Feb. 12, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 6702-edited.doc).

Title: Tbl3 encodes a WD40 nucleolar protein with regulatory roles in ribosome biogenesis.
An original research article

Author: Jindong Wang and Schickwann Tsai

Name of Journal: *World Journal of Hematology*

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The manuscript has been improved according to the suggestions of reviewers:
1 Format has been updated

2 Revision has been made according to the suggestions of the reviewers:

Reviewer 02446126:

Q(1) Is it possible to also see some level of tbl3 protein in nucleoplasm or is it prominent protein of nucleolus?

Answer: Over 99% of the tbl3-egfp protein is seen in nucleoli. We concur with the reviewer that there must be some tbl3 protein in the nucleoplasm. In fact, a very faint fine punctate or lacy pattern is visible in Fig. 3C at high magnifications. This minor fraction may represent a fraction transiting through the nucleoplasm on its way to nucleoli or a fraction with genuine extranucleolar function(s).

Q(2) Localization of tbl3-tagged by EGFP in nucleolus should be verified on endogenous level, by appropriate antibody.

Answer: Since the submission of the manuscript we have become aware of the availability of a commercial anti-tbl3 polyclonal rabbit antibody preparation (raised against a synthetic peptide). The staining pattern of this antibody (Novus Biologicals NBP1-92481) is also predominantly nucleolar with some punctate staining (visible at high magnifications) in other parts of nuclei. It is unclear if the minor punctate staining by this polyclonal antibody preparation represents staining of extranucleolar tbl3, cross-reacting epitopes or fixation artifacts. Regardless, both methods (EGFP-fusion protein localization in live cells and the NBP1-92481 antibody staining in fixed cells) demonstrate that tbl3 is predominantly if not exclusively a nucleolar protein.

Q(3) Overlay of Fig. 3C and 3D to see if tbl3-positive compartment of nucleoli is surrounded by DAPI-dense chromocenters.

Answer: An overlay of tbl3-EGFP and DAPI staining is now included (new Fig. 3E). No obvious relationship is noted.

Q(4) It will be nice to see colocalization of tbl3 with other well-known proteins of nucleolus.

Answer: We performed colocalization with a proven nucleolar protein, Rex, of HTLV 1 that contains a well-characterized nucleolar targeting sequence (NOS; Nosaka et al: Proc Natl Acad Sci USA 86:9798-9802, 1989) and observed that colocalization of tbl3-egfp and Rex NOS.

Q(5) Western blots should also show endogenous tbl3 when authors use an appropriate antibody.

Answer: The polyclonal anti-peptide antibody (Novus Biologicals NBP1-92481) demonstrates a single endogenous tbl3 of ~90kD, thus confirming our finding.

Reviewer 01453224:

Q(1) The relationship between ribosome biogenesis and hematology, which is explained in the last paragraph of DISCUSSION, should be described in INTRODUCTION.

Answer: We have modified the first paragraph of INTRODUCTION to emphasize the connection between ribosomopathy and well-known hematological disorders.

Q(2) In MATERIALS AND MEHTODS, the authors describe that they made two independent shRNAs for Tbl3…..They should show that similar results can be obtained by a second shRNA.

Answer: This was mentioned in MATERIALS AND METHODS. We have also amended the texts and legends of Fig. 5 and 6 in the revised manuscript to the same effect.

Q(3) The experiments in Tbl3-depleted cells suggested that in the absence of Tbl3, either 47S pre-rRNA transcription is upregulated or pre-rRNA processing is repressed…..they should examine whether RNA polymerase inhibitors (e.g. actinomycin D) block the increased in the level of 47S pre-rRNA in Tbl3-depleted cells.

Answer: As actinomycin D will (1) inhibit all three RNA polymerases and therefore introduce additional variables (e.g. by interfering with RNA polynerase II and the expression of most genes) into the system and (2) prevent the incorporation of ^{3}H-labeled uridine into newly synthesized rRNAs, we are afraid that such an experiment will be challenging to perform or interpret.

Q(4) Depletion of Tbl3 in promyelocytes and fibroblasts resulted in a reduced number of cells after several days of culture. Is it due to decreased rate of cell proliferation or increased rate of apoptotic cell death?

Answer: We observed the MPRO control vector- vs. shRNA-transfected cells closely in the first 1-2 weeks after completion of puromycin selection and did not see increased apoptosis (apoptotic MPRO cells display a distinct morphology in phase-contrast microscopy and Wright stain). The viability was 100% in both populations during this period.

Q(5a) In Fig. 4, the subcellular localization pattern of some typical Tbl3 mutants which did not localize to nucleoli should be shown in the figure or described in the text.

Answer: The distribution of non-nucleolus-localizing mutant Tbl3-EGFP proteins appear diffusely in the cytoplasm and nucleoplasm and excluded from the nucleoli. This pattern is indistinguishable from that of EGFP per se. We have added this description to the legend of Fig. 4.

Q(5b) The authors suggest in RESULT and DISCUSSION that Tbl3 is possibly localized to nucleoli through WD40 repeats. This does not make sense to this reviewer.

Answer: We have modified the last sentence in the first paragraph of DISCUSSION to "tbl3 likely associates with additional nucleolar proteins via other topological features (in conjunction with or independent of WD40 protein-protein interactions) and this forms the basis for its nucleolar localization".

Reviewer 01573121:

Q(1) The authors should provide more quantitative data on the lack of an effect of tbl3 depletion on rRNA processing.

Answer: We analyzed the signal volumes of key bands of lane 10 (negative control 1) and lane 12 (shRNA-transfected) in Fig. 5B. We then assigned the signal strength of the 28S rRNA as 1.00 in each

lane to facilitate comparison. The ratio of ^{3}H-uridine-labeled 32S : 28S : 18S rRNAs are 0.47 : 1.00 : 0.66 for negative control 1 and 0.28 : 1.00 : 0.65 for the shRNA group. Thus, there is no evidence of delayed processing in the shRNA group. This is reinforced by the nearly identical 28S : 18S rRNA ratios (1.00 : 0.66 vs. 1.00 : 0.65) . This information is included in the revised text.

Q(2) The authors should measure the decay rate of 47S (using the data of Fig. 5) to confirm that it is not affected by tbl3 depletion.

Answer: The 47S pre-rRNA band intensity was analyzed and a new graph is added (new Fig. 5D). This graph revealed that peak ^{3}H-uridine incorporation occurred earlier (30 vs. 60 minutes) in the shRNA group, consistent with the notion that tbl3 knockdown increases the rate of 47S pre-rRNA synthesis. During the most relevant and analyzable period (i.e. 60 to 90 minute), the decrease of 47S pre-rRNA in the negative control parallels that in the shRNA group. In the later phase (i.e. 90 to 180 minute), there is also no evidence that the decrease of 47S pre-rRNA is delayed in the shRNA group judging from the slopes of decrease.

Q(3) They should also measure the decay rate of mature rRNA by extending the chase time of the experiment shown in Fig. 5.

Answer: One concern was that extending the chase period beyond 3 hours (much longer than the normal processing time) would likely introduce new variables (e.g. different cell cycle progression, cell division, transport and metabolic rate) into the system and therefore complicate the interpretation. Therefore, we focused on the most critical and analyzsable period, i.e. 30-90 or 30-180 min following pulse and chase.

Q(4) The authors mention that longer time of selection of transfectants allows the recovery of growth rate. They should check if this is due to the recovery of 47S level.

Answer: That is the case.

Q(5) The steady-state level of rRNA after depletion of tbl3 should be analyzed by a more quantitative technique.

Answer: We directly quantified rRNAs by spectrophotometry and detected no significant differences in the steady-state levels.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Hematology*.

Sincerely yours,

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