**Title:The role of polycomb group factor- CBX8 in genomic integrity**

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**Abstract:**

The central dogma of biology states that the information in DNA is transcribed to RNA and then translated to proteins, which are the structural and functional units of cells. It is therefore critical that the information contained in the DNA remain accurate and free of errors. However, our genome is continuously exposed to a number of damaging agents, both exogenous and endogenous. Exogenous agents such as radiation (UV, X-rays, gamma-rays), chemicals (chemotherapeutic agents, certain aromatic hydrocarbons, etc) and endogenous agents such as reactive oxygen species, errors in DNA replication are some of the sources that damage DNA and jeopardize the fidelity of the information contained in it. Compromised genomic integrity results in deleterious consequences such as cell injury, cell death or even cancer.

Almost all kinds of cancer are associated with some form of DNA damage (mutations, translocations, insertions, amplifications, deletions etc.) It is therefore no surprise that our cells have evolved to have numerous mechanisms to maintain genomic integrity. Disruptions in these pathways lead to genomic instability and are often associated with cancer. For example, mutations in BRCA1, BRCA2, ATM, p53, BLM, WRN, FANC – important DNA repair genes, are associated with cancer-prone syndromes (Reliene et al, 2007). Furthermore, many of the new anti-cancer agents such as topoisomerase inhibitors, DNA cross linking agents and PARP inhibitors exploit the already dysregulated DNA repair pathways in tumors to selectively target them (Bhattacharyya et al, 2000, Bryant et al 2005, Farmer et al, 2005, Fong et al, 2009). Mechanistically, DNA damage manifests its carcinogenic effects by either down-regulating the expression of tumor-suppressors or by over expression of oncogenes.

**Introduction**

**Polycomb group proteins**

Another mechanism which can potentially alter gene expression, but without altering the DNA sequence itself, is through the epigenetic modification of chromatin. One of the major groups of epigenetic regulators of gene expression is the polycomb group (PcG). PcG genes are thought to mediate transcriptional silencing and have been implicated in the control of embryogenesis, development, stem-cell self renewal, heritable epigenetic states, as well as cell proliferation and cancer (Schwartz et al 2008, Schuettengruber et al, 2007, Jacobs et al, 1999, Sauvageau et al 2008, Nakayama et al 2009).

Furthermore, in order for efficient initiation and execution of DNA repair, repair factors must gain access to the sites of DNA damage necessitating the need for chromatin modification. And once the repair process is complete the chromatin must be restored to its original configuration to maintain the proper gene expression profile. Hence genomic integrity is not just dependent on the DNA repair factors but also on the proper epigenetic modifications of chromatin. Genomic homeostasis is a dynamic process between DNA repair factors and the epigenetic regulators.

**CBX proteins**

Chromodomain stands for *Chr*omatin *O*rganization *Mo*difier domain named for its involvement in the organization of the chromatin (Paro et al 1991). Chromobox (CBX) is the element that encodes the chromodomain. The chromodomain is an evolutionarily conserved domain found in plants, fungi, protists, fish, insects, amphibians and mammals (Eissenberg 2001). Chromodomain superfamily is comprised of 3 major classes on the basis of domain organization: (i) Polycomb, which contains a single chromodomain, (ii) Chromodomain Helicase DNA-binding protein (CHD), which contains paired tandem chromodomains and (iii) HP1 family, containing a chromodomain and the chromo-shadow domain (Lomberk et al 2006).

We decided to focus our attention on the Polycomb group chromobox genes due to the importance of polycomb group complexes in various essential cellular processes as described earlier. Polycomb group proteins (PcG) can be classified based on the complexes they form. There are 3 main complexes: polycomb repressive complex 1 (PRC1), polycomb repressive complex 2 (PRC2) and pleiohomeotic repressive complex (PhoRC) (Schwartz et al 2008, Schuettengruber et al 2007, Kohler et al 2008). PRC2 functions to initiate chromatin silencing by virtue of its histone methyltransferase activity (among others) whereas PRC1 functions to maintain the silent state by binding methylated histones and recruiting more PRC2 complexes thereby spreading the inactivation signal. The functions of PhoRC are not well characterized. CBX proteins belong to the PRC1 complex.

Five CBX proteins belong to the polycomb class – CBX 2, 4, 6, 7 and 8 (hereafter referred to as Pc-CBX to distinguish them from the HP1 and CHD classes of CBX proteins). Their structure and function are poorly characterized. The chromodomain is known to have methyl histone binding properties, particularly H3K27me3, and hence Pc-CBXs are implicated in targeting chromatin modeling complexes such as PRC1 to chromatin (Schwartz et al 2008). At a functional level Pc-CBX proteins have been associated with cell proliferation and cancer (among many other functions). CBX4 has been suggested to be a repressor of the c-myc proto-oncogene activity; over-expression of the wild-type CBX4 protein results in decreased c-myc expression. Expression of mutated protein can lead to cellular transformation (Satijn et al 1997). CBX4 is known to possess SUMO E3 ligase activity for substrate proteins HIPK2 and Dnmt3a (Roscic et al 2006, Li et al 2007). CBX8 over-expression leads to the repression of the INK4A-ARF locus and hence promotes cellular proliferation (Dietrich et al 2007). Similarly, CBX7 has also been shown to extend the lifespan of normal human cells and immortalizes mouse fibroblasts by its repressive effects on the INK4A-ARF locus (Gil et al 2004). In contrast, CBX7 expression was observed to progressively decrease with malignancy grade and neoplasia stage and restoration of CBX7 expression in thyroid cancer cells reduces growth rate (Pallante et al 2008), suggesting CBX7 may function as a tumor suppressor in this disease. In another study, the expression levels of CBX7 inversely correlated with the progression of tumor stage and grade in urothelial carcinomas of the bladder (Hinz et al 2008). Loss of CBX7 expression was associated with increasing malignancy grade in pancreatic adenocarcinoma (Karamitopoulou et al 2010). These diverse observations regarding the functional role of Pc-CBX suggest that the precise role of Pc-CBX in the pathogenesis of cancer is not clear and needs intense further investigation.

**Material and Methods:**

There are many techniques used one of the technics is Western Blotting:

The Western blot (alternatively, protein immunoblot) is an extremely useful analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein. The proteins are then transferred to a membrane, where they are probed (detected) using antibodies specific to the target protein.

There are now many reagent companies that specialize in providing antibodies against tens of thousands of different proteins. This method is used in the fields of molecular biology and biochemistry and other molecular biology disciplines. How Does Western Blotting Work?

Obtain a protein sample you want to analyze, such as cell samples. Lyse the cells to release protein contents. Run these on a gel which separates proteins on the basis of size. Then transfer these gel proteins onto a membrane using electricity. This membrane can then be used to probe for proteins of interest using a primary antibody.

I manage an experience in which preparing cell extract for western blot monitoring experiment level of CBX8 plasmid:

NETN buffer: For 3ml For10ml stock

20 mM Tris (PH 8.0) 60Ml 200ML 1M

150 mM Nacl 450 ML 1.5ML 1M

1mM EDTA 6ML 20ML 0.5

0.5% NP40 15ML 50Ml 100%

1X 1X pretease inhibition (add fresh) 60ML 200Ml 50X

add H2O 2.4ML 8.23Ml

Protocol:

• Aspirate Media (on ice)

• Wash 2X with cold PBS

• Add 200-300 Ml NETN buffer (for 100 mm plate)

• 15 min on ice

• collect in 1.5 ml centrifuge tube

• Senicate

• Wash with 70% ethanol and H2O (last wash H2O)

• Spin 14000 X rmp 10 min 4C

• Collect supernatant in a new tube.

Western blot relies on the primary antibody to detect this protein from the thousands of proteins on your membrane and previously on your gel!

Lane 1 is a protein size marker ladder which shows different *known* sizes of proteins, this can be purchased commercially and the sizes of all the spots are given in a pamphlet. Lane 3 is a PEGFP sample and lane 5 is a EUFP-CBX8. As you can see the protein in lane 3 has a higher expression than the lane 5, which is interesting. Line 7 is POZ-C, Line 9 POZ-C,CBX8, Line 11 PCEXDt1, Line 13 PGEX47 ,CBX8 We can then look at the known protein size from our brochure which we received with the ladder. We then determine that the size of the protein is 80 kDa. Our protein of interest is also 80 kDa. So we know that the western blot worked and that the protein is highly expressed in a cancer sample!

To Detect our Protein: Used an Antibody Against Your Primary Antibody Source, Use an ECL - Chemiluminescence Kit and Film to Get the Results

Steps in Western Blot Analysis:

- Sample Prepartation

- Lysing Buffer

- Antibody

- Lysing Cells

- Gel Preparation

- Running Gel

- Transfer of Gel

- Controls

- Western Blot

-Readout

-Analysis and Interpretation

To Prepare Samples for Western Blot monitoring experiment level of CBX8 plasmid:

Plated 2\*10^5 HEK293T cells in 6-meu plate, For non-adherent cells you can gently pellet them with centrifugation and then wash the pellet with PBS. For adherent cells, simple wash the flask or dish with PBS prior to western blot.

For transfection we add 250 Ml optimem +1 Mg Plasmid and 250 Ml optimem+4 Ml lipofeet ---->we mix for 5 min mix---->20 min---->1Add to cells.

In this experiment the six meu plate is included PEGFP—POz-C(FH)--PGEX4T(GST)--PEGFP-CBX8--POZ-C CBX8—PGEX4T1 CBX8.

Western blot analysis examines proteins, and so we want the proteins to be release from the cells and also prevent the proteins from being cut up by proteases. We need these to western blot:

- to keep things cold! 4C on ice during cell lysis for western blotting

- use Detergent to break up membranes of cells to release proteins

- Buffer

- Inhibitors (both protease inhibitors and phosphatase inhibitors if we will do western blot for phospho-proteins)

SDS and RIPA are detergents for Lysing Cells that are used to solubilize proteins for later analysis using western blotting. This is required as many proteins are either inside the cell or located inside cell membranes, so we need to release these proteins to be able to immunoblot for them. Usually either mouse monoclonal antibodies or rabbit polyclonal antibodies are used for western blotting. SDS-PAGE Gels are gel matrices which are used to separate proteins by size in the presence of electric current. Low percentage gels separate larger proteins whereas higher percentage gels separate smaller proteins better. The problem is that all proteins have a charged associated with them, and in an electrical current this could cause problems. This is solved by the addition of SDS to the protein samples. SDS binds to proteins every few amino acids and neutralizes the charge differences that proteins have. This allows proteins to be separated by size and not by charge.

SDS-PAGE Gel Preparation for Western Blot

- depending on how much protein you will want to load for western blotting, you should use small combs or larger combs.

- the percentage of acrylamide is important. Usually 10% acrylamide is used.

- A resolving gel is used at the bottom with a pH of 8.8

- A stacking gel (4-5%) pH 6.8 is used to pack proteins in together after loading

- Polymerization of gels is increased by the catalysts APS and TEMED which speed up the polymerization reaction (formation and solidification) of the poly-acrylamide gel.

- Load every Samples to prevent sample leaking out of the lane.

⁃ Load every lane and use sample buffer in each (to prevent differences in ).

Gel Electrophoresis

Centrifuge samples for 10 sec after boiling.

Load sample into each lane

Load MW reference

Run gel at 100V (constant voltage) or even better at 40 mA (constant current). Watch protein marker/ladder or dye front for when to stop gel. Constant current gives better and sharper results if you have the time.

- watch for bubbles between glass and under the gel - this means that it is working

- watch for protein migration (should be going down) - if not you have switched the leads and can lose all your samples!

- Initially run slowly through the stacking gel (~ 50 Volts), this gives sharper bands.

Transfer of Proteins to Membrane for Western Blotting

Discusion:

Genomic integrity is central to the maintenance of normal cells and numerous type of cancer are associated with some form of DNA damage induced mutation – including translocation, insertions, amplifications, deletions etc. Another mechanism which can potentially alter gene expression but for DNA repair to initiate the repair factors must gain access to DNA leions for this chromatin. In order for DNA repair to initiates the repair factor must gain access to DNA lesions for this chromatin alteration must occur. Furthermore, once the repair is complete the chromatin must be restored to its original configuration to preserve by both DNA repair pathways and epigenetic regulators.

We have found a polycomb group protein, CBX8, exhibits a strong location to the sites of laser scissors induced DNA damage, suggesting that it might have a role in maintaing genomic integrity live cell imaging using YFP-CBX8 fusion protein demonstrated that CBXXX8 fusion protein demonstrated that CBX8 localizes to sites of DNA damage within 30 seconds peaks at around 2 minutes and returns to its original pre- damage distribution within 15 minutes. Moreover this localization was reduced both in PARP1-/-MEFs, and in cells treated with a PARP inhibitor, suggesting that CBX8 functions downstream of PARP-dependent DNA damage response pathways.

They have identified the recruitment of a novel protein CBX8, to the sites of DNA damage CBX8 has exclusively been studied for its role in epigenetic regulation owing to its part in the larger polycomb group complexes. Our finding that CBX8 localizes to the sites of DNA lesions is very exciting. We will use CBX8 as our prototype and will determine its biochemical structural and functional aspects in the context of DNA damage response.

Literature Cited