Generation of Galectin-3-Binding Protein (LG3BP) F357W Mutant as a Crucial Resource for Characterization of Sulf-2/LG3BP Interaction

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The galectin-3 binding protein (LG3BP) was identified as an interacting partner for extracellular 6-O-endosulfatase Sulf-2. Interaction with LG3BP inhibits Sulf-2 activity, leading to changes of heparan sulfation that is involved in numerous biological processes, including cancer cell proliferation, migration and/or invasion. Generation of LG3BP F357W mutant is based on structure models generated by Dr. C. Barinka. We generated the F357W mutant to test the hypothesis that the mutant LG3BP disrupts Sulf-2-LG3BP interactions and eliminates the LG3BP-mediated inhibition of Sulf-2 activity.

Specific Aims

Generate a mammalian cell line for production of mutant galectin-3 binding protein (LG3BP) to further characterize the interaction between LG3BP and Sulf-2.

Over 1.7 million new cancer cases were reported and 608,000 people died of cancer in the United States in 2021, according to the Center for Disease Control (CDC, 2024). While cancer cases are overall decreasing, there are still 403 new cancer cases for every 100,000 people. This study paves the ground for future studies of LG3BP impact on Sulf-2 mediated carcinogenesis.

Sulf-2 is overexpressed in tumor cells and is described as an oncogene, due to its regulation of heparan sulfate proteoglycan (HSPG) interactions through post-synthetic 6-O-desulfation of the heparan sulfate (HS) chains (Figure 1) (Yang 2022, Rosen, 2010). HSPGs regulate signaling pathways due to their ability to bind to a diverse range of protein ligands (Esko, 2001). HSPGs consist of a core protein with covalently bound HS chains that consist of alternating N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) disaccharide units (Masri, 2017). The HS chains are responsible for interactions with the protein ligands and undergo extensive modifications, including N-deacetylation, N-sulfation, epimerization of GlcA to iduronic acid (IdoA), and sulfation at the 2-O-sulfate, 3-O-sulfate, and 6-O sulfate (Figure 2) (Masri, 2017). Selective post-synthetic 6-O-desulfation by Sulf-2 is one of the key regulators of the protein interaction and, therefore, HSPG function. Prior studies have shown the importance of 6-O-desulfation of HS in regulating the signal pathways of many growth factors, including Wnt (Dhoot, 2001), FGF-1, FGF-2 (Pye 2000), VEGF, SDF-1 (Uchimura, 2006). In addition to oncogenesis, Sulf-2 is also associated with embryonic development.

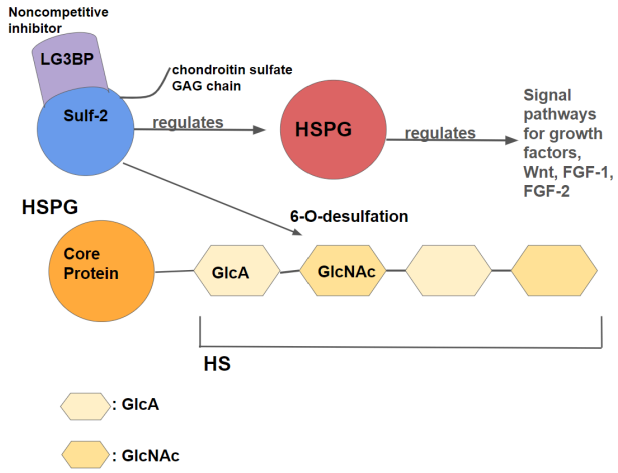

Fig 1. LG3BP/Sulf-2/HSPG relationship. LG3BP, Sulf-2, and HSPG are colored *purple*, *blue,* and *red*, respectively. The HSPG core protein, GlcA, and GlcNAc are colored in *orange* and varying shades of *yellow,* respectively.

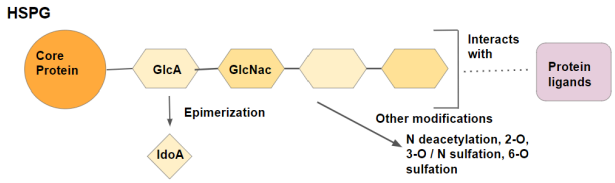

Fig 2. HSPG modifications and interactions. The HSPG core protein, GlcA, GlcNAc, and IdoA are colored in *orange* and varying shades of *yellow,* respectively. Protein ligands are colored in *pink*.

Sulfs consist of a catalytic domain, a cleavable signal sequence, a hydrophilic domain (HD) and a C-terminal region (Vives, 2014). The presence of the HD in Sulfs allows it to bind to HS with high-affinity and specifically catalyze the 6-O-desulfation (Seffouh, 2019, Masri 2017). Sulfs are overexpressed in many types of cancer (Vives, 2014). However, the specificity of Sulfs for HSPG or regulation of Sulf activity remain poorly understood. We hypothesize that the activity of the Sulf enzymes heavily depends on complexes with other proteins and our recent studies of the LG3BP strongly support this hypothesis (ref to Aswinis paper). We pursue, therefore, the long-term goal of deciphering how complexes of the Sulf enzymes regulate HSPG structure and function.

LG3BP (also known as Mac-2 binding protein) interacts with Sulf-2 *in vitro* and inhibits its enzymatic activity (Panigrahi, 2024). LG3BP is a secreted glycoprotein overexpressed in many cancers and identified as a potential therapeutic target (Capone, 2021). LG3BP inhibits the activity of Sulf-2 in a dose-dependent manner, requiring 40 times excess of LG3BP protein for 50% inhibition of Sulf-2 (Panigrahi et. al, 2024). This is in line with the expected *in vivo* concentrations of the two proteins, which suggests an *in vivo* regulation of the Sulf-2 activity by LG3BP. Our laboratory documented that LG3BP/Sulf-2 interactions decrease invasion of head and neck squamous cell carcinoma (HNSCC) cells with an associated fibroblast into Matrigel (Panigrahi, 2024). Recent studies established that Sulf-2 is a proteoglycan carrying a chondroitin sulfate glycosaminoglycan (GAG) chain, which contributes to regulation of its enzyme activity (Masri, 2022). We have also seen that the presence of the chondroitin sulfate on Sulf-2 decreases the binding of LG3BP (Panigrahi, 2024). Subsequent removal of the chondroitin sulfate increases LG3BP affinity for Sulf-2, inducing stronger inhibition of Sulf-2 activity (Panigrahi, 2024). LG3BP is a non-competitive inhibitor of Sulf-2. Our models suggest that it binds to the catalytic domain at a site distant from the active site and the current study is designed to test this hypothesis.

Experimental Procedures

Materials

LG3BP plasmid (OriGene, RC204918), XL10 Gold & Top10 *E.coli* cells (Invitrogen), pHR-CMV-TetO2_3C-TwinStrep_IRES-EmGFP lentiviral vector (Addgene, plasmid # 113884), sequencing, insertion, and mutagenic primers (Thermo Fisher Scientific-Invitrogen Oligos), Lenti-X HEK293T cells (Takara # 632180), Eagle's Minimum Essential Medium (Corning), Freestyle 293 Expression Medium (Invitrogen), packaging (psPAX2, Addgene #

12260) and envelope vectors (pMD2.G, Addgene #12259), polyethylene (polysciences #23966), polybrene (Santa Cruz Biotechnology), Trypsin protease (Thermo Scientific), HEK293F cells, Phusion Plus PCR Master Mix (Thermo Fisher Scientific), HisPur Ni-NTA resin (Thermo Scientific), gravity columns, Amicon Ultra-0.5 and Amicon Ultra-15 (Millipore), Bolt NuPAGE 4-12% polyacrylamide gels (Thermo Fisher Scientific), BSA (NEB), LGALS4BP Mouse Antibody (Protein Tech 60066-1-Ig), Anti-mouse antibody, Cellometer (Nexcelom Bioscience) and other reagents obtained from Thermo Fisher Scientific.

Cell Culture

Wild-type human embryonic kidney HEK293F cells (Invitrogen) were grown in suspension culture in FreeStyle293 medium (Invitrogen) at 37ºC / 5% CO_2 atmosphere on a rotating platform at 140 rpm. Cells were subcultured at 2.5-3 x 10^6 cells/ml by 10-fold dilution in fresh culture medium. HEK293F cells overexpressing wild-type LG3BP with C-terminal TwinStrep-6xHis tags were generated as reported (Panigrahi 2024) and cultured as above. Adherent Lenti-X HEK293T cells (Takara # 632180) were grown in DMEM-F12 medium (Corning) supplemented with 10% FBS and non-essential amino acids (NEAA, Gibco) under 37ºC / 5% CO_2 atmosphere and subcultured by trypsinization at 90% confluence.

Sulf-2 and Mutant LG3BP/Sulf-2 AlphaFold Models

Predicted structures of LG3BP (Uniprot, Q08380) and Sulf-2 (Uniprot, Q8IWU5) were generated in PyMOL. LG3BP and Sulf-2 coloring was done by making the amino acid sequence visible (“SEQ”) and highlighting the expected sequences/portions of the protein. Mutation of LG3BP from F357 (phenylalanine, TTT) to W (tryptophan, TGG) was done through PyMOL’s “Wizard” feature.

Generation of LG3BP F357W Mutant

pCMV6 expression vector containing wild-type LG3BP ORF (Origene # RC204918) was subjected to inverse PCR to introduce F357W mutation using CTCCCTGAGGAGCTCT**GG**GAGCTGCAG TTCAACCTGTCC (forward) and AGAGCTCCTCAGGGAGCATC (reverse) primers (the mutation site is highlighted in bold). The PCR was carried out using DNA Phusion Plus PCR Master Mix (Thermo Fisher Scientific), 10 ng of template DNA and 500 nM of each primer in 20 µl reaction with following steps: initial cycle of 95ºC/30s followed by 25 cycles of 95ºC/30s (denaturation)-60ºC/30s (annealing)-72ºC/5min (extension) and final extension 72ºC/5min. The product of PCR reaction was then subjected to FastDigest DpnI nuclease (Thermo) according to manufacturer’s instructions to remove *dam* methylated wild-type template DNA. The product was verified by 1% agarose gel electrophoresis and visualization by SafeGreen DNA stain (IntellixBio). 2 µl of PCR reaction mix were used to transform recA-deficient Top10 *E.coli* (Invitrogen for In-Vivo Assembly as described (Garcia-Nafria et. al 2016).

Assembly of Lentiviral Vector

pHR-CMV-TetO2_3C-TwinStrep_IRES-EmGFP lentiviral vector (Addgene, plasmid # 113884) was subjected to restriction digest with Xba1 and Kpn1 restriction enzymes and incubated for 3 hours at 37ºC. Restriction digested pHR-CMV-TetO2_3C-TwinStrep_IRES-EmGFP lentiviral vector and LG3BP mutant DNA was isolated with 1% agarose gel electrophoresis and visualization by SafeGreen DNA stain (IntellixBio). Restriction digested pHR-CMV-TetO2_3C-TwinStrep_IRES-EmGFP lentiviral vector and LG3BP mutant DNA bands were excised from the gel and placed in pre-weighted 1.5 ml tubes. DNA from each gel piece was harvested through gel extraction with

the following steps: weigh gel piece, add 3 volumes of QG buffer, incubation at 50ºC/10min, add 1 gel volume isopropanol, transfer to spin column, centrifuge 18,000g/1min, add 500 µl QG buffer (wash), centrifuge 18,000g/1min, add 750 µl PE buffer (wash), incubate 5 min at 37ºC, centrifuge 18,000g/1min, centrifuge empty column at 18,000g/3min, place in clean collection tube, add 30 µl elution buffer (pre-warmed at 50ºC), incubate 5 min at 37ºC, centrifuge 18,000g/3min (Qiagen). Concentration of DNA obtained per sample was measured by NanoDrop Spectrophotometer (Thermo). 2 µl Master Mix, 200 ng of DNA (LG3BP mutant DNA and lentiviral plasmid DNA, molar ratio 2(LG3BP mutant DNA):1(lentiviral plasmid DNA)), and double-distilled water (ddH2O, Thermo) was used for the assembly mixture to a total volume of 10 µl. Assembly mixture was incubated at 50ºC/15 min. The product was verified by 1% agarose gel electrophoresis and visualization by SafeGreen DNA stain (IntellixBio). 2 µl of assembly mixture were used to transform NEB stable competent *E.coli* (New England Biolabs, C3040H) for In-Fusion Cloning (Takara).

Generation of LG3BP Mutant Viral Particles

Transfection mix containing 23.3 µg assembled transfer plasmid DNA, 23.3 µg envelope vector pMD2.G (Addgene # 12259) DNA, 23.3 µg packaging vector psPAX2 (Addgene #12260)) DNA, 175 µl polyethylenimine (PEI), and 325 µl DMEM/F12/SFM was prepared with a DNA:PEI ratio of 1:2:5 and incubated for 30 min. Lenti-X HEK293T cells (Takara # 632180) were transfected with 1 ml of the transfection mix and grown for 3 days in DMEM-F12 medium (Corning) supplemented with 10% FBS and non-essential amino acids (NEAA, Gibco) under 37ºC / 5% CO_2 atmosphere to generate viral particles. Successful generation of particles was confirmed with green fluorescent protein (GFP) fluorescence. Viral particles were then concentrated by centrifugation at 800 x g for 5 minutes through a sterile 0.45 µm syringe filter. Concentrator solution was prepared consisting of 80g PEG-8000, 14g NaCl in 80 ml ddH2O, and 20 ml of 10X PBS (pH 7.4), then brought to a volume of 200 ml, filtered through a 0.2 µM filter, and stored in 4ºC. 1 volume of concentrator was added to every 3 volumes of supernatant and incubated overnight to optimize recovery. The solution was centrifuged at 1600 x g for 60 min at 4ºC. The pellet was resuspended with FreeStyle293 medium (Invitrogen) with 1/10 of original volume then stored in 80ºC until use.

Concentration of viral particles was measured using a sandwich enzyme-linked immunosorbent assay (ELISA). Coating, blocking, standard, detection, and washing was done with 100 µl/well of HIV-I-GAG-p24 antibodies, 100 µl/well of phosphate-buffered saline (PBS)/1% bovine serum-albumin(BSA)/0.05%RDBT/0.01%Tw20, 100 µl/well HIV-I-GAG-p24/STD/0.05%RDBT, 100 µl/well detection antibody, and 0.05% PBS, respectively. Detection antibody was measured with an anti-mouse antibody. 100 µl/well of substrate was added and after 15 min 50 µl/well of H2SO4 was added to stop the reaction.

Infection of HEK293F Cells

HEK293F cells were infected by viral particles as described (Elegheert, 2018). 1 million HEK293F cells were brought to 2 ml with Freestyle 293 medium (Invitrogen) in a six-well plate then incubated for 24 hours at 37ºC / 5% CO_2 atmosphere on a rotating platform at 140 rpm. 1 ml viral particles and 3 µl polybrene (Santa Cruz Biotechnology) was added to the six-well plate and incubated for 3 days at the same conditions mentioned previously. Successful infection was confirmed with green fluorescent protein (GFP) fluorescence. Cells were transferred to 125 ml

flasks once 6-well plate density reached 2.5-3 x 10^6 cells/ml.

Confirmation of Mutant LG3BP in HEK293F Cells

Verification of mutant LG3BP in HEK293F cell media was done with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot analysis. Samples consisted of conditioned media harvested from HEK293F media (negative control), 200 ng purified LG3BP protein from prior study (positive control, Panigrahi et. al, 2024), conditioned media from HEK293F cells containing mutant LG3BP, and conditioned media from HEK293F cells containing wild-type LG3BP. Negative control, HEK293F cells containing wild-type LG3BP, and infected HEK293F cells containing mutant LG3BP consisted of 26.6 µl conditioned media, 10 µl lithium dodecyl sulfate (LDS), and 3.4 µl dithiothreitol (DTT). Positive control consisted of 2 µl purified LG3BP protein, 6 µl double-distilled water, 3 µl LDS, and 1 µl DTT. All samples were incubated for 80ºC / 5 min. Western blot consisted of the following steps: addition of 10 ml primary LG3BP mouse antibody 1:2,500 dilution in 5% bovine serum-albumin (BSA)/1X TBS/0.01% Tween20 overnight, 4x 10 ml TBST (1X TBS, 0.01% Tween20) with incubation for 5 min each wash, addition of 10 ml secondary anti-mouse antibody diluted 1:100,000 with 1 hour incubation, 4x 10 ml TBST (1X TBS, 0.01% Tween20) with incubation for 5 min each wash, addition of horseradish peroxidase substrate (thermo) with incubation for 5 min, followed by imaging with chemiluminescence.

Production and Purification of the Mutant and Wild-type LG3BP

Conditioned media was harvested from viral transduced HEK293F cells after each subculture for purification of protein. Conditioned media was centrifuged at 300 x g/5min then 2400 x g/10min to remove cells and cell debris. A gravity column was used to purify LG3BP wild-type and LG3BP mutant from the conditioned media with the following steps: rotation overnight at 4ºC with PBS/0.5M NaCl/5% glycerol binding buffer and 1 ml of HisPur Ni-NTA resin (Thermo Scientific) (binding), 40 ml PBS/0.5M NaCl/5% glycerol/10 mM Imidazole washing buffer (wash), 15 ml of PBS/0.5M NaCl/5% glycerol/250mM Imidazole elution buffer (elution), and 20 ml binding buffer (equilibrium). This process was repeated 3 more times to achieve higher purification with rotation binding for 1 hour at 37ºC instead of overnight binding. LG3BP purification efficiency and concentrations were measured through SDS-Page Coomassie Blue staining and Western Blots using Bolt NuPAGE 4-12% polyacrylamide gels (Thermo). Solution was replaced with TBS and concentrated 10X using 50K Amicon Ultra-filtration tubes (Millipore).

Co-LG3BP and Sulf-2 Activity Assays

Purified wild-type LG3BP and mutant LG3BP were incubated with Sulf-2 for 30 minutes. 4-MUS and arylsulfatase activity assay were conducted on LG3BP/Sulf-2 mixture. Reaction was done in triplicates with 2.5 µl 1M Tris-HCl pH 7.5, 5 µl 100 mM $CaCl_2$, 0.5 µl 10% Tween 20, 50 ng Sulf-2, 1 mg LG3BP and 10 µl 20mM 4-methylumbelliferyl sulfate (4-MUS) (Alfa Aesar, #J66160). Reaction was incubated at 37ºC. 10 µl aliquots were taken at 0, 0.5, 1, 2, and 4 hours and added to 190 µl 0.5 M $NaCO_3$ in a 96-well plate. Fluorescence was measured at 360 nm (excitation max) / 450 nm (emission max).

Results

F357 (Phenylalanine) is the Predicted Interaction Site for LG3BP-Sulf-2

Recently, we identified LG3BP (gene name G3BP) as an interacting partner and inhibitor of Sulf-2 (Panigrahi et. al, 2024).

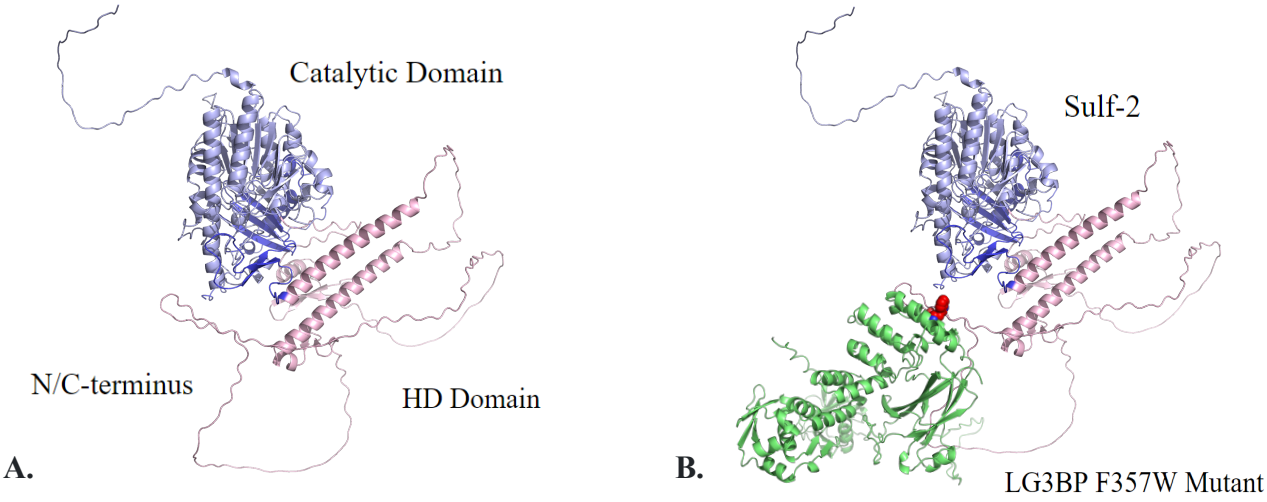

Fig 3. AlphaFold model of LG3BP-F357W/Sulf-2 complex. *A*, Structure of Sulf-2, the catalytic core, the hydrophilic domain, and the N/C-termini of Sulf-2 are colored *light blue*, *pink*, and *blue*, respectively. *B,* The predicted LG3BP-F357W/Sulf-2 complex; LG3BP-F357W is colored *green* and Sulf-2 is colored as in *Panel A*. The F357W (phenylalanine to tryptophan) mutant is colored *red*.

An AlphaFold model of the wildtype LG3BP/Sulf-2 complex with the Sulf-2 alteration can be found in our prior study (Panigrahi et. al, 2024). We developed an AlphaFold model of the LG3BP/Sulf-2 complex and identified F357 (phenylalanine, TTT) on LG3BP as the predicted binding site. We generated an AlphaFold model of the LG3BP/Sulf-2 complex including a mutated version of LG3BP F357 phenylalanine (TTT) to tryptophan (TGG) (Figure 3). We chose tryptophan to disrupt the binding of LG3BP to Sulf-2.

Generation of LG3BP F357W Mutant in Top10 E.coli cell line

From our previous identification of wild-type LG3BP as an interacting partner and inhibitor of Sulf-2 (Panigrahi, 2024), we hypothesized that a mutation to F357 on LG3BP would impact the ability of LG3BP to bind to Sulf-2 and inhibit its activity. To test our hypothesis, we created a CMV plasmid containing a mutated sequence of LG3BP from F357 phenylalanine (TTT) to tryptophan (TGG) (Supplementary Figure 1). We performed site-directed mutagenesis and selected colonies containing our mutant gene (Figure 4). We confirmed successful transformation of the F357W LG3BP mutant CMV plasmid through Genewiz sequencing (Azenta Lifesciences) using sequencing primers CTTCTGTGGCCACACGGTC (forward) and CTTCCAGGGCCAGGAGAGG (reverse).

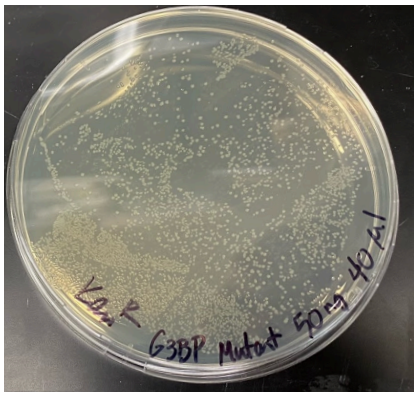

Fig 4. LG3BP-F357W Bacterial Colonies. LG3BP-F357W colonies on kanamycin plates, 50 ng of plasmid DNA in a 40 µl solution.

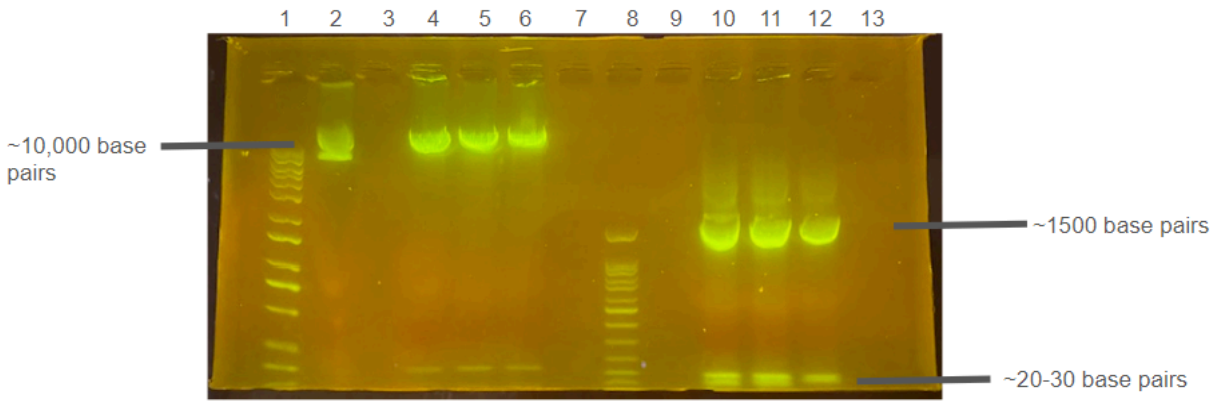

Fig 5. Lentiviral Vector Components Used for In-Fusion Cloning. CMV-pHR control (lane 2) Digested lentiviral plasmid (lanes 4-6) and LG3BP-F357W mutant (lanes 10-12). 20 µl of DNA sample and 4 µl loading dye was loaded lanes 2, 4-6, and 10-12. Lanes 1 and 8 contain 5 µl 1 to 10 kilobase (kb) protein ladders and 5 µl 100 base pairs to 1 kb ladders, respectively. 10 kb, 1500 base pairs, and 20 to 30 base pair bands are shown in lanes 4-6, 10-12, and 4-6 & 10-12, respectively.

Generation of HEK293F Cells Containing LG3BP Mutant

To produce LG3BP protein, we generated a lentiviral vector containing our mutant LG3BP gene to transduce HEK293F cells. The LG3BP mutant CMV plasmid confirmed by Genewiz sequencing (Figure 5) was grown in culture to increase plasmid DNA concentration. Mutant CMV Plasmid DNA was isolated through maxi prep. The mutant LG3BP gene was amplified with CTCCCTGAGGAGCTCTGGGAGCTGCAGT TCAACCTGTCC (forward) and AGAGCTCCTCAGGGAGCATC (reverse) primers. Restriction digest of pHR lentiviral plasmid using Xba1 and Kpn1 was used to create the insertion site for the lentiviral plasmid (Supplementary Figure 2). We obtained clear bands of the mutant LG3BP gene (1437 base pairs) and the restriction-digested pHR-CMV-TetO2_3C-TwinStrep_IRES-EmGF P lentiviral plasmid (Addgene, plasmid # 113884) for in-fusion cloning (Figure 4). The presence of two separate bands in our CMV-pHR control (lane 1) confirms that cleavage of our lentiviral plasmid occurred (Figure 4). The clear bands around our expected base pair size of 1998 base pairs and 10 kilobases (kb) confirmed the successful amplification of mutant LG3BP gene and digestion of lentiviral plasmid, respectively (Figure 6). The bands around 20-30 base pairs are likely primer dimers.

Mutant LG3BP and restriction-digested pHR-CMV-TetO2_3C-TwinStrep_IRES-EmGF P lentiviral plasmid (Addgene, plasmid # 113884) DNA was obtained through gel extraction. Concentration of mutant LG3BP DNA was 22.5, 10.55, and 16.35 ng/µl. Concentration of restriction-digested lentiviral plasmid DNA was 28.6, 17.9, and 21.4 ng/µl. Assembly was performed through in-fusion cloning using the gel extraction samples containing the highest concentrations of DNA for mutant LG3BP DNA and lentiviral plasmid DNA. We checked for the successful assembly of our lentiviral vector using PCR screening with the same sequencing primers as listed above and in addition to Genewiz sequencing (Azenta Lifesciences) using pHR sequencing primers CGGTACCGCGGGCCCGCCACCATGACCC CTCCGAGGCTC (forward) and CAGCACCTCAAGCCCGTCCACACCTGA GGAGTTGG (reverse).

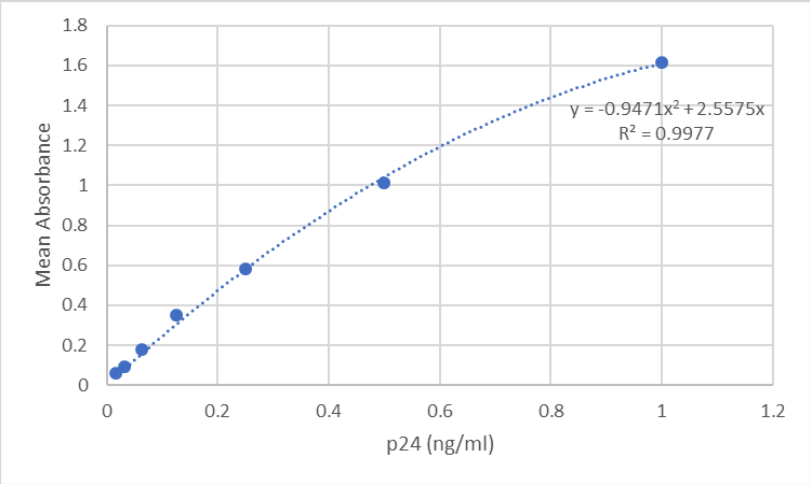

Fig 6. p24 Standard Curve Used to Approximate Amount of Lentiviral Particles. Lentiviral particle quantities were approximated using sandwich ELISA and ran in triplicates; p24-HIV protein was diluted using 2-fold dilutions of 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0 ng/ml.

After successful assembly of our pHR lentiviral vector, we performed lentiviral transduction with our mutant gene to infect our HEK293F target cells. The mutant LG3BP lentiviral vector was added to HEK293T cells to generate lentiviral particles. We confirmed the production of our lentiviral particles using fluorescein isothiocyanate (FITC) to identify the green fluorescence from the GFP tag in the lentiviral plasmid. We used a sandwich ELISA to calculate the approximate number of lentiviral particles. A standard curve was generated from measuring the absorbance levels of 2-fold dilutions (1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0 ng/ml) of p24-human immunodeficiency virus (HIV) protein (Figure 6). Each dilution was done in triplicates and the final OD was averaged to ensure accurate measurement. The zero absorbance value was subtracted from each of the other dilution absorbance levels. p24-HIV protein was used because it is a key component of the HIV viral capsid and can indicate the number of viral particles (Molina et. al, 2023). Dilutions of 100, 1000, 10000, and 100000 were used to measure the mean absorbance and quantify the number of lentiviral particles. Based on the result from the sandwich ELISA, the lentiviral titer was calculated to be 196.2 ng/ml of p24. 1 ng of p24 correlates to $1.25 \cdot 10^7$ total lentiviral particles. Out of the total lentiviral particles, there is a minimum of $1.25 \cdot 10^4$ infectious particles and a maximum of $1.25 \cdot 10^5$ infectious particles. Since the multiplicity of infection (MOI) of the HEK293F cells is 5, 5 infectious lentiviral particles were needed to infect one cell. 490500 cells were needed per LVP mix at minimum, and 4905000 cells were needed at maximum. Based on the sandwich ELISA calculations, we infected 3 million HEK293F cells/ml.

We compared the phase contrast image of the infected HEK293F cells (Figure 7A) to the fluorescent image of the same area (Figure 5B). From comparing these and the overlay of the two images (Figure 7C), we concluded that HEK293F cells were infected with high efficiency.

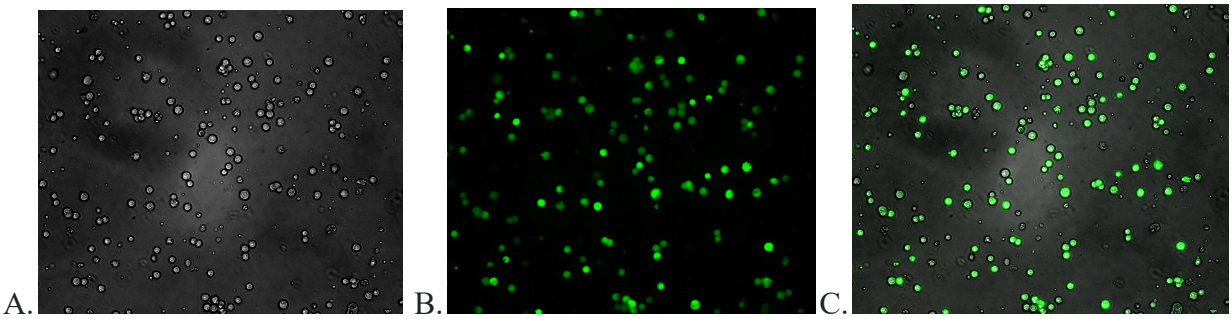

Fig 7. Infected HEK293F cells with LG3BP mutant lentiviral particles. *A*, Phase contrast image of infected HEK293F cells. *B*, Fluorescent image of infected HEK293F cells. *C*, Overlay of phase contrast image and fluorescent image of infected HEK293F cells.

We confirmed successful infection of HEK293F cells with our mutant LG3BP lentiviral vector through Genewiz sequencing (Azenta Lifesciences). After letting the cells grow to 3 million cells/ml, we transferred the cells to a 125 ml flask and subcultured to a 250 ml flask after 3 million cells/ml was reached. We confirmed the production of LG3BP mutant and LG3BP wildtype through SDS-PAGE and Western blotting using primary LG3BP mouse antibody (Figure 8).

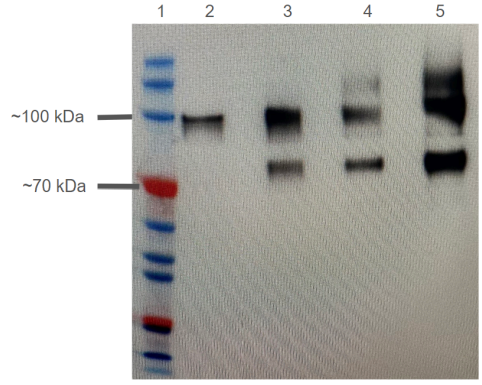

Fig 8. Confirmation of our mutant LG3BP gene in HEK293F cells. SDS-PAGE and Western Blot were done on conditioned media harvested from infected HEK293F cells. 30 µl HEK293F media (negative control), 2 µl LG3BP (positive control), 30 µl HEK293F media containing wildtype LG3BP protein, and 30 µl HEK293F media containing mutant protein were loaded together with protein loading dye to Lanes 2-5, respectively. A 10 kDa to 250 kDa ladder was loaded in Lane 1.

Our results document that we successfully generated the F357W LG3BP mutant in HEK293F cells, a crucial resource for the characterization of the LG3BP-Sulf-2 interaction site.

Discussion

LG3BP is a highly N-glycosylated secreted protein associated with tumor invasion and metastasis (Grassadonia et. al, 2004). LG3BP has been shown to mediate cell-cell adhesion (Inohara et. al, 1996), bind to collagens IV, V and VI, fibronectin and nidogen (Saski et. al, 1998). However, there is no information on the Sulf-2-mediated impact of LG3BP on any living system. We have shown that LG3BP is an interacting partner for Sulf-2 and inhibits its activity with potency that suggests *in vivo* effects (Panigrahi et. al, 2024). At this point, we want to generate a mutant that will not bind and examine the changes in its function. Ultimately, we want to verify whether the disrupted interaction modifies cell biology and design inhibitors that would allow disruption of this interaction *in vivo*.

In this study, we generated a mutant cell line of LG3BP at the predicted LG3BP F357 binding site of the LG3BP/Sulf-2 complex. While we have previously identified LG3BP as an interaction partner of Sulf-2 and inhibitor of Sulf-2 activity, the exact binding site of this interaction has yet to be confirmed. We created an AlphaFold model of the mutant

LG3BP/Sulf-2 complex and identified F357 (phenylalanine, TTT) as the predicted interaction site for LG3BP-Sulf-2. From this model, we hypothesized that a mutation to F357 would impact the ability of LG3BP to bind to Sulf-2 and inhibit its activity. We generated a LG3BP F357W (phenylalanine to tryptophan, TTT to TGG) mutant in the Top10 *E.coli* cell line. We then obtained the necessary lentiviral components needed to assemble the lentiviral vector containing our LG3BP mutant. Using the assembled lentiviral vector, we created lentiviral particles with our LG3BP mutant and infected HEK293F cells to successfully generate HEK293F cells containing our LG3BP mutant.

The generation of these cell lines provides a resource crucial for further characterization of the LG3BP/Sulf-2 interaction site. Further work includes the harvesting of conditioned media from wild-type and mutant LG3BP cells and the purification of mutant and wild-type LG3BP protein. Using purified LG3BP protein and purified Sulf-2 protein obtained from prior studies (Paragrahi, 2024), *in vitro* assays can be performed with the F357W mutant LG3BP/Sulf-2 and wild-type LG3BP/Sulf-2.

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Conflict of interest–The authors declare they have no conflict of interest.

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Supplementary

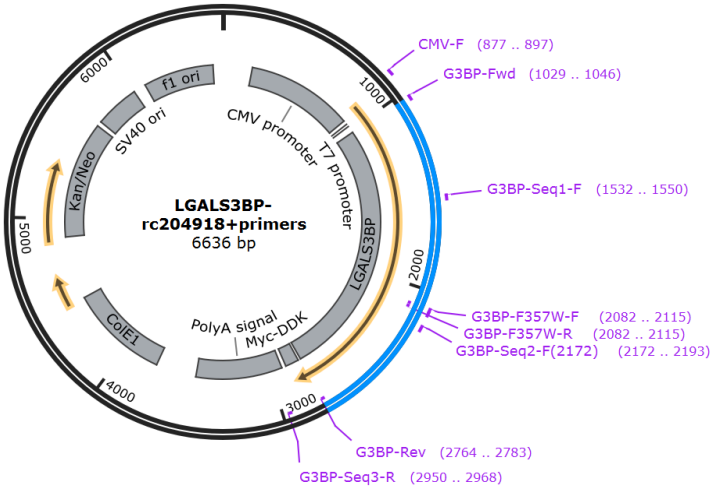

Fig 1S. Design of LG3BP-F357W Plasmid. Original plasmid used for generation of LG3BP-F357W mutant (OriGene, RC204918); includes CMV promoter, T7 promoter (T7), LG3BP F357W mutant (LGALS3BP), poly A signal, Myc-DDK tags, ColE1 plasmid origin sequence (ColE1), Kanamycin resistance (Kan), Simian virus 40 origin (SV40 ori), F1 origin (f1 ori).

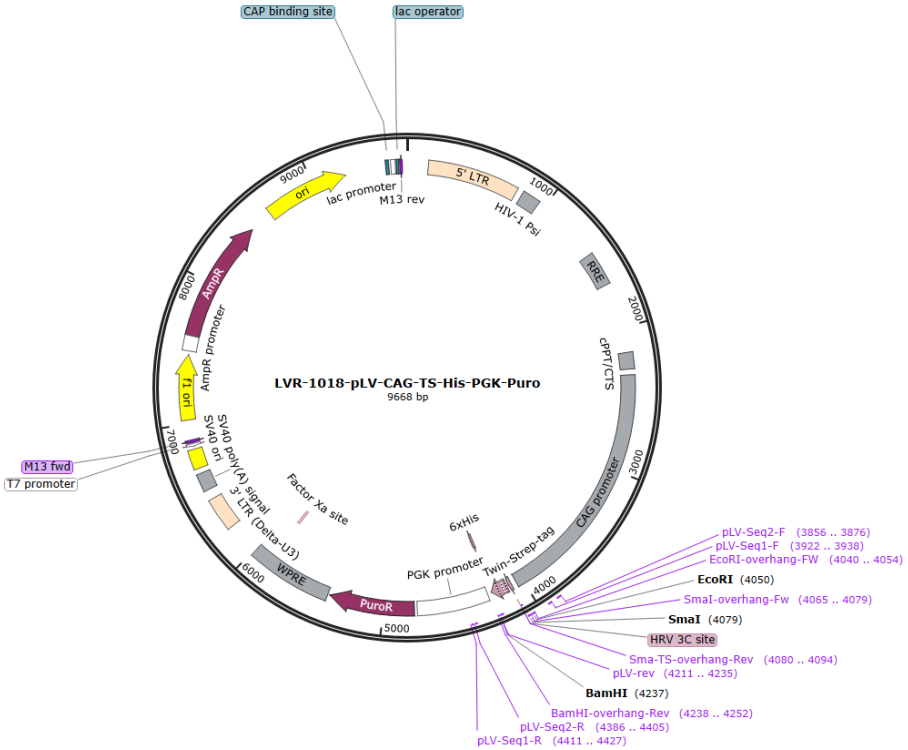

Fig 2S. Design of Lentiviral Plasmid. Plasmid used for generation of lentiviral vector for infection of HEK293F cells; includes CAG promoter, Twin-Strep-tag, 6x histidines (6xHis), Puromycin resistance (PuroR), long term repeats (LTR), poly A signal, Simian virus 40 origin (SV40 ori), ampicillin resistance (AmpR), the lac promoter, and HIV-1 Psl.