Marker-free coselection for successive rounds of prime editing in human cells

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ABSTRACT

Prime editing enables the introduction of precise point mutations, small insertions, or short deletions without requiring donor DNA templates. However, editing efficiency remains a key challenge in a broad range of cell types. We designed a robust coselection strategy to perform successive rounds of genetic changes in human cells using CRISPR-Cas nucleases and prime editors. Through coediting, the recovery of cells modified at a target gene of interest is coupled to selection for dominant alleles of the ubiquitous and essential sodium/potassium pump (Na⁺/K⁺ ATPase). The method improves prime editing efficiency 1.5–10-fold in K562 and HeLa cells, reaching up to 83% of desired alleles in cell populations, for previously optimized prime editing guide RNAs (pegRNAs). Using pegRNAs designed *de novo*, we readily engineered homozygous cell lines with cancer-associated or drug resistant *MTOR* mutations displaying altered mTORC1 signaling. Our approach streamlines the production of cell lines with multiple genetic modifications to create cellular models for biological research and lays the foundation for the development of cell-type specific coselection strategies.

INTRODUCTION

Prime editing (PE) is a genre of genome editing that can be used to install nucleotide substitutions, as well as, short insertions and deletions without requiring donor DNA or double-strand breaks (DSBs)^{1–3}. Prime editors are ribonucleoproteins (RNPs) composed of a Cas9 nickase fused to a reverse transcriptase (RT) and a programmable pegRNAs. The pegRNAs are single-guide RNAs (sgRNAs)⁴ extended at their 3' ends with two motifs required for initiation of reverse transcription and specification of the genomic changes, namely the primer binding site (PBS) and the RT template¹. As compared to sgRNAs for CRISPR nucleases and base editors, the design of pegRNAs is more complex since few PBS and RT template combinations are functional in a broad range of cell lines¹. While the prediction of efficiency of pegRNAs in human cells is making progress^{5,6}, there remains a need to test many permutations empirically to find optimal reagents.

To date, six types of prime editors are used: PE2, PE3, PE3b, PE4, PE5 and PE5b^{1,7}. PE2 relies on an engineered Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) fused to a SpCas9 nickase (H840A) to introduce small genetic changes templated by the pegRNA. After

DNA binding and nicking, PE2 reverse transcribes the intended edit from the pegRNA 3' extension. The resolution of the heteroduplex, by a mechanism that remains incompletely defined, results in the introduction of the intended edit^{1-3,7}. The PE3 strategy relies on an additional sgRNA to nick the non-edited strand and direct DNA repair to preferentially incorporate the intended edit which typically results in higher editing efficiency versus PE2^{1,3}. However, PE3 can yield a low but detectable rate of indels formation at the target site, decreasing product purity^{1,3,8,9}. When permitting, a nicking sgRNA that matches the edited strand can be used to minimize the presence of concurrent nicks, and consequently, indel formation¹. Denoted as PE3b¹, this strategy is not applicable when no protospacer adjacent motif (PAM) is available near the targeted edit. Transient coexpression of a dominant negative DNA mismatch repair (MMR) protein (MLH1dn) along with PE components further enhances efficiency and lowers editing byproducts⁷. This strategy yielded the PE4 (PE2+MLH1dn), PE5 (PE3+MLH1dn), and PE5b (PE3b+MLH1dn) editors⁷. Thus, the optimal PE strategy to adopt varies according to the context and there is a need to develop methods to consistently improve its success rate.

To circumvent these limitations several approaches have been implemented including editing with purified ribonucleoprotein complexes (RNPs)¹⁰, mRNA-based delivery^{7,11}, engineered pegRNAs (epegRNAs)¹², NLS- and codon-optimized prime editors^{7,9,13}, enrichment with puromycin, and fluorescent reporter-based selection¹⁴⁻¹⁶. Nevertheless, these improvements are still dependent on extensive pegRNA optimization, absolute activity remains low, and the streamlined production of homozygous cell lines has yet to be achieved^{1,12}. Hence, further refinements to these approaches are needed to boost the efficiency of PE in human cells^{2,3,7,12}.

We previously reported a robust coselection method for CRISPR nucleases that relies on co-targeting a gene of interest (GOI) and the *ATP1A1* locus to confer dominant cellular resistance to ouabain¹⁷, a plant-derived inhibitor of the ubiquitous and essential Na⁺/K⁺ ATPase^{18,19}. Such an approach derives from the observation that simultaneous targeting of two different loci results in double-editing events that are not statistically independent^{17,20-25}. The strategy has proven to be portable to non-homologous end joining (NHEJ), homology directed repair (HDR), and base editing events in near-haploid, diploid and polyploid cells^{17,26-32}. To further extend the versatility of this method, we took advantage of the extensive mutagenesis and crystallographic studies that

have led to the identification of mutated versions of the enzyme that are resistant to various concentration of ouabain^{18,19,33–35}. We engineered these dominant gain-of-function mutations in *ATP1A1* to confer cellular resistance to discrete levels of ouabain, allowing up to three sequential rounds of selection to be performed, by progressively increasing the concentration of the inhibitor at each step. This approach was adapted to enrich PE events in human cells whilst enabling multiple rounds of coselection. Lastly, we demonstrate that *ATP1A1* can be used as a selectable genomic safe harbor^{36,37} for targeted integration and long-term expression of transgenes.

Hence, we propose an easy-to-implement toolkit to select cells engineered via PE without the use of exogenous selection markers that largely bypasses the need for extensive pegRNA optimization. This approach should be compatible with future improvements of the PE technology and should simplify the generation of isogenic panels of human cell lines with multiple genetic modifications.

RESULTS

Editing *ATP1A1* to confer discrete levels of resistance to ouabain

The main subunit of the Na⁺/K⁺-ATPase (a.k.a sodium-potassium pump), encoded by *ATP1A1*, is a 1023 amino acid long protein containing ten transmembrane domains and five extracellular loops which transports Na⁺ and K⁺ against their electrochemical gradients. It is also the extracellular receptor for cardiac glycosides, a class of drugs that comprises ouabain and digoxin^{18,19,38}. A long history of random mutagenesis studies has mapped amino acid residues that alter ouabain sensitivity^{34,39}. We previously demonstrated that introducing in-frame deletions at *ATP1A1* exon 4, encoding border residues of the first extracellular domain of the Na⁺/K⁺ ATPase, conferred resistance to ouabain and allowed the co-enrichment of NHEJ events at a second locus of interest¹⁷. We also demonstrated that introducing the Q118R and N129D point mutations at *ATP1A1* exon 4 using a single-stranded oligodeoxynucleotide (ssODN) donor and a sgRNA targeting the adjacent intron allowed the enrichment of HDR events at a second locus of interest¹⁷ (**Fig. 1a,b** and **Supplementary Fig. 1**). While point mutations in the first extracellular loop (Q118R/N129D) enabled K562 cells to grow in the presence of ~1000 µM ouabain, cells harboring in-frame deletions only resisted to low doses (<5 µM) (**Fig. 1b**). Since several amino acid substitutions across the protein can confer distinct levels of resistance, we hypothesized that they could be engineered in succession to create variants of the pump that are progressively more resistant. By

targeting *ATP1A1* repeatedly, and increasing the dose of ouabain at each step, one could perform sequential rounds of coselection and engineer cell lines with multiple genetic modifications.

We first tested two mutations (L800K and T804N) located in the third extracellular loop and known to confer an intermediate level of resistance as compared to Q118R and N129D^{34,35,40,41}. We designed a sgRNA to cleave within *ATP1A1* intron 17 (hereafter named G7) in the vicinity of T804 and used a single-stranded oligodeoxynucleotide (ssODN) to introduce either L800K or T804N (**Supplementary Fig. 1**). Cleaving within the intron allows for seamless induction of recombination within the juxtaposed exon and mitigates the negative impact caused by on-target NHEJ-based mutagenesis of this essential gene¹⁷. K562 cells were transfected with sgRNA G7 along with ssODNs, and growth was monitored after the addition of a low dose of ouabain (0.5 μ M) which is sufficient to kill all non-edited cells within 48 hours¹⁷. Only cells bearing the T804N mutation survived selection and titration of ouabain revealed that growth remained robust up to 10 μ M ouabain (**Fig. 1b**). In addition, as observed when DSBs are induced within *ATP1A1* exon 4¹⁷, cleavage within exon 17 selects for in-frame deletions changing the extracellular loops of the pump and conferring resistance to low concentrations of ouabain (**Fig. 1b** and **Supplementary Fig. 1**). Thus, dominant gain-of-function mutations can also be generated by editing the third extracellular loop of the Na⁺/K⁺-ATPase and confer an intermediate level of resistance to the drug.

Robust coselection of cells with CRISPR-driven HDR events via *ATP1A1* T804N

We then tested whether cointroduction of *ATP1A1* T804N could be used to perform coselection for HDR events. First, we targeted an EBFP expression cassette to the *AAVS1* safe harbor³⁶ in K562 cells and selected a homozygous clone to monitor its conversion to EGFP through FACS-based analysis (**Fig. 1c** and **Supplementary Fig. 1**)^{42,43}. Co-targeting *ATP1A1* and the EBFP reporter yielded a marked increase in EGFP positive cells upon low dose (0.5 μ M) ouabain selection (**Fig. 1c**). Similarly, targeted insertion of the coding sequence for fluorescent proteins to *LMNA* and *NPM1*, resulted in successful tagging in ~50% of the selected cells (**Fig. 1d,e**). These levels of improvement are equivalent to the ones observed for coselections using the *ATP1A1* Q118R/N129D mutations¹⁷, indicating that both types of mutations are interchangeable in this context.

***ATPIA1* as a selectable genomic safe harbor**

We then tested whether *ATPIA1* could be used as a safe harbor for targeted integration of transgenes via intron nesting⁴⁴. In our design, the outcome of HDR is the co-introduction of an ouabain resistant allele (T804N) and an expression cassette in antisense orientation within intron 17 to maintain functionality of both *ATPIA1* and the transgene (**Fig. 2a** and **Supplementary Fig. 2**). We first targeted an expression cassette for the fluorescent protein mScarlet-I to *ATPIA1* intron 17 and recovered up to 99% of K562 cells and 88% of HeLa cells expressing the reporter after ouabain selection (**Supplementary Fig. 2**). Targeted integration of the transgenes was confirmed in both cell lines by PCR-based genotyping with primers binding outside the region of homology (out-out PCRs) (**Supplementary Fig. 2**).

A similar strategy was taken to create a K562 cell line containing a reporter for mTOR signaling. The mTOR signaling indicator (TOSI)⁴⁵ was adapted to build a TOSI-mScarlet-I-NLS (mSc-TOSI) fluorescent reporter targeted to the *ATPIA1* locus (**Fig. 2a**). Targeting efficacy was compared to the well-established *AAVS1* safe harbor³⁶. In both cases, we obtained almost pure populations of reporter-expressing cells (**Fig. 2a-d** and **Supplementary Fig. 2**). Active mTORC1 signaling results in rapid phosphorylation of the mSc-TOSI phosphodegron by S6K, ubiquitination, and degradation by the proteasome while its inhibition stabilises the reporter (**Fig. 2e**)⁴⁵. Treatment with rapamycin increased the fluorescence signal confirming the functionality of the reporter construct when expressed from both loci (**Fig. 2f**). In selected cell populations, expression of fluorescent transgenes from the *ATPIA1* locus was slightly less intense but was more homogenous than from *AAVS1* as determined by FACS-based analysis (**Fig. 2** and **Supplementary Fig. 2**). These results suggest that *ATPIA1* can be used as a selectable genomic safe harbor for targeted integration of reporter transgenes and that targeting efficacy is high enough to work with pools of selected cells, thereby bypassing the need to isolate single-cell derived clones.

We then tested whether targeted integration of a transgene at *ATPIA1* intron 17 could be coupled to gene inactivation at a second locus of interest. Using K562 cells stably expressing fluorescent reporter genes, we performed proof-of-concept experiments suggesting that concomitant knockout of a GOI and exogenous expression of its mutant version can be achieved (**Supplementary Fig.**

3). This strategy should facilitate the creation of isogenic panels of cells expressing mutant versions of a gene in a knockout background.

Multiple rounds of coselection with CRISPR nucleases by repeated editing at *ATP1A1*

Having established that T804N and Q118R/N129D are functionally equivalent for coselection, but that edited cells resist to different concentrations of ouabain, we tested if these mutations could be engineered in succession to create variants of the pump that are progressively more resistant. As a starting point, we used a homogenous population of cells expressing mScarlet-I from *ATP1A1* intron 17. This pool was selected with 0.5 μ M ouabain via the co-introduction of T804N (see above and **Supplementary Fig. 2**). We then tagged *NPM1* at its N-terminus by targeted insertion of the coding sequence for mNeonGreen (mNG). Following coselection using 100 μ M ouabain via Q118R/N129D, 57% of cells expressed the mNG-NPM1 fusion based on FACS analysis. Targeted integration was further confirmed by out-out PCR (**Supplementary Fig. 4**). All cells maintained expression of mScarlet-I demonstrating sustained function over two rounds of selection (**Supplementary Fig. 4**). Similarly, it was possible to target an expression cassette for mNG within *ATP1A1* intron 4 into a cell line already expressing mScarlet-I from *ATP1A1* intron 17. Upon selection with 100 μ M ouabain, 93% of cells expressed both transgenes (**Supplementary Fig. 4**). Therefore, this pool of selected cells had two nested and functional expression cassettes within introns of *ATP1A1* as confirmed via out-out PCR (**Supplementary Figs. 2 and 4**). Since NHEJ-mediated editing at *ATP1A1* conferred the lowest level of resistance, performing an initial step of coselection for NHEJ-mediated gene knockout allowed up to three sequential steps of marker-free selection to be performed (**Supplementary Fig. 5**). Taken together, these results demonstrate that *ATP1A1* can be targeted multiple times to perform sequential rounds of marker-free selection by increasing the dose of ouabain at each step. Overall, this efficient and versatile system simplifies the engineering of human cells containing multiple genetic modifications.

Robust coselection for prime editing events

Despite continuous improvements in PE technology, there remains a need to develop universal methods to achieve high levels of editing in bulk populations, especially in MMR-proficient cells^{1,7}. As observed with CRISPR-nucleases and base editors^{17,26} (see also **Supplementary Fig. 6**), we hypothesized that cells that are proficient at completing prime editing at the *ATP1A1* locus

are more likely to harbor a second prime editing event at a GOI. We first designed pegRNAs to install the Q118R and T804N mutations and confirmed that PE3 conferred resistance to ouabain in K562 cells (**Supplementary Fig. 7**). We then constructed tandem U6-driven pegRNA and nick-inducing sgRNA expression vectors to co-target *ATP1A1* and GOIs using a three vectors PE3 system (**Fig. 3a**). We selected eight previously optimized pegRNAs¹ targeting four loci to introduce point mutations, insertions, and deletions to test the impact of coselection in K562 and HeLa S3 cells. These MMR-proficient cell lines are known to display lower PE rates, as opposed to HEK293 cells, and have been extensively used as a model system for PE^{1,7}. K562 cells were transfected with PE3 elements and expanded in the presence or absence of 0.5 μ M ouabain starting at D3 post-transfection until all non-resistant cells were eliminated, which typically takes 10 days. The frequencies of alleles harboring precise modifications markedly increased after coselection for every pegRNA tested, as determined by BEAT⁴⁶ and TIDE⁴⁷ analysis (**Fig. 3b,c**). Co-targeting with either PE3-T804N or PE3-Q118R yielded very similar improvements in editing rates without exacerbating indels byproducts (**Fig. 3b,c**). Comparable trends were observed in HeLa S3 cells despite slightly lower absolute levels of efficacy with the most active pegRNAs reaching 59% editing, as compared to 83% in K562 cells (**Supplementary Fig. 9**). In HeLa S3, some pegRNAs with undetectable activity prior to selection reached between 26% and 31% upon coselection (**Supplementary Fig. 9**). Overall, coselection systematically and reproducibly increased prime editing yields.

Successive rounds of coselection for prime editing in human cells

We tested if cells having undergone a first round of coselection at GOI A with PE3-T804N were competent for a second round of coselection at GOI B with PE3-Q118R (**Fig. 4a**). A pool of K562 cells coselected with PE3-T804N at 0.5 μ M ouabain and harboring the *RUNX1* +1 ATG insertion was used as starting material. This population had 80% and 73% PE alleles at *RUNX1* and *ATP1A1* T804N, respectively (see **Fig. 3b**). A successful second round of coselection, testing six different pegRNAs (GOI B), was then performed via *ATP1A1* Q118R using 100 μ M ouabain (**Fig. 4b**). Similar results were obtained when the entire process was reproduced using a pool of cells with the *EMX1* +1 G to C modification as starting material (**Fig. 4c**). The level of enrichment in these subsequent rounds of PE paralleled the improvements observed in the first rounds of editing, demonstrating that the two steps of co-selection perform equally well and are independent

(compare **Figs. 3** and **4**). Furthermore, editing levels from the first round of coselection remained stable after the second round of coselection, creating cell pools that are highly modified at both GOI A and GOI B (**Fig. 4b,c**). For example, one pool contained 64% and 84% edited alleles at *FANCF* and *RUNXI*, respectively (**Fig. 4b**). Another pool had 78% and 64% editing at *RUNXI* and *EMXI*, respectively (**Fig. 4c**). These data demonstrate that two rounds of PE can be performed sequentially to considerably enrich cells modified at two positions within the genome.

Installation of clinically relevant mutations at *MTOR*

The serine/threonine kinase mTOR is a master regulator of eukaryotic cell growth and metabolism that integrates environmental cues. Given its central role in maintaining physiological homeostasis, mTOR mutations lead to a diverse range of diseases, including cancers^{48–51}. To determine if coselection could streamline the production of homozygous cell lines for functional studies, we designed pegRNAs to install well-characterized and clinically relevant *MTOR* hyperactivating mutations^{49,50,52,53} or rapamycin resistance mutations^{54–56}. While the design and screening of several pegRNAs remains a critical aspect of successful PE^{1,7,12}, we designed and tested only one pegRNA per target based on previously established rules^{1,5} for this proof-of-concept experiment. We designed pegRNAs harboring 25 nucleotides (nts) 3' extensions with 12- to 13-nts PBS and PAM or seed mutations within the RT template to install four hyperactivating mutations (L2431P, E2419K, A2020V, I2017T) and two mutations causing resistance to rapamycin (F2108L, S2035T). To monitor the impact of mTOR variants on signaling activity, PE was performed in a K562 cell line containing the mTOR signaling indicator targeted to *ATPIA1* intron 17 (**Figs. 2** and **5a**).

The mTOR reporter cell line was transfected with the indicated pegRNAs in a PE3 format and cells were treated or not with 100 μ M ouabain starting three days post-transfection. At D14, genomic DNA was extracted and the percentage of PE alleles or indels was quantified. In the selected populations, E2419K increased 4 fold (from 21 to 87%), F2108L went up 5 fold (from 12 to 58%), and I2017T rose 10 fold (from 4 to 39%) (**Fig. 5b**). While undetectable before selection, coselection allowed the detection of 12%, 9%, and 6% of alleles harboring L2431P, S2035T, and A2020V, respectively (**Fig. 5b**). Indels remained low with ($\leq$ 8%) after coselection with all *MTOR* pegRNAs (**Fig. 5b**). Notably, hyperactive mTOR signalling could be detected in coselected cell pools indicating that the process provides the means to evaluate variant causality in populations of

PE edited cells, thus bypassing the need to isolate single-cell derived clones (**Supplementary Fig. 11**). Similarly, rapamycin resistance conferred by the F2108L mutation could also be observed in the coselected population (**Supplementary Fig. 11**). The entire gene editing process was reproduced in HeLa S3 cells. First, targeted integration of the mSc-TOSI reporter within *ATP1A1* yielded a cell pool with 99% of cells expressing the transgene (**Supplementary Fig. 12**). However, in these cells the difference in fluorescence observed by FACS-based analysis was too minimal between the on and off states (+/- rapamycin) to monitor the impact of PE-induced mutations (**Supplementary Fig. 12**). Nevertheless, PCR-based genotyping revealed that the percentage of alleles harboring the E2419K, F2108L, and I2017T mutations increased 5 fold for E2419K (from 17 to 78%), 4 fold for F2108L (from 9 to 39%), and 8 fold for I2017T (from 4% to 30%) (**Supplementary Fig. 12**). The ratio between PE and indels was lower in HeLa S3 cells than in K562s (**Fig. 5b** and **Supplementary Fig. 12**). Larger PE-driven insertions were also observed in gel-based assays in coselected pools of HeLa S3 but not in K562 cells (**Supplementary Fig. 12** and below).

To test whether coselection can facilitate the isolation of homozygous clones, we isolated and characterized single cell-derived K562 clones with E2419K, F2108L, and I2017T mutations at *MTOR*. At D3 post transfection, cells were either selected in pools or plated in methylcellulose-containing media with 100 μ M ouabain to derive clones. This way, PE efficiency can be compared between pools (see **Fig. 5b**) and clones. Genomic DNA was extracted from each clone and the region surrounding the targets were amplified by PCR. Amplicons were analysed on agarose gels and by Sanger sequencing coupled to trace decomposition by BEAT⁴⁶ and TIDE⁴⁷ (**Supplementary Figs. 13** and **14**). Taking all three mutations together, only 5 clones did not have at least one pegRNA-specified allele out of 88 clones analysed (**Table 1** and **Supplementary Fig. 14**). Moreover, 35% (31/88) of the clones were homozygous for the desired mutations. Of note, K562s contain three copies of both *MTOR* and *ATP1A1* (**Supplementary Fig. 14**). Biallelic and monoallelic modifications were observed in 24% (21/88) and 34% (30/88) of the clones, respectively (**Table 1** and **Supplementary Fig. 14**). A wide range of relatively short PE-driven indels were also observed in the clones, including tandem duplications and deletions of sequences found between the two nicks^{7,10} (**Supplementary Fig. 13**). This spectrum of byproducts is dissimilar to the ~1-5bp indels typically observed with Cas9 nucleases⁵⁷. Large (> 50 bp) PE-

mediated insertions and a few large deletions were observed via gel-based assays (**Supplementary Fig. 14**). In fact, most clones (54/57) that were not homozygous for the specified *MTOR* mutations contained undesired indel byproducts (**Table 1** and **Supplementary Fig. 14**). These observations contrast with the relatively low levels of indels detected in cell populations with these pegRNAs (**Fig. 5b**). This suggests that PCR bias in bulk population of cells may lead to an underrepresentation of larger insertions products driven by PE3. Overall, the many genotypes observed at the clone level indicate that polyclonal populations are created during coselection.

The functional impact of PE-mediated *MTOR* mutations was tested in single cell-derived clones. As expected, degradation of the mSc-TOSI reporter was intensified in homozygous clones harboring E2419K or I2017T hyperactivating mutations (**Fig. 5c**). In contrast, monoallelic E2419K and I2017T clones containing inactivating indels at the remaining alleles displayed crippled mTOR signaling despite growing indistinguishably from all other clones (**Supplementary Fig. 15**). This observation suggests that a single edited allele is not sufficient to hyperactivate mTORC1 signaling in K562 cells, highlighting the importance of carefully genotyping PE-generated clones. Complete rapamycin resistance was also observed in homozygous F2108L clones (**Fig. 5d** and **Supplementary Fig. 15**). First and second generation mTOR inhibitors^{56,58} were used to demonstrate the specificity of the F2108L mutations and to show that the reporter still responded to drug treatment after clonal derivation (**Fig. 5d** and **Supplementary Fig. 15**). In addition, all clones used for functional FACS-based assays were homozygous for mSc-TOSI knock-in at *ATPIA1* intron 17 as determined by out-out PCR further indicating that the observed variation in reporter signal were caused by the presence of the desired *MTOR* mutations (**Supplementary Fig. 15**). Note that the wild-type (WT) *MTOR* controls used in these FACS-based assays contained the *ATPIA1* Q118R mutation and were selected with ouabain just like the test samples to mitigate any potential impact of the coselection process itself on the readout of the experiments⁵⁹. Taken together, these data indicate that homozygous clones can be readily isolated via marker-free coselection to characterize pathogenic mutations even when low levels of PE are observed in the starting population.

DISCUSSION

The efficiency of PE fluctuates markedly according to the particular edit, target loci, and cell type^{1-3,7}. Despite recent key improvements, it still requires substantial level of optimization to use the technology⁷ successfully. In this work, we describe a marker-free coselection strategy that substantially and stably improved the outcome of PE for every pegRNAs tested. The extent of enrichment varies with different pegRNAs, but the strategy greatly facilitates the enrichment of both cell pools and homozygous clones to study the impact of variants on gene function. Due to the high efficiency of deriving novel cell lines, multiple independent cell lines can be analysed to ensure that any unexpected result is not from a clonal artifact⁵⁹. The use of well-characterized dominant gain-of-function mutations^{34,35,60,61} in *ATP1A1* introduces minimal modifications to the human genome without detectable effect on cell fitness. For functional studies, any potential impact of the modified sodium-potassium pump on the pathway under study can be easily controlled for using a selected control cell line containing only the *ATP1A1* mutations. Typically, the most efficient strategy for the selection of edited cells is targeting endogenous genes as opposed to the use of exogenous markers^{17,20,21,62}. Nonetheless, alternative methods have been described to enrich for edited cells, such as puromycin-based selection strategy and the use of surrogate reporters^{14,16}. Selecting cells that express all PE components with a selection marker is not sufficient for robust enrichment, while FACS-based cell sorting using exogenous gene markers could be prohibitive for some cell types due to cellular stress^{14,16}.

An improvement made to our previously described strategy¹⁷ is the option to perform successive rounds of coselection to install multiple genetic modifications sequentially to derive cell lines. Multiple edits can be engineered at the pool level followed by isolation of clones of interest or new modifications can be added to a previously characterized clone enabling the rapid creation of isogenic cell lines. As the method is portable to CRISPR nucleases, base editors, and prime editors, different types of genomic changes can be implemented. Potential applications include (i) gene complementation with variants coupled to endogenous knock out, (ii) epitope tagging followed by creation of point mutations, and (iii) rescue experiments to confirm causality between edit and phenotype. To create heavily modified human cell lines for applications such as synthetic biology, one could couple our approach with orthogonal coselection approaches based on auxotrophy or toxins^{20,62}. As coselection is now a proven and established strategy, it appears that other pathways

and systems such as MAPK signaling⁶³, proteasome⁶⁴, translation⁶⁵, and other tissue-specificities⁶⁶ could form the basis of future applications of the methodology.

Although a more thorough characterisation will be required to fully establish its full potential, it is worth noting that *ATP1A1* can be used as a selectable genomic safe harbor for targeted integration of transgenes via intron nesting⁴⁴. Stable transgene expression was achieved with a near 1:1 correspondence between targeted integration and drug resistance, similar to observations made at the *HBEGF* locus²⁰.

While the complete mechanism of action of PE remains to be fully elucidated, DNA mismatch repair (MMR) was identified as a genetic determinant of PE outcome. Specifically, MMR inhibits PE and promotes the formation of undesired indel byproducts⁷. In the context of coselection, clonal analysis revealed a high degree of correlation between editing at *ATP1A1* and modification at three different positions within *MTOR*. As such, only one out of 88 selected clones did not display any gene modification (PE or indels) at *MTOR* and only 5 clones did not have at least one pegRNA-specified allele. This clearly indicates that cells proficient at completing one PE3-driven genomic manipulation have an increased probability of completing a second event at a distinct locus. Moreover, approximately a third (31/88) of selected clones were found to be homozygous for the specified mutations at the *MTOR* locus in the triploid K562 cell line. Interestingly, clones triallelic for the I2017T mutation were observed at a frequency of 30% (8/27) even though PE levels in the pool of selected cells only reached 39%. Thus, when active in a cell, PE is prone to edit more than one allele of its target gene. Taken together, our data suggest that only a fraction of cells in an asynchronous population are competent for PE. Although this observation warrants further investigation, a detailed mechanistic understanding of the PE reaction may help to explain these results. A confounding factor in this analysis may stem from *MTOR*'s essentiality, but we did not observe any growth delay in either pools of cells or when clones developed into colonies in methylcellulose for the various genotypes.

We selected the PE3 strategy to initiate coselection because it is more active than PE2 in human cells^{1,7}. This reduced the time required to obtain a fully resistant population of cells. However, PE3 comes at the expense of product purity and is linked to the formation of indels^{1,7,10}. Genotyping of

single cell-derived clones allowed us to better describe the different types of PE3 byproducts. Our PE3 designs for *MTOR* are all configured with PAMs facing outwards with respect to each other (PAM-out). When combined with the H840A SpCas9 nickase (inactivated HNH domain) it creates DSBs with 3' ssDNA protruding ends ranging from 38 to 50 bases (**Supplementary Fig. 13**). As such, the types of undesirable DNA lesions observed are reminiscent to the repair outcomes occurring when a paired nickase strategy⁶⁷ is used to create DSBs with 3' overhangs^{57,68}. First, the major repair byproducts are direct repeats of the sequences found within the overhangs^{57,68} (**Supplementary Fig. 13**). Mechanistically, fill-in synthesis of the 3' protruding ends by Polα-primase can account for these tandem duplications in a pathway that requires 53BP1^{69–71}. Notably, a CRISPRi screen revealed that *TP53BP1* knockdown drastically reduces the frequency of tandem duplications during PE3 (PAM-out)⁷ further suggesting that editing byproducts are linked to the DSB repair response resulting from presence of concomitant nicks. Secondly, frequent large insertions were clearly observed in clones via-gel based assays and missed by sequencing bulk populations of prime edited cells (**Supplementary Fig. 14**). To our knowledge, these events have not been reported before and may be composed of multiple repeated sequences formed by consecutive rounds of repair and nicking since the target sites are still present in the tandem duplications⁶⁸ (**Supplementary Fig. 13**). Finally, deletions occurring in between the two nicks are larger than normally observed when using WT SpCas9⁵⁷ (**Supplementary Fig. 13**).

Taken together, these data suggest that a competition between PE3 and NHEJ determines the overall efficiency of prime editing. Delaying rapid and predominant NHEJ-mediated repair may improve PE3 yields and purity. DNA end protection mediated by 53BP1^{70,72} could modulate PE3 outcome as 53BP1-deficient cells have delayed repaired kinetics that can lead to a switch in the choice of repair pathway⁷³. Further supporting this view, *TP53BP1* knockdown not only markedly decreases the formation of tandem duplication and deletions but increases the levels of intended edits when PE3 is used in a PAM-out mode⁷. *TP53BP1* knockdown also reduces deletions in the PE3 PAM-in form⁷. This warrant testing of i53 (inhibitor of 53BP1), a genetically encoded inhibitor of 53BP1⁷⁴ to enhance PE3, further delineate its mechanism of action, and compare its outcome to PE5. The impact of modulating NHEJ or MMR on genome integrity remains to be fully explored.

Taken as a whole, the various strategies presented here should further streamline the incorporation of marker-free genetic changes in human cells while being compatible with future improvements in PE technology.

METHODS

Cell culture and transfection

K562 cells were obtained from the ATCC (CCL-243) and maintained at 37 °C under 5% CO₂ in RPMI medium supplemented with 10% FBS, penicillin–streptomycin, and GlutaMAX. HeLa S3 and U2OS cells were obtained from the ATCC (CCL-2.2 and HTB-96) and maintained at 37 °C under 5% CO₂ in DMEM medium supplemented with 10% FBS, penicillin–streptomycin, and GlutaMAX. Cells were routinely tested for the absence of mycoplasma contamination. Ouabain octahydrate (Sigma) was dissolved at 50 mg/ml in hot water, working dilutions were prepared in water and stored at -20°C. Rapamycin (Cayman Chemicals) was dissolved at 10 mg/ml in DMSO, working dilutions were prepared in water and stored at -20°C. AZD8055 (STEMCELL Technologies) was dissolved at 10 mM in DMSO, working dilutions were prepared in water and stored at -20°C. DMSO alone was diluted in water and used as vehicle control. K562 cells (2x10⁵ cells/transfection) were transfected with an Amaxa 4D-nucleofector™ (Lonza) using the SF nucleofection kit (program FF-120) per manufacturer's recommendations. HeLa S3 cells (2x10⁵ cells/transfection) were transfected using the SE nucleofection kit (Standard HeLa program CN-114 and High efficiency HeLa S3 program DS-150). U2OS cells (4x10⁵ cells/transfection) were transfected using the SE nucleofection kit (program DN-100). Amounts of DNA used for each experiment can be found in the **Supplementary material** section.

Cells were treated with the indicated concentrations of ouabain starting 3 days post-nucleofection until all non-resistant cells were eliminated. Typical coselection for prime editing requires 10-14 days and 14-21 days for K562 and HeLa S3 cells, respectively. Timing can vary according to initial modification rates at *ATP1A1* and we recommend using the High efficiency HeLa S3 program (DS-150) for HeLa S3 cells. Simultaneous coselection and cloning was performed in methylcellulose-based semi-solid RPMI medium supplemented with 100 μM ouabain for 10 days. To establish the K562 cell line constitutively expressing EBFP and EGFP from the *AAVS1* safe-harbor, cloning was performed in methylcellulose-based semi-solid RPMI medium supplemented

with 0.5 µg/ml puromycin for 10 days as previously described⁷⁵. Clones were picked and expanded in 96 wells for 3 days and transferred to 24-well plates for another 3 days before genomic DNA extractions. Clones of interest were then cultured in 6-well plates prior to FACS analysis.

Genome editing vectors and reagents

Adenine base editing (ABE) and prime editing (PE) experiments were performed with pCMV_ABE_{max}⁷⁶ (Addgene 112095), ABE8e⁷⁷ (Addgene 138489), ABE8e (TadA-8e V106W)⁷⁷ (Addgene 138495), pCMV-PE2¹ (Addgene 132775), and pU6-pegRNA-GG-acceptor¹ (Addgene 132777). These vectors were gifts from David R. Liu. U6-pegRNA cassette expressing the *ATP1A1*-Q118R and T804N cassettes were cloned upstream of the U6-RFP-acceptor cassette to create *ATP1A1_G4_Q118R_Dual_pegRNA* (Addgene 173199) and *ATP1A1_G6_T804N_Dual_pegRNA* (Addgene 173200). For coselection, pegRNAs targeting GOI were cloned in the dual pegRNA vectors using the same protocol¹. For PE3 nick gRNAs, gRNAs were cloned into an in-house pUC19-U6-BbsI-sgRNA vector. The U6-*ATP1A1* nick gRNA cassettes were cloned upstream of the U6-BbsI-sgRNA cassette to create *ATP1A1_G3_Dual_sgRNA* (Addgene 173202) and *ATP1A1_G8_Dual_sgRNA* (Addgene 178104). For PE3 coselection, nick gRNAs targeting the genes of interest were cloned into the BbsI sites of the dual nick gRNA vectors. The U6-*ATP1A1* gRNA (G2) cassette was also cloned upstream of the U6-BbsI-sgRNA cassette to create *ATP1A1_G2_Dual_sgRNA* (Addgene 173201) for ABE coselections. When required, DNA sequences for the guides were modified at position 1 to encode a G, owing to the transcription requirement of the human U6 promoter. All sgRNA, pegRNA, and ssODN sequences used during this study are provided in the **Supplementary material** section.

For nuclease-based coselections, gRNA sequences were cloned into eSpCas9(1.1)_No_FLAG (Addgene 79877)⁷⁵, eSpCas9(1.1)_No_FLAG_*ATP1A1_G3_Dual_sgRNA*¹⁷ (Addgene 86613) or pX330-U6-Chimeric_BB-CBh-hSpCas9⁷⁸ (Addgene 42230), a gift from Feng Zhang. Plasmid donor sequences used for *ATP1A1* gene knock-in were cloned by restriction cloning in a pUC19 backbone. All plasmid donor sequences contained short homology arms (< 1 kb) and they were modified to prevent Cas9 cleavage. To repurpose the mTORC1 signaling reporter mVenus-TOSI previously developed for mouse⁴⁵, the N-terminal residues (1-82) of the human *PDCD4* gene were codon optimized using the GenSmartTM codon optimization tool, synthesized as a gBlocksTM, and

cloned into ATP1A1_T804N_hPGK1_mScarlet-I_Donor (Addgene 173207) upstream of the mScarlet-I-NLS cassette using AflIII and NcoI. For puromycin selection, the TOSI_mScarlet-I-NLS cassette was transferred to *AAVSI_Puro_PGK1_3×FLAG_Twin_Strep*⁷⁵ (Addgene plasmid 68375). Desalted ssODNs (see **Supplementary material**) were synthesized as ultramers (IDT) at a 4 nmol scale. A series of vectors described in this work (see **Supplementary material**) have been deposited to Addgene.

Flow cytometry

The percentage of fluorescent cells was quantified using a BD LSRII flow cytometer and 1×10^5 cells were analyzed for each condition. When HDR donor plasmids harboring a human *PGK1* promoter were used to target fluorescent gene cassettes to the *ATP1A1* locus, cells were cultured for 21 days to eliminate residual expression from non-integrated reporter cassettes. For mTORC1 signaling assays, cells were treated with the indicated concentration of rapamycin or AZD8055 for 24 hours before analysis. Flow cytometric data visualization and analysis was performed using FlowJo software (Tree Star).

Genotyping

Genomic DNA was extracted with QuickExtract DNA extraction solution (EpiCentre) following manufacturer's recommendations. Primers used in this study and the PCR product sizes are provided in the **Supplementary material** section. All PCR amplifications were performed with 30 cycles of amplification. Sanger sequencing was performed on PCR amplicons to quantify the percentage of edited alleles using BEAT, TIDE, and TIDER^{16,17,21}. Phusion and Q5 high-fidelity DNA polymerases were used for PCR amplification. Kapa-HiFi polymerase was used for out-out PCRs. To detect targeted integration, out-out PCRs were performed with primers that bind outside of the homology regions of the donor plasmids. Wild type K562 genomic DNA was used as a negative control for all out-out PCRs.

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AUTHOR'S CONTRIBUTIONS

Conceptualization, S.L. and Y.D.; methodology, S.L., D.M., J-P.F., C.G., A.D., A.L., E.B., D.A., and Y.D.; investigation, S.L., D.M., J-P.F., C.G., A.D., A.L., E.B., D.A., and Y.D.; writing original draft, S.L.; writing, review and editing, S.L., and Y.D.; supervision, Y.D.; funding acquisition, Y.D. All authors reviewed the manuscript and approved its final version.

COMPETING INTERESTS

The authors declare they have no financial competing interests

Table 1: Distribution of alleles in single cell-derived clones edited at *MTOR* by coselection

<i>MTOR</i>	% of clones with indicated alleles				
mutation	WT	WT + Indels	Monoallelic	Biallelic	Triallelic
			PE*	PE*	PE ^{&}
E2419K	0% (0/30)	0% (0/30)	40% (12/30)	33% (10/30)	27% (8/30)
F2108L	0% (0/31)	6% (2/31)	26% (8/31)	19% (6/31)	48% (15/31)
I2017T	4% (1/27)	11% (3/27)	37% (10/27)	19% (5/27)	30% (8/27)
Total	1% (1/88)	6% (5/88)	34% (30/88)	24% (21/88)	35% (31/88)

*Indels are detected on remaining alleles

[&]Homozygous

a

c

d

e

Figure 1. Coselection for HDR-driven events by coediting *ATP1A1* exon 17. (a) Schematic for the co-enrichment of CRISPR-driven editing events at a GOI. (b) Schematic representation of the *ATP1A1* locus regions targeted by SpCas9. The first and third extracellular loops of the Na⁺/K⁺ ATPase are encoded by *ATP1A1* exon 4 and 17, respectively. The relative levels of resistance to ouabain conferred by different *ATP1A1* mutations in K562 cells are shown. (c) FACS-based quantification of EBFP to EGFP conversion. K562 cells stably expressing EBFP were transfected with nuclease vectors and ssODN donors targeting *ATP1A1* and EBFP. Where indicated, cells were treated with 0.5 μM ouabain starting 3 days post-transfection and expanded until all non-resistant cells were eliminated. (d) FACS-based quantification of HDR-driven tagging of *NPM1* with mNeonGreen. K562 cells were transfected with nuclease vectors and donors targeting *ATP1A1* and *NPM1*. Where indicated, cells were treated with 0.5 μM ouabain starting 3 days post-transfection and expanded until all non-resistant cells were eliminated. (e) Same as in (d) for HDR-driven tagging of *LMNA* with mScarlet-I. *PGK1*, human phosphoglycerate kinase 1 promoter. PA, polyadenylation signal. HA, homology arm. E, exon.

a

c

e

b

d

f

Figure 2. *ATP1A1* as a selectable genomic safe harbor for targeted integration of transgenes.

(a) Schematic representation of the targeted integration of mScarlet-I mTOR Signaling Indicator (mSc-TOSI) in antisense orientation within intron 17 of *ATP1A1*. The T804N mutation is introduced via the left homology arm (HA) and transgene expression is driven by the *PGK1* promoter. (b) FACS-based quantification of mSc-TOSI knock-in at *ATP1A1* intron 17. K562 cells were transfected with SpCas9 and the donor and treated with or without 0.5 μ M ouabain starting 3 days post-transfection until all non-resistant cells were eliminated. (c) Schematic representation of mSc-TOSI targeting to the *AAVSI* locus. (d) FACS-based quantification of mSc-TOSI knock-in at *AAVSI*. K562 cells were transfected with SpCas9 and a donor targeting *AAVSI* and treated with or without 0.5 μ g/ml puromycin starting 3 days post-transfection until all non-resistant cells were eliminated. (e) Schematic of mSc-TOSI degradation under mTORC1 signaling. (f) mSc-TOSI fluorescence intensity after stable integration at the *ATP1A1* and *AAVSI* loci in presence and absence of rapamycin. Where indicated, cells were treated with 500 nM rapamycin or vehicle control for 24 hours before FACS analysis. *PGK1*, human phosphoglycerate kinase 1 promoter. PA, polyadenylation signal. HA, homology arm. E, exon.

Figure 3. Robust coselection for prime editing. (a) Schematic of the dual and tandem U6-driven pegRNAs and sgRNAs expression vectors along with the PE2 expression vector used to target *ATP1A1* and a GOI. (b) PE and indels quantification as determined by BEAT and TIDE analysis from Sanger sequencing. K562 cells were transfected with PE3 vectors targeting *ATP1A1* exon 17 (T804N) and the indicated GOI. Genomic DNA was harvested 3 days post-transfection (before selection) and cells were treated (ouabain) or not (untreated) with 0.5 μ M ouabain until all non-resistant cells were eliminated. (c) Same as in (b) but co-targeting was performed via *ATP1A1* exon 4 (Q118R). These experiments were performed twice and at different times with equivalent results (see also **Supplementary Fig. 8**).

Figure 4. Robust coselection for successive rounds of prime editing. (a) Schematic of the strategy for performing successive rounds of coselection. Cells harboring modifications at *ATP1A1* (T804N) and GOI A are first coselected with 0.5 μ M ouabain. Following the first round, a subsequent round of coselection occurs at GOI B via modification at *ATP1A1* (Q118R) with 100 μ M ouabain. (b) PE and indels quantification as determined by BEAT and TIDE analysis from Sanger sequencing. K562 cells harboring the *ATP1A1*-T804N and *RUNXI* +1 ATG insertion (GOI A) modifications were transfected with PE3 vectors targeting *ATP1A1* exon 4 (Q118R) and the indicated GOI B. Genomic DNA was harvested 3 days post-transfection (before selection) and cells were treated (ouabain) or not (untreated) with 100 μ M ouabain until all non-resistant cells were eliminated. (c) Same as in (b) with K562 cells harboring the *ATP1A1*-T804N and *EMXI* +1 G to C (GOI A) modifications. These experiments were performed twice and at different times with equivalent results (see also **Supplementary Fig. 10**).

Figure 5. Coselection for the installation of clinically relevant mutations at the *MTOR* locus.

(a) Schematic representation of reporter cell line engineering. K562 cells stably expressing the mSc-TOSI reporter from *ATPIAI* intron 17 are first selected with 0.5 μ M ouabain. Then, coselection for PE to insert the indicated mutations at *MTOR* is performed with 100 μ M ouabain. (b) PE and indels quantification as determined by BEAT and TIDE analysis from Sanger sequencing. K562 cells stably expressing the mSc-TOSI reporter were transfected with PE3 vectors targeting *ATPIAI* exon 4 (Q118R) and *MTOR*. Genomic DNA was harvested 3 days post-transfection (before selection) and cells were treated (ouabain) or not (untreated) with 100 μ M ouabain until all non-resistant cells were eliminated. This experiment was performed twice and at different times with equivalent results (see also **Supplementary Fig. 11**). (c) Histogram plot of mSc-TOSI intensity in homozygous single cell-derived K562 clones harboring *MTOR* hyperactivating mutations. (d) Same as in (c) with a homozygous clone harboring the *MTOR*-F2108L rapamycin resistance mutation. Where indicated, cells were treated for 24 hours with 50 nM rapamycin or 50 nM AZD8055 before FACS analysis.