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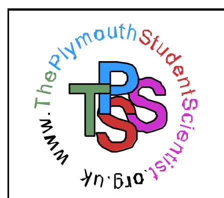

Expression and Localisation of Wave Isoforms in Human Schwann and Schwannoma Cells

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Abstract

Schwannoma, the main tumours associated with Neurofibromatosis Type II, are caused by loss of function mutations in the NF2 gene, encoding for the tumour suppressor Merlin. Schwannoma cells display protrusive structures, due to deregulation of the cytoskeleton. The cytoskeleton is controlled by small Rho GTPases, such as Rac1 and Cdc42, known to be activated continuously in merlin-deficient human schwannoma cells (NF2-/-). Downstream Rac signalling leads to the activaton of Wave signalling complexes, which have been shown to bind and activate the Arp2/3 complex, a crucial regulator of the actin cytoskeleton that is involved in formation of protrusive structures. This investigation used an established NF2 model of primary human Schwann and schwannoma cells to investigate the expression and localisation of Pan-Wave and Wave-2. Using commercially available antibodies, immunocytochemistry (ICC) labelling for Pan-Wave showed an atypical distribution in both human Schwann and schwannoma cells. Western blotting for Pan-Wave in NF2-/- (schwannoma cells) also showed multiple non-specific bands. ICC for Wave2 in human schwannoma showed atypical distribution of this isoform. Western blotting for Wave2 using whole Schwann cell lysate, schwannoma lysate and HL60 cell lysate confirmed the non-specificity of the antibody. Therefore the specificity regarding commercially available antibodies is discussed.

Keywords: NF2 • schwannoma • merlin • GTPase activation • cytoskeleton • Wave isoforms • Westernblot • Immunocytochemistry

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INTRODUCTION

Background

Schwannoma tumours are the hallmark of Neurofibromatosis Type II and are characterized by loss of function mutations in both alleles of the NF2 gene (OMIM 101000) encoding for the tumour suppressor Merlin (Rouleau *et al.*, 1996). Merlin, physiologically expressed in a variety of cell types, is thought to mediate contact inhibition of growth by blocking recruitment of Rac to the membrane (Okada T. *et al.*, 2007) and suppresses Rac dependent signalling (Shaw, RJ. *et al.*, 2001). Moreover merlin blocks signalling through integrins the Ras/Raf/Mek (Lim, JY. *et al.*, 2006) and P13/AKT pathway implicated with proliferation and cell survival (Okada, T. *et al.*, 2007). Loss of Merlin in Schwann cell leads to the formation of schwannoma. Schwanoma cells display abnormalities such as increased proliferation rate, reduced apoptotic response (Utermark, T. *et al.*, 2005), loss of contact inhibition of growth (Lallemande D. *et al.,* 2003; Flaiz C. *et al.,* 2008), increased cell spreading, adhesion and dynamic protrusive structures (Pelton, P.H. *et al.*, 1998) due to increased actin remodelling (reviewed in Hanemann, C.O., 2007).

The formation of protrusive structures, such as membrane ruffles, lamellipodia and filopodia has been shown to be controlled by members of the small Rho GTPase family like Rho, Rac and Cdc42 (Hall, A. *et al.*, 1998). RhoGTPases control the cytoskeleton and as they cycle between an active GTP bound state and an inactive GDP bound state, can be referred to as molecular switches. It is understood that the rate at which GTP is cycled is dependent on GEFs (Guanine exchange factor) and GAPs (GTPase activating proteins) (Hall, A. *et al.*, 2005). Co-ordinated Rac activation has been shown to mediate myelination in normal Schwann cells (Nodari *et al.*, 2007).

Rho GTPase Effectors

Rac and Cdc42 regulate the actin cytoskeleton and the formation of protrusive structures by activation of the Arp2/3 (Actin related protein) complex. The Arp2/3 complex, consisting of seven subunits, is capable of binding to existing actin filaments upon activation (Machesky, L. *et al.*, 1999), thereby inducing the nucleation of new actin filaments implicated with the formation of the highly branched actin filament network in protrusive structures. The Arp2/3 complex becomes activated through WASP (Wiskott-Aldrich syndrome proteins) and WAVE (Wiskott-Aldrich verprolin homologous) proteins (Fig.1), which are downstream effectors of Cdc42 and Rac. Rac is thought to activate Wave indirectly through PIR121 (p53 inducible messenger RNA) (reviewed in Bompard, G and Caron, C., 2004), whereas Cdc42 is capable of binding WASp directly via the GTPase binding domain (GBD) a domain absent in Wave (Miki, H. *et al.*, 1998). WASp, N-WASp and Wave proteins belong to the family of Wiskott-Aldrich syndrom proteins (WASP) and share the VCA module consisting of 3 domains known to be required for Arp2/3 complex activation.

Wave

Wave proteins have been described to exist as three isoforms in mammalian systems (reviewed in Sonderling, S.H and Scott, J.D, 2003), namely Wave1, Wave2 and Wave3.
Expression of isoforms varies between different tissues, cell types respectively, and in rat Schwann cells Wave 2 has been described as the main isoform (Bacon *et al.*, 2007). Each Wave protein is bound in a complex with other proteins such as HSPC300, PIR121 (p53 inducible messenger RNA), Nap1 (Nck-associated protein), and Abi1 (Abl interactor). These pentameric Wave complexes are thought to partially disassemble upon activation. And some isoform specific variation has been described concerning these complexes, and their regulation. Despite these differences PIR121, associated with

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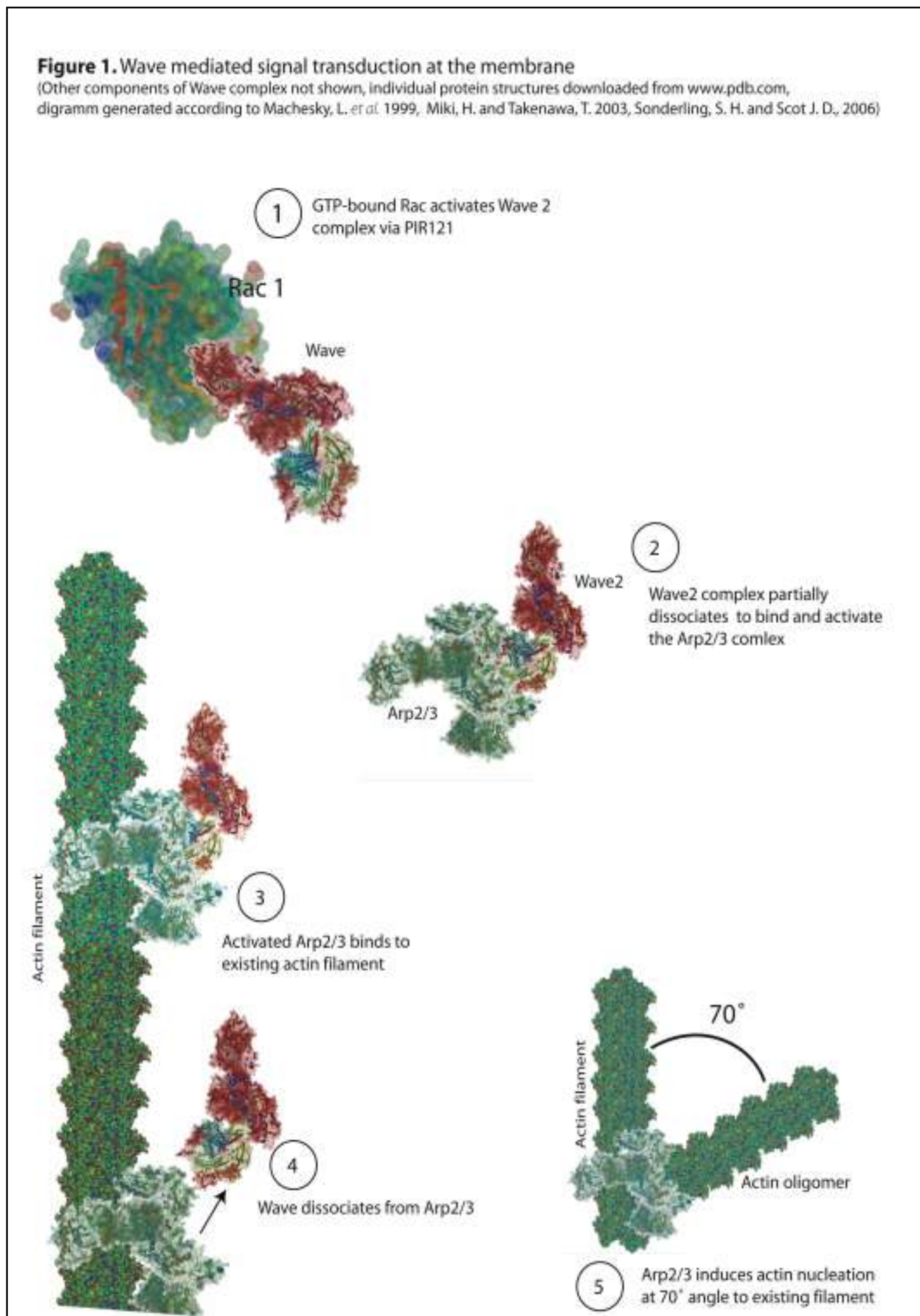

Figure 1. Wave mediated signal transduction at the membrane
(Other components of Wave complex not shown, individual protein structures downloaded from www.pdb.com, digramm generated according to Machesky, L. *et al.* 1999, Miki, H. and Takenawa, T. 2003, Sonderling, S. H. and Scot J. D., 2006)

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all isoforms, has been brought forward as the link between Rac and Wave activation, due to its sequence homology to p140Sra- (specific Rac1-associated protein) (reviewed in Bompard, G and Caron, C., 2004).

The underlying ultimate aim of any study in any disease model is to understand the mechanisms involved and to identify a molecular target for treatment. Molecular targets are proteins involved in signalling pathways, and are activated or expressed at higher levels compared to the wild type. In addition proteins can be mislocalised, altering their wild type function. It was the aim of this project to investigate expression (Western blot analysis) and localisation (Immunocytochemistry) of Wave in the context of human schwannoma (Neurofibromatosis Type 2). In order to obtain comparative data, experiments were also performed in normal Schwann cells. Using this model it has already been shown that Rac and Cdc42 are activated at higher levels leading to higher levels of Arp2/3 activity in schwannoma cells (Flaiz et al., 2007, Kämpchen et al. 2003). By combining westerblot analysis and immunocytochemistry, using the same antibody, results can be qualitative and quantitative verified.

METHODS AND MATERIALS

In vitro schwannoma Model

This model allows the comparison of human primary Schwann cells and Schwannoma cells (donated from Neurofibromatosis II patients), and requires any experiment to be repeated three times in cells with different origin (three pairs of NF2+/+ and NF2-/-).

Not all investigations have involved both cell types. Only if considered favourable have human Schwann cells been used (as these cells are derived from healthy donors and are difficult to obtain.) Some antibody optimization has been performed using RT4 (Rat Schwann cells).

A final experiment in the Wave-2 investigation has been carried out using three pairs of Schwann and schwannoma cells derived from independent origin.

Cell Culture

(All chemicals from Sigma-Aldrich unless indicated otherwise.)

RT4

RT4 (rat schwannoma) cells were cultured from frozen aliquots. Cells were thawed from liquid nitrogen (-273° C) to 37° C gently in a water bath, before being transferred into 10%FCS/DMEM. The suspension was centrifuged for 10 minutes at 1500 rpm. The supernatant was exhausted and the pellet was resuspended in 10%FCS/DMEM, seeded in plastic dishes and incubated at 37° C and 5% CO_2.

Schwann /schwannoma cells from tissue

Schwann and schwannoma cells were cultured according to the methods described by Rosenbaum, C. *et al.* (1998).

Schwann cells (NF2+/+): The nerve was prepared by pulling the fascicles out of the connective tissue (adventitia) and incubated with medium (10%FCS/DMEM, 400U/ml Penstrep, 1.6μM Forskolin, Amphotericin) for 14 days at 37° C/ 10% CO_2. Incubation medium was changed every 3 days.

Schwannoma cells (NF2-/-): Tumours were stripped of connective tissue and incubated with medium (10%FCS/DMEM, 400U/ml Penstrep, 1.6μM Forskolin, Amphotericin) for 1-7 days at 37° C/ 10% CO_2. Incubation medium was changed every 3 days. After incubation for the indicated times, digestion medium containing 10%FCS/DMEM, 400U/ml Penstrep, Dispase (Roche) and Collagenase was added and the nerve/tumour was cut into 1mm pieces and remained incubated in digestion medium for 18h (nerve) or 24 h (tumour) respectively at 37° C /10% CO_2. The pieces were then further broken down using a series of constricted glass Pasteur pipettes and the suspension was centrifuged for 7 minutes at 4000 rpm. The supernatant was exhausted and the pellet resuspended in growth medium consisting

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of 10%FCS/DMEM, 100U/ml Penstrep, 0.5µM IBMX, Amphoterecin, 2.5µg/ml Insulin, 0.5 µM Forskolin and 10nM Beta-Heregulin. Cells were incubated on poly-L-lysine/laminin coated plates (Greiner Bio One) for a maximum time of 1 week at 37° C 10% CO_2. Mediums were changed after 3 days. S-100 (Schwann Cell marker) staining was performed in the 1° or 2° passage, to assess fibroblast contamination (negligible at < 2%).

Antibodies

Anti-Wave/SCAR, rabbit polyclonal (Upstate)

Anti-Wave-2, rabbit antiserum against C-NRGNVNPRKIKTRKE 200 Aa of human Wave-2 protein (Upstate).

Western Blotting

Cell lysis/Protein extraction: In order to extract protein from the cells, also referred to cell lysate. Lysis buffer consisting of DLP, complete inhibitor (1:50) (Roche) and phosphatase inhibitor I and II (1:100) was boiled at 99°C and applied to adherent cells (50µl lysis buffer per 200.000 cells). Cells were scraped down and transferred to a tube, boiled at 99° C for 5 minutes, cooled on ice for 5 minutes and centrifuged for 30 minutes at 3500 rpm and 4° C. Aliquots (20µl) were frozen at -80° C and used in subsequent investigations.

Protein estimation: In order to measure protein amounts. Protein amounts were measured using the Bio-Rad colorimetric assay against a lysis buffer control. This step is necessary when comparing different cells i. e Schwann and schwannoma cell, and is later verified by the loading control.

Electrophoresis(SDS-PAGE): In order to separate proteins by size. Following the standard protocol 1.5mm thick 12% polyacrylamide resolving gels, most suitable for detection of proteins between 20-120 kDa, were poured into Bio-Rad systems and allowed to set for 40 minutes. 4% polyacrylamide stacking gels were poured on top, comb defining the loading wells was inserted and allowed to set for 30 minutes at room temperature. Protein aliquots were boiled in their sample buffer (100mM Tris-HCL, 4% SDS, 20% Glycerol, 0.01% Bromophenol blue) for 5 min. and cooled on ice for 5 min., before being loaded into the wells. Standard marker (containing proteins of known size) was loaded into the most outer well. The gels were run in running buffer containing 3.03 mg/ml Tris, 14.4 mg/ml Glycine, 1mg/ml SDS at 200V, 400mA for 45 minutes until the dye front run off.

Blotting (Transfer): In order to transfer protein from the gel to the membrane. PVDF membranes were cut to gel size and placed on the gel in a blotting sandwich. Blotting was run in transfer buffer containing 3.03mg/ml Tris, 14.4mg/ml Glycine, 200µl/ml methanol and H_2O over night at 85V, 175mA and 4° C.

Protein Detection (following the standard protocol)

Blocking: in order to block unspecific binding sites, membranes were placed in blocking solution (Tris buffered saline-Tween 20 (TBS-Tween) 5% skimmed milk powder, 2% Bovine albumin serum (BSA)) for 1 hour at room temperature on a rotator.

Primary antibody: in order to detect Wave protein, Anti Pan-Wave (1:1000) or anti Wave-2 (1:500) were diluted in blocking solution and/or 1% BSA TBS and incubated with the membrane overnight at 4° C on a rotator. Membranes were washed in TBS-Tween twice for 30 minutes.

Secondary Antibody: in order to label primary antibody, Goat-anti-rabbit HRP conjugated secondary antibody (Bio-Rad) at concentration (1:2500) was incubated in blocking solution with the membrane for 1 hour at room temperature on a rotator. Membranes were washed three times in TBS-Tween for 5 minutes and twice in TBS for 10 minutes

ECL detection: in order to detect protein bound secondary antibody on the membrane, ECL Plus (Amersham) solution was prepared and applied to the membrane for 5 minutes. Membranes were incubated with the film in the darkroom for an initial period of 5 minutes,

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followed by development of the film using a conventional 3- step solution system.
<u>*Densitometry:*</u> In order to compare protein levels, optical density of bands can be measured using the FlourS Multi Imager, and the Quantity One analysis software (Bio-Rad). The data is normalised by dividing each value by the value obtained from each loading control.

Immunocytochemistry *(ICC)*
In order to show intracellular localisation of Wave proteins and to investigate possible co-localisation. Cells were grown on poly-lysine/laminin coated Lab-tek slides, in growth medium. After 3 days, cells were fixed with paraformaldehyde (PFA 4%) for 10 minutes at room temperature. Following the standard protocol cells were washed with TBS, incubated with 0.2% Triton/PBS for 5 minutes at room temperature, washed 3 times with TBS for 5 minutes before blocking with 10% NGS 1%BSA TBS for 45 minutes at room temperature. After washing cells with TBS for 5 minutes at room temperature, slides were incubated with primary antibodies (anti Pan-Wave 1:100, anti-Wave-2 1:100, anti Rac1 1:100) (BD Bioscience) in 1%BSA/TBS overnight at 4º C.
TBS washing step (3 times 5 minutes) was followed by incubation with secondary antibodies (Goat-anti-mouse Alexa Fluor 488 1:500 or Goat-anti-rabbit Cy3 1:500) in 1%BSA/TBS and AlexaFlour 488 labelled Phalloidin (1:100) (Molecular Probes) to stain F-actin, for 40 minutes at room temperature. Following three final washing steps with TBS (5 minutes at room temperature) slides were covered and sealed using vecta shield.

Confocal Microscopy
Subsequent visualisation of fluorescently labelled structures and proteins within the cells was performed using a Zeiss meta510 confocal laser microscope, in conjunction with the Zeiss Imaging Software
.
RESULTS

Pan Wave
Immunocytochemistry labelling for Factin to visualize the cytoskeleton and for Pan Wave was used to determine localisation and distribution of WAVE in both NF2+/+ and NF2-/-. The polarized Schwann cells showed Pan Wave mostly concentrated in the nucleus (Fig.2A), whereas NF2-/- displayed Pan Wave in the nucleus and the cytoplasm where Wave seems to co-localise partly with actin filaments (Fig.2B). In order to verify the specificity of the Pan Wave antibody, western blots using NF2-/- lysate were performed. The western blot analysis for Pan-Wave using NF2-/- whole cell lysate revealed bands at 125 kDa, 84 kDa and 50 KDa (Fig.3).

Wave–2
ICC labelling for Wave-2, F actin and Rac in NF2-/- schwannoma cells was carried out in order to determine localisation and distribution of Wave 2, and to investigate a potential co-localisation of Wave 2 with Rac1. Wave 2 could be found in the nucleus and the cytoplasm (Fig.4A). Fig.4B and at higher magnification (Fig.4C) showed co-localisation (yellow) of Rac1 and Wave-2 at the membrane.
Three pairs of NF2+/+ and NF2-/- at protein concentrations of 5µg and 10µg were used to investigate Wave 2 levels in both cell types. RhoGDI was used as a loading control. Wave-2 was expected to show a single band at 68 kDa. Western blots showed bands at around 75 kDa, 50 kDa, 37 kDa and 30 kDa (Figure 5). Only a very weak band at 68kDa was detected, and protein levels between cell types could not be compared using densitometry analysis.

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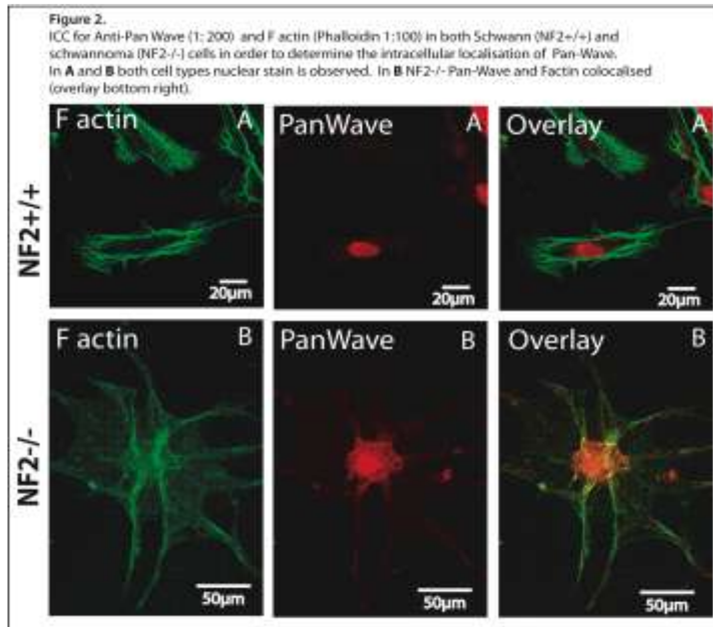

Figure 2.
ICC for Anti-Pan Wave (1: 200) and F actin (Phalloidin 1:100) in both Schwann (NF2+/+) and schwannoma (NF2-/-) cells in order to determine the intracellular localisation of Pan-Wave. In **A** and **B** both cell types nuclear stain is observed. In **B** NF2-/- Pan-Wave and Factin colocalised (overlay bottom right).

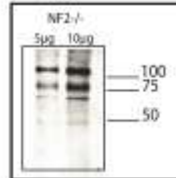

Figure 3.
Western blot with different protein concentrations of NF2-/- (5µg and 10µg) probing with Anti-Pan-Wave (1:1000) revealed three bands at 125kDa, 84 KDa and 60 kDa. Pan-Wave was expected at around 84 kDa.

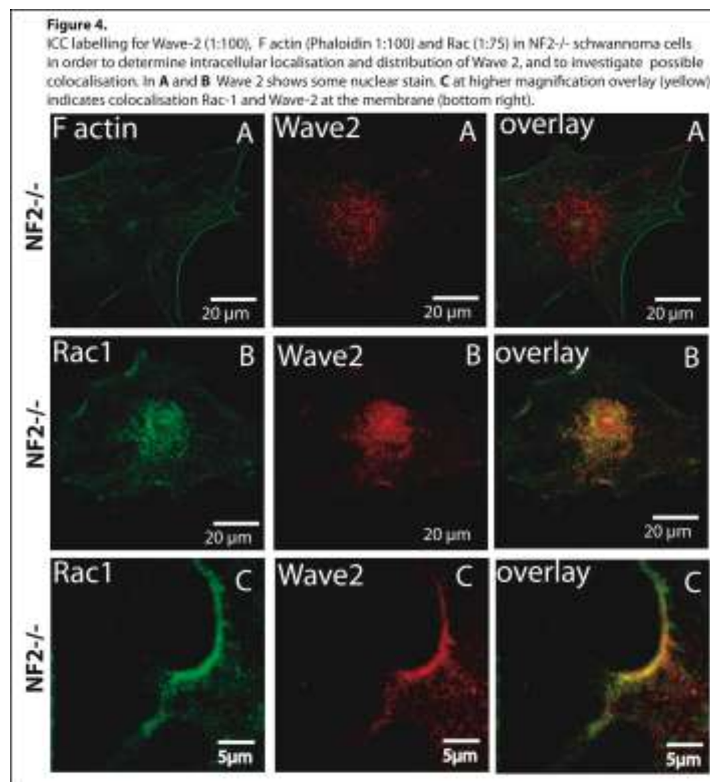

Figure 4.
ICC labelling for Wave-2 (1:100), F actin (Phaloidin 1:100) and Rac (1:75) in NF2-/- schwannoma cells in order to determine intracellular localisation and distribution of Wave 2, and to investigate possible colocalisation. In **A** and **B** Wave 2 shows some nuclear stain. **C** at higher magnification overlay (yellow) indicates colocalisation Rac-1 and Wave-2 at the membrane (bottom right).

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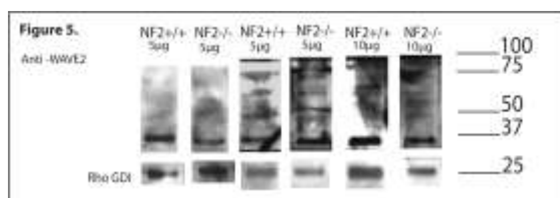

Figure 5. Wester blot to determine Wave 2 levels in 3 pairs of NF2+/+ and NF2-/- at protein concentrations of 5µg and 10µg. Rho GDI (25kD) was used as loading control. Multiple bands were detected. Wave 2 was expected at 68 kD. Main band was found at 37 kDa, another band at around 50 kDa and a weak band at around 68 kDa, which did not give enough contrast for densitometry analysis.

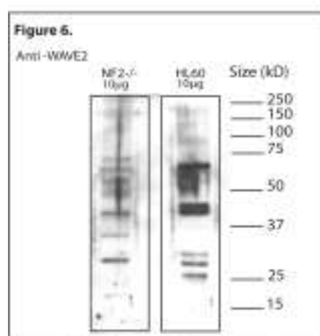

Figure 6. Control western blot to determine specificity of the Wave2 antibody. HL60 cell lysate and NF2-/- at 10µg showed similar multiple bands. Wave- 2 was expected at 68 kDa. Main bands found at around 30kD and 40kD .

To analyse the specificity of the antibody, a control western blot using HL60 cell lysate and NF2-/- lysate was performed (Fig.6). Multiple bands, including a weak band at around 68 kDa, but a main band at around 40 kDa and one band at 30 kDa were detected. ICC in Schwann cells was not performed as the western blot analysis of Wave-2 showed multiple bands in the control lysate, indicative for non-specificity of the antibody.

DISCUSSION

To investigate Wave expression and localisation in human primary schwannoma cells, a Pan-Wave antibody was used initially. Western blot analysis showed multiple bands among which was one at 84 kDa as expected. Multiple bands can occur due to different reasons and as cell lysis and protein extraction involved boiling the samples; this could have lead to partial breakdown of the protein in question, and would offer some explanation to why the bands were lower than expected. Protease and phosphatase inhibitors contained in the lysis buffer should however prevent protein breakdown.

Multiple bands can also be an indication for non-specificity of the Pan-Wave antibody. Specificity can be tested using a blocking peptide. The term “blocking peptide” refers to the original peptide against which the host produced antibodies, which subsequently are extracted from the blood serum. Blocking peptide however was not available for this antibody. In NF2-/- cells Pan-Wave colocalises with F-actin, which can be expected due to observed increase in actin remodelling in schwannoma cells. However, specificity of the antibody is in question, and therefore conclusions taken from this colocalisation are difficult. Moreover a nuclear stain in both cell types was observed. Wave has been put forward to have a potential role in regulation of nucleocytoskeleton dynamics as it has been observed in the nucleus (reviewed in Bompard & Caron, 2004). Nuclear staining however is also an indication for unspecific binding of the antibody, and taking into consideration the results from the western blot analysis this seems more likely. The Pan-Wave antibody is polyclonal, as a number of epitopes are recognised. Wave and WASP share the Arp2/3 binding domains (VDA module), and belong to the same family of proteins and share 85% homology. It is therefore possible that the Pan-Wave antibody binds any of these, very similar proteins. An isoform specific antibody (Wave-2) was expected to provide more specific results. Wave2 was chosen, since it is considered to be the major isoform in rat Schwann cells (Bacon *et al.*, 2007).

ICC labelling for Wave-2 showed some co-localisation of Rac1 and Wave-2. This is to be expected as Rac1 binds and thereby activates Wave-2 at the membrane.

Anti-Wave-2 was expected to give a single band at 68 kDa in western blots in HL-60 cell (a leukocyte cell line) lysate according

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to the manufacturer. Multiple bands obtained in western blots using Schwann and schwannoma cell lysates were lower than expected. To rule out errors within the method, HL-60 lysate was purchased and used to determine the antibody's (Wave-2) specificity, by running a control western blot as described by the manufacturer. However this experiment also revealed several bands. A blocking peptide was not available for the Wave-2 antibody.

It is therefore not possible to verify any of the results obtained from the ICC as the antibody is not specific. For this reason ICC in Schwann cells was not performed.

Since Wave proteins are bound in complexes it would be possible to investigate the expression and localisation of other proteins bound in the Wave-2 complex, such as PIR121, Abi1 or Nap-1, but Abi1 in particular (Innocenti, M. *et al*., 2004) in order to further test antibody specificity.

Further experiments that circumvent the antibody problem could be used. For example, real-time PCR could be used to establish the main Wave isoform at mRNA level in both cell types NF2+/+ and NF2-/-. However, to investigate expression of the isoforms at protein levels, the use of antibodies is indispensable. As the quality of commercial Wave antibodies is in question, some research groups generate their own antibodies in rabbits and other rodents and therefore do not rely on commercial availability. Once work using a particular antibody has been published, sample aliquots can be obtained from these groups. Another approach would be the generation of an own antibody.

RNA Interference (RNAi), a technique to block a protein at transcriptional level, could be utilised to knock down Wave-2 and investigate the effect on cell morphology in NF2-/- schwannoma cells. However this technique is known for its intricacy and brings other variables into consideration (Fire, A. *et al*., 1998).

Further more results from N-WASp investigations (including blocking with wiskostatin) in human schwannoma cells (unpublished data) have indicated that upregulation / activation of the cytoskeletal regulatory proteins is a secondary effect following Rac and Cdc42 activation. Potential molecular targets for the treatment of Neurofibromatosis Type II are therefore likely to be upstream of Rac. Such a target could be PAK (Hirokawa *et al*., 2004), or GEFs and GAPs known to control GTPase activity, like Beta-Pix (ten Klooster *et al*., 2006).

3D cell culture and the generation of spheroids, offer an approach to culture cells in a more lifelike environment for longer periods of time (some cells have been shown to retain key functions for weeks to months). As cells are able to interact, and form cell-to-cell junctions, they have been shown to behave differently in response to their microenvironment. Spheroids have been described to mimic *in vivo* functioning. This has proven particular useful in toxicological investigation preceding any clinical trials. However investigations are not restricted to this aspect and all known investigation methods known in biological sciences can be applied using spheroids. As for Schwann and schwannoma cells, 3D culture method could be employed in addition to the existing model to further verify results gained from investigations. The equipment required to establish such a 3D culture model was estimated at around 10,000 GBP in addition to some expertise perhaps in the form of a PhD project (Xu, J. & Purcell, WM., 2006; Kim, SM. *et al*., 2007).

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