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RESEARCH ARTICLE

REVISED **A mouse model for functional dissection of TAB1 O-GlcNAcylation [version 2; peer review: 2 approved]**

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Abstract

Background: O-GlcNAcylation is a posttranslational modification associated with various physiological and pathophysiological processes including diabetes, cancer, neurodegeneration and inflammation. However, the biological mechanisms underlying the role of specific O-GlcNAc sites and their link to phenotypes remain largely unexplored due to lack of suitable *in vivo* models. TGF- β activated kinase-1 binding protein-1 (TAB1) is a scaffolding protein required for TGF- β activated kinase-1 (TAK1) mediated signalling. A single O-GlcNAc site has been identified on human TAB1 that modulates TAK1-mediated cytokine release in cells.

Methods: Here, we report the generation of the *Tab1*^{S393A} mouse model using a constitutive knock-in strategy. The *Tab1*^{S393A} mice carry a Ser393Ala (S393A) mutation that leads to loss of O-GlcNAcylation site on TAB1.

Results: We did not observe any obvious phenotype in *Tab1*^{S393A} mice. Loss of O-GlcNAcylation on TAB1 has no consequences on TAB1 protein level or on TAB1-TAK1 interaction.

Conclusions: The homozygous *Tab1*^{S393A} mice are viable and develop with no obvious abnormalities, providing a powerful tool to further investigate the role of O-GlcNAc on TAB1 in the inflammatory response in the context of a whole organism.

Keywords

O-GlcNAcylation, TAB1, TAK1 signalling, inflammation

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Any reports and responses or comments on the article can be found at the end of the article.

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REVISED Amendments from Version 1

This version of the manuscript addresses all comments and suggestions by the reviewers. The following changes have been made.

Main text: We have clarified that the Ser393 residue on mouse TAB1 was the sole reported O-GlcNAc site *in vivo* with the addition of two references.

Figure 1: We have added an additional panel that shows the sequence alignment of mouse and human TAB1 covering the C-terminal region. The panel B (originally panel A) has been updated and shows the locations of restrictions sites, primers and probes.

Figure 2: We have added an additional panel that provides the DNA sequences of WT and mutant homozygous *Tab1*^{S393A} mice that confirms the presence of the S393A single point mutation and we amended the methods section accordingly.

Figure 3: We have clarified the unit of the Y-axis that shows now the normalised weights of the hearts in milligram per g of body weight (mg/g) on panel B. We have indicated negative (IgG) and positive controls (macrophage lysates) used for immunoblots in panel C and D, with the original unedited full gels deposited in the Figshare data repository (<https://doi.org/10.6084/m9.figshare.9206567.v1> (Authier, 2019a)).

Any further responses from the reviewers can be found at the end of the article

Introduction

O-GlcNAcylation is a dynamic and reversible post-translational modification on serine and threonine residues of intracellular proteins. It is catalysed by two enzymes, O-linked N-acetylglucosaminyltransferase (OGT) and β -N-acetylglucosaminidase (O-GlcNAcase, OGA), responsible for the addition and removal of the O-GlcNAc group, respectively (Hart *et al.*, 2011). O-GlcNAcylation modulates several cellular processes, such as transcription (Hanover *et al.*, 2012), cell signalling (Muller *et al.*, 2013; Zachara *et al.*, 2004), and metabolism (Ruan *et al.*, 2013). Changes in O-GlcNAc levels are associated with pathological conditions including diabetes, neurodegeneration and cancer (Issad *et al.*, 2010; Lazarus *et al.*, 2009; Slawson *et al.*, 2010). In addition, accumulating evidence suggests that O-GlcNAc modification controls the maturation and activation of immune cells, including T cells, B cells and macrophages (Golks & Guerini, 2008; Li *et al.*, 2019; Machacek *et al.*, 2019).

O-GlcNAcylation has been shown to affect the transforming growth factor (TGF)- β -activated kinase 1 (TAK1) signalling pathway (Mendoza *et al.*, 2008). The TAK1 pathway regulates inflammatory cytokine production and release in response to pro-inflammatory and endotoxin stimuli in macrophages and innate immune cells. TAK1 forms a functional complex with the pseudo-phosphatase TAK1 binding protein-1 (TAB1) and regulates the production of inflammatory molecules through activation of several mitogen-activated protein kinases (MAPKs) including p38 α , extracellular signal-regulated kinases (ERKs) and c-jun kinases, leading to subsequent activation of downstream effectors including the I κ B kinases (IKKs) and the transcription factor NF κ B (Adhikari *et al.*, 2007; Conner *et al.*, 2006; Sato *et al.*, 2005; Shibuya *et al.*, 1996; Shim *et al.*, 2005; Wang *et al.*, 2001). TAB1 is required for TAK1 activation and

downstream signalling events. TAK1 activity can be negatively regulated by p38 α MAPK through a feedback mechanism where p38 α MAPK phosphorylates TAB1, leading to TAK1 activity suppression (Cheung *et al.*, 2003). The TAK1-TAB1 complex has been found to be important *in vivo* for the survival of activated macrophages upon lipopolysaccharide (LPS) stimulation and for the modulation of immune response in T and B cells (Mihaly *et al.*, 2014; Sato *et al.*, 2005).

We previously showed that human TAB1 is modified with N-acetylglucosamine (O-GlcNAc) on a single site, Ser395, in human cells. O-GlcNAcylation of TAB1 is induced under stress conditions and modulates TAK1-mediated cytokine release *in vitro* by increasing TAK1 activation, leading to downstream signalling activation and cytokine production in mouse embryonic fibroblasts (MEFs) (Pathak *et al.*, 2012). However, the *in vivo* biological significance of this single O-GlcNAc site remains to be explored.

Here, we describe the generation of a genome edited constitutive knock-in (KI) mouse model expressing endogenous TAB1 lacking the key residue targeted for O-GlcNAcylation. The Ser393 site, equivalent to Ser395 in the human TAB1 sequence, was abolished by introducing a Ser393Ala mutation using a classical recombinational approach. We demonstrate that this mutation causes the loss of the O-GlcNAc modification on TAB1 without affecting TAB1 protein levels or its interaction with TAK1. Homozygous *Tab1*^{S393A} mice lacking O-GlcNAc on TAB1 were viable with no obvious development abnormalities. This work provides a platform for exploration of O-GlcNAc-dependent functions of TAB1.

Results

Generation of *Tab1*^{S393A} KI mice

We previously showed that human TAB1 is modified with N-acetylglucosamine (O-GlcNAc) on a single site, Ser395 (Pathak *et al.*, 2012). TAB1 sequence is 97% identical between human and mouse. The O-GlcNAc modified Ser395 residue in human is conserved in mice and corresponds to the Ser393 (Figure 1A). The Ser393 residue is the sole reported O-GlcNAc site on mouse TAB1 (Alfaro *et al.*, 2012; Trinidad *et al.*, 2012). To establish an animal model for investigating the role of O-GlcNAc on TAB1 *in vivo*, we generated TAB1 constitutive knock-in (KI) mice carrying a Ser393Ala (S393A) mutation. The *Tab1* gene is located on mouse chromosome 15 and contains 11 exons. The nucleotide sequence encoding the amino acid S393 region is located on exon 10. The targeting vector contained the translation initiation codon in exon 1 and an FRT-flanked puromycin cassette in the intronic sequence between exons 9 and 10. The targeted allele was obtained via homologous recombination in C57Bl/6NTac ES cells. The constitutive KI-point mutation (PM) allele was obtained after *in vivo* Flp recombination (Figure 1B). Positive targeted ES cell clones were confirmed by Southern blot analysis. Correct homologous recombination at 5' and 3' sides was detected in A-A08, A-C04, B-G06 clones (Figure 2A). A single integration site was also confirmed by Southern blot analysis using the *cag* probe, which detects a region located within the CAG_PuroR_pA selection cassette. Correct single

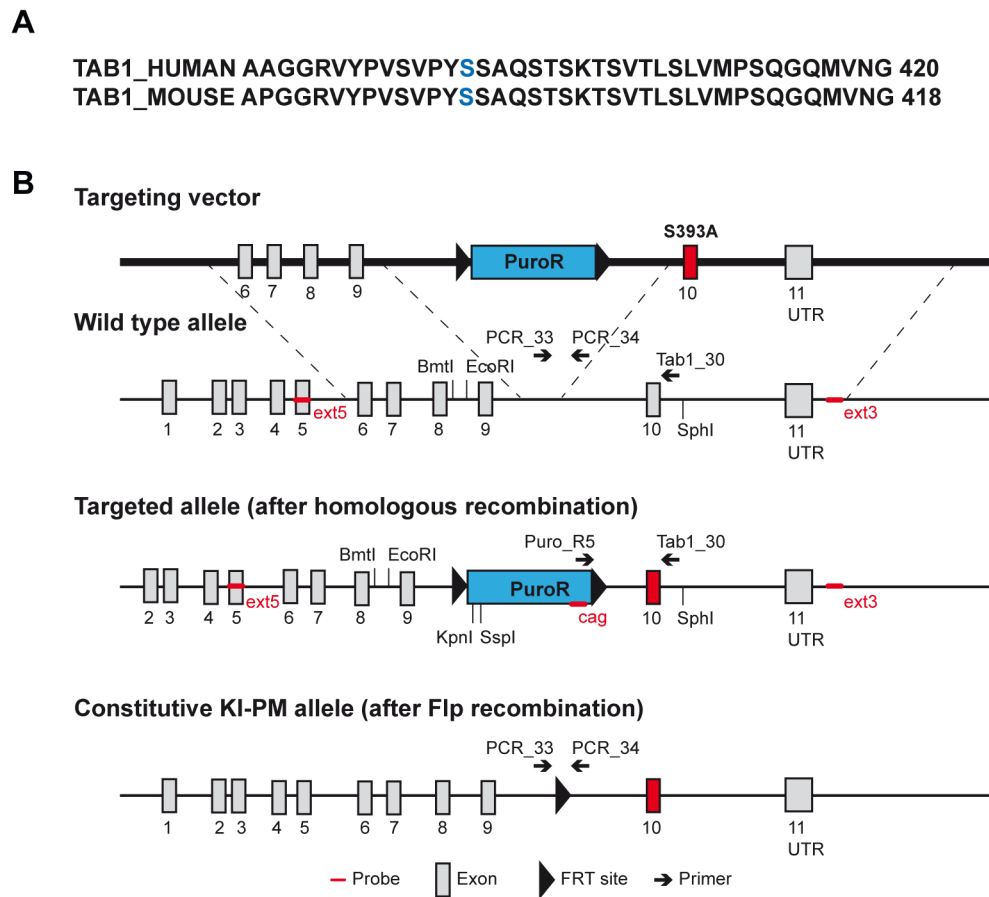


Figure 1. Targeting strategy for the generation of constitutive *Tab1*^{S393A} knock-in (KI) mice. (a) Sequence alignment of human (UniProt Q15750) and mouse (UniProt Q8CF89) TAB1 covering the 382-420aa of the C-terminal domain. The human and mouse O-GlcNAc sites are reported in blue. (b) Schematic representation of the targeting vector containing a flippase recognition target (FRT)-flanked puromycin resistance cassette in intron 9, wild type allele, targeted allele after homologous recombination and the constitutive KI point mutation (PM) allele after flippase (Flp) recombination. The locations of the enzymatic restriction sites, primers and probes are also shown.

integration was detected in A-A08, A-C04, B-G06 clones (Figure 2B upper panels and left lower panel). The insertion of the point mutation was validated by PCR genotyping and detected in all the targeted clones (Figure 2B right lower panel). The A-A08 and A-C04 positive clones were injected into BALB/c blastocysts to generate chimeric mice. Genotyping was performed to identify constitutive KI animals. The presence of the S393A mutation was confirmed by sequencing (Figure 2C). Heterozygous mice were backcrossed to C57BL/6J genetic background and maintained as heterozygotes. Homozygous *Tab1*^{S393A} mice were obtained by breeding heterozygous offspring. Thus, we successfully generated TAB1 S393A KI mice lacking the O-GlcNAc site at Ser393.

***Tab1*^{S393A} KI mice exhibit normal development and survival**
 We used *Tab1*^{S393A/+} heterozygous animals to generate homozygous *Tab1*^{S393A} animals. We obtained 19 WT, 68 *Tab1*^{S393A/+} and 17 *Tab1*^{S393A} animals from heterozygous pairs. Although deletion of TAB1 leads to embryonic lethality and abnormal cardiovascular

development in mice (Komatsu *et al.*, 2002), the homozygous *Tab1*^{S393A} mice lacking O-GlcNAc on TAB1 were viable and developed normally. The *Tab1*^{S393A} mice appeared healthy and similar to WT animals. They showed comparable body weight and heart size to WT at three months of age (Figure 3A and 3B; Authier, 2019b). In addition, newborn pups were obtained by breeding of either male or female homozygous *Tab1*^{S393A} mice with sex-matched WT animals, suggesting that fertility is not altered in these animals.

TAB1 is ubiquitously expressed in mammals (Komatsu *et al.*, 2002; Shibuya *et al.*, 1996). To verify that the S393A mutation leads to absence of O-GlcNAcylation of TAB1 in the transgenic animals, brain tissues were isolated from WT and *Tab1*^{S393A} mice and TAB1 was immunoprecipitated prior to probing with a site specific O-GlcNAc TAB1 antibody (Pathak *et al.*, 2012). As expected, TAB1 was found glycosylated in WT animals, while the S393A mutation led to absence of O-GlcNAc on TAB1 in *Tab1*^{S393A} mice (Figure 3C).

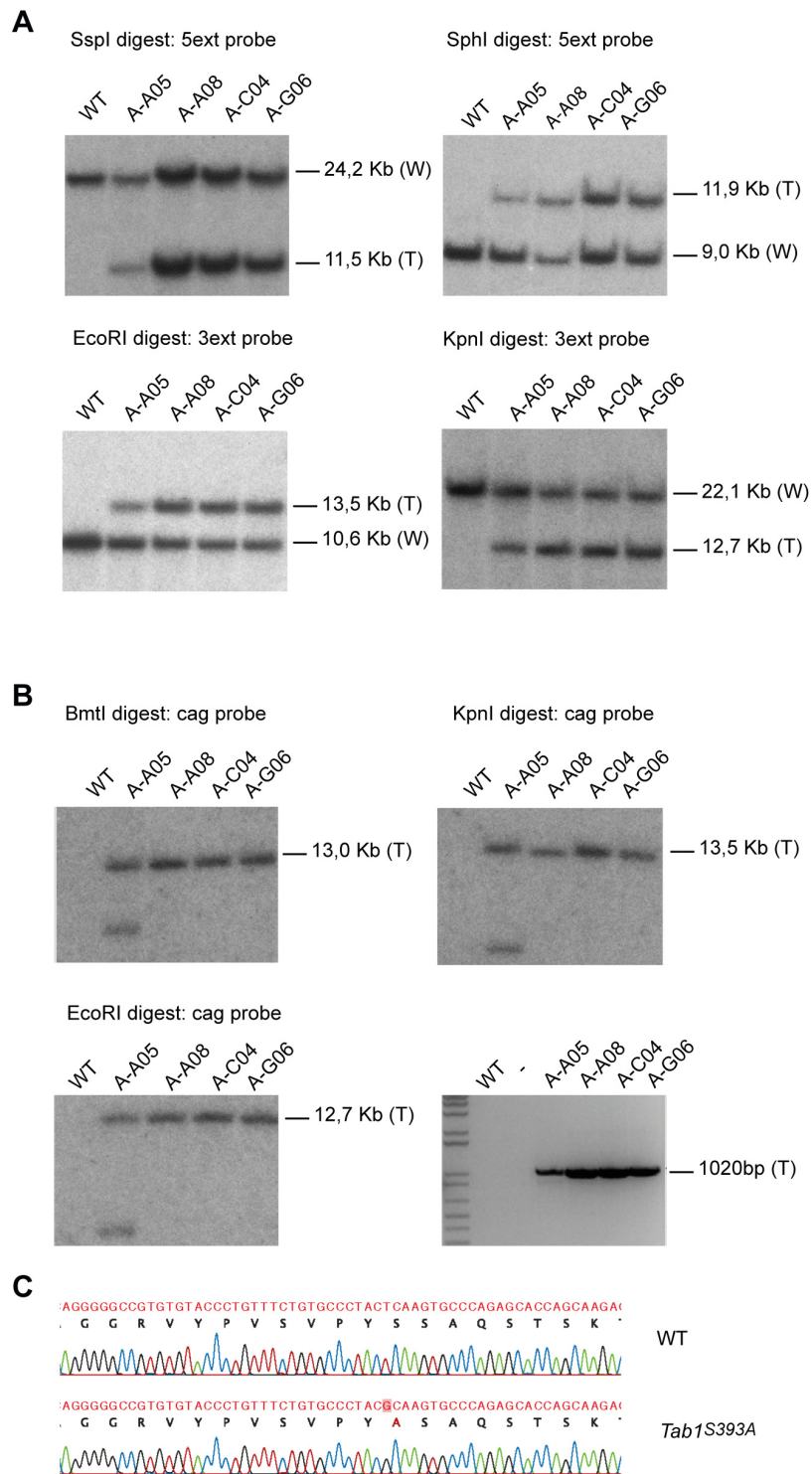


Figure 2. Generation of correctly targeted embryonic stem (ES) clones bearing the constitutive knock-in (KI) S393A point mutation in TAB1. (a) Southern blot of wild-type (WT) and four targeted ES clones (named A-A05, A-A08, A-C04 and A-G06) with 5' ext and 3' ext probes shows correct homologous recombination at the 5' and 3' sides in all clones except for A-C05. The expected molecular weight band for WT (W) and the targeted (T) alleles are shown. (b) Southern blot of WT and four targeted ES clones (named A-A05, A-A08, A-C04 and A-G06) with the cag probe shows single integration in all clones except for A-C05 (upper left and right panels and lower left panel). The expected molecular weight band for the targeted (T) allele is shown. PCR of WT and four targeted ES clones (named A-A05, A-A08, A-C04 and A-G06) shows insertion of the point mutation in all targeted clones (lower right panel). The expected molecular weight band for the targeted (T) allele is shown. (c) Sequencing of the genomic DNA from WT and KI *Tab1*^{S393A} mice confirming the S393A single point mutation (in red) in KI animal.

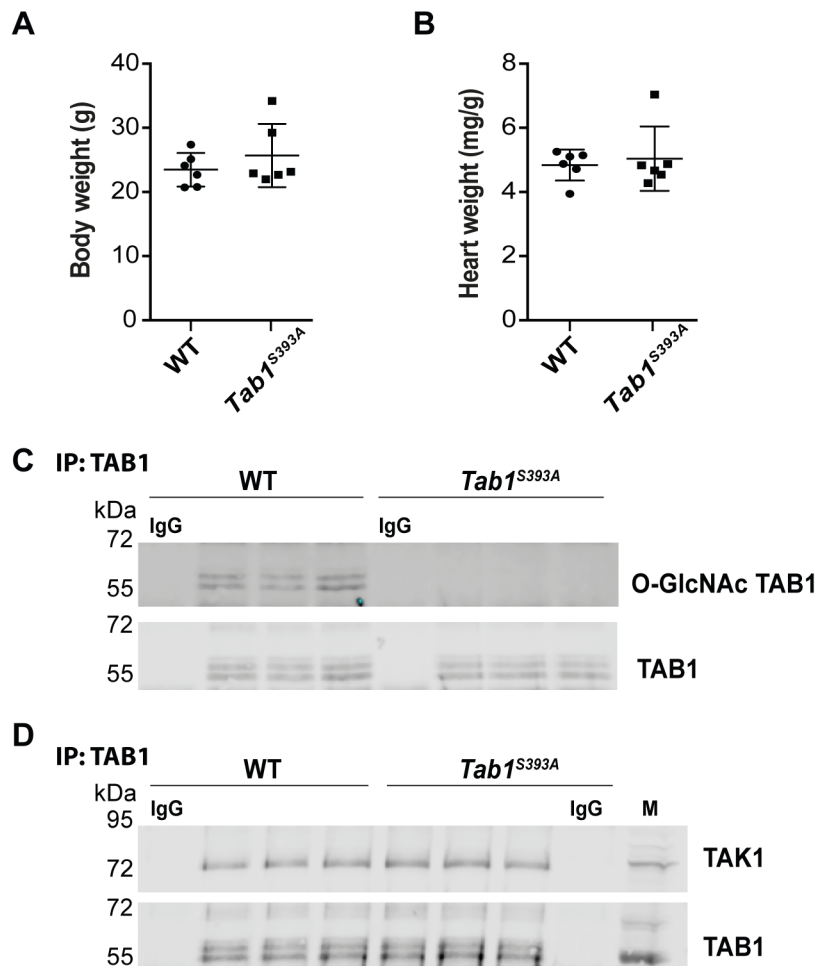


Figure 3. *Tab1^{S393A}* knock-in (KI) mice lacking O-GlcNAcylation on TAB1 exhibit normal development and retained TAB1-TAK1 interaction. (a) Body weight of wild-type (WT) and *Tab1^{S393A}* KI at three months old. Data are expressed as the mean \pm SD (WT, n=6; *Tab1^{S393A}*, n=6), Student's t-test. (b) Heart weight per g of body weight of WT and *Tab1^{S393A}* KI at three months old. Data are expressed as the mean \pm SD (WT, n=6; *Tab1^{S393A}*, n=6), Student's t-test. (c) TAB1 protein immunoprecipitation (IP) and blotting using anti-gTAB1 and anti-TAB1 antibodies in brain tissues of WT and *Tab1^{S393A}* mice shows loss of O-GlcNAcylation of TAB1 in *Tab1^{S393A}* compared to WT animals with similar TAB1 protein levels in both genotypes (WT, n=3; *Tab1^{S393A}*, n=3). (d) TAB1 protein immunoprecipitation (IP) and blotting using anti-TAK1 and anti-TAB1 antibodies in brain tissues of WT and *Tab1^{S393A}* mice shows retention of TAB1-TAK1 interaction in both genotypes (WT, n=3; *Tab1^{S393A}*, n=3). IgG: Isotype control; M: mouse macrophage lysate.

We next investigated the impact of the S393A mutation on TAB1 protein levels. We observed similar levels of TAB1 protein expression in WT and *Tab1^{S393A}* animals suggesting that absence of O-GlcNAc of TAB1 has no detectable influence on TAB1 protein levels (Figure 3C). Thus, *Tab1^{S393A}* KI mice lacking O-GlcNAc on TAB1 exhibit normal development and survival.

O-GlcNAcylation of TAB1 is not required for its interaction with TAK1

TAB1 is found in complex with TAK1 and this interaction is required for TAK1-mediated signalling (Mendoza *et al.*, 2008). To investigate the effect of loss of O-GlcNAcylation on TAB1 on the interaction with TAK1, immunoprecipitation analysis was performed on brain tissue lysates from WT and *Tab1^{S393A}* mice. We found that TAB1 interacts with TAK1 in both WT and

transgenic animals, suggesting that O-GlcNAcylation of TAB1 is not required for its interaction with TAK1 (Figure 3D).

Discussion

Although O-GlcNAcylation was discovered over 30 years ago, our knowledge on the biological processes regulated by site-specific O-GlcNAcylation of specific proteins is limited. Generation of transgenic animal models lacking specific O-GlcNAc sites will help to decipher the role of individual O-GlcNAc sites and associated mechanisms in physiological and pathologic conditions. In the present study we have described the generation of a viable constitutive KI mouse model leading to loss of the sole reported O-GlcNAc site S393 on TAB1 protein, allowing for determining the biological significance of TAB1 O-GlcNAcylation *in vivo*.

A growing body of evidence supports that O-GlcNAcylation is an important modulator of the immune response and inflammation (Golks & Guerini, 2008; Golks *et al.*, 2007; Kearse & Hart, 1991). However, the role of this modification on specific proteins involved in the function and regulation of immune cells remains largely unknown. The TAB1-TAK1 complex regulates the activation, survival and maintenance of activated macrophages upon LPS stimulation *in vivo* (Mihaly *et al.*, 2014). O-GlcNAcylation of TAB1 modulates TAK1-mediated cytokine release in response to IL-1 stimulation or osmotic stress in MEF cells (Pathak *et al.*, 2012). However, these pathways have not been investigated *in vivo*. The *Tab1*^{S393} mice model will allow investigation of the consequences of the loss of O-GlcNAc of TAB1 on TAK1 activation and signalling in the context of a whole organism.

TAB1 is also involved in a negative feedback loop in which p38 α MAPK suppresses TAK1 activation through phosphorylation of TAB1 at two residues; Ser423 and Thr431 (Cheung *et al.*, 2003). The p38 α MAPK is a protein serine-threonine kinase involved in signalling pathways induced by stress. It is activated by transphosphorylation of the TGY motif by MAPK kinases (MKKs) 3, 4 and 6 (Enslin *et al.*, 1998; Raingeaud *et al.*, 1996). Besides being a substrate for p38 α MAPK, TAB1 binds to p38 α , leading to its autophosphorylation of Thr180 and Tyr182 residues (Ge *et al.*, 2002). This mechanism constitutes an alternative activation pathway for p38 α MAPK that has been demonstrated to be independent to the MKK cascade and does not lead to classical p38 α MAPK downstream signalling and inflammatory gene expression (Lu *et al.*, 2006). The autoactivation of p38 α MAPK through TAB1 was observed in pathological conditions, including cardiac ischemia and heart failure and has been shown to aggravate myocardial injury (De Nicola *et al.*, 2018; Li *et al.*, 2005; Shi *et al.*, 2010; Tanno *et al.*, 2003). A recent study has demonstrated that the p38 α -TAB1 interaction can be targeted by small molecules (De Nicola *et al.*, 2018). The same study reported that reduction of infarction volume was observed in a KI mouse model in which residues located in the p38 α docking region of TAB1 were mutated to prevent the p38 α -TAB1 interaction (De Nicola *et al.*, 2018). Thus, the development of strategy to target the TAB1-p38 α MAPK may be therapeutically relevant. Interestingly, the single O-GlcNAc site of TAB1 is located within this p38 α MAPK binding region (DeNicola *et al.*, 2013; Pathak *et al.*, 2012), which is required for p38 α MAPK activation (Ge *et al.*, 2002). It is possible that O-GlcNAc on TAB1 could play a role in modulating the TAB1-p38 α interaction and subsequent p38 α MAPK autoactivation. In the future, both *in vitro* and *in vivo* experiments could be performed to test this hypothesis. *Tab1*^{S393A} mice lacking O-GlcNAc on TAB1 will provide a suitable model to investigate the potential effect of this posttranslational modification on p38 α MAPK signalling during cardiac injury in mice.

In our study, we did not observe any obvious phenotype in *Tab1*^{S393A} mice. Given the role of TAB1 in immune response

and its interactors, it is likely that induction of stress as inflammation could reveal potential phenotypes. We conclude that this model could be a valuable tool to further investigate the functional relevance of the sole reported O-GlcNAcylation site of mouse TAB1 in a whole organism at several stages of development but also in pathological conditions.

Methods

Ethical statement

All efforts were made to ameliorate any suffering of animals by providing appropriate husbandry environment conditions (listed below) in accordance with UK and European Union regulations. All animal studies were conducted in accordance with the Animal (Scientific Procedures) Act 1986 for the care and use of laboratory animals, and procedures were carried out under United Kingdom Home Office regulation and the project licence PAAE38C7B with approval by the Welfare and Ethical Use of Animals Committee of University of Dundee. Mice were humanely killed using a Schedule 1 method as soon as any sign of distress or suffering were observed.

Animal source and husbandry

Mice were maintained on a C57BL/6J (Charles River UK) background and kept in groups of five animals in individually ventilated cages at 21°C, 45–65% relative humidity and a 12h/12h light/dark cycle under specific-pathogen-free conditions in accordance with UK and European Union regulations. Mice had access to a mouse house with sizzle-nest material for bedding and corn cob for nesting and free access to food (R&M3 pelleted irradiated diet) and 0.2 micron sterile filtered water.

Generation of TAB1 constitutive knock-in S393A mice

The TAB1 knock-in (KI) mouse line (C57BL/6NTac-*Tab1*^{tm3593(S393A)Arte}) carrying a S393A mutation was generated by Taconic Artemis GmbH using the targeting strategy illustrated in Figure 1 and based on the NCBI transcript NM_025609_2. The targeting vector contains the translation initiation codon in exon 1 and also contains a flippase recognition target (FRT)-flanked puromycin cassette in intron 9. The targeting vector was generated using bacterial artificial chromosome (BAC) clones from the C57BL/6J RPCIB-731 Tac BAC library and transfected by electroporation into C57BL/6NTac embryonic stem (ES) cell lines. Recombinant ES cell clones were identified by Southern blot using external (5' ext and 3' ext probes) and internal probes (cag). The genomic DNA of the ES cell clones were digested with *SspI* or *SphI* for the 5' ext probe and analysed for 11.5 kb (*SspI* digest) or 11.9 kb (*SphI* digest) fragments for the recombinant allele, in addition to 24.2 kb (*SspI* digest) or 9 kb (*SphI* digest) for the wild-type (WT) allele. For the 3' ext probe, the genomic DNA was digested with *EcoRI* or *KpnI* and analysed for 13.5 kb (*EcoRI* digest) or 12.7 kb (*KpnI* digest) fragments for the recombinant allele, and 10.6 kb (*EcoRI* digest) or 22.1 kb (*KpnI* digest) fragments for the WT allele. Single integration was also confirmed by Southern blot analysis using the cag probe that detects a region located within the puromycin selection cassette. The genomic

DNA of the ES cell clones was digested with *BmiI*, *EcoRI* or *KpnI* restriction enzymes and analysed for single fragments of 13.0 kb (*BmiI* digest), 13.5 kb (*EcoRI* digest) or 12.7 kb (*KpnI* digest) for the recombinant allele. The insertion of the point mutation S393A was validated by PCR genotyping using the Puro_R5 and Tab1_30 primers, recombinant Taq DNA polymerase (EP0402, Invitrogen) and T100 Thermal Cycler (Biorad). The following PCR program was used (98 °C for 2 min, 98 °C for 10 sec, 68 °C for 1 min, 40 cycles, 20 °C for 10 min). Two positive targeted ES cell clones, A-A08 and A-C04, were injected using piezo actuated microinjection pipette with an internal diameter of 12–15 µm into blastocyst cells from 10 superovulated 4/5 weeks old BALB/c female mice (BALB/cAnNTac, Taconic Artemis GmbH), which were later transferred into six pseudopregnant albino NMRI females (BomTac:NMRI, Taconic Artemis GmbH) and 31 chimeric mice were obtained. Chimerism was measured by coat colour contribution of ES cells to BALB/c host (black/white). Based on visual estimation, three highly chimeric (>75%) seven-week-old male mice were selected for further breeding with six age-matched flippase (Flp) deleter C57BL/6 female mice (*C57BL/6-Tg(CAG-Flpe)2 Arte*, Taconic Artemis GmbH) to remove the selection marker. From this breeding, 28 mice reached the weaning stage and were used for mouse genotyping analysis. Genomic DNA was extracted from ear notches collected during the mice identification procedure using an ear punch tool (Braitree Scientific, Inc) and analysed by PCR using recombinant Taq DNA polymerase (EP0402, Invitrogen) and T100 Thermal Cycler (Biorad). The PCR_33 and PCR_34 primers amplified bands of 259 bp and 334 bp for the WT and the constitutive KI, respectively, using the following PCR program (95 °C for 5 min, 95 °C for 30 sec, 60 °C for 30 sec, 72 °C for 1 min, 35 cycles, 72 °C for 10 min). The presence of the point mutation S393A was validated by DNA sequencing using the 10597_35 and 10597_36 primers DNA amplification and the MT93_dig_seq for the sequencing analysis with Applied Biosystems 3730 device (ThermoFisher Scientific). The primers sequences used were: 5' ext probe sense: AAGCTAAGGTGGCCCTCAAGCAC, 5' ext probe antisense: CACCGACATTGGCAACGTAGAG, 3' ext probe sense: TCTAAGGTCAGTGCCTATCATAACG, 3' ext probe antisense: GGACTTAACCTCTCGCTGTAAGACC, Puro_R5: CCGTAAGT-TATGTAACGCGGAACCTCC, Tab1_30: ATTGGAGGCCAAA-GATCCAGAGTCC, PCR_33: TCACTTCTCACCTTACCAGC, PCR_34: AATGGGAGGTGAGAGCTCC, 10597_35: AGGCCAC-TACCTTGGTTTCC, 10597_36: CTGTGTCTAATAAGACTC-CCTACCC, MT93_dig_seq: TGGAGAATTGGAGGCCAAAG.

Body weight measurement and tissue collection

Heterozygous offspring were used for breeding to generate homozygous *Tab1*^{S393A} mice. WT and homozygous *Tab1*^{S393A} mice were housed together in the same cage in groups of five animals. Body weight of six WT mice (three female and three male) and six homozygous *Tab1*^{S393A} mice (four female and two male) were measured at three months of age. Data collection was performed by an independent observer in a blinded manner. For tissue collection, mice were euthanized by carbon dioxide gas exposure in a rising concentration and brain and heart tissue samples were collected, weighted and rapidly snap frozen in liquid nitrogen.

Immunoprecipitation of TAB1 and immunoblotting

Endogenous TAB1 was immunoprecipitated as described previously (Pathak *et al.*, 2012). Briefly, mouse brain tissue from three WT and three homozygous *Tab1*^{S393A} mice was lysed in 50 mM Tris-HCl pH 7.4 0.1 mM EGTA, 1 mM EDTA, 1 % Triton-X100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1 % 2-mercaptoethanol supplemented with protease inhibitors (1 mM benzamide, 0.2 mM PMSF and 5 µM leupeptin) and 10 µM GlcNAcstatin G. Brain lysate was centrifuged with 14,000 rpm for 20 minutes at 4 °C and the protein concentration was determined with Pierce 660 nm protein assay (22660, ThermoFisher Scientific). Cleared brain lysate containing 1mg of input protein was incubated for two hours at 4 °C with 10 µg of anti-TAB1 (sheep polyclonal S823A, Division of Signal Transduction Therapy, DSTT, University of Dundee) or anti-IgG antibody coupled with 10 µl of Dynabeads Protein G (10009D, ThermoFisher). The beads were washed twice with 1 ml of lysis buffer containing 0.25 M NaCl, followed by two washes with 1 ml of 50 mM Tris/HCl, pH 7.5, 50 mM NaCl and 0.1 % (v/v) 2-mercaptoethanol. Eluted samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting (Pathak *et al.*, 2012). Membranes were incubated with primary antibodies specific to in-house produced gTAB1 (rabbit polyclonal, 1:1000, University of Dundee) (Pathak *et al.*, 2012), TAB1 (sheep polyclonal S823A, 1:1000, DSTT) and TAK1 (sheep polyclonal S828A, 1:1000, DSTT) in blocking buffer, 5 % BSA in TBST (Tris-buffered saline with 0.1 % Tween-20) overnight at 4 °C and next with IR680 (catalogue # 925-68024, Li-Cor) and IR800 (RRID: AB_621848, catalogue # 926-32213, Li-Cor) donkey labelled secondary antibodies at room temperature for one hour. Blots were imaged using Li-Cor Odyssey infrared imaging system (Li-Cor). Original unedited gels and details of the controls used can be found here (Authier, 2019a).

Statistical analysis

Statistical analyses were performed with GraphPad Prism version 6 software. For pairwise comparisons of WT and *Tab1*^{S393A} body weight and heart size data, the Student's t-test was used.

Data availability

Underlying data

Figshare: Original unedited gels. <https://doi.org/10.6084/m9.figshare.9206567.v1> (Authier, 2019a)

Figshare: Body weight and heart weight raw data. <https://doi.org/10.6084/m9.figshare.9206546> (Authier, 2019b)

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/) (CC-BY 4.0).

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Kuo-I Lin 

Genomics Research Center, Academia Sinica, Taipei, Taiwan

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 07 October 2019

<https://doi.org/10.21956/wellcomeopenres.16824.r36480>

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Gian F. De Nicola

British Heart Foundation Centre of Excellence, The Rayne Institute, St. Thomas' Hospital, King's College London, London, UK

Authier et al. in their manuscript characterise a new knock-in mouse with a Serine to Alanine mutation on residue 393 of TAB1. This leads to a loss of the only O-GlcNAcylation site on TAB1. They confirm the loss of O-GlcNAcylation using an in house site specific anti g-TAB1 antibody. They go on to characterise the mice at baseline where they do not observe any obvious phenotype.

This new mouse model will be a valuable tool to investigate the role of GlcNAcylation in MAPK signalling in homeostasis and pathological settings.

The manuscript is a brief report on a preliminary characterisation of a new knock-in mouse model at baseline, the characterisation is quite limited and there could be few more experiments that could expand the characterisation but I am not sure whether they would go beyond the scope of the journal.

For instance:

1. it would be interesting to measure protein and mRNA levels of TAB1 in few more tissues and organs to test if O-GlcNAcylation has any role in protein stability.
2. TAB1 knock-out is embryonic lethal, in KOs, heart development and myocardial cell differentiation are impaired, it would be therefore useful to compare basic heart functions (heart rate, blood pressure, LV ejection fraction) between KI and WT at baseline, it could reveal a subtle difference that is exacerbated upon stress.
3. TAB1 is both a substrate and activator of p38alpha which sits downstream of TAK1. It would be interesting to compare the levels of p38alpha and TAB1 phosphorylation to test whether TAB1 ser 393 O-GlcNAcylation affects TAK1 signalling at baseline.

The authors observe that it is likely that the induction of stresses such as inflammation or ischaemia might reveal potential phenotypes. Testing such hypothesis would be of great interest but probably as I mentioned it goes beyond the scope of this journal, nevertheless this new mouse model is a useful tool to investigate the biology of TAB1 O-GlcNAcylation.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: MAPK signalling. protein-protein inhibitors

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 11 Jun 2020

Daan van Aalten, University of Dundee, Dundee, UK

We thank the referees for their critical reading of the manuscript and constructive feedback. Several points for improving the manuscript were made, in addition to good suggestions for further

studies. Our aim of publishing this work in a rapid open access journal such as Wellcome Open Research was to report the availability of this mouse model, facilitating future research to dissect the role of this specific O-GlcNAc site on TAB1. As such, most of the experiments proposed are outside the scope of this manuscript and it is hoped our model will allow such experiments to be conducted in the future.

Reviewer 2:

The manuscript is a brief report on preliminary characterisation of a new knock-in mouse model at baseline, the characterisation is quite limited and there could be few more experiments that could expand the characterisation but I am not sure whether they would go beyond the scope of the journal.

For instance:

1. it would be interesting to measure protein and mRNA levels of TAB1 in few more tissues and organs to test if O-GlcNAcylation has any role in protein stability.
2. TAB1 knock-out is embryonic lethal, in KOs, heart development and myocardial cell differentiation are impaired, it would be therefore useful to compare basic heart functions (heart rate, blood pressure, LV ejection fraction) between KI and WT at baseline, it could reveal a subtle difference that is exacerbated upon stress.
3. TAB1 is both a substrate and activator of p38alpha which sits downstream of TAK1. It would be interesting to compare the levels of p38alpha and TAB1 phosphorylation to test whether TAB1 ser 393 O-GlcNAcylation affects TAK1 signalling at baseline.

We agree with the referee that this mouse model now enables a number of hypothesis as to the role of TAB1 O-GlcNAcylation to be tested, but as the referee suggests, this paper is focused on describing the generation of the model, and demonstration of loss of O-GlcNAcylation. The experiments the referee proposes are excellent suggestions, but beyond the scope of this manuscript.

Competing Interests: No competing interests were disclosed.

Reviewer Report 19 September 2019

<https://doi.org/10.21956/wellcomeopenres.16824.r36321>

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Kuo-I Lin

Genomics Research Center, Academia Sinica, Taipei, Taiwan

This manuscript described a mouse model for studying the function of O-GlcNAcylation of TAB1 by generating a conventional knock-in (KI) mouse in which the single O-GlcNAcylation site at TAB1 Ser 393 was mutated to Ala (*Tab1*^{S393A}). The homozygous mutation mice were viable and had no obvious abnormalities. The authors further showed that loss of O-GlcNAcylation of TAB1 did not affect TAB1 protein level or interaction with TAK1. The authors concluded that this *Tab1*^{S393A} KI mouse line could be

a good tool to study the role of O-GlcNAcylation on TAB1 in inflammation responses. Overall, this knock-in mouse line can be a potentially useful tool to study the importance of O-GlcNAcylation on TAB1 in inflammation. However, there are some major points need to be clarified and addressed:

1. Although the information was described in the Methods, the authors should also indicate the location of the restriction enzyme sites, primers and probes used in Figure 1. This will help the readers to understand the strategy. The authors should also provide the raw data of genomic sequencing containing the sequences encoding 393 Ala in KI and 393 Ser in WT mice.
2. It has been reported that Tab1-deficiency caused embryonic lethality, due to the defects in lung and cardiovascular morphogenesis.¹ The authors showed that the heart weight of *Tab1*^{S393A} mouse and WT mouse is comparable (Figure 3). It is unclear if there are any morphological abnormalities in the heart and lung of *Tab1*^{S393A} mice. Histological analyses of heart and lung tissues should be done here. In addition, Figure 3B shows the heart weight is comparable between *Tab1*^{S393A} and WT mouse at three months old. The unit in Y-axis should be clarified.
3. The title of this report is "A mouse model for functional dissection of TAB1 O-GlcNAcylation". To convince the readers that this mouse model can be used to dissect the function of TAB1 O-GlcNAcylation, the authors should demonstrate the potential biological effects of losing O-GlcNAcylation on TAB1 Ser393.
4. In Figure 3C, the authors used site-specific antibody to detect TAB1 O-GlcNAcylation in mouse cells. Although it has been reported that human TAB1 is O-GlcNAcyated on a single site at Ser395 (which is equivalent to Ser393 in mouse), it's not clear if mouse TAB1 is also only modified by O-GlcNAcylation on a single site or actually on multiple sites. The authors should clarify this point by experiments.
5. In Figure 3D, the authors showed that, as compared with WT TAB1, TAB1^{S393A} interacts normally with TAK1. More appropriate controls, such as the negative control antibody or treatment with LPS, should be included. For example, it has been reported that O-GlcNAcylation of TAB1 is induced upon IL-1 stimulation or osmotic stress.² It will provide better information if the experiments in Figure 3C and Figure 3D can be conducted in cells under stimulation or stress.
6. The authors claimed that this KI mouse line could be a good model for further study of the inflammatory diseases, but mouse brain tissues were used in the co-IP experiments in Figure 3. It will be more informative if immune cells from *Tab1*^{S393A} mice can be included for comparison with WT immune cells.

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 11 Jun 2020

Daan van Aalten, University of Dundee, Dundee, UK

We thank the referees for their critical reading of the manuscript and constructive feedback. Several points for improving the manuscript were made, in addition to good suggestions for further studies. Our aim of publishing this work in a rapid open access journal such as Wellcome Open Research was to report the availability of this mouse model, facilitating future research to dissect the role of this specific O-GlcNAc site on TAB1. As such, most of the experiments proposed are outside the scope of this manuscript and it is hoped our model will allow such experiments to be conducted in the future.

Reviewer 1:

1. Although the information was described in the Methods, the authors should also indicate the location of the restriction enzyme sites, primers and probes used in Figure 1. This will help the readers to understand the strategy. The authors should also provide the raw data of genomic sequencing containing the sequences encoding 393 Ala in KI and 393 Ser in WT mice.

We agree that this suggestion would improve the readability of Figure 1. We have added the locations of restriction sites, primers and probes to the revised figure legend. We have provided sequences of wild type and mutant homozygous mice in an extra panel in Figure 2.

2. It has been reported that Tab1-deficiency caused embryonic lethality, due to the defects in lung and cardiovascular morphogenesis.¹ The authors showed that the heart weight of *Tab1*^{S393A} mouse and WT mouse is comparable (Figure 3). It is unclear if there are any morphological

abnormalities in the heart and lung of *Tab1*^{S393A} mice. Histological analyses of heart and lung tissues should be done here. In addition, Figure 3B shows the heart weight is comparable between *Tab1*^{S393A} and WT mouse at three months old. The unit in Y-axis should be clarified.

This is a valuable suggestion. In the revised Figure 3 we have now data on normalised heart weights. We consider a detailed analysis of heart phenotypes as an interesting research avenue, but beyond the scope of this paper.

3. The title of this report is "A mouse model for functional dissection of TAB1 O-GlcNAcylation". To convince the readers that this mouse model can be used to dissect the function of TAB1 O-GlcNAcylation, the authors should demonstrate the potential biological effects of losing O-GlcNAcylation on TAB1 Ser393.

We have deliberated chosen the wording of this title to indicate that this manuscript reports the characterisation and validation (e.g. loss of O-GlcNAc) of the model, not a detailed functional dissection. We would prefer to leave the title as is.

4. In Figure 3C, the authors used site-specific antibody to detect TAB1 O-GlcNAcylation in mouse cells. Although it has been reported that human TAB1 is O-GlcNAcylation on a single site at Ser395 (which is equivalent to Ser393 in mouse), it's not clear if mouse TAB1 is also only modified by O-GlcNAcylation on a single site or actually on multiple sites. The authors should clarify this point by experiments.

We have added (to Figure 1a) a sequence alignment of mouse and human TAB1 covering the 382-420aa of the C-terminal region that carries the TAB1 O-GlcNAc site. The human O-GlcNAc Ser residue is conserved in mouse and corresponds to the Ser393. We have clarified in the manuscript that the Ser393 residue is the sole reported O-GlcNAc site in mouse TAB1 *in vivo* according to previous studies (Trinidad *et al.* 2012; Alfaro *et al.* 2012). We also replaced the mention of "single O-GlcNAcylation site" on mouse TAB1 "by sole reported O-GlcNAcylation site" from the manuscript.

5. In Figure 3D, the authors showed that, as compared with WT TAB1, *Tab1*^{S393A} interacts normally with TAK1. More appropriate controls, such as the negative control antibody or treatment with LPS, should be included. For example, it has been reported that O-GlcNAcylation of TAB1 is induced upon IL-1 stimulation or osmotic stress.² It will provide better information if the experiments in Figure 3C and Figure 3D can be conducted in cells under stimulation or stress.

We have now added negative (IgG) and positive controls (macrophage lysates) to Figure 3 with the full blots deposited in the Figshare data repository along with the original unedited gels (<https://doi.org/10.6084/m9.figshare.9206567.v1> (Authier, 2019a)). We agree that the effects of loss of TAB1 O-GlcNAcylation can now be studied using this mouse model in different contexts, but this is beyond the scope of this paper.

6. The authors claimed that this KI mouse line could be a good model for further study of the inflammatory diseases, but mouse brain tissues were used in the co-IP experiments in Figure 3. It will be more informative if immune cells from *Tab1*^{S393A} mice can be included for comparison with WT immune cells.

We focused on brain tissues as they provided the largest amounts of TAB1 for biochemical studies.

Competing Interests: No competing interests were disclosed.
