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RESEARCH COMMUNICATION

TRF2, but not TBP, mediates the transcription of ribosomal protein genes

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The TCT core promoter element is present in most ribosomal protein (RP) genes in *Drosophila* and humans. Here we show that TBP (TATA box-binding protein)-related factor TRF2, but not TBP, is required for transcription of the TCT-dependent RP genes. In cells, TCT-dependent transcription, but not TATA-dependent transcription, increases or decreases upon overexpression or depletion of TRF2. In vitro, purified TRF2 activates TCT but not TATA promoters. ChIP-seq (chromatin immunoprecipitation [ChIP] combined with deep sequencing) experiments revealed the preferential localization of TRF2 at TCT versus TATA promoters. Hence, a specialized TRF2-based RNA polymerase II system functions in the synthesis of RPs and complements the RNA polymerase I and III systems.

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The signals that direct the initiation of transcription ultimately converge at the RNA polymerase II (Pol II) core promoter, which is sometimes referred to as the gateway to transcription (for reviews, see Smale and Kadonaga 2003; Goodrich and Tjian 2010; Juven-Gershon and Kadonaga 2010; Kadonaga 2012). The core promoter comprises the stretch of DNA that is typically from −40 to +40 nucleotides (nt) relative to the +1 start site, which is sufficient for accurate transcription initiation. There are a variety of specific sequence motifs that can contribute to the activity of core promoters. These motifs include the TATA box, initiator (Inr), downstream core promoter element (DPE), motif ten element (MTE), TFIIB recognition elements (BREu and BREd), and polypyrimidine initiator (TCT). There are no universal core promoter elements.

Specific core promoter elements can have important roles in biological networks. For instance, the DPE motif is present in nearly all of the promoters of the *Drosophila* Hox genes, and Caudal, which is one of the master regulators of the Hox genes, is a DPE-specific transcriptional activator (Juven-Gershon et al. 2008). In addition, the TCT motif is a core promoter element that is found in most of the ribosomal protein (RP) gene core promoters in *Drosophila* and humans and is important for transcriptional activity (Parry et al. 2010). The TCT motif encompasses the transcription start site from −2 to +6 relative to the +1 start site and is hence located at the same position as the Inr motif. It was found, however, that the TCT motif is functionally distinct from the Inr. For instance, the TCT motif cannot function in lieu of an Inr element and is not recognized by the TBP (TATA box-binding protein)-containing TFIID complex (Parry et al. 2010). These data suggest that there is a distinct transcription system, which does not depend on the canonical TFIID complex, that functions via the TCT motif and is dedicated to the synthesis of RPs. To investigate this question, we examined factors that might mediate transcription from TCT-dependent RP gene promoters and found a requirement for TBP-related factor 2 (TRF2; also known as TLP, TRP, TLF, and TBPL1) (Maldonado 1999; Moore et al. 1999; Ohbayashi et al. 1999 ; Rabenstein et al. 1999; Teichmann et al. 1999; Reina and Hernandez 2007; Goodrich and Tjian 2010; Akhtar and Veenstra 2011) but not TBP. These findings reveal that a specialized TRF2-based transcription system functions in the synthesis of RPs and complements the RNA Pol I and III systems, which produce ribosomal and transfer RNAs.

Results and Discussion

TCT-dependent transcription appears to require TRF2 but not TBP

To investigate the factors that are specifically required for transcription from TCT-dependent RP gene promoters, we used an RNAi depletion assay in *Drosophila* S2 cells to screen candidate proteins for transcriptional activity with TCT-dependent promoters but not a TATA-dependent promoter. Because it appeared that canonical TFIID does not function with the TCT motif (Parry et al. 2010), we were particularly interested in testing the roles of TBP and TBP-related factors in TCT-dependent transcription. Based on its properties, TRF2 was an excellent candidate. TRF2 is widely expressed and has been found to be present in many metazoans (for reviews, see Reina and Hernandez 2007; Goodrich and Tjian 2010; Akhtar and Veenstra 2011). Although TRF2 is related to TBP, it does not bind to TATA sequences, and the DNA sequences, if any, that are directly bound by TRF2 are not known. In *Drosophila*, there are two forms of TRF2, which we term dTRF2S (for short; also known as p75) and dTRF2L (for long; also known as p175) (Kopytova et al. 2006). dTRF2S is identical to the C-terminal 632-amino-acid residues of dTRF2L and is generated by translation initiation from an

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internal ribosome entry site (Kopytova et al. 2006). dTRF2S appears to be more closely related to human TRF2 (hTRF2), which lacks the long N-terminal extension that is present in dTRF2L (Fig. 1A). By ChIP–chip analysis (chromatin immunoprecipitation [ChIP] coupled with microarray analysis) with *Drosophila* S2 cells, dTRF2 (S and/or L) was found to be associated with many RP gene promoters (Isogai et al. 2007). Moreover, RNAi depletion of dTRF2 in *Drosophila* salivary glands was observed to result in a significant reduction in the sizes of the cells and the glands as well as a decrease in the levels of RP gene transcripts (Isogai et al. 2007), but it was not known whether the decrease in RP transcript levels was due to a transcriptional effect or a general growth defect.

Thus, to analyze the role of TRF2 in TCT-dependent versus TATA-dependent transcription, we carried out RNAi depletion analyses of dTRF2 (with dsRNAs corresponding to both S and L forms) or dTBP in S2 cells with TCT-dependent or TATA-dependent reporter genes. We achieved efficient depletion of dTRF2 as well as dTBP, each with two nonoverlapping dsRNAs (Supplemental Fig. S1). The depletion of dTRF2 resulted in a decrease in TCT-dependent transcription but not TATA-dependent transcription. Conversely, depletion of TBP caused a decrease in TATA transcription but not TCT transcription (Fig. 1B). To address the possibility of off-target effects, we performed experiments with a separate set of nonoverlapping dsRNAs for dTRF2 and dTBP and obtained essentially the same results (Supplemental Fig. S2). We additionally tested the effect of TRF2 depletion upon endogenous RP gene transcription via quantitative RT–PCR (qRT–PCR) analysis of intronic RNAs as a measure of newly synthesized transcripts. We examined several RP genes (lacking intronic snoRNAs, which could affect intronic RNA levels) and found that depletion of TRF2 resulted in a stronger decrease in RP gene transcription than depletion of TBP (Fig. 1C). Hence, these findings suggest that TCT-dependent core promoters require TRF2 but not TBP.

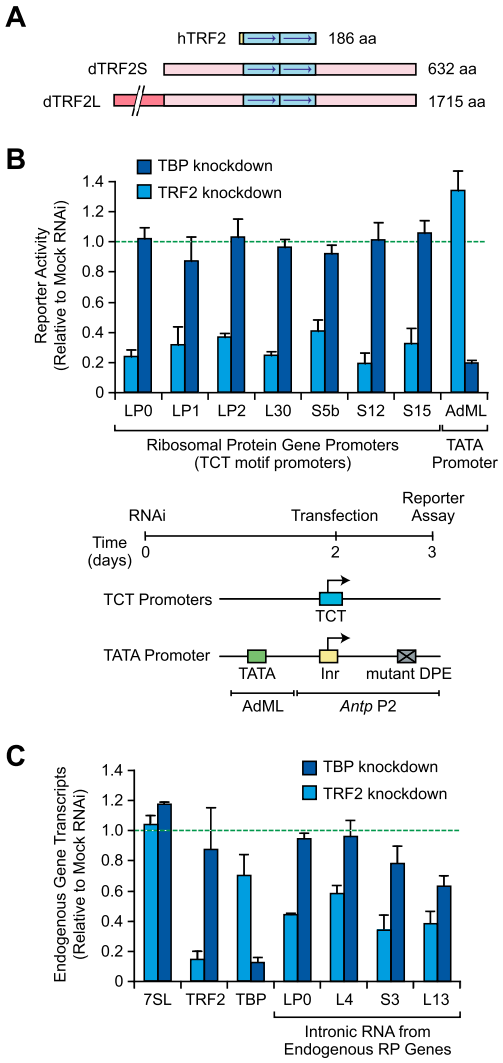

Figure 1. TCT-dependent transcription appears to require TRF2 but not TBP. (*A*) Schematic diagrams of hTRF2 and the two forms of *Drosophila* TRF2 (dTRF2S and dTRF2L). dTRF2S is identical to the C-terminal 632-amino-acid residues of dTRF2L. (*B*) Depletion of TRF2, but not TBP, reduces RP gene expression. *Drosophila* S2 cells were depleted of either TRF2 or TBP by RNAi and then transfected with TCT-dependent or TATA-dependent luciferase reporter genes. The experimental scheme and reporter constructs are depicted at the *bottom* of the figure. The activities of the RNAi-depleted extracts are reported as relative to the activities of mock RNAi-treated control extracts. Error bars represent the standard deviation. (*C*) Analysis of endogenous transcript levels by qRT–PCR. *Drosophila* S2 cells were depleted of TRF2 or TBP, as in *B*. The total RNA was then isolated and analyzed by qRT–PCR. For the RP genes, intronic sequences were used to detect newly synthesized transcripts. We did not analyze RP genes with intronic snoRNA genes, as they could affect the levels of the intronic RNAs. The error bars represent the standard deviation.

Purified TRF2 can mediate TCT-dependent but not TATA-dependent transcription in vitro

To test the specificity of function of TRF2 protein, we performed in vitro transcription experiments with purified TRF2 at TCT-dependent and TATA-dependent core promoters. For these experiments, we synthesized hTRF2 and hTBP with a wheat germ in vitro translation system (Takai et al. 2010) and purified the proteins to near homogeneity (Supplemental Fig. S3). hTRF2 contains the central conserved region of TRF2 and is smaller than dTRF2S (Fig. 1A). To test the activity of the purified hTRF2, we depleted dTRF2 from *Drosophila* nuclear extracts with anti-dTRF2 antibodies (Fig. 2A) and then performed two-template (with TCT-dependent and TATA-dependent promoters) in vitro transcription experiments with the TRF2-deficient extracts. As shown in Figure 2B, the depletion of TRF2 results in an essentially complete loss of transcription from two different TCT-dependent promoters (*RpL30* and *RpLP1*) but has little or no effect on transcription from a TATA-dependent promoter (*Act87E*). We further found that the addition of purified hTRF2 protein to the depleted extracts can partially or nearly fully restore the transcriptional activity that is lost upon depletion of TRF2. In contrast, the addition of purified hTBP did not restore TCT-dependent transcription. We obtained results analogous to those seen in Figure 2B with the *RpS12* (TCT), *RpS15* (TCT), and *hb* P2 (TATA) core promoters (Supplemental Fig. S4). It is also important to note that the TRF2-dependent transcription of TCT-dependent promoters is sensitive to low levels of α-amanitin and hence is mediated by RNA Pol II (Supplemental Fig. S5). We additionally found that *Drosophila* dTRF2S, but not

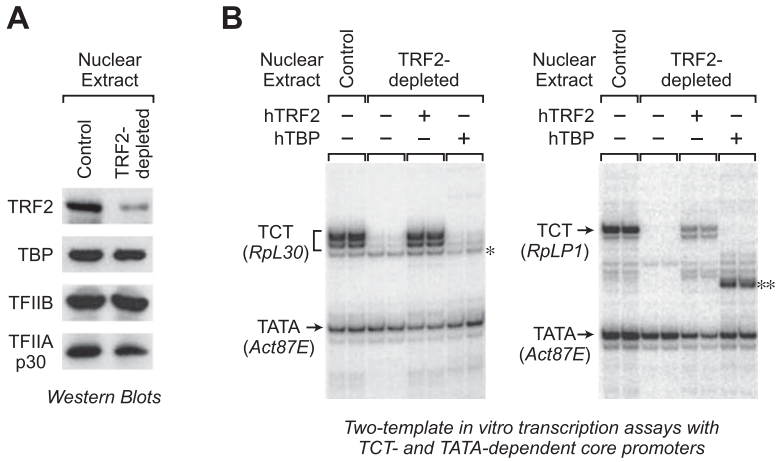

Figure 2. Purified TRF2 is required for in vitro transcription of TCT-dependent genes but not a TATA-dependent gene. (*A*) Immunodepletion of endogenous dTRF2 from a *Drosophila* embryo nuclear extract. The levels of TRF2 (dTRF2S), TBP, TFIIB, and TFIIA (p30 subunit) in TRF2-depleted extracts versus control extracts were monitored by Western blot analysis. We were able to detect dTRF2S but not dTRF2L even though the antibodies were raised against a polypeptide that is shared by both proteins. This effect may be due to inefficient transfer of the dTRF2L protein to the blot, the lack of recognition of dTRF2L by the antibodies, or the absence of dTRF2L in the extract used in the Western blot. (*B*) Purified hTRF2, but not purified hTBP, is able to restore the specific loss of TCT-dependent transcription that occurs upon depletion of TRF2 from a nuclear extract. Two-template in vitro transcription assays were performed with TCT-dependent and TATA-dependent promoters. Reactions were carried out with either TRF2-depleted or control nuclear extracts. Where indicated, purified hTRF2 or hTBP was added to reactions with the TRF2-depleted extracts. The resulting transcripts were detected by primer extension–reverse transcription analysis. The single asterisk denotes a nonspecific transcript. The double asterisk indicates a nonspecific transcript that is observed in the presence of hTBP. This stimulation of nonspecific initiation by hTBP is most likely a consequence of the relatively nonspecific binding of hTBP to DNA. This effect was not observed with dTBP (Supplemental Fig. S7).

Drosophila TBP, activates RP gene promoters in vitro (Supplemental Figs. S6, S7). These results thus provide biochemical evidence of the specificity of both hTRF2 and dTRF2S proteins for TCT-dependent promoters relative to TATA-dependent promoters. In addition, these experiments further reveal that the central conserved region of TRF2, which is the only segment of TRF2 that is shared by hTRF2 and dTRF2S (Fig. 1A), is sufficient for the specificity of its function for TCT-dependent core promoters.

Overexpression of TRF2 or TBP has opposite effects on TCT-dependent and TATA-dependent transcription

To complement the depletion experiments, we investigated the effects of the overexpression of TRF2 or TBP on the TCT-dependent versus TATA-dependent promoters (Fig. 3). To this end, TRF2 or TBP expression vectors were cotransfected into S2 cells with either TCT-dependent or TATA-dependent reporter constructs (for Western blots, see Supplemental Fig. S8). With TCT-dependent promoters, overexpression of TRF2 increases TCT-dependent transcription in a dose-dependent manner, whereas overexpression of TBP has little or no effect. With TATA-dependent promoters, however, overexpression of TRF2 has a negative effect on TATA-dependent transcription, whereas overexpression of TBP increases TATA-dependent transcription. These findings further support the conclusion that TRF2 has a positive role in TCT-dependent transcription, whereas TBP does not. Moreover, both the cell-based and biochemical experiments show that TRF2 has either no effect or perhaps a slight negative effect on TATA-dependent transcription.

TRF2 is localized preferentially to TCT promoters relative to TATA promoters

We next sought to determine whether the genome-wide localization of TRF2 in the organism is consistent with its function in TCT-dependent transcription. We therefore performed parallel ChIP-seq (ChIP combined with deep sequencing) analyses of TRF2 and TBP in early *Drosophila* embryos. At a representative example of a TATA promoter and a TCT promoter with comparable levels of RNA Pol II occupancy, there is a distinct preference for the localization of TRF2 at the TCT core promoter (Fig. 4A). TBP, on the other hand, exhibits a strong peak at the TATA promoter and a weaker, less focused peak at the TCT promoter. This pattern is observed genome-wide, as shown in the heat maps of 171 TATA-containing promoters and 134 TCT-containing promoters (Fig. 4B). There is a sharp preference for TRF2 at TCT-containing promoters and a strong but less absolute preference for TBP at TATA-containing promoters. The analysis of the TRF2 and TBP occupancy at the 87 RP genes additionally revealed a peak of TRF2 near the +1 transcription start site as well as a weaker and broader peak of TBP over the region encompassing the core promoter (Fig. 4C). In all, the ChIP-seq data indicate a strong preference for the localization of TRF2 at TCT core promoters and TBP at TATA core promoters. These findings further reinforce the conclusion that TRF2, but not TBP, functions at TCT-containing promoters.

A specialized TRF2-based transcription system for TCT-dependent transcription

This study reveals that the transcription of TCT-dependent genes uses a TRF2-based transcription system that is distinct from the well-known TBP-based transcription systems. The existence of a specialized transcription system for the TCT-containing RP gene promoters suggests that this system, which functions in the synthesis of RPs, is the complement to the RNA Pol I and RNA Pol III systems, which synthesize ribosomal and transfer RNAs.

The TCT motif is known to be present in *Drosophila* (Parry et al. 2010), zebrafish (Nepal et al. 2013), mice (Perry 2005), and humans (Perry 2005; Parry et al. 2010), and TRF2 is generally present in metazoans (for example, see Reina and Hernandez 2007; Goodrich and Tjian 2010; Akhtar and Veenstra 2011). In contrast, neither TRF2 nor the TCT motif appears to be present in the yeast *Saccharomyces cerevisiae* (for example, see Reina and Hernandez 2007; Goodrich and Tjian 2010; Akhtar and Veenstra 2011; Bosio et al. 2011). It therefore seems likely that the TRF2–TCT system is widely used among metazoans. As might be expected for a protein that is important for RP gene expression, the loss of TRF2 is embryonic-lethal in *Caenorhabditis elegans* (Dantonel et al. 2000; Kaltenbach et al. 2000), *Drosophila* (Kopytova et al.

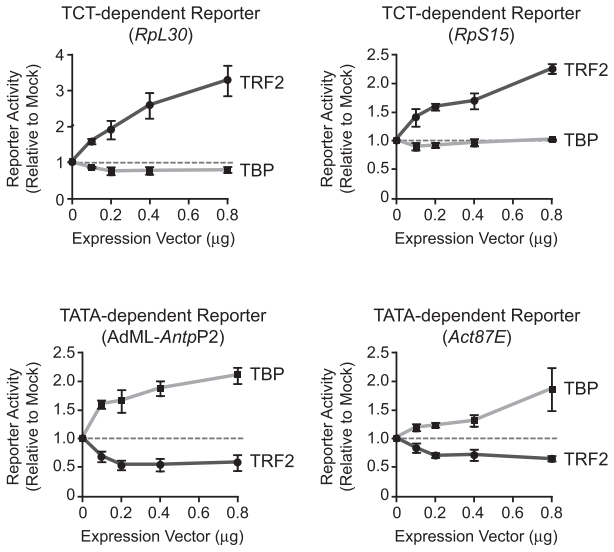

Figure 3. Overexpression of TRF2 increases TCT-dependent but not TATA-dependent transcription, whereas overexpression of TBP increases TATA-dependent but not TCT-dependent transcription. *Drosophila* S2 cells were transfected with a TCT-dependent or TATA-dependent reporter construct along with the indicated amounts of expression vector for either dTRF2S or dTBP. The AdML-*Antp*P2 promoter is identical to the TATA promoter that was used in Figure 1B. Luciferase reporter activities were normalized to those obtained with the empty vector alone. Error bars represent the standard deviation.

2006), *Xenopus* (Veenstra et al. 2000), and zebrafish (Müller et al. 2001). However, in mice, TRF2 is not essential, although it is required for spermiogenesis (Martianov et al. 2001; Zhang et al. 2011; Zhou et al. 2013). It is possible, for instance, that another related protein can compensate for the loss of TRF2 in mice.

Does TRF2 bind to DNA? The ChIP-seq data indicate a peak of TRF2 occupancy in the vicinity of the transcription start site (Fig. 4C) and are hence suggestive of a sequence-specific DNA-binding activity. Nevertheless, to date, sequence-specific DNA binding by TRF2 has not yet been seen. Moreover, with the purified TRF2 that is active for transcription in vitro (Fig. 2), we did not observe sequence-specific DNA binding under an extensive range of conditions with many different template DNAs and methodologies in the absence or presence of different combinations of purified TFIIA and TFIIB. Thus, TRF2 may not bind directly to DNA or, alternatively, may bind to DNA under specific conditions that have not yet been tested.

Last, it is important to note that TRF2 may function in different transcription systems, as such a precedent has been observed with the participation of TBP in RNA Pol I, II, and III transcription systems (for example, see Cormack and Struhl 1992; Hernandez 1993). Thus, the function of TRF2 is probably not restricted to TCT-containing promoters. In support of this hypothesis, it is known that TRF2 is important for the transcription of the histone H1 gene (Isogai et al. 2007), but the histone H1 promoter does not appear to contain a TCT motif. Moreover, TRF2 preferentially occupies the histone H1 gene promoter relative to the core histone gene promoters (Supplemental Fig. 9; Isogai et al. 2007). Ultimately, it is likely that we will find that there are many different transcriptional systems that involve TBP, TRF2, and other factors and that each of these networks serves a specific and important biological function.

Materials and methods

Depletion and overexpression assays in Drosophila *S2 cells*

For reporter assays involving RNAi depletion of TRF2 or TBP, *Drosophila* S2 cells were seeded at 0.2×10^6 cells per well in a 24-well plate, and then

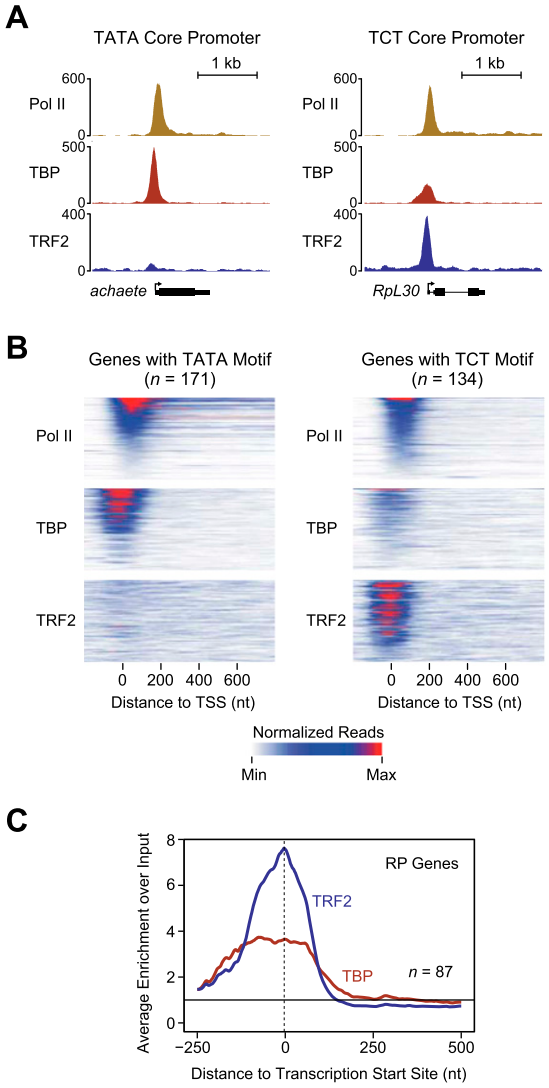

Figure 4. TRF2 is enriched at TCT-dependent promoters in vivo. The occupancy of TRF2 and TBP was analyzed by ChIP-seq experiments with *Drosophila* embryos collected from 2 to 4 h after egg deposition. (*A*) Differential TRF2 occupancy at a TATA promoter and a TCT promoter. Read counts across a representative TATA-containing promoter (*achaete*) and a representative TCT-containing promoter (*RpL30*) with comparable levels of RNA Pol II show that TRF2 is bound at higher levels to the TCT promoter relative to the TATA promoter. (*B*) Heat maps of the ChIP occupancy of Pol II, TRF2, and TBP at 171 genes with a predicted TATA box and at 134 genes with a predicted TCT motif. These two sets of genes were sorted in descending order of Pol II occupancy and are shown in a window from −200 to + 800 nt relative to the +1 transcription start site. The same genes and their order are shown for Pol II, TBP, and TRF2 occupancy. (*C*) TRF2 occupancy is highest near the transcription start site of RP genes. The graph depicts the average enrichments of the TRF2 and TBP ChIP-seq signals over input from −250 to +500 nt relative to the transcription start site for the 87 known RP genes. The dashed line indicates the peak TRF2 signal at −3 nt relative to the transcription start site.

5 μg of dsRNA was added to each well. After 2 d, cells were transfected with 400 ng of reporter plasmids containing the indicated core promoters and the firefly luciferase gene by using Effectene (Qiagen). The dsRNA sequences used to deplete dTBP were described previously (Hsu et al. 2008). The dsRNA sequences used to deplete dTRF2 correspond to positions 3272–3871 (TRF2-A) and 4361–4986 (TRF2-B) relative to the upstream initiating ATG of TRF2 (Kopytova et al. 2006). For overexpression assays, cells were seeded at 0.6×10^6 cells per well in a 24-well plate. After 24 h, cells were transfected with the indicated amounts of expression vector together with 100 ng of the reporter plasmids containing the indicated core promoters and the firefly luciferase gene by using Effectene (Qiagen). When necessary, the total mass of transfected vector was maintained at a constant level by the addition of the compensatory amount of empty vector (pAc5.1) to give a total of 0.8 μg of expression vector per transfection. For depletion and overexpression assays involving reporter genes, cells were harvested 24 h after transfection, and the lysates were assayed for luciferase activity by using Luciferase Assay Reagent II (Promega). The protein concentration of cell lysates was measured by using the Bradford assay (Bio-Rad). To ensure reproducibility of the data, each experimental condition was performed (in triplicate) a minimum of three times.

For the qRT–PCR analysis of RNAs, TRF2 and TBP were depleted in *Drosophila* S2 cells, as above. Total RNA was isolated by using TRIzol reagent (Life Technologies) and then subjected to reverse transcription with the iScript cDNA synthesis kit (Bio-Rad), as recommended by the manufacturers. The resulting cDNAs were analyzed by qPCR by using the Opticon 2 instrument (Bio-Rad). TRF1-dependent, RNA Pol III-synthesized 5S rRNA transcripts were used as a reference for normalization. Each experimental condition was performed independently at least two times in triplicate.

ChIP-seq

ChIPs were performed essentially as previously described (Chen et al. 2013). A detailed description is included in the Supplemental Material. TRF2 and TBP ChIP-seq samples were single-end-sequenced on an Illumina HiSeq 2500 at 51 base pairs (bp). All reads passing the standard Illumina quality filter were aligned to the University of California at Santa Cruz *Drosophila* dm3 reference genome using Bowtie version 1.0.0. Only reads with unique alignments and a maximum of two mismatches were kept. Reads were extended to 110 bp (the estimated insert size of both libraries as determined by a Bioanalyzer), and genome-wide per-base coverage was calculated using R/Bioconductor. The Pol II ChIP-seq data were previously published (Chen et al. 2013). These data were aligned in the same way, and reads were extended to 78 bp.

TATA and TCT gene heat map

Figure 4B used genes with a Pol II enrichment of at least threefold above input in a region from the +1 transcription start site to +100 nt. Predicted TATA-containing genes (171 genes) were selected by the presence of a match to the TATA consensus STATAWAWR (between −60 and the +1 start site). Predicted TCT-containing genes (134 genes) were identified by the existence of a match to the TCT consensus of YYCTTTYY (between −10 and +20 relative to the +1 start site). Pol II, TBP, and TRF2 ChIP-seq signals were plotted (one row per gene) by aligning the genes at the transcription start site in a 5′ (left) to 3′ (right) orientation. The genes were sorted by decreasing total Pol II occupancy in the first 100 nt. The scales for the three factors were independently normalized such that 0 represents no signal and 1 is the signal value at the 99th percentile for the 305 genes plotted.

Average gene analysis

For Figure 4C, the 87 known *Drosophila* RP genes from the Ribosomal Protein Gene Database (Nakao et al. 2004) were matched to their corresponding FlyBase release 5.51 genes and aligned at their annotated transcription start sites. The average enrichment for TRF2 and TBP over a previously published *Drosophila* 2- to 4-h after egg deposition whole-cell extract sample (He et al. 2011) was calculated for each base after normalizing for differences in read count and fragment size. The results were smoothened by using a 9-bp sliding window.

Accession number

TBP and TRF2 ChIP-seq data are available from Gene Expression Omnibus (GEO) under the accession number GSE52029. In addition, a list of the ChIP-seq signals of TBP and TRF2 at each annotated transcript is provided in Supplemental Table 1.

Additional Materials and Methods are included in the Supplemental Material.

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SUPPLEMENTAL MATERIAL

TRF2, and not TBP, Mediates the Transcription of Ribosomal Protein Genes

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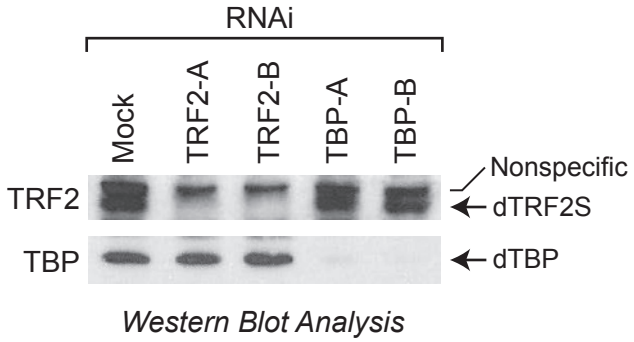
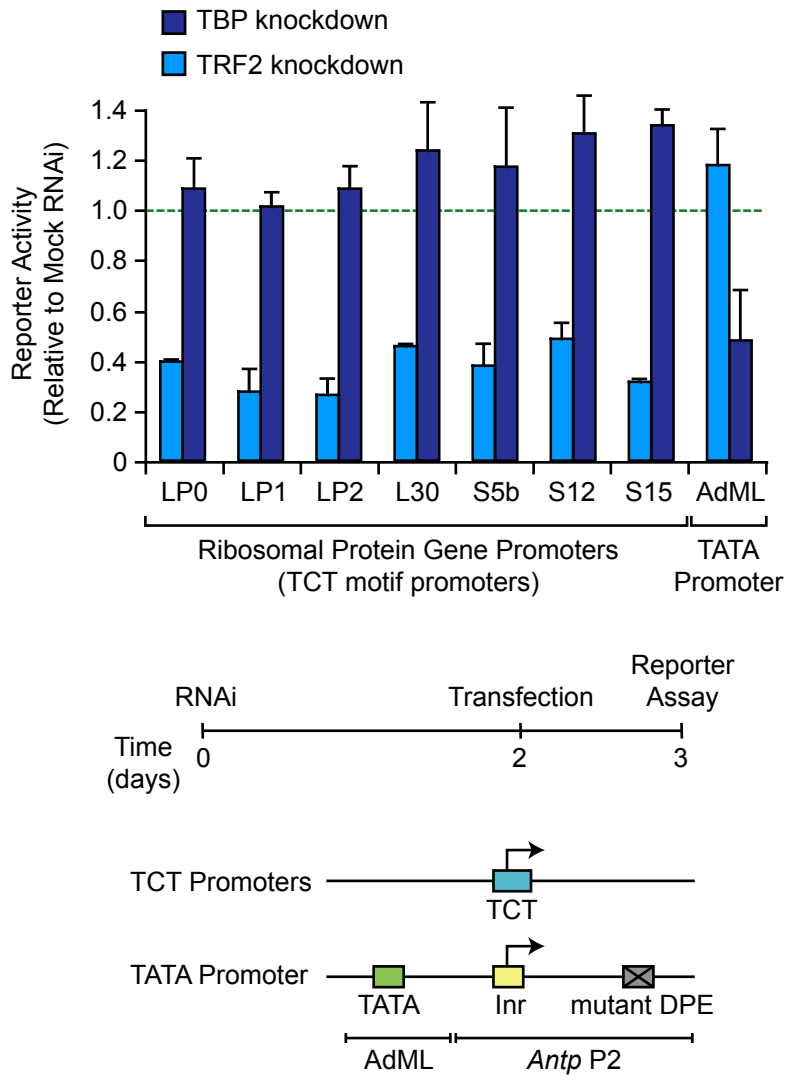
George A. Kassavetis, Julia Zeitlinger, and James T. Kadonaga

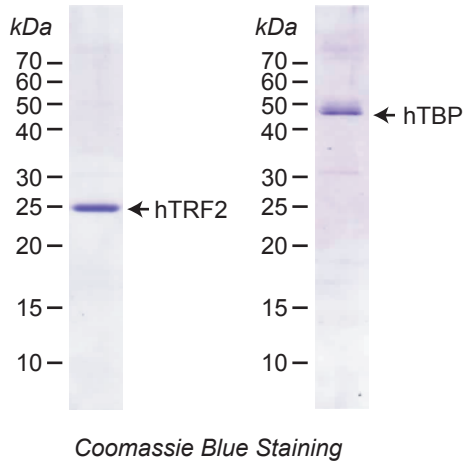
Contains Supplemental Figures 1-9 as well as Supplemental Materials and Methods.
Supplemental Table 1 is in a separate file.



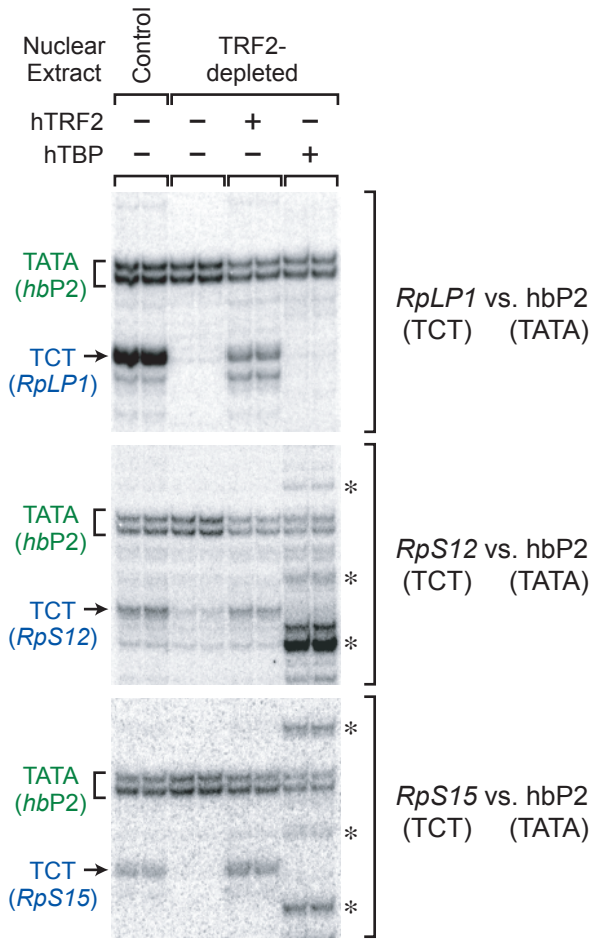
Supplemental Figure S1. RNAi depletion of TRF2 and TBP in *Drosophila* S2 cells. Cells were treated for three days with two non-overlapping dsRNAs that target TRF2 (TRF2-A and TRF2-B) or TBP (TBP-A and TBP-B). The protein levels of dTRF2S and dTBP were assessed by western blot analysis. In these experiments, we were able to detect dTRF2S but not dTRF2L, even though the antibodies were raised against a polypeptide that is shared by both proteins. This effect may be due to inefficient transfer of the dTRF2L protein to the blot, the lack of recognition of dTRF2L by the antibodies, or the absence of dTRF2L in the extract used for the western blot.



Supplemental Figure S2. Two independent dsRNAs for TRF2 and TBP yield nearly identical effects upon TCT-dependent versus TATA-dependent reporter expression. The experimental scheme and reporter constructs are identical to those in Fig. 1 of the main text and are depicted at the bottom of the figure. In this figure, the TRF2-A and TBP-A dsRNAs were used, whereas in Fig. 1 of the main text, the TRF2-B and TBP-B dsRNAs were employed. The activities of the RNAi-depleted extracts are reported as relative to the activities of mock RNAi-treated control extracts. Error bars represent the standard deviation.

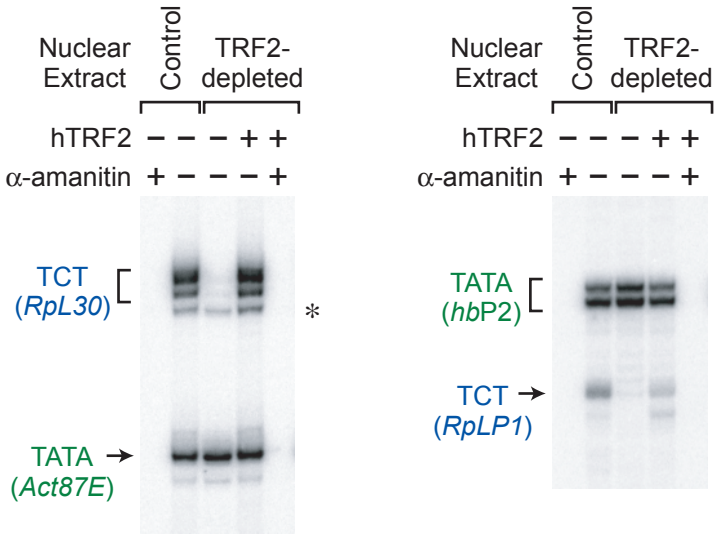


Supplemental Figure S3. Purification of hTRF2 and hTBP. N-terminally His-tagged, full-length, wild-type versions of human TRF2 (hTRF2) and human TBP (hTBP) were synthesized by using an in vitro transcription and translation system (CellFree Sciences, Co., Ltd.; Ehime, Japan) and purified by Ni(II) affinity chromatography. The resulting proteins were subjected to 10% polyacrylamide-SDS gel electrophoresis and visualized by staining with Coomassie Brilliant Blue R-250. The positions of molecular mass markers are indicated.

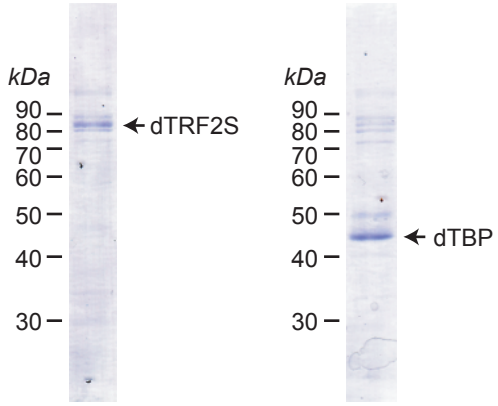


Two-template in vitro transcription assays with TCT- and TATA-dependent core promoters

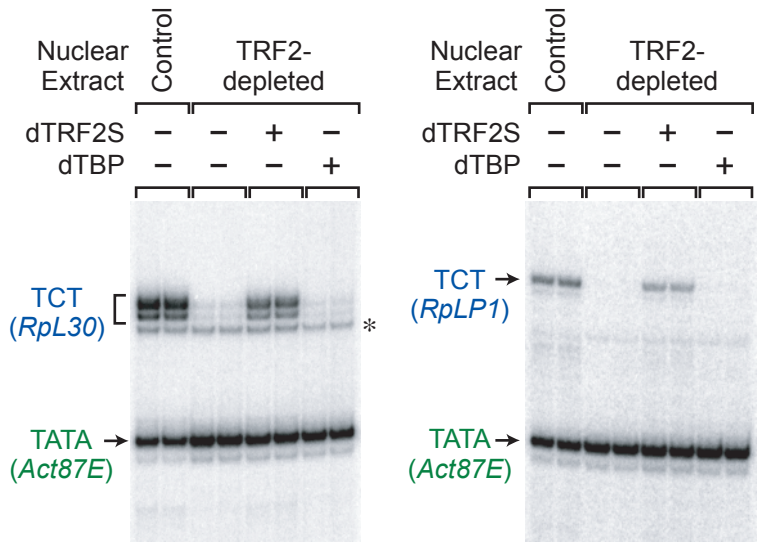
Supplemental Figure S4. Purified TRF2, but not purified TBP, is able to restore the specific loss of TCT-dependent transcription that occurs upon depletion of TRF2 from a nuclear extract. Two-template in vitro transcription assays were performed with TCT- and TATA-dependent promoters, as in Fig. 2 of the main text. In these experiments, *RpLP1*, *RpS12*, and *RpS15* were used as TCT-dependent promoters, and *hb*P2 was used as a TATA-dependent promoter. Reactions were carried out with either TRF2-depleted or control nuclear extracts. Where indicated, purified hTRF2 or hTBP was added to reactions with the TRF2-depleted extracts. The resulting transcripts were detected by primer extension-reverse transcription analysis. The asterisks denote non-specific transcripts that are observed in the presence of hTBP.



Supplemental Figure S5. TRF2-mediated transcription of TCT-dependent promoters is carried out by RNA polymerase II. Transcription reactions were performed as in Fig. 2 of the main text. Where indicated, α-amanitin was included in the reactions at a final concentration of 4 µg/mL. The asterisk denotes a non-specific transcript.

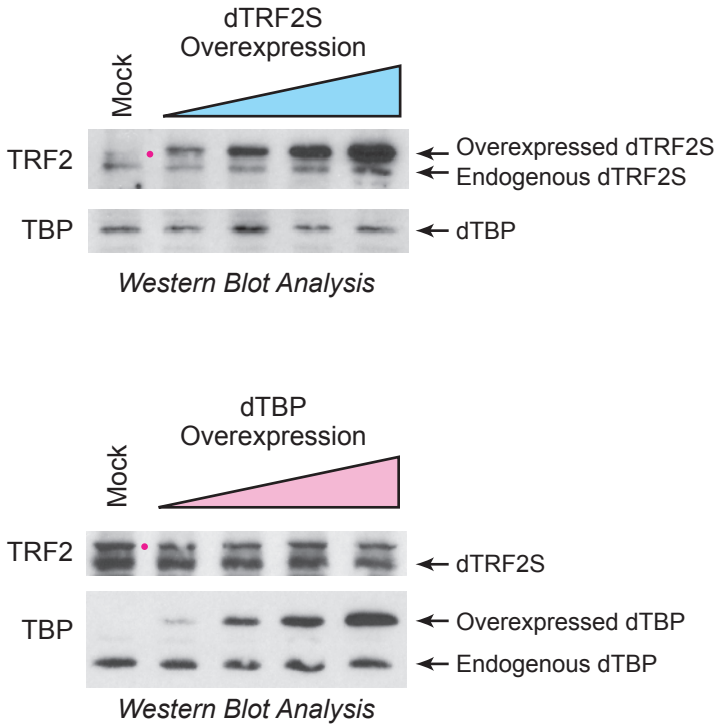


Supplemental Figure S6. Purification of dTRF2S and dTBP. N-terminally His-tagged, full-length, wild-type versions of *Drosophila* dTRF2S and dTBP were synthesized by using an in vitro transcription and translation system (CellFree Sciences, Co., Ltd.; Ehime, Japan) and partially purified by Ni(II) affinity chromatography. The resulting proteins were subjected to 10% polyacrylamide-SDS gel electrophoresis and visualized by staining with Coomassie Brilliant Blue R-250. The dTRF2S was estimated to be approximately 25% pure, and the dTBP was estimated to be approximately 50% pure. The positions of the molecular mass markers are indicated.

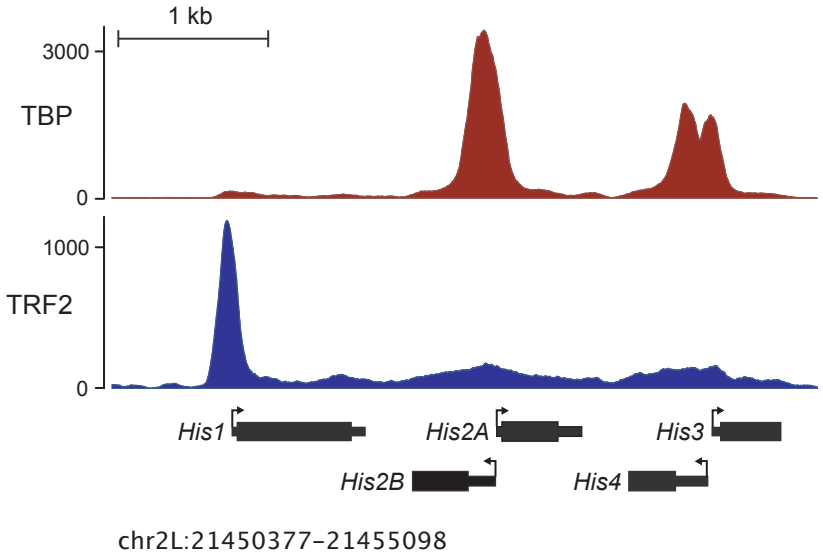


Two-template in vitro transcription assays with TCT- and TATA-dependent core promoters

Supplemental Figure S7. *Drosophila* dTRF2S protein, but not dTBP protein, is able to restore the specific loss of TCT-dependent transcription that occurs upon depletion of TRF2 from a nuclear extract. Two-template in vitro transcription assays were performed with TCT- and TATA-dependent promoters, as in Fig. 2 of the main text. Reactions were carried out with either TRF2-depleted or control nuclear extracts. Where indicated, dTRF2S (10 nM final concentration) or dTBP (10 nM final concentration) was added to reactions with the TRF2-depleted extracts. The resulting transcripts were detected by primer extension-reverse transcription analysis. The asterisk denotes a non-specific transcript.



Supplemental Figure S8. Overexpression of dTRF2 and dTBP in *Drosophila* S2 cells. Cells were transfected with increasing amounts of expression vectors for dTRF2 or dTBP under conditions that are identical to those used in the experiments shown in Fig. 3 of the main text. The levels of overexpressed proteins were detected by western blot analysis with antibodies against dTRF2 and dTBP. The apparent molecular masses of the overexpressed proteins are slightly larger than those of their endogenous counterparts due to the presence of FLAG and HA tags on the overexpressed proteins. The pink dots in the TRF2 western blots of the Mock samples denote an unknown protein that is recognized by antibodies in the dTRF2 antisera. In these experiments, we were able to detect dTRF2S but not dTRF2L, even though the antibodies were raised against a polypeptide that is shared by both proteins. This effect may be due to inefficient transfer of the dTRF2L protein to the blot, the lack of recognition of dTRF2L by the antibodies, or the absence of dTRF2L in the extract used for the western blot.



Supplemental Figure S9. The occupancy of TRF2 and TBP at a histone gene cluster in *Drosophila*. The ChIP-seq analysis is described in Fig. 4 of the main text. The read counts show that TRF2 is localized preferentially to the histone H1 promoter relative to the core histone promoters. In contrast, TBP preferentially occupies the core histone promoters relative to the histone H1 promoter. Because there are multiple repeated copies of the histone gene cluster in the *Drosophila* genome, this figure includes ChIP-seq reads with multiple alignment locations in addition to the uniquely aligning reads.

Materials and methods

Cell culture

Drosophila Schneider S2 cells were cultured at 25°C in Shields & Sang M3 media (Sigma; St. Louis, MO) prepared with yeast extract (Sigma; St. Louis, MO) and Bacto Peptone (BD Biosciences; San Jose, CA) supplemented with 10% heat-inactivated FBS.

Plasmids

Plasmid templates for in vitro transcription experiments were constructed by the insertion of double-stranded oligonucleotides containing core promoter sequences into the PstI and XbaI sites of pUC119. In Fig. 2B and Supplemental Fig. S7, templates containing the following promoters were used: *Act87E* (sequences from −125 to +50 relative to A+1 in the Inr), *RpL30* (sequences from −250 to +100 relative to C+1 in the TCT motif), and RpLP1 (sequences from −250 to +100 relative to C+1 in the TCT motif). In Supplemental Fig. S4, templates with the following promoters were used: *hb*P2 (sequences from −50 to +50 relative to the A+1 in the Inr along with a downstream 24 nt spacer), *RpLP1*, *RpS12*, and *RpS15* (all ribosomal protein gene promoters contained sequences from −50 to +50 relative to the C+1 in the TCT motif). In Supplemental Fig. S5, the templates contained the following promoters: *Act87E* (same as in Fig. 2B), *RpL30* (same as in Fig. 2B), *hb*P2 (same as in Supplemental Fig. S4), and *RpLP1* (same as in Supplemental Fig. S4).

The expression vectors for the in vitro synthesis of TRF2 and TBP were constructed by insertion of their coding sequences into the EcoRV and SpeI sites of pEU-E01-His-TEV-MCS-N2 (CellFree Sciences; Ehime, Japan). The plasmids used as reporter genes in the transfection assays were constructed by the insertion of ribosomal protein gene core promoters (sequences from −50 to +50 relative to C+1 in the TCT motif) or Act87E (sequences from −125 to +50 relative to A+1 in the Inr) into the pGL3-Basic vector (Promega; Madison, WI). The TATA-dependent reporter AdML-AntpP2 and the expression vector for *Drosophila* TBP (pAc5.1-FH-

TBP) were described previously (Hsu et al. 2008). The expression vector for *Drosophila* TRF2 (pAc5.1-FH-TRF2S) was a gift from Dr. Tamar Juven-Gershon (Bar-Ilan University; Israel). The sequences of all subcloned DNA fragments were verified by DNA sequencing analysis.

Antibodies

Antibodies against *Drosophila* TRF2 were generated by immunizing rabbits with a C-terminal polypeptide that corresponds to amino acids 98 to 523 of dTRF2S. The antibodies against *Drosophila* TFIIB, TBP, and TFIIA-L were generated by immunization of rabbits with purified, full-length, recombinant proteins (Kadonaga laboratory, unpublished).

Immunodepletion and in vitro transcription assays

Drosophila embryo nuclear extracts were prepared as previously described (Soeller et al. 1998). To deplete TRF2 from the extracts, TRF2 antiserum (60 μL) was incubated with 30 μL of nProtein A Sepharose 4 Fast Flow (GE Healthcare; Piscataway, NJ; cat. no. 17-5280-01) in 700 μL of binding buffer [250 mM NaCl, 50 mM HEPES-K^+, pH 7.6, 0.2 mM EDTA, 0.1% (v/v) NP-40, 1.4 mM 2-mercaptoethanol, 0.2 mM PMSF] and rotated for 3 h at 4°C. The unbound antibodies were washed off sequentially with 3 x 1 mL of binding buffer and 3 x 1 mL of 0.1 M HEMGN+D [100 mM KCl, 25 mM HEPES-K^+, pH 7.6, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 0.01% (v/v) NP-40, 10% (v/v) glycerol, 1 mM dithiothreitol]. The antibody-bound protein A resin (10 μL) was then applied to the nuclear extract (200 μL) and rotated for 2 h at 4°C. After spinning down the resin, the supernatant was transferred to a new tube for two more rounds of depletion. Proteins in the control (undepleted) and depleted nuclear extracts were monitored by western blot analysis. In vitro transcription assays were carried out essentially as described (Wampler et al. 1990) with control or TRF2-depleted nuclear extracts in the absence or presence of 50 nM human TRF2 or human TBP. The resulting transcripts were subjected to primer extension analysis with the M13 reverse sequencing primer (5'-AGCGGATAACAATTTCACACAGGA). Quantitation of the reverse transcription products was carried out with a Typhoon

phosphorimager (GE Health Sciences). To ensure reproducibility of the data, all experiments were performed a minimum of three independent times.

Protein purification

Preparative scale in vitro transcription-translation reactions were performed with the wheat germ WEPRO7240H expression kit, as specified by the manufacturer (CellFree Sciences; Ehime, Japan), and the reaction products were adjusted to 500 mM NaCl and 15% (v/v) glycerol for storage at –80°C. Upon thawing, the following reagents were added to the samples (~14.5 mL) to the indicated final concentrations: imidazole (20 mM, pH 7), NP-40 (0.01%, v/v), 2-mercaptoethanol (10 mM), PMSF (0.5 mM), pepstatin (1 μg/mL), and leupeptin (1 μg/mL). The mixtures were clarified by centrifugation at 24,000 x *g* for 10 min at 4°C and then loaded onto 0.5 mL Ni-NTA Sepharose columns that were pre-equilibrated in buffer N [30 mM HEPES-Na^+, pH 7.8, 500 mM NaCl, 20 mM imidazole, 0.01% (v/v) NP-40, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol, 0.5 mM PMSF, 1 μg/ml pepstatin, and 1 μg/mL leupeptin]. The columns were washed with 8 mL buffer W [30 mM HEPES-Na^+, pH 7.8, 1 M NaCl, 20 mM imidazole, 0.01% (v/v) NP-40, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol, 0.5mM PMSF, 1 μg/mL pepstatin, and 1 μg/mL leupeptin], 2 ml buffer N, and then sequentially eluted with 0.35 mL, 1.0 mL, 0.6 mL and 0.4 mL buffer E [30 mM HEPES-Na^+, pH 7.8, 500 mM NaCl, 200 mM imidazole, 0.01% (v/v) NP-40, 20% (v/v) glycerol, 10 mM 2-mercaptoethanol, 0.5 mM PMSF, 1 μg/mL pepstatin, and 1 μg/mL leupeptin]. The resulting samples were frozen in liquid nitrogen and stored at –80°C.

ChIP-seq

Chromatin immunoprecipitations (ChIPs) were performed essentially as previously described (Chen et al. 2013). *Drosophila* embryos (800 mg) collected from 2 to 4 h after egg deposition were crosslinked with 1.8% (v/v) formaldehyde in 2.4 mL crosslinking buffer with 7.5 mL heptane for 15 min. After washing two times with PBT-0.125 M glycine, embryos were

transferred to a 7 mL Dounce homogenizer with 5 mL Buffer A1 [15 mM HEPES-K^+, pH 8, 15 mM NaCl, 60 mM KCl, 4 mM $MgCl_2$, 0.5% (v/v) Triton X-100, 0.5 mM dithiothreitol, fresh 1 × Protease Inhibitor Cocktail], and homogenized for five times each with the loose fitting and the tight fitting pestles. Homogenized samples were centrifuged at 1500 x *g* for 3 min at 4°C. The pellet was subsequently washed three times with Buffer A1 and one time with Buffer A2 [15 mM HEPES-K^+, pH 8, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate, 1% (w/v) SDS, 0.5% (w/v) N-lauroylsarcosine, fresh 1 × Protease Inhibitor Cocktail]. After the wash, the pellet was suspended in 2.8 mL of Buffer A2 and aliquoted into four microcentrifuge tubes. The chromatin was then sheared with a Branson sonicator for 8 x 10 sec at Power 3 and subjected to centrifugation at 13,000 x *g* for 10 min at 4°C. The supernatant was the input for ChIP. For beads preparation, 130 μL of Rabbit IgG Dynabeads (Life Technologies; Grand Island, NY) were aliquoted into 4 tubes and washed two times with PBS-0.5% BSA. Beads were incubated with antibodies (10 μL for TBP and 30 μL for TRF2) in 700 μL PBS-0.5%BSA for 2 h at 4°C. After spinning down the beads, 700 μL of sheared chromatin was added to each tube, and the samples were incubated overnight at 4°C. The samples were washed three times with RIPA buffer and eluted with 200 μL elution buffer + 200 μL TE. The eluted chromatin was incubated for 30 min at 37°C with 60 μg RNase and then for 6 h at 65°C to reverse the crosslinks in the presence of 60 μg proteinase K. The DNA was further purified by phenol and phenol-chloroform-isoamyl alcohol extractions and ethanol precipitation. Libraries for the ChIPed DNA were prepared and barcoded with NEBNext DNA Library Prep Master Mix Set for Illumina and NEBNext Multiplex Oligos for Illumina (New England Biolabs; Ipswich, MA). For each step, DNA was cleaned with AMPure XP beads (New England Biolabs; Ipswich, MA) as recommended by the manufacturer.

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