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Identification of the Cyclin Responsible for the Activation of Cancer Dependency CDK11

A. RATIONALE:

According to the National Cancer Institute, over 600,000 people in just the United States die due to cancer every year. Facing a severe public health crisis, significant research efforts are dedicated to finding new and improved treatments for those suffering from cancer. One promising area of research is the identification of cancer dependencies as drug targets. Also called cancer addictions, these dependencies are required for cancer cell proliferation, and without them, the cancer would not be able to survive. One recently discovered cancer dependency in multiple types of cancers is CDK11, a cyclin-dependent kinase whose function in cancer is unknown. CDK11 must first be activated by a cyclin in order to phosphorylate the relevant target proteins, but currently, which cyclin is responsible for this cancer dependency's activation is unknown. The identification of the activating cyclin of CDK11 can help researchers understand the true function of CDK11 as a cancer dependency, characterize the structure of CDK11 itself, synthesize novel therapeutics targeting CDK11, and identify a new potential drug target for cancer therapies.

B. RESEARCH QUESTION(S), HYPOTHESIS(ES), ENGINEERING GOAL(S), EXPECTED OUTCOMES:

Research Question: Which protein is responsible for activating CDK11 in cancer?
Expected Outcome: Is it expected that either cyclin L1, cyclin L2, or both are responsible for activating CDK11 in cancer, and that cyclin D3 is not responsible for activating CDK11 in cancer.

C. PROCEDURES:

New gRNA Recombinant Plasmid Formation

1. Linearize lentivirus CRISPR vector with BsmBI restriction enzyme and digest at 55°C for 3 hrs:

    8µg vector (CRISPR plasmid)
    2µL BsmBI enzyme
    5µL NE buffer 3.1
    XµL ddH_2O
    50µL total

2. Add 1µL of Calf Intestinal Alkaline Phosphatase (CIP) and incubate at 37°C for 1 hour followed by gel purification of digested vector. There should be two bands (11kb and 2kb). Cut and DNA purify the 11kb vector.

3. gRNA cloning Phosphorylate 5' end and anneal each pair of oligos:

    1µL oligo 1 (100µM)
    1µL oligo 2 (100µM)
    1µL 10x T4 ligase buffer (in buffer box)
    6.5µL ddH 2 O
    0.5µL T4 PNK enzyme
    10µL total

4. Annealing process:
    a. Put in thermocycler under "SGRNA" program (Lid Temp 105°C)
        i. 37°C, 30 minutes
        ii. 95°C, 5 minutes
        iii. 90°C, 5 minutes
        iv. 80°C, 15 seconds
            1. Ramp 0.1°C/s
        v. 70°C, 30 seconds
            1. Ramp 0.1°C/s
        vi. 60°C, 30 seconds
            1. Ramp 0.1°C/s
        vii. 50°C, 1 minute
            1. Ramp 0.1°C/s
        viii. 40°C, 2 minutes
            1. Ramp 0.1°C/s
        ix. 30°C, 2 minutes
            1. Ramp 0.1°C/s
        x. 4°C, infinite hold
            1. Ramp 0.1°C/s
    b. Dilute each sample 1:200:
        995µL ddH 2 O
        5µL product

5. Set up ligation reaction below and incubate reaction at room temperature for 30 min:
        XµL 25ng of digested vector
        YµL ddH2O
        1µL phosphorylated and annealed oligo duplex diluted 1:200
        1µL 10x T4 ligase buffer
        1µL T4 ligase
        10µL total

6. Transformation with Stbl3 *E. coli* cells
    a. Warm plates in incubator
    b. Thaw, mix, and add ampicillin; put cells on ice
    c. Add 5µL of the ligation reaction to thawed cells, mix gently
    d. Incubate on ice for 5 minutes
    e. Add 105µL mixture to culture plate
    f. Grow culture overnight

CRISPR/Cas9 gRNA Sequence Validation

1. Take out liquid cultures from shaker from the night before

2. GLYCEROL STOCK: 500µL bacteria + 500µL 50% glycerol; store at -80°C

3. Miniprep
   a. Transfer 1mL of inoculated bacterial culture to eppendorf tube
   b. Spin at 8,000 RPM for 5 min
   c. Resuspend pellet in 250µL Buffer P1
   d. Add 250µL Buffer P2 and mix immediately by inverting tube
   e. Add 350µL Buffer N3 and mix immediately by inverting tube
   f. Centrifuge at 13,000 RPM for 10 min
   g. Apply supernatant from step 6 to QIAprep spin column
   h. Centrifuge at 13,000 RPM for 1 min
      i. Discard flow-through
   i. (Wash) Add 500µL Buffer PB
      i. Centrifuge at 13,000 RP for 1 min
      ii. Discard flow-through
   j. (Wash) Add 750µL Buffer PE
      i. Centrifuge at 13,000 RP for 1 min
      ii. Discard flow-through
   k. Centrifuge for 1 min at full speed
   l. Place column in eppendorf tube
   m. Add 50µL ddH 2 O
      i. Let sit for 1 min
      ii. Centrifuge for 1 min at 13,000 RPM
   n. Place tubes on ice
   o. Determine the DNA concentration using the nanodrop. Pure DNA should have 260/230 and 260/280 ratios above 1.9.

4. Sequence with the U6 primer
   a. SEQUENCING:
      i. Go to MCLab.com
      ii. Click “order sequencing”
         1. Order Easy Format Reaction
      iv. Prep samples according to sample preparation
         1. 100ng/µL concentration for plasmids, 10µL volume
         2. U6 primer for each sample
      v. Fill out and attach form, put samples on a rack and parafilm it so they don’t pop open

5. When sequencing comes back = Check sequencing on SnapGene
   a. Download sequences from MCLab
   b. Open the sequence of the parental plasmid in SnapGene
   c. Actions > Restriction Cloning > Delete Restriction Fragment
      i. Cut with: “BsmBI”
      ii. Check off “and remove 5’ overhangs”

iii. “Clone” (bottom right corner)

d. Tools > “Align multiple sequences”

i. Select the “.ab1” files for each sequence being verified (these include chromatogram)

Lentiviral Generation

1. Prepare Solution A (500ul, keep pipetting order):

20ug Plasmid DNA
25ug Packaging plasmid DNA (PS Pax 2.0)
4ug pVSVG
to 500µL H_2O
62.5µL $CaCl_2$

2. Prepare solution A and Solution B (500ul 2 x HBS) in separate FACS tubes

3. Mix drop wise (A) to (B). Use a pipet aid as means to blow bubbles through solution B. Use a pasteur pipet for drop by drop adding of solution A to solution B while the pipet aid blows bubbles

4. Incubate mixture (1ml) at RT for 15 min

5. 293 cells should be 75-85% confluent, and plated in 9ml DMEM + 10% FBS

6. Add 2.5ul 100mM Chloroquine to each 10cm plate

7. Add precipitate to cells drop wise with mixing cells (caution: be gentle as the packing cells may easily detach from plate)

8. Incubate 10-14 hours then carefully change the media with 10ml DMEM + 10% FBS

9. Virus collection time: 24-72 hours post transfection; highest titers are at 32-50 hours post media change; can also collect virus every 6-8 hrs after media change

10. Collection viral supernatant and add to target cells or freeze at store at -80°C

Test Lentiviral Infections

Day 0: Plate 500,000 cells in a 6 well.

Day 1:

1. Warm serum free media (“DMEM nothing added”) to room temperature.
2. Warm Lipofectamine 3000 reagent and P3000 reagent to room temperature.
3. Aliquot 125µL serum free media to two eppendorfendorf tubes. Label “Tube A” and “Tube B.”
4. Add 2.5g of DNA + 5l P3000 reagent to Tube A.
5. Add 10l Lipofectamine 3000 to Tube B.
4. Add Tube A contents to Tube B.

5. Incubate 10-15 minutes.
6. Add dropwise to a single well. Distribute evenly.

Day 2:
Check for fluorescence; should be visible in 18-24 hours. Change the media 16-24 hours after transfection.

Dropout Assays
1. Transduce and select for stable expression of Cas9 in a cell line.
2. Plate 50,000 cells (or an appropriate amount) per well onto a 12 well plate. Use 1mL media. Make sure to include at least 1 extra well as a no-GFP control.
3. The next day, transduce cell with correct amount of viral supernatant.
4. After two days, change the media.
5. Passage the cells and take the first time point 3 to 5 days post transduction (3 days for fast growing cell lines, 4-5 days for slower lines). To passage – 175ul trypsin, quench with 825ul media. Split 1:3 for a slow growing cell line, split 1:6 for a fast-growing line.
6. For the MacsQuant reading, filter 500ul of the unused cell suspension into a FACS tube (blue cap), and measure percent GFP.
7. Repeat steps 5 and 6 every 3 or 4 days (depending on cellular proliferation)
until a total of 15-20 days is reached.

Flow Cytometry
1. Sort using flow cytometry to select for GFP-expressing transduced cells

Western Blot Validation
HARVEST PROTEIN
1. Day 1:
    a. Plate cells at 300k, ⅛ well for each sample
2. Day 2:
    a. Trypsinize cells, quench, spin down (~3000g, 5min), aspirate supernatant
    b. Resuspend in 500µL PBS, move sample to microcentrifuge tube if not in one already
    c. Spin down again, aspirate supernatant
    d. Resuspend pellet in 100µL of Ripa + Protease/Phosphatase inhibitor
        ii. Recipe: 10mL Ripa + 1 Tablet Protease inhibitor + 1 tablet Phosphatase inhibitor
        iii. Note: can resuspend pellet in less Ripa+inhibitor if you'd like more concentrated protein
    e. Let shake in cold room at max speed for 30-60 minutes
    f. Centrifuge in cold room at max speed for 20-60 minutes
    g. CAREFULLY transfer supernatant to new tube and store at -20

MAKE GEL
1. Choose the correct size spacer plate
    a. I.e. 1.0mm means you load less sample, this is good if your protein is highly expressed; 1.5mm = more sample, for low expressing proteins
2. Wash spacer plate and glass plate with E-pure water + wipe dry with kim wipe

3. Make sure both plates are level and put into the clamp. Put the green holder + glass plates into plastic stand
4. Make the gel mixture recipe under "resolving gel", adding the TEMED last
    a. Dump out water from Step 2A if you did it
5. Invert then add until ~1cm under the green bar of the gel holder
    a. Make sure there are no bubbles
6. Quickly add ~1mL methanol to the top of the gel so that it will even out.
7. Wait until polymerized (~20-30minutes, can verify w/ left over gel)
8. Make resolving gel
    a. Before adding Temed, make sure you have the correct size combs out
9. Gels can be saved for 24 hours overnight, take your gel out of the clamp, wrap your gel in a paper towel and place it in a pipette tip box filled with E-pure water at 4° (write your initials + date on the lid)

QUANTIFYING PROTEINS + CALCULATIONS

1. Make the dilutions given in red so that there is 5µL/well
2. Pipette 4µL of RIPA + 1µL of your protein in triplicate in the top rows (in yellow)
    a. Put samples immediately back on ice
3. In a microcentrifuge tube, mix 1mL DC Reagent A (the Blue soln) + 20µL DC reagent S (the small bottle). (Note: this is just a ratio, can be adjusted up and down depending on your samples)
4. Add 25µL to all of your samples (yellow) and the standards (orange)
    a. Can use a multichannel and reservoir for this
5. Add 200µL DC Reagent B (big brown bottle). Pipette to mix or swirl plate (DON'T make bubbles)
    a. Can use a multichannel and reservoir for this
6. Wait 15 minutes
7. Read on plate reader in Tonks Lab
8. Calculations (if you want to use max. Amount of protein)
    a. Using the concentration from the plate reader, find the sample with the lowest concentration.
    b. Calculate the total amount of protein from this sample that you can get in 25µL
        vii. 25µL can be comfortably loaded into a 1.5mm, 10 well western
    c. Calculate the volume needed for all other samples to have the SAME total protein as the sample from steps 1 and 2
    d. Put this volume in new eppendorfendorfs and refreeze your stock sample
    e. Add ripa to get all your proteins to 25µL volume

SET UP SAMPLES + RUN GEL

1. Turn heating block on to high (~95°)
2. Add 8µL loading dye to samples
3. When block reaches 90+°, add samples
    a. Put on eppendorf hats to prevent caps from popping open during boiling first
4. Boil proteins for 10min
    a. Carefully take off eppendorf hats and briefly centrifuge to spin down condensation
    b. Take out protein ladder to thaw
5. Put your gel in the holder thing, use a buffer dam if necessary
    a. Make sure you are using a holder thing that has the gold electrodes sticking up

6. Fill the middle of the holder thing with Running buffer
7. Load 7-10µL protein ladder in one well of your western
8. Set pipette to 25µL, CAREFULLY load 25µL of protein to wells
    a. Keep track of the order that you load your samples in!
9. Run at 110-120V, check every once in a while, should take ~1.5-2.5 hours to run
    a. Make sure bubbles are rising from center wire

TRANSFER

1. When blue dye front reaches bottom of gel, turn off current
2. Get 2 filter pads, use to cut out membrane
3. Soak 1 filter pad in 1X transfer buffer
4. Soak membrane in Methanol to activate it

a. Transfer to 1X transfer buffer

5. In a transfer case, lay down soaked filter pad
6. Lay down the membrane (NO BUBBLES)
7. Cut stacking gel off of your gel and lay that on the membrane (NO bubbles)
8. Soak 2nd filter pad and lay it on top of sandwich
    a. Optional, if sandwich looks dry, pour a bit of extra transfer buffer on top
9. Lock in and put in transfer machine (note, once cassette is locked DON'T open it! Opening and reclosing cassette often causes bubbles to form
10. Turn on transfer machine
    a. Hit constant A (this tells the machine to keep the current constant
    b. Set current to 1.3A
    c. Make Time = 30-45 min
        viii. Use longer transfer times for larger proteins
    d. Hit run, select cassette slot you are using

BLOCKING > DEVELOPING

1. After transfer, put the membranes in 5% milk for 1 hour for blocking
    a. 5g milk, 100mL TBST
2. After 1 hour, put membrane in primary antibody, cover with tinfoil and put in 4° overnight
3. Day 2:
    a. Take out membrane
        ix. Save primary antibody if expensive by putting in a falcon tube and adding Sodium Azide (on Anand's Bench), 1:100 dilution, i.e. 100µL in 10mL
    b. Wash ~3 times with TBST for ~10min per wash
    c. Add secondary antibody in 5% milk, incubate for 1 hour at room temp
        x. 1:20k is usually a good dilution for secondary rabbit and mouse, so 0.5µL in 10mL milk
    d. Wash ~3 times with TBST for ~10min per wash
    e. On last wash, make Black and White solution (ECL)
        xi. 500µL black + 500µL white per blot
    f. Dump out TBST and add the Black and White solution with a pipette
    g. Bring to developer immediately + develop

STRIPPING

1. After developing, put blots in Ponceau, shake 20min
    a. If this is your first time using the ponceau, you can save it in the glass flas covered in tinfoil
2. Add 5% milk, shake 1 hour (blocking)
3. Add primary antibody in milk
    a. Shake overnight at 4°C
    b. Repeat developing process

Genomic DNA Sequencing

1. Genomic DNA was extracted from sorted A375, HCT116, and MDA-MB-231 cells
2. Oligonucleotides (PCR Primers) encompassing the target sites of tested gRNAs were designed used SnapGene to produce smaller fragments of DNA including the predicted mutations due to knock-out
3. PCR was used to isolate and replicate desired DNA segments
    a. Denaturation: DNA unwound at ~95℃
    b. Annealing: DNA primed at ideal temperatures for each primer
    c. Extension: DNA extended by polymerase enzyme
4. Gel electrophoresis and gel extraction was used to isolate the desired DNA segment from the PCR product
5. DNA was sent to MCLab for sequencing
6. Sequencing results were compared to sequences from the gRNA Sequence Validation step to verify knock-out

D. BIBLIOGRAPHY:

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1. Human participants research: N/A

2. Vertebrate animal research: N/A

3. Potentially hazardous biological agents research:

a. Give source of the organism and describe BSL assessment process and BSL determination

- Human cancer cell lines A375, MDA-MB-231, and HCT116 - BSL1, from ATCC
- Human embryonic kidney cell line HEKFT: BSL1, from ATCC

- Stbl3 *E. coli* strain: BSL1, from Thermo Fisher Scientific
- rDNA: primers from IDT cloned into GFP-expressing backbone vector

b. Detail safety precautions and discuss methods of disposal

- Experimentation with cell lines conducted in a designated Tissue Culture Room with BSL2 containment
    - Use of Nuaire Biological Safety Cabinets Class II Type A/B3 hoods
- Use of disposable lab coats, disposable gloves, and safety glasses
- All cultured material will be disposed of through aspiration and biological waste containers, and any materials with virus are cleaned with 10% bleach first
- The student will receive Biosafety training through the Cold Spring Harbor Laboratories Safety Training Department

4. Hazardous chemicals, activities & devices:

a. Include detail regarding chemical concentrations and drug dosages

- NE Buffer 3.1 - 1X concentration contains 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, and 100 µg/mL BSA
- Agarose - 1% concentration (1 g agarose powder + 100 mL water)
- Ethidium bromide - 10 mg/mL
- T4 ligase buffer - 1X concentration contains 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, and 10 mM DTT
- Glycerol - 50% concentration
- Buffer P1 - 1X concentration contains 50 mM Tris-Cl, 10 mM EDTA, 100 µg/mL RNase A
- Buffer P2 - 1X concentration contains 1% SDS, 200 mM NaOH
- Buffer N3 - 1X concentration contains 4.2 M Gu-HCl, 0.9 M potassium acetate
- Calcium chloride - 30% concentration
- HBS - 2X concentration contains 280 mM NaCl, 50 mM HEPES, 1.42 mM $Na_2HPO_4 \cdot 7H_2O$
- Chloroquine - 10 µM concentration
- RIPA buffer - 1X concentration contains 10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl
- Acrylamide - pure (>99% concentration)
- TRIS buffer - 100 mM
- SDS - sodium dodecyl sulfate 10% concentration
- APS - 10% concentration (1 g ammonium persulfate + 10 mL water)
- TEMED - pure (>99% concentration)
- Methanol - pure (>99% concentration)
- Ethanol - 70% concentration
- Bleach - 8.25% sodium hypochlorite
- Loading dye - 6X concentration includes bromophenol blue, xylene cyanol FF, and EDTA
- TBST - 1X concentration contains 1 L 1X TBS + 1 mL Tween-20

b. Describe the risk assessment process

Risks of chemicals were assessed based on knowledge of prior use and chemical safety information. Risks include spills. Will be cleaned following standard procedure, and when necessary Facilities personnel will assist in cleanup.

c. Supervision
   Student will be supervised by the Designated Scientist and other senior lab members, all of whom have years of lab experience.

d. Safety precautions
   All experiments will always be done with gloves on, and when necesary eye/face protection. Any spills will be reported to the CSHL Facilities department for instruction on disposal or evacuation. Cancer cell manipulation will be performed in a Biological Safety Hood.

e. Methods of disposal
   All chemicals and items used with them will be disposed as Regulated Medical Waste or down the sink as appropriate.