Research Article

Myosin-driven Nucleation of Actin Filaments Drives Stereocilia Development Critical for Hearing

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SUMMARY

The assembly and maintenance of actin-based mechanosensitive stereocilia in the cochlea is critical for lifelong hearing. Myosin-15 (MYO15) is hypothesized to modulate stereocilia height by trafficking actin regulatory proteins to their tip compartments, where actin polymerization must be precisely controlled during development. We identified a mutation (p.D1647G) in the MYO15 motor-domain that initially maintained trafficking, but caused progressive hearing loss by stunting stereocilia growth, revealing an additional function for MYO15. Consistent with its maintenance of tip trafficking *in vivo*, purified p.D1647G MYO15 modestly reduced actin-stimulated ATPase activity *in vitro*. Using ensemble and single-filament fluorescence *in vitro* assays, we demonstrated that wild-type MYO15 directly accelerated actin filament polymerization by driving nucleation, whilst p.D1647G MYO15 blocked this activity. Collectively, our studies suggest direct actin nucleation by MYO15 at the stereocilia tip is necessary for elongation *in vivo*, and that this is a primary mechanism disrupted in DFNB3 hereditary human hearing loss.

INTRODUCTION

Cochlear hair cells are the primary transducers of sound in the mammalian inner ear and are fundamental for hearing. Each hair cell assembles approximately 100 individual stereocilia on its apical surface to form a mechano-sensitive hair bundle. Within each bundle, stereocilia are precisely graded into ranks of ascending height and this staircase architecture is critical for mechano-electric transduction (MET) (Barr-Gillespie, 2015). The regulation of stereocilia size is thus central for hearing and the disruption of hair bundle architecture is a common theme in hereditary deafness (Richardson et al., 2011). Stereocilia develop from microvilli by building a highly cross-linked para-crystalline core of actin filaments as an internal scaffold to confer shape and structural rigidity (Vélez-Ortega and Frolenkov, 2019). As stereocilia develop, the actin core thickens and elongates to reach its mature size, necessitating precise control of actin filament polymerization (Tilney et al., 1992; Kaltenbach et al., 1994; Krey et al., 2020). Actin filaments are uniformly polarized within stereocilia with rapidly growing barbed ends orientated towards the tip, the major site of actin polymerization and filament elongation, and also the site of MET (Flock and Cheung, 1977; Tilney et al., 1980; Beurg et al., 2009). Once stereocilia are fully assembled, actin polymerization continues primarily at the tip compartment, indicating an ongoing plasticity throughout adult life (Schneider et al., 2002; Zhang et al., 2012; Drummond et al., 2015; Narayanan et al., 2015). A number of proteins have been identified within the tip compartment that are essential for stereocilia growth, yet the molecular mechanisms governing actin polymerization are unknown. Identification of this mechanism is central to the acquisition and maintenance of hair cell mechano-sensitivity, and also for understanding hearing loss as the clinical manifestation of a cytoskeletal disease.

A key molecule in establishing stereocilia architecture is unconventional myosin 15 (MYO15), encoded by the gene *Myo15* in mice and *MYO15A* in humans. Mutations in *MYO15A* (MIM #602666) cause DFNB3 recessive hereditary hearing loss in humans (Friedman et al., 1995; Wang et al., 1998; Rehman et al., 2016). MYO15 is a member of the myosin superfamily of P-loop ATPases that generate contractile force on actin filaments to power cellular processes such as cytokinesis, endocytosis and vesicular trafficking (Hartman and Spudich, 2012; Houdusse and Sweeney, 2016). Two MYO15 isoforms are produced in auditory hair cells through alternative mRNA splicing (Liang et al., 1999; Anderson et al., 2000; Fang et al., 2015). A shorter isoform (MYO15-2, also known as MYO15-S) consists of the ATPase ‘motor domain’ and three light chain binding sites that associate with calmodulin-like proteins, in addition to myosin tail homology 4 (MyTH4), Src homology 3 (SH3), and protein 4.1, ezrin, radixin, moesin (FERM) domains (Fig. 1A). A larger isoform (MYO15-1, also known as MYO15-L) is identical to MYO15-2, except for the addition of a 133 kDa N-terminal domain

79 (Fig. 1A). Both MYO15 isoforms localize to the stereocilia tip compartment where actin polymerization
80 is concentrated (Schneider et al., 2002; Belyantseva et al., 2003; Drummond et al., 2015; Fang et al.,
81 2015; Narayanan et al., 2015). The prototypical *Myo15* mutant allele, the *shaker 2* (*Myo15*^{sh2}),
82 prevents both isoforms from accumulating in stereocilia, blocking developmental elongation and
83 causing profound hearing loss from birth (Probst et al., 1998; Anderson et al., 2000; Belyantseva et
84 al., 2003; Fang et al., 2015). The use of an isoform-specific *Myo15* null allele revealed additional
85 functions, with MYO15-2 being necessary for stereocilia developmental elongation, whilst MYO15-1
86 independently maintains the postnatal size of shorter stereocilia (Fang et al., 2015) that are
87 structurally plastic in response to MET (Vélez-Ortega et al., 2017; Krey et al., 2020).
88
89 The MYO15-2 isoform associates with additional proteins essential for stereocilia elongation,
90 and by inference, actin polymerization. The 'elongation complex' consists of epidermal growth factor
91 receptor pathway substrate 8 (EPS8), whirlin (WHRN), G-protein signalling modulator 2 (GPSM2) and
92 G-protein subunit alpha$_{i3}$ (GNAI3). Similar to MYO15-2, these proteins are concentrated at the tips of
93 the tallest stereocilia (row 1) (Mburu et al., 2003; Belyantseva et al., 2005; Delprat et al., 2005; Manor
94 et al., 2011; Zampini et al., 2011; Tarchini et al., 2016; Mauriac et al., 2017; Tadenev et al., 2019).
95 Individual knock-out mouse mutants of *Eps8*, *Whrn*, *Gpsm2* or *Gnai3* recapitulate the *shaker 2*
96 phenotype, exhibit short stereocilia and are profoundly deaf (Mburu et al., 2003; Manor et al., 2011;
97 Zampini et al., 2011; Tarchini et al., 2016; Mauriac et al., 2017; Tadenev et al., 2019). There is strong
98 evidence that a key function of MYO15-2 is to traffic the elongation complex and concentrate it at the
99 stereocilia tips. First, elongation complex proteins are absent from the stereocilia of *Myo15 shaker 2*
100 hair cells, demonstrating their functional dependence upon MYO15-2 *in vivo* (Belyantseva et al., 2005;
101 Delprat et al., 2005; Manor et al., 2011; Mauriac et al., 2017; Tadenev et al., 2019). Second, EGFP-
102 tagged MYO15-2 actively traffics elongation complex proteins along filopodia in cell lines (Belyantseva
103 et al., 2005; Manor et al., 2011; Mauriac et al., 2017). Finally, enzymatic studies of the purified MYO15
104 ATPase domain reveal kinetic adaptations that enable long-range processive molecular trafficking
105 (Bird et al., 2014; Jiang et al., 2021). Together, these data support MYO15-2 delivering the elongation
106 complex to the stereocilia tips, where the elongation complex is hypothesized to regulate actin
107 polymerization. Despite its central role in promoting stereocilia growth, the specific molecular activity
108 of the elongation complex remains unknown.
109
110 Here, we describe a novel *Myo15* mutant mouse ('*jordan*') that exhibits progressive hearing
111 loss resulting from a missense substitution in the MYO15 motor domain. In striking contrast with
112 *shaker 2* hair cells, MYO15 and the elongation complex proteins are still delivered to the stereocilia tip

113 compartment in *jordan* mutant hair cells, but stereocilia fail to elongate properly. These results
114 questioned the sufficiency of the elongation complex to drive stereocilia growth, and prompted us to
115 search for another role of MYO15 independent of delivering the elongation complex. We found that
116 purified MYO15 motor domain protein directly stimulated actin polymerization *in vitro*, and that the
117 *jordan* mutation blocked this activity, whilst only moderately affecting its ability to bind and move along
118 actin filaments. A companion structural study shows that the *jordan* mutation targets the actomyosin
119 binding interface and interferes with the ability of wild-type MYO15 to regulate structural plasticity
120 within the actin molecule itself (Gong *et al.,* bioRXiv). Combined, our results argue that MYO15
121 controls stereocilia elongation by directly regulating F-actin conformation in stereocilia, and that this
122 critical activity is independent of the elongation complex. More broadly, our work suggests that in
123 addition to their classical roles generating force and motility, myosin motors have a physiological role
124 regulating actin polymerization *in vivo*.
125
126 **RESULTS**
127 **A forward genetic screen identifies *jordan,* a *Myo15* allele causing progressive hearing loss**
128 During a recent phenotype-driven ENU-mutagenesis screen (Potter et al., 2016), the MPC190
129 cohort (comprising 83 mice) was identified with 10 mice exhibiting severe hearing loss at 3-months of
130 age (Fig. 1B). A genome scan and single nucleotide polymorphism (SNP) mapping of third generation
131 (G3) deaf mice found linkage to a 16.7 Mb region on Chromosome 11 (Fig. S1A). Whole-genome
132 sequencing of a single deaf mouse identified a high confidence homozygous mutation within the
133 critical interval, consisting of an A-to-G transition at coding nucleotide 4940 of the *Myo15* gene
134 (ENSMUST00000071880). This variant was confirmed by Sanger sequencing (Fig. S1B) and leads to
135 the substitution of an evolutionarily conserved aspartate residue with a glycine (p.D1647G) in the
136 encoded MYO15 protein (Fig. 1C). We named this mutant allele *jordan* ($Myo15^{jd}$).
137
138 To confirm that the $Myo15^{jd}$ substitution causes hearing loss, we performed a genetic
139 complementation test utilizing the *shaker-2* ($Myo15^{sh2}$) deafness allele *in trans* (Probst et al., 1998;
140 Stepanyan et al., 2006). We recorded ABRs from postnatal day 28 (P28) mice and found that
141 compound heterozygous $Myo15^{jd/sh2}$ mice had elevated thresholds of >90 decibel sound pressure level
142 (dB SPL) at all frequencies (Fig. 1D). In contrast, $Myo15^{jd/+}$ and $Myo15^{sh2/+}$ littermates had normal
143 thresholds (< 40 dB SPL) (Fig. 1D). Failure of complementation in $Myo15^{jd/sh2}$ mice confirms that the
144 p.D1647G mutation in *Myo15* is the cause of recessive deafness in the *jordan* pedigree.
145

146 All reported mutant *Myo15* mouse alleles cause profound deafness (MGI:1261811), measured
147 from P14 onwards (Probst et al., 1998; Anderson et al., 2000; Stepanyan et al., 2006; Fang et al.,
148 2015). ABR showed that several *Myo15*$^{jd/jd}$ mice had residual hearing at 3 months (Fig. 1B, red
149 circles), suggesting a distinct mechanism of hearing loss. We investigated this using longitudinal ABR
150 measurements. At 4 weeks of age, *Myo15*$^{jd/jd}$ mice had moderate hearing loss with broadband click
151 ABR threshold of 53 ± 11 dB SPL, compared to normal hearing *Myo15*$^{+/+}$ (26 ± 6 dB SPL) and
152 *Myo15*$^{+/jd}$ (24 ± 6 dB SPL) littermates (Fig. 1E). The hearing of *Myo15*$^{jd/jd}$ mice progressively worsened,
153 with click ABR thresholds of 53 ± 11, 69 ± 9, 80 ± 9 and 81 ± 8 dB SPL at 4, 6, 9 and 12 weeks,
154 respectively (Fig. 1E,F, Fig. S1C-F). Click ABR thresholds for control *Myo15*$^{+/+}$ and *Myo15*$^{jd/+}$
155 littermates ranged between 22 ± 4 and 26 ± 6 dB SPL; showing they did not develop hearing loss with
156 age (Fig. 1E,F, Fig. S1C-F). To investigate outer hair cell (OHC) function we measured distortion-
157 product otoacoustic emissions (DPOAEs), and found they were absent in *Myo15*$^{jd/jd}$ mice at 12 weeks,
158 except for frequencies < 10 kHz, where they were significantly reduced compared to *Myo15*$^{+/+}$ and
159 *Myo15*$^{+/jd}$ littermates (Fig. S1G). The absence of DPOAEs shows that OHC function is impaired in
160 *Myo15*$^{jd/jd}$ mice. As the only known mouse *Myo15* variant to cause progressive hearing loss, the *jordan*
161 allele is an important new model to explore the full spectrum of DFNB3 deafness, which presents
162 heterogeneously as either profound congenital, or progressive hearing loss in humans (Rehman et al.,
163 2016).
164
165 **Stereocilia do not properly elongate in *jordan* mutant hair cells**
166 To investigate the cellular pathology underlying hearing loss in *Myo15*$^{jd/jd}$ mice, we used
167 scanning electron microscopy (SEM) to assess the structure of cochlear hair bundles. In wild-type
168 *Myo15*$^{+/+}$ IHCs and OHCs at P8, stereocilia were graded into a staircase pattern with three distinct
169 rows (Fig. 2A,B). By comparison, bundle heights were reduced in *Myo15*$^{jd/jd}$ littermates, whilst still
170 retaining the staircase architecture (Fig. 2C,D). Quantification of row 1 stereocilia heights showed that
171 *Myo15*$^{jd/jd}$ IHCs and OHCs were significantly shorter than *Myo15*$^{+/+}$ controls (Fig. 2G,H). Furthermore,
172 stereocilia at the lateral edge of *Myo15*$^{jd/jd}$ OHCs, and to lesser extent IHCs, were shorter in height
173 such that the central stereocilia were tallest (Fig. 2C,D). *Myo15*$^{jd/jd}$ hair cells consistently had 1 or 2
174 additional rows of stereocilia (Fig. 2C,D) when compared to the three well-defined rows in *Myo15*$^{+/+}$
175 hair cells (Fig. 2A,B). The additional row phenotype of *Myo15*$^{jd/jd}$ hair cells was similar to *Myo15*$^{sh2/sh2}$
176 hair cells at P8 (Fig. 2E,F), however *Myo15*$^{jd/jd}$ stereocilia lengths were significantly longer when
177 compared to *Myo15*$^{sh2/sh2}$ hair cells (Fig. 2G,H). We conclude that stereocilia in *Myo15*$^{jd/jd}$ hair cells
178 elongate more than *Myo15*$^{sh2/sh2}$ hair cells, but fail to reach normal wild-type heights. The *Myo15*

179 *shaker 2* and *jordan* allelic series shows that mutations in the motor domain led to altered stereocilia
180 heights and explain why *jordan* mice initially have less severe hearing loss than *shaker-2* mice.
181
182 To further investigate the progressive component of hearing loss, we next examined hair
183 bundle morphology at 12 weeks, when *jordan* mice were profoundly deaf (Fig. 1F). Compared with
184 *Myo15*$^{+/+}$, IHC bundles of *Myo15*$^{jd/jd}$ mice had lost their staircase architecture (Fig. S2A) and were still
185 significantly shorter (Fig. S2B). Since the staircase was initially present at P8 in *Myo15*$^{jd/jd}$ IHCs (Fig.
186 2D), these data suggested a postnatal resorption of stereocilia. Consistent with this hypothesis,
187 extremely short stereocilia at the hair bundle periphery were observed at 12 weeks in *Myo15*$^{jd/jd}$ OHCs
188 (Fig. S2C,D). These data argue that the progressive hearing phenotype in the *jordan* mouse was due
189 to postnatal stereocilia bundle degradation. Overall, we conclude that the *jordan* allele causes hearing
190 loss by interfering with both initial elongation of stereocilia, and their postnatal maintenance. This was
191 consistent with the p.D1647G mutation targeting the motor domain of both MYO15 isoforms (Fang et
192 al., 2015).
193
194 **MYO15 traffics the elongation complex in *jordan* hair cells**
195 Hair bundle development requires MYO15 dependent trafficking of EPS8, WHRN, GNAI3 and
196 GPSM2 (the 'elongation complex'), and mutations that prevent MYO15 trafficking (*i.e. shaker-2*) cause
197 a short hair bundle phenotype (Belyantseva et al., 2005; Manor et al., 2011; Mauriac et al., 2017;
198 Tadenev et al., 2019). Our finding that stereocilia lengths in the *jordan* mouse were only marginally
199 longer than the *shaker-2*, led us to hypothesize that MYO15 trafficking was defective in *jordan* hair
200 cells. We used the previously validated pan-MYO15 antibody PB48 (Fig. 1A), that binds an epitope
201 common to all isoforms (Liang et al., 1999; Fang et al., 2015), to detect MYO15 in fixed cochleae from
202 *Myo15*$^{jd/jd}$ mutants and *Myo15*$^{+/jd}$ littermates at P14. As expected, in *Myo15*$^{+/jd}$ mice, PB48 labelling
203 was concentrated at the tips of all stereocilia rows in IHCs (Fig. 3A) (Belyantseva et al., 2003, 2005;
204 Rzadzinska et al., 2004). We confirmed that PB48 did not label the short stereocilia of *Myo15*$^{sh2/sh2}$
205 IHCs at P14 (Fig. 3A), consistent with MYO15 being absent from the bundle (Belyantseva et al.,
206 2003). In stark contrast with the *shaker-2*, we observed PB48 labelling at the tips of IHC stereocilia in
207 *Myo15*$^{jd/jd}$ mice at both P7 and P14 (Fig. 3A,B). These data support the *jordan* and *shaker-2* mutations
208 affecting stereocilia growth by profoundly different mechanisms: the *shaker-2* mutation blocks MYO15
209 trafficking, whilst the *jordan* mutation allows MYO15 to traffic but prevents stereocilia elongation.
210
211 We next investigated if the *jordan* mutation interfered with trafficking of the elongation complex
212 proteins. To test this, mutant *Myo15*$^{jd/jd}$ and control *Myo15*$^{+/jd}$ littermate cochleae at P7 were labelled

213 with validated antibodies to localize WHRN, EPS8, GPSM2 and GNAI3. All of the elongation complex
214 proteins were concentrated at the tips of the tallest stereocilia (row 1) in control *Myo15*$^{+/jd}$ hair cells
215 (Fig. 3C), in agreement with previous work (Mburu et al., 2003; Manor et al., 2011; Zampini et al.,
216 2011; Tarchini et al., 2016; Mauriac et al., 2017). All four proteins in the elongation complex were also
217 targeted to the stereocilia tips in mutant *Myo15*$^{jd/jd}$ hair cells (Fig. 3C), although the signal-to-noise of
218 GPSM2 and GNAI3 was lower, indicating a reduced overall accumulation (Fig. 3C). In older animals at
219 P14, EPS8 and WHRN were still correctly targeted in *Myo15*$^{jd/jd}$ hair cells, however GNAI3 and
220 GPSM2 were no longer concentrated at stereocilia tips (Fig. S3). These data are consistent with
221 GPSM2-GNAI3 operating as a distinct complex with MYO15-EPS8 (Tadenev et al., 2019). We
222 conclude that the initial defective stereocilia elongation in *jordan* hair cells evident at P7 was not due
223 to gross disruption of elongation complex trafficking by MYO15.
224
225 **Actin barbed end capping is unaffected in *jordan* mutant hair cells**
226 The presence of the elongation complex at the stereocilia tips of *jordan* hair cells suggested
227 that the proteins known to stimulate growth were present, but insufficient to drive elongation. We
228 considered whether an overall inhibition of actin polymerization was preventing stereocilia elongation
229 in *jordan* hair cells. Capping proteins (CAPZ, TWF2) are present within stereocilia and regulate
230 filament polymerization by blocking barbed end elongation (Peng et al., 2009; Avenarius et al., 2017).
231 To detect free barbed ends, we monitored the incorporation of TMR-labelled actin monomers in
232 permeabilized hair cells at P6. In control *Myo15*$^{+/jd}$ hair cells, TMR-actin was concentrated at row 2
233 stereocilia tips revealing uncapped barbed ends (Fig. 4A). TMR-actin was not consistently detected at
234 row 1 tips, arguing that barbed ends were inaccessible at that location (Fig. 4A). The *jordan* mutation
235 did not alter this distribution of free barbed ends and we observed a similar incorporation of TMR-actin
236 in *Myo15*$^{jd/jd}$ hair cells compared to controls (Fig. 4A). In striking contrast, TMR-actin was incorporated
237 at the tips of all stereocilia rows in *Myo15*$^{sh2/sh2}$ hair cells, indicating the abnormal presence of barbed
238 ends in row 1, and an overall loss of hair bundle row identity (Fig. 4A). In summary, *jordan* hair cells
239 retain row identity at P6, consistent with MYO15 correctly targeting the elongation complex to the
240 stereocilia tip compartment. These data argue that inappropriate actin filament capping is unlikely the
241 cause of short stereocilia in *jordan* hair cells. Instead, we hypothesized that a stimulatory factor
242 independent of the elongation complex was missing.
243
244 **The *jordan* mutation affects the interaction of MYO15 with actin filaments**
245 The *jordan* missense substitution is in the MYO15 motor domain helix-loop-helix (HLH) motif
246 that forms part of the direct binding interface with the actin filament (Gong *et al.,* bioRXiv). We

247 hypothesized that a defect in MYO15's interaction with actin might underlie the *jordan* phenotype. To
248 explore this, we examined MYO15-2 trafficking along filopodia; actin-based structures that protrude
249 from the periphery of heterologous cells (Belyantseva et al., 2003, 2005). We focused on MYO15-2 as
250 the isoform responsible for stereocilia growth (Fang et al., 2015). In transfected HeLa cells, EGFP-
251 tagged wild-type MYO15-2 accumulated at filopodia tips indicating robust anterograde myosin
252 movement along the filopodia shaft (Fig. 4B). Discrete puncta of MYO15-2 were observed along the
253 filopodia shaft, arising from retrograde actin filament treadmilling (Belyantseva et al., 2005; Kerber et
254 al., 2009; Bird et al., 2017). EGFP alone did not accumulate within filopodia, proving this distribution
255 required active myosin motility (Fig. 4B). In cells expressing the MYO15-2 *jordan* mutant, EGFP was
256 observed diffusely along filopodia shafts and was not concentrated at filopodia tips (Fig. 4B). This was
257 qualitatively similar to cells expressing the MYO15-2 *shaker-2* mutant (Fig. 4B), which was previously
258 shown to not traffic along filopodia (Belyantseva et al., 2005). The inability of the MYO15-2 *jordan*
259 mutant to traffic within filopodia and accumulate at filopodia tips was unexpected and contrary to
260 MYO15 protein accumulating at the stereocilia tips of *Myo15*$^{jd/jd}$ hair cells (Fig. 3A).
261
262 Myosin motors are sensitive to actin filament topology (Nagy et al., 2008; Brawley and Rock,
263 2009; Reymann et al., 2012; Ropars et al., 2016) and we hypothesized that filopodia might not contain
264 the appropriate repertoire of actin-binding proteins (ABPs) to support motility. To test this hypothesis,
265 we used the porcine LLC-PK1-CL4 (CL4) epithelial cell line that generates microvilli and is a more
266 accurate model for stereocilia (Zheng et al., 2010). In CL4 cells transfected with wild-type EGFP-
267 MYO15-2, EGFP positive puncta localized at the tips of microvilli (Fig. 4C, orthogonal projections). In
268 striking contrast to HeLa cells, the MYO15-2 *jordan* mutant also concentrated into microvilli and was
269 indistinguishable from the wild-type in CL4 cells (Fig. 4C). No microvillar accumulation of the MYO15-
270 2 *shaker-2* mutant was observed (Fig. 4C). We conclude that whilst *jordan* and *shaker-2* mutants are
271 both immobile within filopodia, the *jordan* mutant can still actively concentrate in microvilli. These data
272 mimic our findings from hair cells *in vivo*, and support a change in actin binding as being central to the
273 stereocilia growth defect phenotype observed in *jordan* hair cells.
274
275 **The ATPase activity of MYO15 is altered by the *jordan* mutation**
276 To study the interaction between MYO15 and actin filaments in more detail, we characterized
277 the influence of the *jordan* mutation upon ATPase mechanochemistry (Bird et al., 2014; Jiang et al.,
278 2021). Motor domain proteins were expressed in *S. frugiperda* (*Sf*9) insect cells and purified by
279 chromatography (Fig. 5A,C). In contrast to the *shaker-2* variant, which aggregated within *Sf*9 cells,
280 both wild-type and *jordan* motor domains were soluble. Using size exclusion chromatography (SEC),

281 we found that FLAG-purified *shaker-2* motor domain eluted close to the void volume (Fig. 5B). In
282 contrast, the *jordan* motor domain eluted at a similar delay volume to the wild-type (Fig. 5B), which is
283 monomeric (Bird et al., 2014). We conclude that the *jordan* variant does not affect folding, whilst the
284 *shaker-2* caused misfolding, consistent with the mutation being within the ATPase transducer core
285 (Gong *et al.,* bioRXiv).
286
287 To test for differences in enzymatic activity between wild-type and *jordan* motor domain
288 variants (Fig. 5A), we measured steady-state ATP hydrolysis using an enzyme-linked NADH assay.
289 The basal ATPase activity (k_{basal}) of the wild-type motor domain was 0.06 ± 0.01 s^{-1}, measured without
290 F-actin. The addition of F-actin caused a 97-fold increase in ATPase activity to an extrapolated
291 maximum catalytic rate (k_{cat}) of 5.8 ± 0.2 s^{-1} (Fig. 5D). Half-maximal activation of the ATPase activity
292 (K_{ATPase}) was reached at 29.1 ± 2.1 μM F-actin, as previously reported (Bird et al., 2014; Jiang et al.,
293 2021). Using identical assay conditions, the basal ATPase rate of the *jordan* variant was unchanged at
294 k_{basal} = 0.07 ± 0.01 s^{-1}. Strikingly, there was a reduced 7-fold maximal activation to k_{cat} = 0.87 ± 0.04 s^{-1}
295 (Fig. 5D). Furthermore, half-maximal ATPase activation was increased to K_{ATPase} = 114.3 ± 8.2 μM
296 actin, signifying a 4-fold reduction in the apparent actin affinity compared to wild-type.
297
298 The mechanical activity of wild-type and *jordan* motor domains was measured using an *in vitro*
299 gliding filament assay, where actin filaments are propelled across a microscope cover glass
300 functionalized with motor domain protein (Sellers et al., 1993). Due to the reduced apparent affinity of
301 the *jordan* motor domain for actin, we lowered the salt concentration to 10 mM KCl in these assays.
302 The wild-type motor domain robustly propelled actin filaments at 473 ± 67 nm·s^{-1} (Fig. 5E), consistent
303 with previous data (Bird et al., 2014). In contrast, the *jordan* mutant moved filaments at 216 ± 71 nm·s^{-}
304 ^{1}, a 2-fold reduction from the wild-type velocity (Fig. 5E). Overall, we conclude that whilst the *jordan*
305 mutation caused a significant ATPase defect and decreased the motor domain's apparent affinity for
306 actin, the motor domain was still mechanically active. Our data further establish a functional
307 correlation between motor domain activity and the severity of hearing loss in a *Myo15* allelic series.
308 The *jordan* motor domain retained partial activity consistent with this mutation causing intermediate
309 hearing loss, whilst the *shaker-2* motor domain was misfolded and associated with the most severe
310 phenotype.
311
312 **The MYO15 motor domain directly stimulates actin polymerization**
313 Our *in vitro* analysis of purified MYO15 motor activities were consistent with the *jordan* mutant
314 maintaining the ability to traffic the elongation complex and accumulate at stereocilia tips. As

315 stereocilia nevertheless fail to grow normally in *jordan* hair cells, we hypothesized that independent of
316 molecular trafficking, MYO15 had an additional function at the stereocilia tip critical for elongation.
317 Classic biochemical studies of muscle myosin have shown its motor domain (*e.g.* subfragment-1, S1)
318 can directly stimulate actin polymerization *in vitro*; however, the physiological relevance of this activity
319 *in vivo*, if any, is unknown (Yagi et al., 1965; Miller et al., 1988; Fievez and Carlier, 1993; Lheureux et
320 al., 1993). We hypothesized that the MYO15 motor domain could directly stimulate actin
321 polymerization to drive stereocilia elongation, and that this critical activity is disrupted by the *jordan*
322 deafness mutation.
323
324 We tested this hypothesis using pyrene-conjugated globular actin (G-actin) monomers that
325 increase in fluorescence as they polymerize into filamentous actin (F-actin) (Cooper et al., 1983). As a
326 control, 2 μM G-actin was polymerized with 1x KMEI (50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM
327 imidazole) while monitoring pyrene fluorescence. F-actin assembled with an initial lag phase
328 representing the kinetically unfavourable nucleation step (Fig. 6A, red line). When the reaction was
329 repeated with 1 μM wild-type motor domain + 2 μM G-actin + KMEI, actin polymerization initially
330 followed the same trajectory (Fig. 6A, blue line). However, following a delay of ~ 400 seconds, an
331 inflection was observed where the rate of actin polymerization reduced momentarily (Fig. 6A, blue
332 line), before rapidly increasing and reaching steady-state (Fig. 6A,B). We tested if MYO15-stimulated
333 actin polymerization was sensitive to the *jordan* mutation. Under identical experimental conditions
334 using 1 μM *jordan* MYO15 motor domain, the actin polymerization rate was initially indistinguishable
335 from the control reaction. However, following an inflection at ~ 600 seconds, the actin polymerization
336 rate decreased below that of the G-actin alone control (Fig. 6A). We conclude that the wild-type
337 MYO15 motor domain stimulated actin polymerization, whilst the deafness-causing *jordan* mutant
338 blocked this activity.
339
340 With either wild-type or *jordan* motor domains, we observed a delay before the actin
341 polymerization rate deviated from the control, with the delay longer for *jordan* (~ 600s) versus wild-
342 type (~ 400s). We hypothesized this difference arose from residual ATP (70 μM) being introduced
343 from the G-actin storage buffer; ATP binding would prevent the motor domain from occupying the
344 strongly actin bound (rigor) state. Furthermore, we expected this residual ATP to be hydrolysed faster
345 by the wild-type motor domain, compared with the *jordan* mutant (Fig. 5D). To test this, we repeated
346 the pyrene polymerization assay in the absence of free ATP. To ensure G-actin monomers were ATP-
347 bound, G-actin was desalted immediately prior to use to remove free ATP from solution. Using this
348 approach, we measured the polymerization of 2 μM G-actin + KMEI in the absence of free ATP (Fig.

349 6C) and observed kinetics comparable to before (Fig. 6A). Strikingly, pyrene fluorescence immediately
350 increased upon addition of 1 μM wild-type motor to the reaction, and rapidly saturated (Fig. 6C) with a
351 significantly reduced half-time of 124 ± 12 s, compared with 1091 ± 202 s for G-actin + KMEI alone
352 (Fig. 6D). While addition of 1 μM *jordan* motor domain to 2 μM G-actin + KMEI reduced the overall
353 extent of actin polymerization (Fig. 6C), the half-time to saturation was 1000 ± 65 s and not
354 significantly different to G-actin + KMEI alone (Fig. 6D). We conclude that the wild-type MYO15 motor
355 domain strongly accelerated actin filament polymerization in the absence of ATP, implicating the rigor
356 state as critical for this activity. Combined with experiments from our companion study showing that
357 ADP-bound MYO15 also stimulated polymerization (Gong *et al.,* bioRXiv), these data identify the
358 strong actin-binding states as the key MYO15 ATPase intermediates that stimulate actin
359 polymerization.
360
361 **The *jordan* deafness mutation inhibits *de novo* nucleation of actin filaments**
362 To directly visualize how the MYO15 motor domain accelerates polymerization, we performed
363 actin polymerization assays using total internal reflection fluorescence microscopy (TIRFM) where the
364 elongation of individual filaments can be tracked (Fujiwara et al., 2002). A control time-lapse of 1 μM
365 G-actin + KMEI revealed a slow rate of filament nucleation, with short polymers attaching to surface
366 and elongating (Fig. 7A, top row). We repeated the experiment with 1 μM wild-type motor domain and
367 observed a large burst in filament density (Fig. 7A, middle row) that was statistically significant at 480
368 seconds when compared to the actin-alone control (Fig. 7B). In contrast, when polymerization
369 experiments were repeated with 1 μM *jordan* motor domain (Fig. 7A, bottom row), there was no
370 increase in actin filament density above the actin-alone control (Fig. 7C). Using kymographs to track
371 filament barbed ends, we found that the motor domain could influence actin filament elongation rates.
372 In control experiments, barbed ends elongated at 16.2 ± 2.6 $nm \cdot s^{-1}$ (Fig. 7C,D), consistent with
373 previous data using TMR-conjugated G-actin on Cys374 (Kuhn and Pollard, 2005). Elongation rates
374 were significantly reduced by the addition of either wild-type or *jordan* motor domain, to 13.3 ± 4.3
375 $nm \cdot s^{-1}$ and 13.4 ± 3.5 $nm \cdot s^{-1}$, respectively (Fig. 7C,D). The decrease in elongation rate was dependent
376 upon timing of the filament burst (Fig. 7B). When data were binned as pre-burst (< 320 s), the addition
377 of wild-type or *jordan* motor domains did not significantly alter elongation rates compared to the
378 control (Fig. 7E). In contrast, the presence of either wild-type or *jordan* motor domain post-burst (> 720
379 s) both significantly, and equally, reduced elongation rates below the actin alone control (Fig. 7F).
380
381 Nucleation by wild-type MYO15 took an average of 480 seconds to be significantly increased
382 above the spontaneous nucleation rate of G-actin + KMEI alone (Fig. 7B). We considered whether the

383 delayed production of short filaments was caused by MYO15 severing actin filaments that nucleated
384 spontaneously. To test this hypothesis, we repeated our experiments in the absence of free ATP to
385 force the motor domain into rigor binding and accelerate polymerization. Free ATP was desalted from
386 1 μM G-actin monomers; this did not affect barbed-end elongated rates when polymerized by KMEI
387 (Fig. 7D,I). As expected, when 1 μM wild-type motor domain was included in the reaction, there was a
388 potent nucleation of short actin filaments observed within 90 s (Fig. 7G,H), confirming acceleration of
389 nucleation when the motor domain was forced into rigor. Under identical ATP free conditions, 1 μM
390 *jordan* motor domain did not nucleate additional filaments compared with the G-actin + KMEI control
391 (Fig. 7G,H). Similar to experiments where ATP was present, but presumably hydrolyzed (Fig. 7F, >
392 720 s), we saw that both wild-type and *jordan* motor domains significantly reduced barbed-end
393 elongation rates (Fig. 7I). We conclude that the MYO15 motor domain exerts multiple direct effects on
394 actin polymerization, including catalyzing *de novo* filament nucleation and reducing barbed end
395 elongation rates in a nucleotide-dependent fashion. Our data show that MYO15-induced actin
396 nucleation is a key process targeted by the *jordan* deafness mutation, and argue that this activity is
397 critical for stereocilia elongation and hearing.
398
399 **DISCUSSION**
400 Plasticity of the stereocilia actin core is central to hair bundle development, mechano-
401 sensitivity and hearing. Existing evidence that MYO15 moves along stereocilia actin filaments has led
402 to a cargo trafficking model, where the elongation complex is delivered to the stereocilia tips and
403 provides the actin regulation machinery necessary for elongation. Here, we uncover a previously
404 unknown activity of MYO15 that requires the cargo-trafficking model to be re-evaluated. *In vitro*, we
405 show that MYO15 can directly stimulate actin filament nucleation and that this activity is inhibited by
406 the *jordan* mutation, providing key evidence this activity is functionally linked to deafness pathology. *In*
407 *vivo*, the *Myo15 jordan* mutation stunted stereocilia growth, leading to an early-onset, progressive
408 hearing loss in mice. The effect of the *jordan* mutation to inhibit MYO15-induced actin nucleation *in*
409 *vitro*, and block stereocilia elongation *in vivo*, provides the functional link between these two
410 processes. We propose that in addition to trafficking key molecular cargos, MYO15 directly catalyses
411 actin filament nucleation at the stereocilia tip, and that both these processes combined are required
412 for normal stereocilia growth.
413
414 Using purified proteins and reconstituted assays, the MYO15 motor domain exerted multiple
415 effects upon actin filament polymerization *in vitro*. The motor domain: 1) nucleated actin filaments *de*
416 *novo*, and 2) slowed the elongation rate of existing actin filaments. No additional proteins were

417 required, demonstrating that these properties were intrinsic to the purified actomyosin system. The
418 *jordan* mutation completely blocked MYO15-induced actin filament nucleation, whilst filament
419 elongation rates were unaffected relative to wild-type MYO15. We conclude that MYO15's nucleation
420 activity is the key polymerization effect disrupted by the *jordan* deafness mutation. Similar to MYO15,
421 muscle myosin was shown in classic biochemical experiments to stimulate actin filament nucleation *in*
422 *vitro* (Yagi et al., 1965; Miller et al., 1988; Fievez and Carlier, 1993; Lheureux et al., 1993). The
423 MYO15 motor domain shares a common structural fold with muscle myosin (Gong *et al.,* bioRXiv),
424 and although the mechanism of muscle nucleation remains controversial (Lheureux et al., 1993), we
425 believe by homology to muscle that MYO15 nucleates actin through stabilizing inter-subunit contacts
426 that establish the protofilament (Fievez et al., 1997a, 1997b). In our companion study (Gong *et al.,*
427 bioRXiv), the MYO15 motor domain is further shown to influence structural plasticity within the
428 DNaseI-binding loop (D-loop), a sub-domain of actin that regulates the monomer to polymer transition
429 (Dominguez and Holmes, 2011). Combined, our data argue that nucleation is a shared function for at
430 least some members of the myosin superfamily, and that myosin motors can directly influence
431 cytoskeletal plasticity in addition to their canonical role generating force.
432
433 A cellular function for myosin-driven actin nucleation has, to the best of our knowledge, not
434 previously been reported in any tissue. Our study provides evidence that MYO15-driven nucleation
435 contributes to hair cell function *in vivo*, and furthermore is a cause of disease when disrupted. Actin
436 nucleation by MYO15 appears distinct from other nucleation factors, such as formins, Arp2/3, Spire
437 and Cobl (Machesky et al., 1994; Mullins et al., 1997, 1998; Sagot et al., 2002; Quinlan et al., 2005;
438 Ahuja et al., 2007). MYO15-driven nucleation was nucleotide-sensitive, and strongly promoted
439 nucleation under rigor (nucleotide-free) conditions, or when bound to ADP (Gong *et al.,* bioRXiv). Both
440 conditions populate the motor domain into strong actin binding states (Jiang et al., 2021). Conversely,
441 the presence of ATP in assays prevented nucleation and we hypothesize this is due to the motor
442 domain populating a weakly actin-bound state (Jiang et al., 2021). The nucleotide-sensing properties
443 of MYO15 may therefore couple local concentrations of ATP and ADP to actin polymerization. We
444 speculate this activity could form the basis of a tuning mechanism, where dynamic changes in
445 nucleotide concentrations control the height of developing stereocilia. The concentrations of ATP and
446 ADP in stereocilia are unknown, and whilst a creatine kinase ATP generation system has been
447 described in avian hair cells (Shin et al., 2007), its activity in mammals is unclear (Krey and Barr-
448 Gillespie, 2019). In addition to nucleotide state, MYO15-driven nucleation *in vitro* was observed at
449 high protein concentrations equimolar with actin, in agreement with data from muscle myosin (Miller et
450 al., 1988). The concentration of MYO15 in hair cells is unknown, however micromolar quantities may

451 be present at the stereocilia tips, especially as MYO15 actively traffics and self concentrates into this
452 subcellular compartment (Belyantseva et al., 2003). Other unconventional myosin motors critical for
453 hearing, including MYO1C, MYO3A, MYO6 and MYO7A (Avraham et al., 1995; Gibson et al., 1995;
454 Gillespie and Corey, 1997; Hasson et al., 1997; Holt et al., 2002; Salles et al., 2009; Ebrahim et al.,
455 2016), also concentrate in specific stereocilia sub-domains and it is exciting to consider whether they
456 also directly regulate actin polymerization as part of their cellular function.
457
458 In parallel with identifying MYO15 as an actin nucleator, we used the *jordan* mutant mouse to
459 probe sufficiency of the elongation complex to drive stereocilia elongation. Each protein in the
460 elongation complex is critical for growth, but existing models do not explain their molecular activty,
461 except that they must be delivered to the stereocilia tip via MYO15-driven trafficking. This model is
462 based on the *shaker-2* mouse, where stereocilia growth is inhibited and both MYO15 and the
463 elongation complex are absent from the hair bundle (Belyantseva et al., 2005; Delprat et al., 2005;
464 Manor et al., 2011; Zampini et al., 2011; Mauriac et al., 2017; Tadenev et al., 2019). Our data show
465 that MYO15-driven actin nucleation and elongation complex trafficking activities are simultaneously
466 lost in the *shaker-2*, leaving their relative contributions unknown. By contrast, MYO15-driven trafficking
467 of the elongation complex is initially (largely) preserved in the short stereocilia of the *jordan* mouse.
468 These data argue that the elongation complex proteins are necessary, but not sufficient for driving
469 stereocilia growth, and that MYO15-nucleation activity is critical *in vivo*. We cannot exclude the
470 possibility of reduced MYO15 trafficking in *jordan* hair cells; indeed, gliding filament velocities of the
471 mutant motor were ~ 50% of the wild-type *in vitro*. However, there is no evidence that the
472 concentration of MYO15 at the stereocilia tips determines stereocilia length. Neither the elimination of
473 MYO15-1 from shorter stereocilia rows (Fang et al., 2015), nor the over-expression of MYO15-2 alters
474 initial stereocilia lengths (Belyantseva et al., 2003, 2005). We also considered whether the *jordan*
475 mutation might interfere with MYO15 binding to another protein critical for stereocilia growth. Our
476 companion study (Gong *et al.,* bioRXiv) localizes the *jordan* missense substitution to the actomyosin
477 interface, and shows a direct structural effect within the actin monomer itself. Combined with our
478 study, these findings strongly suggest that MYO15 directly accelerates actin nucleation at the
479 stereocilia tip by structurally regulating the actin molecule.
480
481 Our study raises new questions concerning the stability and plasticity of the stereocilia
482 cytoskeleton, processes that must be tightly controlled for mechano-transduction and lifelong hearing
483 (Vélez-Ortega and Frolenkov, 2019). Stereocilia actin filaments are unidirectionally polarized with their
484 fast-growing barbed ends orientated towards the tip compartment, where new actin monomers are

485 incorporated (Flock and Cheung, 1977; Tilney et al., 1980; Schneider et al., 2002; Drummond et al.,
486 2015). Although actin capping proteins also contribute to stereocilia architecture (Peng et al., 2009;
487 Avenarius et al., 2017), we find that excessive barbed end capping is not the cause of short stereocilia
488 in *Myo15* mutant mice. Given the presence of barbed ends, why might actin nucleation be required to
489 extend pre-existing filaments at stereocilia tips? One possible explanation is that stereocilia elongation
490 occurs through direct end-to-end annealing of short actin polymers nucleated by MYO15, rather than
491 through addition of individual actin monomers at the barbed end. Pre-formed actin filaments can
492 anneal into longer filaments *in vitro* (Murphy et al., 1988; Kinosian et al., 1993; Sept et al., 1999), and
493 more recent *in vitro* studies have detected filament extension through incorporation of short polymers,
494 in addition to the dominant mode of monomer addition (Young et al., 2018). Interestingly, endocytosis
495 in yeast is proposed to use end-to-end actin filament annealing rather than monomer polymerization
496 (Okreglak and Drubin, 2010). Though speculative in hair cells, the extension of the stereocilia actin
497 core using short polymer annealing may allow for rapid changes in stereocilia length that are likely
498 needed to continually maintain the sensitivity of the MET machinery (Vélez-Ortega et al., 2017; Krey
499 et al., 2020).
500
501 Given the potent ability of MYO15 to nucleate actin filament polymerization *in vitro*, we infer
502 this activity must be tightly regulated in hair cells. There is extensive evidence for intramolecular
503 regulation throughout the myosin superfamily (Heissler and Sellers, 2016). For example, myosin 5
504 (MYO5A) exists in an autoinhibited conformer where the globular tail domain binds and inhibits motor
505 domain activity; binding of melanophilin (MLPH) to MYO5 releases this autoinhibition to activate the
506 motor domain (Li et al., 2005; Liu et al., 2006; Thirumurugan et al., 2006; Sato et al., 2007; Sckolnick
507 et al., 2013). Similar autoinhibitory regulation mechanisms have been described for other members of
508 the MyTH4 - FERM myosin family, *e.g.*, MYO7A and MYO10 (Umeki et al., 2009, 2011; Yang et al.,
509 2009; Sakai et al., 2011). We hypothesize that part of the elongation complex function may be to bind
510 MYO15 and shift the equilibrium between active and inhibited states. Intramolecular regulation may
511 also explain how different MYO15 isoforms independently exert their effects upon hair bundle
512 architecture (Fang et al., 2015). MYO15-1 and MYO15-2 possess an identical motor domain
513 competent to regulate actin polymerization, but differ by the addition of a 133 kDa N-terminal domain
514 (Fig. 1A). How the N-terminal domain affects the actin nucleation activity of the motor domain is an
515 important future question and will help uncover how the MYO15-1 isoform controls actin
516 polymerization in shorter stereocilia rows with active MET (Fang et al., 2015). In conclusion, we reveal
517 a new function for MYO15 and argue that defective nucleation of actin polymerization interferes with
518 stereocilia architecture and causes progressive hearing loss. We speculate that defective actin

519 nucleation by MYO15 is a central molecular pathology underlying DFNB3 hereditary hearing loss in
520 humans.
521
522 **MATERIALS AND METHODS**
523 **Mice**
524 Pedigree MPC190 was identified from a phenotype-driven mutagenesis screen undertaken at
525 the MRC Harwell Institute (Potter et al., 2016). Briefly, *N*-ethyl-*N*-nitrosourea (ENU) mutagenized
526 C57BL/6J males were mated with wild-type 'sighted C3H' (C3H.Pde6b^{+}) females. Resulting G_1 males
527 were crossed with C3H.Pde6b^{+} females to produce G_2 females, which were screened for the *Cdh23*^{ahl}
528 allele. *Cdh23*$^{+/+}$ G_2 females were backcrossed to their G_1 fathers to generate recessive G_3 pedigrees,
529 which entered a longitudinal phenotyping pipeline that included click box and ABR tests to assess
530 auditory function (Hardisty-Hughes et al., 2010). DNA from mice exhibiting hearing loss, and normal
531 hearing pedigree mates, was prepared from ear biopsies and used for linkage mapping utilizing the
532 Illumina GoldenGate Mouse Medium Density Linkage Panel (Gen-Probe Life Sciences Ltd, UK), which
533 identified a critical interval on chromosome 11. DNA was extracted from mouse MPC190/2.18a and
534 subject to whole genome sequencing employing the Illumina HiSeq platform (Oxford Genomics
535 Centre, Wellcome Trust Centre for Human Genetics). Subsequent alignment to the reference genome
536 (NCBIM38/mm10) identified a homozygous, non-synonymous coding lesion in the *Myo15* gene.
537
538 At the MRC Harwell Institute, mice were housed and maintained at the Mary Lyon Centre
539 under conditions outlined in the Home Office Code of Practice, with all animal procedures licenced by
540 the Home Office under the Animals (Scientific Procedures) Act 1986, UK and approved by the local
541 Ethical Review Committee (PBF9BD884 to MRB). At MRC Harwell, *jordan* mice were crossed to
542 C57BL/6N (*Cdh23*$^{753A>G}$) 'repaired' mice (Mianné et al., 2016) until congenic. Concurrently, *jordan*
543 mice were imported to the NIH and the University of Florida (UF) and maintained on a 'sightless C3H'
544 (C3H.Pde6b^{rd1}) background. Animal procedures were approved the Institutional Animal Care and Use
545 Committees (IACUC) at UF (#201910739 to JEB) and at the NIH (#1263-15 to TBF). Genomic DNA
546 from mouse tail biopsies was used as template in a PCR reaction with primers (5'-
547 CAGGAGGAGTACATCCGGG-3', 5'-AGACCACAGAAGTATCTGGGTCTT-3'). The resulting 161 bp
548 amplicon was analyzed by *MlsI* endonuclease digestion. Resulting restriction fragments lengths
549 unambiguously detected wild-type (161 bp) and mutant (116 bp + 45 bp) alleles.
550
551 **Auditory phenotyping + behavioral testing**

552 Auditory Brainstem Response (ABR) were recorded as previously described (Dunbar et al.,
553 2019). Briefly, mice were anaesthetised via intraperitoneal injection with ketamine hydrochloride (100
554 $mg \cdot kg^{-1}$) and xylazine (10 $mg \cdot kg^{-1}$). Anesthetized mice were placed inside a sound-attenuated
555 chamber (ETS-Lindgren) and recording electrodes (Grass Telefactor F-E2-12) placed sub dermally
556 over the vertex (active), right mastoid (reference), and left flank (ground). ABR responses were
557 collected using TDT system III hardware and BioSig software (Tucker Davis Technology). Stimuli were
558 presented free field from a ES1 transducer (TDT) calibrated at a distance of 1 cm from the right ear.
559 Stimuli consisted of 0.1 ms broadband clicks or 7 ms tone-bursts at 8 kHz, 16 kHz and 32 kHz. Each
560 stimulus was presented at a maximum 90 dB SPL, followed by decreasing steps of 5 - 10 dB SPL until
561 no replicable response peaks were observed. ABR operators were blind to genotype.
562
563 Distortion Product Oto-Acoustic Emissions (DPOAE) were recorded as a terminal procedure in
564 12 weeks old mice, as described (Dunbar et al., 2019). Mice were anaesthetised with a modified
565 ketamine/xylazine solution (see ABR), with the addition of acepromazine (2 $mg \cdot ml^{-1}$, 8% v/v).
566 Anesthetized mice had a section of the pinna removed to access the external auditory meatus. Mice
567 were placed inside a sound-attenuated chamber (ETS Lindgren), and the DPOAE probe assembly
568 was inserted into the ear canal. In-ear calibration was performed before each test. An ER10B+ low-
569 noise probe microphone (Etymotic Research) was used to measure DPOAEs near the tympanic
570 membrane. Tone stimuli were presented via separate MF1 (Tucker Davis Technology) speakers, with
571 f1 and f2 at a ratio of f2/f1 = 1.2 (L1 = 65 dB SPL, L2 = 55 dB SPL). The f1 and f2 tones were
572 presented continuously in specific tone-bursts between 8 and 32 kHz.
573
574 **Scanning electron microscopy**
575 Inner ears were dissected and fixed overnight at 4ºC in 0.1 M phosphate buffer, 2.5%
576 glutaraldehyde (Sigma-Aldrich). Post-fixed ears were decalcified in 4.3% EDTA in phosphate buffer at
577 4ºC, before dissecting out the sensory epithelium. Samples were processed with alternating 1%
578 osmium tetroxide (Agar Scientific) in 0.1 M sodium cacodylate (Sigma-Aldrich) and 1%
579 thiocarbohydrazide (Sigma-Aldrich) treatments. Osmicated samples were dehydrated in graded
580 ethanols (25% to 100%) at 4ºC and stored in 100% acetone until critical point drying with liquid CO_2
581 (EM CPD300, Leica Microsystems Ltd.). Samples were mounted onto stubs using silver paint (Agar
582 Scientific), sputter-coated with platinum (Q150R S, Quorum Technologies) and visualised with a
583 scanning electron microscope (JSM-6010LV, JEOL).
584

585 For morphometric analyses, image pairs from the middle cochlear turn IHC and OHC bundles
586 were captured with a 5° tilt angle difference at a constant working distance of 20 µm. Stereocilia from
587 at least two bundles per animal were measured using ImageJ (https://imagej.nih.gov), with a minimum
588 of 14 stereocilia for each condition. Length measurements were taken from the rear aspect of the hair
589 bundle, so that the length of the tallest stereocilia (row 1) could be measured from the cuticular plate
590 insertion to the tip. Estimates for actual stereocilia length were calculated using a pseudo-eucentric
591 tilting approach (Bariani et al., 2005). A single measure x_1 (length of stereocilium) was taken from the
592 first image and measured again (x_2) on the corresponding 5°-tilted image pair. Perpendicular counter-
593 measures (y_1 and y_2), matched to (x_1 and x_2) were also recorded. Equation 1 was used to estimate
594 uncertainty (ζ) due to plane rotation. Using the uncertainty estimate ζ from Equation 1, every pair of
595 tilted measures (x_1 and x_2) was processed using Equation 2 to obtain a close approximation (ξ) of the
596 true length of each stereocilia measured.
597

598 $$\zeta = \frac{(\Delta y)cos\Delta\varphi + (2y_1(y_1 - \Delta y)/d)sin\Delta\varphi}{(1 + y_1(y_1 - \Delta y)/d^2 sin\Delta\varphi) + \left(\frac{\Delta y}{d}\right)\cos(2\Delta\varphi)} \qquad \xi = \frac{2d - 2\zeta cos\Delta\varphi}{\frac{d}{x_1} + \frac{d}{x_2}}$$

599
600 (Equation 1) (Equation 2)
601
602 Where: ζ = uncertainty estimate, ξ = estimate of true size of structure of interest, $x_{1,2}$ = tilted
603 paired-measures of structure of interest, $y_{1,2}$ = perpendicular counter-measures to measures $x_{1,2}$, Δy =
604 arithmetic difference of counter-measures y_1 and y_2, Δφ = tilting angle (5°), *d* = working distance (20
605 µm).
606
607 In addition, low-magnification (1500 - 2000X) fields of mid-turn sensory epithelium were
608 imaged and the OHC bundles counted and visually categorised as either: 'Intact', 'Abnormal /
609 Damaged' where the bundle had an abnormal shape or was missing stereocilia, or 'Missing Bundle'
610 where a cuticular plate was observed with no stereocilia. Cochleae from four different animals per
611 genotype were examined, with a total of 1344 wild type and 972 *jordan* OHC bundles.
612
613 **Whole mount immunofluorescence and confocal microscopy**
614 Mouse inner ears were fixed in 4% paraformaldehyde (EMS Diasum) in PBS for 2 hours at
615 room temperature, washed in PBS, and then micro-dissected to isolate the cochlear sensory
616 epithelium. Samples were permeabilized in 0.5% (v/v) Triton X-100 in PBS for 30 minutes at RT,
617 followed by blocking in 5% normal goat serum (Sigma-Aldrich), 2% bovine serum albumin (Fraction V,

618 Roche) in PBS for 1 hour at RT. Primary antibodies were diluted in blocking solution and incubated
619 with samples at 4°C overnight. Primary IgG antibodies used were: PB48 rabbit anti-MYO15 (Liang et
620 al., 1999), HL5136 rabbit anti-WHRN (Belyantseva et al., 2005), mouse anti-EPS8 (#610143, BD
621 Biosciences), rabbit anti-GPSM2 (Ezan et al., 2013) and rabbit anti-GNAI3 (#G4040, Sigma-Aldrich).
622 After washing in PBS, samples were labelled with Alexa Fluor 488 conjugated anti-IgG secondary
623 antibodies (Life Technologies) diluted in blocking solution for 1 hour at RT. Samples were co-labelled
624 with rhodamine phalloidin (Life Technologies) and mounted with high-precision #1.5 cover glass
625 (Zeiss) using Prolong Gold (Life Technologies). Images were captured using a 63x objective (1.4 NA,
626 plan apochromat, Zeiss) and a laser scanning confocal microscope (LSM780, Zeiss).
627
628 **Actin barbed end incorporation assay**
629 Cochleae from *shaker-2* or *jordan* mice were dissected at P6 in Leibowitz L-15 media (Life
630 Technologies) and adhered to the inside surface of a petri dish lid (Greiner CellStar 35 x 10 mm). The
631 stria vascularis was left intact, as described (Xiong et al., 2014). Cochleae were cultured for 6 hours in
632 DMEM / F-12 (Life Technologies) supplemented with 1% FBS (Atlanta Biologicals) and 1.5 $\mu g \cdot mL^{-1}$
633 ampicillin at 37°C, 5% CO_2. Cochlea were incubated for 2 minutes at RT in 0.5 μM TMR-actin
634 (Cytoskeleton) diluted in labelling buffer (concentrations in mM): HEPES (20), KCl (138), EGTA (3),
635 $MgCl_2$ (4), ATP (1), DTT (0.5), 1% (w/v) bovine serum albumin (BSA), 0.2 $mg \cdot mL^{-1}$ saponin, pH 7.5.
636 Cochleae were immediately fixed for 30 mins at RT in 4% paraformaldehyde (EMS Diasum) diluted in
637 PHEM buffer (concentrations in mM): PIPES (60), HEPES (25), EGTA (10), $MgCl_2$ (2), sucrose (120),
638 pH 7.0. Fixed cochleae were permeabilized in 0.5% (v/v) Triton X-100 in PBS for 30 minutes at RT,
639 labelled with Alexa 488 phalloidin (Life Technologies), and mounted using a high-precision #1.5 cover
640 glass (Zeiss) with Prolong Gold (Life Technologies). Confocal microscopy images were captured as
641 described above.
642
643 **Expression of EGFP-MYO15-2 in mammalian cells**
644 The pEGFP-C2-Myo15-2 and pEGFP-C2-Myo15-2(sh2) plasmids expressing an N-terminal
645 EGFP fusion with the mouse MYO15 isoform 2 coding sequence (NP_874357.2) were previously
646 reported (Belyantseva et al., 2003). The pEGFP-C2-Myo15-2(jd) plasmid was generated using site
647 directed mutagenesis (QuikChange II, Agilent) to introduce the jordan (c.4940A>G) non-synonymous
648 substitution. All expression plasmids were verified by Sanger sequencing and prepared as endotoxin-
649 free, transfection grade DNA (NucleoBond Xtra Maxi EF, TakaraBio). HeLa cells (#CCL2) were
650 obtained as an authenticated, low passage stock from the American Type Culture Collection (ATCC).
651 LLC-PK1-CL4 (CL4) cells were a kind gift from Dr. James Bartles at Northwestern University. HeLa

652 and CL4 cells were cultured in high-glucose DMEM (#11995, Life Technologies), supplemented with
653 1x GlutaMAX (Life Technologies) and 10% FBS (Atlanta Biologicals), and maintained at 37°C, 5%
654 CO_2. Transfection of HeLa cells or CL4 cells was performed using Lipofectamine 3000 (Life
655 Technologies) according to the manufacturer's protocol. pEGFP-C2 (Clontech) was used as an empty
656 vector control. Cells were plated onto fibronectin (10 $\mu g \cdot mL^{-1}$) coated glass bottom culture dishes
657 (#1.5, MatTek Corp) and allowed to adhere, and in the case of CL4 cells, characteristic island
658 formation could be observed. Cells were fixed in 4% paraformaldehyde and 2% sucrose in PBS for 10
659 mins (EMS Diasum). Fixed cells were permeabilized/blocked in 0.2% (v/v) Triton X-100, 10% (v/v)
660 normal goat serum (NGS) in PBS for 1 hr at RT, followed by labelling with rhodamine phalloidin (Life
661 Technologies) and Hoechst 33342 (Life Technologies) in blocking buffer (2% NGS in PBS). Confocal
662 microscopy images were captured as described above.
663
664 **Actin purification and labelling**
665 Actin was extracted from rabbit skeletal acetone powder (Pel-Freeze, AZ) in chilled G-buffer
666 (concentrations in mM), Tris-HCl (2), ATP (0.2), $CaCl_2$ (0.1), NaN_3 (1), DTT (1), pH 8, using
667 established protocols (Spudich and Watt, 1971). Actin was additionally labelled on Cys 374 using
668 either N-(1-pyrene)-iodoacetamide (Life Technologies), or tetramethylrhodamine-5-maleimide
669 (Adipogen Life Sciences)(Criddle et al., 1985; Fujiwara et al., 2002). F-actin used for steady-state
670 ATPase and gliding filaments assays was purified through two rounds of polymerization and
671 depolymerization using ultracentrifugation. F-actin was dialyzed extensively against MOPS (4), $MgCl_2$
672 (1), EGTA (0.1), DTT (1), NaN_3 (1), pH 7.0, and its concentration measured at 290 nm (ε = 26,600 M^{-}
673 $^{1} \cdot cm^{-1}$) prior to use. Unlabelled, pyrene- and TMR-labelled G-actin for polymerization studies were
674 further purified by size exclusion chromatography (Superdex 200, Cytiva) using isocratic elution in G-
675 buffer. Fractions were exclusively taken from the falling edge of the chromatogram, to ensure the
676 recovery of monomeric actin. Concentrations and dye-labelling efficiency was determined at 290 nm
677 (actin: ε = 26,600 $M^{-1} \cdot cm^{-1}$), 344 nm (pyrene: ε = 22,000 $M^{-1} \cdot cm^{-1}$) and 550 nm (rhodamine: ε = 96,900
678 $M^{-1} \cdot cm^{-1}$). Correction factors were applied for pyrene actin, $A_{corr} = A_{290} - (0.127 * A_{344})$, and separately
679 for rhodamine actin, $A_{corr} = A_{290} - (0.208 * A_{550})$(Fujiwara et al., 2002). Biotinylated skeletal muscle
680 actin (#8109-01, HyperMol, Germany) was rehydrated, dialyzed against G-buffer and cleared by
681 ultracentrifugation for 60 minutes at 100k x *g* prior to use. For actin polymerization experiments
682 performed without free ATP in solution, G-actin stocks (with 0.2 mM ATP) were desalted (PD
683 SpinTrap G-25, Cytiva) into a modified G-buffer (no ATP): Tris-HCl (2), $CaCl_2$ (0.1), NaN_3 (1), DTT (1),
684 pH 8, immediately prior to use. Desalted G-actin (ATP) monomers were stored on ice and used within
685 3 hours.

686

687 **Expression of MYO15 and deafness mutants in *Sf*9 cells using baculovirus**

688 The baculoviral transfer vector pFastbac1 M15-2IQ-EGFP-FLAG, encoding the wild-type
689 mouse MYO15 motor domain (NP_874357.2, aa. 1 - 743) as a C-terminal fusion with EGFP and
690 FLAG moieties, was previously described (Bird et al., 2014). The expressed protein was 114 kDa. The
691 *jordan* and *shaker-2* non-synonymous substitutions were separately introduced into pFastbac1 M15-
692 2IQ-EGFP-FLAG by site-directed mutagenesis (QuikChange II, Agilent) and verified by Sanger
693 sequencing. Plasmid DNA encoding wild-type, *jordan* and *shaker-2* was separately transformed into
694 DH10Bac cells (Life Technologies) and recombinant bacmid DNA prepared following the
695 manufacturer's protocol. First passage (P1) recombinant baculovirus was generated by transfecting
696 *Sf*9 cells (Expression Systems) with bacmid DNA complexed using linear polyethylenimine (PEI MAX,
697 40,000 MW, Polysciences Inc., PA) at a 12:1 (PEI : DNA) molar ratio. *Sf*9 cells were maintained in
698 suspension culture with ESF-921 medium (Expression Systems) in a shaking incubator at 27°C. P1
699 baculovirus was amplified in *Sf*9 cells using low multiplicity of infection (MOI = 0.1) to generate P2
700 baculovirus for expression. Dual-promoter baculovirus expressing mouse UNC45B + HSP90AA1
701 chaperones (Bird et al., 2014), and bovine smooth muscle essential (MYL6, also referred to as
702 MLC17B / ELC) + chicken regulatory (MYL12B, also referred to as MLC20 / RLC) light chains, were
703 previously described (Pato et al., 1996). All baculoviruses were tittered using an end-point dilution
704 assay and the *Sf*9 Easy Titer cell line (Hopkins and Esposito, 2009). To express M15-2IQ, *Sf*9 cells
705 were seeded at a density of 2 x 10^6 cells·mL^{-1} in ESF-921, and infected simultaneously with three
706 baculoviruses at the following MOI: M15-2IQ (5), UNC45B / HSP90AA1 (5), ELC / RLC (5).The
707 myosin chaperones UNC45B and HSP90AA1 we co-expressed to aid folding, in addition to essential
708 (MYL6) and regulatory (MYL12B) muscle light chains to bind the LCBDs (Bird et al., 2014; Jiang et al.,
709 2021). Identical expressions were performed for the *shaker-2* and *jordan* variants. Cells were
710 harvested at 48 - 72 hours post-infection by centrifugation at 500 x *g* and flash frozen in liquid
711 nitrogen.

712

713 **Purification of the MYO15 motor domain**

714 M15-2IQ (wild-type, *jordan*, *shaker-2*) motor domains were purified from frozen *Sf*9 cells
715 following established protocols (Bird et al., 2014; Jiang et al., 2021). Cell pellets were lysed using a
716 Dounce homogenizer in extraction buffer (concentrations in mM): MOPS (10), NaCl (500), EGTA (1),
717 $MgCl_2$(10), ATP (2), PMSF (0.2), DTT (0.1), NaN_3 (1), leupeptin (2 μg·mL^{-1}), protease inhibitor cocktail
718 (Halt EDTA-free; Thermo Scientific), pH 7.2. Cell lysates were cleared for 30 mins at 48k x *g* and the
719 supernatant incubated with FLAG M2 affinity resin (Sigma-Aldrich) for 3 hours at 4°C. FLAG resin was

720 packed into a gravity flow column and washed with a high-salt buffer, MOPS (10), NaCl (500), EGTA
721 (1), $MgCl_2$(5), ATP (1), PMSF (0.1), DTT (0.1), NaN_3 (1), leupeptin (2 μg·mL^{-1}), pH 7.2, followed by a
722 low-salt buffer, MOPS (10), NaCl (60), EGTA (1), PMSF (0.1), DTT (0.1), NaN_3 (1), leupeptin (2
723 μg·mL^{-1}), pH 7.0. M15-2IQ protein was eluted using low-salt buffer supplemented with 0.2 mg·mL^{-1} 3x
724 FLAG peptide (American Peptide, CA). For assessment of hydrodynamic radius, purified motor
725 domains (wild-type, *jordan*, *shaker-2*) were concentrated by centrifugation (10'000 MWCO; Amicon,
726 EMD-Millipore) and directly analyzed using size exclusion chromatography (see below), alongside
727 protein size standards (Thyroglobulin (1), Ferritin (2), Aldolase (3), Conalbumin (4), Ovalbumin (5),
728 Carbonic anhydrase (6), Ribonuclease A (7); HMW + LMW gel filtration calibration kit (Cytiva). For
729 preparative scale protein production, FLAG-eluted motor domains (wild-type + *jordan*) were bound to a
730 strong anion exchanger (5/50 MonoQ GL; Cytiva) using a Purifier 10 chromatography system (GE
731 Healthcare). The column was washed with MOPS (10), NaCl (100), EGTA (1), PMSF (0.1), DTT (1),
732 pH 7.0, and a 160 CV gradient elution performed to 1M NaCl (100% B). Fractions eluting at ~31
733 mS·cm^{-1} were concentrated (10,000 MWCO) and further purified using size exclusion
734 chromatography (Superdex 200, Cytiva) with isocratic elution in MOPS (10), KCl (100), EGTA (0.1),
735 NaN_3 (1), PMSF (0.1), DTT (1), leupeptin (1 μg·mL^{-1}), pH 7.0. The M15-2IQ : ELC : RLC complex
736 (1:1:1) eluted as a single peak and was concentrated (10,000 MWCO) before determining complex
737 concentration at 280 nm (ε = 88,020 M^{-1}·cm^{-1}).
738
739 **ATPase assays**
740 Steady-state actin activated ATPase assays were measured using a NADH-coupled assay, as
741 previously reported (Bird et al., 2014). Briefly, wild-type M15-2IQ (30 nM) or *jordan* (150 nM) protein
742 was assayed in the following reaction buffer (concentration in mM): MOPS (10), KCl (50), $MgCl_2$ (5),
743 EGTA (0.1), MgATP (2), 40 U·mL^{-1} lactate dehydrogenase (Sigma-Aldrich), 200 U·mL^{-1} pyruvate
744 kinase (Sigma-Aldrich), phosphoenolpyruvate (1) (Sigma-Aldrich), NADH (0.2), pH 7.0 at 20° ± 0.1 ºC.
745 Due to its lower activity, a higher concentration (150 nM) of M15-2IQ *jordan* protein was used. The
746 concentration of F-actin in the reaction was titrated from 0 to 100 μM. The absorbance of NADH (ε =
747 6,220 M^{-1}·cm^{-1}) at 340 nm was measured using a dual-beam spectrophotometer (UV-1800, Shimadzu)
748 and the ATP hydrolysis rate calculated from the change in absorbance over time. Hydrolysis rates
749 were corrected for basal M15-2IQ ATPase activity in the absence of F-actin, and also for the ATPase
750 activity of F-actin. ATP hydrolysis rates were fit to the Michaelis-Menten equation to estimate k_{cat} and
751 k_{ATPase} using Prism (GraphPad).
752
753 **Gliding filament motility assay**

754 Motility chambers were assembled by coating a clean cover glass (#1.5) with 0.1%
755 nitrocellulose in amyl acetate (Ladd Research Industries), and attaching it to a microscope slide with
756 two strips of double-sided adhesive tape (Scotch, 3M) to form a channel approximately 3 mm wide.
757 The chamber was incubated for 5 mins in 0.1 mg·mL^{-1} anti-GFP (clone GFP-20, Sigma-Aldrich)
758 diluted with motility buffer (MB) (concentrations in mM): MOPS (20), KCl (10), $MgCl_2$ (5), EGTA (0.1),
759 pH 7.0. The surface was blocked using 1 mg·mL^{-1} BSA (Sigma-Aldrich) diluted in MB and incubated
760 for 1 min. After washing the chamber with MB, 1 μM M15-2IQ (wild-type or *jordan*) diluted in MB was
761 incubated for 1 min to functionalize the anti-GFP coated surface. The chamber was washed with 1
762 mg·mL^{-1} BSA in MB, followed by MB alone. Finally, the chamber was incubated for 2 mins with TMR-
763 phalloidin stabilized actin filaments (5 nM) diluted in MB, and subsequently washed in MB. Filaments
764 were visualized using an inverted epifluorescence microscope (Olympus IX-51) and motility recorded
765 in MB supplemented with 50 mM DTT, 2 mM ATP, 3 mM glucose, 100 μg·mL glucose oxidase, and 20
766 μg·mL catalase at 30 ± 0.5 °C. Actin filament velocities were analyzed as described (Sellers et al.,
767 1993).
768
769 **Polymerization assay**
770 Actin polymerization was measured using G-actin labelled on Cys 374 with N-(1-pyrene)-
771 iodoacetamide (see above), and a cuvette-based fluorometer (PTI Quantamaster 400, HORIBA
772 Scientific) used to excite pyrene at 365 nm and capture fluorescence emission at 407 nm. Gel filtered
773 G-actin (10% pyrene labelled, with free ATP, or desalted) was converted to the physiological Mg^{2+}
774 bound form by addition of 50 μM $MgCl_2$ and 0.2 mM EGTA for exactly two minutes at room
775 temperature. The polymerization reaction was initiated by mixing G-actin (3 x stock) with KMEI buffer
776 (1.5x stock) in a 1:2 ratio, respectively. Myosin (wild-type or *jordan*) was included in the 1.5x KMEI
777 buffer as needed. Final concentrations were 2 μM G-actin and 1 μM myosin, in assay buffer
778 (concentrations in mM): KCl (50), $MgCl_2$ (1), EGTA (1), imidazole (10), pH 7.0 at 25° ± 0.1 ºC. Data
779 were corrected for dead-time and fluorescence recorded until the transient reached plateau, or for a
780 maximum of 3 hours. Fluorescence derivatives with respect to time were processed in Prism
781 (GraphPad). Half times were calculated as described (Hansen et al., 2013).
782
783 **TIRF single-filament polymerization assay**
784 High-tolerance cover slips (24 × 50 mm, #1.5, Marienfeld Superior, Germany) were cleaned by
785 sequential sonication (10 mins each) in 2% Hellmanex III (Hellma, Germany), 1M KOH, 100% ethanol,
786 and finally Milli-Q water. Cover slips were dried under a nitrogen stream and processed for 10 mins
787 under argon plasma (ZEPTO, Diener Electronic, Germany). A mixture of mPEG-silane (2 mg·mL^{-1},

788 Laysan Bio, AL) and biotin–PEG–silane (2 μg·mL^{-1}, Laysan Bio) was prepared in 96% ethanol, 0.1%
789 (v/v) HCl. Cover slips were coated with 100 μL of the mPEG mixture and baked at 70°C for 1 hour.
790 Cover slips were rinsed twice in 96% ethanol, sonicated for 10 mins, followed by two rinses in Milli-Q,
791 sonicated for 10 mins in Milli-Q, and finally dried under a nitrogen stream. Flow chambers were
792 assembled using double-sided sticky tape to create a 3 mm wide channel on a glass slide.
793 Functionalized coverslips were placed over the tape and firmly pushed down to seal the flow chamber.
794 Immediately prior to use, flow cells were washed with buffer T50 (concentrations in mM): Tris·HCl
795 (10), KCl (50), DTT (1), pH 8.0. Flow cells were sequentially washed with 0.1 mg·mL^{-1} (Neutravidin,
796 Thermo Scientific) in T50 for 1 min, followed by a wash with 1 mg·mL^{-1} bovine serum albumin (A0281,
797 Sigma Aldrich) in T50 for 30 s, and a final wash of T50. Experiments were performed in the following
798 reaction buffer (final concentration in mM): KCl (50), $MgCl_2$ (1), EGTA (1), imidazole (10), ATP (0.025),
799 DTT (10), glucose (15), 0.5% methylcellulose, 20 μg·mL catalase, 100 μg·mL glucose oxidase, pH 7.0.
800 G-actin (1 μM total) was included in the reaction with TMR-actin (20%) and biotin-actin (10%) doping.
801 Purified myosin motor domain (1 μM) was optionally added, as needed. The reaction was loaded into
802 the flow cell and immediately mounted onto an inverted microscope (Nikon Ti-E2) equipped with an oil
803 immersion objective (CFI Apochromat TIRF 100x, 1.49 N.A., Nikon) for objective-style total internal
804 reflection fluorescence (TIRF) microscopy (H-TIRF, Nikon). The flow cell was illuminated using a 561
805 nm laser line, and emission filtered using a bandpass filter (ET630/75m, Chroma). Time-lapse images
806 were captured on an EM-CCD camera (iXon Ultra 888, Andor) controlled by NIS-Elements (AR
807 version 5.2, Nikon). Samples were imaged at 21 ± 1°C. The assay dead-time was typically 45 - 60 s,
808 and was included in data analyses.
809
810 Images were pre-processed in FIJI (https://fiji.sc)(Schindelin et al., 2012) by performing
811 background subtraction and image registration (descriptor-based series registration, 2d/3d +t). Actin
812 filament densities were quantified using the Analyze Particle command (size > 3 pixel^2, circularity: 0.0-
813 1.0) to count discrete particles within a 50 x 50 μm region of interest (ROI) that was randomly selected
814 from the field of view. A minimum of 3 experiments, from two independent protein preparations, were
815 analyzed for each condition. Filament elongation rates were calculated from time-lapse TIRF imaging
816 sequences using kymographs generated in Elements Software (version 5.2, Nikon). At least 40
817 filaments from two independent experiments were analyzed for each condition.
818
819 **Statistical analyses**
820 All statistical calculations and non-linear regressions were performed in Prism (GraphPad).

821 Regression fit parameters are quoted as mean ± SEM, and data points as mean ± SD, unless
822 otherwise stated. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.
823

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825 Conceptualization: JEB, MRB
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850

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095

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097

098 **FIGURE LEGENDS**

099

100 **FIGURE 1**

101 **A)** Schematic showing the protein domains of the long (MYO15-1) and short (MYO15-2) isoforms
102 encoded by the *Myo15* gene. The *jordan* and *shaker-2* deafness mutations are shown. **B)** ABR
103 phenotyping of the *jordan* pedigree at 3 months identified 10 mice with statistically elevated hearing
104 thresholds (red circles) for click and at 8, 16 and 32 kHz stimuli, compared to their normal hearing
105 pedigree mates (n = 73, black circles). Statistical outliers were detected using robust regression and
106 outlier removal (red circles, ROUT, Q = 1%). Thresholds of affected mice that did not respond to the
107 highest intensity stimulus (90 dB SPL) are recorded as 95 dB SPL. Data are mean ± SD. **C)**
108 Evolutionary conservation of the aspartate (D) residue of MYO15 altered to glycine (G) in *jordan* mice
109 that causes hearing loss. Residue positions refer to mouse MYO15-1 (NP_034992.2). **D)** ABR
110 recordings of *Myo15*$^{jd/sh2}$ compound heterozygotes at P28 shows profound hearing loss, with
111 thresholds elevated compared with normal hearing *Myo15*$^{+/jd}$ *or Myo15*$^{+/sh2}$ littermates. **E-F)**
112 Longitudinal auditory phenotyping of *jordan* mice at 1- (E) and 3- (F) months of age. ABR recordings
113 show that *Myo15*$^{jd/jd}$ mice (red, n=10) exhibit a progressive, moderate-to-severe hearing loss affecting
114 all frequencies, whereas age-matched *Myo15*$^{+/+}$(black, n=10) and *Myo15*$^{+/jd}$ (grey, n=15) littermate
115 controls have normal thresholds (15 - 45 dB SPL). *Myo15*$^{+/+}$ *vs Myo15*$^{jd/jd}$ comparison, ANOVA with
116 Tukey's multiple comparisons test.

117

118

119 **FIGURE 2**

120 **A-F)** Representative SEM images of stereocilia bundles from *Myo15*$^{+/+}$, *Myo15*$^{jd/jd}$, and *Myo15*$^{sh2/sh2}$
121 OHCs (A,C,E) and IHCs (B,D,F) at P8. In *Myo15*$^{+/+}$ mice, both IHC and OHC bundles display the
122 characteristic staircase architecture with 3 stereocilia ranks of increasing height. IHC and OHC
123 bundles from either *Myo15*$^{jd/jd}$ and *Myo15*$^{sh2/sh2}$ mice are shorter in height than the wild-type bundles.
124 *Myo15*$^{jd/jd}$ stereocilia taper in height towards the periphery of the bundle (white arrows). **G)** Projected
125 heights of tallest (row 1) OHC stereocilia at P8 are 1.2 ± 0.1 µm (*Myo15*$^{+/+}$, 58 stereocilia from 4 mice),
126 0.8 ± 0.1 µm (*Myo15*$^{jd/jd}$, 60 stereocilia from 4 mice), and 0.4 ± 0.1 µm (*Myo15*$^{sh2/sh2}$*,* 50 stereocilia from
127 2 mice). **H)** Projected heights of tallest (row 1) IHC stereocilia at P8 are 2.2 ± 0.2 µm (*Myo15*$^{+/+}$*,* 47
128 stereocilia from 4 mice), 1.3 ± 0.1 µm (*Myo15*$^{jd/jd}$, 47 stereocilia from 4 mice), and 0.9 ± 0.1 µm
129 (*Myo15*$^{sh2/sh2}$*,* 30 stereocilia from 2 mice). Brown-Forsythe and Welch ANOVA with Dunnett's T3
130 multiple comparisons test. Images are from mid-cochlear turn. Scale bars, 1 µm.

131

132 **FIGURE 3**

133 **A,B)** Immunofluorescence (IF) confocal images showing anti-MYO15 (green, PB48) in control *Myo15*$^{+/jd}$,
134 *Myo15*$^{sh2/sh2}$ and *Myo15*$^{jd/jd}$ IHCs fixed at P14 (A), or P7 (B). Phalloidin was used to label F-actin
135 (magenta). **C)** IF confocal images of elongation complex proteins (green) in control *Myo15*$^{+/jd}$ and
136 *Myo15*$^{jd/jd}$ IHCs fixed at P7, overlaid with phalloidin labelled F-actin (magenta). Images are
137 representative of data from at least two independent animals per genotype / antibody, and are shown
138 as maximum intensity projections reconstructed from z-stacks. Scale bars, 10 µm.

139

140 **FIGURE 4**

141 **A)** Actin barbed-end assay in detergent-permeabilized hair cells from mouse cochlear explant cultures
142 at P6. TMR-labelled G-actin (green) was added prior to fixation to identify uncapped barbed ends.
143 Phalloidin labelling of F-actin (magenta) is overlaid. In both *Myo15*$^{jd/jd}$ and littermate *Myo15*$^{+/jd}$ controls,
144 barbed-ends were detected at row 2 stereocilia tips, and at the tips of all stereocilia rows in *Myo15*$^{sh2/sh2}$
145 hair cells. **B)** HeLa cells were transfected with EGFP-tagged *Myo15-2* expression constructs or EGFP
146 alone (green) as indicated, fixed and probed with phalloidin (magenta) and Hoechst (blue). EGFP-M15
147 (wt) trafficked to filopodia tips (red arrowheads), while EGFP-M15 (jd) and EGFP-M15 (sh2) did not.
148 Boxed regions are magnified (inverted grayscale). **C)** CL4 cells were transfected with EGFP-tagged
149 *Myo15-2* and prepared as above. Both EGFP-M15 (wt) and EGFP-M15 (jd) concentrated at microvillar
150 tips, while EGFP-M15 (sh2) did not. Orthogonal projections are shown (inverted grayscale). Images are
151 representative from at least three independent experiments. Scale bars, 2 µm (A); 20 µm (B+C).

152

153

154 **FIGURE 5**

155 **A)** Cartoon of MYO15 motor domains expressed in *Sf*9 cells, consisting of the ATPase and two light
156 chain binding domains (LCBD). **B)** Size exclusion chromatography analysis of FLAG/IEX purified
157 M15(wt) and M15(jd) proteins. M15(sh2) elutes close to the void volume (arrow) and was aggregated.
158 Protein calibration standards (dotted lines), FLAG peptide (asterisk). **C)** SDS-PAGE analysis of SEC
159 purified motor domain proteins. The motor domain (arrow) co-purifies with RLC and ELC light chains
160 for all variants. M15(sh2) was misfolded and extracted from *Sf*9 cells at low yield. **D)** Steady-state
161 ATPase activation of M15(wt) and M15(jd) motor domains measured using a NADH-coupled assay at
162 20 ± 0.1°C. Reactions were performed with [F-actin] as shown. Rectangular hyperbola fits are shown
163 for M15(wt) (blue, k_{cat} = 5.8 ± 0.2 s^{-1}, k_{ATPase} = 29.1 ± 2.1 µM) and for M15(jd) (green, k_{cat} = 0.87 ± 0.04
164 s^{-1}, k_{ATPase} = 114.3 ± 8.2 µM, n = 4). **E)** Frequency histogram of F-actin velocities in a gliding filament
165 assay at 30 ± 0.1°C. Gaussian fits (dotted line) are overlaid for M15(wt) (473 ± 67 nm·s^{-1}, n = 5449

166 filaments, mean ± SD) and M15(jd) (216 ± 71 nm·s^{-1}, n = 2844 filaments). Data are from 2
167 independent experiments.
168
169
170 **FIGURE 6**
171 **A)** Fluorescent time-course of 2 μM G-actin (10% pyrene) measured in a fluorimeter (red trace).
172 Polymerization was induced by addition of KMEI (1x) at t = 0 s. Introduction of 1 μM M15(wt) (blue) at t
173 = 0 s increases in actin polymerization following an inflexion point (arrow), whilst 1 μM M15(jd) (green)
174 does not (arrow). Reactions contains 70 μM ATP carried over from the actin storage G-buffer. **B)** First
175 derivative of pyrene fluorescence with respect to time for G-actin + M15(wt)(blue) and G-actin +
176 M15(jd)(green). **C)** Fluorescent time course of 2 μM G-actin (10% pyrene) induced to polymerize by
177 KMEI buffer in the absence of free ATP (red trace). G-actin monomers were desalted immediately
178 prior to use, ensuring they were ATP bound. Addition of 1 μM M15(wt) (blue) triggers an almost
179 immediate increase in actin polymerization. **D)** Quantification of time to reach half maximal
180 fluorescence (n = 4).
181
182
183 **FIGURE 7**
184 **A)** TIRFM visualization of actin filaments polymerizing on PEG-biotin-neutravidin functionalized cover
185 glass. Polymerization of 1 μM G-actin (20% TMR + 10% biotin labelled) was induced by 1 x KMEI (50
186 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole, pH 7.0) at t = 0 s, in the presence of 25 μM ATP.
187 Representative time-lapses shown for: 1 μM G-actin (top), 1 μM G-actin + 1 μM M15(wt) (middle), and
188 1 μM G-actin + 1 μM mutant M15(jd) (bottom). **B)** Quantification of actin filament density shows delayed
189 nucleation activity of MYO15 in the presence of ATP. **C)** Kymographs of actin filament elongation. **D**)
190 Barbed end elongation rates for G-actin alone (red), G-actin + M15(wt) (blue), G-actin + M15(jd) (green).
191 **E + F)** Elongation rate data (from D) re-binned as before (E), or after (F) nucleation. The G-actin + KMEI
192 control data set (from D) is reproduced identically as a comparator in E and F. **G)** Time-lapse of actin
193 filaments polymerization induced by KMEI at t = 0 s, with no ATP in solution. G-actin (ATP) monomers
194 were prepared by desalting into ATP-free G-buffer. **H)** Actin filament density shows nucleation activity
195 of MYO15 is accelerated in the absence of ATP. **I)** Barbed end filament rates in the absence of free
196 ATP. Reaction deadtimes were typically 50 s and included in quantification. TIRFM images are shown
197 as inverted grayscale. Scale bars are 10 μm (A,G). Data are from 4 experiments, using 2 independent
198 protein preparations.
199

200

201 **FIGURE S1**

202 **A)** Whole genome SNP mapping of genomic DNA from ten MPC190 mice exhibiting hearing loss (red,
203 HL) and three hearing (H) littermate controls. The genotype of each mouse is either homozygous for
204 C57BL/6J (black box), C3H (white box) or heterozygous (grey box) for each marker. The analysis
205 defined a 16.5Mb critical interval on Chromosome 11 between markers rs29410974 and rs26906764
206 (Chr11:50420012-67162951, GRCm38). **B)** Sanger sequencing confirmed the only WGS-identified,
207 coding lesion (arrow) within the critical interval, in exon 17 (ENSMUSE00000244718) of the *Myo15*
208 gene (ENSMUSG00000042678). A nucleotide transition (c.4940A>G) at codon 1647 alters the wild-
209 type aspartate (D) codon to a mutant glycine (G). Example electropherograms are shown for *Myo15*$^{+/+}$,
210 *Myo15*$^{+/jd}$ and *Myo15*$^{jd/jd}$. **C-F)** Minimum ABR detection thresholds for a longitudinal cohort of *Myo15*$^{jd/jd}$
211 mice (n = 10), *Myo15*$^{+/+}$ (n=10) and *Myo15*$^{+/jd}$ (n=15) littermates at 4, 6, 9 and 12 weeks reveal
212 progressive hearing loss. Data points represent individual mice, with the mean value indicated. **** p
213 < 0.0001; two-way ANOVA with Tukey's multiple comparisons test. **G)** Distortion Product Otoacoustic
214 Emission (DPOAE) responses are greatly reduced at 12 weeks in *Myo15*$^{jd/jd}$ mutants (red, n = 10) at
215 all frequencies tested compared with *Myo15*$^{+/+}$ (black, n = 9) and *Myo15*$^{+/jd}$ (gray, n = 14) littermates.
216 Data are mean ± SD.

217

218

219 **FIGURE S2**

220 **A)** SEM analyses of IHC bundles from *Myo15*$^{jd/jd}$ and *Myo15*$^{+/+}$ littermates at 12 weeks. **B)**
221 Quantification of the tallest (row 1) stereocilia at 12 weeks in IHCs from *Myo15*$^{+/+}$ mice (45 stereocilia
222 from 3 animals) and *Myo15*$^{jd/jd}$ mice (45 stereocilia from 3 animals). **** $p < 0.0001$, unpaired, two-
223 tailed *t*-test. **C)** SEM analyses of *Myo15*$^{jd/jd}$ OHC bundles at 12 weeks. OHC bundles were qualitatively
224 categorized as "intact", "abnormal/damaged" or "missing". Representative examples are shown. In the
225 "abnormal/damaged" group, stereocilia were typically resorbed from the periphery of the hair bundle.
226 **D)** Quantification of OHC bundle categories at 12 weeks in *Myo15*$^{+/+}$ (1344 OHCs from 4 animals) and
227 *Myo15*$^{jd/jd}$ littermates (972 OHCs from 4 animals). All images and quantification are from the mid-
228 cochlear turn. Scale bars, 1 μm.

229

230

231 **FIGURE S3**

232 Immunofluorescence (IF) confocal images of anti-EPS8, anti-WHRN, anti-GNAI3 labelling or anti-
233 GPSM2 (green) in control *Myo15*$^{+/jd}$ and *Myo15*$^{jd/jd}$ IHCs fixed at P14. Phalloidin was used to label F-

234 actin (magenta). Images are representative of data from two independent animals per genotype and
235 antibody combination. Scale bars, 10 μm.
236

FIGURE 1

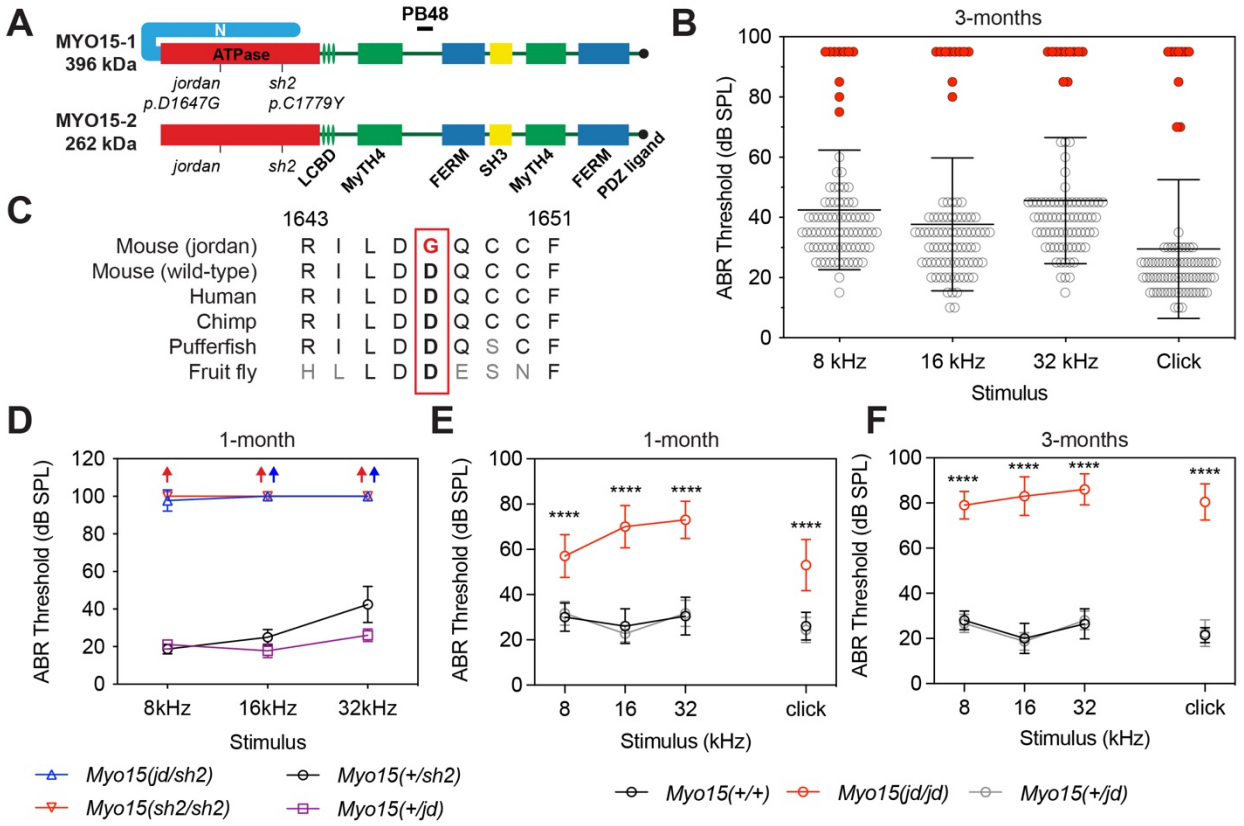

A
MYO15-1
396 kDa
N
ATPase
PB48
jordan
p.D1647G
sh2
p.C1779Y
MYO15-2
262 kDa
jordan
sh2
LCBD
MyTH4
FERM
SH3
MyTH4
FERM
PDZ ligand
B
3-months
ABR Threshold (dB SPL)
8 kHz
16 kHz
32 kHz
Click
Stimulus
C
1643
1651
Mouse (jordan) R I L D G Q C C F
Mouse (wild-type) R I L D D Q C C F
Human R I L D D Q C C F
Chimp R I L D D Q C C F
Pufferfish R I L D D Q S C F
Fruit fly H L L D D E S N F
D
1-month
ABR Threshold (dB SPL)
8kHz
16kHz
32kHz
Stimulus
Myo15(jd/sh2)
Myo15(+/sh2)
Myo15(sh2/sh2)
Myo15(+/jd)
E
1-month
ABR Threshold (dB SPL)
8
16
32
click
Stimulus (kHz)
F
3-months
ABR Threshold (dB SPL)
8
16
32
click
Stimulus (kHz)
Myo15(+/+)
Myo15(jd/jd)
Myo15(+/jd)

FIGURE 2

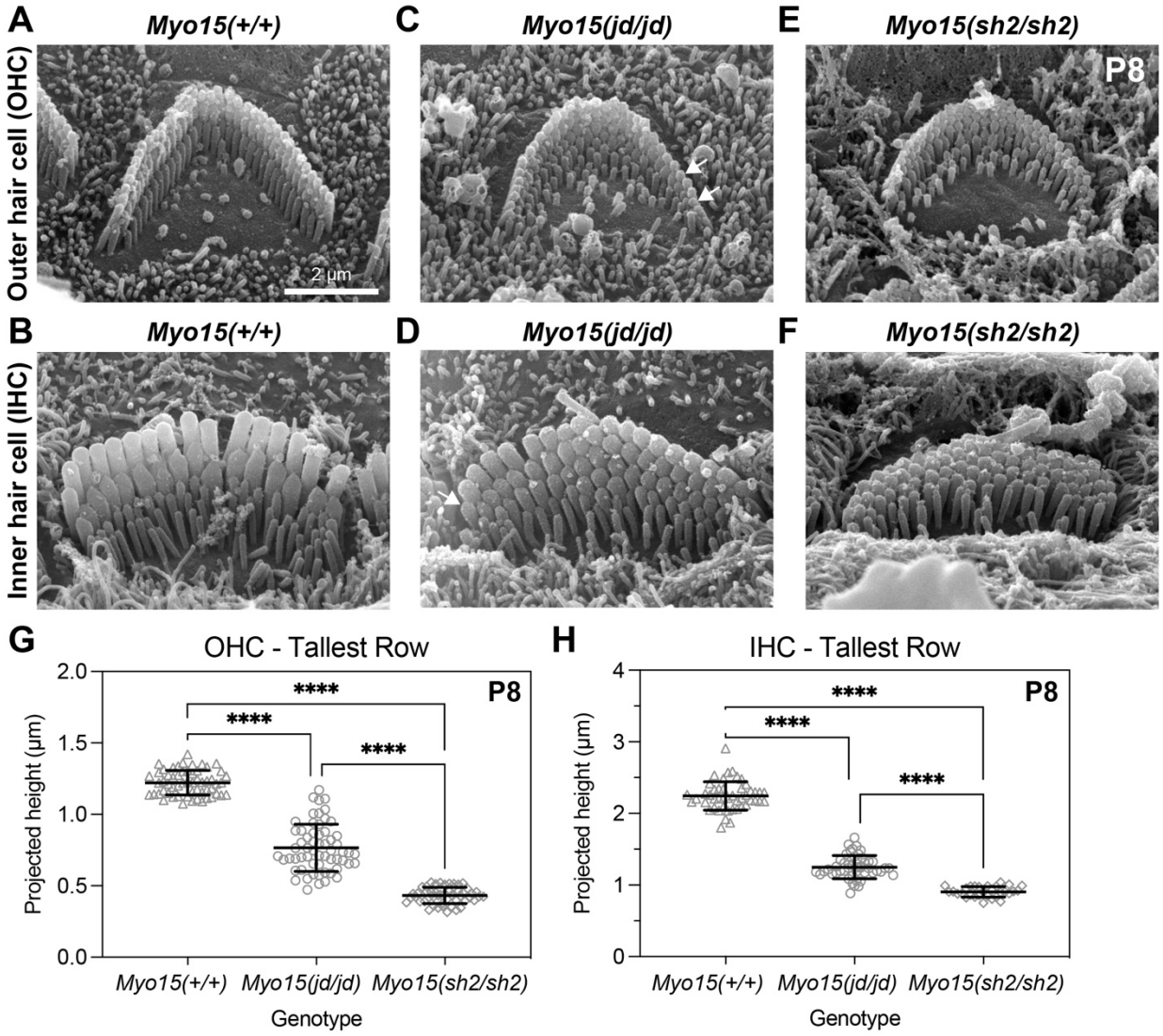

A
Myo15(+/+)
C
Myo15(jd/jd)
E
Myo15(sh2/sh2)
P8
Outer hair cell (OHC)
2 µm
B
Myo15(+/+)
D
Myo15(jd/jd)
F
Myo15(sh2/sh2)
Inner hair cell (IHC)
G
OHC - Tallest Row
P8
****
****
****
Projected height (µm)
2.0
1.5
1.0
0.5
0.0
Myo15(+/+)
Myo15(jd/jd)
Myo15(sh2/sh2)
Genotype
H
IHC - Tallest Row
P8
****
****
****
Projected height (µm)
4
3
2
1
0
Myo15(+/+)
Myo15(jd/jd)
Myo15(sh2/sh2)
Genotype

FIGURE 3

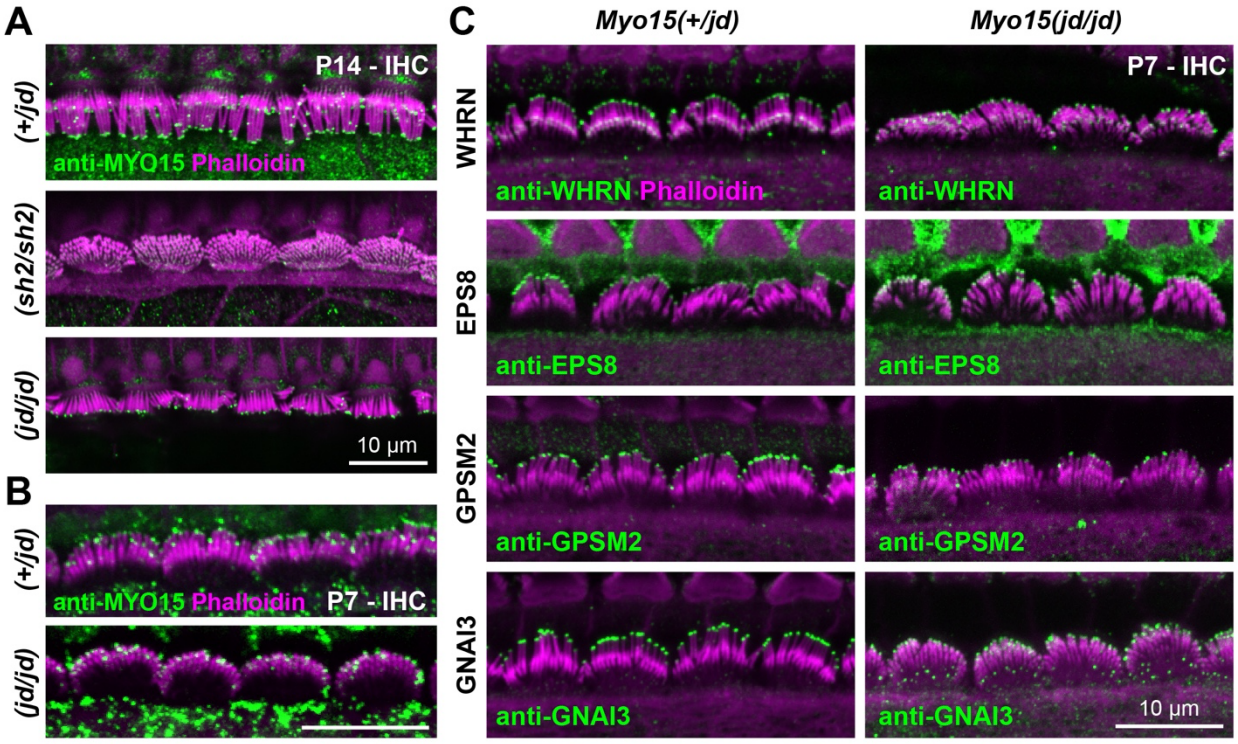

A
P14 - IHC
(+/jd)
anti-MYO15 Phalloidin
(sh2/sh2)
(jd/jd)
10 µm
B
(+/jd)
anti-MYO15 Phalloidin
P7 - IHC
(jd/jd)
C
Myo15(+/jd)
Myo15(jd/jd)
WHRN
anti-WHRN Phalloidin
P7 - IHC
anti-WHRN
EPS8
anti-EPS8
anti-EPS8
GPSM2
anti-GPSM2
anti-GPSM2
GNAI3
anti-GNAI3
anti-GNAI3
10 µm

FIGURE 4

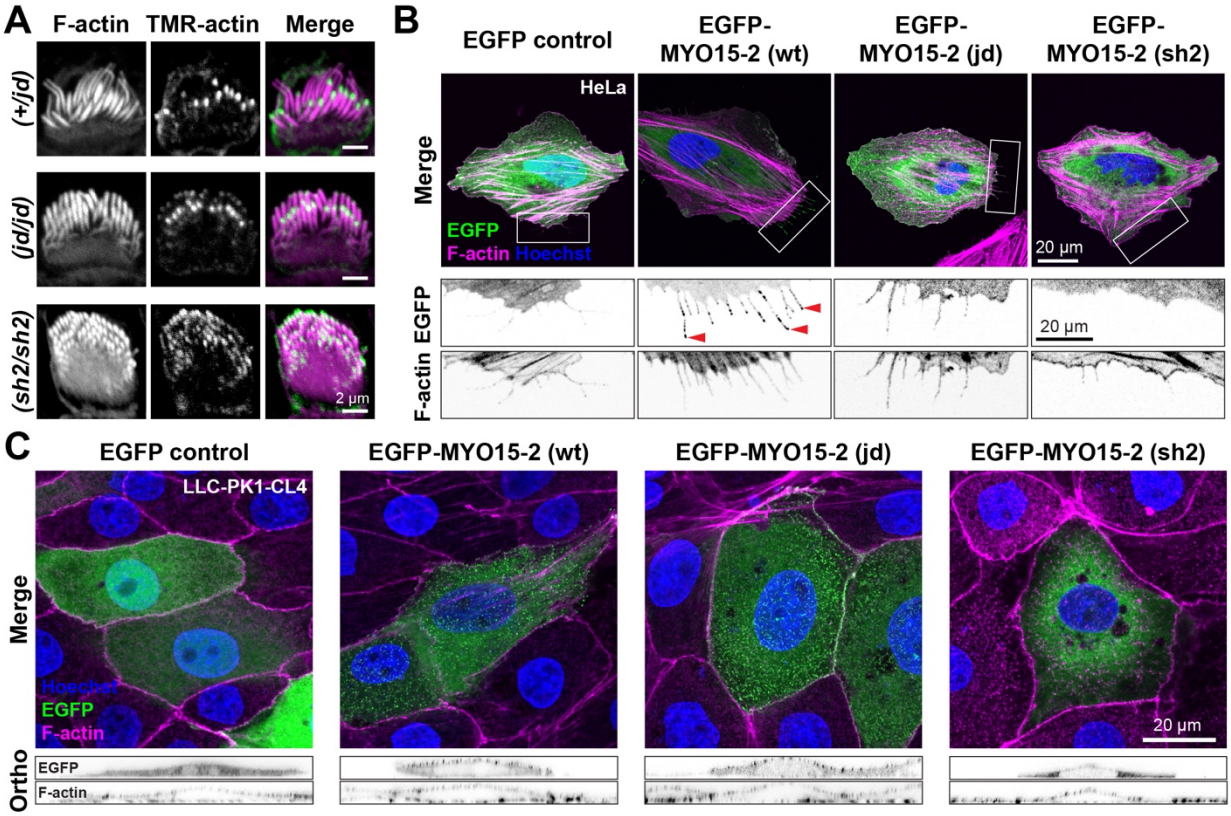

A
F-actin
TMR-actin
Merge
(+/jd)
(jd/jd)
(sh2/sh2)
2 µm
B
EGFP control
EGFP-MYO15-2 (wt)
EGFP-MYO15-2 (jd)
EGFP-MYO15-2 (sh2)
HeLa
Merge
EGFP
F-actin Hoechst
20 µm
EGFP
F-actin
20 µm
C
EGFP control
EGFP-MYO15-2 (wt)
EGFP-MYO15-2 (jd)
EGFP-MYO15-2 (sh2)
LLC-PK1-CL4
Merge
Hoechst
EGFP
F-actin
20 µm
Ortho
EGFP
F-actin

FIGURE 5

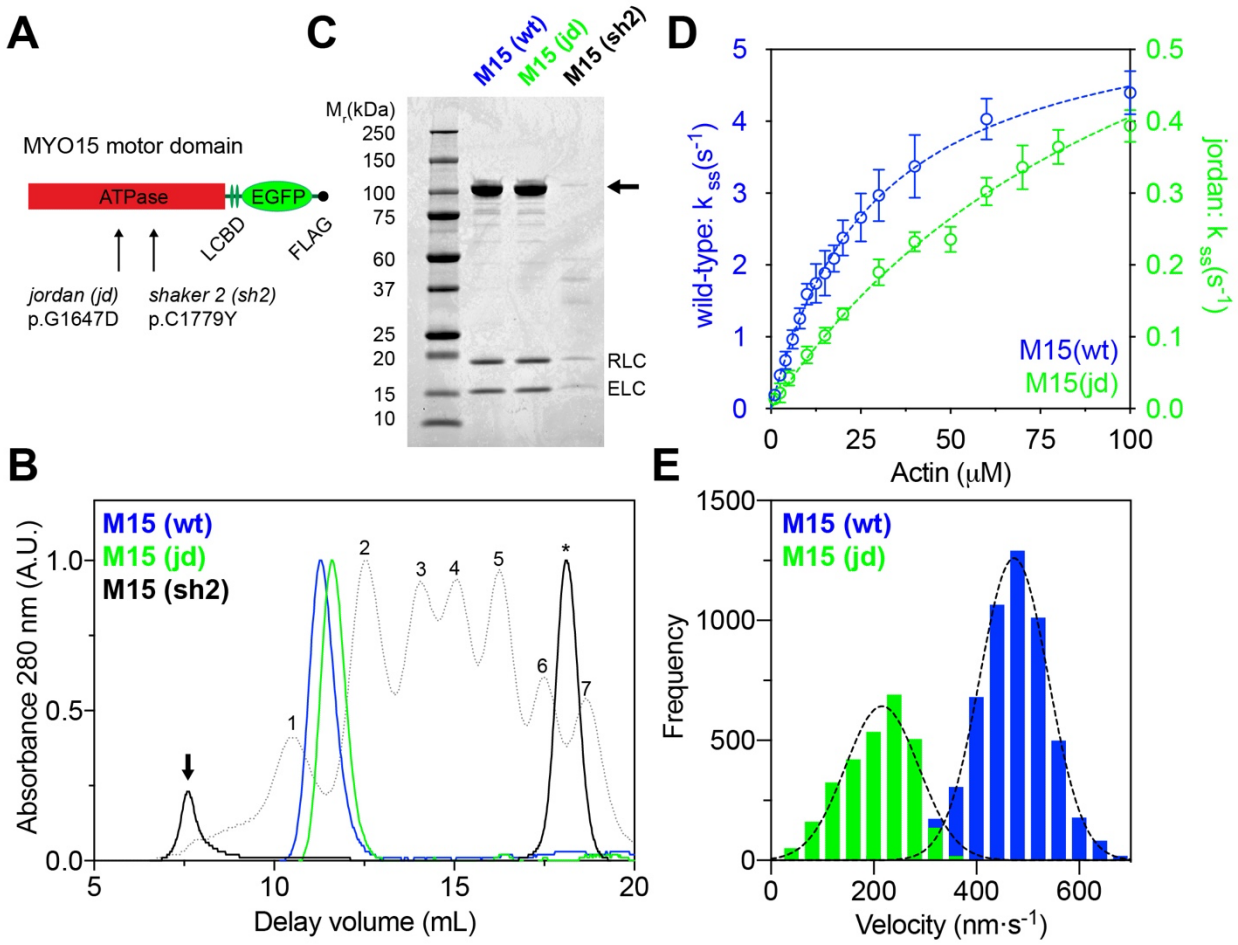

A
MYO15 motor domain
ATPase
EGFP
LCBD
FLAG
jordan (jd)
p.G1647D
shaker 2 (sh2)
p.C1779Y
C
M15 (wt)
M15 (jd)
M15 (sh2)
Mr(kDa)
250
150
100
75
60
37
25
20
15
10
RLC
ELC
D
wild-type: kss(s-1)
jordan: kss(s-1)
M15(wt)
M15(jd)
Actin (μM)
B
M15 (wt)
M15 (jd)
M15 (sh2)
Absorbance 280 nm (A.U.)
Delay volume (mL)
E
M15 (wt)
M15 (jd)
Frequency
Velocity (nm·s-1)

FIGURE 6

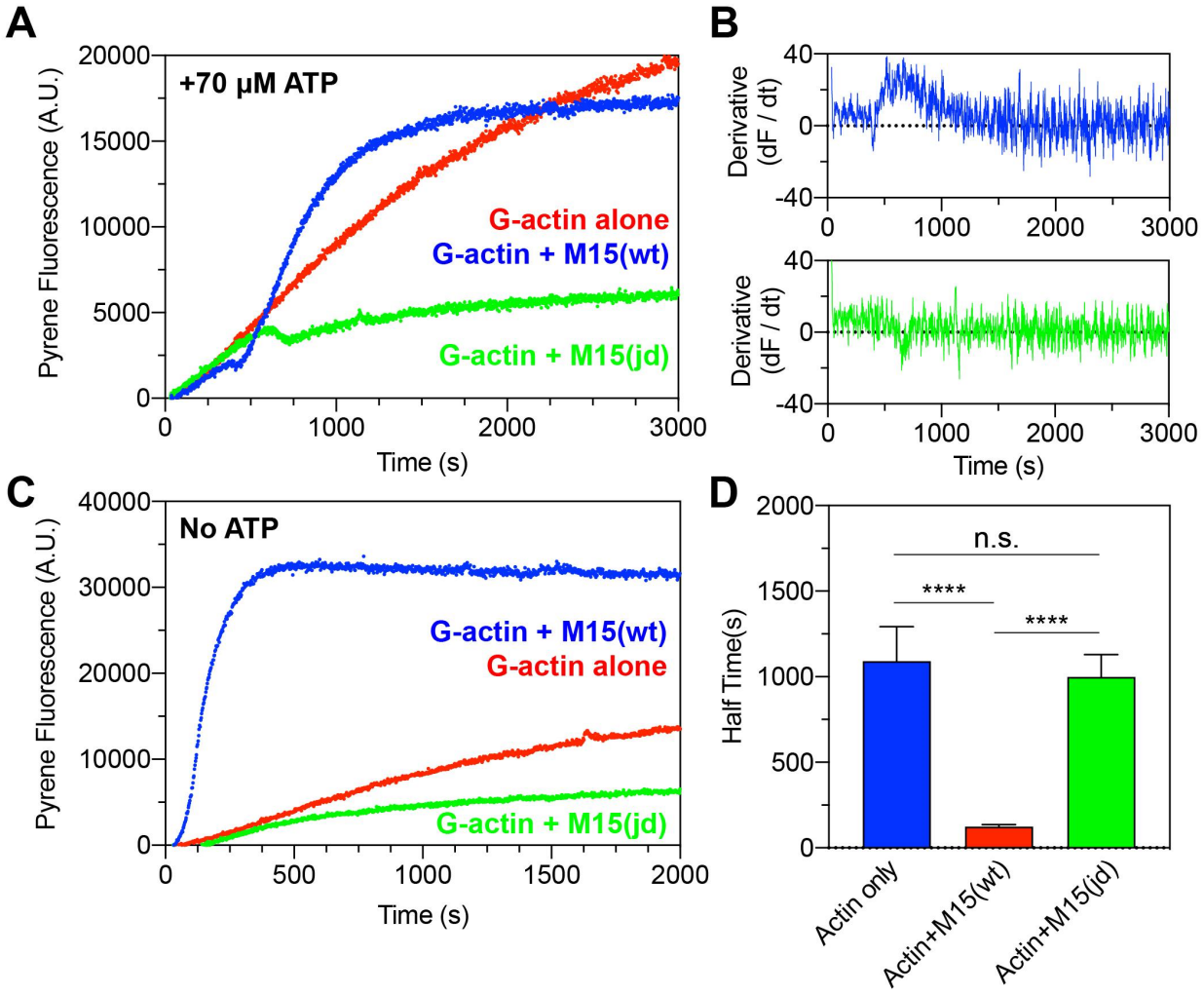

A
+70 μM ATP
Pyrene Fluorescence (A.U.)
Time (s)
G-actin alone
G-actin + M15(wt)
G-actin + M15(jd)
B
Derivative (dF / dt)
Time (s)
C
No ATP
Pyrene Fluorescence (A.U.)
Time (s)
G-actin + M15(wt)
G-actin alone
G-actin + M15(jd)
D
Half Time(s)
n.s.
****
****
Actin only
Actin+M15(wt)
Actin+M15(jd)

FIGURE 7

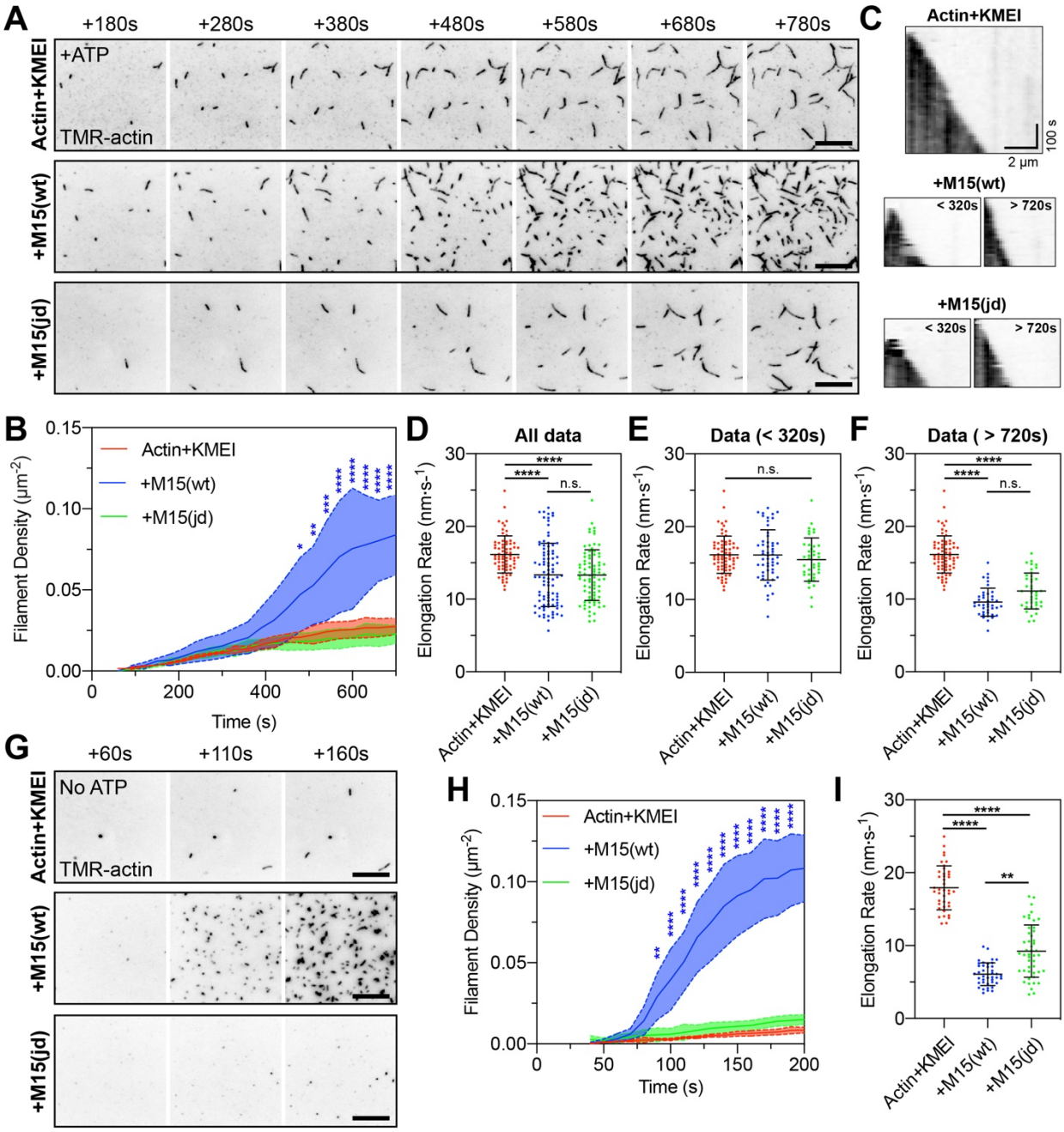

A
+180s
+280s
+380s
+480s
+580s
+680s
+780s
Actin+KMEI
+ATP
TMR-actin
+M15(wt)
+M15(jd)
C
Actin+KMEI
100 s
2 µm
+M15(wt)
< 320s
> 720s
+M15(jd)
< 320s
> 720s
B
Actin+KMEI
+M15(wt)
+M15(jd)
Filament Density (µm-2)
Time (s)
D
All data
E
Data (< 320s)
F
Data (> 720s)
Elongation Rate (nm·s-1)
n.s.
Actin+KMEI
+M15(wt)
+M15(jd)
G
+60s
+110s
+160s
No ATP
TMR-actin
H
I

FIGURE S1

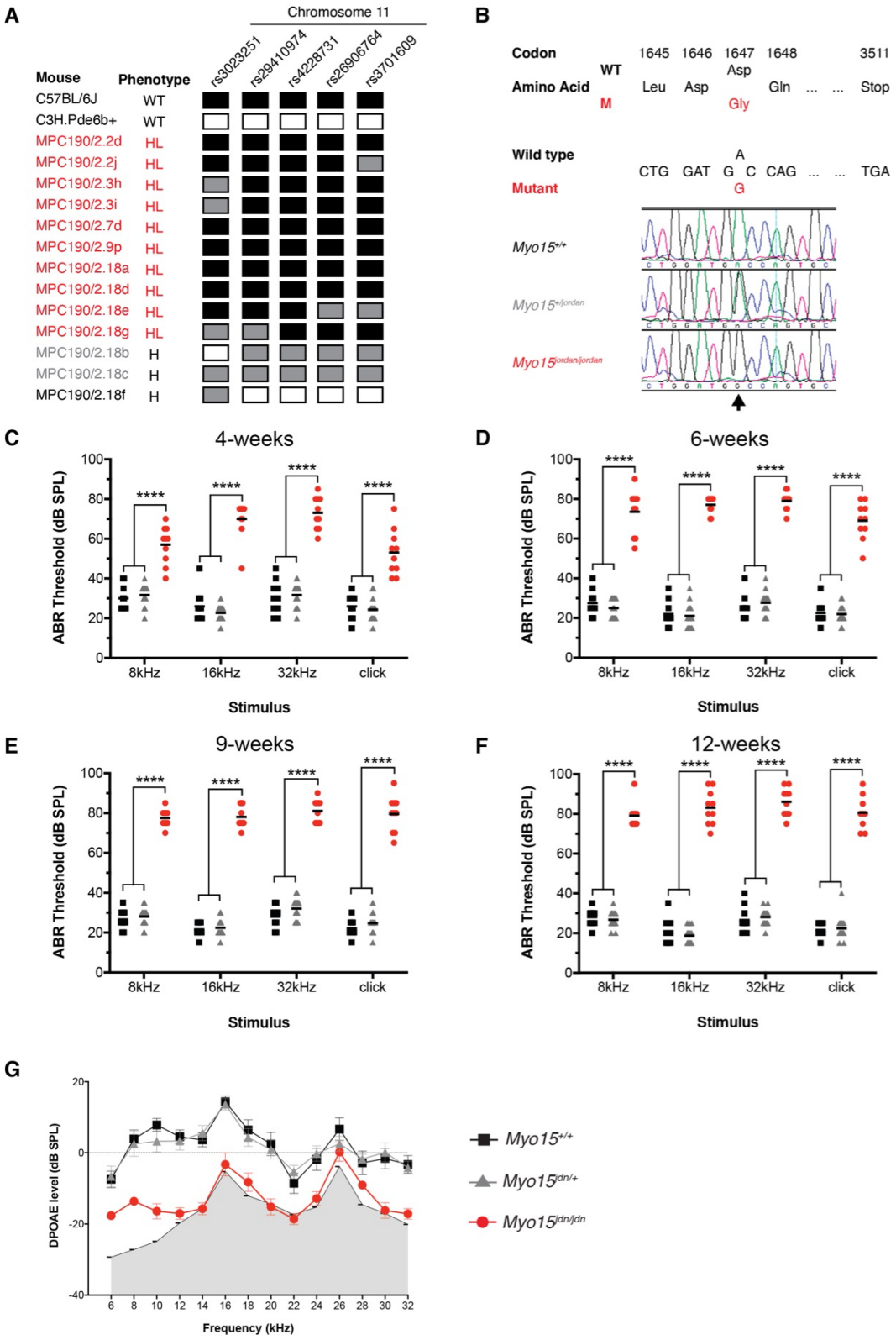

A
Chromosome 11
rs3023251
rs29410974
rs4228731
rs26906764
rs3701609
Mouse
Phenotype
C57BL/6J WT
C3H.Pde6b+ WT
MPC190/2.2d HL
MPC190/2.2j HL
MPC190/2.3h HL
MPC190/2.3i HL
MPC190/2.7d HL
MPC190/2.9p HL
MPC190/2.18a HL
MPC190/2.18d HL
MPC190/2.18e HL
MPC190/2.18g HL
MPC190/2.18b H
MPC190/2.18c H
MPC190/2.18f H
B
Codon
1645 1646 1647 1648 3511
WT
Asp
Amino Acid
Leu Asp Gln ... ... Stop
M
Gly
Wild type
A
CTG GAT G C CAG ... ... TGA
Mutant
G
Myo15+/+
Myo15+/jordan
Myo15jordan/jordan
C
4-weeks
D
6-weeks
E
9-weeks
F
12-weeks
ABR Threshold (dB SPL)
8kHz
16kHz
32kHz
click
Stimulus
****
G
DPOAE level (dB SPL)
Frequency (kHz)
Myo15+/+
Myo15jdn/+
Myo15jdn/jdn

FIGURE S2

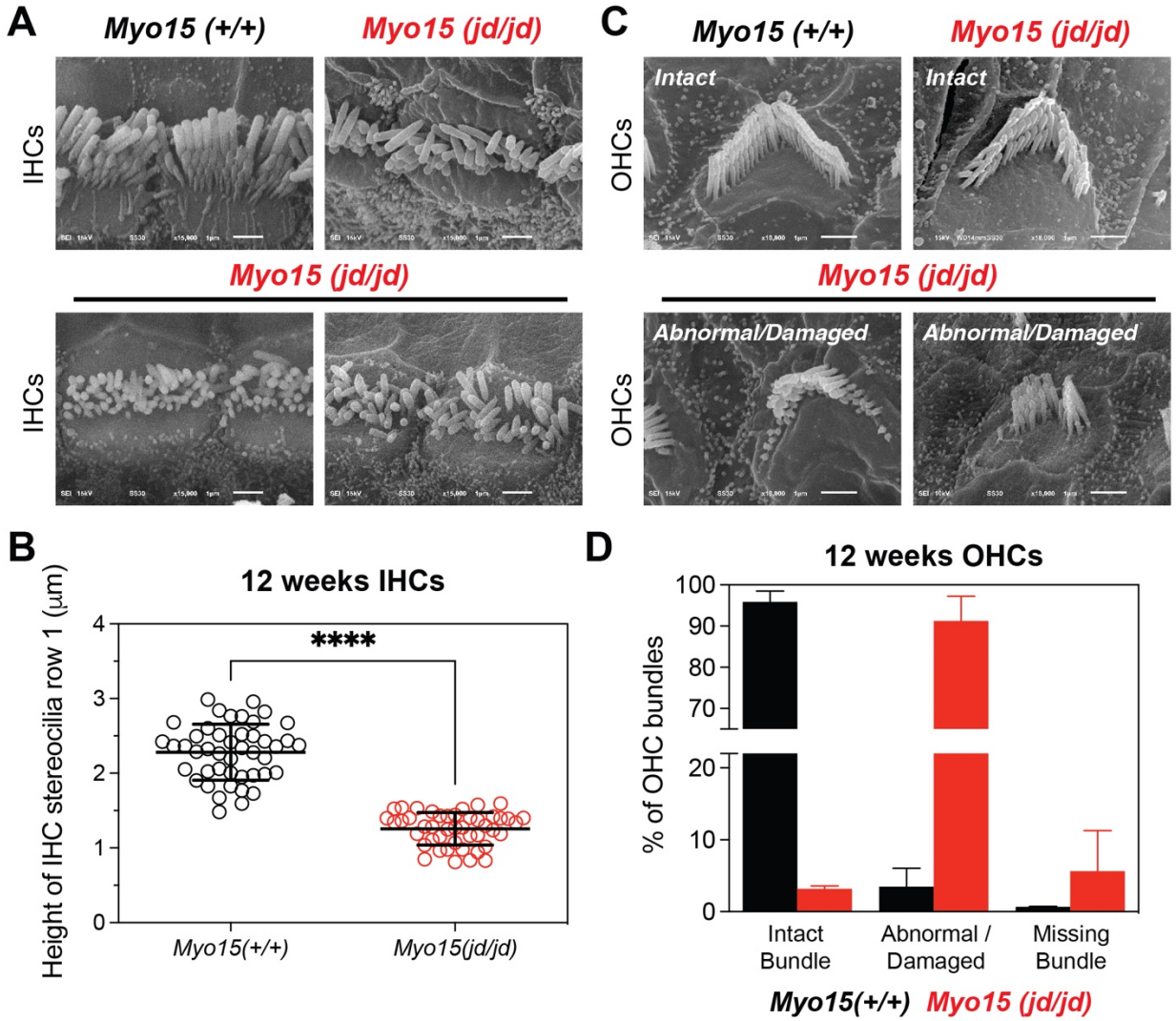
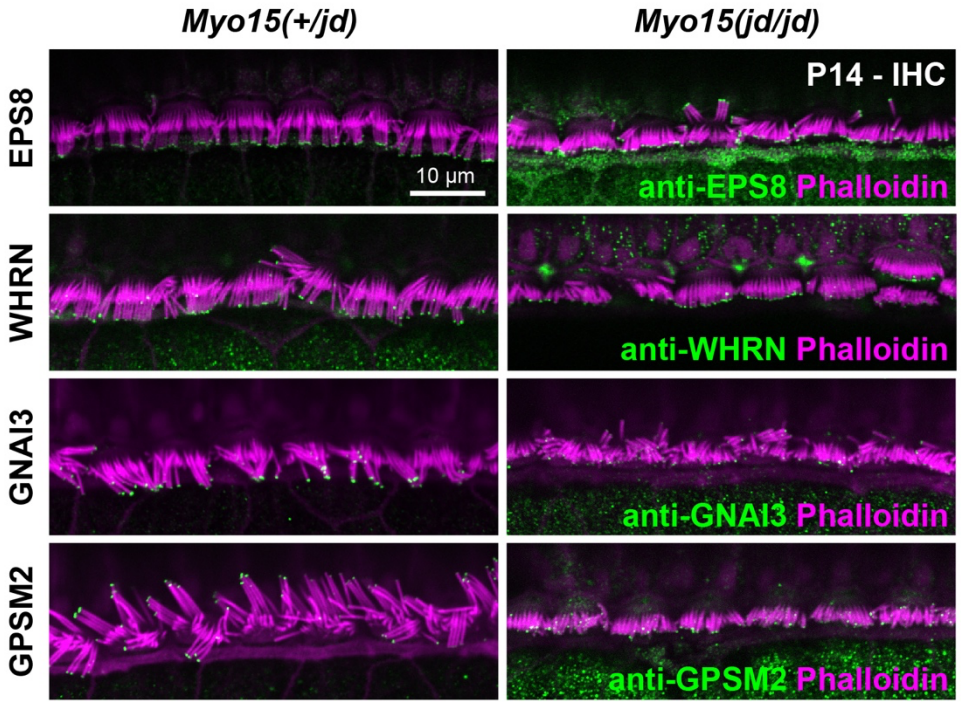

A
Myo15 (+/+)
Myo15 (jd/jd)
IHCs
Myo15 (jd/jd)
IHCs
C
Myo15 (+/+)
Myo15 (jd/jd)
OHCs
Intact
Intact
Myo15 (jd/jd)
OHCs
Abnormal/Damaged
Abnormal/Damaged
B
12 weeks IHCs
Height of IHC stereocilia row 1 (μm)
****
0
1
2
3
4
Myo15(+/+)
Myo15(jd/jd)
D
12 weeks OHCs
% of OHC bundles
100
90
80
70
20
10
0
Intact Bundle
Abnormal / Damaged
Missing Bundle
Myo15(+/+)
Myo15 (jd/jd)

FIGURE S3


Myo15(+/jd)
Myo15(jd/jd)
EPS8
WHRN
GNAI3
GPSM2
P14 - IHC
10 µm
anti-EPS8 Phalloidin
anti-WHRN Phalloidin
anti-GNAI3 Phalloidin
anti-GPSM2 Phalloidin