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1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED Final Progress Report 6/1/05-8/31/06

4. TITLE AND SUBTITLE Genetic Screening of Cells with Enhanced Antibody Production	5. FUNDING NUMBERS W911NF-05-C-0059
6. AUTHOR(S) Roxanne Duan, Ph.D.	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Functional Genetics, Inc. 708 Quince Orchard Rd. Suite 200, Gaithersburg, MD 20878	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U. S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211	10. SPONSORING / MONITORING AGENCY REPORT NUMBER 48758.1-LS-DRP

11. SUPPLEMENTARY NOTES

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12 a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited.	12 b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Here we report the progress made for grant No. W911NF-05-C-0059 between the entire funding period, 6/1/05 and 8/31/06. We have completed the study proposed: to use Functional Genetics' proprietary technology, Randon Homozygous Knock Out (RHKO) to increase antibody production from a production cell line, in this case CHO cells, as measured by Specific Productivity Rate (SPR). We also identified 2 candidate RHKO genes that cause increased SPR. Finally, we report the validation of one of these two genes.

14. SUBJECT TERMS	15. NUMBER OF PAGES
	16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION ON THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	**UNCLASSIFIED**	**UNCLASSIFIED**	**UL**

NSN 7540-01-280-5500

Standard Form 298 (Rev.2-89)
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298-102

Enclosure 1

Final Progress Report
DARPA Grant No. **W911NF-05-C-0059**
Genetic Screening of Cells with Enhanced Antibody Production

By

Roxanne Duan, Ph.D.
Functional Genetics, Inc.
January 22, 2007

Summary

Here we report the progress made for grant No. W911NF-05-C-0059 between the entire funding period, 06/01/05 and 8/31/06. We have completed the study proposed: to use Functional Genetics' proprietary technology, Random Homozygous Knock Out (RHKO) to increase antibody production from a production cell line, in this case CHO cells, as measured by Specific Productivity Rate (SPR). We also identified 2 candidate RHKO genes that cause increased SPR. Finally, we report the validation of one of these two genes.

Specific Aims Proposed

In the proposal submitted to DARPA in March, 2005, Functional Genetics proposed to use its proprietary RHKO (Random Homozygous Knock Out) technology, which inactivates or activates genes at random in a genome wide manner, to improve the specific productivity (pg antibody produced per cell per day) of an antibody producing cell line. This effort is expected to lay the foundation for the engineering of antibody producing cell lines with greatly, i.e 10-20 fold, enhanced specific productivity for industrial use. This could, in turn, dramatically reduce production costs for antibody drugs, including those for Anthrax and Botulinum toxin. We had set forth the following specific aims and timeline:

Timeline	Specific Aim
Month 1-2	1. Construct RHKO vector for CHO or NS0 mAb producing cell line
Month 1-2	2. Develop high throughput FACS screening assay for antibody specific productivity
Month 2-4	3. Generate RHKO libraries of CHO or NS0 mAb producer cell lines
Month 3-7	4. Screen and isolate RHKO clones of CHO or NS0 mAb producer cell clones with enhanced antibody specific productivity
Month 4-8	5. Characterize and validate the isolated RHKO clones with ELISA and functional reversibility assay
Month 5-10	6. Identify and clone the candidate genes whose inactivation or over-expression by RHKO leads to mAb producer cells with enhanced specific productivity
Month 8-12	7. Validate and charactetrize the roles of the candidate genes

Progress

At the beginning of this project, we obtained a stable antibody-producing CHO cell clone, 3559, from our collaborators at Xoma. This cell line has a reported specific productivity of 17 ng/cell/day of a humanized antibody therapeutic. Our goal for this current proposal is to identify genes that, any of which upon inactivation or activation singularly by our RHKO technology, will increase the specific productivity at least three fold, to 51 ng/cell/day or higher. In later projects, reiteration of the RHKO process is expected to generate *10 to 20 fold* higher specific productivity.

Specific Aim 1. Construct RHKO vector for CHO or NS0 mAb producing cell line

RHKO Gene Search Vector (GSV) was constructed on the basis of an MMLV backbone with features (see schematic representation below, Figure 1) that facilitate the genomic integration of GSV as well as selection of the resultant cells.

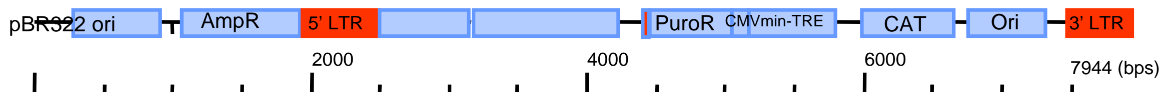


Figure 1. Linear schematic Representation of RHKO Gene Search Vector.

The viral LTRs facilitate the integration of the GSV cassette. The p15A origin (Ori) and Chloramphenicol acetyl transferase (CAT) enables cloning of the GSV-genomic fragment in E. *coli*. The inducible TRE-CMV minimal promoter provides a mechanism for reversing the transcription of the message, hence reversal of the phenotype of interest. We have engineered a Puromycin resistance gene for selecting CHGO cells that have GSV integration, rather than the Neomycin marker in our older version of GSV since the CHO cells expressing a human antibody already possess Neo resistance for antibody over expression.

Specific Aim 2. Develop high throughput FACS screening assay for antibody specific productivity

To screen RHKO libraries for high antibody producers, we needed to develop a high throughput FACS screening assay for initially identify RHKO cells that have higher antibody specific productivity than the beginning specific productivity. We developed a FACS assay based on a reported assay (Brezinsky et al., 2003) that measured the amount of the cell surface associated antibodies. This assay is based on the fact that antibodies

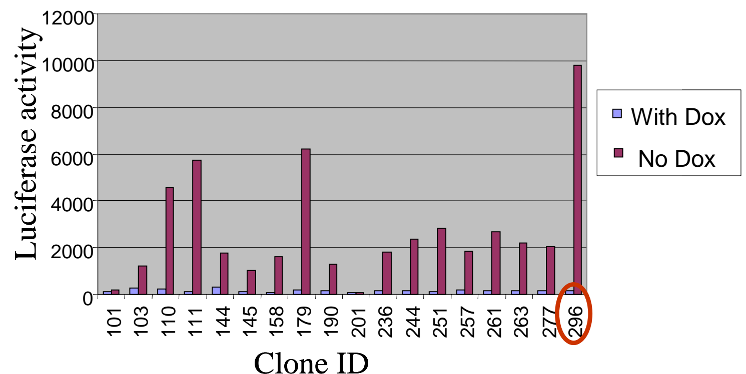
temporarily remain associated with the cell surface, at low temperature, before secretion and that the amount of surface associated antibody is proportional to the specific productivity of secreted antibody (Brezinsky et al., 2003). Thus, we stained the RHKO cells with a goat-anti-human antibody conjugated to fluorescien (FITC) and then used FACS to identify cells that had high level cell surface fluorescence. This assay was established to screen RHKO libraries. See Figure 3. for an example of a typical screen.

Specific Aim 3. Generate RHKO libraries of CHO mAb producer cell lines

To make RHKO libraries, we first needed to make a stable *tet* transactivator cell line using our target CHO antibody producing cells. This is because our RHKO technology is based on a gene insertion event of one allele followed by a tetracycline-regulated antisense inactivation of the second allele (Li and Cohen, 1996). This tetracycline-regulated expression of the antisense allows us to reverse the gene inactivation event and therefore validate the causal relationship between the gene and the observed phenotype, in this case the antibody specific productivity. A cell line with a high *tet* transactivator also allows us to readily distinguish the RHKO cells from the rest of the cells.

We transfected CHO cells with a cDNA vector that carried a *tet* transactivator coding sequence. After selection of positive *tet* transactivator stable cell clones with Hygromycin, we screened the levels of *tet* transactivator activities of 300 cell clones in two separate screening experiments. For this purpose, stable *tet* transactivator cell clones were transiently transfected with a reporter gene, luciferase, that is under the control of a *tet* promoter and then treated with Doxycycline, a tetracycline ananlog. Initially, we obtained a cell clone that showed 10 fold induction of reporter gene activity by Doxycycline. However, the RHKO libraries made with this cell line did not produce stable high producers. Later, we discovered one cell clone, T296, that showed a 70 fold induction of *tet* transactivator activity by Doxycycline (Figure21). We used this T296 cell line to generate RHKO libraries with our retroviral-based Gene Search Vector.

Figure 2. Screening of *tet* transactivator cell lines. Individual cell clones carrying stable *tet* transactivator gene were transiently transfected with a vector containinng a *tet*-regulated luciferase reporter gene. The luciferase activity was measured 48 hours after transfection.



Specific Aim 4 Screen and isolate RHKO clones of CHO mAb producer cell clones with enhanced antibody specific productivity

We have generate 6 independent RHKO libraries with T296 cell line and one with another transactivator cell line, T263, which showed 50 fold induction of *tet* transactivator activity. All RHKO libraries were subjected to at least two to three rounds of FACS selection for cells displayed high fluorescence signals resulted from immunostaining of the secreted antibodies that transiently remain associated with the cell surface at a low temperature. Two most successful libraries, one each from T296 and T263, showed histograms of FACS selected cells that have completely shifted away from the starting cell histogram towards high fluorescence, indicating the secretion of much higher amounts of antibodies than the starting cell population (Figure 3). This high fluorescence was presumably attributed to stable secretion of high amounts of antibodies rather than immunostaining variations since, according to our experience with some of the libraries, the histogram would have shifted back to the base line if the latter was true. We have collected individual cells, into 96 wells, that showed top 3% fluorescence levels in our third round of FACS experiments. These individual cell clones were cultured and analyzed for the reversal of RHKO and their specific productivity rates of antibodies (see Specific Aim 4).

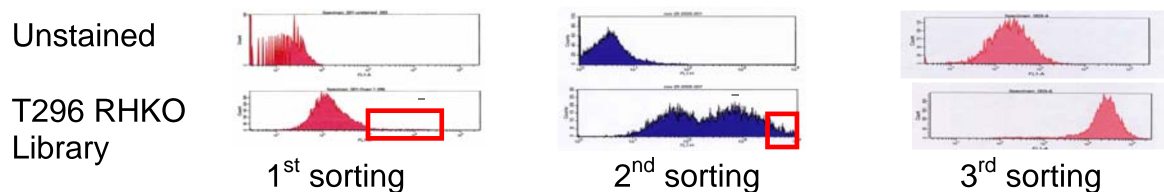


Figure 3. Selection of high antibody producing RHKO cells by FACS. One RHKO library made from stable *tet* transactivator cell lines, T296, that was derived from CHO cells engineered to produce a humanized antibody therapeutic, was stained with goat anti-human IgG-FITC conjugate and then selected by FACS for high antibody producers. Three rounds of FACS were performed. Top 3% cells were selected from each round (red box).

One difficulty encountered in the making of RHKO library was the decrease of transactivator activity in T296 and T263 cell lines over time. While these cell lines displayed 70 and 50 fold induction level of *tet* transactivator activities, respectively, in the beginning, the activities went down to about 10 fold after two months of culturing time. This lowered *tet* transactivator activity has prohibited the making of successful libraries as the selected 'high producers' (high fluorescence cells) reverted back to the starting cell population's fluorescence levels in later rounds of FACS experiments, indicating that even the top 3% fluorescence level that we selected cells at was not high enough or 'far enough' from the starting cells' fluorescence. This 'high' fluorescence level was probably due to immunostaining variation of 'low produces' rather than true fluorescence associated high levels of secreted antibodies. The decrease of the tet transactivator activity is most likely a result of contaminating cells in the clonal selection

process of the stable transactivator cell lines. To correct this problem, we are in the process of making new transactivator cell lines, taking extreme precaution in making sure the cell lines are stable single cell populations.

We have grown the 'high producer' CHO cells clonally, i.e. one cell per well in 96 well plates, for screening of their ability to secrete high levels of antibodies. The next step is to examine whether the amounts of secreted antibodies from these 'high producers' are indeed higher than the starting specific productivity of 17 ng/cell/day. Thus, we have set up a 'sandwiched ELISA' experiment for the secreted human antibody from our CHO cells. The ELISA uses an goat anti-human IgG gamma antibody to capture the secreted human antibody in cell culture supernatants. A horseradish peroxidase conjugated anti-human kappa antibody was then used to specifically recognize the secreted antibody.

Specific Aim 5: Characterize and validate the RHKO cell clones for high antibody secretion by ELISA and functional reversal of RHKO.

1. RHKO reversal:

To ensure that the high antibody secretion *is* a result from RHKO gene disruption event, we perform RHKO reversal experiments in order to pick cell clones for isolation of the RHKO affected gene. Clonal cell populations grown in 96 wells were split into two identical sets. One set of culture was treated with Doxycycline, which reverses the RHKO event by turning off the anti-sense mRNA expression, which is driven by the *tet* promoter in the RHKO Gene Search Vector cassette inserted in the particular gene. This results in the reversal of one allele of the RHKO-disrupted gene to 'normal' status. Depending on the 'gene dosage' required for the gene's function, the functiona of the gene can be completely or partially reversed. This reversal will validate the causal relationship between the RHKO affected gene and the high antibody secretion phenotype. We performed ELISA to measure the amounts of secreted antibodies on 400 clones three days after Doxycyvline treatment. By comparing the amounts of antibodies in the absence or in the presence of Doxycycline, we observed low levels of "RHKO reversal" in some clones. Thus, the ratio of antibodies in the absence versus in the presence of Doxycycline ranged from 1 fold to 2.2 fold). We picked clones that showed 1.7 fold to 2.2 fold reversal of antibody production for later analysis of their Specific Productivity Rates. These RHKO reversal rates seem lower than what we observed in the past in different phenotype selection assays. One reason could be that the cells continued to secrete antibodies for a period of time before Doxycycline treatment took effect on stopping the expression of the anti-sense mRNA. Another potential reason for low RHKO reversal could be that the antibody production level was increased only by about two fold. We did not feel that this observed low reversal will impede the progress since RHKO reversal could be partial at times and that the true validation will come from reproducing the RHKO effect in naïve cells with the isolated RHKO gene. We therefore moved onto measuring the Specific Productivity Rates of selected clones.

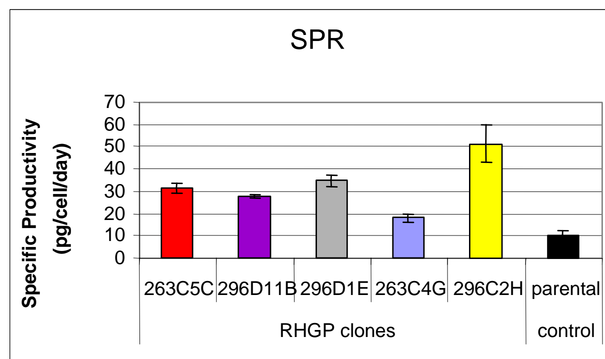
2. Specific Productivity Rates of RHKO cell clones

Our goal of the current project is to increase the Specific Productivity Rates (SPR) of commercial antibody producing cells by three fold. We set out to measure SPR, according to an established procedure (Brezinsky et al., 2003), on the cell clones that show some degree of RHKO reversal experiments. Thus, we determine cell number and measure amounts of secreted antibodies by sandwiched ELISA on different days and then calculate the SPR (pico gram antibody secreted per cell per day) according to the following equation:

$$SPR = \frac{\text{Amount of antibody}}{[(\text{Final cell No.} - \text{initial cell No.}) * \text{days in culture} / \log_e(\text{final cell No.}/\text{initial cell No.})]}$$

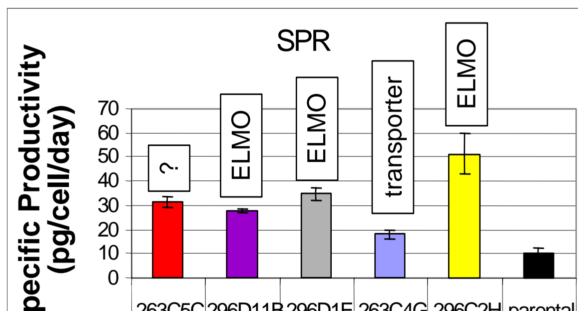
So far we observed SPRs that are 1-6 fold that of the parental cell population (Figure 4). This result along reflects the incomplete reversal of RHKO in our attempt to see RHKO reversal by Doxycycline treatment. At times we observe even higher SPR (up to 20 fold that of the parental SPR). However, these results need to be confirmed, as the experimental procedures are still being optimized. Many factors affect the accurate measurement of SPR, especially when only 3 fold of increase is sought after in the current proposal. These factors include accurate cell number determination, true logarithmic growth of all clones, and accurate ELISA results. For example, we need to improve the assays to insure logarithmic growth of hundreds of clones with good aeration of the 96 well plates, which can be achieved by constant shaking of deep culture wells, mimicking shaker flasks in industrial production.

Figure 4. Specific Productivity Rate of RHKO-antibody in logarithmic growth were cultured in 48 well plates. The amounts of secreted antibodies were measured by ELISA and the cell counts determined at the same time by a Coulter counter SPR was calculated according to Brezinski et al.



Specific Aim 6. Identify and clone the candidate genes whose inactivation or over-expression by RHKO leads to mAb producer cells with enhanced specific productivity

Figure 5. Candidate RHKO genes in RHKO-CHO cell clones with high Specific Productivity Rate. The RHKO genes in five CHO cell clones that showed high SPR were isolated. The candidate gene, ELMO, was identified to be integrated by the RHKO GSV in three cell clone (296-C2H, 296-D1E, and 296-D11B). Another candidate gene, an ion transporter was



By identifying the genome integration site of the GSV, we isolated candidate RHKO genes in five CHO cell clones that showed high SPR (see figure above). The candidate gene, ELMO-1 (Gumienny et al.), was identified to be integrated by the RHKO GSV in three cell clone (296-C2H, 296-D1E, and 296-D11B). We are the first group to clone the CHO ELMO-1 gene. ELMO-1 is highly conserved among mammalian species (Sequences shown below). Another candidate gene, a predicted multi-transmembrane transporter protein, was identified in cell clone 263-C4G. The GSV genome integration site for cell clone 263-C5C was identified but no candidate gene could be assigned as that genomic locus does not have homology to genomic sequences of human, mouse, or any other sequenced

Figure 6. cDNA Sequences of A Candidate RHKO Gene, ELMO-1, that Resulted in Increased SPR. Sequences from top to bottom line, are rat, mouse, CHO, and human

```
Rat Transpor      1 mafgnlacivcklhfisktndnvlsgcgllgdpcpaqrppgilrrpgcpsprpcvcpgggdgarlegalvrrsgaagwlrargfrerpks
Mouse transp        ------------------------------------------------------------------------------------------
CHO ITP Prot        ------------------------------------------------------------------------------------------
human transp        ------------------------------------------------------------------------------------------

Rat Transpor     91 vedlltrgrprlqhnahrcpqgpesrqgrgsgssssaasaqqrraselggrpgrgadeprrlrartssarrpgrggarvtwgwpdspgpe
Mouse transp        ------------------------------------------------------------------------------------------
CHO ITP Prot        ------------------------------------------------------------------------------------------
human transp        ------------------------------------------------------------------------------------------

Rat Transpor    181 hslrpralllgggavpgrppssaalyaplrapralamaidrrreaagsgagrqpapaeengslppgdaaasaplggragsggsaeiqplp
Mouse transp      1 ------------------------------------maidrrreaagsgagrqpapaeengslppgdaaasaplggragsgssaeiqplp
CHO ITP Prot      1 ------------------------------------maidrrreaagsgagrqpppaeengslppgdaaasaplggragsggsaeiqplp
human transp        ------------------------------------------------------------------------------------------

Rat Transpor    271 alhpsggphssccaaaaapslllldydgsvlpflgglgggyqktlvvltwipalfigfsqfsdsflldqpnfwchgagkgtelagatvtg
Mouse transp     55 alhpsggphssccaataapslllldydgsvlpflgglgggyqktlvvltwipalfigfsqfsdsflldqpdfwcrgagkgtelagatvtg
CHO ITP Prot     55 alhpsggphssccaaaaapslllldydgsvlpflgglgggyqktlvvltwipalfigfsqfsdsflldqpnfwcrgagkgtelagstvtg
human transp        ------------------------------------------------------------------------------------------

Rat Transpor    361 rwgdmgnwtspsatpfstaswgttsnrsnssdtpplpsppgkgnndsncechawdygirtglvqnvvskwdlvcdnawkvhvakfsllvg
Mouse transp    145 rwgdmgnwtspsatpfstaswgttsnrsnssdtpplpsppgkgnndsncdchawdygirtgliqnvvskwdlvcdntwkvhiakfsllvg
CHO ITP Prot    145 rwgdmgnwtsppanpfstaawgtasnrsnssdtpplpsppgkgnndsncdchawdygirtglvqnvvskwdlvcdnawkvhiakfsllvg
human transp        ------------------------------------------------------------------------------------------

Rat Transpor    451 lifgylitgciadwvgrrpvllfsvifilifgltvalsvnvtmfstlrffegfclagiiltlyalrielcppgkrfiitmvasfvamagq
Mouse transp    235 lifgylitgciadwvgrrpvllfstifilifgltvalsvnvtmfstlrffegfclagiiltlyalrielcppgkrfiitmvasfvamagq
CHO ITP Prot    235 lifgylitgciadwvgrrpvllfsiifilifgltvalsvnvtmfstlrffegfclagviltlyalrielcppgkrfmitmvasfvamagq
human transp      1 ----------------------------------------------------------------------------mitmvasfvamagq

Rat Transpor    541 flmpglaalcrdwqvlqaliicpfllmllywsifpeslrwlmatqqfesakklilyltqkncvspesdikgvmpelekelsrrpkkvciv
Mouse transp    325 flmpglaalcrdwqvlqaliicpfllmllywsifpeslrwlmatqqfesakklilyltqkncvspesdikgvmpelekelsrrpkkvciv
CHO ITP Prot    325 flmpglaalcrdwqvlqaliicpfllmllywsifpeslrwlmatqqfeaakklilyitqkncvspesdikgvmpelekelsrrpkkvciv
human transp     15 flmpglaalcrdwqvlqaliicpfllmllywsifpeslrwlmatqqfesakrlilhftqknrmnpegdikgvipelekelsrrpkkvciv

Rat Transpor    631 kvvgtrnlwknivvlcvnsltgygihhcfarsmmghevkvpllenfyadyytmasialasclamclvvrflgrrgglllfmiltalasll
Mouse transp    415 kvvgtrnlwknivvlcvnsltgygihhcfarsmmghevkvpllenfyadyytmasialasclamclvvkflgrrgglllfmiltalasll
CHO ITP Prot    415 kvvgtrnlwknivvlcvnsltgygihhcfarsmmghevkvpllenfyadyytmasialasclamcvvvrflgrrgglllfmiltalasll
human transp    105 kvvgtrnlwknivvlcvnsltgygihhcfarsmmghevkvpllenfyadyyttasialvsclamcvvvrflgrrgglllfmiltalasll

Rat Transpor    721 qlgllnligkysqhpdselqlklavgmsdsvkdkfsiafsivgmfashavgslsvffcaeitptvircgglglvlasagfgmltapiiel
Mouse transp    505 qlgllnligkysqhpdselqlklavgmsdsvkdkfsiafsivgmfashavgslsvffcaeitptvircgglglvlasagfgmltapiiel
CHO ITP Prot    505 qlgllnligkysqhpds--------gmsdsvkdkfsiafsivgmfashavgslsvffcaeitptvircgglglvlasagfgmltapiiel
human transp    195 qlgllnligkysqhpds--------gmsdsvkdkfsiafsivgmfashavgslsvffcaeitptvircgglglvlasagfgmltapiiel

Rat Transpor    811 hnqkgyflhhiifacctliciicilllpesrdqnlpeniangehytrqpllshkkgeqpllltnaelkdysglhdvaavgdglsegatan
Mouse transp    595 hnqkgyflhhiifacctliciicilllpesrnqnlpeniangehytrqpllphkkgeqpllltnaelkdysglhdvaavgdglpegatan
CHO ITP Prot    587 hnqkgyflhhiifacctliciicilllpesrdqnlpeniangehytrqpllphkkgeqpllltnaelkdysglhdvaavgdglpegatan
human transp    277 hnqkgyflhhiifacctliciicilllpesrdqnlpenisngehytrqpllphkkgeqpllltnaelkdysglhdaaaagdtlpegatan
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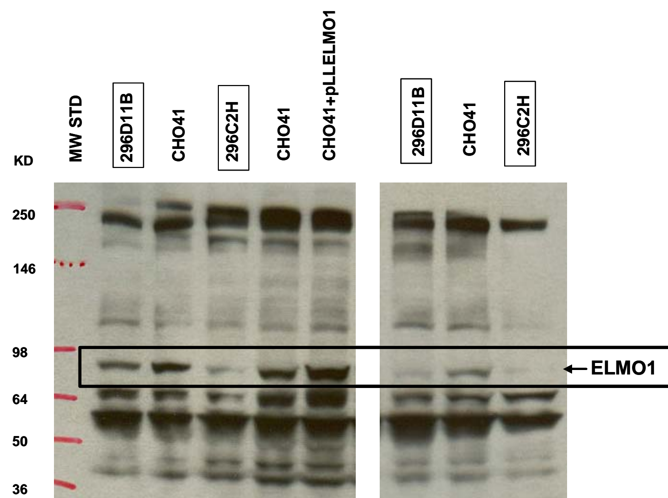
Specific Aim 7. Validate and characetrize the roles of the candidate genes



<u>Figure 7. ELMO Protein Is Down-regulated in RHKO-CHO Cell Clones that Showed Increased SPR.</u> Western blotting showed that ELMO-1 protein level was reduced in RHKO-CHO cell clones (296-C2H, 296-D11B)that had increased SPR, relative to the ELMO-1 protein levels in parental cells (CHO41).

Among the candidate RHKO genes identified, ELMO-1 was identified in three of the five clones. It also has commercially available reagents such as antibodies for protein work . We therefore choose to validate ELMO-1 gene for its role in increasing SPR of an antibody-producing cell. The RHKO GSV integrated into intron 15 of the CHO cell ELMO-1 gene. The direction of the “RHKO anti-sense mRNA” was opposite to that of the ELMO-1 transcript, predicting a “knockout’ (or severe knockdown) phenotype, as opposed to an “over-expression” phenotype. To validate that ELMO, we first examined whether disruption of ELMO-1 gene resulted in the reduction of ELMO-1 protein levels, as would be predicted by the direction of the RHKO anti-sense mRNA. Indeed, ELMO-1 protein levels were reduced in RHKO-CHO cell clones (296-C2H, 296-D11B)that had increased SPR, relative to the ELMO-1 protein levels in parental cells (CHO41) (Figure 7).

We then used anti-sense DNA validate the role of ELMO-1 gen in increasing SPR. When ELMO-1 anti-sense DNA was trasfected into cells, the ELMO-1 protein levels were reduced but not completely ‘knocked out’. There is a good correlation between the existence of anti-sense DNA, reduced protein levels and increased SPR (Figure 8). We then try to further validate ELMO-1 by siRNA. To date, the siRNA results are not

conclusive. This could be due to many technical reasons. Further experiments are needed. We are also trying to add back ELMO-1 into the original RHKO-CHO cell clones such as 296-C2H to examine whether SPR can revert to that of the parental cells.

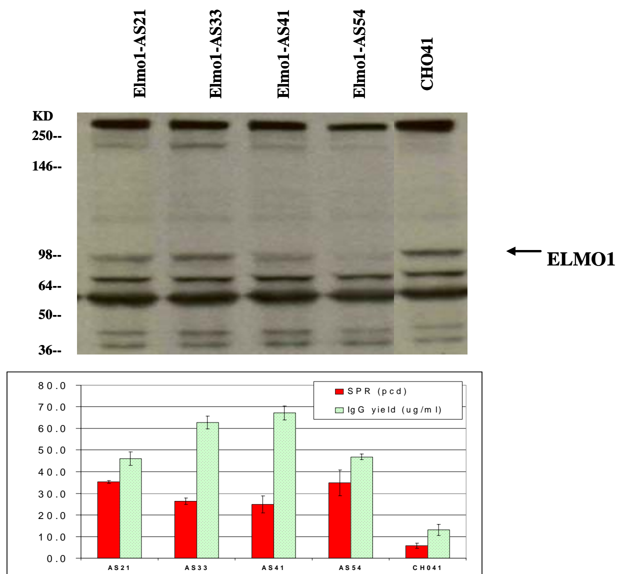


Figure 8. Effect of ELMO-1 Antisense DNA on ELMO-1 protein expression and SPR. CHO41 parental cells were stably transfected with ELMO-1 anti-sense DNA. Individual cell clones were picked and analyzed for ELMO-1 protein levels (top panel) and SPR (bottom panel). In the bottom panel, green bars represent total IgG levels and red bars represent SPR.

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