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TITLE: Multiple Cooperating Oncogenes Drive Recurrent Breast Cancer-Associated Chromosomal Amplifications: Creation of Isogenic Human Cell Line Models

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| 14. ABSTRACT<br><br>Human breast cancers, like other cancers, frequently harbor amplifications of chromosomal regions containing multiple genes. Many such multi-gene amplifications are recurrent across many individual instances of breast cancer, suggesting they contain oncogenes critical for breast cancer development or progression. In some cases, traditional laboratory methods have identified more than one candidate "driver" oncogene within such amplified regions. This research proposal is designed to test the hypothesis that some recurrent amplicons actually function through the concerted action of multiple simultaneously overexpressed oncogenes. We are testing this hypothesis by attempting to create cell line models of such multigene amplifications, prospectively. We have designed an experimental strategy to target a dominantly selectable IMPDH gene to specific chromosomal regions using gene targeting technology. Targeted cells will then be selected in increasing concentrations of the IMPDH inhibitors mycophenolic acid and mizoribine to provide selective pressure for the targeted cells to amplify the targeted region, co-amplifying multiple genes simultaneously. Here we report the design of a functional, efficient gene targeting vector and the successful delivery of the IMPDH selection marker to two different chromosomal loci, ZNF703 and ERBB2, in two different human breast cancer cell lines, MCF-7, and T47D. In MCF-7, we have succeeded in creating subclones with focal, overlapping amplification of regions of chromosome 8p11-12, where ZNF703 is located. We demonstrate amplification by array CGH, digital PCR, and fluorescent in situ hybridization. Amplification is shown to cause coordinate overexpression of co-amplified genes at the mRNA and protein level. |             |                         |                            |                                           |                                           |
| 15. SUBJECT TERMS<br>Isogenic cell lines, chromosomal amplifications, oncogenes, gene targeting                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |             |                         |                            |                                           |                                           |
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## Introduction

Targeted therapy for cancer is based upon the notion that tumors have characteristics that are quantitatively or qualitatively different from normal tissues, or that tumor cells are uniquely dependent, or “addicted”, to certain of these characteristic differences. Genetic changes including somatic mutation, gene amplification, and chromosomal translocation often serve as irreversible drivers of tumor growth and survival—making them attractive targets for therapy. The amplification of ERBB2/Her-2 on chromosome 17q12 in 15% of breast cancers led to the identification of Her-2 as a driver oncogene and successful target for therapy. Other chromosomal regions are recurrently amplified in breast cancer almost as frequently as 17q12: these include 8p11-12 and 11q13. Amplification of 8p11-12 has been shown to confer a high risk of metastasis after primary breast cancer surgery and adjuvant therapy, and it is therefore critical to identify the oncogene(s) driving this aggressive behavior. The identification of a single driver gene in each of these amplicons has been difficult. There is experimental evidence supporting driver gene function for at least six genes in the 8p11-12 amplicon. We hypothesize that many recurrent cancer-associated genomic amplifications encompass multiple genes which *cooperate* as drivers of tumorigenic phenotypes. The ability to model complex genomic events leading to *simultaneous* dysregulation of multiple genes will more accurately represent the biology of these genomic aberrations and lead to more specific targeted therapies. We propose to develop human breast cancer cell line models of common, recurrent breast cancer-associated chromosomal amplification events. We will use adeno-associated virus mediated gene targeting through homologous recombination to introduce a mutant dihydrofolate reductase (DHFR) gene at specific genomic loci, which are sites of frequent, recurrent amplification in human breast cancers. These sites include chromosome bands 8p11-12, 11q13, and 17q12. After identifying homologously targeted clones of cells, we will expose cells to stepwise increasing concentrations of methotrexate to induce amplification of the mutant DHFR locus, which can act as a dominant selectable marker in the presence of wild-type DHFR. Cells that have amplified the mutant DHFR cassette will be assayed for co-amplification of genes in the targeted locus. These amplified cell lines will be compared with their isogenic controls for in vitro and in vivo phenotypes of growth, cellular transformation, and drug resistance.

**Keywords**

Isogenic cell lines, chromosomal amplifications, oncogenes, gene targeting

## Overall Project Summary

### **Aim1. To use somatic cell gene targeting technology to model recurrent breast cancer genomic amplifications in non-tumorigenic human breast epithelial cells and human breast cancer cell lines.**

The objective of this aim is to create derivatives of the MCF10A and MCF7 cell lines with experimentally induced chromosomal amplifications at sites that are recurrently amplified in human breast cancers. Completion of the proposed tasks will result in isogenic cell lines differing in amplification of 8p11-12 or 17q12. In addition we will generate control cells expressing single oncogenes from these chromosomal amplicons.

Timeframe: Months 1-12

Task 1. To create targeting vectors for insertion of the mutant DHFR gene cassette at specific chromosomal locations. We will generate 5' and 3' homology arms from the genomic position to be targeted by PCR using genomic DNA from the cell line to be targeted as a template. Recombinant adeno-associated virus will be produced for each targeting vector by transient transfection of 293T cells.

Month 1

As described in our previous progress reports, this task has taken much longer to accomplish than originally anticipated. Although we were eventually able to target our mutant DHFR/neo selectable marker to several chromosomal loci in three different cell lines, we were unable to establish clones with amplification of the selection cassette. The modest copy number increases we observed in a few clones in our previous report could not be verified on further testing. We were unable to achieve targeting in the mismatch repair-proficient BVEC cells as proposed in our last report. We hypothesized that methotrexate selection was not efficient at amplifying the cassette in our cell lines. As an alternative strategy, we created a new version of the targeting vector with a different selectable marker, E. Coli inosine monophosphate dehydrogenase (IMPDH) (Figure 1). IMPDH is a rate limiting enzyme in de novo purine synthesis. Human cellular IMPDH can be inhibited by mycophenolic acid (MPA). Bacterial IMPDH is orders of magnitude more resistant to MPA than human IMPDH and can therefore serve as a dominant selectable marker<sup>1</sup>. We subsequently designed and investigated a number of other targeting vectors using different dominantly selectable and potentially amplifiable markers. We used various alternative versions of L23F mutant DHFR fused to neoR protein, either via flexible peptide linker or 2A peptide. We were able to target some of these vectors using G418 selection but could not demonstrate expression or activity of the DHFR moiety using two different DHFR antibodies or a 2A-peptide antibody. We concluded that the fusion proteins were inactive as DHFR or that N-terminal placement of the DHFR in the fusion was deleterious to activity. This was likely the explanation for our failure to amplify our original DHFR-neo fusion proteins. Next we tried using E. Coli *gpt*, which can be selected for using a combination of MPA and xanthine as a growth substrate. We successfully targeted constructs with *gpt* to the ZNF703 locus in MCF-7 cells and attempted to amplify them by growing them in HAT medium with MPA and then gradually reducing the amount of xanthine in the medium. We reached a growth limit around 2 micrograms/mL of xanthine but were not able to obtain clones that grew out in these conditions. Next we turned back to our original aim of using the L23F mutant DHFR as our selection marker. However, this time we used it as the selectable marker for targeting as well by replacing the IRES-neoR cassette with an IRES-DHFR L23F cassette. Methotrexate was used as a selection for targeting at a concentration which completely inhibited growth of parental MCF-7 cells (30 nM). We obtained many colonies, indicating that mutant DHFR functions well as a dominant selectable marker in these cells.

Task 2. To identify homologous recombinant cells with targeted insertion of the DHFR cassette. We will employ pooling and PCR-based screening to identify correctly targeted cells. Clones will be infected with Cre-expressing adenovirus to excise the neomycin resistance cassette and single-cell clones will be ensured by limiting dilution.

Months 2-4

We have succeeded in targeting two different chromosomal loci in three different cell lines. In MCF-7 and T47D breast cancer cells, we have successfully targeted the IMPDH gene to the ZNF703 locus on chromosome 8p11-12 (Figure 2 and data not shown). We obtained multiple targeted clones from each of these cell lines, which do not have amplification of this region in the parental cells. We have also succeeded in targeting the IMPDH marker to the ERBB2/Her-2 locus in MCF-7 cells. We have attempted but not yet succeeded in targeting either ZNF703 or ERBB2 in MCF-10A nontumorigenic breast epithelial cells yet, however. Cell types differ in their ability to survive MPA selection, even in the presence of bacterial IMPDH, suggesting their may be functions of human IMPDH that are not complemented by the bacterial gene. Recently, using our DHFR L23F vectors, we have succeeded in targeting the ZNF703 and ERBB2 loci in MCF-7 cells.

Task 3. To amplify the mutant DHFR cassette in targeted cells. This will require isolation of drug resistant cells and culture in stepwise increasing concentrations of methotrexate.

Months 4-9

The use of an alternative selectable marker (IMPDH) required modification of our amplification selection strategy. Since bacterial IMPDH is resistant to MPA, we cannot use increasing MPA doses to select for amplification of our targeted cassette. Instead we developed a dual drug selection strategy. The cells are maintained in MPA to suppress purine synthesis by endogenous cellular IMPDH. A second drug mizoribine inhibits both bacterial and cellular IMPDH. By culturing the cells in both MPA and mizoribine (at increasing concentrations), we can apply selective pressure on the targeted bacterial IMPDH cassette to amplify and overcome the effect of mizoribine. For each clone we have identified a dose range of mizoribine that inhibits growth 50-90%. We then plate the cells in 96 well plates at three to four different concentrations of mizoribine at approximately 1-4 times the IC<sub>50</sub>. With one of the MCF-7 clones targeted at the ZNF703 locus, we observed three subclones with copy number gain, using quantitative droplet digital PCR (Figure 3). In T47D cells, we have six clones that show 2 to 3-fold increase in ZNF703 copy number (Figure 4). We are in the process of selecting and analyzing colonies of MCF-7 cells targeted at ZNF703 that have survived methotrexate selection at 4-8-fold higher concentrations.

Task 4. To characterize mutant DHFR integration sites for co-amplification of adjacent genes using SNP arrays, real time PCR, and microarray gene expression analysis

Months 9-12

We have performed array comparative genomic hybridization (array CGH) on our mizoribine-selected MCF-7 subclones (Figure 5). All show amplification of the targeted ZNF703 locus. In addition they show varying patterns of amplification of adjacent loci. In some cases there are long contiguous regions of copy number gain. In one clone (E8), there is a discontinuous pattern of amplification. Clone G5 shows distal copy number loss telomeric to the region of amplification. This change is expected with the breakage-fusion-bridge mechanism of amplification. All of the patterns we observe in MCF-7 cells have been described in primary breast cancers with amplification of this region. We have confirmed amplification of the targeted region on the single cell level by using fluorescence in situ hybridization (FISH) with probes from the region (Figure 6 and data not shown). We observe overexpression of co-amplified genes by quantitative real time RT-PCR (Figure 7) and of the encoded proteins by western blotting (Figure 8). FISH characterization of the T47D-ZNF703 targeted shows equal increases in signal for the ZNF703 or FGFR1 probe on 8p12 and the chromosome 8 centromeric probe, suggesting that in T47D cells we have selected for whole chromosome gain or polyploidy. This will have to be confirmed by analyzing other chromosomes.

Task 5. To generate MCF-10A and MCF7 cell lines overexpressing single candidate oncogenes.

Months 1-6. This work can be done in parallel to the creation of the amplified cell lines.

5a. Subclone full length cDNAs from commercial sources into uniform lentiviral expression vector.

Months 1-3.

5b. Make recombinant lentiviruses, infect cells, and isolate overexpressing clones. Confirm expression by immunoblotting using specific antibodies. Months 4-6.

We have established MCF-7 and MCF-10A cells overexpressing Her-2.

**Aim 2. To compare the effects of specific chromosomal amplifications with the effects of single overexpressed oncogenes in isogenic cell lines pairs using in vitro assays of growth, invasion, drug resistance, and transformation.**

The objective of this aim is to characterize the phenotypic consequences of amplification of multiple genes simultaneously versus overexpression of a single breast cancer oncogene. Phenotypes of cellular transformation relevant to in vivo tumorigenicity include soft agar colony formation, growth factor independence and invasive behavior. We will also assay drug sensitivity using standard chemotherapeutic agents and tamoxifen. Signal transduction analysis will be used to determine mechanisms of effects on proliferation, invasion, and apoptosis. Timeframe: Months 13-18.

Task 1. Perform growth, invasion, and drug sensitivity assays in MCF10A cells, MCF7 cells, and their amplified or single-oncogene-expressing derivatives.

Months 13-18.

Task 2. Perform immunoblotting analyses of signal transduction through the PI3-kinase and MAPK pathways in control and amplified cell lines.

Months 13-18. This work can be performed in parallel with task 1.

We have begun to characterize our recently derived MCF-7 and T47D ZNF703-amplified cells with growth assays in response to estrogen and tamoxifen (Figure 9). One of the amplified clones appears to be growth stimulated by the antiestrogen tamoxifen, which may go along with the poorer response of 8p11-12 amplified breast cancers to hormonal therapy.

**Aim 3. To study the effect of multigene amplification versus single oncogenes on the in vivo growth of MCF7 breast cancer cells in mouse xenografts.**

The objective of this aim is to study the impact of multigene amplification versus the effect of single candidate genes on in vivo tumor growth phenotypes using the well characterized MCF7 cell athymic mouse xenograft model.

Timeframe: Months 16-24 (including 4 months for regulatory approval).

Number of research subjects: 2 experiments. 10 mice per group. 10 groups (2 independent 17q12 amplified MCF7 clones, MCF7 cells with endogenous DHFR amplification, parental MCF7 cells transduced with empty vector, MCF7 cells overexpressing Her-2, 2 independent clones). Two independent clones of 8p11-12 amplified MCF7 cells, 2 11q13 amplified clones, and two controls (MCF7 and MCF7 with endogenous DHFR amplification). 200 mice total. For MCF10A (Task2) 10 mice per group, 8 groups (MCF10A, MCF10A with endogenous DHFR amplification, two clones each amplified for 17q12, 8p11-12, 11q13). 80 mice total.

Task 1. Xenograft studies using MCF7 cell line and derivatives in female athymic nude mice 4-6 weeks old. Estrogen pellet implantation, tumor cell injection, serial caliper measurements of tumor growth. Statistical analysis.

Task 2. Xenograft studies using MCF10A cell line and amplified derivatives in female athymic nude mice 4-6 weeks old. Tumor cell injection, serial caliper measurements of tumor growth. Statistical analysis.

We have held off on performing xenograft experiments until we can amplify ERBB2 in MCF-7 cells, since we have a better understanding of the expected effects of ERBB2 amplification on tumor growth phenotypes. We have not yet been able to amplify loci in MCF-10A cells.



### **Key Research Accomplishments**

1. Successful re-design of gene targeting constructs to introduce the E. Coli IMPDH or L23F mutant DHFR genes at precise locations in the human genome.
2. Demonstration of successful targeting of the IMPDH and L23F DHFR genes to the ERBB2 and ZNF703 chromosomal loci in two different breast cancer cell lines.
3. Conclusive evidence of focal targeted chromosomal amplification of 8p11-12 in MCF-7 cells, and preliminary evidence of targeted amplification in T47D cells.
4. Testing of additional amplification strategies, including DHFR fusion proteins, E. coli gpt, and L23F mutant DHFR.

## Conclusion

The goal of this research is to develop an isogenic cell line model system to prospectively identify the relative contributions of multiple simultaneously amplified oncogenes with the biological effects of overexpression of single candidate oncogenes. In order to accomplish this task, we have modified existing somatic cell gene targeting technology to deliver a bacterial IMPDH gene into cells at a precisely chosen location. This gene was chosen because it can act as a dominant selectable marker in the presence of endogenous cellular IMPDH. We have succeeded in designing novel gene targeting vectors to deliver the IMPDH or L23F DHFR genes to a chosen locus: ZNF703, CCND1, or ERBB2. We have succeeded in targeting these vectors to all three chromosomal loci, which are in regions amplified in human cancers. We have been able to target two different human breast cancer cell lines, MCF-7 and T47D, with these constructs. We have selected these targeted cell clones in mizoribine, to attempt to force amplification of the bacterial IMPDH and co-amplification of the targeted locus and nearby genes. We have conclusive evidence for focal amplification at 8p11-12 in the targeted MCF-7 cells. Further work will be necessary to characterize the phenotypic consequences of these amplification events. The progress described represents a unique achievement, which if successful going forward, will provide a powerful tool to study the cooperativity of co-amplified genes in cancer. In future work, we will extend the number of targeted/amplified loci and the variety of cell lines used. This will allow us to formally test the hypothesis for oncogene cooperativity, which may have implications for drug development strategies targeting chromosomal amplifications in cancers. In addition, our system will allow us to pursue basic questions about the processes that allow amplifications to occur in cancers.

## **Reportable Outcomes**

Cell lines:

MCF-7 breast cancer cells targeted to ZNF703 with IMPDH gene and amplified subclones

T47D breast cancer cells targeted to ZNF703 with IMPDH gene and amplified subclones

## Other achievements

Pending grant funding based on work described in this award:

1R21CA191262-01 (P.I. Loring)

12/1/14-11/30/16

20% (2.4 cal)

National Cancer Institute

Engineering chromosomal amplifications to identify cooperating oncogenic drivers

The project goal is to model recurrent chromosomal amplification events in breast cancer using gene targeting technology and to test the hypothesis that multigene chromosomal amplifications harbor multiple cooperating driver oncogenes.

**Publications, abstracts, and presentations:**

Oral presentations:

6<sup>th</sup> Annual Safeway Breast Cancer Research Retreat, Mt. Washington Conference Center, Baltimore, Maryland. May 17, 2013.

“Identifying breast cancer oncogenes in recurrent chromosomal amplifications”

Breast Cancer Program seminar, Johns Hopkins University Sidney Kimmel Comprehensive Cancer Center, Baltimore, Maryland. 4/29/14.

“Chromosomal amplifications in breast cancer: clinical significance and preclinical modeling”

American Society of Clinical Oncology Annual Meeting

Chicago, Illinois. June 3, 2014

“Use of isogenic cell line models to predict driver mutation effects on drug sensitivity and resistance”

## References

1. Baccam M, Huberman E. Bacterial IMPDH gene used for the selection of mammalian cell transfectants. *Biotechniques*. 2003;34:1220-1222, 1224, 1226 passim.

Appendices

Supporting Data

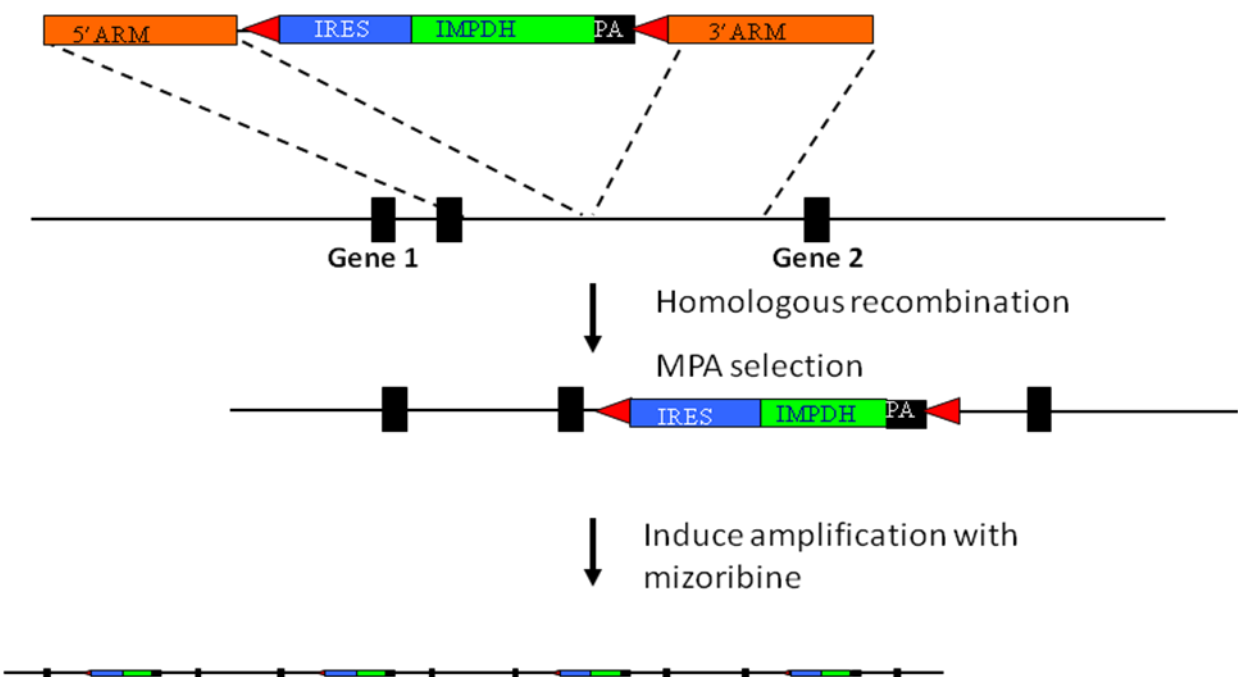


Figure 1. Re-designed bacterial IMPDH gene targeting vector. Triangles represent loxP sites. IRES, internal ribosome entry site. IMPDH, E. Coli IMPDH gene. NEO, neomycin resistance gene. PA, polyadenylation signal.

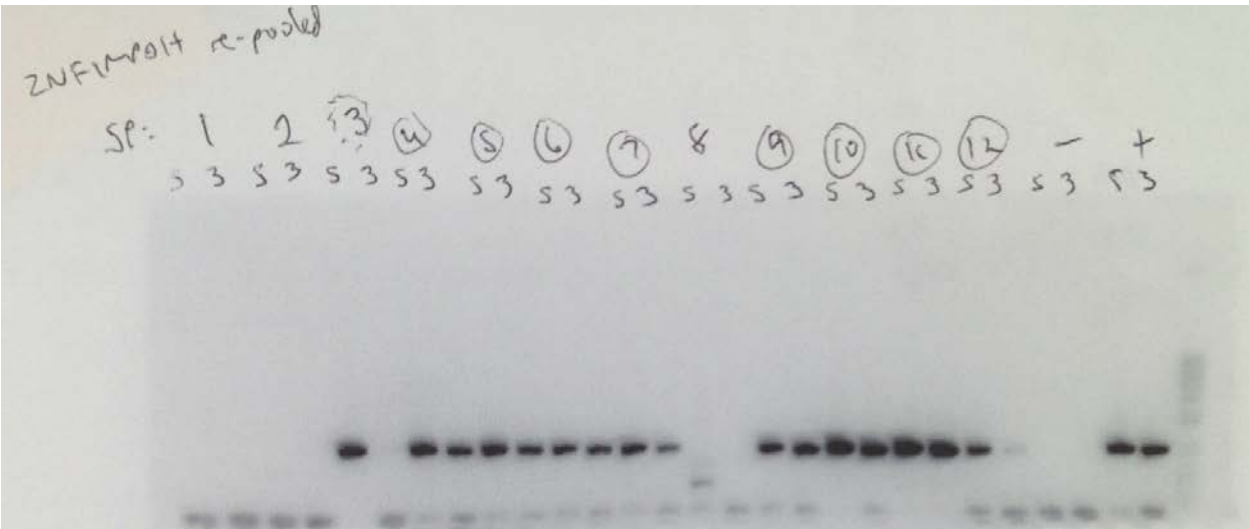


Figure 2. Representative PCR screen for homologous integration of the ZNF703 targeting construct in MCF-7 cells. 5' and 3' screens each use a locus-specific primer that binds to a genomic region distal to the end of the homology arm and a vector specific primer. Depicted are 12 superpools of individual wells with MPA-resistant colonies

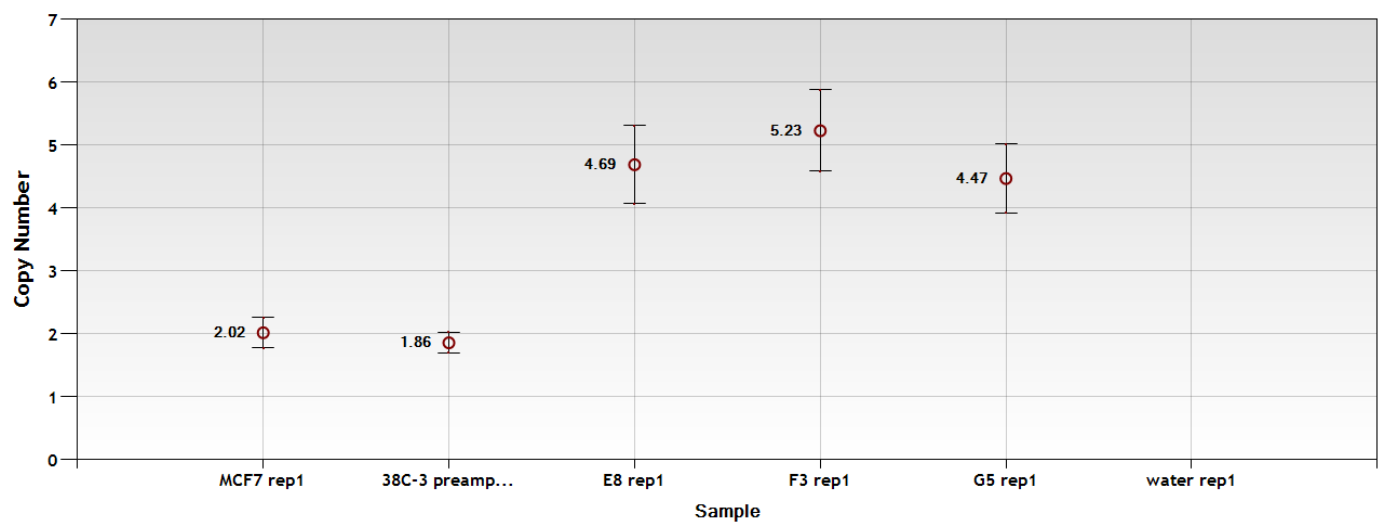


Figure 3. Droplet digital PCR measurement of copy number at the ZNF703 locus with reference to the two copy RPP30 locus. From left to right are parental MCF-7 cells, a targeted clone (38C-3) before mizoribine amplification selection, and three amplified subclones of the 38C-3 targeted clones (E8, F3, G5). The three subclones have an average copy number of ZNF703 that is approximately 2.5-fold higher than the parental clone and non-targeted MCF-7 cells. Bars represent 95% confidence intervals.

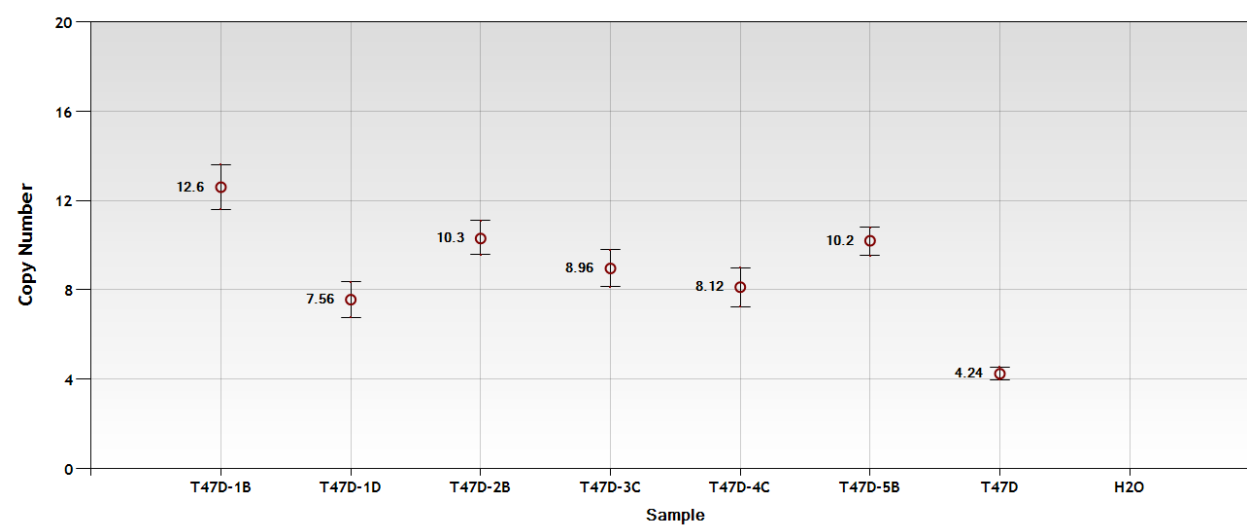


Figure 4. Droplet digital PCR measurement of copy number at the ZNF703 locus with reference to the two copy RPP30 locus. From left to right are six clones of T47D targeted at the ZNF703 locus and parental T47D cells. The targeted clones have an average copy number of ZNF703 that is approximately 2 to 3-fold higher than the parental T47D cells. Note that T47D cells have 4 copies of ZNF703 at baseline, due to tetrasomy for chromosome 8. Bars represent 95% confidence intervals.



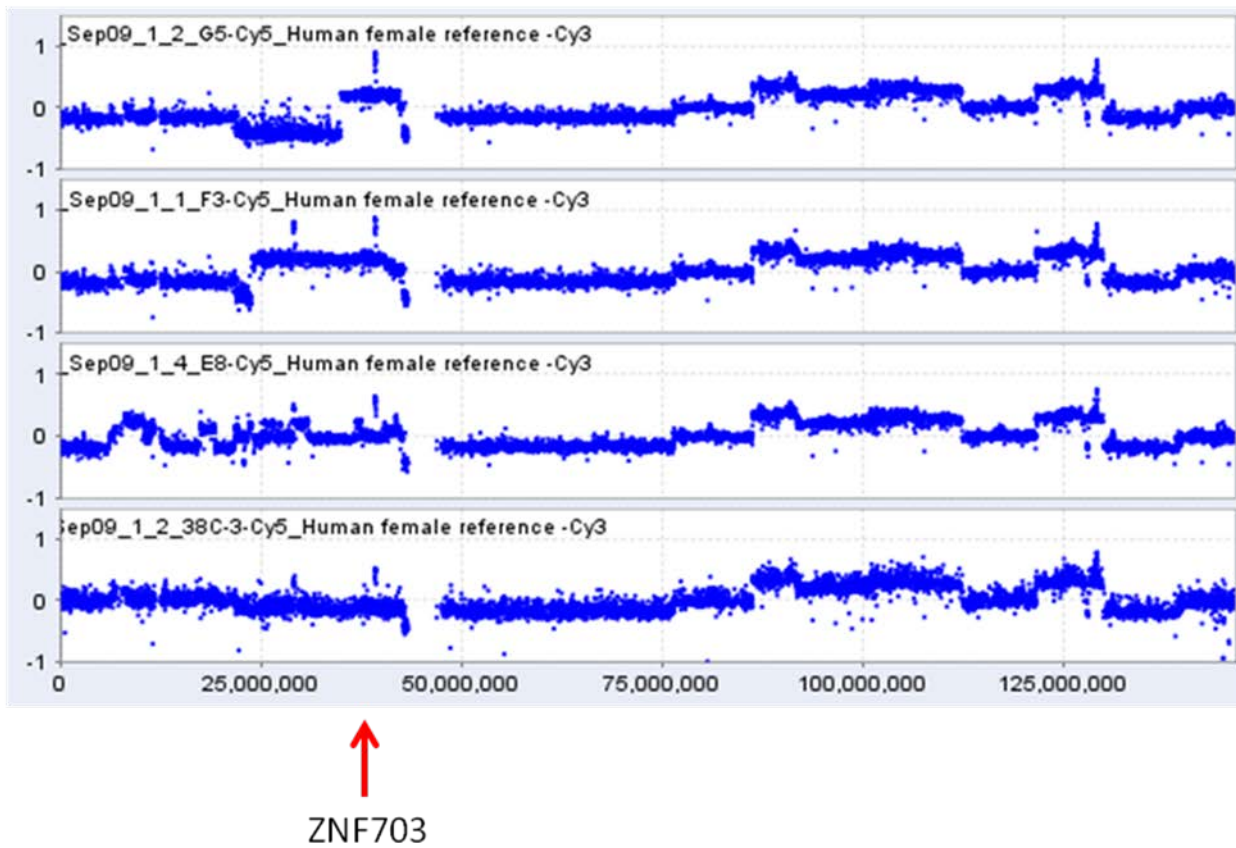
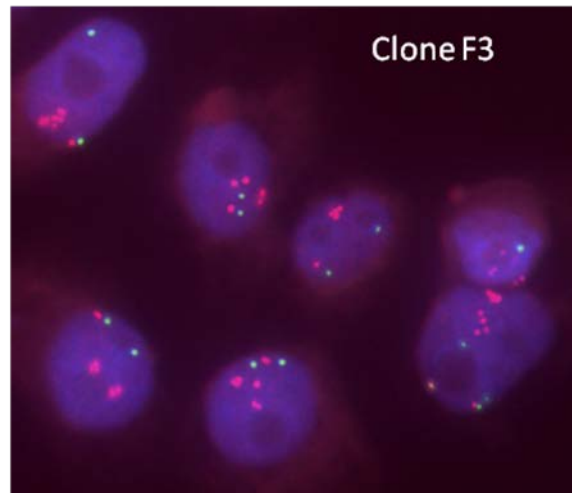
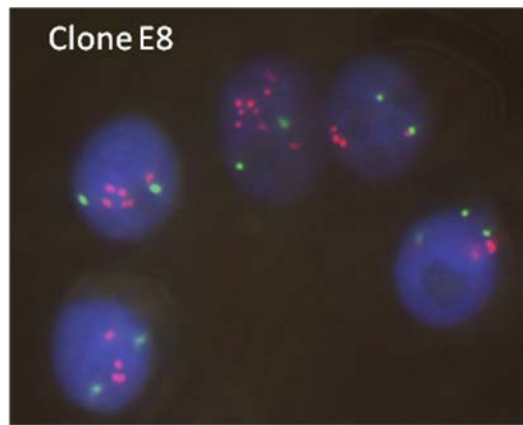
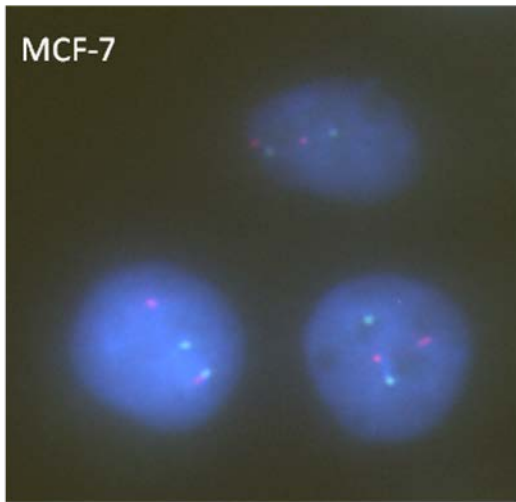


Figure 5. Array CGH of chromosome 8, normalized to copy number of normal human female control genomic DNA. From top to bottom, MCF-7/ZNF703-amplified subclones G5, F3, E8, and the targeted, non-amplified MCF-7 clone 38C3. The y-axis represents  $\log_2$  ratios of copy number, with 0 representing normal diploid copy number. All three subclones show copy number gain around the targeted ZNF703 locus. The size of the amplified region varies between the subclones. F3 has the broadest peak of amplification, whereas E8 has a discontinuous, sawtooth pattern of amplification along the short arm of chromosome 8. Clones G5 and F3 show copy number loss telomeric (to the left) of the amplified region.



Green, cen8  
Red, ZNF703

Figure 6. Interphase FISH on parental MCF-7 cells and amplified ZNF703-targeted subclones E8 and F3. Nuclei are stained with DAPI. The green probe is to chromosome 8 centromeric sequences. The red probe is a BAC in the ZNF703 region on 8p11-12.

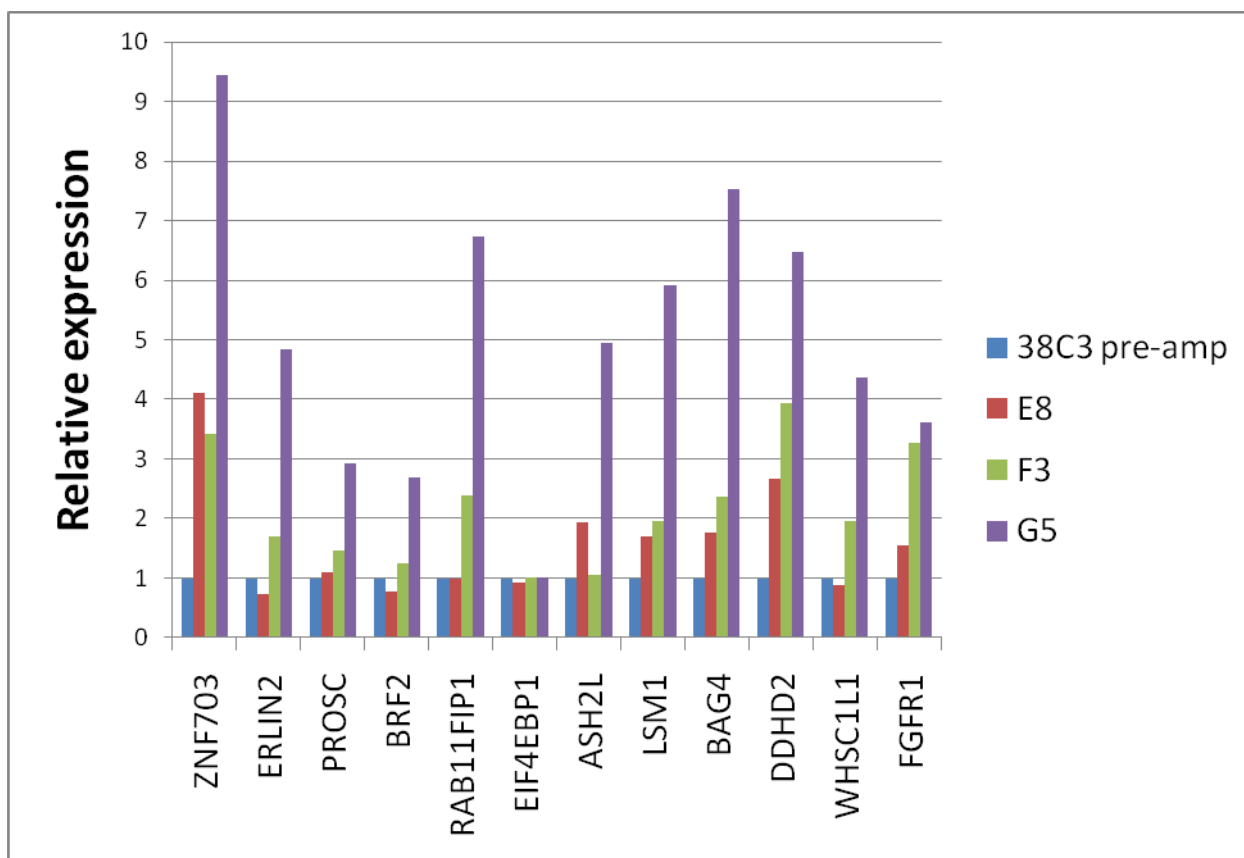


Figure 7. Copy number-associated overexpression of co-amplified genes. Quantitative real time RT-PCR for genes in the 8p11-12 region in their genomic order (ZNF703, telomeric; FGFR1, centromeric). Expression for each gene is normalized to a reference housekeeping gene, TBP. The expression level in the pre-amplified 38C3 clone is set at 1.

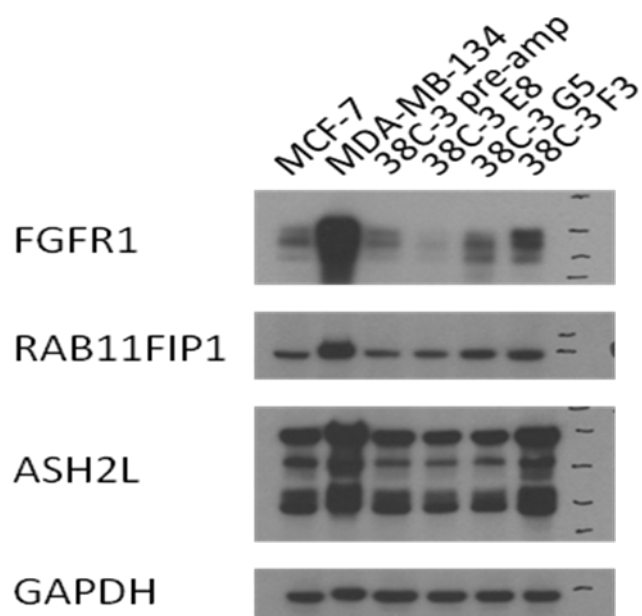


Figure 8. Proteins in the amplified region are overexpressed. Western blot for selected proteins from the 8p11-12 region. Lane 1, MCF-7. Lane 2, MDA-MB-134, a human breast cancer cell line with known amplification of 8p11-12. Lane 3, the targeted, pre-amplified 38C3 clone. Lanes 4-6, amplified subclones derived from 38C3. FGFR1, RAB11FIP1, and ASH2L are genes on 8p11-12 (see Figure 6). GAPDH is used as a reference protein for equal loading.

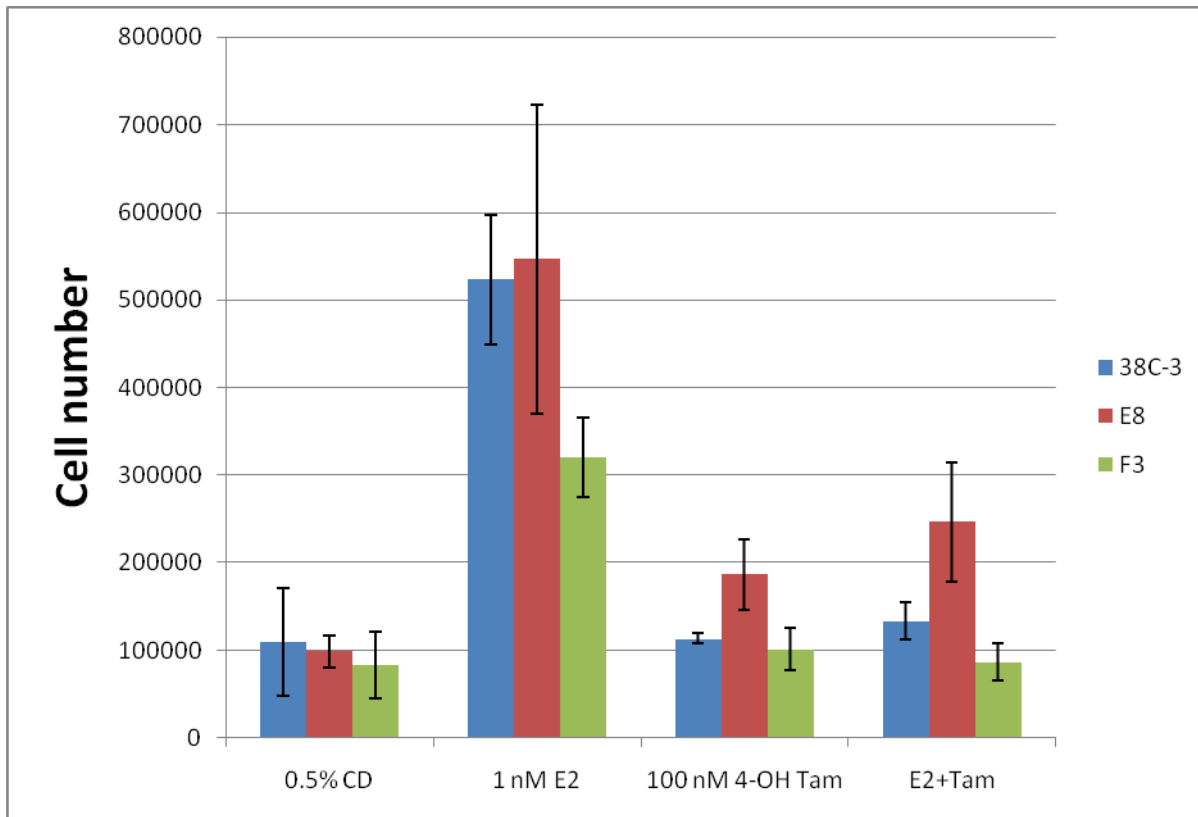


Figure 9. Growth response of 8p11-12 amplified MCF-7 subclones E8 and F3 compared to the pre-amplified 38C3 clone. Cells were grown in low serum (0.5% charcoal dextran treated FBS) with or without supplementation with estrogen (E2) or tamoxifen (4-OH Tam). All clones were growth-stimulated by estrogen, but the E8 clone is also stimulated to grow in response to the antiestrogen tamoxifen.