ANTIBODY VALIDATION ARTICLE

Unexpected lack of specificity of a rabbit polyclonal TAP-L (ABCB9) antibody [v1; ref status: indexed, http://f1000r.es/5ex]

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Abstract

In this article, we describe the surprising non-specific reactivity in immunoblots of a rabbit polyclonal antibody (ref. Abcam 86222) expected to recognize the transporter associated with antigen processing like (TAP-L, ABCB9) protein. Although this antibody, according to company documentation, recognizes a band with the expected molecular weight of 84 kDa in HeLa, 293T and mouse NIH3T3 whole-cell lysates, we found that this band is also present in immunoblots of TAP-L deficient bone marrow-derived dendritic cell (BMDC) whole-cell lysates in three independent replicates. We performed extensive verification by multiple PCR tests to confirm the complete absence of the ABCB9 gene in our TAP-L deficient mice. We conclude that the antibody tested cross-reacts with an unidentified protein present in TAP-L knockout cells, which coincidentally runs at the same molecular weight as TAP-L. These findings underline the pitfalls of antibody specificity testing in the absence of cells lacking expression of the target protein.

This article is included in the Antibody validations channel.

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Introduction

TAP-L (TAP-Like), also known as ABCB9, is an ATP-dependent membrane half-transporter. It belongs, like TAP, the transporter associated with antigen processing, to the ABC transporter family, the members of which transport various molecules across membranes. TAP-L can form homodimers and is located primarily in lysosomes, presumably importing peptides from the cytosol. TAP-L has broad specificity for peptides ranging from a length of 6 to 59 amino acids, with an optimal activity for peptides of 23 residues (Wolters *et al.*, 2005). TAP-L can transport two peptides at a time (Herget *et al.*, 2009). Considering its similarity to the heterodimeric TAP transporters (ABCB2/3) importing MHC class I peptide ligands into the endoplasmic reticulum, TAP-L is a potential candidate involved in antigen presentation by MHC molecules (Bangert *et al.*, 2011). Indeed, the length of the peptides transported by TAP-L (6-59 residues) is compatible with loading of both MHC class I and class II molecules. Moreover, TAP-L is highly expressed in lysosomes of professional antigen presenting cell (APC) lysosomes, and upregulated during differentiation of dendritic cells. However, such a function remains hypothetical, and the biological role of TAP-L is presently unknown.

In this article, we describe experimentation designed to specifically detect the ABCB9 protein in bone marrow-derived dendritic cells (BMDCs) by immunoblot. We purchased a rabbit polyclonal antibody generated by Abcam Company using a synthetic peptide as the immunogen, corresponding to a region between residues 475 and 525 of human ABCB9. This antibody is expected to recognize mouse and human ABCB9 and recommended for immunohistochemistry (IHC), immunoprecipitation (IP) and western blot (WB).

Materials and methods

Mice

C57/BL6 TAP-L KO/WT heterozygous mice ($ABCB9^{tm1\ (KOMP)\ Vlcg}$) were purchased from The Komp Repository at the University of California at Davis, CA 95616 (see the results section for details). Heterozygous mice were bred in our laboratory and inter-crossed to obtain homozygous knock out (KO) mice (TAP-L KO/KO) along with their C57/BL6 wild type (WT) littermates.

BMDC culture

Bone Marrow-derived Dendritic Cells (BMDCs) were generated from precursors isolated from femur and tibia of C57/BL6 WT and TAP-L KO mice and cultured for 6 days in IMDM (Iscove's Modified Dulbecco's Medium) (Sigma Aldrich, St. Quentin Fallavier, France) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine (PAA, Velizy-Villacoublay, France), 100 U/ml penicillin, 100 µg/ml streptomycin (PAA), and 50 µM 2-mercaptoethanol (GIBCO, Cergy Pontoise, France) in the presence of 3% supernatant of J558 hybridoma cells producing GM-CSF (Granulocyte-macrophage colony-stimulating factor) (Inaba *et al.*, 1992).

Sample preparation

On day 6 of culture, WT and TAP-L KO BMDCs (Table 1) were lysed in a buffer containing 20mM Tris-HCl pH 7.4, 150mM NaCl, 5mM $MgCl_2$, 1% NP40 and protease inhibitors (protease inhibitor cocktail, Roche) for 1 h at 4°C. Protein concentration was determined by Lowry's method, a biochemical assay for determining the total level of protein in a solution, using DC Protein Assay Reagents Package™ (BioRad).

Twenty to 200µg protein from total cell lysate was mixed at a volume ratio of 1:1 with 2x Laemmli buffer containing 62.5mm Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 100mM DTT and heated for 10 min at 95°C.

Electrophoresis and western blot (WB)

Reagents are listed in Table 2 and Table 3 and the WB protocol is given in Table 4. The samples were loaded on a 10% acrylamide gel for electrophoresis at 80V. Separated proteins were transferred onto polyvinylidine fluoride (PVDF) membrane (pore size 0.4µm) for 1 h at 75V. The membrane was blocked with 5% BSA (Bovine Serum Albumin) in Tris-Buffered Saline (50mM Tris, 150mM NaCl) containing 0.5% Tween 20 (TBS-T) for 1h at room temperature, then incubated with the polyclonal rabbit ABCB9 antibody (Abcam, Catalog number 86222, Lot number: GR22408–1) diluted 1/2000 in TBS-T with 5% BSA for 1h at room temperature. The membrane was washed four times for 5 min with TBS-T then incubated with a goat polyclonal anti-Rabbit-HRP (Jackson ImmunoResearch Laboratory; Suffolk, UK) secondary antibody diluted 1/5000 in TBS-Tween 5% BSA for 1h at room temperature. An enhanced chemiluminescence (ECL) detection system, Immobilon Western HRP (Millipore, Guyancourt, France) was used for developing the membranes. Images were taken with a CCD camera (Fujifilm, Tokyo, Japan). Three independent experiments were performed.

Results

Seeking to detect the ABCB9 protein, we performed a series of WBs on whole-cell lysates obtained from BMDCs, thought to correspond to an inflammatory subtype of DCs. It has previously been shown that ABCB9 expression by monocyte-derived human DCs is increased under inflammatory conditions (Demirel *et al.*, 2007). To validate specificity of antibody staining, we included TAP-L deficient BMDCs as a negative control. TAP-L KO/WT heterozygous mice ($ABCB9^{tm1\ (KOMP)\ Vlcg}$), in which the region located between nucleotides 5625 and 33216 of the TAP-L gene has been

Table 1. Cells used during the validation assay.

Species	Tissue Type	Strain/Cell line	RRID	Details
Murine	BMDCs	C57/BL6 WT mouse	RRID:MGI_2439598	Female/Male
Murine	BMDCs	C57/BL6 TAP-L KO mouse	RRID:MGI_5636449	Female/Male

Table 2. Reagents used for WB analysis.

Process	Reagent	Manufacturer	Catalogue number	Concentration/Composition
Sample preparation	Lysis Buffer	Homemade		20mM Tris-HCl pH 7.4, 150mM NaCl, 5mM $MgCl_2$, 1% NP40
	DC Protein Assay Reagents Package	BioRad	500-0116	
	Laemmli Buffer 2x	Homemade		62.5mm Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 100mM DTT
Staining	ECL detection system, Immobilon Western HRP	Millipore	WBKLS0500	
Washes/Blocks	Washing Buffer	Homemade		TBS-T: Tris-Buffered Saline (50mM Tris, 150mM NaCl) containing 0.5% Tween 20
	Tween 20	Sigma Aldrich	P1379	
	Blocking buffer	Homemade		TBS-T with 5% BSA
	BSA	Sigma Aldrich	A7906	
Electrophoresis and protein transfer	Acrylamide gel 10%	Homemade		
	Running Buffer	Homemade		25mM Tris, 192mM glycine, 0.1% SDS
	Transfer Buffer	Homemade		10mM CAPS (pH11), 10% Methanol

Table 3. Primary and secondary antibodies.

Antibody	Manufacturer	Catalogue number	RRID	Concentration
Rabbit polyclonal anti-ABCB9	Abcam	86222	RRID:AB_1924743	1/2000
Goat polyclonal anti-Rabbit-HRP	Jackson Immunoresearch	111-035-003	RRID:AB_2313567	1/5000

Table 4. Western Blot Protocol.

Protocol steps	Reagent	Time	Temperature
Sample preparation	Lysis Buffer	1 h	4°C
	Laemmli Buffer 2x	10 min	95°C
Electrophoresis (80V)	Acrylamide gel 10%	1 h	Room temperature
	Running Buffer		
Protein transfer (75V)	PVDF membrane (pore size 0.4 µm)	1 h	4°C
	Transfer Buffer		
Blocking	TBS-T 5% BSA	1 h	Room temperature
Primary antibody	Rabbit anti-ABCB9	1 h	Room temperature
Washes (4 times)	TBS-T	5 min each	Room temperature
Secondary antibody	Goat anti-Rabbit-HRP	1 h	Room temperature
Washes (4 times)	TBS-T	5 min each	Room temperature
Detection	ECL detection system	20 seconds	Room temperature

removed for the insertion of a cassette of 6085bp containing the cDNA conferring resistance to neomycin (Neo), were purchased from The Komp Repository at the University of California at Davis, CA 95616 (see construction of the KO gene; Figure 2A). Heterozygous mice were bred in our laboratory and inter-crossed to obtain homozygous KO mice (TAP-L KO/KO). To our surprise, the ABCB9 antibody recognized a band, with an apparent molecular weight (84kDa) corresponding to that of ABCB9 protein, both in WT and TAP-L deficient BMDCs (Figure 1). Three different immunoblots were performed in three independent experiments.

Given these surprising results, we verified that the TAP-L KO mice were truly deficient for the target gene. We performed a series of polymerase chain reactions (PCRs). Different fragments of the WT allele (located in exons 2, 4, 8, 11 and introns 5, 6, 9) and the expected genomic region in KO mice (located between the upstream or downstream arm and within the Neo cassette) were amplified by PCR.

The following forward (F) and reverse (R) primers were used:

- Ex1-F: 5'-GTAGTAGTGACGCTGGCCTT-3' and Ex1-R: 5'CTTCTGTAGTGTGGCTCCCG-3', located in exon 1 of the WT allele and amplifying a product of 498bp in the WT allele

- Ex2-F: 5'-AGACCTTCCTGCCCTACTACA-3' and Ex2-R: 5'-CAGCAGGCAAACGACGACAA-3', located in exon 2 of the WT allele and amplifying a product of 101bp in the WT allele

- Ex4-F: 5'-CGCCTCACCTCTGATACCAC-3' and Ex4-R: 5'-TGCCGTAGATGTTGGACACC-3', located in exon 4 of the WT allele and amplifying a product of 181bp in the WT allele

- Ex8-F: 5'-CAAGGTGACAGCTCTGGTGG-3' and Ex8-R: 5'-GCCATCCAACAATACACGGC-3', located in exon 8 of the WT allele and amplifying a product of 106bp in the WT allele

- Ex11-F: 5'-GAGACACACGGTGCTCATCA-3' and Ex11-R: 5'-TGTGTTCAGTGTTGCTGGGT-3', located in exon 11 of the WT allele and amplifying a product of 214bp in the WT allele

- INT5-F: 5'-TACTCGGGTGCCACTACCTG-3' and INT5-R: 5'-GGCACATGCCACCTTCAAGT-3', located in intron 5 of the WT allele and amplifying a product of 379bp in the WT allele

- INT6-F: 5'-TGCTTAAAGGCACTCGGTGA-3' and INT6-R: 5'-CTTCGGGGATACCACAGAGC-3', located in intron 6 of the WT allele and can amplifying a product of 371bp in the WT allele

- INT9-F: 5'-TGCCAAGTTTAGTGCCAGGATG-3' and INT9-R: 5'-GCCCAGGACAAAAAAAGCAATC-3', located in the intron 9 of the WT allele and amplifying a product of 371bp in the WT allele

- KOFwd1: 5'-TTGCATGGAGAAGACCCTCC-3', located in the arm upstream of the Neo cassette (Neo upstream arm), and KORvs1: 5'-GAGGGGACGACGACAGTATC-3', located in the Neo cassette and amplifying a product of 465bp in the KO allele

- KOFwd2: 5'-GCAGCCTCTGTTCCACATACACTTCA-3', located in the Neo cassette and KORvs2: 5'-GCTTAGTTCTCTCCCAGACATCCTCC-3', located in the arm downstream of the Neo cassette (Neo downstream arm) and amplifying 425bp in the KO allele.

PCRs were performed in a total volume of 25µl containing: 17.3µl H2O (DEPC treated water, pathogen free, DNase/RNase

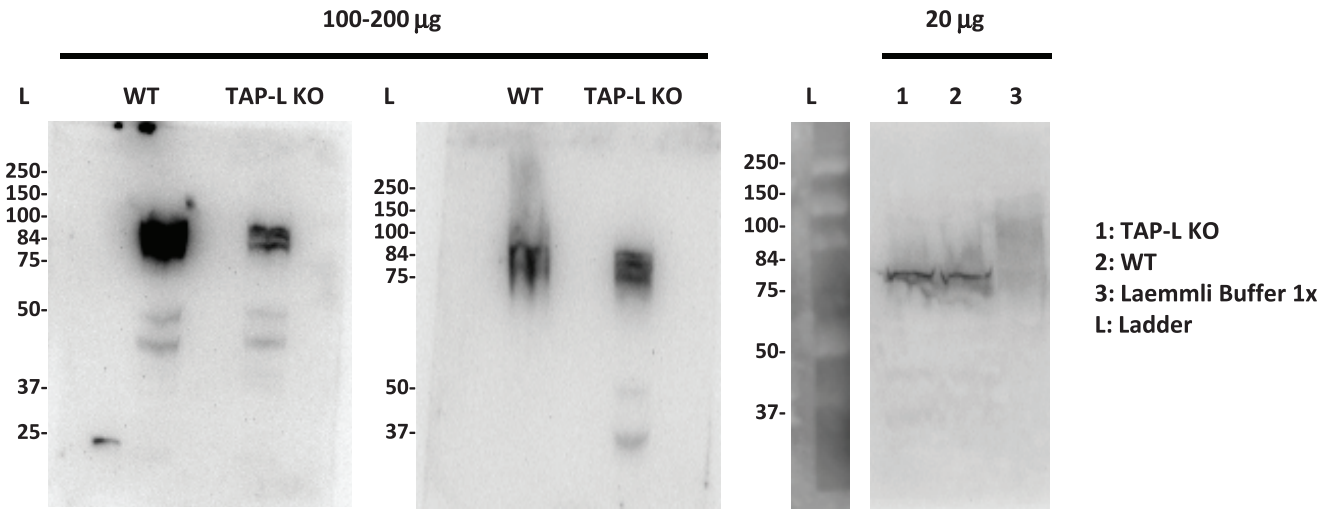

Figure 1. WB anti-ABCB9 on total cell lysates from WT and TAP-L KO BMDCs. 20–200µg of total BMDC cell lysate from WT and TAP-L KO BMDCs was loaded on 10% acrylamide gels. The proteins were transferred onto a PVDF membrane. The rabbit ABCB9 antibody was used to detect the TAP-L protein (84kDa), followed by incubation with an HRP-conjugated goat anti-rabbit secondary antibody. An ECL detection system was used for developing the membranes by chemoluminescence. Three immunoblots from three independent experiments are shown.

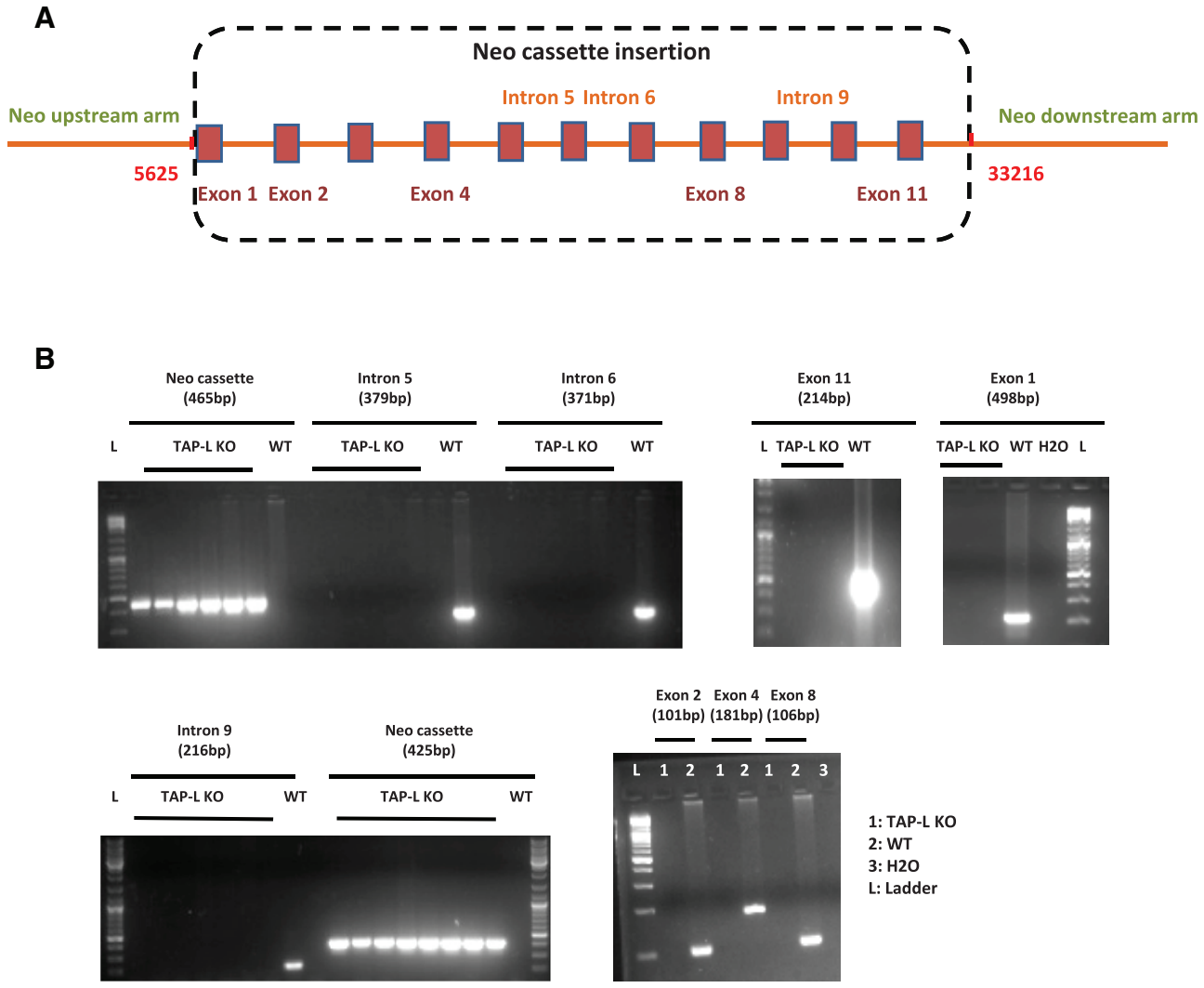

Figure 2. Strategy for genomic invalidation of the TAP-L/ABCB9 gene (**A**) and genotyping of WT and TAP-L deficient mice (**B**). In **B**, multiple KO mice were tested in the PCRs amplifying the Neo cassette and introns 5, 6 and 9. L, DNA ladder (See the results section for details).

Free-Invitrogen), 5 µl 5x GoTaq Green Reaction Buffer (Promega), 0.5µl dNTP (10mM), 20 µM primers, 0.2µl polymerase (5 U/µl) (GoTaq-Promega polymerase) and 1µl DNA or water (negative control). The amplification reaction was performed as follows: for the WT allele, an initial denaturation at 94°C for 5 min, 10 cycles: denaturation 94°C for 15 sec, annealing 65°C for 30 sec, elongation 72°C for 40 sec; 30 cycles denaturation 94°C for 15 sec, annealing 55°C for 30 sec, elongation 72°C for 40 sec; final elongation 72°C for 5 min. For the KO allele: initial denaturation at 94°C for 5 min; 10 cycles: denaturation 94°C for 15 sec, annealing 62°C for 30 sec, elongation 72°C for 40 sec; 25 cycles: denaturation 94°C for 15 sec, annealing 57°C for 30 sec, elongation 72°C for 40 sec; final elongation 72°C for 5 min. The PCR products obtained were migrated on a 1.5% agarose gel containing 10 µg/ml of Ethidium Bromide. Migration was performed in a buffer tank filled with TAE buffer containing 40mM Tris, 20mM acetic acid, 1mM EDTA, pH=8 for 20 min at 120 V and visualization of the PCR products under a UV lamp connected to a photographic device.

The resulting PCR products from multiple KO mice confirmed the absence of the TAP-L gene and the presence of the Neo cassette (Figure 2B), indicating that the TAP-L gene was deleted as expected and that the mice obtained were TAP-L KO/KO. Consequently, the band recognized by the ABCB9 antibody, even though

running at the expected molecular weight, could not correspond to the TAP-L protein.

Conclusion

Collectively, these results show that the commercial ABCB9 antibody recognizes a protein with a molecular weight similar to that of TAP-L. It is impossible to know whether it also recognizes TAP-L. Our findings highlight the importance of verifying commercial antibody specificity using knockout cells. If such cells are not available, lentiviruses encoding target-specific shRNA, which are now readily available for an essentially complete range of proteins, can be used to produce cells that provide informative negative controls.

Author contributions

ML designed, performed and interpreted experiments and wrote the manuscript. PvE designed and interpreted experiments and edited the manuscript.

Competing interests

Both authors confirm that they have no conflict of interest.

Grant information

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Current Referee Status:   

Version 1

Referee Report 29 June 2015

doi:10.5256/f1000research.7017.r9026

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This study relates to antibody specificity for the characterization of ABCB9 (transporter associated with antigen processing-like (TAP-L)) expression. The methods and results are explained in detail and the abstract and title are appropriate for the study. TAP-L knockout/wild type heterozygous mice from a commercial source were crossed to obtain homozygous TAP-L knockout mice. TAP-L deficiency in the knockout is confirmed by PCR. Cell lysates from bone marrow-derived dendritic cells of wild type or TAP-L-deficient mice were subject to immunoblotting analyses with a rabbit polyclonal anti-TAP-L antibody from Abcam (http://www.abcam.com/ABCB9-antibody-ab86222.html). An expected band at 84 kDa is seen. However, similar sized bands are seen in lanes containing lysates from either the wild type or the knockout mice, suggesting that the tested antibody is not specific for TAP-L, at least based on immunoblotting analyses. The commercial vendor should take note of this study. The commercial link also indicates immunoprecipitations and immunohistochemistry as tested applications for the antibody. These applications could also be tested using the knockout cells as negative controls. While the study correctly emphasizes the importance of relevant controls prior to the use of commercial antibodies, it appears that a number of TAP-L-specific antibodies are available from different commercial sources. It would be useful to the reader to know which of the commercial antibodies are in fact specific for TAP-L.

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

Competing Interests: No competing interests were disclosed.

Referee Report 15 June 2015

doi:10.5256/f1000research.7017.r8943

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This in an interesting antibody validation article showing that a rabbit polyclonal antibody raised against a peptide (475-525) within the human TAP-L transporter (84 kDa) apparently cross-reacts with another unknown protein of similar size within TAP-L deficient murine dendritic cells. Since mouse TAP-L has a decent homology to TAP2 (77.5 kDa) and TAP1 (78.9 kDa) the authors should precipitate mTAP1/2 and perform a blot with this polyclonal antibody to prove or disprove that the reported cross-reactivity is to

TAP.

Please follow this link to view the homology of mTAPL(a.a. 475-525) with mTAP2, and with mTAP1:
https://f1000researchdata.s3.amazonaws.com/supplementary/6535/041aa8a5-5260-4b06-929e-b91f6b3c

I have read this submission. I 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.

Competing Interests: No competing interests were disclosed.

Referee Report 15 June 2015

doi:10.5256/f1000research.7017.r9028

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In this article, the authors report the results of studies in which they attempt to validate the specificity of a commercially available antibody to TAP-Like (TAP-L), which is an endosomal peptide transporter from the same family at the prototypical ER TAP proteins. They report that, unexpectedly, the anti-TAP-L antibody exhibits immunoreactivity on samples prepared from TAP-L knock mice.

Suggestions for improvement:

- It would be helpful to know the number of cell equivalents loaded on each lane of the gels. The gels were loaded for equal protein, so the number of cell equivalents is likely similar, but this point should be addressed.
- Figure 1 – Since the purpose of the experiment is to test the specificity of the primary western blot antibody (i.e., anti-TAP-L), it would be appropriate to include a control blot that was probed with secondary antibody only. It is possible that the unexpected reactivity on the western blot is due to the secondary antibody (and that the primary antibody is generating no signal). A blot probed with secondary antibody only would address this possibility.
- The company makes specific note of the peptide used to generate the reagent under analysis. Therefore, it would be interesting of the authors compared this sequence to the protein sequence in the available databases to see if they could identify candidates for the non-TAP-L protein being recognized by the antibody under analysis.

I have read this submission. I 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.

Competing Interests: No competing interests were disclosed.