

Exploring Tumour Suppressor ATRX and Telomere Maintenance in MLLr Leukaemia



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Abstract

The acquisition of limitless replicative potential, is crucial for cancer development. Normally, cells undergo senescence once the telomeres (sequences that protect genes from being lost after replication due to the end replication problem), reach a critically short length. In cancer, two mechanisms are known to maintain telomere length: the upregulation of the enzyme Telomerase and the Alternative Lengthening of Telomeres (ALT) pathway. The ALT pathway has defining features of increased long non-coding telomeric repeat containing RNA (TERRA), extrachromosomal DNA (C-circles), ALT associated PML bodies (APBs), heterogeneous telomere lengths and the loss of the tumour suppressor protein ATRX.

Mixed Lineage Leukaemia-1 (MLL-1) is a histone methyltransferase required for histone 3 lysine 4 trimethylation (H3K4me3) during normal development. The MLL gene is mutated in 70-90% of infant leukaemia and is associated with a poor clinical outcome. The most common MLL gene mutations in leukaemia are gene rearrangements (MLLr). Especially translocations that fuse MLL in-frame with common partners such as AF4, AF9 or ENL, creating novel MLL fusion proteins (MLL-FP). Although ALT is relatively rare in leukaemias, recent evidence of MLL-1 binding to telomeric repeats, coupled with it driving TERRA transcription raises interesting questions as to how telomeres are regulated in MLL fusion driven leukaemias.

Here, we show that telomeric sequences are bound by MLL-1 in MLL-AF4 cell lines. We also show an increase of the telomere transcript, TERRA and other hallmarks of ALT, such as reduction of ATRX and presence of C-circles in cell lines that have the MLL translocation.

Understanding the role of ATRX and telomere maintenance may provide new insight into how these therapy resistant MLLr leukaemias are initiated or maintained.

ACKNOWLEDGMENTS	2
ABSTRACT	3
INTRODUCTION	6
DNA REPLICATION	6
IMPORTANCE OF TELOMERES.....	7
TELOMERASE	8
THERAPIES FOR TELOMERASE.....	8
ALTERNATIVE LENGTHENING OF TELOMERES PATHWAY (ALT)	9
HALLMARKS OF ALT CANCERS	9
ALT CANCERS	13
LEUKAEMIA	13
MIXED LINEAGE LEUKAEMIA.....	15
MLL REARRANGEMENTS	16
PROGNOSIS OF MLLR LEUKAEMIA	16
THERAPIES FOR MLLR LEUKAEMIA	17
MLL IN TELOMERE BIOLOGY	17
PROJECT GOALS	18
METHODS.....	19
CELL LINES AND CULTURE.....	19
CHROMATIN IMMUNOPRECIPITATION (CHIP)	19
ANTIBODIES.....	20
CHIP-QPCR	20
<i>Table 1: SYBR Green Primers</i>	<i>21</i>
<i>Table 2: Sub-Telomere Primers and Telomere Primers.....</i>	<i>22</i>
<i>Table 3: The cycle conditions for ChIP-qPCR</i>	<i>23</i>
RNA EXTRACTION	23
REVERSE TRANSCRIPTION-PCR	23
<i>Table 4: The cycle conditions for cDNA.....</i>	<i>23</i>
<i>Table 5A: RT-PCR SYBR Green Primers (human)</i>	<i>24</i>
<i>Table 5 B: The cycle conditions for RT-PCR SYBR green</i>	<i>24</i>
<i>Table 5C: RT-PCR TaqMan Probes (human).....</i>	<i>24</i>
<i>Table 5D: The cycle conditions for RT-PCR Taqman.....</i>	<i>24</i>
C-CIRCLE ASSAY	25
C-CIRCLE-QPCR	25
<i>Table 6A: Telomere q-PCR Reaction Mixture</i>	<i>26</i>
<i>Table 6B: The cycle conditions for SYBR green QuantiTECT.....</i>	<i>26</i>
RNA FISH	26
NORTHERN BLOT.....	27
WESTERN BLOT.....	27
INTRACELLULAR FLOW	28
<i>Table 7: siRNA.....</i>	<i>29</i>
<i>Cell Lines Information</i>	<i>30</i>
RESULTS	31
MLL ^{WT} AND/OR MLLR WEAKLY BINDS TO TELOMERES.....	31
<i>Figure 1. MLL-N Weakly Binds at Telomeres in SEM cells.....</i>	<i>33</i>
<i>Figure 2. MLL-N Weakly Binds at Telomeres in RS4:11 cells.....</i>	<i>34</i>
<i>Figure 3. MLL-N Weakly Binds at Telomeres in SEM cells.....</i>	<i>35</i>
<i>Figure 4. MLL-N Binding at Sub-telomeres in SEM Cells (Optimization).</i>	<i>36</i>
<i>Figure 5. MLL-AF4 Knockdown in SEM Show No Change at the Telomere and Sub-telomeres. ...</i>	<i>37</i>
<i>Figure 6. MLL^{WT} Knockdown in SEM Show No Change at the Telomere and Sub-telomeres.</i>	<i>38</i>

<i>Figure 7. MLL-AF6 Weakly Binds at the Sub-telomeres in ML-2 Cells.</i>	39
INCREASE IN TELOMERE TRANSCRIPT (TERRA) IN MLLR CELL LINES	40
<i>Figure 8. Increased TERRA in MLLr Leukaemia Cell line RS4;11.</i>	41
<i>Figure 9. Increased TERRA in MLLr Leukaemia Cell line RS4;11.</i>	42
<i>Figure 10. Increased TERRA in MLLr Leukaemia Cell lines.</i>	43
<i>Figure 11. Increased TERRA in MLLr Leukaemia Cell lines.</i>	44
<i>Figure 12. TERRA Specific Signal in Northern Blots.</i>	45
REDUCTION OF TUMOUR SUPPRESSOR PROTEIN ATRX IN MLLR CELL LINES	46
<i>Figure 13. Loss of Tumour Suppressor ATRX in MLLr Cell lines.</i>	47
<i>Figure 14. Loss of Tumour Suppressor ATRX in MLLr Cell lines.</i>	48
<i>Figure 15. Low Intracellular ATRX in MLLr and ATRX Negative Cell lines (Optimization).</i>	49
<i>Figure 16. Low Intracellular Levels of ATRX in MLLr Cell lines.</i>	50
<i>Figure 17. Low Intracellular Levels of ATRX in MLLr Cell lines.</i>	51
ATRAX RNA STATUS IN MLLR CELL LINES	52
<i>Figure 18. No Significant Change in ATRX RNA Expression from Non-Fusion vs Fusion Cell lines.</i>	
<i>Figure 19. No Change in ATRX RNA Expression from MLL-AF4 Patient RNA-Sequence Data.</i>	54
PRESENCE OF ALT HALLMARKS IN MLLR CELL LINES	55
<i>Figure 20. Presence of C-circles in SEM.</i>	56
<i>Figure 21. Presence of C-circles in Non-Fusion and Fusion Cell lines.</i>	57
DISCUSSION	58
MLL ^{WT} AND/OR MLLR WEAKLY BINDS TO TELOMERES	58
INCREASE IN TELOMERE TRANSCRIPT (TERRA) IN MLLR CELL LINES	59
REDUCTION OF TUMOUR SUPPRESSOR PROTEIN ATRX IN MLLR CELL LINES	60
PRESENCE OF ALT HALLMARKS IN MLLR CELL LINES	62
CONCLUSION/FUTURE DIRECTIONS	63
BIBLIOGRAPHY	65

Introduction

In this thesis, we will be exploring how telomeres are maintained in MLLr leukaemia. Maintaining telomeres is well known to be essential for cancerous cells achieving immortality. Telomere regulation in this leukaemia has not been extensively studied. Patients with a translocation in the MLL gene have a poor prognosis, therefore we believe that exploring this less popular area could generate informative data on the nature of the disease, potentially improving current therapies or perhaps better understanding how these leukaemias are initiated.

DNA Replication

For DNA replication to occur, the double strand helix is unwound and separated into single strands by an enzyme called Helicase. During this phase topoisomerases relieve torsional stress caused from the supercoiling of downstream DNA and single strand DNA binding proteins are added to the DNA to prevent them from reannealing. A replication fork is generated (structure of proteins and separated DNA) at sites of active replication. Oversimplifying the proteins involved, there are specific proteins required for replication, such as helicase, DNA polymerase δ , DNA Primase, DNA polymerase α , Ligase, and Topoisomerase. For a single strand of DNA to be replicated DNA polymerase δ (pol δ) needs an existing 3'OH, therefore DNA Primase generates short RNA "primers" at the start of the replication site for which pol δ can use. Pol δ can only synthesize DNA in the 5'→3' direction, therefore one strand of DNA can be continuously synthesized (leading strand) whereas the other strand needs primers added where the DNA was recently opened (lagging strand). Once Pol δ synthesizes the DNA, the primers are removed and DNA polymerase α fills in the remaining DNA. However, there are nicks in the area that was recently filled in, so ligase joins the DNA together (Thömmes and Hübscher, 1990).

A problem that arises with this method of replication, is that once the primers are removed, DNA polymerase cannot synthesize DNA to fill in the 3' end of the leading strand, where replication first began. As a result, at the chromosome ends, DNA is lost after every round of replication, which is critical to the cell. To protect the DNA from being lost, cells developed the ability to add repeats (TTAGGG)_n to the ends of DNA. Consequently, instead of important DNA being lost, the repeats are instead lost. However, this puts a replicative time limit on the cell as once all the repeats are lost the cell senesces or goes into crisis and dies (Olovnikov, 1996). Considering that these repeats are important to a cell's lifespan, it can be understood why there are mechanisms involved to maintain them, and why they can be aberrantly regulated in atypical cells.

Importance of Telomeres

Once replication is completed, the 3' repetitive portion of the DNA is left as an overhang. To avoid being considered damaged DNA, the end is looped (t-loop) and protected by the proteins of the shelterin complex (TRF1/2, POT1, Rap1, TIN2 and RPP1) (Gilson and Géli, 2007). This complex of repetitive DNA and shelterin proteins is referred to as a telomere (Blackburn et al., 2015).

Due to this “end replication problem” in human somatic cells, there is a limit on how many cell divisions can occur before the cell senesces. However, in the germline and stem cells, telomeres are maintained by an enzyme called Telomerase (Xu and Goldkorn, 2016).

In the case that senescence checkpoints are bypassed; the cell continues to divide and the damaged DNA fuses with other DNA in attempts to repair the breaks. This results in crisis, which is defined by several undesirable chromosome fusions and cell death (Capper et al., 2007). Cells that are on the way to becoming malignant achieve immortality by maintaining their telomeres. The most common method of doing this is by upregulating Telomerase (80-

90% of cancers) (Cong et al., 2002). A less common mechanism observed is the Alternative Lengthening of Telomeres (ALT) pathway (10-15% of cancers) (Bryan et al., 1997a).

Telomerase

Telomerase extends telomeric DNA using an internal RNA template. The protein is made up of two components, the RNA template (*TERC*) and the catalytic subunit (*TERT*) that has reverse transcriptase abilities (Greider and Blackburn, 1987). The RNA template is found to be expressed in normal somatic cells, whereas the catalytic subunit is tightly regulated, and an upregulation of it is seen in cancerous cells (Cong et al., 2002). Telomerase functions by extending the 3' overhang (Greider and Blackburn, 1987). Primase adds an RNA primer and DNA pol δ can then fill in the missing DNA. The removal of the RNA primer generates a 3' overhang which is then protected by shelterin.

Therapies for Telomerase

There are a few telomerase targeting therapies currently in phase 1-3 of clinical trials. These include telomerase inhibitors, TERC interference, oncolytic virus targeting the TERT promoter and suicide genes, TERT immunotherapy and telomerase vaccinations (Xu and Goldkorn, 2016). The worry of targeting telomerase is that it will disrupt normal cells that depend on it (germline etc.) and induce telomerase-independent mechanism of extending telomeres (Shay and Wright, 2002).

Alternative lengthening of Telomeres Pathway (ALT)

The ALT pathway is dependent on recombination and uses telomeric DNA as a template to extend telomeres (Nabetani and Ishikawa, 2011). The ALT pathway originates from double strand breaks in telomeric DNA thought to be caused from replication fork collapse. The DNA repair pathway choice in ALT is break induced replication (form of homologous recombination). In this mechanism, proteins, MRE11, Rad50, and NBS1 are important for DNA resection, BRCA1/2, Rad 51, and Rad54 are required for strand invasion and PCNA and POLD3 facilitate extension of the overhang (Dilley et al., 2016; Nabetani and Ishikawa, 2011). Based on where the strand invades, the telomeric DNA can be extended for various numbers of repeats.

Hallmarks of ALT Cancers

There are several characteristics of ALT that can be used to identify whether this pathway is active in cancers (Dilley and Greenberg, 2015). It has been shown that most ALT cancers have increases in the telomere transcript TERRA (Episkopou et al., 2014), ALT associated promyelocytic leukaemia bodies (PML) (Yeager et al., 1999), heterogeneous telomere lengths (Bryan et al., 1995), increased frequency of telomeric sister chromatid exchange (Londoño-Vallejo et al., 2004), extrachromosomal telomeric circular DNA (C-circles) (Henson et al., 2009) and the loss of tumour suppressor protein ATRX (Henson and Reddel, 2010; Lovejoy et al., 2012).

Telomeric Transcript

Although telomeres are known to have heterochromatic marks, they are still actively transcribed (Azzalin et al., 2007). These telomere transcripts also known as TERRA are non-coding RNA that are transcribed from the promoter in the sub-telomere (area upstream from telomere body, closer to the centromere) (Nergadze et al., 2009). TERRA ranges from 100bp

to about 9kb depending on the telomere length (Azzalin et al., 2007). It is not expressed at every chromosome; recent studies have suggested the major contributors of TERRA are from chromosome 20 and Xp (Montero et al., 2016). TERRA is thought to have roles in protecting the telomere (Chu et al., 2017; Silanes et al., 2014), establishing a heterochromatic environment at the telomere (Cusanelli and Chartrand, 2015), telomere replication and elongation (Cusanelli et al., 2013). In the ALT pathway, TERRA has been shown to be highly expressed. Excessive amounts of TERRA can lead to DNA: RNA hybrids (R-loops) (Arora et al., 2014) which ultimately cause replication fork stalling, collapse and generation of double strand breaks (Sollier and Cimprich, 2015). TERRA can be detected by RNA-FISH and Northern blot assays with either fluorescently labelled or ³²P end-labelled telomeric probe (Azzalin et al., 2007; Montero et al., 2016).

ALT Associated PML Bodies

PML bodies are sub-nuclear compartments originally implicated in acute promyelocytic leukaemia (APL). They are known to be important for cell growth, differentiation, protein modifications and degradation (Ching et al., 2005) and contain proteins involved in transcription, DNA repair, and apoptosis (Dellaire and Bazett-Jones; Takahashi et al., 2004; Zhong et al., 2000). When the ALT pathway is active, telomere proteins and DNA are found interacting with PML protein in these bodies (Yeager et al., 1999). Though the exact purpose for why telomere components interact with PML is unclear, it has been suggested that the recombination involved in ALT occur in these bodies (Grobelny et al., 2000). The presence of these ALT-associated PMLs (APBs) can be visualized through immunofluorescence by co-staining with antibodies directed at PML and the shelterin complex (Grobelny et al., 2000).

Heterogeneous Telomere Lengths

Telomere lengths vary from 2kb to over 20kb in ALT positive cancers (Bryan et al., 1997b). In contrast, cells that upregulate telomerase have their telomeres consistently ranging from

5kb-10kb. This phenotype of ALT is thought to occur because of recombination, however the exact mechanism is still unclear. Long telomeres are sometimes shortened through telomere trimming, which could potentially generate the extremely short telomeres seen in ALT. The length can be assessed by terminal restriction fragment analysis or DNA FISH on metaphase spreads.

Telomere Sister Chromatid Exchange

Sister chromatid exchange (SCE) takes place when replicated DNA exchanges DNA with its parental strand (Wilson and Thompson, 2007). This can occur naturally in the process of replication or when the replication fork collapses and generates breaks in the DNA (Wilson and Thompson, 2007). When the DNA exchange occurs at telomeres this is referred to as telomere sister chromatid exchange (T-SCE). There is a higher incidence of T-SCE in ALT cancers compared to normal cells, cells from Bloom Syndrome patients and cancer cells immortalized by telomerase (Rudd et al., 2007). This is suggested to be due to greater DNA damage occurring at telomeres as expected for the extension of telomeres by recombination. In ALT cases, telomeric sequence are exchanged amongst sister chromatids, but evidence suggest that when there is excessive damage at the telomeres, telomeres can also recombine with other chromosomes' telomeres (Cho et al., 2014). T-SCE can be detected through Chromosome Orientation Fluorescence In situ Hybridization (CO-FISH) (Blagoev et al., 2010; Rudd et al., 2007).

C-circles

Extrachromosomal telomeric circular DNA (C-circles) are strongly associated with the ALT pathway (Henson et al., 2009, 2017; Lau et al., 2013). The exact mechanism for how this occurs is unknown but it has been suggested to be a result of telomere trimming. C-circles are partially double stranded circular telomeric DNA that vary in size and number among ALT cancers (Lovejoy et al., 2012). The partial double strand acts as an internal primer for rolling circle

amplification which generates single stranded telomere sequence of greater than 70kb (Henson et al., 2009). This DNA can then be blotted for with ^{32}P labelled telomeric probes or quantified via real time PCR with primers designed at the telomere (Henson and Reddel, 2010; Henson et al., 2009, 2017).

ATRX

The ATRX gene/protein is found to be aberrant in patients with X-linked alpha thalassaemia mental retardation syndrome. This disease is characterized by alpha thalassemia, facial abnormalities, delayed development, genital abnormalities and typically affects males due to it being x-linked recessive (Gibbons, 2006). The exact function of ATRX is yet to be determined, but its proposed roles are in DNA repair, replication, transcription regulation, and chromosome segregation (Clynes et al., 2013; Gibbons, 2006; Koschmann et al., 2016a; Nandakumar et al., 2017). It is a chromatin remodeler that in cooperation with the histone chaperone, DAXX inserts histone variant H3.3 into chromatin to direct several of the functions above. It is known to bind to rDNA and telomeric repeats and have a role in establishing the heterochromatic environment of the genes/ DNA body it regulates (Clynes et al., 2013; Law et al., 2010; McDowell et al., 1999). ATRX exists in isoforms that differ in their 5' ends (around 265kDa & 280kDa) and one that retains intron 11 and truncates the protein, resulting in a 180kDa protein (Gibbons, 2006). ATRX has a DNA binding domain in its N-terminus, known as the ADD (ATRX–DNMT3–DNMT3L) domain and helicase/ATPase domain in its C-terminus (Diagram 1) (Clynes et al., 2013; Gibbons, 2006). Mutations found in the ATRX gene usually occur in the ADD and helicase domains (Gibbons, 2006). Interestingly ATRX is also found to be lost in most ALT positive cancers (Lovejoy et al., 2012; Nandakumar et al., 2017). Its role in ALT cancers is still being investigated however recent studies have indicated that re-introducing ATRX into these cancers reduces the phenotype of ALT (Clynes et al., 2015a; Napier et al., 2015) therefore suggesting a role in tumour suppression. Although this data

suggests ATRX plays a crucial role in preventing the ALT. Loss of ATRX alone is not sufficient to induce ALT (Clynes et al., 2014; Napier et al., 2015).

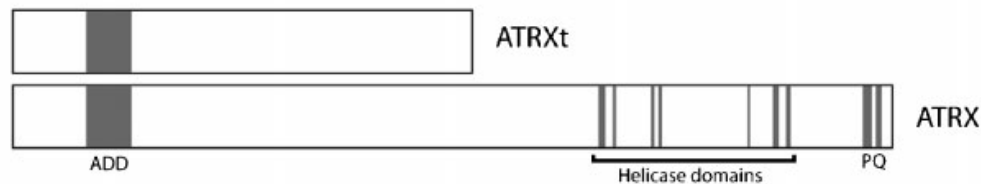


Diagram 1. Schematic of full length and truncated ATRX. Taken from (Gibbons et al., 2008).

ALT Cancers

The ALT pathway is generally found to be active in tumours arising from mesenchymal tissues. Specifically, the peripheral (23%) and central nervous systems (15%), neuroendocrine (40%), bone (62%) and soft tissues (32%). Epithelial malignancies are less commonly shown to have ALT present while hematopoietic malignancies have no reported ALT cases (Heaphy et al., 2011), with the exception of a recent study which reported the presence of C-circles in Chronic Myeloid Leukaemia cases (Samassekou et al., 2013).

Leukaemia

Haematopoiesis is the development of blood cells starting from hematopoietic stem cells that differentiate into common myeloid or lymphoid progenitor cells (Jagannathan-Bogdan and Zon, 2013). The myeloid progenitor cells give rise to granulocyte macrophage progenitors and megakaryocyte erythroid progenitors that produce erythrocytes, platelets, neutrophils, basophils, and eosinophils (Orkin and Zon, 2008). The lymphoid progenitor cells give rise to B-cells, T-cells and natural killer cells (Orkin and Zon, 2008). Leukaemia results from a block in differentiation during haematopoiesis, that then produces many immature abnormal blood

cells that impair the function of normal blood cells. Depending on the rate the disease progresses, the leukaemia are classified as chronic (slow) or acute (fast) and further subdivided as chronic myeloid, or lymphoid (CML, CLL) or acute myeloid or lymphoid (AML, ALL) (Grigoropoulos et al., 2013). CML and CLL are slower progressing and typically occur from more differentiated blood cells whereas ALL and AML progress fast and occur from immature blood cells (Grigoropoulos et al., 2013). AML is the most common occurring acute leukaemia. AML prognosis is characterized as favourable, intermediate and adverse based on the chromosomal abnormalities and additional mutations present. Patients that have translocations on chromosome 8 and 21 (t(8:21)) or t(15:17) alone usually have a favourable outcome whereas having t(9:11), t(8:21) in addition to c-KIT, FLT-ITD, DMT3A or MLL partial tandem duplication result in a significantly poorer survival outcome (De Kouchkovsky and Abdul-Hay, 2016). Novel therapies for favourable AML cases include treating the patients with cytarabine and anthracycline, FLT3-ITD inhibitors, STAT inhibitors, IDH1/2 small molecule inhibitors, DNA analogs, monoclonal antibodies or CAR-T cell therapy (De Kouchkovsky and Abdul-Hay, 2016). ALL is most commonly found in young patients and has an overall 5-year survival of 90% whereas adults are typically worse (Inaba et al., 2013). The majority of ALL cases are B-cell and are characterized by various translocations, such as BCR-ABL, MLL rearrangements (MLLr), ETV6-RUNX1, TCF3-PBX1 and IL3-IGH (Pui et al., 2011; Terwilliger and Abdul-Hay, 2017). T-cell comprise the remainder of ALL and are frequently found to have mutations in NOTCH1, CDKN2A/B, TAL1, TLX3, and PTEN (Pui et al., 2011). Current therapies for ALL involve treating with vincristine, corticosteroids, anthracycline, monoclonal antibodies, proteasome inhibitor, JAK inhibitor, hypomethylating agents, PI3K/mTOR inhibitors, CAR-T cells, and hematopoietic stem cell transplants (Terwilliger and Abdul-Hay, 2017).

Mixed Lineage Leukaemia

A subset of leukaemia that have a poor clinical outcome hold rearrangements in the MLL gene (KMT2A) (Ballabio and Milne, 2012; Hess, 2004). MLL is a histone methyltransferase that deposits methyl marks on histone 3 at lysine 4 (H3K4) residues at target genes which are important during embryonic development and haematopoiesis. These modifications are associated with an active gene transcription of target loci, such as the Hox cluster and cell cycle regulatory genes (Muntean and Hess, 2012). The MLL protein is cleaved by an aspartic protease into two fragments, generating a 320kDa N-terminal protein and an 180kDa C-terminal protein that dimerize via their FYRN and FYRC domains (Hess, 2011). The N-terminal portion contains Menin binding motifs, AT-hooks which bind to AT rich DNA, subnuclear localization motifs, CXXC zinc finger domain that can bind to unmethylated CpG and recruit repressive protein complexes, plant homeodomains (PHD), bromodomain and a FYRN domain. The C-terminal portion has a transactivation domain (TAD), FYRC domain and a SET domain that has methyltransferase activity (Diagram 2A). MLL^N interacts with Menin and LEDGF which direct MLL to regions of interest whereas MLL^C interacts with MOF which is responsible for H4K16 acetylation and RbBP5, WDR5 and ASH2L (Diagram 2B) which are necessary for MLL's methyltransferase activity (Ballabio and Milne, 2012; Hess, 2011).

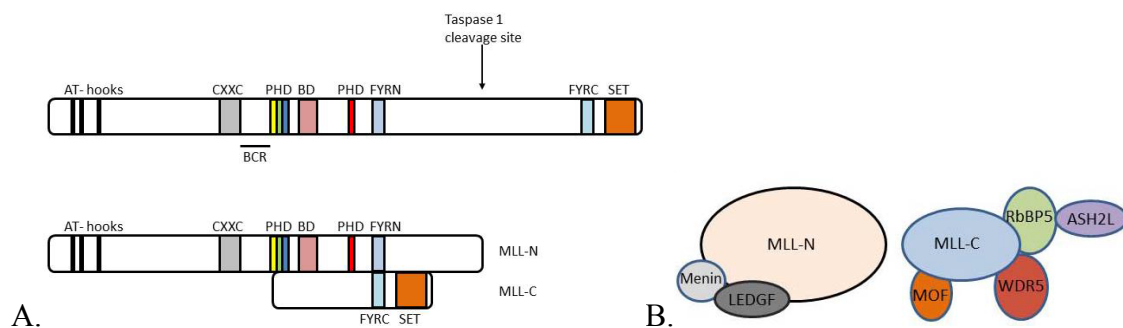


Diagram 2. Schematic of MLL^{WT} and its binding partners. Taken from (Ballabio and Milne, 2012).

MLL Rearrangements

Abnormalities in the MLL gene are most commonly seen as translocations, but partial tandem duplications of the N-terminal portion also occur. Of the various translocation partners (over 120) the most common fusion partners seen in ALL and AML are with AF4, AF9, ENL, AF6 and AF10. The break point cluster region (BCR) in the MLL gene spans 8.3kb from exon 8-12 and in generation of these translocations everything from the PHD domains in MLL^N to MLL^C is lost, generating MLL fusion proteins (MLL-FP). Fusion partners AF9 and ENL recruit DOT1L (a histone 3 lysine 79 methyltransferase), AF4 and AFF4 to MLL target genes. The AF4/AFF4 complex is known to recruit pTEFb (CDK9 and Cyclin1 or 2 heterodimer) to activate RNA polymerase II by phosphorylating the serine 2 hydroxyl residue (Ballabio and Milne, 2012). Although the exact mechanism of MLL-FP activation of gene targets is unknown, these fusion partners function in what is termed as the super elongation complex (SEC) to promote the transcription of MLL target genes through recruitment of DOT1L and RNA pol II. MLL^{WT} and MLL-FP have similar target genes (HOX, MEIS1) (Milne et al., 2005) but MLL-FP are thought to only regulate a subset of MLL targets (Wang et al., 2011).

Prognosis of MLLr Leukaemia

Infants, children and adults harbouring an MLL translocation tend to do poorly in comparison to wild type MLL ALL groups. Infants with MLLr have a 5-year survival of 20-40% in comparison to 60% in non-MLLr ALL. Paediatric MLLr ALL generally have a better prognosis than infant (60%), however, non MLLr paediatric cases are significantly higher, at 92% survival. Adult non MLLr ALL have a slightly better prognosis of 40-45% long term survival as opposed to 35% in MLLr cases, but clearly still present with a poor prognosis. In AML, the prognosis appears to be unfavourable as well, with a 44% 5-year survival as opposed to 55% in non MLLr AML (Winters and Bernt, 2017).

Therapies for MLLr Leukaemia

Currently, there are a few therapies in clinical trials against MLLr leukaemia however the indirect effects and relapse rates are still being investigated. Some of these therapies include FLT3, proteasome, and histone deacetylase (HDAC) inhibitors, hypomethylating agents, CD19 CAR-T cell therapy (patients with ALL relapsed with AML after this treatment (Gardner et al., 2016)), DOT1L, bromodomain, Lysine-Specific Demethylase-1 (LSD1), Polycomb Protein, cell cycle checkpoint, menin, and pTEFb inhibitors (Winters and Bernt, 2017).

Due to the various translocation partners and their prevalence in ALL vs AML it is still difficult to determine the exact role they play in establishing the disease.

MLL in Telomere Biology

Previous studies suggested that MLL has a role in telomere regulation through its binding and promotion of telomeric transcription (Caslini, 2010; Caslini et al., 2009). In this study, the authors looked at MLL binding at telomeres through chromatin immunoprecipitation (ChIP) and DNA blotting for telomere sequence. They saw that in a myelomonocytic leukaemia cell line U937, acute lymphoblastic leukaemia cell lines RS4;11 and ALL-PO that MLL^{WT} appeared to be bound to telomeres. Furthermore, they showed that it was specifically the N-terminal portion of MLL that was responsible for telomere binding, by looking at MLL-AF4, MLL-AF9 and a portion of MLL with the AT hooks. They also observed H3K4 mono, di and tri methylation associated with MLL by ChIP and detected bound telomeric sequences. This suggested to the authors that an active gene signature was present at the telomeres therefore they proceeded to investigate telomeric transcription. They conducted an siRNA knock down of MLL^{WT} and noticed a 28% decrease in TERRA signals via northern blot analysis and a decrease of H3K4 di and tri methylation. The authors suggested MLL^{WT} has a role in telomere

protection as knocking down MLL^{WT} caused DNA damage responses (increased 53BP1) and p53 dependent cell growth arrest.

Project Goals

In regard to MLL^{WT} having a role in telomere maintenance we want to explore this role in the context of MLLr leukaemia. To achieve this, our project aims are to first confirm if MLL^{WT} and or MLLr are binding at telomeres. Subsequently, if these proteins are binding, we want to investigate their role in telomere transcription. Finally, we want to determine the mechanism of telomere lengthening in this leukaemia.

Methods

Cell lines and Culture

SEM, ML-2, KOPN-8, NOMO-1, RS4;11, MV4;11, REH, RCH-ACV, U-937, 697, THP-1, CCRF-CEM were purchased from DMSZ and ATCC. With the exception of SEM, cells were cultured in RPMI 1640 supplemented with 10% FBS and 1% Glutamax. SEM were cultured in IMDM supplemented with 10% FBS and 1% Glutamax.

U2OS, G292, SW39, HeLa-LT, WI-38 Va13 2RA and SaOS2 were purchased from ATCC. U2OS was cultured in DMEM, 10% Tet-free serum, Penicillin, Streptomycin, Glutamine, Neomycin (0.7mg/mL), and Puromycin (0.25mg/500 mL). G292, SW39, HeLa-LT, WI-38 Va13 2RA and SaOS2 were cultured in DMEM supplemented with 10% FBS and Penicillin, Streptomycin and Glutamine.

Chromatin Immunoprecipitation (ChIP)

Approximately 10 million SEM cells were used for ChIP. The cells were initially rinsed in 1x phosphate buffered saline (PBS) and subjected to double fixation with 2mM Disuccinimidyl Glutarate (cat. 80424-50MG-F) and 1% formaldehyde. Fixed cells were then lysed in 1% SDS, 10mM EDTA, 50mM Tris pH 8.1 and sonicated using the Covaris. The sonicated chromatin was then diluted 10x with 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCL pH 8.1, 167 mM NaCl and 1x protease inhibitors. Dynabeads Protein A and G (cat. 100-01D and 100-03D) were added to the chromatin and incubated for thirty minutes at 4°C. One microgram of the desired antibody was added to a millilitre of the chromatin sample and incubated overnight at 4°C and 50µL of the chromatin was saved for a control. More dynabeads were added to the chromatin samples the next day and incubated for 3-5 hours at 4°C. The samples then were washed four times in 1x RIPA buffer (50mM HEPES-KOH pH 7.6, 500mM LiCl, 1mM EDTA, 1% NP-40, 0.7% Na-Deoxycholate) and once with Tris-EDTA, 50 mM

NaCl. Samples were then eluted from the beads, de-crosslinked using 1% SDS, 10mM EDTA, 50mM Tris pH 8.1 and incubated at 65°C overnight. The chromatin was then cleaned and purified with 500µg/mL RNase A, 14mg/mL Proteinase K and the Zymo research kit.

Antibodies

Antibodies used for ChIP experiments were MLL-1 (Bethyl A300-086a, polyclonal), AF4 (Ab31812, polyclonal), TRF1 (C-19, polyclonal) and IgG (CST 2729, polyclonal). Antibodies used for western blots were Histone H3 (Ab1791, anti-rabbit, 1: 20,000, polyclonal), ATRX (39F, anti-mouse, 1:17, monoclonal generated by Richard Gibbons) and alpha-tubulin (Ab7291, anti-mouse, 1:10,000 monoclonal). For intracellular flow the primary ATRX antibody was used at a 1:25 dilution and the secondary Alexa Fluor 647 (A21235, anti-mouse, 1:3000).

ChIP-qPCR

ChIP DNA was validated by looking at known positive target genes of MLL-AF4 and by looking at non-target regions to determine the background signal. ChIP DNA was also used for detection of MLL^{wt} and or MLL-AF4 binding at telomeres and sub-telomeres. Primers designed at telomeric and sub-telomeric regions were taken by (Feretzaki and Lingner, 2017; Henson et al., 2017) and adapted for our use.

The amount of ChIP DNA was calculated by:

$$2^{(CT \text{ of Input} - CT \text{ of ChIP})} \times 5\% \text{ (percent of input taken from total sonicated chromatin)}$$

Table 1: SYBR Green Primers

HOXA9 FORWARD: ATGCTTGTGGTTCTCCTCCAGTTG
HOXA9 REVERSE: CCGCCGCTCTCATTCTCAGC
GNAQ FORWARD: GTCCATCATGGCGTGCT
GNAQ REVERSE: CGGACGGTACTCACCGA
CDK6 FORWARD: TCGAAGCGAAGTCCTCAACA
CDK6 REVERSE: GCTTGGGCAGAGGCTATGTA
18205 FORWARD: GGCTCCTGTAACCAACCACTACC
18205 REVERSE: CCTCTGGGCTGGCTTCATTC

Table 2: Sub-Telomere Primers and Telomere Primers

	DISTANCE TO TELOMERE BODY
1Q TERRA – FORWARD GCATTCCTAATGCACACATGAC	213 BP
1Q TERRA – REVERSE ACCCTAACCCGAACCCTA	137 BP
7P TERRA 2 – FORWARD GAGAGAGGGTTTCACTCTGTTG	568 BP
7P TERRA 2 – REVERSE GGTGGTTCACGCCTGTAAT	481 BP
9P TERRA – FORWARD GAGATTCTCCCAAGGCAAGG	237 BP
9P TERRA – REVERSE ACATGAGGAATGTGGGTGTTAT	138 BP
10Q TERRA 2 – FORWARD ATGCACACATGACACCCTAAA	332 BP
10Q TERRA 2 – REVERSE TACCCGAACCTGAACCCTAA	284 BP
15Q TERRA 2 – FORWARD CAGCGAGATTCTCCCAAGCTAAG	176 BP
15Q TERRA 2 – REVERSE AACCCTAACCACATGAGCAACG	62 BP
15Q TERRA 3 – FORWARD GCAAATGCAGCAGTCCTAATG	112 BP
15Q TERRA 3 – REVERSE GACCCTGACCCTAACCCCTAA	48 BP
17Q TERRA 3 – FORWARD GTCCATGCATTCTCCATTGATAAG	940 BP
17Q TERRA 3 – REVERSE AGCTACCTCTCTCAACACCAAGAAG	889 BP
XpYp TERRA 1 – FORWARD GCGCGTCCGGAGTTTG	305 BP
XpYp TERRA 1 – REVERSE CCACAACCCCAACCAGAAAGA	255 BP
XpYp TERRA 3 – FORWARD AAGAACGAAGCTTCCACAGTAT	125 BP
XpYp TERRA 3 – REVERSE GGTGGGAGCAGATTAGAGAATAAA	56 BP
TELOMERE FORWARD: GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT	
TELOMERE REVERSE: TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA	

Table 3: The cycle conditions for ChIP-qPCR

	95 DEGREES	10 MINUTES
CYCLE (40 CYCLES)	95 DEGREES	15 SECONDS
CYCLE (40 CYCLES)	60 DEGREES	1 MINUTE

RNA Extraction

For RNA extraction, 1-5 million cells were harvested and washed with 1x PBS then lysed, washed, and re-suspended according to the RNeasy kit protocol (Qiagen Cat. 74104). RNA was quantified using the nanodrop and 1 µg was taken for cDNA synthesis, and northern blots.

Reverse Transcription-PCR

cDNA was synthesized from 1 µg RNA using the SuperScript III First Strand Synthesis kit (cat. 180808-051). Random hexamers were used instead of oligo dTs with dNTPs, 5x First-Strand buffer, 0.1M DTT, RNaseOUT and SuperScript III.

Table 4: The cycle conditions for cDNA

25 DEGREES	5 MINUTES
50 DEGREES	60 MINUTES
70 DEGREES	15 MINUTES

*cDNA was diluted 10x before setting up qPCR.

Table 5A: RT-PCR SYBR Green Primers (human)

MLL-AF4 FORWARD 1: AGGTCCAGAGCAGAGCAAAC
MLL-AF4 REVERSE 1: CGGCCATGAATGGGTCATTTC
MLL-AF4 FORWARD 2: ACAGAAAAAAGTGGCTCCCCG
MLL-AF4 REVERSE 2: TATTGCTGTCAAAGGAGGCGG
YWHAZ FORWARD: ACTTTTGGTACATTGTGGCTTCAA
YWHAZ REVERSE: CCGCCAGGACAAACCAGTAT

Table 5 B: The cycle conditions for RT-PCR SYBR green

	95 DEGREES	10 MINUTES
CYCLE (40 CYCLES)	95 DEGREES	15 SECONDS
CYCLE (40 CYCLES)	60 DEGREES	1 MINUTE

Table 5C: RT-PCR TaqMan Probes (human)

ATRX	HS00230877_M1	FAM
ATRX	HS00997525_M1	FAM

Table 5D: The cycle conditions for RT-PCR Taqman

	50 DEGREES	2 MINUTES
	95 DEGREES	20 SECONDS
CYCLE (40 CYCLES)	95 DEGREES	3 SECONDS
CYCLE (40 CYCLES)	60 DEGREES	30 SECONDS

C-circle Assay

DNA from various cell lines were extracted using the Core B DNA purification kit and rehydrated in 20 mM Tris buffer pH 7.4. The extracted DNA (250 ng) was digested using HinfI and RsaI for 3 hours at 37 degrees. Next, rolling amplification was performed with each 100 µL reaction containing 100 mM dATP, dGTP and dTTP, 10mg/mL bovine serum albumin, 1% Tween, 10x φ 29 polymerase buffer, φ 29 Polymerase, milli-Q H₂O, and 150 ng of digested DNA. For controls, samples without Phi polymerase were included (no Phi). The reaction was carried out at overnight at 30 degrees for 8 hours, 65°C for 20 mins and 4°C until blotted.

To quantify the C-circles, the C-circle reaction was diluted in 2x Saline Sodium Citrate (SSC), blotted onto a zeta probe membrane, UV cross-linked and then dried at 80 degrees for 3 hours. The membrane was pre-soaked in 2x SSC then placed in PerfectHyb Plus buffer containing γ³²P-(CCCTAA)₃ labelled telomeric probe at 37°C overnight. The membrane was washed twice with 10x SSC for 5-10 minutes depending on the strength of the radioactive isotope. The membrane was then placed on an autoradiography film for 1-7 days at -80 degrees.

C-circle-qPCR

An alternative and faster method to blotting the C-circle DNA is qPCR. The C-circle Phi and no Phi reactions were diluted 1/4 and 5 µL was taken for the qPCR using the telomere primers mentioned in Table 2. To generate a standard curve, U2OS (known positive sample for C-circles) was used in a dilution range of 100%-0.01%. The reaction mixture and cycle conditions were different from the sub-telomere qPCR, as seen in table 6A&B.

The C-circle signal was normalized by:

$$\frac{\text{signal from phi}}{\text{signal from phi gDNA}} - \frac{\text{signal from no phi}}{\text{signal from no phi gDNA}}$$

Table 6A: Telomere q-PCR Reaction Mixture

TELOMERE F	0.3 μ M
TELOMERE R	0.4 μ M
SYBRGREEN QUANTITECT	1x
DTT	10mM
DIMETHYLSULFOXIDE	0.5 μ L
MQ H ₂ O	FINAL VOLUME TO 20 μ L
DNA	5 μ L

Table 6B: The cycle conditions for SYBR green QuantiTECT

	95 DEGREES	15 MINUTES
CYCLE (33 CYCLES)	95 DEGREES	15 SECONDS
CYCLE (33 CYCLES)	54 DEGREES	2 MINUTES

RNA FISH

Cells were harvested, re-suspended in 1xPBS and cytospun onto coverslips at 1000 rpm for 5 minutes. The mounted cells were then fixed with 4 % formaldehyde, 5% Glacial Acetic Acid, and 1.5M NaCl for 20 minutes at room temperature. After fixation, cells were washed with 1x PBS and incubated in 70% ethanol for five minutes at room temperature. After the incubation, the cells were rinsed in Tris Saline, treated with 0.01% pepsin, post-fixed in 3.7% formaldehyde, dehydrated in 70%, 90% and 100% ethanol and hybridized with the TERRA probe, (TAACCC)₇ /3Cy3 overnight at 37°C. Cells were washed 3x in 2x SSC at 37 °C and once in Tris Saline-Tween and Tris Saline. The coverslips were then dehydrated and mounted onto slides with Vectashield containing DAPI (1 μ g/mL).

Images were Z-projected using ImageJ and then subsequently analysed using CellProfiler 3.0 to count foci and measure foci intensity and area.

Northern Blot

One microgram of extracted RNA was prepared in 50% formamide, 2.2M formaldehyde, and 1x MOPS, denatured at 65°C for 15 minutes and run on a 1.2 % formaldehyde, MOPS agarose, SYBR safe gel at 120V. The RNA was transferred onto a zeta probe membrane overnight, UV crosslinked 3x and dried for 3 hours at 80°C. The probing for TERRA was the same as described in the C-circle assay. The membranes were then quantified by Fiji and the lane intensities were analysed.

Western Blot

Approximately five million cells were harvested, washed with 1x PBS, lysed with RIPA buffer (1M NaCl, 1% NP-40, 1% Na-Deoxycholate, 0.2% SDS, 50mM Tris-HCl pH 7.4) plus protease inhibitor cocktail and syringed 30x with a 25G gauge needle. The supernatant from the samples after centrifugation was collected and mixed with 1M DTT and 1x Loading Dye and incubated at 95 degrees for 5 minutes. The amount of protein loaded in each well was normalized to the sample containing the lowest protein concentration. The samples were run on a 4-12% Bis-Tris Plus Bolt Gel with 1x Bolt MOPS SDS Running Buffer at 100V. The proteins were transferred onto a PVDF membrane using NuPAGE 1x Transfer Buffer with 10% Methanol overnight at 25mA at 4 degrees. The membranes were blocked in 5% milk-Tris Buffered Saline –Tween for an hour at room temperature. The membrane was then incubated in primary antibodies diluted in 2.5% milk-TBST for an hour at room temperature. The membrane was washed 3x in TBST then incubated in secondary antibodies diluted in 2.5% milk-TBST for an hour at room temperature. The membrane was washed 3x times in TBST

and incubated with ECL (reagents A & B) for one minute then exposed using high performance chemiluminescent film.

Intracellular Flow

Around five million cells of interest were collected and washed in 1x PBS then fixed with 1.6% paraformaldehyde for 10 minutes at room temperature. Cells were washed with PBS containing 2% fetal bovin serum and centrifuged at 800g for 3 minutes. Two methods of permeabilization was used. The first being methanol, cells were vortexed while cold methanol was added slowly. The cells were then immediately centrifuged and washed 2x with PBS/ 2% FBS. The second method was washing cells with a wash buffer containing 0.1mg/mL digitonin in PBS and 2% FBS. For the two methods, the cells were either re-suspended in PBS/FBS or digitonin/PBS/FBS to make up a final concentration of 20,000 cells/ μ L. From this cell suspension, 25 μ L was taken for primary antibody incubation or no antibody control. The primary antibody concentration was initially optimized using dilutions from 1:25-1:100. Once the primary antibody was added to the cells, the samples were incubated on ice for 30 minutes. The cells were then washed in either PBS/FBS or digitonin/PBS/FBS, re-suspended in 50 μ L of Alexa Fluor 647 anti-mouse secondary (1:3000) and incubated on ice for 15 minutes in the dark. After the secondary antibody incubation, the cells were washed and re-suspended in 150 μ L of either PBS/FBS or digitonin/PBS/FBS and then run on the Fortessa X20.

siRNA

Approximately 45 million SEM cells were electroporated with 100 μ mol of siRNA at 350V for 10ms. Cells were then plated in fresh media and harvested after 48 hours.

Table 7: siRNA

MLL-AF4 siRNA: siMA6
MLL-AF4 NON-TARGETING siRNA: siMM
MLL ^{WT} siRNA: DHARMACON/L-009914-00
MLL WT NON-TARGETING siRNA: DHARMACON/D-001206-13-05

Cell Lines Information

CELL LINE	FUSION	CANCER CLASSIFICATION	PATIENT AGE/SEX
U937	NON MLLR	AML-ACUTE MYELOID LEUKAEMIA	<i>37 MALE</i>
REH	NON MLLR	ALL-B CELL PRECURSOR LEUKAEMIA	<i>15 FEMALE</i>
RCH-ACV	NON MLLR	ALL-B CELL PRECURSOR LEUKAEMIA	<i>8 FEMALE</i>
697	NON MLLR	ALL-B CELL PRECURSOR LEUKAEMIA	<i>12 MALE</i>
CCRF-CEM	NON MLLR	ALL-T CELL LEUKAEMIA	<i>4 FEMALE</i>
SEM	MLL-AF4	ALL-B CELL PRECURSOR LEUKAEMIA	<i>5 FEMALE</i>
RS4;11	MLL-AF4	ALL-B CELL PRECURSOR LEUKAEMIA	<i>32 FEMALE</i>
KOPN-8	MLL-ENL	ALL-B CELL PRECURSOR LEUKAEMIA	<i>3 MONTH FEMALE</i>
MV4;11	MLL-AF4	AML-ACUTE MONOCYTIC LEUKAEMIA	<i>10 MALE</i>
THP-1	MLL-AF9	AML-ACUTE MONOCYTIC LEUKAEMIA	<i>1 MALE</i>
ML-2	MLL-AF6	AML- ACUTE MYELOMONOCYTIC LEUKAEMIA	<i>21 MALE</i>
NOMO1	MLL-AF9	AML-ACUTE MYELOID LEUKAEMIA	<i>31 FEMALE</i>
SAOS2	NON MLLR	OSTEOSARCOMA	<i>11 FEMALE</i>
U2OS	NON MLLR	OSTEOSARCOMA	<i>15 FEMALE</i>
WI-38 VA13 2RA	NON MLLR	FIBROBLAST SV40 TRANSFORMED	<i>3 MONTHS FEMALE</i>
HeLa	NON MLLR	CERVICAL ADENOCARCINOMA	<i>31 FEMALE</i>

Results

MLL^{WT} and/or MLLr Weakly Binds to Telomeres

MLL^{WT} was shown to bind to telomeres in a previous publication (Caslini et al., 2009). We initially tried to recapitulate these findings so we could explore further what MLL is doing at telomeres. To see if MLL^{WT} or MLL-FPs are binding at telomeres we set up ChIP using SEM cells (a cell line that has both wild type and MLL-AF4 protein). We pulled down chromatin with antibodies targeting the N terminus of MLL and the C terminus of AF4 then blotted the DNA onto a membrane. Telomeric DNA was detected by probing with ³²P-telomere sequences. We observed faint signal from our MLL-N pulldown ChIP DNA but not from the AF4-C pulldown (Figure 1).

We also examined ChIP signal at telomeres using qPCR in RS4;11 cells (MLL-AF4 cell line) and SEM cells, using TRF1 ChIP as a positive control (Figures 2&3). The positive control TRF1 had a high enrichment at the telomeres (3-5% of input), whereas the MLL-N pull down produced a relatively weak signal (0.06-0.1% of input) in comparison to its binding at canonical target sites. However, the signal appeared to be above background as measured by a negative control primer set, Neg-18205. Noticeably, AF4-C pull downs produced a negligible signal at the telomeres, comparable to the DNA blot results.

The sonication step in the ChIP protocol generates around 200bp fragments; it is possible that if MLL is binding at the sub-telomere (a region upstream from telomere body but close to the telomere body) this could produce signal at the telomere in our ChIP qPCR and DNA blot experiments. Therefore, we attempted to establish whether MLL was binding at the telomeres or the sub-telomere. We used primers designed at the sub-telomeres to perform qPCR using ChIP DNA. Primers were initially selected based on the best single in silico PCR product (Figure 4). After the qPCR was completed, the primers with the best melt curve were selected to be the final primers used for experiments. From the ChIP-qPCR data it is evident

that there is a low and inconsistent amount of signal at the sub-telomeres (Figures 2C,3C,5D,6D and 7B) for both the MLL-N and AF4-C pull down.

Given that MLL is weakly binding at the telomere and or the sub-telomeres we proceeded to determine if MLL^{WT} or MLL-AF4 is binding in our cell line (SEM). We performed a knock down of MLL-AF4 using siRNA and achieved an approximately 60% knockdown based on reverse transcription qPCR (Figure 5A). We noticed there was no decrease in MLL binding at the telomere and sub-telomere using our qPCR assay (Figure 5B-D). This lead us to believe that, perhaps, it was solely MLL^{WT} that was binding and not the translocated protein. Consequently, we proceeded to knock down MLL^{WT} using siRNA yet there was no decrease in MLL binding at both the telomeres and sub-telomeres (Figure 6). In addition, we performed a ChIP on an MLL-AF6 cell line without the wild type protein. We observed that the fusion protein weakly bound to sub-telomeres (Figure 7). Our results suggest that there could be weak binding of MLL at the telomere and or sub-telomere, however, it is difficult to determine if it is MLL^{WT} or MLL-FP driven.

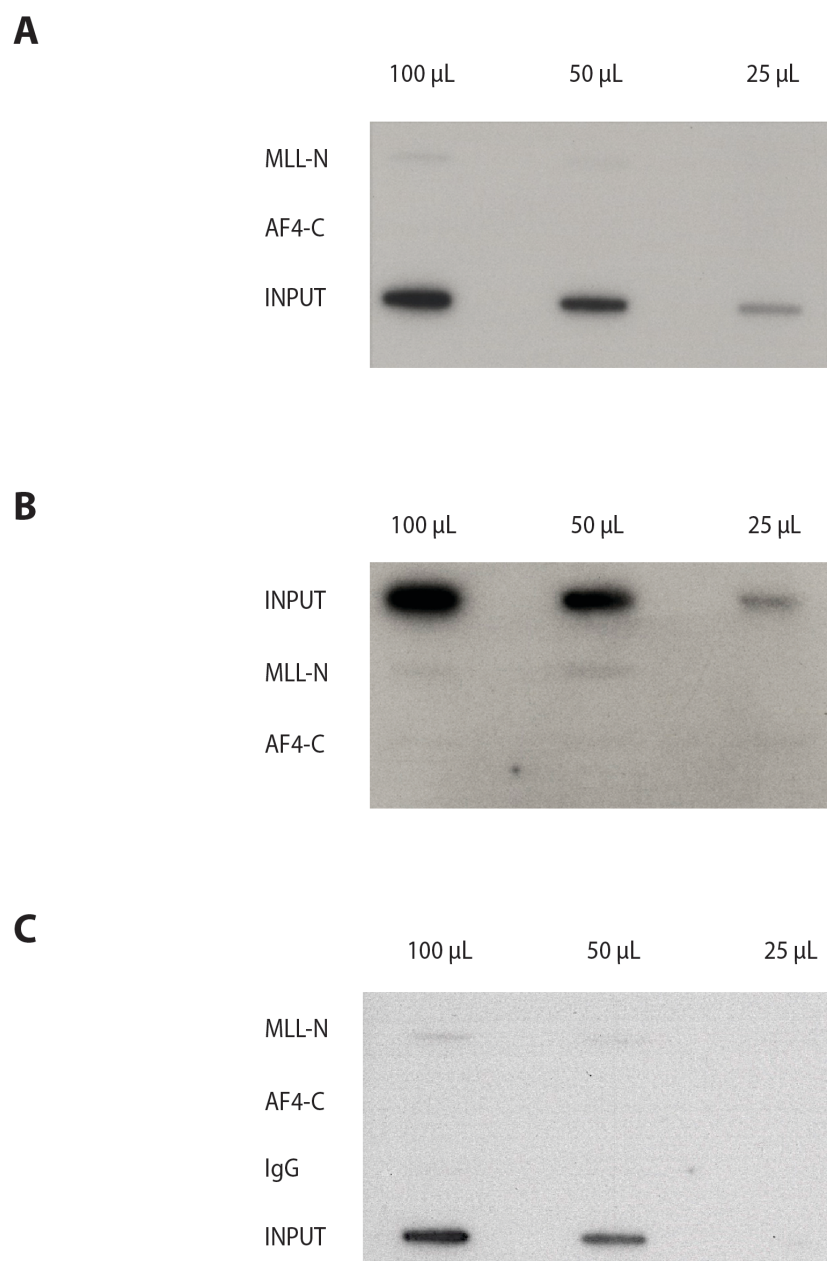


Figure 1. MLL-N Weakly Binds at Telomeres in SEM cells.

MLL-N and AF4-C ChIP DNA was analysed for telomeric sequences in SEM cells (MLL-AF4 cell line) by DNA blotting in three biological replicates with IgG included as a negative control for telomere binding (A-C). 25-100 μ L of ChIP DNA was loaded onto the membrane. The input sample represents 5% of the total chromatin in each pull down. The blots were probed with 32 P-end labelled telomere oligo.

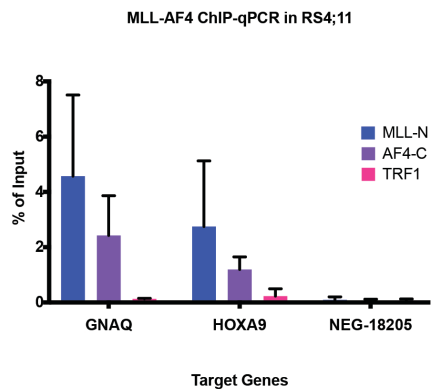
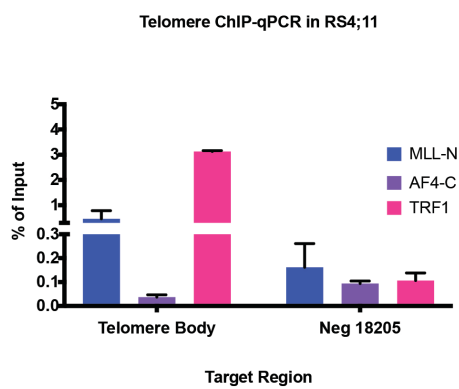
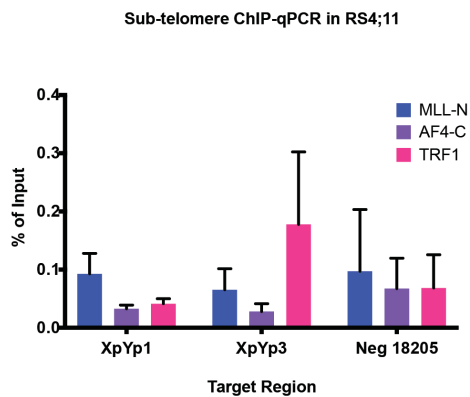
A**B****C**

Figure 2. MLL-N Weakly Binds at Telomeres in RS4;11 cells.

MLL-N, AF4-C and TRF1 ChIP DNA was analysed for telomeric sequences in RS4;11 cells (MLL-AF4 cell line) in three biological replicates. **A.** ChIP-qPCR representing binding at canonical MLL-AF4 target sites; Neg 18205 was included as a negative control to detect ChIP background levels. **B.** ChIP-qPCR representing binding at the telomeres with TRF1 included as a positive control. **C.** ChIP-qPCR representing binding at the sub-telomeres.

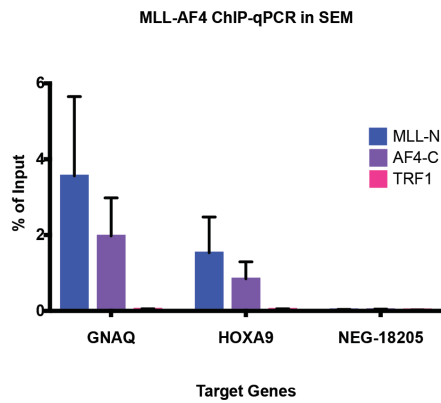
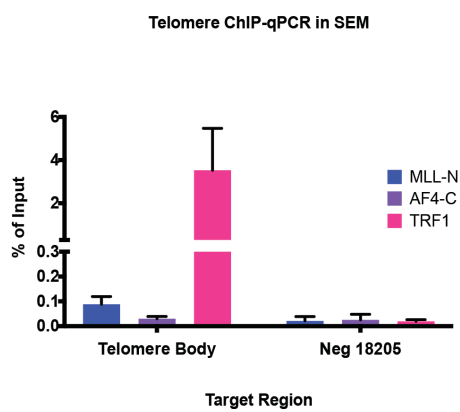
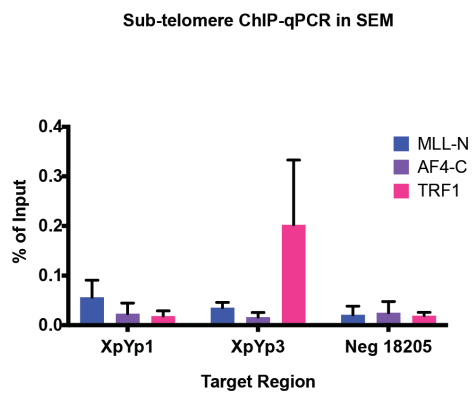
A**B****C**

Figure 3. MLL-N Weakly Binds at Telomeres in SEM cells.

MLL-N, AF4-C and TRF1 ChIP DNA was analysed for telomeric sequences in SEM cells (MLL-AF4 cell line) in three biological replicates. **A.** ChIP-qPCR representing binding at canonical MLL-AF4 target sites; Neg 18205 was included as a negative control to detect ChIP background levels. **B.** ChIP-qPCR representing binding at the telomeres with TRF1 included as a positive control. **C.** ChIP-qPCR representing binding at the sub-telomeres.

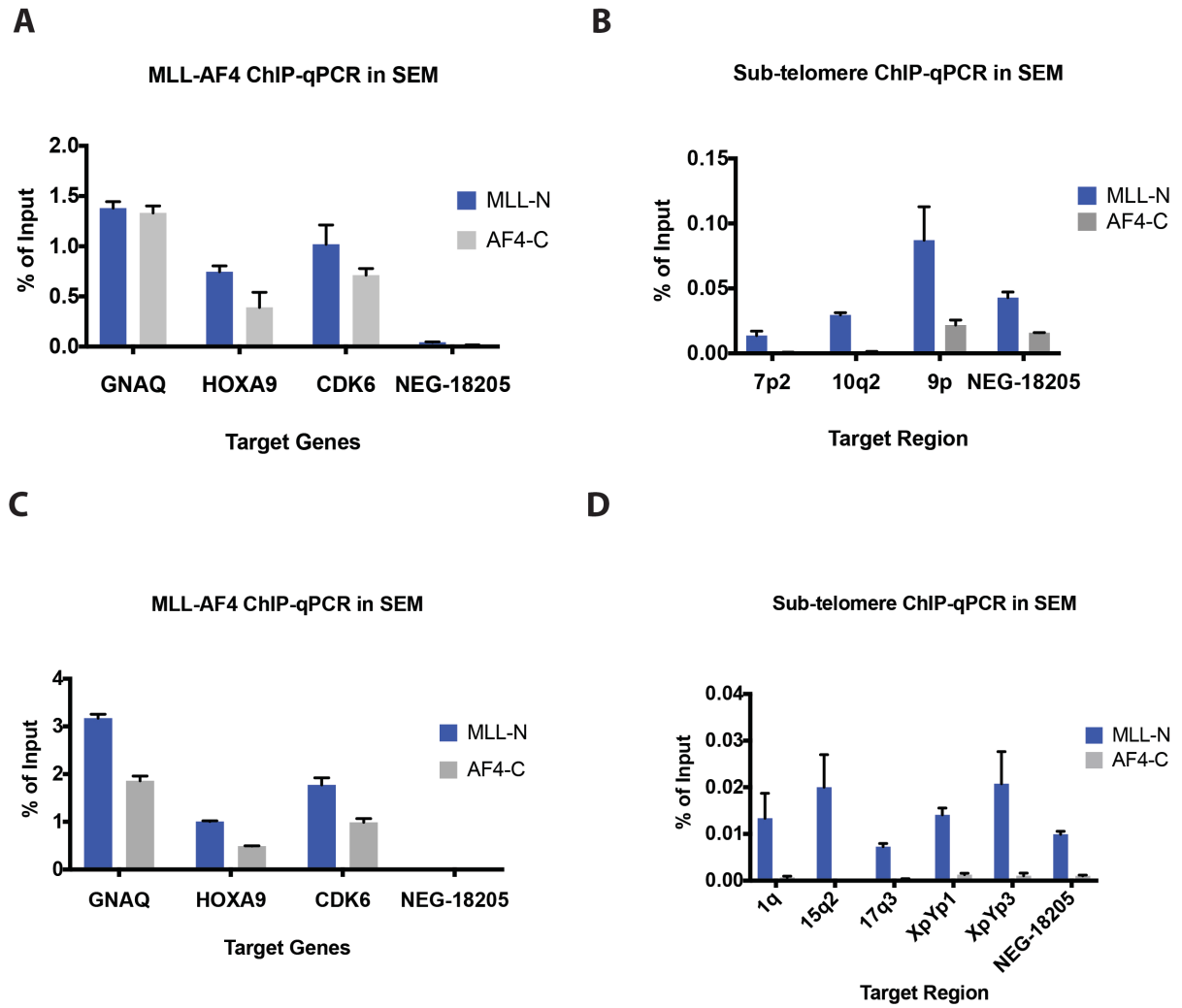


Figure 4. MLL-N Binding at Sub-telomeres in SEM Cells (Optimization).

MLL-N, and AF4-C ChIP DNA was analysed for sub-telomeric sequences in SEM cells (MLL-AF4 cell line) by qPCR in three technical replicates per experiment. **A&C.** MLL-N and AF4-C binding at canonical target sites, GNAQ, HOXA9, and CDK6; Neg 18205 was included as a negative control to detect ChIP background levels. **B&D.** MLL-N, and AF4-C binding at the sub-telomeres of chromosomes 1,7,9,10,15,17 and XpYp.

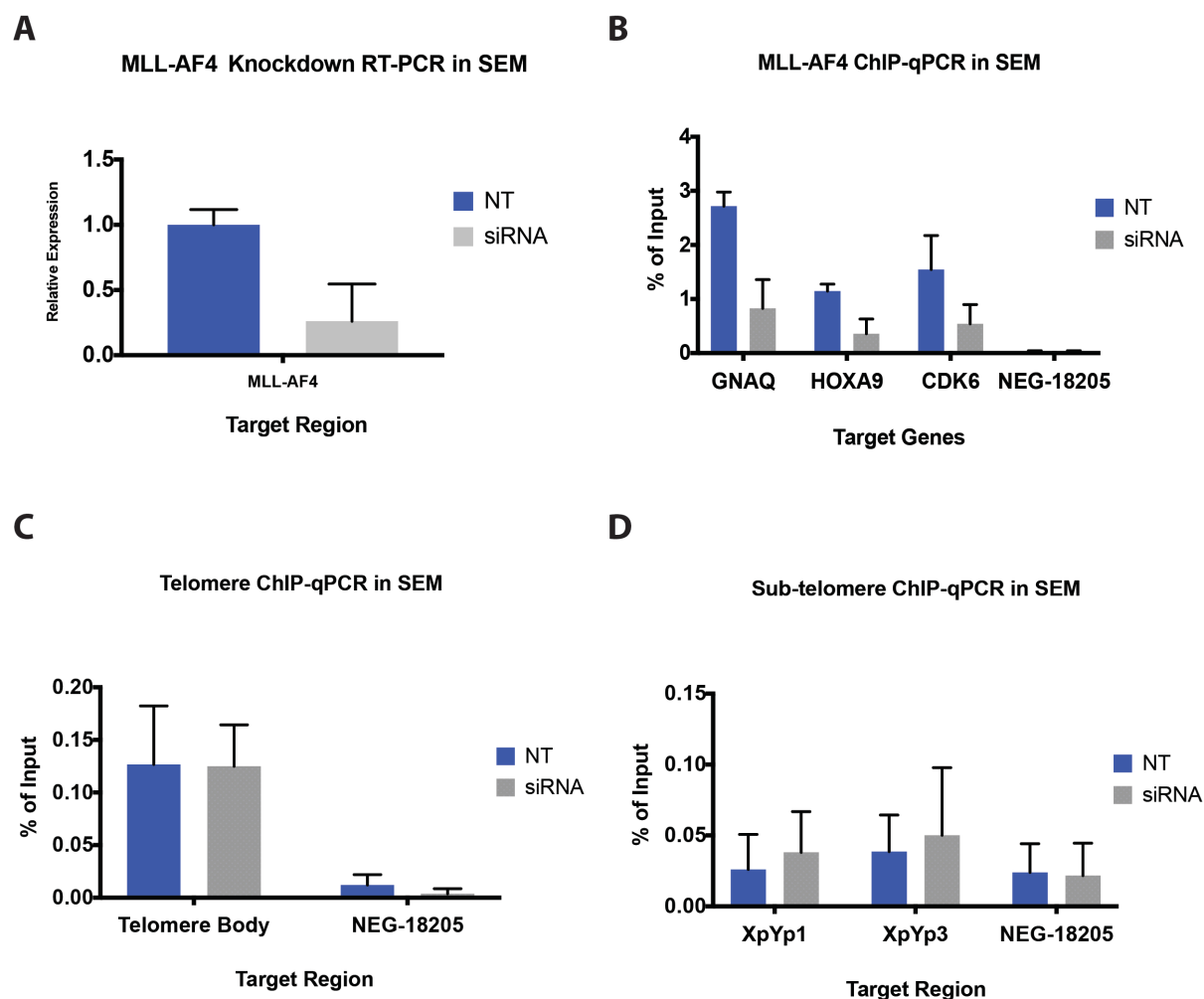


Figure 5. MLL-AF4 Knockdown in SEM Show No Change at the Telomere and Sub-telomeres.

MLL-N ChIP DNA from T.Milne and D.Gracias were analysed for telomeric and sub-telomeric sequences after MLL-AF4 knock down in SEM. **A.** Knockdown efficiency of about 60% after 48hrs in culture post electroporation in three technical replicates. **B.** MLL-N binding at canonical target sites, GNAQ, HOXA9, and CDK6; Neg 18205 was included as a negative control to detect ChIP background levels in three biological replicates. **C&D.** MLL-N binding at the telomere and sub-telomeres in three biological replicates.

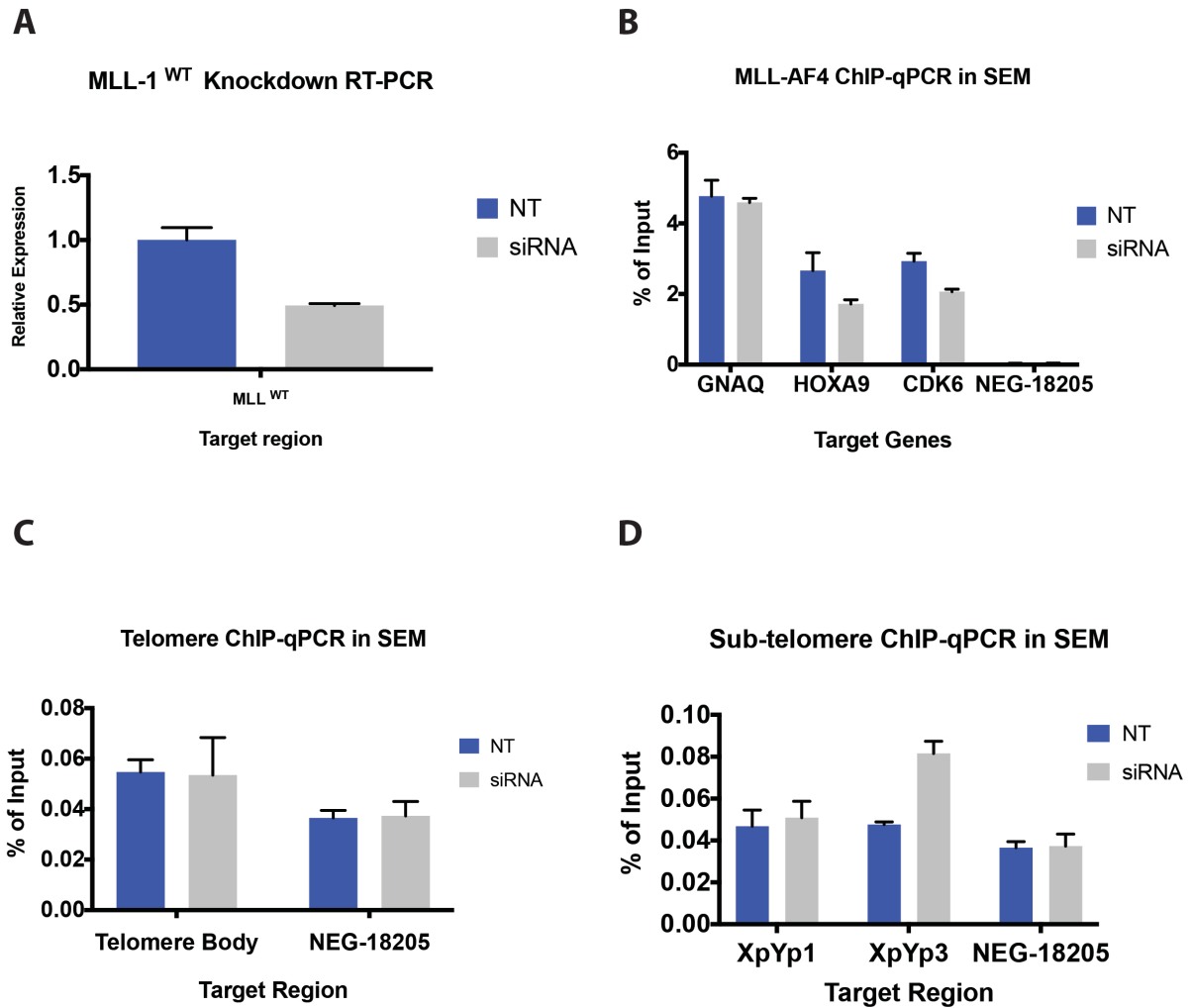


Figure 6. MLL^{WT} Knockdown in SEM Show No Change at the Telomere and Sub-telomeres.

MLL-N ChIP DNA was analysed for telomeric and sub-telomeric sequences after MLL^{WT} knock down in three technical replicates. **A.** Knockdown efficiency of about 50% after 48hrs in culture post electroporation. **B.** MLL-N binding at canonical target sites, GNAQ, HOXA9 and CDK6; Neg 18205 was included as a negative control to detect ChIP background levels. **C&D.** MLL-N binding at the telomere and sub-telomeres.

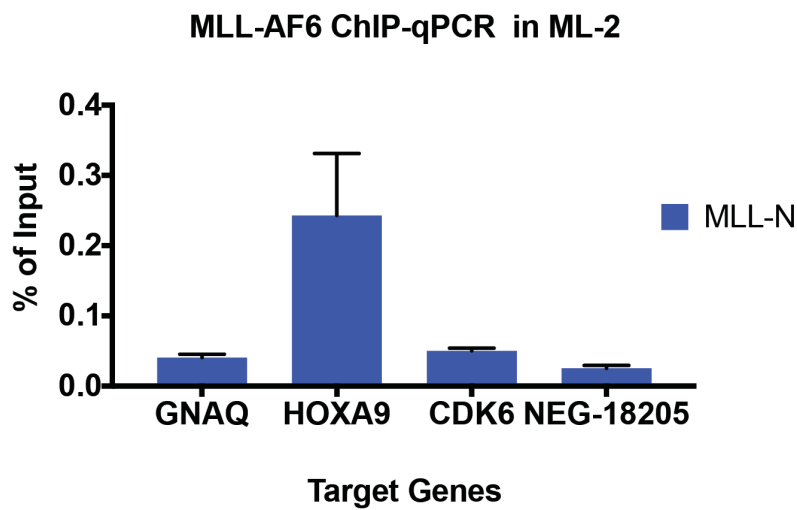
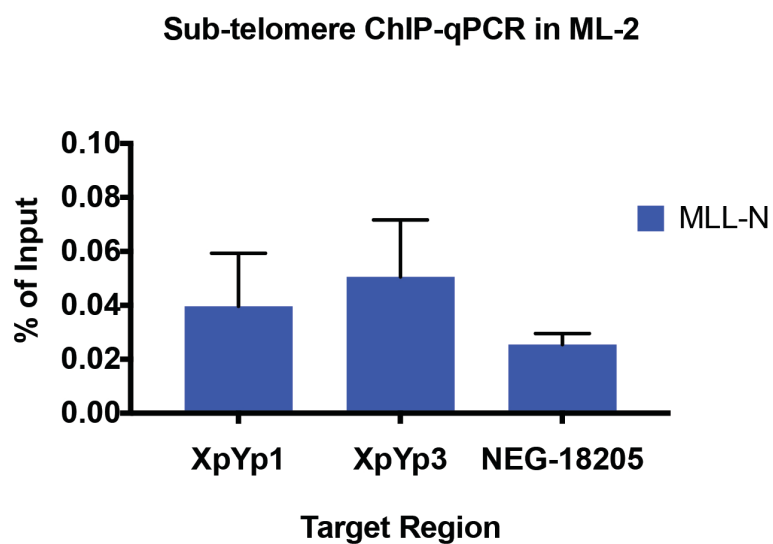
A**B**

Figure 7. MLL-AF6 Weakly Binds at the Sub-telomeres in ML-2 Cells.

MLL-N ChIP DNA from T.Milne was analysed for sub-telomeric sequences in ML-2 cells (MLL-AF6 cell line) in two biological replicates. **A.** ChIP-qPCR representing binding at canonical MLL-AF6 target sites; Neg 18205 was included as a negative control to detect ChIP background levels. **B.** ChIP-qPCR representing binding at the sub-telomeres.

Increase in telomere transcript (TERRA) in MLLr cell lines

Previous reports indicated that telomeric transcription was regulated by MLL^{WT} (Caslini et al., 2009). Therefore, to confirm these findings we investigated TERRA expression in our cell lines by two methods: RNA FISH and Northern blot assays. For RNA-FISH, we included an ALT positive cell line (U2OS) and an ALT negative cell line (SW39) that had high and low expression of TERRA as controls for our assay. We assayed three further cell lines, NOMO-1 (an MLL-AF9 cell line), RS4;11 (an MLL-AF4 cell line) and U937 (a monocytic leukaemia cell line without an MLL translocation) (Figure 8). Our analysis suggested that RS4;11 had more TERRA foci per nuclei (3.7) than NOMO-1(1.5), our non-fusion cell line, U937 (1.7) and our negative control SW39 (2.2) (Figure 9).

To supplement this data, we analysed cell lines for TERRA by a Northern blot assay (Figure 10). Here, we included our positive controls, U2OS and G292, and negative control SW39 alongside the leukaemia cell lines. We observed a slight increase in TERRA in cell lines that had an MLL translocation (Figure 11). In addition, we treated a selected number of samples with RnaseA to confirm that our signal was specific to TERRA RNA (Figure 12).

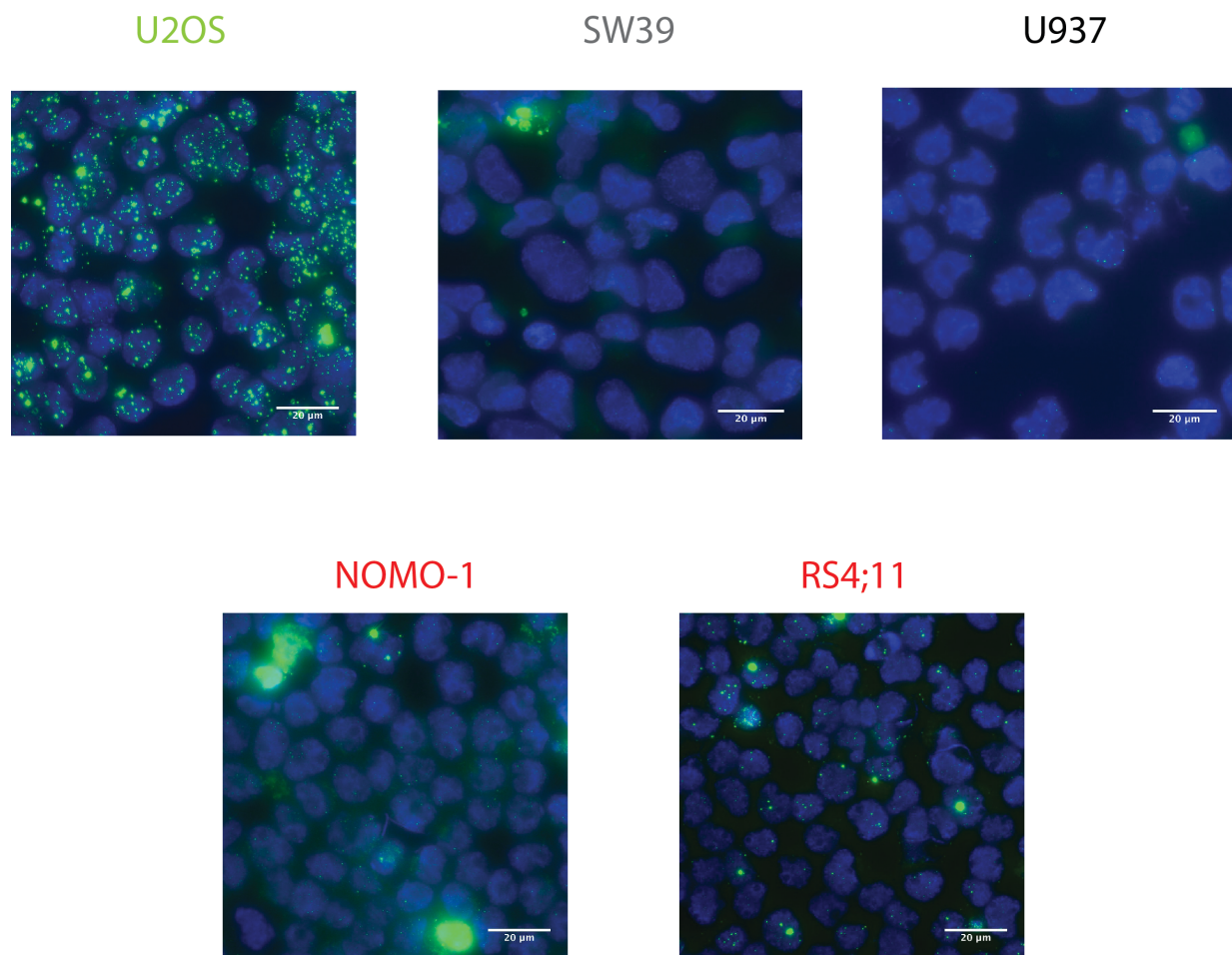


Figure 8. Increased TERRA in MLLr Leukaemia Cell line RS4;11.

A representative image of RNA FISH in an **ALT positive** cell line (U2OS), an **ALT negative** cell line (SW39), a **non MLL-fusion** cell line (U937) and two **MLL-fusion** positive cell lines, NOMO-1 (MLL-AF9) and RS4;11 (MLL-AF4).

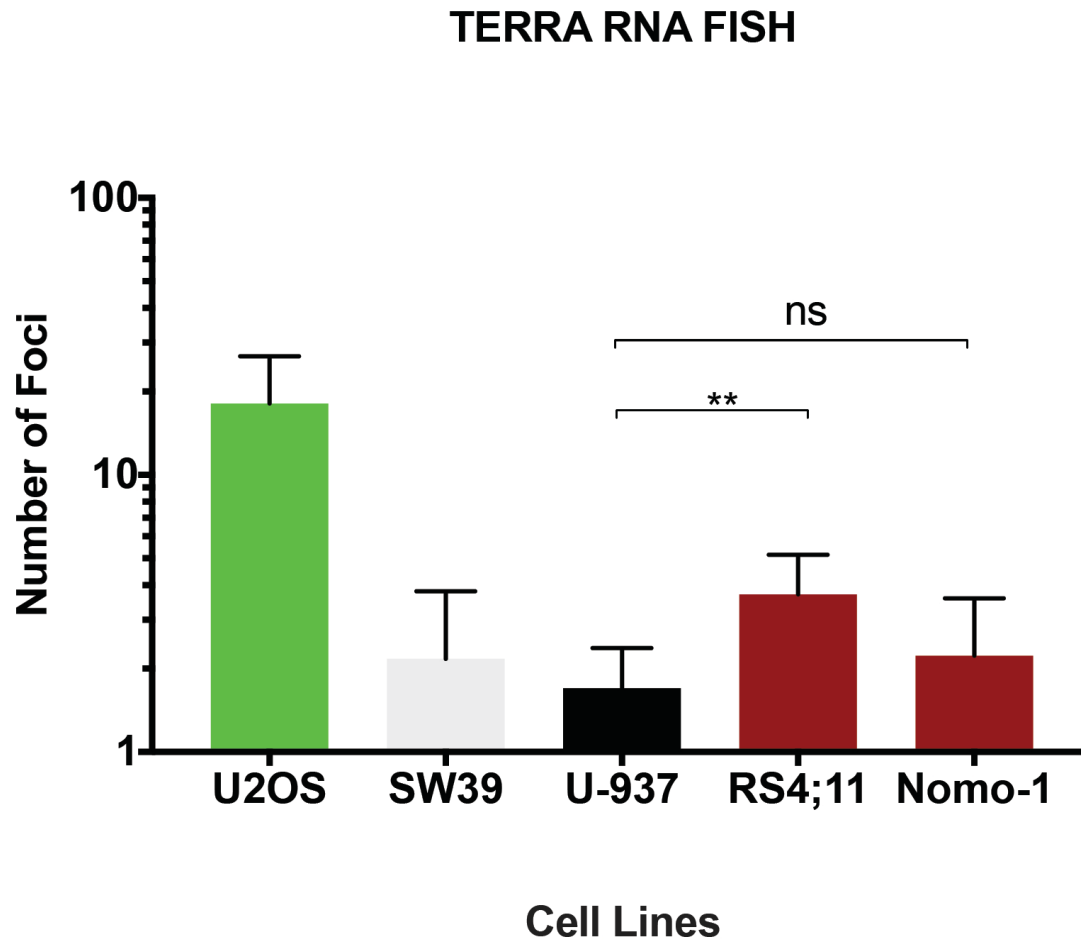


Figure 9. Increased TERRA in MLLr Leukaemia Cell line RS4;11.

Quantification of RNA FISH images using Cell Profiler 3. Average nuclei found per image was 14 in three biological replicates. Graph representing the mean number of foci per cell line, represented in log 10 scale. ** $p < 0.001$. Paired t-test.

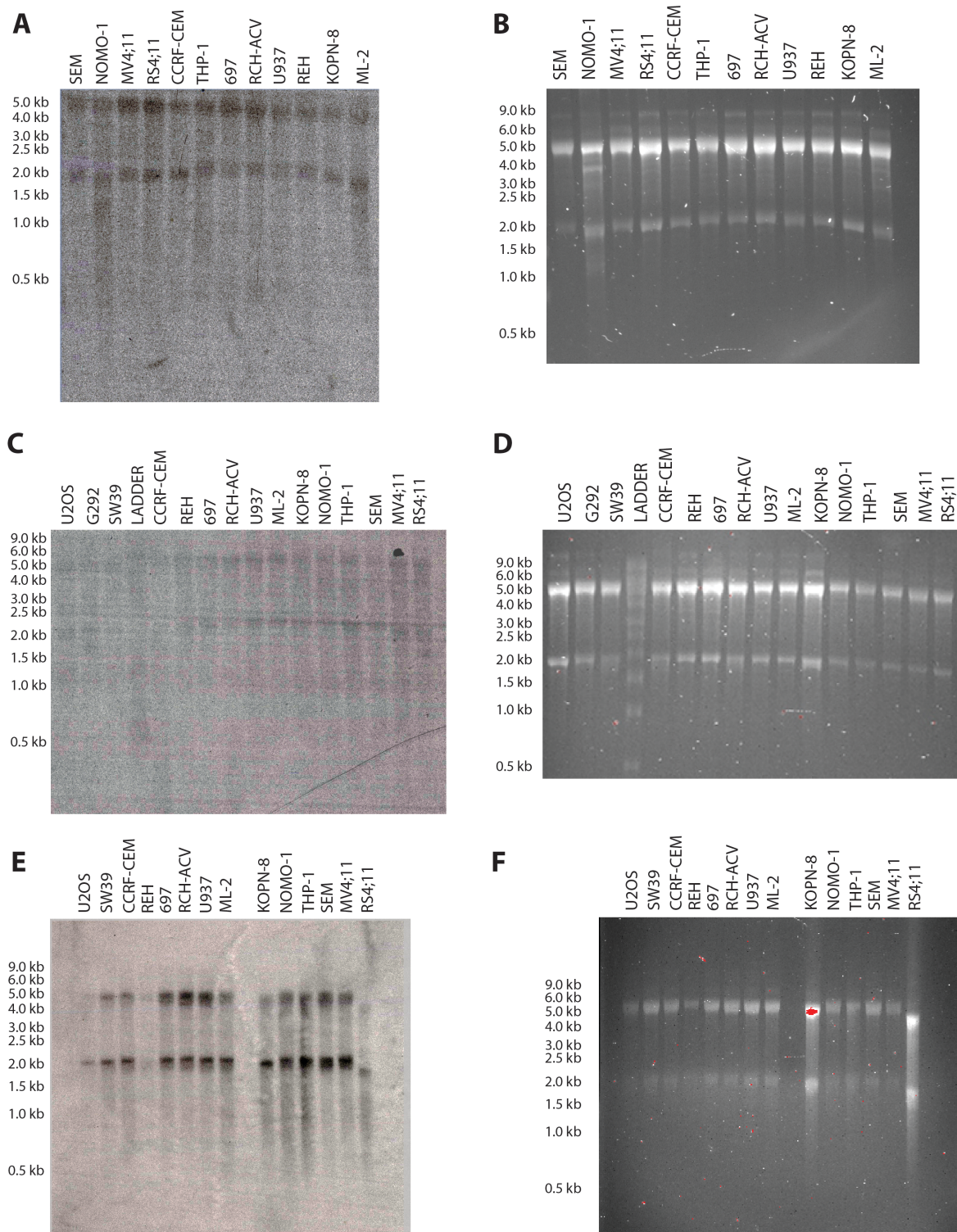


Figure 10. Increased TERRA in MLLr Leukaemia Cell lines.

A, C & E Northern blot membranes of three biological replicates that were used for quantification. **B, D & F.** Total RNA loading control prior to transfer onto membranes.

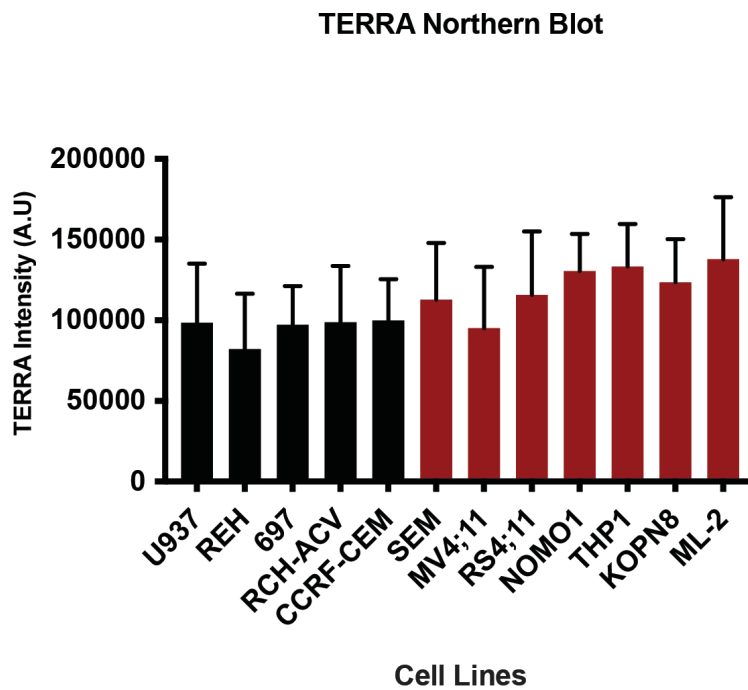
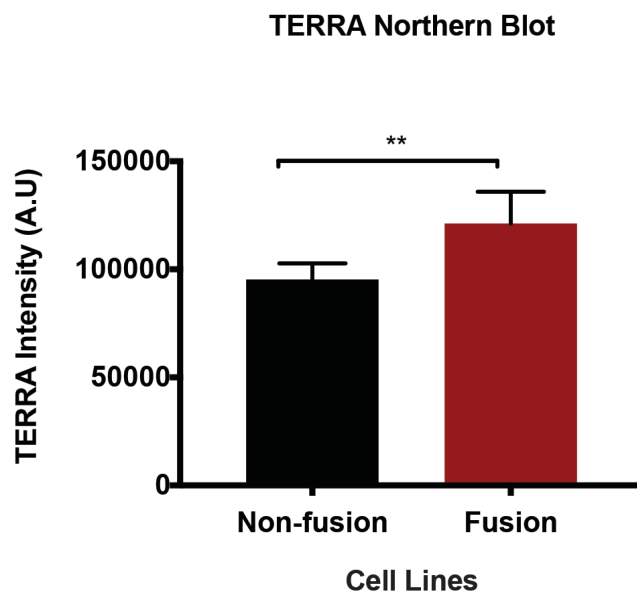
A**B**

Figure 11. Increased TERRA in MLLr Leukaemia Cell lines.

Northern Blot analysis of all the leukaemia cell lines in three biological replicates. The membranes were quantified using Fiji and the intensities were plotted. **A.** Mean TERRA intensity of cell lines. **B.** Mean TERRA intensity of the cell lines categorized as non-fusion vs fusion. ** $p < 0.003$. Welch's t-test.

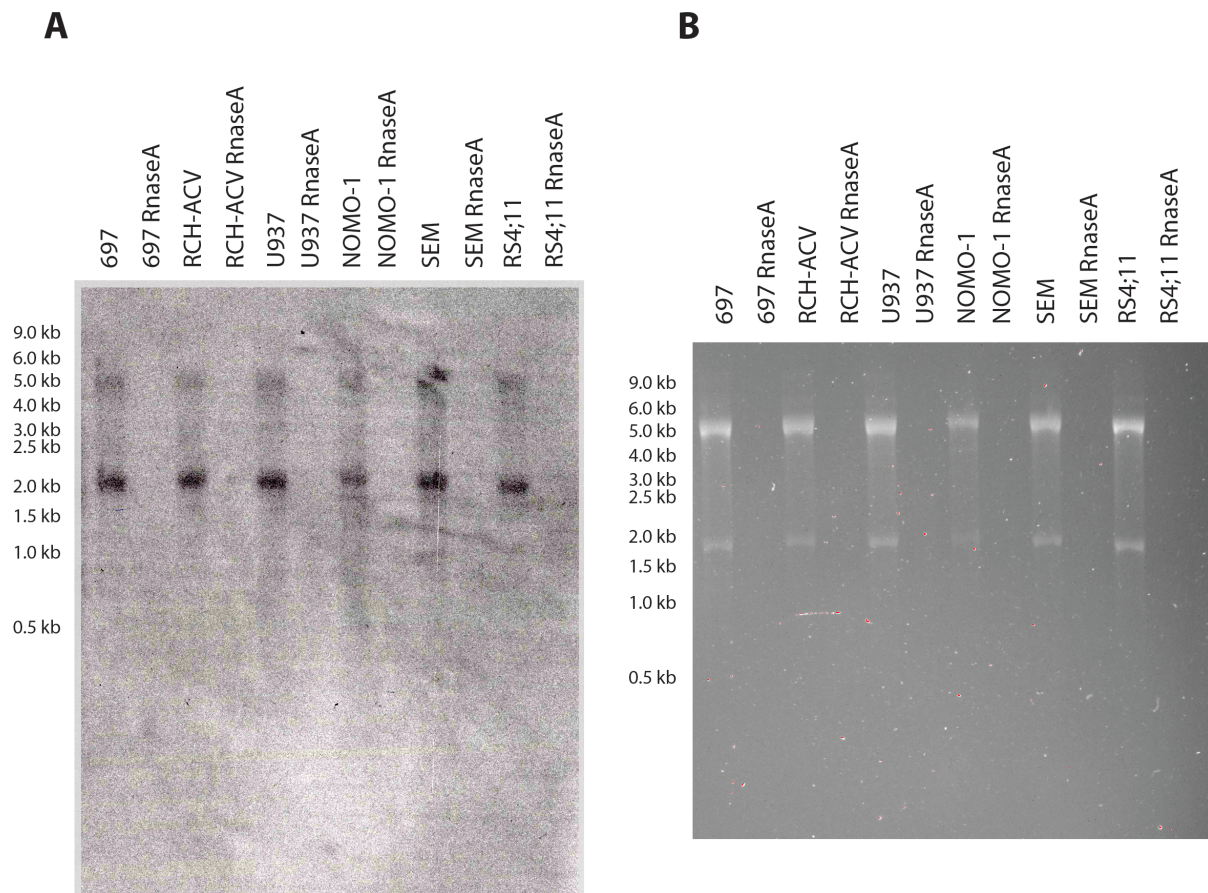


Figure 12. TERRA Specific Signal in Northern Blots.

A. RNA was treated with RnaseA and visualised by northern blot analysis. **B.** Total RNA loading control prior to transfer onto membranes.

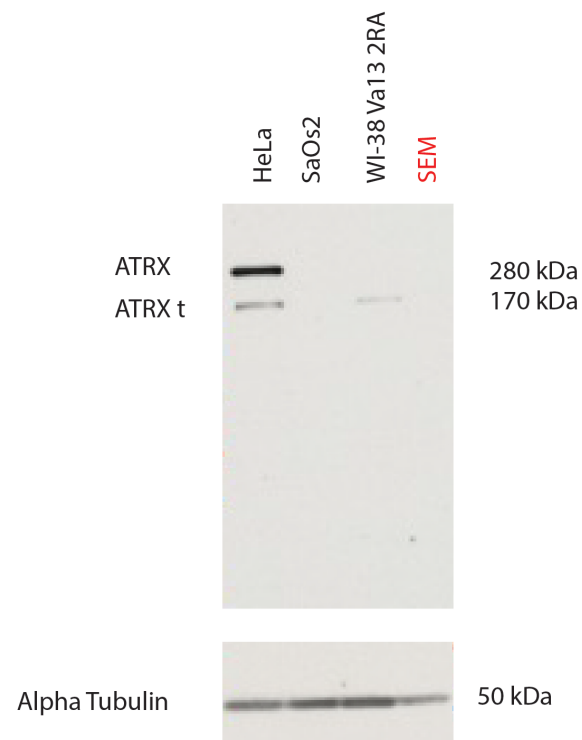
Reduction of Tumour Suppressor Protein ATRX in MLLr cell lines

We next sought to investigate other markers of the ALT pathway in MLLr cells. As ATRX loss is strongly correlated with this pathway, so we questioned the status of ATRX in our panel of cell lines. The presence of ATRX was assayed by western blot analysis in our positive cell line (HeLa), negative cell lines (SaOS2 and WI-38 Va.13 2RA) and MLL-AF4 cell line (SEM). We noticed that in SEM cells, there was a substantial reduction of full length and truncated ATRX protein (Figure 13A). We expanded our cell line panel to include several leukaemia cell lines and found that in two biological replicates, cell lines that had an MLL translocation had undetectable levels of ATRX (Figures 13B & 14).

Next, we sought to look at the intracellular levels of ATRX in our cell lines by flow cytometry. This method provided an easier and quicker means to check the status of ATRX in future MLLr patient samples while giving us more information on the phenotypic profile of the leukaemia. We had to optimize the primary antibody concentration, so we initially took four samples: HeLa and U937, positive controls for ATRX, and SaOS2 and NOMO-1, negative controls for ATRX based on the western blot. We tested three dilutions of antibody for these samples, 1:25, 1:50, and 1:100. It appeared that 1:25 gave us the best separation from our no antibody control, so we chose this dilution to work with going forward (Figure 15).

Thereafter, we looked at two methods of permeabilization (digitonin and methanol) as some antibodies do not work well after methanol permeabilization. According to our data digitonin permeabilization more closely recapitulated our Western blot data (Figure 16A-D; n=2), although methanol was not significantly worse (Figure 17 A-D; n=2). We show with four biological replicates that ATRX was reduced significantly in our MLLr cell lines.

A



B

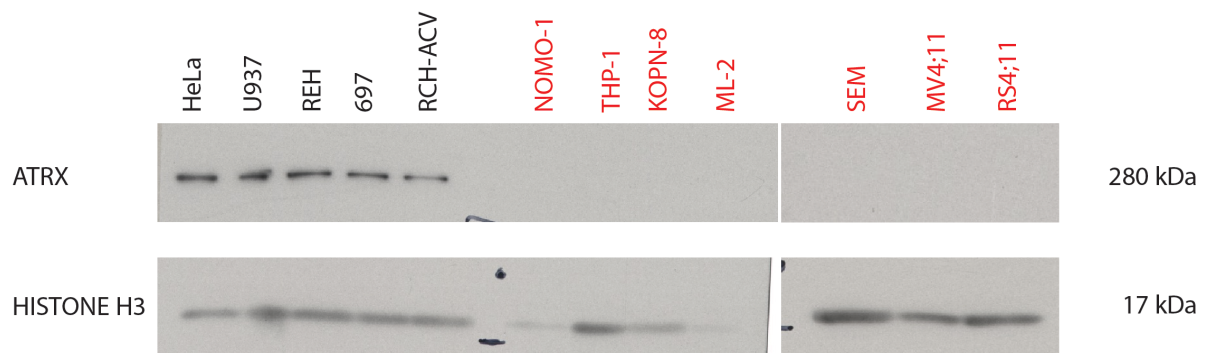


Figure 13. Loss of Tumour Suppressor ATRX in MLLr Cell lines.

A. Western Blot of ATRX positive cell line (HeLa), ATRX negative cell lines (SaOS2 and WI-38 Va13 2RA) and MLL-AF4 cell line SEM with α -tubulin as a loading control. **B.** Western Blot of ATRX in HeLa, and leukaemia cell lines (**non-fusion** vs **fusion**) with histone H3 as a loading control.

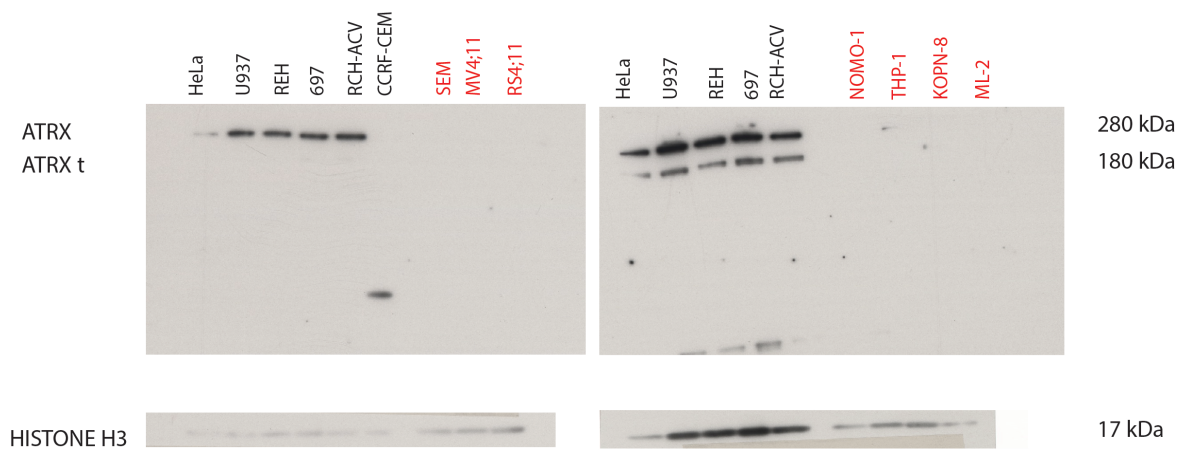


Figure 14. Loss of Tumour Suppressor ATRX in MLLr Cell lines.

Second biological replicate for western blot of ATRX in HeLa, and leukaemia cell lines (**non-fusion** vs **fusion**) with histone H3 as a loading control.

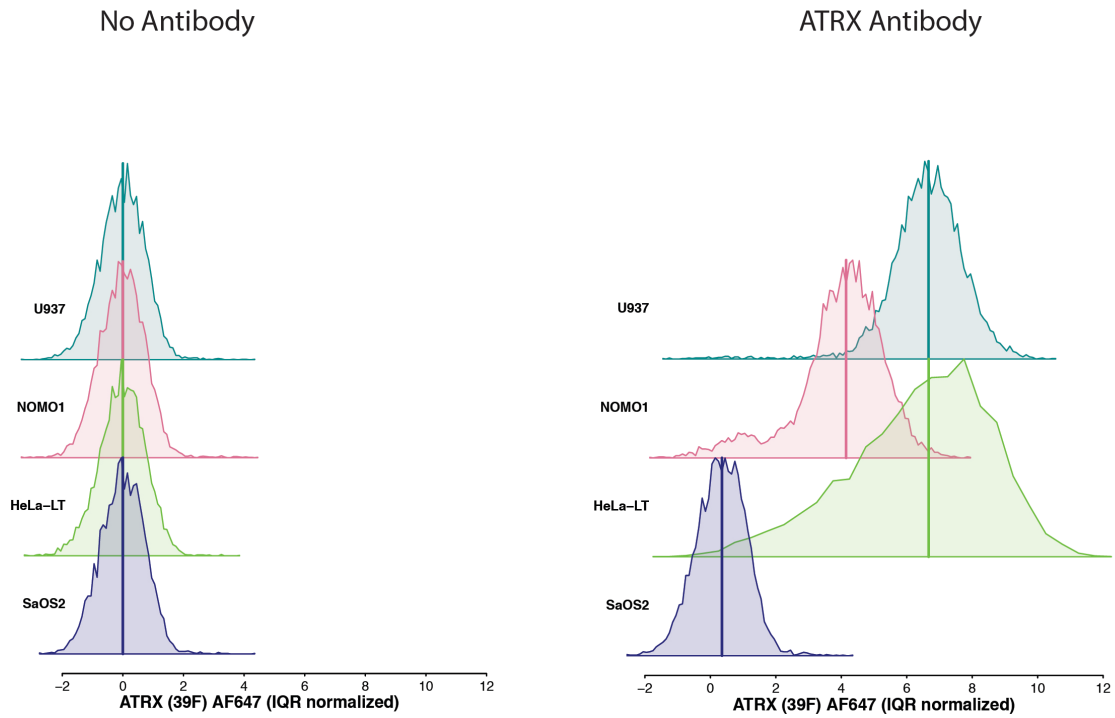


Figure 15. Low Intracellular ATRX in MLLr and ATRX Negative Cell lines (Optimization).

Distribution of ATRX protein using intracellular flow in HeLa and U937 as positive controls and SaOS2 and NOMO-1 as negative controls. The image represents arbitrary ATRX levels (x-axis) in a distribution of cells (y-axis). IQR normalization for each cell line was performed by subtracting the fluorescence of ATRX signal from the median fluorescence of the no antibody control then dividing this value by the interquartile range of the no antibody control (Knapp et al., 2017).

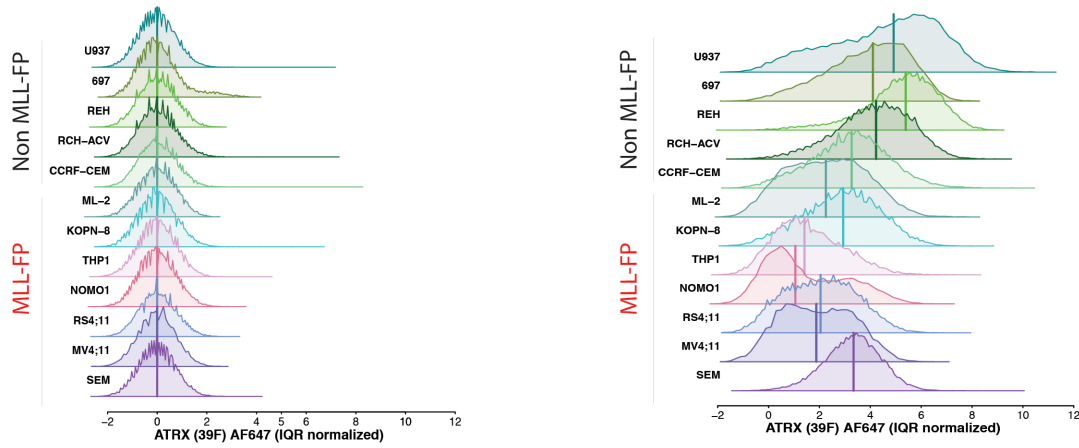
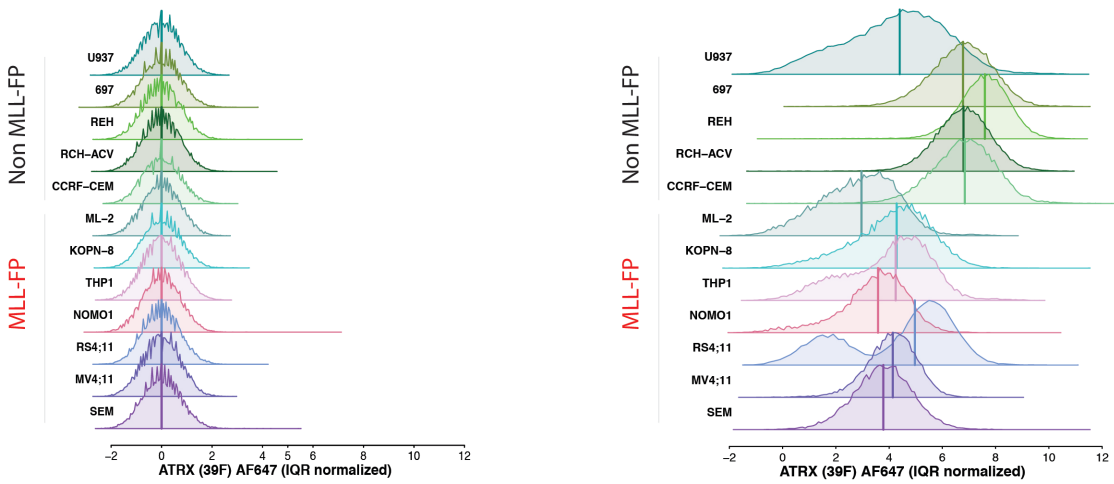
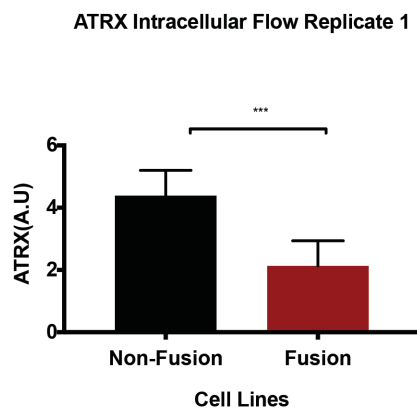
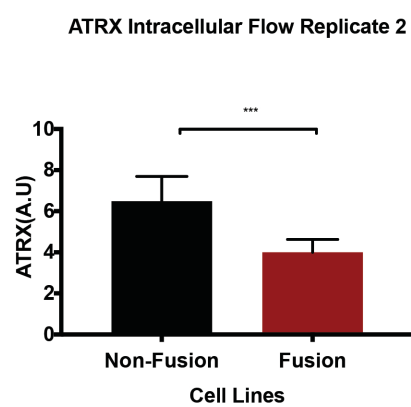
A**B****C****D**

Figure 16. Low Intracellular Levels of ATRX in MLLr Cell lines.

A&B. Intracellular flow for ATRX in the expanded panel for two biological replicates permeabilized with digitonin. **C&D.** The median fluorescence of the cell lines was categorized into **non-fusion** vs **fusion**. *** $p < 0.0009$. Unpaired t-test. IQR normalization for each cell line was performed by subtracting the fluorescence of ATRX signal from the median fluorescence of the no antibody control then dividing this value by the interquartile range of the no antibody control (Knapp et al., 2017).

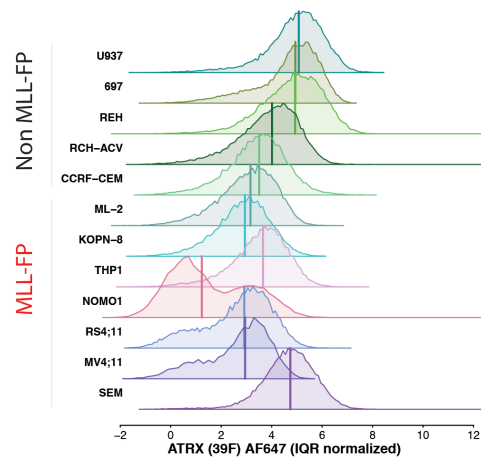
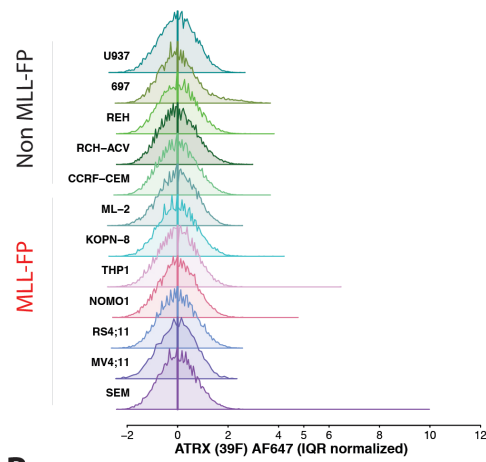
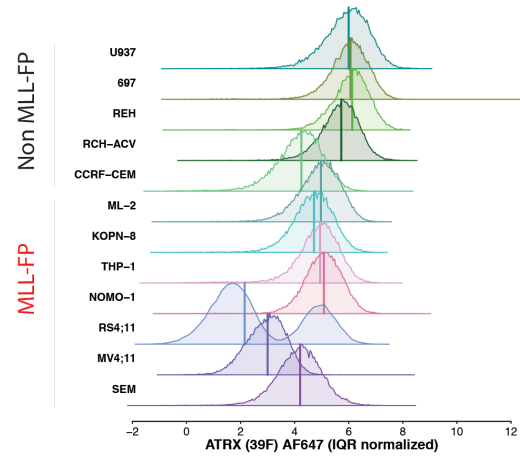
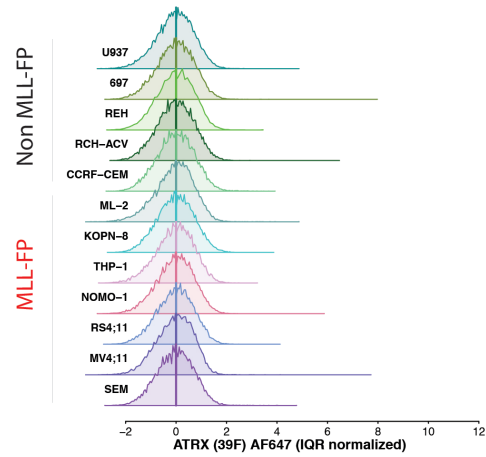
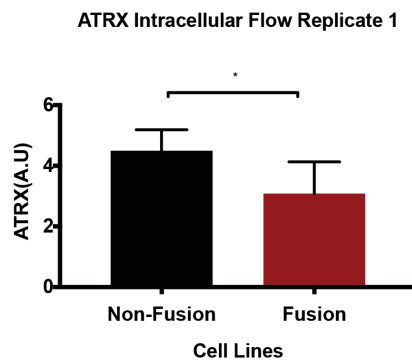
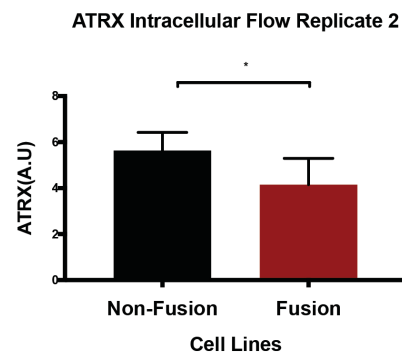
A**B****C****D**

Figure 17. Low Intracellular Levels of ATRX in MLLr Cell lines.

A&B. Intracellular flow for ATRX in the expanded panel for two biological replicates permeabilized with methanol. **C&D.** The median fluorescence of the cell lines was categorized into **non-fusion** vs **fusion**. * $p < 0.03$. Unpaired t-test. IQR normalization for each cell line was performed by subtracting the fluorescence of ATRX signal from the median fluorescence of the no antibody control then dividing this value by the interquartile range of the no antibody control (Knapp et al., 2017).

ATRX RNA Status in MLLr Cell lines

After seeing the reduction of ATRX protein we questioned if this was due to lower mRNA expression. Therefore, we extracted RNA from the leukaemia cell lines and processed it into cDNA which was then used for qPCR. When the ATRX RNA was normalized to the housekeeping gene YWHAZ we observed that there was no significant change of mRNA expression from cell lines with and without the translocation (Figure 18). This finding was validated using MLL-AF4 patient RNA-sequencing data which was kindly shared by Andi Roy and David Knapp (Figure 19). Therefore, it appears that the reduction of ATRX protein is not correlated to the mRNA expression levels and loss of ATRX protein levels in MLLr samples is not simply due to transcriptional regulation of the gene.

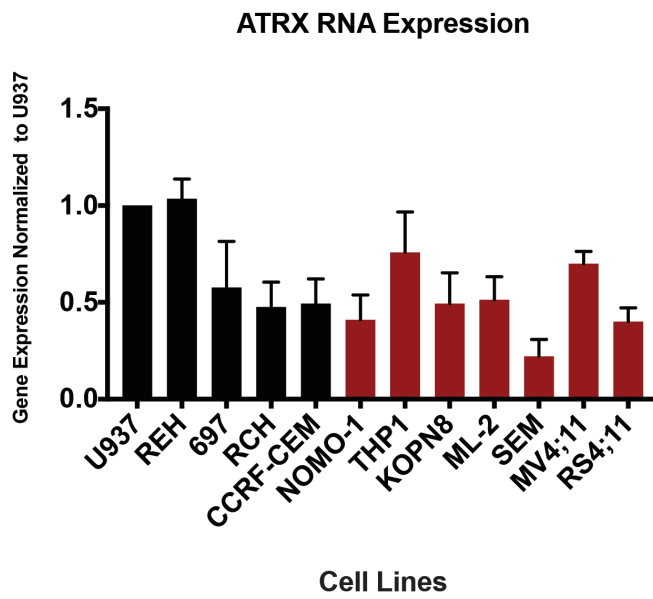
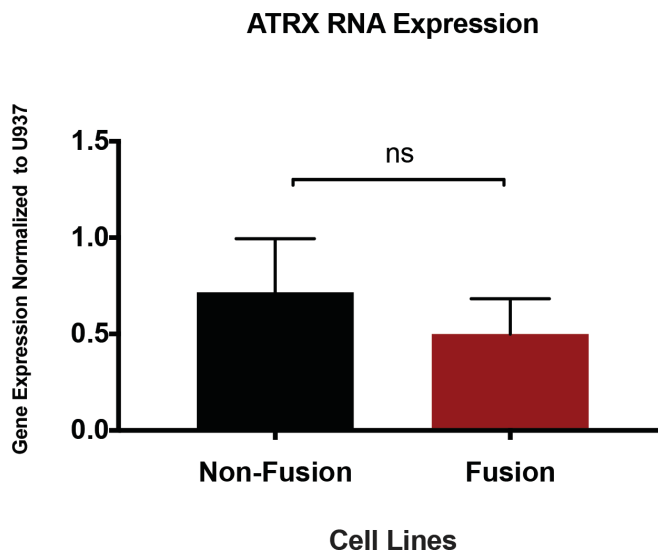
A**B**

Figure 18. No Significant Change in ATRX RNA Expression from Non-Fusion vs Fusion Cell lines.

RT-PCR of RNA extracted from the leukaemia cell lines (**non-fusion** vs **fusion**) normalized to housekeeping gene YWHAZ and then to U-937 in three biological replicates. **A**. The mean gene expression per cell line. **B**. The mean gene expression categorized as non-fusion and fusion. ns. Welch's t-test.

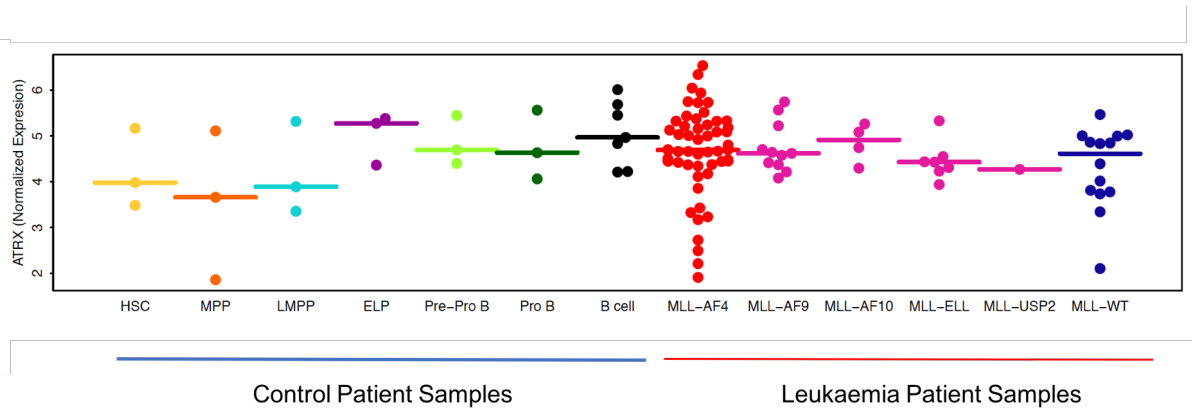


Figure 19. No Change in ATRX RNA Expression from MLL-AF4 Patient RNA-Sequence Data.

RNA from MLL-FP patients were analysed for the presence of ATRX transcripts in comparison to normal haematopoietic progenitors by A. Roy and D. Knapp.

Presence of ALT hallmarks in MLLr cell lines

As a result of the striking observations of reduced ATRX protein and general increase of TERRA in MLLr cell lines we proceeded to look for more hallmarks of ALT. We subsequently assayed for the presence of C-circles in our MLLr cell lines. We performed C-circle amplification of ALT positive cell lines (SaOS2 and WI-38 Va13 2RA) and MLL-AF4 cell line (SEM) and blotted the DNA onto a membrane, which was then probed for telomeric sequence (Figure 20). The results suggested the possibility of C-circles in SEM, however not as robust as SaOS2 or WI-38.

We also performed qPCR using our C-circle amplified DNA as it was more sensitive than radioactivity and could provide a better detection of low number of C-circles (Figure 21). In our qPCR assay we included an ALT positive cell line (U2OS), an ALT negative cell line (SW39), MLLr cell lines SEM (MLL-AF4), NOMO-1 (MLL-AF9), RS4;11 (MLL-AF4), and non-MLLr cell lines (U-937, REH, and 697). For each sample that was amplified by phi polymerase we had a control with no phi polymerase. This served to establish the background signal. We observed that there were a high number of C-circles in U2OS and low number in SW39, as expected for our controls. C-circles were present in our MLLr cell line RS4;11 but not in NOMO-1 and SEM. In addition, U937, 697 and REH displayed C-circle expression. Together, this data indicates that there may be some detectable C-circles in MLLr samples but the presence is not MLL-FP specific.

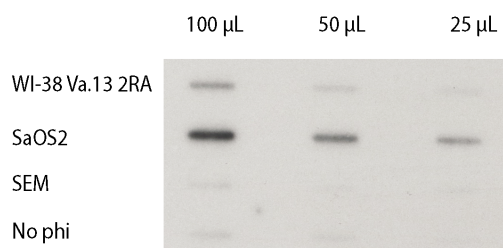
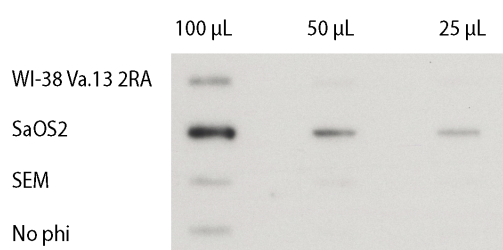
A**B**

Figure 20. Presence of C-circles in SEM.

DNA blot of extracted DNA from C-circle positive cell lines (SaOS2 and WI-38 Va13 2RA), MLLr cell line SEM (MLL-AF4) and a no phi control using SaOS2 extracted DNA from two biological replicates (**A&B**). 25-100 μ L of ChIP DNA was loaded onto the membrane. The blots were probed with 32 P-end labelled telomere oligo.

C-circle Quantification qPCR

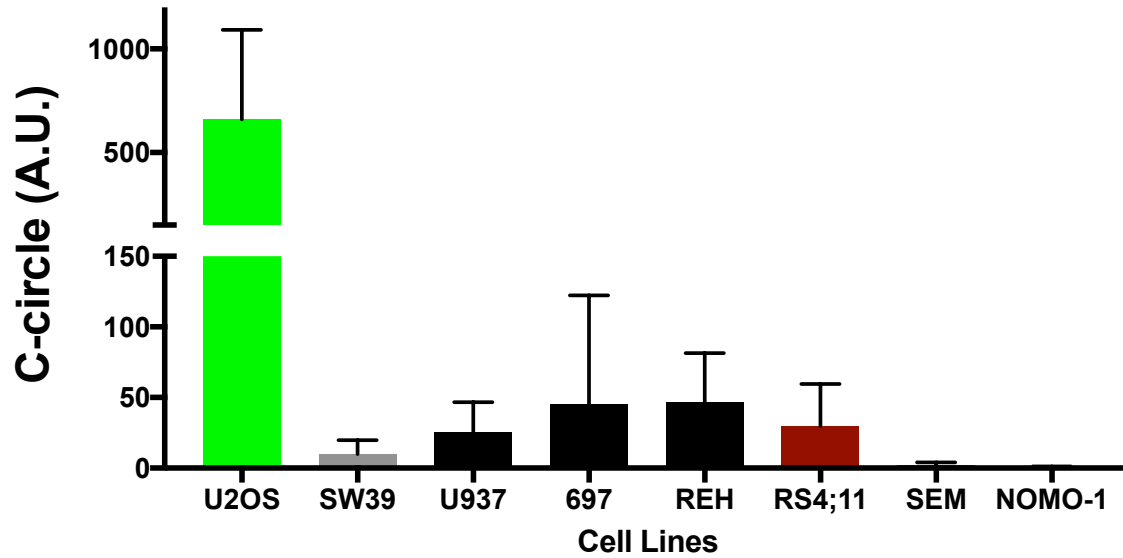


Figure 21. Presence of C-circles in Non-Fusion and Fusion Cell lines.

C-circle qPCR of extracted DNA from **C-circle positive** cell line (U2OS), **C-circle negative** cell line (SW39), **non-fusion** cell lines (U937, REH, 697), **MLL-AF4** cell lines (RS4;11, SEM) and **MLL-AF9** cell line (NOMO-1) in three biological replicates.

Discussion

MLL^{WT} and/or MLLr Weakly Binds to Telomeres

Due to the previous report (Caslini et al., 2009) indicating MLL^{WT} binds at telomeres and the lack of studies in this area we were drawn to explore the function of MLL at the telomeres. To validate the previously published results we repeated the ChIP and DNA blot albeit with a higher cell number (10 million cells) and a different MLL antibody than in the previous study. Even with higher cell numbers, our dot blot signal was quite weak, whereas Caslini and co-workers reported a strikingly strong telomeric signal for having only one million cells. One possibility for this difference in signal between our group and past work could be due to the specific antibody used. Caslini et al generated their own polyclonal antibodies to specific epitopes of the N-terminus of MLL. It is possible that the antibody we used recognized different epitopes, as a result we did not pull down as much chromatin as they did. The weak signal (relative to TRF1 binding and MLL binding at canonical target genes) from our ChIP qPCR for MLL binding at telomeric sequences did however validate our dot blot signal.

Despite our results, we were unsure whether MLL was binding at the sub-telomere or telomere body. We believed there was a possibility that MLL may bind at sub-telomeres due to the presence of CpGs in sub-telomeres. MLL^{WT} and MLL-FP have a CXXC domain that can bind to unmethylated CpGs, therefore this suggested a possible mechanism for how MLL could bind to the sub-telomere. To explore this mechanism, we ran a ChIP-qPCR using primers designed for various chromosome sub-telomeres. Our results at the telomere were consistently above background whereas at the sub-telomere we observed signal comparable to the background therefore suggesting that MLL-N weakly binds at telomeres and not the sub-telomeres. In addition, we noticed that the signal at the sub-telomere was higher the closer the primers were to the telomere body. This further suggests that maybe MLL is binding somewhere near the boundary of the sub-telomere and telomere body.

To this end, we sought to explore if the wild type or fusion protein was binding at the telomere, therefore we knocked down MLL-AF4 or MLL^{WT} in SEM cells with siRNA. We expected to see a decrease in signal at the telomere from the siRNA treated cells. However, we saw no change in signal. This suggested to us that a knock down may not be sufficient to see a change in binding or that there could be compensation from the other allele during each knockdown. Our data in ML-2 cells (an MLL-AF6 cell line with no wild type MLL present) suggests that MLL-FP may bind to telomeres as its binding patterns in the sub-telomeric ChIP qPCR were similar to MLL-N binding in SEM and RS4;11 but we were unable to verify this for MLL-AF4.

Taken together, our results suggest that MLL^{WT} and possibly MLL-FPs bind to telomeres but the binding is relatively weak compared to known binding sites.

Increase in telomere transcript (TERRA) in MLLr cell lines

Previous publications suggest that excessive amounts of RNA have the potential to hybridize with DNA by displacing one of the strands. These RNA: DNA hybrids (R-loops) can cause complications during DNA replication through replication fork stalling and collapse, which ultimately result in double strand breaks. Caslini et al demonstrated that MLL^{WT} had a role in telomeric transcription through the deposition of modifications associated with an active gene transcription. Therefore, observing that MLL-N weakly binds at the telomeres, we questioned whether telomeric transcripts would be highly expressed in cell lines that harbour an MLL translocation as opposed to no MLL translocations. We discovered that in cell lines that have an MLL-FP there was generally a higher expression of TERRA. These results suggest to us that MLL translocations might be driving an aberrant expression of TERRA which could result in R-loop formation at the telomere and genome instability. RNaseH1 was shown to resolve RNA:DNA hybrids at telomeres (Arora et al., 2014). To assay for R-loops we could treat our

cell lines with RNaseH1 and identify TERRA-telomere co-localizations by immuno-FISH. In addition, higher TERRA expression is associated with the ALT pathway therefore we believe this pathway could be active in MLLr leukaemia.

Reduction of Tumour Suppressor Protein ATRX in MLLr cell lines

The reduction of ATRX in our MLLr cell lines is an interesting and exciting observation because ATRX loss has been implicated in the ALT pathway. Our data suggested that MLL translocations correlated with an increase of TERRA which previous studies showed can result in the generation of DNA double strand breaks, a requirement of the ALT pathway (Arora et al., 2014). ATRX has a role in resolving R-loops (Nguyen et al., 2017) and it has been recently shown that introducing ATRX back into ALT cancers reversed the hallmarks of ALT (Clynes et al., 2015b). More importantly ATRX also has a role in the repair of DNA double strand breaks through either non-homologous end joining (NHEJ) or homologous recombination (HR) (Koschmann et al., 2016b; Juhász et al., 2018). Notably, these two observations are mutually exclusive and members of our group are investigating which pathway is controlled by ATRX. However, in the case that ATRX repairs DNA via NHEJ, it is suggested that ATRX loss favours DNA repair by HR, thereby supporting the initiation of the ALT pathway. Conversely, if ATRX directs DNA repair through HR, it is more difficult to explain how the HR pathway is active in ALT cancers without ATRX.

Evidently, ATRX acts as a tumour suppressor through some mechanism of DNA repair and replication, and loss of this protein can promote the recombination based method of telomere lengthening. Therefore, observing more TERRA and significantly reduced ATRX protein in our MLLr cell lines suggests that MLL translocations could be a key factor in the selection of the ALT pathway.

Abnormalities in the ATRX gene generally occur in cancers such as neuroblastoma, pancreatic neuro-endocrine and osteosarcomas (Koschmann et al., 2016b). However, recent evidence reveals ATRX anomalies in patients with chronic lymphoblastic leukaemia (CLL), paediatric acute lymphoblastic leukaemia (p-ALL) and FLT3 subset of acute myeloid leukaemia (FLT3-AML) (Amin et al., 2016; Ding et al., 2017; Lacayo et al., 2004). This suggests that ATRX impairment maybe beneficial to leukaemia especially in the MLLr driven subset.

MLL rearrangements could be driving the reduction of ATRX protein. It is possible that MLL-FP destabilize ATRX protein through the introduction of post-translational modifications (i.e. phosphorylation, ubiquitylation). Alternatively, MLL-FPs could be upregulating microRNAs that target the 3'UTR of ATRX mRNA.

In the case that MLL-FPs are not the cause for low ATRX protein, there is a possibility that ATRX could be generating MLL translocations.

Interestingly, patients that receive etoposide (topoisomerase II inhibitor) treatments have been shown to develop therapy related leukaemia (Andersen et al., 2001; Libura et al., 2005; Pui and Relling, 2000). Studies have indicated that etoposide treatments induce DNA double strand breaks in the break point cluster region of MLL (Canela et al., 2017). Other members of our group generated data indicating that ATRX and topoisomerase II interact with each other. We propose that ATRX could be directing topoisomerase II to sites of DNA torsional stress to relieve the tension and thereby resolving replication fork stalling. In the case where we have reduced ATRX we hypothesize that topoisomerase can no longer be brought to these sites of tension thereby resulting in replication fork collapse and double strand breaks. More importantly, because it has been demonstrated that topoisomerase II has cleavage sites in the break point region of MLL (Stanulla et al., 1997), we believe reduced ATRX maybe generating MLL translocations.

Presence of ALT hallmarks in MLLr cell lines

ATRX loss and general increase in TERRA are indicators of the ALT pathway, therefore it is key to determine whether our MLLr cell lines are immortalized via the ALT pathway. In addition to these two characteristics, ALT cancers can also be identified by the presence/frequency of APBs, heterogeneous telomere lengths and C-circles. Unfortunately, due to the technical issues with antibody and probe optimization, we were unable to generate usable data on the presence of APBs and telomere lengths. However, the presence of C-circles are considered to be a major indicator of the presence of ALT. In the MLL-AF4 cell line RS4;11 we detected low levels of C-circles, although we could not detect C-circles in the MLL-AF4 SEM and the MLL-AF9 cell line NOMO-1. The presence of C-circles was identified in CML (Samassekou et al., 2013) thereby supporting the possibility of ALT in leukaemia as we observed.

Our data also suggests that our ATRX positive cell lines U937, 697 and REH have C-circles. It is possible these cell lines are ALT positive but retain full ATRX protein expression (Lovejoy et al., 2012) or that our qPCR assay is detecting long endogenous telomeres. If the C-circle signal holds true in RS4;11 we can suggest that it is much lower than U2OS simply because ALT positive cancers do not display all characteristics strongly, including some that have extremely low levels of C-circles (Lovejoy et al., 2012).

Our data indicates that there is no relationship between non-fusion and fusion cell lines in C-circle expression. To definitively prove that the ALT is not active in these fusion leukaemias additional assays may be needed.

Conclusion/Future Directions

Our aims going forward are to conclude whether MLLr leukaemia lengthen their telomeres via the ALT pathway and to establish a role for ATRX in MLLr leukaemia.

To achieve our first goal, we will need to optimize the APB, telomere length and t-SCE assay for our leukaemia cell lines. In addition, because the ALT pathway has been shown to coexist with an active telomerase pathway (Perrem et al., 2001) we need to establish whether our cell lines have telomerase activated. This can be verified by performing a telomerase repeated amplification protocol (TRAP) (Fajkus, 2006; Mender and Shay, 2015).

To accomplish our second goal, we first need to sequence our cell lines to identify any mutations occurring in the ATRX gene. Second, we need to identify the status of ATRX in MLLr patient samples. We are currently in the process of optimizing several surface markers with ATRX in CD34- cord blood. We want to include surface markers so we can isolate the leukaemic blasts in the heterogeneous population and then observe ATRX protein expression. In this optimization, we are going to stain cord blood cells with lineage specific surface markers, permeabilize with either digitonin or methanol and then stain for ATRX. Once we know the protocol works well we will analyse MLL-AF4 leukaemia patient samples. Hopefully the results recapitulate that ATRX is reduced in MLLr leukaemia.

Finally, to investigate if ATRX causes MLL translocations we intend on knocking out ATRX via CRISPR-CAS9 in CD34+ cord blood cells. Subsequently we will treat these cells with etoposide and assay for the presence of MLL translocations via MLL break apart probes.

Although there are several questions remaining with this project, we came across very interesting data that can hopefully shed light on the nature of ALT cancers and MLLr leukaemia. Understanding MLL's role in telomere maintenance is attractive as it clearly identified more pathways affected in MLLr leukaemia (TERRA upregulation, ATRX loss, and the presence of C-circles). It would be beneficial to pursue this area as it could identify key

mechanisms involved in these devastating poor prognosis cancers which could potentially be targeted therapeutically.

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