SIAH1 targets the alternative splicing factor T-STAR for degradation by the proteasome

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T-STAR is one of three members of the SAM68 family of RNA-binding proteins that have been shown to be involved in various gene expression pathways including the control of pre-mRNA splicing. We employed a two-hybrid screen to identify proteins that interact with human T-STAR. The predominant interacting proteins were the E3 ubiquitin ligases SIAH1 and SIAH2. We found that SIAH1 bound to an octapeptide sequence in T-STAR targeting it for proteasome-dependent degradation. Rodent T-STAR orthologues (also known as etoile or SLM2) were not targeted for degradation by SIAH1. However a double amino acid substitution of mouse T-STAR that mimics the human SIAH1-binding site brought mouse T-STAR under in vivo control of SIAH1. Using a minigene transfection assay for alternative splicing activity we showed that human T-STAR, like its rodent orthologues can influence splice site choice and that human, but not mouse, T-STAR-dependent alternative splicing is modulated by SIAH1. Western blots of protein from purified germ cells indicated that SIAH1 protein expression peaks in meiosis. In mouse, T-STAR is co-expressed with SIAH1 during meiosis but, in humans, T-STAR is only strongly expressed after meiosis. Comparative sequence analysis showed SIAH-mediated proteasomal degradation of T-STAR has evolved in the primate lineage. Collectively these data suggest that SIAH-mediated down regulation of alternative splicing may be an important developmental difference between otherwise highly conserved T-STAR proteins.

INTRODUCTION

Up to 60% of human genes are alternatively spliced, often during development, and although relatively little is known about what turns this process on or off, in some cases it has been attributed to expression of cell type- and tissue-specific splicing factors (1,2). Regulation of alternative splicing is thought to respond to extrinsic signals (3), and it is also thought to be important for spermatogenesis, as important gene regulators are frequently alternatively spliced in testis (4). T-STAR was originally discovered as an interacting protein of the testis-specific splicing factors RBM and hnRNP G-T (5) and its rat parologue has been shown to cause alternative splicing of various minigenes and to interact with Srp30c, hnRNP L and the glutamate and arginine (ER)-rich domains of splicing associated factors YT521B and SAF-B (6). Although these findings implicate T-STAR in the control of alternative splicing, little is yet known about the signals that regulate either it or other proteins with similar function.

T-STAR is one of three members of the SAM68 family (Fig. 1) of proteins, which are a subgroup of the so-called STAR protein family. These have an extended ‘maxi-KH’ (hnRNPK-homology) RNA-binding domain and are particularly implicated in ‘signal transduction and activation of RNA’ in development (3,7,8). Of these, SAM68 has been most investigated. SAM68 is phosphorylated by both CDK1 and the oncoprotein SRC, stimulates cell cycle progression and is involved in a multitude of processes (reviewed in 7). In contrast, T-STAR generally acts as a growth suppressor that is down-regulated upon immortalization of many cell
actors. Twenty contained the 'seven in absentia homologue' previously reported (5). Fifty-nine of the clones were novel interclones included 36 of T-STAR itself, a homodimerization pre-on a human testis library. One hundred and three true positive hybrid screen using human T-STAR as bait was performed expressed in testis and developing brain (5,6). In order to T-STAR interacts with SIAH1 to control the stability of this protein in development.

which suggests that species-specific mechanisms have evolved in contrast mouse T-STAR is highly expressed in meiosis, reduced until after meiosis consistent with it being a meiotic of human T-STAR. In humans, T-STAR protein expression is gues SIAH1 uniquely reduces the alternative splicing activity of the CD44V5 exon when co-transfected into cells, but that SAM68 family proteins are capable of stimulating inclusion of viral transcripts (reviewed in 7). They also both have SH2- and SH3-binding sites, bind signalling molecules such as the 85 kDa subunit of PI3 kinase (7,10) and they have SH2- and SH3-binding sites, bind signalling molecules such as the 85 kDa subunit of PI3 kinase (7,10) and they

results
T-STAR interacts with SIAH1

T-STAR is an alternative splicing factor that is predominantly expressed in testis and developing brain (5,6). In order to investigate the pathways in which it is involved, a twohybrid screen using human T-STAR as bait was performed on a human testis library. One hundred and three true positive clones included 36 of T-STAR itself, a homodimerization previously reported (5). Fifty-nine of the clones were novel interactors. Twenty contained the ‘seven in absentia homologue’ proteins SIAH1 and SIAH2. These were isolated 18 and two times, respectively. Since human SIAH1 and SIAH2 proteins are 85% identical except in their N-termini, and no differences in their biochemical properties have been reported, we have concentrated our efforts on SIAH1.

To identify the interacting regions of SIAH1 and T-STAR we tested deletion derivatives of them in a directed twohybrid assay (Fig. 2A and B). SIAH1 is a typical E3 ubiquitin ligase with an N-terminal RING finger domain (1–99), a central zinc finger region (99–153) and a C-terminal 130 amino acid (153–282) ‘substrate binding domain’ which is 100% identical between mouse and man (18,19). Only the complete substrate-binding domain (amino acids 153–282), and none of the other regions (including two partial substrate binding domains 153–220 or 178–282) of SIAH1 specifically interacted with T-STAR (Fig. 2A).

To identify the region of T-STAR that is recognized by SIAH1, three separate domains of T-STAR were tested for their ability to bind SIAH1 (Fig. 2B). Only the 79-amino acid RG-rich region (176–254) bound SIAH1 in yeast. Since SIAH1 interaction leads to proteasomal destruction of its interacting partners (see below) we reasoned that it would not form a stable complex in mammalian cells. For this reason, and to confirm this was a molecular interaction that did not require any bridging yeast proteins, we expressed the T-STAR RG-rich region as a fusion protein with GST in Escherichia coli, and used this to specifically pull down in vitro translated SIAH1. Under identical conditions, no binding of SIAH1 was detected to GST alone, and the RG-rich region of T-STAR did not interact with the non-specific protein luciferase (Fig. 2C). Next we systematically mapped the precise SIAH1-binding site in T-STAR by stepwise reduction of T-STAR in the yeast two hybrid assay, and identified the residues RPVGVVVP (amino acids 212–219) as a single high-affinity binding site. Further reduction of this sequence by one amino acid at either the N- or C-terminus caused complete loss of protein binding assayed in the yeast two-hybrid system (Fig. 2B). The interaction with this T-STAR octapeptide was extremely strong (colonies turning blue in less than 5 minutes). To our knowl-edge, this is the shortest peptide known to bind strongly in the yeast two-hybrid system. When these eight residues were removed from the full-length T-STAR sequence (construct T-STARΔ8) binding was greatly reduced, but it was not completely abolished, implying that a further weak SIAH1-binding site exists in T-STAR. A larger deletion, T-STARΔ40, that removed the high-affinity sequence (amino acids 212–219) and 32 adjacent amino acids (220–251) totally prevented SIAH1-binding (Fig. 2B). To investigate the specificity of this interaction, the T-STAR paralogues SAM68 and SLM1 were also tested, as was mouse T-STAR. Surprisingly none of these other proteins bound the substrate-binding domain of SIAH1, which is 100% conserved between mouse and humans (Fig. 2B).

T-STAR is targeted for degradation by its interaction with SIAH1

Although SIAH1 is an E3 ubiquitin ligase that binds and targets substrates for degradation it also binds other molecules
without causing their degradation [e.g. inhibitors (20) and adaptors (21–23)]. To determine if the interaction with SIAH1 has direct consequences for T-STAR protein stability in vivo we cloned both it, its deletion derivatives (Δ8 and Δ40) and SAM68 to create C-terminal fusion proteins with GFP, and co-transfected them into 293T cells with an expression vector internal standard expressing GFP alone to show the relative degradation of the GFP-fusion protein. Analysis of these extracts by western blotting and probing with antisera to GFP showed that GFP-T-STAR was completely degraded and T-STARΔ8 was also mostly degraded after 24 h. Consistent with their inability to bind SIAH1, T-STARΔ40 and SAM68 were not degraded in the presence of SIAH1. GFP itself was also stable in this assay (Fig. 3A).

As the deletion of the high affinity 8-residue motif from T-STAR only marginally affected protein stability we reasoned that either its effects might be masked after 24 h, or that it might be relatively unimportant for T-STAR protein stability. To differentiate between these two possibilities we followed the degradation of various T-STAR constructs in real-time over several hours in mouse oocytes. Oocytes were microinjected with cRNAs corresponding to GFP-SAM68, GFP-T-STAR and both of the deletion constructs. The effects of SIAH1 on the stabilities of the GFP fusion protein expressed in oocytes were then determined by monitoring GFP fluorescence levels after the microinjection of unlabelled SIAH1 cRNA (Fig. 3B and Supplementary Material Fig. A1). GFP-T-STAR and GFP-SAM68 were stable in mouse oocytes over the time course of these experiments since no loss of GFP signal was observed following addition of cycloheximide to block protein synthesis (Fig. 3B and Supplementary Material Fig. A1). Similar to the western data in 293T cells we found that in mouse oocytes GFP-T-STAR was rapidly degraded in the presence of SIAH1 with a half-life of about 1 h. However, in comparison GFP-T-STARΔ8 was much more slowly degraded with a half-life of ~6 h. This suggests that the high affinity octapeptide sequence is required for efficient protein degradation. GFP-T-STARΔ40 was completely stable in the presence of SIAH1 over a 6 h period, consistent with all the determinants for SIAH1 recognition being within the 40mer identified by the yeast two-hybrid assay. We next used this in vivo system to test if this degradation was proteasome dependent by adding in the proteasome inhibitor MG132 (20 μM). Consistent with a proteasome dependent mechanism, this stopped SIAH1 mediated degradation of T-STAR (Fig. 3B).

The protein interaction data suggested that SIAH1 mediated proteasomal degradation might be specific for human T-STAR. To test this, GFP fusions of the T-STAR paralogues SLM1, SAM68, and the mouse and rat T-STAR orthologues were co-transfected into 293T cells with or without SIAH1. We also tested the splicing factor PUF60 (24,25) which was previously reported to be a SIAH1 binding protein (GenBank accession no. U51586). Of the proteins tested, only human T-STAR was significantly degraded in the presence of SIAH1 (Fig. 3C, upper panel).

Since rat T-STAR has been reported to be an alternative-splicing factor which can control the splicing of the CD44V5 exon minigene, we reasoned that human T-STAR

Figure 2. SIAH1 interacts with human T-STAR. The two hybrid constructs used in this study are shown schematically in (A) and (B). The strength of the protein:protein interaction in a LacZ filter-lift assay with SIAH1 is depicted on the right. Turns blue in less than 5 min ++++; strong +++; medium ++; weak ++; barely detectable +/−; no interaction −. (A) T-STAR interacts with the ‘substrate binding domain’ of SIAH1. Protein interaction strengths were tested between full length T-STAR and subregions of SIAH1. (B) SIAH1 interacts with the RG-rich domains of T-STAR. T-STAR paralogues and different regions and mutants of T-STAR were tested for interaction with the substrate-binding domain of SIAH1. (C) Detection of protein interactions by in vitro pull down. Glutathione agarose beads were charged with a GST fusion of the RG-rich region of T-STAR or GST alone and then in vitro translation products of SIAH1 or luciferase were added. After binding beads were washed and boiled and samples were separated by SDS–PAGE prior to autoradiography.
might have similar activity and that SIAH1 co-expression might switch splice site usage (6). To test this we investigated human T-STAR-dependent inclusion of the CD44V5 exon in the absence and presence of SIAH1. All the SAM68 family homologues enhanced inclusion of the V5 exon compared to GFP alone or the PUF60 splicing factor, which had no effect on this substrate. Human T-STAR caused inclusion of the V5 exon but SIAH1 reversed this towards the ratio found in the SIAH1 + GFP-alone transfection (Fig. 3C, lower panel, lanes 1 and 2 compared to lane 14). SIAH1 consistently reduced stimulation of the V5 exon by human GFP-T-STAR but no reduction in V5 incorporation was observed due to the co-expression of SIAH1 with any other GFP fusions (compare adjacent lanes delineated by dotted lines in Fig. 3C lower panel). This indicates that the human T-STAR alternative splicing function is compromised in vivo by SIAH1-mediated degradation.

**A double amino acid substitution in mouse T-STAR renders it susceptible to SIAH1-mediated degradation**

To further define the SIAH1-binding site in T-STAR we created two double amino acid substitutions. First, we altered the SIAH1 binding octapeptide RPVGVVVP to IDVGVVVP but
The T-STAR SIAH1-binding site evolved in the primate lineage

Our data suggested that although human T-STAR was efficiently bound by SIAH1 resulting in proteasomal degradation, mouse T-STAR was neither bound nor degraded. To investigate the nature and timing of the evolution of the SIAH1-binding site, we sequenced the corresponding region of T-STAR in eight primates: chimpanzee, gorilla, black gibbon (apes), macaque, baboon (old world monkeys), spider monkey, white-faced saki (new world monkeys) and slow loris (prosimian). Each of these species had the same sequence as humans (VGVVVP) except the slow loris, which had a single amino acid difference from the human (VGVVVP) (Fig. 4).

SIAH1 and T-STAR expression patterns in testis are consistent with targeted degradation

In mouse, SIAH1 proteins are essential for embryogenesis and probably also in many adult tissues. However, because of redundancy between the Siah1a gene and a nearly identical (rodent specific) copy Siah1b on the X-chromosome, which is shut down during meiosis, mice carrying a targeted deletion of the Siah1a gene survive to adulthood with the only gross defect being an arrest in male meiosis. The implication of this is that SIAH1 is expressed in meiosis, but the cell

### DISCUSSION

The data described in this paper show that human T-STAR is a substrate for SIAH1 binding and directed proteasomal degradation. Destabilization of T-STAR is likely to have significant impact on its function in vivo. Consistent with this we found that all the SAM68 family proteins have alternative splicing activity (including SL1M which had not previously been assigned a function) but that human T-STAR activity is unique among these in being sensitive to SIAH1 (Fig. 3C). SIAH1 proteins are important developmental regulators that are involved in apoptosis and cell cycle control (26,30,31) and have been shown to regulate diverse processes by targeting important developmental molecules for proteolysis, however T-STAR is the first identified splicing factor to be regulated by SIAH1 (Table 1).

Within T-STAR we identified an octapeptide RPVGVVVVP that has a high affinity for SIAH1 in the yeast two-hybrid system and this is the shortest peptide that has been shown to bind to SIAH1 (Fig. 2B). While this work was in progress...
a 22-residue SIAH1-binding peptide containing a similar motif RPVAVMP was discovered in the *Drosophila*-specific protein Phyllopod and a search for Phyllopod-like sequences in known mammalian SIAH1-binding proteins revealed that most known SIAH-binding regions contained a VxP motif (32). The mouse T-STAR sequence (RPVAVGV) differs from the SIAH1-binding region (RPVGVVVP) in human T-STAR at the two underlined positions. It is therefore consistent with the ability of human but not mouse T-STAR to bind SIAH1 that the human SIAH1-binding region (RPVGVVVP) has a VxP motif but the corresponding position of the mouse (RPVAVGV) does not. We also found evidence that the determinants of SIAH1-binding were less than these eight amino acids as in the context of the full length T-STAR protein (in the RP to ID mutant in Fig. 2B) the initial RP dipeptide was dispensable, defining a shorter hexapeptide minimal SIAH1-binding region (VGVVVP). By sequencing this region in various primates we found an identical hexapeptide to human in all new world monkeys, old world monkeys and apes (Fig. 4). The slow lorises has the intermediate sequence (VGVVGP) differing at only one position from the mouse (VAVGVG). While we cannot unambiguously stage the A to G change, the most parsimonious explanation is that the ability of SIAH1 to bind and be degraded by SIAH1 evolved by a G to V change at the conserved VxP position before the split leading to new world monkeys (40 MY ago).

Our data indicate that a high affinity motif is sufficient but not necessary for SIAH1-binding or subsequent destruction of T-STAR, but it is essential for efficient binding and destruction (Figs 2B, 3A and B and especially visible in Supplementary Material movie). We have employed a novel real-time assay in mouse oocytes has not previously been used to quantify rates of SIAH1-mediated destruction. Using this assay we showed that T-STAR is the most potently degraded substrate of SIAH1 discovered so far. This degradation is much more dramatic than those of the previously reported SIAH1 and SIAH2-substrates, synaptophysin or TRAF2 (33,34). In the absence of the octapeptide RPVGVVVP (i.e. in T-STAR8) the half-life of T-STAR in the presence of SIAH1 was 6 h, which is the same as the natural half-life of synaptophysin in the absence of SIAH1.

It is clear that recognition is not sufficient to determine whether a SIAH1-binding molecule is a good substrate for SIAH1-mediated degradation, as PUF60, which binds to SIAH1 in the yeast two-hybrid assay was not degraded in our assay (Fig. 3C). PUF60 may belong to the class of SIAH-binding proteins that act as cofactors, such as *Drosophila* phyllopod which binds to the original SIAH1-like protein SINA and acts as an adaptor protein to cause its associated protein tramtrack to be degraded rather than itself (23). Similar to this, SIAH1 is known to cause indirect degradation of β-catenin through an adapter protein (21,22). Despite the importance of the high affinity site for efficient degradation, we found that GFP fused directly to RPVGVVVP could not be degraded in the presence of SIAH1 in either of our assays (data not shown). It is notable that the A and B type cyclins, which are needed at different times during spermatogenesis (35,36), have short destruction boxes that are only functional in some contexts (37). Data from the mouse T-STAR double point mutant m2h (Figs 2B and 3D) indicate that mouse and human T-STAR share the determinants of competence to be degraded by SIAH1 that lie outside of the human SIAH1-binding region.

Ubiquitin and proteasome mediated degradation are known to be important for spermatogenesis (38). Two of the candidate genes involved in these processes are present on the Y-chromosome—UBE1Y encoding an E1 ubiquitin activating enzyme (39) and DFFRY (USP9Y) encoding a deubiquitinating enzyme; the latter has been found to be deleted in three infertile men (40). We have shown that in male germ cells the E3 ubiquitin ligase SIAH1 is expressed mainly in meiosis (Fig. 5A and B), consistent with the phenotype of the knockout mouse (27). We show here that human T-STAR protein is mainly present post-meiosis, consistent with a delay being caused by the presence of SIAH1 in meiosis, while in mouse T-STAR is predominantly expressed during meiosis, consistent with an inability of SIAH1 to degrade it. T-STAR is the first likely target of SIAH ubiquitin ligases in human spermatogenesis.

Our data suggest that despite being 95.9% identical and 99.4% similar, because of their different expression in meiosis, human and mouse T-STAR are likely to regulate distinct

**Figure 4.** The T-STAR SIAH1-binding region in mammals. The amino acid sequence at the position of the human T-STAR SIAH1-binding region from various mammals are aligned with the minimal six residues defined in our experiments in bold. Differences from the human sequence are highlighted by shading. Approximate evolutionary distance in millions of years from humans is indicated.

<table>
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<tr>
<th>Species</th>
<th>Consensus Sequence</th>
<th>Evolutionary Distance from Humans (MY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chimp</td>
<td>TAREGVVVVPRTPTPRGVLSTRGPVSRGGRG</td>
<td>7</td>
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<tr>
<td>gorilla</td>
<td>TAREGVVVVPRTPTPRGVLSTRGPVSRGGRG</td>
<td>7</td>
</tr>
<tr>
<td>black gibbon</td>
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<td>baboon</td>
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<td>rhesus macaque</td>
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</tr>
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<td>spider monkey</td>
<td>TAREGVVVVPRTPTPRGVLSTRGPVSRGGRG</td>
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<tr>
<td>white-faced saki</td>
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<td>slow loris</td>
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<tr>
<td>rat</td>
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<tr>
<td>mouse</td>
<td>TAREGVVVVPRTPTPRGVLSTRGPVSRGGRG</td>
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species-specific gene expression pathways. Such distinct patterns of protein regulation may be evolutionarily important, particularly given that T-STAR is expressed in the developing brain and the testes. As the brain is the only other significant site of T-STAR expression it is possible that SIAH1-mediated degradation could be responsible for some of the complexity of alternative splicing there (41) and could possibly have been an enabling factor in the evolution of the human brain’s complexity. The genetic control of both sex-specific pathways and alternative splicing have also evolved quickly in mammalian species (42,43). It will therefore be illuminating to uncover alternative splices that are regulated by SIAH1 and T-STAR.

**MATERIALS AND METHODS**

**Cloning**

The regions of open reading frame encoding the amino acids referred to in the text were cloned by PCR into the Eco R1 and Xho1/Sal1 sites of the following plasmids: yeast two-hybrid...
vectors pGBK7T and pGADT7 (Clontech, Palo Alto, CA), GST-fusion expression plasmid pGEX 5X-1 (Amersham, Piscataway, NJ) and pGFP3 which is a eukaryotic expression vector encoding a fusion protein with an upstream EGFP which was constructed by cloning EGFP between the BamH1 and EcoR1 sites of pCDNA3.1 (+ (Invitrogen, Carlsbad, CA).

T-STARΔ8 and T-STARΔ40 were constructed by PCR in two halves such that the two halves overlapped and primed on each other. Oligo 5’GGCTGTAAACTCCTCCTCCCTC3’ was used with 5’GAAGGGAGAGTTACAGCCCGAGGGACGCCAACCTCA3’ for Δ8 and 5’GAAGGGGAGGAGGTATTACAGCCCGAGGGACGCCAACCTCA3’ for Δ40. The T-STAR RP to ID mutation used 5’TTGAGATTGTTAGTACCAGGCCCGAGGGACGCCAACCTCA3’ and 5’AAAGGGGAGAGGATCTACAGCCCGAGGGACGCCAACCTCA3’ for Δ40. The mouse T-STAR m2h mutation was cloned by the same strategy with the oligos 5’AGTCACAGCCAGGCCTGTTGG3’ and 5’GAAGGGGAGGAGTTACAGCCCGAGGGACGCCAACCTCA3’. The mouse T-STAR m2h mutation was cloned by the same strategy with the oligos 5’AGTCACAGCCAGGCCTGTTGG3’ and 5’GAAGGGGAGGAGTTACAGCCCGAGGGACGCCAACCTCA3’. The mouse T-STAR m2h mutation was cloned by the same strategy with the oligos 5’AGTCACAGCCAGGCCTGTTGG3’ and 5’GAAGGGGAGGAGTTACAGCCCGAGGGACGCCAACCTCA3’.

Construcstions were checked by sequencing and where appropriate were also translated in vitro to check the integrity of the reading frames.

Two-hybrid screen
T-STAR in pAS2.1 was used to screen a ‘Matchmaker’ human testis two-hybrid cDNA library (Clontech catalogue no. HL4035AH) as previously described (5). 140 000 colonies were screened. Large colonies were patched on full selection and the standard filter lift assay was performed. Blue strains were grown on again on tryptophan plates to select for the library plasmids. Crude yeast plasmid preps were then transformed into electro-competent E. coli DH5α. Clones were sequenced and transformed back into Y190 with either the bait or pGBK7T vector to test for true positives.

In vitro pull downs
Siah1 (from Siah1 in pGBK7T) and the luciferase control protein were transcribed and translated in TNT-quick T7 reticulocyte lysate (Promega) with 35S methionine. Pull downs were performed as (5) except binding (and washing) was in PBS + 1 mM DTT for 1 h at 4°C.

Protein degradation assay in cell culture and alternative splicing assay
293T cells were grown at 37°C in 5% CO2 in Dulbecco’s MEM with glutamax-1, 10% foetal bovine serum and 1% penicillin-streptomycin (Invitrogen) to 60% confluence and transfected using 5 μl Genejema reagent by the 6 × 35 mm well plate protocol (Strategene) with or without 500 ng SIAH1-HApcDNA, 500 ng of various clones in pGFP3 to be tested, 100 ng pGFP3 as a loading control and 100 ng CD44V5 minigene for the splicing analysis. After 24 h cells were detached with trypsin, washed in PBS and half was resuspended in Laemmli loading buffer and half in Tris-reagent (Sigma). Samples in loading buffer were then sonicated, boiled, cleared by centrifugation, electrophoresed on a 10% acrylamide gel, blotted onto an Immobilon-P membrane (Millipore), probed with anti-GFP ‘living colours’ antibody (Clontech) (1 in 1000) and sheep anti-mouse secondary (Amersham) (1 in 5000) and visualized by ECL.

For the splicing assay, RNA was extracted from the Tri-reagent samples and resuspended in 50 μl of dH2O. One microlitre of this was then used in a ‘superscript one-step RT–PCR’ reaction (Invitrogen) with the following primers InsF 5’CTGGGTGTGGGGAGGTCT3’ and InsB 5’CCACCAGCTCCAGTGTTGTCACA3’. The following program: 50°C 30 min, 94°C 2 min, (94°C 10 min, 59°C 30 s, 72°C 45 s) × 35 cycles, 72°C 10 min. Samples were visualized on a 1.5% agarose gel and quantified by densitometry.

Protein degradation assay in mouse oocytes
Transcription templates were prepared to terminate transcription about 200 nucleotides downstream of the open reading frame by digesting SAM68pGFP3 with Spnl, T-STARpGFP3 constructs with PvuII and SIAH1pGBK7T with HindIII. RNA was transcribed using the ‘mMessage mMachine’ T7 kit (Ambion, Austin, TX) and purified by phenol:chloroform extraction and isopropanol precipitation as recommended, then resuspended in water (1 mg/ml) with 1 U/μl ‘SUPERas-In’ RNase inhibitor (Ambion). GFP-SAM68 or GFP-T-STAR mRNAs were pressure-microinjected into germinal vesicle (GV) stage mouse oocytes using a negative capacitance facility on an electrometer to aid cell penetration, as described previously (44,45). After 3–4 h, SIAH1 cRNA was microinjected into GFP-expressing oocytes that had undergone GV breakdown, a process that occurs spontaneously in mammalian oocytes released from their follicular environment (46). Imaging was performed using a Nikon inverted microscope fitted for epifluorescence as detailed previously (45,47). Image-capture and analysis was performed using MetaMorph software (Universal Imaging Corp., PA). All experiments were repeated at least once, with between six and 12 oocytes per replicate.

Tissue western blotting and testis immunohistochemistry
Mouse tissues for western blotting were dissected and homogenized in 2 × SDS loading buffer containing 4 M urea,
Electrophoresed on a 4–12% NuPAGE bis-tris gradient gel (Invitrogen) and blotted onto an Immobilon-P membrane (Millipore). Western filters were probed with rabbit antiserum generated against T-STAR residues 180–248 (5) affinity-purified and detected as described (63). Goat anti SIAH1 (N-15): sc-5505 was purchased from Santa Cruz. Mouse testes immunological studies (Fig. 5B–F) were performed as described (48). Human testes sections (Fig. 5G and H) were stained with anti-T-STAR and visualized (49).

**Evolutionary study of SIAH binding sites**

The following primers were used to amplify the SIAH1-binding region from genomic DNA. 5' CCTCTTGCTCTGGG AGTGAGAA3' and the degenerate primer 5' G(G/C)T(C/T) A CAGGAGGAGGAAGAGGT3'. PCR products were cloned and sequenced and sequences were deposited in GenBank (accession numbers AV601555–62).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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