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Obligate Ligation-Gated Recombination (ObLiGaRe): custom designed nucleases mediated targeted integration through non-homologous end joining

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Gene Editing by ObLiGaRe

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Abstract

Custom designed nucleases (CDNs) greatly facilitate genetic engineering by generating a targeted DNA double-strand break (DSB) in the genome. Once a DSB is created, specific modifications can be introduced around the breakage site during its repair by two major DNA damage repair (DDR) mechanisms: the dominant but error-prone non-homologous end joining (NHEJ) pathway and the less-frequent but precise homologous recombination (HR) pathway. Here we describe ObLiGaRe, a new method for site-specific gene insertions which uses the efficient NHEJ pathway and acts independently of HR. This method is applicable with both zinc finger nucleases (ZFNs) and Tale nucleases (TALENs) and has enabled us to insert a 15 kb inducible gene expression cassette at a defined locus in human cell lines. In addition, our experiments have revealed a previously underestimated error-free nature of NHEJ and provided new tools to further characterize this pathway under physiological and pathological conditions.

Introduction

The development of CDNs including ZFNs and TALENs has made it possible to perform precise genetic engineering in many cell types and species (Bibikova et al. 2003; Christian et al. 2010; Hockemeyer et al. 2009; Kim et al. 1996; Meyer et al. 2010; Moehle et al. 2007; Porteus and Baltimore 2003; Urnov et al. 2010). CDNs are hybrid endonucleases consisting of a FokI nuclease domain and a DNA binding domain assembled from optimized DNA binding modules that are specific for either single nucleotide (for TALENs) or trinucleotide motifs (for ZFNs). Once introduced into cells, CDNs generate a DSB in the genome at or near the desired modification site and induce DDR to mend the break (Rouet et al. 1994). Repair is largely accomplished by NHEJ in which the two ends are processed and ligated together in a way that is frequently accompanied by nucleotide insertions and deletions. Though highly efficient, NHEJ produces knockout alleles that are often heterogeneous and individual cell clones must be isolated for characterization. Currently, specific gene modification relies on HR in which exogenous DNA fragments flanked by homologous sequences around the DSB site are copied faithfully from a template with defined boundaries (Rouet et al. 1994).

We have successfully applied ZFNs to generate knockout and knock-in alleles directly in mouse zygotes (Cui et al. 2011; Meyer et al. 2010). While optimizing gene targeting conditions, we observed that a donor plasmid can be "ligated" into the genome if it contained the same ZFN recognition site as the targeted genomic locus. It has been reported that short double stranded DNAs with 5' overhangs could be ligated to complementary ends generated after ZFN digestion (Orlando et al. 2010a). This observation has not been further explored probably because it requires the knowledge of the overhangs generated by ZFNs and only insertions of small oligonucleotide have been described (Orlando et al. 2010a). Furthermore, it has also been shown that donor molecules including single strand oligodeoxynucleotides (ssODNs) (Chen et al. 2011; Radecke et al. 2010) and larger external linear sequences can be captured at DSB sites generated by ZFNs (Fung and Weinstock 2011; Gabriel et al. 2011; Li et al. 2011; Mittelman et al. 2009). This feature has been harnessed to track "off-target" effects of the homing endonuclease I-SceI and ZFNs (Petek et al. 2010). Based on these reports and our own observation in mouse embryos, we surmised that it should be possible to directly ligate an external DNA fragment linearized *in situ* by the same ZFNs that target the genome.

We took advantage of the obligated herterodimeric property of the CDNs (Doyon et al. 2011; Miller et al. 2007; Ramalingam et al. 2011; Szczepek et al. 2007) and designed a strategy to achieve efficient and precise gene targeting without homology in the donor plasmid. We named this method ObLiGaRe (Obligate Ligation-Gated Recombination) to reflect the etymologic meaning of the *Latin* verb *obligare* (to bind, to join to). ObLiGaRe should be broadly applicable across different cell types and provides an additional approach for genetic engineering.

Results

ObLiGaRe mediated precise end-joining.

In order to directly ligate an exogenous DNA fragment into the genome, we initially introduced ZFN binding sites into a donor plasmid with the same orientation as in the genome. However we found this strategy often yielded unpredictable products presumably because the same ZFN binding sites were produced after ligation which could then be repetitively digested by the ZFNs, a process that could stimulate end recessing before joining (Pruett-Miller et al. 2008). One essential requirement for ZFN-mediated site-specific digestion using obligated heterodimers is that a pair of ZFNs needs to form heterodimers through the modified FokI nuclease domain after binding to their targeted DNA sequences on the opposite strands. We reasoned that if we altered the orientation of the ZFN recognition sequences in the donor plasmid we could lock the ligation product in a palindrome of identical half ZFN recognition sites which would no longer be sensitive to the same obligated heterodimeric ZFN pairs. We used the well-characterized *AAVS1* ZFN binding site (located in the first intron of *PPP1R12C*) (Hockemeyer et al. 2009) to illustrate this design principle (Fig. 1A). Here we inverted the two half *AAVS1* ZFN binding sites in the vector without changing the orientation of the linker region (Fig. 1A, ObLiGaRe donor). The *AAVS1* ZFNs cut both the genome and the donor plasmid to produce complementary overhangs. After ligation, the newly formed junctions are resistant to further digestion by *AAVS1* ZFNs.

To test our hypothesis we constructed a vector containing the modified *AAVS1* ZFN site followed by a promoterless T2A-puro cassette. Precise ligation of this cassette into the *AAVS1* site in the genome would render the cells resistant to puromycin. We also included a constitutive GFP reporter driven by CAG promoter (Xu et al. 2001) in the vector to track the cells transfected with the plasmid. We transfected the donor plasmid into HCT116 cells and observed an increase in the number of puromycin resistant colonies when the donor plasmid was co-transfected with the plasmid encoding *AAVS1* ZFNs (Supplementary Fig. 1) . We isolated four randomly chosen puromycin resistant, GFP positive clones and examined the vector integration by Southern blot. All four clones were heterozygous insertions of the vector as indicated with an *AAVS1* locus specific probe (int probe, Fig. 1B). In addition, we did not observe random insertions of the vector in the genome when using a vector specific probe (cm probe, Fig. 1B). We then amplified the integration junctions by PCR and directly sequenced the PCR products. We found that 3 out of 4 clones had perfectly ligated junctions as predicted (Fig. 1B). Clone# 1 had a two nucleotide insertion in the linker region at the 5' junction which was not predicted to affect the downstream splicing acceptor (sa) and the expression of puromycin resistant gene (Fig. 1B). Similar results were obtained in RKO cells (Fig. 1B). Interestingly, one of the RKO clones was homozygous for a single copy vector insertion (clone #2). One clone (clone #4) was homozygous for a larger insertion which may represent more than 1 copy of the vector.

We then tested ObLiGaRe in KBM7 cells. KBM7 is a near haploid human myeloid leukemia cell line in which gene targeting has not been reported (Carette et al. 2009;

Kotecki et al. 1999). We co-transfected KBM7 cells with the ObLiGaRe donor plasmid and *AAVS1* ZFN plasmid. Because the transfection efficiency was low, we sorted GFP positive cells by FACS and subjected them to puromycin selection. We characterized 4 puromycin resistant clones and found 3 had the expected insertions at the *AAVS1* locus with no detectable random insertions as judged by Southern blot (Fig. 1B). All clones (#2-4) were hemizygous for the insertion as expected due to the haploid nature of the *AAVS1* locus in KBM7 cells. We then sequenced the PCR amplified 5' and 3' insertion junctions of these clones. In cases that we were unable to directly sequence the PCR products (3' junction in clones #1 and 4, Fig. 1B), amplified junctions were cloned and sequenced. In contrast to experiments in RKO and HCT116 cells, the sequences of the junctions in KBM7 cells were heterogeneous and often involved deletions probably between potential micro-homologies (Fig. 1B). This high proportion of aberrant ligation might due to the unique DDR mechanisms associated with haploid KBM7 cells which are also karyotypically unstable (Andersson et al. 1995; Kobayashi et al. 1998; Kotecki et al. 1999; Skorski 2008).

To determine whether ObLiGaRe is restricted to the *AAVS1* locus and/or *AAVS1* ZFNs, we used *PTEN* specific ZFNs to insert a vector containing a promoterless T2A-mCherry cassette and a CAG-neo selection marker into the *PTEN* locus in HCT116 cells. Because the human genome contains a *PTEN* pseudogene, *PTENP1* (Poliseno et al. 2010) which is also targeted by the *PTEN* ZFNs, we expected to observe insertions at both *PTEN* and *PTENP1*. We isolated 4 G418 resistant clones and examined integration sites at both *PTEN* and *PTENP1* loci by Southern blot. Because we used an autonomous selection marker, we expected that a proportion of G418 resistant clones would result from random insertion of the donor plasmid into the genome. Surprisingly, all clones had targeted insertions in the *PTEN* locus. Clones #1 and 3 were heterozygous and clone #4 was homozygous for the insertion as judged by Southern blot (Fig. 2B). Interestingly, clone #2 had targeted homozygous insertions in both *PTEN* and *PTENP1* loci (Fig. 2B). However, analysis of clone 2 DNA also showed a larger than expected band in the Southern blot probed with a *PTEN* allele-specific probe (Fig. 2B). The size of this band implied it was the result of incorporating two copies of donor plasmid ligated head-to-tail, which was confirmed by further characterization (Supplementary Fig. 2). Clone #1 had an extra insert in the genome as detected by the vector specific probe (cm probe) which could be the result of either random insertion or insertion in other unidentified locus cleaved by *PTEN* ZFNs. We sequenced the 5' junctions at *PTEN* locus in clones #1, 3 and 4 and found they all had precise predicted sequences at the junction between *PTEN* and T2A-cherry (Fig. 2B). We used a TOPO® TA Cloning® strategy to isolate and sequence 5' junctions at both *PTEN* and *PTENP1* loci in clone #2 and identified additional ligation products in this clone, suggesting the two alleles of both genes might have been modified differently (Fig. 2B table).

In order to confirm that ObLiGaRe does not rely on any specific mutations in HCT116 and RKO cells which are known to be defective in DNA mismatch repair mechanisms (Brown et al. 2003), we decided to use a pair of ZFNs to target exon 3 of *Nras* gene in mouse myoblast C2C12 and primary mouse embryonic fibroblast (MEF) cells (Fig. 3). We co-transfected C2C12 cells with either the *Nras* ZFN plasmid alone or with an

ObLiGaRe vector containing a promoterless T2AmCherry cassette and a PGKneo selection marker. We then studied the integration in 4 randomly selected G418 resistant, mCherry fluorescent clones by Southern blot. Although all the clones were mCherry positive, suggesting correct in-frame fusion of T2AmCherry into *Nras* exon 3, only clone #4 had the predicted targeted integration of the vector at the *Nras* locus without additional genomic insertion events (Fig. 3B). Clone #1 had a smaller band hybridizing to the *Nras* specific probe (NR), which might represent a deleted allele present in their largely tetraploid genome (Casas-Delucchi et al. 2011). We sequenced the junctions between *Nras* and mCherry in the genome of clone #4 and confirmed they were perfectly ligated (data not shown). In applying the same strategy to target primary MEF cells, we could not perform clonal selection, so we genotyped pooled cells 3 days after transfection using genomic PCR with primers specific for either the 5' or 3' junctions. We obtained the PCR products expected for correct integration only in cells transfected with the ObLiGaRe vector and *Nras* ZFN plasmid (D+Z, Fig. 3C). We sequenced individually cloned PCR products and found that the majority of the sequences were the precise end joining products at both the 5' and 3' ends (Fig. 3C).

ObLiGaRe mediated insertion of a 15 kb inducible gene expression cassette at the *AAVS1* locus.

Previously, inducible transgene expression from the *AAVS1* locus was achieved by targeting a tetracycline controlled responder in the *AAVS1* locus followed by delivery of the reverse tetracycline transcription activator (rtTA) via lentiviral transduction or gene targeting in a second allele of *AAVS1* (DeKelver et al. 2010; Hockemeyer et al. 2009). We determined whether ObLiGaRe could facilitate insertion of the entire 15 kb inducible transgenic cassette into the *AAVS1* locus in a single step. We inserted the modified *AAVS1* ZFN site at the 5' of an inducible cassette that contains all the components for doxycycline-induced transgene expression in mice (Y Yang, unpublished data) and co-transfected it with *AAVS1* ZFN plasmid in HCT116 cells**.** We screened 18 puromycin resistant clones for correct integration by PCR and identified 14 with the expected PCR product (data not shown). We chose 8 positive clones for further analysis by Southern blot, and showed that they either had heterozygous (clones #1-7) or homozygous (clone #8) insertions of the vector at the *AAVS1* locus (int probe, Fig. 4B). We observed precise end joining at both the 5' and 3' ends in the majority of these 8 clones (Fig. 4B). We also noted that clones #4, 5 and 7 had additional integration events (neo probe, Fig. 4B). Furthermore, we could induce GFP expression by adding doxycycline in all 8 clones as shown with clone# 6 (Fig. 4C).

Mechanism of ObLiGaRe

We speculated that ObLiGaRe might be mediated by NHEJ since it does not require any homology between the donor and the target. To test this hypothesis, we inserted a defective GFP (DGF) harboring a mouse *Nras* ZFN recognition site at the *AAVS1* locus by HR in HCT116 cells (Fig. 5A). We chose one of the correctly targeted heterozygous clones as a reporter (clone#10, Supplemental Fig. 3). We designed two donor plasmids, one had 500-bp homology arms to the DGF and could reconstitute functional GFP by HR; the other had a modified *Nras* ZFN site 5' to a promoterless T2A-mCherry cassette

which would lead to mCherry expression upon insertion by ObLiGaRe (Fig. 5A). We transfected *Nras* ZFN plasmid with both ObLiGaRe and HR donors into the reporter cell line and measured GFP and mCherry positive cells by FACS. We detected 6 times more mCherry positive cells than GFP positive cells in the reporter line but not the parental line (Fig. 5B). All clones derived from the mCherry fluorescent cells presented precise end joining of the mCherry with the defective GFP (data not shown). Interestingly, when we added a potent inhibitor of DNA-PK (a key component of NHEJ (Hollick et al. 2003)) to the medium, the number of mCherry fluorescent cells was significantly reduced whereas that of GFP fluorescent cells was increased ($p<0.001$, Fig. 5C). Conversely, a significant increase of mCherry fluorescent cells with a concomitant decrease of GFP fluorescent cells ($p<0.001$, Fig. 5C) was observed after adding 4 mM caffeine (a nonspecific inhibitor of the ATM and ATR kinases involved in HR) to the medium (Sarkaria et al. 1999). Taken together, these data suggests that ObLiGaRe is mediated via the NHEJ pathway.

Discussion

Targeted gene modification in mammalian cells has been achieved almost exclusively through laborious homologous recombination techniques and has been limited to cells where this pathway seems particularly active. Though CDNs have made gene targeting feasible in cell lines that have low intrinsic HR efficiency, NHEJ is still the dominant DSB repair pathway. As a result, KO alleles generated by NHEJ are obtained at a much higher frequency than KI alleles generated by HR. A deficiency in NHEJ can significantly promote HR as seen in *E.coli* in which NHEJ is negligible (Beumer et al. 2008; Liang et al. 1996; Maresca et al. 2010).

In this study we developed ObLiGaRe, a strategy of site-specific gene insertion utilizing the NHEJ pathway. It applies a similar logic to the one used in unidirectional loxP sites (Oberdoerffer et al. 2003) but maintains all the advantages and flexibility of CDNs. The alternate design of the ZFNs site that we insert in the vector is necessary and sufficient to achieve precise end joining of the vector in the genomic ZFN site. Though less frequent, we also observed aberrant joining products involving small deletions or insertions at the junctions in HCT116 cells (Fig. 1 and 4, Supplementary Table 1). We speculated that these might result from processing alternative overhang types generated by the same ZFNs that were mismatched (Hockemeyer et al. 2009; Orlando et al. 2010a; Smith et al. 2000; Zeevi et al. 2008). Furthermore genetic and cellular context might influence the ligation precision as observed in KBM7 cells (Fig. 1B). Nevertheless, we never observed precise insertion of the vector at targeted genomic locus when a ZFNs site identical to the one present in the genome is introduced in the vector. In fact no integration of a surrogate reporter plasmid bringing a ZFN site identical to the one in the genome was observed in a recent study (Kim et al. 2011). While our manuscript was under revision, Crostea et al. reported that introducing a ZFN site in a donor plasmid could promote its integration into the ZFN targeting site in the genome (Cristea et al. 2012). Because the same ZFN sites were used in donor plasmids, only a minority of genomic insertions had precise 5' and 3' junctions presumably due to repetitive digestions by ZFNs (Cristea et al. 2012). Interestingly, they found that inefficient donor integration could also happen

when the ZFNs used for donor linearization and chromosomal cleavage were not identical. This is in agreement with what Orlando et al. reported previously that double stranded oligos with mismatched overhangs to the DSB generated by ZFNs can be captured though at much lower frequency comparing with those with complementary overhangs (Orlando et al. 2010b). ObLiGaRe should be more efficient under circumstances where precise ligation is critical, for example, to make an in-frame fusion between a reporter and the endogenous gene.

ObLiGaRe eliminates cloning homology arms into the donor vector and does not require any previous knowledge of the overhangs generated by specific CDNs. We are currently modifying the donor plasmids so that the vector backbone can be removed after integration using Cre or Flpe recombinases. Alternatively the ObLiGaRe donors lacking the vector backbone can be generated from minicircle DNA vectors (Kay et al. 2010).

Although we have tested ObLiGaRe mostly with ZFNs in this study, we found it also worked with *AAVS1* TALENs (Supplementary Fig. 4). We also showed that it worked in a variety of cell types including KBM7 and C2C12 cells in which precise gene insertion has not previously been demonstrated. We are currently testing whether it can be used in primary, non-replicating cells in which NHEJ seems to be the predominant pathway to repair DSBs. We are also exploring whether it can work in mouse and zebrafish embryos. Finally we used ObLiGaRe to insert the largest construct in the genome to date by ZFNs (Fig. 3) and we predict that we can use it to deliver even larger constructs, such as BACs, into a pre-defined genomic locus, which is very challenging, if not impossible, to achieve via HR.

Beyond the practical value of our method, ObLiGaRe can be used as a new tool in the study of NHEJ and the crosstalk between NHEJ and HR. Although similar strategies were reported using the I-SceI homing endonuclease (Certo et al. 2011), our strategy allows us to track any endogenous locus that can be targeted by ZFNs. We are interested in using this reporter system to monitor differential utilizations of NHEJ and HR in cells before and after oncogenic transformation. We are also continuing to identify chemical and genetic modulators that influence the cells to choose NHEJ or HR for DDR.

Materials and Methods

Cell culture and transfection

HCT116 and RKO (American Type Culture Collection, VA) were cultured in McCoy's 5A medium (Life Technologies, CA) supplemented with 10% fetal bovine serum (Life Technologies, CA). C2C12 (American Type Culture Collection, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, CA). KBM7 cells, were cultured in IMDM Glutamax (Life Technologies, CA) supplemented with 10% fetal bovine serum. HCT116, RKO, C2C12 and MEFs cells were plated in 6-wells plates (50-80% confluence) and transfected with Lipofectamine LTX according to manufacturer's instructions. Briefly, 2 μg of ZFNs plasmid and 0.5 μg

of ObLiGaRe vector were mixed with 12 µl of Lipofectamine LTX reagent and co-transfected into 5× 10^5 cells. 2 days after transfection cells were transferred to 15cm plates and subject to either puromycin (Life Technologies, CA) selection (0.5 µg/ml) or G418 (Life Technologies, CA) selection (100 µg/ml) the following day. GFP positive, puromycin resistant clones were harvested after 15 days of selection. 10^7 KBM7 cells were electroporated with 8 µg of ZFN plasmids and 2 µg of ObLiGaRe donor using Gene PulserII electroporation system (Bio-Rad, Melville, NY) with electrical settings of 250 V and 950 µF. GFP positive cells were sorted by FACS (FACAria™, Becton Dickinson, NJ) two days after transfection and 100 GFP positives cells were seeded in 12 wells and selected with puromycin (1.0 µg/mL); GFP positives, puromycin resistant clones (pools) were harvested after 10 days of selection.

Comparison of HR to ObLiGaRe

2× 10^5 reporter cells were transfected with 0.2 µg ZFN plasmid, 0.3 µg ObLiGaRe donor (Ob) and 0.5 µg HA donor plasmids (HA), using 4 µL Lipofectamine LTX in 12 wells. The percentage of GFP and mCherry positive cells was measured using FACS cantoII flow cytometer (Becton Dickinson, NJ) 4 days after transfection.
Treatment with Nu7026 (Sigma Aldrich, MO) was started one day before transfection with a final concentration of 10 µM Nu7026 (from a 10 mM stock solution in DMSO) and was continued for 2 days after transfection. ANOVA and Tukey's HSD tests were adopted to perform statistical analysis using R statistics software.

ZFN expression plasmids

ZFNs against the human *PTEN* and *Nras* loci were designed and manufactured by Sigma-Aldrich. ZFNs and TALENs used in this work carried obligate heterodimer forms of the FokI endonuclease. *AAVS1* ZFNs and TALENs were made according to (Hockemeyer et al. 2009; Hockemeyer et al. 2011). The ZFN expression constructs were obtained by Sigma-Aldrich but modified to insert both ZFNs in one plasmids using *ad h.o.c.* recombination (M. Maresca, unpublished). The sequence of linking the *AAVS1* ZFN pair is shown in Supplementary Fig. 5. SURVEYOR® assay was performed according to manufacture instructions (Transgenomic, NE) using the primers below.

PCR primers:

Southern blot internal probe for *AAVS1*	5'-tttctgtctgcagcttgtgg	5'-gggtggaggggacagataaa
AAVS1 5' ObLiGaRe	5'-cccctatgtccacttcagga	5'- tgaggaagagttcttgcagct
AAVS1 3' ObLiGaRe	5'- tggctcattagggaatgctt	5'- acaggaggtgggggttagac
Southern blot probe for *cm*	5'- tcactggatataccaccgttg	5'- tggtctgacagttattacgcc
Southern blot probe for *PTEN*	5'- gctgcagtccattgagcata	5'- gctgtggtgggttatggtct
Southern blot probe for *PTENP1*	5'- attcgtcttctccccattcc	5'- agtgaattgctgcaacatga

PTEN 5' ObLiGaRe	5'- aagaccataacccaccacagc	5'- ttggtcaccttcagcttggc
PTENP1 5' ObLiGaRe	5'- aaagacattatgacaccgcc	5'- ttggtcaccttcagcttggc
Southern blot probe for *Nras*	5'- gttccagtgccctgttcaat	5'- cacaaccacttcccgaaact
Nras 5' ObLiGaRe	5'- ctgttagcgggttgagggta	5'- aagcgcatgaactccttgat
Nras 3' ObLiGaRe	5'- attaatgcagctggcacgac	5'- tggcaaatacacagaggaacc
Southern blot probe for neo	5'- gatcggccattgaacaagat	5'- gcgataccgtaaagcacgag

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Contributions

M.M. and Y.Y. conceived the study and wrote the manuscript. M.M. performed most experiments. V.L. and N.G. performed individual experiments. V.L. performed statistical analysis.

Figure Legends:

Figure 1 ObLiGaRe at the *AAVS1* locus.
(A) Schematic illustration of the ObLiGaRe strategy. ZFN recognition sites are indicated as grey and blue circles with the corresponding sequences displayed in the same color scheme. The predicted joining sequences between the vector and the genome are shown. The insertion of the vector in the *AAVS1* locus will cause a size shift from 6.7 kb (WT) to 14.2 kb (LP) of a HindIII (H) digested fragment. *AAVS1* internal (int) and vector specific probes (cm) are indicated in the map at the hybridization sites.

(B) Southern blot of 4 puro resistant colonies after ObLiGaRe at *AAVS1* locus in HCT116 (upper), RKO (middle) and KBM-7 (lower) with int probe (left) and cm probe (right) with sequences at the 5' and 3' junctions corresponding to each clones listed on the right. Insertions are indicated in red, deletions are indicated as red dotted lines.

WT: wild type; LP: ligation product; sa: splicing acceptor; CM: chloramphenicol resistant.

Figure 2 ObLiGaRe at the *PTEN* locus.
(*A*) Strategy for targeting human *PTEN* and *PTENP*1. *PTEN* ZFN recognition sites are indicated as orange and cyan circles with the corresponding sequences displayed in the same color scheme. The predicted joining sequences between the vector and the genome are indicated. The insertion of the vector will cause a size shift of a BsrGI (B) digested fragment from wild type of 4.1 kb (WT) to the ligation product of 9.5 kb (LP) at *PTEN* locus and 25.7 kb (WT) to 31.0 kb (LP) at *PTENP1* locus. *PTEN* internal probe (PT), *PTENP1* (P1) and vector specific probe (cm) are indicated in the map at the hybridization sites.

(*B*) Southern blots using probes specific for *PTEN* (PT, left), *PTENP1* (P1, middle), and the vector (cm, right) for 4 G418 resistant colonies. P1 probe also could detect exon 1 of *PTEN* which is the lowest signal across all lanes (middle blot). The sequence of the 5' junction between *PTEN* or *PTENP1* and mCherry are indicated in the table. Insertions are indicated in red where "ins" represents a 224bp insertion.

Figure 3 ObLiGaRe in C2C112 and MEF cells.
(*A*) Strategy for targeting *Nras* locus in MEF and C2C12 cells. *Nras* ZFN sites are indicated as violet and red circles with the corresponding sequences displayed in the same color scheme. The predicted joining sequences between the vector and the genome are shown. Primers for PCR detection of the junctions and probes (cm and NR) are indicated. The insertion of the vector will cause a size shift of an NdeI (N) digested fragment from wild type of 6.2 kb (WT) to the ligation product of 11.6 kb (LP) at *Nras* locus. *Nras* specific probe (NR) and vector specific probe (cm) are indicated in the map at the hybridization sites.

(*B*) Southern blot for 4 C2C12 clones expressing mCherry. LP indicates the expected ligation product band upon integration of the vector in the genome. The band lower than the WT band in clone #1 might be a deleted *Nras* allele since C2C12 cells are tetraploid.

(*C*) Genomic PCR products amplified from pools of MEFs after transfection with ObLiGaRe donor alone (D) or with *Nras* ZFN plasmid (D+Z). P1-P2 primers amplify the 5' junction and P3-P4 primes amplify the 3'junction. The table lists the sequences of individually isolated PCR fragments. Deletions are indicated as red dotted lines and insertion in red capital letters.

Figure 4 Introduction of a 15 kb inducible gene expression cassette in the *AAVS1* locus by single step ObLiGaRe.
(*A*) Strategy to introduce an inducible gene expression cassette. ZFN cutting sites are the same as in Fig. 1. The expression of rtTA is controlled by the constitutive CAG promoter while GFP is under the doxycycline responsive promoter (tetO) (Gossen and Bujard 1992). The STOP sign indicates a transcription termination cassette. Int and neo

represent the probes for Southern blot. P1-P2 and P3-P4 are PCR primers used to amplify the 5' and 3' junctions. K: KpnI.

(*B*) Southern blot of 8 positive clones is shown with both internal (int) and vector specific probe (neo). LP: expected ligation product. The sequences for 5' and 3' junctions of the 8 clones are reported in the table on the right with dotted lines indicate deletions.

(*C*) GFP fluorescence is detected by fluorescence microscopy in cells from clone #6 without (-) and with (+) 48 hr treatment of 1 μg/ml doxycycline (dox).

Figure 5 ObLiGaRe is mediated via NHEJ.
(*A*) Illustration of the reporter DGF cassette in the *AAVS1* locus. ZFN sites are the same as in Fig. 3. A functional GFP is generated after HR using the HA donor, while a functional mCherry is inserted upon ObLiGaRe using the ObLiGaRe donor. The two donors are about the same size.

(*B*) Determination of fluorescent cell number after co-transfection of *Nras* ZFN, ObLiGaRe and HA donors by FACS. Detection of GFP and mCherry cells were gated using parental cells (WT, left) which did not show any fluorescent cells upon co-transfection. When the reporter cell line was co-trasfected with the 3 constructs, GFP and mCherry cells were detected in their corresponding gates (DGF, right).

(*C*) Representation of the percentage of GFP and mCherry positive cells out of total live cells in WT HCT116 cells, reporter HCT116 cells without treatment (DGF) or after treatment with 10 μM Nu7026 or 4 mM Caffeine. Error bars: standard deviation; n=3.

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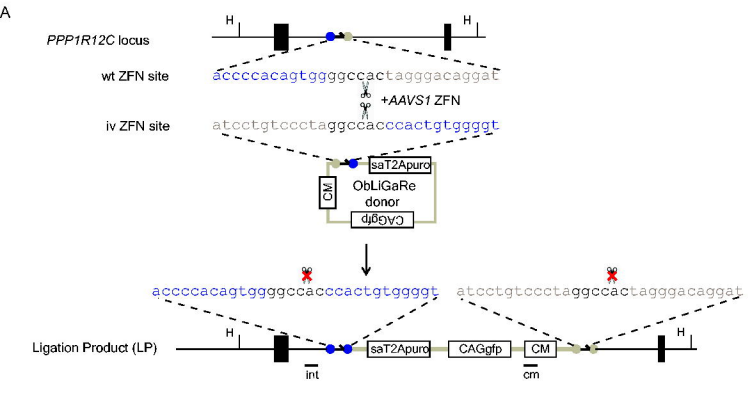
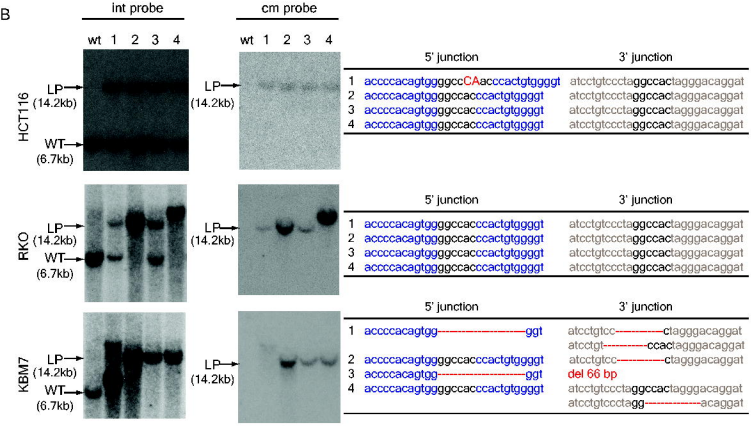
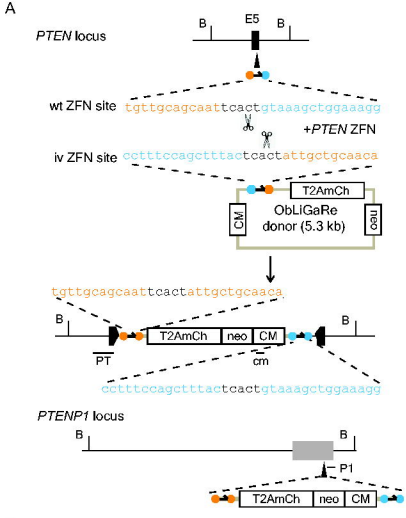
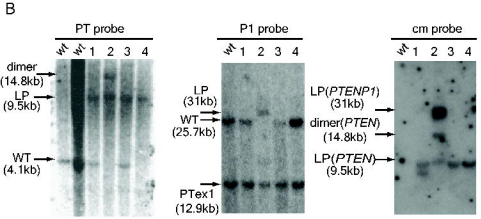




Maresca_Fig.1





	5' junction at *PTEN*	5' junction at *PTENP1*
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2	tgttgcagcaattcactattgctgcaaca	tgttgcagcaattcactattgctgcaaca
	tgttgcagcaattcinsctattgctgcaaca	tgttgcagcaattcactACTattgctgcaaca
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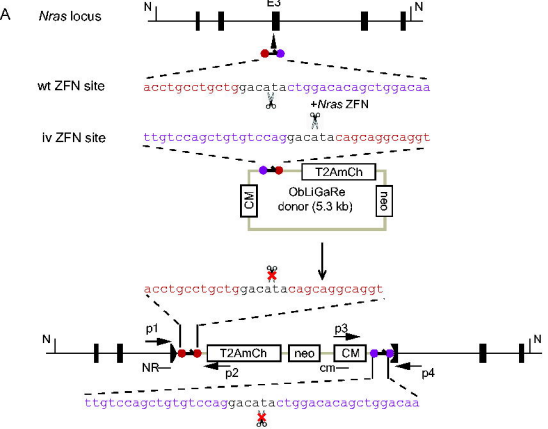

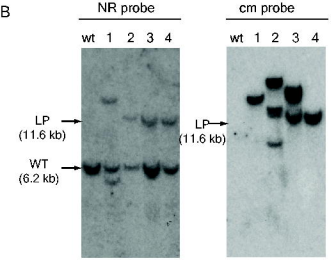

	5' junction	3' junction
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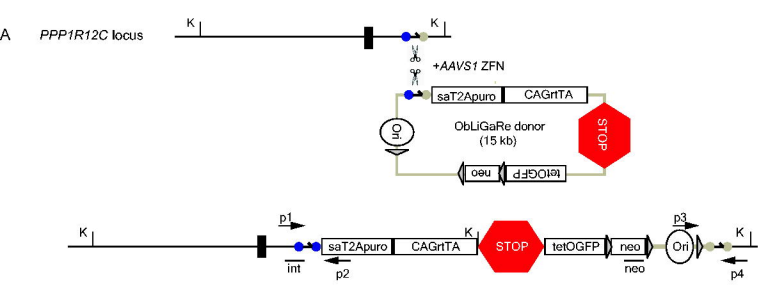
Maresca_Fig. 3



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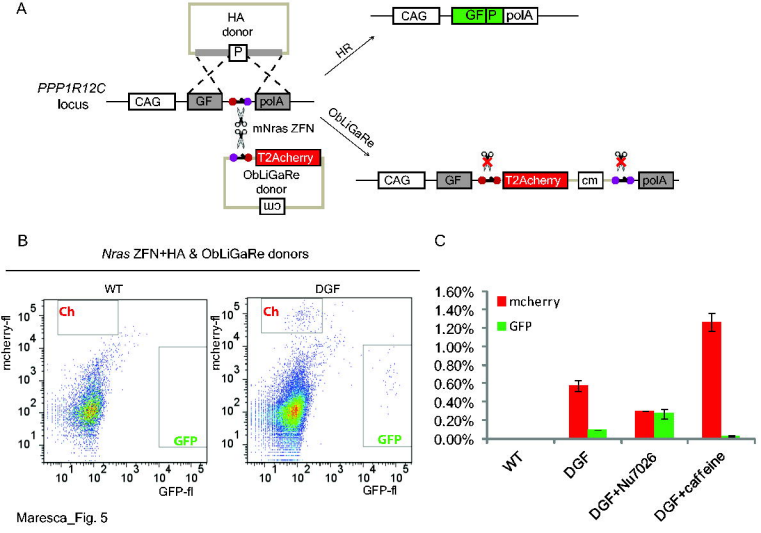

Maresca_Fig. 5