Telomeric repeat-containing RNA (TERRA) constitutes a nucleoprotein component of extracellular inflammatory exosomes

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Telomeric repeat-containing RNA (TERRA) has been identified as a telomere-associated regulator of chromosome end protection. Here, we report that TERRA can also be found in extracellular fractions that stimulate innate immune signaling. We identified extracellular forms of TERRA in mouse tumor and embryonic brain tissue, as well as in human tissue culture cell lines using RNA in situ hybridization. RNA-seq analyses revealed TERRA to be among the most highly represented transcripts in extracellular fractions derived from both normal and cancer patient blood plasma. Cell-free TERRA (cfTERRA) could be isolated from the exosome fractions derived from human lymphoblastoid cell line (LCL) culture media. cfTERRA is a shorter form (~200 nt) of cellular TERRA and copurifies with CD63- and CD83-positive exosome vesicles that could be visualized by cyro-electron microscopy. These fractions were also enriched for histone proteins that physically associate with TERRA in extracellular ChIP assays. Incubation of cfTERRA-containing exosomes with peripheral blood mononuclear cells stimulated transcription of several inflammatory cytokine genes, including *TNFα*, *IL6*, and C-X-C chemokine 10 (*CXCL10*) Exosomes engineered with elevated TERRA or liposomes with synthetic TERRA further stimulated inflammatory cytokines, suggesting that exosome-associated TERRA augments innate immune signaling. These findings imply a previously unidentified extrinsic function for TERRA and a mechanism of communication between telomeres and innate immune signals in tissue and tumor microenvironments.

TERRA | telomere | exosome | innate immunity | cytokine

Telomeres are the repetitive and dynamic DNA structures that play a critical role in controlling cellular replicative capacity and cancer suppression (1, 2). Human telomeric DNA contains 4- to 15-kb double-stranded DNA with a sequence of TTAGGG repeats that are bound by a telomere-specific protein complex, referred to as shelterin (3). Telomere repeats are lost by attrition during DNA replication due to the end-replication problem, and critically short telomeres elicit a DNA damage-associated cell cycle arrest and replicative senescence (3, 4). Telomere repeat loss is thought to be part of a somatic cell senescence program that restricts cellular proliferation and regulates tissue homeostasis. Specialized telomere elongation mechanisms, including activation of the reverse transcriptase telomerase or alternative lengthening of telomeres (ALTs) through recombination, can overcome telomere repeat loss-induced cellular senescence. Telomere dysfunction occurs when abnormally short telomeres fail to induce senescence and is an early hallmark of human cancer. Cells with telomere dysfunction are also known to secrete distinct types of inflammatory cytokines (5, 6), but how telomeres are linked to this phenotype is not well characterized.

Telomere repeat DNA can be transcribed in response to developmental changes and cellular stress conditions (7, 8). Telomeric repeat-containing RNA (TERRA) has been implicated in telomere length regulation and DNA damage signaling (9, 10). TERRA can be found in complexes containing nuclear proteins, including hnRNP1, Pot1, RPA, and HP1 (11, 12) and forms stable RNA-DNA hybrids at telomere DNA repeats (13, 14). TERRA may also form foci in cells that can colocalize with the inactive X chromosome (15, 16) or form aggregates in some cancer cells and tissues (17). TERRA can also form highly stable G-quadruplex structures (18), and these structures have been implicated in telomere length regulation (19). Whether TERRA has additional functions distinct from telomere end regulation is not yet known.

Structured nucleic acids, like TERRA, can have potent effects on innate immune sensing pathways (20). Extracellular forms of repetitive DNA fragments, including telomeric DNA, have been shown to modulate inflammatory cytokine production (21). Furthermore, cell-free nucleic acids can be used as a biomarkers for various diseases, including autoimmunity and cancer (22). Cell-free nucleic acid has been identified in stable protein complexes, as well as encapsulated in microvesicles and exosomes (23–25). Exosomes are small (50–100 nm) vesicles that carry a unique composition of proteins (26), lipids (27), mRNA (28), and miRNA (29). Exosomes form in the endosomal multivesicular bodies of the cytoplasm of various cell types and are secreted into body fluids, including blood plasma (30). Depending on their cellular origin and conditions, exosomes exhibit differential enrichment of components, allowing for specialized functions (23).

Significance

Loss of telomere repeats leads to cellular senescence and the secretion of inflammatory cytokines. How telomere dysfunction is linked to this inflammatory phenotype and its role in aging and cancer is not yet understood. We show here that noncoding telomere RNA transcripts [telomeric repeat-containing RNA (TERRA)] are secreted into the extracellular environment in exosome vesicle fractions. This cell-free TERRA (cfTERRA) is shorter and more stable than intracellular TERRA, is associated with histone proteins, and can induce inflammatory cytokines in responsive cells. These findings suggest that TERRA can have a cell extrinsic function and provide a mechanism through which telomere dysfunction can lead to the activation of inflammatory cytokine signals in the tissue microenvironment through the signaling capacity of cfTERRA.

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Exosomes have been implicated in regulation of the immune response (31), gene expression by transmission of miRNA (32), and pathogen spreading (33). Tumor-derived exosomes promote tumor progression at many levels, either by suppressing antitumor immune responses (34) or by incorporating oncogenic materials (35). Whether telomeres and their derived RNA are involved in intercellular communication through exosome transport has not been studied.

Here, we report the identification of a previously unidentified, small form of TERRA found in the cell-free environment of mouse normal and tumor tissue, human blood plasma, and cell culture medium. This cell-free TERRA (cfTERRA) was highly enriched in exosome fractions that also induced transcription of inflammatory cytokines. These findings reveal a previously unrecognized extracellular localization of TERRA and provide a molecular mechanism through which telomere dysfunction may impact the tissue microenvironment.

Results

Identification of cfTERRA in the Fraction of Exosomes. In a previous study (17) we observed that TERRA formed discrete foci in the nuclear compartment of highly proliferating cells in mouse embryonic cerebellum and brain tumors. We now report that a significant number of TERRA foci localize outside of the nuclear and cellular compartments in tissue sections of a mouse model of medulloblastoma (Fig. 1*A*, *Left*), as well as in developing embryonic brain (Fig. 1*A*, *Right*). Many of these foci were sensitive to RNase treatment, indicating they are mostly telomeric RNA and not DNA fragments (Fig. 1*A*, *Lower*). We also observed TERRA foci forming outside of nuclear compartments in human tissue culture cells, especially in serum-starved human lymphoblastoid cell lines (LCLs) (Fig. 1*B*). Consistent with this, we found that serum-starved LCLs produced higher levels of a shorter form of TERRA (Fig. S1 *A* and *B*). We next asked whether TERRA RNA could be detected in RNA-seq analyses from cell-free RNA derived from plasma samples of normal or cancer patients (Fig. 1*C*). TERRA RNA, as defined by a least six telomere repeats, was detected at relatively high abundance in all samples. RNA with 2 or 3 UUAGGG-repeats were found at much lower read counts, suggesting that most TERRA RNA was derived from longer repeat transcripts (Table S1). While no significant differences between cancer and normal patients were found, read counts for TERRA ranked in the top 20 most frequent transcripts for all RNA-seq reads of extracellular RNA (Fig. 1*C*). These findings indicate that extracellular TERRA is a relatively abundant component of the cell free RNA from human blood plasma.

To investigate the possibility that TERRA was exported to the extracellular compartment, we isolated the microvesicle and exosomal fractions from LCL culture media using differential centrifugation (Fig. 1*D*). We then assayed the total cellular RNA, cellular debris, microvesicle fraction, and exosome fractions for TERRA RNA by Northern blot (Fig. 1*E*). We found that a smaller form of TERRA migrating at ∼200 nt was highly enriched in the exosome fraction. Identical forms of TERRA were identified when exosomes were isolate by ultrafiltration or exosome precipitation reagent (Fig. S1 *C* and *D*). Quantitative RT-PCR (qRT-PCR) with primers situated close (<300 nt) to the subtelomere–telomere junction showed enrichment in exosome fractions relative to total cellular TERRA (Fig. S1*E*). Similar forms of TERRA could be isolated from different cell types, although LCLs produced the highest amounts among the cells tested (Fig. S2*A*). TERRA production correlated with higher levels of fast migrating CD63-positive exosomes (Fig. S2*B*) and did not correlate with cell death or apoptosis (Fig. S2*C*). This form of TERRA (referred to as cfTERRA) was partly resistant to RNase A treatment, forming a diffuse and slower migrating signal on Northern blot (Fig. 1*D*). We did not detect any antisense TERRA, suggesting that this is mostly G-rich single-stranded RNA. The control 18S probe identified 18S RNA in cellular debris and microvesicles, but not in the exosome fraction. These results indicate that cfTERRA is enriched in exosome-like fractions from human LCLs.

cfTERRA Was Protected by a Structure with Similar Density as Exosomes. To better characterize cfTERRA, we fractionated extracellular vesicles on sucrose gradients using tetraspanin CD63 as a marker for exosomes (36) (Fig. 2). We observed that cfTERRA cofractionated with the faster migrating form of $CD63^+$ through the sucrose gradient centrifugation (Fig. 2 *A* and *B*). We examined these fractions by electron cryo-microscopy and observed that most of the spherical exosomes (red arrows) comigrated with cfTERRA and fast migrating CD63 in fraction 9 (density, 1.15 g/mL), along with some other membrane vesicles (green arrows). Although fraction 3 contained the slower mobility (and presumably glycosylated) form of CD63 typically associated with exosomes, there were few exosome structures and many large macromolecular complexes presumably of protein composition (blue arrows) in this fraction. To investigate whether cfTERRA was within exosomes, we compared the RNase sensitivity of cellular TERRA with exosome fraction of cfTERRA (Fig. 2*D*). Although cellular TERRA was efficiently degraded by RNase mixture treatment, cfTERRA was protected from RNase activity when the exosome structure was intact. In contrast, purified cfTERRA from denatured exosomes were mostly degraded by RNase mixture treatment (Fig. 2*D*). Exosome fractions did not contain detectable amounts of control 18S RNA. These findings indicate that cfTERRA cofractionates with the nongylcosylated $CD63^+$ exosome fraction where it remains resistant to RNase treatment either by encapsulation within the exosome or its association with other factors that copurify with exosomes.

cfTERRA Is Bound by Histones in Exosome Fraction. Sucrose gradient fractions enriched in TERRA (fraction 9) and CD63 were analyzed by silver staining of SDS/PAGE and then by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify the protein composition (Fig. 3 *A* and *B*, Fig. S3, and Dataset S1). MS revealed histones and ribosomal proteins, as well as many known exosome components (Fig. S3), including CD81, CD20, and annexin A1 (Fig. 3*B*) (37). Western blot confirmed the enriched levels of histone H3 and H4, as well as CD81, CD63, and LMP1 in the sucrose fractions containing TERRA (Fig. 3*B*). To determine whether cfTERRA is associated with any of the protein components of the exosome fraction, we performed immunoprecipitation assays on these exosomes (Fig. 3 *D*–*F*). We found exosome-associated TERRA could be immunopurified with antibodies to exosome membrane constituents CD81 and to a lesser extent with CD63. TERRA could also be detected in immunoprecipitation (IP) with H3 antibody, suggesting some cfTERRA may associate with chromatin components outside of exosomes (Fig. 3 *E* and *F*). Exosome-associated TERRA was detected at higher levels than 18S RNA relative to total cellular amounts, suggesting that TERRA is selectively enriched in exosomes in LCLs.

To determine whether TERRA was physically associated with any protein constituents found in exosome fractions, we performed extracellular ChIP (Exo RNA-ChIP) assays using formaldehyde cross-linking before exosome lysis (Fig. 4*A*). We found that TERRA was significantly enriched in histone H3 Exo RNA-ChIP and to a much lesser extent with TRF2 (Fig. 4 *B* and *C*). In contrast, 18S RNA was enriched with S6 and H3 cellular RNA-ChIP, but not detectable in Exo RNA-ChIP. We also performed Exo DNA-ChIP on total extracellular fractions (Fig. S4). We found that telomere repeat DNA (both sense and antisense) could be detected in both H3 and TRF2 Exo DNA-ChIPs, whereas α-satellite DNA was enriched only in the H3 ChIP (Fig. S4 *B* and *C*). These findings indicate that chromatin-associated

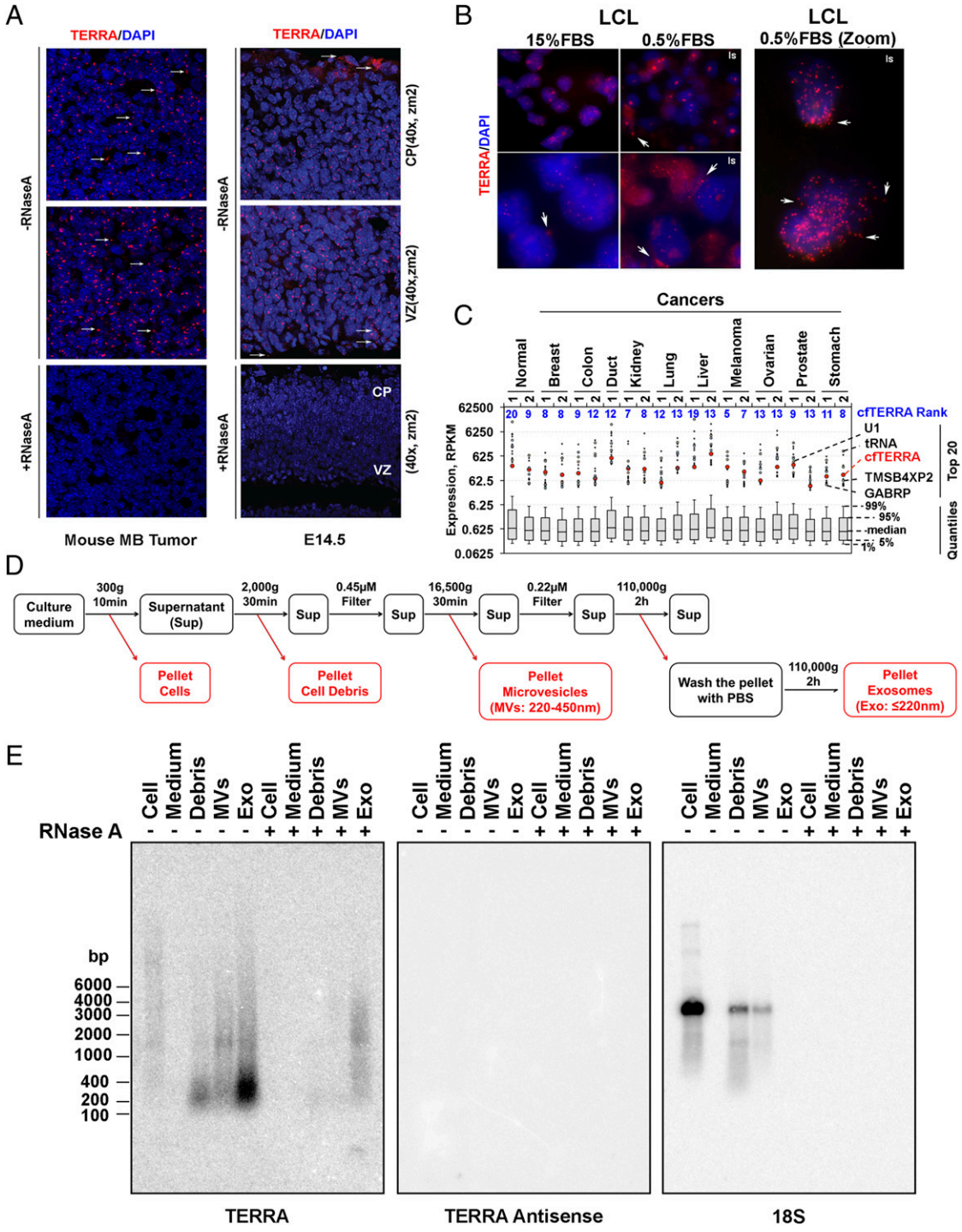

Fig. 1. Identification of cfTERRA in exosome fractions. (*A*) RNA-FISH analysis of TERRA expression on mouse medulloblastoma tumor (*Left*) and embryonic E14.5 brain tissue sections by confocal microscopy. TERRA was stained with $(CCCTAA)_3$ PNA probe in red, and nuclei were counterstained with DAPI in blue on mouse medulloblastoma tissue (*Left*) or cerebral cortex section of E14.5 WT mouse embryo (*Right*). Cortical plate (CP) and ventricular Zone (VZ) are indicated. RNase A treatment eliminates all signals of TERRA (*Lower*). Arrows indicate TERRA signals found outside of nuclei. Images were taken with 40× lens at zoom 2. (*B*) TERRA foci were found outside of nuclei in human lymphoblastoid cell lines grown under normal serum (15%) or serum starved (0.5%) conditions for 24 h before fixation. (*Right*) Zoom image of the same LCL samples. (*C*) RNA-seq analysis of cell-free DNA from various normal and cancer blood plasma samples. TERRA (as defined by six tandem UUAGGG repeats) and its ranks in read counts relative to all other genes. Whisker plots demonstrate distribution of gene expression levels that had at least 10 aligned RNA-seq reads. Dots represent RPKM values for the top 20 expressed genes. Among those, highlighted are cfTERRA and 6 other known genes that appear in the top 20 genes across all samples the most. (*D*) Flowchart for fractionation of culture medium by differential centrifugation. The conditions of each centrifugation or filtering are indicated above the black arrows. Pellets highlighted in red are used for analysis of RNA or proteins. (*E*) Northern blot analysis of TERRA levels in extracellular fractions from LCL culture medium. RNA was isolated from pellets of the differential centrifugation as shown in *C*. Equal mass amounts of RNA (1 μg) were either mock treated (–) or treated with RNase A (100 μg/mL) for 30 min at 37 °C before Northern blot analysis. The blot is hybridized with ^{32}P-labeled probes for TERRA, antisense TERRA, or 18S RNA, or indicated under the blot. Numbers on the left show the position of RNA markers in base pairs.

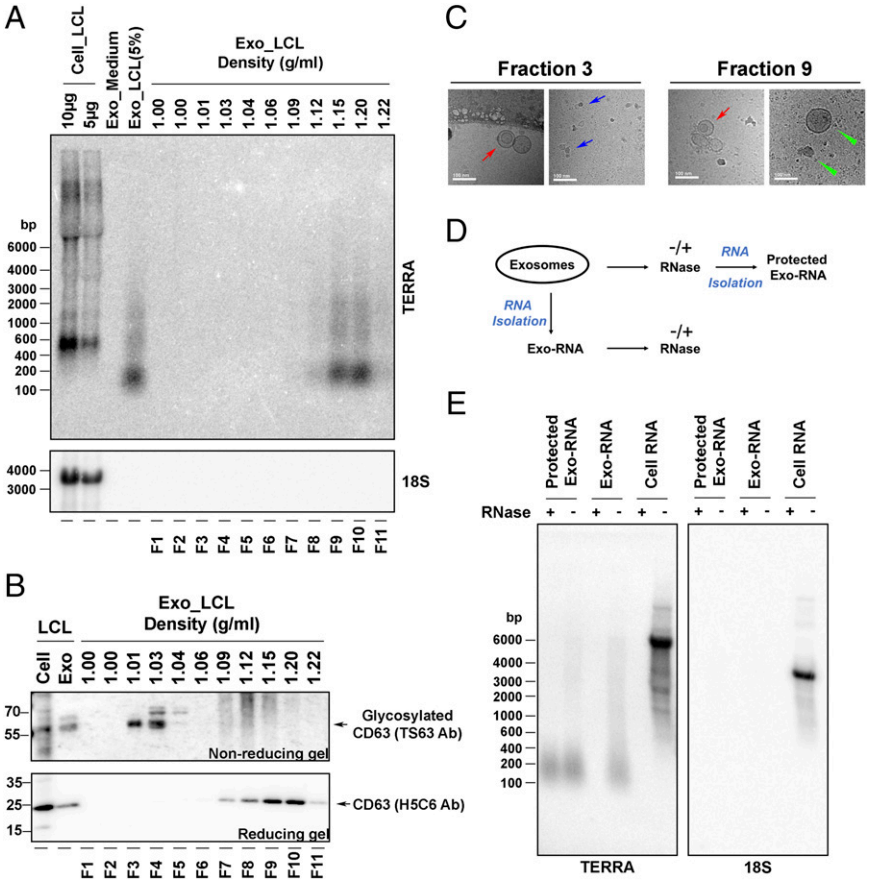

Fig. 2. cfTERRA copurifies with exosomes. (*A*) Northern blot analysis of total cellular RNA (10 and 5 μg) or RNA isolated from total exosome fractions from either fresh media or LCL extracellular media or exosomes that were fractionated on a continuous sucrose gradient (fractions 1–11) were probed for TERRA (*Upper*) or 18S RNA (*Lower*). (*B*) Western blot analysis of sucrose fractions (as shown in *A*) with CD63 antibody using nonreducing (*Upper*) or reducing (*Lower*) SDS/PAGE. The unmodified and glycosylated CD63 mobilities are shown as indicated. (*C*) Electron cryo-microscopy analysis of sucrose fractions 3 and 9. Exosomes are indicated with red arrows, whereas other vesicle structures are indicated with green arrows. The blue arrows indicate a presumed protein macromolecular complexes found in fraction 3. (Scale bars, 100 nm.) (*D*) Schematic of RNase protection assay used in *E*. Exosomes were treated with or without RNase mixture (Protected Exo-RNA) or exosome RNA was first isolated and then treated with or without RNase mixture (Exo-RNA). (*E*) Northern blot of RNA isolated from LCLs or LCL-derived exosomes. Exosomes were pretreated without (–) or with (+) RNase mixture before RNA isolation (Protected Exo-RNA) or treated after RNA isolation (Exo-RNA) and cellular RNA. The isolated RNA was analyzed by Northern blotting and hybridized with ^{32}P-labeled probes for TERRA or 18S RNA as indicated.

DNA fragments enriched with telomeric and α-satellite DNA fragments can be found in extracellular fractions.

cfTERRA Modulates the Transcription of Inflammatory Cytokines in Recipient Cells. Exosomes have been implicated in various types of intercellular communications, including the modulation of inflammatory cytokines and the innate immune signaling (38). We therefore tested whether exosomes from LCLs enriched with cfTERRA could induce transcription for various cytokines and chemokines. We found that cfTERRA-enriched exosome fractions efficiently induced transcription of several cytokines, including *IL6*, *TNFα*, GMCSF, and C-X-C chemokine 10 (*CXCL10*) (Fig. 5 *A* and *B* and Fig. S5*A*). To determine whether cfTERRA levels in exosomes correlated with cytokine activation, we isolated exosomes from cells engineered to produce elevated TERRA levels (Fig. 5*C*). Exosomes were isolated from HCT116 cells transduced with ectopic TRF1(ΔN) or TRF1(ΔN) fused to the transcription activation domain of VP16 [VP16-TRF1(ΔN)]. We validated by Northern blot that VP16-TRF1(ΔN) induced high levels of cellular and exosome-associated TERRA relative to vector and TRF1ΔN only (Fig. 5*D*), suggesting TERRA was induced by the VP16 domain instead of ectopic expression of TRF1(ΔN). Although some 18S RNA was detected in exosomes from vector control samples, no 18S was detected in TRF1(ΔN) or VP16-TRF1(ΔN), and U1 RNA was not detected in any exosome fraction (Fig. 5*D*). Protein levels of cellular TRF1 and exosomal CD63 were monitored by Western blot (Fig. 5*E*). Exosomes normalized by CD63 expression levels were then incubated with peripheral blood mononuclear cells (PBMCs) and assayed for cytokine induction (Fig. 5*F*). We found that exosomes from VP16-TRF1ΔN containing the highest levels of cfTERRA induced the highest levels of cytokine mRNA, including *IL6*, *TNFα*, and *CXCL10* while having no significant effect on control *GUSB* mRNA levels (Fig. 5*F* and Fig. S5*B*). To determine whether TERRA alone is capable of stimulating inflammatory cytokine transcription on recipient cells, we expressed and purified sense or antisense TERRA-containing RNA transcripts, as well as equimolar U6 transcripts, and delivered these in liposomes to either PBMCs (Fig. S6*A*) or IMR90 fibroblasts (Fig. 5 *G* and *H*). We found that synthetic TERRA-containing liposomes selectively stimulated *IL6, CXCL10,* and *TNFa* in IMR90 cells (Fig. 5*H*) and to a lesser extent in PBMCs (Fig. S6*A*). We also found that short synthetic oligonucleotides (36 bp) containing TERRA could partially induce some cytokine production, although not fully recapitulating endogenous exosomes (Fig. S6*B*). Taken together, these findings suggest that exosome-associated cfTERRA may function to modulate cytokine production in recipient cells.

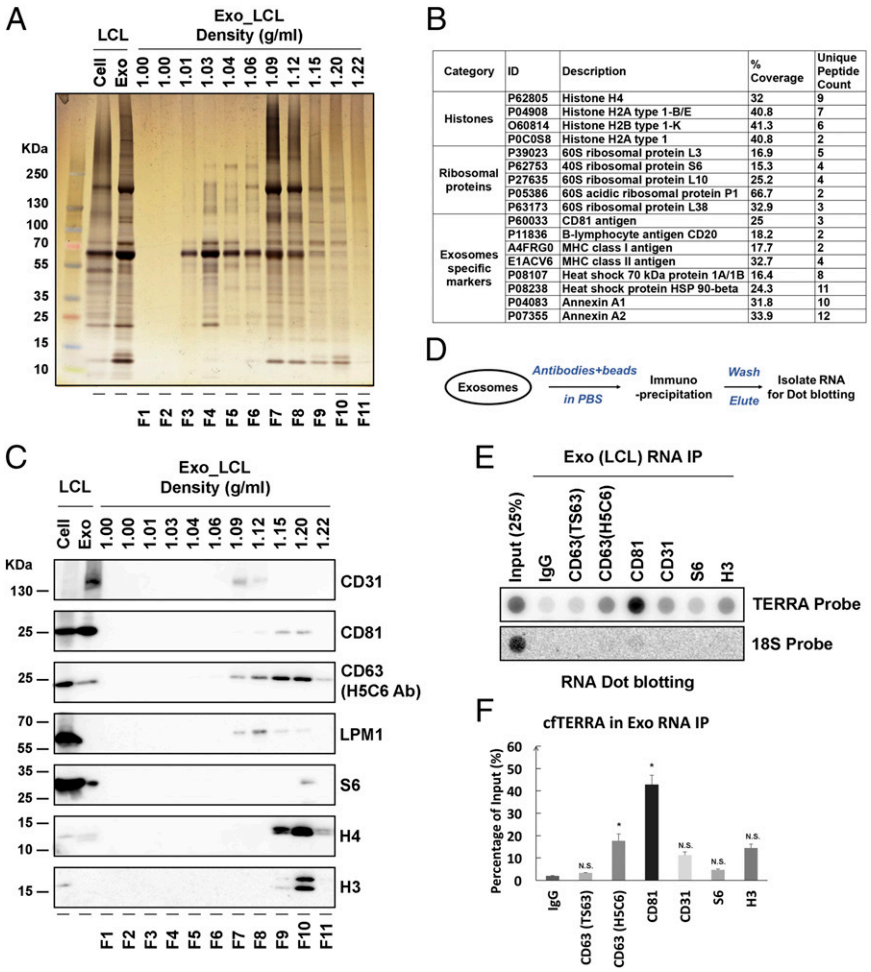

Fig. 3. cfTERRA is associated with exosomes. (*A*) Sucrose fractions collected in Fig. 2 were assayed by SDS/PAGE and visualized by silver staining. Molecular weight of the marker was indicated on the left in kilodaltons. (*B*) Summary of LC/MS/MS data from sucrose fractions F8 and F9. Proteins identified by MS from the major categories of histone, ribosomal protein, or exosome component are shown. Percent coverage and unique peptide counts are indicated. Full list of MS identified peptides is provided in Table S2. (*C*) Western blotting of sucrose factions using antibodies specific for CD31, CD81, CD63, LMP1, S6, histone H4, and H3 antibodies. (*D*) Schema of exosome immunoprecipitation and RNA isolation method used in *D* and *E*. (*E*) Exo RNA IP using antibodies to CD63 (TS63), CD63 (H5C6), CD81, CD31, S6, H3, or control IgG. Isolated RNA was then assayed by dot blotting with TERRA or 18S-specific probes. (*F*) Quantification of three independent replicates of Exo RNA IP as represented in *E*. Error bars, SD.

Discussion

Telomeres have been implicated in the cell intrinsic regulation of senescence (39), as well as in more complex functions, including tissue homeostasis (40) and organismal aging (41). Telomere-associated changes are known to occur in cancerous and precancerous lesions (42), and many of these lesions are known to have a senescence-associated secretory phenotype (SASP) that can drive carcinogenesis (43). Cells with short telomeres produce a distinct pattern of cytokines that has been referred to as a telomere-associated secretory phenotype (TASP), which is distinct from SASP (5, 44). The mechanism through which telomere dysfunction produces extracellular signals relevant to tissue microenvironment, inflammation, and cancer is not completely understood.

Here, we demonstrate that TERRA-derived RNA fragments can be found in the extracellular fraction of mouse tumor and normal embryonic tissue, human blood plasma, and human cell lines in culture. cfTERRA from human LCLs copurified with $CD63^+$ and $CD81^+$ exosome fractions and coprecipitated with histone H3, suggesting that cfTERRA forms a chromosomal-like ribonucleoprotein particle within or associated with exosomes. We showed that exosome fractions enriched in cfTERRA induced inflammatory cytokines from human PBMCs. We also found that synthetic TERRA could induce a similar inflammatory response in human fibroblasts. We conclude that cfTERRA is a component of exosome fractions that can modulate the inflammatory response.

TERRA Is Deregulated in Cancer and Stress Response. TERRA expression can be regulated by developmental and stress-related signals, including DNA damage and viral infection (7, 45–47). Telomere shortening may also increase TERRA expression (48), but it is not clear that senescent cells show a global increase in TERRA levels. We found that TERRA can be enriched in some cancer tissues (17) and is highly induced in cells after infection by herpes simplex virus 1 (HSV1) (47). TERRA has been shown to have several functions at telomeres, including recruitment of telomerase (48), inhibition of telomerase (49), assembly of DNA damage repair proteins (50), and maintenance of telomeric heterochromatin (12). However, TERRA has not yet been implicated in TASP or other related telomere-extrinsic functions.

Telomeres and Immunological Response. Several lines of evidence suggest that telomeric events can impact the innate immune response and tissue microenvironment. Although telomere shortening

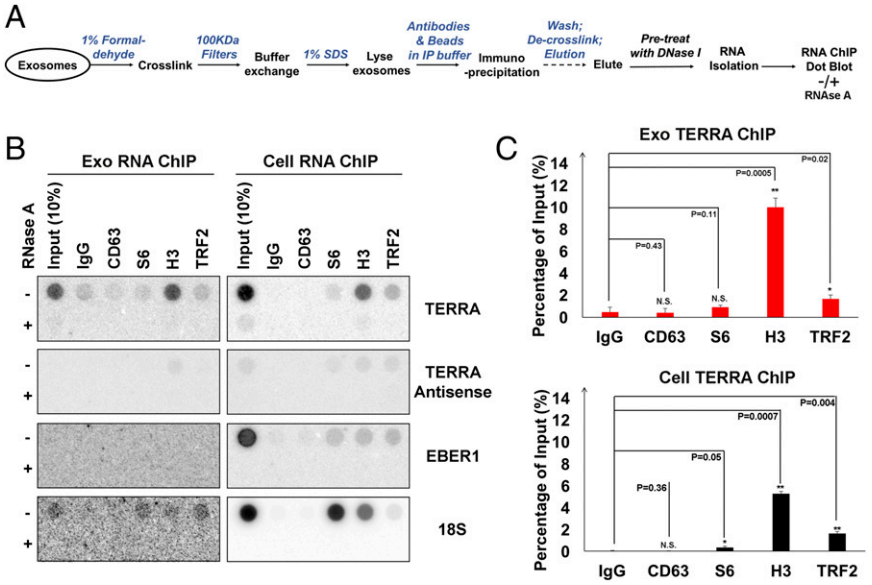

Fig. 4. cfTERRA is associated with histones. (*A*) Schema of Exo RNA ChIP assay. (*B*) RNA ChIP assays were performed with exosomes (Exo) or cellular (Cell) LCLs using antibodies specific for CD63, S6, H3, TRF2, or control IgG. Isolated RNA was treated with either mock (–) or RNase A and then assayed by hybridization with probes for TERRA, TERRA-antisense, EBER1, or 18S, as indicated. (*C*) Quantification of at least three independent TERRA RNA ChIP assays, a representative shown in *B*. Bar graphs represent mean values with SDs. *P* values were calculated by two-tailed Student *t* test: $*P < 0.05$, $**P < 0.01$.

and dysfunction can limit immunological function by restricting proliferation of immune cells, telomere shortening appears to also increase systemic inflammation, including that associated with lupus erythematosus, rheumatoid arthritis, and granulomatous diseases (51). Individuals with short telomeres in leukocytes were found to have elevated biomarkers for systemic inflammation (52). $TERC^{-/-}$ mice with shortened telomeres undergo immune inflammatory response in bone marrow macrophages due to a TLR4-depenent activation of IL6 and TNFα (53). Perhaps related is the finding that telomere shortening in aged human macrophages resulted in impaired STAT5 signaling (54). Telomere uncapping was found to be associated with cellular senescence and inflammation in human arteries (55). Furthermore, malignant cells with elevated TRF2 levels had a decrease in natural killer (NK) cell infiltration in the tumor microenvironment (56). These findings suggest that telomeres contribute directly or indirectly to inflammatory signaling.

Immunological Effects of Telomere Repeat DNA. Synthetic oligonucleotides containing CpG-DNA are known to be potent agonists of innate immunity through activation of Toll-like receptors (TLRs) (57). This activity is thought to reflect the innate immune response to foreign viral and bacterial DNA. Interestingly, synthetic telomere repeat DNA was found to suppress the production of cytokines induced by CpG DNA, as well as by other TLR agonists, including lipopolysaccharides (LPSs) (58) and various polyclonal activators (21, 59). Molecular targets for TTAGGG-repeat oligonucleotides have included STAT1 and STAT4 (60) and the lupus autoantigen Ku (61). Additionally, native DNA from telomerase-deficient mice had reduced capacity to inhibit inflammation compared to that of the control DNA (62), supporting the hypothesis that telomere-rich DNA is immunomodulatory. There have been fewer studies on the immunological effects of telomeric RNAs. However, a recent report showed that telomere RNA forming G-quadruplex structures can induce global changes in gene expression, including suppression of innate immune sensing genes (63).

Components of the Exosome Code. The complex combination of factors that comprise exosomes and the type of recipient cells that sense the exosomes may determine the nature of the signal and response. Specific signaling through exosomes depends on the cell source of the exosomes, as well as the recipient cell receptors. Exosome coding information is provided by the lipid, protein, and nucleic acid composition. Although we did not detect full-length TERRA molecules in exosomes, the smaller processed forms of TERRA are highly enriched in exosomes from various cell types, especially LCLs (Fig. S2). This smaller, processed form of TERRA was also found to be associated with histones, which were also a major protein component of the inflammatory exosome fraction from human LCLs. Although exosomes containing higher levels of TERRA elicited greater cytokine response and purified TERRA molecules can stimulate cytokines, it is not yet clear whether the endogenous cfTERRA in exosomes is the primary immunomodulator in these microvesicles. Nevertheless, we propose that processed cfTERRA associated with histones constitutes an important telomere-derived component of inflammatory exosomes with potential to modulate signaling capacity. Thus, cfTERRA may constitute an important component of a complex, yet incompletely understood exosome code.

Materials and Methods

Plasmids for TERRA Induction. TRF1ΔN (44-439) was cloned from pBSK-hTRF1 (a gift from T. de Lange, Rockefeller University, New York) and inserted either in control Lentivirus vector pLU-CMV-Flag (Protein Expression Facility, Wistar Institute) or Vp16 domain-containing vector pLU-CMV-Flag-Vp16.

Culture Medium Fractionation and Exosome Isolation. The supernatant of the LCL culture was fractionated and prepared for exosomes isolation by differential centrifugation as previously described (64), with some modifications (*SI Materials and Methods*).

ChIP Assays. Cellular ChIP assays were performed as previously described (65).

PBMC Isolation and Cytokine Stimulation. PBMCs were isolated from fresh donated human blood by density gradient centrifugation with Lymphoprep in SepMate-50 tubes (Stemcell Technologies). Liposomes were prepared by incorporating RNA from in vitro transcription or synthesized oligos (IDT) into Lipofectamine 2000 as previously described (66).

Additional methods are included in *SI Materials and Methods*.

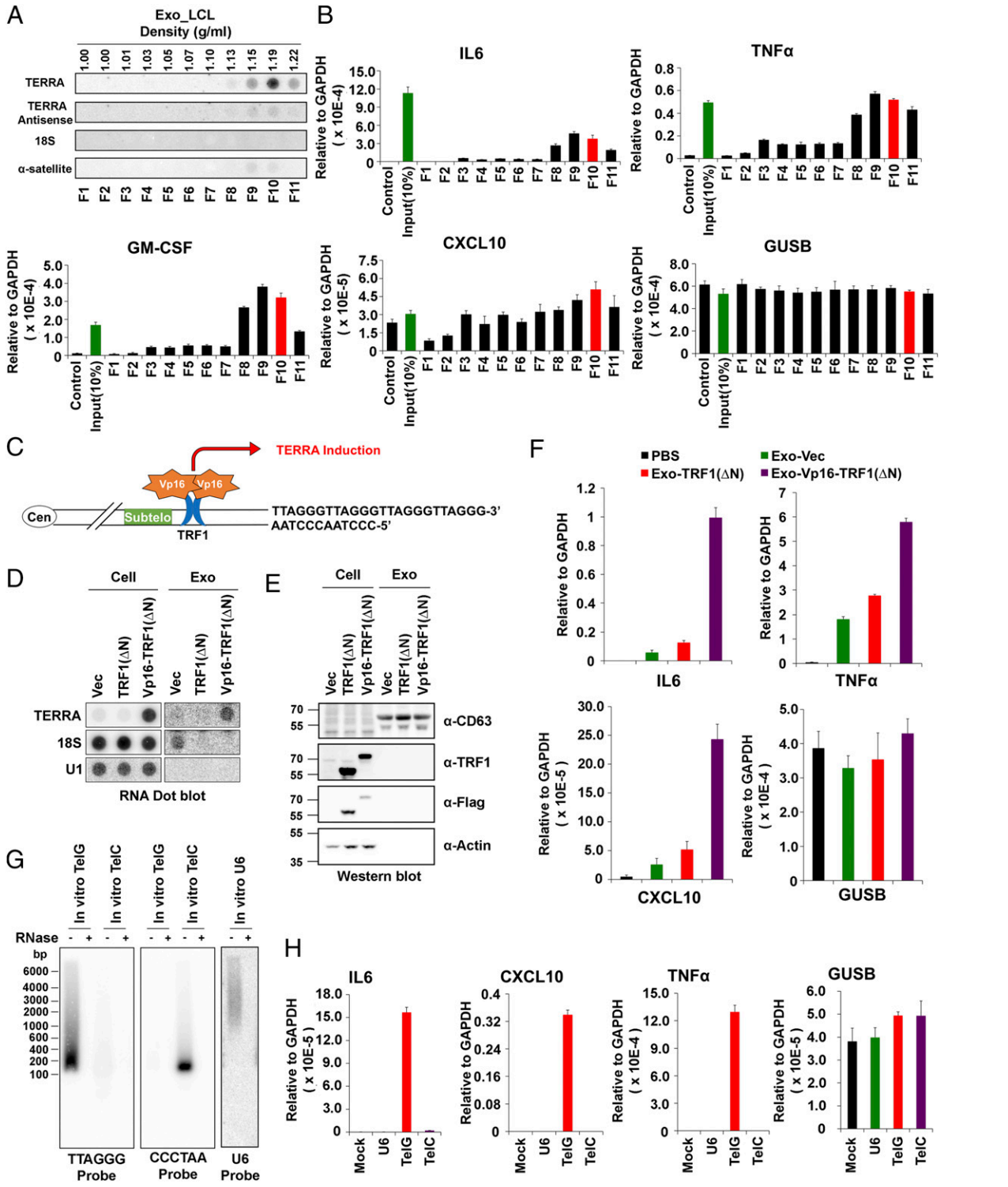

Fig. 5. Exosome-associated TERRA stimulates inflammatory cytokines. (*A*) RNA dot blot analysis of sucrose gradient fractionation of LCL-derived exosomes probed for TERRA (*Upper*), TERRA antisense, 18S rRNA, or alpha-satellite RNA (*Lower*). (*B*) Total exosomes (input) or sucrose gradient fractions were incubated with PBMCs for 3 h and then assayed by qRT-PCR for expression of *IL6*, *TNFα*, *GM-CSF*, *CXCL10*, or control *GUSB* mRNA. Bar graphs represent qRT-PCR values relative to *gapdh* mRNA (mean ± SD) from three independent experiments. (*C*) Schema of VP16-TRF1(ΔN) activation of TERRA. (*D*) RNA dot blot for TERRA, 18S, or U1 RNA from HCT116 cells (*Left*) or exosomes (*Right*) transduced with vector, TRF1(ΔN), or VP16-TRF1(ΔN). (*E*) HCT116 cells transduced as in *D* were assayed by Western blot for CD63, TRF1, FLAG, and Actin. (*F*) qRT-PCR for expression of *IL6*, *TNFα*, *CXCL10*, or control *GUSB* mRNA for PBMCs treated with exosomes derived from HCT116 cells transduced with vector control (green), TRF1(ΔN) (red), VP16-TRF1(ΔN) (purple), or PBS control (black). (*G*) Northern blot of in vitro transcribed TelG, TelC, or U6 RNA treated with control or RNaseA and probed for TERRA (*Left*), TERRA antisense (*Center*), or U6 (*Right*). (*H*) IMR90 cells were treated with liposomes containing TelG or TelC RNA for 24 h and then assayed by qRT-PCR for *IL6*, *TNFα*, *CXCL10*, or control *GUSB* mRNA. Bar graphs represent qRT-PCR values relative to *gapdh* mRNA (mean ± SD) from three independent experiments.

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