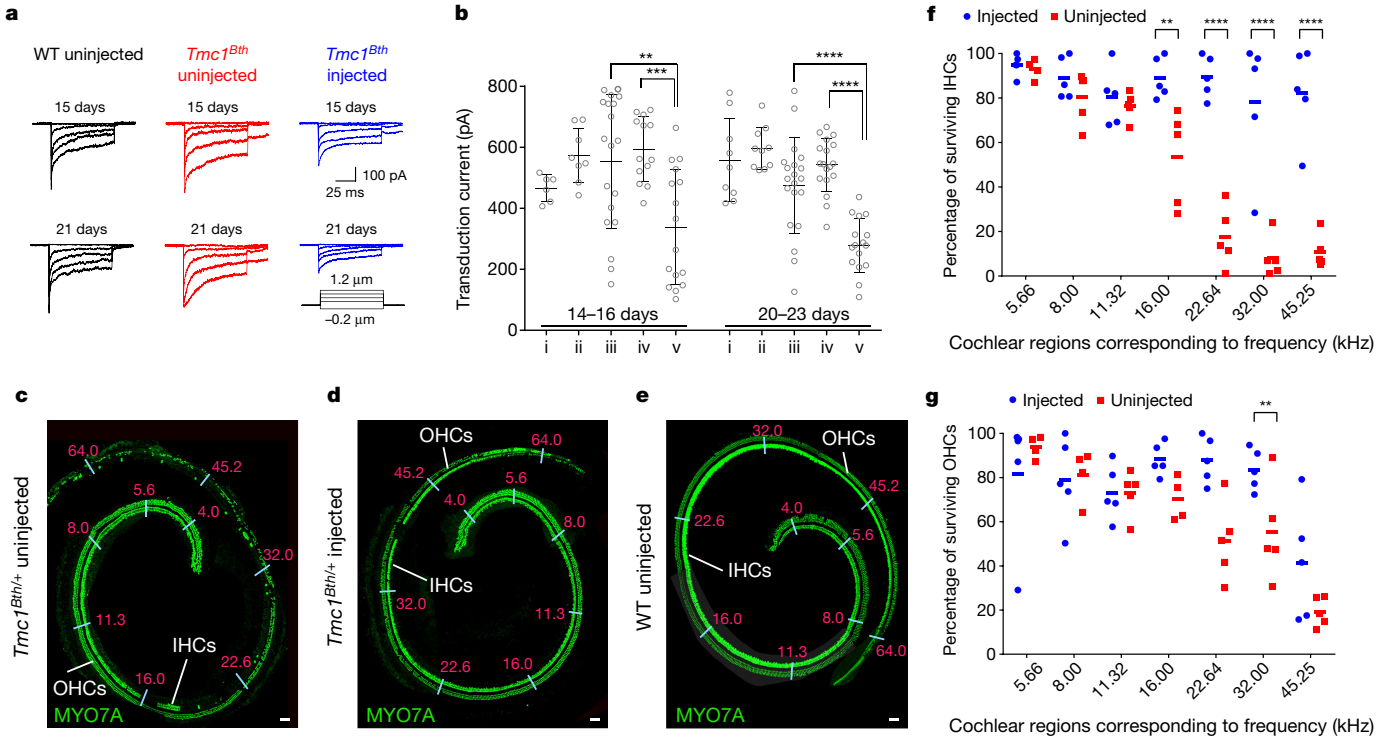

Figure 2 | Effects of Cas9–Tmc1-mut3 sgRNA–lipid injection on hair-cell function and hair-cell survival in mice. a, Representative transduction currents from IHCs of P0–P1 wild-type (WT) or $Tmc1^{Bth/\Delta}Tmc2^{\Delta/\Delta}$ mice that were uninjected, or injected with the Cas9–Tmc1-mut3–lipid complex, 15 or 21 days after injection. **b**, Maximal transduction current amplitudes for 135 IHCs from wild-type C57B/L6 and $Tmc1^{Bth/\Delta}Tmc2^{\Delta/\Delta}$ mice. i, uninjected wild-type C57B/L6 mice; ii, wild-type C57B/L6 mice injected at P1 with Cas9–Tmc1-wt3–lipid; iii, uninjected $Tmc1^{Bth/\Delta}Tmc2^{\Delta/\Delta}$ mice; iv, $Tmc1^{Bth/\Delta}Tmc2^{\Delta/\Delta}$ mice injected at P1 with Cas9–GFP sgRNA–lipid; v, $Tmc1^{Bth/\Delta}Tmc2^{\Delta/\Delta}$ mice injected at P1 with Cas9–Tmc1-mut3–lipid. Data were recorded after 14-23 days. Individual values ($n = 6$–20) are shown; horizontal lines and error bars reflect mean ± s.d. **c**–**e**, Representative confocal microscopy images around the age of eight weeks from an uninjected $Tmc1^{Bth/+}$ cochlea (**c**); the contralateral cochlea of the mouse in **c** injected with Cas9–Tmc1-mut3–lipid complex at P1 (**d**); and an untreated wild-type C3H cochlea (**e**). Numbers in pink indicate approximate frequencies (in kHz) sensed by each region. Scale bars, 50 µm. **f**, **g**, Quantification of IHC (**f**) and OHC (**g**) survival percentages in $Tmc1^{Bth/+}$ mice relative to wild-type C3H mice (100%) eight weeks after Cas9–Tmc1-mut3–lipid injection (blue) compared to uninjected (red) contralateral ears. Individual values are shown; horizontal lines represent mean values of five biological replicates. Statistical tests in **b** are two-population *t*-tests, and in **f**, **g** are two-way ANOVAs with Bonferroni correction: $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

ABR waveform pattern, in injected ears than in uninjected controls (Fig. 3b, c). Together, these results show that injection of neonatal $Tmc1^{Bth/+}$ mice with Cas9–Tmc1-mut3–lipid complexes reduces progressive hearing loss.

To test whether amelioration of hearing loss requires the mutant $Tmc1^{Bth}$ allele-specific sgRNA, we injected Cas9–Tmc1-wt3–lipid complexes targeting the wild-type *Tmc1* allele rather than the $Tmc1^{Bth}$ mutant allele into P1–2 $Tmc1^{Bth/+}$ mice. After four weeks, ABR thresholds in the injected ears were similar to, or worse than, those in the contralateral uninjected ears (Extended Data Fig. 6a; Supplementary Table 1), consistent with the inability of Cas9–Tmc1-wt3 to efficiently disrupt the $Tmc1^{Bth}$ allele (Extended Data Fig. 1e), and possible disruption of wild-type *Tmc1*. Injection of Cas9–sgRNA–lipid complexes targeting an unrelated gene (*Gfp*) did not significantly affect ABR thresholds at most tested frequencies in $Tmc1^{Bth/+}$ mice (Extended Data Fig. 6b). To test whether preservation of cochlear function requires Cas9 nuclease activity, rather than transcriptional interference from Cas9 binding to *Tmc1*, we treated $Tmc1^{Bth/+}$ mice with catalytically inactive dCas9[10] complexed with Tmc1-mut1 sgRNA and observed no evidence of hearing preservation (Extended Data Figs 5d, 6c; Supplementary Table 1). To evaluate the effects of the treatment on normal mice, we injected Cas9–Tmc1-mut3–lipid into wild-type C3H mice. We observed similar or slightly elevated ABR thresholds in injected ears relative to uninjected ears four weeks after treatment (Extended Data Fig. 6d, e), suggesting that Cas9–Tmc1-mut3 does not modify wild-type *Tmc1* efficiently enough to substantially affect hearing. Finally, injection of Cas9 and lipid without sgRNA did not improve ABR or DPOAE thresholds (Extended Data Fig. 6f, g). Collectively, these results establish that hearing preservation depends on sgRNA allele specificity, Cas9 DNA cleavage activity, and the presence of the $Tmc1^{Bth}$ allele. We also characterized the cochlear function of $Tmc1^{Bth/+}$ mice eight weeks after treatment. Mean ABR thresholds following Cas9–Tmc1-mut3–lipid injection remained lower than uninjected controls from 5.7–23 kHz, although the average improvement was lower than at four weeks post-treatment (Extended Data Fig. 4c, d), potentially owing to continued progressive hearing loss in the non-edited hair cells.

As a behavioural measure of hearing rescue, we assessed acoustic startle responses eight weeks after injection. In uninjected $Tmc1^{Bth/+}$ mice, no startle response was detected following stimulation at 120 dB. By contrast, significant startle responses were detected in Cas9–Tmc1-mut3–lipid-injected $Tmc1^{Bth/+}$ mice following stimulus at 110 and 120 dB (Fig. 3d and Extended Data Fig. 4e), demonstrating that hearing preservation upon treatment also preserves an acoustic behavioural reflex.

To evaluate the ability of each of the other $Tmc1^{Bth}$-targeting sgRNAs to mediate hearing rescue *in vivo*, we also injected Tmc1-mut1, Tmc1-mut2, and Tmc1-mut4 complexed with Cas9 into neonatal $Tmc1^{Bth/+}$ cochleae, and observed varying degrees of enhanced cochlear function (Extended Data Fig. 7). Thus, while Tmc1-mut3 resulted in the most robust hearing preservation, other sgRNAs targeting the mutant *Bth* allele also partially preserved cochlear function.

To test whether RNP delivery of editing agents in adult mouse inner ears supports genome editing in hair cells, we injected Cas9–GFP sgRNA–lipid complexes into the cochleae of six-week-old Atoh1–GFP