

# Development of Novel Knock-In Cell Lines with Target Genes Endogenously Tagged by Fluorescent Reporters Utilizing Zinc Finger Nuclease Technology

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## Abstract

Fluorescent protein (FP) tagging is extensively used to provide a visual readout on the protein of interest in the cell. However, current methods of expression often rely on heterologous promoters that result in distorted regulation and expression pattern. Thus, there exists a strong need for a method that can direct specific integration of a reporter into the genome to produce a cell line expressing the corresponding fusion protein controlled by endogenous regulatory pathways.

Zinc finger nucleases (ZFNs) are synthetic proteins engineered to bind DNA at a sequence-specific location and create a double strand break (DSB). The cell then employs the natural DNA repair mechanisms of either error-prone non-homologous end joining (NHEJ) or high-fidelity homologous recombination (HR). We relied on the second DSB repair pathway - HR that enables insertion of a transgene into the targeted region. To utilize HR, a donor template is used that contains the transgene flanked by sequences homologous to the regions on either side of the cleavage site. This donor is co-delivered into the cell along with the ZFNs to fool the cell by presenting the donor in place of the sister chromatid to repair the cut.

By this approach, we tagged cytoskeletal and chromatin genes by integrating a FP sequence into the desired location in the genome. The integration resulted in endogenous expression of the corresponding fusion proteins that show their native characteristic pattern. Four gene loci were tagged: TUBA1B (alpha-tubulin 1b, microtubule), ACTB (beta-actin, actin stress fibers), LMNB1 (lamin B1, nuclear envelope) and HMGA1 (high mobility group AT-hook 1, nucleus). Single cell clones were isolated in U2OS cells with at least one copy of a given gene tagged. Multiplexing was demonstrated by labeling different genes in the same cell line as well as different alleles of the same gene. ZFN mediated gene tagging in knock-in cell lines could provide the basis for development of various high content screening (HCS) assays for compound screening where target gene regulation and corresponding protein function are preserved.

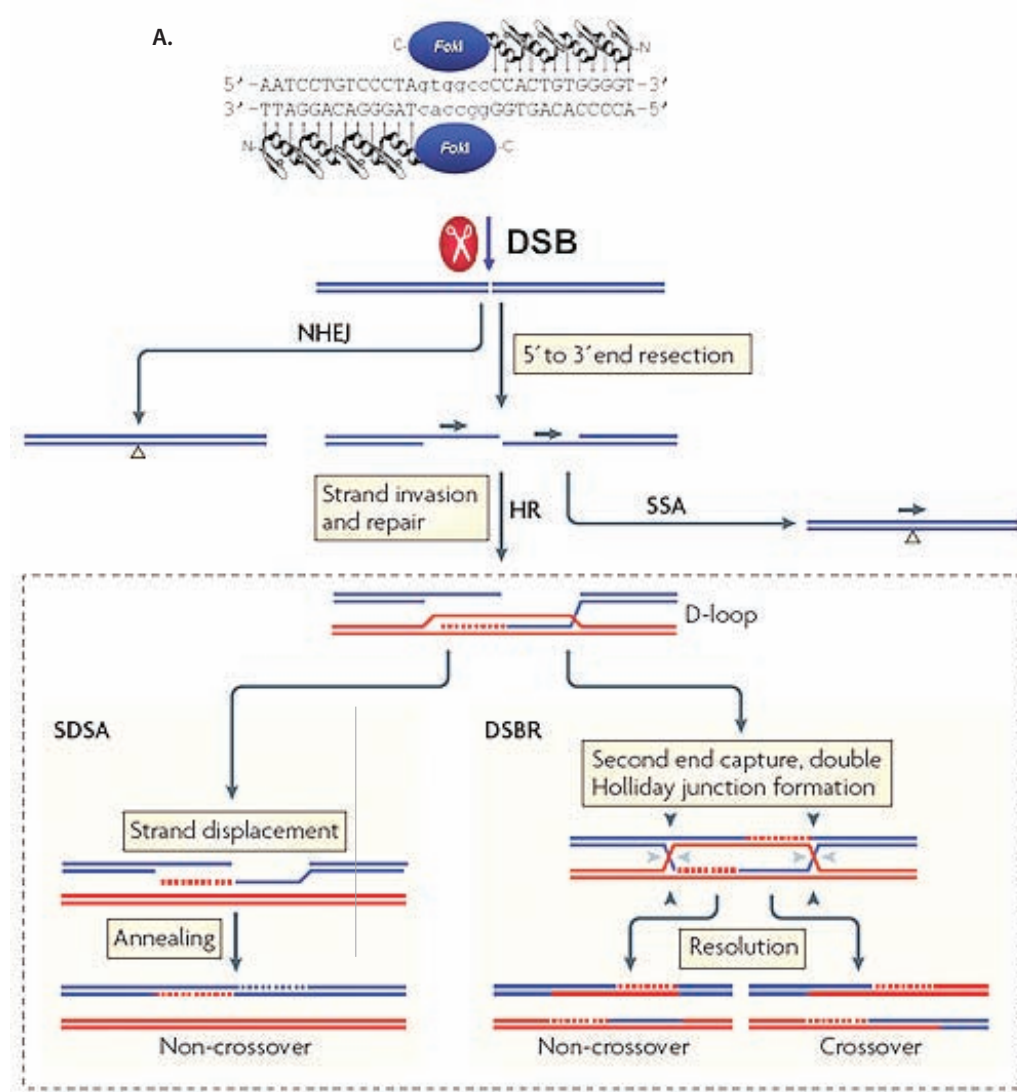
## Introduction

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 DSBs. Thus, 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 HR between a specific genomic target and a given donor plasmid.

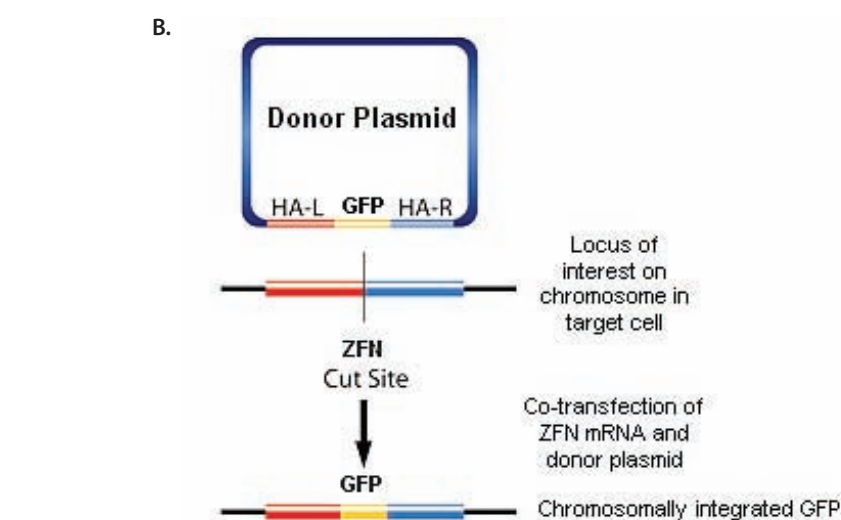
## Materials and Methods

- U2OS (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.
- The cells were imaged with an automated Nikon TE2000-E inverted microscope. BFP: ex 395-410 / em 430-480, GFP: ex 450-490 / em 500-550, RFP: ex 530-560 / em 590-650, 40x/1.4 oil. MetaMorph® was used for image analysis.
- 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



Adapted from Moynahan, M. E. and M. Jasin (2010) *Nat Rev Mol Cell Biol* 11(3): 196-207



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

**A:** ZFNs bind to the target site. Then the FokI endonuclease domain dimerizes and makes a DSB between the binding sites. DSBs are repaired by either an error-prone NHEJ pathway or high fidelity HR. 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.

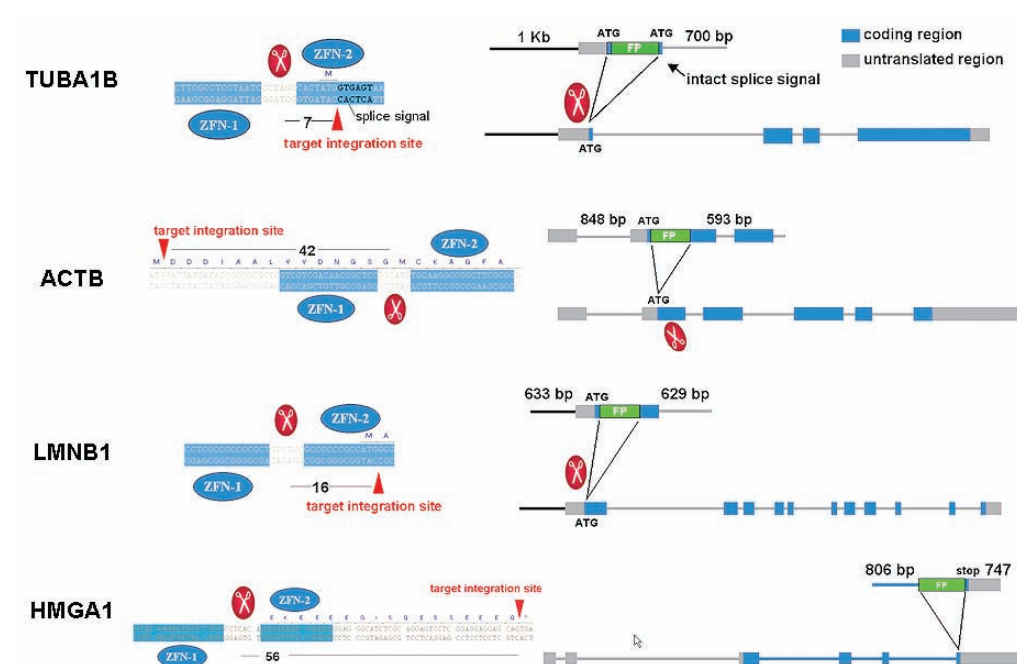
**B:** In the presence of a donor DNA carrying homologous arms (HA-L and HA-R) of the ZFN cut site flanking a FP sequence, HR can use the donor as template to repair a DSB, achieving targeted integration.

## Results

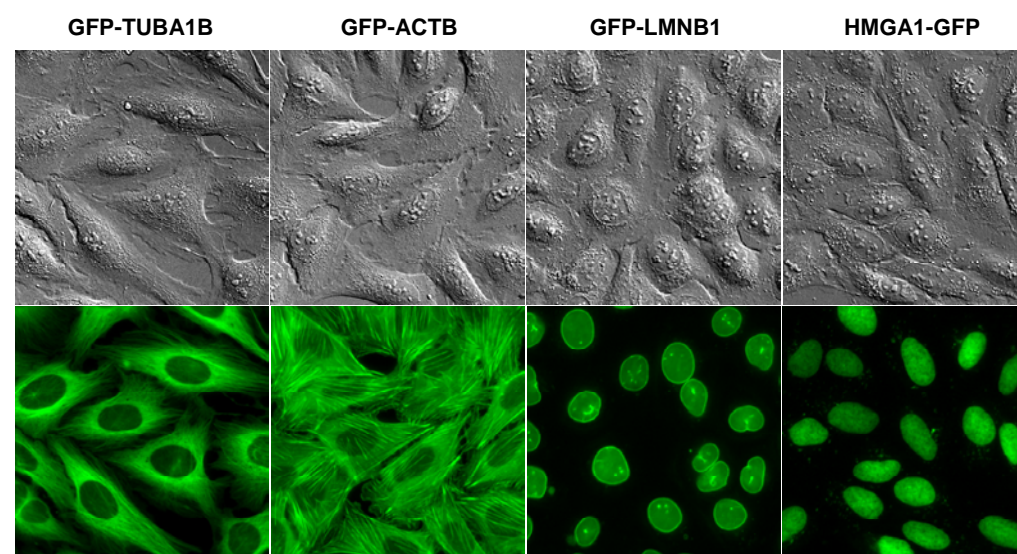
### Successfully Tagged Loci

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 HMGA/HMG-Y isoform A (AT-hook) - a non-histone dsDNA binding protein)	Nucleus (DNA)	6	C	56	0.2 %

**Table 1:** Summary of Tagged Loci in human genome.

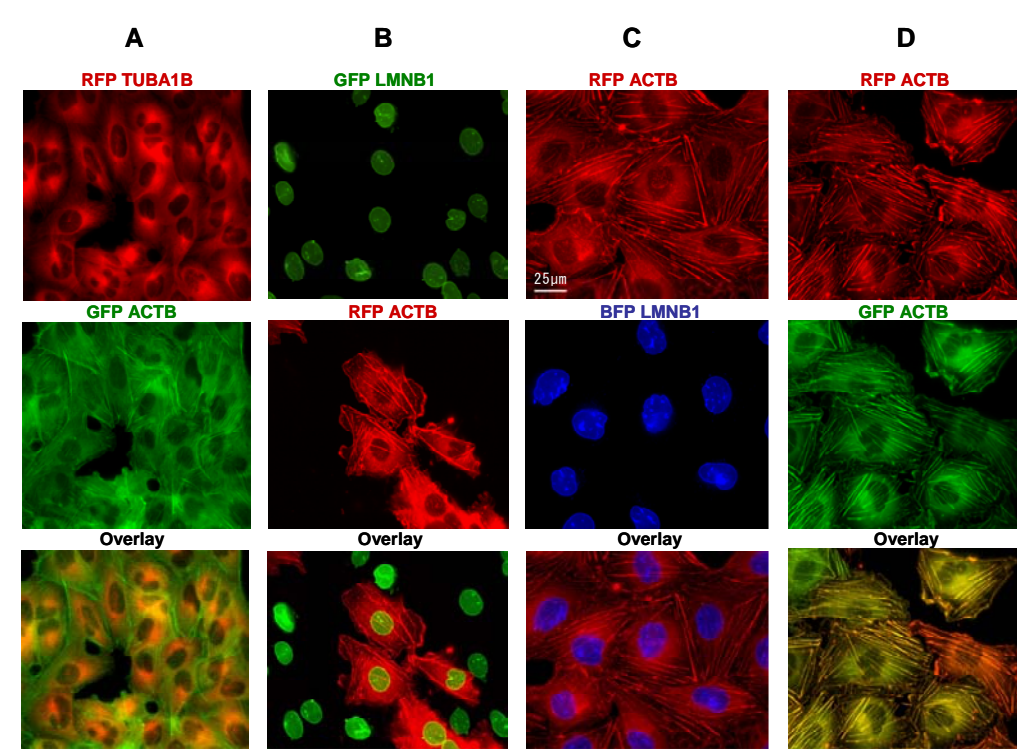


**Figure 2:** Schematics of the four tagged loci showing ZFN binding sites / ZFN cut site with respect to the targeted integration site. The Donor (top) has the homology arms of indicated length and the FP sequence (green).



**Figure 3:** Differential interference contrast (DIC) and fluorescence microscopy images of isolated single cell clones each expressing one of the four genes endogenously tagged with GFP: TUBA1B (α-tubulin 1b, microtubule), ACTB (β-actin, actin stress fibers), LMNB1 (lamin B1, nuclear envelope) and HMGA1 (high mobility group AT-hook 1, nucleus).

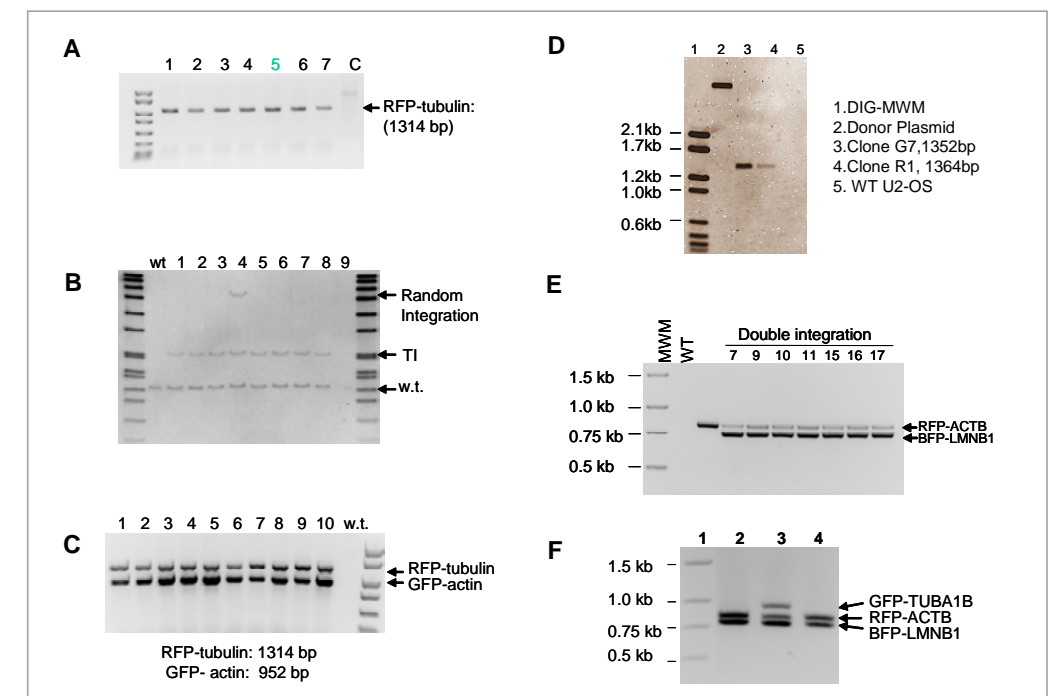
## Trait Stacking



**Figure 4:** Trait Stacking

- RFP-TUBA1B expressing cell line was modified with ZFNs to also express GFP-ACTB.
- GFP-LMNB1 expressing cell line was modified with ZFNs to also express RFP-ACTB.
- RFP-ACTB expressing cell line was modified with ZFNs to also express BFP-LMNB1.
- The ACTB gene has 3 alleles in the U2OS 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.
- U2OS cells expressing both RFP-ACTB and BFP-LMNB1 described in C were further modified with ZFNs to express GFP-TUBA1B.

## Molecular Analysis



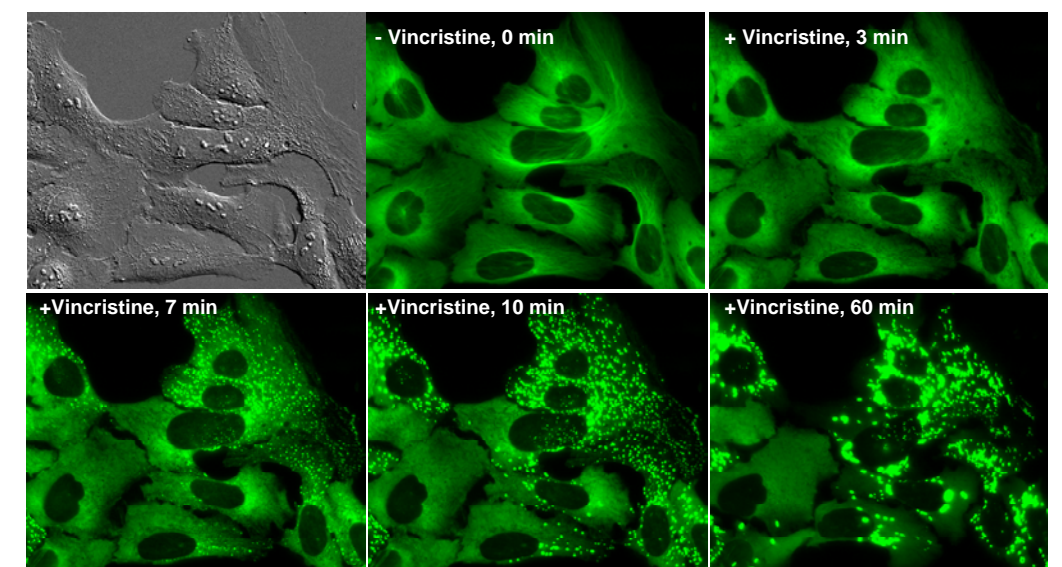
**Figure 5:** Molecular Analysis

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.

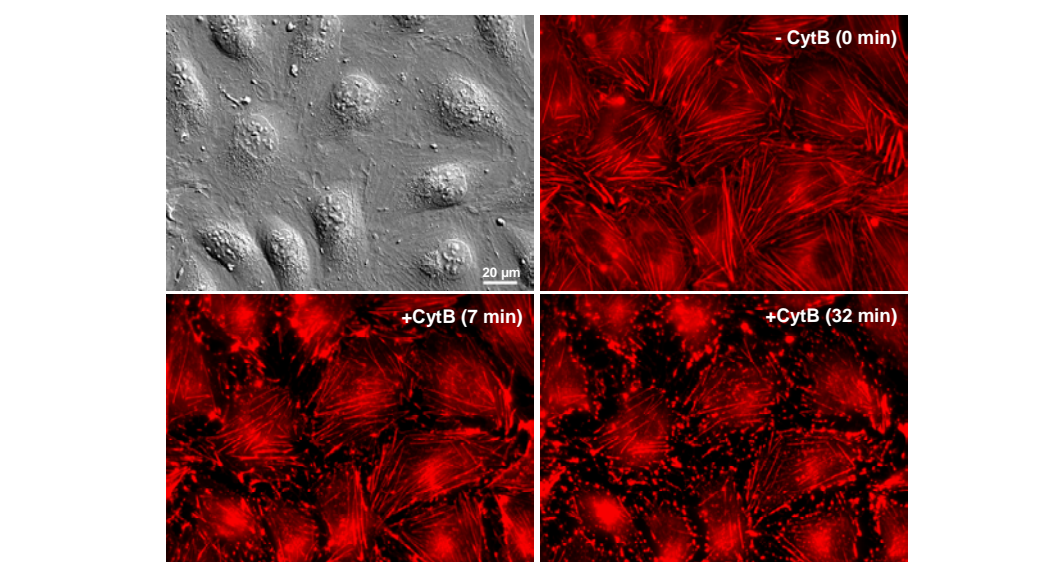
**Left panel: Identification of RFP-TUBA1B clones and subsequent trait stacking.**

- Junction PCR amplification. All 7 clones contained the characteristic integration band at 1314 bp. C is the donor-only control.
- Southern hybridizations were performed on PstI digested genomic DNA isolated from wild type U2OS and nine single cell clones positive for red tubulin fluorescence. Using a DIG labeled tubulin probe, a 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. Out of 9 TUBA1B-RFP clones, only clone #4 contained random integration.
- 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. They were not detected in wild-type control.
- 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. Clone G7 is for GFP and clone R1 is for RFP.
- Junction PCR to confirm double knock-in with RFP-ACTB and BFP-LMNB1 (see Figure 3C). The integration bands for both RFP-ACTB and BFP-LMNB1 were amplified simultaneously in selected clones, whereas only RFP-ACTB can be detected in cells with only RFP-tagged ACTB and no band can be detected in wild-type control.
- Triple knock-in (see Figure 3E) was confirmed by junction PCR. GFP-TUBA1B TI was done in cells that were already RFP-ACTB and BFP-LMNB1 tagged. 1- Marker; 2 - RFP-ACTB / BFP-LMNB1 double knock-in; 3 - GFP-positive sorted triple tagged population; 4: unsorted cells after the third TI.

## Small Molecule Effect



**Figure 6:** Vincristine effect on microtubules. 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. GFP tagged TUBA1B U2OS cells were exposed to 20 μM Vincristine for sixty minutes. As time progressed, tubulin is repolymerized into a crystalline structure.



**Figure 7:** Cytochalasin B effect on actin polymerization. Cytochalasin B is a mycotoxin. It blocks the formation of contractile microfilaments thus inhibiting cytoplasmic division. By blocking monomer addition actin filaments are shortened. RFP tagged ACTB U2OS cells were exposed to 21 μM Cytochalasin B. Over time, shortening of actin filaments can be observed.

## Conclusions

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.

This work shows successful tagging of four individual loci: TUBA1B (α-tubulin 1b, microtubule), ACTB (β-actin, actin stress fibers), LMNB1 (lamin B1, nuclear envelope) and HMGA1 (high mobility group AT-hook 1, nucleus). Labeling of three different genes in the same cell line as well as two different alleles of the same gene are also demonstrated. Future work includes the study of cellular processes, compound screening, and cell-based assay development.

## 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)

## Acknowledgements

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