

# Improving nuclease-and nickase-based homology-directed repair with ZIP CRISPR

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# Abstract

Precise genome editing technologies create the potential for genetic studies and innovative gene therapies. However, the presence of the repair template at the DNA cleavage site could limit the efficiency of homology-directed repair (HDR) editing. On this basis, we explored the possibility of creating an all-in-one RNP cargo containing the guide RNA (gRNA), the Cas9 and the single-stranded oligodeoxynucleotide (ssODN) template. 3D modelling revealed that the ssODN template could interact with the RNP. Here, we present a new CRISPR-Cas9 strategy, called ZIP CRISPR, to reinforce this all-in-one cargo, using complementary extensions of the ssODN template and gRNA, allowing for improved HDR editing efficiency. This new strategy is easy-to-design, easy-to-use, inexpensive and versatile. It increases HDR editing efficiency using Cas9 nuclease up to 12-fold with a mean increase of 5-fold as demonstrated at many *loci* in many cell types. It can also be used with the Cas9 nickase, resulting in HDR editing with minimal InDels, and preventing double-strand break (DSB)-mediated genotoxicity. ZIP CRISPR is a non-viral platform adaptable to targeted DSB (higher HDR editing efficiency) or nick (higher safety) to precisely model and correct a wide range of edits. It is suitable for many biological applications and could be considered for HDR-based gene therapies.

## Background

The CRISPR-Cas9 system induces DNA double-strand breaks (DSBs) at targeted sites to activate mainly two competitive DNA repair pathways: homology-directed repair (HDR), which allows precise editing with a homologous DNA template, and non-conservative NHEJ/MMEJ pathways, which connect two ends of the broken DNA and are often accompanied by random insertions and deletions (InDels)(1). The relatively low efficiency of HDR compared to NHEJ/MMEJ repair has been a major hurdle in achieving precise genome editing (PGE) at desired *loci*. Due to their higher efficiency, to date, the only approved gene therapy using CRISPR technology is based on the NHEJ repair mechanism, which, whilst imperfect, is very efficient(2). Several techniques have been proposed to obtain a higher PGE rate, in particular by modulating repair pathways, either by pharmacological NHEJ/MMEJ inhibition(3–7), or by HDR activation (*via* the expression of HDR actors(8–10), Cas9-fusion proteins to enhance the HDR pathway at the targeted *locus*(11–14) or S/G2 synchronization(15)). While promising, these approaches may alter the normal behavior of the targeted cells, raising substantial safety concerns when explored in a therapeutic context(16). In addition, HDR remains to be optimized for therapeutic applications. The presence of the exogenous DNA template at the DNA cut is critical for HDR editing. For example, fluorescently labeled donor DNA can be used to enrich cells that are likely to be edited *via* HDR(17, 18). Several teams have already done important work to develop Cas9 tools to directly carry the single-stranded oligodeoxynucleotide (ssODN) template on the RNP complex. ssODN donors can be tethered to the Cas9 protein *via* the Cas9-avidin biotin-ssODN system(12), the RNP-DNA (RNPd) system(19), the Cas9-PCV system(20), the Cas9-AeF DBCO-adaptor ssODN system(21), or a platform that relies on thiol-maleimide chemistry and DNA-base pairing(22). Alternatively, ssODNs can be tethered to the guide RNA (gRNA) *via* a S1mplex system and a biotinylated ssODN(23), either with a chemical link between tracrRNA and the ssODN(18) or directly with a chimeric RNA-DNA donor guide(24). However, they mostly

rely on chemical modifications of the Cas9, gRNA, ssODN donor and/or used fusion proteins. Thus, their uses are constrained by chemical synthesis difficulties and risk of destabilizing the Cas9/gRNA interaction.

Crystal structures of *Streptococcus pyogenes* Cas9 revealed a bilobed architecture composed of target recognition and nuclease lobes, accommodating the gRNA:target DNA heteroduplex in a positively charged pocket(25). We therefore sought to determine whether it was possible to introduce the ssODN template together with the RNP into a Cas9/gRNA/ssODN ternary complex. Here, we model an interaction between the ssODN template and the RNP suggesting spontaneous all-in-one cargo containing the gRNA, the Cas9 and the ssODN template. We then developed a new, efficient CRISPR-Cas9 strategy, called ZIP CRISPR, designed to promote the presence of the ssODN template within the cargo and, subsequently, at the editing site, without any chemical modifications. For that, we extended the gRNA and the ssODN template to create a sequence complementarity interaction. This construction is easy-to-design and does not impact RNP activity. It does not require either any specialized and/or expensive chemical modifications or modified Cas9 making it readily transferable to all laboratories. Importantly, it is versatile, allowing increased HDR editing efficiency at many *loci*, in cell lines and primary cells with nuclease. Interestingly, this strategy also unlocked HDR editing by using safer single nickase.

## Results

### **An all-in-one ternary gRNA/Cas9/ssODN template complex seems to form spontaneously and can be reinforced with the ZIP CRISPR strategy**

To better visualize the spatial arrangement between the Cas9/gRNA RNP and the ssODN template used for HDR editing we modelled it. Indeed, while crystal structures of Cas9 are frequently done when the Cas9 is in interaction with the gRNA and/or with the targeted genomic DNA(25), to our knowledge none has been published with Cas9, gRNA and ssODN template (supposed conformation, Fig. 1a, named UNZIP). Using AlphaFold, we modelled this ternary complex with a gRNA/ssODN couple editing *eGFP* locus to obtain BFP sequence. We observed that interestingly, the BFP ssODN template seems to enter the positively charged pocket commonly occupied by the targeted DNA (Fig. 1b top panel). To decipher whether this ssODN localization was due to interactions with the Cas9 and/or with the gRNA, we modelled the binary complexes (Cas9 + gRNA, ssODN+Cas9, and ssODN+gRNA). As expected, modeling highlights gRNA/Cas9 interaction (Fig. 1b, middle panel). Moreover, modelings predict that the ssODN template could also interact with the positively charged pocket of the Cas9 and also to some extent with the gRNA (Fig. 1b bottom panels). Thus, a ternary cargo could be formed between the ssODN template, the Cas9 and the gRNA. To test whether this conformation was *locus* or template-dependent, we first model the interaction when using an absurd template containing only A nucleotides. In this case, a cargo is also predicted, suggesting that it is not template or sequence-dependent (Supplementary Fig. S1a). Then, we modeled tools targeting another *locus* *CFTR*, an endogenous target implicated in cystic fibrosis. AlphaFold software also predicts the formation of a ternary cargo containing the Cas9, the gRNA and the ssODN template (Supplementary Fig. S1b). Taken together, those modelings may indicate that the

ssODN template can interact closely with the Cas9/gRNA RNP complex and may promote the ssODN template supply at the site of editing to favor HDR.

Given this new observation, we wondered whether we could reinforce this potential ssODN/RNP interaction until editing. To that end, we developed a strategy to hybridize the ssODN donor template to the gRNA. For that, we added a 3'-extension (Fig. 1c, light pink in the schema) on the gRNA. This 20nt extension has a similar sequence as the 3'-extension of the first versions of pegRNAs targeting *HEK3 locus* published by Anzalone *et al.* in 2019(26), as we hypothesized that it will not destabilize the gRNA structure. This extension is complementary to a 5'-extension (light blue in the schema) of the 80nt ssODN template for annealing. The RNP complex formed is named ZIP and the control RNP with regular unextended ssODN template and gRNA is named UNZIP. Using AlphaFold for modelings, we confirmed the hybridization of elongated gRNA and ssODN in the ZIP RNP, without impairing Cas9/gRNA/ssODN cargo interactions already observed in the UNZIP regular RNP (Fig. 1d).

## ZIP CRISPR improves precise genome editing (PGE) using Cas9 nuclease

In light of these encouraging predictions, we tested this ZIP construct and its ability to increase HDR editing efficiency. To quantify CRISPR-Cas9-mediated genome editing, we used a rapid flow cytometry assay based on eGFP to BFP conversion(27) (Fig. 2a/b). In the case of HDR editing, the substitution of a histidine in place of a tyrosine at position 66 in the eGFP chromophore shifts its emission toward the blue spectrum(28) (Fig. 2a). HEK293T cells, K562 cells and human foreskin fibroblasts (hFFs) were stably transduced at low multiplicity of infection with a lentiviral construct containing an eGFP/puromycin resistance cassette to obtain less than 10% of eGFP<sup>+</sup> cells. After selection, we obtained cells with one copy of eGFP per cell. Reliable simultaneous quantification of HDR- and imprecise-editing was obtained by flow cytometry one week after edition by measuring the appearance of BFP fluorescence or the loss of eGFP fluorescence, respectively (Fig. 2b). The BFP detection is highly specific to HDR editing (no BFP without template). The eGFP fluorescence loss is mainly due to imprecise editing but can rarely be related to a spontaneous loss of fluorescence or to untransduced cells. Interestingly, overall editing efficiency was not affected by the extended gRNA but PGE (HDR editing) efficiency was significantly increased by 2-fold with ZIP compared to UNZIP RNP nucleofection in eGFP<sup>+</sup>-HEK293T cells ( $14.9\% \pm 0.7$  vs  $8\% \pm 1$  respectively, Fig. 2c/d) and was confirmed in an eGFP<sup>+</sup>-K562 cell line ( $10.2\% \pm 3.8$  vs  $5.2\% \pm 2.6$  respectively, Fig. 2e). These data obtained in two cell lines demonstrated that ZIP CRISPR increases PGE by using CRISPR-Cas9 nuclease, and is associated with a 2-fold increase in the HDR/InDels ratio.

## ZIP CRISPR' improvements

To establish whether ssODN template stability and quantity were critical for PGE, we designed two new ZIP CRISPR RNPs with either double ssODN hybridization on gRNA or elongated ssODN (120nt) to slow

down its degradation. We named these new RNPs ZIP\_Double\_Lock and ZIP\_Long\_Lock (Supplementary Fig. S2a). Indeed, because these modifications could affect gRNA stability and Cas9 interaction, we added a t-lock element (orange in the schema) in the third loop of the gRNA, as previously described(29) to reinforce its stability. This t-lock element alone (control ZIP\_Lock RNP, Supplementary Fig. S2a) did not modify editing efficiency but ZIP\_Double\_Lock RNP, and more efficiently ZIP\_Long\_Lock RNP improved PGE in eGFP<sup>+</sup>-K562 cell line with respectively + 37.2% and + 70% of HDR-edited cells (Fig. 3a and Supplementary Fig. S2b/c). For the ZIP\_Double\_Lock RNP, the lower efficiency compared to ZIP\_Long\_Lock RNP could be due to a steric hindrance when two ssODN templates are present in the RNP complex. The important effect with ZIP\_Long\_Lock RNP was due to elongation (control ZIP\_Long RNP, + 47.3%) combined with the t-lock loop. To create these new systems, we elongated the extension length to 30nt. To determine if the HDR increase observed could have been due to a change in the extension/annealing length (from 20 to 30nt for ZIP\_Long and ZIP\_Long\_Lock RNPs and from 20 to 2\*15nt for ZIP\_Double\_Lock RNP), we created new versions of ZIP\_Long\_Lock RNPs with annealing of 15nt, 20nt or 30nt and demonstrated that this change in annealing length doesn't impact HDR editing efficiency (Supplementary Fig. S2d/e). This suggests that the increase in HDR observed with the ZIP\_Long\_Lock RNP compared to the ZIP RNP is due to the t-lock element in the gRNA and the elongation of the ssODN template. Because template length seems to be an important parameter, we again elongated the ssODN template (170nt, ZIP\_ExtraLong\_Lock RNP, Supplementary Fig. S2f). We also tested the putative impact of hybridization position in the template. For that, we flipped the template with a 3'-extension for hybridization to the gRNA (ZIP\_Inv RNP, Supplementary Fig. S2f). However, neither of these constructs improved HDR editing efficiency compared to the ZIP\_Long\_Lock RNP (Supplementary Fig. S2g/h/i). Altogether, ssODN template elongation (120nt) associated with a t-lock loop in the gRNA (ZIP\_Long\_Lock RNP) was the most efficient system, so we named it ZIPmax and used it for the following experiments. To check whether the increase in HDR editing with the ZIPmax RNP was due to the initial complementary extension sequences chosen, we randomly created a new pair of complementary sequences with the same nucleotides and compared this ZIPmax\_V2 RNP to the ZIPmax RNP. We did not observe any difference in terms of HDR editing rates suggesting that the gRNA/ssODN template complementary extensions, no matter their sequences, are important in ZIP CRISPR (Supplementary Fig. S2j/k). We decided to keep the initial ZIPmax sequences for the following experiments (Fig. 3b). Importantly, the optimisations in the ZIPmax RNP do not alter the Cas9/gRNA/ssODN ternary cargo effect compared with the previous ZIP version (Fig. 3c).

### **ZIPmax benchmarking and validation in endogenous loci**

Then, we evaluated ZIPmax in primary eGFP<sup>+</sup>-hFFs, which are difficult to edit precisely. ZIPmax outperformed the UNZIP control by improving HDR editing efficiency up to 12-fold and the HDR/InDels ratio by 16-fold (Fig. 3d). Given this higher efficiency of ZIPmax, we decided to benchmark it to other strategies to improve HDR editing efficiency. First, we compared it to the S1mplex method proposed by Carlson-Stevermer *et al.* in 2017, because as ZIPmax it uses only commercially available components(23). S1mplex is composed of a biotinylated ssODN template and a gRNA modified to

include an aptamer that binds streptavidin. We challenged our ZIPmax RNP with S1mplex RNP in eGFP<sup>+</sup>-K562 cells and eGFP<sup>+</sup>-hFFs and observed a 2.6 and 2-fold increase in HDR editing rates using the ZIPmax RNP respectively (Fig. 3e/f and Supplementary Fig. S3b/c). Then, we compared the ZIPmax construct to Jin *et al.*'s strategy that demonstrated that small ssODN template extensions can recruit proteins and increase HDR editing efficiency. In particular they demonstrated that a specific 24nt 5'-extension on the ssODN template can recruit RAD51 and increase HDR in several cell types for several targets(8). We compared ZIPmax with an UNZIP condition using an ssODN template with this RAD51 recruitment motif, called UNZIP\_RAD51 (Supplementary Fig. S3a). In eGFP<sup>+</sup>-K562 cells, we observe the same HDR editing efficiency using ZIPmax or UNZIP\_RAD51 RNPs (Fig. 3e) but in eGFP<sup>+</sup>-hFFs we observe that our ZIPmax RNP induces a higher HDR editing rate than the UNZIP\_RAD51 construct, which edits cells as efficiently as UNZIP RNP did (Fig. 3f). Thus, our ZIPmax strategy seems to be more universal and usable in more cell types than Jin *et al.*'s strategy.

Another way to increase the HDR/InDels ratio is to mitigate the DNA repair pathways. To date, the most potent molecule to inhibit the NHEJ pathway is AZD7648, a potent inhibitor of DNA-PKs(4). We decided to challenge the ZIPmax strategy with this compound. Using eGFP<sup>+</sup>-hFFs, ZIPmax RNP provided a 1.7-fold higher precise editing efficiency compared to UNZIP RNP with the optimal conditions of AZD7648 exposure (13.9%  $\pm$  6.5 vs 8.3%  $\pm$  12.1 respectively, Fig. 3g and Supplementary Fig. S4). Importantly, a complementary additive effect was observed with the simultaneous use of ZIPmax RNP and AZD7648, leading to 23.1%  $\pm$  7.3% of HDR-edited cells. Given recent data showing the alarming genotoxicity of AZD7648(30–32), we decided to use only the ZIPmax RNP without AZD7648 in the following experiments.

Next, we tested the ZIPmax RNP to edit the *HBB* and *UROS* endogenous *loci* which are pertinent genomic targets for clinical applications (Fig. 3h/k). Because the t-lock element in the gRNA can improve HDR editing efficiency by itself at some *loci*(29), we used the same gRNA (with t-lock stabilization and 3'-extension) as a control in the UNZIP\_2 RNP to avoid a bias (Fig. 3i). The only difference between UNZIP\_2 and ZIPmax RNPs was the template, i.e. with or without the 5'-extension to anneal (or not) to the gRNA. First, we confirmed by AlphaFold the ternary cargo formation with the UNZIP\_2 construct (Supplementary Fig. S5a). Again, the ssODN template seems to interact with the Cas9 and the gRNA. ZIPmax RNPs targeting *HBB* or *UROS* also form a ternary complex, reinforced by the gRNA/ssODN template hybridization (Supplementary Fig. S5b). In terms of HDR editing efficiency, NGS analysis by Nanopore sequencing across the targeted *HBB* region showed that the seven nucleotides modified in the template (from - 15 to + 13nt around the cut site) were edited with a 2-fold increase in the HDR editing rate with the ZIPmax RNP (from around 7 to 13.5%, Fig. 3j). To check the versatility of ZIP CRISPR in clinically relevant cells, we evaluated it in cord blood-derived CD34<sup>+</sup> hematopoietic stem and progenitor cells (HSPCs) by targeting the exon 4 of *UROS*, an enzyme of the heme biosynthesis (Fig. 3k). In these primary cells, PGE was measured by restriction fragment length polymorphism (RFLP). Digestion occurred after the perfect editing of two nucleotides to create a *SacI* restriction site. The HDR editing

rate was more than 2-fold higher with ZIPmax than with UNZIP\_2 RNP ( $16.5\% \pm 5.1$  vs  $7.3\% \pm 4.6$  respectively, Fig. 3l).

Taken together, these data showed the higher efficacy of the ZIPmax construct in cell lines and primary cells to improve HDR editing efficiency for different targets.

### **CFTR gene editing in human pulmonary basal, bronchial and nasal epithelial cells**

We harvested 'healthy' pulmonary epithelial basal cells to model class I nonsense *CFTR* p.(GlyG542Ter), usually named G542X, responsible for cystic fibrosis (NM\_000492.4(*CFTR*): c.1624G > T, Fig. 4a). Cells were edited with UNZIP or ZIPmax RNPs by nucleofection. The template contained i) a non-coding TAG codon to replace the GGA codon (Gly) and ii) a *SacI* restriction site to allow HDR editing analysis by RFLP and mute the PAM to avoid a Cas9 recut. Analysis revealed a 3-fold higher percentage of HDR-edited alleles with the G542X mutation and the other four nucleotides responsible for the *SacI* cut with ZIPmax compared to UNZIP RNP, reaching up to 22% (Fig. 4b).

We then decided to correct this nonsense mutation, which is not eligible for current pharmacological treatments(33). We obtained nasal and bronchial epithelial cells from a cystic fibrosis patient carrying the G542X mutation in the heterozygous state. For editing, we used a mutation-specific gRNA targeting the G542X-mutated allele to keep the other allele (not mutated at this exon) intact, and a correcting template coding GGA (Gly) instead of TGA (nonsense). We added six additional silent mutations (from -15 to +4nt around the cut site) to avoid the Cas9 recut and analyzed editing by Nanopore NGS sequencing (Fig. 4c). Whereas the UNZIP\_2 system was inefficient to edit these nasal and bronchial epithelial cells, ZIPmax RNP allowed moderate complete (7 modifications) HDR editing (Fig. 4d/e, around 2%) and increased the HDR/InDels ratio (Fig. 4f/g). Regarding the c.1624T > G correction specifically, cells were initially 50% c.1624G. We obtained around 56 or 57% of c.1624G after editing, i.e. 6 or 7% of genomic edition in bronchial and nasal epithelial cells, respectively (Fig. 4h/i). This result, which is higher than for other substitutions, suggests an additive pathway to correct c.1624T, in accordance with previous reports of gene conversion with the endogenous allele in the event of heterozygosity(34, 35). These HDR editing rates could appear low but have to be put into perspective with the overall editing efficiency and to levels needed to get a clinical improvement. Moreover, regarding the literature, these HDR editing rates (6–7% considering only the c.1624T > G correction relevant for therapeutic impact) could be enough to recover CFTR function and lead to clinical benefit for the patients(36, 37).

## **ON-target genotoxicity outcomes of ZIP CRISPR**

Cas9 nuclease is known to induce DSB-dependent ON-target genotoxicity(38, 39). Some recently developed HDR boosters, such as AZD7648, have been shown to exacerbate ON-target genotoxicity, causing frequent kilobase- and megabase-scale deletions, chromosome arm loss, and translocations(30–32). We therefore investigated whether the ZIPmax RNP could similarly modulate this

toxicity. To test this hypothesis, we used our previously published fluorescence-assisted megabase-scale rearrangements detection (FAMReD) system that sensitively detects and quantifies megabase-scale loss-of-heterozygosity(40) (Supplementary Fig. S6a). We designed UNZIP\_2 and ZIPmax RNPs to target *SORCS1* locus. While increasing HDR editing (Supplementary Fig. S6b), ZIPmax did not increase DSB-dependent megabase-scale LOH frequency compared to UNZIP\_2 or to gRNA without template RNPs (Supplementary Fig. S6c). Thus, the ZIPmax strategy appeared to be safer than protocols modulating DNA repair pathways. Finally, ZIP CRISPR using Cas9 nuclease increased the PGE rate in many *loci* and cell types, but InDels and genotoxicity were still present. We thus applied ZIP CRISPR to a DSB-free Cas9 (single Cas9<sup>D10A</sup> nickase), which is known to allow HDR editing with minimal to no InDels but only using plasmid, not RNP transfection(9, 17, 41–50).

## ZIP CRISPR coupled with Cas9 nickase to obtain precise genome editing

To edit cells without DSB and avoid InDels formation, we applied the ZIPmax construct with the t-lock element in the gRNA and a 5'-extended (120nt) ssODN template to Cas9 nickase. The Cas9<sup>D10A</sup> nickase was preferred as the unmodified HNH domain has higher activity(51) and creates less DSBs(52). This new RNP was named ZIPmax\_Nick (Fig. 5a). As expected, UNZIP RNP with nickase (named UNZIP\_Nick) with unextended BFP-ssODN template did not edit eGFP<sup>+</sup>-HEK293T cells, eGFP<sup>+</sup>-K562 cells and eGFP<sup>+</sup>-hFFs or only at very low levels (Fig. 5b/c/d), confirming previous reports showing that it is challenging to edit the genome using a unique nick(42, 53). Importantly, the ZIPmax\_Nick RNP induced a 3-fold increase in PGE (BFP<sup>+</sup> cells) in the eGFP<sup>+</sup>-HEK293T cell line (Fig. 5b) and unlocked PGE in the eGFP<sup>+</sup>-K562 cell line (17-fold increase, Fig. 5c) and in primary eGFP<sup>+</sup>-hFFs (35-fold increase, Fig. 5d).

To confirm these results, we edited an endogenous *locus* (*UROS*) in hFFs with a template containing three substitutions (Fig. 5e). To be sure that the difference between control UNZIP\_Nick and ZIPmax\_Nick RNP was really due to the hybridization and not to other modifications (t-lock or the 3'-extension in the gRNA), we designed a new control with Cas9<sup>D10A</sup> nickase similar to the UNZIP\_2 RNP using nuclease with a gRNA containing the t-lock element and the 3'-extension, but with a 120nt template (without 5'-extension, UNZIP\_Nick\_2 RNP, Fig. 5f). Nanopore sequencing revealed a 4-fold improvement of perfect *UROS* HDR editing (3 concomitant edited bases, Fig. 5g), confirmed by individual base analysis (Fig. 5h) and without any detection of InDels (not shown).

Unlike for the nuclease, the absence of InDels using the nickase was an opportunity to repeat editing of the unedited alleles using the same gRNA to enrich the HDR-edited population as previously described(17) (Fig. 6a). We made three iterative RNP transfections (separated by one week to limit cytotoxicity) with ZIPmax\_Nick in eGFP<sup>+</sup>-hFFs and obtained an interesting additive effect leading to more than 3% of BFP<sup>+</sup> cells (Fig. 6b). We confirmed this additive effect targeting *UROS* in hFFs (Fig. 6c).

Then, to take advantage of our easy-to-design ZIP CRISPR tool, we evaluated the impact of the ssODN template design. When editing through DSBs, it is known that template design and especially length and asymmetry of homology arms can influence HDR editing efficiency(54). Thus, we decided to study the impact of template design when editing through SSBs and compared two asymmetric templates with either the nucleotide modifications on the 5'- (ZIPmax\_Nick template) or on the 3'-extremity (Super ZIPmax\_Nick template) (Fig. 6d). Interestingly, we observed a 2-fold increase in HDR editing in eGFP<sup>+</sup>-hFFs with the Super ZIPmax\_Nick RNP compared to the ZIPmax\_Nick RNP ( $0.71\% \pm 0.45$  vs  $1.58\% \pm 0.62$  respectively, Fig. 6e). Thus, ZIP CRISPR could be a great help to test and optimize template designs. With the Super ZIPmax\_Nick RNP in eGFP<sup>+</sup>-hFFs, we confirmed that iterative transfections led to an additive effect and resulted in more than 5% of HDR-edited cells (Fig. 6f).

### **ZIPmax\_Nick can be combined with 'cell state' modulation to increase nick-based HDR editing**

To further improve HDR editing efficiency and benefit from the versatility of ZIPmax\_Nick strategy, we investigated various pathways known to modulate genome editing. First, we tried to modulate DNA repair pathways. Nick-mediated HDR repair using a single-stranded template is known to be suppressed by RAD51/BRCA2(42). Thus, we transiently inhibited *BRCA2* using a siRNA in eGFP<sup>+</sup>-HEK293T cells and eGFP<sup>+</sup>-K562 cells. In both cell lines, we obtained a transient drop in *BRCA2* mRNA expression (Supplementary Fig. S7a/b). We achieved a 2-fold increase in HDR editing (BFP<sup>+</sup> cells) when *BRCA2* was inhibited during editing (Supplementary Fig. S6c/d). However, it was accompanied by a 3 to 4-fold increase in imprecise editing (InDels, eGFP<sup>-</sup>/BFP<sup>-</sup> cells) in eGFP<sup>+</sup>-HEK293T cells and eGFP<sup>+</sup>-K562 cells (Supplementary Fig. S6e/f), probably due to the conversion of nicks into DSBs. Thus, we decided not to pursue the idea of inhibiting *BRCA2* to avoid DSB-associated genotoxicity. We also tried to inhibit *MLH1* by a CRISPR knock-out to modulate the mismatch repair (MMR) pathway, but we encountered toxicity issues after editing (data not shown).

While HDR editing using the Cas9 nuclease is known to depend on the S/G2 phases, the impact of the cell cycle on nick-mediated HDR editing has been less studied(55). We observed higher HDR editing rates in highly proliferative HEK293T cells than in less proliferative hFFs (Fig. 5b/d), suggesting that editing could preferentially be performed during the S/G2/M phases. To decipher the role of the cell cycle on HDR-based nick repair, and the S/G2/M phases in particular, we modulated hFF's cell cycle. We used palbociclib, a CDK4/6 inhibitor, to reduce the proportion of eGFP<sup>+</sup>-hFFs in the S/G2/M phases during editing (Fig. 7a/b). We observed a 2-fold decrease in HDR editing rates (Fig. 7c and Supplementary Fig. S8a). In contrast, HDR editing efficiency was improved by almost 2-fold (+ 85.4%, Fig. 7c) when using the optimal conditions of CDC7 inhibitor XL413 exposure during editing (Supplementary Fig. S8b/c/d/e/f) to increase the proportion of eGFP<sup>+</sup>-hFFs in S/G2/M phases (Fig. 7a/b). These data suggest that HDR editing with nickase is S/G2/M phase-dependent.

Chromatin access can also be important for the efficiency of CRISPR tools. We modulated chromatin opening by using HDAC inhibitors in eGFP<sup>+</sup>-hFFs with the ZIPmax\_Nick RNP. When we screened several

drugs (valproic acid - VPA -, WT-161 and sodium butyrate - NaBut -) at different doses, we observed a global trend to an increase in HDR editing efficiency in presence of HDAC inhibitors. A significant effect was found with WT-161 at 10nM (+ 86.6%), NaBut at 0.3mM (+ 52.7%) and more importantly with NaBut at 1mM (+ 125%) (Fig. 7d and Supplementary Fig. S8g/h). We validated the effect of NaBut at 1mM editing *UROS* in hFFs (+ 81.5%, Fig. 7e and Supplementary Fig. S8i). Then, we combined NaBut and XL413 exposure. Remarkably, we observed an additive effect, leading to a 7-fold increase in HDR editing compared to the condition without drugs (Fig. 7f and Supplementary Fig. S8j).

Therefore, by reaching detectable HDR editing rates at several *loci* and in several cell types with the ZIPmax\_Nick strategy, we demonstrated that HDR editing with nickase was more efficient during or after replication, and was boosted by HDAC inhibitors and ssODN template design.

## Discussion

HDR editing remains a challenge with CRISPR-Cas9, especially for clinical purposes. The spatiotemporal availability of the HDR template at the cut site during repair is supposed to be a key factor for HDR editing. In this study, we observed in modelings that the RNP complex containing the Cas9 and the gRNA could also incorporate the ssODN template and forms a big all-in-one cargo to facilitate proximity of the different molecular actors for HDR editing. Building on this structural rationale, we engineered a programmable gRNA/donor hybridization strategy designed to reinforce donor template association with the RNP and promote precise genome editing following either DSB- or nick-mediated editing. Using RNPs, we demonstrated that ZIP CRISPR is an HDR-enhancement strategy easy to implement yet effective.

We validated ZIP CRISPR for several targets (*eGFP*, *HBB*, *UROS*, *CFTR*, *SORCS1*) in different cell types (HEK293T cells, K562 cells, hFFs, HSPCs and pulmonary, bronchial and nasal epithelial cells) and for various kinds of edits (substitutions of 2 to 7 bases and deletions) around the cut site (editing window from - 15 to + 15nt depending on the targets). We obtained up to 12-fold improvement in HDR editing when mediated by a DSB (mean of 5-fold) and up to 35-fold (unlocking) when mediated by a single nick compared to a control approach with an unextended ssODN template.

As previously described in the introduction, other strategies have been implemented to increase HDR editing efficiency by bringing the ssODN template with the Cas9/gRNA complex, with a link to the Cas9 or the gRNA, but faced some limitations especially in terms of production, transfection, gene therapy immunogenicity, and the increased costs of producing a gene-editing tool. Here we develop an approach without chemical modifications based on extensions of the gRNAs and ssODN templates to reinforce proximity by annealing and enhance HDR efficiency. Unlike the other approaches, ZIP CRISPR is free from fusion protein or a covalent link between CRISPR-Cas9 components. In particular, we demonstrated in two cell types that our ZIPmax strategy induced higher HDR editing rates than the S1mplex import system published in 2017. While S1mplex strategy was a good solution to increase HDR/InDels ratio without fusion protein for many years, ZIPmax RNP presents several advantages such as an increase in

HDR rate, an easier availability and transfectability of the components (no additional protein to complex) and the possible use of longer ssODN templates (not limited to 60-100nt because of the biotin). Other strategies were developed to increase the concentration of the ssODN template near the DNA break. Polymerase-based DNA writing technologies have been developed to produce large template quantities *in situ* near the targeted *locus*. Prime editing enables precise modification of genomes *via* reverse transcription of an RNA pre-template encoded at the 3' end of the gRNA (a prime editor guide RNA, pegRNA) to produce the ssODN template(26). More recently, a DNA-dependent polymerase-based system known as click editing was developed to amplify the ssODN template with higher fidelity(56, 57). However, these strategies rely on large Cas9 fusion proteins featuring a polymerase activity that still require limiting substrates (nucleotides, MgCl<sub>2</sub>...). In addition, their impact on cells remains largely elusive, and they are complex to design and adapt to new targets.

Importantly, ZIP CRISPR reached high-efficiency HDR editing rates without modulating DNA repair pathways. Indeed, several methods have been proposed to inhibit NHEJ/MMEJ. Small pharmacological molecules (e.g. SCR7, AZD7648, M3814) have been developed to transiently inhibit the NHEJ components (KU70/80, DNA PK, Ligase IV)(3, 4). The concomitant inhibition of NHEJ and MMEJ by polymerase theta (Polθ) inhibitors has also been proposed(5–7). In all studies, these molecules limit the NHEJ/MMEJ pathways and therefore enhance HDR editing efficiency. Recruitment(8) or ectopic expression of RAD51 alone or combined to SCR7(9, 10) can also be used. In the present study, ZIP CRISPR was at least as efficient as AZD7648 exposure to increase the HDR editing rate in hFFs. Interestingly, the efficiency was improved when ZIP CRISPR and AZD7648 were associated. This combined approach could be interesting to obtain higher levels of HDR editing and confirm that the selection of the DSB repair pathway and the recruitment of the DNA template are two main steps limiting the rate of HDR editing. However, modulating DNA repair pathways may not be without effect and could alter genome integrity. For example, one of the most potent NHEJ inhibitors, AZD7648, was recently shown to be genotoxic(30–32) inducing large deletions and translocations. This suggests that DNA repair modulators should be used carefully in clinical studies and that researchers risk overestimating the results of HDR rate(39). Interestingly, ZIP CRISPR did not increase DSB-associated genotoxicity and HDR editing efficiency in hFFs was higher with ZIPmax RNP than with AZD7648 exposure combined with UNZIP RNP. The safety of ZIP CRISPR has to be considered for future clinical applications of CRISPR-Cas9 mediated gene therapy, and ZIP CRISPR should be preferred to NHEJ inhibitors.

To avoid genotoxicity, editing without DSBs is recommended for gene therapy. SSB-based editing with single nickase is feasible but currently relies on plasmid transfection of components to reach detectable HDR edition levels. This lack of efficiency(41, 58, 59) is probably due to the involvement of the single-strand break repair (SSBR)(42) and MMR repair pathways. Indeed, it has been described for other SSB-based CRISPR tools, such as base editing(60) and prime editing(61, 62) that editing was higher in MLH1-deficient cells and the authors have proposed to inhibit MLH1 (by knock out or using dominant negative) or to trick the MMR pathway with the introduction of a second nick on the opposite strand. However, these approaches are toxic or induce more InDels and ON-target genotoxicity(63). Here, ZIP CRISPR

allowed us to obtain HDR editing without InDels, with a single nickase RNP (to avoid any risk of genomic integration), and without polymerase or fusion protein.

Using this new strategy we decipher the mechanisms implicated in nick-mediated HDR editing. HDR repair at nicks is known to be influenced by the transcriptional status of the nicked strand (more efficient on the transcribed strand) and suppressed by RAD51/BRCA2 when using an ssDNA template(42). Here, we confirmed the inhibiting role of BRCA2 on nick-based HDR editing and showed an important increase in imprecise editing, validating BRCA2's importance in maintaining genome integrity(64). We also investigated the impact of cell cycle modulation on nick-based HDR editing, showing that HDR editing was increased in proliferative cells (synchronized in the S + G2/M phases) and reduced in non-dividing cells (synchronized in the G0/G1 phases). Those results are different from a previous study done by Zhang, Davis and Maizels in 2021(55) showing that nicks initiated HDR more efficiently in the G1 than in the S/G2 phases. This could be explained by the different cells (primary fibroblasts in this study vs HEK293T and U2OS cell lines) and the different methodologies used: pharmacological synchronization of cells in a phase of the cell cycle for 48h vs the use of Cas9 fused to degrons, as the previous publication indicated that the G1 phase nicks may persist to be repaired later in cell cycle. We also confirm chromatin opening impact for nick-based HDR editing in line with previous studies done with other CRISPR-based editing tools(65, 66). Further studies will be required to improve the efficiency and decipher the nick-based HDR editing mechanisms.

In this study, we used ZIP CRISPR with ssODN templates shorter than 200nt. However, it would be interesting to use it to transfect larger single- or doubled-stranded DNA. This could be a safer non-viral alternative to the AAV or IDLV template currently used for large gene insertions(67). Further studies would be important to explore this possibility.

## Conclusions

Altogether, ZIP CRISPR is a new programmable chemical-free non-viral versatile strategy that can meet the needs of a wide range of experiments. ZIPmax strategy based on nuclease is the first choice when a higher HDR editing rate is needed and when DSB-associated genotoxicity is acceptable. On the other hand, ZIPmax\_Nick strategy based on single nickase is more suited when a non-genotoxic approach is required and when low HDR editing is sufficient to restore the phenotype, or when iterative editing is feasible. The simplicity of ZIP CRISPR strategy should extend its utility to the investigation of complex biological issues in a wide range of cells and organisms. Since ZIP CRISPR is a modular strategy, it is potentially compatible with several types of templates and Cas (eventually after further optimizations), so it could play a leading role in many areas of research. Importantly, ZIP CRISPR achieves efficient HDR editing and could be considered to unlock non-viral HDR-based gene therapies.

## Methods

### AlphaFold modeling

All structural modelings were performed using the online AlphaFold server. The resulting modelings were subsequently visualized and colored using PyMOL (v3.1.8). For comparative visualization, each modelling was structurally aligned based on the Cas9 region to ensure a consistent orientation across all structures.

## Cell culture

Human embryonic kidney (HEK) cell line immortalized with large T antigen HEK293T (ATCC®, Manassas, VA, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM), low glucose (1g/L), L-Glutamine (1g/L) and pyruvate (Gibco® by Life Technologies™, Carlsbad, USA) supplemented with 10% fetal bovine serum, 100U/mL penicillin and 100µg/mL streptomycin (all from Eurobio™, Courtaboeuf, France).

K562 cell line (ATCC®) was maintained in Roswell Park Memorial Institute (RPMI) Medium 1640, L-Glutamine, 25mM HEPES (Gibco® by Life Technologies™) supplemented with 10% fetal bovine serum (Eurobio™), GlutaMAX (Gibco® by Life Technologies™), 100U/mL penicillin and 100µg/mL streptomycin (Eurobio™).

hTERT immortalized human foreskin fibroblasts (hFFs, ATCC®) were maintained in DMEM, high glucose (4.5g/L), L-Glutamine (1g/L) and pyruvate (Gibco® by Life Technologies™) supplemented with 10% fetal bovine serum (Eurobio™), 1% essential amino acids (Gibco® by Life Technologies™), 100U/mL penicillin, 100µg/mL streptomycin (Eurobio™), 10µg/mL ciprofloxacin (Biogaran™, Colombes, France) and 0.5µg/mL amphotericin B (Sigma-Aldrich®, Saint Louis, USA). A previously established heterozygous *UROS*<sup>+/-</sup> hFF cell line was used for the FAMReD genotoxicity assay (see (40) for details).

Human HSPCs were isolated from the cord blood of healthy donors. Briefly, mononuclear cells were isolated by Ficoll gradients. hCD34<sup>+</sup> cells were purified according to the manufacturer's instructions (Human CD34-Positive Selection kit II from Stem Cell Technologies, Vancouver, Canada) and purity was analyzed by flow cytometry using phycoerythrin-conjugated anti-CD34 antibody (Biolegend, San Diego, USA). Cryopreserved hCD34<sup>+</sup> cells were thawed and cultured in expansion medium consisting in StemSpan SFEM (Stem Cell Technologies) supplemented with Flt3-L (100ng/mL, Peprotech, Carlsbad, USA), SCF (100ng/mL Peprotech), human TPO (100ng/mL Peprotech), vitamin C (0.35mg/mL, Sigma-Aldrich), ciprofloxacin (10µg/mL, Biogaran™) and 100U/mL penicillin and 100µg/mL streptomycin (Eurobio™).

Nasal and bronchial epithelial cell cultures were established from nasal or bronchial brushings, as previously described(68, 69). Nasal and basal epithelial cells were cultured in PneumaCult™-Ex Plus Medium (StemCell Technologies) supplemented with penicillin-streptomycin-Amphotericin B 1X (100X, Gibco).

All cell types were cultured at 37°C, 5% CO<sub>2</sub> in a humidified chamber.

## Construction of eGFP<sup>+</sup> cell models

HEK293T cells, K562 cells and hFFs were transduced with a lentivirus containing the gene encoding eGFP (enhanced green fluorescent protein) and a puromycin resistance gene, under the dependence of the ubiquitous strong constitutive promoter MND (myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted). This lentivirus was produced and titrated by the Vect'UB vectorology platform at Bordeaux University. HEK293T cells, K562 cells and hFFs were transduced at a multiplicity of infection (MOI) of 0.1, 1 and 2, respectively, to obtain a low copy number of the integrated vector. Transduced cells were selected 96h after transduction by adding puromycin (2µg/mL for 48h, Gibco) to their culture medium. Hereafter, these cells are referred to as eGFP<sup>+</sup>-HEK293T cells, eGFP<sup>+</sup>-K562 cells and eGFP<sup>+</sup>-hFFs.

## RNP transfection and gene-editing tools

The crRNAs were designed using CHOP-CHOP software ([chopchop.cbu.uib.no](http://chopchop.cbu.uib.no)). Then the tracrRNA part (eventually modified for one of the loops and lengthwise) was added to reconstitute the gRNA. gRNAs and ssODN templates for HDR correction were ordered from Integrated DNA Technologies (Coralville, USA). Their sequences are given in Supplementary Tables 1, 2 and 3.

Eventually, annealing between gRNA and ssODN template(s) to form gRNA-template hybrids (ZIP, ZIP\_Lock, ZIP\_Long, ZIPmax, ZIP\_Long\_Lock\_hyb15, ZIP\_Long\_Lock\_hyb20, ZIP\_ExtraLong\_Lock, ZIP\_Inv, ZIPmax\_V2, ZIPmax\_Nick, Super ZIPmax\_Nick) or gRNA-templates hybrid (ZIP\_Double\_Lock) was performed by mixing the components either in equimolar proportion (1:1) or with a double template (1:2) for the ZIP\_Double\_Lock. The components were then heated for 5 minutes in a dry bath at 95°C before allowing them to cool at room temperature.

The different components of the CRISPR-Cas9 system (Alt-R® S.p. HiFi Cas9 Nuclease V3 (abbreviated hereafter as Cas9 nuclease) or Alt-R S.p.Cas9 D10A Nickase V3 protein (abbreviated hereafter as Cas9 nickase) both from Integrated DNA Technologies, with either split gRNA and ssODN template or gRNA-template(s) hybrid(s) were combined to form an RNP. They were mixed and then incubated for 10–20 minutes at room temperature. For this, 62pmol (for HEK293T cells, K562 cells and hFFs) or 105pmol (for other cells) of nuclease or nickase Cas9 and 100pmol (for HEK293T cells, K562 cells and hFFs) or 170pmol (for other cells) of gRNA, ssODN template or gRNA-template(s) hybrids for ZIP CRISPR were mixed. Finally, 100pmol of Alt-R Cas9 Electroporation Enhancer solution (Integrated DNA Technologies) were added to the RNP complex to improve electroporation efficiency.

For S1mplex transfection, S1m-gRNA and biotin-ssODN template were ordered from Integrated DNA Technologies. The RNP complex was formed as described in the publication(23). Molecular ratios were conserved but quantities were adapted to use the same quantity of Cas9 as in the other conditions of the experiment.

Cells were transfected by electroporation using the Nucleofector 4D AMAXA electroporation system (Lonza®, Bale, Switzerland). In brief,  $5 \cdot 10^4$  to  $3 \cdot 10^5$  depending on the cell type were resuspended in SF Cell Line 4D-Nucleofector® (HEK293T cells or K562 cells) or P3 Primary Cell Line 4D-Nucleofector®

(hFFs, CD34<sup>+</sup> HSPCs, pulmonary epithelial basal cells, nasal epithelial cells and bronchial epithelial cells) and added to the RNP complex. Then cells were nucleofected using DG-150, FF-120, CZ-167, DO-100 or DC-100 programs, respectively.

Cells were then cultured as described above for 5–15 days depending on the editing analysis method.

## **Edition analysis**

### **HDR and imprecise editing (InDels) analysis by flow cytometry**

For the *eGFP* target, precise editing allows the switch from eGFP to BFP. At least 6 days after editing and before flow cytometry analysis, cells were recovered in a PBS (phosphate buffered saline, Gibco) - EDTA (ethylenediaminetetraacetic acid, 2mM, Sigma-Aldrich) solution. Cells were analyzed for fluorescence using the BD FACSCanto II analyzer (BD Biosciences, Le Pont de Claix, France) at the cytometry platform at University of Bordeaux (UB'FACSility). Excitation wavelengths of 488 and 405nm were used to detect eGFP and BFP fluorescence in the FITC (Fluorescein-5-isothiocyanate) (530/30 filter) and Pacific Blue (450/50 filter) channels, respectively. The data presented are from the analysis of at least 10,000 events. The percentages of eGFP<sup>+</sup>/BFP<sup>-</sup>, eGFP<sup>-</sup>/BFP<sup>+</sup>, and eGFP<sup>-</sup>/BFP<sup>-</sup> correspond to cells unedited, edited by HDR, or imprecisely-edited, respectively. The percentages of BFP<sup>+</sup> or eGFP<sup>-</sup>/BFP<sup>-</sup> cells shown in the graphs were calculated by subtracting the percentages of BFP<sup>+</sup> or eGFP<sup>-</sup>/BFP<sup>-</sup> cells, respectively, from a sample of cells from the corresponding lineage not transfected with RNP when these were non-zero. Then, the percentage of BFP<sup>+</sup>, eGFP<sup>-</sup>/BFP<sup>-</sup> or eGFP<sup>+</sup>/BFP<sup>-</sup> cells were normalized to recover a total of 100%.

### **HDR analysis by RFLP**

If a restriction site is added in the ssODN template sequence, the HDR rate can be measured by RFLP (restriction fragment length polymorphism). At least 5 days after editing, genomic DNA was extracted from cell pellets using Nucleospin® Tissue (Macherey-Nagel, Duren, Germany) according to the manufacturer's protocol. The genomic region flanking the edited site was amplified by PCR (HotStarTaq Plus DNA polymerase, Qiagen®, Venlo, Netherlands) with adequate primers (Supplementary Table 4). PCR products were purified with Nucleospin® Gel and PCR Clean-up (Macherey-Nagel) and digested with SacI restriction enzyme (New England Biolabs, Ipswich, USA) for at least 1h at 37°C. Then, digestion products were loaded into the Agilent® 2200 TapeStation (Santa Clara, USA) capillary electrophoresis at the OneCell platform at University of Bordeaux, using D1000 ScreenTape and D1000 reagents according to the manufacturer's protocol. Quality control of enzymatic digestion efficiency was included in each assay.

### **HDR and InDels analysis by Nanopore sequencing**

At least 5 days after editing, genomic DNA was extracted from cell pellets using Nucleospin® Tissue (Macherey-Nagel) according to the manufacturer's protocol. The genomic region flanking the edited site

was amplified by High-Fidelity PCR (Phusion High-Fidelity DNA Polymerase, New England Biolabs) using 35 cycles under recommended manufacturer's protocol with adequate primers (Supplementary Table 4). A sequencing library was prepared using the Rapid Barcoding Kit 96 V14 (SQK-RBK114.96, Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's protocol. Sequencing was then performed using a MinION Mk1B device and a Flow Cell R10.4.1 (FLO-MIN114) both from Oxford Nanopore Technologies to reach at least 2000 quality-filtered reads per sample (Q score > 8). After sequencing, super-accurate basecalling was performed using MinKNOW (v24.02.6) and FASTQ were aligned on the human reference genome GrCH38 (GCA\_0000001405.15) using Epi2Me (v5.1.9) and the wf-alignment pipeline (v1.1.2). Then, BAMs were analyzed with two custom bioinformatic scripts in Python (v3.8) using pysam (v0.16). Briefly, one searches specific sequences in the BAM corresponding to unedited, HDR edited sequences or eventually predominant InDels seen with IGV (Integrative Genomics Viewer) visualization to determine the HDR editing rate per read, whereas the other one calculates the proportion of each nucleotide for each selected position to determine the HDR editing rate per base, returning these data in xlsx format (pandas v1.1.3). For *CFTR* G542X correction, HDR and InDels rates were determined using nCRISPResso2, according to Mc Farlane *et al*'s publication(70). The command is available in Supplementary Table 5.

## Drugs used

## Cell cycle synchronization

To evaluate the impact of synchronization on HDR editing efficiency, cells were synchronized in the G0/G1 phase by incubation with palbociclib, also named PF-00080665 or PD 0332991 (1 $\mu$ M, Sigma Aldrich) 24h before and 24h after RNP transfection. To synchronize cells in the S phase, they were incubated with XL413 (10, 20 or 40 $\mu$ M, Selleckchem, Houston, USA) for 24h before RNP transfection and 24h after RNP transfection (or at the time indicated in the figures).

Cell cycle analyses were performed to check synchronization efficiency in each experiment. Briefly, cells either synchronized or not were harvested, fixed with 4% paraformaldehyde (for 15 minutes) and permeabilized with Triton 0.5% (Sigma-Aldrich, for 15 minutes). Cells were then incubated overnight with RNase (100 $\mu$ g/mL, MoBiTec, Goettingen, Germany) and then for 15 minutes with propidium iodide (4 $\mu$ g/mL, Biolegend). The samples were examined on a BD Biosciences Accuri C6 Plus flow cytometer and the data were analyzed with BD CSampler™ software (BD Biosciences).

## HDAC inhibition

To evaluate the impact of HDAC inhibition, cells were incubated 24h before and 48h after edition (or at the time indicated in the figures) with either valproic acid (VPA, 0.3, 1 or 3mM, Sigma-Aldrich), WT-161 (0.5, 2 or 10nM, MedChemExpress, Monmouth Junction, USA) or sodium butyrate (NaBut, 0.3, 1 or 3mM, Sigma-Aldrich).

## NHEJ inhibition

To modulate the NHEJ pathway, we incubated the cells 3h before and 48h after edition (or at the time indicated in the figures) with AZD7648 (0.3, 1, 3 or 10 $\mu$ M, MedChemExpress).

## Nick repair pathway inhibition

To modulate the nick repair pathways, HEK293T and K562 cells were transfected either 48h or 72h before edition with a siRNA targeting *BRCA2* (10pmol/mL, #s2085 Life Technologies), respectively. siRNA transfections were performed using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's protocol. To determine the efficiency of siRNA knockdown, *BRCA2* mRNA levels were quantified by RT-qPCR relative to control that assayed glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) transcripts. Briefly, RNA was extracted from cell pellets using Nucleospin® RNA (Macherey-Nagel) according to the manufacturer's protocol. RNA was retro-transcribed into cDNA using high-capacity cDNA reverse transcription (Applied Biosystems, Waltham, USA) according to the manufacturer's protocol. Then qPCR was performed using GoTaq qPCR Master Mix 1X (Promega, Madison, USA) and adequate primers (Supplementary Table 6). Results were analyzed with CFX Maestro 1.0 (BioRad, Hercules, USA).

## FAMReD fluorescent cell quantification

At least 15 days after editing, 0.3mM of 5-aminolevulinic acid hydrochloride (5-ALA, Sigma-Aldrich) was added to hFFs' medium. After 16h of exposure (overnight), cells were washed twice with PBS and placed in fresh medium. Upon LOH, loss of UROS function can be detected by the appearance of fluorescence due to porphyrin accumulation. Following 8h of clearance, fluorescent cells were quantified by flow cytometry. UV-sensitive porphyrins were excited at 488nm and the emitted wavelength was approximately 667nm, detected by the PE-Cy5A PMT channel (FACS Accuri, BD Biosciences). FL-1 is a control green-fluorescent channel used to exclude auto-fluorescent cells.

## Statistical analysis and reproducibility

The experimental data presented are from the analysis of several independent experiments. No statistical method was used to predetermine sample size. No data were excluded from the analyses. The experiments were not randomized. Statistical significance was inferred when necessary and formatted using GraphPad Prism 10.3.1 software. To confirm significance, p-values were calculated when analyzing at least three independent experiments. Exact distinct and independent experiments size is indicated in each legend (n). Results are presented as mean  $\pm$  SD. To compare two groups, the parametric t test (two-sided) or the nonparametric Mann–Whitney test (two-sided) were used when residual distribution was Gaussian/normal or not respectively. If the comparison was done to a condition normalized to 1, the nonparametric Kolmogorov-Smirnov test was used. To compare more than two groups, one-way ANOVA (two-sided) was used and Gaussian/normal residual distribution was checked. If Gaussian/normal residual distribution was not assumed or not validated, Kruskal-Wallis test was used. To compare several conditions in two groups or more, 2way ANOVA was used and Gaussian/normal

residual distribution was checked. If not indicated, the results are not significant. The details (number of independent replicates, statistical test used) are indicated in the legends of the figures.

## Declarations

### Ethics approval and consent to participate

This research complies with all relevant ethical regulations. Human CD34<sup>+</sup> stem and progenitor cells (HSPCs) were isolated from cord blood of healthy donors from Bagatelle Hospital, according to the hospital's ethical institutional review board (Maison de Santé Protestante de Bordeaux, Talence, France) and with the mothers' informed consent.

The patient with cystic fibrosis was recruited at the pediatric cystic fibrosis resource and skills center (CPP 23.00854.000207). Nasal and bronchial epithelial cells were obtained by nasal or bronchial superficial ciliary brushing, respectively, according to the hospital's ethical institutional review board and with the parents' informed consent.

Control subjects (CPP 23.00854.000207) were recruited after surgical resection at the University Hospital of Bordeaux. Bronchial specimens from all subjects were obtained either by fibroscopic bronchoscopy or lobectomy in macroscopically normal areas, as previously described(68). All subjects gave their written informed consent to participate in the study after the nature of the procedure had been fully explained.

### Consent for publication

Not applicable

### Availability of data and materials

All raw data are available upon reasonable request to corresponding authors. Bioinformatic pipelines are available on GitHub: [https://github.com/BioGO-BRIC/ZIP\\_CRISPR\\_code](https://github.com/BioGO-BRIC/ZIP_CRISPR_code) (DOI: 10.5281/zenodo.17602848). Nanopore sequencing data are available on Zenodo: <https://doi.org/10.5281/zenodo.17487199>.

### Competing interests

C.T., J.R., A.B., and F.M.G. declared a patent application EP25305256 filed on February 26, 2025 and claims the use of ZIP CRISPR to increase HDR editing efficiency with CRISPR. The remaining authors declare no competing interests.

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### Authors' contributions

C.T., J.R., F.M.G. and A.B.: design and analysis of data; C.T., F.M.G. and A.B.: drafting and final approval of manuscript; A.B. and F.M.G.: supervision, funding acquisition; C.T., J.R., M.R., V.M., R.E. and S.F.: performing editing experiments and associated analysis; V.M., C.T., C.B.: Nanopore sequencing bioinformatic analysis; C.T., I.L.G.: performing cell cycle analysis; M.R.: AlphaFold modeling; I.L.G.: isolation of HSPCs; T.T., S.B.: obtention of pulmonary epithelial basal cells, nasal epithelial cells and bronchial epithelial cells; C.B., J.B., : helpful discussion in the field of CRISPR-Cas9; S.D., C.B., J.B.: proofreading of manuscript.

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## Figures

Fig. 1

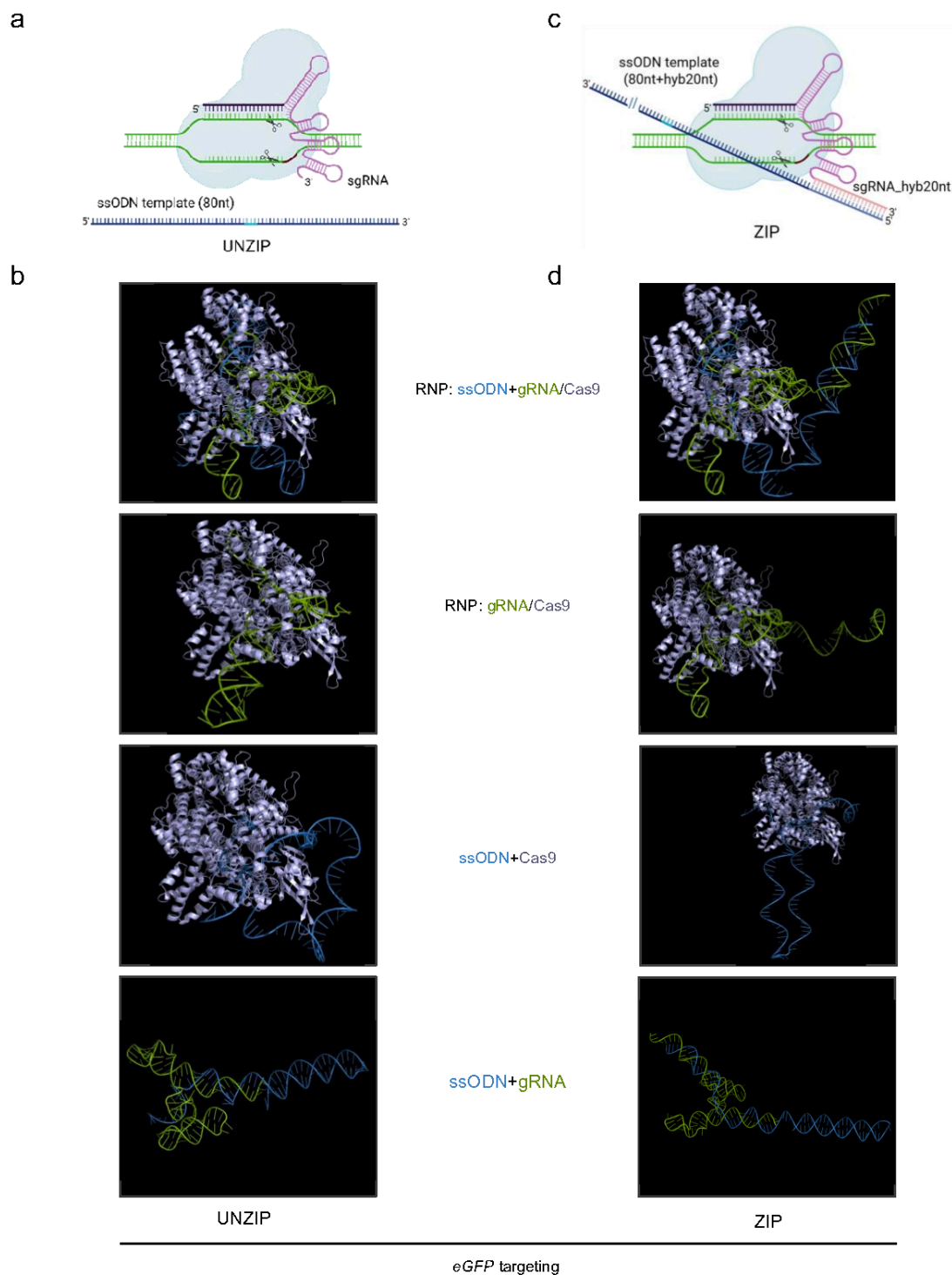


Figure 1

**AlphaFold modelling and ZIP CRISPR strategy to strengthen Cas9/gRNA/ssODN interaction.** **a** Representation of the UNZIP RNP (Cas9 nuclease with unextended gRNA and ssODN template). **b** AlphaFold modelings of either ssODN template and Cas9/gRNA RNP, Cas9/gRNA RNP, ssODN template and Cas9 or ssODN template and gRNA to target *eGFP* with the UNZIP configuration. For all modelings Cas9 is indicated in light purple, ssODN template in blue and gRNA in green. **c** Representation of the ZIP

RNP (Cas9 nuclease with extended ssODN template - in light blue - for hybridization to the extended gRNA - in light pink -). **d** AlphaFold modelings of either ssODN template and Cas9/gRNA RNP, Cas9/gRNA RNP, ssODN template and Cas9 or ssODN template and gRNA to target eGFP with the ZIP configuration. For all modelings Cas9 is indicated in light purple, ssODN template in blue and gRNA in green.

Fig. 2

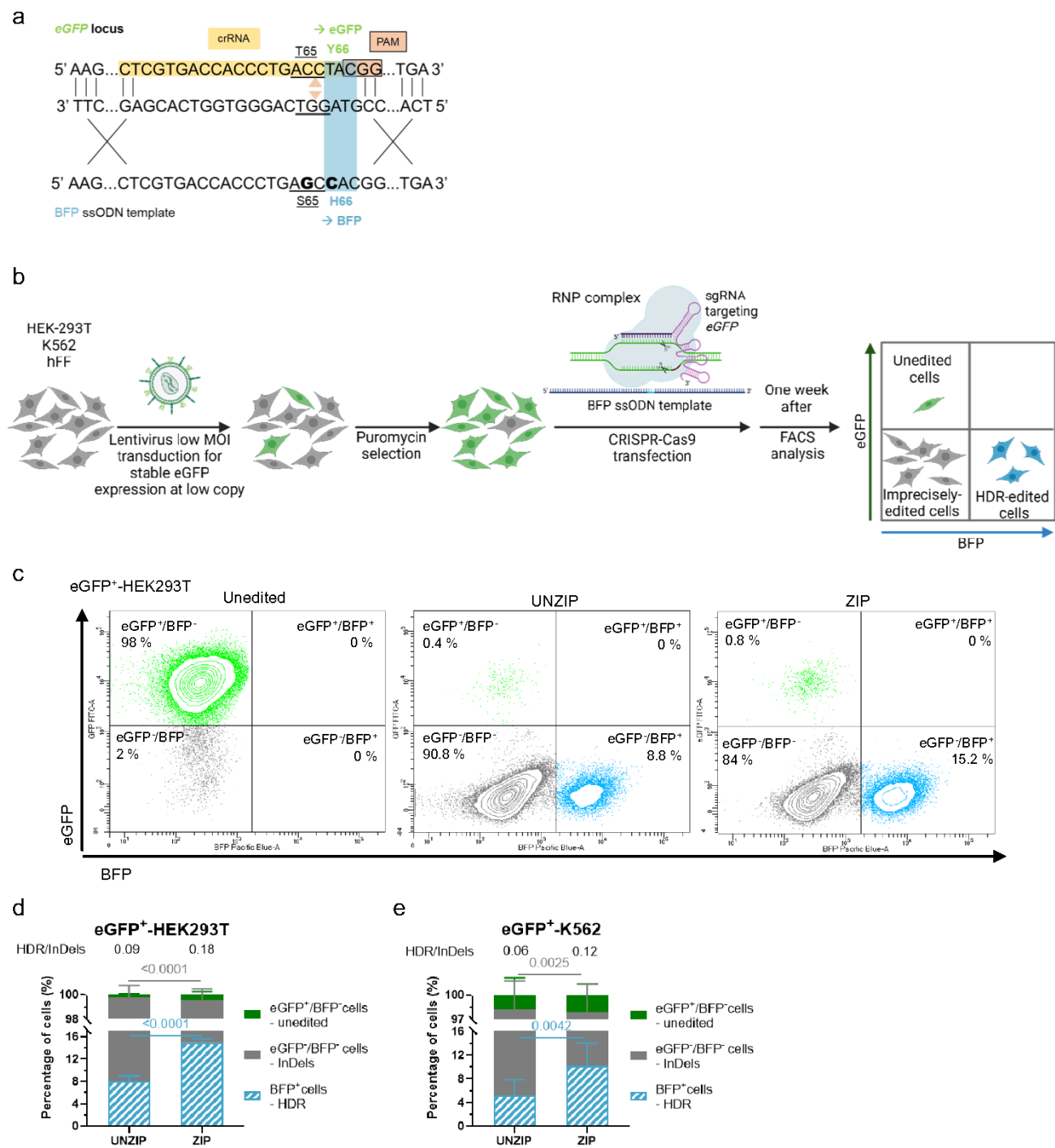


Figure 2

**Set up and validation of ZIP CRISPR to increase HDR editing efficiency with Cas9 nuclease in cell lines.**

**a** Editing strategy to convert eGFP into BFP. T65S and Y66H amino acid substitutions correspond to a shift in the fluorescence excitation and emission spectra of the protein, converting eGFP to BFP. **b** Construction of cell models and result analysis timeline. **c** Illustrative cytometry results of eGFP<sup>+</sup>-HEK293T cells, unedited, or edited by UNZIP or ZIP RNPs. **d and e** Flow cytometry quantification of the edition with Cas9 nuclease with (ZIP RNP) or without (UNZIP RNP) the correction template physically bound to the gRNA in eGFP<sup>+</sup>-HEK293T cells (n=4) and eGFP<sup>+</sup>-K562 cells (n=8), respectively. The proportions of cells expressing BFP (HDR-edited), eGFP (unedited) or neither of these fluorophores (imprecisely-edited, with InDels) one week after transfection are reported. The mean  $\pm$  SD is shown. The HDR/InDels ratio is indicated at the top of the graphs. Statistical significance determined by two-way ANOVA. Schemas created with [BioRender.com](https://BioRender.com).

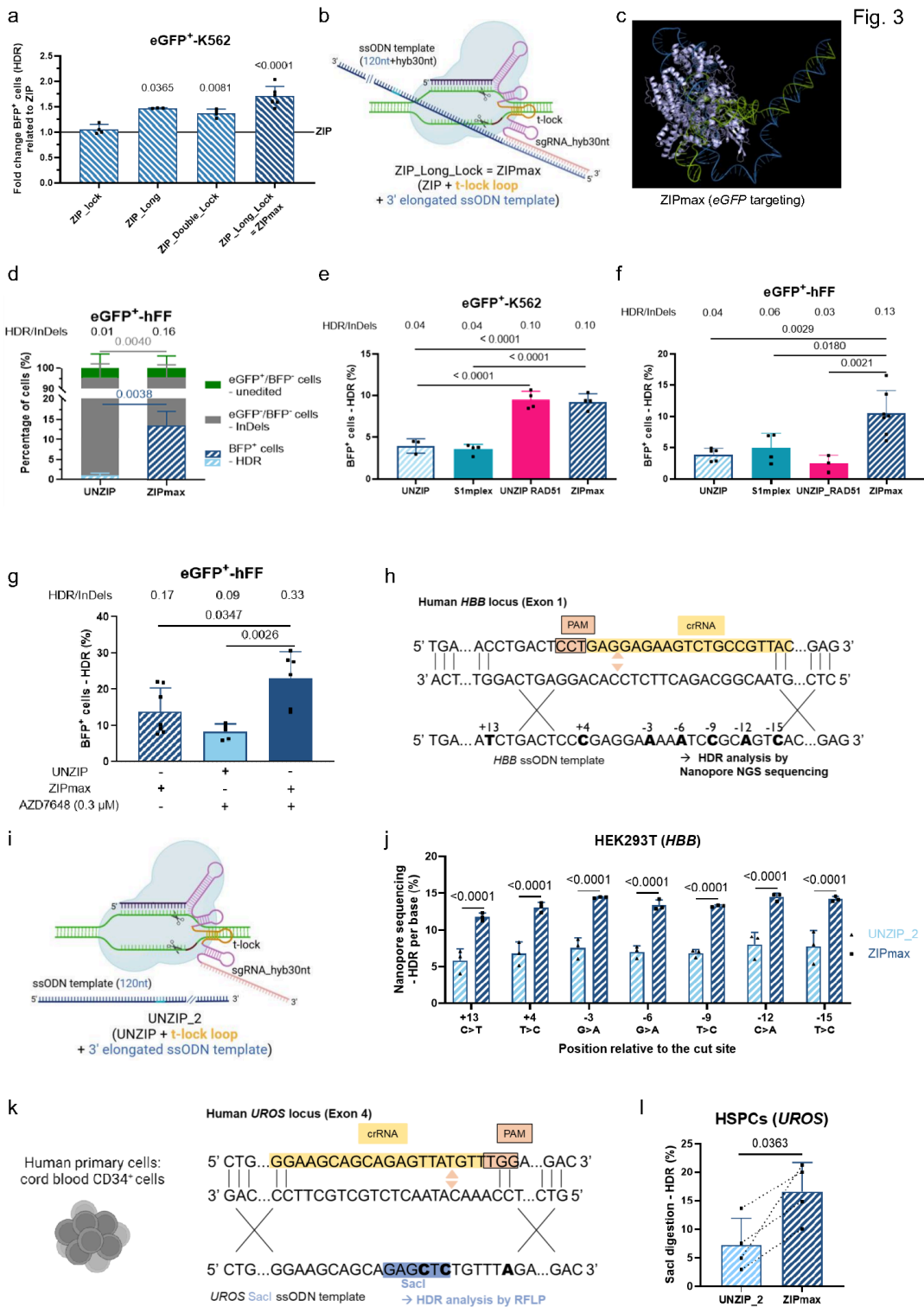


**Figure 3**

**Improvement and benchmarking of ZIP CRISPR to boost HDR editing with Cas9 nuclease in cell lines and primary cells.** **a** Flow cytometry quantification of the HDR edition with Cas9 nuclease with ZIP\_Lock (n=4), ZIP\_Long (n=3), ZIP\_Double\_Lock (n=4) or ZIP\_Long\_Lock (n=6) RNPs in eGFP<sup>+</sup>-K562 cells. The proportions of cells expressing BFP one week after transfection are reported. Fold-changes indicate the fold-increase between the ZIP\_Lock, ZIP\_Long, ZIP\_Double\_Lock or ZIP\_Long\_Lock RNPs relative to the

ZIP RNP. The mean  $\pm$  SD is shown. Statistical significance to the ZIP condition determined by Kruskal-Wallis test. **b** Representation of the ZIP\_Long\_Lock=ZIPmax RNP. **c** AlphaFold modeling of ZIPmax RNP to target *eGFP*. Cas9 is indicated in light purple, ssODN template in blue and gRNA in green. **d** Flow cytometry quantification of the edition with Cas9 nuclease with ZIPmax or UNZIP RNPs in *eGFP*<sup>+</sup>-hFFs (n=5). The proportions of cells expressing BFP, *eGFP* or neither of these fluorophores one week after transfection are reported. The mean  $\pm$  SD is shown. The HDR/InDels ratio is indicated at the top of the graphs. Statistical significance is determined by two-way ANOVA. **e and f** Flow cytometry quantification of the edition with Cas9 nuclease with UNZIP, S1mplex, UNZIP\_RAD51 or ZIPmax RNPs in *eGFP*<sup>+</sup>-K562 cells (n=4) and *eGFP*<sup>+</sup>-hFFs (n=3/4) respectively. The proportions of cells expressing BFP one week after transfection are reported. The mean  $\pm$  SD is shown. The HDR/InDels ratio is indicated at the top of the graphs. Statistical significance is determined by one-way ANOVA. **g** Flow cytometry quantification of the HDR edition with Cas9 nuclease with UNZIP or ZIPmax RNPs and with or without inhibition of NHEJ repair with AZD7648 exposure in *eGFP*<sup>+</sup>-hFFs (n=6). The proportions of cells expressing BFP one week after transfection are reported. The mean  $\pm$  SD is shown. The HDR/InDels ratio is indicated at the top of the graphs. Statistical significance determined by one-way ANOVA. **h** Editing strategy to edit *HBB* in HEK293T cells. **i** Representation of the UNZIP\_2 RNP. **j** Nanopore sequencing to quantify HDR editing per base with UNZIP\_2 or ZIPmax RNPs (n=3). The mean  $\pm$  SD is shown. Statistical significance determined by two-way ANOVA. **k** Editing strategy to edit *UROS* in CD34<sup>+</sup> HSPCs. **l** Capillary electrophoresis of *SacI* digestion products to quantify HDR editing with UNZIP\_2 or ZIPmax RNPs (n=4). The mean  $\pm$  SD is shown. Statistical significance determined by unpaired t-test. Schemas created with [BioRender.com](https://www.biorender.com).

Fig. 4

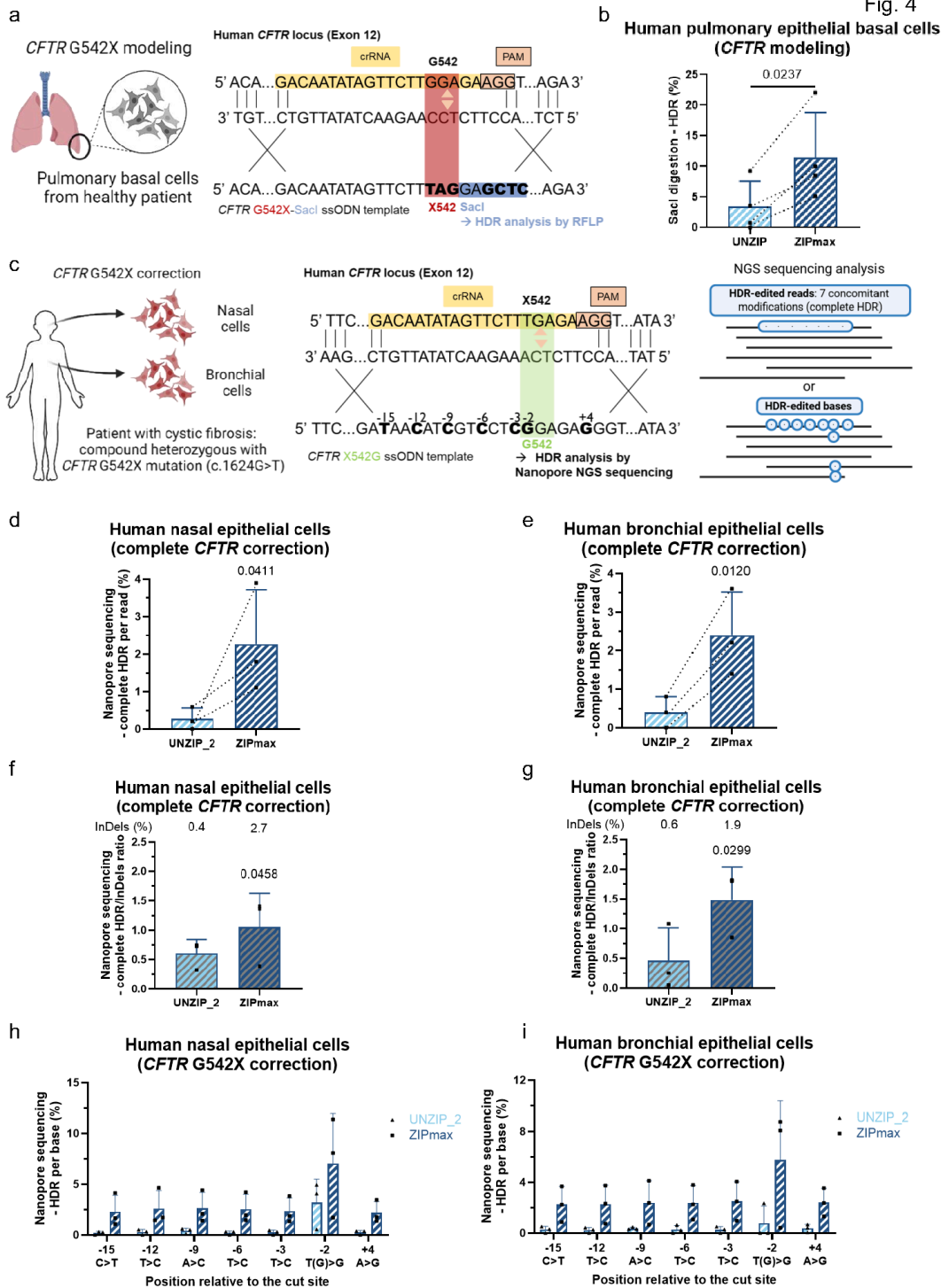


Figure 4

### Validation of ZIP CRISPR technology for *CFTR* editing in pulmonary basal, bronchial and nasal epithelial cells.

**a** Obtention of human healthy pulmonary epithelial basal cells and editing strategy to model *CFTR* G542X mutation and insert *SacI* restriction site. **b** Capillary electrophoresis of *SacI* digestion products to quantify HDR editing with UNZIP or ZIPmax RNPs (n=4). The mean  $\pm$  SD is shown. Statistical significance determined by paired t-test. **c** Obtention of human nasal and bronchial epithelial cells from a

patient with cystic fibrosis carrying the *CFTR*G542X rare mutation and editing strategy i) to correct *CFTR* G542X mutation and ii) to add other silent modifications to prevent re-cut by nuclease Cas9. **d and e** Nanopore sequencing to quantify HDR editing per read (complete HDR, 7 modifications) with UNZIP\_2 or ZIPmax RNPs in nasal or bronchial epithelial cells respectively (n=3). The mean  $\pm$  SD is shown. Statistical significance compared to unedited cells determined by one-way ANOVA. **f and g** Nanopore sequencing analysed with nCRISPResso2 to quantify HDR/InDels ratio with UNZIP\_2 or ZIPmax RNPs in nasal or bronchial epithelial cells respectively (n=3). The mean  $\pm$  SD is shown. Statistical significance compared to unedited cells determined by Kruskal Wallis test. The InDels rate is indicated at the top of the graph. **h and i** Nanopore sequencing to quantify HDR editing per base with UNZIP\_2 or ZIPmax RNPs in nasal or bronchial epithelial cells respectively (n=3). The mean  $\pm$  SD is shown. Schemas created with BioRender.com.

Fig. 5

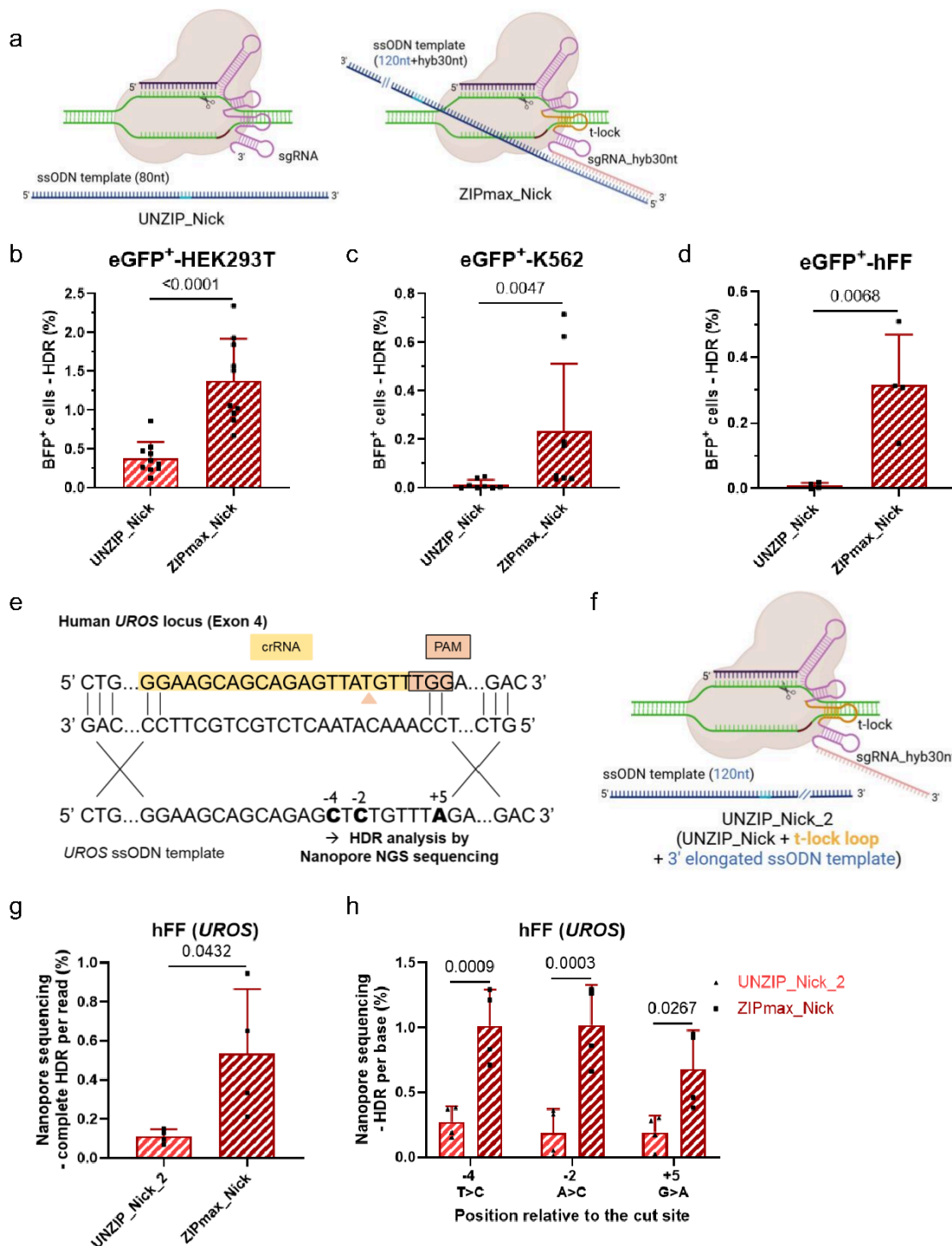


Figure 5

**Increased availability of HDR template with ZIPmax\_Nick is essential to repair nicks by HDR. a**

Representation of UNZIP\_Nick (Cas9 nickase with unextended ssODN template) and ZIPmax\_Nick (Cas9 nickase with 5'-extended ssODN template to hybridize the t-lock and extended gRNA) RNP. **b, c and d** Flow cytometry quantification of the edition with Cas9 nickase with (ZIPmax\_Nick RNP) or without (UNZIP\_Nick RNP) the correction template physically bound to the gRNA in eGFP<sup>+</sup>-HEK293T cells (n=10),

eGFP<sup>+</sup>-K562 cells (n=8), and eGFP<sup>+</sup>-hFFs (n=4) respectively. The proportions of cells expressing BFP (HDR-edited) one week after transfection are reported. The mean  $\pm$  SD is shown. Statistical significance determined by unpaired t-test (eGFP<sup>+</sup>-HEK293T cells, eGFP<sup>+</sup>-hFFs) or Mann-Whitney test (eGFP<sup>+</sup>-K562 cells). **e** Editing strategy to edit *UROS* in hFFs. **f** Representation of the UNZIP\_Nick\_2 RNP (control of the ZIPmax\_Nick RNP with modified gRNA but without gRNA/ssODN template annealing). **g** Nanopore sequencing to quantify HDR editing per read (complete HDR, 3 modifications) with UNZIP\_Nick\_2 or ZIPmax\_Nick RNPs (n=4). The mean  $\pm$  SD is shown. Statistical significance determined by unpaired t-test. **h** Nanopore sequencing to quantify HDR editing per base with UNZIP\_Nick\_2 or ZIPmax\_Nick RNPs (n=4). The mean  $\pm$  SD is shown. Statistical significance determined by two-way ANOVA. Schemas created with BioRender.com.

Fig. 6

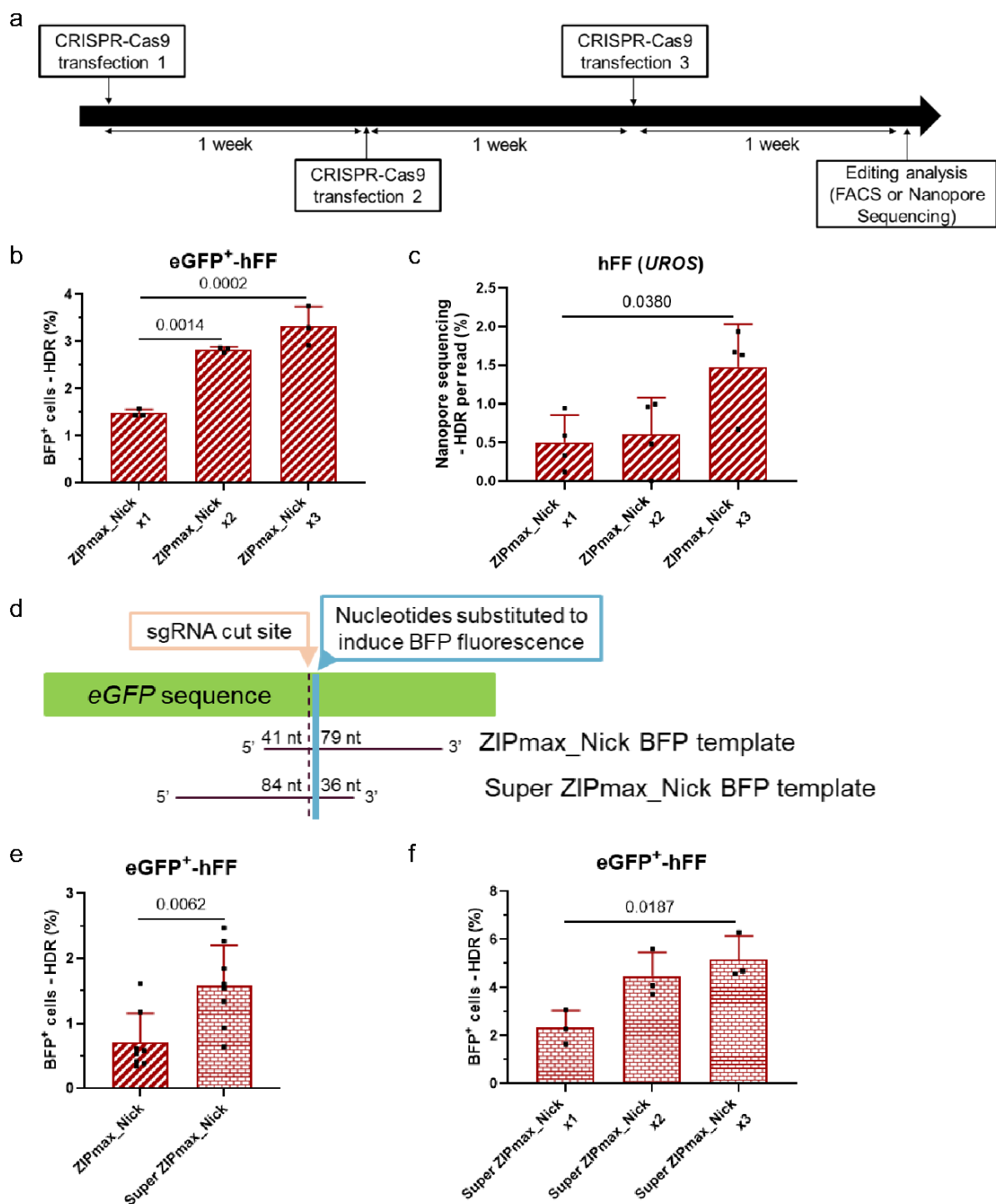


Figure 6

**Iterative editing and ssODN template design improve HDR editing with ZIPmax\_Nick.** **a** Timeline for iterative transfections (RNP nucleofections). **b** Flow cytometry quantification of edition after 1, 2 or 3 transfection(s) with the ZIPmax\_Nick RNP in eGFP<sup>+</sup>-hFFs (n=3). The proportions of cells expressing BFP (HDR-edited) one week after transfection are reported. The mean  $\pm$  SD is shown. Statistical significance determined by one-way ANOVA. **c** Nanopore sequencing quantification of edition after 1, 2 or 3

transfection(s) with the ZIPmax\_Nick RNP in hFFs (n=4). Complete HDR (3 modifications) rates per read are reported. The mean  $\pm$  SD is shown. Statistical significance determined by one-way ANOVA. **d** Representation of BFP-ssODN template position relative to the cut site used for ZIPmax\_Nick or Super ZIPmax\_Nick RNPs. **e** Flow cytometry quantification of edition with ZIPmax\_Nick or Super ZIPmax\_Nick RNPs in eGFP<sup>+</sup>-hFFs RNPs (n=8). The proportions of cells expressing BFP (HDR-edited) one week after transfection are reported. The mean  $\pm$  SD is shown. Statistical significance determined by unpaired t-test. **f** Flow cytometry quantification of edition after 1, 2 or 3 transfection(s) with Super ZIPmax\_Nick RNPs in eGFP<sup>+</sup>-hFFs (n=3). The proportions of cells expressing BFP (HDR-edited) one week after transfection are reported. The mean  $\pm$  SD is shown. Statistical significance determined by one-way ANOVA.

Fig. 7

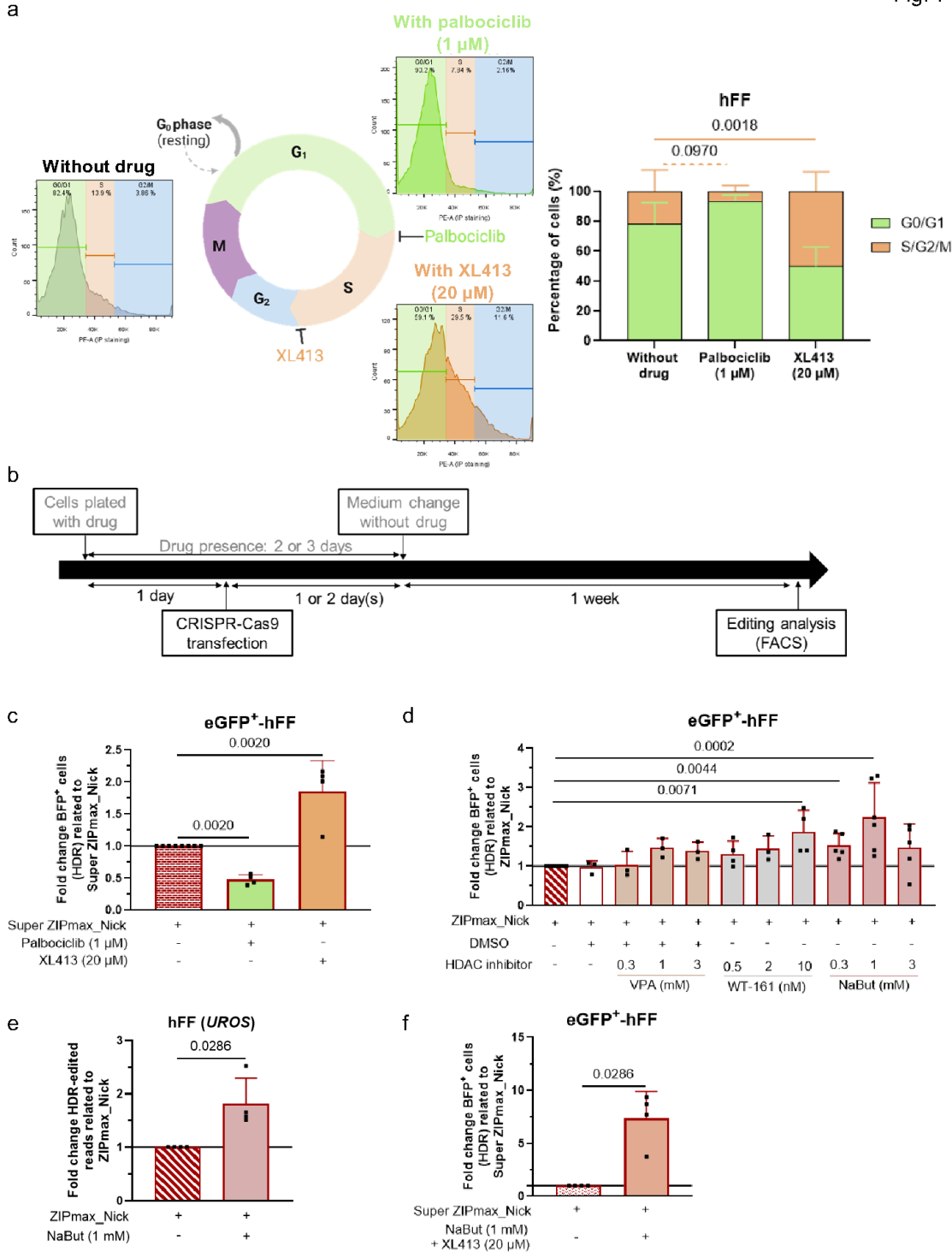


Figure 7

**Modulation of 'cell state' can be associated with ZIPmax\_Nick to increase HDR editing.** **a** Illustrative cell cycle results of eGFP<sup>+</sup>-hFFs with palbociclib, XL413 or without drug exposure (left panel) and flow cytometry quantification of the proportion of eGFP<sup>+</sup>-hFFs in G0/G1 or S/G2/M phases with palbociclib, XL413 or without drug exposure (right panel, n=4). The mean ± SD is shown. Statistical significance to cells without drugs determined by two-way ANOVA. **b** Timeline for transfections (RNP nucleofections) in

presence of drugs. **c** Flow cytometry quantification of HDR edition with Cas9 nickase (Super ZIPmax\_Nick RNP) with or without synchronization of eGFP<sup>+</sup>-hFFs in G0/G1 phase with palbociclib exposure or in S/G2/M phases with XL413 exposure (n=4). The proportions of cells expressing BFP (HDR-edited) one week after transfection are reported. Fold changes indicate fold increases between conditions with or without drugs. The mean  $\pm$  SD is shown. Statistical significance to Super ZIPmax\_Nick RNP without drugs determined by Kolmogorov-Smirnov test. **d** Flow cytometry quantification of HDR edition with nickase Cas9 (ZIPmax\_Nick RNP) with or without VPA (n=3), WT-161 (n=3) or NaBut (n=5) exposure at different doses. The proportion of cells expressing BFP (HDR-edited) one week after transfection is reported. Fold changes indicate fold increases between the conditions with either VPA, WT-161 or NaBut exposure and the condition without drug. The mean  $\pm$  SD is shown. Statistical significance determined by Kruskal-Wallis test. **e** Nanopore sequencing to quantify *UROS* HDR editing per read with Cas9 nickase (ZIPmax\_Nick RNP) with or without NaBut exposure in hFFs (n=4). Fold changes indicate fold increases between the conditions with or without NaBut exposure. The mean  $\pm$  SD is shown. Statistical significance determined by Kolmogorov-Smirnov test. **f** Flow cytometry quantification of HDR edition with Cas9 nickase (Super ZIPmax\_Nick RNP) with or without NaBut and XL413 exposure in eGFP<sup>+</sup>-hFFs (n=4). The proportions of cells expressing BFP (HDR-edited) one week after transfection are reported. Fold changes indicate fold increases between conditions with or without drugs. The mean  $\pm$  SD is shown. Statistical significance to Super ZIPmax\_Nick RNP without drugs determined by Kolmogorov-Smirnov test. Schemas created with [BioRender.com](https://BioRender.com).

## Supplementary Files

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