

# Development of Gene-Specific Knock-in Reporter Cell Lines via ZFN-Mediated Homologous Recombination

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## Introduction

Genome modification of mammalian cells is one of the most challenging and therapeutically important fields that impacts drug-discovery and cell-based assays. At present, gene targeting by homologous recombination (HR) is the standard method utilized for precise genome modification. The most efficient approach by far to facilitate HR in the targeted cell is through formation of site specific DNA double-strand breaks (DSBs). Thus, the Zinc Finger Nuclease (ZFN) technology has gained popularity in the gene targeting field due to its capability to bind DNA and create a DSB in a sequence-specific manner, dramatically increasing the rate of homologous recombination (HR) between a specific genomic target and a given donor plasmid.

Zinc finger nucleases (ZFNs) are engineered proteins that theoretically can be customized/modified to cut near the desired site of integration for any given target gene. To demonstrate ZFN mediated HR for the development of knock-in cell lines, we have targeted various fluorescent reporter sequences into selected cytoskeleton and/or chromatin specific genes using Sigma-Aldrich CompoZr™ ZFN technology.

In our study, four gene loci – ACTB (β-actin, actin stress fibers), TUBA1B (α-tubulin 1b, microtubule), LMNB1 (lamin B1, nuclear envelope) and HMG1 (high mobility group AT-hook, nucleus) – were site-specifically tagged by green, red or blue fluorescent proteins (FP) in several human cell lines. The targeted in-frame integration resulted in endogenous expression of the corresponding fusion proteins that maintained their native expression level and characteristic pattern. For all cell lines (except for the triple tagging), single cell knock-in clones were isolated. These single clones retained stable expression of the FP-tagged proteins through more than twenty passages. ZFN mediated gene tagging in knock-in cell lines may provide the basis for development of various cell-based assays for compound screening where native gene regulation and protein function are preserved.

## Materials and Methods

- U-2 OS (Cat. No. HTB-96TM) cells were obtained from ATCC and cultured according to the product manual.
- Nucleofections were performed with the Amaxa® Nucleofector® device (Cat. No. AAD-1001) and Nucleofector® Kit V (Cat. No. VCA-1003) from Lonza AG according to the product manual.
- Donor plasmids were designed and constructed in house.
- Fluorescent reporter genes were obtained from Evrogen (<http://evrogen.com/products/TagFPs.shtml>).
- CompoZr™ ZFNs were designed and manufactured by Sigma-Aldrich.
- Fluorescent microscopy was performed with a Nikon Eclipse TE2000-E inverted research microscope and MetaMorph® software.
- Unless otherwise indicated, all reagents and materials used in this work were obtained from Sigma-Aldrich (St. Louis, MO USA).

## ZFN targeting mechanism and donor design.

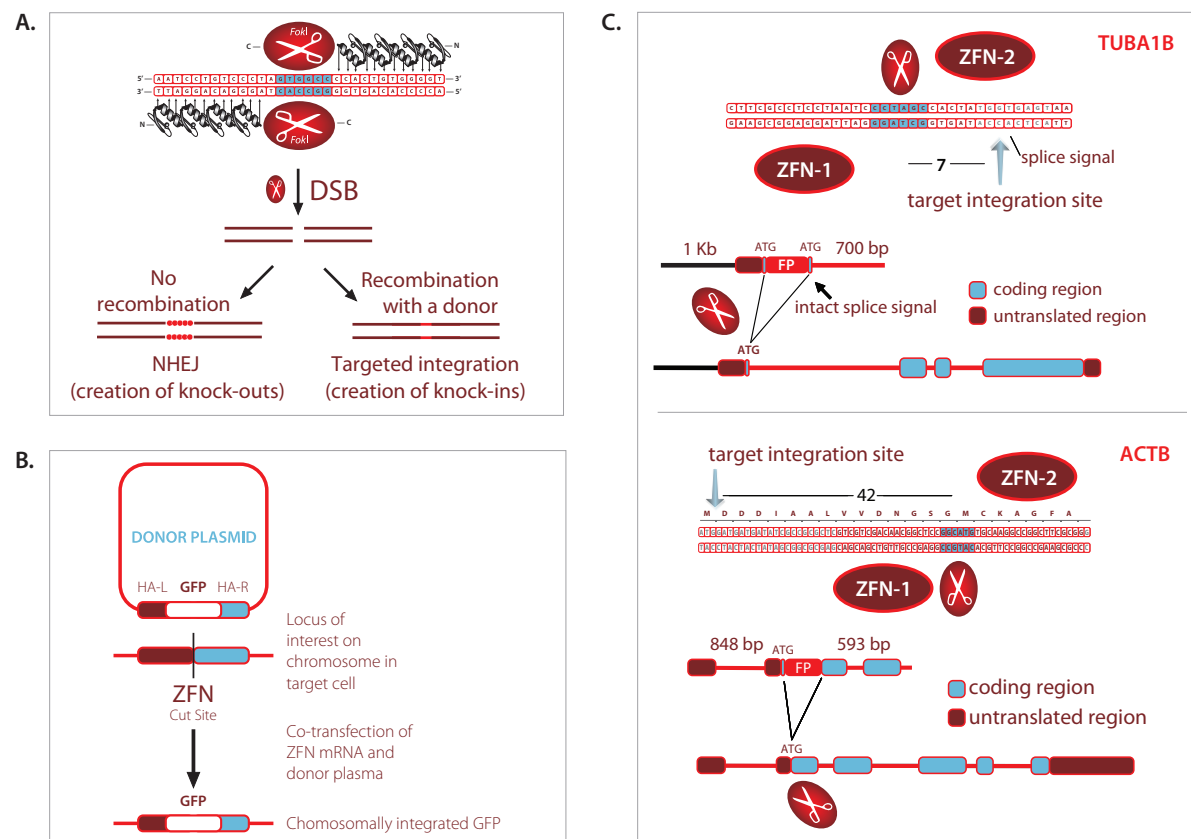


Figure 1: ZFN targeting mechanism and donor design.

- ZFNs bind to the target site. Then the 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 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.
- Generic workflow. The donor plasmid consists of homologous arms (HA-L and HA-R) of the ZFN cut site flanking a fluorescent reporter molecule (GFP).
- Examples to show schematic of CompoZr™ ZFN binding sites/ZFN cut site with respect to the targeted integration site for TUBA1B loci (upper panel) and ACTB loci (lower panel)

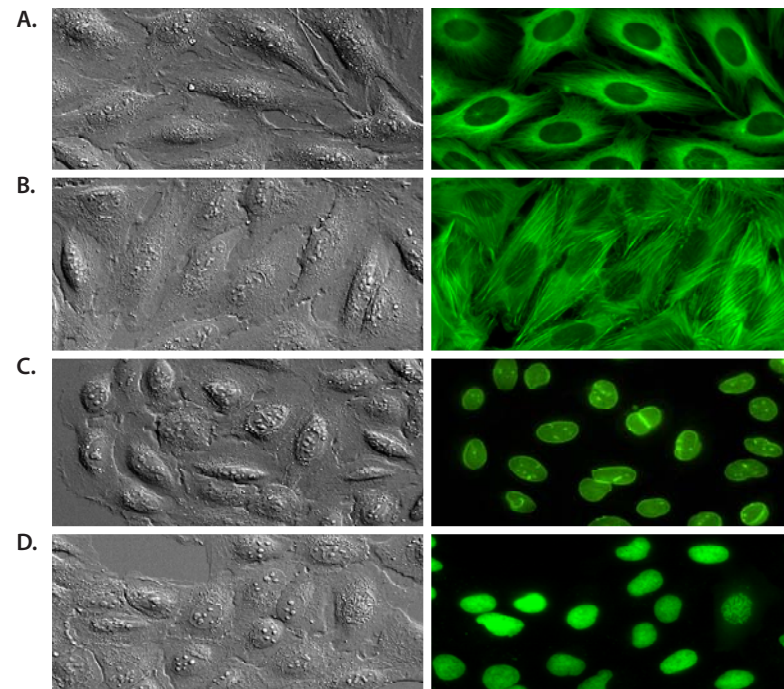
Table 1. Summary of Tagged Loci in human genome

NM_number (gene name, encoded protein)	Organelle	Human Chromosome Number	Terminus	Distance between ZFN cut site and splice site (bp)	Initial GFP Integration Efficiency
NM_006082 (TUBA1B, α-tubulin 1b)	Microtubule	12	N	7	8.0%
NM_001101 (ACTB, β-actin)	Actin Stress Fibers	7	N	42	9.8%
NM_005573 (LMNB1, lamin B1 - key structural component of the nuclear lamina, an intermediate filament meshwork that lies beneath the inner nuclear membrane)	Nuclear Envelope	5	N	16	1.2%
NM_145899 (HMG1, High Mobility Group protein HMG-1/HMG-Y isoform A (AT-hook) - a non-histone dsDNA binding protein)	Nucleus (DNA)	6	C	56	0.2%

## Successfully Tagged Loci.

Figure 2. Successfully Tagged Loci.

- GFP integrated into the TUBA1B locus in U-2 OS cells highlighting tubulin filaments
- GFP integrated into the ACTB locus in U-2 OS cells highlighting actin filaments.
- GFP integrated into the LMNB1 locus in U-2 OS cells depicting the nuclear envelope.
- GFP integrated into the HMG1 locus in U-2 OS cells localized in the nucleus.



## Trait Stacking

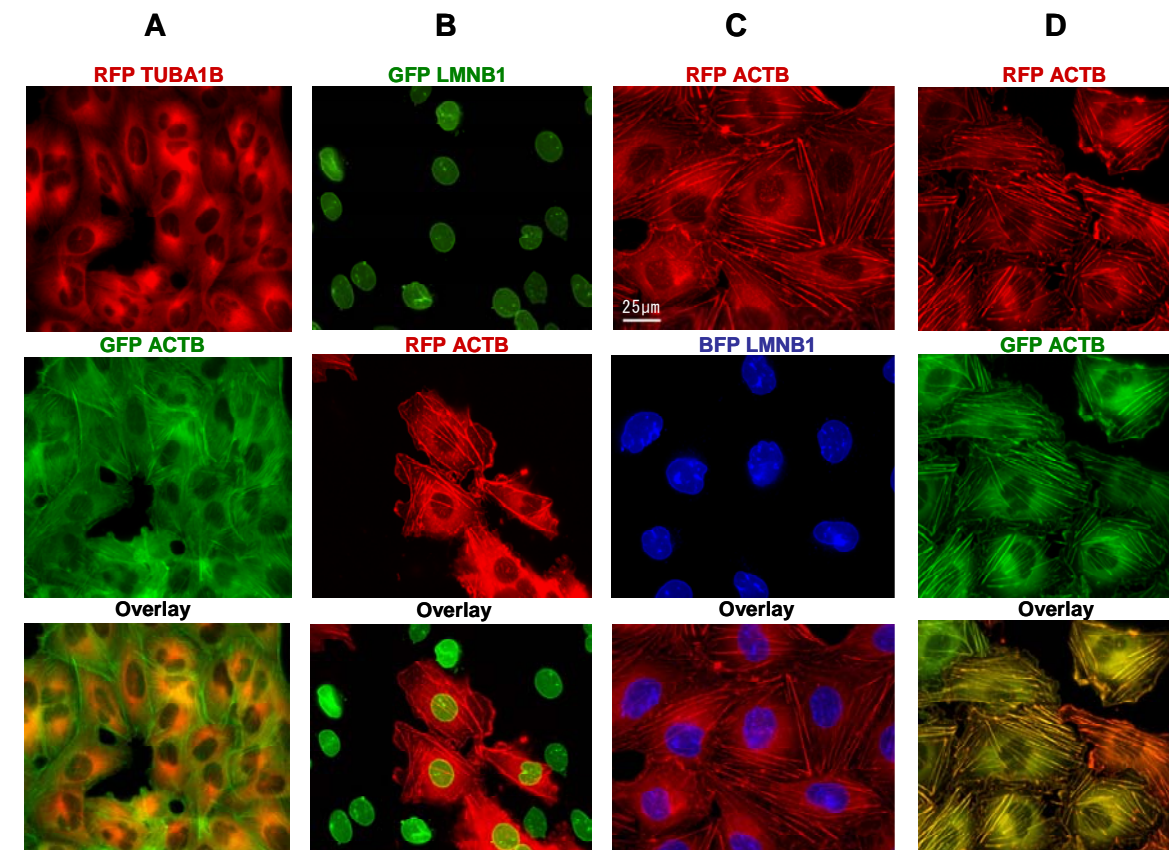


Figure 3: Trait Stacking

- A RFP-TUBA1B expressing cell line was modified with ZFNs to also express GFP-ACTB.
- A GFP-LMNB1 expressing cell line was modified with ZFNs to also express RFP-ACTB.
- A RFP-ACTB expressing cell line was modified with ZFNs to also express GFP-LMNB1.
- The ACTB gene has 3 alleles in the U-2 OS cell line. ZFNs were used to express GFP-ACTB in a cell line already expressing RFP-ACTB resulting in the modification of two alleles of the same gene.
- U-2 OS cells expressing both RFP-ACTB and GFP-LMNB1 described in C were further modified with ZFNs to express GFP-TUBA1B.

## E. Triple Knock-In: GFP-LMNB1, RFP-ACTB and GFP-TUBA1B

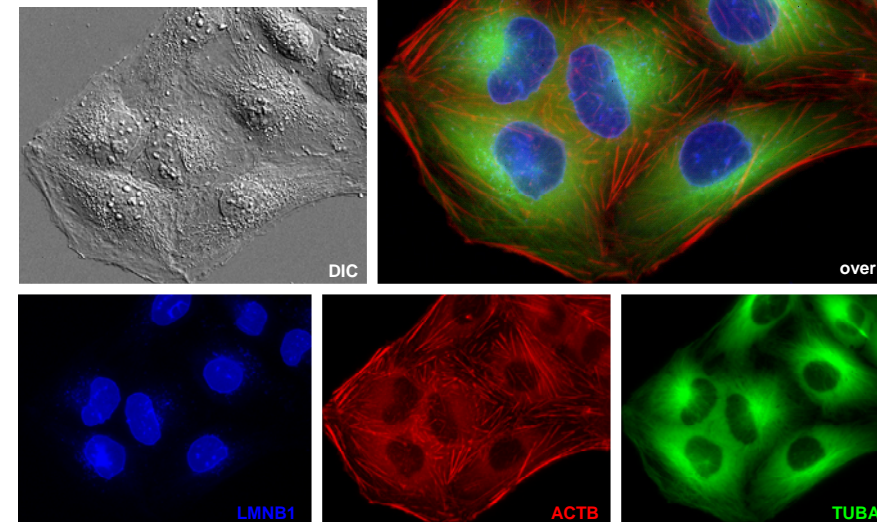


Figure 4: Molecular Analysis. Besides fluorescent imaging, targeted integration was identified by junction PCR and Southern hybridization. In addition, the final clones for all targeted loci were subjected to DNA sequencing to confirm integrated DNA fragments.

Left panel summarized assays to identify RFP-TUBA1B and its subsequent trait stacking. A. Junction PCR amplification. All 7 clones contained the characteristic integration band at 1314bp. C is the donor-only control. B. Southern hybridizations were performed on PstI digested genomic DNA isolated from wild type U-2 OS and nine single cell clones positive for red tubulin fluorescence. Using the tubulin probe, a 1946 bp band represents the wt 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. Out of the 9 TUBA1B-RFP clones, only clone #4 contained an off-target insert. C. RFP-TUBA1B cells were further tagged with GFP-ACTB via ZFN modification (see Figure 3A). The characteristic integration bands for both RFP-TUBA1B and GFP-ACTB were amplified simultaneously by junction PCR in all 10 clones tested, but not detected in wild-type control.

Right panel focused on some molecular analyses to identify RFP/GFP-ACTB and its subsequent trait stacking. D. Southern hybridization, using DIG-labeled GFP and RFP probes, to confirm the single clones for GFP and RFP tagging in ACTB loci. Genomic DNA was digested with NcoI and PstI. G7 is for GFP and R1 is for RFP. E. Junction PCR to confirm double trait stacking on RFP-ACTB and GFP-LMNB1 (see Figure 3C). The integration bands for both RFP-ACTB and GFP-LMNB1 were amplified simultaneously in selected clones tested, whereas only RFP-ACTB can be detected in cells expressing RFP-ACTB and no band can be detected in wild-type control. F. Triple trait stacking (see Figure 3E) was confirmed by junction PCR in GFP-TUBA1B enriched cell population expressing RFP-ACTB and GFP-LMNB1. 1: MWM; 2: Double tagged for RFP-ACTB and GFP-LMNB1; 3: GFP enriched triple tagging population; 4: un-enriched triple tagging population.

## Compound Screening

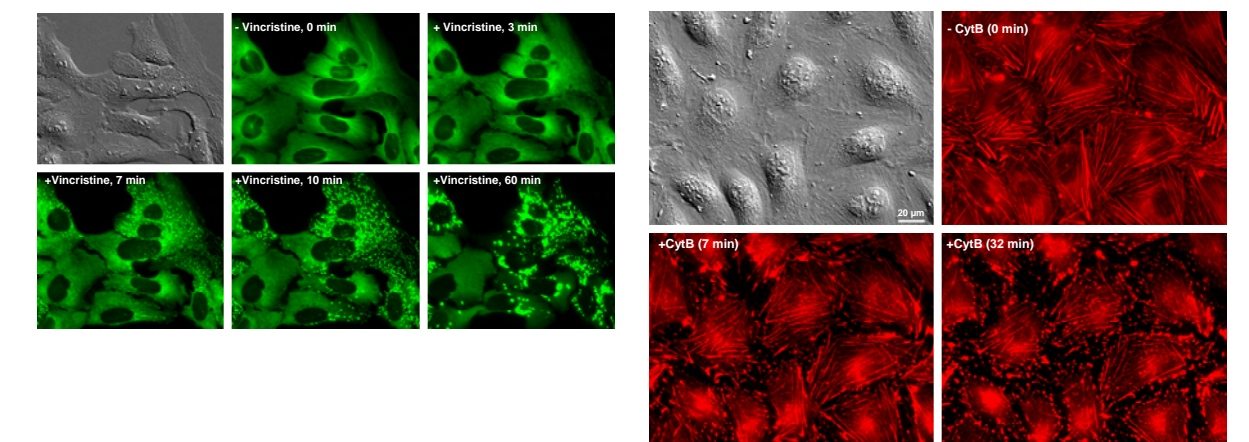


Figure 5: Vincristine time course. Vincristine is a mitotic inhibitor used in cancer chemotherapy. Its mode of action is to bind to tubulin dimers thereby inhibiting the assembly of microtubule structures. 1. GFP tagged TUBA1B U-2 OS cells were exposed to 20 μM Vincristine for sixty minutes. As time progressed, tubulin is repolymerized into a crystalline structure.

Figure 6: Cytochalasin B Time Course. Cytochalasin B is a mycotoxin. It blocks the formation of contractile microfilaments thus inhibiting cytoplasmic division. 2. By blocking monomer addition actin filaments are shortened. RFP tagged ACTB U-2 OS cells were exposed to 21 μM Cytochalasin B. Over time, shortening of actin filaments can be observed.

## Discussion/Conclusion

Until now, fluorescence detection of proteins relied on either exogenous promoters or immuno-techniques requiring cell fixation. With the ZFN technology, it is now possible to create stable integration of a reporter gene into the genome. Unlike fusion proteins generated with an external promoter, the fusion proteins created using the ZFNs are expressed at their physiological level and apparently retain the characteristic expression profile of the endogenous proteins in the cell. The fusion protein can be observed throughout the cell's life cycle.

This work demonstrates successful tagging of four individual loci: TUBA1B (α-tubulin 1b, microtubule), ACTB (β-actin, actin stress fibers), LMNB1 (lamin B1, nuclear envelope) and HMG1 (high mobility group AT-hook 1, nucleus). Also demonstrated are the labeling of more than two different genes in the same cell line as well as two different alleles of the same gene. Future work includes the study of cellular processes, compound screening, and cell-based assay development.

## Endnotes/References

- Lober S; Vulevic B; Correia JJ. (1996) "Interaction of vinca alkaloids with tubulin: A comparison of vinblastine, vincristine, and vinorelbine". *Biochemistry* 35(21): 6806 – 14.
- Theodoropoulos, PA; Gravanis, A; Tsapara, A; Margioris, AN; Papadogiorgaki, E; Galanopoulos, V; Stourmaras, C (1994). "Cytochalasin B may shorten actin filaments by a mechanism independent of barbed end capping". *Biochemical pharmacology* 47 (10): 1875–81.

## Product Offerings

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| Osteosarcoma Cell Line with GFP-tagged α-tubulin 1b (CLL1031) | Osteosarcoma Cell Line with RFP-tagged α-tubulin 1b (CLL1034) |
| Osteosarcoma Cell Line with GFP-tagged β-actin (CLL1032)      | Osteosarcoma Cell Line with RFP-tagged β-actin (CLL1035)      |
| Osteosarcoma Cell Line with GFP-tagged LaminB1 (CLL1033)      |   |

## Acknowledgements

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