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Publication Date
2021-08-23

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ROLE OF PIR-1 PROTEIN IN GENE REGULATION

By

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A capstone project submitted for
Graduation with University Honors

April 20, 2021

University Honors
University of California, Riverside

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ABSTRACT

Non-coding small RNA is untranslated transcripts of RNA molecules that is transcribed from DNA and base-pairs to target mRNA. They regulate gene expression, which not only controls biological functions, but mis-regulation of these sRNAs have been contributed to diseases such as Cancer and Alzheimer's. To study gene expression and defenses against viral infections, short interfering RNAs (siRNAs) will be studied through C. Elegans, since C. Elegans grow similar to mammalian model systems, and many of its functions and genetic makeup is similar to that of humans. To learn about RNAi, it is essential to learn about PIR-1, a protein that interacts with Dicer, (an enzyme that cleaves dsRNA into ssRNA fragments), and studying PIR-1 can contribute in discovering ways to fight virus infection. The approach is to tag C. Elegans employing CRISPR Cas-9 and generate strains that express tagged PIR-1 via microinjection. After extracting PIR-1 protein through generations of C. Elegans, perform co-immunoprecipitation (co-IP), to look into protein-protein interactions and find how PIR-1 interacts with other proteins affecting RNA interference. Since the research could not be performed in the lab due to COVID-19 shutdowns, this is only an introduction, which dives into the background and importance of this topic.

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ACKNOWLEDGEMENTS

I would first like to thank my faculty mentor, Dr. Weifeng Gu, for his tremendous support and longstanding guidance throughout my capstone project and undergraduate career. This was a very complex subject for someone in my shoes and without his help, I would not be in this position. He was also extremely helpful to me as a mentor in aiding in my numerous graduate school applications and letters of recommendations, and I am very thankful for that support. I would also like to thank graduate student, Myung Eun Oh, for spending all those hours with me to teach me about this topic and work with me to improve my skills and techniques in the research lab, and without her help, I would not have the adequate understanding to complete this introductory, yet complex project.

I would also like to thank my family and friends for their endless support, especially during times where I felt overwhelmed with my academic projects and thought that I could not be in this position, turning in this massive capstone project. I am confident that without the help and support of these people, I would not be where I am today.

INTRODUCTION

In humans, only 1.2% or about 20,000 genes encode for a protein, while the other 98.8% is the noncoding portion which contains components that will not be used to encode proteins, but will be synthesized into functional RNA molecules such as tRNA, mRNA, and etc. The non-coding genome was overlooked previously since it had no genetic script input, but recent research suggests that non-coding RNA makes up much more of the genome than the coding counterpart. The non-coding RNA is believed to play a major role in genome stability, disease formation, and anti-viral defense. The main focus of this research is PIR-1, an RNA polyphosphatase that removes the beta and gamma phosphates from a 5' prime RNA transcript. This particular polyphosphatase can only remove phosphate groups from a (di- or tri-) phosphorylated RNA molecule and not from (mono-) phosphorylated RNA transcripts. (Deshpande et al., 1999) The major reason for choosing this particular protein for research is due to the previous research known as co-immunoprecipitation, a popular technique to identify protein-protein interactions by employing antibodies, have showed that PIR-1-Dicer interactions, could take part in RNA interference. RNA interference is a gene silencing process in which double stranded RNA is cleaved by Dicer into small interference RNAs and it's a eukaryotic gene-regulatory pathway that silences the expression of specific genes in response to homologous small double-stranded RNAs.

RNA interference (RNAi) was revealed by Andrew Fire and Craig C. Mello in 1998, (for which they won the Nobel Prize in Physiology or Medicine in 2006), as a process of gene silencing and this is essential since it is in charge of gene regulation and antiviral defenses in eukaryotes. Fire and Mello found out that double stranded RNA was more effective in making interreference than single stranded RNAs, and they also realized that only some molecules of

dsRNA was required to produce an amplified result in interference with endogenous mRNA. (Fire et al. 1998) Since the discovery of RNAi and its regulatory potential discoveries, it is essential to understand that RNAi plays an important role in the elimination of target genes. It was later found out that there are two types of small silencing RNAs called miRNAs and siRNAs. Micro-RNAs or miRNAs are derived from endogenous genes transcribed by RNA Polymerase II as independent units (miRNA genes) or from introns in pre-mRNAs of protein-coding genes. On the other hand, small interfering RNAs or siRNAs are derived from exogenous long double-stranded RNAs, such as RNA viruses, or endogenous "aberrant" transcripts from repeated DNA and transposons. (Duchaine et al. 2006) This shows that inhibition of translation is possible through pretranscription silencing mechanism of RNA interference, where a certain enzyme complex catalyzes DNA methylation at complementary genomic positions to siRNA or miRNA. This also shows how RNAi plays a vital role in defense against not only viruses, transposons and other parasitic nucleotide sequences, but it also has a role in development. The dsRNA interacts with argonaute proteins (AGO), which are proteins that associate with and also function in the production of non-coding RNAs known as Piwi-Interacting RNAs, to make a silencing complex by the name of RISC (RNA induced silencing complex) and it also mediates TGS (Transcriptional Gene Silencing) and PTGS (Post-Transcriptional Gene Silencing). PIR-1 also regulates RNAs and plays vital roles in silencing and modifying RNAs. (Duchaine et al. 2006)

There are two types of RNA interference processes: exogenous RNAi pathway, where it is involved in the silencing of external and foreign genes, and endogenous RNAi pathway, where RNAi is involved in self-gene regulation and transposon silencing. This relates to RNAi since RNA induced silencing complex (RISC) controls RNAi, which is initiated by short dsRNA

molecules in a cell's cytoplasm, where they interact with the catalytic RISC argonaute. (Bernstein et al., 2001) Post-transcriptional gene silencing by miRNAs within RISC can occur in two methods: one, where miRNAs can inhibit translation of target RNAs and two, where miRNAs can induce de-adenylation and mRNA degradation, where removal of poly(A) tail leads to mRNA degradation by endonucleases. RISC is formed by a RNAi process, where a nuclease called Dicer cleaves double stranded RNAs (dsRNAs) into 20-30nt siRNAs, which can form RISC by interacting with numerous argonaute proteins. It is important to note that some miRNAs that hybridize perfectly can cleave and degrade their target mRNA, while some siRNAs that hybridize imperfectly have been shown to inhibit translation. (Duchaine et al. 2006) Also, during the RNA strand selection by RISC, the strand that enters the RISC complex is usually the one whose 5' end of less tightly paired and an unknown protein/enzyme within RISC samples the ends of the duplex. It is also not entirely clear yet how the guide strand is selected by RISC.

There are two notable siRNA mediated gene silencing processes at the transcriptional level. One involves the siRNA directed methylation of DNA (Cytosines within two types of sequences: CpG and non CpG). (Munshi et al., 2009) In plants and some human cells, certain siRNAs can induce sequence-specific promotor methylation and inhibit gene transcription, and localized silencing is recognized by siRNAs like promotor DNA at specific sequences, although it is essential to note that these mechanisms are not fully understood and are still poorly characterized. Another process involves siRNA directed methylation of histones and heterochromatin formation. In animals, yeast, and plants, siRNAs derived from transcripts of repeated DNA in centromeric regions are important to maintain heterochromatin and not to mention, are also used to inhibit transcription within those regions. In this process, the silencing is spread over humungous chromatin domains and a multiprotein complex very similar to RISC

is needed, called RITS, RNA induced transcriptional gene silencing. (Li et al., 2009) Gene silencing is a modification of gene expression which is achieved by changing chromatin structure, DNA methylation, and destabilization of mRNA. The purpose of this project is, even though there is a relationship between Dicer, PIR-1, and RNAi, there is no identified mechanism or pathway, and that is the end goal, to find some interactive components between these factors. This is important since, if a pathway is found to control any non-coding region of DNA or RNA, diseases such as Cancer, diabetes, and also viral infections can be fought in new less harmful methods.

As mentioned before, the C. Elegans PIR-1 protein was previously identified as a binding component of the RNAi factor, Dicer, which not only encodes proteins with multiple functions, but it also binds and processes double stranded RNA substrates into siRNAs or miRNAs that eventually aid argonaute cofactors in their genetic silencing. It is also essential to note, that lab research still has not found the correct complete pathway of how and why Dicer associates with protein like PIR-1, since for example, Dicer is not responsive to the 5' phosphorylation status of its substrates. In this specific research involving C. Elegans, Dicer is involved in numerous small pathways such as the miRNA pathway and both exo-RNAi and endo-RNAi pathways which are triggered by double stranded RNAs. In this pathway, Dicer processes double stranded RNAs into short 23-nucleotide duplex siRNAs that has 3-2nt overhangs and 5' ends, both monophosphorylated. Then these pieced siRNAs are processed into other components such as the Argonaute RDE-1, which also has to recruit RdRP, an RNA dependent RNA Polymerase, to amplify a silencing signal to carry on the full process. (Duchaine et al. 2006) Other than this exogenous RNAi pathway, Dicer also plays a role in small endogenous RNA pathways, but just

like in previous cases, how certain proteins are processed by Dicer and how they acquire their final mono-phosphorylated state are still mostly unknown.

Previous Immunoprecipitation with protein Dicer suggested that PIR-1, a RNA polyphosphatase, interacted with it in some manner, but the exact mechanisms were not solved. However, current research from Dr. Gu's lab shows that the C. Elegans PIR-1 is indeed involved in the silencing of a certain Orsay virus, which is RNAi mediated. His research group demonstrated that a catalytically inactive PIR-1 strain has similar growth defects as the null mutants, which means that PIR-1 does in fact act as an RNA phosphatase to regulate the 5' ends of the tri-phosphorylated ppp-RNAs. (Chaves et al., 2020) This result was also previously predicted by their in vitro observation that the recombinant wild type PIR-1 retains the functions of an RNA polyphosphatase while the catalytically dead PIR-1 could only bind to tri-phosphorylated ppp-RNAs, and not dephosphorylate it. It is also notable that their PIR-1 immunoprecipitation analysis showed that PIR-1 not only interacted with Dicer, but also with other proteins of the ERI complex throughout its development. (Claycomb et al., 2009) In conclusion, these findings suggest that PIR-1 is a conserved main regulator of tri-phosphorylated ppp-RNAs and it also has an essential function in silencing viral ppp-RNAs and also modifying other tri-phosphorylated ppp-RNAs, which can be used in further research for gene regulation and antiviral studies.

Since this field of research is relatively new, previous research suggests that PIR-1 most likely belongs to a class of Dual Specificity Protein Phosphatases, but many other studies have showed that C. Elegans PIR-1 homologs human PIR-1 and Baculovirus RNA triphosphatase has a normal polyphosphatase activity where the beta and gamma phosphates are removed from tri-phosphorylated ppp-RNAs. It is also essential to note that C. Elegans PIR-1 has significant

similarities with the triphosphatase domain of the mRNA capping enzymes, which, unlike the previous example, only removes the the gamma phosphate from the tri-phosphorylated ppp-RNAs, which means that PIR-1 could either belong to the tri-phosphorylated ppp-RNAs group or the novel polyphosphatase group. In order to test further antiviral roles of PIR-1, researchers could introduce viruses, such as the previously mentioned Orsay virus, and if the PIR-1 null mutant promotes the Orsay virus replication in contract to the wild type, there is little evidence to support that PIR-1 can indeed play a role in the antiviral RNAi response. (Chaves et al., 2020)

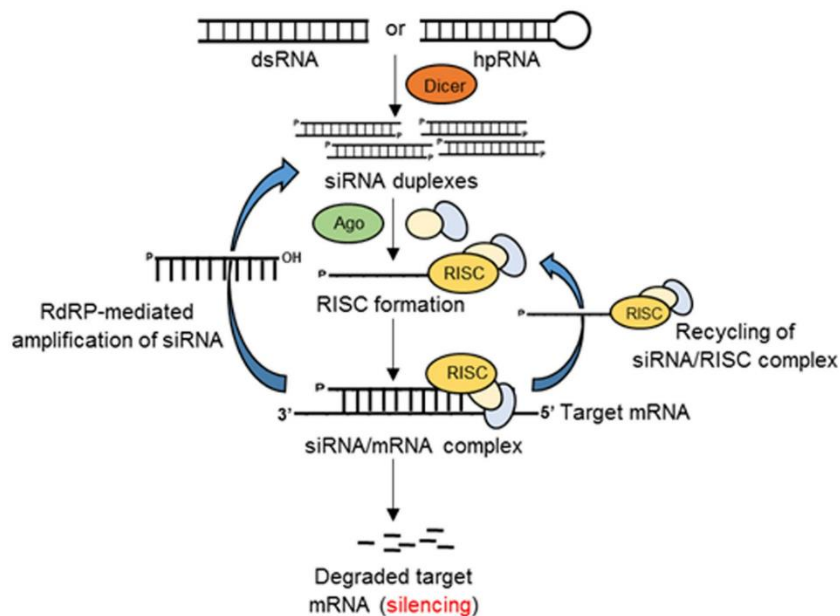

Figure 1. **Pathway of RNAi mediated gene silencing in eukaryotic cells.**

Dicer generates small siRNAs through double stranded RNAs or hairpin RNAs. The RNA guide strand binds with proteins such as Argonaute (Ago) to form RNA-induced silencing complex (RISC). (Majumdar et al. 2017)

IMPORTANCE

The relatively new research on RNA interference is important since RNAi can not only be used to control gene expression, but it is also a potential therapeutic agent for viral and genetic diseases, cancer, and other disorders. However, since it is only a relatively recent discovery in the scientific community, there has to be more understanding regarding its side effects, toxicity, and safe delivery methods before it can be implemented as an accepted drug. RNAi is not only used on animals, but it was also previously used to fight resistance against parasites in crops and other agriculture production.

One of the current leads in RNA interference research shows how RNAi mechanisms play important roles not only in innate antiviral immune responses, but also in the daily regulation of gene expression in cells. In both possible scenarios of invasion by exogenous viruses and mobile endogenous transposons, RNAi is an innate natural defense mechanism, which now enables the organism to fight against infection and also preserve genetic stability. (Ebbesen et al., 2008) Even though in many scenarios, research has shown how arduous it is to introduce short double stranded RNAs into the cells, if the DNA molecules are designed to finally turn into RNA hairpins, these can then be processed to form siRNAs by the nuclease, Dicer. Even though RNAi is a possible means of treatment for diseases such as HIV, the main difficulties that hinder the progress of application is the uncertainty of potential toxicity. For example, in the treatment against hepatitis C virus, HCV, small inhibitory RNAs targeting the internal ribosome site (IRES) and other proteins involved in encoding mRNAs, were shown to inhibit HCV replicon function in cells, and also "cure" cells that replicated HCV replicons (Wilson et al., 2003). However, the main challenge is the delivery of the siRNAs, since in most

of the studies, a hydrodynamic intravenous injection was used, but this method cannot be used in treating human Hepatitis.

RNAi treatment can also be used in the treatment against cancer, since the concept of gene knockout can be used on a protein without harming the cell, and not to mention, chemotherapy destroys tumors by killing both healthy and cancerous cells simultaneously. This relatively new method of treating cancer is far better, since there is not only a lower cost, but also a high target specificity and multiple gene targets that can be silenced without hurting the normal healthy cells. In the study regarding the efficiency of RNAi treatment, siRNAs were targeted against the colon cancer associated gene, beta-catenin, and the results showed decreased invasiveness and proliferation. Also, when the treated cancer cells were transferred to a live mouse, there was an association of a longer survival rate, compared to mice that received unoperated tumors. In another example, silencing of an oncogene (H-ras) led to the tumor growth of human ovarian cancer in a mouse being inhibited, but with the other diseases, the most prevalent problem is related to optimal target delivery and reducing toxicity. (Ebbesen et al., 2008) A great reason to employ RNAi to fight cancer is that it can also be implemented to silence certain pathways that assist in the progress of conventional cancer drugs, such as in the targeting for MDR1, multidrug resistance gene, for not only chemotherapy re-sensitization, but also to silence certain enzymes that repair double stand breaks, so that chemotherapy can proceed without any adverse problems. Even though the clinical studies are relatively new, in the last 4 years, there has been significant progress in different delivery systems and also the progression of RNA interference therapy from pre-clinical to human trials. (Brake et al., 2008) Even though RNAi based cancer treatment is relatively better than chemotherapy, it still has many challenges that it has to overcome before it can be 100 percent successful. One of the

problems is that certain factors such as the heterogeneity status of the tumor, numerous signaling pathways, and other complex feedback loops challenge the gene targeting process in most treatment studies, and not to mention, just as in other treatments, drug resistance is still an issue. Since there is a wide-spread movement to an individual patient-focused treatment plan in the upcoming years, RNAi based therapies will also be based on an individual basis in the future and this plan will consist of a RNAi based triple vaccine that should not only reduce the risk of cancer, but it also focuses on disrupting certain signal pathways. (Rao et al. 2013)

Another disease RNAi technology may be able to control is Alzheimer's Disease, since there is no current effective method to halt its progression, and also, the key factors of the disease is based on nonfunctioning proteins and genetic mutations. Alzheimer's is mainly characterized of a decline of memory, cognition, and negative changes in personality, in a progressive manner, and the main hypothesized causes are extracellular beta amyloid plaques and intracellular neurofibrillary tangles that occur in individuals after age 65. (Mansoori et al. 2014) One of the main hallmarks of this disease is the deposit of the insoluble combined form of Aa, which is a 40-42 amino acid peptide that is processed from a transmembrane protein called APP, that is found to be deposited in the walls of cerebral blood vessels of individuals with Alzheimer's. (Glenner et al. 1989) This Aa peptide can be produced in numerous locations such as the endoplasmic reticulum, Golgi network, and the endosomal system, and another key component of this study is BACE, an APP cleaving enzyme, which is synthesized in the ER and becomes mature on its way to the cell surface. In this RNAi study, it was found that when the recycling of APP was mediated, this resulted in the aggregation of endocytosed APP and also a decline in APP preparation. (Choy et al. 2012)

METHODS

For this type of research, C. elegans is the best choice for a host in order to extract purified PIR-1, since even though Caenorhabditis elegans is a round worm, they have similar tissues to humans, more importantly, their PIR-1 shows high conservation with human PIR-1 (hPIR-1). In a study done in 2006, researchers found that the previously mentioned argonaute proteins (AGO), have various roles in C. elegans, which also supports a mechanism that involves not only had different structural components, but also functionally distinct AGOs that act in a sequence wise fashion during initiation and effector steps of interference RNA. (Yigit et al. 2006) Since C. Elegans lack commercially available antibodies, which are essential for RNAi processes, the only solution is to first tag their PIR-1 protein. Microinjection is used to tag the worms' PIR-1 protein with a roller gene, a dominant phenotype, and this overall process of CRISPR only works if the germ cells are hit and if it hits tissue cells, the roller mutation will not be passed onto the future generations. The goal of microinjection is to tag the worms' PIR-1 with FLAG-degron, and if the procedure is successful, the worm will show two possible ways: 1) The roller phenotype will be visible under a microscope; 2) In terms if genotype, homozygous worms will be responsive to auxin plates, which would induce protein degredation in C., which was a technique researched in 2015 where researchers looked at plants' responsive to auxin and then modified C. Elegans to be responsive to auxin so that they can shut down a gene at any spot. (Zhang et al. 2015) To modify the germ line after finding homozygous "mother" worms, we can perform loads of genotyping to grow tagged strains, extract, and finally purify tagged PIR-1 protein and test via Western Blot. Western Blot is a method to detect certain protein molecules from a mixture of proteins, and in this case, this method can allow to pick a purified PIR-1 from a vast array of others from the worm. After waves of genotyping and testing PIR-1 for purity, co-

immunoprecipitation can be performed to look into protein-protein interaction. At this point, the experiment will include more genetic components such as insertions, deletions, and silencing to find out the interaction between PIR-1 and RNAi's roles in certain viral infections.

In order to purify the PIR-1 protein, the coding sequence of the C. Elegans PIR-1 gene should be integrated into a vector, pET 28a, between two restriction sites. In Dr. Gu's preliminary experiment, pET 28a was integrated between NdeI and BamHI restriction sites, where restriction enzymes will connect with the matching DNA and cleave the DNA, which causes a break in both strands of the DNA molecule. To then express the PIR-1 protein, the colony was grown in 5ml Broth (TB) medium, which contained 50μg/ml Kanamycin and 20μg/ml Chloramphenicol. (Chaves et al., 2020) This culture was then inoculated and allowed to grow at room temperature until it reached a OD600 of 0.5, which indicates the optical density for a certain sample measured at a wavelength of 600nm, and this is a great way to study how a culture a growing based on its growth curve. It is also essential to mention that 600nm was chosen for bacteria since it is not harmful to the culture, unlike other UV wavelengths. The protein expression was then activated using a very small concentration of IPTG (Isopropyl-β-D-Thiogalactopyranoside), usually 0.5mM. IPTG is a biological molecule that mimics allolactose, which is a lactose metabolite that induces transcription of the genes in the lac operon, and this also induces protein expression. Since IPTG is not an innate part of any metabolic pathways, it will not be broken down and used by the body, unlike lactose, and this makes sure that it never gets depleted, making it a more useful inducer than lactose. IPTG also functions as an inducer of the enzyme galactosidase, by inhibiting the repressor and allowing protein expression to occur. After the protein expression was activated by IPTG, the cells were pelleted and suspended in lysis buffer containing ingredients with usual concentrations such as 50mM Tris-HCl (pH 7.5),

0.5 M NaCl, 5mM 2-mercaptoethanol, 5% glycerol, 10 mM imidazole, 0.01% NP40, and 1 mM PMSF. Then the cells were broken down through sonification and centrifugation. After more waves of centrifugation and washes with lysis buffer, the protein is then eluted using imidazole buffer. The column used would be proportional to the size of the protein so that only that certain protein will be eluted and the specific activity will be high. (Chaves et al., 2020)

After getting purified protein, Western Blotting technique was used to test the PIR-1 protein, since there is no commercially available antibody to detect them. Western blotting is used to identify and separate proteins based on molecular weight, and this is performed though gel electrophoresis, where DNA fragments are separated based on their size and an electric current will pull the fragments from the negative anode to the positive cathode. Then more strains would be created that expressed tagged PIR-1 interactors, such as other interacting proteins, and the proteins would then be extracted, purified, and tested. If the proteins are not fully pure, these steps would be performed again, but if they are indeed pure, the next step would be co-immunoprecipitation (co-IP) to look into certain protein-protein interactions. Since co-immunoprecipitation uses a technique of precipitating a protein antigen out of solution, this can be used to concentrate a protein of interest from a crowded sample with thousands of other unwanted proteins.

Since the labs closed down due to COVID-19 during the start of this experiment, research was halted where the C. Elegans was being grown in different cultures, in an attempt to find the worms with the best phenotype and gene of interest. When the labs re-open, the experiment will continue, where proteins will be extracted from roller-gene worms and then purified to test protein-protein interactions, and this will give more information on how PIR-1 plays a role in gene regulation.

Figure 2. **PIR-1 is an RNA Polyphosphatase**.

Taken from Dr. Gu's research involving PIR-1; shows techniques used. **A**. Shows that the active site alignments for PIR-1 orthologs from C. Elegans, Drosophila, and Human, and it shows identical residues. **C**. Single stranded ppp-RNAs were annealed with comp. DNA or RNA, purified, and then digested with Terminator. **E**. Western Blot Analysis to verify that C150S and not WT PIR-1 binds with single stranded ppp-RNAsl shows Histidine-tagged PIR-1. (Chaves et al., 2020)

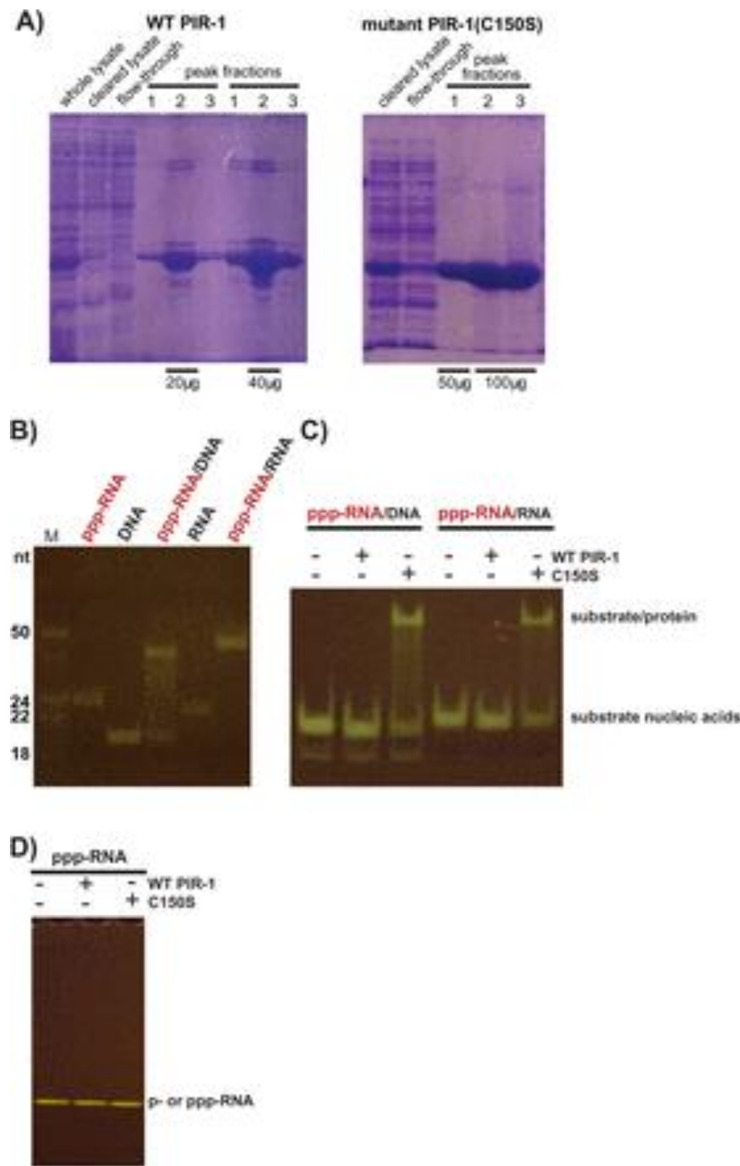

Figure 3. PIR-1 binds ppp-RNAs in double stranded structures.

Taken from Dr. Gu's research involving PIR-1; shows techniques used. **A**. Shows flow-throughs and elution factors during the purification steps, which used SDS-PAGE and Coomassie Blue staining. **B and C**. Shows how different types of PIR-1 were incubated with ds nucleic acids and then resolved on a native 12 % PAGE gel. (Chaves et al., 2020)

DISCUSSION AND PREDICTION

All of the information presented regarding RNAi demonstrates that small RNAs can have directed effects against chromatin and protein expression, and this also shows that the silencing of the genome via RNAi is essential and diverse in its effects to fight against numerous diseases such as Cancer, HIV, and Alzheimer's disease. In such a short time, from 1998 to present, the discovery of RNAi has been proved to be an essential tool for the research of genetic function in organisms, both plants and animals. For example, it is important to mention that RNAi genomic editing technology was used to silence the gene responsible for making a neurotoxin called β-oxalylaminoalanine-L-alanine (BOAA), in leafy vegetables called *Lathyrus sativus*, and this was a major advancement in research and agriculture since this positively affected the people in poor nations that did not have a means to fight this neurotoxin in other ways. RNAi was also employed in Southeast Asia and India, to produce bananas resistant to Banana Bract Mosaic Virus (BBrMV), which also has helped a massive population to survive not only in terms of agriculturally but also economically. (Rodoni et al. 1999) Even though the entire research involving RNAi is still relatively new and most of these pathways is not fully understood, technologies such as RNAi and CRISPR Cas9 will be the future of plant and animal therapeutics, since this can have most of the promising positive results, without many of the adverse side effects. Some of the potential concerns against RNAi is based on its safety, since there can be instances where both the target gene and a similar sequence is repressed, causing "off-target" effects, and this is estimated to have an error rate of 10%. Even with numerous clinical trials involving RNAi, detrimental issues such as toxicity, off target effects, and unknown delivery pathways, have to be overcome before RNAi can be implemented into society as a successful therapeutic option. When focusing on PIR-1, just as previous research demonstrate that it is an

essential RNA phosphatase that plays a vital role in regulating triphosphorylated ppp-RNAs, future research will show how important it is in becoming a target in viral studies.

In this research involving PIR-1, CRISPR Cas9 will be utilized to tag the PIR-1 protein, but in general, there are many similarities and differences between CRISPR and RNAi technologies even though they are both used to silence a target gene. RNAi was first discovered when gene expression was suppressed in plants, and at the time, the entire mechanism was more unknown than it is currently. When Andrew Fire and Craig Mello researched RNAi using C. Elegans, they showed that RNAi used single stranded RNA to silence a target gene, instead of double stranded RNA, but it was also discovered that this occurred in a sequence specific manner. Unlike RNAi, the discoveries of CRISPR were shown through bacterial immunity, where microbes used a nuclease controlled by a guide RNA to cleave viral DNA sequences in an infection scenario, and this cleaved fragment was then stored in the organism's genetic memory, just like antigens are stored in preparation for future pathogen attacks. This mechanism to cleave and edit target genes in a programmed manner was used in 2014, where CRISPR was first used on human cells to inactivate certain genes, and in 2019, it was first experimentally used on a real patient to cure a woman with sickle cell disease. In 2018, CRISPR was even used to make two babies immune to HIV, where He Jiankui, a Chinese doctor and researcher used this technology to delete a gene called CCR5. CCRS is a gene needed by HIV to enter human blood cells, and by deleting this from these babies, they are now not only immune to HIV, but in experimental studies, removal of this gene also supposedly improves the recovery of a human brain after a severe stroke. Even thought the initial goal was accomplished, this act was obviously controversial since this editing can have other side effects on these children, which only time will tell. In the CRISPR mechanism, a guide RNA leads a Cas nuclease to cleave a target DNA

sequence and there is another mechanism where a homology directed pathway is used to knock out target sequences. The main comparison between CRISPR and RNAi is that CRISPR is a knockout, where it permanently silences a target gene in a comprehensive manner, while RNAi is more of a known down, where the target gene's expression is decreased at the mRNA level. The controversy of CRISPR surrounds around the fact that this is a very new innovation in the genomics world, where the effects of a full gene knockout if not fully understood, and this knockout could have adverse irreversible effects on the organism in the future. However, if a target gene is knocked down via RNAi, there is an impermanent, reversible factor, that can somewhat restore protein expression after a variable amount of time, which makes this more usable than CRISPR. One limitation of RNAi silencing is that the targeting system affects more than just the target gene, where unintended RNA target genes are silenced, which has a potential to cause more harm than good. (Cong et al. 2013) If there was more research and funding provided into CRISPR, it would be better option for genetic therapy and disease treatment in the long run, since target gene knockouts are more effective in fully intercepting protein expression and it also would be essential in eliminating fully understood viral pathways of diseases such as HIV.

Numerous future predictions can be made with PIR-1 based on the minimal knowledge available. After purifying the target protein, performing western blots, and co-immunoprecipitation (co-IP) to look into certain protein-protein interactions, PIR-1 can be susceptible to 5' to 3' exonucleases, since it can remove both beta and gamma phosphates of triphosphorylated ppp-RNAs. PIR-1, especially human PIR-1, can probably inhibit or down regulate cell growth, since it is similar to other proteins that have similar functions, but the hardest part will be pinpointing its effects. It is also important to note that PIR-1 will also have

interactions with Dicer, but the numerous pathways will have to be investigated using mutant forms of these proteins, where there has to be trials involving PIR-1 and ones without, so the recruitment of other proteins can be studied in a detailed manner. In conclusion, it is known that PIR-1 is an RNA polyphosphatase that is involved in RNAi mediated antiviral pathways, but its specific effects have to be studied with regulation RNAs, factors such as temperature and transcription factors, and how it is independent or dependent with other involved factors, and based on these results, PIR-1 will be an essential target of research in antiviral and cancer studies.

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