RNA-binding proteins and heat-shock protein 90 are constituents of the cytoplasmic capping enzyme interactome

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Abstract

The N7-methylguanosine cap is added in the nucleus early in gene transcription and is a defining feature of eukaryotic mRNAs. Mammalian cells also possess cytoplasmic machinery for restoring the cap at uncapped or partially degraded RNA 5' ends. Central to both pathways is capping enzyme (CE, RNA guanylyltransferase and 5'-phosphatase, RNGTT), a bifunctional, nuclear and cytoplasmic enzyme. CE is recruited to the cytoplasmic capping complex by binding to a C-terminal proline-rich sequence to the third SH3 domain of NCK adapter protein 1 (NCK1). To gain broader insight into the cellular context of cytoplasmic recapping, here we identified the protein interactome of cytoplasmic CE in human U2OS cells through two complementary approaches: chemical cross-linking and recovery with cytoplasmic CE and protein screening with proximity-dependent biotin identification (BioID). This strategy unexpectedly identified 66 proteins, 52 of which are RNA-binding proteins. We found that CE interacts with several of these proteins independently of RNA, mediated by sequences within its N-terminal triphosphatase domain, and we present a model describing how CE-binding proteins may function in defining recapping targets. This analysis also revealed that CE is a client protein of heat shock protein 90 (HSP90). Nuclear and cytoplasmic CEs were exquisitely sensitive to inhibition of HSP90, with both forms declining significantly following treatment with each of several HSP90 inhibitors. Importantly, steady-state levels of capped mRNAs decreased in cells treated with the HSP90 inhibitor geldanamycin, raising the possibility that the cytotoxic effect of these drugs may partially be due to a general reduction in translatable mRNAs.

Introduction

Cap homeostasis is a cyclical process of cytoplasmic decapping and recapping that impacts a subpopulation of the transcriptome. Previous work classified cytoplasmic capping targets into 3 groups based on conditions that impact the appearance of their uncapped forms (1). One group of mRNAs has a population of ‘natively uncapped’ transcripts that become more susceptible to degradation by XRN1 when cytoplasmic capping is blocked. For another group, the ‘capping inhibited’ mRNAs, uncapped
forms only appear when cytoplasmic capping is blocked. A third group, termed ‘common’ mRNAs, overlaps with the natively uncapped pool but differs in that the representation of uncapped forms increases when cytoplasmic capping is blocked. We showed in (2) that inhibition of cytoplasmic capping had no detectable impact on global poly(A) tail length. Uncapped forms of recapping targets accumulate in non-translating mRNPs, and these have poly(A) tails similar in length to polysome-bound forms of the same mRNAs. As such, recapping is all that is needed to restore these mRNAs to the translating pool. Cytoplasmic recapping also appears to have a role in diversifying the transcriptome by acting on 5′-end-shortened products of post-transcriptional processing (3–7).

Enzymes capable of regenerating caps on cytoplasmic RNAs have been described in diverse eukaryotic systems (8, 9), but the most thoroughly characterized recapping system is that of mammalian cells. In mammals, CE and RNA guanine-7 methyltransferase (RNMT) localize to both the nucleus and cytoplasm to participate in canonical cap synthesis and recapping, respectively (10–12). Cytoplasmic recapping is catalyzed by a complex of enzymes in the same pathway (i.e., a metabolon) that is capable of converting RNA with a 5′-monophosphate end to one with an m7G cap. The core of this complex is NCK1, an adapter protein with 3 SH3 domains and a single SH2 domain. The kinase that generates a 5′-diphosphate recapping substrate binds to the second SH3 domain, and CE binds to the third SH3 domain. The latter interaction is mediated by a C-terminal proline-rich sequence of CE (13). The functional form of RNMT is a heterodimer with RNA guanine-7 methyltransferase activating subunit (RAMAC, formerly called RAM (14)), and the RNMT:RAMAC dimer is recruited into the cytoplasmic capping complex by direct interaction with CE (12). A diagram of the cytoplasmic capping complex is shown in Figure 5.

Immunofluorescence analysis of CE distribution in (10) showed the expected nuclear staining and punctate focal staining of CE throughout the cytoplasm. While it is tempting to think of the latter as focal sites of cytoplasmic recapping, we were unable to confirm this by co-staining for NCK1 due to the uniform distribution of that protein throughout the cytoplasm. This left open the question whether the majority of cytoplasmic CE was in the recapping complex, or if instead only a minor portion of the cytoplasmic protein participated in this process. This question gained significance with the recent identification of a role for cytoplasmic CE in regulating hedgehog signaling by antagonizing protein kinase A (8). To address this, we performed two complementary proteomics analyses of the cytoplasmic CE interactome using cells with stably integrated transgenes expressing cytoplasmically restricted forms of CE. The principal approach looked for proteins recovered with CE carrying an N-terminal sequence that is natively biotinylated in vivo (bio-cCE in (13)). This was complemented by proximity-dependent biotinylation using protein with BirA* substituted for the biotinylation tag (BirA*-cCE, (12)). The combined approaches identified 66 proteins as constituents of the cytoplasmic CE interactome. Strikingly, 52 of these have been characterized as RNA-binding proteins. This analysis also identified HSP90, and we show that nuclear and cytoplasmic CE are client proteins whose levels are dependent on HSP90 chaperone activity.

Results

Generation of cell lines for proteomics analysis

The initial approach to identifying the cytoplasmic CE interactome took advantage of a construct developed in (13) termed bio-cCE, which expresses CE with an N-terminal sequence that is biotinylated in vivo. This protein is restricted to the cytoplasm by deletion of the KRKY nuclear localization signal and addition of the HIV Rev nuclear export sequence (NES), modifications that were detailed in (10). A plasmid expressing this cCE transgene was modified to introduce tetracycline operator elements and was stably transfected into tetracycline-inducible U2OS cells. To control for interacting proteins that may bind to the tags or interact non-specifically, we also generated a cell line expressing a similarly tagged enhanced green fluorescence protein (bio-EGFP). These constructs included a myc epitope tag (Figure 1A). Immunofluorescence images in Figure 1B shows each of these proteins was
undetectable in the absence of tetracycline (-tet) and was only observed in the cytoplasm of tetracycline-treated cells (+tet). Streptavidin Western blotting of cytoplasmic extracts confirmed both proteins were biotinylated. This also showed a small amount of leaky expression of bio-EGFP and no evidence for leaky expression of bio-cCE (Figure 1C). Western blotting of nuclear and cytoplasmic extracts with anti-CE antibody provided additional evidence showing bio-cCE is restricted to the cytoplasm (Figure 1D). Note that cytoplasmic extracts have a cross-reacting protein the same size as bio-cCE that makes the overall induction appear greater than it actually was.

**Identification of cCE interacting proteins**

Stable cells were briefly crosslinked with formaldehyde prior to harvesting to preserve in vivo interactions of proteins with bio-EGFP and bio-cCE (16). As proof of principle, cytoplasmic proteins recovered on streptavidin paramagnetic beads were visualized by SDS-PAGE and Coomassie blue staining (Figure 1E). This provided evidence for the selective recovery of at least 14 proteins with bio-cCE that were not recovered with bio-EGFP. Several proteins were also identified in both samples, likely as contaminants that bound nonspecifically to the streptavidin beads or to the common N-terminal peptide tags.

This experiment was then repeated three times, with bead-bound proteins identified by liquid chromatography coupled to tandem mass-spectrometry (LC-MS/MS) of tryptic peptides. The numbers of peptide spectral matches (PSMs) were tallied for each protein identified, and results are presented in Table 1. For each protein, a cCE enrichment index was calculated as the average number of PSMs for bio-cCE divided by the average number of PSMs for bio-EGFP plus one. The addition of one to the denominator was done to prevent division by zero and to de-emphasize proteins with fewer PSMs. As expected, PSMs for CE (RNGTT) were exclusive to the bio-cCE samples (Supplemental Table S1). These data indicate that cytoplasmic CE interacts with many more proteins than previously thought. Forty-five proteins were identified as having a cCE enrichment index of at least 1.5 (Table 1). Unexpectedly, this list did not include the two known cCE-interacting proteins NCK1 (13) or RNMT (12). The reason for this is unclear; however, we suspect this may indicate that at any given time only a minor portion of cytoplasmic CE associates with the previously described functional recapping complex (see Discussion). Likewise, no obvious candidate protein was identified that may harbor 5’ monophosphate kinase activity, though the possibility of uncharacterized kinase activity in the identified proteins cannot be ruled out.

The most striking observation is that 38 (84%) of the cytoplasmic CE interactors are classified as RNA-binding proteins in a recent comprehensive analysis of the human RNA-binding proteome ((17), Table 1). That study compiled an “integrated human RNA-binding proteome” (ihRBP) containing 978 known poly(A) RNA-binding proteins (18–20) and an additional 775 novel non-poly(A) RNA-binding proteins. In our cytoplasmic CE interactome, 24 proteins are classified as poly(A) RNA-binding, and 14 proteins are non-poly(A) RNA-binding. This list of proteins did not include proteins identified in (21) (mainly RNA polymerase II subunits, regulators of transcription elongation, and protein phosphatase 4 (PP4) subunits) as CE-interacting proteins. That is not altogether surprising since that study used total cell extracts and CE is mostly a nuclear protein (see Figure 1D; also Figure 1D in (10)).

**BioID of the cytoplasmic CE interactome**

In previous work (12) proximity-dependent biotinylation was used to obtain supporting evidence for the presence of cap methyltransferase (RNMT) in complex with cytoplasmic CE. The BirA*-cCE construct used in that study was modified to create a tetracycline-inducible transgene which was stably transfected into U2OS-TR cells. As a specificity control, we also developed U2OS-TR cells with inducible BirA* fused only to the HIV Rev NES and the linker peptides present in BirA*-cCE. These were then used to generate orthogonal data to those obtained above with bio-cCE. The background of proteins detected with BirA* alone was higher than that obtained with bio-EGFP, perhaps as a result of nonspecific biotinylation by freely diffusing protein. When this was taken into account, BioID identified 32 proteins with a cCE...
enrichment index of at least 1.25 (Supplemental Table S1), 11 of which were common to both proteomics analyses (Table 1). Of the 32 proteins identified by BioID, 23 (72%) were classified as RNA-binding in the ihRBP (17) (Supplemental Table 1). Together the combined approaches identified 66 CE-interacting proteins, 52 (79%) of which are represented in the integrated human RNA-binding proteome. Our reasoning for classifying all of these as cytoplasmic CE-interacting proteins is covered in the Discussion.

Validation and characterization of cytoplasmic CE protein interactions

To confirm these results, we selected 6 proteins representing a range of PSMs for further analysis by Western blotting. These included heat shock protein 90 (48 and 85 PSMs for the α and β isoforms, respectively) at the high end, RuvB-like AAA ATPase 1 (RUVBL1) at the low end (5 PSMs), and several in between: fatty acid synthase (FASN), exportin-2 (XPO2/CSE1L), translation elongation factor 2 (EEF2), and RuvB-like AAA ATPase 2 (RUVBL2) (Figure 2A). As a negative control, we also probed the streptavidin-bound material with an antibody against methylosome protein 50 (MEP50/WDR77), which was not identified by our proteomics analysis. With the exception of MEP50 each of these proteins was selectively recovered with bio-cCE but not with bio-EGFP (compare lanes 3 and 5). Although CE is not an RNA-binding protein, it was formally possible that these proteins were co-recovered as a consequence of mutual interactions with RNA molecules. To control for this, the same extracts were treated with micrococcal nuclease (13) prior to binding onto streptavidin beads (lanes 4 and 6). Nuclease digestion had no impact on the recovery of any of these proteins, thus ruling out mutual RNA-binding as a factor in their appearance in the CE interactome.

CE has two catalytic domains, the triphosphatase domain spanning residues 1-210 and the guanylyltransferase domain spanning residues 211-597. We previously showed that a C-terminal proline-rich sequence is required for binding of CE to NCK1 and its recruitment into the cytoplasmic capping complex (13). To narrow down the interaction site on CE the experiment in Figure 2A was repeated using cells transfected with plasmids that separately express residues 2-210 and 211-597. FASN, RUVBL1 and HSP90 were each recovered with residues 2-210 (i.e., the N-terminal triphosphatase domain) but not with residues 211-596 (the C-terminal guanylyltransferase domain, Figure 2B). Given their size (e.g., FASN is 2511 amino acids) and diverse nature, it is unlikely CE-interacting proteins bind simultaneously to the N-terminal domain of CE. Instead, these data suggest cytoplasmic CE has many interacting partners, with each protein representing a separate binding event.

Identification of CE as an HSP90 client protein

HSP90 is an ATP-dependent protein chaperone that helps fold and stabilize a select but diverse group of client proteins, including protein kinases, transcription factors, and disease-related factors such as p53 and Tau (22, 23). HSP90 functions as a chaperone constitutively, but expression of both the α and β isoforms increases in response to protein-denaturing stressors to help stabilize client proteins (24, 25). We were struck by the high PSMs for HSP90 (Table 1), and its parallel identification by BioID suggested that CE might be an HSP90 client protein.

Geldanamycin is a natural product that specifically inhibits HSP90 by binding to its unique ATP-binding site (26). Adding geldanamycin to the medium of cells expressing the bio-cCE transgene reduced the overall level of this protein to 32% of the DMSO-treated control (Figure 3A). This experiment was repeated using non-transfected U2OS cells to determine if geldanamycin had a similar impact on endogenous CE or if the observed loss in Figure 3A resulted from interference with the induction process (Figure 3B). Treating cells with geldanamycin resulted in a 50% loss of nuclear CE and 70% loss of cytoplasmic CE, thus confirming the identity of CE as an HSP90 client protein. In a separate experiment, the effect of geldanamycin inhibition was evident within 3 hr of exposure (Figure 3C), with nuclear CE reduced 80% and cytoplasmic CE reduced 60% after 24 hr.

HSP90 inhibitors are in development as therapeutic agents for cancer and other diseases (26). We therefore looked at the impact of two
additional HSP90 inhibitors with distinct modes of binding to the ATP-binding pocket: radicicol and AT13387 (onalespib), a second generation HSP90 inhibitor that is currently in clinical trials. Nuclear and cytoplasmic CE declined by 30% following treatment with 1 µM radicicol, and nuclear CE continued to decline with increasing concentrations (Figure 3D). Similar results were also observed for onalespib (Figure 3E). With each of these inhibitors, the decline in nuclear CE was greater than that observed for cytoplasmic CE. While the majority of HSP90 localizes to the cytoplasm, a small population has been reported to function in the nucleus as well (27, 28). Thus, the simultaneous loss of nuclear and cytoplasmic CE upon HSP90 inhibition could be due to redistribution of CE via nucleocytoplasmic shuttling or due to direct action of HSP90 on mature nuclear CE.

HSP90 inhibition is associated with decreased steady-state levels of capped mRNAs

CE loss results in apoptosis (29), and while capping is essential for cell growth and viability, to date this process had not been examined for sensitivity to HSP90 inhibition. To address this, we examined the impact of geldanamycin treatment on steady state levels of a number of mRNAs used in previous studies on cytoplasmic capping. In the experiment in Figure 4A, cytoplasmic RNA recovered from control (DMSO) or geldanamycin (GA)-treated cells was spiked with internal controls of uncapped luciferase mRNA and capped mCherry mRNA. Relative levels of these mRNAs were then quantified by RT-qPCR with results normalized to mCherry RNA. As expected, treating cells with geldanamycin had no impact on the relative amounts of the spike-in luciferase control. Geldanamycin also had no impact on the steady-state level of 18S rRNA, which is not a substrate of CE. However, steady-state levels of every mRNA examined declined following geldanamycin treatment. This included mRNAs that are cytoplasmic capping targets (MAPK1, POLR2B, VDAC3, ZNF207) as well as mRNAs that are not targets of cytoplasmic recapping (RPLP0, STRN4).

Cells have a number of surveillance mechanisms for degrading uncapped or improperly capped mRNAs (30, 31), and capping is required for RNA polymerase II transcription elongation and nuclear RNA export (32). Thus, if nuclear capping was diminished in geldanamycin-treated cells, the surviving cytoplasmic mRNAs should still be capped despite their lower levels. eIF4E is highly selective for binding m‘G capped mRNAs (33), and in (12) immobilized eIF4E was used to assay the cap status of mRNAs that survive inhibition of cytoplasmic cap methylation. eIF4E binds mRNAs with differing efficiency, and this was evident by differences in mRNA recovery in Figure 4B. Uncapped luciferase mRNA and 18S rRNA were almost undetectable in the eIF4E-bound fraction. This contrasts with STRN4, which was bound just as efficiently as capped mCherry RNA. Importantly, there was no statistically significant difference in the recovery of capped mRNAs examined here from control or geldanamycin-treated cells. This finding indicates that the decline in each of these species following inhibition of HSP90 is due to a decrease in the steady-state level of capped forms of these mRNAs.

Discussion

The identification in (10) of a cytoplasmic pool of CE led to the discovery of a cyclical process of cytoplasmic decapping and recapping termed cap homeostasis (1). Cap homeostasis maintains the stability and translation of a limited portion of the transcriptome and is facilitated by a complex of enzymes that catalyze the sequential phosphorylation, guanylation, and cap methylation of decapped forms of these mRNAs.

Previous studies of CE-interacting proteins focused on the relationship of CE to transcription and nuclear pre-mRNA processing (see (34) for a recent review). The current study used two complementary approaches to identify the cytoplasmic CE interactome. The first used a form of capping enzyme with a sequence tag that is biotinylated in vivo (bio-CE (13)). This protein is restricted to the cytoplasm by deletion of the four-amino-acid nuclear localization sequence and addition of the HIV Rev nuclear export sequence. Addition of a myc epitope tag facilitated detection by immunofluorescence and Western blotting (Figure 1). Inducible transgenes expressing cytoplasmic CE or EGFP with similar
modifications were stably transfected into tetracycline-inducible U2OS cells, and proteins recovered on streptavidin beads were identified by LC-MS/MS of tryptic fragments. These results were complemented by BioID using an inducible transgene (BirA*-cCE, (12)) that differed from bio-cCE by substitution of BirA* for the biotinylation tag.

These analyses yielded several unanticipated findings. BioID performed in (21) identified a small number of primarily nuclear CE-interacting proteins. This was not surprising as that study used whole cell lysates and nuclear CE is significantly more abundant than cytoplasmic CE. Our study used forms of CE that are restricted to the cytoplasm, and analyses were performed with cytoplasmic extracts that were essentially free of nuclear contamination. In the first approach, bio-cCE was induced to a level that approximates the endogenous protein, and cells were briefly crosslinked prior to lysis to stabilize native interactions (16) and minimize reassociation artifacts (35). We anticipated this would yield a similarly small number of interacting proteins. This instead identified 45 proteins, the majority [38] of which are represented as RNA-binding proteins in the integrated human RNA-binding proteome (17). This was complemented by BioID using stable cells carrying a BirA*-cCE transgene that again was induced to a level similar to that of endogenous cytoplasmic CE. This identified 32 proteins, 23 of which were identified in (17) as RNA-binding proteins. Eleven of the proteins identified by BioID were also among those recovered with bio-cCE (see Supplemental Table S1).

Based on results in (12) and (13) we anticipated also finding NCK1, RNMT, RAMAC, and the kinase that generates a diphosphate capping substrate from 5’-monophosphate RNA. The absence of these proteins from the cytoplasmic CE interactome was puzzling at first. However, this is true only if one considers the cytoplasmic capping complex as the major repository of cytoplasmic CE. Cytoplasmic CE is not particularly abundant, and the number of proteins identified here would reduce the possibility of identifying NCK1 and associated proteins by proteomic methods. That being said, this finding may also shed light on the question of recapping target specificity. In total this study identified 52 RNA-binding proteins in the cytoplasmic CE interactome.

CE does not bind RNA, and results in Figure 2A show that recovery of at least a few of these proteins with CE is independent of RNA. These proteins bind to the N-terminal triphosphatase domain of CE rather than the C-terminal region required for CE binding to NCK1 (Figure 2B). The RNA binding specificities of many of the proteins identified here have yet to be determined, but their sheer number suggests a model (Figure 5) in which target specificity is determined by the binding of capped or perhaps uncapped forms of a substrate RNA by one or more proteins of the CE interactome. Upon binding to CE, the mRNP joins with NCK1 for subsequent recapping. A process such as this is consistent with results in (1), where the off-on cycling of 5’ caps modulates the stability of some mRNAs and the translation of others.

Given the differences in proteins recovered with bio-cCE and those identified by BioID, we were faced with classifying all of these as interacting proteins or limiting this set to the 11 that overlap between both approaches. The predominance of RNA-binding proteins in both datasets was one reason for choosing the inclusive classification, since this provides a testable hypothesis for substrate specificity. The broader classification of interacting proteins was also influenced by results in (12) where cytoplasmic RNMT was biotinylated by BirA*-cCE but its closely associated activating subunit (RAMAC) was not. This led us to suspect methodological differences could in part explain differences in proteins identified here.

Finally, this study identified HSP90 as a constituent of the cytoplasmic CE interactome. As noted above, HSP90 is an ATP-dependent protein chaperone that functions in numerous signal transduction pathways (22). It is also a drug development target for cancer and other disorders (26). HSP90 inhibitors impact a number of downstream proteins; however, none of the studies to date have implicated CE, even though reduced levels of this protein lead to cell death via...
autophagy and apoptosis (29). Like some of the other proteins identified here, HSP90 binds the N-terminal triphosphatase domain of CE (Figure 2B), and its recovery with CE is independent of RNA (Figure 2A). Results with three different HSP90 inhibitors confirmed the dependence of nuclear and cytoplasmic CE on HSP90 activity. Treating cells with geldanamycin resulted in a 68% drop in bio-cCE expressed from the inducible transgene (Figure 4A), a 50% loss of endogenous nuclear CE, and a 70% loss of endogenous cytoplasmic CE (Figure 4C). Radicicol and geldanamycin both target the N-terminal ATP binding domain, and similar results were seen when U2OS osteosarcoma cells were treated with radicicol. Neither of these compounds is currently under evaluation as a therapeutic, but a related compound onalespib (AT13387) is in clinical trials. Onalespib had a greater impact on the level of nuclear CE than cytoplasmic CE. Over 300 proteins have been characterized as HSP90 clients (23), but the loss of CE with HSP90 inhibitors raised the question whether reduced cytoplasmic mRNAs might contribute to observed losses in these proteins’ abundances. The experimental results shown in Figure 4A illustrate that geldanamycin treatment resulted in a statistically significant decrease in steady-state levels of 6 mRNAs that we have studied extensively in our work on cytoplasmic capping. Because geldanamycin treatment reduced the levels of capping target and non-target mRNAs, its effect on steady-state levels was most likely due to decreased nuclear capping. Although this finding needs to be expanded upon, it raises the possibility that decreases in nuclear capping and the consequent decrease in translatable mRNAs might account for the decreases observed for some proteins upon inhibition of HSP90.

Experimental procedures
Plasmid constructs

The generation of plasmid pcDNA4/TO-bio-myc-NES-mCEANLS (bio-cCE; Addgene plasmid #112711) was described in (13). A similar plasmid expressing bio-EGFP (pcDNA4/TO-bio-myc-EGFP; Addgene plasmid #112712) was created by inserting a dsDNA gBlocks Gene Fragment (Integrated DNA Technologies) encoding the the entire bio-myc-NES-EGFP sequence into KpnI-digested pcDNA4/TO (Thermo Fisher V102020) using In-Fusion cloning (Clontech). Plasmid pcDNA3.1/TO-myc-BirA*-cCE (Addgene plasmid #112713) was created by inserting two tetracycline operator sites into pcDNA3.1-myc-BirA*-cCE ((12)) at the same location as in pcDNA4/TO. This was accomplished with the Change-IT site-directed mutagenesis system (Affymetrix) using 5’-phosphorylated forward primer JT93 (TGGGAGGTCTATATAAGCAGAGCTCTCCCC TATCAGTGATAGAGAACCCACT GAAGCTCGA TAGAGATC TGAAGGTCCACTAGAGAAGCTGCA GTTCTCTCGGCTTCTCAGG), which added the HIV Rev nuclear export signal (NES) downstream of BirA*.

The CE deletion constructs pcDNA3-bio-myc-CE 2-210 (Addgene plasmid #112715) and pcDNA3-bio-myc-CE 211-597 (Addgene plasmid #112716) were generated using the Fusion Site Directed Mutagenesis Kit (Thermo Fisher F541) with pcDNA3-bio-myc-CE ((13)) as the template. Mutagenic PCR was performed using 5’ phosphorylated primers JT164 (TGAGGCCTATTATCATGATGTCACC) and JT165 (TGAGTCCTTTTTTCATCATGCTTCTCAGG) for pcDNA3-bio-myc-CE 2-210 and 5’ phosphorylated primers JT166 (GAACGGGTCAAGTGAGCGTTCTCAGG) and JT167 (GGCCTCCATGGCCATAAGCTGCA GTTCTCTCGGCTTCTCAGG) for pcDNA3-bio-myc-CE 211-597. The sequences of all plasmids were confirmed by Sanger sequencing.

Cell culture, cell transfection, and generation of stable cell lines

All cells were grown at 37 °C under 5% CO₂ and were discarded after no more than 15 passages. U2OS-derived cell lines were grown in McCoy’s 5A medium (Thermo Fisher 116600) supplemented with tetracycline-free fetal bovine...
serum (FBS, Atlanta Biologicals S10350) to 10% (v/v). Tetracycline-inducible U2OS (U2OS-TR) cells were described previously in (10). Human embryonic kidney (HEK293) cells were cultured in Dulbecco’s Modified Eagle Medium (Thermo Fisher 11995–073) supplemented with FBS to 10% (v/v). For the experiment in Figure 2B, HEK293 cells were transfected with 10 µg pcDNA3-bio-myc-CE 2-210 or pcDNA3-bio-myc-CE 211-597 per 70% confluent 10-cm dish. After 24 h, cells were crosslinked, harvested, and processed as described below. For HSP90 inhibition experiments, cells were treated with the following compounds prepared in stock solutions with dimethylsulfoxide (DMSO): geldanamycin (Cayman Chemical 13355), radicicol (Cayman Chemical 13089), and onalespib (Cayman Chemical 10655).

To generate stable U2OS-TR cell lines expressing bio-EGFP or bio-cCE, U2OS-TR cells were transfected with either pcDNA4/TO-bio-myc-NES-EGFP or pcDNA4/TO-bio-myc-NES-mCEANLS using FuGene 6 (Promega E2691) according to manufacturer’s instructions. Cells were passaged two days following transfection and then maintained for two weeks in medium containing 600 µg/mL Geneticin (Thermo Fisher R25001). Maintaining selective pressure with 600 µg/mL Zeocin (Thermo Fisher 1029801) at room temperature in PBS containing 4% formaldehyde and 0.2% (v/v) Triton X-100. Coverslips were briefly washed three times with PBS before incubating in IF Block Solution (PBS containing 1% (w/v) BSA and 0.05% (v/v) Triton X-100) for 90 min at room temperature, followed by overnight incubation at 4 °C in IF Blocking Solution containing a 1:1000 dilution of mouse anti-myc antibody (Santa Cruz sc-40). Coverslips were washed three times with IF Wash Solution (PBS containing 0.5 mM MgCl₂ and 0.05% (v/v) Triton X-100) at room temp, 5 min per wash, and then incubated for 60 min in the dark at room temp in IF Block Solution containing 0.75 µg/mL DAPI and a 1:1000 dilution of anti-mouse Alexa Fluor 594 (Thermo Fisher A11005). After washing with IF Wash Solution as before, coverslips were mounted on glass microscope slides with ProLong Gold Antifade Mountant (Thermo Fisher P36930) and incubated overnight in the dark at room temp to allow the mountant to cure. Images were acquired at room temp with a Nikon Eclipse Ti-U inverted microscope fitted with a CFI Plan Apo VC 60X oil immersion objective and a Nikon DS-Qi1 monochrome digital camera. Images were analyzed using Nikon NIS-Elements AR 3.10 software. Specificity of the secondary antibody for the primary antibodies was confirmed by parallel preparation of control coverslips not treated with primary antibody.

Immunofluorescence

Glass coverslips in the wells of a 24-well plate were each seeded with 50,000 stable cells 17 h prior to induction. Expression of bio-EGFP or bio-cCE was induced by addition of tetracycline-HCl to a working concentration of 0.2 µg/mL. After 24 h, medium was removed from the coverslips, and the cells were fixed for 20 min at room temperature in PBS containing 4% formaldehyde and 0.2% (v/v) Triton X-100. Coverslips were briefly washed three times with PBS before incubating in IF Block Solution (PBS containing 1% (w/v) BSA and 0.05% (v/v) Triton X-100) for 90 min at room temperature, followed by overnight incubation at 4 °C in IF Blocking Solution containing a 1:1000 dilution of mouse anti-myc antibody (Santa Cruz sc-40). Coverslips were washed three times with IF Wash Solution (PBS containing 0.5 mM MgCl₂ and 0.05% (v/v) Triton X-100) at room temp, 5 min per wash, and then incubated for 60 min in the dark at room temp in IF Block Solution containing 0.75 µg/mL DAPI and a 1:1000 dilution of anti-mouse Alexa Fluor 594 (Thermo Fisher A11005). After washing with IF Wash Solution as before, coverslips were mounted on glass microscope slides with ProLong Gold Antifade Mountant (Thermo Fisher P36930) and incubated overnight in the dark at room temp to allow the mountant to cure. Images were acquired at room temp with a Nikon Eclipse Ti-U inverted microscope fitted with a CFI Plan Apo VC 60X oil immersion objective and a Nikon DS-Qi1 monochrome digital camera. Images were analyzed using Nikon NIS-Elements AR 3.10 software. Specificity of the secondary antibody for the primary antibodies was confirmed by parallel preparation of control coverslips not treated with primary antibody.

SDS-PAGE and western blotting
Samples in Laemmli Sample Buffer were heated at 95 °C for 5 min and briefly centrifuged before loading onto Mini-PROTEAN TGX gels (Bio-Rad) and electrophoresing at 150 V in Tris-glycine-SDS running buffer. Proteins were transferred to Immobilon-FL (EMD Millipore IPFL00010) PVDF membranes at 100 V for 60 min at 4 °C. Membranes were blocked in Blocking Buffer (Tris buffered saline (TBS) containing 3% (w/v) BSA) for at least 30 min at room temperature before incubating overnight at 4 °C in Blocking Buffer containing primary antibodies at the dilutions listed in the Antibodies section. Membranes were washed three times in TBST (TBS plus 1% Tween 20) at room temperature, at least 10 min per wash. Secondary antibody incubations were performed for 30 min at room temperature in Blocking Buffer, with antibody dilutions listed in the Antibodies section. Membranes were washed again with TBST as before and then imaged with a Li-Cor Odyssey infrared scanner at 700 or 800 nm as appropriate. Using Li-Cor Odyssey software, band intensities were quantified following background correction. Protein abundances were then calculated following normalization with the accompanying loading control shown in each figure. GraphPad Prism 6 software was used to plot individual data points and to perform unpaired two-tailed t-tests, with significant results reported in each figure.

**Antibodies**

Primary antibodies used in this study are as follows: mouse anti-myc (Santa Cruz sc-40; 1:1000 for WB, 1:1000 for IF), rabbit anti-CE/RNGTT (Novus NB1P-49972; 1:500 for WB), rabbit anti-FASN (Proteintech 10624-2-AP; 1:500 for WB), rabbit anti-XPO2/CSE1L (Proteintech 22219-1-AP; 1:1000 for WB), rabbit anti-EEF2 (One World Labs; 1:500 for WB), rabbit anti-HSP90α/β (Santa Cruz sc-4947; 1:2000 for WB), rabbit anti-RUVBL1 (Proteintech 10210-2-AP; 1:1000 for WB), rabbit anti-RUVBL2 (Proteintech 10195-1-AP; 1:500 for WB), rabbit anti-WDR77/MEP50 (Cell Signaling #2823; 1:1000 for WB), mouse anti-α-tubulin (Sigma T6199; 1:5000 for WB), rabbit anti-U2AF35 (provided by Brenton Graveley, University of Connecticut; 1:3000 for WB). Secondary antibodies used in this study are as follows: anti-mouse Alexa Fluor 594 (Thermo Fisher A11005; 1:1000 for IF), anti-rabbit Alexa Fluor 680 (Thermo Fisher A21109; 1:10,000 for WB), anti-mouse Alexa Fluor 680 (Thermo Fisher A21057; 1:10,000 for WB). Qdot 800 streptavidin conjugate (Thermo Fisher Q10171MP; 1:10,000 for WB) was used to detect biotinylated proteins.

**Crosslinking, BioID, and recovery on streptavidin beads**

After 24 h induction with 0.2 µg/mL tetracycline-HCl (Sigma T7660), growth medium was completely removed from 3 or 4 80-90% confluent 15-cm dishes each of bio-EGFP and bio-cCE stable cells. To each dish, 10 mL of PBS containing 0.15% formaldehyde (VWR 0493) was added and incubated at room temp for 10 min. To quench the crosslinking reaction, 1 mL of ice-cold PBS containing 1.25 M ice-cold glycine was added to each dish. Dishes were gently mixed by tilting and incubated for 5-10 min at room temp. The preceding steps were all performed in a staggered, timed manner to ensure consistent treatment. Cells were then washed with PBS and harvested by scraping into PBS, pooling the bio-EGFP and bio-cCE cells into one 1.6-mL tube each. Cytoplasmic extracts were prepared as described in (12, 15). For the experiment in Figure 2A, CaCl$_2$ was added to cytoplasmic extracts to 5 mM, micrococcal nuclease (Thermo Fisher 88216) was added to 0.46 U/mL (or not added as a control), and samples were incubated at room temp for 15 min. To 1.6-mL tubes containing 15 µL (stock slurry volume) of pre-equilibrated MyOne Streptavidin T1 Dynabeads (Thermo Fisher 65601), equal mass amounts of protein (~1000 µg per sample) were added, brought to 1 mL with YO Lysis Buffer (12), and brought to 150 mM upon addition of 30 µL of 5 M NaCl. Tubes were incubated end-over-end for 1 h at 4 °C to bind biotinylated proteins to the beads. The beads were washed 2 or 3 times with 500 µL of ice-cold YO Lysis Buffer containing 150 mM NaCl and then 2 or 3 times with 500 µL of ice-cold 50 mM ammonium bicarbonate, incubating end-over-end at 4 °C for 10 min per wash. After removal of the final wash, beads were resuspended in either 50 mM ammonium bicarbonate (for proteomics analysis) or 2X Laemmli Sample Buffer (Bio-Rad 1610737) containing 5% (v/v) β-mercaptoethanol (for SDS-PAGE). Samples were stored at -20 °C.
BioID, BirA* and BirA*-cCE cells were induced with tetracycline as above and treated as in (12), except that medium was supplemented with 1 µM biotin for 14 h prior to lysis. Cytoplasmic extract preparation and recovery of biotinylated proteins with streptavidin beads were performed as described above.

**Proteomics analysis**

Protein-bound streptavidin beads were incubated in ice cold acetone in -20 °C overnight to precipitate proteins. Samples were centrifuged 16,000 × g for 10 min, and supernatants were discarded. Pellets were reconstituted in 80 µL of 50 mM ammonium bicarbonate containing 0.02% ProteaseMAX surfactant (Promega V2071) and incubated at 95 °C for 5 min. Samples were reduced with DTT at a final concentration of 5 mM at 56 °C for 20 min and alkylated with iodoacetamide (Sigma Aldrich) at a final concentration of 16.5 mM at room temperature in the dark for 15 min. Tryptic digests were performed by adding 2 µL of 1 µg/µL trypsin supplemented with 1 µL of 1% ProteaseMAX surfactant and incubating at 37 °C for 3 h. Trypsin was inactivated upon addition of trifluoroacetic acid (TFA) to a final concentration of 0.5%. The digestion products were centrifuged 16,000 × g for 10 min, and the supernatants were collected and evaporated to dryness. Approximately 0.5 µg of peptides per sample were separated by reversed-phase ultra-performance liquid chromatography (RP-UPLC) on a Waters nanoACQUITY UPLC system and mass analyzed by a Thermo LTQ-Orbitrap Elite fitted with an EASY-Spray ion source. Peptides were loaded on a Waters Symmetry C18 trap column (100 Å, 5 µm particle diameter, 180 µm x 20 mm) and desalted at a flow rate of 20 µL/min followed by analytical separation on a second C18 reverse phase column (75 µm I.D. x 15 cm, PepMap C18, 3 µm, 100Å, nanoViper) at pH 2.4 using 0.1 % formic acid (A1) and acetonitrile with 0.1 % formic acid (B1) as mobile phases in a 90 min gradient. The heated capillary temperature and electrospray voltage on the LTQ-Orbitrap Elite were 275 °C and 1.7 kV, respectively using top 15 data dependent acquisition in positive ion mode.

**Label-free relative quantitation**

Protein identifications were obtained via the Thermo Proteome Discoverer software (v 1.4.1.14) using the Sequest search algorithm and the Uniprot Swissprot canonical *H. sapiens* proteome (as of 09/04/2016) modified to contain our designed protein sequences. Cysteine carbamidomethylation was set as a fixed modification while oxidation of methionines and deamidation of asparagines and glutamines were all set as variable modifications. Proteins were identified at a 5% false discovery rate (FDR) for peptide spectral matches (PSM) and 0.05 protein FDR. Protein groups were filtered to include only those peptides with 99% confidence and a minimum of two peptides per protein group. The complete mass spectrometry dataset is available from the ProteomeXchange Consortium via the jPOST partner repository under the dataset identifier PXD010368.

**Measurement of RNA abundance and cap status**

Triplicate cultures of U2OS-TR cells were harvested after 24-h treatment with either 0.2 µM geldanamycin or equivalent volume of DMSO. From each culture, cytoplasmic RNA was prepared and qualified as described in (12), and reduced levels of CE upon geldanamycin treatment were confirmed by western blot. Cap status analysis was performed by binding of cytoplasmic RNA to recombinant GST-eIF4E K119A as described in (12). Briefly, for each sample, 400-µL mixtures were prepared on ice containing 1 µg of cytoplasmic RNA, 0.2 ng of 5'-triphosphate luciferase RNA (Promega L4561), and 0.2 ng of 5'-m'G-capped mCherry RNA (TriLink) in a buffer containing 150 mM NaCl, 0.1% (v/v) IGEPAL CA-630, 10 mM Tris pH 7.5, 1 mM DTT, and 0.2 U/µL RNaseOUT (Thermo Fisher 10777019). From each mixture, 200 µL was set aside to be processed as input RNA, and the other 200 µL was incubated with bead-bound GST-eIF4E K119A and washed. Input and eIF4E-recovered RNA were purified using the Direct-zol RNA MiniPrep Kit (Zymo Research R2053), eluting with 25 µL RNase-free water. cDNA synthesis and quantitative PCR were performed as described in (12), using the same primers described therein. Additional primers used were as follows: mCherry-F (ATGGTGAGCAAGGGCGAGGAG), mCherry-
R (GCCACCCTTGGTCACCTTCAGC), 18S rRNA-F (CTGAGAAACGGCTACCACATC) and 18S rRNA-R (GCCTCGAAAGAGTCCTGTATTG). Using Bio-Rad CFX Manager 3.1 software, Cq values were determined by regression mode, and the ΔΔCq method was used to calculate relative abundances of each gene normalized to the capped mCherry spike-in control for each input and eIF4E-recovered sample. The ratio of normalized eIF4E-recovered abundance to normalized input abundance was calculated for each biological replicate as a measure of “normalized eIF4E recovery” (Figure 4B). Individual data points are shown, and statistical analyses (unpaired two-tailed t-tests) were done with GraphPad Prism 6 software for each gene, comparing DMSO control and geldanamycin-treated samples. Results with p < 0.05 were considered significant.

Acknowledgments
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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

References
transcriptional RNA cleavage diversifies the eukaryotic transcriptome. *Genome Res.* **20**, 1639-1650


Table 1. Cytoplasmic CE-interacting proteins identified by LC-MS/MS analysis. Listed are cytoplasmic CE-interacting proteins identified by mass spectrometric analysis of samples recovered on streptavidin beads after formaldehyde crosslinking of cells expressing bio-cCE or bio-EGFP. The total number of peptide spectral matches (PSMs) from three independent experiments is listed. Details of each analysis are in Supplemental Table S1. For each candidate interacting protein, a cCE enrichment index was calculated as the average of cCE PSMs divided by the average of negative control PSMs plus one. Column 6 lists proteins that were also identified by BioID and column 7 identifies proteins that appear in the integrated Human RNA-Binding Proteome (17). Proteins in bold were validated by Western blotting (Figure 2).

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Figure Legends

Figure 1. Development of stable cell lines for identifying the cytoplasmic CE interactome. A. Schematic of bio-EGFP and bio-cCE constructs. Each of these proteins has an N-terminal tag that is biotinylated in vivo, a myc epitope tag and the HIV Rev nuclear export sequence (NES). The nuclear localization sequence has also been removed from CE. B. Immunofluorescence of U2OS-TR/bio-EGFP and U2OS-TR/bio-cCE stable cells grown in the presence (upper panels) or absence of tetracycline (lower panels) for 24 h. Cells were stained with mouse anti-myc antibody and co-stained with DAPI and goat anti-mouse Alexa Fluor 594. The figures to the right show the myc (CE/EGFP) and DAPI channels respectively of the areas in white boxes. C. Cytoplasmic extracts from uninduced and doxycycline-treated cells carrying bio-EGFP or bio-cCE transgenes were analyzed by Western blotting with Qdot 800 streptavidin conjugate. D. 10 µg nuclear (nuc) and 30 µg cytoplasmic (cyto) extracts of bio-cCE stable cells grown ±tetracycline were analyzed by Western blotting with antibody against CE or U2AF35. Note that cytoplasmic extracts have a cross-reacting protein the same size as bio-cCE that is evident in the uninduced sample. E. Proteins recovered on streptavidin beads with bio-EGFP or bio-cCE were separated on a 4-20% SDS-PAGE gel and identified by Coomassie blue staining. Dots indicate differentially recovered proteins.

Figure 2. Validation of interactions with cytoplasmic CE. A. Stably transfected U2OS-TR cells were induced as in Figure 1 to express bio-EGFP or bio-cCE. Cytoplasmic extracts were pre-treated with or without micrococcal nuclease prior to incubation with streptavidin beads. 1% of input and 50% of bead-bound material were analyzed by Western blot analysis using streptavidin-800 conjugate (top panel) and antibodies to FASN, XPO2/CSEL1, EEF2, RUVBL1, RUVBL2, HSP90 and MEP50/WDR77. B. The left panel shows the domain structure of CE and its derivatives with the C-terminal NCK1 binding site indicated. TPase = triphosphatase domain, GTase = guanylyltransferase domain. In the right panel, HEK293 cells were transiently transfected with plasmids expressing each of the indicated domains (bio-CE 2-210, TPase domain) or bio-cCE 211-597 (GTase domain). Cells were processed and analyzed as in A. The CE domain constructs (top panel) were detected with an anti-myc antibody, and co-recovered proteins were detected with antibodies to the indicated proteins. The gels used for these blots had an unrelated sample between lanes containing the input and bound fractions. The excised lanes are indicated by a vertical line.

Figure 3. Impact of HSP90 inhibitors on nuclear and cytoplasmic CE. A. Triplicate cultures of U2OS-TR/bio-cCE cells were induced with 0.2 µg/mL tetracycline and maintained in the presence of 0.2 µM geldanamycin (GA) or DMSO vehicle control for 24 h. Cytoplasmic extracts were analyzed by Western blot with anti-myc and anti-α-tubulin antibodies. Quantified, averaged Western blot data (Li-Cor) are shown graphically, with the bar representing the mean and each dot representing an individual replicate of the myc signal normalized to α-tubulin. B. The experiment in A was repeated using cytoplasmic and nuclear extracts from uninduced U2OS-TR cells. Endogenous CE was detected by Western blotting with anti-CE antibody. For graphical analysis, nuclear CE was normalized to U2AF35 and cytoplasmic CE was normalized to FASN, and individual replicates are indicated as in A on each bar graph. C. U2OS-TR cells were treated with 0.2 µM GA for the indicated time prior to harvesting. Nuclear and cytoplasmic extracts were analyzed by western blotting with antibodies to CE, U2AF35 (nuclear extracts) and α-tubulin (cytoplasmic extracts). D and E. U2OS-TR cells were grown for 24 h in the indicated concentration of radicicol (D) or onalespib (E). Nuclear and cytoplasmic extracts were analyzed as in B with normalized data shown beneath the CE Western blots. * = p < 0.05, ** = p < 0.01, and ***= p < .001 by unpaired two-tailed t-test.

Figure 4. HSP90 inhibition causes widespread decrease in the levels of cytoplasmic capped RNAs. Triplicate cultures of U2OS-TR cells were grown for 24 h in the presence of 0.2 µM GA or DMSO vehicle control. Cytoplasmic RNA was prepared from each culture. Mixtures for each sample were
prepared containing 1 µg cytoplasmic RNA and equal amounts of uncapped luciferase RNA and capped mCherry RNA as normalization and cap-separation controls. Mixtures were split in half, with one half processed as input RNA and the other half added to recombinant eIF4E to select for capped RNAs. RNA in each input and eIF4E-recovered sample was purified and analyzed by quantitative reverse transcription PCR for several genes, with relative abundances in each sample normalized to the mCherry control. In A, the normalized abundance of each input sample is presented by a dot, with bars representing mean values. In B, normalized eIF4E recovery was calculated as the ratio of normalized abundances in eIF4E-recovered RNA to input RNA for each gene in each biological replicate. As such, a value of 1.0 represents recovery of an RNA equivalent to that of capped mCherry RNA. Results are presented as in A with each dot indicating an individual replicate. * = p < 0.05 and ** = p < 0.01 by unpaired two-tailed t-test.

Figure 5. Proposed role for CE-binding proteins in cap homeostasis. The large number of RNA-binding proteins in the cytoplasmic CE interactome suggests a model in which recapping target RNAs are defined by binding of one or more CE-interacting proteins. In this model, the proline-rich C-terminus of CE brings the CE-bound mRNP together with NCK1 and the other enzymes needed to restore the cap on uncapped mRNAs.
Figure 1. Development of stable cell lines for identifying the cytoplasmic CE interactome. A. Schematic of bio-EGFP and bio-cCE constructs. Each of these proteins has an N-terminal tag that is biotinylated in vivo, a myc epitope tag and the HIV Rev nuclear export sequence (NES). The nuclear localization sequence has also been removed from CE. B. Immunofluorescence of U2OS-TR/bio-EGFP and U2OS-TR/bio-cCE stable cells grown in the presence (upper panels) or absence of tetracycline (lower panels) for 24 h. Cells were stained with mouse anti-myc antibody and co-stained with DAPI and goat anti-mouse Alexa Fluor 594. The figures to the right show the myc (CE/EGFP) and DAPI channels respectively of the areas in white boxes. C. Cytoplasmic extracts from uninduced and doxycycline-treated cells carrying bio-EGFP or bio-cCE transgenes were analyzed by Western blotting with Qdot 800 streptavidin conjugate. D. 10 μg nuclear (nuc) and 30 μg cytoplasmic (cyto) extracts of bio-cCE stable cells grown ±tetracycline were analyzed by Western blotting with antibody against CE or U2AF35. Note that cytoplasmic extracts have a cross-reacting protein the same size as bio-cCE that is evident in the uninduced sample. E. Proteins recovered on streptavidin beads with bio-EGFP or bio-cCE were separated on a 4-20% SDS-PAGE gel and identified by Coomassie blue staining. Dots indicate differentially recovered proteins.
Figure 2. Validation of interactions with cytoplasmic CE. A. Stably transfected U2OS-TR cells were induced as in Figure 1 to express bio-EGFP or bio-cCE. Cytoplasmic extracts were pre-treated with or without micrococcal nuclease prior to incubation with streptavidin beads. 1% of input and 50% of bead-bound material were analyzed by Western blot analysis using streptavidin-800 conjugate (top panel) and antibodies to FASN, XPO2/CSEL1, EEF2, RUVBL1, RUVBL2, HSP90 and MEP50/WDR77. B. The left panel shows the domain structure of CE and its derivatives with the C-terminal NCK1 binding site indicated. TPase = triphosphatase domain, GTase = guanylyltransferase domain. In the right panel, HEK293 cells were transiently transfected with plasmids expressing each of the indicated domains (bio-CE 2-210, TPase domain) or bio-CE 211-597 (GTase domain). Cells were processed and analyzed as in A. The CE domain constructs (top panel) were detected with an anti-myc antibody, and co-recovered proteins were detected with antibodies to the indicated proteins. The gels used for these blots had an unrelated sample between lanes containing the input and bound fractions. The excised lanes are indicated by a vertical line.
Figure 3. Impact of HSP90 inhibitors on nuclear and cytoplasmic CE. A. Triplicate cultures of U2OS-TR/bio-cCE cells were induced with 0.2 μg/mL tetracycline and maintained in the presence of 0.2 μM geldanamycin (GA) or DMSO vehicle control for 24 h. Cytoplasmic extracts were analyzed by Western blot with anti-myc and anti-α-tubulin antibodies. Quantified, averaged Western blot data (Li-Cor) are shown graphically, with the bar representing the mean and each dot representing an individual replicate of the myc signal normalized to α-tubulin. B. The experiment in A was repeated using cytoplasmic and nuclear extracts from uninduced U2OS-TR cells. Endogenous CE was detected by Western blotting with anti-CE antibody. For graphical analysis, nuclear CE was normalized to U2AF35 and cytoplasmic CE was normalized to FASN, and individual replicates are indicated as in A on each bar graph. C. U2OS-TR cells were treated with 0.2 μM GA for the indicated time prior to harvesting. Nuclear and cytoplasmic extracts were analyzed by western blotting with antibodies to CE, U2AF35 (nuclear extracts) and α-tubulin (cytoplasmic extracts). D and E. U2OS-TR cells were grown for 24 h in the indicated concentration of radicicol (D) or onalespib (E). Nuclear and cytoplasmic extracts were analyzed as in B with normalized data shown beneath the CE Western blots. * = p < 0.05, ** = p < 0.01, and ***= p < .001 by unpaired two-tailed t-test.
Figure 4. HSP90 inhibition causes widespread decrease in the levels of cytoplasmic capped RNAs. Triplicate cultures of U2OS-TR cells were grown for 24 h in the presence of 0.2 μM GA or DMSO vehicle control. Cytoplasmic RNA was prepared from each culture. Mixtures for each sample were prepared containing 1 μg cytoplasmic RNA and equal amounts of uncapped luciferase RNA and capped mCherry RNA as normalization and cap-separation controls. Mixtures were split in half, with one half processed as input RNA and the other half added to recombinant eIF4E to select for capped RNAs. RNA in each input and eIF4E-recovered sample was purified and analyzed by quantitative reverse transcription PCR for several genes, with relative abundances in each sample normalized to the mCherry control. In A, the normalized abundance of each input sample is presented by a dot, with bars representing mean values. In B, normalized eIF4E recovery was calculated as the ratio of normalized abundances in eIF4E-recovered RNA to input RNA for each gene in each biological replicate. As such, a value of 1.0 represents recovery of an RNA equivalent to that of capped mCherry RNA. Results are presented as in A with each dot indicating an individual replicate. * = p < 0.05 and ** = p < 0.01 by unpaired two-tailed t-test.
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RNA-binding proteins and heat-shock protein 90 are constituents of the cytoplasmic capping enzyme interactome

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