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Analysis of cytoplasmic extrachromosomal telomeric DNA in mammalian cells

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Contributions:

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Summary

Chromosomal DNA and DNA in organelles like mitochondria and chloroplasts are the well-known parts of the genome of a eukaryotic cell and generally partition symmetrically in divisions. However, since the early 1960s, it is known that additional DNA entities exist in eukaryotic cells, so-called extrachromosomal DNA (ecDNA). EcDNA can be classified into two classes, i.e. exogenous and endogenous ecDNAs. Exogenous ecDNA (e.g. viral DNA, transfected plasmid DNA) needs to enter into a cell, whereas endogenous ecDNA arises from the chromosomal DNA of a cell. Endogenous ecDNA can be grouped into four types: 1) chromosomal fragments, often a result of mitotic breaks of chromosomes; 2) double minutes, circular DNA molecules containing genes such as oncogenes and drug resistance genes; 3) microDNA, small circular DNA elements (a few hundred base pairs long) containing unique sequences and 4) ecDNA with tandem repetitive sequences such as satellite DNA, ribosomal DNA, and telomeric DNA (tDNA). Endogenous ecDNA occurs ubiquitously in every tested eukaryotic organism from budding yeast to human. However, not much is known about its fate and cellular localization in mammalian cells. Recently, our group dissected how transfected plasmid DNA, which is circular DNA of bacterial origin, is handled by mammalian cells. We showed that it accumulates in the cytoplasm predominantly in one focus surrounded by a special double membrane. Remarkably, up to now at every tested time point after lipofection of plasmid DNA into cells, that DNA was always enclosed by a membrane reminiscent of the nuclear envelope. Further, such DNA cluster maintained, in contrast to chromosomes, membrane association during mitosis and partitioned asymmetrically during mitosis. Therefore, it is interesting to study if endogenous ecDNA also accumulates in the cytoplasm and if it is enclosed by membrane like exogenous plasmid DNA.

Using fluorescence *in situ* hybridization we found that tDNA also exists in the cytoplasm of two different human cancer cell lines, one with and one without telomerase (HeLa, U2OS respectively). The cytoplasmic tDNA occurred in both cell lines in two patterns: 1) one or multiple telomeric FISH signals within a bigger Hoechst-positive structure, similar to a so called “micronucleus” (micronucleus like structure), containing chromosomal fragments (termed mnls-tDNA), 2) any cytoplasmic telomeric FISH signal that does not belong to the first pattern (termed cyto-tDNA). As U2OS cells with cyto-tDNA are about 10 times more frequent in the population (40%) than cells with mnls-tDNA (4%), the quantitative analysis

refers only to cyto-tDNA.

Remarkably this analysis reveals clear similarities to the handling of plasmid DNA, suggesting that both DNA types are handled by a shared machinery. Firstly, about half of the analyzed cyto-tDNA foci co-localized with membrane proteins, which were also reported to be associated with plasmid foci and thus also present at the nuclear envelope. Secondly, a small fraction of the cyto-tDNA colocalized with plasmid DNA in the apparently same cytoplasmic membrane compartment. Both points indicate the presence of a cellular machinery that membrane encloses cytoplasmic DNA irrespective of its origin. Thirdly, like lipofected plasmid DNA, cyto-tDNA was predominantly present in one focus in cells, suggesting that tDNA can be clustered in the cytoplasm. Fourthly, not considering the nuclear pool of tDNA, cyto-tDNA distributed, like plasmid DNA, asymmetrically in U2OS telophase-cells. How cyto-tDNA comes to be present in the cytoplasm and the reason for its presence there is unknown. But, assuming the absence of further “generation” of cyto-tDNA, the finding of the biased partitioning of cyto-tDNA implicates that U2OS cells restrict over time cyto-tDNA to a subpopulation of cells.

To probe for the function of the membrane enclosure, we challenged the function of LEM (LAP2, Emerin, MAN1)-domain proteins. This is a group of transmembrane proteins predominantly present in the inner nuclear membrane and associated with both the cytoplasmic plasmid DNA and cyto-tDNA. For this, we attempted to compete with the function of LEM-domain proteins by overexpression of a soluble LEM-domain. For plasmid DNA, we found that overexpression of that LEM-domain resulted over time in more foci in single cells and that at every assessed time point an increased proportion of small plasmid foci in comparison to the fraction of big foci and in comparison to the measures in the control condition was present. Thus, we propose that the function of LEM-domain proteins around cytoplasmic plasmid DNA is to support the formation and maintenance of big DNA foci. Suggestive of a similar function for LEM-domain proteins around cyto-tDNAs, we found that the number of cyto-tDNA was higher in the perturbation condition. Thus, LEM-domain proteins seem to be relevant for the maintained separation of endogenous and exogenous ecDNA from the nucleus and its chromosomes.

However there were also striking differences compared to the handling of plasmid DNA, suggesting the presence of cellular machinery that differentiates between endogenous and

exogenous DNA: Firstly, the U2OS population falls into different sub-groups of cells as the presence of Lap2β an inner nuclear membrane LEM-domain protein, at cyto-tDNA foci reports. In about one third of the cell population Lap2β was absent from all of the cyto-tDNA foci in a cell, even that of very big foci. In about one third of the cell population, it was present on all cyto-tDNA foci of a cell, even at those of very small sizes. And in one third of the population a mixed situation was present. The frequency of these three patterns suggests that asymmetric divisions occur in the U2OS population. Secondly, at about half of all cyto-tDNA foci a plasmid-like membrane was not detectable, which even might suggest the complete absence of membrane around such cyto-tDNA.

Taken together, this study indicates that there is on the one hand a cellular machinery that membrane encloses possibly any type of cytosolic DNA with a nuclear envelope-like membrane and on the other hand a machinery that distinguishes between endogenous and exogenous DNA in the cytoplasm. Further work will be needed to decipher the link between these two machineries, to identify the mechanism of membrane enclosure of cytoplasmic DNA especially in the light of the formation of a nuclear envelope, and to identify the mechanism and its components for the differentiation between endogenous and exogenous ecDNA.

Zusammenfassung

Chromosomale DNA und DNA in Organellen wie Mitochondrien und Chloroplasten sind gut bekannte Teile des Genomes einer eukaryontischen Zelle und werden im generellen symmetrisch während einer Zellteilung verteilt. Seit den frühen 1960 Jahren ist jedoch bekannt, dass in eukaryontischen Zellen zusätzliche DNA Einheiten existieren, sogenannte extrachromosomale DNA (ecDNA). Es werden zwei Arten von ecDNA unterschieden, nämlich exogene und endogene ecDNA. Exogene DNA (z.B. virale DNA, transfizierte plasmid DNA) muss in eine Zelle eindringen, wogegen endogene DNA aus der chromosomalen DNA einer Zelle entsteht. Endogene DNA wiederum kann in vier Typen unterteilt werden: 1) chromosomale Fragmente, die oft das Resultat von Chomosomenbrüchen sind; 2) Double Minutes, zirkuläre DNA-Moleküle, die z. B. Onkogene und Arzneimittelresistenzgene beinhalten; 3) microDNA, kleine zirkuläre DNA-Elemente (einige hundert Basenpaare lang), die aus einzigartigen Sequenzen bestehen und 4) ecDNA mit tandem repetitiven Sequenzen wie Satelliten DNA, ribosomale DNA und telomerische DNA (tDNA). Endogene ecDNA ist ubiquitär in jedem getesteten eukaryotischen Organismus, von der Bäkerhefe bis zum Menschen, gefunden worden. Es ist jedoch nicht viel über deren Verbleib und zelluläre Lokalisierung in Säugetierzellen bekannt. Vor kurzem arbeitete unsere Gruppe heraus, wie transfizierte Plasmid-DNA, welche zirkuläre DNA bakteriellen Ursprungs ist, von Säugetierzellen behandelt wird. Wir zeigten, das sie im Zytoplasma in überwiegend einem Fokus, der von eine besonderen Doppelmembran umgeben ist, akkumuliert. Es ist bemerkenswert, dass bis jetzt diese DNA zu jedem analysierten Zeitpunkt nach Lipofektion in eine Zelle immer mit einer Membran umgeben war. Solche DNA-Cluster waren im Gegensatz zu Chromosomen auch während der Mitose Membran-assoziiert und verteilten sich asymmetrisch während der Mitose. Daher ist es interessant zu untersuchen, ob endogene ecDNA auch im Zytoplasma akkumuliert und ob sie auch von Membran umgeben ist wie exogene Plasmid-DNA.

Wir fanden mittels Fluoreszenz in situ Hybridisierung (FISH), dass tDNA auch im Zytoplasma von zwei unterschiedlichen Krebszellinien existiert, eine mit und eine ohne Telomerase (HeLa, U2OS, respektive). Die zytoplasmatische tDNA kam in beiden Zelllinien in zwei Mustern vor: 1) ein oder mehrere telomerische FISH-Signale innerhalb einer grösseren Hoechst-positiven Struktur, mit Aehnlichkeit zu einem sogenannten „Mikronukleus“ (mikronucleus like structure), der chromosomale Fragmente enthält (mnls-tDNA genannt), 2)

jegliches zytoplasmatische telomerische FISH-Signal, das nicht zum ersten Muster gehört (cyto-tDNA genannt). Da U2OS Zellen mit cyto-tDNA ungefähr 10 mal häufiger in der Population (40%) waren als Zellen mit mnls-tDNA (4%), wurde die quantitative Analyse nur für cyto-tDNA durchgeführt.

Bemerkenswerterweise offenbart diese Analyse klare Aehnlichkeiten zu der Handhabung von Plasmid-DNA, was daraufhin deuten könnte, dass die beiden DNA-Arten von einer gemeinsamen Maschinerie gehandhabt werden. Erstens, ungefähr die Hälfte der analysierten cyto-tDNA Fokusse kolokalisierte mit Membranproteinen, die auch, wie publiziert ist, mit Plasmid-DNA assoziierten, und die daher auch an der Kernmembran vorkommen. Zweitens, ein kleiner Teil der cyto-tDNA kolokalisierte mit Plasmid-DNA in scheinbar dem gleichen zytoplasmatischen Membran-Kompartiment. Beide Punkte deuten darauf hin, dass es eine zelluläre Maschinerie gibt, die zytoplasmatische DNA unabhängig von ihrem Ursprung mit Membran umgibt. Drittens, cyto-tDNA war, wie lipofizierte Plasmid-DNA, überwiegend in einem einzigen Fokus in Zellen, was suggeriert, dass tDNA im Zytoplasma geclustert wird. Viertens, wenn man den nuklearen Teil der tDNA beiseite lässt, verteilte sich cyto-tDNA in Telophase U2OS-Zellen asymmetrisch. Wie cyto-tDNA in das Zytoplasma kommt und der Grund für ihre dortige Anwesenheit ist unbekannt. Aber, wenn man eine weitere Bildung von cyto-tDNA ausschliesst, impliziert das Resultat der unausgewogenen Verteilung der cyto-tDNA, dass über die Zeit cyto-tDNA auf eine Subpopulation der Zellen beschränkt wird.

Um die Funktion der Membranumhüllung heraus zu finden, testeten wir die Funktion der LEM(LAP2, Emerin, MAN1)-Domänen-Proteine. Dies ist eine Gruppe von Transmembranproteinen, die überwiegend in der inneren Kernmembran vorkommt und mit sowohl mit der zytoplasmatischen Plasmid-DNA als auch mit der cyto-tDNA assoziiert war. Dafür versuchten wir durch Ueberexpression einer löslichen LEM-Domäne mit der Funktion der LEM-Domänen-Proteine zu kompetitieren. Für Plasmid-DNA fanden wir, dass die Ueberexpression der LEM-Domäne mit der Zeit in mehr Fokussen resultierte und dass an jedem analysierten Zeitpunkt mehr kleine Fokusse im Vergleich zu den grossen Fokussen und im Vergleich zu den Resultaten in der Störsituation vorhanden waren. Wir schlagen daher vor, dass die Bildung und Aufrechterhaltung von grossen Plasmid-Fokussen die Funktion der LEM-Domänen-Proteine, die die zytoplasmatische Plasmid-DNA umgeben, ist. Eine ähnliche Funktion wird für LEM-Domänen-Proteine, die mit der cyto-tDNA assoziiert sind, suggeriert, da in der Störsituation mehr cyto-tDNA Fokusse gefunden worden waren. Daher

scheinen LEM-Domänen-Proteine eine wichtige Rolle bei der Aufrechterhaltung der Trennung von endogener und exogener ecDNA von dem Kern und seinen Chromosomen zu spielen.

Es gab jedoch auch gravierende Unterschiede zu der Handhabung von Plasmid-DNA, was auf die Anwesenheit einer zellulären Maschinerie hindeutet, die zwischen endogener und exogener DNA differenziert: Erstens, die U2OS-Population fällt in Untergruppen, wie von der Anwesenheit von Lap2β LEM-Domänen-Protein der inneren Kernmembran, an cyto-tDNA Fokussen reflektiert wird. In ungefähr einem Drittel der Zellpopulation war es an keiner cyto-tDNA einer einzelnen Zelle, es war sogar nicht an sehr grossen Fokussen. In ungefähr einem Drittel der Zellpopulation war es an jeder cyto-tDNA einer Zelle, sogar an sehr kleinen Fokussen. Und in einem Drittel der Zellpopulation lag eine gemischte Situation vor. Die Häufigkeit dieser drei Muster suggeriert, dass asymmetrische Zellteilungen in der U2OS-Population vorkommen. Zweitens, in ungefähr der Hälfte der cyto-tDNA war eine Plasmid-ähnliche Membran nicht detektierbar, was sogar suggerieren könnte, dass gar keine Membran diese cyto-tDNA umgibt.

Zusammengenommen, diese Studie deutet an, dass es einerseits eine zelluläre Maschinerie gibt, die möglicherweise jede DNA-Art mit einer Membran umschliesst, die ähnlich ist wie die Kernmembran, und dass es andererseits eine Maschinerie gibt, die zwischen endogener und exogener DNA im Zytoplasma differenziert. Weitere Arbeit wird nötig sein, um die Verbindung dieser beiden Maschinerien zu entziffern, um den Mechanismus der Membranumhüllung von zytoplasmatischer DNA, besonders im Licht der Bildung der Kernmembran, zu identifizieren, und um den Mechanismus und seine Komponenten zu identifizieren, der die Unterscheidung von endogener und exogener ecDNA etabliert.

1 Introduction

The majority of the eukaryotic genome in a cell is located on the chromosomes, which localize in the nucleus, and in organelles such as mitochondria and chloroplasts. However, it has been known since the early 1960s that additional DNA entities exist within eukaryotic cells, so-called extrachromosomal DNAs (ecDNAs). The definition of ecDNA may vary among different publications but here we focus on the fragments of DNA without centromeres. The centromere is a region of the chromosome at which two sister chromosomal chromatids connect via the kinetochore in order to segregate equally into the two daughter cells.

EcDNA can be classified into two types: exogenous and endogenous ecDNAs, depending on their origin. While exogenous ecDNA enters the cell from extracellular sources, endogenous ecDNA originates from the cells' chromosomal DNA. The following sections summarize these two types of ecDNAs, focusing on their cellular localisation, formation, localization and possible functions as well as on how the cell handles them.

1.1. Existence, formation and cellular localization of ecDNAs

1.1.1. Exogenous ecDNAs

Exogenous DNA can originate from viruses, bacteria or from experimental procedures such as transfection.

1.1.1.1. EcDNA from viruses

Viral DNA is an example of ecDNA. After penetration into the cells' cytoplasm of mammalian cells, the genome of most viruses is transported to the nucleus. Therein, viruses can take the advantage of the hosts' replication and transcription machineries for their own replication as well as for their transmission to subsequent generations of host cells. To access the nucleus, two strategies can be employed. In the majority of cases, viruses deliver their genome into the host nucleus through the nuclear pore complexes (NPCs) (see reviews (Whittaker et al. 2000; Smith & Helenius 2004)). Some viruses such as onco-retroviruses and papillomaviruses use another strategy to access the nucleus. These viruses wait in the

cytosol until the host nuclear membrane breaks down during mitosis (see reviews (Smith & Helenius 2004; Whittaker et al. 2000; Flatt & Greber 2015)). Subsequently, viral genome may integrate into or tether onto the host chromosomes. Viral proteins mediating the tethering of viral DNA to the host chromosomes seem to vary among viruses. For example, Epstein-Bar nuclear antigen-1 protein of the Epstein-Barr virus binds the latent viral origin of replication and the chromosomal EBP2 protein; the E2 protein of human papilloma virus or the bovine papilloma virus binds to the chromosomal Brd4 protein (Poddar et al. 2009).

Interestingly, failure of delivery through nuclear pore complexes can lead to an accumulation of capsid-free DNA molecules in the cytoplasm which might require defense responses of the host cell (discussed in the section 1.3.2) (Wang et al. 2013; Flatt & Greber 2015).

1.1.1.2. EcDNAs from bacteria

In some cases, bacterial pathogens can also invade human cells (see review (Ribet & Cossart 2015). For example, macrophages or M cells of the intestinal Peyer's patches function as professional phagocytes that internalize foreign particles (see review (Ribet & Cossart 2015). One method by which the host cell recognizes bacterial DNA in the cytoplasm is illustrated for *Mycobacterium tuberculosis*. The DNA from *M. tuberculosis* can be exposed to the ubiquitin-mediated autophagy pathway early after phagocytosis. The DNA exposure is due to permeabilization of the phagosomal membrane which is mediated by the bacterial ESX-1 (6-kDa early secretory antigenic target (ESAT-6) secretion system 1) protein secretion system (Watson et al. 2012).

1.1.1.3. EcDNAs from experimental sources

Foreign DNA can be artificially introduced into mammalian cells through different transfection methods such as lipofection (forming a DNA-cationic lipid complex), electroporation (via holes generated in the plasma membrane by electrical pulses) or microinjection (direct delivery of DNA into cells through a micropipette). Strikingly, it was found that plasmid DNA remains predominantly in the cytoplasm of the cell and only a small portion enters the nucleus, regardless of the transfection method (Wang et al. 2016). Nuclear plasmids seem to not be stable and can be extruded to the cytoplasm (Lechardeur et al. 1999; Shimizu et al. 2005; Denoth-Lippuner 2014).

1.1.2. Endogenous ecDNAs

Endogenous ecDNA is excised from chromosomal DNA. Based on their sequences and characteristics, endogenous ecDNAs can be categorized into four types; 1) Non-centromeric chromosomal fragments in micronuclei; 2) double minutes (DMs), which are circular DNAs found in some tumour cells containing oncogenes or drug resistance genes; 3) microDNAs, small circular ecDNA molecules harbouring unique non-repetitive genomic sequences; 4) tandem repetitive sequence-containing ecDNAs, which originate from chromosomal repetitive regions such as telomeric DNA (tDNA), ribosomal DNA (rDNA) and satellite DNA.

1.1.2.1. Non-centromeric chromosomal fragments in micronuclei

Non-centromeric chromosomal fragments in micronuclei are also a type of endogenous ecDNA. They initially result from chromosome breaks during an abnormal mitosis (Fenech et al. 2011). Towards telophase during mitosis these chromosomal fragments are surrounded by a nuclear envelope-like membrane to form micronuclei. These micronuclei are reported to contain very low signals of RNA polymerase II and NPC staining at the non-centromeric chromosomal fragment, suggesting inactive transcription and importation/ exportation to the cytoplasm (Hoffelder et al. 2004; Terradas et al. 2012; Crasta et al. 2012). The characteristics of the NPC in the non-centromeric broken chromosome micronuclei appear to be similar to structures that contain transfected non-centromeric plasmids (Wang Thesis 2015). Conversely, NPCs are found more frequently in membranes of micronuclei containing an entire lagging chromosome (Terradas et al. 2010; Hatch et al. 2013). All of this evidence suggests that there is an association of the NPC and centromeres in micronuclei.

In regards to formation mechanisms, non-centromeric chromosomal fragment containing micronuclei are often formed due to nuclear bridges at anaphase. Nuclear bridges start with DNA double strand breaks (DSBs) and are followed by either misrepair or non-homologous end joining repair that leads to fusion of chromosome ends, and finally gives rise to dicentric chromatids (see review (Terradas et al. 2010)). During the next anaphase, the dicentric chromatid is pulled to the two poles of the mitotic spindle, forming a nuclear bridge. Broken bridges lead to the formation of micronuclei (see review (Terradas et al. 2010). Detailed characterization of the presence of telomeric and centromeric DNA in micronuclei of lymphocytes has shown that the proportion of micronuclei with either a telomere only

label or both centromere and telomere labels was about 62% and 22% of micronuclei, respectively (Lindberg et al. 2007). This indicates that the formation of micronuclei containing a chromosomal fragment occurs more frequently than with an entire chromosome.

1.1.2.2. Double minutes (DMs)

DMs were first detected under the microscope on metaphase chromosome spreads stained with 4’, 6-diamidino-2-phenylindole (DAPI) or Giemsa. They are described as small, spherical, paired DNA molecules lacking centromeres and telomeres (see reviews (Albertson 2006; Shimizu 2009)). DMs can replicate autonomously and are found occasionally in hematopoietic neoplasia and often in human solid tumours, but not in normal cells (see reviews (Gebhart 2005; Shimizu 2009; Shimizu 2011). Oncogenes and drug resistance genes, such as the cMYC oncogene, epidermal growth factor receptor gene and dihydrofolate reductase are observed on DMs. In addition, the genes on DMs are transcribed and probably related to the cancer cell phenotype (Utani et al. 2007).

Mechanisms of DM formation are still not known. However, some models have been proposed (see review (Kuttler & Mai 2007). One such model suggested that small circular DNAs are excised and subsequently either replicated or fused with each other to form large circular DNA (Schimke et al. 1986; Hamlin & Ma 1990). Another model suggests that chromatin fragments from abnormal mitosis are partially replicated and prematurely condensed in S-phase during the subsequent cell cycle. The following DNA break/recombination leads to extrachromosomal entities, which can intranuclear or extranuclear. This model may account for the special structure of DMs, meaning paired minute chromatin bodies (Sen et al. 1989). DMs were found to localize to the nuclear periphery in G1 and appear to move to the internal area of the nucleus within S phase in a COLO 320DM-GFP clone as shown by Shimizu and the colleagues (Itoh & Shimizu 1998; Shimizu 2011). During mitosis, some DMs can hitchhike to the daughter cells on chromosomes. One putative mechanism proposed for this hitchhiking is via unknown molecules of the nucleolus that mediate the binding of the DMs to the chromosome. This hypothesis was based on the observation that DMs are observed in close vicinity with nucleoli in different phases of mitosis (Levan & Levan 1978). Some of the nuclear DMs can be extruded to the cytoplasm after mitosis. Under physiological conditions 10% of cells have DMs in the cytoplasm (Tanaka & Shimizu 2000).

1.1.2.3. MicroDNAs

The most well known example of ecDNA containing unique sequences is microDNAs. MicroDNA was first described by Shibata in 2012 using a multi-step method including displacement amplification with random primers together with rolling circle amplification, cloning and high-throughput sequencing. MicroDNAs are the shortest type of ecDNA, they are about 100–400 bp long, and can exist in double-stranded or single-stranded forms. MicroDNAs contain unique non-repetitive genomic sequences, preferentially in 5'-untranslated regions of genes, exons and in high GC content regions with very short direct repeats (2- to 15-bp) flanking the sequences. It has been proposed that these repeats circularize to form microDNAs. In total, microDNAs in normal tissue comprise 0.1 to 0.2% of chromosomal DNA by weight (Shibata et al. 2012). These microDNAs have been found across different normal tissues types including sperm from adult mice as well as human cancer cell lines such as HeLa S3, U937 and the chicken cell line DT40 (Dillon et al. 2015; Shibata et al. 2012). MicroDNAs are formed either from DNA break repairs during the transcription process or mismatch repairs of replication slippage during DNA replication (Dillon et al. 2015).

1.1.2.4. Repetitive sequence-containing ecDNAs

Repetitive sequence-containing ecDNAs have been found in a variety of eukaryotes by different methodologies (see following). They range in length from several hundred base pairs to tens of kilobase pairs, and are between 0.1 and 2 μm in diameter (see reviews (Gaubatz 1990; Cohen & Segal 2009). Repetitive sequence-containing ecDNAs were primarily discovered by purifying low molecular weight DNA with sedimentation. Sequences of the ecDNAs was detected by cloning using different restriction enzymes for sequence enrichment coupled with probe hybridization with certain repetitive chromosomal sequences or sequencing (Sunnerhagen et al. 1989; Jones & Potter 1985; Flores & Sunnerhagen 1988). Circular ecDNAs were also separated from chromosomal DNA by 2D-gel and repetitive sequences detected by Southern blotting (Cohen et al. 1997; Cohen et al. 2003; Regev et al. 1998). With these approaches, different ecDNAs containing distinct repetitive sequences were found in the same cell type. For example, 5S ribosomal DNA (rDNA), 28S rDNA, 240-bp rDNA spacer, satellite (359-bp satellite), histone H3 coding sequence, heterochromatic tandem repeat in were found in *Drosophila* embryos (Cohen et al. 2003) and B1, L1, B2, IAP, SAT satellites in mouse heart cells (Flores et al. 1988). Homologous recombination

seems to be the most favoured mechanism for the formation of this ecDNA. Below ecDNAs derived from telomeric DNA (tDNA) is introduced as one example.

EcDNAs from telomeric DNA

Telomeres are nucleoprotein complexes capping the ends of eukaryotic chromosomes. The 2-30 kilobase pairs of telomeric DNA in vertebrates comprise tandem arrays of hexa-nucleotides of the TTAGGG sequence with an overhang of 100-200 nucleotide long single stranded DNA of TTTAGGG repeats (the so-called G-strand, the complementary strand is called the C-strand) (Moyzis et al. 1988; Griffith et al. 1999: Moyzis et al. 1988; Griffith et al. 1999). The shelterin complex which is comprised of the proteins TRF1, TRF2, TIN2, Rap1, TPP1, and POT1, is a specific binding protein complex for telomeric DNA (see review (Lange 2005). Three shelterin subunits, TRF1, TRF2, and POT1 directly recognize TTAGGG repeats. They are interconnected by three additional shelterin proteins, TIN2, TPP1, and Rap1 (Lange 2005). One possible way that the shelterin complex protects chromosomal termini is by generating a stable secondary structure called a telomere loop (t-loop). The model for the t-loop structure states that the 3′ overhang invades the duplex-repeat array forming a displacement (D-loop) (de Lange 2004).

In most normal human cells telomeres are shortened with each DNA replication cycle due to the end replication problem. When their length is critically short, cells stop dividing, entering a crisis state that is characterized by genomic instability and massive cell death (Stewart & Weinberg 2002). Stem cells and cancer cells bypass telomere shortening by maintaining the telomere length through telomerase or collective mechanisms known as alternative lengthening telomere (ALT) (Draskovic & Vallejo 2013; Conomos et al. 2013). In the germline, many immortalized cells and about 85% of human cancers, telomere length is prolonged by telomerase which uses an RNA subunit as a template for amplifying telomeres using the reverse transcriptase catalytic subunit (Martínez & Blasco 2011). Some immortalized human cell lines and nearly 15% of human tumours maintain their telomeres in the absence of any detectable telomerase activity by collective ALT mechanisms which are believed to be strongly based on homologous recombination (Navrátilová et al. 2008; Bryan et al. 1997; Draskovic & Vallejo 2013). Dunham and colleagues provided one line of evidence for this. A tagged sequence within the telomeres (containing the neomycin

resistance gene) that initially presented in either two or three telomeres in two different clones of GM847, an ALT cell line (at the population doubling (PD) 23) spread to additional telomeres at PD 63. In contrast, the same tagged sequence within a subtelomeric area in other GM847 clones did not vary with increasing PD (Dunham et al. 2000). Besides having negative telomerase activity, an ALT cell also has hallmarks such as: 1) pronounced heterogeneous telomere length, 2) association of multiple telomeres in promyelocytic leukaemia (PML) nuclear bodies, 3) an abundance of extra-chromosomal telomeric DNA (ec-tDNA), 4) elevated frequency of telomeric sister chromatid exchanges (Conomos et al. 2013; Draskovic & Vallejo 2013).

Abundant amounts of ec-tDNA is one of the hallmarks of ALT cells (Tokutake et al. 1998). Nonetheless, ec-tDNA is occasionally also found in non-ALT cancer cells such as HeLa S3 (Wang 2004), telomerase positive HeLa hTR, HT1080 hTR clones (they contain long telomeres which are induced by overexpression of human telomerase RNA component hTR (Pickett 2009); Chinese Hamster (Regev 1998); and normal human fibroblasts MJ90 cells (Vidacek 2010). Hence, ec-tDNA probably exists in all cell types.

The cellular localisation of ecDNA in general and ec-tDNA specifically, is not clearly known. It is assumed that these DNAs exist primarily in the nucleus because they are formed therein. Some publications report both the nucleus and the cytoplasm as possible localisations of ecDNA. Non-identified sequence ecDNA has been found in the cytoplasmic fraction of HeLa cells treated with cycloheximide or cultured in saturation condition (Smith & Vinograd 1972). EcDNA containing EcoRI restriction enzyme sites was found in the nucleus of *Drosophila melanogaster* cells (Stanfield & Helinski 1976). Ec-tDNA seems to be correlated with ALT-associated PML bodies (APBs) of ALT cells which contain PML protein in addition to many others (Fasching et al. 2007; Nabetani et al. 2004; Yeager et al. 1999). Using a combination of metaphase spread together with immunofluorescence and fluorescence *in situ* hybridization (IF-FISH), Nabetani and colleagues revealed that ec-tDNA colocalises with PML protein adjacent to the chromosomes (Nabetani et al. 2004). Linear ec-tDNA molecules were found in purified APBs from ALT cells by 2D gel electrophoresis combined with Southern blotting (Fasching et al. 2007). Ec-tDNAs were also shown to localize in the cytoplasm of the KMST-6 human cell line by FISH (Tokutake et al. 1998) and by fractionation coupled with gel electrophoresis and Southern blotting in SUSM-1 and KMST-6 cell lines (Ogino et al. 1998).

Yet no quantification of ecDNA and ec-tDNA at their respective localisations was provided at the single cell level.

Ec-tDNAs exist in a variety of forms: double stranded DNA and single stranded DNA with a small double-stranded section (Nabetani & Ishikawa 2009; Henson et al. 2002); as well as in different topologies: circular or linear. The circular form was confirmed by electron microscopy (Cesare & Griffith 2004), rolling circle amplification (Henson et al. 2009; Vidacek et al. 2010; Zellinger et al. 2007) and by 2D gel (Wang et al. 2004). Linear ec-tDNA was also observed by 2D gel (Fasching et al. 2007) and by conventional electrophoresis on different concentrations of neutral agarose gels, respectively (Ogino et al. 1998).

Ec-tDNA seems to be formed only within the telomeric region because only telomeric sequence, not subtelomeric sequence has been detected on ec-tDNA in human and rodent cells by 2D gels hybridized directly with subtelomeric and telomeric probes (Wang et al. 2004). The telomeric DNA signals of ec-tDNA were not changed when subjected to certain restriction enzymes that subtelomeric sequences are sensitive to (Regev et al. 1998). Despite these findings, the mechanism of ec-tDNA formation in mammalian cells still remains elusive. It has been suggested that ec-tDNA is the result of a resolution of a Holiday junction at a t-loop structure (Wang et al. 2004). A Holliday junction can be formed at t-loop structures by extending the 5’ end of the telomere with a D-loop by base pairing. Indeed, the circular ec-tDNA in ALT cells is of similar size to the t-loop region (Cesare & Griffith 2004; Wang et al. 2004). The second possibility for ec-tDNA formation is due to recombination within the telomeric array of a chromosome due to a double-stranded DNA break. This mechanism is also proposed to happen to rDNA in yeast, leading to the formation of extrachromosomal rDNA circles (Takeuchi et al. 2003). Furthermore, high abundance of hybrid telomeric repeat-containing RNA (TERRA) and telomeric DNA at telomeres in ALT cells may also trigger homologous recombination repair pathways, leading to the generation of circular ec-tDNA with C rich sequences (C-circles) (Arora et al. 2014).

1.2. Biological significance of ecDNAs

As described above, various types of ecDNAs exist ubiquitously between different organisms and even within one cell. This raises the question of what the biological significance of ecDNAs is.

1.2.1. Shortening cell life span

Circular ecDNAs derived from rDNA (ERCs) in *Saccharomyces cerevisiae* are aging factors and accumulate in mother cells (Sinclair & Guarente 1997; Denoth-Lippuner 2014). How the aging effect is achieved remains unknown. Some models have been proposed suggesting that the abundance of ERCs in the mother cells might: 1) titrate away binding proteins (Sinclair & Guarente 1997); 2) induce rDNA instability (Ganley et al. 2009); and 3) affect nuclear organization due to their binding with the NPC (Denoth-Lippuner et al. 2014). In agreement with this, the cell cycle length of HeLa cells, which contain a transfected plasmid, is 0.7 hours longer than related cells without plasmid foci. This indicates that the division potential of cells with foreign DNA is reduced (Wang et al. 2016).

1.2.2. Genomic instability and cancer

Chromosomal DNA in micronuclei is pulverized overtime and linked with chromothripsis (an extensive genomic rearrangement), which can induce cancer (Zhang 2015; Crasta 2012). Similarly, DMs promote cancer development (see Introduction, Section 1.1.2.2) (Von Hoff et al. 1992). Ec-tDNA, which is abundant in ALT tumour cells, may also be associated with the proliferation of these cells by involvement in the telomere elongation process. Some evidence for the role of ec-tDNA in the amplification of telomeres has been reported. Artificial plasmid DNA containing telomeric repeats was shown to form long tandem arrays at telomeres when transformed into *Kluyveromyces lactis* yeast cells (Natarajan & McEachern 2002). In the telomerase deficient system of mitochondria with linear DNA in *Candida parapsilosis* yeast cells, single-stranded DNA consisting of concatemeric arrays of telomeric sequence (detected by Southern blotting of native 2D gels) and lasso-shaped molecules representing rolling-circle intermediates (detected by EM) (Nosek et al. 2005).

1.2.3. Genomic plasticity

Evidence for the involvement of ecDNAs in the plasticity of genomes, which allows individuals to responds to changes in the environment, was provided using *S. cerevisiae*. It was shown that clones carrying either a deletion of the general amino acid permease *GAP1*, or the deletion plus extrachromosomal DNA circles containing *GAP1* genes (*GAP1*^{circle}) can survive in different selected nutrient conditions. Indeed the presence of *GAP1* circles was

frequently found in cultures grown for long periods on glutamine as only source of nitrogen, but not in cultures rich in amino acid sources (Gresham et al. 2010; Møller et al. 2015). Thus a gene expressed on ecDNA can provide a fitness advantage in select environments. This holds similarities to the dihydrofolate reductase coding gene expressed on DM being associated with the survival of Chinese Hamster cells in presence of antifolate conditions (see review (Gebhart 2005). Moreover, the presence of ecDNA is associated with the genetic mosaicism (different genotypes) of somatic cells during development. For example, circular ecDNAs are derived from recombination in variable (V), diversity (D) and joining (J) genes in lymphocytes during human development (Okazaki et al. 1987) or microDNAs in different mouse tissues (Shibata et al. 2012; Dillon et al. 2015).

1.3. The fates of ecDNAs in mammalian cells over time

Chromosomes with centromeres segregate equally between daughter cells during normal mitosis in order to pass all necessary genetic information to the progeny. What could be the fates of non-centromeric ecDNAs during the cells' life span, with the knowledge that certain ecDNAs can have impacts on the cell? Little is known about this, however diverse fates of ecDNAs have been observed.

1.3.1. Fates of nuclear ecDNAs

Nuclear ecDNAs can segregate stably to the daughter cells via different means: 1) ecDNAs integrate or hitchhike onto chromosomes like viral DNA or DMs (see above); 2) in *C. elegans* embryos ecDNAs form *de novo* centromeres like injected naked DNA (Yuen et al. 2011). Two micron plasmids in *S. cerevisiae* were proposed to segregate symmetrically by two mechanisms: 1) by hitchhiking on chromosomes through the cohesion complex or 2) by a chromosome independent manner. In the chromosome-independent model, plasmid segregation is still dependent on cohesin-mediated pairing and unpairing but takes place without chromosome assistance (Ghosh et al. 2006).

Asymmetric partition during mitosis is another fate of nuclear ecDNAs. This was observed for plasmid and rDNA circles in *S. cerevisiae.* In the mother cell, these ecDNAs form a complex with nuclear pores (mediated by the SAGA complex) and are hindered from passing to the bud by a diffusion barrier at the bud neck (Sinclair & Guarente 1997; Denoth-Lippuner et al. 2014; Shcheprova et al. 2008).

The third fate is the extrusion of ecDNAs into the cytoplasm during mitosis. This was clearly observed in the cases of micronuclei and DMs (Fenech 2011; Shimizu 2011). Moreover nuclear plasmids were also rapidly expelled from the nucleus into the cytoplasm (Shimizu et al. 2005; Denoth-Lippuner Thesis 2014).

1.3.2. Fates of cytoplasmic ecDNAs

Cytoplasmic ecDNAs can inversely enter into the nucleus. As mentioned previously, some cytoplasmic viral DNAs clearly translocate from the cytoplasm into the nucleus (Whittaker et al. 2000). In case of micronuclei, the incorporation of DNA from the micronucleus into the nucleus seems to take place for micronuclei containing entire chromosomes but not for the those containing chromosome fragments (Terradas et al. 2010). Around 50% of the DNA in micronuclei has been found to re-join the nucleus in the next mitotic cycle, independent of the integrity of the surrounding membrane structure (Hatch et al. 2013).

In immune cells, exogenous or pathogenic DNA can be recognised by different cytoplasmic DNA sensors, leading to different downstream signalling cascades. Cytokine production and inflammasome activation are two well-known immune responses to induce the immune system to react to invading pathogens (Burdette & Vance 2013; Hornung 2014). STING (stimulator of interferon genes) seems to be the critical DNA-sensing signal for immune responses including the two aforementioned reactions (cytokine production and inflammasome activation) and autophagy. Interestingly, STING is not found in every cell. It has been identified predominantly in the thymus, heart, spleen, placenta, lung and peripheral leukocytes and less in the brain, skeletal muscle, colon, small intestines, liver and kidney. STING is also expressed in certain transformed cell lines, including HEK293, A549, THP-1 and U937, while seemingly undetectable in HeLa human cervical cancer cells (Sun et al. 2009; Zhong et al. 2008; Burdette & Vance 2013). However, it is still not clear how foreign DNA is treated at the single cell level. Furthermore, in non-STING expressing cells there might be different pathways to handle such DNA. Different responses may be particularly beneficial for negatively regulating the overstimulation of innate signalling pathways which are known to facilitate autoimmune disease (Yoshida et al. 2005). In addition, it is worth mentioning that these signalling pathways are “population-level” reactions, little is known about the “fate” of cytoplasmic ecDNA at the single cell level.

DNA degradation in the cytoplasm may represent an alternate pathway response to cells en-

countering foreign DNA. The evidence for this is that cytoplasmic DNA almost disappears one hour after injection into MEF $p53^{+/+}$ cells (Shimizu 2005) and after 4 hours in HeLa cells (Lechardeur et al. 1999). Autophagy-associated degradation, which is the result of the fusion of autophagosomes with lysosomes, could account for their clearance. Indeed, double-stranded DNA-like plasmids, synthesized polynucleotides or cytomegalovirus DNA, were found to be associated with microtubule-associated protein 1A/1B-light chain 3 (LC3), a marker of autophagosomes, in human fibroblast cells and immune cells (McFarlane et al. 2011; Watson et al. 2012). In addition to autophagy, TREX1, a 3’ to 5’ DNA exonuclease which digests single-stranded DNA and double-stranded DNA with mismatched 3’ termini, may also be involved in the degradation process of ecDNAs in the cytoplasm (Barber 2011). Currently, there is no direct evidence for this possible fate of cytoplasmic ecDNA.

Foreign DNA can be also isolated in the cytoplasm by a membrane structure. It was shown that, shortly after transfection, dsDNA colocalised with BAF (barrier to autointegration factor) protein and was surrounded by Emerin, an inner nuclear membrane protein in the cytoplasm of the African green monkey kidney CV1 cell line (Ibrahim et al. 2011) and in HeLa cells (Kobayashi et al. 2015). Knockdown of BAF causes a significant decrease in the assembly of membranes and increased association with LC3, suggesting that BAF and the surrounding membrane can be inhibitory for the LC3-mediated autophagy response (Kobayashi et al. 2015). Furthermore, this indicates that non-immune cells, or even in immune cells, there might have developed a system to encapsulate these foreign bodies. Remarkably, our group has recently shown that exogenous DNA of bacterial origin accumulates in the cytoplasm predominantly in one focus when delivered into cells via different transfection methods. Such a focus is surrounded by a special double membrane which is comprised of nuclear envelope elements (Wang et al. 2016; Wang Thesis 2015). Intriguingly, plasmid clusters are partitioned asymmetrically to the daughter cell harbouring the young centrosome. Nevertheless, it remains an enigma if endogenous ecDNA also accumulates in the cytoplasm and if it is enclosed by membrane. This would provide an answer to the question of whether the cell differentiates self (endogenous) and non-self (exogenous) DNA in the cytoplasm or not and would ultimately help understanding about “DNA immune reactions” in single cells, especially non-immune cells

1.4. Aim of the project

I analyse telomeric DNA as a model of endogenous ecDNA in this project and attempt to address specific aims as follows: 1) examine the cytoplasmic localization of endogenous ecDNA; 2) investigate the membrane structure of the cytoplasmic endogenous ecDNAs; 3) investigate the possible roles of the membrane associated with cytoplasmic endogenous (if it exists) and exogenous ecDNAs (i.e. plasmid DNA).

2 Results

2.1. Telomeric DNA exists in the cytoplasm of telomerase-negative and positive cancer cells.

We began by asking whether extrachromosomal telomeric DNA exists in the cytoplasm of human cell lines or not?

As mentioned in the introduction, human duplex telomeric DNA is composed of a G-strand (with guanine-rich tandem repeats of (TTAGGG)n and the complementary C-strand (with cytosine-rich tandem repeats of (AATCCC)n (Moyzis et al. 1988; Griffith et al. 1999). In order to detect telomeric DNA, we performed fluorescence in situ hybridization (FISH) experiments with two fluorescent labelled peptide nucleic acid (PNA) probes: 1) TelC is a C-rich telomere probe for the G-strand and 2) TelG is a G-rich telomere probe for detecting the C-strand. Two different cell lines, HeLa Kyoto (HeLa K) and U2OS, were tested. Both are immortalized cancer cells but maintain telomere length in different manners: by active telomerase in HeLa (Sakamoto 2000), and by alternative mechanisms in U2OS (Bryan et al. 1997). As one character of ALT cells, U2OS is expected to contain an abundant amount of ec-tDNA.

We observed that in addition to FISH signals in the nucleus, which include telomeres and presumably ec-tDNA, there were telomeric DNA FISH signals in the cytoplasm stained by both probes. These signals exist as two forms: 1) one or multiple telomeric FISH signals within a Hoechst-positive structure, reminiscent of a so called “micronucleus” containing chromosomal fragments (micronucleus-like structure), (termed mnls-tDNA); 2) any cytoplasmic telomeric FISH signal that does not belong to the Hoechst-positive structure pattern (termed cyto-tDNA) (Fig. 2.1.A, B, C). In other words, mnls-tDNA foci comprise telomeric DNA and other DNA sequences. It is known that circular extrachromosomal tDNA molecules, if not exclusively, contain mostly telomeric repeats (Wang et al. 2004; Regev et al. 1998). Thus, we propose that mnls-tDNA foci can be a cluster of ec-tDNA molecules with other ecDNA molecules with a different sequence or a micronucleus with broken chromosomal fragments or entire chromosomes. Most of the U2OS (about 90%) and HeLa (nearly

80%) cells with cytosolic TelG FISH signals were cyto-tDNA containing cells (Fig. 2.1.D). In these analysed mnls-tDNA structures a maximum of three telomeric DNA FISH signals existed per structure (data not shown). Since cells containing cyto-tDNA were the majority in the population, we solely quantified the cyto-tDNA FISH signals, unless otherwise specified.

On average, cyto-tDNA was detected in 34.8% of U2OS and 15.9% of HeLa K cells using the TelG PNA probe and 47.8% of U2OS and 6.6% of HeLa K cells using the TelC PNA probe (Fig. 2.1.E). Interestingly, most of the HeLa K and U2OS cells with cyto-tDNA (almost 80% for both cell types) contain one cyto-tDNA per cell with both kinds of probes (Fig. 2.1.F).

In order to eliminate the possibility that FISH signals originated from clustered probes, we used a scrambled probe that had a randomized sequence of the TelG probe, as a negative control. Two-colour FISH using a scrambled PNA probe with green fluorescence and either the TelG in tamra red or the TelC in far red fluorescent PNA probe were applied to HeLa K and U2OS cells, respectively (Fig. 2.1.B and C). The probes were used at the same concentration. The scrambled probe was detected in the cytoplasm of 0% of U2OS and 3.09% of HeLa K cells (Fig. 2.1.B-E). This indicates that the FISH signals in the cytoplasm were not the result of unspecific clusters of the probes in U2OS cells but were, to a small degree, in HeLa K cells.

Next, in order to test if the FISH signals observed in the cytoplasm indeed corresponded to DNA, endonuclease DNase I treatment was used on fixed cells before FISH was performed with the TelG PNA probe. Compared to the negative control, the DNase I treatment condition (0.5 unit/μl for 2 to 2.5 hours at 37°C) reduced 70% of the cytoplasmic and 85% of the nuclear telomeric FISH signals in U2OS (Fig. 2.1.G and H). This corroborates the conclusion that FISH signals with the TelG PNA probe in U2OS are DNA specific. We also tested if FISH signals were RNA although RNase was always employed in our FISH technique. With different RNase concentrations, cyto-tDNA was still detected and frequency of U2OS cells with cyto-tDNA was not altered significantly (data not shown). Moreover, we also rationalized that telomeric RNA molecules are known to remain associated with telomeric heterochromatin post transcriptionally in the nucleus (Azzalin et al. 2007; Schoeftner et al. 2008).

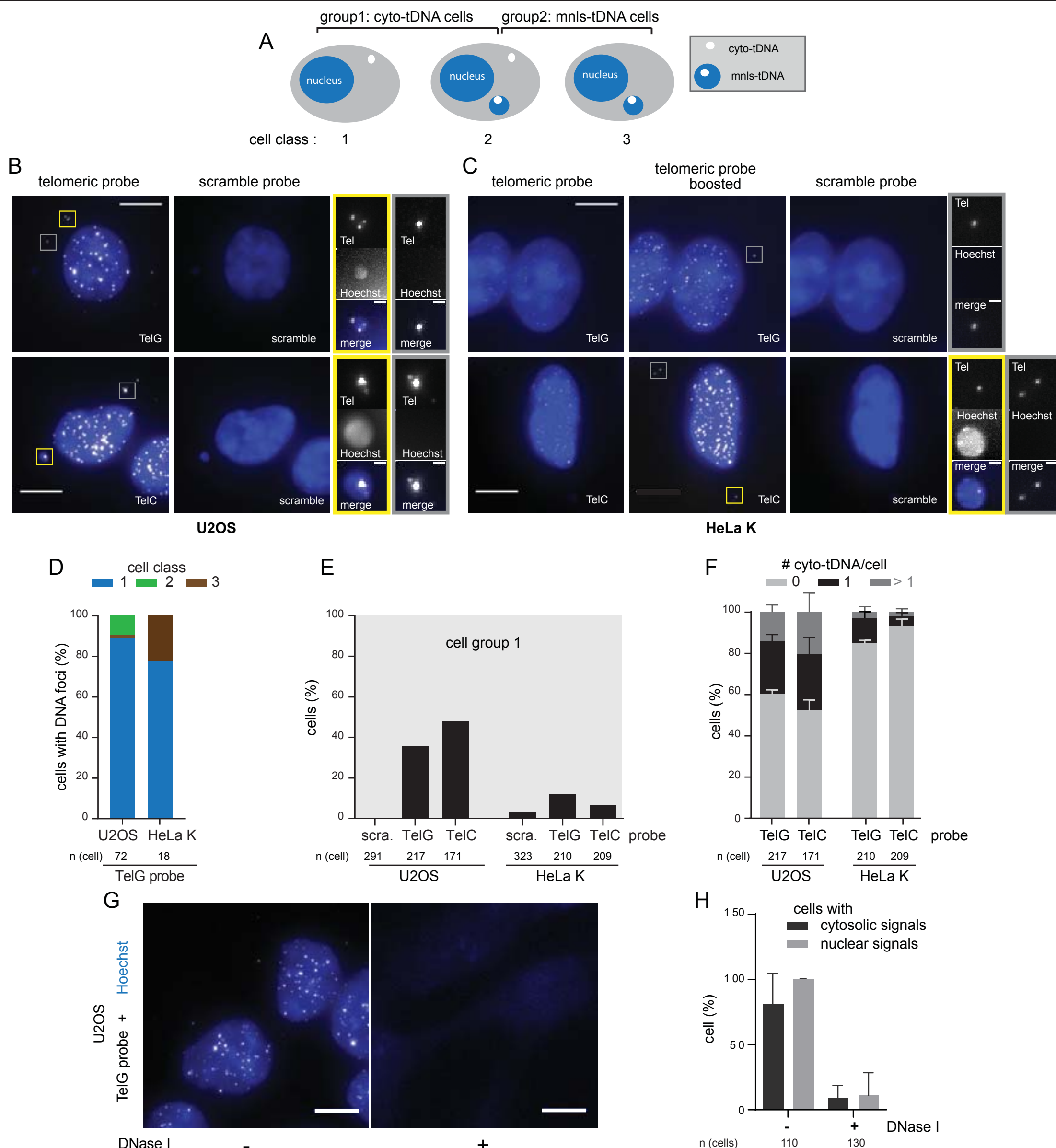

Fig. 2.1. Cytoplasmic telomeric DNA exists in two forms in the cytoplasm with different frequencies in interphase U2OS and HeLa cells.

(A) Scheme of three different interphase cell classes which classified into 2 groups detected by FISH technique: cells contain cyto-tDNA and cells contain mnls-tDNA. White filled circles: FISH signal of a telomeric PNA probe in the cytoplasm. Blue color: Hoechst stained areas (big area: nucleus, small area: mlns-tDNAs). (B and C) Max projected images illustrated for cell class 3 of U2OS (D) and HeLa K (C, lower panel) cells and cell class 1 of HeLa K (C, upper panel) with either TelG or TelC probes combined with scramble probes. Inlets: enlarged areas of yellow (for mnls-tDNA) and gray (for cyto-tDNA) squares of big images. Boost: FISH signals were boosted for HeLa K (C, the second column). (D) Quantification of three cell classes relative to cells containing the cytoplasmic DNA foci (cyto-tDNA and mnl-tDNA) using TelG probe FISH (20-31 and 3-19 foci containing U2OS and HeLa cells/experiment). (E) Percentages of HeLa K and U2OS cells containing cytoplasmic FISH signals (cell class 1+2) using scramble, TelG, TelC probes. “Scra.”: scramble. A minimum of 47 cells per cell line in each experiment. (F) Percentages of HeLa K and U2OS cells with none or one or more than one of cyto-tDNA foci relative to cyto-tDNA containing cells. (G, H) Representative max. projected images and quantification of U2OS cells containing nuclear and cytoplasmic TelG probe FISH signals with and without DNase I treatment. A minimum of 33 cells per condition. (F, H) Error bar: SD; Hoechst stains for double stranded DNA. (B, C, G) Scale bar: 10 μm in big images and 1 μm in inlets. (D-F, H) n: cell number of 3 experiments.

In summary, the percentage of cells containing cyto-tDNA was higher in U2OS, an ALT cell, than in HeLa K, a non-ALT cell. In addition, the intensity of cyto-tDNA in U2OS was on average significantly higher than in HeLa K (Fig. 5.1 in Appendix), suggesting a greater abundance of cyto-tDNA in U2OS. The differences indicate the intrinsic attributes of the two cell lines rather than the accessibility of the two different probes. Due to the greater abundance of cyto-tDNA, U2OS cells were used for the majority of further analyses.

To assess the possibility of cyto-tDNA clustering and to also compare with nuclear telomeric DNA FISH intensities, we measured the intensity of cyto-tDNAs and nuclear telomeric DNA. Intensities of cyto-tDNA foci and nuclear telomeric DNA foci of U2OS were plotted (Fig. 2.2.A). Compared to nuclear telomeric DNA signals, cyto-tDNA signals were in the range of nuclear telomeric DNA, which contain singular or clusters of telomeres and/or nuclear ec-tDNA. Moreover we noticed that there were multiple peaks of cyto-tDNA intensities in the histogram (marked with * in Fig. 2.2.B). This indicates two possibilities: 1) there could be different populations of cyto-tDNAs containing different numbers of telomeric repeats formed by different mechanisms, for example, through t-loop or intra-chromosomal homologous recombination or; 2) cyto-tDNA molecules cluster. However, with the observation that most cyto-tDNA exists as one focus per cell (Fig. 2.1.F), we hypothesized that cyto-tDNA molecules cluster in the cytoplasm.

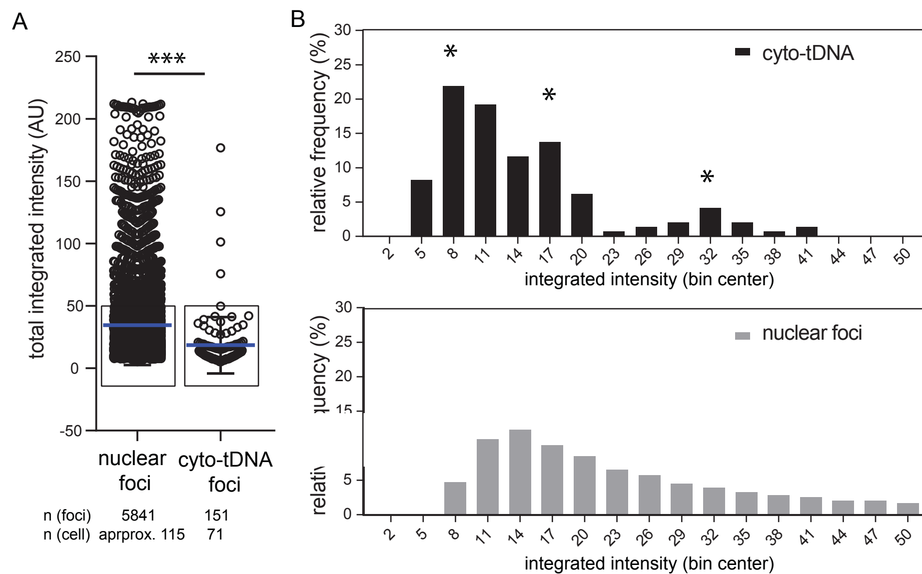

Fig. 2.2. TelG FISH signal intensities of cyto-tDNA foci are in the intensity range of nuclear telomeric FISH signals and distribute with multiple peaks.

(A) Dot plot for intensities of nuclear FISH signals (termed nucleus) and cyto-tDNA signals in interphase U2OS cells. The intensity was measured with Diatrack version 3.05 software, using the same setting for all images (parameters: click subtract background, filtered data: 1, trash dim: 15, trash blurred changing

among images: 0.05-0.07). n: number of measured foci from three experiments. Cells were in cultured less than 15 passages and the cell density was at about 50-60%. Cell images were taken less than one week after the FISH experiment. Blue line: mean values; error bar: SD. (B) Histogram plot for intensity of cyto-tDNA (upper graph) and nuclear telomeric FISH signals (lower graph) in the gray boxes (where contains most of data points of cyto-tDNA). The data area was chosen for a clear presentation of possible peaks (marked with *) of the distribution with the chosen bin width.

2.2. Quantitative analysis of proteins colocalising with cyto-tDNA.

Next, we asked if cyto-tDNA also colocalises with membrane proteins like plasmid DNA does. For a better understanding of the biological significance of cyto-tDNA, we also characterized other proteins, such as telomere binding and DNA binding proteins, with the prior knowledge that they might interact with cyto-tDNA. For this purpose, we performed FISH experiments combined with indirect IF to simultaneously detect telomeric DNA and proteins in different fluorescent colours (indicated specifically in each figure).

2.2.1. C-strand cyto-tDNA is not associated with TRF2, Histone3 (H3) and PML proteins.

First, we wondered whether cyto-tDNA associates with proteins localizing at telomeres. The shelterin component TRF2 which specifically binds to double stranded telomeric DNA (Gison 1997) was assayed first. While the majority of telomeric FISH signals in the nucleus colocalised with TRF2 (98.38%), 70% of mnls-tDNA and 5% of cyto-tDNA co-localised with TRF2, respectively (Fig. 2.3.A).

In mammalian cells Histone3 (H3) is one of the core components of the nucleosome, which has three major variants that are not at the centromere: H3.1, H3.2 and H3.3. Among them, H3.3 is found to be enriched at telomeres (Goldberg et al. 2010). Staining H3 together with telomeric DNA showed that H3 was absent at cyto-tDNA foci but present in the nucleus and mnls-tDNA foci (Fig. 2.3.B). This suggests very low abundance or absence of histone H3 protein with cyto-tDNA.

The promyelocytic leukaemia (PML) nuclear body is one of many subnuclear domains in the eukaryotic cell nucleus and is present in many cells (Bernardi & Pandolfi 2007). PML bodies contain the PML protein and many other proteins involved in a variety of biological processes including DNA damage responses. In ALT cells, some special PML bodies, called ALT-associated PML bodies (APBs), contain (in addition to telomeric DNA), telomeric

repeat-containing RNA (TERRA), telomere-specific proteins and DNA recombination and repair proteins (Yeager et al. 1999; Arora & Azzalin 2015; Nabetani & Ishikawa 2011). Ec-tDNA has also been observed in nuclear APBs (Nabetani et al. 2004; Fasching et al. 2007). Functions of APBs are unknown. However, they are believed to be involved in recombination processes (Draskovic et al. 2009). Therefore, we were also curious to examine whether cyto-tDNA foci are in complexes with APBs or not. By visualizing PML protein and probing telomeric DNA, we observed that in the nucleus 56.98% of PML bodies (n = 623 PML bodies) contain telomeric DNA, whereas PML protein was not detected at cyto-tDNA or at mnls-tDNA foci (Fig. 2.3.C). This suggests there is no association of cyto-tDNA and mnls-tDNA with APBs.

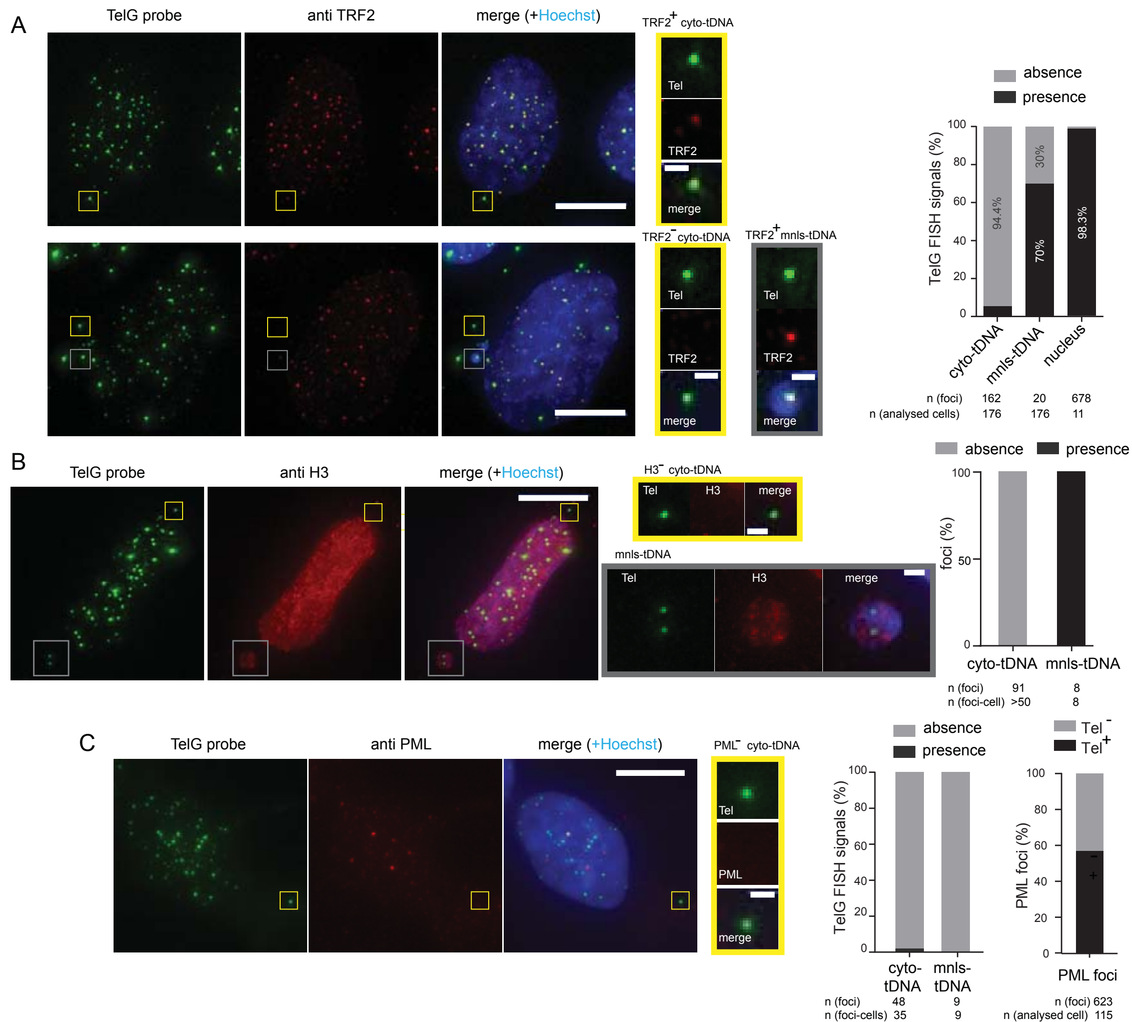

Fig. 2.3. C-strand containing cyto-tDNA does not colocalise with TRF2, Histone 3 (H3) and PML protein in U2OS cells.

IF-FISH mages and quantification of the presence and/or absence (indicated in images) of TRF2 (A), H3 (B), PML (C) at cyto-tDNA and mnls-tDNA. Single-z focus plane of deconvolved images; inlets: enlarged areas of yellow (for cyto-tDNA) and gray (for mnls-tDNA) squares of big images; scale bar: 10 μm in big images and 1 μm in inlets. n: number of foci cells of three experiments, minimum of 9 cyto-tDNA containing cells were analysed in each experiment.

In summary, the difference between mnls-tDNA and cyto-tDNA colocalisation with TRF2 and H3 indicates that mnls-tDNA and cyto-tDNA are different forms of telomeric DNA. It is therefore likely that mnls-tDNA is a chromosomal fragment or an entire chromosome with a preserved heterochromatin structure of telomeres and cyto-tDNA is small telomeric DNA molecules with an altered chromatin structure or associated protein (e.g. cyto-tDNA molecules are mostly single stranded DNA (ssDNA) with very short double stranded (ds) parts). PML protein was not detected in either type of cytoplasmic tDNA, suggesting it is not involved in APBs' function in the cytoplasm.

2.2.2. A fraction of cyto-tDNA is associated with the ER protein Sec61 and inner nuclear protein Lap2β but not Lamin B1 and the nuclear pore complex component protein ELYS.

It is known that cytoplasmic plasmid clusters are engulfed in a membrane structure, which shares many features with the nuclear envelope (Wang Thesis 2015). Therefore, we also tested whether cyto-tDNA also associates with the same type of membrane.

The nuclear envelope is composed of two membranes (Endoplasmic-reticulum- (ER) derived membrane and inner nuclear membrane (INM), the nuclear pore complexes (NPC) and nuclear lamina are associated with the INM (see reviews (Rothballer & Kutay 2012b; Rothballer & Kutay 2012a). Plasmid DNA foci are surrounded with the ER transmembrane protein Sec61 (a component of the mammalian translocon (forming a channel across ER) and ER luminal reporters, Calreticulin and KDEL (Lys (K)-Asp (D)-Glu (E)-Leu (L)) motif containing proteins. In addition the inner nuclear membrane proteins Emerin and Lap2β belonging to the LEM (Lap2-emerin-Man1)-domain protein group (Holmer & Worman 2001) are also present at the DNA foci. However, there is only a partial presence of Lamin B1 and no detection of Lamin A/C and NPCs or the Lamin B receptor (LBR) (Wang Thesis 2015).

In order to examine the possibility that cyto-tDNA associates with a membrane structure like that around plasmid DNA, we tested for the presence of proteins such as Sec61, Lap2β, Lamin B1 and ELYS (a nuclear pore complex component), at cyto-tDNA using the IF-FISH method. Sec61 was transiently overexpressed in U2OS cells. 24 hours after transfection of the plasmid encoding mCherry-Sec61, U2OS cells were probed with the TelC PNA probe and stained with the anti-mCherry antibody followed by the secondary antibody with a green fluorophore.

The result showed that cyto-tDNA FISH signals localised in the same spots with overexpressed mCherry-Sec61 in 47.4% of the analysed foci (Fig. 2.4.A and B), whereas Lap2β colocalised with 54.2% of C-strand containing cyto-tDNA foci (Fig. 2.4.C and D).

We also examined the presence of Lamin B1 (Fig. 2.5 A, B). None of the cyto-tDNAs were found to be associated with these proteins. ELYS is required for the assembly of a functional nuclear pore complex (NPC) on the surface of chromosomes as nuclei form at the end of mitosis (Franz et al. 2007; Rasala et al. 2006). The data showed 100% of cyto-tDNAs were negative for ELYS, suggesting the absence or very low density of NPC at cyto-tDNA (Fig. 2.5.C and D).

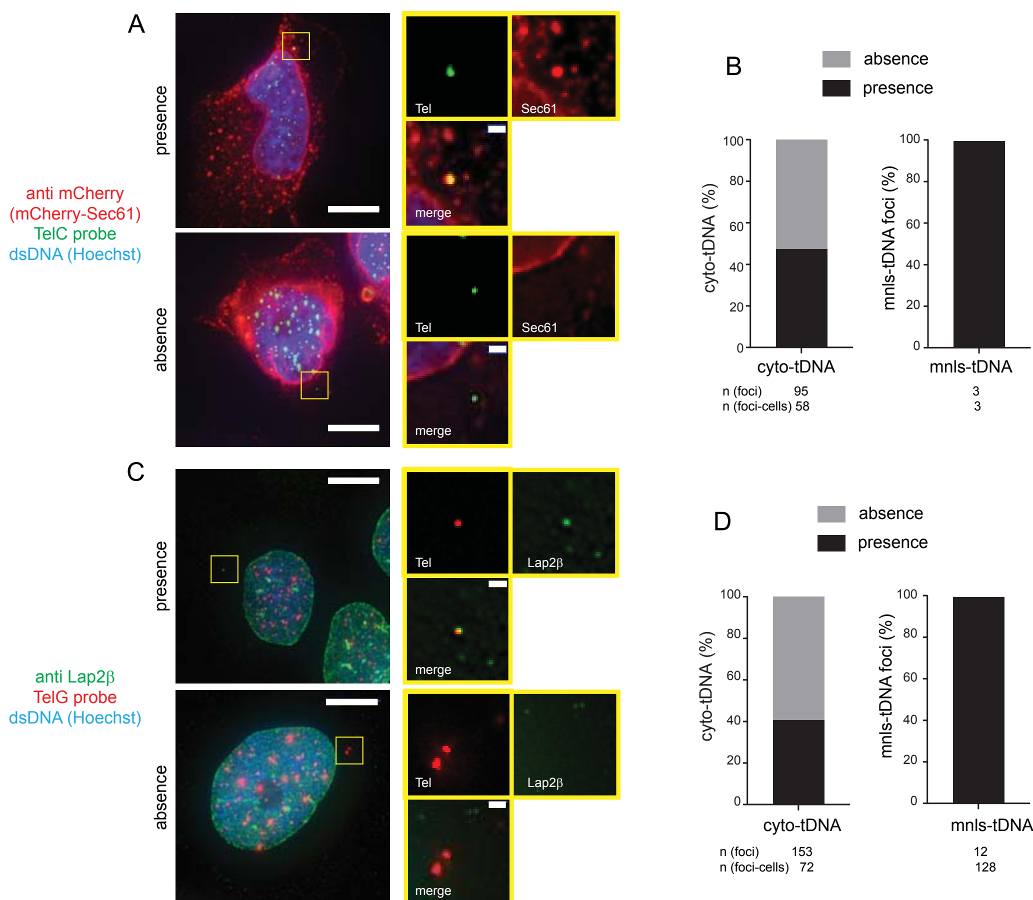

Fig. 2.4. The ER membrane protein Sec61 and inner nuclear membrane protein Lap2β are absent at more than 50% of cyto-tDNA foci in U2OS cells.

Presentative images of relative localization of Sec61 (A), Lap2β (C) with cyto-tDNA foci and the corresponding quantification of two relative localization patterns (presence and absence) of Sec61 (B), Lap2β (D) at cyto-tDNA (left graphs) and mnls-tDNA (right graphs). n: number of foci and either cyto-tDNA or mnls-tDNA containing cells of three experiments, minimum of 10 cyto-tDNA cells were analysed in each experiment.

Collectively, these data suggest that cyto-tDNA can be in contact with a membrane structure that is composed of the ER protein Sec61 and inner nuclear LEM-domain membrane protein Lap2β. This membrane structure seems to lack or have very few copies of Lamin B1 and the NPC component ELYS. Through its' components, this membranous structure seems to resemble the one surrounding transfected plasmids (Wang Thesis 2015). However, unlike the plasmid DNA, which is consistently surrounded by a membrane, the cyto-tDNA associates with Sec61, and Lap2β only in about 50% of the cases. This raises the question of where this heterogeneity could originate.

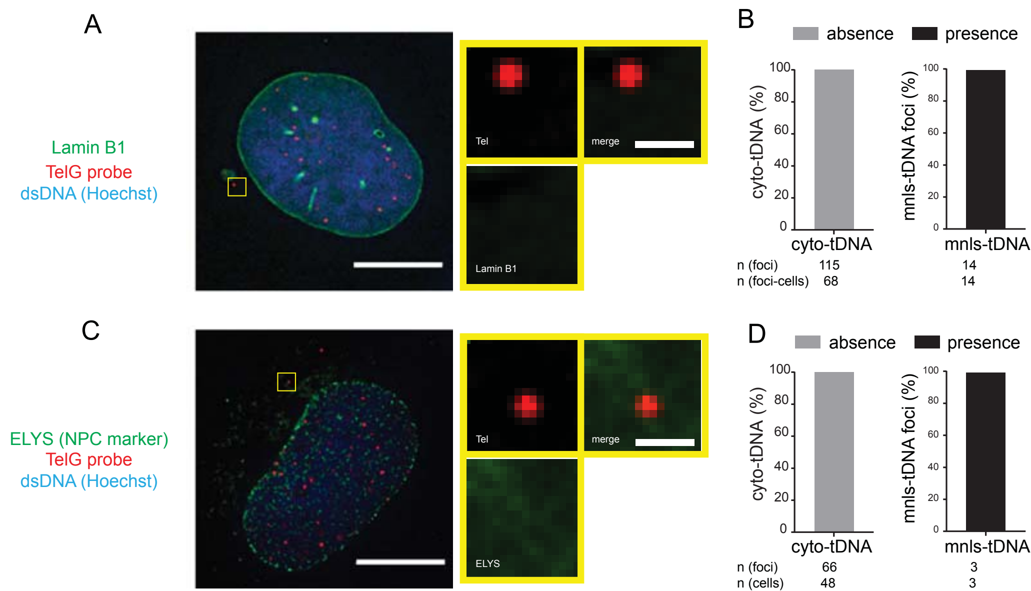

Fig. 2.5. Lamin B1 and ELYS (a nuclear pore complex component) are never detected at cyto-tDNA but always at mnls-tDNA.

Representative images of relative localization of Lamin B1 (A), ELYS (C) at cyto-tDNA foci in U2OS cells. Big images: max. intensity projected of deconvolved images; inlets: enlarged areas of cyto-tDNA in yellow squares of big images, single-z focus plane. Scale bar: 10 µm in big images and 1 µm in inlets; n: cyto-tDNA foci and cells of three experiments; Hoechst staining for dsDNA. Quantification of two qualitative localization patterns (presence and absence) of Lamin B1 (B)and ELYS (D) at cyto-tDNA foci (left graph) and mnls-tDNA (right graph). n: number of foci and either cyto-tDNA or mnls-tDNA foci containing cells of three experiments, minimum of 14 cyto-tDNA cells were analysed in each experiment.

2.2.3. Size/Intensity and form (ds, ss) of cyto-tDNA do not cause the absence of the inner nuclear membrane protein Lap2β.

We tested two factors that we thought could be the reasons for the heterogeneity of Lap2β at cyto-tDNA. The first factor could be the detection limits of the technique. The second possibility is due to different DNA forms of ec-tDNA molecules (dsDNA and ssDNA with a dsDNA).

Size/intensity of cyto-tDNA FISH signals cannot explain the heterogeneity of Lap2β at cyto-tDNA.

We first wondered whether the heterogeneity of Lap2β presence at cyto-tDNA was due to the detection limits of the visualisation method. In order to probe for this, we asked if the presence of Lap2β at cyto-tDNA foci corresponded to the size or the intensity of cyto-tDNA foci.

Interestingly, we observed that in a subset of cells (about 34%), all cytoplasmic cyto-tDNA foci associated with Lap2β. In another subset of cells (about 40%), all cyto-tDNA foci are negative with Lap2β. In a third subset of cells (about 26%), there was a mix of the cyto-tDNA foci with and without Lap2β Fig. 2.6.B). This indicates the heterogeneity of the cell population.

We also measured the intensity of Lap2β positive and negative cyto-tDNA in these cell populations. On average, the Lap2β positive cyto-tDNA foci intensities were not significantly greater than those of the LLap2β negative foci in the first two cell classes containing either all Lap2β positive or negative foci. The difference was significant in the third population of cells. However, we noticed that there seemed to be some outliers in the Lap2β positive group in this cell class (indicated with the dashed line in Fig. 2.6.D). We also had a closer look at the intensities of cyto-tDNA FISH signals in single cells of this cell class in order to avoid the problem of heterogeneous staining of Lap2β in different cells. The results showed that in a single cell, both intense and less intense foci (with maximal intensity differentiating by a factor of 17) colocalised with Lap2β (Fig. 2.6.E). In addition, the results showed that the size of cyto-tDNA was not significantly different between foci with and without Lap2β (Fig. 2.6.A and C). Therefore, size and intensity cannot account for the heterogeneity of

cyto-tDNA colocalised with Lap2β. Thus, the amount of telomeric DNA repeats is not a determinant for the association with membrane.

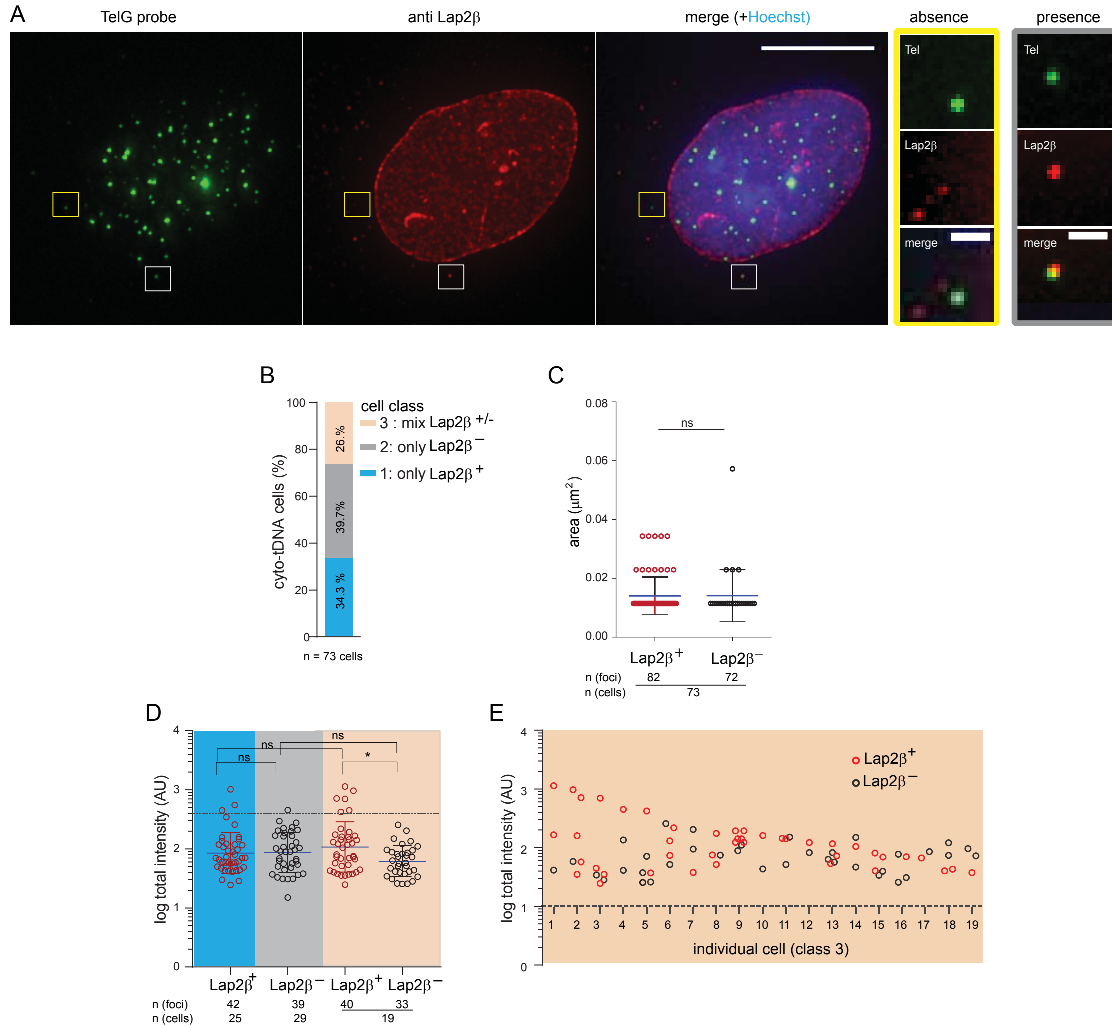

Fig. 2.6. Lap2β colocalisation at cyto-tDNA is independent of size and intensity of cyto-tDNA FISH signals.
(A) Presentative images of two relative localization patterns (presence and absence) of Lap2β at cyto-tDNA foci. Single-z-focus of deconvolved images; inlets: enlarged areas of cyto-tDNA foci in yellow and gray squares in big images; scale bars in big images: 10 µm, in inlets: 1 µm. (B) Quantification of three cell classes relative to cyto-tDNA containing cells. (D) Dot plot of size of Lap2β positive and negative cyto-tDNA foci. Dot plot for fluorescence intensity of Lap2β positive and negative cyto-tDNA foci per cell class (D) and in single cells of the cell class 3 (E). Dash line in (D) is cut off for some outliers. Size and intensity were measured using Diatrack version 3.05 (Vallotton & Olivier 2013) (parameters: click subtract background, filtered data 0.5, trash dim: 15, trash blurred changing among images: 0.05-0.07). (B, C, D) n: number of cells of three experiments; a minimum of 20 cyto-tDNA cells per experiment. (C, D) Blue line: mean values; error bar: SD.

DNA forms (ds, ss) of cyto-tDNA can also not account for the heterogeneity of Lap2β presence at cyto-tDNA.

We next asked whether the different forms of cyto-tDNA molecules could be causal for this heterogeneity. As mentioned in the introduction, extrachromosomal telomeric DNA is known to be present in different forms: ds DNA and partially ss DNA (see review (Henson et al. 2002)).

In a native FISH method, the heat denaturation of dsDNA is omitted; therefore only pre-existing ssDNA can be accessible to the probes. Thus native FISH was employed to detect telomeric ssDNA. The regular denaturing FISH method, which was used in all other FISH experiments in this project, was carried out in parallel. Both samples were also anti-Lap2β stained.

We observed that cyto-tDNA was detected in native FISH, suggesting that there are cyto-tDNA molecules that also contain ssDNA (Fig. 2.7.A and B). The percentage of cells containing cyto-tDNA in native FISH (34.8%) was lower than by regular FISH (57.8%) (Fig. 2.7.B). In addition, about 2.12 versus 1.25 cyto-tDNA foci were detected per cell in regular and native FISH, respectively (n of the foci and cells is shown in Fig. 2.7.C). This suggests an abundant existence of dsDNA cyto-tDNA molecules. Nevertheless, we cannot exclude the possibility of higher accessibility of the probe in regular FISH. Remarkably, there was no significant difference in the localization of Lap2β with cyto-tDNA in regular and native FISH methods (Fig. 2.7.C), indicating that different DNA forms (partial or ds DNA) have no effect on the co-localization with Lap2β.

In summary, the detection limit of the technique and diverse strand forms of cyto-tDNA (ds or partially dsDNA) cannot explain the membrane association heterogeneity of cyto-tDNA. Other factors must be involved. For example, there could be two different cellular machineries handling cyto-tDNA: one set of machinery encloses cyto-tDNA in a membrane structure; another set leads to a different or no-membrane compartment. It would be insightful to further explore what the causes of the heterogeneity of colocalisation of Lap2β with cyto-tDNA are. This could help in understanding the link between these two machineries. Nevertheless, the association with membrane proteins reveals a similarity between endogenous cyto-tDNA and foreign plasmid DNA. It is therefore possible that these two types of ecDNA can colocalise with each other.

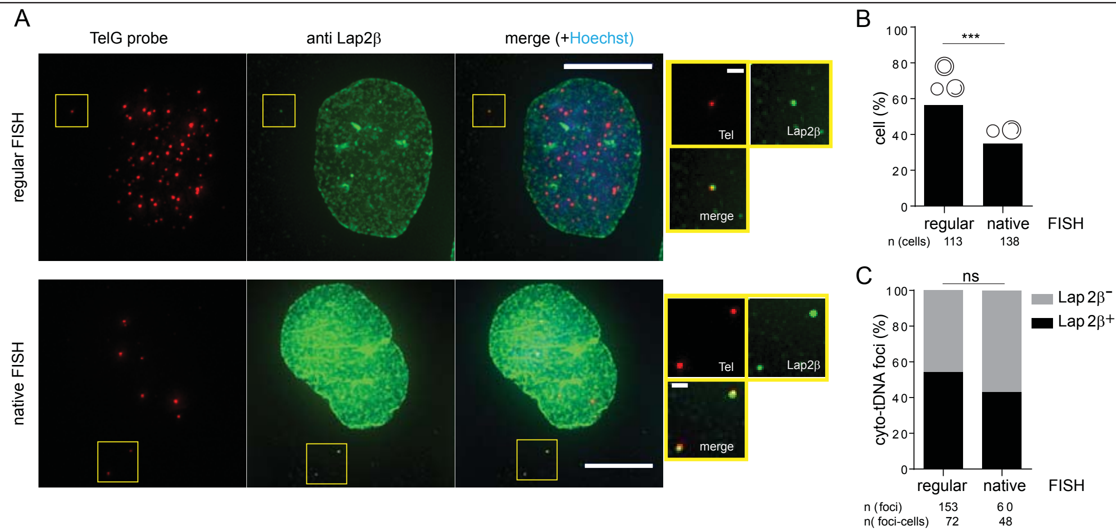

Fig. 2.7. Existence of different forms (ds, partially ssDNA) of cyto-tDNAs has no significant effect on Lap2β colocalisation.

(A) Images of the relative colocalisation pattern of Lap2β at cyto-tDNA in regular and native FISH methods. Big images: max. intensity projected of deconvolved images; inlets: enlarged areas of cyto-tDNA in yellow squares of big images, single-z focus planes. Scale bars in big images: 10 µm, in inlets: 1 µm. (B) Quantification of cells containing cyto-tDNA using regular FISH and in native FISH methods. n: number of analysed cells of three experiments with a minimum of 32 cells in each experiment. (C) Quantification of two quantitative localization patterns (presence and absence) of Lap2β at cyto-tDNA using regular and native FISH methods. Statistics: *Chi-square*, n: number of foci and foci containing cells of three experiments; a minimum of 12 foci and 12 cells in each experiment.

2.3. Cytoplasmic G-strand telomeric DNA and plasmid DNA can occur in the same cellular compartment.

Since cytoplasmic telomeric DNA and plasmid DNA, can both exist in the cytoplasm and are associated with membrane proteins, we wondered if these two entities could cluster together in the same membrane structure. LacO plasmids were transfected into U2OS cells. After 24, 48, and 72 hours, cells were fixed and fluorescence hybridized with LacO and TelC PNA probes, then immuno-stained for Lap2β. About 30% of co-existence cells which contained both LacO plasmid DNA engulfed in Lap2β membrane and cyto-tDNA, harboured co-localization foci of two types of ecDNAs, termed co-localization cells (Fig. 2.8.A, B, C). The percentages of cells with colocalisation remained constant over the 24 h, 48 h, and 72 hour time-course. This result might suggest that cells can cluster ecDNAs in the cytoplasm independent of endogenous or exogenous origin. However, not all cytosolic telomeric foci cluster with the plasmid foci in one membrane compartment in the cell. Maximally, 50% of cyto-tDNA foci colocalised with LacO plasmid DNA clusters (Fig. 2.8.D), indicating that clustering of ecDNAs can occur, but it is not a dominant mechanism. The localisation might be dependent on the proximity of two DNA entities.

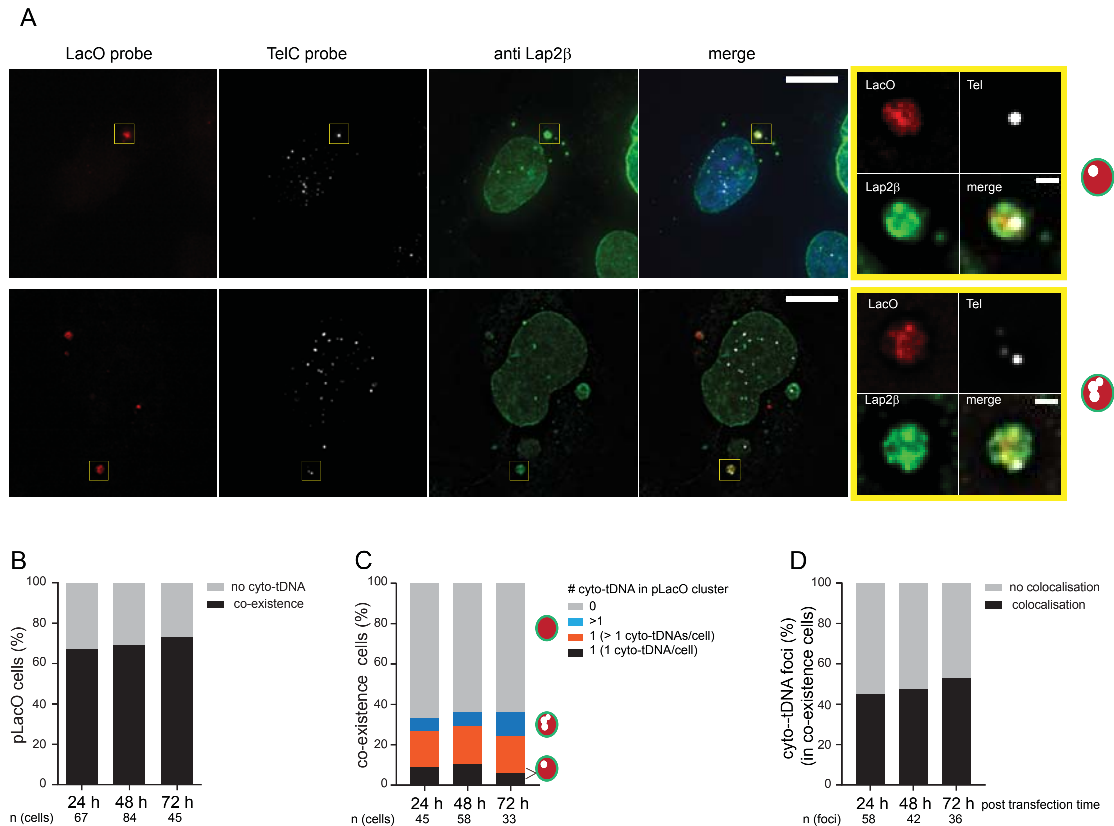

Fig. 2.8. A fraction of cyto-tDNAs colocalize with plasmid DNA in the same cytoplasmic membrane compartment.

(A) Images of the relative colocalisation pattern of pLacO foci with either one cyto-tDNA focus (upper panel) or with multiple cyto-tDNA foci (lower panel). Single-z focus of deconvolved images; inlets: enlarged areas of placO and cyto-tDNA foci in yellow squares of big images; scale bar = 10 μm in big images and 1 μm in inlets. (B) Quantification of cells with cyto-tDNA in Lap2β positive pLacO containing cells 24, 48, 72 hours after pLacO transfection (termed co-existence cells). (C) Cells with different number of cyto-tDNAs in colocalisation with Lap2β positive pLacO clusters (termed colocalisation cells). Scheme for colocalisation of cyto-tDNA and plasmid DNA in the same compartment; green ring: Lap2β; red: pLacO clusters; white: cyto-tDNA. (D) Percentage of cyto-tDNA foci in colocalisation with Lap2β positive pLacO clusters relative to total cyto-tDNA in Lap2β positive pLacO containing cells.

2.4. Cyto-tDNAs partition asymmetrically toward the young centrosome-containing sister cell in fixed telophase U2OS cells.

Since plasmid DNA and cyto-tDNA share many similarities as aforementioned, and since plasmid DNA foci partition asymmetrically in mitosis, we also asked how cyto-tDNA foci distribute into the sister cells after cell division. This could determine if cyto-tDNA is maintained or eliminated from the cell population.

First, we analysed the frequency of cells containing cyto-tDNA in telophase. The results

showed that the frequency of cyto-tDNA in telophase is higher than in interphase cells. The frequency of multiple cyto-tDNA cells in the population also increased (Fig. 2.9.B), suggesting that mitosis might be one mechanism for extrachromosomal telomeric DNA translocation into the cytoplasm. We assessed the partitioning of cyto-tDNAs in fixed telophase U2OS cells containing multiple cyto-tDNA foci. Since the cells containing two or three cytoplasmic foci occurred more frequently in the population of multiple foci containing cells, we focused on these two groups. The data showed that the asymmetric segregation n:0 (n = 2 or 3 foci) was significantly different from the theoretical random expectation (Fig. 2.9.A and C).

However, cells containing one cyto-tDNA focus were a majority group in the population. Therefore, identifying which of the two sister cells the focus co-segregates to allow assessment of the segregation pattern more robustly as well as identifying the specific segregation pattern. Centrosome age is known as one asymmetric maker of the two sister cells (Nigg & Stearns 2011). During S phase, a centrosome duplicates into two centrosomes, one is the so-called “old centrosome” with the grandmother centriole and daughter centriole, the other is the so-called “young centrosome” with mother and daughter centrioles (Nigg & Stearns 2011). The two centrosomes can be differentiated using different protein markers, which primarily localise to the older centrosome. The anchoring protein Outer dense fibre 2 (ODF2), has been reported to localise preferentially at the sub-distal appendages of the grandmother centriole, leading to stronger immunofluorescence signals at the old centrosome than at the young centrosome (Nakagawa et al. 2001; Wang et al. 2016).

We performed IF-FISH for telomeric DNA, Lap2β for nuclear contour, and ODF2 to distinguish old and young centrosomes. Since ODF2 staining had a high background signal,γtubulin (an additional centrosome marker (Moudjou et al. 1996) was also stained (in the same colour with Lap2β was used in addition to unambiguously identify true ODF2 signals. The old centrosome was classified as such if it had a minimal 85% difference in the ODF2 fluorescence intensity compared to the young centrosome (see methods). Counting cells with a cyto-tDNA segregation pattern x:y with the necessary conditions that $x+y>0$ and $x>y$ (excluding ambiguous cells with telomeric FISH signals in the midbody area), there were 66.67% of cells where cyto-tDNAs localised preferentially with the young centrosome-harbouring cell ($p = 0.02$) (Fig. 2.9.D and E). The data suggest that cyto-tDNAs partition asymmetrically in U2OS telophase cells.

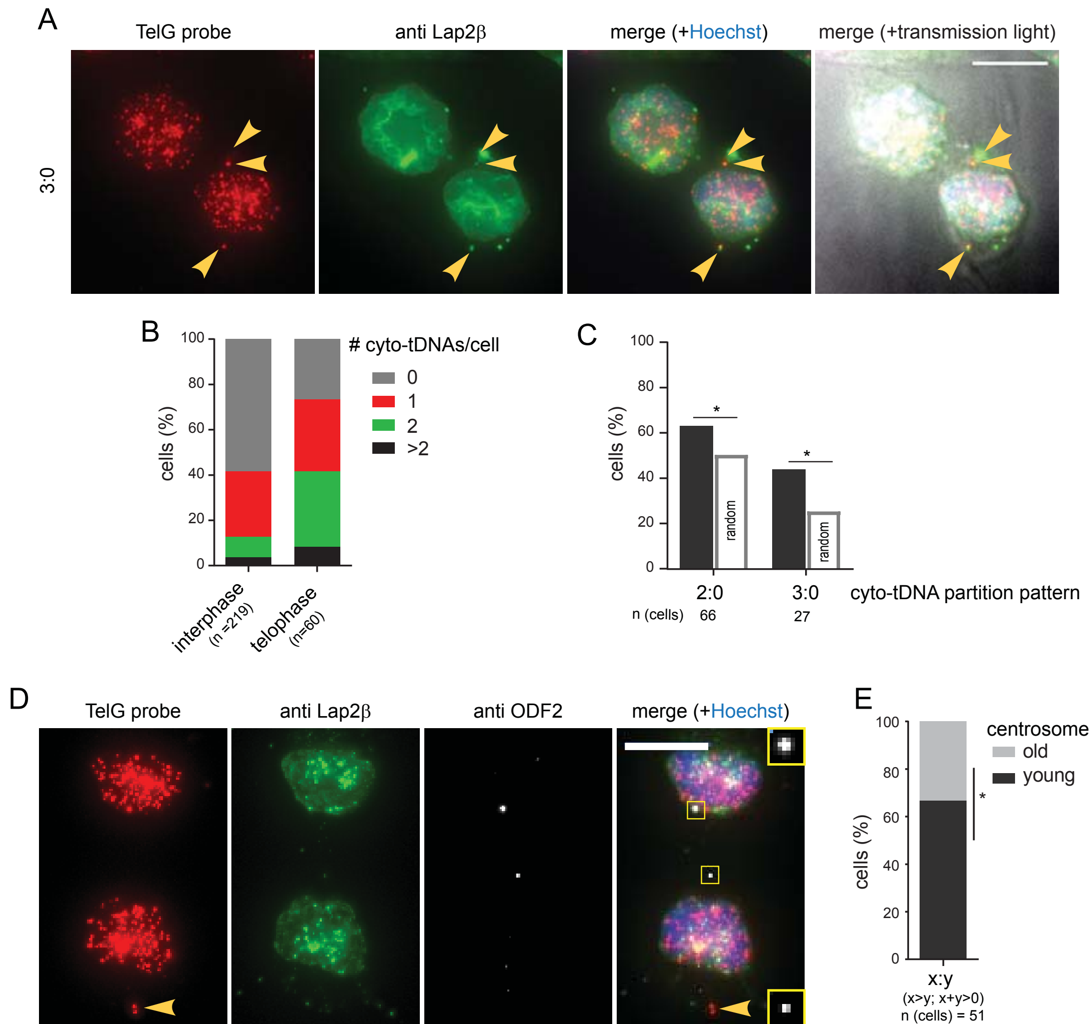

Fig. 2.9. Cyto-tDNA foci in fixed telophase U2OS cells preferentially partition with the young centrosome.

(A) Example images of cells with three cyto-tDNA foci localizing in one sister cell in telophase. (B) Cells with different number of cyto-tDNA foci in interphase and telophase U2OS cells. (C) Cells with n:0 distribution pattern were quantified in cells harboring n cyto-tDNA foci (n = 2 or 3 foci). *Binobimal* test was used to test the experimental data with theoretical random expectation (based on Pascal triangle) in unfilled gray bars. (D) Representative images of a telophase U2OS. Cells were stained with antibodies for Lap2β and γtubulin (both in green fluorescence), ODF2 (in cy5 fluorescence), and probed with TelG probe. Inlets: enlarged areas of the two centrosomes stained with anti-ODF2 antibody in yellow squares of big images; same magnification scale for the images in inlets. (A, D) Yellow arrows point at cyto-tDNA; sale bar: 10 μm in big images. (E) Quantification of the x:y partition pattern of cyto-tDNA foci in fixed telophase U2OS (x>y and x+y>0) in correlation with the young and old centrosomes. Pooled data of 3 experiments, n = 51 cells. *Binominal* test, p= 0.02.

2.5. The role of LEM-domain proteins in the biology of cytoplasmic plasmid and tDNA.

Since membrane proteins can surround both plasmid and cyto-tDNAs, we asked what the role of the membrane is. In order to answer this question, we chose to perturb LEM-domain proteins. As components of the membrane, LEM-domain proteins are known to either directly or indirectly bind DNA via BAF and other linkers (Cai et al. 2001; Poleshko & Katz 2014). Moreover, it was found that knockdown of BAF caused a significant decrease (but not complete elimination) in the assembly of Emerin around dsDNA-coated beads (Kobayashi et al. 2015). We reasoned that BAF knockdown might not be strong enough to disturb the membrane structure due to other connections of LEM-domain proteins with the DNA. Therefore, we aimed to directly perturb the function of LEM-domain proteins in a competition approach, which might provide a stronger effect on the membrane structure around the cytoplasmic DNA foci. The expectation was that the soluble LEM-domain overexpression would compete with the functional LEM-domain proteins present at the cytosolic DNA focus, leading to a detachment of other membrane proteins from DNA focus and/or membrane structure changes therein.

In order achieve this, a DNA construct coding for a soluble LEM domain was used. The construct contained the coding sequence for the LEM-domain of human Emerin fused to GFP and a nuclear export signal (nes), the so-called LEM-nes protein. Overexpressed LEM-nes proteins (in other words, overexpressed LEM-domain) were expected to be soluble and prevalent in the cytoplasm due to the lack of a transmembrane domain (Michael Burger Thesis 2014). In the same experimental set, a GFP-only vector was used as a control (Fig. 2.10.A). We tested the effect of LEM-domain overexpression (OE) on both plasmid DNA and cyto-tDNA.

2.5.1. LEM-domain OE results in more foci in individual HeLa cells and an increased proportion of small plasmid foci over time.

We first tested the effect of LEM-nes on transfected plasmid DNA. LacO plasmid (pLacO) containing an array of 256 LacO repeats was transfected into HeLa cells stably expressing LacI-mCherry-NLS (nuclear localization signal). LacI protein has high affinity for LacO DNA, allowing the visualization of pLacO DNA. The fidelity of this system was previously

validated by our group (Wang Thesis 2015; Wang et al. 2016). HeLa cells were transfected with LEM-nes or the control construct prior to electroporation of pLacO. This transfection method was chosen to avoid the side effect of lipofection. In lipofection DNA is pre-clustered in micelles before entering the cell. However, by any of these transfection methods, LacO plasmid DNA with a known size, formed large cytoplasmic foci with various fluorescent intensities detected by either LacI-XFP indirectly or FISH (with LacO probes) directly. Not only pLacO plasmids, but also any plasmid of known size formed such foci in the cytoplasm (Wang Thesis 2015). This suggests there are different numbers of plasmid DNA molecules in such foci. Cells were fixed 6 h and 24 h after pLacO electroporation (Fig. 2.10.B). The six hour sample was analysed in order to determine the early fate of pLacO after entering the cell, but the time period was also long enough for the cells to attach onto the coverslip after electroporation.

We initially examined where in the cell LEM-nes protein localised. We observed that GFP-LEM-nes could localise to pLacO clusters (Fig. 2.10.C). The preliminary data showed that the frequency of pLacO foci surrounded by Emerin decreased relative to the control at both 6 h and 24 h when in the presence of LEM-domain OE, indicating that the membrane at pLacO foci was affected by the overexpression of the LEM-domain (images in Fig. 2.10.E, quantification not shown). We observed a reduction in the frequency of cells containing pLacO foci at both 6 h and 24 h in LEM-domain OE relative to the control using electroporation (Fig. 2.10.C and D) and lipofection transfection methods (Burger Thesis 2014). As most of the synchronous cells went through a mitotic division between 6 h and 24 h, the reduction of cells harbouring pLacO foci between 6 h versus 24 h in both conditions could be due to asymmetric segregation of plasmid DNA (Wang Thesis 2015). However, the decline is clearer in the LEM-nes than in the control condition, by a factor of 2.2 versus a factor of 1.7, respectively. We also observed that the size of the pLacO foci in LEM-domain OE was smaller than the control condition at any assessed time point. Under the same perturbation conditions, the proportion of small pLacO foci (< 0.4 μm) at 24 h was higher than at 6 h, while in the control little changed between the two time points (Fig. 2.10.F). In addition, more multiple dot cells (> 6 dots per cell) were found in LEM-domain OE than in control cells at 24 hours, 18.75% versus 6.25%, respectively (Fig. 2.10.G).

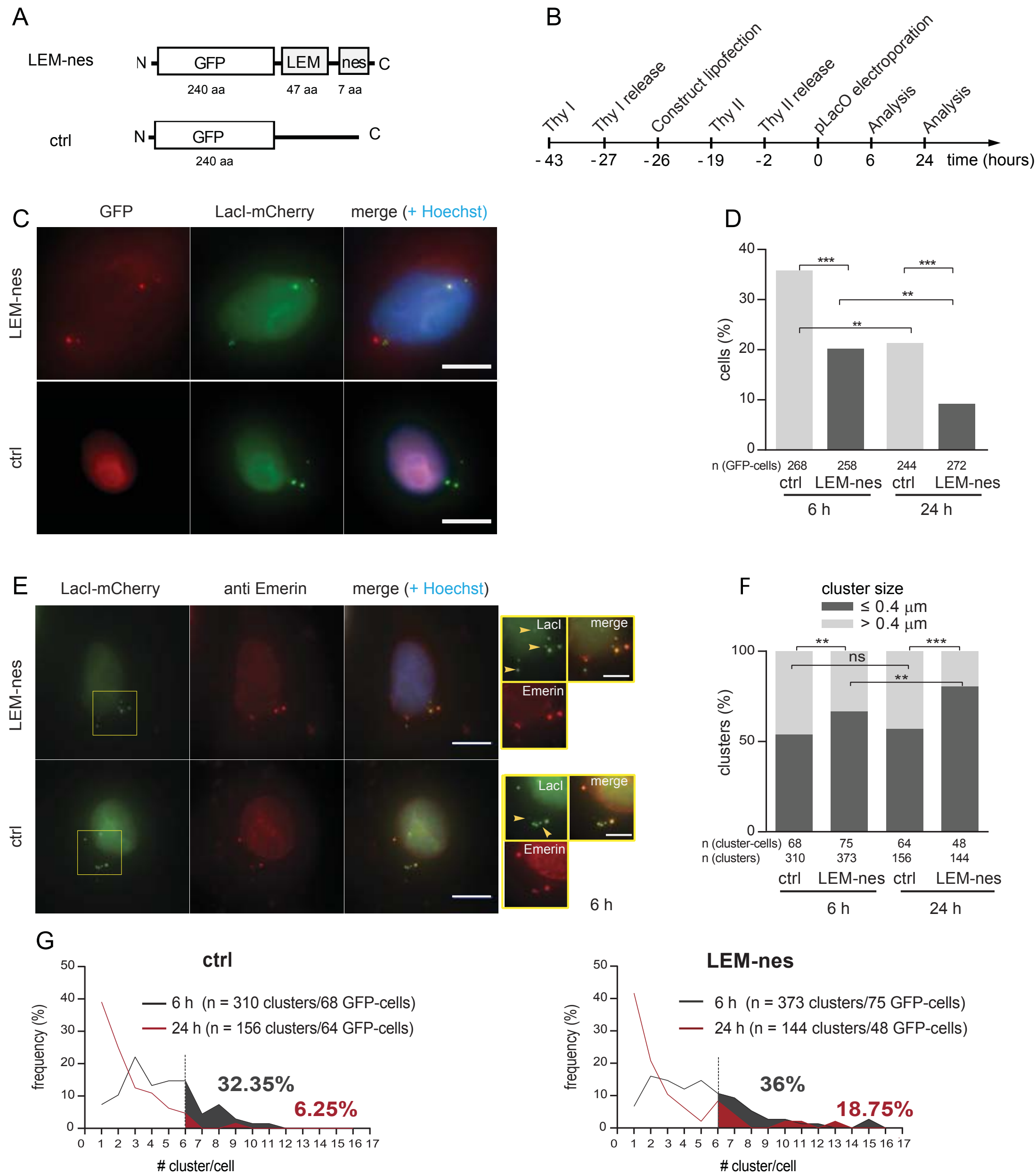

Fig. 2.10. Overexpression of soluble LEM-domain causes more plasmid foci in single HeLa cells and an increased proportion of small foci at different assessed time points.

(A) Scheme of fusion proteins. Ctrl (control): GFP vector; LEM-nes: GFP-LEM-nes construct. (The constructs were created by Michael Burger (Burger 2014)). (B) Scheme of experimental procedure. Thy: thymidine treatment. (C) Presentative images of HeLa cells stably expressing LacI-mCherry lipofected with either LEM-nes or GFP construct, then electroporated with LacO plasmid (pLacO). (D) Cells containing LacO clusters relative to GFP positive cells per condition. (E) Representative images of cells 6 hours after pLacO electroporation per condition. Emerin was stained. Inlets: enlarged area of pLacO foci in yellow squares of big images. Yellow arrows point at Lap2β negative LacO foci. Scale bar: 10 µm in big images and 5 µm in inlets. (F) pLacO cluster size per condition 6 and 24 hours after pLacO electroporation. Size of the pLacO cluster were determined by choosing the longest diameter of the cluster. (G) The frequency of cells with different number of pLacO foci relative to pLacO harboring cells 6 and 24 hours post-transfection in control (left graph) and in LEM-nes (right graph). The frequency of cluster-cells with >5 clusters is written all figures. Dash line: cut off for cells >5 clusteres. Pooled data of 3 experiments; n: number of clusters and cells indicated in the panels. *Chi-square* statistical test shown.

Together, these data support the possibility that large plasmid clusters de-cluster in the presence of overexpressed LEM-domain. In addition, the formation of clusters large enough for visualisation might be hindered. Additionally, pLacO clusters degradation can take place. Further experiments, for example live cell imaging to visualize the pLacO foci de-clustering process, are required to clarify this. Furthermore, it would be insightful to see how the membrane structures actually change after overexpression of the LEM-domain. Is membrane formation at the plasmid DNA foci completely inhibited? Or does overexpression only reduce the concentration of LEM-domain proteins, leading to the destabilization of the structure? Another intriguing question is whether LEM-domain OE affects cyto-tDNA and endogenous ecDNA.

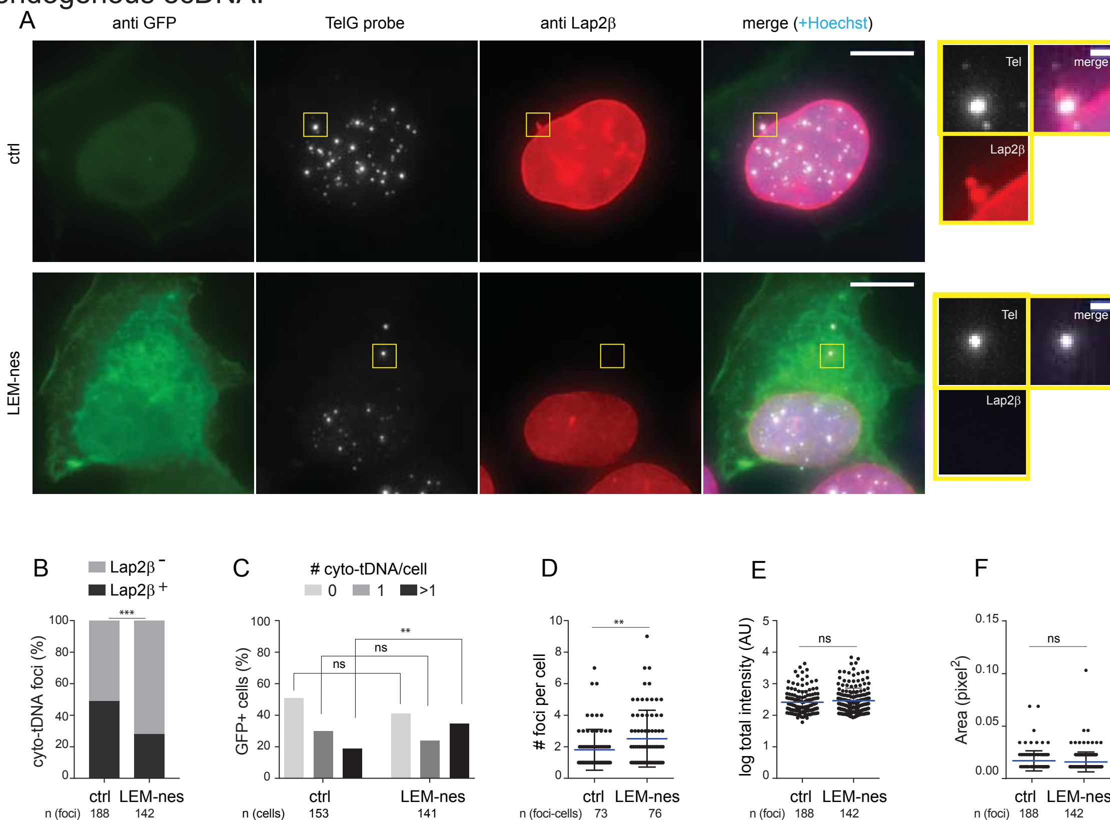

Fig. 2.11. Overexpressed LEM-domain leads to an increased fraction of U2OS cells with more than one cyto-tDNA focus.
(A) Example images of cells 48 hours after transfection of either LEM-nes or ctrl constructs with presence (top panel) and absence (lower panel) of Lap2β at cyto-tDNA in LEM-nes and control conditions, respectively. Inlets: enlarged of cyto-tDNA in yellow squares of big images. Scale bar: 10 μm in big images, 1 μm in inlets. Big images: max. intensity projected, inlets: single-z focus images. (B) Quantification of cyto-tDNA foci with presence and absence of Lap2β per condition. (C) Quantification of cells containing different number of cyto-tDNA foci relative to GFP positive cells. (D) Dot plot for number of cyto-tDNA foci per cell per condition. Dot plot of log (10) total intensity (E) and size (F) of cyto-tDNA foci (Lap2β positive and negative) per condition. (B, C) *Chi-square* statistic test was used. (D, E, F) Data follow Gausian distribution; *t-test* was used; n: number of foci and cells of 3 experiments, 27-60 cells per experiment. (D, E, F) Size and intensity were measured using Diatrack version 3.05 (parameters: click subtract background, filtered data 0.5, trash dim: 15, trash blurred changing among images: 0.05-0.08). Blue line: mean value; error bar: SD.

2.5.2. LEM-domain OE increases the frequency of cells containing multiple cyto-tDNAs.

The LEM-nes or control constructs were transfected into asynchronous U2OS cells. 48 h later the cells were fixed and then hybridized with probes to recognize the C-strand of telomeric DNA. The 48 h time point was analysed due to the observation of the impact of LEM-domain OE on pLacO DNA at that time point (Fig. 2.10). In addition to FISH, Lap2β was stained. The anti-Emerin stain was not examined as immunostaining with this specific antibody was incompatible with FISH.

We observed a decrease in co-localization of Lap2β with cyto-tDNA in LEM-domain OE (27.9%) versus control at 48 h (48.9%), similar to the Emerin effect on pLacO DNA (Fig. 2.11.A, B). Remarkably, the frequency of cells containing more than one focus of cyto-tDNA increased significantly in LEM-domain OE (32.3%) versus control (20.6%) 48 h after transfection (Fig. 2.11.C). Additionally, the number of foci per cell in the LEM-domain OE was significantly higher than in the control (Fig. 2.11.D). These data indicate different change possibilities in LEM-domain OE: 1) de-clustering of cyto-tDNA foci if they are indeed in cluster form or; 2) higher generation of cyto-tDNA.

The size and intensity of cyto-tDNA foci was also measured using the Diatrack v3.05 (Vallotton & Olivier 2013) software. On average, the intensity and size of cyto-tDNA was similar between the two conditions (Fig. 2.11.E and F). By fitting the intensity histogram to a gamma distribution, both data sets (LEM-domain OE and control conditions) fit better to a multiple gamma distribution than a single gamma distribution (R2 =0.89 versus 0.54 in the control; R2 = 0.8 versus 0.65 in LEM-domain OE, Fig. 2.12.B, C). Interestingly, both data sets showed periodicity in their distributions (Fig. 2.12.A). Again this again suggests the possibility of clustering of cyto-tDNA molecules. The multiple gamma distribution fit slightly better with the intensity data set in the control condition than the in LEM-domain OE condition (R2 = 0.89 versus 0.80, Fig. 2.12.C). A single gamma distribution fit better with the LEM-domain OE data than the control (R2 = 0.65 versus 0.54, Fig. 2.12.B). These data suggest a diminished periodicity in the intensity distribution when the LEM-domain is overexpressed. Hypothetically, if cyto-tDNA molecules cluster in the cytoplasm of U2OS cells, the data indicates cluster destabilisation in the LEM-domain OE condition. However, more data points should be added to avoid any possible noise peaks caused by scattered data points and improve confidence in claims about the clustering of cyto-tDNA molecules.

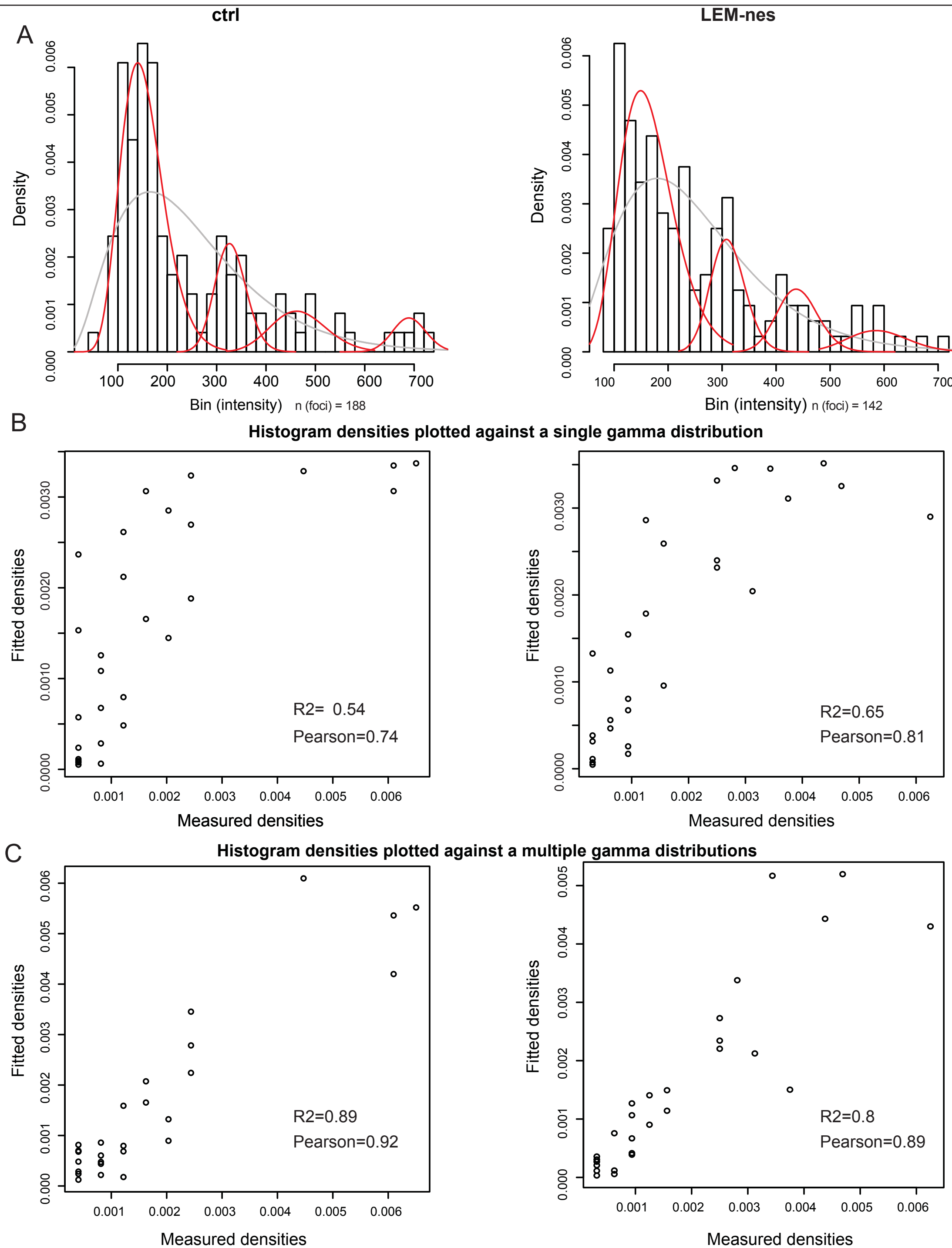

Fig. 2.12. Fluorescence intensity distribution of cyto-tDNA foci in the control fits better with a multiple gamma distribution than that in the LEM-domain overexpression condition.
(A) Fluorescence intensity histograms of cyto-tDNA foci in control (left panel) and LEM-nes (right panel) in the intensity range (0-800 (AU)), containing most of the data points (> 85%). Intensity bin width is 20 AU. Data sets were fitted to a four time gamma distributions (red line) and a single gamma distribution (gray line) using R (R Development Core Team 2012). The boundaries for the periodic distributions were loosely set, allowing the script to find the best local maxima and corresponding peak widths. The histogram densities are plotted against the corresponding density samples from a single gamma distribution fit (B) and a four gamma distribution fits (C). R2 and the Pearson correlation (R) between the histogram densities and the sampled fits were calculated using R. (Data plotting and curve fitting were carried out by Anne Cornelis Meinema)

3 Discussion

3.1.Single-cell FISH analysis exhibits the cytoplasmic localisation of extrachromosomal telomeric DNA in U2OS and HeLa cells.

Using the FISH method to visualize telomeric DNA, we have shown that there are two forms of telomeric DNA in the cytoplasm of U2OS and HeLa cells: 1) one or multiple telomeric FISH signals within micronucleus-like structures (termed mnls-tDNA); and 2) any cytoplasmic telomeric FISH signal that does not belong to the first pattern (termed cyto-tDNA). In this work, we quantified for the first time the presence and abundance of cyto-tDNA in single U2OS and HeLa cells with the FISH method. Cytoplasmic localisation of ec-tDNA was also reported in several previous publications, however no quantification was presented in single cells (Ogino et al. 1998; Tokutake et al. 1998). Rather, the amount of localisation was measured based on the average of cell populations through cellular fractionation coupled with Southern blotting (Ogino et al. 1998). Using the FISH method, the presence of extrachomosomal tDNA (ec-tDNA) in the cytoplasm of a single cell was noticed but not quantified (Tokutake et al. 1998). Interestingly, the observed FISH signals in the cytoplasm were TRF1 negative (Tokutake et al. 1998), which is similar to our cyto-tDNA structure (TRF2 negative). As a result of employing single-cell analysis, we could detect and characterize cytoplasmic localisation as well as assess the abundance of cyto-tDNA in individual cells and at different cell phases. In fact, our analysis reveals the heterogeneity of cyto-tDNA in individual U2OS and HeLa cells (see the following), and this might provide insights into roles or impacts of cyto-tDNA specifically or ec-tDNA at the single-cell level (also discussed in the next section).

3.2. Abundance of cyto-tDNA seems to be correlated with an abundance of total ec-tDNA in the cell.

Cyto-tDNA in the ALT U2OS cells was at a much higher frequency and with greater FISH signal intensity (see Appendix) than in the telomerase positive (non ALT) HeLa cells. This finding is consistent with the characteristic of ec-tDNA, in general, that total ec-tDNA (from nuclear and cytoplasmic pools) are abundantly present in ALT tumor cells and hardly detectable in

telomerase positive cancer and normal cells. Thus, cyto-tDNA may be representative of ec-tDNA for the whole cell or may even be proportional to ALT activity at a certain threshold as shown for partially single-stranded (CCCTAA)n telomeric DNA circles (C-circles) (Henson et al. 2009). If so, the abundance of cyto-tDNA should be 1) much higher for ALT cells than non ALT cells, i.e., many ALT and non ALT cells should be tested; 2) increased upon ALT activation, e.g., a primary cell should be cultured for a long time until overcoming the crisis stage; and 3) decreased if ALT activity is inhibited (Henson et al. 2009).

Although ec-tDNA was not often determined present in non ALT cells, it was still occasionally detected in non ALT cancer and normal cells when using the following techniques: long exposure of 2D agarose gels coupled with Southern blotting of HeLa (Wang et al. 2004) and Chinese Hamster cell extracts (Regev et al. 1998); and rolling-circle amplification of the DNA isolates from normal human fibroblasts MJ90 (Vidacek et al. 2010). Again, these studies all used “ensemble” analysis. Therefore, generally, the detection of ec-tDNA and that of cyto-tDNA may specifically depend on the way of analysing. Single-cell analysis seems to be beneficial for detecting low abundant molecules. Until now, to the best of the author’s knowledge, there is only one publication in the literature quantifying the amount of extrachromosomal DNA molecules in single cells using Halo-FISH, a FISH-based agarose gel technique. With this method, ec-tDNA was gently separated from chromosomes of agarose embedded cells, then discerned by FISH method in certain primary and telomerase-positive cells (Komosa et al. 2015). The analyzed cells were not intact any longer because of the lysation and de-proteination steps before performing FISH. Hence, cellular localisations of ec-tDNA were not observed. Through using the FISH technique on intact cells, we established cytoplasmic localisation of ec-tDNA in individual ALT and nonALT cells, U2OS and HeLa K, respectively. Physiologically, they could in theory be expected in any kind of mammalian cell and might be the result of abrupt telomere shortening as proposed by Vidacek et al. (Vidacek et al. 2010).

3.3. What could be mechanisms for the existence of ec-tDNA in the cytoplasm?

We observed that the faction of U2OS cells containing cyto-tDNA foci were higher in telophase than in interphase. This could suggest that one possible mechanism for the presence of ec-tDNA in the cytoplasm could be that nuclear ec-tDNA molecules lag behind

the main mass of segregating chromosomes based on the lack of centromeres and end up at the cytoplasm after mitosis as the formation of micronuclei. However, we also cannot exclude the possibility of extrusion of nuclear ec-tDNA from the nucleus during interphase as proposed for double minutes (Shimizu et al. 2000; Shimizu 1998; Shimizu 2011). A system that allows tracking telomeric DNA in living cells, such as using CRISPR-Cas9 system (Chen et al. 2013), could help to shed light on their formation time window.

Cyto-tDNA could be the result of nuclear ec-tDNA escaping from the nucleus. The exact localisation of ec-tDNA in the nucleus is not clearly understood thus far. There is evidence for the possibility that ec-tDNA is associated with nuclear APBs (ALT-associated PML (promyelocytic leukemia) bodies) (Fasching et al. 2007; Nabetani et al. 2004). Fasching *et al.* observed linear ec-tDNA in the factions of APB extracts of cystic fibrosis JFCF-6 cells using Southern blotting with TelG probes on 2D agarose gels. Nabetani *et al.* revealed the colocalisation of telomeric DNA, which is next to metaphase, spread on chromosomes with PML protein, a component of APBs, using the IF-FISH method with TelC probes in GM847 cells (Fasching et al. 2007; Nabetani et al. 2004). As such, ec-tDNA with any strand could be expected in theory in APBs. Human APBs localise within the nuclear interior and show no preference to nuclear peripheral localisation (Draskovic et al. 2009). That all said, the function of APBs in ALT cells remains an enigma. There are different hypotheses that have been given (see review (Draskovic & Vallejo 2013)). First, they might serve as a platform for telomeric DNA recombination by sequestering chromosome ends for recombination as clusters of telomeres were found in APBs (Yeager et al. 1999; Draskovic et al. 2009). Therein, ec-tDNA is speculated to either participate in recombination processes to elongate telomeres or is just a by-product of the processes. Furthermore, APBs could also be involved in removing these by-products because there is evidence that PML nuclear bodies could be the sites of intra-nuclear proteolysis (Lallemand-Breitenbach et al. 2001). Second, APBs might prevent inappropriate DNA damage responses by recruiting linear ec-tDNA with unrepaired ends (Fasching et al. 2007). Nonetheless, the localisation of ec-tDNA in APBs still needs to be confirmed. Perhaps there is still a large amount of ec-tDNA that resides somewhere else in the nucleus, such as the nucleoplasm or nuclear periphery, as in the case of localisation of mammalian telomeres. In contrast to yeast telomeres which all cluster and localise at the nuclear periphery (Taddei et al. 2004), only a certain proportion of mammalian telomeres are found at the nuclear periphery while the rest are in the nuclear

interior (Gonzalez-Suarez et al. 2009; Ottaviani 2009; Chuang et al. 2004; Weierich et al. 2003; Molenaar et al. 2003). The anchoring of human telomeres to the nuclear envelope can be mediated by the targeting of subtelomeres containing repetitive DNA sequences, referred to as D4Z4 repeats by LaminA (Guelen et al. 2008; Ottaviani et al. 2009), or directly anchoring telomeres to the nuclear envelope by Lem2, a LEM-domain INM protein (Gu et al. 2017; Braun & Barrales 2016).

3.4. Cyto-tDNA seems to partition asymmetrically in two sister cells during mitosis, suggesting elimination of cyto-tDNA from one of the two daughter cells during mitosis.

The heterogeneity of cyto-tDNA is also exhibited in its asymmetric distribution in the two sister cell halves in telophase U2OS cells. Cyto-tDNA foci distributed preferentially to the young centrosome containing cell halves. This pattern seems to be conserved with the partition of transfected plasmid DNA foci in HeLa and MDCK cell lines (Wang et al. 2016) and may apply to any kind of ecDNA despite its origins and sequences. The mechanism of asymmetric segregation of plasmid DNA could be applied to cyto-tDNA as well where plasmid DNA and the young centrosome are in close proximity before nuclear envelope breakdown and the old centrosome moves away from the young centrosome and plasmid DNA during mitosis (Wang et al. 2016). Quite interestingly, HeLa cells containing cytoplasmic plasmid DNA seem to have longer cell cycles than those without DNA foci, suggesting less division potential of the cells with plasmid DNA (Wang et al. 2016). Correspondingly, in budding yeast *S. cerevisiae,* ERCs, plasmids, aggregated and damaged proteins, and damaged mitochondria are inherited preferentially by the mother cell that harbours the young spindle pole body and is characterized by having reduced division potential (Denoth-Lippurer at el. 2014, Erjavec et al. 2008, Clay et al. 2014, Zhou et al. 2014, Saarikangas and Barral 2015, Aguilaniu et al. 2003, McFaline-Figueroa et al. 2011). Taken together, a number of speculations regarding cyto-tDNA can be drawn: 1) cyto-tDNA also negatively affects cell division potential; and 2) it is removed from one of the sister cells by asymmetric segregation during mitosis in mammalian cells, leading to two subpopulations - with and without cyto-tDNA . If there is a limited amount of cyto-tDNA, eventually it will be eliminated from the population. However, the abundance of cells with cyto-tDNA (50% of interphase U2OS cells) could be explained by a high formation rate. It would be of interest to investigate the biological relevance of cyto-tDNA, ec-tDNA and ecDNA in mammalian cells, for example,

to examine their relationship with aging by comparing the number of divisions and division kinetics of cells with and without them.

3.5. Heterogeneity of cyto-tDNA in membrane compartmentalization.

Through characterizing specific membrane proteins localizing with cyto-tDNA, we found ER proteins (Sec61 and Careticulin (see Appendix)) and Lap2β protein colocalizing with approximately 50% of the cyto-tDNA. This suggests there is a membrane structure composed of certain components of the nuclear envelope surrounding roughly 50% of cyto-tDNA. This membrane structure is reminiscent of the membrane structure surrounding plasmid DNA (Wang Thesis 2015). Remarkably, the membrane seems to be devoid of NPC, LBR, and Lamin B1. The absence of these proteins at plasmid DNA foci was also discussed in Xuan Wang's PhD Thesis (Wang Thesis 2015). In brief, H3 was not found at cyto-tDNA foci could provide a rationale for the absence of LBR because LBR links with DNA through its partner heterochromatin proteins, HP1 and H3, with trimethylation at Lysine 9 (H3K9me3), complex. Consequently, the recruitment of Lamin B1 to the membrane, mediated by LBR, might fail. ELYS is an initiator for NPC formation, which here, was not found at the cyto-tDNA foci and may have resulted in the absence of NPCs. Furthermore, the lack of H3 and ELYS at cyto-tDNA foci also indicated differences in the heterochromatin structure of cyto-tDNA from chromosomal telomeres.

Why does the cell isolate cyto-tDNA and plasmid DNA through a membrane? Theoretically, trapping them in such a membrane compartment might help separate them in space from the chromosomes. The isolation, on one hand, may protect the chromosomes from any threat, and, on the other hand, also safeguard cytoplasmic ecDNA from immediate degradation. Such separation in a membrane structure may be faster and consume less energy than immediate degradation requiring recruitment of degradation systems to the DNA sites or vice versa or an activation of nuclease protein expression. Furthermore, instead of immediate degradation, the membrane might serve as a storage system and/or slow sorting system, allowing the cell to consider whether to either eliminate or keep such ecDNAs. They could in fact be beneficial for individual cells or for the entire population when facing fluctuating environments. For example, aggregated prion-like proteins stored in aged budding yeast cells are proposed to spread to new born cells to promote faster adaptation to the changing

environment (Newby & Lindquist 2013; Saarikangas & Barral 2015). During mitosis, the link to such ER-derived membranes may aid in constraining their movement to ensure asymmetric segregation, as shown for plasmid DNA (Wang Thesis 2015; Wang et al. 2016). From an immunological point of view, this behavior could “calm down” immune responses, or in other words, negative regulation of immune responses because overstimulation of innate signaling pathways may facilitate autoimmune disease (Yoshida 2005). Therefore, it would be very interesting to evaluate whether membrane compartmentalization of cytoplasmic DNA also takes place in single immune cells (which is still not clear so far) or only in STING negative cells. Vice versa, we also can ask whether STING is indeed expressed in ALT cells. Then it would be imagined that cyto-tDNA can be a substrate for DNA sensing pathways, leading to preventing ALT cancer cell from proliferation.

Yet, we also detected nearly 50% of cyto-tDNA foci that were not associated with the aforementioned membrane proteins. It could be that they may reside in different membrane compartments, such as the lysosome. If they are in contact with the lysosome, they could be already undergoing the degradation process (Pu et al. 2016).

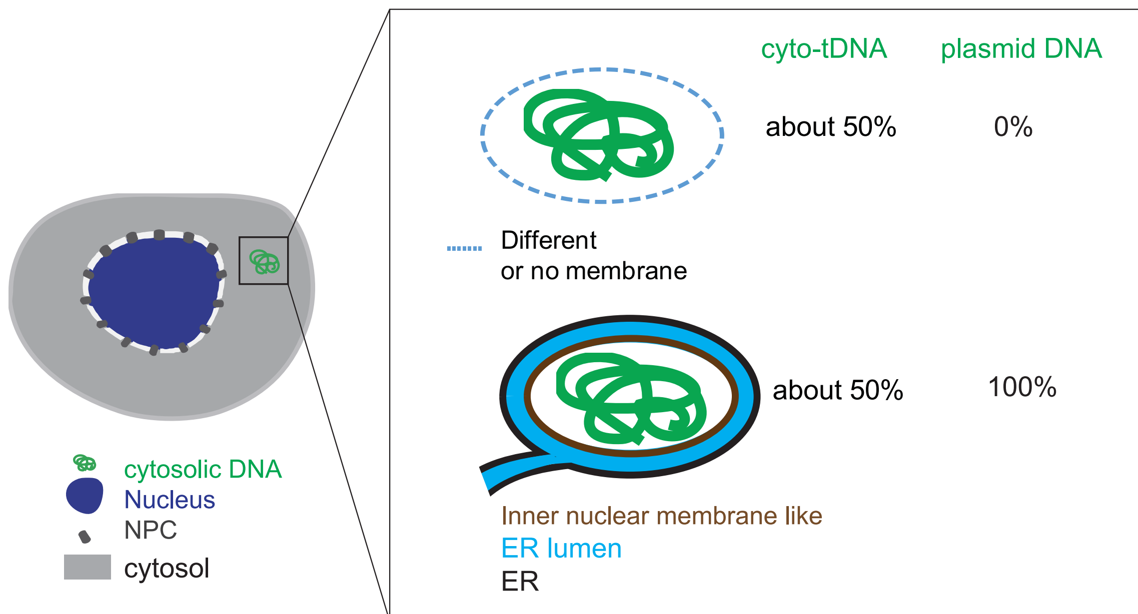

Fig. 3.1. A model: A comparison of membrane association of cyto-tDNA and plasmid DNA in mammalian cells.
A cell with DNA foci in the cytoplasm (left) and the enlarged images of the DNA foci (right).

3.6. Reduced or absence of LEM-domain proteins at plasmid clusters seems to affect the maintenance and/or formation of placO DNA clusters and probably also cyto-tDNA.

We found that overexpression of the soluble LEM-domain of Emerin caused a reduction in the frequency of HeLa cells containing pLacO foci. LEM-domain overexpression led to an increase in the fraction of small foci over time as well as more foci per cell. This data strongly suggested that membrane structure may play a role in plasmid cluster maintenance and probably also formation of plasmid DNA foci. Degradation might take place in addition to de-clustering. In fact, there is a correlation between BAF knockdown with a decrease in Emerin assembly and an increase of LC3 localisation at the DNA beads (Kobayashi et al. 2015). However, degradation alone cannot be the interpretation because it does not explain the result of more foci per cell. In the same line, Wang employed a different method to perturb the membrane structure (Wang Thesis 2015). The principle of this technique was to modify BAF properties locally at the pLacO DNA foci. The expected consequence of this was the detachment of BAF and ultimately LEM-domain proteins via BAF from pLacO DNA foci. Indeed, preliminary data demonstrated a reduction of the enrichment of Emerin at plasmid DNA clusters in the perturbation condition. Moreover, LacI protein intensity (reporter of LacO DNA) was reduced dramatically over time, and ultimately disappeared completely (Wang Thesis 2015). However, it remains to be seen whether the change in LacI intensities really reflects the disappearance of LacO DNA. Altogether, the data presented here along with Wang's data (Wang Thesis 2015) indicates that a reduction or absence of LEM-domain proteins at plasmid clusters can affect the maintenance and/or formation of placO DNA clusters. Thus, LEM-domain proteins seem to play an important role in stabilizing membrane structure at the DNA foci and/or clustering process of such foci.

For cyto-tDNA, de-clustering can also be an interpretation but the circumstance might even be more complicated. Unlike the results with plasmid DNA, the intensity and size of cyto-tDNA were not obviously different between the LEM-domain overexpression and the control. Could it be that only approximately 50% of cyto-tDNA foci associated with the membrane hinder the clear changes to be observed or might other reasons exist? Additionally, what was the basis of the frequency of cells containing cyto-tDNA not changing significantly in the LEM-domain overexpression compared to that in the control? One possibility is that LEM-

domain overexpression somehow enhances the generation of cyto-tDNA in the cytoplasm which compensates for degradation/breaking apart. In fact, LEM-domain proteins have been proposed to have an important part in silencing and nuclear-periphery tethering of repetitive heterochromatin telomeres and centromeres. Silencing of repetitive DNA seems to critical to the prevention of diseases (Padeken et al. 2015). Dissecting functions of different domains of Lem2, which contains two conserved structural domains (the N-terminal LEM-like domain and the C-terminal MSC (MAN1-Src1 C-terminal) domain) in fission yeast shows that the LEM and MSC domains are necessary for tethering and silencing of centromeric chromatin, respectively (Harami et al. 2013; Braun & Barrales 2016). At the telomeres, Lem2 is proposed to mediate both anchoring and silencing through its MSC domain (see review (Braun & Barrales 2016)). Hence, the overexpresssed LEM-domain may also compete with the LEM-domain proteins in the inner nuclear membrane, probably leading to an detachment and/or transcription activation of the telomeres. Consequently telomeric heterochromatin stability may be afftected, resulting in ec-tDNA formation. It would also be intriguing to assess whether transcription could take place in the plasmid or cyto-tDNA in the case of the LEM-domain overexpression as this would provide insights into the functions of membrane compartmentalization of cytoplasmic DNA.

3.7. LEM-domain proteins appear to play an important role in the membrane structure at the plasmid DNA and cyto-tDNA.

How does LEM-domain protein perturbation affect the membrane structure of plasmid DNA and cyto-tDNA foci in the cytoplasm? This is yet to be determined. In mammals, LEM-domain proteins seem to have redundant functions, including tethering chromatin to the nuclear envelope and gene expression regulation as well as nuclear envelope integrity (Brachner & Foisner 2011). It has been recently demonstrated that Lem2 proteins are involved in nuclear envelop closure in fission yeast and humans. Lem2 recruits the ESCRT-II/ESCRT-III (endosomal sorting complexes required for transport III) endohybrid protein Cmp7/ CHMP7 and downstream ESCRT-III proteins to holes in the nuclear membrane during fission yeast/ human cells (Gu et al. 2017). Therefore, one possibility is that our perturbation of LEM-domain proteins caused a breach in the membrane structure surrounding plasmid DNA and cyto-tDNA, leading to the breaking apart of the membrane together with DNA foci. With this, it was shown that overexpressed Lem2p or Man1p in fission yeast causes nuclear

envelope malformation, like appearance of the cytoplasmic spheres and/or the intranuclear membrane stacks at the nuclear periphery. In addition, this “mini-micronuclei” also contain DNA (Gonzalez et al. 2012). The reverse means of perturbing Lem2, Lem2 deletion, also leads to abnormally shaped nuclei in human cell lines (Ulbert et al. 2006). The irregular shapes of the nuclear envelope start with invaginations of the nuclear envelope followed by a severe modification in overall structure (Braun & Barrales 2016). Collectively, the amount of Lem2 or LEM-domain protein could be strictly regulated in the cell and any change can affect the stability of membrane in the nucleus as well as the membrane of cytoplasmic DNA foci.

In addition, it is not known how the LEM-domain overexpression competes with LEM-domain proteins. Are they dynamically exchanged with LEM-domain proteins in interphase or recruited to the pLacO clusters during nuclear envelope assembly after mitosis? Are they competing with the LEM-domain proteins at the time of cluster formation? Michael Burger (Burger Thesis 2014) reported that when pLacO plasmids were transfected by lipofection into the cell before the LEM-nes construct, the frequency of cells containing plasmid DNA were not affected 36 hours post-transfection compared to the control (Burger 2014). This indicates that if pLacO clusters are already in an ER membrane structure, the LEM-domain overexpression seems not able to exchange efficiently with LEM-domain proteins during interphase.

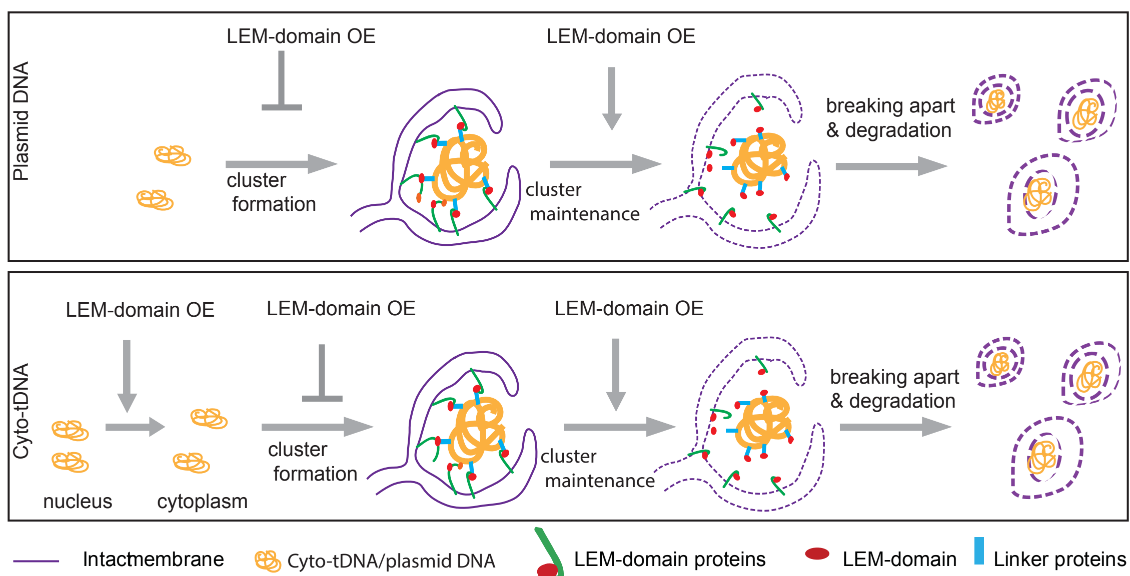

Fig. 3.2. A model: The LEM-domain overexpression affects formation and maintenance of cytosolic plasmid and cyto-tDNA foci.

3.8. Closing remarks

In this thesis work, we have investigated how mammalian cells handle endogenous cytoplasmic ec-tDNA, focusing on cyto-tDNA in comparison with exogenously delivered plasmid DNA through transfection. Cyto-tDNA showed some similarities as well as differences when compared with cytoplasmic plasmid DNA. Examples of similarities include: 1) roughly half of the analyzed cyto-tDNA foci and plasmid DNA co-localised with nuclear membrane proteins; and 2) a small fraction of the cyto-tDNA colocalised with plasmid DNA in apparently the same cytoplasmic membrane compartment . It appears there is cellular machinery such that the membrane encloses possibly any type of cytosolic DNA with a nuclear envelope-like membrane despite their origins. The formation of membrane may be mediated by LEM-domain proteins that can bind indirectly or directly to DNA though BAF which binds with unspecific DNA sequences. Differences between these two ecDNA types were also exhibited: 1) at approximately half of all cyto-tDNA foci, a plasmid-like membrane was not detectable, which might even suggest the complete absence of a membrane around such cyto-tDNA; and 2) while 100% of Hela cells contained 100% plasmid DNA foci surrounded by the membrane, there was a diversity of interphase U2OS cells with this characteristic; and 3) In about one third of the cell population, Lap2β was absent from all of the cyto-tDNA foci in a cell, even that of very big foci, while another third of the cell population featured Lap2β on all cyto-tDNA foci of a cell, even those of very small sizes, and the final third of the population had a mixed situation – either presence or lack thereof of Lap2β . This also suggests a different machinery that distinguishes between endogenous and exogenous DNA in the cytoplasm. How do these machineries coordinate? They may act upon different markers of ecDNA. We demonstrated that single-stranded or double-stranded forms of cyto-tDNA do not distinguish cyto-DNA molecules for being associated with membrane. The determinant for membrane association could be epigenetic markers on the DNA or histones or even the presence of telomere-binding proteins. In this context, human telomeres are known to be enriched with H3K9-me or H4K20-me, low density of acetylated H3 and H4, and bearing no DNA methylation because of the lack of CpG motif on the sequence (see review (Blasco 2007)). Additionally, telomeric-specific binding proteins, such as shelterin mentioned in the Introduction to the present work, might contribute to the recognition of different machineries. There are probably a variety of ways ec-tDNA is formed as described previously, such as replication slippage, dissolving of the t-loop structure, or replication

fork stalling, and these could result in different types of ec-tDNA carrying unique markers that are consequently recognized by certain machineries in the cell. In addition, ALT U2OS cancer cells with mutated genomes can differentiate their population into subpopulations that possess one or both of the active machineries. Using non cancer cell lines or primary cells will solve this problem. Further work is necessary to decipher the link between these machineries, to identify the mechanism of membrane enclosure of cytoplasmic DNA, especially in the light of the formation of a nuclear envelope, and to identify the mechanism and its components for the differentiation between endogenous and exogenous ecDNA. It would also be rather beneficial to examine other types of endogenous ecDNA, such as ecDNA arising from ribosomal DNA or satellite DNA.

4 Material and methods

4.1. Mammalian cell culture

HeLa Kyoto (HeLa K), telomerase positive cells were a kind gift from from P. Meraldi (ETHZ, Switzerland) which was originated from S. Narumiya, Kyoto University, Japan, (http://web.expasy.org/cellosaurus/CVCL_1922). U2OS osteosarcoma cells, ALT cells, were a kind gift from C. Azzalin, ETHZ, Switzerland which was originated from A. Londono Vallejo. All these cells were cultured at 37°C with 5% CO2 in a humidified incubator in the basic medium which is Dulbecco's modified medium (DMEM) with high glucose (cat. 41965-039, Gibco™, Thermo Fischer Scientific) plus 10% FCS (A15-101, PAA) , P/S (100 U/ml penicillin and 100 mg/ml streptomycin) (cat. 15140122, Gibco™, Thermo Fischer Scientific). HeLa cells stably expressing NLS-lacI-mCherry (internal ID: HeLa#C1, RK nr. 126) which were created by X. Wang in our group were cultured in the basic medium plus 5µg/ml blasticine (cat. R210-01, Gibco™, Thermo Fischer Scientific) (Wang Thesis 2015).

4.2. DNase I enzyme treatment prior to FISH

Cells were fixed with methanol for 10 min at -20°C and then washed three time in 1X PBS. Cells were permeabilized with 0.5% Triton X-100 for 10 min, then incubate with 0.5 unit/µl DNase (M0303L, BioConcept) in 1X PBS for 2 to 2.5 h at 37°C .

4.3. Native FISH, regular FISH and IF-FISH

Regular FISH: (was modified from (Lansdorp et al. 1996)) Cells were rinsed briefly in PBS before fixation. The cells were fixed in 2% paraformaldehyde (PFA) in 1x PBS pH 7.4 for 10 min at room temperature (RT) or in 100% methanol for 10 min at -20°C. Cells were rinsed in 1x PBS three times for 5 min and fixed again for 10 min in methanol at -20°C if they were fixed with 2% PFA before. Cells were incubated with 0.2% Triton X-100 for 10 min, then treated PBS containing 20 mg/ml RNAase (EN0531, Thermo Scientific) at 37°C for 30 min to 1 h. PNA probes were diluted to 20 nM concentration in hybridization solution (70% deionized formamide (GHYFOR01, Eurobio), 0.5% blocking reagent (11096176001, Roche), 10 mM Tris-HCl (pH 7.2)). DNA on cell is denatured on heat plate at 80°C for 3 min and then incubated humid chamber in dark for 2 h at RT. Leave at RT in a humid chamber (in the dark) for 2 h. Cells were washed with hybridization wash solution 1 (10mM

tris-HCL (pH 7.2), 70% formamide and 0.1% BSA) for two times, 15 min each time at RT and with hybridization wash solution 2 (100 mM tris-HCL (pH 7.2), 0.15 M NaCl and 0.08% Tween-20) for three times. The nuclei were stained by Hoechst33342 (cat. 62249, Thermo Fisher) for 3 min at RT in 1X PBS and rinsed once with 1X PBS. Coverslips containing cells were mounted with Mowiel with 1.4% w/v DABCO and sealed with nail polish.

Native FISH: was regular FISH without the denaturation step.

For IF-FISH (for both regular and native FISH): After the RNase treatment step, cells were blocked with 5% BSA in 1X PBST for 1 h at RT. Afterwards, cells were incubated in diluted primary antibody in 1% BSA in 1X PBST in a humidified chamber for 1 h at room temperature and afterwards with the secondary antibody (1:500 for each) in 1% BSA/1X PBST for 1 h at room temperature in dark. Cells were fixed with 4% paraformaldehyde for 7 min, then PFA was quenched with 5% BSA in 1X PBS and 20 mM glycine for 30 min. Then cells were hybridized with probes as described above.

4.5. Cell synchronization

HeLa cells stably expressing NLS-lacI-mCherry were synchronised using double thymidine treatment. Cells were treated with thymidine 2 mM for 16 h and released for 8 h. For LEM-nes overexpression experiment in Fig. 2.10, LEM-nes and GFP constructs were lipofected in cells 1 h after the first release. After 8 h of the first thymidine release, the second time with thymidine was treated for 17 h. Thymidine was washed off and 2 h later were electroporated.

4.6. Plasmid transfection

Plasmid preparation

Plasmid DNA was purified from bacterial using QIGEN plasmid kits (cat. 12143 for midi kit or 12125 for mini kit). Afterwards, the plasmid DNA solution was span down for 10 min at maximum speed and the liquid was transferred to new tube (2 ml) and at least 1 volume of Phenol/Chloroform/Isopropanol (25:24:1) was added in. The mixture was span down for 10 min by 12 000 rpm and the aqueous phase was transferred into a new tube. DNA was precipitated by 1/10 5M NH4Ac and 2.5 X volume of pure ethanol mix at -20°C for 30 min. The DNA pellet was collected by centrifugation 10 min at maximum speed at 4°C and washed with ice cold 75% ethanol. The washed DNA pellet was resuspended

in ddH2O of appropriate volume. Plasmid concentration was measured by a NanoDrop Spectrophotometer.

Lipofection: Plasmid was transfected into cells using X-tremeGENE™ 9 DNA Transfection Reagent (cat. 06365787001, Roche). The plasmid: transfection reagent ratio (w:v) was 1:3. Plasmid DNA concentration was 25 ng per (cell culture dish/ plate surface area (cm^2).

Electroporation: Transfection was conducted by an ElectroMicroporator (MP-100, Digital-Bio Technology) with provided buffers. The electroporation parameters were 1000 V, 30 ms and 2 pulses for 10 μl electroporation tips. The plasmid dosage was 250 ng per 10^5 suspension cells in provided R buffer. Electroporated cells with same condition but for different time analysis were collected in a same tube and then splited on coverslips 10^5 cells per a coverslip ϕ 12 mm to ensure an even distribution of electroporated cells for each time point.

4.7. Fixed Cell Imaging

Images were acquired using 60x NA objective 1.42 on a Deltavision microscope (Olympus), equipped with a CoolSNAP HQ2 camera (Roper Scientific) with 0.2 μm steps.

Images acquired from DeltaVision microscope were deconvolved using Softworx (Applied Precision).

4.8. Image analysis

For plasmid DNA cluster size (in Fig. 2.10.F) images were analyzed using software Fiji by choosing the longest diameter of each cluster for measurement.

The intensity and area of telomeric FISH signals per cell (in Fig. 2.2, Fig. 2.11, Fig. 2.12) were measured using Diatrack v3.05; raw images were loaded on Diatrack software. The setting parameters indicated in the specific figures.

Telomeric FISH signals were set with certain display ranges with Fiji for analysis: 100-300 for CCD camera (for images acquired before March, 2016) and 100-1000 for CMOS camera with images (for images acquired from March, 2016).

Colocalisation analysis: Fluorescence signals of stained proteins were boosted until see background level of the cell's cytoplasm. Proteins colocalisation at telomeric DNA was

defined as if the signals at the telomeric DNA were visually higher from the background in the boosted setting.

4.9. Centrosome classification

Max projected images were used to measure fluorescent intensity of Odf2 staining. The young centrosome (Y) is identified as the one with less bright signal and the old centrosome (O) is identified as the one with brighter signal. The areas of two centrosomes and the two adjacent background areas were segmented with same sizes (in squares). The signal ratio y/O was calculated as the background corrected intensity of the young centrosome versus the background corrected intensity of the old centrosome. Cut off level is 0.85.

4.10. Statistics

The statistical methods are indicated in the figure legends. Data were tested with Gaussian distribution for normality (*D'Agostino & Pearson* normality test) ($\alpha=0.05$)), if t-tests were used. If non-normal data was presented, Mann-Whitney test was used. The signs are as follows: *: $p<0.05$; **: $p<0.01$, ***: $p<0.001$, ns: not significant. The Pearson correlation coefficient, r, is to measure of the strength of a linear relationship between measured densities and theoretical fitted densities based. The coefficient of determination, R^2, was the proportion of the variation in a response variable that was explained by a fitted statistical model. $0=<R^2<=1$. The higher the R^2, the better the model fits the data (PrometheusWiki).

Table 4.1. Antibodies

Antigens	supplier	host	fixation	dilution
Calreticulin	Abcam (ab16048)	rabbit	formadehyde	1:500
LBR	Abcam (ab122919)	rabbit	MeOH	1:500
ELYS	Iain Mattaj (EMBL Heidelberg, Germany)	rabbit	formadehyde	1:200
H3	ab1791, abcam	rabbit	formadehyde	1:500
LaminB1	Abcam (ab16048)	rabbit	formaldehyde	1:1000
Mab414	Abcam (ab24609)	mouse	MeOH	1:1000
Odf2	Sigma (HPA001874)	rabbit	MeOH	1:500
Sun1	Millipore (ABT273)	rabbit	MeOH	1:200
Lap2β	BD transduction laboratories (611000)	mouse	formaldehyde	1:500
human PML protein (N terminal epitope corresponding to aa 37-51)	Santa Cruz Biotechnology (sc-966)	mouse	formaldehyde	1:500
mCherry	Abcam (ab167453)	rabbit	formaldehyde	1:1000
GFP	Roche (11814460001)	mouse	formaldehyde	1:1000
mouse IgG, Alexa-Fluor™ 647	ThermoFischer Scientific (A21236)	goat	--	1:500
mouse IgG, Alexa-Fluor™ 594	ThermoFischer Scientific (A11032)	goat	--	1:500
rabbit IgG, Alexa-Fluor™ 647	ThermoFischer Scientific (A21245)	goat	--	1:500
rabbit IgG, Alexa-Fluor™ 594	ThermoFischer Scientific (A11037)	goat	--	1:500
rabbit IgG, Alexa-Fluor™ 488	ThermoFischer Scientific (A11034)	goat	--	1:500

Table 4.2. FISH probes

probes	fluorescent label	sequence	probe type	lot nr	company	received from
telomeric G	Tamra	tamra-OO-TTA GGG TTA GGG TTA GGG	PNA	SP1462-1R	Biosynthesis	C. Azzalin (ETHZ, Switzerland)
telomeric C	Cy5	cy5-OO-CCC TAA CCC TAA CCC TAA	PNA	F1003	PANAGENE	PANAGENE
LacO	alexa 488	alexa488-OO-GAA TTG TGA GCG GAT AAC AAT T	PNA	130902PO-03	PANAGENE	PANAGENE
scramble	alexa488	Alexa488-OO-GGGTAGGAGGTTAGTGTTTTGAGT	PNA	130902PO-01	PANAGENE	PANAGENE

Table 4.3. Plasmids

plasmid	sources (affiliation)	internal ID (pRK#)
mCherry-Sec61β	T. Kirchhausen (Harvard Medical School, USA) (Lu et al. 2009)	932
pEGFP-LEM-NES	M. Buger (Kroschewski group)	1084
pEGFP-C1	P. Meraldi (ETH Zurich, Switzerland)	980
pEGFP-BAF	M. Buger (Kroschewski group)	1075
pLacO	Susan M. Gasser (FMI, Basel, Switzerland) (Rohner et al. 2008)	960

5 Appendix Results

In this section, some preliminary results of less three experiments are reported.

5.1. FISH fluorescent signal intensity in of cyto-tDNA U2OS is higher than in HeLa cells.

FISH fluorescent signal intensity of cyto-tDNA was measured in U2OS and HeLa cells on sum projection images. The average signal intensity of cyto-tDNAs in U2OS is significantly higher than cytosolic telomeric DNAs in HeLa cells. Possibly there are more telomeric DNA molecules in one cyto-tDNA focus or more big molecules with many telomeric repeats in one focus in U2OS than in HeLa cells. This is the additive evidence for higher amount of cyto-tDNA in U2OS then in HeLa cells.

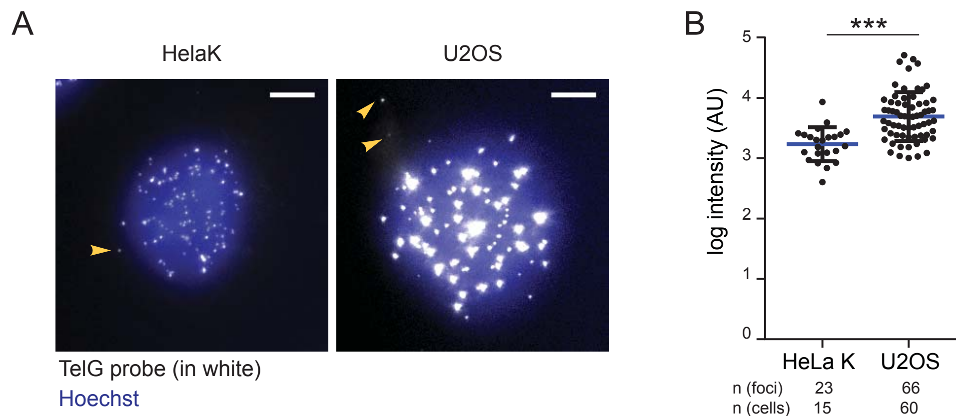

Fig. 5.1. Signal intensities of cyto-tDNA in U2OS is higher than in HeLa.

(A) Max projected FISH images using the same PNA TelG probe concentration (20 nM) in HeLa and U2OS cells. Yellow arrows point at cyto-tDNA. Scale bar: 5 μm. (B) Fluorescence intensities of were measured and subtracted the relative background using manually using Fiji software. Measurement was on sum slice projection pictures; n: cyto-tDNA foci of one experiment.

5.2. Calreticulin, BAF but not LBR, Sun1, nucleoporins with FG repeats colocalise at cyto-tDNA

We have showed that Sec61 (ER transmembrane protein) and Lap2β (inner nuclear transmembrane protein) can colocalized with about 50% of cyto-tDNA foci. Here we preliminarily tested more markers for the membrane structure of cyto-tDNA such as Calreticulin, LBR, Sun1, nucleoporins with FG repeats of NPC. IF-FISH method was carried out to detect concurrently one of these proteins and telomeric DNA.
While Calreticulin, a major chaperone Ca(2+)-binding protein in the lumen of ER (Michalak et al. 2009), LBR (Lamin B receptor) is an inner nuclear protein that links with heterochromatin via HP1 (heterochromatin protein 1) (Güttinger, Laurell, and Kutay 2009). Sun1 and Sun2 in association with nesprins server as a link between the inner and outer membranes of mammalian nuclear envelopes. Sun1 is also a NPC-associated protein (Liu et al. 2007).

We observed about 37.2% of cyto-tDNA foci colocalizing with Calreticulin (n = 43 foci of one experiment), suggesting association of cyto-tDNA with ER protein (Sec61) and ER lumen, thus probably a double membrane structure. We didn't observe any co-localisation of LBR at cyto-tDNA (n = 53 foci of one experiment). One possible explanation for this is HP1 might not be present at cyto-tDNA. In other words, cyto-tDNA is not heterochromatin as telomeres. Nucleoporins with FXFG repeats of NPCs are recognized by mab414 antibody, 94.12% of cyto-tDNA FISH signals were not colocalized with these nucleoporins staining (n = 51 foci of two experiments). In addition 100% of cyto-tDNA foci were Sun1 negative. Almost no detection of the nucleoporins and Sun1 again suggests the absence or very low concentration of NPC at cyto-tDNA. Altogether, the proposed membrane structure at a portion of cyto-tDNA remains the same: double membranes containing ER transmembrane proteins Sec61, luminal ER protein Careticulin and inner nuclear proteins such as LEM-domain proteins, but low copy or absence of NPCs, LBR, Sun1. Again, this seems to be similar to the membrane structure at plasmid DNA.

Overexpressed BAF, one of the linkers of LEM-domain proteins with DNA, colocalised with 25.8% of cyto-tDNA foci (n = 31 foci of one experiment). It is worth to notice that BAF overexpression caused high frequent U2OS cell death. Nevertheless, the presence of BAF at small faction of cyto-tDNA might also account for the presence of LEM-domain protein

which was also not 100% at cyto-tDNA.

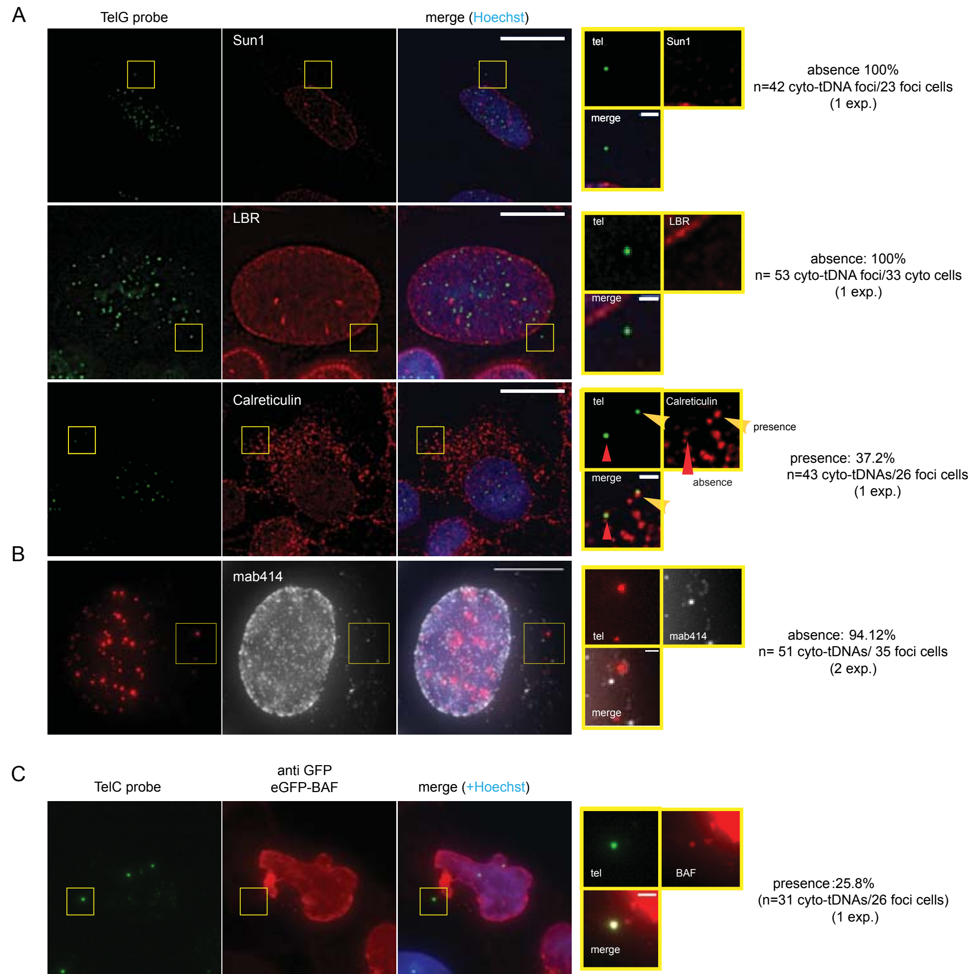

Fig.5.2. Calreticulin, BAF but not LBR, Sun1, nucleoporins with FG repeats seems to colocalise at cyto-tDNA.

Presentative images of the relative colocalisation of indicated proteins with cyto-tDNA using TelG probe (A, B) and TelC probe (B) in U2OS cells. Number of foci and cells as well as the number of experiments were indicated next to the images. Single-z focus of deconvoled images in (A), non-deconvoled images in (B, C). Yellow and red arrowheads in some images points at presence and absence of the protein at cyto-tDNA, respectively. Inlets are enlarged areas of the cyto-tDNA foci indicated with yellow squares of big images. Scale bars in big images: 10 μm, in inlets: 1 μm.

5.3. Drugs inducing replication and translation stress can produces more cyto-tDNA.

Can we modulate the amount of cyto-tDNA? We tested some drugs in order to increase the frequency of cyto-tDNA containing cells in the population and bigger cyto-tDNA foci faction. The cells were treated with cycloheximide (CHX), a well-known protein synthesis inhibitor, and ribonucleotide reductase inhibitor hydroxyurea (HU). Those drugs were tested because it was known that treatment with low doses of HU (50 μm of HU for 4 days) induced about 1.5 fold mount of t-circles in U2OS (Deng et al. 2007) and CHX increases about 20-30 fold of circular ecDNA in general in HeLa and Drosophila cells (Smith and Vinograd 1972; Stanfield and Helinski 1976). The preliminary data showed that with different concentrations and treatment times of CHX and HU, the percentage of cells with cytosolic telomeric DNAs was increased and apparently also the class of the big dots (qualitatively equal to brightest FISH signals in the nucleus) (Fig. 5.3). This suggests that replication and translation stress can produces more cyto-tDNA. Probably this may corresponds with the increase of total extrachromosomal tDNA in the cell.

This might be helpful for further characterization of cyto-tDNA as well as on understanding its formation mechanism. For example, we could compare the membrane structure at new born cyto-tDNA and long-existing cyto-tDNA foci by using drug treatment followed with adding a DNA labeling reagent such as 5-ethynyl-2′-deoxyuridine (EdU) or BrdU, a thymidine analog, in cell culture medium. The new born cyto-tDNA foci can be identified as EdU/BrdU positive.

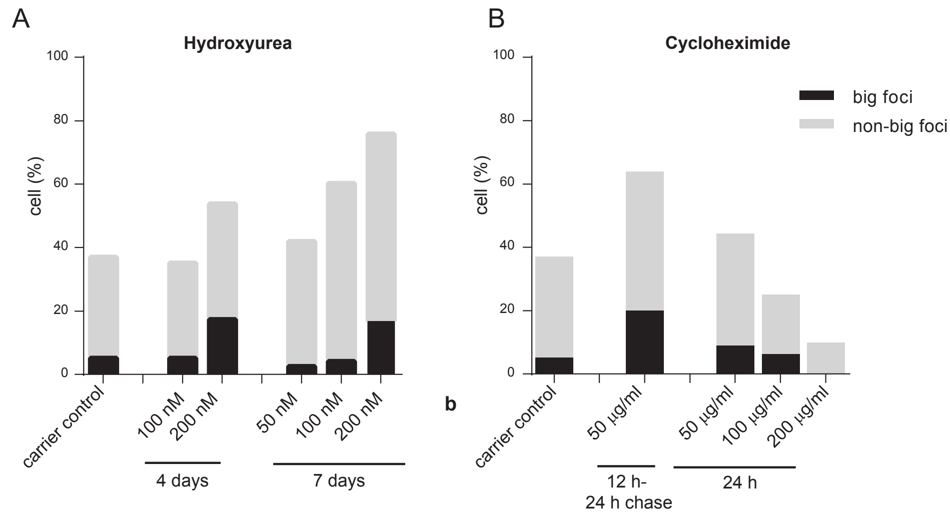

Figure 5.3 Moderate treatment of Hydroxyurea or Cycloheximide can induce more cyto-tDNA in U2OS cells.

Quantification of U2OS cells with cytosolic telomeric DNA and within there with qualitatively big dots (relative to the bright FISH signals in the nucleus). Cell were treated with hydroxyurea (A) or cycloheximide (B) in different concentrations and times prior to fixation. n = 30-63 analysed cells for HU treated conditions and n = 30-117 analysed cells for CHX treated conditions, one experiment.

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