Gene Repair of an Usher Syndrome Causing Mutation by Zinc-Finger Nuclease Mediated Homologous Recombination

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Purpose. Human Usher syndrome (USH) is the most frequent cause of inherited deaf-blindness. It is clinically and genetically heterogeneous, assigned to three clinical types of which the most severe type is USH1. No effective treatment for the ophthalmic component of USH exists. Gene augmentation is an attractive strategy for hereditary retinal diseases. However, several USH genes, like *USH1C*, are expressed in various isoforms, hampering gene augmentation. As an alternative treatment strategy, we applied the zinc-finger nuclease (ZFN) technology for targeted gene repair of an USH1C, causing mutation by homologous recombination.

Methods. We designed ZFNs customized for the p.R31X nonsense mutation in *Usb1c*. We evaluated ZFNs for DNA cleavage capability and analyzed ZFNs biocompatibilities by XTT assays. We demonstrated ZFNs mediated gene repair on genomic level by digestion assays and DNA sequencing, and on protein level by indirect immunofluorescence and Western blot analyses.

Results. The specifically designed ZFNs did not show cytotoxic effects in a p.R31X cell line. We demonstrated that ZFN induced cleavage of their target sequence. We showed that simultaneous application of ZFN and rescue DNA induced gene repair of the disease-causing mutation on the genomic level, resulting in recovery of protein expression.

Conclusions. In our present study, we analyzed for the first time ZFN-activated gene repair of an USH gene. The data highlight the ability of ZFNs to induce targeted homologous recombination and mediate gene repair in USH. We provide further evidence that the ZFN technology holds great potential to recover disease-causing mutations in inherited retinal disorders. (*Invest Ophthalmol Vis Sci.* 2012;53:4140–4146) DOI: 10.1167/iovs.12-9812

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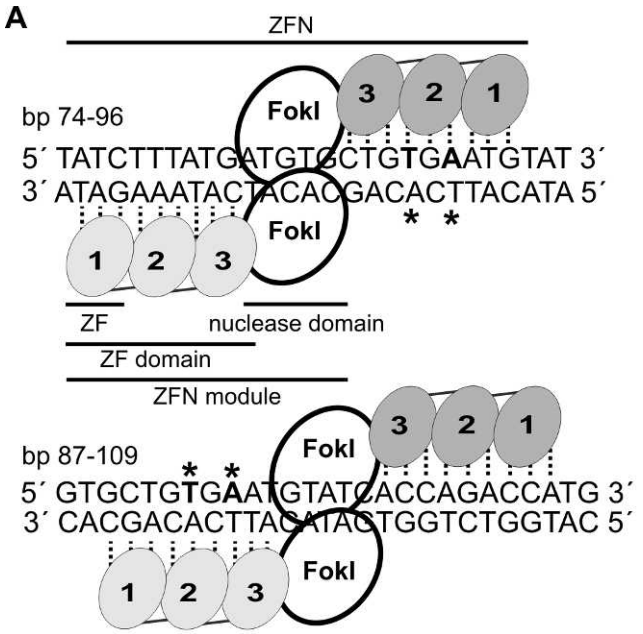
The human Usher syndrome (USH) is the most frequent cause of inherited combined deaf-blindness. USH is clinically and genetically heterogeneous and is assigned to three clinical USH types.[1] The most severe form of the disease is USH1, characterized by profound prelingual hearing loss, vestibular areflexia, and prepubertal onset of *retinitis pigmentosa* (*RP*).[1,2] Five USH1 genes (*USH1B–G*) and their gene products have been identified.[1,2] In the present study, we focused on the *USH1C* gene coding for the scaffold protein harmonin.[3] Harmonin was previously identified as one of the key organizers in the USH protein interactome.[1,2] So far no effective clinical treatment for the ophthalmic component of USH exists.

Gene-based strategies became attractive for the treatment of hereditary retinal degenerations (e.g., gene augmentation by adeno-associated viruses for the treatment of Leber Congenital Amaurosis [LCA]).[4] However, USH is a difficult target for viral gene augmentation approaches; several USH genes exceed the packaging size of therapeutic viruses. In addition, the transcripts of most USH genes are alternatively spliced, and several splice variant isoforms have to be added for an augmentative treatment.[5] In particular, recent expression analysis of *USH1C* in human retinas demonstrated the presence of several harmonin transcripts.[6] The ambiguity concerning the activities of different isoforms hampers viral gene augmentation approaches for USH1C patients.

The most elegant way to treat a genetic disorder is to repair the disease-causing mutation in its endogenous locus.[7] In contrast to other gene-based therapy approaches, in this method, the expression and splicing of the corrected gene remains under endogenous control, the size of the gene is no issue, and the type of the disease-causing mutation does not have to be taken into account. Gene repair, also called genome editing or gene correction, can be achieved by homologous recombination (HR) within the cell. Unfortunately, HR occurs under normal conditions at a very low frequency of 10^{-6} and therefore cannot be used for therapeutic means.[8,9] However, the frequency of HR can be increased several thousand-fold by the introduction of double-strand breaks (DSBs) in the DNA.[10] Zinc-finger nucleases (ZFNs) are engineered genetic tools designed to specifically create DSBs at a preselected genomic target sequence and thereby activate HR.[11,12] ZFN modules are chimeric proteins composed of a DNA-binding domain (ZF domain) fused to the nonspecific nuclease domain of FokI (Fig. 1).[7] The ZF domains can be specifically designed to bind to almost any preselected DNA sequence in the genome.[12] To introduce a DSB, two ZFN modules have to form a dimer in order to activate the FokI nuclease activity (Fig. 1).[7] This requires two ZFN modules, binding opposing targets across a spacer where the FokI domains come together to create the break (Fig. 1).[7,9] In a ZFN module, each zinc-finger subunit (Fig. 1, grey



B

			1	2	3
bp 74-96	bp 87-96 5′- 3′	A	TSGNLVR	QRANLRA	TSGNLTE
		B	ISSNLQR	−II−	−II−
	bp 74-82 3′- 5′	C	RRDELNV	RNDALTE	RSDALRE
		D	−II−	QAGHLAS	−II−
bp 87-109	bp 101-109 5′- 3′	E	ERSHLRE	SKKALTE	HKNALQN
	bp 87-95 3′- 5′	F	TSHSLTE	QLAHLRA	DKKDLTR
		G	−II−	QSSHLLT	−II−

Figure 1. Zinc-finger nucleases for the *Ush1c* p.R31X mutation. (**A**) Zinc-finger nucleases are chimeric proteins consisting of DNA binding zinc-fingers (ZF) fused to the nonspecific cleaving endonuclease FokI (ZFN module). We designed ZFN modules with three DNA binding ZFs (1–3 *grey ovals*, ZF domain). The FokI is only functional when dimerized, making a pair of ZFN modules necessary to form an active ZFN heterodimer and introduce a DNA double-strand break. Segments from bp 74 to 96 and bp 87 to 109 were chosen because one ZF is binding directly to a mutated base pair (*asterisks*). (**B**) In silico screens resulted in seven different potential ZFN modules for p.R31X (A–G).

ovals; ZF) recognizes and binds to 3 base pairs (bp). Cloning of three ZFs in line results in binding to nine consecutive base pairs. Dimerization of two ZFN modules therefore recognizes 18 bp, making binding of the ZFN to DNA strands a likely unique event.[7,11] To repair the DSB during HR, an exogenously introduced DNA rescue plasmid, containing the desired sequence, can be used as a template.[13–15]

Here we designed customized ZFNs, specific for the p.R31X mutation of murine *Ush1c*. We show the activity of ZFN by cleavage of the DNA target sequence. In addition, we demonstrate gene correction of the p.R31X mutation on the genomic level and expression of the repaired gene product, harmonin. These results highlight the capacity of ZFNs to treat individuals with mutations in *USH1C* and other inherited ocular and nonocular diseases as well.

Materials and Methods

Design and Generation of ZFN for *Ush1C-P.r31X* Mutation

The mutated *Ush1c* sequence was screened for putative ZF binding sites with the Zinc-Finger consortium–based software ZiFiT (in the public domain; http://bindr.gdcb.iastate.edu/ZiFiT).[16] Cloning of ZFN by modular assembly was performed as previously described.[17] ZFN consisted of three individual ZF-DNA binding sites fused to the FokI domain (Addgene plasmid 13,426). Resulting ZFN modules were each cloned into an eukaryotic expression vector (Addgene plasmid pST1374) with a nuclear localization signal (NLS) and a Flag-tag to allow detection of the ZFN module.

P.r31X-Ush1c Stable Cell Line and Rescue Plasmid

The p.R31X mutation was introduced into mouse *Ush1c* cDNA by PCR site-directed mutagenesis applying the QuickChange Lightning Site-Directed Mutagenesis Kit (Stratgene, La Jolla, CA). The *Ush1c* and mutated cDNA (bp 1-999) was subcloned in a pDEST expression vector with a C-terminal Myc-tag. Stable p.R31X-*Ush1c* or *Ush1c* cell lines were generated by transfecting plasmids containing either p.R31X-*Ush1c* or *Ush1c* cDNA regulated by a cytomegalovirus (CMV) promoter into Flp in-HEK293 cells and selection by hygromycin B according to manufacturer's protocol (Invitrogen, Karlsruhe, Germany). The rescue plasmid contained the wild type *Ush1c* cDNA (bp 1-999). The sequence was cloned into pGL3-Basic vector (Promega, Fitchburg, WI), which has no promoter.

Cell Culture

Multi-clone p.R31X-*Ush1c* and *Ush1c* cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with GlutaMax supplemented with 10% fetal calf serum (FCS; Invitrogen), 1% penicillin/streptomycin (Invitrogen) at 37°C, and 5% CO_2. Transfections were performed with Lipofectamine LTX and PLUS reagent (Invitrogen) according to manufacturer's protocol. After 6 hours, medium was changed to fresh medium, and cells were grown for 48 to 72 hours.

Assessment of ZFN-Related Cytotoxicity

Stable p.R31X cells were transfected with ZFNs. Forty-eight hours post transfection, ZFN toxicity was assessed by XTT cell viability assay (Roche, Düren, Germany) according to manufacturer's instructions. Assays were performed as triplets. Presented data are an average of five to nine independent experiments. G418 (1 mg/mL) was used as positive control for the assay.

Semiquantitative PCR

Forty-eight-hours post transfection of stable p.R31X cells with ZFNs, genomic DNA was isolated by the High Pure PCR Template Preparation Kit (Roche). As positive control for the cleavage, genomic DNA of stable p.R31X cells was digested with EcoRI. Duplex PCRs were performed in a volume of 50 μL using 12 ng genomic DNA and 10 nmol of each primer/reaction. Cycling conditions were 25 cycles at 94°C for 40 seconds, 60°C for 40 seconds, and 72°C for 1 minute followed by a 7-minute 72°C extension. Harmonin-specific primers flanking the potential cleavage site (forward 5′-ATGGACCGGAAGGTGGCCCGA-3′ and reverse primer 5′ CCTTCTGGGTGCAGACGGTCCAAG-3′) were used. Primer set for GAPDH (forward 5′-TGCACCACCAACTGCTTAGC-3′ and reverse 5′-GGCATGGACTGTGGTCATGAG-3′) was used as internal control for the PCR reaction. PCR products were analyzed on ethidium bromide–stained 1% agarose gels.

TseI Digest

Seventy-two hours post transfection, genomic DNA was isolated from cells transfected with ZFNs and rescue plasmid by the High Pure PCR Template Preparation Kit (Roche). PCRs were performed in a volume of 50 μL, using 300 ng prepared genomic DNA and 10 nmol of each primer per reaction. Cycling conditions were 35 cycles at 94°C for 40 seconds, 60°C for 40 seconds, and 72°C for 1 minute followed by a 7-minute 72°C extension. Forward primer binding in the CMV-promoter of stable transfected p.R31X construct (5′-GTACGGTGGGAGGTCTATAT-3′) and a harmonin-specific reverse primer (5′ CCTTCTGGGTGCAGACGGTCCAAG-3′) were used. Ten microliters PCR product were digested with 0.5 μL TseI (New England Biolabs, Ipswich, MA) for 2 hours at 65°C and digestion analyzed on ethidium bromide–stained 2% agarose gels.

DNA Sequencing

Sequencing of 500 bp PCR products after gene repair was performed by StarSeq (Mainz, Germany). Sequencing results were analyzed, and DNA alignment performed with BioEdit Version 7 (Carlsbad, CA).

Antibodies and Dyes

Monoclonal mouse antibodies to α-tubulin (clone DM1A), Flag (M2) were obtained from Sigma-Aldrich (St. Louis, MO) and Myc (clone 9B11) from Cell Signaling (Danvers, MA). Polyclonal rabbit antibodies against harmonin (H3) were used as previously described.[18] Secondary antibodies conjugated to Alexa 488 or 568 were purchased from Molecular Probes (Leiden, The Netherlands) and DAPI (4′,6-Diamidino-2-phenylindol) from Sigma-Aldrich. For Western blot, secondary antibodies were purchased from Invitrogen or Rockland (Gilbertsville, PA).

Immunofluorescence Microscopy and Western Blot Analysis

Immunofluorescence analyses were carried out on stable transfected p.R31X cells seeded on coverslips, followed by fixation with methanol, and proceeded as previously described.[19] Specimens were analyzed in a Leica DM 6000 B microscope (Leica Microsystems, Bensheim, Germany) and processed with Adobe Photoshop CS (Adobe Systems, San Jose, CA). Harvesting of cells and Western blot analyses were performed as previously described.[20,21]

Results

Design of Specific ZFNs for a Nonsense Mutation in *Ush1C*

In order to design specific ZFNs for target sites in the relevant mutation in *USH1C*, we used the ZiFiT software.[16] We screened the sequence coding for the murine *Usb1c* orthologue, which contains an USH1 disease-causing p.R31X nonsense mutation. In this mutation, the wild type CGA arginine codon (R) at position 91 is altered to a premature stop codon TGA (X). For the ZFN design, using ZiFiT, we applied software settings for ZF modules, which recognize a nine-bp sequence at the putative cleavage site separated by a spacer of five bp (Fig. 1A). Based on these prerequisites, we identified bp 74 to 96 and bp 87 to 109 as targets in the mutated *Usb1c* coding sequence for the design of promising specific ZFNs (Figs. 1A, B). We designed the ZF domains in order that one ZF directly binds to mutated base pairs. We generated a total of seven predicted ZF domains by modular assembly, which were fused with the FokI nuclease domain to form customized ZFN modules.[16,17] This strategy resulted in six matching ZFNs (A/C, A/D, B/C, B/D, E/F, and E/G) and one non-matching control ZFN (E/C) (Fig. 1). We transfected the ZFN modules into HEK293 cells and analyzed their cellular distribution. Immunofluorescence analysis revealed localization of ZFN modules as expected mainly within the nucleus (Fig. 2A).

Next we established a cellular reporter system to monitor ZFN-mediated genome editing of the mutated *Usb1c* in the genomic context. For this, cDNA coding for the N-terminal part of the murine *Usb1c* gene product harmonin, including the p.R31X mutation, the first two PDZ domains (named after PSD-95, DLG, and ZO-1), and a C-terminal Myc-tag, was stably integrated at a single location in the genome of Flp in-HEK293 cells. In parallel, *Usb1c* cells carrying the nonmutated construct were established.

Customized ZFNs Have No Cytotoxicity

Since application of ZFNs is often associated with significant cytotoxicity,[22] we examined potential side effects of ZFNs in the p.R31X reporter system by measuring the cell viability in an XTT-based assay. For this, p.R31X cells were transfected with combinations of ZFN modules suitable to assemble active ZFN heterodimers, and 48 hours post transfection, the relative number of viable cells was determined (Fig. 2B). Transfection of p.R31X cells with ZFN did not lead to a significant alteration in the number of viable cells compared with mock or empty vector-transfected cells, indicating no cytotoxic side effects of the applied ZFNs.

DNA Cleavage Capability of Customized ZFN

To evaluate the cleavage capability of generated ZFNs, the p.R31X cell line was transfected with either matching ZFNs or the control ZFN. Cells were harvested, genomic DNA was purified, and semiquantitative PCR with a primer pair flanking the putative ZFN cleavage site was performed (Fig. 2C). Efficient ZFN-mediated cleavage resulted in either a faint or a complete lack of the PCR product as observed after transfection of ZFN module combinations A/C and E/F (Fig. 2C). In contrast, the transfection of combinations A/D, B/C, E/G, and the control ZFN resulted in a PCR amplicon, indicating that these ZFNs are not able to cleave the target sequence. Transfections of ZFN B/D, however, led to varying results (Fig. 2C). Based on these findings, we focused on the active ZFNs A/C and E/F in the following experiments as these seemed to be the most efficient ZFNs.

Gene Repair Mediated by ZFN-Induced HR

Next we evaluated whether we can stimulate gene repair of the p.R31X mutation mediated by ZFN-induced HR. For this purpose we co-transfected p.R31X cells with ZFN and a rescue plasmid that contains the nonmutated *Usb1c* sequence without any promoter. Cells were harvested, genomic DNA was purified, and a PCR with a primer pair flanking the mutation was performed. To omit amplification of the rescue plasmid, we designed a forward primer, binding within the CMV promoter located at the 5′ end of the integrated p.R31X construct in the Flp in-HEK293 cells, and a reverse primer specific for the *Usb1c* sequence (Fig. 3A). Gene repair was analyzed by independent, complementary experiments. The PCR products were digested with TseI, which only cleaves the *Usb1c* (GCT/GC) but not the mutated p.R31X sequence (GCT/GT). The digestion resulted in a single band (500 bp) in the untransfected p.R31X cells and a double band (200 and 300 bp) in the *Usb1c* cells (Fig. 3B). TseI digestion after transfection of matching ZFN modules A/C and E/F resulted in clear double bands (200 and 300 bp), showing an efficient gene repair of the p.R31X mutation mediated by ZFN (Fig. 3B). Transfection

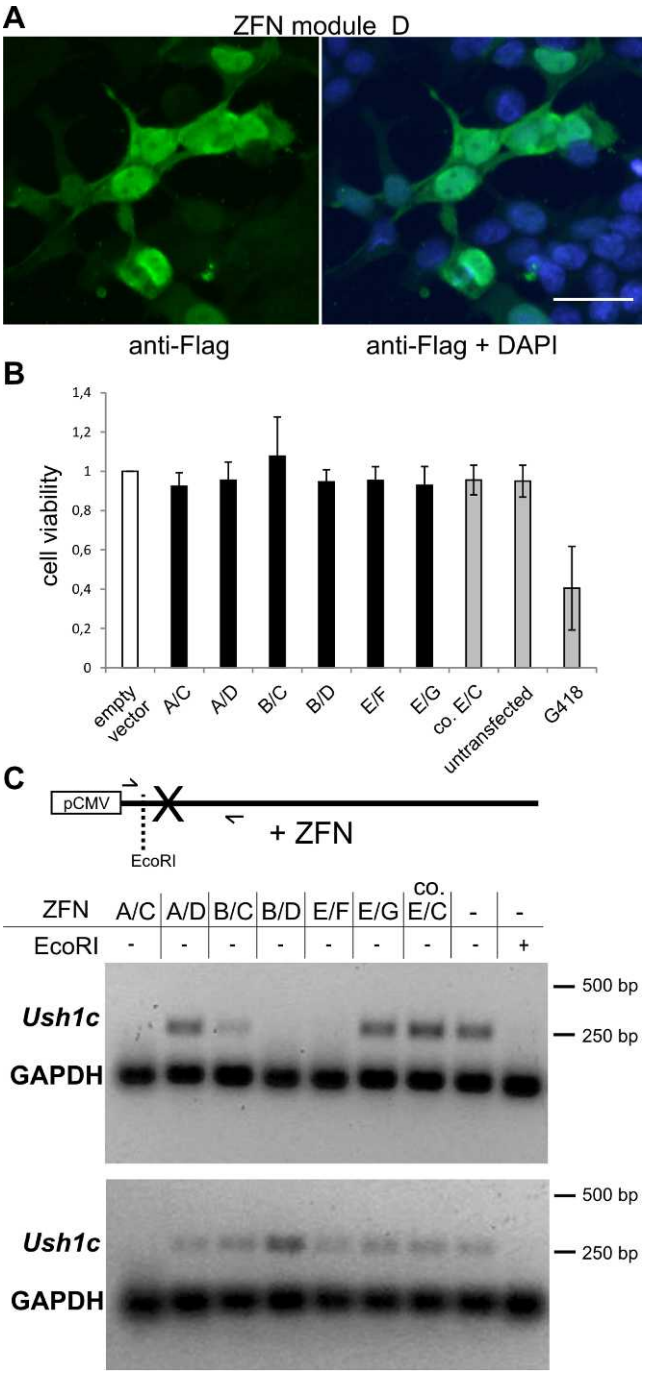


Figure 2. ZFN expression and evaluation in cell culture. (**A**) ZFNs are mainly localized in the nucleus of HEK293 cells, detected by anti-Flag antibody, exemplarily shown for ZFN module D. DAPI, nuclear DNA staining; scale bar: 25 μm. (**B**) ZFN related cytotoxicity was analyzed by measurement of cell viability in XTT assay. Cells transfected with matching or control ZFNs do not show an elevated rate of cell death compared with mock transfected cells or p.R31X cells transfected with the empty vector. Addition of G418 was used as positive control for the assay. (**C**) In vitro cleavage of ZFN. Semiquantitative duplex PCR with genomic DNA of p.R31X cells transfected with ZFN or un-transfected was performed with a primer pair (*arrows*) flanking the putative ZFN cleavage site (X). The two gels represent independent experiments. The faint or complete lack of the PCR product after transfection of A/C and E/F indicated highly efficient DNA cleavage by these ZFNs. In contrast, the transfection of combinations A/D, B/C, E/G, and the control ZFN resulted in a more prominent PCR amplicon, indicating that these ZFNs are not able to cleave the target sequence efficiently.

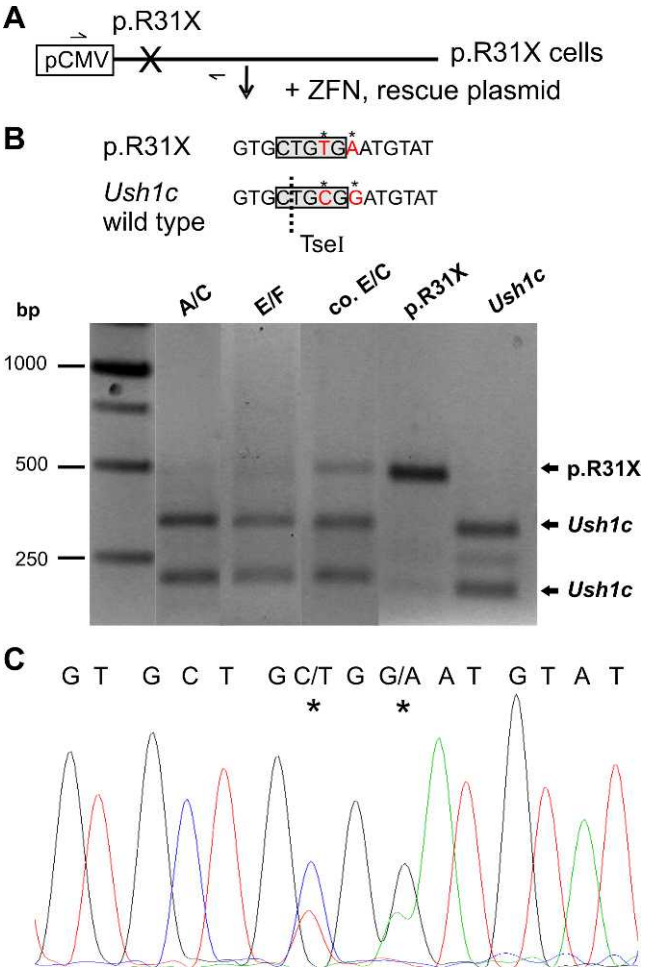


Figure 3. Gene repair mediated by ZFN induced HR on the genomic level. (**A**) Stable transfected p.R31X cells were triple transfected with two ZFN modules and rescue plasmid. PCR was performed on genomic DNA with a primer pair (*arrows*) flanking the mutation (X). (**B**) TseI digestion of PCR products. TseI cleaved only the *Ush1c*, not the mutated p.R31X sequence. Fragment sizes: wild type, 200/300 bp; mutated p.R31X sequence, 500 bp. Transfection with matching ZFN A/C and E/F resulted in gene repair of p.R31X in the *Ush1c* sequence. (**C**) Sequencing of the undigested 500-bp PCR product showed a mix of *Ush1c* and p.R31X sequences, with a higher peak for repaired *Ush1c*. This confirms an alteration of the mutated codon TGA to the wild type CGG, generated from the rescue plasmid by HR, shown for ZFN E/F. *Asterisks* indicate exchanged nucleotides due to ZFN-mediated HR.

of the control ZFN resulted in three bands (200, 300, and 500 bp), indicating only weak repair efficiency of the mutated sequence most likely owing to HR of the rescue DNA with the mutated sequence.

To independently verify these results for repair by HR, we sequenced the undigested 500-bp PCR product, which was amplified using a primer set composed of the CMV promoter forward primer and the *Ush1c* reverse primer. This ensured amplification of the integrated genomic sequence and excluded amplification of the rescue plasmid. Four (A/C) and five (E/F) PCR products after gene repair were sequenced. The results confirmed that the generated PCR product after transfection of matching ZFN was a mix of *Ush1c* and p.R31X sequences represented by the two peaks in the electropherogram (Fig.

Transfections of B/D led to varying results. The EcoRI digestion served as a positive control for the cleavage, and GAPDH amplification served as an internal PCR control.

3B; also, see Supplementary Material and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9812/-/DCSupplemental). In all sequencing results, we observed a higher peak for the sequence of the *Usb1c* site repaired by HR, as shown for ZFN E/F and ZFN A/C (Fig. 3C; Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9812/-/DCSupplemental), respectively. These higher electropherogram peaks for the repaired sequence indicated higher abundance of the repaired nucleotides compared with mutated nucleotides, suggesting efficient gene repair of the p.R31X mutation. Furthermore, in our sequencings we did not observe any further base pair alterations besides the desired sequence repair (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9812/-/DCSupplemental). Although, we cannot completely exclude non-homologous end joining (NHEJ), our data indicate that HR is the preferred repair mechanism for DSB repair.

ZFN-Induced Gene Correction by HR Recovers Harmonin Protein Expression

To demonstrate recovered expression of the wild type *Usb1c* gene product harmonin on protein level we co-transfected p.R31X cells with matching ZFNs and the rescue plasmid. Gene repair was analyzed by indirect immunofluorescence using anti-Myc antibody against the C-terminal tag of the recovered protein and Western blot analyses with antibodies against harmonin (Fig. 4). No Myc-positive cells were detected in un-transfected cells (Fig. 4A, upper panel). In contrast, co-transfection of matching ZFNs (A/C and E/F) with rescue plasmid resulted in Myc-positive cells (Fig. 4A). This clearly indicated gene repair of the mutated *Usb1c* sequence resulting in recovered expression of the harmonin construct.

In Western blots of un-transfected p.R31X cells or p.R31X cells transfected with the control ZFN, no anti-harmonin positive band was detected, indicating that harmonin was not expressed (Fig. 4B). As expected, anti-harmonin Western blot analyses from lysates of *Usb1c* cells revealed a band of about 43 kDa, corresponding to the calculated molecular weight of the harmonin construct (Fig. 4B). A band of the same molecular weight was detected in lysates of p.R31X cells co-transfected with matching ZFNs and rescue plasmid, demonstrating ZFN-induced restoration of harmonin (Fig. 4B).

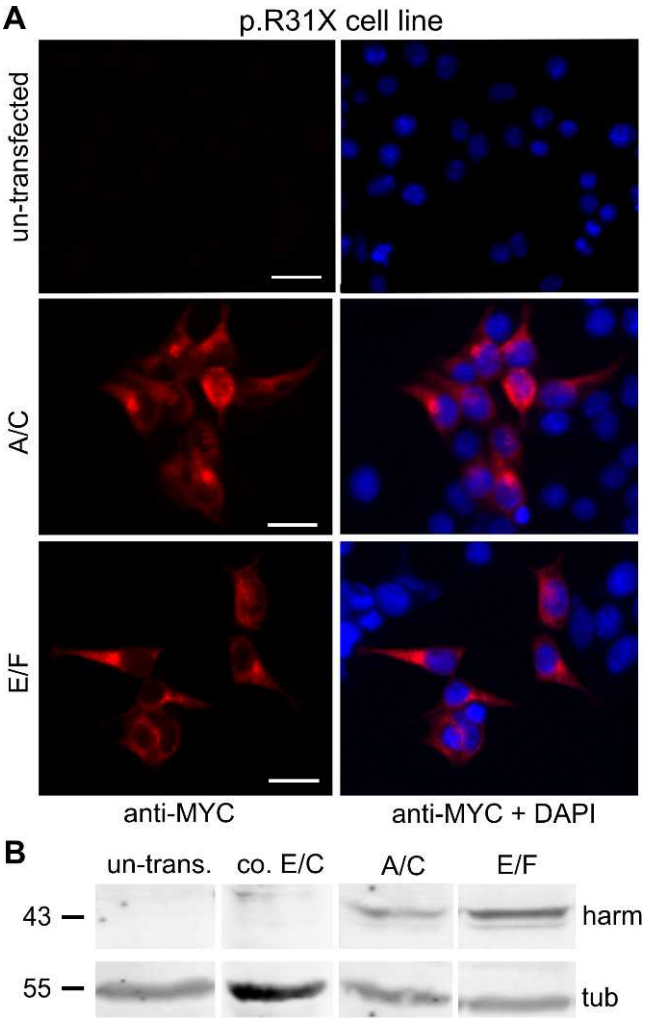


Figure 4. Gene repair mediated by ZFN induced HR on protein level. (**A**) Indirect immunofluorescence analysis of p.R31X cells revealed Myc-positive cells after transfection of ZFN A/C and E/F together with the rescue plasmid. No Myc labeling was detected in un-transfected cells. DAPI, nuclear DNA staining; scale bar: 25 μm. (**B**) Western blot analysis of p.R31X cells, either un-transfected or transfected with ZFN and rescue plasmid. Cell lysates were subjected to Western blot with anti-harmonin and anti-tubulin antibodies (loading control). Harmonin-PDZ2-Myc expression (~43 kDa) was detected by anti-H3 in *Usb1c* wild type cells and in pR31X cells transfected with ZFN A/C and E/F.

Discussion

Continual efforts have brought ZFN technology into the focus of faster and more efficient genome editing at selected predetermined sites in diverse eukaryotic species.[7,11,12,23–25] So far, most of the reported successful applications of the ZFN technology have been for the introduction of targeted modifications in the genome to create transgenic species.[26–31] More recently, a series of key publications has emphasized the significant therapeutic potential of ZFNs, placing this technology into the spotlight in the field of gene-based therapy.[32–34] In our present study, we introduced the ZFN technology using customized ZFNs to induce HR for gene repair of a specific disease-causing mutation responsible for a severe sensoneuronal degeneration, namely, the human Usher syndrome affecting the inner ear and retina. Our data indicate that the designed ZFNs are suitable for gene targeting to induce HR to correct the p.R31X mutation in the *Usb1c* gene.

To ensure the ZFN-mediated introduction of DSBs at the site of the mutation to be repaired, we have designed ZFNs containing one ZF binding directly to the p.R31X-mutated site. Based on this strategy, we selected ZFs that did not always target to GNN triplets, which were thought to be required for efficient binding of the ZF domain to the target DNA.[35,36] Nevertheless, the efficient cleavage and the high biocompatibility of the generated ZFNs indicate that the ZF domain composition out of ZF exclusively recognizing GNN triplets is not crucial, which is in line with previously reported data.[27] The observed differences in the binding and cleavage efficiency of the tested ZFNs are probably owing to varying synergistic effects between adjacent ZFs as previously discussed.[37]

In the present study, we provide proof-of-concept results that designed ZFNs mediate gene correction of a mutated USH gene efficiently through the introduction of site-specific DSBs at the mutation. Cells have developed two, in principle, different mechanisms to repair DSBs: NHEJ and homology-directed repair.[38] Since in NHEJ DNA ends are re-ligated without any use of homology, NHEJ is an error-prone mechanism, often introducing mutagenic changes.[39] In con-

trast, HR is a "copy and paste" mechanism, using a DNA template for the correct repair of DSBs preferred for therapeutic gene correction.[40] In our study, we activate HR providing the rescue DNA as the correct template for repair of ZFN-induced DSB. Our results provide several lines of evidence for the site-specific gene correction of the USH disease-causing p.R31X mutation by HR. The present cleavage assay revealed introduction of the wild type sequence into the mutant p.R31X cell line after application of matching ZFNs in concert with the rescue DNA. Sequence analysis revealed the rescue-specific genotype, indicating that rescue DNA was used as a template to fill up the gap introduced by ZFN-induced DSB. Finally, we demonstrated the recovery of harmonin protein expression by immunocytochemistry and Western blot analysis.

One significant drawback for the therapeutic application of the ZFN technology is the frequently reported toxic side effects of ZFNs introduced by off-target DNA cleavage.[41–43] Nevertheless, based on the results of cell viability tests, we did not observe cytotoxicity of the customized p.R31X ZFNs, indicating no obvious off-target effects. Our findings are in line with previous publications.[14,30]

We customized our ZFNs to target the *Ush1c* gene, with the future goal of correcting mutations causing human USH affecting the sensory neurons in the inner ear and the retina. Although the mechanism of HR has been well-established for cycling cells,[44] only recently was HR identified as a DSB repair pathway in terminally differentiated neurons as photoreceptor cells.[45] The authors of the study state that about 15% of DSB in rod photoreceptor cells are repaired by applying homology-based repair mechanisms, even without adding homologue DNA sequences.[45] This further qualifies our ZFN-based repair approach for correcting USH genes in the eye and ear. The possibility of repairing a disease-causing mutation in one therapeutic intervention instead of continuously treating the symptoms holds great advantages especially for eye diseases. Because of an effective blood-retina-barrier, systemic application of therapeutics to the retina is very challenging.[5]

Gene addition by viral vectors is traded as a promising strategy for the treatment of USH and is in preclinical trials for USH1B[46] (in the public domain; www.oxfordbiomedica.co.uk). Since USH genes frequently are very large and/or have various splice variants, gene addition approaches are very challenging. More recently, we succeeded in introducing translational readthrough-inducing drugs as a therapeutic intervention for USH1 nonsense mutations.[47,48] However, this drug treatment is only suitable for ~12% of USH mutations,[49] and lifelong medication is needed. The presented strategy of ZFN technology has apparent advantages over established gene-based therapy approaches[5] as (1) the repaired gene stays under control of its endogenous promoter, so expression level, splicing, and spatio-temporal expression remain unaffected; (2) no lifelong medication is necessary; (3) the size of the gene affected is not an issue; and (4) the type of the disease-causing mutation is not an issue.

Taken together, the good biocompatibility of our ZFNs and repair of the p.R31X mutation in our cell culture system provide evidence that ZFN-induced HR is suitable for the treatment of inherited genetic disorders, such as USH or related retinal ciliopathies, to cure the affected terminally differentiated sensory neurons in the retina. Conclusively, the ZFN technology holds an extensive potential for the treatment of known mutations in the direction of personalized therapy strategies.

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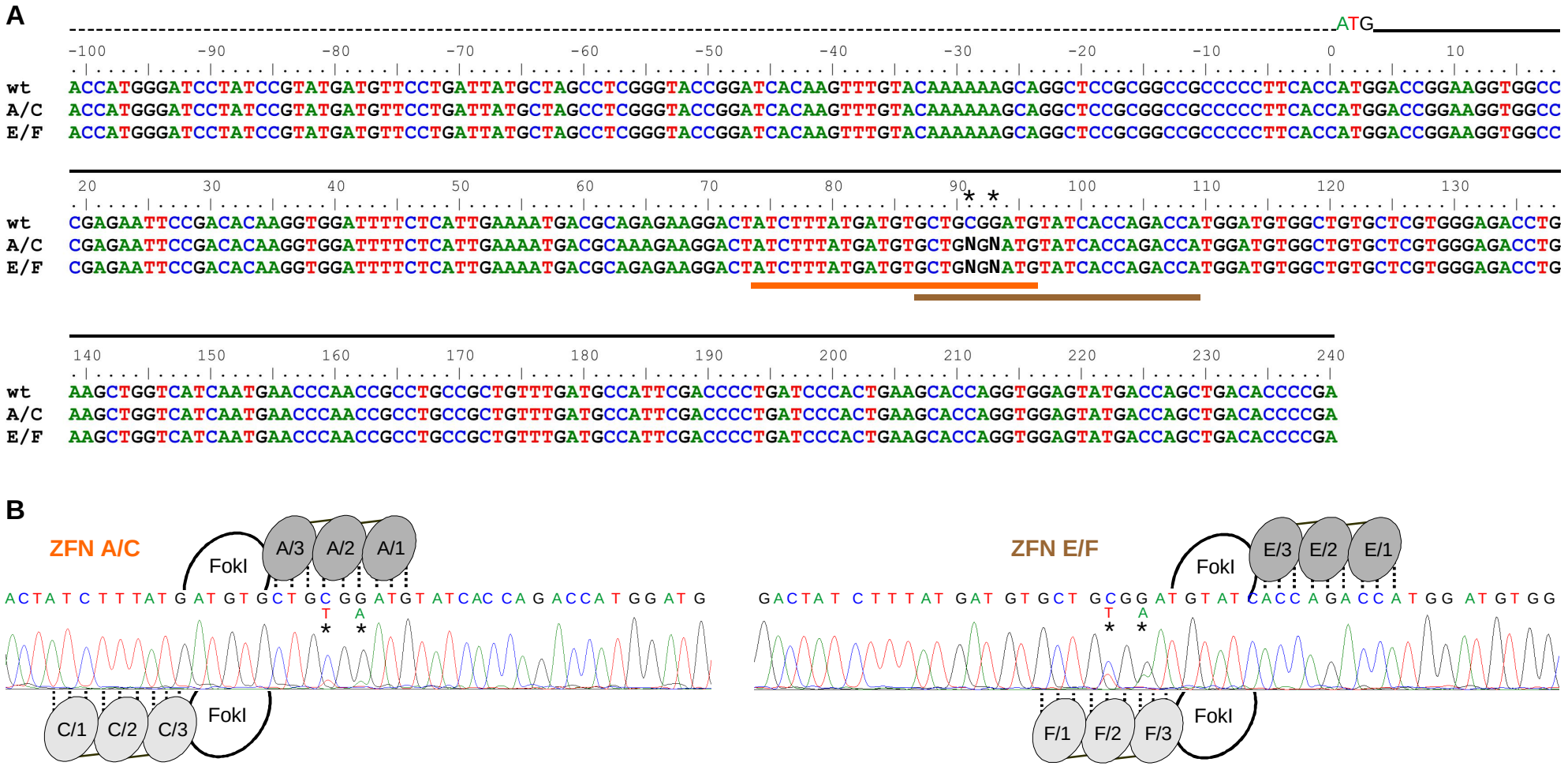

Supplementary Figure S1. ZFN induced gene repair does not alter bp sequences surrounding the p.R31X mutation. (A) Sequence alignment of wild type Ush1c sequence with sequencing results of gene repair after transfection of ZFN A/C and E/F. We observed no further bp alterations 5´and 3´of the desired sequence repair (bp 91 and bp 93). Dotted line: stably integrated vector sequences; solid line: Ush1c sequence up to bp 240; orange bar: ZFN A/C binding site (bp 74-96); brown bar: ZFN E/F binding site (bp 87-109); ATG: Ush1c start codon; asterisks/N: desired sequence repair of the p.R31X mutation. **(B)** Sequencing electropherogram of repaired Ush1c sequence after transfection of ZFN A/C (orange) or E/F (brown). Binding sites of ZF-domains (grey ovals) and spacer for FokI dimerization are indicated.