Optimising Bioproduction Using Zinc Finger Nuclease-Mediated Genome Modification

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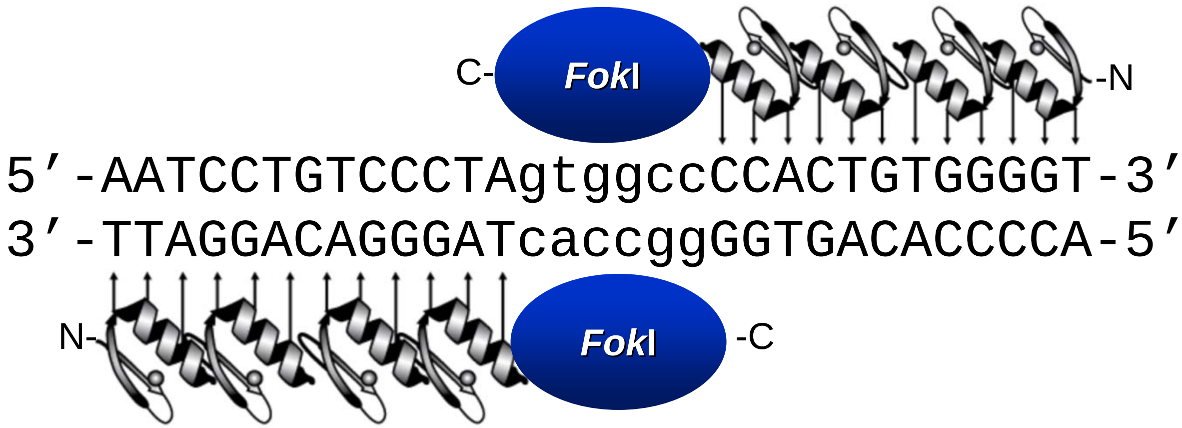
Optimising Bioproduction using ZFNs

Enhancing traits of the host / producer cell line at the level of the *gene*

- Growth & viability
- Glycosylation
- Sialidation
- Auxotrophic selection
- Removal of contaminating / copurifying endogenous proteins
- *All of the above…*

Permanent genetic modification = predictable performance

Zinc Finger Nucleases



ZFN sites are spaced by 5-7bp

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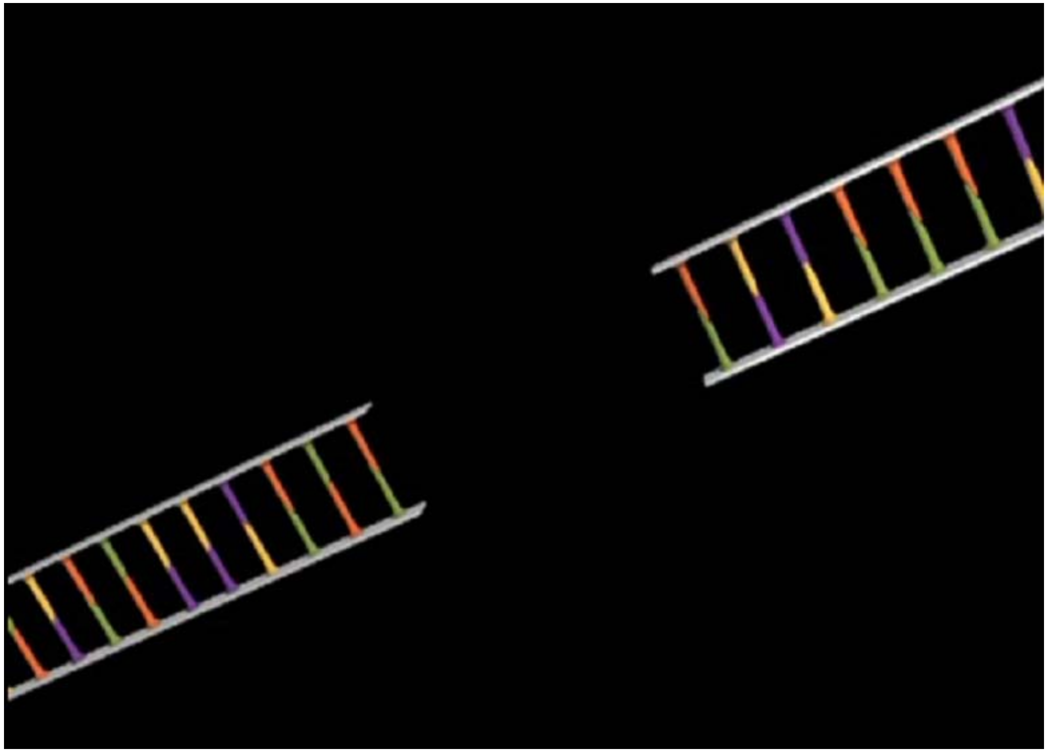
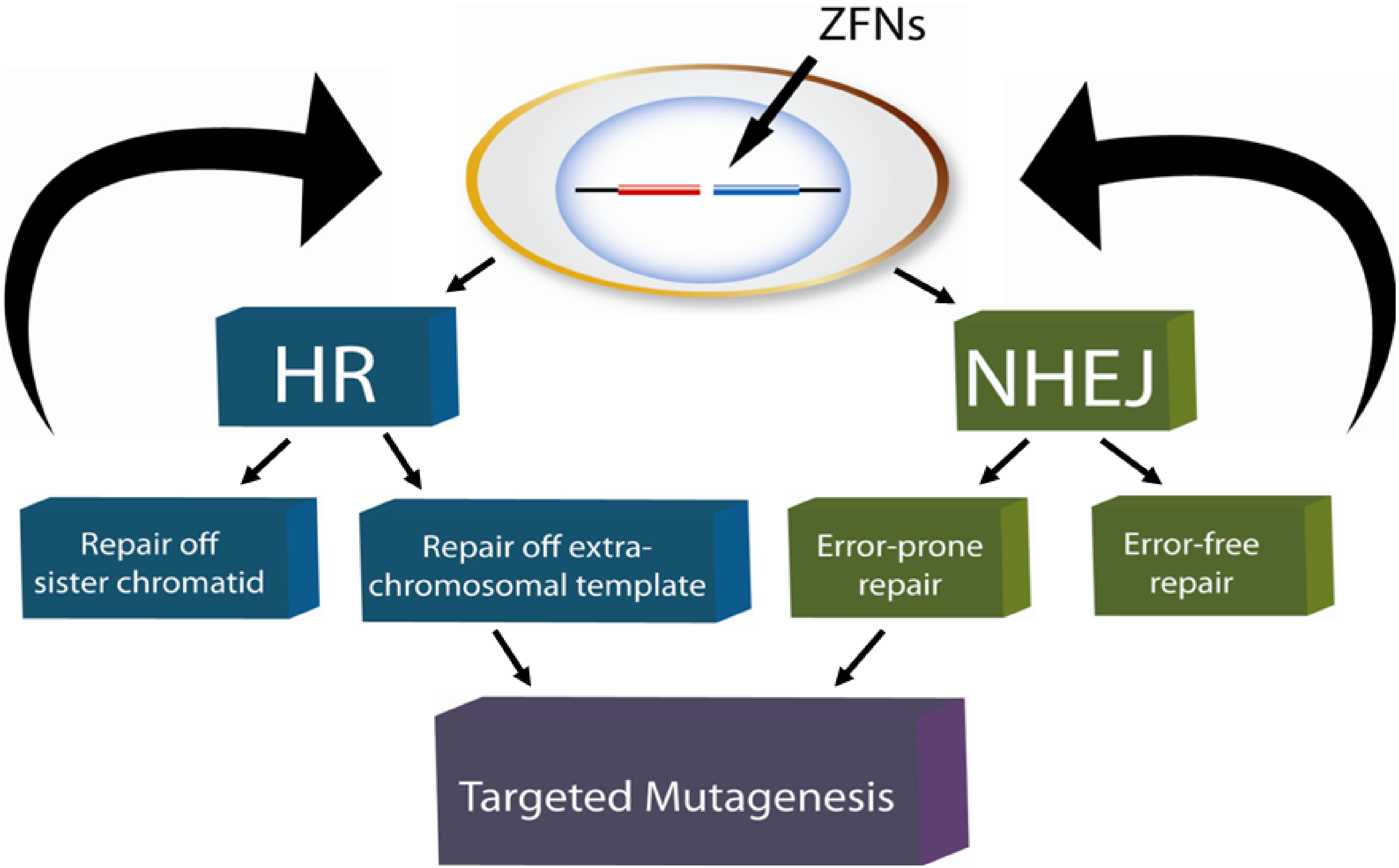
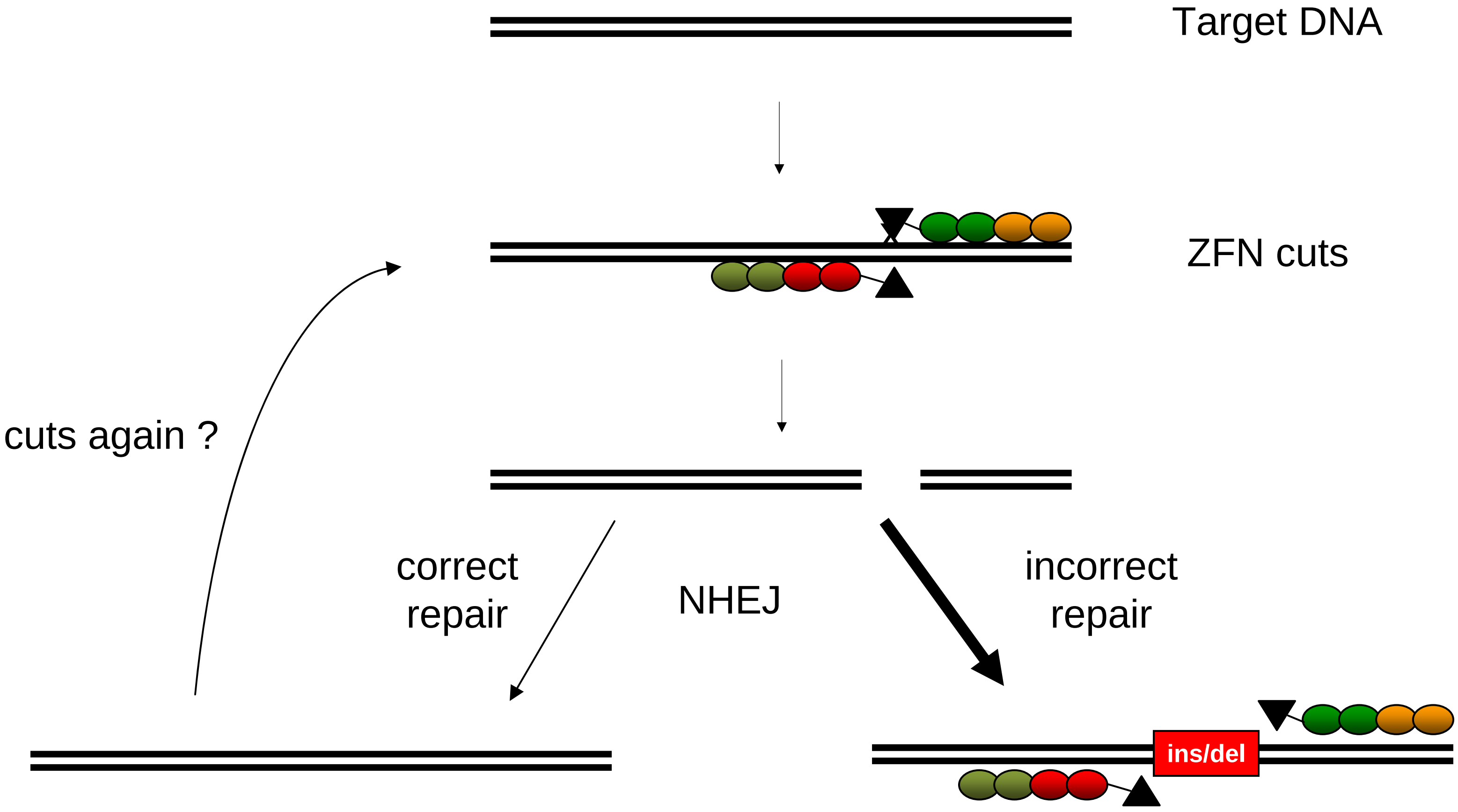
Zinc Finger Nucleases Perform Site-Specific DNA cleavage: The first step in genome modification



Genome Editing Using ZFNs: The Process



ZFN Drives NHEJ to Produce Mutations



Mutant site no longer a substrate for ZFN binding/cleavage (likely a favored outcome)

Work Flow for ZFN-Mediated Gene Knockout

Design, build and validate ZFNs

⬇

Transfect ZFNs into cells

⬇

Dilution clone

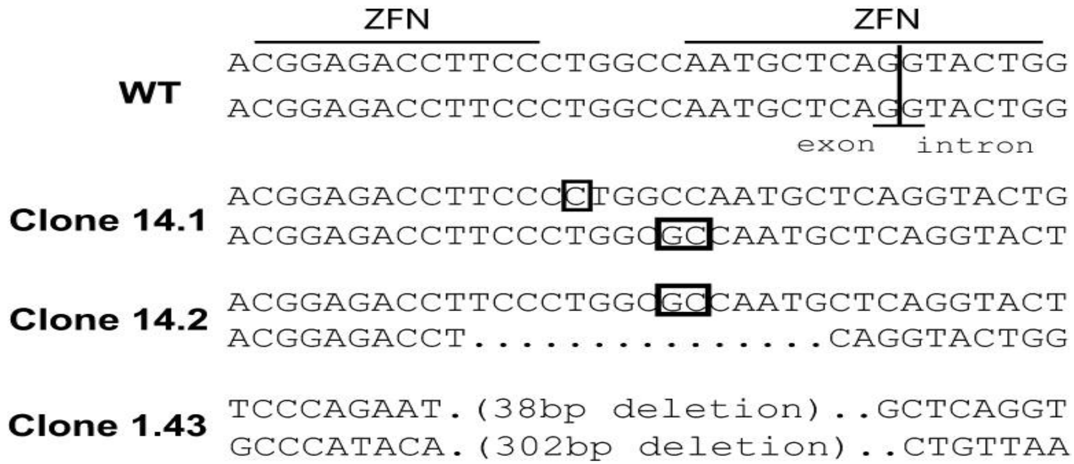
⬇

Screen clones by PCR-based methods

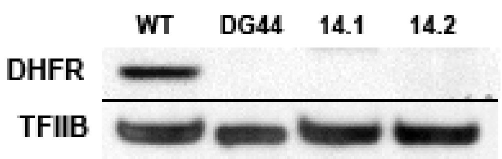
⬇

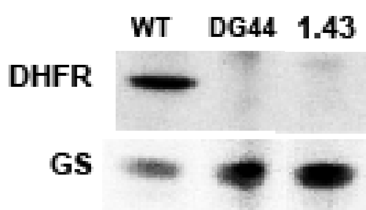
Confirm mutant genotypes by sequencing

CHO DHFR Knockout:
Different Genetic Changes but Same Phenotype



DHFR gene





7 / 418 clones were biallelic mutants (1.6%)

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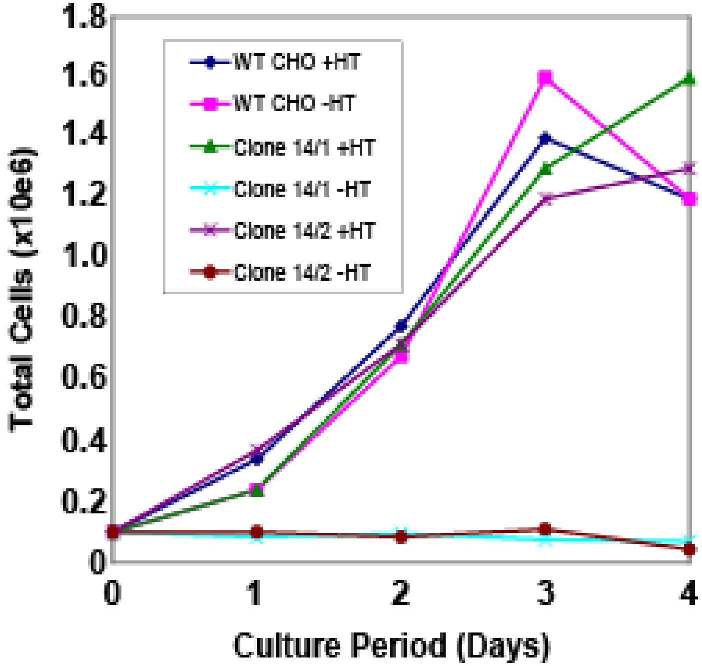
Santiago et al., 2008, *PNAS*, 105(15): 5811

DHFR Knockout in CHO Cells

Knockout at DNA → Protein → Phenotype



ZFN-driven DHFR knockout clones require HT for growth, the desired phenotype.

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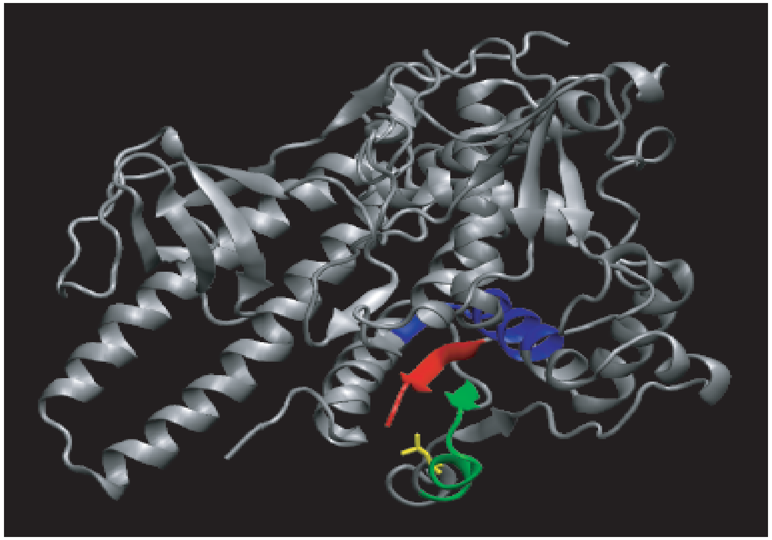
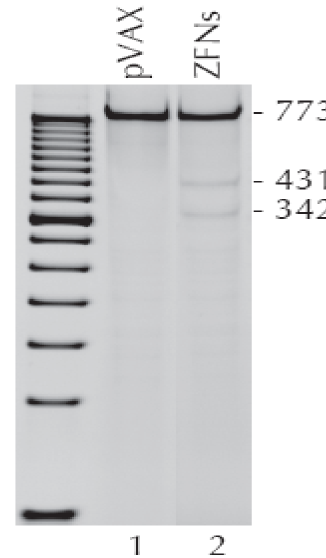
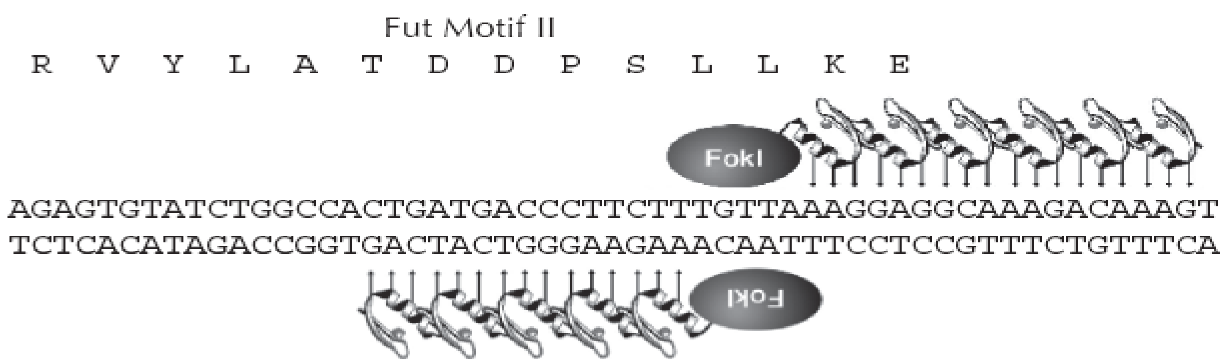
Santiago et al., 2008, *PNAS*, 105(15): 5811

ZFNs Target a Catalytic Domain in FUT8





Cel-I assay



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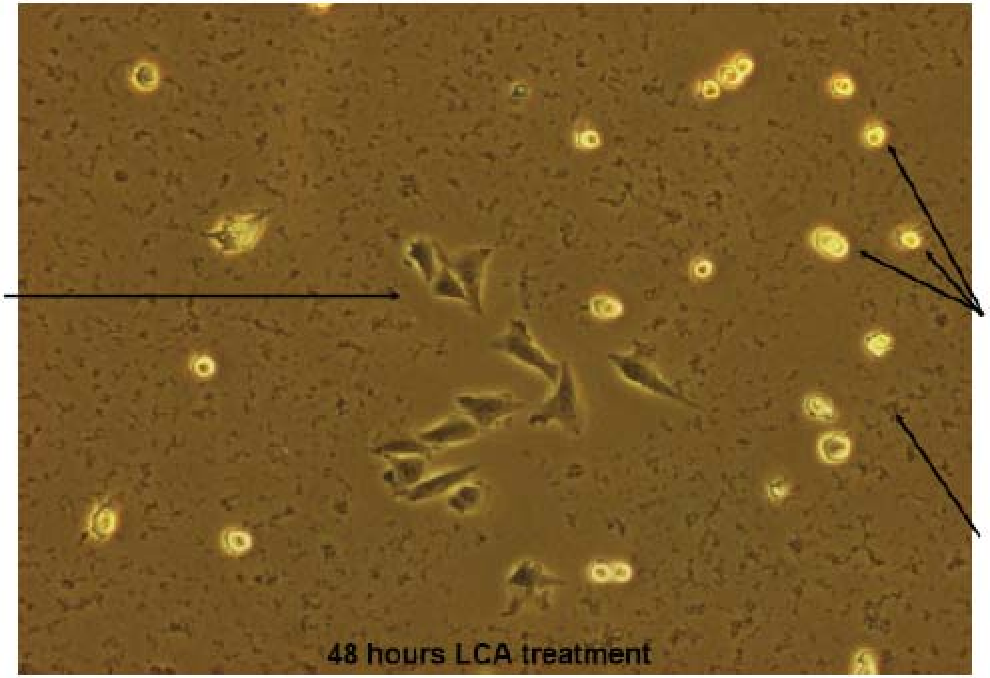
Malphettes, et al., (2010) Biotech. Bioeng. 106, 774-783

LCA Selection Identifies FUT8 KO Clones

FUT8 -/- KO cells (adherent) ALIVE

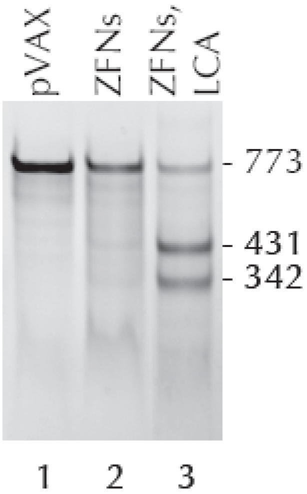


FUT8 +/+ cells (non-adherent) DEAD

LCA-lectin

Malphettes, et al., (2010) Biotech. Bioeng. 106, 774-783

LCA Selection Enriches for FUT8-/- Cells

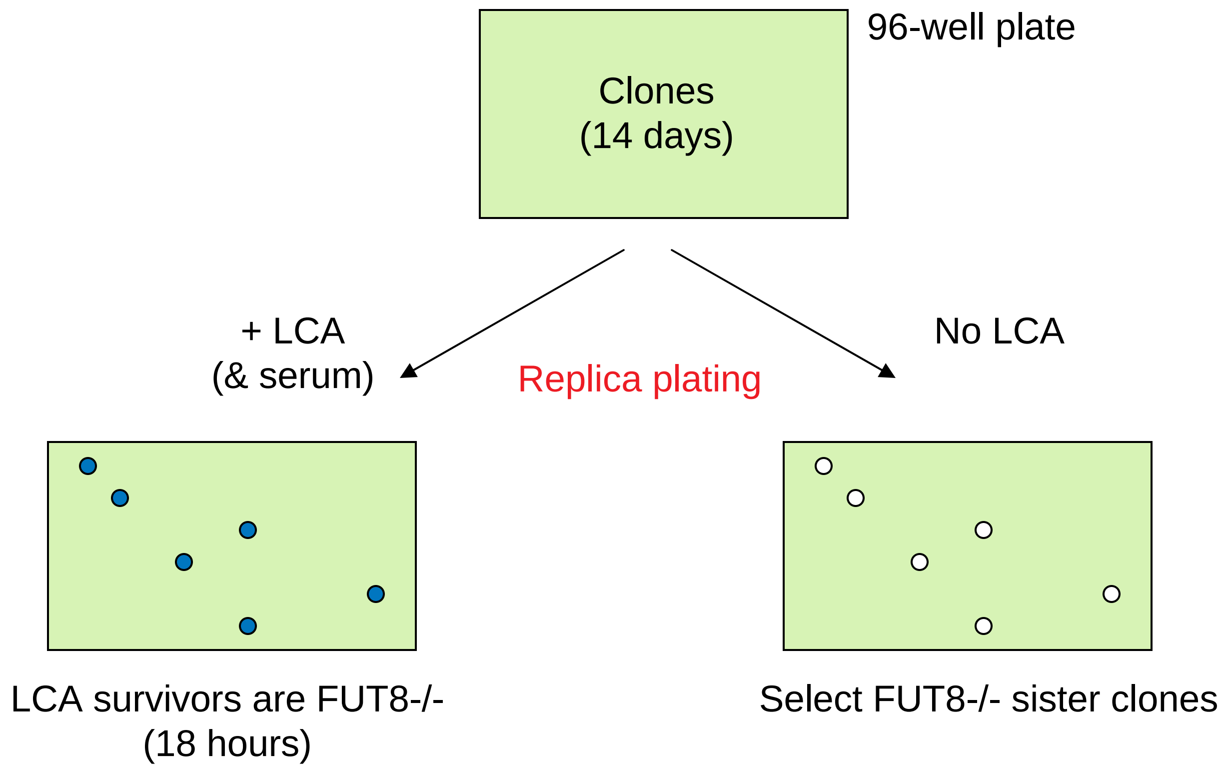


Can dilution clone directly from the enriched pool

Cel-I assay

Malphettes, et al., (2010) Biotech. Bioeng. 106, 774-783

Replica Plating Identifies FUT8-/- Clones without Exposure to LCA



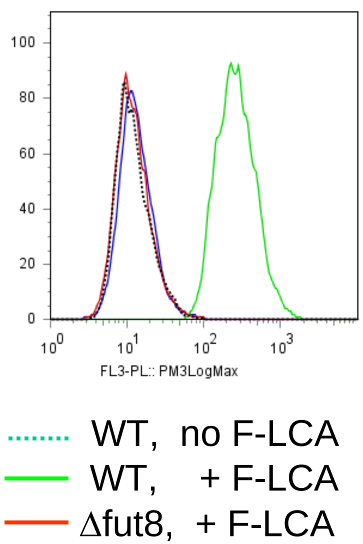
Genotypes of LCA-resistant FUT8-/- Clones

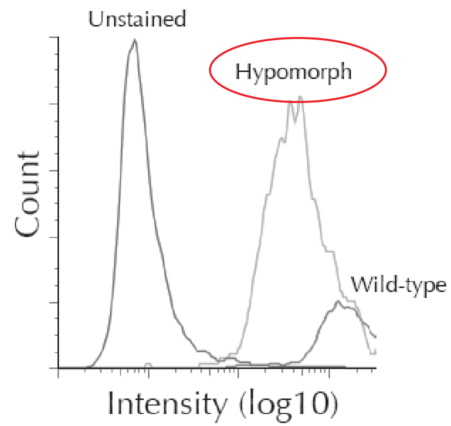
```
Wild-type    AGAGTGTATCTGGCCACTGATGACCCTTCTTT---GTTAAAGGAGGCAAAGACAAAGTAAGT
Clone 1-A    A----------------------------TATAAAGAGGACAAGGGCAAAGACAAAGTAAGT
Clone 1-B    AGAGTGTATCTGGCCACTGATGACCCTTCTTT------AAAGGAGGCAAAGACAAAGTAAGT
Clone 2-A    AGAGTGTATCTGGCCACTGATGACCC---------------------AAAGACAAAGTAAGT
Clone 2-B    AGAGTGTATCTGGCCACTGATGAC-----------------------------AAAGTAAGT
Clone 3-A    AGAGTGTATCTGGCCACTGATGACC-------AGATACAAAGGAGGCAAAGACAAAGTAAGT
Clone 3-B
Clone 4-A    AGAGTGTATCTGGCCACTGATGACCCTTCTTTT-----AAAGGAGGCAAAGACAAAGTAAGT
Clone 4-B    AGAGTGTATCTGGCCACTGATGACCCTTT------GTTAAAGGAGGCAAAGACAAAGTAAGT
Clone 6-A    AGAGCGTATCTGGCCACTGATGACCCTT----------TAAGGAGGCAAAGACAAAGTAAGT
Clone 6-B    AGAGTGTATCTGGCCACTGATGACCCTT-----------AAGGAGGCAAAGACAAAGTAAGT
Clone 7-A    AGAGTGTATCTGGCCACTGATGACCCTTCTTTGTTA--------GGCAAAGACAAAGTAAGT
Clone 7-B    AGAGTGTATCTGGCCACTGATGACCCTTCTTTGTTATTAAAGGAGGCAAAGACAAAGTAAGT
Clone 8-A    AGAGTGTATCTGGCCACTGATGACCCTTCTT-------AAAGGAGGCAAAGACAAAGTAAGT
Clone 8-B    AGAGTGTATCTGGCCACTGATGA----(+189)-----AAAGGAGGCAAAGACAAAGTAAGT
Clone 9-A    -----------------------------------------GGAGGCAAAGACAAAGTAAGT
Clone 9-B    AGAGTGTATCTGGCCACTGATGACCCTTC(+274TGTTAAAGGAGGCAAAGACAAAGTAAGT
Clone 11-A   AGAGTGTATCTGGCCACTGATGACCCTTCTTTGT----------------------GTAAGT
Clone 11-B   AGAGTGTATCTGGCCACTGATGACCCTTCTTTGTTTT-AAAGGAGGCAAAGACAAAGTAAGT
Clone 12-A   AGAGTGTATCTGGCCACTGATGAC-----------------------------AAAGTAAGT
Clone 12-B   ------------------------------------------------------AAGTAAGT
Clone 13-A   AGAGTGTATCTGGCCACTGATGACCCTTCTTTGTT-ATAAAGGAGGCAAAGACAAAGTAAGT
Clone 13-B
Clone 14-A   AGAGTGTATCTGGCCACTGAT------------------------------ACAAAGTAAGT
Clone 14-B   AGAGTGTATCTGGCCACTGATGACCCTTCTTTGTTATTAAAGGAGGCAAAGACAAAGTAAGT
Clone 15-A   AGAGTGTATCTGGCCACTGATGACCCTT-----------AAGGAGGCAAAGACAAAGTAAGT
Clone 15-B   AGAGTGTATCTGGCCACTGATGACCCTTT----------AAGGAGGCAAAGACAAAGTAAGT
Clone 16-A   AGAGTGTATCTGGCCACTGATGA----------------AAGGAGGCAAAGACAAAGTAAGT
Clone 16-B   AGAGTGTATCTGGCCACTGATGACCCTTCTTTGTTT--AAAGGAGGCAAAGACAAAGTAAGT
Clone 17-A   AGAGTGTATCTGGCCACTGATGACCCTT-----------AAGGAGGCAAAGACAAAGTAAGT
Clone 17-B   AGAGTGTATCTGGCCACTGATGAC-----------------------------AAAGTAAGT
Clone 18-A   AGAGTGTATCTGGCCACTGATGACCCTTCTTTT------AAGGAGGCAAAGACAAAGTAAGT
Clone 18-B   AGAGTGTATCTGGCCACTGATGACCCTTCTT----(+67)----GGCAAAGACAAAGTAAGT
Clone 19-A   AGAGTGTATCTGGCCACTGATGACCCTTCTTTGTTATTAAAGGAGGCAAAGACAAAGTAAGT
Clone 19-B   AGAGTGTATCTGGCCACTGATGACCCTT-------------GGAGGCAAAGACAAAGTAAGT
Clone 20-A   AGAGTGTATCTGGCCACTG--------------------------GCAAAGACAAAGTAAGT
Clone 20-B   AGAGTGTATCTGGCCACTGATGAC-----------------------------AAAGTAAGT
Clone 21-A   AGAGTGTATCTGGCCACTGATGACCC---------------------------------AGT
Clone 21-B   AGAGTGTATCTGGCCACTGATGACCC---------------------------------AGT
Clone 24-A   AGAGTGTATCTGGCCACTGATGACCCTTCTTTGTTATTAAAGGAGGCAAAGACAAAGTAAGT
Clone 24-B   AGAGTGTATCTGGCCACTGATGACCCTTCTTTGTTT--AAAGGAGGCAAAGACAAAGTAAGT
```

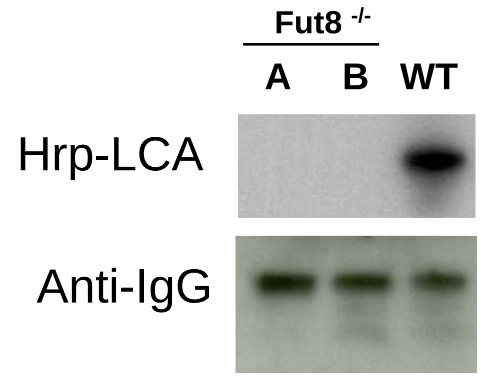
3.5% of clones are FUT8-/-
(25 of 711)

Malphettes, et al., (2010) Biotech. Bioeng. 106, 774-783

Phenotypic Assays for FUT8-/- Cell Lines



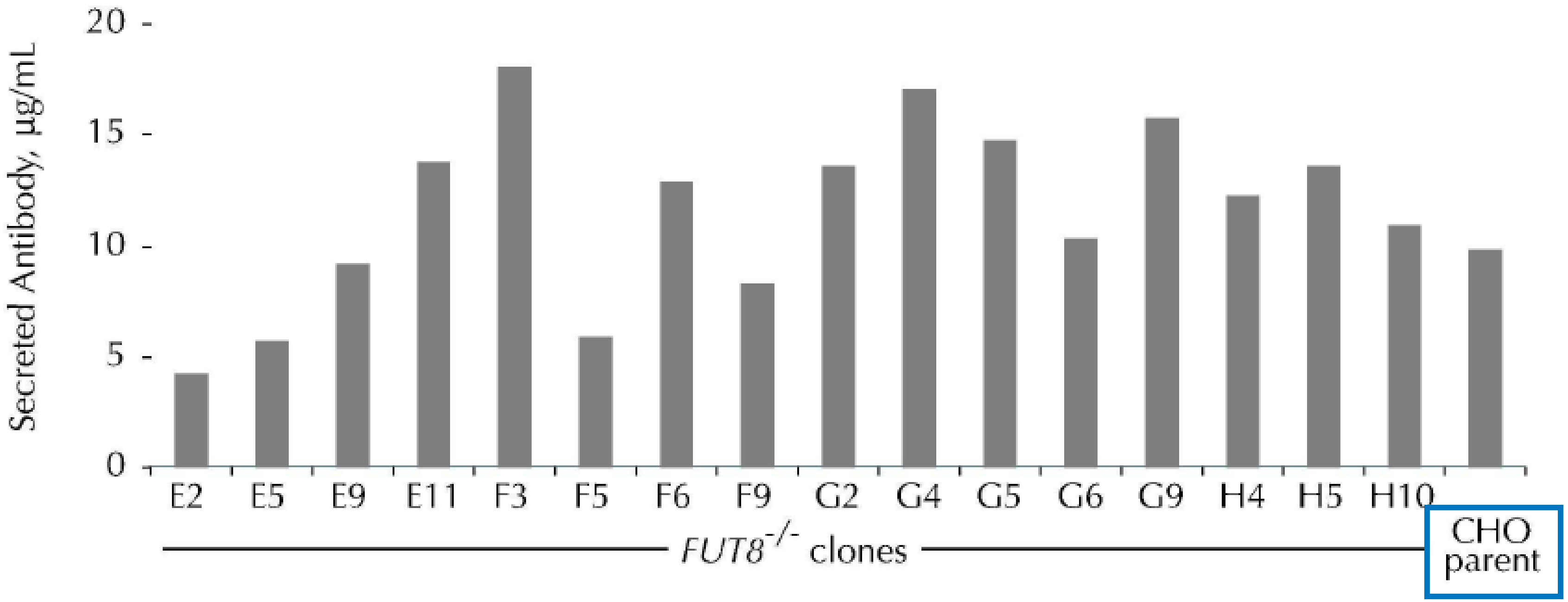




FITC-LCA

HRP-LCA

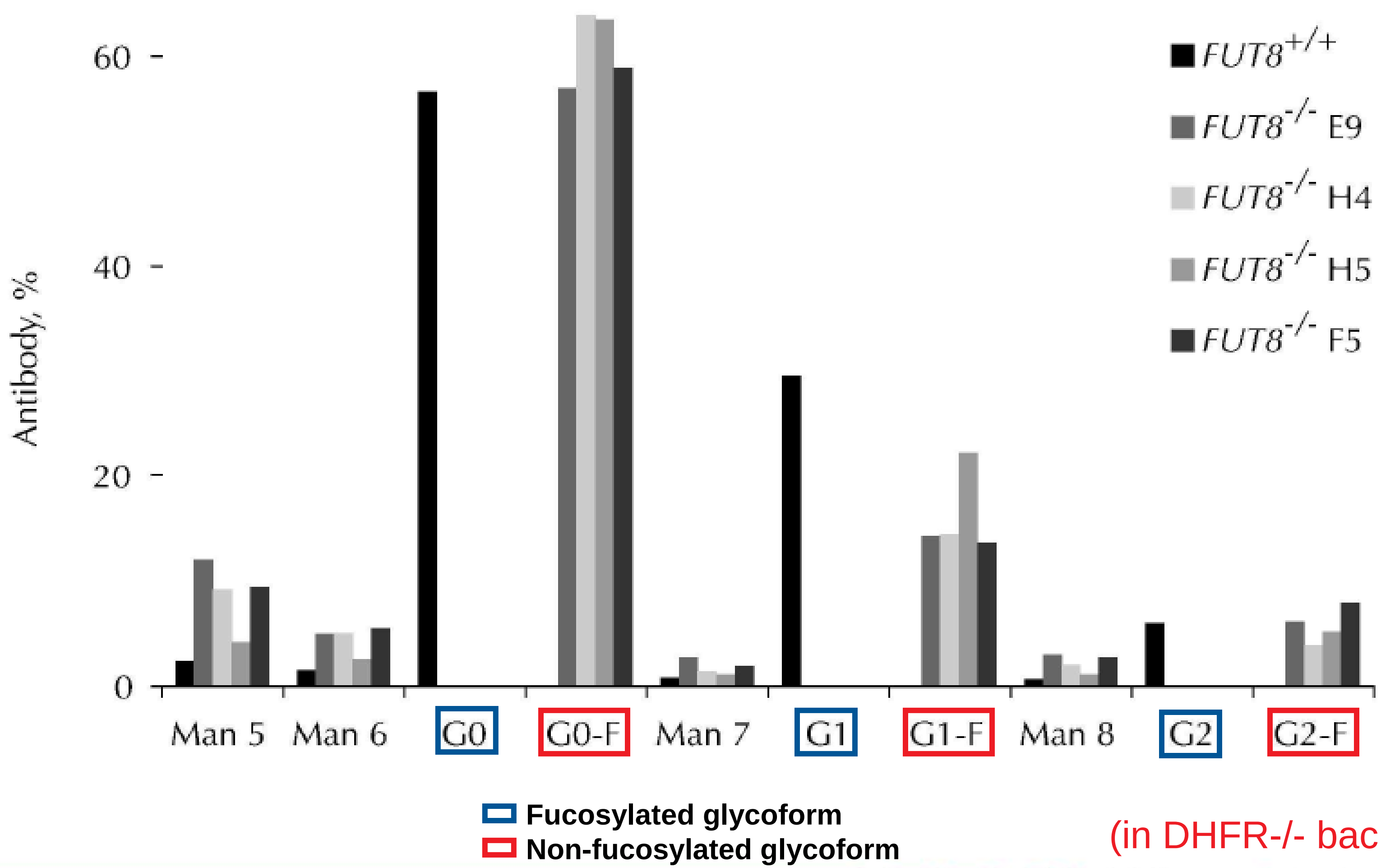
Antibody Production from Transiently Transfected CHO FUT8-/- Clones



(in DHFR-/- background)

Malphettes, et al., (2010) Biotech. Bioeng. 106, 774-783

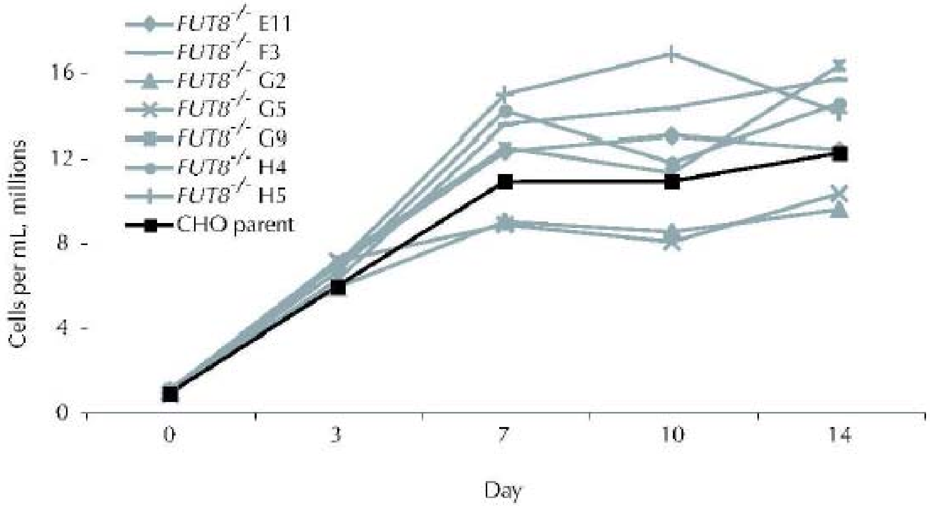
Antibody from Transiently Transfected FUT8-/- Clones is Non-fucosylated



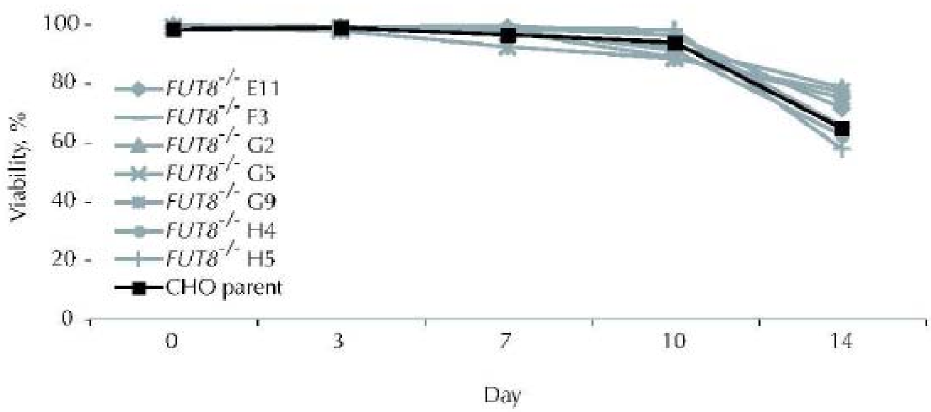
(in DHFR-/- background)

Malphettes, et al., (2010) Biotech. Bioeng. 106, 774-783

Growth & Viability of FUT8-/- Clones



Cell density

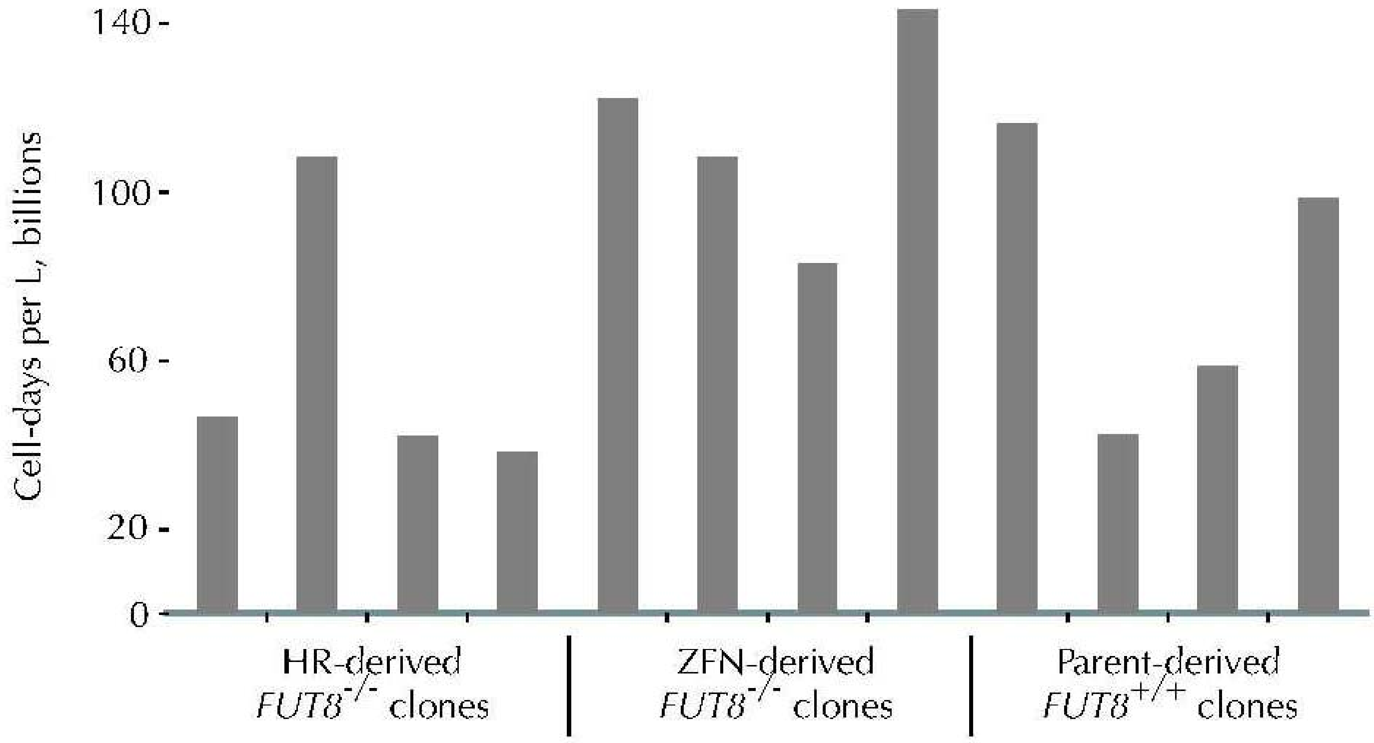


Culture viability

(in DHFR-/- background)

Malphettes, et al., (2010) Biotech. Bioeng. 106, 774-783

Integrated Viable Cell Count:
HR *vs* ZFN-Derived FUT8-/- Clones



(in DHFR-/- background)

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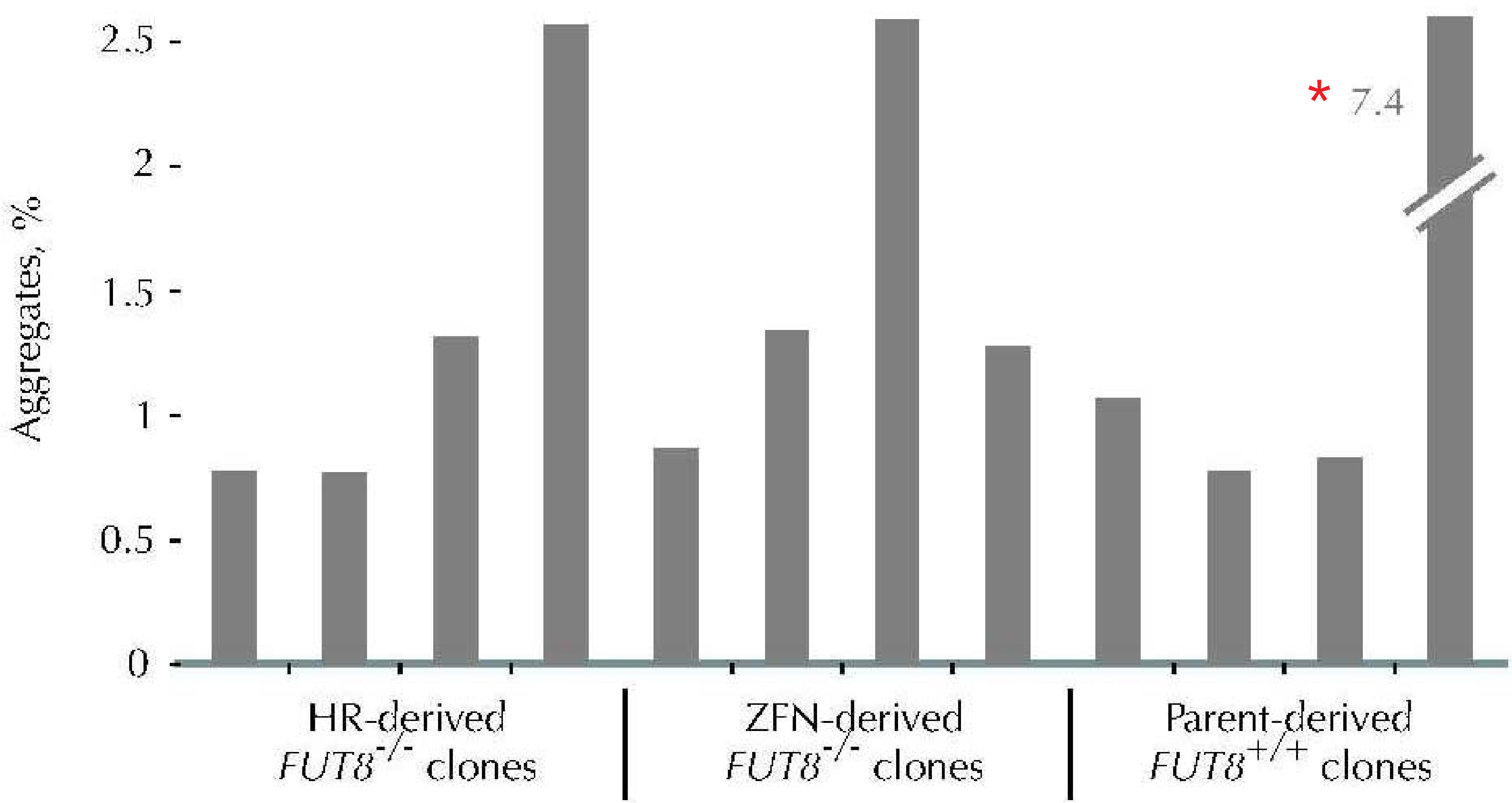
Malphettes, et al., (2010) Biotech. Bioeng. 106, 774-783

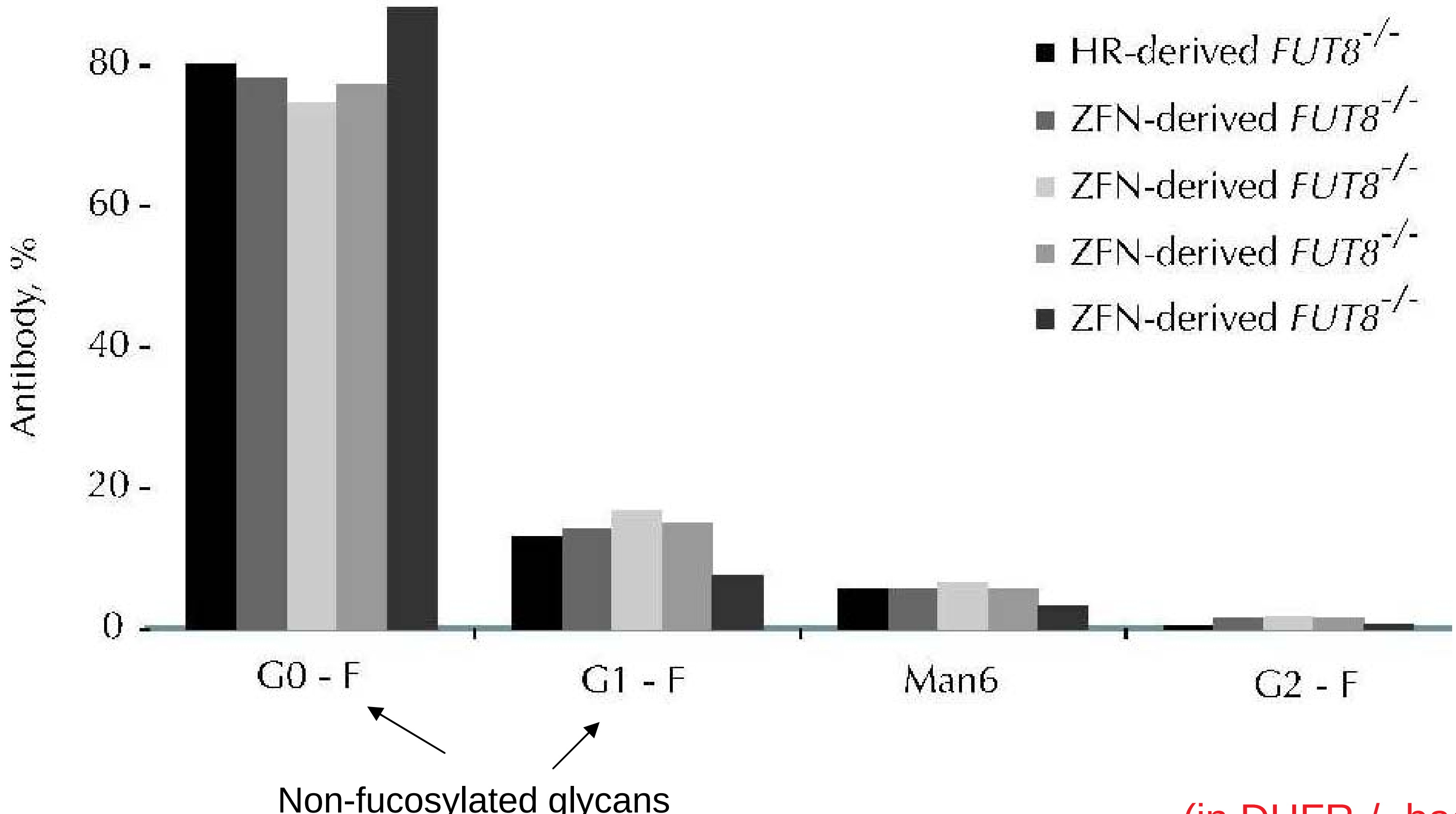
Aggregates:
HR *vs* ZFN-Derived FUT8-/- Clones



(in DHFR-/- background)

Malphettes, et al., (2010) Biotech. Bioeng. 106, 774-783

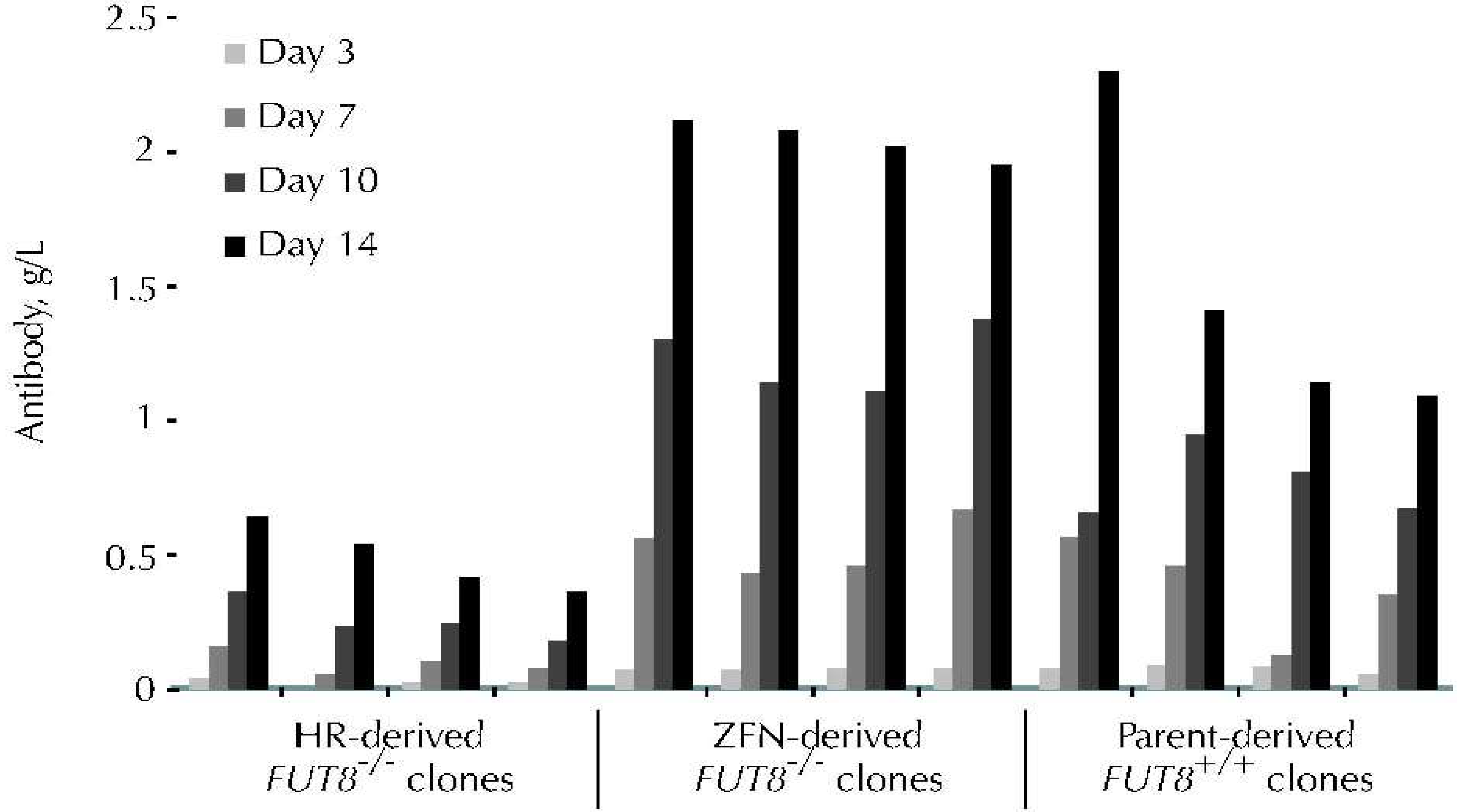
Fucosylation:
HR *vs* ZFN-Derived FUT8-/- Clones



(in DHFR-/- background)

Malphettes, et al., (2010) Biotech. Bioeng. 106, 774-783

Productivity:
HR *vs* ZFN-Derived FUT8-/- Clones



(in DHFR-/- background)

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Malphettes, et al., (2010) Biotech. Bioeng. 106, 774-783

Summary of FUT8 Knockout Study

ZFNs produce FUT8-/- in 3 weeks at a frequency of 5% in the absence of any selection.

Alternatively, populations of ZFN-treated cells can be directly selected to give FUT8-/- pools in as few as 3 days

Compared to wild type production CHO cell line, ZFN-derived FUT8-/- cell lines

- Had similar or better growth profiles
- Were equally transfectable
- Produced equivalent amounts of antibody during transient transfection

Antibodies stably expressed in these FUT8-/- cell line clones

- Completely lacked core fucosylation
- Had otherwise normal glycosylation pattern
- Had equivalent titer and specific productivity distribution to wild type clones
  - Up to 4x higher than commercially available CHO FUT8-/- line

Glutamine Synthetase Knockout in CHO Cells
Enhancing Selection

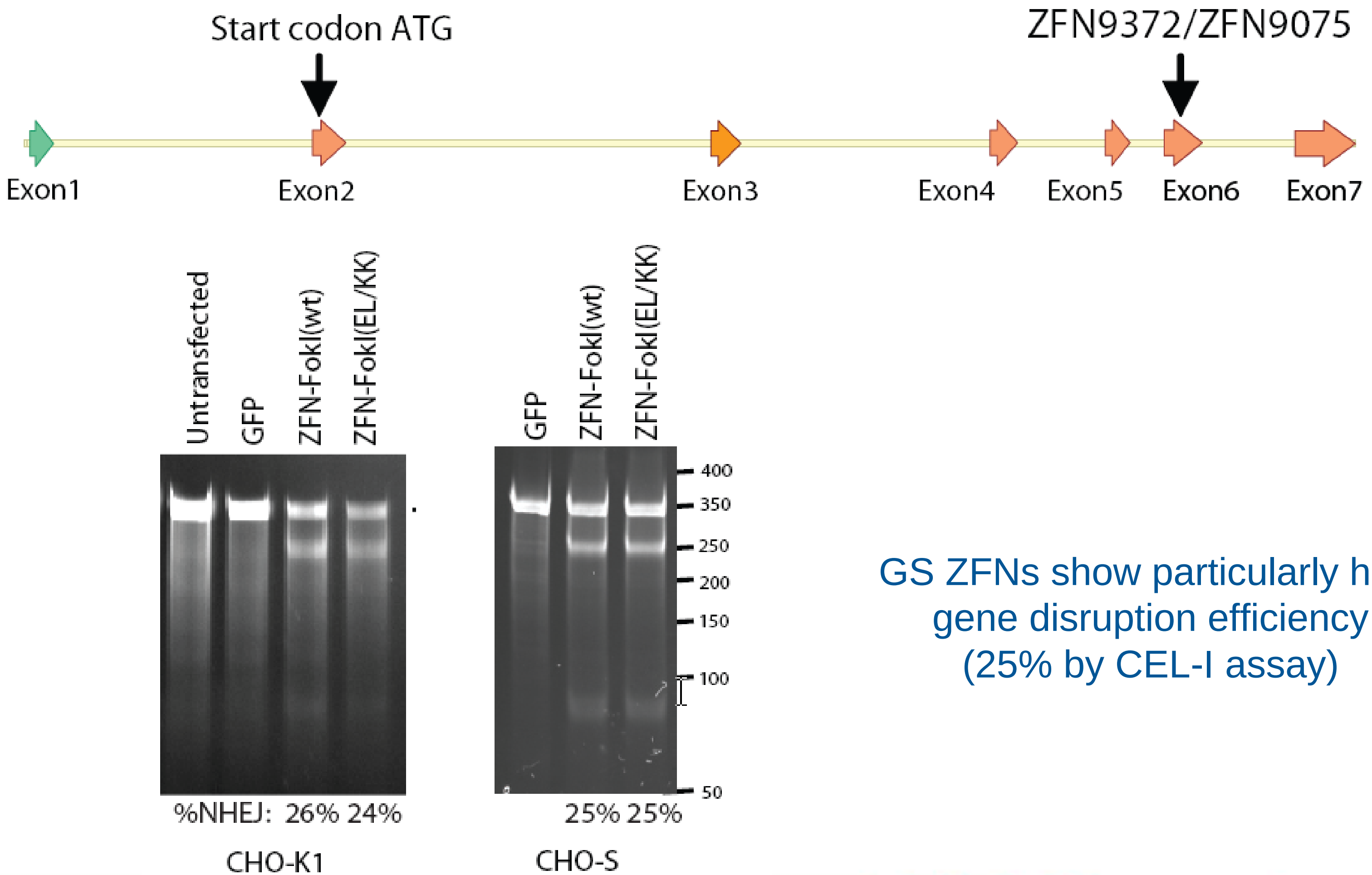
Problem:

- Endogenous GS expression reduces effectiveness of rGS as a selection marker
- Need to add GS inhibitor (MSX) to increase stringency of selection

Solution:

- Knockout endogenous GS
- Cells become completely auxotrophic for glutamine
- Producer clone growth & survival absolutely dependent on GS marker expression (level)

Glutamine Synthetase (GS) Knockout in CHO Cells



GS ZFNs show particularly high gene disruption efficiency (25% by CEL-I assay)

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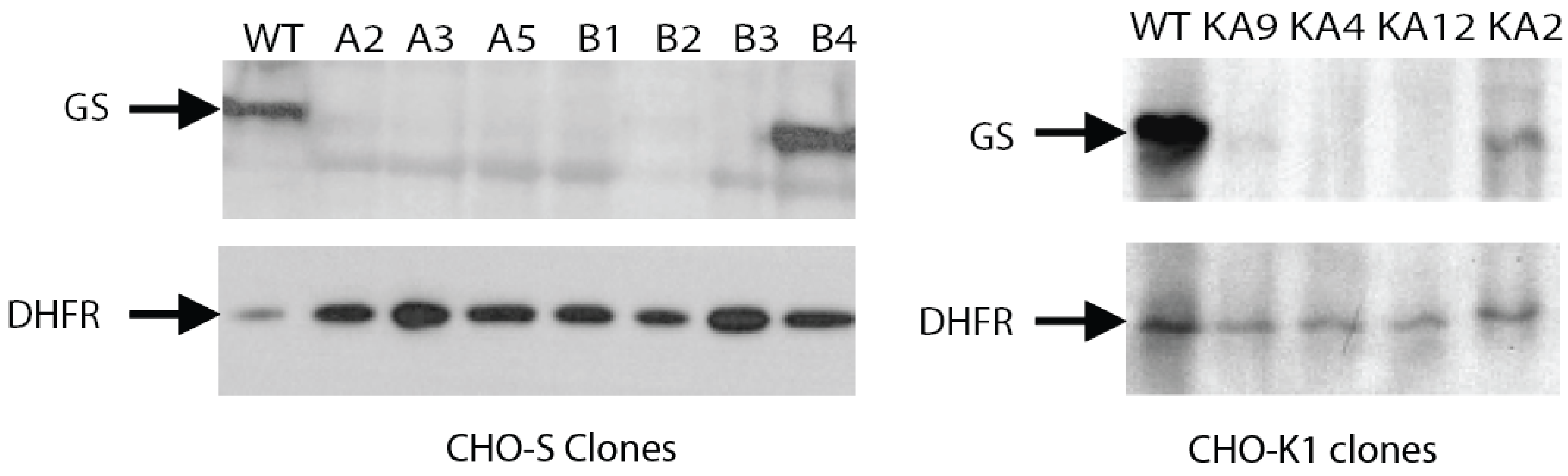
Liu et al, 2010, Biotech. Bioeng. 106, 97-105

Western Blot shows Loss of Glutamine Synthetase Expression



(B4 and KA2 are in-frame deletions of 27 and 6 bp respectively)

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Liu et al, 2010, Biotech. Bioeng. 106, 97-105

Glutamine Synthetase Knockout in CHO Cells

Enhancing Selection

Problem:

- Endogenous GS expression reduces effectiveness of rGS as a selection marker
- Need to add GS inhibitor (MSX) to increase stringency of selection

Solution:

- Knockout endogenous GS
- Cells become completely auxotrophic for glutamine
- Producer clone growth & survival absolutely dependent on GS marker expression (level)

Externally validated platform improvement (in proprietary hosts)

- Decreased timeline by 8 weeks to tox material (Pfizer)
- 8x increase in clones over 2g/l (Lilly)
- Non-producers almost eliminated (Lilly)

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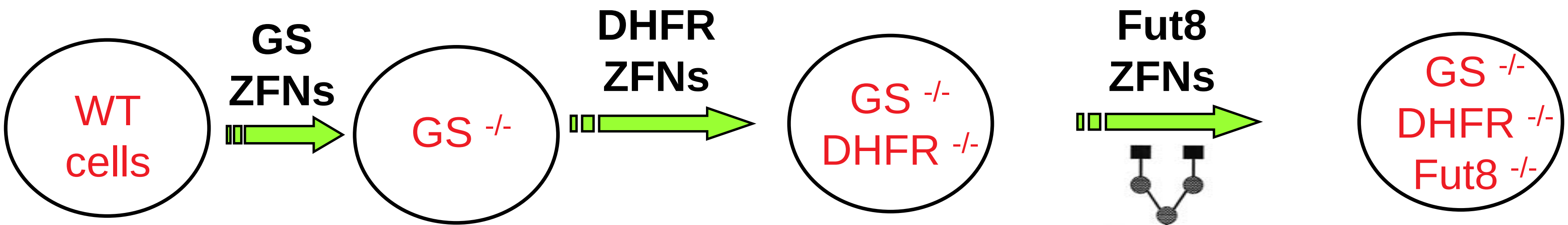
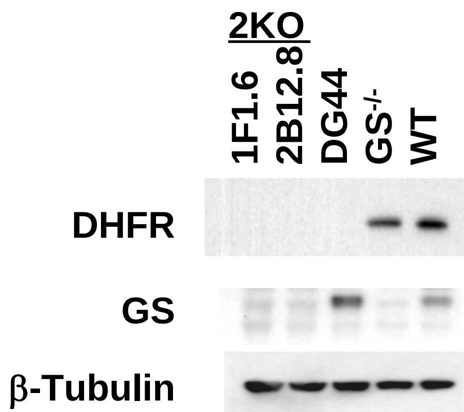
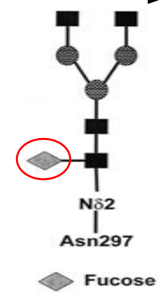
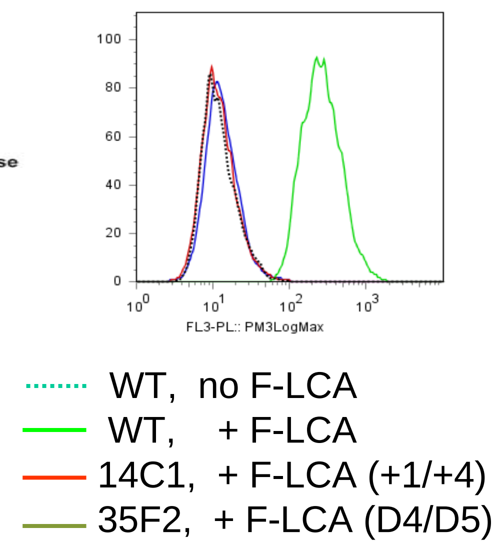
Targeting Multiple Genes in the Same Cells









GS = glutamine synthetase
DHFR = dihydrofolate reductase
FUT8 = α1,6-fucosyltransferase

All three biallelic knockout events were obtained at frequencies of >1% without the use of selection

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Liu, P, *et al*., (2010) *Biotechnology and Bioengineering*, 106(1): 97-105

Reducing Apoptosis in CHO Cell Culture

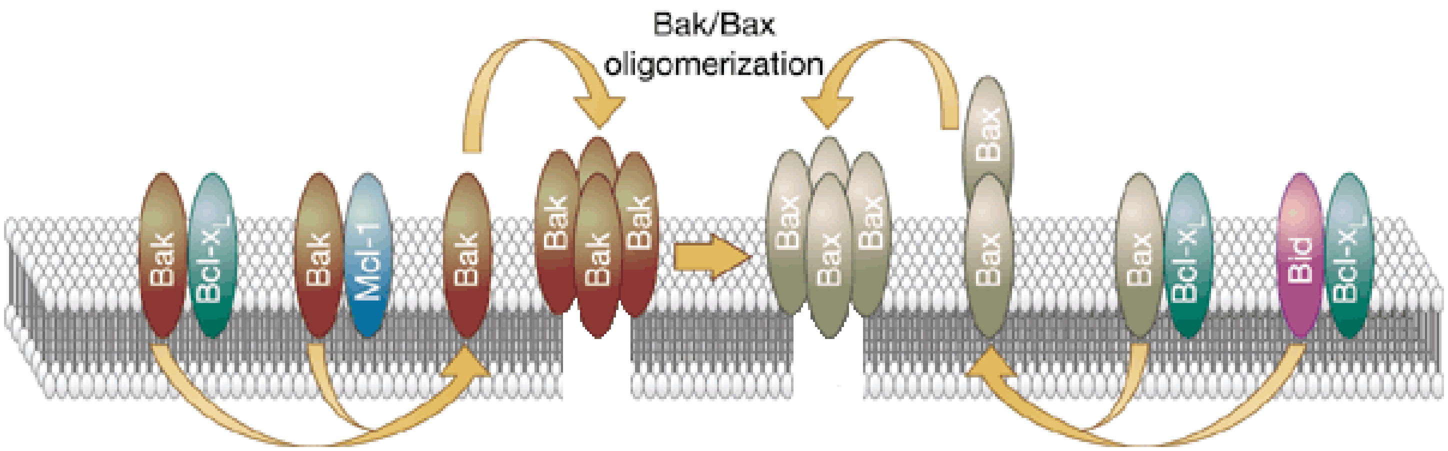
Bak and Bax activate the intrinsic apoptotic pathway by promoting mitochondrial permeability

- Stress response

Overexpression of Bcl2 / Bclx can block Bak and Bax oligomers…but

- Further metabolic stress during production?

Alternate solution: Knockout both Bak and Bax



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Work Flow for Bak/Bax Double Gene Knockout in CHO Cells

Design, build, validate ZFNs

⬇

Transfect cells with Bak ZFNs

⬇

Isolate Bak -/- clones

⬇

Transfect a Bak -/- clone with Bax ZFNs

⬇

Isolate Bax knockout clones

⬇

Bax / Bak expression profile

⬇

Phenotype analysis

Genotypes of Bax and Bak Mutant Alleles

Gene	Clone	Allele	Sequence
			ZFN-L ZFN-R
			L V W I Q D Q G G W
BAX	wild-type	N.A.	CCTCCGAGAGCGGCTG**CTTGTCTGGATC**CAAGAC**CAGGGTGGCTGG**gtgagaccccttagtccttgtcacactttagactagtggttctcaaacttcct
BAX	1-5	13 bp Δ	CCTCCGAGAGCGGCTGCTTGTCT-------------GGGTGGCTGGgtgagaccccttagtccttgtcacactttagactagtggttctcaaacttcct
BAX	8H6-47	46 bp Δ	CCTCCGAGAGCGGCTGCTTGTCTGGATCC--agactagtggttctcaaacttcct
BAX	8H6-71	68 bp Δ	CCTCCGAGAGCGGCTGCTTGTC--aaacttcct
BAX	8H6-97	295 bp Δ	CCTCC------------------------------(212bp)---aaagcaataca
			ZFN-L ZFN-R
			- - - - - - M A S G Q G
BAK	wild-type	N.A.	agGGTGACAGTGCT**GCCAACCAAGGCCTGAAA**GATGG**CGTCTGGACAAG**GACCAGGTCCTCCTAGGCAGGACTGTGATGACTCCCCCTCCCCTTCTGgt
BAK	8H6 allele A	28 bp Δ	agGGTGACAGTG----------------------------CTGGACAAGGACCAGGTCCTCCTAGGCAGGACTGTGATGACTCCCCCTCCCCTTCTGgt
BAK	8H6 allele B	10 bp Δ	agGGTGACAGTGCTGCCAACCAAGGCC----------CATCTGGACAAGGACCAGGTCCTCCTAGGCAGGACTGTGATGACTCCCCCTCCCCTTCTGgt
BAK	8D4 allele A	19 bp Δ	agGGTGACAGTGCTGCCAACCAAGG-------------------ACAAGGACCAGGTCCTCCTAGGCAGGACTGTGATGACTCCCCCTCCCCTTCTGgt
BAK	8D4 allele B	19 bp Δ	agGGTGACAGTGCTGCCAACCAAGG-------------------ACAAGGACCAGGTCCTCCTAGGCAGGACTGTGATGACTCCCCCTCCCCTTCTGgt

BAX mutants made on BAK-/- 8H6 clone background

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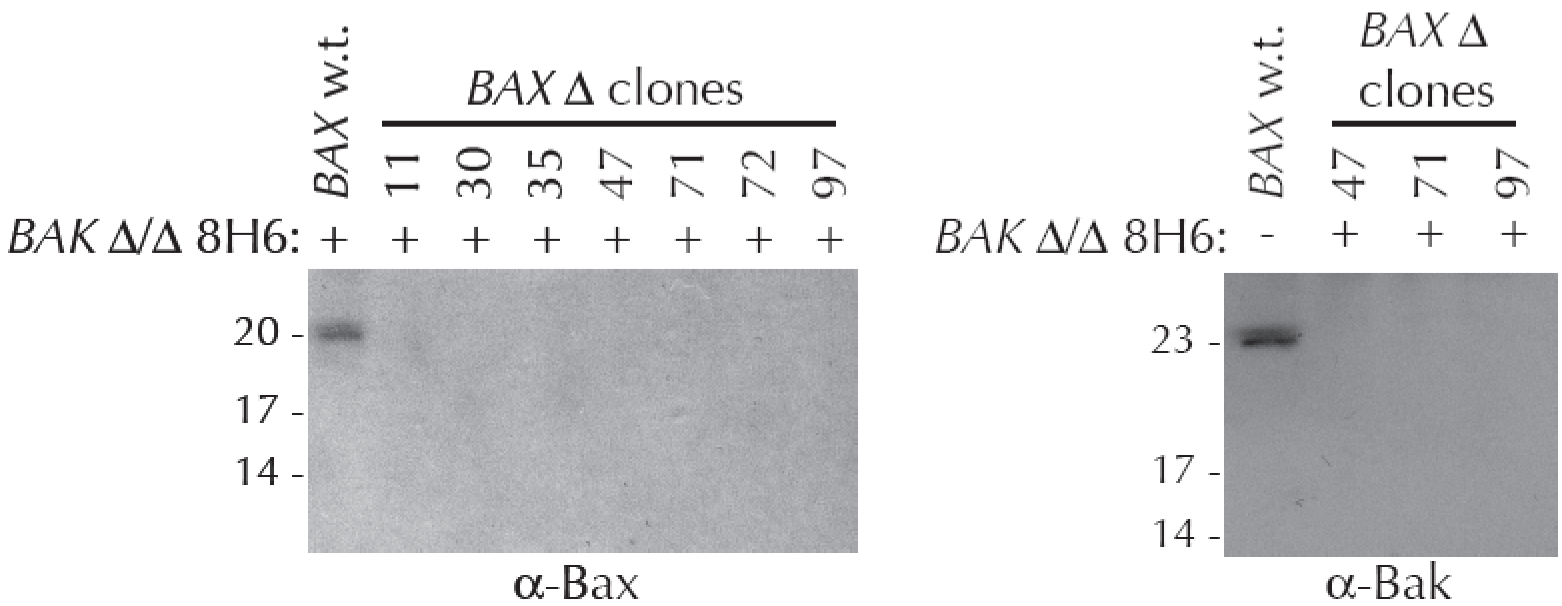
Cost, G, et al., (2010) Biotech. Bioeng. 105, 330-340

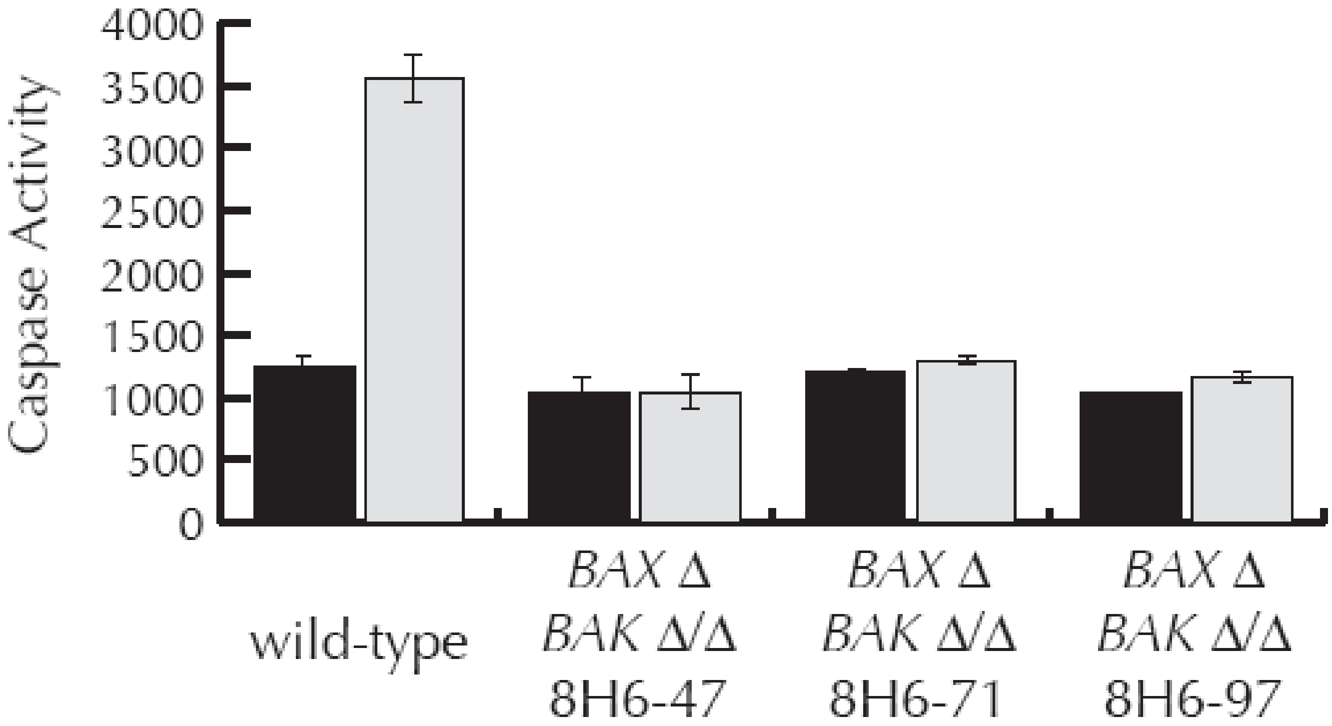
Analysis of Bax/Bak Protein Expression



Western antibody

Cost, G, et al., (2010) Biotech. Bioeng. 105, 330-340

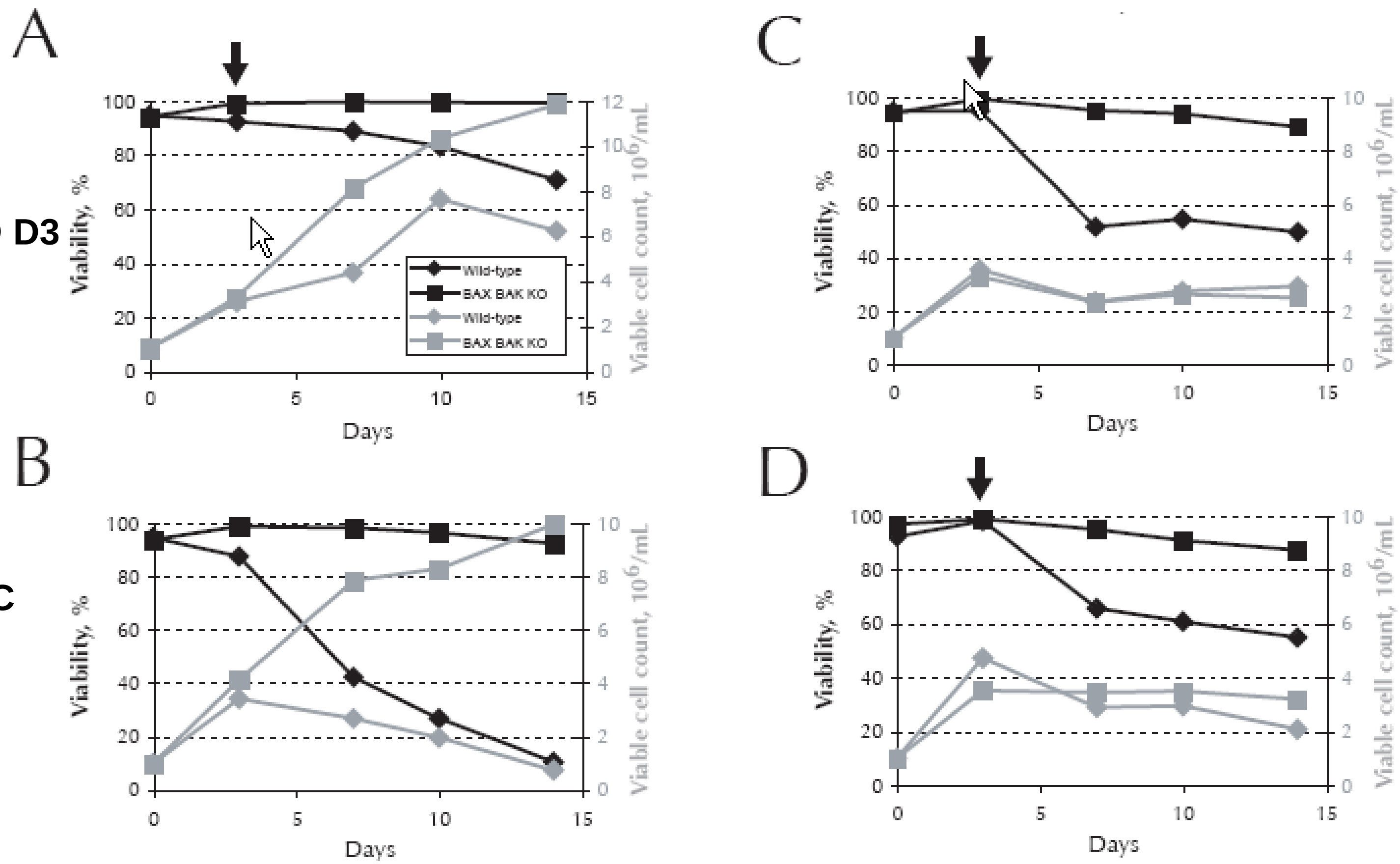
Bax/Bak KO in CHO Cells Inhibits Staurosporine-Induced Apoptosis



Cost, G, et al., (2010) Biotech. Bioeng. 105, 330-340

Viability and Viable Cell Density in Shakeflask Scaledown Model of Large-scale Bioreactor Culture

33 C
BF@ D3



37 C

33 C
BF@ D3
Stauro @ D3

33 C
BF@ D3
NaButy @ D3

WT CHO

Bak/Bax knockout

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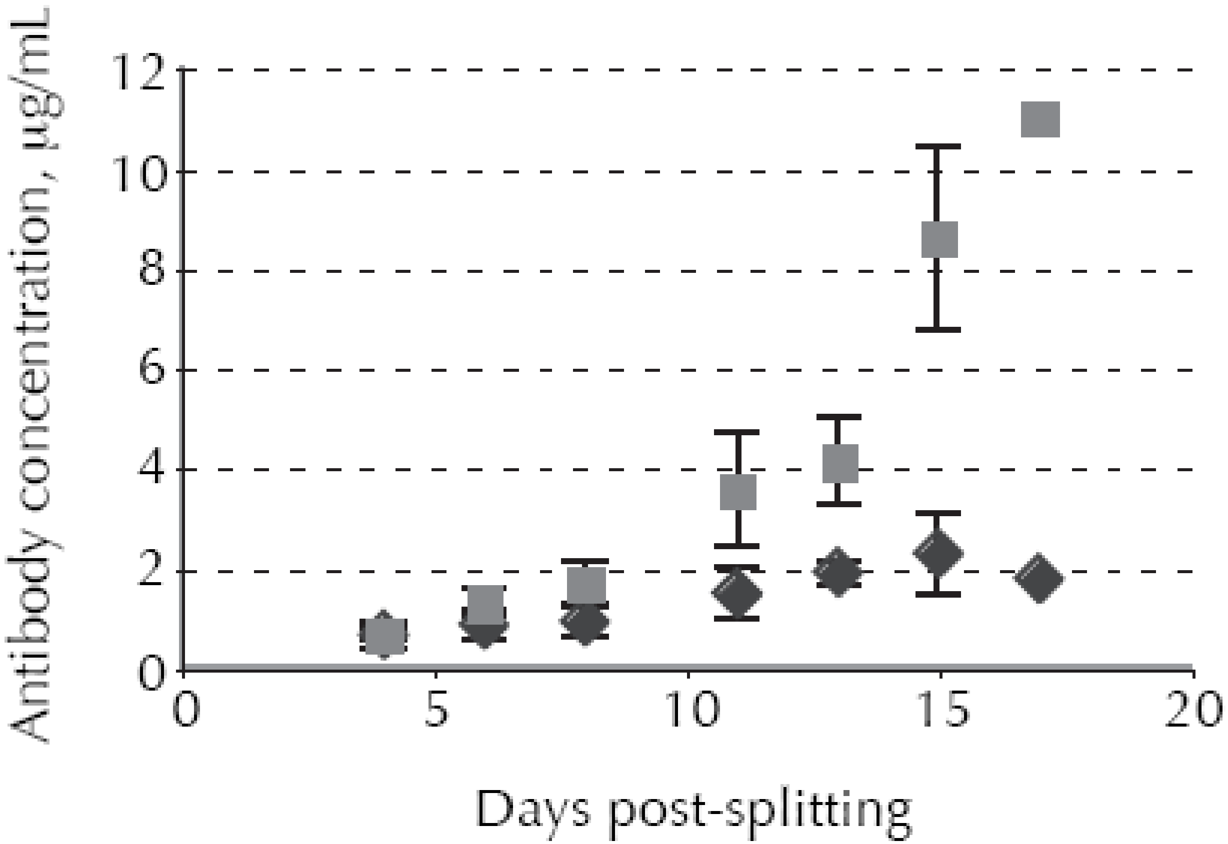
Cost, G, et al., (2010) Biotech. Bioeng. 105, 330-340

Stable Pools of *BAX*$^{-}$ *BAK*$^{-/-}$ CHO cells Produce Higher Levels of Recombinant Antibody

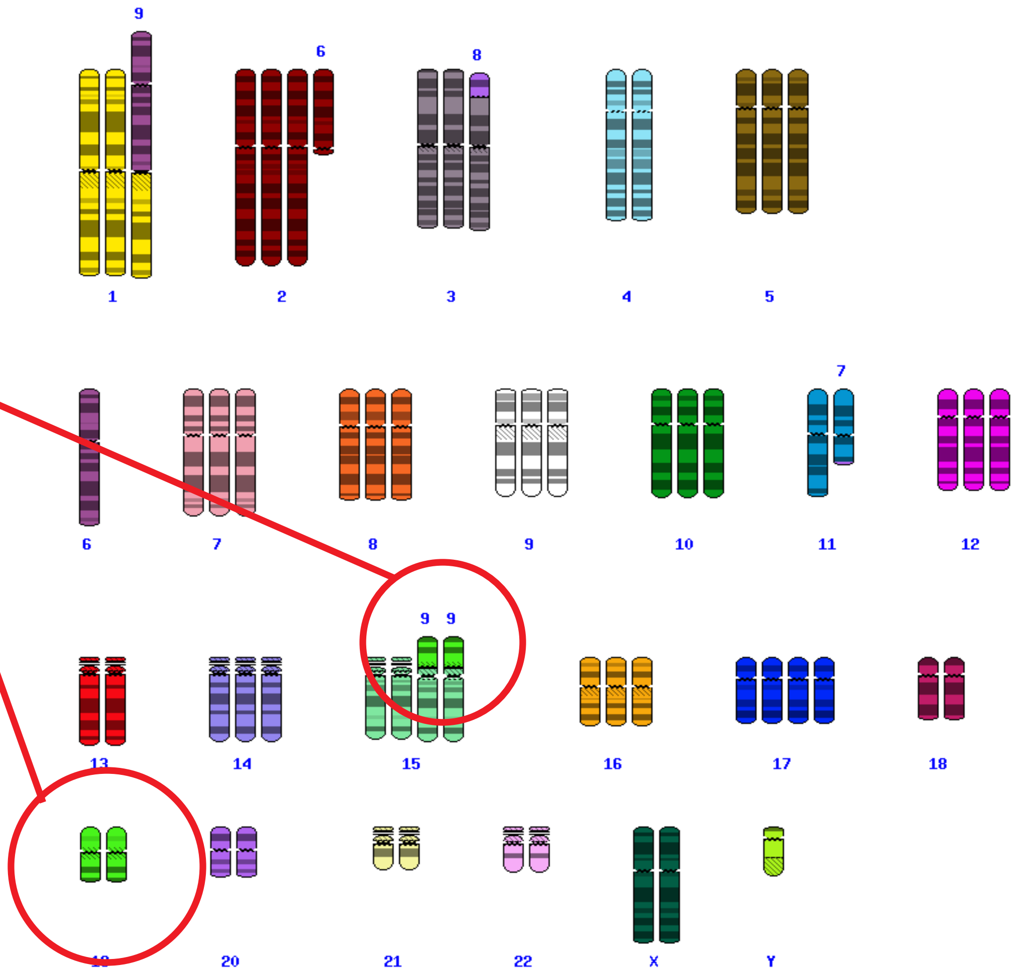


Cost, G, et al., (2010) Biotech. Bioeng. 105, 330-340

Polyploidy is Not a Barrier to ZFNs

BAX 19q13.3

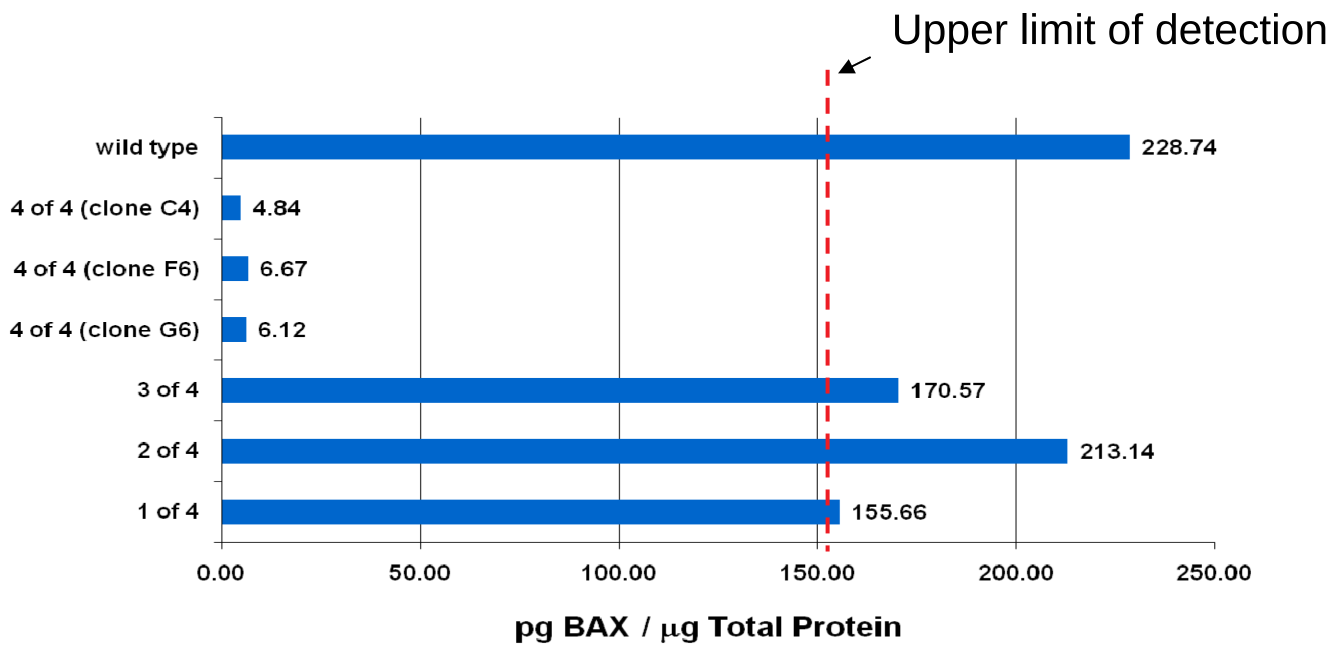
tetraploid



Spectral Karyotyping of A549 cells

Tetraploid BAX Knockout via Single ZFN Treatment in Human A549 Cels

AGGAAGTCCAATGTCCAGCCCATGATGGTTCTGATCAGTTCCGGCACCTTGGTGCACAGGGCCTGTGG	wt
AGGAAGTCCAATGTCCAGCCCATGATGGT--TGATCAGTTCCGGCACCTTGGTGCACAGGGCCTGTGG	-2
AGGAAGTCCAATGTCCAGCCCATGATGGTT----TCAGTTCCGGCACCTTGGTGCACAGGGCCTGTGG	-4
AGGAAGTCCAATGTCCAGCCCATGATG-------TCAGTTCCGGCACCTTGGTGCACAGGGCCTGTGG	-7
AGGAAGTCCAATGTCCAG-------------------TCCGGCACCTTGGTGCACAGGGCCTGTGG	-19



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Suzanne Hibbs, Sigma-Aldrich

Summary

ZFNs are an efficient method for targeted optimisation of bioproduction cell lines

- Growth
- Selection
- Glycosylation / sialidation
- Interfering proteins

Single-treatment complete gene knockout

- No selection
- Polyploidy not a barrier

Externally validation in bioproduction process

Broad cell type utility

What Next for ZFN-Based Cell Engineering…

Product Quality/Stability

- Sialidation consistency
- Sialic acid type (humanised)

Interfering Host Cell Proteins

- Endogenous molecule targets
- Co-purifying host-cell proteins

Targeted Integration

- Hot spot identification & targeting

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Laetitia Malphettes

FUT8 Knockout in CHO Cells

Highly Efficient Deletion of *FUT8* in CHO Cell Lines Using Zinc-Finger Nucleases Yields Cells That Produce Completely Nonfucosylated Antibodies

Laetitia Malphettes,[1] Yevgeniy Freyvert,[2] Jennifer Chang,[1] Pei-Qi Liu,[2] Edmond Chan,[2] Jeffrey C. Miller,[2] Zhe Zhou,[1] Thu Nguyen,[1] Christina Tsai,[1] Andrew W. Snowden,[1] Trevor N. Collingwood,[2] Philip D. Gregory,[2] Gregory J. Cost[2]

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Malphettes, et al., (2010) Biotech. Bioeng. 106, 774-783

Multiple Gene Knockout in Cell Lines

Generation of a Triple-Gene Knockout Mammalian Cell Line Using Engineered Zinc-Finger Nucleases

Pei-Qi Liu,[1] Edmond M. Chan,[1] Gregory J. Cost,[1] Lin Zhang,[2] Jianbin Wang,[1] Jeffrey C. Miller,[1] Dmitry Y. Guschin,[1] Andreas Reik,[1] Michael C. Holmes,[1] John E. Mott,[2] Trevor N. Collingwood,[3] Philip D. Gregory[1]

Liu, P, *et al*., (2010) *Biotechnology and Bioengineering*, 106(1): 97-105

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Reducing Apoptosis in CHO Cell Culture

BAK and *BAX* Deletion Using Zinc-Finger Nucleases Yields Apoptosis-Resistant CHO Cells

Gregory J. Cost,[1] Yevgeniy Freyvert,[1] Annamaria Vafiadis,[2] Yolanda Santiago,[1] Jeffrey C. Miller,[1] Edward Rebar,[1] Trevor N. Collingwood,[1] Andrew Snowden,[2] Philip D. Gregory[1]

Cost, G, et al., (2010) Biotech. Bioeng. 105, 330-340

ZFN-Driven Targeted Gene Knockout
CHO DHFR

Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases

Yolanda Santiago*, Edmond Chan*, Pei-Qi Liu*, Salvatore Orlando*, Lin Zhang†, Fyodor D. Urnov*, Michael C. Holmes*, Dmitry Guschin*, Adam Waite*, Jeffrey C. Miller*, Edward J. Rebar*, Philip D. Gregory*‡, Aaron Klug‡§, and Trevor N. Collingwood*

Santiago et al, 2008, PNAS 105, 5809.

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