

1 **The dyslexia susceptibility gene KIAA0319 influences cilia**  
2 **length, cell migration and mechanical cell-substrate**  
3 **interaction**

4 Rebeca Diaz<sup>\*,1</sup>, Nils M. Kronenberg<sup>\*,2</sup>, Angela Martinelli<sup>1</sup>, Philipp Liehm<sup>2</sup>, Andrew C.  
5 Riches<sup>1</sup>, Malte C. Gather<sup>2</sup>, Silvia Paracchini<sup>1</sup>.

6 \* These authors have contributed equally to this paper

7 <sup>1</sup> School of Medicine, University of St Andrews, St Andrews, KY16 9TF, UK

8 <sup>2</sup> SUPA, School of Physics and Astronomy, University of St Andrews, St Andrews,  
9 KY16 9SS, UK

10 Corresponding authors:

11 Silvia Paracchini [sp58@st-andrews.ac.uk](mailto:sp58@st-andrews.ac.uk)

12 Malte Gather [mcg6@st-andrews.ac.uk](mailto:mcg6@st-andrews.ac.uk)

13

14 **Keywords**

15 **KIAA0319, neurodevelopment, dyslexia, cilia, podosomes, cytoskeleton,**  
16 **CRISPR, force microscopy, mechanobiology**

17 **Abstract**

18 Dyslexia is a common neurodevelopmental disorder with a strong genetic component.  
19 Independent genetic association studies have implicated the *KIAA0319* gene in  
20 dyslexia, however its function is still unknown.

21 We developed a cellular knockout model for KIAA0319 in RPE1 cells via CRISPR-  
22 Cas9n to investigate its role in processes suggested but not confirmed in previous  
23 studies, including cilia formation and cell migration.

24 We found that KIAA0319 knockout increased cilia length and accelerated cell  
25 migration. Using Elastic Resonator Interference Stress Microscopy (ERISM), we  
26 detected an increase in cellular force for the knockout cells that was restored by a  
27 rescue experiment. Combining ERISM and immunostaining showed that KIAA0319  
28 depletion reduced the number of podosomes formed by the cells.

29 Our results suggest an involvement of KIAA0319 in mechanosensing and force  
30 regulation and shows for the first time that podosomes exert highly dynamic,  
31 piconewton vertical forces in epithelial cells.

## 32 **Introduction**

33 Dyslexia is a neurodevelopmental disorder that affects around 5% of school-aged  
34 children and refers to unexpected difficulties in learning to read (Peterson &  
35 Pennington, 2012). In spite of the high heritability of dyslexia (up to 70%), very few  
36 candidate genes have been identified so far (Paracchini et al., 2016). Among those,  
37 *DYX1C1*, *DCDC2*, *ROBO1* and *KIAA0319* are supported by independent, family-  
38 based association studies and have been investigated at functional level (Newbury et  
39 al., 2014; Paracchini et al., 2016). Initial *in utero* gene silencing experiments in rats for  
40 these genes provided strong support for the neuronal migration hypothesis (Paracchini  
41 et al., 2007) first proposed in the eighties (Galaburda et al., 1985). This hypothesis is  
42 based on the observation of subtle cortical anomalies, i.e. heteropias and microgyrias,  
43 in post-mortem brains from individuals with dyslexia ( $n = 8$ ). Such anomalies are likely  
44 to be the result of neuronal migration defects. However, knockout mouse models for  
45 *DYX1C1*, *DCDC2* and *KIAA0319* did not present cortical alterations (Martinez-Garay  
46 et al., 2016; Rendall et al., 2015; Wang et al., 2011). Instead, some of the knockouts  
47 for these genes showed defects in auditory processing (Guidi et al., 2017; Truong et  
48 al., 2014). These results are consistent with defects in auditory processing underlying  
49 dyslexia (Tallal, 1980). Speech sound processing deficits were also described in adult  
50 rats that underwent either *Kiaa0319* or *Dyx1c1* silencing during embryonic  
51 development (Centanni, Booker, et al., 2014; Centanni, Chen, et al., 2014; Szalkowski  
52 et al., 2012; Threlkeld et al., 2007). Explanations for the discordance between  
53 knockdown experiments in rat and knockout mouse models included species-specific  
54 effects, compensatory mechanisms in mouse, or artefacts in shRNA experiments  
55 (Baek et al., 2014; Rossi et al., 2015). The discordance has also led to extensive  
56 reviews of the literature and to revisit the neuronal migration hypothesis (Galaburda,  
57 2018; Guidi et al., 2018).

58 In parallel, new roles for *DCDC2*, *DYX1C1*, *ROBO1* and *KIAA0319* have been  
59 described in cilia biology (Paracchini et al., 2016). Transcriptomic studies showed  
60 differential expression for these genes in ciliated tissue (Geremek et al., 2014; Hoh et  
61 al., 2012; Ivliev et al., 2012). Beyond these studies, a role of *KIAA0319* in cilia biology  
62 for has not been described yet, but cellular and animal *DCDC2* and *DYX1C1*  
63 knockouts presented cilia defects (Chandrasekar et al., 2013; Grati et al., 2015;  
64 Massinen et al., 2011; Schueler et al., 2015; Tarkar et al., 2013), and *ROBO1* has

65 been shown to localize to the cilium of mouse embryonic interneurons (Higginbotham  
66 et al., 2012). Mutations in *DYX1C1* and *DCDC2* have been identified in patients with  
67 ciliopathies, a group of disorders caused by defective cilia and often characterised by  
68 alterations in body asymmetry (Massinen et al., 2011; Schueler et al., 2015; Tarkar et  
69 al., 2013). *DCDC2* stabilizes the microtubules in the axoneme, and its overexpression  
70 causes elongation of the primary cilium. Lower numbers of cilia and atypical cilia length  
71 are common indicators of defects at this cellular structure, and have been implicated  
72 in developmental defects, such as craniofacial abnormalities and malformation of the  
73 CNS (Avasthi & Marshall, 2012). Cilia length is regulated by the interplay between  
74 actin depolymerisation and stabilisation in a tightly regulated process; however, the  
75 exact underlying regulation and involved proteins remain to be fully explained (Avasthi  
76 & Marshall, 2012). Cilia biology has been proposed as a molecular link to explain the  
77 atypical brain asymmetries which are consistently reported for neurodevelopmental  
78 disorders, such as dyslexia (Brandler & Paracchini, 2013). However, *KIAA0319*  
79 cellular function remains largely uncharacterised and most of what is known about this  
80 gene has been described through over-expression and knock-down experiments in  
81 human cell lines and in animal models. *KIAA0319* has been shown to undergo  
82 proteolytic processing, with a possible subsequent role in signalling pathways  
83 (Velayos-Baeza et al., 2010), and inhibits axon growth (Franquinho et al., 2017). A  
84 gene expression analysis in zebrafish showed very high expression in the first hours  
85 of development and specific signal in defined embryonic structures, including the  
86 notochord and the developing eye and optic vesicles (Gostic et al., 2019). *KIAA0319*  
87 encodes a transmembrane protein with five PKD domains (Velayos-Baeza et al.,  
88 2007) (Figure 1A). Such structures have been previously found in cell surface proteins  
89 and are known to be involved in cell-cell and cell-matrix interactions (Bycroft et al.,  
90 1999; Hughes et al., 1995; Ibraghimov-Beskrovnaya et al., 2000).

91 With this work, we seek to shed light on the role of *KIAA0319* in cilia formation and as  
92 a regulator of mechanical forces during cell migration. We generated the first cellular  
93 knockout model of *KIAA0319* in human cells to specifically investigate its role in cilia  
94 biology and neuronal migration, addressing the two main hypotheses currently  
95 proposed. We used retina pigmented epithelium cells (RPE1), which are particularly  
96 suitable to study cilia (Kim et al., 2015; Pugacheva et al., 2007), and studied their  
97 mechanobiology using the recently introduced Elastic Resonator Interference Stress

98 Microscopy (ERISM) (Kronenberg et al., 2017). ERISM allows for continuous imaging  
99 of cell forces with high spatial resolution and over extended periods of time. This is  
100 achieved by growing cells on a substrate that consists of a layer of an ultra-soft  
101 elastomer situated between two semi-transparent, mechanically flexible gold mirrors,  
102 which form an optical micro-cavity. Mechanical force and stress exerted by cells cause  
103 local deformations of this micro-cavity and thus local shifts to its resonance  
104 wavelengths. The resulting interference patterns are analysed by optical modelling in  
105 order to compute a high-resolution displacement map with  $\mu\text{m}$  lateral resolution and  
106 nm vertical displacement resolution (Liehm et al., 2018), which allows for the detection  
107 of forces in the Piconewton range. Unlike many other stress microscopy techniques,  
108 ERISM does not require a zero-stress reference image, so cells can be kept on the  
109 substrate for time-lapse imaging and immunostaining. The low probe light intensity  
110 and mechanical stability of the micro-cavity substrate enable long-term measurements  
111 of cell forces without phototoxic effects or mechanical degradation of the substrate.

112 Our data show that loss of KIAA0319 in RPE1 cells leads to the formation of longer  
113 cilia and to an increase in cellular force. The force phenotype of the wild type was  
114 rescued by expressing a KIAA0319-GFP fusion construct in the knockout cells. Our  
115 data further indicate that KIAA0319 knockout cells form fewer podosomes, a special  
116 type of cell-matrix contact that in the past been has shown to have mechanosensitive  
117 function (Labernadie et al., 2014). Using ERISM we were able to show that these  
118 podosomes exert oscillating, vertical forces, possibly for mechanical probing of the  
119 substrate. Our measurements present the first observation of mechanical activity of  
120 podosomes in epithelial cells. The results of this study show that KIAA0319 knockout  
121 strongly affects the mechanical phenotype of RPE1 cells and suggest a function of  
122 KIAA0319 in mechanosensing and force regulation.

## 123 **Results**

### 124 ***Generation of KIAA0319 KO in RPE1 cell lines***

125 We generated a KIAA0319 knockout model in RPE1 cells by introducing random  
126 modifications in exon 6 with CRISPR-Cas9n based genome editing. The *KIAA0319*  
127 main isoform (NM\_014809) consists of 21 exons and spans 102 kb of human  
128 chromosome 6 (Figure 1A). We generated a biallelic knockout (Ex6KO) by causing  
129 deletions that introduce premature stop codons in the reading frame of *KIAA0319*

130 using paired gRNAs (Figure 1B). The deletion was confirmed by RT-PCR (Figure 1C).  
131 Transcript quantification by qRT-PCR shows that KIAA0319 expression in Ex6KO is  
132 five-times lower than the wild-type ( $t$ -test,  $p < 0.001$ ) consistently with nonsense-  
133 mediated decay (Baker & Parker, 2004) (Figure 1D).

#### 134 ***KIAA0319 knockout cells form longer cilia***

135 For assessment of KIAA0319 involvement in cilia formation, cilia length in RPE1 wild  
136 type (WT) and Ex6KO cells were measured by staining of the cilium-specific protein  
137 ARL13B and analysis of epi-fluorescence images (Figure 2A & B). While a similar  
138 fraction of WT and Ex6KO cells formed cilia (WT: 379/571, 68%; Ex6KO: 271/383,  
139 70%), the cilia in Ex6KO were significantly longer than in the wild type (mean  $\pm$  SEM:  
140 WT: 4.5  $\mu\text{m} \pm 0.1 \mu\text{m}$ ,  $n = 129$ ; Ex6KO: 6.1  $\mu\text{m} \pm 0.2 \mu\text{m}$ ,  $n = 104$ ;  $t$ -test:  $p < 0.001$ ;  
141 Figure 2C).

#### 142 ***Cell morphology, migration speed and force exertion are altered in KIAA0319*** 143 ***knockout cells***

144 We performed a scratch assay on a confluent layer of cells to test collective cell  
145 migration. This assay did not reveal a significant difference between the collective  
146 migratory speed of WT and Ex6KO cells after 24 h (mean cell coverage  $\pm$  SEM: WT:  
147 27.4%  $\pm 4.2\%$ ,  $n = 3$ ; Ex6KO: 30.2%  $\pm 3.5\%$ ,  $n = 3$ ;  $t$ -test:  $p = 0.63$ ; Supplementary  
148 Figure 1).

149 Next, we tested the migration of single cells. WT and Ex6KO cells were plated on  
150 ERISM substrates with an effective stiffness of 6 kPa at a density low enough to  
151 ensure non-confluency and thus allow mapping of the forces exerted by individual cells  
152 (Figure 3A). Ex6KO cells covered a smaller surface area than WT cells  
153 (mean cell area  $\pm$  SEM: WT: 2052  $\mu\text{m}^2 \pm 91 \mu\text{m}^2$ ,  $n = 36$ ; Ex6KO: 1295  $\mu\text{m}^2 \pm 65 \mu\text{m}^2$ ,  
154  $n = 36$ ;  $t$ -test:  $p < 0.001$ ; Figure 3B), even though the shape and morphology of the  
155 cells did not differ. The displacement maps recorded with ERISM (Figure 3A) revealed  
156 that cells from both lines exert similar force patterns on their substrate: pulling was  
157 focused around the two long ends of the cells, perpendicular to the direction of  
158 migration (cells were polarised in a way that the nucleus was positioned posterior to  
159 the direction of migration). Downward compression was observed underneath the  
160 centre of the cells. This displacement pattern is a fingerprint for the exertion of

161 contractile forces by adherent cells (Kronenberg et al., 2017). (See next section for  
162 discussion of the other features in the ERISM map).

163 The migratory behaviour and the associated dynamics of force exertion of WT and  
164 Ex6KO cells were then investigated by taking time-lapse measurements of phase  
165 contrast and ERISM displacement maps in five-minute intervals over a time span of  
166 17 hours (Supplementary Movie 1 & 2). The average speed of single cell migration  
167 was significantly higher for Ex6KO than for WT cells (mean speed  $\pm$  SEM: WT:  
168  $0.33 \mu\text{m min}^{-1} \pm 0.03 \mu\text{m min}^{-1}$ ,  $n = 10$ ; Ex6KO:  $0.44 \mu\text{m min}^{-1} \pm 0.04 \mu\text{m min}^{-1}$ ,  
169  $n = 13$ ,  $t$ -test:  $p = 0.04$ ; Figure 3C). This result contrasted with the results from the  
170 scratch assay for collective cell migration (Supplementary Figure 1). The directness of  
171 the migration was not affected by the KIAA0319 knockout (Supplementary Figure 2A).

172 To assess the mechanical activity of cells, we compute the total volume by which each  
173 cell indents into the substrate and use this as a proxy for the applied force. Comparing  
174 the temporal averages of applied force during migration shows that Ex6KO cells exert  
175 significant stronger contractile forces on the substrate than WT cells (mean indented  
176 volume  $\pm$  SEM: WT:  $167 \mu\text{m}^3 \pm 19 \mu\text{m}^3$ ,  $n = 10$ ; Ex6KO:  $319 \mu\text{m}^3 \pm 39 \mu\text{m}^3$ ,  $n = 13$ ;  $t$ -  
177 test:  $p = 0.004$ , Figure 3D). Figure 3E summarizes the temporal evolution of migratory  
178 speed and applied force for a representative WT and Ex6KO cell, respectively. The  
179 Ex6KO cell showed more pronounced fluctuations in speed and applied force than the  
180 WT cell.

181 To analyse the temporal evolution of mechanical activity in more detail, we computed  
182 the temporal Fourier transform of the data. This revealed that the mean amplitude of  
183 oscillations in both migratory speed and applied force is larger for Ex6KO cells than  
184 for WT cells (amplitude of oscillations in migratory speed increased by mean factor of  
185 1.4 over the analysed frequency range; amplitude of oscillations in indented volume  
186 increased by mean factor of 1.5 over the analysed frequency range; WT:  $n = 11$ ,  
187 Ex6KO:  $n = 13$ ; Supplementary Figure 2B & C). However, neither of the two oscillation  
188 amplitudes were increased at a statistically significant level (mean amplitude of  
189 oscillation in migration speed:  $t$ -test:  $p = 0.06 - 0.54$ ; mean amplitude of oscillation in  
190 indented volume:  $t$ -test:  $p = 0.16 - 0.49$ ). The time traces in Fig. 3E also show that for  
191 the Ex6KO cell occasional single events of high migration speed were correlated with  
192 a drop in exerted force (indicated by red, vertical lines).

193 To validate our findings of the impact of KIAA0319 on cell force exertion, we conducted  
194 a rescue experiment by generating an Ex6KO cell line with stable expression of  
195 KIAA0319-GFP fusion protein (Ex6KO K-GFP; Figure 4A). We also generated a  
196 control line of RPE1 WT cells with the same construct (WT K-GFP). Even though the  
197 KIAA0319 rescue did not recover the reduction in cell area seen for Ex6KO cells  
198 [mean cell area  $\pm$  SEM: WT:  $2315 \mu\text{m}^2 \pm 200 \mu\text{m}^2$ ,  $n = 16$ ; WT K-GFP:  
199  $2299 \mu\text{m}^2 \pm 107 \mu\text{m}^2$ ,  $n = 20$ ; Ex6KO:  $1565 \mu\text{m}^2 \pm 123 \mu\text{m}^2$ ,  $n = 23$ ; Ex6KO K-GFP:  
200  $1297 \mu\text{m}^2 \pm 131 \mu\text{m}^2$ ,  $n = 17$ ; Figure 4B], the level of cell force was restored in Ex6KO  
201 K-GFP cells [mean indented volume  $\pm$  SEM: WT:  $115 \mu\text{m}^3 \pm 14 \mu\text{m}^3$ ,  $n = 16$ ; WT K-  
202 GFP:  $96 \pm 9 \mu\text{m}^3$ ,  $n = 19$ ; Ex6KO:  $186 \pm 20 \mu\text{m}^3$ ,  $n = 24$ ; Ex6KO K-GFP:  
203  $125 \pm 16 \mu\text{m}^3$ ,  $n = 16$ ;  $t$ -test(WT vs. Ex6KO):  $p = 0.01$ ,  $t$ -test(WT vs. Ex6KO K-GFP):  
204  $p = 0.67$ ; Figure 4C)].

### 205 ***RPE1 KIAA0319 WT and Ex6KO show different fine patterns of force exertion***

206 Given the differences in cilia length, cell size, migration speed and exerted force we  
207 reasoned that KIAA0319 knockout might affect cytoskeleton dynamics. To test this  
208 hypothesis, we took phase contrast and ERISM time-lapse measurements of migrating  
209 WT and Ex6KO cells at 5 seconds intervals (Supplementary Movie 3 & 4), and fixed  
210 and immunostained the cells for actin and vinculin immediately after the time-lapse.  
211 Spatial Fourier-filtering of ERISM maps can be used to filter out broad deformation  
212 features associated with the overall contractility of cells and thus resolve finer details  
213 linked to interaction of sub-cellular components, e.g. focal adhesions or podosomes,  
214 with the substrate (Kronenberg et al., 2017). (For further discussion on the  
215 displacement fine-structure in Fourier-filtered displacement maps see Supplementary  
216 Figure 3.) Figure 5A shows phase contrast images, Fourier-filtered ERISM maps and  
217 immunofluorescence microscopy images for a WT and Ex6KO cell. The Fourier-  
218 filtered displacement maps of both cells show small push-pull features that co-  
219 localised with vinculin-rich areas in the immunofluorescence microscopy images.  
220 Insets ii) and iii) in Figure 5A highlight examples of such areas for the WT and the  
221 Ex6KO cell, respectively. Vinculin is enriched in the centre between pulling (red areas  
222 in Fourier-filtered ERISM maps) and pushing (green areas), and actin fibres are  
223 connected to vinculin on the pulling site. Push-pull features in Fourier-filtered ERISM  
224 maps were previously attributed to focal adhesions transmitting contractile, mostly  
225 horizontal forces to the substrate that are generated by the actin cytoskeleton



226 (Kronenberg et al., 2017). In agreement with these earlier observations, in the Ex6KO  
227 cell, the axes defined by the push-pull features co-aligned with the actin fibres that  
228 connect different vinculin-rich sites (see Figure 5A and Supplementary Figure 3). This  
229 push-pull behaviour is also consistent with earlier observations of torque being applied  
230 by focal adhesions (Legant et al., 2013). Since the formation and alignment of stress  
231 fibres is less distinct in the WT cell, the above-mentioned co-alignment of actin,  
232 vinculin and the ERISM push-pull features is also less pronounced for the WT cell. In  
233 agreement with this, the forces exerted by single focal adhesions are smaller in the  
234 WT cell (Figure 5C & D).

235 Beside focal adhesions, the Fourier-filtered ERISM displacement map of the WT cell  
236 also showed tightly confined pushing sites with a diameter of about 2  $\mu\text{m}$  (green-blue  
237 areas highlighted with black arrow heads in Fourier-filtered ERISM map of Figure 5A).  
238 These pushing sites were surrounded by circularly arranged dots of upward pulling  
239 (red areas). Immunocytochemistry analysis showed that the pushing sites  
240 corresponded to actin-rich locations (white arrow heads in epi-fluorescence image of  
241 Figure 5A), whereas pulling around the pushing sites colocalised with vinculin-rich  
242 positions (in inset i) in Figure 5A). This protein arrangement is a hallmark for  
243 podosomes, a cellular adhesion structure that is chiefly formed in monocyte-derived  
244 cells (Linder & Wiesner, 2016) but that has also been reported in spreading and  
245 migrating epithelia cells (Spinardi et al., 2004).

246 The time-lapse measurement revealed that the podosomes exerted an oscillating  
247 vertical force, that reached maximum values of up to 80 pN (Figure 5B). The horizontal  
248 contractile forces exerted by focal adhesions were roughly 100-times larger than the  
249 vertical indentation forces exerted by podosomes (Figure 5B & D). However, while  
250 podosomal pushing was highly dynamic, the horizontal forces originating from focal  
251 adhesions were relatively static and showed little oscillation in force. Focal adhesions  
252 at the leading edge of the cell were chiefly stationary once assembled (top right in  
253 Supplementary Movie 4) and any lateral movement of focal adhesions was confined  
254 to the trailing edge of the cell (bottom left in Supplementary Movie 4).

255 The WT and Ex6KO cell shown in Figure 5 and Supplementary Movie 3 & 4 are  
256 examples illustrating the general differences between the two force transmission  
257 patterns (podosomes and focal adhesion). In total, combined ERISM and

258 immunochemistry measurements were carried out for 33 cells (see  
259 Supplementary Figure 4 and Supplementary Movie 5 & 6 for further examples). While  
260 both WT and Ex6KO cells form podosomes (Supplementary Figure 4), their  
261 prevalence was higher in WT cells; while 63% of the investigated WT cells formed  
262 podosomes (10 out of 16), they were only observed in 18% of Ex6KO cells (3 out of  
263 17).

## 264 **Discussion**

265 We successfully developed a cellular knockout model via CRISPR-Cas9n to study the  
266 potential role of the *KIAA0319* gene in cilia biology and cell migration. Sequencing  
267 confirmed loss-of-function deletions in the sixth exon of *KIAA0319* and qRT-PCR  
268 analysis showed a strong decrease in the expression of *KIAA0319*, consistent with  
269 nonsense mediated decay of the transcript (Figure 1). We set out to characterise this  
270 cellular model to investigate specifically the role of *KIAA0319* in cilia biology and  
271 neuronal migration on the basis of the dominating hypothesis proposed by the  
272 literature (Paracchini et al., 2016) .

273 Although the same fraction of *KIAA0319* Ex6KO and WT cells developed cilia, these  
274 were significantly longer in the knockout (Figure 2C). Cilia biology is emerging as a  
275 contributing factor to a range of diseases, including neurodevelopmental disorders and  
276 dyslexia (Paracchini et al., 2016). Other dyslexia candidate genes have been reported  
277 to affect cilia length. *DYX1C1* knockouts present shorter cilia than the wild type in  
278 zebrafish (Chandrasekar et al., 2013), and overexpression of *DCDC2* increases cilia  
279 length in rat neurons (Massinen et al., 2011). The only evidence in support of a role of  
280 *KIAA0319* in cilia comes from transcriptomic studies (Geremek et al., 2014; Hoh et al.,  
281 2012; Ivliev et al., 2012). Our work is therefore the first study to support a role for  
282 *KIAA0319* in cilia biology in a biological model. In turn, these data further support the  
283 role of cilia in neurodevelopment. When assessing collective cell migration using the  
284 commonly used scratch assay, we did not observe a significant effect of the *KIAA0319*  
285 knockout (Supplementary Figure 1). However, investigation of single cell migration on  
286 soft ERISM substrates showed that single knockout cells move significantly faster than  
287 wild type cells (Figure 3C). These contradictory findings can be explained by several  
288 factors: the apparent stiffness of the ERISM sensor is in the range of soft tissue  
289 (1 to 20 kPa) and significantly different from the stiffness of the cell culture plastic plate

290 in which the scratch assay was performed (~100,000 kPa) (Skardal et al., 2013).  
291 Substrate stiffness has a strong influence on cell migration in vitro (Bangasser et al.,  
292 2017). Furthermore, single and collective cell migration are affected by different  
293 factors. Finally, while cells respond to an acute event, namely local damage, in the  
294 scratch assay, the ERISM assay observes the migration of undisturbed cells.  
295 Additionally, ERISM analysis revealed that the knockout cells exert significantly strong  
296 forces on their substrate compared to the wild type (Figure 3D). A rescue experiment  
297 recovered mechanical activity of the wild type phenotype (Figure 4C) supporting an  
298 involvement of KIAA0319 in cellular forces. In addition to the higher cell forces in  
299 Ex6KO cells, Ex6KO cells showed stronger temporal oscillation of cell force and  
300 migration speed (even though not at a statistically significant level). They also showed  
301 more frequent correlated events of high migration speed and a drop in exerted force.

302 Fluorescent staining revealed the presence of actin-rich spots surrounded by vinculin  
303 rings in the studied cell lines (Figure 5). Previously, such local actin cores have been  
304 reported to associate with podosomes. By combining fluorescent staining with Fourier-  
305 filtered ERISM measurements, we found that the actin cores of podosomes protruded  
306 vertically into the substrate, exerting oscillating forces of up to 80 pN, while  
307 surrounding rings of pulling sites were tightly colocalised with vinculin. To the best of  
308 our knowledge, this is the first report on direct force exertion by podosomes in epithelial  
309 cells. WT cells formed podosomes more often than Ex6KO cells.

310 The link between the functionality of KIAA0319 and the observed phenotypical  
311 changes may originate from the molecular structure of the protein. KIAA0319 is a  
312 transmembrane protein that contains five PKD domains. These domains have been  
313 described in very few human proteins, among which the best characterised is  
314 Polycystin-1 (PC1). PC1 acts as a mechanosensor in the membrane of cilia  
315 (Dalagiorgou et al., 2010), most probably by unfolding of the highly extensible PKD  
316 domains in response to stretching forces. It has been proposed that this unfolding  
317 maintains neighbouring cells in contact during cell movement (Qian et al., 2005). PC1  
318 interacts with the cytoskeleton (Boca et al., 2007) and plays an important role in  
319 adaptative cilia shortening (for example under strong flow) (Besschetnova et al.,  
320 2010). PC1 therefore mediates both cilia length and mechanosensing properties. Our  
321 results suggest that KIAA0319 has a similar function to PC1 affecting both cilia  
322 formation and mechanosensing. Our data show that knockout of KIAA0319 not only

323 results in formation of longer cilia, but also in dysregulation of mechanical forces which  
324 impairs migration behaviour. We observed that higher cell forces lead to increased  
325 fluctuations in the migration pattern, increasing oscillations of cell speed and force.  
326 KIAA0319 knockout also results in the formation of fewer podosomes. Podosomes are  
327 cell-matrix contacts; their function ranges from cell-matrix adhesion and matrix  
328 degradation (facilitating cell invasion) to mechanosensing (Linder & Wiesner, 2016).  
329 They are especially prominent in cells of the monocytic lineage but have also been  
330 reported in migrating and spreading epithelial cells, where they were found to  
331 associate with hemidesmosomes (Spinardi et al., 2004). Hemidesmosomes are  
332 adhesive structures specific to epithelial cells that regulate a wide range of biological  
333 processes, including among others cell migration, exertion of traction force and  
334 mechanosensing (Grashoff et al., 2010; Hiroyasu et al., 2016; Spinardi et al., 2004;  
335 Walko et al., 2015; Zhang et al., 2011). Direct measurements of the forces exerted by  
336 podosomes in epithelial cells have not been reported in the literature so far and our  
337 work shows for the first time that epithelial podosomes mechanically probe the  
338 environment by exerting oscillating forces in the pN-range, similarly to what has been  
339 previously described for podosomes formed by macrophages (Kronenberg et al.,  
340 2017; Labernadie et al., 2014). Podosome formation was reduced in the Ex6KO cells  
341 compared to the WT, which suggests an involvement of KIAA0319 in cellular  
342 mechanosensing.

343 While our work shows that knockout of KIAA0319 affects cytoskeleton dynamics, the  
344 pathways involved in this regulation are not yet known. Earlier studies have also  
345 suggested a link between KIAA0319 function and cytoskeleton regulation. KIAA0319  
346 over-expression inhibits axon growth and KIAA0319 knockout results in neurite  
347 outgrowth (Franquinho et al., 2017), two processes controlled by cytoskeleton  
348 filaments. Genes with roles in microtubule cytoskeleton function have been found to  
349 be associated with other neurodevelopmental disorders including schizophrenia,  
350 depression, bipolar disorder (Marchisella et al., 2016) and autism (Lin et al., 2016).

351 In summary, this study advances our understanding of the cellular function of the  
352 KIAA0319 dyslexia susceptibility gene. Our data contributes to the current debate  
353 about the role of cell migration and cilia biology in dyslexia, showing that the KIAA0319  
354 is involved in mechanosensation and control of cytoskeletal dynamics. These

355 processes are likely to play important roles during brain development and may  
356 contribute to neurodevelopmental disorders.

## 357 ***Materials and Methods***

### 358 ***Cell culture***

359 hTERT-RPE1 cells were generated by transfection with pGRN145, which expresses  
360 hTERT under the control of the MPSV promoter, and were kindly supplied by Dr.  
361 Andrea Bodnar, Geron Inc. Cell lines were cultured in DMEM F12 with 10% of fetal  
362 bovine serum and 1% Penicillin/Streptomycin, or in serum-free media (DMEM F12  
363 with 1% Penicillin/Streptomycin) at 37 °C and 5% CO<sub>2</sub>.

### 364 ***Plasmids***

365 pSPgRNA was a gift from Charles Gersbach (Addgene plasmid #47108) (Perez-  
366 Pinera et al., 2013). pSPCas9n-2A-GFP (pSpCas9n(BB)-2A-GFP (PX461)) was a gift  
367 from Feng Zhang (Addgene plasmid #48140) (Ran et al., 2013). KIAA0319-GFP was  
368 a gift from Antonio Velayos-Baeza (Velayos-Baeza et al., 2008).

### 369 ***Cloning and transfection***

370 KIAA0319 knockout cell lines were generated through a CRISPR-Cas9 double nicking  
371 strategy designed with the web-based tool developed by Hsu and collaborators  
372 (<http://crispr.mit.edu>) (Hsu et al., 2013). This strategy uses Cas9 nickase (Cas9n), a  
373 modified Cas9 in which one of the nuclease domains has been mutated, lowering the  
374 rate of off-target effects compared to Cas9 (Ran et al., 2013). RPE1 cells were  
375 transfected with pSpCas9n(BB)-2A-GFP (PX461) and paired gRNAs, using  
376 Lipofectamine3000 (ThermoFisher). gRNAs were generated by cloning annealed  
377 oligonucleotides containing the protospacer sequence into the chimeric gRNA  
378 sequence in pSPgRNA linearised with BbsI, downstream of a U6 promoter  
379 (Supplementary Table 1). Sequences targeted were AGCCACCCCACAGACTACCA  
380 and TAAATTCCATTCATAGTTGT on KIAA0319 exon 6. pSpCas9n(BB)-2A-GFP  
381 (PX461) contains a GFP expression cassette that acts as indicator of positive  
382 transfection. Twenty-four hours after transfection, 384 individual GFP positive cells  
383 (four 96 well plates) were isolated using Fluorescence Activated Cell Sorting (FACS)  
384 and plated onto 96 well plates coated with Poly-D-Lysine for clonal expansion.

## 385 **Screening**

386 Fifty cells were successfully expanded for further analysis. PCR was performed in all  
387 clones using primers int6-7R and int5-6F, that amplify a 1311 sequence DNA flanking  
388 the site targeted with the gRNAs (Supplementary Table 1). Amplicons were digested  
389 with the restriction enzyme Styl. One of the used gRNAs targets this sequence, hence  
390 mutations caused by this gRNA are likely to eliminate this site. Amplicons from the 7  
391 clones that showed loss of a Styl site upon digestion were cloned into Zero Blunt  
392 TOPO (ThermoFisher K280020) and sequenced using primers SP6 and T7. We  
393 identified one of these lines as a homozygous knockout as it contains two types of  
394 deletions causing frameshifts and premature stop codons.

## 395 **Immunofluorescence**

396 Cells on the ERISM micro-cavity were fixed with 4% paraformaldehyde (PFA) in PBS  
397 at room temperature for 20 minutes. Immediately after fixation, cells were  
398 permeabilised with 0.1% Triton X-100 for 3 minutes and blocked for 30 minutes with  
399 1% BSA in PBS. Cells were then stained for vinculin using anti-vinculin antibody  
400 (Merck Millipore, cat. no. 90227, 1:250 in BSA solution, 1 hour at room temperature)  
401 and for actin using TRITC-conjugated phalloidin (Merck Millipore, cat. no. 90228, 1:500  
402 in BSA solution, 1 hour at room temperature). The nuclei of the cells were stained with  
403 DAPI (Merck Millipore, 1:1,000 in BSA), at room temperature for 3 minutes.

404 RPE1 cells for cilia analysis were cultured on uncoated coverslips for 24 hours with  
405 serum-free media, fixed with 4% PFA for 10 minutes, permeabilised with 0.1% Triton  
406 X-100, blocked with 10% goat serum in PBS, and stained with the ciliary marker  
407 ARL13B Antibody Rabbit polyclonal (17711-1-AP Proteintech) and anti-gamma-  
408 tubulin (Abcam 11316). Under serum starvation, cells stay in G<sub>0</sub> and form cilia. We  
409 measured the length of the cilia manually using ImageJ. To ensure that cilia were  
410 positioned flat against the surface of the cell, only cilia that were completely in focus  
411 were considered.

## 412 **Gene expression quantification**

413 qRT-PCRs were performed using Luna OneStep reagent (NEB) on biological  
414 triplicates. KIAA0319 expression was assessed with primers ex11F and ex12R  
415 (Supplementary Table 1). Analysis was performed by the  $\Delta\Delta C_t$  method using Beta-

416 actin as endogenous control. Results were normalised against expression in WT cells.  
417 Error bars are calculated using the standard deviation of the triplicates ( $2^{\Delta\Delta Ct-s.d} - 2^{\Delta\Delta Ct+s.d}$ ).  
418 s.d).

#### 419 **Western blot**

420 Protein lysates were obtained from all cell lines using RIPA buffer and separated in a  
421 NuPAGE Bis-Tris 4-12% gradient gel (ThermoFisher). Proteins were transferred to a  
422 nitrocellulose membrane, blocked in WesternBreeze blocker (ThermoFisher) and  
423 incubated with primary antibodies anti-GFP (Chromotek #029762) and anti-beta actin  
424 (Sigma). Secondary antibodies were donkey anti-rat and anti-mouse HRP conjugated.  
425 Membranes were developed using SuperSignal WestFemto substrate  
426 (ThermoFisher).

#### 427 **Scratch assay**

428 The scratch assay is a simple way to measure cell migration in vitro and consists on  
429 creating a “scratch” on a confluent layer of cells and quantifying the movement of the  
430 cells over time to close this gap (Liang et al., 2007). Since this test is performed in  
431 serum free culture conditions, which prevent the cells from dividing, it only takes into  
432 account cell movement and not proliferation. Wild type and Ex6KO cell lines were  
433 plated on a 6-well plate. When confluent, the layer of cells was scratched with a pipette  
434 tip creating a straight gap. Cells were then washed with PBS to remove media and  
435 floating cells, and serum free media was added. We took images covering the whole  
436 gap at the time of the scratch (time 0) and after 24 hours. We measured the width of  
437 the scratch using TScratch (Geback et al., 2009), and calculated the mean width for  
438 each cell line after 24 hours.

#### 439 **ERISM measurements**

440 ERISM substrates were fabricated as described previously (Kronenberg, 2017) and  
441 four silicon chambers (surface area: 0.75 x 0.75 cm<sup>2</sup>; Ibidi) were applied. RPE1 cells  
442 were seeded on the ERISM substrate at 1,000 cells per well and kept at 37 °C, 5%  
443 CO<sub>2</sub> culture conditions in DMEM-12 supplemented with 10% FBS and 1%  
444 Penicillin/Streptomycin. WT and Ex6KO cells as well as WT, WT\_K-GFP, Ex6KO and  
445 Ex6KO\_K-GFP cells were investigated in different wells on the same ERISM chip.  
446 Prior to ERISM measurements, cells were cultured for 24 h to allow adhesion to

447 complete. ERISM force measurements were performed and converted into  
448 displacement maps as described before (Kronenberg et al., 2017). To investigate  
449 forces during cell migration, ERISM maps were recorded continuously for 17 h at  
450 intervals of 5 minutes, recording from seven different positions within each of the  
451 respective wells with a x20 objective. To analyse the force exertion patterns, ERISM  
452 measurements were performed at higher frame rate (every 5 s or 2 min) and  
453 magnification (x40 objective). To generate the Fourier-filtered ERISM maps, a FFT  
454 bandpass filter was applied to the raw displacement maps using the ImageJ software.  
455 For cell force analysis, the volume by which migrating cells indent into the ERISM  
456 substrate was calculated using ImageJ. All pixels in the ERISM displacement maps  
457 with indentation of less than 20 nm were set to NaN's (not a number) and the "indented  
458 volume" under each individual cell was calculated as the product of area and mean  
459 indentation. Only cells that moved freely for >4 h (i.e. that were not in physical contact  
460 with other cells) were included in the analysis.

461 The "indentation force" of a single podosome protrusion was calculated by converting  
462 spatial Fourier-filtered ERISM displacement maps with a cut-off frequency of  $0.6 \mu\text{m}^{-1}$   
463 into stress maps using FEM as described in Kronenberg, 2017. Podosome protrusions  
464 were identified in stress maps as isolated, localised indentation surrounded by a ring  
465 of pulling. Indentation force was calculated as the product of indentation area and  
466 mean applied stress at a threshold of 4 Pa. Only structures that colocalise with actin-  
467 dots in the respective immunostaining image were analysed.

468 To calculate the "contraction force" of single focal adhesions, the twist in spatial  
469 Fourier-filtered ERISM displacement maps with a cut-off frequency of  $0.6 \mu\text{m}^{-1}$  were  
470 analysed and converted into the corresponding horizontally exerted contractile forces  
471 as described in Kronenberg, 2017. In short, twisting results from the torque applied by  
472 focal adhesions when transmitting contractile cell forces to the ERISM substrate. The  
473 twisting response of ERISM substrates was calibrated by applying horizontal forces  
474 using AFM. The amount of twisting was found to be directly proportional to the applied  
475 force (6.6 nm of twist per 1 nN of applied force;  $R^2 > 0.99$ ;  $n = 5$  force measurements).  
476 Only twists in ERISM displacement maps that form around vinculin-rich areas in the  
477 respective immunostaining image were analysed.



478 The “directness” of cell migration was calculated as the product of euclidean distance  
479 and accumulated distance relative to the position of the cell at the start of the  
480 measurement. The speed of the cells on the ERISM sensor was measured using the  
481 plugin Manual Tracking on ImageJ (Schneider et al., 2012).

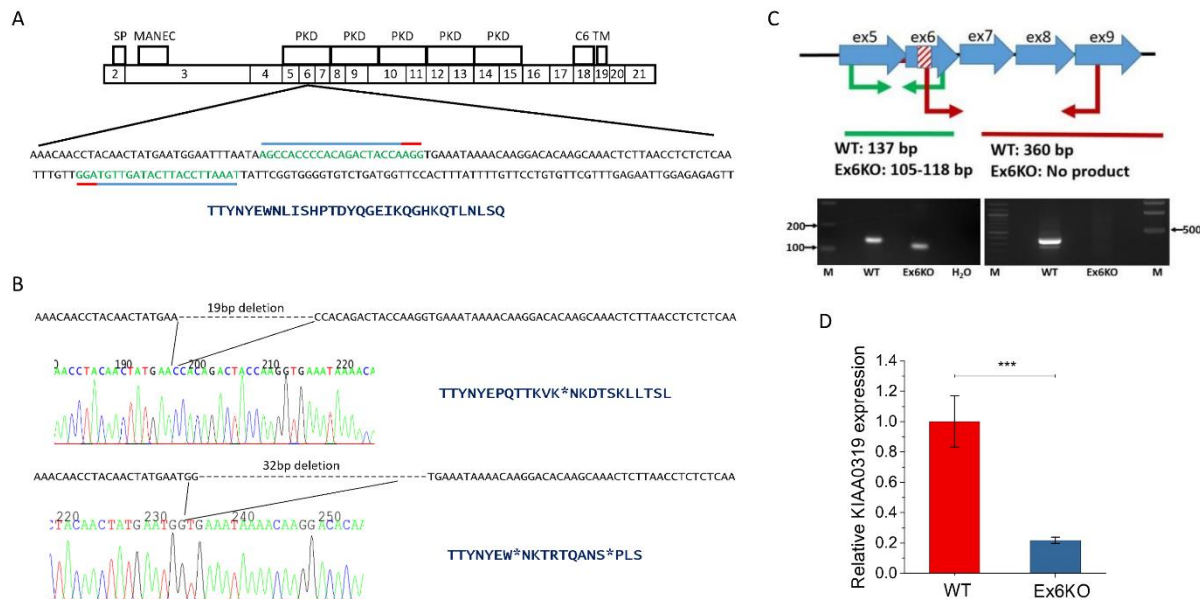
#### 482 ***Generation of cell lines expressing KIAA0319-GFP***

483 RPE1 wild type and Ex6KO were transfected with linearised KIAA0319-GFP plasmid  
484 using Lipofectamine 3000 according to the manufacturer’s specifications. KIAA0319-  
485 GFP contains a neomycin resistance cassette that was used to select cells that had  
486 undergone stable transfection, integrating the construct in their genome. Stably  
487 transfected cells were selected with G418 (Roche) at a concentration of 400  $\mu\text{g ml}^{-1}$   
488 for 2 weeks. Cells tend to lose the expression of the transgene with time (Mutskov &  
489 Felsenfeld, 2004), and after a few passages of this cell line, GFP expression was  
490 detected in only a small percentage of the cells. To enrich cells expressing the  
491 construct, we selected GFP positive cells via FACS. After FACS selection, cells were  
492 kept in culture for 24 hours to allow them to recover, and then plated onto the ERISM  
493 microcavity for measurement.

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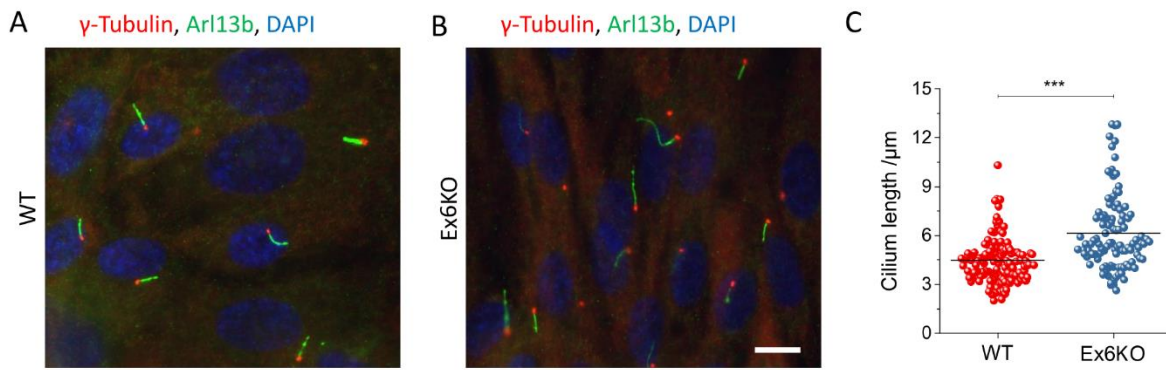
501 **Figures**



502

503 **Figure 1: Generation of a cellular KIAA0319 knock out**

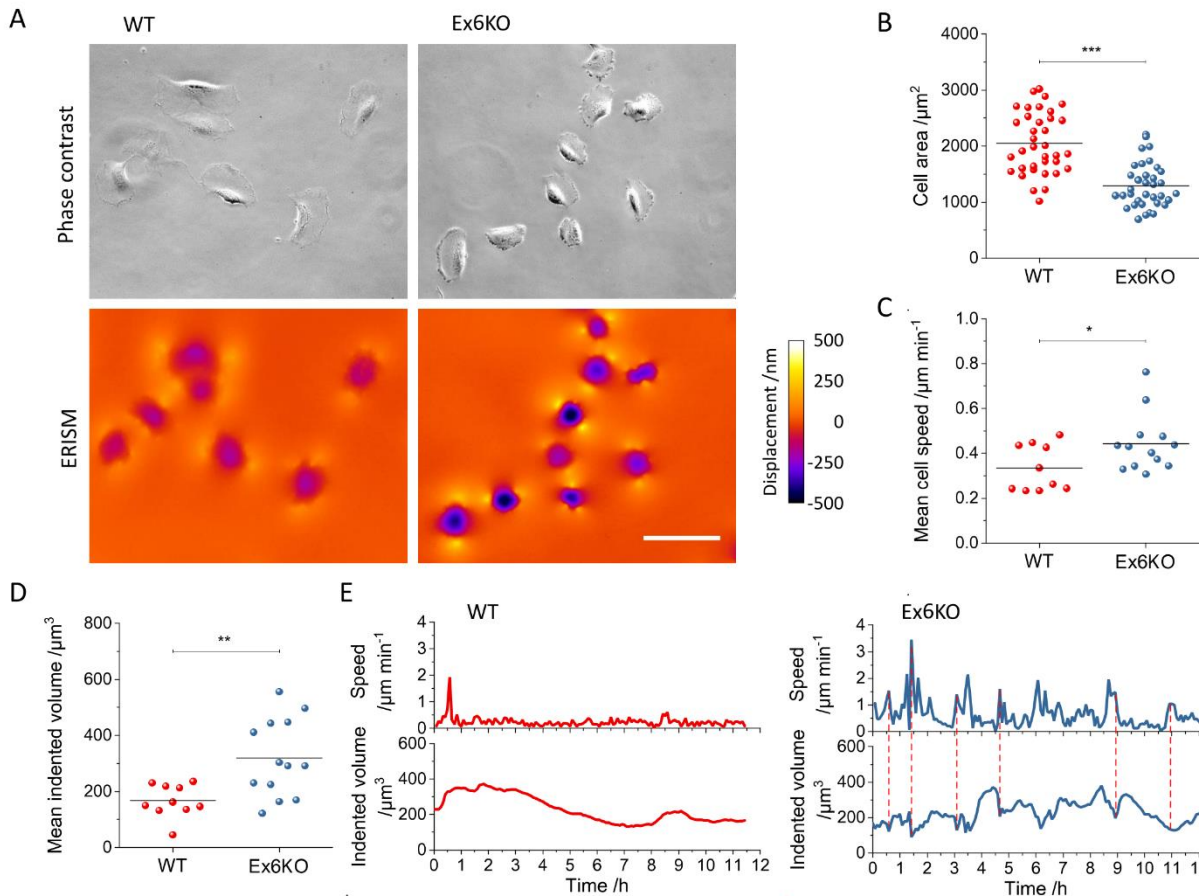
504 **(A)** Top: Structure of Human KIAA0319 (adapted from Velayos-Baeza et al., 2008 and  
505 Ensembl release 94 (Zerbino et al., 2018)). The diagram shows the correspondence between  
506 protein domains and coding exons in KIAA0319. Signal peptide (SP), MANEC domain  
507 (MANEC), PKD domains (PKD), cysteine residues (C6) and transmembrane domain (TM) are  
508 indicated. Bottom: full DNA sequence of KIAA0319 exon 6 with target sequences for the  
509 gRNAs indicated with blue lines. Red lines show the position of the PAM sequences.  
510 Translated sequence of amino acids for the targeted exon is shown below the DNA sequence.  
511 **(B)** Chromatograms of the deletions found in Ex6KO and translated corresponding amino  
512 acids for wild type and knockout cell line. Asterisks indicate premature stop codons. **(C)**  
513 Results of the PCR screening to confirm the deletions in Ex6KO. The cartoon on the left  
514 represents the screening strategy. Two sets of primers were designed to give different bands  
515 in the WT and KO. The stripped area indicates the 19 and 32 base pair (bp) deletions in the  
516 exon 6 of KIAA0319. The first set of primers (Ex\_6R and Ex\_5F) amplifies the region around  
517 the deletion and therefore a smaller band is expected for the KO (105 – 118 bp) compared to  
518 the WT (137 bp). The second pair (Ex9\_R/Ex6delF) has one primer mapping within the  
519 deletion. PCR is expected to give a band of 360 bp in the WT and no product in the KO.  
520 Images below confirm the expected results for both pairs. **(D)** Quantification of KIAA0319  
521 mRNA in WT and Ex6KO by qPCR. KIAA0319 expression is significantly lower in Ex6KO  
522 (Student's *t*-test:  $p < 0.0001$ ), consistent with nonsense mediated decay of the mRNA caused  
523 by the deletion.



524

525 **Figure 2: Analysis of the cilia length**

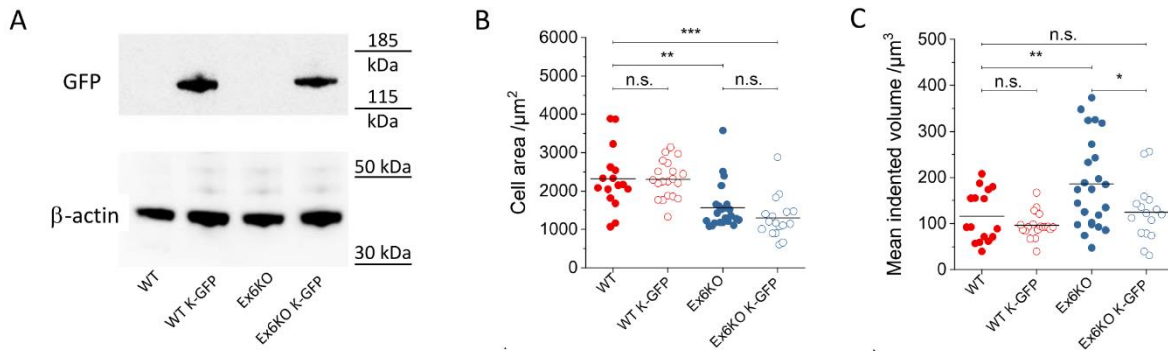
526 Representative immunofluorescence images of RPE1 wild type (A) and Ex6KO (B), stained  
527 for cilia marker Arl13b (green), centrosomal marker  $\gamma$ -tubulin (red), and DAPI (blue). (C) Plot  
528 of the cilia length for wild type ( $n = 129$ ) and Ex6KO cells ( $n = 104$ ). Groups were compared  
529 using the Student's  $t$ -test (\*\* $p < 0.001$ ). Scale bar, 10  $\mu\text{m}$ .



530

531 **Figure 3: Analysis of mechanical activity of RPE1 WT and Ex6KO cells during migration**  
 532 **on an ERISM micro-cavity**

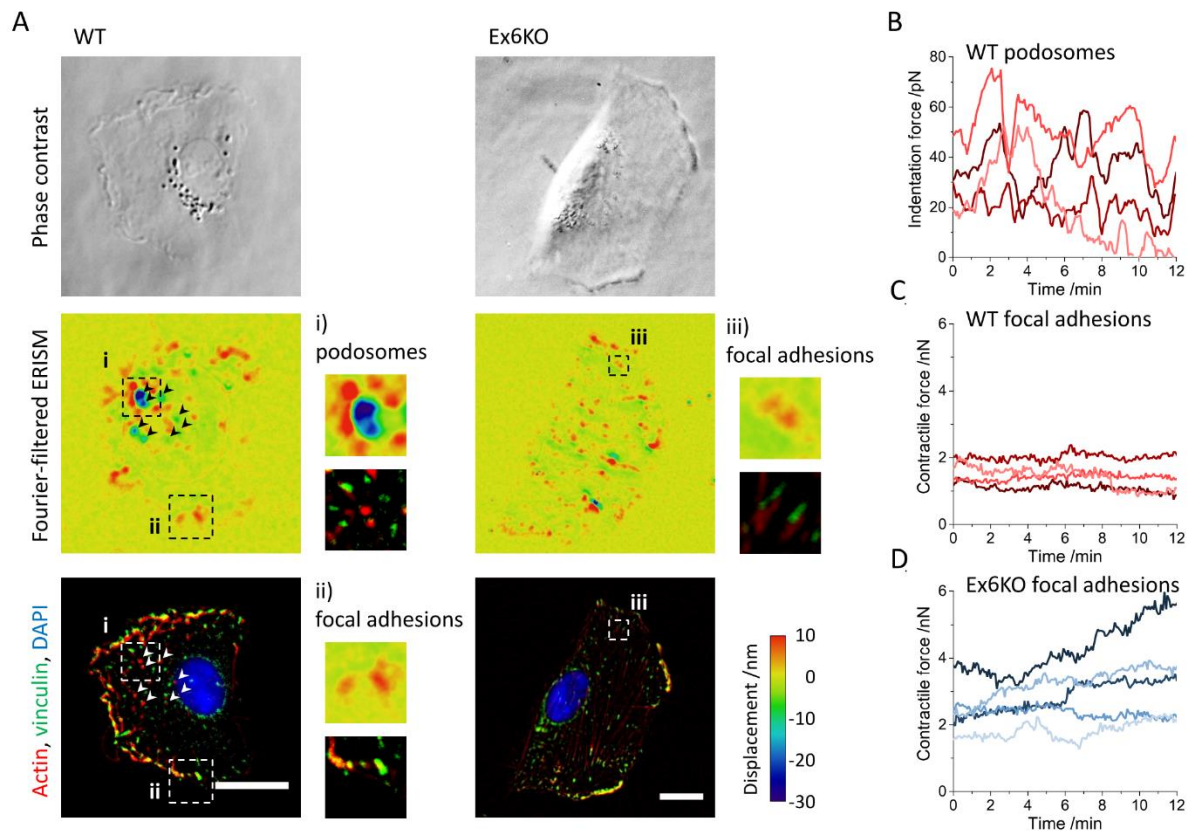
533 **(A)** Phase contrast (upper row) and ERISM micro-cavity displacement maps (lower row) of  
 534 WT (left) and Ex6KO (right) cells. **(B)** Comparison of the surface area covered by WT ( $n = 36$ )  
 535 and Ex6KO ( $n = 36$ ) cells types. **(C)** Comparison of mean speed of WT ( $n = 10$ ) and Ex6KO  
 536 ( $n = 13$ ) cells. **(D)** Comparison of mean indented volume of WT ( $n = 10$ ) and Ex6KO ( $n = 13$ )  
 537 cells. Only cells with free movement for  $>4$  h were included in analysis of speed and indented  
 538 volume. Plots in (B), (D) and (E) show all measured data points and the mean (line). Groups  
 539 were compared using the Student's  $t$ -test ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ). **(E)** Exemplary  
 540 temporal evolution of speed and mechanical activity (using the total volume by which each cell  
 541 indents into the ERISM substrate as a proxy for the applied force) of representative RPE1 WT  
 542 (left panel) and Ex6KO (right panel) cells, following the movement of two individual cells on  
 543 an ERISM micro-cavity for  $>11$  h. Red, vertical lines indicate timepoints when high migration  
 544 speed of Ex6KO cells correlate with a drop in exerted force. Scale bar,  $50 \mu\text{m}$ .



545

546 **Figure 4: Phenotype recovery through KIAA0319 rescue**

547 **(A)** Western blot confirming the presence of a fusion protein (140 KDa) following transfection  
548 with a full length KIAA0319 construct fused to a GFP tag. **(B)** Comparison of area covered  
549 by RPE1 WT, WT K-GFP, Ex6KO and Ex6KO K-GFP cells attached to ERISM micro-cavity.  
550 (WT:  $n = 16$ , WT K-GFP:  $n = 20$ , Ex6KO:  $n = 23$ , Ex6KO K-GFP:  $n = 17$ ) **(C)** Comparison of  
551 mean mechanical activity of RPE1 WT, WT K-GFP, Ex6KO and Ex6KO K-GFP cells during  
552 migration on ERISM micro-cavity. Only cells with free movement for  $>4$  h were included in  
553 the analysis. Plots in B and C show measured data points and the mean (line). (WT:  $n = 16$ ,  
554 WT K-GFP:  $n = 19$ , Ex6KO:  $n = 24$ , Ex6KO K-GFP:  $n = 16$ ) Groups were compared using  
555 the Student's  $t$ -test ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ).



556

557 **Figure 5: RPE1 KIAA0319 WT and Ex6KO cells use different modes of force exertion**

558 **(A)** Phase contrast images (upper row), Fourier-filtered ERISM displacement maps (middle

559 row) and epi-fluorescence images (lower row, red: actin, green: vinculin, blue: nuclear DNA)

560 of a RPE1 WT cell (left column) and an Ex6KO cell (right column). Arrow heads indicate

561 positions of actin-rich cell protrusions (podosomes). The insets i) in the Fourier-filtered

562 ERISM map and the epi-fluorescence image of the WT cell show magnifications of

563 podosome protrusions. The insets ii) and iii) show magnifications of vinculin-rich cell-

564 substrate contacts (focal adhesions) for the WT and Ex6KO cell, respectively. **(B)** Temporal

565 evolution of the indentation force applied by different podosomes of the WT cell shown in A.

566 **(C)** and **(D)** Temporal evolution of the contraction force applied by different focal adhesions

567 of the WT and Ex6KO cell shown in A, respectively. All scale bars: 20  $\mu$ m.

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