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Allosteric Activation of Trypanosomatid Deoxyhypusine Synthase by a Catalytically Dead Paralog

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Background: Deoxyhypusine synthase (DHS) catalyzes the spermidine-dependent modification of translation factor eIF5A. 

Results: Trypanosomatid DHS activity is increased 3000-fold by heterotetramer formation with a catalytically dead paralog, and both gene products are essential for parasite growth.

Conclusion: Trypanosomatid DHS is a complex between catalytically impaired and inactive DHS subunits.

Significance: This activation mechanism uniquely evolved for two independent enzymes within the trypanosomatid polyamine pathway.

Polyamine biosynthesis is a key drug target in African trypanosomes. The “resurrection drug” eflorenthine (difluoromethylornithine), which is used clinically to treat human African trypanosomiasis, inhibits the first step in polyamine (spermidine) biosynthesis, a highly regulated pathway in most eukaryotic cells. Previously, we showed that activity of a key trypanosomatid spermidine biosynthetic enzyme, S-adenosylmethionine decarboxylase, is regulated by heterodimer formation with a catalytically dead paralog (a prozyme). Here, we describe an expansion of this prozyme paradigm to the enzyme deoxyhypusine synthase, which is required for spermidine-dependent hypusine modification of a lysine residue in the essential translation factor eIF5A. Trypanosoma brucei encodes two deoxyhypusine synthase paralogs, one that is catalytically functional but grossly impaired, and the other is inactive. Co-expression in Escherichia coli results in heterotetramer formation with a 3000-fold increase in enzyme activity. This functional complex is also present in T. brucei, and conditional knock-out studies indicate that both DHS genes are essential for in vitro growth and infectivity in mice. The recurrent evolution of paralogous, catalytically dead enzyme-based activating mechanisms may be a consequence of the unusual gene expression in the parasites, which lack transcriptional regulation. Our results suggest that this mechanism may be more widely used by trypanosomatids to control enzyme activity and ultimately influence pathogenesis than currently appreciated.

Trypanosomatid parasites cause several fatal vector-borne human diseases, including the following: human African trypanosomiasis (HAT), 3 American trypanosomiasis (Chagas disease), and leishmaniasis (1). Together, these parasites infect more than 20 million people primarily in tropical and subtropical regions. In particular, Trypanosoma brucei gambiense and T. brucei rhodesiense, the causative agents of HAT, are endemic in 36 countries in sub-Saharan Africa and are responsible for a debilitating neurological disease that invariably leads to death if untreated.

Eflorenthine (difluoromethylornithine) is a suicide inhibitor of the polyamine biosynthetic enzyme ornithine decarboxylase (ODC) (Fig. 1A), which in combination with nifurtimox, is a front line treatment for HAT, demonstrating the importance of polyamine function for parasite growth (2). The cationic polyamines (putrescine and spermidine) are essential for growth of most eukaryotic cells and have been explored as potential targets for the treatment of both infectious disease and cancer (2, 3). Spermidine has been implicated in the regulation of translation and transcription, modulation of chromatin structure, and ion channel function (4, 5). In addition, in trypanosomatids spermidine is used in the synthesis of trypanothione (N3′,N6′-bis(glutathionyl)spermidine), required to maintain intracellular thiol-redox balance (6, 7).

Biosynthesis and metabolism of polyamines are tightly controlled; in mammalian cells regulation is orchestrated by a complex array of transcriptional, translational, and post-translational mechanisms (3, 4) that are generally lacking in trypanosomatids. Instead, these parasites have evolved a novel mechanism to control activity and expression of a key enzyme required for spermidine biosynthesis, S-adenosylmethionine

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\(\text{DHS} \) indicates deoxyhypusine synthase.

3 The abbreviations used are: HAT, human African trypanosomiasis; AdoMetDC, S-adenosylmethionine decarboxylase; ODC, ornithine decarboxylase; DHS, deoxyhypusine synthase; TbDHC5, T. brucei deoxyhypusine synthase (catalytic subunit); TbdHsp, T. brucei deoxyhypusine synthase prozyme (activating) subunit; hsDHS, human DHS; eIF5A, eukaryotic initiation factor 5A; hypA, hypusine modification; eIF5A-h, eIF5A with hypusine modification; BSF, bloodstream form T. brucei; GC7, N2-guanyl-N6-diaminohexanate; Tet, tetracycline; Dox, doxycycline; SKO, single knock-out cell line; cDKO, Tet-regulated conditional double knockout cell line; qPCR, quantitative PCR.
decarboxylase (AdoMetDC) (2). Previously, we found that the functional trypanosomatid AdoMetDC was a heterodimer between a catalytically impaired subunit and a catalytically dead paralog, both of which were essential for cell growth (8, 9). We defined the term prozyme to describe activating subunits that arose via gene duplication of their partner enzyme. Heterodimer formation between AdoMetDC and the AdoMetDC prozyme led to a 1200-fold activation of AdoMetDC activity. Furthermore, the AdoMetDC prozyme protein levels appear to be translationally regulated, suggesting that the two DHS homologs were shown to be essential and to encode a functional trypanosomatid AdoMetDC was a heterotetramer (8, 9). We defined the term prozyme to describe activating subunits that arose via gene duplication of their partner enzyme. AdoMetDC prozyme led to a 1200-fold activation of AdoMetDC activity. Furthermore, the AdoMetDC prozyme protein levels appear to be translationally regulated, suggesting that the two DHS homologs were shown to be essential and to encode a functional trypanosomatid AdoMetDC was a heterotetramer (8, 9).

A specialized yet essential function of the polyamine spermidine in eukaryotic cells is to serve as a precursor for the hypusine modification of eukaryotic initiation factor 5A (eIF5A) (10). Hypusine-modified eIF5A is present in both eukaryotes and archaeb; although its functions are poorly understood, eIF5A is essential in yeast and mammalian cells (11). In bacteria, the eIF5A homolog elongation factor P, which is lysylated instead of hypusinated, was shown to relieve ribosome stalling in the presence of polyproline tracks (12, 13). In yeast, eIF5A associates with translating ribosomes in a hypusine-dependent manner and is required for translation elongation (14, 15). Synthesis of hypusine requires two enzymatic reactions catalyzed by deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase. DHS catalyzes the modification of eIF5A to eIF5A-deoxyhypusine in a four-step NAD⁺-dependent reaction that proceeds through two imine intermediates (Fig. 1A and Scheme 1) (16). The reaction is highly specific and unique to eIF5A. The X-ray structure of human DHS (HsDHS) shows the protein is a homotetramer formed from a dimer of dimers with each containing two active sites at the interface between monomers (17).

Genomes of kinetoplastids such as T. brucei and Leishmania species encode two homologs of HsDHS, one of which appears to be catalytically dead. In Leishmania donovani, one of these homologs was shown to be essential and to encode a functional DHS, although it was significantly less active than the mammalian enzyme (18). The functional role of the second DHS homolog was not established. Here, we examine the roles of both homologs in T. brucei and demonstrate that both are required for optimal enzyme activity. Similar to AdoMetDC, we show that the two T. brucei DHS genes encode one catalytically active DHS subunit and one catalytically dead subunit that associate as a heterotetramer to form the active enzyme commensurate with a 3000-fold increase in catalytic activity. We also show that both genes are essential for parasite growth and infectivity in vivo and that the functional form of DHS in the parasite is the heterotetramer. These data demonstrate that the trypanosomatids have independently evolved an analogous strategy to activate two key enzymes involved in polyamine synthesis through oligomerization with a catalytically dead paralog. Trypanosomatids represent the only known species where this strategy is used to generate the catalytically active species of both DHS and AdoMetDC.

### MATERIALS AND METHODS

#### Ethics Statement
Animal experiments were approved by the Ethical Review Committee at the University of Dundee and performed under the Animals (Scientific Procedures) Act of 1986 (UK Home Office Project License PPL 60/4039) in accordance with the European Communities Council Directive (86/609/EEC). To minimize animal suffering, mice with a terminal parasitemia (>10⁸ cells ml⁻¹) were humanely killed.

#### Anti-DHS Antibody Production
Antibodies were raised in rabbits by Covance Inc., Denver, PA, against recombinant TbdHSc and TbdHSp purified from Escherichia coli (see below). Generation of rabbit polyclonal antibodies to T. brucei dihydroorotate dehydrogenase (TbDHODH) was described previously (19).

#### Multiple Sequence Alignment
DHS sequences were obtained using NCBI BLASTP searches of the kinetoplastid protein database with HsDHS (P49366) as the search query. Sequences were aligned with Clustal Omega (version 1.1.0). Phylogenetic trees were constructed with Mega5 software using the Neighbor-Joining algorithm with Kimura-2 parameters. DHS sequence accession numbers are listed in Fig. 1 and supplemental Fig. S1.

#### Cloning of TbdHSc, TbdHSp, and TbeIF5A
Genes (TriTrypDB accession numbers are as follows: TbdHSp, Tb927.1.870; TbdHSc, Tb927.10.2750; and TbeIF5A, Tb11.03.0410) were PCR-amplified from T. brucei single marker genomic DNA, cloned into pCR®8/GW/TOPO® (Invitrogen), and sequenced (Applied Biosystems Big Dye Terminator 3.1 chemistry and capillary instrumentation) to confirm that no mutations were introduced (see Table 1 for primers).
No nucleotide polymorphisms were identified compared with the published genomic sequence of *T. brucei gambiense*. In addition, the 5′UTRs from both *TbDHSc* and *TbDHSp* genes were cloned by PCR from total RNA using the splice leader sequence as a forward primer and gene-specific reverse primers and sequenced in their entirety (Table 2).

### In Vitro Growth and Transfection of *T. brucei*

Mammalian bloodstream forms (BSF) of *T. brucei* were cultured at 37 °C...
with 5% CO₂ in HMI-11 media supplemented with 10% heat-inactivated tetracycline (Tet)-free fetal bovine serum (Atlanta Bio) as described (20). BSF single marker cells expressing T7 RNA polymerase and Tet repressor were used for genetic experiments (21). Transfection was performed with an Amaxa Nucleofector II as described (22) using NotI-linearized DNA (5 μg) followed by selection with the appropriate antibiotic. Antibiotic concentrations were as follows: 2 μg/ml−1 G418 (Sigma), 2.5 μg/ml−1 pheleomycin (InvivoGen), 2 μg/ml−1 blastidicin S (Sigma), 2 μg/ml−1 hygromycin B (Sigma), and 1 μg/ml−1 Tet (RPI). Cell growth was monitored with a hemocytometer; cell number was defined as cell density × culture volume × dilution factor. For GC7 EC₅₀ determination, cell density was monitored using PrestoBlue (20 μl, Invitrogen) after 72 h of incubation with GC7 (0.01–1000 μM). Chicken serum was used for these studies to avoid polyamine oxidase-mediated toxicity of GC7. Fluorescence (560 nm excitation/590 nm emission) was measured using a Synergy™ H1 Hybrid Multi-Mode Microplate Reader (BioTek), normalized to untreated cells, and data were fitted to Equation 1 using GraphPad Prism.

\[
Y = \frac{Y_{\text{max}}}{1 + (X/X_{\text{EC}_{50}})}
\]  

(Eq. 1)

**TABLE 2**

| UTR sequence of TbDHSc and TbDHSp |
|-----------------------------------|
| 5'UTRs for TbDHSc (Tb927.10.2570) and TbDHSp (Tb927.01.870) were cloned by amplification from Tb427 cDNA using the splice leader sequence as a primer with a reverse primer in the ORF. Sequences of the cloned fragments are displayed. The spliced leader sequence is not shown. |

5'UTR for Tb927.1.870

\[
\begin{align*}
\text{TbDHSc} & \text{ genes (499 bp) were amplified with gene-specific primers (Table 1, flank primer sets). The reverse primer included an overhang complementing the blastidicin resistance gene (19 bases) or hygromycin resistance gene (21 bases). Similarly, the 3’-flanking regions of the TbDHSc (384 bp) or TbDHSp genes (497 bp) were amplified starting directly after the annotated stop codon, and the forward primer included an overhang to complement the resistance gene. The hygromycin resistance gene was amplified from the pLew90 vector (21) and the blastidicin resistance gene from the pLew300 vector (9). Amplified fragments were gel-purified and used in a second PCR with the TbDHSc or TbDHSp nesting primers (Table 1) and the amplified blastidicin or hygromycin resistance gene. PCR mixtures (50 μl) contained the following: 1× Phusion HF buffer, 200 μM dNTPs, 0.5 μM nesting primers, 20 ng of flanking fragment, 50 ng of resistance gene, and 1 unit of Phusion polymerase (New England Biolabs). PCR cycling conditions were as follows: 94°C for 15 s, 65°C for 30 s, and 72°C for 2 min for 30 cycles. This reaction led to the joining of the TbDHSc or TbDHSp flanking regions in a cassette containing the resistance marker for replacement of the allele. The PCR product was gel-purified using the High Pure PCR product purification kit (Roche Applied Science) and transfected into *T. brucei* parasites directly. For transfections, the first allele of the TbDHSc or TbDHSp gene was replaced with the hygromycin resistance gene, and clonal lines were obtained by limited dilution. The resultant single knock-out (SKO) cells were then transfected with the tagless TbDHSc- or TbDHSp-regulatable gene expression constructs and selected using pheleomycin. Confirmation of ectopic expression upon Tet induction was obtained by quantitative PCR (qPCR) using a primer in the ectopic UTR and a primer within the gene (Table 1) and by Western blot using rabbit antiserum to recombinant *T. brucei* DHS. Expression levels from the Tet-induced ectopic copy were considerably higher than from the genomic DHS alleles. SKO cell lines that were confirmed to show good expression from the ectopic gene copy were then used to create conditional double knock-out (cDKO) cell lines by replacement of the second allele with the blastidicin resistance gene. Knock-outs were confirmed by PCR. Tet-regulatable cDKO cell lines were propagated in media containing Tet to maintain expression of TbDHSc or TbDHSp and with G418, hygromycin B, pheleomycin, and blastidicin to maintain selection. To study the effects of DHS knockdown, TbDHSc and TbDHSp cDKO cells were washed three times with Tet-free media prior to plating in fresh media. Growth curves were analyzed for n = 3 biological replicates. 

**T. brucei Mouse Infection Model**—A second independent set of cDKO clones was generated at University of Dundee and used for in vivo studies. Methods were essentially as described (23) using flanking primers and PCR (Table 1) to generate gene replacement constructs for TbDHSc and TbDHSp containing the hygromycin or puromycin resistance genes. These cell lines showed similar in vitro behavior to those characterized in Fig. 2,
Allosteric Activation of Deoxyhypusine Synthase

A and C. Wild-type and cDKO BSF T. brucei parasites were cultured in the absence of selectable drugs for 24 h with cDKO cells grown ± Tet. Cells were used to infect n = 3 mice per group (dosed ± doxycycline (Dox)) by a single intraperitoneal injection of 10⁶ parasites as described (24).

Recombinant Expression of DHS and eIF5A—TbDHSc, TbDHSp, and TbEFSAs gene were cloned into pE-SUMO Kan (Life Sensors) for expression as N-terminal His₆-SUMO-tagged fusion proteins. PCR fragments generated with primers shown in Table 1 were digested with Bsal/XbaI and cloned directly into Bsal-linearized pE-SUMO. Untagged TbDHSp was also cloned into the HindIII-KpnI site of the pT7-FLAGTM-MAT-Tag®-2 vector (Sigma) where the C-terminal His tag was removed by insertion of a stop codon. HDDS (P49366.1) and human eIF5A (HsEIF5A) (P63241.2) sequences were codon-optimized for E. coli, synthesized by GenScript, and cloned into pE-SUMO Kan as above. Genes were expressed in T1 phage-resistant E. coli BL21(DE3) cells selected with kanamycin (50 μg ml⁻¹) for single gene expression or kanamycin (50 μg ml⁻¹) and ampicillin (100 μg ml⁻¹) for co-expression of SUMO-TbDHSc and TbDHSp. Protein expression was induced at 37 °C for 0.5 h, resuspended in Buffer A (50 mM Hepes, pH 8.0, 300 mM NaCl, 50 mM imidazole, 2 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride (PMSF)), and lysed by high pressure disruption (EmulsiFlex-C5, Avestin). Lysate was clarified using a protein assay (Bio-Rad) and a bovine serum albumin hot or a hot-cold mixture ranging in concentration (0.2 to 150 μM), NAD⁺ (0.5–1000 μM), DTT (1 mM), and glycine-NaOH buffer (0.2 M, pH 9.3). Initial rates of velocity data were fitted to Equation 1 using GraphPad Prism. The catalytic rate constant, kcat, was calculated based on active monomer concentration. For GC7 IC₅₀ determination, velocity data were fitted to Equation 1 using GraphPad Prism. Data were collected in triplicate, and error represents the mean ± S.D.

Sedimentation Velocity—TbDHSp-TbDHSc complex (0.5 ml) was prepared at a range of concentrations (A₂₈₀ 0.7, 0.2, and 0.05) in assay buffer (50 mM Hepes, 150 mM NaCl, pH 8.0) and then loaded into ultracentrifuge cells assembled with sapphire windows after overnight incubation at 4 °C. A₂₈₀ and interference data were collected at 20 °C in an An-50 Ti rotor monitored continually for 16 h at 40,000 rpm in an Optima XL-1 ultracentrifuge (Beckman-Coulter). Complete sedimentation was observed by 5 h. Interference data were analyzed using SEDFIT (27) to calculate sedimentation-coefficient distributions (c(s)) and estimate molecular weight. c(s) plots were generated in GUSI.

Protein Quantitation—Protein concentration was quantitated using a protein assay (Bio-Rad) and a bovine serum albumin standard curve, unless otherwise stated.

RNA and DNA Purification—RNA was isolated from T. brucei single marker BSF cells (2 × 10⁶ cells), washed in phosphate-buffered saline (PBS), pH 7.4, and then lysed using TRIzol reagent (Invitrogen) followed by purification with the RNesy mini kit as recommended by the manufacturer (Qiagen). DNA was isolated from T. brucei single marker BSF cells (10⁷ cells) that were harvested and washed twice with PBS, pH 7.4, before being resuspended in DNA lysis buffer (1 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 5 mM NaCl, 20% SDS, 0.1 mg of proteinase K) and incubated for 6 h at 55 °C. RNA was digested using RNase A at 37 °C for 30 min. An equivalent volume of chloroform was added, and the DNA was extracted in the aqueous layer, ethanol-precipitated, and resuspended in 50 mM Tris-HCl, pH 8.0.

Quantification of RNA Levels by qPCR—cDNA was synthesized from isolated RNA using the SuperScript®III first-strand synthesis system (Invitrogen). The reaction mixture (20 μl) contained the following: 2 μg of total RNA, 50 ng of random hexamers, 250 μM dNTPs, 1 × RT buffer, 5 mM MgCl₂, 10 μM DTT, 40 units of RNaseOUT™, and 200 units of SuperScript™III RT. RNA and primers were initially denatured at 65 °C for 5 min before the remaining components were added. The subsequent reaction conditions were as follows: annealing at 25 °C for 10 min, synthesis at 50 °C for 50 min, termination at 85 °C for 5 min, and removal of RNA with RNase H (2 units) at 37 °C for 20 min. The synthesized cDNA was used directly for qPCR without further purification. Relative gene abundance was quantitated using the iQ™ SYBR® Green Supermix and CFX96-Real-Time System by Bio-Rad. The reaction mix (20 μl) contained 100–150 ng of cDNA, 1 × iQ™ SYBR® Green Supermix, and 100 nm primers (Table 1). PCR cycling conditions

DHS Enzyme Activity Assay—Activity was measured at 37 °C in 1 h reactions using a nitrocellulose filter binding assay to detect the incorporation of [³H]spermidine into eIF5A as described (25, 26). Reaction mixture (20 μl) contained recombinant DHS (4–40 μM HsDHSc, 10–40 μM TbDHSc, or 5–20 mM TbDHSc-TbDHSp), eIF5A (0.1–100 μM), and [³H]spermidine (7.5 μM hot or a hot-cold mixture ranging in concentration from 0.2 to 150 μM), NAD⁺ (0.5–1000 μM), DTT (1 mM), and glycine-NaOH buffer (0.2 M, pH 9.3). Initial velocities were fitted to Michaelis-Menten equation using GraphPad Prism. The catalytic rate constant, kcat, was calculated based on active monomer concentration. For GC7 IC₅₀ determination, velocity data were fitted to Equation 1 using GraphPad Prism. Data were collected in triplicate, and error represents the mean ± S.D.
included a one-time initial denaturation at 95 °C for 3 min followed by 40 cycles of the denaturation at 95 °C for 15 s and annealing/extension at 56 °C for 60 s. After completion of the cycles, melt curve analysis was done from 55 to 95 °C in 0.5 °C increments. Relative gene abundance was calculated by ΔΔCT using telomerase reverse transcriptase (TERT) as the reference gene (28).

Western Blot Analysis—Cells (typically 2 × 10⁶) were harvested by centrifugation (2000 × g, 10 min); pellets were washed twice with PBS, pH 7.4 (1 ml), resuspended in Tryp Lysis Buffer (50 mM Hepes, pH 8.0, 100 mM NaCl, 5 mM β-mercaptoethanol, 2 mM PMSF, 1 µg ml⁻¹ leupeptin, 2 µg ml⁻¹ antipain, 10 µg ml⁻¹ benzamidine, 1 µg ml⁻¹ pepstatin, 1 µg ml⁻¹ chymostatin), and lysed with three freeze/thaw cycles. The lysate was clarified by centrifugation (13,000 × g, 10 min, 4 °C), and supernatant (30 µg of total protein) was separated by SDS-PAGE and transferred to a PVDF membrane (iBlot, Invitrogen). The membrane was blocked with 5% milk in Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) and incubated with primary antibody. Primary antibodies anti-TbDHSc (rabbit polyclonal), anti-TbDHSp (rabbit polyclonal), anti-FLAG/M2 (mouse monoclonal, Sigma), or anti-AU1 (mouse monoclonal, Covance) were used at a 1:1000 dilution, anti-FLAG/M2 (mouse monoclonal, Sigma) was used at a 1:10,000, goat anti-rabbit (mouse monoclonal, Covance) or goat anti-mouse antibody conjugated to alkaline phosphatase (Sigma). Protein was detected using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). For a loading control, membranes were stripped and reprobed with antibody to TbdHODH as described previously (19). Membranes were stripped with Restore Western blot stripping buffer (Thermo Scientific, Rockford, IL) for 20 min at RT and rinsed with TBS before blocking again with 5% milk in TBS.

Immunoprecipitation—BSF T. brucei cells (10⁶ cells) co-transfected with the AU1-TbDHSc and FLAG-TbDHSp expression plasmids were induced with Tet for 24 h before harvesting by centrifugation (2000 × g, 10 min). Cell pellet was washed twice with PBS, pH 7.4 (1 ml), resuspended in hypotonic buffer (10 mM Tris, pH 7.5, 2 mM PMSF, 1 µg ml⁻¹ leupeptin, 2 µg ml⁻¹ antipain, 10 µg ml⁻¹ benzamidine, 1 µg ml⁻¹ pepstatin, 1 µg ml⁻¹ chymostatin), and incubated on ice for 1 h followed by three freeze/thaw cycles resulting in lysis and adjusted with salt buffer (10 mM Tris, pH 7.5, 400 mM NaCl) to 80 mM NaCl. Cell lysate was clarified by centrifugation (10,000 × g, 10 min, 4 °C). Total soluble protein (50 µg) was incubated alone or with either mouse monoclonal anti-AU1 antibody (Covance) or mouse monoclonal M2 anti-FLAG antibody (Sigma) (1:150 dilution for both antibodies) for 12 h at 4 °C. Dynabeads® protein A (50 µl, Invitrogen) was added, and the antibody-antigen complex was captured with a magnetic stand. The beads were washed three times with TBS, pH 7.6, and the antibody-antigen complex was eluted with 40 µl of citrate buffer, pH 3. Eluent was neutralized with 0.1 M NaOH (5 µl) before separation by SDS-PAGE and Western blot analysis as described above.

RESULTS

Phylogenetic Analysis of the DHS Genes in the Kinetoplastids—Two distinct clades of trypanosomatid DHS proteins were identified by BLASTP analysis of the translated trypanosomatid genome using HsDH as the search model (Fig. 1 and supplemental Fig. S1). Multiple sequence alignment and comparison of key residues show that one group consists of a protein that contains the catalytic Lys, shown to form the key imine intermediate with substrate (Scheme 1) (10, 17), while the other DHS group lacks the catalytic Lys despite containing many putative substrate-binding residues. The genes encoding these proteins are present on different chromosomes. We refer to the T. brucei gene products as TbdHSc (Tb927.10.2750) reflecting the presence of the catalytic (c) Lys, and TbdHSp (Tb927.1.870) where the enzyme (p) designates an activating function. TbdHSc exhibits 28% amino acid sequence identity with human HsDH but is 92 amino acids larger due to internal expansions. TbdHSp shares 40% identity with HsDH but only 30% identity with TbdHSc. Trypanosoma cruzi has two DHS gene homologs (TcDHSc(B) and TcDHSc(C)) that are closely related and group together on the tree and one copy of the more diverged DHS gene (TcDHSc(A)), whereas Leishmania species and T. brucei contain only a single copy of each gene. BLASTP analysis of eukaryotic DHS proteins showed that Entamoeba species also contain two significantly diverged paralogs of DHS, one with the catalytic Lys that groups with TbdHSc and one without the Lys that groups with TbdHSp. It is not clear if a single gene duplication event led to the generation of both the trypanosomatid DHS and Entamoeba DHS homologs or if they arose from independent events. All other eukaryotes appear to contain either only a single DHS gene or closely related gene duplicates that retain the catalytic Lys and are thus likely to be functionally equivalent and catalytically competent.

TbdHSc and TbdHSp Genes Are Essential for T. brucei Growth—We generated cDKO of the TbdHSc and TbdHSp genes in the T. brucei bloodstream form cells to determine whether one or both of the DHS genes were essential for cell growth. T. brucei is a diploid organism, so for both genes one endogenous locus was replaced with the hygromycin resistance antibiotic selection marker generating the final cDKO cell lines. The presence of the catalytic (c) Lys, and TbdHSp was slower growth by day 4, and to complete parasite clearing by day 6 (Fig. 2, B and D). Likewise, for the TbdHSp cDKO parasites, no detectable TbdHSp RNA or protein was observed 24 h after Tet withdrawal, and cell death occurred by day 8 (Fig. 2, D and E). Cultures were monitored by microscopy for an additional 4 days after cell death, and no live parasites were observed. These data demonstrate that both TbdHSc and TbdHSp are essential for survival of BSF T. brucei in vitro.
Interestingly, in both cDKO cell lines, knock-out of one DHS gene (either TbDHSc or TbDHSp) led to the simultaneous loss of both TbDHSc and TbDHSp proteins (Fig. 2, A and D) despite the finding that the RNA as expected was only depleted for the gene targeted for knockdown (Fig. 2, B and E). These data suggested that TbDHSc and TbDHSp form a complex in the cell and that the individual proteins were not stable when the complex was disrupted.

**DHS Is Essential for Infectivity of T. brucei in Mice**—Mice were infected with TbDHSc and TbDHSp cDKO lines. One set of animals received Dox in their drinking water to maintain expression of the respective DHS proteins, and for the other set

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**FIGURE 1.** Phylogenetic analysis of DHS genes in trypanosomatids. A, spermidine and hypusine metabolic pathway in *T. brucei*. B, partial sequence alignment of DHS from select eukaryotes chosen to include a representative of each of the major eukaryotic lineages in the analysis: Opisthokonta (humans, *Trichoplax*, and *Saccharomyces*); Excavata (trypanosomatids, *Giardia*, and *Naegleria*); Amoebozoa (*Entamoeba* and *Acanthamoeba*); Archaeplastida (*Arabidopsis* and *Chlamydomonas*), and Alveolata (*Perkinsus*). Highlighted in yellow is the catalytic lysine residue. For organisms that contain more than one DHS homolog, duplicates are indicated using consecutive letters (A–C, etc.), except for those where function has been demonstrated in this paper (e.g. *T. brucei* DHSc and DHSp). Gene IDs are as follows: Homo sapiens (P49366); *Trichoplax* adherens (EDV28024.1); *Chlamydomonas* reinhardtii (A, EDP09680.1; B, EDP01029.1); *Perkinsus* marinus (A, EER15074.1; B, EER03596.1). The full sequence alignment is shown in supplemental Fig. S1. C, Neighbor-Joining tree constructed with Mega5.
FIGURE 2. Effects of DHS knockdown on T. brucei growth and survival. Panel 1, effects of TbDHSc knockdown; panel 2, effects of TbDHSp knockdown. A and D, cell growth curve of log(cell number × dilution factor) over time. Data represent an average mean ± S.E. for multiple independent biological replicates. A, TbDHSc cDKO cells (n = 6); D, TbDHSp cDKO cells (n = 3); blue circle, + Tet (0.5 μg/ml); green square, − Tet. Panel inset, representative Western analysis performed with rabbit polyclonal antibodies to the indicated protein (30 μg of total protein); TbDHODH was detected as a loading control. B and E, qPCR analysis of mRNA levels for TbDHSc cDKO cells (B) and TbDHSp cDKO cells (E). The symbol < indicates RNA levels were below the limit of detection. Error bars represent the mean ± S.D. for n = 3 replicates. C and F, Kaplan-Meier survival curves of infected mice (n = 3 per group). C, TbDHSc cDKO; F, TbDHSp cDKO cells; SM (single marker); T. brucei wild-type cells (purple), and cDKO-infected mice treated with (blue) or without (green) Dox.

Tet was removed 24 h prior to inoculation, and mice were not administered Dox. Mice infected with TbDHSc or TbDHSp cDKO lines that received Dox in their water succumbed to parasitemia by day 6 after inoculation and showed an identical time course to mice infected with the control parental cell line (Fig. 2, C and F). In contrast, in the absence of Dox, mice infected with the cDKO of TbDHSc survived to the end of the experiment (day 30), at which time they remained parasite free and were assumed to be cured (Fig. 2C). Mice infected with cDKO of TbDHSp showed a prolonged survival time, but they eventually succumbed to parasitemia on day 24 after infection (Fig. 2F). The relapse of parasitemia in the TbDHSp cDKO infection suggests that a small number of parasites survived most likely through mutation in the Tet promoter, allowing re-expression of the TbDHSp protein, as documented previously for other proteins with this system (21). These data demonstrate that TbDHSc and TbDHSp are essential to sustain an in vivo infection of T. brucei in mice.

TbDHSc and TbDHSp Form a Functional Complex—To determine whether TbDHSc and TbDHSp form a complex, we generated a stable T. brucei BSF cell line that co-expressed N-terminally tagged AU1-TbDHSc and FLAG-TbDHSp. Immunoprecipitation of AU1-TbDHSc from soluble T. brucei lysates using monoclonal antibody to AU1 was performed followed by Western blot analysis with anti-AU1 and anti-FLAG antibody. Both AU1-TbDHSc and FLAG-TbDHSp were detected (Fig. 3A). Likewise, if a monoclonal antibody to FLAG was used for immunoprecipitation, both AU1-TbDHSc and FLAG-TbDHSp were found in the immunoprecipitate (Fig. 3A). The ability of purified recombinant DHS to catalyze the hypusine modification of eIF5A was measured with either HsDHs or human eIF5A as substrate using [3H]spermidine and a previously described filter binding assay (25, 26). The specific activity of purified TbDHSc and TbDHSp was 103-fold lower than the activity of HsDHs and HsIF5A (Table 3), the latter being in agreement with previous reports (29, 30). The low observed activity of TbDHSc was similar to that...
Allosteric Activation of Deoxyhypusine Synthase

Data were collected at fixed substrate concentrations (1 mM NAD⁺, 7.5 μM [3H]spermidine, and 10 μM eIF5A). Error represents the mean ± S.D. for three replicates.

| Substrate  | Tbel5F5A | Hsel5F5A |
|------------|----------|----------|
| Enzyme     |          |          |
| HsDHS      | 0.0029 ± 0.0001 | 0.016 ± 0.0003 |
| TbelDHSc   | 1.8 × 10⁻⁵ ± 2 × 10⁻⁴ | <10⁻⁵ |
| TbelDHSp   | <10⁻⁷   |          |
| TbelDHSc-TbelDHSp | 0.0057 ± 0.0001 | 0.0035 ± 0.0001 |

TABLE 4
Steady-state kinetic parameters for T. brucei heterotetrameric DHS

Variable concentrations of the substrate under determination were used with fixed concentrations (1 mM NAD⁺, 100 μM Tbel5F5A, and 100 μM spermidine) of the other substrates. Error represents the standard deviation for three independent experiments.

| Substrate  | kₐp | k₋a |
|------------|-----|-----|
| NAD⁺       | 82 ± 16 | 0.018 ± 0.001 |
| Tbel5F5A   | 0.7 ± 0.1 | 0.018 ± 0.001 |
| Spermidine | 43 ± 5 | 0.015 ± 0.001 |

was functional on both T. brucei and human eIF5A substrates (Table 3). Substrate titrations were performed using the heterotetrameric TbelDHSc (Table 4) and showed that the kₐp values for Tbel5F5A were similar to what has been reported for HsDHS/Hsel5F5A, although the kₐp values for NAD⁺ and spermidine were ~10-fold higher than reported for HsDHS (30). These data demonstrate that the heterotetrameric TbelDHSc complex is the functional enzyme in T. brucei.

To further characterize TbelDHSc activity, sodium cyanoborohydride was used to trap the imine reaction intermediates (Scheme 1). Reaction mixtures containing [3H]spermidine were treated with sodium cyanoborohydride, TCA-precipitated, separated by SDS-PAGE, and analyzed by autoradiography (Fig. 3D). In reactions containing TbelDHSc-TbelDHSp and Tbel5F5A, two bands were detected as follows: a strong band corresponding in size to Tbel5F5A, and a weaker band corresponding to TbelDHSc (Fig. 3D). These data show that as expected DHS is transiently labeled during the reaction and that the labeled substrate is transferred to Tbel5F5A resulting in deoxyhypusine modification (Fig. 1A and Scheme 1). No labeling of either Tbel5F5A or TbelDHSc was detected for reactions containing only TbelDHSc as the catalyst, again showing that on its own TbelDHSc is highly impaired in catalytic function and that only in complex with TbelDHSp is it fully functional.

GC7 is a structural analog of spermidine and a known inhibitor of HsDHS (31). GC7 inhibited the activity of TbelDHSc-TbelDHSp and the growth of BSF cells at similar concentrations (IC₅₀ = 1.5 ± 0.15 μM and EC₅₀ = 8.0 ± 1.5 μM, respectively). When AU1-TbelDHSc or FLAG-TbelDHSp was overexpressed in BSF cells independently, there was not a significant shift in the EC₅₀ value for GC7 (EC₅₀ = 5–6 μM). However, overexpression of AU1-TbelDHSc and FLAG-TbelDHSp together reduced sensitivity to GC7 (EC₅₀ = 26 ± 3.0 μM), while TbelDHSc and TbelDHSp SKO lines were somewhat more sensitive (EC₅₀ = 3.8 ± 0.4 and 5.5 ± 0.84, respectively). These data suggest that the mechanism of action of cell killing by GC7 is mediated by DHS.

reported for the Leishmania enzyme (18). No activity was detectable for TbdHSc with Hsel5F5A as the substrate. Recombinant TbdHSp showed no activity within the limit of detection with either eIF5A substrate (Table 3).

To assess the activity of the TbdHSc-TbdHSp protein complex, tagless TbdHSp was co-expressed with His₅SUMO-TbdHSc in E. coli. Following purification by Ni²⁺-affinity chromatography, the SUMO tag was removed using Ulp1 protease and the tag-free protein complex further purified by size exclusion chromatography. TbdHSc and TbdHSp were present in approximately equimolar amounts in the peak fraction from this column confirming that the two paralogous gene products form a stable complex (Fig. 3B). Velocity sedimentation and analytical ultracentrifugation (Fig. 3C) revealed a single species of 175 kDa consistent with a 2:2 TbdHSc-TbdHSp heterotramer. The specific activity of the heterotramer was ~3000-fold higher than for the TbdHSc homotetramer, and it
inhibition, providing further evidence that TbDHSc-TbDHSp is the functional DHS species in T. brucei.

DISCUSSION

Regulation and control of gene expression and modulation of enzyme activity are critical aspects of cellular function. Although diverse mechanisms for regulating enzyme activities are well known, we report here a new paradigm for potential enzyme regulation in the trypanosomatids based on activators that are catalytically dead enzyme paralogs termed prozymes. Remarkably, these parasitic protozoa have independently evolved this mechanism in two different steps in the same essential biochemical pathway, the biosynthesis of spermidine and the subsequent hypusine modification of a critical lysine in the translation factor eIF5A. Gene duplication of both trypanosomatid AdoMetDC and DHS led to the evolution of an enzyme activation mechanism in which one paralog retained limited catalytic function and the other lost key catalytic residues, but retained the ability to oligomerize with the catalytic subunit to greatly enhance catalytic activity. A pivotal feature of this model is that observed activation by the prozyme component is dramatically large (1000–3000-fold) and is thus likely to result from cooperative structural changes.

The functional significance of prozyme activation of DHS has been clearly demonstrated by our studies. We show that both TbDHSc and TbDHSp are essential for the growth of mammalian blood form T. brucei and for infection of a mammalian host. Additionally, GC7, a known inhibitor of DHS, was found to have anti-trypanosomal activity. We demonstrate that the functional species of DHS in T. brucei is a heterotetrameric complex between TbDHSc and TbDHSp and that complex formation is required not only for full activity but also for stability of the proteins in the parasite. These data genetically and chemically validate T. brucei DHS as a potential drug target and demonstrate the importance of the functional heterotetrameric DHS complex.

Despite the similarities to the AdoMetDC example, significant differences in mechanism are also present. The two DHS subunits are not stable in T. brucei unless in complex with each other. In contrast AdoMetDC prozyme and AdoMetDC levels are independent, and one can exist stably in excess over the other, which is a significant factor in AdoMetDC regulation in T. brucei (8, 9). T. brucei up-regulates AdoMetDC prozyme protein levels in response to inhibition or knockdown of AdoMetDC. It remains to be determined if T. brucei also regulates levels of DHSp to control deoxyhypusine formation in the parasite. Finally, although the AdoMetDC prozyme mechanism appears novel to the trypanosomatids, BLASTP analysis identified potential DHSp homologs in Entamoeba species in addition to the trypanosomatids. Functional analysis is needed to determine whether the Entamoeba DHSp paralog is also required to activate DHSc in this genus.

Our discovery that T. brucei DHS is activated by a prozyme mechanism adds to the list of unusual and novel mechanisms that cells have evolved to regulate polyamine metabolism or modulate enzyme activity. Polyamine metabolism is tightly regulated in mammals, plants, and yeast, although interestingly, no regulation of DHS has been described (3–5). Regulation occurs through common mechanisms such as transcriptional control but also through novel pathway-specific mechanisms. The intracellular turnover rate of ODC is controlled by expression of a protein inhibitor termed antizyme that targets ODC for degradation by the 26 S proteasome. Antizyme expression is in turn initiated by translational frame-shifting of antizyme mRNA when spermidine levels are high (32), and it is further regulated by the antizyme inhibitor, which is itself an inactive paralog of ODC (33). AdoMetDC expression is controlled by a small ribosome-stalling upstream open reading frame (uORFs) that is also sensitive to spermidine levels (34). Trypanosomatids not only lack these mechanisms but unlike other eukaryotes are also unable to regulate RNA polymerase II transcription (35–37). The protein coding genes typically lack introns (38) and are transcribed as large polycistronic clusters, which undergo 5′-leader splicing of the pre-mRNA (39). Regulation instead occurs during mRNA processing, mRNA degradation, translation, protein processing, and protein turnover (36). Furthermore, as a consequence of the mRNA trans-splicing reaction, 5′-UTRs are short, and translational control by uORFs has not been observed. Thus, the driving force to evolve novel mechanisms to regulate the polyamine pathway in trypanosomatids may have been the paucity of other potential mechanisms. Given the large investment made by cells to control and regulate polyamine levels, together with the number of novel mechanisms that have been uncovered, it is clear that regulation of this pathway is a key cellular function.

Inactive paralogs have been identified in a wide variety of gene families in metazoan species, although they are most prevalent in the kinase, protease, sulfotransferase, and RAS-like protein families (40–43). Inactive paralogs are perfectly poised to play regulatory roles, retaining the ability to bind both ligands and regulatory molecules. It has been shown that when duplicate genes evolve complementary mutations, the ability of cells to maintain both duplicates is enhanced, allowing novel function to evolve (44), thus providing a platform for the evolution of a regulatory function. With the exception of pseudo-kinases, there is still limited functional data on the roles of inactive paralogs. Examples of regulation by both inhibitory and activating mechanisms have been described, although most of the examples involve inhibition or dominant negative effects. The sheer magnitude of the activation observed for T. brucei DHS and AdoMetDC is unprecedented, and the observation that this occurs at two points in the same metabolic pathway is new.

In conclusion, the ability to regulate enzyme activity with a catalytically dead paralog provides cells with another tool for post-transcriptional regulation. Trypanosomatids represent the only known species where this regulatory strategy is available to potentially control the activity of DHS and AdoMetDC. Evolution of the prozyme mechanism in the trypanosomatids may have been driven by the need to control polyamine synthesis and function in an organism that lacks transcriptional control of gene expression and the frame-shifting and uORF-based mechanisms employed by many other eukaryotes. The discovery of this novel enzyme activation mechanism first for AdoMetDC and now for DHS powerfully confirms the importance of polyamines in the parasite, first exemplified by the
Allosteric Activation of Deoxyhypusine Synthase

discovery of trypanothione (6). Our data suggest that the paradigm of enzyme activation by a catalytically dead paralog may be more widespread than currently known. Indeed, many additional examples of this mechanism for the regulation of enzyme function in eukaryotes are likely still undiscovered.

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REFERENCES

1. Stuart, K., Brun, R., Croft, S., Fairlamb, A., Gürtler, R. E., McKerrow, J., Reed, S., and Tarleton, R. (2008) Kinetochoïdists: related protozoan pathogens, different diseases. J. Clin. Invest. 118, 1301–1310
2. Willert, E., and Phillips, M. A. (2012) Regulation and function of polyamines in African trypanosomes. Trends Parasitol. 28, 66–72
3. Casero, R. A., and Pegg, A. E. (2009) Polyamine catabolism and disease. Biochim. Biophys. Acta 1794, 323–338
4. Pegg, A. E., and Casero, R. A., Jr. (2011) Current status of the polyamine research field. Methods Mol. Biol. 720, 3–35
5. Pegg, A. E. (2009) Mammalian polyamine metabolism and function. IUBMB Life 61, 880–894
6. Fairlamb, A. H., Blackburn, P., Ulrich, P., Chait, B. T., and Cerami, A. (1985) Trypanothione: A novel bis(glutathionyl)peroximide cofactor for glutathione reductase in trypanosomatids. Science 227, 1485–1487
7. Willert, E., Oza, S. L., Patterson, S., Spinks, D., Thompson, S., and Fairlamb, A. H. (2009) Dissecting the essentiality of the bifunctional trypanothione synthetase-aminase in Trypanosoma brucei using chemical and genetic methods. Mol. Microbiol. 74, 529–540
8. Willert, E. K., Fitzpatrick, R., and Phillips, M. A. (2007) Allosteric regulation of an essential trypanosome polyamine biosynthetic enzyme by a catalytically dead homolog. Proc. Natl. Acad. Sci. U.S.A. 104, 8275–8280
9. Willert, E. K., and Phillips, M. A. (2008) Regulated expression of an essential allosteric activator of polyamine biosynthesis in African trypanosomes. PLoS Pathog. 4, e1000183
10. Oka, K., Nishimura, K., Zanelli, C. F., and Valentini, S. R. (2010) Functional significance of eIF5A and its hpyusine modification in eukaryotes. Amino Acids 38, 491–500
11. Nishimura, K., Lee, S. B., Park, J. H., and Park, M. H. (2012) Essential role of eIF5A-1 and deoxyhypusine synthase in mouse embryonic development. Amino Acids 42, 703–710
12. Ude, S., Lassak, J., Starosta, A. L., Kraxenberger, T., Wilson, D. N., and Jung, K. (2013) Translation elongation factor EF-P alleviates ribosome stalling at polyproline stretches. Science 339, 82–85
13. Doerfel, L. K., Wohlgemuth, I., Kothe, C., Peske, F., Urlaub, H., and Rodnina, M. V. (2013) EF-P is essential for rapid synthesis of proteins containing polyproline stretches. J. Mol. Biol. 434, 434–438
14. Schuck, P., Perugini, M. A., Gonzales, N. R., Howlett, G. J., and Schubert, D. (2002) Size-distribution analysis of proteins by analytical ultracentrifugation: strategies and application to model systems. Biochem. J. 382, 1096–1111
15. Barendorf, M., and Boshart, M. (2010) Selection of reference genes for mRNA quantification in Trypanosoma brucei. Mol. Biochem. Parasitol. 172, 52–55
16. Sasaki, K., Abid, M. R., and Miyazaki, M. (1996) Deoxyhypusine synthase gene is essential for cell viability in the yeast Saccharomyces cerevisiae. FEBS Lett. 384, 151–154
17. Joe, Y. A., Wolff, E. C., and Park, M. H. (1995) Cloning and expression of human deoxyhypusine synthase cDNA. Structure-function studies with the recombinant enzyme and mutant proteins. J. Biol. Chem. 270, 22386–22392
18. Lee, Y. B., Park, M. H., and Folk, J. E. (1995) Diamine and triamine analogs and derivatives as inhibitors of deoxyhypusine synthase: synthesis and biological activity. J. Med. Chem. 38, 3053–3061
19. Kahana, C. (2009) Regulation of cellular polyamine levels and cellular proliferation by antizyme and antizyme inhibitor. Essays Biochem. 46, 47–61
20. Ivanov, I. P., Firth, A. E., and Atkins, J. F. (2010) Recurrent emergence of catalytically inactive ornithine decarboxylase homologous forms that lack regulatory function. J. Mol. Biol. 70, 22386–22392
21. Clayton, C., and Shapira, M. (2007) Post-transcriptional regulation of gene expression in trypanosomes and leishmanias. Mol. Microbiol. 68, 37–50
22. Boyd, D., Alphy, M. S., Wylie, S., and Fairlamb, A. H. (2012) Chemical, genetic, and structural assessment of pyridoxal kinase as a drug target in the African trypanosome. Mol. Microbiol. 86, 51–64
23. Sienkiewicz, N., Jaroszowski, S., Wylie, S., and Fairlamb, A. H. (2008) Chemical and genetic validation of dihydrofolate reductase-thymidylate synthase as a drug target in African trypanosomes. Mol. Microbiol. 69, 520–533
24. Wolff, E. C., Lee, S. B., and Park, M. H. (2011) Assay of deoxyhypusine synthase activity. Methods Mol. Biol. 720, 195–205
25. Sommer, M. N., Bevec, D., Klebel, B., Fliche, B., Hölscher, K., Freudenreich, T., Hauber, I., Hauber, J., and Mett, H. (2004) Screening assay for the identification of deoxyhypusine synthase inhibitors. J. Biomol. Screen. 9, 434–438
26. Ivanov, I. P., Atkins, J. F., and Michael, A. J. (2010) A profusion of upstream regulatory mechanisms in African trypanosomes. Nature Rev. Microbiol. 8, 8275–8280
27. Brennert, C. M., and Roditi, I. (2007) Highly efficient stable transformation of bloodstream forms of Trypanosoma brucei. FEBS Lett. 578, 802–807
28. Brennert, C. M., and Roditi, I. (2007) Highly efficient stable transformation of bloodstream forms of Trypanosoma brucei. FEBS Lett. 578, 802–807
and Fasel, N. (2004) Genomic organization and gene expression in a chromosomal region of *Leishmania major*. *Mol. Biochem. Parasitol.* **134**, 233–243
40. Pils, B., and Schultz, J. (2004) Inactive enzyme–homologues find new function in regulatory processes. *J. Mol. Biol.* **340**, 399–404
41. Adrain, C., and Freeman, M. (2012) New lives for old: evolution of pseudoenzyme function illustrated by iRhoms. *Nat. Rev. Mol. Cell Biol.* **13**, 489–498
42. Zeqiraj, E., and van Aalten, D. M. (2010) Pseudokinases–remnants of evolution or key allosteric regulators? *Curr. Opin. Struct. Biol.* **20**, 772–781
43. Pei, B., Sisu, C., Frankish, A., Howald, C., Habegger, L., Mu, X. J., Harte, R., Balasubramanian, S., Tanzer, A., Diekhans, M., Reymond, A., Hubbard, T. J., Harrow, J., and Gerstein, M. B. (2012) The GENCODE pseudogene resource. *Genome Biol.* **13**, R51
44. Force, A., Lynch, M., Pickett, F. B., Amores, A., Yan, Y. L., and Postlethwait, J. (1999) Preservation of duplicate genes by complementary, degenerative mutations. *Genetics* **151**, 1531–1545
