

**Final Progress Report 08/09 – Alyson Freeman – The DNA Damage Response in Breast Cancer: Functional Analysis of the Interaction Between CHK2 and a Regulatory Subunit of Protein Phosphatase 2A**

**1- Summary of Project Objectives**

The main goal of this project is to determine the functional significance of the interaction between checkpoint kinase CHK2 and protein phosphatase PP2A. CHK2 plays an important role in the DNA damage response pathway, therefore we proposed to define this interaction to further characterize the CHK2-mediated response. The CHK2-PP2A interaction was originally found in this lab by performing a yeast two-hybrid screen on human mammary gland cDNA using CHK2 as bait. A subunit of PP2A, namely B56alpha (also called B'alpha), was pulled out of this screen. The holoenzyme form of PP2A consists of an A structural subunit, a C catalytic subunit, and one of many B regulatory subunits. It is the B subunits that are thought to give PP2A its specificity. Since both CHK2 and subunits of PP2A have been found to be mutated in breast cancer tumors and cell lines, we hope that by defining this interaction we will be able to shed some light on its importance in breast cancer so that we may exploit it for therapeutic means.

**2- Work Achieved**

*Task 1. To determine the regions in CHK2 and PP2A B56alpha that are necessary for binding and test the hypothesis that binding is modulated by DNA damage:*

- a. *Map the minimal binding regions involved in CHK2 and B56alpha binding using the yeast two-hybrid system*
- b. *Verify the interaction by expressing proteins in mammalian cells*
- c. *Determine any changes in the interaction in breast cancer cells or cells exposed to DNA damage*

We have successfully mapped the binding regions in CHK2 and PP2A B56alpha that are necessary for the interaction using the yeast two-hybrid system. In CHK2, the region that binds to B56alpha consists of amino acids 1-107 which contains the entire SQ/TQ region. In B56alpha, the region that binds to CHK2 is comprised of amino acids 89-322. Unfortunately, this could not be verified in mammalian cells since the SQ/TQ fragment, which is naturally unstructured, could not be expressed.

Co-immunoprecipitation of CHK2 and FLAG-tagged B56alpha verified that they can bind in mammalian cells. Endogenous co-immunoprecipitation was able to show that CHK2 can bind to the PP2A C catalytic subunit as well. This indicates that CHK2 can bind to the potentially active holoenzyme complex. We would have liked to examine the CHK2-B56alpha interaction in breast cancer cell lines. Unfortunately, the reagents available for endogenous IPs were not sufficient.

The binding between CHK2 and B56alpha is disrupted by DNA damage caused by gamma-irradiation (IR) in an ATM-dependent manner. Interestingly, the dissociation of CHK2 and B56alpha is correlated with the phosphorylation of CHK2 at serines 33/35. When these residues are mutated to mimic glutamic acid (S33/35E), the dissociation after IR is increased and when these residues are mutated to alanine to prevent phosphorylation (S33/35A), there is increased binding in mock treated cells. This is indicating that the phosphorylation of CHK2 at S33/35 influences the binding between CHK2 and B56alpha.

The dissociation of CHK2 and B56alpha is an early event after IR that occurs in a dose-dependent manner. The proteins can reassociate hours later. This does not correlate with repair of the DNA damage, however, it does correlate with the dephosphorylation of CHK2 at serines 33/35.

*Task 2. To determine the subcellular localization of PP2A B56alpha and CHK2 in the presence and absence of DNA damage:*

- a. *Use confocal microscopy to determine co-localization of endogenous CHK2 and PP2A B56alpha*
- b. *Determine the subcellular localization of CHK2 and PP2A B56alpha before and after DNA damage using subcellular localization*

- c. *Determine any changes in subcellular localization of mutant CHK2 proteins by expression in cell lines*

Using biochemical fractionation and confocal microscopy, we have determined that CHK2, PP2A B56alpha, PP2A A and PP2A C co-localize to the cytoplasmic and nucleoplasmic compartments. All proteins were abundant in both compartments and no change in localization was seen after exposure to IR.

Recently, a paper was published in which 38 breast cancer cell lines were tested for both CHK2 gene mutations and CHK2 protein expression (1). MCF-7, SUM1315, and T47D have the highest percentage of cells expressing CHK2 in the nucleus, MDA-MD-231 had a low level of expression, and MDA-MD-453 was in the middle. These cell lines also had corresponding levels of CHK2 phosphorylation at threonine 68, which is often used as a marker for CHK2 activity. Therefore, we did not test the localization of CHK2 in these cells.

*Task 3. To determine the extent to which the activities of PP2A and CHK2 change in response to DNA damage:*

- a. *Verify the presence of CHK2 and B56alpha in active enzymatic forms*
- b. *Determine the kinase activity of CHK2 in cells with either overexpressed or knocked-down B56alpha in response to DNA damage*
- c. *Determine the kinase activity of CHK2 mutants in response to DNA damage*
- d. *Determine the phosphatase activity of PP2A in a complex with B56alpha in cells with either overexpressed or knocked-down CHK2 in response to DNA damage*

We first determined whether CHK2 or DNA damage had any effect on PP2A activity. In an *in vitro* kinase assay, we found that CHK2 was able to phosphorylate B56alpha. We then moved on to look at any effect this had on PP2A activity. By examining total PP2A activity in HCT116 and HCT116 CHK2<sup>-/-</sup> cells, we were able to show that PP2A activity is the same in both cell lines. This means that CHK2 does not influence PP2A activity *in vivo*. Next, in 293T cells or 293T cells expressing FLAG-B56alpha, we compared the PP2A activity in mock treated or cells exposed to IR. There was no difference in total PP2A activity or the activity of PP2A complexes specifically containing FLAG-B56alpha after IR.

Next we examined the extent to which PP2A influenced CHK2 activity, first by looking at CHK2 phosphorylation. When PP2A was inhibited with okadaic acid, CHK2 phosphorylation increased dramatically at serine 19, as well as slightly at serines 33/35 and threonine 68. This is indicating that PP2A can dephosphorylate CHK2 at these sites, which may affect its kinase activity.

We also tested the abilities of the S33/35A and S33/35E mutants to be phosphorylated at other sites. In mock treated cells, S33/35E has a dramatic increase in phosphorylation at serine 19 when compared to wild-type CHK2. After IR, S33/35A has less serine 19 phosphorylation and S33/35E has more than wild-type CHK2. Since we have determined that phosphorylation of serines 33/35 can influence B56alpha binding and okadaic acid treatment increases serine 19 phosphorylation, these data indicate that when CHK2 is phosphorylated at serines 33/35, there is a decrease in PP2A binding which allows for an increase in serine 19 phosphorylation.

Interestingly, by overexpressing FLAG-B56alpha, CHK2 activity was decreased after IR as compared to mock treated cells. We also tested the CHK2 mutants S33/35A and S33/35E. In mock treated cells, S33/35A had less activity and S33/35E had more activity than wild-type CHK2. In cells that were exposed to IR, S33/35A had less activity than wild-type and S33/35E had about the same as wild-type. Therefore, PP2A is negatively affecting CHK2 kinase activity.

We also examined CHK2 protein stability since it is known to increase after IR. In the mock-treated cells, S33/35E was much more stable than wild-type CHK2. Also, the S33/35A and S33/35E mutants' stability did not change after exposure to IR, whereas the stability of wild-type CHK2 increased. Therefore, the phosphorylation of CHK2 at serines 33/35 increases CHK2 stability.

*Task 4. To determine the ability to sensitize cells to current therapies by inhibiting either CHK2 or PP2A:*

*Use RNA interference to silence either CHK2, PP2A B56alpha, or PP2A C expression and test the response of cells to current breast cancer therapies doxorubicin, paclitaxel, mitomycin C, and radiation*

We had previously proposed to use okadaic acid to inhibit PP2A and then treat with various cancer therapies. The concentration of okadaic acid that inhibits PP2A without affecting other phosphatases of the cell was unfortunately extremely toxic to the cell so we could not use it in these experiments. We also tried to knockdown PP2A subunits with siRNA but this was unsuccessful as well.

In order to test the sensitivity of CHK2 mutants to cancer treatments, we attempted to utilize the HCT15 cell line that has no functional CHK2. Unfortunately, when we reconstituted wild-type CHK2 in these cells using transfection, there was no difference in cell viability in HCT15 cells transfected with empty vector or vector containing the gene for CHK2 after treatment with IR at various time points. Therefore, this system could not be used.

### **3- Conclusions**

We have identified the B56alpha subunit of protein phosphatase 2A (PP2A) as a CHK2 binding partner and show that their interaction is modulated by DNA damage. B56alpha binds to the SQ/TQ repeat region of CHK2, which is a target of ATM phosphorylation. The induction of DNA double-strand breaks by gamma irradiation causes dissociation of the B56alpha and CHK2 proteins. This dissociation correlates with an increase in the ATM-dependent phosphorylation of CHK2 at serines 33 and 35 in the SQ/TQ region. Indeed, mutating these sites to mimic phosphorylation increases the dissociation after irradiation. CHK2 is able to phosphorylate B56alpha *in vitro*; however, *in vivo*, irradiation has no effect on PP2A activity or localization. Alternatively, PP2A negatively regulates CHK2 phosphorylation at multiple sites, as well as its kinase activity and protein stability. These data reveal a novel mechanism for PP2A to keep CHK2 inactive under normal conditions while also allowing for a rapid release from this regulation immediately following DNA damage. This is followed by a subsequent reconstitution of the PP2A/CHK2 complex in later time points after damage, which may help to attenuate the signal.

### **4- Meetings and Awards**

Poster Presentation Abstract  
AACR 100th Annual Meeting, April 2009

*Protein Phosphatase 2A and CHK2 Binding is Disrupted by DNA Damage*

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Maintenance of genomic integrity is an essential part of every cell's existence. The DNA damage response pathway allows for the repair of DNA breaks caused by genotoxic insults in order to prevent the propagation of mutations that can cause diseases such as cancer. CHK2 is a major effector kinase of this pathway. To further characterize CHK2 via its interacting partners, we performed a yeast two-hybrid screen with CHK2 as bait and screened a human mammary gland cDNA library. We identified a subunit of protein phosphatase 2A (PP2A) as a CHK2 binding partner. The PP2A holoenzyme consists of an A structural subunit, a C catalytic subunit, and one of many B regulatory subunits. We show that the B'alpha subunit interacts with CHK2 in co-immunoprecipitates of endogenous proteins in mammalian cells. The CHK2 and B'alpha interaction is exclusively cytoplasmic and binding is abrogated by DNA damage caused by gamma-irradiation (IR) but not by UV, mitomycin C, or hydroxyurea. We have determined that the extent of CHK2 and B'alpha IR-induced dissociation occurs in a dose-dependent manner. Time course experiments show that dissociation is an early response to IR and reassociation can be detected several hours after IR suggesting a temporal correlation with recovery. CHK2 also binds other B' family members with varying affinities, and these subunits also dissociate from CHK2 after IR. The minimal binding region in CHK2 that interacts with B'alpha includes the SQ/TQ domain, which is a target of ATM-dependent phosphorylation. The phosphorylation of CHK2 at specific sites in this domain

correlates with the dissociation of CHK2 and B'alpha after IR, and this dissociation can be prevented by pharmacologic inhibition of ATM. When these sites are mutated to mimic phosphorylation, there is an increase in CHK2 phosphorylation at other sites. Inhibition of PP2A catalytic activity results in an increase in CHK2 phosphorylation at multiple sites as well, suggesting a scenario in which PP2A acts to keep CHK2 in a dephosphorylated inactive state in the absence of certain kinds of DNA damage.

## **5- References**

1. Wasielewski, M., P. Hanifi-Moghaddam, A. Hollestelle, S.D. Merajver, A. van den Ouweland, J.G. Klijn, S.P. Ethier, and M. Schutte, *Deleterious CHEK2 1100delC and L303X mutants identified among 38 human breast cancer cell lines*. Breast Cancer Res Treat, 2009. **113**(2): p. 285-91