HSP90 and Its R2TP/Prefoldin-like Cochaperone Are Involved in the Cytoplasmic Assembly of RNA Polymerase II

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SUMMARY

RNA polymerases are key multisubunit cellular enzymes. Microscopy studies indicated that RNA polymerase I assembles near its promoter. However, the mechanism by which RNA polymerase II is assembled from its 12 subunits remains unclear. We show here that RNA polymerase II subunits Rpb1 and Rpb3 accumulate in the cytoplasm when assembly is prevented and that nuclear import of Rpb1 requires the presence of all subunits. Using MS-based quantitative proteomics, we characterized assembly intermediates. These included a cytoplasmic complex containing subunits Rpb1 and Rpb8 associated with the HSP90 cochaperone hSpagh (RPAP3) and the R2TP/Prefoldin-like complex. Remarkably, HSP90 activity stabilized incompletely assembled Rpb1 in the cytoplasm. Our data indicate that RNA polymerase II is built in the cytoplasm and reveal quality-control mechanisms that link HSP90 to the nuclear import of fully assembled enzymes. hSpagh also bound the free RPA194 subunit of RNA polymerase I, suggesting a general role in assembling RNA polymerases.

INTRODUCTION

RNA polymerases play fundamental roles in the cell. RNA polymerases I and III synthesize the noncoding RNAs that form the translational apparatus, and their activity is intimately linked to the growth state of the cell (White, 2005). RNA polymerase II synthesizes capped noncoding RNAs as well as all mRNAs. This enzyme is at the heart of gene regulation and is subjected to many controls, including at the level of initiation, elongation, and termination (Fuda et al., 2009).

The activities, structures, and subunit composition of the three major RNA polymerases have been characterized in detail (Cramer et al., 2008). RNA polymerases I, II, and III are composed of 14, 12, and 17 subunits, respectively. The two largest subunits form the catalytic core of the enzyme, while the others are smaller and generally bind on their surface. The three RNA polymerases are related to each other, and this structural similarity is also highlighted by the fact that some subunits are shared by several polymerases, with a few being present in all three enzymes. Despite intensive studies on the structure and regulation of RNA polymerases, relatively little is known about the location and mechanism of their assembly. To date, this question has been principally addressed by live-cell microscopy techniques that used GFP-tagged subunits in mammalian cells. FRAP (fluorescence recovery after photobleaching) studies on RNA polymerase I have indicated that some subunits can either assemble or exchange directly at promoters (Dundr et al., 2002). Furthermore, the exchange/assembly rate depends on the phase of the cell cycle, indicating that assembly of RNA polymerase I in the nucleolus is a way to regulate gene expression (Gorski et al., 2008). Live-cell studies on RNA polymerase II promoters have also revealed a rapid exchange of basal and sequence-specific transcription factors at promoters (Dundr et al., 2002). Moreover, the exchange/assembly rate depends on the phase of the cell cycle, indicating that assembly of RNA polymerase I in the nucleolus is a way to regulate gene expression (Gorski et al., 2008). Live-cell studies on RNA polymerase II promoters have also revealed a rapid exchange of basal and sequence-specific transcription factors at promoters. In yeast, the TATA binding factor TBP and the transcriptional activator Ace1p were shown to rapidly come on and off the chromatin, with a residency time in the range of seconds (Karpova et al., 2008; Sprouse et al., 2008). Moreover, the rapidly cycling Ace1p molecules were shown to be responsible for transcriptional activation. In mammalian cells, glucocorticoid and estrogen receptors were shown to have similarly high exchange rates with their target sites on DNA (McNally et al., 2000; Stenoien et al., 2001). More recently, the use of Drosophila salivary glands with polytene chromosomes and the generation of human cell lines carrying artificial arrays of reporter genes have allowed detailed studies of transcription in living cells (Janicki et al., 2004; Yao et al., 2006). In particular, FRAP studies of GFP-tagged subunits of RNA polymerase II were used to define the dynamics of their binding to promoters and the transcription.
kinetics of this key enzyme. In two studies that used HIV-1 reporters or natural Drosophila genes, an efficient initiation entry mode was found (Boireau et al., 2007; Yao et al., 2007). In contrast, a study that used a Tet-inducible promoter in human cells found that a large fraction of the polymerase exchanges rapidly at promoters, with only a few percent that go into productive elongation (Dargacz et al., 2007). Given the precedent example of RNA polymerase I assembly at its promoter, these data led to the suggestion that RNA polymerase II may also assemble at its promoter and that this could allow a gene-specific regulation of its assembly (Darzacq and Singer, 2008; Hager et al., 2009).

Recently, affinity purification of soluble human RNA polymerase II with TAP-tagged subunits identified a number of polymerase-associated factors of unknown function (Jeronimo et al., 2004, 2007). Four of these factors are homologous to the yeast R2TP complex (Boulon et al., 2008; Zhao et al., 2005), and further studies suggested that they indeed form a complex that contains the R2TP factors plus five prefoldin-like proteins (referred to as R2TP/Prefoldin-like) (Boulon et al., 2008; Cloutier et al., 2009; Sardi et al., 2008). The yeast R2TP protein Tah1 was initially characterized as a cochaperone for Hsp90 (Zhao et al., 2005) and, in agreement, we recently showed that the human Tah1 homolog hSpagh (also called RPAP3 or FLJ21908) is an Hsp90 cofactor (Boulon et al., 2008). Furthermore, Hsp90 and R2TP proteins, including hSpagh, play a key role during the assembly of snRNPs (Boulon et al., 2008; Gonzales et al., 2005; King et al., 2001; Zhao et al., 2008), raising the possibility that the association of hSpagh with RNA polymerase II subunits could be involved in the assembly of this enzyme.

In this study, we used a combination of quantitative MS-based proteomics and fluorescence microscopy to characterize the mechanism of RNA polymerase II assembly in human cells and the role of hSpagh and Hsp90 in this process.

RESULTS

Unassembled RNA Polymerase II Subunits Accumulate in the Cytoplasm

To study assembly of RNA polymerase II, we took advantage of α-amanitin. This drug binds the large Rpb1 subunit of human RNA polymerase II with high affinity. In vitro, this reduces the elongation rate more than 100-fold and makes the polymerase prone to essentially irreversible stalling (Rudd and Luse, 1996). In vivo, this results in transcriptional arrest and concomitant destruction of subunit Rpb1. The other subunits remain intact, suggesting that α-amanitin may result in enzyme disassembly (Nguyen et al., 1996). First, the localization of RNA polymerase II subunit Rpb3 was analyzed by fluorescence microscopy using a U2OS cell line stably expressing a GFP-Rpb3 fusion protein known to incorporate into functional RNA polymerase (Boireau et al., 2007 and see below). The GFP-Rpb3 fusion protein normally accumulates in the nucleus, with only a faint cytoplasmic staining (Figure 1A). However, this changed following α-amanitin addition, with GFP-Rpb3 now accumulating in the cytoplasm. To test whether this resulted either from a failure to import newly synthesized Rpb3 or instead from export of the nuclear subunit, cells were treated with both α-amanitin and leptomycin B (LMB), a specific inhibitor of the exportin CRM1 (Fornerod et al., 1997). This resulted in the retention of GFP-Rpb3 in the nucleus (Figure 1A), indicating that an active export mechanism removes GFP-Rpb3 from nuclei when Rpb1 is degraded.

Surprisingly, LMB prevented nuclear import of Rpb1 (Figure 1A) and made this subunit insensitive to α-amanitin (Figures 1A and S1). Because α-amanitin specifically promotes degradation of the elongating form of RNA polymerase II (Nguyen et al., 1996), the cytoplastic accumulation of Rpb1 most likely represents newly synthesized subunits that were never engaged in transcription elongation. These data suggest that factors required for Rpb1 nuclear import may be retained in the nucleus upon LMB treatment. Since Rpb3 remains nuclear in these conditions, an intriguing possibility would be that Rpb1 needs to be incorporated in fully assembled enzymes before being imported into the nucleus.

To test this hypothesis, RNA polymerase II assembly was prevented by inhibiting de novo synthesis of individual subunits with siRNAs. Depletion of Rpb2, the second largest subunit, prevented nuclear import of Rpb1 (Figure 1B). The pool of Rpb1 accumulated in the cytoplasm was resistant to α-amanitin, indicating that it corresponds to newly synthesized proteins that were never engaged in elongation. Furthermore, analysis with a panel of antibodies that recognize various phosphoisoforms of the CTD heptad-repeat indicated that the cytoplasmic Rpb1 is mostly unphosphorylated (see Supplemental Experimental Procedures and Figure S1). This effect on Rpb1 caused by siRNA-mediated depletion of Rpb2 was not an indirect consequence of transcription inhibition. Indeed, the transcription inhibitors DRB and actinomycin D, which do not trigger subunit degradation (Nguyen et al., 1996), did not induce a cytoplastic accumulation of Rpb1 (Figures 1C and S1). These results were extended by examining siRNA-mediated removal of other RNA polymerase II subunits. Remarkably, depletion of any subunit, including Rpb3, resulted in a similar accumulation of Rpb1 in the cytoplasm (Figures 1D and S1, and see below for quantitative measurements of Rpb1 localization). Together with the fact that degradation of elongating Rpb1 by α-amanitin triggered nuclear export of Rpb3, these data suggested that assembly of RNA polymerase II occurs in the cytoplasm and is an obligatory step prior to nuclear import.

GFP-Tagged Subunits Rpb1 and Rpb3 Have Similar Dynamics at the Transcription Site of an HIV-1 Reporter Gene

Assembly of RNA polymerase I in the nucleolus was demonstrated by FRAP using GFP-tagged subunits (Dundr et al., 2002). Indeed, RNA polymerase I subunits have biphasic recovery curves in the nucleolus, with an initial rapid recovery of about 1 min that is specific for each subunit and that represents exchange of their unassembled form. To further substantiate that RNA polymerase II is assembled in the cytoplasm, rather than at promoters, we determined the dynamics of different polymerase subunits at the transcription site of an HIV-1 reporter gene that was tagged with MS2 sites for visualization in living cells (Fusco et al., 2003; Boireau et al., 2007) (Figure 2). To this end, we used the Exo1 cell line, which has integrated many copies of the reporter gene and was shown to
accumulate high levels of RNA polymerase II at its transcription site (Boireau et al., 2007). We previously observed that in this system, GFP-tagged subunit Rpb3 was initiating transcription efficiently and did not show a rapid exchange phase during the first minute of the recovery (Boireau et al., 2007). However, this did not preclude a different behavior for the other subunits and, in particular, for subunit Rpb1 (Darzacq et al., 2007). To analyze the dynamics of this subunit, we transfected the Exo1 cell line with a GFP-tagged Rpb1 construct that carried an α-amanitin resistance mutation and selected α-amanitin-resistant cell lines to replace the endogenous polymerase by the GFP-tagged one. Remarkably, the analysis of GFP-Rpb1 dynamics by FRAP revealed a recovery rate at the HIV-1 transcription site that was not significantly different from the GFP-Rpb3 subunit, with an absence of an initial rapid exchange phase. This was consistent with the idea that RNA polymerase II was recruited to the HIV-1 promoter as a preformed complex rather than as free subunits.

**Characterization of RNA Polymerase II Assembly Intermediates by SILAC Quantitative Proteomics Using GFP-Rpb3 as a Bait**

Next, a quantitative MS-based proteomics approach was used to characterize the changes in RNA polymerase II complexes in either the absence or presence of α-amanitin, i.e., when RNA polymerase II enzyme is either mostly nuclear and active or mostly cytoplasmic and inactive, respectively (see Figure 1). A triple isotope SILAC labeling scheme was employed (Figure 3A) (Trinkle-Mulcahy et al., 2006). Control U2OS cells were cultivated in medium containing the normal, “light” amino acids, while cells stably expressing GFP-Rpb3 were grown either with “medium” (no treatment) or with α-amanitin for 15 hr (10 μg/ml). Cells were processed for immunofluorescence against Rpb1. Each field is 83.75 × 83.75 μm.

(C) Inhibition of RNA polymerase II transcription by DRB has no effect on the localization of Rpb1. U2OS cells were treated for 15 hr with DRB (100 μM), and the localization of Rpb1 was determined by immunofluorescence (panels α-Rpb1). FISH against polyA + RNAs was performed in parallel to assess the inhibition of transcription (panel PolA+). Each field is 67 × 67 μm.

(D) Depletion of RNA polymerase II subunits leads to the accumulation of Rpb1 in the cytoplasm. U2OS cells were transfected for 48 hr with siRNA against luciferase (siFFL) or the indicated subunit. Cells were processed for immunofluorescence with an antibody against Rpb1. Each field is 83.75 x 117.25 μm and contains few nondepleted cells that show wild-type nuclear Rpb1 levels.
below GFP-Rpb3, while those present above correspond to proteins whose specific copurification with GFP-Rpb3 increased after treatment. Of the 12 known RNA polymerase II subunits, 11 were specifically copurified with GFP-Rpb3, with a high number of peptides identified and quantified (see Table S1). Five of these—subunits Rpb4, Rpb5, Rpb7, Rpb8, and Rpb9—clearly dissociated from GFP-Rpb3 in cells treated with $\alpha$-amanitin, showing that RNA polymerase II complex is, at least in part, disassembled after Rpb1 degradation. In agreement, structural analyses have shown that except for Rpb9, four subunits cited above interact with Rpb3 via subunit Rpb1 (Figure 3B) (Armache et al., 2003; Bushnell and Komberg, 2003). Several additional factors were found to associate with subunit Rpb3 with high specificity, i.e., RPAP1, RPAP2, GrinL1A, GPN1, GPN2, and GPN3, and the SILAC data show that these factors preferentially associate with Rpb3 subunits that are not assembled in a complete RNA polymerase II enzyme (Figures 3A and 3B and Table S1). Systematic yeast two-hybrid assays also revealed an extensive set of protein-protein interactions (Figure 3B and Table S2). Together, these data indicate the existence of a cytoplasmic RNA polymerase II complex formed by subunits Rpb2, Rpb3, Rpb10, Rpb11, and Rpb12 and identify additional factors associated with this complex.

### Large Subunits of RNA Polymerase I and II Associate with the HSP90 Cochaperone hSpagh when Enzyme Assembly Is Prevented

Next, a similar SILAC-based proteomics strategy was used to identify factors bound to newly synthesized Rpb1 in cells treated with $\alpha$-amanitin and LMB (Figure 4A). In this case, an antibody specific for the endogenous Rpb1 subunit was used for immunopurification (antibody PB-7C2), rather than a GFP fusion protein. Treatment of cells with $\alpha$-amanitin and LMB prevented the association of most subunits with Rpb1. While Rpb8 was not much affected, all the other subunits were present in Rpb1 complexes in lower amount, from $\sim$3-fold to $\sim$6-fold (Figure 4A and Table S3). Several additional factors copurified specifically with Rpb1, including some that were previously described (Jeronimo et al., 2007). Remarkably, association of Rpb1 with RPAP2, GPN1, GPN3, GrinL1A, and the R2TP/Prefoldin-like components hSpagh, PFDN2, and UXT was largely unaffected by treatment with $\alpha$-amanitin and LMB, indicating that they all bind Rpb1 prior to its assembly into a complete enzyme. Systematic yeast two-hybrid tests further revealed that UXT interacts with Rpb1 (Table S4) and could thus link it to the R2TP/Prefoldin-like complex (Figure 4D).

Analysis of protein half-lives using pulsed isotope labeling and quantitative MS showed no correlation with loss or enrichment in the immunoprecipitates upon $\alpha$-amanitin treatment (F.M. Boisvert and A.I.L., unpublished data), indicating that changes in complex composition were not due to loss of proteins induced by transcriptional shutdown (data not shown). To further rule out this possibility, we performed a similar SILAC experiment in which we analyzed the status of polymerase assembly in cells treated with actinomycin D. The time of actinomycin D treatment (7 hr) was chosen to match the time of effective transcriptional arrest during $\alpha$-amanitin treatment (Figure S2). For this purpose, a triple-labeling SILAC experiment was performed, where we purified Rpb1 complexes from (1) untreated cells (light amino acids), (2) cells treated with actinomycin D (medium amino acids), and (3) cells treated with $\alpha$-amanitin + LMB (heavy amino acids). The data were displayed in a 2D graph, with SILAC ratios of actinomycin D versus untreated cells on the x axis and ratios of $\alpha$-amanitin + LMB versus untreated cells on the y axis. Again, we observed that $\alpha$-amanitin + LMB induced RNA polymerase II disassembly and association of the Rpb1/Rpb8 dimer with the full R2TP/Prefoldin-like complex (Figure 4B). In contrast, polymerase disassembly was not observed upon treatment with actinomycin D. Although an increased association was observed with the R2TP/Prefoldin-like complex, it was clearly less than in cells treated with $\alpha$-amanitin + LMB (Figures 4B and 4C). Thus,
unassembled Rpb1 is specifically associated with the R2TP/Prefoldin-like complex.

hSpagh is a central component of the R2TP/Prefoldin-like complex that binds HSP90 (Boulon et al., 2008). To determine possible functions of hSpagh and HSP90 in the biogenesis of RNA polymerase II, we first confirmed the binding of HSP90 and hSpagh to Rpb1 by western blotting. Coimmunoprecipitation (coIP) experiments of endogenous proteins in S100 extracts revealed that Rpb1 interacted with hSpagh and HSP90, that hSpagh was associated with HSP90, and that these interactions did not depend on HSP90 ATPase activity (Figure S3). Next, we generated a stable U2OS clone expressing a GFP-tagged version of hSpagh and immunoaffinity-purified GFP-hSpagh. This confirmed that Rpb1 was bound to GFP-hSpagh, even after treating cells with α-amanitin and LMB (Figure 5A). We then depleted the Rpb2 subunit with siRNA. Remarkably, interaction of Rpb1 with GFP-hSpagh increased upon knockdown of Rpb2 (Figure 5B), confirming that hSpagh preferentially binds unassembled Rpb1. Interestingly, hSpagh has also been reported to bind the large subunit of RNA polymerase I, RPA194 (Jeronimo et al., 2007). To test whether this also occurs preferentially on the unassembled form of this subunit, cells were treated with siRNA against the second largest subunit of RNA polymerase I, RPA135, and GFP-hSpagh immunoprecipitates were probed for the presence of RPA194. Indeed, an increased association of RPA194 with hSpagh was seen upon knockdown of RPA135 (Figure 5C), suggesting that hSpagh preferentially associates with free RPA194.

To further characterize the RNA polymerase II complexes associated with hSpagh, we performed SILAC-based proteomic analyses using GFP-hSpagh as bait, and we compared protein complexes from control and α-amanitin-treated cells.
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(Figure 5D and Tables S5 and S6). Components of the R2TP/Prefoldin-like complex were readily identified copurifying with GFP-hSpagh, but only four RNA polymerase II subunits were found, i.e., Rpb1, Rpb2, Rpb8, and Rpb5. Furthermore, α-amanitin-induced destruction of Rpb1 strongly decreased the levels of Rpb2 and Rpb8 copurified with GFP-hSpagh, while levels of Rpb5 remained unchanged. Previous SILAC immunoprecipitation showed that Rpb1 dissociates from Rpb5 but remains associated with hSpagh upon treatment with LMB and α-amanitin (Figure 4A). Altogether, these data suggest that hSpagh associates independently with Rpb5- and Rpb1-containing subcomplexes (Figure 5E).

Interaction of hSpagh with Unassembled Subunit Rpb1 Occurs in the Cytoplasm
Unassembled subunit Rpb1 accumulates in the cytoplasm, suggesting that its interaction with hSpagh may take place in this compartment. To test this possibility, we set up a subcellular corecruitment assay (Figure 6) (Darzacq et al., 2006). In these experiments, hSpagh was artificially targeted to distinct subcellular compartments: either to cytoplasmic P-bodies, by fusion to a fragment of p54/RCK (Minshall et al., 2009), or to nucleoplasmic LacO arrays, by fusion to Laci (Darzacq et al., 2006). Subsequent recruitment of interacting partners to either P-bodies or LacO arrays was then tested to assess their binding to hSpagh in the cytoplasm or the nucleus, respectively. First, corecruitment of GFP-HSP90 was tested. In cells that expressed either p54- or Laci-importin Kpna3 fusion protein as a negative control, GFP-HSP90 localized diffusely and did not concentrate in P-bodies or at the LacO array (Figure 6A). In contrast, in cells that expressed hSpagh fused to either p54 or Laci, GFP-HSP90 accumulated in P-bodies or at the LacO array, respectively (Figure 6B). Next, the localization of Rpb1 was analyzed by immunofluorescence. We found that it was weakly detectable in P-bodies containing p54-hSpagh. However, it accumulated there strongly upon depletion of Rpb2, and this was specific, because no recruitment was seen in cells expressing p54-Kpna3 in the same conditions (Figure 6C). Interestingly, p54 fusion with truncated versions of hSpagh revealed that different domains are responsible for HSP90 and HSP90-α-amanitin treatment or siRNA-mediated Rpb2 depletion (Figures 7A and S1). In addition, quantification of fluorescence microscopy images revealed that GA destabilized specifically the cytoplasmic form of Rpb1 (Figure 7B). This also occurred upon siRNA-mediated depletion of every subunit except Rpb4, Rpb6, Rpb7, and Rpb9 (Figure 7C). We therefore propose that HSP90 is required to stabilize Rpb1 through most of the assembly pathway and, in particular, for the joining of Rpb1 with Rpb8, Rpb5, and with the Rpb2-Rpb3-Rpb10-Rpb11-Rpb12 subcomplex. To then test the role of hSpagh in this process, it was depleted with siRNA. We observed that removal of hSpagh mimicked the effect of HSP90 inhibition: it destabilized the cytoplasmic Rpb1 that accumulated upon depletion of either Rpb2 or Rpb3, but not Rpb6 (Figures 7D and S5). Interestingly, long-term depletion of hSpagh, for 3 days, resulted in the loss of Rpb1, consistent with hSpagh having a role in the assembly of RNA polymerase II (Figure 7E).

DISCUSSION
In this study, we have identified a mechanism for the formation of RNA polymerase II from its subunits, which suggests that the assembly pathway occurs predominantly, if not entirely, within the cytoplasm. Analysis of the SILAC quantitative MS data permits rigorous and comprehensive evaluation of the dynamic behavior of RNA polymerase II complexes, while fluorescence microscopy assays determine their subcellular localization. This identified putative assembly intermediates containing a subset of RNA polymerase II subunits and showed that these complexes occur predominantly in the cytoplasm. Altogether, we propose a model whereby nuclear entry of RNA polymerase II is restricted to fully assembled enzymes. Previous studies on RNA polymerase I postulated the existence of an assembly compartment in the nucleolus (Dundr et al., 2002). For RNA polymerase II, our results indicate that this assembly compartment is the cytoplasm. This model does not exclude the possibility of additional nuclear reassembly mechanisms for disassembled polymerases. However, recycling of RNA polymerase II subunits may also be predominantly a cytoplasmic process, rather than occurring at polymerase II promoters. In agreement, FRAP experiments using GFP-tagged subunits Rpb1 and Rpb3 show virtually identical recovery rates at an HIV-1 promoter, with an absence of a rapid recovery phase during the first minute of the experiment. This indicates that RNA polymerase II is recruited as a preformed complex, allowing for an efficient initiation entry mode. Our data support the idea that assembly of RNA polymerase II is regulated at the level of the entire cell, rather than in a gene-specific manner, at individual promoters.

RNA polymerase II is a central enzyme required for the transcription of essentially all pre-mRNAs in eukaryotic cells. The other main macromolecular machines involved in eukaryotic gene expression, i.e., ribosomes and spliceosomes, are also composed of multisubunit complexes that are largely assembled in compartments distinct from their final functional location. Thus, ribosomes are mostly assembled within nucleoli but function in mRNA translation in the cytoplasm (Boisvert et al., 2007), while snRNPs mature in the cytoplasm but function to remove introns exclusively in the nucleus (Matera et al., 2007).
Figure 4. Unassembled Rpb1 Is Associated with the R2TP/Prefoldin-like Complex

(A) Comparison of Rpb1 complexes when RNA polymerase II assembly is normal or inhibited by α-amanitin and LMB. The experimental design is indicated on the left. It is similar to Figure 2, except that cells are treated simultaneously with α-amanitin and LMB (for 15 hr) and that an antibody against endogenous Rpb1 is used. Legend as in Figure 2.

(B) Transcriptional shutdown does not result in polymerase disassembly (legend as in A). The experimental design is indicated on the left. Legend is as in Figure 2, except that cells were untreated or treated with α-amanitin + LMB (15 hr) or with actinomycin D (7 hr).

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Separating sites of assembly and function might be beneficial in preventing aberrant and potentially disruptive interactions between partially or incorrectly assembled complexes and their substrates.

Our proteomics data identified factors that associate specifically with the putative RNA polymerase II assembly intermediates. Further studies will be required to characterize the precise role of these different factors in RNA polymerase II biogenesis. Interestingly, RAPAP2 might be the key import factor of RNA polymerase II, as it binds the fully assembled enzyme (Jeronimo et al., 2007); it is required for its import and it shuttles in an LMB-dependent manner (data not shown). In this study, we deciphered the function of hSpagh, which interacts with unassembled Rpb1, the largest RNA polymerase II subunit, and which is a cofactor for HSP90. We show that HSP90 stabilizes free Rpb1 and that Rpb1 and HSP90 bind separable domains on hSpagh (Figure S4). This suggests that hSpagh delivers unassembled Rpb1 to HSP90, thereby linking this important chaperone to the assembly mechanism of RNA polymerase II. HSP90 is unusual in that it functions at late stages of protein folding, with clients in a near-native state, whereas most chaperones function at earlier stages to facilitate the folding and maturation of nascent proteins (Wandinger et al., 2008). HSP90 thus appears ideally suited to facilitate assembly of protein complexes. Recent systematic studies on HSP90 have suggested a large number of potential client proteins, with diverse functions, but few in vivo roles have been clearly demonstrated (McClellan et al., 2007; Zhao et al., 2005). Here, we provide evidence that HSP90 and its cochaperone hSpagh coordinate the assembly of RNA polymerase II in the cytoplasm. The precise mode of action of HSP90 will require detailed structural data, but possible roles could be to maintain Rpb1 in a conformation compatible with assembly, to prevent association with incorrect partners, and/or to provide a quality-control mechanism to ensure that the enzyme is properly assembled. Our data also suggest that HSP90 may buffer a transient imbalance in free subunit stoichiometry, which could, for instance, arise upon spontaneous, stochastic fluctuations in gene expression (Raser and O’Shea, 2005).

Purification of tagged hSpagh identified not only Rpb1, but also the large subunits of RNA polymerases I and III (Jeronimo et al., 2007 and this study). By western blot analysis, binding of subunit RPA194 to hSpagh was barely detectable under normal conditions but increased markedly upon depletion of subunit RPA135 (Figure 5C). This indicates that hSpagh preferentially associates with unassembled RPA194, suggesting that HSP90 and hSpagh may be involved in the assembly of all three RNA polymerases. HSP90 and hSpagh are also involved in the production of snoRNPs (Boulon et al., 2008), and they could thus assemble the machineries that produce tRNAs, mRNAs, and ribosomes. These molecules form the translational apparatus, whose activity directly controls production of the cellular mass (White, 2005). We therefore propose that the function of HSP90 in snoRNP and RNA polymerase assembly may contribute to its established role in cell proliferation and oncogenic transformation (Caldwood et al., 2006).

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Antibodies
U2OS cells were cultivated in DMEM plus antibiotics and 10% FBS. Cells were transfected with Ca-P coprecipitates or with lipofectamine and plus reagent (Invitrogen). Drugs were used at the following concentrations: actinomycin D, 5 μg/ml; DRB, 100 μM; z-amanitin, 10 μg/ml; GA, 2 μM; LMB, 15 nM, except for the SILAC experiments, where it was reduced to 8 nM. The sequence of siRNAs is given in the Supplemental Information.

L30-GFP-Rpb3 was described previously (Boireau et al., 2007). The other vectors were made using the Gateway technology (Invitrogen), with donor vectors from the human ORFeome (Lamesch et al., 2007) or generated by PCR. Detailed maps are available upon request. hSpagh TPR1 and TPR2 domains encompassed nucleotides 390–690 and 850–1160, respectively.

Antibodies against Rpb1 recognized the CTD heptads-repeat (both phosphorylated and unphosphorylated forms [Figure S1]) and were mouse monoclonal from Euromedex (PB-7C2) (Soufelfweyershain, Germany). Antibodies against Rpb5 and RPA194 were mouse monoclonals from Euromedex (PB-4H7) and Santa Cruz (C-1), respectively. Antibody against Rpb2 was a goat polyclonal from Santa Cruz (S20). GFP-TRAP beads were previously described and contained a recombinant llama monoclonal and monochain antibody against GFP (Chromotek; Planegg-Martinsried, Germany) (Rothbaue et al., 2008). Tubulin was detected using a rat antibody from Serotec (MCA780) (Colmar, France) and GAPDH using an antibody from Santa Cruz (FL-335).

Two-Hybrid Assays
Two-hybrid assays were performed as previously described (Boulon et al., 2008).

Cellular Imaging
For analyses in fixed cells, cells were grown on glass coverlips, treated as indicated, and fixed in 4% formaldehyde, 1x PBS for 30 min at RT. After permeabilization in 0.5% Triton for 10 min at RT and labeling with the appropriate antibodies, slides were mounted in Vectashield. For quantitative imaging of Rpb1 localization, images were captured with a 40× objective using identical settings. Average gray levels in the cytoplasm and nucleus were measured for a large number of cells (between 80 and 200). Background levels were taken in an area devoid of cells and subtracted to cytoplasmic and nuclear values. Signals from individual cells were then averaged and normalized to the nuclear signal of untreated cells.

For FRAP analyses in live cells, U2OS Exo1 cells were transfected with GFP-Rpb3 and MS2-Cherry vectors, transcription sites were bleached, and fluorescence recovery was recorded as previously described (Boireau et al., 2007). For studies of GFP-Rpb1, U2OS Exo1 cells were transfected with a GFP-tagged z-amanitin-resistant form of subunit Rpb1 (Sugaya et al., 2000), and clones were selected in 5 μg/ml z-amanitin. Individual clones were checked for expression of the HIV-1 reporter gene and GFP-Rpb1, and suitable cells were then transfected with an MS2-Cherry vector and analyzed by FRAP.

Immunoprecipitation
Cells were rinsed in PBS and lysed in HNTG for 20 min at 4°C. Cellular debris was removed by centrifugation (10 min at 20,000 g), and extracts were incubated with appropriate beads (2 hr at 4°C). The beads were washed four times in PBS/Triton 0.1% and resuspended in Laemmli buffer. Bound proteins were analyzed by western blotting. HNTG contained 20 mM HEPES (pH 7.9), 140 mM NaCl, 2 mM EDTA, 10% glycerol, 0.5% Triton X-100, 0.002% SDS, 1% aprotinin, and 1% leupeptin.

Mass spectrometry analysis
Mass spectrometry analysis was performed by the Rapid Mass Analysis Facility at the University of California, Berkeley.

Supplemental Information
The Supplemental Information is available with the online version of this paper.

(C) Unassembled Rpb1 is specifically associated with the R2TP/Prefoldin-like complex. SILAC ratios are from the experiment depicted in (B) and correspond to cells treated with z-amanitin + LMB versus those treated with actinomycin D. The ratios are normalized to that of Rpb1.

(D) Model of the complex containing unassembled Rpb1 (legend as in Figure 3).
Figure 5. hSpagh Preferentially Associates with Unassembled Forms of the Large Subunits of RNA Polymerases I and II

(A and B) hSpagh preferentially binds incompletely assembled subunit Rpb1. U2OS cells stably expressing GFP-hSpagh were subjected to GFP-TRAP immunoprecipitation. Inputs and pellets were analyzed by western blots with the indicated antibodies. Cells were untreated (NT) or treated with α-amanitin and LMB.

(C) GFP-TRAP IP

(D) Increased in α-amanitin vs untreated cells

(E) Decreased in α-amanitin vs untreated cells
Figure 6. hSpagh Interacts with HSP90 and Unassembled Subunit Rpb1 in the Cytoplasm

(A) hSpagh interacts with HSP90 in the nucleus and the cytoplasm. U2OS cells were transfected with the indicated constructs and processed for epifluorescence microscopy. The p54 and Laci constructs were also fused to a red fluorescent protein. Each field is 38 × 38 μm. Insets: zoom on the boxed area (4.3 × 4.3 μm).

(B) hSpagh interacts with subunit Rpb1 in the cytoplasm. U2OS cells were transfected with the indicated construct and siRNAs and then processed for immunofluorescence against Rpb1. Legend is as in (A).

(C) hSpagh fails to recruit Rpb1 subunit at LacO array in the nucleoplasm. Legend is as in (A) and (B).

PROTEOMIC ANALYSES

The SILAC procedures are described in detail in the Supplemental Information.

SILAC PROCEDURE

150 mM NaCl, 1% Triton, 10% glycerol, 1 mM MgCl2, 1 mM EGTA, and protease inhibitors (Roche).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, five figures, and six tables and can be found with this article online at doi:10.1016/j.molcel.2010.08.023.
Figure 7. hSpagh and HSP90 Activity Stabilize Unassembled Cytoplasmic Rpb1 Subunit

(A) Inhibition of HSP90 activity with GA selectively destabilizes the unassembled Rpb1 subunit. U2OS cells were treated for 43 hr with control siRNAs (siFFL) or with siRNA against Rpb2 (siRpb2) and incubated with GA in the last 15 hr (GA, 2 μM) when indicated. Extracts were prepared in HNTG and analyzed by western blotting with antibodies against Rpb1, Rpb2, and tubulin.

(B) Inhibition of HSP90 activity with GA selectively destabilizes cytoplasmic Rpb1 subunit. U2OS cells were treated as above and subjected to immunofluorescence against Rpb1. Each field is 83.75 × 117.25 μm.

(C) All stages of polymerase II assembly are not equally sensitive to GA. The amount of subunit Rpb1 in the cytoplasm and nucleus was quantified as follows. Subunit Rpb1 was labeled by immunofluorescence, and images of cells treated as in (B) were taken with identical microscopic settings. Background was removed, and Rpb1 signals in the nucleus and cytoplasm were normalized to the nuclear signal of Rpb1 in untreated cells. Numbers are averages of more than 80 cells; bars are the standard deviations.

(D) hSpagh stabilizes unassembled cytoplasmic Rpb1. U2OS cells were treated with the indicated siRNA for 43 hr and processed for immunofluorescence against Rpb1. Each field is 83.75 × 117.25 μm.

(E) Destabilization of Rpb1 after long-term depletion of hSpagh. U2OS cells were treated with siRNAs against hSpagh for 48 or 72 hr and analyzed by western blotting with the indicated antibodies.

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