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ANTIBODY VALIDATION ARTICLE

REVISED Immunoblotting validation of research antibodies generated against HS1-associated protein X-1 in the human neutrophil model cell line PLB-985. [v2; ref status: indexed, http://f1000r.es/5qc]

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Abstract

HS1-associated protein X-1 (Hax1) is a 35 kDa protein that is ubiquitously expressed. Hax1 is an anti-apoptotic protein with additional roles in cell motility, and autosomal recessive loss of Hax1 results in Kostmann syndrome, a form of severe congenital neutropenia. Because of the important role of Hax1 in neutrophils we demonstrate here validation of two commercially available research antibodies directed against human Hax1 in the human myeloid leukemia cell line PLB-985 cells. We show that both the mouse anti-Hax1 monoclonal IgG directed against amino acids 10-148 of Hax1 and a rabbit anti-Hax1 polyclonal IgG antibody directed against full-length Hax1 reliably and consistently detect Hax1 during immunoblotting of three different PLB-985 cell densities. Using shRNA mediated Hax1 knockdown, we demonstrate the specificity of both Hax1 antibodies. In addition, our results suggest that the rabbit anti-Hax1 polyclonal antibody provides a stronger intensity in detecting Hax1 protein, with detection in as few as 0.1×10^6 cells in 6 total replicates we have performed.
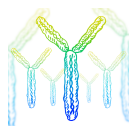
This article is included in the Antibody validations channel.

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Referee Status: ✓✓✓

	Invited Referees		
	1	**2**	**3**
REVISED version 2 published 10 Aug 2015			
version 1 published 10 Jun 2015	✓ report	✓ report	✓ report

1 **Andrew Chalmers**, University of Bath UK

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Comments (0)

Corresponding author: Peter Cavnar (pcavnar@uwf.edu)

REVISED   **Amendments from Version 1**

This revision incorporates suggestions from the referees and includes all updated figures along with additional Dataset 1 and Dataset 2. The following updates and revisions have been incorporated into the publication:

1. All Western blots have been separated to clearly display the goat anti-rabbit 680 channel and the goat anti-mouse 800 channel. This was to further explain and demonstrate the source of background bands observed in the goat anti-rabbit 680 channel.

2. The text was updated to clearly explain that these two antibodies are commercially available. In addition, the buffer compositions of the 6× Laemmli buffer and transfer buffer have been included.

3. Figure 3 now contains quantification of the band intensities of the mouse anti-Hax1 and rabbit anti-Hax1 antibodies from three independent replicates.

4. Figure 4 includes quantification of the band intensities using the mouse anti-Hax1 and rabbit anti-Hax1 antibodies in the control shRNA and Hax1 shRNA cell lines to demonstrate that at the 1×10^6 cell density both antibodies exhibit similar knockdown levels. This demonstrates their specificity.

5. Figure 5 now includes more controls to establish the origin of background bands observed in the goat anti-rabbit 680 blots. We tested rabbit and mouse pre-immune sera to determine if this could be the source of the background. We found that in fact we got more background with the sera. Using secondary antibodies only we show that there is no background observed with the goat anti-mouse 800 antibody but we see a characteristic background pattern using the goat anti-rabbit 680 antibody. Subsequent incubation of these secondary only blots with either the rabbit anti-Hax1 or mouse anti-Hax1 antibodies show the appearance of Hax1. These two combinations clearly demonstrate that the background bands are coming from the goat anti-rabbit secondary antibody.

See referee reports

Introduction

HS1-associated protein X-1 (Hax1) is a 35 kDa protein consisting of 279 amino acids that is ubiquitously expressed[1]. Hax1 has been demonstrated to be a negative regulator of apoptosis in many immune cell types[2–4]. Furthermore, Hax1 has been shown to have additional roles in regulating cell motility and adhesion[5,6], and is overexpressed in many types of cancer[7]. Patients with autosomal recessive mutations in the HAX1 gene have a form of severe congenital neutropenia called Kostmann syndrome. Severe congenital neutropenia is characterized by early recurrent bacterial infections and decreased neutrophil counts in the blood stream[8].

Because of the recent increase in Hax1 investigations, it is important to identify reliable antibodies directed against Hax1. Using the human neutrophil model cell line PLB-985 cells, which can be terminally differentiated into neutrophil-like cells after treatment with DMSO, we demonstrate the applicability and selectivity of two commercially available antibodies against Hax1. A mouse Hax1 monoclonal antibody (BD Biosciences) that is routinely used in publications investigating Hax1[5,6,9–11] directed against Hax1 amino acids 10–148, and a rabbit polyclonal antibody (Proteintech Group, Inc.) directed against the full length Hax1 protein[6].

Materials and methods

Reagent details
Details of all reagents used in the Western blotting procedures can be found in Table 1.

Antibody details
Anti-tubulin (beta-) is a mouse monoclonal IgG1 [E7 was deposited to the DSHB by Klymkowsky, Michael (DSHB Hybridoma Product E7)] and was used as a loading control for all Western blots at a dilution of 1:1000 resulting in a final concentration of 45 ng/mL. Rabbit anti-Hax1 (Proteintech Group, Inc, Table 2) is a polyclonal antibody generated to full length *Homo sapiens* Hax1. The lot number used was 1, and a dilution of 1:1000 was used for all Western blots resulting in a final concentration of rabbit anti-Hax1 of 230 ng/mL. Mouse anti-Hax1 (BD Biosciences) is a mouse monoclonal IgG1 raised against *Homo sapiens* Hax1 amino acids 10–148. The lot number used was 3266979, and a dilution of 1:1000 was used for all Western blots resulting in a final concentration of 250 ng/mL. Goat anti-rabbit IgG IRDye 680LT and Goat anti-mouse IgG IRDye 800CW (Li-Cor Biosciences, Table 2) were used at a dilution of 1:40,000 (25 ng/mL).

Cell culture
PLB-985 cells were maintained in RPMI 1640 (Mediatech, Inc.) supplemented with 10% fetal bovine serum, 60 μg/mL penicillin, and 100 μg/mL streptomycin (Mediatech, Inc.) at a concentration of $0.1–1 \times 10^6$ cells/mL. To differentiate PLB-985 cells into "neutrophil-like" cells 1.25% DMSO (Fisher Scientific) was added to 2×10^5 cells/mL for 6 days. Lentiviral Hax1 shRNA targets were purchased from Open Biosystems. Targets used; Hax1 shRNA (5'-ACAGACACTTCGGGACTCAAT-3') and control shRNA (5'-TGTCTCCGAACGTGTCACGTT-3'). HEK293-FT cells were grown to 70% confluency in a 10cm tissue culture dish for each lentiviral target and transfected using 6μg Hax1, 0.6μg vesicular stomatitis virus (VSV)-G, and 5.4μg cytomegalovirus (CMV) 8.9.1. 72 hour viral supernatant was collected and concentrated using Lenti-X concentrator (Clontech, Inc.) following the manufacturer's instructions. 1×10^6 PLB-985 cells were infected with viral supernatant for 3 days in the presence of polybrene (4 μg/mL, Santa Cruz Biotechnology). Stable cell lines were generated with puromycin (1 μg/mL, Sigma Aldrich) selection.

Immunoblot analysis
- Differentiated PLB-985 cells were counted and 0.1×10^6, 0.5×10^6, and 1×10^6 cells were pelleted by centrifugation.

- Cells were lysed in Triton X-100 lysis buffer with protease inhibitors (25 mM HEPES, pH 7.5, 150 mM $NaCl_2$, 1% TX-100, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 μg/mL pepstatin A, 2 μg/mL aprotinin, 1 μg/mL leupeptin) on ice for 10 minutes and clarified by centrifugation.

Table 1. Details of reagents used for immunoblotting.

Process	Reagent	Manufacturer	Catalogue Number	Concentration
6× Laemmli Protein Loading Buffer	Tris-HCL SDS Glycerol Bromophenol blue DL-Dithiothreitol	Fisher Scientific Fisher Scientific Fisher Scientific Sigma-Aldrich Sigma-Aldrich	Tris base BP152 BP166-100 G33-1 B0126 D0632	375mM 9% 50% 0.03% 0.6M
Protein blotting	0.45μm nitrocellulose pure transfer membrane	Santa Cruz Biotechnology	Sc-201705	
SDS-PAGE Transfer Buffer	1× Tris-Gylcine Electroblotting buffer Methanol	National Diagnostics Fisher Scientific	EC-800 A412-4	1× (25mM Tris-HCL, 192mM glycine) 20% v/v
Wash Buffer, blocking buffer, and Antibody Diluent	Tween-Tris/Saline (T-TS)	Fisher Scientific	Tris base BP152 NaCl S271 Tween-20 BP337	50mM Tris 150mM NaCl 1% Tween -20
Blocking	Bovine Serum Albumin, heat shock fraction	Sigma-Aldrich	A9647	5% in T-TS
Pre-Immune Serum Incubation (Figure 5A)	Rabbit pre-immune serum Mouse pre-immune serum	Sigma-Aldrich Sigma-Aldrich	R9133 M5905	1:1000 1:1000

Table 2. Details of Primary and Secondary Antibodies.

Antibody	Manufacturer	Catalogue number	RRID	Concentration used
Tubulin (beta-)	Developmental Studies Hybridoma Bank	E7-s	RRID:AB_528499	45 ng/mL
Hax1	BD Biosciences	610824	RRID:AB_398143	250 ng/mL
Hax1	Proteintech Group, Inc.	11266-1-AP	RRID:AB_2263720	230 ng/mL
Goat anti-Rabbit IgG IRDye 680LT	Li-Cor Biosciences	926-32221	RRID:AB_621841	25 ng/mL
Goat anti-Mouse IgG IRDye 800CW	Li-Cor Biosciences	827-08364	RRID:AB_10793856	25 ng/mL

- Cellular lysate was then removed and added to 6× Laemmli sample buffer, boiled at 90°C for 5 minutes, and run on 10% SDS-PAGE gels.

- Proteins were then transferred to 0.45μm nitrocellulose membranes (Santa Cruz Biotechnology) at 400mA for 1 hour at 4°C.

- Following transfer, the membrane was blocked in 5% BSA in 1× T-TS for 1 hour at room temperature with gentle rocking.

- Membranes were then probed with mouse anti-tubulin [(beta-) (45 ng/mL)], and either mouse anti-Hax1 (BD Biosciences, 250 ng/mL) or rabbit anti-Hax1 (Proteintech Group, Inc., 230 ng/mL) at room temperature for 1 hour.

- After primary antibody incubation the membranes were washed 3 × 5 minutes with 1× Tris-HCL/NaCl saline buffer (1× T-TS), see Table 1.

- The membranes were incubated with goat anti-rabbit IgG IRDye 680LT and goat anti-mouse IgG IRDye 800CW (Li-Cor Biosciences, 25 ng/mL) at room temperature for 1 hour.

- After secondary antibody incubation the membranes were washed 3 × 5 minutes with 1× T-TS.

- Blots were imaged with an infrared imaging system (Odysssey Fc; Li-Cor Biosciences) using a 2-minute exposure time.

Results

To determine the reproducibility and sensitivity of the mouse and rabbit anti-Hax1 antibodies on the PLB-985 cells, we performed Western blot analysis using three separate cell densities, 0.1×10^6, 0.5×10^6, and 1×10^6 cells. In our research using the PLB-985 cell system, we routinely use $1 \times 10^6 – 10 \times 10^6$ cells in a Western blot. Using beta-tubulin as a loading control our Western blots illustrate an increasing protein concentration in the three samples as would be expected with increasing cell densities. We found that the mouse anti-Hax1 antibody (BD Biosciences) is visible as low as 0.5×10^6 cells, binding to a protein band at the expected Hax1 size with a relative mobility of 35 kDa (Figure 1). In six different experiments (Figure 1 and Figure 4) we found inconsistency in protein detection with the Ms anti-Hax1 antibody. In all blots Hax1 was visible, however with varying degrees of intensity. Conversely, when the rabbit anti-Hax1 antibody (Proteintech Group, Inc.) was used, the antibody gave consistent and robust detection (Figure 2 and Figure 4).

In some cases, Hax1 can be detected in as low as 0.1×10^6 cells using the rabbit anti-Hax1 antibody (Figure 2C). We do not believe the difference between the two antibodies is due to variations in the cell extract or imaging software because when the same cell extract is immunoblotted on two different blots and scanned simultaneously the difference in sensitivity can be observed (Figure 3A). Using the Odyssey imaging system (Li-Cor Biosciences) to measure the intensity of each band, we calculated the intensity ratio of Hax1 relative to the tubulin loading control from three independent blots for each antibody (Figure 3B). In both blots the levels of tubulin are similar, however it is evident that the rabbit anti-Hax1 antibody exhibits a stronger signal compared to the mouse monoclonal antibody. Nevertheless, it should be noted that both antibodies reliably detect Hax1 in differentiated PLB-985 cells.

To demonstrate the specificity of both Hax1 antibodies we generated stably-expressing control shRNA and Hax1 shRNA PLB-985
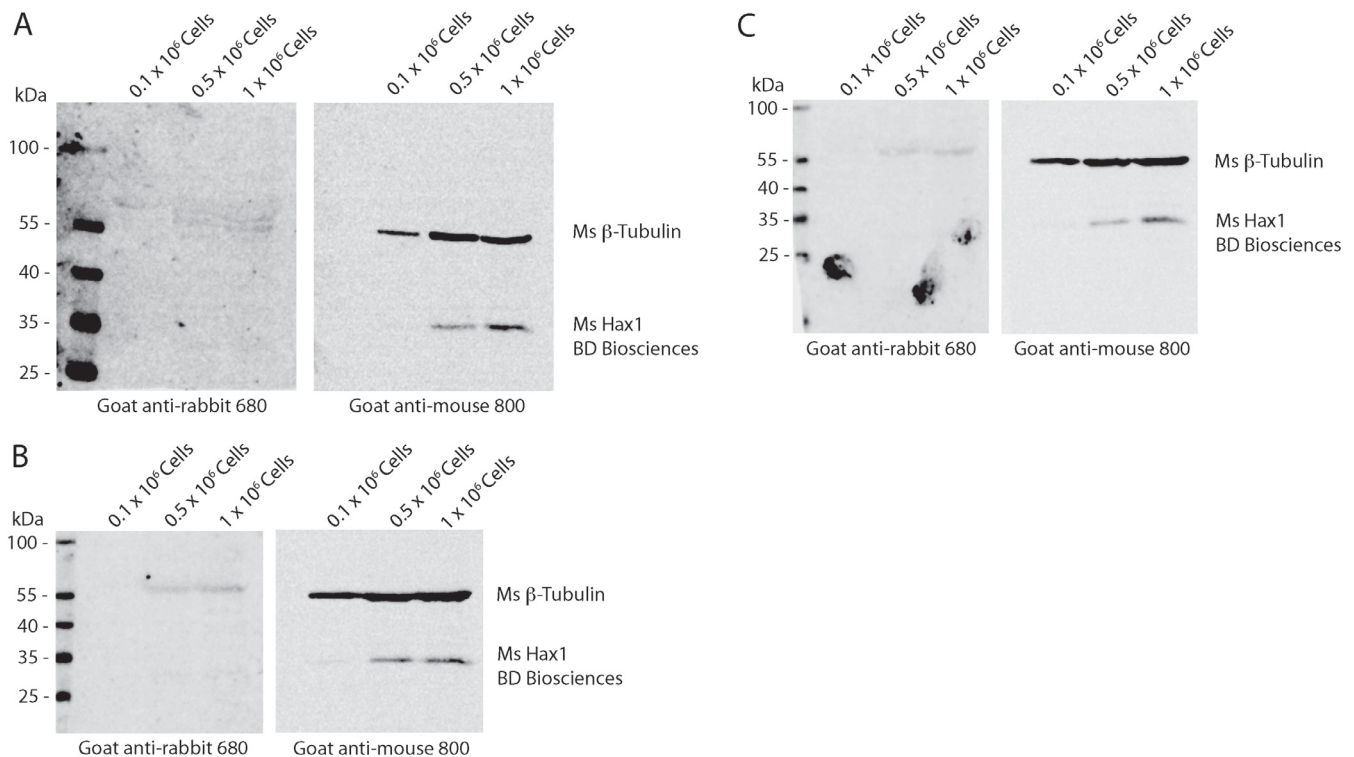

Figure 1. Detection of Hax1 in differentiated PLB-985 cells using a mouse anti-Hax1 antibody. Western blot analysis of differentiated PLB-985 cell lysates from 0.1×10^6, 0.5×10^6, and 1×10^6 cells from three independent replicates. Mouse anti-tubulin (beta-) is used as a loading control and can be seen present at a relative mobility of 55 kDa in the goat anti-mouse 800 channel. Mouse anti-Hax1 detects a band with a relative mobility of 35 kDa as predicted. Hax1 can be detected in densities of 0.5×10^6 and 1×10^6 cells.
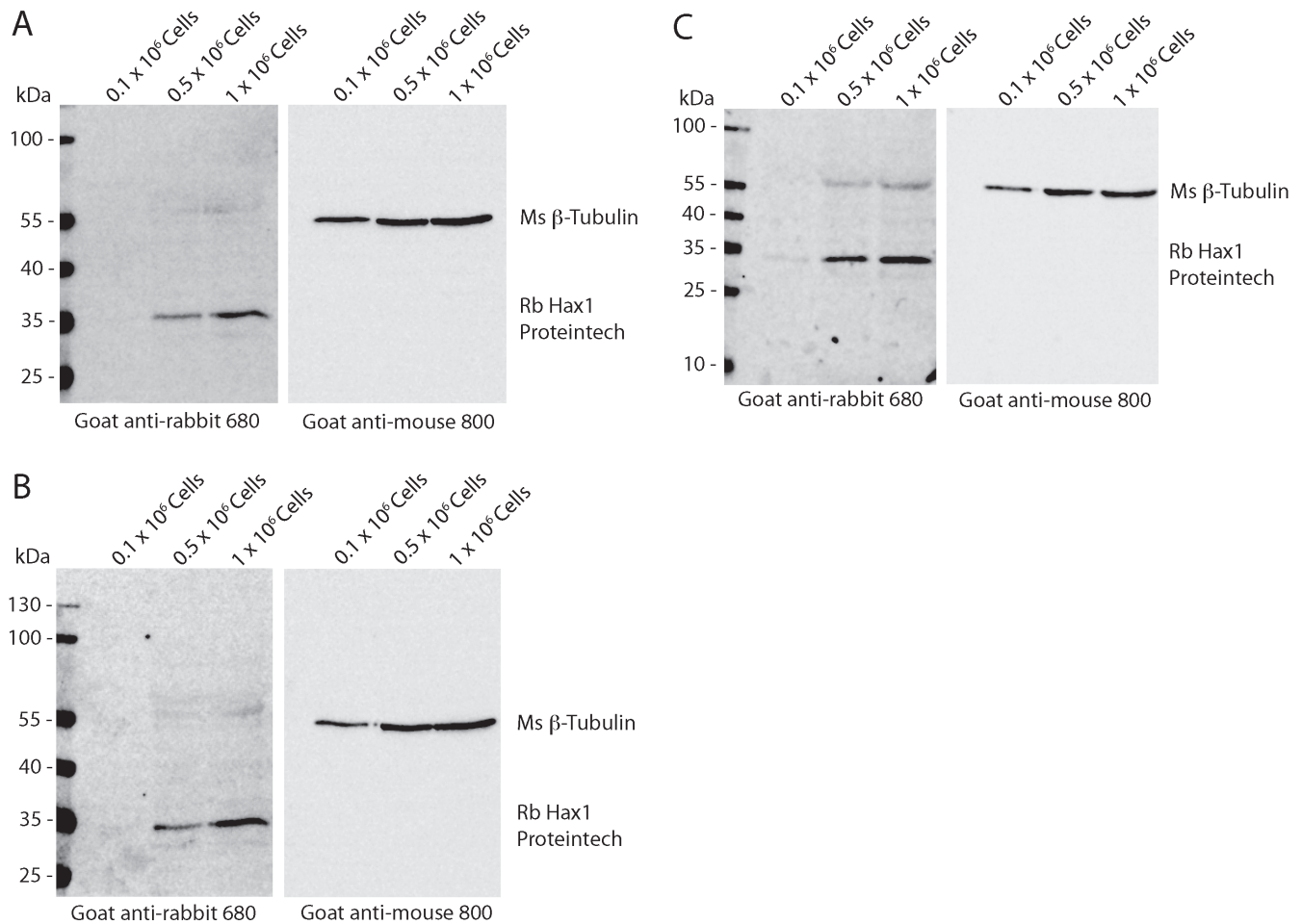
Figure 2. Detection of Hax1 in differentiated PLB-985 cells using a rabbit anti-Hax1 antibody. Western blot analysis of differentiated PLB-985 cell lysates from 0.1×10^6, 0.5×10^6, and 1×10^6 cells from three independent replicates. Mouse anti-tubulin (beta-) is used as a loading control and can be seen present at a relative mobility of 55 kDa. Rabbit anti-Hax1 detects a band at a relative mobility of 35 kDa as predicted. Hax1 can be detected in densities as low as 0.1×10^6 cells (**C**).

cells (Figure 4). As described previously using the mouse anti-Hax1 antibody the control shRNA cells show inconsistent staining intensity, however in these samples the mouse anti-Hax1 antibody is more robust than in the wild-type PLB-985 cells. Both the mouse anti-Hax1 and rabbit anti-Hax1 antibodies show reduced detection in the Hax1-deficient PLB-985 cells. Quantification of the level of Hax1 knockdown is consistent using the two antibodies at 1×10^6 cells. This demonstrates that the antibodies are highly specific for Hax1. In many of the experiments we observed additional background bands in the rabbit 680nm channel. To determine the source of these background bands rabbit and mouse pre-immune serum were tested (Figure 5A). Our results show a unique background pattern using the pre-immune serum that we do not observe on the Hax1 blots. We next performed a Western blot on cells using only the rabbit and mouse secondary antibodies (Figure 5B). The mouse channel does not display any significant background,

however the rabbit secondary antibodies shows a background staining that we observe in the previous blots as well. These blots were then subsequently probed with the rabbit and mouse Hax1 antibodies (Figure 5C). Comparison of the secondary only blot before and after Hax1 antibody incubation demonstrates that the Hax1 antibodies are highly specific and the background we are observing can be attributed to the goat anti-rabbit IgG secondary antibody.

Dataset 1. Raw data for Figure 3 quantification

http://dx.doi.org/10.5256/f1000research.6516.d99343

Comparison of mouse and rabbit anti-Hax1 antibody band intensities in differentiated PLB-985 cells. Quantification of the band intensities from three independent Western blots was measured and the ratios of Hax1 to tubulin were plotted.
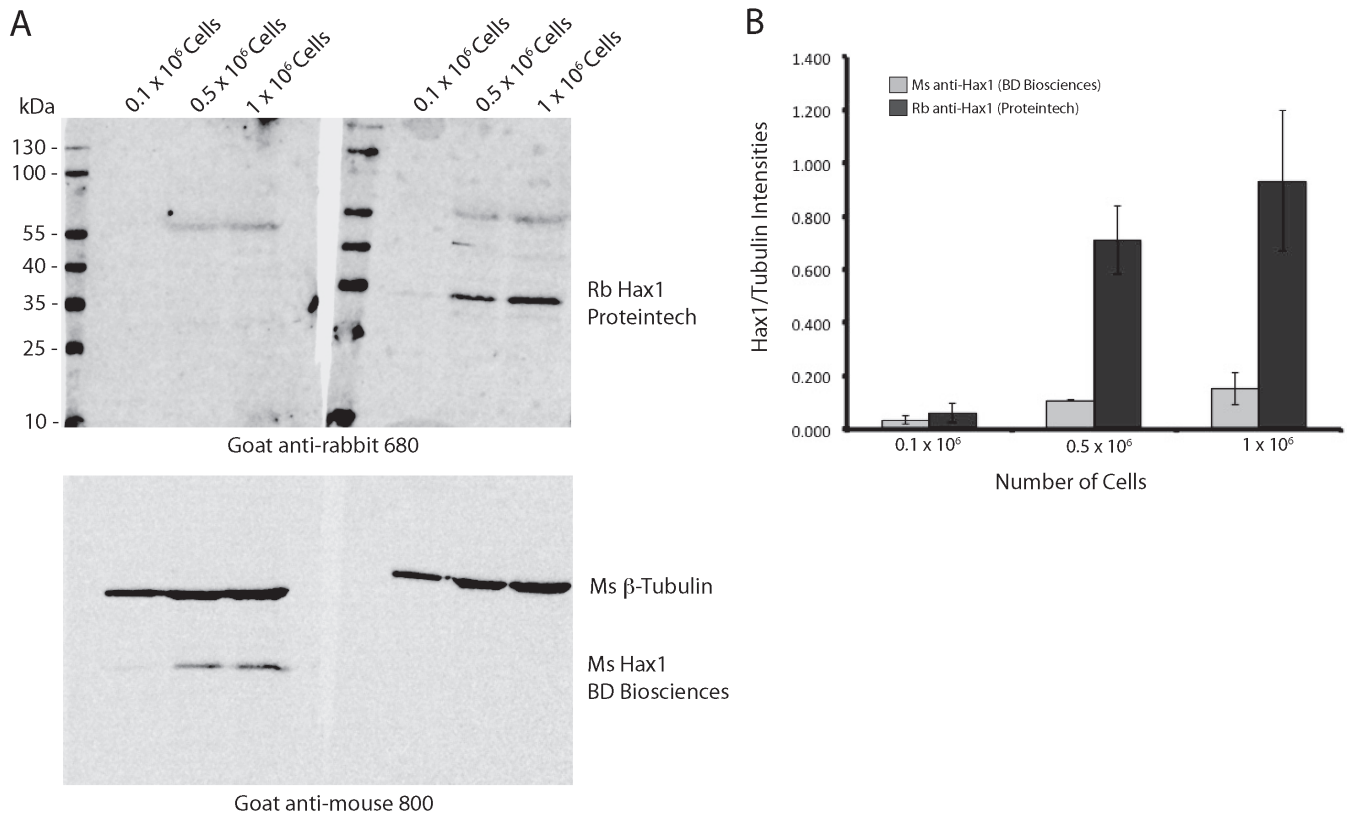
Figure 3. Comparison of mouse and rabbit anti-Hax1 antibodies in differentiated PLB-985 cells. (**A**) Western blot analysis of differentiated PLB-985 cell lysates from 0.1×10^6, 0.5×10^6, and 1×10^6 cells comparing mouse and rabbit anti-Hax1 antibodies. Lysates from the same cell extractions were run on a single SDS-PAGE gel and blotted onto a single nitrocellulose membrane. After transfer, the membrane was divided and probed with either mouse anti-Hax1 or rabbit anti-Hax1. The membranes were imaged simultaneously. (**B**) Quantification of the ratios of Hax1 and tubulin band intensities from three independent blots were measured and plotted. Error bars indicate standard deviation.

Dataset 2. Raw data for Figure 4 quantification

http://dx.doi.org/10.5256/f1000research.6516.d99344

Detection and quantification of Hax1 in control shRNA and Hax1 shRNA expressing PLB-985 cells. Quantification of the band intensities was measured and the ratios of Hax1 to tubulin were plotted relative to the control shRNA ratios for each cell density assayed. An average, standard deviation, and standard error of the mean were calculated for each cell density and each antibody used from three independent replicates.

Conclusion

Here we show validation and comparison results of two commercially available antibodies generated against HS1-associated protein X-1 (Hax1), an anti-apoptotic protein that has a multi-factorial role in regulating cell proliferation and differentiation, cell motility, and cancer. Homozygous loss-of-function of Hax1 results in severe congenital neutropenia, a life threatening loss of circulating neutrophils in the blood stream. Studying the function of Hax1 in primary neutrophils and the neutrophil model cell line PLB-985 will help elucidate the disease pathogenesis of neutropenia

syndromes. We demonstrate that mouse anti-Hax1 (BD Biosciences) and rabbit anti-Hax1 (Proteintech Group, Inc.) are both specific for Hax1. Furthermore we show that as little as 0.5×10^6 differentiated PLB-985 cells can be used to reliably detect Hax1 expression with both of the antibodies. We have evidence that the rabbit anti-Hax1 (Proteintech Group Inc.) results in a more robust and consistent detection of Hax1, likely due to the polyclonal nature of the antibody. Finally, lentiviral knockdown of endogenous Hax1 expression results in loss of Hax1 detection by both mouse anti-Hax1 and rabbit anti-Hax1 demonstrating the specificity of each antibody. In our quantification of Hax1 knockdown we observed variation when the cell densities were low, with 1×10^6 cells giving us the most reliable quantification. In our experiments we observed background bands that we attributed to the goat anti-rabbit 680nm secondary antibody. Therefore we are confident that these antibodies are very specific.

In conclusion we recommend the use of either mouse or rabbit anti-Hax1 antibodies shown here for studies using the PLB-985 cells as a neutrophil model cell line. It is our conclusion that a minimum cell density of 0.5×10^6 neutrophils should be used as
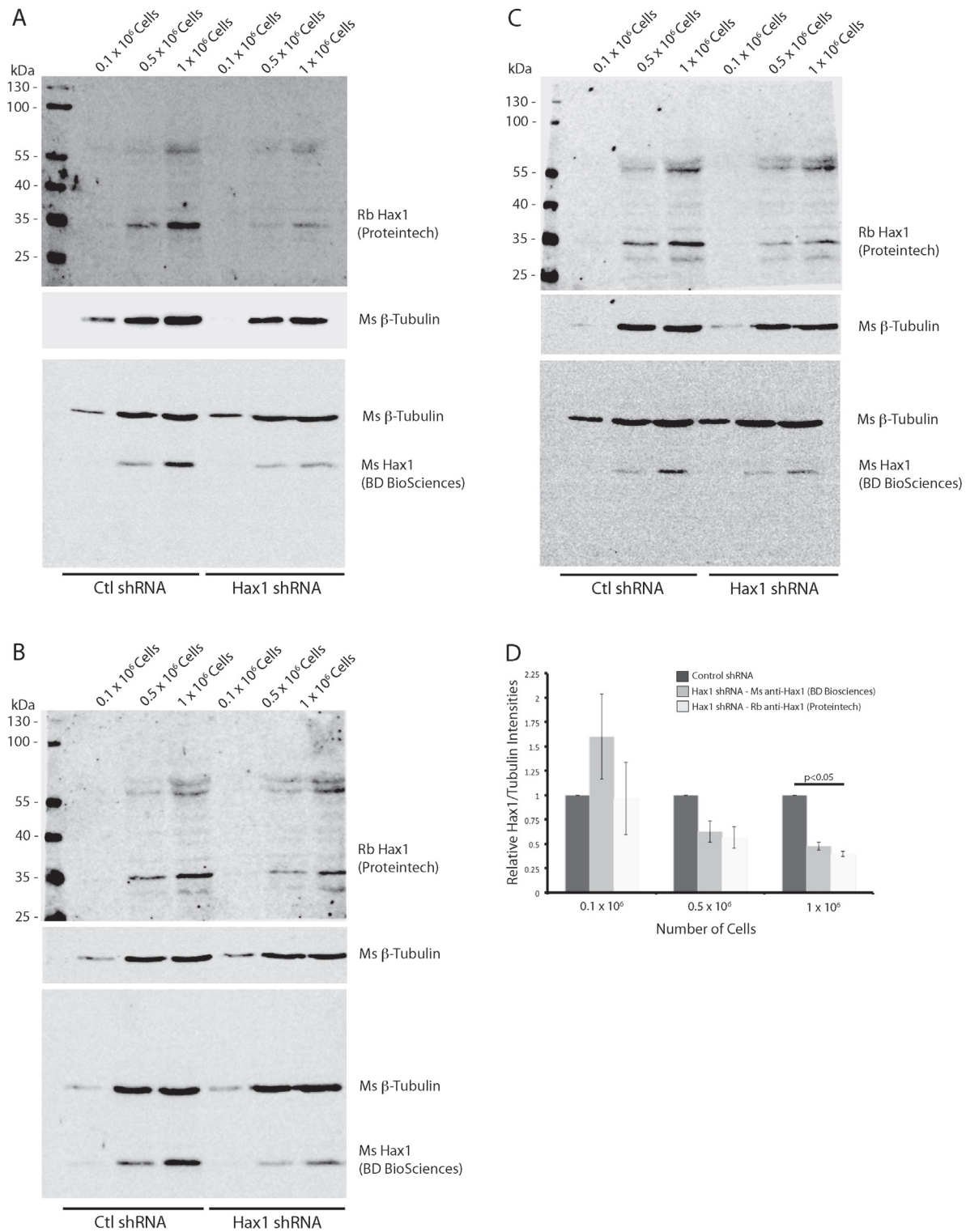
Figure 4. Detection of Hax1 in Control shRNA and Hax1 shRNA expressing differentiated PLB-985 cells using mouse and rabbit anti-Hax1 antibodies. (**A–C**) Western blot analysis of differentiated PLB-985 cell lysates from 0.1×10^6, 0.5×10^6, and 1×10^6 cells expressing either control shRNA or Hax1 shRNA from three independent replicates. Mouse anti-tubulin (beta-) is used as a loading control and can be seen present at a relative mobility of 55 kDa. Both mouse and rabbit anti-Hax1 detects a band at a relative mobility of 35 kDa as predicted. (**D**) Quantification of the band intensities of tubulin and Hax1 relative to control shRNA from three independent Western blots. Error bars indicate standard error of the mean. p values were calculated using paired t-test to assess significance relative to control shRNA.
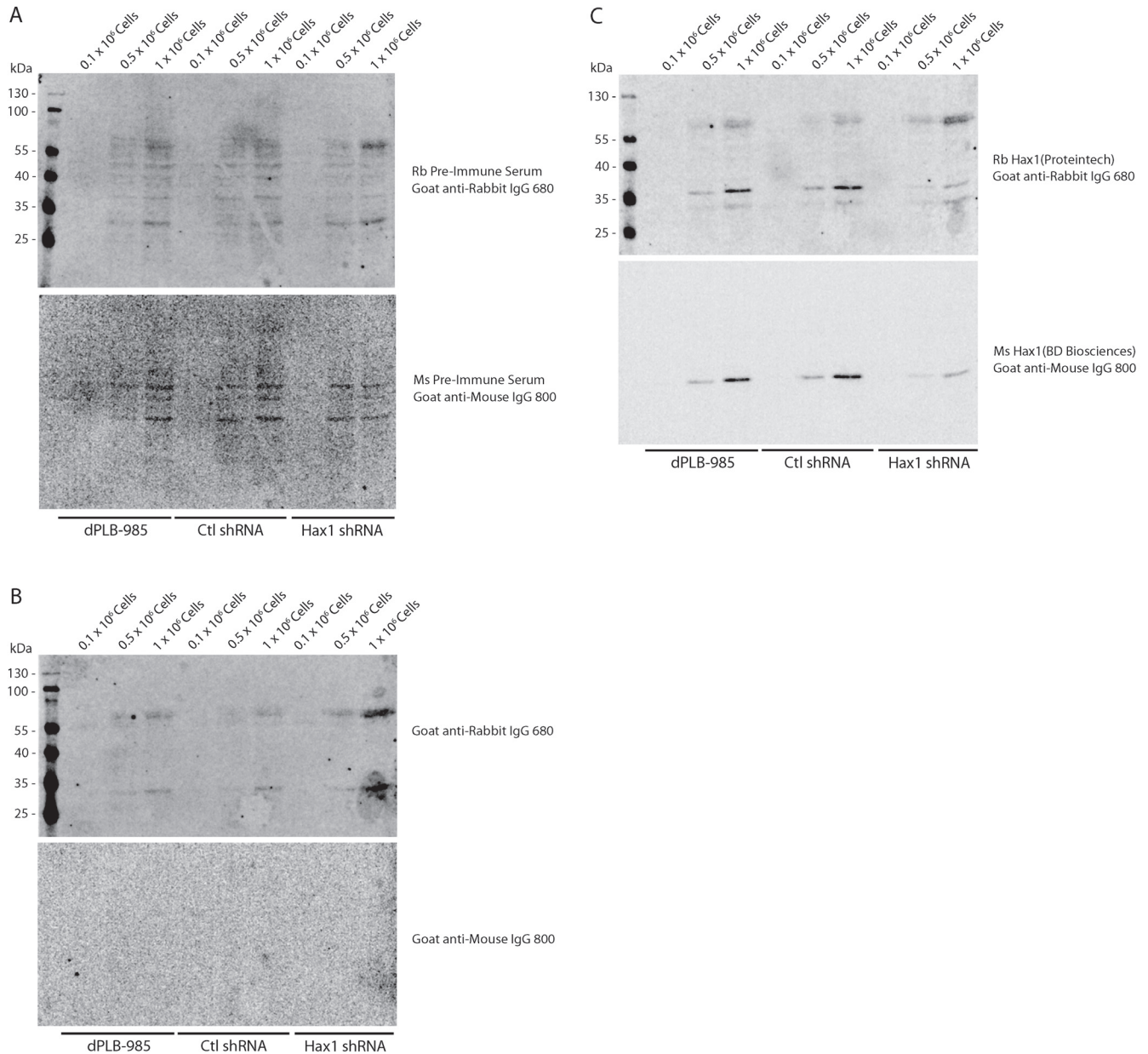
Figure 5. Goat anti-Rabbit IgG secondary antibody only background detection of differentiated PLB-985 cell lysates. (**A**) Western blot analysis using rabbit and mouse pre-immune serum from 0.1×10^6, 0.5×10^6, and 1×10^6 differentiated PLB-985, control shRNA, and Hax1 shRNA cells. (**B**) Western blot analysis using goat anti-rabbit IgG 680LT only on cell lysates from 0.1×10^6, 0.5×10^6, and 1×10^6 differentiated PLB-985, control shRNA and Hax1 shRNA expressing PLB-985 cells. Two predominant background bands can be observed at a relative mobility of 60 and 70 kDa, and one band around 30 kDa. These background bands can also be seen in Figure 1, Figure 2, and Figure 4. (**C**) Subsequent incubation with rabbit and mouse anti-Hax1 from Western blots shown in **B** demonstrate the appearance of the Hax1 band at the predicted 35 kDa size.

a starting point for immunoblotting of Hax1, with greater than or equal to 1×10^6 cells being optimal.

Data availability

F1000Research: Dataset 1. Raw data for Figure 3 quantification., 10.5256/f1000research.6516.d99343[12]

F1000Research: Dataset 2. Raw data for Figure 4 quantification., 10.5256/f1000research.6516.d99344[13]

Author contributions

PC and KI co-wrote and conceived of the article. PC developed the figures. KI performed Western blotting and cell culture. All authors agreed to the final content of the manuscript.

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    PubMed Abstract | Publisher Full Text

12. Kristina I, Cavnar P: **Dataset 1 in: Immunoblotting validation of research antibodies generated against HS1-associated protein X-1 in the human neutrophil model cell line PLB-985.** *F1000Research.* 2015.
    Data Source

13. Kristina I, Cavnar P: **Dataset 2 in: Immunoblotting validation of research antibodies generated against HS1-associated protein X-1 in the human neutrophil model cell line PLB-985.** *F1000Research.* 2015.
    Data Source

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Current Referee Status: ☑ ☑ ☑

Version 1

Referee Report 08 July 2015

☑ **Lawrence L. LeClaire III**

Department of Biochemistry and Molecular Biology, University of South Alabama, Mobile, AL, USA

Kristina and Cavnar present data to validate two commercial antibodies for HSA-associated protein X-1 in a human neutrophil cell line. The authors describe the conditions for the experiments as well as provide sufficient information/protocols for reader about the conditions used in that assays. In addition, the authors present the entire blots and describe the secondary bands that were detected. Only minor changes in the text should be made (below).

1. The commercial source of the antibody should be listed in the abstract. It currently gives the impression that the antibodies used in the study were generated by the authors.

2. The electrophoretic transfer conditions should be listed. Transfer buffer contents (%MeOH) as well as the model of transfer equipment (dry, semi-dry) will cause variability in protein transfer/antibody detection.

3. Did the authors attempt to vary blocking conditions (milk, commercial blocking agents)?

4. Is purified HAX1 available to use as a positive control or to build a standard curve for quantifying total HAX1 in the samples? Not necessary for this publication but would be extremely useful. Figure 3B suggests that detection is close to linear (especially with LiCor technology).

5. Authors should denote the size of bands on an SDS-PAGE as "relative mobility." For example, a band with a relative mobility of 32 kDa was detected...

6. In Figure 3, change "After transfer, the membrane was cut.." To "the membrane was divided"

7. Are the authors certain that the band detected by the secondary antibody is the lower of the two bands in the experiments? A single lane could be divided and one side probed with primary and the other with only secondary to confirm.

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.

Author Response 03 Aug 2015

Peter Cavnar, University of West Florida, USA

Thank you for your kind review. I have revised and submitted a new version of this manuscript. Per your questions I will address each one:

1. I have included the commercial availability of the antibodies in the abstract, introduction, and discussion.

2. The transfer buffer conditions have now been included in Table 1.

3. We have tried blocking with non-fat dry milk and do not find any differences in the selectivity of the Hax1 antibodies, however we did get considerable background from the milk in the goat anti-rabbit 680 channel. We have tested this with a blank membrane. Therefore we will traditionally use 5% BSA to block.

4. We do have bacterial constructs that could be used to purify Hax1. Naturally that was not the purpose of this study, but I concur that these antibodies do behave quite linearly.

5. Thank you for this suggestion, we have included all sizes as a relative mobility in our results and figure legends.

6. We have edited figure 3 legend to reflect this.

7. We have revised figure 5 which demonstrates that the background band that has a relative mobility of 32 kDa is from the secondary antibody.

Competing Interests: No competing interests were disclosed.

Referee Report 06 July 2015

Mautusi Mitra
Department of Biology, University of West Georgia, Carrollton, GA, USA

The paper titled ""Immunoblotting validation of research antibodies generated against HS1-associated protein X-1 in the human neutrophil model cell line PLB-985." by Kristina and Cavnar focuses on the validation of two commercial antibodies generated against the human Hax1 protein. The experiments are in most part well designed. The research approaches, methods and the reagents used for the experiments are well described. The researchers have shown the entire Western blot and have also used Hax1-deficient PLB-985 cell lines to demonstrate the specificity of the two Hax1 antibodies. The data presented in the article strongly indicate that both the antibodies, especially the rabbit anti-Hax1 polyclonal antibody, can be used for specific detection of the human Hax1 protein and also shows the minimum cell density of neutrophils that can be used for immunoblotting experiments.

The manuscript is suitable for indexing, provided the authors address few issues, which are described below:

1. It is not clear if the antibodies were purchased commercially or were generated by the research laboratory with the help of a commercial company. This should be clarified.

2. Hax1 interacts with the polycystic kidney disease protein PKD2, located in the ER membrane/plasma membrane protein. Have the authors checked the efficiency of protein solubilization in their experiments? Have the authors done western analyses on a blot which has proteins transferred from a SDS-PAGE gel that has the intact stacking gel? If the solubilization is incomplete, insoluble proteins will be detected by the respective antibody in the stacking wells in the blot. This experiment can help to confirm the minimum cell density of neutrophils to be used for immunoblotting and might help to improve the sensitivity of the Hax1 detection by these antibodies.

3. The authors should specify the detailed composition of the Laemmli sample buffer as the composition of this buffer varies slightly from lab to lab. Some research labs add 2M-4M urea in the Laemmli buffer for complete solubilization of membrane bound- hydrophobic proteins that have a tendency to precipitate in SDS when heated at 95°C. If urea plus SDS are used for sample solubilization, samples are incubated at room temperature for 30 minutes [as heating causes urea to break down].

4. Have the authors tried immunoblotting with the pre-immune serum to make sure that these antibodies do not have any non-specific interactions? The authors should make this clear in the manuscript.

5. The authors should clearly state the duration of the secondary antibody incubation which is missing in the manuscript.

6. The authors state that the background protein bands are due to the goat anti- rabbit IgG secondary antibody (Figure 5). But there are background bands with the goat anti-mouse IgG secondary antibody (Figure 5). Have the authors performed the same experiment with the goat-anti-mouse secondary antibody? Is the background due to the cell line used in the experiment or due to the specific batch of the antibody? It would be nice to discuss this issue in the discussion section of the manuscript.

7. No explanation was given for the observation why the control shRNA cells show more intense staining intensity than the wild type cells. This should be addressed in the discussion.

8. Figure 3B does not clearly state how many replicates were used for the quantification of the band intensities.

9. The authors should quantify the Hax1 knockdown in the Hax1 shRNA cell line (Figure 4). Without quantification, it is hard to accurately distinguish the specific Hax1 reduction from the background non-specific weak binding of antibodies.

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.

Author Response 03 Aug 2015

Peter Cavnar, University of West Florida, USA

Thank you for your kind review of our manuscript. Below I have addressed your comments:

1. We have clarified in the abstract, introduction, and discussion that these antibodies are commercially available.

2. We have performed these experiments using gels that have not had the stacking gel removed and we do not observe any noticeable signal in the stacking gel. Furthermore, Gallagher *et al.* use a 1% TX-100 lysis buffer in their experiments similar to our lysis conditions. It would be interesting to purify the nuclear extract and compare to the cytosolic fractions to determine this further.

3. The composition of the Laemmli sample buffer has now been added to Table 1.

4. Thank you for this suggestion. Pre-immune sera experiments have been added to figure 5. In short we find that there is increased background using the pre-immune sera that we don't typically see. This demonstrates that the commercially available antibodies are relatively clean after the purification process.

5. The 1 hour incubation of the secondary antibody has been added to the manuscript.

6. The apparent background bands noticed in the mouse channel was a mistake on our part. For many of the figures the rabbit and mouse channels were overlays. This has been fully corrected and every figure now contains separate blots for the mouse and rabbit channels. As can be seen the background is only observed in the rabbit channel.

7. We have repeated these experiments and on average we do not see any significant increase in band intensities between the wild-type PLB-985 cells and the control shRNA cells (see Figure 5C). This could have been variation due to loading in the past figures. We hope the new figures are more clear in this regard.

8. Figure 3B is quantification from three independent replicates. This has been clarified in the figure legend, and the included dataset 1 contains the raw numerical information.

9. This has been completed. Quantifying the knockdown from the 0.1×10^6 and 0.5×10^6 cell samples was highly variable due to the low level of protein available, however at the 1×10^6 cell density quantification was reliable and both antibodies showed a relatively equal level of knockdown.

Competing Interests: No competing interests were disclosed.

Andrew Chalmers
Department of Biology and Biochemistry, University of Bath, Bath, UK

The work presented by Kristina and Cavnar presents data validating two commercial antibodies raised against Hax1. Their experimental approach is well explained and there is a good description of the methods and reagents used. The data is also convincing and well presented, I particularly liked the fact that complete blots are shown and knockdown used to show specificity. Overall, it is a very strong example of an antibody validation paper.

I believe the manuscript is suitable for indexing subject to a few fairly minor changes, which are detailed below:

1. I would make it clear that the antibodies are commercially available in the abstract.

2. It would be worth double checking that details are provided for all of the western blotting reagents and methods. There is plenty of evidence that small changes can alter antibody behaviour and it really helps those trying to reproduce the work. One thing I noticed is that I don't think the contents of Laemmli loading buffer are spelt out, does it have reducing agent in it? Another example is how long were secondaries incubated for?

3. Figure 3- Is the quantification based on one or more experiments- this should be clear in the legend.

4. Dataset 1- should be "plotted" and not "graphed".

5. I was interested in the background which appears to be caused by the secondary antibodies. The authors state it is due to the anti-rabbit antibody, but it seems there is at least some with the anti-mouse staining? I would be interested if this is common with this cell line or is it this set of secondaries- I think this would be worth briefly mentioning in the discussion.

6. The authors should present the quantification of the knockdown (figure 4). If both antibodies are specific then you would predict that they will show roughly similar levels of reduction in signal. This would suggest that the remaining signal is caused by a lack of knockdown rather than low levels of non-specific staining.

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.

Author Response 03 Aug 2015
Peter Cavnar, University of West Florida, USA

Thank you for the kind review of our manuscript. We have addressed your comments below:

1. Commercial availability of the antibodies has now been included in the abstract, introduction, and discussion.

2. We have now included the composition of the Laemmli loading buffer and also our transfer buffer composition to Table 1.

3. Figure 3 includes quantification from three independent experiments.

4. All references to datasets and graphs have been changed to "plotted".

5. This was a mistake on our part in our first submission the two channels were overlays. This has been corrected and all blots have been separated into their subsequent mouse and rabbit channels. We hope it is clear now that the background bands we see are due to the goat anti-rabbit 680 secondary antibody with no detectable background in the mouse channel.

6. This was a great suggestion and has been completed. The quantification is highly variable at low cell densities, however at 1×10^6 cells the level of the Hax1 KD is relatively constant and significant using the mouse or rabbit Hax1 antibodies. This demonstrates the specificity of the Hax1 antibodies used in this study.

Competing Interests: No competing interests were disclosed.