Protein kinase C\(\mathrm{\tau}\) promotes UBF1–ECT2 binding on ribosomal DNA to drive rRNA synthesis and transformed growth of non-small-cell lung cancer cells

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Ribosomes are large ribonucleoprotein complexes that carry out cellular protein production. The synthesis of ribosomes begins with transcription of the ribosomal DNA (rDNA) to generate a 45S pre-rRNA, which is subsequently processed into mature 28S, 18S, and 5.8S rRNAs. These mature rRNAs are then assembled along with the ribosomal proteins into functional ribosomal subunits. rDNA is transcribed by RNA polymerase I (Pol I), which functions in concert with several associated factors, including upstream binding factor 1 (UBF1), a key transcriptional activator of rDNA transcription (1). UBF1 and the Pol I-specific “selectivity” factor (SL1), which contains a TATA-binding protein (TBP), TBP-associated factors, and RRN3 (TIF-1A), together mediate assembly of the pre-initiation complex on the rDNA promoter (2).

The rate-limiting step in ribosome biogenesis is rDNA transcription, and ribosome biogenesis is regulated mainly at the level of rRNA synthesis (3). Furthermore, rDNA transcription is directly linked to elevated protein synthesis, which in turn accommodates cell growth and cell division. The association between altered ribosome biogenesis and cancer is now well-established. Increased rRNA synthesis and ribosome biogenesis are well-known hallmarks of cancer cells, and up-regulated ribosome biogenesis is associated with an increased risk of cancer development (4). Moreover, genetic mutations and oncogenic pathways involved with malignant transformation and cancer progression are associated with increased rDNA transcription. In cancer cells, dysregulation of rDNA transcription is reported to occur by mechanisms involving major oncogenes and tumor suppressors, including c-Myc (5), p53 (6), pRb (7), and PTEN (8). In addition, mitogenic stimuli activate several oncogenic pathways that up-regulate rDNA transcription. For example, the MEK/ERK pathway phosphorylates UBF1 to enhance rDNA transcription (9). Similarly, rDNA transcription is stimulated by growth factor–mediated activation of the mammalian target of rapamycin (mTOR) signaling network, which contributes to the activation of UBF1 and TIF1-A (10, 11). Taken together, these studies show that rDNA transcription is tightly linked to cell growth and is up-regulated in transformed cells by oncogenic mechanisms that control transformed growth.

We previously demonstrated that epithelial cell–transforming sequence 2 (ECT2), a guanine nucleotide exchange factor (GEF) for the Rho-family GTPases, Rac1, RhoA, and Cdc42, is an oncogene in lung cancer (12–16). The ECT2 protein consists of a C-terminal DBL homology and pleckstrin homology (DH/PH) GEF domain and an N-terminal regulatory sequence containing a tandem BRCT domain (17, 18). The linker sequences between the ECT2 N- and C-terminal domains contain two nuclear localization sequences that localize ECT2 to the cell nucleus (19). ECT2 is overexpressed in a variety of human cancers, including the two major subtypes of non-small-cell lung cancers (NSCLC), lung adenocarcinoma (LADC), and lung squamous cell carcinoma (LSCC) (12, 13, 15, 16, 20–27). Functionally, ECT2 regulates multiple signaling pathways that promote NSCLC tumor initiation, cell proliferation, transformed growth, and invasion. Specifically, we demonstrated that in NSCLC cells, a significant pool of ECT2 is mislocalized to the cytoplasm, where it associates with an oncogenic PKC\(\mathrm{\tau}\)-Par6 complex that functions to activate a proliferative RAC1-PAK-

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MEK-ERK signaling axis (15, 16). More recently, we demonstrated that a second, nucleolar pool of ECT2 associates with UBF1 on the promoter region of rDNA in NSCLC cells (12, 13). UBF1-bound ECT2 recruits and activates the small GTPase, Rac1, to rDNA, which in turn stimulates the association of active Rac1 with nucleophosmin (NPM) to drive rDNA transcription, transformed growth, and lung tumor formation in vivo (12, 13). These data revealed for the first time how a key step in ribosome biogenesis, rDNA transcription, is regulated by ECT2 in tumor cells to promote transformation.

In this study, we investigated the underlying mechanism that regulates the association between ECT2 and UBF1 on rDNA. We first assessed the ECT2 domains that mediate the interaction between UBF1 and ECT2. Next, we assessed the upstream activator that drives this interaction. Last, we assessed the importance of the ECT2-UBF1 interaction for UBF1 binding to rDNA and the growth of cancer cells.

Results

The ECT2 BRCT domain is required for UBF1 binding

Our published data established a role for ECT2-mediated rDNA transcription in lung tumorigenesis (12–14, 16). A key gap in knowledge is how the ECT2-UBF1 interaction, which is required for ECT2-dependent rDNA transcription, is regulated in lung cancer cells. The ECT2 protein contains a tandem BRCT domain within its N terminus (17). One of the most well-characterized properties of BRCT domains is their ability to bind phosphorylated proteins (28). The ECT2 BRCT domain binds the mitotic GTPase-activating protein CYK-4, a protein that targets ECT2 to the central spindle during mitosis (29, 30). Biochemical and structural analysis revealed that that make direct contact with CYK-4 disrupts ECT2 binding to CYK-4 and inhibits the mitotic function of ECT2 (29, 30).

Therefore, to assess the role of the tandem ECT2 BRCT domain in UBF1 binding, we expressed either HA-tagged WT ECT2 or a T153A/K195M ECT2 BRCT domain mutant in a panel of NSCLC cells (H358, A549, H1299, and H1703) (Fig. 1). Immunoprecipitation with an anti-HA antibody followed by immunoblot analysis demonstrated that WT ECT2 efficiently co-immunoprecipitated UBF1, whereas the T153A/K195M mutant did not. These data suggest that the ECT2 BRCT domain is important for the interaction between UBF1 and ECT2.

The ECT2 BRCT domain is required for ECT2 binding to rDNA

UBF1 binds rRNA genes and functions in RNA Pol I preinitiation complex formation, transcription initiation, and elongation (2). Our previous studies showed that ECT2 associates with rDNA through a UBF1-dependent mechanism (13). Specifically, we observed UBF1-ECT2 complexes that were most abundant near the rDNA promoter and transcriptional start site of the rDNA genes. Therefore, we next assessed whether the ECT2 BRCT domain is required for UBF1-mediated recruitment of ECT2 to rDNA. For this purpose, we generated H358 and H1703 cells with stable knockdown (KD) of endogenous ECT2 using our previously characterized ECT2 shRNA; a nontarget shRNA that does not target any genes in the human genome served as a negative control (13, 14, 16). In some ECT2 KD cells, we reconstituted ECT2 expression by stably expressing either an HA-tagged WT ECT2 or a T153A/K195M ECT2 mutant in which a silent mutation was introduced to render resistance to shRNA-mediated KD. The empty HA tag vector was used as a negative control for ECT2 reconstitution. Immunoblot analysis revealed efficient loss of endogenous ECT2 using our previously characterized ECT2 shRNA; a nontarget shRNA that does not target any genes in the human genome served as a negative control (13, 14, 16). In contrast, ChiP-qPCR assays on these cells using two independent primer sets directed to sites proximal to the transcriptional start site in the rDNA promoter region to assess UBF1 binding (Fig. 2B, primer sets A and B). Results revealed abundant UBF1 binding to rDNA that was unaffected by either ECT2 KD or repression of WT or BRCT domain mutant ECT2 in ECT2 KD cells (Fig. 2C).
revealed significant binding of WT ECT2, but not the ECT2 BRCT domain mutant, to rDNA (Fig. 2D). Taken together, these data indicate that the ECT2 BRCT domain is not required for UBF1 binding to rDNA but is required for recruitment of ECT2 into the UBF1-ECT2 binding complex near the transcriptional start site of rDNA.

**The ECT2 BRCT domain is required for rRNA synthesis**

The data above suggest that interactions between UBF1 and the ECT2 BRCT domain serve to recruit ECT2 to rDNA to enhance rRNA synthesis in NSCLC cells. To test this hypothesis, we assessed 45S rRNA levels, a proximal measure of rDNA transcription, in NT and ECT2 KD cells expressing vector control or WT or BRCT mutant ECT2 (Fig. 2A and Fig. S1A). qPCR analysis for 45S revealed that ECT2 KD resulted in decreased rDNA transcription, which is restored by expressing WT ECT2 (Fig. 3A), consistent with our published results (12, 13). In contrast, the BRCT domain ECT2 mutant failed to restore rDNA transcription in ECT2 KD cells. These data strongly indicate that rDNA-bound UBF1 recruits ECT2 to rDNA via the ECT2 BRCT domain to promote rDNA transcription.

**The ECT2 BRCT domain is required for NSCLC transformed growth**

ECT2 is functionally important for anchorage-independent growth of cancer cells, including NSCLC cells (12–14, 16, 31–33). Furthermore, we have demonstrated that ECT2-dependent rDNA transcription is required for NSCLC transformation. Therefore, we assessed whether the ECT2 BRCT domain...
domain, which links ECT2 to UBF1-dependent rDNA transcription, is required for anchorage-independent growth of NSCLC cells in vitro. As expected, ECT2 KD cells expressing empty vector exhibited impaired anchorage-independent growth measured by soft agar colony formation when compared with NT control cells (Fig. 3, B and C), consistent with our published results (12–14, 16). Interestingly, expression of exogenous WT ECT2 significantly restored anchorage-independent growth to ECT2 KD cells, whereas expression of the BRCT domain ECT2 mutant did not (Fig. 3, B and C). These results demonstrate that a functional ECT2 BRCT domain is important for the oncogenic activity of ECT2.

**PKCα-mediated phosphorylation of UBF1 generates an ECT2 BRCT domain phosphopeptide-binding motif**

The ECT2 BRCT domain mediates phosphorylation-specific binding of ECT2 to the mitotic GTPase-activating protein CYK-4, a protein that targets ECT2 to the central spindle during mitosis (29, 30). Given the role of the ECT2 BRCT domain in UBF1 binding, we sought to determine whether phosphorylation of UBF1 may mediate UBF1-ECT2 binding interactions. Mass spectrometric analysis of tryptic digests of UBF1 immunoprecipitated from nuclear lysates of H1299 NSCLC cells identified five phosphorylation sites on UBF1: Thr-201, Ser-273, Ser-412, and Ser-493 and Ser-584 (Fig. 4A; see also Table S1 and Fig. S2). These results provide independent validation of a previously reported phosphoproteomic screen indicating that each of these sites is phosphorylated on UBF1 in cells (34, 35) and suggest their physiologic relevance. Inspection of the UBF1 amino acid sequence adjacent to the identified phosphorylated amino acid residues revealed a sequence, S412AMF that conforms to a BRCT domain phosphopeptide-binding motif (SXX(F/W)). Interestingly, Prosite phosphorylation site prediction software suggests that Ser-412 may serve as a consensus phosphorylation site for the oncogenic serine/threonine kinase atypical protein kinase Cα (PKCα). Therefore, we next assessed whether PKCα can phosphorylate UBF1. For this purpose, we combined purified recombinant PKCα with full-length recombinant, bacterially expressed UBF1 in an in vitro kinase assay (Fig. 4B). Results demonstrated that PKCα can phosphorylate UBF1 as detected by immunoblotting with pIMAGO reagent (Fig. 4B). The phosphorylated UBF1 band was excised.
and subjected to MS to identify recovered phosphorylated UBF1 peptides. Analysis using the A-score algorithm threshold of 19, which corresponds to \(99\%\) certainty of a correct assignment, revealed that PKC\(\alpha\) phosphorylated only one of the phosphorylation sites identified in H1299 cells, Ser-412 (Table S1 and Fig. S2). MS analysis of UBF1 recovered from PKC\(\alpha\) KD H1299 cells revealed a loss of Ser-412 phosphorylation indicating that PKC\(\alpha\) is a relevant Ser-412 UBF1 kinase in vivo (Table S1).

**UBF1 Ser-412 phosphorylation regulates UBF1 binding to ECT2**

UBF1 Ser-412 conforms to a BRCT domain phosphopeptide-binding motif, suggesting that it may be involved in mediating the interaction between UBF1 and ECT2. To assess the importance of Ser-412 phosphorylation in binding of UBF1 to ECT2, we mutagenized Ser-412 to an alanine (S412A) to eliminate Ser-412 phosphorylation. We next expressed either FLAG-tagged WT or S412A mutant UBF1 in the same panel of NSCLC cells utilized in Fig. 1 (Fig. 4C). Immunoprecipitation with an anti-FLAG antibody demonstrated that WT UBF1 efficiently co-immunoprecipitated endogenous ECT2, whereas the phosphomutant S412A did not (Fig. 4C). These data indicate that phosphorylation of UBF1 at Ser-412 can regulate binding of UBF1 to ECT2.

**UBF1 phosphorylated at Ser-412 is required for UBF1-mediated recruitment of ECT2 to the rDNA**

UBF1 contains six tandem HMG box domains, motifs that mediate UBF1 binding to rDNA. Ser-412 resides between the HMG box domains 4 and 5 in UBF1, suggesting that Ser-412 phosphorylation could regulate UBF1 rDNA binding. To assess the importance of Ser-412 phosphorylation in UBF1 binding to rDNA, we first introduced a silent mutation into the FLAG-tagged WT and S412A UBF1 cDNAs in Fig. 4C to render them resistant to our previously characterized UBF1 shRNA construct (13). Immunoblot analysis of H358 and H1703 cells with stable KD of endogenous UBF1 and stably transduced with WT UBF1, S412A UBF1, or empty control vector revealed efficient loss of endogenous UBF1 and

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Figure 4. PKC\(\alpha\) phosphorylation of UBF1 generates an ECT2 BRCT domain phosphopeptide-binding motif. A, schematic diagram showing the domain structure of UBF1 and phosphorylation sites identified by MS in UBF1 expressed in NSCLC cells. B, recombinant human UBF1 and recombinant PKC\(\alpha\) alone or in combination were incubated in kinase reaction buffer containing ATP. Phosphorylated proteins were detected by immunoblot analysis using pIMAGO. Mass spectrometry analysis of UBF1 phosphorylated by PKC\(\alpha\) in an in vitro kinase assay identified UBF1 S412 (red) as PKC\(\alpha\) substrate, which conforms to an ECT2 BRCT domain phosphopeptide-binding motif (SXX(F/W)) (see A). C, H1703, H1299, A549, and H358 NSCLC cells expressing FLAG-empty vector (Vec), FLAG-WT UBF1, or FLAG-S412A UBF1 mutant were immunoprecipitated for FLAG, and immunoprecipitates were immunoblotted for ECT2 and FLAG. Confirmation of FLAG-WT UBF1 or S412A mutant UBF1 expression was performed on total cell lysates (Input).
expression of WT and S412A UBF1 to levels similar to NT control cells (Fig. 5A). As expected, ChIP-qPCR analysis revealed that UBF1 KD cells showed a significant decrease in UBF1 binding to rDNA (Fig. 5B). Interestingly, no significant differences were observed in the ability of WT and S412A UBF1 to bind rDNA (Fig. 5B). Next we assessed whether phosphorylation of UBF1 at Ser-412 is required for UBF1 recruitment of ECT2 to rDNA. Consistent with our published results, UBF1 KD significantly decreased ECT2 binding to rDNA, which could be restored by expression of WT UBF1 but not the S412A UBF1 mutant (Fig. 5C). Taken together, these results demonstrate that phosphorylation of UBF1 at Ser-412 contributes little to UBF1 binding to rDNA but is required for ECT2 binding and recruitment to rDNA.

Figure 5. Phosphorylated UBF1 Ser-412 is required for UBF1 recruitment of ECT2 to the rDNA. A, immunoblot analysis of H358 and H1703 NSCLC cells stably transduced with nontarget (NT) RNAi or co-transduced with UBF1 RNAi and FLAG-empty vector (Vec), FLAG-wildtype (WT) UBF1 or FLAG-S412A UBF1 mutant. Shown is ChIP analysis in cells described in A for UBF1 (B) and ECT2 (C) binding to rDNA promoter regions (see Fig. 2): primer set A (solid symbols) and primer set B (open symbols). Data represent the mean ± S.D. (error bars); n = 3. *, p < 0.05 compared with NT; **, p < 0.05 compared with vector; ***, p < 0.05 compared with WT.
UBF1 phosphorylated at Ser-412 is required for rRNA synthesis

UBF1 is essential for transcription of the rRNA genes. Our results show that phosphorylation of UBF1 at Ser-412 does not appear to regulate UBF1 binding to the rDNA promoter. However, UBF1 Ser-412 mediates recruitment of ECT2 to the rDNA, which promotes rDNA transcription, suggesting that phosphorylated Ser-412 may be required for rDNA transcription. To test this hypothesis we assessed 45S rRNA levels in NT and UBF1 KD cells expressing vector control or WT or S412A mutant UBF1 (Fig. 5 A and Fig. S1 B). qPCR analysis revealed that UBF1 KD resulted in decreased rDNA transcription, which could be restored by expression of WT UBF1. In contrast, the UBF1 S412A mutant failed to restore rDNA transcription in UBF1 KD cells (Fig. 6A). Thus, our data indicate that phosphorylation of UBF1 at Ser-412 is necessary for UBF1-dependent transcription of the rDNA.

UBF1 phosphorylated at Ser-412 is required for NSCLC transformed growth

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Discussion

The synthesis of RNA is a major rate-limiting step in ribosome biogenesis, a process that determines the capacity of a cell to synthesize proteins and regulate growth. To sustain uncontrolled growth and proliferation, cancer cells have acquired mechanisms to stimulate rDNA promoter activity. We previously demonstrated that activation of the ECT2 oncogene on rDNA is one such mechanism to promote rDNA transcription and enhance NSCLC transformation. Here, we identify the mechanism by which UBF1 binds and recruits ECT2 to the rDNA promoter to enhance rDNA transcription and drive the transformed phenotype of NSCLC cells.
required for UBF1 binding to the rDNA promoter. Rather, phosphorylated Ser-412 UBF1 serves as a binding site for the ECT2 BRCT domain, thereby regulating recruitment of ECT2 into UBF1-ECT2 complexes at the rDNA promoter. ECT2, in turn recruits its downstream effector Rac1, which activates a Rac1-NPM signaling axis that stimulates rDNA transcription to promote the transformed phenotype of NSCLC cells. Our studies are the first to assess the role of the ECT2 BRCT domain in the transforming activity of ECT2. Our results reveal a PKCα-regulated interaction between ECT2 and UBF1 that drives the oncogenic function of ECT2 in transformed cells.

UBF1 is a member of the high-mobility group (HMG) box protein family, which contains six conserved HMG box DNA-binding domains (37). The first HMG box is reported to be sufficient for the association of UBF1 with rDNA, whereas the other HMG boxes enhance UBF1 binding efficiency (38, 39). UBF1 is often the target for oncogene- and tumor suppressor-induced activation of rDNA transcription. Regulation of UBF1 activity by phosphorylation is a mechanism for regulating rRNA synthesis. For example, phosphorylation of UBF1 at Ser-388 by CDK2/cyclin plays a key role in modulating UBF1 DNA-binding activity and its interaction with other components of the Pol I transcriptional machinery (40). Another report demonstrated that ERK1/2 was able to phosphorylate UBF1 at amino acids Thr-117 and Thr-201 within HMG boxes 1 and 2, thereby preventing their interaction with rDNA (9). Our MS analysis of UBF1 in NSCLC cells revealed phosphorylation at Thr-201, indicating that ERK-mediated phosphorylation at this site may play a role in regulating UBF1 function in these cells. Importantly, our data also revealed phosphorylation at Ser-412 of UBF1. Our subsequent analysis demonstrated that phosphorylation at this site is important for enhanced rDNA and ECT2 oncogenic activity in NSCLC, a conclusion supported by the observation of a lack of ECT2 binding and inhibition of 45S rRNA synthesis and transformed growth by expression of a Ser-412 mutant UBF that cannot be phosphorylated on Ser-412 (S412A).

BRCT domains were initially identified in breast cancer-associated 1 (BRCA1), a breast and ovarian cancer susceptibility gene (41, 42). A large family of proteins that contain BRCT domains have been well-characterized for their ability to recognize phosphopeptides to mediate recruitment and regulation of proteins in signal transduction. The best-characterized role for BRCT domain–containing proteins is in the DNA damage response (43). However, there is increasing evidence to support a role for BRCT domain–containing proteins in other pathways as well. For example, the ECT2 BRCT domain has been shown to play a central role in regulating the physiologic function of ECT2 in cytokinesis. Interestingly, many cancer cells have developed mechanisms to overcome the requirement for ECT2 during cytokinesis, and we and others have clearly demonstrated that the oncogenic function of ECT2 is distinct from its role in cytokinesis (13, 16, 33, 44). Our current data demonstrate that in addition to regulating the physiologic function of ECT2 in nontransformed cells, the ECT2 BRCT domain also plays a key regulatory role in controlling oncogenic ECT2 signaling.

Atypical protein kinase Cα (aPKCα) is an oncogene that regulates a number of signal transduction pathways that promote the transformed phenotype of a variety of cancer cell types, including NSCLC (45–47). We previously identified ECT2 as a direct substrate for PKCα. Specifically, PKCα phosphorylates ECT2, at Thr-328 to regulate the oncogenic activity of ECT2 in NSCLC cells (14), including its function in regulating rDNA transcription (12, 13). Our current results identify another critical substrate for PKCα, UBF1, that drives elevated rDNA transcription in NSCLC cells. This PKCα-mediated mechanism of rDNA activation, mediated through phosphorylation of both
ECT2 and UBF1 to drive their interaction and activation, is reminiscent of the actions of Polo-like kinase 1 (PLK1), a mitotic kinase with critical functions in cytokinesis. PLK1 triggers cytokinesis by phosphorylating CYK-4, thereby creating a phospho-dependent docking site for ECT2 at the central spindle (48). In addition, PLK1 has also been reported to phosphorylate ECT2 to regulate its function in cytokinesis (49). Similar to PLK1 regulation of ECT2 and CYK-4 binding and function in cytokinesis, PKCζ phosphorylation appears to both regulate spatio-temporal activation of ECT2 and promote ECT2-UBF1 binding on rDNA, thereby driving UBF1-dependent rDNA transcription in NSCLC cells. Interestingly, our previous studies have shown that the oncogenic function of ECT2 requires PKCζ-dependent phosphorylation at Thr-328 but not CDK1- or PLK1-mediated phosphorylation of ECT2 at sites required for control of mitotic functions of ECT2 (14). Therefore, phospho-Thr-328 ECT2 and phospho-Ser-412 UBF1 may serve as markers of oncogenic PKCζ signaling in NSCLC cells.

Recently, PKCζ was reported to control c-Myc expression and promote the proliferation of angiosarcoma cells (50). Although rDNA transcription was not assessed in PKCζ-deficient cells in this study, given the well-characterized role of Myc in regulating rDNA transcription, it is plausible that PKCζ may regulate rDNA transcription, at least in part, through regulation of c-Myc expression in angiosarcoma cells. Taken together, these data suggest that PKCζ regulates the function of multiple effectors that stimulate rDNA transcription.

Our results demonstrate that UBF1 Ser-412 phosphorylation is not required for UBF1 binding to the rDNA promoter, strongly suggesting that UBF1 binding to rDNA precedes PKCζ phosphorylation of UBF1 Ser-412. Therefore, our data provide evidence that PKCζ functions within the nucleus of NSCLC cells. Interestingly, mislocalization of PKCζ to both the cytoplasm and nucleus is a common observation in cancer cells. For instance, in normal cervical epithelium, PKCζ is largely localized to cell junctions (51). In contrast, ~37% of cervical cancer cases examined exhibited PKCζ nuclear localization. Furthermore, PKCζ mislocalization to the nucleus is associated with cervical tumor progression and worse prognosis (51). It will be interesting to determine whether nuclear PKCζ in cervical cancer cells also promotes enhanced rDNA through similar mechanisms identified in our current study. Our published studies have demonstrated that PKCζ phosphorylates other targets within the nucleus to promote the transformed phenotype of cancer cells. Specifically, PKCζ phosphorylates the oncogenic transcription factors SOX2 and ELF3 in LSCC and LADC cells, respectively, leading to activation of transcriptional activity (52–55). Indeed, our recent study demonstrated that PKCζ-mediated SOX2 phosphorylation is required for expression of a SOX2 transcriptional program that drives lineage-restricted transformation of lung basal stem cells, a major cell of origin, into LSCC (55).

Ribosome biogenesis and rDNA transcription is a common target of prosurvival and proliferative signaling pathways that are activated in transformed cells. Given the importance of upregulated rRNA synthesis in cancer biology, blockade of this process may be an effective strategy to therapeutically target cancer cells. Recently, small-molecule inhibitors have been developed, such as CX-5461, that specifically target RNA Pol I and rDNA transcription. Interestingly, NSCLC cells exhibiting elevated ECT2-mediated rDNA transcription are highly sensitive to the growth-inhibitory effects of CX-5461 (13). Likewise, we have found that the PKCζ inhibitor, auranofin (ANF) inhibits transformed growth of NSCLC cells, at least in part, by blocking nuclear ECT2-, Rac1-dependent rDNA transcription (13, 56). Vertical blockade, the process of using a combination of inhibitors that target multiple points of a pathway, has been shown to be an effective therapeutic strategy. Perhaps one of the best reported examples of this combination strategy is combined BRAF and MEK inhibition for treatment of NRAS and BRAF-mutant melanomas (57, 58). Therefore, targeting NSCLC cells with ANF and CX-5461, which both act through inhibition of rRNA synthesis, may synergistically and more effectively inhibit the growth of NSCLC cells. ANF and CX-5461 are approved by the Food and Drug Administration and currently being tested clinically in cancer patients (59). Therefore, a synergistic effect of combined therapy with ANF and CX-5461 in preclinical studies can be rapidly translated to the clinic for the treatment of NSCLC patients.

**Experimental procedures**

**Antibody reagents and cell lines**

The following antibodies were used in these studies: ECT2 (Millipore, Danvers, MA, USA), UBF1 (Santa Cruz Biotechnology, Dallas, TX, USA), FLAG epitope (Sigma–Aldrich), HA and β-actin (Cell Signaling, Danvers, MA, USA), and FLAG epitope (Sigma–Aldrich). The H358, H1703, A549, and H1299 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in low-passage culture as recommended by the supplier.

**In vitro kinase assays**

PKCζ in vitro kinase assays were performed as described previously (60). Briefly, purified recombinant bacterially expressed full-length human UBF1 was incubated in the presence or absence of 100 ng of recombinant human PKCζ (Millipore) in reaction buffer (20 mM Tris-HCl pH 7.5, 10 mM CaCl2, 10 mM MgCl2, and 40 μg/ml phosphatidic acid) supplemented with 200 μM ATP for 30 min at 25 °C. Reactions were stopped by the addition of 2X Laemmli buffer. Phosphorylation of recombinant UBF1 was visualized using the pIMAGO-based detection of phosphorylated proteins according to the manufacturer protocol (Millipore–Sigma). Briefly, kinase reactions were boiled, run on SDS-PAGE, and transferred onto polyvinylidene difluoride membrane. Membranes were blocked in pIMAGO blocking buffer overnight followed by incubation in pIMAGO-Biotin reagent for 1 h. After three washes (5 min each) with wash buffer, the membrane was incubated with avidin-horseradish peroxidase for 1 h. After washing three times with 1X TBST, phosphorylation signals were detected by ECL reagent. For loading control, total protein was visualized using Coomassie staining. Specific phosphorylation sites on UBF1 were determined by MS as described below.
Mass spectrometry analysis of UBF1 phosphorylation

UBF1 was immunoprecipitated from Nuclear extracts of H1299 cells as described previously (16). Immunoprecipitated UBF1 was resolved by SDS-PAGE, and the band corresponding to UBF1 was excised and submitted to the Mayo Clinic Medical Genome Facility Proteomics Core for proteolytic cleavage and phosphorylation site analysis by MS. The SDS-polyacrylamide gel bands were prepared for MS analysis using the following procedures. Silver-stained gel bands were destained and reduced with 50 mM tris(2-carboxyethyl)phosphine, 50 mM Tris, pH 8.2, at 55 °C for 20 min and then alkylated with 25 mM iodoacetamide at room temperature for 30 min in the dark. Proteins were digested in situ with 50 μl (0.0025 mg/μl) trypsin (Promega, Madison, WI, USA) in 25 mM Tris, pH 8.2, 0.0002% Zwittergent 3-16 at 37 °C overnight, followed by peptide extraction with 25 μl of 2% TFA and then 50 μl of acetonitrile. The pooled extracts are concentrated to less than 5 μl on a SpeedVac spinning concentrator (Savant Instruments, Holbrook, NY, USA) and then brought up in 0.15% formic acid, 0.05% TFA for protein identification by nano-flow LC electrospray tandem MS, using a Thermo Scientific Q-Exactive Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to a Thermo Ultimate 3000 RSLCnano HPLC system. The digest peptide mixture is loaded onto a 330-nl OPTI-PAK EXP trap (Optimize Technologies, Oregon City, OR) packed with Halo 2.7-μm C18 material. Chromatography was performed using 0.2% formic acid in both the A solvent (98% water, 2% acetonitrile) and B solvent (80% acetonitrile, 10% isopropyl alcohol, 10% water), and a 2–40% B gradient over 43 min at 400 nl/min through a 100 μm × 34-cm PicoFrit column (New Objective, Woburn, MA) hand-packed with Agilent Poroshell 120 EC C18 material. The Q-Exactive Plus mass spectrometer setup was a FT full scan from 340 to 1500 m/z at a resolution of 70,000 (at 200 m/z), followed by HCD MS/MS scans on the top 15 ions having a charge state of +2, +3, or +4, at a resolution of 17,500. The selected ions were placed on an exclusion list for 15 s. The MS1 automatic gain control target was set to 1e6 with 50-ms maximum ion inject time, and the MS2 target was set to 1e5 with a maximum ion inject time of 100 ms.

The MS/MS raw data were searched using PEAKS X (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) set up to search the Swissprot human protein database (January 2020, 20,363 entries), with a concatenated decoy reverse database and assuming the digestion enzyme full trypsin. The fragment ion mass tolerance was set to 0.02 Da, and the parent ion tolerance was limited to 10.0 ppm. Oxidation of methionine and sulfation and phosphorylation of serine, threonine, and tyrosine were specified as variable modifications, and carbamidomethyl cysteine was specified as a fixed modification. A-score values are used to assess phosho-site assignments. Protein identification required a minimum of 2 peptides matched and a z > 10lgP > 20. The peptide spectral matches are filtered at a 0.5% false discovery rate. Phosphopeptide identifications and site assignments were inspected manually.

Plasmids, lentiviral RNAi constructs, cell transduction, and immunoblot analysis

Previously characterized lentiviral RNAi against human ECT2 and UBF1 were obtained from the Sigma–Aldrich Mission short hairpin RNA library, packaged into recombinant lentiviruses, and characterized for target gene KD as described previously (61). A nontarget lentiviral RNAi (NT-RNAi) that does not recognize any human genes was used as a negative control. RNAi target sequences and characterization of the specificity of RNAi reagents were published previously (13, 16). The cDNA of full-length human RNAi-resistant ECT2 described previously (16) was mutagenized to encode an alanine substitution at Thr-153 (T153A) and a methionine substitution at Lys-195 (K195M). A lentiviral plasmid containing full-length UBF1 was obtained from Addgene (plasmid 26672, Watertown, MA). The UBF1 cDNA was rendered RNAi-resistant by introducing silent mutations that disrupt the UBF1-RNAi target site as described previously (61). The cDNA of full-length human RNAi-resistant UBF1 was mutagenized to encode an alanine substitution of Ser-412. Stably transduced NSCLC cell populations were generated as described previously (61). ECT2 and UBF1 RNAi constructs were analyzed for efficiency of target gene KD expression by qPCR and immunoblot analysis as described previously (13, 16). Cell lysates were prepared in radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitor mixtures I and II (Sigma–Aldrich) and subjected to immunoblot analysis as described previously (62). shRNA targeting sequences, qPCR primers, and mutagenesis primers are provided in Fig. S3.

Immunoprecipitations

NSCLC cells were transduced with lentiviral packaged empty vector, WT ECT2, T153A/K195M ECT2, WT UBF1, or S412A UBF1. Cell lysates were subjected to immunoprecipitation and immunoblot analysis as described previously (13).

ChIP

ChIP assays were performed to assess occupancy of UBF1 and ECT2 on the rDNA promoter as follows. Cells were cross-linked with 1% formaldehyde and lysed, and cytoplasmic protein fractions were discarded. Nuclear protein fractions were sonicated to obtain DNA fragments of ~500 bp, as determined by agarose gel electrophoresis and ethidium bromide staining. Precleared supernatants were first incubated with a UBF or IgG control antibody (Santa Cruz Biotechnology) overnight followed by a 3-h (4 °C) incubation with Protein A/G-conjugated agarose beads (Santa Cruz Biotechnology). Protein A/G bead complexes were washed, and protein-DNA complexes were eluted in 1% SDS and 100 mM NaHCO3. For co-occupancy experiments, eluants were subjected to a second ChIP with ECT2 antibody. Cross-links were reversed by incubation overnight in elution buffer containing 200 mM sodium chloride. DNA was extracted, purified, precipitated, and resuspended in Tris-EDTA for qPCR using primer sets designed to amplify the proximal and distal regions of the human rDNA promoter (see
list of primers in Fig. S3). qPCR was performed using the SYBR® Green (Life Technologies, Inc.) dye detection method on an Applied Biosystems ViiA7 thermal cycler. The comparative Ct method was used for quantitation.

**RNA isolation and 45S rRNA expression**

Total cellular RNA was isolated from cells using the RNAeasy Plus Mini Kit (Qiagen, Valencia, CA). 45S pre-rRNA abundance was assessed by qPCR using SYBR Green as described above. 45S pre-rRNA levels were normalized using β-actin.

**Soft agar growth**

Anchorage-independent growth was assayed as described previously (16, 61, 62). Experiments were independently conducted in triplicate at least three times.

**Data availability**

All raw MS data have been deposited in MassIVE with the data set identifier MSV000085269. All other data are contained within the article and associated supporting information.

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**Abbreviations**—The abbreviations used are: rDNA, ribosomal DNA; Pol I, RNA polymerase I; GEF, guanine nucleotide exchange factor; NSCLC, non-small-cell lung cancer; LADC, lung adenocarcinoma; LSCC, lung squamous cell carcinoma; HA, hemagglutinin; KD, knockdown; qPCR, quantitative PCR; PKCα, protein kinase Cα; aPKC, atypical protein kinase C; HMG, high-mobility group; ANF, auranofin; NPM, nucleophosmin.

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