# An Application of Zinc Finger Nuclease Technology to Create Knock-in and Knock-out Cell Lines

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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 to facilitate HR in the targeted cell is through formation of site specific DNA double-strand breaks (DSBs). Thus, Zinc Finger Nuclease (ZFN) technology has gained popularity in the gene editing 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 are engineered proteins that can be customized/designed to cut near the desired site of integration for any given target gene sequence. To demonstrate ZFN mediated HR for the development of knock-in cell lines, we have inserted various fluorescent reporter sequences into selected cytoskeleton and/or chromatin specific genes using Sigma-Aldrich CompoZr™ ZFN technology. We also demonstrate the construction of genetically defined knock-out cell lines. Two pro-apoptotic regulators, Bcl2–associated X protein, BAX, and Bcl2-like protein, BAK, genes were knocked out using ZFNs.

ZFN technology is an ideal tool for the generation of genetically modified cell lines. The straightforward approach to address both diploid as well as polyploid targets is an ideal tool to generate specific gene knockout cell lines. Additionally, ZFN mediated gene tagging 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

### Human Cell Culture

U-2 OS: human osteosarcoma; media: McCoy's 5a (ATCC 30-2007) containing 10% v/v fetal calf serum (Sigma F4135), A549: human lung carcinoma; media: F12 Ham (Sigma N4888) containing 10% v/v fetal calf serum (Sigma F4135) and 2mM L-glutamine (Sigma G7513) (F12 complete media). DLD-1: human colorectal carcinoma cell line; media: RPMI (Sigma R5886) containing 10% v/v fetal calf serum (Sigma F4135), 1mM sodium pyruvate (Sigma 8636), and 2mM L-glutamine (Sigma G7513). SW48: human colorectal carcinoma cell line; media: Dulbecco's Modified Eagle's Medium (Sigma D5671) containing 10% v/v fetal calf serum (Sigma F4135), 1mM sodium pyruvate (Sigma 8636), and 2mM L-glutamine (Sigma G7513) (DMEM complete media).

### Zinc-Finger Nuclease Design and Nucleofection

Sigma-Aldrich uses a proprietary algorithm to design and assemble ZFN molecules. The assembled ZFNs are tested for DNA binding specificity and for ability to cleave at the target site on the chromosome. A pair of ZFNs is required as the endonuclease FokI must dimerize in order to catalize cleavage of double-stranded DNA. The ZFNs were nucleofected into the cells as plasmids (Lonza, Nucleofection Kit V or R).

### Analysis of ZFN Activity

The mismatch-specific nuclease assay, CEL-I (Transgenomics Surveyor Nuclease Kit), was used to confirm ZFN activity. PCR was used to amplify wild-type and mutant DNA in the region of ZFN binding. The DNA was denatured and re-annealed such that the annealing of wild-type DNA strand to a mutant strand will create a mismatch "bubble" that will be recognized and cleaved by CEL-I. The cleavage products were detected by acrylamide gel electrophoresis.

### Generation and Genotyping of Clones

Cells were plated using a limiting dilution protocol, such that statistically one third of a cell was deposited per well in 96-well plate. The resulting clonal cell lines were perpetuated in complete media and genomic DNA isolated to perform CEL-I assay as above. Wild-type PCR amplicon was mixed with clonal amplicon to ensure heteroduplex formation in the presence of a complete homologous knockout. PCR product from CEL-I positive clones was sequenced (Sigma Analytical Lab) to confirm deletion and determine number of affected alleles.

### Fluorescent Reporter Tagged Loci

Fluorescent reporter genes were obtained from Evrogen (http://evrogen.com/products/TagFPs.shtml). Donor plasmids were designed and constructed in house. Fluorescent microscopy was performed with a Nikon Eclipse TE2000-E inverted research microscope and MetaMorph ® software.

### Induction of apoptosis and Annexin V cell staining

DLD1 cells wt, BAX knockout, or BAX BAK double knockout cells were treated with staurosporine (Sigma 55921) at 0µM, 2µM, or 4µM concentration for 2 hours. Annexin V-FITC Apoptosis Detection Kit (Sigma APOAF) was used to stain the cells prior to flow cytometry. The flow cytometry analysis software, FlowjoTM, was used to plot results.

## ZFN targeting mechanism and donor design.

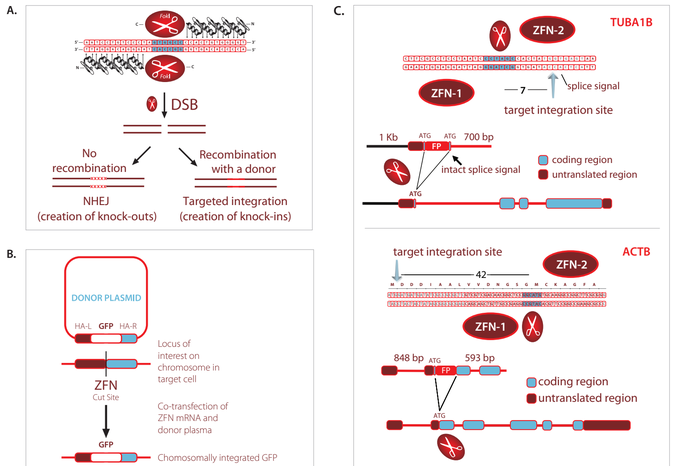



**Figure 1: ZFN targeting mechanism and donor design.**

**A.** ZFNs bind to the target site. Then the FokI endonuclease domain dimerizes and makes a double strand break between the binding sites. DSBs are repaired by either an error-prone non-homologous end joining (NHEJ) pathway or high-fidelity homologous recombination. NHEJ introduces deletions or insertions, which change the spacing between the binding sites such 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.** 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).

**C.** 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 (HMGA1, High Mobility Group protein HMG-I/HMG-Y isoform A (AT-hook) - a non-histone dsDNA binding protein) | Nucleus (DNA) | 6 | C | 56 | 0.2 % |

## Successfully Tagged Loci

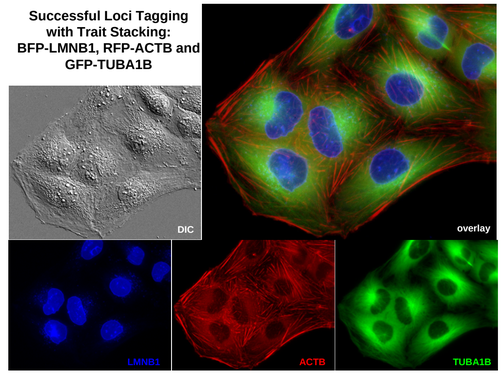



**Figure 2: Trait Stacking.** U-2 OS cells were modified by ZFNs on three loci leading to the simultaneous expression of RFP-ACTB, BFP-LMBN1 and GFP-TUBA1B .

## Compound Screening

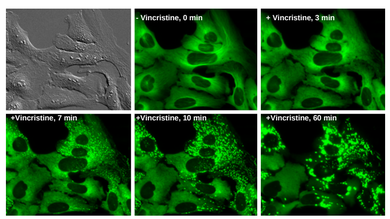



**Figure 3: Vincristine Time Course.** Vincristine is a mitotic inhibitor used in cancer chemotherapy. Its mode of action is to bind to tubulin dimers thereby inhibiting the assembly of microtubule structures.[1] GFP tagged TUBA1B U-2 OS cells were exposed to 20 µM Vincristine for sixty minutes. As time progresses, tubulin is repolymerized into a crystalline structure.

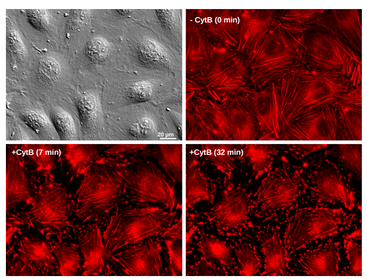


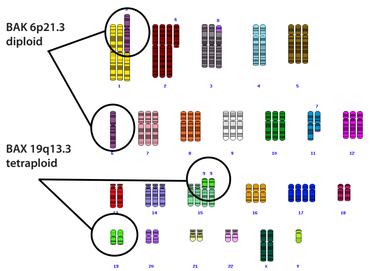

**Figure 4: Cytochalasin B Time Course.** Cytochalasin B is a mycotoxin. It blocks the formation of contractile microfilaments thus inhibiting cytoplasmic division[2]. By blocking monomer addition actin filaments are shortened. RFP tagged ACTB U-2 OS cells were exposed to 21 µM Cytochalisin B. Over time, shortening of actin filaments can be observed.

## Product Offerings

Osteosarcoma Cell Line with GFP-tagged α-tubulin 1b (CLL1031)
Osteosarcoma Cell Line with GFP-tagged β-actin (CLL1032)
Osteosarcoma Cell Line with GFP-tagged LaminB1 (CLL1033)
Osteosarcoma Cell Line with RFP-tagged α-tubulin 1b (CLL1034)
Osteosarcoma Cell Line with RFP-tagged β-actin (CLL1035)
A549 Cell Line BAX -/-/-/- (CLL1017)
A549 Cell Line BAX/BAK (-/-/-/-,-/-) (CLL1019)
DLD1 Cell Line BAX -/- (CLL1006)
DLD1 Cell Line BAX/BAK (-/-,-/-) (CLL1007)

**For additional cell line offerings, please visit**
**wherebiobegins.com/biocells**

## Knockout Cell Lines




Spectral Karyotyping of A549 cells

**A549 BAX (-/-/-/-) BAK (-/-)**

```
BAX locus
TGCACCAAGGTGCCGGAACTGATCAGAACCATCATGGGCTGGACATTGGACTT wt
TGCACCAAGGTGCCGGAACTGATCA--ACCATCATGGGCTGGACATTGGACTT -2
TGCACCAAGGTGCCGGAACTGA----AACCATCATGGGCTGGACATTGGACTT -4
TGCACCAAGGTGCCGGAACTGA-------CATCATGGGCTGGACATTGGACTT -7
TGCACCAAGGTGCCGGA----------------CTGGACATTGGACTT -21

BAK locus
TCCTGCTCCTACAGCACCATGGGGCAGGTGGGACGGCAGCTCGCCATCATCGG wt
TCCTGCTCCTACAGCACCATGGGGCAGG----ACGGCAGCTCGCCATCATCGG -4
TCCTGCTCCTAC----------------GGCAGCTCGCCATCATCGG -22
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**DLD1 BAX (-/-) BAK (-/-)**

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BAX locus (homologous deletion 63bp)
GTGCACCAAGGTGCCGGAACTGATCAGAACCATCATGGGCTGGACATTGGACTTCCTCCGGGGA wt
GT----------------------------------------------------------A -63

BAK locus (homologous deletion 38bp)
TGCATGCCTCCTGCTCCTACAGCACCATGGGGCAGGTGGGACGGCAGCTCGCCATCATCGGGG wt
TGCATGCCTCCTGC----------------------------------CATCATCGGGG -38
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**Figure 5: A549 with Tetraploid BAX Knockout.** Many cell lines are aneuploid and may contain multiple (>2) copies of the target gene. This is not a barrier for ZFN technology. The spectral karyotyping of A549 cell lines is shown. The BAX gene is tetraploid, and BAK gene diploid in A549. Following treatment with ZFNs specific for BAX and BAK, a double knock-out clone containing a unique disruption in all four alleles of BAX and both alleles of BAK, was isolated.

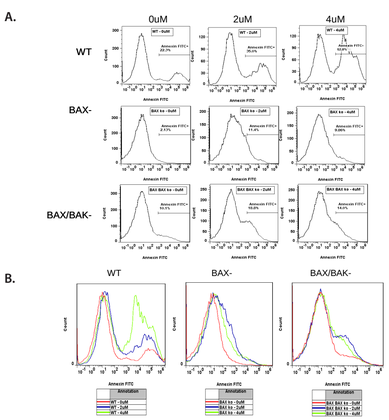


**Figure 6: Induction of Apoptosis and Annexin V Cell Staining.** DLD1 cells, wt, BAX knockout, or BAX BAK double knockout, were treated with the apoptosis effector staurosporine at 0µM, 2µM, or 4µM concentration. Cell survival was determined by staining with Annexin V-FITC and analysis by flow cytometry. The results are shown for the individual populations (A) and overlaid (B). The wild-type cells exhibit a significant shift (B-WT) in Annexin V staining with the addition of staurosporine indicating entry into apoptosis. The BAX knockout cell population exhibits a lesser shift (B-BAX-) in Annexin V staining when 2µM or 4µM staurosporine is introduced. The bulk of the BAX BAK double knockout population does not shift with the addition of staurosporine indicating resistance to entry into apoptosis (B-BAX/BAK-). The observed increase in Annexin V staining in the BAX BAK knockout (A-BAX/BAK-) is likely to be a function of alternate pathways independent of BAX and BAK.

## Discussion/Conclusion

Homologous recombination (HR) is a common tool for genome modification. Our results indicate that HR via ZFN technology is a rapid and reliable method to generate knock-in and knock-out immortalized cells lines.

Our work demonstrates the swift construction of genetically defined cell lines. Two pro-apoptotic regulators, Bcl2–associated X protein, BAX, and Bcl2-like protein, BAK, genes were knocked out using ZFNs in two cell lines, A549 (human lung carcinoma) and DLD-1 (human colorectal cancer). In all cases, including A549 where BAX is tetraploid, knockout lines were successfully generated. An immunometric assay was used to confirm that BAX protein is not produced in the complete knockout cell lines, and protein production was decreased in partial knockout cell lines (data not shown). Cell lines were treated with the apoptotic effector staurosporine, stained with Annexin V and analyzed by flow cytometry. The knockout cell lines were impaired in the induction of apoptosis compared to the wild type cell lines.

We have successfully tagged three individual loci within the same cell line: TUBA1B (α-tubulin 1b, microtubule), ACTB (β-actin, actin stress fibers), and LMNB1 (lamin B1, nuclear envelope). Until now, fluorescence detection of proteins relied on either exogenous promoters or immuno-techniques requiring cell fixation. With 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.

Future work includes the generation of gene-specific knock out cell lines to better understand various types of cancer and the study of cellular processes, compound screening, and cell-based assay development.

## Endnotes/References

1. Lobert S; Vulevic B; Correia JJ. (1996) "Interaction of vinca alkaloids with tubulin: A comparison of vinblastine, vincristine, and vinorelbine". Biochemistry 35(21): 6806 – 14.

2. Theodoropoulos, PA; Gravanis, A; Tsapara, A; Margioris, AN; Papadogiorgaki, E; Galanopoulos, V; Stournaras, C (1994). "Cytochalasin B may shorten actin filaments by a mechanism independent of barbed end capping". Biochemical pharmacology 47 (10): 1875–81.



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