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Letter from the Editors

The Journal of Undergraduate Research was founded in the fall of 2002 with the mission of serving as a forum for original student research. Between those pages have been every possible academic discipline our university has to offer, yet reflecting on how the face of JUR has changed over the past 20 years, one feature has remained consistent: the quality and depth of the works published.

Fundamentally, conducting research requires embarking on a quest for answers. How can we improve existing computer software systems? What happens when different languages first come in contact with each other? What are the most effective methods to assess student burnout? Armed with this knowledge, we can contribute to the betterment of humanity. But it becomes challenging to see the big picture when met with protocols to troubleshoot, funding to obtain, and exams to take. It would be natural for any student to ask, 'is this pursuit worthwhile?'. Becoming an adept researcher, able to think critically and persist through these challenges, to refine one's pursuit, and importantly, to ask for help when needed, requires time and years of practice. That is why we are continuously inspired by the tenacity and grit of our student researchers to not only develop these skills but to reaffirm, year after year, their worth — to contribute to our collective body of knowledge.

In this issue, we highlight seven articles that represent the wide breadth and diversity of research being conducted at the University.

We would be remiss if we did not express our gratitude to our outstanding editorial board, including our managing editors and content editors for their hard work ensuring our articles were of the highest quality, as well as our layout chair, Catherine Lan, and layout editors for assembling the journal design. Every single contribution is prized and appreciated. Finally, we are ever grateful for the support of the Office of Undergraduate Research, Dr. Sina Ghaemmaghami and Ms. Ann Robinson, without which this publication would not be possible.

Research as a student endeavor is a difficult but rewarding undertaking, and we are proud to continue to be the platform that showcases the intellectual capacity of our student body — as we have for the last 20 years, and as we plan to for many more. To our readers, we hope you enjoy the end result of their hard work.

Sincerely,

Michael Christof & Priya Mandava

Editors-in-Chief

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Health Disparities Between English And Non-English Speakers in Rochester, NY

David I. Del Valle Ortiz '25, *Health, Behavior, and Society*
Advised by Courtney Jones, Department of Public Health

The city of Rochester, located in Monroe County, is one of the most populated in Upstate New York, with a population of 210,606 according to the United States Census Bureau estimates as of July 2021.[1] However, when the surrounding towns and cities are taken into consideration, the Census reporter demonstrates Greater Rochester has a population of 1.08 million.[2] Consequently, the bigger the population of a region, the bigger its diversity usually is, and Rochester is no exception to this. In the city of Rochester Health equity report, it was shown that over 63% of the city's population was of minority groups that included Black, Hispanic, Asian, Native American, and others.[3] Furthermore, another factor seen in this equity report[3] was the language diversity of the city, where 8.8% of the total population, or about 16,600, people speak English less than very well, which could mean that most of them believe their English capabilities are not as good as their capabilities in a different language.

Another population that has a solid presence in Rochester and Monroe County is the Hispanic population.[5] According to the article "In a pandemic, medical interpreters bridge the language gap" published in the Democrat and Chronicle, 21,000 patients at the University of Rochester Medical Center listed Spanish as their preferred language.[6] Maria Gallina, one of the Spanish interpreters at URMC, was asked by Democrat and Chronicle how she decided to become a Spanish interpreter to which she responded, "I realized the need to have appropriate communication and to have it be understandable, particularly when the doctors were trying to give instructions or the nurses were trying to give recommendations" (Memmott, 2020).[6] This testimonial shows the importance of adequate resources to reduce the communication barrier between providers and patients when the language of each is different.

The greater Rochester region has a substantial amount of Deaf and Hard of Hearing people when compared to other cities in the U.S.4 According to the Rochester Institute of Technology, Rochester has about 42,674 people that are Deaf or Hard of Hearing, most of them using American Sign Language as their main language.[4] The Deaf population is well within the Rochester community, many of them are graduate students at the University of Rochester, residents at the Medical Center (URMC), researchers at RIT, teachers, and students at the Rochester School for the Deaf, and students from the National Technical Institute for the Deaf (NTID). [4] This shows us how the services available for this community should represent its solid presence and culture in Rochester and its surroundings.

Furthermore, hospitals, clinics, and healthcare centers in Rochester do not just serve the populations of the city but also those of the surrounding counties. Rochester has the only level 1 trauma center in the western Finger Lakes Region, meaning that any trauma that takes place in any of those seventeen counties will probably go through the emergency department of URMC.[7] This highlights, once again, the diversity of patients that are treated at healthcare institutions in the city of Rochester. However, most people in these communities, Deaf, Spanish speaking, and others, have stated that the services they are receiving need to improve and that they are not being treated like the hearing, English-speaking patients.[5,8–9]

Unfortunately, in the last couple of years, there has been an increasing shortage of medical interpreters nationwide.[9] According to an article published in the American Medical Association Ethics Journal, "free clinics, where a large majority of patients with LEP [Limited English Proficiency] receive care, are especially affected by this shortage" (Aitken, 2019).[9] The health inequities present in this population are noticeable in the current statistics published by the Monroe County department of health, where the Hispanic population had some of the highest rates for cancer, diabetes, coronary artery disease, and asthma indicators.[10] In the Hispanic population in Monroe County, 24 out of 10,000 had been hospitalized for asthma between 2017 and 2019, and 25.7 out of 10,000 were hospitalized due to coronary heart disease.[10] Furthermore, 19.3 out of 10,000 had a potentially preventable diabetes short-term complication hospitalization and 6.9 out of 100,000 were diagnosed with cervical cancer among the Hispanic population.[10] In addition, the Hispanic community has an all-cause mortality rate of 645.4 for every

100,000 people.[10]

When we compare this epidemiologic measure to those of the white non-Hispanic hearing population, the rates are very different.[3],[10] The white non-Hispanic population of Monroe County, located mostly in the suburbs outside of the city has a median household income almost double that of the minority populations living in the city, 33,399 vs. 57,479.3 Furthermore, the mostly white population outside of the city has a lower percentage of people with no health insurance (3.8%) or state health insurance (37.9%) while in the minority population in the city of Rochester more than half of the population has state health insurance (52.9%) or no health insurance (6.1%).[3] Analyzing this data, we can see how the inequities in the county also portray its health, but also how the healthcare services between the English and non-English population are very different, leading to differences in their health outcome, lifestyle, and treatment.

Now the question is how this public health issue can be tackled with an equity-based approach. In 2012, the Icahn School of Medicine implemented a program in which medical students received a course in language interpretation as part of their curriculum with the goal that, as physicians, having this background would lead to better interaction with patients that do not have English as their preferred language.[9] This program was found to be successful, both patients and providers felt more comfortable talking to each other, and information about treatment, guidelines, and diagnoses was better understood by the patient.[9] However, this program did not offer an official medical interpreter certification and even though they did have better patient-provider interaction, it was not an official interpretation.[9]

A different program was developed in 2015 at Loyola University of Chicago.[9] In it they would evaluate first- and second-year medical school students that knew another language in addition to English and went through the process of being certified interpreters through the Loyola University Medical Center Interpreter Services.[9] These students went through pre-assessment and multiple training sessions, and shadowed interpreters in clinical settings.[9] When these students complete their certification, they can volunteer at the hospital and clinics that are part of the Loyola University healthcare network, and even those outside of the network nationwide, to gain clinical hours and gain experience since medical school students gain much of theirs through observation and volunteering.[9] Even though the results of this program have not been fully published, it was expected that both patient and provider would be more comfortable, communication would be better both ways, and a positive healthcare outcome was going to be obtained.[9] However, this program had a high cost since the certification obtained by the students was not exclusive to their hospital but official.[9]

For both cases, the Mount Sinai Medical Center and the Loyola University Medical Center, the funding for these programs came from their schools of medicine since their impact was mostly centered in their medical center and clinics.[9] However, if a city or county-wide approach is wanted, funding for this type of program should come from the Department of Public Health. The city of Rochester has a top-ranked medical school with dozens of students, there are also many interns, residents, and fellows working and being trained in the many hospitals and clinics around the county.[4] Furthermore, interpreting certification programs already exist in the Rochester area. Monroe Community College (MCC) offers Spanish interpreter certifications and RIT offers ASL interpreter certifications, but both of these have a high cost-MCC's certification has a cost of $895, and RIT's costs $1600.[11],[12] If the cost of these programs could be covered by URMC and then reimbursed by the Department of Public health, med-school students, interns, fellows, and residents could receive an interpreting certification making them able to better approach patients that do not know English as their main language, and they can even volunteer in their free time and offer their services.

Moreover, another possible way to tackle this communication barrier could be through the creation of a certification funding program with the Monroe County department of health (DOH). People that are residents of Monroe County could apply through the program after it is announced through the media and flyers in specific centers around the city of Rochester that have ASL and Spanish speaking majorities. Certified interpreters would determine a person's eligibility and upon an evaluation and interview, the cost of the person's preferred interpretation program at either MCC or RIT could be fully covered by the DOH. These now certified interpreters can then work as interpreters or volunteers in clinics and hospitals around the county, this way reducing the shortage of interpreters and improving the communication between healthcare workers and patients, but also creating new jobs, and possibly helping to reduce the already high unemployment rate of the city and the county, 11.3%, and 5.9% respectively.[3]

Unfortunately, in moments of seeking healthcare services, the language barrier can be a significant challenge since communication in these processes is key to understanding what is happening, ex-

pressing one's feelings, understanding the next steps to take, and following the recommended guidelines. This communication barrier between healthcare workers, ranging from nurses, doctors, social workers, to even research assistants, can lead to many health inequities because the services given to people that are English speaking will result in better treatment due to better communication while people that speak different languages will have to wait for interpreters or personnel that speaks their language, prolonging their care and treatment in a health setting. Moreover, in the absence of interpreters, or workers that know the patient's language, any information given to them will not be understood at its fullest.

These solutions fit under the social-ecological model of health behavior, specifically at the public policy level.[13] For this program to have the impact wanted, city and county-wide, it should ideally be done through public policy in the Department of Health. The development of this policy would encourage both people to become certified interpreters and providers to use the interpreters to give a better service. The availability of certified interpreters, either volunteers or paid, will also encourage patients to seek the services they need since they can be certain that the communication will be better, and information is going to be clear and understandable to both provider and the patient.

Likewise, these possible solutions fit under both the primary and tertiary levels of prevention.[14] These solutions would allow patients to be able to understand what their provider is informing them and therefore understand the importance of their health checkups, prevention strategies, and possible actions to prevent a disease or illness from arising. Additionally, if a patient already has a diagnosis, the availability of interpreters will improve the medical care or rehabilitation service the patient will receive, which without interpreters would not be as effective. We can say that the availability of interpreters, or providers that can also serve as interpreters, will both help to prevent illness from occurring and minimize the disability of the disease by providing the necessary care.[14]

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About the Author

Growing up in Puerto Rico, we spoke Spanish, but my family had difficulties understanding healthcare resources centered on the English language. In Rochester, this challenge was shared by many, including the Spanish-speaking population as well as the Deaf population that uses ASL. Many times these barriers lead to miscommunication between providers and patients. This topic not only improved my understanding of the situation in Rochester, but also to think of new solutions to this problem. For undergrads interested in research, go for it! Choose a topic you are passionate about and put that time into it. Just do it!

Review: Peripheral Neuroprosthetics for the Treatement Of Phantom Limb Pain

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Introduction

Every year, approximately 200,000 people lose a limb due to acute trauma, infection, vascular disease, and various other reasons (Peterson et al. 2019). While the sudden loss of a limb itself interferes with daily life, many amputees are also plagued by chronic post-amputation pain. Post-amputation pain includes residual limb pain and phantom limb pain (PLP). Phantom limb pain is pain that is felt in the missing part of the limb. Approximately 85% of amputees experience phantom limb pain, but the intensity, duration, and sensation (e.g. burning, tingling) of PLP vary from patient to patient (Peterson et al. 2019). It is still unclear how phantom limb pain develops, although several proposed mechanisms of PLP exist (Peterson et al. 2019).

To understand the proposed mechanisms of PLP, it is necessary to first review the normal pain signaling mechanisms in the spinothalamic pathway and the brain. The perception of pain begins upon encountering a noxious stimulus, resulting in the activation of nociceptors, free nerve endings in the skin, and viscera. The signal then travels through the pseudounipolar Aδ and C fibers into the spinal cord, where they synapse on second-order neurons in the dorsal horn. The second-order neurons decussate before traveling in the ventrolateral column to the thalamus in the brain, where they synapse on third-order neurons, which convey pain signals to the primary somatosensory cortex (S1). S1 exhibits somatotopic mapping, with each area of the body corresponding to an area in the cortex, the size of which is dependent on the frequency of use.

Many proposed mechanisms of PLP focus on hyperactivity in the spinothalamic pathway. When a limb is amputated, neurites sprouting from severed nerves attempt to reinnervate the missing limb, resulting in tangled growths called neuromas. Neuromas often exhibit spontaneous activity due to changes in gene expression and upregulation of sodium channels, leading to an overall uptick in spinothalamic signaling (Peterson et al. 2019). However, studies have shown that simply inhibiting signaling from neuromas only partially eliminates spinothalamic hyperactivity associated with PLP (Peterson et al. 2019). Another peripheral source of this hyperactivity is the dorsal root ganglion (DRG), where the first-order neuron somas in the spinothalamic pathway are located. Studies in animal models have suggested that three-quarters of the hyperactivity associated with PLP stems from the DRG (Peterson et al. 2019).

It is thought that the outlined peripheral mechanisms result in the onset of phantom limb pain, while central mechanisms maintain PLP over time (Peterson et al. 2019). Continuous pain signaling in the periphery causes downstream pain processing structures to become hypersensitive to peripheral pain signals due to the strengthening of synapses through long-term potentiation (Peterson et al. 2019). Central sensitization influences signal processing in the thalamus and cortex, and may rewire the central nervous system such that innocuous stimuli are perceived as painful and lead to disinhibition of natural pain regulation systems (Peterson et al. 2019). In addition, recent studies have indicated that the preservation of the area in S1 corresponding to the amputated limb is associated with PLP, perhaps due to activity in the nerves that once innervated the limb despite the absence of the limb itself (Peterson et al. 2019).

Based on these proposed mechanisms, most existing treatments for PLP attempt to inhibit pain signaling in the spinothalamic pathway. Surgical treatments include neurectomy, dorsal root entry zone lesioning, cordotomy, cryoablation, and coblation (Peterson et al. 2019). Each of these treatments severs the peripheral nerves that once innervated the amputated limb, directly preventing pain signaling. However, since these techniques are highly invasive and vary in efficacy and duration of pain relief among patients, surgical approaches are often a last resort (Peterson et al. 2019).

Other treatments for phantom limb pain rely on the body's built-in pain regulation systems, namely afferent and descending regulation. In afferent regulation, activation of Aβ fibers inhibits pain signaling at second-order spinothalamic neurons in the spinal cord. Aβ fibers are responsible for touch signaling; thus, pressure co-located with noxious stimuli can reduce sensations of pain. In descending regulation, activation of the periaqueductal

gray matter in the midbrain induces the release of endogenous opiates in the spinal cord, which block pain signaling (Peterson et al. 2019). Based on these pathways, many neuroprosthetic treatments for PLP attempt to activate afferent regulation, while pharmacological treatments for PLP (i.e. opioids) often rely on descending regulation (Peterson et al. 2019).

Mirror therapy, a non-invasive treatment for PLP, is based on an alternative theory for the etiology of PLP. After amputation, the brain may continue to send motor commands to the amputated limb but does not receive confirmation of movement; thus, the brain believes the limb is paralyzed, leading to pain. In mirror therapy, the patient watches movements of their intact limb in a mirror in order to perceive the phantom limb under voluntary, painless control. Mirror therapy has been proven an effective short-term method of pain relief, but its long-term efficacy remains unclear (Peterson et al. 2019).

Due to the limited effectiveness of existing treatment options and the debilitating nature of phantom limb pain, a myriad of neuroprosthetic approaches are under development for reducing PLP: two such approaches are stimulation of the dorsal root ganglion and high-frequency stimulation of peripheral nerves.

Targeting the Dorsal Root Ganglion

In a retrospective study of eight patients, Eldabe et al. investigated the efficacy of the Spinal Modulation Axium neurostimulator, a device that stimulates the dorsal root ganglion, in treating PLP. As described in a previous study, the device consisted of up to four quadripolar electrode leads, an implanted neurostimulator, and wireless controllers (Liem et al. 2013). The quadripolar electrode leads were implanted in the epidural space near the appropriate DRG using fluorescent guidance. Following implantation, patients underwent a trial period using an external neurostimulator; if at least 50% pain reduction was achieved, a neurostimulator was implanted for long-term use with a wireless controller (Eldabe et al. 2015).

The device had been developed for the treatment of various types of neuropathic pain; eight patients treated with the device for PLP were identified by the researchers (Eldabe et al. 2015). Six of the patients had undergone various lower limb amputations, and two patients had amputations of the upper limb. All patients had experienced PLP, with or without concurrent pain in the residual limb, that had been unresponsive to pharmacological treatment. Electrode leads were implanted near the L3-S1 DRGs for lower limb amputees and near the C6-C7 DRGs for upper limb amputees (Eldabe et al. 2015).

Neuroprosthetics such as the Spinal Modulation Axium neurostimulator are hypothesized to work based on the principles of afferent regulation (Peterson et al. 2019). Stimulation of the DRG induces a sensation of tingling, called paresthesia (Eldabe et al. 2015). Paresthesia overlapping with the location of the patient's pain produces pain relief; this pain relief is thought to be mediated by the activation of Aβ fibers and afferent regulation (Peterson et al. 2019). In line with this theory, clinicians programmed the stimulation pulse width and frequency for maximal overlap of paresthesia with areas in pain, while the intensity of stimulation was patient-controlled (Eldabe et al. 2015).

The researchers compared baseline measurements of pain intensity, medication intake, and quality of life with post-implantation measurements. Total pain intensity, including both residual and phantom limb pain, was measured using a 0-100 mm visual analogue pain scale (VAS), where 0 mm represents no pain. The type and frequency of medication intake were recorded for six patients, and quality of life was assessed for two patients. Follow-up times ranged from 5 to 24 months post-implantation, with a mean of 9 months (Eldabe et al. 2015).

The results of the study were overall promising, but varied significantly from patient to patient. All patients perceived paresthesia localized to the residual limb, with five patients also reporting paresthesia in or interacting with their phantom limbs. The average baseline VAS measurement was 83.3 mm, which decreased to 38.9 mm in the follow-up measurement. One patient experienced complete pain relief and the disappearance of the phantom limb five months after implantation, while another patient's pain regressed to baseline levels within one month after implantation. However, in two other patients, pain relief gradually decreased over a 24 month period after implantation, which the researchers attributed to poor lead placement. Among the two patients for which quality of life was assessed, significant improvements were observed, while of the six patients whose pain medications were monitored, half decreased the amount or types of medications taken (Eldabe et al. 2015).

While this study was successful in proving the feasibility of DRG stimulation for PLP, several weaknesses exist in its design. Despite focusing on the treatment of PLP, the study's measurements make no distinction between residual limb and phantom limb pain. Whether the average decrease in pain at follow-up was due to a decrease in PLP, residual

limb pain, or both is unclear. Furthermore, the follow-up times varied significantly, making it difficult to determine the long-term efficacy of the device. Notably, the patients that experienced gradual decreases in pain relief had the longest follow-up times. This brings into question whether the pain relief observed in other patients would also last over longer periods of time. Finally, generalizability of the study results cannot be determined from such small sample sizes; the overall study only included eight patients, with medication intake and quality of life measurement sample sizes of only six and two patients, respectively.

On a positive note, the device design and some results of the study were promising. By targeting the DRGs associated with the location of the patient's pain, the device selectively activated afferent regulation in painful regions. All patients experienced paresthesia localized to the residual and/or phantom limb, proving the device provided localized activation of Aβ fibers; however, interfacing at pain processing structures further downstream would not be as amenable to selective activation. In addition, despite variations in efficacy among patients, the results prove the feasibility of treating PLP with dorsal root ganglion stimulation.

Targeting the Peripheral Nerves

Soin and colleagues investigated whether an electrical nerve block could be used to treat post-amputation pain. Chemical nerve blocks have been shown to reduce post-amputation pain, but due to the risk of toxicity, they would not be a fitting long-term treatment option. An electrical nerve block was hypothesized to provide a non-toxic method of achieving similar pain relief, through the usage of high-frequency alternating current (HFAC) to inhibit sodium channels through continuous membrane depolarization (Soin et al. 2015). Through these mechanisms, a conduction block in the appropriate peripheral nerves would inhibit hyperactivity in the spinothalamic pathway, providing pain relief.

Ten lower-limb amputees with chronic, severe post-amputation pain were recruited for the study (Soin et al. 2015). Among other requirements, a key inclusion criterion was a positive response to the injection of lidocaine near the target nerve, defined as at least 50% pain relief following injection. A positive response indicated pain relief via peripheral nerve block was feasible, and participants were then implanted with the device (Soin et al. 2015).

Under general anesthesia, the appropriate peripheral nerves were exposed downstream of the neuroma and wrapped with cuff electrodes; the target nerves were the sciatic nerve for above-the-knee amputees and the tibial and common peroneal nerves for below-the-knee amputees. Each electrode consisted of platinum contacts embedded in silicon, and were self-sizing to prevent nerve compression. Initially, an extension cable, which exited from the anterio-lateral thigh, was used to connect the electrode leads to an external waveform generator. After 9–12 months, the external generator was replaced with an implanted generator placed in a subcutaneous pocket below the rib cage and used in conjunction with a wireless controller (Soin et al. 2015).

In an initial postoperative visit, the optimal stimulation frequency, voltage, and ramp-up period necessary for pain relief were identified. While treatment voltages varied among participants, frequency and ramp-up time was set to 10 kHz and 5 minutes respectively for the majority of participants. The waveform generator was then programmed with the optimal parameters for 30-minute treatment sessions. Three participants did not proceed past the initial postoperative visit: two participants did not experience pain relief with stimulation, and one participant's pain was greatly reduced with a better-fitting prosthetic leg. Of the remaining seven participants, three experienced primarily PLP, and four experienced primarily residual limb pain (Soin et al. 2015).

The participants were instructed to turn on the waveform generator as needed to manage their pain. Each participant was provided with a diary to record pain intensity before and after treatment, pain intensity every three hours while awake, and medication intake. Pain intensity was recorded on a 0–10 numerical rating scale (NRS), with 0 representing no pain. The diaries were reviewed at weekly and then monthly follow-up visits, and pain relief from treatment was quantified as a percentage of pain reduction. Pain interference with day-to-day activities was also measured via the interference subscale of the Brief Pain Inventory (Soin et al. 2015).

In the first three months of treatment, pain was reduced by an average of 75%. In comparison, in the last three months of using the external generator and in the three months after the conversion to implanted generators, patients reported an average of 73% and 75% pain reduction respectively, demonstrating that pain relief was sustained over time. Of the treatment sessions that produced significant pain relief, pain relief was sustained for at least nine hours in 69% of these sessions, and the use of both narcotic and non-narcotic pain medications significantly decreased. The level of pain interference in day-to-day activities also signifi-

cantly decreased, particularly for walking ability (57%) and normal work (59%; Soin et al. 2015).

Overall, the study indicates HFAC peripheral nerve stimulation is a strong candidate for the treatment of post-amputation pain, including PLP. Strengths of the design include the treatment duration and interface location; the device was programmed for 30-minute treatment sessions, followed by an hour-long lockout period (Soin et al. 2015). This setup prevents the nervous system from developing any sort of "tolerance" to stimulation and lengthens the battery life of the device. Furthermore, by targeting the interfacing to the peripheral nerves just proximal from the post-amputation neuroma, the device is minimally invasive and blocks only those signals originating in the residual or phantom limb. However, the location of the neural interface also made the device susceptible to damage. In one participant, two revision surgeries were required to replace damaged electrode leads, believed to be a result of the participant sitting on their short residual limb and causing the damage (Soin et al. 2015). Based on this, it is plausible that placing electrodes too close to a prosthetic leg could also result in damage.

While the study focused on all post-amputation pain, the researchers distinguished between residual and phantom limb pain, allowing the efficacy of the device in inhibiting both types of pain to be assessed. Three subjects experienced primarily PLP, and significant pain reduction was achieved with HFAC stimulation in all three subjects (Soin et al. 2015). Thus, the study successfully proved the feasibility of using an electrical nerve block to treat PLP. However, the sample size of the study was small: only seven participants proceeded to treatment, and PLP was the dominant type of pain in only three participants. Furthermore, the inclusion criteria were not completely accurate in determining whether the device would be effective. Two patients responded to the chemical nerve block but did not respond to HFAC stimulation in the initial postoperative visit (Soin et al. 2015).

Comparison

Due to the retrospective nature of the study conducted by Eldabe et al., the organization of the study was lacking in comparison to that of Soin and colleagues. Follow-up times varied from as little as 5 months to as long as 24 months post-implantation, making it difficult to compare long-term efficacy in different patients. In addition, medication intake and quality of life data were not collected for all participants. In the study by Soin et al., follow-up times were more consistent, as was data collection, allowing for conclusions to be more clearly drawn. However, the sample sizes of the studies were comparable, and they assessed the efficacy of treatment over similar mean timelines. Both used each participant's baseline pain levels for assessing the efficacy of the device in reducing pain, and neither study focused only on phantom limb pain by excluding potential participants who also experienced residual limb pain.

The two neuroprosthetic treatments presented varied in efficacy. DRG stimulation resulted in greater extremes, with some participants reporting near-complete pain relief and other participants reporting minimal pain relief over time. In contrast, the peripheral nerve block using HFAC stimulation provided more consistent results. Speculatively, this difference in consistency may be underlied by the interface locations and mechanisms required for operating each type of neuroprosthetic.

Stimulation of the DRG requires activation of afferent pain regulation pathways to provide pain relief- if the participant's afferent regulation systems are impaired or Aβ fibers incompletely activated, pain relief will be minimal. In contrast, HFAC peripheral nerve stimulation directly blocks the conduction of pain signals in the spinothalamic pathway. With fewer steps leading to pain relief and less reliance on the participant's nervous system, HFAC stimulation provides greater assurances of pain reduction. Furthermore, while implantation of cuff electrodes in the peripheral nerve may leave them susceptible to damage, peripheral nerves are easily accessible for both the initial and potential revision surgeries. In contrast, the DRG is less accessible and the type of electrode used in the Spinal Modulation Axium neurostimulator is more susceptible to migration. As a result, HFAC stimulation of the peripheral nerves led to more consistent pain relief than stimulation of the DRG, which is in line with these differences in device design.

Both studies employed a test to determine whether participants were candidates for the neuroprosthetic, but the test employed by Soin et al. was overall safer and more efficient . In contrast to the trial period in the study conducted by Eldabe et al., which required invasive surgery and a multi-day commitment, the lidocaine test used by Soin et al. was minimally invasive and relatively quick. Thus, given the consistency of results and simplicity of candidacy determination, HFAC peripheral nerve stimulation appears to be the more effective treatment for phantom limb pain when compared to dorsal root ganglion stimulation.

Future Directions

While both studies showed at least some success in reducing phantom limb pain, they were limited by

a small sample size and follow-up time. Future studies should examine the efficacy of each device in larger populations and for longer durations (Eldabe et al. 2015; Soin et al. 2015). In addition, future work should more carefully distinguish between residual limb pain and phantom limb pain. Residual and phantom limb pain may occur by different mechanisms (Peterson et al. 2019), and thus, neuroprosthetic devices may have different effects on each type of pain. Since amputees often experience both PLP and pain in the residual limb, using separate measures for each type of pain could result in a clearer understanding of the efficacy of a given device. In addition, the validity of future study results could be enhanced by including more objective measures of pain reduction; for example, activity in S1 could be monitored to corroborate participant's reports of pain relief.

Both studies included a test to determine whether a prospective subject was a candidate for device implantation, indicating limited treatment generalizability. Generalizability is a common issue in neuroprosthetic treatments of PLP and an obstacle to clinical use (Peterson et al. 2019). A better understanding of the underlying causes of PLP would allow for the development of more generalizable neuroprosthetic treatments. Thus, further studies should also be done to determine the neural mechanisms by which PLP arises (Eldabe et al. 2015) and whether these mechanisms are the same in all PLP patients. If different mechanisms are found, another possible goal would be to find a way to distinguish which form of PLP a particular patient is experiencing.

Future work could also extend the use of neuroprosthetic devices to other types of neuropathic and chronic pain. Peripheral nerve and DRG dysfunction is not exclusive to PLP; therefore, stimulation of the DRG and inhibition of pain signaling in peripheral nerves through an electrical nerve block could lead to useful treatments for other pain disorders.

Finally, regenerative electrodes are being developed to provide more stable neural interfaces in the peripheral nervous system. Regenerative electrodes are designed to be placed near the end of a severed nerve; the nerve then grows through the electrode as it regenerates (Peterson et al. 2019). Electrode migration and potential damage could be mitigated with the use of such electrodes in future neuroprosthetic devices that interface in the peripheral nervous system to treat PLP.

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About the Author

I chose to investigate neuroprosthetics for phantom limb pain as my term paper for BME 415: Neuroscience of Neuroprosthetics. I am also involved in other research at the medical center. My advice for fellow undergraduates interested in research would be to dive deep into topics you are interested in, and to not be afraid to reach out to professors.

The Role of Human THUMPD3 methyltransferase in RNA Modification and Protein Translation

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ABSTRACT

RNA modifications play a multifaceted role in influencing the folding and stability of RNA molecules, thus aiding in the regulation of gene function and cellular metabolism. Mutations in the enzymes responsible for RNA modifications are associated with a wide range of human diseases, so it is important to identify and study the activity of such enzymes. Proteins that contain the THUMP domain, which is involved in RNA modification, are promising candidates. There are at least three THUMP domain-containing proteins in humans: THUMPD1, THUMPD2, and THUMPD3. However, not much is known about their function in RNA modification, especially THUMPD2 and, until recently, THUMPD3. My study shows that besides a previously characterized tRNA substrate, THUMPD3 introduces m2G modification to ribosomal RNA, with the most likely candidate being 28S rRNA. Loss of THUMPD3 is also associated with a decreased growth rate, altered morphology, and changes in sensitivity to a number of translation inhibitors. Further research into the activity of the THUMPD3 protein can reveal its role, as well as the roles of other methyltransferases in protein translation regulatory pathways, opening directions for studies of relevant human diseases.

INTRODUCTION

Post-transcriptional RNA modification is a conserved process across all domains of life. To date, around 170 RNA modifications have been identified, introducing diverse alterations to the four basic RNA building blocks, thereby influencing structure, interactions with proteins, and overall function of RNA molecules.[1] RNA modifications regulate gene expression and other cellular processes, which in turn, influence cellular metabolism.[2] In addition, RNA modifications or the lack thereof have been associated with various human diseases.[3]

Transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) are the two most heavily modified classes of RNAs.[4] tRNAs contain the most diverse set of modifications, including stabilizing hairpin loop structures,[5] extending wobble pairing,[6] regulating the integrity of aminoacylation,[7] and fine-tuning interactions with ribosomes during translation.[8] Ribosomal RNAs, despite having a lower diversity of modifications, are also frequently modified and carry important roles of stabilizing ribosomal structures, enhancing interactions between ribosomes and ligands, serving as "assembly checkpoints" for ribosome biogenesis, and regulating gene expression.[9] Interestingly, for some ribosome-modifying-enzymes (e.g. Dim1/DIM1TL, Emg1/EMG1, and Bud23/WBSCR22), their presence, but not their catalytic activity, is required for rRNA processing.[10] Many tRNA and rRNA modifications, and their modifying enzymes, remain uncharacterized, and RNA modifications remain a dynamic research area open for exploration.

Methylation is one of the major types of RNA modifications, as two-thirds of all modified RNA nucleosides involve the addition of methyl groups.[11] Due to the non-polar nature of a methyl group, such modifications on these residues are likely involved in hydrophobic interactions.[12] Many types of methylation, such as m6A and m5C, are abundant in various RNA species, and as a result, are relatively well-studied.

Another prevalent modification of RNA is N^2-methylguanosine (m^2G), which is present in human tRNAs and rRNAs.[13,14] This modification does not disturb the Watson-Crick C-G base pairing. While the physiological role of this modification in humans has not been elucidated, certain m^2G modifications in E. coli are essential in ribosome biogenesis and binding site stabilization.[9,15] Furthermore, there is computational evidence that tRNAs require m^2G for stabilizing hairpin loop and stem structures.[16] Several methyltransferases responsible for m^2G modifications have been identified in different eukaryotes, including RsmD, a chloroplast rRNA m^2G methyltransferase in Arabidopsis17, and Trm11, which introduces m^2G modification in yeast tRNA.[18] There is a human homolog of this protein, TRMT11, but its role has not been experimentally validated. Despite being widely distributed in RNAs from various organisms, m^2G is not yet well-characterized, partly due to the lack of a high-throughput sequencing-based method to detect this modification.

To better understand RNA modifications and their regulation, it is important to study their modifying enzymes. Candidates of interest include proteins with the THUMP domain, a conserved RNA-binding domain that helps facilitate recruitment of target RNA substrate to the adjacent RNA-modifying domain, and subsequent modification of the bound RNA. The THUMP domain of divergent proteins does not have a consensus sequence, but shares a common alternating α/β fold.[19] The architecture of THUMP domain-containing proteins and their RNA substrates are diverse, and many of them are well-studied with experimentally verified biochemical functions and solved crystal structures. Examples of such proteins include the bacterial ThiI (responsible for the formation of 4-thiouridine),[20] bacterial RlmM (2′O-methylcytosine),[21] archaeal and eukaryotic Pus10 (pseudouridine),[22,23] archaeal CDAT8 (C deamination),[24] as well as archaeal and eukaryotic Trm11 (N2-dimethylguanosine).[18,25]

The crystal structure of ThiI in complex with a truncated tRNA shows that the THUMP domain binds tightly to the 3'-ACCA moiety of the tRNA and makes multiple contacts with the double-stranded acceptor stems. This demonstrates that the THUMP domain is capable of binding to both single-stranded and double-stranded RNA.[26] However, a comparison of crystal structures of two other proteins with the THUMP domain, RlmM (which targets rRNA) and Trm14/TrmN (which targets tRNA), shows that this domain exhibits distinct architecture for RNA recognition in different enzymes,[21] suggesting that the THUMP domain can bind to RNA in a number of ways.

An attempt at purifying the THUMP domain as a standalone protein (specifically, from archaeal methyltransferases PAB1283) shows that it is resistant to further proteolysis and can fold autonomously. This THUMP domain exhibits very low affinity for the substrate tRNA as compared to the full-length protein, suggesting that the domain alone is not fully responsible for RNA binding.[27] However, it is worth noting that Tan1 can bind tightly to tRNA despite having only a THUMP domain.[28] Additionally, while deleting the THUMP domain in Pus10 reduces but does not eliminate RNA binding affinity, the truncation significantly decreases the pseudouridylation rate of the protein. It is proposed that the THUMP domain facilitates catalysis by orienting the RNA substrate to the active site of the modifying domain.[29]

In humans, three THUMP domain-containing proteins have been identified. One of them, THUMPD1, which is homologous to Tan1 in yeast, is involved in tRNA N4-acetylcytidine modification. THUMPD1 is a 39 kDa protein consisting of a single THUMP domain that serves as an adaptor protein and assists the enzyme N-acetyltransferase 10 (NAT10) in acetylating transfer RNAs.[30] Increased expression of THUMPD1 is linked to many cancer types,[31, 32] while a lack of functional THUMPD1 causes hypoacetylation which leads to intellectual disability.[33]

Less is known, however, about the other human THUMP domain-containing proteins, THUMPD2 and THUMPD3. Considering the essential role of THUMPD1 in human development, it is worth exploring related proteins as they might be involved in related pathways. Unlike THUMPD1, THUMPD2 and THUMPD3 have an additional RNA methylase domain. Both proteins have been shown to be interaction partners of TRMT112, an evolutionarily conserved cofactor of several tRNA, rRNA, and protein methyltransferases.[34] Limited studies have been done on THUMPD2, although downregulation in in vitro esophageal squamous cell carcinoma was associated with multidrug resistance.[35]

A recent paper by Yang et al. harnessed a reverse genetic approach to show that when THUMPD3 interacts with TRMT112, it is responsible for m^2G modifications, thus confirming its role as a methyltransferase. Specifically, the complex can introduce m^2G6 in all 26 tested G6-containing human cytosolic tRNAs, as well as m^2G7 in $tRNA^{Trp}$. Like ThiI, the THUMPD3-TRMT112 complex recognizes the 3′-CCA end of tRNAs, although the tRNA tertiary structure is also required for substrate recognition. Furthermore, THUMPD3 is expressed in various human cell lines and mouse tissue, with extremely high expression levels in mouse testis. Finally, THUMPD3 knockout cells exhibit reduced global translation and suppressed cell proliferation.[36]

Protein sequence alignment indicates that the methyltransferase domain of the THUMPD3 protein exhibits partial homology to previously characterized rRNA methyltransferases, including E. coli rlmG and rlmKL (unpublished O'Connell Laboratory data). This suggests the possibility that THUMPD3 might modify another RNA substrate in addition to tRNA. To conclusively study the role of THUMPD3, it is of interest to characterize its function(s) in RNA modification. In this study, we aim to conduct a more unbiased approach to identify the role of THUMPD3 as a modifying enzyme by analyzing total RNA, both to confirm the findings of this study and to identify other potential RNA substrates of the protein. RNA nucleoside modification mass spectrometry analysis revealed a reduction of m^2G modification in both large and small RNAs in CRISPR-Cas9 generated THUMPD3 knockout (KO) cells. Further analysis using fractionated ribosomal

subunits showed that the lack of THUMPD3 is associated with a decreased proportion of m^2G modification in ribosomal RNA samples. Additionally, we observed that THUMPD3 KO cell lines exhibited a slow growth rate with elongated cell morphology and altered levels of resistance to different translation inhibitors, suggesting that THUMPD3 plays a role in protein translation and cell development. Finally, we showed partially successful purification of THUMPD3 when co-expressed with its cofactor for tRNA modification TRMT112.

RESULTS AND DISCUSSION

Construction and Selection for *THUMPD3* KO Cell Lines

To determine the function of THUMPD3 protein in RNA modifications in vivo, we knocked out the *THUMPD3* gene in HeLa cells using CRISPR-Cas9. Cas9 nuclease was loaded with four different gRNA sequences, targeting the coding sequence of exon 1 (Fig. 1A), and transfected into HeLa cells. After transfection and cell recovery, single-cell colonies were picked to identify those with an altered sequence in *THUMPD3*.

Genotyping of candidate cell lines was performed through Sanger sequencing. Specifically, genomic DNA was extracted from candidate clones, from which a 750 bp THUMPD3 exon 1 fragment was amplified with PCR (Fig. 1A). Purified PCR products were sequenced, followed by analysis using Synthego ICE software and assessment for the presence of indel mutations that introduce a frameshift and premature stop codon. Fig. 1B illustrates the Sanger sequence analysis of a successful KO cell line produced with gRNA2, in which *THUMPD3* KO cells were shown to have indels at the CRISPR target site and a high percentage of frameshift mutations. This frameshift created a downstream premature TGA stop codon that effectively knocked out the gene.

Selected *THUMPD3* KO cell lines from ICE analysis were then further tested for THUMPD3 protein expression, or the lack thereof, using Western blot analysis. Protein extracts of the candidate cell lines were prepared using reagents listed in the Materials and Methods section. Aliquots of cell extracts were then denatured and loaded on a protein gel to conduct SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane and probed with a rabbit anti-THUMPD3 primary antibody, then a goat anti-rabbit HRP-conjugated secondary antibody, allowing for immunodetection by chemiluminescent substrates. Cell lines that showed a clear reduction of protein production compared to HeLa wildtype (WT) were selected for further experiments. As shown in Fig. 1C, selected *THUMPD3* KO cell lines had approximately less than 10% THUMPD3 protein expression compared to WT after accounting for equal protein loading by using GAPDH as the housekeeping protein. Three independent KO cell lines were selected in total, 2 and 1 of which were derived from frameshift mutation at the target site of gRNA2 and gRNA3, respectively.

THUMPD3 KO cells exhibit abnormal morphology

The loss of THUMPD3 protein caused alterations in cell morphology. HeLa WT cells normally have a round, polygonal shape, but *THUMPD3* KO cells acquired a thinner, more elongated, and spindle-like morphology (Fig. 1D). HeLa cells are usually elongated in a similar manner, albeit with varying degrees of alteration, when treated with different growth inhibitory compounds.[37-39] The abnormal shape of *THUMPD3* KO cells suggests that the mutation is responsible for growth defects. This observation allows the use of altered morphology as a preliminary selection for KO cell lines, narrowing down the candidates needed to be examined for genotype and protein production.

Loss of THUMPD3 protein expression results in decreased m^2G RNA modification

To analyze potential alterations in RNA modifications due to the loss of THUMPD3 protein expression, total RNA was extracted using TRI Reagent from both HeLa WT and THUMPD3 KO cells (n = 3 independent biological replicates). RNA samples were then hydrolyzed to mononucleosides by a mixture of enzymes as described in Materials and Methods and subjected to liquid chromatography with tandem mass spectrometry analysis (LC-MS/MS) and nucleoside modification analysis. A total of 28 modified and unmodified nucleosides were identified in the hydrolyzed mixtures. The most underrepresented modification in THUMPD3 KO cells was m^2G, which was only 0.6 times that of the HeLa WT cells (Fig. 2B). m^2A was the second most affected modification by THUMPD3 KO after m^2G. The remaining modifications were unchanged or slightly unchanged between WT and KO cells.

Based on these analyses, m^2G is the most reduced modified base in THUMPD3 KO cells. Notably, m^2G nucleosides in THUMPD3 KO cells were only reduced, not completely depleted. Considering that different human guanine methyltransferases have been identified,[40-42] it is a reasonable assumption that there may be additional methyltransferases and THUMPD3-independent pathways that could also methylate the amino group at guanine C2. THUMPD3 was only responsible for the 40% fraction lost from total RNA m^2G in KO cells compared to WT. A possibility for such an enzyme includes

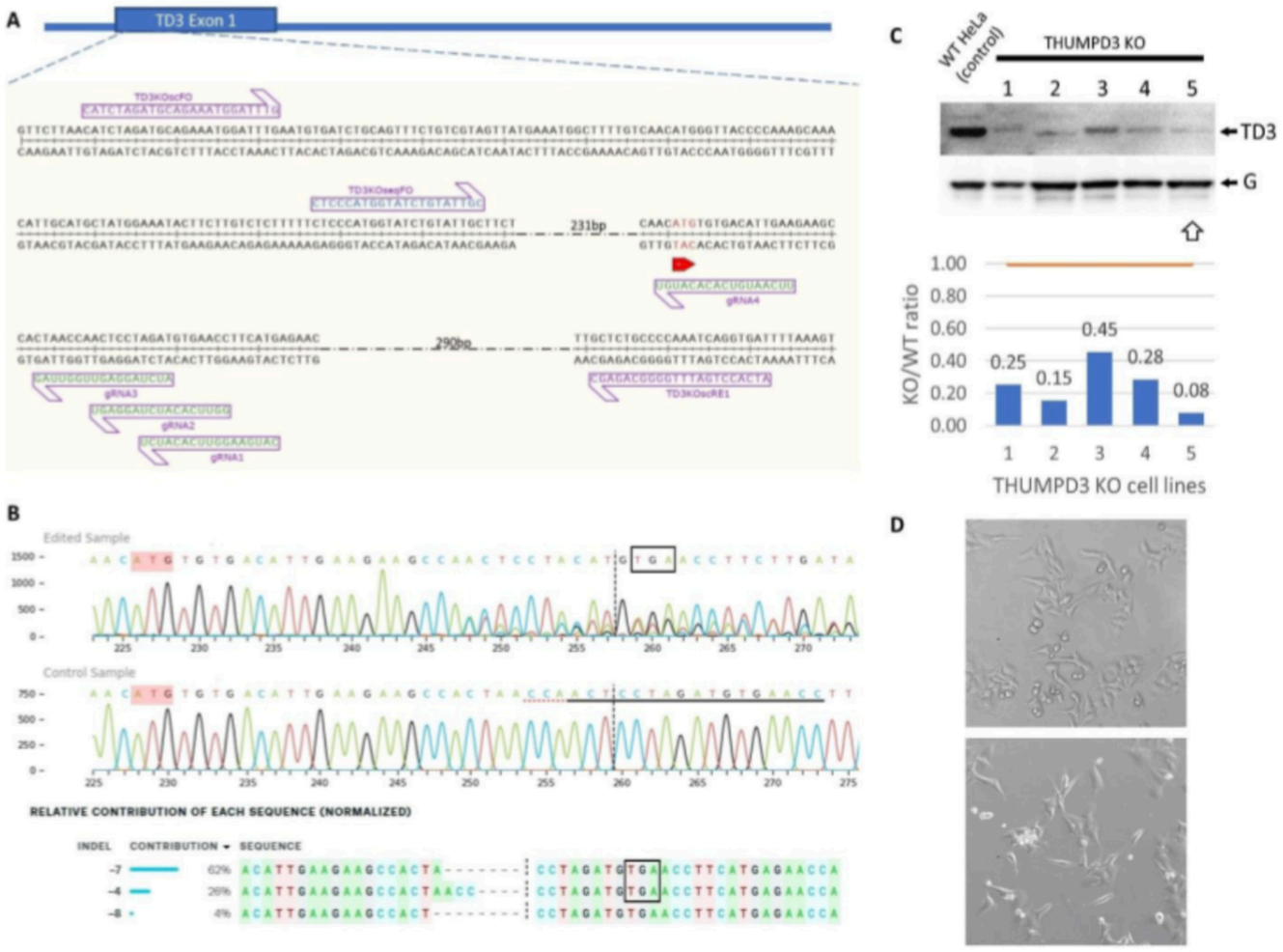

Figure 1. *Construction and selection for THUMPD3 KO cell lines.* ***(A)*** *Partial THUMPD3-containing genomic sequence to illustrate THUMPD3 KO cell line construction using the CRISPR-Cas9. Shown in the figure are gRNA sequences used for knockout (gRNA1, gRNA2, gRNA3, and gRNA4), the primer pair used for PCR amplification of THUMPD3 fragment (TD3KOscFO and TD3KOscRE), and the primer used for Sanger sequencing (TD3KOseqFO). Translation start site of THUMPD3 exon 1 (ATG) is indicated by a red arrow.* ***(B)*** *Analysis of a successful THUMPD3 KO cell line. (Top) Sanger sequencing data of the THUMPD3 exon 1 fragment from the selected THUMPD3 KO cells and HeLa WT cells. The underlined sequence is complementary to the guide RNA (gRNA2 for this sample). The vertical dotted lines indicate Cas9 cleavage site. ATG start sites for both KO and WT sequences are highlighted in red. Premature TGA stop codon in the KO sequence is indicated by a box. (Bottom) ICE Analysis (Synthego) results of the THUMPD3 exon 1 fragment from the selected THUMPD3 KO cells. The dashed regions indicate deletions compared to wildtype sequence. Indel types and their distribution are shown on the left.* ***(C)*** *(Top) Western blot of proteins extracted from HeLa WT cells and potential THUMPD3 KO candidate cell lines, probed with anti-THUMPD3 antibody and anti-GAPDH antibody (as loading control). Observed bands, labelled TD3 and G, indicate the presence (or the lack thereof) of THUMPD3 and GAPDH proteins, respectively. The white arrow indicates a selected successful knockout cell line. (Bottom) Quantification of the ratio of THUMPD3 protein in KO cell lines compared to WT from the Western blot, normalized for GAPDH.* ***(D)*** *Cell morphology altered by the loss of THUMPD3. THUMPD3 KO (top) and HeLa WT (bottom) cells were plated in DMEM medium and grown for 72 hours at 37°C, then photographed under the microscope. Compared to the WT cells, THUMPD3 KO cells are thinner and more elongated in shape.*

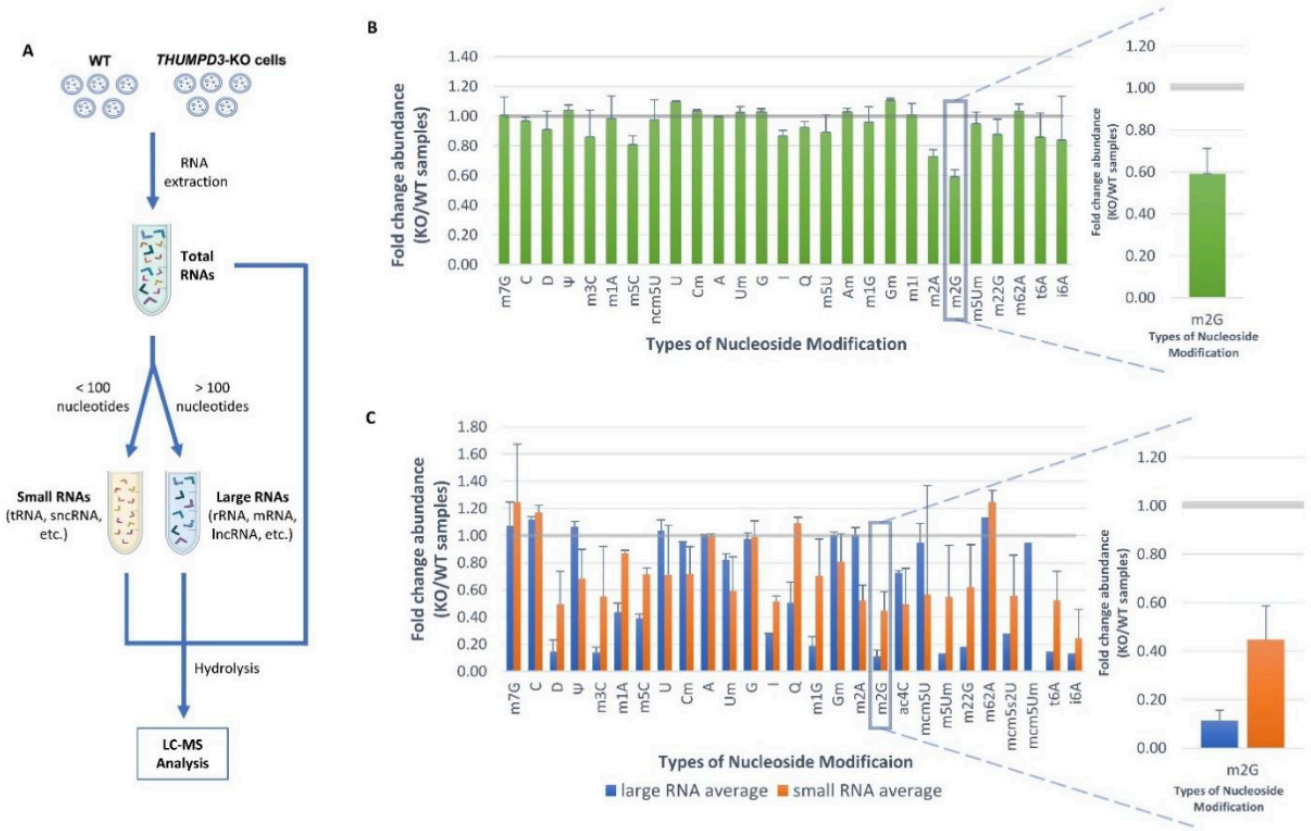

Figure 2. *Analysis of RNA modification landscape in WT and THUMPD3 KO cells.* ***(A)*** *Schematic diagram for RNA modification analysis for different RNA samples using mass spectrometry.* ***(B)*** *LC-MS/MS analysis of RNA modifications for total RNA samples (mean ± s.d. of n=3 independent biological replicates) as well as* ***(C)*** *large and small RNA samples (mean ± s.d. of n=2 independent biological replicates) extracted from HeLa WT cells and three independent THUMPD3 KO cell lines. Modified nucleoside abundance was normalized by the total abundance of standard nucleosides A, C, G, and U. Fold change abundance of each type of modification was calculated as the ratio between the corresponding values of THUMPD3 KO over HeLa WT. The average fold change with standard deviation is shown in the graph. Data points of interest are indicated by blue boxes and enlarged for better visualization (right).*

TRMT11, whose homolog in yeast introduces m2G modification at position 10 in tRNA (although the human protein has not been experimentally characterized).[18] THUMPD2, which contains a methyltransferase domain similar to that in THUMPD3, is another potential candidate.

Another point worth noting is that a small amount of THUMPD3 protein was still produced in THUMPD3 KO cells, as shown in the Western blot experiments (Fig. 1C), which could partially contribute to the remaining m^2G value. However, the reduction of protein expression was significant, providing convincing evidence that the THUMPD3 knockout was responsible for the reduction of m^2G modification.

It is also interesting to consider that the other slightly reduced modifications may be a downstream effect due to the loss of m^2G modification. There are instances where modification of a nearby base can affect the activity of modifying enzymes at the target site.[43-45] This is especially true for heavily modified RNAs, such as tRNAs and rRNAs, where different modifying enzymes can compete or enhance the efficiency of each other, or where pre-existing modifications might change the RNA structure in a way that affects the binding between the substrate and the modifying enzyme, thus altering modification efficiency. Given the observed data, THUMPD3-catalyzed m^2G modification might have the same effect, such as with m^2A or m^5C, which are also slightly reduced in KO cells.

To further investigate how THUMPD3 KO affects RNA modifications and potentially narrow down the substrate of this RNA modification, large (>100nt) and small (<100nt) RNAs were isolated from HeLa WT and THUMPD3 KO cells (n = 2 independent biological replicates) using the mirVana™ miRNA Isolation Kit (Thermo Scientific). This procedure utilizes varying ethanol concentrations to immobilize

RNAs of different sizes on the binding column, allowing the separation of large and small RNAs. Each isolated RNA fraction was then hydrolyzed separately. LC–MS/MS nucleoside modification analysis results of both samples are shown in Fig. 2C. There was more variation in fold change abundance among different modifications in this analysis compared to total RNA samples.

Consistent with the analysis in total RNA, m^2G modified nucleosides were reduced in both large and small RNAs of THUMPD3 KO cells compared to WT (0.11 and 0.45, respectively). The decrease in m^2G in small RNAs (which contain tRNAs) is in agreement with the study by Yang et al., which showed that THUMPD3 was responsible for human tRNA:m^2G modification.[36] In this study, however, our analysis indicated that large RNAs (which include several large rRNAs, mRNAs, and lncRNAs) contained lower levels of m^2G modification as well. Thus, it can be reasonably deduced that besides tRNAs, THUMPD3 is also responsible for the m^2G methylation of another RNA species in this fraction.

There are many human RNA modifying enzymes, including methyltransferases, recorded in literature that have dual specificity; namely, they can target two different RNA substrates. NAT10 is an 18S rRNA N^4–acetylcytidine (ac^4C) acetyltransferase that can introduce the same modification on tRNA with the assistance of the adaptor protein THUMPD1.[30] Specific to methyltransferases, TRMT61B is a mitochondria-specific N^1–methyladenine (m^1A) methyltransferase that can catalyze the formation of both m^1A58 in tRNA and m^1A947 in 16S rRNA.[46,47] Another example is TRMT2B, which is responsible for introducing N^5–methyluridine (m^5U) modification in both mitochondrial tRNA and 12S rRNA at positions 54 and 429, respectively. Therefore, the assumption that THUMPD3 has another substrate other than tRNA is not without basis.

rRNA samples from THUMPD3 KO cell lines contain decreased m2G modifications

Given the LC–MS/MS nucleoside modification analysis above, it is of interest to identify the large RNA species that THUMPD3 modifies. A potential candidate is ribosomal RNA. Considering that rRNAs make up at least 80% of total RNA by weight,[48] the majority of the large RNA fraction would consist of rRNAs.Therefore, the modification of rRNA by THUMPD3 would explain the significant decrease in m^2G in the large RNA fraction as seen in Fig. 2B. Moreover, a methyltransferase containing the THUMP domain in E. coli, rlmKL, introduces m^2G in 23S rRNA at position 2445. This modification is the most important among the cluster of modified nucleosides at the peptidyl transferase center, as knockout of the gene encoding for rlmL results in severely decreased growth.[49] Given the known role of THUMPD3 in human protein translation and cell growth,[36] and that human ribosomal RNAs also contain m^2G modifications,[14] it is not unreasonable to suggest that THUMPD3 may have a similar activity as rlmL in humans.

To make this suggestion, it is necessary to extract enriched rRNA samples from both HeLa WT and THUMPD3 KO cells to analyze their modification profile. First, ribosomes were isolated and purified from the soluble fraction of cell lysate using a 1M sucrose cushion. Other proteins and cellular components with lower molecular weights remained in the cushion, while the heavy ribosomes were pelleted down to the bottom of the tube. Next, intact ribosomes were dissociated into individual large (60S) and small (40S) subunits, which was done by incubating with puromycin in low Mg^{2+} and high salt conditions. A sucrose gradient was then used to fractionate the different ribosomal subunits. Finally, different rRNA species were separately isolated from these ribosomal subunit fractions, using the procedure described in the Materials and Methods section. A schematic diagram for the isolation and fractionation of ribosomal subunits can be found in Fig. 3A.

A_{260} measurements were obtained throughout the subunit fractionation process. The absorbance profile revealed distinct peaks (regions A and B, Fig. 3B), which possibly contained the different dissociated ribosomal subunits. Polyacrylamide gel electrophoresis analyzing the extracted RNAs from these fractions is shown in Fig. 3C. While it was clear that ribosomal RNAs were successfully isolated, the different ribosomal subunits (and therefore the different rRNA species) were not cleanly dissociated.

The rRNA samples were then subjected to hydrolysis and LC–MS/MS nucleoside modification analysis (n = 2 independent replicates), the result from which is shown in Fig. 3D. In fractions from region A as indicated in the A_{260} absorbance profile, m^2G modification was significantly underrepresented in THUMPD3 KO cells, with the KO/WT ratio being as low as 0.45.

This experimental analysis provided strong evidence that rRNA is a substrate for THUMPD3–directed m^2G modification. The procedure used was designed to selectively isolate ribosomes, and therefore the resulting rRNA samples should contain little contamination. Firstly, the sample is unlikely to contain mitochondrial RNAs, as mitochondria were removed along with cell debris when the cell lysate was centrifuged to obtain the soluble fraction. Secondly, the sample should not contain other types of nuclear or cytoplastic RNAs, since the sample was extensively treated with puromycin and a high salt concentration, and went through a sucrose cushion/gradient twice. So, the resulting ribosome samples should contain at most only a small amount of other RNAs or RNA–protein complexes with low molecular weight. The polyacry-

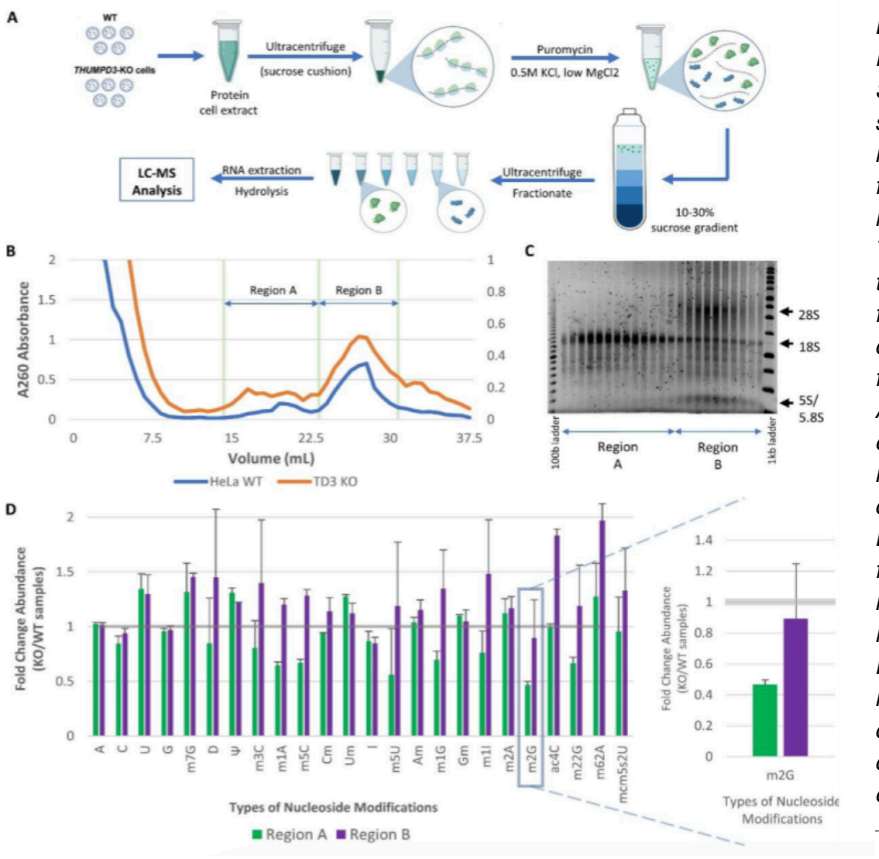

Figure 3. *Identification of the ribosomal RNA substrate of THUMPD3.* ***(A)*** *Schematic diagram for isolation and separation of ribosomal subunits.* ***(B)*** *A260 measurement of the sucrose gradient fractions throughout the process of ribosomal fractionation for HeLa WT and THUMPD3 KO cells. The x-axis runs from the top to the bottom of the gradient. The first peak (before 10mL) is non-ribosomal contamination. The arrows indicate fractions used for further analysis.* ***(C)*** *Analytical gel electrophoresis of rRNAs extracted from the ribosomal fractions of interest, as indicated in panel B. Bands of different rRNA species were indicated.* ***(D)*** *LC-MS/MS analysis of RNA modifications for rRNA samples (mean ± s.d. of n=2) independent replicates extracted from the regions of interest identified in panel B, for HeLa WT and THUMPD3 KO cells. Data normalization and representation was done as described in Fig. 2B, C. Data point of interest is indicated by a blue box and enlarged for better visualization (right).*

lamide gel confirmed the purity of the obtained rRNA samples.

However, the question of which specific rRNA species contain THUMPD3-modified m^2G modifications remains unclear. From the LC-MS/MS data, only RNAs from the fractions in region A, but not B, had the m^2G KO/WT ratio much smaller than 1, indicating only a subset of RNA species were modified by THUMPD3. While an initial look at the polyacrylamide gel might suggest that region A contained mostly 18S rRNA, which would imply that it is the THUMPD3 substrate, a review of existing literature showed that no m2G modification has been identified in the human 18S rRNA.[14,50-52] In E. coli 16S rRNA, three m^2G modifications have been identified, including m^2G966, m^2G1516, and m^2G1516;[9,53] however, none of these modifications are conserved in human 18S rRNA. For example, the corresponding nucleotide of E. coli m^2G1516 is uracil (U) in humans, making it unlikely that the THUMPD3-mediated m^2G modification as identified from this experiment is on the 18S rRNA. On the other hand, m^2G modifications have been visualized in human 28S rRNA, including m^2G729 (ES7L-B), m^2G978 (ES7L:HelixC), m^2G1517 (ML:H27-H32), m^2G4872 (ES39L), and $m^2xp7G4371$ (E-tRNA binding site).[14] This provides a basis for the prediction that the m^2G modification introduced by THUMPD3 is on the 28S rRNA, with potential candidates for its location in the five positions mentioned above.

Although this is not in good agreement with the initial polyacrylamide gel analysis of the extracted RNA samples, it is possible that the resolution of the gel image does not reflect the true RNA species composition. As mentioned above, the separation of the ribosomal subunits was not completely achieved, and, these RNA samples likely did not contain a single type of rRNA. Still, this does not eliminate the possibility that the m^2G modification identified is indeed on the 18S rRNA, though this specific modification has not been previously identified. However, this assumption, if true, would need more experimental analysis to support it.

It should be noted that it is not well understood why several modifications other than m^2G had larger variations in fold change abundance in the LC-MS/MS data of large/small RNA and ribosomal RNA samples (Fig. 2C, 3D). A possible explanation is that the different large/small RNA and ribosomal RNA ratios in independent cell lines affected the separation and collection of the fragments, leading to variation between biological replicates. However, we can still argue for forthe validity of the data for m2G modification. Firstly, high signal intensity for m2G detection indicates that this re-

duction is due to the THUMPD3 KO status and not an artificial signal from variability. Secondly, the reduction of m^2G modified nucleosides is consistent across all rounds of analysis, including total RNA, large/small RNAs, and ribosomal RNAs, as well as across all biological replications.

To validate the conclusion that THUMPD3 introduces m^2G modifications in human 28S rRNAs, the procedure for the separation of the rRNA species needs to be improved. One possible approach is to use a hybridization-based method following ribosome isolation with a sucrose cushion. To isolate each rRNA species separately, corresponding biotinylated DNA probes would be used followed by Streptavidin agarose resin. The DNA probes would be designed to complement a unique sequence of each rRNA, allowing hybridization between the specific pairs of RNA and DNA probe and independent isolation of each specific rRNA.

THUMPD3 KO cells show altered levels of resistance to different translation inhibitors

In their study, Yang and colleagues have shown that THUMPD3 KO cells exhibit reduced cell proliferation and suppressed global translation.[36] To further examine the possible role in the translation of THUMPD3, we analyzed THUMPD3 KO cell growth in the presence of different translation inhibitors. 10^5 cells of both HeLa WT and a THUMPD3 KO cell line were seeded into media with and without different translation-inhibiting drugs, and the number of cells was counted at 24, 48, and 72 hours after seeding.

In drug-free media, the growth rate of THUMPD3 KO notably reduced compared to WT (Fig. 4A), consistent with the findings of Yang et al. While the knockout of this gene was not completely lethal, the loss of THUMPD3 protein caused a growth defect in the cells.

Upon puromycin treatment, the reduction in growth rate by THUMPD3 KO was exacerbated (Fig. 4B). Puromycin mimics the 3' end of a charged tRNA, occupying the ribosomal site A and binding to the growing polypeptide chain in place of the next amino acid, blocking translation.[54] Considering that it has been shown that THUMPD3 methylates tRNAs and is involved in translation,[36] this observation makes sense. It can be assumed that the lack of tRNA m^2G modifications in THUMPD3 KO cells destabilizes its tRNAs, leading to weakened interactions between charged tRNAs and ribosomal site A. This allows puromycin to better compete with tRNAs for binding at the site, ultimately increasing drug sensitivity.

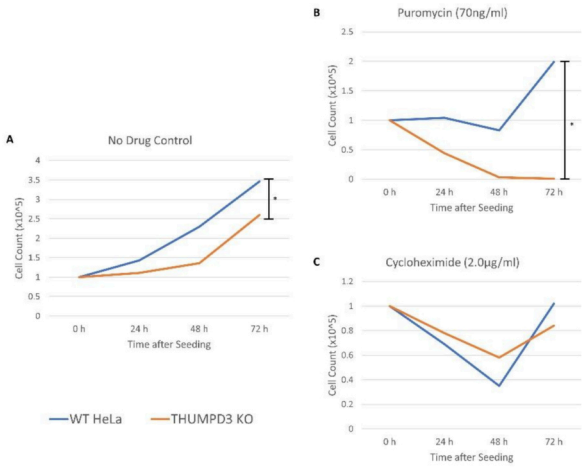

Figure 4. *Growth curve of HeLa WT and a THUMPD3 KO cell line over 72 hours after seeding in* ***(A)*** *normal DMEM media with no drug,* ***(B)*** *with 2.0µg/ml cycloheximide (final concentration), and* ***(C)*** *with 70ng/ml puromycin (final concentration). Cell count was taken after approximately every 24 hours. Interesting differences between the two samples are marked by asterisks.*

Interestingly, when seeded in media with cycloheximide, THUMPD3 KO cells did not decrease in growth rate relative to WT, unlike in the no-drug control (Fig. 4C). In other words, cycloheximide repressed the negative effect on the growth rate of THUMPD3 KO. Cycloheximide blocks translation by binding to the ribosomal E-site and preventing the uncharged tRNA from leaving, in turn, stopping P-site tRNA from translocation and halting elongation.[55]

A possible explanation for this observation is that the lack of THUMPD3-mediated tRNA modification affects the binding between tRNAs and ribosomes, allowing translocation to compete with the binding of cycloheximide at the E-site. Another prediction is that THUMPD3 introduces nucleoside modifications on 28S rRNA at the same or nearby position as where cycloheximide targets. The lack of modification in THUMPD3 KO cells affects the interaction between cycloheximide and the elongation complex, reducing drug sensitivity. There have been instances in which a lack of modification in rRNA at the active sites of the ribosome leads to increased resistance to drugs. For example, in E. coli, suppression of C2 methylation at A2503 on 23S rRNA creates a selective advantage in environments containing the antibiotic tiamulin.[56] E. coli variants lacking the 23S rRNA m^1G745 methyltransferase show a reduced growth and translation rate, but an increased resistance toward the ribosome-targeting antibiotic viomycin.[57] The lack of THUMPD3-mediated m^2G modification on human rRNA may create a similar effect with respect to

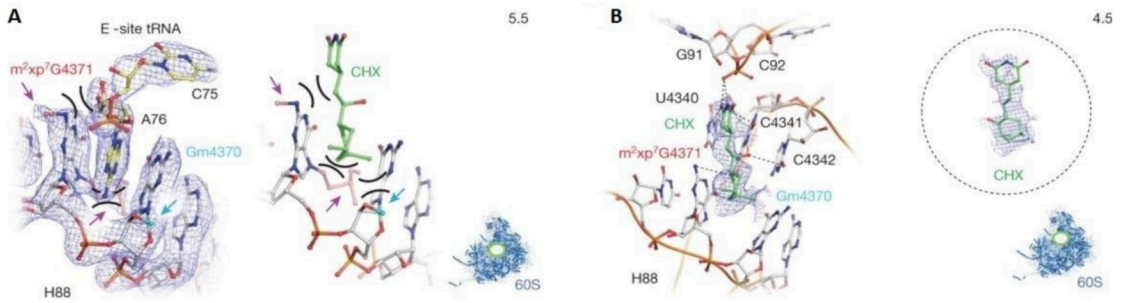

Figure 5. *Chemical modifications in the vicinity of ligand-binding pockets of the human 80S ribosome with bound cycloheximide.* ***(A)*** *The CHX-binding site (tRNA E-site). Right, model of CHX in the high-resolution 80S ribosome structure.* ***(B)*** *CHX density in a subpopulation of the 80S complex. Figures obtained from S K Natchiar et al. Nature 1–6 (2017) doi:10.1038/nature24482.*

cycloheximide resistance.

The latter hypothesis is supported by the presence of a previously identified m^2G modification on the 28S rRNA, m^2xp7G4371, which lies in the E-tRNA binding site. It has been shown that the extra methyl group of m2xp7G4371 generates a hydrophobic contact with cycloheximide (Fig. 5).[14] If this modification is indeed introduced by THUMPD3, it would explain the experimental result observed in THUMPD3 KO cells: the lack of hydrophobic interaction by m^2G destabilizes the binding of cycloheximide into the ribosomal E-site, resulting in the rescue of cell growth when in the presence of the drug.

This data further supports the suggestion that 28S rRNA is the substrate for THUMPD3, and potentially narrows down the specific m^2G position for further investigation. While the accuracy of this predicted model for reduced cycloheximide sensitivity in THUMPD3 KO cells is speculative and requires additional experimental analysis to confirm, it allows for further investigation of the rRNA target of THUMPD3-mediated methylation.

Purification of THUMPD3 expressed alone is prone to truncation

A logical approach to confirm the methylating activity of THUMPD3 and determine THUMPD3-directed modification sites within human rRNA species is through in vitro biochemical assays. To prepare for these assays, it is important to obtain purified THUMPD3 protein.

Various bacterial strains BL21, Rosetta, and Rosetta 2 contain a THUMPD3 expressing construct and were grown in large-scale 75 0mL cultures for protein expression and purification. After 3 hours of IPTG induction, cells were collected and spun down. The collected cell culture was then lysed, and the supernatant and pellet were separated as described in the Materials and Method section.

In the first purification round, affinity chromatography was used. The supernatant was incubated with the HisPur Ni-NTA Resin, allowing the His-tagged THUMPD3 protein to bind to the column. The resin was packed into a column, washed, and the protein of interest was eluted in an imidazole gradient, resulting in eight 750 µL fractions. SDS-PAGE analysis of the eluents for all three bacterial strains was similar, indicating that the choice of bacterial strains for protein expression does not influence the quality of the expressed and purified proteins. One such analysis is shown in Fig. 6A. The expected band for His-tagged THUMPD3 was observed (57 kDa), but there were other bands, suggesting impurities in the product.

Western blot of the eluted fractions showed the signal for the His tag at 57kDa, which is expected for a full-length THUMPD3 protein. However, multiple bands of smaller size were also observed, suggesting that truncation occurred during the protein purification process (Fig. 6B). Although the later eluted fractions contained less truncation and impurities, as shown by both the SDS-PAGE gel and Western blot, the protein concentration is too low for any next-step analysis. Notably, two truncated variants at approximately 38 and 25 kDa were most dominant, as they were present even in the later fractions as observed in the Western blot.

Why truncation occurred was not clear. A specific site on the THUMPD3 protein that is particularly sensitive to degradation by proteases may have led to truncation at that specific location, resulting in the very sharp bands of the truncated proteins, specifically at 25 and 38 kDa (instead of random truncation that would result in a smear on the gel). It is also possible that the THUMPD3 protein was not folded properly in the cell extract, leading to

degradation over time throughout the purification process. Additionally, the protein was expressed in a bacterial system, which might not have provided the proper modifications and processing for mammalian proteins like THUMPD3, leading to misfolding and subsequent degradation.

Attempts to improve this purification trial included using a partially denaturing environment during the affinity chromatography step. To create this environment, 2 M urea was added before separating cell debris. This improved protein solubilization and allowed for the disruption of non-specific protein interactions, therefore removing impurities and truncation from the THUMPD3-containing eluants. After the supernatant passed through the column, it was washed several times with decreasing urea concentrations, theoretically allowing the THUMPD3 protein to refold into its native state. Additional rounds of purification using anion exchange and size exclusion chromatography were attempted to further purify the THUMPD3 protein samples. The best fractions from the affinity chromatography eluents were combined and dialyzed to be used in anion exchange chromatography. Finally, fractions that contain a significant ratio of the full-length protein were combined and concentrated to be used in the size exclusion chromatography with the Superdex 200 Increase 10/300 GL column.

After three rounds of chromatography, while the purity of the protein was improved throughout the process, three bands (full-length His-tagged THUMPD3 and two major truncated variants at 25 and 38 kDa) consistently remained in all independent trials. The SDS-PAGE gel showed no further separation from the chromatography, with identical sets of three bands across all fractions (Fig. 6C). When the Western blot was conducted, a signal for the His tag was again detected at the approximate location of these three bands (data not shown). One possible explanation as to why it is difficult to remove the truncated products is that there may be very strong interactions between the full-length and truncated forms, making the various forms co-migrate and therefore inseparable.

Co-expression with TRMT112 partially improves THUMPD3 purification success

According to Yang et al., THUMPD3 needs to interact with the methyltransferase activator TRMT112 to introduce an m^2G modification in tRNA. Yang and colleagues also found that THUMPD3 is highly oligomeric in vitro, but stays in a monomeric form in the presence of TRMT112.[36] In another study, co-expression with TRMT112 increases the expression of THUMPD3 in U2OS cells, suggesting that TRMT112 stabilizes THUMPD3 in human cells.[34] Indeed, TRMT112 has a major role in stabilizing various methyltransferases.[58,59] For example, in the case of METTL5, the protein has a large hydrophobic surface that is shielded from the surrounding hydrophilic environment by TRMT112 upon complex formation.[59] TRMT112 may have a similar role in the case of THUMPD3, and in the above purification trials, THUMPD3 was not stabilized in the cell extract in the absence of

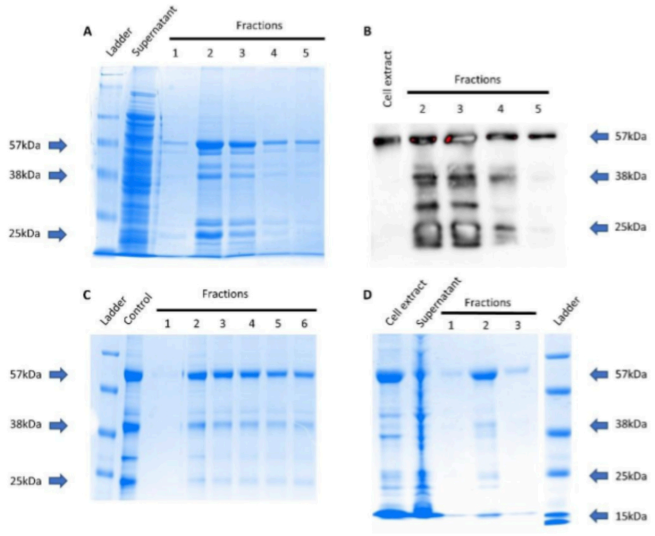

Figure 6. *Expression and purification trials of THUMPD3 protein. Coomassie-blue stained SDS-PAGE gels of eluting fractions from HisPur Ni-NTA Resin from different purification steps are shown in panels A, C, and D.* ***(A)*** *THUMPD3 expression and partial purification of THUMPD3 using affinity chromatography. Bands for His-tagged THUMPD3 protein (57kDa) can be observed across all fractions.* ***(B)*** *Western blot probing for His tag of the eluted fractions from the affinity chromatography step (same as panel A), which detects His-tagged THUMPD3 protein. Signal at 57kDa can be seen in all fractions, confirming the presence of full-length THUMPD3 protein. However, other smaller-sized bands are also observed, which indicate THUMPD3 protein truncation. Two major truncation variants are at approximately 38kDa and 25kDa, which can be seen even in later fractions 4 and 5.* ***(C)*** *Further THUMPD3 purification using a partially denaturing environment, followed by ion exchange and size exclusion chromatography. After the additional purification rounds, the fractions containing less contaminants compared to the first trial in panel A. However, two other bands besides the full-length protein at 25 and 38 kDa were observed, with the proteins of different sizes minimally fractionated. Control is an aliquot of combined fractions from affinity step in panel A.* ***(D)*** *Co-purification of THUMPD3:TRMT112 using affinity chromatography resulted in partial success. Bands for His-tagged THUMPD3 protein (57kDa) can be observed across all fractions. The bands for the truncated forms were fainter, indicating lower concentration relative to the full-length form. The band at 15kDa is expected to be TRMT112.*

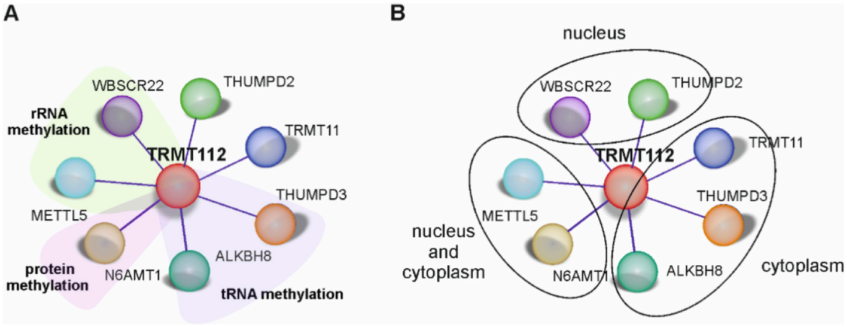

Figure 7. *TRMT112 interactome. MTases interacting directly with TRMT112 are shown together with their* ***(A)*** *known enzymatic activities and* ***(B)*** *cellular localisation. Figures obtained from B Brūmele et al. Int J Mol Sci (2021) doi: 10.3390/ijms222413593.*

TRMT112. Therefore, co-expression and co-purification of THUMPD3 and TRMT112 is a logical approach, with the assumption that by complexing with TRMT112, THUMPD3 would be stabilized and thereby minimize any potential proteolysis.

A pET-Duet plasmid containing the open reading frame of THUMPD3 (containing a 6xHis tag) and TRMT112 was transformed into a Rosetta 2 bacterial strain, with the expression of THUMPD3 confirmed using Western blot (figure not shown). Transformed bacteria were then cultured in 750mL TB media and subjected to affinity chromatography as described in the section above, without the additional step of adding urea. SDS-PAGE analysis showed that this co-purification resulted in some partial purification success, with the full-length protein band clearly showing that it was the predominant species in the sample (Fig. 6D).

A band at 15 kDa was observed in the SDS-PAGE gel, which is most likely the expressed TRMT112. However, a Western blot using anti-TRMT112 antibody provided mixed results in three independent trials (data not shown). It is still being determined whether these results are due to experimental error or a technical error relating to the quality of the antibody used.

The success rate of this purification process is being optimized by varying several parameters and by attempting additional rounds of purification with other chromatography methods. Expression and purification in other systems, including insect cell- and mammalian cell-based systems, will also be considered.

<u>THUMPD3 in the context of the TRMT112-Methyltransferase interactome</u>

As mentioned above, THUMPD3 requires the cofactor TRMT112 to methylate tRNAs. TRMT112 is a small, conserved activator for different rRNA-, tRNA-, and protein methyltransferases.[60,61] In their study, Brūmele and colleagues have identified the interacting partners of human TRMT112, as shown in Fig. 7.[34] Among these, WBSCR22 and METTL5 are rRNA methyltransferases. WBSCR22, a nuclear protein, is responsible for N^7-methylguanine in 18S rRNA and participates in pre-rRNA processing. Interestingly, the protein, but not its catalytic activity, is required for ribosome biogenesis.[62,63] METTL5 is found in both the nucleus and cytoplasm and catalyzes N^6-methyladenosine in 18S rRNA.[59] TRMT112 is also required for the methylation activity of the protein methyltransferase N6AMT1. This protein has two distinct substrates: eukaryotic translation termination factor 1, on which N6AMT1 introduces N^5-methylglutamine modification; and histone 4 lysine 12, which is monomethylated.[64,65] Finally, TRMT112 acts as an activator for tRNA methyltransferases ALKBH8 and THUMPD3. ALKBH8 catalyzes the formation of the wobble mcm^5U from its precursor cm^5U in several tRNAs, as well as further hydroxylation of that nucleoside to form $mchm^5U$ modification.[66] TRMT112 also interacts with THUMPD2 and TRMT11, although the biochemical functions of these proteins have not been experimentally elucidated. The knockout of many of these genes influences different cellular functions, and the deletion of yeast Trm112 itself is associated with reduced growth.[67] It is unclear why these methyltransferases require a common cofactor, but considering that these proteins participate in different steps of translation, it can be speculated that TRMT112 serves as a central 'hub' protein that helps coordinate and regulate this process.

It is reasonable to assume that given the cytoplasmic localization of THUMPD3, it is involved in the later stages of ribosomal biogenesis, similar to METTL5, as opposed to the nuclear WBSCR22. It would be of interest to test whether the rRNA methylating activity of THUMPD3 requires TRMT112, as this would provide insight into the regulation of this catalytic activity and the role of THUMPD3 in the TRMT112 interaction network. It is worth noting that the identified rRNA modification may not be directly catalyzed by THUMPD3, but rather be indirectly caused by another methyltransferase in the TRMT112 interaction network. It is less likely, yet not improbable, that the interaction between this protein and TRMT112 is disturbed by

the lack of THUMPD3 protein in knockout cells, affecting the level of rRNA m^2G. The enzyme in question, if exists, is unlikely to be one of the previously characterized proteins: none of the modifications that they catalyze (m^6A, m^7G, and mcm^5U) were affected by the knockout status of THUMPD3 according to the LC-MS/MS data, suggesting that the activity of their modifiers is not influenced by the lack of THUMPD3. THUMPD2, while its substrate is unknown, is shown to localize in the nucleus, and is thus less likely to be affected by the absence of the cytosolic THUMPD3 protein. This leaves the uncharacterized TRMT11 as the remaining plausible candidate, although its homolog in yeast, Trm11, has been shown to introduce m^2G10 in tRNA.[18] It is also possible that the enzyme that characterizes rRNA m^2G modification is another TRMT112 interacting partner that has not been identified. In general, to fully confirm the methylating activity of THUMPD3 on rRNA, in vitro methyltransferase assays will be conducted.

An interesting direction to explore is to further study how THUMPD3 is regulated in the context of the TRMT112 interaction network. The first step will be to generate TRMT112 single and THUMPD3-TRMT112 double KO, as well as other double KO cell lines among the members of the interactome, and compare their RNA modification profile with WT and THUMPD3 KO cell lines. Given the important role of TRMT112 partners and methylation in various cellular processes, such further studies would allow for a better understanding of RNA processing as well as relevant clinical applications.

Future directions

For the long-term goals of this project, to confirm THUMPD3-mediated rRNA-modifying activity in vitro, the purified protein, once obtained, will be used in methyltransferase assays with RNA species obtained from THUMPD3 KO cells as a substrate to characterize the THUMPD3 activity in vitro.

Experiments will then be conducted to uncover the specific position(s) of the modification. Potential candidate THUMPD3's binding sites include identified m^2G sites from published human rRNA cryo-EM structures as discussed above, especially the $m^2xp7G4371$ in the cycloheximide binding pocket. This information will be used to design primer extension assays, in which the identified RNA substrates are reverse transcribed in vitro in close proximity to predicted substrate positions. The presence of m^2G modification will decrease the read-through activity of reverse transcriptase, and the resulting truncated product will help locate its position. Partial hydrolysis of rRNA and fragment LC-MS/MS nucleoside modification analysis would also be conducted in the process of identifying the modification position(s).

Another goal is to analyze the role of THUMPD3 and THUMPD3-directed m^2G modifications in the context of the TRMT112 interactome. A good starting point would be to generate THUMPD2 and TRMT11 KO cell lines and double KO cell lines with THUMPD3, and analyze their RNA modification profiles. As there has not been much research on these two proteins, relationships between these proteins that impact m^2G or other guanine modifications on rRNA may be uncovered.

Finally, it is of interest to establish the connection between the biochemistry of THUMPD3-mediated m^2G modifications and the observed phenotype of THUMPD3 KO cells. The results in this project serve as the initial step towards investigating the biological effects of the diminished m^2G on cellular processes, including translation, with the hope that it would lead to the discovery of underlying causes of related diseases in humans.

MATERIALS AND METHODS

Construction of THUMPD3 KO Cell Lines

THUMPD3 KO cell lines were constructed from HeLa cells using a CRISPR-Cas9 Gene Knockout kit (Synthego). Four different guide RNAs were designed to target THUMPD3 exon 1 (Figure 1). To prepare for transfection, HeLa cells were grown in cell tissue culture medium (DMEM with pyruvate, L-glutamine, and fetal bovine serum containing penicillin and streptomycin) to 75% confluency, then collected using trypsin treatment. RNP complexes consisting of Cas9 nuclease and gRNA were assembled in R buffer according to the manufacturer's instructions, with a Cas9:gRNA ratio of 1:9, and incubated for 10 minutes at room temperature. For each transfection, 5µl of cell suspension containing approximately 10^5 cells was added, and then the mixture was subjected to Neon electroporation using the standard Immortalized Cell Neon Electroporation Protocol. After transfection, cells were transferred to pre-warmed 12-well dishes and incubated at 37°C. After cells reached confluency, they were treated with trypsin and collected into fresh DMEM. Cell counts were determined using a hemocytometer, and serial dilutions were performed accordingly to seed them into 96-well plates (200µl of media per well), such that each well contained approximately one single cell. After 24 hours, wells with single cells were selected, left to grow into single-cell colonies, collected, and re-seeded in 6-well plates. To confirm successful THUMPD3 knockout, we determined the genotype of each cell line. Genomic DNA of cells from each

well was extracted using the Wizard® SV Genomic DNA Purification System (Promega). A 762bp genomic DNA fragment containing the predicted Cas9 site was amplified by polymerase chain reaction (PCR), then sequenced by Sanger sequencing and analyzed for indels using Synthego ICE software (https://ice.synthego.com). The sequence of the primers for amplification and sequencing are shown in Figure 1. THUMPD3 KO cell lines with the highest out-of-frame indel proportions were selected. The morphology of both HeLa WT and THUMPD3 KO cells was investigated using the Nikon light microscope.

Western Blot Analysis of THUMPD3 Protein Expression in WT and KO Cell Lines

To confirm the knockout status of the selected THUMPD3 KO cell lines, a Western blot was conducted to analyze THUMPD3 protein expression levels. HeLa WT cells and THUMPD3 KO cells were lysed by incubating with cell extract buffer, which contained 1% TRITON X-100, 1 mM DTT, and 200x protease inhibitor in 1x Phosphate-Buffered Saline (PBS), for 15 minutes. The mixtures were then incubated with 2 µL of 5 mg/mL DNase I at 37°C for 10 minutes and subjected to 1-minute sonication. The 10 µL supernatant aliquots of the spun-down mixtures were denatured with 10 µL of 2x SDS loading buffer for 10 minutes and loaded into Novex™ NuPAGE 10% Bis-Tris Gels. SDS-PAGE was conducted in Bis-Tris running buffer at 75 V for 5 minutes, then increased to 150 V for the remainder of the run. Proteins were then transferred onto the nitrocellulose membrane in NuPAGE transfer buffer, using the semi-dry Trans-Blot Turbo Transfer system (Bio-Rad), at 2.5 V for 40 minutes. After blocking with 5% non-fat milk solution in 1xPBST (0.05% Tween20 in 1x PBS), the membrane was incubated with anti-THUMPD3, rabbit polyclonal primary antibody (ABclonal) for 1 hour at room temperature. The membrane was then washed with PBST three times before being incubated with goat anti-rabbit, HRP-conjugated secondary antibody (SantaCruz) at room temperature for 1 hour. All antibody treatments were performed according to manufacturer recommendations. After five PBST washes, the membrane was then treated with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific). Imaging was performed using a BIO-RAD Molecular Imager GelDoc™ XR+ imager.

Loading of protein was normalized by measuring the expression of a housekeeping protein, GAPDH. After imaging for THUMPD3 protein expression, the membrane was then treated with a stripping solution (25 mM Glycine, 1% SDS, pH 2.0) twice, each for 15 minutes. Antibody incubation and chemical treatment were repeated, using rabbit anti-GAPDH antibody (ProteinTech) as the primary antibody.

Isolation of Total Cellular RNA

Total RNA was extracted from HeLa WT and THUMPD3 KO cells using TRI Reagent (Molecular Research Centre, Inc.), following the manufacturer's instructions. Approximately 107 cells were lysed by resuspending in 500 µL of cell extract buffer (as described above) and solubilized on ice for 2 minutes. 1.25 mL of TRI Reagent was then mixed with the samples and incubated at room temperature for 5 minutes. 0.4 mL of chloroform was then added to the mixtures, homogenized, and further incubated for 5 minutes at room temperature. The RNA-containing upper aqueous phase of the spun-down mixture was collected and incubated with 0.5 mL of isopropanol for 10 minutes. After centrifugation, total RNA precipitated into a white gel-like pellet, which was washed in 2 mL of 75% ethanol and resuspended in 20 µL RNase-free water. RNA concentration was measured using NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific).

Extraction of Small and Large RNAs

Separation of small (<100 nt) and large (>100 nt) RNAs from both HeLa WT and THUMPD3 KO cells was done using the mirVana™ miRNA Isolation Kit (Thermo Scientific). Approximately 10^7 cells were pelleted and washed with cold 1xPBS, then lysed by vortexing in 600 µL of Lysis/Binding Solution. To extract small RNAs from the samples, the lysate was incubated with 60 µL miRNA Homogenate Additive for 10 minutes on ice, then mixed thoroughly with 600 µL Acid-Phenol:Chloroform. After centrifugation, the upper aqueous layer was collected, thoroughly mixed with 200µl 100% ethanol, and spun down into the RNA binding Cartridge at 10,000 xg for 15 seconds. The low ethanol concentration allowed small RNAs to pass through the filtrate and immobilized large RNAs in the filter. To isolate the small RNAs, a volume of 100% ethanol equal to 2/3 of the filtrate was added to the flow-through, which was then passed through a second RNA binding Cartridge. This time, the higher ethanol concentration kept the small RNAs bound to the filter. The column was washed with the Wash Solutions (700 µL miRNA Wash Solution 1, then twice with 500 µL Wash Solution 2/3), and small RNAs were eluted with 70 µL pre-heated 95°C nuclease-free water. The columns containing large RNAs were washed and eluted similarly to collect the RNA fractions.

Isolation of Ribosomes and Polysomes

The protocol was adapted, with adjustments, from

Rivera et al., 2015.[68] Approximately 2 g of WT and THUMPD3 KO cells each were cultured in DMEM, and then collected by centrifugation (4,000 xg for 3 minutes). The cell pellet was resuspended in Breaking Buffer (20 mM Tris pH7.6, 50 mM KCl, 10 mM MgCl2, and 1X protease inhibitor cocktail) at a 1:5 (w:v) ratio. The suspension was then homogenized in a glass homogenizer using about 30 strokes with a loose-fitting Teflon pestle. The mixture was then incubated for 20 minutes at 4˚C before RNase-free DNase and NP-40 detergent was added to a final concentration of 2 µg/mL and 0.1% respectively. The mixture was further homogenized with an extra 10 strokes, and then set aside for another 10 minutes at room temperature. After a quick incubation in ice, cell debris was separated by centrifugation at 4 ˚C using an SS-34 rotor at 14,000 g for 30 min, and the supernatant was collected. This crude lysate was then layered with a 1.1 M sucrose solution in Breaking Buffer at a 1:1 (v:v) ratio. The ribosomes were pelleted at 4˚C, 40,000 rpm for 18 hours at 4 ºC using a Beckman SW41 rotor.

Dissociation of Ribosomes into Large and Small Subunits

The protocol is adapted, with adjustments, from Rivera et al., 2015.[69] The pellet of ribosomes and polysomes, obtained from the ribosomal isolation step, was solubilized in Buffer A (20 mM Tris pH7.6, 50 mM KCl, 4 mM MgCl2, 0.2 mM EDTA, 0.25 M sucrose) and diluted to 100-200 A260 a.u. The solution was then incubated with 1 mM puromycin at 4ºC, and subsequently at 37ºC for 15 min at each step. Ribosomal subunits were separated by ultracentrifugation in a 10-30% sucrose gradient (500 mM KCl, 4 mM MgCl2, 20 mM Tris-HCl pH 7.5, and varying sucrose concentration) at 4˚C, 20,000 rpm, 18 hours using a Beckman SW28 rotor. The sucrose fractions are collected at 750 µL per fraction, with A260 and A280 absorbance of each fraction measured. 50 µL aliquots of the fractions were used in Western blot analysis to determine the presence and ribosomal proteins.

Isolation of rRNA from Ribosomal Subunits

Combined solutions of detected ribosomal fractions were diluted with PBS at a 1:1 (v:v) ratio. A 1:1 (v:v) ratio of phenol-chloroform was added to the mixtures, homogenized, and further incubated for 5 minutes on ice. The RNA-containing upper aqueous phase of the spun-down mixture was collected. 1:1000 (v:v) of glycoblue and 2.5:1 (v.v) of pure ethanol were added to the supernatant and incubated overnight. After centrifugation, the precipitated RNA was air-dried and resuspended in 20 µL RNase-free water. RNA concentration was measured using NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific).

RNA Hydrolysis and LC-MS/MS Analysis of RNA Modifications

RNA hydrolysis of RNA samples of interest was performed using the protocol described by Su et al..[70] For each sample, 10µg of RNA was mixed with 10 µL RNA hydrolysis solution (250 mM Tris-HCl at pH 8.0, 5 mM MgCl2, 0.5 mg/mL BSA, 1 U μL^{-1} of benzonase, 0.01 U μL^{-1} phosphodiesterase I, 0.1 U μL^{-1} alkaline phosphatase in RNase-free water), with RNase-free water added to bring the volume to 50µl. The mixtures were incubated at 37ºC for 3 hours, then cold centrifuged using a 10,000-MW cut-off spin filter (Amicon) for 15 minutes at 16,000 xg. The filtrates, which contained clean hydrolyzed RNAs, were collected. RNA modifications in the hydrolyzed RNAs were analyzed using separation onto Dionex Ultimate 3000 UHPLC (Hypersil Gold 2.1 column) coupled to a Q Exactive Plus mass spectrometer (Thermo Scientific).

Growth Curves and Translation Inhibitor Assay

10^5 cells of each cell line, HeLa WT and THUMPD3 KO, were seeded in 12-well plates containing 2 mL of DMEM medium mixed with 10 µL of each translation inhibitor solution per well at final concentrations: Cycloheximide at 2.0, 0.4 and 0.08 µg/mL, and Puromycin at 70, 14 and 2.8 ng/mL, done in three replicates. Control wells contained 10µL of water instead of a drug solution. Plates were incubated at 37ºC. Attached cells from each replicate were removed by trypsin treatment and collected approximately after 24, 48, and 72 hours after seeding, and the number of cells was counted using a TC20 Automated Cell Counter (BioRad).

Cloning of THUMPD3 and TRMT112 Gene into Protein Expression Vector

For a single-protein expression vector, THUMPD3 ORF was amplified from the cDNA construct by tailed PCR using the primers listed below, generating a DNA fragment of 1557 bp.

Forward primer: TCTCAATATTTGTGACATTGAAGAAGCCAC

Reverse primer: TCTCGTCGACTCATCATTCTTTGCATTGCCAAAG

Primers were designed so that the gene was flanked with 5' SspI and 3' SalI restriction sites. The PCR product was purified using the Monarch® PCR & DNA Cleanup Kit (NEB) as suggested by the manufacturer. The vector used was the pET-based 6xHis-TEV LIC cloning vector (1B; Addgene), which was prepared from the corresponding transformed E.coli strain using Wizard® Plus SV Minipreps DNA Purification System (Promega), linearized with SspI

and SalI restriction enzymes (NEB), and gel-purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) using product instruction procedure. Ligation was performed by incubating a mixture of 150 ng vector and 50 ng of THUMPD3 PCR product at 14°C overnight with 1 µL T4 DNA ligase (NEB) in x10 Ligase Buffer (NEB). Ligated products were tested by agarose gel electrophoresis for completeness of the ligation reaction. Ligation mixtures were then transformed into 100 µL of XL1-Blue competent cells by co-incubating on ice for 30 minutes, heat shocked at 42°C for 30 seconds, and letting cells recovered in 350 µL LB media at 37°C for 1 hour, and plated on kanamycin-containing LB plates (50 µg/µL). No-insert linearized vectors were transformed as well to use as a negative control, and uncut vectors were used as the positive control. Selected colonies were inoculated in LB media containing 50 µg/µL kanamycin overnight at 37°C. Plasmids were extracted using the Promega Miniprep kit. Positive clones, if identified, would be screened by restriction digests for the presence of THUMPD3 inserts. Positives would show a larger 6 kb band on the gel as opposed to vector only, which would show a smaller 5 kb band.

For the co-expression vector, TRMT112 ORF was amplified from the cDNA construct by tailed PCR using the primers listed below, generating a DNA fragment of 410 bp.

Forward primer:

TGACAGATCTGGCGACATGAAACTGCTTAC

Reverse primer:
TGACCTCGAGGCACAATCAACTCTCA-CAACTCTCAGTTTCC

Primers were designed so that the gene was flanked with 5' BglII and 3' XhoI restriction sites. The vector used was the pETDuet-1 expression vector (Novagen). The TRMT112 gene was cloned into the vector using the same procedure as described above. The 6xHis-tagged THUMPD3 ORF was transferred from the 1 B vector to the pET-Duet-1 vector using the flanking XbaI and SalI restriction sites.

Protein Expression and Purification using Affinity Chromatography

Various E. coli strains (BL21, Rosetta, and Rosetta 2), with THUMPD3 plasmid or THUMPD3:TRMT112 pET-Duet plasmid were grown at a large scale (750 mL) in TB media containing 50 µg/µL kanamycin until the culture reached OD_{600} 0.5, and induced with 0.5mM IPTG for 3 hours. An aliquot of this culture was taken to test for successful protein expression using Western blot. The remainder of the culture was then centrifuged at 8,000 xg for 10 minutes to collect the cell pellet. This pellet was then resuspended in cell extract buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM MgCl2, 10 mM imidazole), and incubated with 1 mg/mL lysozyme at 37 oC for 15 minutes. The buffer was then adjusted to 1M NaCl, 1x Protease inhibitors (Sigma), and 1% Triton-X, and this mixture was allowed to be solubilized in ice for 15 minutes. Finally, 100µg/ml of DNase I and RNase A each were added, again incubated at 37oC for 20 minutes. In an alternative procedure, the addition of 2M urea to lysis buffer was used. The lysed cells were then spun down at 14,000 xg for 30 minutes. The supernatant was mixed with 400 µL HisPur Ni-NTA Resin (Pierce; equilibrated with cell extract buffer) at 4°C for 20 minutes. Resin was washed with 3 aliquots of 4 mL cell extract buffer containing decreasing NaCl (and urea, if applicable) concentration after each wash, and His-tagged THUMPD3 was eluted using elution buffers (cell extract buffer with 1 M NaCl, and imidazole ranging from 10 mM to 500 mM, increased after each elution) with 8 aliquots of 400 µL.

Protein Purification using Ion Exchange and Size Exclusion Chromatography

Two buffers were prepared, including buffer A (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5mM MgCl2, 5% glycerol, 0.05% DDM) and buffer B (50 mM Tris-HCl pH 8.0, 1 M NaCl, 5mM MgCl2, 5% glycerol, 0.05% DDM). Combined affinity fractions containing THUMPD3 protein selected for further purification from the affinity chromatography step were dialyzed overnight in buffer A. The sample was then loaded onto a HiTrap® Q High-Performance anion exchange column (pre-equilibrated with buffer A). A gradient mixture of buffer A and B, starting from 100% buffer A and ending with 100% buffer B, was used to elute THUMPD3 in 500 µL fractions. Combined affinity fractions containing THUMPD3 protein selected for further purification from the ion exchange chromatography step were concentrated to 750 µL and subjected to size-exclusion chromatography with Superdex 200 Increase 10/300GL column. Specifically, buffer B was used to equilibrate the column. After the sample was loaded onto the column, the same buffer was used to elute THUMPD3 in 500 µL fractions. The buffer flow rate was kept constant at 0.6 ml/min, and the A260 (amount of DNA) and A280 (amount of protein) were monitored in real time during the elution process. A total of approximately 25 mL of eluent was collected. 10 µL aliquots of samples at each purification step were used in SDS-PAGE and Western blot analysis to determine the presence and purity of THUMPD3.

SDS-PAGE and Western Blot Analysis of THUMPD3 Protein Expression

To test the solubility of bacterially expressed THUMPD3, protein cell pellets were lysed in buffer with 1% Triton X-100, treated with RNase A and DNase I and centrifugated 14,000 ×g for 10 min at 4°C. 10µl supernatant aliquots were mixed with 10 µL 2x SDS loading buffer. For the pellet, samples were solubilized in 1% Triton X-100, 1% sodium deoxycholate in 1x SDS loading buffer. Samples were then denatured at 95°C for 10 minutes and then loaded onto 10% SDS-polyacrylamide gels (prepared using ProtoGel from National Diagnostics stock solution following the manufacturer's protocol). SDS-PAGE was conducted in Tris-glycine SDS running buffer (25 mM Tris-Cl, 250 mM glycine, 0.1% SDS) at 75 V for 5 minutes, then increased to 150 V for the remainder of the run. For Coomassie staining, the gel was then incubated in fixing solution (10% AcOH, 40% MeOH) for 10 minutes, then in staining solution (10% AcOH, 40% MeOH, 0.2% Coomassie Blue) for 1 hour, and finally destained using the same solution as the fixing solution until the desired contrast was achieved. Imaging was performed using a BIO-RAD Molecular Imager GelDoc™ XR+ imager. For Western blot, the same protocol described above for analysis of protein expression in cell lines was used, with anti-His mouse polyclonal primary antibody and goat anti-mouse HRP-conjugated secondary antibody.

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Cult of Isis Frescoes as Narratives at the Temple of Isis in Pompeii

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Cult of Isis Frescoes as Narratives at the Temple of Isis in Pompeii

Isis, originally an Egyptian goddess who possessed magical powers and healing abilities,[1] was a figure of great intrigue and admiration in the Classical world. As with the entirety of Egypt, the land, religion, writing, and culture were viewed as exotic and mystical. The interest in Isis was partially a product of European "Egyptomania,"[2] the fixation and obsession that European societies had with Egypt. An example of Egyptomania can be seen in Figure 1, a hieroglyphic stela, which was found displayed in the Temple of Isis in Pompeii. This tablet narrates various stories of pharaohs from Egypt, ranging from the last dynasty to the conquest of Alexander the Great. Therefore, it is strange why this artifact would be found in a temple specifically dedicated to the goddess Isis, because the tablet is unrelated to Isis. Since Romans could not read complex hieroglyphs, it can be assumed that they displayed this in their temple as they were fascinated by the "Egyptianess" of it.

Figure 1. *Hieroglyphic stela.* (Photograph from Museo Archeologico Nazionale di Napoli, https://mann-napoli.it/wp-content/uploads/2021/04/2-Stele-geroglifica-inv.-1035-scaled-1.jpg).

The cult of Isis was considered an exotic and foreign religious group in Rome. Scholar Eric M. Orlin stated that "Egyptian rites and Egyptian culture were welcomed at Rome but clearly marked as exotic, enabling a clearer definition of Roman identity."[3] This separation of Roman and Egyptian culture allowed for a clear outline of what made Romans truly Roman by explicitly demarcating what was not Roman, in this case, Egyptian culture. This made Egyptian culture seem exotic to Romans rather than synonymous with their own culture, which can be seen in the frescoes discussed in this paper. Through analysis of the frescoes, this paper will highlight how the Romans looked at the Egyptians in light of their foreignness, and how they were made appealing to Romans. Generally, Isis was viewed by Romans andGreeks as the ultimate healer and mother goddess. This is partially due to her roles in traditional Egyptian mythology, with Isis magically healing her husband Osiris, the god of the underworld. She was also the mother of the prominent falcon-headed god Horus, who was intrinsically connected to the throne of Egypt.[4]

Both of the frescoes discussed below were found in the ekklesiasterion section of the Isis temple in Pompeii, and are titled "Isis and Io at Canopus" and "Worship of Osiris' Sarcophagus." "Isis and Io at Canopus," seen in Figure 2, depicts the Greek Io and Egyptian Isis meeting at the Nile river port in the Egyptian city of Canopus. The "Worship of Osiris' Sarcophagus," seen in Figure 3, portrays an idyllic nature scene where a ceremony honoring Isis' husband, Osiris, is taking place. This ekklesiasterion room was where "worshippers of Isis gathered and ritual banquets were held,"[5] and was the size of "a large hall…dedicated to meetings of the initiates."[6] Moreover, there was a portico-style courtyard where the temple sat, a purgatorium that led to the sacred Nile water basin, and a sacrarium, a smaller room where initiates could meet and look

[1] George Hart, *The Routledge Dictionary of Egyptian Gods and Goddesses* (London: Routledge, 2005), 79.
[2] Bob Brier, "Egyptomania!" *Archaeology* 57, no. 1 (2004): 16–22.
[3] Eric M. Orlin, "Octavian and Egyptian Cults: Redrawing the Boundaries of Romanness," *The American Journal of Philology* 129, no. 2 (2008): 248.
[4] Hart, 80.
[5] Salvatore Nappo, *Pompeii: Guide to the Lost City* (London: Weidenfeld & Nicoloson, 1998), pp. 89-91.
[6] "Temple of Isis," *Pompeii Sites*, http://pompeiisites.org/en/archaeological-site/temple-of-isis/.

at the frescoes.[7] These aspects of the Isis temple are relayed from the viewpoint of Romans interpreting a foreign religion. The temple itself was not that different from a normal Roman temple in terms of architectural design, but had subtle Egyptian elements that made the culture palpable to Romans. This notion can also be seen in the frescoes as they are painted in a Roman style with the figures having understated indications of being Egyptian. These indications include the animals around the figures or Egyptian-style patterns or objects being present in the images.

With the popularity of the cult among the lower classes in the Roman Empire,[8] some interesting details about these frescoes can be observed. This cult provided a hopeful alternative to the mainstream state Roman religion, where things like a positive life after death and magical healing properties would be offered. Since initiates into the cult most likely could not read texts about Isis, these frescoes served as narratives to teach members about aspects of the goddess and the cult. These depictions show stories about Isis, including her relationship with other deities, geographical features, and religious connections. They acted as chronicles in an aesthetically pleasing and artistic way to demonstrate why Isis was such an important deity and why they should worship her.

Isis and Io at Canopus

Beginning with one of the most famous fresco paintings in the Temple of Isis in Pompeii, "Isis and Io at Canopus" (Figure 2) depicts the goddess Io being welcomed into Egypt by the goddess Isis. Io, pictured on the left being carried on the shoulder of a Nile river god, is shown with horns reminiscent of a heifer. This is due to her connection with white heifers since she was transformed into one

Figure 2. *Fresco Depicting Isis and Io at Canopus*. (Photograph from Museo Archeologico Nazionale di Napoli, Quadro con Io a Canopo, https://mann-napoli.it/wp-content/uploads/2021/04/11-Quadro-con-Io-a-Canopo-inv.-9558-IO-E-CANOPO-scaled-1.jpg).

[7] Ibid
[8] Orlin, 235.

by Zeus to protect her from the wrath of his wife, Hera. In traditional Greek mythology, Io was a princess who Zeus fell in love with. After being turned into a heifer, she supposedly wandered all over the Earth and was restored to her original form when she traveled to Egypt.[9] Isis is on the right with some cult followers behind her, depicted with a snake and a crocodile, which are animals often associated with Egypt.. Her followers are holding sistri, musical instruments connected with the Isis cult. She is also portrayed in a seemingly Greco-Roman style, with light skin, a laurel wreath atop her head, and a Roman-style dress. Additionally, this scene is supposed to take place in the Egyptian city of Canopus, which was a port town used for trade with Greece. It was also the site of an extensive temple to the Greco-Egyptian god Serapis, who was associated with a bull, and Osiris, who was Isis' husband.[10]

However, an aspect of this story that adds an interesting layer to this fresco is that the Greeks believed that Io and Isis were the same goddess, while the Egyptians only worshiped her as Isis.[11] Therefore, this fresco may represent both the connection between Greek and Egyptian ideologies and stories and the Roman interpretation of this connection. This theory also connects with the location in the painting, Canopus, since the aforementioned god Serapis and Osiris were famously worshiped there and it was a center of trade between Greece and Egypt. Specifically, Isis' contribution to Osiris' resurrection and healing was a very important aspect of the myth and to the cult of Isis, while Serapis was a Greco-Egyptian deity. All of these connections between Greece and Rome established a direct relationship between Europe and Egypt as the Roman cult of Isis was partly a perception of Greek interpretations of Egyptian religion. As the scholar Robert Turcan states, the Isis cult "did not arrive in the pure and raw state of their country of origin. [It] had…undergone the effects of Hellenic filtration, both in their imagery and the form of their liturgy, even in the very structure of their initiatory ceremonies."[12] This Greek-Egyptian relationship is depicted in this fresco by directly showing Io, representing Greece, touching Isis, representing Egypt, and Io being delivered to Isis at the meeting point between the two civilizations, Canopus. The Romans were famous for adopting foreign, most famously Greek, gods and goddesses into their own systems of worship, and interpreting their stories to fit their own culture. Here we see this with the Isis cult, with most depictions of Isis not looking distinctly Egyptian. She instead often resembles a Roman woman or goddess with details that indicate that she is Isis, as in this fresco with the snake, crocodile, and cult followers present.

Another aspect of the myth that surrounds this fresco is Io's transformation into an animal and then back into a human through Isis' influence is not isolated to this story. An additional narrative where these transformations occur is in Apuleius' Metamorphoses; the main character of this work, Lucius, is accidentally turned into a donkey by a witch from Thessaly, and it is only when he travels to Egypt and joins the cult of Isis that he is transformed back into a human. Right after he transforms back into a human, the surrounding people:

> were amazed, and the faithful bowed down before this public manifestation of the power of the great goddess [Isis], the ease with which the transformation was accom plished and its miraculous conformity with the nocturnal visions; and raising their hands to heaven, loudly and with one voice they bore witness to the goddess's marvellous beneficence.[13]

In this piece, there is a great contrast between positive magic as opposed to harmful magic. Isis was viewed as the supreme entity of holy and healing magic while the Thessalian witch's magic was seen as damaging. In this transformation scene, it is portrayed as easy for Isis to perform this miracle, and her generosity is highlighted. This glorification of Isis' magic is in contrast to the Thessalian witch, Pamphile, who is described as evil. Lucius is warned to "watch out for the wicked wiles and criminal enticements of that woman Pamphile, the one that's married to Milo…Never lower your guard. They say she's a top-class witch, mistress of every kind of graveyard spell."[14] This witch in Thessaly, a place famous for witchcraft in Greece,[15] serves to highlight the superiority of Isis' positive magic by demonstrating Isis' benevolence and the devotion of her followers.[16]

It is notable that both Io and Lucius experience salvation when they travel to Egypt, which, in this fresco, is meant to be understood by the cult followers as the land of positive Isis magic. Since both Apuleius' Metamorphoses and the story of Io contain these sentiments of Egyptian salvation, it can

[9] Apollodorus, *The Library*, trans. Sir James George Frazer (Cambridge, MA: Harvard University Press; London, William Heinemann Ltd, 1921), II.I.
[10] Christian H. Bull, "Prophesying the Demise of Egyptian Religion in Late Antiquity: The Perfect Discourse and Antoninus in Canopus," *Numen* 68, 2-3 (2021): 191.
[11] Aaron J. Atsma, "IO - Argive Princess & Nymph of Greek Mythology," Theoi Project, 2017, https://www.theoi.com/Heroine/Io.html.
[12] Robert Turcan, *The Cults of the Roman Empire,* trans. Antonia Nevill (Oxford, UK: Blackwell Publishers, 1996), 5.
[13] Apuleius, *The Golden Ass or Metamorphoses*, trans. by E. J. Kenney (New York: Penguin Books, 2004), XI. XIII.
[14] Ibid., II.V.
[15] Ibid., Introduction, 3.
[16] Stavros Frangoulidis, *Witches, Isis and Narrative: Approaches to Magic in Apuleius' Metamorphoses* (Berlin: De Gruyter, 2008), 5-6.

be assumed that this was an idea circulating throughout the Greco-Roman world. For a Roman member of the Isis cult to view this fresco, depicting the relationship between Europe and Egypt, it is suggestive that this relationship was commonplace. It is meant to show the Romans the holy deliverance that following an Egyptian cult could bring, and would therefore encourage devotion to Isis and her native land.

The Adoration of the Mummy of Osiris

Advancing to the second fresco, the "Adoration of the Mummy of Osiris" also depicts this attempt to make Egypt and Isis comprehensible to a Roman audience. It was located directly next to the aforementioned fresco of Io and Isis,[17] and provides another aspect of the Isis narrative intended to inspire and amaze the cult members. This scene focuses on the specific story of the resurrection of Osiris, Isis' husband, and the importance of this mystical event. According to Plutarch's version of this legend, Osiris' brother, Seth, betrayed Osiris by creating a sarcophagus sized to Osiris' exact measurements that was offered at a banquet to anyone who fit inside it. Once Osiris climbed in, the sarcophagus was sealed and thrown into the Nile. Isis finds the chest and while she leaves it alone, Seth discovers it and cuts Osiris' body into fourteen parts and scatters it throughout the Nile Valley. Isis then goes and finds each part and magically reassembles and heals Osiris.[18] In this fresco, it seems that this scene of resurrection is yet to happen and Osiris is still sealed in this sarcophagus.

The fresco is painted in a very ethereal and aesthetically pleasing way, with an Egyptian-style temple or portal in the middle that holds a decorated sarcophagus and a hierogrammateus. It also contains a type of Egyptian priest, holding a tray of offerings toward the sarcophagus.[19] Furthermore, there are other Egyptian stylistic or cultural references in this fresco, such as the fisherman on the river. This is done in a European romantic style based on the color choices and painting form. There is not a notable difference between other Roman frescoes and this one in terms of style, similar to the aforementioned Io and Isis fresco where they both resemble Roman women.

Additionally, the hazy style and resurrection scene of Osiris in this fresco may cause the viewer to posit life's mysteries, such as the relationship between life and death, specifically with "the magnificent sacral architecture and breathtaking nature…intimate that the mysteries of life and of the

Figure 3. *Fresco depicting ceremony in honor of the sarcophagus of Osiris.* (Photograph from ArchaiOptix, *Wall-painting - idyllic landscape with sarcophagus of Osiris*, October 6, 2018, https://commons.wikimedia.org/wiki/File:Wall_painting_-_idyllic_landscape_with_sarcophagus_of_Osiris_-_Pompeii_%28VIII_7_28_-_sanctuary_of_Isis_-_ekklesiasterion%29_-_Napoli_MAN_8570_-_01.jpg).

[17] David L. Balch, "The Suffering of Isis/Io and Paul's Portrait of Christ Crucified (Gal. 3:1): Frescoes in Pompeian and Roman Houses and in the Temple of Isis in Pompeii," *The Journal of Religion* 83, no. 1 (2003): 48.
[18] Hart, 123-4.
[19] Frederick E. Brenk, "A Gleaming Ray: Blessed Afterlife in the Mysteries," *Illinois Classical Studies* 18 (1993): 162.

goddess are ultimately indistinguishable."[20] The misty background and exotic Egyptian ceremony taking place reflect the mysterious aspect of this cult and portray how this scene is related to death and rebirth through magical means. This will not only cause the viewer to think about the overall death and rebirth cycle, but specifically the Egyptian cycle of death and rebirth. The fresco does not portray Osiris after he is resurrected but instead chooses to depict the scene right before, which includes a sarcophagus. Mummies are stereotypically very Egyptian, and it would not have been as compelling or foreign if Osiris' sarcophagus was not included.

From gazing at this fresco, a follower of the Isis cult would gain a sense of the holy salvation that this cult allows. With Osiris depicted in "such an unreal atmosphere, a sudden, unexpected, and supernatural transition from death to life seems to await Osiris and all who follow his mysteries,"[21] this fresco highlights the resurrection of Osiris and its relation to Isis by demonstrating that these initiates are choosing to follow the deity that made the impossible possible: reversing the seemingly permanent state of death. As mentioned above, it is noteworthy that Osiris is still encased in his sarcophagus and not yet in his earthly form, and that instead of the ribbons being tied tightly around the sarcophagus they appear to be loose and ready to be broken. These aspects of the fresco offer a sense of anticipation and excitement about what the initiates know is upcoming, and provide evidence as to why they should follow Isis. By studying this fresco, they can imagine this astonishing resurrection and healing process that is about to be completed, and imagine for themselves what it would be like.

Conclusion

Similar to how European Roman Catholics created grand stained glass windows for their churches and cathedrals for instruction and adoration, these fresco paintings were meant to have a similar purpose. They portray positive narratives of Isis and Egypt and provide explanations of why the initiates of the Pompeii cult were dedicated to this deity. Just as "Catholic social teaching [provides] visual images that educate, challenge, and nourish the viewer…they function both as a form of catechesis and as sources for students of…Catholic history,"[22] these frescoes acted as narratives for teaching initiates about the mysteries of Isis. They depict Isis' relationship with other deities such as Io and Osiris, while creating an appealing Roman version of this Egyptian goddess to be consumed by the followers. As such, the presence of the grand frescoes of "Isis and Io at Canopus" and the "Adoration of the Mummy of Osiris" in one of the main meeting areas of the temple, the ekklesiasterion, demonstrated some of the "the deepest and most consoling meaning of the Isis religion…resurrection, redemption, and survival after death in a better world."[23]

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[20] Ibid., 162.
[21] Ibid., 162-3.
[22] Joseph F. Chorpenning, "[About the Cover]: Catholic Social Teaching in the Stained Glass," *American Catholic Studies* 115, no. 4 (2004): 80.
[23] O. Elia, Pompei III-IV: Le pitture del Tempio di Iside, Monumenti della pittura anlica scoperti in Italia. Sezione terza: La pittura ellenistico-romana (Rome 1941) 33-4. Quoted in Brenk, 163.

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About the Author

I got involved with this research when I was enrolled in CLST294: Ancient Rome in Context, where I actually got to travel to Italy with two professors in the Religion & Classics Dept. I supplemented that course with this independent study about the Cult of Isis in the Roman Empire because that was something I was very interested in before, during, and after the trip to Italy! Some advice for other students pursuing research would be to not limit yourself based on what the stereotypical definition of "research" is. Research in the Humanities looks very different from STEM research, but that doesn't mean that you shouldn't pursue it because it is just as important!

On the Role of "Therefore" in Argumentative Discourse

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In natural language, causality is everywhere. From the order in which information is delivered, to verbal or gestural emphasis, plethoras of unspoken content are nonetheless relayed to our conversational partners. Upon hearing "I went outside. It rained. I got soaked", the most natural inference is that the speaker was soaked by the rain, and not by an errant miscreant with a water gun. However, there are also conversational instances in which we wish to make very clear exactly what causal relation applies between the events that transpired, upon which we employ argument connectives such as 'then', 'hence', 'thus', and the object of my reflection: 'therefore'. For formal language connectives, whether complex expressions can be formed depends solely on the truth-values of the expressions involved. But for natural language counterparts, there are often additional parameters for felicitous use. 'Despite' gets transcribed as a variant of 'and' (&), although 'φ&Ψ' does not carry the nuance of an apparent contradiction between propositions in 'φ, despite Ψ'. Further pragmatic criteria for felicitous use suggests itself beyond purely semantic considerations. In the case of argument connectives, not all inferences by virtue of form[1] appear to register as actual felicitous inferences which can be made. I will seek to explore what about 'therefore' contributes to its peculiarity and discern criteria for its correct use in discourse.

1. The inference problem

In the most simplistic of terms, use of 'therefore' tends to mean "A is true. B is true. C is in some way supported by the truth of A and B, so C is true." What could "support" be? Robert Stalnaker[2] uses a structure called the direct argument to evidence the differences between what is syntactically implied and the inference made by the speaker.

(1) Either the butler did it, or the gardener did it. Therefore, if the butler didn't do it, the gardener did. (Stalnaker, 1975)

At first glance, this appears straightforward. The premise of the argument introduces a disjunction, of which at least one disjunct must be true, and the conclusion narrows down the identity of the culprit following from the assumption that one disjunct was false. The conclusion is an indicative conditional, informing us of the truth of some consequent from the truth of an antecedent. Problems arise when trying to quantify the nature of the implication made: if we afford that the indicative conditional is logically equivalent to the material conditional, written as A→B, which has somewhat counterintuitive truth conditions.

(2) The butler did it. Therefore, if the butler didn't do it, the gardener did. (Stalnaker, 1975)

In this world, we are given the culpability of the butler as fact. The subsequent falsity of the antecedent is enough to make the conditional true. But we do not wish for this occurrence of the conclusion to be true: there is no information which necessarily incriminates the gardener! If we want to the indicative conditional in (1) to be true, there must be a way of distinguishing the indicative from the material conditional to avoid nonsensical results like that in (2). To preserve the direct argument, it would appear that we need a different way to quantify its conditional.

Although the scope of conditionals far exceeds argumentative discourse, they present an excellent starting point for understanding the distinction between reasonable inference, the pragmatic notion of arguments whose conclusions we accept as following from their premises, and entailment, the semantic notion of an argument being valid if it is impossible for the premises to be true without making the conclusion true. We want pragmatic arguments to be formally true, without making paradoxical or incomplete formal constructions acceptable for use.

2. Interaction of 'therefore' with other operators

'Therefore' can be found in two types of arguments: categorical and suppositional. In categorical arguments, the truth of the premises is affirmed in the world of the argument; in suppositional cases, they are supposed, or assumed. This may be modeled as jumping over to the nearest possible world in which the premises can be satisfied. Unlike other connectives which link propositions, 'therefore' appears to link contextual elements. The semantic truth of a phrase is not always re-

[1]Referring to Grice's (1975) notion of conventional implicatures
[2]Stalnaker, R. (1975). "Indicative Conditionals." Philosophia, 5 (3), pp. 269-288

quired to affirm a logically following conclusion:

(3) I got soaked by the rain. Therefore, I dried my hair.

Nothing about the semantic truth-conditions of my getting soaked leads to drying my hair; and yet, it seems perfectly acceptable. Stalnaker proposes that "a conditional statement is an assertion that the consequent is true, not necessarily in the world as it is, but in the world as it would be if the antecedent were true." The meaning of 'therefore' statements appears to be equivalent to modal conditionals, such as:

(4) If I got soaked by the rain, I must have dried my hair.

Allowing for such an equivalence, we can understand 'therefore' as a pragmatic notion, highlighting causal relations in either actual or hypothetical sets of worlds. The mental operation in (4) would be along the lines of "If I got soaked by the rain, my hair would have been wet. I would have wanted to dry my hair. Without cause to behave otherwise, I would have dried it."

'Therefore' also appears to differ in scope from other logical connectives and structures. Kocurek and Pavese[3] (2022) note that 'therefore' appears to have restrictive syntax concerning its embedding within complex sentences. It can embed under conjunction but not disjunction, as demonstrated in (5).

Furthermore, 'therefore' can embed under modals and negation but appears to take wide scope, even when its syntactic placement should lead to narrow scope (see (6)).

(5)

a. I got soaked by the rain and I therefore dried my hair.[4]

b. I got soaked by the rain. Either I therefore either dried my hair or I therefore did nothing. → There cannot be two contradictory reasonable inferences! Inconsistent.

(6)

a. I got soaked by the rain. I might therefore dry my hair.

→ Appears equivalent to "Therefore, I might dry my hair."

b. It wasn't raining. I will not, therefore, need to dry my hair. → Appears equivalent to "Therefore, I will not need to dry my hair."

What do the evasions in (5) and (6) signify for argumentative discourse? When we make an argument, the aim is generally to prove a point, to show that some state of affairs is evident from existing or hypothetical evidence. We do not aim to change the face of reality, or create new information, but to bring to light what should already be available to others, if they are able to make the same logical deductions. As 'therefore' does not create new truth-functional relations, we might classify it as more of a meta-discourse operator, interacting with speaker intention and what is communicated, rather than the propositional content of what is being stated. This stronger role might explain why it evades efforts to restrict its meaning at the propositional level.

Concerning the cases in (6), Kocurek and Pavese note that it is possible to force 'therefore' to take narrow scope by embedding it into a conjunction. However, they note that the relationship between causally linked elements seems to be preserved, even when the conjunction is negated. The reasonable inference from 'therefore' still projects out, unlike analogous modals.

(7) a. It is not the case that I got soaked by the rain and therefore dried my hair. → Drying my hair would still be the intended course of action should it get wet.

b. It is not the case that I got soaked by the rain and must have dried my hair. → The second conjunct is not dependent on the first and the entailment disappears.

Furthermore, the entailment signaled by 'therefore' cannot be referred to by demonstratives, as evidenced in (8). Only target the truth-value of the narrative can be targeted, rather than the reasonable inference from the affirmation of the antecedent. The entailment is undamaged.

(8)

A: "Mary is English and therefore brave." (from Grice, 1975)

B: "That is false."

The cases listed so far have served to illustrate the unusual behavior that 'therefore' displays when interacting with other restrictive structures. Argument connectives appear to convey particularly strong relations, which are difficult to encroach upon with the usual restrictions of scope, negation, and denial via demonstrative. Having listed ways 'therefore' does not work, we shall now consider

[3]Kocurek, A.W., Pavese, C. (2022) "The Dynamics of Argumentative Discourse." J Philos Logic, 51, pp. 413–456. https://doi.org/10.1007/s10992-021-09636-2.
[4]The sequence of observations in this section is a less interesting restitution of Kocurek & Pavese (2022), simplified and commentated to provide background for my characterization of 'therefore' as a meta-discourse operator. A thorough perusal of their work is highly recommended for a better explanation of the unusual behavior described.

what is required for its use in argumentative discourse.

3. Anaphoric 'therefore'

Argument connectives are employed to highlight a point following from other points. As such, if there are no previous claims in the conversational discourse, unprompted 'therefore' statements (8a) appear confusing and nonsensical. While not false, 'therefore' statements linking two unrelated declarative propositions as seen in (8b) don't register as fully correct. To be employed correctly, 'therefore' must have some kind of referent; unlike pronouns, the referent is a relevant state of affairs rather than a specific individual or occurrence.

(9)

a. Therefore, I ate dinner.

b. The sky is blue. Therefore, I ate dinner.

Kocurek and Pavese propose a test to ensure that 'therefore' statements don't add any new information to the conversational context: an utterance of the form "therefore, φ" is felicitous if and only if the information encoded by φ is already part of the information state of the conversational context, and is undefined otherwise. We can thus affirm that (9b) fails because the relevant information was not available in the information state between author and reader. The test also allows for entailment to project from negated 'therefore' statements if the 'therefore' affirmation follows from information previously available. Consequently, existing information needs to be in the form of truth-functional propositions, like declarative sentences. However, 'therefore' expressions themselves can be in the form of imperatives (for example, in the case of

a recommendation based on evidence) or questions (such as when an interrogation aims to highlight a seeming lack of causal relation, or including a rhetorical question in telling a story). This supports analysis of 'therefore' as a meta-discourse connective, since it follows that if there is no informational referent to connect to the pronouncement, no relation can be expressed[5].

Their model includes extensions of 'therefore' use, with imperatives encoding a preference relation along with the possible highlighting and causal functions, with the clause that the preference relates two states of information supported by the state of the world—avoiding the caveat of 'reasonable' inference to impossible things. Interrogatives, on the other hand, would take on a partitioning role of the context between worlds, distinguishing those in which the causality succeeds, and those where it does not. While these extensions succeed in representing dimensions of 'therefore' use, it is more interesting to note that non-declarative dimensions are still characterized by the truth-values of meta-discourse criteria, even though imperatives and interrogatives are concerned by possibility of existence and truth rather than prior information.

Lastly, it has been theorized that 'therefore' shares traits of performative speech; similarities include the requirement for pre-existing information for 'therefore,' which might be analogous to preemptive Searlean felicity conditions for performative speech, as well as the importance of speaker intention in highlighting information with 'therefore' and performing an action in the latter. But if we restrict 'therefore' statements from introducing new information, it is unclear how compatible this may be with performative speech creating new realities.

4. Stalnaker's dynamic model

Returning to Stalnaker and the indicative conditional, we can begin modeling dynamic arguments. Stalnaker's issue was allowing for reasonable inference that is not logically equivalent to the material conditional, to avoid paradoxical conclusions that did not line up with common use of 'therefore.' His strategy was to distinguish between semantic validity and pragmatic correctness with a possible worlds model. Within our world, the conversational common ground contains several propositions that are unquestioned (either being indisputably true, or not-at-issue and accepted, or both); these agreements characterize the context set, a set of possible worlds in which the same propositions are held. The nearby possible worlds are thereby the closest, or the most salient, with respect to hypothetical constructions arising within discussions in our present world. Stalnaker sifts out reasonable inference with the use of an inference pattern called "Or-to-If." Or-to-If stipulates that from a disjunction AvB, it follows syntactically that ~A→B[6].

He holds that consequents of reasonable inferences are admitted if the antecedent is admitted by the context. In the case of Or-to-If, AvB is accepted

[5]This also applies to traditional connectives: to be well-formed according to the rules of syntax, connectives like '&' and 'v' need two formulas to connect. '&φ' is meaningless (barring perhaps a transcription of incorrect speech).
[6]The discerning reader will remark that this is simply an application of a derived rule in symbolic logic, the Law of the Conditional, which shifts between conditionals and their logical equivalents.
[7]While their theory is admirably constructed and reasonably effective, along their journey Kocurek and Pavese (2022) accumulate a vast number of discarded formalisms upon which the discerning reader need not dwell. I found sections 4.1-4.3 to be particularly useful in understanding their argument.

if the context c admits A&~B or if c admits ~A&B, meaning that the context admits only the restrictive 'v'. The result of updating the context with AvB thus creates a new context c' that is incompatible with ~A&~B, and compatible with either of the restrictive forms that c was compatible with. Hence, the truth-conditions for the conditional expression are met if c admitted ~A&C, and the conclusion can be felicitous. The context's processing of the conditional's antecedent creates specific truth conditions that satisfy one row of the material conditional's truth conditions, but not the rest. This results in an expression which looks like the material conditional, but truth-factually corresponds to only one of its three possible relations of antecedent to consequent.

Stalnaker's argument is interesting because it incorporates the pragmatic notion of conversational context, and applies it to the formal notion of possible worlds. He holds that utterance of an antecedent within a world triggers a selection function extending to the context set: this is how we are able to make pronouncements about hypothetical situations, with many things remaining part of the common ground. He disentangles the very different pathways undergone to get the conclusions in (1) and (2): in (1), the antecedent of the 'therefore' statement leads to the selection of a world compatible with the existing context, and yields a distinct result expressed in conditional form. In (2), the same conditional results from punching in truth-values and getting the same conditional, which encodes for more possible truth-values (two possibilities compared to one). This work sets up the possibility of exploring relations between worlds.

5. Modeling dynamic argumentative discourse: Kocurek and Pavese

Having established that felicitous 'therefore' statements cannot spring into existence without precedence, we need to determine what grounds can support such statements. Crucially, it was brought up that referent information has to be already present in the conversational discourse. However, as friends of absent-minded storytellers will know, it can be difficult to recall what is admitted in the information state, and that state changes throughout the exchange.

(10) I got soaked by the rain. Hours later, I showered. Therefore, I dried my hair.

Comparing (10) with (3), it is not clear what sequence of events is meant to serve as a referent. It may be that I dried my hair as a result of the rain; or I may have let it dry in the intermittent hours, and dried it after showering. To be felicitous, the ambiguity should be eliminated; one option might be to have the referent be the most recent and most salient object in the discourse.

Kocurek and Pavese's solution[7] can be compared to accommodation of contexts for presuppositions, and envisions the construction of labeled layers of context throughout a conversational exchange. Hypotheticals and suppositions trigger the creation of a new level, at which the added claims are accepted. Information flows upwards, so the parties' previously-accepted truths will still apply, unless stated otherwise. This allows treatment of both categorical and suppositional uses of 'therefore': if the conclusion is categorical, the relevant antecedent information is contained at the most fundamental level of the interlocuters' accepted beliefs, and the resulting 'therefore' statement is true at all levels of the conversation. If the conclusion is suppositional, it is situated at an elevated level of the context and will only be held as true at levels where its antecedent claims are accepted, and those following upwards. It is important to note that levels are continuously being created and populated throughout the conversation, following the idea that 'therefore' statements in dynamic entailments are noncommutative—one cannot state 'therefore, φ' and then list the relevant evidence for φ. It is difficult to imagine a purely static 'therefore' entailment: much of the argumentative power appears to be derived from the causal and highlighting roles, which become weakened or confused when the order of utterances is changed, which is permitted by commutative static entailment.

When navigating the conversational discourse, speakers and listeners must endeavor to clarify the level of supposition to which they are referring; else their conclusions might become ambiguous and lack proper setup. Additionally, this model allows us to represent fallacious arguments with far-reaching conclusions, by showing the discrepancy between the levels at which their claims might be accepted as true, and the levels to which they extend the claims. One weakness may be whether this structure holds for inductive instances of 'therefore,' and cases with inappropriately qualified arguments, where it is unclear of whether the assumptions made provide sufficient ground for conclusion. It may be that in such cases, 'therefore' expresses the speaker's thoughts on the likelihood of the relation between propositions, rather than the truth of the relation itself. This avenue invites further consideration to distinguish between 'therefore' as a marker of factual states of informa-

'therefore' as a marker of factual states of information, and an expression of speaker opinion.

6. Conclusion

'Therefore' exhibits many peculiarities: it takes wide scope over modals and negation when it syntactically should not, and refuses to be picked out by demonstratives. It exhibits anaphoric behavior and requires a referent, yet the referent is difficult to pin down in words. From Stalnaker's analysis, we take ideas of indicative conditionals (including 'therefore' statements) reaching out to possible worlds, and of contexts processing truth-conditions to create direct inference arguments that are intuitively sound, but formally invalid. From Kocurek and Pavese, we can reflect upon the specific contextual interactions that can occur within layers of our own world, and reflect on the criteria for upper-layer conclusions to pass into reality.

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About the Author

As a student of philosophy, I am drawn to the interplay between logic and language. This interest has led me to pursue a combination of logic sciences, semantics and pragmatics courses in order to study the ways in which communication and meaning arise from language. This project, which started as my capstone paper for Pragmatics, allowed me to draw on my logic background and explore a new dimension of language.

For other undergrads: find what you're passionate about, no matter how strange.

The Role of PDE10A in DOX-induced Cardiotoxicity

Sparsh Kumar '24, *Multidisciplinary Studies*
Advised by Si Chen, Aab Cardiovascular Research Institute

Introduction

Background and Current Therapies

Cardiovascular diseases (CVDs) are a leading cause of death in the United States and globally. They cause approximately 39% of deaths annually (1). Epidemiologic studies have found that CVD is prevalent in adults over the age of 60 years and that it is largely preventable (7). CVD is an umbrella term for disorders affecting circulation and the heart, including heart failure and coronary artery disease. Atherosclerosis, the buildup of fat and cholesterol-based plaques on the arterial walls, underlies most CVD. The buildup of plaque in arterial walls can be due to multiple factors and can start in early childhood, eventually resulting in coronary complications in adulthood (7). Cardiovascular risk factors that promote the development of CVD can include diabetes, high blood lipid levels, obesity, lifestyle choices such as physical inactivity, high blood pressure, and high cholesterol.

Trials focusing on primary preventative measures for cardiovascular disease have demonstrated significant results. For example, studies investigating the treatment of hypertension and high cholesterol through pharmaceuticals and diet have found beneficial effects (7). Similarly, increased consumption of fruits and vegetables, decreased consumption of saturated fats and free sugars, and increased activity levels reduce the risk of cardiovascular disease and act as a primary prevention method. All of the prevention measures listed are also used for secondary prevention measures. In addition to those listed above, low-dose aspirin, beta-blockers, and ACE inhibitors are also established secondary prevention interventions (8). Although multiple prevention techniques are available to treat CVD, it remains one of the leading causes of death. Therefore, it is vital to develop more effective therapies that can reduce the morbidity and mortality of CVD.

Molecular Mechanisms

Pre-existing health conditions such as hypertension, diabetes, and acquired cardiomyopathies can overwork the heart, leading to the progression and development of heart failure. Heart failure is characterized by cardiac hypertrophy (enlargement of the heart muscle), cardiac cell death, and fibrosis (scarring of the heart tissue), all of which eventually lead to a significant decline in cardiac function (2). Myocardial hypertrophy is a response of the heart to pathological or physiological stimuli. Physiological stimuli can include growth, pregnancy, or exercise, which can trigger the heart to increase in size to maintain the body's increased demands. Hypertrophy due to physiological stimuli is not associated with fibrosis of the myocardial tissue. On the other hand, pathological stimuli, such ascardiac injury, hypertension, or neurohormonal activation, lead to the hypertrophy of the cardiac muscle and are associated with fibrosis. Hypertrophy is also related to disadvantageous changes in the extracellular matrix and unfavorable ratios of cardiac apoptosis and regeneration (2). Overall, myocardial hypertrophy is a major predictor of the progression of heart disease.

On the molecular level, pre-existing health conditions like hypertension are associated with putting biomechanical stress on the heart muscle, which can stimulate specific G-protein coupled receptors, triggering the synthesis of cyclic nucleotides and activation or inhibition of multiple downstream targets. This activation can eventually lead to increased protein synthesis and cardiac remodeling or molecular and cellular changes in the heart, changing heart and cell size. For instance, due to persistent biomechanical stress, the heart will undergo pathological remodeling, resulting in heart muscle enlargement. However, this type of remodeling is associated with fibrosis, not with a greater cardiac output. It decreases the heart's function by reducing its structural adaptation, leading to dilation and heart failure. This type of cardiac remodeling is also related to cardiac cell death, elongation, and contractile dysfunction (2).

In addition to cardiomyocyte loss and elongation, chronic stress can trigger cardiac fibroblasts to convert into highly proliferative or reproductive fibroblasts that can migrate. This leads to fibrosis of the heart tissue contributing to the development of heart failure, as mentioned previously (3). Even with existing treatments and interventions, cardiovascular disease continues to be a significant cause of morbidity and mortality. However, there have been significant advances in the knowledge regarding molecular pathways and mechanisms involved in pathological hypertrophy and remodeling of the heart (2). Therefore, closer examination of

the molecular pathways involved in the deterioration of the heart muscle can provide critical answers for developing effective therapies.

PDEs

Homing in on the molecular pathways associated with cardiac remodeling, cyclic nucleotide signaling plays a significant role in this aspect. Any disturbance in the homeostatic levels of cyclic nucleotides results in the progression of various diseases (3). Cyclic nucleotide phosphodiesterases (PDEs) are enzymes responsible for the degradation of cyclic nucleotides like cAMP and cGMP. By degrading these molecules, PDEs can alter the intracellular levels of cyclic nucleotides, therefore playing a role in the duration of signals in the cell and, in turn, affecting cyclic nucleotide signaling and cardiac remodeling. Of the 11 PDE gene families, PDE10A was first isolated as a cAMP-cGMP dual phosphodiesterase with high brain expression. This was discovered by measuring mRNA expression of the PDE10A protein throughout the rat brain. It was found that this protein was overexpressed by striatal medium spiny neurons (4). This led to the development of multiple PDE10A inhibitors targeted at treating neurodegenerative diseases like Huntington's and Schizophrenia. It should be noted that multiple PDE10A inhibitors have been tested in humans and passed phase I clinical trials. Consequently, the safety of these inhibitors has been established for use in humans.

Preliminary studies have explored the molecular mechanism of PDE10A and its potential effects in the cardiovascular setting. It was found that there is an upregulation of PDE10A activity in diseased hearts, as seen in Figure 1. These data suggest a therapeutic possibility through the inhibition of this phosphodiesterase. However, the mechanism of PDE10A in the heart is mainly unknown. Therefore, investigating the role of PDE10A-mediated downstream signaling in pathological cardiac remodeling is of interest and can also present more information about the pathway, which can help develop targeted therapies.

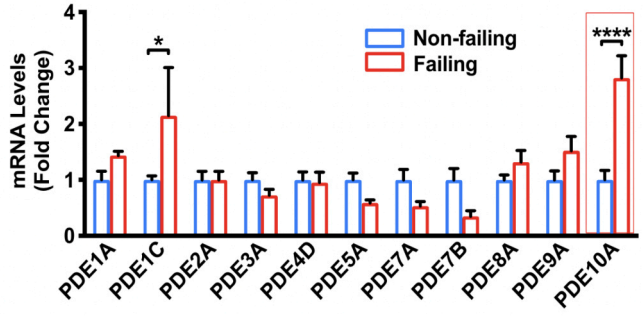

Figure 1) Expression of Different PDEs in Diseased and Normal Hearts

Furthermore, the effects of a PDE10A inhibitor on cardiomyocyte hypertrophy were also investigated. To do this, hypertrophic stimuli such as angiotensin II were inserted into adult mice, and then cardiomyocyte hypertrophy was analyzed by measuring the cell surface areas. It was found that cell hypertrophy in angiotensin II stimulated cardiomyocytes, when combined with the PDE10A inhibitor TP-10, was essentially abolished. Overall, the hypertrophic effects associated with the upregulation of PDE10A were attenuated after adding its specific inhibitor. In addition, it was also found that the TP-10 inhibitor was very specific to its substrate, PDE10A, eliminating the possibility of off-target effects with the use of the inhibitor. The inhibitor was also used with knockout mice models to confirm these results. It was observed that TP-10 displayed no additional effect on the hypertrophic-induced cardiomyocytes, showing that the protective effects of TP-10 are through PDE10A inhibition and not due to another mechanism (3).

Overall, it has been established that PDE10A directly promotes the pathogenesis of cardiomyocytes (3). Also, TP-10 is a selective inhibitor against PDE10A and is associated with reversing or abolishing the cardiac remodeling related to high PDE10A levels.

Cardiotoxicity and Cancer

Cardiovascular diseases are also a leading cause of mortality in cancer patients. Continuation of cancer treatments for an extended period of time has harmful effects on patients' organ systems, particularly the cardiovascular system (9). It can result in increased atherosclerosis, eventually progressing into cardiovascular disease. Even though heart disease and cancer are usually considered two independent conditions, they contribute to 50% of the overall fatalities in the United States. Some risk factors for CVD and cancer include lifestyle choices like smoking, obesity, hypertension, diabetes, and physical inactivity. Anticancer therapies like radiotherapies, chemotherapies, and hormonal cancer treatments can enhance or further CVD when combined with cardiovascular risk factors such as physical inactivity and increased body weight (9). Overall, cardiotoxicity is a limitation of anticancer therapy and contributes to the lower survivorship of cancer patients.

A specific and successful anticancer therapy is doxorubicin (DOX). While successful as an anticancer therapy, DOX is associated with cardiotoxicity that can present itself as cardiomyopathy or the inability of the heart to pump blood effectively to the rest of the body. This can eventually lead to cardiac dysfunction as well as heart failure. Therefore, developing interventions or strategies to im-

prove this chemotherapy is essential. DOX-induced cardiotoxicity is associated with cellular events that eventually lead to cardiomyocyte death via apoptosis and necrosis. Some cellular events include increased reactive oxygen species (ROS) production, which can stimulate the sarcoplasmic reticulum (SR), increasing intracellular calcium levels. This increase in calcium levels can supplement ROS production. Calcium-releasing sites on the SR in cardiomyocytes are located near the mitochondria, and, due to the oxidative stress via ROS, the intracellular calcium levels in the mitochondria can increase past their threshold. This results in the release of cytochrome C and apoptotic factors from the mitochondria, leading to cardiomyocyte death. DOX-induced cardiotoxicity is also associated with increased protein degradation and changes in regulating proteins involved in contraction, the SR, and functioning of the mitochondria. Changes in these protein levels can cause cardiac atrophy and dysfunction (5). Altogether, DOX-induced cardiotoxicity can lead to the pathogenesis of cardiovascular disease and, eventually, heart failure.

As mentioned earlier, PDE10A plays a vital role in the pathogenesis of cardiomyocytes. In addition, it has been found that there is a high level of PDE10 in colon and lung cancer as well as colorectal and lung cancer growth (6). This was done by collecting tissue samples from mouse models with induced colon and lung cancers. Tissue samples indicated that PDE10 is overexpressed in mice with colon tumors compared to normal mice without cancer. This suggests an unrecognized relationship between PDE10 and the cardiotoxicity associated with anticancer therapies. As seen in Figure 2 and as mentioned before, there is a known relationship between PDE10A and myocardial hypertrophy. Additionally, doxorubicin treatment is associated with cardiotoxicity, including cardiac atrophy and cell death, however, the mechanism of this interaction is now known. Therefore, I plan to investigate not only the role of PDE10A in tumor growth and the cardiotoxicity accompanying chemotherapy but also if using a PDE10A-specific inhibitor (TP-10) can demonstrate the protective effects against cardiac remodeling as observed in the preliminary studies mentioned above.

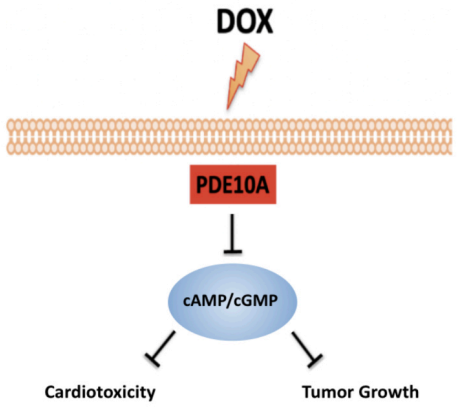

Figure 2) Proposed mechanism of the role of PDE10A in DOX-induced cardiotoxicity

To evaluate this hypothesis, several experiments were conducted to determine if PDE10A plays a role in the cardiovascular complications observed in anticancer therapies. Firstly, basal levels of PDE10A expression in cultured cell lines and animal models treated with or without doxorubicin were determined. The purpose of this experiment was to gather insight about the normal expression levels of PDE10A in cells after chemotherapeutic treatment and whether upregulation of the protein is observed, similar to cells in diseased hearts. In addition, an in vitro study was performed to investigate the effects of PDE10A inhibition on DOX-induced cardiotoxicity.

Methods

Mice on a C57/B16J background were utilized and categorized into different groups. Mice were either treated with the PDE10A inhibitor, TP-10 or had the PDE10A gene knocked out to measure the impact of PDE10A deficiency. These two groups were further divided to receive or not receive DOX treatment. After the treatment period, the heart tissues of the mice were harvested. The impact of the treatment was determined by measuring PDE10A mRNA levels and PDE10A protein levels as well as by analyzing the cell surface areas of the cardiomyocytes. mRNA levels were measured through RT-qPCR, protein levels through western blot, and cell surface areas were measured on Fiji, an image processing system based on a larger Java program called ImageJ. After individual measurements of all cells were obtained, these values were averaged and compared to experimental and control group values to determine treatment effects. For each experimental group, approximately 150 cells were analyzed and averaged. This was also done in an in vitro study using two ovarian cancer cell lines, OCC1 and A2780, to assess the impact of TP-10 and DOX treatment. Additionally, mice were injected with ovarian cancer xenograft samples and then subjected to PDE10A inhibition and DOX treatment to inspect the effects of inhibition on tumor size.

Data and Results

PDE10A levels in DOX-treated mice

An upregulation of PDE10A protein levels was observed in mouse hearts after the DOX treatment. Figure 3 displays a western blot analysis of the PDE10A protein levels in control and DOX-treated hearts. PDE10A should reveal a band at around 95 kD on the western blot, as seen in Figure 3. Darker bands indicate a higher expression of the protein in the sample. Therefore, it can be observed that the DOX-treated mice hearts exhibited higher protein expression levels when compared to mice hearts that did not receive this treatment. The membrane was also incubated in GAPDH to ensure the protein loading was accurate when performing the western blot. The data suggest that DOX-induced hearts exhibit a higher level of PDE10A protein on average compared to no treatment.

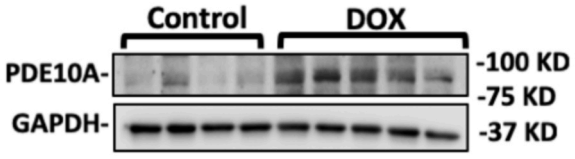

Figure 3) Western blot analysis for PDE10A in control and DOX-treated mice hearts.

PDE10A levels in DOX-treated cancer cells

A similar trend was observed in an in vitro study using two ovarian cancer cell lines, A2780 and OCC1. These two cancer cell lines were cultured and split into four groups. These groups include control (no treatment), DOX-treated cells, TP-10 treated cells, and DOX and TP-10 treated cells. The different treatments were performed for six hours. After the treatment period, mRNA levels of the PDE10A protein were measured. It can be seen from Figure 4 that when DOX is added to the A2780 cells, the mRNA levels of the PDE10A protein are significantly increased, similar to the in vivo results. However, this trend was not observed in the OCC1 cell line. The mRNA levels were significantly decreased after DOX was added to the cultured cells.

Effects of TP-10 on cancer cells

The effects of the PDE10A inhibitor, TP-10, on the cancer cell lines, were also determined. As seen in Figure 4, when TP-10 and DOX were added to the OCC1 cells, the mRNA expression of PDE10A was decreased. This indicates that TP-10 can reduce the protein levels of PDE10A, which has been associated with protective effects on the heart, as mentioned before. However, the same trend was not observed in the A2780 ovarian cancer cell line.

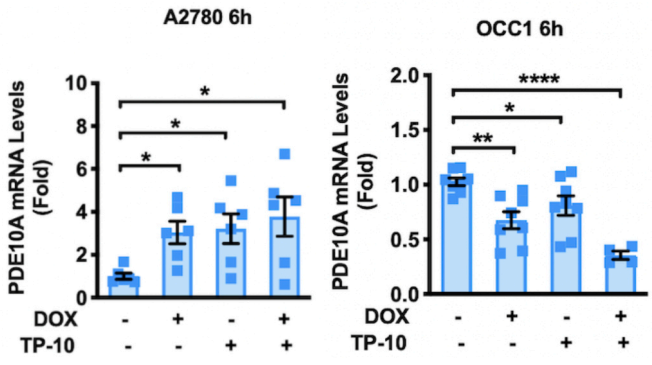

Figure 4) mRNA levels of PDE10A in ovarian cancer cell lines with various treatments

PDE10A levels in xenograft samples

PDE10A protein levels were also measured in A2780 xenograft samples. A2780 cells were injected into mice, and, after ten days, the developed tumors were isolated. The animals were also injected with either DOX or TP-10, both of these reagents, or none. Consistent with the A2780 cell line results, PDE10A protein levels were upregulated after the DOX and TP-10 treatment, as seen in Figure 5. The protein levels were slightly decreased when both DOX and TP-10 were added to the animals compared to only the DOX treatment.

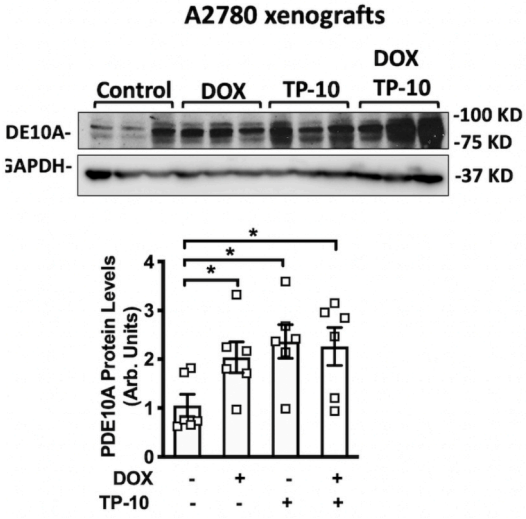

Figure 5) Western blot analysis of PDE10A in A2780 xenograft samples.

Tumor volume changes in xenograft samples:

The tumor growth was measured in mice implanted with the A2780 xenograft samples. It can be seen from Figure 6 that, when treated with TP-10 or DOX, the tumor growth effects are significantly decreased compared to the control samples. When administered together, these effects are amplified,

exhibiting a greater efficacy in attenuating tumor growth.

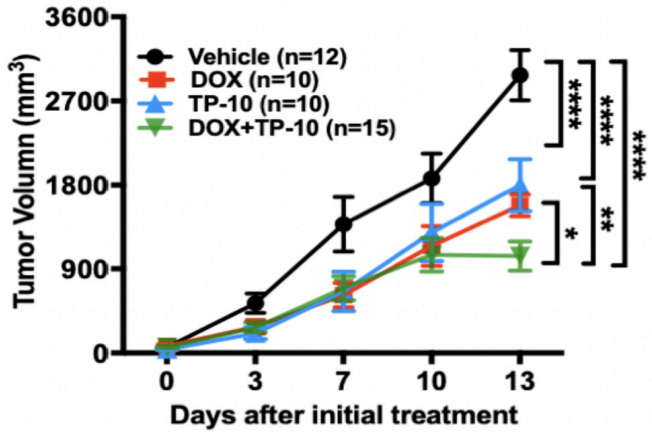

Figure 6) Tumor volume progression in A2780 xenograft models.

Discussion

Through the experiments, it can be established that PDE10A plays a role in DOX-induced cardiotoxicity. This was established through the in vivo study, which showed an upregulation in PDE10A levels in mice hearts treated with DOX, suggesting that PDE10A has some contribution to the cardiotoxicity observed. The data also shows that, when PDE10A is inhibited or is deficient, the negative effects of DOX on cardiac functioning are significantly decreased both in vivo and in vitro.

Moreover, PDE10A inhibition decreased tumor cell growth and potentiated the anticancer effects of DOX therapy, indicating a greater efficacy of the chemotherapy. As mentioned before, the TP-10 inhibitor has been used in clinical trials, and its safety has been established in humans, which makes it easier to employ in further studies. Overall, the findings suggest a potential role for PDE10A inhibitors in decreasing the negative effects of anticancer therapies on cardiovascular functioning.

Future Work

More research is needed to investigate the molecular mechanisms and pathways involved in DOX-induced cardiotoxicity. Also, the intersection between DOX and the PDE10A molecular pathway is of interest. Using different PDE10A inhibitors is also something that can be done in the future to investigate the specificity and whether particular inhibitors are more effective. In addition, an in vivo study with the inhibition of PDE10A can be undertaken and compared to the in vitro results from the ovarian cancer cell line to determine any observable differences.

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About the Author

Due to my family history of cardiovascular disease, I have always been interested in research in this area. Therefore, I started working in the Cardiovascular Research Institute (CVRI) at the Yan Lab during my second year at U of R. I was extremely interested in investigating the pathogenesis of heart failure. Specifically, I was interested in investigating whether certain intermediates of the pathway can be intervened upon through medication to disrupt the pathway, and eventually prevent adverse heart outcomes. I also recently started working on a clinical study examining intolerance to statin medications among cardiology patients to get more exposure to research further in the translational science spectrum. My advice for undergraduates interested in research is to get a broad range of experience and to get involved early!

How Confucianism and Daoism influence the characterization of fox spirits in Chinese classics SouShenJi 搜神记 during the Six Dynasties Period

Xuewen Geng '23, *Modern Languages and Cultures*

Advised by Elizabeth Weber, Department of Modern Languages and Cultures

Introduction

Fox spirits play an important and diverse role in the history of Chinese literature, and their positions remain prominent across East Asia (Johnson; Uther). They are a unique class of fictional figures that remain popular and favored by the audience across time, from the pre-Qin period (先秦时期) to contemporary media. Throughout their existence over thousands of years, the figure of the fox spirit has undergone frequent development and alteration according to the varying social atmosphere and cultural ideas of contemporary societies (Ren; Zhang). One of the most important periods for the development of this figure is the Six Dynasties period (六朝时期), when the genre of zhiguai (志怪), which specifically focuses on the existence or explanation of unnatural events, emerged and became popular (Campany). This genre inherited and continued the bizarre mythologies and religious worshiping rituals from previous dynasties, and simultaneously served as the foundation for future advancement of Chinese tales and fiction (Liu ch. 2; Shang). The different classes of fox spirits that were common in later times, such as the scholar male foxes, the seductive female foxes, and the elderly powerful evil foxes, all have origins in stories from the Six Dynasties period (Huntington 10-13). It is believed that both the chaotic social dynamics and the superstitious beliefs that were spreading during the later Han Dynasty (汉朝; before the start of the Six Dynasties Period) contributed to the development of this zhiguai genre, as people needed these strange tales to provide explanations for the "supernatural" events they experienced (Dewoskin 21-23; Fyler 29-30). In this paper, I would like to focus specifically on the influence of two social ideas, Confucianism and Daoism, on the characterization of fox spirits during the Six Dynasties period: which characteristics of fox spirit figures have emerged and how are they associated with the concept of these two social ideas? Although other materials will also be considered, I will focus my analysis on SouShenJi (搜神记) by Gan Bao (干宝; 1 – 336) from the Jin Dynasty (晋朝) as it both serves as a collection of stories prior to the Jin Dynasty and also as the largest and most influential source of new stories collected or written during the Six Dynasty period (Fang 2149-2151; Cheng 5-7). If not noted otherwise, the translations of quotations in this paper are done by the author of this paper based on the cited original Chinese materials.

Background on the development of the figure of fox spirits before the Six Dynasties period

The figure of fox spirits has been a focus of Chinese traditional literature and has occupied a uniquely prominent position in Chinese culture (Hungtington 2-3). Myths about foxes were assumed to be prevalent starting from the early times of Ancient China, as one of the earliest fox records can be dated to the early Qin dynasty from Shanhaijing (山海经)[1]. Shanhaijing is a collection of Ancient Chinese mythologies that explain the causes of natural events, historical events, and culture of ethnic peoples. In some of the chapters of this collection of ancient myths[2], a nine-tailed fox from Qingqiu (青丘) was mentioned as a monstrous creature that can eat humans and can also be eaten by humans to grant them the power of dispelling evil creatures ("There is a beast that looks alike to fox with nine tails, nine heads, and tiger claws. Its name is Long Zhi. It sounds like human infants and can prey humans.")[3]. Despite these horrendous images, however, there are also descriptions about virtuous fox figures during the same period of time that are considered to originate from the reproduction worship of local ancient clans. For example, Nv Jiao 女娇, the wife of Da Yu 大禹, was recorded to be a member of the clan of Tushan 涂山氏, which is believed to be a clan that worshiped white nine-tailed foxes because they were powerful symbols of reproduction. Although different sources describe their encounter and their marriage slightly differently, most of the materials include descriptions of how, when he was in Tushan, Da Yu saw a nine-tailed white fox and heard about the clan of Tushan singing about the good fortunes white foxes can bring[4]. This encounter was interpreted as a sign of Da Yu's later fortune of solving

the flood problem and becoming the king of Xia kingdom. In addition, it is also one of the earliest descriptions of foxes as auspicious beasts (often referred to as Rui Shou 瑞兽 in Chinese), which might have influenced the ritual of viewing foxes (especially uncommonly large or white foxes) as auspicious beasts during the Han dynasty (Kang ch. 1).

However, even though foxes were viewed as auspicious beasts during this period of time, earning them the respect and honor as the divine signs that confirm the great ruling ability of the current emperor, figures that are considered to be evil, the opposite of auspicious beasts, are more famous and popular in later dynasties, such as the seductive female fox figures that become dominant in stories after the Tang dynasty. This demonization (妖化) of the image of foxes took place around the late Han dynasty and the following Six Dynasties period (Dewoskin 21; Huntington ch. Introduction). In the following sections, I will present how a combination of social ideas (Confucianism and Daoism) has contributed to this transition, gendering, and sexualization in the fox spirit figure.

Confucianism, the auspicious beasts, and the Position of Foxes

The previously discussed attention given to auspicious beasts and other divine signs suggest a widespread public belief in the omnipotent Divine (天) during the Han dynasty. The Divine was often associated with the ruling family, as they were considered to be selected, promoted, and monitored by the Divine. Thus, divine signs that show the satisfaction of the divine to the current king and the ruling family can strengthen their positions. In order to utilize this mindset, the ruling class of the Han dynasty allowed a very relaxed social atmosphere for the spread of superstitious ideas and religions (Liu ch. 2; Chou ch. 3–4), which were strongly related to the later high respect and honor toward Confucianism, the state orthodoxy (国教) during the Han dynasty (Chou ch. 3). This prominent position of Confucianism can not be discussed without mentioning the most important Confucius figure during the Han dynasty, Dong Zhongshu (董仲舒 179 B.C. – 104 B.C.), who put forward the philosophical idea about the connection between the Divine and humans (天人感应). Following the previously mentioned logic, this idea of connection strengthens the authenticity of the Han emperors by suggesting that the Divine, mostly described as universal laws by philosophers prior to Dong, actually has thoughts and wills and is the omnipotent god that monitors and rules the world. Thus, he is suggesting that the human emperors of the Han dynasty were specially chosen and monitored by the Divine In addition, those signs sent by the divine indicate its attitudes toward the current emperor. For example, the spotting of auspicious beasts shows the Divine's satisfaction with the current emperor. He believes it is the courtiers' responsibility to correctly interpret those signs and make suggestions to the emperor accordingly (Puett ch. 8). Naturally, this revised version of Confucianism was favored by the Han emperors, and consequently, the emperors focused on the searching of auspicious beasts from all around the country and submission of them to the court to reconfirm the emperor's great ability. This belief in divine signs and foxes as auspicious signs might have reinforced the divine figure of foxes and contributed to the later close association between foxes and divine power. This interesting association can even be found in stories in which foxes were considered antagonists (for example, FengShenYanYi 封神演义). But since most of these types of fox stories occurred during the Ming dynasty, much later in time compared to our time of interest, the Six Dynasties period, we won't go into depth here.

During the rise of Confucianism, foxes, along with many other creatures, were mentioned in Confucius's works as beings with humanized virtues that fit into Confucian values in order to explain the world by applying Confucian standards and structures. For example, in Bai Hu Tong 白虎通 from the Han dynasty, foxes were described as a symbol for gentlemen (君子[5]) because "when foxes died they face where they were born, this shows how a gentleman should not forget his origin[6]." In Shuowen jiezi 说文解字, Xu Shen (许慎) concludes that "there are three virtues in foxes. First, their color is uniform; second, their bodies are shaped smaller on the front and bigger on the end; third, when they die they face their cave."[7] The first two points are interpreted as virtues because Confucians believe uniform color represents intelligence of balancing and this body shape represents a strict social standard as the sizes are in strict order (smaller at the front and bigger at the end). These Confucian descriptions of natural or interpreted behaviors of foxes integrated humanized characteristics, virtues or vices, into foxes to reflect the authors' moral opinions on similar behaviors or personalities in human society.

Thus, besides reinforcing the auspicious beast role of the foxes that originated from primitive reproduction worship and their rarity (the white foxes or the nine-tailed foxes), Confucianism during this time period also elaborated on foxes' connection with the divine by describing them as morally virtuous animals. In addition, the humanization of the

characters of foxes by Confucianism might provide a social foundation for the later closer association between foxes and humans in the ways of transformation and demonization (妖化 in Chinese), as people widely believed in the similarities between foxes and humans.

Superstitious beliefs and the birth of zhiguai genre during the Six Dynasties period

Besides Dong's philosophical idea, the public acceptance of using supernatural causes to explain natural events also promoted the spread of other superstitious ideas among the public. Beliefs that integrated ancient worshiping and social ideas from the prior Qin dynasty got widespread during the Han dynasty and continued to develop during the Six Dynasties period. The Six Dynasties period (六朝 222 A.D. – 589 A.D.) includes the six dynasties located in Southern China and the five dynasties located in Northern China between the Han dynasty and the Sui dynasty. They are often discussed as a whole time period instead of individual dynasties because of the frequent wars and the resulting replacement of emperors and changing state lines. This chaotic time period required the public to turn to religion or superstitious beliefs to explain occurring events as the changing political and social dynamics could not be trusted anymore (Dewoskin 21–23).

The bloom of superstitious beliefs, due to both the constant wars and the previously widespread religious and social ideas, promoted the birth and development of zhiguai tales during the Six Dynasties period. Zhiguai tales includes mostly short stories about supernatural events, such as the occurrence of ghosts and spirits. Typically, collections of these zhiguai stories, either written by the authors or collected by them, were spread and had a chance to be preserved. Although the original copies of most of the collections from the Six Dynasties period were lost, copies from later dynasties may have been found and served as the source for the current published versions of these zhiguai collections. One of the most famous and largest zhiguai collections is SouShenJi by Gan Bao from the Jin dynasty, including 20 chapters and 454 stories (Cheng 5–6). It will also be the major source of analysis for this paper because of the diverse and enormous descriptions of fox spirits and spirits in general included in this collection.

The authors of zhiguai fiction often believed in the authenticity of the stories they wrote. So instead of treating them as fiction, they treated them as historical records of true events, even though they acknowledged that it is hard to test the integrity of their stories as they often collect them from secondary sources (Berry 4–5). The introduction Gan Bao wrote for SouShenJi, shows that at least some authors recognized the potential bias in treating these folklores as true records. Gan Bao wrote:

"Although the author [Gan Bao himself] used classics and documents to validate historical records in this book and tried to collect folklores that were nearly lost in the street, the author was not the first-handed witness of these stories. So, how can the author guarantees there are no false accounts?. .. Even the historical records of each country can not guarantee their authenticity, not to talk about this booklet about ghosts and monsters! … If there are false records in this book that are inherited from prior stories, it can not be concluded as the author's fault. If those stories that were collected by the author himself in recent years still contain false records, then the author is willing to take the embarrassment and shame from the public along with the other authors and scholars that are much nobler."[8] – SouShenJi Introduction

From the above quotation, we notice that although the possibility of false accounts in these stories is understood, the ultimate pursuit for these stories is the same as what is expected for historical records: guaranteed authenticity. This is why Gan Bao stated he will still feel ashamed if his tales were later found to be false accounts. I propose that the reasons for such pursuit not only correlate with the lack of development of fiction as a genre (that only historical or authentic records were valued) but also correlate with the superstitious beliefs that promoted these stories.

Superstitious beliefs and zhiguai stories reinforced each other because people that believed in supernatural events were also interested in records and descriptions about them, while people that read more descriptions of supernatural events might believe in their true existence as well. The key to maintaining this reinforcing relationship is that zhiguai stories cannot be fictional, or at least their authors cannot claim them to be fictional. The authenticity of these stories needs to be stated and confirmed so that while reading them, people will believe in their existence and thus subscribe to superstitious beliefs. This function of zhiguai stories has been mentioned by Gan Bao in his introduction of SouShenJi: "... At least this work has enough evidence to show the beliefs of immortals and Dao are not false accounts." This suggests one of the functions of zhiguai stories is indeed to provide evidence for superstitious beliefs, and also shows the influence of these superstitious beliefs on people during the Six Dynasties period.

People tend to subscribe to these beliefs in the first place because they provide good explanations for the supernatural events they witnessed during that period of time, which are often related to the lives and deaths of humans. This specific interest might correlate with the chaotic political dynamics of that time period, during which consistent wars between different countries cast negative influences on the normal lives of people. This desire of obtaining a stable life fits with the rise of religious Daoism, which provides a religious foundation and structure for the creation of the spirit figures in zhiguai stories.

The religious Daoism and the existence of fox spirits

The immortals and Dao (神道) Gan Bao mentioned in SouShenJi best described the ideas from the religious Daoism (道教). Although often referred to interchangeably as Daoism, religious Daoism is distinct from philosophical Daoism (道家) established by Laozi and Zhuangzi during the Chunqiu period (春秋时期 770 B.C. – 476 B.C.). Philosophical Daoism advocates the power of "doing nothing" (无为) by obeying the natural laws, while religious Daoism advocates taking active steps to pursue longevity or even immortality by essentially breaking the natural laws. Religious Daoism was first founded during the late Han dynasty by integrating the ideas from both philosophical Daoism, Confucianism, and other prevalent social ideas like Wu-Xing (五行) and yin-yang (阴阳) (Pregadio ch. 2.4). Because of the diverse ideas it was based on, religious Daoism has often been referred to when interpreting and explaining supernatural events and thus, its name has been closely associated with such events. It has been included in the term- immortals and Dao (神道) – to generalize supernatural beings. One of their central ideas is that while Dao constitutes the laws of the world, Jing Qi (精气) constitutes the beings of the world, thus humans can somehow achieve immortality by learning to practice their Jing Qi through more efficient ways. This idea inherited a lot of prior philosophical ideas on the constitution of living things and the constitution of the world, and is essential to the appearance of spirits (精怪，妖怪) in the history of Chinese literature.

In Chapter 6 of SouShenJi, there is a short discussion about why spirits existed on Earth, which shows how Daoism influences the formation of the concept of spirits:

"Spirits and monsters are formed because Jing Qi has attached to normal objects. When Jing Qi messed up the inner of the objects, the external appearance of the objects will change as well. The external appearances of the spirits or the monsters represent the differences they caused within the objects. As all objects originated from Wu-Xing, they shared similarities within the five basic things (appearance, speaking, observation, listening and thinking)." – SouShenJi Chapter 6 Discussion about spirits and monsters[10]

The idea presented here, that a change in Jing Qi can cause changes in various aspects of an object, was consistent with the religious Daoism's idea that Jing Qi is useful in maintaining lives. The wording in this explanation also suggests that spirits and monsters have not been viewed as particularly evil things, but instead, as natural results of a combination of Jing Qi and objects. They also shared many similarities with normal living beings because they shared origins from the same Wu-Xing. This suggests a more neutral attitude toward monsters or spirits compared to the later demonization of these figures, although people do recognize spirits are not the same as those normally occurring living beings.

A more specific discussion on how the Qi from Wu-Xing influences the characteristics of the life it forms has been carried out in Chapter 12 pf SouShenJi. Within this discussion, foxes were specifically mentioned:

"There are five Qi that originated from Wu-Xing in the world. Everything is originated from these five Qi…Beings from the sky like to stay in the sky. Beings from the ground like to stay in the ground. Beings from certain temporal ranges like to stay within that range. Every life form obeys its classification… Foxes that live over a thousand years can turn into beautiful girls…. This all required longevity to achieve. " – SouShenJi Chapter 12 Discussion about the changes of Qi within Wu-Xing[11]

This discussion introduces how Wu Xing serves as a classification for beings and constitutes the natural laws. Although the importance of obeying the classification and natural laws is emphasized, the importance of achieving longevity has also been mentioned. This reconfirms that this idea might originate from religious Daoism as longevity and immortality have been the major pursuits. Most importantly, it describes the power that was brought by longevity – the ability to transform into humans.

Fox spirits gaining the Transformation ability

The description that as one achieves certain longevity, their power will increase, is consistent with the basic idea of religious Daoism as longevity was believed to bring humans closer to immortality

and bring them supernatural power (神通). According to this, beings other than humans might acquire the ability to transform into humans in the same way, by simply living long enough. This idea of ancient beings transforming into spirits (物老成精) might promote the diversity of spirits in zhiguai tales, as not only animals but plants and tools can also become spirits. There is even a story about a conversation between a spoon spirit and a pillow spirit in SouShenJi[12]. The rules that the power of one grows stronger when one becomes older also enable the growth of the transformation ability of the spirits, which might enable the spirits to become more and more alike to humans as they grow older.

Aligning with this rule, foxes that were in different phases of this transformation process have been depicted in zhiguai tales, and even in SouShenJi specifically. First, there are foxes that have the physical appearance of normal animals but carry signs or warnings to humans to show their understanding of the human world. For example, Chapter 3 of SouShenJi depicts how the howling of a fox represents a warning of unfortunate events that in the end, saved a family of lives[13]. Next, there are stories in which the fox spirits are only able to transform into humans in dreams, which SouShenJi also includes examples for: In Chapter 15, there is a story about how a white fox is spotted in the grave of Yi Shu (奕书) and has been hurt on its left foot. The fox then appeared as an elderly man with white hair in the dream of the king of Guangchuan and punished him on the left foot too[14]. Then, there are lots of depictions of foxes that transform entirely into humans even during the daytime, in which the foxes appear to be less animal-like but more human-like as more humanized features were depicted. This transformation ability into humans broadens the potential roles of spirits in zhiguai fiction and is a crucial part of their features, because spirits now can not only serve as a vague metaphorical representation of moral standards for human society but can also serve as a direct carrier of these characteristics of humans.

Besides how fox spirits' ability to transform into humans, specifically beautiful girls, can be obtained through living long enough as described in the above quotation of Chapter 12 of SouShenJi, another character can be observed in these fox spirits that might indicate another way of gaining the ability of transformation. In chapter 18 of SouShenJi, there are several stories depicting knowledgeable scholarly fox figures (typically male). For example, a very knowledgeable guest scholar of Dong Zhongshu forecast an upcoming rain and was thus been recognized as a fox spirit[15], and an old scholar who suddenly disappeared turned out to be a white-browed fox[16]. These figures correspond to the other way of achieving immortality that the religious Daoism suggests, by cultivating (修炼) themselves. They often cultivate themselves by analyzing and thinking about the classics of Daoism and trying to understand more about the Dao that exists within everything in the universe. Thus, fox spirits' wide interest in the classics of philosophy and religion (especially Confucianism and Daoism) might also be a feature that is necessary or required for them to acquire the ability of transformation according to the doctrines of the religious Daoism. The gendering of fox figures associated with this method, that those scholarly foxes are overwhelmingly male, might be caused by certain social biases and judgements on females being scholars during the time of writing.

The Gendering and Sexualization of the fox figures, and the Seductive Acts

Besides the differences in the ways of achieving longevity, a more distinct gendering of the fox spirits appears within the ways of depicting the seductive behaviors in foxes. As Daoism focused their structures of understanding the nature on the balance of yin and yang, they have used this balance to explain many natural phenomena, such as the existence of females and males and days and nights. So it is not surprising that religious Daoism has integrated this concept to sex and describes sex lives as a way of cultivation. They consider sex as the most explicit practice of the balance between yin and yang. This group of religious ideas is called Sexual Acts (房中术). These acts mostly focused on how males should absorb yin and nourish yang (采阴补阳) during sex instead of the other way around because Daoists believe yin is nourished naturally during sex while yang needs to be supplemented (Valussi 445-446). Thus the acts that teach people to do the other way around, to absorb yang and nourish yin (采阳补阴), are considered to be forbidden and wicked. They are described as Seductive Acts (媚术) that will mentally blind the males and make them willing to nourish the females, even with the cost of their lives.

There is a correlation between these female foxes that seduce human males and the seductive acts described in Daoism, as the process is described in similar details. In Chapter 18 of SouShenJi, there is a story about a seductive fox named A Zi (阿紫), who seduced a soldier named Wang Lingxiao and keeps having sex with him in a tomb cave:

During the Han Dynasty and the time of the Emporer Xian (196 A.D.- 220 A.D.), Chen Xian from the Kindom Pei was the captain of the Xihai county.

His subordinate Wang Lingxiao ran away for no reason. Chen planned to kill him, but not long after that, Wang escaped and ran away again. Chen noticed Wang didn't come back for a long time, thus imprisoned his wife. His wife told him the truth about what she knew. Chen said: "It must be Mei (媚) that lured him. We must try to find him." He then ordered several soldiers with hound dogs to search around the city. They found Wang in an empty tomb. The monster flew once it heard the sound of men and dogs. Chen ordered others to help Wang to his wife, but Wang already appeared to look like a fox instead of a human. He didn't speak any other human language instead of crying for A Zi. A Zi must be the name of that fox. After several days, Wang started to become better and told the others: "When the fox first came, it disguised as a fine lady at the corner of my house, and lured me more than once. I then came with her and did the things that husbands and wives should do. I always went back to her house with her, and wouldn't be waken even if there were dogs around." He said that was the most joy in the world. One Daoist said that is Mei from the mountains. Mingshanji described: "Fox was the vixen from ancient times. Her name was A Zi and later transformed into a fox. Because of that, monsters alike her always called themselves A Zi." [17]

Although this story is one of the longer zhiguai tales, it is still short in contemporary views, and it didn't contain a detailed description of what actually happened between the fox and Wang. But it described that after Wang and the fox formed a sexual relationship, Wang became fox-like in his appearance. His senses became weaker and only knew to cry "A Zi" when they found him. This story is very similar in structure and in descriptions to a group of later fictions that focus on describing how a female fox seduced a human male and lured him to his death by absorbing his life energy. It can serve as an example showing a direct association between fox spirits and seductive acts depicted in religious Daoism. Furthermore, a Daoist appears at the end of the story and explains the nature of this monster, which suggests the profound knowledge of Daoists when it comes to spirits and monsters and hints that these creatures are consistent with the Daoism ways of perceiving the world.

It is worth noticing that during the Six Dynasties period, this seductive female figure has not yet established its dominant position in the figures of fox spirits, however, the observed gendering in the ways of cultivation of fox spirits shows the basis of the later more profound female sexualization in the figures of fox spirits.

Conclusion

In the end, I would like to summarize the association between the development of the figures of fox spirits and the ideas of Confucianism and Daoism I made in this paper. I first argued that the rise of Confucianism strengthened the auspicious beast figure of the foxes, which laid a foundation for further descriptions of the humanized features of foxes. I then presented how the spread of superstitious beliefs led to the birth of the genre of zhiguai, and how ideas from religious Daoism influence the formation of animal spirits in fiction. Following the influence associated with religious Daoism, I explained how two important features of the figure of fox spirits, the transformation ability into humans and the seductive power of female foxes to human males, are associated with specific ideas from religious Daoism. As a result, a clear pathway of the development of the fox spirit figures during the Six Dynasties period has been shown.

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About the Author

I have gotten involved in this research because of the Take5 program. I highly recommend students who have a strong academic interest outside of their majors to know more about this wonderful chance to spend one tuition-free year on courses you are excited about! I was always fascinated by mythology and As a Chinese, I was always interested in the fox worshipping ritual and the history behind it. So I didn't hesitate to propose this subject as an independent study when I was doing my take5 program in comparative literature and film studies.

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