Canonical mRNA cap binding proteins have distinct roles in regeneration from the fish specific ortholog *eif4e1c*

Honors Research Thesis

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Abstract:

Zebrafish are valuable models for cardiac regeneration due to their remarkable ability to regenerate heart tissue. Our research focuses on the eIF4E family of translation initiation factors, which are suspected to play a role in this process. While canonical eIF4E is found in all terrestrial vertebrates and is essential for cell viability, zebrafish also contain an additional ortholog that is unique to aquatic vertebrates, called *eif4e1c*. Previously, we showed that deletion of *eif4e1c* impaired growth, survival, and heart regeneration. It is unclear whether these phenotypes result from the reduced total level of cap-binding proteins-or if *eif4e1c* has specialized functions. To investigate further, we used CRISPR to make zebrafish deletion mutants for canonical *eif4ea* and e*if4eb*. Concurrent deletion of *eif4e1c* and *eif4ea* resulted in a further mortality and size deficits when compared to the solo *eif4e1c* mutants. Compound mutants of *eif4e1c* and *eif4eb* found similar growth defects, but no exacerbation of mortality. The eIF4E family of translation initiation factors are required for viability in eukaryotes from yeast to mice. Surprisingly, deletion of both *eif4ea* and e*if4eb* did not result in any obvious phenotypic changes in survival, growth, nor heart size. However, it was found that the loss of canonical *eif4ea* or *eif4eb* alone results in improved cardiomyocyte regeneration, and impaired fin regeneration. Compound mutants of a canonical and *eif4e1c* lose these regeneration phenotypes suggesting a "goldilocks" threshold where *eif4e1c* begins to compensate fully for the loss of canonical factors. This is to say, there may be a required ratio – canonical factors to *eif4e1c* – which allows for compensation. These results suggest *eif4e1c* can fully compensate for the loss of the canonical EIF4E factors, while the canonical factors only partially compensate for

the loss of *eif4e1c*. This suggests that the fish specific *eif4e1c* ortholog is a bona fide translation initiation factor with additional functions that impact growth and survival.

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INTRODUCTION

Zebrafish are excellent models for studying regeneration. Many groups covet them for the insight they provide into limb regrowth by the formation of a blastema of stem-like cells. This regenerative process was first described in Thomas Hunt Morgan's book *Regeneration in Teleosts* in 1900. This phenomenon has been studied extensively since and has led to the discovery of yet another zebrafish regenerative capability: cardiac regeneration.

Ischemic heart disease is the leading cause of death in the United States (WHO, 2024) and results in irreversible cumulative damage to the heart. Upon cardiac injury, the contractile cells called cardiomyocytes are replaced by non-contractile tissues, fibroblasts, which proliferate and fill in gaps by depositing scar tissue (Rog-Zielinska et al. 2016). Zebrafish avoid this cardiac

scarring through a proliferation of pre-existing cardiomyocytes which dedifferentiate, proliferate, and then redifferentiate into their contractile form (Jopling et al. 2010).

Many different labs are investigating the molecules involved in this proliferative programming in zebrafish to better understand the prerequisites of regenerative medicine. Of these labs, the majority focus on the relative abundance of mRNAs through targeted *in situ* hybridization and other unbiased approaches based on RNA sequencing (RNAseq) (Goldman et al., 2017; Honkoop et al., 2018; Wu et al., 2016). It is important to note that while mRNA is useful measurement of gene expression, it is not the sole determinate of protein production. In only about 40% of genes do mRNA levels correlate with the amount of protein translated (Buccitelli and Selbach, 2020). This disconnect suggests that there is also some post-transcriptional regulation involved (Ma et al. 2018). One important post-transcriptional regulator of gene expression is initiation of translation (Kong and Lasko, 2012; Wang et al., 2020a). The classic pathway for translation initiation is recognition of 5' methylated caps on mRNA (Borden and Volpon 2020).

The canonical cap binding protein called eIF4E is responsible for the initiation of loading and its expression levels can regulate the translation of specific mRNA subgroups (Davis et al., 2019; Truitt et al., 2015). The eIF4E pathway is considered canonical because it is required in all eukaryotes. In mice, *Eif4e* is required for development which restricts its study to mainly cell culture models (Sénéchal et al., 2021). Partial knockdown in heterozygote mice, however, has shown resistance to certain cancers (Truitt et al., 2015). Intriguingly, this factor is also upregulated in the growing heart muscle of neo-natal mice although its regulation therein is not well documented (Chen et al., 2022). It should also be noted that alongside eIF4E, is a second ortholog exists called eif4e1b that represses translation in both Xenopus and zebrafish (Minshall

et al., 2007; Robalino et al., 2004). Further, another ortholog, *eif4e1c* exists in all aquatic vertebrates (Joshi et al., 2005; Zhang et al., 2018). Zebrafish have two paralogs of canonical eIF4E called *eif4ea* and *eif4eb,* and are likely the product of a whole-genome duplication event around 380 mya in teleost fish (Taylor et al., 2001).

Previous profiling studies of *eif4e1c* revealed that the expression of this fish-specific ortholog increases during regeneration (Goldman et al., 2017). In subsequent studies of this cap binding protein, knockout mutants of *eif4e1c* revealed decreased size, decreased cardiomyocyte proliferation following injury, and decreased cardiomyocyte number as well as increased mortality (Rao et al., 2023). The phenotypes resulting from deletion of *eif4e1c* suggest two possible models: the phenotypes were caused by a reduction in overall translation irrespective of the family of cap binding proteins *or* there is some unique function to the *eif4e1c* protein which is responsible for these phenotypes.

To test this, we created CRISPR knockouts of *eif4ea, eif4eb,* and *eif4e1c* and observed phenotypes to better understand the relationship between these three cap binding proteins. Knockout mutants of the canonical factors demonstrated that *eif4e1c* can fully compensate for their absence. Thus, the *eif4e1c* phenotypes are unique to *eif4e1c,* and are not simply the result of overall reduced translation. However, compound deletion mutants of *eif4e1c* and *eif4ea* (or *eif4eb*) potentiated *eif4e1c* phenotypes. Due to the worsening phenotypes with each subsequent knockout, triple mutants were made and found that fish lacking all three factors died after 15 days post fertilization.

Materials and Methods:

Statistics

To ensure data collected was relevant, an α error probability of 0.05 was used to determine if differences were statistically significant. Outliers were removed based on husbandry observations which found a *bully* (the largest fish) and *runt* (the smallest fish) in every tank of 12 fish. To adjust for these outliers, the largest and smallest fish of every 12 fish was removed. All graphs were made in Prism.

Fish Strains

The Ekwill strain of zebrafish (*Danio rerio)* was used for this entire study. Zebrafish were not separated by sex during development.

CRISPR mutant production and genotyping – Pairs of sgRNA fused to tracRNA were assembled with Cas9 and injected into newly fertilized zygotes using the IDT DNA system. For *eif4ea,* sgRNA targeted exon 3 (TGT CAA ACT TTG AGA TGA GA) and exon 7 (CAT GTG ACT GGT ATC CAA TG) that created a 4,262bp deletion. For *eif4eb*, sgRNA targeted exon 2 (GAT CTC AGA CGA GAG CAA TC) and exon 3 (CTC CAA ATT TGA CAC CGT AG) that created a 560bp deletion. Independent lines from different injections were crossed to one another to homozygous mutant alleles and minimize potential for homozygosis of possible off target mutations that would be confounding. We created two lines for *eif4ea*, one with a perfect deletion (L2) and the other had an insertion of a single G (L1). For *eif4eb* we created four lines. Two with *eif4eb* alone and two that were injected into *eif4e1c* carriers to create double mutants since both *eif4eb* and *eif4e1c* are on the same chromosome. For the *eif4eb* solo mutants, one had an insertion of GGATTTTTGGG (L4) and the other deletion was perfect (L5). For the double mutants one allele was perfect (L5) and the second had an insertion of GGG (L8). For genotyping *eif4ea* mutants we used two oligos that bridge the mutation 5'-ACT TGT TCT TGG TGG TGG AAC CGC and 5'-TCT AGA GGA GTT ATC ATC CCC AAC CCC plus a third

internal oligo that recognizes the wildtype allele (5'-GGT CAC TTT AGG ATC TGT TCA CCC TGC). For genotyping *eif4eb* mutants we used two oligos that bridge the mutation (5'-TGT TCA AAC TGG CTG CTT TAG TGT GGA and 5'-AGC GAG TAG TCA CAT CCT GAC ATC AG) plus a third internal oligo that recognizes the wildtype allele (5'-GCA AAA CAT GGC AGG CCA ACC). Deleted domains from both *eif4ea* fish as well as *eif4eb* provided for loss of function of these genes (Fig 1 A,B).

Length and weight measurements:

Zebrafish (3-4 mpf) were anesthetized in phenoxyethanol (800λ) measuring length using a ruler estimated to the nearest millimeter from the tip of their mouth to the center of the bifurcation of their caudal fin. They were then blotted dry with Kimwipes and weighed on an analytical scale. Fish of all genotypes were raised and housed together, and measurements were taken shortly after genotyping.

Zebrafish cardiac regeneration experiments:

Zebrafish were placed in Tricaine and then placed dorsal side down in the carved groove of a sponge for surgery. Iridectomy scissors were used to open the ventral integument and pericardial sac. Gentle pressure was applied to the abdomen to expose the heart before around 20% of the apex was removed with curved scissors to allow surface tension to aid in clotting process (Poss et al., 2002).

Quantification of CM proliferation:

10 µL of a 10mM solution of EdU diluted in PBS was injected into the abdominal cavity of injured zebrafish once every 24 hours for three days (4-6 dpa). Hearts were removed 7 dpa, embedded and cryosectioned. Sectioned hearts of 10-12 µm heart sections were washed 4x5 min

in 1mL of 1x PBT with 0.1% Tween-20. Slides were blocked for 1 hour at 37 ℃ in NCS-PBT (10% Newborn Calf Serum (Sigma N4762), 90% PBT, and 1% DMSO). Slides were then incubated with Boster DZ01398 (Rabbit polyclonal anti-Mef2aa 1:500) overnight at 4 ℃. Slides were then incubated in anti-rabbit-488(1:200) for 2 hours at room temperature and then re-washed in PBT. Slides were then imaged on a confocal microscope (Zeiss).

The number of $Mef2^+$ and $Mef2^+EdU^+$ cells were counted using MIPAR image analysis software and the CM proliferation index was calculated as the number of $Mef2^+EdU^{+/}$ $Mef2^+$ cells (Sosa et al., 2014). CM proliferation was averaged across 1-3 appropriate sections per heart to produce tabled values.

Quantification of CM count:

Sectioned hearts of 10-12 μm heart sections were washed 4x5 min in 1mL of 1x PBT with 1% Tween-20. Slides were blocked for 1 hour at 37 ℃ in NCS-PBT(10% Newborn Calf Serum (Sigma N4762), 90% PBT, and 1% DMSO). Slides were then incubated with [Primary Antibody] overnight at 4 ℃. Slides were then incubated in [Secondary Antibody] for 2 hours at room temperature. Slides were then re-washed in PBT. Slides were then imaged on a confocal. The number of $Mef2^+$ cells were counted using MIPAR image analysis software and CM count values were averaged across 1-3 sections per heart for tabled values.

Fin regeneration analysis:

Fish (3-4 mpf) were anesthetized in phenoxyethanol (800λ), and caudal fins were amputated right below the center of the fins where the lobes diverge using a razor blade. The removal was approximately halfway between the body and tip of the caudal fin. The zebrafish were then left to regenerate for 4 days before the fins were imaged using a 10X objective. Fin regrowth was

measured from site of injury to the most distal portion of the blastema using Zeiss microscopy software.

RESULTS AND DISCUSSION

The gene encoding for *eif4e1c* can fully compensate for loss of *eif4ea* and *eif4eb*

In zebrafish, deletion of *eif4e1c*, further referred to as Δ*eif4e1c,* causes increased mortality, growth deficits, and impaired cardiomyocyte growth (Rao et al.,2023). To determine whether these phenotypes are specific to *eif4e1c,* a stable CRISPR fish line was created for both *eif4ea* and *eif4eb* (Fig 1 A,B). These will be referred to as Δ*eif4ea* and Δ*eif4eb*. Unlike Δ*eif4e1c*, the Δ*eif4eb* and Δ*eif4ea* zebrafish showed no such growth deficits into adulthood (Fig 1C,D). Likewise, there was no observed effect on cardiomyocyte numbers in Δ*eif4ea* and Δ*eif4eb* zebrafish (Fig 1E). Together this suggests that *eif4e1c* can fully compensate for the loss of either of the canonical factors and suggests that Δ*eif4e1c* phenotypes are in fact unique and not due to an overall reduction in translation.

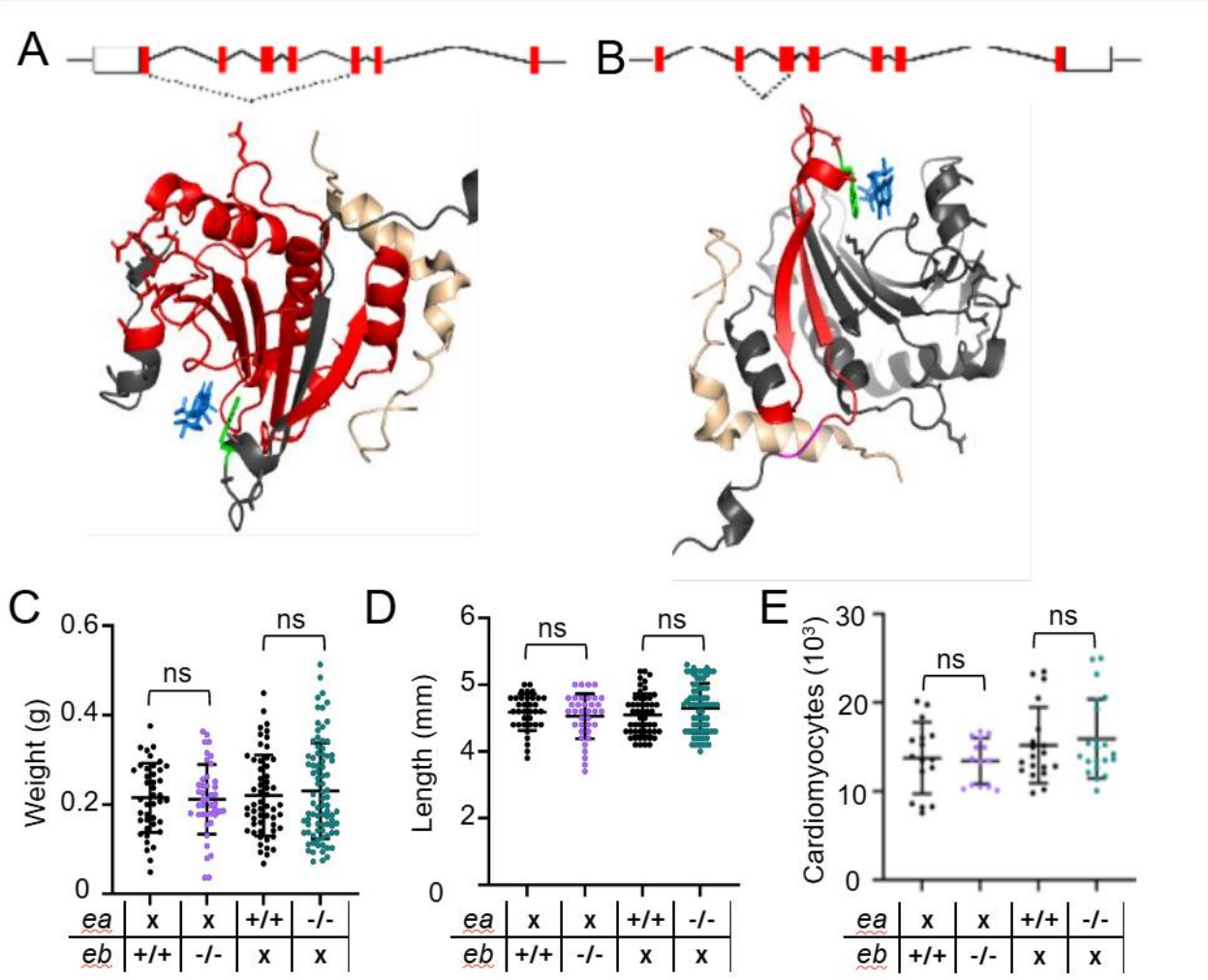

*Figure 1: (*A) Cartoon of *eif4ea* mutant CRISPR-mediated design, and model of EIF4EA crystal structure. Red sections map deleted portion of EIF4EA which results in null mutation. (B) Cartoon of *eif4eb* mutant CRISPR-mediated deletion design, and model of EIF4EB crystal structure. Red sections map deleted fragments in knockout mutants which removed cap binding portions of EIF4EB. (C) Fish were grown up together, genotyped at 3 months then weighed before 4 months after drying as much as possible (eb black average wildtype = 0.2200 g, mutant=0.2307 g; Tukey's multiple comparisons test, nsP=0.9084; n=52,73; ea black average wildtype=0.2150 g, ea mutant=0.2116 g; Tukey's multiple comparisons test, nsP=0.9984; n=35,36). (D) Fish were then measured from tip of jaw to bifurcation of caudal fin(eb black average wildtype = 25.47 mm, mutant = 26.48 mm; Tukey's multiple comparisons test, nsP= 0.3537 ; n= 124; ea black average wildtype= 25.47 mm, ea mutant=25.28 mm; Tukey's multiple comparisons test, nsP= 0.8710 ; n= 71). (E) The number of Mef2c-positive cells were counted with MIPAR. Eb black average wildtype =3038, mutant = 3184; Tukey's multiple comparisons test, nsP=0.9387; n=19,21; ea black average wildtype= 2748, mutant = 2682; Tukey's multiple comparisons test, nsP=0.9959;n=16,14).

Compound deletion of *eif4e1c* and either canonical factor potentiates the *eif4e1c* phenotypes

To observe interactions between *eif4e1c* and canonical factors, compound mutants were bred. Deletion of *eif4ea* alongside *eif4e1c* results in a 54% drop in weight relative to Δ*eif4e1c* alone [p=<0.0001], and a 22% drop in length [p=<0.0001]. There were 26% fewer cardiomyocytes relative to Δ*eif4e1c* [p=0.0178](Figure 2B,C,D). Nearly all observations were so striking that fish were distinguishable before any measurements or genotyping took place. In addition to developmental defects, zebrafish viability was 28% of what was observed for Δ*eif4e1c* alone (Fig 2A). This exacerbated phenotype of compound mutation was consistent for Δ*eif4eb* and Δ*eif4e1c* compound mutants in all categories except for mortality. For Δ*eif4eb* and Δ*eif4e1c,* mortality was in line with those observed by single deletion of *eif4e1c.* The potentiation of Δ*eif4e1c* phenotypes with the additional loss of a canonical factor suggests that the canonical *eif4ea* and *eif4eb* partially compensate for loss of *eif4e1c.*

A

ea	+/+	+/+	+/+	+/-	+/-	+/-	-/-	-/-	-/-
e1c	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-
Obs	156	243	95	266	455	150	107	204	27
Exp	107	214	107	214	428	214	107	214	107
R	4.73	1.98	-1.16	3.55	1.03	-4.37	0	-0.68	-7.73
χ^2	121	p=	1.66E-22						

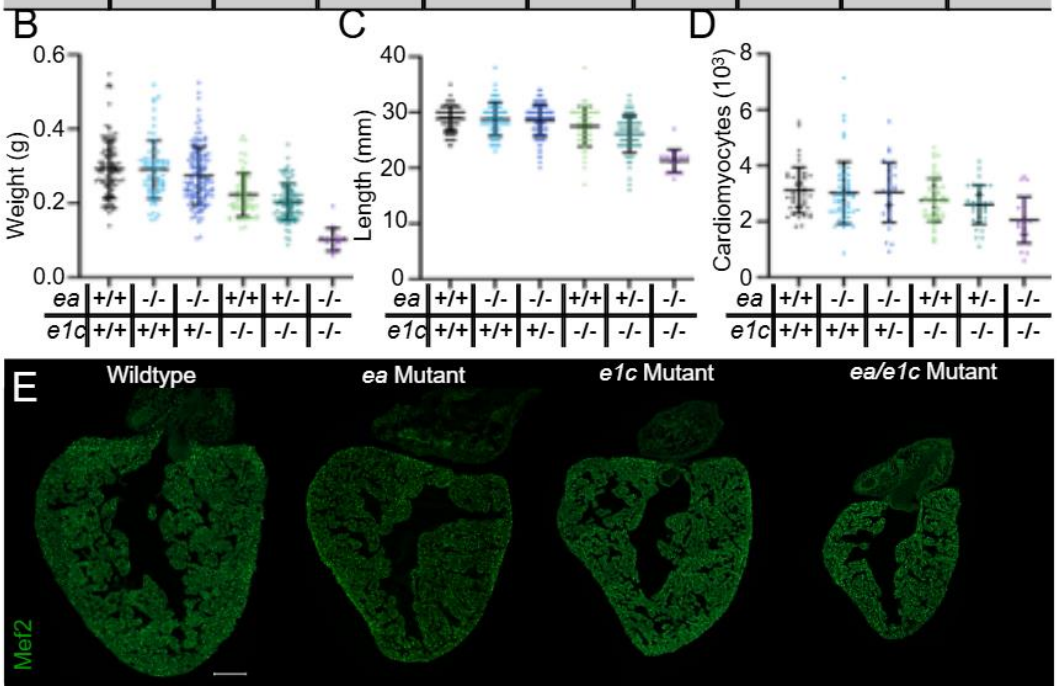

Figure 2: (A) Fish were genotyped and ratios of different genotypes were found. *Eif4e1c* single mutants vs Δ*eif4ea*/Δeif4e1c mutants were compared (E1c single obs = 95, exp = 107; r = -1.16; ea/e1c double obs = 27, exp = 107 r= -7.73; p = 1.77*E^{-22}). (B) Fish were grown up together, genotyped at 3 months then weighed before 4 months after drying as much as possible *(*Δ*eif4e1c green average = 0.2225 g,* Δ*eif4e1c*/Δ*eif4ea* pink average = 0.1017 g; Tukey's multiple comparisons test (ANOVA), *P= <0.0001 ; n= 71,22). (C) Fish were then measured from tip of jaw to bifurcation of caudal fin *(*Δ*eif4e1c* green average = 26.83 mm, Δ*eif4e1c*/Δ*eif4ea* pink average = 21.09 mm; Tukey's multiple comparisons test, **P= <0.0001 ; n= 71,22). (D) The number of Mef2-positive cells were counted with MIPAR. E1c green average = 2763 , Δ*eif4ea*/Δ*eif4e1c* pink average = 2055; Tukey's multiple comparisons test, P=0.0178; n=61,24). (E) Images of sectioned stained hearts of mutations of wildtype, Δ*eif4ea*, Δ*eif4e1c*, and compound Δ*eif4ea*/Δ*eif4e1c*.

Mutants lacking canonical cap binding proteins and *eif4e1c* die in the juvenile stages

Because there was a marked exacerbation of each of the previous Δ*eif4e1c* phenotypes, triple knockout mutants were bred using heterozygous mutant carriers. The triple mutants

survived in Mendelian ratios through the larval stages (Fig 3A). To determine whether they survived due to some other compensatory translation initiation mechanism or survived due to maternal deposition, a time course was performed. Zebrafish triple mutants were bred and subsequently genotyped at various days post fertilization(dpf). The fish grew up in Mendelian ratios until 15 dpf after which no more triples were found (Fig 3A) suggesting that maternal deposition is responsible for maintaining the triple mutants through their larval stages. This suggests that outside of *eif4e1c* and the canonical factors, there are no other compensatory programs available.

ea	+/+	+/+	+/+	+/-	+/-	+/-	-/-	-/-	-/-			Trip
eb	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-			M
e1c	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-	χ^2	p	R
5 dfp	13	15	12	23	28	10	22	10	9	33	5.6E-5	.067
11 dfp	10	35	8	26	44	17	16	38	15	16	.03	.555
15 dfp	15	31	12	30	61	16	27	25	15	18	.02	.131
1 mpf	24	32	8	39	76	18	24	30	0	38	5.4E-6	-3.94
2 mpf	14	37	9	37	48	5	17	18	0	47	1.2E-7	-3.39
Adult	59	81	33	133	229	20	51	82	0	148	4E-28	-6.55

Figure 3: Time course of genotypes depicting morality of the triple mutants. Triple mutants follow Mendelian ratio until 15 dpf. After 15 dpf, no triple mutants were recovered [1 mpf $p<0.0001$; 2 mpf <0.0001; Adult $p<0.0001$].

Compound deletion of canonical cap binding proteins have no observed deficits in viability, growth, and cardiomyocyte size.

To determine if the mortality seen from deletion of the triple mutants was due to the loss of canonical factors alone, heterozygotes were crossed to produce compound Δ*eif4ea* and Δ*eif4eb* mutants (referred to as canonical double mutants). Shockingly, canonical double

mutants not only survived in Mendelian ratios, but they exhibited none of the previous phenotypes in weight, length nor number of cardiomyocytes and were indistinguishable from wildtype [(Fig 4 A,B,C)]. This suggests that *eif4e1c* can fully replace the loss of canonical factors.

Single deletion of canonical factors improves cardiomyocyte proliferation

To better understand the role of canonical translation initiation factors and *eif4e1c* in cardiac regeneration, single and double canonical mutants were mated, and phenotypes were observed. No phenotype with regards to cardiomyocyte regeneration when assessing compound mutants of *eif4ea* and *eif4eb* was observed (Fig 4D). However, there was a marked improvement in cardiomyocyte proliferation in the absence of either of the cap binding proteins singularly (Fig 4D). Loss of either of the two canonical factors alone improved cardiomyocyte proliferation. This suggests that there is a partial redundancy in the canonical factors where they hold a dose dependent inhibitory, yet necessary, function. It may be that one canonical factor is required for *eif4e1c* to fully realize its capabilities, but two of them enact inhibition of the same pathway. In this way, the canonical factors may inhibit one another in the pathway of cardiac regeneration.

Single deletion of canonical factors improves fin regeneration

Similarly to cardiomyocyte proliferation, there was no phenotype with regards to fin regeneration when assessing compound mutants of *eif4ea* and *eif4eb* (Fig 4E). However, the singular loss of either of the canonical cap binding proteins inhibited fin regeneration. This suggests that they may individually inhibit fin regeneration but are neutralized by the presence of their other canonical counterparts.

The single mutant phenotype suggests an antagonistic relationship between the two canonical factors.

The relationship between *eif4ea* and *eif4eb* is unclear. The data suggests that the ratio of the two factors determines the phenotype, not the absolute levels of cap binding proteins. When both are present, they inhibit one another's action. When one is missing, the other is free to go about its regulatory goals. However, when both are missing, the overall function returns to the baseline. In their new regulatory paths, the presence of one another destabilizes their novel activity. This may suggest that - supported by the fact that the absence of the canonical factors, no phenotypes are observed - in exchange for new regulatory roles, the canonical factors have abandoned their roles as principal translation initiation factors.

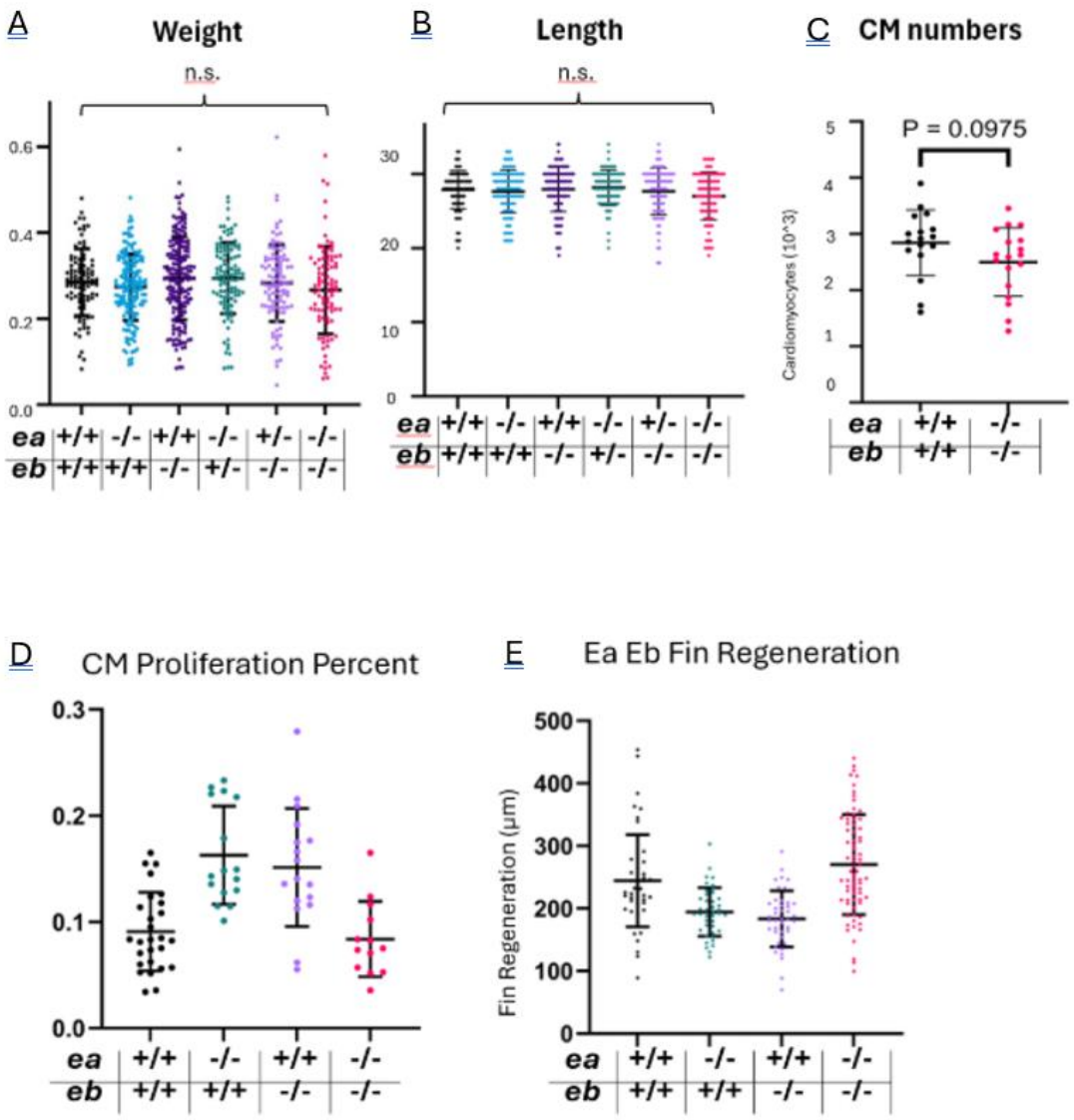

*Figure 4: (*AB)Fish were grown up together, genotyped at 3 months then weighed before 4 months after drying as much as possible (There was no significant difference between weights or lengths; Tukey's multiple comparisons test (ANOVA), *p=ns; n= 96: 102: 111: 178: 161: 90). (C) The number of Mef2-positive cells were counted with MIPAR. (Wildtype black average = 2809, Δeif4ea/Δeif4eb compound mutant pink average = 2516; Unpaired T-test, *p= 0.0975 ; n= 21:21). (D) Hearts were injured and allowed to recover for 4 days before daily injection of EdU and extraction. The number of Mef2-positive cells colocalized with EdU cells were counted using MIPAR. (Wildtype black average = 0.09084 vs Δeif4ea/Δeif4eb pink average =0.08384; Tukey's multiple comparisons test (ANOVA), P=0.9645; n=27:13; Wildtype black average = 0.09084 vs Δeif4ea green average = 0.1629 or Δeif4eb average = 0.1514; Tukey's Multiple Comparisons test (ANOVA), p1=<0.0001 ; n=27:16, p2=<0.0001; n=27,17). (E) Various mutants fins were cut just below the bifurcation, and after 4 days, their blastema was measured using a Zeiss microscopy software. (Wildtype black average = 244.3 µm vs Δeif4ea/Δeif4eb pink average =170.1 µm; Tukey's multiple comparisons test (ANOVA),

P=0.1774; n=50:73; Wildtype black average = 244.3µm vs Δeif4ea green average = 194.4 µm or Δeif4eb average = 183.4 µm; Tukey's Multiple Comparisons test (ANOVA), P1=<0.0013 ; n=50:41, P2=<0.0001; n=50:42).

Future Studies:

This study provides a strict genetic understanding of these proteins. While a genetic understanding is useful, it only helps with understanding surface-level phenotypes. To understand how these phenotypes occur, the proteins involved in these processes should be investigated. It should be noted that translation initiation factors like those described in this manuscript have been reported to modify some ribosomal subunit conformations (Jackson et al., 2010). CryoEM structural analysis when *eif4e1c* is bound would help to elucidate if these changes occur.

When *eif4e1c* is lost, the canonical factors can partially compensate for the loss of *eif4e1c.* Upon compound mutation with a canonical factor, *eif4e1c* phenotypes are exacerbated. This is likely because the canonical deletion mutants are true mutants which have lost the function of the canonical proteins. As a result, the remaining genes may not be able to fill gaps in translation normally filled in tandem by the canonical factors together. However, there still could be some muted, incomplete compensation. For example, eb could be upregulated transcriptionally in the ea mutant. Further studies should investigate how the levels of these transcripts are affected by deficits in any of these proteins. qPCR of each of the mutant combinations would help to identify if any of the homologous genes are regulated.

Finally, understanding which RNAs are differentially translated would be important in understanding the mechanism underlying these phenotypes. Additionally, Ribosome profiling could be performed to understand what RNA transcripts are differentially translated in the

mutants. Also, the knock-in of a tag on the cap binding proteins could be used for a pull-down to help understand which RNAs they touch.

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