

ZFN Assisted Targeted Integration to Develop Knock-in Reporter Cell Lines

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Abstract

Sigma-Aldrich CompoZr™ zinc finger nuclease (ZFN) technology is a fast and reliable way to manipulate the genome in a targeted fashion. In the majority of cases ZFNs have been used to create gene knock-outs utilizing non-homologous end joining (NHEJ) – the main pathway of double-strand break (DSB) repair in somatic cells. Here we relied on the second DSB repair pathway - homologous recombination (HR) to tag organelle specific genes by integrating a fluorescent reporter sequence into the desired location in the genome. The integration resulted in endogenous expression of the corresponding organelle marker fusion proteins that have the advantage of easy and fast detection due to their native high expression level and characteristic organelle pattern. Three gene loci were tagged: LMNB1 (lamin B1, nucleus), TUBA1B (α-tubulin 1b, microtubules), & ACTβ (β-actin, actin stress fibers). Green and red fluorescent proteins were used as reporters. Single cell knock-in clones were isolated in U2OS cells with one copy of a given gene tagged. ZFN mediated gene tagging in knock-in cell lines could provide the basis for development of various cell-based assays for compound screening where native gene regulation and protein function are preserved.

Introduction

Zinc Finger Nucleases (ZFNs) are fusions of zinc finger proteins (ZFPs) and a nuclease domain, such as the DNA cleavage domain of a type II endonuclease, *FokI*. The ZFPs provide binding specificity, and the nuclease domain dimerizes and cleaves the DNA to generate double-strand breaks (DSBs), which are detrimental to the cell. The cell uses two main pathways to repair DSBs: high-fidelity homologous recombination (HR) and error-prone non-homologous end joining (NHEJ) (Figure 1A). ZFNs have been primarily used to create gene knockouts in mammalian cell lines (Figure 1B) and various species including zebrafish, rats, flies, and worms. ZFNs have also been shown to increase the HR rate several orders of magnitude (Figure 1E). Here we report successful targeted integration of GFP and RFP at multiple loci in the U2OS human cell line, creating fluorescently labeled fusions of LMNB1 (lamin B1), TUBA1B (α-tubulin 1b), & ACTβ (β-actin) that are endogenously regulated.

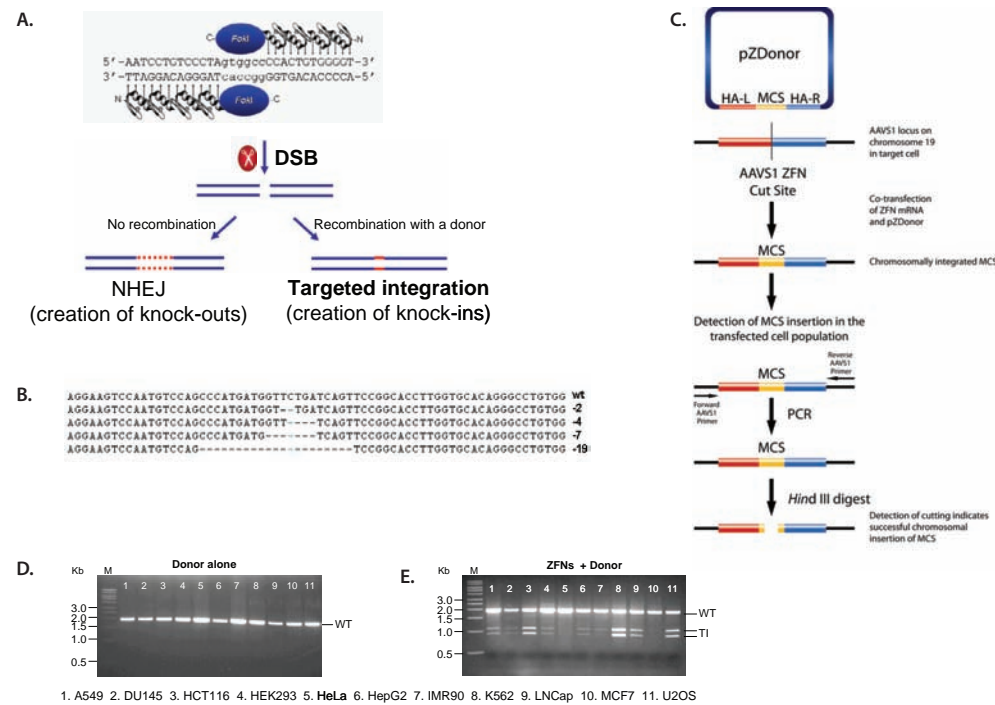


Figure 1: ZFN targeting mechanism. **A.** ZFNs bind to the target site, and *FokI* endonuclease domain dimerizes and makes a double strand break (DSB) between the binding sites. DSBs are repaired by either an error-prone NHEJ pathway or high-fidelity homologous recombination. NHEJ introduces deletions (see Figure 1B) or insertions, which change the spacing between the binding sites so that ZFNs might still bind but dimerization or cleavage cannot occur. In the presence of a donor DNA carrying homology flanking the target site, homologous recombination can use the donor as template to repair a DSB, achieving targeted integration. **B.** Allelic sequences from a *Bax* (pro-apoptotic Bcl-2-associated X protein) null clone in the tetraploid A549 cell line created by CompoZr™ ZFNs targeting *Bax*'s exon 5. Numbers to the right of the sequence represent the bases deleted from wild type *Bax* gene. **C.** CompoZr™ Targeted Integration Kit – AAVS1 (CT11) workflow. The pZDonor consists of homologous arms (HA-L and HA-R) of AAVS1 loci flanking a MCS (multiple cloning site) containing *HindIII* restriction enzyme site. *HindIII* digested PCR products were initially amplified from eleven cell lines nucleofected with either donor alone (**D**) or donor + AAVS1 ZFN DNAs (**E**). In wild type cells, there is no *HindIII* site in the AAVS1 PCR-amplified region. However, DNA from cells with targeted integration (TI) will have one *HindIII* site in the AAVS1 PCR-amplified region; digestion will result in two bands both approximately 1 Kb in size. Note 1) no targeted integration occurred without ZFNs and 2) targeted integration varies among the eleven cell lines, with K562 having the highest amount and MCF7 with the least.

Results

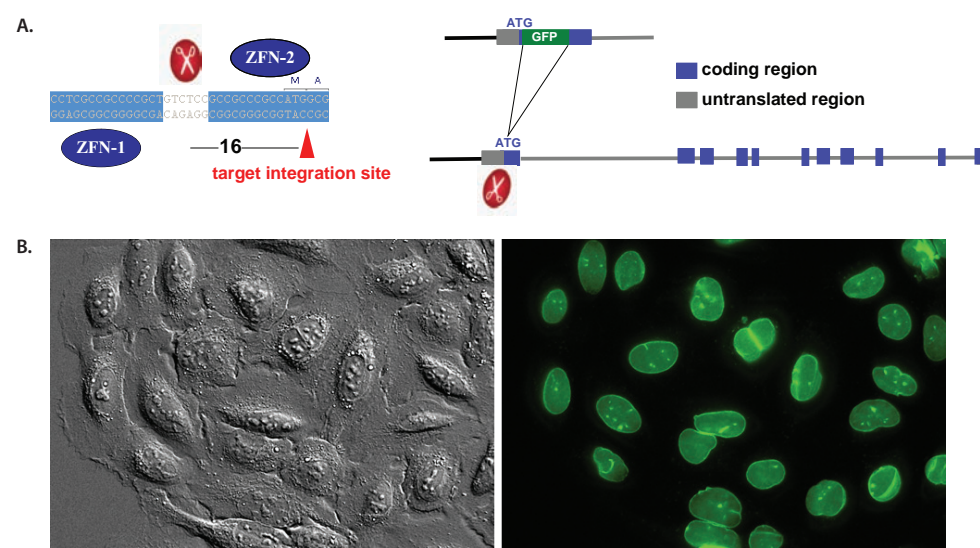


Figure 2: Targeted integration of LMNB1 in U2OS. **A.** Schematics of the LMNB1 CompoZr™ ZFN binding sites/ZFN cut site with respect to the targeted integration site (left) and of the LMNB1 loci and green donor (right). Lamin B1 is an inner nuclear membrane protein. **B.** DIC differential interference contrast (left) & fluorescence microscopy image (right) of endogenously labeled LMNB1.

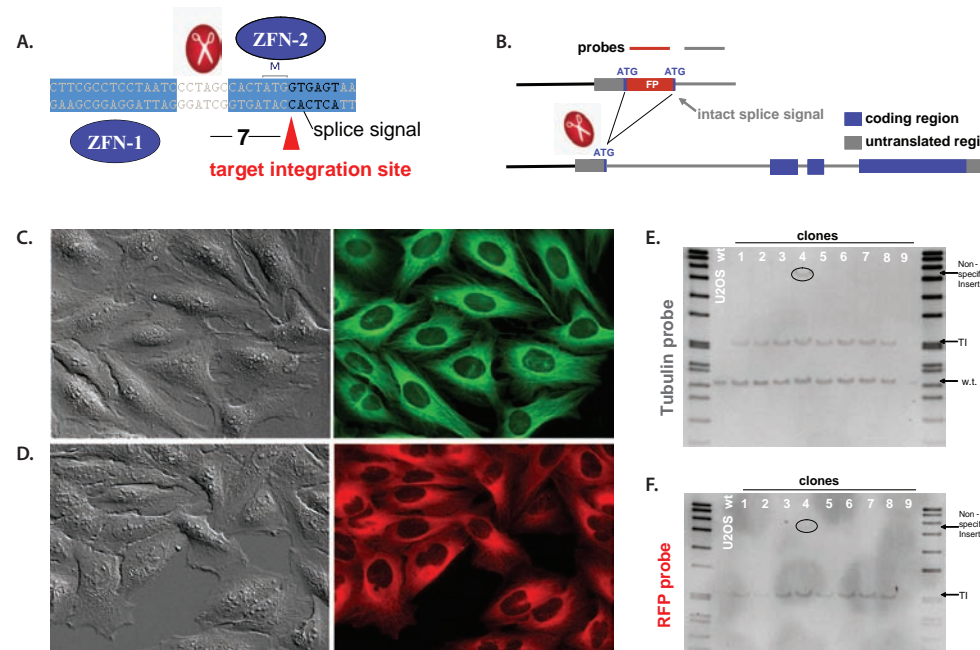


Figure 3: Targeted integration of TUBA1B. **A.** Schematic of the TUBA1B CompoZr™ ZFN binding sites/ZFN cut site with respect to the targeted integration site. **B.** Schematic of the TUBA1B loci and donor(s) including location of DIG-labeled probes used in the Southern blots. DIC & fluorescence microscopy images of endogenously labeled TUBA1B with GFP (**C**) and RFP (**D**) in U2OS cells. Southern hybridizations were performed on DNA isolated from wild type U2OS and nine single cell clones positive for red tubulin fluorescence (**E** & **F**). Genomic DNAs were digested with *PstI* overnight. (**E**) Using the tubulin probe, 1946 bp band represents the tubulin genomic DNA with addition of RFP while the 1219 bp band represents wt tubulin genomic DNA. Targeted integration (TI) did not occur at every allele, note the presence of both bands in the lanes 1-9. (**F**) Using the RFP probe, hybridization to wild type U2OS DNA did not occur. However, the RFP probe did hybridize to the DNA from the RFP-TUBA1B clones. Out of the 9 TUBA1B-RFP clones, only 1 clone (#4) contained an off-target insert.

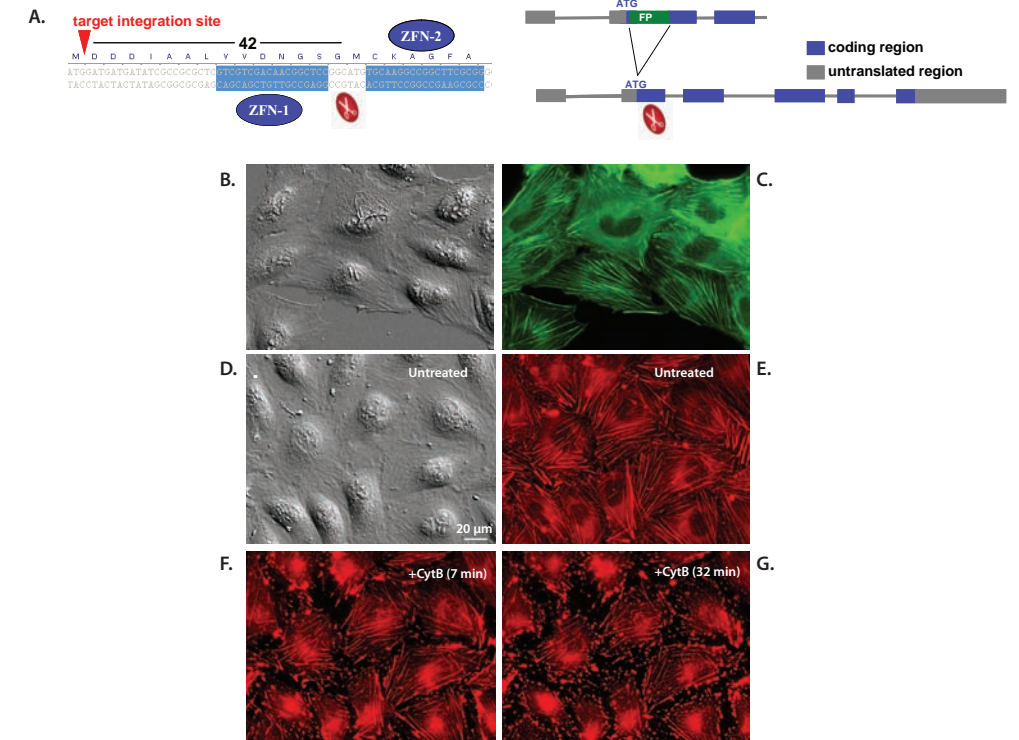


Figure 4: Targeted integration of ACTβ. **A.** Schematics of the ACTβ CompoZr™ ZFN binding sites/ZFN cut site with respect to the targeted integration site (left) and the ACTβ loci with respect to the ACTβ donor(s) (right). DIC (**B** & **D**) & fluorescence microscopy images of GFP-ACTβ (**C**) and RFP-ACTβ clones (**E**, **F** & **G**) in the U2OS cell line. 21 μM cytochalasin B added to the RFP-ACTβ clone for 7 minutes (**F**) and 32 minutes (**G**). Cytochalasin B is a metabolite that disrupts microfilaments.

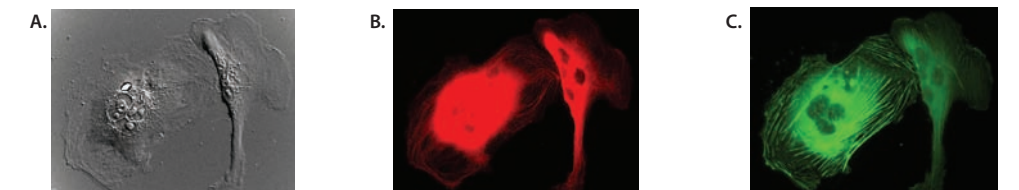


Figure 5: Trait Stacking: TUBA1B & ACTβ. Tubulin-RFP clone 7 (see Fig. 3, E & F) nucleofected with ACTβ CompoZr™ ZFNs and ACTβ green donor. DIC (**A**) & fluorescence microscopy images from the same field of view. **B.** Red fluorescence from RFP integrated into the *TUBA1B* loci. **C.** Green fluorescence from GFP integrated into the *ACTβ* loci.

Summary

In the past, ZFNs have been primarily used to create knock out cell lines. Instead we chose to utilize ZFNs to permanently integrate a reporter gene into an organism's genome. Unlike fusion proteins generated with an external promoter, the fusion proteins created using the ZFNs are expressed at a physiologically relevant level in the cell.

Here we successfully tagged the loci of 3 organelle markers (lamin B1, α-tubulin 1b, & β-actin) with fluorescent reporter genes using CompoZr™ ZFNs. Future work includes demonstrating that the marker expression is regulated by endogenous regulatory pathways, trait stacking, and the labeling of additional gene targets.

ZFN technology is an ideal tool for generation of unique cell lines and allows the design of experiments never thought possible before.

Acknowledgments

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