5 DISCUSSION:

5.1 Development of high throughput *in situ* hybridization for adult DRGs.

DRG is a complex tissue with different cell types. In order to find out the expression pattern of genes that are restricted to a subpopulation of DRG neurons, we developed a high through put whole mount *in situ* technique. This is a highly efficient strategy to screen many genes for a subpopulation specific expression in the DRGs of adult mice. In a complex tissue like the DRG, this screening method was useful when we had to screen large number of candidate genes, checked for their expression in subpopulation specific manner. That is because we directly visualize the expression of our genes of interest. In addition, this has advantages over using *in situ* hybridization on sections because of its ease of processing as well as it is less cumbersome. For the time we require to screen the expression pattern of one or two genes by this method we would be able to screen the expression pattern of 200 cDNAs with our new method. This new method is also more cost effective than conventional techniques. However, this high-throughput method is not suitable for optimizing *in situ* signals because, each cDNA probe must be hybridized with identical conditions to all other clone, unlike *in situs* performed on sections.

We found genes that are expressed in a sub population of DRG neurons whose expression is influenced by BDNF. The Majority of the clones did not give any expression or were ubiquitously expressed. There was no selection method used for decreasing the background. Normally background is decreased by eliminating the clones that hybridize with probes for ubiquitously expressed transcripts. (Niedhardt, et al., 2000, Wertz and Herrmann, 2000).

One of the cDNAs we found in our screen was already reported to be expressed by DRG neurons. Sncg is reported to be present in DRG cultures and axonal concentration of Sncg was reported to be influenced by neurotrophins like BDNF and NT3 (Willis, et al., 2005). In addition, many other genes SPOCK1 that is involved in cell adhesion (Weaver, et al., 2006), Pyridoxal kinase, Progestin and adipoQ receptor family member V (involved in G-protein coupled receptor protein signaling pathway), Tubulin ß5 involved in microtubule based process (Bhattachraya and Cabral, 2004), Annexin 6 that is involved in calcium ion transport, a membrane protein Tmeme50a whose function is not yet know were also found to have a subpopulation specific expression pattern.

Though the library was made to enrich transcripts that are down-regulated in BDNF mutant mice, we could find transcripts that were up regulated as well. It was interesting to note that many genes were influenced by BDNF gene haploinsufficiency. In addition to known genes

we could find transcripts that had no annotations or coded for predicted proteins (A230083H22). Many reports (Cao, et al., 2004, Evans, et al., 2002, Jiang, et al., 2002) show identification of predicted transcripts using enriched library using suppression subtractive hybridization (SSH).

For some of the transcripts, hybridization was observed by both sense and anti-sense probes. This could be because of the presence of repeat sequences in the genome or might be having some transcripts which had the complementary sequences in the cells it self (like mirco RNA mediated regulation). BDNF is known to regulate mirco RNAs (Shratt, et al., 2004, Tchurikov and Kretova, 2007).

As functional loss is seen only in SA fibers, these could be the transcripts affecting the SA neuron properties. By qPCR experiments it was observed that the subtracted library contained up-regulated genes that were 30% increased in comparison to controls. However, many genes were only slightly down-regulated (10%). During the suppression, some molecules are amplified linearly. It might be possible that these transcripts are present in the subtracted library (Diatchenko, et al., 1996).

To determine if the identified transcripts are directly influenced by the addition of BDNF, through Trk B, we stimulated DRG cultures with BDNF. No change in any of the identified transcripts was observed. This suggests that these transcripts were down-regulated as an indirect consequence of BDNF insufficiency.

5.2 Analysis of NT3+/-//NT4-/- mice

It is reported that SA, DH and sympathetic neurons require NT3 (Ernfors, 1994, Airaksinen, 1996). NT4 is shown to affect DH (Stucky, 2002). In my case I was using NT3+/-//NT4-/- mice. Electrophysiology experiments were done to investigate the effect of these neurotrophins in sensory DRG neurons. There was no change observed in the sensitivity of these neurons to different mechanical stimuli. Further experiments need to be done to verify this. Electron microscopy results indicated a profound loss of both myelinated and unmyelinated axons in the purely sensory saphenous nerve. It is reported that NT3+/- animals loose large diameter and Aδ axons (Airaksinen, 1996). This suggests that the remaining neurons might not be influenced by the absence of NT3 and NT4. It is reported that among cutaneous afferents expressing trk A and trk C showed no overlap and trk B expressing cells also expresses trk A or trk C (McMahon, et al., 1994). It is also reported that there is a distinct population of neurons that does not expresses trk A, trk B, trk C or P75 (Wright and Snider,

1995). The neurons that are not affected in NT34 mutants could be the ones not expressing trk B, trk C or p75.

We were interested to find out molecules that were expressed in the lost neurons. We followed a two-pronged strategy. First, using a subtracted suppressive hybridization (SSH) cDNA library enriched for down-regulated transcripts in NT34 mutant DRGs and second, by using expression profiling of NT34 mutant and control adult DRGs. Cao, et al., (2004) reported that genes that were not represented on affymetrix gene chip microarrays could be identified by SSH. In addition, they also reported that some genes were not efficiently detected irrespective of their copy numbers. One independent round of gene chip experiment was also performed with enriched pool of transcripts (SSH method) for the down-regulated genes and its control. Genes that were increased by 2.5 fold were taken to be further analyzed. 118 genes were found to be increased compared to the unsubtracted sample. It was observed that only two genes, secreted phosphoprotein 1 (SPP1) and synaptogamin 11, were found to be increased in the enriched pool for the down-regulated transcripts. Their expression was observed to be down-regulated in NT34 mutant DRGs compared to control DRGs in the normal affymetrix experiments. This indicated that the subtraction procedure was successful. Out of these 118 genes, that were enriched; about 48 were cloned and *in situs* performed. Only a few were found to be expressed in a subpopulation specific manner. The staining observed was light suggesting that the copy number of these transcripts might be less. It is also reported that the copy number and the expression level observed from affymetrix experiments does not correlate well for all the genes (Cao, et al., 2004).

Comparison of the expression profile of adult NT34 mutant and control DRGs were also performed using affymetrix gene chips. 83 genes were found to be down-regulated, whereas 50 genes were observed to be up-regulated. Because of the lower efficiency to identify transcripts that are having less copy number (Cao, et al., 2004, Evans, et al., 2002, Jiang, et al., 2002,) one of the selection criteria used was to directly compare the signal intensity. Transcripts that showed signal intensities that were 1.5 times increased or decreased in the NT34 mutant DRGs in comparison to the control DRGs and that were called present or marginally present in two out of three independent experiments were taken into consideration for further analysis. In the *in situ* hybridizations, only 28% of the down-regulated genes showed a subpopulation specific expression pattern. But in case of up regulated genes, about 92% of them were expressed in subpopulation specific manner. It could be possible that as there is a huge loss of neurons, the RNA obtained is only from this surviving subpopulation.

Subtracted libraries were made and spotted onto high density filters as well as cultured in 384 well plate format. In our previous experiment, done with subtracted BDNF library, we

observed the presence of both down-regulated as well as up-regulated genes. Making use of this property, these filters were screened with both forward subtracted and reverse subtracted probes. In other words, the library was made to enrich the down-regulated clones and was spotted onto the filter. This was screened using both probes that were enriched for down-regulated transcripts and up-regulated transcripts. Transcripts seen to be enriched were selected were checked for their expression pattern in adult DRGs. This procedure was used for reducing the background that was not enriched (Niedhardt, et al., 2000, Wertz and Hermann, 2000).

It was observed that Persyn was observed to be down-regulated in the affymetrix experiment and was also found in the subtracted library screen. Receptor (calcitonin) activity modifying protein 2, was found in the subtracted library and was observed to be up-regulated. Ramp2 is reported to be influencing the specificity of calcitonin receptor like receptor (CRLR). CRLR transported with Ramp2 functions as adrenomedullin receptor (McLatchie, et al., 1998). This was observed to be up-regulated in NT34 mutants.

Many genes were found to be down-regulated in the affymetrix experiments. Matrix proteins like SPP1, CD44, receptor molecule like Gabbr1, kinases like Dapk1, AK1, were found to be expressed in a subpopulation. Among these, 114131_at (Affymetrix ID) was seen to be up-regulated in qPCR experiments. Function of this transcript is yet to be annotated.

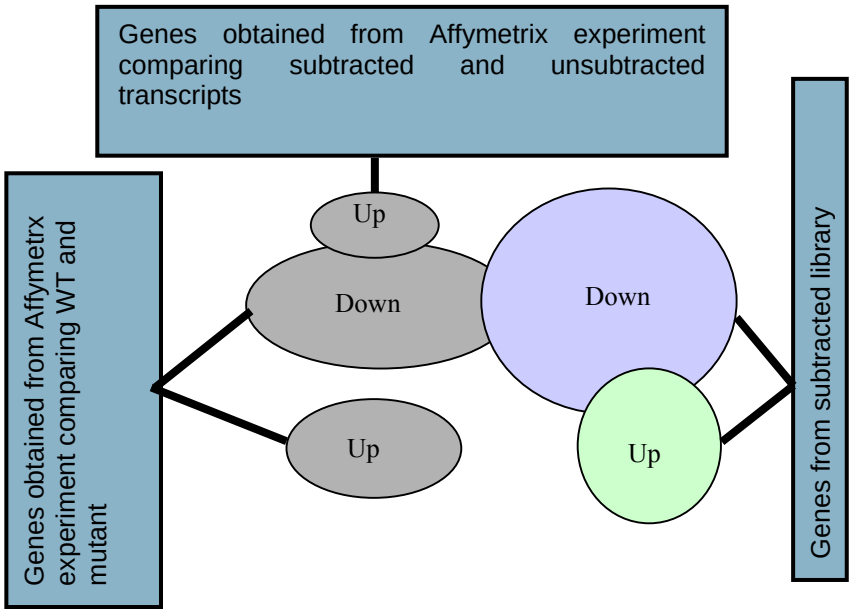

Fig. 41. Scheme showing the overlap of different genes between different experiments

Transcripts that were found to be up-regulated in affymetrix experiments included KcnV1 that modulates the activity of Kv2 and Kv3 channels (Hugnot, et al., 1996, Salinas, et al., 1997),

kinases like Cnsk1e, Camk4, BDNF were found to be expressed in a subpopulation specific manner.

A schematic diagram of for all the observations are given in Fig. 41. NT34 mutants, seemed not to be recognizing the rough surface of sandpaper in surface grid tests. Tests for the discrimination of different grids was not successful as these animals were lethargic.

After this initial screening study, further electrophysiological, behavioral and molecular biological studies are needed understand the role of proteins that are expressed in a subpopulation of DRG neurons, for their involvement in mechanotransduction if there is any.

5.3 Analysis of SPP1 mutant mice

Data obtained here indicates that the expression of SPP1 is mostly in medium sized neurons in mouse DRGs. The diameter of majority neurons expressing SPP1 was observed to be between 24 and 33 μm. This is similar to the reports that show colocalization of SPP1 expression in neurons that are positive for neurofilament 200. CGRP and IB4 positive neurons were not found to be expressing SPP1 (Marsh et al., 2007, Ichikawa, et al., 2001). This indicates that they are absent in the subpopulation of small cells.

Electrophysiological studies indicated an increase in the stimulus response of AM mechano fibers. AM fibers are not the largest neurons in the DRGs and this correlates with our observation of SPP1 expression in adult DRG neurons in medium sized cells. Further studies to increase the number of units are needed to confirm this data. This observation led us to carry out behavioral experiments on these animals.

Mechanical withdrawal latency and thermal withdrawal latency were measured, and no change was observed between the SPP1 mutant and the control animals. Marsh, et al., (2007) showed an increase in the mechanical withdrawal latency indicating that they are less susceptible mechanical stimuli. Our data is not in accordance to their findings. We used an automatic measuring device, which is less prone for any manual errors in comparison with their method, but worked on the same principle. But we also observed that the thermal withdrawal threshold of SPP1 mutant and the control animals were same.

To check the ability of these animals to discriminate different surfaces, grid based tactile acuity tests were performed. Out of three different grids with spatial frequency of 250, 500 and 750 μm SPP1 mutants seemed to be not recognizing the smallest grid surface. As only 8 animals were used further experiments are to be done to confirm this observed effect (Wetzel, et al., 2007).

All this data together does not suggests that SPP1 is involved in mecahnotransduction but there may be subtle effects on mechanosensation. SPP1 binds to may integrins, elastin and other extracellular matrix proteins in addition to CD44. It mediates many different pathways by the binding with different integrin molecules (Fig. 42). The SPP1 mutants are viable but they have problems only in tissue remodelling after injuries (Liaw, et al., 1998).

CD44, a transmembrane molecule for which SPP1 is a ligand was also seen to be down-regulated and with a cell specific expression pattern (Fig.43). CD44-/- mice are also viable. The CD44 binds to integrin ligands extracellularly and with cytoskeletons present intracellularly mediated through ERM (erzin-radixin-moesin) along with SPP1. CD44 was observed to be expressed in small diameter neurons. CD44-/- animals and double mutants of CD44 and OPN could be analyzed to investigate the role of CD44-OPN complex found in mammals that links the ECM to the cytoskeleton. This becomes important as CD44 was observed to be expressed exclusively in small diameter neurons, which has a free nerve ending in different layers of epidermis. Also binding of elastin-SPP1-CD44 could also be considered as a complex that could act like a "spring" which could modulate gating ion channel in a subpopulation of DRG sensory neurons.

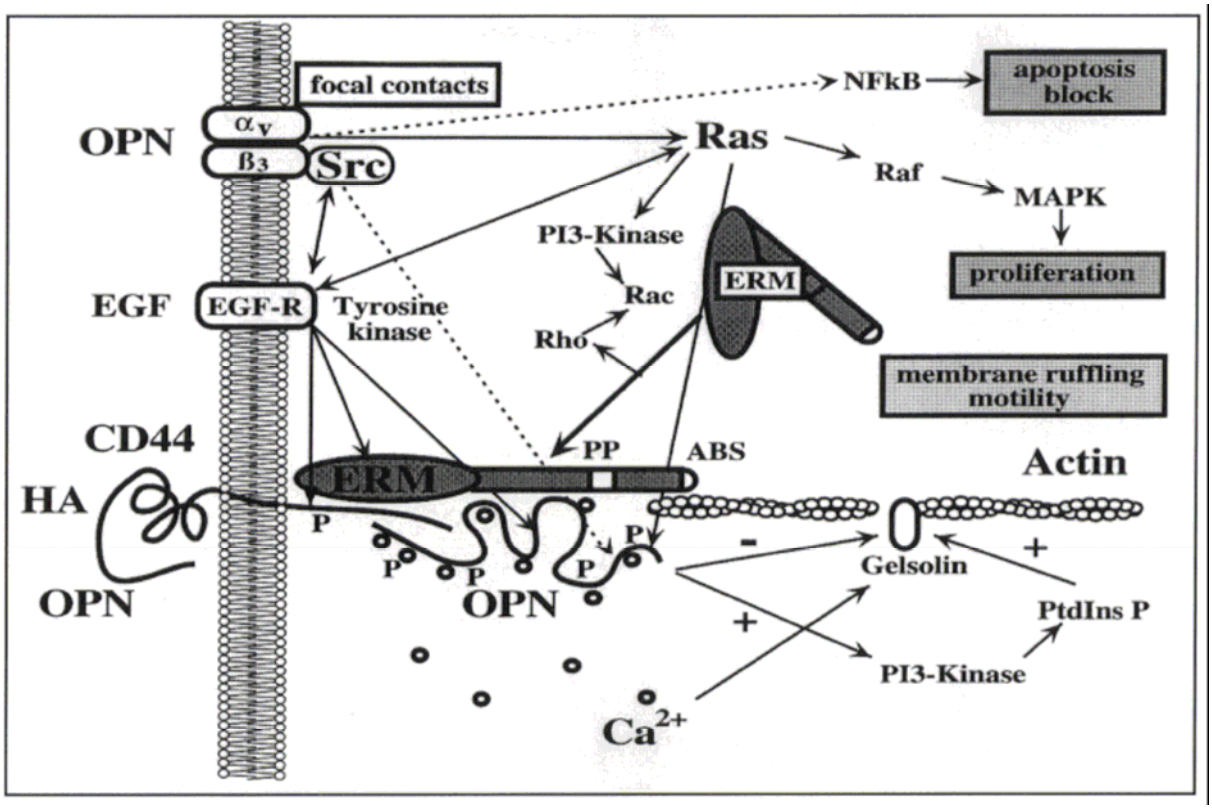

Fig: 42. Picture depicting the association of intracellular osteopontin with the CD44-ERM complex to the cytoskeleton and extracellular osteopontin association with ECM. Diff signaling pathways are also seen. Adapted and modified from Sodek, et al., (2000).

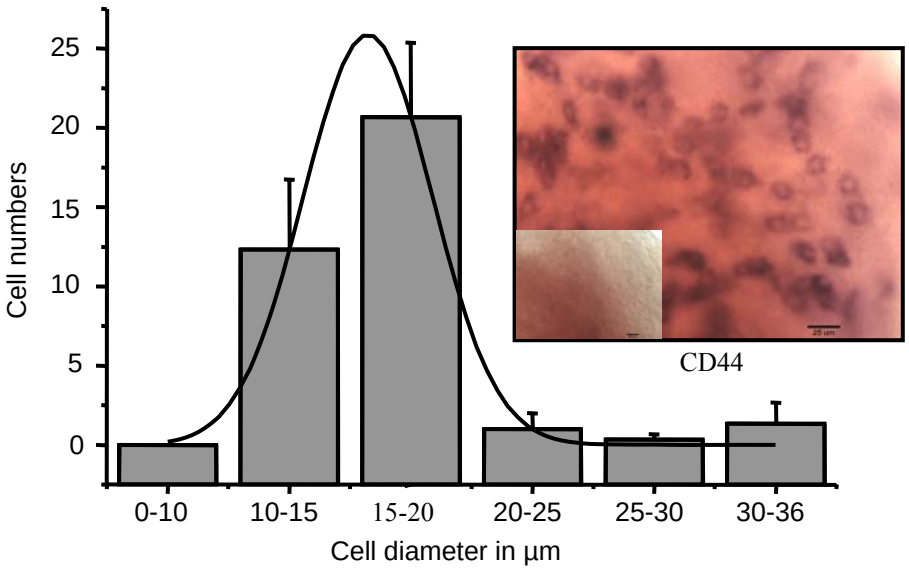

Fig: 43. Expression of CD44 in DRG neurons cells

5.4 Putative extracellular binding partners of ASIC3

ASIC3 (acid sensing ion channel 3/ACCN3/DRASIC), was shown to play a role in mechanotransduction (Price., et al., 2001, Chen., et al., 2002, Mogil., et al., 2005, Page., et al., 2005, 2007, Lumpkin and Caterina, 2007, Hughes., et al., 2007). It is one of the members in

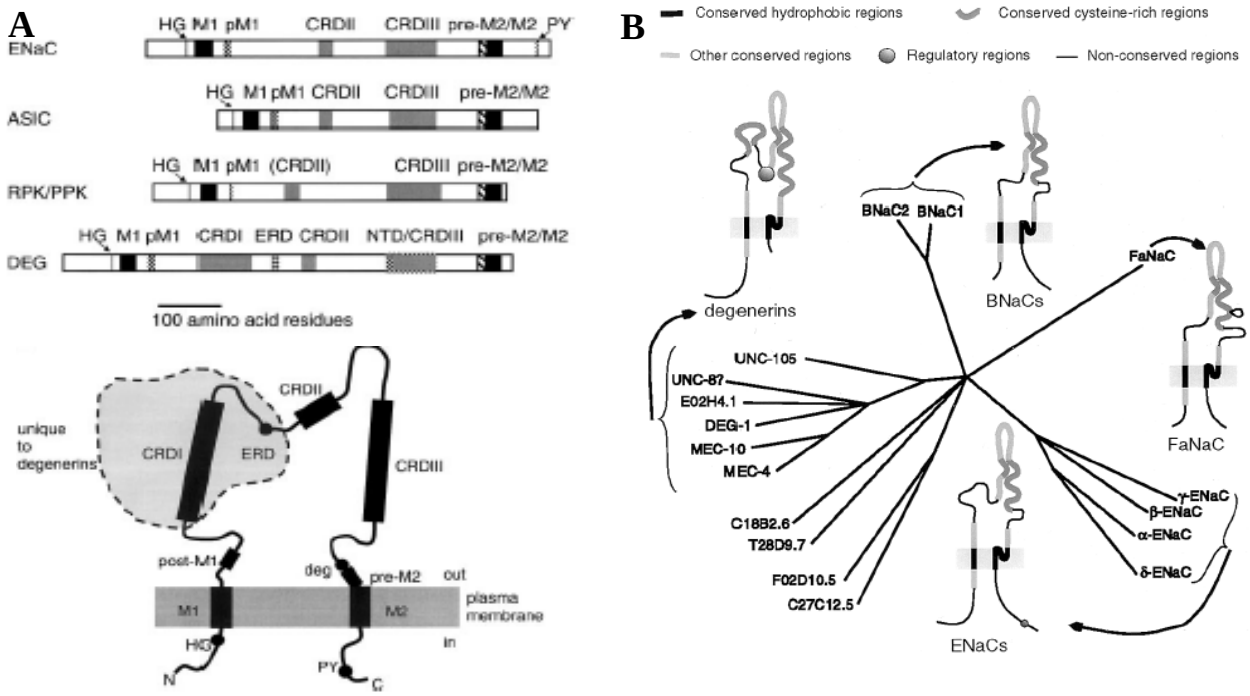

Fig. 44. Conserved domain structure of DEG/ENaC family. A. M1 and M2 are transmembrane domains, HG is His-Gly motif with in a conserved amino terminal, pM1 is post transmembrane1, CRD is Cysteine rich domain, pre-M2 is hydrophobic domain directly preceding M2. ASIC channels don't have the CRDI and ERD (extracellular regulatory domain), which is unique to degenerins. (Adapted and modified from Kellenberger and Schild, 2002). B is adapted and modified from Garcı´a-An˜overos., et al., (1997).

ASIC subfamily of DEG/ENaC (Degenerin/Epithelial sodium channels) family of ion channels (Fig.44). Like other members in the family, ASICs also have a large extracellular domain with conserved cystein rich domains and glycosylation sites, which could bind to the extracellular matrix. A phage display library made from skin derived cDNA was used for identifying putative binding partners.

Among many genes that were found to be present only in the population of phages that were adsorbed by ASIC3 expressing oocytes, keratin 10 was present higher in number, compared to others that were sent for sequencing. Other interesting candidate genes obtained were hypothetical protein similar to secreted protein acidic and rich in cysteine (SPARC), hypothetical protein containing ankyrin repeat domain (Ankrd44) and chloride intracellular ion channel 5 (CLIC5).

DNA sequencing was done commercially as single read. So the sequences delivered were not checked with the chromatogram. When the sequences are obtained from the sequencer, it is observed that adjacent higher signal intensity sometimes mask the lower intensity peak, thereby giving the sequence out put without the nucleotide corresponding to the lower peak. This could eventually lead to frame shift when the obtained sequences were translated to find out the binding motifs. In order to overcome this problem, all three reading frames of the sequences of phages adsorbed to ASIC3 expressing *Xenopus* oocytes as well as control oocytes were generated and taken as a database. Individual sequences were blasted to the above database to get the phages that had common short stretches of amino acid sequences. (Ramkrishna Sompallae is duely acknowledged for writing the algorithm for this purpose). Amino acid sequences of groups of phages that had adsorbed on to ASIC3 expressing oocytes alone were further analysed to determine the putative binding motifs. Only Keratin 10 expressing phages were fitting to this criterion. Among the different short streches of amino acid sequences, those that are present in keratin 10 are “KYRRT”, “DLKGQI”, “NANVLLKID” and “RGG”.

5.4.1 Keratin 10

Some of the phages had insert sizes upto 856 bp keratin 10 sequence. Smaller clones had sequence similarity to different regions of the bigger clones. Sequencing was commercially done (Hot shot or extended hot shot, Seqlab, Goettingen). Sequences thus obtained were not corrected after checking the chromatogram. After aligning the DNA sequences, common sequences of all the clones was used to construct the following amino acid sequence.

DPNQAGGKIKEWYEKHGNSSQREPRTTANTTKPSRTLRGRSSP.QLTMPTCCCRLTMP
AWQLMTSG.NTRMR.PCARAWRPTSMACAGYWMS.PLASLTWKCRSKV.TKSWPT.R

RTTKRKLAAALE.LVNPSKDPFRGPKGLTSYSSAAASFLFVVLL.VGQLFVQTFDLHFQ
VRLAKGQLIQYPAQAIDVGLHALAQGHLILVFQPEVISCQAGIVNLQQHVGIVSCQGE
DLPLKVLDGFVVFAVVPGLSLA.VAMLLVPLLDFTLQLEFGSPSIT

It was interesting to note that the amino acid sequence obtained after taking the most common DNA sequence, did not give the common amino acid sequences present in the phages that were adsorbed to oocytes expressing ASIC3 except, the short stretch "VLL"

Cytosekletal keratin intermediate filaments are the major differentiation product of keratinocytes. They constitute about 85% of terminally differentiated cells. These tonofilaments extends from cell periphery into the cell body. They align with filaments that extend through the desmosome from other neighbouring cells that constitute tension in the system. But near the nucleus they are less straight that indicates that the tension varies in different sub cellular space. About 30 different keratin are divided in to two subtypes. Type I (acidic) consists Keratin 5/14 expressed in basal layers and Type II (neutral) Keratin 1/10 expressed in spinous layer (Yang, et al., 1999).

The forces at the interface of ion channel and the lipid membrane is important for gating (Kung, 2005). The network of these keratin tonofilaments could play an important role in the distribution of mechanical forces when applied on to the skin that could change the membrane tension opening the channel directly or indirectly. In this context keratin 10, which seems to bind to ASIC3 channel assumes significance though it has to be verified.

5.4.2 SPARC (Secreted Protein Acidic and Rich in Cysteine)

SPARC is one among the proteins known as matricellular proteins. Matricellular proteins are a group of modular, extracellular proteins that bind to extracellular matrix proteins as well as to cell surface receptors, or to other molecules such as cytokines and proteases that interact, in turn, with the cell surface (Bornstein, 1995). Some of the other members of this category of proteins include members of tenascin protein family, thrombospondin and osteopontin (Bornstein and Sage, 2002). Structure and different functions of SPARC is summarized in fig. 45.

As ASIC3 seems to bind to C-terminus of a hypothetical protein similar to SPARC (C-terminus being extracellular), which could bind with cells and matrix, this could be acting like a link between ASIC3 and the extracellularmatrix and further experiments are needed confirm this assumption.

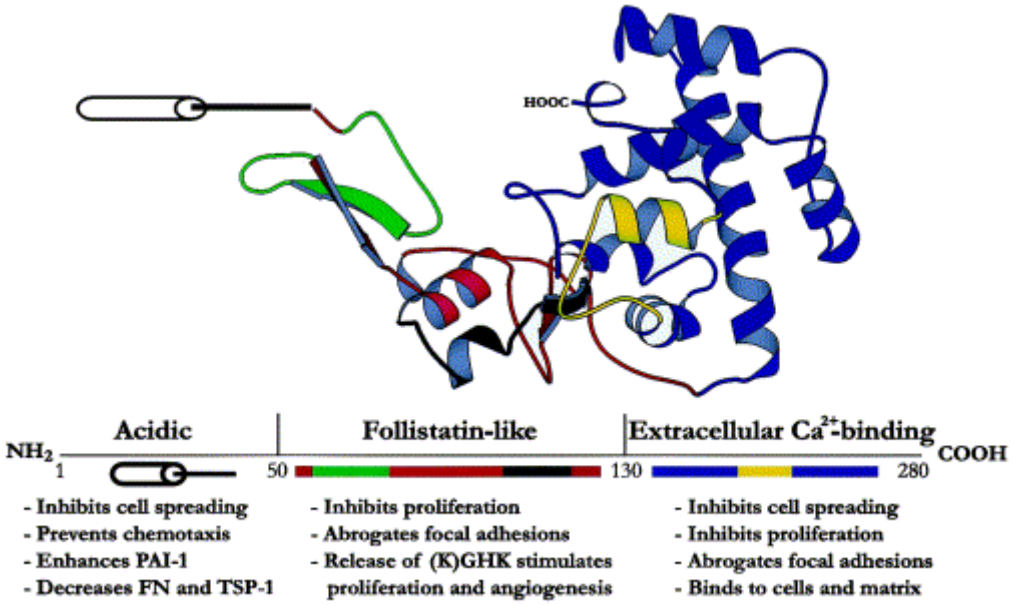

Fig. 45. Structure and function relationship of different regions in human SPARC. (Adapted and modified from Brekken, et al., 2001)

5.4.3 Chloride intra cellular ion channel

CLIC5 is a member of Chloride intra cellular channel family of proteins. Different members of the family are observed to have different subcellular distributions (Berryman and Bretscher, 2000). CLIC5 was isolated from human placental proteins that binds to ezrin, a major membrane-cytoskeleton linking protein in human placental microvilli. Later on it was also shown to be abundant in hair cell steriocilia associated with radixin (Gagnon, et al., 2006), a member of ERM (Ezrin-radixin-moesin) proteins thought to be linking actin microfilaments to the plasma membrane plasmamembrane specialized structures like microvilli and membrane ruffles (reviewed in Bretscher, et al., 1997). Though CLIC5A was shown to function like a chloride selective channel *in vitro*, cells overexpressing the channel was not found to have increased plasma membrane anion permeability. This was also observed to be associated with ezrin (Berryman, et al., 2004). Further studies are needed to confirm the interaction between ASIC3 and CLIC5A as a link between ASIC3 and the cytoskeletal proteins. It could as well function as a mechanosensitive channel because of its interaction with cytoskeletal proteins as it was not activated in *in vivo* situation (Berryman, et al., 2004). Exon structure of *CLIC5* is given in fig. 46.

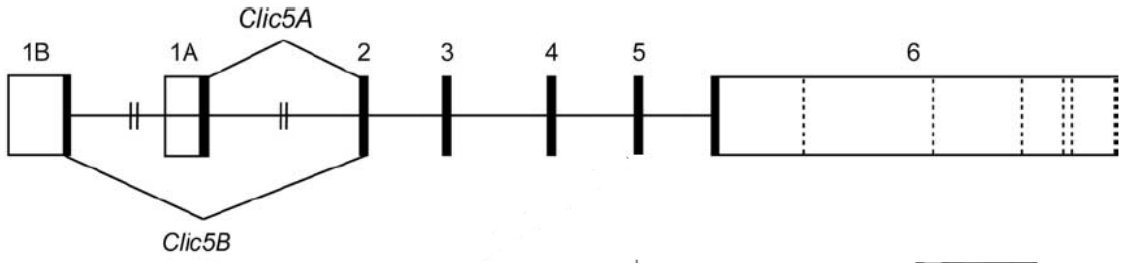

Fig.46. Exon structure of *CLIC5* gene. Dotted lines in exon 6 denotes the alternate poly(A) sites in the 3' untranslated region. Dark rectangles indicate the coding region. (Adapted and modified from Gagnon, et al., 2006).

5.4.4 Ankrd44

The ankyrin repeat is one of the commonly observed motifs in protein databases which are involved in many cellular functions (reviewed in Mosavi., et al., 2004, Table 10).

Ankyrin repeat protein	Number of repeats	Function	Partner(s)
Ankyrin	24	Intracellular adaptor	α-Na,K-ATPaseL1CAMs, CD44, Cl−/HCO3-anion exchanger (AE1–3), NaCh, Clathrin heavy chain, Rh and RhAG
ANKRA	3	Endocytosis	Megalin
Ankrd2	4	Regulation of muscle stress response	Titin
BARD1	3	Inhibition of polyadenylation	CstF50
BCL-3	7	Oncoprotein and transcriptional regulator	JAB1, Pirin, Tip60, Bard1, B3BP
CARP	4	Regulation of muscle stress response	YB1, Titin
cpSRP43	4	Chloroplast signal recognition particle	LHCP
DARP	4	Regulation of muscle stress response	Titan
GABPß	5	Transcriptional regulator	GABP α
Gankyrin	6	Oncoprotein and regulator of retinoblastoma protein (Rb)	CDK4/Cyclin-D complex, Rb, and S6-ATPase
IκB	6 or 7	Transcriptional regulators	NFκB, PTP-BAS (IκB α only)
Integrin linked kinase	4	Cell–cell adhesion	PINCH
INK4	3, 4, or 5	Tumor suppressors and cell-cycle regulators	Cdk4/6, NFκB, Tax
Mbp1	4	Late G1 transcription factor	Clb2/Cdc28 kinase
Myotrophin/V1	3	Development	CP, NFκB
MYPT1	8	Regulation of myosin Phosphorylation	Protein phosphatase-1c
NPR1	4	Transcriptional regulator	AHBP-1b and TGA6
Notch	7	Cell-fate determination	Deltex, CSL, PCAF and GCN5, SKIP, YY1, MAML1
P85	6	Regulation of actin cytoskeleton	Protein Phosphatase-1Δ
RNase L	9	Ribonuclease	2'-5' oligoadenylates
RFXANK	4	Transcriptional regulator	RFXAP and CIITA
Shank	7	Synapse density scaffolding	Sharpin,α-fodrin
Swi4	4	Late G1 transcription factor	Clb2/Cdc28 kinase
Swi6	4	Late G1 transcription factor	Stb1

Tankyrase	24	Poly(ADP-ribose) polymerase	TRF1, IRAP, Grb14, TAB182, NuMA, Mcl-1
Tvl-1	4	Signal transduction adaptor	Raf-1
53BP2	4	Tumor suppression	p53, Protein phosphatase-1, Bcl-2, NFκB

Table: 10. Confirmed protein-protein interactions mediated by ankyrin repeats. (Adapted and modified from Mosavi, et al.,2004)

Ionchannels like TRP have multiple ankyrin repeats in the cytoplasmic domain (Caterina., et al.,1997, Tominaga and Julius, 2000) unlike ASICs. The finding of Ankrd44 as a candidate ASIC3 binding protein could be interesting as it could regulate the properties of the functional channel like NHERF-1, an adaptor protein (Davel., et al.,2006).

Further studies need to be carried out to confirm the interaction of ASIC3 to these proteins or these domains and to verify its effects on the channel function. .