CBX2 AND DNA DAMAGE REPAIR:

DEVELOPMENT OF CBX2-SPECIFIC REAGENTS

By

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ABSTRACT OF THE THESIS

CBX2 and DNA Damage Repair: Development of CBX2-Specific Reagents

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DNA damage is inevitable, however, methods of DNA repair exist which allows cells to recover, or die, depending on the severity of the damage. Homologous recombination (HR) is one type of DNA damage repair that has the ability to repair double-stranded breaks (DSBs) in a high-fidelity manner. Unrepaired DSBs can led to cell death or genomic instability.

BRCA1is a tumor suppressor protein that is involved in the cellular response to DNA damage. Studies show that BRCA1 accumulates in S and G2 phases of the cell cycle, and after DNA damage re-localizes to nuclear repair foci. Cells that lack BRCA1 are unable to repair DNA using HR, and therefore tend to use more error-prone mechanisms.

A human auto-antisera, PIKA, first characterized in 2003 by William Earnshaw et al., recognizes proteins which contain chromodomains, and co-localize with BRCA1, before and after DNA damage. Chromodomains are critical features of the HP1 group of proteins, and the polycomb group (PcG) proteins. Prior work has suggested that the

protein being recognized by the PIKA anti-sera, was CBX2, a member of the PcG and the polycomb repressive complex 1 (PRC1).

Unfortunately, the antibodies commercially available for CBX2 do not recognize both isoforms of CBX2, the truncated and the full-length. The purpose of this project was to generate plasmid constructs containing coding sequences for the CBX2 full-length protein using the directional cloning technique. A vector encoding a GST-CBX2 SL fusion protein was successfully generated, and conditions for expression and extraction of this protein from bacterial culture were optimized. Progress has been made in isolating a cDNA for the full length CBX2 isoform to generate both GST-fusion proteins and a GFP-tagged protein. By generating a proper CBX2 antibody for the full-length protein, we hope to show that this protein is involved in the normal response to DNA damage.

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<u>Introduction:</u>

DNA Damage

DNA damage is inescapable and inevitable due to both endogenous sources, such as lesions resulting from normal cell metabolism, and exogenous sources, such as genotoxic stress (Li and Heyer, 2008; Borges et al., 2008; Shrivastav et al., 2008; Weterings and Chen, 2008). Due to the inevitability of DNA damage, cells have evolved the ability to sense and respond to the occurrence of DNA damage. The cellular response to DNA damage includes, arrest of the cell cycle from checkpoint and then activation of DNA repair pathways. A variety of different DNA repair mechanisms have evolved to deal with different classes of DNA damage, such as, single stranded breaks, double stranded breaks, single base excision, nicks, deletions, etc. The mechanisms include, but are not limited to: base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end joining (NHEJ). For purposes of this project, we focused on the DNA double strand break repair.

Double Stranded Breaks and Damage Repair

Double stranded breaks (DSBs) are considered the most hazardous because when not repaired, they are lethal to the cell. If DSBs are not repaired, not only can cell death result, but also chromosomal changes, including deletions and translocations, which are hallmarks of genome instability, and an initial step in carcinogenesis (Li and Heyer, 2008; Shrivastav et al., 2008; Weterings and Chen, 2008). Because of the gravity of DSBs, cells have evolved several mechanisms to repair them, including, homologous recombination (HR) and non-homologous end joining (NHEJ). HR is error-free with

high fidelity but requires a DNA template for repair, whereas, NHEJ does not require a template, but has a high error rate (Lo et al., 2003; Li and Heyer, 2008). Cells have evolved groups of proteins whose function is to sense DSBs, and arrange repair of the lesion (Borges et al., 2008; Shrivastav et al., 2008; Weterings and Chen 2008).

Induction of DSBs activate the ATM and ATR kinases, which then have the ability to activate multiple repair pathways. One pathway involves ATM/ATR phosphorylating and thereby activating the checkpoint proteins (Chk1 and Chk2), which then phosphorylate BRCA1. At the specific sites of damage, ATM and ATR can phosphorylate the histone variant H2AX; focal phosphorylation of H2AX is considered a key initial marker of DNA DSB. DNA DSB also perturbs local chromatin structure; this alteration of chromatin structure near DNA DSB unmasks normally cryptic methylated histone residues, allowing focal binding of the repair/checkpoint protein 53BP1. Therefore, phospho-H2AX and 53BP1 are both valid indicators of DNA damage (see Figure 2 and 3). This accumulation of p-H2AX and 53BP1 at sites of DSB then is thought to nucleate the formation of DNA repair complexes that include proteins such as BRCA1, BRCA2, NBS, RAD51, BACH and PALB2. These proteins are localized in discrete nuclear foci that form after ionizing radiation and are thought to indicate sites of DNA repair. Many of these repair proteins, such as BRCA1, are also intimately involved in checkpoint activation. Intriguingly, many of these repair proteins that localize to sites of DNA DSB are also breast cancer tumor suppressors. These include BRCA1, BRCA2, PALB2, BACH1, BARD1, ATM, and CHK2.

Both BRCA1 and BRCA2 are tumor suppressors for breast, ovarian, and other cancers (Shivji and Venkitaraman, 2004). These mutations carry a lifetime risk of 50-

80% for developing breast cancer, and 20-40% for developing ovarian cancer (Venkitaraman, 2001; Venkitaraman, 2002). BRCA1 has been shown to act in both DNA repair and cell cycle regulation (Venkitaraman, 2001).

Cells that are BRCA1 deficient and incur DSBs repair the DNA via error-prone mechanisms, rather than the normal method of HR when BRCA1 is present (Scully et al., 1999). BRCA1 deficient cells also have sensitivity to ionizing radiation. It has been shown that BRCA1 participates in DSB repair by HR, and cells lacking BRCA1 instead repair DNA via NHEJ (Moynahan et al., 1999; Venkitaraman, 2001). Repairing DNA using NHEJ is more error-prone then HR, and can then lead to chromosomal aberrations as well as a greater mutation rate (Venkitaraman, 2003; Borges et al., 2008; Shrivastav et al., 2008).

S Phase Localization

BRCA1 is a nuclear protein that accumulates in S and G2 phases of the cell cycle, and is localized in distinct nuclear foci (Scully et al., 1996; Shrivastav et al., 2008). After incurring damage from ionizing radiation or other agents, ATM and ATR kinases phosphorylate BRCA1 (Scully et al., 1996; Venkitaraman, 2001). In response to DNA damage, BRCA1 moves from its S phase nuclear foci to "repair foci" where BRCA1 can be seen to co-localize with many proteins that are involved in DNA repair including RAD51, FANC, and BACHI (Scully et al., 1996; Venkitaraman, 2001). Despite the evidence that BRCA1 is needed for the normal response to DNA damage, BRCA1's actual role in this process still has yet to be determined (Venkitaraman, 2001; Shrivastav et al., 2008).

Polymorphic Interphase Karyosomal Association (PIKA)

Interestingly Dr. Ganesan and colleagues have observed that PIKA (polymorphic interphase karyosomal association) antisera which was characterized by William Earnshaw et al., recognizes a chromodomain motif that co-localizes with BRCA1, both in S phase foci and in repair foci following DNA damage (Saunders et al., 1991). PIKA was isolated from patients that have the autoimmune disorder CREST (Saunders et al., 1991). The PIKA antiserum was used to identify and then clone the human homologs of the Drosophila HP1 protein (Saunders et al., 1993; Saunders et al., 1996; Hill, 2005). The epitope that was recognized was the chromodomain of the HP1 proteins, and therefore the antisera could be used as an antibody against the chromodomain motif (Saunders et al., 1993; Saunders et al., 1996). The fact that PIKA co-localized with BRCA1 both before and after DNA damage, suggests that a chromodomain containing protein co-localizes with BRCA1 and may be playing a role in DNA repair response.

Chromodomains

Chromodomains (chromatin organization modifier) are highly conserved amongst proteins that have roles in chromatin silencing, the HP1 group of proteins, and also the polycomb group (PcG) proteins (Pearce et al., 1992; Gecz et al., 1995; Jones et al., 2000; Ruddock-D'Cruz et al., 2008). The PIKA antisera did not co-localize with the HP1 antibodies, suggesting that another chromodomain containing protein must be co-localizing with the PIKA antisera. Work previously done by Sarah Hill while at Harvard University characterized a specific protein, termed POPE-1, which was recognized by PIKA (Hill, 2005). She was able to show co-localization of POPE-1 and BRCA1 both before and after DNA damage. After the genome was annotated, POPE-1 was identified

as CBX2, a chromodomain containing protein, and a member of PcG. CBX2 is also a

member of the Polycomb Repressive Complex (PRC1), which is thought to inhibit

SWI/SNF mediated chromatin remodeling, and directly interacts with BMI-1 (Shao et al.,

1999; Cao et al., 2002).

CBX2 has two isoforms, found by alternative splicing, one that is short-length or

truncated, and one that is full-length (see Figure 1). The full-length CBX2 isoform is the

human homolog of the murine M33. The significance of the shorter isoform is not yet

clear.

PcG and PRC1

PcG proteins transcriptionally repress developmentally important genes

throughout rounds of cell division. They also have been shown to maintain "cellular

memory" of the differentiated cell state (Pirrota, 1998; Iwama et al., 2005; Bernstein et

al., 2006). The chromodomain of PcG specifically binds tri-methylated lysine 27 on

Histone 3 (Cao et al., 2002; Bernstein et al., 2006; Bracken et al., 2006; Papp and Muller,

2006). Suggestions have been made that the interaction of binding to K27me on H3

recruits PRC1 to the PRC2 targets (Cao et al., 2002).   After PRC2 methylates the active

chromatin and PRC1 is recruited to the targets, the chromatin becomes transcriptionally

silenced (Shao et al., 1996; Cao et al., 2002).
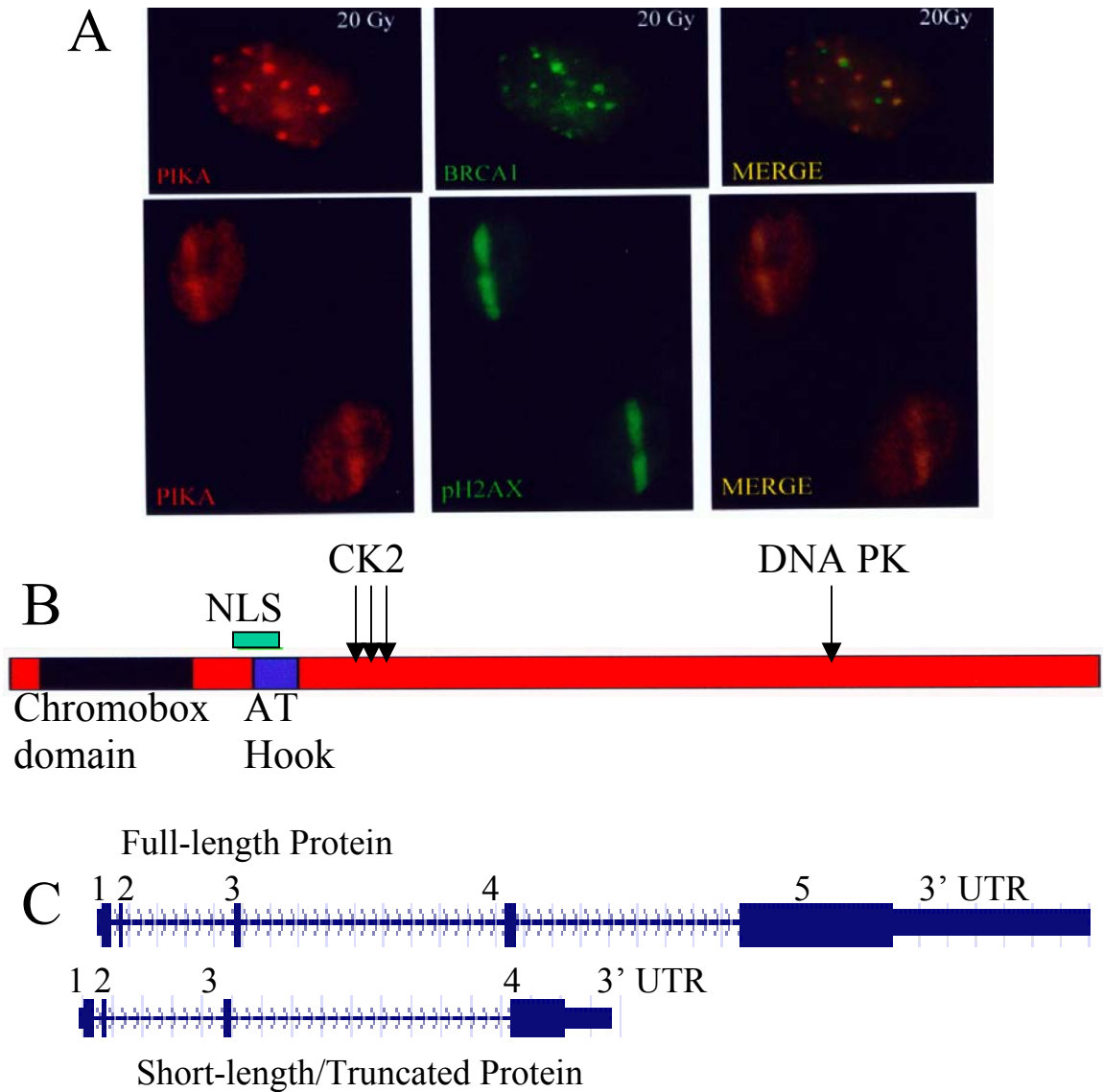
Figure 1. Localization of PIKA in HeLa cells; and CBX2's domains and isoforms.

A) Top-Co-localization of PIKA (red) and BRCA1 (green) at sites of DNA damage induced by 20Gy of ionizing radiation. Bottom- Co-localization of PIKA (red) and pH2AX (green) at sites of DNA damage induced by UV Laser Scissors. B) Diagram of CBX2 and its functional domains (Hill, 2005). C) Top- Full-length isoform of CBX2 containing a small portion of exon 4, and all of exon 5. Bottom- Short-length isoform of CBX2 containing all of exon 4, and none of exon 5.
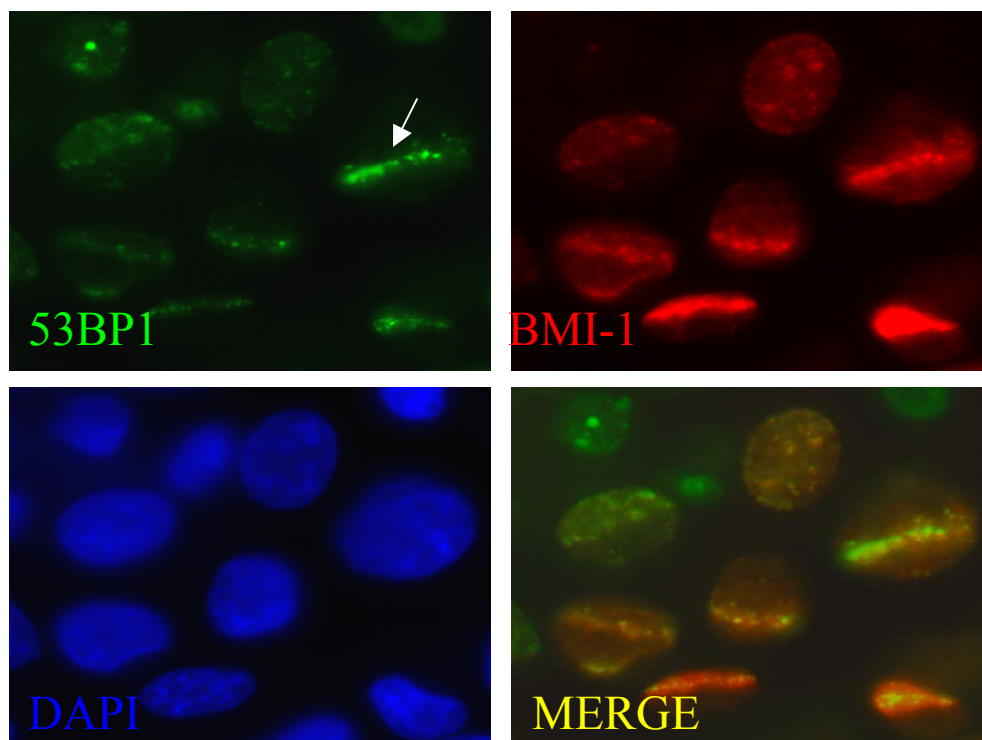
Figure 2. 53BP1 co-localizes with BMI-1 to sites of DNA damage induced by UV Laser Scissors. Immunofluorescence done by the Ganesan lab at the CINJ have demonstrated that BMI-1 localizes to sites of DNA damage induced by UV Laser Scissors using immunofluorescence microscopy. The above pictures show HeLa cells that have been exposed to laser scissors, and then co-stained with 53BP1 (top left, green) and BMI-1 (top right, red) antibodies, and DAPI nuclear staining (bottom left, blue). It can be seen that BMI-1 co-localizes with 53BP1 at sites of DNA damage induced by laser scissors.
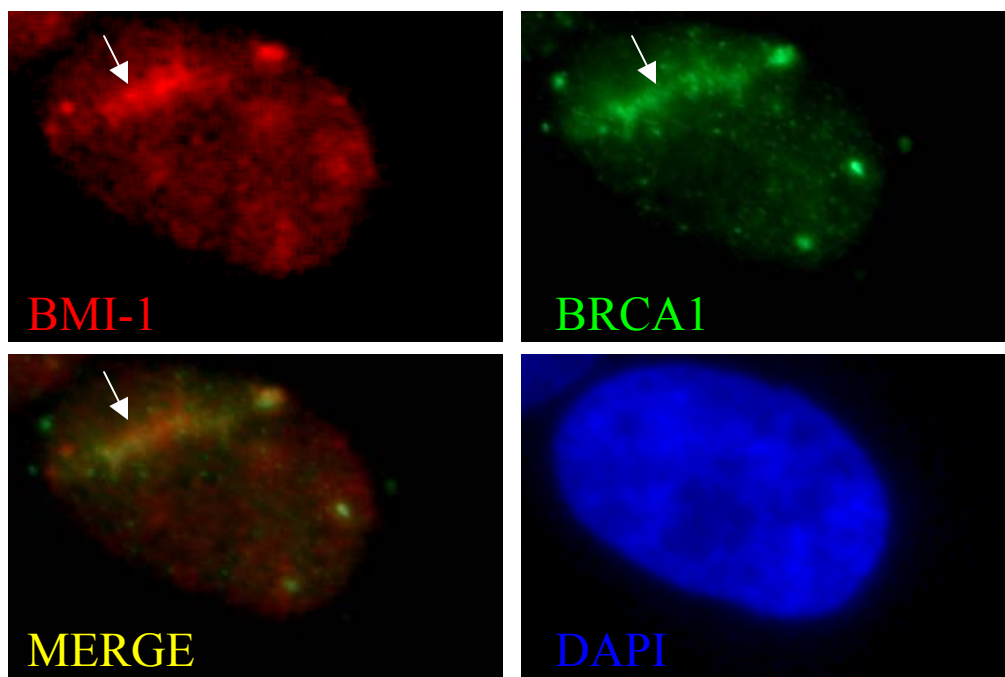
Figure 3. BRCA1 co-localizes with BMI-1 to sites of DNA damage induced by UV Laser Scissors. Immunofluorescence done by the Ganesan lab at the CINJ have demonstrated that BMI-1 and BRCA1 co-localize to sites of DNA damage induced by UV Laser Scissors. The above images show HeLa cells exposed to laser scissors and co-stained with BMI-1 (top left, red) and BRCA1 (top right, green) antibodies, and DAPI nuclear staining (bottom right, blue). BMI-1 co-localizes with BRCA1 at sites of DNA damage induced by laser scissors.

PRC1 and PRC2 Components

The PRC1 component BMI-1 has recently been shown to localize to specific sites of DNA damage produced by laser scissors (Ganesan lab, data not yet published)(see Figure 2 and 3). The chromodomain-specific PIKA antisera also localized to sites of DNA damage; as CBX2, a BMI-1 binding partner, may be the relevant target of PIKA antisera, this implies that CBX2 is also recruited to sites of DNA damage. These observations suggest that multiple components of PRC1 are being recruited to sites of DNA damage, and may therefore be playing a role in the DNA repair response. In order to show that PRC1 is indeed localizing to sites of DNA damage, and not just a few of the components of the complex, each component needs to be demonstrated to localize with phosphorylated H2AX, or another protein such as RAD51, which indicates DNA damage.

CBX2 Isoforms and Antibodies

Despite Sarah Hill's previous work with CBX2 (POPE-1), the polyclonal antibody for CBX2 she generated that functions for immunofluorescence is not currently available. Some antibodies for CBX2 are commercially available, however, the antibodies were raised against regions of Exon 4 present in the short-length or truncated isoform of CBX2 that are not necessarily conserved in the full-length isoform (Figure 1). As mentioned before, studies still have yet to show that the short-length CBX2 isoform is actually expressed in mammalian cells; therefore, to be able to identify the presence of CBX2 at sites of DNA damage, an antibody must be created that recognizes the full-length isoform of CBX2. The hope is that once CBX2 and the PRC1 complex is shown

to localize with BRCA1 at sites of DNA damage, the function of the PRC1 proteins and

BRCA1 may be better elucidated.

Purpose and Aims of this Project

There is a pressing need for specific antibodies that could recognize both the

short-length isoform and the full-length isoform of CBX2 in HeLa cells. One step to

achieving this goal is to generate fusion proteins encoding the relevant portions of CBX2

that can be used to generate specific antibodies. The specific aims of this project were

three-fold: 1) create a GST fusion protein for the short-length isoform of CBX2, 2) create

a GST fusion protein for the full-length isoform of CBX2, and 3) create a GFP construct

for retroviral transfection to probe for the CBX2 protein. In order to make all of these

constructs, the technique of directional cloning was employed. The construct that

contains the sequence that codes for the CBX2 short-length isoform GST-fusion protein

was successfully made. However, the other two constructs are still in the process of

validation.

Materials and Methods:

Initial Plating of CBX2 Short-Length Clone

Clone ID 3626683 was received from Open Biosystems in DH10β cells, which contained the CBX2 short length fragment cDNA (CBX2 SL). A small sample was scraped with an inoculating loop and then streaked onto LB Agar plates with a final concentration of 25ug/mL of chloramphenicol. These plates were placed in 37ºC and grown overnight.

Maxi Prep

The QIAfilter Plasmid Maxi Kit from QIAGEN was used to prepare Maxi Prep samples. Maxi prep was done using QIAGEN's protocol for Maxi Prep. This method allowed for large-scale purification of plasmid DNA.

Agarose Gel Electrophoresis

All DNA, PCR, and Colony PCR samples were run on 1% agarose gels, and visualized by ethidium bromide staining. To make the gel, 0.5g GenePure LE Agarose from ISC BioExpress was mixed with 50mL 1XTAE, obtaining a final concentration of 1% agarose. The solution was then heated to a boil in a microwave and then poured into a gel casting case, a gel comb was inserted, and allowed to solidify.   The gel was then loaded into a Mupid-exu Submarine Electrophoresis System chamber from Advance, with an Intelligent Power Supply. The first lane of the gel was loaded with O'Gene Ruler 1kb DNA Ladder from Fermentas. Samples typically of 20uL DNA were loaded with 5uL of 5X Gel Loading Dye. The gel was run with 50 volts for 45-60 minutes. The gel was then stained with 7uL EtBr in 1XTAE Buffer, enough volume to cover the gel, for 10 minutes on a tilter. In order to visualize, a Molecular Imager FX from BioRad was used

with UV Light. To capture the images, the Quantity One 4.5.0 program was used, and printed with a Sony Digital Capture Printer.

Quantification of DNA Samples

The DNA samples were quantified using the Nano Drop ND-1000 Spectrophotometer. First 1uL of double distilled water was loaded and this initialized the spectrophotometer. Next, 1uL of the solution the DNA was diluted or eluted in was loaded, this acted at the blank. Then, to read the DNA sample, 1uL of sample was loaded and quantified.

Gel Extraction and Gel Elution

Any band of interest was extracted from the agarose gel using a clean razor. Then the QIAquick Gel Extraction Kit from QIAGEN was used to extract and elute the DNA from the agarose gel, using QIAGEN's QIAquick Gel Extraction Protocol.

Primer Design

Forward and reverse primers were designed for all CBX2 fragments, which includes: the short length fragment (primers CBX2 forward/reverse Open), the full-length fragment (primers CBX2 forward/reverse FL), and retroviral construct fragment (primers CBX2 forward/reverse Retro). The primers for the short length fragment and for the retroviral construct fragment were designed using accession number BC004252 from GenBank. An EcoRI and XhoI restriction enzyme recognition site was included in the forward and reverse primers, respectively, for both the short and full-length fragments. A BamHI and SalI restriction enzyme recognition site was included in the forward and reverse primers, respectively, for the retroviral fragment of CBX2. Once designed, the primers were ordered from Sigma.

Table of Primers:

1	CBX2 forward Open (SL)	5' AGAATTC GAC TGG CGG CGG GCG C 3'
	CBX2 reverse Open (SL)	5' ACTCGAG CA GGG ACA GTG CCC GG 3'
2	CBX2 forward FL	5' AGAATTC ATG GAG GAG CTG AGC AGC G 3'
	CBX2 reverse FL	5' ACTCGAG GTA ATG CCT CAG GTT GAA G 3'
3	CBX2 forward Retro	5' AGGATCC GAC TGG CGG CGG GCG CCG C 3'
	CBX2 reverse Retro	5' AGTCGAC CA GGG ACA GTG CCC GGA GG 3'

PCR

For PCR's done with the short length fragment, the reaction was as follows: 1-2uL Template DNA, 4uL Forward Primer, 4uL Reverse Primer, 15uL 2X Hot-Start IT Taq Master Mix (USB), 6uL PCR Grade Nuclease-free Water (Roche); 35 cycles, annealing temperature 73ºC for 30 seconds, and extension temperature 72ºC for 1min 20 seconds. These same reactions were used to PCR lift the CBX2 fragment from the Open Biosystems clone.

Digestion of CBX2 fragments DNA using Restriction Enzymes

All restriction enzyme digestions were done using Fast Digest Enzymes from Fermentas. The protocol for Double Digestion with Fast Digest Enzymes from Fermentas was used with EcoRI and XhoI for the short and longer length CBX2 fragments, and BamHI and SalI were used for the retroviral CBX2 fragment. Reactions consisted of: DNA 6uL, Fast Digest enzyme #1 2uL, Fast Digest enzyme #2 2uL, 10X Fast Digest buffer 2uL, DEPC Treated Water from Invitrogen 8uL. Incubated at 37ºC for 1hour on a heat block to digest the DNA. Inactivated the enzymes at 80ºC for 5-10 minutes.

Digestion of pGEX4T-1 and pLXSP vectors using Restriction Enzymes

The pGEX4T-1 vector from GE Healthcare was double digested using the Fast

Digest restriction enzymes EcoRI and XhoI. Reaction consisted of: 6uL of the pGEX4T-

1 DNA, 4uL of EcoRI, 2uL of 2X Buffer, 8uL of DEPC Treated Water from Invitrogen

in tube 1. In tube 2 we mixed 6uL of the pGEX4T-1 DNA, 4uL of XhoI, 2uL of 2X

Buffer, 8uL of DEPC Treated Water from Invitrogen. Both tubes were incubated in a

37ºC water bath for 10-15min. The tubes were then mixed together in order to achieve a

double digestion, and then put back into the water bath for another 20-25 minutes. Heat

inactivation was achieved by heating the sample for 5-10 minutes on a heat block at

80ºC. The same methodology was used for double digestion of the pLXSP vector from

Dr. Shen's lab; however, the enzymes used were BamHI and SalI.

Another method used followed the protocol from Fermentas for Double Digestion

with FastDigest Enzymes. 2uL (pGEX4T-1 or pLXSP), 2uL 10X Buffer, 1uL Enzyme 1,

1uL Enzyme 2, 14uL DEPC, heated for extended time: 1hour in 37ºC water bath. Heat

inactivated at 80ºC for 5-10 minutes.

Ligation of vectors and inserts

Ligation of the double digested vectors (digested with EcoRI and XhoI for the SL

and FL, and BamHI and SalI for the retroviral) pGEX4T-1/pLXSP and the double

digested (digested with EcoRI and XhoI for the SL and FL, and BamHI and SalI for the

retroviral) CBX2 fragments using Quick T4 DNA Ligase from New England BioLabs.

The protocol for Quick Ligation from New England BioLabs was used. Components of

the reaction were 1uL of Quick T4 DNA Ligase from New England Biolabs, 10ul 2X

Quick T4 DNA Ligase Reaction Buffer, 3uL double digested vector sample, 9uL CBX2

double digested sample. The reaction was done at room temperature for 10 minutes, and then placed on ice until cooled.

Transformation of construct into DH5α Subcloning Efficiency Competent Cells

Transformation of the newly ligated constructs was done using DH5α competent cells subcloning efficiency from Invitrogen. The transformation protocol from Invitrogen was followed. To begin the protocol, 50uL of competent cells was mixed and chilled on ice with 2-5uL of the ligated construct for 30 minutes. The cells were then heat shocked for 20 seconds in 42ºC. Immediately after heat shock, the cells were put on ice for an additional 2 minutes. After 2 minutes, 950uL of S.O.C. medium was added to each tube, and placed in a 37ºC shaker for 1 hour. After 1 hour, the transformed bacteria were plated onto LB-amp agar plates, and grown in a 37ºC incubator overnight.

Miniprep

Individual colonies were picked from LB-amp plates and used to inoculate 3mL of LB media with ampicillin at a final concentration of 100ug/mL and grown overnight in a 37ºC shaker. The GeneJet Plasmid Miniprep Kit from Fermentas was used to isolate the plasmid DNA from each culture preparation.

DNA Sequencing

The samples to be sequenced were sent to the UMDNJ Core Facilities in Piscataway, NJ for DNA sequencing. Samples consisted of 5uL of DNA sample and either 1uL of the respective forward primer, or 1uL of the respective reverse primer.

Transformation into BL21-Codon Plus Competent Cells

Transformation of the construct into BL21-Codon Plus Competent Cells was done using the Transformation Protocol with BL21-CodonPlus Competent Cells from

Stratagene and plated on LB-amp agar plates. These colonies were then used for Colony

PCR and subsequent protein expression experiments.

Colony PCR

Colony PCR was done using 10uL 2X Hot Start-IT (for SL and retroviral

constructs) 1-2uL respectively of the Forward Primer, 1-2uL respectively of the Reverse

Primer, and 1 colony of the DH5α or BL21 transformation. For the retroviral construct,

an additional 7uL of DEPC water was added. For the SL and retroviral construct, PCR

conditions were standard except for 73ºC for 30 seconds for annealing, and 72ºC for

1minute and 20 seconds for extension, and 35 cycles.

Induced Bacterial Culture Preparation

First, an overnight culture of LB-amp media inoculated with BL21 cells

transformed with the construct of interest were grown overnight in a 37ºC shaker. In the

morning, a new smaller culture of LB-amp, along with IPTG (final concentration 1.0mM)

was inoculated with 500uL of the overnight culture, and grown to varying OD600's

ranging from 0.3-0.8. Negative control samples were grown in the same manner, but

without the addition of IPTG. To these samples, 2X Laemmli Sample Buffer was added

and heated at 90ºC for 10 minutes, then loaded onto the SDS-page gels. Some samples

also had PIC and DTT added prior to heating.

Bug Buster Protein Extraction

Fresh 1.5mL cultures of BL21 cells transformed with the construct of interest,

which had been induced with IPTG (final concentration 1.0mM), were collected. The

protocol from Novagen for Soluble Fraction Bug Buster Protein Extraction Reagent was

followed. After collection of the culture, the sample was centrifuged 14,000-16,000 x g

for 10 minutes. The supernatant was removed, and 300uL of Bug Buster Protein Extraction Reagent was used to re-suspend the pellet. The mixture was then incubated at room temperature for 20 minutes on a rotating mixer. The sample was then centrifuged 16,000 x g for 20 minutes at 4ºC. The supernatant and pellet were both used as samples for SDS-page gels by combining with 2X Laemmli Sample Buffer, and boiling to 90ºC for 10 minutes prior to loading.

Sonication of Bacterial Culture Samples

Bacterial cultures of BL21 cells transformed with the construct of interest, which had been induced with IPTG (final concentration 1.0mM), were collected. Samples were centrifuged to pellet by spinning at 8,000 rpm, 4ºC for 10 minutes. The pellet was then re-suspended in 1mL of 1% TritonX-100 in PBS and 5uL of Protease Inhibitor Cocktail (PIC) was added. Samples were then sonicated using a Branson Sonifier 450 with a Microtip at a duty cycle of 30% intensity, and with an output of 25, for 10 pulses, with a 1 minute break, and repeated 4 times. Samples were kept in ice to avoid overheating of the sample. After sonication, 50uL of 10X PIC and 5uL of PMSF was added. The samples were then centrifuged at 10,000rpm, 4ºC for 10 minutes.

Glutathione Sepharose 4B (Bead) Purification

The final supernatant from the sonicated bacterial samples was used for the Glutathione Sepharose 4B (GE Healthcare) protein purification. For every 1mL of supernatant, approximately 100uL of beads was added. The samples were then incubated for 30 minutes on an end-over-end rotator in 4ºC. Samples were then centrifuged at 1,000rpm, at 4ºC for 10 minutes. To the pellet, 125uL of 1% TritonX-100 in PBS was added, along with 1uL PIC and 2uL PMSF and then mixed. Then the samples were

boiled 2 times at 90ºC for 10 minutes. The samples were then centrifuged again

1,000rpm, at 4ºC for 10 minutes. The supernatant was mixed with 2X Laemmelli Sample

Buffer, heated at 90ºC for 10 minutes, and then loaded to the SDS-page gel.

6-10% SDS-page Gels

6-10% Tris-Glycine SDS-polyacrylimide Gel Electrophoresis (SDS-page) gels

were made to 1.5mm thickness. Gels were run in 1X Tris/Glycine/SDS Buffer (10X

from BioRad) at 100-120V in a Mini PROTEAN 3 cell chamber (BioRad) using a Basic

Power Pac power source (BioRad) for approximately 1.5-2 hours. After running, the gels

were used either for Simply Blue Safe Staining, Coomassie Staining, or transferred to

nylon membranes.

Simply Blue Safe Staining and Coomassie Staining

To stain SDS-page gels, two methods were used: Simple Blue Safe Staining or

Coomassie Staining. Invitrogen's Simply Blue Safe Stain was used by first removing the

gel from the glass slide casing. The gel was then rinsed in double distilled water, 3 times,

5 minutes each on a tilter. The gel was then incubated on a tilter for 1 hour in 20mL of

Simply Blue Safe Stain. Lastly, the gel was rinsed with double distilled water, 3 times,

15 minutes each. All gels were photographed using a personal digital camera DiMage

G600 from Konica Minolta.

In order to stain with Coomasie, the gel was removed from the glass slide casing

and rinsed with double distilled water, 2 times, 3 minutes each. The Coomasie stain

consisted of: 9mL of 0.2% Coomasie blue and 50% methanol, 2 mL of Acetic Acid

(Glacial), and 9ml of distilled water. Enough stain was added to the gel in order to cover

it, and then placed on a tilter covered overnight. In order to lift the stain for better

visualization, the gel was de-stained using a De-staining Wash. The De-staining Wash consisted of: 25mL Methanol, 65mL distilled water, and 10mL Acetic Acid (Glacial). The wash was changed about every 30-45 minutes until the bands were considered light enough for photography.

Western Blot

After the 6-10% SDS-page gels were removed from glass casings, the protein had to be transferred to nylon membranes using the semi-dry transfer method. Millipore Immobilon-P nylon Transfer Membranes were cut to sizes approximate to the gels, then washed in methanol for 20 seconds, then distilled water for 2 minutes, then soaked in cold semi-dry transfer buffer (25mM Tris base, 192mM Glycine, 20% Methanol, distilled water to volume) for at least 5 minutes. Four pieces of gel blot paper, slightly larger then the gels were also soaked in cold semi-dry transfer buffer. The stacking from the bottom to the top was as followed: 2 blot papers, nylon membrane, SDS-page gel, then 2 blot papers. A BioRad Trans Blot SD Semi-Dry Transfer Cell was used with a Basic Power Pac power source (BioRad) for transfer. The transfer was run at 20V for approximately 45 minutes up to 1 hour.

After proper protein transfer to the membrane, the membrane was blocked in about 50mL of 5% milk/PBST (5% powdered non-fat dry milk, 1X PBS to volume) at 4ºC on a slow moving shaker, overnight. The next step was to do primary antibody incubation on a tilter. For the Monoclonal anti-GST soup (from Dr. Bing Xia) a 1:5,000 dilution was used in 2mL of 5% milk/PBS for 45 minutes at room temperature, and for the Rabbit polyclonal to GST ab9085 (from Abcam) a 1:500 dilution was used in 4mL of 5% milk/PBS for 2 hours at room temperature on a tilter. Next, the membranes were

washed in PBST (20mL Tween20 and 980mL 1X PBS), 3 times, 5-10 minutes each on a tilter. Secondary antibody incubation was done using goat anti-mouse IgG-HRP (Upstate) and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) 1:2,000 dilution, respectively, for 1 hour on a tilter. The membranes were again washed in PBST, 3 times, 5-10 minutes each, room temperature on a tilter.

Chemoluminescence reagents #1 and #2 from Amersham Biosciences were mixed, 2.5mL each, then applied to the membrane in a dark room. The reagents were agitated slightly every minute for 5 minutes. After 5 minutes of incubation, the membrane was allowed to drain a bit, then placed in a clear plastic sheet protector. Lastly, Kodak Biomax MS Scientific Imaging film was exposed to the membrane in a dark room in a Fisher Biotech Electrophoresis Systems Autoradiography Cassette for varying exposure times. To develop the film, a Hope Micro-Max X-ray developer was used with an Automixer from White Mountain Imaging.

HeLa cell Preparation

HeLa cells were grown in Dulbecco's Modification of Eagle's Medium (DMEM) from Invitrogen supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin, in 37ºC, 5%CO_2 water-jacketed incubator to 80% confluence.   Media was aspirated and cells were washed with PBS. The PBS was then aspirated from the cells, and RNA isolation followed.

RNA Isolation and Collection

The protocol from Invitrogen using the Trizol Reagent was followed. Homogenization was done using the Cell Grown in Monolayer option. Two other methods of RNA collection were also used, both being kits. The Stratagene Absolutely

RNA Miniprep Kit was completed following the protocol. Also, the RNeasy Mini Kit from QIAGEN was used, with its included protocol.

First Strand cDNA Synthesis and RT-PCR

The protocol Transcriptor First Strand cDNA Synthesis Kit from Roche was used to make the first stand of cDNA from the RNA collections. Reverse Transcriptase-PCR was done using the protocol included in the kit from Roche.

Results:

Initial Isolation of CBX2 Short-length Clone

In order to generate a construct with the DNA sequence that encodes the short length CBX2 protein, a clone ID no. 3626683 was received from Open Biosystems, which contained the cDNA sequence corresponding to GenBank acquisition number BC004252 in a pOTB7 vector backbone (Figure 4). The clone was plated onto LB Agar plates containing a final concentration of 25ug/mL of chloramphenicol and grown overnight. From these plates individual colonies were then isolated and used to make small cultures in preparation for Minipreps. The GeneJet Plasmid Miniprep Kit from Fermentas was used to isolate the plasmid DNA coding for the CBX2 short length (CBX2 SL) protein (Figure 5A).
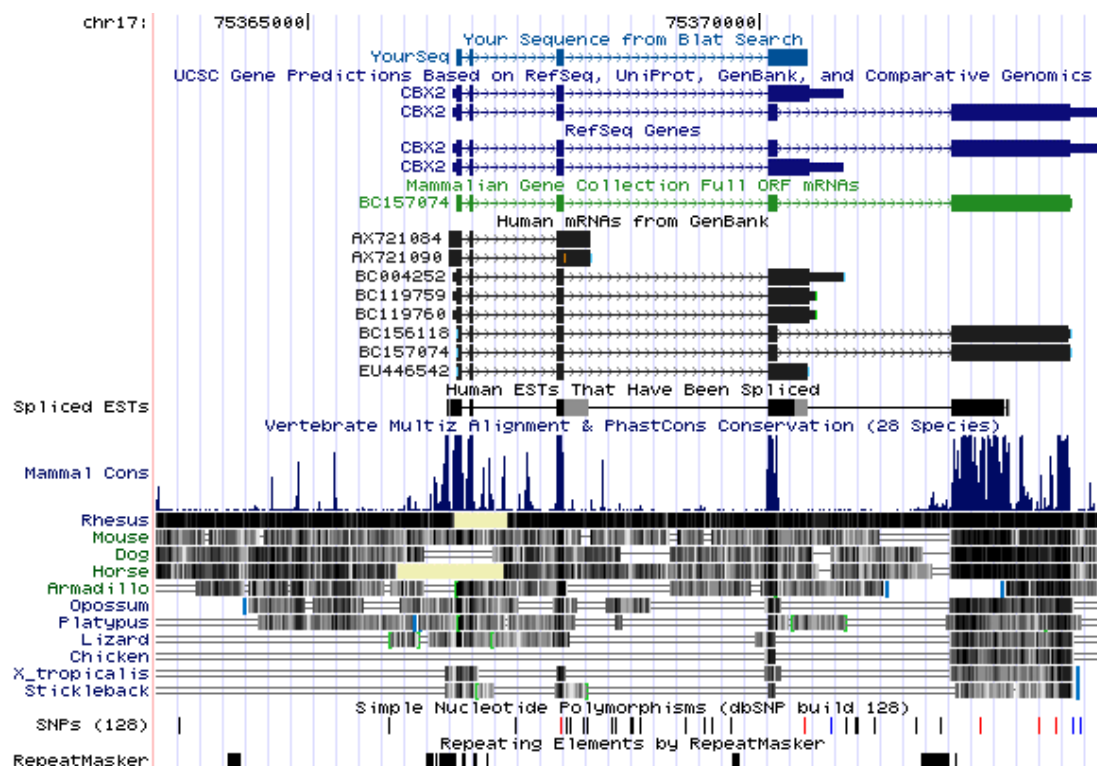
**Figure 4. Blat using UCSC Genome Browser of GenBank acquisition number BC004252.** Sequence BC004252 coded for the CBX2 SL isoform. From this Blat, it can be seen that the top sequence (BC004252) codes for the short length isoform of CBX2 rather than the full-length isoform. The entirety of exon 5 is not present in the short-length isoform.

Creation of Fragments for Ligation

To release the CBX2 SL fragment from the pOTB7 vector backbone, the isolated plasmid was double digested with EcoRI and XhoI restriction enzymes. Two different sets of restriction enzymes were used for double digestions: FastDigest restriction enzymes from Fermentas that complete digestion within 5 minutes, and standard restriction enzymes that can take up to 2 hours for proper digestion. Effective double digestions were seen from both the FastDigest restriction enzymes and the standard restriction enzymes as seen by the two bands on the agarose gel (Figure 5A). However, the FastDigest restriction enzymes were more efficient because they completely digested all the vector DNA, and released the 1.1kb fragment (lower band) from the vector backbone (top). The standard restriction enzymes left some of the vector only singly digested (top band, linear vector), thereby not releasing the 1.1 kb fragment of interest.

The bands of interest, 1.1kb, were excised and then gel eluted to isolate the DNA. Samples were sent for sequencing to confirm that we had the proper fragment, and that we received the proper clone from Open Biosystems. Once the sequencing information was received, the Sequence Scanner v1.0 from Applied Biosystems and UCSC Browser were used to verify we had the proper sequence that codes for the CBX2 short length isoform (Figure 6).

To obtain the vector backbone for the GST fusion protein construct and create sticky ends for ligation purposes, double digestion of the pGEX4T-1 plasmid was done using the FastDigest Enzymes EcoRI and XhoI from Fermentas. The protocol from Fermentas was followed for Double Digestion with FastDigest Enzymes. The digested plasmid was then run on a 1% agarose gel, extracted, and gel eluted (Figure 5B).
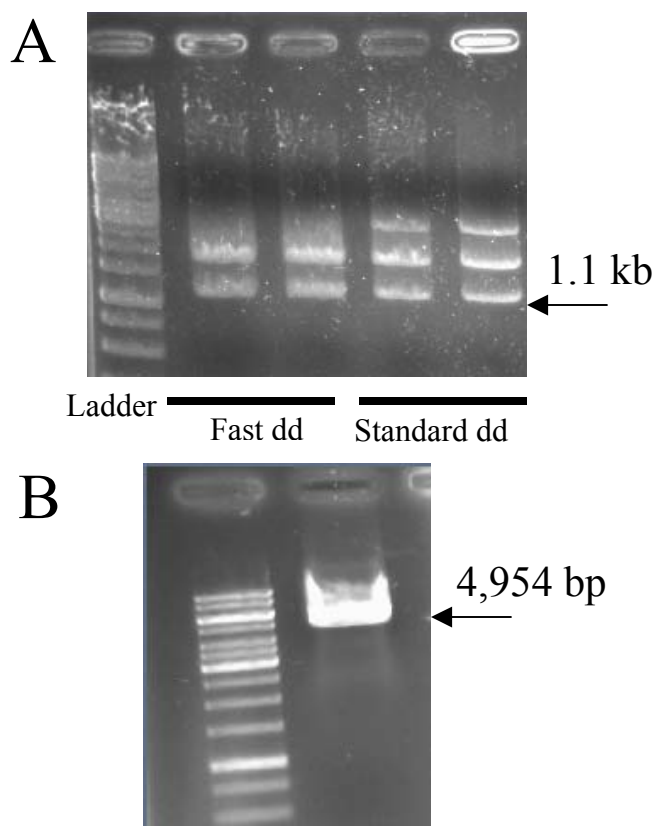
Figure 5. Creation of Fragments for the CBX2 SL construct. A) Double Digests of Open Biosystems Clone to excise CBX2 SL fragment. The lowest bands of both the Fast double digest (meaning the FastDigest enzymes from Fermentas were used) and the standard double digest (meaning the standard restriction enzymes were used) were sent for sequencing and confirmed. The top band of the standard double digests is the vector singly digested and thereby yielding the vector linear. The top band in the FastDigest and the middle band in the standard digest samples, are both the vector backbone that contained the CBX2 SL fragment. B) pGEX4T-1 double digested with EcoRI and XhoI, yielding a size of 4,954 bp. Once double digested sticky ends are created on the vector backbone, which then makes it ready for ligation with the double digested CBX2 SL fragment.
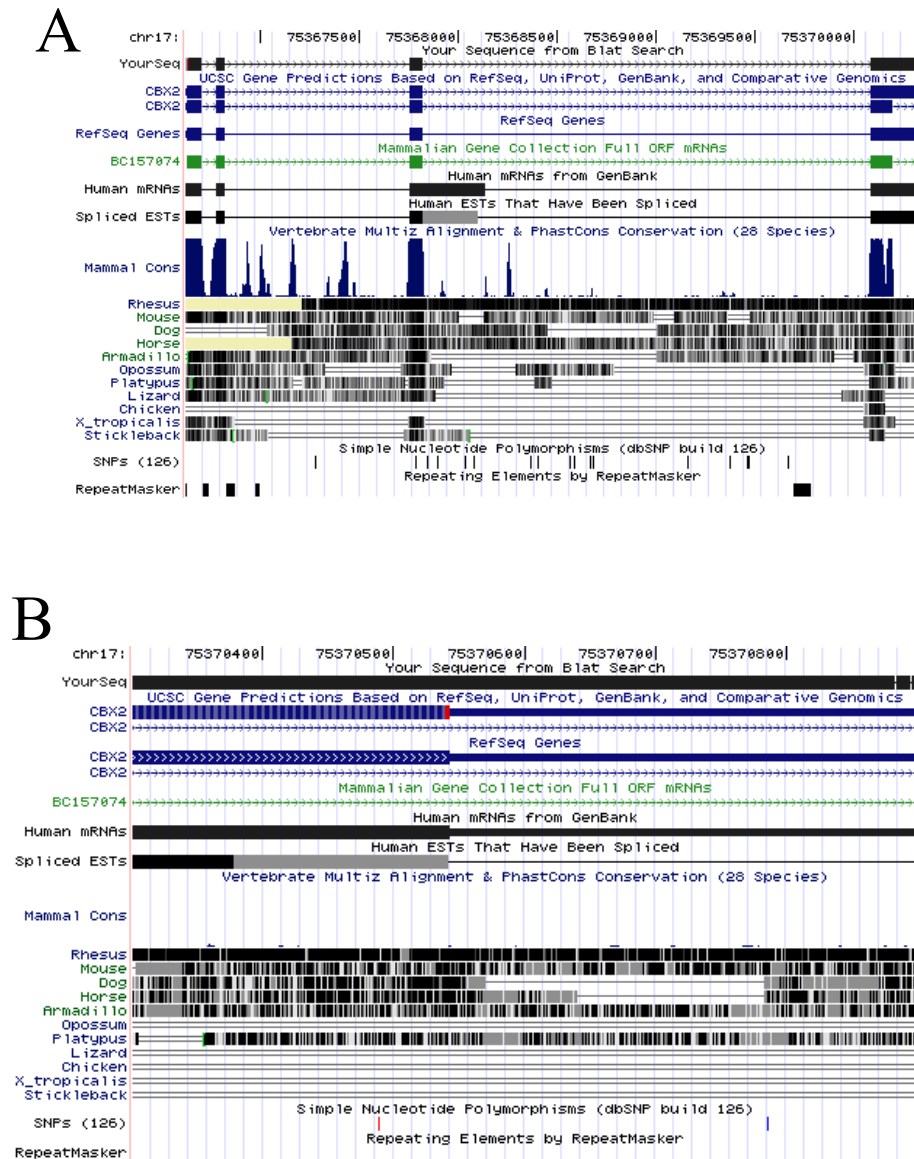
Figure 6. Blat using the UCSC Genome Browser of the Sequencing Data from the CBX2 SL fragments. A) N-terminus of CBX2 SL. The Blat shows that the 5' end of CBX2 SL fragment that was sent for sequencing does indeed code for the exons 1-4. B) C-terminus of CBX2 SL. The Blat shows that the 3' end of CBX2 SL fragment that was sent for sequencing does indeed code for the 3' end of only the truncated CBX2 protein, and not the full-length CBX2 protein. The sequence encodes for the entire exon 4, and none of exon 5. From the blats we can verify we have the right clone.

Ligation of CBX2 SL insert and vector, Transformation into DH5α and BL21 cells

The two fragments, each having sticky ends, were then ligated using Quick T4 DNA Ligase from New England BioLabs. This ligation product was then used to transform into DH5α competent cells. The transformed cells were plated onto LB agar plates containing ampicillin and grown overnight. The colonies that grew were confirmed to be positive by Colony PCR using the primers for the CBX2 SL fragment (Figure 7A). The colonies positive for the new fragment were then used to inoculate small cultures in preparation for Miniprep to isolate the plasmid DNA. The DNA from the Miniprep was used to do PCR to again confirm that the positive colonies had the insert (Figure 7B). The samples that were confirmed positive for the insert were then sent for sequencing and the data allowed us to verify that we had proper ligation.

In order to isolate a large amount of the newly constructed pGEX-CBX2 SL, cultures were set-up in preparation for Midiprep. Once the DNA was isolated, the construct was transformed into BL21 cells. To check for proper transformation, colony PCR was done, and confirmed that all colonies that grew were positive for containing the CBX2 SL fragment (Figure 7C). To confirm proper ligation and orientation of the fragment in the construct, single and double digests using EcoRI and XhoI were made of the construct (Figure 7D). If the ligation did not occur properly, and in the proper direction, the restriction enzyme sites would not have reformed. Without the restriction enzymes sites reforming, the sites would not be cleavable. From the gels we can see the sites are cleavable by the restriction enzymes because 2 bands are seen, the insert, and the vector backbone which released the insert. Both the insert and the backbone are of the expected sizes.
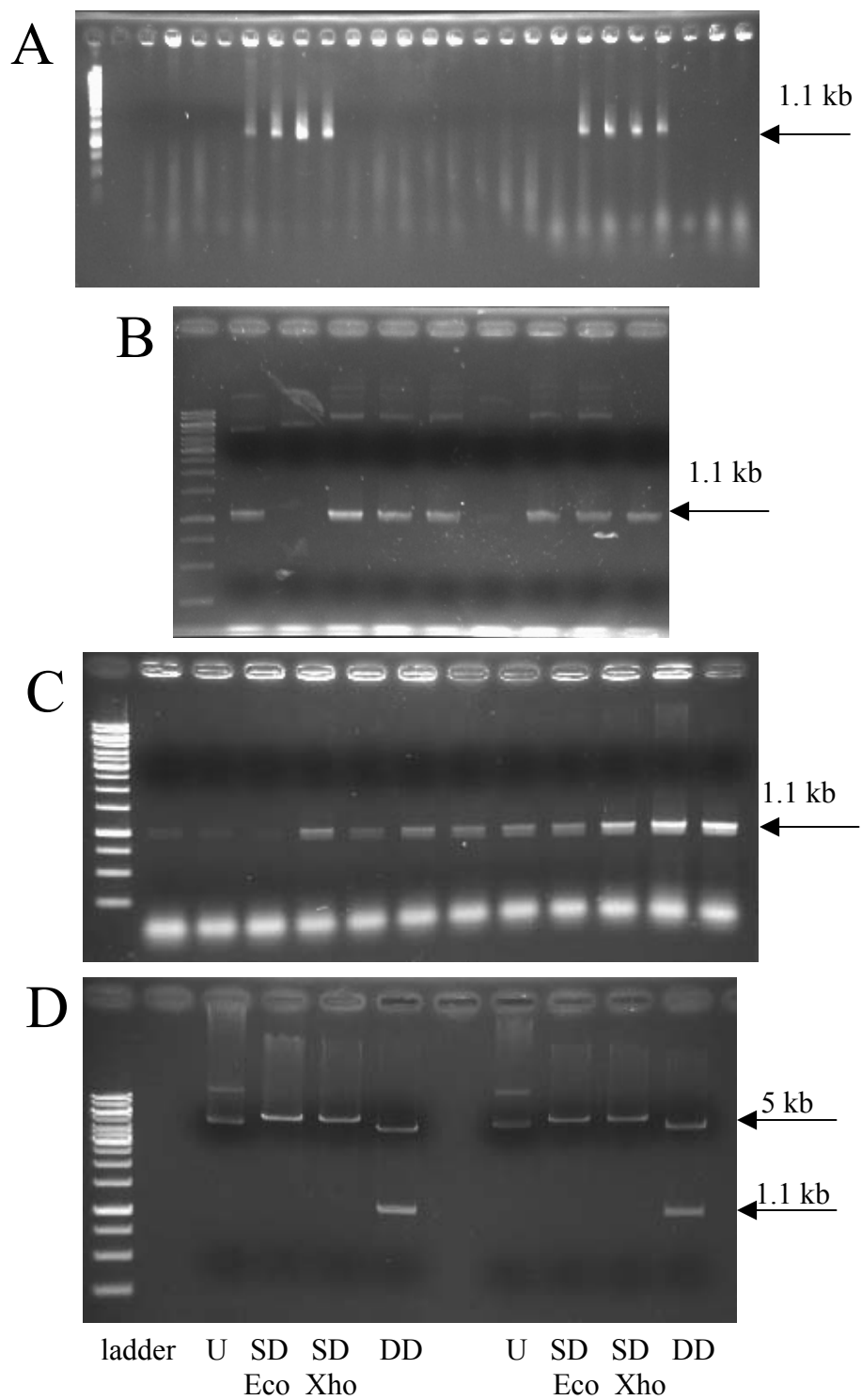
Figure 7. Confirmation of pGEX – CBX2 SL construct. A) Colony PCR on 24

colonies transformed into DH5α cells. Colonies 1-24: 8 confirmed positive

(#5,6,7,8,18,19,20,21) for containing the CBX2 SL fragment. These colonies were then

used as the source to inoculate small cultures for Minipreps of the plasmid DNA. B)

PCR product of Minipreps from transformation into DH5α cells. A few of the positive

samples, seen by the presence of the band at 1.1kb, were sent for sequencing to make

sure the fragment was indeed transformed into the cells. This was confirmed with the

sequence data (not shown). C) Colony PCR of BL21 cell transformation. Colonies that

grew after the transformation were tested, and confirmed positive for having the CBX2

SL insert, seen by the presence of a band at 1.1kb. D) Confirmation of proper construct

and transformed construct into BL21 cells via Single and Double Digestions. Single and

double digests of the construct using the restriction enzymes EcoRI and XhoI were

performed. Double digestions of the construct allowed the insert of CBX2 SL fragment

(1.1kb) to be released from the vector backbone. The proper ligation therefore reformed

the restriction enzyme sites and yielded them cleavable by the restriction enzymes.

Protein Induction, SDS-page gels, and Western Blot

After making the GST fusion protein construct, we wanted to confirm that the

fusion protein is expressed and optimize conditions for protein expression. After finding

out the expression of the protein, we wanted to determine how to best extract the protein

from the bacterial cells.

BL21 cells carrying the construct pGEX-CBX2 SL encoding the GST-CBX2 SL

fusion protein were incubated with IPTG (isopropyl-beta-D-thiogalactopyranoside) to

induce expression of the fusion protein. IPTG is generally used to induce the expression

of cloned genes that are under the control of the *lac* operon. Samples of BL21 cells

before and after IPTG expression were obtained and boiled in SDS-containing sample

buffer to extract all bacterial protein. These samples were run on a 6% SDS-page gel and

stained with Coomassie overnight. Analysis showed banding in the expected size range

(63.9kD), and therefore efforts to properly express the protein continued (not shown).

Another 6% SDS-page gel was run with various samples, transferred to a membrane and

processed for Western blotting using a monoclonal mouse anti-GST antibody obtained

from Dr. Bing Xia at the CINJ, (Figure 8).   From the Western blot we can see an obvious

expression of the GST fusion protein at 63.9kD, in BL21 cells induced with IPTG.

To determine the optimal cell density that will give the best protein expression,

BL21 cells were grown to different cell densities in liquid culture as determined by

OD600. A 10% SDS-page gel was run containing samples grown to various OD's

ranging from 0.4 - 0.8 after induction with IPTG. After running all the samples on a

SDS-page gel, the protein was then transferred from the gel to a membrane, and this was

followed by Western blot using a rabbit polyclonal anti-GST antibody. The Western

blots showed good expression of the GST tagged protein at its expected size, 63.9 kD

(Figures 9). The findings were that the fusion protein is expressed similarly across all the

OD600's tested; therefore we decided to use OD600=0.6-0.8.

In order to extract the fusion protein from the bacterial cells, two methods were

used: The Bug Buster Protein Extraction Reagent, and sonication with detergent. Bug

Buster is a product that contains lysozyme, which disrupts the cell wall of *E. coli* and

thereby liberates the active protein. The reagent utilizes a detergent mix that does not

denature the protein. Bacterial pellets were suspended in the Bug Buster Reagent

according to manufacturers protocol and then centrifuged to obtain a soluble fraction and

a pellet. Aliquots of the supernatant and pellet fractions were run on SDS-page and

subject to Western blotting using a GST antibody. We can see from the Western blot that

no detectable GST protein was seen in the soluble fraction after treatment with Bug

Buster implying that Bug Buster Protein Extraction Reagent is not effective for liberating

our fusion protein from these cells.

Another method to extract bacterial protein is to use mechanical disruption of the

cell walls using sonication and a detergent-containing buffer. Again BL21 cells were

induced with IPTG, pelleted and re-suspended in a Triton 100X containing buffer and

subjected to sonication as described in Methods. After sonication, the samples were

centrifuged and supernatant and pellets were collected and processed for western blotting

using a GST antibody. Western blotting shows that sonication with detergent was

reasonably effective in releasing the protein into the supernatant (SS) (Figure 9), although

significant protein remained in the pellet fraction. Either increasing the time of

sonication, the strength of detergent, or the duty cycle applied could increase the amount of GST protein released into the soluble fraction.

Affinity purification of GST CBX2-SL fusion protein using glutathione sepharose beads

The construct that was made using the pGEX4T-1 vector contains a GST tag in the N-terminus, which allows for protein purification using Glutathione Sepharose beads. BL21 cells were grown to OD600= 0.6 and 0.8 after induction with IPTG, and the soluble protein extracted using sonication as described above. The sonicated supernatant was then incubated with glutathione-sepharose beads. After incubation with beads, a sample of the unbound supernatant was removed and saved (fraction SB). The pellet was washed, and bound protein extracted by boiling (fraction F). Both fractions were then processed for western blotting using the anti-GST antibody. The Western blot showed good expression of the GST tagged protein at its expected size, 63.9 kD in the 0.6F and 0.8F samples (Figures 10). However, some protein remained unbound after incubation with glutathione-sepharose beads (0.6SB and 0.8 SB). Either increasing the amount of beads used, or the incubation time could optimize the purification with the Glutathione Sepharose beads.

Sample 1   Sample 2
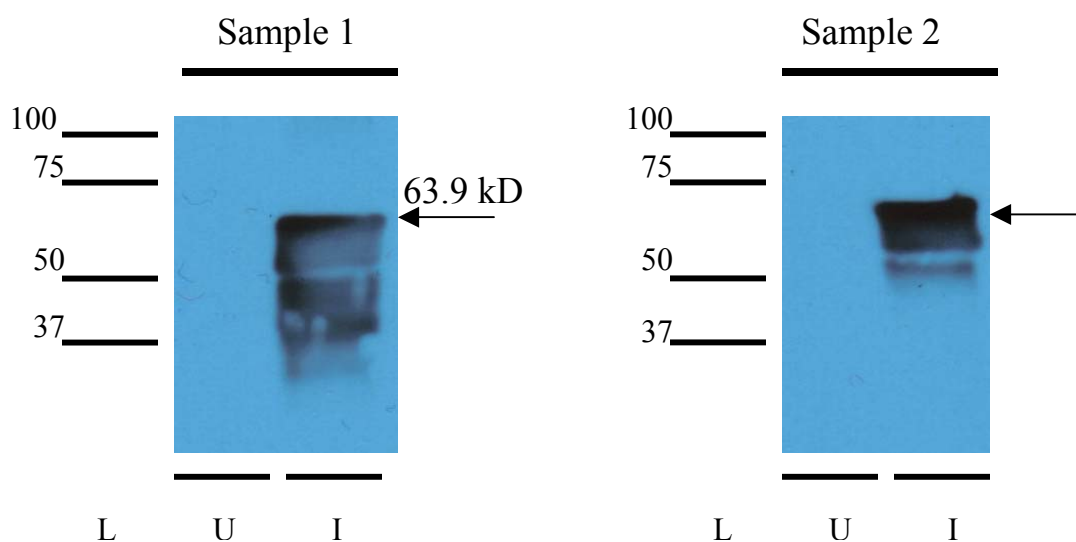
Figure 8. 6% SDS-page gel with Western Blot using Monoclonal Mouse anti-GST supernatant. L-ladder, U-uninduced sample, I-induced sample. We can see that in the uninduced sample there is no expression of the fusion protein. We can see in the induced samples that the fusion-protein is easily detected after induction with IPTG. The black arrows indicates the size of interest, 63.9kD. Although there is slight variation, expression of the fusion protein seems to be comparable between both samples 1 and 2.
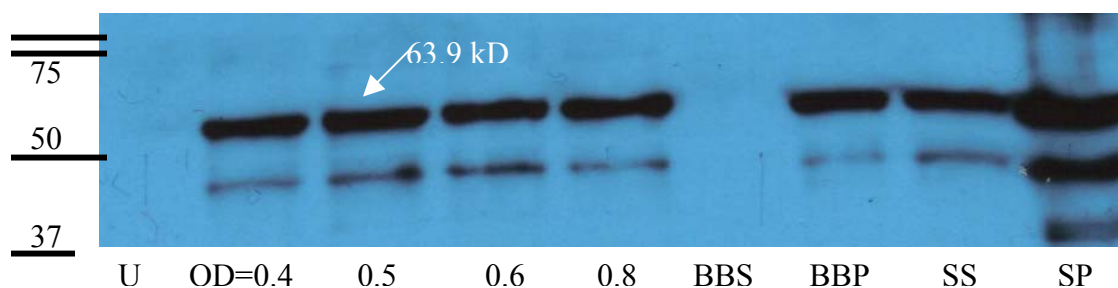
Figure 9. 10% SDS-page gel with Western Blot using anti-GST antibody –

Optimizing Protein Induction and Protein Extraction Conditions.

U- uninduced sample (negative control), 0.4 to 0.8- OD600 of sample after being induced

with IPTG, BBS- Bug Buster Reagent supernatant, BBP- Bug Buster Reagent pellet, SS-

sonicated supernatant, SP- sonicated pellet. The white arrow indicates the band of

interest, 63.9 kD, which is the size of the fusion protein containing the CBX2 SL

fragment. The uninduced sample acted as a negative control for this experiment.
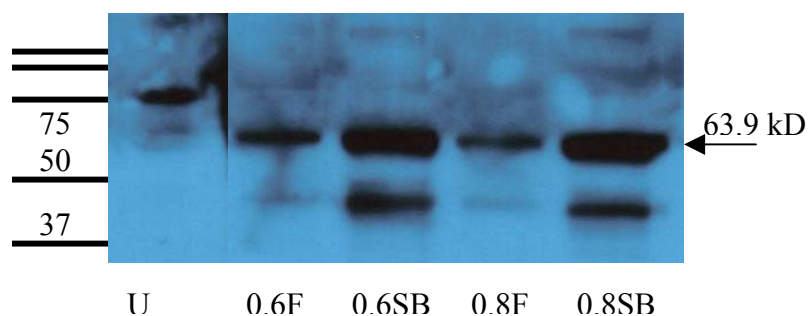
Figure 10. 10% SDS-page gel with Western Blot using anti-GST antibody –

Optimizing Glutathione Sepharose Bead Purification.

BL21 cells were induced with IPTG, grown to 0.6 or 0.8 OD, and subjected to sonication

to extract soluble proteins. Soluble fraction was then incubated with glutathione

sepharose beads. Protein bound to the beads was then extracted by boiling (samples 0.6 F

and 0.8 F), aliquots of the unbound supernatant were also analyzed (0.6 SB and 0.8 SB).

U- uninduced sample (negative control), 0.6F and 0.8F- protein eluted from Glutathione

beads by boiling, 0.6SB and 0.8SB- the unbound supernatant from the beads. Also

samples were run on SDS-page and processed for Western blotting using an anti-GST

antibody. The black arrow indicates the band of interest, size 63.9 kD. The uninduced

sample acted as the negative control for this experiment.

Creation of CBX2 FL fragment

To design the primers for the CBX2 FL fragment (CBX2 forward/reverse FL), the GenBank sequence NM_005189 was used. The primers contained overhangs for the EcoRI/XhoI restriction enzyme sites in the forward/reverse primers respectively. These primers were compared to the annotated genome by blatting from UCSC Genome Browser to check for similarity to other sequences (Figure 11). These primers proved to be specific for full-length CBX2.

In order to create a fragment that contains the sequence that encodes the CBX2 full-length (CBX2 FL) protein, RNA had to be collected from HeLa cells. Three different methods were used: the Trizol Reagent, Stratagene Absolutely RNA Miniprep Kit, and the RNeasy Mini Kit. After isolating the RNA, First Strand cDNA synthesis was done using Reverse-Transcriptase PCR. The cDNA served as the template DNA for PCR, where the conditions were varied in order to find the best annealing temperature with the highest stringency for the designed primers (CBX2 forward/reverse FL) (Figure 12A). Following PCR, the product was run on a 1% agarose gel and visualized. This band was then excised from the gel, and is currently being sequenced. To confirm the positive results, the PCR was repeated with newly collected RNA (Figure 12B). We can see that the Trizol Reagent proved to be the most effective technique for isolating RNA from the HeLa cells, which is seen on the agarose gel as the 1.5 kb band (CBX2 FL) in the "X" samples. From these few experiments, we can see that we can obtain the proper sequence that encodes for the CBX2 FL protein straight from the RNA of HeLa cells. Further experiments will be performed, modeled after the work done with the CBX2 SL fragment and the pGEX-CBX2 SL fusion protein.
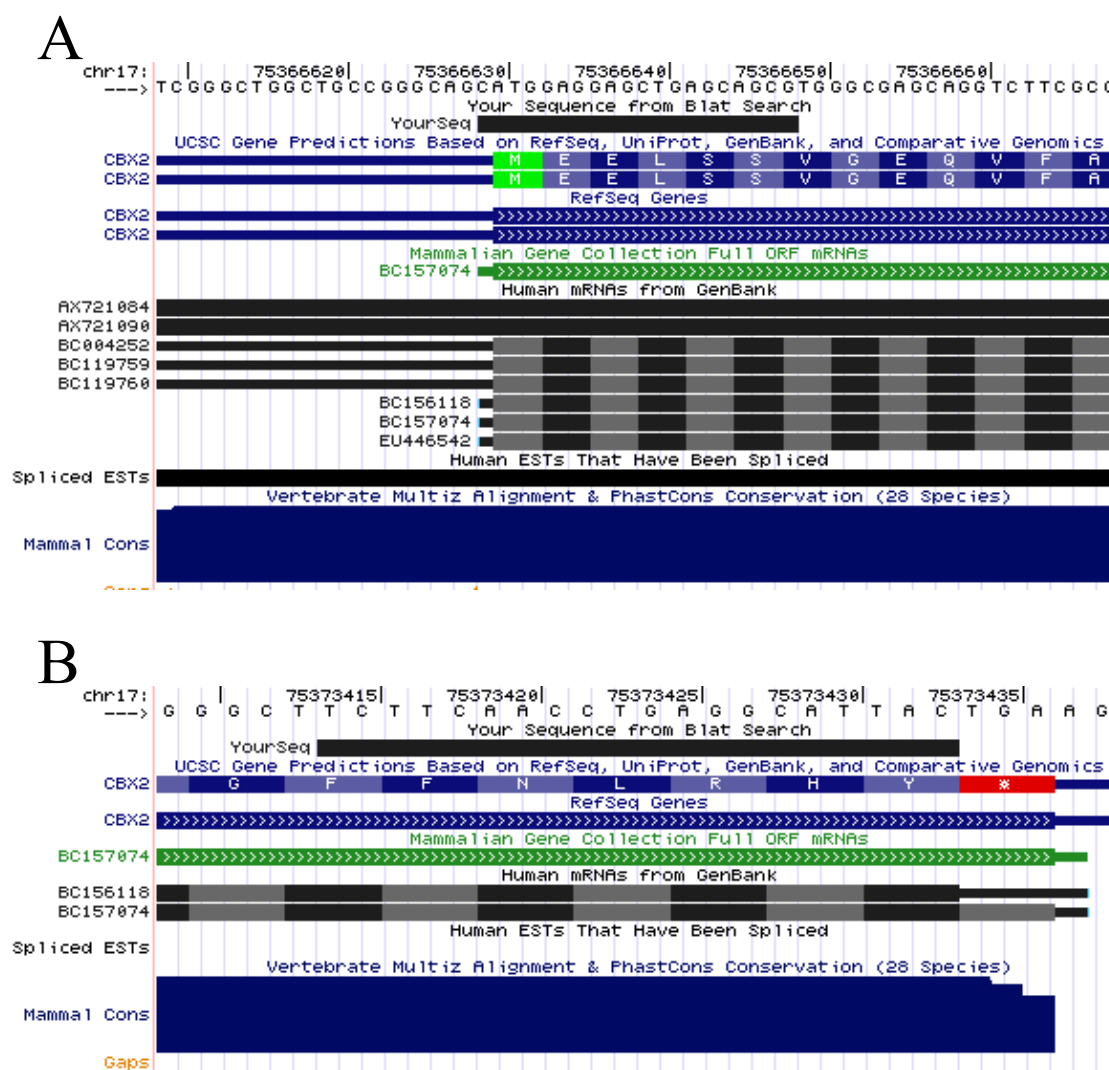
Figure 11. Blat using the UCSC Genome Browser of the primers designed for the CBX2 FL fragment. A) CBX2 forward FL primer. The Blat shows that the 5' end of CBX2 FL fragment is the only sequence recognized by the CBX2 forward FL primer. B) CBX2 reverse FL primer. The Blat shows that the 3' end of CBX2 FL fragment is the only sequence recognized by the CBX2 reverse FL primer. From the blats we can verify we will only be PCR lifting the CBX2 FL fragment, and no other sequences.
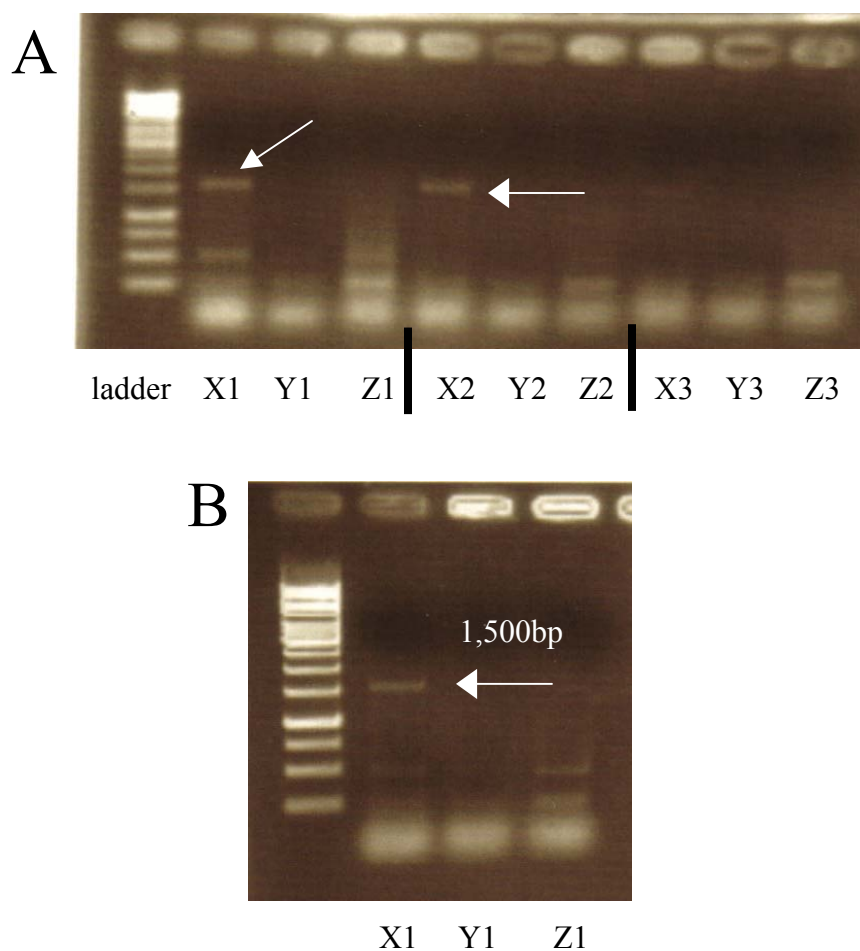
Figure 12. PCR Products of First Strand cDNA synthesis of CBX2 FL from RNA.
RNA Collection Technique: X- Trizol Reagent, Y- Stratagene Kit, Z – QIAGEN Kit,
PCR Conditions for annealing temperature: 1-62°C, 2-67°C, 3-70°C. A) PCR product of
First Strand cDNA synthesis from RNA. X1 and X2 showed the band of interest at
1,500bp. From this, we can see that the Trizol Reagent proved to be the best technique
for RNA collection. The white arrows indicate the band of interest, sized at 1,500bp. B)
PCR product of First Strand cDNA synthesis from RNA repeated. We wanted to repeat
the experiment with freshly collected RNA to confirm the results, and it can be seen that
X1 shows the band of interest at 1,500bp.

Creation of fragments for the Retroviral construct pLXSP-CBX2 SL

In an effort to make a construct that can be used immediately, we plan to make a construct containing a GFP tag to be used in a Retroviral Expression System. The pLXSN Retorviral Expression Vector, GenBank Accession No.: M28248, from Clontech Laboratories, Inc. was obtained from the laboratory of Dr. Shen at CINJ. The Shen lab had changed the Neomycin resistance to Puromycin resistance for easier growth in mammalian cells, as well as inserted an approximately 1.1kb fragment. Upon receiving the vector, now pLXSP, it was plated on LB agar plates with a final ampicillin concentration of 100ug/mL. Once colonies grew, several were isolated for Miniprep, and the DNA for the pLXSP vector was obtained.

The DNA isolated from the Minipreps of the pLXSP vector was then double digested with BamHI and SalI, and run on a 1% agarose gel. The correct band of approximately 5.9kb was excised and then gel eluted (Figure 13B). This effectively removed the 1.1kb insert.

The desired DNA fragment, CBX2 SL, was PCR lifted from the Miniprep of the Open Biosystems done earlier for the pGEX constructs, using primers designed to have BamHI and SalI overhangs (CBX2 forward/reverse Retro). The product of this PCR was then run on a 1% agarose gel, the band of interest sized at approximately 1.1kb was excised, and then gel eluted (Figure 13A). This gel elution of the CBX2 SL DNA, having BamHI and SalI overhangs was then double digested using these same restriction enzymes, BamHI and SalI.

Although this vector allows for ligation with the CBX2 SL fragment at this time, Dr. Shen's lab has seen very poor expression of GFP using this system.
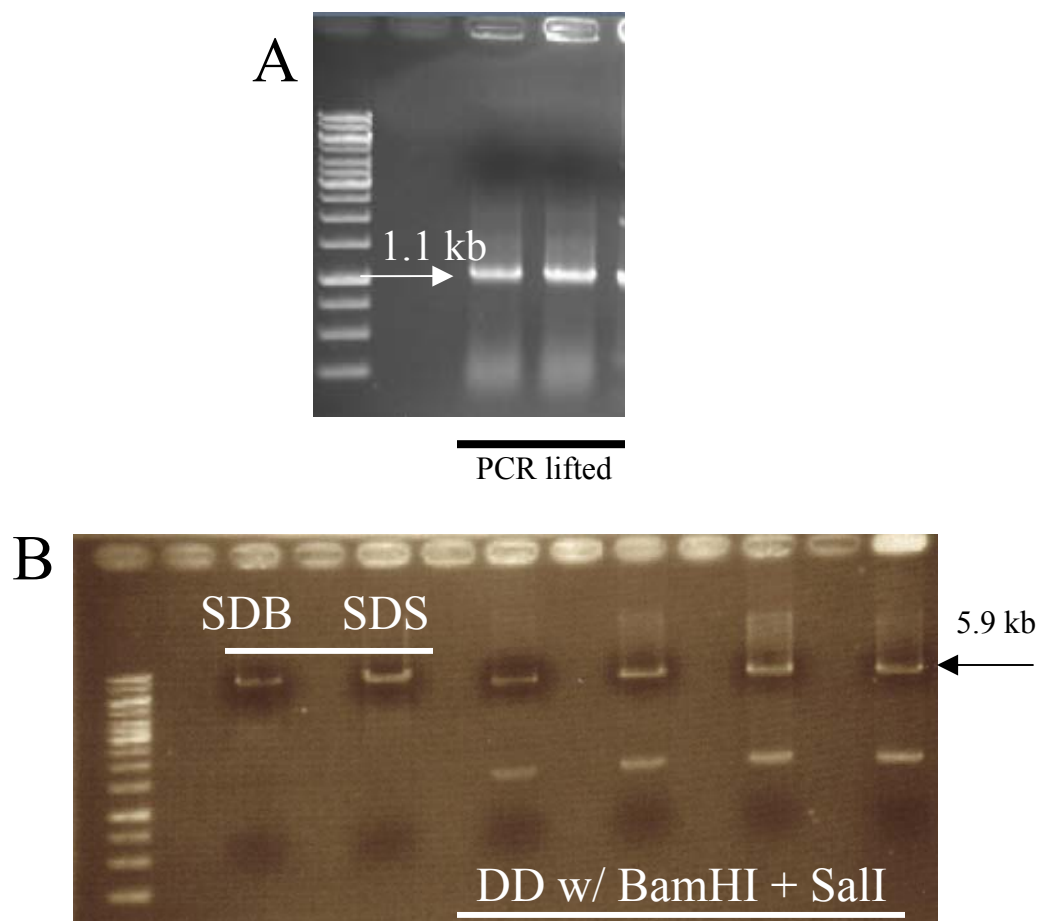
Figure 13. Creation of fragments for the Retroviral construct pLXSP – CBX2 SL.
SDB- Single digest with BamHI, SDS- Single digest with SalI, DD- double digest with
both BamHI and SalI. A) PCR lift of CBX2 SL from Open Biosystems with primers
having BamHI and SalI overhangs. Bands of interest can be seen at 1.1kb indicated by
the white arrow. B) Single and double digests of pLXSP with BamHI and SalI. The
vector once digested released the insert from Dr. Shen's lab (1.1kb), and is not of interest
for this project. The band of interest indicated by the black arrow, once double digested,
was 5.9kb, which is the pLXSP vector.

Discussion:

This project was motivated by data showing that the PIKA antisera recognizes proteins with chromodomains which co-localize with BRCA1 and redistributes to DNA damaged sites. Work previously done by Sarah Hill in 2005 identified a novel protein, which contained a N-terminal chromodomain recognized by PIKA. This protein showed great homology to the murine polycomb protein M33, or CBX2, a member of the polycomb group of proteins. These findings suggest that PcGs play a role in a DNA repair pathway. However, a new antibody to CBX2 is needed to confirm a potential role of CBX2 in the DNA repair pathway.

In an effort to make a rabbit polyclonal custom antibody that would recognize both the truncated/short-length (SL) and the full-length (FL) CBX2 protein, plasmid constructs were made containing both of the coding sequences for the CBX2 isoforms. The vector used for constructing the fusion protein contained a Glutathione S-transferase (GST) tagged vector (pGEX4T-1), which would allow for easy purification, detection, and expression of the GST fusion protein

The pGEX – CBX2 SL Construct

First, the intention was to make an antibody that would recognize the N-terminal exons (1-4) found in both the SL and FL isoforms of CBX2. A construct was made using the technique of directional cloning with the SL sequence of CBX2 and the pGEX vector. From the samples run on the SDS-page gels, we can see several things. First, we were able to determine that varying the OD600 after inducing with IPTG did not yield differing levels of expression for the fusion protein. This allowed us to consistently use an OD600=0.8 for future experiments. Once we obtained the results from the protein

extraction experiments, we were able to clearly see that the Bug Buster Protein Extraction Reagent was not effective at all, however, mild detergent sonication was reasonably effective. We can conclude that some of the protein still remained in the pellet of the sonicated sample, but also, some of the protein was released from the cell under these conditions. In order to optimize the yield of extracted protein from the sonicated sample, a couple of parameters could be adjusted. Either the pulse length used to sonicate the sample could be increased, or the strength of the detergent could be increased.

For the fusion protein purification, conditions can also be optimized to increase yields. By examining the results, we see that the sample removed from the supernatant of the GS bead purification still contained a sizeable amount of unbound GST fusion protein. This issue can be fixed simply by increasing the amount of GS beads, and therefore the surface area, added to the initial sample, which would allow for a greater amount of fusion protein to bind to the GS beads.

Intentions with this construct are to continue optimizing expression of the protein if necessary, and then send the plasmid construct to an outside company for generation of rabbit polyclonal antibody to CBX2 SL. The GST protein can then be used to affinity purify this polyclonal antisera. Arrangements have been made with Innovative Research Incorporated for the expression and purification of our protein, immunization and bleeding of the rabbits, and then lastly, antibody purification.

The CBX2 FL fragment

Another construct is also in progress using the GST tagged vector, pGEX4T-1. It is possible that the CBX2 SL antibody will not recognize both the SL and FL CBX2 isoforms, due to the lack of a significant portion of exon 4 in the CBX2 FL isoform.

Therefore, efforts have been made to also make a pGEX – CBX2 FL construct. Although this plasmid construct is not complete, the directional cloning technique has proved to be effective for the purpose of making the pGEX – CBX2 constructs and will therefore be used, with aforementioned optimization adjustments. Experiments thus far have yielded a fragment of 1,500 bp, which is the expected size of the CBX2 FL fragment. Because we feel that we have obtained the appropriate fragment, using primers specific for this sequence, the sample is being sequenced for confirmation. The CBX2 FL fragment not only can be used to make tagged constructs, but also as a general tool for laboratory experiments.

The next steps with this fragment will be to ligate it with the pGEX vector, as done with the pGEX – CBX2 SL construct. Then transformation into expressive bacteria will follow, and then protein induction, expression, and purification. The plasmid construct will also be sent to Innovative Research Incorporated for rabbit polyclonal antibody purification.

The Retroviral pLXSP – CBX2 SL and FL construct

For the purpose of immediate immunofluorescence, a retroviral construct using the GFP tagged vector pLXSP with CBX2 SL is in progress. Using this vector allows for the direct transfection of the retroviral construct into packaging cells, collection of the virus, and then infection of target cells, which in this case are HeLa cells. By using this GFP tagged construct, immunofluorescence data regarding localization of CBX2 after DNA damage can be quickly obtained without the need for specific antibodies. Unfortunately, the pLXSP vector has been seen to have poor expression by another lab, and therefore the intension is to use an EGFP vector to complete this construct. Since we

believe we have effectively isolated the CBX2 FL fragment, efforts in the future will also be made to make a construct with the CBX2 FL fragment and the EGFP vector. By making GFP constructs with both the CBX2 SL and FL fragments, we should quickly be able to determine which isoform is expressed in mammalian cells, and its localization after DNA damage.

Antibodies for Specific Exons

The constructs we have generated include epitopes found in both the short and long isoforms of CBX2. To obtain antibodies specific to either the CBX2 SL or FL isoforms, one could target regions of exons unique to each isoform. Therefore, to target the CBX2 SL isoform, an antibody that will only recognize the C-terminus of exon 4, a region not conserved in the FL isoform, can be affinity purified. To develop an antibody that will only recognize the CBX2 FL isoform, the entirety of exon 5 can be targeted since this exon is not conserved in the SL isoform. By excluding the regions conserved by both isoforms, antibodies can be affinity purified that are specific to only one isoform. This technique is another way in which the determination can be made of whether or not the CBX2 SL isoform is actually expressed in mammalian cells.

Experiments with Polyclonal CBX2 SL and FL Antibodies

Once the CBX2 antibodies are received, they will be used for immunofluorescence (IF) studies. DNA damage will be introduced to HeLa cells from UV Laser Scissors, and IF will be used with the CBX2 antibodies to study the protein's response and localization after DNA damage.

Future Experiments

Additional work done by the Ganesan lab at the Cancer Institute of New Jersey with UV laser scissors, have been able to show that BMI-1, also a member of the PRC1, localizes to DNA damaged sites indicated by phosphorylated H2AX, and also co-localizes with BRCA1 (Figure 1 and 2). EZH2, a member of the PRC2, has also recently been shown to co-localize with BRCA1 at sites of DNA damage (data not yet published). Once the CBX2 polyclonal antibody is obtained for both isoforms, immunofluorescence will be done which we hope will show the co-localization of CBX2 and BMI-1 with BRCA1 at sites of DNA damage caused by UV laser scissors. By showing the localization of PRC1 to sites of DNA damage, along with BRCA1, we hope to begin to elucidate the function of BRCA1 and the PRC1 proteins at sites of DNA damage.

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