds and Tcr in TRD. t haplotypes encode several Tcds (only *Tagap1* (*Tcd1a*), *Tcd1b* and *Tcd2* are indicated; wild-type alleles are not shown) and Tcr, acting upstream of Smok1 kinase involved in controlling flagellar behavior. Tcds act in all sperm, whereas Tcr is restricted to t sperm. Tagap1 is a hypermorph of a negative regulator of Rho, which inhibits Smok1. Tcd1b is an amorph or hypomorph of an activator of Rho or of an inhibitor of Smok1, acting epistatically to Tagap1. Both mutations additively or synergistically hyperactivate Smok1, an effect that is further enhanced by Tcd2. This negative effect of Tcds on flagellar behavior, which is effective in all sperm, is counterbalanced by Tcr, which is restricted to t sperm. Thus, Tcr rescues only t sperm, giving them an advantage in fertilization and promoting transmission of the t haplotype to offspring. Arrows symbolize activation; thick arrows, hyperactivation; bars, inhibition; green arrow, normal signaling; magenta arrow, impaired signaling.

nontransgenic littermates (combined data: 88% versus 80% t^6 offspring, respectively; $P < 0.01$). Therefore, an increase in dosage of wild-type *Tagap1* in testis phenocopied a Tcd, strongly supporting the idea that the t loci of *Tagap1* encode Tcd1 activity.

To confirm this result, we created a loss-of-function allele. We disrupted the *Tagap1* gene by inserting a selection cassette into exon 5, resulting in premature termination of the transcript (**Fig. 3c,d,f**). The translation product is predicted to be truncated upstream of the RhoGAP domain. This allele, called *Tagap1*^{tm3Bgh}, was bred in *trans* to the t^6 haplotype. We tested transmission of the t^6 chromosome in littermates carrying either the wild-type allele or *Tagap1*^{tm3Bgh} in *trans* to t^6 (**Fig. 3e** and **Table 1**). Complementary to results from the transgenic lines, the transmission ratio of t^6 from *Tagap1*$^{tm3Bgh/+}$ t^6/+ males was significantly lower than that from *Tagap1*$^{+/+}$ t^6/+ litter mates (69% versus 84% t^6 offspring, respectively; $P < 0.001$; **Table 1**).

The combined data show that *Tagap1* has a role in TRD. Taken together, the genetic and molecular data suggest that the t loci of *Tagap1* encode Tcd1 activity.

A large deletion in the wild-type chromosome, T^{22H}, phenocopies *Tcd1*, leading to the suggestion that *Tcd1* must be an amorph or hypomorph[13]. But here we showed that a hypermorphic allele of *Tagap1* phenocopies a Tcd located in the *Tcd1* region. This discrepancy could be reconciled by recent data suggesting the existence of two separate distorter loci, *Tcd1a* and *Tcd1b*, which are both lacking from T^{22H} (ref. 14). Our data support this finding, as the gain- and loss-of-function alleles that we analyzed change the transmission rate of t^6 much less than does the partial t haplotype t^{h51}, which carries *Tcd1* (8–15% for *Tagap1* versus >27% for t^{h51}; ref. 3). Some but not all of this difference may be due to variation in the genetic background, inadequate expression of the transgene construct or a stronger effect of the *Tagap1* t loci. But two distorter loci in t^{h51}, which act additively or synergistically, provide a plausible explanation for this difference. Because one of these distorters, encoded by the t loci of *Tagap1*, is a hypermorph, we suggest that the other is an amorph or hypomorph. Taking the T^{22H} phenotype into account, we predict that the latter gene in its wild-type form encodes an inhibitor of *Smok1*, acting upstream or epistatically to the G protein controlled by *Tagap1*.

In summary, we suggest that one or a combination of the t loci of *Tagap1* produce *Tcd1a*. The contribution of each of the four loci and the exact mechanism by which they produce a hypermorph remains to be explored in detail.

Notably, the segregation distorter (*Sd*) of *Drosophila melanogaster* also encodes a mutant form of a GTPase-activation protein, RanGAP, which is C-terminally truncated, leaving the GAP domain intact[15]. Sd-RanGAP is mislocalized in the cell. It accumulates in the nucleus, and this fact is important to its function as a distorter[16,17]. Tagap1^{t1} is also C-terminally truncated. Future work should address whether this fact also has a role in the subcellular localization of this protein and how it contributes to its function as a distorter.

We previously proposed that the Tcds act upstream of *Smok1* (ref. 7). In our model, *Smok1* activity is enhanced by the action of Tcds in all spermatozoa derived from t/+ males, which triggers abnormal flagellar behavior. This negative effect of the Tcds is counterbalanced by Tcr, which is restricted to cells expressing *Smok1*^{Tcr}. Thus, Tcr rescues only t sperm, and + sperm remain dysfunctional. This gives t sperm an advantage in fertilization. The findings presented here allow us to refine this model with respect to the role of Tcd1 (**Fig. 4**).

Tagap1 links Rho signaling to TRD in the mouse for the first time to our knowledge. A previous report has provided evidence for a role of Rho GTPases in sperm motility, and the Rho-binding protein Rhophilin and its interaction partner Ropporin were found in the flagellum[18–20]. The identification of a Tcd increases our understanding of the molecular principles of TRD and promotes the investigation of the role of Rho signaling in sperm motility.

METHODS

Mice and genetics. To map *Tagap1* on genomic DNA, we used the following genotypes: t^{w12}/t^{w12}, t^{h2}/t^{h2}, t^{h49}/t^{h49}, t^{h51}/t^{h51}, T^{OR}/t^{w5}, T^{OR}/t^6, t^6/+. t^{w5} and t^{w12} are complete t haplotypes. t^{h49}, t^{h51}, t^6 and t^{h2} are partial t haplotypes derived from rare recombination events between a t haplotype and a wild-type chromosome; they carry subregions of the t complex in the wild-type form. t^{w12}, t^{h49}, t^{h51} and t^{w5} carry *Tcd1*, and t^6 and t^{h2}, which is derived from t^6, lack *Tcd1* activity. We carried out Southern-blot analysis of *Kpn*I-digested genomic DNA using standard procedures[21] using fragments corresponding to positions 942–3,001 of the *Tagap1* cDNA as a 3′ probe and corresponding to positions 124–942 as a 5′ probe. We genotyped mice for the transmission of t^6 by PCR using primers for the marker *Hba-4ps*. We generated transgenic lines in the inbred strain FVB/N by pronuclear injection of construct DNA using standard procedures. We generated the *Tagap1*^{tm3Bgh} allele in BALB/c embryonic stem (ES) cells obtained by B. Ledermann (Basel Institute for Immunology)[22]. We back-crossed transgenic and knockout lines for several generations to the strain BTBR/TF-+tf/+tf before testing them for distorter activity. Animal experiments were approved by the ethics committee of the Regierungspräsidium Freiburg, registration number T-00/28.

Transcript analysis. We obtained the 5′ ends of *Tagaps* and *Tagap1* by 5′ rapid amplification of cDNA ends using the GeneRacer kit (Invitrogen). We carried out standard reverse transcription with 1 μg of total RNA using AMV-RT (Promega). We carried out RNase protection assays using standard procedures[23]. We synthesized the *Tagap1* probe *in vitro* in the presence of [^{32}P] UTP from a fragment derived from the *Tagap1* cDNA by PCR and cloned it in pCRII TOPO (Invitrogen). The *Gapdh* control RNA was transcribed from the pTRI-GAPDH template (Ambion). We carried out northern-blot analysis using standard techniques using 8 μg of single purified poly(A)$^+$ RNA isolated with the Fast Track system (Invitrogen). We carried out quantitative PCR analysis on an ABI PRISM 7900 HT SDS (Applied Biosystems). As a reference, we determined *Gapdh* expression using the mouse GAPDH assay (Applied Biosystems).

Gene targeting and transgene constructs. We constructed the targeting vector by ligating the left and right arms, both derived by PCR amplification of genomic DNA, to the PGK-neo cassette. We carried out culture, electroporation, selection, isolation of ES-cell clones, DNA preparation in 96-well plates and Southern-blot analysis in accordance with standard procedures[24]. The transgenic construct *Tg(Tagap1)H1Bgh* consists of the angiotensin converting enzyme (*Ace*) testis promoter and transcription start (extending from –91 to +17 bp), driving expression in elongating spermatids[12], followed by the complete open reading frame of wild-type *Tagap1*, its 3′ untranslated region and the SV40 polyadenylation signal, replacing the *Tagap1* poly-A signal sequence. This transcription unit is flanked by tandem copies of the chicken β-globin insulator[25]. We identified transgenic mice by PCR.

***In vitro* GAP assays.** We produced the catalytic domain of wild-type Tagap1, small G proteins and the C-terminal polypeptide of Tagap1 (serving as negative control) as GST-fusion proteins in *Escherichia coli* BL21 using the pGEX vectors. To quantify the relative amount of proteins used, all preparations were adjusted relative to a BSA standard. We carried out GAP assays in triplicate (G proteins at 6 nM; Tagap1 at 15 nM) essentially as described[26]. Oligonucleotide sequences and conditions for PCR experiments are listed in **Supplementary Table 1** online.

Accession codes. GenBank: *Tagap1*, NM_145968.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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