A coactivator trap identifies NONO (p54^{nrb}) as a component of the cAMP-signaling pathway

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Signal transduction pathways often use a transcriptional component to mediate adaptive cellular responses. Coactivator proteins function prominently in these pathways as the conduit to the basic transcriptional machinery. Here we present a high-throughput cell-based screening strategy, termed the “coactivator trap,” to study the functional interactions of coactivators with transcription factors. We applied this strategy to the cAMP signaling pathway, which utilizes two families of coactivators, the cAMP response element binding protein (CREB) binding protein (CBP)/p300 family and the recently identified transducers of regulated CREB activity family (TORCs1–3). In addition to identifying numerous known interactions of these coactivators, this analysis identified NONO (p54^{nrb}) as a TORC-interacting protein. RNA interference experiments demonstrate that NONO is necessary for cAMP-dependent activation of CREB target genes *in vivo*. Furthermore, TORC2 and NONO complex on cAMP-responsive promoters, and NONO acts as a bridge between the CREB/TORC complex and RNA polymerase II. These data demonstrate the utility of the coactivator trap by identification of a component of cAMP-mediated transcription.

transcription | signal transduction | cell-based screen | RNA polymerase II | transducer of regulated cAMP response element-binding protein

Transcription is regulated by large multisubunit complexes that can be grouped into three general categories; DNA binding proteins, coregulators (coactivators and corepressors), and basal transcriptional components. DNA binding proteins recognize discrete sequences or response elements within promoters and in general function as scaffolds that direct the recruitment of coregulatory proteins. Coregulators in turn function as a conduit to the basic transcriptional machinery. Coregulators may also influence gene expression via intrinsic enzymatic activity or by recruitment of other enzyme activities (e.g., acetylation, methylation, poly ADP-ribosylation, ubiquitination, sumoylation, or ATP-dependent remodeling complexes) capable of modifying both transcriptional proteins and chromatin (1). Thus, determining the interaction networks of coregulators recruited by transcription factors and the accompanying enzymatic activities is necessary for understanding the complexities of gene expression.

The cAMP signal-transduction pathway activates transcription by stimulating interactions between cAMP response element binding protein (CREB) and two coactivator families, CREB-binding protein (CBP)/p300 and transducers of regulated CREB (TORCs) (2–4). CREB–CBP/p300 interaction occurs when elevations in intracellular cAMP liberate protein kinase A (PKA) catalytic subunits (PKA_c) from PKA regulatory subunits. PKA_c directly phosphorylates serine 133 in the kinase-inducible domain of CREB, increasing the affinity of CBP/p300 for CREB (5, 6). CBP/p300 interacts with components of the RNA polymerase II (RNA pol II) complex to facilitate transcription and contains intrinsic acetyltransferase activity speculated to facilitate transcriptional activation by affecting chromatin structure (7–10).

TORC recruitment to CREB-bound promoters by PKA_c is less direct. Under basal conditions, TORC1 and TORC2 are phosphorylated by AMP/SNF kinases (11, 12) and bound by 14-3-3 proteins that sequester TORCs in the cytoplasm. As levels of cAMP rise, PKA_c phosphorylates SNF kinases, inhibiting their phosphorylation of TORCs. Dephosphorylated TORC1 and TORC2 are then released from 14-3-3 proteins, translocated to the nucleus, and bound to CREB (12, 13). Remarkably, the phosphorylation status of TORCs and cytoplasmic retention by 14-3-3 can serve to integrate converging cellular signals. For example, hormone and energy-sensing pathways converge on TORC2 phosphorylation to modulate glucose output via CREB-mediated hepatic gene expression (11). Furthermore, in excitable cells, TORC phosphorylation functions as a coincidence detector that funnels cAMP and calcium-signaling pathways to CREB-dependent transcription (12).

TORCs function as robust transcriptional activators at cAMP-responsive promoters (3). Ectopic expression of TORCs bypasses normal regulatory mechanisms and activates reporter genes many hundredfold, suggesting that TORCs either have intrinsic enzymatic activity or recruit catalytic proteins. Recent studies demonstrate that TORC2 mediates target gene activation in response to cAMP in part by cooperative interactions with CBP (14, 15); however, CBP/p300 recruitment cannot fully explain how TORCs robustly activate transcription. Moreover, the contribution of each of these coactivators varies, depending on the cAMP-responsive promoter (15).

To better understand the mechanisms by which TORCs influence cAMP-mediated transcription, we sought to identify a more complete cohort of functional TORC-interacting transcription factors. We developed the “coactivator trap,” a high-throughput screen with a functional readout for interactions. Here we apply our screening method to the cAMP signaling pathway and identify NONO (p54^{nrb}) as a component of cAMP signaling that interacts with TORCs to coordinate transcription by recruiting RNA pol II.

Results

A Functional Transcription Factor Trap Identifies Proteins Interacting with TORC Coactivators. The coactivator trap was developed to screen for interacting partners of TORCs in a mammalian cell

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Fig. 2. cAMP signaling stimulates TORC–NONO complex formation. (a) (Left) Endogenous NONO coimmunoprecipitates with TORC2. TORC2 was immunoprecipitated by using affinity-purified 638A, 3363, or 3364 α -TORC2 antibody (TORC-IP) or beads alone (Control-IP) from cell lysates stimulated with forskolin (+) or vehicle (–) and were immunoblotted (IB) with α -NONO or α -p65/NF κ B. (Right) Pre-IP of NONO and p65/NF κ B protein (2% of input) is shown. (b) (Left) FRET analysis of interaction of TORC and NONO. Shown are representative pseudocolored fluorescence images of cells cotransfected with CFP–TORC and GFP–NONO expression plasmids and treated with DMSO (control) or forskolin and IBMX for 1 h. (Scale bar, 10 μ m.) (Right) Percentage of overlap in fluorescence energy of CFP–TORC and GFP–NONO expression plasmids in control and forskolin-treated cells ($n = 5$ experiments; mean $\pm$ SEM; each experiment was the average of 10 measurements). (c) (Left) FRET/GFP ratio as captured in pseudocolored fluorescence images of transfected cells after control and forskolin treatment for 1 h. (Scale bars, 10 μ m.) Shown are FRET efficiency (Center) and the distance between CFP donor and GFP acceptor (Right) between CFP–TORC and GFP–NONO in control and forskolin-treated cells ($n = 5$ experiments; mean $\pm$ SEM). Data are from at least five regions of interest (ROI) per treatment group from three independent experiments.

filtering criteria in secondary assays. Approximately 90% of the observed interactions were confirmed in independent assays, indicating that this method generates an $\approx$ 10% false positive rate. By using this criteria, several factors were found to be strongly activated in the coactivator trap by TORCs. NONO (p54^{nrb}) was of particular interest because a previous study implicated NONO in cAMP signaling (21). Furthermore, we analyzed immunoprecipitated TORC2 cellular protein complexes in a parallel study by using nano-liquid chromatography coupled to tandem mass spectrometry (nLC-MS/MS). This study also revealed interactions of NONO protein with TORC2 after stimulation with the adenylate cyclase agonist forskolin, which elevates intracellular cAMP levels (SI Fig. 6).

cAMP Signaling Stimulates TORC–NONO Complex Formation. We performed endogenous coimmunoprecipitation experiments focusing on TORC2, which is natively expressed in HEK293T cells. Lysates from cells stimulated with forskolin or DMSO vehicle as a control were immunoprecipitated with three different antibodies that recognize endogenous TORC2. Complete immunodepletion of TORC2 from cell lysates resulted in consistent recovery of cellular NONO in a TORC2 containing complex,

whereas a non-TORC interacting factor, NF κ B/p65, was not present in the TORC2 immunoprecipitate. Moreover, forskolin stimulation resulted in a marked increase in NONO recovery (Fig. 2a).

The formation of an endogenous TORC–NONO complex prompted us to explore whether TORC2 and NONO were colocalized in the cell. Under basal conditions, immunocytochemistry of endogenous TORC2 and NONO demonstrated little colocalization. However, elevated cAMP levels stimulated by forskolin treatment resulted in nuclear colocalization of endogenous proteins (SI Fig. 7). Similar to the endogenous proteins, GFP–NONO was present exclusively in the nucleus under basal conditions and exhibited a marginal $27 \pm 8\%$ overlap with CFP–TORC on the basis of pixel area quantification. In contrast, elevations in cAMP produced a robust $80 \pm 8\%$ overlap ($P < 0.05$) between the two proteins (Fig. 2b). Förster FRET was performed to quantify NONO and TORC molecular proximity in response to forskolin. Quantitation of the FRET/GFP fluorescence ratio revealed that forskolin induced a 8.3-fold increase in the transfer of energy from the donor to the photobleached acceptor (0.09 ± 0.05 control vs. 0.71 ± 0.14 forskolin), demonstrating an interaction between TORC and NONO (Fig. 2c). Moreover, measurement of this interaction revealed that forskolin stimulation brought the proteins into close proximity (2.5 ± 0.08 nm), whereas unstimulated cells were near the upper limits of our detection (10 nm) (Fig. 2c). Collectively, these results demonstrate that cAMP stimulates the formation of a nuclear complex between TORC and NONO.

NONO Plays Essential Roles in cAMP-Dependent Transcription. If NONO is a critical component of the CREB–TORC complex, then depletion of NONO should effectively silence TORC-dependent gene activation and, consequently, cAMP signaling. To examine the effects of NONO knockdown, four siRNA oligonucleotides targeting NONO were each tested for their ability to block cAMP activation of the CRE-containing *EVX1* promoter (SI Fig. 8) (22). The four siRNA molecules targeting NONO varied in their efficacy to block forskolin induction, which strongly correlated (Pearson correlation, $r = 0.77$) with decreases in NONO protein levels (SI Fig. 8). Remarkably, the most potent NONO siRNA blocked cAMP induction at levels similar to siRNA molecules targeting CREB and TORC2 (Fig. 3). None of the siRNAs blocked TNF α -mediated activation of the IFN promoter, demonstrating specificity of the NONO siRNA (Fig. 3). NONO protein depletion was confirmed by Western blot analysis (Fig. 3). Therefore, NONO is necessary for cAMP-dependent transcriptional activation.

NONO Is Recruited to cAMP-Responsive Promoters by TORC2 and Tethers TORC2 to RNA Pol II. The identification of a TORC–NONO nuclear complex and our RNA interference data identifying NONO as a necessary component for cAMP-signaling prompted us to assess whether NONO occupied endogenous cAMP-responsive promoters and whether this occupation was forskolin-dependent. To determine whether NONO was present at endogenous cAMP-dependent promoters, we used ChIP assays with antibodies directed against CREB, TORC2, and NONO. Like CREB and TORC2, NONO was detected on the endogenous CRE-containing *NR4A2* promoter in the presence of forskolin (Fig. 4a). Multiple independent ChIP experiments were conducted and analyzed by real-time PCR to confirm results obtained by standard PCR (Fig. 4b). Based on these results we hypothesized that disrupting interactions between TORCs and NONO should impair the regulation of endogenous CREB target genes. Indeed, knockdown of NONO attenuated the induction of *NR4A2* and *FOS* mRNA in response to forskolin, comparable to effects observed with CREB and TORC2 siRNAs (Fig. 4c). However, depletion of NONO had no effect

Fig. 3. Nono plays essential roles in cAMP-dependent transcription. Shown is the effect of Nono knockdown by siRNA on the cAMP-responsive *EVX1* luciferase (Top) versus control *IFN* luciferase (Middle) reporters after forskolin or $TNF\alpha$ stimulation, respectively, in HEK293T cells. (Bottom) Western blot showing amounts of protein in cells treated with siRNA ($n = 3$ wells; mean $\pm$ SEM).

on *YWHAH*, a constitutively active gene not responsive to elevations in intracellular cAMP.

Previous studies demonstrated that Nono can bind to the C-terminal domain (CTD) of RNA pol II (23, 24). Therefore, TORC2–Nono interactions may represent uncharacterized conduits from TORCs to the basic transcriptional machinery. ChIP assays were performed to determine whether Nono bridges the CREB–TORC complex to the CTD of RNA pol II by using antibodies that recognize the CTD of RNA pol II regardless of its phosphorylation status. Two endogenous CRE-containing promoters, *NR4A2* and *FOS*, demonstrated increased RNA pol II occupancy after forskolin stimulation (Fig. 4d). Moreover, knockdown of Nono selectively impaired cAMP-dependent recruitment of RNA pol II (Fig. 4d), whereas a constitutively active gene, *YWHAH*, did not have an attenuation of RNA pol II recruitment after Nono knockdown. Collectively, these data demonstrate Nono-dependent recruitment of RNA pol II to cAMP-regulated promoters.

Fig. 4. Nono recruits RNA pol II to cAMP-responsive promoters. Nono is present at a cAMP-responsive promoter along with CREB and TORC2. (a) ChIP of the *NR4A2* and *GAPDH* promoters from HEK293T cells using anti-CREB (Left), anti-TORC2 (Center), anti-Nono (Right), or anti-GAL4-specific antisera as a negative control. Preimmunoprecipitation (Pre-IP) control DNA is also shown. (b) Quantification of precipitated *NR4A2* promoter by TaqMan real-time (qRT) PCR ($n = 3$ experiments; mean $\pm$ SEM; each experiment was the average of three measurements). Occupancy of the target protein on the *NR4A2* promoter is expressed relative to the *GAPDH* promoter. (c) RNA from HEK293T cells transfected with siRNAs for CREB, TORC2, Nono, or NS control was quantified by quantitative RT-PCR using *YWHAH*, *NR4A2*, and *FOS* mRNA-specific TaqMan probes. Fold changes in endogenous *YWHAH*, *NR4A2*, and *FOS* mRNA levels after treatment with forskolin or DMSO vehicle for 45 min are graphed. Nono siRNA abolishes cAMP-dependent transcriptional activation but not constitutive activity of *YWHAH* ($n = 3$ wells; mean $\pm$ SEM). (d) Knockdown of Nono protein attenuates RNA pol II recruitment to cAMP-responsive promoters. ChIP assay of *YWHAH*, *NR4A2*, *FOS*, and *GAPDH* promoters from HEK293T cells using anti-RNA pol II antibody. Fold occupancy of RNA pol II on the target promoter is expressed relative to the *GAPDH* promoter ($n = 3$ experiments; mean $\pm$ SEM).

Discussion

The Coactivator Trap Identifies New cAMP Pathway Components. TORC coactivators are integral components of the cAMP signaling pathway and regulate numerous biological processes, including hepatic gluconeogenesis, adaptive mitochondrial biogenesis, and long-term synaptic plasticity (11, 25, 26). The identification of TORC-interacting transcription factors sheds light on the mechanisms used by TORCs to influence these diverse biological processes. To this end, we developed and applied the coactivator trap assay to identify interacting partners of the TORC family of CREB coactivators.

Although the coactivator trap uncovered many putative interactions, we chose to investigate NONO (p54^{nrb}) because it was strongly activated by TORCs1–3, and an independent experimental methodology, mass spectrometry of coimmunoprecipitated proteins, also identified NONO (p54^{nrb}) as a TORC-interacting partner. It has been previously demonstrated that NONO is a multifunctional protein implicated in transcription and RNA processing. With respect to transcriptional regulation, NONO was previously shown to bind to thyroid hormone receptors (TR) and retinoid X receptors (RXR) (27) and the Spi-1/PU.1 transcription factor (28) to regulate transcription from their respective target promoters. Moreover, the possible roles for NONO in cAMP-dependent regulation have been previously suggested. Specifically, NONO is recruited to the cytochrome *p450 hCYP17* promoter by the steroidogenic factor-1 (gene symbol *NR5A1*) in response to elevations in intracellular cAMP (21), but it is unclear whether TORCs or CREB are part of this complex. Therefore, we hypothesized that NONO may play a role in cAMP signaling through interactions with TORCs.

Our studies demonstrate that NONO is an integral component of the cAMP pathway that tethers TORCs to RNA pol II to regulate cAMP-responsive genes. First, we showed that endogenous NONO forms complexes with TORC2 in response to cAMP and together they are assembled on endogenous CRE-containing promoters. Moreover, RNA interference experiments showed that NONO is required for cAMP-dependent activation of CREB target genes *in vivo*. Our observation that NONO facilitates the recruitment of RNA pol II to cAMP-responsive genes supports previous studies indicating that NONO mediates transcription by providing a direct physical link to the RNA pol II CTD (23, 24). Collectively, the data support a mechanism by which NONO is recruited to cAMP-dependent promoters by TORCs and subsequently couples TORC proteins to the basal transcriptional machinery, thereby enabling transcriptional responses to elevations in intracellular cAMP. Although previous studies also demonstrate that NONO binding to the RNA pol II CTD cotranscriptionally regulates premRNA processing, it remains to be determined whether TORC:NONO interactions regulate cotranscriptional processing of cAMP-dependent transcripts.

Development of the Coactivator Trap. Coactivator proteins encompass a subset of transcriptional modulators whose actions are mediated by DNA binding proteins. For example, the coactivators p300 and CBP modulate transcriptional responses to cAMP by their CREB-dependent recruitment to promoters (2). However, there are also dozens of other *trans* factors, including Clock, HIF1 α , STAT1, ATF4, FOXO1, NFE2, Neuro D, NF-Y, and members of the nuclear hormone receptor family that are able to bind CBP/p300 (20). Most of these interactions were discovered serendipitously or by trial and error, because each factor binds its own distinct regulatory element. To date, a generic system has not been available to investigate these issues. To overcome this limitation and to parallelize the process of identification of functional transcription factor/coactivator interactions, we devised the coactivator trap. We reasoned that coactivators would stimulate the transcription of a distinct cohort of these transcription factors and that this screen could be used to determine transcription factor/coactivator pairs.

Although, the coactivator trap assay has many benefits, such as interrogation of transcription factors for interactions on a massively parallel scale, some bona fide protein–protein interactions may not function properly in this context. Although this technique offers a method to efficiently interrogate and identify interacting transcription factors on a large, parallel scale, the assay does have inherent limitations. To account for false positives, putative interactions have to be experimentally validated using standard biochemical procedures. False-negative

errors could potentially arise from poor expression of the fusion protein or because of missing cofactors not present in the mammalian screening line. Along the lines of the latter point, many coactivators or inhibitors function by recruiting DNA-modifying enzymes, such as HATs and HDACs, that will not affect a luciferase plasmid and therefore will not show in the assay. Therefore, although the coactivator trap represents a powerful method to identify transcription factor/coactivator interactions, caution should be used in interpreting the primary screening results, especially in regard to null observations.

Experimental Procedures

Amplification, Purification and Cloning of Transcription Factors. Human clones were identified by surveying the expert annotation systems, such as Panther (29) and GO annotation (by ≈ 20 categories) at the National Center for Biotechnology Information (NCBI) EntrezGene (LocusLink in 2003), combined with the filtered keyword searches over the NCBI annotation. The resulting list of 4,083 human Celera and public transcripts and transcript predictions was followed by a similarity search in the available clone sequences by BLAST (30). We were able to select 1,428 human transcription factor clones from the Mammalian Gene Collection (MGC), and, of these, we were able to successfully subclone and sequence-verify 837 clones. The CM-GAL4 expression vector was created using standard cloning. Purified plasmids were normalized and spotted into 384-well plates at a concentration of 10 ng per well.

Generation of Expression Plasmids for FRET Microscopy. The C-terminally tagged expression plasmid pECFP-TORC1 was generated by PCR amplification of the entire human TORC1-coding region by using published primer sequences (13) and was cloned into the BamHI and BglII sites of pECFP-N1 (Clontech).

High-Throughput Transfection and Reporter Assay. HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin). Reverse transfection was carried out using the arrayed transcription factor library collection containing 10 ng of transcription factor CM-GAL4 fusion cDNA per well. Serum-free DMEM (20 μ l), containing test cDNA (cDNAs encoding *TORC1*, *TORC2*, or *TORC3*), the reporter GAL4::luciferase, and Eugene 6 (Roche Diagnostics) was allocated into each well. After a 30-min incubation at room-temperature DMEM 20% FBS (20 μ l) containing 10⁴ 293T cells was dispensed into each well. Cells were cultured for 24 h in a humidified incubator at 37°C in 5% CO₂. BrightGlo (Promega) reagent (35 μ l) was added to each well, and luciferase luminescence was measured with an Acquest plate reader (LJL Biosystems).

RNA Interference. RNA interference and luciferase activity assays were performed as described in ref. 22, normalizing to activity from Rous sarcoma virus (RSV)- β -galactosidase expression plasmid. Where indicated, 20 nM siRNA, 100 ng of reporter vector, and 25 ng of RSV- β -galactosidase expression plasmid per well were cotransfected. Seventy-two h after transfection, cells were treated for 4 h with 10 μ M forskolin or vehicle (DMSO).

Real-Time PCR. RNA was extracted from 293T cells with TRIzol (Invitrogen), and reverse transcription was conducted with SuperScript III reverse transcriptase (Invitrogen), following the manufacturer's protocol. PCR amplification reactions were run in triplicate in 96-well optical microplates in an ABI Prism 7900 HT SDS instrument (Applied Biosystems). Differences in mRNA expression levels between the nonspecific and gene-specific siRNA-treated cells during control and forskolin stimulation were measured by relative quantification with the Comparative $\Delta\Delta$ Ct method (Applied Biosystems) and were normalized against *GAPDH* levels. Probe sequences are listed in [SI Table 1](#).

Antibodies. Antiserum 3363 and 3364 against human TORC2 was generated using peptide [H]-CAETDKTLKQSWDSKKAG-[NH₂] at Covance. Other antibodies included NONO (Bethyl), CREB (Upstate Biotechnology), monoclonal anti-NONO (Upstate Biotechnology), anti-RNA polymerase II (Upstate Biotechnology), anti-GAL4 (Santa Cruz Biotechnology), and tubulin (Sigma).

Coimmunoprecipitation Assays. To coimmunoprecipitate endogenous proteins, 293T cells were stimulated with 10 μ M forskolin in culture medium for 20 min and cells were washed with cold PBS and lysed with 1 ml of cold 1% Nonidet P-40 lysis buffer for 30 min at 4°C. Lysates were pelleted 10,000 $\times$ g for 20 min at 4°C, and the supernatant was incubated with protein G Sepharose beads (East Coast Biologicals) for 2 h at 4°C. Precleared lysates were