Rationale

- Control of DNA repair is essential in preventing cellular transformation leading to cancer. The laboratory explores the roles of Ajuba in controlling an important tumor suppressor DNA repair system relying on the ATR kinase. Understanding how Ajuba participates in the control of ATR is important for our knowledge of how a cell can become a cancer cell and may provide strategies for cancer therapy. Ajuba exerts an inhibitory activity on ATR in the nucleus in S phase, but it exhibits a complex intracellular life cycle, spending most of its time in the cytoplasm. We wish to understand the events necessary for the control of the nucleo-cytoplasmic transport of Ajuba throughout the cell cycle in order to study the events important for its function in DNA repair.

Research question

- What are the domains within Ajuba that control nuclear import and export?

Hypothesis

- Ajuba possess a determinate sequence that drives the protein into the nucleus.

Expected outcomes

- Either LIM or pre-LIM will enter the nucleus; one will at the nucleus, one at cytoplasmic.

Procedures

1. Test pre-LIM and LIM segments of Ajuba on IMR90 and HeLa II cells
    a. Culture human cell lines IMR90.
    b. Transfect IMR90 cells with C1, D3, and vector
        i. Mix OptiMEM and Lipofectamine 2000.
        ii. Mix OptiMEM and C1 plasmid (Ajuba's LIM segment) / D3 plasmid (Ajuba's preLIM segment) / vector.
        iii. Combine the liposomes with plasmids / vector.
        iv. Add the final mixture to the cells.
    c. Microscopy (immunofluorescence)
        i. Fix and permeabilize the cells
        ii. Add antibodies 6696 (rabbit anti-Ajuba) and 9E10 (mouse anti-MYC) to detect Ajuba and MYC-tag (recognized by the 9E10 antibody),then incubate at 4°C overnight
        iii. Add anti rabbit FITC (re-emits green light) and anti mouse TRITC (re-emits red light) to detect antibodies 6696 and 9E10
        iv. See under a microscope
    d. Western blots (protein expression)
        i. Use lysis buffer, NaCl, and cold dH2O with microfuging to extract pure proteins from the cells after transfection.
        ii. Dilute the supernatant liquid with PBS
        iii. Run polyacrylamide gel (PAGE)

2. Construct preLIM segment of Ajuba and test it on HeLa II cells
    a. Polymerase Chain Reaction (PCR) to get pre-LIM without NES
        i. Add template DNA, 2 primers that define the exact sequence needed, nucleotides (dNTPs, dA, dT, dC, dG), and polymerase enzyme Tag into one tube. Make two same samples and one control with no DNA template.
        ii. Put the tubes into PCR machine, set 30 cycles (98°C for 2 minutes to warm up, then 98°C for 30 seconds, 45°C for 15 seconds, and 72°C for 1 minute).
    b. Gel electrophoresis
        i. Make the gel with agnose and TBE, add ethidium bromide.
        ii. Add standard DNA, PCR products with loading dye, and PCR product without DNA with loading dye into the wells.
        iii. Run the gel at 80 volts for 1 hour.
        iv. Take a picture of the result under UV light.
    c. Purify the PCR products
        i. Use buffer PB, buffer PE, and pure water to wash the PCR products in sequence.
        ii. Centrifuge the mixture each time.
    d. Restriction digest
        i. Use enzymes BamHI and XhoI to cut the PCR products.
        ii. Run gel electrophoresis to vector and the PCR products, cut them under UV light.
        iii. Use buffers and microfuge to extract the DNA fragments and vector from the gel blocks.
    e. Clone plasmids into the pLPC-MYC vector
        i. Transformation
    f. Microscopy (immunofluorescence)
        i. Fix and permeabilize the cells
        ii. Add antibodies 6696 (rabbit anti-Ajuba) and 9E10 (mouse anti-MYC) to detect Ajuba and MYC-tag (recognized by the 9E10 antibody),then incubate at 4°C overnight
        iii. Add anti rabbit FITC (re-emits green light) and anti mouse TRITC (re-emits red light) to detect antibodies 6696 and 9E10
        iv. See under a microscope
    g. Western blots (protein expression)
        i. Use lysis buffer, NaCl, and cold dH2O with microfuging to extract pure proteins from the cells after transfection.
        ii. Dilute the supernatant liquid with PBS
        iii. Run polyacrylamide gel (PAGE)

Risk and safety

- Potential hazardous biological agents to be used in this experiment include ethidium bromide, which contained in safety cabinet and disposed of in appropriate biohazard waste, and E.coli bacterial cells, which are used for molecular cloning.
- To minimize risk, protective equipment including latex gloves and lab coat will be used. Cell culture will be performed using a sterile biosafety cabinets class II and the cells will be incubated in adequate CO2 incubator.

Data analysis

- Analyze from pictures obtained.

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Addendum

- The IMR90 cells were not transfected well. In addition, LIM segment of Ajuba was detected through immunofluorescence microscopy. As a result, these were not included in the research paper.
- In order to confirm that the nuclear export is a part of the cell cycle of Ajuba, Leptomycin B was used to block the NES of endogenous Ajuba in HeLa II cells.