Crumbs is Required for the function and morphology of fly Johnston's organ.

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Introduction

A *Drosophila* antenna is a tripartite organ, which consists of Johnston's organ (JO) in the second segment. Johnston's organ is involved in various mechanosensory functions including the detection of touch, gravity, wind, and sound (Heinzel and Gewecke, 1987; Camhi and Johnson, 1999; Staudacher et al., 2005; Budick et al., 2007; Sane et al., 2007; Bender and Frye, 2009; Kamikouchi et al., 2009; Yorozu et al., 2009; Sun et al., 2009). Johnston's organ is gaining attention for hearing or detecting sound in last few decades. Like other chordotonal organ, during development of the larvae, JO develop from the single organ precursor cell (SOPs). These SOPs are developmentally specified by the proneural gene atonal (ato) (Jarman et al., 1993, 1995), which is conserved in vertebrate and is responsible for cochlear hair cells of vertebrate ears (Bermingham et al.,1999; Millimaki et al., 2007).

The building block of the JO is scolopidia. Each JO consists of 230-240 scolopidia. Each scolopidium consists of: neuron, scolopale cell, cap cell, dendritic cell and ligament cell (reviewed in Eberl, 1999; Jarman, 2002). Each JO scolopidium typically contains 2-3 primary mechanosensory neurons (Boo and Richards, 1975; Todi et al., 2004; Ai et al., 2007). The sensory neurons are bipolar, bearing a proximal axon and an apical dendrite whose outer segment represents a characteristic feature of a typical non-motile cilium with a 9+0 axoneme (Uga and Kuwabara, 1965; Boo and Richards, 1975; Sarpal et al., 2003). The cilia contain a dilated structure known as ciliary dilation and it is filled with paracrystalline material. The basal part of the sensory cell contains, long, regularly cross-striated ciliary rootlets, which extend from the base of the dendritic outer segments through the dendritic inner segments into the perikarya. The scolopale cell is an intracellular cytoskeletal tubular cylinder, typifies the innermost auxiliary cell, which, extends from the apical part of the dendritic inner segments of the sensory cells to the cap cell. The scolopale cell forms an endolymph-space around the cilium and produces actin-based rods that serve as a strengthening force along the scolopidia (Field and Matheson, 1998; Todi et al., 2004). Apical to the scolopale is the cap cell, which is

enveloped by the attachment cell, which also connects a scolopidium with the cuticle at the segment joint. Bundles of longitudinally oriented microtubules are characteristic structural elements of the attachment cell. These microtubules are associated with electron-dense filaments. Adherent junctions link attachment cell, scolopale cell and dendritic inner segment of the sensory cells.

Any disarrangement of the cells described above may lead to a defective Johnston's organ. Like various sensory organs, division of labor (Arendt et al., 2009) is also seen in Johnston's organ and various cells are assigned for various functions. Several genes contribute for formation of various cells and disruption of function of any of them may lead to a defective JO. Sensory mechanism localizes to the cilia, thus any mutation affecting the cilia lead to deafness. Mutation affecting cilia include *touch insensitive larva B* (*tilB*) and *smetana* (*smet*) (Kernan *et al.*, 1994, Eberl *et al.*, 2000, Todi *et al.*, 2004). Molecules present at the base of the cilia include Uncoordinated (*Unc*), which is a coiled coil protein (Baker et al., 2004; Martinez-Campos *et al.*, 2004) and mutants having defect in this gene have defect in hearing. Defective motor protein like anterograde kinesin motor subunits *kinesin-like protein 64D* (*Klp64D*), Myosin VIIA encoded by *crinkled* (*ck*), (Todi *et al.*, 2005), *Kinesin associated protein* (*DmKAP*) (Sarpal *et al.*, 2003), retrograde cytoplasmic dynein motor encoded by *beethoven* (*btv*) (Eberl *et al.*, 2000), affects the function of JO. The anterograde IFT-B particle proteins no mechanoreceptor potential B (nompB) (Han et al., 2003), *outer segment protein 2* (*oseg2*; also called *osm-1*) *outer segment protein 5* (*oseg5*) (Avidor-Reiss *et al.*, 2004) the retrograde IFT-A particle proteins *oseg1* and *reduced mechanoreceptor potential A* (*rempA*; also called *oseg3*) (Avidor-Reiss *et al.*, 2004). The ciliary transcription regulator *Drosophila regulatory factor X* (*dRfx*) (Durand *et al.*, 2000, Dubruille *et al.*, 2002) is associated with cilia and rootlet formation and disruption of this gene also affects the function of JO. Besides ciliary and motor protein, protein belongs to transient receptor potential (TRP) family ion channels like, No mechanoreceptor potential C (*nompC*), nanchung (*nan*), and inactive (*iav*) are essential for the hearing and the mutant show a defective JO phenotype. *nompC*, a member of the TRPN subfamily required for bristle organ mechanotransduction (Walker *et al.*, 2000), and contributes to auditory sensitivity,

but its absence does not abolish hearing completely (Eberl *et al.*, 2000). Besides, some of the genes required for JO formation are also involved in joint formation suggesting that both structures share similar developmental genes (Dong et al., 2003; Göpfert et al., 2003; Ebacher et al., 2007). Further it would be interesting to study the role of proteins involved in junction formation in Johnston's organ.

Various phenotypes described so far in Johnston's organ. In myosin VIIA mutant the scolopidia appear in a disorganized array and the cap cell fail to encircle the cilia. In *Rfx* mutant, cilia and axial filament (rootlet) do not develop (Dubruille et al., 2002). Besides cilia, the scolopidia also appear disorganized in *Rfx* mutant (Durand *et al.*, 2000, Dubruille et al., 2002). Cut is a transcription factor and the scolopidia do not differentiate in the absence of cut. In *nompA* mutant scolopidia are less organized and cap cell fail to encircle the cilia (Chung et al., 2001). In *Klp64D* mutant dendritic cap appears to be empty (Sarpal et al., 2003). In Beethoven (*Btv*) mutant, ciliary dilation is increased and the paracrystalline material found to be absent in this mutant and is replaced by vacuoles (Eberl et al., 2000). Mutations like *uncoordinated* (*unc*) and the *Drosophila pericentrin-like protein* (*D-PLP*), affect basal bodies or centrioles and thus disrupt hearing (Baker *et al.*, 2004, Martinez-Campos *et al.*, 2004). Although various phenotypes described for various parts of the JO, a mutation, which affects the junction, is scanty. Adherens junctions are pivotal for cell-cell communication and maintaining the proper morphology of various tissues. Lack of a study about junctional phenotype in JO and importance of junction in maintaining the morphology warrants study of a junctional protein in this organ.

Crumbs (Crb) a transmembrane protein, known to be involved in the epithelial cell polarity of the embryo. In *Drosophila* embryo, Crb is required for ectodermally derived epithelia, where it controls assembly of a continuous ZA and maintenance of apical-basal polarity (Grawe et al., 1996; Tepass, 1996; Tepass and Knust, 1990; Tepass and Knust, 1993). Besides embryo, Crb is required for remodeling of AJs and maintaining tissue integrity of the malphighian tubule (Campbell et al., 2009; Harris and Tepass, 2008). Furthermore, Crb is also known to be involved in modeling zonula adherens (ZA) of

photoreceptor cells of *Drosophila*. Besides formation of ZA, Crb also regulate the length of the stalk membrane and protect the photoreceptor from light-dependent degeneration (Izaddoost et al., 2002; Johnson et al., 2002; Pellikka et al., 2002). This function is conserved from *Drosophila* to human being. In humans, loss of function of *CRB1* leads to Leber congenital amaurosis (LCA), and retinitis pigmentosa (RP) type 12. In RP12 patients, preservation of para-arteriolar retinal pigment epithelium (PPRPE), RP with Coats-like exudative vasculopathy, and early onset of RP without PPRPE (den Hollander et al., 1999; den Hollander et al., 2004; Gosens et al., 2008; Richard et al., 2006) is observed. Loss of CRB1 function in the mouse impairs the integrity of the outer limiting membrane (OLM) resulting in the delamination of parts of the photoreceptor layer and neuronal cell death (Mehalow et al., 2003; van de Pavert et al., 2004). In zebrafish neuroepithelia, Crb also regulate the apical basal polarity by aligning the adherens junctions properly. Like *Drosophila* photoreceptor, zebrafish mutant for Crb2a exhibits shorter inner segment, which is the homologous organ of stalk membrane (Omori and Malicki, 2006). Various phenotypes observed for different species suggests Crb have different function in different photoreceptors. Since eye is the most dominant sensory organ of our body and function of Crb is conserved from *Drosophila* to human being we expect Crb may have a role in other sensory organs as well.

Sensory receptor of eye and the ear possess highly specialized apical surface. From the structural point both share the common homology in terms of their structure. Receptor cells in the ear possess an apical bundle of actin-filled stereocilia that transduce mechanical stimuli (reviewed by Hudspeth, 1989), whereas the photoreceptors of the eye have an outer segment that contains membrane disks specialized for detection of light. Besides structure, in terms of development in *Drosophila*, both antennae and eye develop from the single organ precursor cells (SOPs). Similarly, in vertebrate the ear and eye are thought to have evolved from a common ancestral ciliated cell (Popper et al., 1992). Besides structure and development, genetic studies identified various molecules that are involved in both hearing and vision. Some of the molecules are conserved throughout evolution. In humans, mutations in these genes cause diseases like retinitis pigmentosa (RP), one of the most prevalent inherited disorders (Ahmed et al., 2003). One of the

known retinitis pigmentosa is RP65, which is caused due to Myo VIIA causes hearing abnormality in *Drosophila*. Very similarly RP25 mutation caused due to eys/spam resulted mutilation in the photoreceptor of both human being and *Drosophila*. Besides vision in *Drosophila* eys/spam mutation consequences in a impaired chordotonal/Johnston's organ in *Drosophila* (Cook et al., 2006). One of the most severe/incurable type of retinitis pigmentosa is RP 12, which is caused due to a mutation in CRB1. Individuals suffering from this disease become legally blind at the age of 40s or 50s. Besides the role on maintaining the photoreceptor, Crb is also required for maintain ing the adherent junction. In JO all the cells are interconnected by means of adherens junction. Based on the structure of JO and role of Crb, the current study is to uncover the function of Crb in the JO of *Drosophila*.

Results

Localisation of Crb in JO organ

To view the distribution of Crb in JO, we examined the adult wild type JO using the Crb 2.8a antibody. Along with the Crb antibody we also use Alexa conjugated Pholloidin to envision the scolopale rod. Scolopale rod is an actin rich structure, which provide strength to the scolopidia. Florophore conjugated pholloidin binds to actin thus visualize the scolopale rod. Crb staining is abundantly found in the intervening region between the scolopale cell and cap cell and the scolopale cell and neuronal cell. To check the specificity of the antibody we test the labeling of pholloidin and Crb in *crb* clone. Pholloidin staining in *crb* clone reveals the presence of scolopidia. However, the scolopidia looks disorganized in *crb* clone in comparison to the wild type. The basal part of the scolopale cell appears splayed in mutant clone. In many cases scolopidia are uprooted and they fall one over the other. Furthermore, Crb labeling is absent from the *crb* clone indicating the specificity of the antibody. Disorganised scolopidia is a very common phenotype in JO and is observed by various mutants. The disorganised scolopidia suggests Crb may have a role in the maintenance of JO.

Ultrastructure of JO in *crb* Mutant

To complement the confocal microscopy analysis, we examined the wild type and mutant of Johnston's organ by electron microscopy. The Johnston's organ in the second antennal segment was used for the analysis of electron microscopy study. The Longitudinal sections of the chordotonal organ from the wild type revealed prominent ultrastructure, including a dendritic cap, scolopale, cilia, and ciliary rootlets (Fig. 2). In each scolopidium, the dome-shaped scolopale surrounds cilia whose tips are connected to the dendritic cap. Cross-sections of the organ at different positions revealed different structures at different level in wild-type scolopidia. We check the structure at various level in case of both *crb* and wild type scolopidia.

At the most apical region of the scolopidia cap cells are found in transverse section of wild type. In case of *crb* mutant, cap cell are often mislocalised and hard to find. After few micron basal to the cap cell, cap cell encircle the cilia in case of the wild type scolopidia (Fig 2A). Actin rich scolopale rod encircles the cap cell more proximally. The cap has a circular cross-sectional profile enclosing the tips of both dendrites (Fig 2A). In *crb* mutant, the cap often encloses the cilia incompletely, with one cilium remaining outside it (Figure 2A'). Mutant caps may also be divided into several compartments, each hosting a cilium or being empty in some scolopidium. Similar defects are observed in Myosin 7a mutant flies (Todi et al., 2005). More basally after the cap cell, the scolopale rod encircles the electron dense structure called ciliary dilation in case of the wild type scolopidia (Fig.2B). The ciliary dilation is also observed in case of the *crb* mutant and remains unaffected (Fig.2B'). More proximally the scolopale rod encircle both the cilia in wild type scolopidia. In transverse section of the wild type cilia a typical non-motile cilia is seen. This has an arrangement of 9+0 (Fig.2C). In case of *crb* mutant the cilia remains unaffected and the similar arrangement like the wild-type is observed for the mutant. In wild-type scolopidia the scolopale space remains empty where as in mutant the scolopale space contains numerous membranous structures (compare fig 2C and 2C'). A very similar shaded structures at the base of the stalk membrane of the *crb* mutant

photoreceptor. These structures have never been reported in other mutants. More proximally, at the base of the scoloplae rod four basal bodies are found in both wild type and mutant scolopidium. At the base, a pair of ciliary rootlets with regular repetitive structures is visible. In higher magnification, basal bodies are clearly identifiable at the base of cilia. The apical basal body gives rise to the cilia whereas the proximal basal body forms the ciliary rootlet (Fig 2). At the most basal part of the scolopale rod in *crb* mutant the shape of the scolopale rod appears different and the membrane detaches from the scolopale rod. At the basal part of the scolopale cell there are adherent junctions of the neuron (Fig.2D). In case of the *crb* mutant the adherent junctions still form (Fig.2D'). In case of the wild type there is some electron dense structure encircling the rootlet. Rootlets are found to be missing from many of the scolopidium in case of *crb* mutant. More proximally the neurons and it's cell body appear in case of *crb* mutant. The neurons and the cell body appears apoptotic in case of *crb* mutant. In many cases they loses it's shape and spread allover. Most of the scolopidia loses it's connection from the scolopale cell or the amount of neurons found to be reduced in case of the *crb* mutant.

We examined scolopidial ultrastructure and detected morphological anomalies associated with the scolopale cell and neurons. Such anomalies could perturb sound transduction in *crb* mutant flies by affecting scolopidia that remain attached to the a2/a3 joint. Furthermore, because the apical juncture is believed to occur through the cap and because the cap cell is detached scolopidia function may get affected in *crb* mutant.

Junctional proteins localization in wild type and *crb* mutant

Various cells within a tissue are connected to one another by means of extracellular matrix and cellular junctions (Yap et al., 1997). Junctions are required for the physical adhesion of cells to their surroundings and thus play a fundamental role in determining and maintaining tissue organization. From the morphological point of view, adherens junctions include focal adhesions, desmosomes, hemidesmosomes, and the zonula adherens (ZA) junctions (Farquhar & Palade 1963, Burridge et al 1988, Garrod 1993, Gumbiner 1996, Burridge & Chrzanowska-Wodnicka 1996). Molecular analyses of these

junctions indicate that they constitute various classes responsible for different functions of the cell. Among them a classical cadherin adhesion molecule as its main adhesive component play an important role in the maintenance of various tissue. Some of the important function may include the adhesive region of synaptic junctions in the CNS (Fannon & Colman 1996, Uchida et al 1996), autotypic junctions in the myelin sheath (Fannon et al 1995), and the intercalated discs found between myofibers (Volk & Geiger 1986). Besides cadherin mediate cell-cell recognition events and in association with the actin cytoskeleton, bring about morphological transitions that underlie tissue formation and maintain tissue architecture in the adult organism. Cadherin interact with catenin and thus form the cadherin-catenin complex, which is essential for the adhesion. The requirement for β-catenin in AJ formation has also been demonstrated in *Drosophila* embryos, by analyzing mutations in the β-catenin homologue Armadillo (Cox et al 1996). β-catenin is often proved to be an dispensable molecule for cadherin-mediated adhesion. Besides adhesion the junctional molecules also play an important role in cell polarity.

Crb is known to be involved in the junction formation although in case of photoreceptor junctions remain unaffected. Similar to the finding of the photoreceptor the junctions form in case of JO of the *crb* mutant. Based on the importance of cadherin and catenin in maintaining the junctions we check for the expression pattern of *D*E-cadherin and Armadillo in Johnston's organ. To visualise the whole scolopidia pholloidin along with antibodies raised against horseradish peroxidase (HRP) was used. HRP stains neuronal membranes, which appear to recognize sugar residues on multiple glycoproteins (Jan and Jan, 1982; Sun and Salvaterra, 1995; Seppo et al., 2003). Anti-HRP antigens also accumulate in the ciliary dilation and sensory dendrites in wild-type scolopidia (Husain et al., 2006). Pholloidin binds to actin thus the actin rich area of the scolopidia can be visible (Fig.3A,A',A''). Using the same (pholloidin and HRP) labeling in corresponding mutant clone reveals reduced amount of HRP labeling or very disorganized neuronal cell along with the scolopale cell (Fig.3B,B',B''). The labeling of pholloidin and anti-HRP makes it easy to find the proper localization of *D*E-cadherin. In wild type, *D*E-cadherin is expressed at the junction between the scolopale cell and cap cell and the cap cell and neuronal cell. Longitudinal section through the JO revealed two

labeling in two different places (Fig 3C,C',C''). The localization is in agreement with the junction observed under EM in the corresponding level (Fig 2). In *crb* mutant the signal of *D*E-cadherin is reduced and mislocalised from the corresponding region (Fig 3D, D',D''). Junctional molecule Armadillo is also expressed in JO and has similar expression as *D*E- cadherin. In *crb* mutant Armadillo is reduced and mislocalised (data not shown). Although the junctional molecule like *D*E- cadherin and armadillo is reduced in *crb* mutant under electron microscope there is not much alteration in the ultrastructure of junctions revealed. Reduced amount of *D*E-Cadherin and Armadillo, indicate the junctional complexes do not form properly in the absence of Crb. In photoreceptor cells Crb is required for the formation and stablisation of the junctions thus regulate the morphogenesis of the photoreceptor cells. Like photoreceptor cells, in JO probably Crb is required to ensuring junctional integrity. As a consequence the junctions are not strong enough to do the function and the functional units appear to lose its strength resulting disorganized functional units in mutant.

Besides junction microtubule, organizing center (MTOC) also play an important role in maintaining the cell polarity and morphogenesis. It has been suggested that gamma-tubulin may be a universal component for establishment of microtubule polarity (Oakley, 1992). In the mature terminally differentiated cell (mammalian photoreceptor cell), the cytoplasmic microtubules of the cell body appear to originate from the basal body (Pagh-Roehl et al., 1991). Furthermore, the striated rootlet extends from the basal body into the inner segment to the level of the outer limiting membrane (Sjöstrand, 1953), and may continue through the cell body and axon down to the synaptic terminal (Spira and Milman, 1979). The function of striated rootlets remains largely unknown, although it is likely that they may be involved in the association of the basal body with the cytoskeleton (Lemullois et al., 1991). We also check for the formation of basal body in JO by checking expression of the gammatubulin. There are four basal bodies present in JO organ. The distal basal body gives rise to the cilia where as the proximal basal body gives rise to the rootlet (EM observation). In mammalian photoreceptor cell, the striated rootlet proximal to the basal body is involved in the nucleation of inner segment microtubules. Gamma tubulin (Oakley et al., 1990; Joshi et al., 1992) being an evolutionarily conserved and ubiquitously expressed protein we expect a similar role for

MTOC could be proposed for JO. Wild type labeling of the gamma tubulin revealed the presence of basal bodies at the base of the scolopale rod. All the four basal body appear as a bright dot in wild type JO (Fig. 3E, E', E''). It is difficult to distinguish all the four at the confocal level. In case of the mutant the intensity of the dots appear to be reduced and all the four basal bodies can be observable under confocal microscope (Fig. 3F, F', F''). Reduced amount of gamma tubulin suggests that either the centriole pairs themselves or the areas to which gamma tubulin binds are much reduced. To determine if the reduced gamma-tubulin labeling reflects the altered centriole structure in *crb* mutant JO we check for the basal body in *crb* mutant. Although reduction in gamma tubulin is reported from Unc-GFP spermatocytes and resulted in defective basal body (Baker et al., 2002) reduction of gammatubulin does not alter the structure in case of *crb* mutant scolopidia.

Localisation of Chordotonal organ protein in *crb* mutant

Spacemaker or EYS is an extracellular sensory protein and the agrin/perlecan-related protein known to have a role in mechanosensory organ. This mechanosensory protein fills the extracellular space (scolopale space) surrounding the cilia. This scolopale space is more vulnerable to osmotic pressure changes and thus the function is to protect the cell from mechanosensory damage. Spam further functions as a mechanical barrier that protects MRN from deformation (Cook et al., 2008). Spam protein concentrates at two specific sites in MRN: one, right at the interface between the MRN cell body and the lymph space, the very domain that collapses at high temperature in mutant animals and at a second site close to the ciliary dilation, possibly helping sustain the two ciliary processes at the proper position (Cook et al., 2008; Lee et al., 2008). From the structural point of view spam could be an interactor of Crb. In Crb mutant photoreceptor cell inter rhabdomereal space is reduced indicating amount of spam may be affected in *Crb* mutant. Furthermore, the function of the receptor lymph inside the scolopidia is to provide strength to the scolopidia. The disorganised scolopidia of the *Crb* clone further implies spam may be affected in *Crb* mutant. We further check for the expression pattern of spam in both wild type and *Crb* mutant. In agreement with expression pattern described in wild type, spam is present in the two different part of scolopale cell (Fig 4A,A',A''). In

case of *Crb* mutant the space between the localization of two different expression of spam seems to be reduced representing spam is mislocalised in *Crb* mutant or the lumen length of the scolopale cell is reduced in *Crb* mutant, which is agreement with the finding of photoreceptor cell (Fig 4B, B', B''). Mislocalised spam in the scolopale space may result in a lesser strength to the scolopidia resulting disorganized scolopidia of the mutant.

DCX-EMAP is a microtubule associated protein and is essential for the mechano transduction in *Drosophila* sensory cilia. Thus necessary for auditory transducer gating and amplification in *Drosophila* chordotonal receptor cells (Bechstedt et al., 2010). The expression pattern of DCX-EMAP is restricted only to ciliary dilation. Mutant of this protein shows severe uncoordinated behavior alongwith deafness. Being a hearing organ protein we check for the expression in both wild type and mutant clone. In wild type JO in agreement with the previous results, DCX-EMAP is localized to the ciliary dilation (Fig.4C, C', C''). In a longitudinal section through the scolopidia the DCX-EMAP labeling appears at the same level and is nicely aligned in wild type scolopidia. In case of the mutant, besides having disorganized scolopidia DCX-EMAP is still identified (Fig.4D, D', D''). Presence of hearing protein DCX-EMAP in *crb* mutant indicates that, hearing may be still possible in case of this mutant.

To know further about the function of the JO we check for the localistaion of various proteins involved in the function of chordotonal organ. *Drosophila* hearing requires transducer components, which includes transient receptor potential (TRP) -family ion channels. Three members of the TRP family have been shown to be expressed in the neurons of *Drosophila* JO and proved to be involved in hearing of flies, *i.e* the TRPN1 channel, no-mechanoreceptor-potential C (NOMPC) and two interacting TRPV members Nanchung (Nan) and Inactive (Iav), of which the later are deemed to form a heteromultimeric Nan-Iav channel (Walker et al., 2000; Kim et al., 2003; Gong et al., 2004). We check for the expression of some protein involved in TRP channel (IAV and NOMPC). In agreement with the results published before our labeling shows that NOMPC, a protein of TRPPV channel, localizes to the distal cilium in wild type JO (Fig. 4E, E', E''). NOMPC is known to be involved in signal transduction and anterograde

transportation. NOMPC is further serve as an element, acting as a ‘‘gating spring’’ to transmit forces arising from cuticle deformations to the gate of the channel (Howard and Bechstedt, 2004). The finding of Gopfert et al., 2006, where electrical responses are attenuated and mechanical responses are diminished in NOMPC mutants, further strengthens the finding of Howard and Bechstedt 2004. In *Crb* mutant NOMPC seems to be present however, mislocalised (Fig. 4F, F’, F’’). This could be due to the disorganized scolopidia observed in *Crb* mutant. Although it is not very clear at this moment, which is the subsequence of the other, the mislocalised NOMPC may affect the functional aspects of the chordotonal organ of the *Crb* mutant.

The basal part of the scolopidia seems to be affected in *Crb* mutant. HRP staining reveals discontinuous staining in the proximal part of the scolopidia. Electron microscopy of this area revealed the presence of rootlet and neurons. Long, striated ciliary rootlet or axial filament extends from the basal bodies through the neuronal cell body and into a basal process that is attached to a ligament or epidermal cell (Hartenstein, 1988; Wolfurm, 1992, 1997). Striated rootlets are characteristic feature of most ciliated cells and some are capable to act in a calcium-dependent manner (Salisbury, 1998). We check for the expression of proteins present in the rootlet. Rootlet components include isoforms of centrin/caltractin, a calmodulin-related protein that regulates rootlet contractility (Wolfurm, 1992; 1997). Rootletin, a large coiled- coil protein required to maintain mammalian sensory cilia (Yang et al., 2002; 2005). Recently the fly homolog of rootletin CG6129 is also reported from *Drosophila* although the function still needs further investigation (Cachero et al., 2011). *Drosophila* has genes encoding two centrin isoforms and a rootletin homolog. No mutations affecting either of these proteins have been identified, but the rootlet as well as the cilium is absent in Rfx mutants (Dubrullie, 2002), so some RFX transcriptional targets must be involved in its assembly. We check for the health of the rootlet using centrin and actinin antibody. We found the expression of pattern of centrin in the wild type rootlet, is in agreement with the former finding Wolfrum,) (Fig. 4G, G’, G’’). This labeling is founding to be mislocalised in *Crb* mutant indicating the rootlets are affected in *Crb* mutant (Fig. 4H, H’, H’’). In nomarski picture such area appear like a hole and the structures correspond to that region is found to be

missing. Although rootlets are not known to be involved in hearing this may be a supportive structure to provide strength to the scolopidia and help in the contraction of the scolopidia. JO being a chordotonal organ, contraction is required for the proper functioning of the JO. Loss of rootlet resulted in loss of strength to the scolopidia resulting disorganized scolopidia and result in improper functioning.

Behavioral and Functional Defects in *crb* Mutant

The structural defect of the *crb* mutant and mislocalised mechanosensory protein suggests a defect in behavior of the *crb* clone. We check for the behavioral defect of *crb* mutant to check for the chordotonal mechanosensory organs (Eberl et al., 2000). We check for the gravity test for the *crb* mutant. In such experiment wild type fly, *Iav* , wild type fly with mechanical damaged antennae along with *crb* clones were used. The test is performed in a gravity-sensing device as explained in (Kamikouchi, 2009). Based on the performance, the flies were subjected five times for each test. All the results were plotted in a graph. Based on the graph *crb* mutant gravity sensing is somewhat in between the wild type and the *Iav* mutant (Fig.5A). The intermediate step of the behavioral tests indicates the mechnosesnory effect is not completely abolished in case of the *crb* mutant. The neural basis of *Drosophila* gravity-sensing and hearing chordotonal organs is stretch receptors that transduce movement or vibration of limb and body segments. Since the functional units or scolopidia are disorganized probably all the information cannot process properly resulting in an impaired JO in *crb* mutant.

To assay the function of JO in the *crb* mutant, we recorded air puff compound potentials from the antennae. Individual wild-type and mutant flies were exposed to near-field air puff while extracellular potentials were recorded from their antennae (Fig. 5B). In either case the airpuff could rotate the antennae 45 degree and spin the A3 (Funniculus) form it's original place. In wild-type controls, a standard pulse stimulus evoked compound potentials with an average peak-to-peak amplitude of …. (Fig. 5B). In *crb* mutant, responses to stimuli of the same intensity were reduced to an average of …. (Fig. 5B). Although many of the mutants showed reduced activity and severe uncoordination, the reduction in response amplitude is unlikely to be due to general

"sickness," because three especially healthy and vigorous mutants gave similarly reduced responses (average amplitude ….V). Because antennal response represent an aggregate response from the many scolopidia in JO, the response amplitude reduction may reflect either loss of function in a subset of scolopidia or an overall partial loss of function. Nevertheless, this result indicates that *crb* is required for normal function of proper functioning of the chordotonal organs.

Role of Crb in the development of all chordotonal organ.

Johnston's organ is the largest chordotonal organ in *Drosophila*. Expression of Crb in JO suggests Crb may have a role in all chordotonal organ. Both structure and functional analysis of *crb* clone suggests Crb affects the JO to certain extend. To know more about the function of Crb in chordotonal organ we check for the expression pattern in embryonic chordotonal organ. Like adult, embryo chordotonal organs are built of a variable number of units called scolopidia. Each scolopidium comprises distinctive types of cells, which have been tentatively identified as homologous to the different cells of the external sensilla (Schmidt, 1969). An individual scolopidium is innervated by one or more bipolar sensory neuron(s). Dendrites are enclosed within the scolopale cell, a homologue of the thecogen cell of an external sensillum. Like the thecogen cell, the scolopale cell secretes an extracellular sheath around the dendritic tip. Additionally, it forms the scolopale, an intracellularly located framework of densely packed microtubules surrounded by an electron-dense material. Apically, the scolopale cell is connected to the elongated cap cell, which anchors the scolopidium in the epidermis. The cap cell might be homologous to either the trichogen or tromogen cell of an external sensillum. The soma of the sensory neuron is enclosed within a soma sheath cell. Although this is the general organization of the scolopidia there are various types of chordotonal organ present in different segments of the embryo. Depending of the localization and their arrangement of number they are named differently (Hartensiein, 1988). We check for the expression pattern of Crb in all types chorodotonal organ. Crb is expressed in all types of chordotonal organ (Fig-6). Chrodotonal organ starts forming from sensory organ precursor cell. Crb expression starts appearing as soon as the chrodotonal organ form. To know the proper localization pattern of Crb we co-stain the chordotonal organ with

neuron specific antibody, 22c10 (Hartenstein, 1988) (Fig-6). The costaining of 22c10 indicates Crb is expressed apically to the neuron of all types of chordotonal organ. To further confirm the localization of Crb we costain the scolopale cell with Crb and spacemaker. Spacemaker is known to mark the scolopale cell. This labeling further confirms the labeling of Crb in the scolopale cell.

Which part of Crb is responsible for the phenotype of Johnston's organ

The *Drosophila* Crb is composed of a large extracellular domain and 29-epidermal growth factor (EGF)-like repeats, four Laminin A globular domain like repeats (Tepass et al., 1990). The small cytoplasmic domain of 37 aminoacids known to interact with Sdt and Yurt. Expression of the cytoplasmic domain of Crb is able to rescue the defects in rhabdomere elongation in the photoreceptor cell (Johnson et al., 2002). Surprisingly, most of the polarity functions in *crb* mutant are rescued by expression of its transmembrane and short cytoplasmic domains, suggesting that the major interactions regulating cell polarity and shape in the embryo are mediated by the 37 intracellular amino acids of this large (2,139 amino acids) protein (Wodarz et al., 1995). This hypothesis is reinforced by the observation that a nonsense mutation in the *crb* ^{8F105} allele, prevent the translation of the last 23 amino acids of the cytoplasmic tail, produces a severe loss of function phenotype (Wodarz et al., 1993) and this phenotype is indistinguishable from Crb null allele phenotype (Jiirgens et al., 1984). In this allele there is still Crb protein synthesized but shows abnormal localisation in both eye and embryo (Tepass et al., 1990). The cytoplasmic domain of this mutant contains only 14 amino acid residues. The transmembrane domain and the positively charged amino acid residues flanking it on the cytoplasmic side are not suggesting that it still retains the characteristics of an integral membrane. Furthermore, it has been shown that the MAGUK family member Stardust (Sdt) binds to the last four amino acid residues (ERLI) of the cytoplasmic tail of Crb, along with the PSD-95/DLG/ZO-1 (PDZ) domain protein Discs-lost (Dlt) (Bhat et al., 1999; Klebes and Knust, 2000; Bachmann et al., 2001; Hong et al., 2001). These two proteins are both required for epithelial polarity, and thus Crb, together with Dlt and Sdt,

defines a membrane–associated complex in the apical cytocortex of epithelial cells that is necessary for the proper generation of the polarized phenotype.

Expression of either full-length Crb or its membrane bound extracellular domain results in an expansion of stalk membrane in the photoreceptor. Based on the published results, we asked which part of the Crb is responsible for the phenotypes that are seen in the JO of Crb mutant. For this we checked the phenotype of the JO in a weaker allele of crb i.e. crb^{8F105}. We also checked the expression of Crb protein in the JO of crb^{8F105} allele. Interestingly in agreement with the previous finding there is still some amount of Crb found in this allele. The phenotype of the crb^{8F105} in JO appear weaker then the crb^{11A22} which, is a complete null allele for Crb. The disorganized scolopidia and abnormal actin filament of crb^{11A22} is found to be missing in this weaker allele (Fig. 8A, B, C, D). However, like crb^{11A22} neuronal abnormality is observed in this allele. Junctions present in between the scolopale cell and the neuronal cell look very normal like W^- (Fig. 8C). The cap cell still fails to encircle the cilia completely in some cases like crb^{11A22}. The base of the scolopidia also appeared splayed in this mutant like crb^{11A22}. Defective morphology observed in the JO may be attributed to the extracellular domain. In human being, the extracellular domain is known to be responsible for light dependent degeneration (den Hollander, 1999; Lotery et al., 2001). We check for the behavioral defect in this mutant and the functional defect as well. We check for some old flies (20days) to check for the degeneration defect. This phenotype do not change much even after 20days. This result is in agreement with the result found in the compound eye of the same allele.

Discussion

Crb is essential for the maintenance of Johnston's organ

To date, little is known about the junctional molecules required for establishing the JO. Significantly, our findings emphasize that; Crb a transmembrane protein is required for the morphogenesis and function of JO. First and foremost, Crb is expressed near the junction of scolopidia. The loss of Crb results in poorly formed junctions, although the junctions are still present and junctional proteins still accumulated near the junctions. The

base of the scolopale rod appears splayed as observed in *Ck* mutant. However, removal of Crb does not affect the formation of scolopidia. Moreover, our confocal analysis demonstrates that the disorganised scolopidia is not due to degeneration but due to a developmental defect. Our data further suggests that the roles of Crb is to coordinate and direct the morphological changes of the actin cytoskeleton.

Another intriguing observation from our studies is the role of Crb in photoreceptor versus hearing organ. The loss of Crb in JO does not result in noticeable defects in scolopidia formation, in stark contrast with the situation for the eye. Why is the difference? One possibility is that the organization of the JO versus the compound eye. In case of compound eye all the functional units are well separated from each other and there are numerous cells, which connect them to each other. In case of JO the functional units are piled one on top of the other and there are numerous empty spaces present in the JO. There are two junctions near the apical and basal region of the scolopidia to hold it properly which is required to stretch the functional units properly. In case of JO although junctions are form loss of junctional complex from the junctions may lead them to prevent from proper functions. As a result the junctions do not have proper strength to hold the socolopedia resulting disorganized scolopidia in the JO. In contrast, the photoreceptors have junctions throughout the length of the ommatidia. In case of the mutant photoreceptor, the junctions become discontinuous in the proximal part of the retina (Izaddost et al., 2002; Pellika et al., 2002). As a result the photoreceptor cells cannot elongate throughout the length of the retina. Due to lack of proper stretching of the photoreceptor the photoreceptor appear bulky resulting shorter and wider rhabdomere in case of *crb* mutant. In JO since both the junctions required for the proper stretching a phenotype like shorter and wider scolopidia as observed in ommatidia is scanty.

Besides disorganised scolopidia, numerous structural deformities are also observed in case of *crb* mutant. The scolopale rod, which is the apical compartment of the scolopidia, formed although they appear to be disorganized. In comparison to the apical compartment, the basal parts seem to be more affected. The neuronal cell seems to lose its shape and seem to be significantly less in number. If we compare this phenotype to the

phenotype observed in the photoreceptor then there is some similarity. In case of photoreceptor the proximal part of the photoreceptor is empty. In case of JO organ the basal part of the scolopidia are affected which is the neuronal part of the JO. Neurons are required for the transduction of signals. Reduced/malformed neuronal cell may affect the signal transduction or hearing in *crb* mutant flies. Each scolopidia is formed of a scolopale cell, which is filled with extracellular matrix. The function of the extracellular matrix is to provide the strength to the scolopidia. In case of *crb* mutant shorter lumen and defective scolopale space may affect additional strength of the scolopidia.

Besides, the cap cell fails to encircle the cilia completely in some scolopidia. This type of phenotype is observed in *Ck* and *NOMPA* mutant (Todi et al., 2005). The incomplete encircling of the cap cell in *crb* mutant may be due to the bending of the scolopidia. In case of *crb* mutant, the ciliary dilation and the protein required for it appears to be fine. The scolopale cell appears to be splayed at the level of basal body although the cilia, which are required for the signal transduction, appear to be fine. Defective cilia always result in a defective signal transduction. In case of *TilB* and *smet* mutant, the cilia are affected and in many cases the cilia do not form (Kernan *et al.*, 1994, Eberl *et al.*, 2000, Todi *et al.*, 2004). In such cases under electrophysiology the mutant fails to initiate the hearing. Role of Crb is studied in various vertebrate photoreceptors, which is a ciliated organ. So far, cilia defect is not reported from any species. In agreement with the previous study Crb do not affect the cilia in case of JO.

In case of JO, very large junctions are formed near the base of the scolopale rod. However, the junctional proteins appear to be reduced. Furthermore, the organelle, which appears to be affected mostly in *crb* mutant, is the rootlet. The number of neuronal cell is reduced. Neuronal cell over proliferate and thus loses its shape. Rootlet, which is found inside the wild type neuronal cell, is found to be absent in some part of the neuron. Apoptotic structures also appear inside the neuronal cell body. Proteins necessary for the rootlet like actinin and centrin is found to be mislocalised in *crb* mutant. Gamma tubulin amount is found to be reduced. Gmama tubulin is known to interact with some parts of the rootlet in vertebrate photoreceptor. Furthermore, gamma tubulin is required for the proper extension of the rootlet. Reduced amount of gamma tubulin may account for the

discontinuous rootlet in *crb* mutant resulting discontinuous neuronal cell. Rootlets are further known to function as a supporting structure and acts as a railroad in transmitting signals form the scolopidia to the neuron. The contraction of the rootlets act in a calcium-dependent manner. Proteins in the rootlet like centrin/caltractin (a calmodulin-related protein) regulate further the contractility of the rootlet. Mislocalised centrin from the rootlet may affect the proper functioning of the rootlet in *crb* mutant. All the structural defects altogether reflect in a functional and behavioral phenotype in *crb* mutant.

Besides scolopale cell and rootlet neurons are also affected in *crb* mutant. Although disorganised scolopidia phenotype is reported from various mutations associated with JO, reduced amount of neuron is not reported before. Disorganized scolopidia may be due to mislocalised junctional protein form the junctions. Crb is known to have a role in the junction. Loss of Crb results loss of junctional proteins from the junctions. Junctions are required to stretch the scolopidia properly in empty space. Loss of junctional molecule or defective junctions fails to hold the scolopidia properly as a result they are bent and often uprooted from the junctions and fall over another scolopidia. This disorganized phenotype could be explained as a loss of function of Crb from the junctions. Furthermore, mislocalised scolopidia may affect the strength of *crb* mutant.

Less amount of neuron in *crb* mutant still needs further investigation. However, JO is a ciliated neuron. In case of vertebrate photoreceptor cell, the photoreceptor cell over proliferate thus loses it's shape and form psudorosset ultimately reducing the number of photoreceptor nuclei. Although there is not similar study from hearing organ of vertebrate JO being a ciliated organ we may apply the same principle to JO. This hypothesis still needs further investigation.

Materials and Methods

Fly stocks and reagents

*white (*w*)* flies were used as wild-type control throughout the experiment. Eyes and ocelli

mosaic for crb^{11A22}, a protein null allele, or crb^{8F105}, which encodes a truncated Crb protein (Wodarz, 1993) were generated by crossing *yw ey*-FLP;;FRT82B *w*+ cl3R3/TM6B females (Newsome, 2000) to w;;FRT82B *crb*/TM6B males.

Transmission Electron Microscopy

Fixation of adult eyes for transmission electron microscopic analysis was performed as previously described (Richard, 2006). Essentially, fly heads of appropriate genotypes were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde overnight at 4°C. Heads were washed with PBS, followed by a secondary fixation with 2% osmium for one hour at room temperature in the dark. Heads were dehydrated in 50%, 70%, 90%, 2 x 100% ethanol and 2x propylene oxide. Heads were infiltrated with a mixture of resin and polypropylene oxide overnight at room temperature, and embedded in pure resin. Samples were allowed to polymerise at 60°C overnight. Semithin sections of 500 nm were cut (Leica Ultracut Microsytems) and stained with toluidine blue. Ultrathin sections of 70 nm were cut from the appropriate areas with a diamond knife and stained with 2% uranyl acetate and lead citrate for contrast enhancement. Digital images (Morada digital camera SiS) were taken using a FEI Tecnai 12 Bio Twin electron microscope operated at 80 kV, and mounted using Adobe Illustrator CS4.

Antibodies and Immunofluorescence analysis

Staining of adult antennae were done essentially as follows. Heads were cut with a razor blade and the proboscis was removed. Heads were fixed for 40 minutes in Stefanini's fixative at room temperature in shaking condition, washed three times with phosphate buffered saline (PBS) for 15 minutes each, and infiltrated at 4 °C with 10% sucrose in PBS for 2 hours and in 25% sucrose solution overnight. Heads were embedded in mounting media and frozen over dry ice. 12 µm sections were stained as described (Richard, 2006). The following primary antibodies were used: rat anti-Crb2.8 (1:500; E. Theilenberg and E. Knust, unpublished), rat anti-DE-cadherin, mouse anti-Armadillo, mouse anti-Spacemaker (1:50; Developmental Studies Hybridoma Bank, DSHB), DCX EMAP (1:100) (Howard lab), NOMPC (1:100) (Howard lab), anti-rabbit gammtubiluin (1:200) Sigma, Anti rabbit alpha actinin (1:200 Sigma), Anti-centrin (1:200 Sigma). Cy3-

conjugated goat anti-HRP (1:100; Dianova) was used to label neuronal cells in the ocelli. Alexa 568- and Alexa 488-conjugated secondary antibodies (Molecular Probes, Inc.) were applied at 1:200 dilution. Alexa 647-conjugated phalloidin (1: 40; Invitrogen) was used to mark filamentous actin. Confocal images were taken on a LSM Meta confocal microscope (Carl Zeiss). All images were processed with Fiji software and mounted using Adobe Illustrator CS4.

Gravitactic behavior test

The negative gravitaxis was assayed as described by using a countercurrent device (Inagaki et al., 2010) in darkness. 20-30 flies were used in each assay. The flies were given choices 5 times to stay at the bottom of the tube or climb up. After the assay, the flies distributed into 6 tubes in the device (N_1, N_2, N_3, N_4, N_5, N_6. N_k is the number of flies in *kth* tube). The numbers of flies in the 1st and 2nd, 3rd and 4th, 5th and 6th were summed up into fraction 1, 2 and 3, respectively. The distribution of these 3 fractions was shown in a bar graph (Figure 5A,B,C). The partition coefficient was calculated as described (Inagaki et al., 2010) and shown as mean ± standard deviation.

$$Cf = \frac{N_2 + 2N_3 + 3N_4 + 4N_5 + 5N_6}{5 \times (N_1 + N_2 + N_3 + N_4 + N_5)}$$

Extracellular recording of the fly antenna

An extracellular recording technique was used to assay the function of Johnston's organ. Briefly, the adult fly (2-3 days) was anesthetized on ice for 10 min. A fly was introduced into a pipette tip and encouraged to walk up the tip by aspiration until it was trapped in the tip. The pipette tip was trimmed under a stereomicroscope so that the fly head was exposed. The fly head was then fixed by wax. The pipette was mounted vertically on a ball and socket stage which could provide ±25° tilt and 360° rotation (M-RN-86, Newport). To stimulate the Johnston's organ, a capillary was placed next to the arista of antenna to deliver the air puff. The ball and socket stage can be adjusted accordingly to allow the capillary to deliver the air puff from approximately the same angle. The air puff usually moved the arista for about 45°. A sharp tungsten electrode (tip

diameter 1 μm) was inserted into the joint of the first and second segment of antenna (Figure 5D). A reference electrode was inserted into the fly eye. The difference in voltage between the reference electrode and the recording electrode was amplified by a differential amplifier (DAM50 Bio-Amplifier, WPI). Labview 8.6 was used to program the software to record the signal. The data were sampled at 10 kHz and stored in computer for the off-line analysis. The amplitude of the highest spikes in response to every air puff stimulus was used to quantify the response intensity. The recordings on at least 5 flies were averaged to get the mean value that was then used for the statistics.

Staining of embryo

Drosophila embryos were collected as described By Muller, 2008. In brief, flies were kept in egg collection cages with apple juice plates. Plates with embryos were collected and dechorionated fro 3 min in a 3% sodium hypochlorite solution. Dechorionated embryos were transferred to a wire mesh basket and rinsed with tap water. Embryos were fixed for 20mins in 4% formaldehyde on an overhead mixer. Afterwards, embryos were transferred to glass scintillation vial, containg 4ml 4% formaldehyde in 1xPBS pH7.4 and 4ml n-hepatane, which ensures that embryos float between two phases. After fixation, the lower formaldehyde layer was replaced by 100% methanol. Subsequently, embryos were devitellinised by vortexing the vial for 20sec. Devitellinised embryos were collected from the methanol phase with a Pasteur pipette and transferred to an eppendrof tube with 100% methanol. Embryos were washed three times with 100% methanol to remove all n-heptane and formaldehyde. Eventually, embryos were either either used directly for immunohistochemistry or stored at -20^{o}C. Embryos were permeabilised by washing three times for 20mins in PBST (PBS+Tween 20). Permeabilised embryos were blocked by washing three times 20 mins in PBST with 5%NHS, before incubation with rat anti-Crb (1:500) diluted in 5%NHS and PBST for overnight at 4^{o}C. The primary antibody was removed and the embryos were washed three times for 20mins in PBST before incubation with secondary antibody. Alexa conjugated secondary 647 antibody alongwith cy3 conjugated HRP (1:100) were diluted with 5% NHS diluted in PBST and incubate at room temperature for 2 hours. Afterwards, the antibody were removed and the embryos were washed three times 20mins each in PBS. Eventually embryos were

mounted on Glycerin Propyl gallate.