C to U Editing Stimulates A to I Editing in the Anticodon Loop of a Cytoplasmic Threonyl tRNA in Trypanosoma brucei

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**Recommended Citation**

Rubio, M.A.T., Ragone, F.L., Gaston, K.W., Ibba, M. and Alfonzo, J.D. (2006) C to U editing stimulates A to I editing in the anticodon loop of a cytoplasmic threonyl tRNA in Trypanosoma brucei. *J. Biol. Chem.* 281, 115-120. https://doi.org/10.1074/jbc.M510136200
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Comments
This article was originally published in *Journal of Biological Chemistry*, volume 281, in 2006.
https://doi.org/10.1074/jbc.M510136200

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C to U Editing Stimulates A to I Editing in the Anticodon Loop of a Cytoplasmic Threonyl tRNA in *Trypanosoma brucei*

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Editing of tRNAs is widespread in nature and either changes the decoding properties or restores the folding of a tRNA. Unlike the phylogenetically dispersed adenosine (A) to inosine (I) editing, cytosine (C) to uridine (U) editing has only been previously described in organellar tRNAs. We have shown that cytoplasmic tRNA<sup>Thr</sup>(AGU) undergoes two distinct editing events in the anticodon loop: C to U and A to I. In vivo, every inosine-containing tRNA<sup>Thr</sup> is also C to U edited at position 32. In vitro, C to U editing stimulates conversion of A to I at the wobble base. Although the in vivo and in vitro requirements differ, in both cases, the C to U change plays a key role in A to I editing. Due to an unusual abundance of A34-containing tRNAs, our results also suggest that the unedited and edited tRNAs are functional, each dedicated to decoding a specific threonine codon. C to U editing of cytoplasmic tRNA expands the editing repertoire in eukaryotic cells, and when coupled to A to I changes, leads to an interrelation between editing sites.

The degeneracy of the genetic code is implied in the need for 61 sense codons to specify 20 different amino acids and, with the exception of methionine and tryptophan, each amino acid is encoded by more than one codon (1). This discrepancy between codon and amino acid numbers was first explained by Crick’s wobble hypothesis, which invoked flexibility between the first anticodon and third codon positions during decoding (2). Since the inception of the wobble rules, over 100 posttranscriptional modifications have been described with the largest number affecting the anticodon of tRNA (3, 4). As anticodon modifications accrue, new findings lead to a constant reinterpretation of the wobble rules to include novel effects on tRNA function. Although some anticodon modifications play key roles in translational fidelity and efficiency (1, 5), anticodon-sequence alterations that permit decoding of multiple codons are part of a growing number of posttranscriptional changes collectively known as tRNA editing. Thus decoding changes imparted by tRNA editing provide a mechanism to effectively accommodate genetic code degeneracy. To date, well characterized anticodon editing events include editing of C34 to lysidine of methionyl tRNAs in bacteria, which permits decoding of AUA codons as isoleucine (6, 7), cytidine (C) to uridine (U) editing in eukarya, which reassigns tRNA<sup>Thr</sup> and tRNA<sup>Trp</sup> to new codons in mitochondria (8, 9), and adenosine (A) to inosine (I) editing, which expands tRNA decoding capacity and is found in organisms from each of the three domains of life (4, 10).

Although inosine was first discovered over 40 years ago in tRNA (11), its involvement in codon alterations in eukaryotes was first demonstrated by the discovery of A to I editing in mRNAs (12, 13). Inosine in mRNA expands the number of proteins that can be encoded from a single gene and is a significant source of genetic diversity (14). In tRNA, adenosines at the first position of the anticodon (A34, wobble position) are almost universally changed to inosine by hydrolytic deamination of the 6-amino group of the base (4, 15). This editing reaction is so efficient that under steady-state conditions, A34-containing tRNAs are difficult to detect, tRNA<sup>Thr</sup> from *Mycoplasma*, thus far, being the only naturally occurring exception (16).

C to U editing of tRNA is less prevalent and, until now, restricted to eukaryotic organelles. In marsupial mitochondria, a single C to U editing event at the second position of the anticodon (C35) changes a tRNA such that it recognizes aspartate in place of glycine codons (9, 17–19). Some evidence supports the requirement for methylation and pseudouridylation reactions prior to editing (19). C to U editing in this system is also required for creating the proper substrate for further modification of the first anticodon position (G34) (19). This C to U editing also generates structural features important for aminoacyl-tRNA synthetase recognition (9). The only other example of C to U editing of tRNA occurs in the mitochondria of trypanosomatids (8), where the nucleus-encoded tryptophanyl tRNA (tRNA<sup>Trp</sup>) is transcribed with a CCA anticodon. A subpopulation of this tRNA is imported into the mitochondrion, where RNA editing of C34 creates the U<sub>34</sub>CA anticodon required to translate the UGA tryptophan codons (8). Following mitochondrial import, tRNA<sup>Trp</sup> undergoes an unprecedented number of posttranscriptional modifications, which, as in the marsupial system, may play a role in editing specificity (8, 20).

In the current study, we have shown that the cytoplasmic tRNA<sup>Thr</sup>- (AGU) of *Trypanosoma brucei* undergoes two distinct editing events in the anticodon loop, where A34 is changed to inosine and C32 is changed to uridine. We demonstrated that C to U editing at position 32 affects the efficiency of A to I editing of the anticodon. These findings represented the first example of C to U editing of tRNAs outside organelles and demonstrated an interrelation between two different editing sites in a single anticodon loop. Unlike most organisms, we also reported an abundance of unedited tRNAs, which are substrates for aminoacylation in vivo. Together, our findings have raised new and important questions about the prevalence of tRNA editing in eukaryotes and demonstrated a functional role for double editing of tRNAs in trypanosomatids.

**MATERIALS AND METHODS**

**Cell Culture and Preparation of Cell-free Extracts—*T. brucei***
cells were grown in SDM-79 medium supplemented with 10% fetal bovine serum (Fisher) and 10 g/ml hemin (Calbiochem). Exponentially grow-

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*This work was supported by a grant from the American Heart Association (AHA) (to J. D. A.) and by an AHA predoctoral fellowship (to K. W. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*The on-line version of this article (available at http://www.jbc.org) contains two supplemental figures.

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ing cultures (2 × 10^6 cells/ml) were harvested by centrifugation at 4,000 × g and washed with phosphate-buffered saline. The resulting pellets were suspended in buffer containing 50 mM Heps, pH 8.0, 50 mM KCl, 2.5 mM EDTA, 1 mM DTT. The suspension was sonicated with a Sonifier 450 sonicator (Branson) using a microprobe at 50% output for a total of five intervals with 1-min rest between sonication. The resulting lysate was initially spun at 10,000 rpm in a Beckman Coulter Avanti J-25 centrifuge at 15 min at 4 °C followed by a 30-min centrifugation at 100,000 × g in a Beckman Coulter Optima L-90K ultracentrifuge Type 60TI at 4 °C. To the clarified lysate, glycerol was added to a final concentration of 20% and stored frozen in 4 mg/ml aliquots at −80 °C.

cDNA Synthesis and Amplification by PCR—RNA was isolated from cells (total RNA) and/or nuclear fractions by the guanidinium thiocyanate/phenol/chloroform extraction method (21) and as described previously by us (22). RNA was further treated with RNase-free DNase I (Promega). Two picomoles of reverse oligonucleotide primer (57R: 5′-AGGCCACTGGGGGATCGAACCC-3′) complementary to the 3′-end of tRNA 35S(AGU) was added to 5 μg of total or nuclear RNA with 10 μM of all four deoxynucleotide triphosphates and heated at 65 °C for 5 min and then quick-cooled at 4 °C for 1 min followed by the addition of 1 μl of SuperScript™ II reverse transcriptase (RT) in 1× first strand buffer and incubation at 50 °C, as described (Invitrogen). Following the RT reaction, the cDNA was amplified from 1 μl of the RT reaction as a template in a 100-μl PCR reaction with 40 pmol of forward (56F: 5′-GGCCGTTAGCTCAATGGCAGAG-3′) and 40 pmol of reverse (57R) oligonucleotide primers. PRK reactions were performed using Taq DNA polymerase and incubated in a thermal cycler using a program consisting of a 94 °C denaturation step, a 50 °C annealing step for 40 s, and an elongation step of 72 °C repeated for a total of 20 cycles, following manufacturer’s instructions (PerkinElmer Life Sciences). Controls included a mock reaction in which the RT was left out of the reaction and used as a negative control to test for DNA contamination in the RNA samples and a reaction in which total genomic DNA was used as a template serving as a positive control for amplification. RT-PCR products were cloned into pCR2.1-TOPO (Invitrogen). Independent clones were isolated after transformation of DH5α Escherichia coli and sequenced using Sequenase™ Version 2.0 DNA polymerase (USB), per the manufacturer’s instructions. The dideoxynucleotide terminated sequencing reactions were separated in a 6% acrylamide/7 M urea denaturing gel, and the resulting sequences were sequenced to establish editing levels.

In Vitro Editing Assays—in vitro transcribed tRNAs with internally incorporated [α-35S]ATP were heated in water at 70 °C for 3 min and allowed to cool to room temperature. After 1 min, reaction buffer was added to a final concentration of 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 2.5 mM MgSO4, 0.1 mM EDTA (pH 8.0), 2 mM dithiothreitol, and the mixture was allowed to cool for an additional 5 min. The reaction was started by the addition of cell extract and incubated at 27 °C. For the time course experiments, a large reaction (446 μl) containing 40 pmol of RNA (200,000 cpm) in reaction buffer was assembled. As a negative control, an aliquot of 49 μl was transferred into a separate tube and incubated at 27 °C for 480 min. To the remaining 397-μl reaction mix, 8.1 μl of cell extract was added and incubated at 27 °C. Eight individual aliquots of 50 μl were removed after 1, 30, 50, 120, 180, 240, 360, and 480 min, respectively. Each sample was extracted using an equal volume of phenol (previously saturated with 10 μl Tris-HCl, pH 8). The RNA in the aqueous phase was recovered after precipitation with a 0.1 volume equivalent of 3 M sodium acetate (pH 5.2), 2.5 volumes of ethanol and incubated at −20 °C. After centrifugation, the resulting pellet was dissolved in 30 mM ammonium acetate and 10 mM zinc acetate containing 0.4 units nuclease P1 in a 20-μl reaction (MPBiomedicals). The digestion reaction was incubated at 37 °C for at least 12 h. The reaction was dried in a SpeedVac DNA 110 concentrator system (Savant) for 10 min under high heat. The dried sample was resuspended in 3 μl of double distilled H2O, where 1 μl (13.33 pmol) was spotted and dried individually onto a cellulose TLC sheet (EMD Chemicals). On the same sheet, 2.5 μl of a cold mix containing adenosine 5′-monophosphate and inosine 5′-monophosphate was spotted in a separate lane and used as cold markers. The TLC was allowed to develop using liquid chromatography in Solvent C (0.1 M sodium phosphate (pH 6.8): ammonium sulfate-n-propyl alcohol (100:60:2, v/v/v)). The TLC plate was allowed to dry and was then exposed to a Phosphorlmager™ screen. The resulting images were visualized and quantified using an Amersham Biosciences Storm™ imaging system with an ImageQuant™ program (Amersham Biosciences). Cold markers were visualized by a hand-held ultraviolet lamp at 260 nm and used to assess the relative migration of the 35S-labeled individual nucleoside 5′-monophosphates from the radiola beled samples. Two-dimensional TLC was used to further confirm the relative positions of nucleoside 5′-monophosphates assignments. The first dimension of the TLC plate was developed in Solvent A (isobutyric acid:25% ammonium hydroxide:H2O; 50:1.1:28.9, v/v/v). The TLC plate was removed and allowed to dry before separation in the second dimension by developing in Solvent B (isopropanol:acetonitrile:concentrated HCl: water, 68:18:14, v/v/v) or solvent C (0.1 M sodium phosphate (pH 6.8): ammonium sulfate-n-propyl alcohol, 100:60:2, v/v/v). Nucleotide assignments were made using published maps (23).

In Vitro Aminoacylation and Oxidation Assays—for corroboration of the editing state of aminoacylated species, total aminoacyl-tRNAs were extracted under acidic conditions (using phenol equilibrated with 0.3 M sodium acetate, pH 4.5, and 10 mM EDTA), ethanol-precipitated, and resuspended in 10 mM sodium acetate, pH 4.5, and 1 mM EDTA. The RNA was then split into two fractions. One fraction was deacylated by incubation at 37 °C for 1 h in a basic buffer (10 mM Tris, pH 9.0) followed by oxidation of the 3′-ribose by treatment with 40 mM NaPO4 in ice for 90 min. The second fraction was directly oxidized by NaPO4 followed by deacylation as above. Both fractions were individually polyadenylated by incubation of the RNA at 37 °C for 45 min in buffer containing 20 mM Tris, pH 7.0, 50 mM KCl, 0.7 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, 0.1 mg/ml bovine serum albumin, 10% glycerol, 500 μM ATP, and 1,700 units of yeast poly-A polymerase in 100 μl of reaction buffer. The reaction was then supplemented with 30 μl of 5 × E. coli poly-A buffer (200 mM Tris, 7.0, 1 M NaCl, and 25 mM MgCl2), 15 μl of 5 mM ATP, 1 μl of 0.1 M DTT, 3.5 μl of MnCl2, and 3 units of E. coli poly-A polymerase and incubated further for 45 min at 37 °C. The reactions were phenol-extracted and ethanol-precipitated. Both reactions were then used in RT-PCR reactions. First, a 3′-specific primer specific for tRNAThr(AGU) in a 100-μl PCR reaction as above. One μl of this reaction was used as a template for a second PCR reaction in which both primers were specific for tRNA 35S(AGU) (AGU). The resulting product was purified, cloned into pCR2.1-TOPO (Invitrogen), and transformed into E. coli, and individual clones were sequenced to establish editing levels.

For in vitro aminoacylation, all assays were performed at 37 °C as follows. A 35S-pre-reaction mixture was first prepared containing 100 mM Heps (pH 7.5), 25 mM KCl, 10 mM MgCl2, 10 mM ATP, 5 mM DTT, 15 μM in vitro transcribed tRNA 35S variants (see “Results” for details),

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2 The abbreviations used are: DTT, dithiothreitol; RT, reverse transcriptase.
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RESULTS

In the search for examples of interdependence between different editing sites, we focused on the formation of inosine at the wobble base (position 34) of tRNAs in *T. brucei*. In these organisms, eight different tRNA species contain an encoded A at the first position of the anticodon (A34). These tRNAs are proposed to undergo A to I editing to allow the decoding of the C-ending threonine codon (ACC) in the genome and must be formed by editing. In *T. brucei*, it is proposed to undergo A to I editing, where the wobble position inosine can then decode the remaining threonine codon by wobbling. Arrows indicate the position of the primers (56F and 57R) used in the RT and PCR reactions. The short arrow denotes the edited position.

and 28 μM l-[^3-3H]threonine. The reaction was started by the addition of 10 μl (1 μg/μl total protein) of *T. brucei* extract. Eight-μl aliquots were removed periodically and spotted onto 3MM filter disks presoaked in 5% trichloroacetic acid (w/v), washed three times in 5% trichloroacetic acid (w/v), rinsed in ethanol, and dried, and the remaining radioactivity was quantified by scintillation counting.

A to I editing of tRNA^Thr(AGU) allows decoding of the C-ending threonine codon. A, the four threonine codons used in trypanosomatid translation and their respective tRNAs. A possible decoding of the GCA codon by UGU wobbling is shown in brackets. The arrows indicate the sequence polarity. Isoaccepting tRNAs, which may decode the ACU, ACA, and ACM codons, are genomically encoded. No tRNA that may decode the remaining codon (ACC) is encoded in the genome and must be formed by editing. B, tRNA^Thr(AGU) is proposed to undergo A to I editing, where the wobble position inosine can then decode the remaining threonine codon by wobbling. Arrows indicate the position of the primers (56F and 57R) used in the RT and PCR reactions. The short arrow denotes the edited position.

The findings above raised questions as to a possible connection between the two processes. First, we tested for A to I editing in vitro. A ^32P-labeled tRNA^Thr(AGU) was generated by *in vitro* transcription whereby every adenosine is radioactively labeled. This substrate was then incubated for various times with total cell-free extracts from *T. brucei*. Following this incubation, the labeled tRNA was gel-purified followed by digestion with nucleases. The nucleotide mixture generated by the nuclease treatment was then separated by TLC, followed by digestion with nuclease P1. The nucleotide mixture generated by the nuclease treatment was then separated by TLC, and inosine generated during the assay. We found that >50% of aden-
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FIGURE 3. tRNA^{Thr}(AGU) is efficiently edited in vitro. tRNA^{Thr}(AGU) was labeled by in vitro transcription in the presence of [α-32P]ATP. The labeled tRNA was incubated with total T. brucei extracts for increasing lengths of time followed by nuclease P1 digestion and separation by TLC. A, TLC analysis of the reaction in the presence or absence of extract, where pA and pl denote the positions of unlabeled 5'-AMP and 5'-IMP used as markers and visualized by UV shadowing (not shown). B, a reaction where a similar tRNA as in A but containing a G34 was used as a control for specificity. This reaction also served as a background control. The relative fraction of pA converted to pl was calculated by dividing the amount of radioactivity in the pl spot by the sum of the radioactivity in the pA + pl spots (pl/pA + pA). The specific percent conversion at a single site (i.e. A34) was then calculated by normalizing the amount of radioactivity at A34 to the total number of label adenosines (n = 14), where conversion at one site will yield a maximum theoretical value of 7.7% (or 1/1.3 possible adenosines). The specific yield of pl was then calculated by dividing the percent total by the relative percentage at one site or %pI/34/34 × 100, where the theoretical maximum of 7.1% equals 100% conversion A to I conversion at position 34.

osine 34 was efficiently converted to inosine by the T. brucei extract under the assay conditions described (Figs. 3A and 6C, and data not shown). No detectable inosine was observed in a control reaction using a substrate in which A34 was changed to G34, indicating that the observed A to I conversion is specific for position 34 (Fig. 3B). A two-dimensional-TLC was also performed to confirm the identity of the reaction products (Fig. 4).

We recently proposed an interdependence model to explain the connection between editing and modification of tRNA^{Thr} in trypanosomatid mitochondria (24). We have now expanded this model to include cytoplasmic tRNAs in these organisms. We propose that double editing of tRNA^{Thr} occurs in a sequential manner, where editing at one position affects subsequent editing at a second position (Fig. 5). To test a possible connection between the two sites, we created in vitro transcribed tRNA substrates representing the unedited tRNA and a possible intermediate in the editing reaction (Fig. 5, I and II) so that every adenosine in the various tRNAs is radioactively labeled. Upon incubation of the different substrates with cell-free extracts, we found that the C32-containing substrate could support editing; however, a similar substrate in which C32 was replaced by U32 (Fig. 5, II) supported editing with reproducibly higher efficiency and significantly higher initial rate (compare Fig. 3A and Fig. 6, A and C). We also found that tRNA substrates, in which C32 was replaced by A or G, showed no stimulation (data not shown). Taken together, the in vivo observation that every inosine-containing tRNA^{Thr} is also edited at position 32 and the observed in vitro stimulation of inosine formation led us to conclude that editing at one site affects subsequent editing at a second site and that indeed, the two editing events are interrelated.

In our model, editing at position 32 occurs first, and it promotes efficient A to I editing at position 34 of the anticodon. If C to U editing at position 32 occurs first, this may impart subtle changes in the loop structure, providing the proper substrate for further editing at position 34 (Fig. 5, I). In this scheme, C to U editing may also affect tRNA aminoacylation in addition to modulating the ability of the tRNA to undergo further A to I editing. Alternatively, C to U editing may affect translational efficiency by affecting A to I formation, thus regulating wobbling. Recent evidence supports a role for 134 as a key determinant for synthetase recognition of tRNA^{Thr} in yeast (25). To test the possibility that A to I editing affects charging of tRNA^{Thr}, substrates were generated corresponding to the two partially edited intermediates (Fig. 5, I and II) and incubated with partially purified synthetase fractions from T. brucei in the presence of °H-labeled threonine. We found no significant difference in aminoacylation efficiency when the in vitro transcriptions were compared with native tRNA (see Supplementary Fig. 2). However, in vitro, the presence of C32, in the A34-containing tRNA (the unedited tRNA), supported a reproducible 2-fold difference in aminoacylation when compared with a similar substrate with a U at position 32. Interestingly, similar experiments performed with either substrate but with a G at position 34 supported similar charging efficiencies as the unedited tRNA (Fig. 7). The observed in vitro aminoacylation efficiency rules out the possibility that the differences in editing levels in vitro between the various substrates could be due to problems in the global folding of the in vitro transcribed tRNA substrates when compared with native substrates.

To further assess the editing state of the aminoacylated tRNAs in vivo, we designed a coupled oxidation/polyadenylation assay. In this assay,
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RNA fractions were isolated under acidic conditions and then subjected to a combination of in vitro oxidation, polyadenylation, and RT-PCR (see “Materials and Methods”). In these reactions, oxidation by sodium periodate led to formation of a dialdehyde at the 3′-end of uncharged tRNAs, whereas the 3′-end of aminoacylated tRNAs is protected from oxidation by the covalently attached amino acid. The oxidized tRNA is not a substrate for polyadenylation, whereas following deacylation, only the charged tRNA will have an intact 3′-end and will thus serve as a substrate for poly-A polymerase (Fig. 8A). Under these conditions, we observed an RT-PCR product when total RNA was oxidized as described, whereas no product was detected in a similar reaction, where the total RNA was deacylated prior to oxidation to de-protect every tRNA present in the mixture (Fig. 8B). The product from the reaction above was then purified, cloned, and sequenced. We found that, as in the in vitro situation, both the edited and the unedited tRNA were substrates for aminoacylation, where the majority of the charged species (23 out of 30 clones) corresponded to that of the double-edited tRNA (Fig. 8C).
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DISCUSSION

We previously proposed an interdependence model for editing and modification in tRNA (24). This model suggests that editing and modifications at multiple sites act in concert to help achieve the degree of substrate specificity that different systems demand. To further expand this model, we have focused on the process of inosine formation in the tRNAs of trypanosomatids. Here we have described the first example of two different editing events in a single tRNA anticodon loop, whereby positions 32 and 34 of tRNA<sup>Thr</sup> undergo C to U and A to I editing, respectively. The finding that every inosine-containing tRNA also undergoes C to U editing at position 32 (5′ of the wobble position) raised important questions as to what role the two editing events play in the function of this tRNA. In vitro, every I34-containing tRNA<sup>Thr</sup>(AGU) also has the C to U change at position 32. By establishing an A to I editing assay, we have demonstrated that C to U stimulates A to I editing in vitro, indicating interdependence between the two editing sites. The fact that these cells might be able to regulate A to I editing of tRNAThr<sup>Thr</sup>(AGU) indicates interdependence between the two editing sites. The fact that the C32-containing tRNA edited by the A to I reaction occurs so efficiently in vivo suggests that the A to I reaction is required for inosine formation in vivo. Additional evidence supports the view that posttranscriptional modifications play an essential role in achieving tRNA functional uniformity helping offset differences among various aminoacyl-tRNAs regarding their binding to the ribosome (34). We could also envisage a situation in which C to U32 editing is not only required for inosine formation in vivo but also enhances translational efficiency by providing the necessary changes for structural tRNA uniformity during translation. However, an in vitro translation system is not currently available for trypanosomatids, and answering these important questions will thus await further experimentation.

Acknowledgments—We thank J. Ringhart, D. Still, K. Fredrick, J. Hanson, and all members of the Alfonzo laboratory for insightful discussions and suggestions.

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*J. Biol. Chem.* 2006, 281:115-120.
doi: 10.1074/jbc.M510136200 originally published online November 3, 2005

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