### Statistical analysis

Tumor curves and measurement comparisons between more than three groups were assessed by one-way ANOVA with Bonferroni multiple-comparison test. Single-measurement comparison between two groups was evaluated by two-sided Student's *t*-test. *p* values of $<0.05$ were considered statistically significant. Except where noted, experiments were performed in triplicate, and averages ±SD of the relative distributions are reported.

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*Abbreviations:* **FAK**: focal adhesion kinase · **PE**: phycoerythrin · **Trm**: tissue-resident memory · **VLA**: very late antigen

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# RNA Polymerase III Regulates Cytosolic RNA:DNA Hybrids and Intracellular MicroRNA Expression *

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*Running Title: *POL III links cytosolic RNA:DNA hybrids to miRNAs*

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**Keywords:** Cancer, Innate Immunity, DNA damage, RNA polymerase III, microRNA, RNA:DNA hybrids, RNase H, S9.6 antibody, RNA transport, Human

**Background**: RNA:DNA hybrids exist in the nucleus and mitochondria, but not in the cytosol except viral infection.
**Results**: RNA:DNA hybrids exist in the cytosol of various human cells is mediated by RNA polymerase III, where RNA polymerase III regulates the microRNA machinery.
**Conclusion**: Cytosolic RNA:DNA hybrids is regulated by RNA polymerase III.
**Significance**: Previous unknown cytosolic RNA:DNA hybrids may have physiological relevance to miRNA machinery and RNA transport.

## ABSTRACT

**RNA:DNA hybrids form in the nuclei and mitochondria of cells as transcription-induced R-loops or G-quadruplexes, but only exist in the cytosol of virus-infected cells. Little is known about the existence of RNA:DNA hybrids in the cytosol of virus-free cells, in particular cancer or transformed cells. Here, we show that cytosolic RNA:DNA hybrids are present in various human cell lines, including transformed cells. Inhibition of RNA polymerase (III), but not DNA polymerase abrogated cytosolic RNA:DNA hybrids. Cytosolic RNA:DNA hybrids bind to several components of the miRNA machinery-related proteins including AGO2 and DDX17. Furthermore, we identified miRNAs that were specifically regulated by RNA polymerase III, providing a potential link between RNA:DNA hybrids and the miRNA machinery. One of the target genes, exportin-1, was shown to regulate cytosolic RNA:DNA hybrids. Taken together, we reveal previously unknown mechanism by which the RNA polymerase III regulates the presence of cytosolic RNA:DNA hybrids and miRNA biogenesis in various human cells.**

RNA:DNA hybrids can occur during transcription and replication of DNA (1). The DNA primase generates short RNA:DNA fragments during replication of the lagging strand (2,3). Short hybrids also form during the transcription of DNA by RNA polymerases. In contrast, long RNA:DNA hybrids are events that can occur during stalling of the RNA polymerase or during replication of mitochondria DNA (4). Stalling of the RNA polymerases can lead to the formation of R-loops, which consist of long RNA:DNA hybrids and the displaced non-template DNA strand. Long RNA:DNA hybrids also occur in G-quadruplexes, which promote class switch recombination in B cells (5). Recent evidence suggest that R-loops and G-quadruplexes may occur more frequently than previously assumed and interfere with gene expression and threaten genome stability (6-8).

While many studies have focused on the generation of nuclear RNA:DNA hybrids, it is unclear how nuclear RNA:DNA hybrids are resolved and their role in diseases related to genomic instability such as cancer.

We recently found the presence of ssDNA and double-stranded (ds) DNA in the cytosol of B-cell lymphoma cells (9). Inhibition of ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) kinases, which initiate the DNA damage response (DDR) lead to the disappearance of cytosolic DNA. Conversely, the levels of cytosolic ssDNA and dsDNA increased in response to DNA damage suggesting that constitutive nuclear DNA damage and the ensuing DDR induces the presence of cytosolic ssDNA and dsDNA in B-cell lymphoma cells. Cytosolic DNA in B-cell lymphoma cells induced STING-dependent DNA sensor pathways leading to the expression of ligands for the activating immune receptor NKG2D (9). Delocalized DNA is important for innate immune recognition of pathogens and recent reports suggest that TLR9 and the NLRP3 inflammasome sense pathogen-derived RNA:DNA hybrids in dendritic cells (10-13). However it is not known if RNA:DNA hybrids exist in the cytosol of non-infected cells.

RNA polymerase III (POL III) is the largest RNA polymerase consisting of 17 subunits including a DNA binding site (14-16). It catalyzes the transcription of genes required for transcription and RNA processing such as tRNAs, ribosomal 5S rRNA, and U6 snRNAs. It also transcribes short interspersed elements (SINEs) and repeated elements in the human genome (14). *POL III* expression is regulated by oncogene products, tumor suppressors such as p53, and POL III-associated transcription factors (17-19). Consistent with these observations, POL III activity is increased in many cancers including melanomas, myelomas and carcinomas (20). Although POL III is mostly present in the nucleus (20,21), cytosolic POL III was proposed to play a role in the sensing of AT-rich DNA via the retinoic acid inducible gene I (RIG-1) pathway (22-24). Despite the regulation of *POL III* by genes associated with tumorigenesis, little is known about the role of POL III in cellular function of transformed cells.

Here, we identified the presence of cytosolic RNA:DNA hybrids in immortalized and transformed human tumor cells. Chemical inhibition of POL III abrogated the presence of cytosolic RNA:DNA hybrids in cells. Cytosolic RNA:DNA hybrids were bound by miRNA-machinery-associated proteins such as DDX17 and AGO2. We also identified POL III-regulated intracellular miRNAs in lung cancer A549 cells. In summary, we demonstrate that the constitutive presence of cytosolic RNA:DNA hybrids in a variety of cell lines, and this accumulation depends on RNA POL III in at least A549 lung carcinoma.

## EXPERIMEI TAL PROCEDURES

*Cells* – The human lung adenocarcinoma (A549), colorectal adenocarcinoma (LoVo and HT29), colorectal carcinoma (HCT116), acute monocytic leukemia (THP-1), human cervix carcinoma cell line (HeLa), and normal lung tissue derived (MRC-5) cell lines were purchased from ATCC (USA). Cells were grown in Dulbecco's modified Eagle's medium (Nacalai Tesque, Japan), supplemented with 10% fetal bovine serum (Cell Culture Bioscience, Japan), 1% penicillin/streptomycin (Nacalai Tesque), and 2% HEPES (Life Technologies, Japan). Cells were maintained with 5 μg/ml of Plasmocin (Invivogen, USA) to prevent mycoplasma infection.

*Reagents and cell treatments* – Cytarabine (Ara-C) was purchased from Wako Chemicals (Japan). Aphidicolin (APH), RNA Pol III inhibitor, ML-60218 and Leptomycin B was purchased from Calbiochem (Germany). ATM inhibitor, KU60019 (Tocris Bioscience, United Kingdom) and ATR inhibitor, VE821 (Axon Med Chem, Netherlands) were used at 10 μM. PicoGreen dsDNA reagent (Life Technologies, USA) was used at a 1:100 dilution. Mitotracker (Life Technologies) was dissolved in DMSO and used at 500 nM. Fixed cells were treated with 0.5 U/ml RNase H (NEB, USA) for 3 hrs at 37°C.

*Immunofluorescence studies* – Cells were fixed with 4% paraformaldehyde for 10 min, and permeabilized in 0.2% Triton X-100 for 15 min. Non-specific sites were blocked with 2% goat serum and 1% BSA in 0.2% Triton X-100 for 1 h. Transfected cells were stained with anti-COX IV antibody (ab16056, Abcam, United Kingdom), anti-POLR3G (LS-C163858, LS Bio, USA), or anti-DDX17 (19910-1-AP, Proteintech, USA). The RNA:DNA hybrid-specific S9.6 antibody was a kind gift of Dr. D. Koshland, University of

California, Berkeley (25). Secondary polyclonal antibodies used were Alexa Fluor 488 F(ab')2 Fragment of Goat Anti-Mouse IgG (H+L) (Life Technologies) and Alexa Fluor 555 F(ab')2 Fragment of Goat Anti-Rabbit IgG (H+L) (Life Technologies). PicoGreen staining of DNA and MitoTracker Red CM-H2XRos staining of mitochondria was performed according to the manufacturer's instructions (Life Technologies). Cells were stained with 2 μg/ml of Hoechst for 10 min and mounted in mounting medium (Dako, USA). Cell images were taken with a Leica TCS SP2 laser confocal scanning microscope (LCSM), and analyzed using Volocity (Version 6.2.1) and Imaris. Micrographs show cells representative of total cell populations. Scale bar represents 10 μm.

*Transfection* – A549 cells were transfected with siRNA of *POLR3G* (Qiagen, Netherlands) using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) according to manufacturer's instructions. AllStars Negative Control siRNA (Qiagen) was used as a control in transfection (siNEG), and its sequence was proprietary. The siPOLR3G sequences used were: siPOLR3G_1, 5'-AAGGCACACCACTCACTAA TA-3', siPOLR3G_2, 5'-TCAGAGTACTCAAGT GTACAA-3'.

*Immunoblotting* – Cells were lysed in cold RIPA buffer (Nacalai Tesque) and lysates were electrophoresed in 4-12% NuPAGE Bis-Tris gel (Life Technologies) before blotted onto PVDF membranes. Antibodies specific to DDX17 (sc-86409, Santa Cruz, USA), AGO2 (C34C6, Cell Signaling Technology, USA), and GAPDH (M171-3, MBL), and horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) were used to develop the blots, with Immobilon Western Chemiluminescent HRP Substrate (Milipore, Germany). Digital images were acquired using ImageQuant LAS 500 (GE Healthcare, United Kingdom).

*Immunoprecipitation and mass spectrometry* – 2 x $10^6$ A549 cells were seeded into 100-mm dishes and fixed in 1% paraformaldehyde (Nacalai Tesque) for 10 min, followed by treatment with 125 mM of Glycine (Wako, Japan) for 5 min. Cells were fractionated using MitoSciences cell fractionation kit (MS861, MitoSciences, USA). The cytosolic fraction was precleared by incubation with 5 μl of Protein G–Sepharose beads (GE Healthcare) at 4°C for 20 min on a rolling shaker. The cleared supernatant was incubated at 4°C overnight on a rolling shaker with 10 μg/ml of RNA:DNA hybrid antibody and 10 μl of Protein G–Sepharose beads. Immunoprecipitates were washed subsequently with RIPA buffer, low salt buffer (20 mM Tris-HCL pH 8.1, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2mM EDTA), high salt buffer (20 mM Tris-HCL pH 8.1, 600 mM NaCl, 0.1% SDS, 1% Triton X-100, 2mM EDTA), final wash (20 mM Tris-HCL pH 8.0, 0.1% SDS, 1% Triton X-100, 1 mM EDTA) and TE buffer. Beads were resuspended in TE buffer with 1% SDS and incubated at 65°C overnight to release protein complexes for subsequent gel electrophoresis. For mass spectrometry, similarly processed cell lysates were immunoprecipitated with RNA:DNA hybrid antibody, and silver stained using Silver Stain Plus Kit (Bio-Rad, USA) according to the manufacturer's instruction. Bands of interest were cut out and sent for mass spectrometry analysis at the Osaka University mass spectrometry facility.

*MiRNA microarray analysis* – A549 cells were treated with 10 μM of RNA POL III inhibitor for 24 hrs, or subsequently treated with 10 μM Ara-C or DMSO for 15 hrs. DMSO-treated cells served as a control. Total RNA was extracted by Trizol (Life Technologies) and labelled using a 3D-Gene miRNA labeling kit. The labelled RNA was hybridized to a human miRNA V19 microarray chip containing 2019 miRNA probes and analyzed on a ProScanArray™ microarray scanner (Toray Industries, Japan). MiRNA profiles were provided as sample-wise median-normalized data by Toray. Data were further normalized using all-sample quantile normalization protocol using the corresponding Bioconductor package developed by Bolstad et al. (26). Original miRNA profiles consisted of 2019 miRNA probes, of which only a small fraction showed significant expression in any of these experiments. After replacing the missing valued data (no expression observed) by the minimum of all observed expression values, miRNA probes that showed at least 3-fold differential expression between any pair of 4 experiments, were used for further quantitative analysis. Identified miRNA sequences were used to obtain predicted gene targets, as acquired from public domain resource, mir-DIANA (27). A P-value threshold of 0.05 and MicroT threshold of 0.8 was applied.

*MiRNA expression analysis* – After RNA extraction, cDNA was synthesized using miScript II RT kit (Qiagen). MiRNA levels were analysed using assay kits for mature miR-4499 (Qiagen), precursor (includes detection for precursor and primary miR) miR-4499 (Qiagen), TaqMan primary miR-4499 (Life Technologies), and quantified by qPCR using iTaq Universal SYBR Green Supermix (BioRad), or TaqMan Gene Expression Master Mix (Life Technologies). Precursor miR expression was determined as per the equation referenced in (28).

*Quantitative Real-time RT-PCR* – Performed as described in (9). The following primers were used: *XPO1*-5', 5'-AGGTTGGAGAAGTGATGC CA-3'; *XPO1*-3', 5'-GCACCAATCATGTACCC CAC-3'; *KPNB1*-5', 5'-GACCGACTACCCAGA CAGAG-3'; *KPNB1*-3', 5'-GACTCCTCCTAAG ACGACGG-3'; *NUP153*-5', 5'-GCCCAAATCTT CCTCTGCAG-3'; *NUP153*-3', 5'-GAAAGGAG CCACTGAAGCAC-3'; *HPRT1*-5', 5'-CCCTGG CGTCGTGATTAGTG-3'; *HPRT1*-3', 5'-TCGA GCAAGACGTTCAGTCC-3'.

*Statistical analysis* – For statistical analysis, one-tailed Student's t-test ($P < 0.05$) was used unless otherwise stated, after data were tested positive for normality by Shapiro-Wilk test. For data that failed normality test, non-parametric Mann-Whitney Wilcoxon Rank-Sum test was used. Error bars represent standard error unless otherwise stated in the figure legends. A P-value of $* < 0.05$ was considered statistically significant.

## RESULTS

*Presence of RNA:DNA hybrids in the cytosol of human cells* – We previously reported the presence of cytosolic ssDNA and dsDNA in cancer cells using specific antibodies and the vital dye PicoGreen, which can detect dsDNA and RNA:DNA hybrids (9,29,30). Here, we sought to test if RNA:DNA hybrids are present in the cytosol of human cancer cell lines. PicoGreen stained DNA in the cytosol of the human lung carcinoma cell line A549 and other cancer cell lines (Fig. 1, *A* and *B*, and Fig. 2, *A* and *B*). Three-dimensional surface rendering of confocal images showed that the majority of extranuclear DNA is present outside of mitochondria (Fig. 1*B*). To analyze if RNA;DNA hybrids contribute to the PicoGreen signals in the cytosol, A549 cells were treated with RNase H, which degrades RNA in RNA:DNA hybrids, prior to staining with PicoGreen (31). Pretreatment of cells with RNase H abrogated the cytosolic PicoGreen signals (Fig. 1, *A-C*). As expected, the staining of nuclear genomic DNA by PicoGreen was not changed.

To further investigate the presence of cytosolic RNA:DNA hybrids, we stained cells using the RNA:DNA hybrid-specific S9.6 antibody (25). In agreement with the PicoGreen, S9.6 staining of tumor cells A549, LoVo, HCT 116, HT29, HeLa, THP-1, and human fetal lung fibroblast (MRC-5) cells showed the presence of RNA:DNA hybrids in the cytosol and to a lesser extent in the nucleus (Fig. 1, *D* and *E,* and Fig. 2, *A* and *B*). As RNA:DNA hybrids can also form during replication of mitochondrial DNA (32), we co-stained cells with the mitochondria-specific vital dye MitoTracker. Three-dimensional surface rendering of confocal images showed that the majority of RNA:DNA hybrids were localized outside of mitochondria (Fig. 1*E*). Pre-treatment of cells with RNase H prior to S9.6 staining significantly reduced the cytosolic RNA:DNA hybrid staining (Fig. 1, *D-F*). S9.6 staining only partially co-stained with PicoGreen suggesting that PicoGreen stains cytosolic dsDNA and RNA:DNA hybrids in A549 cells (Fig. 1*G*). Consistent with this possibility, staining of A549 cells using a dsDNA-specific antibody showed the presence of cytosolic dsDNA, which partially co-localized with the cytosolic PicoGreen staining (Fig. 2*C*). In summary, our data show that RNA:DNA hybrids are constitutively present in the cytosol of all tested cells.

*Presence of cytosolic RNA:DNA hybrids depends on RNA Polymerase III* – RNA:DNA hybrids can occur during transcription of DNA (33). In addition, cytosolic DNA is transcribed by RNA polymerase III in the cytosol and potentially in the nucleus into a RNA:DNA hybrid and dsRNA intermediate (22,23). To understand the mechanism by which cytosolic RNA:DNA hybrids are generated and regulated, A549 cells were treated with POL III inhibitors prior to staining with S9.6 or PicoGreen (34). Treatment of A549 cells with the POL III inhibitor ML-60218 decreased the cytosolic RNA:DNA hybrid staining at doses above the published half-maximal inhibitory concentration ($IC_{50}$), but had no effect on nuclear PicoGreen staining, but affected RNA:DNA hybrid staining at above $IC_{50}$ (Fig. 3). Treatment of A549

cells with α-amanitin, a POL II inhibitor, was toxic on cells as compared to POL III inhibitor ML-60218.

To investigate if genetic effect of POL III inhibition also reduced RNA:DNA hybrid levels, A549 cells were transfected with siRNA against POLR3G (siPOLR3G_1 and siPOLR3G_2), a subunit of the RNA POL III complex, or negative control siRNA (siNEG). Knockdown of POLR3G protein expression resulted in disappearance of cytosolic RNA:DNA hybrids by immunofluorescence staining, consistent with reduced POLR3G mRNA gene expression (Figure 4). In contrast, siNEG did not affect cytosolic RNA:DNA hybrid levels, nor POLR3G expression. This corroborated with previous results of POL III chemical inhibition, which decreased the presence of RNA:DNA hybrids.

In contrast to POL II, which is exclusively localized in the nucleus, a fraction of POL III is present in the cytosol. To investigate if cytosolic POL III contributes to the generation of RNA:DNA hybrids in the cytosol, we co-stained A549 cells for POLR3G, a subunit of the POL III complex, and RNA:DNA hybrids (35). No significant co-staining of POLR3G and S9.6 or PicoGreen was observed in the cytosol of A549 cells (Fig. 5*A*). Furthermore, POLR3G was largely localized in the nucleus suggesting that the presence of cytosolic RNA:DNA hybrids depends on nuclear POL III activity.

*Presence of cytosolic RNA:DNA hybrids is independent of DNA damage* – RNA:DNA hybrids can cause stalling of the replication fork and formation of dsDNA breaks (4,36,37). To test if stalling of replication forks and the associated DNA damage contributes to the presence of RNA:DNA hybrid in the cytosol, A549 cells were treated with cytarabine (Ara-C), a genotoxic DNA replication inhibitor used to treat leukemia, and aphidicolin, an inhibitor of DNA polymerases (38). Treatment with Ara-C or aphidicolin had no effect on the level of cytosolic RNA:DNA hybrids in A549 cells (Fig. 5, *A* and *C*). Moreover, Ara-C treatment did not increase the co-localization of POLR3G and RNA:DNA hybrids (Fig. 5, *A* and *B*).

To test if the cellular response to DNA damage is required for the presence of cytosolic RNA:DNA hybrids, we inhibited ATM and ATR, two kinases that initiate the DDR. We previously found that the presence of dsDNA in the cytosol of B-cell lymphoma cells depends on the DDR (9). In contrast, inhibition of the DDR had no effect on the presence of RNA:DNA hybrids in the cytosol (Fig. 5*D*). Hence, unlike cytosolic dsDNA, RNA:DNA hybrid levels in the cytosol depend on POL III, but not DNA damage or the DDR.

*Cytosolic RNA:DNA hybrids bind members of the miRNA processing machinery* – To examine whether RNA:DNA hybrids interact with proteins in the cytosol, we performed immunoprecipitation experiments using the RNA:DNA-specific S9.6 antibody on cytosolic extracts of A549 cells. A fraction of the extracts was treated with RNase H before analysis to verify RNA:DNA hybrid-specific binding of proteins. Analysis by mass spectrometry identified DEAD (Asp-Glu-Ala-Asp) box polypeptide 5/17 (DDX5/DDX17), Argonaute (AGO 2), and Breast cancer 1 (BRCA-1) as proteins that immunoprecipitated in an S9.6-dependent manner (Fig. 6*A*). All three proteins are part of the miRNA processing machinery (39). Immunoblot analyses of immunoprecipitated proteins showed that DDX17 binding was consistent with the mass spectrometry analysis (Fig. 6*B*). AGO2 is a part of the miRNA-mediated DDR (40), and increases interaction with repair molecules during double-strand break (DSB) repair (41). Ara-C treatment increased interaction of S9.6 with AGO2 proteins (Fig. 6*C*). Consequently, components of the miRNA processing machinery are found to interact with cytosolic RNA:DNA hybrids, in the absence and increased interaction in the presence of DNA damage.

*RNA Polymerase III regulates the expression of specific miRNAs* – Next we sought to gain insights into the POL III-dependent mechanisms leading to the presence of cytosolic RNA:DNA hybrids. Our data support the possibility that POL III-mediated transcription of miRNAs is associated with the presence of cytosolic RNA:DNA hybrids. POL III is able to transcribe a subset of miRNAs in a cell-type-specific manner (42), or interact with canonical genes within a chromosome location that also encodes miRNAs (43). To determine if a subset of miRNAs are dependent on POL-III, we examined the POL III-dependent miRNA expression profile in A549 cells by comprehensive miRNA array analysis. To distinguish POL III effects from RNA:DNA hybrid-induced DNA damage, cells were treated with the genotoxic DNA replication inhibitor Ara-C or the POL III inhibitor

ML-60218. A total of 81 differentially expressed miRNAs were identified after comparison across the treatment groups (Fig. 7*A*). Strikingly, treatment of cells with POL III inhibitor resulted in significant down-regulation of only four miRNAs: miR-615-5p, miR-1178-5p, miR-4499, and miR-5571-3p (Fig. 7, *A* and *B*). The expression of miR-615-5p, miR-1178-5p, and miR-5571-3p was also decreased after Ara-C treatment, suggesting that the expression of these miRNAs is also downregulated by DNA damage. In contrast, miR-4499 is likely to be transcribed directly by POL III as Ara-C had no effect on its expression. Surprisingly, the expression of ten miRNAs was upregulated after treatment with the POL III inhibitor ML-60218, but not Ara-C (Fig. 7, *A* and *B*). To confirm if POL III directly regulated miR-4499 expression, miRNA expression of miR-4499 was measured after POL III treatment. Mature miR-4499 expression decreased after treatment, corresponding to miRNA microarray data (Fig. 7*C*). Precursor miR-4499 (pre-miR-4499) also decreased significantly while primary miR-4499 (pri-miR-4499) was not significantly affected (Fig. 7*C*). This suggested that POL III does not directly transcribe pri-miR-4499, but might regulate Drosha processing of pre-miR-4499 to affect mature miR-4499 expression.

In summary, our data indicate that POL III regulates the expression of a limited number of miRNAs, which may contribute to the presence of cytosolic RNA:DNA hybrids in A549 cells.

To investigate all the potential transcripts targeted by POL III-modulated miRNAs, a miRNA target prediction and pathway analysis was performed using the DIANA-miRPath software (http://www.microrna.gr/miRPathv2.) (27). Among the top ranked pathways, RNA transport and RNA surveillance pathways were identified to contain predicted genes that are targeted by two or more ML-60218-induced miRNAs (red boxes), while other transcripts are potentially targeted by a single miRNA (yellow boxes) (Fig. 8*A*). Hence, RNA transport or stability may contribute to the presence of RNA:DNA hybrids. To test the predictability of the DIANA-miRPath software, three genes *KPNB1*, *XPO1*, and *IGUP153* were selected for mRNA expression detection after POL III inhibition. Gene expression of *XPO1* and *IGUP153* were found to be downregulated by 1.6-fold and 3.3-fold respectively (Fig. 8*B*).

*Exportin 1 regulates transport of RNA:DNA hybrids from the nucleus to the cytosol* – Of the identified genes that were downregulated in response to RNA POL III inhibition, *XPO1* encodes for exportin 1, a protein involved in RNA transport and miRNA processing (44,45). To test if exportin 1 is involved in transport of nuclear RNA:DNA hybrids to the cytosol, cells were treated with Leptomycin B (LMB), an XPO1 inhibitor. Increasing concentrations of LMB decreased accumulation of cytosolic RNA:DNA hybrids, while cytosolic COX IV remained unchanged significantly (Fig. 9), suggesting that XPO1 function is required for the presence of RNA:DNA hybrids.

## DISCUSSION

Here we show the existence of endogenous RNA:DNA hybrids in the cytosol of a variety of human cells, including cancer cells. We previously found that ssDNA and dsDNA in the cytosol of B-cell lymphomas depended on DNA damage and the ensuing DDR (9). In contrast, treatment of cells with genotoxic agents or blocking of the DDR had no effect on the levels of cytosolic RNA:DNA hybrids suggesting the presence of RNA:DNA hybrids is regulated by different pathways. Consistent with this conclusion, inhibition of POL III lead to the disappearance of RNA:DNA hybrids in the cytosol. Interestingly, POL III inhibitors also abrogated cytosolic PicoGreen staining, which stains cytosolic dsDNA and in A549 cells, suggesting that POL III contributes to the presence of dsDNA in A549 cells. It is possible that POL III dependent R-loops, which contain RNA:DNA hybrids, contribute to the presence of cytosolic dsDNA in tumor cells by stalling replication forks, which results in DNA damage and the activation of the DDR (37). In agreement with this possibility, it was recently shown that the DDR is activated in cells that are deficient in the R-loop resolving enzyme *Rnaseh2* (46). Furthermore, overexpression of *Rnaseh1*, which degrades the RNA strand in RNA:DNA hybrids, decreased the levels of cytosolic ssDNA and dsDNA in tumor cells (Shen et al., submitted).

In previously published studies, virus or bacteria-derived cytosolic RNA:DNA hybrids were described in human cells (12,13). Our data show that RNA:DNA hybrids can also exist in the cytosol of non-infected cells. All the cells used in our study

were cultured under sterile conditions and were mycoplasma free. Cells were further treated with Plasmocin to exclude potential undetected mycoplasma contaminations suggesting that cytosolic RNA:DNA hybrids are not a result of infection of the tested cells. RNA:DNA hybrids were also reported to form in the mitochondrial genome of non-infected cells (47,48). Co-staining of cells with mitochondria-specific dyes or marker, showed that extramitochondrial RNA:DNA hybrids are present in human cells. Furthermore, inhibition of POL III, which has not been found to localize to mitochondria or extranuclear RNA:DNA hybrids, abrogated the presence of RNA:DNA hybrids. In addition, exportin-1 inhibition of nuclear export decreased cytosolic RNA:DNA hybrids, suggesting RNA:DNA hybrids are derived from the nucleus. In summary, our data suggest that nuclear POL III activity is required for the presence of RNA:DNA hybrids in the cytosol of non-infected cells.

Each polymerase in the RNA POL family of proteins has defined transcriptional roles (49). POL I transcribes ribosomal RNA, POL II transcribes protein-encoding mRNA, while POL III transcribes 5S rRNA, tRNA, and certain retroelements. Non-coding RNAs can be transcribed by all three polymerases in eukaryotic cells (50). Strikingly, the inhibition of POL III, but not DNA polymerases, abrogated the presence of cytosolic RNA:DNA hybrids suggesting that POL III transcripts are required for the existence of such structures in the cytosol. In contrast, the level of nuclear RNA:DNA hybrids was not decreased in response to POL III inhibition for PicoGreen stains, as well as at low inhibition for RNA:DNA hybrid antibody stains, consistent with previous reports that the majority of RNA:DNA hybrids in the nucleus are transcribed by POL II (51).

Immunoprecipitation experiments suggest that cytosolic RNA:DNA hybrids bind to the DDX17-containing DROSHA miRNA complex (52). It is therefore conceivable that cytosolic RNA:DNA hybrids consist of POL III-transcribed miRNAs. Analysis of the miRNA expression profile showed that POL III modulates the expression of a small number of miRNAs, most of which were upregulated upon POL III inhibition, suggesting a complex regulation of these miRNAs by POL III in A549 cells. Surprisingly, POL III inhibition reduced the expression of only four miRNAs. Three out of the four miRNAs were also downregulated by Ara-C, which does not modulate the levels of cytosolic RNA:DNA hybrids. Hence, our data suggest that cytosolic RNA:DNA hybrids consist of very limited number of POL III-transcribed miRNAs, possibly only miR-4499. However miR expression of miR-4499 after POL III treatment suggested that POL III does not directly transcribe pri-miR-4499, and POL III may instead affect Dicer processing of precursor miR-4499. Alternatively, it is conceivable that POL III-modulated miRNAs target transcripts of genes, which regulate the presence of cytosolic RNA:DNA hybrids. Consistent with this possibility, many POL III modulated miRNAs potentially target RNA transport and mRNA stability pathways. Two out of three selected potential targets from the pathways were shown to be downregulated after POL III inhibition, consistent with previous results of miRNA overexpression that would suppress gene expression of target genes. Out of the affected target genes, exportin-1 inhibition was also shown to prevent accumulation of cytosolic RNA:DNA hybrids, suggesting that RNA POL III may have multiple functions in the generation of RNA:DNA hybrids, and also possibly regulating miRNAs that modulate the transport of nuclear RNA:DNA hybrids into the cytosol. Cytosolic RNA:DNA hybrids may also be generated by the transcription of cytosolic DNA by POL III, which was reported to detect cytosolic DNA and induce type I interferons through the RIG-I pathway (20-22). However, POL III did not co-localize with cytosolic DNA or cytosolic RNA:DNA hybrids. Furthermore, inhibition of POL III did not reduce type I interferon transcript levels (data not shown) consistent with the conclusion that neither POL III nor other type I interferon-inducing sensors recognize DNA in the cytosol of A549 cells. In agreement with this observation, no known cytosolic DNA or RNA sensor precipitated with RNA:DNA hybrids. Finally, it is conceivable that reverse transcription of POL III-transcribed retroelements produces RNA:DNA intermediates in the cytosol similar to replicating RNA viruses (6,53,54).

In summary, we show that POL III activity leads to the presence of RNA:DNA hybrids in human cells. The functional role of RNA:DNA hybrids remains to be further investigated, but RNA:DNA hybrids may modulate cellular functions or

contribute to immune recognition by activating TLR9 or the NLRP3 inflammasome in dendritic cells (12,13).

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## FOOTNOTES

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[2]The abbreviations used are: NKG2D, natural killer group 2, member D; POL, polymerase; rRNA, ribosomal RNA; tRNA, translational RNA; RIG-1, retinoic acid inducible gene I; BRCA1, Breast cancer susceptibility gene 1; POLR3G, polymerase (RNA) III (DNA directed) polypeptide G; DDX5, DEAD (Asp-Glu-Ala-Asp) box polypeptide 5; DDX17, DEAD (Asp-Glu-Ala-Asp) box polypeptide 17; AGO2, argonaute 2; Ara-C, cytarabine; APH, aphidicolin; KPNB1, Karyopherin (Importin) Beta 1; XPO1, exportin-1; NUP153, Nucleoporin 153kDa.

## FIGURE LEGENDS

FIGURE 1. **RNA:DNA hybrids exist in the cytosol of human lung cancer cells.** *A*, The human lung carcinoma cell line was stained with 10 μl/ml of the vital dsDNA-specific dye PicoGreen (Green) for 1 hr and 100 nM of the mitochondria-specific vital dye MitoTracker (Red) for 30 minutes. Samples shown in lower row were pretreated with 0.5 U/ml of RNase H. *B* and *C*, 3D isosurface rendering (*B*) and quantification (*C*) of PicoGreen staining in the nucleus and cytosol of images shown in (A). One-tailed Wilcoxon test was performed. Error bars represent SEM, *P < 0.05 (*D*) A549 cells were stained with the RNA:DNA hybrid-specific antibody S9.6 (Green) and the mitochondrial marker COX IV (Red) in the presence of Hoechst (Blue). Cells shown in the lower row were pretreated with 0.5 U/ml of RNase H before staining. *E* and *F*, 3D isosurface rendering (*E*) and quantification (*F*) of RNA:DNA hybrid staining of images shown in (*D*). *G*, 3D isosurface rendering of staining of A549 cells with PicoGreen (Green), RNA:DNA hybrid-specific antibodies (Red) and Hoechst (Blue). One-tailed Wilcoxon test was performed. Error bars represent SEM, *P < 0.05.

FIGURE 2. **Presence of cytosolic RNA:DNA hybrids in human tumor cell lines**. *A*, The human colorectal carcinoma cell lines LoVo, HCT 116, and HT29, the human acute monocytic leukemia cell line THP-1, the human cervix carcinoma cell line HeLa, and the human normal lung tissue derived cell line MRC-5, were stained for the presence of RNA:DNA hybrids (Red) and Hoechst (Blue). *B*, The colorectal adenocarcinoma cell lines LoVo, HCT116 and HT29 were stained with PicoGreen (Upper row). Bright field images (DIC) of cells are shown in the lower row. *C*, 3D isosurface rendering of confocal images A549 cells stained for the presence of dsDNA (green) and RNA:DNA hybrids (Red) in the presence of Hoechst dye (Blue). Colocalization of dsDNA and RNA:DNA hybrids (Yellow) was determined by Volocity computational image analysis.

FIGURE 3. **RNA Pol III is essential for the presence of cytosolic RNA:DNA hybrids.** *A*, A549 cells were treated with the indicated concentration of RNA Pol III inhibitor ML-60218 for 3 hrs, before staining with 10 μl/ml PicoGreen (Green) for 1 hr and 100 nM of the mitochondria-specific vital dye MitoTracker (Red) for 30 minutes. *B*, Cytosolic and nuclear intensity quantification of images shown in (*A*). Two-tailed Wilcoxon test was performed. Error bars represent SEM, *P < 0.05. *C*, A549 cells were treated with indicated concentration of RNA Pol III inhibitor ML-60218 for 3 hrs, before staining of cells with RNA:DNA hybrid-specific S9.6 antibodies (Green) and Hoechst (Blue). *D*, Cytosolic and nuclear intensity quantification of images shown in (*C*). Two-tailed Wilcoxon test was performed. Error bars represent SEM, *P < 0.05.

FIGURE 4. **Genetic knockdown of RNA Pol III decreases cytosolic RNA:DNA hybrid levels.** *A*, A549 cells were transfected with 25 nM of siNEG (negative control siRNA), or siRNA against POLR3G

(siPOLR3G_1, siPOLR3G_2). 72 hrs after transfection, cells were stained for RNA:DNA hybrids (Green), POLR3G (Red), and Hoechst (Blue). *B*, Some cells in (*A*) were subjected to RNA isolation for measurement of *POLR3G* mRNA expression with respect to *HPRT1*. One-tailed Student's t-test was performed. Error bars represent SEM, *P < 0.05.

FIGURE 5. **Levels of cytosolic RNA:DNA hybrids are not modulated by genotoxic replication inhibitors.** *A*, A549 cells were treated with 10 μM of the genotoxic DNA replication inhibitor Ara-C for 15 hrs, and stained RNA:DNA hybrids (Green) and POLR3G (Red) in the presence of Hoechst (Blue). *B*, A549 cells treated with Ara-C as in *A*, and stained with 10 μl/ml PicoGreen (Green), POLR3G-specific antibody (Red), and Hoechst (Blue). *C*, A549 cells were treated with 4 μM of aphidicolin (APH), an inhibitor of DNA polymerase for 15 hrs, and stained with RNA:DNA hybrids (Green) in the presence of Hoechst (Blue). *D*. A549 cells were treated with 10 μM of ATM inhibitor (ATMi), or ATR inhibitor (ATRi), or both for 15 hrs. Cells were stained with RNA:DNA hybrids (Red) in the presence of Hoechst (Blue).

FIGURE 6. **Cytosolic RNA:DNA hybrids interact with microRNA machinery proteins.** *A*, Cytosolic fractions of A549 cells were subjected to immunoprecipitation using RNA:DNA hybrid-specific S9.6 antibodies. A part of the cytosolic fraction was pretreated with 0.5 U/ml RNase H. Immunoprecipitated proteins were detected by SDS-PAGE gel electrophoresis and silver staining. Indicated bands were analyzed by mass spectrometry. *B* and *C*, A549 cells were treated with DMSO or 10 μM Ara-C for 15 hrs, and harvested for cell fractionation after fixation. Cytosolic fractions were subjected to immunoprecipitation with RNA:DNA-specific S9.6 antibodies. Immunoblot analysis of immunoprecipitated proteins probed with antibodies specific for DDX17 (*B*) and AGO2 (*C*).

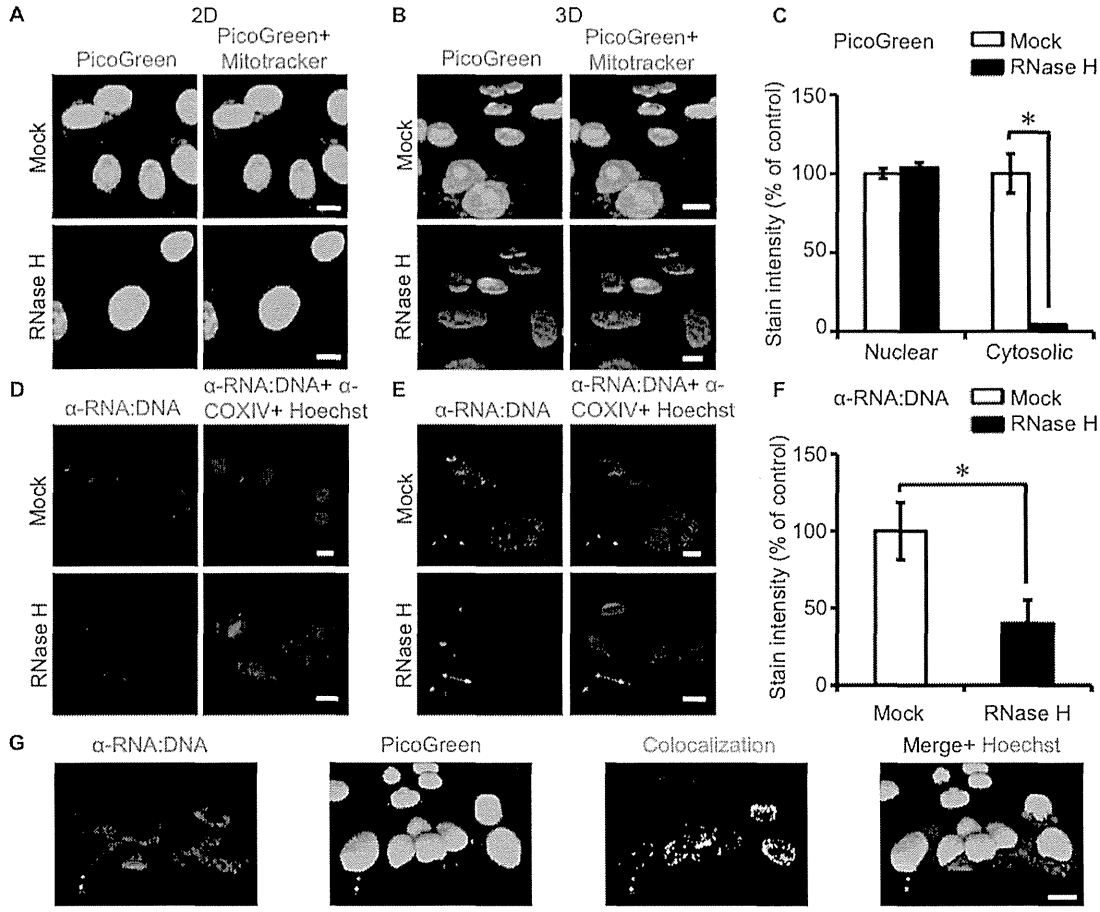
FIGURE 7. **Deregulation of microRNAs by RNA Pol III inhibition**. *A*, A549 cells were treated with 10 μM of the RNA Pol III inhibitor ML-60218 (RPIII inh.) or DMSO for 24 hrs. Some cells were further treated with Ara-C for 15 hrs. Heat map of global miRNA expression profile from total RNA extracted after treatment is shown. All expression profiles were quantile-normalized and then fold values with reference to control (DMSO) were used for plotting this heatmap. 81 probes had at least 3-fold differential expression in any pair of conditions and were pre-selected for this plot. *B*, Scatter-plot of miRNA expression profiles between control (DMSO) and ML-6028-treated samples. Decision surface was plotted for at least 3-fold change to or from control. Highly upregulated and downregulated miRNAs after RPIII inhibitor treatment were identified in the table (lower columns). *C*, A549 cells were treated with 10 μM of the RNA Pol III inhibitor ML-60218 (RPIII inh.) or DMSO for 24 hrs. Expression levels of primary, precursor, and mature miR-4499 were examined from isolated RNA. Two-tailed Student's t-test was performed. Error bars represent SEM, *P < 0.05.

FIGURE 8. **RNA transport and mRNA surveillance pathways are potential targets of RNA polymerase III modulated miRNAs.** *A,* List of predicted miRNAs regulated by POL III were analyzed for potential gene targets by using online resource DIANA-miRPath. Yellow boxes indicate genes that are regulated by one miRNA in response to POL III inhibition. The red boxes indicate potential target genes regulated by more than one miRNA upon POL III inhibition. *B*, mRNA expression levels of three potential genes, each targeted by more than one miRNA. Total RNA was extracted from A549 cells after treatment with 10 μM of the RNA Pol III inhibitor ML-60218 (RPIII inh.) or DMSO for 24 hrs, and subjected to real time PCR analysis. Normalization was done with respect to *HPRT1* housekeeping gene, and further compared against DMSO-treated cells. One-tailed Wilcoxon test was performed. Error bars represent SEM, *P < 0.05.

FIGURE 9. **Exportin 1 function is required for the presence of cytosolic RNA:DNA hybrids.** *A*, A549 cells were treated with the indicated concentration of Exportin 1 inhibitor, Leptomycin B (LMB) for 16 hrs, before staining with RNA:DNA hybrid-specific S9.6 antibodies (Green), COXIV (Red) and Hoechst (Blue). *B*, A549 cells were treated with increasing concentration of LMB (0, 10, 20, 30, and 40 nM) for 16 hrs,

before staining with RNA:DNA hybrid-specific S9.6 antibodies, COXIV, and Hoechst. Microscope images were quantified for cytosolic and mitochondrial intensity of cells. Two-tailed Student's t-test was performed. Error bars represent SEM, *P < 0.05.

FIGURE 1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PicoGreen+ Mitotracker
Mock
RNase H
B
3D
PicoGreen
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RNase H
C
PicoGreen
Mock
RNase H
Stain intensity (% of control)
150
100
50
0
*
Nuclear
Cytosolic
D
α-RNA:DNA
α-RNA:DNA+ α-COXIV+ Hoechst
Mock
RNase H
E
α-RNA:DNA
α-RNA:DNA+ α-COXIV+ Hoechst
Mock
RNase H
F
α-RNA:DNA
Mock
RNase H
Stain intensity (% of control)
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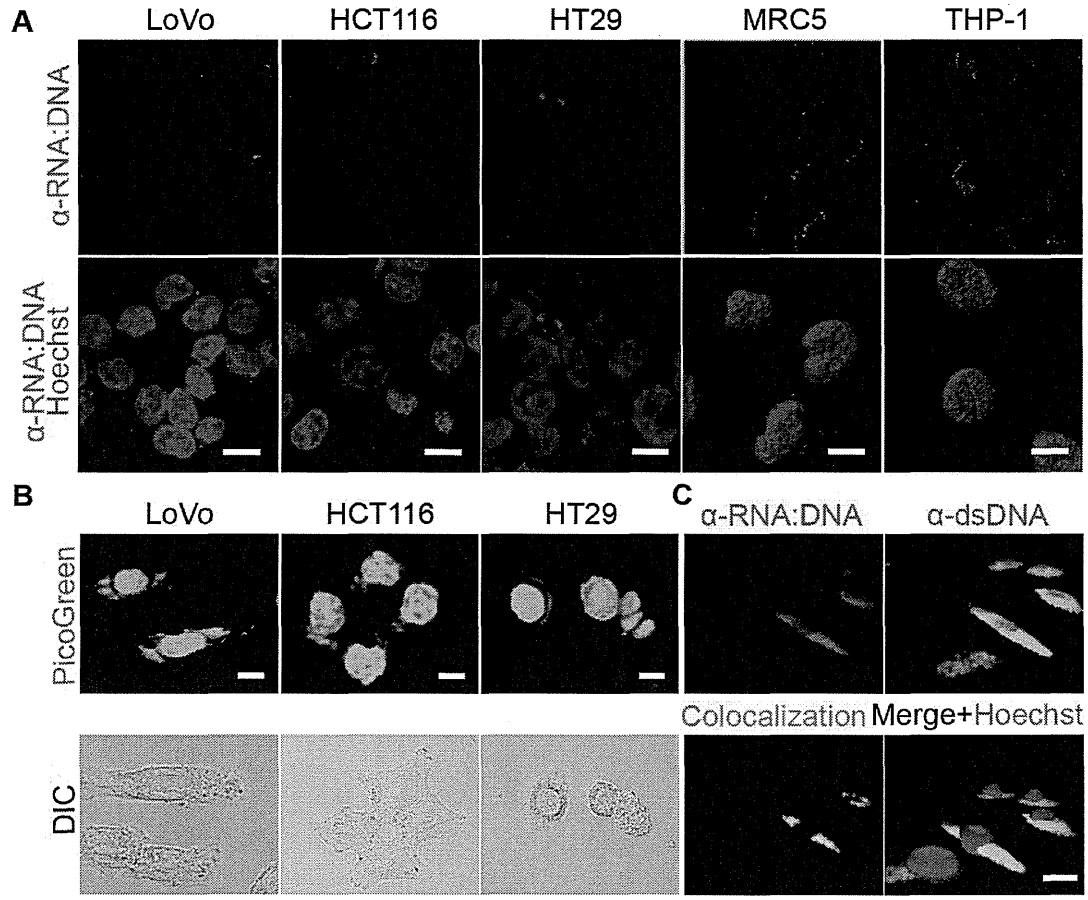
100
50
0
*
Mock
RNase H
G
α-RNA:DNA
PicoGreen
Colocalization
Merge+ Hoechst

FIGURE 2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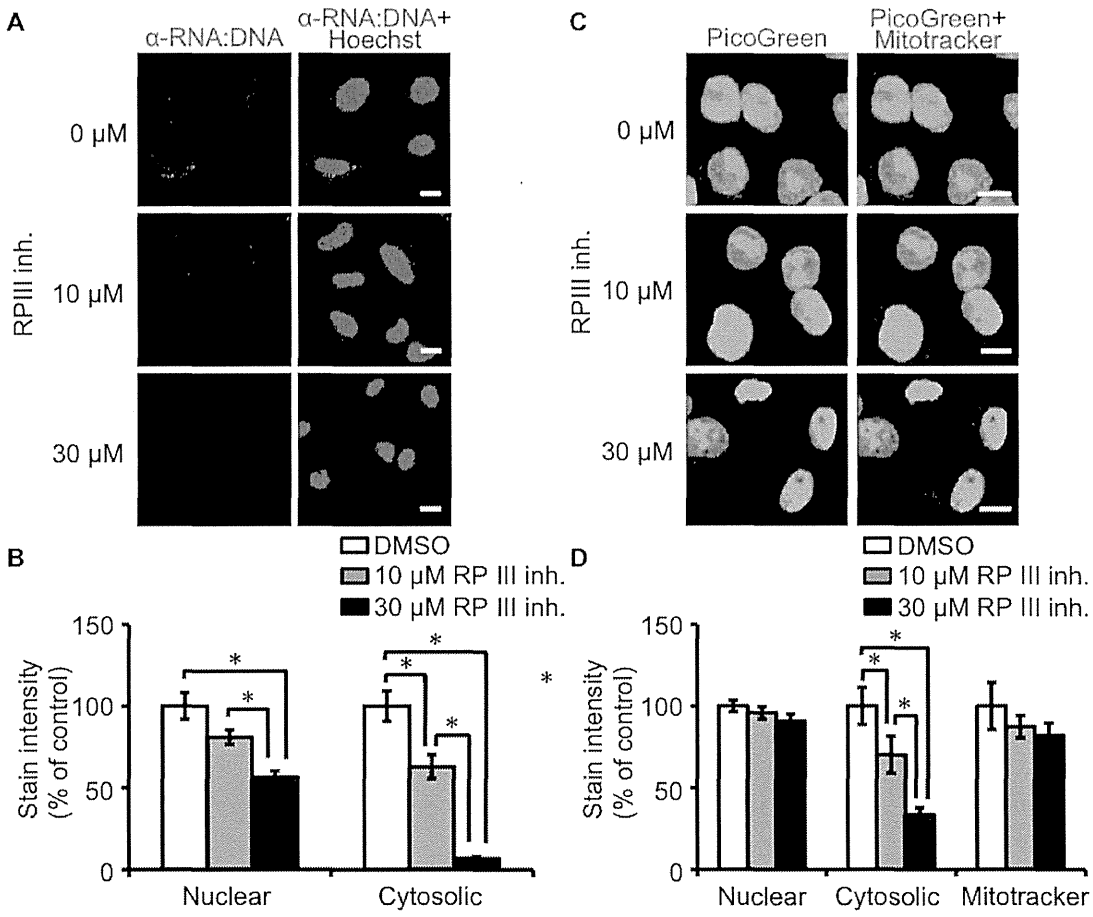
PicoGreen
DIC
C
α-RNA:DNA
α-dsDNA
Colocalization
Merge+Hoechst

FIGURE 3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α-RNA:DNA+ Hoechst
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10 μM
30 μM
RPIII inh.
C
PicoGreen
PicoGreen+ Mitotracker
0 μM
10 μM
30 μM
RPIII inh.
B
DMSO
10 μM RP III inh.
30 μM RP III inh.
Stain intensity (% of control)
150
100
50
0
Nuclear
Cytosolic
D
DMSO
10 μM RP III inh.
30 μM RP III inh.
Stain intensity (% of control)
150
100
50
0
Nuclear
Cytosolic
Mitotracker

FIGURE 4

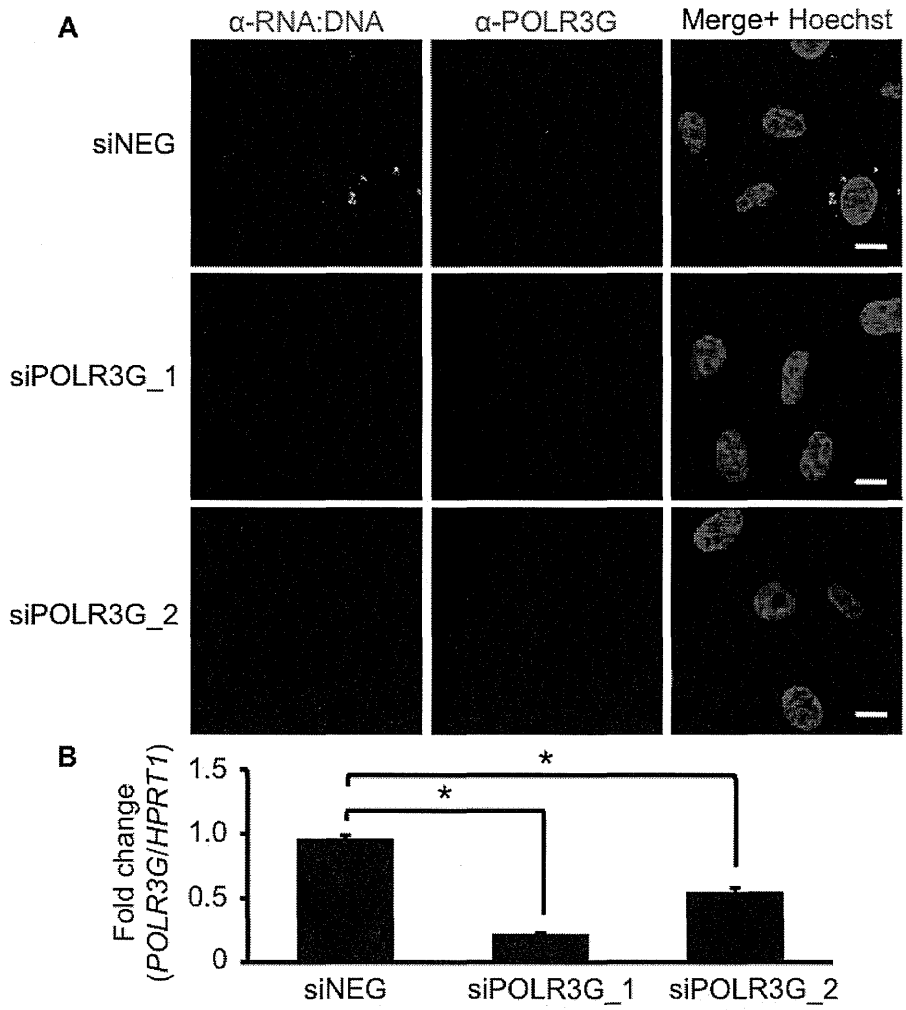
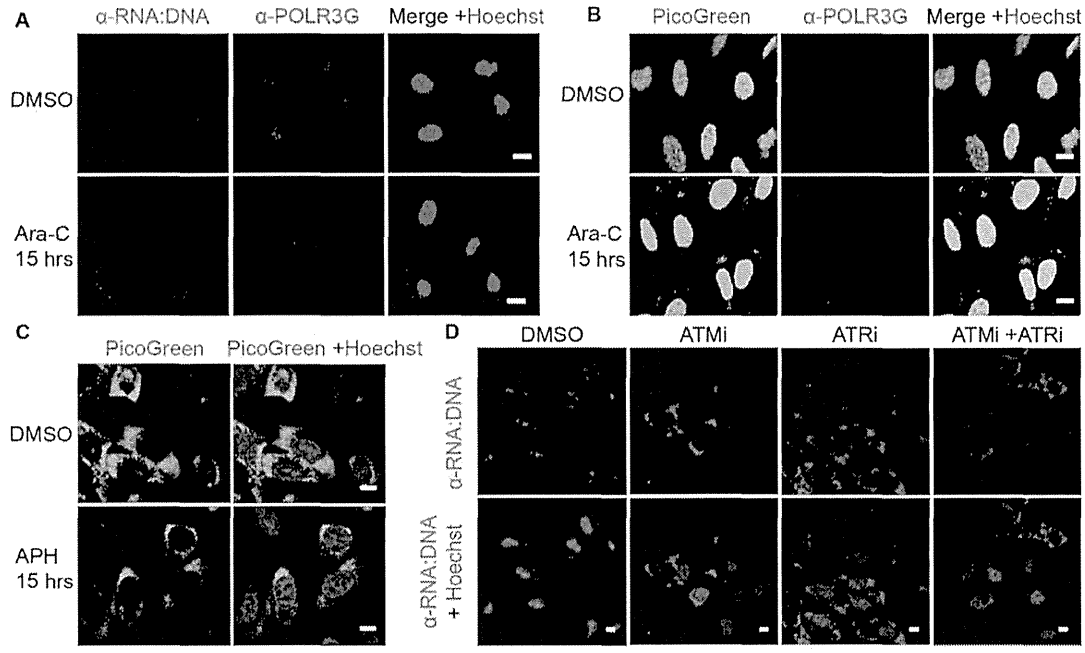



FIGURE 5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α-RNA:DNA
α-POLR3G
Merge +Hoechst
DMSO
Ara-C
15 hrs
B
PicoGreen
α-POLR3G
Merge +Hoechst
DMSO
Ara-C
15 hrs
C
PicoGreen
PicoGreen +Hoechst
DMSO
APH
15 hrs
D
DMSO
ATMi
ATRi
ATMi +ATRi
α-RNA:DNA
α-RNA:DNA
+ Hoechst