The transcription factor TRF2 has a unique function in regulating cell cycle and apoptosis

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ABSTRACT

Background: Diverse biological processes and transcriptional programs are regulated by RNA polymerase II (Pol II), which is recruited by the general transcription machinery to the core promoter to initiate transcription. TRF2 (TATA-box-binding protein-related factor 2) is an evolutionarily conserved general transcription factor that is essential for embryonic development of *Drosophila melanogaster*, *C. elegans*, zebrafish and *Xenopus*. Nevertheless, the cellular processes that are regulated by TRF2 are largely underexplored.

Results: Here, using *Drosophila* Schneider cells as a model, we discovered that TRF2 regulates apoptosis and cell cycle progression. We show that TRF2 knockdown results in increased expression of distinct pro-apoptotic genes and induces apoptosis. Using flow cytometry, high-throughput microscopy and advanced imaging-flow cytometry, we demonstrate that TRF2 regulates cell cycle progression and exerts distinct effects on G1 and specific mitotic phases. RNA-seq analysis revealed that TRF2 controls the expression of *Cyclin E* and the mitotic cyclins, *Cyclin A*, *Cyclin B* and *Cyclin B3*, but not *Cyclin D* or *Cyclin C*. To identify proteins that could account for the observed regulation of these cyclin genes, we searched for TRF2-interacting proteins. Interestingly, mass spectrometry analysis of TRF2-containing complexes identified GFZF, a nuclear glutathione S-transferase implicated in cell cycle regulation, and Motif 1 binding protein (M1BP). TRF2 has previously been shown to interact with M1BP and M1BP has been shown to interact with GFZF. Furthermore, available ChIP-exo data revealed that TRF2, GFZF and M1BP co-occupy the promoters of TRF2-regulated genes. Using RNAi to knockdown the expression of either M1BP, GFZF, TRF2 or their combinations, we demonstrate that although GFZF and M1BP interact

with TRF2, it is TRF2, rather than GFZF or M1BP, that is the main factor regulating the expression of *Cyclin E* and the mitotic cyclins.

Conclusions: Our findings uncover a critical and unanticipated role of a general transcription factor as a key regulator of cell cycle and apoptosis.

Keywords

Basal transcription machinery, RNA polymerase II, gene expression, TATA box-binding protein (TBP), TBP-related factor 2 (TRF2), cyclin genes.

BACKGROUND

Multiple biological processes and transcriptional programs are regulated by RNA polymerase II (Pol II). The initiation of transcription of protein-coding genes and distinct non-coding RNAs occurs following the recruitment of Pol II to the core promoter region by the general/basal transcription machinery (1-4). The core promoter, which directs accurate initiation of transcription and encompasses the transcription start site (TSS), may contain short DNA sequence elements/motifs, which confer specific properties to the core promoter (1, 4-10). The first step in the recruitment of Pol II to initiate transcription is the binding of TFIID, which is composed of TATA-box-binding protein (TBP) and TBP-associated factors. Remarkably, although TBP is considered a universal general transcription factor, robust Pol II transcription is observed in mouse TBP-/- blastocysts, indicating the existence of TBP-independent Pol II transcription *in vivo* (11). The complexity of transcription is also manifested by the existence of diverse transcriptional regulators,

among which are the TBP family members. There are three TBP family members in *Drosophila melanogaster*: TBP, TRF1 and TRF2 (reviewed in (12-16)). TRF1, the first *Drosophila* TBP family member identified, is insect specific (17). An evolutionary conservation analysis indicated that TRF2 (also known as TLP (TATA-like protein), TLF (TBP-like factor), TRP (TBP-related protein) and TBPL1 (TBP-like 1)), is highly conserved in evolution (12, 18-22) and is present in all bilaterian organisms, but not in any of the non-bilaterian genomes available (19). It was further discovered that TRF2, which is involved in Pol II transcription, evolved by duplication of the TBP gene (19). Yet, unlike TBP and TRF1, TRF2 does not bind TATA-box containing promoters (19, 20, 22). There are two *Drosophila* TRF2 protein isoforms that result from an internal translation initiation: the evolutionarily conserved short isoform (632 aa; typically referred to as "TRF2") and a long *Drosophila*-only isoform (1715 aa), in which the same short amino acid sequence is preceded by an N-terminal domain (23). TRF2 affects early embryonic development of *Drosophila*, *C. elegans*, zebrafish and *Xenopus*, differentiation and morphogenesis (23-32). Mouse TRF2 is essential for spermiogenesis (33-35).

One of the open questions in the transcriptional regulation field is what are the cellular functions of TRF2. Despite its importance in development, the cellular processes that are regulated by TRF2 remain largely underexplored. To identify and characterize the cellular processes that are regulated by TRF2, we used *Drosophila* S2R+ cells as a model and knocked-down the expression of TRF2. We discovered that reduced expression of TRF2 (but not TBP or TRF1) results in apoptosis and increased expression of key, yet not all, pro-apoptotic genes (including *rpr, hid, p53*), suggesting this is not a general stress response. Surprisingly, not only that TRF2 regulates apoptotic cell death, reduced expression of TRF2 (but not its family

members, TBP or TRF1), exerts distinct effects on G1, G2/M and specific mitotic phases, as demonstrated by quantitative high-throughput imaging flow cytometry. We further discovered that TRF2 controls the expression of *Cyc E* and the mitotic *Cyc A*, *Cyc B* and *Cyc B3* genes. Using mass spectrometry analyses of TRF2-interacting proteins and available ChIP-exo data, we demonstrate the co-occupancy of TRF2, GFZF (GST-containing FLYWCH zinc-finger protein) and M1BP (motif 1 binding protein) in the majority of promoters bound by each of the three factors. Remarkably, the promoters of the TRF2-regulated mitotic cyclins and *Cyclin E* are bound by the three factors, whereas the promoters of *Cyclin C* and *Cyclin D*, which are not regulated by TRF2, are not bound. Furthermore, the Motif 1 sequence element is enriched in the promoters of genes bound by all three proteins, suggesting the involvement of GFZF and M1BP as co-factors in TRF2-regulated cell cycle progression. Moreover, we demonstrate that TRF2, rather than M1BP or GFZF, is the main factor that regulates the expression of the mitotic cyclins and *Cyclin E*. Importantly, while general/basal transcription factors might be viewed as having a somewhat “generic” role, our findings emphasize the unique, unanticipated functions of *Drosophila* TRF2 as an essential factor for cell cycle progression and apoptotic cell death.

RESULTS

Knockdown of TRF2 expression results in apoptotic cell death and induced expression of key pro-apoptotic genes

The TBP-related transcription factor TRF2 is a key general/basal transcription factor (reviewed in (12-16)), yet the cellular processes that are regulated by TRF2 remain largely underexplored. To investigate the cellular functions of TRF2, we used

Drosophila S2R+ cells as a model, and knocked down its expression by RNAi using non-overlapping dsRNA probes (Additional file 1: Figure S1a). TBP knockdown was used as a control throughout this study. The resulting reduction in protein expression was verified by western blot analysis (Additional file 1: Figure S1b, c). We consistently observed significant cell death following TRF2 knockdown, as evident by microscopic examination and by the reduced amounts of total RNA purified from TRF2-RNAi treated cells, as compared to mock-treated cells. To specifically investigate whether TRF2 plays an important role in apoptosis, we performed Annexin V/PI analysis to detect early and late apoptotic cell death by flow cytometry analysis. PI is excluded from cells with intact membranes, while dead and damaged cells have membranes that are permeable to PI. Annexin V binds phospholipids that are exposed in cells undergoing apoptosis. Hence, cells that are both Annexin V and PI negative are considered viable, while cells that are in early apoptosis are Annexin V positive and PI negative, and cells that are in late apoptosis or already dead are both Annexin V and PI positive. S2R+ cells were incubated for three days with either one of the four non-overlapping dsRNA probes directed against *Trf2*, a dsRNA probe against *Tbp*, a dsRNA probe against *Trf1* or a dsRNA probe against the homeodomain transcription factor *exd*, as a negative control. Cells were harvested and stained with Annexin-V FITC/PI. Approximately 30% of cells stain positive for Annexin V following TRF2 knockdown by the four different dsRNA probes, ~2 fold higher than the mock and the exd-RNAi treated cells (Fig. 1). Notably, cell death induced by TBP or TRF1 dsRNA probes (an average of 20%) was not as pronounced as the cell death induced by either of the four TRF2 dsRNA probes. These findings emphasize the unique effects of TRF2, as compared to TBP, on apoptosis.

147 To identify the targets that are unique to TRF2, we used RNAi to knockdown
148 either TRF2 or TBP in *Drosophila* S2R+ cells and performed RNA-seq analysis at
149 two time points, 48h and 72h. In order to further understand how TRF2 knockdown
150 results in cell death, we searched our RNA-seq data for pro-apoptotic genes that are
151 regulated by TRF2. The inhibitor of apoptosis protein (IAP) family has already been
152 shown to play an important role in cell survival (reviewed in (36-38)). *Drosophila*
153 Death-associated IAP-1 (DIAP1), a key member of the IAP family, inhibits apoptosis
154 by binding to the active Caspase 9-like Dronc, and functioning as an E3-ubiquitin
155 ligase to promote its degradation. The *reaper* (*rpr*), *head involution defective* (*hid*),
156 and *grim* genes encode IAP antagonists that bind DIAP1, disrupt its interactions with
157 caspases and target it for degradation, resulting in caspase activation and cell death.
158 Scylla (*scyl*) has been implicated in developmental cell death (39). Indeed, the RNA-
159 seq analysis revealed a significant upregulation of *rpr* and *scyl* (Additional file 2:
160 Table S1). We thus decided to knockdown either TRF2 (probes #1 and #2), exd,
161 TBP or TRF1 and examine by reverse transcription-qPCR the expression of multiple
162 genes implicated in the apoptotic machinery: the IAP antagonists *rpr*, *hid* and *grim*
163 (reviewed in (37)), *scyl* (39), the BCL-2 family members *buffy* (40) and *Death*
164 *executioner Bcl-2* (*Debcl*) (41-44), and the Caspase 9-like *Dronc* (reviewed in (37)).
165 Remarkably, reducing the expression levels of TRF2 (but not exd, TBP or TRF1)
166 resulted in increased expression levels of *rpr*, *hid* and *scyl* (Fig. 2a). The detected
167 increase in *Buffy* expression levels was not statistically significant. The expression of
168 *grim*, *Debcl* and *Dronc* was not significantly altered by TRF2 knockdown (Fig. 2a),
169 suggesting that this is not a general stress response, and that TRF2 specifically
170 regulates the expression of distinct, but not all, pro-apoptotic genes.

The p53 tumor suppressor gene is a key regulator of both cell cycle progression and programmed cell death (reviewed, for example, in (45, 46)). p53 is expressed at low levels in S2R+ cells (www.flybase.org). Surprisingly, the RNA-seq analysis revealed its upregulation upon TRF2 knockdown (Additional file 2: Table S1). Examination of p53 expression by reverse transcription-qPCR following knockdown of either TRF2 (probes #1 and #2) or TBP reproduced the upregulation trend of p53 expression by TRF2. Notably, TBP knockdown did not alter the expression of p53, manifesting the unique characteristics of gene regulation via TRF2. Interestingly, it was previously demonstrated that p53 regulates the expression of *rpr* and *hid*, which in turn, induce apoptosis (47, 48). Hence, it is possible that the observed upregulation of *rpr* and *hid* following the knockdown of TRF2 (Fig. 2a, b) is mediated via p53. Unfortunately, multiple attempts to reduce the levels of endogenous p53 by two different dsRNA probes, were unsuccessful. Thus, one cannot exclude the possibility that the increased expression of p53 following TRF2 knockdown, may partially contribute to the upregulation of *rpr* and *hid* expression.

TRF2 exerts distinct effects that are independent of TBP

TRF2 may repress transcription involving TBP or TFIID, probably by recruiting TFIIA (20, 49). One can suggest that TRF2 is, in some respects, antagonistic to TBP, and the observed upregulation of pro-apoptotic genes following TRF2 knockdown (Fig. 2) results from TBP activity, which is no longer obstructed by TRF2. To examine whether the observed upregulation of *rpr* and *hid* results from TBP activity, we overexpressed TBP in S2R+ cells that were either incubated with TRF2 dsRNA probes or mock treated. Although overexpression of TBP was clearly evident (>100

196 fold; Additional file 3: Figure S2), no differences in the levels of *rpr* and *hid* were
197 observed upon its overexpression (Additional file 3: Figure S2). Furthermore,
198 overexpression of TBP following TRF2 knockdown did not alter *rpr* or *hid* expression.
199 Thus, we conclude that the observed upregulation of *rpr* and *hid* following TRF2
200 knockdown results from the activity of TRF2 as a unique transcription factor, rather
201 than a TBP antagonist.
202
203 **Knockdown of endogenous TRF2 expression results in altered cell cycle**
204 **distribution and G1 arrest**
205 To identify specific cellular processes that are regulated by TRF2, we performed
206 Gene Ontology (GO) terms analysis of genes that were either downregulated or
207 upregulated following TRF2 knockdown (using string.db.org). Surprisingly, we
208 discovered that genes that were downregulated following TRF2 knockdown are
209 enriched for cell cycle and mitotic cell cycle processes, while genes that were
210 upregulated following TRF2 knockdown are enriched for response to stimulus and
211 stress (Additional file 2: Table S1). Interestingly, while the number of genes that were
212 downregulated was only half of the number of the upregulated genes following TRF2
213 knockdown (337 vs. 684 genes, respectively), the enrichment scores ($-\log_{10}$(P
214 values)) of the downregulated genes were 5-fold higher. We thus decided to
215 examine the effects of TRF2 knockdown on cell cycle distribution. To knockdown the
216 expression of the endogenous genes, S2R+ cells were incubated for three days with
217 dsRNA probes against *Trf2*, *Tbp*, *Trf1* or *exd*, as a negative control. Cells were
218 harvested, fixed and analyzed by flow cytometry. Control S2R+ cells display a
219 normal profile with an average of ~31% of cells in G1 phase and with an average of
220 ~34% of cells in G2/M phase, similarly to mock treated cells (which were processed

similarly, but were not incubated with any dsRNA; Fig. 3). Following TRF2 knockdown by either one of the four dsRNA probes, we observed a distinct decrease in the fraction of cells in G2/M phase (an average of ~20%), as well as the fraction of cells in S phase (an average of ~20%) with a concomitant increase in the fraction of cells in G1 phase (an average of ~55%). Remarkably, these effects are unique to TRF2 knockdown, as the knockdown of its family members TBP or TRF1 resembles the cell cycle distribution of control and mock treated cells (Fig. 3).

Our RNA-seq analysis reveals subsets of genes that are involved in S and G2/M phases. In addition, the cell cycle analysis indicates that TRF2 plays a role in S and/or G2/M phases. To determine if TRF2 affects cell cycle progression to S phase, S2R+ cells were arrested in G1 with 1mM hydroxyurea (HU) for 18h following knockdown of either TRF2 (dsRNA probes #1 and #2) or TBP, and then released to cycle by replacing the medium with fresh medium. As can be seen in Figure 4, HU treatment (0h) resulted in accumulation of cells in G1 (~55%). Two hours following the release, mock treated cells returned to cycle (indicated by the decrease in the number of cells in G1), and the fraction of cells in S and G2/M increased. Similarly, cells in which TBP was depleted by RNAi, returned to cycle. Surprisingly, unlike mock or TBP RNAi-treated cells, cells in which TRF2 was depleted by either one of the two probes, remained in G1 (~55%) and did not return to cycle (Fig. 4, Additional file 4: Figure S3). Moreover, even 8h following the removal of HU, cells in which TRF2 was knocked-down remained G1-arrested. Our findings imply that endogenous TRF2 is involved in progression into S phase.

TRF2 regulates the expression of specific cyclin genes

To further explore the connection between TRF2 and cell cycle progression, we turned to our RNA-seq data for cell cycle-related genes that may be influenced by knockdown of TRF2. We discovered that following TRF2 knockdown, the expression of the *Cyc A*, *Cyc B*, *Cyc B3* and *Cyc E* cell cycle regulators was significantly reduced (over 2.3-fold). Notably, the expression levels of *Cyc C* and *Cyc D* were unchanged (Additional file 2: Table S1). In order to verify the effect of TRF2 knockdown on the expression of these genes, reverse transcription-qPCR analysis of endogenous cyclin genes was performed on mock, TRF2 (probes #1 and #2), exd, TBP or TRF1 RNAi-treated cells. Knockdown of TRF2 by either probe #1 or #2 significantly reduces the expression of *Cyc A*, *Cyc B* and Cyc *B3*, as compared to mock treated cells, much more than knockdown of TBP or TRF1 (Fig. 5a). As there was a difference between the effects of probe #1 and #2 on *Cyc E* expression, 4 non-overlapping TRF2 dsRNA probes were used to assess the effect of TRF2 knockdown on *Cyc E* expression (Fig. 5b). Notably, each of the 4 non-overlapping TRF2 dsRNA probes reduces *Cyc E* expression. Unlike *Cyc D* (which is required for G1 progression (50, 51)), the expression of *Cyc E* (which promotes G1-S transition (52, 53)) was reduced following TRF2 knockdown (Fig. 5a, b). We next tested whether TRF2 knockdown affects the protein levels of Cyc A, Cyc B and Cyc E. Unfortunately, we were unable to detect endogenous Cyc A and Cyc B protein expression using publicly available anti- *Drosophila* Cyc A and Cyc B antibodies (data not shown). Remarkably, using anti-*Drosophila* Cyc E antibodies, we observed a distinct reduction in Cyc E protein levels following knockdown of TRF2 (but not TBP), using both TRF2 probes (Fig. 5c), further suggesting that TRF2 regulates G1-S transition by modulating the expression of *Cyc E*.

TRF2 regulates distinct mitotic phases

We were intrigued by the downregulation of the mitotic cyclin gene expression (*Cyc A*, *Cyc B* and *Cyc B3*) following TRF2 knockdown (Fig. 5a) and decided to explore the effect of TRF2 downregulation on mitotic phases. To this end, we knocked-down the expression of TRF2 or TBP and stained cells for mitotic chromatin (anti-phospho-Histone H3 (Ser10)), DNA (Hoechst) and filamentous Actin (Phalloidin). These allowed us to analyze the fraction of cells in mitosis following TRF2 knockdown (Fig. 6a). To determine the number of mitotic cells, we developed a pipeline to automatically detect the Hoechst and phospho-Histone H3 signals. Notably, there was a reduction in the number of cells undergoing mitosis following TRF2 knockdown, as compared to mock or TBP RNAi treated cells (Fig. 6b).

To explore the effect of TRF2 on G2/M, we sought to synchronize cells in G2/M. Unfortunately, we were unable to synchronize cells in G2/M in a reversible manner (see methods), and thus we could not perform G2/M block-release experiments. Nevertheless, we succeeded in discerning the effects of TRF2 on specific mitotic phases by employing advanced imaging-flow cytometry analysis (ImageStreamX mark II imaging flow-cytometer, Amnis Corp, Seattle, WA, Part of EMD Millipore). Imaging-flow cytometry analysis combines the high-quality imaging and functional insights of microscopy with the speed, sensitivity, and phenotyping abilities of flow cytometry. We knocked down the expression of TRF2 or TBP and stained cells with anti-phospho-Histone H3 (Ser10) antibodies and Hoechst. A total of 40,000 cells of each treatment were analyzed by an ImageStream flow cytometer to determine the number of cells in each mitotic phase, according to their nuclear morphology (Fig. 6c-f, Additional file 5: Figure S4, Additional file 6: Table S2). Remarkably, although 40,000 cells were analyzed in each experiment, only a few

hundred cells were mitotic, and following TRF2 knockdown there was an even bigger reduction in the total number of mitotic cells (Fig. 6b, d). Notably, despite the overall reduction in the mitotic cell population, knockdown of TRF2 (but not TBP) resulted in a significant accumulation of cells in anaphase and telophase (Fig. 6e, f).

To validate the accurate identification of mitotic cells, we used Colchicine as a control. Colchicine treatment resulted in accumulation of cells in mitosis, specifically in anaphase (Fig. 6e). Multiple studies have shown that Colchicine disrupts the metaphase to anaphase transition (see for example, (54)), yet, the increase in anaphase has also been documented (55). Furthermore, it is established that different cell types behave differently during mitosis in the presence of drugs that disrupt microtubules function (56). Interestingly, morphological examination of the Colchicine-treated S2R+ cells indicated aberrant DNA staining patterns and mal-oriented clumped chromosomes in prophase, metaphase and anaphase cells (Additional file 7: Figure S5), in line with the absence of a spindle. Notably, mal-oriented un-centered chromosomes, such as those observed in Colchicine-treated cells, were not observed in TRF2-RNAi treated cells.

The effects of TRF2 knockdown on cyclin gene expression correlates with the promoter occupancies of TRF2 and its co-factors GFZF and M1BP

To better understand how TRF2 regulates the expression of the cyclin genes, we turned our attention to TRF2-interacting proteins. TRF2 was recently shown to interact with M1BP (motif 1 binding protein) (57). Interestingly, M1BP was recently demonstrated to interact with GFZF, a nuclear glutathione S-transferase protein that has been implicated in cell cycle regulation (58). We suspected that GFZF may interact with TRF2. Indeed, using FLAG immuno-affinity purification from FLAG-HA

TRF2-inducible S2R+ cells followed by mass spectrometry analysis, we discovered that both M1BP and GFZF are in complex with the evolutionarily conserved TRF2 (also known as short TRF2), but not with the long *Drosophila*-only TRF2 isoform or with TBP (Table 3 and Additional file 8: Table S3). This prompted us to examine the occupancy of TRF2, M1BP and GFZF in the vicinity of the TSSs (-100 to +100 relative to the TSS) of TRF2-regulated cyclin genes, using publicly available TRF2, M1BP and GFZF ChIP-exo analyses in *Drosophila* S2R+ cells (GSE97841, GSE105009) (57, 58). We examined the number of bound sites, the average peak scores and the maximum peak scores of cyclin genes and several ribosomal protein genes for comparison (Table 4). As expected, M1BP, TRF2 and GFZF co-occupy the promoters of the ribosomal protein genes. Interestingly, while M1BP occupies the -100 to +100 regions of all the examined cyclin genes, both TRF2 and GFZF occupy the -100 to +100 regions of *Cyc A*, *Cyc B*, *Cyc B3* and *Cyc E*, and to a lesser extent the promoters of *Cyc C* and *Cyc D*, which are not regulated by TRF2. The occupancies of the three proteins is especially striking in the vicinities of the *Cyc B* and *Cyc B3* TSSs. Thus, the effects of TRF2 knockdown on cyclin gene expression generally correlate with the occupancies of both TRF2 and GFZF in the -100 to +100 regions of *Cyc A*, *Cyc B*, *Cyc B3* and *Cyc E.*

To characterize the co-occupancies of TRF2, GFZF and M1BP in a genome-wide manner, we examined the binding of each factor to *Drosophila* promoter regions (± 50 bp relative to FlyBase annotated TSSs). Remarkably, a major fraction of promoters is bound by all three transcription factors (Fig. 7a). Reassuringly, the co-bound promoters include the TRF2-regulated *Cyc A*, *Cyc B*, *Cyc B3* and *Cyc E*, but not *Cyc C* and *Cyc D* promoters, which are not regulated by TRF2.

346 To better decipher the characteristics of the co-bound promoters, we used MEME
347 (59) to detect enriched sequence motifs. Interestingly, the top enriched motif in
348 promoters that are bound by all three proteins (Fig. 7b) closely resembles Ohler
349 Motif 1 (60), also detected in M1BP ChIP-exo analysis (57). We next analyzed the
350 core promoter composition of the co-bound promoters, using the ElemeNT algorithm
351 (61). Strikingly, the co-bound promoters are depleted for the TATA-box motif and
352 enriched for the TCT and Motif 1 core promoter elements, as compared to the
353 genomic distribution of core promoter elements (Fig. 7c).
354 To examine the contribution of M1BP and GFZF to the effect of TRF2
355 knockdown on cyclin gene expression, we used RNAi to knockdown the expression
356 of either M1BP, GFZF, TRF2 or their combinations. The use of each of the dsRNA
357 probes resulted in a significantly reduced expression of the targeted gene (Fig. 7d).
358 Surprisingly, M1BP knockdown resulted in increased expression of *Trf2*, *gfzf* and
359 *Cyc E*. Since both TRF2 and M1BP were previously shown to affect the expression
360 of ribosomal protein genes (32, 62), we tested whether their knockdown affects the
361 expression of several ribosomal target genes, namely, *RpL30*, *RpLP1* and *RpLP2*.
362 While TRF2 and GFZF knockdown did not affect their expression, M1BP knockdown
363 resulted in significantly increased expression of *RpLP2* (Additional file 9: Figure
364 S6a). Notably, this effect was not general, but rather specific to distinct cyclin and
365 ribosomal protein genes (Fig. 7d, e and Additional file 9: Figure S6), as the
366 expression of *CG12493* and *Sgll*, two previously identified M1BP targets (62), was
367 reduced following M1BP knockdown (Additional file 9: Figure S6b).
368 The expression levels of *Cyc A* and *Cyc B* were specifically reduced following TRF2
369 knockdown, but not following GFZF or M1BP knockdown (Fig. 5a and Fig. 7d, e).
370 *Cyc D* expression was not affected by either of these single factor knockdowns (Fig.

7d), as in Figure 5. As can be observed by TRF2 knockdown, as well as the combined knockdown of TRF2, GFZF and M1BP, the expression pattern of *Cyc A* and *Cyc B* are mostly influenced by TRF2 knockdown. *Cyc E* exhibits a composite pattern: it is reduced following TRF2 knockdown, but the combined knockdowns of TRF2 and M1BP or GFZF seem to restore its expression as compared to mock treatment. Notably, *Cyc D* expression pattern is the least affected by the different knockdown combinations.

Taken together, these data suggest that the observed effects of TRF2 knockdown on cell cycle progression (Figs. 3, 4 and 6) are, at least partially, mediated by the reduced expression of *Cyc E*, *Cyc A* and *Cyc B* following TRF2 knockdown (Figs. 5 and 7d, e). Importantly, the co-occupancy of TRF2, GFZF and M1BP in the promoters of these cyclin genes, the enrichment of Motif 1 in their promoters and the expression patterns of the cyclin genes following the knockdown of either TRF2 alone, or in combination with GFZF and/or M1BP, imply that GFZF and M1BP may serve as co-factors in TRF2-regulated cell cycle progression. Yet, it is TRF2 that is the major transcription factor regulating the expression pattern of the abovementioned cyclin genes.

DISCUSSION

In this study, we discovered that knockdown of the general/basal transcription factor TRF2 results in increased expression of the *rpr*, *hid* and *p53* pro-apoptotic genes, which is in line with the observed increased apoptotic cell death following TRF2 knockdown and with the extensively characterized involvement of p53 in apoptosis,

G1 arrest and G2 arrest (reviewed, for example, in (45)). A recent study has demonstrated that human TRF2 interferes with MDM2 binding and ubiquitination of p53, leading to p53 protein stabilization (63). Our results provide evidence for another level of p53 regulation, *i.e.*, transcriptional regulation. Furthermore, in a similar manner to the p53-MDM2 negative feedback loop in vertebrates (reviewed in (45)), activation of *Drosophila* p53 transcriptionally activates the *companion of reaper* (*corp*) gene (64-66), which in turn, negatively regulates its activity (67). The fact that *corp* expression in *Drosophila* S2R+ is rather low and not altered by TRF2 knockdown (Additional file 2: Table S1), may provide support for p53-independent regulation of *rpr* and *hid* by TRF2.

Furthermore, we examined the expression of multiple additional genes implicated in apoptosis following TRF2 knockdown. *Scylla* is pro-apoptotic (39) and its upregulation is in line with the observed cell death following TRF2 knockdown. Buffy, a *Drosophila* Bcl2 family member, however, has been shown to act in an anti-apoptotic manner, but has also been shown to cause a G1-S arrest (40). Thus, its upregulation following TRF2 knockdown may contribute to the observed G1-S accumulation of cells. These effects are in line with studies suggesting the existence of TRF2-regulated transcriptional systems (10, 16, 32, 68, 69) and are unlikely to represent a general stress response, as the expression of *Debcl* and *Dronc* is not affected by TRF2 knockdown.

Interestingly, we discovered that TRF2 knockdown results in accumulation of cells in G1 and in reduction in the number of cells in S and G2/M phases. G1/S transition is regulated by Cyclin E activity, while S phase and G2/M transition are regulated by Cyclin A activity, and transition into and within mitosis is regulated by

418 the activities of Cyclin B and Cyclin B3. Remarkably, TRF2 knockdown in S2R+ cells
419 resulted in reduced expression of *Cyc E*, *Cyc A*, *Cyc B* and *Cyc B3* (but not *Cyc D*),
420 suggesting that TRF2 regulates cell cycle progression by modifying the expression of
421 specific cyclins. The reduced expression of *Cyc A*, *Cyc B* and *Cyc B3* and the
422 reduction of the number of cells undergoing mitosis following TRF2 knockdown, are
423 in line with previous studies, which demonstrated inhibition of nuclear mitotic entry in
424 *Drosophila* embryos following simultaneous knockdown of *Cyc A*, *Cyc B* and *Cyc B3*
425 (70). Interestingly, Cyclin A and Cyclin B have previously been shown to inhibit
426 metaphase-anaphase transition, whereas Cyclin B3 promotes it (71). Thus, the
427 specific accumulation of cells in anaphase and telophase observed by imaging flow
428 cytometry (Fig. 6e, f), could result from the reduced expression of *Cyc A* and *Cyc B*
429 following TRF2 knockdown (Fig. 5). Notably, to the best of our knowledge, this study
430 is the first to employ imaging-flow cytometry in the analysis of *Drosophila* cells.

431 To examine whether TRF2-interacting proteins could account for the observed
432 regulation of *Cyc E*, *Cyc A*, *Cyc B* and *Cyc B3* (but not *Cyc D* or *Cyc C*), we
433 searched for TRF2-interacting proteins. TRF2 has been shown to interact with M1BP
434 (57) and M1BP has been shown to interact with GFZF, a nuclear glutathione S-
435 transferase implicated in cell cycle regulation (57). Our proteomic analyses revealed
436 that both M1BP and GFZF preferentially interact with TRF2, but not with TBP (Table
437 3 and Additional file 8: Table S3). Remarkably, examination of publicly available
438 TRF2, M1BP and GFZF ChIP-exo data from *Drosophila* S2R+ cells (57, 58),
439 indicated that the effects of TRF2 knockdown on the expression of cyclin genes
440 correlate with TRF2, GFZF and M1BP co-occupancies of the promoters of the TRF2-
441 regulated cyclin genes (Table 4), in line with the reported regulatory effects of
442 GFZF. Genome-wide examination of promoters bound by all three transcription

443 factors revealed the enrichment of the TCT and Motif 1 core promoter elements.
444 Whereas one would expect the TCT to be enriched as TRF2 and M1BP have
445 previously been implicated in the regulation of ribosomal protein genes (32), the
446 enrichment of Motif 1 within promoters bound by all three factors indicates a shared
447 function for TRF2, GFZF and M1BP. Notably, in our experimental system, TRF2
448 knockdown by RNAi resulted in a two-fold reduction in *Trf2* levels (Fig. 2a, Additional
449 file 2: Table S1). Under these conditions, we did not detect any change in ribosomal
450 protein gene expression (Additional file 2: Table S1). Hence, the reduced expression
451 of *Cyc A*, *Cyc B* and *Cyc E* following a two-fold reduction in TRF2 expression, does
452 not result from a general inhibition of protein synthesis. As direct binding of TRF2 to
453 DNA could not be demonstrated (32), it is likely that TRF2 indirectly regulates the
454 expression of these cyclin genes and that there are TRF2-associated factors that
455 enable DNA binding. Our analysis suggested that GFZF and M1BP could serve as
456 such factors for specific TRF2-regulated processes. Interestingly, the expression
457 patterns of the cyclin genes following the knockdown of either TRF2 alone, or in
458 combination with GFZF and/or M1BP, indicated that among these three factors,
459 TRF2 is the major contributor to the expression pattern of *Cyc A*, *Cyc B* and *Cyc E*.
460 Moreover, while *Cyclin A*, *Cyclin B* and *Cyclin E* expression levels are reduced
461 following TRF2 (but not GFZF) knockdown, the expression levels of both *Cyclin A*
462 and *Cyclin B* , but not *Cyclin E*, are reduced following the combined knockdown of
463 TRF2 and GFZF (Fig. 7d, e), suggesting that the reduced expression levels of *Cyclin*
464 *A* and *Cyclin B* are not mediated via *Cyclin E*.

465 Notably, mouse TBP was recently shown to remain bound to mitotic
466 chromosomes during mitosis of mouse embryonic stem cells (mESCs), and to recruit
467 a small population of Pol II molecules to mitotic chromosomes (72). Nevertheless,

active Pol II transcription occurs in the absence of mouse TBP, whereas Pol I and Pol III, are significantly reduced (11, 72). It remains to be determined whether *Drosophila* TBP is bound to mitotic chromosomes during mitosis. As we did not observe significant effects on cell cycle progression or mitosis following *Drosophila* TBP knockdown (Figs. 3, 4 and 6), it is likely that *mouse* TBP may exert different functions as compared to *Drosophila* TBP, perhaps via its associated proteins.

The effects of TRF2 knockdown on cell cycle progression and apoptosis of *Drosophila* cultured S2R+ cells are in line with the early embryonic lethality of TRF2 knockout flies. TRF2 has also been shown to be essential for embryonic development of *C. elegans*, zebrafish and *Xenopus*. It remains to be determined whether knockdown of TRF2 in cellular systems from these species results in similar effects.

CONCLUSIONS

Taken together, using *Drosophila* cells as a model system, we discovered that the knockdown of TRF2, rather than TBP or TRF1, regulates apoptosis and cell cycle progression via distinct target genes. Importantly, we discovered that TRF2 is associated with the GFZF and M1BP proteins, and that TRF2, GFZF and M1BP co-occupy the promoters of the TRF2-regulated cyclins. Furthermore, we show that TRF2, rather than GFZF or M1BP, is the major contributor to the expression pattern of *Cyc E*, *Cyc A* and *Cyc B*. Importantly, while a general transcription factor may be regarded as having a “generic function”, our findings emphasize the unique, unanticipated functions of *Drosophila* TRF2 as an essential factor for specific major cellular processes.

MATERIALS AND METHODS

Drosophila melanogaster Schneider S2R+ Cells

Drosophila melanogaster Schneider S2R+ adherent cells were cultured in Schneider's *Drosophila* Media (Biological Industries) that was supplemented with 10% heat-inactivated FBS and Penicillin 100 units/ml Streptomycin 0.1mg/ml (Biological Industries).

Generation of dsRNA probes

All dsRNA probes were chosen based on http://www.dkfz.de/signaling/e-rnai3// and http://www.flyrnai.org/snapdragon as described in (69). Primer sequences used for the generation of dsRNA probes are provided in Table 1. DNA fragments corresponding to each dsRNA were subcloned into both pBlueScript SK+ and KS+. The dsRNA probes were generated by PCR amplification of the DNA using T7 and T3 primers, followed by *in vitro* transcription of templates in both pBlueScript orientations using T7 RNA polymerase. Resulting RNA products were annealed to generate the dsRNA probes.

RNA interference (RNAi)

For 6 well plate, 1.25×10^6 cells/well were resuspended and seeded in empty Schneider's *Drosophila* Media (Biological Industries) with 30µg/ml dsRNA directed against different genes for 1 hour. Next, two volumes of complete medium were added to the wells and cells were incubated for 3 additional days.

Western blot analysis

Knockdown of TRF2 and TBP was verified by western blot analysis using anti-TRF2 and anti-TBP polyclonal antibodies (generous gift from Jim Kadonaga). Cyclin E levels were analyzed by the 8B10 antibodies (generous gift from Helena Richardson) (73). The levels of Actin or γ-Tubulin, as a loading control, were detected using either mouse monoclonal anti-Actin (Abcam, 8224) or anti- γ-Tubulin (Sigma, GTU-88) antibodies. Anti-Cyclin A and -Cyclin B concentrated monoclonal antibodies (Developmental Studies Hybridoma Bank, A12 and F2F4, respectively) were tested as well, however no endogenous proteins were detected, possibly due to technical limitations.

TBP expression vector

The coding sequence of *Drosophila* TBP was amplified by PCR and cloned with an N-terminal Flag-HA tag into the pAc5.1 vector (Life Technologies) using cDNA from S2R+ cells as template and the following primers: Forward (containing an XhoI site, underlined)
5' CCGCTCGAGGACCAAATGCTAAGCCCCA 3' and reverse (containing an AgeI site, underlined) 5' AGCACCGGTTTATGACTGCTTCTTGAACTTCTTTAA 3'
Plasmid sequence was verified by sequencing.

RNAi-coupled overexpression

For 6 well plate, 1.25×10^6 *Drosophila* S2R+ cells/well were resuspended and seeded in empty medium with 30µg/ml dsRNA directed against TRF2 for 1 hour. Next, two volumes of complete medium were added to the wells and cells were incubated for 3 days. Three days post dsRNA treatment, cells were transfected with the TBP-pAc expression vector (930 ng) or an empty vector control using the Escort IV reagent

(Sigma). Media was replaced 18-24 hrs post transfection. Cells were harvested 36-48 hrs post transfection and RNA was purified and analyzed by RT-qPCR. Each qPCR experiment was performed in triplicates. The graphs represent an average of 3 independent experiments. Error bars represent SEM.

RNA-seq analysis

S2R+ cells were treated with dsRNA probes against *Trf2* (probe #1) and *Tbp*, and harvested at two time points - 48h and 72h post RNAi treatment. For each time point, a matched mock control was collected separately.

RNA was extracted using Quick-RNA™ MiniPrep (Zymo Research), and 800ng of each sample was purified using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490). Libraries were prepared using NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770), following the manufacturer's instructions. NEBNext Multiplex Oligos for Illumina (NEB #E7335, NEB #E7500, NEB #E7710, NEB #E7730) were used. Libraries were pooled and a 1% PhiX library control was added. Single-end sequencing was performed on an Illumina NextSeq 500 machine. Reads were aligned to dm6 genome build using STAR (version 2.6.0a), and htseq-count (version 0.5.1p3, (74)) was used to count the reads mapped to each gene. Differential expression analysis of conditions was performed using the DESeq2 R package (75). Only genes with adjusted p-value < 0.1 were considered for subsequent analysis. GO terms analysis was carried out using STRING v11 (76). For all experiments, three independent biological replicates were compared and merged for subsequent analysis. RNA-seq Data is available at GSE133685.

RT-PCR

Total RNA was isolated using the PerfectPure RNA Cultured Cell kit (5 PRIME) or Quick-RNA^{TM} MiniPrep (Zymo Research). One microgram of the total RNA was reverse-transcribed into cDNA with M-MLV (Promega) or qScript Flex cDNA Kit (Quanta). Control reactions lacking reverse transcriptase were also performed to ensure that the levels of contaminating genomic DNA were negligible. Quantitation was performed by real-time PCR to determine the transcription levels of the endogenous genes. The expression levels were compared to *Gapdh2*. Primer sequences for real-time PCR are provided in Table 2. For all quantifications, the error bars represent ±S.E.M of at least 3 independent experiments; NS, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$. Statistical analyses were performed on log-transformed relative quantification (RQ) values using one-way ANOVA followed by Tukey's post hoc test, unless otherwise stated in the figure legend.

Flow cytometry analysis

For cell cycle distribution by Propidium Iodide (PI) staining, cells were harvested following 72h incubation with dsRNA, centrifuged for 5 min at 300g and fixed with 80% ethanol at 4ºC overnight. Before subjecting the cells to flow cytometry, the cells were centrifuged for 5 min at 300g, washed in 1 ml of Phosphate-buffered saline (PBS) and incubated for 40 min at 4°C. The cells were then stained in PBS containing 50 µg/ml PI (Sigma) and 50 µg/ml RNase A (Roche). After incubation for 15 min at room temperature, fluorescence was measured using a FACSCalibur Becton Dickinson flow cytometer.

For analyzing apoptosis by Annexin V and PI staining, cells were harvested 72h following incubation with dsRNA probes and stained with Annexin V and PI

(MEBCYTO Apoptosis Kit; MBL). Fluorescence was measured using a FACSCalibur Becton Dickinson flow cytometer.

For G1 phase cell arrest by hydroxyurea (HU) and BrdU (5-Bromo-2´-Deoxyuridine)-PI staining, 72h following incubation with dsRNA, the medium was replaced with medium containing a final concentration of 1mM HU for 18h (77) (for control cells, the medium was replaced with a fresh medium). BrdU (40μM final concentration) was added to the medium for 2h. Cells were released from HU by medium replacement and, at 0, 2, 4, 6 or 8 hours following the release, cells were harvested, centrifuged and fixed with 80% ethanol at 4ºC overnight. Following fixation, cells were stained with FITC *(Fluorescein isothiocyanate)*-conjugated anti-Brdu antibodies (BD) and PI according to the provided protocol, and fluorescence was measured using BD FACSARIA III. All flow cytometry data was analyzed using the FlowJo software. Statistical analyses of flow cytometry and imaging flow cytometry data were performed in SPSS using two-tailed Students t-test. The number of times each experiment was repeated, is detailed in the figure legends.

It is of note that unfortunately, we were unable to synchronize cells in G2/M in a reversible manner using either Nocodazole or Colchicine, which cause microtubules depolymerization. Specifically, Nocadazole did not arrest the S2R+ cells in G2/M, while Colchicine, which has been used since the 1950s to inhibit mitotic progression, did cause enrichment of mitotic cells (Fig. 6b, d). However, this effect was irreversible (data not shown). Thus, Colchicine could not be used for G2/M block-release synchronization experiments.

616 **Immunostaining for fluorescence microscopy and Imaging flow cytometry**
617 **analysis**

618 Cells were RNAi-treated as described above. On day 4, 2ml of fresh medium was
619 added to the wells. To enrich for G2/M, as a control, Colchicine (Sigma) was added
620 to a final concentration of 350ng/ml. On the following day, the cells were harvested
621 and fixed with 4% Paraformaldehyde (PFA)/PBS (30 min), washed in PBST (PBS
622 containing 0.5% Triton x), blocked with PBS containing 1% BSA and 1% serum (1h),
623 and incubated with phospho-Histone H3 (Ser10) antibody (1:200, Cell Signaling
624 Technology #9701) for 1h at RT, followed by overnight at 4°C. Cells were washed,
625 stained with the secondary antibody (1:500, DyLight 488, ab96883), and then
626 counter-stained with 10µg/ml Hoechst 33342 (Sigma). For microscopy analysis,
627 samples were also stained for filamentous Actin with 3.5µM Acti-stain 670 Phalloidin
628 (Cytoskeleton, Inc. Cat. # PHDN1). Following staining, samples were subjected to
629 imaging flow cytometry, confocal microscopy or wide-field fluorescence microscopy
630 analysis.

631

632 **Microscope image analyses**

633 High resolution images were acquired using a Leica SP8 confocal microscope, and
634 high-throughput images for quantitative analysis were acquired using a Leica DMi8
635 microscope. Three separate experiments were performed and captured at 20x
636 magnification. For each treatment, approximately 275 frames were acquired and
637 analysed. The total number of Alexa 488 anti-phosphor-Histone H3 (Ser10) (PH3)
638 labeled cells, and the total number of Hoechst stained cells, were calculated using
639 the Fiji distribution of ImageJ.
640 Analysis workflow:

641 1. The raw PH3 channel images were enhanced using brightness and contrast, and
642 then the background was subtracted by reducing the Gaussian blurred filtered
643 image of the enhanced image. Next, a median filter was applied to smoothen the
644 image and an Otsu threshold was applied to get the binary image of all mitotic
645 nuclei. Finally, watershed was implemented to separate touching nuclei. Mitotic
646 cells were counted, eliminating small debris and noise.
647 2. To analyze the Hoechst channel, the background was subtracted by reducing the
648 Gaussian blurred filtered image of the original image. Next, a Moments threshold
649 was applied to get the binary image of total nuclei. Finally, watershed was
650 implemented to separate touching nuclei. Nuclei were counted while eliminating
651 small debris and noise.

652 The ratio between the number of mitotic cells and the total number of cells yields the
653 mitotic index for each treatment.
654 All manipulations in the images were made evenly across the entire field.
655 The Fiji macros will be shared upon request.
656
657 **Multispectral imaging flow-cytometry (IFC) analysis**
658 Cells were imaged using multispectral imaging flow cytometry (ImageStreamX mark
659 II imaging flow-cytometer; Amnis Corp, Seattle, WA, Part of EMD Millipore). Each
660 experiment was performed 3 times. In each experiment, at least 40,000 cells were
661 collected from each sample, and data were analyzed using the image analysis
662 software (IDEAS 6.2; Amnis Corp). Images were compensated for fluorescent dye
663 overlap by using single-stain controls. Imaging flow cytometry results were analyzed
664 by calculation of a set of parameters, termed “features”, performed on a defined area
665 of interest, termed “mask”. The serial gating strategy to identify the mitotic cell

population was as follows: Single cells were first gated using the area and aspect ratio features on the bright-field (BF) image (the aspect ratio, which indicates how round or oblong an object is, is calculated by division of the minor axis by the major axis). Uncropped cells were gated using the centroid X (the number of pixels in the horizontal axis from the upper left corner of the image to the center of the mask) and area features. Focused cells were gated using the Gradient RMS feature, as previously described (78) (Additional file 5: Figure S4a-c). Following this standard gating series, the mitotic cell fraction of the entire cell population was identified using the staining intensity for PH3 AF488 (channel 2), and mitotic cells were gated as the high intensity population of PH3 staining within all focused cells (Additional file 5: Figure S4d,e).

For a more complex analysis, we performed a second gating series. Focused cells were first gated for G2/M based on DNA (Hoechst) intensity (Additional file 5: Figure S4) and then gated for mitotic cells, as previously, by high PH3 intensity. To further subdivide into the specific cell division phases, we gated according to nuclear morphology based on the spot count and aspect ratio intensity features (see Additional file 5: Figure S4f, g for detailed masking and gating). As it was previously shown that serine 10 of histone H3 becomes dephosphorylated during telophase (79), the telophase population was derived from the negative PH3-stained cells, based on the BF circularity feature and DNA aspect ratio intensity (see Additional file 5: Figure S4h for detailed masking and gating). Full details of all masking, features and analysis strategies are included in the legend of Additional file 5: Figure S4.

Identification of unique TRF2-interacting proteins

To identify the proteins that are in complex with TRF2 (the evolutionarily conserved short TRF2), we used inducible FLAG-HA-tagged TRF2 S2R+ cells (69). As

controls, we used inducible S2R+ for FLAG-HA-long TRF2 (69) or FLAG-HA-TBP (generated as in (69)). Cells were either induced by copper sulfate or left untreated. Protein extracts were prepared and TRF2-containing complexes were immuno-precipitated using anti-FLAG M2 affinity gel (Sigma). Following the IP, TRF2-containing complexes were released with a FLAG peptide (Sigma). Samples were resolved by SDS-PAGE. Proteins that were purified from TRF2-induced cells were separated to two samples: proteins larger or smaller than 40 kDa. Samples were subjected to Mass spectrometry analyses (The Smoler Protein Research Center, Technion). Briefly, samples were digested by trypsin, analyzed by LC-MS/MS on Q-Exactive Plus (ThermoFisher) and identified by the Discoverer software (with two search algorithms: Sequest (ThermoFisher) and Mascot (Matrix science) against the *Drosophila melanogaster* section of the NCBI non-redundant and Uniprot databases, and a decoy database (in order to determine the false discovery rate). All the identified peptides were filtered with high confidence, top rank, mass accuracy, and a minimum of 2 peptides. High confidence peptides have passed the 1% FDR threshold. Semi-quantitation was done by calculating the peak area of each peptide. The area of the protein is the average of the three most intense peptides from each protein. The results are provided in Additional file 8: Table S3.

Visualization of publicly available ChIP-exo data

A genome browser session (https://genome.ucsc.edu/s/Anna%20Sloutskin/dm3_ChIP_Exo) based on available TRF2, GFZF and M1BP ChIP-exo bedgraph files (GSE97841, GSE105009) (57, 58) was created. The session contains an “Overlap” track (the ChIP-exo peaks that were identified as overlapping in the ±50bp window relative to FlyBase TSS) and the

716 "trustedTSS" track that is based on 5' GRO-seq (GSE68677) and PRO-Cap
717 (GSM1032759) data. The relevant interval (±50bp or 100bp) is indicated.
718

719 **List of abbreviations**

720 ChIP-exo - chromatin immunoprecipitation combined with exonuclease digestion
721 followed by high-throughput sequencing
722 DPE - downstream core promoter element
723 GFZF - GST-containing FLYWCH zinc-finger protein
724 M1BP - Motif 1 binding protein
725 Pol II - RNA polymerase II
726 TBP - TATA-box-binding protein
727 TRF2 - TBP-related factor 2
728 TSS - Transcription start site
729
730

731 **Declarations**

732 **Ethics approval and consent to participate**

733 Not applicable
734

735 **Consent for publication**

736 Not applicable
737

738 **Availability of data and materials**

739 RNAseq data generated during the current study is available in the GEO repository
740 (GSE133685).

741 ChIP-exo data analyzed during the current study was downloaded from the GEO
742 (GSE97841, GSE105009).
743
744 **Competing interests**
745 The authors declare that they have no competing interests
746
747 **Funding**
748 This work was supported by grants from the Israel Science Foundation to T.J.-G.
749 (no. 798/10 and no. 1234/17).
750
751 **Authors' contributions**
752 A. Kedmi prepared RNA samples and O. Yaron and A. Sloutskin performed RNA-
753 seq experiments. T. Doniger and A. Sloutskin analyzed the RNA-seq experiments
754 and performed bioinformatics analysis. A. Kedmi, N. Epstein, L. Gasri-Plotnitsky and
755 D. Ickowicz performed and/or analyzed flow cytometry experiments. A. Kedmi, A.
756 Sloutskin and D. Ideses performed reverse transcription-qPCR analysis. I. Shoval
757 and Z. Porat advised and performed the analysis of ImageStream® experiments, A.
758 Kedmi and I. Shoval performed and analyzed fluorescence microscopy experiments.
759 E. Darmon and D. Ideses performed western blot analyses. A. Sloutskin performed
760 the statistical analysis. A. Kedmi, A. Sloutskin and T. Juven-Gershon designed the
761 study, planned experiments, analyzed results, and wrote the manuscript with input
762 from all authors.
763
764
765

766 **Acknowledgements**

767 We thank Jim Kadonaga for the generous gift of anti-TRF2 and anti-TBP antibodies,
768 for invaluable advice and assistance and for critical reading of the manuscript. We
769 thank Helena Richardson for the generous gift of anti-Cyclin E antibodies. We thank
770 Moshe Oren, Doron Ginsberg, Ze'ev Paroush, Sivan Henis-Korenblit, Shaked Cohen
771 and Itay Lazar for invaluable advice and assistance. We thank Dr. Jennifer Israel-
772 Cohen for assistance with statistical analysis. We thank Doron Ginsberg, Yaron
773 Shav-Tal, Galit Shohat-Ophir and Orit Adato for critical reading of the manuscript.
774 We thank Orit Adato and Sarit Lampert for technical assistance.

775

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Figure Legends

Fig. 1.

Trf2 Knockdown enhances early and late apoptosis in S2R+ cells. *Drosophila* S2R+ cells were incubated for three days with dsRNA directed against *Trf2*, *exd*, *Tbp* and *Trf1*. To examine whether *Trf2* knockdown triggers apoptotic cell death, cells were harvested following 72 h and stained with Annexin-V FITC and PI. **a** FACS analysis of a representative experiment. **b** Average percentages of cells undergoing early and late apoptosis. (n=4, *$0.01 < p \leq 0.05$, **$0.005 \leq p \leq 0.01$, ***$p < 0.005$, two-tailed Students t-test; comparison to mock).

Fig. 2.

The expression levels of distinct pro-apoptotic genes increase following knockdown of TRF2. *Drosophila* S2R+ cells were incubated for three days with dsRNA directed against TRF2, exd, TBP and TRF1. RNA was isolated from the cells and reverse transcribed to cDNA. qPCR experiments were used to analyze the RNA levels of the endogenous genes: **a** *Trf2*, *TBP, TRF1*, *rpr*, *hid*, *grim*, *scyl*, *Buffy*, *Debcl* and *Dronc* and **b** *Trf2*, *p53* and *TBP.* qPCR experiments were performed in triplicates, and the graph represents the average of three to eight experiments. Error bars represent the SEM. *p< 0.05, one-way ANOVA followed by Tukey's post hoc test as compared to the mock treatment of the relevant gene.

Fig. 3.

Trf2, but not *Tbp* or *Trf1* knockdown, affects cell cycle distribution. *Drosophila* S2R+ cells were incubated for three days with dsRNA directed against *Trf2*, *Tbp*, *Trf1* and *exd*. Cells were fixed with 80% ethanol and stained with Propidium-Iodide (PI) for

flow cytometry (FACS) analysis. **a** Cell cycle distribution histograms of a representative experiment. **b** Average cell cycle distribution determined by FACS analyses of eight independent experiments (*0.01 < $p \leq 0.05$, **0.005 ≤ $p \leq 0.01$, ***$p < 0.005$, two-tailed Students t-test; comparison to mock).

Fig. 4.

TRF2 is involved in S phase progression. *Drosophila* S2R+ cells were incubated for three days with dsRNA probes directed against *Trf2* or *Tbp*. Next, cells were either left untreated or treated with 1mM Hydroxyurea for 18h. The cells were allowed to resume cell cycle for 2h, 4h, 6h or 8h in fresh medium containing 40µM 5-Bromo-2´-deoxyuridine (BrdU), and were then fixed with 80% ethanol overnight and analyzed by FACS using BrdU-PI staining. Each histogram plots the PI fluorescence intensity (representing DNA content) on the X-axis, and cell count on the Y-axis.

Fig. 5.

Knock down of *Trf2* expression by RNAi reduces the expression of cyclin genes. *Drosophila* S2R+ cells were incubated for three days with dsRNA probes directed against *Trf2*, *exd*, *Tbp* and *Trf1*. RNA was isolated from the cells and reverse transcribed (RT) to cDNA. Real-time PCR (qPCR) experiments were used to analyze the RNA levels of the endogenous genes: **a** *Trf2*, *CycA*, *CycB*, *CycB3*, *CycC*, *CycD* and **b** *CycE*. As there were differences in *CycE* expression following RNAi with probe #1 compared to probe #2, four non-overlapping probes were used to knockdown *Trf2* expression towards the analysis of *CycE* expression. qPCR experiments were performed in triplicates, and the graph represents the average of

three to eight experiments. Error bars represent the SEM. *p< 0.05, one-way ANOVA followed by Tukey's post hoc test as compared to the mock treatment of the relevant gene. **c** Western blot analysis following TRF2 and TBP knockdown in S2R+ cells, using anti-TRF2 polyclonal antibodies and anti-Cyc E monoclonal antibodies. Actin was used as a loading control.

Fig. 6.

TRF2 regulates cell cycle progression through mitosis. *Drosophila* S2R+ cells were incubated for three days with dsRNA directed against TRF2 or TBP. Cells were then fixed with 4% PFA and stained with a phospho-Histone H3 (Ser10) antibody (PH3, mitotic marker; green), Hoechst (DNA visualization; Blue) and Phalloidin (filamentous Actin visualization; red). **a** Representative confocal microscopy images of *Drosophila* S2R+ cells in different mitotic phases. **b** Comparison of mitotic indices following each treatment, calculated based on microscopic analysis. Shown are the averages of three independent experiments, in which a total of 100,000-800,000 cells were analyzed for each treatment (*$0.01 < p \leq 0.05$, **$0.005 \leq p \leq 0.01$, ***$p < 0.005$, two-tailed Students t-test; comparison to mock). **c** Representative images obtained by imaging flow cytometry analysis. **d** Comparison of mitotic indices following each treatment, calculated based on imaging flow cytometry. Shown are the averages of three independent experiments, in which a total of 40,000 cells were analyzed for each treatment (*$0.01 < p \leq 0.05$, **$0.005 \leq p \leq 0.01$, ***$p < 0.005$, two-tailed Students t-test; comparison to mock). **e-f** Distribution of mitotic phases among all mitotic cells, based on imaging flow cytometry. Shown are the averages of three independent experiments, in which a total of 40,000 cells were analyzed for each treatment (*$0.01 < p \leq 0.05$, **$0.005 \leq p \leq 0.01$, ***$p < 0.005$, two-tailed Students t-

test; comparison to mock). Filled triangles indicate aberrant chromosomal morphology in Colchicine-treated cells. Phospho-Histone H3 Ser10-positive cells (**e**), were analyzed separately from the Phospho-Histone H3 Ser10-negative cells undergoing mitosis (**f**). Phospho-Histone H3 Ser10-negative cells undergoing mitosis (as identified by the imaging flow cytometer) were defined as cells in telophase (f). Notably, cell counts of telophase cells likely include Phospho-Histone H3 Ser10-negative doublet cells, which, even using the high-resolution Imagestream, could not be distinguished from telophase cells.

Fig. 7.

CycA and *CycB* expression is affected by TRF2 knockdown, and less by GFZF and M1BP. **a** Schematic representation of genes containing at least one binding site of the specified transcription factor at ±50bp relative to its TSS, as determined by FlyBase. ChIP-exo data was retrieved from GSE97841 and GSE105009. **b** Top enriched motif among the 4331 commonly bound promoters, as detected by MEME analysis. Its resemblance to Ohler Motif 1 is depicted by the motif logo derived by M1BP ChIP-exo (57). **c** The 4331 commonly bound regions were analyzed for core promoter composition. This promoter group was found to be depleted for the TATA-box motif and enriched for dTCT and the Motif 1 core promoter elements. p-values were adjusted using Bonferroni correction. ***$p< 10^{-5}$. **d-e** *Drosophila* S2R+ cells were incubated for three days with dsRNA probes directed against *Trf2* (probe #1), *gfzf*, *M1BP* or their combinations. RNA was isolated from the cells and reverse transcribed (RT) to cDNA. Real-time PCR (qPCR) experiments were used to analyze the RNA levels of the endogenous of *Trf2*, *gfzf* and *M1BP*, as well as *CycA*, *CycB*, *CycD* and *CycE* genes, as indicated. **d** Single knockdowns of *Trf2*, *gfzf* or *M1BP.* **e** Knockdown of multiple genes, as indicated. qPCR experiments were performed in

triplicates, and the graph represents the average of 4 independent experiments. Error bars represent the SEM. *p< 0.05, one-way ANOVA followed by Tukey's post hoc test as compared to the mock treatment of the relevant gene.

Additional files

Additional file 1: Figure S1. .pdf

A) Schematic representation of dsRNA TRF2 probes used in this study. *B*) Western blot analysis following TRF2 and *C)* TBP knockdown in S2R+ cells, using anti-TRF2 and anti-TBP polyclonal antibodies, respectively. Actin and γ-Tubulin were used as loading controls.

Additional file 2: Table S1. .xlsx

GO terms analysis of the RNA-seq data. Summary, as well as the exact GO terms are provided, according to the datasheet name. Analysis was performed sing STRING. Only genes with pAdj <0.1 were considered. DEseq2 output is presented for either *Trf2* or *Tbp* knockdown, as compared to mock at 72h post silencing.

Additional file 3: Figure S2. .pdf

TBP overexpression does not result in induction of pro-apoptotic gene expression. *Drosophila* S2R+ cells were depleted of *Trf2* by RNAi and then transfected with either pAc-empty (mock) or TBP expression vector. Cells were harvested 36–48h following transfection, RNA was purified and reverse transcribed to cDNA for RT-qPCR analysis. qPCR experiments were performed in triplicates, and the graph represents the average of 3 experiments. Error bars represent the SEM. *p< 0.05,

1129 two-tailed Students t-test; comparison to the same treatment without TBP
1130 overexpression.
1131

1132 **Additional file 4: Figure S3. .pdf**
1133 TRF2 is involved in S phase progression. *Drosophila* S2R+ cells were incubated for
1134 three days with dsRNA probes directed against *Trf2* or *Tbp*. Next, cells were either
1135 left untreated or treated with 1mM Hydroxyurea for 18h. The cells were allowed to
1136 resume cell cycle for 2h, 4h, 6h or 8h in fresh medium containing 40μM BrdU, and
1137 were then fixed with 80% ethanol overnight and analyzed by FACS using BrdU-PI
1138 staining. The PI fluorescence intensity (representing DNA content) is plotted on the
1139 X-axis (linear scale), and the BrdU-FITC fluorescence intensity (representing BrdU
1140 incorporation into the DNA) is plotted on the Y-axis (log scale).
1141

1142 **Additional file 5: Figure S4. .pdf**
1143 Gating and masking strategy for imaging-flow cytometry data analysis. *A*) Cells were
1144 gated for single cells, using the area and aspect ratio features on the BF image. *B*)
1145 Centered and uncropped cells were gated based on the centroid X (the number of
1146 pixels in the horizontal axis from the upper left corner of the image to the center of
1147 the mask) and area features. *C*) Focused cells were gated, using the Gradient RMS
1148 feature, as previously described (78). *D*) The G2/M population was gated out of the
1149 focused cells, based on DNA (Hoechst) intensity. *E*) Mitotic cells were gated from the
1150 G2M population, as the high intensity population of pH3 staining, based on the
1151 intensity feature of pH3 and Max pixel feature of pH3 (the largest value of the
1152 background-subtracted pixels contained in the input mask). *F*) To include only single
1153 positive pH3 stained cells, doublet cells were eliminated by gating the single cells

from the mitotic population, using the area and aspect ratio features of the BF. *G*) To discriminate between the different mitotic phases subpopulations, several masks were created: 1. A morphology mask that includes all pixels within the outermost image contour. 2. A threshold mask that includes the highest intensity pixels (indicated as percentages). 3. A range mask that selects components in an image within a selected size (μm), was used to eliminate small components. Furthermore, the following features were used on the combined masks:

1. Spot count - the number of connected components in an image.

2. Aspect ratio intensity - the aspect ratio weighted for fluorescence intensity.

These masks were combined and the features were calculated and plotted as follows:

Spot count of the combined mask: range (threshold 75%, M07) 20-5000, was plotted against the aspect ratio intensity of the combined mask: range (Threshold(Morphology(M07), 82%) 15-5000.

The prophase population was defined as having a more circular nuclear staining (aspect ratio intensity should be high), and was hence gated as one nuclear spot count with aspect ratio intensity bigger than 0.6. On the other hand, the metaphase population was defined as having a more elongated DNA distribution and was gated as one nuclear spot count with aspect ratio intensity smaller than 0.6. Finally, the anaphase population was gated as cells with two nuclear spots having aspect ratio intensity less than 0.6. (80). *H*) As serine 10 of histone H3 becomes dephosphorylated during telophase, the telophase population was derived from the negative pH3-stained cells. To identify telophase pairs, an object mask was created (which segments images to closely identify the area corresponding to the cell). The circularity feature (which measures the degree of the mask's deviation from a circle)

of the object mask (BF), was plotted against the aspect ratio intensity of the M07 DNA mask. Telophase cells were gated as having the lowest BF circularity and as the most elongated, based on DNA stain (lowest aspect ratio intensity (M07)).

Additional file 6: Table S2. .doc

Number of cells in each mitotic phase within phospho-Histone H3 Ser10-positive mitotic cells or telophase.

Additional file 7: Figure S5. .pdf

Drosophila S2R+ cells treated with Colchicine display aberrant chromosomal morphology. Colchicine-treated cells were fixed with 4% PFA and stained with a phospho-Histone H3 (Ser10) antibody (green), Hoechst (DNA visualization; blue) and Acti-stain 670 Phalloidin (filamentous Actin visualization; red). *A*) Shown are representative images of cells in different mitotic phases obtained by imaging flow cytometry analysis. *B*) Quantitation of cells with aberrant DNA morphology within mock and colchicine-treated cells using imaging flow cytometry analysis. Prophase- and metaphase-gated cells within either mock or colchicine-treated S2R+ cells, were further gated using calculations of the following features:

1. The Delta centroid XY feature (which measures the distance in microns between the centroid feature of two images using the user provided masks) was calculated using the BF default mask and Hoechst channel mask of the 60% most highly intense pixels. Cells with centered nucleus will get a lower value while polar located nucleus will get a higher value. 2. The Max contour position feature (the location of the contour in the cell that has the highest intensity concentration; the score is between 0 to 1, with 0 being the object center and 1 the object perimeter). To

distinguish between central vs. polar location of the dividing nucleus, the Delta-centroid XY was plotted against the Max contour position, and polar DNA was gated as having the highest values of both features. Depicted are the analyses of cells in prophase and metaphase. Cells in anaphase are not shown due to the low number of cells observed in anaphase (Additional file 6).

Additional file 8: Table S3. .xlsx

Mass-spectrometry data for short TRF2, long TRF2 and TBP proteins.

Additional file 9: Figure S6. .pdf

Knockdown of *M1BP* specifically elevates the expression of *CycA* and ribosomal protein genes. *Drosophila* S2R+ cells were incubated for three days with dsRNA probes as indicated in the legend. RNA was isolated from the cells and reverse transcribed (RT) to cDNA. Real-time PCR (qPCR) experiments were used to analyze the RNA levels of the endogenous genes. qPCR experiments were performed in triplicates, and the graph represents the average of 3 experiments ± SEM. *A) CycA* expression levels are reduced following *Trf2* knockdown, but not *gfzf* or *M1BP* knockdown. *p< 0.05, one-way ANOVA followed by Tukey's post hoc test as compared to the mock treatment of the relevant gene. *B)* Additional ribosomal genes are influenced by *M1BP* knockdown, however not all genes are upregulated in response to *M1BP* knockdown. *CG12493* and *sgll* genes were previously shown to be downregulated upon *M1BP* knockdown (62).**p< 0.01, two-tailed Students t-test; compared to mock treatment.

Table 1.

Primers for generation of dsRNA probes

dsRNA probe	Forward primer	Reverse primer
Trf2 #1	ATAGGTACCGGCAACCGGCAGTAAAAATA	ATAACTAGTACTCCACATTTGATCCCTGC
Trf2 #2	ATACTCGAGAACAGAAGGAGCAGCATCGT	ATAACTAGTTATTTTTACTGCCGGTTGCC
Trf2 #3	ATAGGTACCAAGGAGAACCAATCGCCGAAT	ATAACTAGTATTAGAAGAACTTAAGCGATC
Trf2 #4	ATACTCGAGCAATCTGACTTGAATCCCGG	ATAACTAGTTCATCTGAAGCTTGTCGCG
exd	ATAACTAGTTCGATGGTGCTGACAATGCC	ATAGGTACCGGGGCTTAGATCCTGATGGAG
Trf1	GGGGTACCGGACAGGGATAATGTGGCTG	AAACTAGTGGCTTGACCATGCGATAGAT
Tbp	GGGGTACCACATGATGCCCATGAGTGA	AAACTAGTAATGGGGAATATCTTGTCGAAG
rpr	AAGGTACCACGAAAGAAAAGTGTGTGCG	AAACTAGTTGCAATTTTTAGCCAACTTCG
scyl	AAGGTACCTACTACGCTGCTGACGAGGA	AAACTAGTATCACCATTAGTTGGTGGGCG
p53	AAGGTACCGATGCTGCAGGACATTCAGA	AAACTAGTCTCGGCTATCATTGCTCTCC
M1BP	GGGGTACCATATTAACACGAAACACCGGG	AAACTAGTACCTTGGTGTCGTCGATCTC
gfzf	GGGGTACCTCAGCATCTGTTCCACTTCG	AAACTAGTGTGTGTGAATGTGGGTCGAG

Table 2.

Real-time PCR (qPCR) primers

Gene	Forward primer	Reverse primer
Gapdh2	TTCCTCAGCGACACCCACTC	ATGACGCGGTTGGAGTAGCC
Trf2	GGAATCGTCTTCTGGGGACT	GACGACTCCTGTTGGCTTTG
CycA	TGGGCACGGCAGCTATGTAT	CCTGCGCCTTGGTGTAACTG
CycB	CGAGCACCATACGATGTCCA	TTGAGCAAGTGCAGCGACAG
CycB3	TCCCAGAGACTGCTCCAAGC	CATGGCGTAGTGGGACACCT
CycC	CACCGATGTCTGCCTGCTC	GCACGATCTCCTGGACCTTG
CycD	AGGTCGAGGAGAAGCACCAC	CCTCGGCACACACTTCCAT
CycE	CTCGGTTTTGAGCCTCCATC	AGACAACGGGCGAGGTGTAG
Tbp	TCAGCTCCGGCAAGATGGTG	GCAGGGAAACCGAGCTTTTGG
Trf1	AGAAGCTGGGATTCCCCGTA	GCACGTGGTTGAGGTTCTCC
exd	GCGAAATCAAGGAGAAGACCGTCC	CCTCGGCAATCAGCATGTTGTCC
rpr	CATACCCGATCAGGCGACTC	GTGTACTGGCGCAGGGTTTC
hid	CGACCTCCACGCCGTTATC	GCTCTGGTACTCGCGCTCAT
grim	TTTGGCCCAGATCTTCTGCT	GCATCAGTCACGTCGTCCTC
Debcl	ACAGCATGGGCGAGGAACT	ATGTCGCTGTCCTCCAGCTC
scyl	ATAATCCGCGTGTCGGAGAA	CCGTATCCGAATCGACCTTG
buffy	TTCTCAGGGTCGTTGCCTGT	TGGAGGTGGAGCCCAGTATG
p53	TTAGCGTTGAGCCTTTGACG	CAGGGGGACTACAACGGAAA
M1BP	AATTTGGCTGCGAACTCTGT	CAGCGGCCACAGTACTTACA
gfzf	GAACCCACCGGATATGTCAC	TGCTGGCAGGGTCTTAAGTT
RpLP2	GACATGGGCTTCGCTCTCTT	GTGAACGGATGGGTGCTACA
RpS12	CAAGCGTCAGGCTGTTCTGT	CAGCTTCTTGTGCGAGTCCA
CG12493	GACAACCAATTGGATCAGGAAAG	AGATTCACCATCGGGCATATT
sgll	ATTCGAAGGATGAGCCAAGG	CCAGTCTCGGAATACACAGAAG
RpL30	CAAATACTGCCTGGGCTACA	TACTCGATCTCGGACTTCCTC
RpLP1	CACTTCGACATGTCCACCAA	CCTTCAGGATGGTGTTGATCTT

Table 3.

Enrichment of TRF2, TBP, M1BP and GFZF in FLAG-immuno-affinity purified complexes from inducible S2R+ cells

Name of purified (/co-purified) protein	Enrichment of purified (/co-purified) proteins within		
	Short TRF2-associated proteins	Long TRF2-associated proteins	TBP-associated proteins
TRF2	78.695	6.128	-
TBP	-	-	18.3
M1BP	1.795E7	-	-
GFZF	1.683E7	-	-

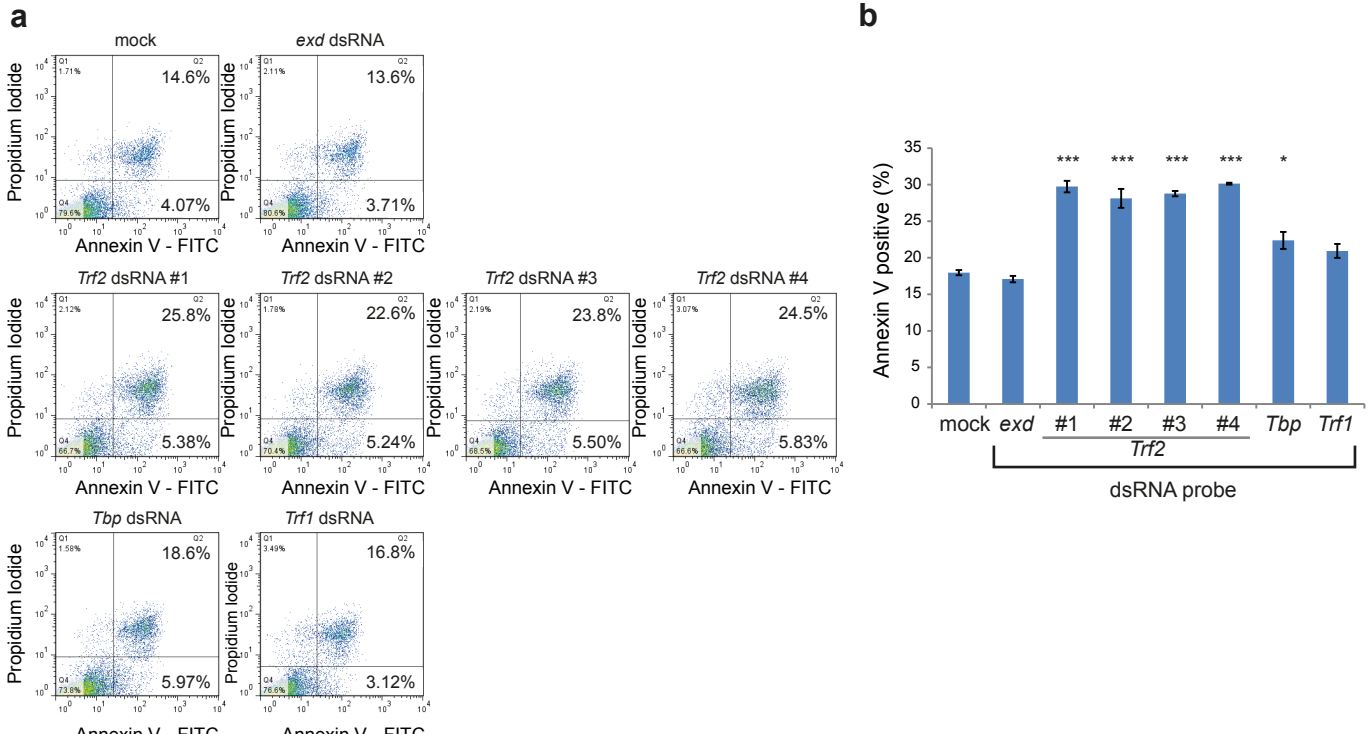
The values in the table represent the enrichment of each purified (/co-purified) protein in the indicated sample. The enrichment was calculated as the ratio between the mass spectrometry area (the average of the three most intense peptides from each protein) of the induced sample and the un-induced sample.

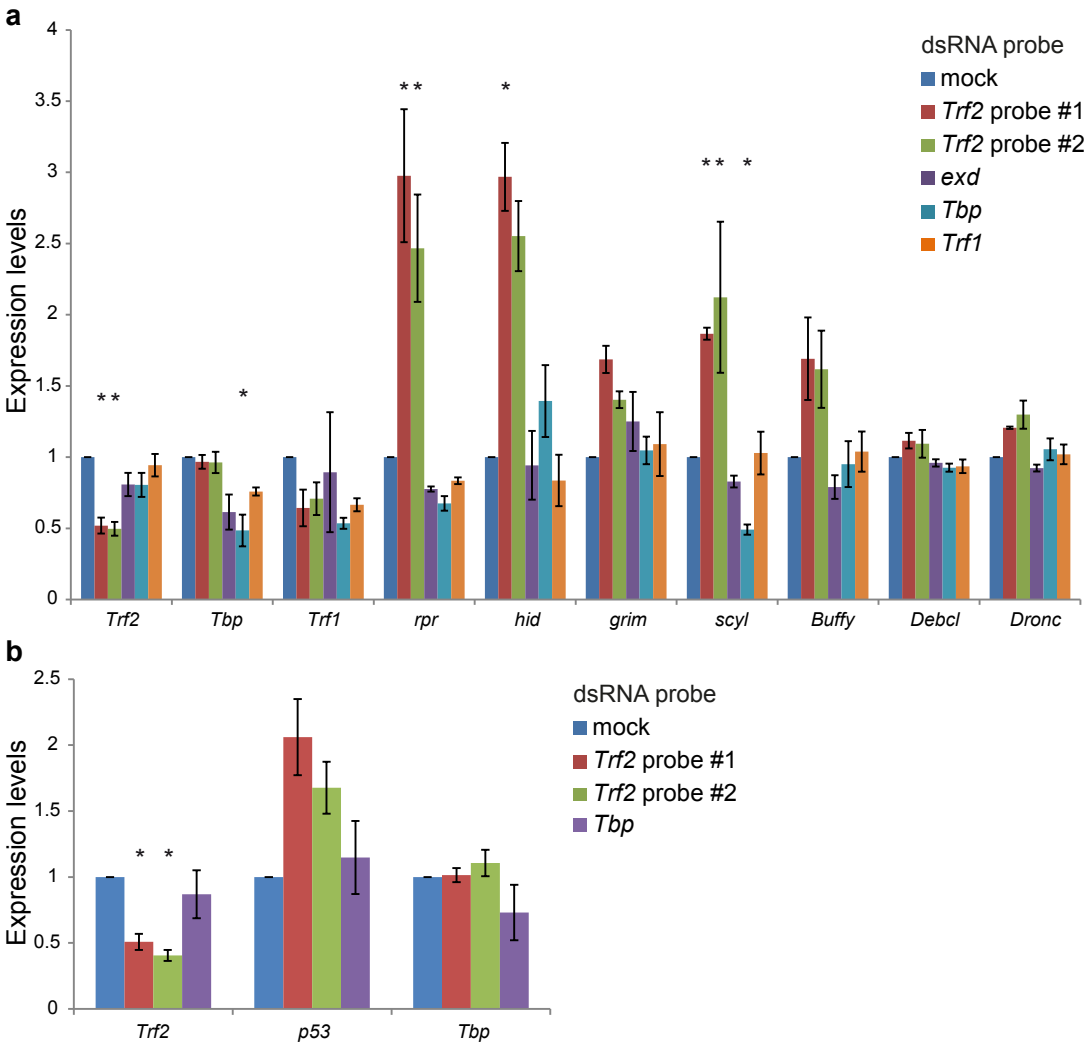
Table 4.

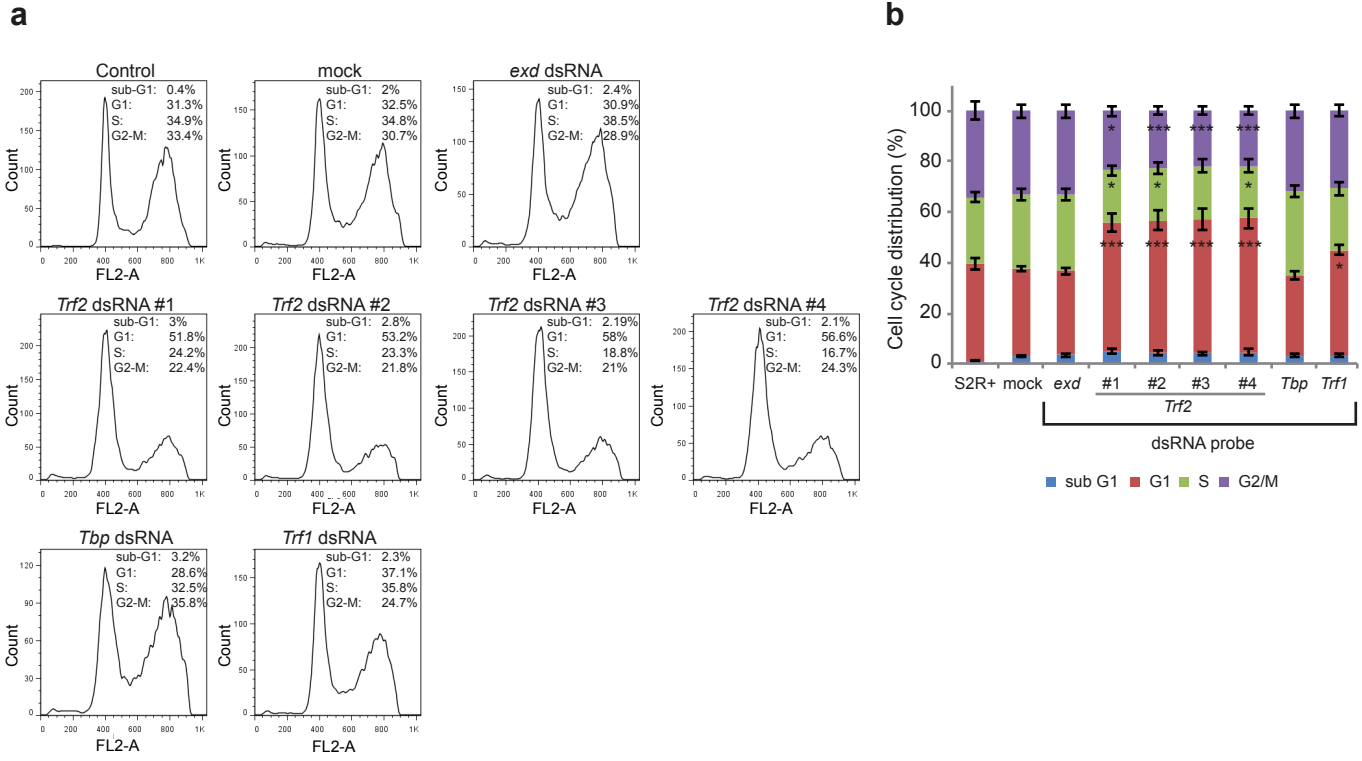
TRF2, M1BP and GFZF occupancy within -100 to +100 relative to the TSSs

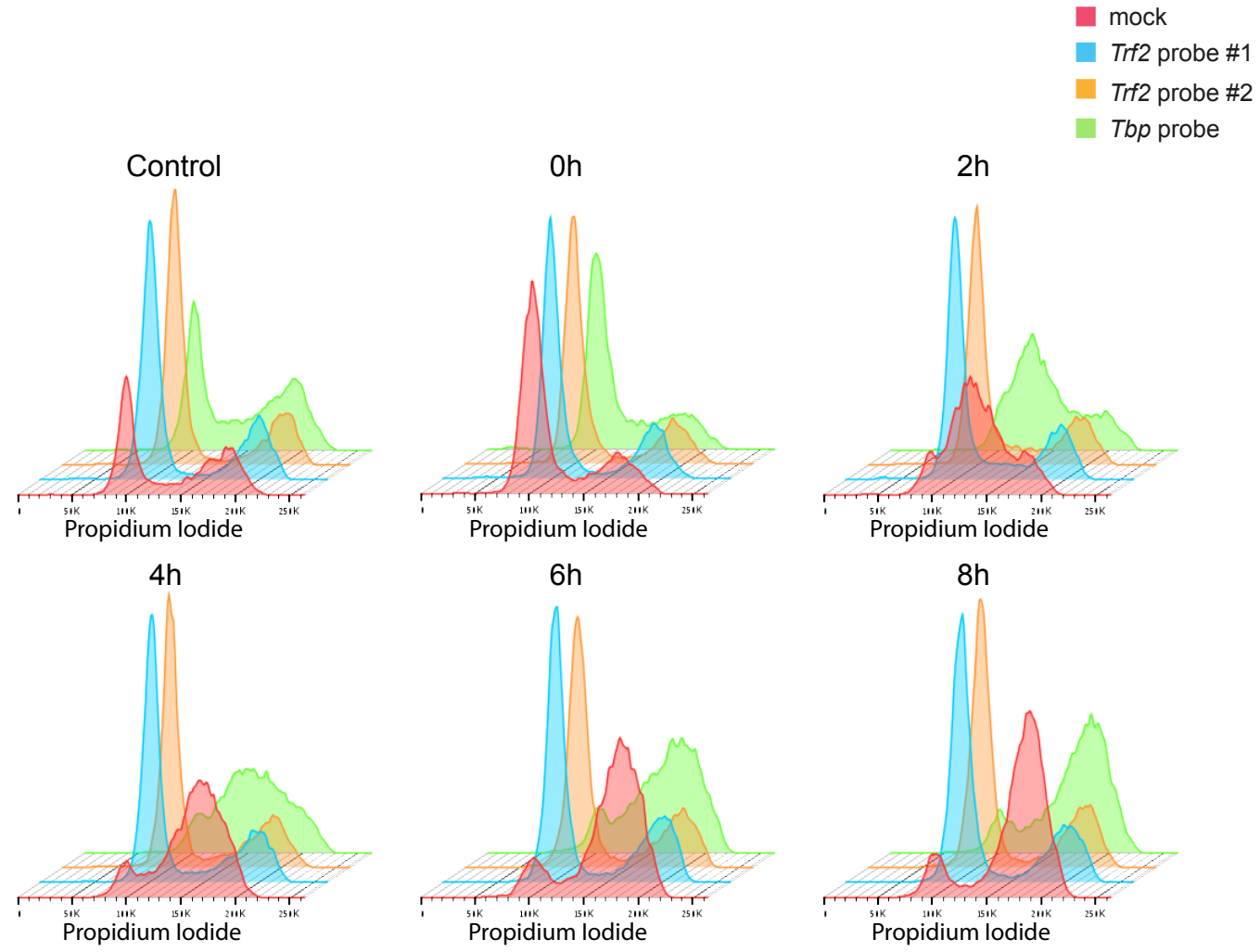
Gene name	TSS Location	TRF2			M1BP			GFZF		
		# of bound sites	Max. peak score	Avg. peak score	# of bound sites	Max. peak score	Avg. peak score	# of bound sites	Max. peak score	Avg. peak score
CycA_1	chr3L:11826617	1	54.16	54.16	59	126.93	30	2	112.85	87.78
CycA_2	chr3L:11826310	1	36.11	36.11	1	20.04	20.04	0	0	0
CycB_1	chr2R:18694432	15	150.45	38.11	47	106.89	26.72	3	114.64	62.1
CycB_2	chr2R:18694437	15	150.45	38.11	51	106.89	25.28	3	114.64	62.1
CycB_3	chr2R:18694449	15	150.45	38.11	60	106.89	27.28	3	114.64	62.1
CycB3	chr3R:20696533	4	90.27	40.62	128	140.29	36.69	9	157.63	72.05
CycE_1	chr2L:15746609	2	66.2	42.13	1	6.68	6.68	0	0	0
CycE_2	chr2L:15748123	4	60.18	31.6	8	53.44	30.9	1	136.14	136.14
CycC_1	chr3R:10715915	4	36.11	24.07	4	66.81	23.38	1	42.99	42.99
CycC_2	chr3R:10715922	4	36.11	24.07	3	66.81	26.72	1	42.99	42.99
CycD_1	chrX:15803691	2	42.13	24.08	7	46.76	20.04	0	0	0
CycD_2	chrX:15803682	2	42.13	24.08	7	46.76	20.04	0	0	0
RpL5	chr2L:22429377	74	210.63	51.56	142	140.29	33.07	29	148.68	48.36
RpL7	chr2L:10201108	23	210.63	50.50	121	374.11	62.44	5	118.23	65.92
RpL23	chr2R:18741912	38	132.4	46.25	200	327.35	51.27	68	449.61	99.00
RpL30	chr2L:19009229	21	102.31	30.38	141	233.82	47.81	16	231.08	76.58
RpLP1	chr2L:419957	14	156.47	44.28	148	180.38	37.78	19	195.25	74.10
RpLP2	chr2R:12473638	48	234.71	67.08	175	173.70	37.37	8	175.55	65.16

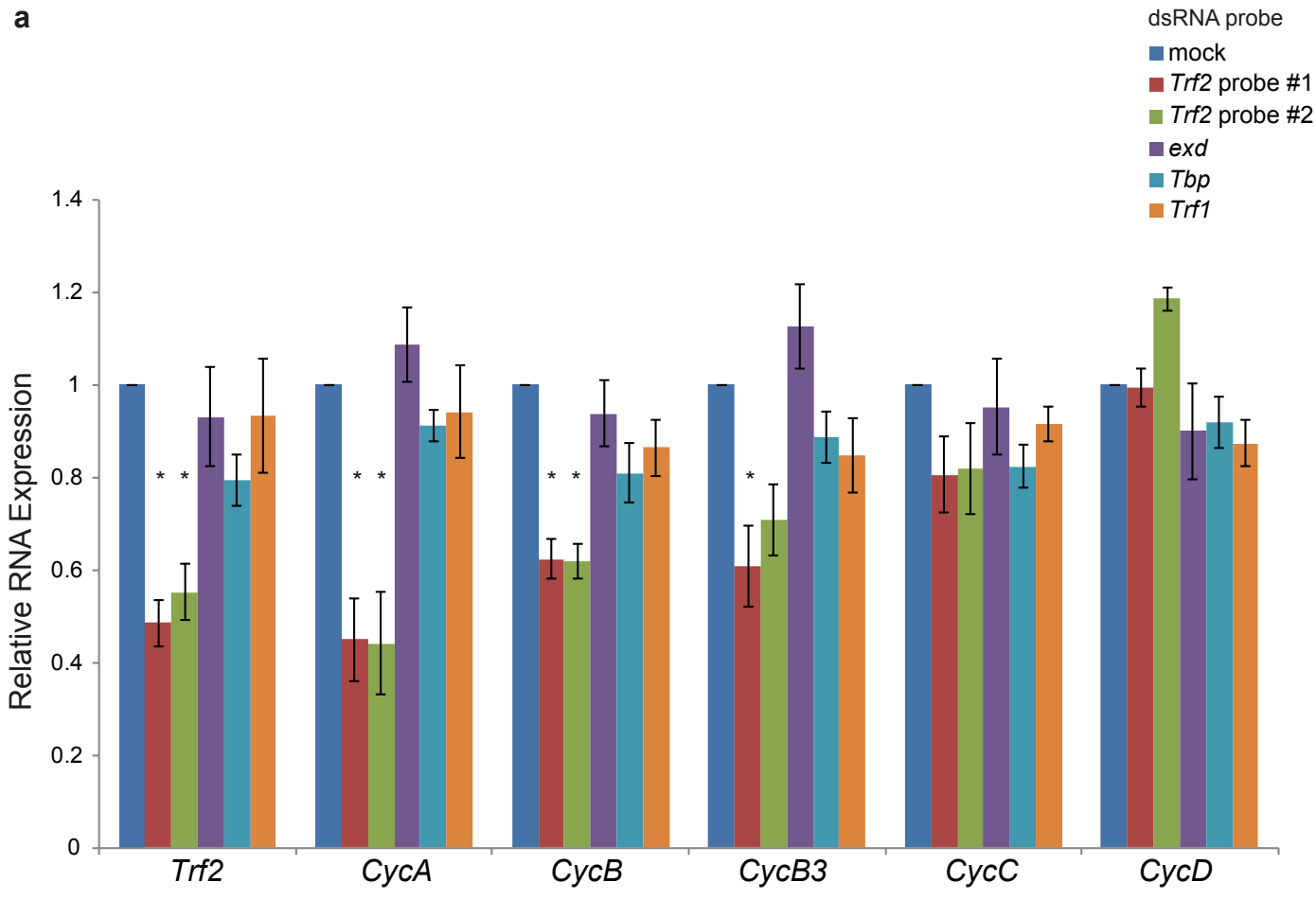
TRF2, M1BP and GFZF occupancy data (number of bound sites, the maximum peak scores and the average peak scores) was retrieved from ChIP-exo experiments performed in *Drosophila* S2R+ cells (GSE97841 and GSE105009) (64, 65). Due to variations in TSSs obtained by different methods, we relate to -100 to +100 relative to the TSSs peaks from available 5'GRO-seq and focused TSS analysis in S2 cells (GSE68677 and http://labs.biology.ucsd.edu/Kadonaga/drosophila.tss.data/) and PRO-cap analysis in S2 cells (GSM1032759).

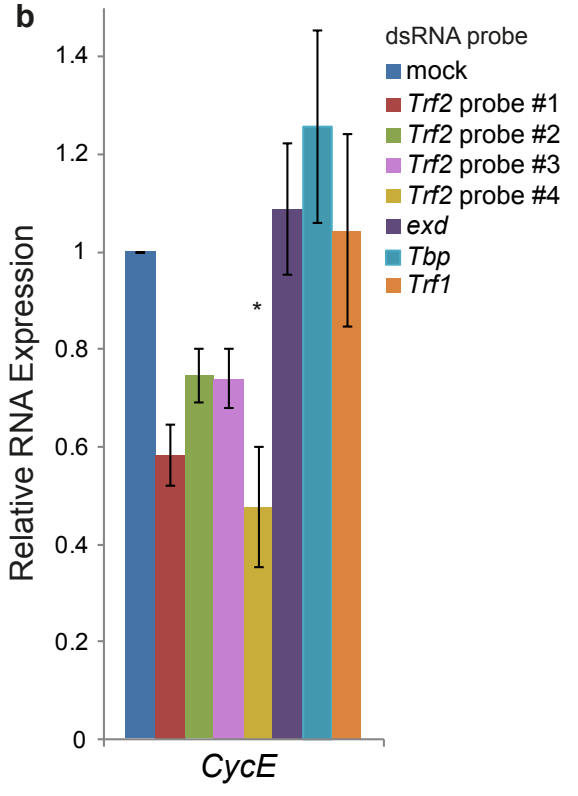

a
mock
14.6%
4.07%
exd dsRNA
13.6%
3.71%
Trf2 dsRNA #1
25.8%
5.38%
Trf2 dsRNA #2
22.6%
5.24%
Trf2 dsRNA #3
23.8%
5.50%
Trf2 dsRNA #4
24.5%
5.83%
Tbp dsRNA
18.6%
5.97%
Trf1 dsRNA
16.8%
3.12%
Propidium Iodide
Annexin V - FITC
b
Annexin V positive (%)
0
5
10
15
20
25
30
35
***
***
***
***
*
mock
exd
#1
#2
#3
#4
Tbp
Trf1
Trf2
dsRNA probe

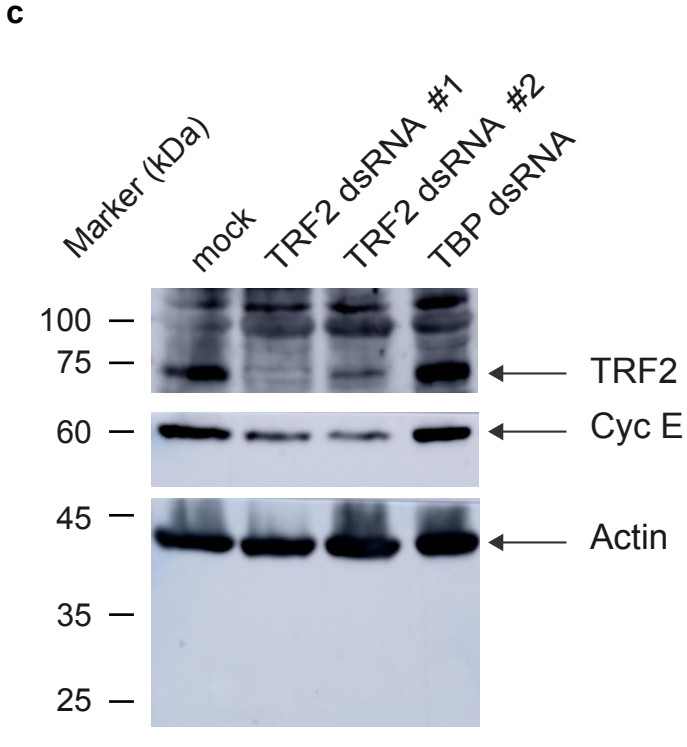

a
Expression levels
dsRNA probe
mock
Trf2 probe #1
Trf2 probe #2
exd
Tbp
Trf1
Trf2
Tbp
Trf1
rpr
hid
grim
scyl
Buffy
Debcl
Dronc
b
Expression levels
dsRNA probe
mock
Trf2 probe #1
Trf2 probe #2
Tbp
Trf2
p53
Tbp

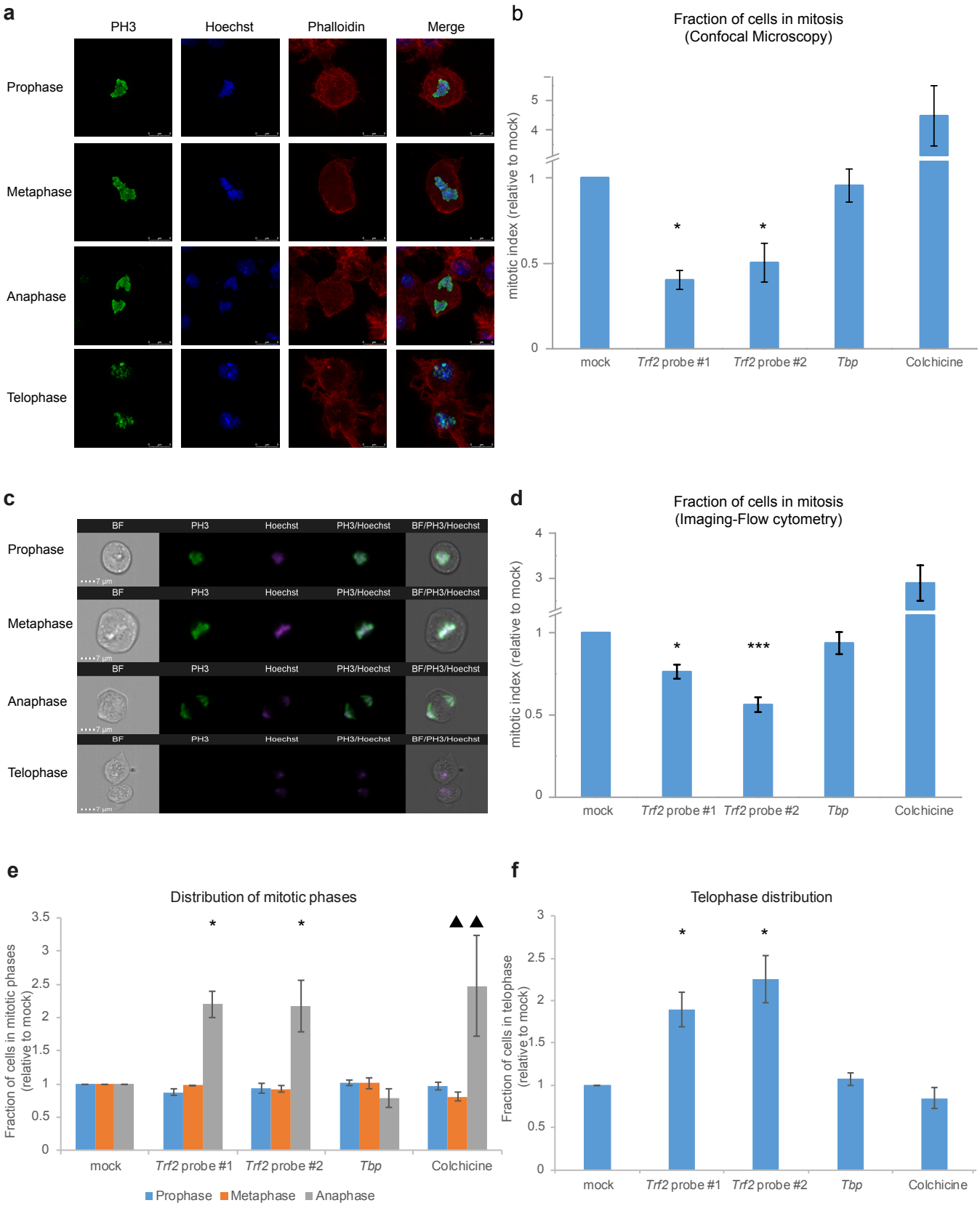

a
Control
sub-G1: 0.4%
G1: 31.3%
S: 34.9%
G2-M: 33.4%
mock
sub-G1: 2%
G1: 32.5%
S: 34.8%
G2-M: 30.7%
exd dsRNA
sub-G1: 2.4%
G1: 30.9%
S: 38.5%
G2-M: 28.9%
Trf2 dsRNA #1
sub-G1: 3%
G1: 51.8%
S: 24.2%
G2-M: 22.4%
Trf2 dsRNA #2
sub-G1: 2.8%
G1: 53.2%
S: 23.3%
G2-M: 21.8%
Trf2 dsRNA #3
sub-G1: 2.19%
G1: 58%
S: 18.8%
G2-M: 21%
Trf2 dsRNA #4
sub-G1: 2.1%
G1: 56.6%
S: 16.7%
G2-M: 24.3%
Tbp dsRNA
sub-G1: 3.2%
G1: 28.6%
S: 32.5%
G2-M: 35.8%
Trf1 dsRNA
sub-G1: 2.3%
G1: 37.1%
S: 35.8%
G2-M: 24.7%
Count
FL2-A
b
Cell cycle distribution (%)
S2R+
mock
exd
#1
#2
#3
#4
Tbp
Trf1
Trf2
dsRNA probe
sub G1
G1
S
G2/M

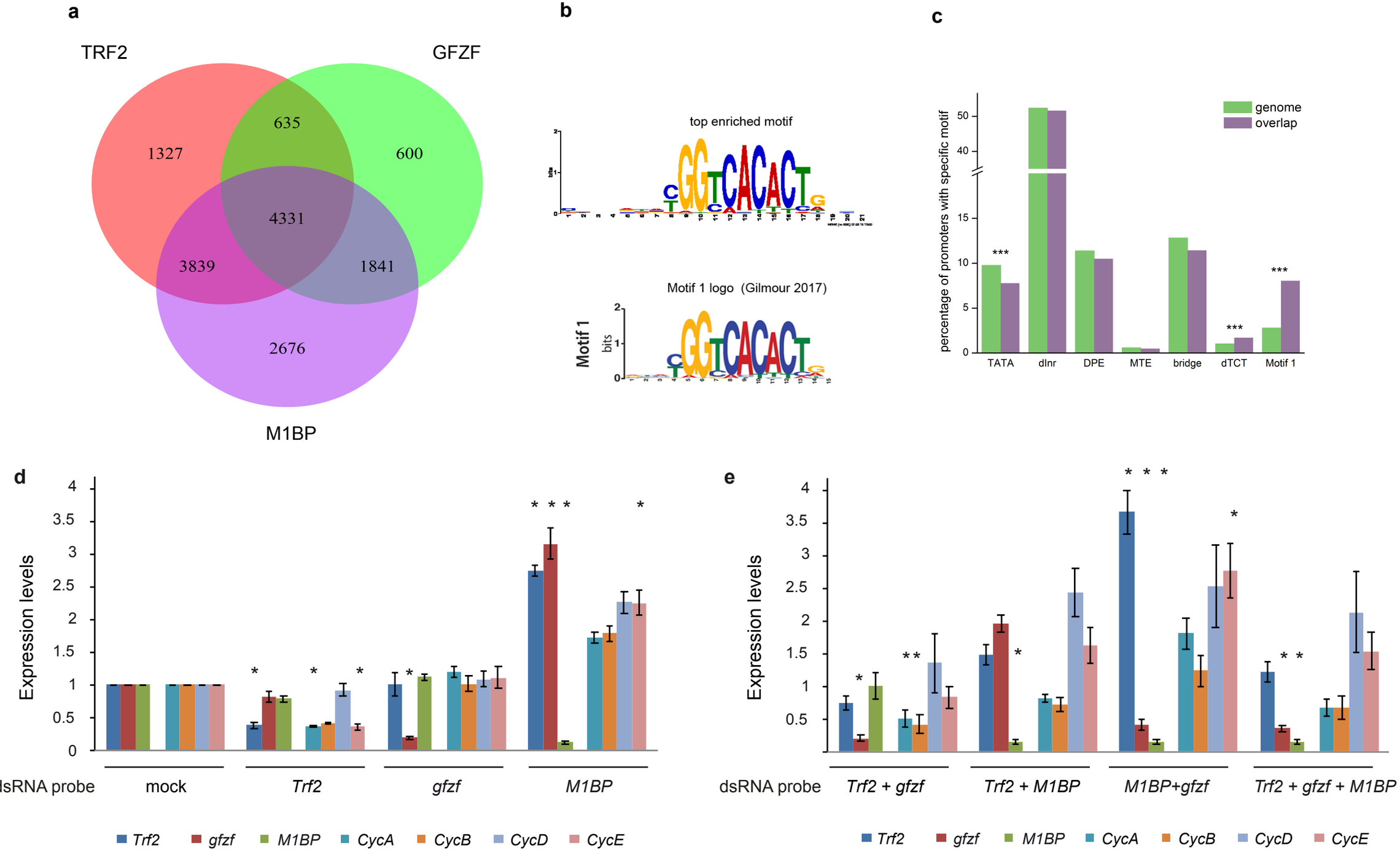

mock
Trf2 probe #1
Trf2 probe #2
Tbp probe
Control
0h
2h
4h
6h
8h
Propidium Iodide


a
dsRNA probe
mock
Trf2 probe #1
Trf2 probe #2
exd
Tbp
Trf1
Relative RNA Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Trf2
CycA
CycB
CycB3
CycC
CycD


b
dsRNA probe
mock
Trf2 probe #1
Trf2 probe #2
Trf2 probe #3
Trf2 probe #4
exd
Tbp
Trf1
Relative RNA Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CycE


c
Marker (kDa)
mock
TRF2 dsRNA #1
TRF2 dsRNA #2
TBP dsRNA
100
75
60
45
35
25
TRF2
Cyc E
Actin


a
PH3
Hoechst
Phalloidin
Merge
Prophase
Metaphase
Anaphase
Telophase
b
Fraction of cells in mitosis
(Confocal Microscopy)
mitotic index (relative to mock)
mock
Trf2 probe #1
Trf2 probe #2
Tbp
Colchicine
c
BF
PH3
Hoechst
PH3/Hoechst
BF/PH3/Hoechst
Prophase
Metaphase
Anaphase
Telophase
d
Fraction of cells in mitosis
(Imaging-Flow cytometry)
mitotic index (relative to mock)
e
Distribution of mitotic phases
Fraction of cells in mitotic phases
(relative to mock)
Prophase
Metaphase
Anaphase
f
Telophase distribution
Fraction of cells in telophase
(relative to mock)


a
TRF2
GFZF
M1BP
1327
635
600
4331
3839
1841
2676
b
top enriched motif
Motif 1 logo (Gilmour 2017)
Motif 1
bits
c
genome
overlap
percentage of promoters with specific motif
TATA
dInr
DPE
MTE
bridge
dTCT
Motif 1
d
Expression levels
dsRNA probe
mock
Trf2
gfzf
M1BP
Trf2
gfzf
M1BP
CycA
CycB
CycD
CycE
e
Expression levels
dsRNA probe
Trf2 + gfzf
Trf2 + M1BP
M1BP+gfzf
Trf2 + gfzf + M1BP
Trf2
gfzf
M1BP
CycA
CycB
CycD
CycE