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Functional Analyses of the Kek5 Intracellular SLIM, CO1, in *Drosophila* and Disease Implications in Humans

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ABSTRACT

Controlled and orchestrated cell signaling pathways are paramount for proper cellular and organismal development and function. Bone Morphogenetic Protein (BMP) signaling represents one of the most highly conserved pathways whose dysregulation in humans leads to skeletal, cardiovascular, and metabolic diseases, as well as numerous types of cancer. Model organisms, like *Drosophila*, provide a way to characterize the function of such conserved cell signaling proteins and pathways *in vivo*. Kekkon5 (Kek5) is a transmembrane regulator of BMP signaling in *Drosophila*. Strikingly, Kek5 was recently shown to putatively contain an intracellular motif found in the human Deleted in Colorectal Cancer (DCC) receptor, which regulates DCC activity. In this MQP I focused on determining if this short linear motif (SliM) functions similarly to regulate Kek5's activity. Specifically, the GAL4/UAS system was used in transgenic *Drosophila* to compare the function of wild type Kek5 and a variant lacking this SLiM, Kek5$^{\Delta CO1}$, at the organismal and cellular levels. Together, these results indicate that, as in DCC, the CO1 SLiM is indeed critical for regulating Kek5 activity and provides a path forward for future studies. Developing treatments for people who have metabolic, cardiovascular and other diseases from disrupted signaling pathways, including BMP, will ultimately require a detailed understanding of the function of these pathways and their dysregulation in patients.

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1.0 INTRODUCTION

1.1 Overview

Controlled and orchestrated cell signaling pathways are paramount for proper cellular and organismal development and function. When cell signaling pathways are disrupted in humans, diseases such as cancer, asthma, and diabetes can occur (Grainger & Brugge, 2015). In humans and other organisms, proper cell signaling function is also needed for an organism to develop from conception to adulthood. In the model organism *Drosophila melanogaster*, I am studying the function of the transmembrane protein Kekkon5 (Kek5), which has been implicated in cell signaling pathways such as the Bone Morphogenetic Protein (BMP) pathway and in cell adhesion (Evans et al., 2009). I am also examining the importance of the "PDL" amino acid motif within the cytoplasmic domain of Kek5 through the Gal4/UAS system.

1.2 LIGs and the Kekkon Family

Thousands of proteins in prokaryotes and eukaryotes contain leucine rich repeats (LRRs) (Bella et al., 2008). Leucine rich repeats are protein sequences with a repetitive pattern, the eleven amino acid sequence, LxxLxLxxNxL (Bella et. Al., 2008). Due to their repetitive nature, proteins with LRRs form a solenoid structure (Fig. 1). This solenoid shape is thought to facilitate protein-protein binding interactions through its flexibility, as well as tendency to dimerize (Kobe & Deisenhofer, 1995; Bella et al., 2008).

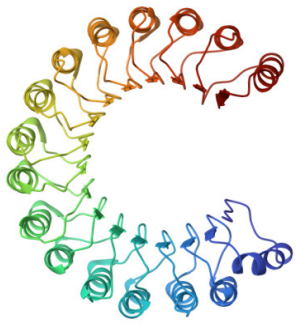

Figure 1. This shows the solenoid shape of a LRR structure, from a porcine ribonuclease inhibitor (Kobe & Deisenhofer, 1996).

Along with LRRs, many proteins identified in metazoans, including *Drosophila melanogaster*, contain Immunoglobulin (Ig) domains. Ig domains are characterized by a disulfide bridge connecting two antiparallel beta sheets (Williams & Barclay, 1988). Within animals, Ig domain proteins are the largest family of surface receptors (Watson et al., 2005). Despite the large quantity of proteins containing LRRs or Ig domains, few proteins (deemed **LIGs**) have been identified in metazoans containing both LRRs and Ig domains. In *Drosophila melanogaster* there are only nine LIG proteins, while humans have thirty-six LIGs (MacLaren et al., 2004).

Within the nine LIG proteins currently identified in *Drosophila*, six (Fig. 2) are members of a subclass of related transmembrane proteins known as the Kekkon (Kek) family, Keks 1, 2, 3, 4, 5, and 6. The Keks are defined by a common structure: on the extracellular side they contain seven LRRs which are flanked on either side by cysteine caps and followed by a single Ig domain. On the intracellular side no identifiable catalytic motifs have been uncovered (MacLaren et al., 2004). Kek1 was the first of the six proteins to have its function determined and was shown to be an inhibitor of the Epidermal Growth Factor Receptor (EGFR)

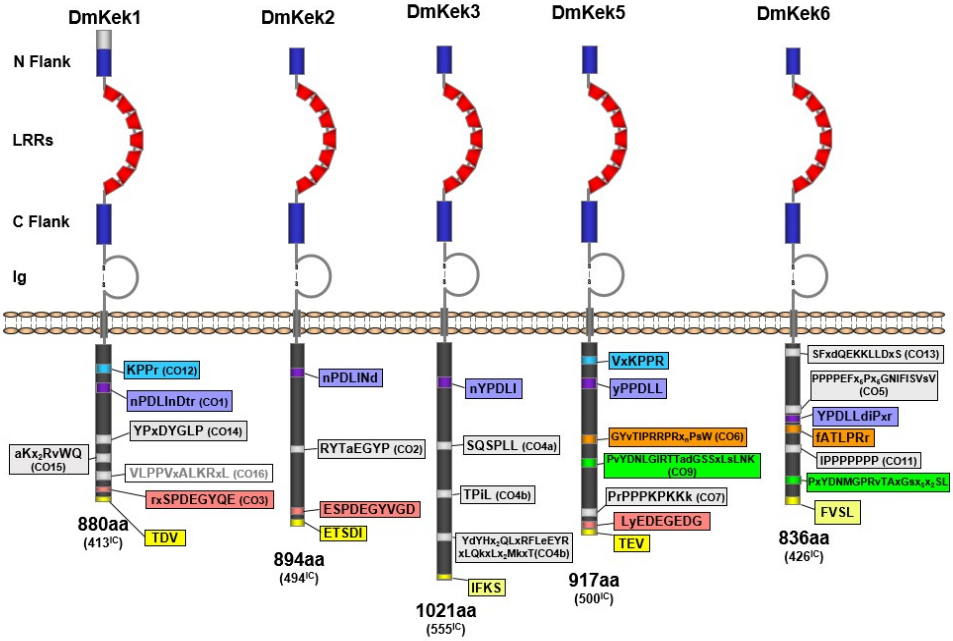

Figure 2. Graphic overview of the Kek family and conserved SLiMs. Adapted from O'Reilly, 2020.

(Ghiglione et al., 1999). Later, Kek5 was found to be a regulator of Bone Morphogenetic Protein (BMP) signaling and Kek6 was found to have a role in regulating synaptic plasticity (Evans et al., 2009; Ulian-Benitez et al., 2017).

Despite the Kek family being identified in the late 1990s, there is little published research on the function of the intracellular portions of the proteins. However, the conservation of small amino acid motifs within the intracellular regions across the Kek family strongly supports a function for the intracellular domain of the proteins (O'Reilly, 2020). These conserved amino acid motifs have been identified previously and are depicted in Fig. 2 (MacLaren et al., 2004; O'Reilly, 2020).

1.3 SliMs

SliMs, or **S**hort **Li**near **M**otifs, are small sequences of amino acids likely serving as interaction sites within a protein (Van Roey et al., 2014). SliMs were originally identified as having functionality in intrinsically disordered regions of proteins (Van Roey et. al., 2014). The SliMs in these regions were found to either modulate protein-protein interactions or help modify proteins post-translationally (Van Roey et. al., 2014). Previously numerous conserved SliMs were identified within the intracellular domains of the Kek family (MacLaren et al., 2004; O'Reilly, 2020). Within the *Drosophila melanogaster* Kek family, only one SliM was conserved across all six proteins. This SliM, defined as CO1, is approximately 6-10 amino acids long depending on which Kek and in all Keks contains a fingerprint sequence of Proline-Aspartic Acid-Leucine (PDL) (O'Reilly, 2020). The presence of the C01 SliM in all Kek family members suggests that

this motif, CO1, may impart an important functional property to all members of the protein family (**Table 1**; O'Reilly, 2020).

Protein	CO1	CO2	CO3	CO4	CO5	CO6	CO7	CO8	CO9	CO10	CO11	CO12	CO13	CO14	CO15	CO16
Kek1	+	-	+	-	-	-	-	-	-	-	-	+	-	+	+	+
Kek2	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Kek3	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-
Kek5	+	-	+	-	-	+	+	-	+	-	-	+	-	-	-	-
Kek6	+	-	-	-	+	+	-	+	-	-	+	-	+	-	-	-
Dmag5/6	+	-	+	-	-	+	+	+	+	-	-	-	+	-	-	-

Table 1. Conservation of SliMs across the Kek family within *Drosophila melanogaster* and an outgroup of *Daphnia magna*. Adapted from O'Reilly, 2020.

1.4 Kek5: A Multifunctional Protein?

Kek5 was found to be a mediator of Bone Morphogenetic Pathway (BMP) signaling (Evans et. al., 2009). *In vivo*, both loss-of-function (LOF), null mutants, and gain-of-function (GOF) misexpression experiments showed wing defects, specifically, posterior cross vein abnormalities in adult flies. Vein patterning in *Drosophila melanogaster* is known to be sensitive to fluctuations in BMP ligand distribution (Ralston & Blair, 2005). Further experiments, including epistasis and expression studies on pMad and dSRF (downstream components of BMP signaling) in Kek5 LOF and GOF pupal wings demonstrated that Kek5 is a regulator of BMP signaling (Evans et. al., 2009).

A large body of unpublished data from the Duffy lab has also pointed to Kek5 having numerous other GOF effects. At the organismal level these include effects on wing development and adhesion, bristle formation, eye development, leg development and viability (Evans, 2006; Menon, 2013). At the cellular level, GOF effects were observed on adherens junctions, cell size, cell extrusion and programmed cell death (Evans, 2006; Menon, 2013). The wide array of organismal and cellular effects associated with Kek5 misexpression coupled with the presence of numerous intracellular SLiMs supports a model in which Kek5 has multiple and possibly distinct

activities. To address a multi-function hypothesis, further work was previously conducted to assess the role of the intracellular SLiMs (Evans, 2006; Menon, 2013). In this work, misexpression studies with multi- and single SLiM deletion variants suggested the SLiMs CO1 and CO6 may regulate the function of Kek5 (Evans, 2006; Menon, 2013). Moreover, O'Reilly, 2020, discovered that CO1 shares sequence similarity to an intracellular motif within the Deleted in Colorectal Cancer (DCC) receptor, known as the Frazzled receptor in *Drosophila*. Interestingly, this motif (termed P1 in DCC), appears to function in toggling DCC's activity between two states – axonal attraction or repulsion (Boyer et. al., 2018).

In summary, CO1 represents a conserved SLiM, is found in all Kek family members, is within the P1 motif in DCC which is known to regulate receptor activity and appears to cause organismal effects through misexpression of Kek5 multi-SLiM deletion variants (including deletion of CO1) that are more severe than observed with the wildtype protein (Evans, 2006; Menon, 2013). Together, these results suggest the possibility that Kek5 activity is modulated by the CO1 motif. Given this, a single SLiM deletion variant lacking only CO1 was constructed and transgenic *Drosophila* were generated by a former MQP student (Miller, 2021). *With these transgenics, my goal was to investigate the functional effects of CO1 through a single SLiM deletion variant (named ΔCO1) on Kek5 activity in vivo and determine if CO1 does indeed act as a regulatory switch for Kek5.*

To address this goal, I set out to accomplish the following specific objectives:

- Map all transgenics Kek5 inserts (WT and ΔCO1) and create stable stocks.
- Use the GAL4/UAS misexpression system to compare the activity and localization of $Kek5^{\Delta CO1}$ and $Kek5^{WT}$ in numerous *in vivo* organismal and cellular assays, as well as an *in vitro* assay.
- Generate an *in silico* 3-D model of the Kek5 intracellular domain to investigate the predicted structure of SLiMs, particularly CO1.

2.0 MATERIALS AND METHODS

2.1 Chromosomal Mapping of Selected Kek5 Variant Strains

2.1.1 Stock Lists

Building off the most recent prior MQP work of (Miller, 2021), stock lists were created of all Kek5^{WT}, Kek5$^{\Delta CO1}$, and Kek5^{K6CO1} UAS responder lines in the Duffy lab. All WT and ΔCO1 strains that had not yet been mapped were selected for mapping.

2.1.2 Balancer Crosses

Virgin females were collected from both w^- ; TM3, Sb/ Cx^D stock flies (BSC#3607) and Sp/CyO stock flies (BSC#8379). w^- ; TM3, Sb/ Cx^D flies have a third chromosome Balancer (TM3) with a dominant marker of stubble bristles (Sb[1]) balanced over another dominant marker, Cx^D with wings that are outheld. w^- ; wg^{Sp}/CyO flies provide a second chromosome Balancer (CyO) with a dominant marker of curly wings balanced over another dominant marker with extra sternopleural bristles (wg^{Sp}). The UAS responder lines all carried the mini-white gene on the UAS responder transgene, which allowed for the UAS responder transgene to be tracked by the presence of eye color.

Table 2: Genotype and stock number information for the balancers I used to map responder lines.

BSC Stock Number	**Balancer Genotype**
3607	w^{1118}; TM3, Sb^1/CxD
8379	w^{1118}; wg^{Sp}/CyO

After collecting virgin females from both balancer stocks, crosses were set up with male UAS-Kek5^{WT} and UAS-Kek5$^{\Delta CO1}$ responder lines at 25^{o}C. The male F1 progeny of these crosses with the responder transgene and balancer chromosome, phenotypically either stubble or curly wings AND non-white eyes, were placed into new vials for their respective lines.

2.1.3 Out Crosses

From the Balancer crosses, the selected male F1 progeny for each respective responder line with each respective Balancer (II or III chromosome) were outcrossed to virgin w^{1118} females flies (BSC Stock #3605). The F2 progeny were then observed using the 2nd and 3rd chromosome balancer markers to determine which chromosome the responder line segregated away from and thereby determine chromosomal location. Lines that resulted in only F2 females exhibiting eye color were presumed to be on the first, X, chromosome.

2.1.5 Stable Stock Generation

Once mapped, depending on which chromosome the responder line was on, responder males were bred to virgin females of the balancer stocks. Virgin F1 progeny containing the responder over the appropriate balancer were then mated to establish stable stocks.

2.2 Organismal Misexpression Studies

2.2.1 Initial Driver Selection

Different driver strains of *D. melanogaster* were ordered and shipped to Worcester Polytechnic Institute from the Bloomington Drosophila Stock Center. Based on a literature review, Gal4 drivers that could create easy-to-track phenotypes or influence wing development were chosen.

2.2.2 Selection of Representative Responder Lines

Males for $Kek5^{WT}$ and $Kek5^{\Delta CO1}$ responder lines were bred to virgin females with GMRGal4, apGal4/CyO, and ptcGal4 genotypes in vials at 27°C. The F1 generation carrying the driver and responder were graded on a phenotypic scale of 0-10 (0 = wild type and 10 = severe) for de-novo mutant phenotypes. Once these scores were analyzed, two representative responder lines were chosen for $Kek5^{\Delta CO1}$ and one representative responder line was chosen for $Kek5^{WT}$. An explorative cross for the A9 driver was also conducted with selected $Kek5^{WT}$ and $Kek5^{\Delta CO1}$ responder lines, to assess general phenotypic effects.

2.2.3 Confirmation of Ap and Ptc Driver Effects

Phenotypic effects with the Ap and Ptc GAL4 drivers were then reconfirmed with the selected representative responder lines. These crosses were performed with all possible gender configurations (male drivers crossed to virgin female responders, and virgin female drivers crossed to male responders). The resulting F1 phenotypes were scored for severity.

2.3 Viability Studies

The effect of misexpression on adult viability was quantified by crossing the PtcGAL4 driver by the selected responder lines for $Kek5^{WT}$ and $Kek5^{\Delta CO1}$, and additionally a new $Kek5^{WT}$ line referred to as "LL-Kek5" (BSC# 95293, Genotype: w[*]; P{y[+t7.7] w[+mC]=UAS-kek5.L}attP40/CyO) at 27°C. PtcGal4 virgin females were first crossed to w^{1118}; wg^{Sp}/CyO males to create the necessary F1 generation of PtcGal4/CyO flies which were then mated to the respective responder line. Vials were flipped periodically every few days. All eclosed F1 were scored and relative viability was calculated as below (X indicated specific transgene line).

$$\text{Relative Viability} = \frac{\#\ \text{UAS Kek5}^{X}\ /\ \text{PtcGAL4 progeny}}{\left(\frac{\#\ \text{UAS Kek5}^{X}\ /\ \text{CyO} + \#\ \text{PtcGAL4/ CyO progeny}}{2}\right)}$$

2.4 In Vitro Misexpression Kek5

Transfections were done according to standard Duffy lab procedures. Briefly, a 6-well plate was seeded with S2* cells at 5 x 10^6 cells per well in 2mL of Schneider's media + 10% FBS one day prior to transfections. On day zero of transfection each well was rinsed 2X with sterile 1X PBS and then 1.6mls of Schneider's media + 10% FBS was added. Transfection mixes consisting of 4uL of ArmGal4 (driver), 4 uL of selected responder (UAS construct), and 92 uL of EC buffer were pre-mixed in an Eppendorf tube. 6.4uL of Enhancer was then added and incubated at room temperature for 15 minutes, followed by 8.0 uL of Effectene Transfection reagent. After 10 minutes, 0.6ml of Schneider's media + 10% FBS was added to each transfection mix which was then added to each well (Fig. 4). The plate was placed in an incubator at room temperature and allowed to grow for 10 days. Every 2-3 days, pictures of the cells were taken under a fluorescence dissecting microscope.

Kek5^{WT} Construct Number: T109	**Kek5$^{\Delta CO1}$** Construct Number: T110	**Kek5$^{\Delta 1}$** Construct Number: T22
Kek5$^{\Delta 123}$ Construct Number: T21	**Kek5$^{\Delta 2}$** Construct Number: T27	**Kek5$^{\Delta 3}$** Construct Number: T26

Figure 3. 6-well plate experiment schematic of which construct was added to each well.

2.5 Larval Dissections

Imaginal wing discs from third instar larvae expressing different Kek5 responder lines under PtcGal4 or ApGal4 were dissected out in a nine well glass dish in 1X PBS and fixed for 15 minutes in PEMP (0.1M PIPES, 2mM MgSO4, 1mM EDTA, 0.5% Tween20). After fixation, the wing discs were rinsed two times in 1X PBS, and stored in 70% glycerol/PBS. Discs were then mounted onto glass slides for microscope viewing.

3.0 RESULTS

3.1 Using the GAL4/UAS System for Kek5 Misexpression Studies

The goal of my research was to investigate the functional effects of the CO1 SLiM deletion variant on Kek5 activity *in vivo* and determine if CO1 does indeed act as a regulatory switch for Kek5. Gain-of-Function, specifically misexpression, studies provide a powerful way to investigate the function of genes and have been used previously in addressing Kek5's function *in vivo*. To address my goal, I similarly used a misexpression approach with the GAL4/UAS system (Brand & Perrimon, 1993; Duffy, 2002). An overview of the system is provided in Figure 4. Briefly, the gene of interest (responder) is placed under the control of GAL4 binding sites (UAS). Strains containing the responder transgene are then crossed to strains expressing GAL4 in various patterns, drivers. In the resulting progeny containing both the driver and the responder, the presence of GAL4 activates expression of the responder, target gene, in a defined temporal and spatial pattern (Fig. 4). Additionally, the Gal4/UAS system is temperature dependent, allowing for further control of misexpression by modulating the level of responder expression (Duffy, 2002).

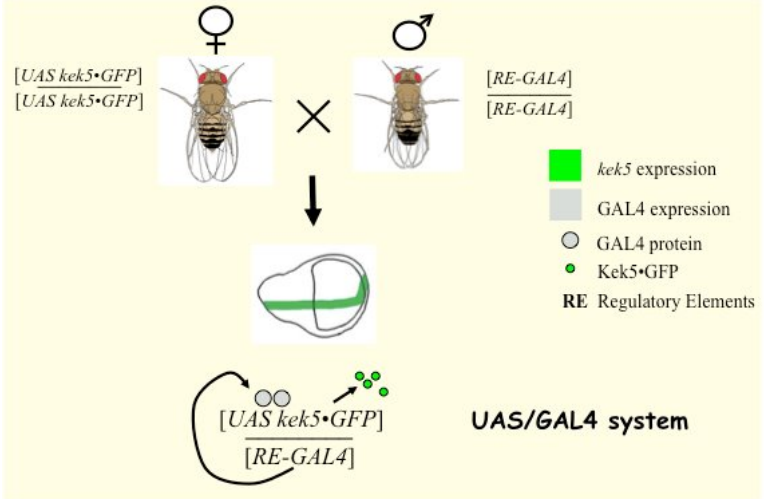

Figure 4. Overview of the GAL4/UAS system.

3.2 Mapping Kek5$^{\Delta CO1}$ and Kek5^{WT} transgenic lines

Since transgene expression can be subject to position effects, even with the GAL4/UAS system, I set out to map multiple insert lines for both the Kek5^{WT} and Kek5$^{\Delta CO1}$ responders (refer to Materials and Methods for details). A total of seventeen Kek5^{WT} and Kek5$^{\Delta CO1}$ responder insertions were mapped, eight and nine lines respectively (Tables 3 & 4). Each line that had been mapped to a 2nd or 3rd chromosome was then established as a homozygous stock (responder/responder genotype) or a heterozygous stock with a balancer (responder/balancer genotype).

Kek5^{WT} Responder Line	Chromosome
W396Cm2-2m	2
W396Cm2-3m	3
W396Cm1-4m	3
W396CF8-1F	X
W396Cm8-1m	3
W396BF5-1F	X
W396BF6-2F	X
W396Cm1-2m	2

Table 3. Kek5^{WT} responder lines and their respective chromosomal locations.

For insertions mapping to the X chromosome, an X chromosome Balancer stock was ordered (y^1, arm^1/FM7c, Bar) from the Bloomington *Drosophila* stock center, but due to time constraints balanced stocks have not yet been established for the X chromosome insertions.

Kek5$^{\Delta CO1}$ Responder Line	Chromosome
W416Em11-F1	X
W416Em5-m1	2
W416Dm12-m1	2
W416Dm13-F2	3
W416EF9-F1	3
W416Dm10-m1	3
W416CF1-F1	2
W416BF10-F2	X
W416AF3-F1	2

Table 4. Kek5$^{\Delta CO1}$ responder lines and their respective chromosomal locations.

3.3 Assessing Kek5$^{\Delta CO1}$ and Kek5^{WT} Activity in vivo

3.3.1 Selection of Representative Responder Lines

Prior work in the Duffy lab utilized the drivers Patched-GAL4 (ptcGAL4), Apterous-GAL4 (apGAL4), and A9-GAL4 for misexpression studies in the wing, and the Glass Multimer Reporter-GAL4 (GMRGAL4) in the eyes. Table 5 summarizes the drivers and relevant information on their targeted expression.

Bloomington Stock Center (BSC) Number	Driver Name	Driver Acronym	Driver Genotype	Primary expression pattern
#2017	Patched	Ptc>	w*; P[w^{+}, GawB ptc$^{559.1}$]^{II}	Anterior-Posterior margin in all imaginal discs
#3041	Apterous	Ap>	y[1] w[1118]; P{w[+mW.hs]=GawB}ap[md544]/CyO	Dorsal compartment in wing imaginal disc
#1104	Glass multimer reporter	GMR>	w[*]; P{w[+mC]=GAL4-ninaE.GMR}12	Eye imaginal disc
#8761	A9	A9>	P{w[+m*]=GAL4}A9, w[*]	Wing and haltere discs

Table 5: Gal4 driver strains and expression patterns.

To assess the general level of activity associated with each responder construct (Kek5^{WT} and Kek5$^{\Delta CO1}$), all second and third chromosome insertions lines and a control responder containing only GFP (both Kek5 responders contain C-terminal GFP fusions) were tested for organismal effects by the PtcGAL4, ApGAL4 and the GMRGAL4 drivers. After examining the F1 generation of each driver/responder line cross, the phenotype severity of each genotypic group

Ptc Driver				
Responder	Insert	Driver	Phenotype Severity	Noticed Lethality?
Control	GFP-2X	Ptc	0	No
WT	W396CF8-1F	Ptc	1	No
	W396Cm1-2m	Ptc	3	No
	W396Cm2-2m	Ptc	8	No
	W396Cm2-3m	Ptc	5	No
ΔC01	W416AF3-F1	Ptc	3	Yes
	W416CF1-F1	Ptc	all dead	Yes
	W416Dm12-m1	Ptc	3	Yes
	W416Dm13-F2	Ptc	5	Yes
	W416EF9-F1	Ptc	all dead	Yes
	W416Em5-m1	Ptc	all dead	Yes

Figure 5. Severity ranking for PtcGAL4 by Kek5^{WT} and Kek5$^{\Delta CO1}$.

of offspring was ranked (Figures 5-7). Some crosses produced only a few offspring of the correct genotype making it difficult to rank the phenotypic severity in adults, but this also demonstrated that misexpression of the relevant responder resulted in significant lethality. The phenotypic severity was based on the number of bristles for PtcGAL4 crosses (a previously characterized phenotype for PtcGAL4/Kek5 misexpression), overall wing structure for ApGAL4 crosses, and the "rough eye" for GMRGAL4 crosses.

After comparing the organismal phenotypic severity scores for the same responders with different drivers, I decided to proceed with responder line W396cm2-2m to represent Kek5^{WT} and with responder lines W416CF1-F1 and W416Dm13-F2 to represent Kek5$^{\Delta CO1}$. This decision was made based on what lines were most likely to be an overall representative of

Ap Driver				
Responder	**Insert**	**Driver**	**Phenotype Severity**	**Noticed Lethality?**
Control	GFP-2X	Ap	0	n/a
WT	W396CF8-1F	Ap	1	n/a
	W396Cm1-2m	Ap	9	n/a
	W396Cm2-2m	Ap	3	n/a
	W396Cm2-3m	Ap	8	n/a
ΔC01	W416AF3-F1	Ap	3	n/a
	W416CF1-F1	Ap	3	n/a
	W416Dm12-m1	Ap	never did cross	never did cross
	W416Dm13-F2	Ap	5	n/a
	W416EF9-F1	Ap	never did cross	never did cross
	W416Em5-m1	Ap	8	n/a

Figure 6. Severity ranking for ApGAL4 by Kek5^{WT} and Kek5$^{\Delta CO1}$.

GMR Driver				
Responder	**Insert**	**Driver**	**Phenotype Severity**	**Noticed Lethality?**
Control	GFP-2X	GMR	0	No
WT	W396CF8-1F	GMR	2	No
	W396Cm1-2m	GMR	4	No
	W396Cm2-2m	GMR	4	No
	W396Cm2-3m	GMR	4	No
ΔC01	W416AF3-F1	GMR	3	No
	W416CF1-F1	GMR	3	No
	W416Dm12-m1	GMR	3	No
	W416Dm13-F2	GMR	2	No
	W416EF9-F1	GMR	2	No
	W416Em5-m1	GMR	never did cross	never did cross

Figure 7. Severity ranking for GMRGAL4 by Kek5^{WT} and Kek5$^{\Delta CO1}$.

the relative severity levels for Ptc and Ap drivers. Due to the relatively mild phenotypic effects observed with GMRGAL4 it was not used in future experiments.

3.3.2 Comparative Organismal Analyses of Kek5$^{\Delta CO1}$ and Kek5^{WT} Misexpression

Prior work in the lab with multi-domain deletions of Kek5 SLiMs, inclusive of CO1, indicated that misexpression of these variants with PtcGal4 typically resulted in lethality, in contrast to misexpression of wildtype Kek5. However, whether or not this effect was due to a deletion of a single SLiM or the cumulative effect of multiple SLiM deletions was unknown. With representative lines for the CO1 single domain deletion and wildtype Kek5 in hand, I addressed this question. The representative lines for Kek5$^{\Delta CO1}$, Kek5^{WT}, and a line with wildtype Kek5 lacking the C-terminal GFP tag (obtained from the BDSC), were all crossed to PtcGAL/CyO progeny scored and the relative viability of the Kek5 misexpression progeny was calculated (Figure 8). As shown in Figure 8, misexpression of Kek5$^{\Delta CO1}$ in both lines tested led to a clear decrease in viability relative to the wildtype Kek5 responders.

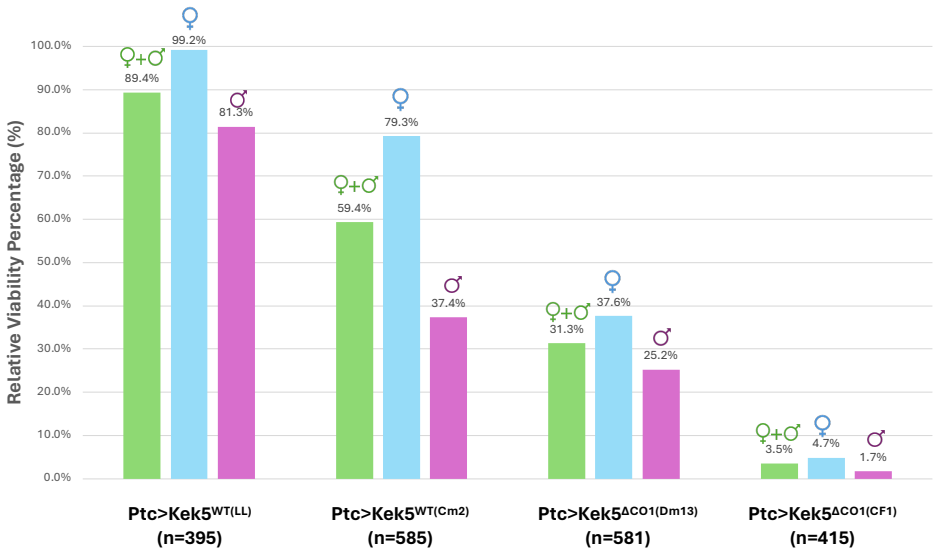

Figure 8. Misexpression of Kek5$^{\Delta CO1}$ results in decreased viability relative to Kek5^{WT}.

To confirm the representative lines exhibited the same organismal phenotypic effects as

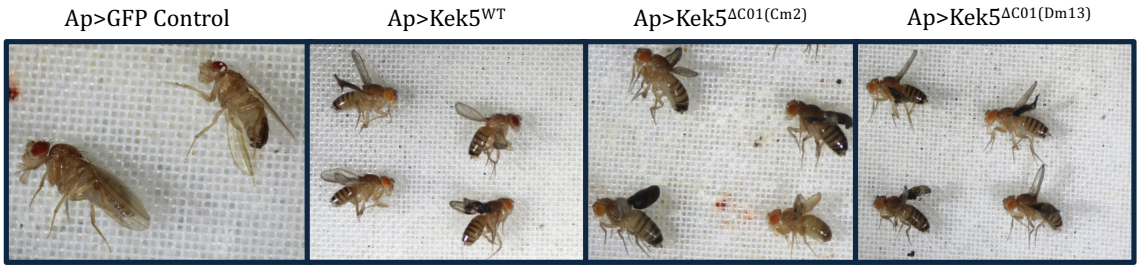

Figure 9. Kek5 misexpression results in severe wing defects.

observed in the earlier assessment crosses, I examined the bristle phenotypes in the Kek5 misexpressing progeny from the viability crosses. Consistent with the initial assessment, the PtcGAL4/Kek5 variant progeny exhibited the same phenotypic severity. Similarly, I retested the representative Kek5 responder lines with the ApGAL4 driver to confirm the wing phenotypes seen in the assessment experiments. Consistent with the earlier results, all ApGal4/Kek5 variant progeny had abnormal phenotypes, with wings that were rotted, black, or malformed (Figure 9).

Similar to the distinct effects on viability observed with PtcGAL driven misexpression of Kek5 multidomain variants lacking the CO1 SLiM, it was previously noted that their misexpression in the wing with A9GAL4 also leads to a distinct phenotypic effect at the organismal level. A9GAL4 driven misexpression of wildtype Kek5 results in an upward wing cupping phenotype, while CO1 deletion variants lead to a dramatically distinct downward wing cupping phenotype (Ernst, 2010). Given this, I wanted to determine if the Kek5^{CO1} deletion variant showed the same downward curling phenotype observed with the Kek5 multidomain variants lacking the CO1 SLiM.

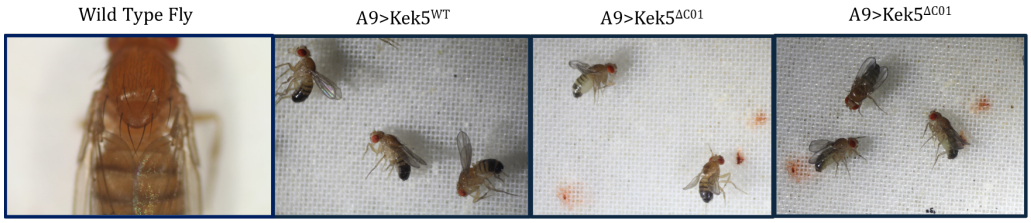

Figure 10. Kek5$^{\Delta CO1}$ and Kek5^{WT} upward cupping phenotype with A9GAL4

The representative Kek5^{wt} and Kek5^{CO1} lines were crossed to APGAL4 and misexpressing progeny scored for their wing phenotype. In contrast to the multidomain deletions, the Kek5^{CO1} variant exhibited an upward cupping wing phenotype similar to that observed with Kek5^{WT} (Figure 10).

In addition to observing phenotypic effects at the organismal level, the cellular effects of Kek5^{wt} and Kek5^{CO1} misexpression were examined. Prior work had demonstrated that misexpression of Kek5 and multidomain SLiM variants leads to cellular extrusion (Menon, 2013). To determine if the same activity was observed with Kek5$^{\Delta CO1}$, imaginal discs misexpressing it were dissected and examined. As shown in Figure 11, cellular extrusion, raised and bumpy areas, was seen on the imaginal wing discs in cells specifically where Kek5^{WT} or Kek5$^{\Delta CO1}$ misexpression was being driven by the PtcGAL4 and ApGAL4 drivers (Fig. 11).

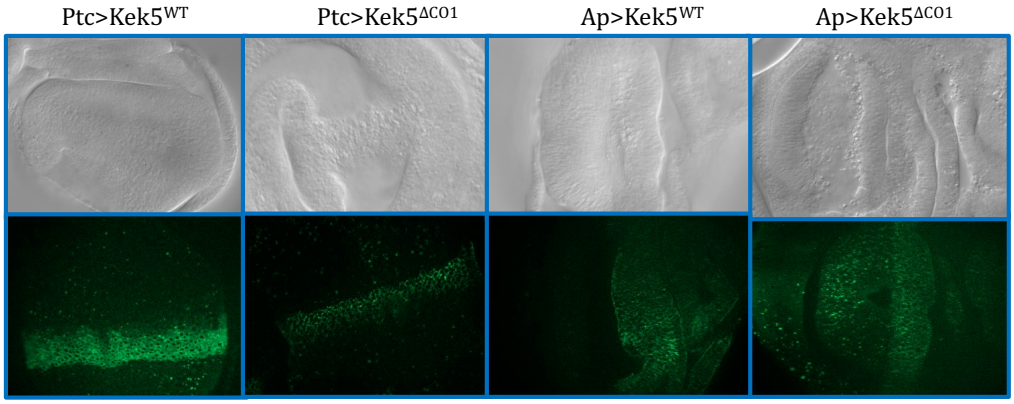

Figure 11. Misexpression of Kek5^{WT} or Kek5$^{\Delta CO1}$ in wing imaginal discs results in cellular extrusion.

3.4 Assessing Kek5$^{\Delta CO1}$ and Kek5^{WT} Activity in vitro

In earlier work from the lab, preliminary studies in cell culture suggested that misexpression of Kek5 SLiM multidomain deletions lacking CO1, but not Kek5^{WT}, led to cell death, providing a possible explanation for the decreased viability seen at the organismal level when CO1 is deleted (Lajeunesse, 2017). To test this, misexpression of wildtype Kek5 and a series of deletion variants were misexpressed in cell culture. Cells were examined for Kek5 expression (using the C-terminal GFP tag) at daily intervals for ~10 days. In the Kek5^{WT} and all the SLiM deletion variants consistent Kek5 expression was observed at all time points. Figure 12 shows three time points, days four, six, and seven post-transfection, indicating no loss of Kek5 expression for any of the responders over time (Figure 12).

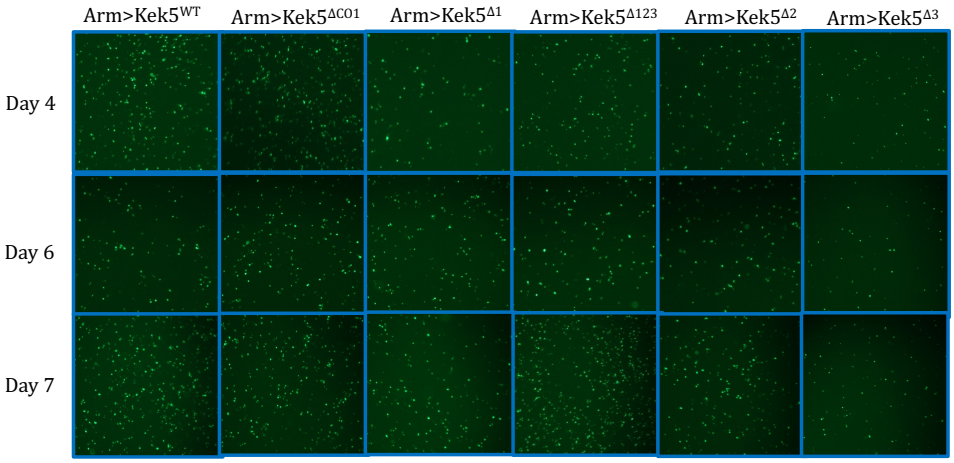

Figure 12. Cellular misexpression of Kek5 and SliM deletion variants.

3.5 In Silico Modeling the Intracellular Structure of Kek5 SLiMs

My results above indicate that misexpression of Kek$^{\Delta CO1}$ causes a large decrease in viability relative to wildtype Kek5. This indicates that loss of CO1 deletion results in a GOF, rather than LOF effect on Kek5 activity. Given that the CO1 SLiM is similar to the P1 motif of DCC, one model is that it functions analogous to P1 and is essential to correctly regulate Kek5 activity between two states. To provide structural insight into this, I used AlphaFold to generate an *in silico* 3-D model of the Kek5 intracellular domain, focusing on the predicted structure of SLiMs, particularly CO1. Consistent with the possibility of distinct states of activity, the intracellular domain appears extremely unstructured and likely to be capable of adopting numerous configurations (Figure 13). The location of the CO1 SLiM is highlighted in green, with the transmembrane domain represented as the blue alpha helix.

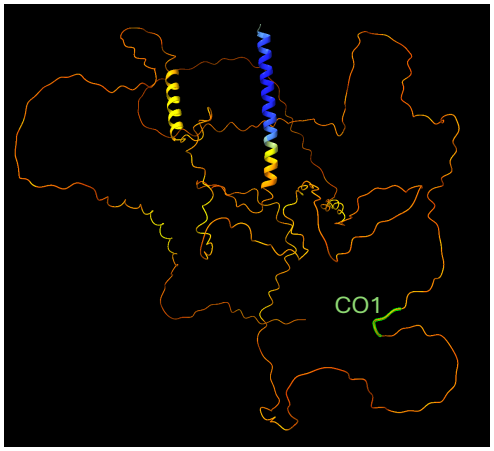

Figure 13. Predicted Structure of Kek5 intracellular domain.

4.0 DISCUSSION

The overall goal of my work was to investigate the functional effects of the CO1 SLiM on Kek5 activity *in vivo* and determine if CO1 does indeed act as a regulatory switch for Kek5. To accomplish this I mapped Kek5^{CO1} and Kek5^{WT} responder lines, created stable stocks, and used the GAL4/UAS misexpression system to compare their activity *in vivo* organismal and cellular assays, as well as an *in vitro* assay. Finally, I generated an *in silico* 3-D model of the Kek5 intracellular domain to investigate the predicted structure of SLiMs, particularly CO1.

The most striking result was that misexpression of Kek5$^{\Delta CO1}$ led to a dramatic reduction in the relative viability of flies compared to wildtype Kek5 misexpression, 3.5% to 59.4%, respectively. The only difference between these two groups was the absence or inclusion of the CO1 domain. Often deletions within a protein typically lead to a loss of activity, generating inert, nonfunctional molecules. If this was true for the CO1 deletion, then Kek5$^{\Delta CO1}$ would be a LOF allele with no activity and its misexpression would not result in any effect on viability. In contrast, the absence of the CO1 domain results in a large reduction in viability indicating it is actually a GOF allele relative to wildtype Kek5. One reason may be that its deletion causes Kek5 to lock into one of two states, analogous to loss of P1 in DCC. In DCC, P1 acts to toggle DCC between repulsion and attraction (Boyer et. al., 2018). If Kek5 similarly has two distinct active states, deleting the CO1 domain may disrupt Kek5's normal regulation, causing it to become fixed into one state. This dysregulation results in a GOF effect with respect to activity and subsequent dramatic decrease in viability when the CO1 deletion is expressed with the PtcGAL4 driver.

Some additional evidence I found to back up this hypothesis is that Kek5$^{\Delta C01}$ does not appear to be lethal when expressed in a cellular assay. The lack of lethality of the protein when expressed *in vitro* could imply that Kek5$^{\Delta C01}$ only becomes toxic when implicated in pathways that

involve multiple cells, such as cellular adhesion pathways. *In vivo* Kek5^{WT} and Kek5$^{\Delta C01}$ phenotypes under the control of ApGal4 displayed deformed and rotting wings. In some of these wings, there were blisters filled with fluid. When wing discs expressing the CO1 deletion and wildtype Kek5 under ApGal4, both genotypes showed unusual cellular extrusion on the surface of the wing discs consistent with disrupted cellular adhesion. In the literature, wing blister phenotypes can be associated with mutated laminins (Martin et. al., 1999), molecules important for cellular adhesion and viability. Wing blister phenotypes have also been associated with integrin molecules (Fristom et. al., 1994) where intervein cells hold the two surfaces of the wings together through an integrin anchor. It is possible the Kek5 interacts with molecules like laminins or integrins, or perhaps intervein cells, and a CO1 domain deletion results in a altered active state that prevents its ability to toggle it function with adhesion molecules throughout development.

Certainly, cellular adhesion pathways are not the sole function of Kek5, as it has already been found to be associated with BMP function (Evans et. al., 2009). However, the evidence from the experiments I conducted lend some support to a possible connection between Kek5 and cellular adhesion pathways. It is possible for proteins to have more than one function, and for those functions to be dramatically different. Known as "protein moonlighting", these proteins may have both enzymatic functions and receptor functions (Jeffrey, 2018). Could Kek5 fall into the category of a moonlighting protein? The presence of multiple different SLiMs within Kek5, and the broader Kek family, provides further evidence for distinct activities and such a categorization.

4.1 FUTURE DIRECTIONS

My work supports a model where the CO1 SLiM regulates Kek5's ability to toggle between two states of activity. Deletion of the CO1 SLiM then results in Kek5 remaining locked in one state of activity. A prediction from this model is that despite a CO1 deletion, additional sequences within Kek5 are critical for its activity in this locked state. Are these sequences the other SLiMs within the intracellular domain, and more broadly what is the molecular function that Kek5 is performing in either state? From this perspective, it would be interesting to create Kek5 variants that lack both a CO1 and other SLiMs, such as CO6. Although a variety of multidomain deletions have been studied, none represent dual domain deletions with CO1. With my results demonstrating a central role for CO1 in regulating Kek5 activity, it will be important to use the CO1 deletion as a base for additional variants. If a dual SLiM deletion variant of Kek5 is created that has no function (no phenotypic effects) when misexpressed relative to wildtype Kek5, this would imply that the second SLiM is necessary for Kek5 function. Additionally, testing such a variant, $Kek5^{\Delta CO1+CO6}$ for example, with different GAL4 drivers, could reveal a variant that is non-functional under one driver, but still functional under the other. This would provide further evidence to the idea that Kek5 may be a moonlighting protein ultimately with the ability to regulate distinct cellular pathways.

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APPENDIX

Appendix 1: Professional Writing MQP Report

Implementing an American Rescue Plan Act Grant At Three Free Medical Programs

Authors: Lauren Averka, Kellie Bushe, Kylie Doehring, Kelly Heffernan
Advisor: Brenton Faber, PhD (WPI)

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Abstract

This project worked with three programs allied with The Worcester Free Care Collaborative: Epworth Methodist Free Medical Program, Akwaaba Free Medical Program, and Worcester Islamic Center Social Services (WICSS). These Free Medical Programs (FMPs) are an important health safety net for individuals who are uninsured, underinsured, or are otherwise hindered from having access to medical care. In response to the COVID-19 pandemic, fiscal recovery funds were used to create the American Rescue Plan Act (ARPA) Grant for the reimbursement of vital community services across the country. The three programs were awarded a grant from this program in Fall 2023. Research has shown that populations belonging to specific geographical, racial, and economic demographics that use free medical services were disproportionately affected by COVID-19, and the ARPA Grant aims to help reimburse the FMPs who were placed under extra strain during the pandemic. Our team reviewed deliverables from the United States Department of Treasury which outlined the purpose of the grant, the parameters that qualified patient visits, the need to track forms for unduplicated submissions, and the timeline of the grant implementation. This project's goal was to create iterations of documentation that met the needs of each FMP to ensure smooth implementation of the grant funding process. After six months of work, the team raised approximately $10,000, or one-third of the reimbursement-based grant. The implementation strategy included a sustainability plan that enables the three FMPs to continue with the grant program until their full reimbursement targets are met. Throughout this project, writing was used as the main tool to support grant implementation and sustainability.

Chapter 1: An Introduction to Free Medical Programs

Section 1.1 The Establishment of Free Medical Programs (FMPs)

The Haight-Ashbury Medical program was the first free medical program (FMP) established in the United States during the height of the "Summer of Love" in 1967 ("The 1960s Drugs and the Birth of the Free Program Movement", 2015). The 1960s marked a time of social, cultural, and political change in the United States as a result of the ongoing Vietnam War, leading to many counterculture ideals. Paralleling the Summer of Love in 1967 was the widespread experimentation and use of drugs such as LSD and marijuana, leading to increased health concerns and the need for accessible medical care (Smith et al., 1971). The district of Haight-Ashbury in San Francisco, California was a focal point within the counterculture movement, attracting an influx of young citizens to the district. In response to the increasing population and emerging drug epidemic, the Haight-Ashbury Medical program was established by Dr. David Smith and healthcare volunteers, centered around the new ideal that "healthcare is a right, not a privilege ("The 1960s, Drugs and the Birth of the Free Program Movement," 2015)

The Haight-Ashbury Medical program influenced additional FMPs to open in the region and eventually nationwide. Within the same year of the Haight-Ashbury Medical program's opening, five additional FMPs opened in the region, and 28 more by 1968 (Fletcher, 1982). As more FMPs were established across the region and spread across the country, the National Free Program Counsel was formed in 1975, encompassing 400 free programs nationwide ("The 1960s, Drugs and the Birth of the Free Program Movement," 2015). Early FMPs struggled to obtain licenses to practice medicine. This often resulted in relying on a lead physician to claim the FMP as a private practice operating under their medical license which risked potential liability for the program ("The 1960s, Drugs and the Birth of the Free Program Movement," 2015). Additionally, medications often came from drug representatives' samples and all equipment and supplies were donated from local hospitals. Despite these challenges, FMPs have continued to grow across the US and are still widely utilized by patients without access to formal medical care, underserved communities, and un-or-underinsured populations. It has been documented that more than 1200 free programs are currently in practice across 49 states ("The 1960s, Drugs and the Birth of the Free Program Movement," 2015).

Approximately 1.8 million Americans nowadays receive care from these programs annually, with no trends indicating a decrease in the use of their services (Hu, 2016). The private health care insurance system within the United States limits access to quality, equitable, and affordable healthcare. As a result, over 8.4% of Americans or approximately 27.6 million people reported not having health insurance in 2022 (*U.S. Uninsured Rate Dropped 18% During Pandemic*, 2023). Further, a Commonwealth Fund survey conducted before the COVID-19 pandemic revealed 27% of non-geriatric adults were underinsured, 24% struggled to pay medical bills, 12% initiated a change to their "way of life" to pay a medical bill, and 23% were enrolled

in long-term plans to pay off medical bills (Himmelstein & Woolhandler, 2021). With rising costs of healthcare and insurance plans, an increasing number of Americans are unable to afford basic healthcare needs which highlights the need for alternative healthcare options like FMPs across the country.

Section 1.2 The Need for Free Medical Programs In The U.S.

In the United States in 2022, 11.5% of Americans lived in poverty as reported by the U.S. Census Bureau (Shrider & Creamer, 2022). For American citizens living in poverty, federal programs like Medicaid and the Children's Health Insurance Program (CHIP) exist to help provide insurance options (Medicaid.gov, 2024). In 2011, Medicaid was expanded under the American Care Act, yet states were allowed to opt out of the Medicaid expansion which eroded some people's ability to obtain affordable health care covered by insurance (Dickman et. al., 2017).

For new immigrants and non-US citizens living in the United States, they must wait five years after acquiring legal residence status to qualify for Medicaid and CHIP (Healthcare.gov, 2024) which prolongs the possibility of having no health insurance. Those who are living in the United States as undocumented cannot qualify for Medicaid (medicaid.gov, 2024). However, some states such as Massachusetts still provide state assistance to undocumented residents through programs like MassHealth (Mass.gov, 2024).

Due to factors like poverty and residence status, millions of people in America are uninsured, which directly impacts their ability to receive medical care (Dickman et. al., 2017). With the average cost of life-saving medicine like an Epipen costing upwards of $600.00 without insurance, people who cannot afford to pay out of pocket or who do not qualify for government-aided insurance are at a disadvantage in their health outcomes (Epipen Pricing, 2016).

Free medical programs provide a healthcare option for those without insurance, as well as those who still may not be able to pay for uncovered costs with certain insurances like Medicaid. In 2010, 1.8 million people utilized FMPs in the United States showcasing the need for this health safety net (VanderWielen et. al., 2015). At FMPs, patients can receive a variety of services through volunteer professional health providers trained in primary care, preventative medicine, physical therapy, and ophthalmology (Arenas et. al., 2019). Patients can also receive physicals or vaccines that are required for a job or school, or medications like insulin. With those who are uninsured having worse health outcomes than insured people (Wilper et. al., 2009), FMPs can help vulnerable populations have continuous access to healthcare when they need it.

Section 1.3 Exploring the Current State of Free Medical Programs

According to the National Association of Free & Charitable Clinics (with clinics functioning similarly to free medical programs), there are currently over 1,400 Free & Charitable Clinics in the United States (National Association of Free & Charitable Clinics, 2024). Each

FMP is unique in the services they offer, funding means, and access to a volunteer network. Yet despite this, all FMPs often fill a healthcare gap identified by medical professionals or community members and share a mission to serve a majority of uninsured patients (VanderWielen & Ozcan, 2015). The network of FMPs in the U.S. has much variety in both the size and associated funding of each program; this plays a large role in the types of services each program can offer and the number of medical providers and/or volunteers at their disposal (Isaacs & Jellinek, 2007).

Section 1.4 Addressing Limitations of Free Medical Programs

FMPs have a wide variety of services they offer to patients, with most conducting general physical exams and providing testing and treatment for chronic conditions (e.g., diabetes and high blood pressure) and minor medical problems (e.g., headaches, sore throats, cough/colds, stomach issues). Some programs may also provide prescription assistance programs, pharmacy services, and certain gynecological services (AMA Foundation, 2016). Program-to-program disparity in services is affected by the general lack of specialty care stemming from the inability to access specialists, medicine, and malpractice coverage (Isaacs & Jellinek, 2007). Specialist services include psychiatrists, orthopedists, urologists, rheumatologists, and dentists. This leaves programs only able to provide certain services, with some being dictated by the surrounding community's needs (VanderWielen & Ozcan, 2015).

Since FMPs operate under a non-profit organizational format, they primarily rely on volunteers to stay open and provide services to patients (Darnell, 2011). These volunteers come from a variety of backgrounds that help fulfill all functions in a medical program. This staff may be made up of volunteer physicians, licensed healthcare professionals, and non-licensed medical personnel. It’s also common for nurses, nurse practitioners, physician assistants and, on a smaller level of frequency, social workers and psychologists to volunteer at FMPs (AMA Foundation, 2016).

Funding of FMPs primarily can be categorized into categories of; sponsorship, fundraising, grants, and donations to stay open. Sponsorship of a FMP can be done by individuals or organizations such as hospitals, medical associations, secular community organizations, faith-based entities, and foundations that were established as a result of a hospital sale. Sponsorship may also dictate the mission and services provided at the program. Fundraising for FMPs often includes community outreach through annual fundraising drives and outreach to individuals, businesses, and other organizations. A major source of funding for FMPs is through grants, which can be from businesses, foundations, and government organizations. Grants however require a proposal, budget, and narrative, as well as compliance with the terms of the grant and any related agreements, making them less accessible for all programs (AMA Foundation, 2016). The funding of FMPs is unique to each program, and often various levels of funding emerge for different FMPs, with some even “living hand to mouth” (VanderWielen & Ozcan, 2015; Isaacs & Jellinek, 2007).

Section 1.5 Benefits to the Community Free Medical Programs Provide

There is a large uninsured population in the United States, making up approximately 46 million people, who are often forced to forego needed healthcare due to prohibitive costs. FMPs, which make up part of the "health safety net" for uninsured people in need of healthcare, offer basic services for little or no cost to patients. Although FMPs are one of the few options available to uninsured and underinsured people, they have been widely overlooked and rarely studied. This has caused the characterization of the free medical program sector to be largely impossible.

A national survey of all known FMPs sponsored by the Agency for Healthcare Research and Quality was conducted in 2010 that attempted to characterize FMPs based on standard criteria and learn more about the communities they serve. It was found that free programs provide both preventative and general medical care for approximately 10% of the uninsured, working-age adult population that seek care (Darnell, 2010).

Due to the understudied nature of free health programs, it remains a challenge to quantify the impact that free health programs have on communities that utilize them. However, patients of free health programs report high degrees of satisfaction with primary care and routine women's health services received at free programs, especially when compared to the degree of satisfaction with other, traditional, healthcare options made more affordable with insurance. Patients of FMPs report a high intent to continue seeking care at free programs. FMPs also help to alleviate some of the strain on emergency rooms, as patients of free programs reported that the emergency room was their only other option for primary healthcare (Gertz et. al., 2011).

There are various types of FMPs in the United States, including independent FMPs, church-run FMPs, and student-run FMPs. Independent FMPs are usually better staffed, open more days of the week, and have larger budgets than the other types of FMPs. Each kind of free program offers different levels of care depending on the resources available to them, which can be limited due to staffing constraints. Student-run programs are mostly evenly distributed throughout the country instead of primarily in areas with higher need. This is most likely due to the distribution of medical schools throughout the country (Gertz et. al., 2011).

Chapter 2: The Project

Section 2.1.0 Need Statement

In Worcester, Massachusetts, three FMPs—Epworth, Akwaaba, and WICCS—operate locally, providing essential services to uninsured individuals. These programs are part of the Worcester Free Program Coalition, a group of seven free medical programs that serve as a vital health safety net for the community. Their primary goal is to offer medical assistance to those facing obstacles in accessing conventional healthcare systems.

In response to the COVID-19 pandemic, fiscal recovery funds were allocated by the federal government to establish an American Rescue Plan Act (ARPA) Grant. A component of the grant reimburses community programs for essential services provided during and after the COVID-19 pandemic. The three programs we partnered with, Epworth, Akwaaba, and WICCS, received a $49,230 grant from the City of Worcester, in partnership with the ARPA program to reimburse services provided during and in the reconstruction period after the COVID-19 pandemic. The grant reimbursed the programs for services provided for Worcester residents who have been disproportionately impacted by the COVID-19 pandemic. The grant's guidelines, as outlined by the documentation provided by the Federal Office of the Treasury and the City of Worcester, specify various criteria for program visits to qualify for coverage. These criteria include ethnicity, race, address, household income per size, participation in government programs, and specific services received during the visit. For each qualifying visit, the free medical program will be reimbursed $128.24 until the full $49,230 is exhausted.

Despite each program being unique in their size, patient volume, funding and more, this network of FCs needed our team to design and implement a documentation system within the constraints of the ARPA grant to secure federal funding.

Section 2.1.1 Mission Statement & Project Objectives

When the team began this project we created a mission statement that encapsulated what we hoped to accomplish and embody during the duration of this project. It is as follows;

Mission Statement:
To support accessible, sustainable and free healthcare in Worcester for populations disproportionately affected by COVID-19.

In addition to our broader mission statement to guide our efforts our team identified a technical goal statement. This technical goal statement is derived from the more quantitative aspect of our project to give the team tangible aspirations. It is;

Technical Goal Statement:
To design and implement a documentation system within the constraints of an ARPA grant to secure federal funding for three free public health programs in Worcester, Massachusetts.

Our technical goal statement and mission statement both are aligned with the primary goals of the FMPs. To further develop the scope of our project the team created project objectives we wanted to fulfill by the conclusion of the project. With these project objectives the team achieved both our technical goal and mission statement. These objectives are identified below:

1. Determine a reasonable fiscal end goal by identifying trends from data taken at each of the three programs.

2. Determine how each program functions by assessing the means of organizing information and administrative procedures, leadership approaches and who will take responsibility for the ARPA forms.
3. Identify the ways in which writing can be and is applied through our project.

As previously discussed in section 2.1, the ARPA grant was created in response to the disproportionate effects of the COVID-19 pandemic on Worcester, MA residents. This grant is derived from fiscal recovery funds and aims to reimburse free medical programs for qualifying medical visits. The FMPs involved in the grant include; Epworth, Akwaaba, and WICCS. As noted above, the total amount for this grant is $49,230.00 and $128.24 per participant allowing for 384 unduplicated qualifying individuals' visits at any of the three Worcester Free Medical Services to be reimbursed to the program. The grant's guidelines specify various criteria for program visits to qualify for coverage. These criteria include ethnicity, race, address, household income per size, participation in government programs, and specific services received during the visit. Included in Appendix A is the ARPA grant form used in the free programs.

Our project was responsible for the implementation of the grant as well as the tracking of patients seen by all programs under the grant to ensure no duplicate patients are covered by the grant. This is accomplished by the group via a "crosswalk" stored on the secure WPI browser. The crosswalk is an Excel sheet where data entry is completed for collected forms from programs that have been checked and qualify for the grant. This data entry properly guarantees the programs can use the grant under the state's guidelines, such that no duplicates will be sent to the state for reimbursement. The crosswalk holds the following information from each qualifying patient; date of visit, patient number, name, patient identification number, and program visited. There is a blank version of the crosswalk to showcase its format in Appendix E.

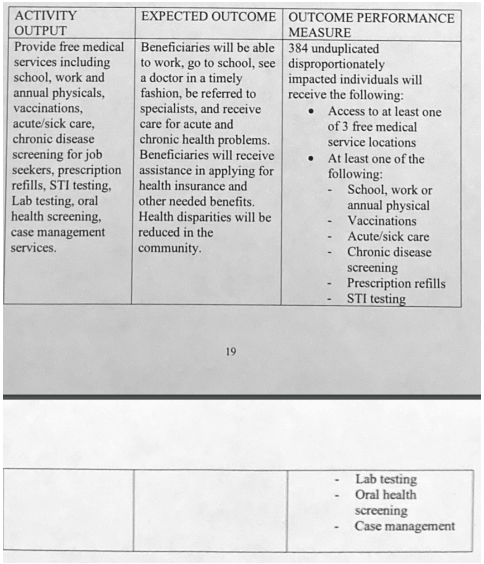
Once the crosswalk was developed the team created an additional cover page to be used alongside the forms at the three FMPs to assign patients an identification number and obtain their name. This cover page contains information explaining what the form is for and helps patients make informed decisions on their participation in the grant. Although the cover page contains their name, it is not sent to the City with the actual ARPA grant forms but is shredded once the patient information is archived in the crosswalk. There is a blank copy of the cover page in Appendix A.

Despite having data entry means and privacy concerns covered, the team had to account for variation in each free medical service and how that variation impacted the successful implementation of the grant forms. This stemmed from various functional formats of the FMPs, their staff involvement, and even the program's ability to distribute the grant forms. Our team created long-term solutions for the three medical service programs; this meant that although our project concluded at the end of this spring, the FMPs participating in the grant are still able to operate while collecting reimbursement money through their own means. In doing so, the team had to account for the unique struggles each FMP has to implement a sustainable solution.

Section 2.2.0 Methodology/Approach Overview

Section 2.2.1 Iterations of the Form

The original ARPA Grant outlines a "Performance-Based Payment Plan" in which the beneficiaries of the grant- the three free programs- will provide select services to 384 unduplicated eligible patients living in Worcester, who have been impacted or disproportionately impacted by the COVID-19 public health emergency or its negative economic impacts. The Performance-Based Payment plan lays out specific activities, outcomes, and performance measurements that ensure the grant is directly benefiting the appropriate patients. Figure 1 displays the eligibility requirements for a patient visit, with each eligible patient visit equating to a total of $128.24 per participant every month for successful completion of the outcome performance measure.



ACTIVITY OUTPUT	EXPECTED OUTCOME	OUTCOME PERFORMANCE MEASURE
Provide free medical services including school, work and annual physicals, vaccinations, acute/sick care, chronic disease screening for job seekers, prescription refills, STI testing, Lab testing, oral health screening, case management services.	Beneficiaries will be able to work, go to school, see a doctor in a timely fashion, be referred to specialists, and receive care for acute and chronic health problems. Beneficiaries will receive assistance in applying for health insurance and other needed benefits. Health disparities will be reduced in the community.	384 unduplicated disproportionately impacted individuals will receive the following: • Access to at least one of 3 free medical service locations • At least one of the following: - School, work or annual physical - Vaccinations - Acute/sick care - Chronic disease screening - Prescription refills - STI testing
		- Lab testing - Oral health screening - Case management

Fig. 1. A summary of eligibility requirements directly from the state for each patient visit under "Outcome Performance Measure".

A method to track patient visits and evaluate the eligibility of patients for the grant was developed by the team based on the criteria outlined in Fig. 1. Due to the distinct operational and cultural considerations at each FMP, the medical programs were first observed by the team to gather information on how each program operated, the cultural and linguistic considerations at each program, and the patient and healthcare team workflow. It was observed that while each program had distinct cultural considerations, patient populations, and overall workflow, the general structure of how a patient arrives at the program, receives care, and exits the program were similar at each site. Due to this observation, a form regarding grant funding eligibility was created to be completed by a patient or their guardian at the time that the patient arrived to receive care from either Epworth, WICs, or Akwaaba.

Layout, language, and word choice were carefully considered when developing and iterating on the form throughout the grant implementation process. Due to the differing patient

populations at each program, it was important to ensure the form was written in a way that was comprehensible and in language that was easy to read and understand. This included not using overly technical or complex terms but instead, using laypeople's terminology. Additionally, it needed to be made clear to the patient that any sensitive information would be kept confidential. **See Appendix A** for our full grant form.

Section 2.2.2 Implementing Sustainability Practices

Implementing a sustainable process each program could follow to correctly gather and report eligible visits was important to establish as a result of the student team's graduation being prior to the end of the grant implementation period. To assist the programs with this process an infographic, instructional document, and "grant importance" document were developed. The infographic and supporting documents represent three ways to communicate how to fill out the form and why the form is important for the programs to allow them to receive grant funding without the help of the MQP team.

Infographic

The infographic created by the team is a collection of imagery and instructional language that provides five steps to follow in order for the programs to fill out and complete the grant-eligibility form without outside assistance (**See Appendix B**). The form includes two main sections: purpose and process steps. The purpose section is a small text box that briefly explains the rationale and importance for the programs to complete the forms. The process steps section outlines the five steps in an easy-to-follow format, including imagery and color coding.

Instructional Document

The instructional document included by the team is a written step-by-step procedure for the programs to follow that details how the program can complete the grant eligibility form. The form includes two main sections: purpose and steps by program. The purpose section is a short, five-line paragraph that again outlines the importance and rationale for the programs to complete the forms in order to receive funding (**see Appendix C**).

"Grant Importance" Document

Due to feedback from Akwaaba that the program was struggling to fill out the forms without MQP team assistance, a one-page "Grant Importance" document was developed by the team. The document is titled "What the ARPA Grant Can Do For Your Medical Service" and uses both written language, varying font settings, and visual graphics in order to easily display the importance of receiving grant funding for the program by completing the form. The written component explains that eligible visits will award the program with $126.24 per visit, with each form taking less than five minutes to complete. Figure 1 displays the visual graphic used within the Grant Importance document that shows how one eligible form completed by the program will gain $126.24 towards the program. See **Appendix D** for the Grant Importance document.

Section 2.3.0 Results

Section 2.3.1 Quantitative Results

Over the time of this project (November 2023 to March 2024), 94 patients' visits qualified for the ARPA grant. With each qualified visit equaling $128.24 in grant funds, the three programs collectively have been awarded $12,054.56. As the maximum amount of funding available through the grant is $49,230, this means that 25% of available ARPA grant funds have already been distributed to the programs over the course of four months. If the rate of qualifying form acquisition and submission to the government remains the same from this point in time onwards, the grant should be fully distributed to all programs within 12 months. With the grant ending in July of 2025, there should be ample time for form collection and submission so the grant can be fully distributed if our methodology is followed.

The Worcester Free Care Collaborative, which includes the health programs of Akwaaba, WICS, and Epworth, cares for over 5,000 patients annually (WFCC, 2024). These programs run entirely on private donations and grants, like the ARPA grant our project focused on (WFCC, 2024). Although not all 5,000 patients qualify for the ARPA grant, their care is improved by the funds the program receives from those visits that do qualify.

Section 2.3.2 Qualitative Results

Through volunteering at the programs, team members of this project observed the structure and organizational methods of each FMP. From there, a grant form including a top explanation sheet in different languages was developed (appendix A). After the initial attempt to implement this grant form into the admission process of each program, it was clear that each program would have different needs. It was initially challenging to implement the form due to the program's volunteer workers' requiring a greater understanding of the grant form and its purpose. The team created additional written resources in order to increase effective communication between us and the program volunteer workers. These additional written resources took the form of an infographic (Appendix B), an instructional document for implementation (Appendix C), and a document explaining the importance of the grant to FMP volunteers (Appendix D). Ultimately, the team learned that communication was increased with the FMPs through clearly written, well-distributed infographics and instruction sheets.

Writing in non-profit settings is challenging due to the vast amount of genres utilized in this type of organization. For example, non-profit writing includes genres such as memos, instruction manuals, grant proposals, grant reports, annual reports, media-related documentation, and more (Jones et. al., 2020). Non-profit organizations' success relies heavily on effective communication internally, with those who utilize their services, as well as with partners in the private sector and the government.

Section 2.3.3 Impacts on the Community

After implementing the grant for the government and finding patients who qualify, we could see the effect the grant would have on both the free programs and the greater Worcester community. The money raised has the potential to enable the programs to continue to run and acquire necessary resources, allowing them to continue to serve populations in Worcester that were disproportionately affected by COVID-19 and those that rely on free programs as their primary source of healthcare. The grant provides support for the free programs and makes it possible for the free programs in Worcester to operate as a healthcare safety net as they were intended. With more funding, FMPs can purchase the medical supplies they need. They could also start additional programs running out of their spaces such as food banks and free clothing supply centers. More funding for FMPs means improved patient health and well-being, all of which also help the entire Worcester community.

In addition to the funding allowing the FMPs to continue to run and acquire necessary resources, the patients have a direct effect from the implementation of the grant. Many patients across all free clinics were able to receive work and school physicals, vaccinations, tuberculosis testing, prescription refills, and other crucial services like dental and dermatological care. With the patients being able to have increased access to these services provided by the FMPs, they are able to return to work, which might have been affected by the COVID-19 pandemic, school, and be in better health.

Chapter 3: An Anthropological Lens on Writing for Non-Profit Programs

Section 3.1 Project Impacts Through Writing

Our project had the unique ability to combine our written skills developed from coursework into a tangible outcome that touched many people in the Worcester community. Unlike what most assume writing is capable of, our project helped to change the city around us that we have spent the last four years living in, and will have a lasting impact even after the conclusion of this project. Most notable is the way the three free programs we worked with are impacted in both their ability to take on such roles in the grant process, as well as the benefits from such work to keep their programs open and improve the experiences of the individuals who visit.

To highlight the impact of this project, our team helped the three free Worcester night programs receive $128.24 per qualified visit in reimbursement funding to the programs. This number only includes patients who qualified for grant reimbursement; however, there are many more people being served by the grant implementation and who will feel the positive effects of this grant. This funding provides the Worcester Care Collaborative Inc. with the necessary means

to better serve their patients and improve patient experiences. Writing has the ability to turn the possibility of grant funding for the local free programs into reality and create sustainable practices for all types of programs. Several different aspects of writing can be seen throughout the process of this project and although some may seem obvious others constitute further thought and reflection to see their impacts. Further discussed in the following sections is the way in which writing through the teams' experiences and perspectives made our project both successful and impactful.

Section 3.2 Writing as Negotiation

A large part of this project was communication back and forth with the City of Worcester and negotiation the team completed as a means of writing. At the start of the project, the City provided deliverables on information regarding the guidelines of the grant, initial forms to be filled out, and other information surrounding the grant and its purpose. The team had to negotiate back and forth in developing a form that was usable in the program's setting and satisfied the needs and requirements the city had for the grant. This process was in part negotiation with the programs, figuring out what worked and what didn't work for them through assessing the needs of each individual program to create a form that would be usable. And as that negotiation with the City settled, the next negotiation with the programs began to be at the forefront as we helped them use the forms and take responsibility for their use. Asking struggling, or understaffed, programs to do more work required negotiating with volunteers. The team negotiated with programs to increase their use of the forms by making the how-to guides for the program staff to facilitate their understanding of what the grant could do for their program. The next negotiation was with the patients, helping them to understand the form they were filling out, which was complicated by frequent language barriers. Asking personal questions about income, ethnicity, the government programs used, and the reason for visit can feel invasive and takes communicating a commitment to patient confidentiality (ensuring that their private information will not be given to the government) as well as conveying the positive intent of the grant. When proper negotiation was implemented the team found success and both the programs and patients were on board with participation in the grant.

Section 3.3 Writing for Instantiating Need

In some ways, writing can constitute a form of reality. People sign legal contracts that bind them to work for a company for a set time, prevent them from disclosing information about sensitive issues, or marry another person. When writing in the free health program setting, documenting patient visits through the grant qualification form helps to prove that there is a need for free programs. By gathering other information through standard patient intake forms at the front desk, programs can prove that their patients need their services. Programs can gather data on why exactly their patients are using their programs, what illnesses or health problems are most often encountered, and how frequently people come back to the same program.

Without the forms and documentation systems we implemented in our MQP, the City would not be able to track qualified visits. In this way, our documentation systems help instantiate the need for grant money, and therefore the need for free health programs that heavily rely on grant money. In the future, the data from our documentation systems could be analyzed to determine if there are other populations that may benefit from alternate grants from the government. These grants may not cover the same qualifications as this ARPA grant, but instead cover other currently under-resourced populations that also utilize Worcester's free health programs.

Section 3.4 Writing Constitutes Culture

Writing documentation for free programs helps to put sensitive issues into perspective. Writing about how some people do not have the same access to healthcare as others, or how many people were disproportionately affected by COVID-19, makes systematic flaws in the healthcare industry known to a wider audience, ultimately increasing awareness of systematic issues and improving cultural sensitivity.

The three FMPs eligible for the COVID-19 relief ARPA grant- Epworth Clinic, Worcester Islamic Center Clinic, and Akwaaba Clinic- serve patients from distinct and varying cultural backgrounds. Adapting to the cultural and linguistic needs of each FMP was extremely important to maximize the effectiveness of the form and respect the patients at each clinic.

Section 3.5 Writing is Disincentive

As a means of communication in this project, writing needed to be understandable to individuals with varying levels of literacy and education. Complex language can disincentivize and discourage a patient from participating in filling out the form. Due to the complex technical language used within the original ARPA Grant, the team needed to develop a rewritten version of the ARPA Grant through a simplified Grant Eligibility Form. Word choice and formatting were carefully considered to ensure the form was understandable for all patients at each of the free programs. Additionally, language can disincentivize patients from filling out a form if it is not in their first language. The team also translated the form into multiple languages as an option for patients at the free programs.

Section 3.6 Writing is Ethics

Deliberate verbiage and word choice within the Grant Eligibility form were necessary to ensure all patients had an equal understanding and opportunity to complete the form. When writing is too complex or not understood by all audiences, some individuals may not be able to access the benefits resulting from the written work. Complex language and certain words or phrases can also insinuate biases within the written work and wrongfully assume the work will be understood. Additionally, if not all audiences have an understanding of a written work,

writing can effectively be used against certain populations and decrease equity. For example, having a written work translated into only one language can effectively limit all individuals speaking other languages to read and benefit from the written work.

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Appendices

Appendix A: Grant Form

In English

This free medical program is participating in a City of Worcester grant that can reimburse us for your visit. Your identifying information will not be shared with the city, but we do need to ask you some questions on page two to see if your visit qualifies.

My visit can be used towards the grant.

Print name: _____ Date: _____

Participant Identification Number: _____

En español

Este programa médico gratuito participa en una subvención de la ciudad de Worcester que puede reembolsarnos su visita. Su información de identificación no se compartirá con la ciudad, pero necesitamos hacerle algunas preguntas en la página dos para ver si su visita califica.

Mi visita se puede utilizar para la subvención.

Nombre impreso: _____ Fecha: _____

Número de identificación del participante: _____

Em português

Este programa médico gratuito é subsidiado pela cidade de Worcester e pode oferecer reembolso pela sua visita. As suas informações pessoais não serão compartilhadas com a cidade, mas precisamos fazer algumas perguntas na segunda página para determinar se a sua visita é elegível para o subsídio

Minha visita pode ser usada para a concessão.

Nome impresso: _____ Data: _____

Número de identificação do participante: _____

—---------- PAGE 2 BEGINS —----------

City of Worcester in partnership with Worcester Evening Free Medical Service Program Inc (Epworth), Worcester Islamic Center Social Services, WICSS) Free Medical Program, Akwaaba Free Health Program

COMMUNITY PROJECTS AND PROGRAMS COMPLIANCE FORM FOR SLFRF FUNDING

The participant/guardian should complete this form regarding program eligibility. Several regulations require that we determine eligibility for participants receiving services paid for, in part, but the State and Local Fiscal Recovery Funds (SLFRF), which are provided by the United States Department of the Treasury. The service, or contract, provider should retain this form for monthly reporting requirements as well as for on-site monitoring visits.

INFORMATION PROVIDED ON THE FORM IS KEPT CONFIDENTIAL AND IS NOT SHARED WITHOUT YOUR PERMISSION EXCEPT AS REQUIRED BY THE US DEPARTMENT OF THE TREASURY TO CONFIRM INCOME ELIGIBILITY OF PARTICIPANTS IN SLFRF FUNDED PROGRAMS. THE CITY OF WORCESTER HAS THE RIGHT TO VERIFY ELIGIBILITY.

PARTICIPANT INFORMATION

Address: _____

(Street, City, and Zip Code required)

SELF-DECLARATIONS

Please state your ethnicity and race from the boxes below.

Ethnicity *(please select only one)*

- ☐ Hispanic or Latino
- ☐ Not Hispanic or Latino

Race *(please select only one)*

- ☐ White
- ☐ Black/African American
- ☐ Asian
- ☐ American Indian/Alaska Native
- ☐ Native Hawaiian/Other Pacific islander
- ☐ American Indian/Alaskan Native *and* White
- ☐ Asian *and* White
- ☐ Black/African American *and* White
- ☐ American Indian/Alaskan Native *and* Black/African American
- ☐ Other Multi-racial _____

HOUSEHOLD INCOME INFORMATION

1) Circle the household size below and proceed to question 2.

Household size	1	2	3	4	5	6	7	8

Income Limits	$50,310	$59,160	$74,580	$90,000	$105,420	$120,840	$136,260	$151,680

2) For the household size circled above, is your income **below** the income amount listed?
Please Circle **YES** or **NO**

—------- PAGE 3 BEGINS —----------

Do you partake in any of the following government programs? <u>Please check *ALL THAT APPLY*</u>

- ☐ Children's Health Insurance Program (CHIP)
- ☐ Childcare Subsidies through the Child Care and Development Fund (CCDF) Program
- ☐ Medicaid
- ☐ National Housing Trust Fund (HTF), for affordable housing programs only
- ☐ Home Investment Partnerships Program (HOME), for affordable housing programs only
- ☐ Temporary Assistance for Needy Families (TANF)
- ☐ Supplemental Nutrition Assistance PRogram (SNAP)
- ☐ Free and Reduced-Price Lunch (NSLP) and/or School Breakfast (SBP) programs
- ☐ Medicare Part D Low-income Subsidies
- ☐ Supplemental Security Income (SSI)
- ☐ Head Start and/or Early Head Start
- ☐ Special Supplemental Nutrition Program for Women, Infants, and Children (WIC)
- ☐ Section 8 Vouchers
- ☐ Low-Income Home Energy Assistance Program (LIHEAP)
- ☐ Pell Grants

<u>PARTICIPANT CERTIFICATION</u>

I certify that the above information is true and correct to the best of my knowledge,

Participant/Guardian: _____ **Date:** _____

(signature)

==========

TO BE COMPLETED BY THE MEDICAL PROGRAM

<u>SERVICES PROVIDED</u>

The following is to certify that the patient received a service eligible for City of Worcester reimbursement under SLFRF. By signing below, this certifies that the patient received one or more of the services below while at the free medical program listed. Individuals that can certify on behalf of the program(s) include licensed providers, RNs, medical students, dental technicians, case managers, and registration personnel.

Participant Identification Number: _____

Seen at

- ☐ Epworth (WEFMSP Inc)
- ☐ WICSS

Care Provided

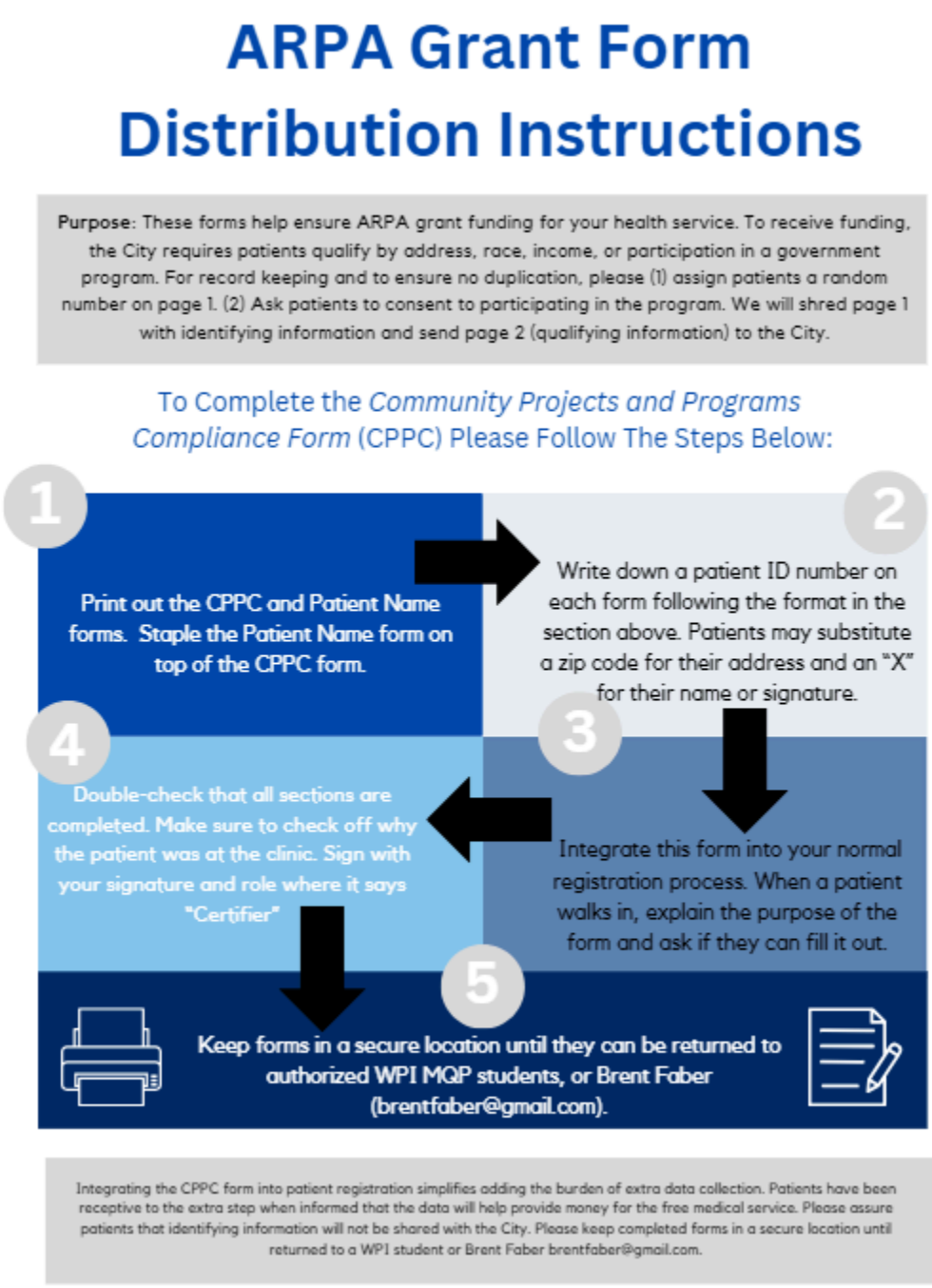
- ☐ School, work, or annual physical
- ☐ Vaccinations
- ☐ Acute/sick care
- ☐ Prescription refills
- ☐ STI testing
- ☐ Lab testing
- ☐ Oral health screening

☐ Akwaaba ☐ Chronic disease screening ☐ Case management

Certifier Name _____ Title _____

Signature of Certifier _____ Date _____

Appendix B: Infographic



Appendix C: Instructions for program

ARPA Grant Form Distribution Instructions

Purpose

By distributing these forms, you are helping to ensure ARPA grant funding for Worcester's free public health programs. To receive funding, the state requires patients at the program fill out the Community Projects and Programs Compliance Form (CPPC). Patients need to be assigned a random number upon being handed the two forms so the form can be entered into a secure database. This number should follow the format (month-day-year-starting number of the day).

EX) If starting on January 25th, 2024, write 1252401 for the first patient, 1252402 for the second, etc.

Steps by Program

1. Print out both the CPPC and Patient Name forms.
2. Staple the Patient Name form on top of the CPPC form.
3. Write down a patient ID number on each form following the format above.
4. When a patient walks into the program, explain the purpose of the form to them and ask if they would like to fill it out. Integrate this form into your normal registration process.
    a. Example script to explain this form: “I am helping to secure more governmental funding for this program. If you would like to, you can fill out this form which will go to the government. If your visit qualifies, the program here will receive money from the government to continue serving the community. Filling out this form is completely optional and you can stop at any time you do not feel comfortable. Nothing is required of you, and by filling out this form you will not have to give money to the program. This only helps to support more funding for the program from the government.” Make sure to ask for their consent and ensure they know they can stop filling out the form if they want to. Choosing to not participate in this will NOT impact their medical care.
5. If they say yes and would like to fill it out, you can give them a pen and clipboard if necessary. If they have any questions, you can explain as they go through it or sit next to them and help them fill it out.
6. Once they have completed the form (double-check that all sections are completed properly), ask them why they are visiting today.
7. Fill out the back section of the CPPC form. Make sure to check off why the patient was at the program, and sign off with your signature where it says “Certifier”.
8. Keep forms in a secure location until they can be returned to authorized WPI MQP students, or Brent Faber (brentfaber@gmail.com).

Appendix D: Importance Document

What the ARPA Grant Can Do For Your Medical Service

The ARPA Grant gives the Worcester Evening Free Medical Service Program, Inc. the opportunity the receive reimbursement for patient visits that qualify according to specific grant-defined deliverables. These qualifiers include; ethnicity, race, address, household income per size, participation in government programs, and specific services received during the visit. Completed forms will be collected by ARPA grant team and submitted to the city. Money will be reimbursed to the clinic.

- **$49,230 total** Grant money available to receive **by 2025.**
- **$128.24 per qualifying form**
- **Less than 5 minutes** to fill out the form
- Clinics are only responsible for the completing the forms and holding them until they are collected

1 Eligible Completed Form

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$126.24 towards clinic

Appendix E: Blank copy of crosswalk format