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Dynamic recruitment of transcription factors and epigenetic changes on the ER stress response gene promoters

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ABSTRACT

Response to stresses that alter the function of the endoplasmic reticulum is an important cellular function, which relies on the activation of specific genes. Several transcription factors (TFs) are known to affect this pathway. Using RT–PCR and ChIP assays, we studied the recruitment of promoter-specific TFs, general TFs and epigenetic marks in activated promoters. H3-K4 di- and tri-methylation and H3-K79 di-methylation are present before induction. H3 acetylation is generally high before induction, and H4 acetylation shows a promoter-specific increase. Interestingly, there is a depletion of histone H3 under maximal induction, explaining an apparent decrease of H3-K4 tri-methylation and H3-K79 di-methylation. Pol II is found enriched on some promoters under basal conditions, unlike TBP and p300, which are recruited selectively. Most genes are bound by XBP-1 after induction, some before induction, presumably by the inactive isoform. ATF6 and CHOP associate to largely different set of genes. C/EBPβ is selective and binding to the CHOP promoter precedes that of XBP-1, ATF6 and CHOP. Finally, one of the ER-stress inducible genes analyzed, HRD1, is not bound by any of these factors. Among the constitutive TFs, NF-Y, but not Sp1, is found on all genes before induction. Intriguingly, siRNA interference of the NF-YB subunit indicates transcriptional impairment of some, but not all genes. These data highlight a previously unappreciated complexity of TFs binding and epigenetic changes, pointing to different TFs-specific pathways within this broad response.

INTRODUCTION

A stress that targets the endoplasmic reticulum (ER) triggers a cellular response termed the unfolded protein response (UPR) (1,2). Several different external stimuli lead to UPR causing the dangerous presence of malfolded proteins in the ER. The cellular reaction includes an initial burst of translational attenuation and a rapid communication to the nucleus, where a robust transcriptional response is elicited. Finally, as in the case of many other noxious stimuli, the cells commit suicide through apoptosis. The key to our understanding of the UPR response is the relatively few genes that are specifically activated by the stimulus.

Upon treatment of mammalian cells with thapsigargin (Tg), which depletes the ER calcium by blocking the ATPase pump, a number of genes are activated (2). Among these, the most widely studied is Grp78/Bip, an ER chaperone that binds to unfolded proteins and facilitates the activity of ER stress transducers [(3) and references therein]. The molecular mechanisms of induction have been studied by *in vivo* footprinting, transfections with promoter constructs, EMSAs and, recently, chromatin immunoprecipitation (ChIP) assays (4–10). The Grp78 promoter contains multiple copies of the bipartite ER stress response element (ERSE), constituted by CACGC and CCAAT boxes; the latter is bound by NF-Y, the former by several factors, including YY1, ATF6 and TFII-I. A recent study revealed that some factors are constitutively bound, whereas others are loaded after the ER stress (10). Additional ER stress genes include CHOP (GADD153), a C/EBP family transcription factor (TF) [(11–15); reviewed in Ref. (16)] and Herpud (17). ERSEs, or similar elements, have been found in these promoters as well.

ATF6 is a key component of the transcriptional response to ER stress; it has two isoforms, α and β, with divergent transcriptional activation domains (5–9,18,19). ATF6α, the better characterized of the two, is an ER transmembrane protein, a fraction of which relocates to the Golgi and undergoes

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proteolytic cleavage after ER stress (18). This triggers nuclear translocation of the N-terminal part, which then activates target genes, including Grp78. While ATF6(N) is unable to bind directly to DNA, it can activate the ERSE by forming a complex with NF-Y in a manner dependent on the CCACG part of the ERSE.

Another TF partaking in the ER stress response is XBP-1, originally identified in expression library screenings with an MHC class II X-box element (20) and later associated by genetic experiments to crucial functions in plasmacells (21). KO mice die *in utero* because of liver failure (22). In general, it acts on UPR elements, which are found upstream of genes that control the ER-associated system that degrades unfolded proteins (23). These elements are separated and act independently from ERSEs: indeed, transcription from the latter is activated in the absence of XBP-1 (24). It is unclear, at present, which are the direct targets of XBP-1, and nothing is known about the kinetics of binding *in vivo*.

The ER stress induces two members of the C/EBP Basic-Leucine Zipper family, which regulate several physiological processes related to cell differentiation and proliferation: CHOP (16) and C/EBPβ (25,26). CHOP KO studies indicate an important role in protection from apoptosis following ER stress (27,28). The human and hamster CHOP promoters function through ERSEs and an element resembling a UPRE. The former requires NF-Y and ATF6, and the latter, possibly, ATF4 (13–15).

Most ER stress promoters contain the widespread CCAAT box which is bound and regulated by the trimeric factor NF-Y, composed of three subunits, NF-YA, NF-YB and NF-YC, all necessary for DNA-binding [(29) and references therein]. NF-YB and NF-YC contain histone fold motifs common to core histones: dimerization is essential for NF-YA association and sequence-specific DNA-binding. Typically, NF-Y is not a strong activator on its own, but rather it helps neighbouring TFs in binding DNA and recruiting coactivators. ChIP experiments determined that NF-Y is dynamically bound to cell-cycle promoters (29) and to Grp78 in the absence of stimuli (10).

A further issue concerns histones modifications following gene activation. The only available data are on Grp78, and they indicate the presence of tri-methyl K4 of H3 under unstressed conditions and a dramatic increase in H4 acetylation following Tg (10). The state of other histone modifications were not determined.

To shed light on the molecular mechanisms of the ER stress response, we used RT–PCR to recapitulate the kinetics of ER stress genes induction, and ChIP assays to investigate the binding of TFs and of general transcription factors (GTFs), as well as modifications of histones on several induced genes.

MATERIALS AND METHODS

Cell lines, transfections and western blot

HepG2 and HCT116 cells were grown in DMEM with 10% FCS. Induction was performed with Tg (300 μM) according to standard conditions, for the indicated times. An siRNA interfering oligonucleotide for NF-YB (GGACAGCAUGAAUGAUCAUtt) was transfected in 10^5 HCT116 cells in 24-wells plates with Lipofectamine (Gibco BRL USA). Cells were harvested 48 h later, nuclear and whole cell extracts prepared and RNA extracted. Extracts were assayed in western blot analysis with anti-YB and anti-vinculin antibodies under standard conditions.

RT–PCR analysis

RNA was extracted using an RNA-Easy kit (Quiagen, Germany), according to the manifacturer's protocol, from HepG2 and HCT116. For cDNA syntesis, 2 μg RNA were used with the M-MLV-RT kit (Invitrogen, USA). Semi-quantitative PCR analysis was performed with primers detailed in Supplementary Data 1.

Chromatin immunoprecipitation

ChIP assays were performed as described (29). HepG2 cells ($0.5/1 \times 10^7$) were washed in PBS and incubated for 10 min with 1% formaldehyde; after quenching the reaction with Glycine 0.125 M, cells were sonicated and chromatin fragments of an average length of 1 Kb recovered by centrifugation. Immunoprecipitations were performed with ProtG-Sepharose (KPL, USA) and 3–5 μg of the indicated antibodies: NF-YB [Purified rabbit polyclonal; Ref. (29)]; ATF6 (Active Motif 40962), C/EBPβ (Active Motif 39307), p53 (Active Motif 39041), Sp1 (Santa Cruz 59×), TBP [Purified mouse polyclonal; Ref. (30)], XBP-1 (Santa Cruz 7160), CHOP (Santa Cruz 575), p300 (Santa Cruz 585×), anti-Pol II (Santa Cruz 9001), Sp1 (Santa Cruz 59), acetyl-H3 (Upstate 06-599), acetyl-H4 (Upstate 06-759), H3-K4-me2 (Abcam 6000), H3-K4-me3 (Abcam 8580), H3-K79-me2 (Abcam 3594), H3-K9-me2 (Abcam 7312) and H3-K27-me3 (Abcam 6002). ProtG-Sepharose was blocked twice at 4°C with 1 μg/μl salmon sperm DNA sheared at 500 bp length and 1 μg/μl BSA, for 2 h and overnight. Chromatin was precleared by adding ProtG-Sepharose for 2 h at 4°C, aliquoted and incubated with the antibodies overnight at 4°C. Several semi-quantitative PCRs were performed with the primers described in Supplementary Data 2. The gels (6/10 depending from the samples) were scanned with a Typhoon 4000 instrument and values within the linear range of amplifications were plotted as fold-enrichment over the negative control antibody (Flag, Sigma). Standard deviations were <20%.

RESULTS

Kinetic induction of ER stress genes

Several genes are induced after ER stress. We performed kinetic analysis of various genes by treating human hepatoma HepG2 cells with Tg for different times: 1, 4, 8 and 24 h. Figure 1 shows semi-quantitative RT–PCR analysis of the different ER stress induced mRNAs. Most of the genes analyzed do show induction, with a peak between 4 and 8 h. In some cases—PDI, Calreticulin and Grp94—the basal levels are already substantially visible, and the induction is small. Among the TFs known to be involved in ER stress, CHOP is rapidly induced from very low levels, already after 1 h; C/EBPβ is present, it increases at 4 h and remains high at 24 h; XBP1 shows a rapid appearance of the active, spliced isoform [Termed S in Figure 1; see Refs (19,21)]. ATF6

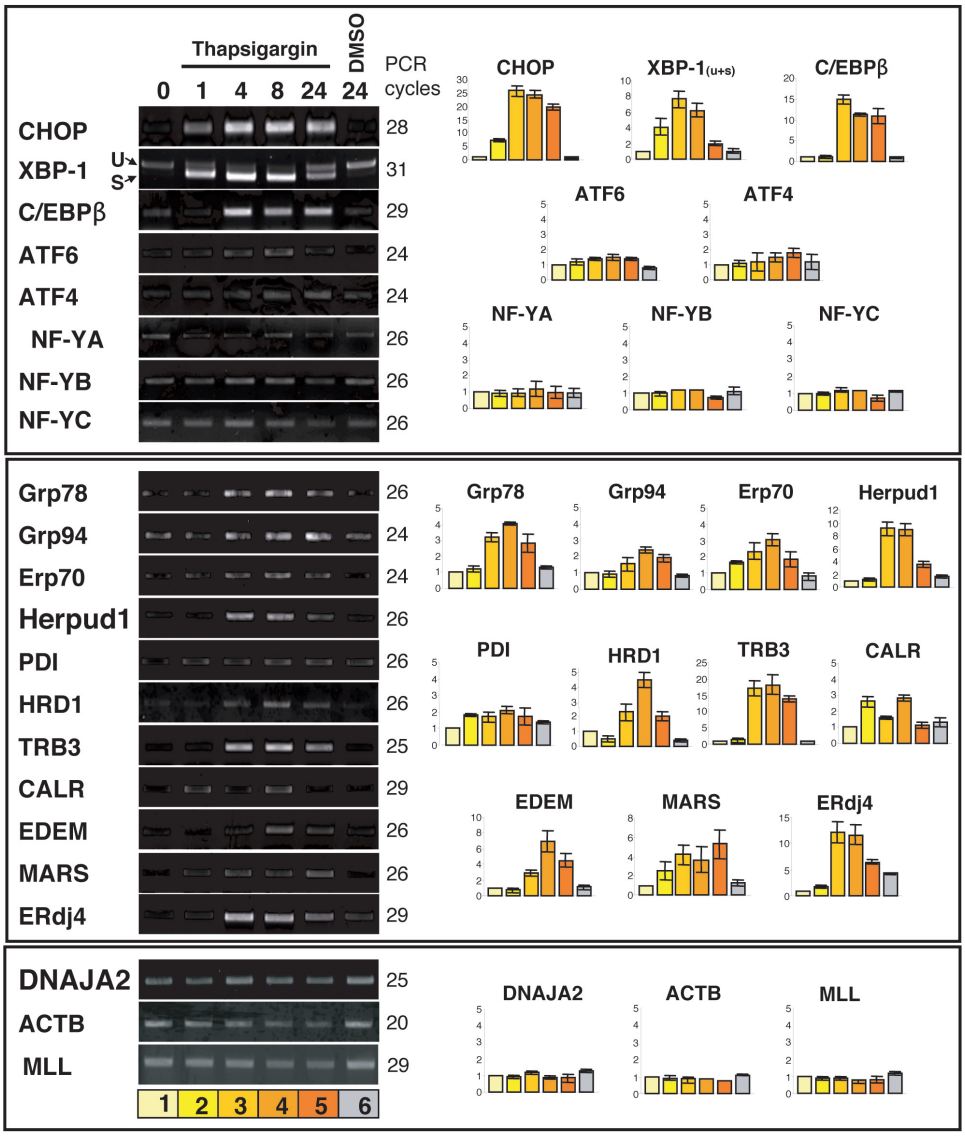
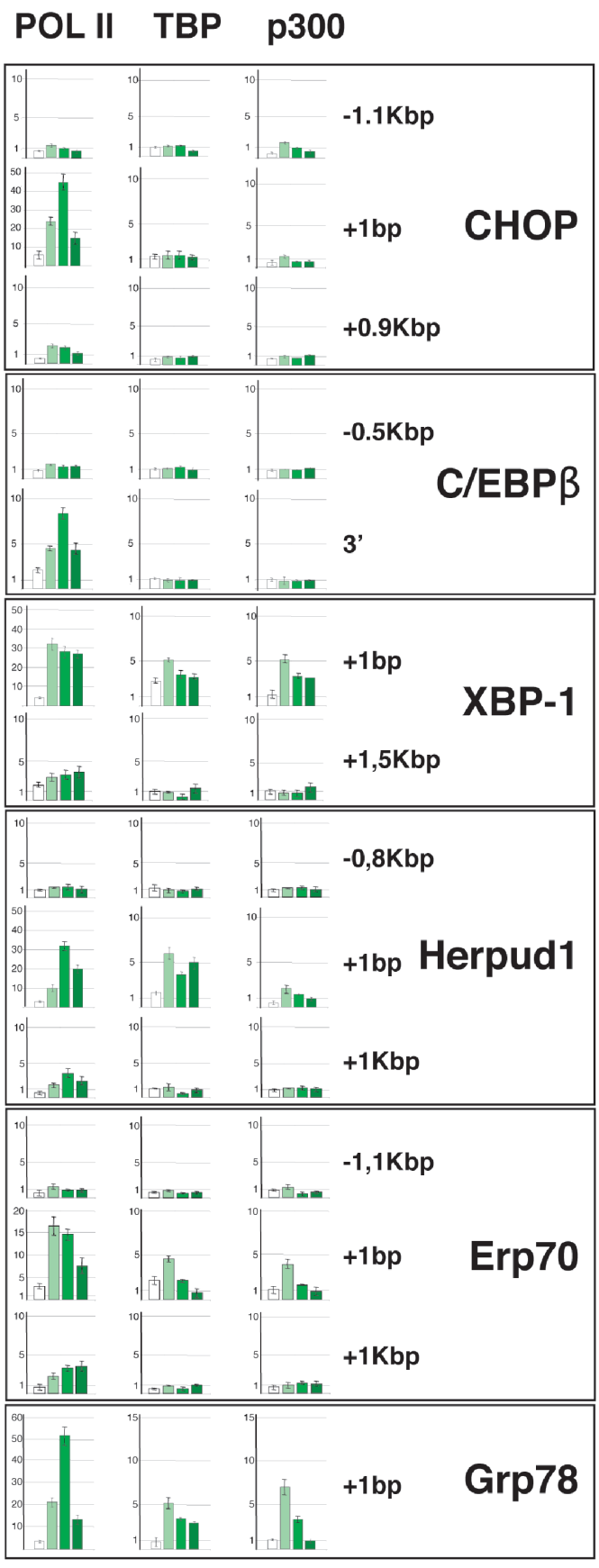


Figure 1. Induction of ER stress genes. Semi-quantitative RT–PCR analysis of ER stress induced genes in HepG2 cells treated for the indicated hours with thapsigargin (Tg). A control 24 h treatment with DMSO is shown on the right. The PCR cycles are shown for each gene, to indicate the relative abundance of the mRNAs analyzed. Quantification of the RT–PCRs are shown in appropriate panels. The colors refer to the different time points analyzed.

and ATF4 mRNA levels change modestly. Several controls were run in parallel: none of the NF-Y subunits changed significantly, nor DNAJA2, β-actin or MLL.

Recruitment of Pol II, TBP and p300

In the same cellular context, we evaluated the association of RNA Pol II, TBP and of the p300 HAT coactivator to representative promoters in ChIP assays. For CHOP, Herpud1, Erp70 and XBP-1, we evaluated different areas of the genes; in particular, for C/EBPβ, a downstream region which is the site of TFs binding and regulation [Marked 3′ in Figure 2, see Ref. (26)] and the −500 region, since we were unable to PCR amplify the core promoter with several primers sets. At 0 h, Pol II binding is low, but above background on all promoters. The CHOP core promoter, for example, shows a selective 6-fold enrichment with the Pol II antibody. Association to all promoters increases considerably at 1 and 4 h, then it declines. This is true for the genes induced with a slower kinetics, including C/EBPβ in the 3′ region. In general, this behaviour correlates with the RT–PCR analysis: the recruitment at 1 h precedes mRNAs accumulation. p300 is transiently inducible, at 1 h, on all genes except C/EBPβ and CHOP. Interestingly, TBP is also inducible with rapid kinetics, but it is not detected before induction on any of the targets. The upstream regions of CHOP, Herpud1 and Erp70 were negative for the proteins considered, whereas downstream regions showed a small, but reproducible induction of Pol II. Interestingly, the enrichment was lower than in the core promoters. These data indicate that there is some Pol II association before induction and strong recruitment after, while TBP and p300 are not ubiquitous and observed only after induction.



Histone modifications on ER stress-induced genes

It has been shown that H4, but not H3 acetylations, as well as H3-K4-me3 increase upon induction of Grp78 in Hela cells (10). We performed ChIP analysis with several anti-histones antibodies in HepG2. Indeed, we confirm that basal H3 acetylation is high on essentially all promoters, and modestly increases upon induction, while H4 acetylation increases considerably (Figure 3, Note the different scales of fold-enrichments over the Flag control). In some promoters—CHOP, Erp70, HRD1 and Herpud1—both H3 and H4 acetylation is induced. As for histone methylations, H3-K4-me2 was not scored under normal conditions and somewhat increased, radiply on XBP-1, slowly on other promoters, reaching a peak at 8 h. The most profound effects, and the highest overall levels, are observed in the coding sequences of all genes, whereas upstream regions remain poorly enriched. H3-K4-me3, a marker of active genes, is high in unstressed cells; surprisingly, it drops at 4 h on CHOP, Herpud1 and Erp70, rebounding strongly at 8 h. This behaviour is not seen on C/EBPβ −500 and 3′ regions, XBP-1 or HRD1. Note that the CHOP and Herpud1 usptream regions hardly tri-methylated under basal and early induction, and becomes positive after 8 h. As for H3-K79-me2, it is measurable and constant at 0, 1 and 4 h and induced at 8 h on all promoters. Interestingly, however, the stronger positivity is found in the coding regions, while upstream regions are essentially negative. Taken together, these data highlight a level of 'pre-activation' of epigenetic marks on ER stress genes.

We were intrigued by the finding that H3-K4-me3 decreases upon induction on some promoters. In light of the recent findings that H3 is evicted from Heat Shock genes of yeast (31) and *Drosophila* after promoter induction (32), we decided to control the overall enrichment for H3 by using an antibody against unmodified H3. We run a control to quantify the amount of immunoprecipitated DNAs in the different ChIPs, by amplifying a repetitive satellite region: there is no enrichment on any of the factors analyzed (Figure 4). The overall amount of H3 immunoprecipitated at 1 and 4 h is decreased in CHOP (Core Promoter), at 4 h on Herpud1, as well as Grp78/94 and Erp70. These are specifically the regions and times where the decrease of H3-K4-me3 is scored (Figure 3). Parallel ChIPs with negative epigenetic marks, such as H3-K9-me2 and H3-K27-me3, are essentially negative (Figure 4). We normalized the levels of H3 modification for the amount of immunoprecipitable unmodified H3 (Supplementary Data 3): indeed, no reduction of modifications was score, in particular H3-K4-me3. We conclude that the decrease in H3-K4-me3 and the lack of H3 hyperacetylation is largely due to H3 displacement during maximal transcriptional effort.

Figure 2. Recruitment of GTFs. Kinetic ChIP analysis of Pol II, TBP and p300 recruitment on representative ER stress promoters in HepG2 cells. Several semi-quantitative PCRs were analyzed, scanned and the data plotted against the negative control (Flag antibody). Values are represented as fold-enrichment over the Flag control. The bars of the standard deviations result from the quantification of several PCRs within the linear range. Note the different scales in some of the panels. The basal, uninduced levels are in white, 1 h in light green, 4 h in green and 8 h in dark green.

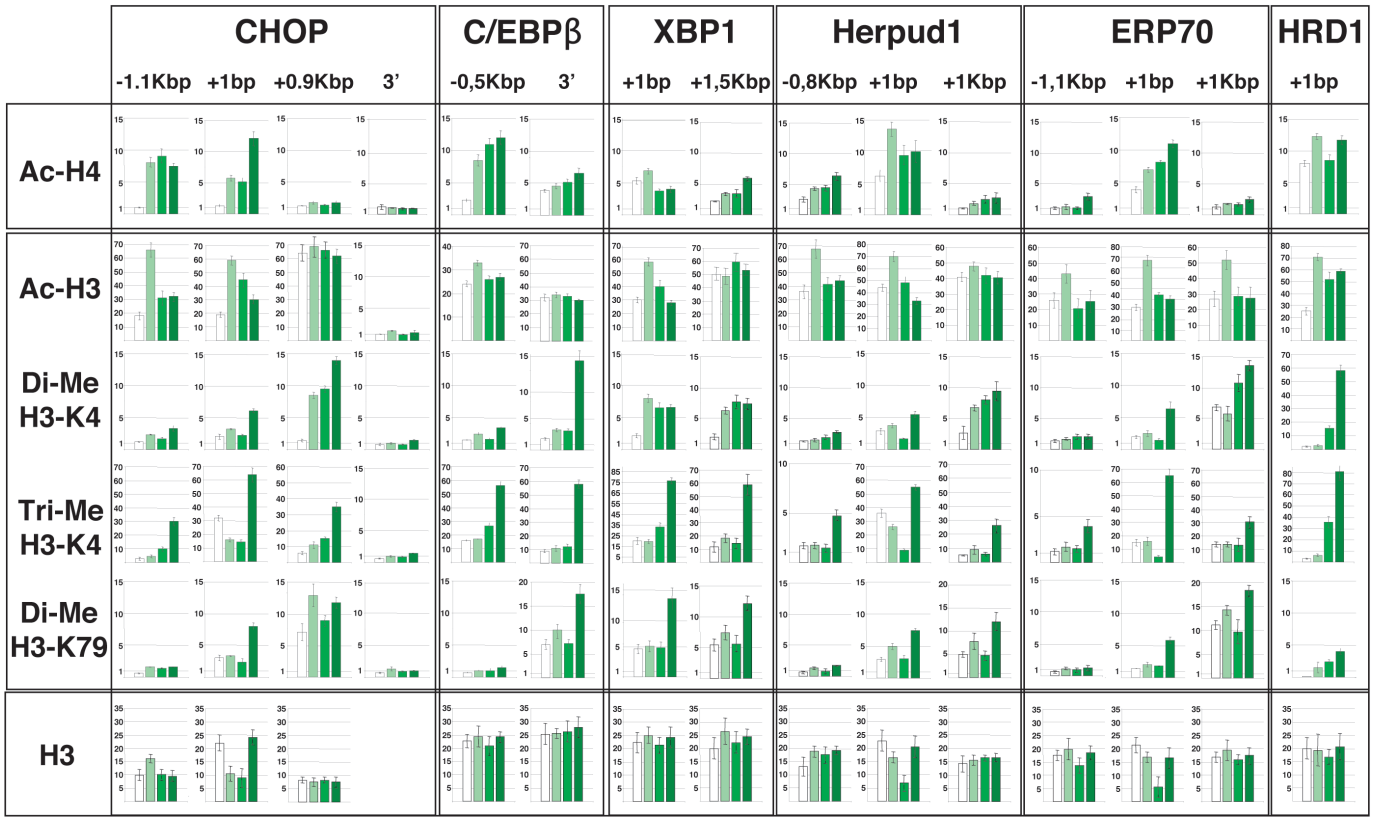


Figure 3. Changes in histones acetylation and methylation upon ER stress. Kinetic ChIP analysis of HepG2 cells as in Figure 2, with antibodies directed anti-AcH4, anti-AcH3, H3-K4-me2, H3-K4-me3, H3-K79-me2 and unmodified H3. The results shown are expressed as enrichment over the negative control antibody.

Dynamic binding of TFs to ER stress genes

Next, we performed ChIPs of several TFs known to play a role in the ER stress response, namely XBP-1, CHOP, ATF6 and C/EBPβ, on the genes considered previously. Figure 5 shows the quantification of the PCR analysis (see Supplementary Data 4 for representative semi-quantitative analysis). None of the factors binds to the uninducible DNAJA2 promoter. In unstressed conditions, XBP-1 binds to Grp78/94, XBP1 Erp70 and PDI. In many cases, association is consistent with the splicing switch resulting in the appearance of the transcriptional competent isoform (see Figure 1). Interestingly, however, we observe XBP-1 on the functionally similar Grp78/94, ERP70 genes, as well as on its own promoter. CHOP is essentially absent at 0 h, with the exception of PDI, in keeping with its low level of expression in unstimulated cells; it is still negative at 1 h, and later binds to C/EBPβ, ERdj4, MARS, ERP70, Calr, CHOP. ATF6 is progressively recruited to CHOP Grp94 and Erp70 at 1 h, to Grp78, Herpud1 at 4 h, to XBP-1, EDEM and Calr at 8 h. It binds to none of the other promoters. Finally, C/EBPβ associates to Erp70, Calr, MARS and Herpud1. It is particularly relevant that it is found on CHOP at 1 h, when the gene starts to be induced, before ATF6 and XBP-1. These data indicate a complexity for TFs binding to activated promoters: XBP-1 is the most widespread; C/EBPβ is quite selective; CHOP and ATF6 show inducible and largely non-overlapping targets selectivity. Remarkably, none of the factors associated to the inducible HRD1.

NF-Y is constitutively bound to ER stress promoters

Two factors, Sp1 and NF-Y, are generally involved in many inducible systems (10,33). We then assayed these TFs in ChIPs; we also analyzed p53, because (i) it is involved in the apoptotic response to ER stress (34 and reference therein) and (ii) it is related to p73, which has been involved in some aspects of the ER stress response (35). Note that HepG2 cells have wt p53 alleles. The kinetic analysis is shown in Figure 6: NF-Y is bound before induction to Grp78, consistent with the data published in Hela cells (10), and also to all other inducible genes, with the exception of TRB3, MARS and C/EBPβ, promoters that indeed have no CCAAT boxes. Sp1 is enriched on CHOP, XBP-1, Calreticulin and PDI, p53—weakly—on the latters. Upon induction, all NF-Y genes remain positive and Sp1 is still on CHOP at 1 and 4 h and PDI at 1 h. p53 is found on PDI at 1 and 4 h. Note that in the case of CHOP and C/EBPβ, other regions of the genes were devoid of these factors. The control repetitive satellite region shows no enrichment on any of the factors (Figure 6A, lower panel). We further analyzed NF-Y binding with an antibody specific for NF-YA: Figure 6B shows that all targets positive for NF-YB are also scored with the anti-NF-YA, both before and after induction, while C/EBPβ and

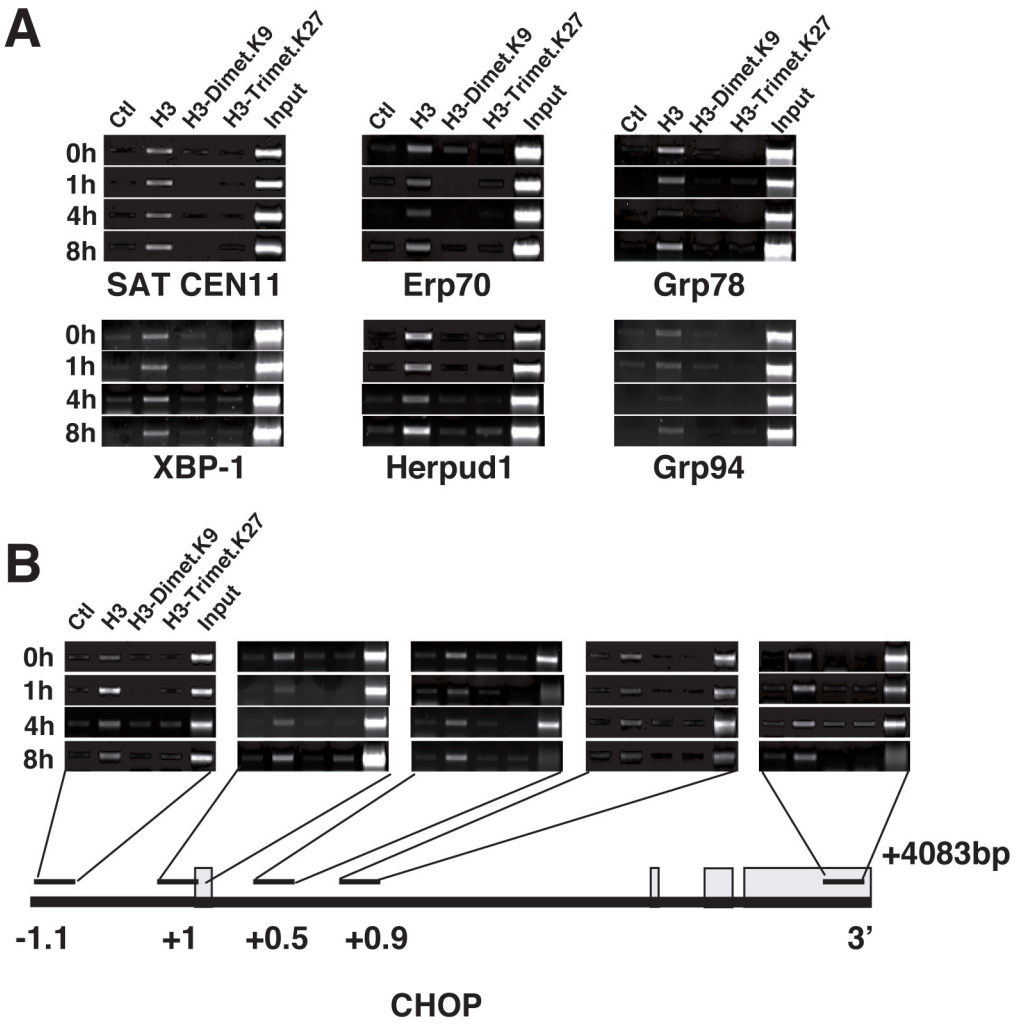


Figure 4. H3 recovery in ChIP assays under ER stress. (**A**) Kinetic ChIP analysis with the indicated antibodies against unmodified H3, H3-K9-me2 and H3-K27-me3 in HepG2 cells, treated for the indicated times with Tg. The different targets were PCR-amplified with specific primers. A control satellite centromeric 11 region was added in the upper left panel. (**B**) Same as (A) on different regions of the CHOP gene, a scheme of which is depicted.

TRB3 remain negative; this confirms the results obtained with NF-YB, and indicate that the trimer, and not only the histone-fold dimer, is associated to promoters.

Effects of NF-YB functional inactivation

Because of the widespred binding of NF-Y, we wished to ascertain whether its functional inactivation hampers gene expression. We failed to transfect HepG2 cells at high efficiency with several NF-YB siRNA oligonucleotides (data not shown). We then switched to HCT116 colon carcinoma cells and transfected them with an NF-YB siRNA oligonucleotide. As shown in Figure 7A, the NF-YB subunit was considerably reduced, albeit not abolished, in transfected cells, either uninduced or induced for 2 and 4 h with Tg. The results of RT–PCRs on ER stress genes are shown in Figure 7B. Induction is promptly observed in this cellular context as well (Figure 7B, right panels). While most of the genes bound by NF-Y are severely impaired in their induction upon siRNA treatment, others showed minor effects, if any. The CCAAT-less uninducible MLL was used as an internal control for this analysis; furthermore, we controlled TRB3, MARS and C/EBPβ, all NF-Y-less promoters according to the ChIP analysis of Figure 6: none of these gene showed any significant change in trancription after NF-YB inactivation (Figure 7B, lower panel). Given the surprising insensitivity of some of the CCAAT promoters to NF-YB inactivation, we verified binding of NF-Y in HCT116 cells: Figure 7C confirms that the factor is indeed present under uninduced conditions, ruling out fundamental differences in the NF-Y binding behaviour in HCT116 and HepG2 cells. We therefore conclude that there is a hierarchy of NF-Y dependancy within the ER stress genes.

DISCUSSION

The basis of our work relied upon genetic observations suggesting that several TFs are involved in the ER stress response. The picture emerging is one of remarkable complexity at the level of TFs binding. Our data rule out simplistic 'master' gene activation models, favouring a highly

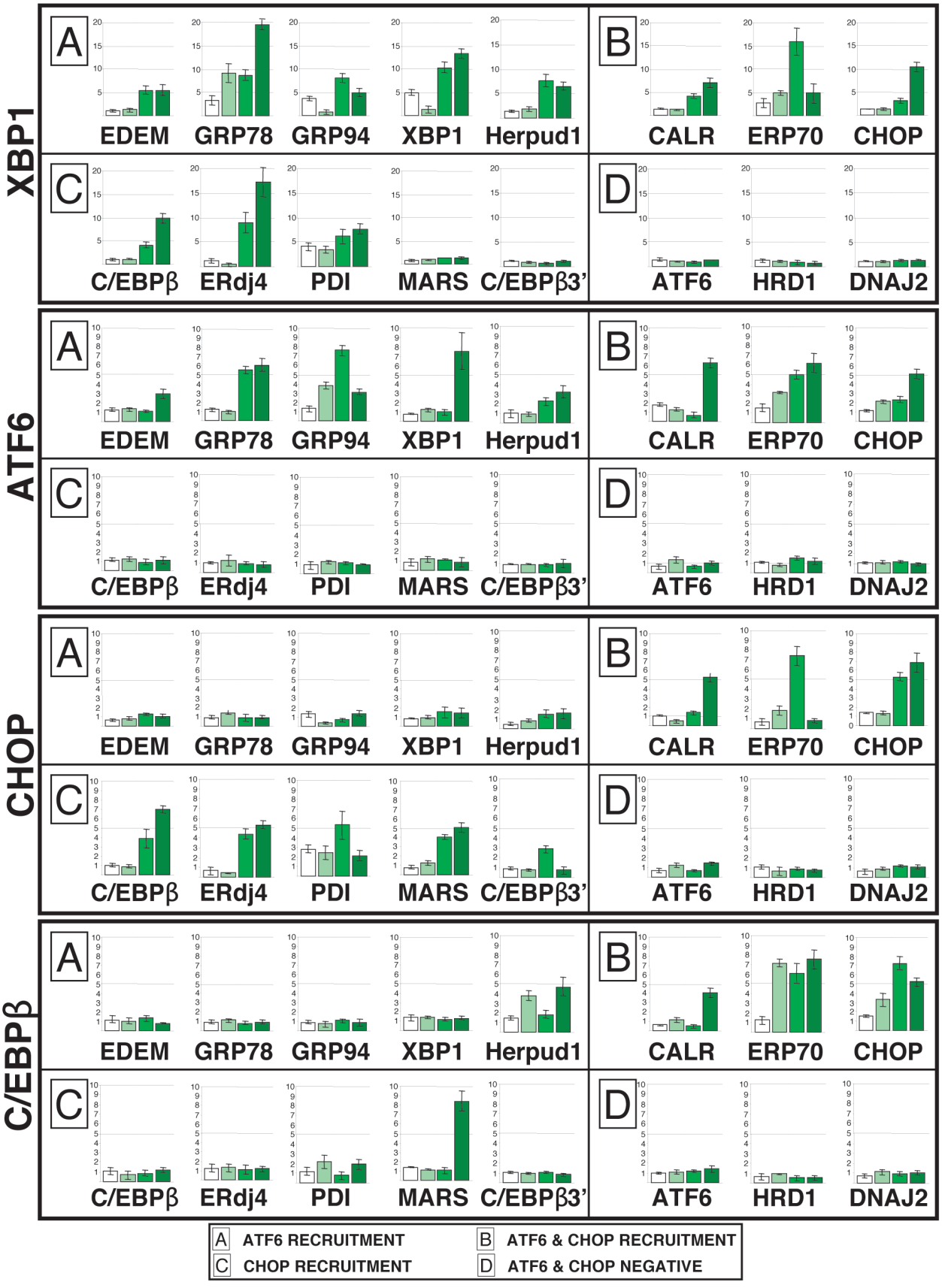


Figure 5. Binding of ER stress induced TFs to ER stress promoters. Kinetic ChIP analysis of XBP-1, CHOP, ATF6 and C/EBPβ to ER stress promoters in HepG2 cells. Chromatin was prepared as in Figures 2–4 under growing and 1, 4 and 8 h post ER stress induction.

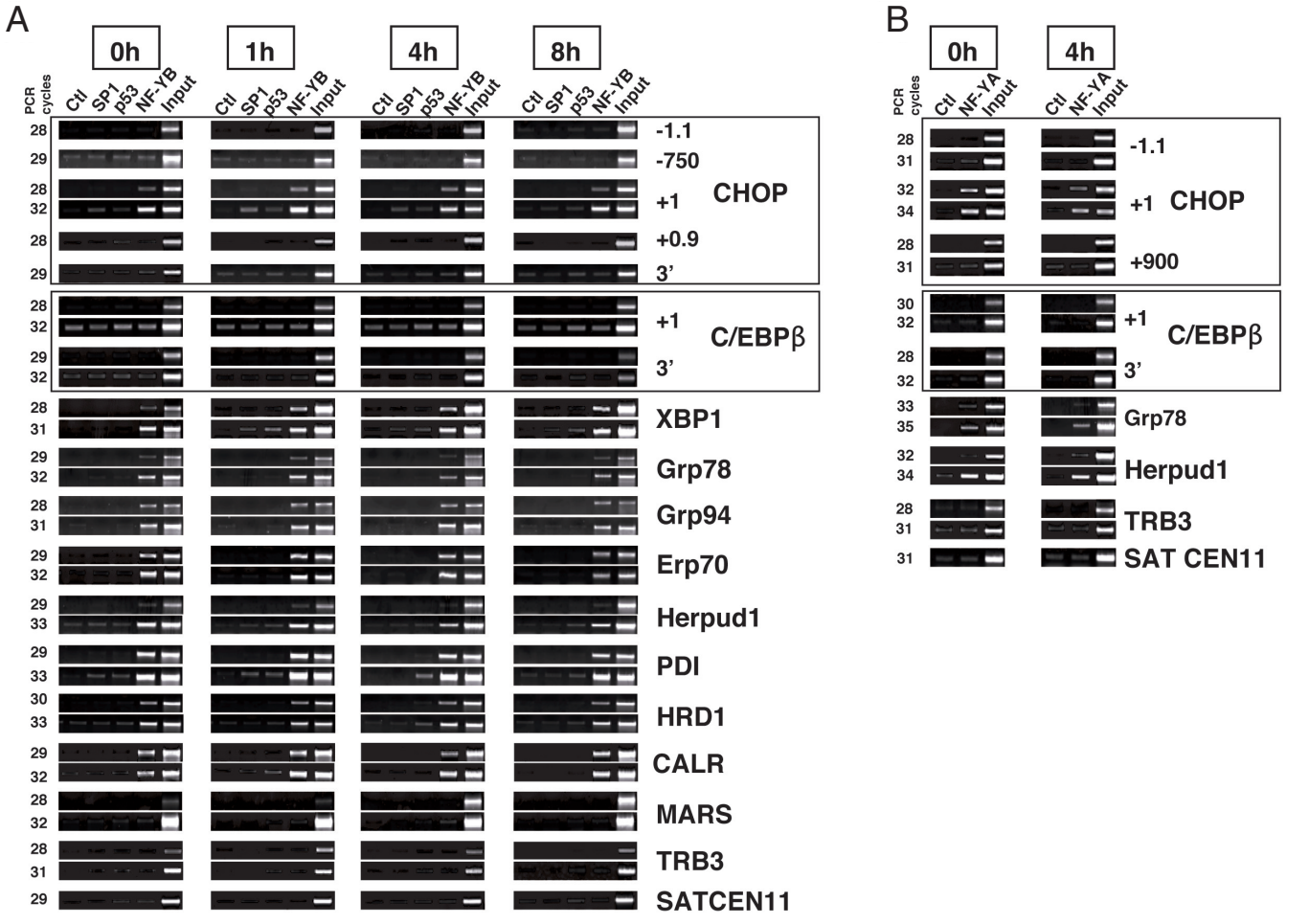


Figure 6. Binding of NF-Y, Sp1 and p53 to ER stress promoters. ChIP analysis of NF-Y, Sp1 and p53 in HepG2 cells. Chromatin was prepared from cells treated for the indicated times with Tg. Two semi-quantitative PCR amplifications are shown for most targets. The number of PCR cycles are shown on the left.

integrated and cooperative way of operating a global response.

ER stress TFs

Biochemical and genetic studies clearly established that the UPR and, specifically, the ER stress response is quite complex. Not surprisingly, therefore, a number of TFs were shown to be involved. According to our analysis, there are two categories of TFs: pre-bound (NF-Y, C/EBPβ and XBP-1) and recruited (CHOP and ATF6). Interestingly, XBP-1, ATF6 and CHOP display different modes of activation: XBP-1 through alternative splicing, generating an isoform, XBP-1s, capable of activating promoters (19,23). Therefore, although we cannot prove it with specific reagents, the XBP-1 bound at promoters before induction is most likely the inactive isoform, which gets substituted by XBP-1s after induction. This might be part of a regulatory mechanism, not only in terms of further recruitment of cofactors after induction, but also to keep the promoter at bay under normal growing conditions (36). On the other hand, ATF6 is regulated through proteolytic cleavage, nuclear translocation and DNA-binding (8,37). Finally, the CHOP gene is induced by transcription (16). Indeed, after ER stress, there is rapid CHOP mRNA accumulation (Figures 1 and 7) and subsequent activation of genes that are either bound by ATF6 or CHOP. Only in the cases of CHOP, Erp70 and Calreticulin do we see these two TFs bound together. In general, our ChIP data are consistent with dependency of ER stress genes from the various TFs considered. XBP-1 does bind constitutively to PDI (Figure 5), and indeed XBP-$1^{-/-}$ cells show an impaired Tg response for PDI (24). ATF6 targets were identified by microarray profiling of Tg-induced Hela cells, as genes encoding proteins resident in the ER and directly involved in protein folding (18,38): our data are consistent with this analysis, as *in vivo* association is found on Erp70/Grp78/Grp94. Moreover, PDI, which is poorly induced under our experimental conditions, is not bound by ATF6.

The model emerging is one of distinct and partially converging transcriptional signals operated by the four TFs considered (Figure 5). We observe three promoters which are bound by all TFs: Erp70, Calr and CHOP. More specifically, the ATF6 and CHOP pathways are largely not overlapping. XBP-1 is more promiscuous. Interestingly, at the opposite end of the spectra, TRB3 and HRD1 are devoid of the TFs considered here, yet they are clearly inducible by ER stress.

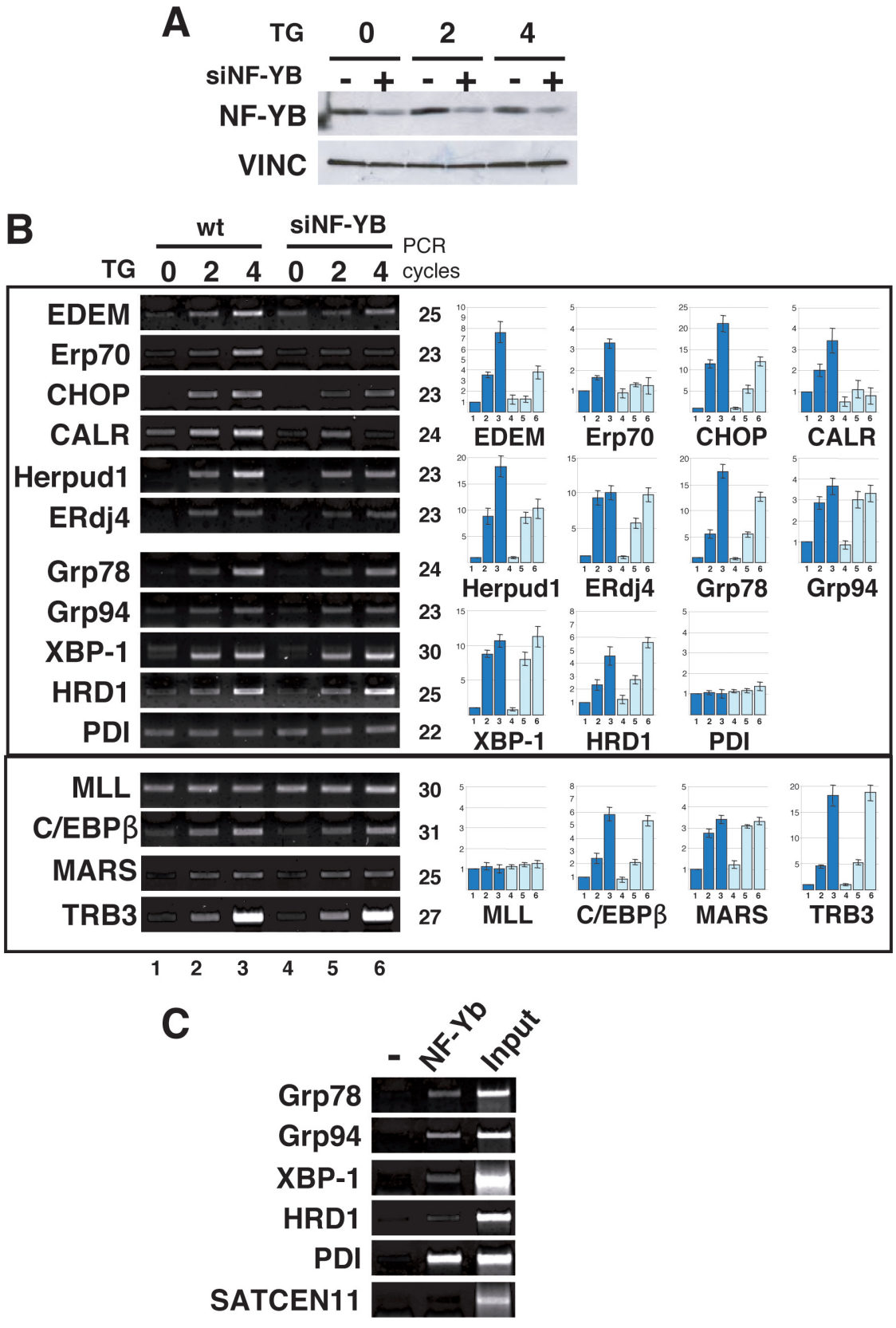


Figure 7. NF-YB inactivation: induction of ER stress genes. HCT116 cells were transfected with an siRNA oligonucleotide that targets the NF-YB subunit of NF-Y. (**A**) Western blot analysis NF-YB (and of the control Vinculin) with extracts from uninduced and ER stress induced cells. (**B**) RT–PCR analysis of ER stress response genes in HCT116 cells treated as in (A). In the lower panel, the analysis of genes with CCAAT-less promoters are shown. (**C**) ChIP analysis of HCT116 cells in uninduced conditions with anti-YB antibodies. The indicated ER stress promoters were analyzed by semi-quantitative PCR analysis.

This suggests that either the crucial binding sites reside away from the core promoters, or that other TFs not considered here, such as ATF4 (39), might be involved.

It is clear that CHOP is a nodal point of the UPR response. One key issue is what is activating CHOP initially: ATF6 and XBP-1 are not bound to the promoter region at 1 h, when transcription has gone up already. In keeping with this, XBP-$1^{-/-}$ cells and ATF6i inactivated ones have normal CHOP induction (24). Thus, these two TFs are unlikely to play a major role in CHOP initial activity. At 4 h, all TFs, including CHOP itself are bound and transcription maximal. Hence, other factors are important, such as NF-Y and C/EBPβ, possibly through modifications by a signal cascade, so that they are more efficient in recruitment of other TFs and GTFs. It should be noted that CHOP and C/EBPβ belong to the same class of TF and they are able to heterodimerize: thus, our data are consistent with a model of feed-back loop regulation.

GTFs recruitment and epigenetic changes

Pol II is weakly, but definitely enriched over controls in several promoters under unstressed condition, which is consistent with the detectable levels of transcripts. Interestingly, Pol II is observed at the 3′ enhancer site of C/EBPβ and, albeit at lower levels, in the coding regions of XBP-1, Herpud1 and Erp70. TBP is hardly enriched under basal conditions, and there is a clear increase in promoters association soon after induction. There is little evidence of TBP binding at the C/EBPβ enhancer: this might be understandable, considering its specific role on core promoters, which we were unable to analyze in this gene. TBP was absent in CHOP, a TATA-less promoters. It is unclear whether these data are due to masking of epitopes, or outright absence: in this latter case, it is possible that complexes other that the TBP-containing TFIID, such as STAGA/TFTC/PCAF, might be involved in activation (40). As for TBP kinetics of association, two types of scenarios emerged from previous studies. (i) In the HSP70 systems, which has similarities to the one used here, TBP is already bound to the core promoter before induction: Pol II firing takes place within one-third of a minute, following HSF association (41). (ii) In slower systems, TBP is recruited at a relatively late stage—β-IFN—or in cyclical fashion, as in the Estrogen Receptor response (42,43). Our data are clearly in line with these latter systems.

Tri-methylation of H3-K4 and di-methylation of H3-K79 are normally high, and negative epigenetic marks, such as H3-K27-me3 are absent. A notable difference between these two marks is the fact that H3-K79-me2 is more localized in the coding regions, whereas H3-K4-me3 spreads over upstream locations. This indicates local predisposition to activation, as also suggested by high levels of H3 acetylations. H4 acetylation is remarkably induced: consistent with this, p300 is also absent in unstressed cells, and differentially recruited with kinetics similar to H4 acetylation, suggesting that this HAT is involved in the process. On the other hand, there are promoters—CHOP, C/EBPβ—where robust H4 acetylation is manifest in the absence of p300 binding, an indication of other HATs operating. These data should be considered from another perspective: the decrease in methylation, and the lack of H3 hyperacetylation upon induction, are reconciled by the finding that H3 is generally decreased after stress: when normalized for unmodified H3 levels, the decrease is balanced and reversed (Supplementary Data 3). Therefore, our data suggest that H3 is either depleted, or substituted by a variant H3. A similar scenario was recently observed on the highly inducible Heat Shock promoters both in yeast and *Drosophila* (44,45). In the latter case, Schwartz and Ahmad (32) established that H3.3 replaces H3 upon induction and that transcription is required for this to happen. Considering the tight relationships of H3 with H4, which is most likely not evicted according to the data on its vast hyperacetylation (Figure 3), the substitution model is particularly attractive. Therefore our data extend previous conclusions that H3 incorporation into nucleosomes is highly dynamic (i) to an additional, large family of inducible genes and (ii) to mammalian cells.

Role of NF-Y in the ER stress response

We were not surprised to find NF-Y on almost all genes analyzed in HepG2 and in HCT116. We were equally unsurprised to find it under unstressed conditions, since this has been described in a numer of systems *in vivo*, including genes of sterol metabolism and the Heat Shock response (10,46–49). NF-Y is not further recruited after stress. Thus, it belongs to the restricted set of marks defining a pre-active state of promoter configuration. Interestingly, Pol II has some access to promoters before induction, and NF-Y is apparently crucial in this mechanism (48,49). We establish for the first time that at least two subunits are present: NF-YB and NF-YA. This is a sensitive point, since NF-YB, and the tightly associated partner NF-YC, are histone-like subunits capable to interact with H3-H4 *in vitro* (50), and NF-YA is required for sequence-specific binding of the trimer (51). One of the hypothesis was that the local chromatin structure could be 'organized' by the histone-like dimer, and NF-YA would be recruited later, following stress, or any induction stimulus in other systems. NF-YA would then stabilize the complex on CCAAT, providing an additional activation domain and leading to further recruitment of co-factors (29,52). Our data indicate that this is not the case.

It is somewhat surprising that functional inactivation of NF-YB has not such a general effect on induction of ER stress response genes: while some genes are hit, specifically in their induced levels, others are not. There are several explanations to account for this result. The first is that RNAi having been incomplete, there is enough of the NF-YB subunit left to support the bulk of transcription. This is supported by the finding that NF-YB is in excess with respect to NF-YA: hence, the stoichiometric balance of the CCAAT-binding trimer should not be impaired severely; we might not have obtained an effective lowering of the CCAAT-binding activity, but rather only of the histone-like dimer. We note that NF-YC is also in excess (data not shown) and, therefore, the siRNA procedure likely impairs dimer stoichiometry as well. Furthermore, several genes do show a clear decrease. One final issue that will need to be addressed is what the relationships are between active chromatin marks, as described in many studies and confirmed here, and the presence of the TFs analyzed here.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Shen,X., Ellis,R.E., Lee,K., Liu,C.Y., Yang,K., Solomon,A., Yoshida,H., Morimoto,R., Kurnit,D.M., Mori,K. *et al.* (2001) Complementary signaling pathways regulate the unfolded protein response and are required for *C. elegans* development. *Cell*, **107**, 893–903.
2. Schroder,M. and Kaufman,R.J. (2005) The mammalian unfolded protein response. *Annu. Rev. Biochem.*, **74**, 739–789.
3. Reddy,R.K., Mao,C., Baumeister,P., Austin,R.C., Kaufman,R.J. and Lee,A.S. (2003) Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. *J. Biol. Chem.*, **278**, 20915–20924.
4. Li,W.W., Sistonen,L., Morimoto,R.I. and Lee,A.S. (1994) Stress induction of the mammalian GRP78/BiP protein gene: *in vivo* genomic footprinting and identification of p70CORE from human nuclear extract as a DNA-binding component specific to the stress regulatory element. *Mol. Cell. Biol.*, **14**, 5533–5546.
5. Yoshida,H., Haze,K., Yanagi,H., Yura,T. and Mori,K. (1998) Identification of the *cis*-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *J. Biol. Chem.*, **273**, 33741–33749.
6. Roy,B. and Lee,A.S. (1999) The mammalian endoplasmic reticulum stress response element consists of an evolutionarily conserved tripartite structure and interacts with a novel stress-inducible complex. *Nucleic Acids Res.*, **27**, 1437–1443.
7. Li,M., Baumeister,P., Roy,B., Phan,T., Foti,D., Luo,S. and Lee,A.S. (2000) ATF6 as a transcription activator of the endoplasmic reticulum stress element: thapsigargin stress-induced changes and synergistic interactions with NF-Y and YY1. *Mol. Cell. Biol.*, **20**, 5096–5106.
8. Yoshida,H., Okada,T., Haze,K., Yanagi,H., Yura,T., Negishi,M. and Mori,K. (2001) Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6alpha and 6beta that activates the mammalian unfolded protein response. *Mol. Cell. Biol.*, **21**, 1239–1248.
9. Parker,R., Phan,T., Baumeister,P., Roy,B., Cheriyath,V., Roy,A.L. and Lee,A.S. (2001) Identification of TFII-I as the endoplasmic reticulum stress response element binding factor ERSF: its autoregulation by stress and interaction with ATF6. *Mol. Cell. Biol.*, **21**, 3220–3233.
10. Baumeister,P., Luo,S., Skarnes,W.C., Sui,G., Seto,E., Shi,Y. and Lee,A.S. (2005) Endoplasmic reticulum stress induction of the Grp78/BiP promoter: activating mechanisms mediated by YY1 and its interactive chromatin modifiers. *Mol. Cell. Biol.*, **25**, 4529–4540.
11. Bartlett,J.D., Luethy,J.D., Carlson,S.G., Sollott,S.J. and Holbrook,N.J. (1992) Calcium ionophore A23187 induces expression of the growth arrest and DNA damage inducible CCAAT/enhancer-binding protein (C/EBP)-related gene, gadd153. Ca^{2+} increases transcriptional activity and mRNA stability. *J. Biol. Chem.*, **267**, 20465–20470.
12. Wang,X.Z., Lawson,B., Brewer,J.W., Zinszner,H., Sanjay,A., Mi,L.J., Boorstein,R., Kreibich,G., Hendershot,L.M. and Ron,D. (1996) Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein CHOP/GADD153. *Mol. Cell. Biol.*, **16**, 4273–4280.
13. Fawcett,T.W., Martindale,J.L., Guyton,K.Z., Hai,T. and Holbrook,N.J. (1999) Complexes containing activating transcription factor (ATF) cAMP-responsive-element-binding protein (CREB) interact with the CCAAT-enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response. *Biochem. J.*, **339**, 135–141.
14. Ubeda,M. and Habener,J.F. (2000) CHOP gene expression in response to endoplasmic-reticular stress requires NFY interaction with different domains of a conserved DNA-binding element. *Nucleic Acids Res.*, **28**, 4987–4997.
15. Ma,Y., Brewer,J.W., Diehl,J.A. and Hendershot,L.M. (2002) Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response. *J. Mol. Biol.*, **318**, 1351–1365.
16. Oyadomari,S. and Mori,M. (2004) Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ.*, **11**, 381–389.
17. Kokame,K., Kato,H. and Miyata,T. (2001) Identification of ERSE-II, a new *cis*-acting element responsible for the ATF6-dependent mammalian unfolded protein response. *J. Biol. Chem.*, **276**, 9199–9205.
18. Okada,T., Yoshida,H., Akazawa,R., Negishi,M. and Mori,K. (2002) Distinct roles of activating transcription factor 6 (ATF6) and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) in transcription during the mammalian unfolded protein response. *Biochem. J.*, **366**, 585–594.
19. Yoshida,H., Matsui,T., Yamamoto,A., Okada,T. and Mori,K. (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell*, **107**, 881–891.
20. Liou,H.C., Boothby,M.R., Finn,P.W., Davidon,R., Nabavi,N., Zeleznik-Le,N.J., Ting,J.P. and Glimcher,L.H. (1990) A new member of the leucine zipper class of proteins that binds to the HLA DR alpha promoter. *Science*, **247**, 1581–1584.
21. Calfon,M., Zeng,H., Urano,F., Till,J.H., Hubbard,S.R., Harding,H.P., Clark,S.G. and Ron,D. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature*, **415**, 92–96.
22. Reimold,A.M., Etkin,A., Clauss,I., Perkins,A., Friend,D.S., Zhang,J., Horton,H.F., Scott,A., Orkin,S.H., Byrne,M.C. *et al.* (2000) An essential role in liver development for transcription factor XBP-1. *Genes Dev.*, **14**, 152–157.
23. Reimold,A.M., Iwakoshi,N.N., Manis,J., Vallabhajosyula,P., Szomolanyi-Tsuda,E., Gravallese,E.M., Friend,D., Grusby,M.J., Alt,F. and Glimcher,L.H. (2001) Plasma cell differentiation requires the transcription factor XBP-1. *Nature*, **412**, 300–307.
24. Lee,A.H., Iwakoshi,N.N. and Glimcher,L.H. (2003) XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol. Cell. Biol.*, **23**, 7448–7459.
25. Chen,C., Dudenhausen,E.E., Pan,Y.X., Zhong,C. and Kilberg,M.S. (2004) Human CCAAT/enhancer-binding protein beta gene expression is activated by endoplasmic reticulum stress through an unfolded protein response element downstream of the protein coding sequence. *J. Biol. Chem.*, **279**, 27948–27956.
26. Chen,C., Dudenhausen,E.E., Chen,H., Pan,Y.X., Gjymishka,A. and Kilberg,M.S. (2005) Amino acid limitation induces transcription from the human C/EBPbeta gene via an enhancer activity located downstream of the protein coding sequence. *Biochem. J.*, **391**, 649–655.
27. Zinszner,H., Kuroda,M., Wang,X., Batchvarova,N., Lightfoot,R.T., Remotti,H., Stevens,J.L. and Ron,D. (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev.*, **12**, 982–995.
28. Oyadomari,S., Koizumi,A., Takeda,K., Gotoh,T., Akira,S., Araki,E. and Mori,M. (2002) Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J. Clin. Invest.*, **109**, 525–532.
29. Caretti,G., Salsi,V., Vecchi,C., Imbriano,C. and Mantovani,R. (2003) Dynamic recruitment of NF-Y and histone acetyltransferases on cell-cycle promoters. *J. Biol. Chem.*, **278**, 30435–30440.
30. Mantovani,R., Tora,L., Moncollin,V., Egly,J.M., Benoist,C. and Mathis,D. (1993) The major histocompatibility complex (MHC) Ea promoter: sequences and factors at the initiation site. *Nucleic Acids Res.*, **21**, 4873–4878.
31. McKittrick,E., Gafken,P.R., Ahmad,K. and Henikoff,S. (2004) Histone H3.3 is enriched in covalent modifications associated with active chromatin. *Proc. Natl Acad. Sci. USA*, **101**, 1525–1530.
32. Schwartz,B.E. and Ahmad,K. (2005) Transcriptional activation triggers deposition and removal of the histone variant H3.3. *Genes Dev.*, **19**, 804–814.

33. Abdelrahim,M., Liu,S. and Safe,S. (2005) Induction of endoplasmic reticulum-induced stress genes in Panc-1 pancreatic cancer cells is dependent on Sp proteins. *J. Biol. Chem.*, **280**, 16508–16513.
34. Li,J., Lee,B. and Lee,A.S. (2006) Endoplasmic reticulum-stress induced apoptosis: Multiple pathways and activation of PUMA and NOXA by p53. *J. Biol. Chem.*, **281**, 7260–7270.
35. Terrinoni,A., Ranalli,M., Cadot,B., Leta,A., Bagetta,G., Vousden,K.H. and Melino,G. (2004) p73-alpha is capable of inducing scotin and ER stress. *Oncogene*, **23**, 3721–3725.
36. Tirosh,B., Iwakoshi,N.N., Glimcher,L.H. and Ploegh,H.L. (2005) Rapid turnover of unspliced XBP-1 as a factor that modulates the unfolded protein response. *J. Biol. Chem.*, **281**, 5852–5860.
37. Haze,K., Yoshida,H., Yanagi,H., Yura,T. and Mori,K. (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol. Biol. Cell.*, **10**, 3787–3799.
38. Yamamoto,K., Yoshida,H., Kokame,K., Kaufman,R.J. and Mori,K. (2004) Differential contributions of ATF6 and XBP1 to the activation of endoplasmic reticulum stress-responsive *cis*-acting elements ERSE, UPRE and ERSE-II. *J. Biochem.*, **136**, 343–350.
39. Ohoka,N., Yoshii,S., Hattori,T., Onozaki,K. and Hayashi,H. (2005) TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. *EMBO J.*, **24**, 1243–1255.
40. Muller,F. and Tora,L. (2004) The multicoloured world of promoter recognition complexes. *EMBO J.*, **23**, 2–8.
41. Lebedeva,L.A., Nabirochkina,E.N., Kurshakova,M.M., Robert,F., Krasnov,A.N., Evgen'ev,M.B., Kadonaga,J.T., Georgieva,S.G. and Tora,L. (2005) Occupancy of the *Drosophila* hsp70 promoter by a subset of basal transcription factors diminishes upon transcriptional activation. *Proc. Natl Acad. Sci. USA*, **102**, 18087–18092.
42. Lomvardas,S. and Thanos,D. (2001) Nucleosome sliding via TBP DNA binding *in vivo*. *Cell*, **106**, 685–696.
43. Metivier,R., Penot,G., Hubner,M.R., Reid,G., Brand,H., Kos,M. and Gannon,F. (2003) Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell*, **115**, 751–763.
44. Erkine,A.M. and Gross,D.S. (2003) Dynamic chromatin alterations triggered by natural and synthetic activation domains. *J. Biol. Chem.*, **278**, 7755–7764.
45. Zhao,J., Herrera-Diaz,J. and Gross,D.S. (2005) Domain-wide displacement of histones by activated heat shock factor occurs independently of Swi/Snf and is not correlated with RNA polymerase II density. *Mol. Cell Biol.*, **25**, 8985–8999.
46. Bennett,M.K. and Osborne,T.F. (2000) Nutrient regulation of gene expression by the sterol regulatory element binding proteins: increased recruitment of gene-specific coregulatory factors and selective hyperacetylation of histone H3 *in vivo*. *Proc. Natl Acad. Sci. USA*, **97**, 6340–6344.
47. Imbriano,C., Bolognese,F., Gurtner,A., Piaggio,G. and Mantovani,R. (2001) HSP-CBF is an NF-Y-dependent coactivator of heat shock promoters CCAAT boxes. *J. Biol. Chem.*, **276**, 26332–26339.
48. Kabe,Y., Yamada,J., Uga,H., Yamaguchi,Y., Wada,T. and Handa,H. (2005) NF-Y is essential for the recruitment of RNA polymerase II and inducible transcription of several CCAAT box-containing genes. *Mol. Cell. Biol.*, **25**, 512–522.
49. Duan,Z., Stamatoyannopoulos,G. and Li,Q. (2001) Role of NF-Y in *in vivo* regulation of the gamma-globin gene. *Mol. Cell. Biol.*, **21**, 3083–3095.
50. Caretti,G., Motta,M.C. and Mantovani,R. (1999) NF-Y associates H3-H4 tetramers and octamers by multiple mechanisms. *Mol. Cell. Biol.*, **19**, 8591–8603.
51. Romier,C., Cocchiarella,F., Mantovani,R. and Moras,D. (2003) The NF-YB/NF-YC structure gives insight into DNA binding and transcription regulation by CCAAT factor NF-Y. *J. Biol. Chem.*, **278**, 1336–1345.
52. Jin,S. and Scotto,K.W. (1998) Transcriptional regulation of the MDR1 gene by histone acetyltransferase and deacetylase is mediated by NF-Y. *Mol. Cell. Biol.*, **18**, 4377–4384.