The Role of Acetylated Microtubules in Mechanosensation in Mice

Thesis submitted in partial fulfilment of the requirements of
five year BS-MS Dual Degree Programme

By

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Under the guidance of

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CERTIFICATE

This is to certify that this dissertation entitled "**The Role of Acetylated Microtubules in Mechanosensation in Mice"** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by **Kalyanee Shirlekar** at EMBL Monterotondo under the supervision of **Dr Paul Heppenstall**, Group Leader at EMBL Monterotondo during the academic year **2015-16.**

Signature of the Supervisor   Date: 28/03/2016

Dr Paul Heppenstall

DECLARATION

I hereby declare that the matter embodied in the report entitled "**The Role of Acetylated Microtubules in Mechanosensation in Mice"** are the results of the investigations carried out by me at the **European Molecular Biology Laboratory, Monterotondo** under the supervision of **Dr Paul Heppenstall** and the same has not been submitted elsewhere for any other degree.

Signature of the Student   Date: 28/03/2016

Kalyanee Shirlekar

ABSTRACT

The molecular mechanisms of force transduction in mammalian sensory neurons are largely unexplored. In this project, the effect of Alpha tubulin acetyltransferase 1 (Atat1), an enzyme which acts to imbue microtubules with a highly conserved post-translational modification (PTM) by acetylating the lysine 40 residue of α-tubulin was studied on a behavioural and cellular level. Using a conditional knockout (KO) for Atat1, which specifically removes the enzyme from the peripheral nervous system (PNS), the function of this enzyme, was investigated. It was shown in various behavioural tests that the KO mice show a profound deficit in mechanosensation and nociception. On a cellular level, a strong sub-membrane localization of the acetylated tubulin can be found in around ~ 80% of the wild type DRG cells. There is an absence of this sub-membrane 'ring' in DRGs taken from Atat1^{cKO} animals. Thus, we posit that the absence of acetylation causes an increase in microtubule rigidity, which renders the neuronal membrane stiffer in KO mice leading to touch insensitivity. This phenotype can be rescued in cultured DRG cells from the KO background by expression of a plasmid construct which uses an amino acid substitution in tubulin to genetically mimic tubulin acetylation. In light of these results we have recently been exploring options to mimic the KO phenotype in WT DRG cells *in vitro*, which could provide novel therapeutic strategies for conditions like mechanical allodynia.

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INTRODUCTION

'Seeing is believing but feeling's the truth' - Thomas Fuller, Gnomologia, 1732

When a mother holds her new born in her hands, the touch of the mother is comforting for the child. An owner caressing its dog, the brush of a gust of wind past your arms, the tickling sensation of a feather, the prick of a needle, cold water running from the shower, the touch of your shirt on a sun burnt area of the body. How does one perceive these different sensations?

Touch is one of five sensory modalities which allow an organism to extract important information from its environment. Touch or mechanosensation can be defined as the conversion of a mechanical stimulus into electrochemical signals i.e. membrane depolarization and firing of action potentials so as the transfer the sensation of touch, pain, pressure, texture, vibrations etc. From bacteria to mammals, all organisms respond to touch stimuli. The skin is sensitive to varying degrees of force exerted upon it. These stimuli are sensed by a complex of protein partners termed the mechanotransduction apparatus, which span across the membrane of mechanosensitive cells. This complex consists of a variety of molecular players including ion channels, the cell membrane, extracellular proteins and cytoskeletal elements.

Our skin consists of different types of specialised receptors which convert the external stimuli into electrochemical signals for our brain to understand in its own language. This system is called the somatosensory nervous system. The somatosensory system can detect a wide array of stimuli ranging from the sensation of touch, pain, itch and temperature, to the overall sense of bodily orientation termed proprioception. Thus somatosensation can be broadly divided into 4 types of perception systems namely: nociception, thermosensation, mechanosensation and proprioception. Sometimes there is overlap between these sub groups.

Peripheral Organization of the somatosensory system

As summarized by Pichon and Chesler, 2014, somatosensory neurons gather as groups into bodies called the dorsal root ganglion (DRGs) which have 2 outgoing terminals from a single axon. One going to the central nervous system (the spinal cord) and another going to the skin or muscle spindles. Hence, these neurons are termed pseudounipolar. (Kandel *et al.*, 2012, Purves *et al.*, 2012). Somatosensory neurons are broadly classified into 4 fiber types, namely the C fibres, the Aβ, the Aδ and the proprioceptors (Aα) depending upon their level of myelination, cell body size and relative conduction velocities. The C fibres are the slow conducting, unmyelinated fibers with a conduction velocity of 2m/s, and are generally considered to be responsible for the transmission of slow pain sensation. The Aβ fibres are the low threshhold mechanoreceptors and have very high conduction velocities due to heavy myelination. The Aβs have 4 types of distinct nerve ending morphologies in the skin, namely the Merkel cells, the Meissener's corpusles, the Panifician corpuscles and the Ruffini's endings. All of which detect distinct forms of physical stimuli, for example Merkel cells detect light touch while Meissner's corpuscles detect vibration or pressure (Pichon and Chesler, 2014). The proprioceptors are also highly myelinated and have fast conduction velocities of 30-70 m/s. The Aδs are lightly myelinated and have faster conduction speeds than the C fibres. This class of fibres are generally sensitive to temperature, pain and itch (Kandel *et al*, 2012, Purves *et al*, 2012). These fibres are also responsible for the inflammatory sensation (Basbaum *et al*, 2009). These fibres can be further characterised into peptidergic and non-perptidergic fiber types, rapidly adapting and slowly adapting LTMRs etc. But broadly, all these classes of receptors make up the somatosensory system. In this project, we have characterised a touch deficit phenotype in a strain of mice which lacks the enzyme Atat1 in all of the fibre types outlined above.
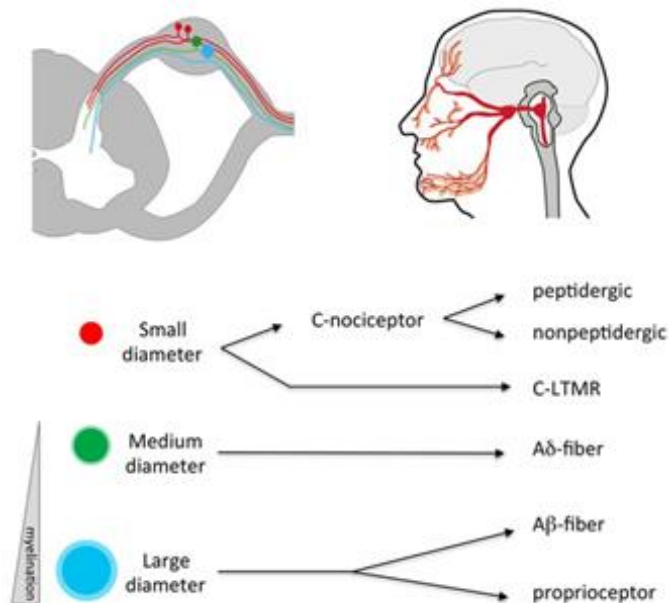
Figure 1 : Sensory system anatomy showing connections emerging from the DRGs and types of sensory afferents classified according to their diameters. (Image taken from the Pichon and Chesler, 2014)

History of Mechanosensation:

In the course of evolution, the emergence of mechanotransduction dates back to eubacteria and archaea highlighting the primitive nature of the mechanosensory faculty. These early organisms developed mechanisms through which they could withstand the environmental challenge of rainfall, by evolving defences against dehydration or over hydration respectively. The first mechanosensitive channel in bacteria and archaea arose as a mechanism for cell protection and survival, and subsequently developed and diversified as these organisms evolved to live in different environments. (Kung, 2005) It is in bacteria where the studies of the mechanosensitive channels (MS) began. In MS channel mutants of *E. coli*, cells were shown to be lysed following a very mild osmotic shock (Martinac B. *et al*., 1987) This was due to the inability of the mutant channels to regulate the response of the organism to the stimulus.

Later, extensive studies were carried out in *Caenorhabditis elegans* and *Drosophila melanogaster*. Genetic screens were carried out to identify the genes associated with mechanical deficits in *C. elegans.* (Chalfie *et al.,* 1989) (Du *et al.*, 1996) These were

the mechanosensitivity or 'mec' genes. Genetic mutants were identified that showed varying degrees of disruption of force transmission in mechanosensitive cells. Some of the genes found to be responsible for these deficits were mec-7 which codes for α tubulin and mec-12 which codes for β tubulin. Also, mutants of the gene mec-4 showed a deficit in mechanosensory currents recorded from *C. elegans* touch receptor neurons when the animal was touched. A major phenotype observed was because of lack of the mec-17 gene, which also resulted in a severe mechanosensitivity deficit especially in the touch receptor neuron (TRN) functions and neurite outgrowth. (Topalidou *et al.*, 2012, Zhang *et al.*, 2002) Mec-17 codes for an acetyltransferase which selectively acetylates the lysine 40 residue on α tubulin subunit of the tubulin heterodimer. (Akella et al., 2010) (Shida, Goodman, & Nachury, 2010)This acetylation occurs in the inner lumen of the microtubule protofilaments, and is the only PTM thus far identified on the luminal side of microtubules. (Soppina *et al.,* 2012) (Nogales *et al.*, 1998)

Mechanosensation and Cytoskeletal Organization:

Microtubules are one of the 3 biopolymers which make up the cytoskeleton of cells, the other two being actin and intermediate filaments respectively. Composed of α and β tubulin subunits, microtubules have been shown to influence cell shape and cytoskeleton tension sensing dynamics. (Brangwynne *et al.*, 2006) Microtubules are approximately 100 times more rigid than actin filaments (Gittes *et al.*, 1993) and hence play a critical role in regulating cell membrane malleability, and nerve outgrowth stabilization. (Zheng *et al.*, 1993) The importance of acetylation of the lysine 40 residue in tubulin in touch sensitive neurons found in *C. elegans*, opened new avenues of understanding into the mechanism of force transduction in mechanosensation.

Mechanosensitive Ion Channels:

Mechanosensitive ion channels are major mechanosensors of force which alter their conformations in open or closed states according to the membrane tensions. The mechanosensitive channels have been extensively studied in the model system *C. elegans.* The mechanosensitive transmission channels coupled with the cytoskeletal

elements have been studied in the TRNs to show the burst of action potentials after these cells are subjected to force. In work which knocked out or knockdown these channel proteins a reduction in mechanosensitivity to varying degrees was observed. The transient receptor potential (TRP) family of proteins is a vast collection of transmembrane proteins which confer sensitivity to a wide array of stimuli. (Montell C., 2005) The most common TRP channels involved in mechanosensation are TRPV, TRPA and TRPN (Lin & Corey, 2005) The TRPA1 type of channel functions to detect nociceptive type stimuli in mice. (Corey DP et al., 2004) Defects in hearing, touch and proprioception have been observed in *Drosophila* mutants for NompC which is the homolog of TRPN which is found in *C. elegans*. (Kahn-Kirby *et al.*, 2006) Up to recently, this channel was one of very few mechanically gated ion channels found in higher organisms. Two new mechanically sensitive ion channels were identified in 2010, termed 'Piezo' after the Greek word for press or squeeze, these channels are expressed in mice. (Coste *et al.*, 2010) Piezo1 which is expressed in vascular tissue and Piezo2 which is expressed in DRG cells, as well as on peripheral sensory organs such as Merkel cells are Ca^{2+} activated mechanically sensitive ion channels. (Hsu e*t al.*, 2015) Work on Piezo1 has shown that this channel exquisitely sensitive to cell membrane tension (Gottlieb, Sachs *et al.*, 2012). Lack of Piezo2 in adult sensory neurons has also been shown to give rise to touch and proprioceptive deficits in mice (Ranade *et al.,* 2014) (Woo *et al.*, 2015)

Like Piezo 1, the 2 pore domain K+ channels: TRAAK and TREK1/TREK2 have been also been shown to respond to mechanosensitive stimuli by detecting tension and deformation on the plasma membrane. (Brohawn, Campbell, & MacKinnon, 2014)

The TRAAK and TREK channels do not require direct linkages to the cytoskeleton to function. This also seems to be the case for Piezo1 (Cox et al., 2016) However, it was demonstrated sometime back (W. Zhang et al., 2015) that the ankyrin repeats from the cytoplasmic domain of the NompC channel found in *Drosophila* were necessary for the mechanical sensitivity of this channel. These elements connect the channel to the microtubule cytoskeleton below. This work demonstrated for the first time that an ion channel can be directly linked to the basal cytoskeleton, and that this binding is essential for some channels function. Thus as summarized in the *figure 2*, the cytoskeleton, the cell membrane and the mechanosensitive ion channels form the

major components of the mechanotransduction machinery which directly and indirectly cause force transmission into the cells.
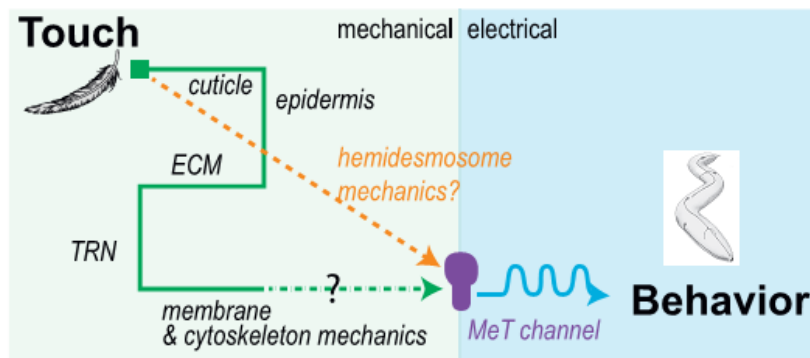

Figure 2: The hypothetical mechanotransduction pathway in *C. elegans* which largely still unexplored. Image taken from Krieg, Dunn, & Goodman, 2015

More about Mec-17:

The mammalian ortholog of the *C. elegans* gene mec-17 is known as the alpha tubulin acetyltransferase-1 (Atat1) which encodes for the enzyme N-acetyltransferase in mice. Atat1 which is a Gcn5-related N-acetyltransferase, was first identified as an α-tubulin acetyltransferase in *Chlymadomonas.* (Shida *et al.*, 2010) Atat1 acetylation is a highly conserved post-translational modification. Previous organisms had shown a deficit of mechanosensation when this gene was knocked out. (Topalidou *et al.*, 2012) In extensive studies carried out in *C. elegans*, it has been shown that mec-17 exclusively acetylates α-tubulin at the lysine 40 residue. This is the only post translational modification known to be present on the luminal side of the microtubules. (Akella et al., 2010) It has been shown that, mec-17 is essential for maintenance of the touch receptors neurons (TRNs) in *C. elegans* (Y. Zhang et al., 2002) though it still remains unknown how absence of mec-17 affects the microtubules and also causes lack of mechanosensation in the TRNs.

Thus, this led us to explore whether or not a similar deficit in mechanosensation would be observed after specifically knocking out Atat1 from the PNS of mice.
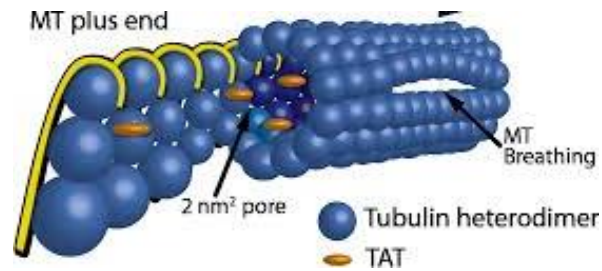
Figure 3: Protofilament of a microtubule with TAT on the luminal side. Image adapted from www.cytoskeleton.com

Hence, a conditional knockout of Atat1 was created in the lab with a peripheral nervous system specific *Advillin-Cre* driver (termed Atat1[cKO]) which results in the absence of acetylation of the lysine 40 residue (K40) of α-tubulin in all the peripheral neuronal fibres, DRGs and the trigeminal ganglion. The gene Atat1 had loxP sites on either side which caused the deletion of the gene in the presence of Cre recombinase. Atat1[Control] were used as a control for the behavioural experiments without the loxP cassettes and thus the gene Atat1 could not be knocked out.

In order the study the behavioural phenotype of the Atat1[cKO] mouse, several behavioural tests for mechanosensation, nociception, thermoception and proprioception were performed to see which somatosensory receptor fibre types were affected. We found that there was a drop in responses in the low threshold mechanoreceptors. On carrying out anti-acetylated staining in DRG, we found a major ring-like structure of acetylated tubulin under the membrane of Atat1[Control] cells. Super resolution microscopy was also performed to investigate the architecture of α-tubulin in control and KO cells. To understand this touch deficit on a neuronal level, we carried out a series of experiments to challenge the microtubule structure of the control and KO DRG cells by giving them a mechanical shock and subsequently studying their responses. To see if we could rescue the lack of acetylation in the KO cells, we created a genetic mutant of tubulin termed K40Q Tub (lysine substituted for glutamine). This amino acid substitution mimicked the acetylated lysine 40 residue of α-tubulin in terms of charge and structure. The transfected KO cells were also given the hyperosmotic shock which caused shrinking of the microtubular network.

In respect to current attempt to utilise a reduction in α-tubulin acetylation as a potential therapy for mechanical allodynia we followed work from Montagnac et al., 2013. In this study they show that the clathrin coated pits (CCPs) on the cell membrane are major

sites of attraction for the Atat1 enzyme. Thus, we went about creating a peptide which is composed of 80 amino acids of the Atat1 enzyme which was shown to bind to the AP2 (adaptor protein 2) domain of CCPs. By saturating these sites with this peptide we hope to reduce Atat1 acetylation at the periphery of DRG by disrupting the localisation of the enzyme.

AIMS AND OBJECTIVES

- To characterize the Atat1^{cKO} phenotype on a behavioural level.
- To understand the molecular mechanisms behind the reduction of mechanosensation in sensory neurons by studying the microtubule dynamics.
- To investigate whether the lack of mechanosensation in DRGs is rescuable by amino acid substitution and to design novel therapeutics for mechanical allodynia.

MATERIALS AND METHODS

Behavioural Assays

1. Mechanosensitive Tests:

Cotton Swab Test: The mice were placed in a plexiglass box atop a base with a mesh like structure. These mice were habituated for 30 minutes in the apparatus. The hind paw of the mice was gently stroked by a cotton swab and a response of paw withdrawal or licking was noted.

Tape Test: The mice were placed into the plexiglass box and habituated for 15 minutes. A 3cm long piece sticky tape was gently placed on the back of the mice along the spinal column and the number of responses of the mice was measured. If the mice were aware of the presence of the tape on their back, scratching, itching and shaking were considered response. This test was carried out for duration of 5 minutes.

2. Nociceptive Tests:

Von Frey Test: The mice were placed into the plexiglass box with a mesh-like base and habituated for 30 minutes. The hind paw of the animal was mechanically stimulated with Von Frey filaments ranging from 0.02g to 1g and responses were measured as the presence or absence of paw withdrawal.

Tail Clip Test: An alligator clip was taken with the tips covered in rubber tubing in order to reduce tissue damage. It was calibrated to exert 400g of force. The clip was attached to the base of the tail of Atat1^{Control} and Atat1^{cKO} mice. Animals were placed in plexiglass containers and the time required for the mouse to realise the presence of the clip which was indicated by biting, vocalization or grasping was measured.

3. Thermoceptive Tests:

Acetone Drop Test: The mice were once again placed in plexiglass containers and habituated for duration of 15 minutes. A drop of cold acetone was squirted onto the hind paw of the animal and a response was scored based on a paw withdrawal, licking and / or jumping.

Hot Plate Test: Mice were placed into the pre heated hot plate chamber and were subjected to temperatures 45 degrees and 55 degrees. Jumping, licking, biting the tails, flicking of paw was considered a response. Latency of the response was measured between the Atat1^{Control} and Atat1^{cKO} mice. In case of no response, mice were removed from the plate after 30 seconds to avoid any tissue damage.

4. Proprioceptive Tests:

Grid Walk Test: Mice were made to walk on a grid for duration of 2 minutes and their movement was recorded. The number of slips on the grid of all 4 paws per number of steps was recorded.

Rotarod Test: A device as shown in the figure below was used for the rotarod test in which the rod rotates with a certain speed. The speed and time can be adjusted by software on the connected computer. The base of the device has sensors which can detect if an animal falls. Mice were habituated with the apparatus and given a trial round at 5RPM beforehand and then subjected to different speeds starting from 5RPM to 25 RPM. The time required for the animal to fall from the rod was measured.

DRG Cultures:

DRG medium: DMEM, P/S, 10% FBS

Collagenase medium: DMEM+ 20ul Collagenase

Coating for the plates with 35 mm glass coverslips: A brief UV treatment was given to the plates before adding 200 μl of poly-Lysine (1:3) for 3-4 hours at room temperature and laminin (1:50) for 1 hour at 37ºC. After each step a wash of water was given.

Around 25-30 DRGs were collected in PBS from an adult mouse and centrifuged at 1000x g for 3'. Next, they were suspended in collagenase medium at 37°C for 25 min in the thermomixer. After centrifuging the cells to remove the collagenase medium, they were incubated in 1ml of trypsin 0.05% EDTA at 37ºC for 25 min. The cells were triturated to separate from each other and resuspended in DRG Medium. They were filtered through the 40 μm strainer in 5 ml, centrifuged and resuspended into a smaller volume of DRG medium. 10ul was placed in each dish and 90ul of DRG media was added to the cultures after 40 minutes.

Super Resolution Microscopy:

DRG cultures were sent to the Microscopy Facility at EMBL Heidelberg for super resolution microscopy. The cells were fixed and stained for α-tubulin. The cells were then washed 3 times with PBS for 10 min and then mounted for STORM imaging. At the time of imaging cells were overlaid with STORM blinking buffer: 50mM Tris pH 8.0, 10 mM NaCl, 10% Glucose, 100 U/ml Glucose Oxidase (Sigma-Aldrich), 40ug/ml Catalase (Sigma-Aldrich). The analysis of microtubule (MT) network morphology was done using the open source software CellProfiler.

Immunofluorescence Staining and Ring counts:

DRG cultures cells were washed once with PBS and fixed for 15 min in cytoskeleton buffer (CB) pH 6.3 containing 3% paraformaldehyde, 0.25% triton and 0.2% glutaraldehyde at room temperature. Cells were then washed 3 times with PBST (0.3% triton). Samples were then subsequently blocked with 5% normal goat serum (NGS) in PBS for 1 h at room temperature. Cells were then placed overnight at 4°C with primary anti α-tubulin (1:1000) (Sigma-Aldrich, T9026) or anti-acetylated-α-tubulin (1:1000) (Sigma-Aldrich, T7451 in PBS. Cells were then washed with PBS and incubated for 1h with fluorescently labelled secondary antibodies (1:1000) (Alexa

Fluor 546 Life technologies) for 1h at room temperature. Images were acquired using a 40 X objective on a Leica SP5 confocal microscope.

Shrinking Experiments:

Cultured DRGs were loaded with 500nM SiR Tubulin2 for 1h at 37°C and/or 2/3M calcein dye (Invitrogen C3100MP) for 30 min at 37°C. The cells were then transferred to imaging buffer (10 mM Hepes pH 7.4, 140 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 5mM D-glucose) at 320mOsm. Following a 5 min acclimatization period the cells were subjected to a 440mOsm (osmolarity adjusted with mannitol) hyperosmotic shock for 3 min. In case of FM1-43, after 3 days of peptide treatment, 10 uM of the dye was added to the cells and shrinking was carried out immediately (380mOsm). Imaging was carried out using a Leica SP5 resonant scanner

Cloning:

Site directed Mutagenesis of Tubulin-Tomato to produce the K40Q mutation

The tubulin-tomato plasmid was modified using the efficient 'Site directed Mutagenesis Kit' from Agilent. It is an easy to follow protocol in which primers were pre-designed with the base pair mutation causing the change in amino acid from Lysine (AAG) to Glutamine (CAG).

The PCR with the mutated primers was run with the high fidelity Pfu enzyme. Later Dpn1 treatment is given to the PCR product to purify the new plasmid which is transformed into the XL-10 Gold cells. The colonies were screened using restriction digestion and the mutation was verified by sequencing the plasmids.

Cloning of AP2P-TAT into petm11 vector-

A pre-made petm11-IL31 SNAP construct was taken from the lab and restriction digestion was carried using BamH1 and Xho1 in Buffer 3.1. Synthetic DNA was ordered from GeneArt Gene Synthesis containing the 307-387 peptide sequence coding nucleotide sequence+ the TAT peptide coding sequence with restriction sites BamH1 and Xho1 on either side. Both the constructs were ligated using the T4 ligation buffer and enzyme and transformed into highly competent cells –DH5alpha electro

max. Colonies were screened for the correct size plasmid and later sequenced for confirmation. This construct – petm11-SUMO-AP2P-TAT was then expressed into the BL21 Rosetta strain as petm11 is a bacterial vector.
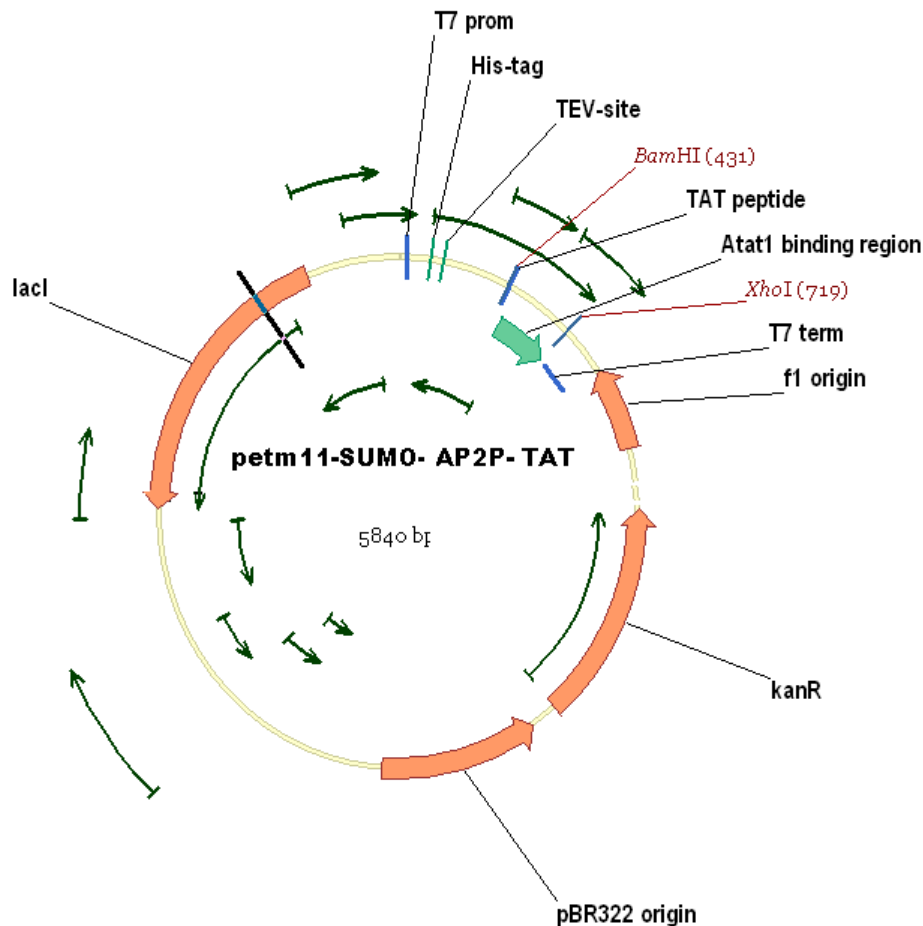

Figure 4: petm11-SUMO-AP2P-TAT plasmid, adapted from Heppenstall group, EMBL.

Protein expression:

A colony from the transformed plate was chosen and inoculated overnight in 10mL of LB+Kan. Next day, 1.5 ml from the inoculum was added to 50 ml of LB+Kan and grown at 37℃ for 2-3 hours till the OD reached 0.6-0.8. Protein expression was induced by the addition of 0.5 mM IPTG at 30° C for 4 hours in shaking. The final inoculum for centrifuged at 4500rpm for 30 minutes at 4℃ and the pellet was resuspended into the lysis buffer to store at -20 °C overnight.

Lysis Buffer	Elution buffer (EB)	Washing Buffer (WB)
50 mM Tris/HCl pH 8.0, 137 mM NaCl, 5 mM Imidazole, 20mM β-mercaptoethanol, protease inhibitors.	50 mM Tris/HCl pH 8.0, 137 mM NaCl, **400mM** Imidazole, 5 mM ß-Mercaptoethanol	50 mM Tris/HCl pH-8.0, 137 mM NaCl, 20 mM Imidazole, 5mM β-mercaptoethanol,

Table 1: Ingredients present in the Lysis Buffer, Elution Buffer and the Washing Buffer.

Protein Purification:

The pellet was thawed on ice and sonicated 3-4x for 30 s, 30 s break (till the solution was clear). The clear solution was then centrifuged at 13200 rpm at 4℃ and the supernatant was collected. Meanwhile, 500 µl of Ni-NTA beads were equilibrated with 15 column volumes (CV) of ddH2O and then with 15 CV of Lysis buffer in the special columns. The beads were then incubated with the supernatant for 1 hour at room temperature in a rotor. The beads were then washed with the 3CV of wash buffer 3x-5x and checked with Bradford solution (BioRad Protein Assay). The protein was then eluted 3x in 1CV of EB. A SUMO-cleavage reaction was set up overnight by the addition of SenP2 protease at 4 °C which got rid of the SUMO tag, His Tag from the AP2P+TAT peptide.

Buffer Exchange Chromatography:

As the protein was eluted in 400 mM imidazole, to reduce imidazole amounts from the protein, buffer exchange was done. 15CV of dH2O and 15 CV of WB was passed through the columns. 2.5 ml of the elution was added to the column and the flow through was collected. The excess of imidazole being a short molecule got eluted while the AP2P peptide got stuck in the column matrix. Pass 3.4 ml of the WB to elute out the protein.

To remove the SUMO tag cleaved from the protein, it was again incubated with the Ni-NTA beads and the flow through was collected which contained AP2P+TAT peptide.

Quantification of the protein:

Protein was quantified using the Nanodrop 2000. Coommassie staining was performed for half hour and destaining over night to show the band size around 10.44 kDa as expected.
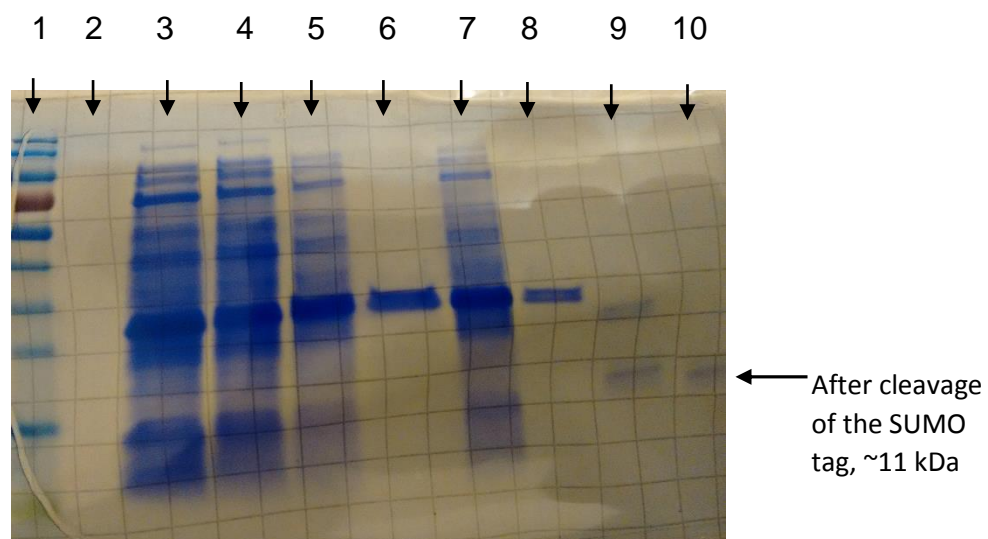

Figure 5: Lane 1- protein ladder, lane 2- blank, lane 3- supernatant after lysis and centrifuge, lane 4- flow through after incubation with beads, lane 5- first wash, lane 6- second wash, lane 7- Ni beads+protein, lane 8- elution before cleavage, lane 9- cleavage reaction, 2 bands- cleaved and uncleaved protein, lane 10- cleaved protein. (~11 kDa)

AP2P treatment of the cells

DRG cells were dissected and cultured from WT mice as mentioned above. 24 hours after cultures, AP2P peptide was added to the cells in varying concentrations from 1uM and 10uM respectively every day. The cells were fixed and stained as mentioned above for acetylated tubulin after 3 days of treatment and imaged on a Confocal Leica microscope at 40x.

RESULTS

Behavioural Experiments -

In order to characterize the extent of the phenotype of the transgenic mouse Atat1^{cKO}, a few behaviour tests were conducted. As the knockout of the acetyl transferase was driven by the advillin promoter, behavioural tests were carefully chosen from the literature which would indicate a difference in any of the somatosensory perceptions of the mice. The somatosensory system of mammals can be classified further into mechanosensation, thermoception, nociception and proprioception

Types of Somatosensation	Behavioral Tests
Mechanosensation	Cotton swab, tape test
Nociception	Von Frey, tail clip test
Proprioception	Rotarod, grid walk
Thermoception	Hot plate, acetone drop

Table 2: The above table lists the different behavioural assays performed on the aTAT1^{cKO} and Atat1^{Control} mice.

Behaviour Tests for Low Threshold mechanoreception:

According to the previous data of the lab, the mice showed a significant difference in detection of low threshold mechanosensation which was indicated by the cotton swab and the tape test. These results established a strong phenotype of the KO mice. In the tape test, the Atat1^{cKO} mice showed a clear drop in the number of responses to the presence of a sticky tape on their back unlike the Atat1^{Control} which more were frequently trying to remove the tape. In the cotton swab test, the number of responses for the brush of the cotton swab on their paw were significantly less in the KO mice compared to their counterparts which can been seen in the graphs.
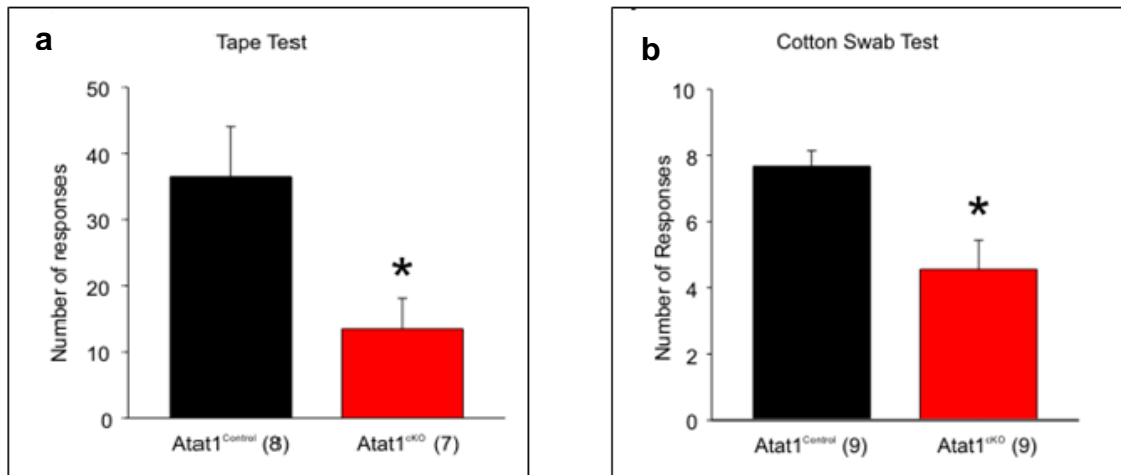
Figure 6: a) The bar chart for the tape test shows that Atat1^{cKO} mice showed significantly less response events over the 5 minute counting period (t-Test, P<0.05). **b)** The bar chart for the cotton swab test shows that Atat1^{cKO} mice had significantly less response events then Atat1^{Control} counterparts (t-Test, P<0.01)

Behaviour Test for Nociceptive receptors:

Previous data in the lab also showed significant differences in a nociceptive test with the Von Frey filaments. The detection frequency in the KO animal were much more than control animals showing that the mice had very little response even to painful stimuli.

In continuation with the previous data, we performed the tail clip test which was the best read out for the delay of detection in painful stimuli on the tails of the KO mice. The control mice started biting and licking the clip as soon as it was placed while the KO mice took a substantially significant amount of time to realise the presence of the painful stimulus on their tails.
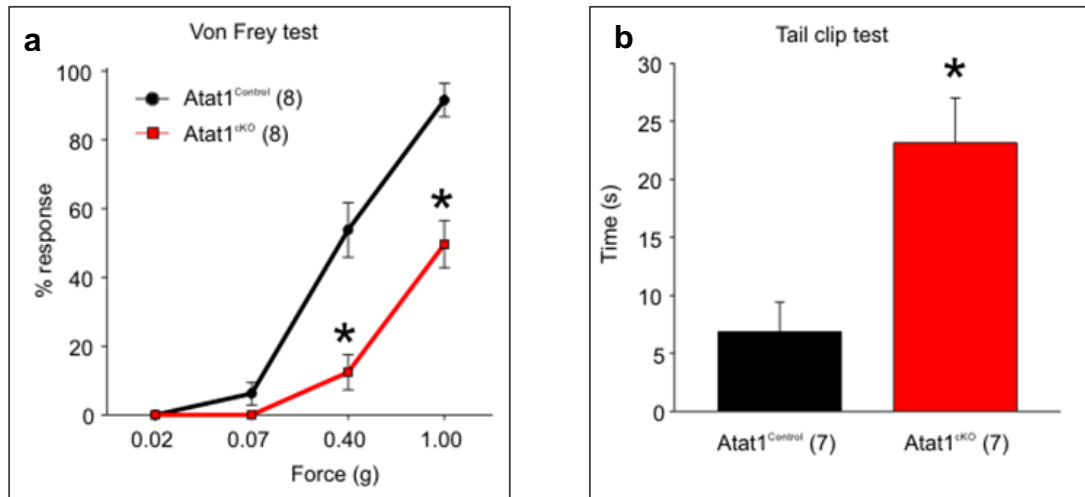
Figure 7: a) The bar graph for the von Frey thresholds shows significantly lower frequency in Atat1^{cKO} animals compared to the control group. (Two-way RM ANOVA, Holm-Sidak method, P<0.001). b) The bar graph showing latency to awareness of a clip attached to the base of the tail. Atat1^{cKO} animals took significantly longer to respond to the stimulus (t-Test, P < 0.01).

Behaviour Tests for Thermoreceptors:

To characterize the effects of deletion of Atat1 gene on the temperature sensing populations, we performed the hot plate test in which the animals were placed on a hot plate which was heated up to 45°C and 55°C. The TRPV1 channel present in some sensory neurons activates at 45°C. The following graphs show that no significant change was obtained between the KO and control animals at 55°C. We did not find any responses from both the sets at 45°C. Thus lack of acetylation did not affect the abilities of the mice to detect noxious thermal stimuli. We also performed cold stimuli tests in which ice cold acetone was squirted on the hind paw of the animals and the response measured paw withdrawal or licking was recorded. In another test, we placed the animal on a cold plate at 2°C. In both the tests, the KO and the control animals showed similar responses. No significant difference was observed as there was no difference in the responses from the animals.
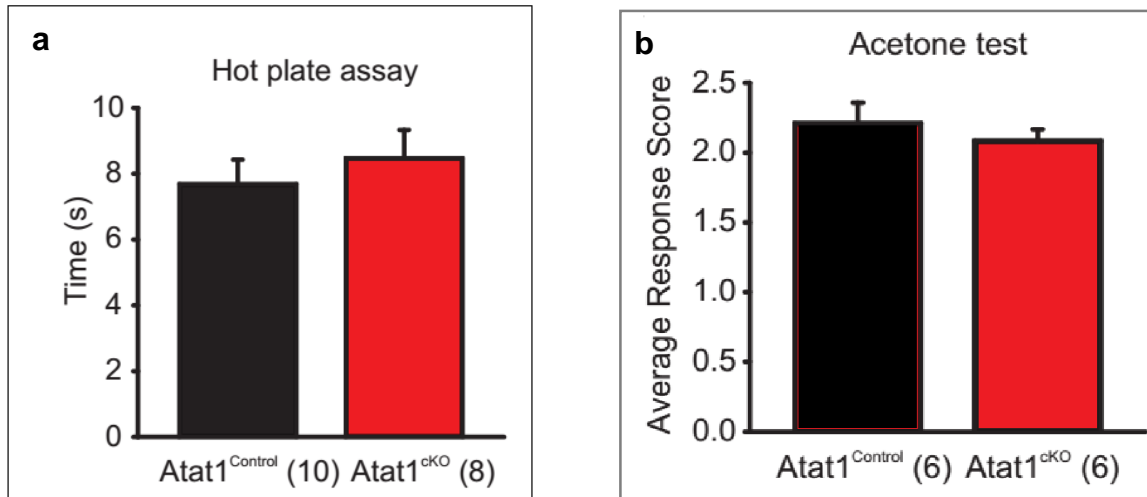
Figure 8: a) There was no significant difference found in the responses recorded to noxious heat between Atat1[cKO] and Atat1[Control] animals (t-Test, P>0.05). b) Similarly, no significant difference was obtained for the responses of both the groups to the ice cold acetone. (t-Test, P>0.05)

Behavioural Tests for Proprioceptors:

The next set of behavioural tests were performed to check for proprioceptive deficits in the Atat1[Control] and Atat1[cKO] mice. The rotarod test which is used to give a read out for the locomotor/balance deficits also did not show any significant difference between the sets. The amount of time that the animal remains on the rotating rod at different speeds was assessed. A lack of any significant difference between the groups indicates that there is no proprioceptive deficit in the KO animals which showed that lack of Atat1 did not affect the proprioceptive population. The second test which is the grid walk test was performed to test the animals for fine motor control in which the animals walked on a grid. The number of steps taken and the number of slips were measured and show that both control and the KO mice performed the same way.
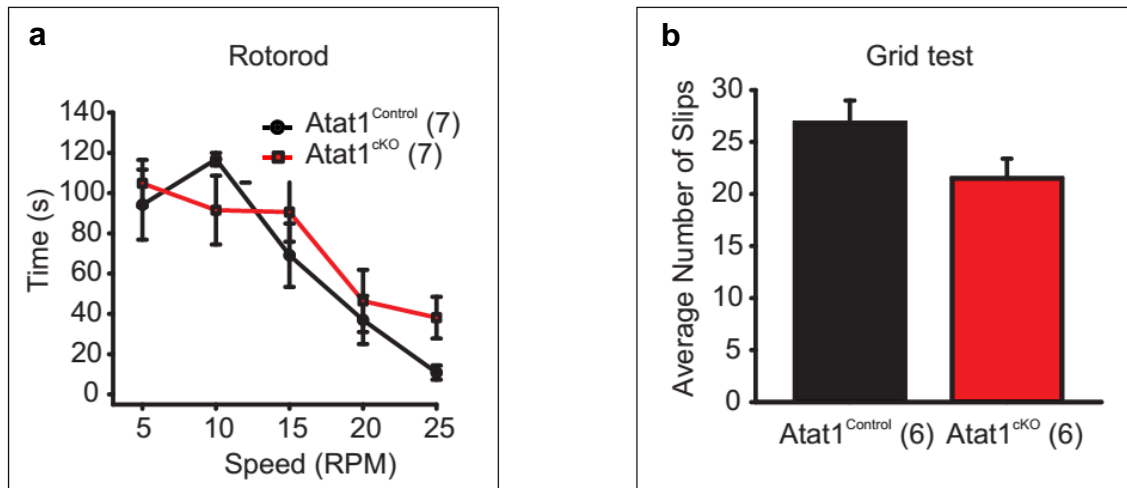
Figure 9: a) No significant difference was seen in the Rotarod test between the Atat1^{cKO} and Atat1^{Control} (Two-Way RM ANOVA, Holm-Sidak method, P> 0.05). Error bars indicate s.e.m. b) The grid test showed that both the groups showed similar responses indicating no difference. (t-Test, p>0.05)

Thus these results indicate that the Atat1^{cKO} mice have a strong mechanosensation and nociception deficit while the other fibre types responsible for thermal sensation and proprioception were completely unaffected. The touch insensitivity phenotype observed with the Atat1^{cKO} animals was further analysed on a neuronal level in order to figure out the mechanism behind this touch sensation deficit.

Sub-membrane localization of Acetylated Tubulin in WT DRG:

As the acetylation is occurring on α-tubulin, microtubules were the obvious target of study for further experiments. Experiments were performed on the dorsal root ganglion (DRG) cells extracted from adult control and KO mice.

In our KO mouse, due to the absence of the α-tubulin acetyl transferase enzyme gene which was driven through the advillin promoter, all the neurons from the peripheral nervous system lacked this particular enzyme. Hence in order to study the distribution of acetylation of tubulin particularly in the peripheral nervous system, we stained the control DRGs for anti-acetylated tubulin. The figure below shows the field of DRGs having a strong sub membrane band for acetylation.
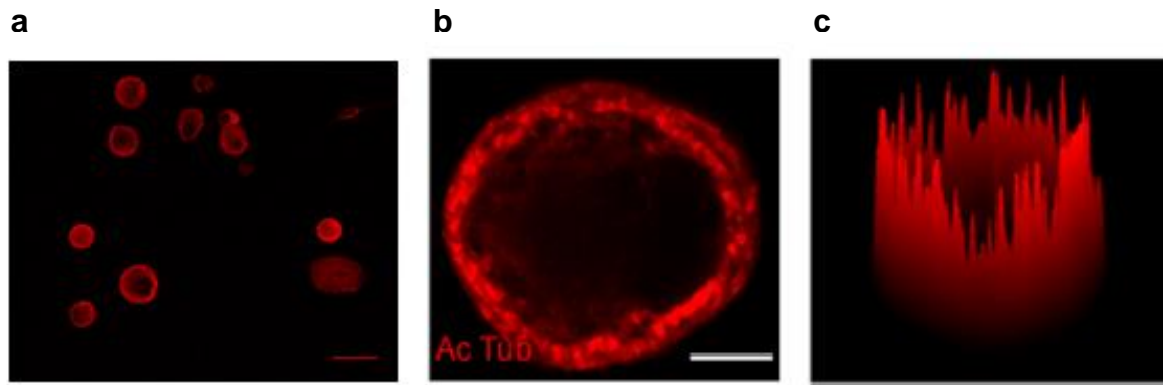
Figure 10: a) WT DRGs stained for anti-acetylated tubulin. b) Cell body of one control DRG highlighting the ring structure. c) Surface plot for the control DRG cell in b.

The DRGs contain a population of neurons which include mechanoreceptors, nociceptors, thermoreceptors and proprioceptors. Multiple stainings were done with cultured DRGs and cell counts were done for ringed and non-ringed cells. It was observed that most of the cells i.e. around 80% of them had rings in wild type DRG neurons. This sub-membrane localization of Atat1 was a very clean and prominent phenotype observed in the control neurons which was completely absent in the KO neurons.

Shrinking Experiments:

Next, we decided to analyse whether the absence of acetylation was affecting the gross morphology of tubulin in the DRG. But there was no significant change observed in the lengths and numbers of the microtubules in the fibroblasts. Dissociated DRG cells were sent to the Microscopy Facility of EMBL Heidelberg for super-resolution microscopy. These cells were stained for α-tubulin and multiple images were taken of individual cells to study the α-tubulin distribution. The structure of microtubules based on number of branch points, lengths, crossing density, angular variance etc. were studied using computational analysis and we found no significant difference in the morphology of the tubulin cytoskeleton from both the groups. The image below shows samples for the Atat1^{Control} and Atat1^{cKO} DRG cells respectively.
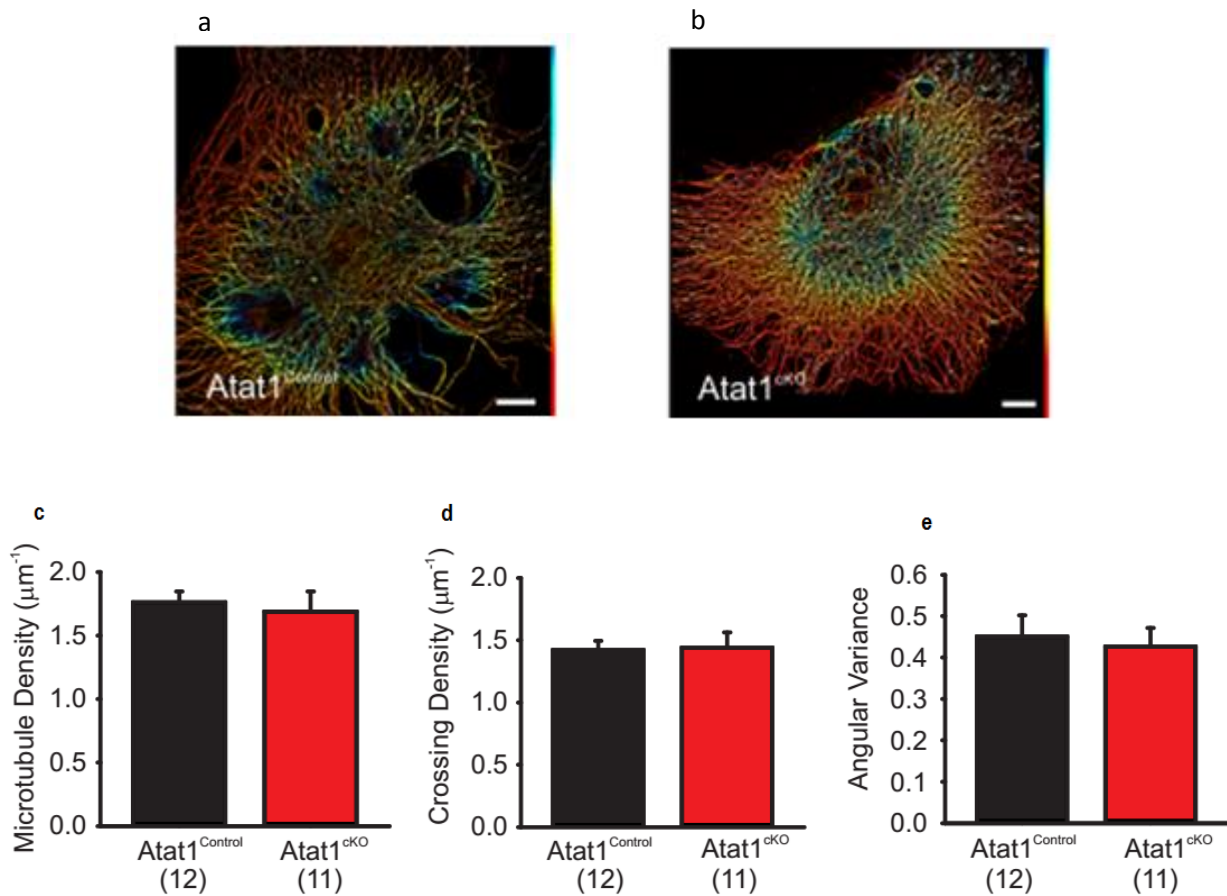
Figure 11: a) Super-resolution microscopy image of one Atat1[Control] cell stained against α-tubulin colour coded by depth from the objective red being closest. b) Super-resolution microscopy image of a Atat1[cKO] cell stained against α-tubulin c,d,e) No significant difference was found in microtubule density, crossing density and angular variance between the Control and KO cells.

In the next set of experiments, we decided to see if lack of acetylation on tubulin had any indirect effect on distribution of actin filaments in the dorsal root ganglion. Even though the acetylation substrate was different, it has been shown that (Choudary *et al.*, 2009) acetylation is a post-translational modification for cortactin as well as Arp2/3 complex which is an actin nucleator. Both the elements, microtubules and actin filaments form the fundamental basis of cytoskeleton of any cell and play a major role at the force transduction apparatus. The following images show that no particular difference was observed in the actin filament architectures of both the neuronal types. The lack of Atat1 did not result in any visible difference in the actin distribution of the Atat1[Control] and Atat1[cKO] neurons.
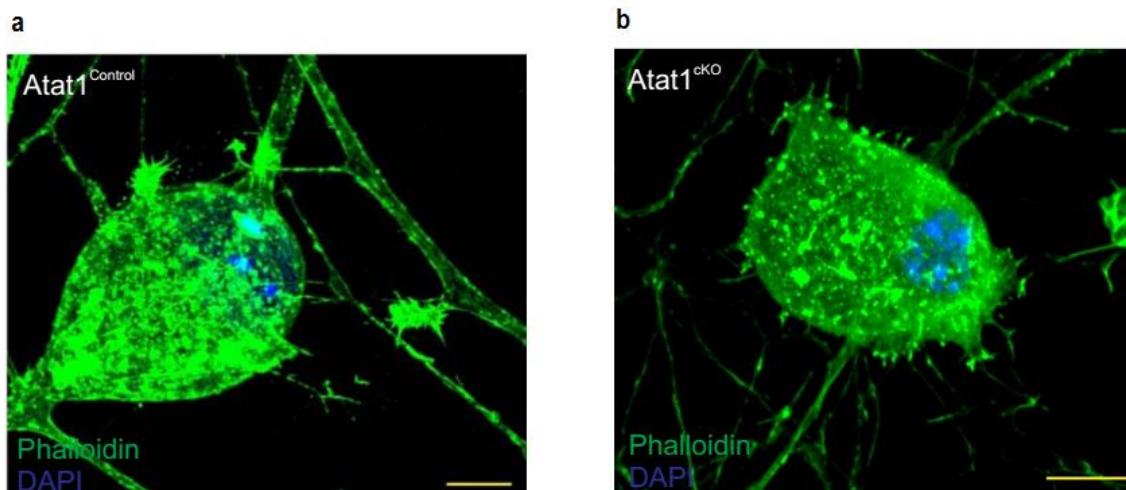
Figure 12: a) The image shows an Atat1^{Control} DRG cell stained for phalloidin which marks the actin network or the cell. B) The image shows phalloidin staining of an Atat1^{cKO} DRG. No significant difference was seen in between both the images.

As the super resolution microscopy did not yield with any differences in the results, we decided to explore our options of what exactly was the absence of acetylation to the K40 residue of α-tubulin doing to the neurons. The lack of acetylation was the factor responsible for the touch sensation deficit observed in mice. Thus we hypothesized that; acetylation plays a significant role in changing the malleability of the mechanosensory neurons by either influencing the microtubular rigidity and/or altering the cell membrane stiffness. In order to test our hypothesis, we decided to subject the cells to external mechanical stimulus that would cause changes in the cytoskeletal architecture. The dissociated cultured DRG neurons were subjected to a hyperosmotic shock which caused shrinking of the cells. We expected that if there was some difference in microtubular rigidities of both the cell types, shrinking of the cells would be an apt way to assess this phenomenon.

On the other hand, Atomic Force Microscopy was performed in the lab of our collaborators which showed that the membrane of the cells lacking acetylated tubulin was much stiffer than the wild type cells. Secondly, electrophysiology data from the lab showed that there was significant difference in the conduction velocities of sensory neuronal fibre types between Atat1^{Control} and Atat1^{cKO} mice. The KO mice had delayed generation of action potentials than the control cells for the RA, SA fibres

strengthening our hypothesis that lack of acetylation modifies the rigidity of the microtubules in the sensory fibres.

The DRGs taken from Atat1[Control] and Atat1[cKO] mice were cultured for a couple of days and then labelled with a peculiar dye Sir-tubulin-2 (C8) (kindly provided by Kai Johnsson lab) which labelled the tubulin in the cells. It is a silicon rhodamine fluorophore which binds to tubulin because of docetaxel. This dye has been shown to bind to stable microtubules with very high specificity. On measuring the 'Int Dent' which is the selected area of the cell times the fluorescence of that area of the control and KO cells, a significant difference was observed between the two types. The Integrated Density of the control cells was significantly higher than KO cells suggesting that the shrinking of the KO cells was much more constrained than in control WT samples.

Thus the acetylation of tubulin was altering the microtubule shrinking in the neurons. The Atat1 enzyme acetylates the K40 residue of α-tubulin. In order to confirm that this was the reason of altered microtubular rigidity, we performed a rescue experiment. A genetic mimic of α-tubulin was created with the lysine residue replaced with glutamine. Glutamine mimics acetylated lysine both in charge and structure. DRG cells from the KO mice were transfected with the K40Q tubulin mutant to observe their microtubule rigidity changes.

Generation of the K40Q mutant of Tubulin using site-directed mutagenesis-
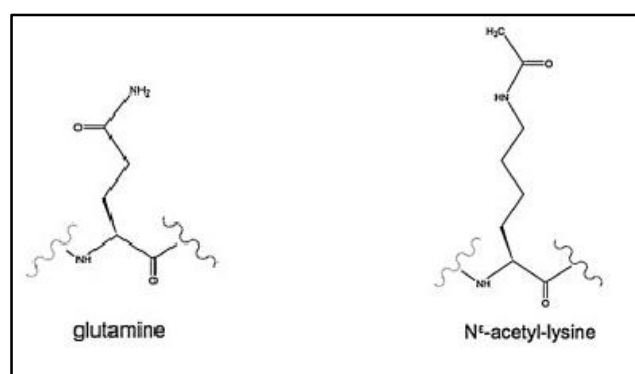

Figure 13: Taken from Kamieniarz and Schneider, 2009

The K40Q mutation was generated as mentioned in the materials and methods. These types of genetic mimics are common in the field to show significance of a particular amino acid.

Transfection of K40Q tubulin tomato into KO DRG cells:

Transfections were carried out using Lipofectamine 2000. The transfected cells were labelled with the Sir-Tubulin Dye were also given hyper osmotic shock and their shrinking was measured. The following graphs shows significant increase in the integrated density of the K40Q transfected cells showing that the rigidity of the cells was restored causing more shrinking of the cells.

Calcein Labelling:

Another set of experiments with similar motive were performed in which cells were labelled with a fluorophore called calcein and then were subjected to hyper osmotic shock. (440osm) Calcein is a dye which fluoresces more when its molecules come together. It can be seen from the graph below that the percentage shrinking in the cells is significantly low in the control as well as the K40Q transfected cells while the KO cells have higher percentage suggesting that the malleability of the KO cells is highly reduced compared to the control cells.
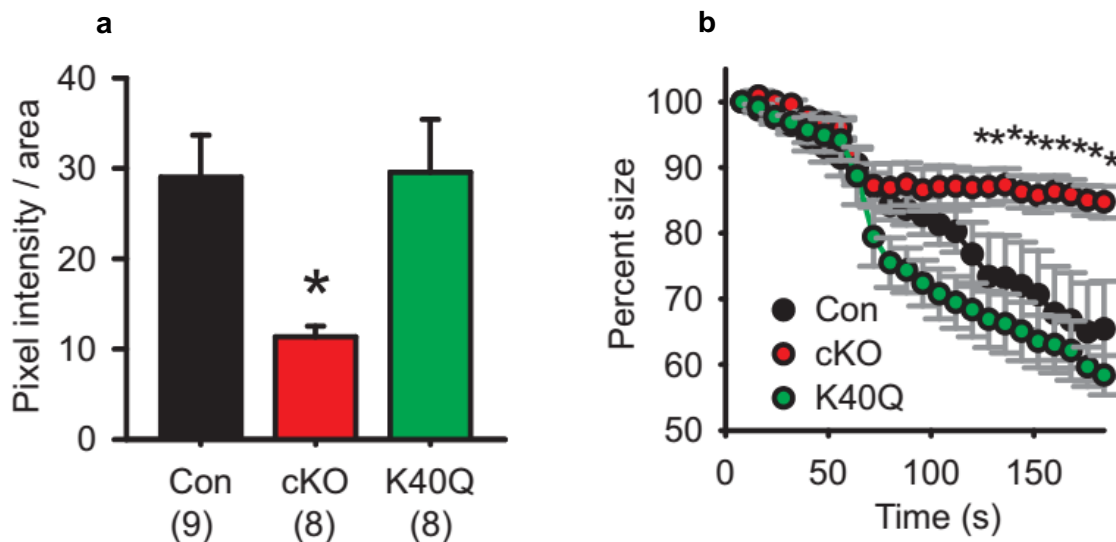

Figure 14: a) The bar chart above summarising osmotically induced microtubule compression in DRG neurons from Atat1[Control], Atat1[cKO], and Atat1[cKO] neurons transfected with tubulin-K40Q. There is significantly less compression in Atat1[cKO] than Atat1[Control] neurons, which is rescued by transfection of tubulin-K40Q (ANOVA on ranks, multiple comparison Dunn's Method, P<0.05). Error bars indicate s.e.m. b) The graph above shows the relative shrinkage of axonal outgrowths from Atat1[Control] and Atat1[cKO] DRG loaded with calcein (2μM) in response to a hyperosmotic shock over time. (ANOVA on ranks, multiple comparison Dunn's Method, P<0.05).

AP2P production and purification

The AP2P peptide is part of the Atat1 enzyme from 307 to 387 amino acid sequence which was shown to interact with the hinge domain of α adaptin in CCPs by (Montagnac et al., 2009). They propose in the paper that there is a positive corelation between the K40 acetylation levels and CCPs and hence Atat1 catalyses microtubule acetylation at the CCPs. Thus this could be utilised to show that acetylation of K40 is a regulated process and also allow us to block the sites at the CCPs thereby rendering WT cells as KO. This also strengthened our argument of the strong sub membrane localization of acetylated tubulin in the DRG cells.

We decided to produce this peptide in bacterial cells using the petm11-SUMO vector. The petm11-SUMO plasmid is a prokaryotic vector with chloramphenicol + kanamycin resistance. The cells that were used for transformation of the plasmid for protein expression and purification were the BL21 Rosetta DE3 strain. This strain carries the chromosomal copy of the T7 polymerase gene required for the T7 promoter under the lacUV5 operon. Thus IPTG is used as in inducer for the protein production which switches on the lac operon. The TAT peptide is a cell membrane penetrating short peptide which facilitates uptake of other materials like DNA, protein etc along with it. Thus the AP2P peptide which has a linker in between the TAT peptide was facilitated into the cells with a strong efficiency.
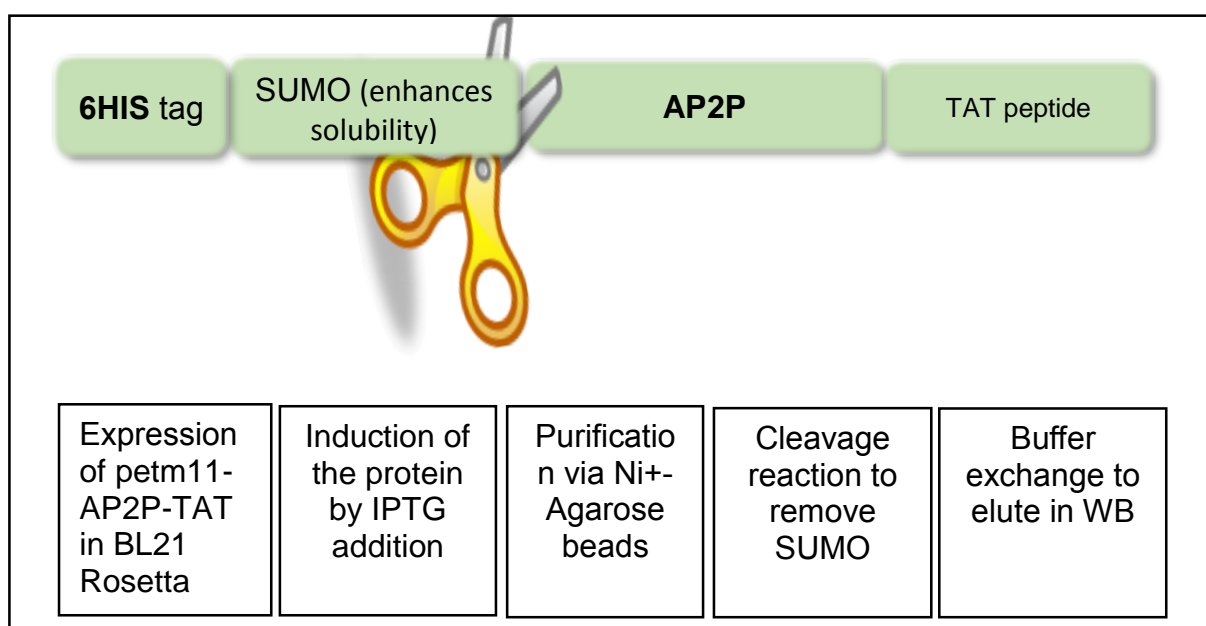

Figure 15: Schematic of the AP2P purification protocol.

Treatment of WT DRGs with AP2P

We tested the activity of the peptide by giving WT DRGs a 4 day treatment of this protein with different concentrations and stained for acetylated tubulin. As it is evident from the graph below that the number of cells with rings was highly reduced for the 10uM treatment compared to 1uM and controls.
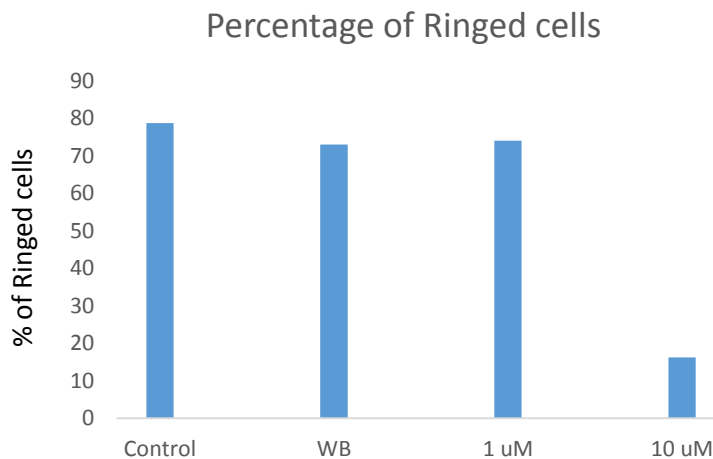

Figure 16: Number of cells which had rings were measured in all 4 groups to show that on addition of 10 uM AP2P, there was sudden drop in the ring counts compared to the control, WB and 1 uM peptide. n = 80-90 per set.

In the next set of experiments, we wanted to check whether the disruption of the ring structure as a result of decrease in the acetylation levels of microtubules was modulating the rigidity of the microtubules and the cell membrane as well.

In order to check this, we first subjected the control and KO cells to the hyperosmotic shock. These cells were labelled with a dye called FM1-43. This dye enters inside the cells via the mechanosensitive ion channels. Thus the amount of dye entering the cell would give us a readout for change in stiffness of the cell membrane affecting the opening of the ion channels. This control experiment established the base levels for both the conditions especially the KO cells. The next step is to conduct the shrinking experiment for AP2P-treated cells which we would expect to behave like the KO cells.

FM1-43- Shrinking Experiments:

Atat1^{Control} and Atat1^{cKO} cells were labelled with FM1-43 which is a rapidly spreading dye and within a couple of minutes binds to the membranes of the cells. The following graph shows significant difference between the Atat1^{Control} and Atat1^{cKO} cells labelled

with FM1-43. On giving a hyperosmotic shock, the number cells which showed a stark change in fluorescence are much more in the control cells than the KO cells indicating once again that the membrane flexibility of the control cells is much more than the KO cells.
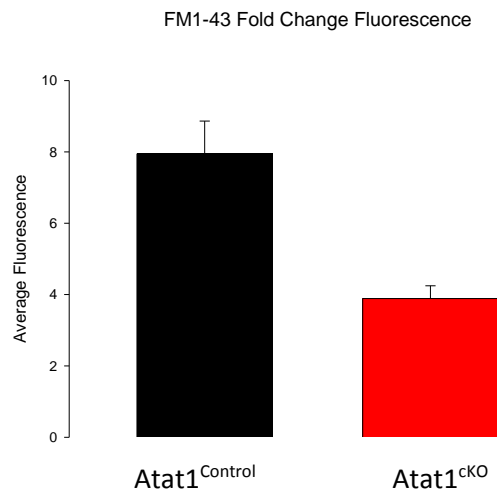
FM1-43 Fold Change Fluorescence

Figure 17: The chart above shows fold difference in the FM1-43 dye uptake of the control (black) and KO (red) DRG cells when given a mechanical shock. n= 50-60

The final experiment was with DRGs treated with AP2P for 3-4 days and then labelled with FM1-43 dye just before giving them the hyper osmotic shock. This would strengthen our claim that acetylation of K40 is a regulated process as well as that addition of AP2P is causing significant drop in acetylation levels of microtubules. This would be the *in vitro* experiment showing whether the phenotype of touch insensitivity could be reversed or not in the control cells. This experiment is still being done and we do not have results for it yet.

DISCUSSION

Since the beginning of studies in mechanosensation, from the primitive organisms like bacteria to higher organisms like mammals, acetylation of tubulin has been shown to be important component in force transduction machinery. The TRNs in *C. elegans* have been shown to be highly enriched in acetylation of K40 in alpha tubulin. (Akella *et al* 2010) Though the function of this acetylation has never been fully characterised in mechanosensation, the literature suggests that it is an important factor. Atat1 or mec-17 in *C. elegans* has expression levels even higher than the fundamental unit of microtubules i.e. α and β tubulins.

The conditional knockout of Atat1 was created in the lab beforehand. As the Cre recombinase was driven by the advillin promoter, this was a novel system to check the functionality of acetylation on all sensory neuronal fibre types including nociceptors and mechanoreceptors. The behavioural assays conducted on the mice which were Atat1^{cKO} and the respective controls which had the Atat1 gene functioning, validated that the phenotype observed was highly restricted to mechanosensation and mechanically induced high threshold pain. Thermal pain and proprioception was not affected by lack of acetylation. The tests like Cotton swab and Tape test, which gave a clear read out for low threshold mechanoreception showed significant latency or lack of response in the mice indicating that the ability of the mice to recognize innocuous stimuli was highly compromised. The von Frey and tail clip tests also showed that the mice lacked the ability to sense mechanical pain as well. To complete the story with respect to somatosensation, the hot plate and acetone drop tests did not show any difference in response. This led us to conclude that the heat sensing and cold temperature sensing machineries were not affected and possibly are not dependent on the acetylated microtubule rigidity as much as the other fibre type machineries. Lastly, the rotarod test which was used to investigate locomotory and balance deficits and the grid walking test which was for fine motor control also gave equivalent results in both the types of animals.

Even though lack of Piezo2, a mechanosensitive ion channel was shown to cause proprioceptive deficits in mice, (Patapaotium *et al*, 2015) we were unable to see any deficits in out Adv-Cr- Atat1^{cKO} model. The paper does not shed any light upon the mechanism of Piezo2 mode of function on this deficit developed, it is one of the

strongest phenotypes recorded by a possibly stretch gated mechanosensitive ion channel. With reference to this, we plan to look for fine motor deficits in the Deleter Cre-Atat1-KO (Kalebic et al., 2013) animal which has not been recorded before. In the literature of touch insensitivity phenotypes thus far recorded, our KO model has the strongest phenotypes ever found, both in terms of the decrease in mechanical sensitivity and the broadness of the fibre types affected.

Descending down from a behavioural level to a neuronal level, we wanted to explore what exactly was happening inside the neurons in the absence of acetylation. Thus to begin with, we carried out staining against acetylated tubulin using a monoclonal antibody in control DRGs and this showed us the ring-like structures just below the cell membranes. This led us to believe that the rise in force sensing thresholds of the mechanoreceptors could be directly linked with this band under the membrane. Secondly, was this lack of acetylation on the luminal side of the tubulin affecting the microtubular network? To verify this claim, super resolution microscopy was carried out on Atat1^{Control} and Atat1^{cKO} cells. Along with the help of Dr. Tischer using various automated analysis measures, we concluded that there was no significant difference in tubulin architecture which was causing the phenotype. This again strengthened our claim that lack of acetylation on alpha tubulin is the major cause of touch insensitivity and not in gross morphology defect in the cells. Furthermore, even though Atat1 has alpha tubulin as a target, it has been shown that cortactin and Arp2/3 are acetylation substrates for other acetylating enzymes. To investigate if there was any difference in the actin architecture, we did phalloidin staining to again show that both Atat1^{Control} and Atat1^{cKO} had no difference between their actin networks.

Now we wanted to investigate how acetylation is affecting the microtubule network. For this we decided to give a mechanical shock to the cells by subjecting them a hyper-osmotic shock. We observed that the shrinking of the cells was much less in the KO cells compared to the control cells. The integrated density or the fluorescence per area gave us a read out that the KO cells were more resistant to change in shape caused by shrinking. Thus this experiment gave us an answer to our question that lack of acetylation was changing the flexibility of the membrane by modulating the rigidity of the microtubules. There was more resistance to bending of the microtubules in KO neurons. The calcein experiment which quantified cell compression validated our argument again while the K40Q tubulin rescue transfections again proved that this

flexibility could be restored by using the acetylation amino acid mimic. Thus going down to the cellular level, we were able to show that the deficit of touch sensation was because the touch sensing thresholds of the mechanoreceptors were highly raised. More force would be required to produce action potentials compared to the control cells. This was also supported by the AFM data which clearly showed that the KO cells had stiffer cell membranes and thus required higher force to induce action potential formation. Thus, in a nutshell we can say that more rigid microtubules and stiffer cell membranes together indirectly or directly raised the thresholds of the force transduction machinery leading to touch insensitivity in the mice.

As mentioned earlier, we hypothesized that the AP2P (AP2 binding peptide) peptide synthesized externally, could block the acetylation sites on the CCPs resulting into decrease in acetylation levels in the neurons in vitro. This experiment was designed firstly to show that acetylation is a regulated process and secondly, if this works we could turn control cells into KO cells in terms of membrane rigidity. This meant that this peptide could be a potential drug for treating mechanical allodynia or commonly known as hypersensitivity as a response to light touch.

When the AP2P was put the DRG cells *in vitro,* we observed that there was a stark drop in the number of rings in the cells at 10 uM. This means even though the turnover rate of microtubules is slow, the peptide could be blocking the sites of acetylation at the CCPs in turn reducing the entry of Atat1 entering the lumen of microtubules to acetylate K40 of alpha tubulin. Even though this is pure speculation, we saw that there was severe lack of the ring structure. This could mean either our peptide was functioning as we thought it would or it was indirectly disrupting the ring structure.

Lastly, the control experiment with FM1-43 dye strengthen our claim that KO cells have stiffer membranes. The FM1-43 dye binds to the cell membrane and enters the cells through recycling endocytic vesicles and mechanosensitive ion channels. We see in the graph that cells turn more fluorescent in the control than in KO suggesting again that the membranes of KO are more rigid thus limiting the channel opening and closing and also entering of the dye. In order to see if, the AP2P treated control cells indeed show a phenotype like the KO cells, experiments are still being done using the FM1-43 dye. In case we see large differences between the dye uptakes, it could mean that

the cells are turning touch insensitive *in vitro.* Thus, this would open doors for us to try out the effects of the peptide *in vivo.*

Thus to conclude, microtubule acetylation is a key factor for regulating the basic operation of mechanically sensitive neurons by significantly affecting the membrane rigidity of these cells. Therefore, we have for the first time defined a role for this highly conserved PTM in a higher organism.

CONCLUSIONS:

1. The Atat1^{cKO} mice show profound defects in mechanosensitivity as well as in nociception while proprioception and thermoception remain unharmed.

2. Absence of Atat1 in neurons does not cause any morphological defects in the neurons.

3. Removal of Atat1 causes microtubular rigidity of neurons to increase and a resulting increase in cell membrane rigidity.

4. Addition of AP2P causes a decrease in mechanosensitivity *in vitro.*

FUTURE PROSPECTIVES

1. We wish to proceed with the *in vivo* experiments with AP2P to deliver it locally inside the cells using microemulsion creams in the control mice.
2. We also wish to carry out proprioceptive behaviour tests in the Deleter Cre-Atat1-KO line of mice where Atat1 is knocked out from all the cells of the organism.
3. We wish to try several behaviour tests in the Spared Nerve Injured mice model and see the effects could be altered by application of the AP2P locally.
4. We wish to study the interaction of the microtubules with the known mammalian mechanosensitive ion channel-Piezo2. This includes co-transfection of Piezo2-GFP and Tubulin-Tomato into MEFs perform FRET/FRAP on it.

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