Extracellular Inflammatory Signaling from Dysfunctional Telomeres

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in Partial Fulfillment of the Requirements for the Degree of

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DEDICATION

With love and gratitude to my wife, Shiyu Zhu, for her love, support, patience, encouragement, and believe in me …

Also to my parents, parents-in-law, and friends for all their support.

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LIST OF ABBREVIATIONS

TERRA: Telomere repeats-containing RNA

cfTERRA: Cell-free TERRA

SASP: senescence-associated secretory phenotype

TASP: telomere-associated secretory phenotype

DAMPs: damage-associated molecular pattern molecules

TAMP: telomere-associated molecular pattern

TIFs: Telomere dysfunction-induced foci

ALT: Alternative lengthening of telomeres

Shelterins: TRF1, TRF2, POT1, TPP1, TIN2 and RAP1

RNAPII: RNA polymerase II

Exo 1: Exonuclease 1

TERT: telomerase reverse transcriptase

H3K9me3: H3K9 trimethylation

HP1: Heterochromatin Protein 1

ORC1: origin recognition complex subunit 1

DNMT3b: DNA methyltransferase 3b

HSF1: heat shock factor 1

NRF1: nuclear respiratory factor 1

NHEJ: non-homologous end-joining

GBM: glioblastoma multiforme

COPD: Chronic obstructive pulmonary disease

IPF: idiopathic pulmonary fibrosis

PRRs: pattern recognition receptors

LINEs: long interspersed nuclear elements

SINEs: short interspersed nuclear elements

HSATII: Human satellite II

PRRs: Pattern recognition receptors

TLRs: Toll-like receptors

PBMCs: peripheral blood mononuclear cells

CML: chronic myeloid leukemia

EVs: extracellular vesicles

MVBs: multivesicular bodies

ESCRT: endosomal sorting complex required for transport

RNAi: RNA interference

hnRNPA2B1: ribonucleoprotein A2B1

HCV: Hepatitis C Virus

HBV: Hepatitis B virus

HSV1: Herpes simplex virus 1

MHV68: murine gammaherpesvirus 68

RIG-I: retinoic acid inducible gene I

NK: natural killer

LCL: lymphoblastoid cell line

CXCL10: C-X-C chemokine 10

LPS: lipopolysaccharides

ABSTRACT

Telomere dysfunction describes the catastrophic damage at telomeres, which often leads to genomic instability at the cellular level. There is rising evidence showing that telomere dysfunction also influences the extracellular environment with the inflammatory response. However, little is known about the molecular mechanism of this dysfunctional telomere-associated inflammation. In this dissertation, we identified extracellular forms of Telomeric repeat-containing RNA (TERRA), and demonstrated it might play a role in mediating the crosstalk of telomere dysfunction and inflammation. We found this cell-free TERRA (cfTERRA) is present 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. By characterizing extracellular fractions of the human lymphoblastoid cell line (LCL) culture media, cfTERRA is shown as a shorter form (~200 nt) of cellular TERRA and co-purifies with CD63- and CD81-positive exosome vesicles that could be visualized by cryo-electron microscopy. Mass spectrometry and extracellular chromatin immunoprecipitation (ChIP) assays revealed that regular cfTERRA was physically interacting with histones and telomeric DNA. Incubation of cfTERRA-containing exosomes with peripheral blood mononuclear cells (PBMCs) 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. The levels of cfTERRA and DNA damage marker γH2AX were

increasingly incorporated into the exosomes during telomere dysfunction. These dysfunctional telomere-derived exosomes activated a more robust transcription of inflammatory cytokines in PBMCs. These findings imply a previously unknown extrinsic function of TERRA and a potentially molecular mechanism of communication between telomeres and innate immune signaling in tissue and tumor microenvironments.

CHAPTER 1: Telomere repeat-containing RNA (TERRA) is deregulated during telomere dysfunction

Telomere repeat-containing RNA (TERRA) is the long non-coding RNA transcribed from telomeres and is involved in many aspects of telomere maintenance (1). Loss of telomeric DNA causes a cell cycle arrest that is a hallmark of cellular aging, whereas cancer cells overcome this replicative senescence by activating telomere elongation mechanisms (2). During tumorigenesis, we found that TERRA expression is often upregulated as shown in a mouse model for medulloblastoma (3). The induction of TERRA expression is also observed during the telomere dysfunction caused by HSV-1 infection (4). These compelling data suggest that TERRA levels may reflect the status of telomere maintenance, and provide a potential biomarker for dysfunctional telomeres under certain types of stress. However, it is still unknown what is the functional significance of TERRA induction for cellular senescence, tumorigenesis, and viral infection. In this chapter, I will review the current understanding of the roles of TERRA in telomere maintenance, and propose the hypothesis that TERRA plays an extrinsic role in mediating the crosstalk between telomere dysfunction and inflammation through the exosomes secretion.

1.1 Telomere maintenance determines cellular aging and tumorigenesis

Telomeres are chromatin structures that protect the ends of linear chromosomes from genetic and structural damage (5). As demonstrated in Figure 1.1, telomeric DNA consists of 4-15kb double-stranded DNA with the sequence of TTAGGG repeats and a six-subunit protein complex, named shelterin, which regulates telomere length and structure (6). Due to the nature of DNA replication, the length of telomeric DNA is shortened at each cell division (7). When telomeres decrease below a critical length, the

cell may lose the ability to proliferate further and undergo replicative senescence or programmed cell death (8). Thus, normal telomere shortening not only explains the molecular mechanism of cellular aging in somatic cells, but also provides a suppressive pressure on tumorigenesis by limiting the replication capacity.

Cancer cells overcome this anti-proliferative barrier by activating telomere elongation mechanisms (9). Most cancers activate telomerase, the enzyme that synthesizes telomeric DNA by reverse transcription, whereas 10-15% of human cancers use a homologous recombination-mediated replication, referred to as Alternative Lengthening of Telomeres (ALT) mechanism (10). Telomerase activity is also required for sustaining the replication capacity in embryonic stem cells and germline cells, but becomes limited in adult stem cells or is restricted to somatic cells (11). Mutations in telomerase components are associated with several premature aging syndromes such as dyskeratosis congenital and aplastic anaemia due to accelerated telomere shortening (12).

Not only is accelerated telomere shortening deleterious for development and aging, but also shortened telomeres promote tumorigenesis due to increasing level of genetic instability (13). There would be enhanced chromosome fusion because short telomeres lose the ability to bind with the shelterin complex (14). The major DNA-binding proteins within the shelterin complex include the double-strand specific factors TRF1 and TRF2, and the single-strand binding protein POT1 (15). They cooperatively recruit other shelterins components (RAP1, TIN2, and TPP1), to facilitate telomere replication and structural integrity necessary for telomere stability (16). TRF2 and POT1 protect

telomere integrity from DNA damage signaling by repressing ATM and ATR kinases, respectively (17). Loss of the Shelterin proteins leads to a strong DNA damage response phenotype, evidenced by an increase in 53BP1 and γ-H2AX telomere dysfunction-induced foci (TIFs) (18). The repair of the dysfunctional telomeres leads to chromosome end-to-end fusions, which increases genomic instability and the potential onset of cancer (19).

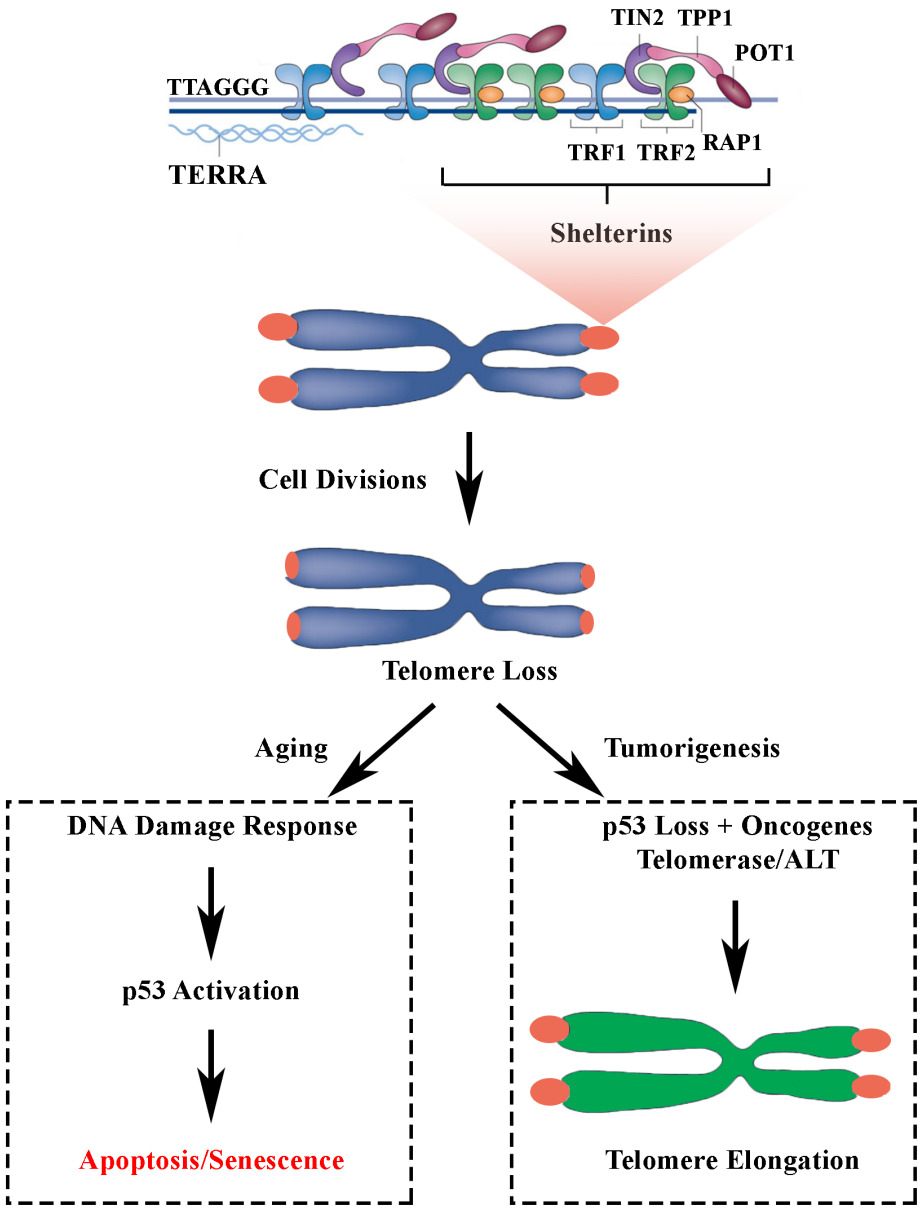

Figure 1.1 Telomere maintenance determines cellular aging and tumorigenesis (Modified based on (20)). Mammalian telomeres are the nucleoprotein structure at the end of linear chromosomes, which consist of repetitive DNA in the sequence of TTAGGG and protein complex named Shelterins (TRF1, TRF2, POT1, TPP1, TIN2 and RAP1). TERRA are the non-coding RNA transcripts generated from telomeres. Shelterins protect the telomeric DNA and prevent DNA damage response. With repeated cell divisions, telomeric DNA is shortened. Cellular aging results from p53 mediated apoptosis or senescence in response to telomere loss. On the other hand, loss of p53 along with other oncogenes promotes tumorigenesis, which activates telomerase or ALT pathway for telomere elongation.

1.2 The roles of TERRA in telomere maintenance

Telomeres are shown to express a group of non-coding RNAs containing the telomeric UUAGGG sequence from all eukaryotic cells, referred to as TERRA (21) (22). The transcribed TERRA is heterogeneous in length ranging from 100 to 9,000 bases, whereas RNAs with the anti-sense telomeric sequence are negligible in mammals (21). TERRA transcription is mediated by subtelomeric CpG-island promoters and RNA polymerase II (RNAPII) from most chromosome ends (22). The majority of TERRA (>90%) is not polyadenylated at their 3'-end, and polyadenylation of TERRA molecules increases their half-life (23). However, only the non-polyadenylated TERRA is associated with telomeric chromatin in the nucleus (23). Therefore, polyadenylation of TERRA molecules determine their stability and localization (23). There is also a small fraction of TERRA localized in the cytoplasm, but no clear function of this cytoplasmic TERRA has been studied (22,23).

The recent studies of TERRA provide new insights into the telomere maintenance. Elevated TERRA levels correlate with abnormally short telomeres as shown in the autosomal recessive ICF syndrome (Immunodeficiency, Centromeric region instability, Facial anomalies), which implies that TERRA may negatively regulate telomere length (24). Indeed, TERRA accelerates telomere shortening by promoting the telomere resection by Exonuclease 1 (Exo 1) (25). Overexpression of TERRA could also accumulate telomeric RNA-DNA hybrids, which interfere with telomeric DNA replication in the normal and telomerase positive cells (26). Also, TERRA levels are inversely correlated with telomerase activity during stem cell differentiation and cancer

progression (22). TERRA interacts with telomerase reverse transcriptase (TERT) protein, and is predicted as a direct inhibitor of telomerase in vitro (27). However, this inhibitory effect on telomerase is still controversial because overexpression of TERRA is observed in several types of cancers and does not affect telomerase activity in human cancer cells (28). TERRA even recruits telomerase to short telomeres for elongation as shown in yeast (29). In ALT-positive cancer cells, TERRA is supporting the recombination-based elongation mechanism by forming the telomeric RNA-DNA hybrids (R-loop) structure (30). TERRA depletion or removal of the telomeric R-loop result in telomere shortening and cellular senescence(31). These findings support the model that TERRA plays an essential role in regulating telomere length.

TERRA is also involved in the epigenetic regulation of telomeric chromatin, and forms an integral component of telomeric heterochromatin (32). Normal telomeric chromatin is rich of heterochromatic epigenetic marks, featuring histone H3K9 trimethylation (H3K9me3), heavily methylated CpGs and Heterochromatin Protein 1 (HP1), which tightly packs the telomeric chromatin to repress undesirable lengthening of telomeres (33). Telomere shortening leads to a change in the architecture of telomeric chromatin with the loss of heterochromatic features (34). Disruption of the heterochromatic environment results in the induction of TERRA, which interacts with TRF2 to recruit origin recognition complex subunit 1 (ORC1) and HP1 to telomeres (32). TERRA also interacts with histone methyltransferases Suv39h1 and 2 to increase the H3K9me3 levels at telomeric chromatin (35). TERRA depletion causes loss of HP1 and H3K9me3 at the telomeres, confirming the role of TERRA in facilitating telomeric heterochromatin

formation (35). As heterochromatin is established at telomeres, the epigenetic environment inhibits TERRA transcription, which provides a negative feedback mechanism in the regulation of TERRA expression (36).

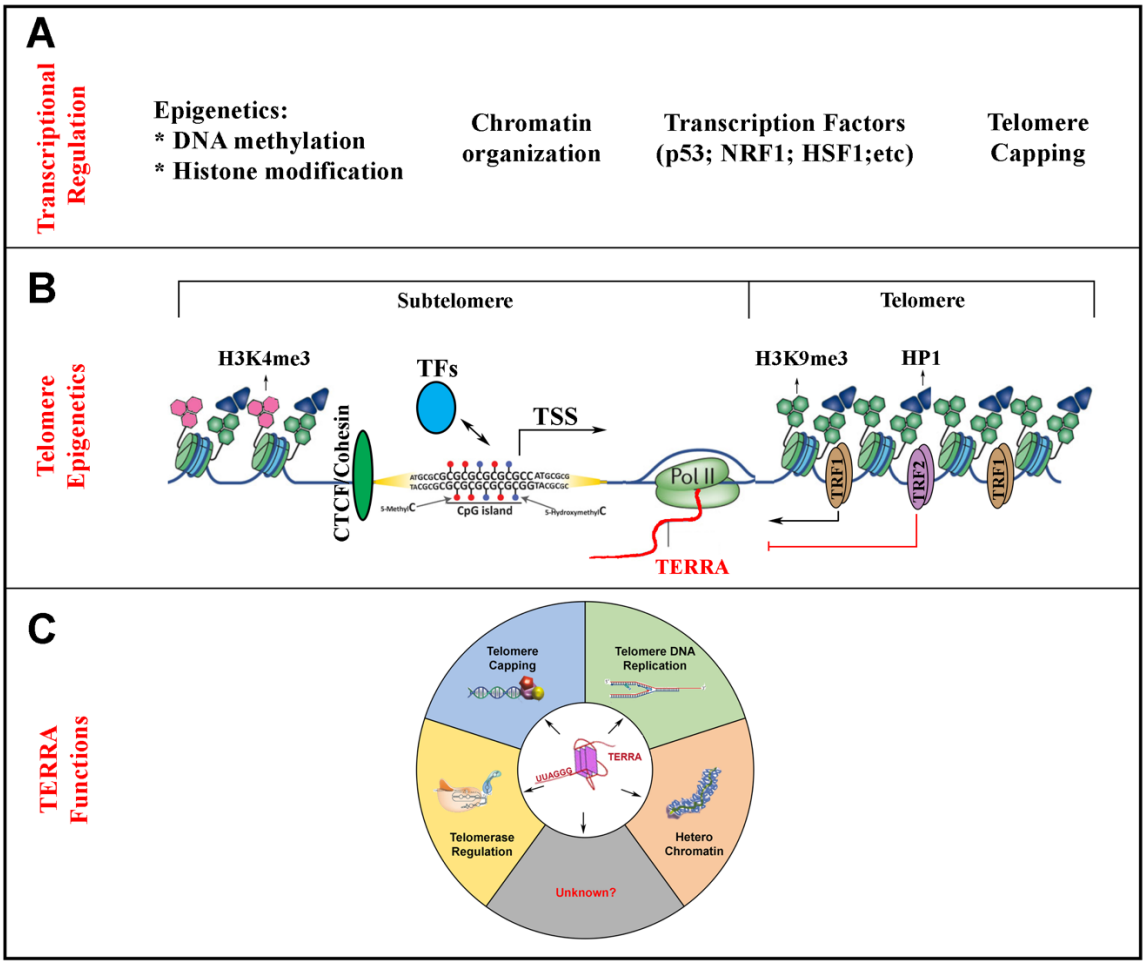

Figure 1.2 Summary of the transcriptional regulation and functions of TERRA (Modified based on (33) (37) (38)). (A) This chart summarizes that the transcription of TERRA is subject to at least the four levels of regulation including epigenetics, chromatin organization, transcription factors and telomere capping. (B) This diagram displays the basic features in subtelomeres and telomere that relate to transcriptional regulation of TERRA. (C) This diagram outlines the core functions of TERRA in controlling telomerase activity, telomere capping, telomeric DNA replication, heterochromatin formation, and other unknown functions.

1.3 Transcriptional regulation of TERRA in normal and dysfunctional telomeres

The transcription of TERRA is primary determined by the epigenetic status of telomeric chromatin (Figure 1.2) (39). Heterochromatic marks H3K9me3, H3K27me3, and HP1 are constitutively present in normal telomeres, which are silenced with a minimum of TERRA expression (36). However, transcriptionally active histone modifications H3K4me3 and H3K27ac are found at TERRA promoters and telomeric repeats, suggesting the epigenetic status of telomeric chromatin is dynamically regulated (40). For example, telomere elongation increases the levels of H3K9me3 and HP1a to repress TERRA transcription(35). Meanwhile, DNA methylation provides another mechanism for the epigenetic regulation of TERRA transcription (41). The CpGs in TERRA promoters are generally methylated by DNA methyltransferase to maintain a low level of TERRA at healthy telomeres (42). Patients with ICF caused by DNA methyltransferase 3b (DNMT3b) mutations display hypomethylation of TERRA promoters, TERRA overexpression and short telomeres (24), which supports the significance of epigenetic regulation of TERRA transcription.

Subtelomere chromatin organization and telomere capping also regulate the transcription of TERRA. We previously found the chromatin organizing factor CTCF, and sister-chromatid cohesion cooperatively binds to most human subtelomeres, which are adjacent to RNAPII-binding sites and TERRA promoters (Figure 1.2) (43). Depletion of CTCF or cohesion subunit Rad21 result in loss of RNAPII binding, decrease in TERRA transcription, and uncapping of TRF1 and TRF2 (43). Telomere uncapping induced by TRF2 depletion increases TERRA levels (36), whereas TRF1 interacts with RNAPII to

support TERRA transcription (22). TERRA induction by TRF2 depletion is also attributed to the telomeric epigenetics mediated by and transcriptional regulator MLL, which is a histone methyltransferase to induce H3K4me3 at telomeres (44). These findings suggest telomeric epigenetics, subtelomere chromatin organization, and telomere capping is essential for the transcriptional regulation of TERRA.

During telomere dysfunction, TERRA induction is mediated by several stress response transcriptional factors. Our recent study shows that tumor suppressor p53 binds to several human subtelomeres to induce histone H3K9 and H3K37 acetylation and downstream TERRA expression in response to DNA damage caused by etoposide, which prevents the accumulation of DNA damage at telomeres (45). p53 can also cooperate with MLL in mediating the TERRA induction resulting from TRF2 depletion (44). Similarly, TERRA is induced by heat shock factor 1 (HSF1) to protect telomeric integrity during heat shock stress (46). Also, nuclear respiratory factor 1 (NRF1) is shown to activate TERRA transcription during endurance exercise, which provides a novel connection between TERRA and metabolism (47). The complete motifs analysis found at least 50 transcriptional regulatory elements in TERRA proximal promoters (36), suggesting there is a complicated regulatory network for TERRA transcription. The function of dysfunctional telomere-induced TERRA is not entirely understood. It is currently believed that TERRA works as a scaffold to recruit Suv39h1 and LSD1-MRE11 complex, and promote non-homologous end-joining (NHEJ)-mediated repair in dysfunctional telomeres (48).

Since tumorigenesis often provokes telomere dysfunction, there is an intriguing hypothesis in developing TERRA as an early biomarker for neoplastic and pre-cancerous lesions. When examining medulloblastoma tumorigenesis in mice, we found TERRA expression was highly upregulated in tumor tissues, but not adjacent normal tissues (3). Consistently with this result, high levels of TERRA were associated with low-grade glioblastoma multiforme (GBM), which is mostly telomerase negative and shows better prognosis in a cohort study (49). As tumors progress to a high-grade stage, TERRA expression is often inhibited by promoter methylation induced by telomerase activity (50), which may counteract the potentially inhibitory effect by TERRA. These findings suggest that TERRA levels may reflect the telomere dysfunction in the early stage of tumorigenesis before telomerase activity is elevated. Further cohort studies with different types of low-grade tumors samples would be informative to establish the correlation of TERRA expression levels and tumor progression.

1.4 The association between dysfunctional telomeres and inflammation

At the physiological level, there is an association between dysfunctional telomeres and inflammation, which contributes to several chronic diseases. Chronic obstructive pulmonary disease (COPD) (51) and idiopathic pulmonary fibrosis (IPF) (52) are two chronic lung diseases caused by sustained inflammation induced by telomere dysfunction. Dysfunctional telomeres induce senescence (53), which generates the senescence-associated secretory phenotype (SASP) featuring the secretion of numerous proinflammatory cytokines, chemokines, growth factors, and proteases (54). Although SASP was believed as the primary source of inflammatory cytokines, telomere

dysfunction also induces another inflammatory profile, named telomere-associated secretory phenotype (TASP) found in chronic myeloid leukemia (CML) model (55). One noticeable difference than SASP is TASP-producing cells are still proliferating with pre-shortened telomeres (55). It is still unclear what is the signaling pathway for producing TASP. One possibility is from the non-canonical activity of telomerase, which interacts with the p65 subunit of NF-kB to activate NF-kB signaling for the inflammatory cytokine expression (56). Another possibility is that innate immune system senses the damage at dysfunctional telomeres to initiate the inflammatory response.

Innate immunity provides a cellular defense to both foreign pathogen and endogenous danger signals (57). These signals are detected by a group of receptors that recognize their targets based on a unique pattern. The damage-associated molecular pattern molecules (DAMPs) are endogenous danger signals generated during cellular stress (58). Since the pattern recognition receptors (PRRs) are common for foreign pathogen and endogenous danger signals, DAMPs may present a similar pattern as a pathogen (59). In this sense, the pathogen-like elements are more susceptible to PRRs detection. For instance, non-coding LINE1 RNA (long interspersed nuclear elements) is the endogenous retrotransposon originally from retroviruses and is typically silenced in somatic cells (60). Environmental stress activates the transcription of LINE1 (61), which is then sensed by Toll-like receptors 7 and 8 (TLR7/TLR8) for innate immune signaling (62). Abnormal expression of LINE1 is correlated with autoimmune diseases including lupus nephritis and Sjögren's syndrome (63). As telomeres are considered as another element

with viral origin in the human genome (64), it is intriguing to consider whether TERRA may function as a danger signal to activate innate immunity.

On the other hand, low-grade chronic inflammation accelerates telomere shortening, and drives the so-called "inflammaging" process (65). Diabetes provides a clinically relevant example of a inflammaging disease with low-grade chronic inflammation and telomere attrition in peripheral blood mononuclear cells (PBMCs) (66 ,67). In patients with chronic kidney diseases, elevated inflammation is also correlated with reduced telomere length and telomerase activity in PBMCs (68). Interestingly, the kidney is one of the tissues with the most abundant in TERRA expression in mice (22). Since TERRA is often induced during telomere dysfunction and affects telomerase activity, it would be interesting to investigate whether these inflammaging diseases display TERRA dysregulation.

1.5 The immunoregulatory function of exosomes

Exosomes are small extracellular vesicles (EVs) secreted from cells to mediate intercellular communication (69). In contrast to other EVs, exosomes are between 30 - 100 nm in size, originate from multivesicular bodies (MVBs) and containing specific markers such as CD63, CD9, CD81, Alix, and Tsg101 (70). Depending on their cellular origin and conditions, exosomes exhibit differential enrichment of proteins, lipids, mRNA, and microRNA, which allow them to deliver specific signals to the recipient cells (71). Since exosomes are found in the secretome of the many types of cells, the analysis of exosomes derived from human body fluids will provide valuable information for

diagnosis and prognosis of disease development (72). In term of the function of exosomes, they have been implicated in the regulation of the immune response (73), gene expression by miRNA (74), and pathogen spreading (75).

The biogenesis and uptake of exosomes primarily utilize multiple steps of the endosomal pathway, although many other pathways are also involved in exosomes metabolism (76). Exosomes are formed by inward budding into late endosome to form the MVBs in the cytoplasm (77). During this inward budding process, selected proteins and RNAs are sorted and enriched in the exosomes (78, 79). The endosomal sorting complex required for transport (ESCRT) machinery is involved in protein sorting (78). ESCRT machinery also crosstalks with GW-bodies, which is part of the RNA interference (RNAi) pathway, to sort miRNA into exosomes (79). The protein heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) also plays a critical role in loading RNA into exosomes by recognizing a specific motif (EXOmotifs GGAG) in the miRNAs (80). The matured exosomes in MVBs are either sorted to lysosomes for degradation or sent to the plasma membrane for secretion. The secretion of exosomes is mediated by the GTPase family RAB27 (81). The exosomes are released into extracellular fluid or travel through the circulating system to the recipient cells (82). Exosomes enter the recipient cells via endocytosis. Upon entering the early endosome, the functional signals are processed and delivered (82). Although endocytosis is the predominant mechanism for cell entry, exosomes can also enter cells by membrane fusion or receptor-mediated internalization (83).

Exosomes have been demonstrated to regulate both innate and adaptive immune system, which result in either beneficial or detrimental effect, depending on the context (84, 85). The immunostimulatory activity of exosomes mostly beneficial in supporting the host immune system (86). Hepatitis C Virus (HCV) infected cells release exosomes containing viral RNA. When the exosomes are recognized by non-permissive plasmacytoid dendritic cells, endosomal TLR7 is activated which in turn activates the innate immune system to limit viral replication (87). All the antigen-presenting cells are shown to release exosomes harboring either MHC I or II to support the T cell activation in vitro or in vivo (88, 89). Conversely, exosomes can induce immunosuppression (90), which is beneficial to prevent an autoimmune response. During mammalian pregnancy, placenta-derived exosomes secrete several immunosuppressive factors, which increases fetal tolerance within the mother (91). However, the immunosuppressive effect of exosomes would be detrimental if utilized by a pathogen or tumor. For instance, hepatitis B virus (HBV) can transfer the viral components through exosomes inhibiting the retinoic acid inducible gene I (RIG-I) mediated innate immune response in natural killer (NK) cells (92). Similarly, nematode parasites release exosomes containing several miRNAs to turn down the host innate immunity (93). In addition, tumor-derived exosomes promote tumor progression at many levels, either by suppressing anti-tumor immune responses (94) or by spreading oncogenic materials (95).

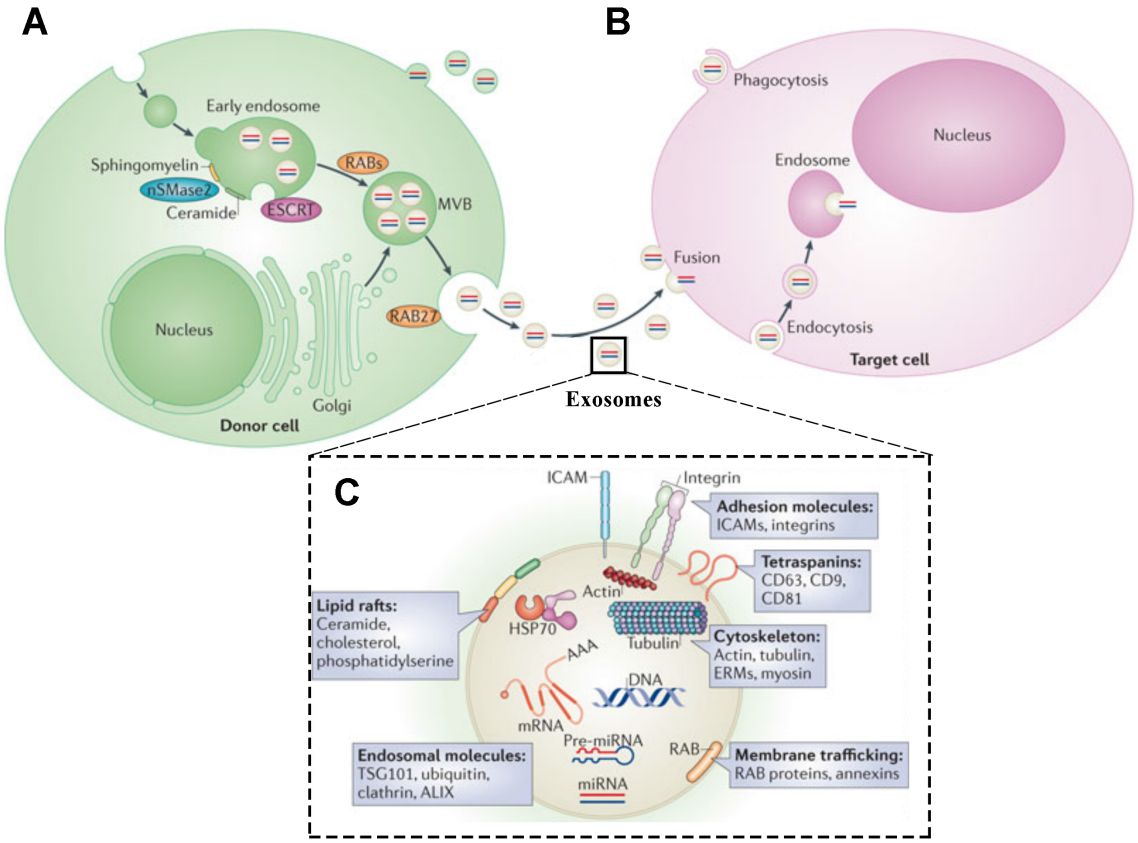

Figure 1.3 The metabolism of exosomes (Modified based on (96)). (A) The biogenesis of exosomes. The exosomes are formed by inward budding at early endosome, and matured in the multivesicular bodies (MVBs) for secretion. (B) The uptake of exosomes by target cells through fusion, endocytosis, and phagocytosis. (C) Zoom in of the exosomes to indicate the markers and components.

1.6 Scope of this dissertation

Based on the previous introduction, we already know that telomere maintenance determines cellular aging and prevents tumorigenesis. TERRA play critical roles in telomere maintenance (1), and is often upregulated during telomere dysfunction (3). Since dysfunctional telomeres have shown to produce a telomere-associated secretory phenotype (TASP) (55), we wonder whether TERRA is involved in the activation of TASP. In our previous studies of mouse medulloblastoma (3), we found TERRA foci

localized outside of the nuclear and cellular compartments in the tumor tissue sections. Therefore, we hypothesized that dysfunctional telomere-induced TERRA might represent a telomere-specific damage signal; this TERRA could be secreted by exosomes to induce the inflammatory cytokine signaling in recipient cells. The hypothesis was tested in this dissertation, and presented in Chapter 2 and Chapter 3. Our ultimate goal is to understand the pathological roles of TERRA induction in inflammaging, and develop a TERRA-based biomarker for detecting telomere dysfunction in the early stage of tumorigenesis or age-associated inflammatory diseases.

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CHAPTER 2: Telomeric repeat-containing RNA (TERRA) constitutes a nucleoprotein component of extracellular inflammatory exosomes

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2.1 Abstract

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 CD81-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.

2.2 Significance statement

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.

2.3 Introduction

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 telomeres (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 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) (24) (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. 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.

2.4 Results

2.4.1 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. 2.1A, Left), as well as in developing embryonic brain (Fig. 2.1A, Right). Many of these foci were sensitive to RNase treatment, indicating they are mostly telomeric RNA and not DNA fragments (Fig. 2.1A, 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. 2.1B). Consistent with this, we found that serum-starved LCLs produced higher levels of a shorter form of TERRA (Fig. 2.3A 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. 2.1C). 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 1). 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. 2.1C). 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. 2.2A). We then assayed the total cellular RNA, cellular debris, microvesicle fraction, and exosome fractions for TERRA RNA by Northern blot (Fig. 2.2B). 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. 2.3C 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. 2.4). Similar forms of TERRA could be isolated from different cell types, although LCLs produced the highest amounts among the cells tested (Fig. 2.5A). TERRA production correlated with higher levels of fast migrating CD63-positive exosomes (Fig. 2.5B) and did not correlate with cell death or apoptosis (Fig. 2.5C). 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. 2.2B). 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.

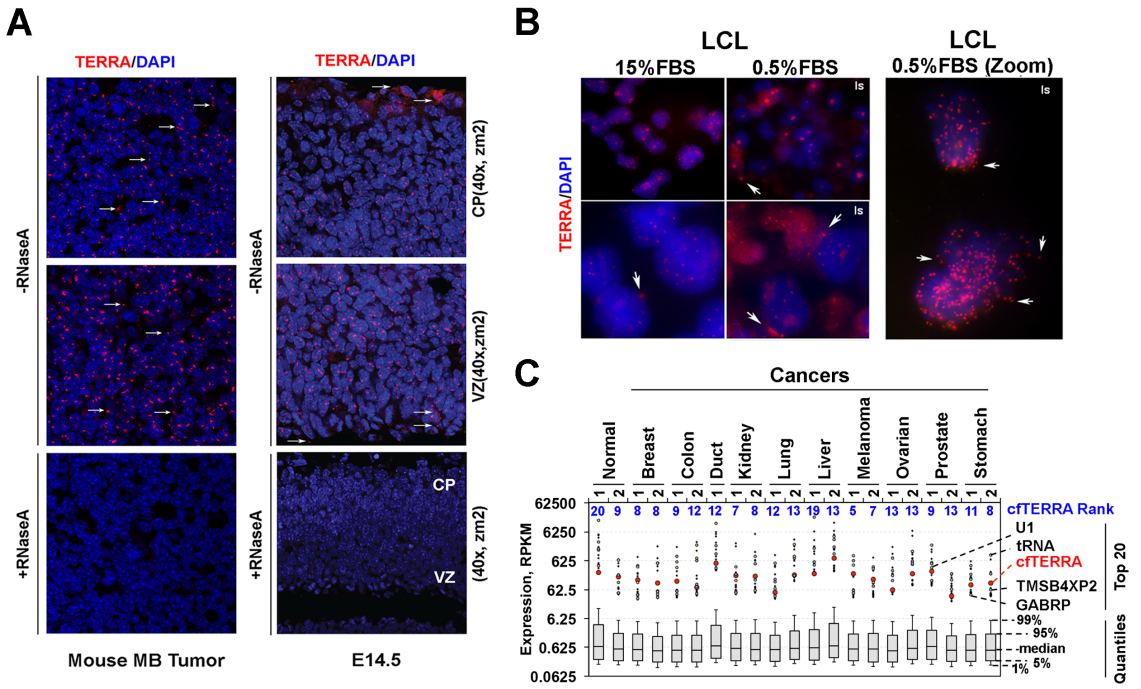


Figure 2.1 Identification of TERRA signals in mouse tissue and human plasma. (A) RNA-FISH analysis of TERRA expression on mouse medulloblastoma tumor (Left) and embryonic E14.5 brain tissue sections by confocal microscopy. TERRA was stained with $(CCCTAA)_3$ PNA probe in red, and nuclei were counterstained 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.

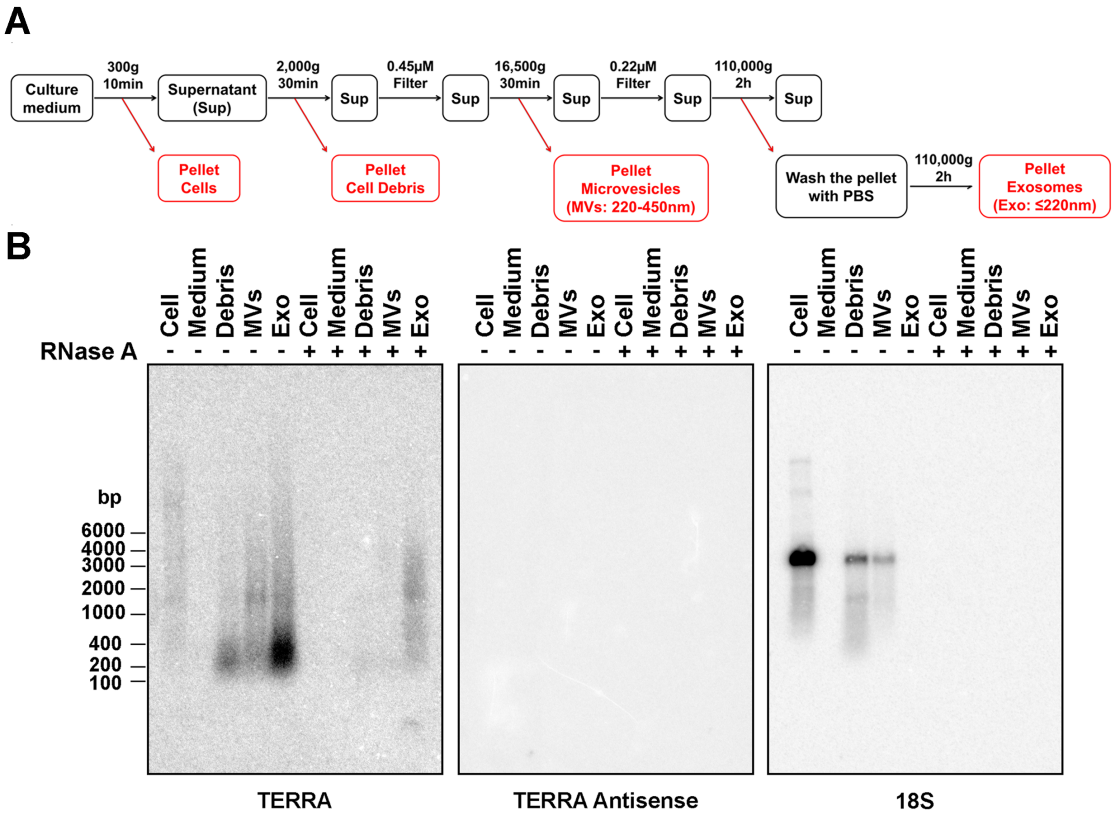


Figure 2.2 Characterization of cfTERRA in extracellular fractions from cell culture. (A) Flowchart for fractionation of culture medium by differential centrifugation. The conditions of each centrifugation or filtering are indicated above the black arrows. Pellets highlighted in red are used for analysis of RNA or proteins. (B) 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 A. 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.

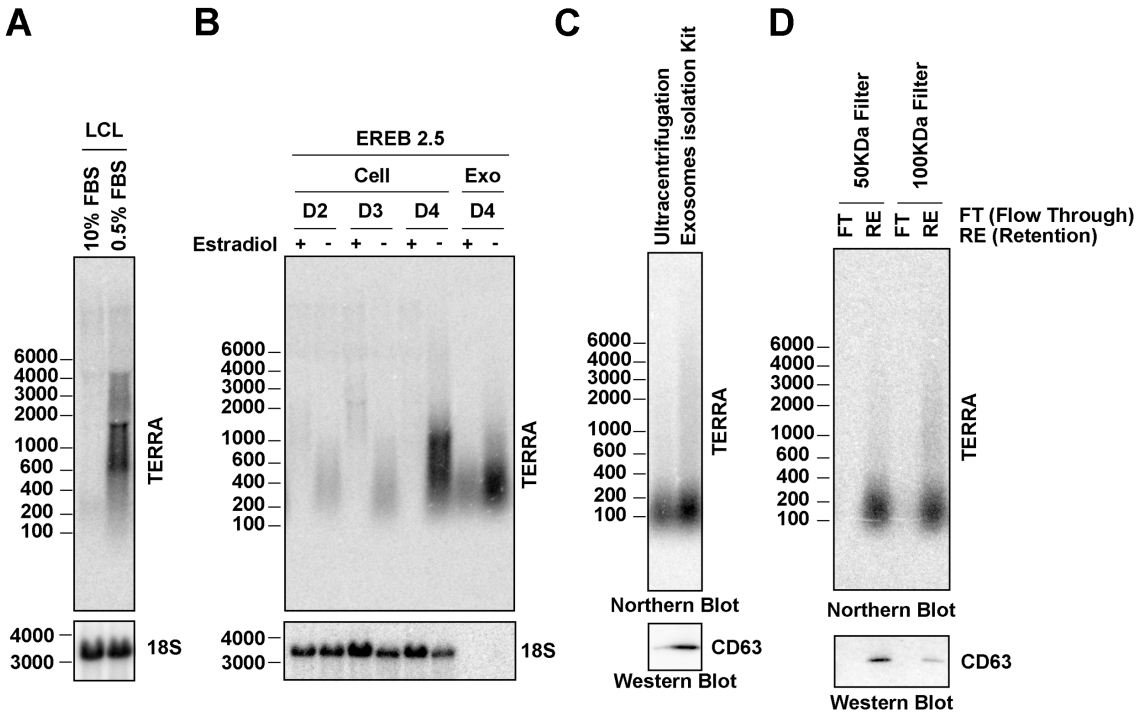


Figure 2.3 Growth arrest induces cfTERRA production in LCLs. (A) Northern blot showing total cellular TERRA levels in LCLs grown in 15% or 0.5% serum for 24 h. (B) Northern blot showing total cellular TERRA or exosome fraction for cfTERRA in EREB LCLs with or without estradiol treatment required for EBV EBNA2 expression and cell proliferation. (C) Comparison of two exosomes isolation methods to purify cfTERRA complex. Exosomes were isolated from equal volume of LCL culture medium (10 mL) by either ultracentrifugation or exosomes isolation kit (Invitrogen). RNAs were isolated from 80% of recovered exosomes and analyzed for cfTERRA by Northern blotting. The remaining exosomes (20%) were assayed by Western blotting with CD63 antibody. (D) Comparison of the recovery of cfTERRA complex by filters with different molecular weight cutoff. Equal amount of exsomes was loaded on the centrifugal filters with either 50- or 100-kDa cutoff. RNA were isolated from either flow through (FT) or retention (RE), and analyzed for cfTERRA by Northern blotting. Proteins of each fraction were TCA precipitated and assayed by Western blotting with CD63 antibody.

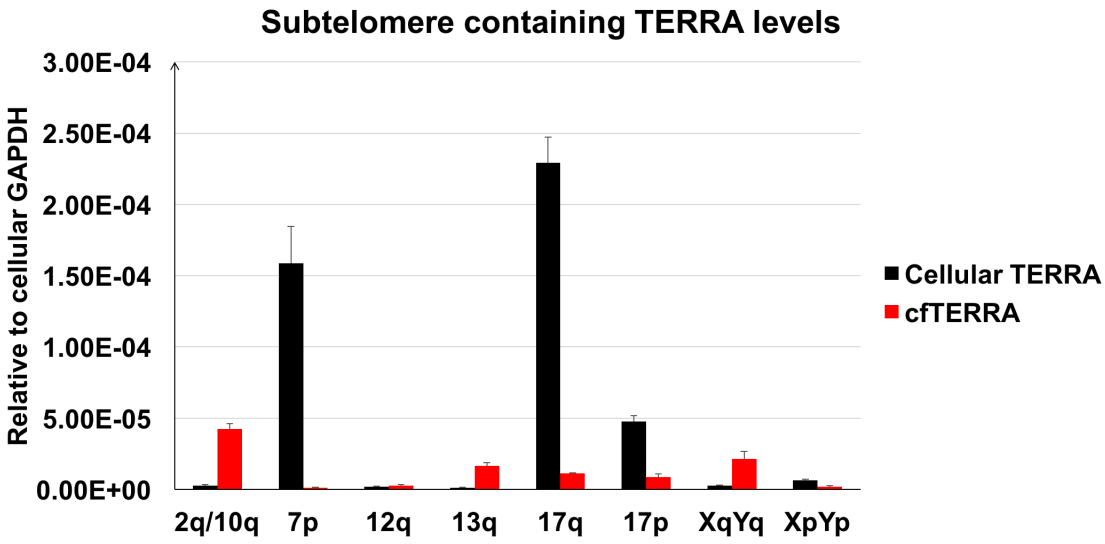
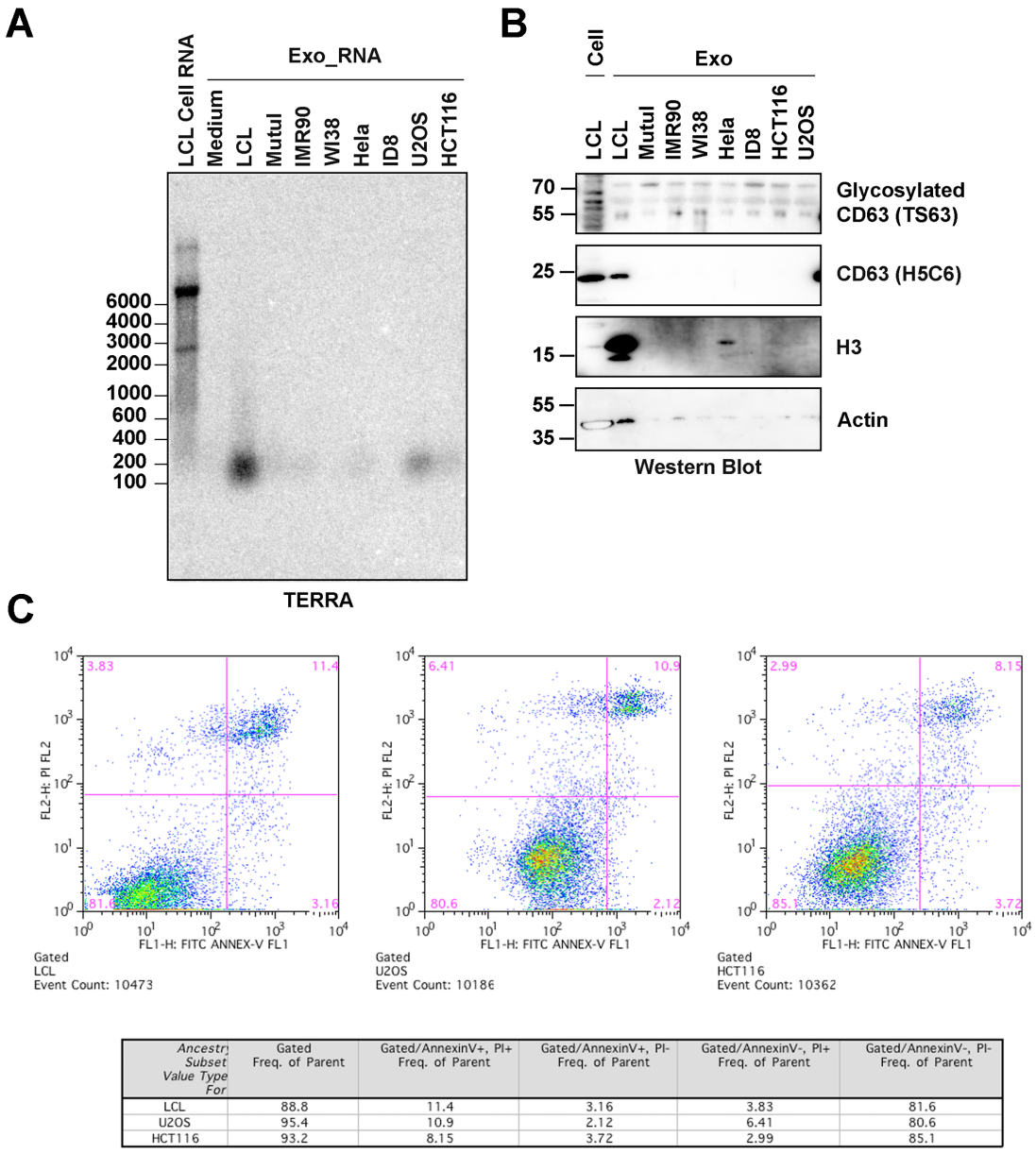


Figure 2.4 Subtelomeric origins of cfTERRA. (A) qRT-PCR for quantification of subtelomere containing TERRA levels in 1 µg of total RNA from LCL cells and exosomes. The subtelomere containing TERRA levels were determine using ΔCT method relative to cellular internal control GAPDH. The distances of the primers to specific telomere track were indicated below.



Ancestry Subset Value Type For	Gated Freq. of Parent	Gated/AnnexinV+, PI+ Freq. of Parent	Gated/AnnexinV+, PI- Freq. of Parent	Gated/AnnexinV-, PI+ Freq. of Parent	Gated/AnnexinV-, PI- Freq. of Parent
LCL	88.8	11.4	3.16	3.83	81.6
U2OS	95.4	10.9	2.12	6.41	80.6
HCT116	93.2	8.15	3.72	2.99	85.1

Figure 2.5 cfTERRA was released with different levels in other cell lines. (A) Comparison of cfTERRA levels across several cell lines in exosomes. Exosomes were isolated from indicated cell lines by ultracentrifugation. RNAs were purified from exosomes with equal amount of proteins and analyzed for cfTERRA by Northern blotting. (B) Western blot analysis of exosomes as shown in A. Equal amount of exosomal proteins were analyzed by Western blotting with CD63, H3, or Actin antibodies. (C) Apoptosis analysis of cell lines used for exosome isolation. After 48-h culture of the indicated cell lines in conditional medium, cells were stained with FITC-Annexin V and PI and analyzed by flow cytometry. Flow cytometry profile present as FITC-Annexin V staining in the x axis and PI in the y axis. The number in the upper right quadrant represents the percentage of late apoptotic cells in each cell line.

2.4.2 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. 2.6). We observed that cfTERRA cofractionated with the faster migrating form of CD63+ through the sucrose gradient centrifugation (Fig. 2.6 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. 2.7). 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. 2.7B). 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.

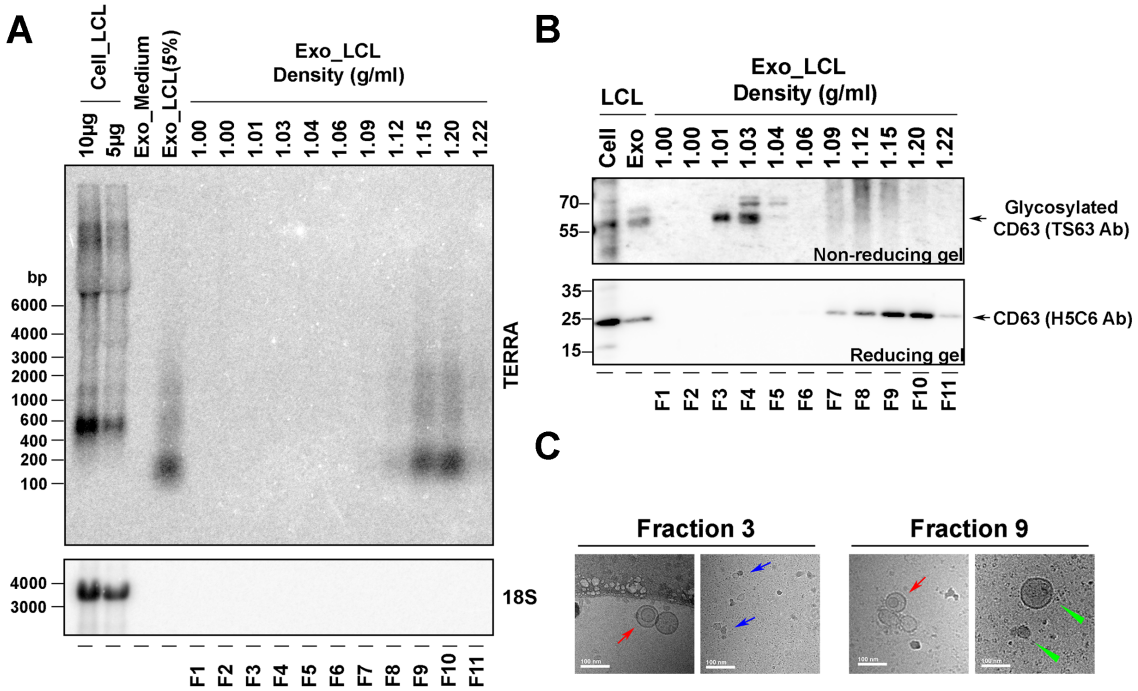


Figure 2.6 cfTERRA co-purifies with exosomes. (A) Northern blot analysis of total cellular RNA (10 and 5 µg) or RNA isolated from total exosome fractions from either fresh media or LCL extracellular media or exosomes that were fractionated on a continuous sucrose gradient (fractions 1–11) were probed 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.)

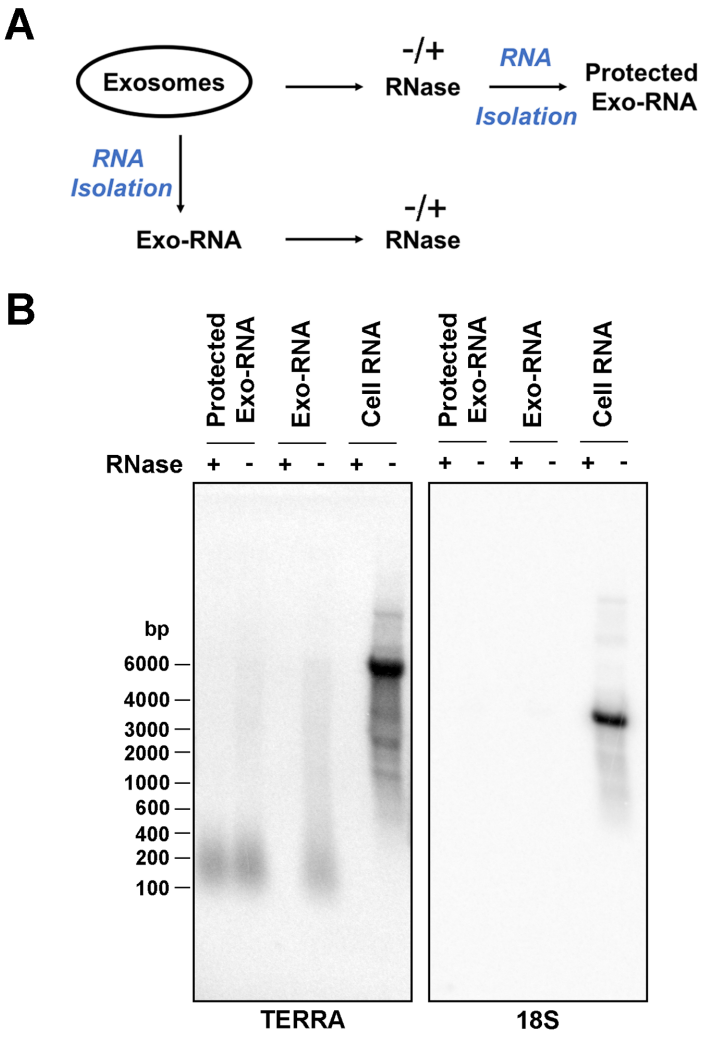


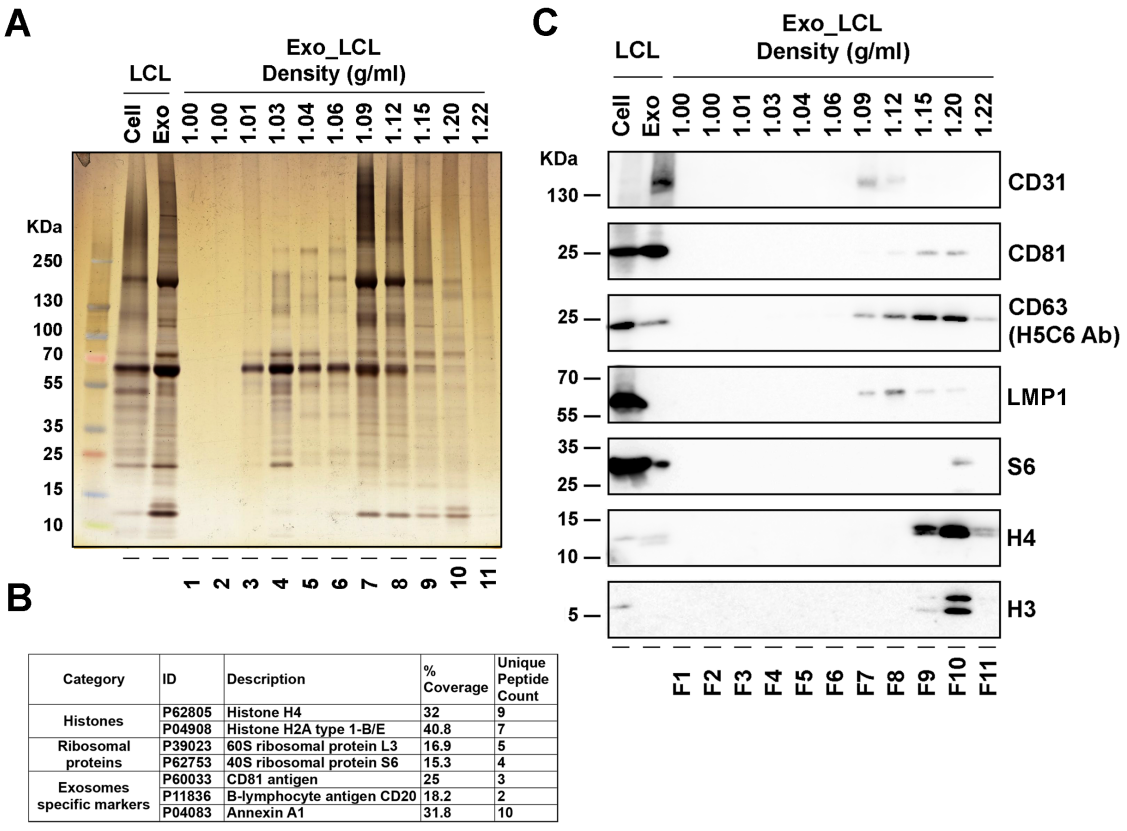
Figure 2.7 cfTERRA is protected by exosomes. (A) Schematic of RNase protection assay used in B. Exosomes were treated with or without RNase mixture (Protected Exo-RNA) or exosome RNA was first isolated and then treated with or without RNase mixture (Exo-RNA). (B) Northern blot of RNA isolated from LCLs 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.

2.4.3 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. 2.8A and B, Fig. 2.10, and Dataset S1). MS revealed histones and ribosomal proteins, as well as many known exosome components (Fig. 2.10), including CD81, CD20, and annexin A1 (37) (Fig. 2.8B). 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. 2.8C). To determine whether cfTERRA is associated with any of the protein components of the exosome fraction, we performed immunoprecipitation assays on these exosomes (Fig. 2.9). 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. 2.9 B and C). 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. 2.11A). We found that TERRA was significantly enriched in histone H3 Exo RNA-ChIP and to a much lesser extent with TRF2 (Fig. 2.11 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. 2.12). 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. 2.12 B and C). These findings indicate that chromatin-associated DNA fragments enriched with telomeric and α-satellite DNA fragments can be found in extracellular fractions.



B

Category	ID	Description	% Coverage	Unique Peptide Count
Histones	P62805	Histone H4	32	9
	P04908	Histone H2A type 1-B/E	40.8	7
Ribosomal proteins	P39023	60S ribosomal protein L3	16.9	5
	P62753	40S ribosomal protein S6	15.3	4
Exosomes specific markers	P60033	CD81 antigen	25	3
	P11836	B-lymphocyte antigen CD20	18.2	2
	P04083	Annexin A1	31.8	10

Figure 2.8 cfTERRA is associated with exosomes. (A) Sucrose fractions collected in Fig. 2.6 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 2. (C) Western blotting of sucrose factions using antibodies specific for CD31, CD81, CD63, LMP1, S6, histone H4, and H3 antibodies.

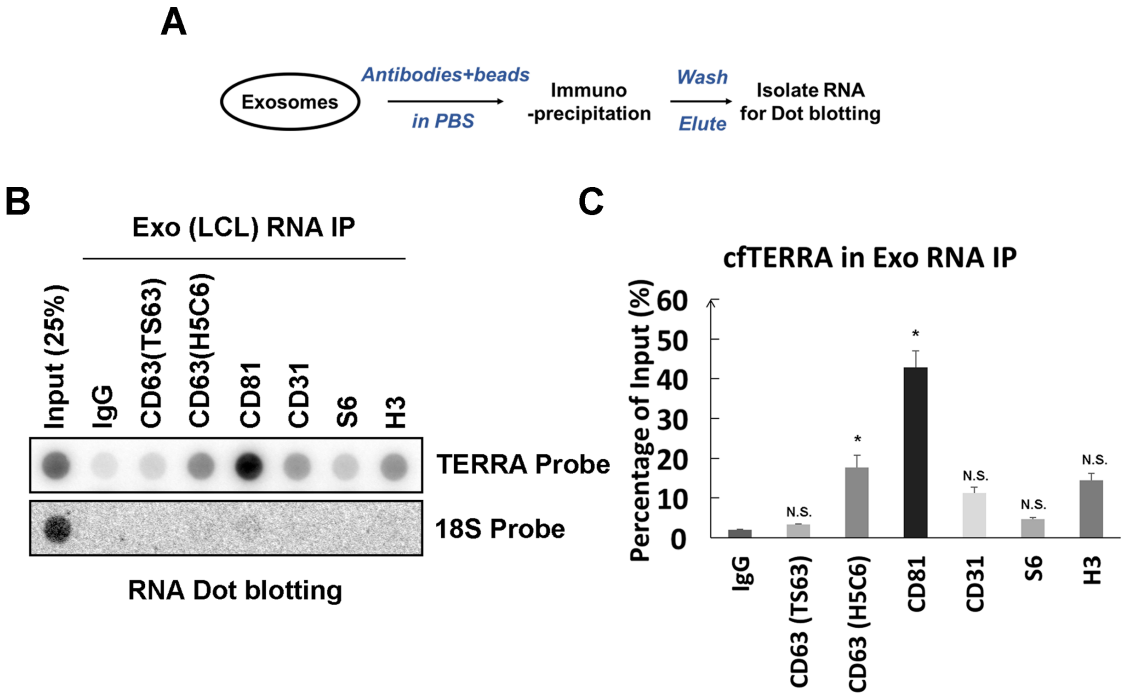


Figure 2.9 cfTERRA is more associated with CD81-positive exosomes. (A) Schema of exosome immunoprecipitation and RNA isolation method used in B and C. (B) Exo RNA IP using antibodies to CD63 (TS63), CD63 (H5C6), CD81, CD31, S6, H3, or control IgG. Isolated RNA was then assayed by dot blotting with TERRA or 18S-specific probes. (C) Quantification of three independent replicates of Exo RNA IP as represented in B. Error bars, SD.

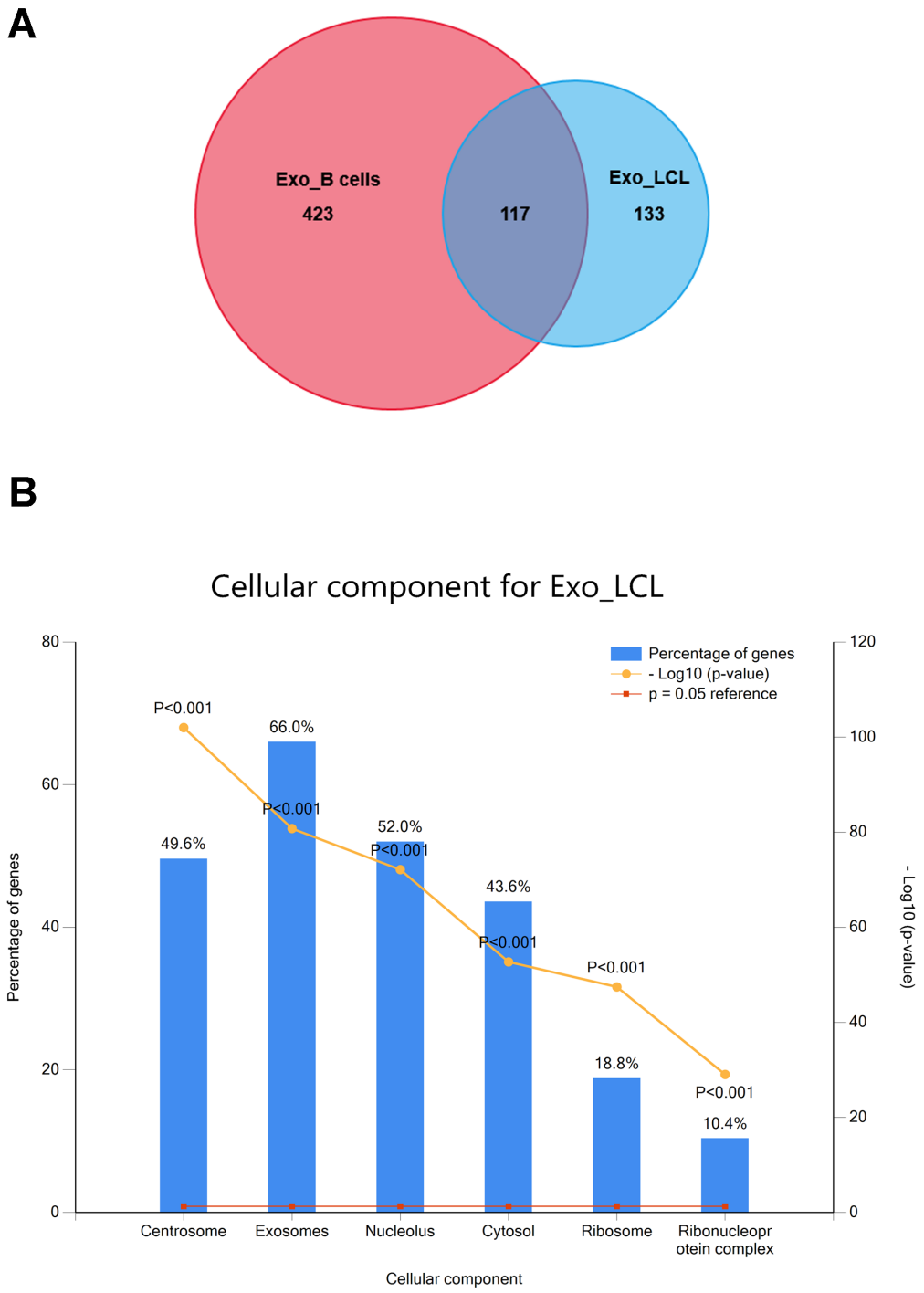


Figure 2.10 Functional enrichment analysis of proteins identified by LC-MS/MS. (A) The Venn diagram shows 201 proteins of the top 250 proteins identified in LCL exosomes (Exo_LCL) overlap with that found in 12 other studies of exosome proteins in the Vesiclepedia database. (B) Enrichment of cellular component for LCL exosomes (Exo_LCL). The percentages of genes that function in different cellular components are shown of the top 250 proteins identified by LC-MS/MS.

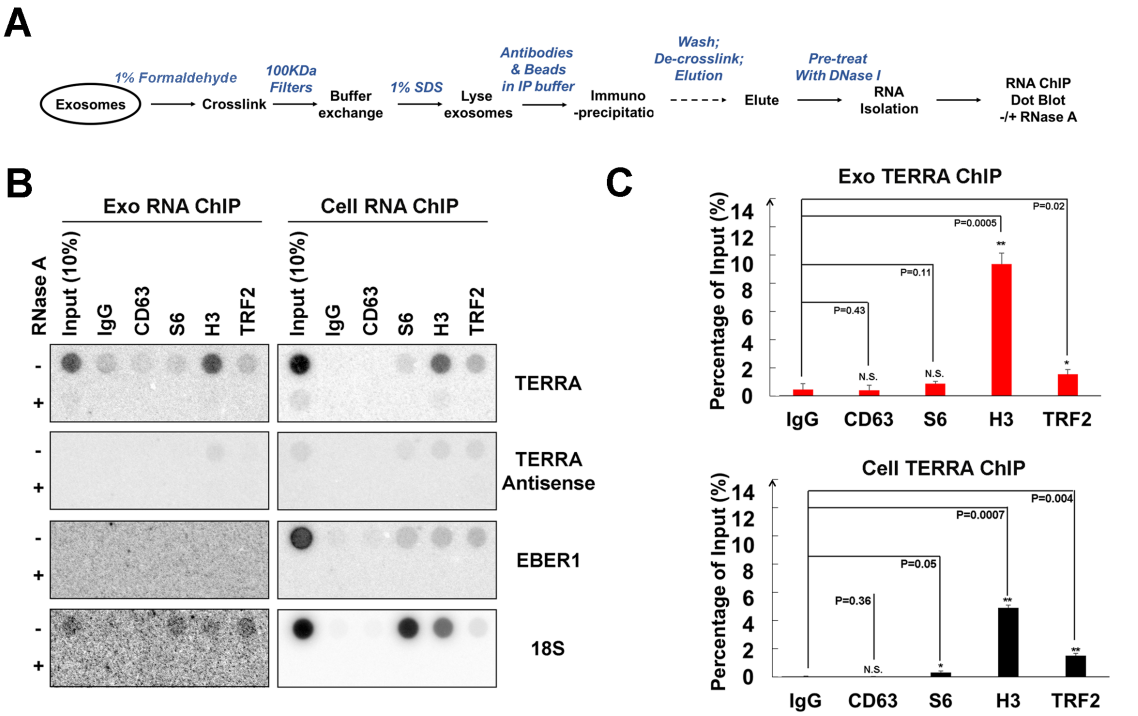


Figure 2.11 cfTERRA is associated with histones. (A) Schema of Exo RNA ChIP assay. (B) RNA ChIP assays were performed with exosomes (Exo) or cellular (Cell) LCLs using antibodies specific for CD63, S6, H3, TRF2, or control IgG. Isolated RNA was treated with either mock (−) or RNase A and then assayed 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.

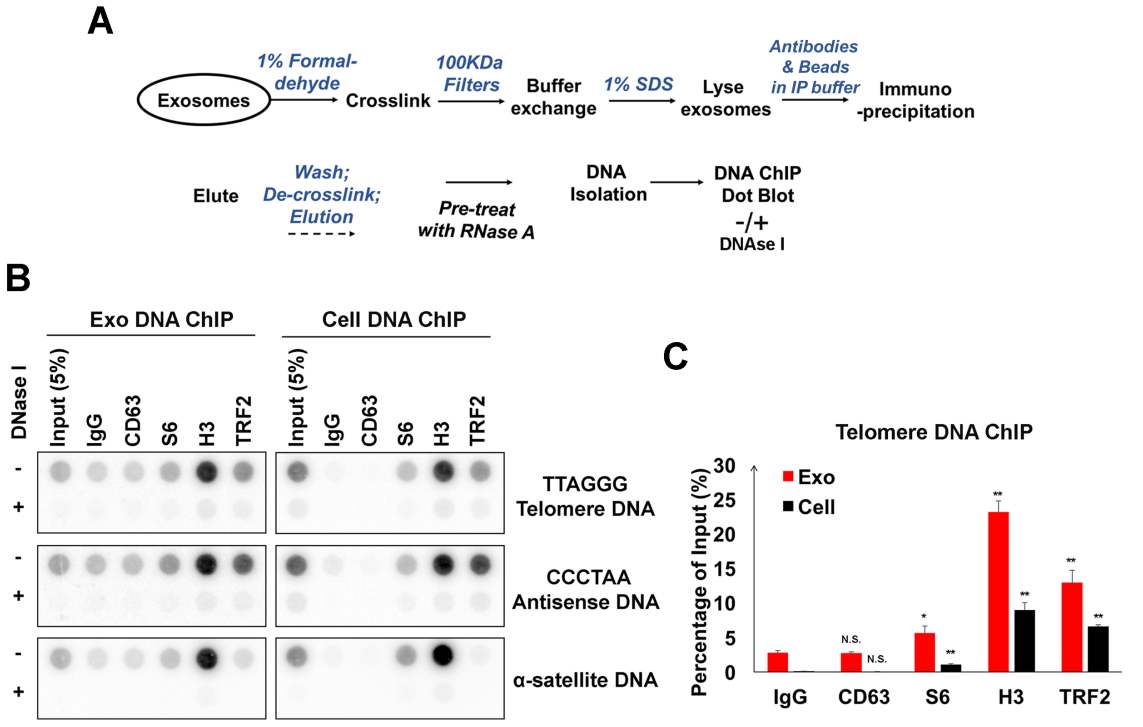


Figure 2.12 Exosomes contain chromatin-associated DNA. (A) Schema for Exo DNA ChIP assays. (B) DNA dot blots for Exo DNA ChIP assays. ChIP was performed with antibodies to CD63, S6, H3, TRF2, or control IgG. (C) Quantification of three independent DNA ChIP assays for telomeric DNA from exosomes (red) or cells (black). Bar graphs represent mean values with SDs. P values were calculated by two-tailed Student t test: *P < 0.05, **P < 0.01.

2.4.4 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. 2.13 and Fig. 2.16A). To determine whether cfTERRA levels in exosomes correlated with cytokine activation, we isolated exosomes from cells engineered to produce elevated TERRA levels (Fig. 2.14A). 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. 2.14B), 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. 2.14B). Protein levels of cellular TRF1 and exosomal CD63 were monitored by Western blot (Fig. 2.14C). Exosomes normalized by CD63 expression levels were then incubated with peripheral blood mononuclear cells (PBMCs) and assayed for cytokine induction (Fig. 2.14D). 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. 2.14D and Fig. 2.16B). 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. 2.17A) or IMR90 fibroblasts (Fig. 2.15 A and B). We found that synthetic TERRA-containing liposomes selectively stimulated IL6, CXCL10, and TNFa in IMR90 cells (Fig. 2.15B) and to a lesser extent in PBMCs (Fig. 2.17A). We also found that short synthetic oligonucleotides (36 bp) containing TERRA could partially induce some cytokine production, although not fully recapitulating endogenous exosomes (Fig. 2.17B). Taken together, these findings suggest that exosome-associated cfTERRA may function to modulate cytokine production in recipient cells.

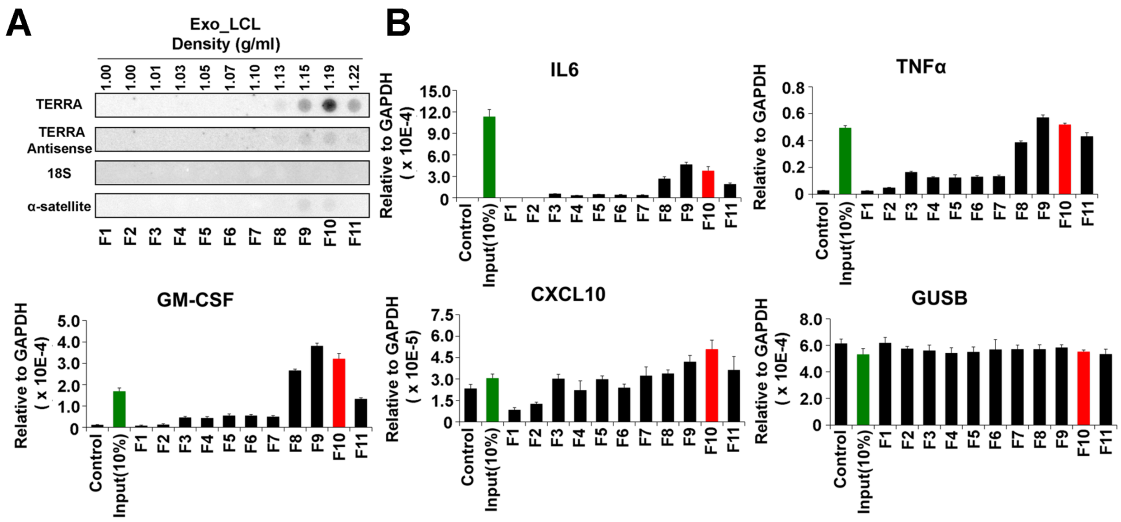


Figure 2.13 TERRA-containing exosomes stimulate inflammatory cytokines. (A) RNA dot blot analysis of sucrose gradient fractionation of LCL-derived exosomes probed for TERRA (Upper), TERRA antisense, 18S rRNA, or alpha-satellite RNA (Lower). (B) Total exosomes (input) or sucrose gradient fractions were 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.

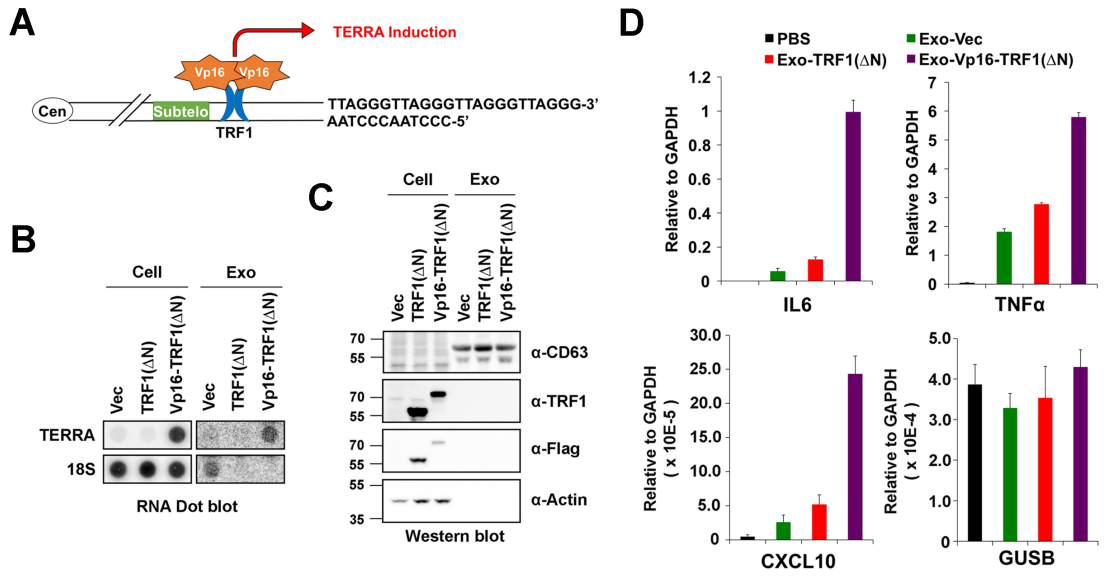


Figure 2.14 Inducing TERRA levels in exosomes promotes inflammatory cytokine expression. (A) Schema of VP16-TRF1(ΔN) activation of TERRA. (B) RNA dot blot for TERRA, 18S, or U1 RNA from HCT116 cells (Left) or exosomes (Right) transduced with vector, TRF1(ΔN), or VP16-TRF1(ΔN). (C) HCT116 cells transduced as in B were assayed by Western blot for CD63, TRF1, FLAG, and Actin. (D) 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).

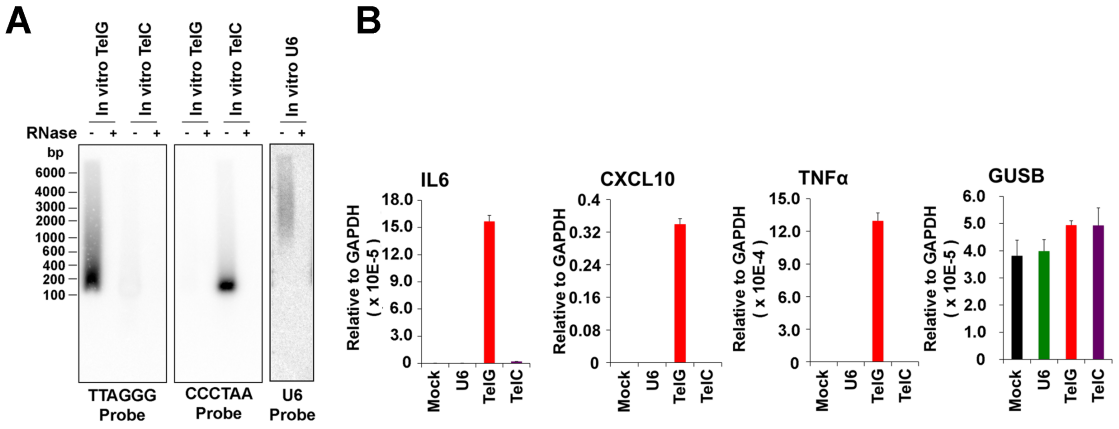


Figure 2.15 TERRA-mimic liposomes stimulate inflammatory cytokines. (A) Northern blot of in vitro transcribed TelG, TelC, or U6 RNA treated with control or RNaseA and probed for TERRA (Left), TERRA antisense (Center), or U6 (Right). (H) IMR90 cells were treated with liposomes containing TelG or TelC 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.

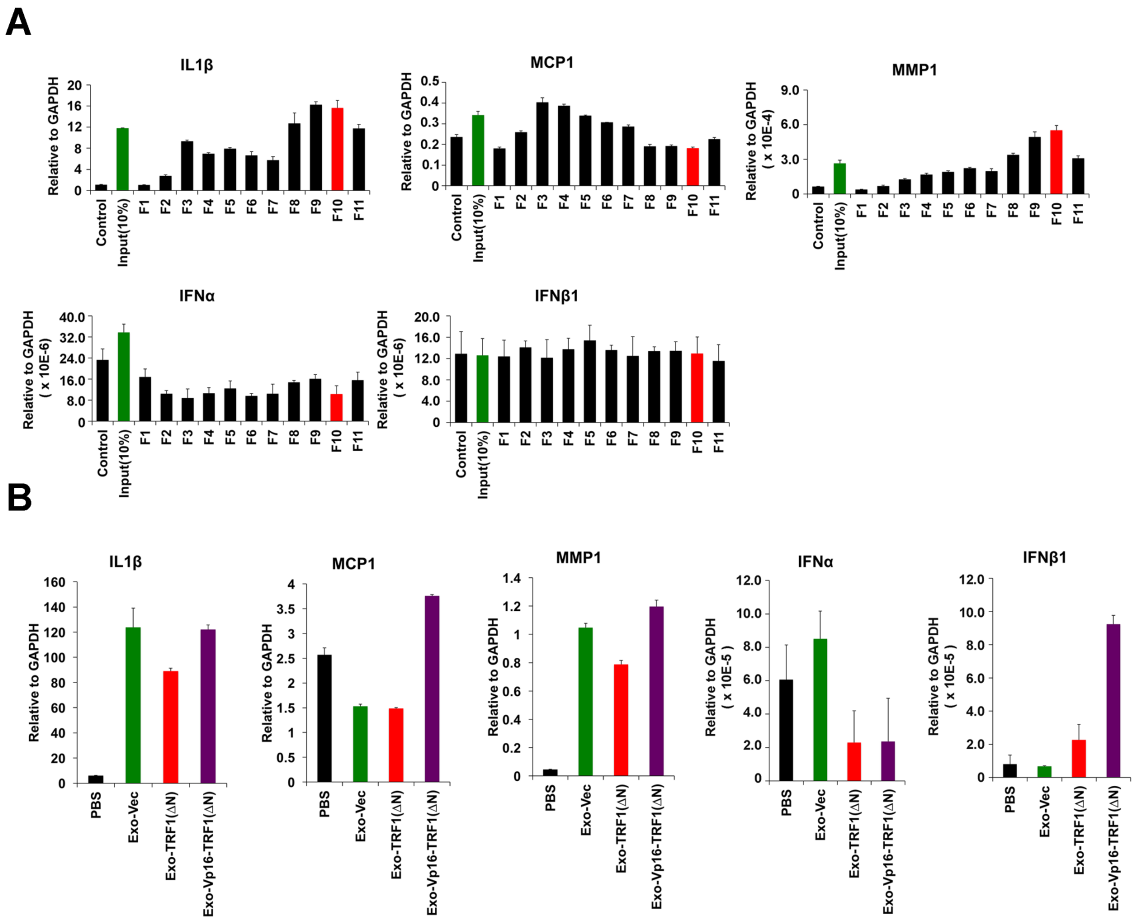


Figure 2.16 Exosome-associated TERRA stimulates inflammatory cytokines. (A) Experiments described in Fig. 2.13B where sucrose fractions from LCL exosomes were used to treat PBMCs and then assayed for induction of cytokine gene transcription, including mRNA for IL1B, MCP1, MMP1, IFNα, and IFNβ1. (B) Experiments described in Fig. 2.14 C–E include addition cytokine genes IL1B, MCP1, MMP1, IFNα, and IFNβ1.

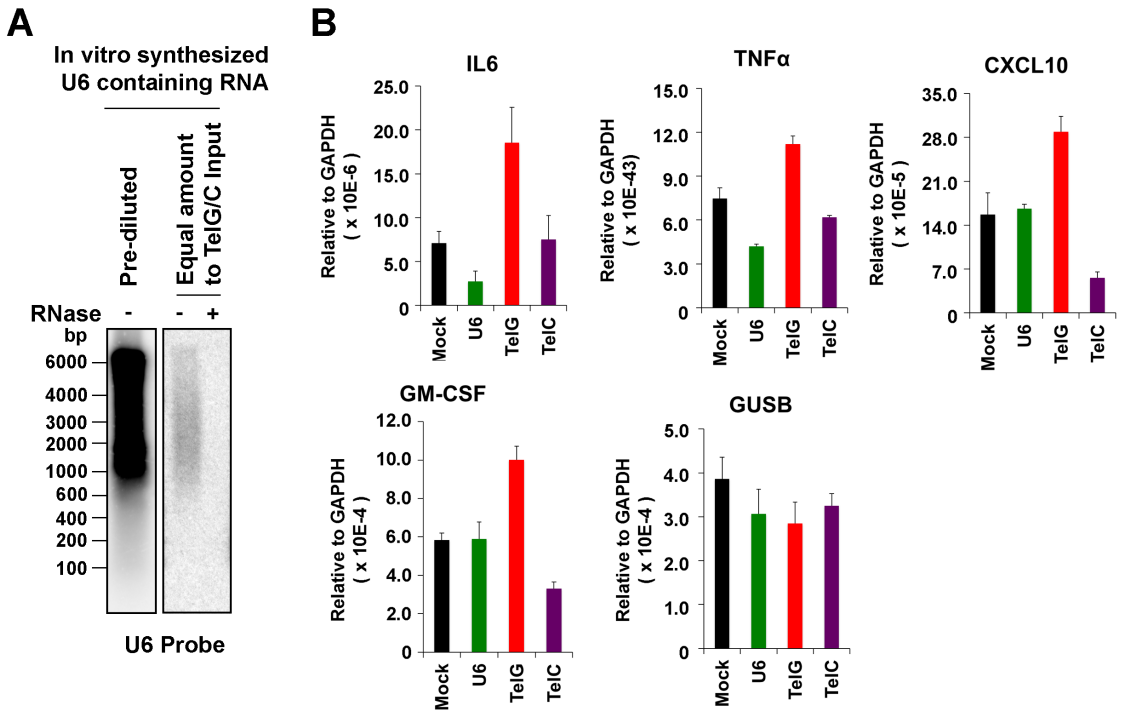


Figure 2.17 Additional cytokine expression profile stimulated by TERRA-mimic liposomes (A) In vitro transcribed U6 RNA is shown by Northern blot in undiluted and diluted amounts used for molar comparison with TelG or TelC as shown in Fig. 2.15 G and H. (B) PBMCs treated with liposomes containing mock, U6, TelG or TelC in vitro transcribed RNA were assayed by qRT-PCR for activation of cytokines IL6, TNFα, CXCL10, GM-CSF, or control GUSB.

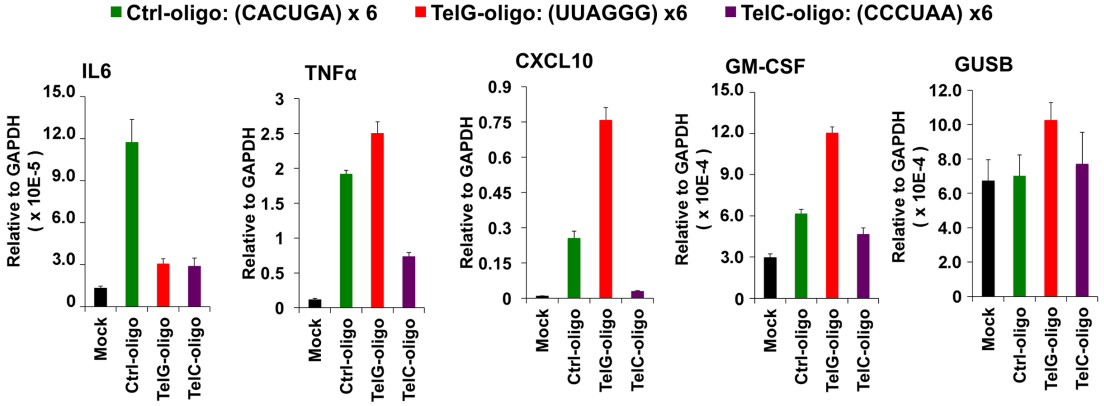


Figure 2.18 Synthetic TERRA stimulates cytokine production. Synthetic 36-nt RNA oligonucleotides for TERRA (TelG), antisense TERRA (TelC), or control (CACUGA)6 were delivered in liposomes to PBMCs and assayed for IL6, TNFα, CXCL10, GM-CSF, or control GUSB mRNA expression.

2.5 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.

2.5.1 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 (45) (46) (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 (7) 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.

2.5.2 Telomeres and immunological response

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

2.5.3 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).

2.5.4 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. 2.5). 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.

2.6 Tables

Table 1 RNA-seq analysis for TERRA

Sample	5/6 reps	2 rep at 5'/3'	% of 5/6	unique	full.gen	part.gen	part.sub
Normal 1	1177	21	1.8%	13	7	8	8
Normal 2	957	32	3.3%	22	13	17	17
Breast 1	672	14	2.1%	14	9	11	11
Breast 2	1189	29	2.4%	25	19	20	20
Colon 1	893	17	1.9%	16	9	9	9
Colon 2	1001	22	2.2%	15	7	8	8
Duct	1951	24	1.2%	21	10	13	13
Kidney 1	728	18	2.5%	11	5	6	6
Kidney 2	704	27	3.8%	19	10	12	12
Liver 1	1129	14	1.2%	14	4	6	6
Liver 2	2094	18	0.9%	15	6	10	10
Lung 1	427	21	4.9%	18	12	13	13
Lung 2	846	25	3.0%	20	9	10	10
Melanoma 1	1743	23	1.3%	21	8	13	13
Melanoma 2	1829	33	1.8%	16	7	12	12
Ovarian 1	399	24	6.0%	22	12	14	14
Ovarian 2	2633	36	1.4%	20	8	9	9
Prostate 1	1128	17	1.5%	15	11	12	12
Prostate 2	328	15	4.6%	13	6	8	8
Stomach 1	783	28	3.6%	24	12	19	19
Stomach 2	789	23	2.9%	23	9	13	13

We searched all of the samples for reads with no more than two telomeric repeats at the 5’ or 3’ end of the read. Those full reads were then aligned against complete human genome and the number of reads mapped to unique sites are indicated (unique). The full counts data are presented in the table. 5/6 reps, number of reads with 5/6 telomeric repeats; 2 at 5’/3’, total number of reads with two, but no more than two, telomeric repeats at the 3’ or 5’ end; % of 5/6, percent of two-repeat reads over 5/6 telomeric repeats; unique, number of unique reads (unique sequence) that had no more than two telomeric repeats.

Table 2 List of oligonucleotides used for qRT-PCR and ChIP-qPCR

Gene name	Primer sequences
GAPDH	Forward primer 5′-TGGGCTACACTGAGCACCAG-3′
	Reverse primer 5′-GGGTGTCGCTGTTGAAGTCA-3′
IL6	Forward primer 5′-CTTTTGGAGTTTGAGGTATACCTAG-3′
	Reverse primer 5′-GCTGCGCAGAATGAGATGAGTTGTC-3′
TNFα	Forward primer 5′-CAGCCTCTTCTCCTTCCTGAT-3′
	Reverse primer 5′-GCCAGAGGGCTGATTAGAGA-3′
IL1β	Forward primer 5′-TCAGCCAATCTTCATTGCTCAA-3′
	Reverse primer 5′-TGGCGAGCTCAGGTACTTCTG-3′
CXCL10	Forward primer 5′-GAAAGCAGTTAGCAAGGAAAGGT-3′
	Reverse primer 5′-GACATATACTCCATGTAGGGAAGTGA-3′
GM-CSF	Forward primer 5′-TCTCAGAAATGTTTGACCTCCA-3′
	Reverse primer 5′-GCCCTTGAGCTTGGTGAG-3′
IFNα	Forward primer 5′-CCGTGCTGGTGCTCAGCTA-3′
	Reverse primer 5′-TGGGTCTGAGGCAGATCACA-3′
IFNβ1	Forward primer 5′-ACTGCCTCAAGGACAGGATG-3′
	Reverse primer 5′-AGCCAGGAGGTTCTCAACAA-3′
MCP1	Forward primer 5′-AGTCTCTGCCGCCCTTCT-3′
	Reverse primer 5′-GTGACTGGGGCATTGATTG-3′
MMP1	Forward primer 5′-GCTAACCTTTGATGCTATAACTACGA-3′
	Reverse primer 5′-TTTGTGCGCATGTAGAATCTG-3′
2q (234-293)	Forward primer 5′-GCCTTGCCTTGGGAGAATCT-3′
10q (396-455)	Reverse primer 5′-AAAGCGGGAAACGAAAAGC-3′
7p (770-831)	Forward primer 5′-GGAGGCTGAGGCAGGAGAA-3′
	Reverse primer 5′-CAATCTCGGCTCACCACAATC-3′
12q (352-419)	Forward primer 5′-ATTTCCCGTTTTCCACACTGA-3′
	Reverse primer 5′-CTGTTTGCAGCGCTGAATATTC-3′
13q (152-210)	Forward primer 5′-GCACTTGAACCCTGCAATACAG-3′
	Reverse primer 5′-CCTGCGCACCGAGATTCT-3′
17q (864-939)	Forward primer 5′-AGCTACCTCTCTCAACACCAAGAAG-3′
	Reverse primer 5′-GTCCATGCATTCTCCATTGATAAG-3′
17p (348-431)	Forward primer 5′-GGGACAGAAGTGGATAAGCTGATC-3′
	Reverse primer 5′-GATCCCACTGTTTTTATTACTGTTCCT-3′
XqYq (109-162)	Forward primer 5′-CCCCTTGCCTTGGGAGAA-3′
	Reverse primer 5′-GAAAGCAAAAGCCCCTCTGA-3′
XpYp (243-311)	Forward primer 5′-CCACAACCCCACCAGAAAGA-3′
	Reverse primer 5′-GCGCGTCCGGAGTTTG-3′

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CHAPTER 3: The crosstalk of telomere dysfunction and inflammation through cell-free TERRA containing exosomes

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3.1 Abstract

Telomeric repeats-containing RNA (TERRA) are telomere-derived non-coding RNAs that contribute to telomere function in protecting chromosome ends. We recently identified a cell-free form of TERRA (cfTERRA) enriched in extracellular exosomes. These cfTERRA-containing exosomes stimulate inflammatory cytokines when incubated with immune responsive cells. Here, we report that cfTERRA levels were increased in exosomes during telomere dysfunction induced by the expression of the dominant negative TRF2. The exosomes from these damaged cells also enriched with DNA damage marker **γ**H2AX and fragmented telomere repeat DNA. Purified cfTERRA stimulated inflammatory cytokines, but the intact membrane-associated nucleoprotein complexes produced a more robust cytokine activation. Therefore, we propose cfTERRA-containing exosomes transport a telomere-associated molecular pattern (TAMP) and telomere-specific alarmin from dysfunctional telomeres to the extracellular environment to elicit an inflammatory response. Since cfTERRA can be readily detected in human serum it may provide a useful biomarker for the detection of telomere dysfunction in the early stage of cancers and aging-associated inflammatory disease.

3.2 Introduction

Telomeres are repetitive DNA elements that protect the ends of linear chromosomes from catastrophic damage(1). Telomeric repeats are assembled into a dynamic nucleoprotein complex, collectively termed Shelterin, which regulates telomere repeat length, stability, and function(2). As cells divide and age, telomere repeat DNA shortens by attrition due

to the end-replication problem. Excessive cell divisions or replication stress can lead to critically short telomeres incapable of forming a functional Shelterin complex. These uncapped telomeres elicit a DNA damage response and cell cycle arrest(3). In stem cells and most cancer cells, the telomere repeats are restored by the telomere-specific reverse transcriptase Telomerase(4). In the absence of Telomerase, these dysfunctional telomeres induce cellular senescence and drive the process of organismal aging(5). Programmed senescence is an important control on tissue homeostasis and an innate barrier to cellular immortalization and carcinogenesis (6). However, excessive or premature senescence can induce a pathogenic tissue microenvironment due to secretion of inflammatory molecules (7). This senescence-associated secretory phenotype (SASP) is characterized by the secretion of proinflammatory cytokines, chemokines, growth factors, and proteases (8). However, the molecular mechanisms driving SASP are not well-understood. A recent study described a 'telomere-associated secretory phenotype' (TASP) (9), which produces a distinct inflammatory pattern associated with pre-shortened telomeres, suggesting that telomere dysfunction may be directly linked to senescence-associated inflammation.

Telomere uncapping can result in the increase production of telomeric repeats-containing RNA (TERRA) (10,11). TERRA is a heterogeneous ensemble of non-coding RNAs generated from multiple different telomeres, and comprises 100 to 9,000 bases of telomere repeats in mammals (12). Due to the unique G-rich sequence, TERRA is folded into G-quadruplex structure to interact with numerous proteins that regulate telomere maintenance (13). We previously found that TERRA interacts with shelterin TRF2 and promotes telomeric heterochromatin formation by mediating the interaction of TRF2 and

ORC (14). Others have found that TERRA can inhibit telomerase, bind to hnRNPA and MRE11, and form stable RNA-DNA hybrid R-loops at telomeres (15,16). Elevated TERRA levels also correlate with the alternative lengthening of telomeres (ALT) phenotype (17,18), and chromosome instability syndromes linked to deficiencies in DNMT3b-associated DNA methylation (19). Whether TERRA also participates in telomere-associated inflammatory secretion was not known.

Recently, we found a cell-free form of TERRA (cfTERRA) that constituted a nucleoprotein component of extracellular microvesicular exosomes in cancer cell culture and human blood plasma (20). These cfTERRA-containing exosomes were strong inducers of inflammatory cytokines in peripheral blood mononuclear cells (PBMCs). We also demonstrated that synthetic TERRA was able to stimulate inflammatory cytokines when delivered by liposomes. Here, we describe recent advances in the role of TERRA during telomere dysfunction, and discuss how cfTERRA may mediate the crosstalk between telomere dysfunction and inflammation.

3.3 Results and Discussion

3.3.1 TERRA is highly enriched in human exosomes

TERRA levels increase in response to telomere uncapping (10). To recapitulate telomere uncapping in cell culture models, we expressed $TRF2^{WT}$ or the dominant negative form of TRF2, $TRF2^{\Delta B\Delta M}$, shown previously to form telomere-specific DNA damage (21). We compared protein and RNA levels in cellular and exosomal fractions after BJ-hTERT

cells were transduced with either vector, TRF2, or TRF2$^{\Delta B\Delta M}$ expressing lentivirus infection (Figure 3.1). As expected, TRF2 proteins were exclusively associated with cellular fractions and not enriched in exosomes, while CD63, a marker for exosomes, was found exclusively associated with exosome fractions (Figure 3.1A). Interestingly, DNA damage marker **γ**H2AX was modestly enriched in the exosome fractions of the TRF2$^{\Delta B\Delta M}$ transduced cells (Figure 3.1A, lower panel). Total cellular TERRA levels were measured by Northern blot (Figure 3.1B). We found that cells transduced with TRF2$^{\Delta B\Delta M}$ expressed elevated levels of TERRA (Figure 3.1B), consistent with previous reports showing elevated TERRA after TRF2 depletion (10). We previously demonstrated that exosomal TERRA is smaller than cellular TERRA, and is associated with a small percentage of telomeric DNA and histone protein components (20). Using identical extraction methods described previously, we measured exosome-associated TERRA by dot blot (Figure 3.1C). We found high levels of TERRA in all exosome fractions relative to cellular levels and compared to 18S RNA. In contrast, Alu transcripts were found at similar levels in cellular and exosome fractions, indicating that they are not selectively enriched in these exosomes (Figure 3.1C, lower panel). Cells expressing TRF2$^{\Delta B\Delta M}$ showed a modest increase in cellular and exosomal TERRA levels, as well as trace amounts of anti-sense TERRA (Figure 3.1C). These findings indicate the dominant negative TRF2-associated telomere uncapping can induce cellular TERRA and increase the relative amount of TERRA and anti-sense TERRA in exosomes.

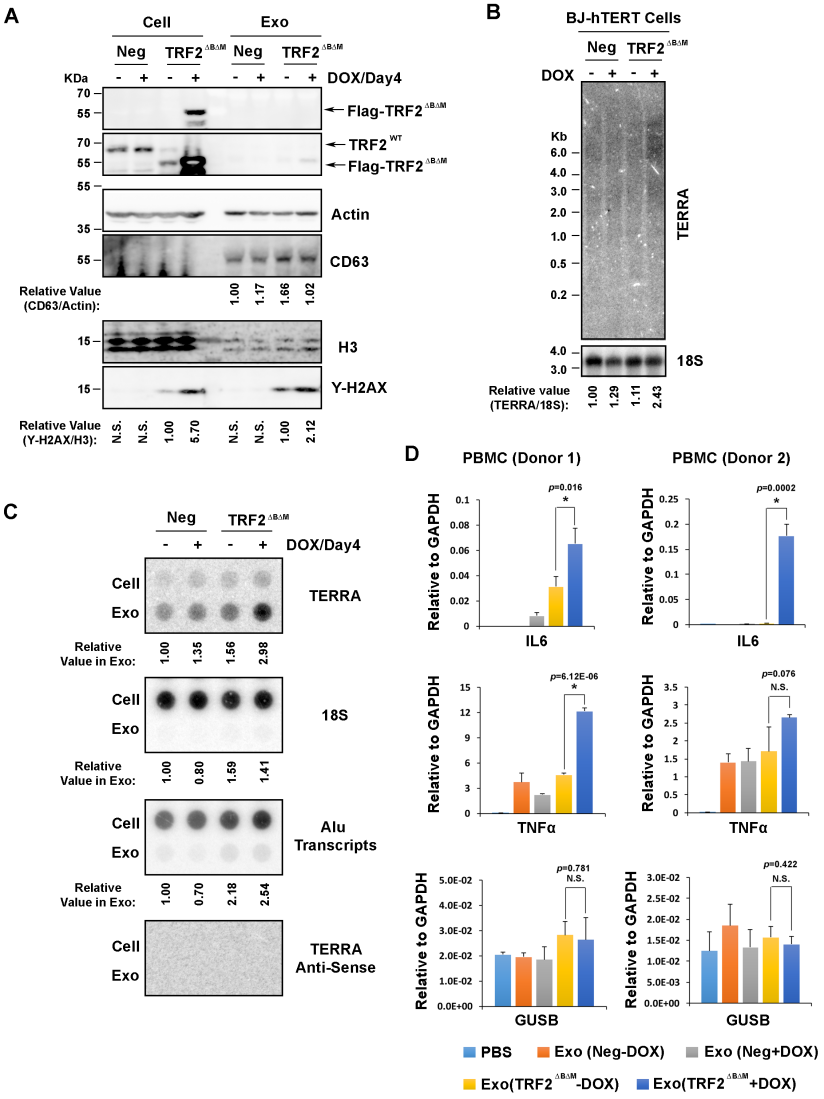

Figure 3.1 Induction of cellular and cell-free TERRA by the dominant negative TRF2. (A) Western blot analysis of lysates from BJ-hTERT cells (Cell) or purified exosomes (Exo). N-terminal FLAG tagged TRF2^{WT} or TRF2$^{\Delta B\Delta M}$ were transduced into BJ-hTERT cells by lentiviral infection. Total protein lysates were collected from transduced cells or purified exosomes at 6 days post lentiviral infection. Western blotting was performed with equal amounts of total protein lysate (15µg) for each sample, using antibodies specific for FLAG (Sigma), Actin (Sigma), CD63 (Abcam), or **γ**H2AX (Millipore). (B) Northern blot analysis of cellular TERRA in the transduced BJ-hTERT cells, as shown in (A). Isolated cellular RNA (10µg) was used for each sample, and hybridized with ^{32}P-labeled probes for TERRA, or 18S RNA as indicated. Numbers on the left show the position of RNA markers in base pairs. (C) RNA dot blot analysis of RNA isolated from BJ-hTERT cells (Cell) or purified exosomes (Exo), as shown in (A). Equal amounts of RNA (1µg) were assayed by dot blotting, and hybridized with probes for TERRA, TERRA-antisense, 18S, or Alu transcripts, as indicated.

3.3.2 Inflammatory properties of exosomal TERRA

Exosomes from human lymphoblastoid cell lines (LCLs) were also found to have elevated levels of TERRA containing exosomes. We found that these exosomes also contain telomeric DNA that may form stable structures, including DNA-RNA hybrids with TERRA. Extraction of DNA or RNA from various fractionations across a density gradient centrifugation revealed that both telomere repeat-containing DNA and RNA could be isolated from exosome-containing fractions that peak in F9 and F10 (Figure 3.2A, top panels). G-rich strand DNA and RNA were enriched, but C-rich DNA could also be detected in fractions F9 and F10. We then tested whether either exosomes, or exosome-associated RNA or DNA could induce an inflammatory response when incubated with peripheral blood mononuclear cells (PBMCs) (Figure 3.2B and C). Exosomes enriched with TERRA from F10 were found to induce the highest levels of IL6 and TNFα whereas no significant effect was caused in control GUSB mRNA levels (Figure 3.2D). Purified exosome F10-associated RNA containing highest levels of TERRA was found to partly induce IL6 and TNFα, while only trace activities could be found in the purified DNA fractions. Interestingly, others have found that purified telomeric DNA can inhibit innate immune response (22). These findings suggest that the RNA component from exosome is a more potent inflammatory molecule than telomeric DNA. However, neither RNA nor DNA was as active as the complete exosome fraction, suggesting that additional factors, or intact membrane-associated nucleoprotein complexes are required for robust activation of the inflammatory response.

Based on these data and our recently published study (20), we propose that telomere-induced stress leads to the upregulation of TERRA that is processed into smaller fragments and secreted in exosome-like vesicles. These TERRA containing exosomes are relatively stable and circulate in extracellular spaces, including blood and plasma. We have shown that the TERRA containing exosomes can induce a robust inflammatory response in human PBMCs leading to the production of various cytokines, including IL6 and TNFα (Figure 3.2B and C). We propose that TERRA containing exosomes are a significant source of SASP and TASP, as telomere dysfunction is known to occur in senescent cells. The function of this inflammation may be to recruit macrophages to consume and eliminate senescing cells and their byproducts from tissue microenvironments (Figure 3.3). Excessive SASP can be a source of pathogenic inflammation which may alter the normal physiology of neighboring cells and tissue (23). It is well known the chronic inflammation leads to a tumorigenic environment. Telomere dysfunction induced inflammation is also observed when TRF2 is depleted in mouse embryonic skin (24) and alveolar stem cells (25). We suspect that TERRA containing exosomes are a significant component of this inflammatory signaling pathway.

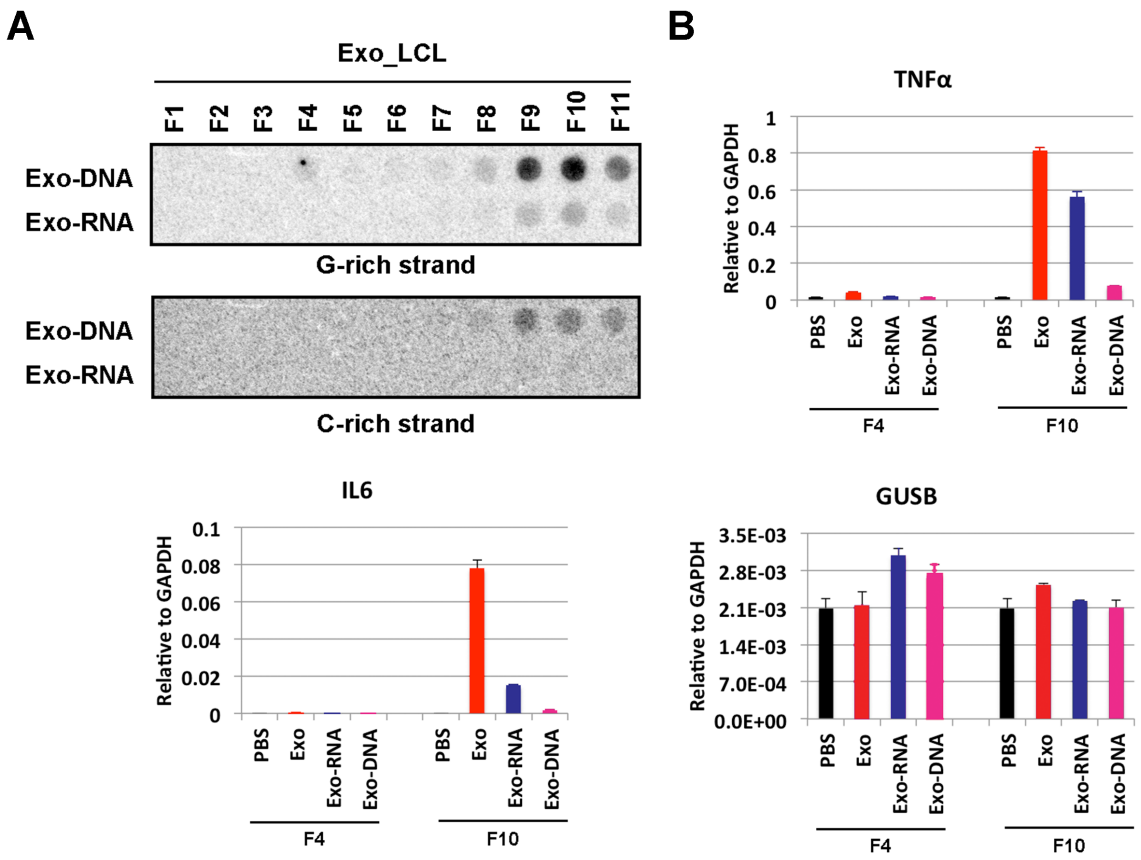

Figure 3.2 cfTERRA containing exosomal RNA constitutes more activity over DNA in stimulating inflammatory cytokines.

(A) Dot blot analysis of telomeric repeats in exosomal RNA (Exo-RNA) and DNA (Exo-DNA) from sucrose gradient fractionation of LCL-derived exosomes. The blot was probed for G-rich telomeric strand (Upper) or C-rich telomeric strand (Lower).

(B-D) PBMCs were incubated with PBS, exosomes (Exo), Exo-RNA, or Exo-DNA from indicated sucrose gradient fraction 4 (F4) and fraction 10 (F10), and then assayed by qRT-PCR for expression of IL6 (B), TNFα (C) or control GUSB mRNA (D). Bar graphs represent qRT-PCR values relative to GAPDH mRNA (mean ± SD) from three independent experiments.

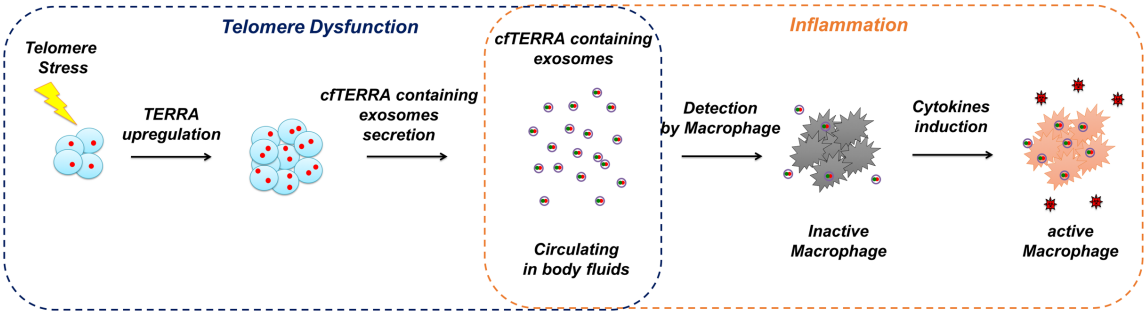

Figure 3.3 Model of cfTERRA containing exosomes as telomere-specific alarmins containing telomere-associated molecular patterns (TAMP) that mediate the crosstalk of telomere dysfunction with inflammation.
Telomere-related stress induces TERRA expression and the secretion of cfTERRA through exosomes. These cfTERRA containing exosomes are released in tissue microenvironment and circulating in body fluids. Immune cells (macrophages) detect cfTERRA containing exosomes as damage signals, and produce inflammatory cytokines to eliminate cells with dysfunctional telomeres.

3.3.3 cfTERRA biogenesis

TERRA is transcribed predominantly by RNA polymerase II (26) and regulated by several mechanisms, including developmental status (27), telomere length (11), epigenetic state (28), and stress (29). Numerous studies showed that telomere dysfunction induces changes in cellular TERRA levels (10,30,31). Depletion of TRF2 resulted in up-regulation of TERRA (10). Loss of TRF2 disrupts the telomere T-loop structure (32), activates ATM-mediated DNA damage response (33), and leads to telomere fusions through non-homologous end joining (NHEJ) (34). Increase expression of TERRA may be a built-in feedback mechanism to protect telomeres by generating RNA-DNA hybrids and promoting homologous recombination with other telomere repeats (16). This telomeric homologous recombination is observed in telomerase negative ALT cells. As TERRA expression is linked to telomere uncapping and dysfunction, its elevated expression correlates well with telomere damage and stress-response.

How the smaller extracellular forms of cfTERRA are generated is not known. cfTERRA expression levels typically correlate with levels of cellular TERRA, so it is likely that the cfTERRA is co-regulated with cellular TERRA production. We found that cfTERRA was typically ~200 nt in length, distinctly smaller than cellular forms of TERRA. cfTERRA can be amplified by RT-qPCR primers situated in unique sequences of the adjacent subtelomeres, suggesting that cfTERRA arises from chromosome terminal repeats. As there are many interstitial telomere repeat sequences (ITS) of 200 nucleotide length, it is also possible that some cfTERRA arises from these shorter interstitial repeats in some stress conditions. The shorter RNA forms suggest that cfTERRA are aborted, incomplete transcripts, or post-transcriptionally processed from longer forms of cellular TERRA. cfTERRA is typically detected in higher abundance than cellular TERRA. This may be due to cfTERRA being more stable, a breakdown product of full-length cellular TERRA, or an alternative, aberrant form that is rapidly eliminated through extracellular vesicle transport. We also observed that cfTERRA is co-purified with extracellular telomeric DNA (G and C-rich strands), and is associated with histone, as detected by extracellular ChIP assays with histone H3 specific antibodies (20). Others have reported that nucleosomes and telomeric DNA can be found in cell-free fractions, and are considered contaminants of exosomes. We addressed this concern by demonstrating the exosome-associated cfTERRA is highly resistant to RNAse, while the majority of chromatin DNA can be eliminated by DNase. Thus cfTERRA is either within exosomes, or in a nuclease protected form that is co-purified with exosomes. While cfTERRA may be a jettisoned by-product of stressed cells or part of apoptotic chromatin debris, we are struck by its stability and abundance in cells and tissues. We therefore consider the intriguing

possibility that this debris carries inherent telomere-specific signaling information to surrounding cells.

3.3.4 cfTERRA as telomere-specific alarmin, or TAMP

The term "alarmins" has been given to a family of endogenous molecules that signal cellular and tissue damage (35). Many nuclear proteins and DNA structures function as alarmins. Extracellular forms of HMGB1, an otherwise abundant chromatin associated protein, is considered a Danger Associated Molecular Pattern (DAMP) because it can be secreted at high levels in apoptotic cells and trigger macrophage cytokine production(36). Similarly, release of unmethylated CpG DNA is referred to as a Pathogen Associated Molecular Pattern (PAMP) because it appears at high levels during viral and bacterial infections and triggers a robust immunologic response(37). We suggest that cfTERRA is a telomeres-specific DAMP, or TAMP. cfTERRA by itself, or in its endogenous form associated with other exosome components, including histones, is a potent activator of IL6 and TNFα from human PBMCs. This represents a direct and specific communication of telomere dysfunction through DAMP-like signaling. It will be interesting to determine if the cytokine profile and innate immune response to TAMPs are distinct from other DAMPs, such as HMGB1. It is conceivable that a low level of cfTERRA serves as a healthy signal in the absence of other DAMPs, but that elevated levels by themselves, or in association with other DAMPs may initiate pathogenic inflammatory environments, including those observed in telomeropathies, like idiopathic pulmonary fibrosis and bone marrow failure syndromes.

3.3.5 cfTERRA as potential biomarker for telomere dysfunction and associated human disease

cfTERRA is highly stable, and relatively easy to detect by sequencing or hybridization. We were able to identify cfTERRA foci in human blood and serum by RNA-seq and dot blotting. We also detected elevated levels of TERRA in human tumor tissue, and increase incidence of cfTERRA foci in tumor tissue sections from mouse models of medulloblastoma (20). As cfTERRA can be found at high levels in normal human plasma, it is unlikely that cfTERRA can serve as a single molecular entity for diagnosis. However, it remains possible that cfTERRA in combination with other DAMPs or biomarkers, may reveal early stage pathogenesis associated with telomere-dysfunction in early stage cancers and senescence-associated inflammatory disease.

3.4 Acknowledgments

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CHAPTER 4: Materials and methods

4.1 Materials

Cell Culture.

LCLs were grown in RPMI medium 1640. Human colon cancer cell line HCT116 (ATCCVA) was grown in McCoy' 5A medium. Human fibroblast IMR90 and BJ-hTERT cells (ATCC) were grown in DMEM. All these mediums were supplemented with 10% (vol/vol) FBS and 1 × penicillin-streptomycin. All cells were cultured in a 5% CO2 incubator at 37 °C. Serum starvation stress was induced by growth in serum containing 0.5% FBS for 24 h. When preparing cultures for exosomes purification, cells were washed twice with PBS and cultured in conditional medium. The conditional medium replaced the normal FBS with 10% exosome-depleted FBS, which was subjected to ultracentrifugation at 150,000 × g for 18 h to deplete exosomes in FBS.

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. TRF2$^{\Delta B\Delta M}$ (45-454) was cloned from pLU-Flag- TRF2$^{\Delta B\Delta M}$, and inserted into the pInducer20 vector by 2-step Gateway cloning.

Lentivirus was produced from 293T cells by cotransfecting the constructs with viral packaging vectors PMD2.G and psPAX2 and harvested 48 h after transfection. For

TERRA induction, 5×10^6 HCT116 cells were infected with 10 mL Lentivirus overnight in the presence of 2 μg/mL Polybrene (Sigma). Infected cells were selected by 2.5 μg/mL Puromycin (Sigma) 48 h after infection. After 2 d of selection, cells were then washed twice with 1× PBS and cultured 3 d in conditional medium for exosomes purification.

4.2 Methods

Culture Medium Fractionation and Exosomes Isolation.

The supernatant of LCL culture was fractionated and prepared for exosomes isolation by differential centrifugation as previously described with some modifications (1). Briefly, LCLs were grown in conditional medium for 3 d with cell density maintained around 0.5 $\times 10^6$ cells/mL. Cells were harvested by centrifugation at 300 × g for 10 min. The supernatant was collected and centrifuged at 2,000 × g for 30 min to pellet cell debris. The supernatant was subsequently filtered through a 0.45-μm filter (Millipore) and centrifuged at 16,500 × g for 30 min to pellet large microvesicles. The supernatant was further filtered through a 0.22-μm filter (Millipore) and subjected to ultracentrifugation at 110,000 × g (T45i rotor; Beckman) for 2 h to pellet exosomes. To remove potential contaminated proteins, the exosome pellet was washed once with PBS and repelleted by ultracentrifugation at 110,000 × g for 2 h. All pellets were resuspended in 100 μL PBS and kept at −80 °C until ready for use. All of the centrifugations were performed at 4 °C. The same procedures were used in preparing exosomes from culture medium of HCT116 cells.

Sucrose gradient separation of exosomes was performed as previously described with some modifications (1). The sucrose gradient was poured 1 d before use to generate a continuous 0.25–2 M sucrose solution in 20 mM Hepes buffer (pH 7.4) at 4 °C. Exosomes were isolated from 800 mL LCL culture and resuspended in 2 mL of 20 mM Hepes buffer (pH 7.4). After loaded on the top of sucrose gradient, exosomes were ultracentrifuged at 210,000 × g for 18 h at 4 °C. After the ultracentrifugation, 1-mL fractions were collected from the top, and the density of each fraction was determined by weight. Particles were pelleted from each fraction by centrifugation at 110,000 × g for 2 h in 4 °C, resuspended in 100 μL PBS, and kept at −80 °C until ready to use.

ChIP Assays.

Cellular ChIP assays were performed as previously described. Exosome ChIP assays (ExChIP) were developed based on cellular ChIP assays with some modifications (2). For exosome RNA ChIP assays, exosomes were isolated from 800 mL LCL culture and resuspended in 4 mL PBS. Cross-linking was performed by adding formaldehyde to a final concentration of 1% to exosomes, followed by 125 mM glycine to quench cross-linking. To remove the cross-linking reagents, exosomes were subjected to buffer exchange by 100 kDa MWCO Amicon Ultra 4 mL device (Millipore) with 5 volumes of non-SDS buffer B [50 mM Tris-HCl (pH 8.1), 10 mM EDTA] and concentrated to 1 mL for 10 ChIP materials. After buffer exchange, exosomes were added with protease inhibitor mixture and 50 U/mL SUPERasein (Ambion), and lysed by SDS to a final concentration of 1%. The lysates were diluted 10-fold into IP buffer [0.01% SDS, 1.1%

Triton X-100, 1.2 mM EDTA, 16.7 mM Tris (pH 8.1), 167 mM NaCl, protease inhibitor mixture, 50 U/mL SUPERasein] for an IP reaction at 4 °C overnight.

Each immunoprecipitated complex was pulled down by magnetic protein A/G beads (Pierce) by rotating at 4 °C for 2 h and washed five times (1-mL wash, 10 min each) in ChIP-related wash buffer at 4 °C. The immunoprecipitates were eluted by addition of 200μL elution buffer (1% SDS, 0.1 M NaHCO3, SUPERasein) and rotated for 15 min at room temperature. Elutes were placed at 65 °C for 2 h to reverse cross-linking and subjected to RNA isolation with TRIzol reagent as describe for cell-free RNA isolation. For exosome DNA ChIP assays, the same procedures were performed without adding SUPERasein, and ChIP DNA was isolated by phenol-chloroform extraction and treated with RNase A as previously described. Antibodies used in this study include rabbit IgG (Santa Cruz Biotechnology), CD63 antibody (H5C6; Developmental Studies Hybridoma Bank), ribosomal protein S6 antibody (C-8; Santa Cruz Biotechnology), histone H3 antibody (catalog no. 39163; Active Motif), and TRF2 antibody. All ChIP assays were done in triplicate to obtain the SD.

ChIP DNA or RNA was analyzed by dot blotting. The samples were mix with denaturing solution (50% formamide, 7% formaldehyde, 1× SSC), incubated at 65 °C for 20 min, and followed immediately by cooling down on ice. The denatured samples were mixed with ice-cold 20× SSC to a final concentration of 10× and loaded onto GeneScreen Plus blotting membranes using a dot-blotting apparatus. The membrane was cross-linked and hybridized with a 32P-labeled probe with the procedures same as described in Northern

blotting. Radioactive signals were quantified using ImageQuant 5.2 software (Molecular Dynamics).

Exosomes RNA Immunoprecipitation.

Exosome RNA immunoprecipitation was performed by incubating 100 μg LCL exosomes with 5 μg of each antibody and beads complex in isolation buffer (1× PBS, 0.1%BSA) by rotating overnight at 4 °C. Each immunoprecipitated complex was washed three times (1 mL wash, 10 min each) in isolation buffer. The immunoprecipitates were eluted by addition of 100μL elution buffer (1× RIPA, SUPERasein) and rotated for 15 min at room temperature. RNA was isolated by adding 900 μL TRIzol reagent to elutes and analyzed by dot blotting as described above.

The antibodies used in this study included mouse IgG (Santa Cruz Biotechnology), CD63 antibody (H5C6; Developmental Studies Hybridoma Bank), CD63 antibody (TS63; Invitrogen), CD81 antibody (M38; Invitrogen), CD31 antibody (P2B1; Developmental Studies Hybridoma Bank), ribosomal protein S6 antibody (C-8; Santa Cruz Biotechnology), and histone H3 antibody (catalog no. 39163; Active Motif). The antibodies were pre–cross-linked with N-hydroxysuccinimide (NHS)-activated magnetic beads (Pierce) per the manufacturer's instruction.

Blood Plasma Cell-Free RNA Isolation and RNA-seq Analysis.

The blood plasma was obtained from 2 normal subjects and 19 cancer patients, consisting of 10 different cancers that include breast, prostate, colon, duct, kidney, liver, melanoma, ovarian, and stomach. Total cell-free RNA was isolated from blood plasma with the formalin-fixed, paraffin-embedded (FFPE) RNA purification kit (Norgen Biotek) per the manufacturer's instructions. The RNA-seq library was constructed by the Script-Seq strand-specific total RNA sample preparation kit (Illumina) per the manufacturer's instructions and sequenced by illumina genome analyzer IIx. Reads from 36-bp RNA-seq runs were aligned against human genome hg19 using the Tophat 2.0 algorithm, and RPKM (reads per kilobase of transcript per million mapped reads) values for all transcripts from the University of California, Santa Cruz (UCSC) database were estimated.

Additionally, all 36-bp reads were tested for maximum number of telomeric motif repeats in sense orientation. Only reads containing five and six (of six possible) perfectly matched motif repeats were considered. RPKM values were calculated assuming average cfTERRA length of 200 bp. All genes with at least 10 reads (on average, ~28,000 per sample) were ranked by RPKM values of their most expressed transcript. The cfTERRA ranks were reported along with visual representation of the 1st, 5th, 50th, 95th, and 99th quantiles for gene distribution of RPKM expression values for every sample.

RNA in Vitro Transcription.

The in vitro transcription of TERRA was performed using the MEGAscript T7 kit (Ambion) using linearized plasmids containing 250-bp telomeric repeats with a T7 promoter at either the 3' end for sense TelG RNA or at the 5' end for antisense TelC RNA (a gift from H. C. Riethman, Wistar Institute, Philadelphia). U6-containing RNA was synthesized using the linearized pU6-neo-TET plasmid (Addgene plasmid #51286) as a template. Plasmid templates were removed by Turbo DNase I after the transcription reaction. RNA was gel purified, and 50 ng was used in RNase digestion for Northern blot.

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). Briefly, the blood was placed on top of Lymphoprep in SepMate-50 tubes and centrifuged at 1,200 × g for 10 min. PBMCs from the top layer were harvested and washed twice with PBS. Cells were resuspended in serum-free RPMI-1640 medium supplemented with 1 mM sodium pyruvate, 10 mM Hepes (pH 7.25), 1× Glutamax, and penicillin-streptomycin. For experiments, cells were seeded in 24-well nonadhering plates at a density of 1.5 × 106 cells/mL in each well and stimulated with exosomes or RNA-containing liposomes. These liposomes were prepared by incorporating RNA from in vitro transcription or synthesized oligos (IDT) into Lipofectamine 2000 as previously described (2). After

stimulated for 3 h, PBMCs were collected for RNA isolation by TRIzol reagent as described earlier. The stimulation of IMR90 cells was performed in the same way as that in PBMCs, except that IMR90 cells were seeded in six-well plates at a density of 0.5 × 106 cells per well and cultured overnight in conditional medium with 10% exosome-depleted FBS. RNA oligos used in this study are as follows:

Control oligos: 5'-CACUGACACUGACACUGACACUGACACUGACACUGA-3'

TelG RNA oligos: 5'-UUAGGGUUAGGGUUAGGGUUAGGGUUAGGGUUAGGG-3'

TelC RNA oligos 5'-CCCUAACCCUAACCCUAACCCUAACCCUAACCCUAA-3'

Electron Microscopy.

After sucrose gradient separation of exosomes, fractions were submitted to the Electron Microscopy Resource Laboratory (EMRL) at University of Pennsylvania for electron microscopy imaging. Three microliters of each fraction was applied to lacey carbon films on 400-mesh copper transmission electron microscopy (TEM) grids. Excess liquid was blotted away with filter paper until a thin vicinal film remained. The liquid containing the fractions contents was vitrified in liquid nitrogen and cooled with liquid ethane by plunge freezing. Samples were observed at -180 °C using a Gatan 626 cryo-transfer holder on a FEI Tecnai 12 transmission electron microscope operating at 120 KeV. Images were collected digitally on a Gatan US1000 CCD bottom-mounted camera.

Telomeric RNA FISH on Tissue Sections.

Telomeric RNA FISH on tissue sections (7–12 μm) was prepared as previously described (3). Briefly, fresh frozen sections were fixed in 4% paraformaldehyde (PFA), followed by acetylation. For RNase A treatment, the slides were incubated in PBST with 100 mg/mL RNase A at 37 °C for 45 min and washed three times with PBS. All slides were prehybridized in hybridization buffer (50% formamide, 5× SSC, 5× Denhardts, 25 mg/mL yeast RNA, 0.5 mg/mL salmon sperm DNA) for 1 h at 37 °C and followed by hybridization with probe overnight at 37 °C. The probe used for TERRA detection is a Tamra-(CCCTAA)3 PNA probe (Panagene). After hybridization, slides were washed in the following sequence for 5 min at each step with gentle shaking: 2× SSC, 50% formamide for three times at 39 °C; 2× SSC for three times at 39 °C; 1× SSC once for 10 min at room temperature; and 4× SSC once at room temperature. Cellular nucleus DNA was counterstained with 0.1 g/mL DAPI in 4× SSC and 0.1% Tween-20, washed in 4× SSC, and mounted with mounting media. Slides were visualized by a Nikon E600 Upright microscope (Nikon Instruments) with ImageProPlus software (Media Cybemetrics) and Adobe PhotoShop CS5 for image processing.

RNA Isolation.

Cellular and cell-free RNA was isolated with TRIzol reagent (Invitrogen) per the manufacturer's instructions. For cellular RNA, the isolation was performed as previously described. For cell-free RNA, 50 μL of each culture fraction (cell debris, microvesicles,

or exosomes) was mixed with 450 µL TRIzol, followed by adding 100 µL chloroform. The mix was centrifuged at 12,000 × g for 15 min at 4 °C, and the aqueous phase was collected. RNA was precipitated at −20 °C for at least 1 h by mixing the aqueous phase with an equal volume of isopropanol precipitation and 1.5 µL GlycoBlue (Ambion) and collected by centrifugation at 20,000 × g for 30 min. RNA precipitates were washed with 75% ethanol, air dried, and resuspended in Nuclease-free water. These samples were treated with 1 U DNase I for 30 min at 37 °C, followed by DNase I inactivation in the presence of EDTA at 65 °C for 5 min. RNA concentration was determined by Nanodrop-2000 (Thermo Fisher). The same procedures were used in isolating cell-free RNA from sucrose fractions, RNA ChIP elute, in vitro transcribed RNA, and exosomes of HCT116 cells. For RNase treatment, RNA was incubated with the indicated RNase at 37 °C for 1 h. The RNase mixture used in this study was a combination of 100 ng/µL RNase A, 1 U/µL RNase H, and 1 U/µL RNase T1.

Northern Blot and RNA Detection.

RNA was electrophoresed on an agarose formamide gel and transferred onto a nylon membrane for Northern blotting analysis. The amounts of RNA loaded were normalized based on the purpose of each experiment as indicated in the figure legends. Briefly, each RNA sample was denatured in NorthernMax formaldehyde load dye (Ambion) for 15 min at 65 °C and separated by 1.2% agarose formamide gel in 1× Mops buffer (Ambion) at 5 V/cm. As the size of cfTERRA is ~200 bp, electrophorese was stopped when bromophenol blue dye reached 5 cm of a 9 cm agarose formamide gel. After

electrophorese, RNA samples were transferred to GeneScreen Plus blotting membranes (Perkin-Elmer) with 10× SSC and UV cross-linked onto membrane at 125 mJ in UV Stratalinker 2400 (Stratagene). For RNA detection, the blot was hybridized with a 32P-labeled probe using Church buffer (0.5 N Na-phosphate, pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA) for 18 h at 50 °C. The blot was washed twice in 0.2 N Na-phosphate, 2% SDS, and 1 mM EDTA at room temperature and once in 0.1 N Na-phosphate, 2% SDS, and 1 mM EDTA at 50 °C. Radioactive signals were collected by phosphor-imager (Amersham Biosciences). Images were visualized with a Typhoon 9410 Imager (GE Healthcare). To reprobe the blot, the membrane was stripped in 1% SDS, 0.1× SSC, and 40 mM Tris·HCl (pH 7.5) and hybridized with another probe under the same conditions.

The probes were 5' end-labeled with 32P by T4 polynucleotide kinase (NEB) per the manufacturer's instructions. The probe sequences are as follows:

- TERRA: 5'-TAACCCTAACCCTAACCCTAACCC-3';
- Antisense TERRA: 5'-TTAGGGTTAGGGTTAGGGTTAGGG-3';
- 18S: 5'-CCATCCAATCGGTAGTAGCG-3';
- U6: 5'-GCAGGGGCCATGCTAATCTTCTCTGTATCG -3'
- The alpha satellite probe was a mixture of three oligos:
  - 5'-CATTAAAACAAGACAGAAGCATTCTCAGAAACTCCTTTAGATGTCTGCA-3';
  - 5'-TGGACATTTGGAGCTCTTTTAGGCTATCGGTTGAAAAGGAAGTATCTTCA-3';
  - 5'-TTTCTTTTGATAGTGCAGTTTTGAAACATTCTTTTTAAAAAATCTGCAG-3'.
- The EREB1 probe was a mixture of two oligos:
  - 5'-CGATAAGCTTAAAACATGCGGACCACC-3';
  - 5'-AAGCAGAGTCTGGGAAGACAACCA-3'.

Protein Analysis.

For cellular proteins, cells were lysed in RIPA buffer containing protease inhibitor mixture by rotating at 4 °C for 30 min and centrifuged at 14,000 × g at 4 °C for 15 min. Exosomal proteins were obtained by lysing the membrane with 1% SDS in the presence of the protease inhibitor mixture. The protein concentration was determined by the Bradford assay (BioRad). Total proteins were separated on a 8–16% Tris-Glycine gel (Invitrogen).

For Western blotting, the gel was transferred to a nitrocellulose membrane (BioRad). The membrane was first stained with Ponceau S to reveal the relative loading in each lane and blocked in 5% nonfat dry milk in Tris-buffered saline with Tween-20 (TBST). Then, it was probed with primary antibodies against CD63 (H5C6 for nongylcosylated form), CD63 [MEM-259 (Abcam) for the gylcosylated form], histone H4 (L64C1; Cell Signaling Tech), H3, S6, and TRF2 as indicated. After washing three times with TBST, the blot was incubated with HRP-coupled secondary antibody and developed with Luminata HRP detection reagent (Milipore).

Before submitting for MS, the gel was stained by Silver Stain Plus (BioRad). The samples corresponding to cfTERRA signals were digested in solution with trypsin. The digested protein sample was analyzed by LC-MS/MS on a Q Exactive Plus mass spectrometer. MS/MS spectra generated from the MS were searched against a human database using SEQUEST. The proteins identified by LC-MS/MS were sorted by

normalized spectra count (spectra count/molecular weight) to determine which proteins are unique and abundant as shown in Dataset 1. The top 250 proteins in the LCL exosomes were analyzed by the FunRich program and compared with exosome proteins in the Vesiclepedia database.

RT-PCR Analysis of RNA.

RT-PCR experiments were performed as previously described (3) with some modifications. Briefly, 1 μg RNA was reversed transcribed with random hexamers by Super Script IV First-Strand Synthesis System (Invitrogen) in a 20-μL reaction. Synthesized cDNA was diluted with 80 μL Nuclease-free water and analyzed by real-time PCR with a SYBR green probe with the QuantStudio 6 Flex System (Life Technologies) using the standard curve method. Relative RT-PCR was determined using the ΔCT method relative to the internal control GAPDH.

Primer sequences used for real-time PCR are listed in Table 2.

Statistics.

Statistical analyses were carried out by paired Student t tests unless otherwise stated. P values and significance levels are annotated in the figures and described in the figure legends.

4.3 References

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CHAPTER 5: Final discussion

In the studies of this dissertation, we demonstrated that a cell-free form of TERRA is secreted through exosomes, and represents a telomere-specific molecular pattern (TAMP) from dysfunctional telomeres to activate the innate immune signaling in the recipient cells. This dissertation is the first study that characterized the extrinsic role of TERRA in mediating the crosstalk between telomere dysfunction and inflammation. Here, I will discuss the significance of this study in the context of aging and tumorigenesis, and the therapeutic potentials of cfTERRA-containing exosomes.

5.1 Telomere dysfunction occurs during aging and tumorigenesis

Telomere dysfunction results from telomere shortening, uncapped telomeres, or DNA damage at telomeres, which occur during aging and tumorigenesis (1) (2). Telomere shortening is an inevitable event of aging due to the nature of DNA replication in linear chromosomes (3). Short telomeres lose the ability to bind shelterin complex, which protects the telomeric DNA from being recognized as DNA damage (4). Persistent DNA damage response activates the p53 to induce apoptosis or senescence (5). Loss of functional p53 increases genomic instability as repair of the dysfunctional telomeres results in chromosome fusions by the NHEJ repair machinery, which promotes tumorigenesis (6). Oncogene expression often induces DNA replication stress, which is demonstrated to promote telomere dysfunction in precancerous lesions (7). Detecting dysfunctional telomeres may provide insight into premature aging and tumorigenesis.

5.2 Dysfunctional telomeres cause TERRA deregulation

There is accumulating evidence that TERRA expression is deregulated during telomere dysfunction induced by uncapped telomeres (8), tumorigenesis (9), and viral infection (10). Depletion of TRF2 induces telomere uncapping and dysfunction, which is associated with TERRA induction (8). In Chapter 3, we found the dominant negative form of TRF2 showed a similar effect, suggesting that dysfunctional telomeres promote TERRA transcription. The induction of TERRA transcription could be due to DNA damage response as a result of p53 activation, as we previous found p53 directly bound to subtelomeres to regulate TERRA transcription (11). Other transcription factors, such as HSF1 (12) and NRF1 (13) have been shown to stimulate TERRA transcription in response to variable cellular stress, suggesting TERRA might represent a stress response signal from telomeres. Similar to telomeres, other repetitive regions of genome are transcribed under the cellular stress. For instance, short interspersed nuclear elements (SINEs) are silenced in somatic cells, but activated during murine gamma herpesvirus 68 (MHV68) infection (14). Interestingly, we found herpes simplex virus 1 (HSV-1) infection triggers a profound TERRA induction (10). It seems that these repetitive non-coding RNAs may share some unknown roles during certain cellular stress response.

5.3 TERRA represents a telomere-specific molecular pattern (TAMP)

Considering the dysfunctional telomere-induced TERRA, we hypothesized that TERRA represents a telomere-specific molecular pattern (TAMP) to activate the innate immune signaling. The innate immune system detects endogenous damage and pathogens

initiating self-defense mechanisms (15). Since many of the repetitive non-coding RNAs are considered of viral origin (16), many of them are immunogenic including LINE1 (17), SINEs (14) and human satellite II (HSATII) RNAs (18) when induced during stress. In our studies, cfTERRA-containing exosomes and TERRA-mimic oligonucleotides activate innate immune signaling in PBMCs, which supports our hypothesis. A recent study presented another result suggesting that TERRA induction did not affect gene expression in 2D culture of cancer cell lines, but suppressed innate immune related genes when cultured in 3D (19). Indeed, we did not notice significant changes in cytokines when inducing TERRA in 2D cultures of HCT116 cells (Figure 5.1B), but observed an increase of cytokines when TERRA was induced in 2D culture of normal fibroblast cells (Figure 5.1C). These findings suggest that the immunoregulatory activity of TERRA may depend on the cell-type or cellular context. It is necessary to further test the role of TERRA in regulating immune system *in vivo* in the future.

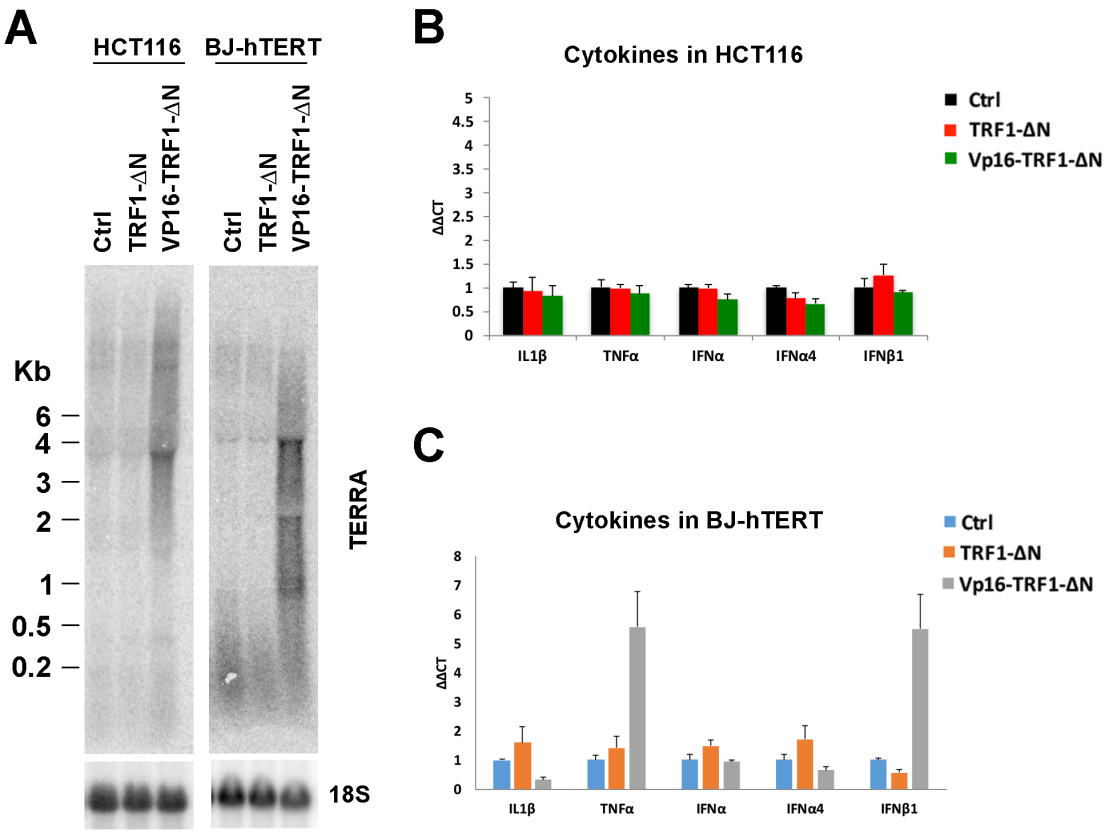

Figure 5.1 Cytokine expression profile in response to TERRA induction in cancerous HCT116 and normal fibroblast BJ-hTERT cell lines. (A) Northern blotting to examine the TERRA induction by Vp16-TRF1-ΔN in HCT116 and BJ-hTERT cell lines. (B) Cytokine expression profile in HCT116 after TERRA induction. (C) Cytokine expression profile in BJ-hTERT after TERRA induction.

The cfTERRA in the exosomes is a unique form of TERRA. Compared to endogenous cellular TERRA, cfTERRA were smaller and more resistant to RNaseA treatment as shown in Figure 2.2B and Figure 5.2. In Figure 5.2, we characterized the total nucleic acids isolated from exosomes, and found that both cfTERRA and telomeric DNA were associated with exosomes. This cell-free telomeric DNA was 200-4,000 nt and sensitive to DNase I treatment, whereas cfTERRA was ~200 nt and only sensitive to DNase I and NaOH treatment. Also, it requires the RNase cocktails, which include RNaseA, RNaseT1 and RNaseH, to completely digest cfTERRA (Figure 4.2), suggesting cfTERRA may be

modified or in a unique secondary structure to preserve the stability during the transfer by exosomes. Determination of the modification and structure of cfTERRA may provide more information on the immunoregulatory activity and biogenesis. In addition, the biogenesis of cfTERRA-containing exosomes may be affected by lysosomal and autophagy pathways. During telomere dysfunction, cfTERRA and telomeric DNA may be excluded from nucleus for degradation in lysosome or autophagosome. As the lysosomal and autophagy pathways cross talk with the endosomal pathway (20), cfTERRA and telomeric DNA may be partially incorporated into exosomes. It would be interesting to test how lysosomal and autophagy pathways affect cfTERRA secretion by exosomes.

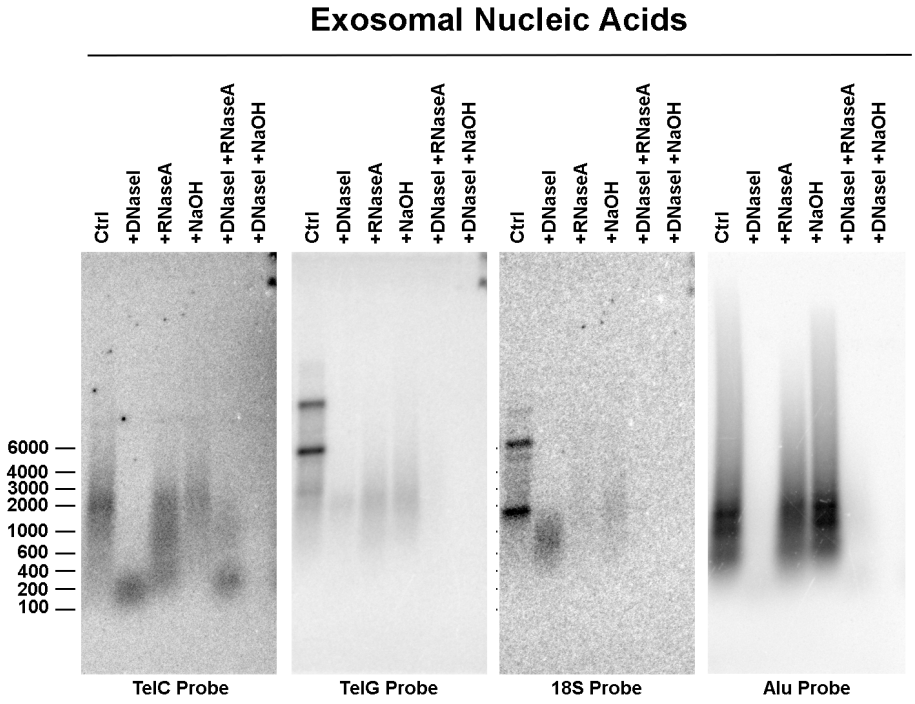

Figure 5.2 Nuclease characterizations of exosomal nucleic acids. The total nucleic acids isolated from exosomes were treated with DNase I, RNase A, NaOH, or their combination. After treatment, the nucleic acids were examined by Northern blotting.

Another future direction is to determine the molecular details of the innate immune signaling pathway in response to cfTERRA-containing exosomes. Since exosomes are endocytosed, TLR7 and TLR8, single-stranded RNA sensors localized in endosome (21), may play a role in detecting cfTERRA delivered by exosomes. TLR7/TLR8 is shown to detect the LINE1 induced during environmental stress and in autoimmune diseases including lupus nephritis and Sjögren's syndrome (22). It would be worthwhile to test whether TLR7/TLR8 knockdown affects the innate immune signaling in response to cfTERRA-containing exosomes, along with other RNA pattern recognition receptors (PRRs) such as TLR3, RIG-I and MDA5 (23). Meanwhile, telomeric DNA oligoes are shown to inhibit TLR9-mediated innate immune signaling (24). The presence of telomeric DNA in exosomes may provide the inhibitory mechanism to limit innate immune response. The fine balance of the immune response is vital to avoid autoimmunity (25). A long noncoding RNA lincRNA-Cox2 is an example, which showed regulatory activity in both activating and inhibiting immune response (26).

5.4 Chronic inflammation promotes on cellular aging and tumorigenesis

Chronic inflammation promotes cellular aging and tumorigenesis (27). The nfkb1 knockout mice develop low-grade chronic inflammation and display the markers of dysfunctional telomeres and accelerated aging (28). As mentioned in Chapter 1, this low-grade chronic inflammation gives rise to telomere attrition associated with diabetes (29) and chronic kidney diseases (30), as examples of "inflammaging" diseases (31). The effect of inflammation on tumorigenesis is more complicated (32). Acute inflammation is tumor suppressive, as the inflammatory cytokines recruit immune cells to eliminate

precancerous lesions (33). For instance, GM-CSF recruits DC cells to enhance its anti-tumor activity by presenting tumor antigens to T cells (34). However, chronic inflammation creates a tumor microenvironment that is favorable for tumor progression. Constant exposure to GM-CSF could switch it from tumor suppressive to stimulatory, as it promotes metastasis of breast cancer (35). IL6 is another cytokine that promotes tumor growth by activating the pro-survival JAK/STAT signaling pathway (36). Since cfTERRA-containing exosomes are able to activate inflammatory cytokine signaling, prolonged exposure would promote cellular aging and tumor progression. It is intriguing to test this hypothesis using *in vivo* studies in the future.

5.5 The therapeutic potentials of cfTERRA-containing exosomes

Because exosome analysis is relatively non-invasive, cfTERRA-containing exosomes may serve as a useful biomarker for the detection of telomere dysfunction, which often occurs in precancerous lesions or aging-associated inflammatory disease. Due to the limited samples in our cell-free RNA-seq, we were not able to draw a statistical conclusion of cfTERRA-containing exosomes in association with a particular cancer type at this point. Future cohort studies of clinical samples would be informative to establish the correlation. Notably, Umezu T. et al. confirms our observation of cfTERRA, and shows that cfTERRA levels are correlated with cellular TERRA expression in myelodysplastic syndrome (37). Besides, there are several vaccines have been developed by re-engineering exosomes due to the immunogenic property of exosomes (38). As cfTERRA is highly stable and immunostimulatory, it would be interesting to learn whether cfTERRA could be applied to the exosomes-based vaccine development.

Together, this dissertation described a previously unrecognized connection between telomere dysfunction and inflammation through cfTERRA-containing exosomes. We characterized the molecular details of the cfTERRA-containing exosomes, and found cfTERRA was a shorter form (~200 nt) of cellular TERRA, and mostly enriched in the CD81-positive exosome vesicles. These exosomes contained the nucleoprotein complex formed by cfTERRA, telomeric DNA and histones. Increasing levels of cfTERRA and DNA damage-induced histone γH2AX were incorporated into the exosomes during telomere dysfunction. These elevated cfTERRA levels in exosomes activated a robust transcription of inflammatory cytokines in PBMCs, suggesting cfTERRA may represent a telomere-specific molecular pattern (TAMP) from dysfunctional telomeres to activate the extracellular inflammatory signaling. Since cfTERRA is detectable in human blood plasma, we also proposed that it may provide an informative biomarker for telomere dysfunction in precancerous lesions or aging-associated inflammatory disease.

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