Endoplasmic Reticulum Stress-responsive Transcription Factor ATF6α Directs Recruitment of the Mediator of RNA Polymerase II Transcription and Multiple Histone Acetyltransferase Complexes*§

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Background: Transcription factor ATF6α is a master regulator of genes induced by endoplasmic reticulum stress.

Results: ATF6α can recruit Mediator and histone acetyltransferase complexes to promoter DNA via interactions with overlapping sites in the activation domain of ATF6α.

Conclusion: ATF6α sequences essential for gene activation recruit Mediator and histone acetyltransferases.

Significance: Learning how coregulators communicate with DNA binding transcription factors is important for understanding gene regulation.

The basic leucine zipper transcription factor ATF6α functions as a master regulator of endoplasmic reticulum (ER) stress response genes. Previous studies have established that, in response to ER stress, ATF6α translocates to the nucleus and activates transcription of ER stress response genes upon binding sequence specifically to ER stress response enhancer elements in their promoters. In this study, we investigate the biochemical mechanism by which ATF6α activates transcription. By exploiting a combination of biochemical and multidimensional protein identification technology-based mass spectrometry approaches, we have obtained evidence that ATF6α functions at least in part by recruiting to the ER stress response enhancer elements of ER stress response genes a collection of RNA polymerase II coregulatory complexes, including the Mediator and multiple histone acetyltransferase complexes, among which are the Spt-Ada-Gcn5 acetyltransferase (SAGA) and Ada-Two-A-containing (ATAC) complexes. Our findings shed new light on the mechanism of action of ATF6α, and they outline a straightforward strategy for applying multidimensional protein identification technology mass spectrometry to determine which RNA polymerase II transcription factors and coregulators are recruited to promoters and other regulatory elements to control transcription.

Endoplasmic reticulum (ER) stress activates signal transduction pathways implicated in a variety of human diseases, including atherosclerosis, diabetes, and neurodegeneration (1–3). A major signaling pathway activated by ER stress is the unfolded protein response, which is triggered by accumulation of misfolded proteins in the ER and serves to protect cells from ER stress in part by down-regulating synthesis of some ER-des tined proteins and by up-regulating expression of proteins involved in ER protein folding.

A critical downstream event in the unfolded protein response signal transduction pathway is activation of transcription of a collection of ER stress response genes by a mechanism that depends on the basic leucine zipper transcription factor ATF6α (1–3). In unstressed cells, ATF6α resides in the ER as a type II transmembrane protein. In response to ER stress, ATF6α is transported to the Golgi apparatus, proteolytically processed by the site 1 and site 2 proteases, S1P and S2P (4–8), and then released into the nucleus, where it binds together with constitutively expressed transcription factors including NF-Y and Yin-Yang 1 (YY1) to ERSEs in the promoters of ER stress response genes (4–11). Ectopic expression of the proteolytically processed form of ATF6α is sufficient to activate the ER stress-induced transcriptional program in unstressed cells, arguing that ATF6α acts as a master regulator of ER stress response genes (4, 9, 12, 13).

Although cell biological studies have provided significant insight into the mechanism by which ATF6α is proteolytically processed, the mechanism by which ATF6α regulates gene transcription remains incompletely understood. The transcription factor ATF6α is a master regulator of genes induced by endoplasmic reticulum stress.

The abbreviations used are: ER, endoplasmic reticulum; ERSE, ER stress response element; ATAC, Ada-Two-A-containing; b-ZIP, basic leucine zipper; dNSAF, distributed normalized spectral abundance factor; HAT, histone acetyltransferase; MudPIT, multidimensional protein identification technology; NF-Y, nuclear factor Y; Pol II, RNA polymerase II; SAGA, Spt-Ada-Gcn5 Acetyltransferase; MBIP, MAP3K12-binding inhibitory protein; TFII, transcription factor II; CREB, cAMP-response element-binding protein; TRRAP, transformation/transcription domain-associated protein; TR, thyroid receptor.

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3 The abbreviations used are: ER, endoplasmic reticulum; ERSE, ER stress response element; ATAC, Ada-Two-A-containing; b-ZIP, basic leucine zipper; dNSAF, distributed normalized spectral abundance factor; HAT, histone acetyltransferase; MudPIT, multidimensional protein identification technology; NF-Y, nuclear factor Y; Pol II, RNA polymerase II; SAGA, Spt-Ada-Gcn5 Acetyltransferase; MBIP, MAP3K12-binding inhibitory protein; TFII, transcription factor II; CREB, cAMP-response element-binding protein; TRRAP, transformation/transcription domain-associated protein; TR, thyroid receptor.
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processed and transported to the nucleus, comparatively little is understood about how it activates transcription of ER stress response genes. ATF6α has an N-terminal transcription activation domain and a C-terminal DNA binding domain, whose affinity for its binding sites in ERSEs can be enhanced through interactions with NF-Y (11, 12, 14, 15). Upon ER stress, multiple Pol II coregulators including the histone acetyltransferases (HATs) CREB-binding protein (CBP)/p300 and SAGA, the protein arginine methyltransferase PRMT1, and the INO80 chromatin remodeling complex have been shown to be recruited to ERSEs in the promoter of HSPA5 and other stress response genes (10, 16–18). Results of prior studies suggest that PRMT1 and the INO80 complex are recruited through YY1 (10, 17), but it is not known whether the ATF6α activation domain also contributes to recruitment of these or other coregulators.

In this study, we applied a combination of biochemical and multidimensional protein identification technology (MudPIT)-based mass spectrometry approaches to investigate the role of ATF6α in recruitment of Pol II coregulatory proteins to the ERSEs of the HSPA5 gene. Below we present our findings, which are consistent with the model that ATF6α activates transcription at least in part by orchestrating the recruitment of a collection of Pol II coregulators, including the Mediator, SAGA, and ATAC complexes, to the ERSEs of its target genes. Dissection of the mechanism by which ATF6α recruits Pol II coregulators argues that Mediator and HAT complexes are recruited through interactions with nonidentical, but overlapping, regions of the ATF6α transcription activation domain. Taken together, our findings shed new light on the biochemical mechanisms underlying ATF6α-dependent transcription activation, and they provide a relatively straightforward strategy for exploiting the sensitivity of MudPIT mass spectrometry to determine how particular DNA binding transcription factors recruit Pol II coregulators to genes.

**Experimental Procedures**

**Materials**—pET28a-Gal4-VP16 AD, which encodes a fusion protein containing residues 1–94 of the Gal4 DNA binding domain fused to residues 412–490 of the VP16 activation domain, was a gift from Jerry Workman (Stowers Institute). pGEX-TRLBD, which encodes GST fused to amino acids 1–94 of the Gal4 DNA binding domain, was a gift from Jerry Workman (Stowers Institute). In Vitro Transcription—Biotinylated DNA fragments containing the wild type HSPA5 promoter (−282 to +147) or HSPA5 promoter lacking the TATA-box sequence TATAAAG were generated by PCR, gel-purified, and immobilized on Dynabeads. HeLa cell nuclear extracts were prepared as described (20) except that nuclei were extracted with 0.24 M KCl and dialyzed into 20 mM Hepes, pH 7.9, 20% glycerol, 0.2 mM EDTA, 1 mM DTT, 0.02% Nonidet P-40 containing 0.1 mg/ml bovine serum albumin (BSA) and 150 μg/ml poly(dI-dC) in a total volume of 80 μl. This mixture was then added to ~450 fmol of immobilized HSPA5 promoter fragment on Dynabeads that had been pre-equilibrated with PB containing 0.1 mg/ml BSA. The mixture was incubated a further 30 min at 30 °C with occasional mixing. The beads were washed twice with 100 μl of PB containing 0.1 mg/ml BSA and once with PB lacking BSA. Bound proteins were eluted with SDS sample buffer (2% SDS, 63 mM Tris-Cl, pH 6.8, 10% glycerol, 0.0025% bromphenol blue, 1.25% β-mercaptoethanol) at 99 °C for 5 min and analyzed by Western blotting. For MudPIT analysis, 800-nl binding reactions contained 100–200 μl of nuclear extracts, 25 pmol of GST-ATF6α, and ~4.5-pmol DNA fragment on Dynabeads. Beads were washed twice with 100 μl of PB containing 0.1 mg/ml BSA and once with PB lacking BSA. Bound proteins were then eluted by incubating with 2% SDS, 50 mM Tris-Cl, pH 8.8, 1.25 mM β-mercaptoethanol at 70 °C for 10 min.

In Vitro Transcription—Biotinylated DNA fragments containing the wild type HSPA5 promoter (−282 to +147) or HSPA5 promoter lacking the TATA-box sequence TATAAAG were generated by PCR, gel-purified, and immobilized on Dynabeads. HeLa cell nuclear extracts were prepared as described (20) except that nuclei were extracted with 0.24 M KCl and dialyzed into 20 mM Hepes, pH 7.9, 20% glycerol, 0.2 mM EDTA, 100 mM KCl, 1 mM DTT, and 0.5 mM PMSF. Transcription reactions were performed in two stages. First, 25-μl binding reactions containing 10 μl of nuclear extract, pH 7.9, 10% glycerol, 0.1 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 0.5 mM DTT, 0.25 mM PMSF, 0.1 mg/ml BSA, 12 μl of dialyzed nuclear extract, and ~450 fmol of immobilized DNA fragment, with or without GST-ATF6α, were incubated for 30 min at 30 °C. Unbound proteins were removed, and beads were equilibrated in transcription buffer by washing twice with 10 μl Hepes, pH 7.9,
10% glycerol, 0.05 M KCl, 6 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT, 0.25 mM PMSF. To initiate transcription, beads were resuspended in 25 µl of the same buffer containing 40 units of RNasin® (Promega) and 0.4 mM each of ATP, CTP, GTP, and UTP. After 90 min at 30 °C, reaction products were analyzed by primer extension as described (21) using an oligonucleotide with the sequence GCT TCC CTC TCA CAC TCG CG.

GST-ATF6α Binding Assays—GST-ATF6α expression plasmids were introduced into pET41 and transformed into BL-21 DE3 cells. Cells from a single freshly transformed colony were grown to an A₆₀₀ of about 0.5, and protein expression was induced by adding isopropyl-1-thio-β-d-galactopyranoside to 1 mM. Cells were grown overnight at 16 °C with shaking at 130 rpm. Cells were harvested at 6000 × g and resuspended in 20 ml of 50 mM Tris-Cl, pH 7.9, 300 mM NaCl, 10% glycerol, 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 mM DTT. After lysis with a French press, the cell suspension was clarified by centrifugation for 30 min at 100,000 × g at 4 °C, and the resulting supernatant was brought to 0.1% Triton X-100. GST-tagged proteins were purified from cell lysates using glutathione-Sepharose™ 4B beads (GE Healthcare) using standard methods. Purified proteins were exchanged into buffer containing 40 mM Hepes-Cl, pH 7.9, 0.05% Triton x-100, 1 mM MgCl₂, 0.1 mM NaCl, 1 mM DTT (GB buffer) using Zeba desalt spin columns (Thermo Scientific).

~6 pmol of GST-ATF6α or GST-ATF6α deletion mutants were mixed with 10–15 µl of HeLa-S3 nuclear extract in GB buffer in a total volume of 80 µl, incubated at 30 °C for 30 min, and then added to 20 µl of glutathione-Sepharose™ 4B beads (GE Healthcare) using standard methods. Purified proteins were exchanged into buffer containing 40 mM Hepes-Cl, pH 7.9, 0.05% Triton x-100, 1.5 mM MgCl₂, 0.1 mM NaCl, 1 mM DTT (GB buffer) using Zeba desalt spin columns (Thermo Scientific).

Mass Spectrometry—Proteins were identified using a modification of the MudPIT procedure (22, 23). TCA-precipitated proteins were urea-denatured, reduced, alkylated, and digested with endoproteinase Lys-C (Roche Applied Science) and modified trypsin (Roche Applied Science) as described (22). Peptide mixtures were loaded onto 100-µm fused silica microcapillary columns packed with 5-µm C₁₈ reverse phase (Aqua, Phenomenex), strong cation exchange particles (PartiSphere SCX, Whatman), and reverse phase (24). Loaded microcapillary columns were placed in-line with an LTQ ion trap mass spectrometer equipped with a nano-LC electrospray ionization source (Thermo Scientific). Fully automated MudPIT runs were carried out on the electrosprayed peptides as described (23). Tandem mass (MS/MS) spectra were interpreted using SEQUEST (25) against a database of 37,394 human proteins (downloaded from NCBI on July 02, 2009), 4228 proteins from Escherichia coli BL21 strain (downloaded from NCBI on January 10, 2010), and 177 sequences from usual contaminants (human keratins, IgGs, proteolytic enzymes) and complemented with the sequences of each of the GST-ATF6 constructs used in this study. In addition, to estimate false positive discovery rates, each nonredundant sequence was randomized (keeping amino acid composition and length the same), and the resulting “shuffled” sequences were added to the “normal” database (doubling its size) and searched at the same time.

Peptide/spectrum matches were sorted and selected using DTASelect (26) with the following criteria set. Spectra/peptide matches were only retained if they had a DeltaCn of at least 0.08 and a minimum XCorr of 1.8 for singly charged, 2.0 for doubly charged, and 3.0 for triply charged spectra. In addition, peptides had to be fully tryptic and at least 7 amino acids long. Combining all runs, proteins had to be detected by at least two such peptides or one peptide with two independent spectra. Peptide hits from multiple runs were compared using CONTRAST (26). To estimate relative protein levels, distributed normalized spectral abundance factors (dNSAFs) were calculated for each nonredundant protein or protein group using the following equations as described by Zhang et al. (27)

\[
dNSAF_i = \frac{\sum_{j=1}^{N} dSAF_{ij}}{N}
\]

with

\[
dSAF_i = \frac{\sum_{m=1}^{M} uSpC_i m \times sSpC_i}{L_i}
\]

in which shared spectral counts (sSpC) are distributed based on spectral counts unique to each protein i (uSpC) divided by the sum of all unique spectral counts for the m protein isoforms that shared peptide j with protein i and where dSAF is the unnormalized distributed spectral abundance factor, and L refers to the length of each protein i in amino acids.

RESULTS AND DISCUSSION

ATF6α-dependent Recruitment of Mediator and HAT Complexes to the HSPA5 ERSEs in Vitro—ATF6α activates transcription of ER stress response genes by binding sequence specifically to its binding sites in the ERSEs of their promoters. The promoter of the HSPA5 gene contains three ERSEs located ~60 bp upstream of the transcription start site (9) (Fig. 1A). In an attempt to identify in an unbiased way Pol II transcription regulatory proteins recruited by ATF6α to the HSPA5 promoter, we coupled DNA affinity purification with MudPIT mass spectrometry. MudPIT has proven to be a highly sensitive and reproducible means of identifying proteins in complex mixtures. In a MudPIT experiment, a mixture of proteins is first digested into peptides, which are then fractionated by multidimensional HPLC and analyzed by tandem mass spectrometry without first isolating individual proteins from gels (23). Previous studies have shown that for many proteins in a MudPIT dataset, the number of spectra from peptides of that protein is a function of its length and abundance. Consequently, the relative amount of a particular protein in different samples can
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To begin to investigate the function of ATF6α in regulation of ER stress response genes, we immobilized on streptavidin-Sepharose beads a biotinylated DNA fragment containing HSPA5 promoter sequences from −282 to +147 of the transcription start site (Fig. 1A); this portion of the HSPA5 gene includes the ERSEs, core promoter, and early transcribed region. In preliminary experiments, we confirmed that the immobilized HSPA5 promoter supports ATF6α-activated transcription. To do so, we incubated the bead-bound HSPA5 DNA fragment with HeLa cell nuclear extracts, with or without the addition of recombinant ATF6α, to allow preinitiation complex formation. After washing to remove unbound proteins, beads were incubated with ribonucleoside triphosphates, and transcription was measured using primer extension. As shown in Fig. 1B, a fragment containing the wild type HSPA5 promoter supported ATF6α-activated transcription, whereas a fragment from which the TATA-box was deleted did not.

To identify proteins recruited to the HSPA5 promoter in an ATF6α-dependent way, we incubated the bead-bound, wild type HSPA5 DNA fragment with HeLa cell nuclear extracts, with or without the addition of recombinant ATF6α. After washing, bound proteins were eluted with buffer containing SDS and analyzed by MudPIT. As illustrated in the heat map of Fig. 1C, MudPIT identified subunits of the Mediator and several HAT complexes, including the SAGA, ATAC, and TRRAP-TIP60 complexes, as proteins recruited to the HSPA5 DNA fragment by ATF6α. The Mediator complex is a very large, multisubunit complex that is required for proper regulation of the majority of Pol II genes and is thought to contribute to transcriptional regulation by acting directly on Pol II and other components of the general transcription apparatus. Although its detailed mechanism(s) of action are poorly understood, it has critical roles in multiple stages of transcription, from assembly and function of the Pol II initiation complex to control of transcription elongation (reviewed in Refs. 31–34). The human SAGA, ATAC, and TRRAP-TIP60 HATs are also large multisubunit complexes. SAGA and ATAC share a common catalytic core that includes either of two closely related acetyltransferases, GCN5 or p300/CBP-associated factor (PCAF); each also includes a collection of additional subunits specific to one complex or the other (reviewed in Refs. 35–38). Among the SAGA subunits is the transformation/transcription domain-associated protein (TRRAP), which is in turn shared by the TRRAP-TIP60 HAT complex (39–41). SAGA, ATAC, and TRRAP-TIP60 catalyze acetylation of nucleosomal histones, and all contribute to transcriptional regulation at least in part by regulating histone acetylation in chromatin (35–38).

The heat map shows the ratio of dNSAFs of Mediator and HAT subunits bound to immobilized HSPA5 promoter DNA in the presence (+) or absence (−) of ATF6α. Immobilized HSPA5 promoter binding assays were performed with or without the addition of purified ATF6α, and bound proteins were detected by MudPIT mass spectrometry. See supplemental Table 2 for supporting data.

ATF6α recruminates a collection of Pol II coregulators to the HSPA5 promoter in vitro. A, diagram showing avidin bead-bound, biotinylated HSPA5 promoter fragment used in assays for ATF6α-dependent recruitment of Pol II coregulators to promoter DNA. The circle indicates the position of the avidin bead. B, assembly of functional preinitiation complexes on immobilized HSPA5 promoter DNA. Preinitiation complexes were assembled with or without ATF6 bead. C, heat map showing the ratio of dNSAFs of Mediator and SAGA subunits of the Mediator and several HAT complexes, including the SAGA, ATAC, and TRRAP-TIP60 complexes, as proteins recruited to the HSPA5 DNA fragment by ATF6α. The Mediator complex is a very large, multisubunit complex that is required for proper regulation of the majority of Pol II genes and is thought to contribute to transcriptional regulation by acting directly on Pol II and other components of the general transcription apparatus. Although its detailed mechanism(s) of action are poorly understood, it has critical roles in multiple stages of transcription, from assembly and function of the Pol II initiation complex to control of transcription elongation (reviewed in Refs. 31–34). The human SAGA, ATAC, and TRRAP-TIP60 HATs are also large multisubunit complexes. SAGA and ATAC share a common catalytic core that includes either of two closely related acetyltransferases, GCN5 or p300/CBP-associated factor (PCAF); each also includes a collection of additional subunits specific to one complex or the other (reviewed in Refs. 35–38). Among the SAGA subunits is the transformation/transcription domain-associated protein (TRRAP), which is in turn shared by the TRRAP-TIP60 HAT complex (39–41). SAGA, ATAC, and TRRAP-TIP60 catalyze acetylation of nucleosomal histones, and all contribute to transcriptional regulation at least in part by regulating histone acetylation in chromatin (35–38).

The heat map shows the ratio of dNSAFs of Mediator and HAT subunits bound to immobilized HSPA5 promoter DNA in the presence (+) or absence (−) of ATF6α. The MudPIT results can be summarized as follows. First, all of the 30 Mediator subunits detected were enriched in the presence of ATF6α. Twelve Mediator subunits were detected only when binding reactions included exogenous ATF6α, and an additional eight Mediator subunits were enriched by more than 10-fold. Similarly, several of the Pol II subunits detected were seen only in the presence of ATF6α. In addition to subunits of Mediator and Pol II, we also observed increased binding to the HSPA5 DNA fragment of subunits of the SAGA, ATAC, and TRRAP-TIP60 complexes. Finally, all three subunits of the ERSE binding transcription factor NF-Y were detected with similar dNSAF values when binding reactions were performed with or without added ATF6α, consistent with previous studies indicating that NF-Y can be detected by chromatin immunoprecipitation at the HSPA5 ERSEs in the absence of ER stress (10, 11).

The ATF6α-dependent recruitment of Mediator, SAGA, and ATAC to the HSPA5 promoter was investigated further in assays using Western blotting to monitor recruitment of repre-
sentative subunits of each complex. Consistent with the results of MudPIT experiments, recruitment of the Mediator subunit Med6 was almost completely dependent on exogenously added ATF6α, whereas binding of SAGA subunit ADA2b and ATAC subunit MBIP was enhanced upon the addition of ATF6α. Although not detected in our MudPIT datasets, we also observed that subunits of Pol II general initiation factors, including TFIID (TAF6), TFIIB, TFIIE, and TFIIF, contribute to its binding to the HSPA5 promoter. Additional factor(s) present in the nuclear extracts also contribute to its binding to the HSPA5 promoter.

It was shown previously that ATF6α-dependent transcription in cells requires a functional ERSE (9, 12). Importantly, recruitment of ATF6α and Pol II coregulatory proteins to the HSPA5 promoter fragment that lacked the ERSEs (HSPA5ΔERSE) was substantially reduced (Fig. 2A, compare lanes 1–4 with lanes 5–8). We note, however, that binding of the ATAC subunit MBIP to HSPA5 was less dependent than MED6 or ADA2b on added ATF6α or an intact ERSE, suggesting that it has more nonspecific DNA binding activity and/or that additional factor(s) present in the nuclear extracts also contribute to its binding to the HSPA5 promoter.

ATF6α-dependent Recruitment of Mediator and HATS to HSPA5 Promoter Depends on Both Its DNA Binding and Its Transcription Activation Domains—Previous studies have shown that the ATF6α transcription activation domain resides within its first 150 amino acids (12, 15), whereas its basic leucine zipper family DNA binding domain lies between residues 308 and 373 (4). To determine which ATF6α regions are required for recruitment of Mediator and HATs to the immobilized HSPA5 promoter, binding reactions were performed using wild-type and various ATF6α deletion mutants (Fig. 2C). Consistent with the earlier findings, deletion of a portion of the ATF6α DNA binding domain prevented it from binding the HSPA5 promoter, whereas deletion of the transcription activation domain had no effect on DNA binding. Arguing that the previously mapped ATF6α activation domain plays a key role in recruitment of Mediator and SAGA to the HSPA5 promoter in our assays, deletion of either the DNA binding domain or the transcription activation domain led to a dramatic decrease in ATF6α recruitment to the immobilized promoter. In addition, although there was a significant background of ATAC subunit MBIP even in the absence of ATF6α, the modest but reproducible ATF6α-dependent increase in the amount of MBIP recruited to the immobilized promoter required both the ATF6α DNA binding and the transcription activation domains. We also observed a modest increase in NF-Y recruitment in the presence of full-length ATF6α, consistent with previous evidence that ATF6α activation can lead to a ~2-fold increase in NF-Y recruitment to the HSPA5 ERSE by chromatin immunoprecipitation (11) and suggesting that co-occupancy of ERSEs by ATF6α and NF-Y may lead to an increase in the DNA binding affinity of NF-Y.

**FIGURE 2.** ATF6α-dependent recruitment of Mediator and HAT complexes to HSPA5 ERSEs requires ATF6α DNA binding and activation domains. A, ERSE dependence of ATF6α and Pol II coregulator binding to the HSPA5 promoter. Immobilized template recruitment assays were performed with either wild type (WT) or ΔERSE HSPA5 promoter DNA and varying amounts of ATF6α. Bound proteins were detected by Western blotting using the designated antibodies. B, ATF6α recruits components of the Pol II preinitiation complex to the HSPA5 promoter. C, diagram of GST-ATF6α fusion proteins used in D. WT, wild type; AD, transcription activation domain; DBD, DNA binding domain. D, effect of deleting ATF6α DNA binding or transcription activation domains on HSPA5 promoter binding. Immobilized HSPA5 promoter recruitment assays were performed with WT HSPA5 and the indicated GST-ATF6α fusion proteins. Input (10% of total) and bound proteins were detected by Western blotting with the indicated antibodies.
The ATF6α Transcription Activation Domain Is Necessary and Sufficient for Mediator and HAT Binding—To begin to address the mechanism of ATF6α-dependent recruitment of the Pol II transcription machinery to the HSPA5 promoter in our assays, we sought to determine whether ATF6α is capable of interacting with any of these Pol II transcription regulatory proteins in the absence of promoter DNA. To accomplish this, HeLa cell nuclear extracts were incubated with GST-ATF6α or one of several GST-ATF6α mutants (Fig. 3A). GST-ATF6α and associated proteins were enriched using glutathione-Sepharose and analyzed by MudPIT mass spectrometry and Western blotting. As shown in Fig. 3, B and C, these experiments revealed that GST fusion proteins containing the ATF6α transcription activation domain can interact with Mediator, Pol II, and each of the various HAT complexes recruited by ATF6α to the HSPA5 promoter in the experiments in Figs. 1 and 2, as well as with p300, which has been shown previously to be recruited to the HSPA5 ERSEs in response to ER stress (10). Binding of these Pol II coregulators to ATF6α was dependent on the ATF6α transcription activation domain because (i) GST-ATF6α (1–150) bound as much or more of the coregulators as did GST fused to full-length ATF6α and (ii) neither Mediator nor HAT complexes bound to GST-ATF6α (151–326), which lacks the transcription activation domain. Finally, although we were able to detect binding of Medi-
ator and HATs to the immobilized ATF6α transcription activation domain in the absence of HSPA5 promoter DNA, we did not detect any of the Pol II general initiation factors in these experiments (data not shown), suggesting that recruitment of these proteins by ATF6α occurs in the context of preinitiation complexes assembled on DNA.

Mediator and HAT Complexes Bind to Nonidentical but Overlapping Regions of ATF6α Transcription Activation Domain—We next sought to define in more detail portions of the ATF6α activation domain required for its interaction with Mediator and the various HAT complexes using the series of GST-ATF6α fusion proteins diagrammed in Fig. 4A. As shown in Fig. 4, B and C, there was little or no interaction between the Mediator complex and GST fusion proteins containing ATF6α (1–43) or ATF6α (20–60). ATF6α fragments containing residues 20–80 and 44–150 bound less well to Mediator than ATF6α (1–150), whereas very similar amounts of Mediator were recovered after glutathione-Sepharose chromatography of proteins that bound GST fusion proteins containing ATF6α (20–100) and the full-length ATF6α activation domain (residues 1–150). In contrast, maximal binding of the HAT complexes to ATF6α was observed only to GST-ATF6α (1–150), suggesting that (i) Mediator and the HAT complexes interact with ATF6α through overlapping but nonidentical surfaces and (ii) optimal binding of the HAT complexes to ATF6α requires a larger region of the transcription activation domain.

Thureau et al. (15) previously noted that the first 100 amino acids of the ATF6α transcription activation domain exhibit some sequence similarity to the VP16 transcription activation domain. Most similar within this region is an 8-amino acid sequence, DFDLDLMP, that closely resembles a VP16 sequence, DFDLDMLG, referred to as VN8 (15). In VP16, the VN8 sequence is necessary for transcription activation (44, 45). In addition, mutations in the VN8 sequence have been reported to abolish interaction of VP16 with the Mediator complex (46), and mutations in the VN8-like domain of ATF6α led to a 5-fold
reduction in ATF6α-dependent activation of a luciferase reporter driven by the HSPA5 ERSEs (15).

To explore in more detail the potential functional relationship between the ATF6α and VP16 transcription activation domains, we first asked whether the VP16 activation domain competes with ATF6α/H9251 for interaction with Mediator and HAT complexes. Remarkably, adding increasing amounts of a Gal4-VP16 transcription activation domain fusion protein to HeLa cell nuclear extracts prevented binding of GST-ATF6α/H9251 (1–150) to Mediator and Pol II but not to the HAT complexes, as revealed by MudPIT and confirmed by Western blotting (Fig. 5, A and B). That Pol II was depleted along with Mediator is consistent with the possibility that ATF6α-dependent recruitment of Pol II is via a Mediator-Pol II holoenzyme complex. Importantly, the observation that the HAT complexes remain associated with ATF6α in the presence of Gal4-VP16 indicates that they can associate with ATF6α independent of Mediator.

We next considered the possibility that the ATF6α VN8-like sequence might contribute to Mediator and/or HAT recruitment. Indeed, we observed that mutating the ATF6α VN8-like sequence to DADALLP led to a dramatic decrease in the interaction of ATF6α with Mediator (Fig. 5, C). Adding Gal4-VP16 to binding reactions containing the ATF6α VN8-like mutant caused little or no further reduction in the amount of Mediator bound. In addition, mutation of the ATF6α VN8-like sequence substantially reduced the interaction of ATF6α with SAGA and ATAC, although Gal4-VP16 does not prevent binding of ATF6α to SAGA and ATAC.
In complementary experiments, we observed that micromolar concentrations of a 32-amino acid peptide containing four repeats of the ATF6α VN8-like sequence blocked binding of GST-ATF6α to Mediator, whereas higher concentrations of the peptide were needed to block binding to SAGA (Fig. 6A). Furthermore, we observed that even at millimolar concentrations, the VN8-like peptide had little effect on thyroid hormone (T3)-dependent binding of Mediator to the activation domain of thyroid receptor (TR), supporting the specificity of inhibition. Importantly, the interactions between ATF6α and the HAT complexes to the ATF6α transcription activation domain, but that additional sequences outside of the VN8-like region make a more significant contribution to the ATF6α-HAT interaction.

Finally, we compared the effect of the VN8-like peptide on ATF6α-dependent recruitment of Mediator, SAGA, and the TFIIID component of the Pol II preinitiation complex with the HSPA5 promoter in vitro. As shown in Fig. 6B, concentrations of peptide that had little effect on SAGA binding strongly reduced binding of Mediator to the HSPA5 promoter. Notably, the VN8-like peptide reduced TFIIID binding to the HSPA5 promoter in parallel with its reduction of Mediator binding, consistent with the possibility that Mediator contributes to ATF6α-dependent TFIIID recruitment to the promoter.

Summary and Perspectives—In this study, we present findings that shed new light on the mechanism of action of ATF6α. By exploiting a combination of biochemical and MudPIT-based mass spectrometry approaches, we have obtained evidence that the ATF6α transcription activation domain can recruit a collection of Pol II coregulators to the ERSEs in the promoter of the ER stress response gene HSPA5. These Pol II coregulators include the Mediator complex as well as several HATs, including the SAGA, ATAC, and TRRAP-TIP60 complexes and p300. These findings are consistent with previous studies reporting that p300 and the SAGA complex exhibit increased occupancy at the HSPA5 ERSEs following induction of the ER stress response with thapsigargin (10, 18). Similarly, in chromatin immunoprecipitation experiments, we have observed increased binding of the Mediator subunit Med26 at the HSPA5 promoter in cells subjected to ER stress (data not shown).

In structure-function experiments dissecting the mechanism by which ATF6α recruits coregulators, we observe that its binding to Mediator and HAT complexes depends on nonidentical but overlapping regions of its transcription activation domain. Importantly, the interactions between ATF6α, Mediator, SAGA, and other HATs described in this study depend strictly on ATF6α domains shown previously to be essential for its transcription activity in cells (12, 15). Of particular note, mutation of three residues in the ATF6α VN8-like sequence, which has been shown to be important for ATF6α activation domain function (15), led to a dramatic decrease in binding of ATF6α to Mediator, SAGA, and ATAC. Thus, our findings are consistent with the model that ATF6α regulates transcription of ER stress response genes at least in part by recruiting these Pol II coregulators to ERSEs.

In the course of these experiments, we observed that the well characterized VP16 transcription activation domain competes with ATF6α for binding to the Mediator but not to the HAT complexes, suggesting that the ATF6α and VP16 transcription activation domains might bind to the same or overlapping surfaces on Mediator. In light of previous studies indicating that VP16 binds to Mediator through its Med25 subunit (46–51), we are now investigating the possibility that ATF6α might also bind to Mediator through Med25. In the future, these studies should provide a deeper understanding of the mechanism by which ATF6α facilitates recruitment of Mediator and other Pol II coregulators to the genes it regulates. Finally, our success in combining DNA
affinity chromatography and MudPIT mass spectrometry to identify proteins recruited by ATF6α to the HSPA5 promoter suggests that this approach might be generally applicable in future studies aimed at determining how particular DNA binding transcription factors recruit Pol II coregulators to genes to regulate transcription.

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