1 **Title:**

2 **USH2A gene mutations in rabbits lead to progressive retinal degeneration and**
3 **hearing loss**

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23 **Abstract**

24 Mutations in USH2A gene are responsible for the greatest proportion of hearing and
 25 vision loss among individuals with Usher Syndrome (USH) and for autosomal
 26 recessive non-syndromic retinitis pigmentosa. Mutations on USH2A exon 13 account
 27 for more than 35% of the disease causing USH2A variants including the most
 28 prevalence point mutation, c.2299delG, a frameshift mutation. The lack of a clinically
 29 relevant animal model has been a bottleneck for the development of therapeutics for
 30 USH2A related vision loss. Using CRSPR/Cas9 technology, this study establishes a
 31 rabbit line carrying an USH2A frameshift mutation on exon12 (equivalent to human
 32 USH2A Exon 13) as a novel mammalian animal model of USH2A. The bi-allelic
 33 mutant rabbits exhibit hyper reflective signals in FAF indicating RPE damage and
 34 OCT changes indicating photoreceptor degeneration as early as 4 months of age.
 35 ERG signals of both rod and cone function were reduced in the USH2A mutant rabbits
 36 starting from 7 months old and further decreased at 15-22 months old, indicating
 37 progressive retinal photoreceptor degeneration, which is further confirmed by retinal
 38 histopathology examination. ABR examination showed moderate to server hearing
 39 loss in the USH2A mutant rabbits. These results indicated that disruption of USH2A
 40 gene in rabbits is sufficient to induce hearing loss and progressive photoreceptor
 41 degeneration. To our knowledge, this is the first mammalian animal model of USH2
 42 which closely recapitulates the phenotype of retinitis pigmentosa in human patients.
 43 This study supports the use of rabbits as a clinically relevant animal model to
 44 understand the pathogenesis and to develop novel therapeutics for Usher Syndrome.

45 **Introduction**

46 Usher syndrome (USH) is an autosomal recessive genetic disorder resulting in
47 hearing loss, progressive visual impairment and, in some types, balance issues¹. The
48 major ocular symptom of patients with USH is a disease called retinitis pigmentosa
49 (RP). RP causes the light-sensing photoreceptor cells in the retina to gradually
50 deteriorate, initially resulting in night blindness, followed by tunnel vision, and severe,
51 permanent, progressive vision loss. More than 400,000 people are affected by USH
52 worldwide, accounting for about 50 percent of all hereditary deaf-blindness cases².
53 USH is classified into three subtypes (I, II, and III), which are distinguished by severity
54 and age of onset of deafness, presence or absence of vestibular dysfunction, and age
55 at onset of RP. Among them, type II (USH2) is the most common subtype,
56 characterized by hearing loss from birth and progressive vision loss that begins in
57 adolescence or adulthood. USH2 may be caused by mutations in any of three genes:
58 USH2A, GPR98, and DFNB31, with USH2A mutations being the most prevalent,
59 present in approximately 70% of USH2 cases³. Mutations in the USH2A gene are
60 also a cause of some forms of RP without hearing loss (i.e., non-syndromic RP)⁴.
61 More than 700 pathogenic USH2A mutations have been identified, as reported in the
62 LOVD database (<http://www.lovd.nl>). Mutations in exon 13 account for approximately
63 35% of all USH2A cases, including the two most recurrent mutations in USH2A,
64 c.2299delG (p. Glu767fs*21) and c.2276G>T (p. Cys759Phe)⁵. Despite extensive
65 research, there is no cure for USH2 yet. Hearing aids provide benefits to USH2
66 patients who have moderate to severe hearing loss; however, efforts to mitigate the

67 progressive visual loss caused by RP have been disappointing. Most individuals with
68 USH2 RP will eventually suffer from severe, progressive vision loss. Therefore, there
69 is a pressing unmet clinical need to develop novel therapeutics for USH2.

70

71 Mouse and zebrafish models of USH2A have been developed. Unfortunately,
72 phenotypes observed in retinas of USH2A-USH2 patients are not faithfully replicated
73 in mouse models carrying USH2A mutations⁶⁻⁸. USH2A knock out mice suffer from
74 hearing loss but only manifested weak and very late onset retina degeneration
75 phenotype. The zebrafish models exhibit early retinal degeneration phenotypes.
76 However, their features of adult photoreceptor regeneration as well as the distance
77 from humans may pose problems in translational studies⁹. Therefore, development of
78 an alternative mammalian model of USH2, which more closely approximates human
79 physiology, function, and anatomy, and importantly RP pathogenesis is of prime
80 importance and may accelerate translating discoveries from animal models into
81 clinical therapies and interventions for the disease.

82

83 Rabbits, compared with mice, are closer to humans in terms of phylogenesis,
84 anatomical features, physiology, and pathophysiological responses¹⁰⁻¹⁴, and are used
85 as a classic lab animal species to develop novel therapeutics for humans and refine
86 medical and surgical equipment^{13,15-19}. Historically, retinal degeneration has been
87 studied in rhodopsin Pro347Leu transgenic rabbits, a model of RP²⁰⁻³¹. Recently, the
88 emerging gene editing technology in rabbits has greatly increased their value to

89 biomedicine, motivating our efforts to develop rabbits that carry the disease causing
 90 mutations found in human patients, as models to replicate human diseases more
 91 precisely³²⁻³⁴. In this study, we report the development of USH2A rabbits by
 92 CRISPR/Cas9. This novel model is expected to greatly facilitate both the basic and
 93 translational studies of USH.

94

95 **Materials and Methods**

96 **Animals**

97 New Zealand White (NZW) rabbits were purchased from Covance or Charles River.
 98 The animal maintenance, care and use procedures were reviewed and approved by
 99 the Institutional Animal Care and Use Committee (IACUC) of the University of
 100 Michigan. All procedures were carried out in accordance with the approved guidelines
 101 and were performed in accordance with the ARVO (The Association for Research in
 102 Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and
 103 Vision Research. All efforts were made to minimize suffering. All the methods were
 104 carried out in accordance with the approved guidelines.

105 **Reverse transcription polymerase chain reaction (RT-PCR) and real time PCR** 106 **analysis.**

107 Total RNA from retina, sclera, brain, liver, kidney, and bone marrow were isolated
 108 using the RNeasy kit (Qiagen). Reverse transcription was used to generate cDNA
 109 (SuperScript® III First-Strand Synthesis System, Thermo Fisher Scientific, 18080-05)

110 as template for RT-PCR and real time PCR. For real time PCR analysis, samples
111 were analyzed on a BioRad CFX Connect™ Real-Time PCR Detection System and
112 amplification was detected using the SYBR green method (BioRad, SYBR green
113 supermix). PCR primers are as following: RTF1: 5'-aattcaggccagtgaagtg-3', RTR1:
114 5'-gccagaaagaggattgcag-3'; RTF2: 5'-ggagaagaagagggtgtgct -3'; RTR2: 5'-
115 gactctccactggaagctga-3'. Rabbit 18S rRNA or GAPDH expression was used as
116 internal control. The RT-PCR products were purified and subject to Sanger
117 sequencing.

118 **Scanning electron microscopy**

119 The neuroretinas obtained from perfused rabbits were postfixed by immersion in 2.5%
120 glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 2 h at room temperature. The
121 samples were dehydrated in ethanol, dried to critical point, and fractured along a
122 plane passing through the long axis of the photoreceptors. The fragments were
123 mounted on aluminum stubs with double-adhesive carbon tape and examined under a
124 scanning electron microscope.

125 **Histopathology**

126 To euthanize the rabbits, euthanasia solution (Euthanasia, 0.22 mg/kg, 50 mg/mL,
127 VetOne, ID, USA) was injected into the rabbit intravenously through the marginal ear
128 vein. The eyeballs were harvested and fixed in Davidson's fixative solution for 24 h.
129 The sample was then cut into 5 mm pieces and embedded in paraffin. The sample
130 was sectioned to a thickness of 4 µm using a Leica Autostainer XL (Leica Biosystems,

131 Nussloch, Germany) and stained with hematoxylin and eosin (H&E). The H&E slides
132 were observed using a Leica DM600 light microscope (Leica Biosystems, Nussloch,
133 Germany) and the images were captured using a BF450C camera.

134 **CRISPR reagents**

135 The Cas9 expression plasmid JDS246 was obtained from Addgene. Cas9 mRNA was
136 transcribed *in vitro*, capped and polyadenylated using the T7 mScript™ Standard
137 mRNA Production System (C-MSC100625, CELLSCRIPT, Madison, WI). Guide
138 RNA (gRNA) was designed using CRISPOR software³⁵, synthesized as chemically
139 modified (2'-O-Methyl at 3 first and last bases, 3' phosphorothioate bonds between
140 first 3 and last 2 bases) single strand gRNA (sgRNA EZ Kit, Synthego). The target
141 sequence on rbUSH2A is shown in Fig. 2A. Cas9 mRNA and sgRNA were diluted in
142 RNase-free TE buffer (1mM Tris-Cl pH 8.0, 0.1mM EDTA), stored at -80 °C in 10 µl
143 aliquots, and were thawed and kept on ice before microinjection.

144 **Rabbit genome editing**

145 Methods of rabbit genome editing has been described previously in detail³⁶. Briefly,
146 pronuclear stage rabbit embryos were injected with approximately 2-5 pL RNase-free
147 TE buffer (1mM Tris-Cl pH 8.0, 0.1mM EDTA) containing 150 ng/µl Cas9 mRNA, 50
148 ng/µl sgRNA and 50 ng/µl donor oligo. Injected embryos were washed three times in
149 embryo culture medium, which consisted of Earle's Balanced Salt Solution (E2888,
150 Sigma) supplemented with non-essential amino acids (M7145, Sigma), essential
151 amino acids (B-6766, Sigma), 1 mM L-glutamine (25030-081, Life Technologies), 0.4

152 mM sodium pyruvate (11360-070, Life Technologies) and 10% FBS. Twenty to thirty
153 embryos were surgically transferred to oviducts of each synchronized recipient doe.
154 For gRNA validation, instead of transferring to recipients, the injected embryos were
155 washed and cultured in the medium at 38.5 °C in 5% CO₂ for additional 2-3 days until
156 they reach blastocyst stage.

157 **Detection of gene editing events**

158 For gRNA *in vitro* validation, PCR products amplified the targeted USH2A gene region
159 were purified with a PCR purification kit. The purified PCR products were mixed in a
160 Eppendorf tube with Cas9 protein and gRNA to be tested as following: 10XNEBuffer
161 3.1, gRNA (30 nM final), Cas9 Nuclease, *S. pyogenes* (M0386S) (~30 nM final),
162 substrate PCR products (3 nM final), and Nuclease-free water to total reaction volume
163 of 30 µl. The reaction solution was incubated at 37°C for 30 minutes and analyzed by
164 gel electrophoresis.

165 For *in vivo* testing, injected embryos developed to blastocyst stage in culture were
166 collected in 1.5 ul water individually and the whole genome was replicated using a
167 REPLI-g® Mini Kit (Qiagen, Germantown, MD) following the manufacturer's protocol.
168 For rabbit genotyping, genomic DNA was isolated from the newborn kits ear skin.
169 Genomic DNA was then amplified by PCR using corresponding primers: Primers for
170 indels detection: forward (F), 5'-tctgcagtagcattgtttgtgatt-3', reverse (R),
171 5'-gtcccagtctcatcacagttacaa-3'; Primers for NGS sequencing: F,
172 5'-agccctgccagtgtaacctc-3', R, 5'-agtgactgagcctgctgtgttg-3'; Primers for OT1 detection:

173 F, 5'-gaggtacaagcagggtagaagg-3', R, 5'-gaatgaacatggcctgggacct-3'; Primers for
174 OT2 detection: F, 5'-gagagctggactggaagaggag-3'; R, 5'-agggtacttctgtgcgttcg-3';
175 Primers for OT3 detection: F, 5'-tcaggagtgaatcagcagatacaa-3', R,
176 5'-ttcggcttattcaggaaagaaatg-3'. PCR products were purified and subjected to T7E1
177 assay, Sanger sequencing, and NGS (MGH CCIB DNA core). NGS data were
178 analyzed using CRISPResso2 software³⁷.

179 **Rabbit eye examination and imaging procedure**

180 A comprehensive examination of the eyelids, conjunctiva, cornea, anterior chamber,
181 iris, and lens was performed using before imaging by slit lamp bio-microscopy
182 (SL120, Carl Zeiss, Germany). Fundus photography, fundus autofluorescence (FAF),
183 fluorescein angiography (FA), and indocyanine green angiography (ICGA) were
184 employed to evaluate the vascular network of the retina and choroid. Briefly, after
185 pupil dilation, a clinical fundus camera (TRC-50EX, Topcon Corporation, Tokyo,
186 Japan) was used to acquire fundus photography, FAF, FA, and ICGA. Fluorescein
187 sodium (0.2mL, 10% solution) (Akorn, Lake Forest, IL, USA) and indocyanine green
188 (0.5 mg/kg, 5 mg/mL, HUB Pharmaceuticals LLC, Patheon, Italy) were injected in the
189 rabbit marginal ear vein. Photographs were captured immediately after injection up to
190 10 minutes to capture early, middle, and late phase angiography images. There was a
191 5-minute interval between the two kinds of angiography tests.

192 Spectral domain optical coherence tomography (OCT) imaging was performed as
193 described previously³⁸. Briefly, two superluminescent light emitting diodes with a

center wavelength of 905 nm were used to illuminate the surface of the cornea and focused on the fundus by the rabbit eye optics. The average power of the OCT probing light was 0.8 mW. The lateral and axial resolutions are 3.8 μm and 4.0 μm , respectively. The system can achieve an imaging depth of 1.9 mm. The rabbits were put on a custom-built platform, and the eye position was adjusted under the ophthalmic lens using a CCD camera to visualize the region of interest.

Electroretinography (ERG)

Full field ERG (ff-ERG) was performed after pupillary dilation. After 60 minutes of dark adaptation, rabbits were anesthetized. After topical anesthesia, ERG-Jet contact lens electrodes (The Electrode Store, Enumclaw, WA, USA) were applied. Corneal hydration was maintained with a 2.5% hypromellose ophthalmic demulcent solution (Akorn Inc, Lake Forest, IL, USA). A pair of reference electrodes and a ground electrode (needle electrodes, The Electrode Store) were placed subcutaneously behind the bilateral ears and in the scruff, respectively. All animal handling was done under dim red light. ERGs were recorded with a Ganzfeld configuration using the LKC UTAS 3000 electrophysiology system (LKC Technologies, Gaithersburg, MD, USA). ERG responses were amplified at 2500 gain at 0.312-500 Hz and digitized at a rate of 2000 Hz. Scotopic ERGs were recorded at a dim flash intensity of 0.01 cd.s/m^2 to obtain the rod isolated ERG and at 3.0 cd.s/m^2 to obtain the combined rod-cone ERG. After 10 minutes of light adaptation to a white 32 cd/m^2 rod suppressing background, photopic ERGs were recorded at a flash intensity of 3.0 cd.s/m^2 . For ERG analyses, the a-wave amplitude was measured from the pre-stimulus baseline to the trough of

216 the a-wave, and the implicit time of the a-wave was measured from flash onset to the
217 trough of the a-wave. The b-wave amplitude was measured from the trough of the
218 a-wave to the peak of the b-wave, and the b-wave implicit time was measured from
219 flash onset to the peak of the b-wave. ERG recording was performed using a xenon
220 white flash, 1000 Hz sampling frequency, 0.312 - 300 Hz cut off filter, 500 ms
221 recording time, 10 ms baseline prior to flash, and no notch filter was used.

222 **Acoustic auditory brainstem responses (ABR)**

223 Auditory sensitivity of the animals is evaluated by recording auditory brainstem
224 responses to acoustic stimuli. Rabbits are anesthetized with xylazine and ketamine
225 (and butorphanol to prolong anesthesia) and placed on a warm water-circulating
226 heating in a sound attenuated chamber. Needle electrodes are placed under each
227 pinna: test ear (reference) and contralateral ear (ground) and at the vertex (active) of
228 the animal's head to record the neural output. Tucker Davis Technologies (TDT)
229 System III hardware and SigGen/BioSig software (TDT, Alachua, FL USA) is used to
230 present the stimulus and record responses. Tones are delivered through an EC1
231 driver (TDT, aluminum-shielded enclosure made in house), with the speculum placed
232 just inside the tragus. Stimulus presentation is 15 ms tone bursts, with 1 ms rise/fall
233 times, presented 10 per second. Up to 1024 responses are averaged for each
234 stimulus level. Responses are collected for stimulus levels in 10 dB steps at higher
235 stimulus levels, with additional 5 dB steps near threshold. Thresholds are interpolated
236 between the lowest stimulus level where a response was observed, and 5 dB lower,

237 where no response is observed. ABR thresholds and suprathresholds will be tested at
238 3 or 4 frequencies (between 4 – 24 kHz). ABR sessions may last 45 - 60 min.

239

240 **Results**

241 **Usherin is highly conserved in rabbit and human**

242 The rabbit USH2A gene has two isoforms: (i) the short transcript containing 23 exons
243 that encodes 1543 amino acids (Ensemble transcript ID: ENSOCUT00000014751.4);
244 and (ii) the long transcript contains 74 exons encodes 5202 amino acids (NCBI
245 Reference Sequence: XM_008268426.2). Analysis of protein sequences of usherin
246 revealed that Usherin is highly conserved in human and rabbit, with 84% identify
247 score for long isoforms and 85% identity score for short isoforms, and 91% positive
248 score for both isoforms (**Fig.1A**). To our interest, as shown in **Fig. 1B**, rabbit exon12
249 of USH2A is of the same length of human exon13, both are 642 bp long, which
250 notably is an in-frame length that is suitable for exon deletion-based therapy³⁹. We
251 examined the expression profiles of USH2A gene in rabbits by real-time PCR using
252 two pairs of primers: (i) Pair 1 on exon11 and exon12, that detects both short and long
253 isoforms; and (ii) Pair 2 on exon50 and exon51, that detects only the long isoform. As
254 shown in **Fig. 1C**, both isoforms of USH2A are exclusively expressed in the rabbit
255 retina, but not in any other organs/tissues examined.

256

257 **Calyceal processes like structures in rabbits**

258 It has been reported that photoreceptor calyceal processes (CPs) are present in the
259 retina of primates but absent from mice, suggesting that the presence/absence of CPs
260 maybe the cause of the difference in visual phenotype between USH1 human patients
261 and mice models⁴⁰. It is possible that this structure (i.e. CPs) is also critical to the eye
262 pathogenesis in USH2, as USH1 and USH2 proteins function together in higher order
263 protein complexes⁴¹. Electron microscopy imaging results indicate that CP-like
264 structures exist in rabbit retina (**Fig. 1D**).

265

266 **Production of USH2A mutant rabbits**

267 In efforts to model USH2 in rabbits, we chose to knock in the USH2A c.2299delG
268 mutation, the most prevalent USH2A frameshift mutation found in human USH
269 patients⁵, into rabbit genome. We designed four gRNAs targeting exon 12 of the rabbit
270 USH2A gene (**Fig.2A**). All four sgRNAs could cut their target in test tubes efficiently
271 with Cas9 protein (**Fig.2B**). T7E1 and Sanger sequencing assay showed that sgRNA1
272 achieved high efficiency of cleavage in rabbit embryos (**Fig.2B**). The guide RNA1
273 selected was co-introduced to rabbit embryos with Cas9 mRNA and a donor single
274 stranded DNA harboring the intended c.2299delG mutation and 50 nucleotides
275 homologue arms on each side. Totally 60 injected embryos were surgically
276 transferred into the oviduct of two synchronized recipients. All 9 term kits were
277 identified as USH2A mutant animals with 1 of them carrying 15.08% of the
278 c.2299delG mutation detected by ear skin deep sequencing (**Fig.2 C&D**,
279 **supplementary Fig.1**). these data demonstrate that USH2A mutant founder rabbits

280 can be produced by CRISPR/Cas9 very efficiently. Sperm DNA analysis by targeted
281 deep sequencing in founder that carries the c.2299delG mutation show a high
282 percentage of presence of this mutant allele (10.22% HDR, **Fig.2D**). Among the
283 knockout founders, one male (Founder#2) was mated with two wildtype rabbits
284 producing 19 kits, 9 of which carried frameshift indels mutations predicted to cause
285 premature stop codon (+14bp, -11bp, and -1 bp, **Fig.3B**). these data show that both
286 knock-in and knockout USH2A rabbits are germline transmitting.

287

288 **Off target analysis**

289 To test the off-target effects, we chose the top three potential off-target sites predicted
290 by the gRNA design software to test off-target effects, in which the predicted off-target
291 sites were PCR amplified and analyzed with T7E1 assay and confirmed by Sanger
292 sequencing. No indels were detected at OT2 and OT3 in any of the nine F1 rabbits
293 (**Fig. 3C**), whereas at off-target1 (OT1), two of the nine F1 generation rabbits showed
294 indels (GCTG to CTC) that are located 32 bp away from the predicted Cas9 cleavage
295 site (**Fig.3C, Supplementary Fig.2**). As this region is an intergenic region, it is more
296 likely a natural polymorphism. Nevertheless, to avoid the potential adverse effects,
297 these two F1 rabbits carrying the OT1 site indels were excluded from the breeding
298 program.

299

300 **Exon 12 mutation in rabbits result in nonsense-mediated mRNA decay**

301 Upon sexual maturation, one of the F1 rabbit carrying del11 mutation was used to
 302 establish the USH2A KO (USH2A^{-/-}) line. This mutation is predicted to cause a
 303 premature stop codon, which may lead to the nonsense-mediated mRNA decay and
 304 the production of non-functional truncated Usherin. Real time PCR detection of the
 305 USH2A transcripts in the retina tissue isolated from a founder animal carrying high
 306 percentage of indels mutation (Founder #2 in **Fig.2D.**) and a USH2A KO homozygous
 307 rabbit. As shown in **Figure 4**, the expression of USH2A transcripts was decreased
 308 more than 50% in the founder animal retina compared with wildtype controls, and the
 309 expression level further decreased to less than 30% that of wildtype controls in the
 310 USH2A KO retina, indicate nonsense-mediated mRNA decay did happened in the
 311 USH2A KO rabbits. We expect that the introduced mutation results in decreased
 312 mRNA level and the residual mRNA carrying the mutation leading to premature
 313 termination of usherin translation.

314 **Eye phenotype of the USH2A rabbits**

315 As shown in **Figure 5A**, USH2A KO rabbits had normal retinal and choroidal
 316 vasculature. In addition, ophthalmic examination confirmed that all USH2A rabbits
 317 had ophthalmoscopically normal and healthy corneas, anterior chambers, and clear
 318 lenses. There was no difference in the fundus appearance between WT and USH2A
 319 KO rabbits. It should be noted that these USH2A KO animals were on an albino
 320 background, and the characteristic bone spicule pigmentation of the retina seen in RP
 321 eyes would therefore not be expected. FA and ICGA imaging shows normal retinal

322 and choroidal vascular morphology. In contrast, hyper-FAF spots were detected in the
323 retina of the USH2A KO rabbits as early as 4 months old. OCT images also indicated
324 changes at the photoreceptor layer with hyper-reflective foci at the level of the
325 photoreceptor IS and OS segments as early as 4 months old.

326 OCT imaging was performed on both WT and USH2A KO rabbits as illustrated in
327 Figure 5B. B-scan OCT image obtained from WT rabbits show normal and healthy
328 retina with the different retinal layers such as the nuclear layers, plexiform layers,
329 photoreceptors, retinal vessels (RVs), choroidal vessels (CVs), RPE, inner limiting
330 membrane, and sclera. No evidence of photoreceptor or RPE disruption or damaged
331 was observed in WT rabbits. On the other hand, hyper-reflective foci were noted at
332 the level of the photoreceptor inner and outer segments and RPE in USH2A rabbits at
333 4 months old as marked by red dotted circle, which increased over time with more
334 disruptions at 8 and 12 months old.

335 **Loss of retinal function in USH2A KO rabbits**

336 To investigate whether the disruption of USH2A gene causes retinal abnormalities,
337 visual function of USH2A KO and WT control rabbits were compared by ERG
338 analyses at different ages under scotopic and photopic conditions. At age 7 months,
339 the USH2A KO rabbits displayed rod response (scotopic 24 dB), scotopic combined
340 rod and cone response (scotopic 0dB), and photopic response b-wave amplitudes
341 (**Fig.6A,B Fig. S3**) and 32 Hz flicker amplitudes (**Fig. 6C,D**) significantly lower than
342 WT counterparts. The responses of USH2A KO rabbits were 10% to 20% lower than

343 those of WT control ($P=0.0456$ and 0.042 respectively by t-test) as shown in **Figures**.
 344 **6B, 6D** and **Fig. S3**. At age 15-22 months, scotopic b-wave amplitudes and 32 Hz
 345 flicker amplitudes became further reduced with the responses of USH2A KO more
 346 than 50% lower than those of WT controls ($P =0.0073$ and 0.0031 respectively by
 347 t-test). This age dependent decline suggests progressively loss of photoreceptor
 348 function in USH2A KO rabbits. Implicit time did not show a significant difference
 349 between WT and USH2A KO over time (**Fig. S4**).

350

351 **USH2A rabbit histopathology showed reduced photoreceptor nuclei in the ONL**

352 To look for photoreceptor degeneration characteristics of Usher-associated retinitis
 353 pigmentosa, the number of photoreceptor nuclei in the outer nuclear layer (ONL),
 354 which represents the rod and cone cell bodies, of a 16 months USH2A KO rabbit was
 355 compared with age matched control rabbits. Figure **6A** shows the H&E image of WT
 356 (left) and USH2A KO (right). These H&E images clearly show thinner ONL layer in the
 357 USH2A KO rabbit retina compared with the age matched wildtype control. **Figure 7B**
 358 represent the overview of a rabbit retinal section illustrating the locations of optic
 359 nerve (Myelinated region) and visual streak as well as the 10 spots for ONL nuclear
 360 number counting in **Figure 7C**. We found that the USH2A KO rabbit has reduced
 361 photoreceptor nuclear numbers throughout the whole retina compared to the age
 362 matched controls, indicating photoreceptor cell degeneration which explained the
 363 reduced ERG signals in **Figure 6**.

364

365 **USH2A KO rabbits showed moderate to severe hearing loss**

366 USH2 affects both hearing and vision. In this study, auditory brainstem response
367 (ABR) was tested in three USH2A KO rabbits at 5 months old. As shown in figure 7,
368 ABR threshold at three different frequencies tested (4 kHz, 12 kHz, and 16 kHz) were
369 all increased by 2-3 times in the USH2A KO rabbits compared with wildtype control
370 rabbits ($p=0.0465$, $n=3$) (**Fig. 7**), indicating moderate to severe hearing loss in these
371 rabbits.

372

373 **Discussion**

374 Usher syndrome type 2 is characterized by hearing loss and early adulthood-onset of
375 RP. Retinal degeneration in patients is apparent by fundus examination and the
376 progressive reduction in ERG amplitudes over the course of the disorder. A targeted
377 Ush2a knock-out mouse demonstrates only mild retinal degeneration with late age of
378 onset⁶. A spontaneous mutant mouse model, Kunming, shows a rapid, early-onset
379 retinal degeneration, but contains mutations in two genes known to be involved in
380 inherited retinal dystrophies: Ush2a and Pde6b⁴⁷. Recently, bright light induction has
381 been reported to be able to induce the damage of the rod photoreceptors in several of
382 the USH1 and USH2 mouse models. However, these experiments were in a 129 Sv/j
383 background, which is inherently more sensitive to light-induced photoreceptor cell
384 damage^{48,49}. Overall, these models only showed slightly reduced rod function

385 indicated by reduced scotopic b wave amplitude, but no cone function reduction was
386 detected. This is probably due to their late onset features, as cones are affected later
387 in the disease course compared with rods. Due to the short life span of the mice, it is
388 very difficult to study USH2 disease in mouse models. Here, we have successfully
389 established USH2A KO rabbit line. We found that targeted disruption of the USH2A
390 gene in rabbits resulted in hearing loss and severe retinal degeneration starting as
391 early as 4 months of age, which is equivalent to human adolescence, and showed
392 progressive retinal degeneration mimicking the RP in USH2 patients. To our
393 knowledge, it is the first genetic preclinical large animal model that manifest eye
394 phenotype of USH2.

395 In humans, the location of highest acuity in the retina is a circular area termed the
396 fovea which boasts the highest concentration of cones, the lowest concentration of
397 rods, and much smaller receptive field sizes for all cells. The area of greatest acuity in
398 rabbit retina is not a single point, but rather an elongated “streak” running across the
399 retina. The presence of the visual streak made rabbits very useful for studying retinal
400 degenerative diseases in contrast to mice. As shown in figure 6, the reduction of the
401 ONL nuclear numbers in USH2A KO rabbits were more apparent in the visual streak
402 area (point 6), suggesting loss of cone photoreceptors. It is reported that severe visual
403 phenotype seen in syndromic USH2A patients compared with the non-syndromic
404 USH2A patients could relate to a greater extent of cone dysfunction indicated by
405 significantly reduced 30Hz-flicker ERG amplitudes⁵⁰. In this study, in addition to the
406 reduced rod function detected by the reduced scotopic b wave amplitudes in ERG, our

407 USH2A KO rabbit models also showed significantly reduced 32Hz-flicker ERG
408 amplitudes, which mimicking the cone function reduction in human patients.

409 In conclusion, we have succeeded in generating a rabbit model of USH2. Although
410 further studies are needed to fully characterize the natural history of hearing loss and
411 retinal degeneration phenotype in USH2A KO rabbits and to determine the exact
412 mechanism of photoreceptor dysfunction observed in this model, we believe that this
413 USH2A mutant rabbit model will serve as a useful large animal model with which to
414 study the pathophysiology of RP in USH and develop novel treatments. The
415 successful replication of the RP phenotype in USH2A rabbit models in this study as
416 well as the extension of gene targeting technology to rabbits by CRISPR/Cas9
417 technology motivating efforts to develop rabbit models for other types of Usher
418 Syndrome as well as other hereditary retinal diseases.

419

420 **Figure legends**

421 **Figure 1. USH2A is conserved in rabbit and human. (A).** Rabbit vs human Usherin
422 protein long isoform sequence alignment using BlastP program. **(B).** Genomic DNA
423 sequence alignment of rabbit exon 12 vs human exon 13 using BlastN program. **(C).**
424 Real-time PCR detection of USH2A variants in adult rabbit organs. Primer pair
425 1(RTF1,RTR1) detects both variant 1 and variant 2 (v1+v2, blue bar); Primer pair
426 2(RTF2,RTR2) detects variant 2 only (orange bar). Values were normalized to18S
427 rRNA expression. Y axes show the fold change relative to the expression level in

428 brain. Error bars represent Standard Deviation. **(D)**. Scanning electron microscopy
429 shown Calyceal Processes like structures (CPLs) in rabbit photoreceptors. IS/OS:
430 inner and outer segments of rod and cone photoreceptor cells.

431

432 **Figure 2. Production of USH2A mutant rabbits. (A)**. Illustration of CRISPR/Cas9
433 mediated targeting strategy to produce rbUSH2A mutant rabbits. **(B)**. Four candidate
434 sgRNA were designed and tested in vitro (left panel). sgRNA1 was validated in rabbit
435 embryos using T7E1 analysis (right panel). In right panel, each lane representative
436 one injected embryos. The PCR product of 479 bp will be cleaved into 238 bp and 241
437 bp bands if the embryos have indels generated at the gRNA target. M, NEB 100 bp
438 DNA ladder. **(C)**. Production of the USH2A mutant founder rabbits through embryo
439 transfer. **(D)**. Representative NGS analysis of the founder rabbits ear biopsy showed
440 high frequencies of both indels (NHEJ) and knock-in (HDR) mutations (left and mid
441 panel). The germline transmission of the mutations was confirmed by targeted deep
442 sequencing in semen collected from the knock in founder.

443

444 **Figure 3. USH2A KO line establishment and off-target analysis**

445 **(A)**. Breeding of the founder male rabbit mated with two female wildtype rabbits. **(B)**.
446 Mutated USH2A DNA sequence and the predicted protein sequence found in the F1
447 generation USH2A KO rabbits. **(C)**. Detection of off-target indels using T7E1 analysis
448 in F1 generation USH2A mutant rabbits with a wild-type rabbit as control. Red arrow

heads showing the indels detected at off-target 1 locus in F1 generation kits #2 and #6.
M, NEB 100 bp ladder DNA marker; On, on-target sequences; Off, off-target
sequences; Nucleotides in red color indicate the mismatches of the gRNA and the
potential off-target sequence. NGG/NGA PAMs were highlighted by underlines.

453

Figure 4. USH2A expression in USH2A KO rabbit retina. Real-time PCR detection
of USH2A variants in retina tissue of a USH2A KO rabbit using the primer pair 1 in
Fig.1, which detects both long and short variants of USH2A. Values were normalized
to GAPDH RNA expression. Y axes show the fold change relative to the expression
level in wild type controls (3 animals). Error bars represent Standard Deviation.

459

Figure 5. Retinal imaging in USH2A KO rabbit. **A).** Fundus photographs
(Fundus), fundus autofluorescence (FAF), indocyanine green angiography (ICGA),
and fluorescein angiography (FA) images obtained from a USH2A KO rabbit at
4-month-old to 12-month-old demonstrating hyper-FAF spots (arrows). **B).** Spectral
domain optical coherence tomography (OCT) images of a USH2A KO rabbit at
4-month-old to 12-month-old demonstrating hyper-reflective foci at the level of the
photoreceptor IS and OS segments in the photoreceptor layer (red dotted circles).

467

Figure 6. Progressive retinal degeneration in USH2A KO rabbits

469 **A).** Representative rod ERGs recorded at scotopic -24 dB flash from a 15-month-old
 470 USH2A KO rabbit with an age matched wild-type rabbit as Control. **B).** Full field
 471 electroretinography (ERG) demonstrated a significant reduction in rod response
 472 amplitude at 7 months that is further reduced at 15-22 months in USH2A KO rabbits
 473 compared with age matched wildtype (WT) rabbits. **C).** Representative ERGs
 474 recorded at 0 dB 32Hz flicker demonstrating cone response from a 15-month-old
 475 USH2A KO rabbit with an age matched wild-type rabbit as control. **D).** A significant
 476 reduction in amplitude of cone ERGs by 7 months that is consistent and further
 477 reduced at 15-22 months were recorded in USH2A KO rabbits compared with age
 478 matched wildtype (WT) rabbits. ICGA: indocyanine green angiography, FA:
 479 fluorescein angiography, RVs: Retinal vessels. ILM: Inner Limiting Membrane. RPE:
 480 Retinal pigment epithelium. CVs: Choroidal Vessels. OD: Right eye; OS: Left eye.
 481 Data analyzed by unpaired t-test, * $p < 0.05$, ** $p < 0.01$.

482

483 **Figure 7. Histopathology analysis of USH2A KO rabbit retina. A).** Representative
 484 retinal section prepared from a 16 months USH2A KO rabbit stained with H&E show
 485 reduced retinal layer thickness compared with age-matched wildtype (WT). Scale
 486 bars 75 μ m. **B).** overview of a rabbit retinal section illustrating the locations for ONL
 487 nuclear number counting in Panel C. **C).** Counting of ONL layer nuclei numbers in a
 488 defined field of view (150 μ m along the retina layer) at different locations on the retina
 489 indicated in panel B. Error bars represent SD. RGC: Retinal Ganglion cell layer; IPL:
 490 Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer

491 nuclear layer; IS/OS: Inner and Outer segments of rod and cone photoreceptor cells;

492 RPE: Retinal pigment epithelium; CL: Choroid layer.

493 **Figure 8. Hearing loss in USH2A KO rabbits.** Auditory brainstem response (ABR) test

494 demonstrating significant hearing loss in the USH2A KO rabbits at 5 months old compared

495 with wildtype (WT) control rabbits (p=0.0139, n=3).

496

497

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633

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641 **Author contributions statement**

642 D.Y, Y.M.P., Y.P and Y.E.C conceived the experiments. D.Y, Y.M.P, V.P. N, J.S, Y. L,
 643 D.P, D.D, J.X, and J.Z, conducted the experiments. D.Y, Y.M.P, K.T.J and Y.E.C
 644 analyzed the results and wrote the manuscript. All authors critically reviewed the
 645 manuscript.

646 **Additional information**

647 **Competing financial interests:** The authors declare no competing financial interests.

Figure 1. USH2A gene is highly conserved in rabbits and humans

Figure 2. Production of USH2A mutant rabbits

Figure 3. USH2A KO line establishment and off-target analysis

A

Male	Female	Kits	USH2A mutant	Germline transmission
Founder #2	Wild-type	19	9	Yes

B

Genotype	DNA sequence	Predicted Protein sequence
Wildtype	GAGTGTAAAAAGAAGCCAGAG-----GACTTCAGTGCG	ECKKEARGLQCDTCRENFYGLDVTD.....X
<i>c. 2315_2325 del11</i> <i>p. R773Vfs*17</i>	GAGTGTAAAAAGAAG-----CAGTGCG	ECKKEA VRHLQRELLWFGCHRLX
<i>c.2321_2334ins14</i> <i>p. G774Efs*106</i>	GAGTGTAAAAAGAAGCCAGAG TAACCTTTGTTACT GACTTCAGTGCG	ECKKEAR VTFTDFSATPAERTF.....X
<i>c.2321insA</i> <i>p.G774Efs*20</i>	GAGTGTAAAAAGAAGCCAGAG ----- A GACTTCAGTGCG	ECKKEAR ETSVRHLQRELLWFGCHRLX

C

Figure 4. USH2A expression in USH2A KO rabbit retina

Figure 5. Retinal imaging in USH2A KO rabbit

B

Figure 6. Progressive retina degeneration in USH2A KO rabbits

Figure 7. Photo receptor loss in USH2A KO rabbit

Figure 8. Hearing loss in USH2A KO rabbits

Supplementary Figure 1. NGS sequencing of USH2A KO founder rabbits

Kits	#1	#2	#3	#4	#5	#6	#7	#8	#9
Frameshift allele (%)	98.6	99.8	64.1	95	51.1	60.7	86.2	71.4	30.1
Inframe allele (%)	1.4	0.2	35.9	5	48.9	39.3	13.8	28.6	69.9
Ki allele (%)	0.01	15.08	0	0	0	0	0	0	0.08

Supplementary Figure 2. Germline transmission of the mutant alleles in F1 generation rabbits (A) and Sanger sequencing of the OT1 off-target mutation (B). Ot1: the off-target binding sequence. The 32 bp shadow part indicated the distance from the indels mutation (double peaks) site to the Cas9 cleavage site (3 bp upstream of the AGG PAM sequence)

A

- Wt GAAGC-----CAGAG-----GACTTCAGTGCACACCTGCAGAGAGAACTTTT
- (+14bp) GAAGC-----CAGAG**TAACCTTTGTTACT**GACTTCAGTGCACACCTGCAGAGAGAACTTTT (Kits #1,2,3,9)
- (+11bp) GAAGC**CCAGAGGACTT**CAGAG-----GACTTCAGTGCACACCTGCAGAGAGAACTTTT (kits # 4,5,6,7)
- (-26bp) GAAGC-----CAGAG-----AACTTTT (Kits #8)

B

Supplementary Figure 3. ERG Amplitudes in USH2A KO rabbits

Supplementary Figure 4. ERG implicit time in USH2A KO rabbits

Supplementary table 1

	Target sequence
sgRNA1	GGTGTCGCACTGAAGTCCTC <u>TGG</u>
sgRNA2	CAGAGAGAACTTTTATGGTT <u>TGG</u>
sgRNA3	CACTCACACTGCCCAGAAAG <u>AGG</u>
sgRNA4	GAGTGTAAGAAAAGAAGCCAG <u>AGG</u>

Supplementary table 2

Primers	Sequence
F1	TCTGCAGTAGCATTGTTTGTGATT
R1	GTCCCAGTCTCATCACAGTTACAA
F2	ATTGCAATTTTGGATTTA
R2	ACGTTGGGTCTACAGAGGCACT
dsF	AGCCCTGCCAGTGTAACCTC
dsR	AGTGACTGAGCCTGCTGTGTTG
RTF1	AATTCAGGCCAGTGCAAGTG
RTR1	GCCCAGAAAGAGGATTGCAG
RTF2	GGAGAAGAAGAGGGTGTGCT
RTR2	GACTCTCCACTGGAAGCTGA