RNA-induced silencing complex (RISC) Proteins PACT, TRBP, and Dicer are SRA binding nuclear receptor coregulators

Andrew D. Redfern^{a,b,1}, Shane M. Colley^{a,1}, Dianne J. Beveridge^{a,1}, Naoya Ikeda^{a,1}, Michael R. Epis^a, Xia Li^a, Charles E. Foulds^c, Lisa M. Stuart^a, Andrew Barker^a, Victoria J. Russell^a, Kerry Ramsay^a, Simon J. Kobelke^a, Xiaotao Li^c, Esme C. Hatchell^{a,b}, Christine Payne^d, Keith M. Giles^a, Adriana Messineo^a, Anne Gatignol^e, Rainer B. Lanz^c, Bert W. O'Malley^{c,2}, and Peter J. Leedman^{a,b,2}

^aLaboratory for Cancer Medicine, University of Western Australia (UWA) Centre for Medical Research, Western Australian Institute for Medical Research (WAIMR), Perth, WA, Australia; ^bSchool of Medicine and Pharmacology, University of Western Australia, Perth, WA, Australia; ^cDepartment of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX; ^dDepartment of Laboratory Medicine, Royal Perth Hospital, Perth, WA, Australia; and ^eVirus-Cell Interactions Laboratory, Lady Davis Institute for Medical Research, McGill University, Montreal, QC, Canada

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The cytoplasmic RNA-induced silencing complex (RISC) contains dsRNA binding proteins, including protein kinase RNA activator (PACT), transactivation response RNA binding protein (TRBP), and Dicer, that process pre-microRNAs into mature microRNAs (miRNAs) that target specific mRNA species for regulation. There is increasing evidence for important functional interactions between the miRNA and nuclear receptor (NR) signaling networks, with recent data showing that estrogen, acting through the estrogen receptor, can modulate initial aspects of nuclear miRNA processing. Here, we show that the cytoplasmic RISC proteins PACT, TRBP, and Dicer are steroid receptor RNA activator (SRA) binding NR coregulators that target steroid-responsive promoters and regulate NR activity and downstream gene expression. Furthermore, each of the RISC proteins, together with Argonaute 2, associates with SRA and specific pre-microRNAs in both the nucleus and cytoplasm, providing evidence for links between NR-mediated transcription and some of the factors involved in miRNA processing.

noncoding RNA | androgen action | prostate cancer

MicroRNAs (miRNAs) are 22-nt noncoding RNAs that regulate gene expression by associating with the multiprotein RNA-induced silencing complex (RISC) and guiding RISC to silence specific mRNA species by mRNA degradation or translational inhibition (1). miRNAs are derived from large primary RNA transcripts, which are processed in the nucleus by Drosha/Pasha into ~70-nt precursor (pre-miRNA) duplexes. These duplexes are exported to the cytoplasm, and their conversion to mature 22-nt miRNAs is mediated by a transactivation response (TAR) RNA binding protein (TRBP)–Dicer complex (1), which provides a platform for RISC assembly and aids recruitment of Argonaute 2 (Ago2), the catalytic enzyme of RISC required for miRNA processing (2, 3). Protein kinase RNA (PKR) activator (PACT) is also integral to the RISC complex and associates with Dicer (4). TRBP and PACT are critical for efficient miRNA processing, because their depletion results in decreased mature miRNA levels and target gene silencing (2, 4, 5).

Ligand-activated signaling by nuclear receptors (NRs) plays a key role in the promotion of human tumorigenesis. NR coregulators are a group of >300 molecules that act to fine tune ligand-induced gene transcription by acting as either coactivators [e.g., steroid receptor coactivator-1 (SRC-1)] or corepressors [e.g., nuclear receptor corepressor1 (NCoR1)] (6). One of the NR coregulators, steroid receptor RNA activator (SRA), is unique, because its RNA transcript functions to coactivate NR activity (7–9).

Several links between miRNA pathways and NR action have been reported. miRNAs can regulate NR signaling at different levels, including targeting NRs [e.g., estrogen receptor- α (ER α)] (10, 11) or NR coregulator transcripts (e.g., amplified in breast cancer 1) (12). Conversely, NRs regulate an array of miRNAs in endocrine tissues (13). For example, ER α can regulate miRNA-21 expression at the

transcriptional level (14) and the first step of miRNA processing (15), and a reduction of Dicer and Drosha/Pasha expression through glucocorticoids can regulate miRNA processing (16). Specific RISC members may also function in the nucleus. There is nuclear association of Ago1 and Ago2 with the progesterone receptor gene (3), Dicer is associated with nuclear ribosomal DNA chromatin, and posttranscriptional silencing can occur in nuclei (17). To date, however, NR coregulators have not been directly implicated in RISC activity or miRNA biogenesis and processing.

Here, we show that the RISC proteins PACT, TRBP, and Dicer, together with PKR, are SRA binding NR coregulators that are recruited to the promoters of hormone-regulated genes and regulate the expression of downstream target genes. In addition, we show that specific pre-miRNAs and SRAs associate with RISC members in both the nucleus and cytoplasm and provide evidence of nuclear pre-miRNA processing.

Results

PACT, TRBP, and PKR Are SRA–Stem Loop Substructure 1 Binding Proteins.

SRA RNA contains multiple stem loop substructures (STRs), several of which contribute to its NR transactivation activity, including SRA-STR1 (Fig. 1A) and SRA-STR7 (9). SRA-STR7 is bound by two NR corepressors: SRA stem loop interacting RNA binding protein and silencing mediator for retinoid or thyroid-hormone receptors (SMRT)/histone deacetylase (HDAC)-associated repressor protein (18). Using radiolabeled SRA-STR1 in RNA electrophoretic mobility shift assays (REMSAs) and UV cross-link (UVXL) assays with protein extracts from MCF-7 and MDA-MB-468 breast, and HeLa cancer cell lines, we identified specific nuclear SRA-STR1 protein complexes in each cell line (Fig. S1A and B). We next used the SRA-STR1 stem loop as bait in a yeast three-hybrid (Y3H) screen of a human breast cancer cDNA library and identified the dsRNA binding proteins (dsRBPs) PACT and TRBP as potential binding partners (Fig. 1B). In our studies, we included PKR, because it is another core member of the dsRBP family. A close correlation exists between these dsRBPs and the M_r of the SRA-STR1 protein complexes observed in UVXL (PACT, 35 kDa; TRBP, 40 kDa; PKR, 68 kDa) (Fig. S1B).

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¹A.D.R., S.M.C., D.J.B., and N.I. contributed equally to this work.

²To whom correspondence may be addressed. E-mail: peter.leedman@waimr.uwa.edu.au or berto@bcm.edu.

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Fig. 1. PACT, TRBP, and PKR are SRA-STR1 binding proteins. (A) mFOLD 2° structure plot of SRA-STR1 (<http://mfold.rna.albany.edu/?q=mfold>). (B) Domain structures of PACT and TRBP clones isolated by Y3H screen and for PKR (not isolated). Black line, length of isolated clone; DB, dsRNA binding domain (RBD); PAD, PKR RBD; black bars in PACT and PKR, amino acid mutations. (C) SRA mRNA copurification with PACT and PKR in HeLa cells detected by IP-RT-qPCR. P, SRA expression plasmid; S/N, supernatant. (D) SRA and miRNA-16 mRNA copurification with transfected FLAG-tagged proteins in HEK-293T cells detected by IP-RT-qPCR. Error bars = SD.

We next investigated the interaction between SRA and the dsRBPs and found that SRA RNA coimmunopurified with PACT and PKR in HeLa cells (Fig. 1C) and also FLAG-tagged TRBP in HEK-293T cells (Fig. 1D). In addition, SRA also copurified with Dicer and Ago2. miRNA-16 (positive control) copurified with TRBP, Dicer, and Ago2. To ascertain the specificity of the SRA/dsRBP interactions, we investigated the association of another dsRNA, 7SK (19), with these proteins in LNCaP prostate cancer (PCa) cells. Although 7SK RNA copurified with PACT and Dicer, relatively more SRA was associated with each of these proteins (Fig. S1C). Furthermore, SRA, but not 7SK, copurified with the androgen receptor (AR). In REMSA studies, a PACT-SRA-STR1 complex was formed with GST-PACT, which was supershifted with a PACT antibody (Fig. S1D). GST-PKR p20, a truncated PKR fusion protein containing the dsRBDs, formed a complex with SRA-STR1, which supershifted with a PKR antibody. In contrast, no binding was observed with GST or antibody alone. These studies indicated multimeric SRA complexes, and we next tested the coregulator properties of the dsRBPs.

PACT, PKR, and TRBP Modulate SRA-Coactivated NR Signaling. To investigate the coregulator potential of PACT, PKR, and TRBP, we used hormone-responsive luciferase (Luc) reporters in transactivation studies. PKR was a repressor of SRA-mediated ER signaling by estrogen-response element (ERE)-Luc in HeLa cells (Fig. 2A) and consequently, anticipated PACT overexpression to exacerbate this repression. However, PACT augmented the SRA-mediated increase in reporter activity. Combined PACT and PKR overexpression resulted in a negation of their activating and repressive effects, respectively. Consistent with the ability of TRBP to inhibit PKR action, overexpression of TRBP also augmented the SRA-mediated transactivation. Similar findings were observed in LNCaP cells transfected with the prostate-specific antigen (PSA)-Luc reporter and treated with dihydrotestosterone (DHT) (Fig. S2A). We examined the effects of the dsRBPs on thyroid, vitamin D, and progesterone [PR-B; mouse mammary tumor virus (MMTV)] receptor response elements in HeLa cells, and we found that PACT augmented and PKR inhibited the SRA-mediated signaling for each pathway (Fig. S2B). In contrast, although TRBP

coactivated PR-B activity similarly to the ER and PSA reporters, it did not augment thyroid or vitamin D receptor reporter activity.

To investigate if the actions of the dsRBPs are dependent on SRA, we compared dexamethasone (Dex)-induced glucocorticoid receptor reporter activity in HeLa cells treated with siRNAs directed against GFP or SRA (Fig. 2B and Fig. S2C). In GFP-treated samples (expressing basal levels of endogenous SRA), PACT and TRBP were activators, and PKR was a repressor of Dex-induced transactivation, consistent with the data described above. When SRA was depleted from the cells, there was a significant reduction in PACT and TRBP coactivation (Fig. 2B), supporting the hypothesis that SRA plays an important role to mediate the coregulatory effects of these dsRBPs. However, reporter activity also decreased with PKR, suggesting that the coactivating activity of SRA influences the role of PKR in NR transactivation.

We next explored the effect of each dsRBP on Luc mRNA expression in HeLa cells with or without cotransfected SRA. SRA induced an increase of Luc mRNA expression (Fig. 2C), consistent with the transactivation studies. PACT overexpression also resulted in an increase in Luc mRNA expression, and cotransfection of exogenous SRA augmented this effect. Interestingly, we found PKR to be an SRA-mediated transcriptional coactivator, which contrasts with its activity as a translational repressor. TRBP overexpression influenced Luc mRNA expression only in the presence of additional SRA. Similar findings were observed in LNCaP cells (Fig. S2D).

To further characterize the role of PACT and PKR as NR coregulators, we examined the effect of mutants (Fig. 1B) on Dex-induced glucocorticoid response element (GRE)-luc reporter activity. With increasing doses of WT PACT, a dose-dependent increase in SRA-mediated coactivation was observed (Fig. 2D). When PACT D3, a mutant that lacks the third RNA binding domain necessary for PKR activation (PAD domain), was cotransfected, there was an increase in GRE-luc activity. In contrast, cotransfection of PACT K84A and PACT K88A has point mutations designed to disrupt RNA binding, and GRE-luc activity was reduced compared with the largest concentration of WT PACT. These data suggest that the coactivating effect of WT PACT on NR reporter activity is a composite of activation through its own RNA binding domain as well as activation of PKR. We explored the effect

Fig. 2. PACT, PKR, and TRBP modulate SRA-coactivated nuclear receptor signaling and regulate downstream target genes. (A) HeLa cells were cotransfected with ERE-Luc reporter, hER α , combinations of FLAG-tagged dsRBP expression vectors (Fig. 1B) and SRA, or empty vectors. Cells were lysed after 10 nM E₂ treatment for 8 h, and Luc assay was performed. * P < 0.05 relative to SRA alone. (B) HeLa cells were transfected with SRA or GFP siRNA for 3 d and GRE-Luc reporter and FLAG-tagged dsRBPs for 1 d before treatment with 10 nM Dex for 8 h and Luc assay was performed. * P < 0.05 and ** P < 0.005 relative to no cotransfected dsRBP; *** P < 0.05 GFP siRNA relative to SRA siRNA for each dsRBP. (C) RT-qPCR of Luc mRNA in HeLa cells cotransfected with GRE-Luc, FLAG-tagged dsRBPs, and SRA or empty vector. Posttransfection, cells were treated for 2 h with 10 nM Dex before RNA extraction. * P < 0.05 and ** P < 0.005 relative to no SRA cotransfection. (D) PACT or its mutants were transfected into HeLa cells (as above), and effects on SRA-coactivated GRE-Luc activity were determined after 8 h of 10 nM Dex. Black bar, increasing WT PACT concentration (highest concentration was the concentration of the mutants used). * P < 0.05 relative to SRA alone. (E) PKR or its mutants were transfected into HeLa cells (as above), and effects on SRA-coactivated GRE-Luc activity were determined after 8 h of 10 nM Dex. Black bar, increasing WT PKR concentration (highest concentration was the concentration of mutants used). * P < 0.05 relative to SRA alone. (F and G) LNCaP (F) or MCF-7 (G) cells were transfected with PACT, PKR, TRBP, or GFP siRNA for 3 d before 10 nM DHT or E₂ for 2–4 h. Gene expression (relative to GAPDH) quantified by RT-qPCR. * P < 0.05 and ** P < 0.005 relative to GFP siRNA with hormone. For all data shown, error bars = SD; they are representative of three independent experiments.

of SDM1 (site-directed SRA mutant that reduces the transactivation activity of SRA) (9) on the ability of PACT to coactivate. In contrast to WT SRA, SDM1 cotransfection abrogated the coactivation of PACT (Fig. S2E). For PKR, we examined the effects of dsRNA binding (K60A and K64A) and kinase (K296R and p20) domain mutations on GRE-luc reporter activity. In contrast to a dose-dependent increase in the inhibition of GRE-luc reporter activity observed with WT PKR, each PKR mutant had little effect on reporter activity (when cotransfected at a concentration comparable with the largest dose of WT PKR) (Fig. 2E). These data suggest that both the RNA binding and kinase functions of PKR are required to repress GR signaling. Furthermore, at the level of transcription, transfection of WT or p20 PKR increased Luc mRNA levels, and expression of the dsRNA binding mutants PKR K296R and K60A had no impact on Luc mRNA (Fig. S2F). Because PKR can inhibit protein synthesis through the inhibition of eukaryotic initiation factor (eIF)-2 α phosphorylation and translation in response to cellular stress (20), we examined if the repressive effect of PKR overexpression in Luc assays could be attributed to an inhibition of Luc protein synthesis. We overexpressed each dsRBP in HeLa cells and assessed eIF-2 α phosphorylation status, and no significant changes were observed (Fig. S2G). Furthermore, endogenous NR levels were not affected by overexpression of WT or mutant PKR (Fig. S2A and H) or overexpression of WT or mutant PACT and TRBP (Fig. S2A and I). Taken together, these data also support that the effects of PKR in NR signaling are SRA-mediated and consist of an early phase of transcriptional coactivation followed by a later phase of translational repression.

PACT, PKR, and TRBP Modulate a Range of NR-Regulated Genes. To further validate the role of PACT, PKR, and TRBP in NR coregulation, we decreased their expression in cancer cells and assessed downstream endogenous target gene expression. After siRNA-mediated reduction of PACT and PKR in LNCaP cells, expression

of the DHT-regulated genes PSA, NKX3.1 (a homeobox protein), and v-maf musculoaponeurotic fibrosarcoma were substantially reduced (Fig. 2F). PSA mRNA expression was also abrogated when TRBP levels were reduced. Similar results were observed in MCF-7 cells, whereby reduction in dsRBP expression abrogated the coregulator-mediated increase in mRNA of the estrogen (E₂)-regulated genes nuclear receptor interacting protein (NRIP), thrombospondin 1 (THBS1), and Ret proto-oncogene (Fig. 2G). NRIP and THBS1 were included, because both have been shown to be SRA targets (21). Together, these data support our overexpression studies and validate a role for the SRA binding dsRBPs as NR coregulators that control the expression of multiple downstream target genes.

PACT, PKR, and TRBP Are Recruited to Hormone-Regulated Promoters.

To explore the possible mechanisms by which dsRBPs regulate transcription, we performed ChIP assays with different target promoters. Using LNCaP cells, PACT, PKR, TRBP, AR, SRC-1, and NCoR were all recruited to the PSA promoter after DHT treatment (Fig. 3A). In addition, DHT-induced NR/coregulator association was detected by coimmunoprecipitation in LNCaP cells (Fig. S3A). Furthermore, in MCF-7 cells, PACT was present at the pS2 promoter in the presence and absence of E₂ (Fig. S3B). We then depleted SRA, PKR, or PACT from LNCaP cells to assess their effect on PSA promoter occupancy. DHT-induced cofactor enrichment at the PSA promoter was reduced with SRA siRNA and completely abrogated with siRNA to PKR or PACT (Fig. 3B). Together, these data support the capacity of the dsRBPs to be recruited to NR promoters and suggest a subtle stoichiometry for these coregulators to mediate NR transactivation.

Dicer Is an NR Coregulator, and It Is Recruited to Hormone-Responsive Promoters. Based on our observations that two members of RISC (PACT and TRBP) are SRA binding NR coregulators and that SRA coimmunopurified with Dicer (Fig. 1D), we investigated if

Fig. 3. Dicer is an NR coregulator, which together with PACT, PKR, and TRBP, is recruited to the PSA promoter. (A) ChIP-qPCR assay in LNCaP cells of AR, PKR, PACT, TRBP, SRC-1, and NCoR recruitment to PSA promoter after 45 min of 10 nM DHT. (B) ChIP-qPCR assay for PSA promoter occupancy of AR, PKR, and PACT in LNCaP cells after SRA, PKR, PACT, or GFP siRNA for 3 d before treatment with 10 nM DHT for 45 min. (C) LNCaP cells were treated with 10 nM DHT for 24 h, whole-cell or nuclear proteins were extracted, and expression of Dicer, AR, tubulin, and actin was assessed by immunoblotting. (D) Cells were transfected with a GRE-Luc (HeLa), PSA-Luc (22Rv1), or MMTV-Luc (MCF-7) reporter along with empty vector or FLAG-tagged Dicer and in MCF-7 cells PR-B. After incubation with 10 nM Dex, DHT, or progesterone (P4) for 24 h, Luc activity was measured. (E) LNCaP or MCF-7 cells were transfected with Dicer or GFP siRNA for 3 d before 10 nM DHT or E_2 for 2–4 h. Gene expression (relative to GAPDH) quantified by RT-qPCR. * $P < 0.05$ and ** $P < 0.005$ relative to GFP siRNA with hormone. (F) ChIP-qPCR assay examining PSA promoter occupancy in LNCaP cells transfected with GFP, SRA, PKR, PACT, or Dicer siRNA for 3 d and treated with 10 nM DHT for 45 min. For all data shown, error bars are SD and representative of three independent experiments.

Dicer was also an NR coregulator. We observed nuclear expression of Dicer in LNCaP cells (Fig. 3C), suggesting it may function within the nucleus. In transfection assays using different NR-Luc reporters and cell lines, overexpression of Dicer augmented ligand-induced reporter activity (Fig. 3D). Additionally, siRNA-mediated reduction of Dicer from cancer cells was associated with a significant decrease in expression of downstream androgen- and estrogen-regulated target genes (Fig. 3E). Dicer was recruited to the PSA promoter in LNCaP cells after DHT treatment (Fig. S3C). Furthermore, siRNA-mediated reduction of SRA, PKR, and PACT all impacted on the DHT-induced enrichment of Dicer at the PSA promoter (Fig. 3F and Fig. S3D). The DHT-mediated recruitment of AR, PACT, and PKR to the PSA promoter (Fig. 3A and B) was

lost in cells with reduced Dicer (Fig. 3F and Fig. S3D). In sum, these data suggest that Dicer is an NR coregulator that plays an important role in regulating transcriptional activation in cancer cells.

Pre-miRNAs Are Associated with the NR Coregulator RISC Proteins.

Our data suggest that members of RISC (PACT, TRBP, and Dicer) have additional functions in the nucleus as NR coactivators. We next showed abundant expression and colocalization of these RISC proteins, along with Ago2, in both the nucleus and cytoplasm of 22Rv1 PCa cells (Fig. 4A and Fig. S3E). We postulated that the nuclear localization of the RISC proteins reconstituted a nuclear RISC complex and hypothesized that these proteins may interact with and/or regulate the processing of specific pre-miRNAs. We performed immunoprecipitation–quantitative RT-PCR (IP-RT-qPCR) studies in nuclear and cytoplasmic lysates from LNCaP cells, focusing on pre-miRNA-21 and pre-miRNA-18a, both of which have been implicated in PCa (22). These pre-miRNAs exhibited the expected cytoplasmic association with each RISC protein, and as hypothesized, nuclear interaction was also observed (Fig. 4B–E).

SRA and pre-miRNA Processing. Our data suggest that the functional role of the RISC proteins as NR coregulators in the nucleus is enhanced by SRA. We hypothesized that SRA may act as an RNA binding scaffold for the RISC proteins in both the nucleus and the cytoplasm. To investigate this proposed interaction, we performed IP-RT-qPCR assays in LNCaP nuclear and cytoplasmic fractions and observed that SRA RNA copurified with PACT, Dicer, TRBP, and Ago2 in both fractions (Fig. 5A). We next used a pre-miRNA-Luc processing assay to explore the potential of SRA to regulate

Fig. 4. Nuclear association of pre-miRNAs with the RISC proteins. (A) Cytoplasmic (cyto) and nuclear (nucl) extracts from 22Rv1 cells treated ± 10 nM DHT for 24 h were immunoprecipitated with rabbit IgG or TRBP antibody. Input lysates and IP samples were immunoblotted for Dicer, PACT, or Ago2. (B–E) IP-RT-qPCR assay in cytoplasmic (cyto) and nuclear (nucl) extracts from LNCaP cells to detect copurification of pre-miRNA-21 and pre-miRNA-18a with PACT, Ago2, Dicer, and TRBP. Error bars = SD. * $P < 0.05$ and ** $P < 0.005$ relative to IgG. $n = 3$ independent experiments.

dicing. In this assay, LNCaP cells were treated with siRNA to Dicer or SRA and cotransfected with a pre-miRNA-Luc expression construct and a Luc reporter. In cells with either reduced Dicer or SRA, there was an increase in Luc reporter activity, indicative of a decrease in the processing of pre-miRNA-Luc into the mature miRNA-Luc to target the reporter for degradation (Fig. 5B).

To further explore the nuclear role in pre-miRNA biogenesis, we performed *in vitro* pre-miRNA processing assays. We *in vitro* generated pre-miRNA-21 and observed that it was effectively processed by recombinant Dicer (Fig. 5C). We then examined the effectiveness of pre-miRNA-21 processing in cytoplasmic and nuclear fractions from LNCaP cells and found that pre-miRNA-21 was processed into mature miRNA-21 in both fractions (Fig. 5D). A similar effect was observed for prelet-7c processing (Fig. S44). Taken together, these data suggest that, in PCa cells, pre-miRNA processing can occur in the nucleus, and it raises the possibility that SRA, by virtue of its scaffold-like properties, may facilitate the function of nuclear RISC proteins in both NR signaling and pre-miRNA processing.

Discussion

These findings define transcriptional roles for the PACT/PKR/TRBP/Dicer family of dsRBPs in NR signaling and also highlight the evolving functional role of SRA as an atypical NR coactivator that is also associated with the RISC proteins.

We have shown in cancer cells that PACT is a SRA binding NR coactivator and that it is recruited to the promoter of hormone-regulated genes to mediate transactivation of downstream target genes. When PACT is depleted from cells, there is a reduction in

promoter occupancy of NRs and other dsRBP coregulators, suggesting that optimal transactivation requires PACT at the promoter. Removal of PACT's third RNA binding motif, which is necessary to activate PKR (23), enhances its transcriptional coactivation. These data highlight a functional role for PACT, supporting the notion that it may be a key transcriptional regulator of endocrine hormone signaling and the concept that overexpression of PACT in cancer could promote tumorigenesis (24). This implication of PACT in endocrine signaling is consistent with its reported role in postnatal anterior pituitary proliferation (25). Our findings add to the repertoire of functions of PACT, which includes the regulation of retinoic-acid-inducible gene 1 expression as part of the antiviral response (26), and ear development and hearing (27).

TRBP binds Dicer and PACT through its C-terminal domain, and it binds to PKR through its dsRBDs, blocking the inhibitory effect that PKR has on translation (28). Our data place TRBP as an NR coactivator that is actively recruited to hormone-responsive promoters for the regulation of downstream target gene expression in cancer cells. Other studies also implicate TRBP in cancer and oncogenesis: a mutation of the *TARBP2* gene has been identified in specific cancers, which results in aberrant Dicer expression and miRNA processing (29), and TRBP phosphorylation regulates the expression of growth-promoting miRNAs (30).

The mechanism by which PKR may activate genes is unclear, although some indirect links with NF- κ B-dependent pathways exist (31). Our data show that PKR-mediated regulation of NR signaling is complex, whereby it can function as a transcriptional coactivator and a translational inhibitor, and its function is also influenced by its kinase activity and level of SRA expression.

Although Dicer has been associated with ribosomal DNA chromatin in mammalian cells (32), it has not been previously linked to NR signaling pathways. Our data suggest a regulatory role for Dicer as an NR coactivator in hormone-regulated transactivation. Dicer is recruited to the PSA promoter in response to DHT treatment, and when depleted from cells, there is decreased promoter occupancy by coregulators. We would, therefore, predict that overexpression of Dicer in PCa cells would augment AR signaling and promote tumor cell growth. Indeed, Dicer expression is increased in PCa and correlated with clinical stage, lymph node involvement, and Gleason score (33).

Our data support the concept of SRA acting as a nuclear scaffold for multiple NRs and NR coregulators (34). Furthermore, based on the observation that SRA associates with RISC proteins in the cytoplasm and nucleus, we suggest that SRA may aid the assembly of protein complexes and have a role in miRNA biogenesis. SRA could also sequester members of RISC and modify their availability for coregulator or miRNA processing activities. Indeed, HIV-1 TAR RNA can sequester TRBP, whereby excess TAR RNA interferes with RISC/Dicer function and modifies miRNA expression (35).

There is increasing recognition of the roles that steroid hormones play in the regulation of miRNA biogenesis. A recent study showed that ER α prevents conversion of primary RNA transcripts to pre-miRNAs by associating in an E₂-dependent manner with the Drosha complex (15). In addition, Dex induces a reduction in Dicer/Drosha/Pasha expression in lymphocytes, resulting in decreased miRNA processing (16). Our data suggest nuclear roles for the cytoplasmic RISC members PACT and TRBP as SRA-interacting NR coregulators. Additional studies are needed to determine if the nuclear presence of the RISC proteins will also mediate nuclear RNA silencing, which has been described in other species (36). Furthermore, given the capacity of SRA to modulate dicing, its nuclear and cytoplasmic interactions with the RISC proteins require additional elucidation along with an understanding of how hormones, such as DHT, may impact on the associations between the RISC proteins and pre-miRNAs in both the cytoplasm and nucleus.

In summary, these studies identify previously undescribed linkages between transcriptional activation, NR coregulators of endocrine signaling, and the miRNA processing machinery. We show a nuclear function for several dsRBP RISC proteins. In addition, we show that SRA is a component of the nuclear and cytoplasmic dsRBP RISC complex and can facilitate recruitment of these dsRBPs to hormone-responsive promoters. Finally, our data

Fig. 5. Nuclear pre-miRNA processing in LNCaP prostate cancer cells. (A) IP-RT-qPCR for SRA copurification with PACT, Dicer, TRBP, and Ago in cytoplasmic (cyto) and nuclear (nucl) fractions. * $P < 0.05$ and ** $P < 0.005$ relative to IgG. (B) pre-miRNA-Luc processing assay. Cells were transfected with SRA, Dicer, or GFP siRNA for 2 d and further cotransfected for 1 d with a BLOCK-iT Pol II pre-miRNA-Luc vector, an empty pre-miRNA-report firefly Luc reporter vector, and a Renilla Luc construct. Cells were lysed at 24 h, and Luc activity was measured (normalized to Renilla). * $P < 0.005$ relative to GFP siRNA. (C) *In vitro* pre-miRNA processing assay. Labeled pre-miRNA-21 was incubated with recombinant Dicer (0.01, 0.1, or 1 U for 1 or 24 h) subjected to denaturing PAGE and imaged. (D) *In vitro* pre-miRNA processing assay. Labeled pre-miRNA-21 incubated for 24 h with 10 or 100 ng LNCaP cell cytoplasmic and nuclear extracts was treated as in C. Error bars are SD and representative of three independent experiments.

provide mechanistic insights into the potential for nuclear RISC proteins to act as oncogenes in hormone-dependent cancers and thereby, may provide inroads for the exploration of potential therapeutics.

Materials and Methods

Y3H Screen, Plasmids and Cloning, REMSA, UVXL Assay, in Vitro pre-miRNA Processing, and Statistical Analysis. Details are in *SI Materials and Methods*.

Cell Culture. HeLa, MDA-MB-468, MCF-7, LNCaP, HEK-293T, and 22Rv1 cells were obtained from ATCC and cultured as per the supplier's recommendations.

FLAG Transfection, Cellular Fractionation, IP, and RT-PCR/qPCR Assay. HEK-293T cells were transfected with 10 μ g FLAG-tagged proteins before IP and RT-qPCR analysis. HeLa or LNCaP cells were lysed, nuclear and cytoplasmic fractions were generated, and IP was performed with specific antibodies. Copurifying RNA or pre-miRNA was extracted, and RT-qPCR analysis was performed. Specific details of protocols, antibodies, and primers used are outlined in *SI Materials and Methods*. The primers used for Luc, PSA, NKX3.1, v-maf musculoaponeurotic fibrosarcoma, NRIP, THBS1, Ret proto-oncogene, GAPDH, Dicer, SRA, TRBP, PACT, and actin gene expression are detailed in *SI Materials and Methods*. qPCR data analyses was performed using the $\Delta\Delta$ Ct method (37).

Western Immunoblotting. Cytoplasmic and nuclear proteins were prepared as described in detail in *SI Materials and Methods*, resolved on NuPAGE Bis-Tris gels (Invitrogen), and transferred to PVDF plus membranes (Roche). Membranes were probed with various antibodies and detected using ECL Plus (GE Healthcare). Details of antibodies used are outlined in *SI Materials and Methods*.

RNAi. HeLa, LNCaP, or MCF-7 cells were transfected with 33–100 nM PACT, PKR, TRBP, Dicer, SRA, or GFP siRNA (SMARTpool; Dharmacon) using Lipofectamine

2000 (Invitrogen) according to the manufacturer's instructions for 3 d before cell harvest for downstream analysis.

ChIP qPCR Assays. MCF-7 or LNCaP cells were treated with E_2 (100 nM) or DHT (10 nM) for 45 min. Cells were fixed with formaldehyde, lysed, and sonicated, resulting in soluble chromatin. IP was performed with input chromatin and specific Abs, which are detailed in *SI Materials and Methods*; 2 μ L recovered DNAs were assayed using 2 $\times$ SYBR Green PCR Master Mix and 200 nM primers specific to either the pS2 or PSA promoters. Primer sequences are detailed in *SI Materials and Methods*. Analyses of the ChIP-qPCR data were performed as described (38) and processed using the $\Delta\Delta$ Ct method (37), and promoter occupancy was expressed relative to IgG control = 1.

Transfection and Luc Assays. HeLa or LNCaP cells were transfected using FuGENE6 (Roche), Lipofectamine 2000 (Life Technologies), or TransIT (Mirus). All transfections contained similar final DNA concentrations by mass as detailed in *SI Materials and Methods*. For pre-miRNA processing assays, 10 ng/well either miRNA-neg- or miRNA-luc-validated control vectors (Invitrogen), together with 100 ng pmIR-REPORT (Ambion) and 10 ng Renilla Luc vector, were transfected. Posttransfection, cells were treated with hormones for 8–24 h (details in *SI Materials and Methods*) and lysed with passive lysis buffer. Firefly or Renilla Luc activity was measured using a Luciferase Assay System (Promega).

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