Identification of Intracellular Localization determinants of Ajuba in Human Cells

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Abstract

Cells possess molecular mechanisms that sense DNA damage, leading to DNA repair or cell death. Because cancer cells are often defective in DNA repair and exhibit high genomic instability, understanding DNA repair mechanism is important to discover the early events that can lead to cancer at the cellular level. This study focuses on Ajuba, a protein that is involved in DNA repair control in human cells. Ajuba possesses a complex intracellular trafficking pattern: it enters and exits the nucleus during the S phase. A nuclear export sequence (NES) is found in Ajuba's preLIM region. In addition, no nuclear localization sequence (NLS) has been found. Ajuba is composed of a LIM region, which contains three highly related tandem LIM domains, and a preLIM region. This study sought to gain more understanding of Ajuba's nuclear import and export determinants. It involved transfecting three truncations of the Ajuba DNA, including a preLIM segment, a preLIM without NES segment, and a LIM segment, into the human cell line Hela II and analyzing the expression and intracellular localization of these alleles, in order to map the nuclear import and export determinants of Ajuba. Among the truncations, preLIM without NES segment was obtained using PCR during the project, the other two segments were provided. Leptomycin B was used to block the nuclear export sequence of endogenous Ajuba in Hela II cells. Immunofluorescence microscopy using anti-Ajuba antibodies showed that the preLIM without NES segment of Ajuba is located in the nucleus, and the preLIM segment is located both in the nucleus and the cytoplasm. This suggests the nuclear import determinant is located within the preLIM segment without NES. Blocking nuclear export with Leptomycin B led to accumulation of endogenous Ajuba in the nucleus of Hela II cells, which confirms that the export of Ajuba depends on the NES present in the preLIM region. In addition, the molecular weights of the preLIM and preLIM without NES expression are about the same as shown in Western blot; therefore, the transfected proteins are expressed at the same level between the constructs.

Introduction

Cells possess molecular mechanisms that sense DNA damage, leading to DNA repair or cell death. Because cancer cells are often defective in DNA repair, and exhibit high genomic instability, understanding these events is important to discover the early mechanisms that can lead to cancer at the cellular level (Chatterjee and Walker, 2018).

ATR kinase is involved in sensing DNA damage and activating DNA damage checkpoints, leading to cell cycle arrest (Sancar et al., 2004). Ajuba proteins negatively regulate ATR activation to prevent DNA damage response in the S phase in human cells by directly interacting with RPA70 in the replication system (Fowler et al., 2018). RPA70 is a protein that stabilizes single-stranded DNA intermediates that are formed during DNA replication or upon DNA stress (Uniprot.org, n.d.).

Ajuba sequester Hippo core kinase complex, thereby limits the activation of YAP pathway in proliferating cells. (Jagannathan et al., 2016). Hippo tumor suppressor pathway is a central signalling pathway for controlling organ size during development by regulating cell apoptosis and proliferation. The hippo pathway is also essential for tissue regeneration and repair in response to injury in adult organisms. (Jagannathan et al.)

Ajuba is located at different parts of a cell depending on the cell cycle. In the cytoplasm, Ajuba is involved in cell-cell adhesion; in the nucleus, it is involved in the DNA damage response. Ajuba's localization in the nucleus increases and then decreases over time in S phase (Fowler et al.).

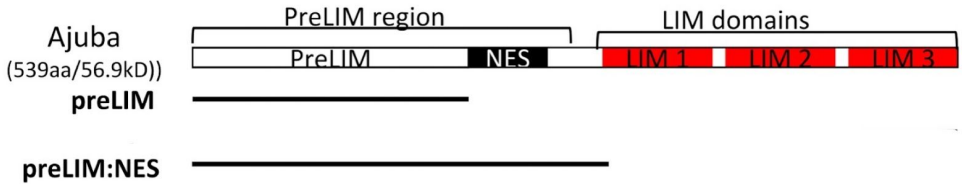
Ajuba proteins possess two different regions: a LIM region and a preLIM region. There are three highly related tandem LIM domains located at Ajuba's LIM region. These LIM domains are cysteine-rich, and they control protein-protein interaction (Schimizzi and Longmore, 2015). The first two LIM domains are responsible for the regulation of proliferation, whereas the last LIM domain affects differentiation decisions (Kanungo et al., 2000).

The LIM domain is present in various kinds of eukaryotic proteins that perform various cellular roles such as regulators of gene expression, cell adhesion, and signal transduction (Kadrmas and Beckerle, 2004). The preLIM region contains a nuclear export sequence

(Schimizzi and Longmore). The research goal was to determine the location of Ajuba's nuclear import localization sequence.

Results

At first, PCR was used to obtain preLIM without NES segment of Ajuba. PreLIM and LIM segments of Ajuba are obtained from the Loayza Lab. To make sure the product was the exact segment, restriction analysis and DNA sequencing was conducted. After that, preLIM, preLIM without NES, and LIM segments are introduced into Hela II cell lines. In the end, immunofluorescence microscopy and western blot were used to analyze the results of transfection -- localization and protein expressions of the transfected segments in cells.



Gel test for the preLIM without NES segment of Ajuba through PCR

Fig.1 was obtained after the restriction analysis of the PCR product. As seen in the picture, in comparison with the DNA ladder, the sizes of vector and the PCR product are as expected. The insert is about 840 base pairs.

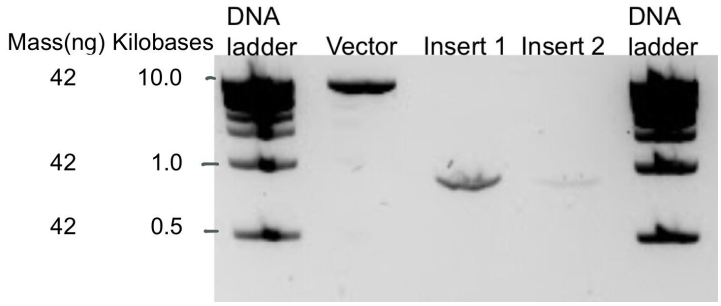


Fig.1. Comparison of the preLIM without NES segment of Ajuba with DNA ladders

PCR sequence

Insert 1 and the vector were ligated; the plasmid formed was then sent to sequence. B-prelim-rev-rc, B-pipc-minus-rc, 208A_005...E01, and 208A_007...G01 are sequences using

different restriction enzymes, including BamHI, XhoI, positive and negative primers. They formed a consensus sequence that is identical to the reference Ajuba sequence on top.

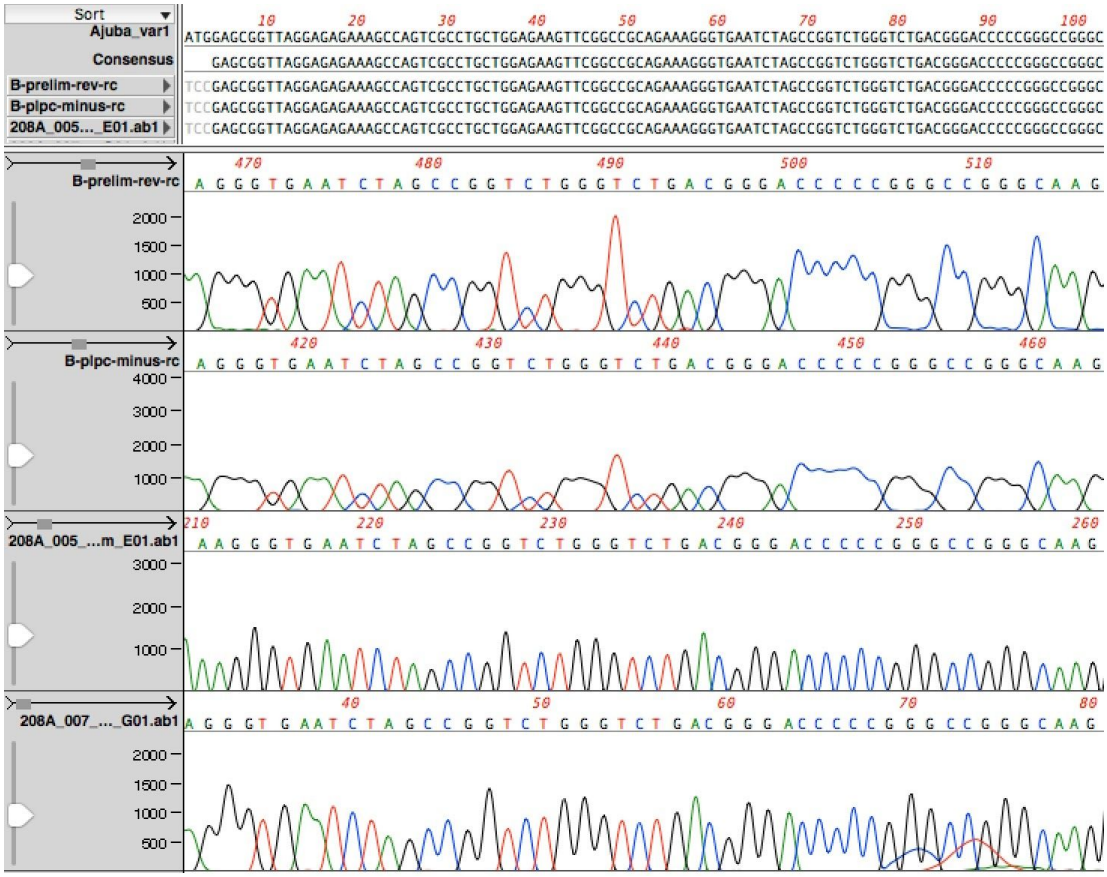
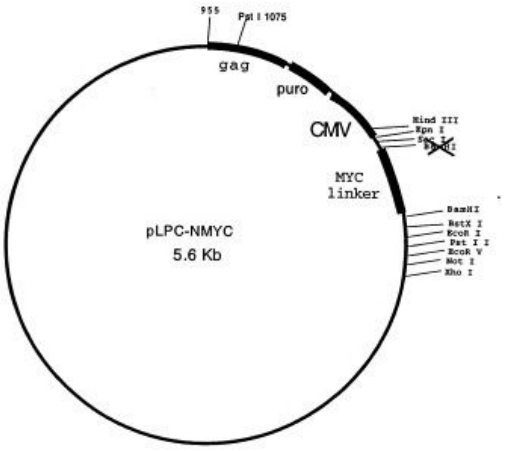


Figure 2: Sequence of plasmid treated with four different primers

Localization of the transfected Ajuba fragments

Hela II cell were transfected with plasmids contained preLIM and preLIM without NES fragments of Ajuba, and vector-only control.



(Fowler et al.)

As shown in Fig.3, the number of the cells that were transfected with plasmids containing preLIM and preLIM without NES fragments emitting green and red signals is around 10% of the cells emitting blue signals. Figures shown are representative among all pictures obtained. Antibody DAPI emitting blue signals detected the localization of the nucleus. Anti-Ajuba rabbit antibody 6696 and anti-MYC mouse antibody 9E10 were used. Then, the anti-Ajuba was detected with an anti-rabbit FIFC conjugated, which emits green signal; the anti-MYC was detected with an anti-mouse TRITC conjugated, which emits red signal. Cells stained with anti-Ajuba antibody 6696 and anti-MYC show color in the same places as the DAPI stain. The preLIM segments emitted signal over the larger area than is seen with DAPI staining. Cytoplasm is the area detected around the nucleus. The preLIM signals can be detected both in the cells' nucleus and cytoplasm. The preLIM without NES fragment staining located about the same area as the DAPI staining. The vector-only control provides a comparison for the MYC antibody. The anti-Ajuba FITC detects endogenous Ajuba in the Hela II cells transfected by the vector-only control, emitting green signal. The signals for exogenous preLIM and preLIM without NES fragments were adjusted stronger; therefore, the endogenous Ajuba is not shown in preLIM and preLIM without NES pictures.

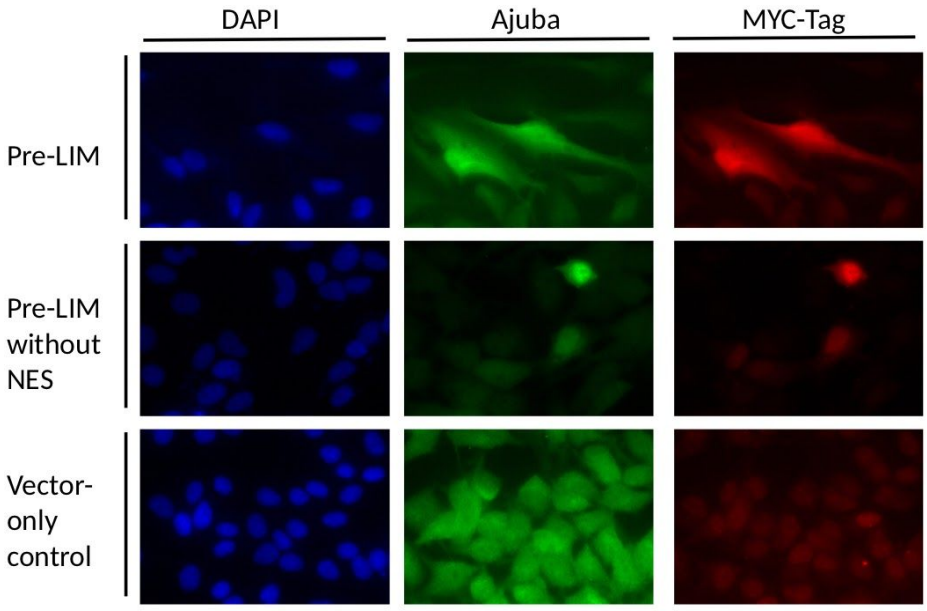


Figure 3: Immunofluorescence microscopy images of preLIM, preLIM without NES, and Vector-only control after treated with anti Rabbit FITC and anti Mouse TRITC

Western blot probed with the anti-MYC antibody

Westerm blot was done to measure the molecular weights of the transfected proteins. Hela II cells were used. The blot in Fig.4 was probed with the anti-MYC antibody. Western blot determined the molecular weight of the transfected protein. The transfected protein is detected. The molecular weights of the transfected proteins are 43kD and 34kD, and the amounts produced by the cells were similar among the various fragments. Therefore, the difference in weight between the two expressions is about the weight of the nuclear export sequence (NES).

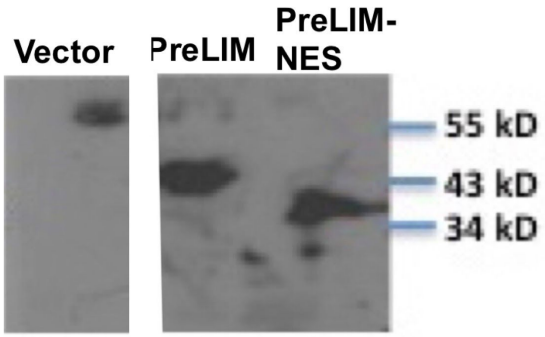


Figure 4: Western blot probed with 9E10 anti MYC

Localization of Ajuba after treated with Leptomycin B

Leptomycin B blocks nuclear export. Hela II cells were treated with Leptomycin B for 1

hour and 2 hours. As shown in Fig.5., Ajuba was located both in the cytoplasm and the nucleus before the treatment. Because the green signals emitted by secondary antibody Rabbit FIFC were located both in and around the place where the blue signals (DAPI, detects nucleus) were detected. After the treatments of Leptomycin B for 1 hour and 2 hours, less Ajuba was detected in the cytoplasm. The green signals were located only at the place where the blue signals were detected.

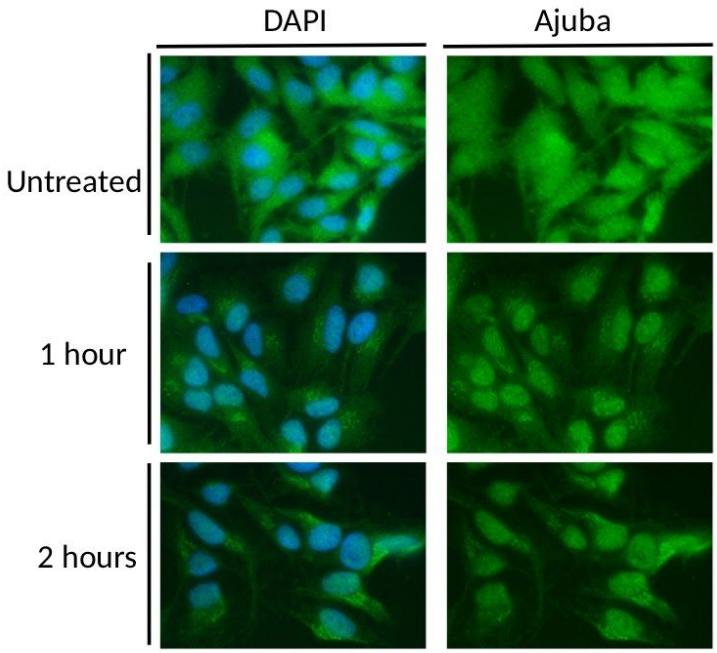


Figure 5: Immunofluorescence microscopy images of Hela II cells after treated with Leptomycin B for 1 hour and 2 hours

Discussion

The preLIM sequence of Ajuba without NES was constructed through PCR. PreLIM and LIM sequence of Ajuba were obtained before the experiment. PreLIM without NES product was tested along with a vector in comparison with two DNA ladders. Fig.1 shows the restriction digest was successful, because the size of the PCR product was 840 base pairs, as expected.

A plasmid was created by ligating the preLIM without NES fragment and the vector. Then, it was cloned by using E-colis. Fig.2 shows the sequence of the plasmid. The consensus sequence can be identified as the preLIM without NES sequence of Ajuba, because its actual size is

identical to its predicted size.

Hela II cells were transfected with plasmids containing preLIM and preLIM without NES fragments of Ajuba. Anti-Ajuba rabbit antibody 6696 and anti-MYC mouse antibody 9E10 were used. Then, the anti-Ajuba was detected with an anti-rabbit FIFC conjugated, which emits green signal; the anti-MYC was detected with an anti-mouse TRITC conjugated, which emits red signal. As shown in Fig.3, all cells in three pictures are in line with each other on each line. Since the preLIM construct is present all over the cell, including the nucleus and cytoplasm, it is imported and exported in and out of the nucleus. Whereas the preLIM without NES is all in the nucleus. Therefore, the sequence that is left out in the second construct contains a determinant for nuclear export with nuclear import occuring for both portions of Ajuba. The LIM sequence was not transfected effectively.

Western blot was conducted on transfected Hela II cells with plasmids containing preLIM and preLIM without NES fragments of Ajuba. Anti-MYC antibody was used. As suggested in Fig.4, the amounts produced by the cells were similar among the various fragments. The result confirms the efficiency of the transfection.The transfection with plasmids containing LIM domains of Ajuba was also conducted; however, it was not effective.

Leptomycin B is a drug known to inhibit nuclear export. As shown in Fig.5, this experiment shows nuclear export is part of the life cycle of Ajuba in the cell. because after treating the Hela II cells for 1 and 2 hours, Ajuba was accumulated in the nucleus.

Conclusion

Ajuba has been known to have a complex intracellular pattern, and its localization in the nucleus increases and then decreases overtime during the S phase (Fowler *et al*., 2018). The preLIM construct is imported and exported in and out of the nucleus. Whereas the preLIM without NES is all in the nucleus. It can be concluded that the nuclear localization sequence is located within the preLIM segment of Ajuba without NES.

Future Research

Additional fragments such as smaller pieces of the preLIM without NES fragment would be

studied to narrow down the trafficking determinants in Ajuba. The result would be more universal if the same experiments were done on different cell lines such as HTC 75 cells, another type of cancer cell, and IMR90, normal human cells.

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