Investigating a role for telomeric transcription in cancer



Sam Dunkley

St. Catherine's College

University of Oxford

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Abstract

A constitutive hallmark of cancers is the extension of telomeric DNA. Approximately 85% of cancers extend their telomeres through the upregulation of the enzyme telomerase; whereas the remaining proportion use a recombination-based method known as Alternative Lengthening of Telomeres pathway (ALT). Tumours that exhibit the so-called 'ALT' pathway include: pancreatic neuroendocrine tumours, astrocytoma and osteosarcoma. The prognosis for many of these tumours is poor and the development of novel therapies is therefore an important unmet need.

Recent work has shown, however, that ALT cancer cells exhibit some characteristic hallmarks which include; a near universal loss of the chromatin remodelling factor ATRX and increases in the transcription of a long non-coding RNA from telomeres, denoted TERRA. This project aims to determine: a) do increases in TERRA transcription facilitate the ALT pathway and b) could targeting of the TERRA transcript be a viable therapeutic approach for the treatment of ALT cancers?

Consistent with a role for TERRA in facilitating ALT we demonstrate that ALT positive cancer cells exhibit changes in the nuclear distribution of TERRA, with clear telomeric foci detectable via RNA-FISH. Furthermore, we demonstrate that the use of LNA GapmeR antisense oligonucleotides can be used as a strategy to deplete TERRA levels. Importantly depletion of TERRA correlated with a loss in C-circles, a major marker of an active ALT pathway. Future work will determine how this affects long-term viability and whether this provides potential for therapeutic intervention in ALT-positive cancers.

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List of Abbreviations

- ALT - Alternative Lengthening of Telomeres
- APNBs - ALT-associated promyelocytic leukaemia body
- ATM - Ataxia Telangiectasia
- ATR – ATR Serine/Threonine Kinase
- ATRX - Alpha Thalassemia/Mental Retardation Syndrome X-Linked
- BIR - Break Induced Replication
- BSA - Bovine Serum Albumin
- CO-FISH - Chromosome Orientation Fluorescence *in situ* Hybridization
- COUP-TF2 – COUP Transcription Factor 2
- CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats
- CRISPRi - CRISPR interference
- CtIP - C Terminal Interacting Protein
- DAPI - 4′,6-diamidino-2-phenylindole
- DDR - DNA Damage Response
- DEPC - Diethyl pyrocarbonate
- DMEM - Dulbecco's Modified Eagle Medium
- DNA - Deoxyribonucleic Acid
- DOX - Doxycycline
- DSB - Double Stranded Break
- DTT - Dithiothreitol
- EDTA - Ethylenediaminetetraacetic acid
- ETOH - Ethanol
- EXO1 - Exonuclease 1
- FBS - Foetal Bovine Serum
- FISH - Fluorescence *in situ* hybridization
- gDNA - genomic DNA
- GFP- Green Fluorescent Protein
- H3K27me3 - Histone 3 Lysine 27 tri-methylation
- H3K9me3 - Histone 3 Lysine 9 tri-methylation
- H4K20me1 - Histone 4 Lysine 20 mono-methylation
- HCl - Hydrochloric acid
- HR - Homologous recombination
- KRAB - Krüppel associated box
- LNA - Locked Nucleic Acids
- NaCl - Sodium Chloride
- NURD-ZNF827 – Nucleosome Remodelling Deacetylase Zinc Finger protein 827
- ONR - Orphan Nuclear Receptor
- PBS - Phosphate Buffered Saline
- PBST - Phosphate Buffered Saline Tween
- PCNA - Proliferating Cell Nuclear Antigen
- PFA - Paraformaldehyde
- PML - Progressive multifocal leukoencephalopathy
- PRC2 - Polycomb Repressor Complex 2

- qPCR - Quantitative PCR
- RFC - Replication Factor C
- RIPA - Radioimmunoprecipitation assay buffer
- RNA - Ribonucleic Acid
- RNAPII - RNA Polymerase II
- RPM - Revolutions Per Minute
- SCE - Sister Chromatid Exchange
- SSC – Saline-Sodium Citrate
- TBS - Tris Buffered Saline
- TBST - Tris Buffered Saline Tween
- TERC - Telomerase RNA Component
- TERRA - Telomere Repeat containing RNA
- TERT - Telomere Reverse Transcriptase
- TR4- Testicular Receptor 4
- TRF1 - Telomeric Repeat Binding Factor 1
- TRF2 - Telomeric Repeat Binding Factor 2
- TS - Tris Saline
- TST - Tris Saline Tween
- varTERRA - Variant Telomere Repeat containing RNA
- Xist - X-inactive specific transcript

1. Introduction

Cancers can be defined by a number of hallmarks including: apoptotic evasion, increased angiogenesis, invasion and metastasis of surrounding tissue as well as perceived cellular immortality (Hanahan & Weinberg 2000). Within each cancer subtype variation in genomic and tissue-specific hallmarks contribute to a differing aetiology and prognostic outcome in cancer patients. Conventional treatment is broad-spectrum and causes damage to normal cells as well as cancer cells, producing negative side effects for the patient. Thus, therapeutically targeting cancers on a genomic or tissue-specific level will reduce the genotoxic effects to non-cancerous cells, currently induced by systemic treatment options, such as chemotherapy. One constitutive hallmark held by cancer cells is the reconstitution of telomeric repeats; elongating telomeres at chromosome ends (Hanahan & Weinberg 2000). The extension of telomeres in cancers allows longer replication periods and thus further tumour growth.

1.1 Cellular Senescence and the End Replication Problem

Cellular senescence is an inherent process designed to limit the replicative capacity for cells to divide (Campisi 2014). This process is considered irreversible and occurs in response to aging of the cell or oncogenic stimuli; limiting the potential development into a cancerous cell (Campisi 2007; Sager 1991). The process of senescence involves the exit from the cell cycle, with drastic changes in gene expression and organisation of the chromatin landscape to a heterochromatic state as a result (Campisi 2014). Additionally, physiological expression of molecules such as growth factors and proinflammatory cytokines are induced/reduced as a result of exit from the cell cycle (Campisi 2014). The causes of cellular senescence have been widely characterized with

telomere shortening, genomic damage, mitogens, epigenomic damage, tumour suppressor and pro-proliferation signal imbalance stimulating the transition of an actively proliferating cell, into that of characteristic senescence (Campisi 2014).

Telomeres are nucleoprotein structures formed from multiple TTAGGG DNA repeats that comprise the ends of our chromosomes. Their primary function is to protect the genome from degradation by cellular nucleases (Shammas 2011). Telomeric shortening is conducive to reaching cellular senescence and lends to the notion that cells have a finite replicative potential known as the Hayflick limit (Hayflick 1965). With each cell replication the telomeric sequence is incompletely replicated, leading to the loss of sequence and the reduction of telomere length; this incomplete replication is termed the End Replication Problem (de Lange 2009). Once cells reach a critical limit of telomere size, senescence is induced and cells exit the cell cycle. Telomere shortening is therefore important for limiting the replicative ability of fast-cycling cells.

1.2 Telomeric shortening leads to genomic instability

During semi-conservative replication at telomeres, incomplete replication due to insufficient priming on the leading strand, leads to the production of 3' overhangs at the ends of DNA (Blackburn et al. 1978) . To cellular processes this overhang is recognised as a double strand break (DSBs) (de Lange 2009). Double strand break repair at telomeres could lead to telomere fusions and consequently genomic instability (Shay & Wright 2012) within normal and cancer cells. The signalling pathways involved in recognising double strand breaks are the ATM and ATR pathway. The ATM (Ataxia Telangiectasia modified) kinase pathway is directly activated by unprotected DNA ends, whereas the ATR (Ataxia Telangiectasia with Rad3 mediated)

pathway is induced as a result of 5' resection at double strand breaks. Stimulation of these pathways leads to induction of a DNA damage response, which could be potentially costly to telomere ends and local genomic information.

Importantly, a complex of proteins known as the Shelterin complex must protect telomere ends from being recognised as a double stranded break in need of repair (De Lange 2005). The Shelterin complex formed from six specific telomere associated proteins manipulates telomeric DNA in a dynamic fashion to evade recognition as a DSB and therefore protects chromosomes (De Lange 2005). This complex is crucial to limiting the DNA damage response at the ends of chromosomes

1.3 The Alternative Lengthening of Telomeres Pathway (ALT)

Cancers employ one of two methods to extend their telomeres and thus extend their capacity to replicate without concern for replication toxicity. The majority of cancers use an enzymatic-based approach, in which the normal cellular enzyme Telomerase is over-expressed. Telomerase is comprised of a reverse transcriptase known as TERT and a telomerase RNA known as TERC, which acts as a primer template for extension of telomeres, by TERT (Wu et al. 2017). This enzyme adds additional copies of the canonical TTAGGG repeat to the telomeric ends of DNA. Approximately 80% of cancers use this technique for telomeric extension (Min et al. 2017). The remaining proportion uses a non-canonical method in extension of their telomeres and thus their replicative ability. The Alternative Lengthening of Telomeres pathway (ALT) is a recombination-based method in which neighbouring telomeres act as a template for extension. This form of extension is not truly understood but it has been suggested that

ALT may be employing a modified version of Break Induced Replication to produce telomeric extension (BIR) (Dilley et al. 2016).

Break-induced replication requires a single ended double stranded break, such as the end of telomeric DNA (Llorente et al. 2008). The first step of BIR is strand invasion and requires Rad proteins, such as Rad51 to mediate invasion in a similar fashion to invasion seen in canonical homologous recombination (Llorente et al. 2008). For strand invasion to occur a homologous sequence is needed to act as a template for subsequent replication. After strand invasion occurs a replisome is formed at the site of invasion causing association of DNA polymerase δ (DNA Pol δ) and subsequent copying of the template strand in order to repair the break (Dilley et al. 2016). This process leads to sequence extension and loss of heterozygosity within the copied sequence (Dilley et al. 2016). In ALT cancers a modified replisome is seen in replication at telomeres (Dilley et al. 2016). In order for BIR to occur at ALT telomeres, replication factor C (RFC) acts to bring proliferating cell nuclear antigen (PCNA) to sites of strand invasion at telomeres (Dilley et al. 2016). After this sensing stage, Pol δ initiates unidirectional replication through its catalytic subunit POLD3 (Dilley et al. 2016). Dilley et al. noted that this sensing complex was required for BIR in ALT, but the process was independent of some canonical proteins seen in homologous recombination such as Rad51 and ATM (Dilley et al. 2016).

In ALT cancers telomere extension mediated by a modified BIR method could be leading to long extended telomeres seen in ALT cancers. Breaks within telomeres combined with homologous sequences provided by neighboring chromosomes could prime BIR and thus extensive replication by DNA polymerase δ; moving along

telomeric repeats until terminating replication at the ends of telomeres. Longer telomeres could thus act as templates for further replication and provide ALT cancers with abnormally long telomeres. These ALT cancers are thus able to divide for longer than normal cells, by extending their Hayflick Limit and avoiding cellular senescence.

ALT positive cancers are primarily found in cell types associated with the central nervous system and give rise to tumours such as Astrocytomas and Glioblastomas (Heaphy et al. 2011). ALT positive cancers can also been found in soft tissue subtypes such as Osteosarcomas and Liposarcomas (Heaphy et al. 2011).

1.4 Hallmarks of ALT Cancers

ALT-associated Cancers harbour their own subclass of defining features, allowing for diagnostic and prognostic value. However, each hallmark is not universal for each ALT-cancer, with many showing-varying levels of defining characteristics or absence thereof. Complementary to this, intra-tumoral heterogeneity confers differing levels of diagnostic/prognostic hallmarks on a tumour-by-tumour basis, contributing to variation across ALT cancers as well as within a single tumour population.

Consistent hallmarks of ALT cancers include: variant telomeric repeat sequences, extrachromosomal telomeric C-circles, ALT-associated promyelocytic Leukaemic nuclear bodies (APNBs), high telomeric sister-chromatid exchange rate, extended telomeres and chromatin compaction at telomeres. The most documented feature of ALT cancers is abrogated presence of the chromatin remodeller protein ATRX; through mutation, truncation, aberrant expression or deletion in 98% of ALT cancers (Lovejoy et al. 2012).

1.4.1 Variant Telomeric Repeat Sequences

Human telomeres are composed of repetitive sequence, the purpose of which is to allow non-detrimental degradation of the genome during cellular proliferation as previously described. The canonical telomeric repeat sequence $[5'\text{-}TTAGGG\text{-}3']_n$ composes the majority of human telomeres (Harley et al. 1990). However it has been described that variant telomeric repeat sequences such as TCAGGG, TTGGGG and TCAGGG amongst others, can be found interspersed between canonical repeats (Allshire et al. 1989; Baird et al. 1995). ALT-telomeres have been shown to contain variant telomeric repeat sequences on a larger scale, with the variant TCAGGG repeat highlighted as the most abundant across ALT-cancers (Conomos et al. 2012). In a fibroblast-derived ALT positive cell line WI38-VA13-2RA, the TCAGGG repeat accounted for 21.3% of telomeric sequence (Conomos et al. 2012) . Conversely, ALT-positive U-2OS osteosarcoma cells do not have the variant repeat interspersed throughout their telomeres (Conomos et al. 2012). The presence of variant telomeric repeat sequences may have a role in potentiating the ALT pathway. Indeed, binding of the Shelterin component TRF2 has been shown to have a reduced affinity for the variant repeat, potentially compromising its role in protecting telomeres (Conomos et al. 2012). Furthermore, the variant repeats have been shown to create high affinity binding motifs for the Nuclear Orphan Receptors COUP-TF2 and TR4 which in turn can recruit the NURD-ZNF827 histone deacetylase complex, which is thought to potentiate ALT through alterations in the chromatin environment (Conomos et al. 2014). Changes in the chromatin environment thus promotes telomere-telomere recombination in this setting (Conomos et al. 2014).

1.4.2 ALT-associated promyelocytic Leukaemic nuclear bodies (APNBs)

PML-Nuclear bodies are unique structures found within normal cellular nuclei (de Thé 2010). They are primarily composed of PML and SP100 proteins, along with additional factors such as SUMO (Chung et al. 2012). PML-Nuclear bodies have many roles within a cell including: transcriptional regulation, induction of apoptosis or senescence, stem cell maintenance and DNA repair (de Thé 2010). Cells employing the ALT-pathway for extending their telomeres exhibit their own unique subset of PML nuclear bodies known as ALT-associated promyelocytic Leukaemic nuclear bodies (APNBs) (Yeager et al. 1999). APNBs are telomere associated PML-bodies and likely represent the sites of telomeric recombination. APNBs can be found co-localising with the Shelterin protein TRF2 within ALT-positive cell lines via widefield and confocal imaging (Chung et al. 2012).

1.4.3 Extrachromosomal Telomeric C-circles

An indicative quantifiable biomarker in ALT cancers is the presence of extrachromosomal telomeric DNA. Single stranded telomeric DNA has been identified in APNBS in ALT cancers but has not been observed in cells expressing telomerase or in normal phenotypic populations (Bøe et al. 2006; Grudic et al. 2007). A presence of partially doubled stranded telomeric DNA has been noted in ALT positive cell lines (Nabetani & Ishikawa 2009). These were composed of a complete circularised c-rich strand, hybridised with an incomplete g-rich strand, in a circular fashion, hence C-circles. The incomplete G-rich strand acts as a primer for a polymerase known as Φ29 DNA polymerase (Henson et al. 2009). Through rolling circle amplification Φ29 DNA polymerase is able to produce single-stranded DNA oligomers of varying heterogeneity greater than or equal to 70 kb, at a linear rate (Dean et al. 2001). Importantly, as the

polymerase is self-priming, there is no need for additional primers, which would allow for amplification of the telomeric repeats and thus increase background signal, confounding results. Through blotting techniques c-circles can be detected and used as a quantitative read-out of ALT cancers. C-circle levels in ALT-positive cancers vary, with U-2OS and SAOS2 osteosarcoma cell lines showing high levels of C-circles, whereas WI38-VA13-2RA fibroblast cells, comparatively showing lower levels of C-circles.

1.4.4 Extended telomeres

Due to the predicted nature of telomeric extension by Break-induced replication in ALT cancers, ALT positive cancers hold telomeres of heterogeneous length ranging up to 20 kb in length (Bryan et al. 1995). This was shown through Southern Blotting and confirmed through telomere fluorescent *in situ* hybridisation (Telomere FISH) of metaphase spreads (Henson et al. 2002). ALT cell lines showed great telomere length using probes designed to the telomere, comparative to normal cells (Henson et al. 2002).

1.4.5 High Sister-Chromatid Exchange Rate

ALT-associated cancers often exhibit a high level of telomere sister-chromatid exchanges (tSCEs), which are produced from the resolution of double strand breaks in a replication dependent manner and involve the crossover of chromosome ends (Rudd et al. 2007). The presence of SCEs can be determined through chromosome orientation fluorescence *in situ* hybridization (CO-FISH). Telomeric CO-FISH showed SCEs to have a greater abundance in ALT cells than in normal cells, indicating a recombination based method is occurring at telomeres (Londono-Vallejo et al. 2004).

1.4.6 Alpha Thalassemia/Mental retardation syndrome X-linked (ATRX)

ATRX syndrome is a condition in which patients are characterised by alpha-thalassemia, facial abnormalities, genital deformation and severe psychomotor retardation (Gibbons et al. 2008). ATRX, a member of the SNF2 family, is a chromatin remodeller protein that's mutation/deletion is the constitutive cause of ATRX syndrome (Gibbons et al. 2008). Interestingly, in cancers employing the ALT pathway, a near universal loss/mutation of ATRX is seen (Clynes et al. 2015). Re-instating ATRX in cell lines with loss or mutation of ATRX was shown to supress the ALT pathway; demonstrated by a reduction in phenotype and a shortening of telomeres (Clynes et al. 2015). These results indicate a prudent role for ATRX in ALT-positive cancers.

Additionally, ATRX has been linked to cohesion resolution during mitosis (Ramamoorthy & Smith 2015). For cohesion resolution to occur, poly (ADP-ribose) polymerase tankyrase 1 needs to localise to telomeres and stimulate resolution (Ramamoorthy & Smith 2015). In the absence of ATRX, poly (ADP-ribose) polymerase tankyrase 1 is sequestered by a histone variant known as macroH2A1.1 (Ramamoorthy & Smith 2015). This regulation of tankyrase-mediated resolution of cohesion during mitosis, indicates ATRXs role in preventing sister chromatid recombination. Confirming this notion, in ALT cell populations the absence of ATRX leads to a high rate of telomere sister chromatid exchange as previously described.

1.4.7 Chromatin Compaction at Telomeres

In a normal cellular environment Telomeres are packaged by nucleosomes, linked through a 40bp section of telomeric DNA (Makarov et al. 1993; Lejnine et al. 1995). This gives the configuration of telomeric chromatin that is compressed in a fashion that is greater than the rest of the chromatin landscape (Lejnine et al. 1995). Consistent with greater compaction, telomeres show increased levels of heterochromatic marks such as H3K9me3 and H4K20me leading to a heterochromatin state and therefore a reduction in transcription (Mattiussi et al. 2014). ALT positive cell lines, however, showed a reduced association of H3K9me3 at ALT telomeres; producing reduced compaction in these cell lines (Mattiussi et al. 2014). As a result of this reduction in compaction they also noted an increase in telomeric transcription within ALT cell populations (Mattiussi et al. 2014). This open chromatin confirmation in ALT telomeres may therefore be contributing to telomeric transcription which may intern have a role in recombination in ALT cell lines (Mattiussi et al. 2014).

1.5 Telomeric Repeat Containing RNA (TERRA)

Telomeric repeat containing RNA (TERRA) has only been identified by groups in the last decade. TERRA is a long non-coding RNA of heterogeneous length transcribed from the C-rich strand of both telomeric and sub-telomeric repeat sequences; producing a transcript of repeating 5'-UUAGGG'-3' (Luke et al. 2008). TERRA is transcribed by RNA polymerase II (RNAPII) starting within the sub-telomeric region and progressing towards the end of the telomere, for this reason the initial transcript of TERRA is homogenous, with varying heterogeneity as transcription moves through into the telomeric repeat sequence (Luke et al. 2008). Due to the nature of transcription from the telomeres, transcript lengths vary greatly in size and can range up to 20 kb,

depending on the size of the telomere it is transcribed from (Luke & Lingner 2009; Bryan et al. 1995). ALT cancers, with long heterogeneous telomeres therefore produce TERRA transcript of greater length. Transcription of TERRA by RNAPII was confirmed by Schoeftner & Blasco who using α-amanitin, an inhibitor of RNAPII, showed that TERRA transcription levels were reduced upon inhibition of RNAPII (Schoeftner & Blasco 2008). TERRA can be found within the genome as well as at telomeres and has described roles in telomerase regulation, epigenetic changes and heterochromatin promotion, development and has an indicated role in replication (Luke et al. 2008).

1.5.1 TERRA at Telomeres

TERRA is found within the nuclear RNA fractions and not in other sub-cellular compartments and was shown to be at telomeres through RNA fluorescent *in situ* hybridization (RNA-FISH) (Luke et al. 2008). However, only a proportion of telomeres displayed TERRA signal in interphase and metaphase cells (Azzalin et al. 2007; Schoeftner & Blasco 2008). The presence of TERRA at a subset of telomeres could suggest a role for TERRA in a transient fashion, precluding a particular structural or regulatory role in *cis* or *trans*.

1.5.2 TERRA in Telomerase regulation

TERRA has been implicated in regulating telomerase, in a manner dependent on telomere length with longer telomeres leading to longer TERRA transcripts (Schoeftner & Blasco 2008). The method of telomerase inhibition by TERRA is not fully understood, however, studies in which a TERRA mimic was introduced to cells, lead to down-regulation of telomerase (Schoeftner & Blasco 2008). This could be an

indication of the low levels of TERRA seen in telomerase positive cancers and normal tissues.

1.5.3 TERRA in Development

TERRA has been shown to localise with both X and Y chromosomes in a male stem cell and both X chromosomes in female stem cells (Zhang et al. 2009). During development, TERRA localises only to the inactive chromosome, suggesting a role in heterochromatin organization at these sites (Zhang et al. 2009). This is furthered by TERRA having been shown to localise with the X-inactive specific transcript (Xist), involved in silencing of one X chromosome in female stem cells and the X chromosome in male stem cells (Schoeftner & Blasco 2008).

1.5.4 TERRA in Heterochromatin promotion

Recently TERRA has been shown to be crucial in facilitating H3K9me3, H3K27me3 and H4K20me3 heterochromatic marks at telomeres (Montero et al. 2018), therefore influencing a repressive state at telomeres. TERRA was shown to accomplish this by binding the Polycomb repressor complex 2 (PRC2) and recruiting the complex to telomeres (Montero et al. 2018). PRC2 then facilitates H3K27 tri-methylation, necessary for the stimulation of H4K20 and H3K9 tri-methylation (Montero et al. 2018). These changes in nucleosome methylation lead to a heterochromatic state, induced by the TERRA transcript.

1.5.6 TERRA in telomere lengthening

Interesting recent work has shown that TERRA may have an important role in stimulating telomere extension at critically short telomeres (Graf et al. 2017). TERRA

has been described to increase at critically short telomeres, where it forms hybrid structures known as R-loops (Graf et al. 2017). R-loops are composed of a DNA-RNA-DNA complex in which one strand of DNA is displaced (Arora et al. 2014). The localisation of TERRA and formation of R-loops at critically short telomeres was noted to be important for stimulation of the DNA damage response (DDR). This was shown through increased presence of Rad51 at critically short telomeres, indicating that Homology directed repair might be occurring (Graf et al. 2017). RNaseH1 over expression, utilized to degrade DNA:RNA hybrids was shown to ablate this response.

1.6 The relationship between ATRX and TERRA

Alterations in ATRX protein expression appear to alter the regulation of Telomeric repeat containing RNA (TERRA) transcription. There is ongoing debate in the literature about the relationship between the two factors. Absence or mutation of ATRX has been documented to cause an up-regulation of TERRA transcription (Clynes et al. 2015; Goldberg et al. 2011; Flynn et al. 2015). However, other sources claim ATRX dysregulation informs a down-regulation of TERRA transcription (Eid et al. 2015). Furthermore, Mattiussi et al. described no change in TERRA levels as a result of ATRX loss (Mattiussi et al. 2014). Initial experiments in this project will aim to confirm whether ATRX loss/mutation causes an upregulation or a down regulation of TERRA and therefore whether TERRA has any bearing in ALT-cancers.

The relationship between ATRX and TERRA has been described to be antagonistic. According to Chu et al. TERRA and ATRX are responsible for regulation of hundreds of genomic loci (Chu et al. 2017). TERRA was described to activate genomic loci, whereas ATRX was described to repress expression at these loci (Chu et al. 2017). They

also showed a direct interaction by electrophoretic mobility shift assays, in which TERRA binds to ATRX to disrupt ATRX's interaction with telomeric DNA (Chu et al. 2017).

1.7 Is TERRA de-regulation facilitating recombination in ALT?

ATRX absence has been shown to lead to increased TERRA levels in the G2/M phase of the cell cycle, suggesting that ATRX has an important role in regulating TERRA levels during the cell cycle, which is then perturbed in ALT upon ATRX loss (Flynn et al. 2015). If TERRA is upregulated in ALT-positive cancers as a result of ATRX loss, it might indicate a role for TERRA in the ALT pathway during replication. It is known that TERRA forms RNA:DNA hybrids on the leading strand of telomeric DNA displacing a single strand of DNA (Arora et al. 2014). R-loop formation could lead to potential abrogation for replication in cancer cells. The formation of R-loops on the leading stand could cause replication fork stalling and thus the formation of genomic instability, with the potential to progress to the production of a double stranded break. At ALT telomeres therefore, an increased presence of TERRA during G2/M and formation of double-stranded breaks could therefore produce one-ended breaks required for BIR to occur. Knocking down the TERRA transcript may therefore aid in understanding whether TERRA has a role in replication at ALT telomeres.

1.8 Knocking-down the telomeric transcript

If TERRA is upregulated in ALT cancers and if it is facilitating recombination in this subset; abrogation of the levels of TERRA transcript should cause an alteration in phenotype. Effective knockdown of the TERRA transcript may therefore cause changes in the hallmarks associated with ALT cancers. In the past it has been difficult to deplete

the TERRA transcript due to the nature of its size and repetitive sequence (homologous to the telomeric repeat). Recent work, however, has shown TERRA knockdown through the use of LNA GapmeRs (Chu et al. 2017).

To initiate transcriptional degradation of TERRA two systems will be put in place within an ALT cell: LNA GapmeR knockdown and LwaCas13a transcriptional degradation. These will be initiated on both a transient and stable level, respectively. After confirmation of effective knockdown, through RNA fluorescent *in situ* Hybridisation (RNA-FISH) and Northern blotting, in these systems, any changes to the hallmarks of ALT cancers will be noted. If TERRA is in fact facilitating recombination in ALT, a reduction in phenotype, for example reduced levels of extrachromosomal C-circles, will indicate a role for TERRA in ALT cancers.

1.8.1 Knocking down TERRA with LNA GapmeRs

Knocking down the TERRA transcript would potentially allow for regulation of the ALT phenotype. LNA GapmeRs will be targeted to the TERRA transcript through sequence homology. LNA GapmeRs are antisense oligonucleotide sequences flanked by locked nucleic acid (LNA) RNA bases. LNA bases are locked by conjugation of the 2^{nd} position oxygen with the 4^{th} position carbon, conferring resistance to degradation by exonucleases. LNA GapmeRs form hybrids with their target sequence and upon hybrid formation RNaseH1 mediates cleavage of the target strand, thus knocking down the target transcript. LNA GapmeRs designed to both the canonical and variant telomeric repeat sequence will allow for effective and sustained knockdown of the TERRA transcript. Changes to the hallmarks of ALT cancers as a result of

knockdown will therefore imply whether TERRA is playing a role on ALT cancers. A summary of this method can be seen in Figure 1.1.

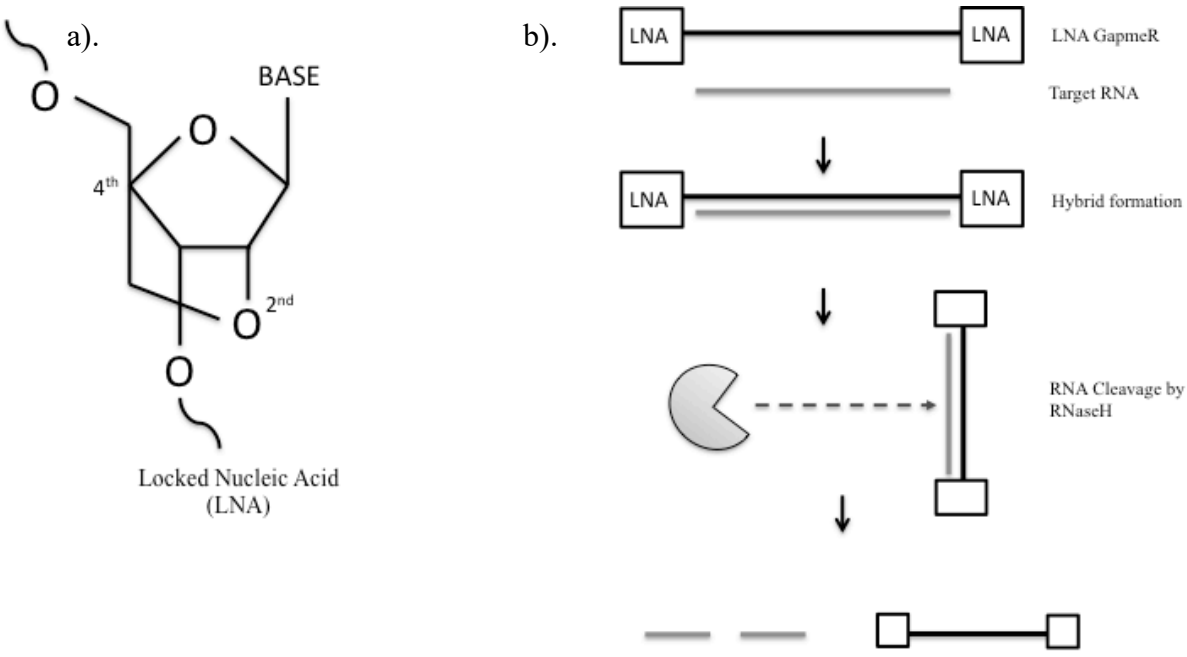


Figure 1.1 Transcript knockdown through LNA GapmeR mediated degradation. a) Structure of a Locked Nucleic Acid (LNA) b) Mechanism of RNaseH1 mediated cleavage of target RNA.

1.8.2 Degrading the TERRA transcript with LwaCas13a

Conventional CRISPR methods have been based on Cas9 a DNA targeting enzyme derived from a bacterial population. Existing methods of RNA interferences such as siRNAs have been effective in the knockdown of cellular RNA, but often harbour off-target effects (Jackson et al. 2003). Therefore, the need for a CRISPR based system in which specific targeting of selective RNAs with little to no off-target effects is required. LwaCas13a derived from *Leptotrichia wadei* was identified for RNA knockdown in an interference assay based in *Escherichia coli* (Abudayyeh et al. 2017). LwaCas13a is

able to cleave RNA through the presence of two HEPN (higher-eukaryotes and prokaryotes nucleotide-binding) cleavage domains (Abudayyeh et al. 2017). They also noted that once expressed in mammalian cells, targeted LwaCas13a using short guide RNAs allowed for comparable levels of knockdown to conventional methods as well as increased specificity for the targeted RNA (Abudayyeh et al. 2017). It was also possible to invoke targeted RNA binding using a catalytically inactive LwaCas13a, comparable to catalytically inactive dCas9 (Abudayyeh et al. 2017).

Targeting TERRA with LwaCas13a would therefore allow for selective and efficient knockdown of the TERRA transcript. LwaCas13a constructs will be utilised with short targeting guides designed to both the canonical and variant telomeric repeat and therefore offer a selective targeting of TERRA in ALT-positive cancers. As before, changes to the hallmarks of ALT would indicate TERRAs role in ALT cancers. A summary of this method of knockdown can be seen in Figure 1.2 below.

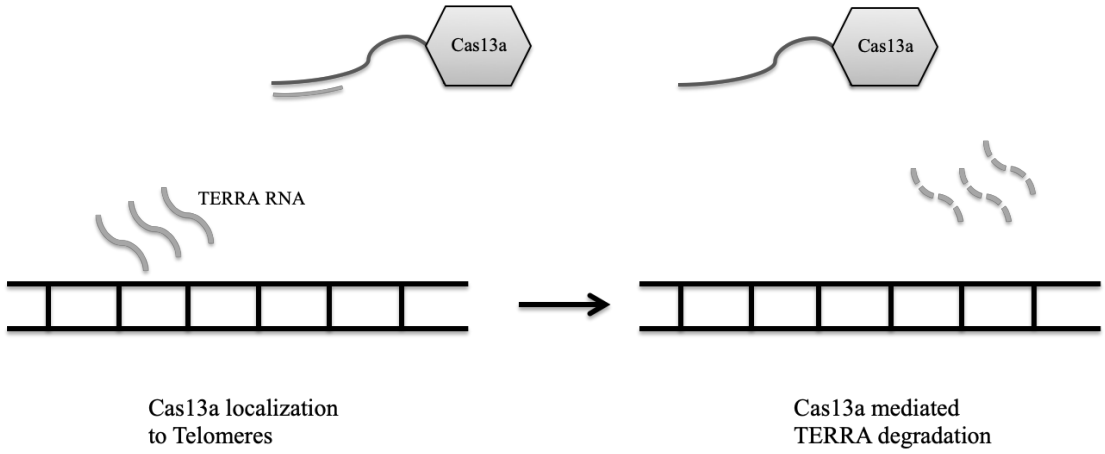


Figure 1.2 Cas13a mediated transcript degradation. Localisations to Telomeres through guide RNAs and consequential degradation of local RNA.

1.9 Potential therapeutic interventions in ALT cancers

The Alternative Lengthening of Telomeres pathway is a cancer specific pathway that is not utilised by normal cells. Conventional treatment options in cancers are generally non-specific and cause damaging effects to normal non-cancerous cells. By elucidating the mechanism of ALT in telomeric maintenance further, progress can be made in therapy of this unique subclass of cancer.

The identification of variant telomeric repeats within ALT cancers could be used diagnostically either through telomeric sequencing of patient genomes, or the design of canonical and variant repeat RNA-FISH probes, highlighting the differing presence of the repeats in patient samples.

If in fact TERRA upregulation is facilitating recombination in ALT cancers, therapeutic intervention into the regulation of TERRA transcript/transcription will help to alleviate the extension of telomeres in ALT cancers; causing the cancer cells to progress towards a senescent state instead of a highly replicative state. LNA GapmeRs designed to both the canonical and variant TERRA transcripts could be used in a therapeutic setting. Similarly using a TERRA targeting Cas13a to actively degrade high levels of transcript, could aid in limiting recombination in ALT cancers.

1.10 Model of Work

A summary of the understanding and experimental design of this project is displayed in the following model (Figure 1.3).

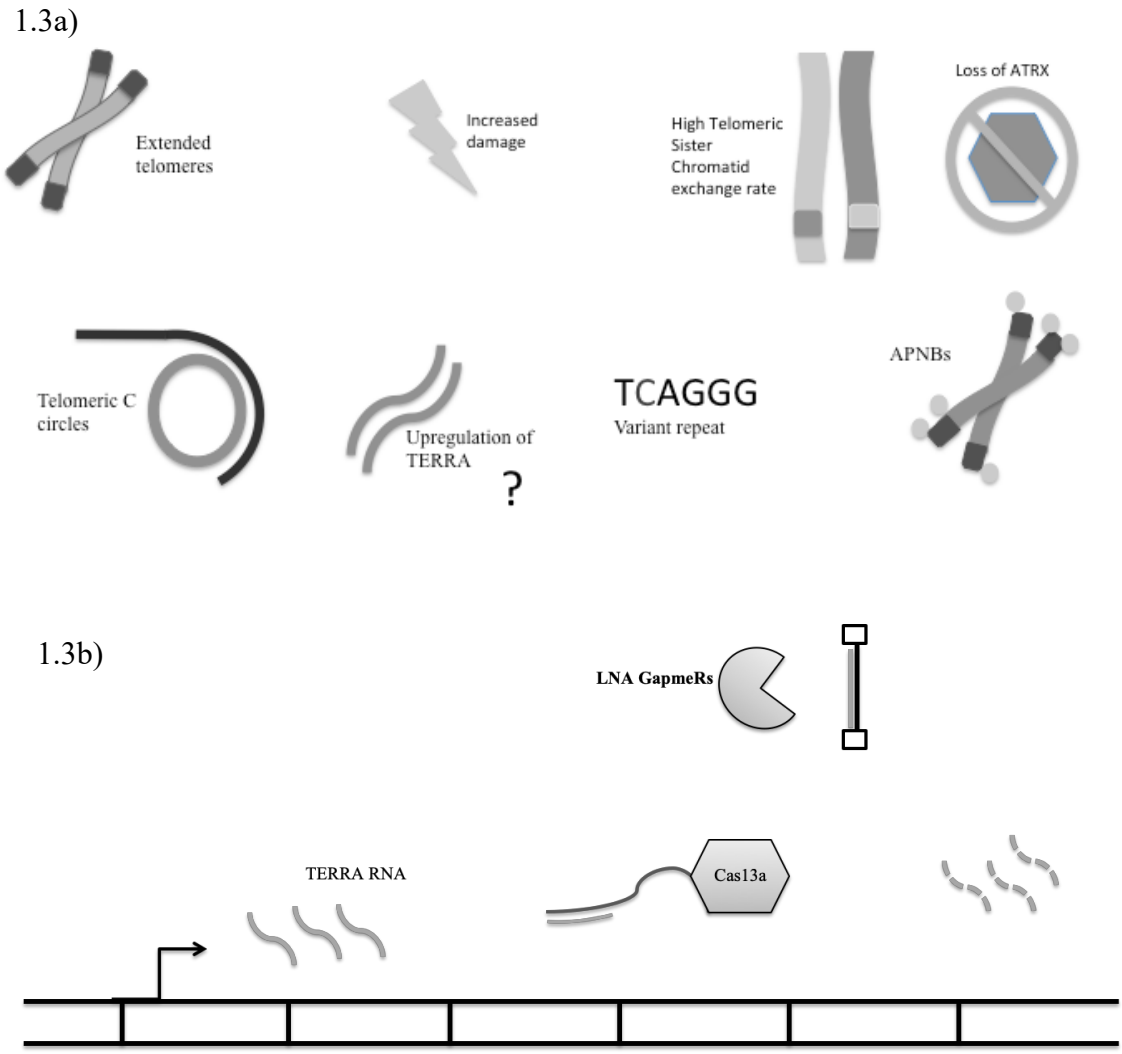


Figure 1.3 Model of Work. a) Investigating TERRA upregulation as a hallmark of ALT cancers b) Knockdown/Inhibition of TERRA transcript and observing any changes to the hallmarks of AL T cancers (a) as a result.

2. Materials and Methods

2.1 Cell Culture

Cells were grown in incubators at 37°C and supplemented with 5% CO_2. Standard media was composed of: Dulbecco's Modified Eagle Medium, 10% Foetal Bovine Serum, 1% PenStrep and 1% Glutamine. U-2OS 22/3 media was composed of: Dulbecco's Modified Eagle Medium, 10% Tet-Free Foetal Bovine Serum, 1% PenStrep, 1% Glutamine, Geneticin (0.35 g/500 ml) and Puromycin (10 mg/ml). RPE-1 Media was composed of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, HEPES 1:1, 10% Foetal Bovine Serum, Sodium Bicarbonate, and Hygromycin B (10 mg/ml). Finally, Hela-SNAP Media was composed of: Dulbecco's Modified Eagle Medium, 10% Foetal Bovine Serum, 1% PenStrep, 1% Glutamine, Puromycin (10 mg/ml) and Blastocidin S (5 μg/ml). Population maintenance was promoted through passaging of cells and removal of waste products.

2.1.1 Cell lines

A panel of ALT-positive and ALT-negative cell lines were used for comparison and are listed below:

ALT Positive

WI38-VA13-2RA: Lung fibroblast cell line, unstable karyotype, hyperdiploid, SV40 transformed - Standard Media

U-2OS 22/3: Bone epithelial cell line from an Osteosarcoma patient. This cell line contains a doxycycline-induced cDNA for ATRX, allowing for ATRX to be switched on experimentally - U-2OS Media

G292: Bone fibroblast cell line, from an Osteosarcoma patient - Standard Media

SAOS2: Bone epithelial cell line, from an Osteosarcoma patient - Standard Media

SW26: A sub-clone of the IMR90 lung fibroblast cell line that was found to be ALT positive through screening of ATRX status. It was found to be telomerase & ATRX null (Min et al. 2017) - Standard Media

ALT Negative

SW39: A sub-clone of the IMR90 lung fibroblast cell line that maintains its ATRX status and is telomerase positive (Min et al. 2017a) - Standard Media

KNS42: Paediatric glioblastoma cell line, telomerase positive - Standard Media

SF188: Paediatric glioblastoma cell line, telomerase positive - Standard Media

HELA LT: Cervix epithelial cell line, telomerase positive with extensive telomeres at a critical level - Standard Media

RPE-1: Retinal pigmented epithelium cell line, immortalized with hTERT, characteristically normal cell line - RPE-1 Media

WI38: Lung fibroblast cell line, telomerase positive, normal cell line - Standard Media

Others

HELA -Snap Scrambled Clone 11: Cervix epithelial cell line, telomerase positive with a Snap-tagged histone H3.3 and a scrambled shRNA - Hela SNAP Media

HELA -Snap shATRX Clone 5: Cervix epithelial cell line, telomerase positive with a Snap-tagged histone H3.3 and an shRNA targeted to ATRX. Knockdown of ATRX shRNA2 with sequence: 5'-GTCTGTGTTGGCTGATATT-3', which is targeted to the ADD domain - Hela SNAP Media.

2.2 Cell passaging and experimental seeding

In order to split cell populations for experimental design, cells were trypsinised using Trypsin-EDTA solution. Cell number and viability was assessed by the Advanced Image Cytometer NucleoCounter® NC-3000™ by Chemometec.

2.3 Lipid based Transfection

Cells were seeded at a density of $5x10^3$ cells per coverslip, with 4 coverslips per well in a 6 well plate. Transfections were carried out using the jetPRIME® transfection reagent kit by Polyplus transfection®. The transfection mix was composed of 200 µl of jetPRIME buffer and 2 µg of plasmid DNA per well. This was gently vortexed for 10 seconds before adding 4 µl of jetPRIME reagent (per well) and then vortexed again for a further 10 seconds. Following an incubation of 10 minutes at room temperature, the transfection mix was added to each well of the 6-well plate (200 µl per well). After incubation at 37°C for 4 hours the media was replaced; cells were then fixed/harvested after 24-48 hours.

2.4 Immunofluorescence

Cells were grown on coverslips for 24 hours at a density of $5x10^3$ cells per coverslip. Media was removed from the wells and followed by a wash with PBS. The cells were then subjected to pre-permeabilization with ice-cold 0.5% Triton X-100 (in PBS) for 1 minute on ice. After a rinse in PBS cells were fixed with 4% paraformaldehyde (in PBS) for 15 minutes at room temperature. Coverslips were then washed 4 times in PBS before permeabilization with 0.5% Triton X-100 (in PBS) for 5 minutes at room temperature. After 4 washes with PBS, coverslips were incubated in blocking solution (2% BSA, 0.1% Tween in PBS) overnight at 4°C. Indirect immunostaining was then

performed using primary antibody, diluted in blocking solution to the desired concentration, overnight at 4°C or for 1 hour at room temperature. Coverslips were then washed 4 times in PBST (PBS with 0.1% Tween) for 5 minutes each time. Secondary antibody staining for 1 hour followed using Alexa-Fluor secondary antibodies; diluted to the required concentration in blocking solution. 3 washes for 5 minutes and 1 wash for 40 minutes in PBST preceded mounting the coverslips on slides with Vectashield (with DAPI).

2.5 RNA Fluorescent in situ hybridization (RNA-FISH)

Method was adapted from Fluorescence in situ Hybridisation (FISH), Protocols and Applications (Bridger & Volpi 2010). The composition of required reagents can be found within Table 2a. The protocol is established through Fixation, Hybridisation and Detection of fluorescent probes.

Fixation: Cells were seeded onto coverslips at a density of $5x10^3$ cells per coverslip. After growth for 24 hours, coverslips were washed in PBS. Cells were then fixed in 200 µl of 4% paraformaldehyde for 20 minutes. After this period coverslips were washed 3 times in PBS for 5 minutes each time at room temperature. Coverslips were then stored in 70% ethanol at -20°C until further use.

Hybridisation: Coverslips from freezer were placed into fresh 70% ethanol at room temperature for 5 minutes. After a rinse in room temperature Tris-Saline, coverslips were incubated in 0.01% Pepsin for 4 minutes at 37°C, followed by a rinse in DEPC treated water for 1 minute. Post-fixation for 20 minutes in Paraformaldehyde-PBS preceded a 10 minute wash in PBS. The coverslips were then dehydrated in 70%

ethanol, 90% ethanol and 100% ethanol for 3 minutes each before allowing to air dry. Whilst air-drying the hybridisation solution was made by combining: 1 µl probe, 4 µl of DEPC water and 45 µl of hybridisation buffer (Table 2a). Coverslips were treated with 8 µl of hybridisation solution overnight at 37°C in a humidity chamber.

Detection: Coverslips were washed 3 times in 2X SSC for 10 minutes at 37°C. Next, 2 washes for 5 minutes at room temperature in Tris-Saline-Tween preceded 1 wash in Tris-Saline for 5 minutes at room temperature. Dehydration of coverslips was then carried out in 100% ethanol for 3 minutes before mounting in Vectashield (with DAPI) on slides. Images were acquired on the DeltaVision Widefield Microscope at 60-100x, with 30 Z-stacks taken.

Solution	Components
DEPC treated milliQ Water	10 Litres of milliQ Water
	10 ml Diethyl pyrocarbonate (DEPC)
Tris-Saline	10X NaCl
	DEPC treated water
	2M Tris-HCL
Tris-Saline-Tween	10X NaCl
	DEPC treated water
	2M Tris-HCL
	Tween-20
Hybridisation Solution	Formamide
	20X SSC
	10 mg/ml Salmon sperm
	50X Denharts Solution
	1M Phosphate buffer
	0.5M EDTA pH 8.0
	DEPC treated water
20X SSC	SSC diluted in DEPC treated water

Table 2a. RNA-FISH solution compositions

2.6 Immuno-Fluorescent in situ Hybridisation

This technique combines protein detection as seen in conventional immunofluorescence, with RNA detection as described above. This allows for RNA comparisons to be made in the presence or absence of protein; also offering insights into the localisation of protein and RNA within a cell. Cells were seeded and fixed by methods described above. However, Ethanol washes as seen in the RNA-FISH protocol was excluded to limit protein denaturation that can occur as a result of alcohol treatment. Following fixation, cell permeabilization was implemented through incubation of 0.5% Triton X-100. After three washes with PBS coverslips were blocked in blocking solution for 15 minutes - composed of 1% Bovine Serum Albumin with 25 U/ml RNAsin. Primary and Secondary antibodies were diluted in blocking solution and micro-fuged at top-speed for 20 minutes at 4°C. Primary antibody staining for 45 minutes at room temperature, precluded 4 washes in PBS for 5 minutes each time. Secondary antibody staining for 45 minutes at room temperature followed initial staining. After secondary staining coverslips were rinsed in PBS before post fixing in 4% PFA. After 2 washes in PBS for 5 minutes coverslips were rinsed in 2xSSC before proceeding to Hybridisation & Detection of RNA-FISH probes as described previously (with the exclusion of ethanol washes). Images were taken on the DeltaVision Widefield Microscope at 60-100x.

2.6 Western Blotting

Cells were pelleted and then thoroughly washed with PBS. PBS was then removed and the pellet re-suspended in high salt RIPA buffer on ice for 25 minutes. The solution was consequently syringed using a 25-gauge needle. The supernatant was transferred to a fresh tube after centrifugation at 13,000 RPM for 10 minutes at 4°C. Concentration was assessed using the NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer with 1% Bovine serum albumin (BSA) as a reference point, before dilution to an equal concentration in 200 µl of high-salt RIPA buffer. After measurement 160 µl of 2X loading buffer and 40 µl of 1M Dithiothreitol (DTT) was added to the supernatant and consequently denatured at 95°C for 5 minutes.

Samples were run on Bolt™ 4-12% Bis-Tris Plus Gels in 20X Bolt™ MOPS SDS Running Buffer at 100 V for roughly 2 hours until the dye front progressed to the end of the gel. The transfer was carried out using a transfer stack comprised of sponges and Wattman paper, with a PVDF membrane (activated by methanol) completing the stack. The transfer process was completed at 4°C at 25 mA, 300 V with constant amperage for 17 hours.

Membranes were blocked for 1 hour in 5% skimmed milk. Primary antibody staining was conducted in 2.5% milk for 1 hour at the desired antibody concentration. Primary antibodies can be found in Table a of appendix c. 3 washes of TBST followed primary staining, before incubation with secondary antibodies for 1 hour at the desired concentration, made in 2.5% milk (appendix b). After 3 washes with TBST blots were developed using SignalFire™ ECL and exposed onto Amersham ® Hyperfilm film in a dark room for varying amounts of time.

2.9 RNA extraction

RNA was extracted from cell pellets using the RNeasy Min Kit by Qiagen; following the protocol provided by the manufacturer.

2.10 RNA concentration

When RNA concentrations were too low for the required protocol, RNA was concentrated using an ethanol precipitation method. 3M Sodium Acetate was added to each sample at a ratio of 0.1 volumes. Pre-chilled 100% ethanol was consequently added at a ratio of 3 volumes. Finally, 20ng of glycogen was added before vortexing. The solutions were then precipitated at -80°C overnight before centrifugation at 13,000 RPM at 4°C for 30 minutes. The pellets were then washed twice in 500 µl of ice cold 75% ethanol before spinning at 13,000 RPM at 4°C for 10 minutes after each wash. The pellet was then dried through a short spin and air-dried. Finally, the pellet was re-suspended in the desired volume of nuclease-free water.

2.6 RNase Treatment

Samples/Coverslips were treated with 200 U of RNaseA by Thermofisher for 1 hour at 37°C before proceeding with the experimental procedure.

2.11 cDNA synthesis

RNA extracts were reverse transcribed to cDNA using Superscript III Reverse Transcriptase, with an oligo-dTT primer set. Samples were prepared on ice and conditions were designed to minimise RNase contamination. Initial denaturation preceded the reaction at 65°C for 5 minutes before reverse transcription at 55°C for 60 minutes, followed by enzyme inactivation for 15 minutes at 70°C. Reactions were

comprised of the reagents found in Table 2b for the initial denaturation followed by the addition of the components for the reverse transcription reaction.

<table>
<tr><td rowspan="4">Denaturation</td><td>Gene specific primer</td><td>2 pmol</td><td rowspan="4">65°C for 5 mins</td></tr>
<tr><td>RNA</td><td>3 µg or 1 µg</td></tr>
<tr><td>dNTP mix</td><td>0.5 mM</td></tr>
<tr><td colspan="2">Total volume
13 µl</td></tr>
<tr><td rowspan="5">Reverse Transcription</td><td>1X First Strand buffer</td><td>To volume</td><td rowspan="5">55°C for 60 mins</td></tr>
<tr><td>DTT</td><td>5 mM</td></tr>
<tr><td>SuperScript III RT</td><td>200 U</td></tr>
<tr><td>H20</td><td>To volume</td></tr>
<tr><td colspan="2">Total volume
7 µl</td></tr>
<tr><td colspan="4">Enzyme inactivation 70°C for 15 minutes</td></tr>
</table>

Table 2d. cDNA synthesis conditions, for reverse transcription from RNA extractions

2.12 Quantitative-PCR

The reaction was carried out using Power SYBR Green PCR Master Mix using a StepOne Plus qPCRmachine. Each sample was adjusted through three independent biological replicates and adjudicated through the use of two technical replicates as well as a no template control for all primer pairs. This allowed for the exclusion of any

outlying data, including false data formed from primer dimers in the no template controls. The reaction mix for the reaction can be found within Table 2c. Plates were sealed with adhesive film and subjected to the qPCR cycle of: 95°C for 15 seconds (denaturation), 60°C for 1 minute (annealing & extension) for 40 cycles, with a final dissociation cycle to provide melt curve analysis.

Diluted cDNA	2 µl
Forward primer	5 pmol
Reverse primer	5 pmol
1X Power SYBR Green PCR Master mix	To volume
H_2O	To volume
To a volume of 10 µl	

Table 2c. Reaction mix for qPCR reactions,

2.13 TaqMan Gene Expression Assays

To assess the levels of RNaseH1 on a transcriptional level, TaqMan Gene expression assays were used. TaqMan assays are pre-designed primer and probe sets. The TaqMan system relies on two specific primers combined with two fluorescent probes that binds the minor groove of B-form DNA. Probes are labelled with a fluorophore at the 5' end and a non-fluorescent quencher at the 3' end. Upon probe incorporation during the PCR reaction the quencher is lost through probe degradation allowing the fluorophore to fluoresce. If the probe is not incorporated and thus not degraded the quencher prevents fluorescence. Pre-designed and optimized conditions allow for a quick quantitative readout of the levels of target gene transcription. cDNA was synthesized as before and combined as described in Table 2d. Reactions were then

run on an Applied Biosystems 7500 Fast Real-Time PCR System to the conditions described in Table 2d.

Component	20 µl Reaction Mix	Stage	Temperature	Time
20X TaqMan® Gene Expression Assay - RNaseH1	1	Hold	50	2 minutes
		Hold	95	20 seconds
2X TaqMan® Gene Expression Assay Master Mix	10	40 Cycles	95	3 seconds
			60	30 seconds
cDNA template 50ng	4			
RNase free-water	5			

Table 2d Components and reaction settings for TaqMan gene expression assays

2.13 Northern Blotting

For TERRA RNA electrophoresis, RNA was extracted using the RNeasy mini kit by Qiagen; following the protocol provided. As large quantities of RNA were required for the process, cell populations were grown to higher titres. RNA was eluted from the column with 15 µl of RNase Free Water. Extracts from the same cell line were pooled to yield a high concentration of RNA in a smaller volume. Therefore, the final sample concentration was between 1 µg ≤ X ≤5 µg in no more than 5 µl. Samples were normalised to the same concentration through dilution. Sample buffer was added to each pooled extract to a final volume of 10-15 µl depending on the RNA concentration. After denaturation for 15 minutes at 65°C 1X loading buffer was added to the sample mix. Samples were then loaded onto a 1.2% agarose-formaldehyde gel and run at 120V until the bromo-phenol-blue in the loading buffer had run ≥8cm from the well. The reagents for this protocol can be found in Table 2e.

1.2% Agarose-Formaldehyde gel	37% Formaldehyde	18.6 ml
	1.2g Agarose dissolved in 10ml 10X MOPs and 70.4ml sterile H_2O	
	Ethidium bromide	To a final concentration of ~0.05 µg/ml
MOPS 10X pH 5.0 (500ml)	MOPS	20.8 g
	3M NaAc (pH 5.5)	9.1 ml
	0.5M EDTA (pH 8.0)	9 ml
	Sterile H_2O	To 500 ml
Loading Buffer	50% Glycerol	To desired volume for sample concentration
	0.05% Xylencyanol	
	0.05% Bromophenol blue	
Running Buffer	1X MOPS in sterile H_2O	To fill tank
Sample Buffer (for 10 samples)	100% Formamide	50 µl
	37% Formaldehyde	18 µl
	1X MOPS	10 µl
	Sterile H_2O	20 µl

Table 2e. Composition of reagents for Northern Blotting techniques

After gel separation, RNA was transferred overnight onto Zeta-probe membrane Blots were UV-cross linked before proceeding to radioactive probing. The Telo-C probe $(CCCTAA)_3$ was used to identify levels of TERRA found in RNA extracts. The probe was labelled with γ^{32}P-ATP through composition of the following: 10X T4 polynucleotide kinase reaction buffer, 10µM Telo-C oligo, γ^{32}P-ATP, T4 polynucleotide kinase and H_2O. The labelling mix was thus incubated at 37°C for 30 minutes. Roche G-25 Sephadex spin columns were used to purify the reaction mix. The labelled probe was then added to PerfectHyb Plus hybridization buffer by SIGMA and incubated with the blot at 37°C overnight in a hybridization oven. Blots were rinsed in 2X SSC before a longer wash in 10X SSC until background signal was lost at the edges of the blot but maintained in areas where the RNA resided. Blots were then exposed with Amersham™ autoradiography films at -80°C before development. Semi-quantitative analysis of Northern blots was compiled using Fiji, allowing for quantification of intensity and size distribution of the probed RNA.

2.14 Electroporation

Cells were counted to approximately 5 million populations and then subjected to electroporation. Cells were electroporated with the desired dosage using the MicroPulser^{TM} Electroporator. Before using valuable LNA GapmeRs, electroporation condition optimization was assessed using known siRNAs to ATRX. Successful knockdown was depicted through western blot analysis of ATRX. Conditions for LNA GapmeR electroporation was thus 350V for 10ms. Following a 15-minute post-electroporation incubation at room temperature, cells were seeded onto coverslips in 6-well dishes for 12 hours prior to fixation.

2.15 Nucleofection

LNA GapmeRs were introduced into the cells through Nucleofection. This process requires specialist kits developed by Lonza designed on a cell line by cell line basis. Due to this, only three cell line kits were available from my cell panel - Hela, U-2OS and WI38. One million cells per sample were counted and spun at 90xg for 10 minutes. The resulting cell pellet was resuspended in Nucleofector® Solution before addition of the LNA GapmeRs and subsequent Nucleofection. Kit R and Nucleofector® program V-001 were used for WI38, whereas for U-2OS Kit V and program X-001 was used. After Nucleofection cells were seeded onto 6 well plates for image analysis or RNA extraction.

2.15 siRNA Transfection

RNAi Max Lipofectamine reagent was used for transfection of siRNAs. $8x10^5$ cells were seeded onto 10 cm dishes containing glass coverslips. Cells were allowed to grow for 24 hours before transfection. siRNA was transfected according to the protocol

provided for the RNAi Max Lipofectamine reagent. 48 hours post transfection cell pellets were taken and coverslips were fixed before probing.

2.15 C-Circle Assay

Cells populations were harvested and genomic DNA was extracted using the PUREgene - Core B kit by QIAGEN. 20 mM Tris Buffer (pH7.4) was used instead of rehydrating the DNA pellets in Tris-EDTA buffer as described by the protocol. DNA concentrations were then diluted to ~50 ng/µl in 20mM Tris buffer (pH 7.4). DNA was then digested for 3 hours at 37°C; the reaction mix is described below in Table e. The C-circle reaction was then composed using the quantities listed in Table e and incubated at 30°C for 8 hours, followed by 20 minutes at 65°C.

Genomic DNA	250 ng
NEB buffer 4	5 µl
HinfI	0.1 µl
RsaI	0.1 µl
10 mg/ml BSA	0.5 µl
RNaseA	0.5 µl
MilliQ H2O	To a final volume of 50 µl

Digested gDNA	150 ng
100mM dATP	1 µl
100mM dGTP	1 µl
100mM dTTP	1 µl
1% TWEEN	5 µl
10X Φ29 buffer	5 µl
10 mg/ml BSA	1 µl
Φ29 DNA Polymerase	3.75 µl
MilliQ H_2O	To a final volume of 100 µl

Table e. C-circle digestion and amplification components.

After the C-Circle reactions were performed, samples were slot blotted onto a Zeta-probe membrane via a vacuum manifold and probed with radioactively labelled oligos as described in the Northern Blotting protocol. A quantitative read-out could then be obtained using Fiji software.

2.16 Imaging

The majority of imaging was carried out using the DeltaVision widefield microscope. Images were taken at 60-100X magnification. Thirty Z-stacks were attained per image. Images were then projected to maximum intensity using Fiji and subjected to further analysis in Fiji and in CellProfiler.

2.17 Image analysis

Images were assessed using Fiji and CellProfiler. CellProfiler is an imaging analysis software, which allows the number of foci in each nucleus to be counted (Carpenter et al. 2006). The basic model relies on a parent-child relationship, in which the nucleus is referred to as the parent and the foci (as individuals) are referred to as children. For example, 1 nucleus would be categorised as 'Parent 1'; if the nucleus had 6 foci, each foci would be labelled individually as 'Child A, B, C, D, E, F'. This allows for each parent-child nucleus to be related and recorded. The software follows this principle based on the staining of the cells. The parent nucleus is identified through parameters set on the DAPI stain, whereas the child signal is defined based on parameters set on each probe channel. This software therefore allows high-throughput quantitative analysis of otherwise qualitative input.

3. Results

3.1 Is TERRA upregulated in ALT-positive cell lines?

The first question we asked was whether TERRA transcription was upregulated in ALT cancers. To answer this a combination of Northern blotting and RNA-FISH was used to distinguish differences in TERRA between a panel of ALT-positive and ALT-negative cancers. Northern blotting allowed a semi-quantitative perspective on TERRA expression, whereas RNA-FISH allows an insight into any distributional differences in TERRA localisation between ALT-positive and ALT-negative cell lines. Initial experiments aimed to set-up and optimize RNA-FISH in my cell lines. This involved tweaking various stages of the RNA-FISH protocol on a cell line by cell line basis. For example, one protocol step involves treatment with Pepsin, which acts to cleave proteins, however, as my cell lines were primarily adherent and grown on coverslips, Pepsin treatment denatured protein contacts with the glass coverslip, causing cells to slough off for the later stages of the protocol. After treatment with reduced concentrations of Pepsin, with similar results, it was decided that Pepsin treatment was not entirely necessary and was no longer used as part of the protocol; this aided the issue of cell loss. Other examples of optimization for my cell populations involved altering wash steps, denaturation using alcohols and optimizing hybridization. These optimization steps enabled consistent clear foci to be produced in all cell lines examined. Figure 3.1 shows how optimization steps produced a clear read-out of TERRA foci through RNA-FISH.

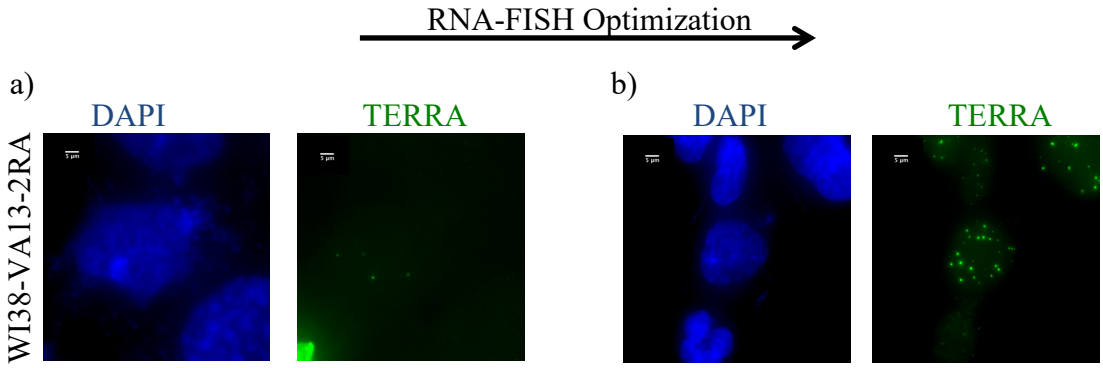
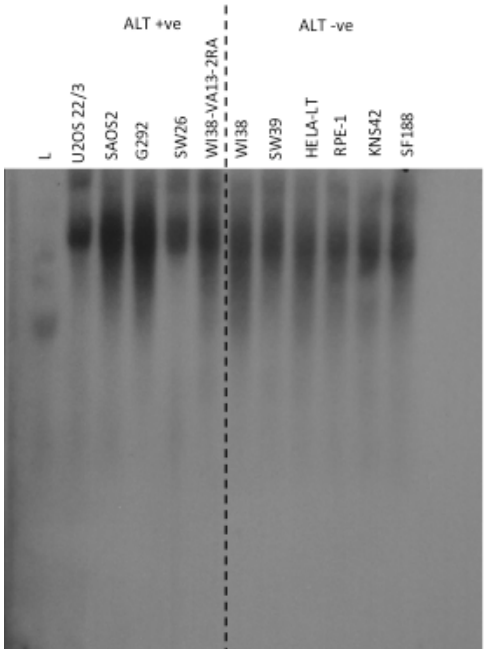


Figure 3.1. RNA-FISH optimization in ALT-positive WI38-VA13-2RA cells. a) First attempt, Nuclei with background staining of salt crystals, poor TERRA output b) Post optimization RNA-FISH images. All images were taken on the widefield DeltaVision® microscope at 60X magnification.

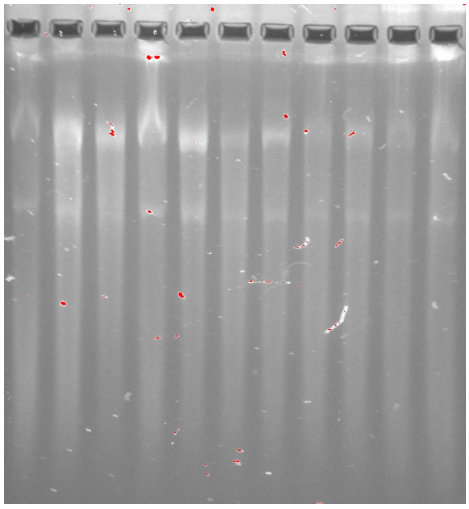
3.1.2 TERRA expression in ALT-positive and ALT-negative cell lines

RNA was extracted from cell populations, quantified and then prepped for Northern blotting; around 2 µg of RNA was loaded onto a formaldehyde gel and run at 120V until the bromo-phenol blue had run 8cm from the well. Following this, loading was assessed through chemiluminescence detection, and then blotted onto PVDF membrane by capillary action. Membranes were then probed using Telo-C probe with sequence $(CCCTAA)_3$ labelled with γ ^{32}P-ATP and exposed onto autoradiography films. This was repeated three times across a panel of ALT-positive and ALT negative cell lines. Relative intensities of TERRA expression on a cell line basis can be seen in Figure 3.2. Northern blotting quantification across three replicates indicated no obvious difference in TERRA intensity between ALT-positive and ALT-negative cell lines. Additional blots can be found in appendix A supplementary Figure 1. Authenticity of signal was controlled through RNaseA treatment, in which TERRA signal is lost as a result of treatment (Figure 3.3).

a)



b)



c)

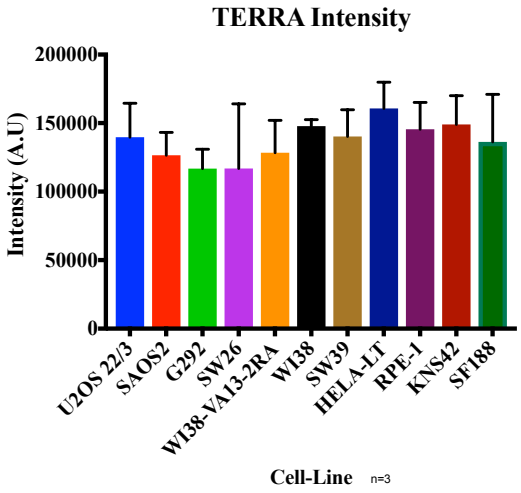


Figure 3.2 Northern blot analysis of TERRA RNA from a panel of ALT-positive and ALT-negative cell lines a) probed northern blot of TERRA using γ^{32}P-ATP tagged TeloC probe b) loading of blot c) Quantification of TERRA intensity from 3 replicates.

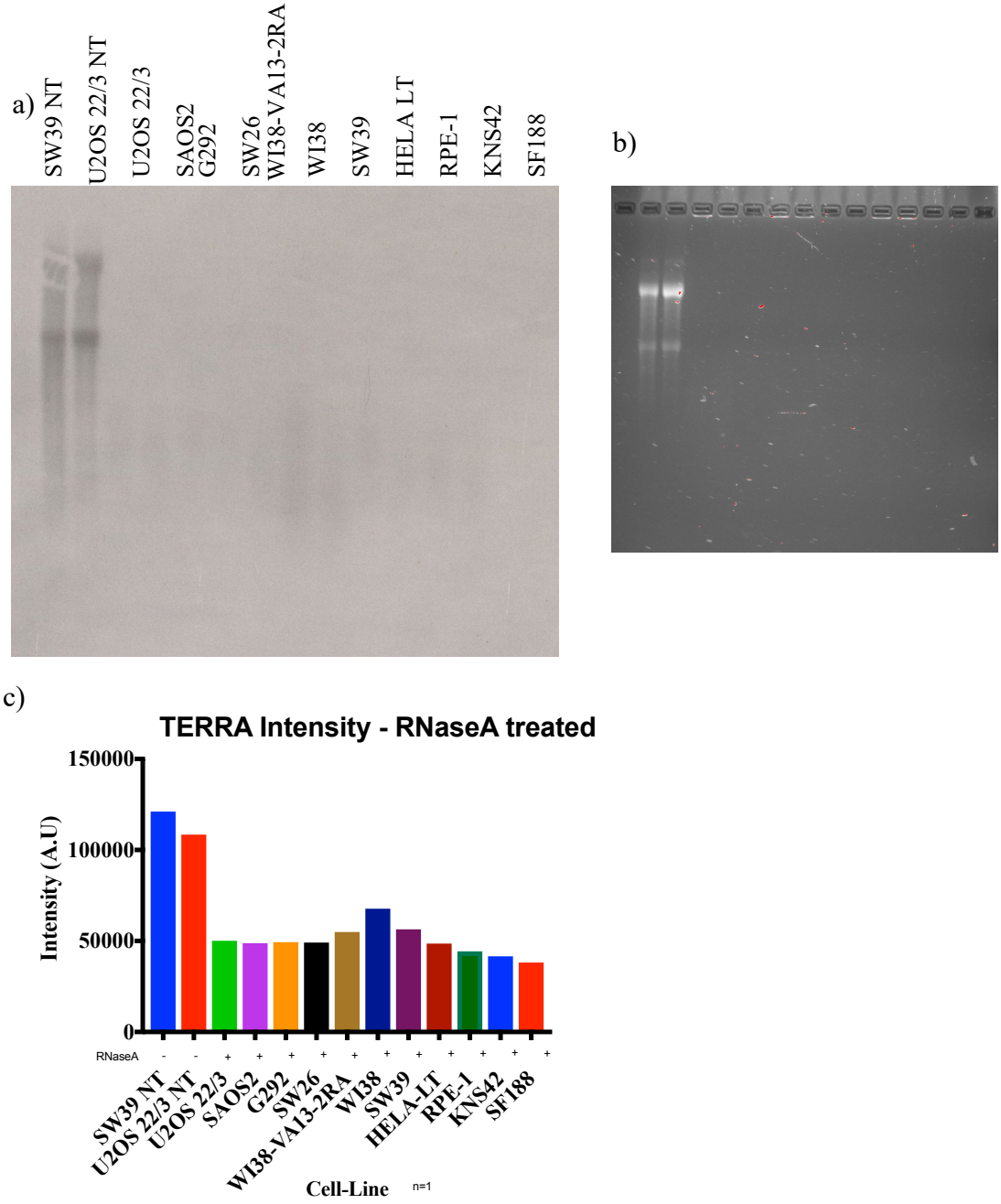


Figure 3.3 Northern blot analysis of TERRA RNA from a panel of ALT-positive and ALT-negative cell lines treated with RNase. Untreated SW39 & U2OS 22/3 show validity of northern a) probed northern blot of TERRA using γ^{32}P-ATP tagged TeloC probe b) loading of blot c) Quantification of TERRA intensity from 1 replicate.

3.1.3 Distributional change of TERRA seen in ALT-positive cell lines

Five ALT-positive cell lines and five ALT-negative cell lines were assessed for distribution of TERRA through RNA-FISH. Cells were grown on coverslips, fixed and hybridised with a fluorescently tagged FISH probe with sequence $(TAA\ CCC)_5$ designed to the canonical TERRA repeat sequence and then detected before mounting on slides. Strikingly there is a universal change in TERRA distribution seen between the ALT-positive and ALT-negative groups (Figure 3.4). The ALT-positive cell lines, such as WI38-VA13-2RA, display distinct TERRA foci, whereas the ALT-negative cell lines show diffuse staining when imaged under the same exposure conditions. This distributional difference was seen through multiple replicates. Image analysis via CellProfiler clearly showed significant differences between the number of foci found in ALT-positive cells and the number found in ALT-negative (* $p<0.05$) (Figure 3.5). Signal readout was controlled for by RNaseA treated controls, which clearly shows a decrease in the number of foci detected post treatment in ALT-positive cell lines (* $p<0.05$) (Figure 3.5). The number of foci found in ALT-negative cell lines did not change as a result of RNaseA treatment, indicating background signal in these replicates. Individual replicates can be seen in appendix A supplementary Figure 2.

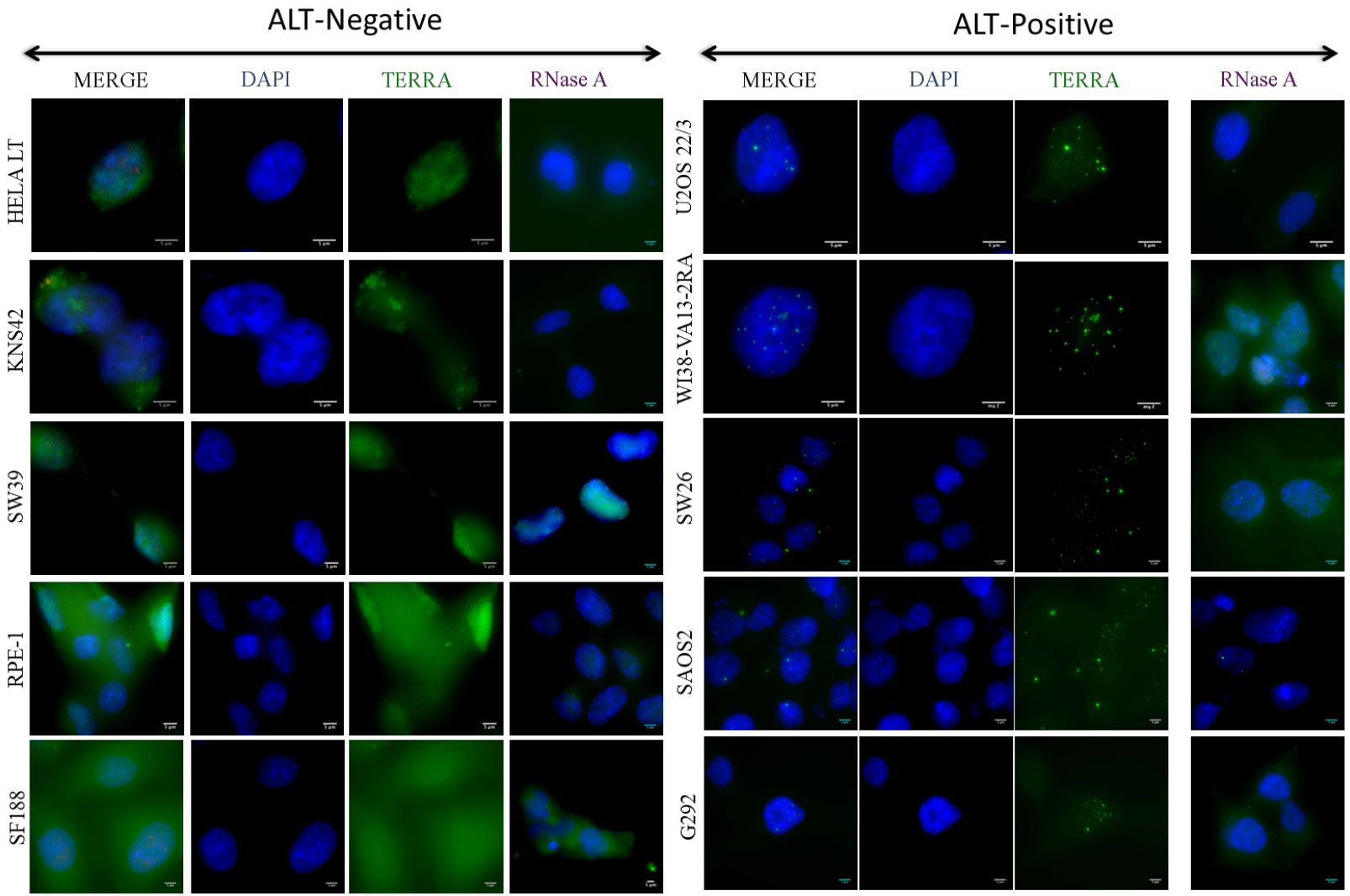


Figure 3.4 RNA-FISH of a panel of ALT-Negative (left) and ALT-positive (right) cell lines. Blue indicates the nuclear DAPI stain, whereas green indicates TERRA signal. Images were taken at 60-100x magnification on the widefield DeltaVision® microscope.

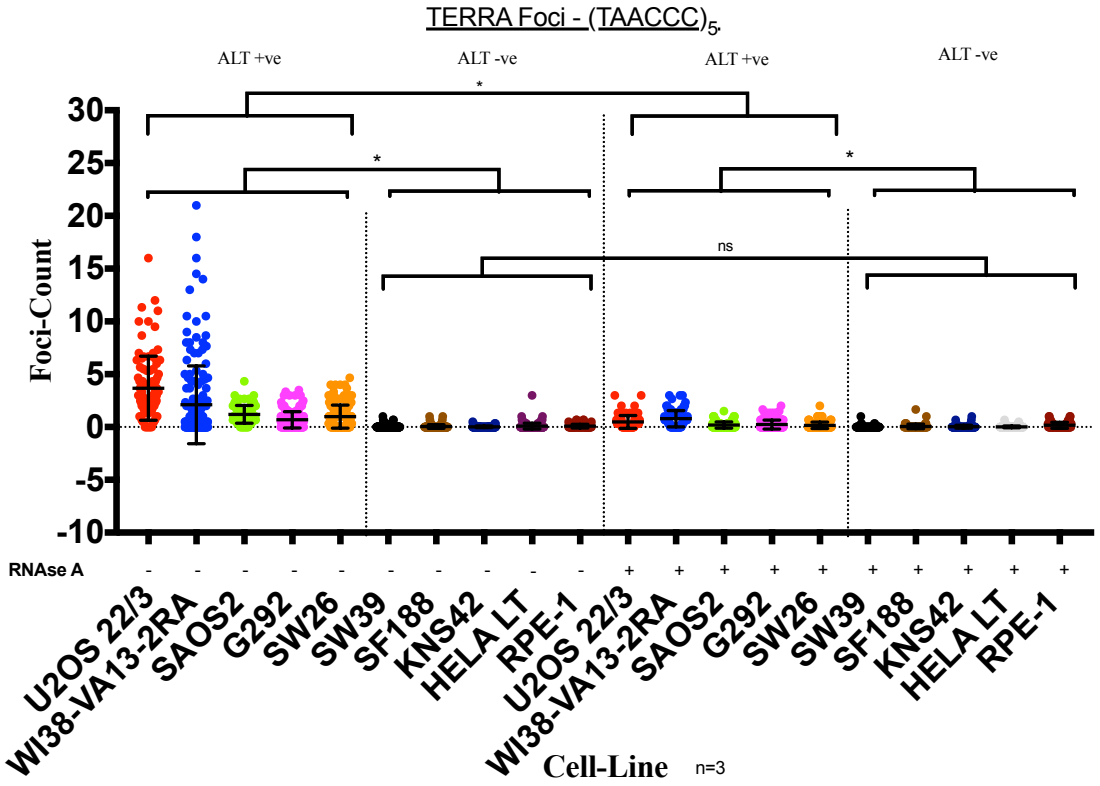


Figure 3.5 Cell profiler analysis of number of canonical TERRA foci seen in a panel of ALT-positive and ALT-negative cell lines. Roughly 100 nuclei were imaged per replicate at 60X magnification on the widefield DeltaVision® microscope. Probe (TAA CCC)$_5$. Canonical TERRA foci count significantly increased between ALT-positive and ALT-negative cell lines ($p < 0.05$). RNaseA controls significantly decrease the number of Canonical TERRA foci (* $p < 0.05$).*

3.1.4 TERRA foci found at Telomeres in ALT-positive cell lines

The presence of TERRA foci in ALT-positive cancers seemed conducive of TERRA localisation to telomeres as described previously. In order to examine whether the TERRA foci seen in my panel of ALT cancers agreed with localisation of TERRA to telomeres, co-localisation of TRF2 and TERRA was investigated through Immuno-

FISH. TRF2 is a member of the Shelterin complex found at telomeric ends and therefore acts as a readout of telomere distribution within the nucleus. As described previously RNA-FISH took meticulous optimization in order to produce a positive readout, this was also the case for Immuno-FISH. The Immuno-FISH protocol starts with protein detection through antibody staining, followed by FISH probe hybridization and detection, before mounting. Ethanol washes used throughout the RNA-FISH protocol, however, were detrimental to protein detection. The alcohol washes likely denatured the proteins and antibodies involved. Therefore, after omittance of alcohol denaturation steps, colocalization of TRF2 and TERRA was seen in WI38-VA13-2RA cells (Figure 3.6). TERRA foci did not co-localise with every TRF2 foci and therefore every telomere, this is indicative of TERRA acting elsewhere in the genome.

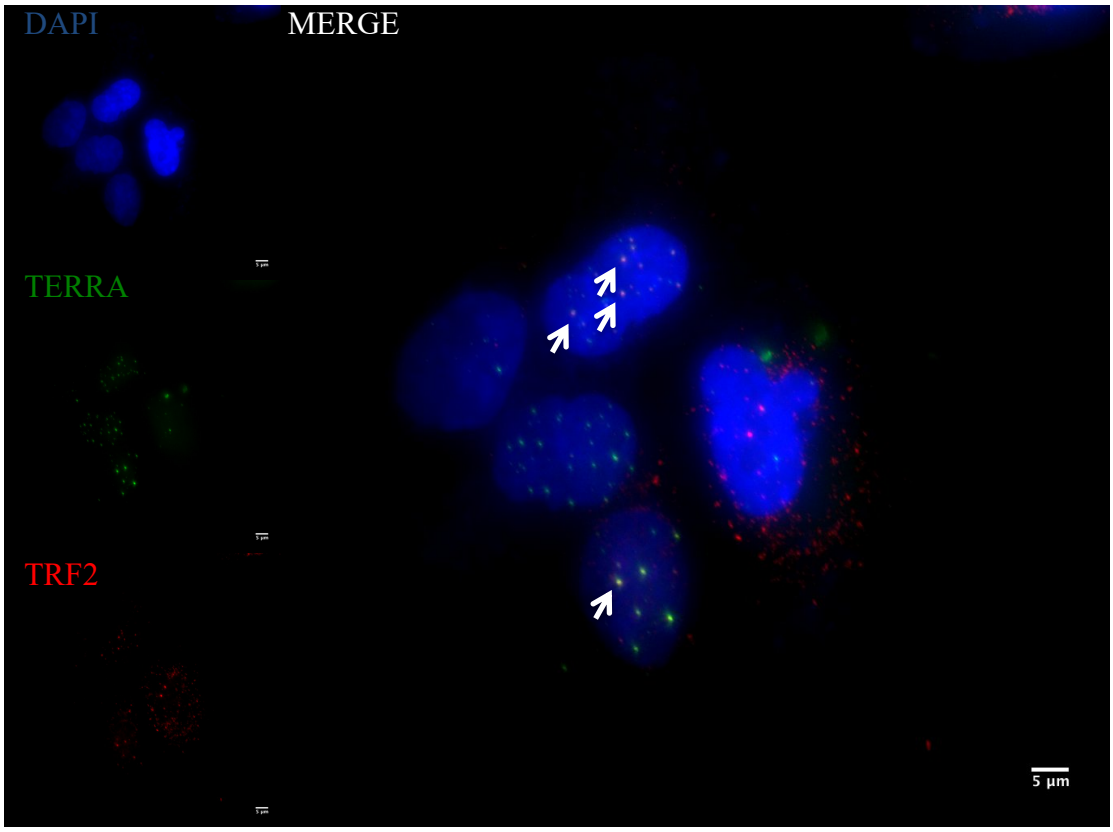


Figure 3.6 Immuno-FISH detecting TERRA RNA (green) and telomere associated protein TRF2 (red) in WI38-VA13-2RA cells. Arrows indicate co-localisation of signals. Images were taken on the widefield DeltaVision® microscope. n=2

3.1.5 TERRA foci formation is not dependent on ATRX status

In order to assess whether functional ATRX loss in ALT-positive cells was producing the distributional change of TERRA foci, both knockdown of ATRX in an ALT-negative cell line and reconstitution of ATRX in an ALT-positive cell line was implemented. In ALT-negative Hela-H3.3-SNAP shATRX (5) cells which contain an shRNA targeted to the ADD domain of ATRX, production of TERRA foci was not seen (Figure 3.7). This was controlled for off target effects of the shRNA by comparison to a scrambled shRNA Hela-H3.3 - SNAP clone (11). Additionally, RNaseA treated controls allowed removal of background signal. Confirmation of the ATRX status of these cell lines can be seen in Figure 3.7c. Individual replicates can be found in appendix A supplementary Figure 3.

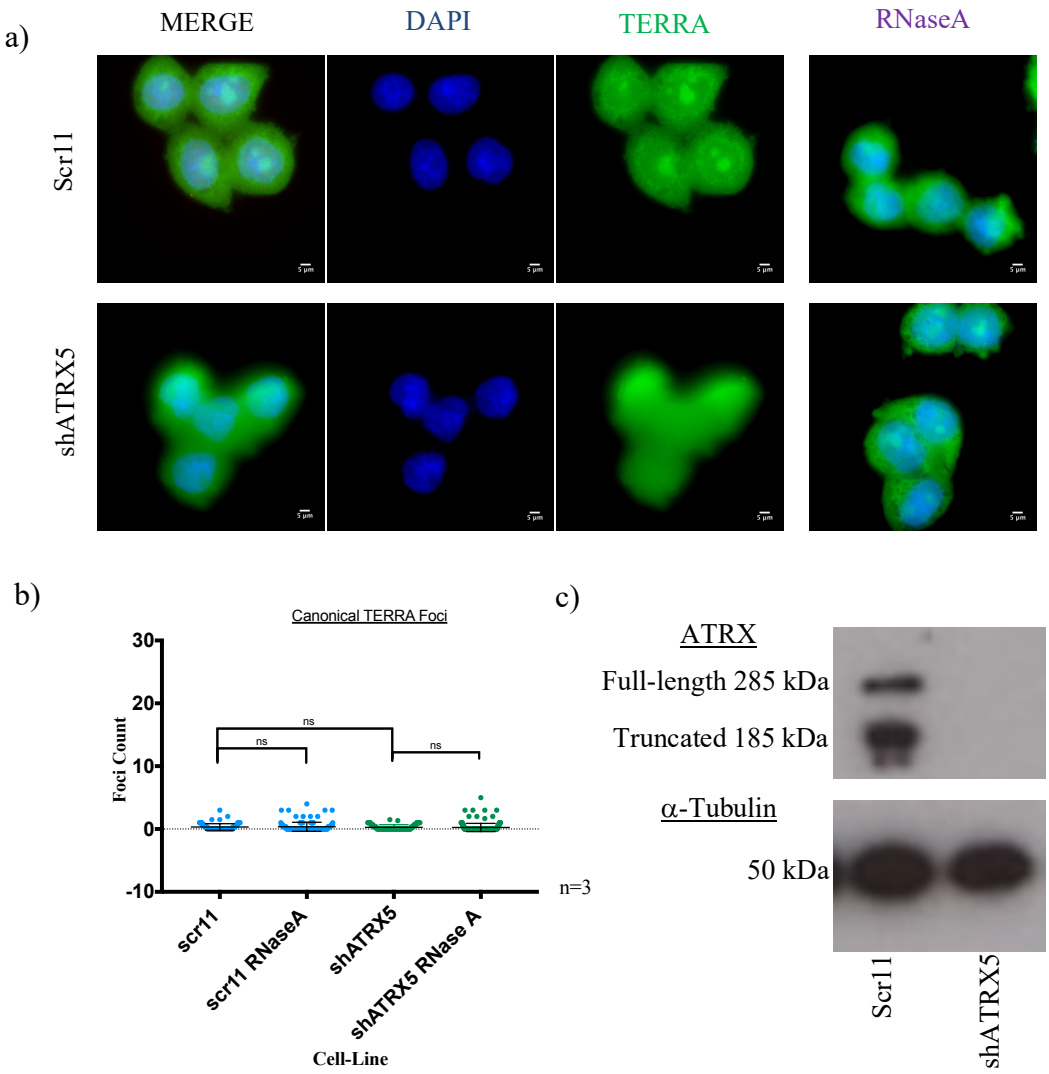


Figure 3.7 ATRX knockdown does not induce TERRA distribution in Hela-SNAP knockdown cells. a) Representative images taken at 60X magnification b) CellProfiler analysis of three replicates c) Western blot showing ATRX expression

Reconstitution of ATRX was carried out in ALT- positive U2OS 22/3 cells; these cells contain a doxycycline inducible cDNA of ATRX. Cells were incubated with doxycycline for 4 days and then assessed by RNA-FISH. Expression of ATRX (confirmed by western blotting Figure 3.8c) caused a decrease in TERRA foci across the replicates (** $p<0.01$) (Figure 3.8). Individual replicates can be found in appendix A supplementary Figure 4.

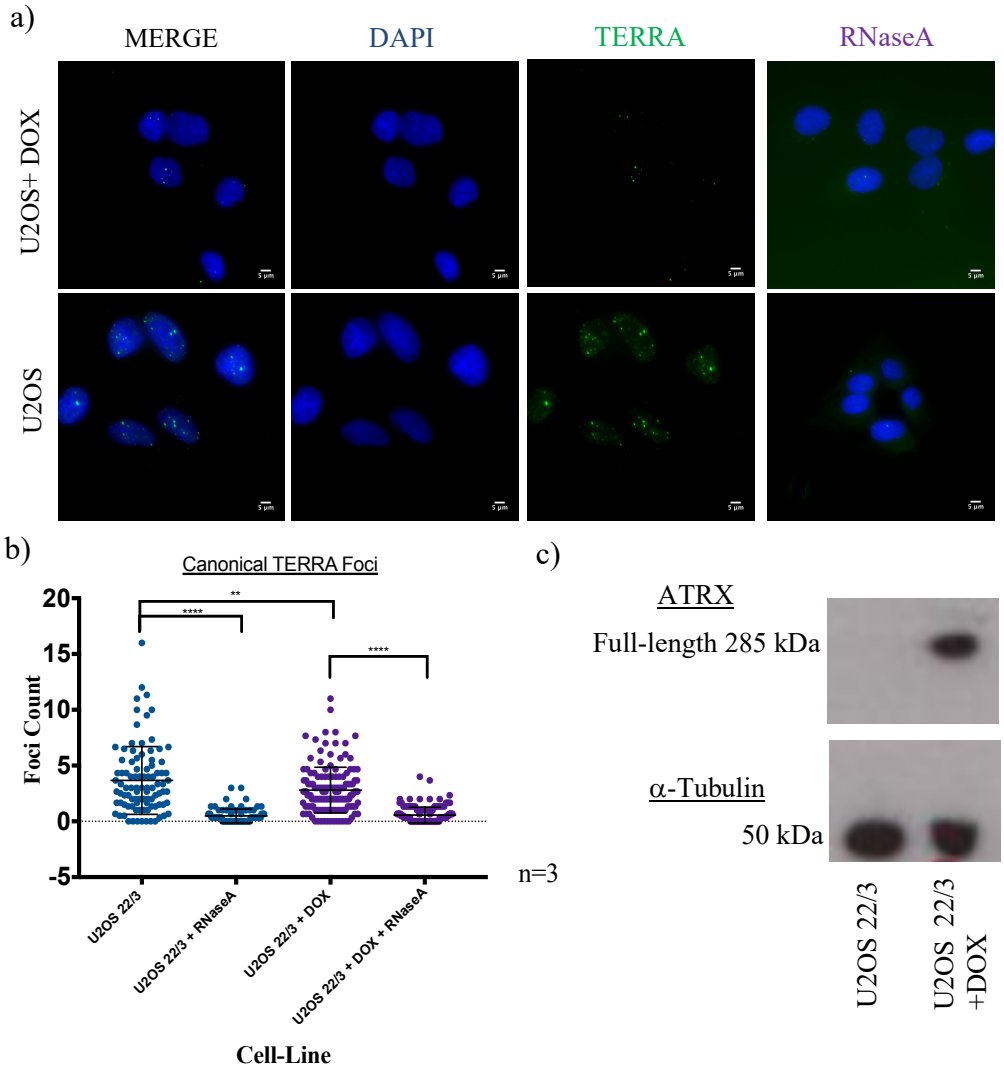
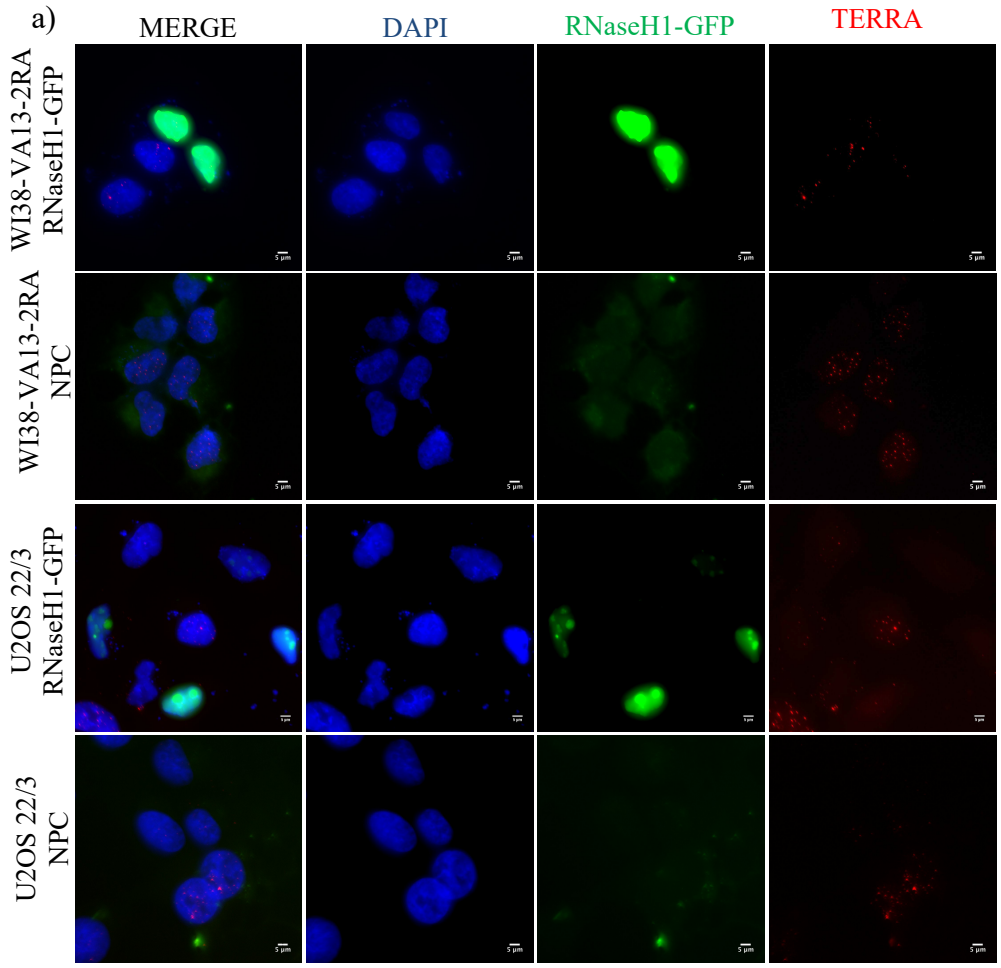


*Figure 3.8 Expression of ATRX in U2OS22/3 cells causing a decrease in TERRA foci (** $p<0.01$). a) Representative images b) CellProfiler analysis c) Western blot showing ATRX expression.*

3.1.6 TERRA Foci in ALT-positive cell lines are R-loops

RNaseH1 selectively degrades DNA:RNA hybrids, which TERRA has previously been reported to form. Over-expression of RNAseH1 was implemented through transient transfection of pEGFP-RNASEH1 (Addgene plasmid # 108699) (Bubeck et al. 2011). Treated populations were imaged based on GFP expression and analysed accordingly through CellProfiler. In ALT-positive U2OS 22/3 and WI38-VA13 positive cells the number of canonical TERRA foci decreased in cells expressing pEGFP-RNASEH1, comparative to the non-plasmid control populations (*** $p<0.001$) (Figure 3.9). This was confirmed through CellProfiler analysis (Figure 3.9). Individual replicates can be found in appendix A supplementary Figure 5.



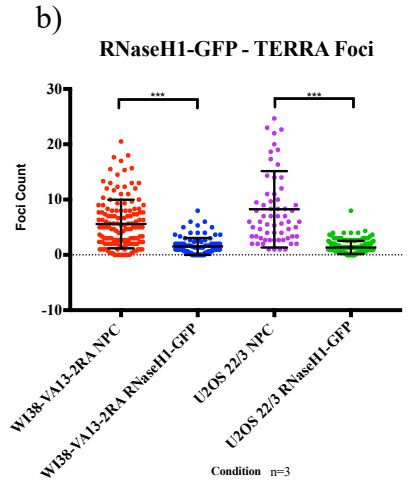


*Figure 3.9 Overexpression of RNase-GFP reduces TERRA foci. a) Representative images taken at 60X magnification b) CellProfiler image analysis, where *** indicates p<0.001*

3.2 Using RNA-FISH as an indicator of ALT-positive cell lines

The variant telomeric repeat TCAGGG is found interspersed throughout the telomeres of some ALT-positive cell lines, namely WI38-VA13-2RA cells, which have TCAGGG repeats within 21% of their telomeres (Conomos et al. 2012). However, U2OS cells, a well characterized ALT cell line has been described to not have this variant telomeric repeat sequence. ALT-positive cell lines were screened using a FISH probe designed to the variant telomeric repeat sequence $(TGACCC)_5$, in order to establish whether the variant sequence could be exploited as a marker to identify ALT-positive cell lines. Additionally, this probe was trialed in the panel of ALT-negative cell lines to act as a control for probe specificity. The variant probe showed clear foci in WI38-VA13-2RA cell populations as well as ALT-positive G292, SW26 and SAOS2 cell lines (Figure 3.10). Consistently U2OS 22/3 did not show foci along with ALT-negative cell lines such as RPE-1. CellProfiler image analysis clearly shows the presence of variant TERRA foci in ALT-positive cell lines (** $p<0.01$) (Figure 3.11). As before RNaseA controls show this signal is valid (* $p<0.05$) (Figure 3.11). This identifies the variant telomeric repeat RNA-FISH probe as a potential tool for identifying ALT-positive cancers. Individual replicates can be found in appendix A supplementary Figure 6.

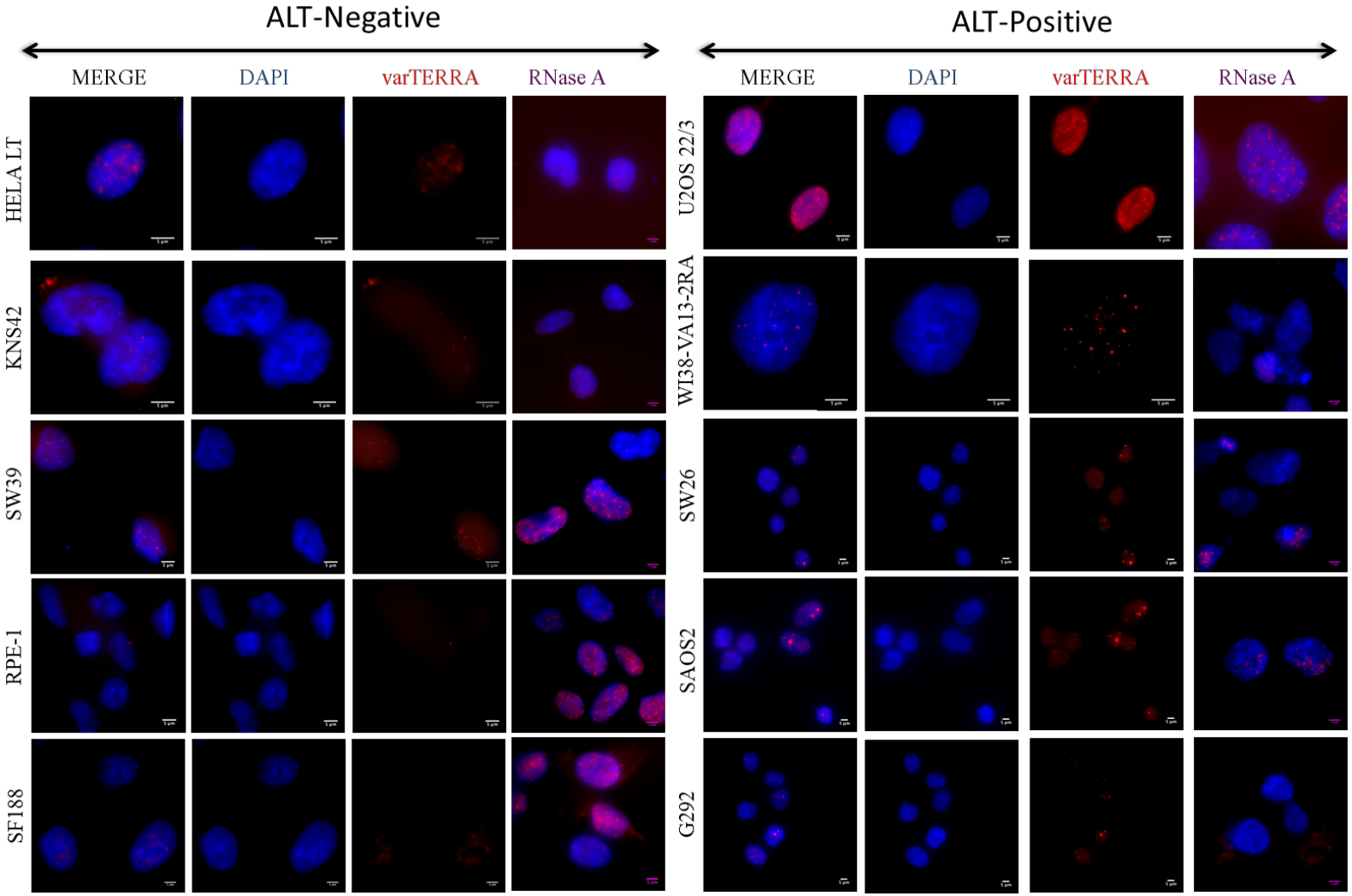


Figure 3.10 RNA-FISH of a panel of ALT-Negative (left) and ALT-positive (right) cell lines. Blue indicates the nuclear DAPI stain, whereas red indicates variant TERRA signal. Images were taken at 60-100x magnification on the widefield DeltaVision® microscope.

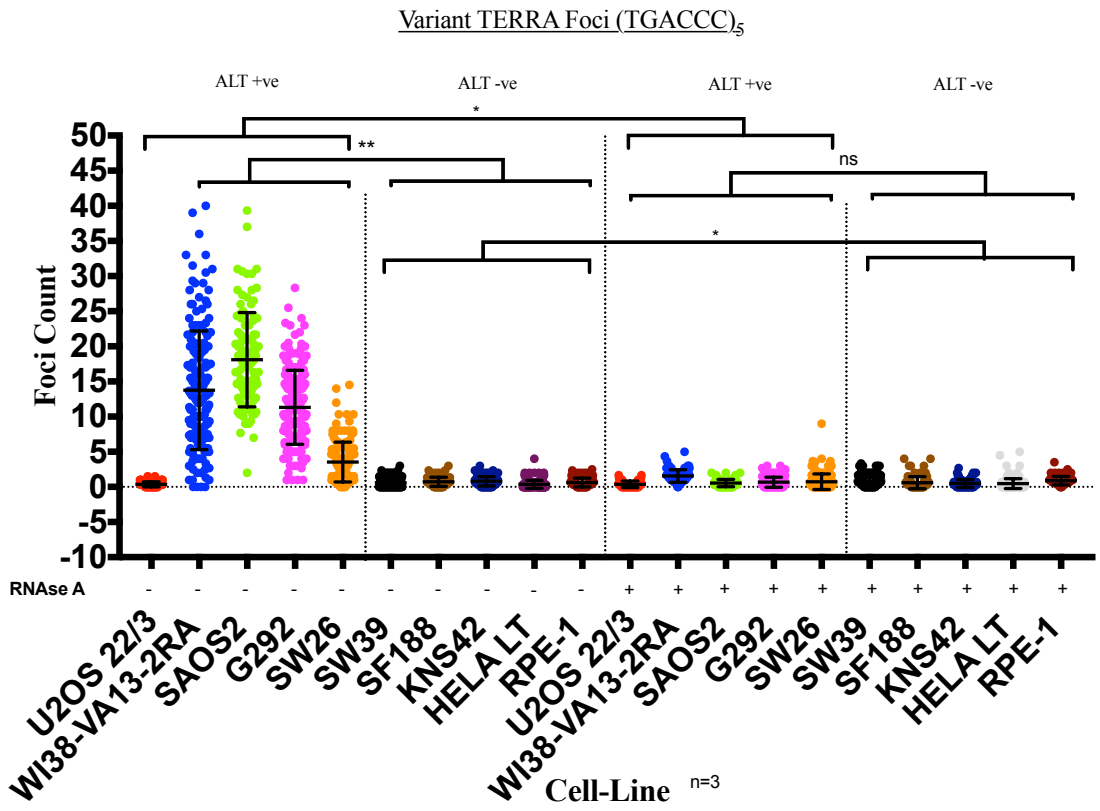


*Figure 3.11 Cell profiler analysis of number of variant TERRA foci seen in a panel of ALT-positive and ALT-negative cell lines. Roughly 100 nuclei were imaged per replicate at 60X magnification on the widefield DeltaVision® microscope. Variant TERRA foci count significantly increased between ALT-positive (excluding U2OS 22/3) and ALT-negative cell lines (** $p < 0.01$). RNaseA controls significantly decrease the number of variant TERRA foci (* $p < 0.05$) but does not significantly change the number found in ALT-negative cell lines.*

3.3 Knocking down the TERRA transcript

Previous reports (Arora et al. 2014) and data presented here suggest that TERRA is forming R-loops at the telomeres of ALT cells. R-loops are predicted to be recombinogenic structures and may therefore have a role in facilitating recombination at telomeres (Arora et al. 2014). Based on this premise we predicted that knocking out/down the TERRA transcript should facilitate a change in the ALT phenotype.

3.3.1 Knocking down the TERRA transcript through LNA GapmeRs

As previously described, LNA GapmeRs work by hybridising to the target RNA through sequence homology of an antisense DNA oligonucleotide sequence. Upon formation of an RNA:DNA hybrid, the RNA strand is cleaved by RNaseH1. The LNA GapmeR is resistant to nucleases through locked nucleic acids (LNA) flanking the antisense oligonucleotide sequence, this allows the LNA GapmeR to bind to its target RNA and influence knockdown for longer timescales than conventional siRNAs. LNA GapmeRs were designed to both the canonical and variant repeat sequence of TERRA in the hope of robustly knocking down the RNA transcript (Table 3a). Concentrations and incubation times of LNA GapmeR treatment were based on a paper published in Cell in 2017 (Chu et al. 2017).

LNA GapmeR	Sequence
TERRA KD	5’ - TAACCCTAACCCTAAC – 3’
varTERRA KD	5’- TGACCCTGACCCTGAC – 3
Scrambled Control	5’- AACACGTCTATACGC – 3’

Table 3a. LNA GapmeR sequences. GapmeRs designed to the canonical and variant repeat - synthesized by Exiqon.

We initially sought to optimize how best to transfect the LNA GapmeRs into our cell populations. I started by inducing transportation through the cell membranes through lipid-based techniques. I trialled a number of transfection reagents including Jet-prime® and Lipofectamine 2000, varying both the concentrations and incubation periods of treatment. I used RNA-FISH as a readout of TERRA levels in order to assess whether the transfection had been successful. However, by RNA-FISH I saw no knockdown of TERRA at both 4 μM and 8 μM of LNA GapmeR (not shown).

After exhausting methods for lipid-based transfection I moved on to use Electroporation as a means of moving the LNA GapmeRs across the cell membrane. Electroporation conditions were trialled through the use of siRNAs designed to knockdown ATRX and assessed via western blots. After successful knockdown of ATRX, the optimized conditions were used to assess whether Electroporation was viable for knockdown of TERRA. Visually it appeared that TERRA may have been knocked down so multiple images were acquired and analysed through Cell profiler. However, image analysis by CellProfiler showed no significant knockdown between TERRA targeted GapmeR and the scrambled control (not shown).

The final technique we tested was Nucleofection; an electroporation-based technique combined with specific transfection reagents. Nucleofection kits come specially designed from Lonza and for this reason there was only two kits available for my cell lines: WI38-VA13-2RA and U2OS. 1 million cells were nucleofected per condition and allowed to settle on dishes for varying time periods.

A distributional change in TERRA transcript size was seen via northern blotting after treatment with 4 μM for 6 hours (Figure 3.12). Quantifying bands and relating the ratio of large fragments to small fragments showed a shift from a greater proportion of large transcripts in the scrambled control to a greater proportion of smaller fragments in the knockdown populations (Figure 3.12c). We conclude that nucleofection was the most effective transfection method to achieve knockdown of TERRA.

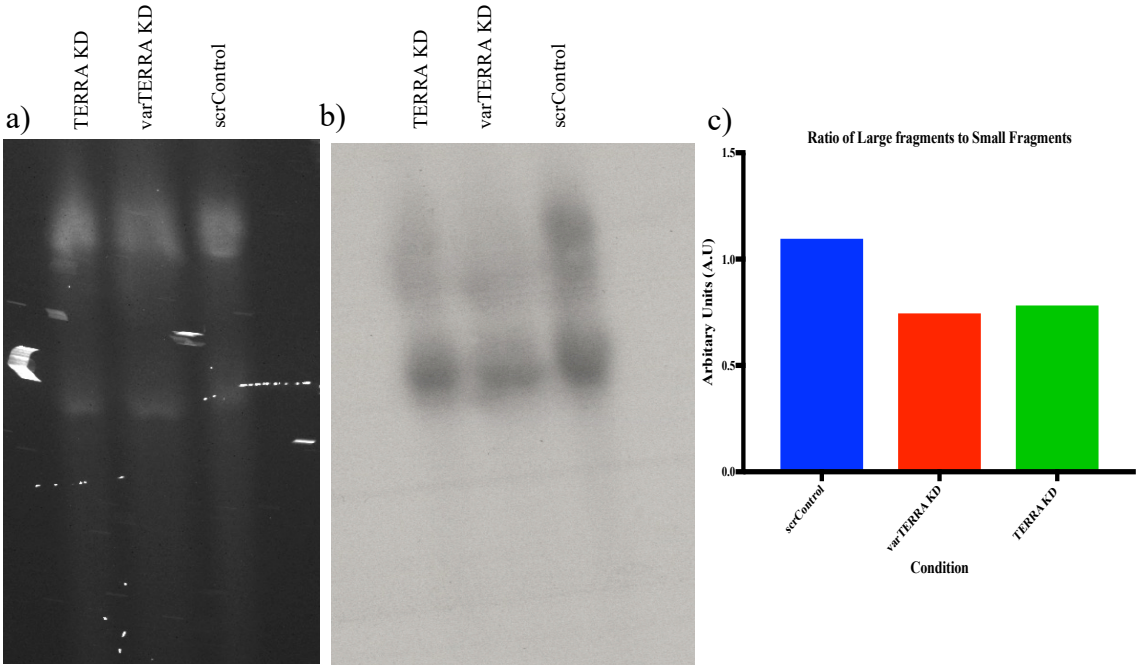


Figure 3.12. Northern blot analysis of 4μM 6hr LNA GapmeR nucleofection in WI38-VA13-2RA cells. a) Gel loading b) Probed northern blot c) Northern blot quantification.

Through RNA-FISH we sought to elucidate the kinetics of knockdown through LNA GapmeRs; cells were fixed 6 hours, 12 hours and 24 hours post nucleofection with 4 μM of LNA GapmeR and assessed via imaging and analysis. Unfortunately, the 4 μM 6-hour knockdown seen through northern blotting could not be replicated by RNA-FISH as the cells had not had enough time to settle on imaged coverslips; distorting any images that were taken. Knockdown was seen 12 hours post nucleofection via RNA-

FISH (* $p<0.05$) and mirrored via northern blotting techniques (Figure 3.13, 3.14). However, both visually and through image analysis a knockdown was not seen at 24 hours post transfection suggesting recovery of the transcript (Figure 3.13).

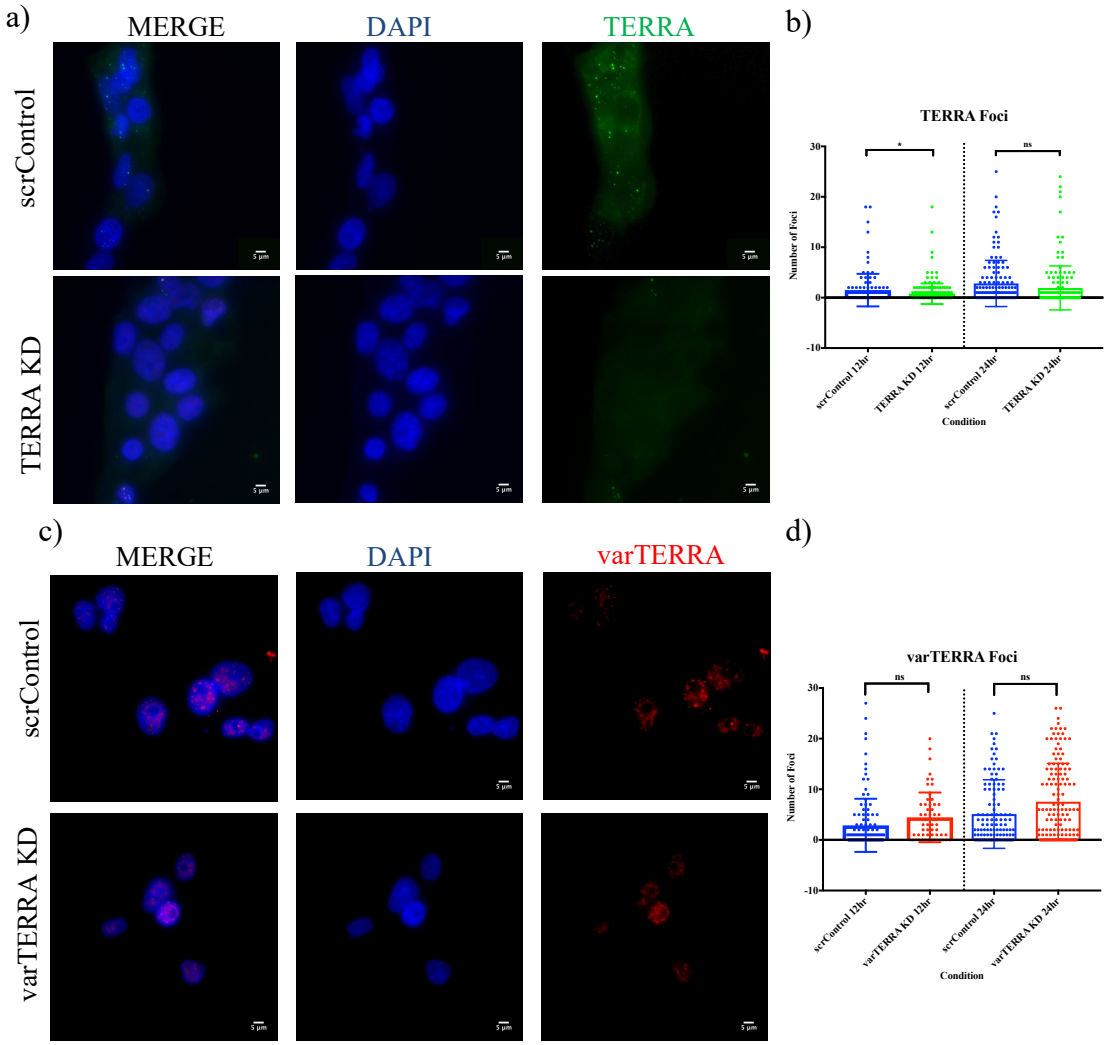


Figure 3.13. RNA-FISH analysis of 4μM 12-hour LNA GapmeR nucleofection in WI38-VA13-2RA cells. a) Representative image of canonical TERRA knockdown with a scrambled control b) Quantification of 12 & 24 hour canonical TERRA knockdown c) Representative image of variant TERRA knockdown with a scrambled control d) Quantification of 12 & 24 hour variant TERRA knockdown.

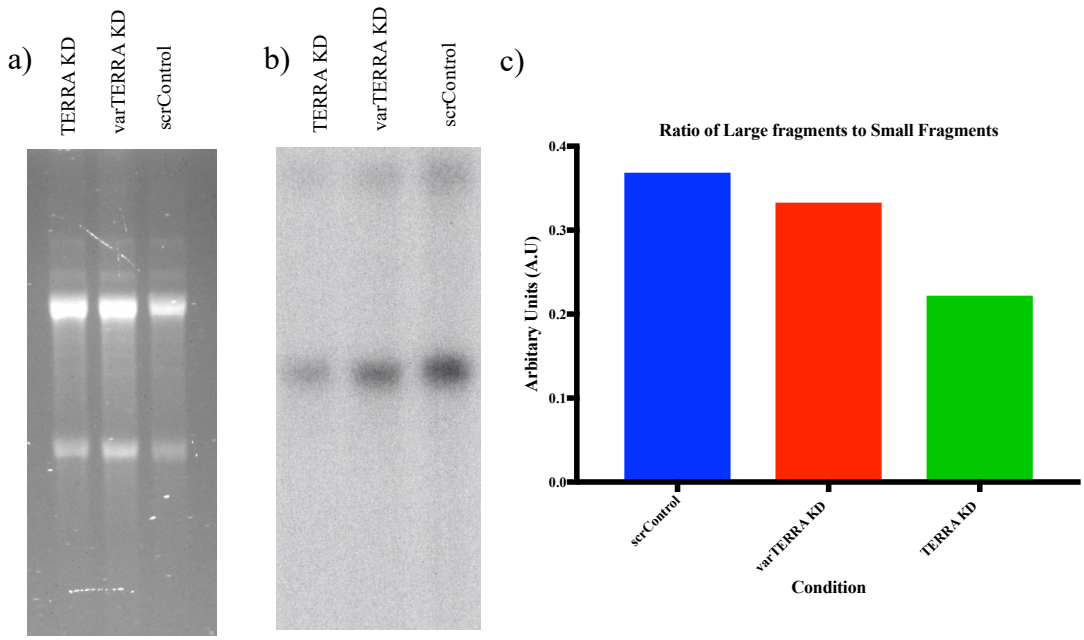


Figure 3.14. Northern blot analysis of 4 μM 12hr LNA GapmeR nucleofection in WI38-VA13-2RA cells. a) Gel loading b) Probed northern blot c) Northern blot quantification.

As previously reported, increasing the concentration of the LNA GapmeR caused a greater reduction in TERRA levels (Chu et al. 2017). Therefore, investigations in to whether this was the case in ALT-positive cell line WI38-VA13-2RA were made. Cells were nucleofected as before for 12 hours with 8μm of LNA GapmeR, to both the canonical and variant repeat, as well as a combination knockdown and scrambled control. Three replicates indicated that TERRA was significantly altered from larger fragments to smaller fragments through this treatment as compared to the scrambled control (* $p<0.05$) (Figure 3.15). The combination and variant targeted knockdown reduced the proportion of larger fragments; however, this was not statistically significant over the three replicates (Figure 3.15). Individual replicates can be seen in supplementary Figure 7.

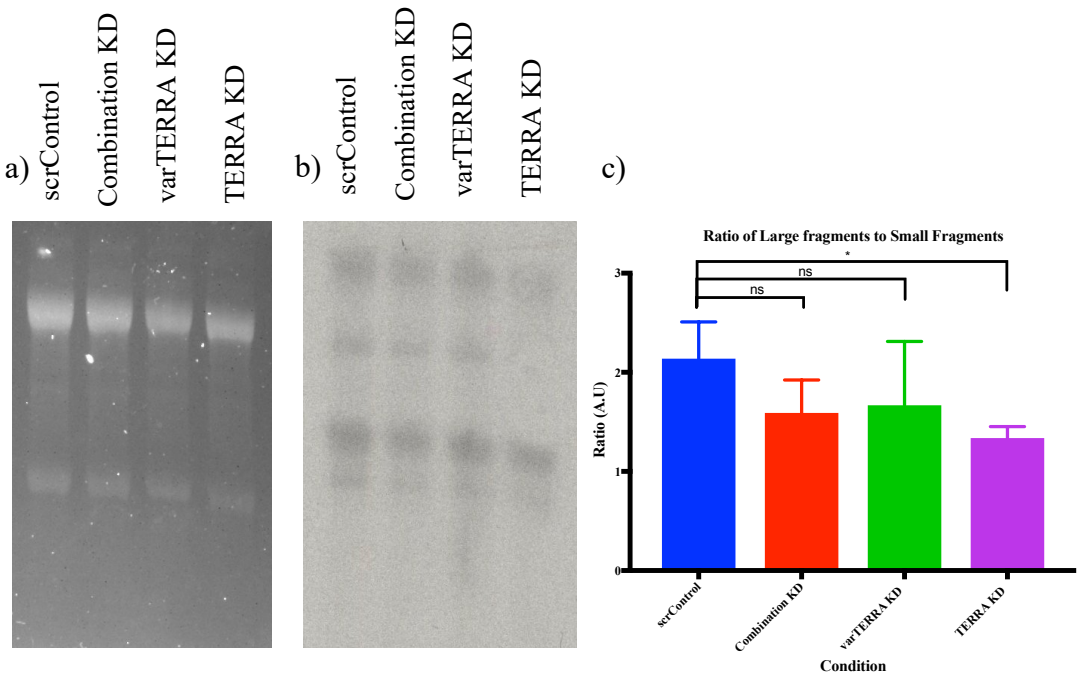


Figure 3.15 Northern blot analysis of 8μM 12hr LNA GapmeR nucleofection in WI38-VA13-2RA cells. a) Example gel loading b) Example probed northern blot c) Northern blot quantification of three replicates.

Importantly, the specificity of the variant repeat targeting LNA GapmeR was tested in ALT-negative Hela LT cells, which should not contain the variant repeat. Specificity was assessed in comparison with the canonical targeting antisense oligonucleotides, along with the scrambled control and a non-treated control (Figure 3.16). As expected the effect of the variant knockdown did not mimic that of the canonical knockdown.

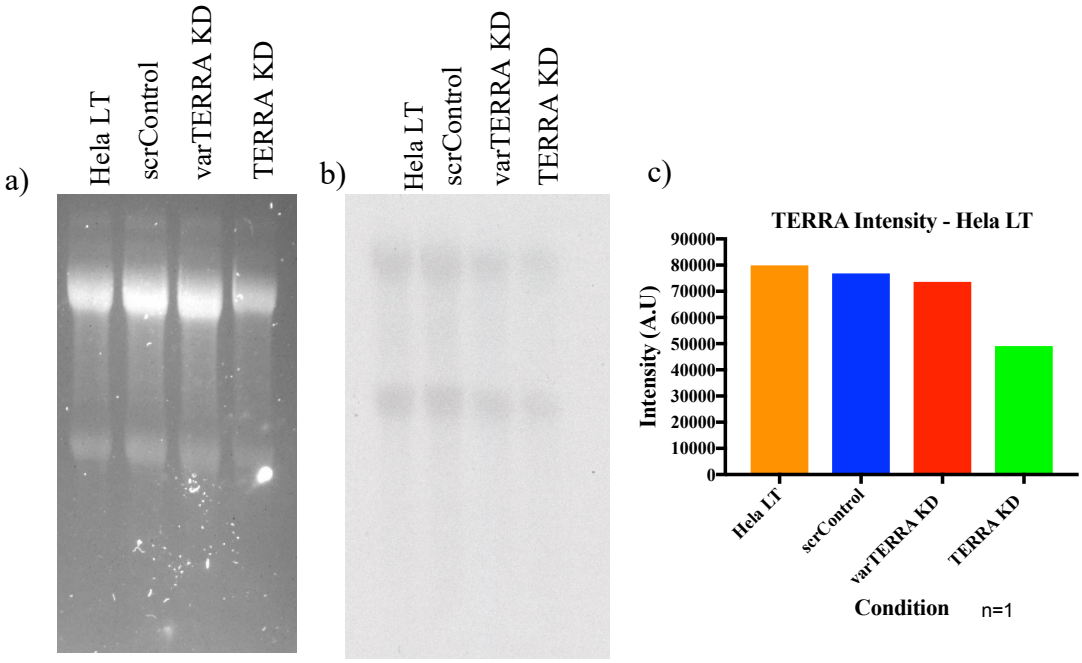


Figure 3.16. LNA GapmeR treatment of ALT-negative cell line Hela LT. a) loading b) probed northern blot c) Northern blot quantification

3.4 Knocking down TERRA using LNA GapmeRs perturbs the ALT phenotype

After successfully producing a shift in TERRA transcript size through transfection of LNA GapmeRs, shown by a reduction in longer fragments, and reduction of TERRA foci, we sought to determine whether this impacted the ALT phenotype by investigating the hallmarks of ALT. C-Circles are partially double stranded extra-chromosomal DNA circles which are characteristically present in ALT cancers. C-circles are constitutive biomarkers of ALT cancers, so investigating whether changes in TERRA transcript size affected the production of C-circles in ALT cancers was the first avenue of exploration. The c-circle assay is semi-quantitative and relies on self-priming rolling circle amplification to amplify off the single stranded proportion of the C-circle. Cells were

nucleofected with 8μM of LNA GapmeR for 12 hours, following which gDNA was extracted and amplified through rolling circle amplification. Samples were then blotted onto membrane and probed using a telomeric specific probe labelled with γ-P^{32}, before development on autoradiography films. The experiment was controlled through non-treated samples as well as using a scrambled control LNA GapmeR, with background signal subtracted through no-polymerase treated samples (no-phi). Knockdown was implemented to both the variant and canonical repeat as well as targeting TERRA by a combination of the two LNA GapmeRs.

Consistently throughout the repeats (n=3) the canonical TERRA knockdown produced a significant drop in ALT-positive WI38-VA13-2RA C-Circle levels (** $p < 0.01$), to a level comparable with the SW39 ALT-negative cell line (Figure 3.17). Targeting the variant TERRA repeat produced a small decrease in C-circles (not statistically significant), with a slightly higher reduction seen when using a combination of the canonical and variant repeat LNA GapmeRs (not statistically significant) (Figure 3.17). Individual replicates can be found in appendix A supplementary Figure 8. This data indicates that knockdown of TERRA affects a constitutive biomarker of ALT cancers, and therefore TERRA is playing some role in ALT cancers.

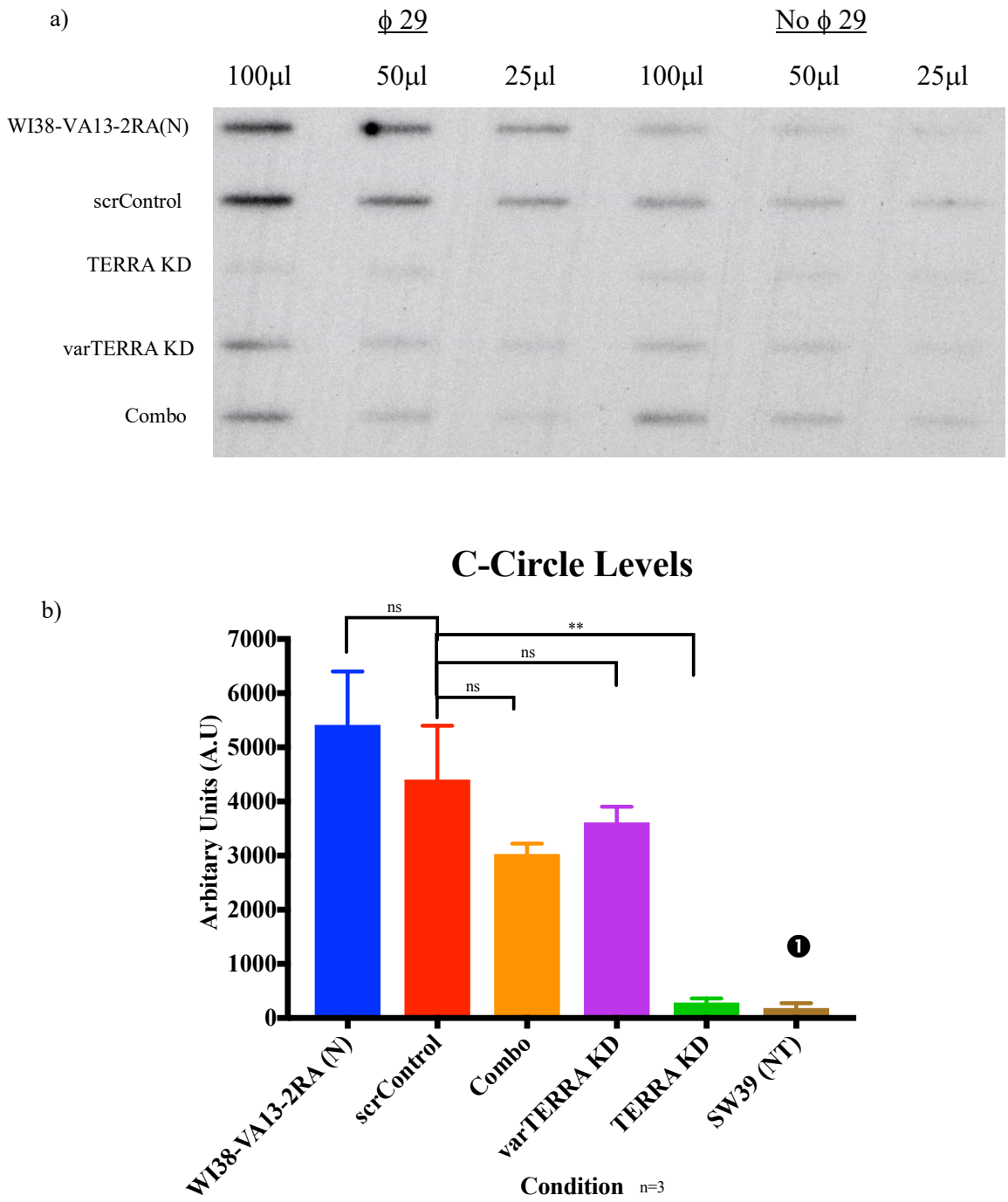


*Figure 3.17. C-Circle levels change as a result of TERRA knockdown in WI38-VA13-2RA cells. a) Example slot blot, with amplification (ϕ 29) vs no amplification (No ϕ 29) in a dilution series. b) Quantification of three replicates (ϕ 29 - No ϕ 29) where: ** p<0.01 ns – non-significant, N – nucleofected, NT- non-treated and ❶ - 1 replicate.*

3.5 Knocking down the TERRA transcript using LwaCas13a

The knockdown produced by LNA GapmeRs was short lived, so we sought to establish a method of TERRA knockdown which would allow us to assess cell viability and further phenotypic changes in ALT-cancers for longer periods. Therefore, the second technique utilized to degrade the TERRA transcript was LwaCas13a. As mentioned previously Cas13a degrades RNA through HEPN cleavage domains, and has reduced off target effects when compared with siRNAs (Abudayyeh et al. 2017). Localising Cas13a to the telomeric repeats would allow for RNA degradation in a selected region of the genome, limiting global RNA degradation effects. Initial experiments aimed to identify suitable guide sequences for localising constructs to the telomeric repeats. In work previously published by Gilbert et al. guide sequences which localised dCas9 fused with a fluorescent protein to the canonical repeat of telomeres were produced (Gilbert et al. 2012). Therefore, utilizing a guide that would localise constructs to the variant telomeric repeat could be beneficial for both identification and targeting of ALT cancers. The plasmid Alt-Tel-Ruby is a dCas9 fused with a red-fluorescent protein (RFP) and a guide targeting the variant telomeric repeat. Co-localisation of Alt-Tel-Ruby with TRF2 via immunofluorescence was seen in an ALT-positive cell line WI38-VA13-2RA post transfection (Figure 3.18). Co-localisation was not seen in an ALT negative cell line Hela LT (Figure 3.18), indicating specificity of the guide for variant repeats within the telomere. We concluded that these guide sequences were suitable for targeting both the canonical and variant telomeric repeat. Philip Hublitz (WIMM genome editing facility) therefore synthesized Cas13a constructs with specificity for the ALT variant repeat sequence. One plasmid containing the guide sequence and one plasmid containing Cas13a-GFP was transiently transfected into cell populations. Unfortunately, transient transfection did not show a knockdown in TERRA foci number

when transfected with Cas13a (Figure 3.19). It also showed a localisation of the Cas13a-GFP to what appeared to be the nucleolus, implying the Cas13a-GFP was being overexpressed and perhaps wasn't localising correctly to telomeres.

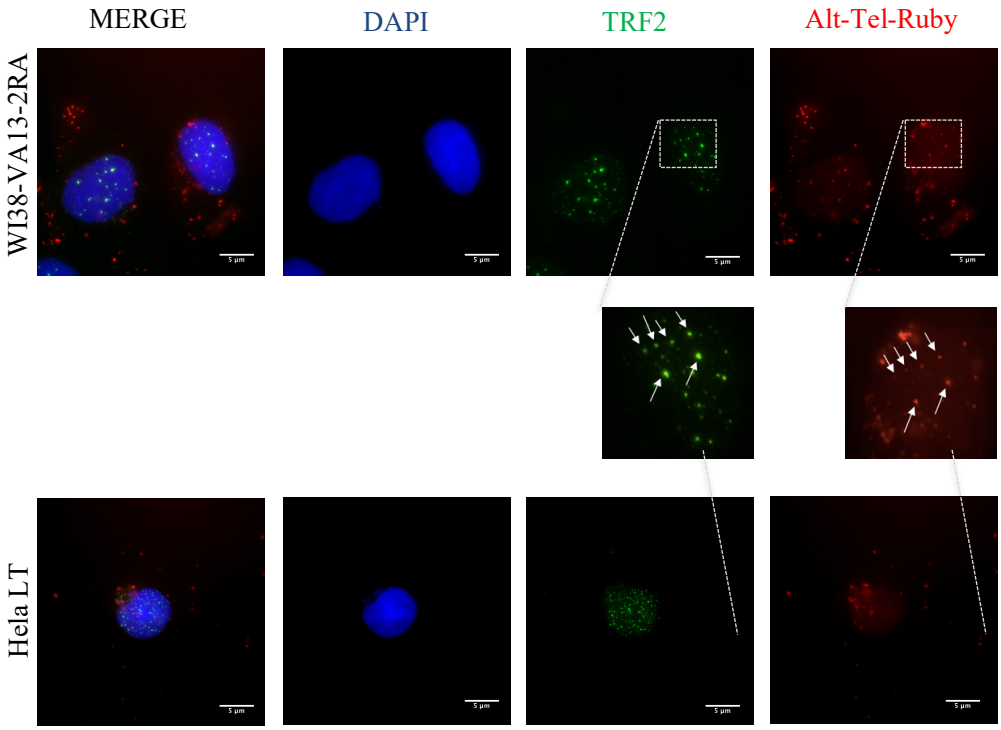


Figure 3.18. Guide sequence selection, with TRF2 in green and Alt-Tel-Ruby in red. Colocalization is indicated trough white arrows. Images were taken at 100X.

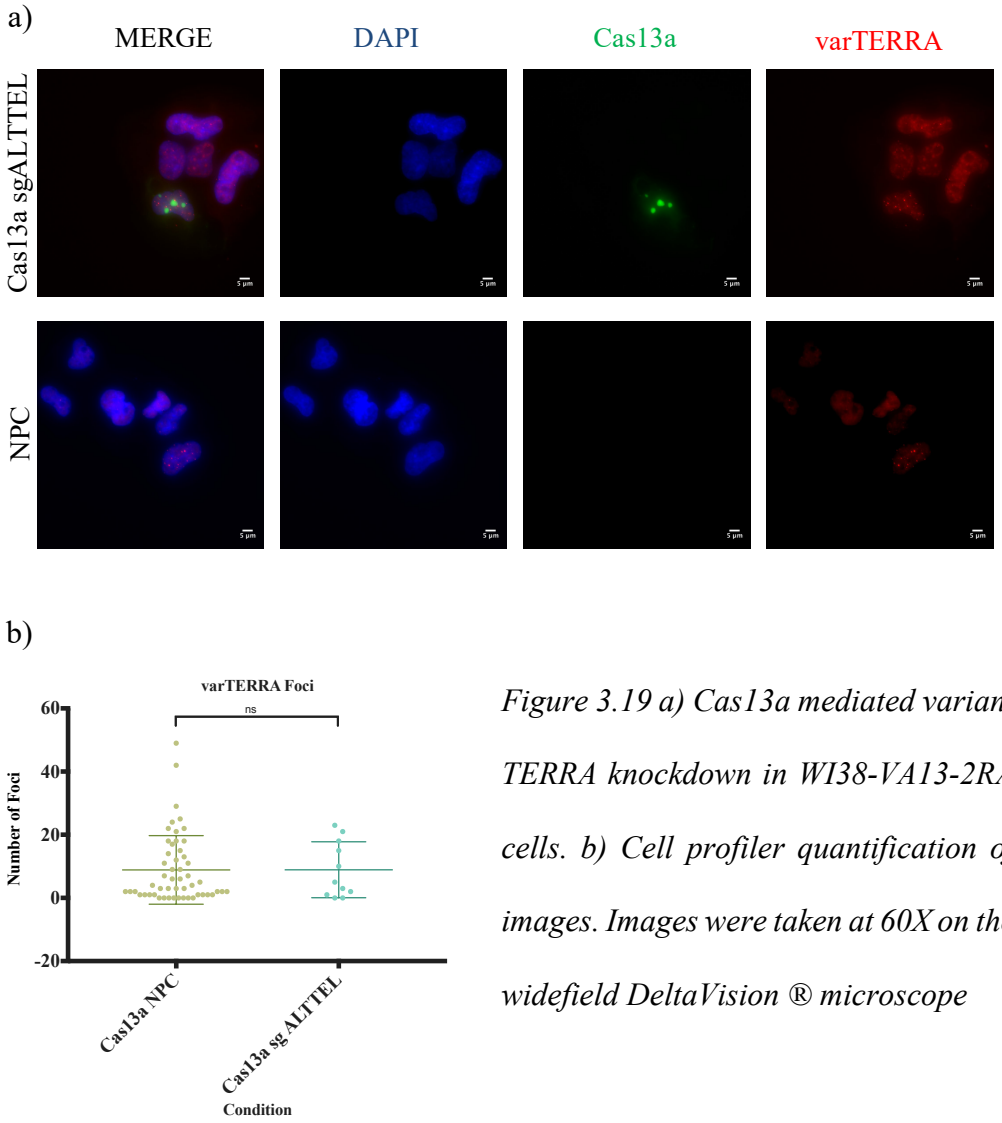


Figure 3.19 a) Cas13a mediated variant TERRA knockdown in WI38-VA13-2RA cells. b) Cell profiler quantification of images. Images were taken at 60X on the widefield DeltaVision ® microscope

To address this issue, we are currently generating stable cell lines containing a Tet-inducible Cas13a-GFP plasmid, which would allow both prolonged expression of Cas13a-GFP as well as modulation of expression levels.

4. Discussion

The unique hallmarks of cancers employing the Alternative lengthening of telomeres pathway (ALT) are an attractive target for therapeutic intervention and study. Telomeric repeat containing RNA (TERRA) appears to hold precedence in ALT cancers, the mechanism of its action, however, is yet to be understood. TERRA through its previously shown interactions with the leading strand of telomeric DNA to form an R-loop, might be facilitating recombination in ALT cancers. This work aimed to investigate whether TERRA transcription was upregulated in ALT-positive cell lines, and whether this upregulation was dependent on ATRX expression. Additionally, this study aimed to determine a role for TERRA in recombination by reducing transcript levels and investigating changes to the phenotype of ALT cancers as a result. A resulting phenotypic change would identify TERRA as a viable therapeutic target for the treatment of ALT cancers.

4.1 TERRA differs in distribution between ALT-positive and ALT-negative cancers

The recent identification of RNA transcribed from the telomeric repeats has been hotly investigated. In ALT cancers TERRA has been described to be up-regulated, down-regulated or unchanged by conflicting sources (Clynes et al. 2015; Goldberg et al. 2011; Flynn et al. 2015; Eid, M.-V. Dematteï, et al. 2015; Mattiussi et al. 2014). The data we present here suggests that although the overall levels of TERRA RNA appear similar in ALT-positive and ALT-negative cells, however, the distribution of TERRA dramatically changes between the two populations. ALT-positive cancers displayed localised, punctate TERRA foci when probed with both the canonical and variant

TERRA repeat. This highlighted a distributional change in TERRA foci in ALT-positive cancers, consistent with localisation of TERRA foci at telomeres. We report that a proportion of TERRA foci appear to co-localise to telomeres as shown by overlap with TRF2 a member of the Shelterin complex found at the ends of telomeres. These findings indicate that in ALT-positive cancers the distributional change observed whereby TERRA localises to telomeres may indicate that TERRA has a functional role at ALT telomeres.

4.2 TERRA foci distribution was not induced by ATRX loss

ATRX loss is a major hallmark of ALT cancers and we therefore considered the possibility that the loss/mutation of ATRX in ALT-positive cell is causing the change in distribution of TERRA foci seen across the panel of ALT-positive cell lines. Therefore, TERRA distribution was examined in an ALT-negative cell line upon knockdown of ATRX. The distribution of TERRA did not change upon knockdown of ATRX, indicating that the loss of ATRX is not required to cause the change in TERRA foci distribution seen in ALT-positive populations. This complements previous studies in which it was shown that ATRX loss alone is not sufficient to induce the ALT pathway (Eid, M. Demattei, et al. 2015).

4.3 ATRX expression in ALT changes TERRA distribution

ATRX re-expression in ALT-positive U-2OS cells was previously reported to be sufficient to supress the ALT phenotype (Clynes et al. 2015). We therefore reasoned that re-expression of ATRX might affect TERRA distribution in this genome context. Image analysis showed a small significant decrease in the number of TERRA foci as a result of ATRX re-expression. Given that the loss of TERRA foci was small, further

replicates need to be investigated as well as the kinetics of TERRA loss upon ATRX expression, to confirm this data.

4.4 TERRA Foci are associated with RNA:DNA hybrids

R-loops are comprised of three stranded structures in which one strand of DNA is displaced allowing a RNA component to hybridise with its complementary DNA sequence (RNA:DNA hybrid) (Sollier & Cimprich 2015). Arora et al. have previously shown that TERRA forms R-loops in ALT-positive U-2OS cell populations (Arora et al. 2014). Overexpressing RNaseH1 in WI38-VA13-2RA cells showed reduction in TERRA foci. This indicates that the foci seen in ALT-positive cell lines are indicative of R-loops as RNaseH1 mediates cleavage of the RNA-DNA hybrids. In fact ATRX has been linked to supressing R-loop formation at telomeric repeat regions (Nguyen et al. 2017). Loss of ATRX, seen in ALT cancers, caused an increase in R-loop formation, whereas reconstitution of ATRX reduced this change (Nguyen et al. 2017). The mechanism by which ATRX modulates R-loop levels is currently unknown and is an interesting topic of future study. R-loop presence in two ALT-positive ATRX null cancer cell lines could therefore mark them as a hallmark of ALT cancers and lend to the idea that TERRA influences recombination by forming R-loop structures at telomeres.

4.5 TERRA knockdown can be achieved through LNA GapmeR treatment

TERRA forming recombinogenic R-loops in ALT-positive cell lines suggests that TERRA may facilitate the ALT pathway as previously suggested (Arora et al. 2014). We reasoned that abrogation of the TERRA transcript might therefore influence the ALT phenotype. Knockdown of TERRA is difficult due to the length and abundance

of the transcript, however, it was demonstrated that LNA GapmeRs could be used to knockdown the TERRA transcript (Chu et al. 2017). We therefore sought to confirm that LNA GapmeRs could be used to knockdown the canonical TERRA transcript as well as the variant TERRA transcript. LNA GapmeRs were designed to both the variant and canonical repeat, however, we were unable to achieve a full knockdown of TERRA. A shift in the size of TERRA distribution and not a full knockdown could be explained based on the sheer size of the TERRA transcript. ALT-positive cancers phenotypically harbour long heterogeneous telomeres so transcription off these telomeres produces TERRA transcripts of up to 20 kb in length (Luke & Lingner 2009). LNA GapmeRs are around 20 nucleotides in length so are dwarfed in comparison to TERRA transcripts. LNA GapmeR binding at one point of the TERRA transcript, followed by RNaseH1 mediated cleavage of the RNA would split the transcript into two pieces. This is because RNaseH1 is only an endonuclease, so it thus cleaves in the middle of the homology and does not degrade the rest of the transcript (Sulej et al. 2012). The likelihood of repeated association to the same transcript and consequent cleave of by RNaseH1 is reduced as the resulting proportion of transcript fragments increases. This notion may explain why a full knockdown is not seen and why a distributional change from long to short transcripts is depicted by northern blotting. We do, however, report a significant loss of TERRA foci as determined through RNA-FISH, suggesting that the level of depletion we achieved was sufficient to disrupt R-loop formation at ALT telomeres.

The LNA GapmeR did not show a knockdown for treatment longer than 12 hours. This was disappointing as we expected that the increased resistance to exonucleases provided by the LNA bases, might allow a sustained knockdown for longer periods and

thus allow insight as to whether TERRA knockdown facilitates changes to cell viability. We do not know, however, if LNA GapmeRs remain bound to RNA fragments after cleavage or dissociate to re-anneal to other target sequences. If LNA GapmeRs do remain in complex with RNA fragments, eventually saturation of the pool of LNA GapmeR on TERRA fragments, will allow transcription to return the levels of TERRA back to normal state. This may explain why TERRA knockdown through LNA GapmeR treatment is only observed for a short amount of time.

4.5.1 The variant repeat targeting LNA GapmeR targets to a lesser extent

ALT cancers uniquely harbour variant telomeric repeat sequences, making the variant repeat an attractive target for therapy. We therefore sought to target the variant repeat through LNA GapmeR knockdown. Targeting the variant repeat showed a smaller distributional change in variant-TERRA knockdown than the canonical repeat targeting LNA GapmeR. This was to be expected as the variant repeat accounts for a smaller proportion of the telomeric repeat sequence. In WI38-VA13-2RA ALT-positive cells the proportion that the variant repeat occupies in the telomere is 21%, so a greater knockdown than the canonical was not expected when targeting ALT cancers with the variant LNA GapmeR (Conomos et al. 2012). Importantly, however, transfection of the variant-targeting LNA GapmeR did not produce a knockdown in an ALT-negative cell line, Hela LT. This data showed specificity of the LNA GapmeR designed to the variant telomeric repeat. This highlights a potential therapeutic opportunity for exploiting the variant telomeric repeat for targeting ALT cancers, if headway can be made in producing a sustained knockdown of TERRA with greater efficiency.

4.8 Does TERRA facilitate the ALT pathway in ALT-positive Cancers?

The data presented here shows that TERRA is differentially distributed in ALT-positive cancers when compared with ALT-negative cancers. This distribution can be telomere associated and implies a role for TERRA at these loci. Knockdown of longer length TERRA transcripts produced a decrease in the ALT constitutive biomarker, telomeric C-circles. The aetiology of c-circle production in ALT cancers is unknown, however, it has been proposed that c-circles are a by-product of telomere trimming (Pickett & Reddel 2012). Levels of C-circles are highly predictive of an active ALT pathway and the data presented here therefore suggests that the formation of TERRA R-loops might be influencing this biomarker. ALT cancers have been previously described to have increased spontaneous replicative stress at their telomeres (Min et al. 2017b). TERRA forming R-loops on the leading strand of DNA may exacerbate replicative stress via replication fork stalling and consequent fork collapse in ALT cancers (Arora et al. 2014; Min et al. 2017b). Replicative stress caused by TERRA R-loops could therefore provide the one ended DSBs necessary for BIR to occur (Dilley et al. 2016). The resulting outcome being extension of heterogenous telomeres and production of extrachromosomal telomeric DNA in the form of C-circles through telomere trimming (O'Sullivan et al. 2014; Pickett & Reddel 2012).

Additionally, conclusions cannot be drawn on a single phenotypic change, investigations into any changes to other hallmarks of ALT cancers such as high telomeric sister chromatid exchange needs to be examined. By implicating TERRA in other phenotypic attributes, further insight into a role for this long non-coding RNA can be drawn.

4.9 Implications of study

This study has indicated a role for the telomeric transcript, TERRA in ALT positive cancers. It also preludes further investigation into a potential role for TERRA in these cancers.

4.9.1 Exploiting the variant telomeric repeat for diagnostic purposes

ALT positive cancers may be vulnerable to different therapies when compared with telomerase positive cancers. Indeed, loss of ATRX has previously been shown to confer sensitivity to PARP inhibitors, Hydroxyurea and Telomestatin (Huh et al. 2016; Clynes et al. 2014; Watson et al. 2013). As such there is likely to be an important need to stratify cancers based on their ALT status. This work has shown the variant telomeric repeat as a potential diagnostic marker of ALT cancers. Through design of an RNA-FISH probe targeted to the variant repeat ALT-positive cell lines such as WI38-VA13-2RA were identified through image analysis. The specificity of the probe was confirmed by probing of ALT-negative cell lines which showed no variant TERRA foci in image analysis. This was further confirmed when no visible foci were seen in U2OS 22/3 cells which do not harbour the telomeric repeat sequence as previously described by (Conomos et al. 2012). The variant repeat could thus be used to screen patient samples for ALT-positivity and stratify these cancers for treatment.

4.9.2 Using LNA GapmeRs in a therapeutic setting

This study highlighted that LNA GapmeRs could be used to target both the canonical and variant TERRA repeat for degradation to a certain degree. Antisense oligonucleotides have been used in clinical trials to treat hypercholesterolemia and hypercoagulation (Beaudet & Meng 2016; Wittrup & Lieberman 2015). Rossiello et al.

firstly used antisense oligonucleotides to limit the number of DNA damage response RNAs (DDRNAs) transcribed from both the C-rich and G-rich strand of telomeric DNA (Rossiello et al. 2017). They confirmed knockdown using a modified qPCR reaction. They next applied this principle to a mouse model, in which a an inducible TRF2 knockout is stimulated by Tamoxifen treatment (Rossiello et al. 2017). The loss of TRF2 preludes unprotected telomeres, which in turn stimulates a DNA damage response reaction (Rossiello et al. 2017). They noted that after treatment with antisense oligonucleotides designed to both the C-rich and G-rich DDRNAs, the number of 53BP1 (a DNA damage marker) foci decreased compared to untreated mouse populations (Rossiello et al. 2017). This study confirms that antisense oligonucleotides like LNA GapmeRs could be used *in vivo*. However, the concentrations of LNA GapmeR used to cause a marginal knockdown of the TERRA transcript *in vitro* would be hard to replicate *in vivo.* This further highlights the need for improved knockdown efficiency of LNA GapmeRs for longer periods of time, at reduced concentrations. It would be conceivably impractical for patients to require multiple injections of LNA GapmeRs as effects only last 12 hours or so. It also furthers a need for a different technique, which would provide sustained knockdown of the TERRA transcript.

4.10 Future directions

This project highlights the need for further exploration into a role for telomeric transcription in cancers employing the Alternative Lengthening of Telomeres pathway.

4.10.1 Is TERRA distribution as a result of DNA Damage?

Extensive telomeres ranging up to 20 kb in length, found in ALT-positive cell lines are susceptible to increased instability and therefore DNA damage. A recent paper

described the presence of DNA-Damage Response RNAs at telomeres (DDRNAs) (Rossiello et al. 2017). These are short RNAs produced transcriptionally at the sites of DNA damage, in which RNA polymerase II is able to directly transcribe across the double stranded break in a unidirectional manner producing damaged induced long non-coding RNAs (dilncRNAs). dilncRNAs are consequently processed by Dicer and Drosha complexes to produce the resulting DDRNAs (Rossiello et al. 2017). For DDRNA production, the group also showed that resection at the site of DNA-damage needed to occur (D'Alessandro et al. 2018). This resection was dependent on exonuclease one (EXO1) and/or C-terminal interacting protein (CtIP) (D'Alessandro et al. 2018). As ALT-telomeres are prone to damage due to their extensive length, the distribution of TERRA foci in ALT-positive cancers could be as a result of the presence of DDRNAs. To test this theory, abrogation of EXO1 and/or CtIP using siRNAs could be enforced. siRNA mediated knockdown should limit resection at sites of damage and therefore limit the production of DDRNAs, thus causing decrease of TERRA foci seen in ALT cell lines.

4.10.2 Investigating other hallmarks of ALT cancers

TERRA knockdown and exploration into other hallmarks of ALT-positive cancers such as heterogeneous telomeric lengths and high telomeric sister chromatid exchange need to be investigated. The findings showed here in which C-circle levels decrease a as result of TERRA knockdown will be further solidified, should other hallmarks of ALT cancers be abrogated. Therefore, future work should delve further into the ALT phenotype, in order to gain a more comprehensive insight into how TERRA affects ALT.

4.10.3 Assessing the kinetics of TERRA knockdown on the ALT phenotype

This work has not fully investigated the kinetics and resulting cell viability of TERRA knockdown in ALT cancers. Further work should focus on understanding how the ALT phenotype changes overtime as a result of transcript depletion as wells as assessing whether this change in TERRA level affects the viability of ALT cells and therefore ALT cancers.

Assessing the kinetics of knockdown could be investigated further by an inducible Cas13a construct; Philip Hublitz at the end of this project developed an inducible system, but stable clones were not made in time for investigation. The plasmid maps for both the canonical and variant targeting inducible system can be seen in appendix B. This is a one-plasmid system containing both the guide RNA and the Cas13a body. The induction of this construct is under the control of a Tet-on promoter. Upon treatment of stable lines with doxycycline the promoter is switched on leading to production of the guide and the Cas13a domain. This system would allow for selective targeting of TERRA for longer periods of time than the LNA GapmeRs and thus grant a method for assessing kinetics. Further to this, switching on TERRA degradation for longer periods would make it possible to elucidate changes in cell viability as a result of transcript degradation. This is a powerful tool and can be stably introduced into a wider range of ALT cell lines than is available for Nucleofection of LNA GapmeRs. Additionally, both the variant and canonical repeat could be targeted through this method, allowing TERRA knockdown specificity in ALT cancers to be introduced.

4.10.4 Inhibiting telomeric transcription

One other option to reduce TERRA levels in ALT cancers would be to selectively inhibit telomeric transcription. This could be achieved through a CRISPR inhibition system (CRISPRi), a system in which an enzymatically dead Cas9 (dcas9) is fused to a repressor protein. This protein leads to transcriptional down-regulation of the target gene through recruitment of additional factors or changes in chromatin compaction at the target locus. We have a CRISPRi system, in which the dCas9 is fused to a KRAB domain (Appendix C - plasmid maps). Krüppel-associated box (KRAB) domains acts as a scaffold for the recruitment of co-repressor proteins such as histone deacetylases (HDACs) and heterochromatin protein 1 (HP1) (Urrutia 2003). KRAB domain proteins have known roles in in transcriptional repression, such as Kid1, which reduces synthesis of RNA by RNA polymerase I (Urrutia 2003). This principle when applied to telomeric transcription could lead to down-regulation of the TERRA across the telomeres if the dcas9-KRAB complex were to bind to regions of the sub-telomeric and telomeric body through guide RNAs. In a study by Gilbert et al. using emission from GFP as a readout of inhibition, they showed that CRISPRi targets within the gene body of GFP (not only at the promoter region) allowed for reduction of transcription of GFP (Gilbert et al. 2012). Therefore, constructs binding to the main telomere body and not just at the subtelomeric locus, should still in theory lead to impaired TERRA transcription lending to a potential use for CRISPRi in reducing telomeric transcription.

4.11 Concluding Remarks

This study has offered an insight into a role for telomeric transcription in cancers employing the Alternative lengthening of Telomeres. Further work should focus on answering how transcript knockdown for sustained periods affects long term viability of cancer cell lines and whether this can be used for therapeutic gain. A proposed model is described below (figure 4.1).

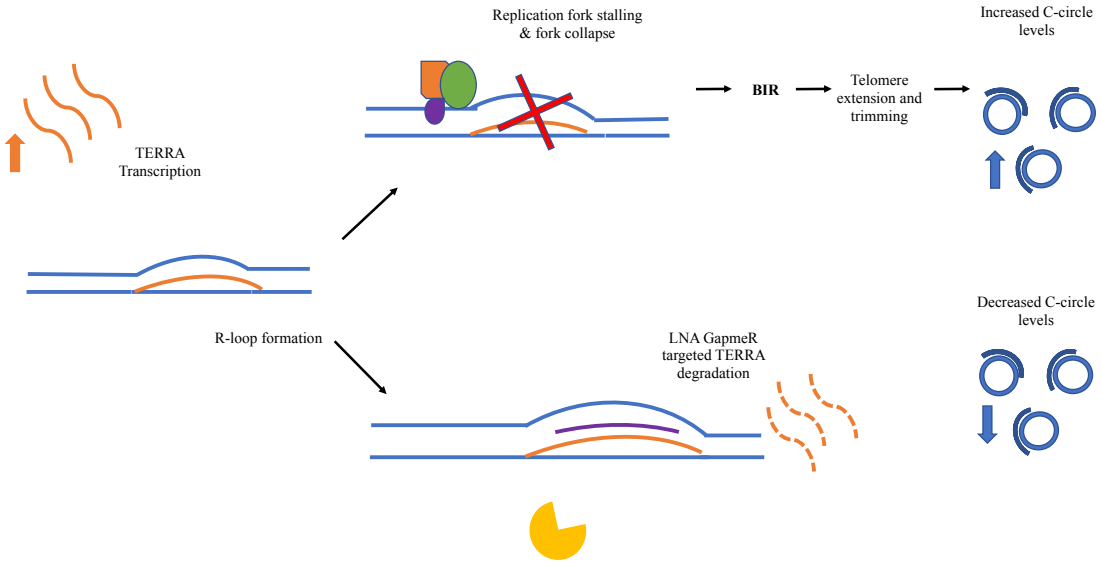


Figure 4.1 Proposed model of TERRA mediated telomere extension in ALT-positive cancers, where LNA GapmeR treatment perturbs the ALT phenotype.

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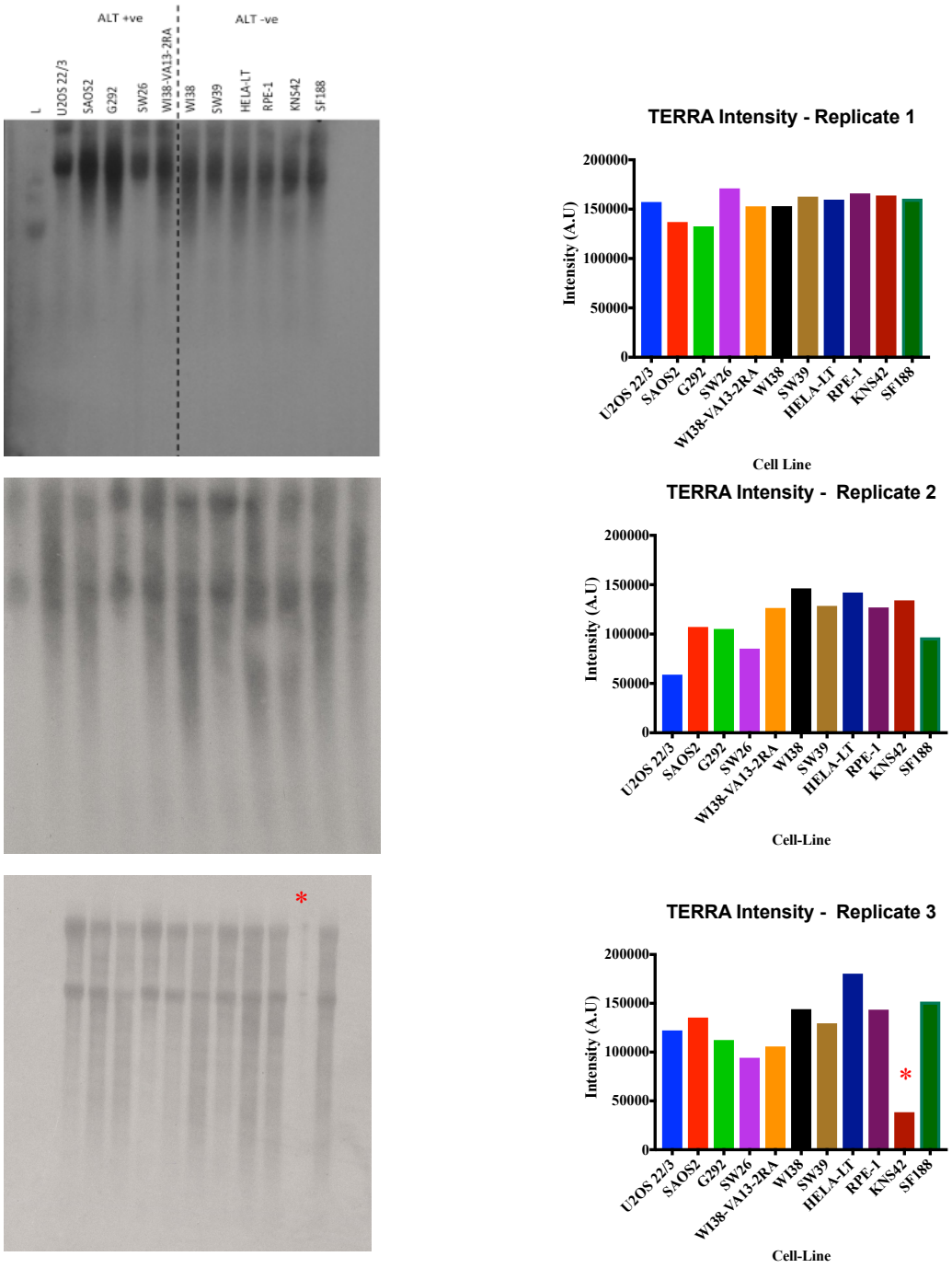
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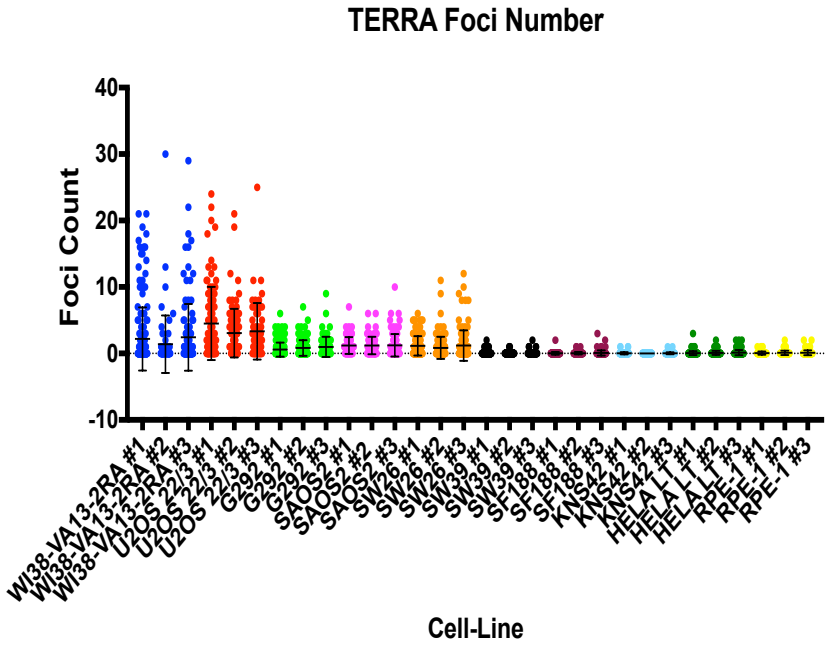
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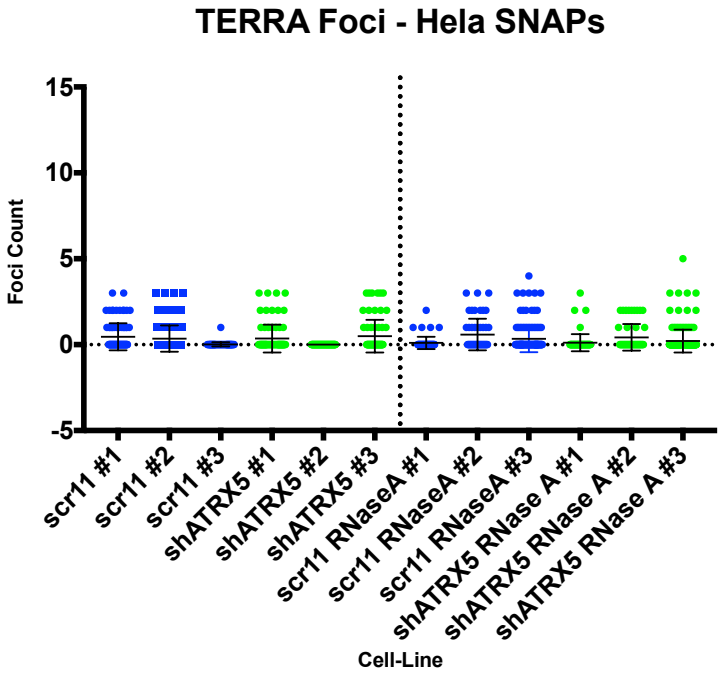
Appendix A - Supplementary Data



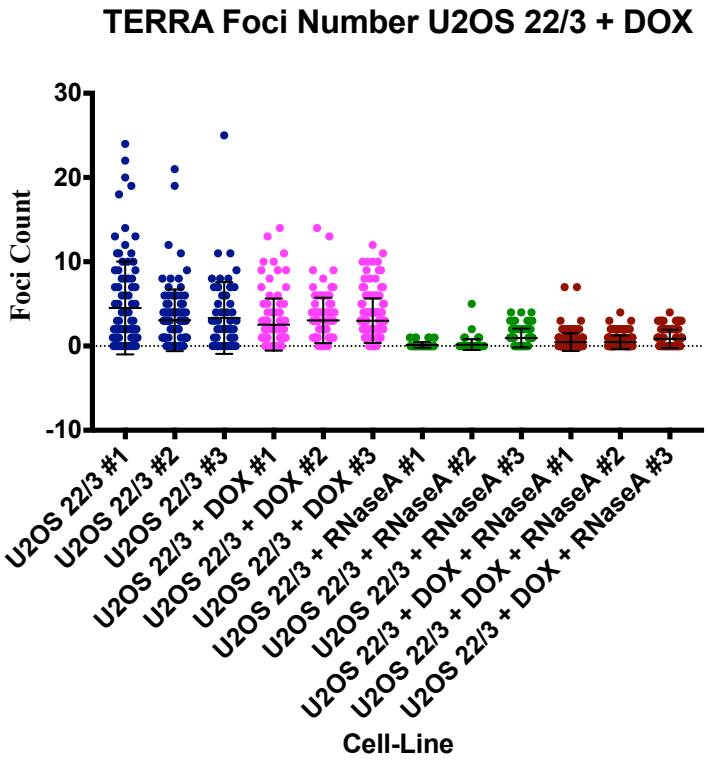
*Supplementary figure 1. TERRA expression northern blot replicates. Loading order is the same throughout the blots, differing exposure time. * indicates degraded sample, which was excluded from grouped analysis.*



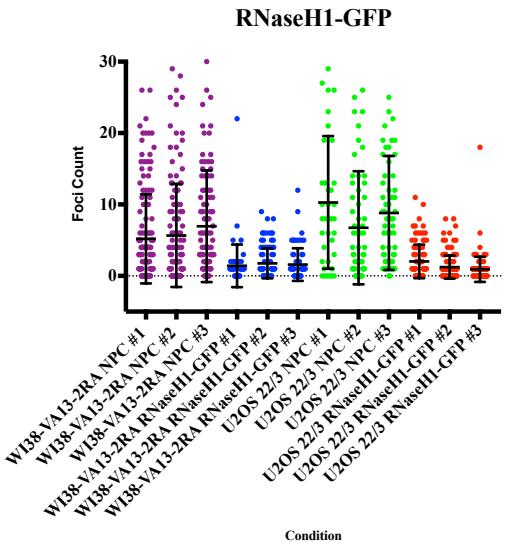
Supplementary figure 2. Canonical TERRA foci count from individual replicates from the panel of ALT-positive and ALT-negative cell lines



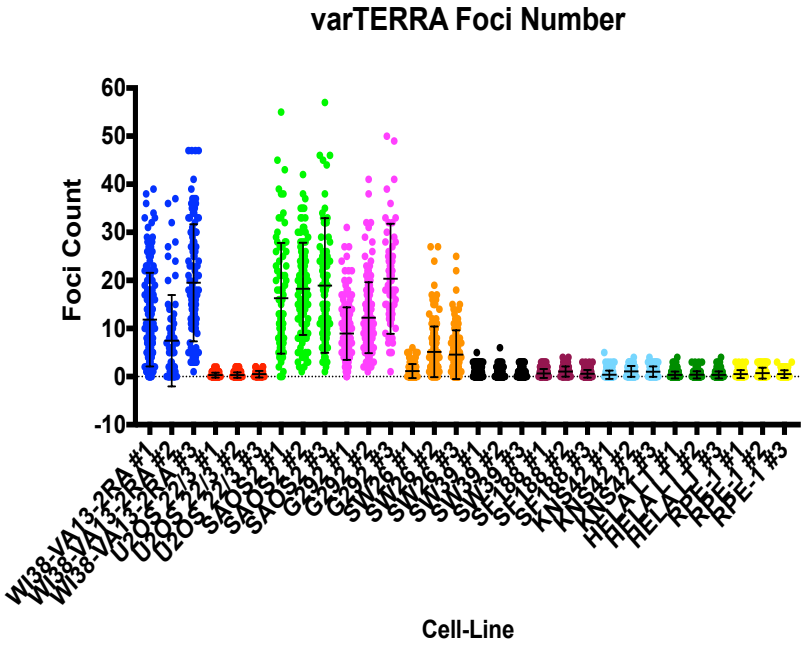
Supplementary figure 3. Canonical TERRA foci count from individual replicates.



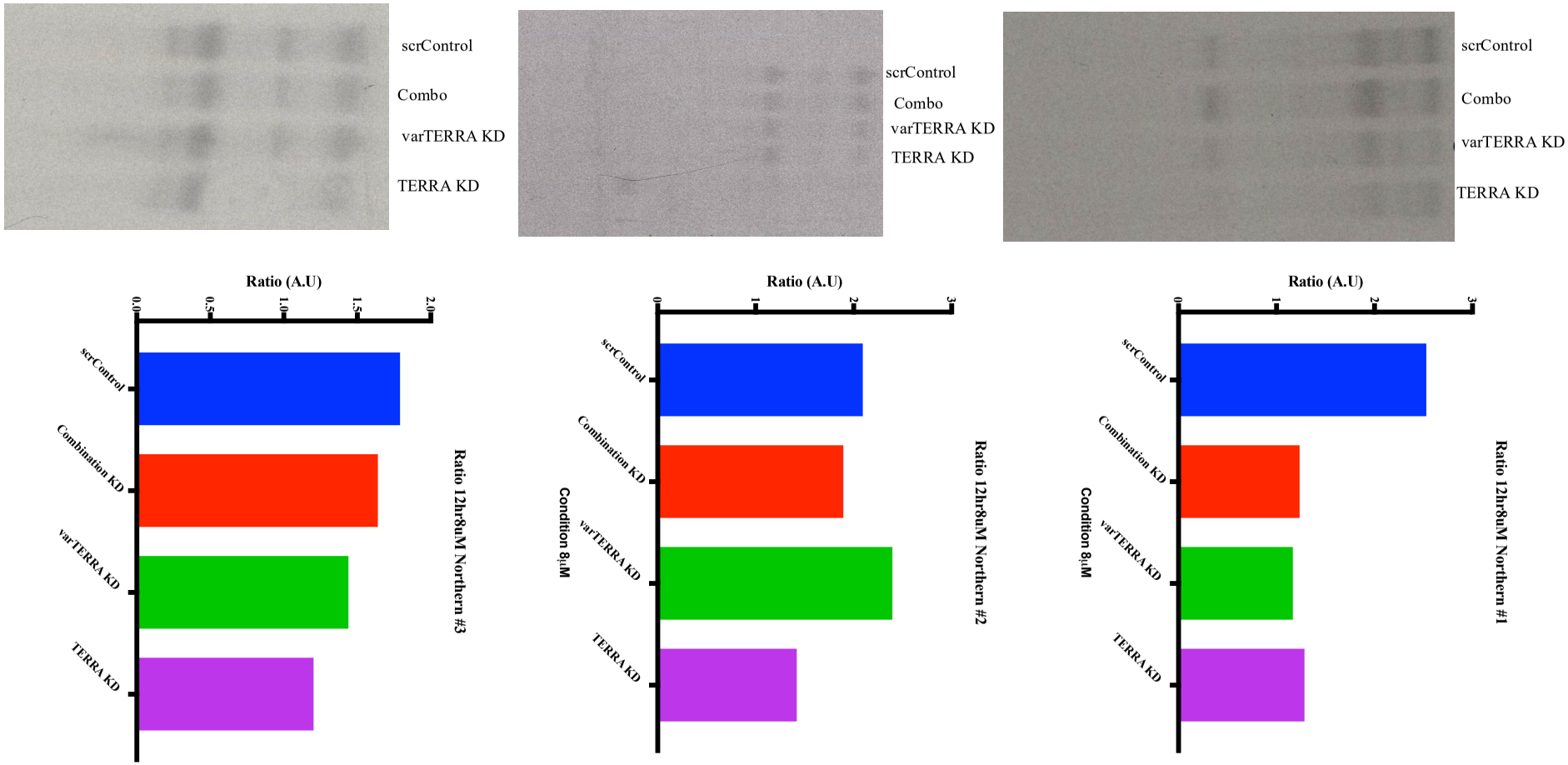
Supplementary figure 4. Canonical TERRA foci count from individual replicates.



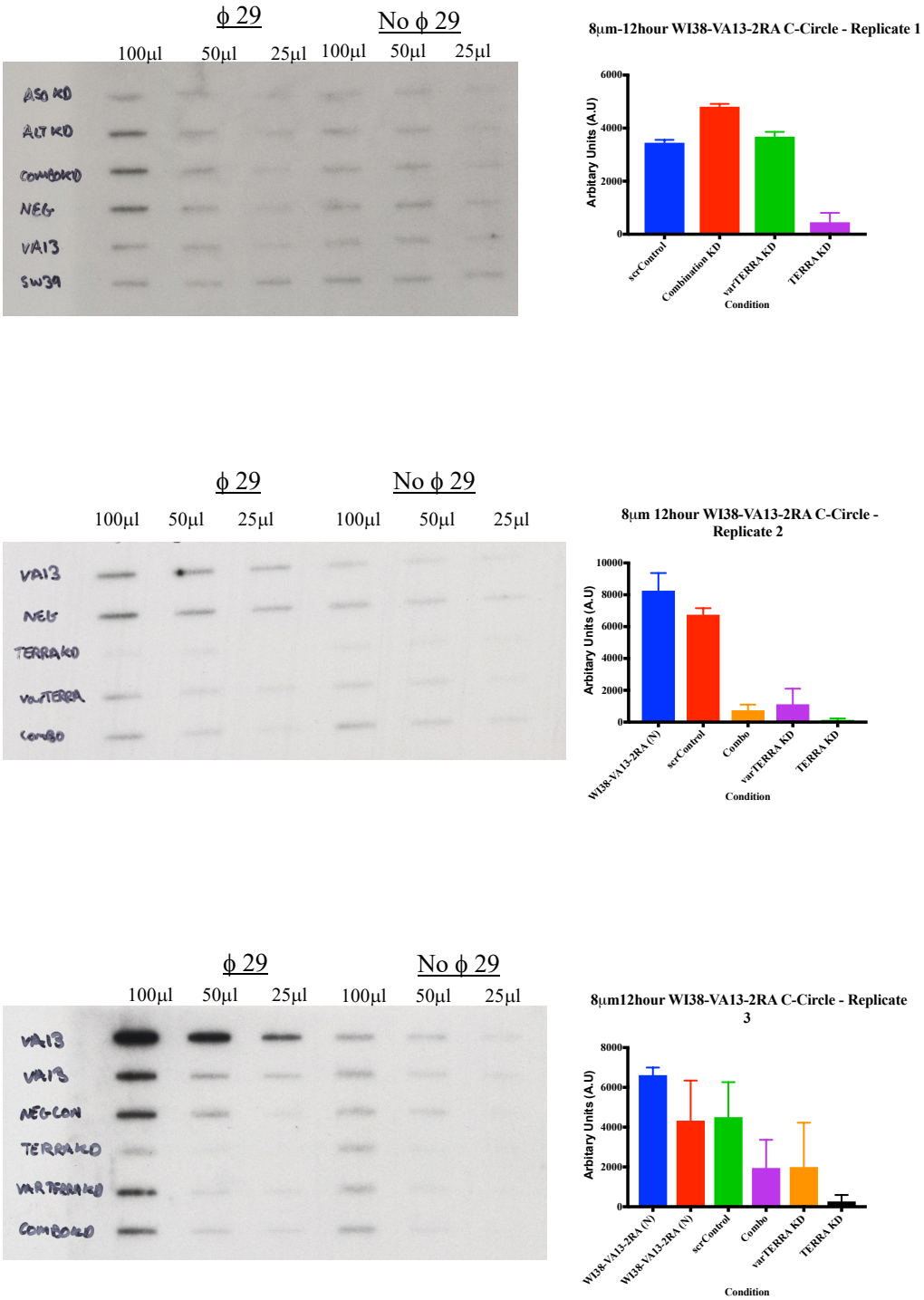
Supplementary figure 5. Canonical TERRA foci count from individual replicates.



Supplementary figure 6. Variant TERRA foci count from individual replicates from the panel of ALT-positive and ALT-negative cell lines



Supplementary figure 7. LNA GapmeR 8μM for 12 hours treatment replicates. All treatments were carried out in WI38-VA13-2RA cells.



Supplementary figure 8. C-circle blot replicates after treatment with LNA GapmeRs 8µM for 12 hours. Where 'ASO' is the canonical targeting LNA GapmeR and 'ALT' is the variant targeting LNA GapmeR. All treatments were carried out in WI38-VA13-2RA cells.

Appendix B - Antibodies, Probes and Oligonucleotides

Antibodies:

- **ATRX - 39F:** mouse, monoclonal, 1 in 500, REF: 151888 **WB**
- **ATRX**: rabbit, polyclonal, 1 in 500, Abcam ab97508 **WB**
- **Alpha-Tubulin:** mouse, monoclonal, 1 in 10,000, Abcam ab7921 **WB**
- **EXO1:** rabbit, monoclonal, 1 in 500, Abcam ab82533**WB**
- **TRF2:** mouse, monoclonal, 1in 200, Novus Biologicals 4A794.15 **IF**

Probes:

- **TERRA probe 1:** green, Integrated DNA Technologies, TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC/3AlexF488N/ **RNA-FISH**
- **TERRA RNA-FISH probe 2:** red, Integrated DNA Technologies, TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC/3Cy3Sp/ **RNA-FISH**
- **Variant TERRA:** red, Integrated DNA Technologies, TGA CCC TGA CCC TGA CCC TGA CCC TGA CCC TGA CCC TGA CCC /3Cy3Sp/ **RNA-FISH**

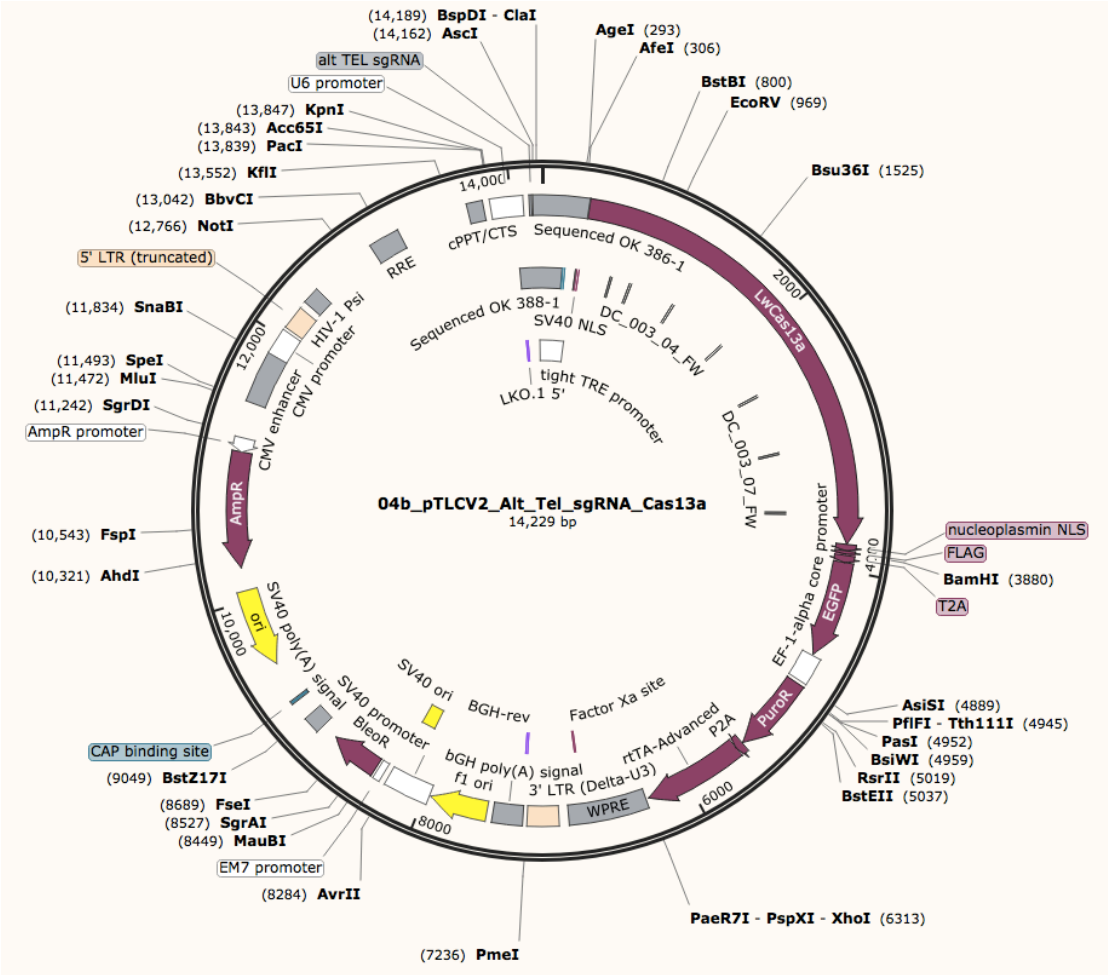
Primers:

- Oligo$(dT)_{12\text{-}18}$ Primer: REF 18418012, Thermofisher, cDNA
- **GAPDH Primer:** F-AACAGCGACACCCATCCTC R-CATACCAGGAAATGAGCTTGACAA, Housekeeping gene, SIGMA, **qPCR**
- **RNASEH1:** Hs00268000_m1, TaqMan Gene Expression Assays, Thermofisher **qPCR**

LNA GapmeRs:

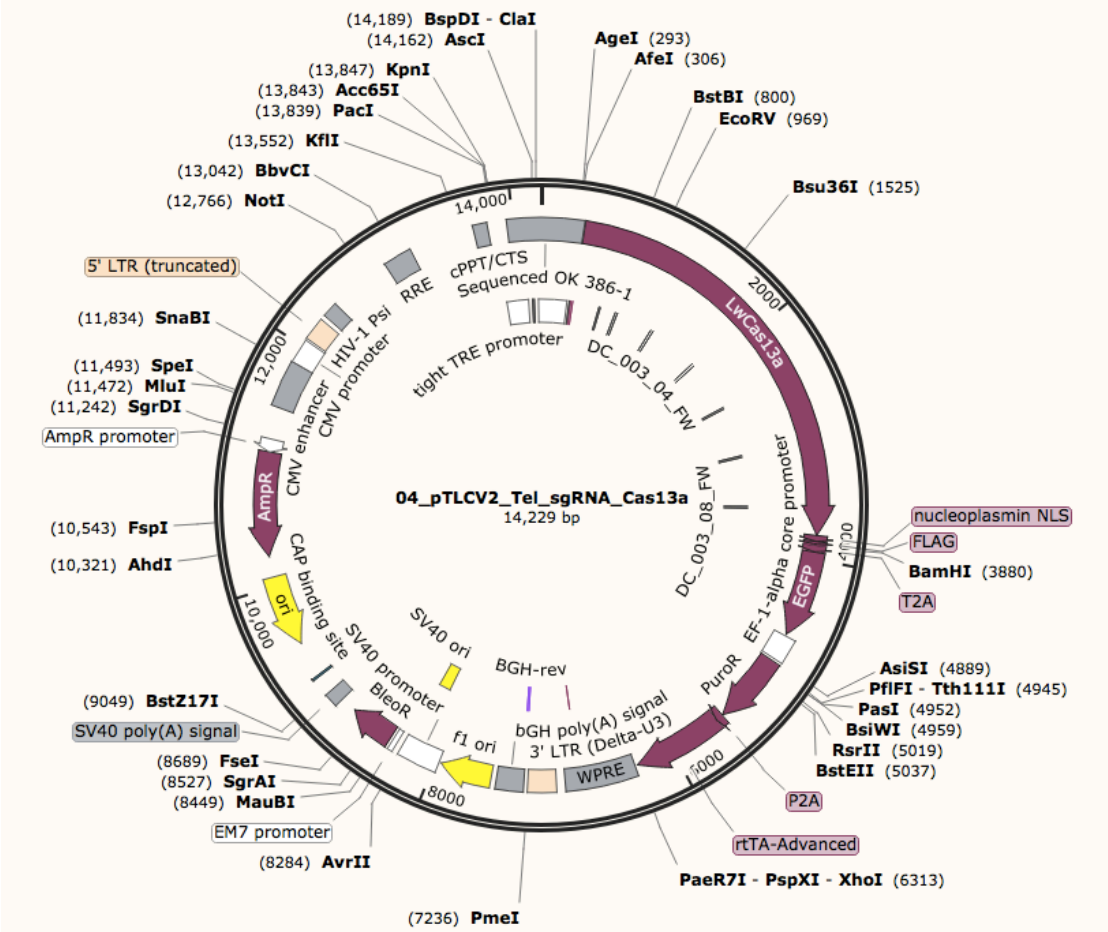
- **NEGATIVE CONTROL A:** REF LG00000002-DDA, Qiagen, **Nucleofection**
- **ASO-TERRA_1:** REF LG00196402-DDA, Qiagen, **Nucleofection**
- **ALT_TERRA_1 GAPMER:** REF LG00193312-DDA, Qiagen, **Nucleofection**

Appendix C - Plasmid Maps



Inducible Cas13a construct – Variant

- Guide to variant telomeric repeat sequence (Alt_Tel)
- Doxycycline promoter allows inducible expression
- GFP tagged
- Zeomycin resistance
- Created by Philip Hublitz



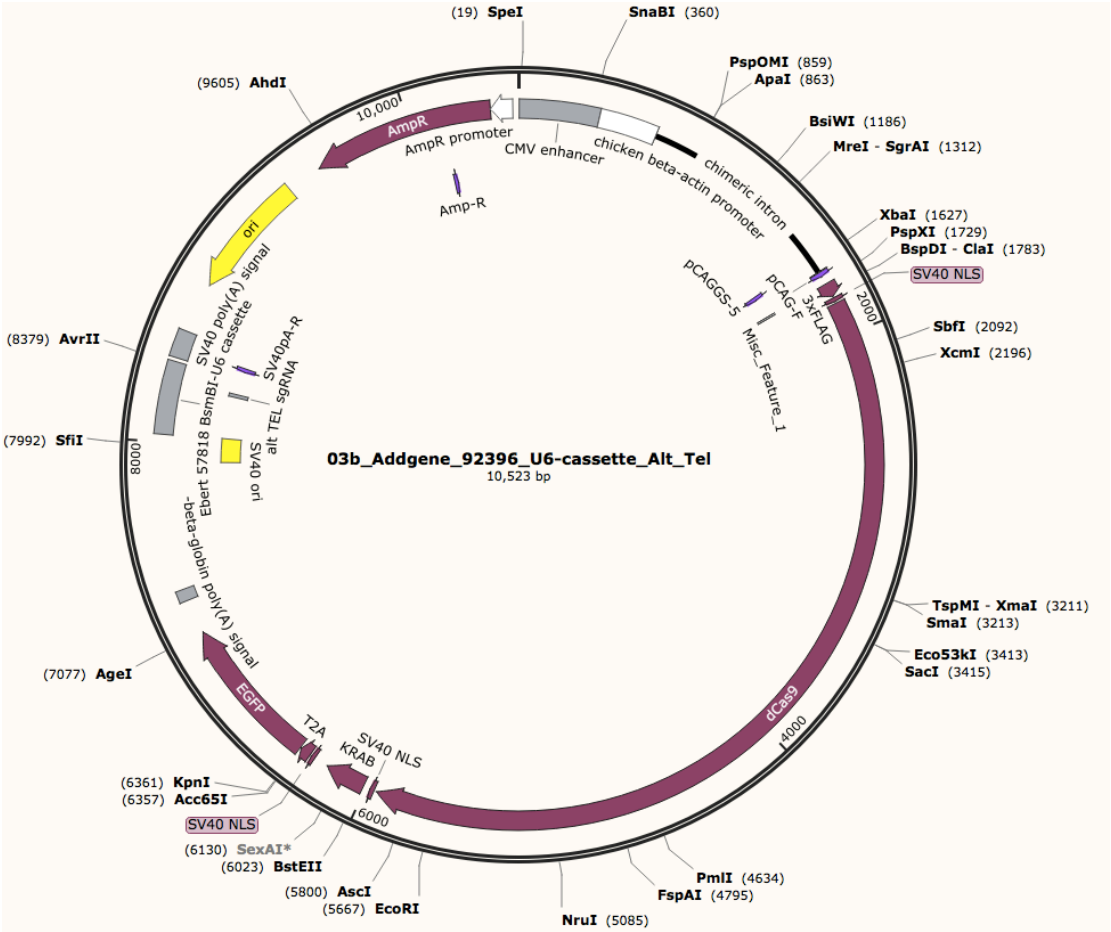
Inducible Cas13a construct - Canonical

- Guide to canonical telomeric repeat sequence (Tel)
- Doxycycline promoter allows inducible expression
- GFP tagged
- Zeomycin resistance
- Created by Philip Hublitz



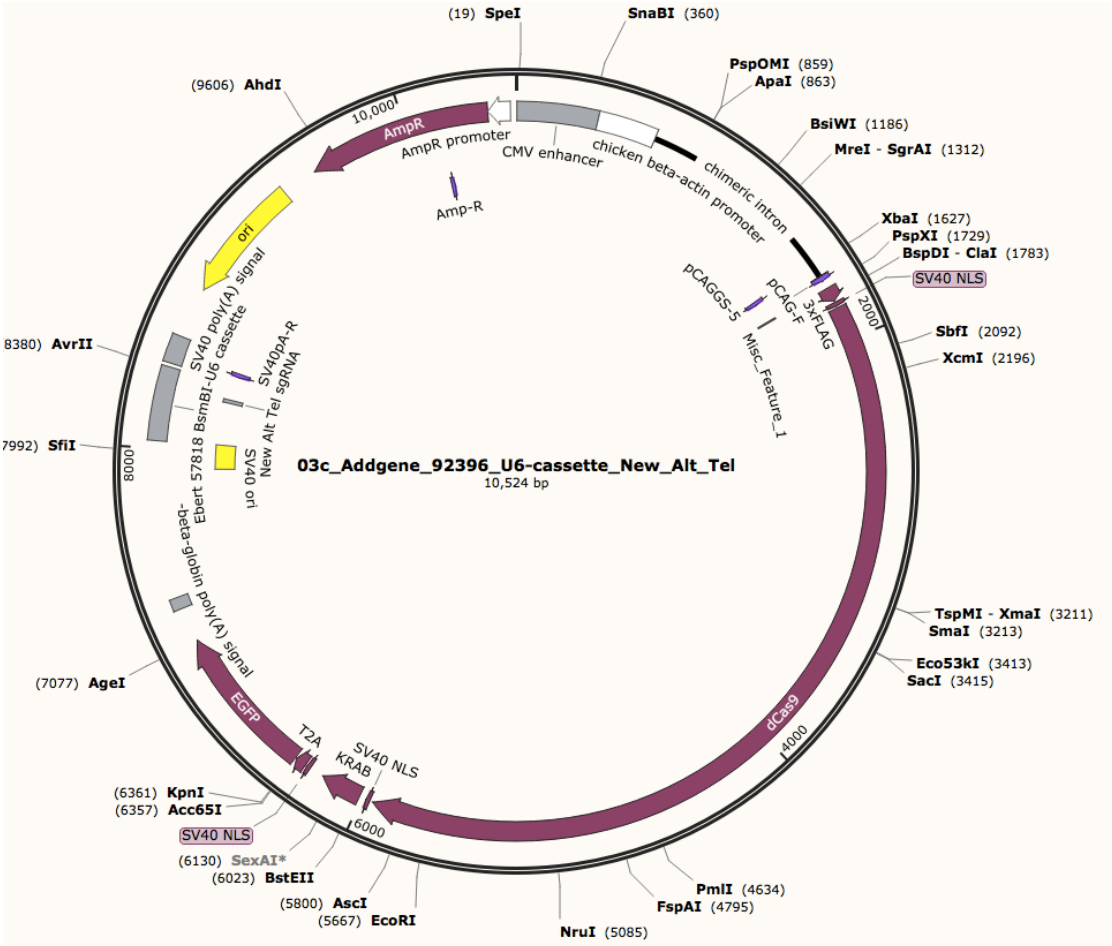
CRISPRi Construct 1 - Canonical

- Guide to canonical telomeric repeat sequence (Tel)
- GFP tagged
- KRAB repressor domain
- Puromycin resistant
- Created by Philip Hublitz



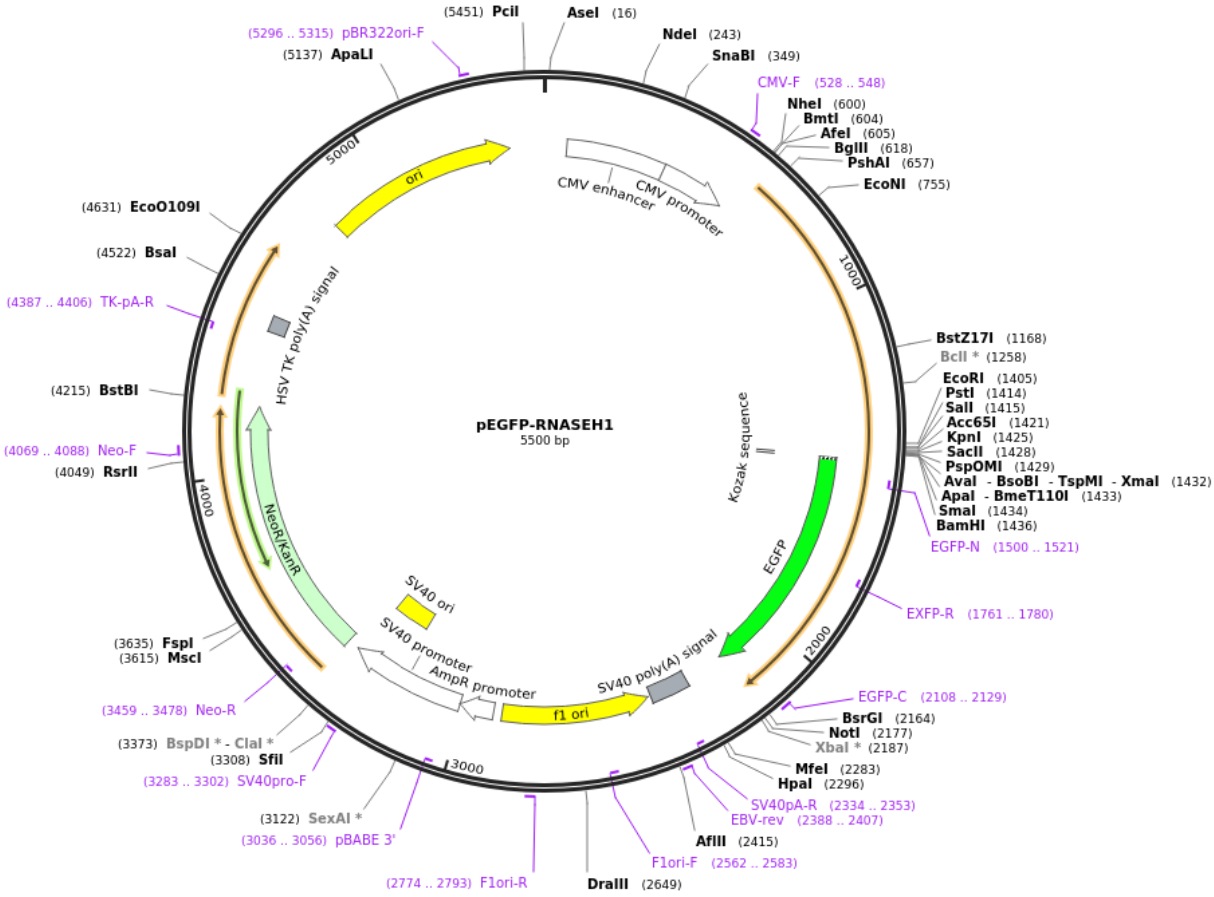
CRISPRi Construct 2 – Variant

- Guide to variant telomeric repeat sequence (Alt_Tel)
- GFP tagged
- KRAB repressor domain
- Puromycin resistant
- Created by Philip Hublitz



CRISPRi Construct 3 – New Variant

- New guide sequence to variant telomeric repeat sequence (New_Alt_Tel)
- GFP tagged
- KRAB repressor domain
- Puromycin resistant
- Created by Philip Hublitz



pEGFP-RNASEH1

- pEGFP-RNASEH1 a gift from Andrew Jackson (Addgene plasmid # 108699) (Bubeck et al. 2011)
- Neomycin resistant

Appendix D - Equipment and Software

Equipment

- Corning® CellBIND® 25cm^2 Flask: Canted neck. REF CLS3289-200EA
- Corning® CellBIND® 75cm^2 Flask: Canted neck. REF CLS3290-100EA
- Corning® CellBIND® 6-well Clear Multiple Well Plates, Flat Bottom, with Lid, Sterile REF 3335
- Corning® CellBIND® 24-well Clear Multiple Well Plates, Flat Bottom, with Lid, Sterile REF 3337
- Corning® Gosselin™ Petri Dish 100 x 15 mm, No Vent, Sterile REF: BH90B-102
- DeltaVision Elite imaging system by GE Healthcare Life Sciences
- Invitrogen by Thermofisher scientific: Mini gel tank
- Labnet International Inc.: Accublock ™ digital dry bath. REF D1100
- Labnet International Inc.: Prism™ Microcentrifuge. REF C2500
- ThermoFisher-Scientific: NanoDrop-Lite Spectrophotometer. REF ND-LITE
- Bio-Dot® SF Apparatus REF 1706542
- ThermoFisher-Scientific: Bolt™ 4-12% Bis-Tris Plus Gels, 10-well. REF NW04120BOX
- ThermoFisher-Scientific: Western blotting filter paper, 7cmx8.4cm, thickness 0.83mm REF 84783
- ThermoFisher-Scientific: Nitrocellulose Membrane, 0.45 µm, 8 cm x 8 cm. REF 88025

Software

- CellProfiler 2.2.0 by BroadInstitute
- SoftWoRx by GE Healthcare Life Sciences version 6.5.2
- Fiji by ImageJ
- SnapGene Viewer