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Chaperonin TRiC/CCT Modulates the Folding and Activity of Leukemogenic Fusion Oncoprotein AML1-ETO*

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AML1-ETO is the most common fusion oncoprotein causing acute myeloid leukemia (AML), a disease with a 5-year survival rate of only 24%. AML1-ETO functions as a rogue transcription factor, altering the expression of genes critical for myeloid cell development and differentiation. Currently, there are no specific therapies for AML1-ETO-positive AML. While known for decades to be the translational product of a chimeric gene created by the stable chromosome translocation t(8;21)(q22;q22), it is not known how AML1-ETO achieves its native and functional conformation or whether this process can be targeted for therapeutic benefit. Here, we show that the biosynthesis and folding of the AML1-ETO protein is facilitated by interaction with the essential eukaryotic chaperonin TRiC (or CCT). We demonstrate that a folding intermediate of AML1-ETO binds to TRiC directly, mainly through its β-strand rich, DNA-binding domain (AML-(1–175)), with the assistance of HSP70. Our results suggest that TRiC contributes to AML1-ETO proteostasis through specific interactions between the oncoprotein's DNA-binding domain, which may be targeted for therapeutic benefit.

Acute myeloid leukemia (AML)[4] is characterized by the uncontrolled proliferation and incomplete differentiation of a malignant clone of myeloid stem or progenitor cells (1). The majority of cases can be categorized based on the stable, non-random chromosome translocation they contain. The most common translocation in AML is t(8;21)(q22;q22), which juxtaposes portions of two genes, AML1 and ETO, generating the fusion oncoprotein AML1-ETO (2). The AML1 gene encodes a critical transcription factor that regulates a variety of genes involved in proliferation and differentiation of many cell types, including those within the hematopoietic system (3, 4). On the other hand, the ETO (eight twenty-one) protein is a protein harboring transcriptional repressor activities (5). The DNA-binding domain of AML1 is located within the amino-terminal portion of AML1-ETO and is fused in-frame to portions of the ETO gene containing dimerization and zinc finger motifs shown to interact with nuclear receptor co-repressors such as the N-CoR (nuclear receptor co-repressor)/SMRT (silencing mediator for retinoid and thyroid receptors) complex and HDAC (histone deacetylase) (6–8). Thus, AML1-ETO functions as a rogue transcriptional repressor, rather than as a transcriptional activator.

Unfortunately, there are currently no specific treatments for AML1-ETO-positive leukemia and the prognosis for this disease remains dismal. However, it has been shown that targeting of AML1-ETO using small interfering RNAs (siRNAs) supports normal myeloid differentiation of t(8;21)-positive leukemic cells (9), which highlights AML1-ETO as a direct clinical target to treat AML. Interestingly, inhibition of heat shock protein 70/90 (HSP70/90), two major proteostasis regulators, has shown antileukemic effects in AML1-ETO-positive cells (10, 11), suggesting that AML1-ETO might rely on chaperones to fold and function properly. However, there has been little direct evidence to support this hypothesis.

The proper folding of proteins into their native conformation in the crowded milieu of the cell is facilitated by molecular chaperones, a network of cellular proteostasis regulators (12). Chaperonins, an important class of molecular chaperones, are large, multi-subunit complexes that form stacked, double-ring structures with a central cavity in each ring (13). During a folding reaction, the rings of a chaperonin can close through large conformational changes driven by ATP hydrolysis, thereby providing an isolated environment for client proteins to fold (14, 15). TRiC (T-complex protein-1 ring complex), also known as CCT (chaperonin containing TCP-1), is an essential 1 MDa eukaryotic chaperonin composed of eight homologous but distinct subunits (CCT 1–8) (16, 17), arranged in a specific order (18). TRiC has the unique ability to fold many essential proteins that cannot be folded by simpler chaperone systems, most notably actin and tubulin. Indeed, this chaperonin plays a central role in the cytosol by assisting the folding of ~10–15% of all newly synthesized polypeptides (19). Furthermore, TRiC has

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[4] The abbreviations used are: AML, acute myeloid leukemia; ETO, eight twenty-one; N-CoR, nuclear receptor co-repressor; SMRT, silencing mediator for retinoid and thyroid receptor; HDAC, histone deacetylase; CCT, chaperonin containing TCP-1; TRiC, T-complex protein-1 ring complex; RRL, rabbit reticulocyte lysate.

been suggested to play a role in cancer cell development by modulating the folding and activity of client proteins involved in oncogenesis, such as the tumor suppressor proteins Von Hippel-Lindau (VHL) (20, 21) and p53 (22), as well as the pro-oncogenic protein STAT3 (signal transducer and activator of transcription 3) (23). Of note, the expression levels of TRiC in leukemic cells are higher than in normal hematopoietic cells (24), suggesting a potential contribution of TRiC to leukemogenesis through its interactions with leukemia-causing oncoproteins.

We demonstrate in this report that the leukemogenic fusion oncoprotein AML1-ETO is a novel TRiC client. Its folding is facilitated by direct interaction with TRiC, mainly through its β-strand rich, DNA-binding domain (AML-(1–175)), and this interaction is assisted by HSP70.

Experimental Procedures

Plasmids—All of the AML1-ETO domain sequences described here were subcloned from pCDNA3-flag-AML1-ETO (Addgene) into the respective vector described in each section.

Stable AML1-ETO Inducible Human Embryonic Kidney (HEK293) Cells—The pEF1a-Tet3G tetracycline-responsive mammalian expression vector was obtained from Clontech. AML1-ETO with N terminus 3×Flag-tags was cloned into this vector using SalI and NdeI restriction sites. pEF1a-Tet3G AML1-ETO plasmid was transfected by Xfect Transfection Reagents (Clonetech) into HEK293 tet-on 3G (Clontech) that was maintained and selected at 37 °C in 5% CO_2 in DMEM with 10% fetal bovine serum, 1% penicillin/streptomycin, glutamax, and 10 nM G418. After 2 days incubation, puromycin was subsequently added to the medium at a final concentration of 10 nM. After 2 weeks, viable colonies were picked and confirmed for protein expression by immunoblotting with anti-Flag (M2 sigma) 24 h after adding doxycycline to the culture media at a concentration of 10 nM. We also confirmed the expression of protein in cytoplasmic and nuclear fractions, separated using CelLytic™ NuCLEAR™ Extraction Kit (Sigma-Aldrich).

Protein Purifications: Bovine and Human TRiC—Bovine TRiC was purified from bovine testes as previously described (25). To achieve extra purity, TRiC fractions were incubated with 1 mM ATP for 15 min at 37 °C and then reprocessed by Mono-Q HR 16/10 (GE Healthcare) and Superose 6 10/300 GL columns (GE Healthcare) in sequence. TRiC's folding activity was assessed by luciferase refolding as previously described (26). Human TRiC was purified from HELA cells in a similar way to bovine TRiC, except for cell lysis, which was performed as previously described (27).

Full-length AML1-ETO and DNA Binding Domain (DBD, AML-(1–175))—The coding sequence of human full-length AML1-ETO was subcloned with a C-terminal 6His-tag in pET28 (Novagen) expression vector and the domain sequence corresponding to 1–175 AML1-ETO was inserted with an N-terminal flag tag into pET-His-2HR-T(Addgene) expression vector. Each plasmid was delivered to Rosetta DE3 (EMD Bioscience) cultured at 37 °C. The cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an optical density of 0.7 at 600 nm. After growing for 4 h at 37 °C, the cells were harvested, resuspended in buffer at pH 8.0 containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1% Triton X-100, and 100 μg/ml lysozyme, and lysed by sonication. The insoluble fraction containing either AML1-ETO or AML-(1–175) as inclusion bodies was collected by centrifugation. The harvested pellet was washed five times through cycles of sonication and centrifugation in resuspending buffer. The washed pellet was solubilized in buffer A at pH 8.0 (1× PBS, 100 mM NaCl, 5 mM imidazole, 10% glycerol, and 6 M guanidinium hydrochloride (Gn-HCl)). After centrifugation, the supernatant was applied onto a Ni-NTA column (GE his-trap HP) equilibrated in the same buffer. The column was washed successively with buffer A, and proteins were eluted from the column with buffer B (buffer A with 300 mM imidazole). The proteins were concentrated proteins using a centrifugal concentrator (Milipore) and were then applied to Superose 6(GE) pre-equilibrated with buffer 1× PBS, at pH 8.0 (100 mM NaCl, 10% glycerol, and 6 M guanidinium hydrochloride (Gn-HCl)). Corresponding AML1-ETO or AML-(1–175) fractions were concentrated and stored at −80 °C until further use.

TRiC Immunodepletion in RRLs—Rat monoclonal anti-CCT1 antibody (91a, Santa Cruz) or rabbit IgG control antibody (Abcam; 6 μg) were added to rabbit reticulocyte lysates and incubated at 4 °C for 4 h. The immune complexes were bound by protein A-agarose and removed by centrifugation. This process was repeated twice.

In Vitro Transcription and Translation—Each sequence encoding either full-length AML1-ETO or truncations corresponding to individual domains (1∼175, 176∼364, 365∼490, 491∼615 or 616∼752) was amplified by PCR and subcloned into pEntr/D-topo (Invitrogen) Gateway entry vector by Topo cloning and subsequently recombined with pDest17 expression vector (Invitrogen) by recombinase cloning. The forward primer of 176–364 has two extra methionines to compensate for the number of methionines labeled by ^{35}S. pCMV6-β-Actin (Origene), pet15-STAT3 (23), and pCMV6-AML1a (Origene) were included as controls. *In vitro* transcription and translation reactions were carried out using [^{35}S]methionine and a rabbit reticulocyte lysate (RRL) system (TNT T7 Quick-Coupled Transcription/Translation System; Promega, Madison, WI) with the constructs containing the insert of full-length or domains of AML1-ETO according to the manufacturer's instruction for 30 min at 30 °C, and terminated by the addition of 2 mM puromycin, 5 mM EDTA, and 1 mM azide. Protein translation was confirmed by SDS-PAGE followed by autoradiography.

Immunoprecipitation-In RRLs—10 μl of each RRL reaction containing a ^{35}S labeled protein of interest was diluted to 100 μl with buffer (25 mM Tris, pH 7.5, 10% glycerol, 5 mM ETDA, 100 mM NaCl, 1 mM azide) and treated with antibodies to TRiC (mixture of rabbit anti-CCT2 and anti-CCT5) or rabbit anti-human IgG control antibody (Abcam, Cambridge, MA) for 1 h on ice. The mixture was incubated with 30 μl of protein A-Sepharose (Dynabead, Invitrogen) and gently agitated in a cold room for 1 h. The immune complexes were pelleted by magnet (Invitrogen) and then washed 6 times with buffer containing 0.5% Triton X-100. The protein samples were separated by SDS-PAGE and visualized by autoradiography; In HEK293

cells: ~2×10^6 HEK293-AML1-ETO cells were seeded on 100 mm plates and induced with 1 nM doxycycline when the cells reached ~80% confluence. After 24 h of incubation, the cells were detached by pipetting and harvested by centrifugation. Cell lysate was prepared using a Dounce homogenizer. Other materials and procedures were identical to those for RRLs described above. The precipitated immune complexes were separated by SDS-PAGE and blotted with different antibodies depending on the protein of interest; Flag (M2, Sigma), HSC/HSP70 (Santa Cruz), HSP90 (Santa Cruz), beta actin (Sigma) and CCT1 (91a, Santa Cruz); Of purified proteins: Purified human TRiC was diluted to 0.1 μM with MQA-based buffer (MQA 10% glycerol, 1% PEG8000). Purified HSP70 (Abcam) was added to TRiC buffer to yield a 1:1 molar ratio of HSP70 to TRiC. The TRiC-HSP70 mixture was pre-warmed at 30 °C in a water bath for 30 min and then full-length or 1–175 of human AML1-ETO denatured in 6 M Gn-HCl was diluted 100-fold into 0.1 μM purified TRiC with and without HSP70, resulting in a 5:1 molar ratio of substrate to TRiC. The binding mixture was incubated at 30 °C for 30 min and the insoluble fraction was removed by centrifugation. Only the supernatant was used for immunoprecipitation experiments following the same protocol as for RRLs as described above. The precipitated immune complexes were separated by SDS-PAGE and blotted using appropriate antibodies against proteins of interest as described above.

Pull-down and Mass-spectroscopy Analysis—Stable His-StrepII-AML1-ETO expression in HEK293 was prepared as described above Protein complex purification and mass spectrometry analyses were performed as previously described (28). The sample was digested while still bound to the Ni-NTA resin, by treating with 8 M urea, 5 mM TCEP in 100 mM ammonium bicarbonate (15 min at 50 °C), followed by the addition of 10 mM iodoacetamide (30 min at room temperature). The samples was diluted to 2 M urea, 50 mM ammonium bicarbonate, and digested with 1 μg of trypsin (Promega) (overnight at 37 °C). The supernatant was adjusted to 0.5% TFA and desalted online using a C_{18} peptide macrotrap column (Michrom Bioresources). The sample was fractionated by size exclusion chromatography as previously described to enrich for crosslinked peptides (29). Fractions were analyzed on an Orbitrap Velos mass spectrometer (Thermo Scientific). Precursor ions were measured in the Orbitrap at 30,000 resolution. The six most intense precursor ions were selected for HCD dissociation, and the product ions were measured in the Orbitrap analyzer at 7,500 resolution. Peaklists were searched using Protein Prospector against the 2013.06.27 version of the human SwissProt database (540546 entries) with the sequence of the tagged protein construct appended.

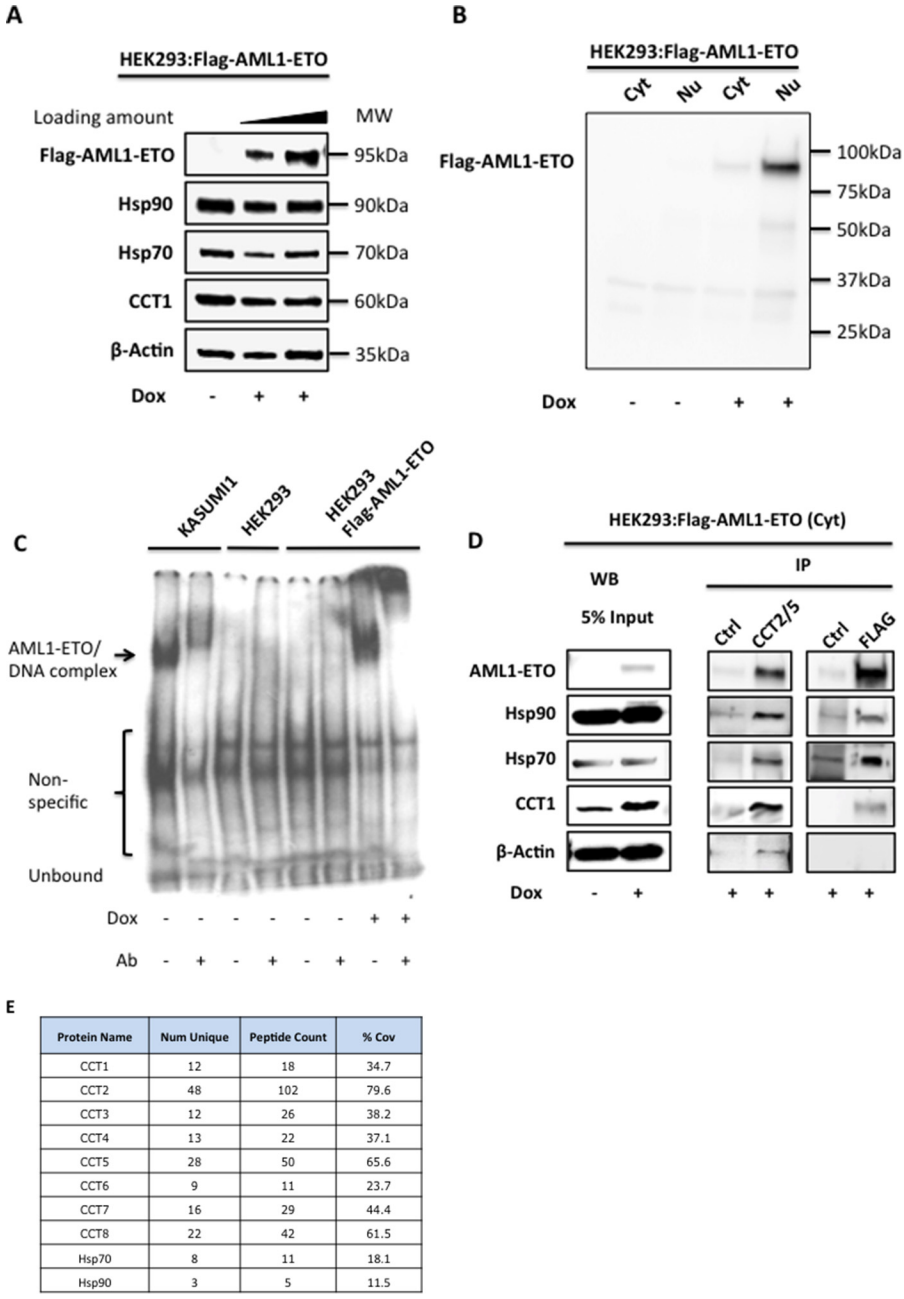
Gel-shift DNA Binding Assays—Gel-shift assays were performed as described above using 10 μg of whole cell extract of AML1-ETO from induced and uninduced HEK293 cells as well as from KASUMI-1 cells (NIH) as a positive control (30). Briefly, cell extracts were incubated with ^{32}P-labeled duplex oligonucleotide AML1 binding elements from RUNX3 for 30 min at 37 °C temperature and then in ice for 30 min in binding buffer (20 mm HEPES, pH 7.9, 0.5 mM EDTA, 2 mm DTT, 10% glycerol, 50 mm KCl, 1 μg of poly (dI-dC) (Thermo pierce)). Supershift experiments were performed using 1 μg of antibody against the N-terminal AML1 (Cell Signaling) or the C-terminal of ETO (Santa Cruz). Protein/DNA complexes were separated on 4% (or 5%) polyacrylamide gels equilibrated in 0.5× TBE. Gels were dried and analyzed by autoradiography.

In Vitro Protein Refolding—Chemically denatured full-length AML1-ETO in 6 M GnHCl was rapidly diluted 100 fold into refolding buffer (25 mM HEPES at pH 7.5, 100 mM KOAc, 10 mM $Mg(OAc)_2$, 2 mM DTT, 10 mM creatine phosphate, 200 mM NaCl, 10% glycerol), containing TRiC, HSP70, and/or 1 mM ATP resulting in a 5:1 molar ratio of substrate to TRiC. The mixture was incubated at 30 °C for 1 h and examined by gel shift DNA binding assay as described above.

Results

AML1-ETO Binds to TRiC Directly and Their Interaction Is Facilitated by HSP70—To begin examining the interaction of AML1-ETO with TRiC in human cells, we engineered the human embryonic kidney cell line HEK293 to allow for inducible expression of AML1-ETO in response to doxycycline. AML1-ETO induction did not affect cell viability, nor did it change the levels of actin, HSP70, HSP90, or TRiC proteins (Fig. 1*A*). In our cell line, as previously reported for HEK293 cells (7), AML1-ETO also localizes predominantly to the nucleolus in our cell line (Fig. 1*B*) and is biologically active in terms of DNA binding (Fig. 1*C*) (31). We first investigated the interaction between TRiC and AML1-ETO by immunoprecipitation using the cytoplasmic fraction of doxycycline-induced HEK293 cells. Immunoblotting of anti-CCT2/CCT5 immunoprecipitates demonstrated the presence of AML1-ETO, as well as actin and HSP70/90, known to interact with TRiC (27, 32, 33). In a reciprocal immunoprecipitation experiment using anti-FLAG-antibody, TRiC co-precipitated with FLAG-AML1-ETO, along with HSP70 and HSP90, while actin was not detected (Fig. 1*D*). To further investigate TRiC putative interaction with AML1-ETO, we undertook a global proteomics approach to identify the AML1-ETO protein-protein interactions. We enriched the AML1-ETO interactome by tandem purification using a StrpII/6His-tag and performed mass spectroscopy. In agreement with results from our immunoprecipitation experiments, we found all eight CCT subunits with high frequency and also identified HSP90 and HSP70 in this interactome (Fig. 1*E*). These results show that AML1-ETO interacts with major elements of the protein folding machinery, specifically TRiC and HSP70/HSP90, inside human cells.

Having solid evidence substantiating the interaction between TRiC and AML1-ETO in human cells, we then tested for their direct binding by immunoprecipitation using purified human TRiC (hTRiC) and denatured AML1-ETO. AML1-ETO chemically denatured in GnHCl was rapidly diluted with buffer containing purified TRiC. Most of AML1-ETO immediately precipitated as pellet and was removed by centrifugation; however, a portion remained in the supernatant and co-precipitated with TRiC during immunoprecipitation. Furthermore, adding purified HSP70 to the buffer (shown to interact with AML1-ETO and TRiC in human cells) increased the fraction of AML1-ETO that co-precipitated with TRiC (Fig. 2, *A* and *B*). In light of previous studies showing that HSP70 mediates unfolded protein delivery to chaperonins (34), these results suggest that



Protein Name	Num Unique	Peptide Count	% Cov
CCT1	12	18	34.7
CCT2	48	102	79.6
CCT3	12	26	38.2
CCT4	13	22	37.1
CCT5	28	50	65.6
CCT6	9	11	23.7
CCT7	16	29	44.4
CCT8	22	42	61.5
Hsp70	8	11	18.1
Hsp90	3	5	11.5

FIGURE 1. **AML1-ETO interacts with TRiC in human cells.** *A*, doxycycline (*Dox*) was added in culture to induce Flag-AML1-ETO expression. Each protein was detected by Western blot (*WB*) as indicated. *B*, cells were fractionated into cytoplasmic (*Cyt*) and nuclear (*Nu*) contents. The amount of AML1-ETO is visualized by Western blot. *C*, protein-DNA binding activity was determined by electrophoresis mobility gel shift assay (EMSA). KASUMI-1 was used as a positive control, while WT HEK293 served as a negative control. Either anti-ETO or anti-FLAG was added to super-shift the AML1-ETO-DNA complex. *D*, CCT2/5 and Flag-AML1-ETO were immunoprecipitated from the cytoplasmic fraction in HEK293 cells. Non-immunized rabbit antibody served as negative control (*Ctrl*). Proteins of interest in the cytoplasmic fraction and immunoprecipitated proteins were detected by Western blot with specific antibodies as indicated. *E*, protein complexes from the HEK293 with Strep-His-AML1-ETO-expression were affinity-purified by a strep-II/His-tag and analyzed by mass spectrometry. In the AML1-ETO interactome, number of unique peptides detected (Num unique), number of peptide detected (Peptide count), and % coverage (% cov) of CCT1～8, HSP70 and HSP90 are shown.

while denatured AML1-ETO can by itself bind to TRiC directly, and that HSP70 enhances this direct interaction.

TRiC Interaction with AML1-ETO Is Early in Its Translation and Is Required for Biogenesis in RRL—Previous studies have shown that TRiC-substrate interactions are typically early translational and transient (19). To test whether this is also the case for AML1-ETO association with TRiC, we performed immunoprecipitation experiments at various time points using rabbit reticulocyte lysates (RRL). Endogenous TRiC was immunoprecipitated from RRL aliquots containing an AML1-ETO expression plasmid, at various time points after *de novo* transcription/translation and quenching (Fig. 3*A*). In agreement with our results from immunoprecipitation experiments using HEK293 cells, ^{35}S radio-labeled AML1-ETO also immunoprecipitated with TRiC in a cell-free translation system, further substantiating their direct interaction. Of note, the interaction between AML1-ETO and TRiC was transient, as suggested by markedly reduced levels of AML1-ETO in TRiC immunopre-

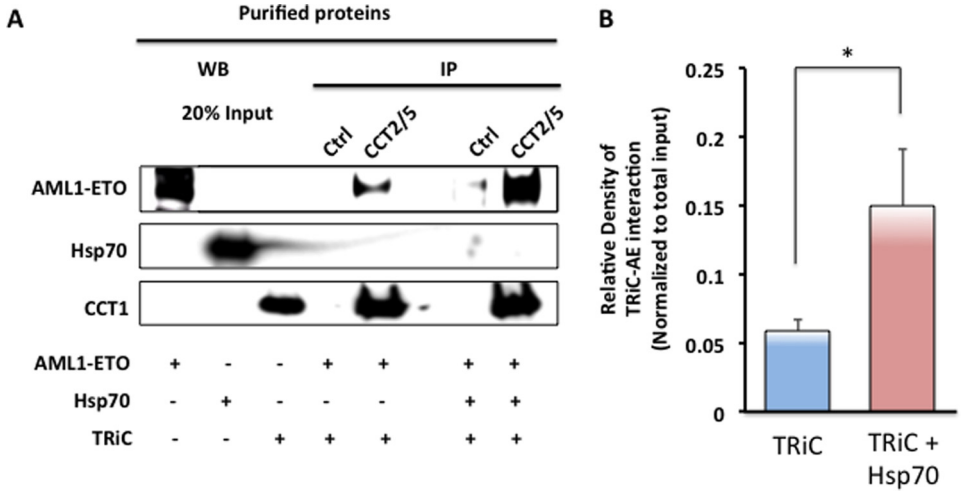


FIGURE 2. **HSP70 facilitates the direct association of AML1-ETO with TRiC.** *A*, chemically denatured AML1-ETO was diluted into physiological buffer containing purified human TRiC and/or HSP70 at a final molar ratio of TRiC:HSP70:AML1-ETO of 1:1:5. TRiC was immunoprecipitated from the reaction mixture with CCT2/5 antibodies. Each protein of interest was detected by Western blot with specific antibodies as indicated. *B*, histogram showing the average and S.D. of three independent experiments. Significance is shown by *t* test with *p* value less than 0.05.

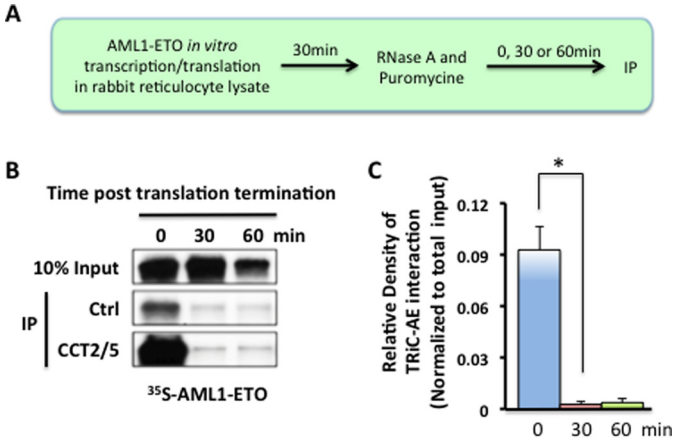


FIGURE 3. **Early-translational AML1-ETO interacts with TRiC.** *A*, experimental scheme is shown. [^{35}S]methionine-labeled AML1-ETO was synthesized in RRL. Addition of RNase A and puromycin quenched *de novo* protein translation after 30 min from initiation of protein synthesis. The reaction lysate was further incubated for additional 0, 30, and 60 min, respectively, and then CCT2/5 were immunoprecipitated from the lysate at each time point. *B*, input and immunoprecipitated ^{35}S-labeled AML1-ETO were detected by autoradiography. *C*, histogram showing the mean and standard deviation of two independent experiments.

cipitates 30 min after termination of the transcription/translation reaction, time during which AML1-ETO protein levels remained constant (Fig. 3, *B* and *C*). Considering TRiC's paramount role in protein folding, our results suggest that AML1-ETO folding intermediates have high affinity for TRiC, both during and immediately after protein translation.

A TRiC interaction early in translational has been shown to be required for the biogenesis of some essential proteins in cells (35). To test whether TRiC's interaction with AML1-ETO polypeptides is coupled to the oncoprotein biogenesis, we immunodepleted TRiC from RRL and tested for AML1-ETO generation. TRiC immunodepletion selectively removed TRiC from RRL (Fig. 4*A*), and the depleted lysate remarkably lost its ability to produce AML1-ETO and STAT3 (23). On the other hand, β-actin did not show a significant change in protein expression (36) (Fig. 4*B*). Notably, adding purified TRiC back into TRiC-depleted RRL reconstituted AML1-ETO protein synthesis in a dose-dependent manner (Fig. 4*C*). These findings indicate that TRiC contributes to AML1-ETO's biogenesis in RRL, most likely through early-translational interactions.

TRiC Restores the DNA Binding Activity of Denatured AML1-ETO—The early translational association of AML1-ETO with TRiC strongly suggests that the chaperonin might play a role in promoting the folding of AML1-ETO into its native conformation. To further test this, we examined the DNA binding ability of full-length AML1-ETO in the presence of TRiC. Denatured AML1-ETO was diluted into refolding buffer containing purified human TRiC. The reaction mixture was incubated with an AML1-binding DNA probe labeled with ^{32}P and analyzed by electrophoresis mobility shift assay (EMSA) with native PAGE (31). KASUMI-1 cell lysates served as a positive control, showing normal DNA binding activity for native, endogenous AML1-ETO. On the other hand, wild-type HEK293 served as a negative control (Fig. 5*A*). Rapid dilution of denatured AML1-ETO in physiological buffer lead to immediate precipitation and no DNA binding activity in the absence of TRiC. However, when incubated with TRiC, AML1-ETO displayed increasing DNA binding activity in a dose-dependent manner (Fig. 5, *B* and *C*). Unexpectedly, addition of ATP or HSP70, and incubation time variations, did not improve AML1-ETO DNA binding activity compared with TRiC alone (Fig. 5*B*). Taken together, these results demonstrate that TRiC can refold denatured AML1-ETO and restore its DNA binding activity *in vitro*. Interestingly, our finding suggests that the refolding of denatured AML1-ETO by TRiC is independent of ATP hydrolysis, indicating that the direct contact occurring in the initial recognition complex suffices for substrate refolding. This implies that TRiC helps to fold AML1-ETO along a pathway different from the canonical ATP-dependent model for assisted-protein folding by chaperonins (37–39).

In addition to using EMSA to examine the activity of denatured AML1-ETO, we also identified the location of TRiC and AML1-ETO on native PAGE by Western blot with specific antibodies. Consistent with previous results, denatured AML1-

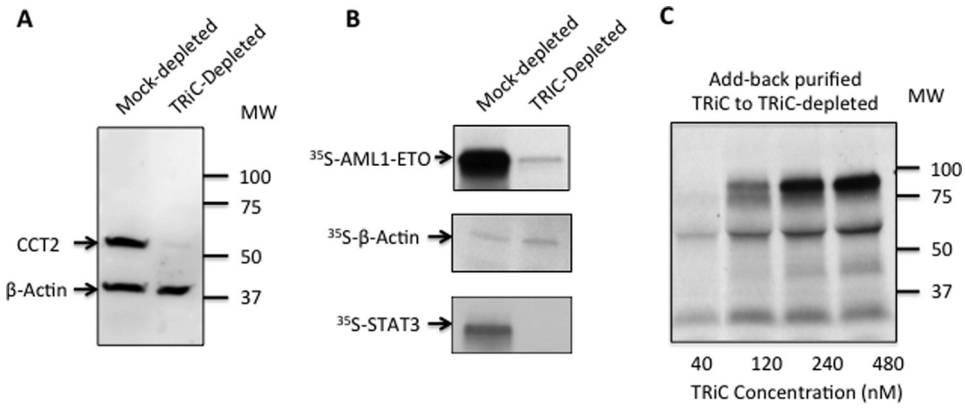


FIGURE 4. **TRiC is required for AML1-ETO biogenesis in RRL.** *A*, RRL was immunoblotted with CCT2 antibody following TRiC depletion (TRiC-depleted) or control (mock-depleted). Actin is shown as a positive control for specific TRiC depletion. *B*, β-actin, AML1-ETO and STAT3 were translated in both mock depleted and TRiC-depleted RRL in the presence of [^{35}S]methionine followed by SDS-PAGE and autoradiography. *C*, AML1-ETO was translated in TRiC-depleted RRL following the addition of increasing amounts of purified bovine TRiC in the presence of [^{35}S]methionine followed by SDS-PAGE and autoradiography.

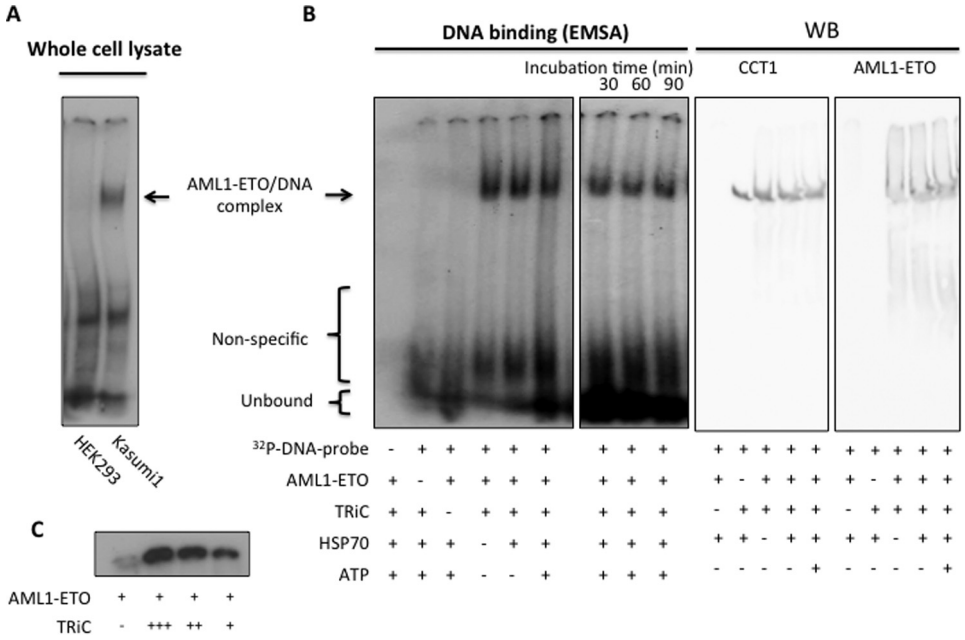


FIGURE 5. **TRiC restores AML1-ETO DNA binding activity and active AML-(1–175) forms a stable complex with TRiC.** The DNA binding activity of AML1-ETO was examined by electrophoresis mobility assay (EMSA) using a ^{32}P-labeled AML1-ETO-binding duplex DNA probe. *A*, to evaluate the binding of the ^{32}P-DNA probe by AML1-ETO, wild type HEK293 is shown as a negative control and KASUMI-1 as positive control. *B*, denatured AML1-ETO, DNA probe, and TRiC form a complex. The location of ^{32}P-DNA is visualized by autoradiography, while TRiC and AML1-ETO were detected by Western blot. *C*, AML1-ETO displayed increasing DNA binding activity in a dose-dependent manner (+: 0.05 μM, ++: 0.1 μM, and +++: 0.2 μM of TRiC).

ETO by itself was not soluble and could not enter the gel in the absence of TRiC. However, in the presence of TRiC, AML1-ETO entered the gel and migrated, as expected. Interestingly, the positions on the native gel for DNA, AML1-ETO and TRiC overlapped and remained unchanged in the presence of ATP, indicating that the AML1-ETO-DNA complex co-migrated with TRiC independently of TRiC ATP usage (Fig. 5*B*). Of note, ATP hydrolysis by TRiC is typically required for substrate folding and release (37, 38). Yet, our results suggest that while AML1-ETO is folded by TRiC to achieve its native function, ATP hydrolysis alone does not suffice to trigger its release from the chaperonin. This stable interaction using purified proteins differs from our results using a cell-free translational system, where TRiC interaction with AML1-ETO was transient.

AML1-ETO Binds to Multiple TRiC Subunits—While TRiC eight subunits are homologous, they are also highly divergent in their apical domains, which confer substrate binding specificity (25, 40). Therefore, AML1-ETO binding to TRiC is likely to occur via a specific subset of CCT subunits. To identify these, we mixed RRL expressing a single radiolabeled CCT subunit (1 through 8, one at a time) with denatured Flag-AML1-ETO followed by immunoprecipitation with anti-Flag antibody. The immunoprecipitant was analyzed by SDS-PAGE and autoradiography (Fig. 6*A*). These experiments revealed that CCT subunits 2, 3, 4, 6, and 7 interact with AML1-ETO but subunits 1, 5, and 8 do not (Fig. 6*B*). In particular, CCT4 and 6 showed the most reproducible interaction with denatured AML1-ETO. These results suggest that multiple but not all TRiC subunits interact with AML1-ETO with different affinity (Fig. 6*C*).

AML1-ETO Binds to TRiC Predominantly through Its DNA Binding Domain—AML1-ETO consists of five distinct functional domains: DNA-binding (DB), transactivation (TAF),

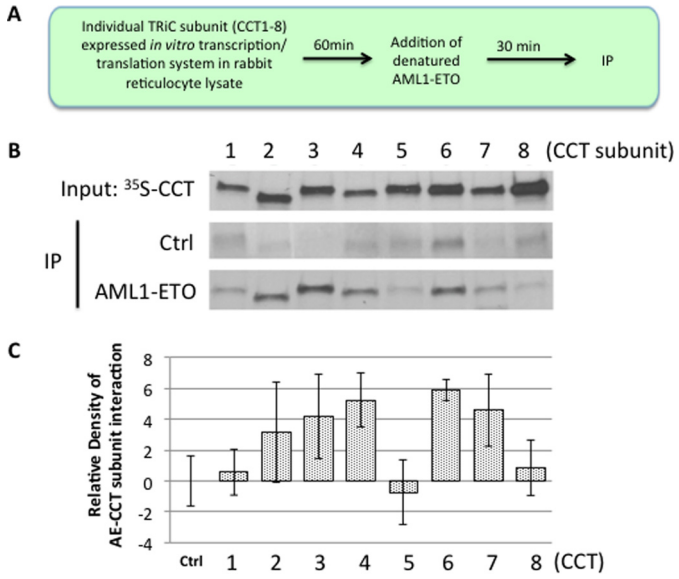


FIGURE 6. **AML1-ETO binds to the multiple TRiC subunits.** *A*, experimental scheme is shown. *B*, eight individual ^{35}S-labeled TRiC subunits were expressed in an *in vitro* translation system (RRL). Chemically denatured AML1-ETO was added into lysate containing single TRiC subunits, one at a time. The interactions between single TRiC subunits and AML1-ETO were identically tested using the same co-IP protocol, as described ("Experimental Procedures"). *C*, histogram shows the mean and standard deviation of three independent experiments.

linker, oligomerization (NHR), and zinc-finger (Zn-F) (Fig. 7*A*) (6). To identify the AML1-ETO domains that bind to TRiC, we performed TRiC immunoprecipitation from RRL expressing individual AML1-ETO domains, one at a time. Of the individual domains, the DBD appears to have the strongest association with TRiC (Fig. 7, *B* and *C*). Although the three-dimensional structure of full-length AML1-ETO is unsolved, there are either x-ray crystallography or NMR structures for the individual domains (Fig. 7*A*) (41–44). Of note, the DBD of AML1-ETO is composed exclusively of β-strands, a common structural motif across TRiC substrates well known to require assistance from chaperones to fold properly (19). As a reciprocal validation, we examined the association of wild-type AML1a (45) with TRiC and demonstrated that AML1a also associates with TRiC.

We next tested for direct interaction between the DBD (AML-(1–175)) and TRiC *in vitro*. Flag-AML-(1–175) was expressed in *Escherichia coli* and purified from inclusion bodies. Denatured AML-(1–175) was diluted into buffer without or with TRiC. Rapid dilution into buffer without TRiC results in formation of insoluble AML-(1–175) aggregates, which can be removed by centrifugation or filtration. In contrast, AML-(1–175) remains soluble when diluted in buffer containing TRiC, suggesting that the chaperonin stabilizes this AML1 fragment in folding intermediate conformations. Solubility is further increased when HSP70 is added to the reaction mixture, suggesting that HSP70 aids TRiC in preventing denatured AML-(1–175) from aggregating (Fig. 8). Thus, in agreement with our results for full-length AML1-ETO, the solubility of AML-(1–175) is directly dependent on its ability to bind TRiC. We further examined the association of AML-(1–175) to TRiC using the same immunoprecipitation approach previously described and found that AML-(1–175) also binds to TRiC directly and that HSP70 also facilitates their interaction, as we found for full-length AML1-ETO. Interestingly, we could not detect any direct association between TRiC and HSP70 under our experimental conditions (Fig. 9).

Discussion

Inside cells, the folding of proteins into their native conformation largely relies on assistance from molecular chaperones. Like most cancerous cells, AML cells show higher expression levels of the eukaryotic chaperonin TRiC/CCT. Although this chaperonin is known to assist the folding of some oncoproteins, such as STAT3, and thereby contributes to their pro-oncogenic functions, our current understanding of how oncogenic fusion proteins achieve their folded and functional conformations in living cells is still very limited. Here we demonstrate that AML1-ETO, the most common leukemogenic fusion protein in AML, interacts with major components of the protein folding machinery; namely, TRiC, HSP70, and HSP90. We show that TRiC directly binds to AML1-ETO, thereby contributing to its folding, biogenesis, and native activity. We also discovered that AML1-ETO binds to TRiC via its β-strand rich, DNA-binding domain (AML-(1–175)).

Several recent studies have analyzed the effects of targeting TRiC on the level and function of multiple TRiC clients. They found that depletion of TRiC from cells results in the misfolding of β-actin, tubulin, p53, and STAT3, which in turn affects their functional activity. For example, in the case of cytoskeletal proteins, TRiC depletion ultimately causes loss of polymerization activity for actin and tubulin. For p53, TRiC depletion leads to decreased p53-protein interactions and, in the case of STAT3, it induces a decrease in its ability to be phosphorylated (22, 23, 35). Here we exhaustively demonstrate that TRiC is strictly required for AML1-ETO synthesis in RRLs (Fig. 4) and contributes to AML1-ETO refolding (Fig. 5). These findings provide proof-of-principle that targeting TRiC, or more specifically targeting the interaction between TRiC and AML1-ETO, may provide a novel approach to reducing AML1-ETO levels for therapeutic benefit in AML1-ETO-positive AML. This possibility warrants further investigation by conducting studies analogous to those summarized above for p53 and STAT3 in hematopoietic cell lines, such as KASUMI-1 cells, commonly used as a model system for AML1-ETO-positive AML (46).

Our observation that TRiC folds AML1-ETO under nucleotide-free conditions (Fig. 5) cannot be completely explained by the classical model for chaperonin-mediated protein folding. According to this model, one or more cycles of ATP hydrolysis are usually necessary for productive protein folding (47). Rather, our data is consistent with the "out-of-cage" refolding model recently proposed for GroEL and TRiC, which does not require ATP (48). In this recent model, GroEL and TRiC bind substrates through their apical domains to assist their folding, without the need for ATP hydrolysis and subsequent substrate encapsulation. Our results highlight AML1-ETO as the first native human substrate of TRiC discovered to be capable of folding in an ATP-independent manner.

We have exhaustively demonstrated that TRiC interacts directly with AML1-ETO primarily through the latter's DNA

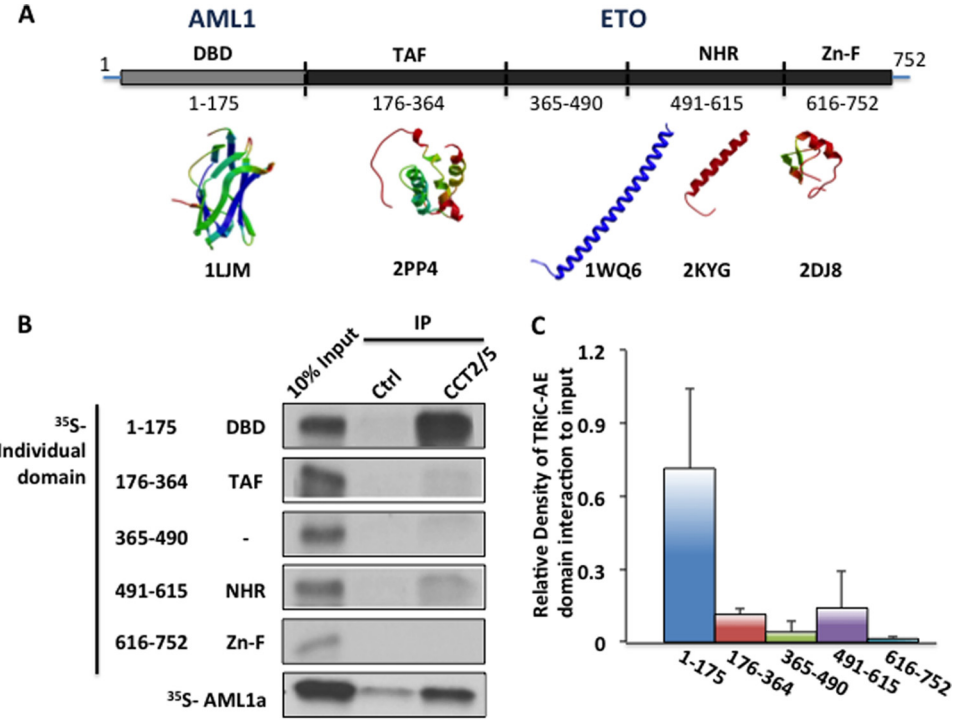


FIGURE 7. **The TRiC binding domain within AML1-ETO maps predominantly to its DNA binding domain.** *A*, schematic depiction of the five AML1-ETO domains with their corresponding structure and PDB ID: DNA-binding (*DB*), transactivation (*TAF*), linker, oligomerization (*NHR*), and Zn-finger (*Zn-F*). AML1 is depicted in *gray* and ETO in *black*. *B*, TRiC was immunoprecipitated using antibodies to CCT2/5 or control antibody (Ctrl) from RRL containing expression constructs with inserts encoding each of the five domains or wild type AML1a in the presence of [^{35}S]methionine. *C*, histogram showing the mean and S.D. of two independent experiments.

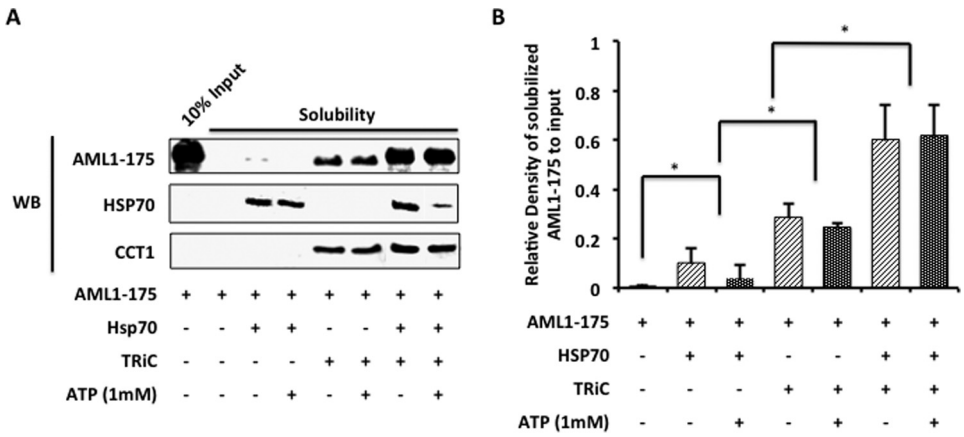


FIGURE 8. **TRIC contributes to the solubility of the denatured DNA binding domain (AML-(1–175)).** *A*, chemically denatured Flag-AML-(1–175) was diluted 100-fold into buffer containing TRiC without or with HSP70. The insoluble fraction was removed by centrifugation and filtration. The soluble fraction was separated by SDS-PAGE and analyzed by Western blot with anti-FLAG, HSP70, or CCT1. *B*, Western blot intensity was quantified and analyzed statistically by *t* test based on two independent experiments.

binding domain. Therefore, it would be of interest to determine whether the association between TRiC and AML1-ETO can be modulated in the presence of AML1-ETO DNA-binding elements. It also would be interesting to determine the effects of such modulation on AML1-ETO downstream protein-protein interactions, and whether these could be exploited for therapeutic benefit. Experiments to address these issues are underway.

Our refolding experiments using purified proteins *in vitro* show that folded AML1-ETO remains associated with TRiC throughout the chaperonin ATP hydrolysis cycle (Fig. 5), in contrast to actin and luciferase which are released from TRiC in the presence of ATP (26). On the other hand, the interaction of AML1-ETO with TRiC is transient in our cell-free translational system (Fig. 3). Thus, it is tempting to speculate that RRL contains co-factors required for AML1-ETO to dissociate from TRiC, as seen for tumor suppressor VHL, which requires Elongin-B/C to be released from TRiC after protein folding is completed (49). Based on our observations, TRiC not only assists protein folding but might also work as a regulatory hub by holding onto transcription factors until other proteins release them. This additional layer of complexity we have uncovered for TRiC action on AML-(1–175) could reflect a more general mechanism, perhaps common to other TRiC sub-

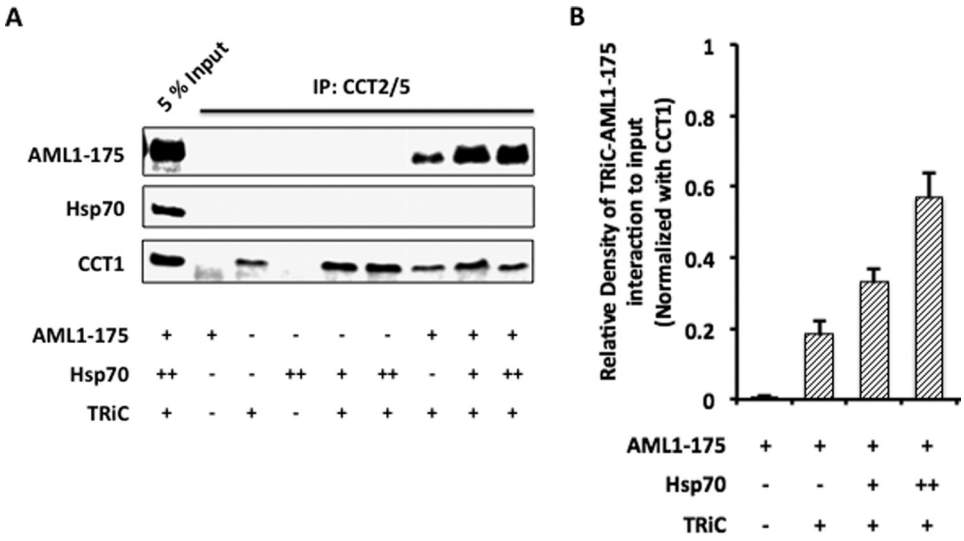


FIGURE 9. **The DNA binding domain (AML-(1–175)) of AML1 directly associates with TRiC.** *A*, chemically denatured Flag-AML-(1–175) was diluted into TRiC or HSP70-containing buffer (+: 0.5, ++: 1 molar ratio of HSP70 to TRiC) and immunoprecipitated using antibodies to CCT2/5. Immunoprecipitates were analyzed by Western blot for Flag, HSP70, and CCT2. *B*, histogram showing the mean and S.D. of two independent experiments.

strates, which might facilitate regulation by co-factors during chaperonin-assisted protein folding. Thus, it would be extremely interesting to investigate the question of how AML1-ETO is released from TRiC (20).

Author Contributions—S. R., W. C., and D. J. T. designed the project. S. R. and M. K. performed biochemistry experiments. A. B. and M. T. performed mass-spec analysis. S. R., J. G., W. C., and D. J. T. wrote the manuscript with input from all other co-authors.

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