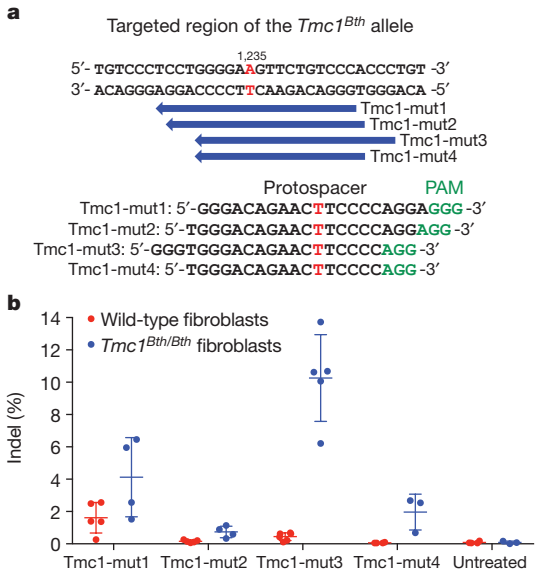

Figure 1 | Design of a genome-editing strategy to disrupt the *Tmc1*^{Bth} mutant allele. a, SpCas9 sgRNAs were designed to target the mutant *Tmc1*^{Bth} allele, in which T1235 is changed to A (red). The protospacer (blue arrows) of each *Tmc1*^{Bth}-targeting sgRNA contains a complementary T (red) that pairs with the T1235A mutation in the *Tmc1*^{Bth} allele, but that forms a mismatch with the wild-type *Tmc1* allele. **b**, Lipid-mediated delivery of Cas9–sgRNA complexes into primary fibroblasts derived from wild-type or homozygous *Tmc1*$^{Bth/Bth}$ mice. Purified Cas9 protein (100 nM) and 100 nM of each sgRNA shown were delivered using Lipofectamine 2000. Indels were quantified by HTS. Individual values ($n = 3$–6) are shown; horizontal lines and error bars represent the mean values ± s.d. of three or more independent biological replicates.

By contrast, all tested sgRNAs edited the wild-type *Tmc1* locus much less efficiently in wild-type fibroblasts (0.066–1.6% indels) (Fig. 1b). Notably, Cas9–Tmc1-mut3 modified the mutant *Tmc1*^{Bth} allele 23-fold more efficiently than the wild-type allele (Fig. 1b and Extended Data Fig. 1d). We also prepared three corresponding wild-type *Tmc1*-targeting sgRNAs (Tmc1-wt1, Tmc1-wt2 and Tmc1-wt3) that lack the T1235A mutation. These sgRNAs edited wild-type fibroblasts on average tenfold more efficiently than *Tmc1*$^{Bth/Bth}$ fibroblasts (Extended Data Fig. 1e), confirming that the observed allele selectivities did not arise from the inability of the wild-type *Tmc1* allele to be edited.

We tested 17 cationic lipids for their ability to deliver the Cas9–Tmc1-mut3 RNP into *Tmc1*$^{Bth/Bth}$ fibroblasts. Several lipids supported substantial modification of the target locus, including RNAiMAX (7.7%), CRISPRMAX (8.9%), and Lipofectamine 2000 (12%) (Extended Data Fig. 2). By contrast, treatment of wild-type fibroblasts with Cas9–Tmc1-mut3 and the same set of 17 lipids resulted in low (≤0.5%) indel rates (Extended Data Fig. 2a, b). These results suggest that the target mutant *Tmc1* locus can be preferentially disrupted by Cas9–guide RNA complexes.

Exposure of cells to Cas9–sgRNA agents typically results in the modification of both on-target and off-target loci[10,23]. We used both the GUIDE-seq method[23] and computational prediction[24] to identify potential off-target loci that could be modified by exposure to Cas9–Tmc1-mut3. Ten off-target sites containing up to six mismatches in the protospacer region of Tmc1-mut3 sgRNA were identified by GUIDE-seq (Extended Data Fig. 3a). None of these off-target loci are known to be associated with hearing function (Extended Data Table 1a). We measured the indel frequency at each off-target site by high-throughput sequencing (HTS) in *Tmc1*$^{Bth/Bth}$ primary mouse fibroblasts treated with Cas9–Tmc1-mut3 following plasmid DNA nucleofection or RNP delivery. Plasmid nucleofection resulted in 0.68–8.1% indels at nine of the ten GUIDE-seq-identified off-target sites (Extended Data Fig. 3b and Extended Data Table 1a). By contrast, after RNP delivery, modification of only one off-target site (*off-T1*, 1.2% indels) was detected (Extended Data Fig. 3b), consistent with our earlier findings that RNP delivery greatly reduces off-target editing compared with DNA delivery[15]. Among the computationally predicted off-target sites[24], only the two (*off-T1′* and *off-T2′*) that were also identified as off-targets by GUIDE-seq were observed to undergo modification (Extended Data Table 1b). Together, these results suggest that delivery of Cas9–Tmc1-mut3 RNP complexes into *Tmc1*$^{Bth/Bth}$ cells leads to minimal off-target modification, and that phenotypes affecting hearing are unlikely to arise from off-target modification.

To evaluate the ability of the Cas9–Tmc1-mut3 sgRNA complex to target the *Tmc1*^{Bth} allele in hair cells *in vivo*, we complexed Cas9–Tmc1-mut3 sgRNA with Lipofectamine 2000 and injected the resulting mixture into the scala media of neonatal mice by cochleostomy. Neonatal cochlear hair cells produce TMC1 and TMC2, both of which can enable sensory transduction. To isolate the effect of editing the *Tmc1*^{Bth} allele, we injected the Cas9–Tmc1-mut3 sgRNA–lipid complex into *Tmc1*$^{Bth/Δ}$*Tmc2*$^{Δ/Δ}$ mice[16], to avoid transduction current contributions from TMC2 or wild-type TMC1. We recorded sensory transduction currents from inner hair cells (IHCs) after injection with the Cas9–Tmc1-mut3–lipid complex, or with a control targeting an unrelated *Gfp* gene. We observed a significant decline in transduction current amplitudes in *Tmc1*$^{Bth/Δ}$*Tmc2*$^{Δ/Δ}$ mice following injection with Cas9–Tmc1-mut3–lipid complexes, consistent with disruption of the *Tmc1*^{Bth} allele in sensory hair cells *in vivo*, but not with Cas9–GFP sgRNA–lipid complexes (Fig. 2a, b).

In *Tmc1*$^{Bth/+}$*Tmc2*$^{+/+}$ mice (referred to hereafter as *Tmc1*$^{Bth/+}$ mice), IHCs undergo progressive death, followed by the outer hair cells (OHCs)[21]. To determine the effect of Cas9–Tmc1-mut3 sgRNA on *Tmc1*$^{Bth/+}$ hair cell survival, we injected Cas9–Tmc1-mut3–lipid complex into the scala media of mice on postnatal day 1 (P1) and removed the injected and uninjected cochleae after eight weeks. Uninjected ears exhibited substantial loss of IHCs and partial degeneration of OHCs (Fig. 2c, f, g) compared with wild-type ears (C3HeB/FeJ (C3H) mice, which are the genetic background of the *Tmc1*$^{Bth/+}$ mice) (Fig. 2e). In injected ears, survival of IHCs and OHCs was significantly enhanced (Fig. 2d, f, g). Stereocilia bundles were observed on surviving IHCs in injected ears, but were absent in uninjected ears in the basal and middle turns (Extended Data Fig. 4a). These results suggest that Cas9–Tmc1-mut3–lipid injection *in vivo* promotes hair cell survival in *Tmc1*$^{Bth/+}$ mice. The strong differences between treated and untreated ears suggests that sporadic disruption of *Tmc1*^{Bth} may benefit not only edited hair cells, but also surrounding hair cells, consistent with previous findings[25].

To study the effect of Cas9–Tmc1-mut3–lipid injection on cochlear function in *Tmc1*$^{Bth/+}$ mice, we measured auditory brainstem responses (ABRs), which represent the sound-evoked neural output of the cochlea, as well as distortion product otoacoustic emissions (DPOAEs), which measure the amplification provided by OHCs[21]. In uninjected ears of *Tmc1*$^{Bth/+}$ mice, we observed profound attenuation of cochlear neural responses, with ABR thresholds ranging from 70–90 dB at four weeks of age, compared with 30–50 dB for wild-type C3H mice (Fig. 3a and Extended Data Fig. 4b). The elevations in DPOAE thresholds at this time in *Tmc1*$^{Bth/+}$ mice were smaller than the elevations in ABR threshold (Extended Data Fig. 5a), consistent with reports that IHCs are more severely affected than OHCs in *Tmc1*$^{Bth/+}$ mice[21]. Four weeks after Cas9–Tmc1-mut3–lipid injection, treated *Tmc1*$^{Bth/+}$ ears showed substantially enhanced cochlear function, with lower ABR thresholds relative to uninjected ears at all frequencies below 45 kHz (Fig. 3a). Significant ($P \leq 0.001$) hearing preservation was detected from 8 to 23 kHz, with average ABR thresholds 15 dB lower for treated ears than untreated contralateral ears (Fig. 3a; Supplementary Table 1). DPOAE thresholds were slightly elevated in the injected ears, consistent with OHC damage, perhaps from the injection procedure (Extended Data Fig. 5). We also observed greater ABR wave 1 amplitudes, and a more normal