LIM Protein Ajuba Directly Interacts with Replication Protein A to Prevent ATR DNA Damage Response

Sandy Wan Shan Fowler

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LIM protein Ajuba directly interacts with Replication Protein A to prevent ATR DNA damage response

By Sandy Wan S. Fowler

A dissertation submitted to the Graduate Faculty in Biology Program in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2017
This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement of Doctor of Philosophy.

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ABSTRACT

*Lim Protein Ajuba directly interacts with Replication Protein A to prevent ATR DNA damage response.*

By Sandy Wan S. Fowler

Advisor: Dr. Diego Loayza

Integrity of the human genome is essential for viability and proliferation of human cells. Intrinsic (endogenous replication stress) or extrinsic (UV, chemotherapy drugs) agents threaten the stability of the genome by generation of single stranded (ss) DNA or double stranded (ds) DNA breaks. The DNA damage response (DDR) pathways are conserved in evolution and constitute systems that perform the surveillance, signaling, and repair of the damage in the nucleus. Unchecked and accumulation of DNA damage can lead to deleterious effects such as replication fork collapse, chromosome fusion and breakage. The dysregulations of DNA damage response pathways are hallmarks of tumorigenesis. ATR (ataxia telangiectasia mutated and rad-3 related) is the protein kinase activated by ssDNA damage. ATR is essential for life and is recruited by ssDNA bound replication protein A (RPA). RPA, the major mammalian ssDNA binding complex with three different subunits: 70, 32, and 14, and is essential for replication, recombination and repair. The majority of ssDNA binding activity resides in the large subunit (RPA70), which directly binds to ssDNA though it’s central OB fold domains. It
was established that LIM protein Ajuba inhibits unscheduled ATR response, and associates with RPA. However, it was unclear whether the association occurred directly, or through additional factors. I found that the Ajuba-RPA interaction is direct, through the RPA70 subunit, and likely occurs in the nucleus during S phase. Upon replication stress, Ajuba-RPA interaction was reduced and Ajuba was shuttled out of the nucleus during replication stress. In addition, I mapped the regions that likely mediate this direct contact to the first LIM domain of Ajuba and the ssDNA binding OB folds of RPA70. These findings revealed additional facets of ATR signaling regulation, and underscored the implications of LIM proteins in genome integrity and tumorigenesis.
Thesis summary:

This thesis work focuses on the characterization of the interaction between LIM domain protein Ajuba and OB fold protein RPA. Previously, it was shown that Ajuba is critical for inhibiting ATR DNA damage response and is a RPA interacting protein. Although LIM domains have not shown to have interacting partner consensus, published work showed POT1 (OB fold protein), and TRIP6 (LIM domain protein) directly interact to prevent DNA damage response at the telomeres in human cells. This work explores the nature of interaction between Ajuba and RPA, the regulation of this interaction during unperturbed cell cycle and during replication stress. The findings form this work adds a new facet to the complex regulation of ATR activation and contributes to the establishment of an interacting consensus between LIM domain and OB fold proteins in repression of DNA damage response.

First, whether Ajuba can be found with all the RPA subunits and co-localized with RPA in the nucleus would be addressed. Secondly, Ajuba depletion in an unsynchronized cell population led to accumulation of S phase cells, I asked if Ajuba nuclear localization and co-localization with RPA are cell cycle dependent. Thirdly, whether Ajuba-RPA interaction would be affected by replication stress would be examined.
To fully characterize if Ajuba directly interacts with RPA or if this interaction is mediated by another protein, I employed *in vitro* transcription coupled translation system with His-tag pulldown assay to address this with truncation mutants of both proteins. Finally, the relevance of this work and the current model developed form this project would be discussed.
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CHAPTER 1: ATR KINASE IN DNA DAMAGE RESPONSE PATHWAY

DNA DAMAGE RESPONSE AND GENOME INTEGRITY

Eukaryotic genomes are constantly threatened by agents or stresses that compromise their stability and integrity such as UV, DNA damaging reagents (hydroxyurea or aphidicolin), endogenous replication stress, and telomere deprotection. The DNA damage response (DDR) pathways are essential to overcome these genotoxic stresses. The DDR signaling pathways are kinase cascades that can lead to cell cycle arrest, apoptosis, and DNA repair \(^1,2\).

There are two major DDR pathways in mammalian cells, which are activated by different types of DNA damage. ATR (Ataxia Telangiectasia mutated Rad-3 related) and ATM (Ataxia Telangiectasia Mutated) are activated by single stranded DNA (ssDNA) breaks and double stranded DNA (dsDNA) breaks respectively \(^2\). In particular, the ATR kinase has shown to be essential for cell viability \(^3\). On the other hand, ATM is non-essential for cell survival \(^4\). The ATR kinase is a master regulator in many essential pathways and found to be mostly active in S phase. It has been found to signal DNA damage response (DDR), regulate cell cycle progression and protect the genome from mitotic catastrophe \(^3,5\).
DDR activation is found dysregulated in early stage cancer cells to induce apoptosis or senescence. This prevents further proliferation and cellular transformation events from taking place. The suppression or mutation targeting members of the DDR pathway members favors cellular transformation and tumorigenesis in mice \(^6\),\(^7\). Thus, the DDR pathway is essential to maintain genome integrity and prevent the rise of potential cancerous cells in the human body.
PHOSPHATIDYLINOSITOL 3-KINASE LIKE KINASE (PI3KK) IN DNA DAMAGE

PI3KK family members include, in addition to PI3K itself, ATR, ATM, DNA-PKcs (DNA Protein Kinase catalytic subunits), and mTOR (mammalian Target Of Rapamycin). These kinases play critical roles in regulation of the cell cycle, DNA damage signaling, DNA repair, and cellular metabolism in the human cell. ATR is the master regulator of cell cycle progression and DNA damage signaling \(^8\). ATM is important for dsDNA damage signaling and repair \(^9\). DNA-PKcs has been shown to be involved in dsDNA break detection and repair, along with the Ku complex Ku70/80 \(^2\). mTOR functions as the major mammalian cell nutrient sensor \(^10\).

Among the PI3KK family members, ATR, ATM, and DNA-PKcs are directly recruited to DNA damage sites by DNA damage sensor proteins \(^2\). This leads to the activation of the kinase and subsequent activation of downstream targets.

ATM and DNA-PKcs are activated by dsDNA damage. The Ku complex senses and binds to dsDNA, and recruits DNA-PKcs and PARP-1 to the damage site to orchestrate downstream signaling and DNA repair. ATM is recruited by the MRN complex (Mre11-Rad50-Nbs1) binding to dsDNA damage site. The MRN complex exhibits exonuclease activity that participates, along with EXO1, to the resection of the 5’ end, leading to the production of a 3’ ssDNA overhang. This allows ssDNA-
binding proteins to bind to the overhang to facilitate homologous recombination (HR) \(^2\) \(^{11}\).

<table>
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Table 1. PIKKs involved in DNA damage response.

Unlike ATM and DNA-PKcs, ATR is required for cell vitality. ATR knockout mice have been shown to be embryonic lethal \(^8\) \(^3\). ATR is mainly activated through ssDNA accumulation produced by endogenous replication stress, UV irradiation, or treatment with DNA damaging agents such as hydroxyurea and aphidicolin \(^2\) \(^{12}\) \(^{13}\) \(^{14}\). In addition to DNA damage signaling, ATR has been shown to be involved in S-M phase progression, regulate origin firing, prevents replication fork collapse, direct cell cycle delay, and respond to mechanical stress \(^{15\text{-}19}\).

Taken all together, PIKKs are crucial components for cell survival. Mutations and deregulations of the pathways that inappropriately activate or inhibit these kinases may give rise to tumorigenesis.
**ACTIVATION OF ATR PATHWAY**

In replicating cells, ATR was found to protect genome integrity through limiting origin firing, preventing premature exit from S phase, and inhibiting the collapse of the replication complex\(^3\). ATR has been found to be the master regulator for the intra-S and G2/M phase checkpoints in the cell cycle \(^5,8\). It was demonstrated in DT40 cells (chicken lymphoma cell line) that complete depletion of the ATR protein results in mitosis catastrophe due to inappropriate entry into mitosis \(^18\). The most extensively studied role of ATR is its activation during DNA damage events and the consequences thereof.

Mutations of ATR was demonstrated to be embryonic lethal \(^3\). Hypomorphic mutation of ATR has been documented to manifest in human patients as the Seckel Syndrome. Patients exhibit severe developmental defects, dwarfism, and microcephaly. In addition, mutations in downstream targets of ATR confer genome instability in human cells and in mouse \(^3,8\). Also, it has recently been reported that an autosomal dominant mutation in ATR that elevates the occurrence of esophageal cancer in patients \(^20\). This heterozygous point mutation that substitutes the glutamine at amino acid 2144 to arginine. Further analysis speculates that this mutation prevents ATR from being efficiently activated, thus allowing other mutations to accumulate, leading to tumorigenesis \(^8\).
Studies have shown that efficient ATR activation (figure 1) requires: 1) accumulation of ssDNA bound RPA complexes $^{16,21}$, 2) recruitment of cofactors $^{22}$, and 3) the presence of a replication fork $^{23}$.

The uncoupling of the MCM helicase from DNA polymerase at the replication fork, as well as some instances of telomere deprotection, have been shown to trigger ATR activation by generating long stretches of ssDNA. RPA, the major ssDNA binding protein in eukaryotes, is a heterotrimeric complex and binds to
ssDNA regardless of the sequence. This accumulation of RPA bound ssDNA recruits other protein complexes necessary for efficient ATR activation at the damage site such as: 9-1-1 complex, TopBP1, and Rad17-RFC. RPA protects the ssDNA from forming secondary structures. More importantly, RPA presents itself as a “docking site” for the localization and recruitment of other proteins and is itself a downstream target of ATR through the RPA32 subunit.

ATR and its mandatory partner ATRIP are recruited to the damage sites through direct interaction with the N-terminus of the large subunit of the RPA complex, RPA70. TopBP1 possesses the AAD that allows physical contact with ATR-ATRIP to activate ATR. It directly interacts with RPA and the 9-1-1 complex through its BRCT domains. The 9-1-1 (Rad9-Rad1-Mus1) complex is structurally similar to PCNA (Proliferating Cell Nuclear Antigen), and functions to stabilize the dsDNA-ssDNA junction during DNA damage. Rad17-RFC complex is also recruited to the dsDNA-ssDNA junction. Following this, ATR phosphorylates its downstream targets to halt the progression of the cell cycle, repair the DNA damage, or induce apoptosis.

RPA binding to ssDNA represents a crucial step in the ATR signaling pathway. RPA possesses protein domains called OB folds that allows the complex to bind to
ssDNA with high affinity (1-5 nM range). This initial step must be strictly regulated to prevent unwanted ATR activating proteins’ recruitment. Another OB fold containing protein that prevents unwanted DDR is POT1 (protection of telomeres 1); it binds to the single stranded overhang at the telomeres to protect them from being recognized as DNA damage sites, specifically preventing activation of ATR at telomeres \(^{29}\).
OLIGONUCLEOTIDE/OLIGOSACCHARIDE BINDING (OB) FOLDS

OB folds are protein domains that allow direct DNA or protein interaction. It has been suggested that OB fold proteins function as genome guardians in human cells by participating in various DNA metabolic pathways. Specifically, RPA and POT1 both contain different numbers of OB folds that allow the proteins to bind ssDNA as well as interact with proteins involved in DNA replication, and damage signaling. POT1 possesses two N-terminal OB-folds that together constitute the DNA binding domain of the protein, and a third C-terminal OB-fold essential for the association with shelterin through direct interaction with the TPP1 subunit.

Figure 2. Classical OB fold structure. (Figure adopted from 34)
The general secondary structure of an OB fold is depicted in Figure 2. An OB fold consists of five beta strands, themselves separated by loops (L12, L3, L45) formed of residues that make direct contacts with the DNA. The structure may contain an alpha helix. The tertiary structure of OB fold proteins has been found to contribute to DNA binding sequence specificity\(^ {34,35}\). This has been shown through the structural determination of POT1 binding to ssDNA that the two N-terminal OB folds of POT1 enforce a structure to the single stranded DNA itself as a “kink”, which occurs through sequence specific binding to TTAGGGAATG\(^ {35,36}\). On the other hand, structural information of RPA has shown that RPA70 ssDNA binding OB folds exhibit a single channel unlike POT1, thus accounting for its lack of sequence specificity\(^ {31}\).
**Replication Protein A (RPA)**

RPA is the major mammalian ssDNA binding protein. It is a heterotrimer; composed of subunits: 70, 32, and 14. As a complex, RPA can bind to 8-30 nt of ssDNA with a binding affinity of 2 nM with 30dT-ssDNA *in vitro*³⁷,³⁸, although it has been shown that RPA shows a preference for poly-pyrimidine oligos ³⁷. The expression of each subunit remains unchanged and remains a complex throughout the cell cycle ³⁹. RPA is found to associate with chromatin as a trimetric complex ⁴⁰.

Each of the subunits contains a different number of OB folds; these OB folds are essential for ssDNA binding and allow RPA to interact with proteins (Figure 3) involved in checkpoint signaling, DNA replication, and DNA repair pathways ²,⁴¹.

![Figure 3. RPA subunits domain map. RPA70 contains 4 OB folds; RPA32 contains 1 OB fold with multiple phosphorylation sites at its N-terminus; RPA14 contains 1 OB fold. The main function of each OB fold is indicated above each OB fold.](image-url)
RPA70 contains four OB folds; the central OB folds (OB A and B) are tandem repeats and the major core domains that directly interact with ssDNA. Mutation or deletion of OB A or B result in a loss of function RPA. OB A and B alone exhibit low affinity for ssDNA. It has been suggested that the binding of OB A and B to ssDNA causes a conformation change, leading to increased affinity for ssDNA as a complex. Mutation in OB fold A has been suggested to drive tumorigenesis in mice. Either OB A or OB B alone has been shown to exhibit comparable affinity for ssDNA as the heterotrimer. When combined, the ssDNA binding affinity dramatically increased in vitro. In addition to direct ssDNA binding, OB folds A and B also participate in protein-protein interactions with DNA damage repair proteins, such as Rad51, WRN (Werner Syndrome helicase), and BLM (Bloom’s Syndrome helicase). OB F is located at the N-terminus of RPA70 and functions as the recruitment domain for RAD9, TOPBP1, and ATR-ATRIP, which are essential for ATR activation. Truncation or mutation in OB F confers inability to activate DDR and failure to recruit DNA repair proteins upon replication stress. In addition, mutations in OB F results in DNA damage agent hypersensitivity. OB C is located at the C-terminus of RPA70 and contributes to interaction with other subunits and the stability of the complex.
RPA32 contains one OB fold, OB D, and multiple phosphorylation sites at its N-terminus. The phosphorylation pattern is complex, and its significance still poorly understood, but is intimately correlated with DNA damage and activation of repair pathways. OB D participates in ssDNA binding and protein interactions. Although the ssDNA binding affinity of OB D alone is quite low, it has been suggested that the binding of OB A/B to the ssDNA facilitates the binding of OB D. Phosphorylation of serine/threonine residues at the N-terminus of RPA32 has been extensively studied in yeast and mammalian cells. Phosphomimetic and un-phosphorylatable mutants have been generated to dissect the function of this post-translational modification on DNA replication and DNA repair.

![Figure 4](image.png)

Figure 4. Phosphorylation sites at the N-terminus of RPA32. Bold amino acid residues represent phosphorylatable sites and the kinases shown to phosphorylate the residue. (Figure adopted from Villar et al. 2015.)

Phosphorylation of RPA32 has been proposed to influence the localization and DNA binding affinity of the RPA complex as well as protein-protein interactions. It has been shown that phosphorylation of RPA32 can be dependent on cell cycle...
stages and DNA damage response pathway activation. During unperturbed cell cycle, the cyclin-CDK complexes can phosphorylate RPA32 at S23 and S29\textsuperscript{16,25}. During DNA damage, RPA32 becomes hyper-phosphorylated. The hyper-phosphorylated form of RPA32 refers to phosphorylation at residues S4, S8, S21, S23, and S33\textsuperscript{26}. Hyper-phosphorylated RPA32 is detected during replication stress and prevents RPA from associating with replication machinery\textsuperscript{25}. S33 phosphorylation has been shown to be ATR specific \textit{in vitro} and \textit{in vivo}\textsuperscript{4}, this modification has been suggested to induce a conformational change to modulate RPA binding to damage sites and allow checkpoint proteins to associate with RPA. Thus, specific phosphorylation forms of RPA32 can present as a “read-out” for DDR.

RPA14 contains one OB fold, OB E. To date, it has been shown that RPA14 stabilizes the complex, and is not directly involved in ssDNA binding\textsuperscript{51}. 
**Protection of Telomeres 1 (POT1)**

POT1 is an OB fold containing protein that belongs to the shelterin complex. The shelterin complex is composed of six proteins: TRF1, TRF2, RAP1, TPP1, TIN2, and POT1\(^{52}\). The shelterin complex functions to protect the telomeric overhangs from being recognized as DNA damage sites, prevents exonuclease access at the chromosome end, and modulates the activity of telomerase\(^{33,52}\). TRF1 and TRF2 are responsible for binding to the double stranded telomeric DNA and prevent ATM activation at telomeres\(^{53}\). POT1 binds to the ssDNA overhang and prevents ATR signaling at the telomeric region, likely by preventing binding of RPA to the telomeric overhang\(^{54}\).

TRF1 and TRF2 have been shown to protect telomeres from ATM activation\(^{53-55}\) by binding to the dsDNA telomeric sequences and inhibiting ATM autophosphorylation by virtue of a direct interaction with TRF2\(^{55}\). The loss of TRF1 or TRF2 leads to DNA damage response at telomeres, with convergence of DNA repair factors at telomeres and formation of telomere dysfunction induced foci (TIFs), and telomeric fusion between different chromosomes. TIN2 functions to stabilize TRF2 binding to telomeres and tethers TPP1-POT1 to the shelterin complex\(^ {56}\).
Figure 5. Shelterin complex at the telomeres. TRF1 and TRF2/Rap1 bind to the dsDNA junction of telomeric DNA. TIN1 and TPP1 tether POT1 to the shelterin complex. POT1 binds to the ssDNA overhang of telomeres. (Figure adapted from de Lange et al. 2005)

Amongst the shelterin components, POT1 is the most conserved member in eukaryotes, highlighting its essential function at the telomeres across species (Table 2)\textsuperscript{57}.

Table 2. Percent conservation (similarities) of POT1 and other proteins across different species. Human (Hs), mouse (Mm), chicken (Gg), frog (XI), or fission yeast (Sp). (Table adapted from Linger et al. 2009)
POT1 contains three OB folds: one at the C-terminus, and two at the N-terminus. The C-terminal OB fold ensures direct contact between POT1 and TPP1 while the N-terminus OB folds bind to telomeric ssDNA TTAGGG repeats \(^{29,56}\).

Humans possess a single POT1 gene like most vertebrates. Knockdown of POT1 in human cells led to accumulation of TIFs, this suggests POT1 functions to inhibit DDR at telomeres \(^{29}\). Further, although significant telomere fusion was not observed, POT1 depletion led to elongated telomeres, suggesting its role in negatively regulating telomerase activity \(^{29,58,59}\).

On the other hand, mouse contains two POT1 genes that produce POT1a and POT1b. It has been shown that POT1a exhibits activity to repress ATR at the telomeres, while POT1b functions to modulate telomeric end processing \(^{60}\). The ability of POT1 to block ATR activation was shown in double knock out POT1 a/b mouse embryonic fibroblasts, where deletion of both genes led to accumulation of RPA at telomeres and activation of ATR at all telomeres \(^{61}\). Further, POT1 has

![Diagram of POT1 OB folds](image-url)
been shown to modulate the nuclease activity at the chromosome ends to regulate end processing \(^{62}\). In humans, mutations in POT1 predisposes the carrier to leukemia and melanomas \(^{53}\). Therefore, the human POT1 gene is a tumor susceptibility locus. Specifically, it has been shown that mutations in the N-terminal OB folds of POT1 are associated by tumor incidence, and this is due to decrease POT1 binding affinity to the telomeres \(^{63-66}\).

Our lab has shown that LIM protein TRIP6 can directly interact with POT1 through a yeast two-hybrid screen and immunoprecipitation from human cell extracts \(^{67,68}\). Specifically, TRIP6 physically comes in contact with the N-terminal OB folds of POT1. The depletion of TRIP6 leads to TIFs in the nucleus, but had modest effect on cell cycle arrest or apoptosis \(^{68}\). This suggests that there are proteins that share redundant functions in the cell.
**Lin11, Isl1, Mec3 (LIM) Proteins**

The LIM proteins were originally defined as a family of over 60 proteins containing at least one common identifiable domain called LIM domain (Lin11, Isl1 and Mec3). LIM proteins are conserved only in eukaryotes. Individual LIM domains are composed of approximately 55 amino acids with 8 highly conserved residues, mostly cysteine or histidine, which function to coordinate the zinc ion. In human, LIM proteins can contain from 1-5 LIM domains. LIM proteins are further subdivided into 13 families depending on the number and arrangements of their LIM domains. Each LIM protein contains various numbers of LIM domains, each LIM domain is composed of two zinc fingers. The LIM domain was discovered in Lin11 (C. elegans cell-lineage protein), Isl1 (Rat insulin gene enhancer binding protein), and Mec3 (C. elegans mechanosensory neuron specification protein).
In my work, I focused on the Zyxin family, which is characterized by three tandem repeats of LIM domains present in the C-terminal half of the protein. The N-terminus is conserved among the Zyxin family members, although to a lower extent, and contains a nuclear export sequence responsible for dynamic trafficking of the protein between the nucleus and the cytoplasm. The Zyxin family members have complex cellular trafficking patterns and diverse roles, from transcription factors to actin associating proteins. LIM proteins are conserved only in eukaryotes. Zyxin family members are: Trip6, Ajuba, LPP (lipoma preferred partner), Migfilin, WTIP (WT1 interacting protein), LIMD1 (LIM domain containing protein 1), Zyxin.
Our laboratory has shown TRIP6 as a POT1 interacting partner. My work focuses on Ajuba, which acts similarly to TRIP6 by binding to an OB fold protein, RPA. TRIP6 and Ajuba have been described as scaffolding proteins that can travel between the cytoplasm and the nucleus to exert different functions. Both proteins have been shown to implicate in tumor proliferation, cancer cell migration, and prevent DNA damage response \(^71-73\).
**Ajuba**

The *AJUBA* gene is located at chromosome 14 that encodes Ajuba with the molecular weight of 55kDa. Ajuba contains multiple phosphorylation sites at the PreLIM region (Uniprot) and has shown to be a substrate of multiple cell cycle kinases. The pre-LIM region of Ajuba lies between residues 1 and 337, and the three LIM domains are between position 337-397, 398-460 and 461-538. The NES sequence is a seven-residue sequence at positions 289-297 in the pre-LIM domain, which allows the protein to be shuttled between the nucleus and cytoplasm. Ajuba has been shown to exert different functions in different cellular compartments. At steady state, the protein appears to be mostly cytoplasmic as observed by immunofluorescence. Truncation mutants of Ajuba have shown that the PreLIM region localizes in the cytoplasm and the LIM domains reside in the nucleus.
Ajuba contains 3 LIM domains at its C-terminus; each LIM domain is composed of two zinc fingers that are essential for protein-protein interaction. Ajuba also contains a NES (nuclear export signal) at its PreLIM region to facilitate its shuttling between the nucleus and cytoplasm.

Ajuba has been shown to be involved in mitotic commitment, cell fate determination, cell-cell adhesion, and transcriptional regulation. It has been shown that interaction between Ajuba and Aurora A kinase (AURKA) is essential for AURKA activation and subsequent mitosis entry. Ajuba was shown to associate with microtubules and localize at kinetochores with Aurora B kinase to modulate the mitosis checkpoint. Further, Ajuba was shown to be a target of cyclin dependent kinase 2 (CDK2), and phosphorylation mutant allele of Ajuba conferred decrease in cell proliferation.

Ajuba is found frequently truncated in esophageal squamous cell carcinoma (ESCC). Additional evidence has shown Ajuba mutation in head and neck small cell carcinoma (HNSCC) conferred sensitivity to cell cycle kinase inhibitors.
These observations support a role for Ajuba as a tumor suppressor. Our work suggests Ajuba suppresses ATR signaling in human cells and it is an interacting partner of RPA \textsuperscript{73}.

Collectively, recent evidence published indicates that Ajuba contributes to inhibition of DDR, cancer cell proliferation, and cell cycle progression. This thesis project would investigate the role of Ajuba in the repression of DDR.
**TRIP6**

Thyroid receptor interacting protein 6 (Trip6) has been shown to implicate in various pathways. *TRIP6* is located at chromosome 7 and the TRIP6 protein is 50 kDa. TRIP6 is ubiquitously expressed in the human body. The general protein domain distribution is very similar to Ajuba: a PreLIM region at the N-terminus, a NES, and three LIM domains at the C-terminus. Mutation analyses have shown that the LIM domains of TRIP6 localize the protein in the nucleus, while the PreLIM region retains the protein in the cytoplasm. Trip6 has been found to be involved in cell-cell signaling, telomere protection, and transcription regulation. The expression of TRIP6 was found upregulated in Ewing’s sarcoma and contributed to cell proliferation and migration. TRIP6 is also found highly expressed in precancerous lesions and in renal, colorectal, and mammary cancers.

TRIP6 was found to be present at a subset of telomeres in human cells and associate with the Shelterin complex. It has been shown that TRIP6 directly interact with the OB folds of POT1 through its LIM domains, and protects the telomeres from inappropriately eliciting DDR. It still remains to be addressed how TRIP6 protects telomeres and whether it can exert similar function by interacting with other ssDNA binding proteins.
CHAPTER 2: PROJECT OBJECTIVE/FOCUS

Our work has shown that association between LIM protein TRIP6 and OB fold protein prevents inappropriate DDR at the telomeres. Recently, we have been able to show that another Zyxin family member, LIM protein Ajuba, prevents unwarranted DDR in human cells, thus protects against apoptosis. Depletion of Ajuba by siRNA led to cell cycle delay and ATR activation. Ajuba was shown to associate with replication protein A (RPA), an OB fold containing complex, although it was not clear whether this interaction was direct, or mediated by other factors. Further, if direct interaction is shown between Ajuba and RPA complex, which RPA subunit mediates this contact was unknown.

In my thesis work, I hypothesized that Ajuba protects the cell from unscheduled ATR signaling through direct interaction with RPA. The central focus of this project was to study the modality of the Ajuba-RPA association. The specific interest is to understand the modulation of the signaling cascade in the initiating step when RPA is bound to ssDNA.

Based on the paradigm of the TRIP6-POT1 interaction, I speculated that Ajuba directly interacts with RPA in the nucleus. The direct interaction between Ajuba and RPA was probed using in vitro pulldown assays. Co-immunofluorescence and co-immunoprecipitation were employed to examine where this interaction takes
place in the cell. Further, *in vitro* pulldown assays using truncation mutants were used to identify direct protein interaction.

My work leads to a model for ATR activation, in which Ajuba is a repressor of ATR signaling and RPA is a critical platform to initiate this cascade. In this context, the Ajuba-RPA interaction is expected to reduce during replication stress to allow efficient ATR activation. Additionally, the intracellular shuttling of Ajuba was examined under conditions of DNA replication stress.

During DNA replication, extensive amount of ssDNA is present in the nucleus and can be recognized as DNA damage sites. RPA is present in the nucleus during S phase to bind to ssDNA at the lagging strand. I have found that Ajuba is a direct interacting partner of RPA. It is possible that Ajuba associates with RPA during S phase to prevent ATR activation. I have laid the groundwork as well to address whether the Ajuba-RPA interaction is specific to S phase, and to investigate whether Ajuba is active at every cell cycle, even though full answers to these questions will require further studies.

Collectively, my work provides significance to the interaction between Ajuba and RPA, and leads to further characterization of this interaction during normal and stressed cell cycle.
CHAPTER 3: MATERIALS AND METHODS

Recombinant DNA construction

For *E. coli* expression Ajuba (ThermoSci, MHS6278-202807864) and Trip6 (GE Healthcare, MHS6278-202758539) cDNAs were PCR with primers containing EcoRI and Xhol sequences and ligated into pET-NT-His vector (Invitrogen). Ajuba-HIS-5’ (5’-GTC CAG AAT TCT GAG CGG TTA GGA GAG AAA GCC-3’), Ajuba-HIS-3’ (5’-GTA CAC TCG AGT CAG ATA TAG TTG GCA GGG GG-3’), Trip6-HIS-5’ (5’-GCT ACG AAT TCT TCG GGG CCC ACC TGG CTG CCC-3’), Trip6-HIS-3’ (5’-GAC TTC TCG AGT CAG CAG TCA GTG GTG ACG GT-3’).

For *in vitro* translations Ajuba and Trip6 was amplified using PCR with primers, Ajuba-HIS-5’ and Ajuba-HIS-3’, digested with EcoRI and Xhol, and cloned into pcDNA 3.1/His-B. RPA subunits were amplified with PCR using primers with EcoRI digestion sites and cloned into pCMV TNT vector (Promega). RPA70-5’(5’-GTA TAT GAA TTC ATG GTC GGC CAA CTG AGC GAG-3’), RPA70-3’ (5’-GTA TAT GAA TTC TTA TTC TGC ATC TGT GGA-3’), RPA32-5’ (5’-GTA TAT GAA TTC ATG TGG AAC AGT GGA TTC GAA-3’), RPA32-3’ (5’-GTA TAT GAA TTC ATG TGG AAC AGT GGA TTC GAA-3’), RPA14-5’ (5’-GTA TAT GAA TTC ATG GTG GAC ATG ATG GAC TTG-3’), RPA14-3’ (5’-GTA TAT GAA TTC ATG GTG GAC ATG ATG GAC TTG-3’). POT1 was amplified with PCR
with primers containing Xhol digestion sequence into pCMVTnT vector. POT1-5’ (5’-GTA TCC TCG AGA TGT CTT TGG TTC CAG CAA C-3’), POT1-3’ (5’-GTA TCC TCG AGT TAG ATT ACA TCT TC TGCA AC-3’).

Ajuba truncation mutants were produced using PCR with primers containing EcoRI and Xhol digestion sites into pcDNA3.1-B/NT-His vector (Invitrogen, product #). LIM-HIS, aa 337-538: LIM-HIS-5’ (5’-CAC ACG AAT TCT GGC ACC TGT ATC AAG TGC AAC-3’), Ajuba-HIS-3’. PreLIM-HIS, aa 1-337: Ajuba-HIS-5’, PreLIM-HIS-3’ (5’-GTA CAC CTC GAG TCA GCC GAA GTA GTC CTC CCT GGC-3’). PreLIM/LIM1-HIS, aa 1-397: Ajuba-HIS-5’, PreLIM/LIM1-HIS-3’ (5’-GTA CAC CTC GAG TCA CTG AAA CCC TGA AAA CAG-3’). PreLIM/LIM12-HIS, aa 1-460: Ajuba-HIS-5’, PreLIM/LIM1+2-HIS-3’ (5’-GTA CAC CTC GAG TCA AGC ATA ATT TTT GTG GTA-3’).

RPA70 truncation mutants were produced using PCR with primers containing EcoRI digestion sequences into pCMVTnT vector. RPA70-OBABC, aa 169-612: OBABC-5’ (5’-CAC AAC GAA TTC ATG GGT CCC AGC CTG TCA CAC-3’), RPA70-3’. RPA70-OBF, aa 1-169: RPA70-5’, OBF-3’ (5’-CAC AAC GAA TTC TCA TGC AGC TTT TCC AAA TGT CTT-3’). RPA70-OBB+C, aa 302-612: OBBC-5’ (5’-CAC AAC GAA TTC ATG GAT TTC AGC GGG ATT GAT GAC-3’), RPA70-3’. RPA70-OBF+A, aa 1-169: RPA70-5’, OBF+A-3’ (5’-CAC AAC GAA TTC TCA GAA ATC AAA CTG AAC CGT-3’). RPA70-OB½ABC, aa
Expression of recombinant proteins in E. coli.

Ajuba-His and Trip6-His plasmids were transformed in BL21 Star™ (DE3) cells (Invitrogen, C6010-03) and grown in 5ml LB with ampicillin (100mg/ml) (Sigma, A-9518) overnight in cell shaker at 37°C. Inoculated 1L of LB with ampicillin next day with overnight culture and grown to cell density OD₆₀₀ 0.5. IPTG (Sigma, I5502-5G) was added to the culture to achieve final concentration of 0.5mM and incubated in shaker for three hours at 37°C or overnight at 28°C.

Affinity purification of His-tagged proteins

Ni-NTA beads (BioRad, 1560123) were washed with dH2O and with lysis buffer (50mM Na₂HPO₄, 300mM NaCl) twice. Beads were transferred into a small column and kept at 4°C. Bacteria pellet was collected by centrifugation and lysed with lysis buffer with protease inhibitor cocktail (Roche). Cell lysate was slowly transferred into the column and washed with wash buffer (50mM Na₂HPO₄, 300mM NaCl). His-tagged proteins were eluted with elution buffers (50mM Na₂HPO₄, 300mM NaCl, 100-150mM imidazole) containing concentration gradient of imidazole (concentrations). Fractions of the elution were collected and analyzed by SDS-PAGE and silver staining.
**Silver staining**

Silver staining was performed according to manufacturer instructions (BioRad).

**Cell culture and drug treatments**

HTC75 and IMR90 (human diploid lung fibroblasts) (ATCC® CCL-186™) cell lines were employed. HTC75 is a derivative of HT1080 (human fibrosarcoma). IMR90 was used at population doubling 30. HTC75 cells were cultured in DMEM (Cellgro, 10-017-CV) with 10% BCS (HyClone, SH30072.03), 1% penicillin and streptomycin (Cellgro, 30-002-CI) and 1% L-glutamine (Gibco, 25030-081).

IMR90 cells were cultured in DMEM with 20% FBS (ClonTech, 631106), and 1% penicillin and streptomycin. Both cell lines were grown in cell culture incubator at 37°C with 5% bone dry CO₂.

Hydroxyurea was added to the media to achieve a final concentration of 2mM. Cells were collected after 24 hours of treatment. Double thymidine block was performed by adding final concentration of 2mM thymidine to the media. Cells were synchronized for 18 hours and was rinsed with media to release for 10 hours. Thymidine was added the second time for 18 hours. Cells were rinsed with media to release for hours indicated. Cell cycle profile was accessed by FACS.

Cells were grown on glass cover slips to 70% confluency and leptomycin B was added for a final concentration of 10ng/ml. Cells were incubated for 3 hours at 37 °C before collection.
**Co-Immunoprecipitation**

Cells were collected by trypsinization and pelleted after centrifugation at 1500xg for 10mins at 4°C. Cell pellets were washed with PBS (pH= 8.0) twice. 0.5ml of lysis buffer (50 mM Tris-HCl pH 7.4/1% Triton X-100/ 0.1% SDS/150 mM NaCl/1 mM EDTA/1 mM DTT/1mM PMSF, with protease inhibitor cocktail (Invitrogen)). Nuclear proteins were extracted by addition of 25ul (5M stock) of NaCl and incubated on ice for 20 minutes. Lysates were diluted by adding 500ul of ice-cold dH₂O. The samples were subjected to centrifugation at 18,000xg for 10 minutes (4°C). The supernatants were collected. Protein G Sepharose beads were blocked with BSA and *E. coli* DNA for 3 ½ hours at 4°C. At the same time, 5ul of antibody targeting the protein of interest was added to 200ul of whole cell lysate and incubated at 4°C for 3 ½ hours. 32ul of blocked beads was added to each sample and incubated for 30 minutes at 4°C. Beads were washed five times with 1ml of ice cold lysis buffer without protease inhibitor. 50ul of loading buffer was added to each sample and subjected to Western blot analysis.

**Co-immunofluorescence**

HTC75 and IMR90 cells were grown on glass coverslips and were washed twice with PBS at room temperature. Fixed cells with 2% formaldehyde in PBS for 10 minutes at room temperature. Washed cells twice with PBS and permeabilized with 0.5% NP-40 in PBS for 10 minutes. Washed cells with PBS twice. Blocked
with PBG for 30 minutes at RT. Added primary antibody diluted in PBG (Ajuba 1:2500, RPA70 1:2500) and stored at 4C overnight. Washed with PBG three times and incubated with fluorescent secondary antibody diluted in PBG for 45 minutes at RT (Anti-rabbit 1:1000, Anti-mouse 1:1000). Washed with PBG twice and incubated the coverslip with DAPI in PBG at 100 ng/ml. Coverslips were mounted on microscope slides with embedding media and sealed with nail polish.

**FACS (Fluorescent Activated Cell Sorting)**

Cells were collected and fixed in PBS-EDTA and ethanol. Staining solution (Triton X-100, RNase A, propidium iodide) was used to stain cells.

**In vitro co-translation**

1ug of Ajuba/Ajuba mutant plasmid and 1ug of RPA subunit/RPA70/RPA70 mutant plasmid were added into the same reaction. The *in vitro* translation protocol was performed as described in manual from manufacturer (Promega, TNT© T7 Coupled Reticulocyte Lysate System, L4610) with 35S-methionine (PerkinElmer).

**His-tag affinity pulldown**

Ni-NTA beads were washed with dH2O and TBST (pH=8.0, 0.01% Tween-20). The beads were blocked with 2% BSA (Sigma) (10mg/ml) in TBST for 2 hours at room temperature. 20ul of blocked beads were added to each sample and incubated
at room temperature for 30 minutes. Beads were washed with 100ul TBST six times and loading buffer was added directly to beads.

**Autoradiography**

SDS-PAGE gels were dried with BioRad Gel Dryer at 80°C for two hours. The dried gels were put into a phosphor storage cassette overnight (Amersham). The storage screen was visualized with Phosphor imager (Typhoon 9410).

**Autoradiography quantitation**

Radioactive signals were measured using ImageQ 2D analysis software. The signals were normalized to the number of methionine in each protein and the signal of His-tagged proteins. We assume that each pulldown constitutes a 1:1 ratio of Ajuba:RPA subunit. Pulldown efficiencies were calculation using the ratio of expected protein pulldown signal to the observed signal. Graphs and statistical significance were produced and calculated using Prism Graph Pad software.

**Cell fractionation**

Cells were collected and washed with PBS and washed twice in buffer A (10mM HEPES, pH 7.9, 10mM KCl, 1.5 mM MgCl2, 0.34M sucrose, 10% glycerol, 1mM DTT, and protease inhibitor cocktail) and centrifuged for 14000xg for 10 minutes at 4C. The supernatant was collected as the cytoplasmic proteins (CP). The pellet was resuspended with buffer B (3mM EDTA, 0.2 mM EGTA, 1mM DTT, and
protease inhibitor cocktail) and centrifuged for 5 minutes at 1700xg, 4C. The supernatant (soluble nucleoplasm, NP) and the pellet (chromatin bound, CB) were collected.

To obtain the soluble chromatin fraction, the nuclei pellet was resuspended with buffer A with micrococcal nuclease and 1mM CaCl2 and incubated at 37°C for 1 minute, then centrifuged for 5 minutes at 1700xg, 4C.

**siRNA transfection**

HTC75 cells were grown in complete DMEM media without antibiotics 24 hours prior to transfection. Lipofectamine reagent (Invitrogen) was used to transfec Ajuba siRNA #3 (5’- GCAGCUGAGUGAUGAGGAAUU-3’) and incubated with cells for 48 hours at 37°C before collection.

**Western blot analysis**

Samples were prepared as described above and 4x loading buffer was added to each sample to run on 10% SDS PAGE gels at 120V. Gels were transferred onto nitrocellulose membranes at 90V for 90 minutes.
CHAPTER 4: CHARACTERIZING AJUBA’S INTERACTION WITH REPLICATION PROTEIN A IN VIVO

Introduction

The ATR pathway is pivotal to signal DNA damage to orchestrate cell cycle delay and damage repair. Cells rely on this pathway to maintain genome integrity and to prevent the passage of erroneous genetic information. The ATR response is a signaling cascade and each step has to be precisely coordinated to ensure the full execution of the downstream effects. The initial step of ATR activation is the binding of RPA to the ssDNA region. This accumulation of RPA-ssDNA further recruits other components such as the 9-1-1 complex, TopBP1, and ATR-ATRIP to elicit the DDR signaling pathway. Upon DNA damage ATR is autophosphorylated\(^8^5\), and Chk1 is phosphorylated at S296 and T345\(^8^6\), RPA32 is phosphorylated on residues T21 and S33 by ATR\(^2^5\). This signaling cascade would result in DNA damage repair, cell cycle delay, and apoptosis\(^3^,^8\).

Due to the dire consequences from activating the ATR pathway, it is necessary for the cell to activate this pathway only when needed. The cell cannot sustain ATR activation during every S phase, because this will ultimately put its proliferation potential to a halt. Our laboratory has published observations documenting the critical interactions between LIM proteins (Ajuba, Trip6) and OB fold proteins.
(RPA, POT1) in maintaining genome integrity \(^{68,73}\). In particular, Ajuba was found to suppress inappropriate DNA damage response during normal cell cycle.

![Figure 9](image)

Figure 9. Ajuba co-immunoprecipitates with RPA32 and depletion of Ajuba leads to RPA32 phosphorylation. A) co-immunoprecipitation of Ajuba and RPA32 in HTC75 (human fibrosarcoma) and IMR90. B) RPA32 (human diploid fibroblasts) phosphorylation at T21 upon Ajuba depletion by siRNA. (Figure from Kalan S. et al 2013)
Knockdown of Ajuba resulted in severe reduction in cell viability and cell cycle delay, with enrichment for cells in S phase. Further, Ajuba was found to immunoprecipitate with RPA32 in unperturbed human cells and down regulation of Ajuba resulted in RPA32 phosphorylation (Figures 9 and 10). Induction of DNA damage induced foci was observed after Ajuba depletion using immunofluorescence (Figure 10). In this experiment, RPA32 S33-phospho foci serve as a strong indicator for DDR activation. Previous report showed that
53BP1 foci, and Chk1 phosphorylation were detected upon Ajuba depletion as well. Thus, Ajuba plays a key role in DDR regulation and we hypothesized that Ajuba exerts its function through interacting with RPA32. Hence, we sought to explore the relationship between RPA and Ajuba.

We have established a model in which Ajuba negatively regulates the ATR pathway through interaction with RPA. We probed the interaction between the two proteins *in vivo* by asking whether Ajuba can be found interacting with the RPA complex in the cell and if this is taking place inside the nucleus. In addition, we aimed to elucidate whether Ajuba and RPA interact more frequently during DNA replication. We also sought to investigate the effects of DNA damage on Ajuba-RPA relationship: whether the two proteins remain to associate with each other and whether Ajuba is present in the nucleus during DDR.
Results:

AJUBA INTERACTS WITH THE RPA COMPLEX

To address the relationship between Ajuba and RPA, we asked if Ajuba interacts with the whole RPA complex or just individual RPA subunits in the cell. We performed immunoprecipitation of Ajuba using antibodies against the three RPA subunits, followed by Western blot detection for Ajuba. Here, two cell lines were employed: HTC75 (human fibrosarcoma) and IMR90 (human diploid fibroblasts). HTC75 is a derivative of HT1080 cell line (human fibrosarcoma) and harbors no known mutation in ATR mediated DNA damage response proteins. IMR90 is used to investigate the whether the Ajuba-RPA interaction takes place in an untransformed cell line.
Figure 11. Ajuba is found immunoprecipitated with the RPA heterotrimer in HTC75 (human fibrosarcoma) cells. (Top) Co-immunoprecipitation of Ajuba by RPA subunits. (Bottom) Co-immunoprecipitation of RPA subunits with Ajuba antibody. (n=3)

Ajuba was found to immunoprecipitate with all three subunits of the RPA complex in untreated HTC75 cells (Figure 11). We observed that only a small amount of Ajuba immunoprecipitated with each of the RPA subunits compared to the amount in total lysate (5% input), estimated at 2-5% of total Ajuba in my blots. This suggests that Ajuba interacts with the whole RPA complex in cells, but that the pool of Ajuba in association with RPA constitutes a minor fraction.

Further, we looked if Ajuba is co-localized with RPA in the nucleus by co-immunofluorescence in unsynchronized cells. We observed a sub-population of cells displayed co-localized signals of Ajuba and RPA70 inside the nucleus in HTC75 and IMR90 cells (Fig. 12). In the cells that showed a detectable signal for Ajuba in the nucleus, we found 1-3 foci of co-localization between Ajuba and RPA70 (Fig. 12) in approximately 30% of cells.
HTC75 cells treated with leptomycin B, which retains proteins inside the nucleus due to inhibition of exportin 1-dependent nuclear export, showed increase colocalization events of Ajuba and RPA70 (Figure 13). This suggests that Ajuba colocalizes with RPA70 in the nucleus in cancer cells as well as primary untransformed cells. In addition, forced retention of Ajuba in the nucleus enhances this event in cells.
Figure 13. Sequestering Ajuba nuclear export increases co-localization with RPA70. (Top) Co-immunofluorescence of Ajuba and RPA70 in unsynchronized HTC75 cells treated with Leptomycin B for 3 hours (n=2). (Bottom) Quantitation of cells exhibited >3 foci of Ajuba and RPA70 colocalization in unsynchronized cells (UT) and cells treated with leptomycin B (+ Lept. B). (n= 1)

Although leptomycin B treatment did increase Ajuba-RPA70 colocalization event (from 20% in untreated cells to 50% in leptomycin B treated cells), there was a significant number of cells that did not display this pattern. This could be
explained by regulation of the localization of Ajuba in the nucleus, for instance by cell cycle checkpoints.

S phase is the only phase in the cell cycle that implicates production of ssDNA in the nucleus that requires the binding of RPA, we hypothesized that colocalization with Ajuba would be detectable mostly during that period. Possibly, interaction with RPA would occur during this vulnerable time of DNA replication to prevent inappropriate or excessive ATR activation. To clarify this point, cells were synchronized to G1/S border with double thymidine block\textsuperscript{88}. Thymidine was washed off with PBS and replaced with fresh media without thymidine. Subsequently, cells were released into S phase. Importantly, it was shown that the majority of RPA exists in the nucleoplasmic fraction, thus washing the cells with Triton-X 100 prior to fixing would extract the majority of nucleoplasmic RPA and leaving behind the chromatin bound RPA fraction in the nucleus. Therefore, cells were collected at 2-hour time points, washed with Triton-X 100, and subjected to co-immunofluorescence with Ajuba and RPA70 antibodies to examine whether the co-localization increases during S phase.
Unsynchronized

0 hour

2 hours

4 hours

6 hours

Ajuba nuclear localization

$p<0.001$

Percentage of cells

hours release from dT block
Figure 14. Immunofluorescence of Ajuba nuclear localization during S phase. HTC75 cells were synchronized to G1/S border with double thymidine block and released into S phase. (Top) Increase of Ajuba nuclear localization during S phase. Cells were collected at the time indicated and washed with Triton-X 100 twice to extract nucleoplasmic proteins. Ajuba (green), nuclei (DAPI, blue). Scale bar indicates 10um. (Bottom) Quantitation of cells exhibited high Ajuba signal in nuclei at each indicated time point.

As shown in Figure 14, strong Ajuba signal was observed in only in 33.02% of unsynchronized HTC75 cells. When the cells were synchronized with double thymidine (dT) block, at 0 hour, the percentage of cells that exhibited clear Ajuba signals in the nucleus increased to 35.77%. Upon release from the dT block, strong nuclear Ajuba signals were found in 54.41% and 68.93% of cells after 2 and 4 hours release, respectively. Ajuba’s presence in the nucleus began to reduce at 6 hours after dT release, with 46.8% of cells showed nuclear Ajuba signals. Upon analysis, cells that exhibited strong Ajuba nuclear signal at 4 hours after released from dT block is significantly more abundant compared to unsynchronized cell population. This indicates that Ajuba’s nuclear import or retention in the nucleus is regulated by S phase progression through an unknown mechanism.
Double thymidine release

Unsynchronized

0 hour

2 hours

4 hours

6 hours

DAPI | Ajuba | RPA70 | Merge

Unsynchronized

0 hour

2 hours

4 hours

6 hours
Figure 15. Co-Immunofluorescence of Ajuba and RPA70 in the nucleus during S phase. HTC75 cells were synchronized to G1/S border with double thymidine block and released into S phase. (Top) Increase of Ajuba-RPA70 co-localization during S phase. Cells were collected at the time indicated and washed with Triton-X 100 twice to extract nucleoplasmic proteins. Ajuba (green), RPA70 (red), nuclei (DAPI, blue). Scale bar indicates 10um. (Bottom) Quantitation of cells exhibited >3 foci of Ajuba-RPA70 signal in the nucleus at each indicated time point. *Experiment was done with the assistance of Baila Schochet.

We probed for Ajuba-RPA interaction in dT block and release HTC75 cells. Quantitation of cells that exhibited >3 foci of Ajuba-RPA70 co-localization at each cell cycle release time point after synchronization is depicted in the bottom panel of Figure 15.

Figure 15 shows an increase of co-localization events occur in HTC75 cells from 2 hours to 6 hours (Table 3), with the 4 hours time-point exhibiting the highest percentage of cells with Ajuba-RPA foci in the nucleus (61.94%). 27.48% of
unsynchronized (untreated) cells showed 3< co-localized foci, which is comparable to the percentage of cells at 6 hours time-point (27.44%). Interestingly, at 0 hour, similar percentage of cells (17.65%) was found compared to unsynchronized population (27.48%). In addition, 34.32% of cells from 2 hours release from dT block showed co-localization signals. At 4 hours release, 54.83% of cells showed co-localized foci. This suggests that Ajuba-RPA interaction is strongly enhanced during mid-S phase. This data complements figure 14 that show gradual increase of Ajuba accumulated in the nucleus.

PCNA (Proliferating Cell Nuclear Antigen) is the sliding clamp that functions at the replication fork to tether DNA polymerase along the template DNA 89. RPA is recruited to replication forks to bind to ssDNA and has been shown to colocalize with newly synthesized DNA and PCNA 90. We speculate that Ajuba may be protecting the replication fork through local inhibition of ATR activation. Co-immunofluorescence was employed to address whether Ajuba can be found at sites of replication.
Figure 16. Ajuba can be found at some replication sites. Co-immunofluorescence of PCNA and Ajuba in synchronized HTC75 cells with double thymidine block and release. Cells were collected at the time indicated and washed with Triton-X 100 twice to extract nucleoplasmic proteins. Ajuba (green), PCNA (red), nuclei (DAPI, blue). Scale bar indicates 10um.

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<th>Ajuba</th>
<th>PCNA</th>
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Figure 16 shows HTC75 cells that are synchronized with dT block and release. At 0 hours and in unsynchronized cells, low levels of Ajuba and PCNA are found in the nucleus. Upon release into S phase, an increase of nuclear Ajuba signals is found concomitantly with an increase of PCNA signal. Although preliminary,
Ajuba is observed to be present at a subset of replication sites. This suggests that Ajuba may exert its function to repress local ATR activation.

Collectively, we found that Ajuba can be found with the RPA trimeric complex and partially colocalizes with RPA70 in the nucleus. This colocalization event in the nucleus was enhanced with leptomycin B treatment. In addition, cell nuclei exhibited strong Ajuba signals upon dT block and release at 4 hours (mid-S phase cells) compared to untreated cells. The observation of increase Ajuba nuclear localization during S phase was complemented with the concomitant increase of Ajuba-RPA70 colocalization signals. Further, Ajuba is found to be present at a subset of replication sites. These data suggest that Ajuba may exert its function during DNA replication at a selected population of replication sites to inhibit local ATR activation.
AJUBA-RPA INTERACTION DURING DNA DAMAGE

The interaction between Ajuba and RPA sparked the question whether this interaction is important during replication stress. In previous publication, the knockdown of Ajuba led to inappropriate unleashing of DDR, cell cycle delay, and apoptosis in human cells. This introduced Ajuba as a negative regulator in the DDR pathway. Hydroxyurea (HU) is a ribonucleotide reductase inhibitor that halts S phase progression by depleting the nucleotide pool inside the cell. Treatment with HU arrests cells at G1/S phases due to stalled replication forks and ssDNA accumulation in the nucleus. Here, we employed HU to explore how Ajuba-RPA relationship is affected in cells when confronted with DNA replication stress. Ajuba possesses a NES, which enables it’s shuttling from the nucleus. We aim to investigate if the shuttling of Ajuba is triggered by replication stress using cell fractionation and co-immunofluorescence.
REPLICATION STRESS DISRUPTS AJUBA-RPA INTERACTION

A. HTC75 cells

<table>
<thead>
<tr>
<th></th>
<th>UT</th>
<th>24 hr 2 mM HU</th>
<th>DOUBLE THYMIDINE (S-phase)</th>
<th>Bedeck only</th>
<th>Unnreated</th>
<th>24 hr 2 mM HU</th>
<th>DOUBLE THYMIDINE (S-phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP Ajube</td>
<td>RPA32</td>
<td>GapDH</td>
<td>RPA32</td>
<td>GapDH</td>
<td></td>
<td></td>
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</tbody>
</table>

IMR90 cells

<table>
<thead>
<tr>
<th></th>
<th>UT</th>
<th>24 hr 2 mM HU</th>
<th>DOUBLE THYMIDINE (S-phase)</th>
<th>Bedeck only</th>
<th>Unnreated</th>
<th>24 hr 2 mM HU</th>
<th>DOUBLE THYMIDINE (S-phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP Ajube</td>
<td>RPA32</td>
<td>GapDH</td>
<td>RPA32</td>
<td>GapDH</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>HTC75</th>
<th>UT</th>
<th>24 hr HU</th>
<th>dT 2 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>64%</td>
<td>58.85%</td>
<td>12.1%</td>
</tr>
<tr>
<td>S</td>
<td>24%</td>
<td>41.14%</td>
<td>78.7%</td>
</tr>
<tr>
<td>G2/M</td>
<td>11%</td>
<td>0%</td>
<td>1.62%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>UT</th>
<th>24 hr HU</th>
<th>dT 4.5 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>63%</td>
<td>55.5%</td>
<td>20%</td>
</tr>
<tr>
<td>S</td>
<td>8%</td>
<td>44%</td>
<td>73%</td>
</tr>
<tr>
<td>G2/M</td>
<td>29%</td>
<td>0%</td>
<td>4%</td>
</tr>
</tbody>
</table>
Figure 17. Ajuba-RPA interaction reduces upon replication stress. A) Co-immunoprecipitation of Ajuba and RPA32 in HTC75 and IMR90 cells. Cells were treated with 2mM of hydroxyurea (HU) for 24 hours. Double thymidine block synchronized the cells to S phase. 5% of total lysate was loaded as input. B) FACS analysis of cells in panel A. C) Co-immunoprecipitation of Ajuba and RPA70 in HTC75 and IMR90 cells. *Experiment was performed with the assistance of Sampada Kalan.

We performed co-immunoprecipitation in HTC75 and IMR90 cells that were unperturbed/untreated, or treated with 2mM HU for 24 hours, or dT blocked and released into S phase. Figure 17A shows RPA32 can be immunoprecipitated by Ajuba in both untreated and S phase cells. Ajuba-RPA32 interaction is significantly reduced in cells treated with 2mM HU. FACS analysis (Figure 17B) shows majority (64% and 63% for HTC75 and IMR90 cells, respectively) of untreated cells are in G1 phase. 76.7% and 73% of the cell populations were synchronized to S phase with double thymidine block and release. Treatment with 2mM with HU arrested 41% (HTC75) and 44% (IMR90) of the cells in S phase. We observed similar results between Ajuba and RPA70 when cells were treated
with HU (Figure 17C). Ajuba’s association with RPA complex in S phase cells but not in cells with replication stress argue Ajuba-RPA interaction is maintained in proliferating unperturbed cells. Enforced DDR through treatment with HU led to the interruption of this interaction. We propose that dissociation of Ajuba-RPA is essential for full activation of ATR response.

Moreover, this data suggests Ajuba interacts with RPA during S phase and DNA damage in the cell disrupts this interaction. This dissociation does not appear to be a mere consequence of cells being in S phase, since synchronizing cells in S phase by itself does not lead to a reduction.
AJUBA IS TRANSLOCATED OUT OF THE NUCLEUS UPON DNA DAMAGE

Ajuba has been shown to shuttle between the nucleus and the cytoplasm to carry out its various functions 78. I am interested in elucidating whether Ajuba is still present in the nucleus during DNA damage. I employed co-immunofluorescence to probe for RPA70 and Ajuba’s presence in the nucleus with and without the treatment of HU in HTC75 cells.
Figure 18. Ajuba nuclear signals reduced after hydroxyurea treatment. Co-immunofluorescence of Ajuba and RPA70 in HTC75 (top) and IMR90 (bottom) cells treated with and without HU. Cells were treated with 2mM HU for 24 hours. Scale bar indicates 10µm.
We found that the amount of Ajuba in the nucleus is significantly reduced upon HU treatment in HTC75 and IMR90 cells (Figure 18). We observe punctate foci of RPA70 in HU treated cells, indicating activation of DDR. Quantitation of cells that exhibit nuclear Ajuba signals with and without HU treatment is depicted in Table 3. We found that both cell lines exhibit similar percentage of cells containing nuclear Ajuba signals in an unsynchronized population. HU treatment led to a 37% decrease of nuclear Ajuba signal in cell nuclei in both cell types. This suggests that the absence of Ajuba in the nucleus is important for DDR in cancerous and non-transformed cell types.

<table>
<thead>
<tr>
<th></th>
<th>- HU</th>
<th>+ HU</th>
</tr>
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<tbody>
<tr>
<td>Ajuba signal</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>in the nucleus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTC75</td>
<td>46.92%</td>
<td>53.08%</td>
</tr>
<tr>
<td>IMR90</td>
<td>46.36%</td>
<td>53.63%</td>
</tr>
</tbody>
</table>

Table 3. Percentage of cells exhibit Ajuba signal in the nucleus with or without treatment of HU (2mM, 24 hr).

This led to the speculation that Ajuba is shuttled out of the nucleus during DDR.

To address this point, we performed cell fractionation to probe for Ajuba in different cellular compartments with and without DNA damage.
Figure 19. Chromatin bound Ajuba is reduced after hydroxyurea treatment. (Left) untreated cells. (Right) cells were treated with hydroxyurea (2mM) for 24 hours. CP: cytoplasmic, NP: nucleoplasmic, CB: chromatin-bound. Experiment was done with the assistance of Sampada Kalan.

We found that in untreated cells, small amounts of Ajuba and RPA32 are present in the chromatin bound (CB) fraction at very low amounts. We note that Ajuba appears as a lower molecular weight band in the chromatin fraction. Presently, we have not characterized this discrepancy, and cannot distinguish the presence of a specific Ajuba isoform in the nucleus (isoform 3 has a compatible molecular weight as per the NCBI website) from a non-specific signal. In addition, this lower molecular weight (MW) band could represent the unphosphorylated species of
Ajuba 74. More work is required to confirm the presence of Ajuba on the chromatin using this assay. The nucleoplasm fraction shows a marked higher amount of RPA32 compared to the chromatin fraction, and Ajuba is not detected in the NP. It has been shown that PARP supports DNA replication, DNA repair, as well as chromatin remodeling 95,96, thus PARP (polyADP ribose polymerase) is found at the CB fraction without significant cleavage in untreated cells. After treatment with HU, most of RPA32 is found in the chromatin bound fraction as expected due to formation of extensive ssDNA in the nucleus. Ajuba is not found in the chromatin bound fraction, and the lower MW band increases in the cytoplasmic fraction (Figure 8). PARP cleavage is apparent in the chromatin bound fraction after HU treatment indicative of apoptosis 97,98. Although preliminary, this data suggest that Ajuba is present on the chromatin during unperturbed cell cycle and transported out of the nucleus during DDR. It is possible that the low amount of Ajuba seen in the chromatin fraction could be retained there through interaction with RPA, and replication stress (HU) reduced the Ajuba-RPA association, leading to efficient NES-dependent export of Ajuba to the cytoplasm.
CHAPTER SUMMARY/DISCUSSION:

Here, I presented data of Ajuba’s interaction with the RPA complex *in vivo* during unperturbed cell cycle and replication stress. The two proteins co-localized within the nucleus in a subset of unsynchronized HTC75 cells, cell cycle synchronization by dT block and release led to a significant increase of co-localization events compared to unsynchronized cells (figures 12, 13, 15). The percentage of synchronized cells that showed strong nuclear Ajuba signals complemented with the percentage of cells that exhibited Ajuba-RPA70 colocalization foci (Figures 14 & 15). In addition, Ajuba is found to colocalize at a subset of replication sites (figure 16). These data suggest that nuclear entry of Ajuba and Ajuba-RPA interaction are upregulated in S phase, implicating Ajuba’s function to inhibit ATR response during DNA replication.

We found that Ajuba-RPA interaction is reduced after HU treatment by Western blot analysis (Figure 17). We did not observe detectable Ajuba signal inside the nucleus upon HU treatment (figure 18, table 4). Further, cell fractionation data argues that Ajuba is shuttled out of the nucleus under replicative stress conditions (figure 19), underscoring its critical role in preventing inappropriate ATR activation through interaction with the RPA complex. The reduction of the Ajuba-RPA interaction within the nucleus and the shuttling of Ajuba from the nucleus could be part of ATR activation during replication stress. These results suggest the
presence of Ajuba in the nucleus is pivotal to modulating the ATR response pathway.
CHAPTER 5: CHARACTERIZING THE INTERACTION BETWEEN AJUBA AND REPLICATION PROTEIN A IN VITRO

Introduction:

The RPA complex is a heterotrimer: RPA70, RPA32, and RPA14. Each subunit has been shown to interact with a list of different proteins though its OB folds. RPA70 possesses four OB folds: F, A, B, and C. Studies have shown that OB folds A and B are the main DNA binding domains, with OB fold A exhibiting the highest affinity for ssDNA. In addition, other studies have shown OB fold A can also interact with proteins involved in DNA metabolism such as Werner syndrome helicase (WRN), Bloom syndrome helicase (BLM), and Xeroderma Pigmentosum Group A-Complementing Protein (XPA). OB fold F has been shown to function as a docking site for numerous proteins involved in check point activation and DDR. Finally, OB fold C facilitates in subunit interaction.

RPA32 contains only one OB fold that is involved in subunit interaction with RPA70. RPA14 contains one OB fold that functions in subunit interaction.

The LIM protein Ajuba possesses a pre-LIM region at the N-terminus and three LIM domains at the C-terminus. Published experiments have shown that the LIM domains of Ajuba are important for protein-protein interactions. The pre-LIM domain of Ajuba is proline rich and includes a NES. The three LIM domains are tandem repeats and each LIM domain is consists of two zinc fingers.
The role of Ajuba in maintenance of genome integrity was shown in our previous work which proposed a model that Ajuba is implicated in the suppression of ATR signaling through interaction with RPA (Figure 20).

![Diagram]

Figure 20. Model of Ajuba’s function in preventing unscheduled DDR. It is not known if Ajuba directly interacts with RPA. Figure adapted from Kalan S. et al.

To further investigate the relationship between RPA and Ajuba, we sought to elucidate the nature of interaction between the two proteins. The co-immunoprecipitation shown in Figures 11 and 17 do not address whether the RPA-Ajuba interaction is through direct contacts, or mediated by other factors. Therefore, I sought to determine whether the interaction between Ajuba and RPA complex is direct. To address this question, I employed two approaches: first, to produce His-tagged Ajuba in E. coli for in vitro binding assays. Second, to use
rabbit reticulocyte *in vitro* transcription/translation system for Ajuba and RPA subunits for pull downs. Although only the second strategy proved informative, I will document both approaches in this thesis.
Results:

Ajuba and TRIP6 were cloned into pET/NT-His vector under the lac promoter so the 6xHis tag would fuse to the N-terminal of the proteins. Protein production was induced by adding 2mM IPTG and incubated at 28C overnight. Bacterial cultures were collected and lysed. The whole cell lysate was incubated with Ni$^{2+}$-NTA beads, washed with lysis buffer and buffer containing 10-30mM of imidazole. Finally, the His tagged proteins that are bound to nickel beads was be eluted with elution buffer containing high concentration of imidazole (100-150mM).

Production and purification of recombinant LIM proteins from E. coli

To address direct interaction between OB fold and LIM proteins, we first attempted to purify each target protein by affinity chromatography. Each target gene was cloned into IPTG inducible promoter with peptide tags. For LIM proteins, Ajuba and Trip6 were cloned into pET-NT His vector. The constructs were transformed into BL21 cells and production of recombinant proteins was induced with the addition of 2mM IPTG overnight at 28 degrees Celsius. To confirm the production of His-tagged proteins, whole cell lysates were prepared and analyzed with Western blot (Figures 21).
Two liters of BL21 cells were lysed after induction with IPTG overnight and incubated with 5ml of 50% slurry Ni\textsuperscript{2+}-NTA beads for 3 hours at 4 degrees Celsius. The beads were transferred into a column in cold room (4 °C), and washed with lysis buffer and non-denaturing buffers containing increasing concentrations of imidazole to elude the bound proteins. Fractions were collected for each wash and elution, and were analyzed with Western blot and silver staining.

As shown in Figure 22, Ajuba-His was eluted with high yield in the wash fractions 1 and 2 (25mM imidazole). Unfortunately the purity of the fractions containing high Ajuba concentrations was not adequate for further use. Additional attempts were made employing lower concentrations of imidazole in the wash buffer; this also led to impurities in the final elution fractions. Identical protocol was performed with TRIP6-His yielding slightly better results (Figure 23). We carried TRIP6 with this protocol to use as a LIM protein control for Ajuba, as well as possible binding assays with POT1.
Figure 22. Purification of His-Ajuba with affinity chromatography using Ni-NTA beads. (Top) Western blot (bottom) silver staining of Ajuba-His purification fractions. Positive control-mammalian cell lysates with Ajuba (not tagged). 6xHis-Ajuba total lysate was loaded in 20ul and 40ul of volume. All other lanes were loaded with 20ul of fraction. Wash buffer (30mM imidazole), Elution buffer (100mM imidazole).
However, this method proved to be inefficient in protein production and time consuming. Thus, I alternated to the \textit{in vitro} transcription/translation system.
**AJUBA-RPA INTERACTION USING IN VITRO TNT SYSTEM**

The *in vitro* transcription/translation system (Promega) contains rabbit reticulocyte lysate, and proteins that are necessary to provide the appropriate environment for transcription and translation to take place in the reaction mixture. It contains all the amino acids but methionine, which can be supplied separately. The methionine employed here is radioactively labeled with $^{35}\text{S}$, thus the proteins produced are radioactive and able to be visualized by autoradiography. Recombinant proteins produced would be subjected to affinity pulldown with Ni$^{2+}$-NTA beads.

![Diagram](image)

Figure 24. Schematic of His-tag pulldown assay with *in vitro* co-translated proteins. A) Ajuba-6xHis plasmid (red circle) and RPA subunit plasmid (green circle) were added to the same *in vitro* TnT reaction with $^{35}\text{S}$-methionine. B) Ni$^{2+}$ beads are added to the co-translation reaction and pulls down RPA subunit that directly associate with His-tag Ajuba.
Each of the RPA subunits was cloned into pCMVTrnT vector (no tag) separately, and Ajuba was cloned into a mammalian CMV N-terminal His tag vector. 6xHis-Ajuba was co-translated with each of the RPA subunits in the same reaction, and then Ni$^{2+}$ beads were added to pulldown the proteins (Figure 24). If the two proteins interact directly, then we would expect the presence of both proteins in the autoradiography, detected in the pellet fraction.
AJUBA DIRECTLY INTERACTS WITH RPA70

We found strong signal of RPA70 pulled down with His tagged Ajuba (Figure 25A). We did not observe RPA32 (Figure 25A) or RPA14 pulled down with His-Ajuba (data not shown). This data suggests that Ajuba directly interacts with RPA70 \textit{in vitro}. In addition, quantitative analysis of the autoradiography showed that His-Ajuba can pulldown RPA70 with a signal at least four-fold higher than the background seen with RPA32. Ajuba does pull down a very low amount of RPA32, and I speculate that this can be largely due to unspecific interaction, but I do not exclude the possibility that Ajuba can directly interact with RPA32 though a minor interaction surface (Figure 25B). Therefore, I conclude that the association of Ajuba with the RPA complex is direct, and that the interaction occurs through RPA70.
Figure 25. Ajuba directly interacts with RPA70 in vitro. A) Autoradiography- His-tag pulldown of in vitro translated Ajuba-His with RPA70 or RPA32. B) Quantification of pulldown efficiency between RPA70 and RPA32 with Ajuba-His. Pulldown efficiency is calculated by normalizing pulldown signals with input and the number of methionine each mutant/protein contains.
This finding was intriguing because RPA70 is known to contain the site for the assembly of the ATR activating complex. Thus, I sought to dissect the interaction between the two proteins by mapping the regions that facilitate the contact. To do this, I made deletions in the Ajuba and RPA70 cDNAs to map the interaction domain.
AJUBA-RPA70 INTERACTION IS MEDIATED BY C-TERMINAL OB FOLDS

The direct interaction between Ajuba and RPA70 has prompted me to probe the regions on both proteins that are responsible for this direct contact. It has been shown the N-terminal OB fold (OB fold F) of RPA70 functions as a docking site for various DDR checkpoint proteins such as ATRIP, p53, and Rad9. OB fold F has not been shown to have significant contribution to the protein’s DNA binding affinity. On the other hand, the three OB folds located at the C-terminus are mainly responsible for DNA binding (OB fold A and B) and subunit interaction (OB fold C). Further, it was shown that proteins can interact with RPA70 through OB fold A and B, such as BLM (Bloom Syndrome helicase), and Rad51.

To identify the region on RPA70 mediating this interaction, we first constructed truncation mutants of RPA70, splitting the N-terminal OB fold (OB F) and three of the C-terminal OB folds (OB ABC) (Figure 26).
We began to study the binding relationship between FL His-Ajuba and RPA70 mutants by employing \textit{in vitro} translation system and His-tag pulldown assay. Figure 27 shows a strong interaction between FL His-Ajuba and OB ABC of RPA70. On the other hand, we did not observe a strong pulldown between OB F and Ajuba.
We generated additional truncation mutants of the C-terminal OB fold of RPA70 to further probe for the region mediating this protein-protein interaction (Figure 28). We deleted OB fold A from OB ABC to generate OB BC mutant, and
pulldown assay showed that the binding with FL His-Ajuba has reduced drastically (figure 28A) in contrast to the pulldown of OB ABC (figure 28A). This suggests that OB fold A is the main binding region with Ajuba and prompted me to further confirm the essential role of OB fold A in this interaction.
I constructed a mutant harboring half of OB fold A with OB folds B and C and assessed the binding with FL His-Ajuba. Although the intensity of OB ½ ABC being pulled down is quite low, Figure 28B shows OB ½ ABC can be pulled down by FL His-Ajuba with low efficiency. This suggests that a truncated form of OB fold A may contain a remaining point of contact with Ajuba, but that the main region for the binding resides in the N-terminal portion of OB fold A, which was truncated in this construct.

In order to test this proposition, and to ask whether OB A is sufficient for binding, I cloned OB F with the full OB A (OB F+A) to assess whether FL His-Ajuba would pull down this fusion protein. Surprisingly, the presence of OB fold A did
not increase the amount of mutant pulled down (figure 28C). This suggests that OB fold A by itself may not be sufficient to maintain interaction with Ajuba. It is possible that the folding of this particular fusion protein used is not presenting a proper configuration for binding. Therefore, the structure of OB folds ABC may be essential for direct contact between RPA70 and Ajuba, with OB fold A containing the actual interaction surface.

Figure 29. Pulldown efficiencies of RPA70 mutants by full length His-Ajuba. Pulldown efficiency is calculated by normalizing pulldown signals with input and the number of methionine each mutant/protein contains.

Quantitation of autoradiography revealed that OB ABC alone was sufficient to recapitulate the binding with Ajuba compared to FL RPA70 (figure 29). On the other hand, OB F has shown to have the least affinity towards FL Ajuba.
Truncation of half of OB fold A yielded a 30% decrease in pulldown efficiency and omitting the entire OB fold A (OB BC) resulted in a drastic 80% decrease of pulldown efficiency. This shows that OB fold A is mostly responsible for the contact between Ajuba and RPA70. However, the OB F+A mutant that contains the entire OB fold A did exhibit an increase of 15% in pulldown efficiency in contrast to OB F alone, although this increase was not found to be statistically significant. Overall, OB folds ABC are essential for the interaction between Ajuba and RPA70.
**LIM DOMAIN 1 OF AJUBA IS REQUIRED FOR EFFICIENT INTERACTION WITH RPA70**

I then sought to identify the regions on Ajuba responsible for the Ajuba-RPA70 interaction. I constructed truncation mutants of Ajuba and cloned into a mammalian CMV/NT-6xHis vector (Figure 30). Each of the mutants was co-translated with FL RPA70 and subjected to affinity pulldown assay. Our lab has previously shown that LIM protein TRIP6 directly interacts with another OB fold protein POT1 at telomeres through the C-terminal LIM domains of the molecule.  

![Figure 30. His-tagged Ajuba truncation mutants domains map.](image)

I speculate LIM protein Ajuba directly interacts with the OB fold protein RPA through its LIM domains. To test this hypothesis, I generated constructs containing
just the PreLIM domain, and, separately, another containing LIM domains 1,2,3. These truncation mutants were subjected to His-tag pulldown with FL RPA70.

Figure 31. LIM domains exhibit stronger pulldown with RPA70 than the PreLIM region. Autoradiography of His-tag pulldown with Ajuba mutants A) LIM domains and B) PreLIM region and FL RPA70. *All the samples from each figure is from the same experiment. Lanes were cropped to facilitate the presentation of data.
The autoradiography (figure 31A) showed that LIM domains (LIM-His) are able to pull down FL RPA70, although with weak efficiency. Visually, no significant amount of RPA70 being pulled down by the PreLIM region is seen (figure 31B). However, when comparing the amount of RPA70 and LIM domains proteins pulled down, we observed that a significant amount of LIM domains protein is required to pull down the low amount of RPA70 (figure 31A, 33). Complementing this data, quantification of PreLIM region pulldown efficiency of FL RPA70 resembles background levels (figure 33).

We considered the possibility that the interacting region of Ajuba with FL RPA70 was severed in the particular design used to address this question.
To address this, I constructed additional truncation mutants that harbor the PreLIM region coupled with one or two LIM domains. If the interacting region was abrogated in the previous mutants (LIM domains and PreLIM region), an increase of RPA70 in the pulldown would be expected. Indeed, figures 32A and B show that the additional LIM domains onto the PreLIM region did enhance the pulldown of FL RPA70 compared to PreLIM region or LIM domains alone (figure 33). This suggests the first LIM domain of Ajuba plays a crucial role in mediating direct contact with RPA70.

Figure 32. Addition of LIM domain 1 to the PreLIM region enhanced pulldown with RPA70 compared to PreLIM region alone. Autoradiography of His-tag pulldown with Ajuba mutants A) PreLIM+LIM1, B) PreLIM+LIM12. *All the samples from each figure is from the same experiment. Lanes were cropped to facilitate the presentation of data.
Figure 33. Pulldown efficiencies of FL RPA70 by Ajuba mutants. Pulldown efficiency is calculated by normalizing pulldown signals with input and the number of methionine each mutant/protein contains.

As seen in figure 33, quantitative results support the visual findings of the pulldowns. A 16% increase of pulldown efficiency compared between PreLIM+LIM1 mutant and LIM domains only was observed. The LIM domains displayed two-fold pulldown efficiency increase (20%) in contrast to PreLIM region. Interestingly, adding LIM domain 1 onto the PreLIM region significantly increased the pulldown efficiency from 10% (PreLIM only) to 36% (PreLIM – LIM1). Further, an additional LIM domain (PreLIM+LIM12) (38%) did not increase the pulldown efficiency of FL RPA70 further compared to adding the first LIM domain only (36%). This suggests that the presence of PreLIM region may facilitate the direct interaction with RPA70, perhaps through the maintenance of proper protein folding.
Collectively, these data entail the PreLIM region along with the LIM domain 1 are important for Ajuba-RPA70 contact. However, I was not able to narrow down a domain with similar efficiency of pull down than the full length of Ajuba, but these results do point to an important domain containing the first LIM domain of the protein. LIM domains 2 and 3 did not appear to be involved in the binding to RPA70.
CHAPTER SUMMARY/ DISCUSSION:

My data describes that the LIM domains of Ajuba are essential for the interaction with RPA70. In particular, addition of the first LIM domain (PreLIM-LIM1, 36%) to the PreLIM region increased the pulldown efficiency by three-fold compared to PreLIM region (10%) (figure 33). These finding is in agreement with previous publication that describes the LIM domains of TRIP6 mediate direct contact with the ssDNA binding OB folds of POT1. I speculate that the PreLIM region facilitates the direct interaction through contributing to proper protein folding of Ajuba. Another possibility is that the PreLIM region with LIM domain 1 mediates this direct contact with RPA70. Moreover, it is possible that additional folding issues prevents full activity of the PreLIM-LIM1 truncation mutant in its binding to RPA70, thus resulting in decrease of pulldown efficiency.

In addition, it is intriguing that Ajuba directly contacts the major ssDNA-binding domain of RPA70, OB fold A. Although we do not exclude OB fold B also contribute to the interaction, we conclude that OB fold A is the major interface for direct interaction with Ajuba. Overall, these findings uncover the domains that mediate direct interaction between RPA70 and Ajuba. I speculate that the two proteins interact directly in cells though the domains mapped here (figure 34).
Central region of Ajuba (including LIM domain 1) and OB fold A of RPA70 mediate the direct interaction between the two proteins \textit{in vitro}. Linear depiction of PreLIM region and LIM domains in Ajuba, OB folds in RPA subunits. Bracket and bold black arrow represent the regions responsible for direct interaction between Ajuba and RPA70. Numbers represent the amino acid positions of each domain. Arrows indicate the position truncated in mutants. Blue arrows depict interactions between RPA subunits OB folds. Circle with P on RPA32 indicates multiple phosphorylation sites on RPA32.

An extension of these studies would involve the test of these ideas by generating point mutations instead of truncations, in both the RPA70 and Ajuba cDNAs to pinpoint specific residues that mediate this interaction.
CHAPTER 6: DISCUSSION & CONCLUSION

Ajuba is pivotal for unperturbed cell cycle progression, since the depletion of Ajuba lead to cell cycle delay and induce DNA damage foci\(^{73}\) (Figure 10). During normal cell cycle, Ajuba is found to exhibit nuclear localization (HTC75 cells, Figure 14), and colocalize with the RPA complex in the nucleus of HTC75 and IMR90 cells (figures 11 & 12). I found the retention of Ajuba in the nucleus and colocalization events are enhanced by S phase synchronization by dT block and release, or by leptomycin B treatment (Figures 13, 14, 15, and table 3). Ajuba is also found at a subset of replication sites during S phase (PCNA) (Figure 16).

These data support the role that Ajuba is essential for S phase progression. It was shown that RPA complex colocalizes with replication centers during S phase\(^{103}\). I would speculate that Ajuba interacts with chromatin bound RPA, though not explicitly demonstrated here. It was published that the RPA complex exists in the nucleoplasmic fraction and chromatin fraction, and treatment with mild detergent (Triton-X 100) removes the nucleoplasmic portion of RPA, retaining the chromatin bound fraction\(^{103}\). In addition, extensive work has established the role of the RPA complex in replication sites\(^{21}\). Thus, I propose that Ajuba interacts with RPA to inhibit local DDR at replication forks, and possibly also at endogenous sites of DNA damage that spontaneously form during S phase.
Once Ajuba is depleted in the cell, it allows DDR factors to be recruited to the RPA-ssDNA at a subset of replication forks that triggered a global activation of ATR response. Whether Ajuba depletion activates ATM response has not been fully investigated, although no activation of Chk2 was ever observed (DL and SK, unpublished). I hypothesize that the subset of Ajuba associated replication sites at possibly difficult to replicate regions or common fragile sites. However, this notion has not yet been tested and remains speculative.

The reduction of Ajuba-RPA interaction upon replication stress (figures 17, and table 4) entails that the dissociation of Ajuba-RPA interaction is necessary for DDR. Both cell lines showed similar percentages of cells with nuclear Ajuba signals with and without HU treatment (table 4), suggesting Ajuba probably exert the same function in both cell lines to repress unwanted DDR. Therefore, the effects seen appear to be indicative of an important cellular pathway, and do not pertain to a tumor phenotype. The apparent lack of Ajuba signal in the nucleus during replication stress (HU) underscores the point established from previous works that Ajuba functions as a DDR antagonist (figure 18 & 19). The lower MW Ajuba band found in chromatin bound fraction in unperturbed cells most likely represent an isoform of Ajuba or a unphosphorylated species of the protein. The former proposition is supported by isoform 3 of Ajuba (NCBI), which displays
similar MW shown on the Western blot but contains a distinct C-terminus. Further confirmation is needed to ensure whether the lower MW band represent isoform 3. The direct interaction between Ajuba and RPA70 (figures 25, 27, 28, 29) complements the previous finding of direct contact between LIM protein and OB fold protein 67,68. Ajuba shares the interacting domain on RPA70 with proteins implicate in DNA repair such as Rad51 104. One model could be that Ajuba modulates RPA70 ssDNA-binding affinity and implicate in DNA repair pathways. It has been shown that reconstitution of RPA coated-ssDNA activates ATR response in Xenopus egg extracts 105, highlighting the importance of RPA-ssDNA structures in DDR. I hypothesize that in order to prevent the accumulation of RPA bound ssDNA at the replication fork, Ajuba may increase the “off” rate of RPA on ssDNA to maintain a minimal amount of RPA at the replication site to evade unnecessary ATR activation.

On the other hand, the central region of Ajuba was shown to mediate this contact (figures 31, 32, 33). This region was previously uncharacterized and did not fit my initial hypothesis of LIM domains being the mediator to directly contact OB fold proteins. The significance of this region in preventing DDR remains to be elucidated. These findings contribute to the role of LIM proteins in maintenance of genome integrity and regulation of DDR.
The data presented here have added important information to our previous model of the function of Ajuba in ATR damage response pathway depicted in figure 35.

Figure 35. Proposed model of Ajuba in the ATR pathway. A) General depiction of the ATR pathway activation. B) During unperturbed cell cycle, Ajuba directly interacts with RPA70 to prevent unscheduled ATR activation. Question marks represent unknown mechanisms as to whether this interaction inhibits the phosphorylation of ATR-ATRIP, or RPA, or the recruitment of other DDR proteins.

I would propose that Ajuba exerts its function at the initiating stage of the ATR pathway, when RPA-ssDNA is present in the cell (figure 35A). Specifically, Ajuba directly binds to RPA70 (figure 35B). This interaction may be inhibiting the phosphorylation of RPA32 or ATR-ATRIP, or it may prevent the recruitment of DDR factors, or dissociate the RPA complex from ssDNA. Further, my findings
argue this direct interaction takes place during S phase of the cell cycle, to prevent unscheduled local ATR response at the replication fork. This leaves an important question unanswered: how does the response get back to the uninduced state after activation? One model would include RPA32 phosphorylation as the primary event in this context: spontaneous de-phosphorylation after repair would allow for the re-association of Ajuba with RPA, allowing for the resumption of ATR inhibition and termination of the signaling. Ajuba could therefore be important in two aspects of the pathway: initial induction at the local sites of damage, as well as resumption of the uninduced state during S phase. In summary, the direct association between LIM protein Ajuba and RPA70 is critical for cell cycle progression and genome stability.
Figure 36. The most frequent Ajuba alterations across different types of cancer. Ajuba expression is amplified in approximately 17% of neuroendocrine prostate cancer (NEPC, 1st column) and around 6% of all prostate cancers that were examined. Ajuba is frequently mutated in Head and Neck Small Cell Carcinoma (HNSCC), 6.3% and 5.7% respectively (2nd and 3rd column). Each color bar indicates distinct types of alteration of Ajuba. Cancer type: light blue (neuroendocrine prostate cancer), burgundy (Head and Neck Small Cell Carcinoma), purple (pancreatic cancer). *Image adapted from cBioportal.
Figure 36 shows the most common alterations that were characterized in different cancer types. Importantly, Ajuba expression is found to be amplified in neuroendocrine prostate cancer. Specifically, around 17% of exhibited high Ajuba expression. Further, Ajuba is upregulated in pancreatic cancer, ovarian cancer and lung adenocarcinomas. Thus, in addition to my findings presented here, Ajuba may contribute to cancer cell proliferation and tumorigenesis through repression of ATR signaling pathway.

On the other hand, Ajuba is found to be frequently mutated in Head and Neck Small Cell Carcinoma (HNSCC). As seen in Figure 37, Ajuba is often altered in the mid-region that includes LIM domain 1. In addition, most of the mutations identified are either missense or truncations.

Figure 37. Mapping of Ajuba mutations found in various cancers. Each post color resembles the type of mutation found at that specific site as indicated below the domain map. *Image adapted from cBioprotal.
This suggests that Ajuba mutations can promote cancer in certain cell types. Based on the findings in this thesis, Ajuba truncation can lead to defects in DNA replication or alterations in RPA metabolism in the cell which can cause genome instability. Thus, overexpression and mutation of Ajuba can contribute to tumorigenesis in specific cell types.

Collectively, the findings of this project contribute to the understanding of the “double lives” of Ajuba in the cytoplasm and nucleus. In other words, cytoplasmic Ajuba functions to associate with focal adhesion sites and implicates in cell migration, while nuclear Ajuba is involved in the repression of DNA damage response during S phase. Further, the shuttling of Ajuba out of the nucleus is speculated to aid efficient ATR activation. It is possible that Ajuba can exert distinct functions by using different regions (i.e. PreLIM, LIM domains) to mediate protein interactions. However, functional separation of Ajuba’s protein interaction domains remains to be performed to substantiate such claim.
CHAPTER 7: FUTURE DIRECTION

A major point that was not addressed in this project was whether Ajuba interacts with RPA bound to ssDNA. The investigation was hampered by difficulty in protein purification using the *E. coli* system, thus not being able to quantitate and control the amount of proteins that will be employed in electro-mobility shift assay. Thus, it will be important to assess whether Ajuba can influence the binding affinity of RPA for the DNA. This remains to be one of the on-going works in the lab to directly investigate the relationship between Ajuba and RPA complex on ssDNA.

The phosphorylation status of RPA32 has been shown to modulate RPA complex recruitment and function\(^{25,27,48}\). Although not explored here, I hypothesize that Ajuba may play a role in preventing DDR induced phosphorylation of RPA32. During replication stress, DDR induced phosphorylation of RPA32 can potentially decrease the complex affinity for Ajuba, and thus leading to full activation of ATR. On the other hand, Ajuba may function to dampen local ATR response after the damage has been repaired through interacting with RPA70. Further investigation is needed to confirm if DDR induced phosphorylated species of RPA complex exhibit lower affinity for Ajuba and if Ajuba is only present at newly repaired damage sites.
Additionally, the RPA70 interacting interface on Ajuba does not exactly coincide with the POT1 interacting domains identified on TRIP6; although LIM domains is the major contributor to the direct interaction for Ajuba-RPA70, and TRIP6-POT1. A well-defined region of interaction on Ajuba could not be identified through truncation mutants. The interacting domains mapped here provide the framework for generating point mutants within these regions, which will provide more precise information about the interaction between Ajuba and RPA70.

Additional experiments showed that Ajuba does not directly interact with POT1 (data not shown). I suspect that this is due to the difference in tertiary structure of the ssDNA-binding OB folds on POT1 and RPA70, which also mediate direct interaction with LIM proteins. POT1 N-terminal OB folds exhibit a “kink” binding to ssDNA, while RPA70 OB folds A and B display as a linear groove. This difference may contribute to distinct interacting partners.

It was shown that TRIP6, LPP, but not Zyxin were found localized at telomeres, this entails a differential local recruitment of LIM proteins to sub-nuclear regions. However, the possibility that TRIP6 can interact with RPA is not excluded here due to the transient presence of RPA at the telomeres during S phase.

Collectively, I propose that TRIP6 functions at the telomeres and Ajuba functions throughout other genomic regions to prevent unwanted DDR, potentially displaying the same paradigm in their associations and functions. Further
examination will be needed to confirm the two LIM proteins’ roles at sub-genomic regions.
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