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Short communication

Evidence that J-binding protein 2 is a thymidine hydroxylase catalyzing the first step in the biosynthesis of DNA base J☆

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ABSTRACT

The genomic DNA of kinetoplastid parasites contains a unique modified base, β-D-glucosyl-hydroxymethyluracil or base J. We recently reported that two proteins, called J-binding protein (JBP) 1 and 2, which regulate the levels of J in the genome, display features of the family of Fe(II)–2-oxoglutarate dependent dioxygenases and are likely to be the enzymes catalyzing the first step in J biosynthesis. In this study, we examine the effects of replacing the four conserved residues critical for the activity of this class of enzymes on the function of *Leishmania tarentolae* JBP2. The results show that each of these four residues is indispensable for the ability of JBP2 to stimulate J synthesis, while mutating non-conserved residues has no consequences. We conclude that JBP2, like JBP1, is in all probability a thymidine hydroxylase involved in the biosynthesis of base J.

The genomic DNA of kinetoplastid parasites like *Leishmania* sp. and *Trypanosoma* sp., contains an unusual modified base, β-D-glucosyl-hydroxymethyluracil or base J, which replaces a fraction of thymines [1,2]. J localizes to the telomeric repeats and other repetitive DNA sequences, and its distribution varies somewhat between different trypanosomatids: in the bloodstream (mammalian stage) form of *T. brucei*, about 50% of J is found in the telomeres, while the modification is absent in the procyclic (insect stage) form [2,3]. In *Leishmania*, 98% of J localizes to the telomeres [4].

Base J appears to be synthesized in two steps. First, a thymine in the DNA is converted into hydroxymethyluracil (HMU) by a thymidine hydroxylase and second, a glucosyl transferase adds a glucose group to the HMU [5]. We have identified two proteins, called JBP1 and JBP2, which regulate the levels of base J in the genome [6–8], and recently we proposed that these proteins actually represent the thymidine hydroxylases responsible for HMU formation in the DNA [9]. Our proposal arose from the finding of an amino acid signature in JBP1 and JBP2 conserved in members of the Fe(II)- and 2-oxoglutarate (OG) dioxygenase/hydroxylase family. Replacement of these conserved residues in JBP1 abolished its J-restoring activity in *JBP1−/− T. brucei* [9]. We have now determined whether these residues are also essential for the function of JBP2.

The region homologous between JBP1 and 2 (about 270 amino acids; 34% identity, 47% similarity) is located at the N-terminal parts of the proteins and includes the amino acid signature mentioned above (for alignment of the homologous regions, see [9]). In addition to this region, JBP2 contains a SWI2/SNF2 chromatin remodeling domain in its C-terminus [8]. There are no indications that JBP2 can bind to base J like JBP1 does [6]. Rather, it associates with chromatin independently of the presence of J, most likely by means of the SWI2/SNF2 domain [8]. This difference in binding substrates reflects the differences in the suggested roles of the JBPs: JBP2 is thought to control *de novo* thymine hydroxylation, while JBP1 functions in maintenance and amplification of J synthesis [8,9]. Interestingly, whereas both JBP1 and 2 can be readily knocked-out in *T. brucei* [7,10], JBP1 in *Leishmania* is essential [11].

We chose to study JBP2 from *Leishmania tarentolae*, as the mutational analysis of JBP1 was also performed with the protein from this species [9]. To clone the *L. tarentolae JBP2* (*LtJBP2*) locus, genomic DNA was digested with various restriction enzymes, size-fractionated, blotted and hybridized with a *L. major JBP2* (accession number CT005253) probe generated by PCR. A single band of approximately 8 kb hybridized with the *JBP2* probe following an EcoRV digest. EcoRV DNA fragments of ~8 kb were then cloned into an *E. coli* cloning vector, and the *LtJBP2* containing colonies were identified by colony hybridization. The insert was sequenced by primer walking. Constructs for ectopic expression of LtJBP2 were generated by subcloning the full-length *LtJBP2* into *Leishmania* and *T. brucei* expression vectors (the vectors have been described in

Abbreviations: JBP, J-binding protein; HMU, hydroxymethyluracil; OG, 2-oxoglutarate; TRF, terminal restriction fragment; BrdU, bromodeoxyuridine.

☆ *Note:* Nucleotide sequence data (*Leishmania tarentolae JBP2*) reported in this paper is available in the EMBL database, accession number FM242183.

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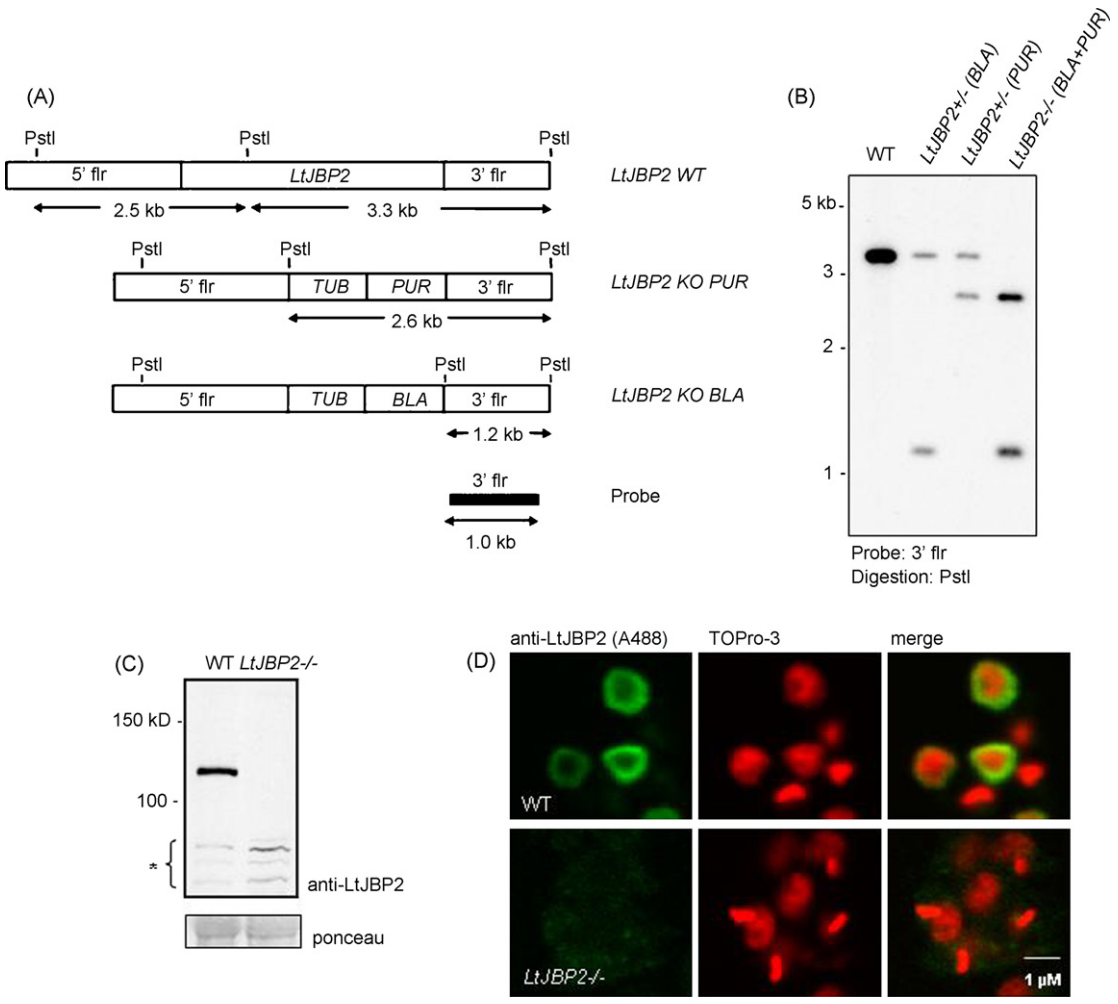

Fig. 1. Generation of *LtJBP2*−/− cells. (A) A schematic presentation of the *LtJBP2* locus and the inactivation constructs used in this study (not in scale). PstI restriction sites and the position of the *LtJBP2* 3′-flr (flanking region) probe used for Southern blotting are shown. *TUB*, alpha-tubulin intergenic sequence from *L. enriettii*; *PUR*, puromycin N-acetyltransferase; *BLA*, blasticidin S-deaminase; KO, knock-out. (B) Southern blot to show the genotypes of WT, *LtJBP2*+/− cells carrying the two different knock-out constructs (*BLA* and *PUR*) and *LtJBP2*−/− *(BLA*+*PUR)* cells. Genomic DNA was digested with PstI, run in an agarose gel, blotted and hybridized with the *LtJBP2* 3′-flr probe. To generate the knock-out cell lines, 1×10^8 *L. tarentolae* (TarII, cultured as in [10]) cells were transfected with 1–5 μg DNA using the Amaxa Nucleofector II electroporator and the Amaxa Human T-cell buffer kit. In our hands, this transfection method improves the transfection efficiency 5- to 10-fold in comparison to conventional methods (data not shown). The cells were allowed to recover for 18–24 h before starting selection with the appropriate antibiotics. (C) Western blot to show the specificity of the rabbit polyclonal anti-LtJBP2 antiserum. The antibody recognizes a single major band of about 120 kD in WT but not in *LtJBP2*−/− cells. Star indicates weak, aspecific bands observed in both cell types. Western blot was performed as in [9]. Ponceau-staining of the blot is shown as loading control. (D) Fluorescent microscopy images to demonstrate the nuclear localization of LtJBP2 in WT cells and the lack of signal in *LtJBP2*−/− cells. The cells were fixed with 1% formaldehyde, blocked in 1× Blocking solution (Roche), and the primary (anti-LtJBP2) and secondary [Alexa 488 (A488)-conjugated goat-anti rabbit (Invitrogen)] antibodies were incubated in 3% BSA/PBS at 37 °C for 1 h and 30 min, respectively. After the antibody incubations, the cells were washed for 3× 5 min with PBS. Finally, the samples were mounted in antifade solution (Vectashield; Vector Laboratories) containing TOPro-3 DNA stain (Invitrogen). The images were captured with a Leica TCS SP2 AOBS confocal microscope.

references [9,11]). We originally intended to use *T. brucei* procyclic cells for this study. These cells would form an optimal system for studying the functionality of the LtJBP2 mutants for several reasons: they completely lack J, do not endogenously express JBP2, and ectopic expression of *T. brucei* JBP2 results in J synthesis in them [8]. Unexpectedly, however, our repeated attempts to express LtJBP2 in the procyclic *T. brucei* cells were unsuccessful (data not shown). Therefore, we had to turn to *L. tarentolae* for further studies.

To generate inactivation constructs for *LtJBP2*, the 5′- and 3′-flanking sequences of *LtJBP2* were subcloned into *Leishmania* expression vectors coding for puromycin and blasticidin resistance genes (the vectors have been described in [11]) (Fig. 1A). Using a conventional knock-out strategy, we successfully inactivated both of the *LtJBP2* alleles (Fig. 1B). To be able to study the expression levels and localization of LtJBP2, we raised a rabbit polyclonal antibody using as immunogen a His-tagged full-length LtJBP2, expressed in and purified from *E. coli*. The resulting antibody recognizes a single major band of the expected size (120 kD) in WT but not in *LtJBP2*−/− cells (Fig. 1C). Immunofluorescent staining with the antibody reveals a nuclear staining in WT cells, while only background signal is detected in *LtJBP2*−/− cells (Fig. 1D).

Thus, unlike JBP1, JBP2 is dispensable for *Leishmania*. The *LtJBP2*−/− cells grow normally and lack major morphological abnormalities, but the amount of J in their DNA gradually drops, reaching a four- to eightfold reduction in comparison to WT over a period of approximately 6 months (Fig. 2A). Thereafter, the J levels in the *LtJBP2*−/− cells remain stable for a period of at least 2.5 years of continuous culturing, the longest observation point that we have. Quantitatively, this reduction resembles the phenotype of the *T. brucei JBP2*−/− [10], but the kinetics of the decline are different. Interestingly, while our previous data strongly suggest that base J is essential in *Leishmania* [11], the results with the *LtJBP2*−/− cells now show that 25% or even less (four- to eightfold reduction) of the WT J levels are sufficient for normal growth and viability.

As the vast majority of base J is found in the telomeres in *Leishmania* [4], we tested whether some aspects of telomere biology

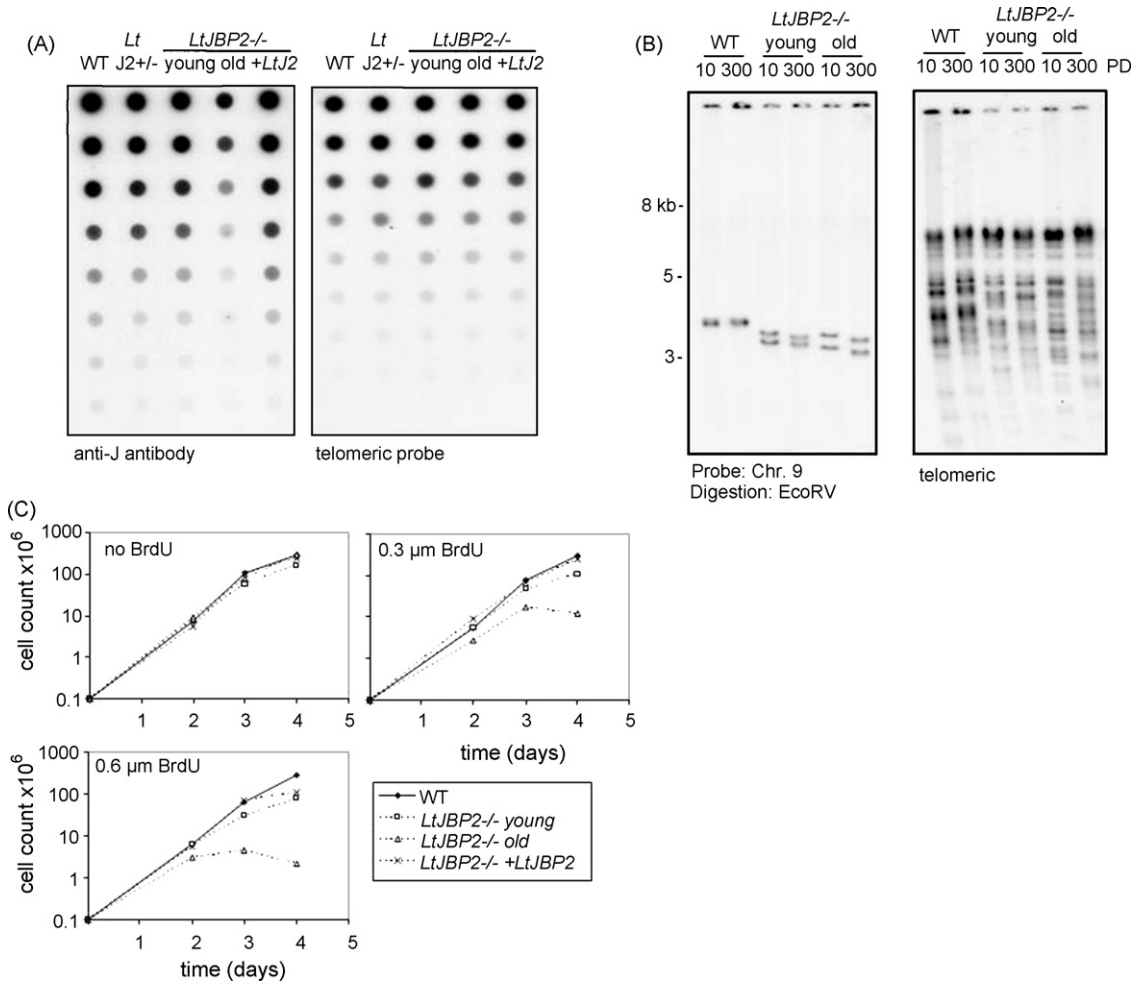

Fig. 2. Properties of *LtJBP2*−/− cells. (A) A dot blot showing the levels of J in WT, *LtJBP2*+/− (*LtJ2*+/−), young (0–5 months in culture) and old (>6 months in culture) *LtJBP2*−/− cells and *LtJBP2*−/− + *LtJBP2* (*LtJBP2*−/− + *LtJ2*) cells (old *LtJBP2*−/− cells carrying an ectopic copy of *LtJBP2*). The dot blot was performed as in [9]; briefly, serially (twofold) diluted genomic DNA (starting with 200 ng) from the indicated cell lines was spotted on a membrane that was incubated with anti-J antibodies, followed by HRP-conjugated secondary antibodies. The signal was visualized by ECL. The membrane was thereafter probed with a $(TTAGGG)_3$ telomeric probe to control for equal loading. (B) Analysis of telomere length in time in WT and young and old *LtJBP2*−/− cells. The indicated cell lines were cloned out by plating on soft-agar plates, the resulting clones were grown for 300 population doublings (PD) and genomic DNA samples were collected after every 50 PD. Only the first (10 PD, the amount of PDs needed for a colony picked from a soft-agar plate to grow into a culture that can be used for DNA isolation) and the last (300 PD) time points are shown. To be able to study a single telomere, genomic DNA was digested with EcoRV and a probe specific for the subtelomeric region of chromosome 9 (chr 9; left panel) was used. This sequence remains attached to the telomere after EcoRV digestion, as described in [11]. The same blot was thereafter stripped and probed with the telomeric probe to show all telomeres (right panel). (C) Growth curves in the absence and presence of BrdU. 1×10^5 cells of the indicated cell lines were inoculated in medium containing 0, 0.3 or 0.6 μM BrdU, and the cell count was determined after 2, 3 and 4 days of incubation. Data from a representative experiment is shown.

are affected by the low amounts of J in the *LtJBP2*−/− cells. Terminal restriction fragment (TRF) analysis (performed as in [12]) showed no gross alterations in the average telomere length of the *LtJBP2*−/− cells of any age (data not shown). Probing the TRF-blots with the anti-J antibodies demonstrated that base J is lost universally from all telomeres, with a slight tendency of the shortest ones to be affected first (data not shown). Further, fluorescence *in situ* hybridization with a telomeric probe revealed no differences in the subnuclear localization of the telomeres in WT and *LtJBP2*−/− cells (data not shown). To study the telomere length regulation in more detail, we cloned out WT and *LtJBP2*−/− cells of different age, let the cells grow for 300 generations (approximately 3 months) and took a sample for telomere analysis after every 50 cell divisions. While in WT cells the telomeres slowly grow, as expected [12], telomere shortening took place in the *LtJBP2*−/− cells (Fig. 2B). This phenotype was present already in the young knock-out cells, which still have almost WT levels of J, and was not aggravated in the old knock-out cells (Fig. 2B). The significance of this phenomenon, however, remains unclear. As we have observed no net loss of telomeric material in the *LtJBP2*−/− cells even over prolonged periods of time (Fig. 2A, B and data not shown), the shortening either must be compensated for by periods of telomere growth or there should be a selective pressure favoring the survival of cells with long telomeres. The latter option appears unlikely, though, as even the old *LtJBP2*−/− grow at rates comparable to WT (see below).

While the telomeric phenotype of the *LtJBP2*−/− cells is rather mild, we found that these cells display dramatic hypersensitivity to bromodeoxyuridine (BrdU) (Fig. 2C), a thymidine analogue that lowers J levels in kinetoplastids by an unknown mechanism [2]. This sensitivity is concentration dependent and correlates with the age (and thus J levels) of the knock-out cells (Fig. 2C): the growth rate of young (0–5 months in culture) *LtJBP2*−/− cells in the presence of 0.6 μM BrdU is on average 30% (±10%, $n = 8$) of the growth rate in the absence of BrdU, while for old (>6 months in culture) *LtJBP2*−/− cells, this growth rate is only 0.1% (±0.04%, $n = 12$). This difference, as well as the difference between WT and old *LtJBP2*−/− cells [the growth rate of WT cells in 0.6 μM BrdU is 90 ± 10% ($n = 12$) of the growth rate in the absence of BrdU] is highly significant ($p < 0.001$ and $p < 0.0001$, respectively). We believe that the BrdU sensitivity is

Table 1
The LtJBP2 mutants used in the study.

Mutant 1	V482[a]A + L548Q
Mutant 2	R478A
Mutant 3	H465A
Mutant 4	D416A
Mutant 5	H414A

[a] The numbers refer to the positions of the amino acids in LtJBP2.

not caused by the lack of LtJBP2 *per se*, but related to the reduced levels of J in the knock-out cells. The mechanisms of the BrdU-induced growth defect will be subjects of future studies.

As shown in Fig. 2A and C, the major phenotypes of the *LtJBP2*−/− cells (reduced levels of J, BrdU sensitivity) can be completely reversed by the presence of an ectopic copy of *LtJBP2* and thus, the knock-out cells can be used as a system to study the functionality of LtJBP2. We therefore generated *LtJBP2* mutants by site-directed mutagenesis, using the WT *LtJBP2* construct for expression in *Leishmania* as a template. We replaced the four amino acids (2× His, 1× Arg and 1× Asp) critical for the function of the Fe(II)–2OG dependent dioxygenases by Ala (Table 1; mutants 2–5). We also designed a control *LtJBP2* mutant, in which a non-conserved Val in the vicinity of the critical residues is replaced by Ala. This construct was found to contain a random Leu to Gln mutation at position 548 in addition to the intended one (Table 1; mutant 1). The expression constructs for WT LtJBP2 and the five mutant-LtJBP2s were transfected into *LtJBP2*−/− cells, and cell lines stably producing the corresponding proteins were generated by selecting for paramomycin resistance. Expression levels of JBP2 were analyzed by Western blotting with anti-LtJBP2 antibodies. All the cell lines expressed a protein of the expected size (Fig. 3A), and the expression levels of LtJBP2 mutants 2–5 were repeatedly found to be higher than those of WT or mutant 1 (Fig. 3A). Immunofluorescent staining showed that all the mutant proteins localized to the nucleus in a pattern resembling that of the WT LtJBP2 (Fig. 3B). Thus, we conclude that the mutations introduced did not affect the folding or trafficking of LtJBP2 in the cells.

To examine the levels of J in the various LtJBP2-expressing cell lines, serially diluted genomic DNA was spotted on a membrane and the amount of J was determined by anti-J antibodies as described previously [9]. The expression of WT and mutant 1 LtJBP2 fully restored the WT J levels, but the amount of J in the cell lines expressing any of the other mutants was comparable to that in the parental *LtJBP2*−/− cells (Fig. 3C). These data strongly suggest that replacing any of the hallmark residues of the Fe(II)–2OG -dependent dioxygenases destroys JBP2's ability to stimulate J synthesis. To verify the results in another assay, we grew the cell lines in the presence of 0.6 μM BrdU for 4 days and determined the cell count thereafter. Cells expressing WT or mutant 1 LtJBP2 reached a density of 1×10^8 cells/ml in the course of the experiment, while the *LtJBP2*−/− cells and cells expressing LtJBP2 mutants 2–5 exhibited serious growth defects (Fig. 3D).

The results of this study show that the key residues conserved in the family of Fe(II)–2OG-dependent dioxygenases are critical for the function of LtJBP2, as they are for LtJBP1 [9]. LtJBP2 carrying a mutation in any of these residues is expressed and has a subcellular localization similar to that of the WT protein, but is unable to restore the levels of J in *LtJBP2*−/− cells. In addition, the sensitivity of the *LtJBP2*−/− cells to BrdU is unaffected by the expression of these mutant proteins, while WT and a control mutant LtJBP2 completely complement both of these phenotypes. Curiously, the non-functional mutants seem to be expressed at a higher level than WT or control mutant LtJBP2, suggesting that there is a selective pressure against high levels of functional JBP2 in *L. tarentolae*.

The hallmark amino acid signature of the Fe(II)–2OG-dependent dioxygenases is a unique feature of this class of enzymes, and required for full catalytic activity of all the family members studied thus far [13]. Hence, the results presented here lend further support to our earlier notion that JBP1 and JBP2 belong to the family of Fe(II)–2OG-dependent dioxygenases and could thus be the thymi-

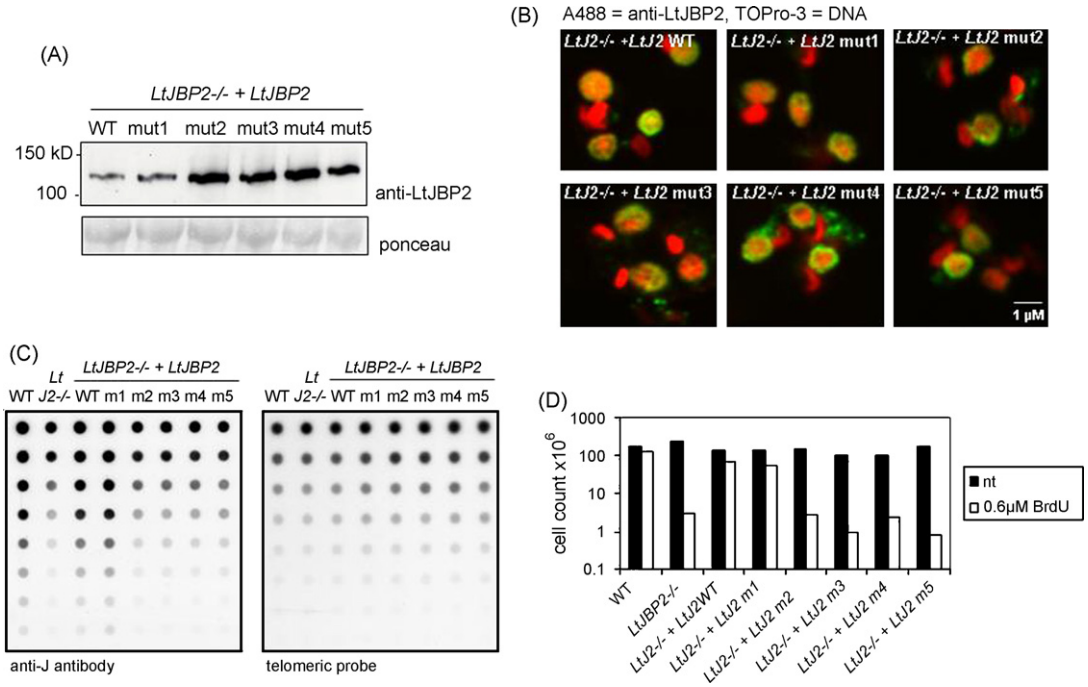

Fig. 3. Expression, sub-cellular localization and functionality of the LtJBP2 mutants. (A) Western blot to illustrate the expression levels of WT and the different mutant (mut) versions of LtJBP2, ectopically expressed in *LtJBP2*−/− cells (*LtJBP2*−/− + *LtJBP2*). The key to the numbering of the mutants is presented in Table 1. Ponceau-staining is shown as a loading control. (B) Immunofluorescent staining with anti-LtJBP2 antibodies to show the nuclear localization of the LtJBP2 variants. Cell lines (*LtJ2*−/− + *LtJ2* WT/mut) are the same as in (A). Only merged images of the LtJBP2 (Alexa 488) and the TOPro-3 signals are shown. The staining and imaging were performed as in Fig. 1. (C) Dot blot analysis of the levels of J in WT, *LtJBP2*−/− and the different *LtJBP2*−/− + *LtJBP2* cell lines. (D) Growth in the absence and presence of 0.6 μM BrdU. 1×10^5 cells of the indicated genotypes were plated −/+ BrdU and cell densities were determined after 4 days of culturing. Data from a representative experiment is shown.

dine hydroxylases responsible for the first step in J biosynthesis [9]. Efforts to directly demonstrate the enzymatic activity of these proteins *in vitro* have thus far failed, but the accumulating *in vivo* evidence motivates further studies.

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