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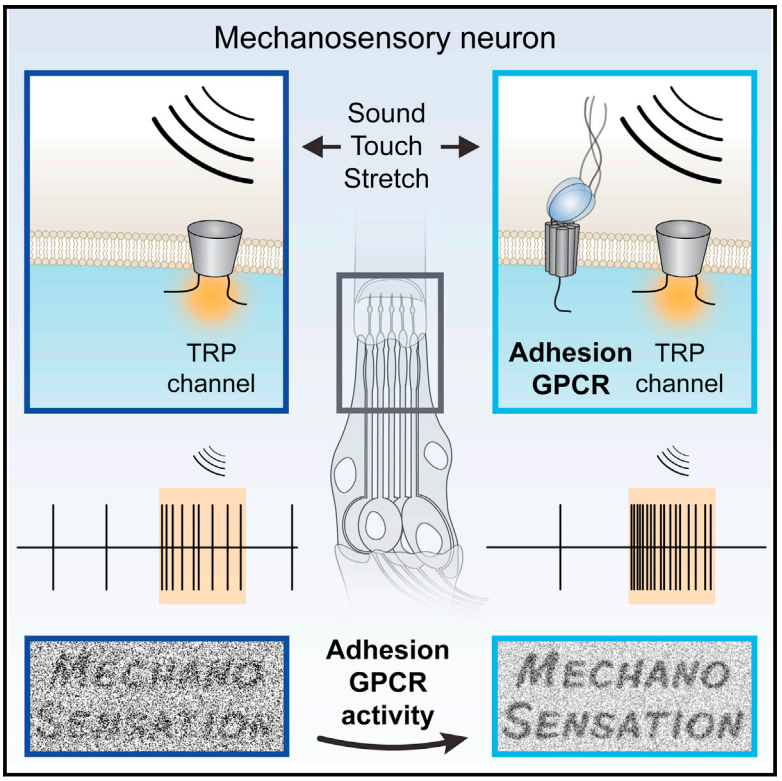
The Adhesion GPCR Latrophilin/CIRL Shapes Mechanosensation

Graphical Abstract



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In Brief

The receptive repertoire of adhesion GPCRs has thus far proved elusive. Scholz et al. analyzed a *Drosophila* mutant lacking Latrophilin/CIRL, a prototype member of this receptor class. They establish that the aGPCR Latrophilin/CIRL participates in the recognition of mechanical signals by determining the sensitivity of mechanosensory neurons.

Highlights

- Latrophilin/CIRL is required for mechanosensory neuron function
- Latrophilin/CIRL modulates sensitivity of neuronal mechanosensation
- Latrophilin/CIRL interacts with TRP channels
- In vivo role for a GPCR in mechanosensation is demonstrated

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The Adhesion GPCR Latrophilin/CIRL Shapes Mechanosensation

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SUMMARY

G-protein-coupled receptors (GPCRs) are typically regarded as chemosensors that control cellular states in response to soluble extracellular cues. However, the modality of stimuli recognized through adhesion GPCR (aGPCR), the second largest class of the GPCR superfamily, is unresolved. Our study characterizes the *Drosophila* aGPCR *Latrophilin/dCirl*, a prototype member of this enigmatic receptor class. We show that *dCirl* shapes the perception of tactile, proprioceptive, and auditory stimuli through chordotonal neurons, the principal mechanosensors of *Drosophila*. *dCirl* sensitizes these neurons for the detection of mechanical stimulation by amplifying their input-output function. Our results indicate that aGPCR may generally process and modulate the perception of mechanical signals, linking these important stimuli to the sensory canon of the GPCR superfamily.

INTRODUCTION

Because of the nature of their activating agents, G-protein-coupled receptors (GPCRs) are established sensors of chemical compounds (Pierce et al., 2002). The concept that GPCRs may also be fit to detect and transduce physical modalities, i.e., mechanical stimulation, has received minor support thus far. In vitro observations showed that, in addition to classical soluble agonists, mechanical impact such as stretch, osmolarity, and plasma membrane viscosity may alter the metabotropic activity of individual class A GPCR (Chachisvilis et al., 2006; Mederos y Schnitzler et al., 2008; Zou et al., 2004). However, the ratio and relationship between chemical and mechanical sensitivity and the physiological role of the latter remain unclear.

Genetic studies have indicated that adhesion GPCRs (aGPCRs), a large GPCR class with more than 30 mammalian members (Fredriksson and Schiöth, 2005), are essential components in developmental processes (Langenhan et al., 2009). Human mutations in aGPCR loci are notoriously linked to pathological conditions emanating from dysfunction of these underlying mechanisms, including disorders of the nervous and cardiovascular systems, and neoplasias of all major tissues (Langenhan et al., 2013). However, as the identity of aGPCR stimuli is unclear, it has proven difficult to comprehend how aGPCRs exert physiological control during these processes.

Latrophilins constitute a prototype aGPCR subfamily because of their long evolutionary history. Latrophilins are present in invertebrate and vertebrate animals (Figures 1A and 1B; Fredriksson and Schiöth, 2005), and their receptor architecture has remained highly conserved across this large phylogenetic distance (Figure 1A). The mammalian Latrophilin 1 homolog was identified through its capacity to bind the black widow spider venom component α-latrotoxin (α-LTX; Davletov et al., 1996; Krasnoperov et al., 1996), which induces a surge of vesicular release from synaptic terminals and neuroendocrine cells through formation of membrane pores (Orlova et al., 2000; Rosenthal and Meldolesi, 1989). Latrophilin 1/ADGRL1 was suggested to partake in presynaptic calcium homeostasis by interacting with a teneurin ligand (Silva et al., 2011) and in *trans*-cellular adhesion through interaction with neurexins 1β and 2β (Boucard et al., 2012). Further, engagement of Latrophilin 3/ADGRL3 with FLRT proteins may contribute to synapse development (O'Sullivan et al., 2014). The role of Latrophilins in the nervous system thus appears complex.

Here we have used a genomic engineering approach to remove and modify the Latrophilin locus *dCirl*, the only Latrophilin homolog of *Drosophila melanogaster*. We report that *dCirl* is required in chordotonal neurons for adequate sensitivity to gentle touch, sound, and proprioceptive feedback during larval locomotion. This indicates an unexpected role of the aGPCR Latrophilin in the recognition of mechanosensory stimuli and provides a unique in vivo demonstration of a GPCR in mechanoception.

RESULTS

Genomic Engineering of a *dCirl* Null Allele

Drosophila melanogaster possesses a single Latrophilin homolog *dCirl/CG8639* (Figure 1C). We pursued a genomic engineering strategy to generate an incontestable *dCirl* null allele by homologous recombination. We replaced a fragment containing the complete *dCirl* open reading frame (ORF), part of the 5′ intergenic region encoding the putative *dCirl* promoter, and the 5′ and 3′ UTRs with an *attP* site for subsequent phiC31-mediated

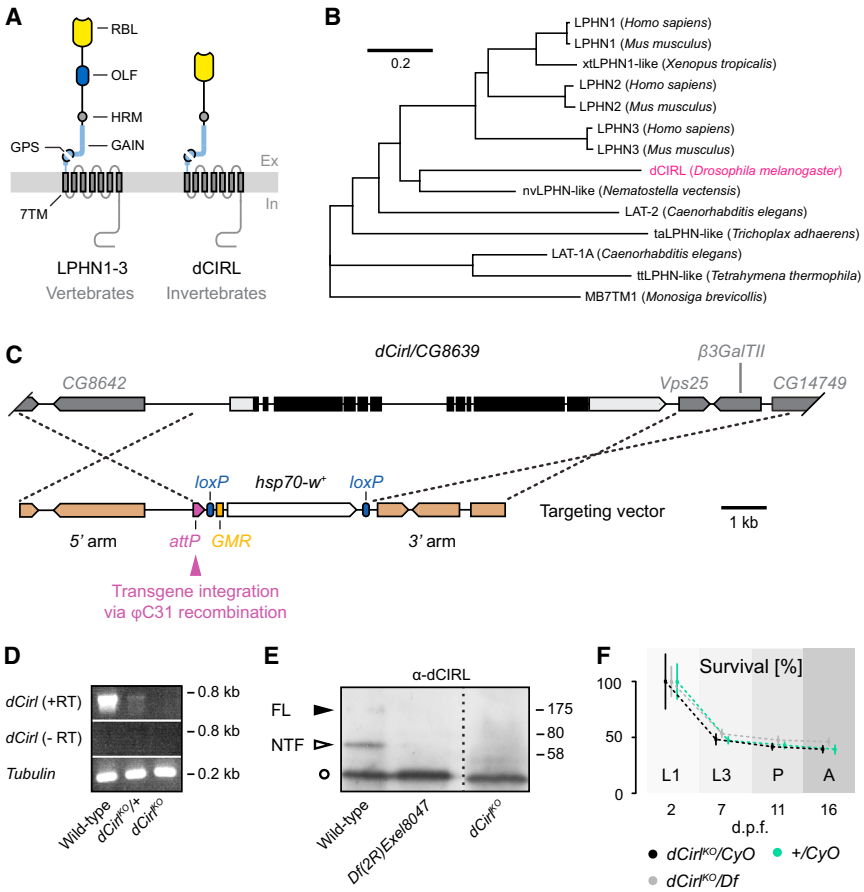

Figure 1. Construction of a *dCirl*^{KO} Allele and a Modifiable *dCirl* Locus

(A) Conserved domain structure of the Latrophilin subfamily of aGPCR containing RBL, OLF (present only in vertebrates), HRM, GAIN, and 7TM domains (N→C order).

(B) Phylogenetic analysis of dCIRL shows ancient conservation of Latrophilins from ciliates to humans.

(C) Genomic organization and targeting strategy of the *dCirl/CG8639* locus. See also Figures S1A–S1C.

(D and E) Confirmation of the *dCirl*^{KO} allele. (D) RT-PCR shows loss of *dCirl* transcripts in the *dCirl*^{KO} strain. (E) Anti-dCIRL antiserum detects no signal in protein extracts from *dCirl*^{KO} and *Df(2R)Exel8047* flies. In extracts of WT flies, two specific bands are detected that correspond to full-length (~180 kDa; closed arrowhead) and autoproteolyzed dCIRL (~70 kDa; open arrowhead). An unspecific signal (open circle) detected by the antiserum served as loading control. FL, full-length; NTF, N-terminal fragment. See also Figures S1D and S1E.

(F) Lethality phase analysis of *dCirl*^{KO} animals. Survival rates of *dCirl*^{KO} animals are indistinguishable from genetic controls throughout larval (L1, L3), pupal (P), and adult (A) stages. d.p.f., days post-fertilization. Data are represented as mean ± SEM.

transgene insertion, and a floxable *hsp70-white* selection cassette (Figures 1C and S1). A recombinant strain, termed *dCirl*^{KO}, was selected and backcrossed into w^{1118} background for 15 generations before further analyses.

First, we established that *dCirl*^{KO} is a null allele by transcribing cDNA libraries from *dCirl*^{KO} and control flies, which showed no residual transcript in *dCirl*^{KO} homozygotes (Figure 1D). We prepared protein extracts from WT larvae and larvae homozygous for *dCirl*^{KO} or *Df(2R)Exel8047*, a small deficiency uncovering the *dCirl* locus. Immunodetection with a polyclonal antiserum raised against a peptide in the extracellular domain (ECD) of dCIRL showed two bands corresponding to the full-length (approximately 185 kDa) and autoproteolyzed receptor (N-terminal fragment [NTF], approximately 77 kDa; C-terminal fragment [CTF], approximately 108 kDa) cleaved at the GPS motif in WT extracts. Both bands were absent from samples prepared from *dCirl*^{KO} and *Df(2R)Exel8047* homozygotes (Figure 1E). We conclude that *dCirl*^{KO} is a protein null allele.

dCirl Is Required for Coordinated Locomotion

To investigate whether *dCirl* exerts developmental functions, we conducted a lethal phase analysis. Intriguingly, *dCirl*^{KO}/*Df(2R)Exel8047* transheterozygotes developed indistinguishably from controls throughout embryonic, larval, and adult phases, indicating that *dCirl* is not essential for overt development and viability in *Drosophila* (Figure 1F).

During this investigation, we noted that *dCirl*^{KO} larvae exhibit a conspicuous crawling pattern and travel less distance than controls (Movie S1). While in control larvae the forward motion phase occupies the majority of the motility cycle, *dCirl*^{KO} animals spent extended amounts of time in head swing episodes, which resulted in increased pausing of larvae and decreased net crawling distances (Figures 2A and 2B; Table S1; Movie S1). This defect was rescued to the WT level by reinsertion of a genomic fragment at the *attP* site in the *dCirl*^{KO} strain generating *dCirl*^{Rescue} larvae (Figure 2B; Table S1; Movie S1).

These results imply an unexpected role of *dCirl* in shaping locomotion. In addition, they validated our genomic engineering approach to remove *dCirl* function and to generate allelic variants of the *dCirl* locus.

dCIRL Is Expressed in Chordotonal Neurons

Larval crawling is a complex behavior controlled through motor and central pattern generator neurons of the CNS (Jan and Jan, 1976; Suster and Bate, 2002) and adjusted by sensory feedback from afferent neurons of the peripheral nervous system (Caldwell et al., 2003; Cheng et al., 2010).

To determine in which cells *dCirl* is expressed, we conceived a *dCirl* transcriptional reporter allele (*dCirlp*^{GAL4}) that contained an optimized *gal4.2::p65* cassette at the start codon of the genomic *dCirl* ORF (Figure 2C). Among other neuron types (Figure 2D; data not shown), *dCirlp*^{GAL4} > *UAS-mCD8::GFP* expression was most prominent in larval pentascolopidial chordotonal organs (lch5; Figures 2D and 2E). Chordotonal organs (cho) are compound

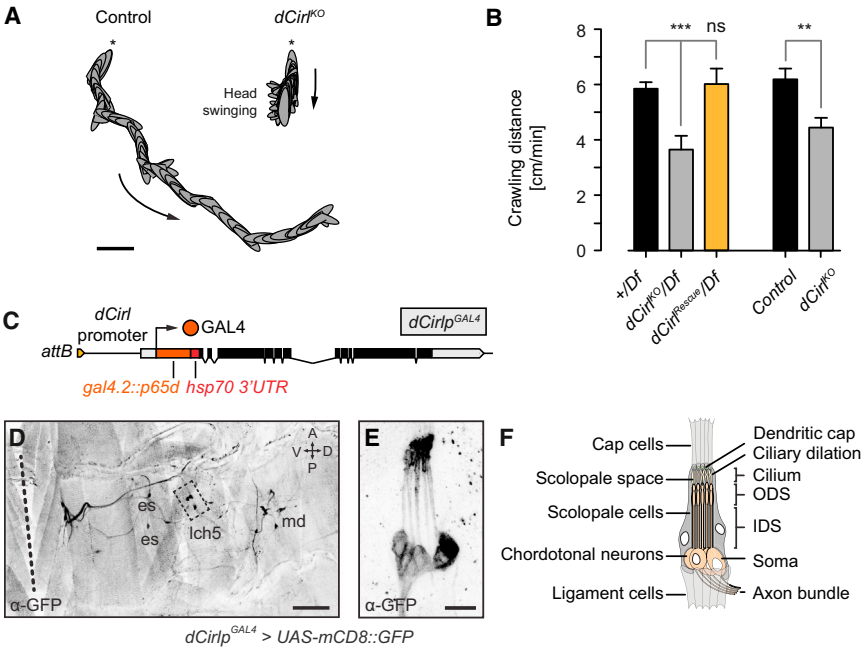

Figure 2. *dCirl* Is Required for Larval Locomotion and Expressed in lch5 Chordotonal Neurons

(A) Loss of *dCirl* results in increased pausing and excessive head swing behavior. Reconstructions of 60 frames for each genotype representing 36 s of recording. Arrows indicate direction of crawling motion, and asterisks mark the start frames. Scale bar represents 5 mm. See also Movie S1.

(B) Quantification of crawling distance. Data are represented as mean ± SEM. See also Table S1 and Movie S1.

(C) Transgene structure of transcriptional reporter (*dCirlp*^{GAL4}).

(D) *dCirlp*^{GAL4} expresses in several peripheral sensory neuron types including type I and type II neurons. Dashed line indicates midline. es, external campaniform sensilla; lch5, pentascolopidial organ; md, multidendritic neurons. Scale bar represents 100 μm. The dashed rectangle is magnified in (E).

(E) Strong *dCirlp*^{GAL4} expression observed in lch5 lateral chordotonal organs. Scale bar represents 20 μm.

(F) Anatomy of a third instar larval pentascolopidial organ. IDS, inner dendritic segment; ODS, outer dendritic segment.

sensory structures that govern the perception of a wide range of mechanical inputs in *Drosophila*, including proprioceptive stimuli, gentle touch, and sound/vibration (Figure 2F; Kernan, 2007).

dCirl Is Required for the Function of Chordotonal Organs

To elucidate whether *dCirl* contributes to chordotonal function, we obtained an established score of the responsiveness of larvae toward gentle touch. In this assay, homozygous and heterozygous control larvae exhibited tactile sensitivities comparable to previously published control genotypes (Caldwell et al., 2003; Kernan et al., 1994; Yan et al., 2013). We found that *dCirl*^{KO} animals indeed exhibited diminished touch sensitivity (Figure 3A; Table S2). This was specifically due to loss of *dCirl*, as the phenotype in *dCirl*^{KO}*/Df(2R)Exel8047* transheterozygotes remained indistinguishable from *dCirl*^{KO} homozygotes, whereas *+/Df(2R)Exel8047* animals exhibited normal touch sensitivity. Further, in *dCirl*^{Rescue} larvae, touch sensitivity was rescued to control level (Figure 3A; Table S2).

To exclude that *dCirl*'s impact on locomotion was conferred via other neurons, we performed a cell-specific rescuing assay with a *20xUAS-dCirl* genomic rescuing transgene, in which the *dCirl* promoter region was replaced with an optimized *20xUAS-IVS* promoter cassette (Figure 3B). We selected *GAL4* drivers with expression domains in motor neurons, (*ok6-GAL4*), in all type II sensory neurons (*21-7-GAL4*), and only in chordotonal neurons (type I sensory neurons; *iav-GAL4*). With this set of *GAL4* lines, we drove expression of the *20xUAS-dCirl* rescuing transgene in the *dCirl*^{KO} background and scored for touch sensitivity. Intriguingly, the sensory deficit was rescued only upon *dCirl* re-expression in chordotonal neurons (Figures 3C and S2A; Table S2). Similarly, chordotonal neuron-specific expression of *dCirl* restored normal crawling of *dCirl*^{KO} larvae (Figures 3D and S2B; Table S1).

The Structure of Chordotonal Neurons Appears Unaffected in *dCirl*^{KO} Mutants

Chordotonal organs and their monociliated type I sensory neurons possess an intricate structure that is relevant for their mechanosensory properties (Figure 2F; Eberl and Boekhoff-Falk, 2007). Consequently, we performed immunostainings to assess the subcellular location of the established chordotonal marker proteins NOMPC (*no mechanoreceptor potential C*) and EYS/SPAM (*eyes shut/spacemaker*) in chordotonal cilia of *dCirl*^{KO} larvae and found no differences in extent, location, or structure of these markers (Figures 3E–3H, S2C, and S2D).

Also, *iav-GAL4* positive chordotonal neuron somata, dendrites, and axonal projections revealed no morphological abnormalities (Figures S2E and S2F; Table S3). Altogether, these results demonstrate that *dCirl* is dispensable for the development and morphology of chordotonal sensory neurons.

dCirl Modulates the Absolute Electrical Activity of Chordotonal Neurons in Response to Mechanical Stimuli

To directly interrogate the function of chordotonal neurons, vibration stimuli were delivered to the cap cells of lch5 using a piezoelectrically actuated glass probe (Figure 4A). The probe tip was placed at the cap cells of the lch5, which are mechanically linked to the apical portions of chordotonal neurons and scolopale cells through an extracellular matrix (dendritic cap; Chung et al., 2001) and septate junctions (Carlson et al., 1997), respectively (Figures 2F and 4A). While applying frequencies from 100–1,500 Hz (Figure S3A), we simultaneously recorded action currents from the axon bundle directly after its exit from the challenged lch5 (Figure 4A).

Without mechanical stimulation, *dCirl*^{KO} lch5 were spontaneously active, albeit at a slightly lower frequency than in WT

animals (Table S4A). Vibration triggered an increase in action current frequencies of WT Ich5, consistent with previous work (Figures 4B and 4C; Table S4A; Zhang et al., 2013). In our preparation, peak activity of neuronal responses was reached at stimulation frequencies around 900 Hz (Figures 4B and 4C; Table S4A). Most intriguingly, *dCirl*^{KO} larvae displayed significantly lower absolute action current frequencies across the entire stimulation spectrum (Figures 4B and 4C), which was fully compensated through the *dCirl*^{Rescue} allele (Figure S3B; Table S4B).

dCirl Modulates the Relative Mechanosensory Response of Chordotonal Neurons

Sensory perception and encoding rely on the ability to contrast evoked from spontaneous activity in the principal sensory neuron. We thus quantified the proportional chordotonal response toward mechanostimulation, i.e., the ratio between evoked and spontaneous spiking activity (R_d; Figure S3A), as a measure of the chordotonal neurons' facility to distinguish signal from noise. The discrimination ratio R_d in control Ich5 neurons peaked around 900 Hz, suggesting that signal perception and/or encoding is most effective in this range of mechanostimulation. In contrast, *dCirl*^{KO} chordotonal neurons showed largely reduced R_d values over several vibration frequencies (Figure S3D; Table S4C). To detail the response profile across the entire stimulation spectrum, we statistically compared the R_d values for any pair of stimulation frequencies and derived discrimination matrices illustrating blurred mechanosignal discrimination in the absence of *dCirl* (Figures 4D, 4E, S3E, and S3F). This demonstrates that, besides modulating the absolute spiking activity of chordotonal neurons, *dCirl* is also necessary for their relative response toward mechanical stimuli.

Intriguingly, when *dCirl* was re-expressed in mutant chordotonal neurons through the *iav-GAL4* driver, the phenotype was partially rescued: the relative mechanosensory responses were re-established (Figures S3D and S3G), while absolute firing frequencies were not recovered (Figure S3C). This implies that *dCirl* cell autonomously modulates the relative response to mechanical stimulation, likely through the metabotropic activity of its CTF. In addition, *dCirl* may be required in other cells, e.g., for an intercellular homodimeric interaction (Prömel et al., 2012), to regulate absolute spiking frequency. Alternatively, as production of the receptor through the GAL4/UAS system is unlikely to restore endogenous expression levels, this may preclude the generation of a physiological evoked response frequency from the neurons.

dCirl Is Required for the Larval Startle Response to Sound Stimuli

To corroborate the impact of *dCirl* on Ich5 function with an independent assay, we examined the startle-freeze reaction of larvae toward a pure sine wave tone of 900 Hz (Zhang et al., 2013). We observed that *dCirl*^{KO} larvae exhibited diminished startle response scores at all sound pressure levels (SPLs) tested (Figures 4F and 4G; Table S5). This defect was rescued in *dCirl*^{Rescue} animals (Figure 4F; Table S5). Remarkably, at lower (60 dB) and higher (90 dB) sound pressure level (SPL), the responses of *dCirl* and control neurons converged (Figures 4F and 4G; Table S5). This indicates that principal sound detection, i.e., the mechanotransduction complex, functions without *dCirl* but that its activation threshold appears increased in *dCirl*^{KO} larvae and is SPL dependent.

Taken together, *dCirl*^{KO} larval responses toward vibrational and acoustical stimulation place the function of dCIRL at the level of mechanotransduction or spike initiation of chordotonal neurons and upstream of synaptic transmission.

dCirl Genetically Interacts with Components of the Molecular Mechanotransduction Apparatus

In order to test this model, we evaluated genetic interactions of *dCirl* with the mechanotransduction machinery. We constructed double mutants of *dCirl*^{KO} in combination with hypomorphic alleles of the TRP channel subunits TRPN1/NOMPC (*nompC*^{f00642}; Sun et al., 2009) and TRPV/Nanchung (*nan*^{36a}; Kim et al., 2003), which forms a heteromeric complex with the subunit IAV (Inactive) in the proximal cilium of chordotonal neurons (Gong et al., 2004). In an epistasis assay, we tested crawling distances of singly and doubly mutant larvae.

Similar to *dCirl*^{KO}, also *nompC*^{f00642} and *nan*^{36a} mutants displayed locomotion activity but traveled less than control animals (control > *nompC*^{f00642} > *dCirl*^{KO} > *nan*^{36a}; Figure 5A; Table S1). This allowed us to study the genetic interaction of *dCirl* with either *trp* channel subunit. Interestingly, we did not observe simple additivity of crawling distance deficits in *dCirl*^{KO}, *nompC*^{f00642} and *dCirl*^{KO}; *nan*^{36a} double mutants (Figure 5A; Table S1). Instead, *dCirl*^{KO} animals behaved epistatically to *nompC*^{f00642} and *nan*^{36a}, implying that *dCirl* acts upstream of the *trp* subunits. Consistent with this model, overexpressing a *nompC::GFP* fusion transgene under *iav-GAL4* control in *dCirl*^{KO} larvae partially rescued the *dCirl*^{KO} crawling defect (Figure 5A). Intriguingly, our analysis also showed that removing *dCirl* from *nompC*^{f00642} or *nan*^{36a} backgrounds results in inverse outcomes, i.e., decreased and increased crawling distances, respectively (Figure 5A). This suggests that *dCirl* enhances *nompC* activity while curtailing *nan* function. These experiments demonstrate that *dCirl* genetically interacts with essential elements of the mechanotransduction machinery in chordotonal cilia.

The *dCirl* Promoter Contains *cis*-Regulatory Elements Required for Specialization of Mechanosensory Cilia

Genes involved in the functionalization of chordotonal cilia into a mechanosensory structure are controlled through recognition sites for the transcriptional activators RFX and Fd3F (Figures S4A–S4D; Newton et al., 2012).

We analyzed the 2.2-kb intergenic region upstream of the *dCirl* translational start site for the presence of RFX and Fd3F binding sites (Emery et al., 1996; Laurençon et al., 2007). Indeed, in the 200-bp fragment upstream of the *dCirl* start codon, we identified a pair of RFX and Fd3F recognition sites (Figure 5B), which were also conserved in other *Drosophila* species (Figures S4E and S4F).

This promotes the notion that the aGPCR *dCirl* is part of a gene set, which functionalizes the cilium of chordotonal neurons into a mechanosensitive subcellular compartment.

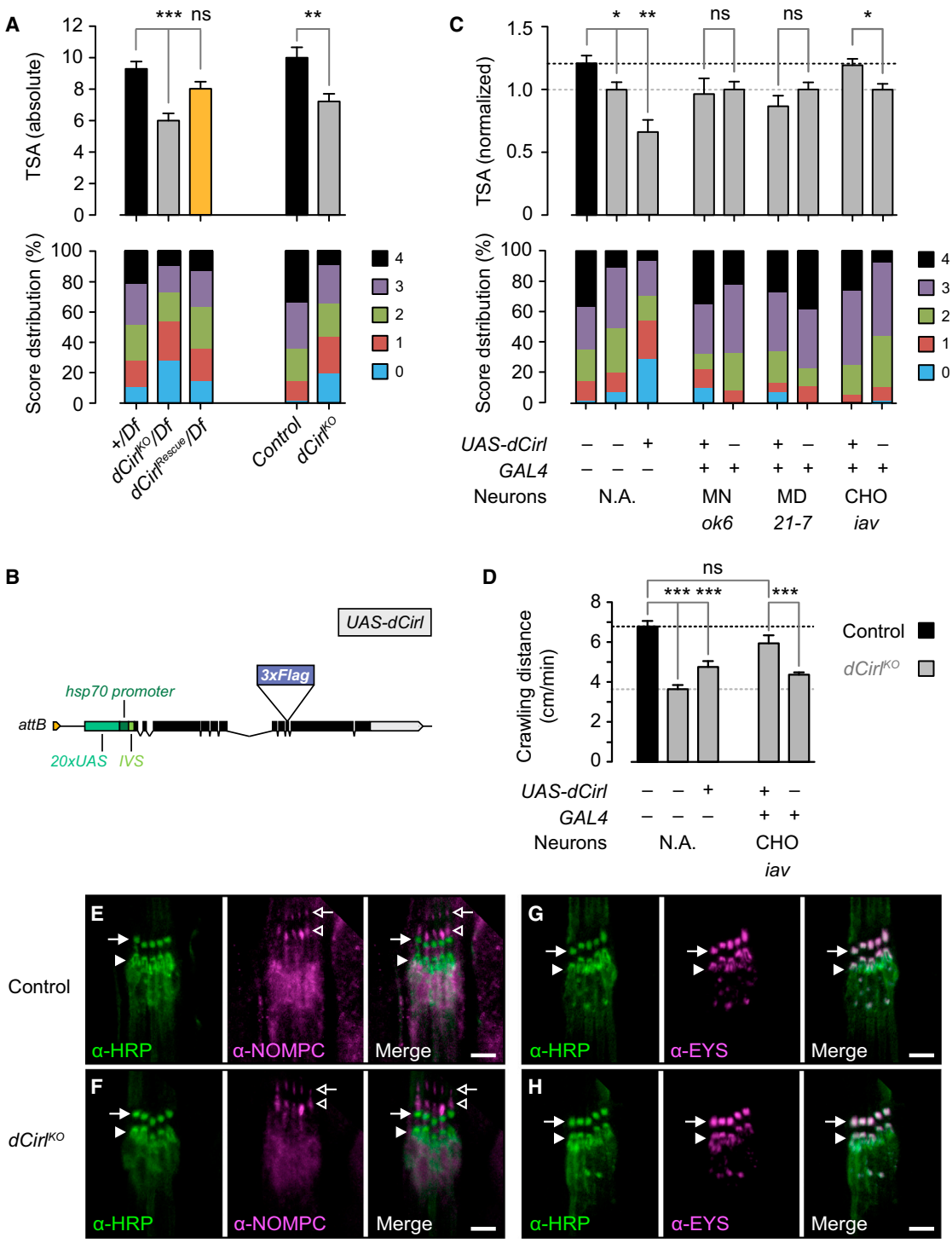

Figure 3. *dCirl* Is Required for Mechanosensation through Chordotonal Organs

(A) Loss of *dCirl* causes reduction in touch sensitivity. The upper shows the averaged results per genotype from 4-fold testing of individual larvae, and the lower contains the score distribution. 0, no response; 1, pause; 2, recoil; 3, retraction and deviation from stimulus <90°; and 4, retraction and deviation from stimulus >90°. See also Table S2.

(B) Structure of *UAS-dCirl* rescuing transgene.

(C) Cell-specific rescue reveals *dCirl* function is specifically required in chordotonal organs (CHO) for full-touch sensitivity, but not in multidendritic (type II; MD) or motor neurons (MNs). Dashed line in black indicates performance of WT, and dashed line in gray indicates performance of $dCirl^{KO}$ animals. See also Figure S2 and Table S2.

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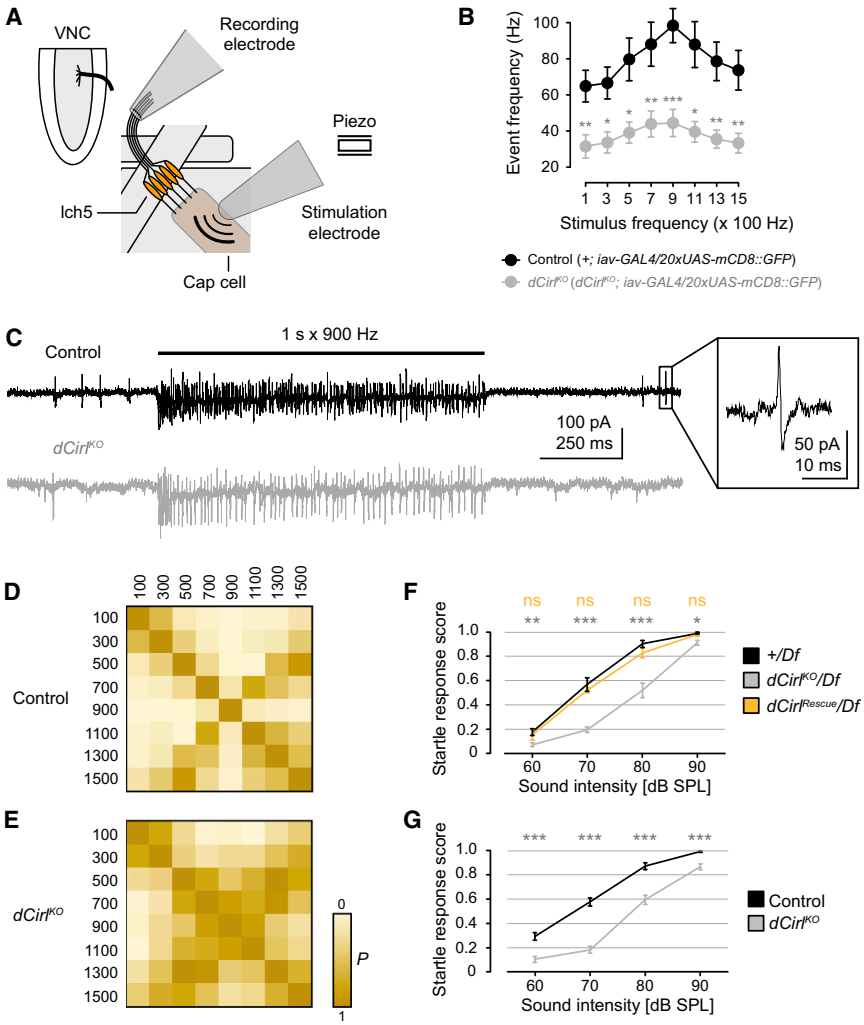

Figure 4. *dCirl* Is Necessary for the Physiological Response to Mechanical Stimulation in Larval Chordotonal Organs

(A) Preparation to probe Ich5 neuron responses to mechanical stimulation.

(B) Quantification of action current frequencies evoked by mechanical stimulation. See also Table S4.

(C) Representative recordings from Ich5 axons of control and $dCirl^{KO}$ animals at 900-Hz stimulation. Boxed region shows a spontaneous event.

(D and E) Statistical comparisons of R_d values (color coded). Adjacent vibration stimuli elicit significantly different relative spiking responses in control Ich5 (D), whereas *dCirl* removal blurs mechanosignal discrimination. See also Figures S3E–S3G.

(F and G) Larval startle responses toward a 900-Hz sine sound of increasing intensity. Hemizygous (F) and homozygous (G) $dCirl^{KO}$ animals show a reduced startle response (F); p values (versus +/*Df*) are indicated above each data point colored according to the genotype. See also Table S5.

Data in (B), (F), and (G) are represented as mean ± SEM.

DISCUSSION

Latrophilin/dCirl Modulates Mechanosensation

In the current analysis, we provide multiple lines of evidence to support that Latrophilin/*dCirl*, one of only two aGPCRs in the fly, is a critical regulator of mechanosensation through chordotonal neurons in *Drosophila* larvae:

(1) Larval chordotonal organs respond to tactile stimuli arising through gentle touch, mechanical deformation of the larval body wall and musculature during the locomotion cycle, and vibrational cues elicited through sound (Caldwell et al., 2003; Hughes and Thomas, 2007). We determined that registration of all these mechanical qualities is reduced in the absence of *dCirl* based on behavioral assays.

(2) We established that behavioral defects can be rescued by re-expression of *dCirl* in chordotonal neurons, one of several cell types with endogenous *dCirl* expression.

(3) Mechanically stimulated Ich5 neurons lacking *dCirl* responded with action currents at approximately half the control rate across a broad spectrum of stimulation frequencies, providing direct functional evidence for a role of *dCirl* in chordotonal dendrites, the site of mechanotransduction and receptor potential generation, or somata, where action potentials are likely initiated (Kernan, 2007). Further, the ability of chordotonal neurons to generate mechanical responses relative to their background spike activity appears to be modulated by *dCirl.*

(4) Combining $dCirl^{KO}$ with strong hypomorphs of *trp* homologs, ion channels that are directly responsible for the conversion of mechanical stimulation into electrical signals within chordotonal neurons (Cheng et al., 2010; Gong et al., 2004; Kim et al., 2003; Yan et al., 2013; Zhang et al., 2013), implies that *dCirl* operates upstream of them.

(5) The *dCirl* promoter contains a RFX/Fd3F transcription factor signature that implicates *dCirl* in the mechanosensitive specialization of sensory cilia (Newton et al., 2012).

(D) Re-expression of *dCirl* only in chordotonal organs rescues the crawling defect. Data in upper (A), (C), and (D) are represented as mean ± SEM. See also Figure S2 and Table S1.

(E–H) Markers HRP, EYS/SPAM, and NOMPC in WT and $dCirl^{KO}$ larval chordotonal neurons are indistinguishable. NOMPC/TRPN1 (E and F) is located in the distal cilium (open arrows), including the ciliary dilation (open arrowheads). HRP (E–H) and EYS/SPAM (G and H) form a collar around the cilium (closed arrows) beneath the ciliary dilation and mark the inner dendritic segment membrane (closed arrowheads). Scale bars represent 5 μm. See also Figure S2 and Table S3.

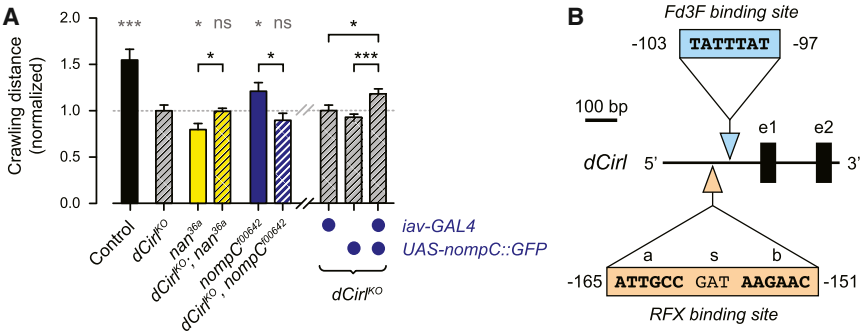

Figure 5. *dCirl* Genetically Interacts with the Mechanotransduction Machinery of Chordotonal Cilia

(A) Normalized score of larval crawling distance for epistasis testing between *dCirl* and *trp* homologs *nompC* and *nan*. Dashed line in gray indicates performance of $dCirl^{KO}$ (left) and $dCirl^{KO}$; *iav-GAL4*/+ animals (right). Data are represented as mean ± SEM. See also Table S1.

(B) Location and sequence of putative RFX-binding (orange arrowhead) and Fd3F (blue arrowhead) binding sites identified in the *dCirl* promoter region. Half-sites a and b of the X box motif recognized by RFX transcription factors (in bold) are separated through a three-nucleotide spacer (s). e1, exon 1; e2, exon 2. See also Figure S4.

On the basis of these results, we propose that *dCirl* partakes in the process of mechanotransduction or spike initiation and transmission to promote sensory encoding.

Adhesion GPCRs: A Class of Metabotropic Mechanosensors

The classical model of GPCR activation has become the archetypical example for cellular perception of external signals. It comprises soluble ligands that bind to the extracellular portions of a cognate receptor, whereby receptor conformation is stabilized in a state that stimulates metabotropic effectors. Thus, GPCRs are primarily regarded as chemosensors due to the nature of their activating agents. The concept that GPCRs may also be fit to detect and transduce physical modalities, i.e., mechanical stimulation, has received little support thus far.

aGPCRs display an exceptional property among the GPCR superfamily in that they recognize cellular or matricellular ligands (Hamann et al., 1996). To date, only one ligand has proved adequate to induce intracellular signaling (Paavola et al., 2014), whereas for the vast majority of ligand-aGPCR interactions this proof either failed or is lacking (Langenhan et al., 2013). This implies that sole ligand recognition is generally not sufficient to induce a metabotropic response of aGPCRs. Thus, in addition to ligand engagement, our results suggest that mechanical load is a co-requirement to trigger the activity of dCIRL, a prototypical aGPCR homolog.

Recent findings place aGPCRs in the context of mechanically governed cellular functions (Yang et al., 2013), but how mechanical perception through aGPCR activity impinges on cell responses has not yet been established. In addition, the molecular structure of aGPCR is marked by the presence of a GPCR autoproteolysis inducing (GAIN) domain (Araç et al., 2012), which plays a paramount role in signaling scenarios for aGPCRs (Prömel et al., 2013). This domain type is also present in PKD-1/Polycystin-1-like proteins, which are required to sense osmotic stress and fluid flow in different cell types and are thus considered bona fide mechanosensors (Retailleau and Duprat, 2014). In addition, studies on EGF-TM7-, BAI-, and GPR56-type aGPCRs further showed that proteolytic processing and loss of NTF may figure prominently in activation of the receptors' metabotropic signaling output (Okajima et al., 2010; Paavola et al., 2011; Yang et al., 2011) and that mechanical forces exerted through receptor-ligand contact are required for receptor internalization (Karpus et al., 2013).

dCirl is not the only aGPCR associated with mechanosensation. *Celsr1* is required during planar cell polarity establishment of neurons of the inner ear sensory epithelium (Curtin et al., 2003). Similarly, the very large G-protein-coupled receptor 1 (VLGR1) exerts an ill-defined developmental role in cochlear inner and outer hair cells, where the receptor connects the ankle regions of neighboring stereocilia (McGee et al., 2006). In addition, VLGR1 forms fibrous links between ciliary and apical inner segment membranes in photoreceptors (Maerker et al., 2008). Both cell types are affected in a type of Usher syndrome, a congenital combination of deafness and progressive retinitis pigmentosa in humans, which is caused by loss of *VLGR1* function (Weston et al., 2004). Although present evidence derived from studies of constitutively inactive alleles suggests a requirement for *Celsr1* and *VLGR1* aGPCR for sensory neuron development, their putative physiological roles after completion of tissue differentiation have remained unclear and should be of great interest.

Outlook

In the current model on *dCirl* function, aGPCR activity, adjusted by mechanical challenge, modulates the molecular machinery gating mechanotransduction currents or the subsequent initiation of action potentials and ensures that mechanical signals are encoded distinctly from the background activity of the sensory organ. Thereby, *dCirl* shapes amplitude and kinetics of the sensory neuronal response. Linking adequate physiological receptor stimulation to downstream pathways and cell function is an essential next step to grasp the significance of aGPCR function and the consequences of their malfunction in human conditions. The versatility of the *dCirl* model now provides an unprecedented opportunity to study the mechanosensory properties of an exemplary aGPCR and to uncover features that might prove of general relevance for the function and regulation of the entire aGPCR class.

EXPERIMENTAL PROCEDURES

Immunohistochemistry and Imaging of Chordotonal Neurons

Wandering third instar larvae were dissected in ice-cold Ca^{2+}-free HL-3 (Stewart et al., 1994), fixed in 4.0% paraformaldehyde for 10 min, blocked for 30 min

in PBT (PBS with 0.1 % Triton X-100; SigmaAldrich) containing 5% normal goat serum, and immunostained according to established protocols (Schmid and Sigrist, 2008). Preparations were incubated with the primary antibody at 4°C overnight and washed and incubated with secondary antisera for 2 hr at room temperature. Each incubation step was followed by two short washes and 3 × 20 min washes in PBT (0.05 % Triton X-100). The blocking step was carried out over night at 4°C using 1% PBT (1% Triton X-100) containing 2% BSA and 5% normal goat serum (NGS). Primary antibodies were added to fresh blocking solution and incubated for 24 hr at 4°C. Next, samples were washed four times for 30 min with PBS containing 0.1 % Tween. Secondary antibodies were diluted in PBS (0.1% Tween, 2% BSA, 5% NGS) and used for incubation overnight at 4°C. Samples were washed four times for 30 min with PBS containing 0.1% Tween and stored in Vectashield over night before mounting.

In each experiment, different genotypes were stained under the same conditions. Antibody dilutions used in the study are detailed in the Supplemental Experimental Procedures. Confocal image stacks were obtained with a line-scanning confocal LSM 5 system (Zeiss) equipped with a 1.25 numerical aperture 63× oil-immersion objective.

Chordotonal Neuron Recordings

Male third instar larval preparations were immersed in extracellular saline (Zhang et al., 2013), and the lch5 was exposed by gentle removal of overlying muscles. The axon bundle was cut with fine scissors and sucked into a recording electrode to measure action currents. Mechanical stimuli of increasing frequency were delivered using a piezo-coupled, fire-polished glass electrode placed at the lch5 cap cells.

In all electrophysiological recordings, genotypes were blinded. Further details of patch clamp and chordotonal neuron recordings are provided in the Supplemental Experimental Procedures.

Behavioral Assays

Larval Crawling Paradigm

The locomotion paths for each genotype were video recorded for 0.5 to 2 min using a digital camera. Briefly, wandering third instar larvae were positioned in an agarose (1%)-filled petri dish. Subsequently, movies were used to track the crawling path of single larvae. Digital measurements of traveled distances were obtained using the wrMTrck plugin (J.S. Pedersen, http://www.phage.dk/plugins/wrmtrck.html) for ImageJ (NIH).

Touch Sensitivity Paradigm

External touch sensitivity was tested on single third-instar larvae raised at 25°C. During linear locomotion in a petri dish (5 cm in diameter), larvae were gently touched with the tip of a von Frey filament (0.3 mN) on their anterior thoracic segments (Kernan et al., 1994), and a scoring system by (Caldwell et al., 2003) was applied and is described in more detail in the Supplemental Experimental Procedures. Genotypes were blinded before scoring.

Startle Response Paradigm

Larvae of different genotypes were challenged with a series of 10 tones of 900 Hz; their startle reaction was video recorded and a startle response score obtained according to Zhang et al. (2013). Videos were shuffled and genotype blinded before scoring. For details, see the Supplemental Experimental Procedures.

Statistical Analysis

Two-tailed Mann-Whitney tests of data sets against a respective WT background control were performed using Prism 5 (GraphPad Software) or SigmaPlot (Systat Software). In the figures, asterisks denote the level of significance: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Data are presented as mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, five tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.04.008.

AUTHOR CONTRIBUTIONS

N.S. analyzed proprioception, hearing, lch5 morphology, and genetic interactions of *dCirl* mutants. J.G. assessed touch sensation and lch5 structure. C.G. and D.L. performed electrophysiological recordings. R.F. constructed the *dCirl* null allele. V.L. built devices for behavioral scoring. R.J.K. and T.L. conceived the project, designed experiments, analyzed data, and coordinated the study. T.L. wrote the manuscript with assistance from all co-authors.

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