Epigenetic regulation of TERRA transcription and metacyclogenesis by base J in *Leishmania major*.

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ABSTRACT

The hyper-modified DNA base J helps control termination of Pol II transcription at polycistronic transcription units (PTUs) in *T. brucei* and *L. major*, allowing epigenetic control of gene expression. The Telomere Repeat-containing RNA (TERRA) is synthesized in *T. brucei* by Pol I readthrough transcription of a telomeric PTU. While little is understood regarding TERRA synthesis and function, the hyper-modified DNA base J is highly enriched at telomeres in *L. major* promastigotes. We now show that TERRA is synthesized by Pol II in *L. major* and loss of base J leads to increased TERRA. For at least one site, the increased TERRA is by Pol II readthrough transcription from an adjacent PTU. Furthermore, Pol II readthrough defects and increased TERRA correlate with increased differentiation of promastigotes to the infectious metacyclic life stage and decreased cell viability. These results help explain the essential nature of base J in *Leishmania* and provide insight regarding epigenetic control of coding and non-coding RNA expression and parasite development during the life cycle of *L. major*.

INTRODUCTION

Trypanosomatids are a group of early-diverged eukaryotes responsible for multiple diseases including African sleeping sickness and leishmaniasis. The protozoan parasites, which include *Trypanosoma brucei* and *Leishmania major*, progress through life stages by cycling between an insect vector and a mammalian host. Thus, gene expression control is needed for the parasites to rapidly adapt to fluctuating conditions in their environment. Unlike most eukaryotes, the genome of trypanosomatids is composed of genes ordered into long polycistronic transcription units (PTUs) that are transcribed from a single promoter at the 5' end of the cluster (1-4). The arrangement of functionally unrelated genes into co-transcribed gene clusters has made it difficult to envision regulated gene expression at the level of RNA polymerase II (Pol II) transcription in trypanosomatids. However, recent work characterizing the mechanism and epigenetic control of Pol II transcription termination in *T. brucei* and *L. major* demonstrated a mechanism for control of individual gene expression within a polycistronic system (5-9).

Termination of Pol II transcription in humans and yeast is by the PTW/PP1 complex that includes protein phosphatase 1 (PP1), PP1 interactive-regulatory protein (PNUTS) and the DNA binding protein Tox4 (10-17). Critical to this process is PP1-dependent dephosphorylation of Pol II leading to decreased Pol II elongation, enhancing the capture by the torpedo RNA exonuclease allowing Pol II dissociation and termination (10,12,17,18). A similar PJW/PP1 complex was identified in *T. brucei* and *L. major* and shown to be involved in Pol II termination at the end of PTUs genome-wide via PP1-PNUTS mediated dephosphorylation of Pol II (5,7,19,20). PP1-PNUTS is presumably recruited to termination sites via association with the base J-DNA binding protein, JBP3 (5,19). Base J consists of a glucosylated thymidine (21) found in the nuclear DNA of trypanosomatids at Pol II transcription termination sites (22,23) and shown to represent an epigenetic mark involved in Pol II transcription termination in *T. brucei* and *L. major* (8,9,23-26). J biosynthesis occurs through the hydroxylation of thymidine in DNA by the thymidine hydroxylase JBP1 or JBP2 (27), forming hydroxymethyluridine, followed by the transfer of a glucose by the glucosyltransferase enzyme JGT (28-31). Addition of dimethyloxalylglycine (DMOG) to the growth medium inhibits hydroxylase activity of JBP1/2 and thus, decreases base J levels in the parasite genome (27). While base J is not essential in *T. brucei* (22,32), it appears to be essential in *Leishmania* since JBP1 KO cells are not viable (33) and reduction of base J using DMOG can lead to a severe growth phenotype (9). Reduction of J in *Leishmania* and *T. brucei* using genetic KO of JBPs or via DMOG led to readthrough transcription at termination sites genome-wide, identical to termination defects seen in PP1-PNUTS complex mutants linking base J with dephosphorylation of Pol II, suggesting a critical role for J in transcription termination (8,23-26). Histone variant H3V co-localizes with J at termination sites in *T. brucei* (22) and *L. major* (9) and both epigenetic marks may independently function to promote termination, such that the combined loss of J and H3V results in a synergistic increase in read through transcription (8,9,25,26). For several PTUs in *T. brucei* and *L. major*, J/H3V are found to promote Pol II termination prior to the end of the gene cluster, leading to silencing of the downstream genes (8,9,24). Loss of J/H3V, or components of the PP1-PNUTS complex results in readthrough transcription and de-repression of the

downstream genes (5-9,19). This has suggested 'premature' termination of Pol II transcription within a trypanosomatid PTU as a novel mechanism of regulating the transcription of individual genes. Genes regulated by Pol II termination include genes that are developmentally regulated and code for proteins involved in optimal growth during infection of the mammalian host (9,26). The de-repression of specific genes by Pol II readthrough in *L. major*, and the associated *in vitro* growth defects, is thought to explain the essential nature of J in Leishmania (9).

Base J synthesis is developmentally regulated in *T. brucei*, *T, cruzi* and *Leishmania* (34-37). However, *T. brucei* is unique where J is restricted to a single life stage; the infective mammalian bloodstream form where approximately 50% of base J localizes to telomeres (36,38). The most telomere-proximal PTU in some chromosomes of *T. brucei* represent the bloodstream form VSG expression site (VSG ES) involved in regulated expression of the trypanosome surface antigen gene, VSG. Allelic exclusion of VSG expression and periodic switching of the expressed allele allows trypanosomes to evade the host immune system, in a process known as antigenic variation. The VSG ESs are transcribed by Pol I. Only one Pol I promoter is transcriptionally active at a time, while the others are repressed, allowing monoallelic VSG expression. J is found in the silent VSG ESs and is highly enriched in the telomeric repeats, replacing ~14% of T in $(CCCTAA)_n$ and ~36% in $(TTAGGG)_n$, and repeats upstream the Pol I promoter (35,36). The Pol II readthrough defects seen in base J/H3V mutants (8,25,26) and PP1-PNUTS complex mutants (5,7) are associated with derepression of the silent VSG ES. Rather than an independent function of base J controlling Pol I transcription of the telomeric VSG ESs, the TbPP1 KD indicates Pol II termination defects at the chromosome core leads to Pol II extending into the Pol I transcribed telomeric PTUs (7). These results suggest a key function of base J in BS form *T. brucei* is to control pervasive Pol II transcription and maintain monoallelic expression of VSG.

Monoallelic control of VSG expression extends to the downstream telomeric repeat that is transcribed into telomere repeat-containing RNA (TERRA), which is prone to form RNA:DNA hybrids (R-loops) with the telomeric DNA (telomere R-loops or TRLs)(39). Studies in mammalian cells and yeast indicate TERRA (and TRLs) as a key player of telomere biology and genome integrity (39,40). In *T. brucei*, TERRA has been shown to form TRLs (41) and higher levels of TERRA and TRLs were associated with higher levels of telomeric and subtelomeric double strand breaks and enhanced rate of recombination based VSG switching (41-43). TERRA is transcribed in bloodstream form *T. brucei* by Pol I as a readthrough product when the enzyme continues into the telomeric repeats downstream of the VSG in the active ES (41,44,45). Transcription of TERRA by Pol I is unique to trypanosomes. TERRA is transcribed by Pol II in mammals and yeast (46,47) and subtelomeric promoters identified (48-50). Therefore, in contrast to human and yeast cells where multiple telomeres can be transcribed by Pol II (51), TERRA is transcribed in *T. brucei* from a single telomere and by Pol I. However, transcription of TERRA from the active ES is increased by Pol II readthrough transcription in the TbPP1 mutant (7). These results suggest J may also help control the levels of TERRA in BS form *T. brucei*.

In the *L. major* life cycle the parasites transform into three main morphologically distinct developmental stages (amastigote, promastigote, and metacyclic promastigote). Amastigotes that reside in mammalian macrophages transform into promastigotes in the alimentary tracts of their invertebrate vector (sand flies). Promastigotes multiply in the insect midgut, migrate to the insect's stomodeal valve and differentiate into the infective non-dividing metacyclic promastigote form (52). This differentiation process, called metacyclogenesis can be replicated in vitro in axenic culture (53). Here, while log phase procyclic promastigotes enter the stationary phase a fraction of the parasites differentiate into metacyclics. While a fraction of base J is localized and functional at Pol II termination sites in the *Leishmania* genome, DNA hybridizations indicate >90% of the total J is localized at telomeres/subtelomeres (54), suggesting an important telomere function. Analysis of blocked restriction digestion suggested developmental regulation of telomeric J in *L. major* with reduced levels in metacyclics (37). While little is known regarding TERRA synthesis and function in Leishmania, it has been shown to be developmentally regulated with increased levels in metacyclics and synthesized from multiple telomeric ends (37,44). While no Pol II promoter has been described, RT-PCR analysis of specific telomeric ends in *L. major* have detected TERRA synthesis in chromosomes with a PTU transcribed towards the telomere as well as those that have a unit directed away from telomeres (44). Therefore, TERRA is the potential product of readthrough Pol II transcription of PTUs as well as subtelomeric localized promoters.

Base J regulation of Pol II termination and parasite growth, along with the modulation of telomeric J and TERRA transcript levels throughout the life cycle in *L. major,* suggest a J regulatory mechanism of TERRA expression and an essential stage-specific role in the life cycle of these early-branching eukaryotes. We now show that TERRA is synthesized by Pol II in *L. major* and loss of base J leads to increased TERRA. Modulating base J levels in vivo using DMOG inhibition of thymidine hydroxylase activity and/or deleting H3V can lead up to ~100-fold increase in TERRA. RT-PCR analysis indicates some level of this increase is due to Pol II readthrough transcription from an adjacent PTU. Thus, base J control of Pol II transcription termination regulates TERRA synthesis. The phenotypes we describe here suggest that epigenetic control of Pol II termination and TERRA synthesis may explain the essential nature of base J in Leishmania. The ~20-fold increase in TERRA in base J mutants is associated with a ~10-fold increase in metacyclogenesis and the ~100-fold increase in TERRA is associated with decreased cell viability. The impact of these studies on understanding to the biological role of the unusual non-coding TERRA RNA in *Leishmania* will be discussed.

MATERIALS AND METHODS

Parasite culture

Promastigote *Leishmania major* strains, wild type and the H3V KO, were grown in M199 medium, supplemented with 10 % FCS at 26°C as previously described (9). DMOG treatment of cells was

performed by supplementing media with 2.5 mM or 5 mM DMOG for 5 days. Control cells were treated with an equal amount of vehicle (DMSO).

Metacyclogenesis

We used the lectin peanut agglutinin (PNA) protocol to purify metacyclic parasites from stationary-phase promastigote of *L. major* (55-57). *L. major* promastigotes are agglutinated by peanut lectin while metacyclics are not. Briefly, promastigotes were grown for 7 days to reach stationary phase. Parasites were then harvested at 2000g for 10 min, washed and resuspended in complete medium at 2 x 10^8 cells/ml. Peanut lectin was added to a final concentration of 50ug/ml and incubated at room temperature for 30 min. Agglutinated parasites (promastigotes) were cleared by centrifugation at 200g for 10 min. Metacyclic stage parasites (PNA-) were recovered from the supernatant by centrifugation at 2000g, resuspended in PBS and counted.

Anti J immuno-precipitation

For quantitating the levels of base J in telomeres, genomic DNA was sonicated and anti J immunoprecipitation was performed as previously described (22). Each J-IP experiment was performed in triplicate and immunoprecipitated J containing DNA analyzed by telomere quantitative PCR. Input DNA was used as a positive control for PCR.

Nuclear run-on

1.5 x 10^8 cells were washed 2x in ice cold PBS and washed 1x in ice cold lysis buffer (10 mM Tris pH 7.5, 3 mM $CaCl_2$, 2 mM $MgCl_2$) and resuspended in 10 ml lysis buffer. NP40 was added to a final concentration of 0.5%, transferred to a Dounce homogenizer and broken with 50 strokes with a tight pestle. Nuclei were harvested after centrifugation for 10 min at 1400 x g, washed with 10 ml lysis buffer and resuspended in 50 microliter lysis buffer. Nuclei were then incubated after addition of H_2O or α-amanitin at a final concentration of 1 mg/ml for 10 min on ice and 5 min at $30^{O}C$. Nuclei were pelleted and resuspended in 100 microliter transcription mixture (100 mM Tris pH 7.5, 25 % glycerol, 0.15 mM Spermidine, 0.5 mM Spermine, 2 mM DTT, 2 mM $MgCl_2$, 4 mM $MnCl_2$, 50 mM NaCl, 50 mM KCl, 2 mM ATP, 2 mM GTP, 2 mM CTP, 10 μM UTP, 2 mM 5-Ethynyl Uridine, 50 U Superasin and 10 μg / ml Leupeptin) in the presence or absence of 1 mg/ml α-amanitin. After incubation for 20 min at $30^{O}C$ nuclei were lysed in TriPure Isolation Reagent. RNA was prepared and 3 μg was subjected to click chemistry (ThermoFisher, C10365) according to the manufacturer's instructions. Briefly, incorporated EU residues were biotinylated and purified with magnetic streptavidin beads. cDNA was generated with bound biotinylated RNA on the beads as described below with strand specific oligonucleotides. WT cells have the blasticidin gene inserted into the rRNA locus (6), providing a Pol I transcribed control.

Northern analysis

Total RNA was isolated with Tripure Isolation Reagent. For Northern blot analysis, 10 μg of Turbo DNase treated total RNA was separated on a 1.2 % agarose gel (1 x MOPS, 2.8 % formaldehyde), transferred to a nitro cellulose filter in 20 X SSC and UV cross-linked. Radiolabeled oligonucleotides were generated and hybridized in an aqueous hybridization buffer with 100 μg/ml yeast tRNA at 60°C. A random primer labeled β-Tubulin probe was generated and hybridized in a 40 % (v/v) formamide hybridization mix with the addition of 10% (w/v) dextran sulfate and 100 μg/ml salmon sperm DNA at 42°C. Final washes were performed with 0.3 X SSC / 0.1 % SDS at 60°C for 30 min.

Quantitative RT-PCR analysis

Total RNA was isolated with Tripure Isolation Reagent (Roche). cDNA was generated from 0.5-1 μg Turbo™ DNase (ThermoFisher) treated total RNA with Superscript™ III (ThermoFisher) according to the manufacturer's instructions with either oligo dT primers or strand specific oligonucleotides. Quantification of Superscript™ III generated cDNA was performed using an iCycler with an iQ5 multicolor real-time PCR detection system (Bio-Rad). Triplicate cDNA's were analyzed and normalized to tubulin cDNA. qPCR oligonucleotide primers combos were designed using Integrated DNA Technologies software. cDNA reactions were diluted 20-fold and 5 µl was analyzed. A 15 µl reaction mixture contained 4.5 pmol sense and antisense primer, 7.5 µl 2X iQ SYBR green super mix (Bio-Rad Laboratories). Standard curves were prepared for each gene using 5-fold dilutions of a known quantity (100 ng/µl) of WT gDNA. The quantities were calculated using iQ5 optical detection system software.

Quantification of telomeric DNA in a sample was performed using a telomeric primer pair that specifically amplifies telomeric hexamer repeats without generating primer dimer-derived products (58). Additional bases at the 5' end of each primer also blocks them from initiating DNA synthesis in the middle of the PCR product in subsequent cycles.

TERRA RT-PCR analysis

For the RT-PCR analysis to determine the origin of TERRA, total RNA was isolated and Turbo™ DNase treated as described above, except DNase I digestions were repeated two additional times. Reverse transcription was performed using TELC20 as the primer and PCR amplified. To reduce endogenous priming by the telomeric primer TELC20 in the RT reaction, the denaturation step was performed by heating to 90°C for 1 min followed by cooling to 55°C at 0.5 ^{O}C / sec. For PCR, equal amounts of (strand specific) cDNA was used with Ready Go Taq Polymerase (Promega).

RESULTS

Base J regulation of TERRA synthesis by Pol II transcription.

TERRA is transcribed by Pol II in mammalian cells and yeast (46,47), but its transcription in *T. brucei* is resistant to α-amanitin and sensitive to the Pol I inhibitor BMH-21 supporting transcription by Pol I during polycistronic transcription of the telomeric VSG ES PTUs (7,41,44,45,59). To characterize the

transcription of TERRA in *L. major*, we utilized isolated nuclei to label and quantify newly synthesized RNA. As a non-radioactive label we used 5-Ethynyl Uridine (5-EU). After the labeling reaction, we prepared total RNA from permeabilized cells, performed click chemistry with biotin, precipitated biotinylated RNA with paramagnetic streptavidin beads, reversed transcribed TERRA using the CCCTAA repeat containing TELC20 oligo (7) and quantified TERRA by qPCR (58). In control reactions, we preincubated permeabilized cells for 15 min with 1 mg/ml α-amanitin, a potent inhibitor of Pol II. The inhibition of newly synthesized TERRA, versus the Pol I transcribed Blasticidin control gene, by α-amanitin (Figure 1) supports transcription of TERRA by Pol II in *L. major*, similar to yeast and human TERRA.

The role of base J in regulating Pol II transcription termination and the enrichment of the modified base in telomeres in *Leishmania*, suggest a role of base J in TERRA synthesis. To address this, we wanted to study the effects of J loss on TERRA synthesis in *L. major*. We have previously demonstrated the ability to titrate the levels of J in the *L. major* genome using the thymidine hydroxylase inhibitor DMOG (9,27). Increasing concentrations of DMOG led to decreasing total levels of base J, including at termination sites, and defects in Pol II termination resulting in pervasive Pol II transcription of the *L. major* genome (9,27). Since it is thought that >90% of J in *Leishmania* is localized to telomeres, we first wanted to directly assess the loss of J in telomeres by DMOG. Using J-IP and telomeric qPCR, we now confirm that inhibition of J synthesis following parasite growth in 5mM DMOG for 5 days, in contrast to the DMSO control, leads to the significant loss of J from telomeres in *L. major* (Figure 2A).

To determine if the control of Pol II transcription termination by base J is involved in the transcription of telomeric repeats, we first did Northern blotting to examine the TERRA level in DMOG treated cells. Total rRNA level was detected by ethidium bromide staining as a loading control. Following treatment with 5mM DMOG, the TERRA level was significantly higher (~ 20-fold) than the control DMSO treated cells (Figure 2B). Consistent with the effects of DMOG concentration on levels of base J (9,27), 2.5mM DMOG led to an intermediate increase (~10-fold) in TERRA level. Analysis of the hybridization signals (including by Phosphoimager analysis, not shown) indicates that depletion of base J causes no detectable change in TERRA size. This can more easily be visualized in the 2.5mM DMOG treated cells (Figure 2B). To allow precise and sensitive measurement of the TERRA level, we performed telomeric specific qRT-PCR, which showed that TERRA levels increased ~20- to ~40-fold upon the induction of J loss in the DMOG titration (Figure 2C). We have previously demonstrated that H3V regulates J synthesis in *L. major*, but where deletion of H3V reduced J levels at termination sites at the end of PTUs with little consequence on Pol II termination and gene expression (9,24). It seems that the remaining levels of J in the H3V KO were sufficient for Pol II termination at these sites. However, DMOG treatment of the H3V KO resulted in greater reduction in J levels at termination sites than DMOG treatment of wild-type parasites that is reflected by more significant Pol II termination defects (9). First, we wanted to confirm the effects of J loss on Pol II termination at a specific termination site on chromosome 22 (cSSR 22.3). While the loss of J in the H3V KO does not lead to detectable levels of Pol II readthrough nascent RNA, significant levels of

readthrough is seen in wild type cells treated with DMOG that is increased further in the H3V KO + DMOG (Figure 2D). Furthermore, while the loss of J in the H3V KO is not sufficient to cause Pol II termination defects at PTUs genome-wide it does lead to ~20-fold increase in TERRA that can be increased further to >100-fold by subsequent DMOG treatment (Figure 2C). Similar to the effects of DMOG on base J levels and Pol II readthrough transcription genome-wide, DMOG treatment of the H3V KO resulted in significantly higher effects on TERRA than DMOG treatment of wild type parasites. Therefore, the loss of base J in wild type *L. major* promastigotes in two different ways, DMOG inhibition of thymidine hydroxylase activity or deletion of H3V, caused an increase in levels of TERRA. The results show that base J regulates TERRA transcript levels in *L. major* promastigotes. The results from the H3V KO also suggest that increases in TERRA can occur without detectable Pol II readthrough transcription at PTU termination sites genome-wide.

Subtelomeres of *T. brucei* lack Pol II promoters driving TERRA synthesis. Rather, TERRA is a product of Pol I readthrough from the active BES into the downstream telomeric repeats (41,44). But, Pol II termination defects at termination sites within the chromosome core lead to Pol II extending into the active VSG ES increasing TERRA (7). Detection of nascent telomeric RNA in *L. major* extending from the final ORF of a PTU to TERRA, suggests that at least some fraction of the total TERRA in wild-type *L. major* promastigotes is due to Pol II readthrough (44). To determine if Pol II transcriptional readthrough of subtelomeric PTUs can help explain the regulation of TERRA by base J, we performed telomere strand-specific RT-PCR for a specific telomeric end of *L. major* (Figure 3). After using the CCCTAA repeat containing TELC20 oligo as a primer in the reverse direction, we used primers specific for the subtelomeric region of the right arm of chromosome 1 to help characterize the origin of TERRA (Figure 3A). First, we analyzed the presence of nascent TERRA readthrough product using Tel20 and a 5' primer specific for the 5'-UTR of the final gene of the PTU. While the nascent RNA is not detected in the primary PCR reaction, nested RT-PCR revealed the TERRA precursor RNA in WT cells only upon the loss of base J (+DMOG) (Figure 3B). Interestingly, the levels of TERRA precursor decreases slightly with increasing levels of DMOG. These results show that at least some fraction of the TERRA transcripts produced following the loss of base J is due to Pol II termination defects and readthrough RNA extending from telomeric proximal PTUs. To explore TERRA expression from this telomeric end representing potential products of a subtelomeric Pol II promoter, we utilized the A3 subtelomeric PCR primer that binds ~1kb from the start of the telomeric repeat. Interestingly, we detect the ~1 kb TERRA RNA species in WT cells (-DMOG) that does slightly increase with DMOG. The detection of this TERRA transcript in WT -DMOG cells that lack readthrough nascent TERRA RNA suggests an endogenous Pol II promoter within the subtelomeric region. The relatively small increase in steady-state TERRA levels by DMOG by this analysis is to be expected when assaying a specific telomeric end out of a total 72 ends in the *L. major* genome. With multiple telomeric ends thought to be expressing TERRA in promastigotes (37,44), only small increases in transcription at each telomere are needed to explain the 20-fold increase in total TERRA. Taken together, the results show that base J regulation of TERRA transcript levels in *L. major*

promastigotes involves the control of Pol II transcription termination either from proximal PTUs and/or independent Pol II promoter.

Control of metacyclogenesis by base J regulated Pol II transcription.

As mentioned above, treatment of wild type *L. major* with DMOG decreases J levels below a certain cutoff leading to read-through transcription, and the degree of readthrough transcription in these cells is stimulated by further loss of J upon the deletion of H3V (9). The increased readthrough transcription in the H3V KO +DMOG is accompanied by significant reductions in promastigote parasite growth, consistent with the proposed essential nature of J in *Leishmania*. Observation of these cells under the microscope suggested the loss of J and Pol II readthrough leads to morphological changes associated with the formation of non-dividing metacyclics. To address this further, we quantitated the levels of metacyclics in cultures of *L. major* promastigotes treated with DMOG by negative selection with peanut agglutinin. We detect a significant increase in numbers of metacyclic promastigotes (PNA-) upon the loss of J in WT cells treated with DMOG, that increases slightly further upon the additional loss of J in H3V+DMOG (Figure 4), a phenomenon that closely mimics the increases in Pol II readthrough transcription at PTUs and associated gene expression changes (9) and the increased TERRA (Figure 2). Interestingly, while WT + DMOG leads to ~30-fold increase in TERRA and ~10-fold increase in metacyclogenesis, the H3V KO has no readthrough termination or metacyclic defects even with ~20-fold increase in TERRA. However, the maximal increase in TERRA (~100-fold increase) in the H3V KO + DMOG is associated with a significant growth defect (9). The results suggest that the control of Pol II termination by base J and expression of protein coding genes at the end of PTUs genome-wide and non-coding RNA TERRA is associated with the control of the differentiation of *L. major* promastigotes to the non-dividing infective metacyclic life stage and decreased cell viability. Where increased metacyclogenesis is more closely related with the de-repression of genes in PTUs rather than increased TERRA, but high levels of TERRA are associated with decreased parasite viability.

DISCUSSION

In this study, we provide the first characterization of base J function in regulating Pol II transcription of TERRA in *L. major*. We have previously described a conserved role for J regulating Pol II transcription termination and expression of genes within polycistronic gene clusters in trypanosomatids and suggested that the essential nature of J in *Leishmania* is related to its role in repressing these specific protein coding genes. Based on the studies here, we now include the role of J in regulating TERRA synthesis and metacyclogenesis in *L. major* via control of Pol II transcription termination. The studies also suggest that the essential nature of J is more closely related to its function in regulating TERRA synthesis and J regulation of protein coding genes within PTUs is involved in the control of metacyclogenesis.

We and others have detected a nascent RNA representing sequence from the final ORF in the chromosome and TERRA, suggesting Pol II readthrough as a source of TERRA in WT *L. major* cells (44).

The characterized role of J controlling pervasive Pol II transcription in Leishmania genome-wide, supports the conclusion that readthrough defects in the *L. major* cells treated with DMOG can lead to increased TERRA transcription, regardless of the orientation of annotated telomeric PTUs. RNA-seq and RT-PCR analyses have indicated multiple telomeric ends as sites of TERRA synthesis in *L. major*, including ones with a gene orientated away from the telomeric repeats (37,44). While TERRA synthesis at telomeres with a gene orientated in the opposite direction could be a result of readthrough from a distant PTU promoter, especially in cells that lack base J, it is more likely that Pol II promoters for TERRA exist in *Leishmania* subtelomeres. Many Pol II promoters in trypanosomatids are adjacent to another divergently arranged promoter allowing divergent PTU transcription. We have also demonstrated the ability of PP1-PNUTS mediated termination to control non-productive transcription from bi-directional Pol II promoters in *T. brucei* (5). Therefore, at least for telomeric sites initiating PTU transcription away from the chromosome end, an adjacent opposing activity likely exists for TERRA synthesis. Analysis of the hybridization signals on the northern blot indicates that depletion of base J causes no detectable change in TERRA size. This suggests that the increase in TERRA is not due to effects of J on the extent of Pol II elongation within the telomeric repeat. While Pol II transcription of TERRA in *L. major* may originate from readthrough transcription from a distant promoter, and potentially via a promoter within the subtelomere, we believe J helps control TERRA levels by termination of transcription prior to the telomeric repeats.

Studies on the telomere position effect on gene silencing in yeast has indicated subtelomeres can control transcription in many ways, primarily involving control of chromatin structure (60,61). These mechanisms may play a role in telomere transcription of TERRA. For example, Sir-complex-dependent chromatin modifications have been shown to be a key regulatory mechanism limiting the global levels of TERRA in yeast. In mammalian cells, promoters have been found in CpG-islands within subtelomeric regions of human chromosomes and where TERRA transcription initiation is controlled by the chromatin state and DNA methylation (48,62,63). Subtelomeres in yeast can also help limit TERRA transcript levels by regulating Rap1-mediated pathways (64). Rap1 mutants have increased levels of TERRA, presumably by the loss of transcription repression by reduced recruitment of Sir complex components as well as by decreased Rat1-dependent degradation (64). Increased TERRA transcription, and formation of telomeric R-loops, was detected in *T. brucei* cells depleted of TbRap1 (41). TbRap1 is also essential for cell viability and VSG silencing and VSG switching (41,65,66). Furthermore, chromatin modifications play a role in silencing transcription of TERRA in the silent VSG ESs by the native telomeric Pol I promoter as well as any leaky Pol II elongation from a distant promoter. While Pol II termination defects in *T. brucei* leads to Pol II transcription into all the telomeric PTUs, TERRA synthesis is still restricted to the active VSG ES (7). Pol II readthrough is terminated just before the telomeric repeats in the silent VSG ESs presumable by the repressive telomeric chromatin. Reb1, an essential transcription factor in yeast, can bind subtelomeres and control TERRA transcription directly, where the reb1 mutant has 75-fold higher levels of TERRA (43). It is presumed that Reb1 represses TERRA levels by a roadblock transcriptional termination mechanism, independent of Sir-complex regulation of chromatin. While base J may provide

some level of steric block for Pol II elongation in trypanosomatids, we believe the main mechanism involves control of Pol II-CTD phosphorylation and torpedo dissociation of the polymerase from the DNA template at termination sites (5-7). Further studies aimed at identifying promoter regions of TERRA and the contribution of base J to controlling TERRA synthesis from these subtelomeric sites and chromosome internal termination sites will need to be performed in order to fully ascertain the mechanism of epigenetic regulation of TERRA biogenesis in this organism.

Analysis of the *L. major* lifecycle has previous suggested a link between base J, TERRA and metacyclogenesis. A majority (>90%) of the total genomic base J in *Leishmania* is localized to telomeres and upon differentiation of promastigotes to metacyclics the levels of J are thought to decrease (37)(while levels of TERRA increase (37,44). By studying the consequence of J loss in wild type *L. major* and the H3V KO, we now describe a clear link between J regulation of Pol II transcription termination and expression of TERRA and metacyclogenesis. Our previous transcriptome analysis of these cells identified 17 protein coding genes regulated by base J associated with an *in vitro* growth defect. The results here allow us to take a closer look at the gene expression changes that correlate with base J, metacyclogenesis and cell viability. We identified 31 genes upregulated at least 2-fold upon DMOG induced loss of J in WT and/or H3V KO (9). 10 of these changes are likely indirect (for example, by induction of parasite differentiation and/or other gene expression changes) since their genomic locations do not fit the J-mediated premature termination model (9). 17 of the remaining genes that fit the J-mediated silencing model exhibit expression changes that correlate with the DMOG induction profile; where further increased expression in the H3VKO + DMOG is associated with increased Pol II readthrough transcription and decreased cell viability. We now show J regulation of Pol II transcription in *L. major* is similarly associated with TERRA synthesis and conversion of promastigotes to metacyclics. Demonstrating that the loss of base J by either DMOG treatment or deletion of H3V led to significant increases in TERRA and a nascent transcript extending from a telomeric proximal PTU into the telomeric repeat, confirms its role in epigenetic regulation of TERRA via control of Pol II termination. The mechanistic link between base J and metacyclogenesis in *L. major*, due to control of Pol II termination and expression of protein coding genes and/or non-coding TERRA, may not be as clear. However, the fact that we see an increase in TERRA in the H3V KO without corresponding measurable effects of Pol II termination at chromosome internal PTUs or effects on metacyclogenesis provides some interesting clues. While the levels of J remaining at PTU termination sites in the H3V are sufficient for Pol II termination, apparently the decreased levels of J at telomeres leads to increased TERRA; demonstrating the ability of H3V/J to independently regulate Pol II transcription of TERRA and chromosome internal protein coding genes. This separation of TERRA synthesis at telomeres from readthrough transcription within PTUs supports the idea that for at least a subset of telomeres in *L. major* TERRA is regulated by its own core promoter as in other model organisms including humans and yeast. More importantly, the stimulation of TERRA independent of genome-wide PTU readthrough transcription and metacyclogenesis in the H3V KO indicates that the stimulated differentiation to metacyclic promastigotes following the loss

of J by DMOG is likely a consequence of changes in gene expression resulting from termination defects at PTUs rather than the increased TERRA.

Surprisingly, decreasing the levels of base J in *L. major* promastigotes, either with DMOG or deleting H3V, leads to a ~20-fold increase in TERRA without significant defects in cell viability. In model organisms such as yeast and humans, increases in TERRA and associated telomere R-loops is typically associated with lethal phenotypes (including telomere loss and genome instability). As mentioned above, TbRap1 control of TERRA and telomeric R-loops is essential for trypanosome cell viability, where a 11-fold increase in TERRA was associated with growth arrest within 24h in BS form *T. brucei*. (41,65,66). This differential phenotypic effect of increased TERRA may be explained by TERRA acting in cis and where the 11-fold increase in *T. brucei* represents activation of one telomeric site, while the 20-fold increase in *L. major* is presumably distributed among several of the 76 telomeric ends. Interestingly, while we demonstrate the ~20-fold increase in TERRA in *L. major* promastigotes without obvious defects in promastigote viability other than potentially help stimulate differentiation to the metacyclic life stage (WT+DMOG), the endogenous increase in TERRA that occurs during differentiation to the infective forms correlates with the detection of R-loops at different chromosome ends (37). Further studies will need to be done in order to characterize the consequence of increased TERRA on telomere R-loops and genome stability in *L. major*. However, while the ~20-fold increase in TERRA had no significant effects on parasite growth, the subsequent ~100-fold increase in TERRA in the H3V KO -/+ DMOG, in contrast to the ~4-fold increase in PTU readthrough transcription and gene expression and minimal increase in metacyclogenesis, strongly correlates with the appearance of a severe growth defect (8). The enrichment of ~90% of the total J at telomeres in *Leishmania* suggested a key biological function in telomere biology. The results presented here suggest that the effects of base J on TERRA expression, potentially combined with differentiation to non-dividing metacyclic promastigotes, may explain why the JBP1 KO was unable to be generated and the essential nature of J in Leishmania. Finally, the ability of DMOG to alter TERRA levels in vivo without the need of other functional genetic approaches, provides a useful tool to elucidate TERRA function and synthesis in Leishmania. Regulating J synthesis may provide a unique way to manipulate TERRA specifically in vivo without simultaneously altering additional telomere components.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Figure Legends

Figure 1. Analysis of TERRA synthesis in *L. major* promastigotes. Isolated nuclei were incubated with a transcription cocktail containing 2 mM EU for 20 min. Post click chemistry, newly synthesized biotinylated RNA was purified with paramagnetic streptavidin beads and reverse transcribed with strand specific oligonucleotides. qPCR was performed with telomeric oligonucleotides and normalized with RNA Pol I transcribed blasticidin specific primers. Control reactions contained 1 mg/ml of the RNA Pol II inhibitor α-amanitin. Two biological replicates were analyzed in triplicate and the value without α-amanitin was set to 1. Means and standard deviations are plotted.

Figure 2. Depletion of base J results in a higher TERRA level. (A) Levels of base J in telomeres in *L. major* promastigotes treated with DMOG were determined by J-IP and telomere qPCR. The percent of telomeric DNA IP'd was calculated from three independent experiments and normalized to levels in WT set to one. Standard deviations are shown. (B) Northern analysis of TERRA in *L. major* promastigotes treated with and without DMOG. Total RNA was isolated from cells incubated with or without DMOG and equal amounts were hybridized with the (CCCTAA)n (TelC4) probe. Right, ethidium bromide stain of the gel as a loading control. (C) Quantitation of TERRA RNA in *L. major* promastigotes (wild type, WT; and H3V KO) treated with and without DMOG by telomeric qRT-PCR. Fold changes in TERRA RNA + and – DMOG were calculated by qPCR from two biological replicates, measured in triplicate. Means and standard deviations are plotted. (D) Strand-specific RT-PCR analysis of readthrough transcription. Above; Schematic representation (not to scale) of cSSR 22.3 illustrating the nascent RNA species assayed by RT-PCR. The dashed red arrow indicates read through transcription past the transcription termination site (TTS). Arrows indicate the location of primers utilized for strand-specific RT-PCR analysis. Below; Read through transcription on the top strand for the indicated cell lines (-/+ DMOG) was quantitated by performing site-specific cDNA synthesis using primer RT illustrated in the diagram above, followed by PCR using primers A and B. Abundance was normalized using tubulin (a gene specific primer against tubulin was added to the same cDNA synthesis reaction with primer RT, followed by PCR using tubulin primers). Fold increase in nascent readthrough RNA species relative to background levels in WT + DMSO is based on A+B qPCR analysis, normalized to tubulin qPCR. Error bars indicate the standard deviation between two biological replicates analyzed in triplicate.

Figure 3. TERRA is transcribed as a Pol II readthrough transcript in WT and J depleted cells. (A) Schematic representation of *L. major* chromosome end and telomeric transcriptome. Telomeric repeats are in black and subtelomeric sequences in white. The final gene in the telomeric PTU is represented by the grey box and the 3'UTR indicated by the hatched box. The dashed arrow indicates the gene orientation and direction of transcription. Grey line above represents Pol II transcription of the telomere in WT cells. The black line represents the proposed extent of Pol II transcription of the subtelomeric loci upon depletion of base J. The large precursor RNA is processed to generate TERRA.

Oligonucleotides used for RT-PCR are indicated by arrows pointing in the 5'-3' direction. The sketch is not in scale. (B) Total RNA was purified from *L. major* cells incubated without and with DMOG and reverse-transcribed using TelC20 as the RT primer. RT products were PCR amplified using primers specific to subtelomeric region of the left arm of chromosome 1 and the PCR products were separated on agarose gels. Left panels: PCR reactions using U5'+TelC20 primers. A portion of this PCR reaction was used in subsequent nested PCR reactions using U5'+A1 or U5'+A2 oligonucleotides.
Right panel; PCR reaction using A3+TelC20. Blasticidin RT-PCR provides a loading control.

Figure 4. Base J regulation of metacyclogenesis. Metacyclic promastigotes were enriched by negative selection with peanut agglutinin following treatment of procyclic promastigotes without and with 5mM DMOG for 5 days. Fold change in percent of cells remaining are expressed relative to the WT minus DMOG control (set to one). KO, H3V KO. Means and standard deviations from at least three independent experiments are plotted.

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Fig 1

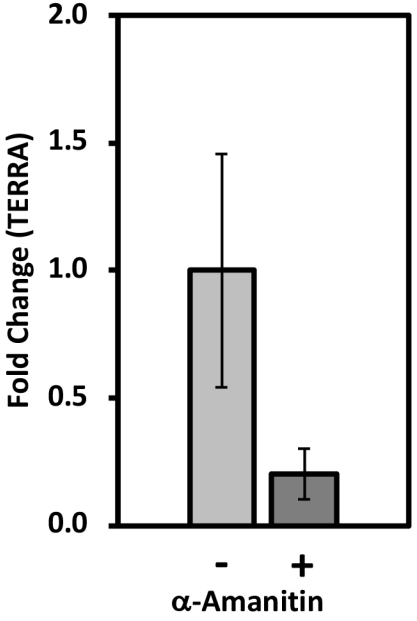


Fig 2

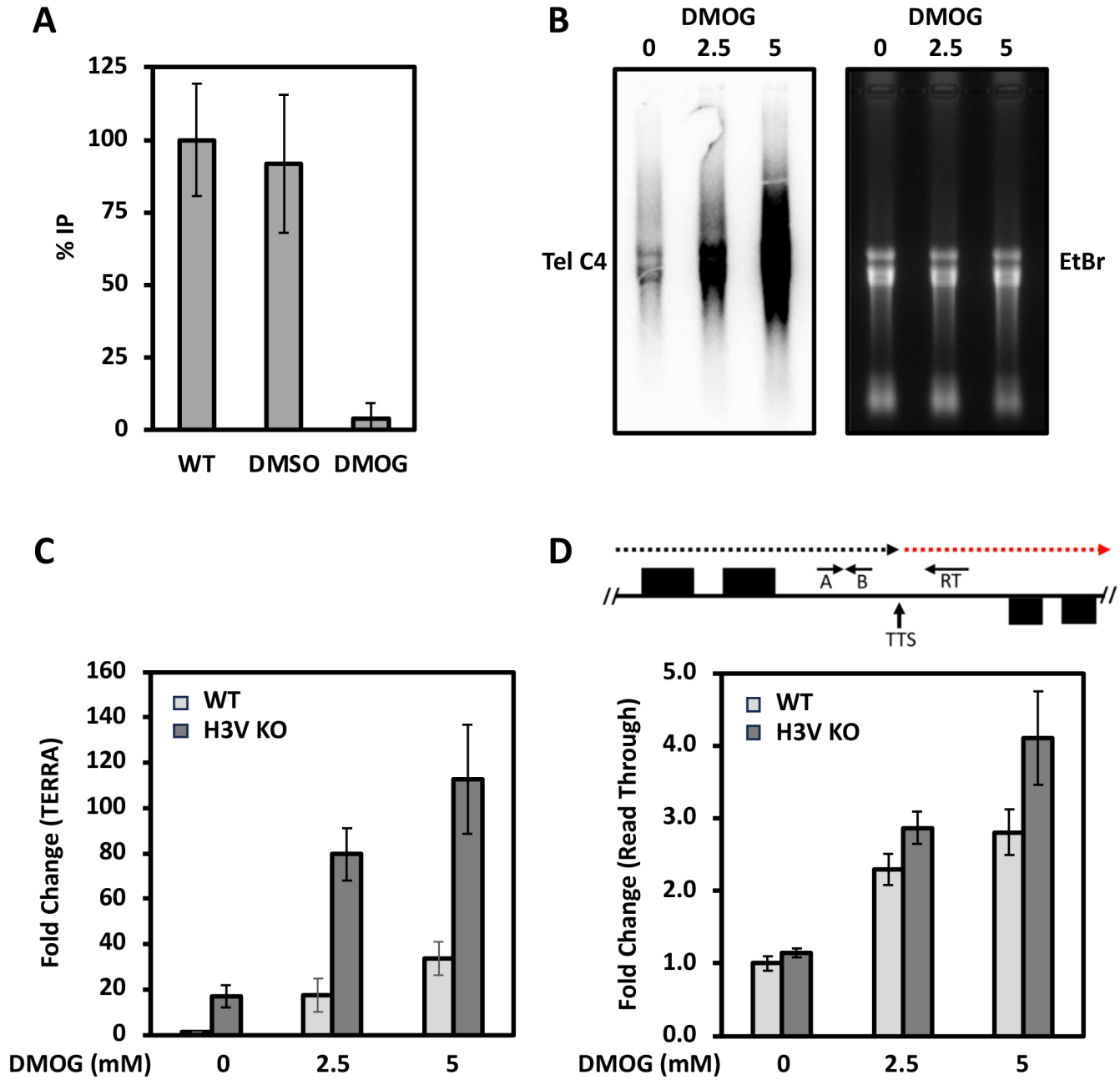
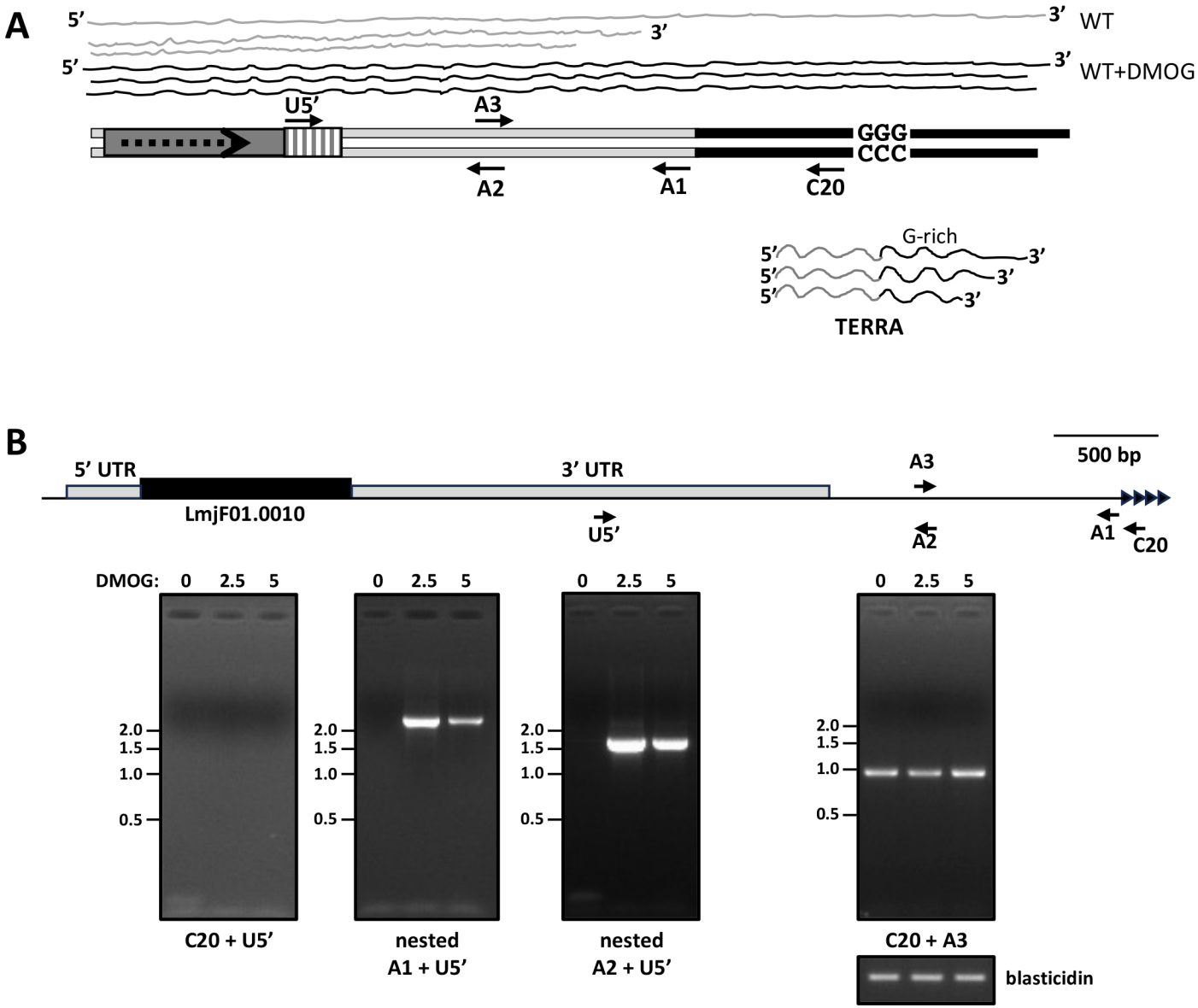

A
125
100
75
50
25
0
% IP
WT
DMSO
DMOG
B
DMOG
0
2.5
5
DMOG
0
2.5
5
Tel C4
EtBr
C
160
140
120
100
80
60
40
20
0
Fold Change (TERRA)
WT
H3V KO
DMOG (mM)
0
2.5
5
D
A
B
RT
TTS
5.0
4.0
3.0
2.0
1.0
0.0
Fold Change (Read Through)
WT
H3V KO
DMOG (mM)
0
2.5
5

Fig 3


A
5'
3'
WT
5'
3'
WT+DMOG
U5'
A3
GGG
CCC
A2
A1
C20
G-rich
5'
3'
5'
3'
5'
3'
TERRA
B
5' UTR
3' UTR
500 bp
A3
LmjF01.0010
U5'
A2
A1
C20
DMOG: 0 2.5 5
0 2.5 5
0 2.5 5
0 2.5 5
2.0
1.5
1.0
0.5
C20 + U5'
nested
A1 + U5'
nested
A2 + U5'
C20 + A3
blasticidin

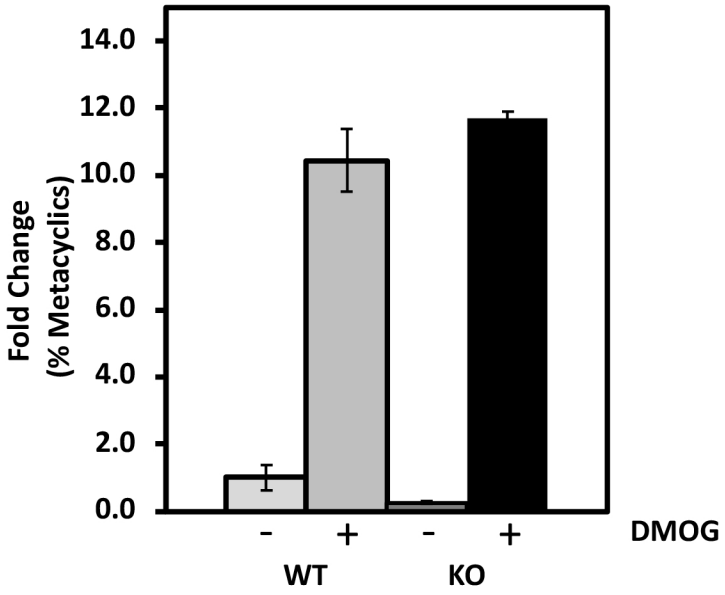



Fig 4