



Characterization of the bipartite degron that regulates ubiquitin-independent degradation of thymidylate synthase

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Synopsis

TS (thymidylate synthase) is a key enzyme in the *de novo* biosynthesis of dTMP and is indispensable for DNA replication. Previous studies have shown that intracellular degradation of the human enzyme [hTS (human thymidylate synthase)] is mediated by the 26S proteasome, and occurs in a ubiquitin-independent manner. Degradation of hTS is governed by a degron that is located at the polypeptide's N-terminus that is capable of promoting the destabilization of heterologous proteins to which it is attached. The hTS degron is bipartite, consisting of two subdomains: an IDR (intrinsically disordered region) that is highly divergent among mammalian species, followed by a conserved amphipathic α -helix (designated hA). In the present report, we have characterized the structure and function of the hTS degron in more detail. We have conducted a bioinformatic analysis of interspecies sequence variation exhibited by the IDR, and find that its hypervariability is not due to diversifying (or positive) selection; rather, it has been subjected to purifying (or negative) selection, although the intensity of such selection is relaxed or weakened compared with that exerted on the rest of the molecule. In addition, we have verified that both subdomains of the hTS degron are required for full activity. Furthermore, their co-operation does not necessitate that they are juxtaposed, but is maintained when they are physically separated. Finally, we have identified a 'cryptic' degron at the C-terminus of hTS, which is activated by the N-terminal degron and appears to function only under certain circumstances; its role in TS metabolism is not known.

Key words: proteasome, relaxed purifying selection, thymidylate synthase, ubiquitin-independent

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INTRODUCTION

The pyrimidine biosynthetic enzyme TS (thymidylate synthase; EC 2.1.1.45) catalyses the reductive methylation of dUMP by N^5N^{10} -methylenetetrahydrofolate, to form TMP and DHF (dihydrofolate) [1,2]. In being the sole *de novo* source of TMP for cellular DNA replication and repair, the enzyme has been an effective target for chemotherapeutic drugs used in the treatment of cancer [1,2]. Anti-metabolites such as 5-fluorouracil and raltitrexed form TS substrate analogues that bind to and inhibit the enzyme, leading to reduced thymidylate pools and apoptosis. X-ray crystallographic studies of the TS polypeptide from an

evolutionarily wide range of eukaryotic and prokaryotic species have shown that the enzyme is highly conserved in terms of the location of secondary structural elements and overall three-dimensional (3D) conformation [3–6].

In previous work, we demonstrated that the intracellular stability of TS is increased by the binding of inhibitory ligands [7], a phenomenon that potentially constrains the effectiveness of TS-directed anti-cancer agents. Subsequent studies showed that the human enzyme [designated hTS (human thymidylate synthase)] is degraded by the 26S proteasome in a ubiquitin-independent manner [8–11]. Most proteasomal substrates require ligation of ubiquitin moieties to be recognized and degraded by the proteasome [12]. However, an increasing number of substrates

Abbreviations used: CHX, cycloheximide; eGFP enhanced green fluorescent protein; FEL, fixed effects likelihood; GFP green fluorescent protein; hA, α -helix; HLH, helix-loop-helix; hTS, human thymidylate synthase; IDR, intrinsically disordered region; ODC, ornithine decarboxylase; PUR prokaryotic ubiquitin-like protein; REL, random effects likelihood; SNAP, Synonymous Non-synonymous Analysis Program; TS, thymidylate synthase.

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do not require ubiquitylation, including ODC (ornithine de-carboxylase) [13,14], tumour suppressor p21^{Waf1/Cip1} [15,16], the NF- κ B (nuclear factor κ B) regulator I κ B α (inhibitor of κ B α) [17,18], apomyoglobin [19] and the yeast transcription factor Rpn4 [20], among others. Thus, hTS is a model for ubiquitin-independent protein turnover.

Studies in our laboratory have shown that an N-terminal segment of hTS spanning the first 45 residues is required for degradation, defining the region as a degron [9–11]. The hTS degron is portable, in that it is capable of destabilizing a heterologous protein to which it is attached [9–11]. Furthermore, it is bipartite in nature, comprising of two co-operating subdomains: an IDR (intrinsically disordered region) spanning residues 1–27, and an amphipathic α -helix (designated hA) at residues 31–42 [9]. The IDR is hypervariable in amino acid sequence among mammalian species [11], although its disordered nature has been conserved [4–6,11]. A free, unmodified N-terminal amino group and an arginine–arginine motif at residues 10–11 are required for IDR activity in promoting proteasomal degradation [10,11]. The hA segment, which is much more highly conserved among species [11], requires maintenance of an α -helical conformation for its degradative function [9]. Interestingly, the hTS degron is active when placed at either the N- or C-terminus of the reporter substrate [9]. The specific roles of the two subdomains in regulating hTS proteolysis have yet to be determined.

In the present report, we have carried out further analysis of the structure and function of the hTS degron. We have examined the origin of inter-species hypervariability within the IDR, and find that its tolerance of amino acid substitution derives from relaxed purifying selection, as opposed to positive Darwinian selection. We also show that co-operation between the IDR and hA does not require that the two elements be juxtaposed, but is maintained when they are physically separated. Finally, we have identified a ‘cryptic’ degron at the C-terminus of the hTS polypeptide that appears to be active only under particular circumstances.

EXPERIMENTAL

Bioinformatics

Sequences corresponding to the coding regions of TS mRNAs from 16 mammalian species were downloaded from the Ensembl database [21] and aligned using ClustalW2 [22,23]. Species included human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), orang-utan (*Pongo abelii*), marmoset (*Callithrix jacchus*), tarsier (*Tarsius syrichta*), rhesus monkey (*Macaca mulatta*), mouse (*Mus musculus*), rat (*Rattus rattus*), guinea pig (*Cavia porcellus*), cat (*Felis catus*), dog (*Canis lupus*), cow (*Bos taurus*), horse (*Equus caballus*), pika (*Ochotona princeps*), rabbit (*Oryctolagus cuniculus*) and dolphin (*Tursiops truncatus*). A 40-codon insertion within the cow and dog sequences, corresponding to an additional exon and likely representing a cDNA copy of an incompletely processed transcript, were removed from the analysis.

Extents of synonymous and non-synonymous substitutions within the coding regions of mammalian TS transcripts were determined for all pairwise comparisons among the 16 species using SNAP (Synonymous Non-synonymous Analysis Program), as available on the HIV database website. SNAP estimates the numbers of synonymous substitutions per synonymous site (d_S) and non-synonymous substitutions per non-synonymous site (d_N), corrected for multiple hits [24,25]. Cumulative behaviour plots for both types of substitutions along the coding regions were constructed using the ‘xyplot’ option of SNAP.

Identification of codons where $d_S \neq d_N$ was carried out by the FEL (fixed effects likelihood) and REL (random effects likelihood) methods, as provided on the Datamonitor webserver [26]. A P -value of 0.1 was set as the cut-off for FEL.

Prediction of secondary structure was done using PSIPRED, as provided on the Bioinformatics Group Server of University College London. PSIPRED is a two-stage neural network that predicts protein secondary structure based on position-specific scoring matrices [27].

Plasmid constructs

All constructs for mutant analysis (shown in Figure 1) were generated using standard techniques and were verified directly by DNA sequencing. The parental expression plasmid was pMP610, containing a full-length eGFP [enhanced GFP (green fluorescent protein)] cDNA under the control of a CMV (cytomegalovirus) promoter. For selection of transfectants, an *Escherichia coli* TS cDNA under the control of a murine stem cell virus LTR (long terminal repeat) promoter, optimized for expression in mammalian cells by modification of codon-usage, was included in the plasmid [28]. Mutagenesis was carried out by standard PCR-based protocols. Details of all plasmid constructions are available upon request.

Cell culture and transfection

Cell line RJK88.13, a TS-deficient derivative of V79 Chinese hamster lung cells [29], was maintained in DMEM (Dulbecco’s modified Eagle’s medium) (Cellgro) containing 4.5 g/l glucose and supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals) and 10 μ M thymidine. Cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere.

For generation of stable transflectants, RJK88.13 cells were transfected with the indicated expression plasmids using Lipofectamine™ 2000 according to the manufacturer’s instructions. Transfectants were selected in thymidine-free medium containing the nucleoside transport inhibitor dipyridamole (5 μ M; Sigma-Aldrich), pooled and maintained in mass culture.

Determination of protein half-lives

Half-lives were assessed following the addition of 50 μ g/ml CHX (cycloheximide; Acros Organic) to the growth medium for the indicated times. Cells were harvested by scraping, and lysed in M-Per® buffer (Pierce) containing 10 mM DTT (dithiothreitol), 2 mM 2-mercaptoethanol, 5 mM PMSF, 200 μ g/ml aprotinin,

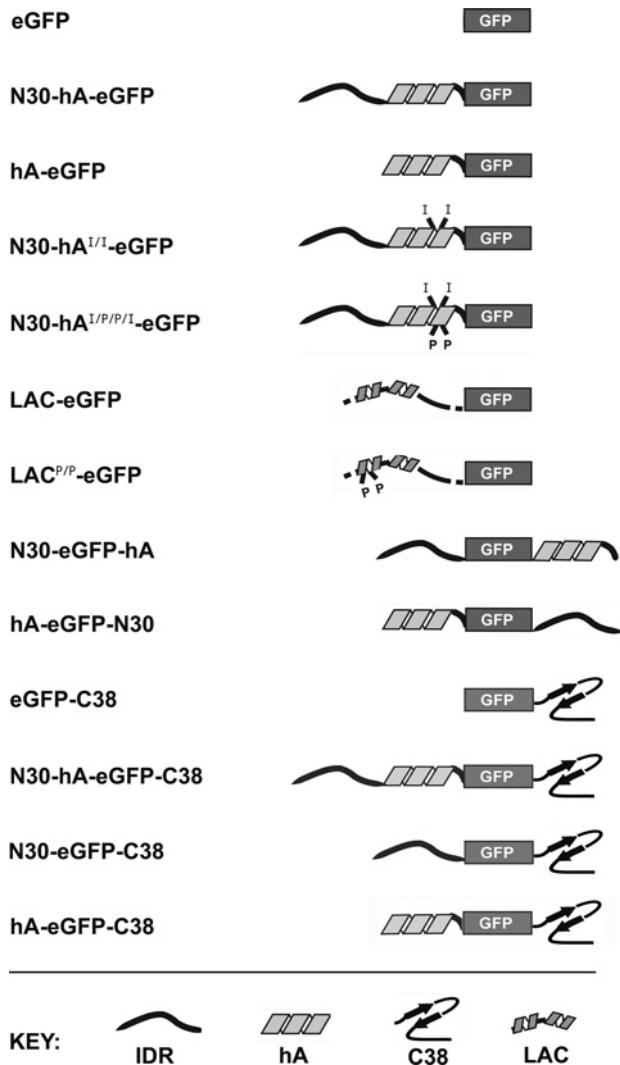


Figure 1 Constructs used in the present study

The eGFP fusion constructs utilized in the current study are shown. Schematic depictions of the various segments are indicated in the KEY at the bottom, including the disordered region (IDR), the amphipathic α -helix (hA) and the C-terminal element (C38) of hTS, as well as the N-terminal region of the *E. coli lac* repressor (LAC).

100 μ g/ml pepstatin and 50 μ g/ml leupeptin. Crude lysates were centrifuged at 15000 g for 1 h at 4 °C, and protein concentrations in the resulting extracts were quantified using the Bio-Rad assay reagent with BSA as a standard.

Immunoblotting was performed by standard techniques. Following SDS/PAGE, fractionated proteins were transferred to nylon membranes, and probed with a monoclonal antibody to GFP (Santa Cruz Biotechnology; catalogue no. sc-9996). To control for equal loading, blots were reprobed with an anti-actin monoclonal antibody (Sigma-Aldrich, Clone AC-40). The antigen–antibody complexes were visualized using appropriate secondary antibodies with the ECL® (enhanced chemiluminescence) kit (Amersham Biosciences). Densitometry was carried

out using ImageJ software maintained by the National Institutes of Health. A 2-fold dilution series of each extract was included on the blots for calibration and to correct for film exposure times. All values were normalized to actin concentrations on the same blots. Data were plotted according to first-order decay kinetics, and half-lives were determined from the slopes of the resulting lines. Each half-life determination was carried out at least twice with independently prepared cells.

RESULTS

Origin of amino acid sequence divergence within the IDR

The amino acid sequence of the IDR within the TS degron is highly divergent among mammalian species [11]. Despite such divergence, the region's role in degradation is maintained, so long as a free, unblocked N-terminus and an arginine–arginine motif at residues 10–11 are present [10]. This, along with the observation that both the IDR and hA can be replaced with segments from unrelated proteins, led us to suggest that it is the overall structure, rather than the specific primary sequence, that is critical to degron function [9].

In order to gain a deeper understanding of IDR hypervariability and its origin, we aligned and compared the coding regions of the TS mRNAs from 16 mammalian species (Supplementary Figure S1 at <http://www.bioscirep.org/bsr/033/bsr033e015add.htm>). The IDR (encoded by nucleotides 1–81 of the human transcript) spans a maximum of 31 codons, including small insertions/deletions in some species. The remainder of the transcript (encoded by nucleotides 82–939 of the human, which includes hA) spans 286 codons and contains no insertions/deletions. Within the IDR, only 16 nucleotide sites (17.8%, not including the initiator Met codon) are completely conserved among the species; most of these are located within the region encompassing residues 5–8 (Supplementary Figure S1). In contrast, 556 sites (64.8%) within the body of the polypeptide are conserved. The difference in degree of nucleotide conservation between the IDR and the remainder of the molecule is highly significant ($P < 10^{-16}$), and indicates a dramatically higher level of divergence for the IDR-encoding portion of the mammalian TS transcript.

Cumulative behaviour plots (Figure 2) indicated that synonymous substitutions are distributed equally throughout the length of the mRNA (Figure 2). They occur at a rate of 0.227 ± 0.0079 per codon within the region encoding the IDR ($R^2 = 96.96\%$; 95% CI = 0.211–0.243) and 0.233 ± 0.00073 per codon within the region encoding the body of the polypeptide ($R^2 = 99.72\%$; 95% CI = 0.231–0.234). Thus, no significant differences were observed between the two domains ($P = 0.40$). In contrast, non-synonymous substitutions are distributed in a biphasic manner (Figure 2), with the rate being 0.395 ± 0.011 per codon within the IDR ($R^2 = 97.97\%$; 95% CI = 0.372–0.418), as compared with 0.0660 ± 0.0005 per codon within the body of the molecule

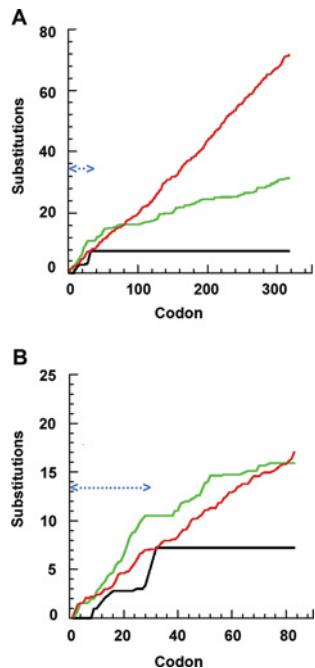


Figure 2 Cumulative behaviour of synonymous and non-synonymous substitutions along the coding region of TS mRNA

Accumulation of amino acid substitutions within the TS polypeptide for the 16 mammalian species, as determined by SNAP is plotted along the coding region of the mRNA (see the Experimental section). Codon numbering refers to hTS. The entire coding region (313 codons) is shown in (A), whereas a closer view of the first 80 codons is shown in (B). The plot for synonymous substitutions is in red, while that for non-synonymous substitutions is in green. The black line shows accumulation of insertions/deletions. The IDR is indicated by a double-arrowed dashed line (<-->).

($R^2 = 98.09\%$; 95 % CI = 0.065–0.067). Thus, a 6–7-fold higher rate of accumulation of non-synonymous substitutions is observed for the IDR ($P < 10^{-16}$).

Within the body of the polypeptide, the occurrence of non-synonymous substitutions is 3.5-fold lower than for synonymous substitutions (i.e. 0.0660 compared with 0.233 per codon, respectively; $P < 10^{-16}$) (Figure 3). This indicates strong negative selection constraining amino acid substitutions throughout most of the TS polypeptide. In contrast, within the IDR-encoding region, the rate for non-synonymous substitutions is 1.7-fold higher than for synonymous substitutions (0.395 compared with 0.227 per codon, respectively; $P < 10^{-16}$) (Figure 3). This may reflect positive Darwinian selection, whereby amino acid substitutions within the IDR are beneficial or adaptive, and are therefore favoured. Alternatively, it could be a consequence of relaxed or weakened intensity of negative (or purifying) selection, indicating a greater tolerance of amino acid changes.

To distinguish between these two possibilities, we determined the proportions of non-synonymous substitutions per non-synonymous site (d_N) and synonymous substitutions per synonymous site (d_S), and calculated d_N/d_S ratios. Purifying, or negative, selection is indicated when such ratios are <1 , while

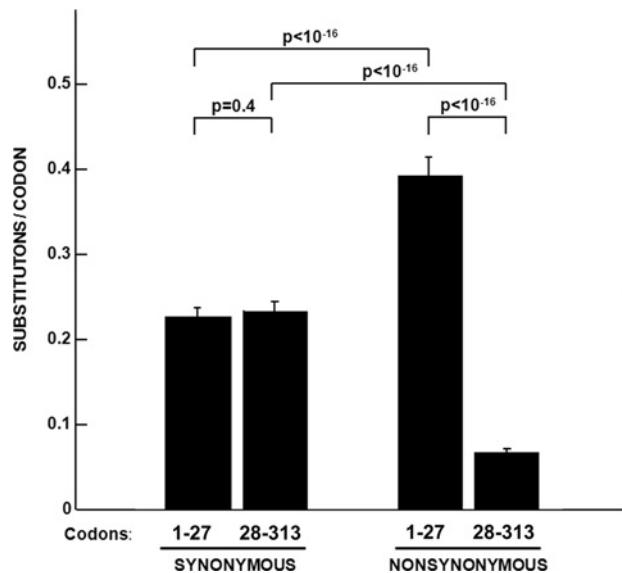


Figure 3 Rates of accumulation of synonymous and non-synonymous substitutions along the TS mRNA

Slopes of the plots in Figure 2 were determined by linear regression. Codons 1–27 represent the IDR of hTS, while codons 28–313 represent the rest of the polypeptide. Bars indicate values \pm S.E.M. P values are shown for various comparisons, as indicated by the brackets.

positive selection is indicated when they are >1 ; d_N/d_S ratios equal to 1 indicate neutrality, or an absence of selection. The d_N and d_S values for each of the 120 pairwise combinations among the 16 mammalian species were determined for both the IDR and the body of the polypeptide (see Supplementary Table S1 at <http://www.bioscirep.org/bsr/033/bsr033e015add.htm>). Average d_N/d_S ratios were 0.5953 ± 0.4433 for the IDR and 0.0745 ± 0.0334 for the body of the molecule. Thus, the IDR exhibited an 8-fold higher ratio ($P < 10^{-16}$) that was still significantly less than 1.0 ($P < 10^{-16}$). This indicates that amino acid substitutions within the disordered segment are constrained by negative selection, albeit at a relaxed or milder intensity as compared with the remainder of the polypeptide. No evidence for positive Darwinian selection was detected.

As an independent test of this conclusion, we utilized an approach that identifies individual codons under positive or negative selection. REL is a maximum likelihood strategy that determines if d_N and d_S are significantly different at each codon along a mRNA [26]. The approach failed to detect any positively selected sites in either the IDR or the body of the TS polypeptide. In contrast, 10.0 % of sites within the IDR (3 of 30, not including the initiator Met codon) and 36.4 % of sites within the body of the polypeptide (104 of 286) were identified as being under negative selection. The difference in the fraction of negatively selected codons between the IDR and the body of the polypeptide is statistically significant ($P < 10^{-4}$), and indicates that the intensity of negative selection is diminished in the former.

In all, our analysis indicates that positive Darwinian selection is not a major contributor to high levels of sequence divergence

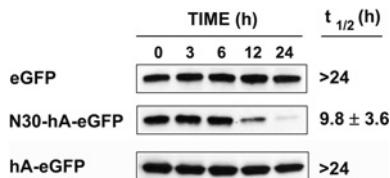


Figure 4 hA is inactive on its own as a degron

The indicated plasmid constructs (see the text and Figure 1) were stably transfected into cell line RJK88.13 and treated with CHX. Decreases in protein concentrations were monitored over time by Western blotting (see the Experimental section for details). A representative blot is shown for each construct. The half-lives ($t_{1/2}$) are shown to the right of the corresponding blots, and are presented as means \pm S.D.

within the IDR of mammalian TS. Rather, the region is constrained by negative selection, although such selection is relaxed as compared with that exerted upon the rest of the polypeptide.

The IDR and hA are both required for degron activity

In previous investigations [11], we found that a fusion protein containing the IDR of hTS (residues 1–30, denoted N30) appended to an eGFP reporter is resistant to degradation, as is the parental eGFP reporter itself. Inserting hA into N30–eGFP between the IDR and the reporter results in a protein (N30–hA–eGFP; see Figure 1) that is significantly destabilized (Figure 4 and [11]). On the basis of these findings, we concluded that both the IDR and hA are required for degradation. However, the possibility that hA on its own functions as a degron was not formally tested. To do this, we measured the half-life of a polypeptide, hA–eGFP, in which hA alone was ligated to eGFP (Figure 1). A stably transfected cell line expressing hA–eGFP was generated (see the Experimental section), and its half-life was measured by a CHX chase assay. As seen in Figure 4, hA–eGFP, like the parental eGFP substrate, is quite stable, having a half-life >24 h. Thus, hA is inactive on its own as a degron. We conclude that neither the IDR nor hA is capable of acting alone in promoting degradation; both segments are required, and must co-operate for full degron function.

A helical conformation is required for degron function

Earlier, we observed that insertion of helix-disrupting proline substitutions at residues 40 and 41 within the hA segment of fusion protein N30–hA–eGFP stabilized the molecule, indicating that a helical conformation is required for maximal degron function [9]. This was the case regardless of whether the degron was at the N- or C-terminal end of the reporter [9]. As a more rigorous test of this requirement, we examined an eGFP-fusion protein containing a very strong degron derived from a mutant hTS molecule. Polypeptide N30–hA^{I/I}–eGFP (see Figure 1) contains isoleucine substitutions at positions 39 and 42 within hA, rendering the protein very unstable, with a half-life of about 0.8 h [9]. We used the secondary structure prediction tool PSIPRED [27] to assess the potential effect of proline substitutions on the



Figure 5 Predicted impact of amino acid substitutions on the helical propensity of hA segment

The helical propensity of the region spanning residues 24–50 of wild-type hTS and mutants I39/I42, P40/P41 and I39/P40/P41/I42 were determined using PSIPRED [27]. For each molecule, the amino acid sequence is shown; targeted substitutions within mutated segments are indicated in red. The predicted secondary structure is shown below each sequence; black lines indicate coiled regions, magenta cylinders indicate α -helices and yellow arrows indicate β -strands. The blue bars at the bottom show the relative confidence for the predictions at each position along the sequence, with the height and depth of blue indicating greater confidence. The predictor accurately identified hA of wild-type hTS, known from X-ray crystallographic studies [4,5].

helical propensity of the hA segment in both N30–hA^{I/I}–eGFP and in its wild-type counterpart N30–hA–eGFP. As seen in Figure 5, for both the wild-type and mutant segments, an α -helical domain spanning residues 31–42, which is the precise location of hA as detected by X-ray crystallography [4,5], was predicted with high confidence for both segments. In contrast, loss of the helical conformation was predicted when proline residues were introduced at positions 40 and 41 (Figure 5). Thus, the secondary structure of hA is likely to be significantly disrupted by insertion of proline substitutions at positions 40 and 41.

With this information, we measured the half-life of polypeptide N30–hA^{I/P/P/I}–eGFP, which is a derivative of N30–hA^{I/I}–eGFP containing proline substitutions at sites 40 and 41 within hA (see Figure 1). As shown in Figure 6(A), N30–hA^{I/P/P/I}–eGFP had a half-life >24 h, indicating it to be significantly more stable than its parent, N30–hA^{I/I}–eGFP. This provides strong verification of the notion that promotion of degradation by hA segment requires a helical conformation.

To assess the generality of this conclusion, we tested the requirement for α -helix in a completely different system. We have shown that the N-terminal domain of the *Escherichia coli lac* repressor, which contains an HLH (helix-loop-helix) motif spanning residues 9–29, functions as a degron [9]. Deletion of the first helix of the HLH motif (residues 9–17) stabilizes the polypeptide [9], whereas deletion of the second (residues 22–29) has no effect (K. Barbour, unpublished work); thus, the first helix drives the region's degron activity. To test if an α -helical conformation is

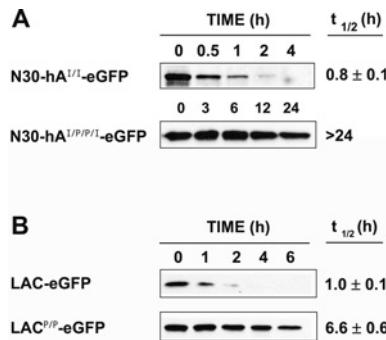


Figure 6 Degron activity requires a helical segment

The indicated plasmid constructs were stably transfected into cell line RJK88.13 and treated with CHX (see the text and Figure 1). Decreases in protein concentrations over time were monitored by Western blotting (see the Experimental section for details). A representative blot is shown for each construct. The half-lives ($t_{1/2}$) are shown to the right of the corresponding blots, and are presented as means \pm S.D.

required, we introduced proline substitutions at residues 13 and 14 of the first helix, resulting in LAC^{PP}-eGFP (see Figure 1). PSIPRED correctly predicted the wild-type LAC region to have an α -helical conformation spanning residues 9–17, which was abolished by insertion of proline at sites 13 and 14 (results not shown). Figure 6(B) shows that LAC^{PP}-eGFP exhibited a half-life of ~ 7 h, which is significantly longer than the parental LAC-eGFP molecule. Thus, as with hA of hTS, full degron function requires maintenance of a helical conformation.

Juxtaposition of the IDR and hA is not required for co-operative degron function

The hTS degron exerts its degradative function when placed at either the N-terminal or C-terminal end of the substrate [9]. Given its bipartite nature, it was of interest to ask if the two co-operating segments, i.e. the IDR and hA, must be juxtaposed. We generated and tested a fusion construct (denoted N30-eGFP-hA) having the IDR at the N-terminus and hA at the C-terminus (see Figure 1). As shown in Figure 7, N30-eGFP-hA had a half-life of 1.4 h, which is considerably shorter than that exhibited by the N30-hA-eGFP polypeptide. Thus, the two segments of the hTS degron co-operate even when placed at opposite ends of the target substrate. We also tested a protein (termed hA-eGFP-IDR; see Figure 1) having the opposite orientation, i.e. with hA at the N-terminus and the IDR at the C-terminus. Interestingly, hA-eGFP-N30 was very stable, exhibiting a half-life >24 h (Figure 7). Thus, it may be that the ability of the two degron components to co-operate when separated is position-dependent, although further studies are necessary to verify this interpretation.

The C-terminal end of TS contains a ‘cryptic’ degron

Previously, we observed that blocking the N-terminal amino group of hTS, either with a histidine-tag or by mutations that promote N- α -acetylation, results in a very stable polypeptide [8]. We interpreted this as indicating that initiation of proteasomal de-

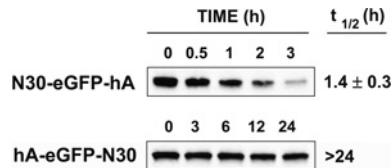


Figure 7 The IDR and hA co-operate when physically separated

Plasmid constructs were stably transfected into cell line RJK88.13 and treated with CHX (see the text and Figure 1). Decreases in protein concentrations over time were monitored by Western blotting (see the Experimental section for details). A representative blot is shown for each construct. The half-lives ($t_{1/2}$) are shown to the right of the corresponding blots, and are presented as means \pm S.D.

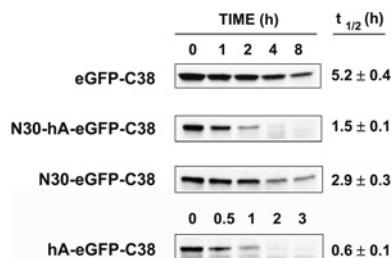


Figure 8 A ‘cryptic’ degron at the C-terminus of hTS

Plasmids expressing (see the text and Figure 1) were stably transfected into cell line RJK88.13 and treated with CHX. Decreases in protein concentrations over time were monitored by Western blotting (see the Experimental section for details). A representative blot is shown for each construct. The half-lives ($t_{1/2}$) are shown to the right of the corresponding blots, and are presented as means \pm S.D.

gradation occurs at the N-terminal end, and proceeds in the $N \rightarrow C$ direction [8]. Of interest was the finding that certain mutant hTS molecules (e.g. those with a P303L substitution) are stabilized by blocking the C-terminal end, suggesting that degradation of these mutant molecules may occur from the C-terminal end and proceed in a $C \rightarrow N$ direction [8,10]. Thus, the C-terminal region of hTS may contain a ‘cryptic’ degron that is activated in certain mutant polypeptides.

To test whether the C-terminal domain of hTS functions as a degron, we generated a polypeptide, termed eGFP-C38, containing the C-terminal 88 amino acids of hTS (residues 276–313) appended to eGFP (see Figure 1). The region includes a short, two-stranded β -sheet formed from residues 277–282 and 295–300. Figure 8 shows that the half-life of this molecule was 5.2 h, indicating that the hTS domain promotes degradation of the reporter, perhaps to a greater extent than the N-terminal degron (see Figure 4). Ligation of the N-terminal degron (i.e. residues 1–45) to eGFP-C38 results in a polypeptide, denoted N30-hA-eGFP-C38 (see Figure 1), that has a half-life of 1.5 h, indicating it to be even more unstable than eGFP-C38 (Figure 8). Thus, the C-terminal region of hTS functions as a degron, the activity of which is enhanced by the presence of the N-terminal degron at the other end of the substrate.

To determine if one or the other of the two subdomains comprising the N-terminal degron enhances the activity of the C38

element, we analysed polypeptides containing either the IDR or hA, neither of which is active on its own, appended to eGFP–C38. Polypeptide N30–eGFP–C38 (see Figure 1) exhibited a half-life of 2.9 h (Figure 8), indicating that the IDR modestly destabilizes eGFP–C38. Polypeptide hA–eGFP–C38 (see Figure 1) had a very short half-life of 0.6 h (Figure 8), indicating that hA induces potent destabilization of the substrate. We conclude that both the IDR and hA element, which by themselves are inactive in promoting degradation, promote the degron activity of the C38 element.

DISCUSSION

In earlier studies, we showed that the intracellular stability of TS is increased by the binding of inhibitory ligands [7], a finding that prompted an examination of the mechanism of TS degradation. We showed that proteolysis of the human enzyme (designated hTS) is carried out by the 26S proteasome, and occurs in a ubiquitin-independent manner. Furthermore, it is mediated by a portable, bipartite degron comprised of an IDR and an amphipathic α -helix located at the enzyme's N-terminal end [8–11]. A striking characteristic of the IDR is the occurrence of extensive sequence divergence among mammalian species [11]. Despite a high rate of amino acid substitution during evolution, the IDR has maintained its disordered character and its role in degron function. Thus, it is overall structure (or lack thereof) that is critical to degradation [9]. The specific amino acid sequence is less important, so long as it does not promote order.

It is well-recognized that IDRs typically evolve more rapidly than ordered regions, resulting in greater extents of amino acid substitution [30–32]; however, the forces driving such high substitution rates, specifically the role of positive against negative selection, are not well defined. Our analysis showed that the mean ratio of rates of non-synonymous to synonymous substitutions among pairwise comparisons of TS polypeptides from a variety of mammalian species is about 8-fold higher for the IDR as compared with the rest of the polypeptide; however, the ratio is still significantly <1 (Supplementary Table 1). Thus, although the disordered domain is more tolerant of amino acid substitutions than is the remainder of the molecule, it is still subject to negative selection. This conclusion is corroborated by the use of codon-based methods of analysis, which showed that the fraction of negatively selected codons is significantly reduced in the IDR as compared with the body of the polypeptide, and that few, if any, codons within the IDR are positively selected. In all, positive Darwinian selection appears to play little if any role in the high extents of amino acid variation within the IDR of mammalian TS.

Neither the IDR nor hA on its own is capable of promoting degradation (Figure 4). Interestingly, degron activity does not require that the two components be juxtaposed, in that they maintain the ability to co-operate when the IDR is at the N-terminus and hA is at the C-terminus (Figure 7). It is of interest that degron activity

is lost when the two elements are in the opposite orientation, i.e. when the IDR is at the C-terminus and hA is at the N-terminus (Figure 7). This may indicate a position-dependency between the two segments when physically separated. However, other interpretations are possible. For example, it may be that when placed at the N-terminus, hA is blocked or modified in some fashion that inhibits its degradative role. The fact that the helical element promotes degradation of the newly identified 'cryptic' degron at the C-terminal end of TS (see Figure 8) suggests that it is functional. However, additional studies will be required to delineate whether or not the TS degron components do exhibit position-dependency when separated.

The 'cryptic' degron spanning residues 276–313 at the C-terminus of hTS (Figure 8) was predicted from the properties of certain mutant hTS molecules analysed in earlier studies [8,10]. This degron appears to be active only when large deletions or certain amino acid substitutions are introduced into the hTS polypeptide [8,10]. The region has a small β -sheet composed of two strands spanning residues 277–282 and 295–300. The importance of this structural element is unknown. The 'cryptic' degron may play a role in regulating hTS degradation under circumstances where the N-terminal degron is either blocked or inactivated. Definition of such circumstances and their physiological implications will require further studies.

It is formally possible that some of the unstable chimaeric proteins produced and examined in the present study have acquired a ubiquitin-dependent mode of degradation, resulting in shortened half-lives. We consider this unlikely, since we find no evidence of high-molecular-mass ubiquitinated isoforms in any of the Western blots we have analysed. This holds true even when cells were grown in the presence of the proteasome inhibitor MG132, which generally causes accumulation of ubiquitinated forms.

The requirement for disordered domains (and, in some cases, co-operating helical elements) may be a general theme among ubiquitin-independent substrates. Degradation of mammalian ODC is governed by an intrinsically disordered 37-residue region at the C-terminal end of the polypeptide [13,33]. Yeast ODC contains a disordered 45-residue domain at its N-terminal end that functions as a ubiquitin-independent degron and that is followed by a predicted α -helix [14]. The ubiquitin-independent degron of Rpn4, which is 80 amino acids in length, is located at the molecule's N-terminus and is predicted to consist of a 15–20-residue disordered domain followed by a structured region [20]. Finally, analysis of apomyoglobin degradation in a purified system *in vitro* has shown that a disordered element in the middle of the polypeptide, in co-operation with helical segments located at the N-terminus, is required for proteolytic breakdown [19]. In all, these studies indicate that ubiquitin-independent degradation requires flexible, disordered regions.

Our finding that TS proteolysis requires both a disordered and an ordered segment is consistent with the so-called 'two-component' model of proteasomal function [34,35]. In this model, degradation is viewed as occurring in two phases: proteasome recognition and binding followed by initiation of polypeptide insertion into the proteolytic chamber. For ubiquitin-dependent substrates, the first phase (recognition/binding) is

mediated by covalently attached polyubiquitin moieties that recognize one or more subunits of the proteasomal complex [35–37]. For ubiquitin-independent substrates, residues within the target polypeptide chain are utilized [13]. The second phase (initiation) involves unfolding of the proteasome-bound substrate, ‘threading’ of the substrate through the pore that leads to the proteolytic chamber, and entry into the chamber itself, where proteolysis ensues [33,35]. These steps are typically governed by disordered segments that are often (although not always) located at one or the other end of the protein [33,37]. With regard to TS, the helical hA segment may be responsible for proteasome binding, whereas the IDR guides substrate unfolding and chamber entry. Specific amino acids within each domain (e.g. Arg¹⁰/Arg¹¹ within the IDR, His³⁹/Arg⁴² within hA) represent critical residues that mediate the respective functions of the degron sub-elements.

Co-operation between disordered and helical segments, such as in TS, has been observed for the PUP (prokaryotic ubiquitin-like protein) of actinobacteria. Similar to ubiquitin, PUP ligation to proteins targets them for degradation by a proteasome-like complex [38]. Detailed structural studies have shown that upon ligation to a target substrate, PUP forms what is essentially a ‘two-component’ degron in which a helical C-terminal region binds regulatory subunits of the proteasome, whereas a disordered N-terminal region governs initiation of chamber entry [39]. Sequence alignments indicate that the helix-forming region of the PUP degron is more conserved among bacterial species than is the disordered region [40]. Such differential rates of evolution for disordered against the ordered degron sub-regions was also observed for TS in the present study.

AUTHOR CONTRIBUTION

Karen Barbour and Franklin Berger designed the research plan and analysed data; Karen Barbour and Yang-Yang Xing carried out the experiments; Edsel Peña carried out the statistical analysis of the data; and Franklin Berger wrote and prepared the paper with the assistance of Karen Barbour.

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SUPPLEMENTARY DATA

Characterization of the bipartite degron that regulates ubiquitin-independent degradation of thymidylate synthase

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See the following pages for Supplementary Figure S1 and Supplementary Table S1.

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|            |                           |                                                                 |              |
|------------|---------------------------|-----------------------------------------------------------------|--------------|
| HUMAN      | 1                         | ATGCCCTGTGGCCGGCTCGGA-GCTG---CCGC GCCGG CCCC TTGCCCC CGC ACAGGA | 56           |
| CHIMPANZEE |                           | ATGCCCTGTGGCCGGCTCGGA-GCTG---CCGC GCCGG CCCC TTGCCCC CGC ACAGGA |              |
| ORANGUTAN  |                           | ATGCCCTGTGGCCGGCTCGGA-GCTG---CCGC GCCGG CCCC TTGCCCC CGC ACAGGA |              |
| MACAQUE    |                           | ATGCCCTGTGGCCGGCTCGGA-GCTG---CCGC GCCGG CCCC TTGCCCC CGC ACAGGA |              |
| MARMOSET   |                           | ATGCCCTGTGGCCGGCTCGGA-GCTG---CCACGCCAGCCCTCGCAGCCC GCG CAGGA    |              |
| TARSIER    |                           | ATGCCCTGTGGCCGGCTCGGA-GCTG---CAGCGCCCACCC CGC GCG CAGGA         |              |
| MOUSE      |                           | ATGCTGGTTGGTGGCTCGGA-GCTG---CAGT-----CCGATGCTCAGCA              |              |
| RAT        |                           | ATGCTGGTTGAAGGCTCTGA-GCTG---CAGT-----CCGGTGCTCAACA              |              |
| RABBIT     |                           | ATGCCCGCCGCCGGCTCTGA-GCTG---CCG-----TCGCCGCCACGGCGCAGGA         |              |
| PIKA       |                           | ATGCCGGCTGCCGGCTCGGA-GCTG---CCGCTCCCGCCTTCGCCGCCACAGCACAGCA     |              |
| COW        |                           | ATGCCAGCGGCCGGCTCGGA-GCCG---TCGCGCCCGCGTCGCCGCCGGCGTGCAGGA      |              |
| DOLPHIN    |                           | ATGCCCGCCGCCGGCTCGGA-GCTG---CCGC CGCCGATGTCGCCGCCGCGCAGGA       |              |
| HORSE      |                           | ATGCCAGCCTCGGCTCGGA-GCTG---CAGCGCCCGCGTCGCCGCCGCCGCGGGGA        |              |
| DOG        |                           | ATGCCCGCCCCCGGCTCGGA-GCTG---CAGCGCCCGCGTCGCCGCCGCCGCGCAGAA      |              |
| CAT        |                           | ATGCCCGCTCCCGGCTCGGA-GCTG---CAGCGCCCGCCGCGCAGCTGCCGAGCAGAA      |              |
| GUINEA_PIG |                           | ATGCCAGTTGCCGGCTCGATGCCGCCGACTGCCGACTGCCGACAGCACAGGA            |              |
|            | **** *      ***** * * * * |                                                                 | *      * * * |

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HUMAN	57	GCGGGGACGCCGAGCCCGTCCGCCG-----CACGGGGAGCTGCAGTACCTGGGGCA	107
CHIMPANZEE		GCGGGGACGCCGAGCCCGTCCGCCG-----CACGGGGAGCTGCAGTACCTGGGGCA	
ORANGUTAN		GCGGGGACGTCGAGCCCGTCCGCCG-----CACGGGGAGCTGCAGTACCTGGGGCA	
MACAQUE		GCGGGGACGCCGAGCCACGCCGCCG-----CACGGGAGACTGCAGTACCTGGGGCA	
MARMOSET		GCTGGACGCCAGCCCGGCCCT-----CACGGGGAGCTGCAGTACCTGGGGCA	
TARSIER		GCAGGGCGCCGAGCCGCCGCC-----CACGGGGAGCTGCAGTACCTGGGAGCA	
MOUSE		GCTGAGCGCGGAAGCCCCACGG-----CATGGAGAACCTCAGTACCTGAGGCA	
RAT		GCCACGCAAGGAAGCCCCCAG-----CATGGAGAACCTCAGTACCTGAGGCA	
RABBIT		GCAGGGCGCCGAGGCCGCCGCC-----CACGGGGAGCTGCAGTACCTGGGGCA	
PIKA		GCAGGGTACCGAGGCACGCCGCC-----CACGGGGAGCTGCAGTACCTGGGGCA	
COW		GCAGAGCGCCGAGCCGCCGCCGCCGCC-----CACGGGGAGCTGCAGTACCTGGGGCA	
DOLPHIN		GCAGGGCGCCGAGTCGCCGCCGCCGCC-----CACGGGGAGCTGCAGTACCTGGGGCA	
HORSE		GCAGGGGCTCGGAACCGCGGCCCT-----CACGGGGAGCTGCAGTACCTGGGGCA	
DOG		GCCGC CGC GCG -----CGTCCGCAGCCGCCCTCCACGGGGAGCTGCAGTACCTGAGGCA	
CAT		GCAGGGGCGCCGAACCGCAGCCGAGCCGCCACCCACGGGGAGCTGCAGTACCTGGGGCA	
GUINEA_PIG		CCCAGGGCCCGAGCCCTTGGCGCCG-----CATGGGGAGCTGCAGTACCTGCCGCA	
	*	*** *** *** *** **** *** ***	

Figure S1 Alignment of coding regions of mammalian TS mRNAs

The amino acid coding region of the TS mRNA from each of 16 species was downloaded and aligned (see the Experimental section for details). Numbers refer to the human transcript, with the initiation codon set as 1. Completely conserved nucleotides are marked by stars (*). The region encoding the IDR, corresponding to nucleotides 1–81 of the hTS transcript, is indicated by a tilde (~).

Figure S1 **Continued**

HUMAN	288	AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	347
CHIMPANZEE		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
ORANGUTAN		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
MACAQUE		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
MARMOSET		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
TARSIER		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
MOUSE		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
RAT		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
RABBIT		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
PIKA		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
COW		CAACGCTAAAGGAACCTCTTCCAAGGGAGTCAAATGGATCCCGAGA	
DOLPHIN		AAACGCTAAAGGAACCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
HORSE		AAATGCTAAAGGAACCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
DOG		AAACGCTAAAGGAACCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
CAT		AAACGCTAAAGGAACCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
GUINEA_PIG		AAATGCCAAAGAACCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
	***	***	***
HUMAN	348	CTTTTGGAACAGCCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	407
CHIMPANZEE		CTTTTGGAACAGCCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	
ORANGUTAN		CTTTTGGAACAGCCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	
MACAQUE		CTTTTGGAACAGCCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	
MARMOSET		CTTTCTGGATAGCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	
TARSIER		CTTTTGGAACAGCCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	
MOUSE		CTTTCTGGACAGCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	
RAT		CTTTTGGAACAGCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	
RABBIT		CTTTTGGAACAGCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	
PIKA		ATTTCTGGACAGCCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	
COW		CTTCCTGGATGGCTGGCTTCTCGACAGAGCTGAAGGGGATTAGGCCAGTTATGG	
DOLPHIN		CTTCCTGGACAGCCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	
HORSE		CTTCCTGGACAGCCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTACGG	
DOG		CTTCCTGGACAGCCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	
CAT		CTTCCTGGACAGCCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	
GUINEA_PIG		CTTCCTGGACAGCCTGGATTCTCACCCAGAGAACAGGGACTTGGGCCAGTTATGG	
	***	***	***
HUMAN	408	CTTCCAGTGGAGGCATTTGGGGCAGAACATACAGAGATATGGAATCAGATTATTCAAGGACA	467
CHIMPANZEE		CTTCCAGTGGAGGCATTTGGGGCAGAACATACAGAGATATGGAATCAGATTATTCAAGGACA	
ORANGUTAN		CTTCCAGTGGAGGCATTTGGGGCAGAACATACAGAGATATGGAATCAGATTATTCAAGGACA	
MACAQUE		CTTCCAGTGGAGGCATTTGGGGCAGAACATACAGAGATATGGAATCAGATTATTCAAGGACA	
MARMOSET		CTTCCAGTGGAGGCATTTGGGGCAGAACATACAGAGATATGGAATCAGATTATTCAAGGACA	
TARSIER		CTTCAATGGAGGCATTTGGGGCAGAACATACAAAGATATGGAATCAGATTATTCAAGGACA	
MOUSE		TTTCAATGGAGGCATTTGGAGCAGAGTACAAAGATATGGAATCAGATTACTCGGGACA	
RAT		ATTCCAGTGGAGACATTTGGAGCAGACTACAAAGATATGGAATCAGATTACTCGGGACA	
RABBIT		CTTCCAGTGGAGGCATTTGGTCAGAACATACAAAGATAAGGACTCAGATTATTCAAGGACA	
PIKA		CTTCCAGTGGAGGCATTTGGGGCAGAACATACAAAGATAAGGACTCAGATTATTCAAGGACA	
COW		CTTCCAGTGGAGGCATTTGGGGCTGAATACAAAGATATGGAATCAGATTATTCAAGGACA	
DOLPHIN		CTTCAGTGGAGGCATTTGGGGCAGAACATACAAAGAGATGGATTCAAGATTATTCAAGGACA	
HORSE		CTTCAGTGGAGGCATTTGGGGCAGAACATACAAAGAGATGGATTCAAGATTATTCAAGGACA	
DOG		CTTCAGTGGAGGCATTTGGGGCAGAACATACAAAGATAAGGATTCAAGATTATTCAAGGACA	
CAT		CTTCAGTGGAGGCATTTGGGGCAGAACATACAAAGATAAGGATTCAAGATTATTCAAGGACA	
GUINEA_PIG		CTTCCAGTGGAGGCATTTGGGGCTGAATACAAAGATAAGGACTCAGATTACTCAAGGACA	
	***	***	***

Figure S1 **Continued**

HUMAN	468	GGGAGTTGACCAACTGCAAAGAGT GATTGACACC ATCAA ACCCTGACGACAGAAG	527
CHIMPANZEE		GGGAGTTGACCAACTGCAAAGAGT GATTGACACC ATCAA ACCCTGACGACAGAAG	
ORANGUTAN		GGGAGTTGACCAACTGCAAAGAGT GATTGACACC ATCAA ACCCTGACGACAGAAG	
MACAQUE		GGGAGTTGACCAACTACAAAGAGT GATTGACACC ATCAA ACCCTGACGACAGAAG	
MARMOSET		AGGAGTTGACCAACTGCAAAGAGT GATTGACACC ATCAA ACCCTGATGACAGAAG	
TARSIER		AGGAGTTGACCAACTGCAAAGAGT GATTGACACC ATCAA ACCCTGATGACAGAAG	
MOUSE		AGGAGTAGACCAGCTGCAAAAAGT GATTGACACC ATCAA ACCCTGATGACAGAAG	
RAT		AGGAGTAGACCAGCTGCAAAAAGT GATTGACACC ATCAA ACCCTGATGACAGAAG	
RABBIT		AGGAGTAGACCACACTGCAAGGT GATCGATACC ATCAA ACCCTGATGACAGAAG	
PIKA		AGGAGTAGACCAGCTGCAAAAAGT GATCGATACC ATCAA ACCCTGATGACCCGAG	
COW		AGGAGTAGATCAACTGCAAAAAGT GATCGACACA ATCAA ACCCTAACGACAGAAG	
DOLPHIN		AGGAGTAGATCAACTGCAAAAAGT GATTGACACA ATCAA ACCCTGACGACAGAAG	
HORSE		AGGAGTAGACCAACTGCAAAAAGT GATTAACACA ATCAA ACCCTGACGACAGAAG	
DOG		AGGAGTCGACCAGCTGCAAAAAGT GATTGACACA ATCAA ACCCTGACGACAGAAG	
CAT		AGGAGTAGATCAACTGCAAAAAGT GATTGACACA ATCAA ACCCTGATGACAGAAG	
GUINEA_PIG		AGGAGTTGACCAACTGCAAGGT GATTGACACC ATCAA ACCCTGATGACAGAAG	
		***** *	
HUMAN	528	AATCATCATGTGCGCTTGGAAATCCAAGAGATCTTCTCTGATGGCGCTGCCTCCATGCCA	587
CHIMPANZEE		AATCATCATGTGCGCTTGGAAATCCAAGAGATCTTCTCTGATGGCGCTGCCTCCATGCCA	
ORANGUTAN		AATCATCATGTGCGCTTGGAAATCCAAGAGATCTTCTCTGATGGCGCTGCCTCCATGCCA	
MACAQUE		AATCATCATGTGCGCTTGGAAATCCAAGAGATCTTCTCTGATGGCGCTGCCTCCATGCCA	
MARMOSET		AATCATCATGTGCGCTTGGAAATCCAAGAGATCTTCTCTGATGGCGCTGCCTCCATGCCA	
TARSIER		GATCATCATGTGCGCTTGGAAATCCAAGAGATCTTCTCTGATGGCGCTGCCTCCATGCCA	
MOUSE		AATCATCATGTGCGCTTGGAAACCAAAGAGATCTTCTCTGATGGCGCTGCCTCCATGCCA	
RAT		AATCATCATGTGCGCTTGGAAACCAAAGAGATCTTCTCTGATGGCGACTGCCTCCTTGCA	
RABBIT		AATCATCATGTGCGCTTGGAAATCCAAAGACCTTCTCTGATGGCGCTGCCCTCGTGCA	
PIKA		AATCATCATGTGCGCTTGGAAATCTAAAGACCTTCTCAGATGGCCCTGCCCTCGTGCA	
COW		AATCATCCTGTGTGCTTGGAAATCCAAGAGATCTGCTCTCATGGCCCTCCCCCATGCCA	
DOLPHIN		AATCATCCTGTGTGCTTGGAAATCCAAGAGATCTGCTCTCATGGCCCTACCCCGTGCA	
HORSE		AATCATCCTGTGTGCTTGGAAATCCAAGAGATCTTCTCTCATGGCTCTGCCCTCGTGCA	
DOG		AATTATTCTGTGTGCTTGGAAATCCAAGAGATCTTCTCTGATGGCCCTACCTCCGTGCA	
CAT		AATCATCCTGTGTGCTTGGAAATCCAAGAGATCTTCTCTGATGGCCCTGCCCTCGTGCA	
GUINEA_PIG		AATTATCATGTGCGCTTGGAAACCGAAAGATCTTCTCTGATGGCGCTGCCTCCCTGTCA	
		*** *	
HUMAN	588	TGCCCTCTGCCAGTTCTATGTGGTGAACAGTGAGCTGCCAGCTGTACCAAGAGATC	647
CHIMPANZEE		TGCCCTCTGCCAGTTCTATGTGGTGAACAGTGAGCTGCCAGCTGTACCAAGAGATC	
ORANGUTAN		TGCCCTCTGCCAGTTCTATGTGGTGAACAGTGAGCTGCCAGCTGTACCAAGAGATC	
MACAQUE		TGCCCTCTGCCAGTTCTATGTGGTGAACAGTGAGCTGCCAGCTGTACCAAGAGATC	
MARMOSET		TGCCCTCTGCCAGTTCTACGTGGTGAACGGTGAGCTGCCAGCTGTACCAAGAGATC	
TARSIER		TGCCCTCTGCCAGTTCTACGTGGTGAACGGTGAGCTGCCAGCTGTACCAAGAGATC	
MOUSE		TGCCCTCTGTCAATTCTACGTGGTGAATGGGAGCTGTCTGCCAGCTTACAGCGGTC	
RAT		TGCCCTCTGTCAATTCTACGTGGTGAATGGGAGCTGTCTGCCAGCTGTACCAAGAGATC	
RABBIT		TGCCCTCTGCCAGTTCTACGTGGTGAATGGGAGCTGTCTGCCAGCTGTACCAAGAGATC	
PIKA		TGCTCTCTGCCAGTTCTACGTAGTGAACGGGAGCTGTCTGCCAGTTGTACCAAGAGATC	
COW		CGCCCTCTGCCAGTTCTACGTGGTGAATGGGAGCTGTCTGCCAGTTGTACCAAGCGGTC	
DOLPHIN		TGCCCTCTGCCAGTTCTACGTGGTGAACGGTGAGCTGCCAGCTGTACCAAGAGATC	
HORSE		TGCCCTCTGCCAGTTCTACGTGGTGAACGGTGAGCTGCCAGCTGTACCAAGAGATC	
DOG		TGCCCTCTGCCAGTTCTACGTGGTGAACGGTGAGCTGCCAGCTATAACCAAGAGATC	
CAT		TGCTCTCTGCCAGTTCTACGTGGTGAATGGTGAACGGTGAGCTGCCAGCTGTACCAAGAGATC	
GUINEA_PIG		CACCCCTCTGCCAGTTCTACGTGGTGAACGGGAAATTGCTCTGTCACTGTACCAAGCGCTC	
		* *	

Figure S1 Continued

Figure S1 **Continued**

Figure S1 **Continued**

Table S1. Synonymous (d_s) and nonsynonymous (d_N) nucleotide substitutions for the IDR and the body of the TS polypeptide
 Values for each of the 120 pairwise comparisons among 16 mammalian species were determined, and d_N/d_s ratios were calculated.

Species 1	Species 2	IDR			Body		
		d_N	d_s	d_N/d_s	d_N	d_s	d_N/d_s
Human	Chimpanzee	0.0000	0.0441	0.0000	0.0045	0.0159	0.2830
	Orang-utan	0.0355	0.1946	0.1824	0.0030	0.0321	0.0935
	Macaque	0.0629	0.2880	0.2184	0.0060	0.0948	0.0633
	Marmoset	0.1011	0.4321	0.2340	0.0205	0.1341	0.1529
	Tarsier	0.1113	0.4674	0.2381	0.0290	0.2979	0.0973
	Rabbit	0.1257	0.5646	0.2226	0.0267	0.3083	0.0866
	Pika	0.2201	0.6355	0.3463	0.0354	0.4987	0.0710
	Cow	0.1953	0.3235	0.6037	0.0361	0.4854	0.0744
	Dolphin	0.1590	0.3699	0.4298	0.0236	0.3595	0.0656
	Horse	0.1833	0.6655	0.2754	0.0386	0.2948	0.1309
	Dog	0.2150	0.3490	0.6160	0.0299	0.3998	0.0748
	Cat	0.2609	0.4975	0.5244	0.0306	0.4741	0.0645
	Mouse	0.3041	1.0397	0.2925	0.0321	0.5807	0.0553
	Rat	0.3536	2.8876	0.1225	0.0329	0.6567	0.0501
	Guinea pig	0.3908	1.1119	0.3515	0.0571	0.6529	0.0875
Chimpanzee	Orang-utan	0.0355	0.1410	0.2518	0.0015	0.0267	0.0562
	Macaque	0.0536	0.2567	0.2088	0.0045	0.1009	0.0446
	Marmoset	0.0913	0.3941	0.2317	0.0190	0.1405	0.1352
	Tarsier	0.1113	0.3904	0.2851	0.0275	0.2905	0.0947
	Rabbit	0.1257	0.4661	0.2697	0.0252	0.3008	0.0838
	Pika	0.2201	0.5416	0.4064	0.0338	0.4695	0.0720
	Cow	0.1953	0.2590	0.7541	0.0345	0.4860	0.0710
	Dolphin	0.1590	0.3014	0.5275	0.0220	0.3599	0.0611
	Horse	0.1833	0.5653	0.3243	0.0370	0.2874	0.1287
	Dog	0.2150	0.2784	0.7723	0.0314	0.3827	0.0820
	Cat	0.2490	0.4546	0.5477	0.0321	0.4747	0.0676
	Mouse	0.3041	1.0397	0.2925	0.0306	0.5480	0.0558
	Rat	0.3536	2.8876	0.1225	0.0345	0.6208	0.0556
	Guinea pig	0.3908	0.9414	0.4151	0.0554	0.6176	0.0897
Orang-utan	Macaque	0.0916	0.1433	0.6392	0.0030	0.0948	0.0316
	Marmoset	0.1312	0.1995	0.6576	0.0175	0.1341	0.1305
	Tarsier	0.1524	0.1979	0.7701	0.0259	0.2902	0.0892
	Rabbit	0.1489	0.3010	0.4947	0.0236	0.2851	0.0828
	Pika	0.2437	0.4582	0.5319	0.0322	0.4886	0.0659
	Cow	0.2413	0.2590	0.9317	0.0330	0.5056	0.0653
	Dolphin	0.2027	0.1270	1.5961	0.0205	0.3427	0.0598
	Horse	0.2351	0.3856	0.6097	0.0354	0.2721	0.1301
	Dog	0.2665	0.0993	2.6838	0.0299	0.3823	0.0782
	Cat	0.2978	0.3041	0.9793	0.0306	0.4546	0.0673
	Mouse	0.3756	1.0397	0.3613	0.0290	0.5582	0.0520
	Rat	0.4363	2.5045	0.1742	0.0329	0.6199	0.0531
	Guinea pig	0.4513	0.8025	0.5624	0.0538	0.6168	0.0872
Macaque	Marmoset	0.0907	0.2635	0.3442	0.0144	0.1992	0.0723
	Tarsier	0.1308	0.2612	0.5008	0.0259	0.3457	0.0749
	Rabbit	0.1722	0.3041	0.5663	0.0236	0.3239	0.0729
	Pika	0.2421	0.3904	0.6201	0.0322	0.5083	0.0633
	Cow	0.2165	0.3295	0.6571	0.0291	0.5525	0.0527
	Dolphin	0.1794	0.1837	0.9766	0.0205	0.4026	0.0509

Table S1 Continued

Species 1	Species 2	IDR			Body		
		d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S
Marmoset	Horse	0.2054	0.4870	0.4218	0.0315	0.3379	0.0932
	Dog	0.2386	0.2177	1.0960	0.0330	0.4264	0.0774
	Cat	0.2130	0.4226	0.5040	0.0337	0.4637	0.0727
	Mouse	0.3406	1.3061	0.2608	0.0259	0.5575	0.0465
	Rat	0.3926	1.6170	0.2428	0.0298	0.6072	0.0491
	Guinea pig	0.4272	0.9178	0.4655	0.0530	0.6581	0.0805
	Tarsier	0.1304	0.2029	0.6427	0.0346	0.3005	0.1151
	Rabbit	0.1489	0.3010	0.4947	0.0299	0.3472	0.0861
	Pika	0.2413	0.5592	0.4315	0.0401	0.5483	0.0731
	Cow	0.2158	0.3326	0.6488	0.0369	0.5504	0.0670
	Dolphin	0.1788	0.1853	0.9649	0.0296	0.4027	0.0735
Tarsier	Horse	0.2286	0.4099	0.5577	0.0386	0.3490	0.1106
	Dog	0.2251	0.2524	0.8918	0.0423	0.4450	0.0951
	Cat	0.2583	0.3490	0.7401	0.0422	0.4492	0.0939
	Mouse	0.3055	1.3061	0.2339	0.0343	0.6084	0.0564
	Rat	0.3926	2.5914	0.1515	0.0377	0.7319	0.0515
	Guinea pig	0.4256	0.9313	0.4570	0.0585	0.6890	0.0849
	Rabbit	0.0600	0.1073	0.5592	0.0275	0.3435	0.0801
	Pika	0.1316	0.4674	0.2816	0.0364	0.5635	0.0646
	Cow	0.1103	0.2635	0.4186	0.0322	0.6074	0.0530
	Dolphin	0.0780	0.1290	0.6047	0.0275	0.4243	0.0648
Rabbit	Horse	0.0957	0.3326	0.2877	0.0354	0.3577	0.0990
	Dog	0.1098	0.1868	0.5878	0.0370	0.3853	0.0960
	Cat	0.1590	0.3099	0.5131	0.0361	0.3844	0.0939
	Mouse	0.2683	1.0892	0.2463	0.0314	0.6311	0.0498
	Rat	0.3154	1.8318	0.1722	0.0361	0.6540	0.0552
	Guinea pig	0.3975	0.9178	0.4331	0.0514	0.7208	0.0713
	Pika	0.0604	0.4608	0.1311	0.0170	0.3563	0.0477
	Cow	0.1517	0.2947	0.5148	0.0268	0.5869	0.0457
	Dolphin	0.0397	0.1643	0.2416	0.0213	0.4503	0.0473
Pika	Horse	0.1827	0.3916	0.4665	0.0229	0.3729	0.0614
	Dog	0.1773	0.2180	0.8133	0.0346	0.4419	0.0783
	Cat	0.2561	0.3658	0.7001	0.0283	0.4367	0.0648
	Mouse	0.3213	1.1765	0.2731	0.0190	0.5587	0.0340
	Rat	0.3597	1.1861	0.3033	0.0236	0.5908	0.0399
	Guinea pig	0.3501	1.0714	0.3268	0.0339	0.6528	0.0519
	Cow	0.2218	0.4586	0.4836	0.0373	0.7123	0.0524
	Dolphin	0.1590	0.4452	0.3571	0.0314	0.6124	0.0513
	Horse	0.2068	0.6655	0.3107	0.0355	0.5008	0.0709
Cow	Dog	0.2275	0.4693	0.4848	0.0434	0.6735	0.0644
	Cat	0.2978	0.5429	0.5485	0.0346	0.6620	0.0523
	Mouse	0.3213	0.9241	0.3477	0.0354	0.6200	0.0571
	Rat	0.2901	1.1861	0.2446	0.0401	0.7198	0.0557
	Guinea pig	0.4780	0.7037	0.6793	0.0518	0.7532	0.0688
	Dolphin	0.1505	0.0954	1.5776	0.0229	0.3044	0.0752
	Horse	0.2286	0.2064	1.1076	0.0284	0.3442	0.0825
	Dog	0.2127	0.1585	1.3420	0.0409	0.4518	0.0905
	Cat	0.2583	0.2138	1.2081	0.0306	0.4513	0.0678
	Mouse	0.3701	0.8740	0.4235	0.0361	0.8683	0.0416
	Rat	0.4232	1.9237	0.2200	0.0385	0.7946	0.0485

Table S1. Continued

Species 1	Species 2	IDR			Body		
		d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S
Dolphin	Guinea pig	0.3817	0.6208	0.6149	0.0515	0.7589	0.0679
	Horse	0.1815	0.2064	0.8794	0.0299	0.1974	0.1515
	Dog	0.1650	0.0492	3.3537	0.0314	0.2922	0.1075
	Cat	0.2349	0.1544	1.5214	0.0283	0.2793	0.1013
	Mouse	0.3041	1.0397	0.2925	0.0282	0.7807	0.0361
Horse	Rat	0.3536	1.6805	0.2104	0.0314	0.7932	0.0396
	Guinea pig	0.3817	0.7301	0.5228	0.0490	0.7717	0.0635
	Dog	0.1521	0.2177	0.6987	0.0474	0.2588	0.1832
	Cat	0.1797	0.2758	0.6516	0.0386	0.2795	0.1381
	Mouse	0.3423	0.7940	0.4311	0.0322	0.6407	0.0503
Dog	Rat	0.4273	0.9607	0.4448	0.0362	0.6450	0.0561
	Guinea pig	0.5244	0.6816	0.7694	0.0500	0.6355	0.0787
	Cat	0.1290	0.1355	0.9520	0.0244	0.2369	0.1030
	Mouse	0.3089	0.7739	0.3991	0.0346	0.6765	0.0511
	Rat	0.2870	1.6841	0.1704	0.0362	0.7140	0.0507
Cat	Guinea pig	0.5769	0.6649	0.8676	0.0523	0.6959	0.0752
	Mouse	0.3503	0.7920	0.4423	0.0290	0.7893	0.0367
	Rat	0.4019	0.9313	0.4315	0.0306	0.8020	0.0382
Mouse	Guinea pig	0.5456	0.6053	0.9014	0.0506	0.7445	0.0680
	Rat	0.1618	0.4879	0.3316	0.0045	0.1833	0.0245
	Guinea pig	0.6132	0.9435	0.6499	0.0331	0.7597	0.0436
Rat	Guinea pig	0.5573	1.2745	0.4373	0.0363	0.8011	0.0453

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SUPPLEMENTARY DATA

Characterization of the bipartite degron that regulates ubiquitin-independent degradation of thymidylate synthase

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See the following pages for Supplementary Figure S1 and Supplementary Table S1.

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|            |                           |                                                                |              |
|------------|---------------------------|----------------------------------------------------------------|--------------|
| HUMAN      | 1                         | ATGCCCTGTGGCCGGCTCGGA-GCTG---CCGCGCCGGCCCCCTGCCCCCGCCGCACAGGA  | 56           |
| CHIMPANZEE |                           | ATGCCCTGTGGCCGGCTCGGA-GCTG---CCGCGCCGGCCCCCTGCCGCCGCACAGGA     |              |
| ORANGUTAN  |                           | ATGCCCTGTGGCCGGCTCGGA-GCTG---CCGCGCCGGCCCCCTGCCGCCCTCCGCACAGGA |              |
| MACAQUE    |                           | ATGCCCTGTGGCCGGCTCGA-GCTG---CCGCGCCCGCCCCCTTGCAAGCCCACAGGA     |              |
| MARMOSET   |                           | ATGCCCTGTGGCCGGCTCGA-GCTG---CCACGCCAGCCCCCGCAGCCCACAGGA        |              |
| TARSIER    |                           | ATGCCCTGTGGCCGGCTCGA-GCTG---CAGCGCCCACCCCTCGCCGCCGCGCAGGA      |              |
| MOUSE      |                           | ATGCTGGTTGGTGGCTCGA-GCTG---CAGT-----CCGATGCTCAGCA              |              |
| RAT        |                           | ATGCTGGTTGAAGGCTCTGA-GCTG---CAGT-----CCGGTGCTCAACA             |              |
| RABBIT     |                           | ATGCCCGCCGGCTCTGA-GCTG---CCG-----TCGCCGCCACGGCGCAGGA           |              |
| PIKA       |                           | ATGCCGGCTGCCGGCTCGA-GCTG---CCGCTCCCGCCTTCGCCGCCACAGCACAGCA     |              |
| COW        |                           | ATGCCAGCGGCCGGCTCGA-GCCG---TCGCCGCCGCCGCGCAGGA                 |              |
| DOLPHIN    |                           | ATGCCCGCCGCCGGCTCGA-GCTG---CCGCGCCGATGTCGCCGCCGCCGCGCAGGA      |              |
| HORSE      |                           | ATGCCAGCCTCGGCTCGA-GCTG---CAGCGCCCGCCGCGCAGGA                  |              |
| DOG        |                           | ATGCCCGCCCCCGGCTCGA-GCTG---CAGCGCCCGCCGCGCAGGA                 |              |
| CAT        |                           | ATGCCCGCTCCCGGCTCGA-GCTG---CAGCGCCCGCCGCGCAGGA                 |              |
| GUINEA_PIG |                           | ATGCCAGTTGCCGGCTCGATGCCGGCTCG-ACCTTCTGCCGCGACTGCAGCACAGGA      |              |
|            | **** *      ***** * * * * |                                                                | *      * * * |

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HUMAN	57	GCGGGGACGCCGAGCCCGTCCGCCG-----CACGGGGAGCTGCAGTACCTGGGGCA	107
CHIMPANZEE		GCGGGGACGCCGAGCCCGTCCGCCG-----CACGGGGAGCTGCAGTACCTGGGGCA	
ORANGUTAN		GCGGGGACGTCGAGCCCGTCCGCCG-----CACGGGGAGCTGCAGTACCTGGGGCA	
MACAQUE		GCGGGGACGCCGAGCCACGCCGCCG-----CACGGGAGACTGCAGTACCTGGGGCA	
MARMOSET		GCTGGACGCCAGCCGCCG-----CACGGGGAGCTGCAGTACCTGGGGCA	
TARSIER		GCAGGGCGCCGAGCCGCCG-----CACGGGGAGCTGCAGTACCTGGGGAGCA	
MOUSE		GCTGAGCGCGGAAGCCCCACGG-----CATGGAGAACTCCAGTACCTGAGGCA	
RAT		GCCACGCAAGAACGCCGCAG-----CATGGAGAACTCCAGTACCTGAGGCA	
RABBIT		GCAGGGCGCCGAGGCCGCCG-----CACGGGGAGCTGCAGTACCTGGGGCA	
PIKA		GCAGGGTACCGAGGCACGCCGCC-----CACGGGGAGCTGCAGTACCTGGGGCA	
COW		GCAGAGCGCCGAGCCGCCGCCGCCG-----CACGGGGAGCTGCAGTACCTGGGGCA	
DOLPHIN		GCAGGGCGCCGAGTCGCCGCCGCCG-----CACGGGGAGCTGCAGTACCTGGGGCA	
HORSE		GCAGGGGCTCGGAACCGCGGCC-----CACGGGGAGCTGCAGTACCTGGGGCA	
DOG		GCCGCGCCG-----CGTCCGCAGCCGCCACGGGAGCTGCAGTACCTGAGGCA	
CAT		GCAGGGGCGCCGAACCGCAGCCGCCACGGGAGCTGCAGTACCTGGGGCA	
GUINEA_PIG		CCCAGGGCCCGAGCCCTTGGCGCCG-----CATGGGGAGCTGCAGTACCTGCCGCA	
	*	*** *** *** *** **** * ***	

Figure S1 Alignment of coding regions of mammalian TS mRNAs

The amino acid coding region of the TS mRNA from each of 16 species was downloaded and aligned (see the Experimental section for details). Numbers refer to the human transcript, with the initiation codon set as 1. Completely conserved nucleotides are marked by stars (*). The region encoding the IDR, corresponding to nucleotides 1–81 of the hTS transcript, is indicated by a tilde (~).

Figure S1 **Continued**

HUMAN	288	AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	347
CHIMPANZEE		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
ORANGUTAN		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
MACAQUE		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
MARMOSET		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
TARSIER		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
MOUSE		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
RAT		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
RABBIT		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
PIKA		AAATGCTAAAGAGCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
COW		CAACGCTAAAGGAACCTCTTCCAAGGGAGTCAAATGGATCCCGAGA	
DOLPHIN		AAACGCTAAAGGAACCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
HORSE		AAATGCTAAAGGAACCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
DOG		AAACGCTAAAGGAACCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
CAT		AAACGCTAAAGGAACCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
GUINEA_PIG		AAATGCCAAAGAACCTGTCTCCAAGGGAGTCAAATGGATCCCGAGA	
	***	***	***
HUMAN	348	CTTTTGGAACAGCCTGGATTCTCACCCAGAGAAGAAGGGACTTGGGCCAGTTATGG	407
CHIMPANZEE		CTTTTGGAACAGCCTGGATTCTCACCCAGAGAAGAAGGGACTTGGGCCAGTTATGG	
ORANGUTAN		CTTTTGGAACAGCCTGGATTCTCACCCAGAGAAGAAGGGACTTGGGCCAGTTATGG	
MACAQUE		CTTTTGGAACAGCCTGGATTCTCACCCAGAGAAGAAGGGACTTGGGCCAGTTATGG	
MARMOSET		CTTTCTGGATAGCTGGATTCTCACCCAGAGAAGAAGGGACTTGGGCCAGTTATGG	
TARSIER		CTTTTGGAACAGCCTGGATTCTCACCCAGAGAAGAAGGGACTTGGGCCAGTTATGG	
MOUSE		CTTTCTGGACAGCTGGATTCTCACCCAGAGAAGAAGGGACTTGGGCCAGTTATGG	
RAT		CTTTTGGAACAGCTGGATTCTCACCCAGAGAAGAAGGGACTTGGGCCAGTTATGG	
RABBIT		CTTTTGGAACAGCTGGATTCTCACCCAGAGAAGAAGGGACTTGGGCCAGTTATGG	
PIKA		ATTTCTGGACAGCCTGGATTCTCACCCAGAGAAGAAGGGACTTGGGCCAGTTATGG	
COW		CTTCCTGGATGGCTGGCTTCTCGACAGAGCTGAAGGGGATTAGGCCAGTTATGG	
DOLPHIN		CTTCCTGGACAGCCTGGATTCTCACCCAGAGAAGAAGGGGACTTGGGCCAGTTATGG	
HORSE		CTTCCTGGACAGCCTGGATTCTCACCCAGAGAAGAAGGGGACTTGGGCCAGTTACGG	
DOG		CTTCCTGGACAGCCTGGATTCTCACCCAGAGAAGAAGGGGACTTGGGCCAGTTATGG	
CAT		CTTCCTGGACAGCCTGGATTCTCACCCAGAGAAGAAGGGGACTTGGGCCAGTTATGG	
GUINEA_PIG		CTTCCTGGACAGCCTGGATTCTCACCCAGAGAAGAAGGGGACTTGGGCCAGTTATGG	
	***	***	***
HUMAN	408	CTTCCAGTGGAGGCATTTGGGGCAGAATACAGAGATATGGAATCAGATTATTCAAGGACA	467
CHIMPANZEE		CTTCCAGTGGAGGCATTTGGGGCAGAATACAGAGATATGGAATCAGATTATTCAAGGACA	
ORANGUTAN		CTTCCAGTGGAGGCATTTGGGGCAGAATACAGAGATATGGAATCAGATTATTCAAGGACA	
MACAQUE		CTTCCAGTGGAGGCATTTGGGGCAGAATACAGAGATATGGAATCAGATTATTCAAGGACA	
MARMOSET		CTTCCAGTGGAGGCATTTGGGGCAGAATACAGAGATATGGAATCAGATTATTCAAGGACA	
TARSIER		CTTCAATGGAGGCATTTGGAGCAGAGTACAAGAGATATGGAATCAGATTATTCAAGGACA	
MOUSE		TTTCAATGGAGGCATTTGGAGCAGAGTACAAGAGATATGGAATCAGATTACTCGGGACA	
RAT		ATTCCAGTGGAGACATTTGGAGCAGACTACAAGAGATATGGAATCAGATTACTCGGGACA	
RABBIT		CTTCCAGTGGAGGCATTTGGTCAGAATACAAAGATAAGGACTCAGATTATTCAAGGACA	
PIKA		CTTCCAGTGGAGGCATTTGGGCAGAATACAAAGATAAGGACTCAGATTATTCAAGGACA	
COW		CTTCCAGTGGAGGCATTTGGGCTGAATACAAAGAGATGGATTCAAGACTTCAAGGACA	
DOLPHIN		CTTCAGTGGAGGCATTTGGGCCGAATACAAAGAGATGGATTCAAGACTTCAAGGACA	
HORSE		CTTCAGTGGAGGCATTTGGGGCAGAATACAAAGAGATGGATTCAAGACTTCAAGGACA	
DOG		CTTCAGTGGAGGCATTTGGGGCAGAATACAAAGAGATGGATTCAAGACTTCAAGGACA	
CAT		CTTCAGTGGAGGCATTTGGGGCAGAATACAAAGAGATGGATTCAAGACTTCAAGGACA	
GUINEA_PIG		CTTCCAGTGGAGGCATTTGGGGCTGAATACAAAGAGATGGACTCAGATTACTCAAGGACA	
	***	***	***

Figure S1 **Continued**

HUMAN	468	GGGAGTTGACCAACTGCAAAGAGT GATTGACACC ATCAAACCAAC CCTGACGACAGAAG	527
CHIMPANZEE		GGGAGTTGACCAACTGCAAAGAGT GATTGACACC ATCAAACCAAC CCTGACGACAGAAG	
ORANGUTAN		GGGAGTTGACCAACTGCAAAGAGT GATTGACACC ATCAAACCAAC CCTGACGACAGAAG	
MACAQUE		GGGAGTTGACCAACTACAAAGAGT GATTGACACC ATCAAACCAAC CCTGACGACAGAAG	
MARMOSET		AGGAGTTGACCAACTGCAAAGAGT GATTGACACC ATCAAACCAAC CCTGATGACAGAAG	
TARSIER		AGGAGTTGACCAACTGCAAAGAGT GATTGACACC ATCAAACCAAC CCTGATGACAGAAG	
MOUSE		AGGAGTAGACCAGCTGCAAAAAGT GATTGACACC ATCAAACCAAC CCTGATGACAGAAG	
RAT		AGGAGTAGACCAGCTGCAAAAAGT GATTGACACC ATCAAACCAAC CCTGATGACAGAAG	
RABBIT		AGGAGTAGACCAC TGCAGAAGGT GATC GAT ACC ATCAAACCAAC CCTGATGACAGAAG	
PIKA		AGGAGTAGACCAGCTGCAAAGAGT GATC GAT ACC ATCAAACCAAC CCTGATGACCCGAG	
COW		AGGAGTAGATCAACTGCAAAGAGT GATC GACACA ATCAAACCAAC CCTAACGACAGAAG	
DOLPHIN		AGGAGTAGATCAACTGCAAAGAGT GATTGACACA ATCAAACCAAC CCTGACGACAGAAG	
HORSE		AGGAGTAGACCAACTGCAAAGAGT GATTAACACA ATCAAACCAAC CCTGACGACAGAAG	
DOG		AGGAGTCGACCAGCTGCAAAGAGT GATTGACACA ATCAAACCAAC CCTGACGACAGAAG	
CAT		AGGAGTAGATCAACTGCAAAGAGT GATTGACACA ATCAAACCAAC CCTGATGACAGAAG	
GUINEA_PIG		AGGAGTTGACCAACTGCAAAGAGT GATTGACACC ATCAAACCAAC CCTGATGACAGAAG	
		***** *	
HUMAN	528	AATCATCATGTGCGCTTGGAAATCCAAGAGATCTTCTCTGATGGCGCTGCCTCCATGCCA	587
CHIMPANZEE		AATCATCATGTGCGCTTGGAAATCCAAGAGATCTTCTCTGATGGCGCTGCCTCCATGCCA	
ORANGUTAN		AATCATCATGTGCGCTTGGAAATCCAAGAGATCTTCTCTGATGGCGCTGCCTCCATGCCA	
MACAQUE		AATCATCATGTGCGCTTGGAAATCCAAGAGATCTTCTCTGATGGCGCTGCCTCCATGCCA	
MARMOSET		AATCATCATGTGCGCTTGGAAATCCAAGAGATCTTCTCTGATGGCGCTGCCTCCATGCCA	
TARSIER		GATCATCATGTGCGCTTGGAAATCCAAGAGATCTTCTCTGATGGCGCTGCCTCCATGCCA	
MOUSE		AATCATCATGTGCGCTTGGAAACCCAAAAGATCTTCCCCTGATGGCAGTGCCTCCATGCCA	
RAT		AATCATCATGTGCGCTTGGAAACCCAAAAGATCTTCCCCTGATGGCAGTGCCTCCATGCCA	
RABBIT		AATCATCATGTGCGCTTGGAAATCCAAAAGACCTTCTGATGGCGCTGCCCTCGATGCCA	
PIKA		AATCATCATGTGCGCTTGGAAATCCAAAAGACCTTCTCAGATGGCCCTGCCCTCGATGCCA	
COW		AATCATCCTGTGTGCTTGGAAATCCAAGAGATCTGCTCTCATGGCCCTCCCCCATGCCA	
DOLPHIN		AATCATCCTGTGTGCTTGGAAATCCAAGAGATCTGCTCTCATGGCCCTACCCCGTGCCA	
HORSE		AATCATCCTGTGTGCTTGGAAATCCAAGAGATCTGCTCTCATGGCTCTGCCCTCGATGCCA	
DOG		AATTATTCTGTGTGCTTGGAAATCCAAGAGATCTGCTCTCATGGCCCTACCTCCGTGCCA	
CAT		AATCATCCTGTGTGCTTGGAAATCCAAGAGATCTGCTCTCATGGCCCTGCCCTCGATGCCA	
GUINEA_PIG		AATTATCATGTGCGCTTGGAAACCCGAAAGATCTTCTCTGATGGCGCTGCCTCCCTGTCA	
		*** *	
HUMAN	588	TGCCCTCTGCCAGTTCTATGTGGTGAACAGTGAGCTGCCAGCTGTACCAAGAGATC	647
CHIMPANZEE		TGCCCTCTGCCAGTTCTATGTGGTGAACAGTGAGCTGCCAGCTGTACCAAGAGATC	
ORANGUTAN		TGCCCTCTGCCAGTTCTATGTGGTGAACAGTGAGCTGCCAGCTGTACCAAGAGATC	
MACAQUE		TGCCCTCTGCCAGTTCTATGTGGTGAACAGTGAGCTGCCAGCTGTACCAAGAGATC	
MARMOSET		TGCCCTCTGCCAGTTCTACGTGGTGAACGGTGAGCTGCCAGCTGTACCAAGAGATC	
TARSIER		TGCCCTCTGCCAGTTCTACGTGGTGAATGGGAGCTGTCTGCCAGCTTACCAAGGGTC	
MOUSE		TGCCCTCTGTCAATTCTACGTGGTGAATGGGAGCTGTCTGCCAGCTGTACCAAGGGTC	
RAT		TGCCCTCTGTCAATTCTACGTGGTGAATGGGAGCTGTCTGCCAGCTGTACCAAGGGTC	
RABBIT		TGCCCTCTGCCAGTTCTACGTGGTGAATGGGAGCTGTCTGCCAGCTGTACCAAGGGTC	
PIKA		TGCTCTCTGCCAGTTCTACGTGGTGAACGGGAGCTGTCTGCCAGCTGTACCAAGGGTC	
COW		CGCCCTCTGCCAGTTCTACGTGGTGAATGGGAGCTGTCTGCCAGCTGTACCAAGGGTC	
DOLPHIN		TGCCCTCTGCCAGTTCTACGTGGTGAACGGTGAGCTGCCAGCTGTACCAAGGGTC	
HORSE		TGCCCTCTGCCAGTTCTACGTGGTGAACGGTGAGCTGCCAGCTGTACCAAGGGTC	
DOG		TGCCCTCTGCCAGTTCTACGTGGTGAACGGTGAGCTGCCAGCTGTACCAAGGGTC	
CAT		TGCTCTCTGCCAGTTCTACGTGGTGAATGGGAGCTGTCTGCCAGCTGTACCAAGGGTC	
GUINEA_PIG		CACCCCTCTGCCAGTTCTACGTGGTGAACGGGAAATTGCTCTGTCACTGTACCAAGGGTC	
		* *	

Figure S1 Continued

HUMAN	648	GGGAGACATGGGCCTCGGTGCTTCACATGCCAGCTACGCCCTGCTCACGTACAT GGGAGACATGGGCCTCGGTGCTTCACATGCCAGCTACGCCCTGCTCACGTACAT GGGAGACATGGGCCTAGGTGCTTCACATGCCAGCTACGCCCTGCTCACACAT GGGAGACATGGGCCTAGGTGCTTCACATGCCAGCTACGCCCTGCTCACACAT GGGAGACATGGGCCTAGGTGCTTCACATGCCAGCTACGCCCTGCTCACACAT AGGAGACATGGGCCTGGGTGACCTTCACATGCCAGCTATGCCCTGCTCACACAT GGGGGACATGGGCCTGGGTGCCCCCAACATTGCCAGCTACGCCCTGCTCACCTACAT AGGAGATATGGGTCTGGCGTCCCCCAACATTGCCAGCTATGCTCTGCTCACCTACAT AGGAGATATGGGTCTGGGTGCCCCCAACATTGCCAGCTATGCTCTGCTGACCTACAT GGGAGACATGGGCCTGGCGTCCCCCAACATTGCCAGCTATGCCCTGCTCACCTACAT GGGAGACATGGGCCTGGCGTCCCCCAACATTGCCAGCTACGCCCTGCTCACCTACAT AGGAGACATGGGCCTGGGTGCCCCCAACATTGCCAGCTACGCCCTGCTCACCTACAT GGGAGACATGGGCCTGGCGTCCCCCAACATTGCCAGCTACGCCCTGCTCACCTACAT AGGAGACATGGGCCTGGGTGACCTTCACATGCCAGCTACGCCCTGCTCACCTACAT AGGAGACATGGGCCTGGCGTCCCCCAACATTGCCAGCTACTCCCTGCTCACCTACAT GGGGGACATGGGCCTGGGTGCCCCCAACATTGCCAGCTACGCCCTGCTCACCTACAT *****	707
HUMAN	708	GATTGCGCACATCACGGGCCTGAAGCCAGGTGACTTTATACACACTTGGGAGATGCACA 767 GATTGCGCACATCACGGGCCTGAAGCCAGGTGACTTTATACACACTTGGGAGATGCACA GATTGCGCACATCACGGGCCTGAAGCCAGGTGACTTTATACACACTTGGGAGATGCACA GATTGCGCACATCACGGGCCTGAAGCCAGGTGACTTTATACACACTTGGGAGATGCACA GATCGCgcACATCACGGGCCTGAAGCCAGGTGACTTTATACACACTTGGGAGATGCACA GATCGCACACATCACAGGCCTGAAGCCAGGTGACTTTATACACACTTGGGAGATGCACA GATTGCAACATATCACAGGCCTGCAGGCCAGGTGATTTGTCACACTTGGGAGATGCACA GATTGCAACATATCACAGGCCTGCAGCCGGGTGATTTGTCACACTTGGGAGATGCACA GATCGCgACGTCACCGGCCTGAAGCCAGGTGATTTGTCACACTTGGGAGATGCTCA GATTGCGCATGTCACTGGACTGAAGCCAGGTGACTTTGTCACACACTCTGGGAGATGCACA GATCGCACACATCACGGACCTGAAGCCAGGTGACTTCGTCACACTCTGGGAGATGCACA GATCGCACACATCACGGGCCTGAAGCCAGGTGACTTTGTCACACACTTGGGAGATGCACA GATCGCACACATCACGGGCCTGAAGCCGGGTGACTTTGTCACACTTGGGAGATGCACA GATCGCACACATCACAGGCCTGAAGCCAGGTGACTTCACACACTTGGGAGATGCGCA GATCGCACACATCACAGGCCTGAAGCCAGGTGACTTCACACACTTGGGAGATGCGCA GATTGCGCACGTCACGGGCCTGCAGCCAGGTGACTTTGTCACACACTTGGGAGACGCA *****	
HUMAN	768	TATTTACCTGAATCACATCGAGCCACTGAAAATTCTCAGCTTCAGCGAGAACCCAGACCTTT 827 TATTTACCTGAATCACATCGAGCCACTGAAAATTCTCAGCTTCAGCGAGAACCCAGACCTTT TATTTACCTGAATCACATTGAGCCACTGAAAATTCTCAGCTTCAGCGAGAACCCAGACCTTT TATTTACCTGAATCACATCGAGATTTAAAAGTTACTCTTCAGCTTCAGCGAGAACCCAGACCTTT TATTTACCTGAATCACATTGAGCCACTGAAAATTCTCAGCTTCAGCGAGAACCAAGACCTTT TATTTACCTGAATCATATAGAGCCGCTGAAAATTCTCAGCTACAGCGAGAACCAAGACCTTT CATTTATCTGAATCATATTGAGCCACTGAAAATTCTCAGCTACAGCGAGAACCAAGACCTTT TATTTACCTGAACCACATTGAGCCCTCTGAAAATTCTCAGCTTCAGCGAGAACCAAGACCTTT TGTTTACCTGAATCACATCGAGCCACTCAAAGTGCAGCTTCAGCGAGAACCAAGACCTTT CATTTACCTGAATCACATCGAGCCACTGAAAATTCTCAGCTTCAGCGAGAACCAAGACCTTT CATTTACCTGAATCACATTGAGCCGCTGAAAATTCTCAGCTTCAGCGAGAACCAAGACCTTT TATTTACCTGAATCACATTGAGCCGCTGAAAATTCTCAGCTTCAGCGAGAACCAAGACCTTT TATTTACCTGAATCACATTGAGCCGCTGAAAATTCTCAGCTCCAGCGAGAACCAAGACCTTT TATTTACCTGAATCACATTGAGCCGCTGAAAATTCTCAGCTCAACGAGAACCAAGACCTTT CATTTACCTGAATCACATTGAGCCGCTGAAAATTCTCAGCTCAACGAGAACCAAGACCTTT *****	

Figure S1 **Continued**

Figure S1 **Continued**

Table S1. Synonymous (d_s) and nonsynonymous (d_N) nucleotide substitutions for the IDR and the body of the TS polypeptide
 Values for each of the 120 pairwise comparisons among 16 mammalian species were determined, and d_N/d_s ratios were calculated.

Species 1	Species 2	IDR			Body		
		d_N	d_s	d_N/d_s	d_N	d_s	d_N/d_s
Human	Chimpanzee	0.0000	0.0441	0.0000	0.0045	0.0159	0.2830
	Orang-utan	0.0355	0.1946	0.1824	0.0030	0.0321	0.0935
	Macaque	0.0629	0.2880	0.2184	0.0060	0.0948	0.0633
	Marmoset	0.1011	0.4321	0.2340	0.0205	0.1341	0.1529
	Tarsier	0.1113	0.4674	0.2381	0.0290	0.2979	0.0973
	Rabbit	0.1257	0.5646	0.2226	0.0267	0.3083	0.0866
	Pika	0.2201	0.6355	0.3463	0.0354	0.4987	0.0710
	Cow	0.1953	0.3235	0.6037	0.0361	0.4854	0.0744
	Dolphin	0.1590	0.3699	0.4298	0.0236	0.3595	0.0656
	Horse	0.1833	0.6655	0.2754	0.0386	0.2948	0.1309
	Dog	0.2150	0.3490	0.6160	0.0299	0.3998	0.0748
	Cat	0.2609	0.4975	0.5244	0.0306	0.4741	0.0645
	Mouse	0.3041	1.0397	0.2925	0.0321	0.5807	0.0553
	Rat	0.3536	2.8876	0.1225	0.0329	0.6567	0.0501
	Guinea pig	0.3908	1.1119	0.3515	0.0571	0.6529	0.0875
Chimpanzee	Orang-utan	0.0355	0.1410	0.2518	0.0015	0.0267	0.0562
	Macaque	0.0536	0.2567	0.2088	0.0045	0.1009	0.0446
	Marmoset	0.0913	0.3941	0.2317	0.0190	0.1405	0.1352
	Tarsier	0.1113	0.3904	0.2851	0.0275	0.2905	0.0947
	Rabbit	0.1257	0.4661	0.2697	0.0252	0.3008	0.0838
	Pika	0.2201	0.5416	0.4064	0.0338	0.4695	0.0720
	Cow	0.1953	0.2590	0.7541	0.0345	0.4860	0.0710
	Dolphin	0.1590	0.3014	0.5275	0.0220	0.3599	0.0611
	Horse	0.1833	0.5653	0.3243	0.0370	0.2874	0.1287
	Dog	0.2150	0.2784	0.7723	0.0314	0.3827	0.0820
	Cat	0.2490	0.4546	0.5477	0.0321	0.4747	0.0676
	Mouse	0.3041	1.0397	0.2925	0.0306	0.5480	0.0558
	Rat	0.3536	2.8876	0.1225	0.0345	0.6208	0.0556
	Guinea pig	0.3908	0.9414	0.4151	0.0554	0.6176	0.0897
Orang-utan	Macaque	0.0916	0.1433	0.6392	0.0030	0.0948	0.0316
	Marmoset	0.1312	0.1995	0.6576	0.0175	0.1341	0.1305
	Tarsier	0.1524	0.1979	0.7701	0.0259	0.2902	0.0892
	Rabbit	0.1489	0.3010	0.4947	0.0236	0.2851	0.0828
	Pika	0.2437	0.4582	0.5319	0.0322	0.4886	0.0659
	Cow	0.2413	0.2590	0.9317	0.0330	0.5056	0.0653
	Dolphin	0.2027	0.1270	1.5961	0.0205	0.3427	0.0598
	Horse	0.2351	0.3856	0.6097	0.0354	0.2721	0.1301
	Dog	0.2665	0.0993	2.6838	0.0299	0.3823	0.0782
	Cat	0.2978	0.3041	0.9793	0.0306	0.4546	0.0673
	Mouse	0.3756	1.0397	0.3613	0.0290	0.5582	0.0520
	Rat	0.4363	2.5045	0.1742	0.0329	0.6199	0.0531
	Guinea pig	0.4513	0.8025	0.5624	0.0538	0.6168	0.0872
Macaque	Marmoset	0.0907	0.2635	0.3442	0.0144	0.1992	0.0723
	Tarsier	0.1308	0.2612	0.5008	0.0259	0.3457	0.0749
	Rabbit	0.1722	0.3041	0.5663	0.0236	0.3239	0.0729
	Pika	0.2421	0.3904	0.6201	0.0322	0.5083	0.0633
	Cow	0.2165	0.3295	0.6571	0.0291	0.5525	0.0527
	Dolphin	0.1794	0.1837	0.9766	0.0205	0.4026	0.0509

Table S1 Continued

Species 1	Species 2	IDR			Body		
		d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S
Marmoset	Horse	0.2054	0.4870	0.4218	0.0315	0.3379	0.0932
	Dog	0.2386	0.2177	1.0960	0.0330	0.4264	0.0774
	Cat	0.2130	0.4226	0.5040	0.0337	0.4637	0.0727
	Mouse	0.3406	1.3061	0.2608	0.0259	0.5575	0.0465
	Rat	0.3926	1.6170	0.2428	0.0298	0.6072	0.0491
	Guinea pig	0.4272	0.9178	0.4655	0.0530	0.6581	0.0805
	Tarsier	0.1304	0.2029	0.6427	0.0346	0.3005	0.1151
	Rabbit	0.1489	0.3010	0.4947	0.0299	0.3472	0.0861
	Pika	0.2413	0.5592	0.4315	0.0401	0.5483	0.0731
	Cow	0.2158	0.3326	0.6488	0.0369	0.5504	0.0670
	Dolphin	0.1788	0.1853	0.9649	0.0296	0.4027	0.0735
Tarsier	Horse	0.2286	0.4099	0.5577	0.0386	0.3490	0.1106
	Dog	0.2251	0.2524	0.8918	0.0423	0.4450	0.0951
	Cat	0.2583	0.3490	0.7401	0.0422	0.4492	0.0939
	Mouse	0.3055	1.3061	0.2339	0.0343	0.6084	0.0564
	Rat	0.3926	2.5914	0.1515	0.0377	0.7319	0.0515
	Guinea pig	0.4256	0.9313	0.4570	0.0585	0.6890	0.0849
	Rabbit	0.0600	0.1073	0.5592	0.0275	0.3435	0.0801
	Pika	0.1316	0.4674	0.2816	0.0364	0.5635	0.0646
	Cow	0.1103	0.2635	0.4186	0.0322	0.6074	0.0530
	Dolphin	0.0780	0.1290	0.6047	0.0275	0.4243	0.0648
Rabbit	Horse	0.0957	0.3326	0.2877	0.0354	0.3577	0.0990
	Dog	0.1098	0.1868	0.5878	0.0370	0.3853	0.0960
	Cat	0.1590	0.3099	0.5131	0.0361	0.3844	0.0939
	Mouse	0.2683	1.0892	0.2463	0.0314	0.6311	0.0498
	Rat	0.3154	1.8318	0.1722	0.0361	0.6540	0.0552
	Guinea pig	0.3975	0.9178	0.4331	0.0514	0.7208	0.0713
	Pika	0.0604	0.4608	0.1311	0.0170	0.3563	0.0477
	Cow	0.1517	0.2947	0.5148	0.0268	0.5869	0.0457
	Dolphin	0.0397	0.1643	0.2416	0.0213	0.4503	0.0473
Pika	Horse	0.1827	0.3916	0.4665	0.0229	0.3729	0.0614
	Dog	0.1773	0.2180	0.8133	0.0346	0.4419	0.0783
	Cat	0.2561	0.3658	0.7001	0.0283	0.4367	0.0648
	Mouse	0.3213	1.1765	0.2731	0.0190	0.5587	0.0340
	Rat	0.3597	1.1861	0.3033	0.0236	0.5908	0.0399
	Guinea pig	0.3501	1.0714	0.3268	0.0339	0.6528	0.0519
	Cow	0.2218	0.4586	0.4836	0.0373	0.7123	0.0524
	Dolphin	0.1590	0.4452	0.3571	0.0314	0.6124	0.0513
	Horse	0.2068	0.6655	0.3107	0.0355	0.5008	0.0709
Cow	Dog	0.2275	0.4693	0.4848	0.0434	0.6735	0.0644
	Cat	0.2978	0.5429	0.5485	0.0346	0.6620	0.0523
	Mouse	0.3213	0.9241	0.3477	0.0354	0.6200	0.0571
	Rat	0.2901	1.1861	0.2446	0.0401	0.7198	0.0557
	Guinea pig	0.4780	0.7037	0.6793	0.0518	0.7532	0.0688
	Dolphin	0.1505	0.0954	1.5776	0.0229	0.3044	0.0752
	Horse	0.2286	0.2064	1.1076	0.0284	0.3442	0.0825
	Dog	0.2127	0.1585	1.3420	0.0409	0.4518	0.0905
	Cat	0.2583	0.2138	1.2081	0.0306	0.4513	0.0678
	Mouse	0.3701	0.8740	0.4235	0.0361	0.8683	0.0416
	Rat	0.4232	1.9237	0.2200	0.0385	0.7946	0.0485

Table S1. Continued

Species 1	Species 2	IDR			Body		
		d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S
Dolphin	Guinea pig	0.3817	0.6208	0.6149	0.0515	0.7589	0.0679
	Horse	0.1815	0.2064	0.8794	0.0299	0.1974	0.1515
	Dog	0.1650	0.0492	3.3537	0.0314	0.2922	0.1075
	Cat	0.2349	0.1544	1.5214	0.0283	0.2793	0.1013
	Mouse	0.3041	1.0397	0.2925	0.0282	0.7807	0.0361
Horse	Rat	0.3536	1.6805	0.2104	0.0314	0.7932	0.0396
	Guinea pig	0.3817	0.7301	0.5228	0.0490	0.7717	0.0635
	Dog	0.1521	0.2177	0.6987	0.0474	0.2588	0.1832
	Cat	0.1797	0.2758	0.6516	0.0386	0.2795	0.1381
	Mouse	0.3423	0.7940	0.4311	0.0322	0.6407	0.0503
Dog	Rat	0.4273	0.9607	0.4448	0.0362	0.6450	0.0561
	Guinea pig	0.5244	0.6816	0.7694	0.0500	0.6355	0.0787
	Cat	0.1290	0.1355	0.9520	0.0244	0.2369	0.1030
	Mouse	0.3089	0.7739	0.3991	0.0346	0.6765	0.0511
	Rat	0.2870	1.6841	0.1704	0.0362	0.7140	0.0507
Cat	Guinea pig	0.5769	0.6649	0.8676	0.0523	0.6959	0.0752
	Mouse	0.3503	0.7920	0.4423	0.0290	0.7893	0.0367
	Rat	0.4019	0.9313	0.4315	0.0306	0.8020	0.0382
Mouse	Guinea pig	0.5456	0.6053	0.9014	0.0506	0.7445	0.0680
	Rat	0.1618	0.4879	0.3316	0.0045	0.1833	0.0245
	Guinea pig	0.6132	0.9435	0.6499	0.0331	0.7597	0.0436
Rat	Guinea pig	0.5573	1.2745	0.4373	0.0363	0.8011	0.0453

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