

# Transcription of histone gene cluster by differential core-promoter factors

Yoh Isogai,<sup>1</sup> Sündüz Keles,<sup>2</sup> Matthias Prestel,<sup>3</sup> Andreas Hochheimer,<sup>3</sup> and Robert Tjian<sup>1,4,5,6</sup>

<sup>1</sup>Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, California 94720, USA;

<sup>2</sup>Department of Statistics, Department of Biostatistics, and Department of Medical Informatics, University of Wisconsin at Madison, Madison, Wisconsin 53706, USA; <sup>3</sup>Adolf-Butenandt-Institut, Molekularbiologie, 80336 Munich, Germany;

<sup>4</sup>Howard Hughes Medical Institute, University of California at Berkeley, Berkeley, California 94720, USA; <sup>5</sup>Li Ka-Shing Center for Biomedical and Health Sciences, University of California at Berkeley, Berkeley, California 94720, USA

The 100 copies of tandemly arrayed *Drosophila* linker (H1) and core (H2A/B and H3/H4) histone gene cluster are coordinately regulated during the cell cycle. However, the molecular mechanisms that must allow differential transcription of linker versus core histones prevalent during development remain elusive. Here, we used fluorescence imaging, biochemistry, and genetics to show that TBP (TATA-box-binding protein)-related factor 2 (TRF2) selectively regulates the TATA-less *Histone H1* gene promoter, while TBP/TFIID targets core histone transcription. Importantly, TRF2-depleted polytene chromosomes display severe chromosomal structural defects. This selective usage of TRF2 and TBP provides a novel mechanism to differentially direct transcription within the histone cluster. Moreover, genome-wide chromatin immunoprecipitation (ChIP)-on-chip analyses coupled with RNA interference (RNAi)-mediated functional studies revealed that TRF2 targets several classes of TATA-less promoters of >1000 genes including those driving transcription of essential chromatin organization and protein synthesis genes. Our studies establish that TRF2 promoter recognition complexes play a significantly more central role in governing metazoan transcription than previously appreciated.

[**Keywords:** TATA-box-binding protein (TBP); TBP-related factor 2 (TRF2); core-promoter recognition; histone gene cluster; ChIP-on-chip; chromatin organization]

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Core promoters serve as the platform for the assembly of transcription initiation complexes critical for specifying accurate and regulated RNA synthesis. The eukaryotic cellular RNA polymerase II (Pol II) machinery has evolved to recognize multiple core-promoter elements such as the TATA box, Initiator, and DPE (Smale and Kadonaga 2003). Indeed, studies of metazoan core promoters revealed considerably greater *cis*-element diversification than previously expected. For example, TATA boxes, which were thought to be the most widely distributed prototypic core-promoter element recognized by the general transcription factor TBP (TATA-box-binding protein)/TFIID (consisting of TBP and TBP-associated factors, TAFs), are found in <20%–30% of annotated promoters in *Drosophila* and human. Instead, the majority of core promoters fall into various distinct TATA-less categories (Ohler et al. 2002; Gershenson and Ioshikhes 2005; Jin et al. 2006). Consistent with diversified core-

promoter structures, recent studies identified a family of TBP-related factors (TRFs) (Crowley et al. 1993; Dantonel et al. 1999; Maldonado 1999; Ohbayashi et al. 1999; Rabenstein et al. 1999; Berk 2000; Tupler et al. 2001; Hochheimer and Tjian 2003), but their potential core-promoter recognition functions have remained elusive.

Metazoan cells have been found to use a diversified set of TBP-related molecules that display altered DNA-binding specificities (Hochheimer and Tjian 2003). In *Drosophila*, TRFs have been implicated in promoter-selective transcription for both Pol II and Pol III gene promoters (Hansen et al. 1997; Holmes and Tjian 2000; Takada et al. 2000; Hochheimer et al. 2002; Isogai et al. 2007). However, a comprehensive analysis of TRFs in promoter-selective recognition of Pol II core promoters has not been performed. Our earlier studies found that a multisubunit TRF2-containing complex includes the transcription factor DREF and is involved in targeting a subset of promoters containing the DNA replication-related element (DRE) (Hochheimer et al. 2002). The *PCNA* gene promoter contains such a DRE (Hirose et al. 1993) and represents a novel tandem core-promoter class composed of two distinct transcriptional start sites, each

<sup>6</sup>Corresponding author.

E-MAIL [jmlim@berkeley.edu](mailto:jmlim@berkeley.edu); FAX (510) 643-9547.

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of which appears to be subject to regulation either by the TRF2/DREF complex or TBP/TAFs. While TRF2 recruitment to the core promoter via DREF may account for a subset of TRF2-dependent promoters, TRF2 is also found in complexes lacking DREF. For example, TRF2 and DREF display only a limited set of overlapping sites in *Drosophila* Schneider cells visualized by immunofluorescence staining (Y. Isogai and R. Tjian, unpubl.), suggesting that TRF2 may be playing multiple roles—some in conjunction with DREF and others independent of DREF. We therefore surmised that there may be additional important TRF2 target promoters that remained uncharacterized.

In order to gain a more comprehensive map of potential TRF2-dependent promoters, we conducted a genome-wide analysis of TRF2 recognition sites both by polytene chromosome staining as well as chromatin immunoprecipitation (ChIP) coupled with high-density tiling microarray detection (ChIP-on-chip). These approaches have revealed several important target genes that illustrate how TRF2 is used as an alternative core-promoter recognition factor. First, we provide biochemical and genetic evidence that two distinct sets of core-promoter recognition factors are responsible for directing transcription of the nucleosome core histone genes (*H2A/B* and *H3/H4*) and the linker histone *H1*. Genome-wide ChIP-on-chip analysis revealed that TRF2 recognizes and binds *in vivo* to a large number of TATA-less core promoters. Importantly, a majority of these TATA-less promoters are selectively recognized by TRF2, but not by TBP. Moreover, with salivary gland-specific depletion of TRF2, we found that TRF2 participates in regulation of chromatin organization and cell growth, by controlling *Histone H1* and ribosomal protein gene expression. Taken together, these data establish that TRF2 is responsible for differentially recognizing and regulating a subset of TATA-less promoters that have shed the requirement for TBP through the usage of novel core-promoter structures. Remarkably, even coordinately expressed gene clusters such as the histone complex have evolved mechanisms to be differentially regulated by alternative core-promoter recognition machinery.

## Results

### *Linking histone gene cluster to TRF2*

In an attempt to obtain an initial, relatively low-resolution picture of how TRF2 might be directly involved in promoter-selective transcription *in vivo*, we used polytene chromosome staining to examine both TBP and TRF2 occupancy on salivary gland DNA. We found that TRF2 localization is largely nonoverlapping relative to the more uniform localization of TBP, with one prominent exception at a locus proximal to the chromocenter that stained intensely with both TBP- and TRF2-specific antibodies (Fig. 1A). Interestingly, this genomic region was not stained by anti-DREF antibody, suggesting that DREF is not recruited to this locus (data not shown). Because of the particularly intense antibody staining as well as the cytological location, we reasoned that the

observed signal may correspond to the histone genes, a gene cluster that is amplified to ~100 copies per haploid genome (Lifton et al. 1978; Matsuo and Yamazaki 1989). Therefore, we used fluorescence *in situ* hybridization (FISH) to confirm the histone gene cluster locus and simultaneously determined if both TBP and TRF2 colocalize to the histone gene cluster by immunostaining. The unusually bright staining by anti-TBP and TRF2 antibodies indeed were found to overlap at the histone gene cluster DNA (Fig. 1B), establishing that both TBP and TRF2 are resident at this locus.

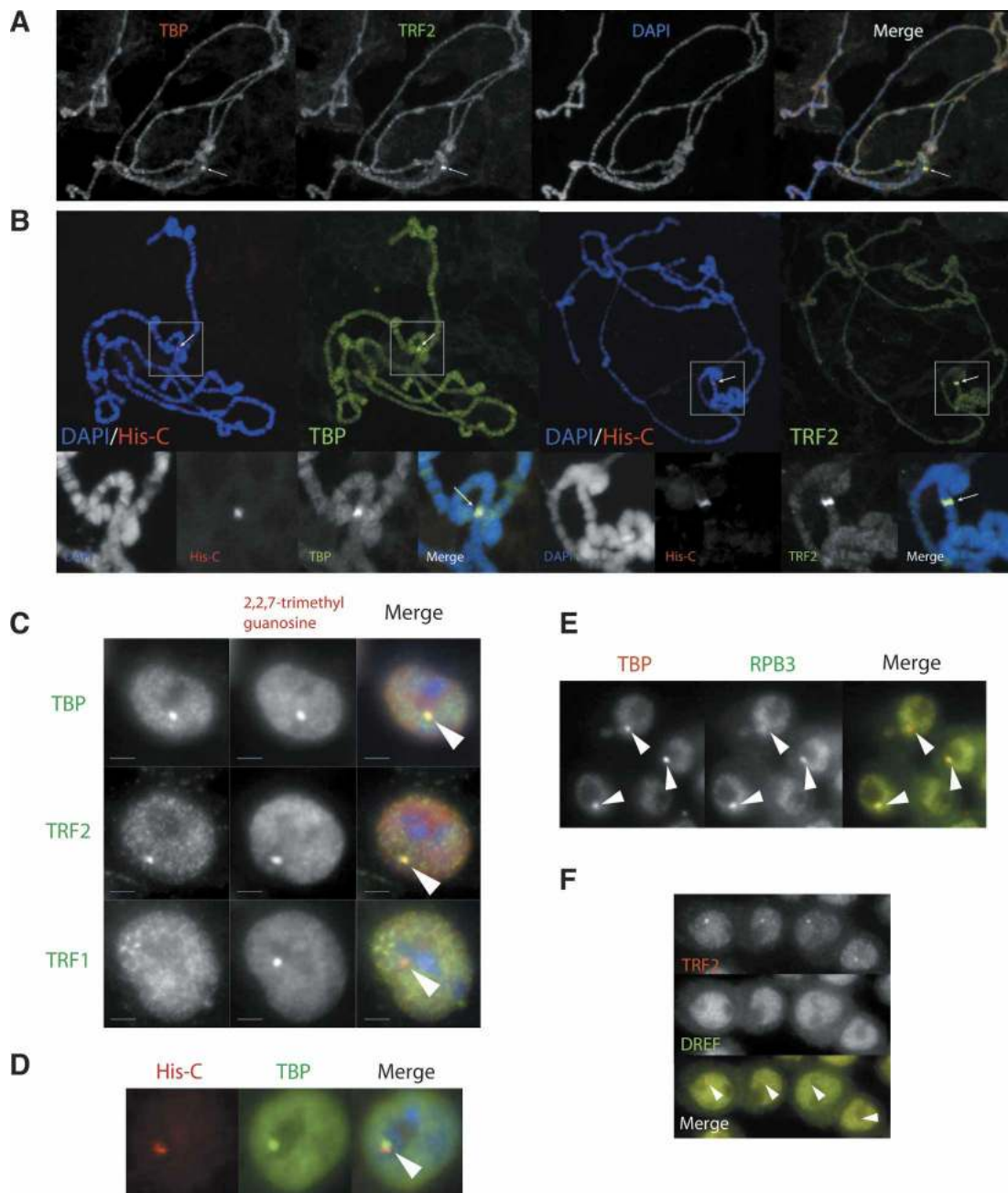
### *TBP and TRF2 colocalize at the histone locus in multiple cell types*

To further probe TBP and TRF2 localization in other cell types, we examined TBP/TRF2 localization in the interphase nuclei of both S2 and Kc cells, which are derived from *Drosophila* embryos. Interestingly, while a majority of TBP and TRF2 proteins appeared distributed uniformly in a punctate pattern throughout the nucleoplasm of these interphase cells, both TBP and TRF2 can be clearly seen to colocalize at a specific nuclear spot in the vicinity of the nucleolus (Fig. 1C). This nuclear body appears to accumulate a specific subset of the transcription machinery including Pol II (Fig. 1E), but not TRF1 or DREF (Fig. 1C,F). Since the histone gene cluster has recently been physically linked to the histone locus body (HLB) (Liu et al. 2006), an antibody against 2,2,7-trimethylguanosine (TMG), which marks small nuclear RNAs that accumulate in the HLB (Liu et al. 2006), was used to confirm that the site of TBP and TRF2 accumulation corresponds to the HLB. Indeed, intense TMG accumulation is found coincident with the TBP/TRF2 localization (Fig. 1C). Using DNA FISH in combination with immunostaining of TBP, we found that this specific nuclear body is invariably juxtaposed to the histone gene locus (Fig. 1D). Therefore, both TBP and TRF2 in *Drosophila* cells appear to be specifically targeted to the HLB, a nuclear body that is tightly linked to histone gene expression.

### *The Histone H1 promoter is selectively recognized by TRF2*

The apparent colocalization of TBP and TRF2 at the histone cluster at this relatively low resolution suggests that histone genes may represent another member of the tandem core-promoter class recognized by both TBP and TRF2. To test this hypothesis more directly, we examined the potential involvement of TBP and TRF2 at the histone locus at higher resolution by probing the individual core-promoter regions of *H1*, *H2A/B*, and *H3/H4* genes (Fig. 2A). Using a ChIP assay with extracts derived from S-phase-synchronized *Drosophila* Kc cells (Fig. 2B), we found that TBP and TRF2 are recruited to the histone gene cluster in a segregated fashion. Remarkably, TBP selectively occupies *H2A/B* and *H3/H4* core-promoter regions, but not *H1*, while TRF2 was found to occupy only the *H1* promoter, and not *H2A/B* and *H3/H4* (Fig. 2C). RNA Pol II is recruited to all five classes of histone

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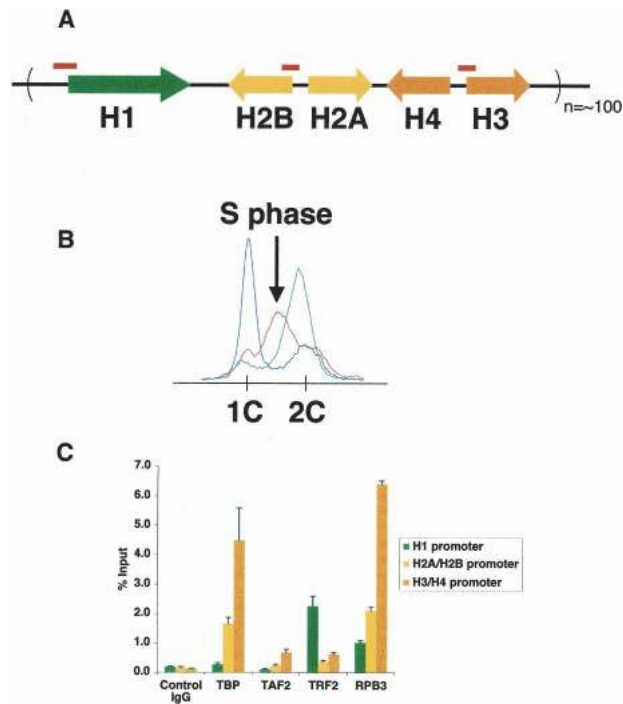


**Figure 1.** Localization of TBP and TRF2 at the histone gene cluster and the HLB. (A) Polytene chromosomal staining of TRF2 (green) and TBP (red). DNA (blue) is stained by DAPI. Arrows mark the cytological locus stained intensely both by anti-TRF2 and anti-TBP antibodies. (B) Colocalization of TBP and TRF2 at the histone cluster. Polytene immunostaining combined with DNA FISH probing the histone gene cluster. (Left) TBP immunostaining (green) combined with histone gene cluster (His-C) DNA FISH (red). (Right) TRF2 immunostaining (green) combined with histone gene cluster DNA FISH (red). DNA (blue) was stained with DAPI. The magnified view for the histone cluster is displayed below. The arrows mark the site of the histone gene cluster indicated by the FISH signals. (C) Colocalization of TBP and TRF2 at the HLB. *Drosophila* S2 cells doubly stained with anti-TMG antibodies (red), a marker for the HLB, and anti-TBP-, TRF1-, or TRF2-specific antibodies (green). (D) Intranuclear clustering of TBP and the histone gene cluster. S2 cells stained with anti-TBP antibody (green) coupled with DNA FISH marking the histone gene cluster (red), indicated by the arrow. (E) Pol II localization at the HLB. *Drosophila* Kc cells are stained with anti-TBP (red) and anti-RPB3, a Pol II subunit (green), antibodies. Arrows indicate the HLB marked by the TBP staining. (F) TRF2 accumulates at the HLB independent of DREF. *Drosophila* S2 cells are stained with anti-DREF (green) and anti-TRF2 (red) antibodies.

gene promoters, consistent with the S-phase-specific transcription of the histone genes. As expected, TAF2, a subunit of the TFIID complex, does not occupy the His-

tone *H1* gene promoter but is detectable at core histone promoters. TFIIA, which is known to bind directly to both TBP and TRF2 (Rabenstein et al. 1999), was also





**Figure 2.** Selective recruitment of TRF2/TBP in the histone gene cassette. (A) Schematics of the histone gene cassette. Positions of primer sets used to amplify the H1, H2A/B, and H3/H4 promoters are indicated by red bars. (B) Fluorescence-activated cell sorting (FACS) profile of synchronized *Drosophila* Kc cells. S-phase cells (purple) used for C are indicated by the arrow. G1 cells and G2 cells are indicated by blue and green, respectively. (C) Segregation of TBP and TRF2 recognition sites within the histone cluster. ChIP assays using S-phase-synchronized Kc cells. The graph represents the occupancy of TBP, TAF2, TRF2, and RPB3 at H1 (green), H2A/B (yellow), and H3/H4 (orange) core-promoter regions.

recruited to all five classes of histone promoters (data not shown). These striking findings suggest that even within a gene cluster, individual members may be regulated by distinct core promoter recognition machinery.

Since histone genes are generally expressed in an S-phase-dependent manner, we wondered whether the recruitment of TBP and TRF2 may correlate with different cell cycle phases. Therefore, we conducted ChIP experiments examining the status of TBP, TRF2, and Pol II occupancy at the H1 and H3/H4 promoters in G2 and S phases (Supplementary Fig. S2). Interestingly, while Pol II recruitment at the histone genes appears to be S-phase-dependent and a slight enrichment was observed for TBP at the H3/H4 promoters, TRF2 recruitment at the H1 promoter remained robust and invariant during all phases of the cell cycle. *Drosophila* cells can apparently use different strategies for transcriptional activation wherein recruitment of a core-promoter component such as TRF2 to the H1 promoter is not the rate-limiting step. This is in contrast to the more conventional situation observed at the heavy metal-inducible *MtnA* promoter, in which recruitment of TFIID provides a key step for activation (Marr et al. 2006).

### TRF2 is required for Histone H1 transcription

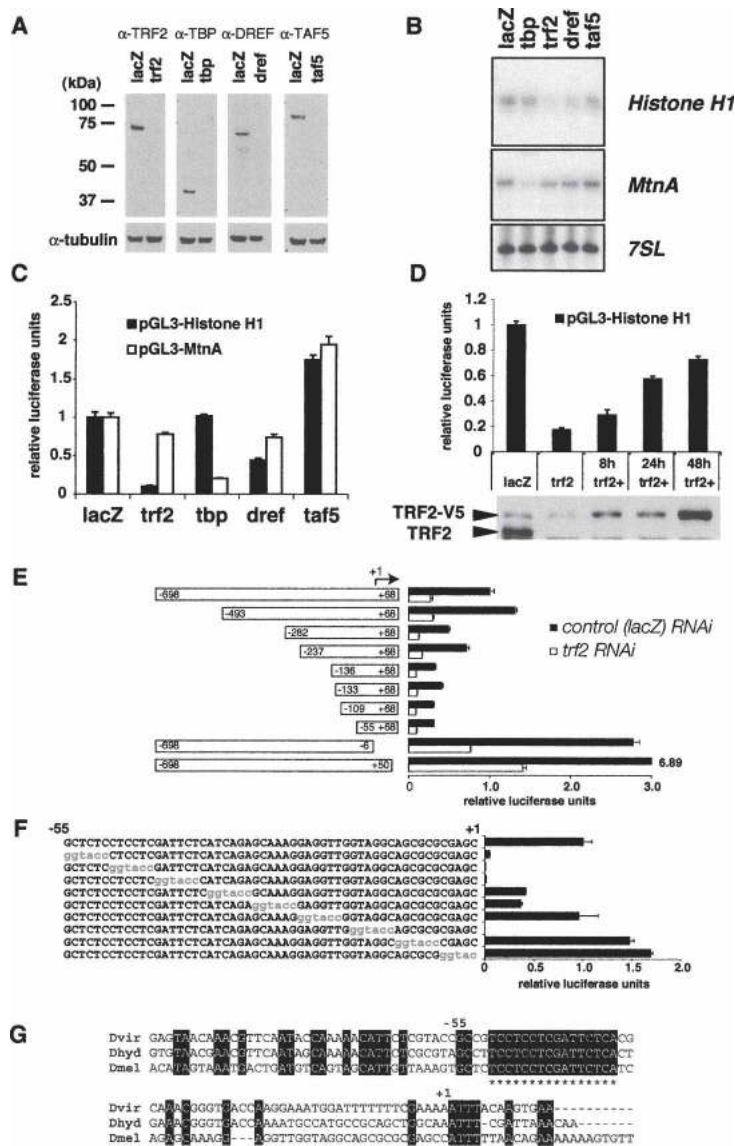
Since *Histone H1* has apparently been singled out for recognition and binding by TRF2, we next probed whether TRF2, but not TBP, is actually responsible for potentiating the transcription of the *Histone H1* gene. First, we used an efficient RNA interference (RNAi)-mediated knockdown assay in S2 cells to target TBP and TRF2 as well as other select subunits of TBP- or TRF2-containing complexes to determine whether the promoter activity of the *Histone H1* gene requires TBP or TRF2 in *Drosophila* cells (Fig. 3A). After efficiently depleting TBP or a TAF (TAF5) by RNAi, *Histone H1* steady-state mRNA levels remained largely unaffected (Fig. 3B). In contrast, RNAi depletion of TRF2 resulted in a significant and reproducible down-regulation of endogenous levels of *Histone H1* mRNA as well as a *Histone H1* reporter gene activity (approximately fivefold). Near complete depletion of DREF resulted in a more modest reduction (approximately twofold) of endogenous H1 mRNA (Fig. 3B) as well as H1 reporter activity (Fig. 3C). Since DREF does not appear to be recruited to the histone cluster and consensus DRE elements have not been found in the *Histone H1* promoter, this observed effect may be indirect. Such an indirect down-regulation was expected since DREF depletion induces a cell cycle arrest by regulating a large number of DNA replication-related genes (Hyun et al. 2005). As an important control, we found that the integrity of the general Pol II transcriptional machinery remained largely unaffected upon TRF2 depletion. For example, heavy metal-inducible transcription from the TBP/TFIID-dependent *Metallothionein A* (*MtnA*) gene (Marr et al. 2006) remained robust even after near complete depletion of TRF2 (Fig. 3B). Assaying the effects of depleting TRF2 or TBP using luciferase reporter assays instead of measuring endogenous *Histone H1* or *MtnA* confirmed our results (Fig. 3C). In addition, using a stable S2 cell line carrying an inducible V5-tagged *Trf2* gene, we were able to efficiently (~80%) rescue *Histone H1* promoter activity by re-expressing exogenous TRF2 protein for 48 h (Fig. 3D). These data, taken together, suggest that *Histone H1*, unlike *PCNA*, is driven by a novel class of core promoters that are TRF2-dependent and TBP-independent.

### The TATA-less Histone H1 promoter is driven by TRF2

Using the *Histone H1* promoter fused to the luciferase reporter, we next mapped a minimum fragment of the *cis*-regulatory region that retains TRF2-dependent transcription in S2 cells. *Histone H1* promoter activity decreases gradually with progressive deletion of upstream promoter-proximal sequence. However, a core-promoter region 55 bp upstream of the transcriptional start site is sufficient to support TRF2-dependent promoter activity (Fig. 3E). Linker scanning mutagenesis experiments revealed at least two elements between -55 and +1 that are critical for *Histone H1* promoter activity (Fig. 3F). One site, located from -55 to -40, is TC-rich and is highly

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**Figure 3.** TRF2 regulates *Histone H1* promoter activity. (A) Western blot showing efficient RNAi-mediated knockdown of TRF2, TBP, TAF5, and DREF.  $\alpha$ -Tubulin was used as a loading control. (B) Endogenous *Histone H1* mRNA level is dependent on TRF2. Primer extension analysis of *Histone H1*, *MtnA*, and *7SL* RNA transcripts. Ten micrograms of nuclear RNA isolated after RNAi-mediated depletion of indicated transcription factors were used to perform a primer extension reaction using specific primers for *Histone H1*, *MtnA*, and *7SL* RNA. (C) Cloned *Histone H1* promoter activity is dependent on TRF2. Luciferase reporter analysis using the *Histone H1* promoter and the *MtnA* promoter. Luciferase reporter activity calibrated to pAct5C-hRenilla activity was plotted after dsRNA-mediated depletion of transcription factors indicated. (D) Rescue of *Histone H1* activity by exogenous TRF2 expression. (Top) Luciferase reporter activity of pGL3-*Histone H1* using S2 cells with inducible TRF2-V5 expression under the control of *MtnA* promoter. During 4 d of RNAi treatments, some cells received an extra dose of TRF2 protein under the control of *MtnA* promoter. The 2 d of dsRNA treatment were sufficient to cause a threefold reduction of pGL3 reporter activity (data not shown). (lacZ) Control RNAi; (trf2) *trf2* RNAi; (trf2+) *trf2* RNAi with re-expression of TRF2-V5 for the indicated number of hours. (Bottom) Western blot using anti-TRF2 antibody indicating the efficient RNAi of TRF2 and re-expression of TRF2-V5. (E) TRF2-dependent activity of the *Histone H1* promoter is localized to the proximal promoter region. Truncations of -698 to +68 of the *Histone H1* promoter fused to luciferase were generated and tested for activity in either *lacZ* control or *trf2* RNAi backgrounds. The smallest fragment (-55 to +68) retained TRF2-dependent activity. (F) Identification of two proximal sequence boxes critical for the *Histone H1* promoter activity by linker scanning mutagenesis. Luciferase activity from various *Histone H1* promoter (-698 to +68) templates harboring mutations in the -55 to +68 region (shown in red) is plotted in the graph. (G) A promoter-proximal, TC-rich box critical for the *Histone H1* activity is highly conserved among *Drosophila* species. ClustalW sequence alignments of the proximal *Histone H1* promoter from *Drosophila melanogaster* (Dmel), *Drosophila hydei* (Dhyd), and *Drosophila virilis* (Dvir). The conserved sequences are marked by asterisks.



conserved among different *Drosophila* species (Fig. 3G). Importantly, this core-promoter region of *Histone H1* lacks a canonical TATA box found in core histone promoters. Taken together, these data suggest that selectivity of the *Histone H1* gene is at least in part mediated by specific interactions between TRF2 and this TATA-less core promoter (see Fig. 6A, below). In addition, our attempts to test direct binding of recombinant TRF2 alone to this promoter-proximal sequence have thus far not been successful, suggesting that TRF2 likely requires additional partners to recognize and bind these *cis*-regulatory elements.

#### Genome-wide high-resolution survey of TRF2 target core promoters

Differential regulation of linker and core histone genes in the histone gene cluster represents the first example

of a bona fide target gene that appears to be selectively regulated by TRF2 but not by TBP. This finding suggests that TRF2 can be used to drive expression from two different types of core-promoter structures; i.e., tandem TRF2/TBP core promoters such as those represented by the promoter proximal regions of *PCNA*, as well as the TRF2-dependent but TBP-independent *Histone H1* promoter. This led us to examine whether the mechanisms used to control *Histone H1* expression might support a more versatile role of TRF2 in core-promoter recognition and transcriptional regulation than had previously been anticipated, especially given the mouse knockout studies that suggested a rather restricted role of TRF2 in spermiogenesis (Zhang et al. 2001; Martianov et al. 2002). To obtain a more global picture of core-promoter recognition mediated by TRF2, we screened for TRF2 as well as TBP and RNA Pol II-binding sites in S2 cells throughout the entire *Drosophila* genome. Using the ChIP-on-chip

experimental platform we recently developed (Isogai et al. 2007), >1000 highly statistically significant binding sites for TRF2 were newly identified in addition to *Histone H1* (Fig. 4A; Supplementary Table S8). A majority of the binding sites map to known 5' ends of Pol II genes, and 60% of these TRF2 sites appear to also colocalize with RNA Pol II (Fig. 4A,B). This suggests that, unlike other classes of TRFs (i.e., TRF1 and TRF3), TRF2 may play a major role in RNA Pol II transcription. Genomic regions recognized by TRF2 as well as TBP appear to be distributed evenly throughout different chromosomes, except for chromosome 4. Interestingly, TRF2 was found to occupy only eight sites on chromosome 4, while TBP occupies 95 sites, most of which, however, are not occupied by Pol II in S2 cells. This observation is reminiscent of unusual chromatin and gene organization observed previously for chromosome 4 (Sun et al. 2000; Larsson et al. 2004), whose functional significance remains unclear.

Consistent with our polytene chromosome staining, the majority (~80%) of the TBP- and TRF2-binding sites mapped by high-resolution ChIP-on-chip were distinct and nonoverlapping, suggesting that TRF2 likely plays a critical and independent role in the recognition of a large number of genes that appear to be TBP-independent (Fig. 4A,C). However, ~20% of the TRF2- and TBP-binding regions actually overlap (Fig. 4C). This finding suggests that the occurrence of tandem TBP and TRF2 core promoters such as was found for the *PCNA* promoter (Hochheimer et al. 2002) may also be quite widespread in the *Drosophila* genome. While genes linked to the core promoters targeted by TRF2 appeared to encode products of diverse cellular functions, it became apparent that TRF2 likely targets a cluster of ribosomal protein genes that appear to be coregulated (Table 1). Indeed, one of the most prominent targets of TRF2 belongs to a specific set of ribosomal protein genes (Supplementary Table S1). This finding suggests that TRF2 may be used to coordinate expression of related classes of functionally interdependent gene sets. Thus, our studies reveal that TRF2 targets a novel class of TBP-independent genes and suggest that this TRF may also be responsible for regulating distinct sets of coordinately controlled genes that are critical for proteins carrying out biochemically related events (i.e., protein synthesis).

#### *TRF2-bound promoters are associated with a distinct repertoire of core-promoter elements*

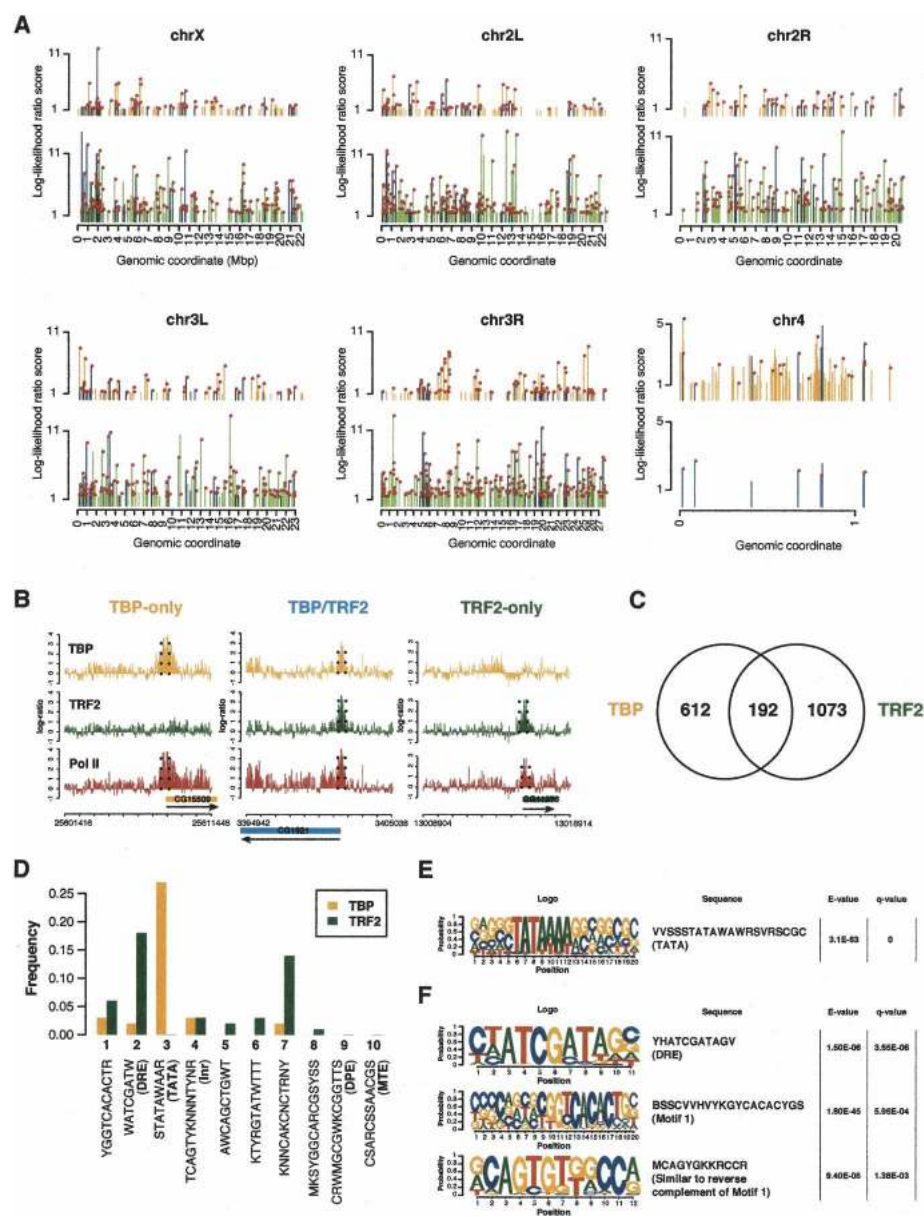
In order to better understand the differential and selective core-promoter recognition by TRF2 revealed by our genome-wide survey, we first analyzed core promoters (-100 to +50) uniquely bound by TRF2 versus TBP by scanning the 10 most commonly found core-promoter sequence motifs reported for *Drosophila* (Ohler et al. 2002). This analysis revealed distinct core-promoter element preferences associated with TRF2- and TBP-bound promoters (Fig. 4D). As expected, TBP-bound promoters exhibit a strong enrichment for TATA boxes, with >85% of the first top 100 TBP target promoters containing variants of TATA boxes (data not shown). In contrast, TRF2-

bound core promoters are virtually devoid of TATA boxes. Instead, TATA-less categories of core-promoter motifs appear preferentially associated with TRF2. Among the most enriched motifs is the DRE element (~20%), which serves as a binding site for DREF, a subunit of one class of TRF2 complexes. Therefore, it seems evident that TBP- and TRF2-containing initiation complexes are differentially utilized for recognition of distinct core-promoter classes. Another unbiased analysis using de novo motif-finding platforms (Bailey and Elkan 1995; Isogai et al. 2007) confirmed that these distinct sequence elements uniquely linked to TBP versus TRF2 data sets are highly statistically significant. Again we see that TATA boxes are tightly linked to occupancy by TBP but not TRF2 (Fig. 4E). In contrast, DRE and Motif 1 are highly linked to occupancy by TRF2 over TBP (Fig. 4F). Thus, differential targeting of TRF2 to TATA-less classes of core promoters may, at least in part, rely on these discriminating sequence elements recognized by either TBP/TAF- or TRF2-containing initiation complexes.

#### *In vivo requirements of TRF2 in fly embryogenesis and organ development*

The identification of a large number of TRF2 recognition sites that appear TBP-independent prompted us next to examine whether these recognition sites represent bona fide functional targets of TRF2. Importantly, we asked whether these diverse TRF2-bound promoters drive specific pathways in vivo during fly development. Since our earlier studies in which TRF2 was depleted during embryogenesis resulted in lethality (Kopytova et al. 2006; M. Prestel and A. Hochheimer, unpubl.), we targeted depletion of TRF2 by RNAi in specific tissues, for example, by using a salivary gland-specific GAL4 driver. Salivary gland-specific expression of *Trf2* double-stranded RNA (dsRNA) resulted in more than fivefold reduction of *Trf2* mRNA, while TRF2 protein was reduced to near undetectable levels (Fig. 5H). Although these mutant *Drosophila* embryos developed to the third instar larval stage (L3), a majority of them failed to pupate or died during pupal stages. There appears to be no apparent size differences between mutant and wild-type larvae at L3, suggesting that a significant level of larval development remains unaffected by specific depletion of TRF2 in the salivary gland (Fig. 5A,C). Interestingly, however, salivary glands in the TRF2-depleted larvae display severe growth defects (Fig. 5A,C). Importantly, this defect appears to be cell growth-related rather than proliferation-deficient since cell numbers remained constant between mutant and wild-type glands (~120 cells) (Fig. 5B) but with a dramatic reduction in cell and organ size. Most notably, nuclei in the TRF2-depleted glands appeared underdeveloped, consistent with a failure to successfully polytenize chromosomes (Fig. 5C). These specific developmental defects attributable to TRF2 depletion support the notion that critical biological pathways impinging on the growth of certain organs may be selectively regulated by TRF2.





**Figure 4.** Genome-wide distribution of TRF2/TBP recognition sites. (A) Overview of TBP/TRF2/Pol II-binding sites in the *Drosophila* genome. The coordinates are in megabase pairs (Mb). TBP-binding sites are indicated in orange, TRF2-binding sites are indicated in green, and sites bound both by TBP and TRF2 are indicated in blue. Pol II (RPB3) binding for each peak is indicated by a circle (in magenta) at the tip of each peak. (B) Spatial structure of TRF2/TBP binding to core promoters. Three examples of the binding profiles of TRF2 and TBP on genomic tiling arrays. (Left) 5' end of the CG15509 gene that is selectively bound by TBP and Pol II (RPB3). (Middle) 5' end of CG1921 gene that is bound simultaneously by TBP, TRF2, and Pol II. (Right) 5' end of CG11276 gene that is selectively bound by TRF2 and Pol II. The regions specified by broken lines are selected as binding regions by our statistical data analysis program TileHGMM. (C) Genome-wide survey of TBP- and TRF2-binding sites. Venn diagram showing the overlap between TBP and TRF2 occupied genomic regions. (D) Motif scanning analysis of core-promoter regions of TRF2- and TBP-bound promoters. Core-promoter sequences spanning  $-100$  to approximately  $+50$  relative to the annotated gene start sites of the top 100 promoters uniquely bound by either TBP or TRF2 (also occupied by Pol II) were probed for the presence of the 10 most commonly found core-promoter motifs (Ohler et al. 2002). We adopted the IUB nomenclature for the nucleotide sequences for degenerate base positions. (E) List of significant sequence motifs for TBP-bound core promoters by de novo motif finding. The *E*-value represents the significance of the motif among the TBP data set, whereas the *q*-value represents the discrimination from the TRF2 data set. (F) List of significant sequence motifs for TRF2-bound core promoters by de novo motif finding. The *E*-values represent the significance of the motif among the TRF2 data set, whereas the *q*-values represent the discrimination from the TBP data set.

**Table 1.** Top 25 TRF2-bound core promoters

Rank	Chromosome	Region start	Region end	Nearest gene annotation	TBP	Pol II	Distance to gene start
14.64	2L	12427220	12427724	CG5446-RA		●	0
14.46	X	420596	420981	vnd-RA*	●		26881
14.09	2L	13394753	13395199	RpL24-RA		●	0
14.00	2L	9967051	9967531	Dref-RA/RpL13-RA		●	0
13.18	X	421935	422379	vnd-RA*	●		25483
13.13	3L	16082622	16083125	CG12272-RA*		●	4320
12.98	3R	1480106	1480622	CG2185-RA		●	0
12.26	X	908047	908549	CG14629-RA	●	●	0
11.51	2R	14957177	14957688	RpL11-RA		●	0
11.47	2L	452609	453136	crq-RA crq-RB		●	0
11.33	X	2131009	2131499	CG2918-RA		●	0
11.28	3R	19877264	19877772	CG5991-RC	●	●	0
11.26	X	10589845	10590353	CG32676-RA/ras	●	●	0
10.94	2L	479476	479993	cbt-RA	●	●	0
10.90	2L	10200920	10201403	Rip $\alpha$ -RA/RpL7-RA			0
10.65	X	1868731	1869234	CG4406-RA*	●	●	3232
10.65	3L	3401287	3401789	sty-RB	●	●	0
10.63	2L	19004746	19005247	RpL30-RA		●	0
10.44	3R	5167209	5167724	CG33188-RB CG33188-RA	●	●	0
10.42	3L	10837008	10837479	tna-RB*			6295
10.10	X	8934376	8934903	CG7033-RC		●	0
10.06	2L	18698335	18698841	CG10343-RA*	●	●	554
10.04	3L	3182084	3182608	gry-RA gry-RB/CG32276-RA		●	0
9.83	2L	11003694	11004189	RpL9-RB		●	0
9.67	3L	13015459	13015986	RpS4-RA		●	0

The table depicts the top 25 core promoters recognized by TRF2. The asterisks indicate binding sites that likely represent unannotated core promoters since current gene annotations do not align well with the TRF2-binding sites (separated by >0.5 kb). When a binding site corresponds to promoters driving two opposite directions (bidirectional promoter), both genes are indicated, separated by a slash (/). The complete list including additional data on precise coordinates can be found in the Supplemental Material.

### *Aberrant organization and structure in TRF2-deficient polytene chromosomes*

Since the *Histone H1* gene was identified as a direct TRF2 target gene in *Drosophila* cultured cells, we wondered if some of the defects in the mutant salivary gland may derive in part from a lack of the linker histone H1. We therefore visualized chromatin structure and integrity using polytene chromosomes, where interphase chromatin is amplified 100- to ~1000-fold through endoreduplication (Andrew et al. 2000). While polytene chromosomes of wild-type salivary glands consist of uniformly thick bundles of endoreplicated chromatin bearing distinct banding patterns due to unique regions of condensed and decondensed chromatin, the chromosomes from TRF2-depleted salivary glands exhibited very few discernible banding patterns and are interspersed with numerous thin chromatin fibers that appeared improperly folded (Fig. 5D). To determine if this severe disorganization of TRF2-depleted polytene chromosomes may partially be due to alterations in chromatin structure, *Histone H1* mRNA levels in total RNA derived from control and mutant salivary glands were analyzed using RT-PCR. Indeed, *Histone H1* mRNA levels are significantly down-regulated, while *Histone H3* mRNA levels remain intact (Fig. 5E). Importantly, H1 protein levels in TRF2-depleted salivary glands are also severely down-regulated if not completely abolished (Fig. 5H). Taken together, our data provide both in vivo and in

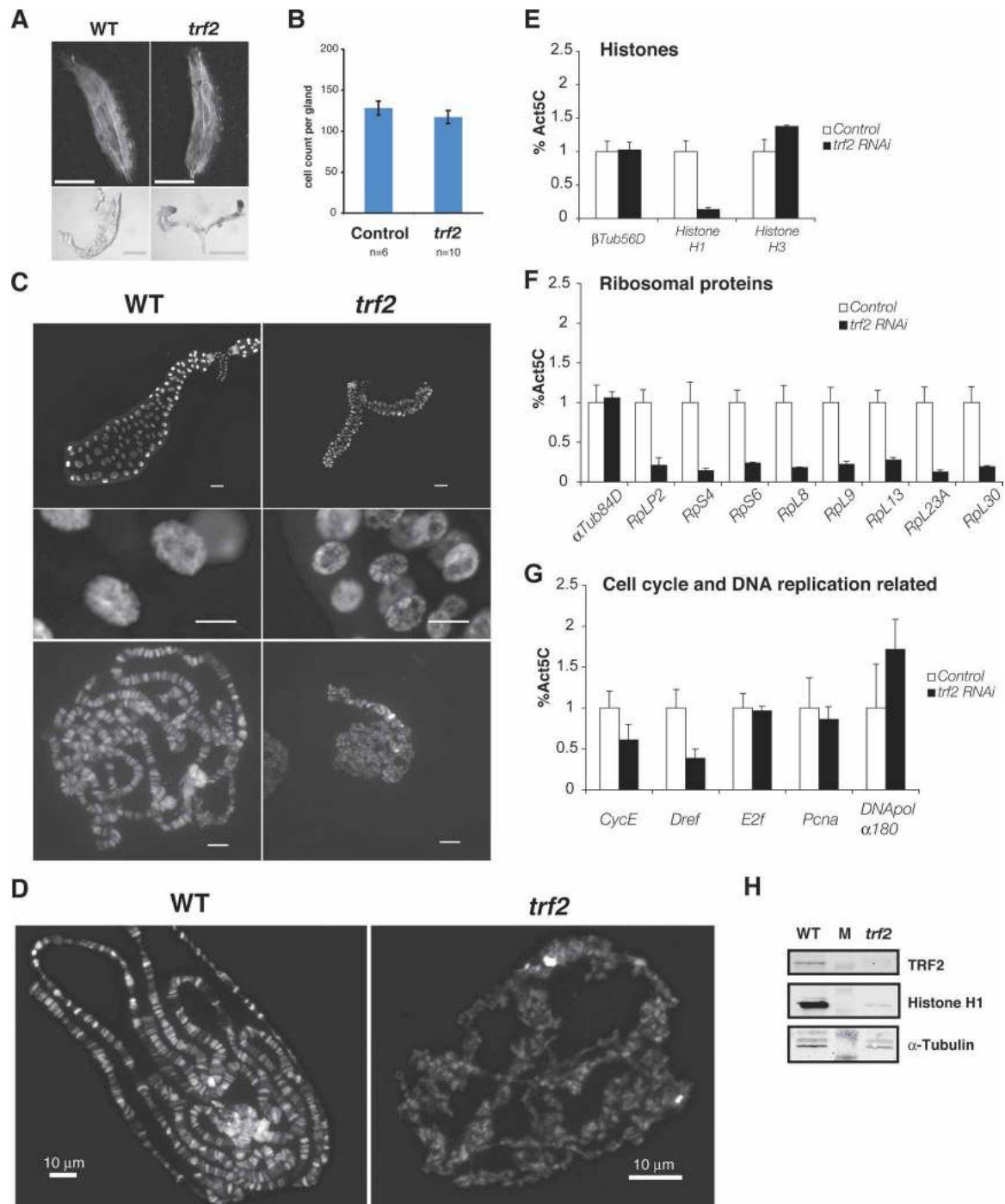
vitro evidence that a differential regulation of *Histone H1* versus the other core histone genes during fly development is dependent on the selective usage of an alternative core-promoter recognition machinery that harbors TRF2 rather than TBP/TFIID. These studies also provide in vivo evidence that H1 does indeed play a critical role in the maintenance of chromatin integrity as had been postulated and shown in vitro (Thoma et al. 1979).

### *TRF2 is required for expression of a cluster of ribosomal protein genes*

The targeted depletion of TRF2 protein in salivary gland cells enabled us to examine whether a variety of TATA-less classes of core promoters bound by TRF2 in S2 cells are indeed dependent on TRF2 activity. Especially, we were intrigued by the finding that TRF2-depleted salivary glands are defective in cell growth because our previous CHIP-on-chip data suggested that one of the major classes of TRF2 target genes encodes ribosomal proteins (Table1; Supplementary Table S5), which appear to be driven by TATA-less core promoters (Supplementary Fig. S6). In *Drosophila*, mutations in ribosomal protein genes lead to proliferation and growth defects (Lambertson 1998). To examine if TRF2 is responsible for driving TATA-less ribosomal protein gene promoters, we conducted RT-PCR analysis of a dozen ribosomal protein genes expressed in salivary glands. Indeed, TRF2-deficient salivary glands display dramatically impaired lev-



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**Figure 5.** TRF2 is required for cell growth and chromatin organization. (A) Salivary-specific depletion of TRF2 affects salivary gland growth. Shown is a comparison between control (WT) and mutant (*trf2*) L3 larvae (top) and dissected salivary glands (bottom). Bar, 0.5 mm. Note that only half of each pair is shown for the control salivary glands. (B) Cell counts of control and *trf2* mutant salivary glands. (C) DNA stain of control (WT) and mutant (*trf2*) salivary gland polytene chromosome. Bars: top, 0.1 mm; middle, 25  $\mu$ m; bottom, 10  $\mu$ m. Note that only half of a pair is shown for the control salivary glands. (D) DNA staining of control (WT) and mutant (*trf2*) polytene chromosome spreads. (E–G) RT–PCR analysis of mRNAs in TRF2-depleted salivary glands. Error bars represent the standard deviation from triplicates. (H) Western blot probing TRF2, Histone H1, and  $\alpha$ -Tubulin in wild-type and TRF2-depleted salivary glands. M lane represents a marker lane.

els of ribosomal protein mRNAs (Fig. 5F). In contrast, when we tested mRNA levels of several cell cycle and DNA replication-related genes including *CycE*, *Dref*,

*E2f*, *Pcna*, and *DNAPol $\alpha$ 180*, they appear largely unaffected (*CycE*, *E2f*, *Pcna*, and *DNAPol $\alpha$ 180*) or exhibit only a modest down-regulation (*Dref*) in the TRF2-de-

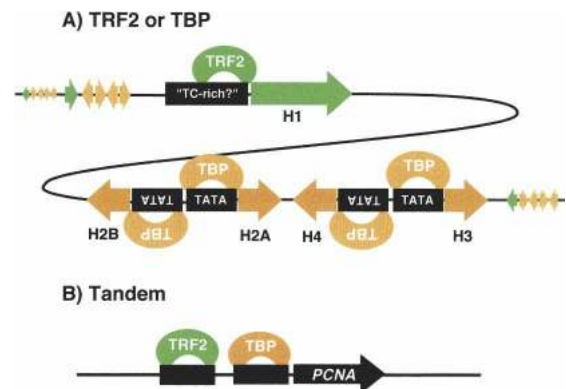
pleted cells (Fig. 5G). Therefore, the cell growth defects exhibited by salivary gland-specific depletion of TRF2 are likely linked to down-regulated protein synthesis caused by reduced levels of ribosomal proteins. Taken together, our *in vivo* analysis of TRF2 function in salivary gland cells suggests that TRF2 may be critically involved in several fundamental cellular functions including chromatin organization as well as protein synthesis, while underreplicated polytene chromosome may represent indirect effects.

## Discussion

### *Regulation of histone genes via differential core-promoter targeting by TRF2 and TBP*

In *Drosophila*, the five histone genes are found in a cluster that is tandemly amplified ~100 times (Lifton et al. 1978; Matsuo and Yamazaki 1989). Despite the need to coordinate histone gene expression during the cell cycle, the ratio of linker and core histones can vary dramatically within each cell, among different tissues, and during embryonic development (Holmgren et al. 1985; Rudell and Jacobs-Lorena 1985; Ner and Travers 1994). This observation suggested that *Histone H1* gene expression may be differentially regulated relative to the patterns of core histone gene transcription. Here, our genome-wide survey of TRF2 target sites uncovered the finding that the histone gene cluster contains both TBP and TRF2 recognition sites. Most strikingly, these two core-promoter recognition factors are segregated within the histone cluster with TBP targeted to the core histone (H2A/B, H3, and H4) promoters, while TRF2 selectively directs transcription of the linker histone H1. This finding reveals a novel mechanism in which *Histone H1* gene expression may be differentially regulated relative to the patterns of core histone gene transcription (Fig. 6A).

The finding that a TRF2-containing preinitiation complex is responsible for *Histone H1* expression while the prototypic TBP/TFIID complex directs transcription of the core histones suggests that the expression of the linker histone H1 and core histones must be uncoupled under certain circumstances, possibly in a developmental-specific and cell type-specific manner. The analysis of TRF2-depleted salivary gland polytene chromosomes suggests that this is indeed the case. Remarkably, the polytene chromosomes in TRF2-deficient cells exhibited severe defects in chromosome organization and structure reminiscent of the failure to form 30-nm fibers in H1-depleted chromatin (Thoma et al. 1979; Fan et al. 2005; Maresca et al. 2005). Given that the *Drosophila* genome encodes only one H1 subtype compared with five to six in mammals (Fan et al. 2005), it is interesting that the H1 knockdown via TRF2 depletion resulted in a severely altered chromatin structure, which represents another *in vivo* evidence that histone H1 is indeed linked to organization of chromatin structure. Importantly, these TRF2-depleted cells appear to specifically down-regulate *Histone H1* mRNA while leaving core histone transcripts intact. These findings suggest that TRF2 must serve as a key component of the transcrip-



**Figure 6.** Model of promoter-selective transcription in *Drosophila* mediated by TBP/TRF2. In *Drosophila*, at least three types of core-promoter architecture became apparent in this study. (A) A core promoter such as the TATA-less *Histone H1* promoter is recognized and regulated uniquely by TRF2. In contrast, core-histone promoters harbor TATA boxes and are targeted uniquely by TBP. (B) Another type of core-promoter architecture is a tandem type, where both TRF2 and TBP/TFIID occupy proximal promoter regions, such as the ones seen in the *PCNA* promoter.

tional initiation complex evolved to differentially control linker histone versus core histone expression.

### *Targeting specific transcriptional complexes to distinct nuclear compartments*

Transcription of nonpolyadenylated histone genes appears to be associated with a specific nuclear body (the HLB) through a physical coupling between the HLB and the histone gene cluster locus (Liu et al. 2006). The HLB is loaded with RNA synthesis and processing machinery, possibly serving as a “factory” for histone mRNA production. Thus, in order to rapidly produce histone transcripts during embryogenesis, *Drosophila* appears to have adapted an elegant strategy that involves tandemly amplified gene cassettes sequestered within a distinct nuclear address (the HLB). Interestingly, it appears that only specific subsets of transcription factors are deposited in the HLB. For example, among the three TBP paralogs in *Drosophila* (TBP, TRF1, and TRF2), only TRF2 and TBP that are used for linker and core histone transcription, in addition to Pol II, are “preloaded” within the HLB, perhaps to facilitate rapid as well as differential linker versus core histone transcript production. Therefore, the histone gene cluster presents an important paradigm wherein a distinct nuclear body loaded with specific transcriptional as well as post-transcriptional machinery becomes dedicated to the purpose of coordinating and differentially regulating five essential genes.

### *TRF2, a key recognition factor for TATA-less core promoters*

Our high-resolution genome mapping of TRF2 recognition sites using the CHIP-on-chip platform has revealed >1000 novel binding sites, with 80% distinct from and 20% overlapping with TBP-binding sites. These results suggest that the TRF2-dependent and TBP-independent

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*Histone H1* promoter is not an exception. Indeed, the H1 case may represent a more general case for how TRF2 can serve as an alternative core-promoter recognition factor at many Pol II genes. A comprehensive and detailed sequence motif analysis of the *Drosophila* genome revealed that TRF2-bound promoters significantly lack TATA boxes, while the TATA box is tightly correlated with TBP-binding sites. Instead, TRF2 appears to selectively recognize promoters containing other distinct core-promoter elements such as Motif 1, DRE, and Motif 7 (Ohler et al. 2002). In addition, functional analysis of transcripts derived from TRF2-depleted salivary glands confirmed that TRF2 activity is indeed required for directing these TRF2 target promoters (Fig. 5; Supplementary Fig. S3). Thus, our genome-wide analysis significantly strengthens the emerging picture that TRF2 likely evolved to recognize and regulate a large class of TATA-less core promoters.

One question concerning TRF2 function in promoter recognition is whether TRF2, like TBP, can directly recognize and bind to a distinct core-promoter element. TRF2 is likely to possess very different DNA-binding specificities from TBP since the amino acid residues critical for TATA-box recognition have been altered in TRF2 (Dantonel et al. 1999; Ohbayashi et al. 1999; Rabenstein et al. 1999). However, all our attempts to experimentally identify a direct TRF2-binding sequence have thus far failed. Similarly, our most recent computational efforts using TRF2 ChIP-on-chip data sets failed to identify any strong consensus core-promoter motifs comparable with the prototypic TATA box with its approximately -30-bp location relative to the start of transcription. Instead, we identified motifs such as the DRE and other uncharacterized elements with no set common position relative to the transcriptional start site. These findings are, however, consistent with our previous studies (Hochheimer et al. 2002) in which TRF2 failed to bind the core promoter by itself. Instead, it appears that TRF2 recruitment to at least a subset of core promoters relies on specific interactions between TRF2 and various other sequence-specific DNA-binding proteins, such as DREF. However, unlike previous studies, our genome-wide survey of TRF2- and TBP-binding sites in *Drosophila* revealed a considerably more comprehensive picture of how TRF2 may be used as an alternative core-promoter recognition factor. Importantly, mixing and matching various enhancer-binding factors (i.e., sequence-specific DNA-binding factors) and alternative core-promoter recognition factors (i.e., TFIID vs. TRF2) appears to be a powerful and perhaps common strategy for metazoan organisms to diversify transcriptional outputs.

#### *Widespread occurrence of tandem core promoters in Drosophila*

Our genome-wide ChIP-on-chip analysis also provides strong evidence that metazoan organisms make much more use of tandem core promoters containing both TFIID and TRF2 recognition sites than might have been anticipated (Fig. 6B). Whether or not this type of dual

core-promoter structure represents a case of redundant pathways or is subject to selective and differential regulation of downstream targets remains unclear. Interestingly, two previously characterized TRF2 targets (*PCNA* and *DNApol $\alpha$ 180*) appear unaffected when TRF2 is depleted in salivary glands, possibly due to the ability of such dual core promoters to use alternative transcription complexes. Thus, the possibility that TRF2 may be used in lieu of TBP/TFIID to diversify transcriptional outputs in response to specific signals cannot be ruled out. It would be of interest for future studies to determine how these two distinct core-promoter recognition factors TBP/TRF2 operating at dual tandem promoters may be coordinated. Are these core-promoter recognition complexes at tandem core promoters recruited by common or distinct activator proteins? Since salivary gland depletion of TRF2 protein resulted in developmental defects, TRF2 may be necessary to selectively up-regulate genes required for specific developmental pathways.

#### *Evolutionary conservation of TRF2 functions in metazoans*

The identification of direct TRF2 target genes in the present study has revealed a striking link between TRF2 and specific biological processes such as chromatin organization and protein synthesis. Since TRF2 is conserved among many metazoan organisms, its role in various model organisms has been of considerable interest. Several studies found that inactivating TRF2 in nematode, fly, fish, and frog all resulted in lethality due to a block in embryogenesis (Dantonel et al. 2000; Kaltenbach et al. 2000; Veenstra et al. 2000; Muller et al. 2001; Kopytova et al. 2006). In contrast, germ cell-specific functions of TRF2 have also been reported for *Drosophila* and mice (Martianov et al. 2001; Zhang et al. 2001; Kopytova et al. 2006). In particular, while TRF2-null mice appear to display a modest non-Mendelian ratio of inheritance, the major defect manifests as a lack of spermiogenesis (Martianov et al. 2001; Zhang et al. 2001). Although these studies revealed that TRF2 provides nonredundant functions during development, these genetic studies were unable to link TRF2 to selective core-promoter recognition functions in vivo. For instance, direct TRF2 target genes responsible for these previously observed phenotypes have not been identified or characterized. The identification of histone H1 and ribosomal proteins as key gene products misregulated in TRF2-depleted *Drosophila* organs not only provides candidate TRF2 target genes responsible for the chromatin defects observed in TRF2-depleted *Drosophila* germ cells (Kopytova et al. 2006), but also underscores the potential role of TRF2 in other organisms. For example, TRF2-null mice display a major defect in chromocenter formation in spermatids (Martianov et al. 2002). This suggests that, consistent with TRF2-mediated H1 regulation in *Drosophila* somatic cells, TRF2 may also target genes that are essential for chromatin structure in mammalian gonads. However, the precise molecular targets and mechanisms of TRF2 action may differ. Indeed, a recent report points to the



involvement of a human DREF homolog in regulating transcription from a TATA-box-containing histone H1 promoter in human cells (Ohshima et al. 2003). In contrast, in *Drosophila*, we found that the H1 gene is TATA-less and does not appear to be regulated by DREF.

In addition, our finding that *Drosophila* TRF2 directs the expression of a large number of gene products critical for essential cell function such as growth (i.e., ribosomal subunits and histones) would be consistent with the lethality associated with the loss of TRF2 in most organisms. These findings also suggest that in mammals TRF2 may play an important role regulating essential cell functions in tissues other than testis. The biological context of TRF2 usage as an alternative core-promoter recognition factor may well be more universal than we had anticipated.

## Materials and methods

### Antibodies

The rabbit polyclonal antibody against TRF2 was generated using GST fusion proteins of the C-terminal nonconserved region of *Drosophila* TRF2. Rabbit polyclonal serum against TBP was generated using his-tagged TBP. These sera were extensively purified using antigen affinity chromatography. RPB1- and RPB3-specific antibodies were similarly purified from anti-holo-Pol II antibodies (Skantar and Greenleaf 1995) using antigen affinity columns. Anti-DREF, anti-TAF5, and anti-TAF2 antibodies were described previously (Weinzierl et al. 1993; Hirose et al. 1996; Marr et al. 2006). Anti-TMG antibody was purchased from Oncogene.

### Cell culture

*Drosophila* S2 and Kc cells were grown in M3-BPYE medium (*Drosophila* Resource Center). Synchronization of Kc cells was conducted as described (MacAlpine et al. 2004). Log-phase cultures ( $\sim 2 \times 10^6$  cells per milliliter) of Kc cells were first incubated with 0.2 nM ponasterone A for 24 h to obtain G2 cells. Cells were then rinsed with  $1 \times$  PBS three times, resuspended in an equal volume of fresh M3-BPYE medium containing 1.5 mM hydroxyurea, and cultured for an additional 18 h to obtain G1/S cells. These Kc cells were rinsed again with  $1 \times$  PBS three times, resuspended in fresh M3-BPYE, and cultured for an additional 2–3 h to obtain S-phase cells. To confirm the synchrony of cell populations, fluorescence-activated cell sorting (FACS) sorting with propidium iodide was conducted as described (Darzynkiewicz and Juan 1997) using a staining buffer containing 1 mg/mL glucose, 20  $\mu$ g/mL propidium iodide, and 1 mg/mL RNase A in  $1 \times$  PBS. RNAi was conducted by treating cells with 10  $\mu$ g of dsRNA per  $0.4 \times 10^6$  cells per well seeded in a 24-well plate or 100  $\mu$ g of dsRNA per  $5 \times 10^6$  cells seeded in a 10-cm plate, and assays were conducted after the indicated number of days. For RNAi in S2 cells, the template for dsRNA synthesis was derived from amplifying the 5' untranslated region (UTR) of the *Trf2* gene from *Trf2* cDNA clone (Rabenstein et al. 1999). For dsRNA against DREF, we used sequences spanning 1716–2124 (relative to ATG), and dsRNA for TBP and TAF5 have been described (Marr et al. 2006). Reporter plasmids were transfected simultaneously using Effectene (Qiagen) for luciferase reporter assays as per the manufacturer's instructions.

### Measurement of Histone H1 promoter activity

Primer extension analysis was performed using nuclear RNAs isolated from *Drosophila* S2 cells after 5 d of dsRNA treatment.

*Histone H1* and *7SL RNA* were detected in nuclear RNA, and *MtnA* transcripts were detected in total RNA from cells treated for 2 h with 0.5 mM  $\text{CuSO}_4$ . Nuclear RNA was prepared using methods described previously (Herold et al. 2003). RNA was purified using TRI-reagent (Sigma). The pGL3-H1 promoter was constructed by inserting –698 to +68 of the *Histone H1* promoter into pGL3(R2.1)-Basic vector (for Fig. 3C,D) or pGL3-Basic vector (for Fig. 3E,F) (Promega). Various H1 promoter constructs harboring deletions and mutations were generated using pGL3-H1 vector by exonuclease digestions (Henikoff 1984) and site-directed mutagenesis (Promega), respectively. Luciferase reporter assays with the *Histone H1* promoter and the *MtnA* promoter in Figure 3C were conducted after 5 d of dsRNA treatment. The luciferase assays in Figure 3E were performed after 3.5 d of dsRNA treatment. As an internal standard, we used phRL-null plasmid (Promega) harboring the Act5C distal promoter (pAct5C-hRenilla). The rescue experiments shown in Figure 3D were conducted using S2 cells harboring a TRF2-V5 expression cassette from the *MtnA* promoter. The cells were first transfected with the pGL3-H1 reporter as well as pAct5C-hRenilla internal control plasmids and were treated with either control lacZ or *trf2* (5'UTR) dsRNA. The re-expression of TRF2-V5 was induced by the addition of  $\text{CuSO}_4$  to a final concentration of 0.05 mM, for the indicated length of time prior to the harvest. The cells were harvested simultaneously on day 4, and luciferase activities were assayed. Each luciferase assay was conducted in triplicate, and standard deviation is indicated by error bars in the graphs.

### Cytology

Polytene chromosomal staining and DNA FISH were conducted as described (Lavrov et al. 2004). Immunostaining and DNA FISH in S2 and Kc cells were described previously (Marr et al. 2006). For RPB3 immunostaining in Kc cells, cells were first treated with 2 mM thymidine overnight, which increased the S-phase population by 10%–20%. FISH probes were generated using the pCR4 vector containing 5 kb spanning the histone cluster (Lifton et al. 1978) using methods described previously (Marr et al. 2006).

### ChIP and ChIP-on-chip

The ChIP experiments in this study were conducted essentially as described (Isogai et al. 2007). A formaldehyde concentration of 1.0% was used for cross-linking S-phase-synchronized Kc cells, and 0.5% was used for asynchronous S2 cells for the ChIP-on-chip experiments. We conducted ChIP-on-chip experiments using affinity-purified TRF2, TBP, and Pol II antibodies. Pol II ChIP-on-chip experiments were conducted using RPB1 and RPB3 antibodies, and the merged data set was used for the subsequent analysis throughout this study, unless otherwise indicated. Detailed description of ChIP, ChIP-on-chip assays, and statistical analysis of the data with the TileHGMM platform (Keles 2007), and de novo sequence analysis using MEME (Bailey and Elkan 1995) and cosmo (Bembom et al. 2007), can be found elsewhere, as they have been described previously (Isogai et al. 2007).

### De novo sequence analysis

To perform de novo sequence analysis, we first extracted nonoverlapping data sets of TBP- and TRF2-binding sites that are co-occupied by Pol II and are within 500 bp of a known gene start site. Each data set was analyzed by MEME and cosmo, and the resulting significant motifs ( $E$ -value  $< 1$ ) were collected as a combined data set. We then scored the sequences in each data set by each motif in the combined motif data set using the

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PATSER program (Hertz and Stormo 1999). For each motif, we compared the mean of the two score distributions in TBP- and TRF2-bound data sets with a Wilcoxon rank sum test. After obtaining a *P*-value from this test for each motif, we adjusted the *P*-values for multiplicity, converting them to *q*-values (Storey 2002), and reported the discriminating motifs with a *q*-value <0.01.

#### Generation of *trf2* RNAi flies

A DNA fragment comprising *Trf2* coding sequence from 24 to 586 bp was amplified by PCR using *Trf2* cDNA (Rabenstein et al. 1999) and XbaI-site-containing oligonucleotides GGTCAG CATTCTAGAGGCCAATTTGAACGGCGG and CGTGCT CATTCTAGACAATTGGCTGGTGAAGGT. The PCR product was cut with XbaI and cloned as inverted repeats in two consecutive steps into the plasmid pWIZ (Lee and Carthew 2003) using XbaI-compatible AvrII and NheI sites. pWIZ:Trf2 was used as a template to transcribe intron-spliced hairpin RNA under the control of Gal4/UAS to induce *Trf2* gene silencing. pWIZ:Trf2 was introduced into the germline by *P*-element-mediated transformation (Spradling and Rubin 1982). Two independent transformant lines were established and mapped. We subsequently crossed homozygous pWIZ:Trf2 females to males carrying homozygous copies of the salivary gland driver GAL4<sup>2314</sup> (gift of J. Großhans). Females with a UAS-GFP construct served as a control.

#### Immunoblot using salivary gland extracts

Glands were dissected and boiled immediately in 2× Laemmli buffer, followed by benzonase nuclease treatment for 1 h at room temperature. The wild-type (WT) lane contains four pairs of salivary glands boiled in Laemmli buffer, and the *trf2* lane contains six pairs of *trf2* mutant salivary glands. The blot was probed with anti-α-tubulin (DM1A; Sigma), anti-TRF2, and anti-H1 (gift of J. Kadonaga), followed by secondary antibodies (IRDye 800CW Donkey anti-Rabbit IgG and IRDye 800CW Donkey anti-Mouse IgG; Biomol). The blot was imaged using a LI-COR Odyssey scanner.

#### Preparation of RNA from salivary glands of *trf2* RNAi larvae

Thirty pairs of salivary glands from *trf2* RNAi fly lines arrested at L3 or control larvae were dissected and lysed in Trizol reagent (GIBCO) and subsequently purified by RNeasy plus kit (Qiagen). Salivary glands from L3 larvae of the GAL4-driver line 2314 crossed to UAS-GFP flies served as a control. Five-hundred nanograms of total RNAs were used for reverse transcription using SuperScript III reverse transcriptase (Invitrogen) per the manufacturer's instructions. These cDNAs were subjected to quantitative PCR using Opticon 2 (MJ Research). We used TBP-dependent Act5C transcripts as an internal standard for calibration. The primer sequences used for the amplification are available on request.

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