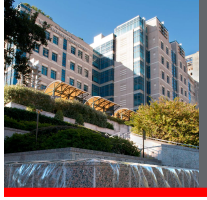

Investigating the Function of Arginine Methylation at Two Mutated Sites of CBP by CARM1

Authors: N. Dawson[1], J.W. Hwang[2], M. A. Santos[3]

[1]*Department of Epigenetics and Molecular Carcinogenesis, The University of Texas MD Anderson Cancer Center, Houston, TX*

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Background:

What are CARM1 and CBP?

- Co-activator Associated Arginine Methyltransferase 1 (CARM1)/Protein Arginine Methyltransferase 4 (PRMT4)
- CREB Binding Protein (CBP), long protein encoded in the *CREBBP* gene
  - Similar to the P300 protein, both are co-activators necessary for activation of transcription and is associated with hundreds of proteins

Methylation?

- Methylation is an important mechanism for turning "on" or "off" a gene for transcription processes across cell divisions
  - Involves the transfer of a CH3 chemical group
- CARM1 methylates arginine residues in CBP in 2 of 5 domains in CBP, IBiD and KIX

Mutations in DLBCLs:

- CBP along with its counterpart, p300 are frequently mutated in DLBCLs (Diffuse Large B-Cell Lymphoma)
  - DLBCLs are a common form of lymphoma that effect older people
  - DLBCLs with genetic lesions are sensitive to inhibition of CARM1 activity, causing synthetic lethality
- Here we are investigating the function of arginine methylation at two mutated sites of the IBiD domain of CBP by CARM1
- Our experiments revolve around the synthesis of point mutations at IBiD: RKA and RKB (Arginine to Lysine Point Mutation)

Methods:

Cell Culture:

- Standard cell culture procedure was done for U2OS, Osteosarcoma cell lines.
- Cultured cells regularly in a 10mm dish every three days at 70% confluency, 37C

Transfection and Co-IP:

- Transfection: Adding plasmid into mammalian cells
- Using Opti-Mem, TransIt-2020; transfected with **pCMV6-CBP-IBiD-WT** and **pCMV6-CBP-IBiD-RKA** Plasmids respectively; U2OS WT and RKA

Single RKA Co-IP:

- We harvested cells after transfection, lysed and did protein quantification using a Bradford Assay analysis
- Incubation of Flag antibody should attach to the protein of interest and interacting proteins
- Used 70ul beads, the weight of the beads brings down the bead-antibody-protein complex
- We added x2 LSB; further disassociates antibodies + bead and its proteins attached. Boiling future denatures the proteins
- Performed Standard Western Blot Procedure After Successful Mutagenesis, We repeated CO-IP with synthesized 2RK mutants (figures 3-5)

Methods:

Site-Directed Mutagenesis:

- Our point mutation is focused on the change from R-Arginine to K-Lysine.
- Synthesis of 2RK mutant
- PCR was done with Q5 hot start enzyme
  - Forward (mutated) Primers (RKB-F)
  - Reverse (mutated) Primers (RKB-R)
  - Template: pCMV6-CBP-IBiD (mutated with RKA)
  - Polymerase: Q5 hot start Mastermix (high fidelity)
  - Overnight PCR (18 cycles)
- Treatment with *DpnI*
  - Recognizes methylation in Template plasmid, removing it, leaving us with the PCR product.

E Coli transformation with NEB Competent cell:

Transformation: Adding ectopic DNA into E. Coli for DNA amplification of our desired plasmid

- After cycling through Ice and Heat shock to further open comp cell for plasmid integration, we added media and spread on Kanamycin agar plate
  - Kanamycin is the antibiotic resistant gene of our desired plasmid
- Checked for colonies, inoculated, harvested, performed midi-prep and sent for DNA Sequencing

Cloning for Lentiviral Plasmid:

- Lentivirus can integrate the plasmid with the DNA, and has greater efficacy than transient transfections
- Enzyme cutting for Inserts:
  [backbone, promoter, insert]
  pCMV6-**CBP-IBiD** / pCMV6-**CBP-KIX**
- Cut at *BamHI* and *NotI*, removed IBiD and KIX inserts from plasmid to create two hybrid plasmids with the backbone of PHR-SFFV and inserts of IBiD and KIX respectively
- Enzyme Cutting of Backbone:
  [backbone, promoter, insert]
  pHR - SFFV - GFP (1-10)
- Cut at *BamHI* and *NotI* and keep the backbone and promoter of pHR-SFFV

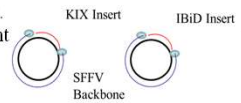

Figure 2: Image of hybrid plasmid generated:
Two enzymes *BamHI* and *NotI* are in light blue. The SFFV backbone is in dark blue. The KIX and IBiD inserts are in red.

- Ran 1% agarose gel which separates backbone from insert (figure 6)
- Cut gel using UV Tray (SFFV backbone and iBiD and KIX inserts) and extracted DNA from gel (For DNA measurements, refer to Results section)
- After T4 Ligation (joining DNA nicks), these hybrids become **pHR-SFFV-CBP-KIX** and **pHR-SFFV-CBP-IBiD** lentiviral plasmids
- NEB stable comp. cell transformation
- Spread well on agar plates of Ampicillin plates; resistant gene of SFFV plasmid

Results:

CO-IP Western Blot:

- Results indicated inconclusive evidence with RKA
- We would need to see more band from IP WT and IP RKA FLAG-IBiD to believe that CARM1 has no band (in red)
- We repeated with generated 2RK mutant after successful Mutagenesis

Site-Directed Mutagenesis:

- (DNA Sequence not shown)
- Successful synthesis of 2RK mutants

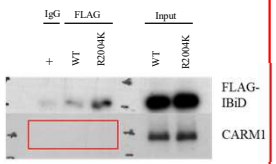

Figure 3: Co-IP Western Blot.
IP: IgG negative control, WT Flag, RKA Flag.
Input: WT and RKA. Empty IP CARM1 band (boxed in red). First line incubated with FLAG antibodies to check IBiD interaction. Second line incubated with CARM1 antibodies to check for CARM1 interaction.

CO-IP Western Blot with 2RK:

- Negative IgG and Positive B-Actin Controls as expected
- CARM1 weakly interacts with RKA, RKB, and 2RK in IP. We can believe this because FLAG-IBiD is positive among IP and Input (Figure 4)
- NFIB-me2a is known to be methylated by CARM1 so its positivity among groups means that CARM1 is actively methylating (Figure 5)

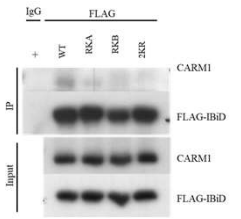

Figure 4: Co-IP Western Blot with 2RK.
IP: IgG (negative control), WT, RKA, RKB and 2RK. Input: WT, RKA, RKB and 2RK. First line incubated with CARM1 antibodies to check for CARM1 interaction. First line incubated with FLAG antibodies to check IBiD interaction.

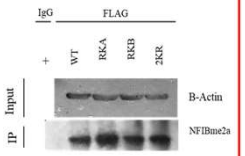

Figure 5: Co-IP Western Blot with 2RK.
Input: WT, RKA, RKB and 2RK.
Primary Antibody: Beta Actin, secondary antibody: Anti-Mouse.
IP: IgG (negative control), WT, RKA, RKB and 2RK. Primary Antibody: NFIB-me2a (created by lab colleague), secondary antibody: Anti-Rabbit.

Results:

Cloning for Lentiviral Plasmid

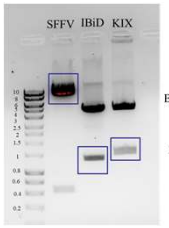

Figure 6: 1% Agarose gel:
DNA separated backbone from inserts. Boxed in blue is where we cut the gel using a UV tray. Cut out SFFV backbone and IBiD and KIX inserts.

After melting gel and extracting DNA we measured our concentrations (Results had very small concentrations but enough to proceed)

1. IBiD: 11.119 ng/ul purity: 0.78
2. SFFV: 29.936 ng/ul purity: 1.32
3. KIX: 5.634 ng/ul purity: 1.42

- After checking colonies, we were not able to proceed with Mini-prep and DNA Sequencing because there were too many non-specific colonies in Ampicillin agar plate.
- Ampicillin powder was expired, next steps is to repeat Lentiviral cloning

Conclusion:

- CARM1 found to methylate mutated residues at two sites in the CBP-IBiD domain
- Leads to more knowledge about the consequences of frequent mutations in DLBCLs

Looking ahead:

- We will do more Co-IP with single point mutations and the now generated 2RK mutant
- Continue to look at interactions between CARM1 and mutated sites in CBP
- Cycloheximide-chase experiment for checking protein stability
- Repeat Lentiviral Plasmid Cloning

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