Akt activation enhances ribosomal RNA synthesis through casein kinase II and TIF-IA

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Transcription initiation factor I (TIF-IA) plays an essential role in regulating ribosomal RNA (rRNA) synthesis by tethering RNA polymerase I (Pol I) to the rDNA promoter. We have found that activated Akt enhances rRNA synthesis through the phosphorylation of casein kinase IIα (CK2α) on a threonine residue near its N terminus. CK2 in turn phosphorylates TIF-IA, thereby increasing rDNA transcription. Activated Akt also stabilizes TIF-IA, induces its translocation to the nucleolus, and enhances its interaction with Pol I. Treatment with AZD8055, an inhibitor of both Akt and mammalian target of rapamycin phosphorylation, but not with rapamycin, disrupts Akt-mediated TIF-IA stability, translocation, and activity. These data support a model in which activated Akt enhances rRNA synthesis both by preventing TIF-IA degradation and phosphorylating CK2α, which in turn phosphorylates TIF-IA. This model provides an explanation for the ability of activated Akt to promote cell proliferation and, potentially, transformation.

PKB/Akt | acute myelogenous leukemia

The regulation of ribosomal RNA (rRNA) synthesis is central to cell proliferation and is therefore responsive to a number of signaling pathways that are, in turn, responsive to the metabolic requirements of the cell (1, 2). Among these pathways is the PI3K/Akt pathway, which also coordinates the synthesis of ribosomal proteins and rRNA. The stimulatory effect of activated Akt on rRNA synthesis was, until recently, thought to be mediated by the mammalian target of rapamycin (mTOR). However, recent data have demonstrated that inhibition of mTOR activity by rapamycin has little effect on rRNA synthesis, although sustained inhibition of both Akt and mTOR markedly suppress both rRNA synthesis and ribosome biogenesis by limiting RNA polymerase I (Pol I) loading onto DNA and by reducing pre-rRNA processing (3). These observations support the view that Akt may play a critical role in controlling ribosomal biogenesis, but the mechanism by which it does so has not been defined.

Transcription initiation factor I (TIF-IA), a mammalian homolog of yeast Rrn3p, plays a central role in the transcription of ribosomal DNA genes. TIF-IA interacts with the TBP-containing factor TIF-IB/SL1 and both are required to recruit Pol I to the rDNA promoter and generate a productive transcription initiation complex (4, 5). TIF-IA is phosphorylated at multiple sites by a variety of protein kinases, with both positive and negative effects on its ability to initiate transcription (6–8), and is ubiquitinated and degraded by the proteasome (9). TIF-IA expression is also essential to maintaining nucleolar architecture and cell viability (10). Hence, TIF-IA is a key intermediate in the overall regulation of rRNA synthesis.

Casein kinase II (CK2), a tetrameric holoenzyme composed of two catalytic subunits (α and α′) and two regulatory subunits (β), is a highly conserved protein serine/threonine kinase that also plays an important role in the regulation of proliferation. A number of interactions occur between CK2 and the PI3K/Akt pathway: CK2 phosphorylates and inhibits PTEN (phosphatase and tensin homolog deleted on chromosome 10) activity, which in turn activates Akt (11); CK2 physically associates with Akt (12) and phosphorylates it on Ser129 (13), contributing to Akt activation (14); and the CK2–Akt interaction functions in the Wnt/β-catenin signaling to promote cancer progression (15). Thus, the interaction of Akt and CK2 is well established and also plays an important role in regulating cell proliferation and cell transformation.

We document a unique role for activated Akt in stimulating TIF-IA–regulated rRNA expression by phosphorylating CK2 on a threonine residue near the N terminus, which in turn phosphorylates and activates TIF-IA. The resulting enhancement of rRNA synthesis is independent of mTOR activation. We conclude that these additional roles of activated Akt play a physiologically important role in the overall regulation of rRNA synthesis and cell proliferation.

Results

Activated Akt Enhances rRNA Synthesis. We noted that the levels of pre-rRNA synthesis in leukemic cells from patients with acute myelogenous leukemia (AML) correlate with the expression of phosphorylated Akt (p-Akt). Results from a representative experiment demonstrate that pre-rRNA expression is significantly reduced ($P < 0.01$) in cells with reduced levels of p-Akt compared with cells expressing higher levels of p-Akt (Fig. 1*A*, *Left*, and Fig. S1 *A* and *B*). Moreover, in cells with lower levels of p-Akt the interaction between Pol I and rDNA is reduced (Fig. 1*A*, *Right*, and Fig. S1 *A* and *C*). To determine whether p-Akt plays a causal role in the regulation of rRNA synthesis, we expressed Akt-myr and Akt activated by a double mutation (S473D, T308D) in 293T cells, which resulted in a four-to fivefold increase in rRNA synthesis and a six- to sevenfold increase in the binding of Pol I/TIF-IA to rDNA (Fig. 1 *B* and *C*). Similar results were obtained using two additional sets of rDNA primers (Fig. S1*E*). In contrast, a reduction in Akt expression from siRNA transfection markedly

Significance

Ribosomal proteins are synthesized in the nucleolus under the control of a number of repetitive DNA elements and are required for cell proliferation. Cancer cells frequently contain mutations that activate the phosphoinositide 3-kinase/Akt signaling pathway. This study shows that activation of Akt enhances the transcription of ribosomal genes by stabilizing a protein, transcription initiation factor I (TIF-IA), which is essential for the transcription of ribosomal DNA. Activated Akt also increases ribosomal RNA synthesis by phosphorylating casein kinase 2, which in turn phosphorylates and enhances the activity of TIF-IA. These results demonstrate new mechanisms by which the activation of Akt can promote tumor cell proliferation and further support the targeting of activated Akt as a potential therapy for certain cancers.

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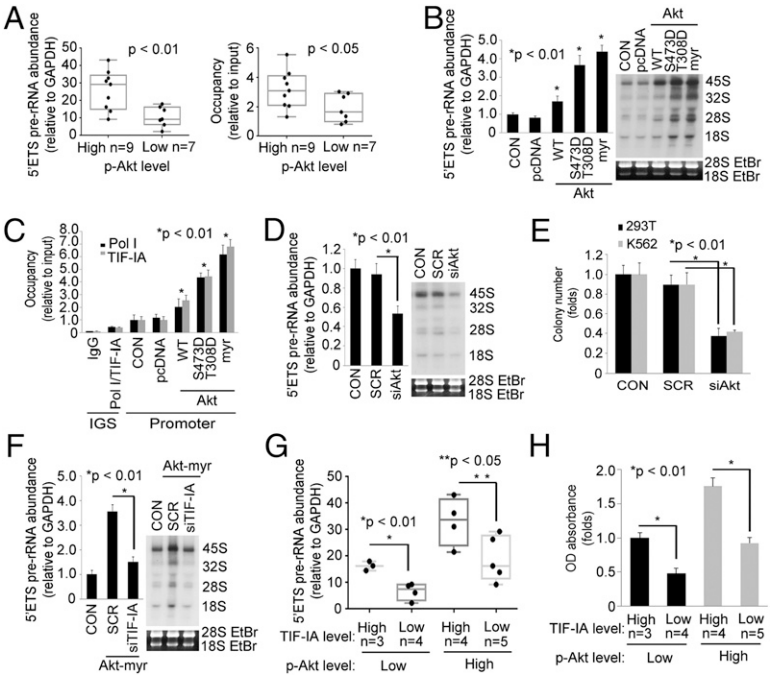

Fig. 1. Regulation of rRNA synthesis by activated Akt. (*A*) Relationship between p-Akt expression and pre-rRNA synthesis level in AML cells. RNA was extracted from 16 AML patient samples for measurement of 5′ ETS pre-rRNA (*Left* and Fig. S1*B*) and ChIP assay was performed to measure the level of Pol I recruited to rDNA promoter (*Right* and Fig. S1*C*). GAPDH (*Left*) or 10% input (*Right*) was used as the internal control and qPCR was performed in triplicate ($n = 3$). Measurements of p-Akt were carried out using densitometry on Western blots and divided into low and high expression relative to the average value for all samples (Fig. S1 *A* and *D*). The bar indicates the average value and the ends of the whiskers represent minimum and maximum values. Significance was determined using the Student *t* test. (*B* and *C*) Effect of Akt overexpression on rRNA synthesis; 293T cells were transfected with pcDNA3 vector control, Akt-WT, Akt-S473D/T308D or Akt- myr for 24 h. (*B*, *Left*) RNA was extracted for qPCR; (*Right*) RNA labeling assay with [^{32}P]. (*C*) ChIP assay with anti-Pol I or anti–TIF-IA antibodies. Values for qPCR represent the mean ± SD of triplicate determinations. (*D* and *E*) Effect of Akt depletion on rRNA synthesis and cell proliferation. (*D*) 293T cells were transfected with a mixture of siAkt1 (20 nM) and siAkt2 (20 nM) for 36 h: (*Left*) qPCR, (*Right*) [^{32}P] RNA labeling. Values shown for qPCR experiments represent the mean ± SD of triplicate determinations. ChIP assay and Western blot controls are shown in Fig. S1*F*. (*E*) 293T or K562 cells were transfected with a mixture of siAkt1 (20 nM) and siAkt2 (20 nM) for 18 h and plated in soft agar. Colonies were counted after 10 d of culture. (*F*) Effect of TIF-IA depletion on the Akt-myr enhancement of rRNA synthesis; 293T cells were transfected with siSCR or siTIF (20 nM) for 18 h, after which cells were transfected with AKT-myr and extracted for qPCR and RNA labeling at 24 h. (*G* and *H*) Correlation of TIF-IA and p-Akt expression levels with pre-rRNA synthesis and cell survival in primary AML cells. Data were analyzed as in *A*. For cell-survival experiments, the mixture of samples in each group were seeded in 96 well-plate for 48 h and a MTT assay was performed.

decreased rRNA synthesis (Fig. 1*D* and Fig. S1 *F* and *G*) and also inhibited cell survival and colony formation (Fig. 1*E* and Fig. S1*H*). To determine whether TIF-IA is an intermediary in the effects of activated Akt on rRNA synthesis, we transfected 293T cells with Akt-myr in the absence or presence of TIF-IA. Depletion of TIF-IA almost completely eliminated the effects of activated Akt on pre-rRNA synthesis and cell survival (Fig. 1*F* and Fig. S1*I*). In primary cells from patients with AML, there is a correlation between low expression of TIF-IA and reduced levels of rRNA synthesis and cell survival (Fig. 1 *G* and *H*, and Fig. S1 *A* and *D*). These data suggest that activated Akt regulates rRNA synthesis through TIF-IA.

Akt Expression Regulates Mdm2-Mediated Stability of TIF-IA. Treatment of 293T cells with MG132, an inhibitor of the 26S proteasome, increases the expression of TIF-IA (Fig. S2*A*). Mdm2 (mouse double-minute 2), a prolific E3 ubiquitin ligase, colocalizes with TIF-IA in the nucleus (Fig. S2*B*). Therefore, we asked whether Mdm2 regulates the level of TIF-IA. Indeed, overexpression of Mdm2 increases the ubiquitination of endogenous TIF-IA and reduces TIF-IA protein levels in a dose-dependent manner (Fig. 2*A*). Overexpression of Mdm2 also increases exogenous Myc–TIF-IA ubiquitination and decreases Myc–TIF-IA protein (Fig. S2*C*). However, expression of the C464A mutant of Mdm2 that lacks E3 ubiquitin ligase activity (16) has no effect on TIF-IA levels (Fig. 2*B*). This result was confirmed using Mdm2-knockout mouse embryonic fibroblast (MEF) cells. Compared with MEF WT, knockout of Mdm2 increases TIF-IA expression. Reexpression of the C464A mutant in Mdm2 knockout cells does not affect the level of TIF-IA (Fig. S2*D*, *Left*). Importantly, Nutlin-3, which inhibits the interaction between Mdm2 and p53 (17), reverses the effects of endogenous Mdm2 on TIF-IA levels (Fig. 2*C*). Endogenous TIF-IA is ubiquitinated in WT MEF cells, but not in Mdm2-knockout MEF cells or in cells expressing the C464A mutant (Fig. S2*D*, *Right*). Finally, the expression of Mdm2 in Mdm2-knockout cells induces ubiquitination of endogenous TIF-IA (Fig. S2*E*). These results confirm that Mdm2 is an E3 ligase for TIF-IA and regulates its expression.

We then asked whether activated Akt protects TIF-IA from Mdm2-induced degradation. Overexpression of Akt-myr markedly inhibits TIF-IA degradation (Fig. 2*D*). Conversely, depletion of Akt by siRNA markedly reduces TIF-IA protein expression (Fig. 2*E*) and its half-life following cycloheximide treatment (Fig. 2*F*). Moreover, reduced Akt expression enhances and overexpression of Akt inhibits the ubiquitination of both endogenous and exogenous TIF-IA (Fig. 2*G* and Fig. S2*F*). These data demonstrate that Akt regulates TIF-IA stability by preventing its ubiquitination and degradation.

Akt and CK2 Cooperate to Increase rRNA Synthesis. We confirmed that there is no direct interaction between TIF-IA and Akt and that Akt does not directly phosphorylate TIF-IA (Fig. S3 *A* and *B*). In a search for intermediaries in the Akt–TIF-IA pathway, we noted that CK2 holoenzyme interacts with and phosphorylates Akt at Ser129. Previous reports have also shown that CK2 phosphorylates TIF-IA at Serine 170/172, leading to its dissociation from Pol I (18). Reduced levels of CK2 decrease rRNA synthesis activity in 293T cells (Fig. S3*C*), as does inhibition of CK2 activity using CK2 inhibitor I (Fig. S3*D*). Coexpression of CK2 and Akt-myr robustly enhances rRNA synthesis compared with overexpression of either construct alone (Fig. 3*A* and Fig. S3*E*), whereas depletion of CK2 from Akt or Akt-myr–transfected cells decreases rRNA synthesis by ∼50% (Fig. S3*F*). Overexpression of CK2 in control or in Akt-depleted 293T cells (Fig. S3*G*) demonstrates that activation by CK2 is dependent on Akt expression. These experiments support a role for the Akt/CK2 interaction in enhancing rRNA synthesis and suggest the possibility that TIF-IA is an essential downstream component of Akt/CK2 regulation of rRNA synthesis.

Phosphorylation of CK2 by Akt Regulates rRNA Synthesis. To determine the effects of Akt activation on CK2, 293T cells were transfected with Akt or Akt-myr and their binding with GST-labeled CK2 protein was determined using a GST pull-down assay. Both Akt-WT and myr-Akt coprecipitate with CK2 (Fig. 3*B*). A single consensus phosphorylation site for Akt on CK2α surrounds threonine 13 (RARVY-T) (Fig. S4*A*). We examined the in vitro kinase activity of Akt using a series of recombinant

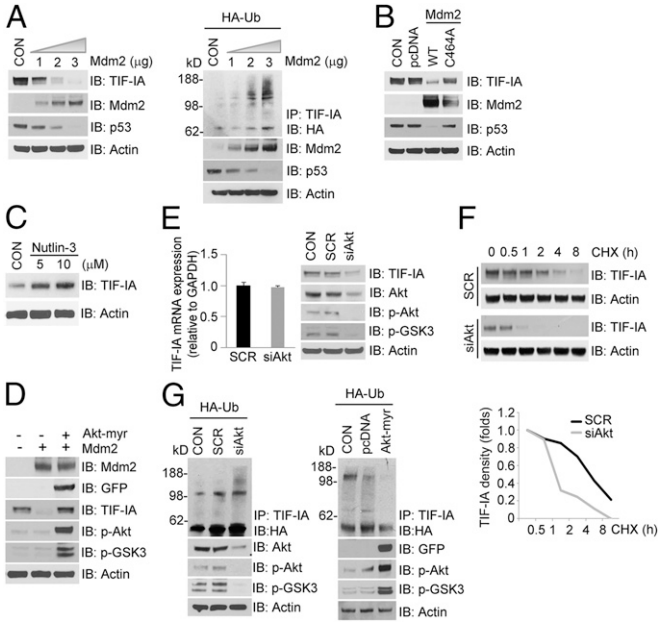

Fig. 2. Effect of Akt on TIF-IA stability. (*A*) Effect of Mdm2 overexpression on TIF-IA expression and ubiquitination; 293T cells were transfected with the indicated construct encoding Mdm2 (*Left*) or cotransfected with HA-Ub (*Right*) for 24 h. Western blot and ubiquitination assays were performed. (*B*) Effect of expression of Mdm2 WT and catalytically inactive Mdm2 C464A on TIF-IA expression; 293T cells were transfected constructs for 24 h before Western blot analysis. (*C*) Effect of Nutlin-3 on TIF-IA expression. 293T cells were treated with the indicated concentrations of Nutlin-3 for 24 h. (*D*) Effect of Akt-myr on Mdm2-induced TIF-IA degradation. (*E*) Effect of Akt depletion on TIF-IA mRNA and protein levels; 293T cells were transfected with a mixture of siAkt1 (20 nM) and siAkt2 (20 nM) for 36 h. (*F*) Effect of Akt depletion on TIF-IA stability; 293T cells were transfected with siSCR or siAkt for 48 h. The cells were then treated with CHX (10 μM) for the indicated times: (*Upper*) Western blot and (*Lower*) TIF-IA density measurements. (*G*) Effect of Akt depletion and Akt overexpression on endogenous TIF-IA ubiquitination; 293T cells were cotransfected with siSCR or siAkt and HA-Ub (*Left*) or with pcDNA vector or Akt-myr and HA-Ub (*Right*) for 24 h. The cells were then treated with MG132 (10 μM) for an additional 10 h.

GST-CK2α proteins mutated at T13 and at S338 as a control (Fig. S4*B*). Akt strongly phosphorylates CK2α in relation to a known positive control substrate, glycogen synthesis kinase (GSK3) (Fig. 3*C*). Phosphorylation was markedly diminished in kinase assays carried out with all T13 mutations of CK2α, but not with the control S338 mutation (Fig. 3*D*).

We next generated a pRS3-CK2-T13D mutant that mimics CK2α phosphorylation and a pRS3-CK2-T13A mutant that cannot be phosphorylated by Akt. Overexpression of CK2-T13D robustly enhances TIF-IA and Pol I recruitment to rDNA as well as rRNA synthesis, whereas CK2-T13A markedly diminished these effects (Fig. 3*E*). These data support the conclusion that phosphorylation of CK2α at T13 by activated Akt is an important mediator of rRNA synthesis.

CK2-Akt Regulates rRNA Synthesis Through TIF-IA. Decreasing endogenous TIF-IA expression in Akt-myr and CK2 cotransfected cells (Fig. 4*A*) abolishes the stimulation of rRNA synthesis by Akt/CK2 and disrupts the effect of CK2-T13D on rRNA synthesis (Fig. 4*B*). These results suggest that TIF-IA is an essential factor in CK2-Akt–regulated rRNA synthesis. Studies by Bierhoff et al. (18) have shown that CK2 phosphorylates TIF-IA on S170 and S172. As shown in Fig. 4 *C* and *D*, introducing TIF-IA–containing mutations at S170 and S172 into cells in the absence of endogenous TIF-IA expression substantially reduces the ability of CK2-T13D to increase Pol I/TIF-IA binding to rDNA and rRNA synthesis.

Effect of Akt on TIF-IA Localization and Activity. Immunostaining reveals that overexpression of Akt-myr induces TIF-IA translocation from the nucleus to the nucleolus and its colocalization with Pol I at the site of rRNA synthesis (Fig. 5*A*). Additional 2D and 3D images are shown in Fig. S5 *A*–*C*. To determine whether activated Akt regulates TIF-IA activity through this translocation, we show that GFP-AKT-myr expression increases the interaction of both endogenous and exogenous TIF-IA and Pol I (Fig. 5*B*). In the presence of Akt-myr, overexpression of TIF-IA enhances Pol I/TIF-IA binding to rDNA (Fig. 5*C*). Akt-myr also enhances rRNA synthesis when TIF-IA is introduced (Fig. 5*D*). Furthermore, nucleolar translocation separates TIF-IA from Mdm2, providing a potential explanation for the decrease in its ubiquitination resulting from Akt-myr expression (Fig. 5*E* and Fig. S5*D*).

Akt and CK2 Regulate rRNA Synthesis Independent of mTOR Signaling. Treatment with rapamycin, an inhibitor of mTORC1 activity, did not abolish rRNA synthesis, whereas a knockdown of Akt using siRNA was far more effective. We therefore compared the effects of AZD8055, an inhibitor of both Akt phosphorylation and mTORC activity (19, 20), with those of rapamycin. AZD8055 strongly reduces the expression of both p-P70 (S6K) and p-Akt, whereas rapamycin only reduces p-P70(S6K). AZD8055 treatment also reduces rRNA synthesis in 293T cells to a far greater extent than does rapamycin (Fig. 6*A* and Fig. S6*A*, *Left*). In addition, AZD8055 treatment markedly reduces the enhancement of rRNA synthesis mediated by Akt-CK2 but rapamycin has less effect (Fig. 6*B* and Fig. S6*A*, *Right*). Finally, AZD8055 more potently inhibits cell survival (Fig. S6*B*) and depletion of Akt with siRNA more strongly inhibits both pre-rRNA synthesis and cell survival than does depletion of mTOR (Fig. S6*C*).

AZD8055 treatment also results in the dissociation of Akt and CK2 in pull-down experiments (Fig. 6*C*, *Right*) and in in vitro experiments using cell lysate (Fig. 6*C*, *Left*) and inhibits the effect of CK2-WT on rRNA synthesis to a greater extent than that of CK2-T13D (Fig. 6*D*). Thus, AZD8055 inhibits the interaction of activated Akt with CK2 and abolishes the enhancement of rRNA synthesis through CK2 while also inhibiting TIF-IA translocation to the nucleolus induced by Akt-myr (Fig. 6*E* and Fig. S6*E*; 2D images are shown in Fig. S6*D*). AZD8055 also reverses the effects of Akt on protein stability while increasing the ubiquitination of both endogenous and expressed TIF-IA (Fig. 6*F*, *Left*, and Fig. S6*F*), and disrupting the protective effect of Akt-myr on TIF-IA ubiquitination (Fig. 6*F*, *Right*). In primary leukemic cells, AZD8055 concomitantly decreases p-Akt, pre-rRNA synthesis, and cell survival (Fig. 6*G* and Fig. S7 *A* and *B*). The addition of the Pol I inhibitor, CX-5461, did not enhance the inhibition of pre-RNA synthesis (Fig. S7 *C* and *D*).

Discussion

Mdm2, originally identified as the p53 ubiquitin E3 ligase (21), has a number of other substrates, including PSD-95, the insulin-like growth factor receptor, the androgen receptor, arrestin, p300, FOXO3a, and GRK2. We now present strong evidence that TIF-IA is a substrate for Mdm2-mediated ubiquitination. This evidence includes the efficacy of transfected Mdm2 in reducing the level of TIF-IA protein; the increase in TIF-IA protein expression with the Mdm2 inhibitor, nutlin3; the increase in TIF-IA expression in Mdm2 knockout MEF cells compared with WT; and the increase in TIF-IA expression in cells treated with the proteasome inhibitor MG132.

To define the upstream signaling pathways that regulate the stability and activity of TIF-IA, we examined the role of Akt activation. Akt has been shown to regulate ribosome biogenesis at multiple levels and to interact with both mTORC1 and c-Myc to stimulate 5′ ETS (external transcribed sequence) pre-rRNA transcription (3). Although a number of studies have attested to

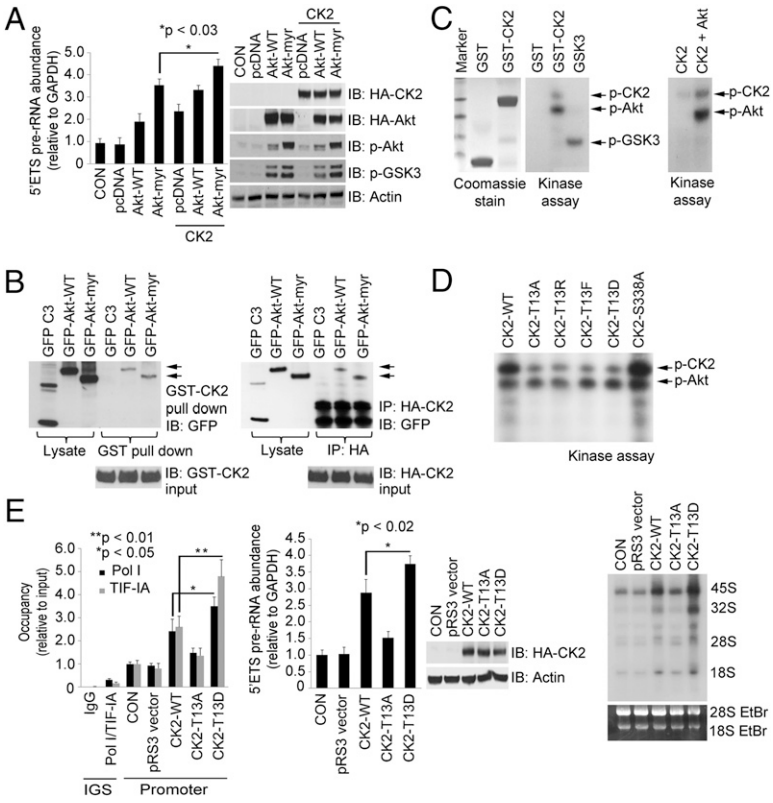

Fig. 3. Akt interacts with and phosphorylates CK2α to enhance rRNA synthesis. (*A*) Effects of activated Akt and CK2 expression on rRNA synthesis; 293T cells were cotransfected with vector control or pRS3-CK2 and the Akt constructs indicated. RNA and protein were extracted at 24 h for Q-PCR (*Left*) and Western blot (*Right*). Values shown for all qPCR experiments represent the mean ± SD of triplicate determinations (n = 3). (*B*, *Left*) Interaction of Akt with CK2 in cell lysate; 293T cells were transfected with GFP vector, GFP-AKT-WT, or GFP-AKT-myr for 24 h. Next, 500 μg of cell lysate were incubated with 1 μg recombinant GST- CK2α for 2 h at 4 °C, immunoprecipitated using GST beads, and a Western blot probed with anti-GFP antibody. (*Right*) Coimmunoprecipitation of CK2 and Akt; 293T cells were cotransfected with HA-CK2α and vector control, GFP-Akt-WT, or GFP-Akt-myr for 24 h. CK2 was immunoprecipitated using anti-HA antibody and the Western blot was immunoblotted with anti-GFP antibody. (*C*) Phosphorylation of CK2α by Akt. Next, 2 μg recombinant GST or GST-CK2α protein were incubated with 0.5 μg recombinant active Akt, [^{32}P]ATP, and 50 μL kinase buffer, as described in *Materials and Methods*. (*Left*) Coomassie stain of recombinant proteins; (*Center*) kinase assay using GSK3 as a positive control; (*Right*) autoradiogram of kinase assay of CK2α alone or with activated Akt. (*D*) Akt phosphorylates CK2α at threonine 13. CK2-T13 and CK2-S338 were mutated as described and the corresponding recombinant proteins purified. Next, 2 μg of each protein were used as substrate in the kinase assay. Results of autoradiography are shown. (*E*) CK2-T13D enhances rRNA synthesis whereas CK2-T13A has minimal effect; 293T cells were transfected with CK2-WT, CK2-T13D, or CK2-T13A for 24 h. ChIP assay was carried out with anti-Pol I or anti–TIF-IA antibodies (*Left*) and measurement of 5′ ETS pre-rRNA or [^{32}P] labeling (*Center* and *Right*) are shown. Values shown for all qPCR experiments represent the mean ± SD of triplicate determinations (n = 3).

the role of the mTOR pathway as the major link between nutrient availability, cell growth, and rRNA synthesis (22–24), recent work has revealed that inhibition of mTORC1 by rapamycin does not ablate rRNA synthesis, whereas inhibition of Akt results in a much more pronounced decrease in 5′ ETS pre-RNA levels (3). Our data support the central role of activated Akt in markedly enhancing the effects of TIF-IA on pre-rRNA synthesis. Moreover, the data provide definitive evidence that activated Akt prevents Mdm2-induced ubiquitination of TIF-IA and facilitates the translocation of nuclear TIF-IA to the nucleolus. Thus, the more-specific role of activated Akt in enhancing pre-rRNA synthesis is mediated, at least in part, by its ability to stabilize TIF-IA and to increase the localization of TIF-IA to the region of active rDNA transcription in the nucleolus.

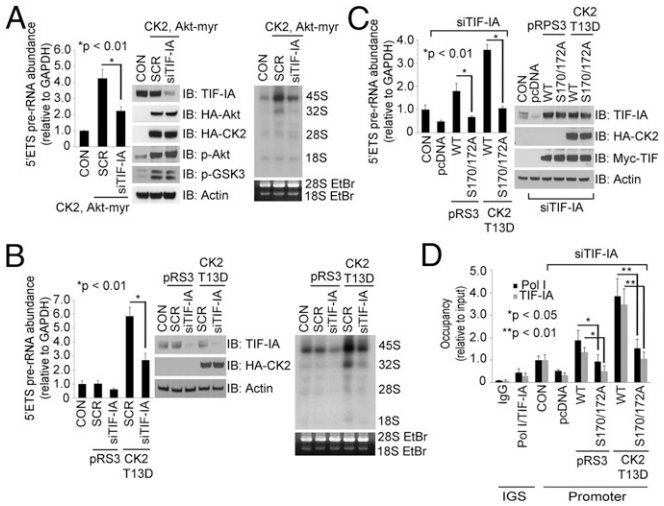

Fig. 4. Effect of TIF-IA on Akt-CK2–regulated rRNA synthesis. (*A*) Effect of TIF-IA depletion on the Akt-CK2 enhancement of rRNA synthesis. 293T cells were transfected with siSCR or siTIF-IA (20 nM) for 18 h, following which the cells were cotransfected with AKT-myr and CK2. (*B*) Depletion of TIF-IA disrupts CK2-T13D–mediated enhancement of rRNA synthesis. 293T cells were transfected with siSCR or siTIF-IA for 18 h and cells were continuously transfected with vector control or CK2-T13D for an additional 24 h. (*C* and *D*) Effects of CK2-T13D on TIF-IA S170/S172A mutant. 293T cells were transfected with siTIF-IA for 18 h and cells were continuously cotransfected with CK2-T13D and TIF-IA (WT or S170/172A mutant) for 24 h. The 5′ ETS pre-rRNA measurement (*C*) and ChIP assay with anti-Pol I or anti–TIF-IA antibodies (*D*) were performed.

In searching for additional components that cooperate in the regulation of this pathway, we investigated the role of CK2 as a ubiquitous protein kinase that has a multiplicity of effects on cell proliferation and survival. CK2 resides in the nucleolus, as well as in the nucleus and cytoplasm, regulates a large number of nucleolar proteins (25) and plays a major role in nucleolar compartmentalization (26). CK2 also regulates the transcriptional activities of all three RNA polymerases (25, 27–29). In the case of Pol I, CK2 has been found in the rRNA promoter region by ChIP assay and coimmunoprecipitates with the Pol I transcription initiation complex (30, 31). CK2 also physically interacts with TIF-IA and phosphorylates serine residues 170 and 172 (18). This phosphorylation step appears to be essential for the dissociation of TIF-IA from Pol I, and the subsequent dephosphorylation of TIF-IA by the phosphatase FCP1 enables reassociation of the proteins and facilitates ongoing Pol I transcription (18). Thus, it is the dephosphorylated form of TIF that is responsible for Pol I binding. In the present study, we confirm a direct physical interaction between Akt and CK2 in 293T cells and demonstrate that CK2 is essential for the Akt-mediated stimulation of rRNA synthesis. In addition, we show that the phosphorylation of CK2α by Akt on threonine 13 is an important component of this effect. Moreover, phosphorylation by CK2α at the S170 and 172 sites on TIF-IA is required for this stimulation.

Although CK2 is able to phosphorylate and activate Akt, the reverse has not been demonstrated, to our knowledge. Although

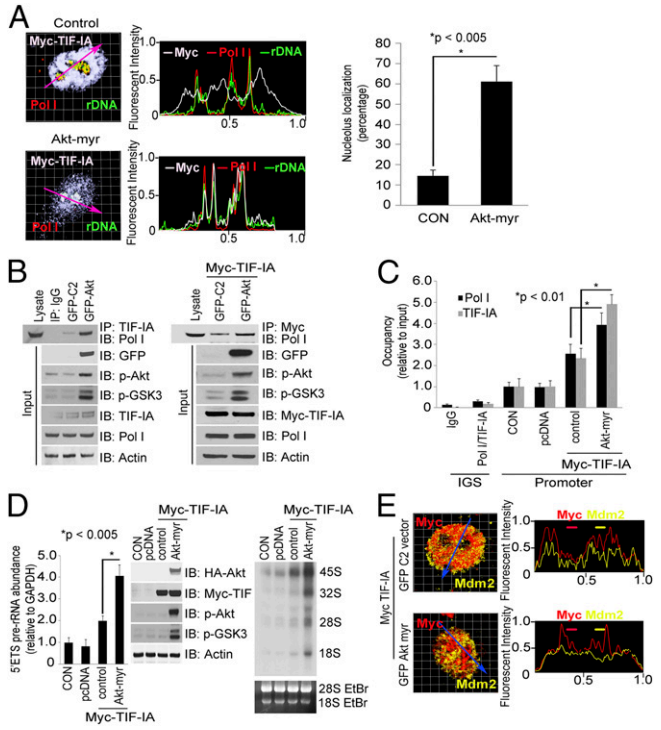

Fig. 5. Effect of Akt on TIF-IA localization and rRNA synthesis. (*A*) Effects of Akt-myr on the localization of TIF-IA with Pol I; 293T cells were cotransfected with Myc-TIF-IA in the presence of vector control or Akt-myr for 18 h. Cells were stained with anti-Myc, anti-Pol I antibodies and rDNA by immunofloruesence-FISH assay. Three-dimensional images are visualized for Myc, Pol I, and rDNA using confocal immunofluorescence microscopy. Fluorescent intensity was measured across the lines shown and fluorescence intensity plots along the lines for each panel shown on the left. The percentage of cells ($n = 200$) that have nucleolar translocation of TIF-IA are shown (*Right*). (*B*) Effect of Akt-myr on TIF-IA interaction with Pol I. 293T cells were transfected with vector control or GFP-Akt-myr (*Left*) or cotransfected with Myc-TIF-IA and vector or GFP-Akt-myr (*Right*) for 24 h. TIF-IA or Myc–TIF-IA was immunoprecipitated using anti–TIF-IA or anti-myc antibody and immunoblotted with anti-Pol I antibody. (*C*) Effect of Akt-myr on the binding of TIF-IA or Pol I to rDNA; 293T cells were transfected with Myc-TIF-IA in the presence of vector control or Akt-myr for 24 h. ChIP assay was performed as described in *Materials and Methods* with anti–TIF-IA or anti-Pol I antibody. (*D*) Effects of coexpression of AKT-myr and TIF-IA on rRNA synthesis; 293T cells were cotransfected with TIF-IA without or with Akt-Myr for 24 h. Values for qPCR represent the mean ± SD of triplicate determinations ($n = 3$). (*E*) Effects of Akt-myr on the localization of TIF-IA with Mdm2; 293T cells were cotransfected with Myc–TIF-IA in the presence of GFP-vector control or GFP-Akt-myr for 18 h. (*Left*) Myc and Mdm2-stained images. Fluorescent intensity was measured across the lines shown. (*Right*) Fluorescence intensity plots along the lines for each panel shown on the left.

the kinase activity of the CK2 holoenzyme is thought to be largely constitutive, there is structural evidence that the N terminus of the CK2α subunit is in contact with the "activation loop" of the protein and may function in a regulatory manner similar to the β-regulatory subunit (31, 32). In addition, post-translational modifications of CK2 are known to affect its interaction with a variety of other proteins (25, 32). In summary, our data support a requirement for the physical association of Akt with CK2 and the "activation" of CK2α through T13 phosphorylation as an upstream event that potentiates TIF-IA–mediated rRNA transcription.

Alterations in the PI3K/Akt axis are known to be both associated with and causal of oncogenesis (33, 34). Although cellular transformation has been most frequently related to the dysregulation of protein translation as a consequence of mTOR activation (35, 36), the role of Akt in enhancing ribosomal biogenesis is receiving increasing attention (3, 37). The up-regulation of rRNA synthesis is a prerequisite for all tumors and presents a unique opportunity for therapeutic intervention (38). This observation is particularly applicable to hematologic malignancies, such as acute

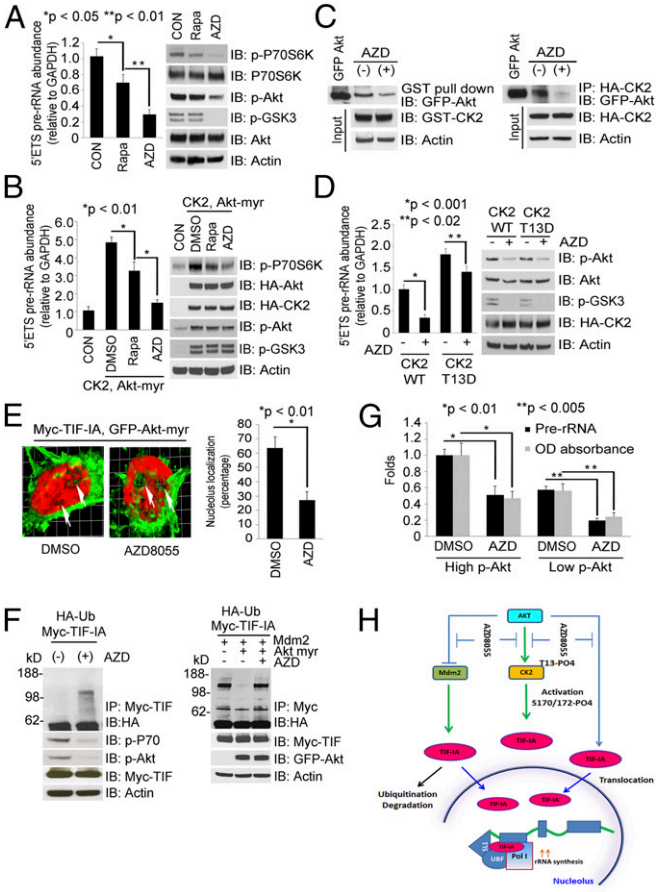

Fig. 6. AKT/CK2/TIF-IA signaling regulates rRNA synthesis independent of the mTOR pathway. (*A*) Effect of AZD8055 or rapamycin on rRNA synthesis; 293T cells were treated with 20 nM rapamycin or 20 nM AZD8055 for 3 h. (*B*) Relative effects of AZD8055 and rapamycin on AKT-CK2–mediated rRNA synthesis; 293T cells were cotransfected with CK2 and Akt for 24 h and treated with rapamycin or AZD8055 for 3 additional hours. (*C*) Effect of AZD8055 on Akt-CK2 interaction; 293T cells were transfected with GFP-Akt or cotransfected with GFP-Akt and HA-CK2. (*Left*) Five-hundred micrograms of cell lysate were incubated with 1 μg recombinant GST-CK2, immunoprecipitated using GST beads, and a Western blot probed with anti-GFP antibody. (*Right*) Coimmunoprecipitation of CK2 and Akt using anti-HA antibody and the Western blot was immunoblotted with anti-GFP antibody. (*D*) Lack of effect of AZD8055 on CK2-T13D-enhanced rRNA synthesis. 293T cells were transfected with CK2-WT or CK2-T13D for 24 h and the cells were treated with AZD8055 for 3 h. (*E*) Effect of AZD8055 on Akt-mediated translocation of TIF-IA; 293T cells were cotransfected with GFP-Akt-myr and Myc-TIF-IA for 24 h, treated with AZD8055 or no drug, and stained with anti-Myc antibody and DAPI. The white arrow shows the localization of TIF-IA in the nucleolus. Percentage of cells which have TIF-IA translocation to nucleolus was shown as bar graph (*Right*). (*F*, *Left*) Effects of AZD8055 on the ubiquitination of TIF-IA. (*Right*) Prevention of Mdm2-mediated ubiquitination of TIF-IA by Akt and reversal by AZD8055; 293T cells were transfected as indicated and treated with AZD8055 or no drug. The ubiquitination assay was performed using anti-Myc and anti-HA antibodies. (*G*) Effect of AZD8055 on pre-rRNA synthesis and cell survival in AML patient samples. Groups of AML cells ($n = 5$ each) expressing low or high levels of pAkt were cultured and treated with DMSO or AZD8055 for 3 h (for qPCR) and for 48 h (for MTT). qPCR and MTT assays were performed as shown. Western blot of p-Akt level is shown in Fig. S7*A*. (*H*) Schematic model of the regulation of TIF-IA activity by Akt.

leukemia, where constitutive activation of the PI3K/Akt axis is associated with a poorer survival (39, 40). The role of activated Akt in stabilizing the TIF-IA protein provides a potential mechanism for this observation and may explain the elevation in rRNA synthesis observed in these cells. Although the regulation of rRNA synthesis remains complex and the target of multiple regulatory pathways, it is apparent that activated Akt plays a major role both through its effects on TIF-IA protein turnover and its phosphorylation of CK2α (Fig. 6*H*), and therefore remains an important therapeutic target.

Materials and Methods

RNA Isolation and Quantitative RT-PCR. Total cellular RNA was isolated using the RNAeasy Plus mini kit (Qiagen). For quantitative RT-PCR (qRT-PCR), reactions were performed in triplicate with primers specific for 5′ ETS pre-rRNA or TIF-IA (target sequences for siRNAs are shown in Table S1; specific primer sequences in Table S2). qRT-PCR was carried out on a 7900T Fast real-time PCR system (Applied Biosystems).

Akt Kinase Assay. Two micrograms of CK2α or GST-purified protein were incubated with recombinant active Akt (Millipore) and 10 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences) in 50 μL of kinase buffer. Reactions were incubated at 30 °C for 1 h. Proteins were separated on NuPAGE 4–12% gradient gels (Invitrogen) and phosphorylated proteins were visualized by autoradiography.

ChIP Assay. Precleared chromatin was incubated overnight by rotation with 4 μg of Pol I antibody or IgG antibody as a negative control. Inmunoprecipitates were resuspended in 50 μL TE buffer. Inputs and immunoprecipitated DNA samples were quantified by qPCR. qPCR was carried out on a 7900T Fast real-time PCR system (Applied Biosystems) using SYBR green as the detection fluorophore. Primers are listed in Table S2.

RNA Labeling and Analysis. The cells were washed and incubated in phosphate-free DMEM (Gibco) supplemented with 10% (vol/vol) FBS for 2 h followed by 1-h label with 0.5 mCi [^{32}P] orthophosphate (Perkin-Elmer). Total RNA was extracted with TRIzol (Life technology). RNA (10 μg) was separated on a 1.2% Mops formaldehyde gel. Gels were dried and visualized by autoradiography.

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