Human Mediator Subunit MED26 Functions as a Docking Site for Transcription Elongation Factors

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SUMMARY

Promoter-proximal pausing by initiated RNA polymerase II (Pol II) and regulated release of paused polymerase into productive elongation has emerged as a major mechanism of transcription activation. Reactivation of paused Pol II correlates with recruitment of super-elongation complexes (SECs) containing ELL/EAF family members, P-TEFb, and other proteins, but the mechanism of their recruitment is an unanswered question. Here, we present evidence for a role of human Mediator subunit MED26 in this process. We identify in the conserved N-terminal domain of MED26 overlapping docking sites for SEC and a second ELL/EAF-containing complex, as well as general initiation factor TFIID. In addition, we present evidence consistent with the model that MED26 can function as a molecular switch that interacts first with TFIID in the Pol II initiation complex and then exchanges TFIID for complexes containing ELL/EAF and P-TEFb to facilitate transition of Pol II into the elongation stage of transcription.

INTRODUCTION

Messenger RNA synthesis begins with recruitment of RNA polymerase II (Pol II) and other components of the transcription apparatus to the promoter to form a preinitiation complex, followed by ATP-dependent unwinding of the DNA and transcription initiation. Following initiation, Pol II moves away from the promoter, ultimately leading to establishment of a productive elongation complex. Although for many years most research focused on mechanisms that control the preinitiation and initiation stages of transcription, transcription of many genes is also regulated during transcript elongation.

Early studies showed that transcription of genes including the human c-myc oncogene, the Drosophila heat shock gene hsp70, and the integrated HIV-1 provirus is regulated by promoter-proximal pausing, in which Pol II initiates transcription but pauses or arrests downstream of the transcription start site (Saunders et al., 2006). Release from the pause is a rate-limiting step that can be regulated by DNA-binding transcription factors or, in the case of HIV transcription, by the viral RNA-binding transactivator Tat. Recently, genome-wide studies demonstrated the presence of promoter-proximally paused Pol II near the 5’ ends of a large fraction of genes, suggesting that regulated promoter-proximal pausing and release are general features of Pol II elongation (Guenter et al., 2007; Kininis et al., 2009; Muse et al., 2007; Nechaev et al., 2010; Rahl et al., 2010; Zeitlinger et al., 2007).

The duration of pausing or arrest during early elongation can be controlled by multiple transcription elongation factors that influence the elongation competence of Pol II. DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) function together to induce pausing (Wada et al., 1998; Yamaguchi et al., 1999). Release of paused Pol II in turn depends on phosphorylation of the Pol II CTD, SPT5, and perhaps other factors by positive transcription elongation factor b (P-TEFb; composed of the cyclin-dependent kinase CDK9 and CYCLIN T1/T2) (Lis et al., 2000; Marshall et al., 1996; Peterlin and Price, 2006; Wei et al., 1998). Also implicated in this process are additional transcription elongation factors, including members of the eleven-nineteen lysine-rich in leukemia (ELL) family and their binding partners ELL-associated factors (EAFs) 1 and 2, which together act directly to increase the rate of elongation by Pol II in vitro and regulate expression of hsp70, HIV-1, and other genes in cells (Byun et al., 2009; Kong et al., 2005; Lin et al., 2010; Shilatifard et al., 1996; Smith et al., 2008). Evidence suggests that these factors contribute not only to release of paused Pol II but also to subsequent events occurring during transcription elongation, including cotranscriptional RNA processing (Martinic et al., 2009; Ni et al., 2004).

P-TEFb and ELL/EAF family members function as components of larger “super-elongation complexes” or SECs that contain the additional coregulators AFF4, AFF1, ENL, and AF9 (He et al., 2010; Lin et al., 2010; Sobhian et al., 2010; Yokoyama...
In HIV-1-infected cells, Tat activates HIV-1 transcription by recruiting P-TEFb, ELL/EAF, and SEC family members through interactions with the TAR sequence in the 5’ end of the nascent HIV-1 transcript (He et al., 2010; Peterlin and Price, 2006; Sobhian et al., 2010); however, mechanisms responsible for bringing these factors to host genes in uninfected cells remain poorly defined.

Mediator is an evolutionarily conserved coregulatory complex that was first identified in yeast (Kim et al., 1994; Koleske and Young, 1994). In metazoan, Mediator is composed of some 30 distinct subunits (Sato et al., 2004). Mediator exists in multiple, functionally distinct forms that share a common core of subunits and can be distinguished by the presence or absence of a kinase module composed of CYCLIN C and isoforms of MED12, MED13, and CDK8. Kinase module has been implicated in both transcriptional repression and activation (Taatjes, 2010).

In metazoan a subset of Mediator contains an additional subunit, MED26. MED26-containing Mediator copurifies with only a small amount of kinase module, but near-stoichiometric Pol II (Mittler et al., 2001; Sato et al., 2004; Taatjes et al., 2002). MED26-containing Mediator appears to play a key role in transcriptional activation (Mo et al., 2004; Näär et al., 2002; Ryu et al., 1999); however, to our knowledge, the mechanism(s) by which MED26 contributes to this process is not known. Here, we present evidence supporting the model that MED26 functions in part by recruiting ELL/EAF- and P-TEFb-containing complexes, including the SEC, to a subset of human genes. We show that the human Mediator complex can recruit ELL/EAF- and P-TEFb-containing complexes to promoters via a direct interaction with the N-terminal domain (NTD) of MED26. The MED26 NTD also binds TFIID, and TFIID and elongation complexes interact with MED26 through overlapping binding sites. In addition we show the following: (1) in cells wild-type MED26, but not a MED26 mutant that prevents binding of Mediator to elongation factors, regulates transcription of c-myc and other genes; and (2) MED26 knockdown interferes with elongation factor recruitment. We propose that the MED26 NTD may function as a molecular switch that contributes to the transition of Pol II into productive elongation.

RESULTS

Enrichment of Elongation Factors and TFIID in Mediator with MED26

As part of a MudPIT-based proteomic analysis of the human Mediator complex, we generated human cell lines expressing Mediator subunits with FLAG epitope tags, immunopurified (IPed) FLAG-Mediator subunits and their associated proteins, and identified proteins in anti-FLAG eluates by MudPIT mass spectrometry. In MudPIT datasets the number of spectra corresponding to peptides from a particular protein correlates with the protein’s abundance and length. The relative amount of a particular protein in a sample can be estimated from a normalized spectral abundance factor, or NSAF (Zhang et al., 2010).

Proteomic analysis previously allowed us to define a complete set of human Mediator subunits (Sato et al., 2004). In-depth analysis of our MudPIT datasets revealed that additional proteins with roles in transcription were consistently enriched in FLAG-MED26 Mediator preparations, relative to Mediator purified through FLAG-MED19 (Figure 1A, and see Table S1 available online) or other core Mediator subunits (data not shown). Among these were initiation factor TFIID and transcription elongation factors including ELL and EAF family members, P-TEFb (CDK9, CYCLIN T1/T2), and MLL translocation partners AFF1, AFF4, AF9, and ENL, all of which are components of the SEC (He et al., 2010; Lin et al., 2010; Sobhian et al., 2010). Also enriched were two previously uncharacterized proteins, KIAA0947 and NARG2. The NSAF values of these proteins were ~5%–20% of the average NSAF of core Mediator subunits, suggesting that they bind only a fraction of Mediator in solution.

Docking Sites for TFIID and Elongation Factors in the MED26 NTD

TFIID, SEC components, and KIAA0947 and NARG2 could be enriched in FLAG-MED26 Mediator because they bind directly to MED26 or MED26-associated Mediator. Alternatively, because ELL/EAF can interact stably with Pol II in vitro (Banks et al., 2007; Shilatifard et al., 1997), and MED26-containing Mediator is enriched in Pol II, some or all of these proteins might be associated with Pol II rather than Mediator or MED26.

To begin to address these possibilities, we asked whether the same or distinct MED26 regions are necessary for interaction with Mediator and Pol II and with TFIID, SEC components, and KIAA0947 and NARG2. The most highly conserved region of MED26 is its NTD, which is similar to the NTDs of the elongation factors TFIIS and ELONGIN A and, based on an NMR structure of the TFIIS NTD, can be modeled as a globular domain composed of a four-helix bundle (Booth et al., 2000). In the MED26 C terminus are approximately 10 amino acids that are conserved from insects to mammals, whereas a more extended C-terminal region of ~100 amino acids is conserved in vertebrates (Bourbon, 2008).

We generated human cell lines stably expressing the series of FLAG-MED26 mutants diagrammed in Figure 1B and identified proteins associated with each by MudPIT and western blotting (Figures 1A and 1C). The MED26 C-terminal domain (residues 421–600) is sufficient for its assembly into Mediator and interaction with Pol II. The most highly conserved C-terminal amino acids are critical for these interactions because deletion of the last eight amino acids from the MED26 C terminus disrupted MED26 binding to Mediator and Pol II. Whether MED26 makes direct contacts with Pol II and/or binds Pol II indirectly due to preferential association with a form of Mediator that binds Pol II is unclear; however, we believe that the latter possibility is more likely because we have not detected binding of purified Pol II to free MED26 in vitro (data not shown). Neither ELL nor EAF family members copurified with Pol II bound to Mediator containing MED26 lacking the NTD (Figures 1A and 1C, lanes 9 and 10), suggesting that the ELL/EAF-binding site on Pol II is blocked when Pol II is bound to Mediator.

The MED26 NTD does not bind Mediator or Pol II. However, it does copurify with TFIID, P-TEFb subunits, ELL and EAF family members, other SEC components, and NARG2 and KIAA0947 (Figures 1A and 1C, lanes 6–10). Binding of these proteins is specific for the MED26 NTD because they do not copurify with the TFIIS- or ELONGIN A-NTDs (Figure 1D). Thus, the conserved
MED26 N- and C-terminal regions are functional domains that support interactions with TFIID and transcription elongation factors and with Mediator and Pol II, respectively. Although we did not detect TFIID in association with MED26 mutants that lack the NTD in our MudPIT analyses, we detected small amounts of TFIID subunit TBP in association with these mutants.
by western blotting (Figure 1C), perhaps due to interactions between TBP and other Mediator subunits (Cai et al., 2010; Larivière et al., 2006). However, we cannot rule out the possibility that the MED26 C terminus can also bind weakly to TFIID.

ELL and EAF Are Components of at Least Two MED26 NTD-Associated Complexes

To define the complexes that interact with MED26, we generated cell lines stably expressing individual FLAG-tagged SEC components, NARG2, and KIAA0947. Analysis of proteins copurifying with each protein argues that the MED26 NTD binds at least two types of complexes that share ELL and EAF family members. The first corresponds to the SEC and includes P-TEFb, AFF4 and/or AFF1, and AF9 and/or ENL (Figures 2A and 2B, lane 4), and the second lacks SEC components but includes KIAA0947 and NARG2 (Figures 2A and 2B, lanes 2 and 3). We detect only small amounts of Mediator subunits by MudPIT in FLAG immunoprecipitates (IPs) from cells expressing SEC subunits, most notably FLAG-AFF4 (data not shown), suggesting that only a small fraction of the SEC is stably associated with Mediator. Confirming the interaction of ELL/EAF1 with KIAA0947 and NARG2, recombinant ELL, EAF1, and KIAA0947 specifically co-IPed with FLAG-NARG2 when the four proteins were coexpressed in insect cells. The function of this novel complex will be the subject of a future study.

Figure 2. ELL/EAF-Containing Complexes Bind the MED26 NTD

(A) MudPIT of proteins bound to SEC components, NARG2, and KIAA0947.
(B) Western blotting of proteins associated with FLAG-tagged NARG2, KIAA0947, and AF9.
(C) Binding of NARG2 and KIAA0947 to ELL/EAF1. FLAG-IPed complexes from baculovirus-infected insect cells expressing the indicated proteins were analyzed by western blotting. Asterisk indicates IgG.
(D) Binding of EAF1 to the MED26 NTD. Western blotting of FLAG-IPed complexes or lysates (Input) from baculovirus-infected insect cells, using anti-FLAG (M2) antibodies and Alexa Fluor 680-labeled anti-mouse IgG (light chain-specific) (red) or rabbit anti-cMyc antibodies and IR Dye 800-labeled goat anti-rabbit IgG (green).
bound the FLAG-MED26 NTD when the two proteins were coexpressed in insect cells in the absence of ELL; EAF2 also bound the MED26 NTD in the absence of ELL (data not shown). In contrast, ELL bound the MED26 NTD only in the presence of an EAF family member. These findings suggest that EAF proteins function as adaptor molecules that link the MED26 NTD to ELL/EAF-containing complexes.

**A Role for the MED26 NTD in Mediator-Dependent Recruitment of Transcription Elongation Factors to Promoters In Vitro**

Our observation that the MED26 NTD directly binds both TFII D and ELL/EAF-containing complexes suggested that MED26 NTD-Mediator. (D) MED26 ΔNTD-Mediator (Med[F-MED26 ΔNTD]) does not recruit EAF1 to a promoter. (E) Free MED26 is not sufficient for EAF1 recruitment. In lanes 1–4, assays contained either free full-length MED26 or MED26-Mediator. Lanes 5–8 show amounts of free MED26 and MED26-Mediator in binding assays. (F) Free MED26 NTD blocks Mediator-dependent EAF1 recruitment. Assays were performed with MED26-Mediator, with or without free MED26 NTD (His-MED26-NTD).

might contribute to transcriptional regulation at least in part by helping to recruit them to the genes they regulate. Carey and coworkers (Johnson et al., 2002) showed that Mediator enhances recruitment of TFII D to a promoter bound by a DNA-binding transactivator, GAL4-VP16. To ask whether the MED26 NTD is responsible for this activity of Mediator, we employed similar immobilized template assays (Figure 3A), using Mediator complexes purified from cells expressing wild-type FLAG- MED26 or MED26 that lacks the NTD but includes residues 421–600 (MED26 ΔNTD). Both MED26- and MED26 ΔNTD-containing Mediator enhanced GAL4-VP16-dependent binding of TFII D to an immobilized promoter (Figure 3B), arguing that the MED26 NTD is dispensable for TFII D recruitment in this assay and that additional contact(s) between Mediator and TFII D is responsible for this activity.

We next tested the ability of Mediator to recruit EAF-containing complexes to promoters in the presence of either GAL4-VP16 or the DNA-binding transactivator HNF4x. As shown in Figures 3C and 3D, the ELL/EAF1 complex or EAF1 alone was recruited to promoters containing GAL4- or HNF4x-responsive elements in the presence of their cognate activator only when binding reactions included wild-type Mediator. However, EAF1 was not recruited to either promoter in binding reactions containing MED26 ΔNTD-Mediator (Figure 3D).

To exclude the possibility that free MED26 protein in Mediator preparations is responsible for EAF1 recruitment, we compared
the ability of free MED26 and FLAG-MED26-IPed Mediator to recruit EAF1 to an immobilized promoter. Unlike Mediator-associated MED26, free MED26 was not recruited by HNF4α to the immobilized template and, accordingly, did not recruit EAF1 (Figure 3E). Furthermore, excess free MED26 NTD (residues 1–113) competes with Mediator for EAF1 recruitment (Figure 3F). Thus, Mediator can recruit EAF1 or EAF1-associated complexes to immobilized promoters via direct interaction of the MED26 NTD with EAF1.

**Elongation Factors and TFIIID Bind to Overlapping Surfaces of the MED26 NTD**

As described above, the MED26 NTD is closely related in sequence to the TFIIIS NTD and, based on an NMR structure of the TFIIIS NTD, has been modeled as a four-helix bundle (Booth et al., 2000). To explore whether elongation factors and TFIIID bind to distinct or overlapping surfaces of the MED26 NTD, we generated a series of MED26 NTD mutants in which various predicted surface residues were changed to alanine and asked whether we could identify mutations that differentially affect MED26 NTD interactions with ELL/EAF-containing complexes and with TFIIID.

We observed that a GAL4-MED26 NTD fusion protein acts as a strong activator of a luciferase reporter driven by five GAL4 responsive elements upstream of the AdML promoter in transient transfection assays (Figure S1A). GAL4-MED26 NTD with alanines in place of predicted surface residues R61 and K62 or K74 and K75 were expressed well but failed to activate the luciferase reporter, whereas GAL4-MED26 NTD D13A was as active as the wild-type GAL4-MED26 NTD. As shown in Figure S1B, GAL4-MED26 NTD and GAL4-MED26 NTD(D13A) could both recruit TFIIID and EAF1 to immobilized DNA, whereas GAL4-MED26 NTD(R61A,K62A) and GAL4-MED26 NTD(K74A,K75A) could not. These residues are also critical for MED26 NTD interactions with both elongation factors and TFIIID in cells because EAF1, ELL, CDK9, CYCLIN T1/T2, or TFIIID subunits were not detected by western blotting in FLAG-IPs from cells stably expressing FLAG-MED26 NTD(R61A,K62A) or FLAG-MED26 NTD(K74A,K75A) (Figure 4A).

We next tested the ability of FLAG-MED26(R61A,K62A) to assemble into Mediator and to interact with elongation factors and TFIIID when expressed in human cells. As expected, both wild-type and mutant MED26 assembled into Mediator, and Mediator containing the MED26 mutant failed to bind to elongation factors (Figure 4B). In addition, Mediator containing MED26(R61A,K62A) could not recruit EAF1 to a promoter (Figure 4C). Finally, Mediator containing MED26(R61A,K62A) could still interact with TFIIID subunits (Figure 4B), consistent with our evidence that deletion of the MED26 NTD does not interfere with Mediator’s ability to enhance recruitment of TFIIID to a promoter in vitro (Figure 3B). We note that there was a modest decrease in the amount of TBP and TAF6 that co-IPed with Mediator purified from cells expressing FLAG-MED26(R61A,K62A); however, it is not clear whether this reflects a small decrease in the affinity of the mutant Mediator for TFIIID or a decrease in expression of TFIIID subunit(s) needed for the interaction because TAF6 expression was decreased in these cells.

Taken together, these observations argue that ELL/EAF-containing complexes and TFIIID bind to the same or overlapping surfaces of the MED26 NTD, even though interactions of TFIIID with surface(s) outside of this domain are most important for its interaction with Mediator. In addition, they provide further support for the idea that EAF family proteins function as adapters that link ELL-containing complexes to Mediator via the MED26 NTD because MED26 double-point mutations that block interactions with EAF1 in vitro also interfere with binding of the MED26 NTD or of intact Mediator to subunits of ELL/EAF-containing complexes in cells.

These observations suggested that Mediator might not be able to bind simultaneously to EAF1 and TFIIID. Consistent with this possibility, EAF1 recruitment to an activator-bound promoter was substantially decreased when binding reactions contained TFIIID (Figure 4D), raising the possibility that prior to initiation and/or the receipt of an appropriate signal, the MED26 NTD might be masked by interaction with TFIIID and only become available to recruit ELL/EAF-containing complexes upon release of this interaction.

**MED26 Regulates Cell Proliferation and Gene Expression**

To study MED26 function in cells, we generated stable human 293T embryonic kidney cell lines expressing nontargeting or one of three different doxycycline (Dox)-inducible short hairpin RNAs (shRNAs) targeting the 3′UTR of endogenous med26. Dox treatment silenced MED26 protein expression in cell lines expressing each of the Dox-inducible MED26 shRNAs (Figure 5A). Induction of any of the three MED26 shRNAs reduced cell proliferation, whereas nontargeting shRNA had no effect (Figures 5B and 5C). Proliferation of mouse ES cells was also inhibited by transfection of med26 siRNAs, but not nontargeting siRNA or med6, cdk8, or nontargeting siRNAs (Figures S2A and S2B).

To identify MED26 target genes, we used Affymetrix expression arrays to analyze mRNA expression in 293T cells from which MED26 had been depleted by transient transfection of one of three different siRNAs (Figure 5D). Of the approximately 14,550 well-characterized genes on the array, more than 10% were significantly affected (adjusted p value ≤ 0.05) after MED26 knockdown by all 3 siRNAs. Interestingly, among the genes most negatively regulated by MED26 depletion were c-myc (myc), hsp70 (hspa1α), and snail2 (snai2) (Table S2). As noted earlier, c-myc and hsp70 were among the first examples of genes shown to be regulated during the transition from early elongation to productive elongation (Saunders et al., 2006). In addition, results of a recent genome-wide analysis provide evidence that Pol II is enriched at the 5′ end of the snai2 gene in mouse ES cells (Rahl et al., 2010), and the snai2 ortholog snail is one of many developmentally regulated genes controlled by promoter-proximal pausing in Drosophila (Zeitlinger et al., 2007).

Quantitative RT-PCR analysis confirmed that steady-state expression of these and other genes was decreased following transfection of all three MED26 siRNAs (Figure 5E and Figure S2C). Upon heat shock, synthesis of new Hsp70 mRNA was delayed in cells transfected with MED26 siRNA (Figure 5F),...
indicating that MED26 regulates not only steady-state but also newly induced gene expression.

**Effect of MED26 Depletion on SEC Recruitment and Pol II CTD Phosphorylation at the c-myc and hsp70 Genes**

To begin to investigate potential roles of MED26 in SEC recruitment in cells, we focused on two examples: steady-state expression of the c-myc gene and heat shock-induced activation of the hsp70 gene. Endogenous MED26, P-TEFb kinase CDK9, and SEC component AFF4, as well as several exogenously expressed SEC components (FLAG-ELL, AFF1, and AF9) (Figures 6A and 6B, and Figure S3A), are detected by chromatin immunoprecipitation (ChIP) at the c-myc gene in human cells. These observations suggest that the SEC contributes to c-myc gene regulation and are consistent with previous evidence that P-TEFb regulates c-myc expression (Glover-Cutter et al., 2009; Montanuy et al., 2008). MED26, like AFF4, ELL, AFF1, and AF9, is detected by ChIP throughout the body of the c-myc gene in these cells, even though TBP occupancy is limited to the promoter region as expected (Figure S3A).

Upon siRNA-mediated depletion of MED26, we observed little change in the amount of total Pol II (detected using an antibody that recognizes an epitope in the N terminus of RPB1) or TFIID subunit TBP at the c-myc promoter region. About 2 kb downstream from the transcription start site, Pol II occupancy was decreased by about 50%, similar to the reduction in c-Myc

![Figure 4. MED26 NTD Mutants Fail to Bind ELL/EAF-Containing Complexes or TFIID](image-url)
mRNA after MED26 knockdown. Occupancy of SEC components AFF4 and CDK9 at the promoter and throughout the body of the gene (Figure 6B) was also decreased, consistent with evidence that the MED26 NTD contributes to recruitment of elongation factors, but not TFIID, to promoters in vitro. Promoter escape and transcript elongation by Pol II are associated with phosphorylation of the Pol II CTD by P-TEFb and other kinases (Bartkowiak et al., 2010; Buratowski, 2009; Kim et al., 2002; Marshall et al., 1996). Depletion of MED26 dramatically decreased the amount of Ser2- and Ser5-phosphorylated Pol II throughout the c-myc gene (Figure 6B). In control experiments we confirmed that MED26 knockdown had no significant effect on expression of Pol II subunit RPB1 or any of the SEC components tested (Figure 6D).

Prior to heat shock, an initiated but paused Pol II is present in the promoter-proximal region of the hsp70 gene (Core and Lis, 2008). Upon heat shock, Mediator (Park et al., 2001) and SEC components (Lin et al., 2010; Lis et al., 2000; Smith et al., 2008) are rapidly recruited to the hsp70 gene, and the paused polymerase is released into productive elongation, allowing additional Pol II to be recruited to the gene (Boehm et al., 2003; Lis, 1998). Similarly, we detect Pol II and TBP at the promoter of the hsp70 gene prior to heat shock (Figure 6C and Figure S3B). Consistent with our observation that there is detectable heat shock gene expression in non-heat-shocked cells, a small amount of Pol II (Ser2- and Ser5-phosphorylated), as well as AFF4 and CDK9, could be detected in the body of the gene prior to heat shock (Figure 6C). Following 7.5 or 60 min of heat shock, Mediator subunits MED26 and MED1 and SEC components CDK9 and AFF4 were recruited (Figures 6A and 6C, and Figure S3B). As observed at c-myc, the ChIP signal for MED26 and, to a lesser extent, MED1 extended into the body of the hsp70 gene (Figure S3B). After 60 min of heat shock, we observed substantial release of Pol II into the body of the gene and recruitment of new Pol II to the promoter (Figure S3B). To focus on the effect of MED26 depletion on early events in heat shock gene induction, we performed ChIP using chromatin from cells that had or had not been subjected to just 7.5 min of heat shock. MED26 depletion had little effect on the amount of total Pol II or of Ser2- or Ser5-phosphorylated Pol II throughout the hsp70 promoter before heat shock (Figure 6A). However, after heat shock, MED26 depletion led to a significant reduction in new Pol II recruitment to the hsp70 promoter, accompanied by

Figure 5. Effect of MED26 Depletion on Cell Proliferation and Gene Expression
(A) Dox-inducible shRNA-mediated silencing of endogenous MED26 expression in 293T cells stably expressing different shRNAs. Cells were incubated with or without Dox for 48 hr, and cell lysates were analyzed by western blotting.
(B and C) MED26 depletion decreases cell proliferation in 293T cells stably expressing Dox-inducible shRNAs. Cell number is expressed relative to cell number at day 5 in cultures without Dox.
(D) Western blots for endogenous MED26 or tubulin 48 hr after transfection of nontargeting (control) or MED26 siRNAs.
(E) Effect of siRNA-mediated MED26 depletion on gene expression.
(F) Effect of MED26 depletion on Hsp70 induction by heat shock. Data points are the average of three independent experiments; error bars show standard deviation. See also Figure S2 and Table S2.
decreased occupancy of AFF4 and CDK9 and of Ser2- and Ser5-phosphorylated Pol II throughout the body of the hsp70 gene. Indeed, in heat-shocked cells depleted of MED26, the amount of total and CTD-phosphorylated Pol II, AFF4, and CDK9 in the body of the gene remained similar to or only modestly higher than that observed in non-heat-shocked cells (Figure 6C).

Together, these findings suggest that MED26 contributes to Mediator activity at multiple stages of transcription. During transcription of c-myc at steady state, MED26 seems particularly important for events after Pol II recruitment, including SEC recruitment and CTD phosphorylation. However, during activation of hsp70, MED26 knockdown not only decreases SEC recruitment, CTD phosphorylation, and release of Pol II into the body of the gene but also leads to a significant decrease in new Pol II recruitment to the promoter. The effect of depleting MED26 on Pol II distribution at hsp70 is very similar to what
was observed previously by Adelman et al. (2005) in experiments examining the effect of depleting the elongation factor TFIIS from Drosophila S2 cells. They attributed the decrease in new Pol II recruitment to failure of paused Pol II to move away from the promoter and make room for new Pol IIs to become associated with the gene; however, it seems likely that some of the effect we observe is due to defects in Mediator-dependent recruitment of new Pol II after MED26 knockdown.

A Role for the MED26 NTD in Regulating Cell Proliferation and Gene Expression

To assess the potential contribution of the MED26 NTD to regulation of cell proliferation and gene expression in cells, wild-type MED26 or MED26(R61A,K62A) was stably expressed in HEK293T cell lines expressing Dox-inducible shRNAs targeting MED26. Because the MED26 shRNAs target sequences in the med26 3′UTR, exogenously expressed MED26 mRNAs, which do not include the 3′UTR, should not be affected by the MED26 shRNA.

Even before depletion of endogenous MED26, the proliferation rate of cells expressing wild-type MED26 was moderately higher than that of control cells or cells expressing MED26(R61A,K62A) (Figure 7A, left panel). After depletion of endogenous MED26, cells expressing mutant MED26 grew more slowly than control cells after shRNA induction, suggesting that MED26(R61A,K62A) is a dominant-negative inhibitor of MED26 function in cells when endogenous MED26 is limiting.

To investigate further the contribution of the MED26 NTD to gene expression, we used cell lines expressing Dox-inducible MED26 shRNAs to test the requirement for the NTD in expression of several genes that were downregulated in cells transiently transfected with MED26 siRNAs. As a control, we also included cyclin D2, which was unaffected by MED26 knockdown in microarrays. The reduction in gene expression upon induction of MED26 shRNA in these cell lines was not as great as the reduction seen in cells transiently transfected with MED26 siRNAs.
due either to less-efficient depletion of MED26 by the shRNA in these cell lines or to leaky expression of shRNA even without Dox induction.

The results of these experiments are consistent with a role for the MED26 NTD in expression of each gene tested except for the control cyclin D2 gene; however, the responses of these genes to exogenously expressed MED26 differed (Figure 7B). MED26 shRNA reduced expression of c-myc and slc7a11 to a similar extent. In both cases, exogenously expressed wild-type MED26 blocked the effect of depleting endogenous MED26 but led to only a modest increase in gene expression relative to control. In contrast, snail2 expression was much higher in wild-type MED26-expressing cells than in control cells even before induction of MED26 shRNA, suggesting that MED26 is limiting for snail2 expression in human embryonic kidney cells. In each case, MED26(R61A,K62A) had a dominant-negative effect because its expression led to an approximately 2-fold decrease in mRNA levels whether or not the MED26 shRNA was induced.

To explore further the contribution of the MED26 NTD to gene regulation, we asked whether overexpression of wild-type MED26 or MED26(R61A,K62A) differentially affects Pol II distribution or CTD phosphorylation at the c-myc gene; these experiments were performed using the same HeLa cell lines in which we showed that both wild-type and mutant MED26 are fully capable of assembling into Mediator. Overexpression of wild-type but not mutant MED26 led to a small decrease in the amount of total Pol II at the c-myc promoter and a concomitant small increase in total Pol II further downstream, consistent with the possibility that more Pol II is released from the promoter region in cells overexpressing wild-type MED26 (Figure 7C). In addition, overexpression of wild-type but not mutant MED26 increased the amount of Ser2- and Ser5-phosphorylated Pol II at the promoter and throughout the body of the gene. Notably, the effect of MED26 overexpression on Ser2 phosphorylation was more pronounced than the effect on Ser5 phosphorylation. Thus, a MED26 NTD mutation that prevents the interaction of Mediator with transcription elongation factors interferes with MED26 functions in cell proliferation and gene regulation.

**DISCUSSION**

In this report, we show that the conserved NTD of MED26 functions as a docking site on Mediator for at least two types of complexes that contain members of the ELL/EAF family of transcription elongation factors: the multimeric SECs, which also contain P-TEFb and the MLL translocation partners AFF4, AFF1, ENL, and AF9, and an additional ELL/EAF-containing complex of as yet unknown function.

In addition to ELL/EAF-containing complexes, the MED26 NTD contains a binding site for TFIIID. However, consistent with evidence that yeast Mediator, which lacks MED26, binds TBP through contacts with MED8 and other subunits in the head module (Cai et al., 2010; Lariévi`ere et al., 2006), mutation of the MED26 NTD does not affect Mediator’s ability to enhance binding of TFIIID to a promoter, arguing that the MED26 NTD is not solely responsible for Mediator’s interaction with TFIIID.

Together with our observation that prior binding of Mediator to TFIIID at a promoter prevents Mediator from recruiting Pol II elongation factors via the MED26 NTD, this finding raises the possibility that the MED26 NTD is a molecular switch that interacts first with the Pol II initiation complex through direct interactions with TFIIID and then exchanges TFIIID for Pol II elongation factors to facilitate productive elongation and/or other elongation-associated processes modulated by these factors (Figure 7D). Consistent with this model, MED26 knockdown has little or no effect on TBP occupancy at the c-myc and hsp70 promoters.

There is precedent for the idea that Mediator has a role in controlling events that occur during transcription elongation. Deletion of MED23 from mouse ES cells eliminates expression of the serum-response gene egr1 due to (1) loss of Mediator recruitment and (2) failure to release Pol II from the promoter region (Balamotis et al., 2009; Wang et al., 2005). In yeast, Mediator subunits are detected by ChIP not only at promoters but also in the coding regions of some genes (Andrau et al., 2006; Zhu et al., 2006). In human cells the Mediator kinase module subunit CDK8 has been localized to coding regions of genes it activates (Donner et al., 2007, 2010). Similarly, we show that MED26 can be detected in the body of both the c-myc and hsp70 genes. Thus, interactions between MED26 and the SEC might contribute not only to SEC recruitment near the transcription start site but also to SEC recruitment or retention throughout elongation. Finally, several recent studies suggest a role for the Mediator kinase module in recruiting P-TEFb following activation of egr1 and other serum-response genes (Donner et al., 2010) and the thyroid hormone receptor-activated gene dio1 (Belakavadi and Fondell, 2010). Although the relationship between MED26 and kinase module in gene regulation remains to be determined, there is little overlap between the genes most affected by manipulating MED26 expression in our studies and those found previously to be most affected by manipulating CDK8 expression (Donner et al., 2007, 2010). This, together with evidence for the existence of both free kinase module and of forms of Mediator containing either MED26 or kinase module, raises the possibility that MED26 and the kinase module act via distinct mechanisms to recruit elongation factors to different genes and/or under different conditions.

Finally, our results have implications for mechanisms underlying misregulation of gene expression in mixed lineage leukemias caused by translocations of the MLL gene to one of a large number of translocation partners. Among these translocation partners are the SEC components ELL, AFF4, AFF1, AF9, and ENL. The finding that in normal cells these proteins are found together in the SEC, together with recent evidence that MLL fusion proteins themselves can assemble into larger, SEC-like complexes (Lin et al., 2010; Yokoyama et al., 2010), is consistent with the model that inappropriate gene activation in mixed lineage leukemias may be due to mistargeting of SEC-like complexes by MLL fusion proteins (Lin et al., 2010; Mueller et al., 2009; Yokoyama et al., 2010). Our demonstration that the SEC binds Mediator through the MED26 NTD suggests that mistargeting of the SEC by MLL fusion proteins could also lead to inappropriate activation of some genes by recruitment of Mediator and Pol II to aberrant chromosomal locations.
EXPERIMENTAL PROCEDURES

Cell Culture, Cell Lines
Parental HeLa S3 and Flp-In 293 cells (Invitrogen, Carlsbad, CA, USA) and their derivatives were cultured as described (Cai et al., 2007; Sato et al., 2004). Construction of expression plasmids and generation of cell lines are described in Extended Experimental Procedures.

Western Blotting, Immunoprecipitation, and Affinity Purification
Antibodies used are described in Extended Experimental Procedures. Protein complexes were purified from cell lines stably expressing FLAG-tagged proteins using anti-FLAG (M2) agarose (Sigma) as described (Takahashi et al., 2009).

Mass Spectrometry
Proteins were identified using MudPIT; for details see Extended Experimental Procedures. NSAFs (Zhang et al., 2010) were used to estimate relative protein abundance.

Im mobilized Template Assays
Biotinylated DNA fragments from the plasmids pG5MLT or pREx4MLT, which contain tandemly repeated GAL4- or HNF4a-responsive elements, respectively, were generated and bound to Dynabeads (Dynal). Recruitment assays were performed essentially as described (Takahashi et al., 2009).

Gene Expression Analysis and ChIP
Total RNA from HEK293T cells transfected with nontargeting control or MED26 siRNAs or from cell lines stably expressing MED26 shRNAs, with or without MED26 rescue constructs, was used to measure genome-wide gene expression with Affymetrix U133A plus 2.0 expression arrays or to measure expression of individual genes by qPCR. ChIP assays were performed with normal IgG or the indicated antibodies, and precipitated DNA was measured by qPCR. Primer sets, methods for gene expression and ChIP analyses, and siRNAs and shRNAs are detailed in Extended Experimental Procedures.

ACCESSION NUMBERS
Microarray data are deposited in GEO under accession number GSE28715.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, three figures, and two tables and can be found with this article online at doi:10.1016/j.cell.2011.06.005.

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EXTENDED EXPERIMENTAL PROCEDURES

Mass Spectrometry
Identification of proteins was accomplished using a modification of the multidimensional protein identification technology (MudPIT) procedure (Florens and Washburn, 2006; Washburn et al., 2001). TCA-precipitated proteins were urea-denatured, reduced, alkylated and digested with endoproteinase Lys-C (Roche) followed by modified trypsin (Roche) as described (Washburn et al., 2001). 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 (McDonald et al., 2004). Loaded microcapillary columns were placed in-line with an LCQ or LTQ ion trap mass spectrometer equipped with a nano-LC electrospray ionization source (ThermoFinnigan). Fully automated MudPIT runs were carried out on the electrosprayed peptides, as described by (Florens and Washburn, 2006). Tandem mass (MS/MS) spectra were interpreted using SEQUEST (Eng et al., 1994) against a database of a database of 30709 human proteins (downloaded from NCBI on 2009-10-27), and complemented with 177 sequences from usual contaminants (human keratins, IgGs, proteolytic enzymes). In addition, to estimate false positive discovery rates, each sequence was randomized (keeping AA composition and length the same) and the resulting “shuffled” sequences were added to the “normal” DB (doubling its size) and searched at the same time.

Peptide/spectrum matches were sorted and selected using DTASelect (Tabb et al., 2002) with the following criteria set: spectra/peptide matches were only retained if they had a DeltaCn of at least 0.08, and minimum XCorr of 1.8 for singly-, 2.0 for doubly- 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 2 such peptides, or 1 peptide with 2 independent spectra. Under these criteria, the overall false discovery rate was 0.18%, suggesting a > 99.82% confidence level for positive protein identifications. Peptide hits from multiple runs were compared using CONTRAST (Tabb et al., 2002). To estimate relative protein levels, Normalized Spectral Abundance Factors (NSAFs) were calculated for each detected protein (Florens et al., 2006; Paoletti et al., 2006; Zhang et al., 2010; Zybailov et al., 2006). The NSAF for a protein k is proportional to the amount of the protein present in the sample and is calculated using the formula:

\[
\langle\text{NSAF}\rangle_k = \frac{(\text{SpC}/L)_k}{\sum_{i=1}^{n}(\text{SpC}/L)_i},
\]

where SpC = spectral count, L = protein length in amino acids, and i = all proteins detected in the MudPIT runs.

Plasmid Construction and Generation of Stable Cell Lines

cDNAs encoding full-length and various mutant versions of human MED26 (NM_004831.3) or human EAF1 (NM_033083.6), ELL2 (NM_012081.5), AFF4 (NM_014423.3), CDK9 (NM_001261.3), or TAF7 (BC032737) were introduced by retroviral transduction into a HeLa S3 cell line stably expressing the mouse ecotropic retrovirus receptor (mCAT-1) (Albritton et al., 1989). Full-length human NARG2 (NM_024611.4), KIAA0947 (NM_015325.1), and AF9 (IMAGE clone ID: 5298142) were amplified from HeLa cell total RNA or obtained from the American Type Culture Collection, subcloned into pcDNA5-FRT with epitope tags, introduced into Flp-In 293 cells using the Invitrogen Flp-in system, and clonal stable cell lines were established following the manufacturers’ instructions.

Clonal HEK293T cell lines stably expressing TRIPZ lentiviral Dox-inducible shRNAmirs (Open Biosystems, Huntsville, AL), either nontargeting (RHS4743) or targeting the med26 3’-UTR (#1, RHS1764-9695596; #2, RHS1764-9394914; #3, RHS1764-9209087), were generated according to the manufacturer’s instructions. To generate cell lines stably expressing Dox-inducible shRNA with MED26 rescue constructs, HEK293T cells expressing med26 shRNA #3 were stably transformed with pcDNA3.1/Hygro carrying wild-type or mutant MED26 with an N-terminal epitope tag.

Antibodies for Western Blotting and Immunoprecipitation

Anti-Flag (M2) and anti-HA (HA-7) antibodies were from Sigma; monoclonal anti-TBP (ab818), anti-TAF6 (ab51026), anti-AFF4 (ab57077) anti-AF9 (ab60083) antibodies were from Abcam; monoclonal anti-TAF1 (sc-735), anti-TAF4 (sc-736), anti-CDK9 (sc-13130) antibodies and rabbit polyclonal anti-GAL4 (sc-577), anti-Cyclin T1 (sc-8127) antibodies were from Santa Cruz; rabbit polyclonal anti-Cyclin T2 (A301-678A) antibody was from Bethyl. Monoclonal anti-ELL antibody has been described (Lin et al., 2010). Mouse monoclonal anti-EAF1 antibody was a gift from Michael Thirman (Department of Medicine, University of Chicago).

ES Cell Culture

ES cells (KH2) were cultured in 6 well tissue culture plates in Glasgow Modified Eagle Medium containing 10% fetal bovine serum, 1/100 (v/v) L-glutamine ( Gibco 25030-024), 1/100 (v/v) nonessential amino acids (Gibco 11140-035), 0.1 mM 2-mercaptopethanol (Gibco 31350-010) and 1000 U/ml LIF (Chemicon, No. ESG1107).
siRNA Transfections
HEK293T cells in 6 well tissue culture plates (~1 x 10^6 cells/well) or 10 cm dishes (~2 x 10^6 cells/dish) were transfected with 25 nM siRNAs targeting human med26 (Ambion/Applied Biosystems, #4, s18074; #5, s18075; #6, s18076) or 25 nM siGENOME NON-TARGETING siRNA Pool #2 (Dharmacon D-001206-14) using X-tremeGENE siRNA Transfection Reagent (Roche) and grown for 48 hr. ES cells were transiently transfected with siRNA targeting mouse med26 (Ambion/Applied Biosystems, #7: cat. number s89018; #8; cat. number s89019, #9, cat. number s89020). siRNA designed against mouse med6 (ON-TARGET plus SMART pool, L-055846-01), siRNA targeting mouse cdk8 (ON-TARGET plus SMART pool, L-053848-00) or siCONTROL NON-TARGETING siRNA (Dharmacon) using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen).

Luciferase Reporter Assays
HEK293T cells were cotransfected with 1 μg of the GAL4-responsive luciferase reporter plasmid pG5-Luc (Promega), 100 ng of the renilla luciferase control plasmid pRL-tk (Promega), and 250 ng effector plasmid expressing wild-type or mutant GAL4-Med26 NTD using FuGene 6 reagent (Roche). Firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter assay kit (Promega). Each experiment was performed in triplicate; error bars shown in the figure represent standard deviations.

Cell Proliferation Assays
5 x 10^4 293T cells stably expressing Dox-inducible shRNAmir were seeded on 10 cm dishes and cultured in DMEM containing 10% Tet-free FBS with or without 2 μg/ml doxycycline. The number of live cells was determined by counting using a haemocytometer after trypan blue staining at the indicated times. Data are average from three independent experiments.

Gene Expression Analysis
Total RNA was isolated using the RNeasy Protect Mini Kit (QIAGEN). For RT-qPCR, total mRNA was reverse transcribed using the iScript Select cDNA Synthesis Kit (Biorad), and real-time PCR reactions were performed using an iCycler iQ Real-Time PCR Detection System and iQ SYBR green supermix (Bio-Rad). Ct values were normalized to GAPDH. Primer sequences are listed in PCR primers. For genome-wide siRNA analyses, total RNA was amplified and labeled using the MessageAmp III RNA Amplification Kit (Ambion), and aRNA was hybridized to Affymetrix U133A plus 2.0 expression arrays. Hybridized arrays were washed, stained, scanned according to standard procedures (Affymetrix). Affymetrix CEL files were processed in the R statistical environment and normalized using RMA (Irizarry et al., 2003). The linear modeling package Limma (Smyth, 2004) was used to derive gene expression coefficients and calculate p values. p values were adjusted for multiple hypothesis testing using the method of Benjamini and Hochberg (Benjamini and Hochberg, 1995). Lists of the most highly affected genes (Table S2) were generated by applying a log2-fold change cutoff of ≤ –0.5 or ≥ 0.5 to data prefiltered for p value; these included 97 well-characterized genes that were downregulated and 196 that were upregulated after cells were treated independently with all three of the siRNAs.

Chromatin Immunoprecipitations
Cells from one 10 cm dish (~1 x 10^5) of HEK293T cells or HeLa cells grown to 70%–80% of confluence were used for each immunoprecipitation. Cells that were non-heat shocked or heat shocked at 42°C for the indicated times were cross-linked with 1% formaldehyde in PBS for 20 min at room temperature. Cells were resuspended and lysed in lysis buffer (0.2% or 0.5% SDS, 10mM EDTA, 150 mM NaCl, 50 mM Tris-HCl pH 8.0), and were sonicated with a Bioruptor Sonicator (Diagenode) for 30 s at the maximum power setting to generate DNA fragments of ~150-400 bps. Sonicated chromatin was incubated at 4°C overnight with 5–15 μg of normal IgG or specific antibodies. Antibodies used were as follows: CDK9 (sc-8338, Santa Cruz), AFF4 (A302-539A, Bethyl); Pol II total Rbp1 (N-20, sc-899, Santa Cruz); Pol II Rbp1 CTD phospho Ser5 (ab5131, Abcam); Rpb1 CTD phospho Ser2 (H5; ab24758, Abcam with Upstate IgG-IgM linker antibody 12-488, Millipore), FLAG M2 (F3165, Sigma), MED26 (sc-48776, Santa Cruz), MED1 (sc-5334, Santa Cruz) or TBP (ab51841, Abcam). Then, salmon sperm DNA-protein A agarose (16-157, Millipore) was added and incubated for 2 hr at 4°C. Beads were washed 2 times with IP buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100), 2 times with high-salt buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100), 1 time with LiCl buffer (250 mM LiCl, 20 mM Tris-HCl pH8.0, 1 mM EDTA, 1% Triton X-100, 0.1% NP40 and 0.5% NaDOC), and two times with TE buffer. Bound complexes were eluted from the beads with 100 mM NaHCO3 and 1% SDS by incubating at 50°C for 30 min with occasional vortexing. Crosslinking was reversed by overnight incubation at 65°C. Immunoprecipitated DNA and input DNA were treated with RNase A and Proteinase K by incubation at 45°C. DNA was purified using the QIAquick PCR purification kit (28106, QIAGEN) or MinElute PCR purification kit (28006, QIAGEN). Immunoprecipitated and input material was analyzed by quantitative PCR. ChIP signal was normalized to total input.

Production of Recombinant Proteins
N-terminally 6xHis-tagged MED26 NTD (residues 1-113), GAL4-VP16, HNF4α, GAL4 DNA binding domain (1-97) or GAL4 DNA binding domain fused to MED26 NTD (GAL4-MED26 NTD) and derivatives were expressed in BL21(DE3) CodonPlus Escherichia coli (Stratagene) and purified as described (Yao et al., 2006). For production of recombinant proteins in Sf9 or Sf21 cells, epitope-tagged ELL, EAF1 and MED26 were subcloned into pBacPAK8 expressed using the BacPAK system (Clontech). Full-length or deletion mutants of NARG2 and KIAA0947 were subcloned into pFastBac HTb with epitope tags and expressed singly or together with the
BAC-to-BAC system (Clontech). Baculovirus infections, Sf9 or Sf21 culture, and affinity purifications were performed as described (Yao et al., 2006).

**PCR Primers**

**Primers Used for ChIP-qPCR**

HSPA1A (−50)

5'- TCTGATTGGTCCAAGGAAGGCTG-3' (forward)
5'- TTTCCCTTCTGAGCCAATCAGGA-3' (reverse)

HSPA1A (+492)

5'- AGGTGATCAACGCCGGGAC-3' (forward)
5'- ATCTCCTCGGGGTAGAATGC-3' (reverse)

HSPA1A (+898)

5'- GGGTGGGGAGGACTTTGACAACAGG-3' (forward)
5'- TGGCTGATTGTGCTTTGTG-3' (reverse)

HSPA1A (+1486)

5'- GACGCAGATCTCCACCACCT-3' (forward)
5'- GCCCCAACAGATTGCTCT-3' (reverse)

HSPA1A (+1895)

5'- AAGATCGAGGCGGCAGACGAGGAA-3' (forward)
5'- TCCTCTTGTGCTCAAACCTCTC-3' (reverse)

MYC (−3 kb)

5'- AACCTGAAGTCGGTCGGTAATC-3' (forward)
5'- GGAAGTCGCTCTGCTGGAATTACTACA-3' (reverse)

MYC (+83)

5'- TTCTCGAGGGCTGCGGAAAA-3' (forward)
5'- CTGCCCCGTGGTAATGCTCT-3' (reverse)

MYC (+548)

5'- AAACCGTAAAGACGCGAGTGCCA-3' (forward)
5'- TGCTCAATAGCGCAGGATGGAGAA-3' (reverse)

MYC (+1170)

5'- AACCTGGGTCCTGCTTGCAGGAATTGAC-3' (forward)
5'- TCAACCGATTCAAGGCGGAC-3' (reverse)

MYC (+2243)

5'- ACTCGGTGAGCCGTATTTCTACT-3' (forward)
5'- GCAGCAGCTTCAATTTCCAGGA-3' (reverse)

**Primers Used for Expression Analysis**

Human GAPDH

5'- TCGACAGTCAGCCGCATCTTCTT-3' (forward)
5'- GCCCAATACGACCAATCCCGTGTA-3' (reverse)

Human MYC

5'- ACAGCTACGGAACCTTGTGGT-3' (forward)
5'- CAGCCAAGTGTGGTAGGATT-3' (reverse)
Human HSP70
5'-TGTGGACAAAGTGTCAAGGTA-3' (forward)
5'-TCCTCTTGGCTCAAACGTCT-3' (reverse)

Human BMP2
5'-CAACCATGGATCGTGAAGT-3' (forward)
5'-CCAGCTGTGCATCTTGGTGCAA-3' (reverse)

Human SLC7A11
5'-TCTTATGGTTGCTTCCTCTCCCTC-3' (forward)
5'-ACCACCTGGGTTGTGTCCCATA-3' (reverse)

Human SNAIL2
5'-TTTCTGGGCTGGCCAAACATAAGC-3' (forward)
5'-ACAAAGAGGTAATGTGGGTCCA-3' (reverse)

Human JUN
5'-AGATGAACCTCTTGTGCTGCT-3' (forward)
5'-ACACTGGGCAGGATACCCAAACAA-3' (reverse)

SUPPLEMENTAL REFERENCES


Figure S1. Identification of Residues Critical for Interaction of MED26 NTD with EAF1 or TFIIID, Related to Figure 4

(A) Activation of a GAL4-dependent luciferase reporter by GAL4-NTD. Relative luciferase activities were measured in extracts of cells transiently transfected with expression plasmids encoding empty vector (vector), the GAL4 DNA binding domain (GAL4) or the indicated GAL4-MED26 NTD proteins.

(B) Mutation of MED26 NTD residues R61,K62 or K74,K75 interferes with recruitment of EAF1 or TFIIID to immobilized GAL4x5-MLT template. Binding reactions included EAF1 or TFIIID, and either GAL4 DNA binding domain or the indicated GAL4-MED26 NTD fusion proteins.
Figure S2. Effect of MED26 Depletion on ES Cell Proliferation and Gene Expression, Related to Figure 5
(A) Knockdown of endogenous MED26 and other Mediator subunits in ES cells. 3 days after transfection with the indicated siRNAs, cells were lysed and subjected to immunoblotting with anti-MED26, CDK8, or MED6 antibodies.
(B) MED26 knockdown inhibits ES cell proliferation. 4 days after transfection with the indicated siRNA, KH2 ES cells were stained with a cell-permeable green fluorescent dye to identify live cells (Live-Dead Cell staining kit, BioVision, Cat.# K501-100).
(C) Effect of MED26 depletion on hsp70, bmp2 and jun expression in HEK293T cells. Gene expression was measured by real time qPCR and normalized to gapdh. Data are averages from three independent experiments; error bars show standard deviations.
Figure S3. Localization SEC and Mediator Subunits on the c-myc and hsp70 Genes, Related to Figure 6

(A) Occupancy of MED26 and endogenous or exogenously expressed SEC components on the c-myc gene. (B) Recruitment of Pol II, Mediator subunits and SEC components to the hsp70 gene by heat shock. Bars indicate average ChIP/input obtained from two biological replicates, error bars show the data range.