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The FUSE/FBP/FIR/TFIIH system is a molecular machine programming a pulse of *c-myc* expression

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FarUpStream Element (FUSE) Binding Protein (FBP) binds the human *c-myc* FUSE *in vitro* only in single-stranded or supercoiled DNA. Because transcriptionally generated torsion melts FUSE *in vitro* even in linear DNA, and FBP/FBP Interacting Repressor (FIR) regulates transcription through TFIIH, these components have been speculated to be the mechanosensor (FUSE) and effectors (FBP/FIR) of a real-time mechanism controlling *c-myc* transcription. To ascertain whether the FUSE/FBP/FIR system operates according to this hypothesis *in vivo*, the flux of activators, repressors and chromatin remodeling complexes on the *c-myc* promoter was monitored throughout the serum-induced pulse of transcription. After transcription was switched on by conventional factors and chromatin regulators, FBP and FIR were recruited and established a dynamically remodeled loop with TFIIH at the P2 promoter. In XPB cells carrying mutant TFIIH, loop formation failed and the serum response was abnormal; RNAi depletion of FIR similarly disabled *c-myc* regulation. Engineering FUSE into episomal vectors predictably re-programmed metallothionein-promoter-driven reporter expression. The *in vitro* recruitment of FBP and FIR to dynamically stressed *c-myc* DNA paralleled the *in vivo* process.

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Introduction

The FarUpStream Element (FUSE) Binding Protein (FBP) and the FBP Interacting Repressor (FIR) have been reported to regulate *c-myc* transcription through the general transcription factor TFIIH. These proteins bind to FUSE, a DNA segment 1.7 kb upstream of the P2-start site that melts *in vitro* in response to stable or dynamic supercoiling forces (Michelotti *et al*, 1996; He *et al*, 2000; Kouzine *et al*, 2004). Though FBP and FIR have been speculated to be recruited to *c-myc* by transcriptionally generated supercoils to feedback regulate transcription as components of a hypothetical molecular 'cruise control', the *in vivo* evidence supporting this speculation has been lacking (Duncan *et al*, 1994; Michelotti *et al*, 1996; Liu *et al*, 2000, 2001).

c-Myc is a bHLH-zip master regulator of as many as 10–15% of genes through an estimated 25 000 binding sites in the human genome (Levens, 2002, 2003; Cawley *et al*, 2004). A crucial regulator of cell proliferation, growth, differentiation, senescence, and death (Spencer and Groudine, 1991; Marcu *et al*, 1992), the *c-myc* promoter itself receives input from dozens of diverse transcription factors channeled onto a large assortment of *cis* elements. As Myc protein and mRNA are each short-lived and scarce, Myc levels would seem likely to fluctuate in resting, normal cells, where it is almost ubiquitous (Spencer and Groudine, 1991; Grandori *et al*, 2000). Small changes in Myc levels alter cellular and organismal physiology, and contribute to pathologies, especially cancer (Trumpp *et al*, 2001; de la Cova *et al*, 2004; Moreno and Basler, 2004; Secombe *et al*, 2004). Myc levels in primary cells are modulated by serum starvation and repletion (Kelly and Siebenlist, 1986), though this maneuver fails in most tumor cell lines. No single signal in serum accounts for the full response of the *c-myc* promoter, nor has a final common pathway for convergent signals been revealed.

The *in vivo* operation of the FBP–FIR system and its mechanistic integration with conventional transcription factors at the *c-myc* promoter were studied during reactivation of serum-starved fibroblasts. These cells were monitored for *c-myc* expression, chromatin organization, and factor binding *in vivo*. These data suggest that *c-myc* becomes activated in two stages following serum re-addition. First, conventional components turn on transcription; thereafter regulation is transferred to a molecular subsystem using FBP and FIR both to monitor the supercoiling forces at FUSE, and to feedback regulate ongoing transcription by looping to TFIIH at the major P2 promoter. In *Xeroderma pigmentosum* B, where TFIIH is mutated, loop formation fails and *c-myc* is improperly programmed. The operation of this mechanism was further supported by using FUSE to re-engineer the output of the metallothionein promoter *in vivo*, as well as by using dynamic supercoils to license FBP and FIR recruitment to FUSE *in vitro*.

Results

FBP binding to DNA requires sustained supercoiling

FBP binds only when FUSE is single-stranded or embedded in supercoiled DNA, so some process or factor must first distort

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or destabilize the double helix (Duncan *et al*, 1994; Bazar *et al*, 1995; Michelotti *et al*, 1996; Kouzine *et al*, 2004). The torsional stress generated by the counter-rotation of the template and RNA polymerase during transcription melts FUSE even in linear templates (Kouzine *et al*, 2004). To test whether these transient dynamic supercoils melt FUSE long enough to bind FBP and FIR, an experiment was devised to observe recruitment to and release of these proteins from binding sites in linear templates upstream of promoters where the intensity of ongoing transcription was modulated.

T3 and T7 RNA polymerases were used to transcribe linear templates *in vitro* under conditions shown to drive dynamic supercoiling of upstream DNA (Figure 1A). FUSE was placed between divergent promoters in order to absorb the mutually reinforcing, negative supercoils trailing each polymerase (Kouzine *et al*, 2004). The rate of transcription, and hence the amount of torque applied to the template, was tuned with limiting GTP. Following transcription in the presence of FBP, formaldehyde was added both to terminate the reactions and to crosslink DNA-bound protein. The fixed protein–DNA complexes were harvested with anti-FBP and analyzed by PCR as for chromatin immunoprecipitation (ChIP). Low-level RNA synthesis supported little FBP binding (Figure 1B, α-FBP panel, lanes 4–6); at least 200 μM GTP was required for maximal FBP–template interactions (lanes 7 and 8) corresponding to the threshold of dynamic supercoiling required to FUSE (Kouzine *et al*, 2004). Stopping transcription (by chelating the Mg^{2+} obligatory for transcription, but not for FBP–DNA interaction) rapidly evicted most bound FBP (lane 9), as FUSE reverted to B-DNA (Kouzine *et al*, 2004). These results were confirmed by real-time PCR (Figure 1B, FBP IP graph).

To verify that releasing torsional stress discharged DNA-bound FBP, FBP–FUSE complexes in supercoiled plasmids were probed with $KMnO_4$ to assess DNA melting with or without subsequent linearization of the plasmid by rapid restriction enzyme digestion. Indeed the FUSE melting induced by FBP in supercoiled DNA vanished upon linearization (Figure 1C, lanes 2, 6, and 11). Therefore, torsional stress initiates and sustains FBP binding to FUSE.

Transient torsional stress allows sustained binding of FIR to FUSE

FIR was originally identified through protein–protein interaction with FBP. Owing to its tandem RRMs, a structural motif that binds single-stranded nucleic acids (usually RNA, but sometimes DNA), FIR was also tested for binding to dynamically stressed DNA (DeAngelo *et al*, 1995; Ding *et al*, 1999; Liu *et al*, 2000; Kielkopf *et al*, 2004). Although FIR did not bind to linear DNA, just like FBP, transcription by T3 and T7 RNA polymerases from divergent promoters

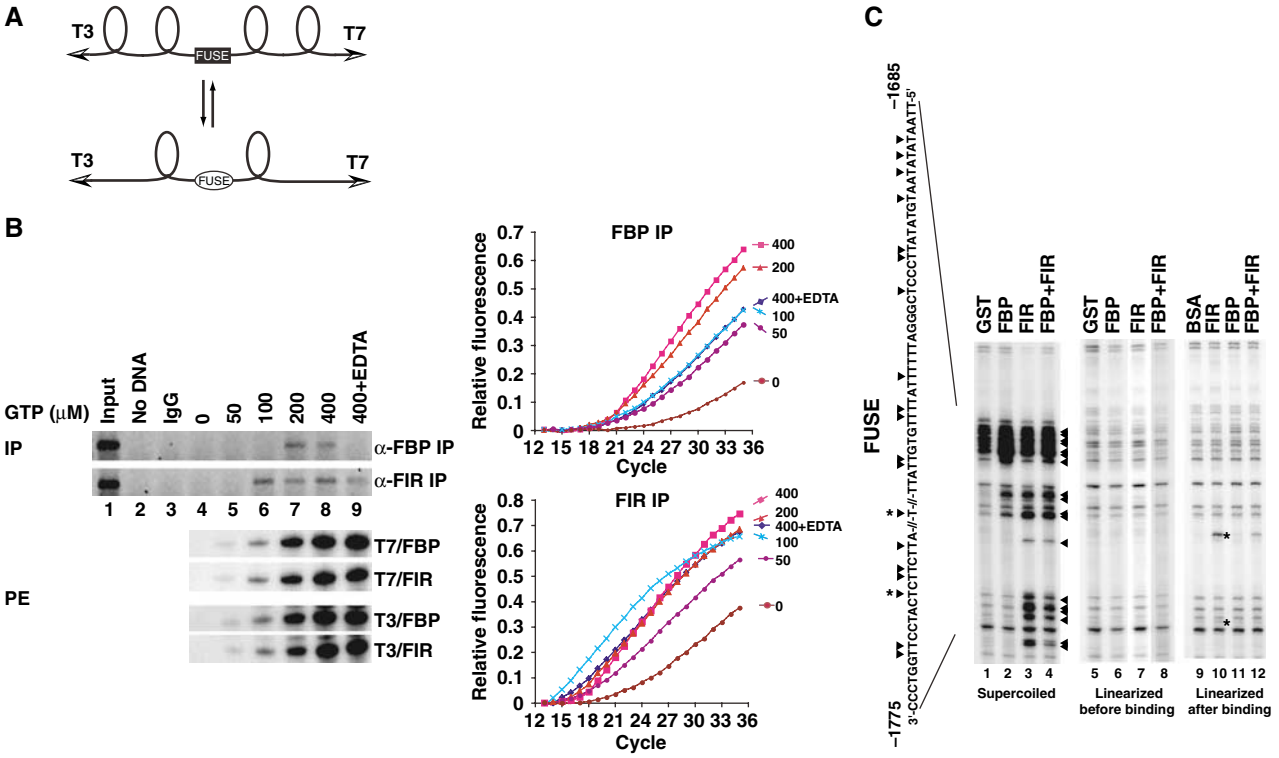

Figure 1 DNA binding by FBP requires sustained superhelical stress, whereas transient stress is sufficient for FIR. (**A**) Schematic drawing of liner *in vitro* transcription template. Active transcription, even in linear templates, develops dynamic supercoils that convert duplex FUSE (box) into single-stranded DNA (oval) (Kouzine *et al*, 2004). (**B**) Negative torsional stress (supercoiling) was dynamically generated by T3 and T7 RNA polymerase transcription *in vitro* of linear templates in the presence of FBP (α-FBP IP panel) or FIR (α-FIR IP panel). Primer extension (PE, lanes 4–8) showed increased transcription with increasing (GTP). EDTA was added to stop transcription prior to fixation (lane 9) to test if ongoing transcription was required for FBP or FIR binding. Immunoprecipitated DNA was amplified (IP, lanes 1–9). Real-time PCR profile of the same DNA is shown on the right. (**C**) *In vitro* $KMnO_4$ footprints of FBP and FIR binding to supercoiled or linear DNA. Hyper-reactive residues are marked with triangles; hyper-reactive residues resistant to linearization are marked with *. Recombinant GST, FBP, and/or FIR were incubated with supercoiled (lanes 1–4 and lanes 9–12) or linear (lanes 5–8) DNA-containing FUSE. After binding, all samples received $MgSO_4$ (2 mM final) and *Xho*I was added in lanes 9–12 for 5 min before $KMnO_4$ treatment.

enabled binding to FUSE when situated between the start sites. Full FIR binding occurred at 100 μM GTP, somewhat lower than for FBP (Figure 1B, α-FIR panel, lanes 3–8). The FIR–FUSE complex was more resistant to transcriptional arrest by Mg^{2+} chelation than was FBP–FUSE (lane 9, also real-time PCR graph). To confirm that the supercoiling authorizing FIR–FUSE complex formation was dispensable thereafter, FIR was bound to FUSE in supercoiled plasmids and then $KMnO_4$ treated with or without prior linearization. FIR elicited prominent $KMnO_4$ reactivity at many bases throughout the FUSE region only on supercoiled, but not on linear DNA (Figure 1C, lane 3 versus lane 7). The FIR-induced hyper-reactivity of FUSE in supercoiled DNA endured (at least partially) after linearization (Figure 1C, lane 10). Thus, FIR only binds to relaxed DNA if the reaction pathway first passages through a supercoiled intermediate. This sort of reaction scheme typically suggests a kinetic barrier to direct complex formation; in this case, the melting of a large stretch of DNA to expose multiple contacts is probably a prerequisite for FIR binding.

If the binding properties of FBP and FIR to transcriptionally torqued templates *in vitro* are paralleled *in vivo*, then FBP binding should be strictly dependent on ongoing transcription. FBP would be recruited to active promoters, but are absent at inactive promoters. FIR too would be recruited to active promoters, but the ability of FIR to remain engaged after DNA relaxation would prolong its repressive influence even as torsional stress waned. The dynamics of the FUSE/FBP/FIR system were interrogated at the human *c-myc* promoter, mindful of these considerations.

A complex mix of factors precedes FBP and FIR recruitment to the c-myc promoter

As *c-myc* is abnormally regulated in most tumors and cell lines, primary human fibroblasts were studied to relate changes in FBP and FIR binding and the recruitment of other *c-myc* regulators with the dynamics of *c-myc* expression. The well-described serum response of *c-myc* in these primary cells is not modified by neoplastic antecedents (Waters *et al*, 1991). First, the fibroblasts were made quiescent by prolonged serum starvation. Then serum was restored, and *c-myc* mRNA levels, and binding by FBP, FIR or other factors, were monitored at various times. *c-myc* expression, virtually absent in serum-starved cells, peaked 2h after serum re-feeding and then rapidly declined to baseline (Figure 2A) as reported (Dean *et al*, 1986; Kelly and Siebenlist, 1986). To monitor the flux of factors binding to *c-myc* regulatory sequences using ChIP, PCR primers were designed to probe: (1) the major promoter P2, (2) the Far Upstream Element (FUSE), and (3) the mid-region between P2 and FUSE (Figure 2E).

Despite the dynamic changes in *c-myc* mRNA levels, ChIP revealed that RNA polymerase was loaded at P2 throughout the experiment (Figure 2B, lane 12), consistent with the paused RNA polymerase shown to reside there by nuclear run-on and *in vivo* footprinting (Bentley and Groudine, 1986;

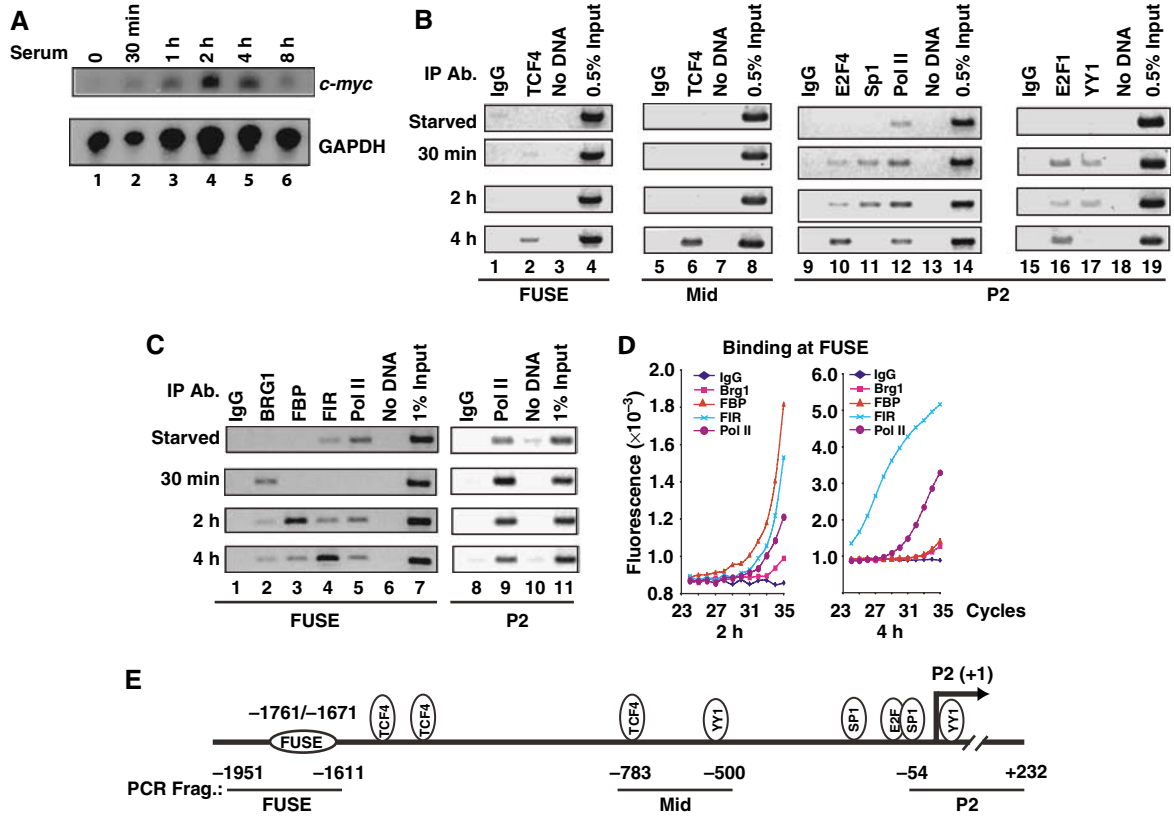

Figure 2 Complex transcription factor choreography precedes FBP and FIR recruitment to the *c-myc* promoter. Human Hs68 primary fibroblasts were cultured in DMEM without FBS for 5 days and then stimulated with 10% serum; cells were harvested for RNA or ChIP at the indicated times after adding serum. (**A**) RNase protection assay measuring *c-myc* levels. (**B, C**) Chromatin from starved and stimulated cells was immunoprecipitated with indicated antibodies. (**D**) Real-time PCR amplification of the same ChIP DNA samples shown in (C) (2 and 4 h). (**E**) Schematic representation of transcription factor binding sites and primer sets for PCR amplification.

Spencer and Groudine, 1991; Marcu *et al*, 1992; Strobl and Eick, 1992). The *c-myc* promoter remained substantially charged with polymerase even after 7 days of serum starvation (Supplementary Figure S2), so the near absence of *c-myc* transcription must reflect, at least in part, inefficient mobilization of the paused polymerase.

Several transcription factors implicated in *c-myc* regulation were tested by ChIP to see if any were recruited early enough to trigger release of the P2-paused RNA polymerase and whether their binding paralleled the peak of expression. Among the factors reported to regulate *c-myc*, E2F1, E2F4, YY1, Sp1, and TCF4 were tested (Thalmeier *et al*, 1989; DesJardins and Hay, 1993; Riggs *et al*, 1993; He *et al*, 2000); all were absent from the *c-myc* promoter in serum-starved cells (Figure 2B, starved). Upon induction, these factors displayed various patterns of recruitment and release from the promoter. Moving in parallel, Sp1 and YY1 bound quickly and exited early (Figure 2B, lanes 11 and 17). E2F1 and E2F4 were also bound rapidly, but remained throughout *c-myc* induction and decline; E2F1 levels were relatively steady, while E2F4 occupancy gradually increased (Figure 2B, lanes 10 and 16). As *c-myc* expression waned, TCF4 was recruited to its two binding sites, one near FUSE and the other within the mid-region (Figure 2B, lanes 2 and 6). The differential flux of these factors argues against a rigid enhanceosome snapped together from synchronously activated components (Maniatis *et al*, 1998; Merika and Thanos, 2001). Rather, these results suggest that different stages of the serum-induced *c-myc* expression peak coincide with the binding of different sets of factors.

When *c-myc* is silent, double-stranded FUSE is fixed upon a nucleosome (Michelotti *et al*, 1996), and so FBP cannot bind, reinforcing the gene's closed state. The positive action of the BRG-1 chromatin-remodeling complex has been hypothesized to open FUSE to help re-start *c-myc* transcription (Chi *et al*, 2003). ChIP was performed to monitor the binding of FBP, FIR, and the BRG-1 near FUSE. Following prolonged serum starvation (5 days), residual FIR binding remained at FUSE after BRG-1 and FBP were lost (Figure 2C and Supplementary Figure S1); after 7 days of serum starvation FIR too was undetectable (Supplementary Figure S1). At 30 min post-stimulation, BRG-1 was recruited to FUSE when neither FIR nor FBP was present. At the peak of *c-myc* expression 2 h post-stimulation, FBP binding crested, FIR started to return, and BRG-1 binding waned. Thereafter, while *c-myc* expression dropped, FBP lingered, BRG-1 receded further, and the level of FIR at FUSE rose dramatically (Figure 2C and D). Once recruited to FUSE, FIR may be stabilized both through protein–protein interactions with FBP and by direct DNA binding (see below) within a ternary complex. Binding of FBP and FIR endured through later stages of serum stimulation (Supplementary Figure S2), when cells make the transition to steady-state growth. Thus, the FUSE region contributes different factors to *c-myc* regulation at different phases of the activation cycle.

As FIR was undetectable at FUSE after 7 days of serum starvation (Supplementary Figure S1) though *c-myc* remained off, some overarching mechanism must convert short-term repression into an enduring shutoff. This same mechanism would need to be reversed upon re-activation, and seemed likely to involve chromatin.

Chromatin remodeling accompanies shutdown and reactivation of FUSE

The pulse of BRG-1 recruitment to FUSE suggested that chromatin remodeling there may occur soon after serum stimulation (Figure 2C, lane 2). Therefore, the nucleosome ladder surrounding FUSE was examined using partial micrococcal nuclease digestion, Southern blots and indirect end-labeling. The regular nucleosome ladder extending through the FUSE region in serum-starved cells (Figure 3A, lanes 1–2) became progressively indistinct as *c-myc* became activated (Figure 3A, lanes 5–8). As *c-myc* expression increased, the margins between nucleosomes 4 and 5 became poorly delineated, though the bulk nucleosome ladders in these same samples were regular and not overdigested (Figure 3A, ethidium bromide panel). The strong hybridization of the probe with the high-molecular-weight DNA upstream of FUSE demonstrates the absence of nuclease hypersensitivity in this region. Therefore, the blurring of the ladder represents attenuated and heterogeneous cleavage of the linker between nucleosomes 4 and 5, as noted previously (Michelotti *et al*, 1996), most likely reflecting partial protection by newly recruited factors or movement and heterogeneous positioning of nucleosome 5.

To see if the expression or inactivity of the *c-myc* gene is associated with distinct chromatin states at FUSE, the boundaries of nucleosome 5 were compared using LM–PCR of Myc-nonexpressing IMR32 neuroblastoma cells versus U2OS cells that make Myc. In this experiment, extensive micrococcal nuclease digestion trimmed DNA to the margins of mononucleosomes that were purified for further analysis. Southern blots of IMR32, where the FUSE is double-stranded *in vivo* (Michelotti *et al*, 1996), revealed normal micrococcal nuclease cleavage of the linker between nucleosomes 4 and 5, yielding a ladder extending through and beyond the FUSE (Figure 3B). LM–PCR mapping of IMR32 mononucleosomes showed FUSE wrapped over 'nucleosome-5' (Figure 3C). This arrangement would oppose melting and physically exclude FBP and FIR. In cells continuously expressing Myc (U2OS osteosarcoma cells and SW13 BRG-1-deficient adrenal cortical carcinoma cells), FUSE is nucleosome-free (Figure 3D) and bound by FBP and FIR as in other Myc-expressing cells (Figure 3E). The SW13 results reveal that some mechanism, perhaps related to transcriptionally generated supercoils, permits FUSE to remain nucleosome-free and to bind FBP/FIR without BRG-1.

If transcription drives melting, then FUSE should be devoid of FBP/FIR when *c-myc* is shut off. Indeed, both FBP and FIR were not found at the duplex FUSE in transcriptionally silent *c-myc* in IMR32 neuroblastoma cells, though both proteins were expressed at usual levels (Figure 3F). PCR of DNA recovered from ChIP of IMR32 using anti-FBP failed to amplify FUSE, though DNA harvested with anti-histone H4 amplified this sequence properly (Figure 3F). Moreover, FBP was evicted from FUSE following transcription inhibition with α-amanitin or DRB in HeLa cells, most likely reflecting the loss of sufficient untwisting stress to maintain single-strandedness at FUSE (data not shown) (He *et al*, 2000).

Remodeling of a TFIIH-mediated loop between FUSE and P2

FUSE-bound factors would influence gene expression most directly by looping to the transcription machinery at the P2

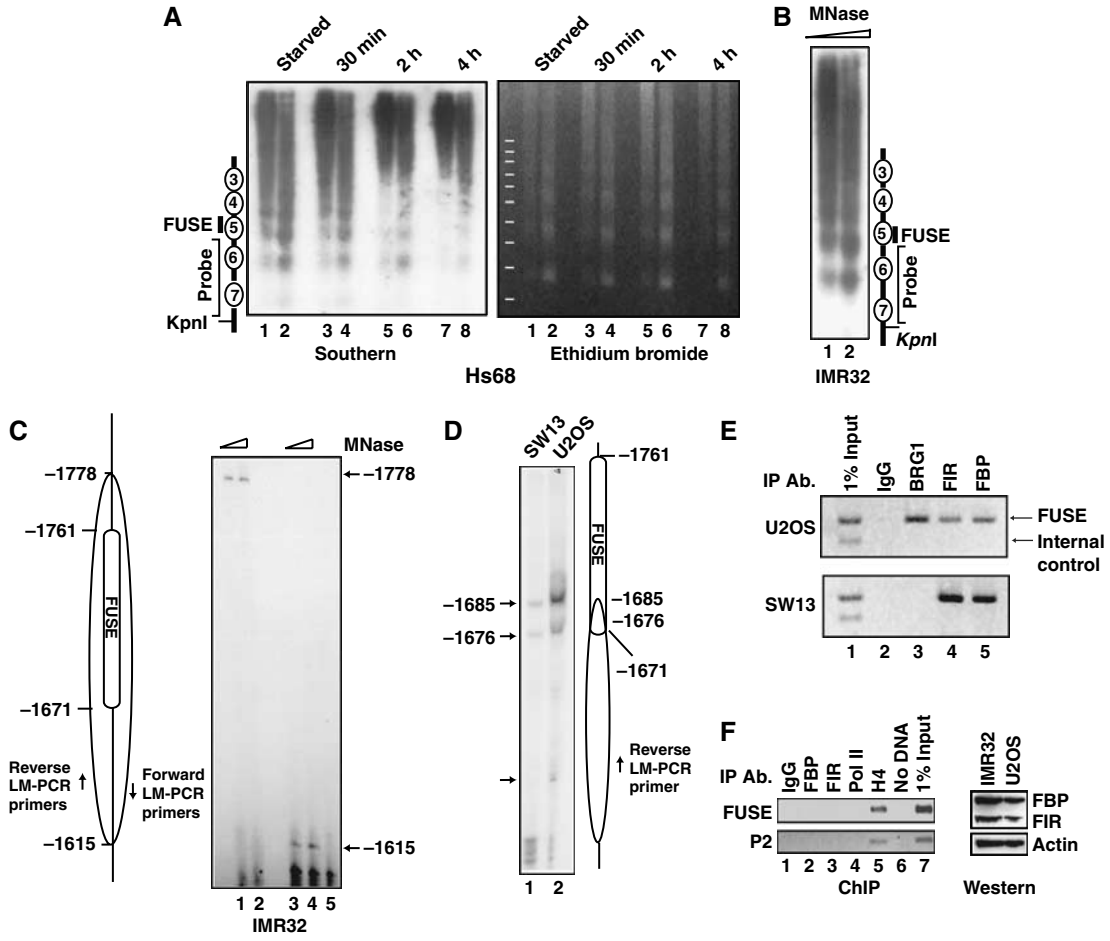

Figure 3 Chromatin remodeling accompanies shutdown and reactivation of FUSE. (**A**) Southern blot of DNA from MNase-treated nuclei from serum-starved (lanes 1 and 2) or stimulated (lanes 3–8) primary fibroblasts reveals imprecise positioning of the FUSE nucleosome in activated cells. Nucleosomes are numbered as in Michelotti *et al* (1996). Nuclei were digested with MNase, and then cleaved with *Kpn*I, separated electrophoretically and analyzed by Southern blot with a *c-myc* probe. (**B**) Southern blot showing nucleosome ladder around FUSE in IMR32 nuclei. (**C**) Fine-mapping of the FUSE-masking nucleosome. Mono-nucleosome-sized DNA from MNase-digested IMR32 nuclei was gel purified and subjected to LM–PCR. The 5′ (lanes 1 and 2) and 3′ (lanes 3 and 4) boundaries were defined by reverse and forward primer sets, respectively. No PCR product was obtained without adapter ligation (lane 5). (**D**) LM–PCR reveals imprecise repositioning of the nucleosome near FUSE in SW13 or U2OS cells expressing *c-myc*. (**E**) ChIP comparing FBP/FIR binding to FUSE in the presence (U2OS) or absence (SW13) of the BAF complex. (**F**) Transcription is necessary to hold FBP and FIR at FUSE. ChIP showing failure of FBP and FIR to bind FUSE in IMR32 cells, where *c-myc* is permanently silenced, although FBP and FIR are expressed at similar levels (Western panel).

promoter (Bentley and Groudine, 1986; Spencer and Groudine, 1991; Marcu *et al*, 1992; Strobl and Eick, 1992). As the aliphatic amino groups on proteins are more reactive with formaldehyde than the exocyclic amino groups of bases, the same treatment crosslinking protein to DNA might trap the hypothesized protein–protein bridge between FUSE and P2 (McGhee and von Hippel, 1975); if so, antibodies that precipitate P2-bound factors should also pull down FUSE-bound proteins. (In a similar manner, the androgen receptor couples the PSA enhancer and promoter in the presence of hormone (Shang *et al*, 2002).) Indeed, ChIP of serum-starved and re-stimulated fibroblasts using α-RNA polymerase II recovered FUSE efficiently only at the time points when FBP and/or FIR bound FUSE (Figure 2C); as expected, P2-DNA was recovered throughout the whole time course (Figure 2C, P2 panel). RNA polymerase II has also been associated with *c-myc* upstream sequences using ChIP-on-chip (Kim *et al*, 2005).

ChIP on HeLa cells using a panel of antibodies confirmed looping between FUSE and P2. PCR revealed similar patterns with DNAs recovered by antibodies against FUSE or P2 factors (Figure 4A, P2 and FUSE panels), whereas nonspecific or irrelevant antibodies returned no signal. The co-recovery of FUSE with P2 sequences was not due to precipitation of DNA fragments spanning both sites, because mid-region sequence failed to amplify (Figure 4A, mid panel).

Most simply, FUSE would connect to P2 through a transcription-dependent protein–protein bridge. This bridge would be expected to include TFIIH, the only promoter-bound transcription component that interacts with FBP and/or FIR (Liu *et al*, 2000, 2001).

When TFIIH is mutated, FUSE-P2 loop formation fails and c-myc is improperly regulated

If FBP and FIR at FUSE are linked with TFIIH at P2, then TFIIH p89/XPB helicase mutations that impair FBP activation and FIR repression might also impede loop closure and deregulate *c-myc* (Liu *et al*, 2001). Therefore, ChIP was performed in XPB-mutant, EBV-immortalized lymphoblasts to compare looping after complementing by episomally

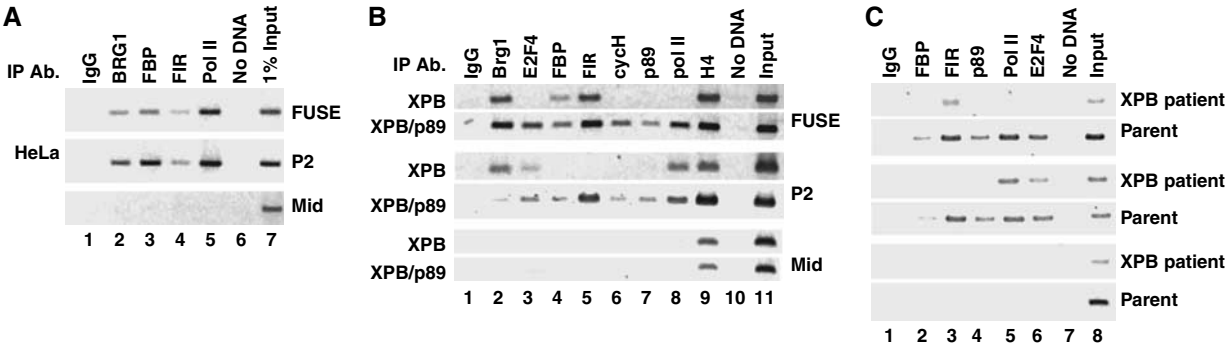

Figure 4 TFIIH-mediated loop between FUSE and P2. (**A**) ChIP with indicated antibodies of HeLa chromatin. Amplicons were described in Figure 2E. (**B**) Mutation of the p89 (XPB) TFIIH subunit disrupts the loop. ChIP comparing log-phase XPB lymphoblasts (GM02252) and complemented cells. (**C**) An independent p89 (XPB) mutation also disrupts the loop. ChIP of *c-myc*-bound factors from log-phase primary fibroblasts from an XPB patient (GM13025) and parent (GM13028).

encoded, wild-type p89/XPB, or with empty vector. With the functionally complemented cells (XPB/p89 panels), antibodies to FUSE (FBP and FIR) or P2 factors (RNA polymerase II, TFIIH, and E2F4) each recovered sequences from both regions. In contrast, with mutant TFIIH (XPB panels), anti-FUSE-factors recovered only FUSE and anti-P2-factors recovered only P2 (Figure 4B). Thus, looping required wild-type p89/XPB. The recovery of P2 but not FUSE using α-RNA polymerase II in XPB cells argues that polymerase II does not bind directly to FUSE. TFIIH was underloaded at P2 in the XPB-mutant lymphoblasts as noted previously (Weber *et al*, 2005). Mid-region sequence was not recovered irrespective of TFIIH status, demonstrating that co-recovery of FUSE with P2 sequences was not due to inadequate DNA fragmentation. The restoration of the looping in the corrected XPB lymphoblasts strongly argues that loop failure results directly from mutation of p89.

To insure that FUSE-P2 loop failure was not peculiar to the XPB mutation in the EBV-immortalized lymphoblasts, primary fibroblasts from a kindred with a different XPB mutation were investigated with ChIP. Fibroblasts from a patient with autosomal recessive XPB disease (GM13025) or from the patient's father (GM13028) were interrogated using anti-FUSE or anti-P2 factors. With paternal fibroblasts, FUSE and P2 sequences always co-precipitated, just as with normal fibroblasts, HeLa cells or functionally corrected XPB lymphoblasts. In contrast, with the patient's cells just like the mutant XPB lymphoblasts, anti-FUSE factors pulled down only FUSE, but not P2, and *vice versa* (Figure 4C). Moreover, the P2 region in the patient's cells was deficient in p89/XPB, again similar to the mutant lymphoblasts. Therefore, p89/XPB mutation leads to inefficient recruitment and/or retention of TFIIH at P2, and FBP/FIR-TFIIH loop failure.

FBP/FIR-TFIIH loop failure prolongs serum-induced c-myc expression

Mutation of p89/XPB is accompanied by inefficient recruitment or premature discharge of TFIIH at the *c-myc* promoter and by ineffective communication of FUSE-bound factors with the promoter. What are the consequences of these deficits on *c-myc* expression? Belying dramatic promoter changes due to mutated TFIIH, in XPB lymphoblasts, steady-state Myc levels are only modestly disturbed. However, cell-to-cell variation is exacerbated (Weber *et al*, 2005). Although this failure to hold Myc levels to close tolerances may be significant, a more generalized defect in the induction or shutdown of *c-myc* might amplify oncogenesis by the well-described DNA-repair deficit in XPB disease (van Steeg and Kraemer, 1999). The *c-myc* response to serum starvation and re-addition was compared between fibroblasts of the XPB patient, his similarly afflicted brother and both phenotypically normal parents. Whereas the parents' cells showed a normal pulse of serum-induced *c-myc* expression, induction was attenuated and shut-off delayed in the cells of both patients (Figure 5A). Lacking input from FUSE-bound factors, and without full TFIIH occupancy at P2, the peak of *c-myc* transcription was mismanaged.

Apparently, the TFIIH/FBP/FIR system helps to integrate and coordinate the flux of regulators patterning *c-myc* output. The sequence of events at the *c-myc* promoter—(1) conventional transcription factor recruitment to the promoter proximal region with concurrent chromatin remodeling at FUSE; (2) activation of the paused polymerase; (3) FBP-recruitment synchronous with peak transcription; followed by (4) FIR recruitment and (5) FBP discharge as the promoter shuts down—evokes the notion that the FUSE–FBP–FIR axis helps to generate a pulse of *c-myc* transcription. If so, then this chronology suggests that FUSE-bound FBP acts as a second-stage booster that delimits its own activity by recruiting FIR to re-establish basal expression.

Knockdown of FIR sustains c-myc activation

If FIR were not recruited to FUSE, then no signal would direct TFIIH to restore baseline *c-myc* transcription. Therefore, the conventional transcription factors recruited upon serum activation would be expected to sustain a broad plateau of persistent *c-myc* expression. To test this prediction, serum-starved fibroblasts were transfected with siRNAs designed to knockdown FIR or FBP, and then re-activated with serum. FIR-siRNA treatment virtually eliminated FIR-protein (Figure 5B, FIR panel); in cells lacking FIR, *c-myc* was expressed in a broad plateau (Figure 5C, siFIR panel) as predicted and as seen with mutant TFIIH. Thus, the FIR-TFIIH nexus shuts down the pulse *c-myc*.

FBP was less efficiently knocked down than was FIR (Figure 5B). The redundancy of the FBP family precluded interpretation of partial FBP deficiency. Reduction of FBP in the presence of normal levels of the more potent activators

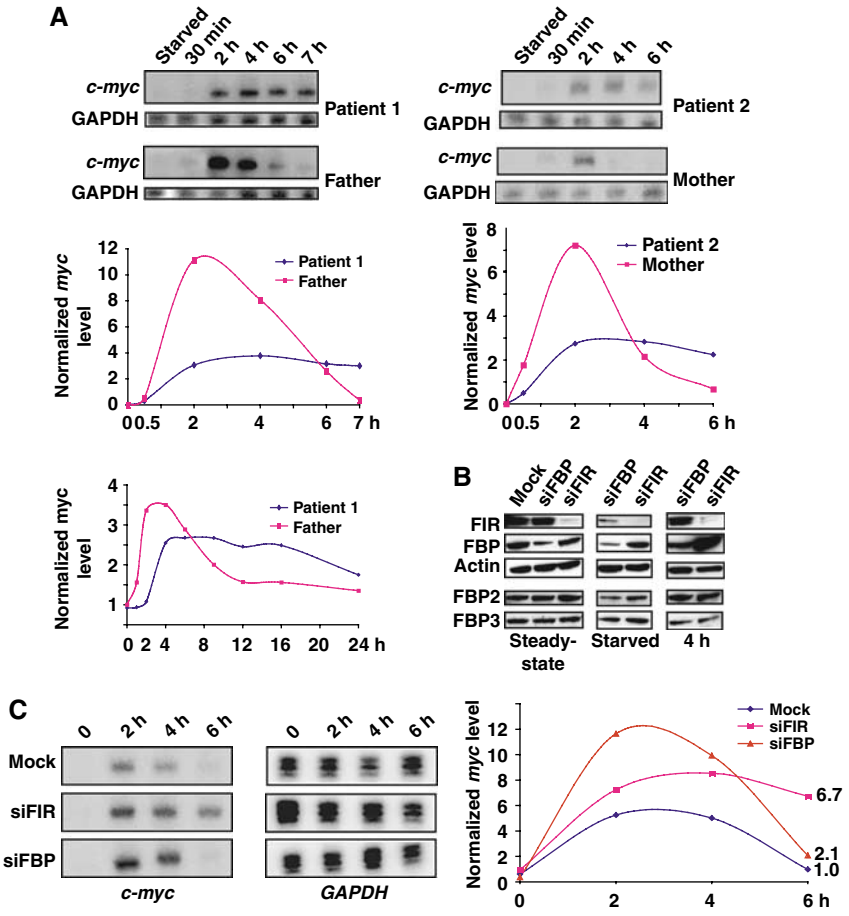

Figure 5 Looping between FUSE–FBP–FIR and TFIIH is essential for the normal response of *c-myc* to serum. (**A**) Independent RNase protection assays comparing the *c-myc* serum response of primary fibroblasts of XPB patients or their parents. *c-myc* mRNA levels were analyzed by ImageQuant and normalized to GAPDH (graph). *c-myc* level (normalized to γ-tubulin) was also monitored throughout a 24-h period by real-time RT–PCR (bottom). (**B**) Steady-state, serum-starved human Hs68 primary fibroblasts were transfected with siRNA to FIR or FBP. Starved cells were harvested before or after 4 h of serum repletion and subjected to Western blot. (**C**) RNase protection assay showing the serum response of *c-myc* in mock, siFBP, or siFIR transfected Hs68 cells (left). *c-myc* levels were analyzed by ImageQuant and normalized to GAPDH. Relative *c-myc* levels at 6 h post-serum induction are shown on the plot (right).

FBP2 and FBP3 (Duncan *et al*, 1996) (H-J Chung, D Levens, unpublished data) might even be expected to increase *c-myc* expression (Figure 5C, siFBP panel). (FIR, in contrast, has no protein siblings.)

If the FUSE system behaves *in vivo* as it does *in vitro*, then it possesses the essential components of a machine that first senses the intensity of ongoing transcription through supercoil-driven, FUSE deformation, and then provides positive or negative feedback via FBP and FIR to transcription complexes within *c-myc*'s expanded proximal promoter.

Using FUSE to reprogram the metal response of the metallothionein (MT) IIA promoter

The complexity of *c-myc* regulation confounds rigorous *in situ* investigation of the operation of the FUSE/FBP/FIR system. To test the notion that FUSE responds to the intensity of ongoing transcription in a simplified, *in vivo* setting, FUSE was placed in an episome between divergent, identical metal-inducible MT IIA promoters (Figure 6A; Karin *et al*, 1984). This arrangement reprises the *in vitro* experiments, where the high-level dynamic supercoiling generated between divergently transcribing promoters melt FUSE, and recruit FBP and FIR. These matched promoters were used to drive GFP and RFP to quantify reporter expression *in vivo*, in individual cells.

If FUSE is a *cis*-sensor of ongoing transcription, it should be without influence when supercoiling to low to melt the element; that is, basal MT IIA promoter activity should not be sensitive to FUSE. Indeed, in the absence of inducing metal, reporter expression was similarly low whether or not the episomes included FUSE (Figure 6B, uninduced). Constitutively, FUSE is neither an enhancer nor an operator.

If FUSE behaves *in vivo* as it does *in vitro*, when the reinforcing negative supercoils trailing the diverging RNA polymerases surpass the FUSE-melting threshold, then reporter expression should be qualitatively altered. Metal-induced reporter expression was compared between episomes with or without FUSE. MT IIA promoter output was dramatically reprogrammed by FUSE. For the first several hours, as metal-induced transcription intensified, FUSE activated progressively stronger, up to 10-fold (Figure 6C and D, 3 kb separation, ratio shown as inset).

If FUSE responds to dynamic supercoils, then reducing the generation, transmission, or accumulation of torsional stress should attenuate its activity. For example, increasing the separation between the divergent promoters should dump

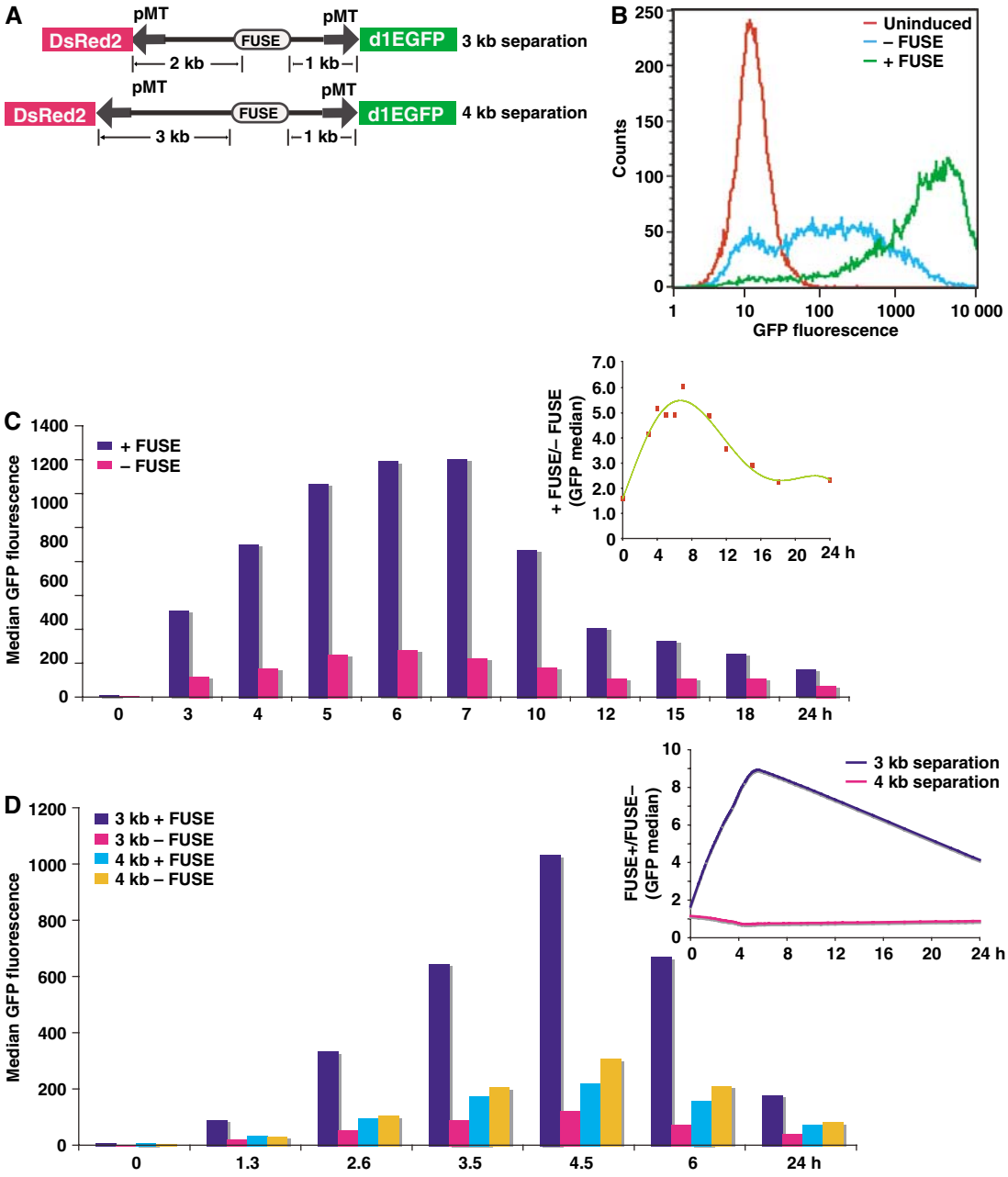

Figure 6 The FUSE–FBP–FIR system re-programs transcription from heterologous promoters. (**A**) Schematic drawing of reporters. (**B**) Raji cells carrying episomally encoded reporters under the control of identical, divergent metallothionein promoters (A, 3 kb separation) were induced with 90 μM Zn^{2+} for 4 h; fluorescence was analyzed by flow cytometry and a representative histogram of GFP fluorescence is shown. (**C**) Attenuation of FUSE activation after peak expression. Time course of median GFP fluorescence from cells with the 3 kb-separated promoters following Zn^{2+} induction. Ratio of +FUSE/−FUSE GFP fluorescence is also plotted (inset). (**D**) Median GFP fluorescence plot of either 3 or 4 kb separated reporters, with or without FUSE, at indicated time points. The ratio of the medians of GFP fluorescence with (+FUSE) or without (−FUSE) FUSE is also shown (inset).

the dynamic supercoils into a larger zone, eventually lowering the effective superhelical density below the threshold required to melt FUSE. Indeed, a sharp FUSE-dependent threshold was crossed as the spacer separating the MT IIA promoters was increased from 3 to 4 kb (Figure 6D, 3 kb + FUSE versus 4 kb + FUSE). (Decreasing the separation below 3 kb did not further augment the FUSE effect, nor did FUSE influence expression at 4 kb or beyond, data not shown.) Importantly, these insertions did not alter the topography of any of the *cis*-elements, including FUSE—driving GFP expression (Figure 6A). Beyond the FUSE melting threshold, RFP transcription was similarly FUSE-unresponsive (data not shown). The spacer (derived from *Bacillus subtilis* DNA) was not predicted to respond to torsional stress. As expected, FUSE was also without influence upstream of a single MT IIA promoter (data not shown).

If FUSE modifies the metal-induced MT IIA-promoter-driven transcription in accord with its inferred role in the serum response of *c-myc*, then the positive influence of this element on reporter expression should not be sustained, but should decline, thereby programming a pulse of expression. Therefore, the influence of FUSE on reporter expression

was monitored following peak expression. The reporter activity revealed attenuation of FUSE potency following peak activity, as predicted (Figure 6C and D). (The 2-h half-life of the GFP reporter precluded a steeper descent from peak expression.)

Attacking FIR with RNAi augmented and prolonged FUSE-driven expression when the element was interposed between the divergent promoters (data not shown), just as it did at the *c-myc* locus.

Discussion

The results above invite consideration of this scheme to describe transcription at the *c-myc* promoter:

First, one or more transcription factors responding to an initiating stimulus (−li) binds the *c-myc* promoter. Depending on the nature and strength of the signal, and the cell type, single factors acting repetitively or multiple factors acting concertedly advance the P2-paused complex. With serum, Sp1 is among the first proteins recruited, though multiple transcription factors are probably activated by this treatment. The resistance of the *c-myc* promoter to inactivation by pathological chromosomal alterations or genetically engineered changes including all manner of mutations, insertions, substitutions, and deletions (Krumm *et al*, 1995a, b) may indicate that there is no unique and obligatory pathway for *c-myc* induction. Rather, multiple pathways drawn from a variety of elements and factors may prime *c-myc* transcription.

As the density of bound factors increases, the paused polymerase is ratcheted closer to promoter escape (Figure 7A). Only after the paused polymerase exits the promoter can the recruited factors direct re-initiation; the paused polymerase is a powerful check on overactivation. Concurrently, the *c-myc* priming factors attract complexes that modify and remodel chromatin. For example, Sp1 (or another BAF-binding factor) recruits chromatin-remodeling machinery to the *c-myc* upstream regulatory sequences (Liu *et al*, 2002). FUSE is one among the targets for remodeling.

BRG-1 mobilizes a FUSE-masking nucleosome. Remodeling forces combined with the torsional stress of incipient transcription melts FUSE, allowing FBP to bind (Figure 7B). Via TFIIH, FBP acts as a second-stage booster to drive transcription up to peak levels. As FBP is approximately five times more abundant than FIR, mass-action-driven kinetics most likely favor more rapid recruitment of the former to FUSE. FBP-binding and peak transcription together fully melt FUSE and conscript FIR through interactions with FBP as well as with DNA across this broadly melted segment (Figure 7C and D). As FIR slows transcription, the dynamic stress at FUSE falls, ejecting FBP, but not FIR (Figure 7E), concordant with the *in vivo* and *in vitro* findings presented here. With this scheme, the absence of functional TFIIH causes both the FBP boost and the FIR brake to fail, explaining the broad plateau of *c-myc* transcription seen following serum addition in XPB cells.

In this scheme, the FBP, FIR, and TFIIH constitute a 'molecular servomechanism'. Servomechanisms sense the output of a machine (such as an engine) and modify future output according to its current performance. The minimal components of a servo are a sensor coupled to positive and negative actuators. This molecular servo uses FUSE melting to sense the intensity of ongoing transcription (Kouzine *et al*, 2004), and FBP and FIR as effectors to provide positive or negative feedback to TFIIH at the promoter. Note that this servo cannot initiate transcription. Conventional factors (such as Sp1) and chromatin-opening complexes must prime the system. Although it is possible to drive the promoter without a functional 'servo', normal regulation is not maintained, Myc levels fluctuate (Weber *et al*, 2005), and the temporal profile of the *c-myc* transcription is improperly regulated, as shown here.

Using FUSE to reprogram the output of divergent MT IIA promoters in episomes dramatizes the distinct roles of the starter- and servomechanisms. FUSE is unable to influence reporter output until the onset of metal-stimulated transcription, and FUSE action is completely dependent on a permissive topography of promoters and chromatin in order to respond to the supercoils emanating from nearby promoters.

Reinforcing supercoils from *two* nearby promoters were required for FUSE to work in engineered episomes. Yet, *c-myc* is expressed *in vivo* mainly from the P2 promoter 1.7 kb from FUSE. Though the architectural elements that license FUSE action at *c-myc* in the absence of divergent promoters have not been fully identified, several possibilities may be imagined. Depending on its detailed architecture, an overarching topological domain might delay the dissipation of the dynamic supercoils first generated upon activation of the paused transcription complex. Within such an overarching domain, threading the template through TFIIH as the transcription complex translocates would pump supercoils directly into the subdomain closed by the FUSE-bound FBP–TFIIH interaction, obviating further need for the overarching domain. Looping between one CTCF site at −2 kb and another immediately downstream of the start site occurs in repressed cells (A Abdullayev and V Lobanenkov, personal communication) (Filippova *et al*, 1996; Gombert *et al*, 2003) that transiently trap the dynamic supercoils first generated upon activation of the paused polymerase. Such a role for CTCF would be in accord with its well-described insulator and self-association properties (Burgess-Beusse *et al*, 2002; Pant *et al*, 2004).

If the dynamic supercoils pumped into the FBP–TFIIH bounded loop were concentrated into the internucleosomal linker regions (a reasonable expectation, since the fixed DNA trajectory on the nucleosome should be less accommodating to additional supercoils), then torsional stress would be restricted to 500 bp—about 50 helical turns—of linker between P2 and FUSE. Translocation of the transcription machinery (including TFIIH) through just 20–40 nucleotides (2–4 turns) of the extended *c-myc* proximal promoter would drive FUSE through its full repertoire of biphasic melting (2–4 turns/50 turns $= \sigma - 0.04 - 0.08$) (He *et al*, 2000).

Pulses of transcription from P0 or P1, each nearer to FUSE than P2, might help to melt FUSE at lower levels of overall transcription. According to the local topography of *cis*-elements, promoters, and chromatin features, the FBP–FIR system operating through other FUSE-like elements may prove a versatile tool to tune transcription. The mechanics of the servomechanism are governed by the elastic properties (bending and twisting) of DNA; therefore, modifications that alter the strength and length of DNA–histone tail interactions would alter the performance of this molecular device. It is

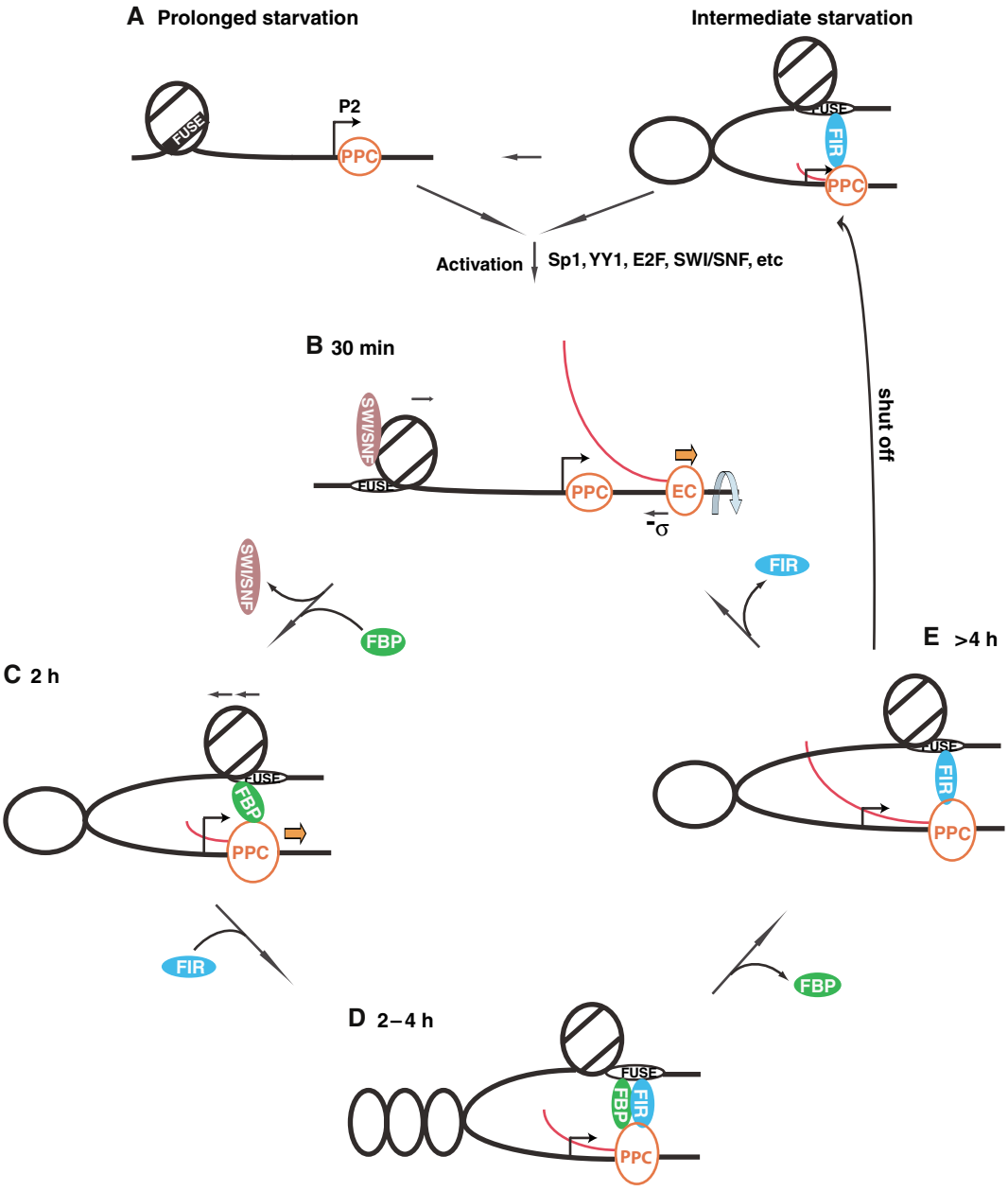

Figure 7 Proposed scheme for molecular servomechanism for regulation of *c-myc* transcription. (**A**) Without serum, *c-myc* transcription shuts off, FUSE becomes masked by a nucleosome, and only a trace of FIR remains. With prolonged starvation, FIR entirely disengages from FUSE. Even when shut off, a Pre-Promoter Escape Complex (PPC) remains paused within the promoter proximal region (P2). (**B**) The earliest factors binding to the promoter upon stimulation advance the PPC to a full Elongation Complex (EC), the FUSE-masking nucleosome is remodeled to expose FUSE. Negative supercoiling ($-\sigma$) generated by EC, combined with remodeling forces, initiate FUSE melting. (**C**) Melted FUSE recruits FBP, which drives transcription up to peak levels by looping with TFIIH. (**D**) FBP activity leads to negative supercoil accumulation within the topologically closed FBP–TFIIH loop. High $-\sigma$ fully melts FUSE and conscripts FIR through protein–protein and protein–DNA contacts. (**E**) As FIR represses transcription, the dynamic stress at FUSE falls, ejecting FBP. The FIR–TFIIH connection allows only basal transcription; so the polymerase at a low rate dissociates from TFIIH to become an EC. Without sufficient activation, the superhelical stress eventually dissipates, and the *c-myc* promoter reverts back to the silent state in (A). For illustrative purposes, only the FUSE-masking nucleosome is shown.

likely that molecular servomechanisms will be found at genes encoding short half-life, low-abundance products.

Materials and methods

Tissue culture

Human Hs68 primary fibroblasts, HeLa, U2OS, SW13, and IMR-32 cells were cultured in DMEM with 10% FBS. Hs68 cells were cultured without serum for 5 days and stimulated with 10% FBS. Cells were harvested at indicated time points for RNA and ChIP. Primary fibroblasts from XPB patients (GM13025, GM13026) and their parents (GM13027, GM13028) were cultured in DMEM with 15% FBS. Lymphoblast XPB (GM02252) and complemented cells were described previously (Weber *et al*, 2005).

c-myc mRNA quantitation

RNase Protection Assay was performed as described (Collins *et al*, 2001). Autoradiographs were analyzed with ImageQuant. Universal Probe Library (Roche) probes 77 and 25 were used for qRT–PCR for *c-myc* and γ-tubulin, respectively.

Chromatin immunoprecipitation and real-time PCR

ChIP was performed as described (Weinmann and Farnham, 2002). PCR was performed using Platinum PCR Supermix (Invitrogen) for 35 cycles with FUSE primers, 30 cycles for P2 and mid-primers. Real-time PCR was performed with Platinum SybrGreen qPCR kit (Invitrogen) with the same primers.

Nucleosome mapping and $KMnO_4$ footprinting

LM–PCR nucleosome mapping at FUSE was performed as described (Carey and Smale, 2000), except that mono-nucleosomal DNA was gel purified prior to ligation. For *in vitro* $KMnO_4$ footprinting, 10 ng of recombinant GST, FBP, or FIR was incubated with 25 ng of either supercoiled or linear DNA in 20 mM HEPES, pH 7.9, 0.1 mg/ml BSA, and 15 mM NaCl for 15 min. $MgSO_4$ was then added to 2 mM in all samples and *Xho*I was added as indicated. $KMnO_4$ treatment was performed 5 min post-*Xho*I addition. $MgSO_4$ (2 mM) is sufficient for complete digestion of supercoiled DNA.

In vitro transcription and DNA IP

Accumulation of negative supercoils in linear DNA by T3 and T7 polymerase was documented (Kouzine *et al*, 2004). Transcription rate was limited by GTP. Highly purified recombinant FBP or FIR was included. After 10 min of transcription, formaldehyde (0.5% final (v/v)) was added to crosslink protein to DNA and stop transcription. To stop transcription prior to fixation, EDTA was added to 20 mM and incubated for 5 min before addition of formaldehyde. The reaction was immunoprecipitated with either α-FBP or α-FIR. PCR was performed with Platinum PCR Supermix for 22 cycles. Real-time PCR was performed with Platinum SybrGreen qPCR kit.

RNA interference

Stealth siRNA to FIR or FBP was synthesized by Invitrogen. Hs68 cells were serum starved for 2 days and siRNA (20 nM) was transfected with Lipofectamine 2000 and cultured in DMEM without serum for 2.5 days before serum stimulation. Cells were harvested for RNA or protein.

Flow cytometry analysis of zinc-induced GFP expression

FUSE or an irrelevant 91 bp fragment was placed between two divergently transcribed, identical metallothionein promoters driving either RFP or destabilized GFP. Spacers of different length were placed between the promoters and FUSE. Raji cells carrying the episomes plasmid were selected and cultured in RPMI-1640 with 10% FBS and 50 μg/ml hygromycin. Transcription was induced by addition of $ZnSO_4$ to a final of 90 μM. GFP fluorescence was analyzed by a flow cytometer.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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